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ISOLATION, IDENTIFICATION AND ASSESSMENT OF ANTIMICROBIAL, ANTIOXIDANT, ANTIDIABETIC AND ANTI-INFLAMMATORY ACTIVITY OF HEAT- KILLED POSTBIOTIC FROM LACTOBACILLUS STRAIN

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ABSTRACT

The study focuses on the isolation and identification of Lactobacillus from yogurt samples. Later, postbiotics were obtained as heat-killed postbiotics from the isolated Lactobacillus strain. And to evaluate its in vitro antimicrobial, antioxidant, antidiabetic, and anti-inflammatory properties. Compared to probiotics, postbiotics are safe to use because they are inactivated. Postbiotics consist of active bioactive metabolites that act as therapeutic agents.

Keywords: Postbiotics, Lactobacillus, therapeutic agents, invitro activity and bioactive metabolites

1. INTRODUCTION:

Postbiotics are defined as bioactive compounds produced during the fermentation process by probiotics, postbiotics encompass a wide variety of non- viable microbial cells, cell components, and metabolic byproducts that confer beneficial effects to the host, Unlike probiotics, which are live micro-organisms, postbiotics are inactivated making them a safer alternative for individuals with compromised immune systems, such as those undergoing chemotherapy, surgery or suffering from chronic illness **[1]**. The term 'postbiotics' encompasses various bioactive molecules, including organic acids, bacteriocins, polysaccharides, short- chain fatty acids, enzymes, proteins and vitamins. These components are known for their diverse biological activities, which include antimicrobial, anti-inflammatory, anti cancer and immunomodulatory effects. For instance, short- chain fatty acids produced during fermentation have been shown to play a crucial role in maintaining gut health, enhancing insulin sensitivity, and modulating immune responses **[2]**.

Antimicrobial activity refers to the ability of a substance to inhibit the growth of or kill pathogenic microorganisms, including bacteria, viruses, and fungi. In the context of postbiotics, this activity is primarily attributed to the bioactive compounds produced during the fermentation process by probiotic microorganisms. These compounds, such as bacteriocins, organic acids, and short-chain fatty acids, exhibit potent antimicrobial properties that can effectively combat a range

of clinical pathogens, including *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi*. The mechanisms underlying the antimicrobial effects of postbiotics are diverse. They may involve the disruption of microbial cell membranes, alteration of metabolic pathways, and inhibition of essential enzymatic activities within the pathogens. The multifaceted approach not only helps in controlling infections but also contributes to maintaining a balanced gut microbiota, which is crucial for overall health. The research conducted in this study aims to elucidate these antimicrobial properties through well-established methods, such as the well diffusion method, to assess the efficacy of various postbiotic samples against clinically relevant pathogens **[3]**.

Antioxidant activity is another critical aspect of postbiotics that has gained prominence in health research. Antioxidants are compounds that neutralize free radicals—unstable molecules that can cause oxidative stress, leading to cellular damage and contributing to various chronic diseases, including cancer, cardiovascular diseases, and neurodegenerative disorders. Postbiotics, through their rich composition of bioactive metabolites, have been shown to exhibit significant antioxidant properties **[4]**.

The antioxidant activity of postbiotics is typically evaluated using assays such as the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay, which measures the ability of a substance to donate electrons to free radicals, thereby neutralizing them. The current study aims to quantify the antioxidant capacity of different postbiotic samples, providing insights into their potential role in mitigating oxidative stress and promoting health.

The antidiabetic activity of postbiotics is an emerging area of interest, particularly in the context of the global rise in diabetic prevalence. Postbiotics have been shown to influence glucose metabolism, enhance insulin sensitivity, and improve overall glycemic control, making them valuable in the management of diabetes. The bioactive compounds present in postbiotics, such as short-chain fatty acids, play a crucial role in modulating metabolic pathways associated with insulin production and glucose homeostasis **[5]**.

Research indicates that postbiotics can enhance insulin secretion from pancreatic beta cells, improve insulin resistance in peripheral tissues, and promote the utilization of glucose by cells. This multifaceted approach not only aids in managing blood sugar levels but also contributes to reducing the risk of diabetes-related complications **[6]**.

The anti-inflammatory properties of postbiotics can be attributed to several mechanisms. One of the primary ways postbiotics exert their effects is through the modulation of immune responses. For example, certain postbiotics have been shown to inhibit the production of pro-inflammatory cytokines, thereby reducing inflammation. A study highlighted that the postbiotic *Faecalibacterium prausnitzii* significantly decreased the levels of inflammatory markers in patients with Crohn's disease, suggesting its role as an anti-inflammatory. By exploring the anti-inflammatory activity of postbiotics, this research aims to explain their significance as a natural strategy for promoting health and preventing inflammation-related diseases **[7]**.

The present study aims to investigate the antimicrobial, antioxidant, antidiabetic and anti-inflammatory activities of postbiotics.

2. MATERIALS AND METHODS:

2.1 Sample collection: Yoghurt is a fermented milk product. Yogurt is purchased in nearby local store, Coimbatore. Later it was stored in a refrigerator until use.

2.2 Isolation: 1 ml of sample was cultured in 9 ml of MRS broth and incubated for 38-38 hours at 37°C. Later, a loopful of cultured broth was isolated on an MRS agar plate and incubated for 38-48 hours. Bacterial colonies were subsequently subcultured to obtain pure cultures and stored in MRS agar plates/slants for future studies **[8]**.

2.3 Identification:

2.3 Gram staining and microscopic observation: Using the gram staining kit, the isolated bacteria were examined under a microscope at 45x and 100x magnifications **[9]**.

2.4 Catalase test: To conduct the test, a drop of 3% hydrogen peroxide was added to a single isolated colony that had been streaked on the glass slide. The abundance of oxygen indicated that the bacteria survived the catalase test **[9]**.

2.5 Biochemical tests: The biochemical characterization, such as indole, methyl red, Voges-Proskauer, and oxidase, is performed by following the Bergey's manual **[10]**.

2.6 MALDI TOF: Pure cultures were obtained from yogurt samples and were identified using MALDI-TOF-MS **[11]** with slight modifications, which is matrix-associated laser desorption/ionization time-of-flight mass spectrometry (Vitek MS Prime).

2.7 Postbiotic extraction – heat killed cells: Isolated strains were cultured in a suitable medium at a controlled temperature of 32°C for a specific duration of 18 hours. After the incubation period, the bacterial cells are harvested from the culture medium, and then the harvested cells are washed in saline solution to remove any residual impurities from the growth medium. The washed cells are resuspended in distilled water to prepare them for heat treatment. Later the resuspended cells are subjected to heat treatment at a temperature of 80°C for duration of 20 minutes. This step is crucial as it effectively kills bacteria while preserving their beneficial properties, allowing them to function as a postbiotic **[12]**.

2.8 Anti bacterial activity - well diffusion method: The antibacterial activity (well diffusion method) was studied by using clinical pathogens *Staphylococcus aureus*, *Streptococcus pyogens*, *Bacillus cereus, Klebsiella sp., Salmonella sp*., and *E. coli*, which were obtained from Royalcare Super Specialty Hospital, Coimbatore. The clinical isolates are inoculated into nutrient broth and incubated at 37°C for 18 hours. The clinical pathogens were swabbed over sterile Muller-Hinton agar plates. Then a 6 mm well was made in MHA plates. 25 µl, 50 µl, 75 µl, and 100 µl of heat killed postbiotic were loaded in a well and incubated at 37°C for 24 hours **[13]**.

2.9 Antioxidant activity – DPPH: The 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity was performed by following the method of **[14]** with slight modifications. Aliquot 25 µl, 50 µl, 75 µl, and 100 µl of DPPH (1 ml of menthol) was added to 0.5 ml of postbiotic samples and shaken. The glass tubes were then placed at 4°C without any light for 30 minutes. Following the incubation at room temperature in the dark for 30 minutes, absorbance was read by a UV-VIS spectrophotometer at 517 nm against a blank. The percentage of DPPH radical scavenging effect was calculated using the equation below:

$$\text{DPPH} = \frac{\text{A blank- A sample}}{\text{A blank}} \times 10$$

2.10 Antidiabetic activity: The α-amylase inhibition assay was performed as described by **[15]** with slight changes. Added 390 ml of 0.02 M phosphate buffer of pH 7 as a positive control. Different concentrations of test samples with 10 µl of alpha amylase were then pre-incubated at 37°C for one hour. Added 1% iodine solution and 5 ml of distilled water were measured OD at 565 nm. The test was done, and the (α-amylase) anti-diabetic inhibitory activity was calculated as follows:

$$\text{Percentage inhibition:} \frac{\text{(O.D. of control – O.D. of sample)}}{\text{O.D. of control}} \times 100$$

2.11 Anti inflammatory activity: The protein denaturaion method was performed as described by **[16]** with slight modifications. 500 µl of 1% bovine serum albumin was added to 10 µl, 20 µl, 30 µl, 40 µl, and 50 µl of the test sample. The mixture was incubated at 37°C for 10 minutes. For a duration of twenty minutes, all the test tubes containing the reaction mixture were incubated in the water bath at 51°C. Following the incubation period, the test tubes were allowed to cool down to room temperature, and the absorbance at 660 nm was compared to the blank. Aspirin was used as a standard positive control. By using the following formula, the percentage of inhibition was calculated:

$$\text{Percentage inhibition:} \frac{\text{100 - (O.D. of test – O.D. of control) x 100}}{\text{O.D. of control}}$$

3. RESULTS:

3.1 Identification of bacterial strain: From the isolated yogurt sample, microbial strains were successfully identified as Lactobacillus after 48 hours of isolation. The appearance of colonies was whitish cream on the surface of the MRS agar plate. On microscopic observation, the culture showed gram-positive rods, leading to the biochemical characterization that revealed that the isolated strain tested negative for the indole and catalase tests, while it was positive for the methyl red test, indicating its ability to produce stable acid from glucose fermentation. Additionally, the oxidase test returned negative results, further supporting the classification of the isolated strain. These biochemical characteristics, combined with morphological observation, were performed as mentioned in Bergey's manual, followed by the MALDI-TOF results, facilitating the isolated microbial strain being identified as *Lactobacillus pentosus*.

Table 1: Morphological identification	
Colony appearance	**Whitish small colonies**
Gram staining	+
Microscopic observation	**Rods**

Table 2: Biochemical Characterization	
Indole	+ve
Methyl red	+ve
Voges Proskauer	-ve
Citrate	-ve
Catalase	-ve
Oxidase	-ve

3.2 Antibacterial activity: The antimicrobial activity of heat-killed postbiotics was evaluated by the agar well diffusion method. A range of clinical pathogens, including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Bacillus cereus*, *E. coli*, *Salmonella typhi*, and *Klebsiella pneumoniae*, were used. The results demonstrated a notable zone of inhibition for several tested organisms. Specifically, it exhibited significant antimicrobial activity against gram-positive bacteria such as *Staphylococcus aureus* and *Streptococcus*, with maximum inhibition observed at lower to higher concentrations, whereas *Bacillus cereus* has shown a zone of inhibition at 75 µl and 100 µl. For gram-negative bacteria, including E. coli and *Salmonella typhi*, effective inhibition was shown, particularly at elevated dosages, but in *Klebsiella pneumoniae*, a good zone of inhibition was also shown at all the concentrations.

Table 3: Evaluation of antibacterial activity					
S. No.	Pathogens	Concentration (25µl) Zone of inhibition (mm)	Concentration (50µl) Zone of inhibition (mm)	Concentration (75µl) Zone of inhibition (mm)	Concentration (100µl) Zone of inhibition (mm)
1	*Staphylococcus aureus*	-	14.3	15.8	16.1
2	*Streptococcus pyogens*	10.2	18	18.8	20.3
3	*Bacillus cereus*	-	-	13	15.3
4	*E. coli*	-	8.4	10	10.6
5	*Salmonella typhi*	-	12	13.8	15.1
6	*Klebsiella pneumoniae*	13.2	15.5	16	18.7

3.3 Antioxidant activity: As observed, the antioxidant activity of the postbiotic sample was assessed using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The result showed a concentration-dependent increase in antioxidant activity. At a concentration of 25 µl, the sample exhibited a DPPH scavenging effect of 43.56%, which significantly increased to 69.24% at the highest concentration of 100 µl. These findings indicate that the postbiotic cell-free supernatant possesses notable antioxidant properties, particularly at elevated concentrations.

Table 4: Assessment of Antioxidant activity				
S.No	Concentration	OD Value of Blank	OD Value of Sample	% OF DPPH
1	25 µl	1.398	0.789	43.56
2	50 µl	1.398	0.466	66.66
3	75 µl	1.398	0.454	67.52
4	100 µl	1.398	0.430	69.24

3.4 Anti- diabetic activity: The antidiabetic activity of postbiotic cell-free supernatant was used as a standard and was evaluated through its inhibitory effect on alpha-amylase activity. The results indicated in enzyme activity. At a concentration of 10 µl, postbiotic samples have shown inhibition of 32% of inhibition, which postbiotic samples are progressively increased to 75.38% at the highest concentration of 50 µl, and standard acarbose increased to 87% at 50 µl. This result suggests that postbiotic cell-free supernatant exhibits antidiabetic properties, particularly at higher concentrations.

Table 5: Assessment of Antidiabetic activity				
S. No.	**Concentration**	**Standard (Acarbose)**	**OD Value (0.455)**	**Sample**
1	**10 µg**	**32%**	**0.455**	**08.57%**
2	**20 µg**	**48%**	**0.331**	**27.25%**
3	**30 µg**	**55%**	**0.255**	**44.70%**
4	**40 µg**	**72%**	**0.213**	**53.15%**
5	**50 µg**	**87%**	**0.112**	**75.38%**

3.5 Anti- inflammatory activity: The anti-inflammatory activity of the sample, which is derived from heat-killed postbiotics, was assessed using the protein denaturaion method. The results indicated a concentration-dependent inhibition of protein denaturaion, with the demonstrating significant anti-inflammatory effects at various concentrations. At the lowest concentration tested, the sample exhibited an inhibition rate of 18.30%, which increased progressively to 79.00% at the highest concentration of 50 µg/ml, whereas aspirin was used as a standard, which showed an inhibition rate of 88% at the same concentration.

Table 5: Assessment of Anti-inflammatory activity				
S. No.	**Concentration**	**% Inhibition of Standard (Aspirin)**	**OD Value (0.008)**	**% Inhibition Sample**
1	**10 µg/ml**	**33%**	**0.155**	**18.30%**
2	**20 µg/ml**	**44%**	**0.210**	**25.25%**
3	**30 µg/ml**	**58%**	**0.352**	**43.00%**
4	**40 µg/ml**	**79%**	**0.550**	**68.00%**
5	**50 µg/ml**	**88%**	**0.640**	**79.00%**

4 DISCUSSION:

Postbiotics are a bioactive compounds produced by lactic acid bacteria during fermentation process. It also offers promising therapeutic benefits and possess various health promoting properties **[17]**.

The morphological identification of the isolated bacterial strain revealed distinct characteristics, with gram staining indicating the predominance of gram-positive bacteria and rod shape being observed **Table 1**, thus providing a preliminary classification for further analysis. In **Table 2**, the biochemical characterization that was conducted on the isolated strain was indole negative, methyl red positive, Voges proskauer, citrate, catalase, and oxidase negative.

Postbiotics exhibit antimicrobial properties through so many mechanisms, involving the production of bioactive metabolites that inhibit the growth of pathogenic microorganisms. One of the key compounds of postbiotics is bacteriocins, which are antimicrobial peptides produced by lactic acid bacteria **[18]**. Heat-killed postbiotics, derived from probiotics that have been rendered non-viable through heat treatment, exhibit significant antimicrobial properties that can be harnessed for various applications. These postbiotics retain bioactive compounds, such as cell wall fragments and metabolites, which can inhibit the growth of pathogenic microorganisms. **Table 3** indicates that heat-killed postbiotics can disrupt the cell membranes of harmful bacteria, thereby reducing their viability.

The assessment of antioxidant activity using the DPPH assay revealed that the tested samples exhibited varying degrees of radical scavenging ability. The sample at higher concentration demonstrated a significant reduction in DPPH absorbance, indicating potent antioxidant properties. In **Table 4**, the

sample at 100µl concentration has shown a scavenging effect, suggesting its effectiveness in neutralizing free radicals.

In evaluation of the anti-diabetic activity, a significant inhibitory effect on alpha-amylase, an enzyme crucial for carbohydrate digestion, was revealed. In **Table 5**, the observed activity is compared to the standard acarbose, and the sample has also shown strong potential for anti-diabetic properties.

The assessment of the anti-inflammatory properties of the postbiotic sample demonstrated a notable ability to inhibit protein denaturaion, which is a key indicator of inflammation. In **Table 6**, it was indicated that the sample exhibited a concentration-dependent effect, with inhibition rates increasing from lower to higher concentration.

5 CONCLUSIONS:

In the current study, the yogurt sample was isolated and identified as Lactobacillus. The postbiotic samples have shown potent results on antimicrobial, antioxidant, antidiabetic, and anti-inflammatory properties. The findings indicate that postbiotics could serve as a valuable natural source of therapeutic agents.

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Conflict of interest

There is no conflict of interest in this research paper.

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