

## ISOLATION AND CHARACTERIZATION OF PROBIOTICS (LACTOBACILLUS) FROM ANDHRA PICKLES (BAPATLA)

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### ABSTRACT

Fermented foods are recognised as natural reservoirs of beneficial microorganisms, particularly lactic acid bacteria (LAB) with probiotic potential. The present study focused on the isolation and preliminary characterization of *Lactobacillus* species from traditionally prepared Andhra pickles collected in Bapatla, Andhra Pradesh, India. Five homemade pickle samples were analyzed, with fresh curd used as a positive control. Samples were processed using serial dilution and enrichment in de Man, Rogosa, and Sharpe (MRS) broth under anaerobic conditions to promote selective growth of LAB. Preliminary identification was carried out using Gram staining, catalase testing, and carbohydrate fermentation assays. Microscopic examination revealed Gram-positive, rod-shaped, catalase-negative bacteria capable of fermenting glucose with acid production, confirming characteristics typical of *Lactobacillus* spp. Semi-quantitative estimation based on serial dilution

turbidity indicated the presence of LAB in all pickle samples, although in lower abundance compared to curd. Pickle samples showed detectable growth up to  $10^{-3}$ – $10^{-4}$  dilutions,

whereas curd demonstrated growth up to  $10^{-7}$  dilutions, suggesting a richer LAB population in dairy fermentation. The findings demonstrate that traditional Andhra pickles serve as a source of viable lactic acid bacteria, though at comparatively lower densities than dairy products. This highlights the potential of regionally fermented vegetable products as alternative probiotic sources. Further molecular identification and functional probiotic assays are recommended to confirm strain-level identity and evaluate health-promoting properties. This study provides baseline data supporting the microbiological value of indigenous fermented foods and encourages future exploration of non-dairy probiotic resources.

**KEYWORDS:** Probiotics, *Lactobacillus*, Andhra pickles, fermented foods, lactic acid bacteria, traditional fermented foods, indigenous fermentation, antimicrobial activity, acid tolerance, bile salt tolerance, gut health, functional foods, microbial characterization, probiotic screening, natural probiotics, food microbiology, gastrointestinal survival, South Indian pickles.

#### ABBREVIATIONS

ATCC – American Type Culture Collection

CFU – Colony Forming Units

°C – Degree Celsius

DNA – Deoxyribonucleic Acid

g – Gram

H<sub>2</sub>O<sub>2</sub> – Hydrogen Peroxide

hrs – Hours

LAB – Lactic Acid Bacteria

mL – Millilitre

mg – Milligram

min – Minutes

MRS – de Man, Rogosa and Sharpe medium

MRS-Ac – MRS Broth supplemented with Sodium Acetate

NaOH – Sodium Hydroxide

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

pH – Potential of Hydrogen

rpm – Revolutions Per Minute

sp. – Single species (unspecified)

spp. – Multiple species

μL – Microlitre

v/v – Volume per Volume

w/v – Weight per Volume

% – Percentage

## 1. INTRODUCTION

The term "probiotic," first introduced in 1965, refers to microorganisms that confer health benefits on the host. Most current definitions centre on the principle that probiotics must be alive when administered and able to confer specific health benefits. The World Health Organization and the Food and Agriculture Organization of the United Nations have specified that, for a microorganism to be recognized as a probiotic, it must belong to one of the following genera: *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Saccharomyces*, or *Pediococcus*, and be a representative of the species most cited in the scientific literature. Furthermore, probiotics, the focus of an intensive search for novel strains, should preferably originate from locally fermented foods. Another probiotic criterion is survival at a pH of 2.0 for 120 minutes or longer, and 0.3% bile concentration or higher. Additionally, the selected strain must adhere to intestinal cells, produce antimicrobial substances, have a safety status, and possess therapeutic properties. Lactic acid bacteria (LAB) comprise a physiologically diverse group of bacteria from various genera, such as *Lactobacillus*, *Streptococcus*, *Lactocaseibacillus*, *Leuconostoc*, *Lactococcus*, *Oenococcus*, and *Enterococcus*, that are characterized by the absence of catalase activity, the inability to metabolise citrate, and the production of lactic acid as the main end-product of carbohydrate fermentation. Among these, the genus *Lactobacillus* is both the most diverse and the most widely used as a starter culture for dairy- and non-dairy fermented foods (Kunchala et al., 2016). Andhra Pradesh and Telangana state regions have many types of fermented foods, including vegetable pickles, especially in the coastal area of Bapatla. Andhra salty, spicy fermented mixed vegetable pickle, well known to housewives in Bapatla town, is prepared by using several locally available vegetables, including Dried bottle gourd, green chilies, carrots, and edible oils. Besides, PROBIOTIC Processed food is selected (Bapatla) City pickles are known to be very famous for their quality, taste and long shelf-life. The study focused on isolating *Lactobacillus* spp. For this reason, the selected district is Bapatla.

## 2. LITERATURE REVIEW

Lactobacillus species constitute one of the main genera of lactic acid bacteria isolated from human foods and environments. In 1965, specific criteria were proposed to determine the probiotic nature of Lactobacillus strains. Certain fermented vegetables contain these lactobacilli, but the presence of Lactobacillus from these foods has yet to be reported in the southern state of India, specifically in Andhra Pradesh or Bapatla. Therefore, a systematic literature review of probiotic Lactobacillus strains isolated from fermented vegetables was conducted to find a theoretical framework that meets the Probiotics 2022 criteria. Furthermore, the evaluation of probiotic characteristics of several Lactobacillus isolates from Andhra pickles was investigated to fill regional and food gaps and provide awareness and future reference. Some studies reported a few microbes from Andhra pickles, but no one reported Lactobacillus species.

## 3. MATERIALS AND METHODS

India has been home to a large variety of pickles, especially in the southern and northeastern regions. Lactic acid bacteria (LABs) are the major functional food components in fermented foods like pickles. LABs not only enhance shelf life and quality but also impart many health benefits to fermented foods. Andhra pickles, being one of the most popular fermented foods, still lack scientific investigations on the isolation and screening of LABs. A special survey was conducted to collect different types of Andhra pickles (Bapatla), which underwent processing and isolation of lactic acid bacteria (LABs) from different types of pickles prepared by traditional methods. Industrial importance for LABs is their conversion and preservation of food components into other useful forms. This conversion occurs during the fermentation of food for shorter or longer periods of time. Most of the LABs show probiotic and other health benefits. The concept and word have become quite popular in recent decades of time and have gained much importance in the scientific community. Probiotic concepts and terminology have become quite popular in recent decades and have gained significant importance in the scientific community and in everyday life. Probiotics are live microorganisms that confer health benefits, mainly in the gastrointestinal tract when taken in adequate amounts. The Lactobacillus genus of LABs is highly emphasized in probiotics. Probiotic LABs get protected in fermented food by encapsulation, and as they are consumed with food, they are effectively delivered to the intestine. Probiotic LABs have been incorporated into pickles. India has been home to a wide variety of pickles, especially in the southern and northeastern regions. Lactic acid bacteria (LABs) are the major functional food

components in fermented foods like pickles. LABs not only enhance shelf life and quality but also impart many health benefits to fermented foods. Andhra pickles, being one of the most popular fermented foods still lacks scientific investigations on the isolation and screening of LABs. A special survey was conducted to collect different types of Andhra pickles (Bapatla), which underwent processing and isolation of lactic acid bacteria (LABs) from different types of pickles prepared by the traditional method. Industrial importance for LABs is their conversion and preservation of food components into other useful forms. This conversion occurs during the fermentation of food for shorter or longer periods of time. Most of the LABs show probiotic and other health benefits. The concept and word have become quite popular in recent decades of time and have gained much importance in the scientific community. The concept and word have become quite popular in recent decades of time and have gained much importance in the scientific community and also in day-to-day life. Probiotics are live micro-organisms that confer health benefits, mainly in the gastrointestinal tract when taken in adequate amounts. The *Lactobacillus* genus of LABs is highly emphasized in probiotics. Probiotic LABs get protected in fermented food by encapsulation, and as they are consumed with food, they are effectively delivered to the intestine. Probiotic LABs have been incorporated into pickles.

### **3.1 Chemicals, Reagents and Culture Media**

All chemicals, reagents, glassware, and microbiological media used in this study were obtained from the laboratories of Bapatla College of Pharmacy, Bapatla, Andhra Pradesh, India. All media were prepared according to standard microbiological procedures and manufacturer guidelines.

### **3.2 Sample Collection**

Five different traditionally prepared homemade pickle samples were collected from separate households in Bapatla, Andhra Pradesh. Samples were transported to the laboratory under hygienic conditions and processed immediately for microbiological analysis.

Fresh homemade curd was used as a positive control for comparison of lactic acid bacteria (LAB) abundance.

### **Preparation of peptone water**

Peptone water is essential for the preparation of the sample.

**Table 1: Composition of peptone water.**

Component	Quantity
Peptone	10 g
Sodium chloride	5 g
Distilled water	Up to 1000 mL
Final pH	7.2 ± 0.2

**Procedure**

Peptone water was prepared by dissolving 10 g of peptone and 5 g of sodium chloride in 1000 mL of distilled water. The pH was adjusted to 7.2 ± 0.2, and the medium was sterilized by autoclaving at 121°C for 15 minutes before use.

**Fig 1: Peptone water.****3.3 Sample Preparation**

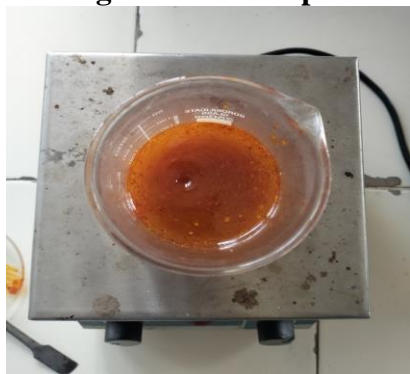
Before sample preparation, the pickles were rendered oil-free by pressing them with sterile filter paper, as shown in Figures 1 and 2. Ten grams of each pickle sample was dissolved in 90 mL of sterile peptone water to obtain a  $10^{-1}$  dilution. Homogenization was carried out using a magnetic stirrer for 30 minutes. The homogenized sample was then used for the inoculation process to ensure uniform microbial distribution. A similar preparation was carried out for the curd sample.



**Fig 2: Pickle sample.**



**Fig 3: Oil separation from pickle.**



**Fig 4: Homogenization of sample solution by magnetic stirrer.**



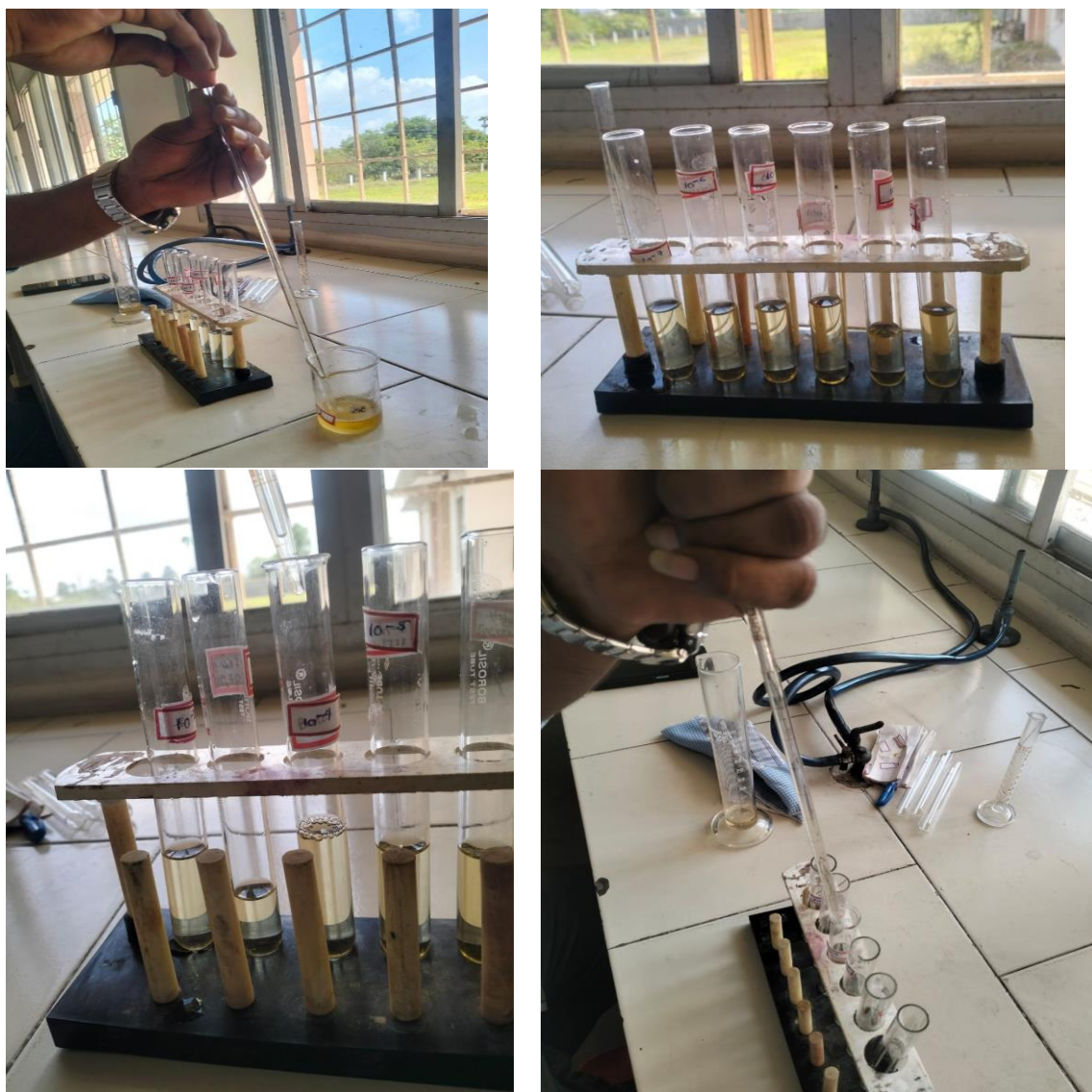
**Fig 5: Homogenous pickle sample.**



**Fig 6: curd sample.**

### 3.4 Serial Dilution

A series of tenfold serial dilutions was prepared from each homogenized sample. From the  $10^{-1}$  dilution, 1 mL was transferred into 9 mL of sterile diluent to obtain the  $10^{-2}$  dilution. This process was repeated sequentially up to  $10^{-7}$  dilution. These dilutions were used for enrichment and microscopic analysis of LAB.



**Fig 7,8,9,10: Serial dilutions of pickle sample.**

### 3.5 Preparation of MRS Broth (1000 mL)

de Man, Rogosa, and Sharpe (MRS) broth was used as a selective enrichment medium for lactic acid bacteria.

#### Composition per Litre

**Table 2: Composition of MRS Broth.**

Component	Quantity (g/L)
Peptone	10.0
Meat extract	8.0
Yeast extract	4.0
Dextrose (Glucose)	20.0
Dipotassium hydrogen phosphate	2.0
Sodium acetate	5.0
Triammonium citrate	2.0

Magnesium sulfate	0.2
Manganese sulfate	0.05
Tween 80	1 mL
water	1000 ml
Final pH	6.2–6.5

### Procedure

1. All components were weighed accurately and dissolved in approximately 900 mL of distilled water.
2. Tween 80 was added and mixed thoroughly.
3. The final volume was adjusted to 1000 mL using distilled water.
4. The pH was checked and maintained between 6.2 and 6.5 by using 0.1 N NaOH
5. The medium was dispensed into culture tubes and sterilised by autoclaving at 121°C for 15 minutes.
6. After cooling, the medium was stored at 2–8°C until use.



Fig 11: Preparation of MRS broth.



Fig 12: Preparation of MRS broth.



Fig 13: Measurement and adjustment of the final PH of MRS broth medium. The medium was adjusted to 6,65 using 0.1N NAOH for the growth of lactic acid bacteria.



**Fig 14: autoclaving MRS Broth medium.**

### 3.6 Isolation and Enrichment of Lactic Acid Bacteria

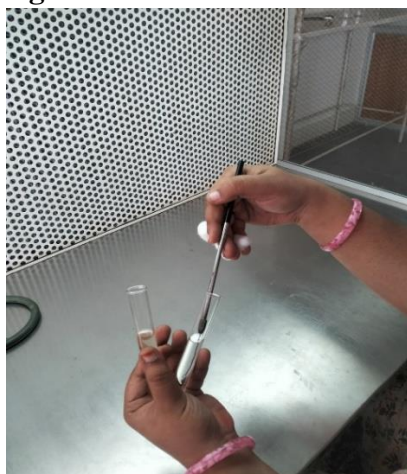
Aliquots from appropriate serial dilutions of each sample were inoculated into sterile MRS broth tubes supplemented with 0.4% (w/v) sodium acetate (MRS-Ac). The inoculated tubes were incubated under anaerobic conditions at 30 °C for up to 72 hours. Favorable for lactic acid bacteria. Subculturing was carried out in freshly prepared MRS broth to enrich the bacterial population.



**Fig. 15: Laminar air flow unit.**



**Fig16: Sterilization of the loop.**



**Fig 17: inoculation of pickle sample in MRS broth media.**



**Fig 18: incubation of inoculated medium.**

### 3.7 Biochemical Characterization of Isolates

#### Catalase Test

A small amount of fresh bacterial culture of the pickle and curd sample was transferred onto a clean glass slide using a sterile loop. One drop of 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was added to the smear. The slide was observed immediately for bubble formation, indicating oxygen release. But no bubbles are formed. The same procedure is applied to the curd sample.

#### Carbohydrate Fermentation Test

Carbohydrate fermentation was assessed using glucose fermentation broth containing phenol red as a pH indicator and a Durham tube for gas detection. The broth was sterilized and inoculated with the test culture under aseptic conditions. Tubes were incubated at 37°C for 24–48 hours. A colour change from red to yellow indicated acid production due to carbohydrate fermentation, while gas accumulation in the Durham tube indicated gas production. The same procedure is applied for curd sample.

#### Gram Staining

A smear of the enriched culture was prepared on a clean glass slide and heat-fixed. The smear was flooded with crystal violet for 1 minute and rinsed with distilled water. Gram's iodine was applied for 1 minute, followed by gentle washing. Decolorization was performed using 95% ethanol for 15–20 seconds, and the slide was rinsed immediately. Safranin was then added as a counterstain for 1 minute, washed, air-dried, and observed under oil immersion (100× objective) using a compound microscope. The same procedure is applied to the curd sample.



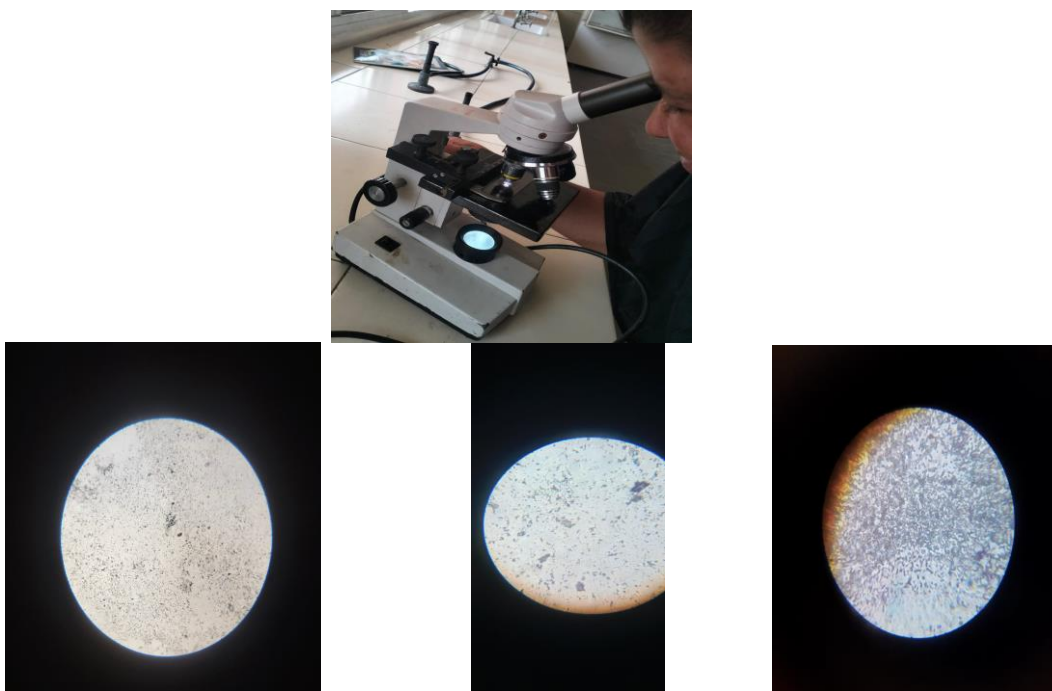


**Fig 19,20,21,22: Gram staining of the pickle sample.**

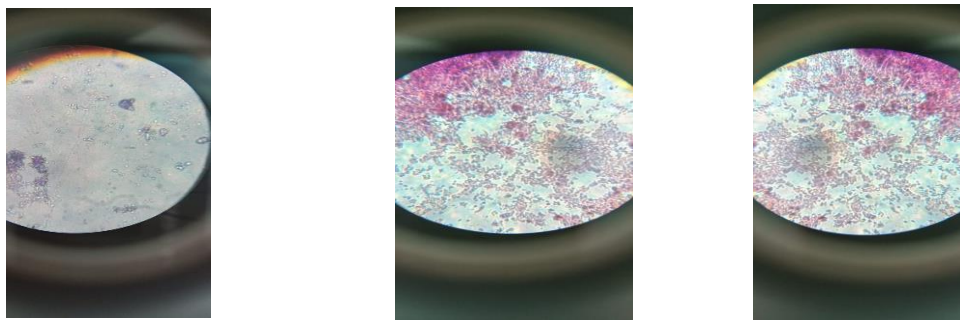
### 3.8 Microscopic Examination

Gram staining was performed on enriched cultures and original samples. Slides were observed under 10 $\times$ , 45 $\times$ , and 100 $\times$  magnification using a compound microscope.

Gram-positive rod-shaped bacteria consistent with lactic acid bacteria morphology were observed. The abundance of LAB in pickle samples was comparatively lower than that observed in curd samples.



**Fig 23,24,25: Microscopic images of gram-stained sample in 10x, 45x, and 100x magnification of the microscope.**



**Fig 26,27,28: Microscopic images of a Gram-stained sample in 10x, 45x, and 100x magnification of the microscope.**

## **4. RESULTS AND DISCUSSION**

### **4.1 Isolation and Enrichment of Lactic Acid Bacteria**

Following enrichment in MRS broth, visible turbidity was observed in all pickle samples as well as in the curd control, indicating the presence of viable microorganisms. However, the intensity of turbidity was noticeably higher in the curd sample compared to the pickle samples.

### **4.2 Biochemical Confirmation of Lactic Acid Bacteria**

The bacterial isolates obtained from pickle and curd samples were subjected to basic biochemical tests for confirmation of lactic acid bacteria (LAB). Gram staining revealed purple-colored, rod-shaped bacteria under oil immersion microscopy, indicating Gram-positive morphology. Catalase testing showed no bubble formation upon addition of hydrogen peroxide, confirming the isolates were catalase-negative.

In the carbohydrate fermentation test, the inoculated glucose broth showed a colour change from red to yellow, indicating acid production. No significant gas accumulation was observed in the Durham tubes. These characteristics are consistent with lactic acid bacteria, particularly members of the genus *Lactobacillus*.

### **4.3 Microscopic Examination**

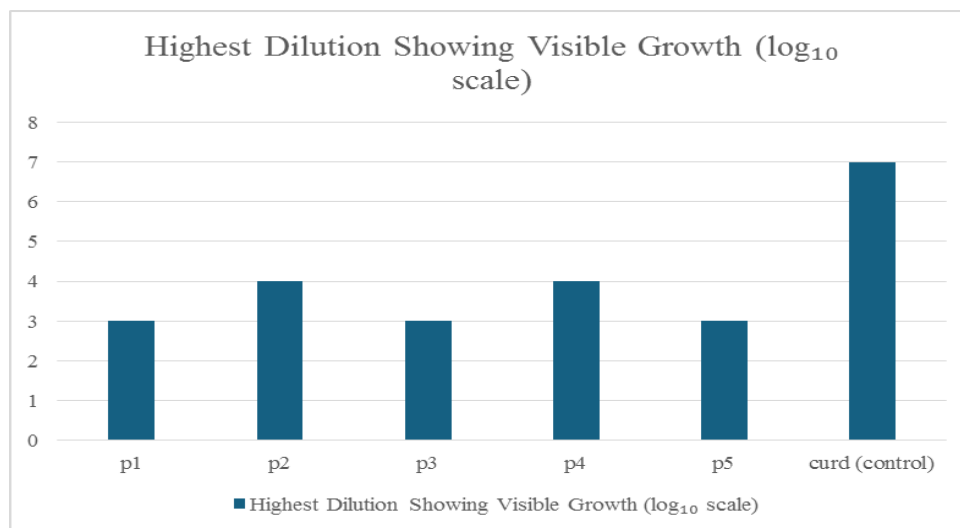
Gram staining of enriched cultures revealed Gram-positive rod-shaped bacteria, characteristic of lactic acid bacteria (LAB). These organisms were observed in both pickle and curd samples. Microscopic fields from curd samples showed a higher density of bacterial cells, whereas pickle samples showed fewer and more scattered cells.

#### 4.4 Semi-Quantitative Estimation of LAB by Serial Dilution

Serial dilution analysis was performed to estimate the relative abundance of lactic acid bacteria (LAB) in pickle samples and curd. Visible growth in MRS broth was observed only up to lower dilution levels in pickle samples, whereas the curd sample showed growth at much higher dilution levels. Pickle samples demonstrated turbidity up to  $10^{-3}$ – $10^{-4}$  dilutions, indicating low to moderate LAB concentrations. In contrast, curd exhibited growth up to  $10^{-7}$  dilution, suggesting a substantially higher LAB load. Since colony-forming unit (CFU) enumeration was not performed, these observations represent a semi-quantitative comparison rather than exact bacterial counts.

**Table 3: Semi-Quantitative Presence of Lactic Acid Bacteria Based on Serial Dilution Growth.**

Sample Code	Source	Highest Dilution Showing Visible Growth	Relative LAB Abundance	Observation
P1	Homemade Pickle 1	Up to $10^{-3}$	Low	Weak turbidity in MRS broth
P2	Homemade Pickle 2	Up to $10^{-4}$	Moderate	Slightly higher turbidity than P1
P3	Homemade Pickle 3	Up to $10^{-3}$	Low	Sparse bacterial presence
P4	Homemade Pickle 4	Up to $10^{-4}$	Moderate	Noticeable but low growth
P5	Homemade Pickle 5	Up to $10^{-3}$	Low	Similar pattern to P1 and P3
C	Curd (Control)	Up to $10^{-7}$	Very High	Dense turbidity and abundant LAB



**Figure 29s: Semi-quantitative comparison of lactic acid bacteria (LAB) abundance in pickle samples and curd based on the highest dilution level, showing visible growth in MRS broth.**

Table 1 and Figure 1 together illustrate the marked difference in LAB abundance between pickle samples and curd. While all pickle samples contained detectable LAB, their growth was limited to lower dilution levels. The curd sample consistently showed growth at higher dilutions, confirming it as a richer source of viable lactic acid bacteria.

#### 4.5 Comparative Observation

The difference in the highest dilution levels showing growth clearly indicates that curd contains a substantially higher concentration of LAB compared to pickle samples. Although traditional pickles harboured viable lactic acid bacteria, their population density was considerably lower than that of dairy-derived fermented food.

#### CONCLUSION

The present investigation confirmed that traditionally prepared Andhra pickles from Bapatla harbour viable lactic acid bacteria with characteristics consistent with *Lactobacillus* species. All pickle samples demonstrated the presence of Gram-positive, rod-shaped, catalase-negative, glucose-fermenting bacteria, which are key traits of probiotic LAB. Although the bacterial load in pickles was lower than in curd, the consistent detection of LAB across different samples indicates that these fermented vegetable products can act as natural, non-dairy sources of beneficial microbes. The semi-quantitative dilution analysis highlighted a clear difference in microbial abundance between dairy and vegetable fermentations, but it also emphasized that traditional pickling practices support the survival of functional LAB. This opens opportunities for exploring Andhra pickles as a source of novel probiotic strains adapted to plant-based environments. However, the study was limited to morphological and basic biochemical characterization. Advanced molecular identification, safety assessment, and in-vitro probiotic property evaluation (acid tolerance, bile tolerance, antimicrobial activity, and adhesion ability) are necessary before considering therapeutic or commercial applications. Overall, this work provides foundational scientific evidence that supports the probiotic relevance of traditional Andhra pickles and encourages further research into region-specific fermented foods as valuable contributors to functional nutrition and gut health.

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