Received Date: March 2025 Revised: April 2025 Accepted: June 2025

Comparative study of MRS Agar and GYP- Calcium carbonate Agar for Isolation of Lactic Acid Bacteria from indigenous yogurt (*Ju Ju Dhau)* of Bhaktapur, Nepal

Arabinda Kumar Singh, Jarina Joshi, Roji Karki, Om Pant and Pramod Poudel*

Central Department of Biotechnology, Kirtipur, Tribhuvan University, Nepal

*Corresponding Author: pramod.poudel@cdbt.tu.edu.np

Doi : https://doi.org/10.3126/ppj.v5i1.85809

Abstract

Lactic acid bacteria (LAB) are important for food fermentation like yogurt and probiotic use. The isolation and enumeration of Lactic Acid Bacteria (LAB) are fundamental steps in studying the microbial diversity and probiotic potential of traditional fermented Ju Ju Dhau dairy products. LAB play a vital role in the fermentation process by producing lactic acid, which contributes to flavor, texture, and preservation in Ju Ju Dhau *of Bhaktapur, Nepal which is king of curd. The present study aimed to investigate the efficiency of MRS(de Man, Rogosa, Sharpe) agar and GYP-Ca agar (Glucose-Yeast-Peptone with $CaCO_3$) in isolating LAB from fermented food samples. Results showed that while MRS agar offers broad-spectrum isolation, GYP-Ca agar provides selective and cost effective isolation of strong acid producers through visual detection by halo formation. Samples of indigenous yogurt were serially diluted and cultured on both media under anaerobic conditions at 37°c. The colonies showed convex, creamy colour less than 1mm of LAB morphology. The Gram staining reaction conformed that all Lab are gram positive and catalase negative. Results indicated that both media supported the growth of diverse LAB populations, including species of Lactococcus,* Lactobacillus, Leuconostoc, *and* Pediococcus. *However, GYP-Ca agar demonstrated higher selectivity by suppressing non-LAB contaminants and producing clear zones around colonies due to calcium carbonate hydrolyze support to identification. MRS agar, while effective, showed slightly lower colony differentiation and higher background flora.*

Keywords: Lactic acid bacteria, MRS agar, GYP-Ca agar, culture media, isolation, Catalase

1. Introduction

Yogurt is a fermented milk product obtained by lactic acid fermentation through the action of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, which shall be viable and abundant in the final product." (Codex Alimentarius, 2003). The term yogurt

defined by Tamime & Robinson "Yoghurt is a coagulated milk product resulting from the fermentation of milk-specific bacteria that produce lactic acid, giving rise to a characteristic texture and flavor" (Tamime & Robinson, 2007).

Primary by-products are those compounds which are directly produced during lactose fermentation by LAB. The most abundant by-product of yogurt fermentation, lactic acid reduces the pH of milk, leading to casein coagulation and inhibiting the growth of pathogens: LAB can produce acetic acid and volatile compounds that contribute to the characteristic flavor and aroma of yogurt (*Leroy & De Vuyst, 2004).* Exopolysaccharides (EPS): Some LAB strains produce EPS during fermentation, improving yogurt texture, stability, and acting as dietary fiber with prebiotic potential *(Ruas-Madiedo & de los Reyes-Gavilán, 2005).* Carbon Dioxide Heterofermentative LAB may produce CO_2, which contributes to a lighter texture in certain fermented dairy products *(Zhou et al., 2020).*

Secondary compounds are produced by microbial metabolism of milk proteins, lipids, or carbohydrates and have notable health-promoting properties: Proteolytic activity by LAB releases peptides from casein with antihypertensive, antioxidant, immunomodulatory, and antimicrobial effects.*(Korhonen & Pihlanto, 2006).* LAB can produce bacteriocins small antimicrobial proteins that inhibit pathogens such as *Listeria monocytogenes* and *Salmonella spp.(Gänzle, 2015)* . Short-Chain Fatty Acids (SCFAs) may be metabolized into SCFAs like acetate and butyrate which improve colon health and modulate immune responses.*(Rivière et al., 2016).* Probiotic strains in yogurt can enhance immune system activity by stimulating production of cytokines and interferons (e.g., IFN-γ), which play antiviral roles in the gut mucosa.*(Matsuzaki et al., 2000)*

Lactic acid bacteria (LAB) are gram positive, non-spore, acid-tolerant, and facultatively anaerobic microorganisms. Among fermented foods, yogurt and its traditional variants hold a prominent place due to their health benefits, sensory qualities, and probiotic potential. Indigenous yogurt varieties, such as *Ju Ju Dhau*—a delicacy from Bhaktapur, Nepal. The harbor a rich microbial consortium, including LAB strains with unique genetic and functional properties. Understanding the LAB community within these traditional dairy products is not only essential for preserving local fermentation practices but also critical for bioprospecting novel strains with technological and therapeutic value.

The term probiotic is a relatively new word meaning “for life” and it is currently used to name bacteria associated with beneficial effects for humans and animals. The observation of the positive role played by some selected bacteria is attributed to Eli Metchnikoff, the Russian born Nobel Prize recipient working at the Pasteur Institute at the beginning of the last century, who suggested that "The dependence of the intestinal microbes on the food

makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Metchnikoff, 1907).

MRS agar can sometimes lack selectivity, leading to the concurrent growth of non-LAB microorganisms, especially when samples contain diverse microbial flora. Additionally, the high cost of some ingredients in MRS media may be a limiting factor in resource-constrained settings or during large-scale screening.

Given the increasing interest in exploring microbial biodiversity in traditional fermented foods, there is a need to assess the comparative effectiveness of these media in isolating LAB. Several studies have shown varying recovery rates of LAB strains depending on the type of culture media employed. However, comprehensive studies that directly compare the performance of MRS agar and GYP-Ca agar in isolating LAB from traditional fermented dairy products like *Ju Ju Dhau* remain limited.

The microbial ecosystem of *Ju Ju Dhau* is believed to be influenced by traditional preparation techniques, raw fresh milk and materials, local environmental factors, and the absence of commercial starter cultures. These factors contribute to the development of a unique microbial community, dominated primarily by LAB. Accurate identification and isolation of these indigenous LAB strains can aid in the development of autochthonous starter cultures, enhance the safety and shelf-life of traditional yogurt, and support the functional food industry by introducing novel probiotics with strain-specific health benefits.

This study aims to evaluate and compare the efficiency of MRS agar and GYP-Ca agar in isolating LAB from *Ju Ju Dhau*, focusing on parameters such as colony count, morphological differentiation, selectivity, and ease of identification.

The outcomes of this study are expected to contribute to the standardization of LAB isolation protocols, promote the use of local fermented products as sources of probiotic bacteria, and support ongoing efforts in food biotechnology and probiotic research. In the present investigation, lactic acid bacteria were the isolated and identified from curd in order to use them as starter for developing probiotic ju ju dhau yogurt.

2. Materials and Methods

2.1 Sample Collection

Fresh samples of *Ju Ju Dhau* were collected aseptically from Bhaktapur, Nepal. Samples were transported to the Central Department of Biotechnology, TU, Kirtipur, Nepal laboratory in cold chain (ice box) and processed within 6 hours of collection to ensure microbial viability.

2.2. Preparation of Media

Two culture media GYP-Ca agar and MRS agar were used for the isolation of LAB:

- MRS agar: Standard formulation with glucose, peptone, yeast extract, and sodium acetate.

  GYP-Ca agar: Glucose (2%), yeast extract (0.5%), peptone (1%), and $CaCO_3$ (0.5%) for halo detection of acid production.

Table 1. Comparision of the media composition of commercial MRS and inhouse GYP media

Ingredients g/L	**MRS**	**GYP**
Polipeptone	10	
Peptone		5
Meat extract	10	
Yeast extract	5	10
D-Glucose/ D-Fructose	20	10
Tween 80	1.08	0.5
Dipotassium phosphate	2	
Sodium acetate	5	2
Ammonium citrate	2	
Magnesium sulphate	0.2	0.2
Manganese sulphate	0.05	0.01
Ferrous sulphate		0.01
Sodium chloride		0.01
Sodium azide		0.05
Cyclohiximide		0.05
Bacteriological agar	15	12
Calcium carbonate		5
pH at 25 ^{o}C	5.7 ± 0.1	
Reference	HiMedia	

The Ingredients (g/L) are required to prepare the MRS agar: Dextrose (Glucose) 20.000 gm/ltr , Proteose peptone: 10.000 g/L ,Yeast extract: 5.000 g/L, Sodium acetate 5.000 g/L, 2-Phenylethyl alcohol 3.000 g/L, Ammonium citrate 2.000 g/L, Dipotassium hydrogen phosphate 2.000 g/L,Magnesium sulphate 0.100 g/L,Manganese sulphate 0.050 g/L,Bromocresol green 0.040 g/L, Cycloheximide 0.004 g/L, Agar 15. g/L (HiMedia Laboratories,2025).

The ingredient(g/L) are required to prepare the GYP-Ca agar.

2.3. Isolation of lactic acid bacteria

After, weighing and homogenization of each sample, 1:10 dilution was subsequently made using sterile normal saline (0.85%) followed by making a 10 fold serial dilution. The 0.1 mL

from each dilution was then sub-cultured aseptically into MRS (deMan Rogosa and Sharpe) agar (Guessas and Kihal 2004), and GYP-Ca agar using pour plate technique, all plates were then incubated at 37˚C for 24-48 hours in anaerobic condition to provide an optimal environmental for growing *LAB.*

Only the Gram positive rod and cocci shape isolates were then purified by streak plating using the MRS medium. After several subcultures, finally the single colony of LAB was isolated by observing their colony morphology and Gram staining, catalase and motility test. The culture was kept in MRS agar, and GYP-Ca slant and stored at 4 °C for long term storage (Hawaz, 2014). Colonies were counted using a digital colony counter. LAB colonies were identified based on typical colony morphology (small, round, white/cream-colored) .Clear zones on GYP-Ca agar due to $CaCO_3$ hydrolyze

2.4. Identification of lactic acid bacteria

The isolated colony was identified using Gram staining, biochemical tests (Chris *et al*., 2006).

2.4.1. Biochemical identification

Gram staining:

For each isolated strain, the normal protocol for the Gram staining test was followed (Mannan et al., 2017). A smear of a single colony was taken on a clean glass slide, which was dried by air, followed by fixation with heat. Following heat fixation of the smear, it was flooded with a crystal violet solution for one minute, then rinsed with water. Subsequently, Gram's iodine mordant solution was applied and left on the smear. Decolorization was achieved using 95 % ethyl alcohol for10-20 sec, followed by rinsing with water. Counterstaining was performed by applying Safranin for 60 sec, followed by another rinse with water, before examination under 100X magnification.

Catalase test

A fresh culture was prepared on a clean glass slide, onto which a drop of 3 % hydrogen peroxide was applied and thoroughly mixed. Catalase positivity was shown by the production of bubbles, and catalase negativity by the absence of bubbles or froth (Mannan et al., 2017)

Strains with Gram positive, catalase negative and no motility were further used for identification (Sharpe, 1979). The LAB isolates were characterized based on its phenotypic characters such as cellular morphology using Gram staining and biochemistry (catalase test). The isolates that confirmed as Gram-positive and catalase negative.

3. Results and Discussion

3.1. Isolation of lactic acid bacteria

Bacteria were isolated from MRS agar GYP-ca agar media at 37°C under anaerobic conditions for 48 hours in incubator. The isolated colonies were then screened for further identification. The isolates CFU were given below in fig.1 and fig 2 of petriplate. The table contained 9 CFU total suspected in MRS agar and GPY –Ca contained 5 CFU isolates of LAB suspected per plate.

MRS agar	total 9 CFU suspected
GYP-Ca Agar	5 CFU LAB

3.2. Identification of lactic acid bacteria species

The complete findings of morphological and biochemical characterization of the isolates are presented in below.

Both the isolates gave purple/violet color with staining showing Gram positive nature (Fig. 3). Because Gram positive cell wall contain thick layer of peptidoglycan with numerous teichoic acid cross linking which resists the decolorization, thus remaining purple in color. The isolates coming from MRS and GYP agar plates were cocci except few rods with long and rounded ends. They appeared mostly as a chain of 3-4 cells or single or in pairs. Colony count and morpholgy study in GYP_ca Agar and MRS Agar. The table contained as per morphology, motility and gram staining results. The identified LAB colony in MRS agar were five and in GYP-Ca were five.

MRS agar	Total 9 CFU suspected and 5 LAB present
GYP-ca Agar	5 CFU LAB

Figure 1. Culture in GYP-ca agar media

From this petriplate, total 5 cfu were isolated from clear zone area .

Figure 3. Culture in MRS agar

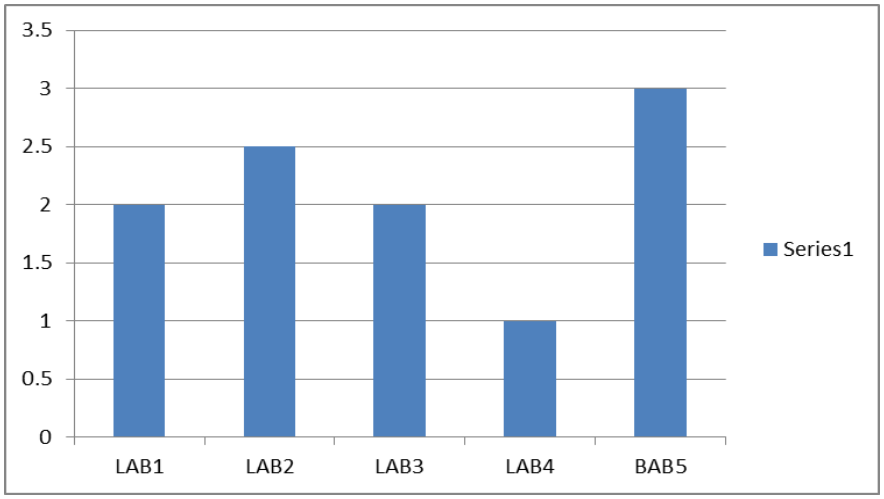
Figure 2. Gram Staining at 100X microscope

No bubble was observed indicating that the isolated bacteria are catalase negative and could not mediate the decomposition of H_2O_2 to produce oxygen. It is well known that *Lactobacillus* is catalase negative. Similar results are reported by Mithun *et al.,* (2015).

The isolation and characterization of LAB from traditional fermented products *Ju Ju Dhau* provide valuable insights into microbial diversity and food biotechnology. The results of this study demonstrated that both MRS and GYP-Ca agars were effective in recovering LAB from yogurt samples, with notable differences in colony clarity, selectivity, and visual screening clear zone.

GYP-Ca agar yielded consistently similar colony counts as well as MRS agar across in similar dilutions. GYP-Ca agar contains Sodium azide which blocks the electron transport chain in Gram-negative bacteria and in MRS agar already contained sodium acetate as a selective agent This is likely due to its ability to suppress non-acid-producing microbes via pH and $CaCO_3$ indicators. The calcium carbonate will be hydrolyzed by lactic acis which makes clear zone. The clear halo formation around LAB colonies on GYP-Ca allowed for rapid identification of acid producers, a feature not evident on MRS agar. These findings are consistent with previous studies, such as the lactic acid produced by LAB, forming clear zones around colonies. And suitable for isolating LAB from yogurt, cheese, and other fermented products studied by Holzapfel & Schillinger (1992)

Sample isolated from GYP agar	Clear Zone size formed in mm
LAB1	2 mm
LAB2	2.5 mm
LAB3	2 mm
LAB4	1 mm
LAB5	3 mm



The branded MRS agar, while nutrient-rich and traditionally preferred for growing fastidious LAB, showed slightly lower selectivity and higher background microbial growth. Nonetheless, it remains a robust medium for maintaining and subculturing LAB due to its comprehensive nutritional profile.

The use of GYP-Ca agar is especially alternative form of MRS agar relevant for developing countries, as it is inexpensive, easy to prepare, and does not require complex ingredients . These attributes make it an ideal alternative for routine LAB screening, especially where large-scale isolate collection is needed.

4. Conclusion

In conclusion, while both MRS and GYP-Ca agars are valuable for LAB isolation, GYP-Ca agar is a cost-effective, selective, and visually efficient method for identifying acid-producing lactic acid bacteria in traditional dairy products. GYP-Ca agar yielded consistently similar colony counts as well as MRS agar across all dilutions.. GYP-Ca agar contains Sodium azide which blocks the electron transport chain in Gram-negative bacteria and in MRS agar already contains sodium acetate as a selective agent *(De Man, Rogosa, & Sharpe, 1960)."*Although some problem in selection of LAB and GYP-Ca Agar also contain

sodium acetate which inhibits the growth of spoilage bacteria. The calcium carbonate was hydrolyzed by lactic acid which made a clear zone near to a colony of Lab.

The observed differences align with prior studies (Springer, 2018) that recommend $CaCO_3$-supplemented media for selective isolation of high-performing LAB which was verified by our result. Using both in parallel enhances strain discovery for fermentation and probiotic development.

5. Acknowledgement

We acknowledge Central Department of Biotechnology Kirtipur, Nepal for providing us chemicals and reagents as well as laboratory facilities to complete our work on comparision and analysis of the growth of lactic acid bacteria from indigenous yogurt (*Ju Ju Dhau)* of Bhaktpur , Nepal.

6. References

1. Codex Alimentarius. (2003). *Codex standard for fermented milks (CODEX STAN 243-2003)*.FAO/WHO
2. Tamime, A. Y., & Robinson, R. K. (2007). *Yoghurt: Science and technology* (3rd ed.). Woodhead Publishing
3. Leroy, F., & De Vuyst, L. (2004). Lactic acid bacteria as functional starter cultures for the food fermentation industry. *Trends in Food Science & Technology*, 15(2), 67–78. https://doi.org/10.1016/j.tifs.2003.09.004
4. Ruas-Madiedo, P., & de los Reyes-Gavilán, C. G. (2005). Invited review: Methods for the screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. *Journal of Dairy Science*, 88(3), 843–856.
5. Zhou, K., Fu, M., Ren, Y., & Jiang, Y. (2020). Lactic acid bacteria: Important roles in food fermentation and potential application in food industry. *Foods*, 9(6), 722. https://doi.org/10.3390/foods9060722
6. Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality. *International Dairy Journal*, 16(9), 945–960
7. Gänzle, M. G. (2015). Lactic metabolism revisited: Metabolism of lactic acid bacteria in food fermentations and food spoilage. *Current Opinion in Food Science*, 2, 1–6. https://doi.org/10.1016/j.cofs.2014.12.005
8. Rivière, A., Selak, M., Lantin, D., Leroy, F., & De Vuyst, L. (2016). Bifidobacterial colonization and modulation of the gut microbiota by prebiotics: A review. *Nutrients*, 8(3), 98. https://doi.org/10.3390/nu8030098
9. Matsuzaki, T., Yamazaki, R., Hashimoto, S., Yokokura, T., & Saeki, K. (2000). The effect of lactic acid bacteria on the production of cytokines and activation of NK cells in mice. *Microbiology and Immunology*, 44(10), 729–734. https://doi.org/10.1111/j.1348-0421.2000.tb02576.
10. Metchnikoff E. *The prolongation of life: Optimistic studies*. Trans. Mitchell PC. New York: G. P. Putnam's Sons; 1907.
11. HiMedia Laboratories. (2025) *MRS Agar (M641) technical data sheet*. Mumbai, India: HiMedia Laboratories Pvt. Ltd. Retrieved from https://www.himedialabs.com

12. Guessas, B., & Kihal, M. (2004). Characterization bacteria isolated from Algerian arid zone raw goats' milk. *African Journal of Biotechnology, 3*(6), 339–342.
13. 12. Hawaz, E. (2014). Isolation and identification of probiotic lactic acid bacteria from curd and in vitro evaluation of its growth inhibition activities against pathogenic bacteria. *African Journal of Microbiology Research, 8*(13), 1419–1425. https://doi.org/10.5897/AJMR2013.6426
14. 13..Chris, A., Mamo, G., & Assefa, F. (2006). Isolation and characterization of lactic acid bacteria from traditional fermented milk of Ethiopia. *International Journal of Food Microbiology, 107*(2), 111–116. https://doi.org/10.1016/j.ijfoodmicro.2005.08.018
15. 14.Mannan, S. J., Saeed, F., Ahmad, A., & Ahmed, W. (2017). Characterization of lactic acid bacteria isolated from fermented foods for probiotic properties. *Journal of Food Safety, 37*(3), e12328. https://doi.org/10.1111/jfs.12328
16. 15.Mithun, S., Prabhakar, P. K., & Hegde, S. (2015). Characterization of lactic acid bacteria from fermented foods and their potential as probiotics. *International Journal of Advanced Research in Biological Sciences, 2*(12), 154–160.*
17. K.H. Schleifer, W. Ludwig, Phylogeny of lactic acid bacteria, *FEMS Microbiology Reviews* 16 (1995) 449–460. https://doi.org/10.1016/0168-6445(94)00082-M..
18. Holzapfel, W. H., & Schillinger, U. (1992). Production and use of GYP-Ca medium for the isolation of lactic acid bacteria. *International Journal of Food Microbiology*, 16(1), 45–57. https://doi.org/10.1016/0168-1605(92)90045-L
19. De Man, J. C., Rogosa, M., & Sharpe, M. E. (1960). A medium for the cultivation of lactobacilli. *Journal of Applied Bacteriology*, 23(1), 130–135. https://doi.org/10.1111/j.1365-2672.1960.tb00188.
20. Springer, Improved selective isolation of lactic acid bacteria using calcium carbonate–supplemented media, *Journal of Microbiological Methods*, 147 (2018) 45–52. https://doi.org/10.1016/j.jmmb.2018.03.005.