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ISOLATION OF LACTOBACILLUS STRAIN AND ITS INVITRO INVESTIGATION OF ANTIMICROBIAL, ANTIOXIDANT AND ANTIDIABETIC EFFICACY OF POSTBIOTIC CELL FREE SUPERNATANT

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ABSTRACT

The study focuses on the isolation and characterization of probiotic bacteria from homemade yogurt and explores the potential health benefits of postbiotics derived from the probiotics. Probiotic bacteria are recognized for their positive effects on gut health, yet their live nature poses certain risks. In general, postbiotics, which include non-viable microbial cells and various metabolites produced during fermentation, have gained attention for their safety and bioactive properties. This research involved the isolation of lactic acid bacteria through a systemic approach, utilizing morphological and biochemical characterization and MALDI-TOF mass spectrometry for accurate identification of the strains. The resulting postbiotic cell-free supernatants were evaluated for their antimicrobial, antioxidant, and antidiabetic activities. The findings revealed substantial antibacterial effects against pathogenic bacteria

such as *Escherichia coli* and *Salmonella enterica* with distinct zones of inhibition correlated to the concentration of the sample. Additionally, antioxidant capacity was quantified through the DPPH assay, revealing the ability to neutralize free radicals. Further, the inhibition of α-amylase activity was performed, and results indicated potential benefits in carbohydrate metabolism and glycemic control. This study contributes to the therapeutic implications of postbiotics for human health.

Keywords: Yogurt, lactic acid bacteria, postbiotics, DPPH and α- amylase

1. INTRODUCTION:

Yogurt consists of probiotic bacteria, which are live microorganisms that offer health benefits to the host, particularly in gut health. Morphological identification and biochemical characterization are very important for understanding the functionality and efficacy of probiotic bacteria. Biochemical characterization also involves evaluating the safety of probiotic bacteria, including their resistance to gastrointestinal conditions and their ability to adhere to intestinal mucosa.

To determine bacterial species, Bergey's Manual is important to identify morphological, biochemical, and physiological characteristics, which are essential for accurate identification. It also helps in reference for phenotypic characterization, which is vital for confirming the identity of isolated probiotic bacterial strains **[1]**.

[2] Introduces postbiotics as beneficial metabolites produced by probiotics, which focuses on their stability and therapeutic advantages. It emphasizes that postbiotics can enhance gut health without the challenges associated with live probiotics. the secondary metabolites of probiotics into various forms of postbiotics, such as organic acids and exopolysaccharides, which contribute to the positive health outcomes.

Postbiotics, a term that refers to the bioactive compounds produced by probiotics, have collected significant attention in recent years due to their potential health benefits and therapeutic applications. Postbiotics also include non-viable microbial cells, cell wall components, and various metabolites that can utilize positive biological effects on the host. Research has demonstrated that the postbiotics derived from lactic acid bacteria contribute to gut health and reduce the risk of infections **[3]**.

Postbiotics can inhibit the growth of pathogenic bacteria by producing substances like bacteriocins and organic acids. Recent research indicates that postbiotics exhibit activity against the harmful pathogens **[4]**.

Postbiotics are the bioactive compounds produced during fermentation that have emerged as a promising area of research due

to their potential health benefits, including antimicrobial properties. Postbiotics show significant antibacterial activity against various pathogens, contributing to health benefits such as the prevention of infections and the maintenance of gut microbiota balance, thereby enhancing overall immune function and promoting gastrointestinal health **[5]**.

As postbiotics do not contain any live microorganisms, they are safer for use in immunocompromised individuals and reduce the risk of infections associated with live probiotics. It also provides various health benefits, including modulation of the immune system, enhancement of gut barrier function, and maintenance of gut microbiota balance. They also play a crucial role in maintaining gut homeostasis, influencing the gut-brain axis, and providing protective effects against infections **[6]**.

Recent research demonstrated that postbiotics can modulate oxidative stress by scavenging free radicals and enhancing antioxidant enzyme activity. The fermentation process of probiotic bacteria transforms substrates into bioactive compounds that exhibit strong antioxidant activities, indicating a symbiotic relationship between postbiotics and their substrates. The bioconversion of the postbiotic compounds during fermentation results in both the reduction of harmful oxidants and the production of beneficial metabolites **[7]**.

Several studies have reported that the antioxidant activity of specific postbiotics is derived from various probiotic strains. The mechanisms through which postbiotics exert their antioxidant effects are multifaceted. They can enhance endogenous antioxidant systems by upregulating the expression of genes responsible for antioxidant enzyme production **[8]**.

Emerging research indicates that postbiotics may play a crucial role in the management of diabetes mellitus by modulating gut microbiota and influencing systemic metabolic processes. Studies have suggested that postbiotics derived from lactic acid bacteria, can enhance insulin sensitivity, reduce blood glucose levels, and alter the composition of the gut microbiota favorably **[9]**.

The current study focuses on the isolation of probiotic bacteria from yogurt and obtaining postbiotic cell-free supernatant. Further to evaluate the antimicrobial, antioxidant, and antidiabetic potential of postbiotics in in vitro conditions.

2. MATERIALS AND METHODS:

2.1 Sample preparation: Homemade yogurt was prepared using the lactic acid bacterial

starter culture and allowed to ferment overnight at room temperature. Later it was transported to the laboratory and stored in the refrigerator until use.

2.2 Isolation: The yogurt samples were diluted at 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} ratios in the sterile distilled water. Then 50 µl of diluted samples were spread on MRS agar plates and incubated at 37°C for 48 hours. For pure culture, subsequent streaking was performed and again incubated at 37°C for 48 hours **[10]**.

2.3 Gram staining: A single colony of bacterial culture was picked and smeared on a clean glass slide, allowed to air dry, and heat-fixed on the smeared glass slide. Followed by heat fixing, the glass slide was flooded with crystal violet, Gram's iodine solution, decolorized with ethyl acetate, counterstained with safranin, and rinsed with water. The smear was then examined under 100x magnification **[11]**.

2.4 Biochemical characterization: Biochemical characterizations of isolated bacterial strains were identified by performing IMViC tests, catalase, and oxidase **[12]**.

2.5 Phenotypic level identification: Phenotypic identification was performed using a mass spectrometry system using matrix-associated laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), which is VITEK MS PRIME **[13]**.

2.6 Postbiotic preparation: Isolated cultures are inoculated in 5 ml MRS broth and incubated at 37°C overnight in a shaking incubator. After the overnight incubation, the cultures were centrifuged at 6000 rpm for 10 minutes at 4°C. The supernatant was then collected in a sterile conical flask and refrigerated until it was ready to use **[14]**.

2.7 Antimicrobial activity: Antimicrobial activity of postbiotic cell-free supernatant was performed using the agar well diffusion method. For antimicrobial activity, the pathogenic bacteria *Streptococcus bovis*, *Enterococcus faecalis*, *Escherichia coli*, and *Salmonella enterica* were obtained from Royalcare Super Specialty Hospital, Coimbatore. Broth culture of pathogens was taken and swabbed in sterile Muller Hinton agar plates, and a 5 mm well was punched using a sterile pipette tip. Later, the obtained postbiotics were added to the wells in 20 µl, 40 µl, 50 µl, 80 µl, and 100 µl, and then the plates were incubated at 37°C for 24 hours **[15]**.

2.8 Antioxidant activity: The DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical-scavenging method was used to evaluate the antioxidant activity. A DPPH solution of 3 mg/ml was prepared in methanol **[16]**. 400 µl

of postbiotic sample and control distilled water were filled in separate vials. 1 ml of DPPH solution was added approximately in vials. At 517 nm, the sample's absorbance was determined using a UV-Vis spectrophotometer. The percentage of antioxidant (DPPH) was calculated using the below equation.

$$\% \text{ of DPPH} = \frac{\text{Absorbance of blank- Absorbance of sample}}{\text{Absorbance of blank}} \times 100$$

2.9 Antidiabetic activity: Antidiabetic activity of postbiotic cell-free supernatant was evaluated using α-amylase inhibitory activity **[17].** 10 µl of α-amylase solution was added to different concentrations of postbiotic samples, and it was pre-incubated for 10 minutes at 25°C. The starch solution of 10µL along with reaction mixture was reincubated for 1 hour at 25°C. 390 ml of 0.02 M phosphate buffer as a positive control with pH 7. The reaction was determined with the addition of 0.1 ml of iodine solution and 5 ml of distilled water to the reaction mixture. Later, the reaction mixture was boiled and allowed to cool, and the OD value of absorbance was measured at 565 nm. The inhibition was evaluated using the following calculation.

$$\text{Inhibition\%} = \frac{\text{(Absorbance of blank- Absorbance of sample)}}{\text{Absorbance of control}} \times 100$$

3. RESULTS:

3.1 Morphological and biochemical characterization: The yogurt samples were diluted in distilled water and inoculated in MRS agar plates. By observing the isolated bacteria under a microscope, seen as gram-positive, rod-shaped bacilli in 100x **(Table 1)**. Biochemical characterization of the probiotic strains shows that indole and methyl red are positive, whereas Voges proskauer, citrate, oxidase, and catalase are negative. Morphological and biochemical characteristics are shown in **(Table 2)** and are suspected to be *Lactobacillus sp*.

3.2 Phenotypic identification using MALDI- TOF: After biochemical characterization, the isolated bacterial strains underwent phenotypic identification performed using the MALDI-TOF MS

method, which shows that the isolated strain is *Lactobacillus pentosus*.

3.3 Antimicrobial activity: The postbiotic cell-free supernatant was tested for its antimicrobial activities in the agar well diffusion method, where the zone of inhibition is measured and tabulated in **(Table 3)**. The pathogenic bacterial strains used for antimicrobial activity are *Streptococcus bovis*, *Enterococcus faecalis*, *Escherichia coli*, and *Salmonella enterica*.

The largest zone of inhibition is noted in *Enterococcus faecalis* from the concentration of 50 µl onwards. Followed by *Enterococcus faecalis, Escherichia coli* has shown a good zone of inhibition from the concentration of 50 µl, but compared to *Enterococcus*, the zone of inhibition in *E.coli* is small in size. *Streptococcus bovis* and *Salmonella enterica* have no zone of inhibition in the concentrations of 25 µl and 50 µl, which shows growth inhibitory activity from the concentration of 75 µl. For all four strains, the zone increases according to the concentration of the sample.

3.4 Antioxidant activity: The antioxidant activity of postbiotic cell-free supernatant was evaluated using DPPH radical scavenging activity, which was calculated and tabulated in **(Table 4)**.

The results indicated that the sample exhibited free radical scavenging capabilities that effectively reduced the DPPH radicals **(Figure 1)**, indicating that at a concentration of 100 µl, the postbiotic sample exhibited a percent inhibition of DPPH radicals exceeding 75 µl of the sample concentration, demonstrating the antioxidant potential.

3.5 Antidiabetic activity: The antidiabetic activity of the postbiotic sample was assessed by evaluating its inhibitory effect on alpha-amylase activity. The result demonstrated a clear dose-dependent response **(Figure 2)**, with significant inhibition observed at varying concentrations of the postbiotic sample **(Table 5)**.

Table 1: Morphological characterisation	
Colony appearance	Whitish cream
Gram staining	Positive
Microscopic observation	Rods

Table 2: Types of biochemical tests	
Indole	Positive
Methyl red	Positive
Voges proskauer	Negative
Citrate	Negative
Catalase	Negative
Oxidase	Negative

Table 3: Zone of inhibition of antimicrobial activity					
S. No.	Pathogens	Zone of inhibition			
		25 µl	50 µl	75 µl	100 µl
1.	*Streptococcus bovis*	-	-	12.2mm	15mm
2.	*Enterococcus faecalis*	-	12mm	14.4mm	17.1mm
3.	*Escherichia coli*	-	8.5mm	10mm	21mm
4.	*Salmonella enterica*	-	-	12.3mm	14.7mm

- No zone of inhibition

Table 4: Antioxidant activity			
Concentration	OD Value of Blank	OD Value of Sample	% OF DPPH
25 µl	1.398	0.529	62.16
50 µl	1.398	0.564	59.65
75 µl	1.398	0.616	55.93
100 µl	1.398	0.692	50.50

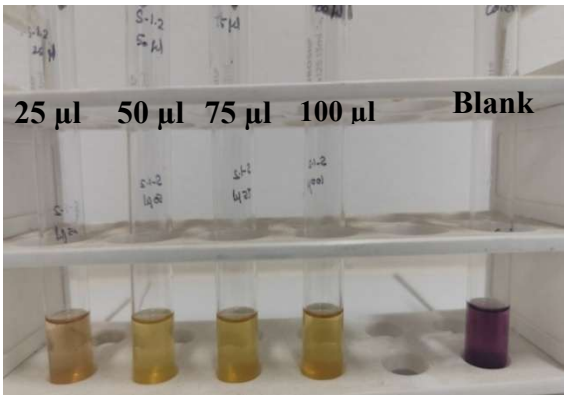

Figure 1: Antioxidant activity

Table 5: Antidiabetic activity			
Concentration	Standard (Acarbose)	OD Value (0.495)	Sample
10 µg	32 %	0.423	17.02 %
20 µg	48 %	0.348	30.00 %
30 µg	55 %	0.270	45.45 %
40 µg	72 %	0.212	57.17 %
50 µg	87 %	0.110	78.00 %

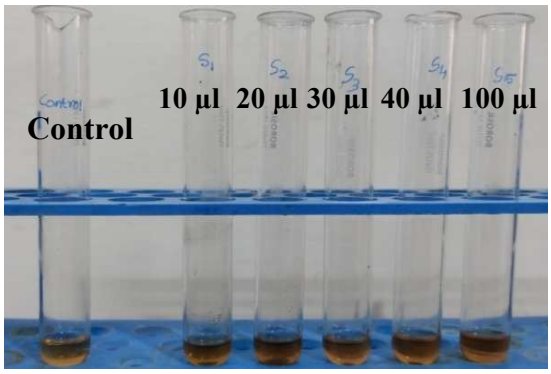

Figure 2: Antidiabetic activity

Specifically, at the highest tested concentration of 50 µl, the sample exhibited an inhibition rate of 78.00%, indicating potent anti-diabetic properties. In comparison with the standard alpha-amylase inhibitor, acarbose displayed an inhibition rate of 87.00% at an equivalent concentration of 50 µl. An intermediate concentration of the postbiotic sample from 20 µl to 40 µl also yielded notable inhibitory effects, ranging between 30.00% and 57.17%, which shows the gradual increase in bioactivity correlating to the concentration of the sample.

4. DISCUSSION:

In the present study, homemade yogurt was selected as the primary source, owing to its rich microbial diversity and traditional significance in various cultures. The yogurt sample were prepared from boiled milk, allowing for optimal growth conditions of lactic acid bacteria, which are integral to probiotic activity. The selection of materials for sample collection emphasized the importance of using non-commercial, homemade products, as they typically harbor a more diverse microbiota compared to industrially produced alternatives.

Isolation was performed using de Man, Rogasa, and Sharpe (MRS) broth, a selective medium conducive to the growth of lactic acid bacteria. This method facilitated the proliferation of desired strains while inhibiting nontargeted microbial species. Following an incubation period, the transfer of cultures to MRS agar plates enabled the differentiation of distinct colonies, which were then subjected to sub culturing to achieve purity. This meticulous isolation process ensured that the probiotic strains maintained their integrity and viability, which is essential for subsequent analysis.

Identification of the isolated strains involved a combination of morphological observation **(Table 1)**, biochemical tests, and MALDI-TOF MS for specific genus identification. Gram staining provided initial insights into the cellular characteristics, while biochemical characterization **(Table 2),** including the methyl red and catalase tests, allowed for further classification based on metabolic profiles. The MALDI-TOF MS method emerged as a critical methodological enhancement, providing information that enables precise identification of isolated bacterial strains at the genus and species level. The identified lactobacillus strains probiotic characteristics are consistent with those reported in other studies. For example, similar antimicrobial activities of Lactobacillus isolates from curd and human milk against foodborne and human pathogens have been documented **[18]**.

Apart from dairy products, the successful isolation and characterization of lactococci from plant materials and investigation of their probiotic properties provide further support for exploring diverse sources of beneficial microbes **[19]**.

The production of postbiotics, particularly the cell-free supernatant, was achieved through centrifugation of the isolated probiotic bacteria, which allowed to get the bioactive compounds without affecting the presence of viable bacterial cells.

In the present study, the antimicrobial activity was assessed using standard well-diffusion method. The efficacy of postbiotics was tested against the *Streptococcus bovis, Escherichia coli, Enterococcus faecalis,* and *Salmonella enterica* was evaluated by measuring the diameter of inhibition zones **(Table 3)**. The strains exhibited different zones of inhibition and depending on the concentration of the sample; it underscores the specificity of the antimicrobial action associated with the postbiotic cell-free supernatant.

The findings **(Table 4)** suggest the postbiotics cell-free supernatant possesses substantial antioxidant properties, thereby affirming its potential application in enhancing oxidative stress resistance and promoting health through antioxidant mechanisms.

In the current study, the antidiabetic activity observed in postbiotic samples is the result indicate a marked reduction in α-amylase activity with increasing concentration of the sample **(Table 5),** suggesting their potential to lower glucose levels. These findings show the potential of postbiotics as functional agents in the management of diabetes.

5. CONCLUSION:

The study effectively demonstrated a structured methodology for the sample collection, isolation, and identification of probiotic bacteria from yogurt samples. Following this selective isolation on MRS media enabled the differentiation of lactobacilli and laid the groundwork for thorough analysis.

Morphological and biochemical characterization. Provided valuable insights into the metabolic capabilities of the isolated strains. The use of gram staining and a suite of biochemical tests, including IMViC, catalase, and oxidase, allowed us to understand the isolate's taxonomy and functionality. Moreover, the MALDI-TOF technique further confirmed the species identity of the isolated strain.

This research shows the postbiotic capacity of inhibiting the growth of pathogenic bacteria, as evidenced by the measurable zone of inhibition against various bacterial strains,

including *E. coli, Streptococcus bovis, Enterococcus faecalis,* and *Salmonella enterica*. These suggest that the postbiotics may serve as effective natural antimicrobial agents.

The investigation into the antioxidant activity of postbiotics revealed significant radical Scavenging potential as evidenced by the DPPH assay results. These findings show the relevance of postbiotics in enhancing oxidative capacity and stability.

The antidiabetic activity revealed a notable capacity to inhibit alpha amylase activity in a concentration-dependent way. The postbiotics showed an impressive reduction in enzyme activity, indicating their potential to modulate carbohydrate metabolism and support glycemic control.

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COMPETING INTEREST

The authors have no competing interest to declare.

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