

## Isolation Of Probiotic Strains From Gahat Dal (Horse Gram): “A Comprehensive Study On Characterization, Functional Properties, And Probiotic Potential For Nutraceutical Applications”

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### Abstract

**Introduction:** The present study concerns the isolation and characterization of Lactic acid bacillus sp. (LAB), isolated from fresh pulse horse gram/ Kulthi dal/ Gahat dal. Lactic acid bacteria sp. (LAB) are an important source of bioactive metabolites and enzymes. This research aimed to isolate and identify potential probiotic strains from Gahat Dal (Horse Gram) and comprehensively characterize their functional properties for potential nutraceutical applications.

**Methodology:** Gram staining and biochemical analyses were conducted to assess the microbial characteristics and identify the isolates. The strains were further evaluated for their probiotic potential, including resistance to gastric acidity, bile tolerance, and adhesion to intestinal epithelial cells. The isolated strain was evaluated for isolation and purification on MRS plates, Gram staining, Catalase test, and morphological examination under microscope, Biochemical test such as IMVIC, starch hydrolysis, carbohydrate fermentation. Antimicrobial activity of cell-free supernatants from presumptive LAB isolates was evaluated by centrifugation against Gram-positive, Gram-negative, LAB, mold, and yeast strains.

**Results:** Twenty-one colonies isolated and characterized from the culture plate and all were gram positive, catalase negative, and six isolates showed the most potent lactic acid bacillus strain characteristics. Overall, LAB sp. isolated from cereal showed potential technological applications and should be further evaluated.

**Keywords:** Lactic acid bacillus, Crop, Protein, Minerals, Vitamins, Gram staining, Catalase test.

### Introduction

#### Lactic Acid Bacillus (LAB) sp.

Despite the many kinds of lactic acid bacteria (LAB), only a fraction of them is used in food fermentation. LAB is usually isolated from dairy products, but fresh cereals recently have become very important because they also act as reservoirs of LAB. As a result, lab strains must adapt to atypical sources such as cereal crop ecosystems, which differ among species and strains. This has posed challenges to the ability of LAB strains to adapt to atypical sources such as cereal crop ecosystems, which also differ significantly among species and strains. Thus, fresh cereals contain wide range of LAB sp. Antimicrobial, enzymatic and adhesive activities were determined for LAB sp. isolates collected from fresh cereal sources. Horse gram is an underexploited legume crop grown in different adverse climatic conditions (Contente *et al.*, 2024). It occupies a significant niche in human diet comprising high protein content, minerals and vitamins. Rich in non-nutritive bioactive substances it is known for its nutritional value that reduces risk of various diseases. Therefore, horse gramme has great potential as a therapeutic agent for the treatment of kidney stones, urinary tract infection, piles, common colds, throat infection or fever and other conditions was widely acknowledged by folk, alternative, and traditional medicine (Liang *et al.*, 2024).

The assessment of LAB as biocontrol agents has gained popularity as a sustainable method of food protection (Medeiros *et al.*, 2024) proposed screening starter cultures among the autochthonous microbiota of vegetables and fruits because these strains are more likely to provide extended shelf life while maintaining the desired nutritional, rheological, and sensory characteristics. The antimicrobial activity against naturally occurring spoilage microorganisms on lettuce and apples as well as pathogens that were inoculated was also observed by these investigators. The GRAS category of organisms includes Gram-positive bacteria that are distinguished by their non-pathogenic nature, absence of endospore formation, catalase negativity, and Gram-positive status (Sherpa *et al.*, 2024). Lactic acid bacteria have been reported to be present in a wide range of sources, including the gastrointestinal and vaginal tracts, fermented food products (fermented milk, cereals, fruits, and vegetables), and raw biotic matrices (grains, crops, plant surfaces, and silages).

According to (Sornplang *et al.*, 2016), the main use of LAB is in food fermentation, where it further reduces food spoilage and inhibits pathogenic microbes through their antimicrobial activities, such as the production of bacteriocin. Despite these similarities, the production of fermented foods and drinks varies greatly across the globe, with notable differences in substrates, products, and microbe types. Consequently, a number of studies have been conducted to distinguish and

isolate LAB from diverse and unique natural food matrices (Fontana *et al.*, 2013). LAB are used as probiotics due to their numerous health advantages, which include lowering cholesterol, preventing cancer, acting as an antidiabetic, modifying immunity, defending against infections, lowering the risk of disease, and improving the microbiota in the gastrointestinal tract. Because LAB strains can decrease mycotoxin and increase bioavailability, most of the strains that are used as probiotics today are also used as biocontrol agents. These strains are classified under the *Lactobacillus* and *Bifidobacterium* genera (Zhang *et al.*, 2024). Probiotic microbes are selected based on several factors, such as their impact on the sensory qualities of products, their technological qualities (cell growth, stability, and viability in raw food substrates), their functional characteristics (cell auto-aggregation, cell-surface hydrophobicity, bacteriocin production, immunomodulation, antimicrobial activity, and safety), and their functional characteristics. However, in vivo studies and clinical trials are required to validate these features (Martínez *et al.*, 2024).

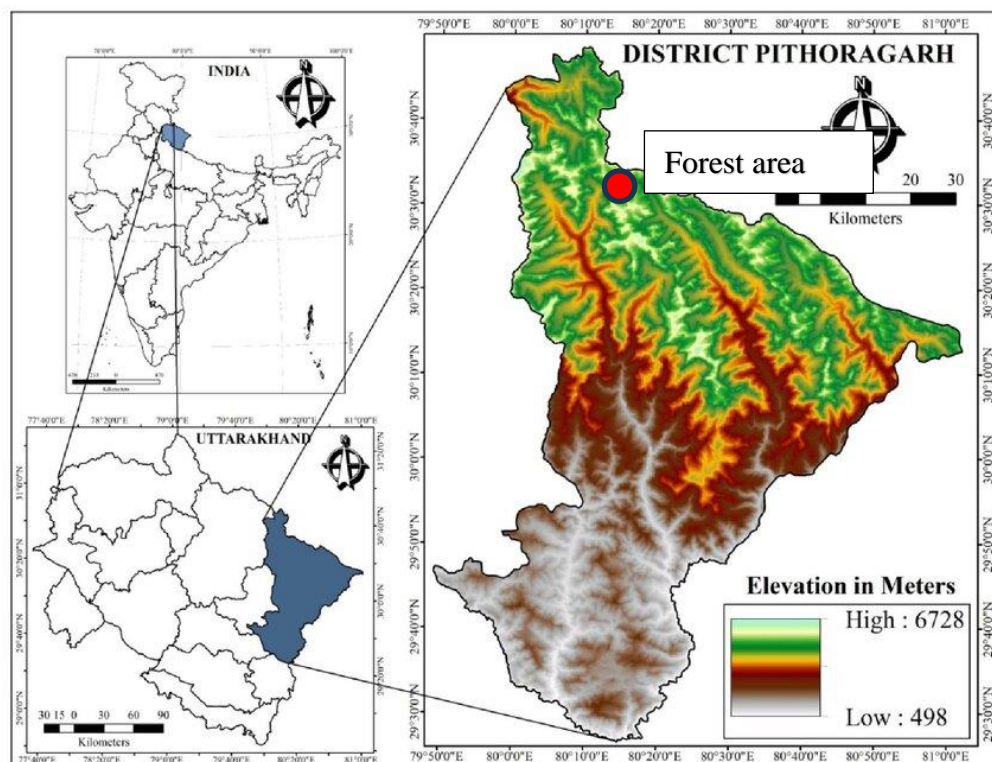
### **Gahat Dal (*Macrotyloma Uniflorum* Variety)**

An underappreciated pulse crop that grows well in a range of unfavorable climates is horse gramme. Other names for Horse Gramme (Gahat) include Hurli, Moth beans, Kulith, kulthi, and Madras Gramme. This disc-shaped lentil comes from the Kumaon region of Uttarakhand. The legume known as horse gramme, or *Macrotyloma uniflorum*, is indigenous to tropical southern Asia. It is also known as Madras gramme, gahat, hurali, and kulthi bean. Its distinct flavour and texture make it a popular ingredient in a wide variety of cuisines (Chin *et al.*, 2024). It is an important part of human nutrition and is a rich source of vitamins, minerals, and protein. Apart from its nutritional worth, the existence of non-nutritive elements has been linked to a decreased likelihood of several illnesses. These bioactive substances have significant effects on metabolism and/or physiology. Fibre, phytic acid, phenol acid, and enzyme/proteinase inhibitors are a few examples of these (Hernández *et al.*, 2024).



**Fig 1 shows Horse Gram Pulse sample**

Horse gramme has been recognized by folk, alternative, and traditional medicine as a potential treatment for various ailments, including fever, common colds, kidney stones, urinary tract infections, piles, and throat infections. Since the nutraceutical concept was introduced and health consciousness has grown, there has been a greater demand for nutraceutical and functional foods (Mashayekh *et al.*, 2024). The isolation and use of potential antioxidants from legumes, such as horse gramme, has recently increased because they may lower the risk of intestinal illnesses, diabetes, coronary heart disease, dental caries, and other ailments. According to (Nenciarini *et al.*, 2024), the horse gramme's potential as a source of compounds and food is the reason for the growing interest in nutraceutical food. This review supports this claim with recent scientific findings.



**Fig 2: Map with marked Horsegramme pulse from Kumaon region of Uttarakhand (Courtesy: [https://in.images.search.yahoo.com/search/images;\\_ylt=AwrKDpvDC7pld48RZxu9HAX.;\\_ylu=c2VjA3NIYXJjaARzbGsDYnV0dG9u;\\_ylc=X1MDMjExNDcyMzAwNQRfcgMyBGZyA21jYWZlZQRmcjIDcDpzLHY6a](https://in.images.search.yahoo.com/search/images;_ylt=AwrKDpvDC7pld48RZxu9HAX.;_ylu=c2VjA3NIYXJjaARzbGsDYnV0dG9u;_ylc=X1MDMjExNDcyMzAwNQRfcgMyBGZyA21jYWZlZQRmcjIDcDpzLHY6a))**

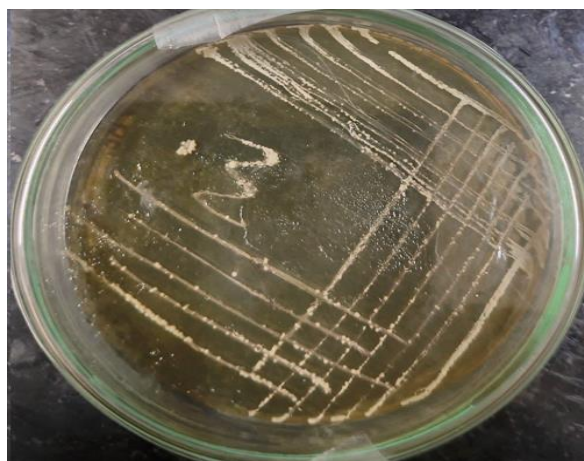
## Materials and methodology

### 1. Sample collection

Horse gram occupies an important place in human nutrition and has rich source of protein, minerals, and vitamins. It is an underutilized pulse crop grown in wide range of adverse climatic conditions especially in India and Himalayas. Kulthi ki dal, is a disk-shaped lentil which was collected from Pithoragarh, Kumaon Mandal of Uttarakhand in a sterile plastic bag.

### 2. Isolation of Lactic acid bacteria:

To isolate the lactic acid bacteria unconventional source such as Gahat dal were collected from Pithoragarh region. The sample was placed in sterile bag and transported to the research laboratory. 1 gm of Gahat dal sample measured aseptically and grind in with help of mortar-pastel with mixing distil water, 1 ml of Gahat solution (uniform paste) was suspended in test tube containing 9 ml of distilled water and subjected to 10-fold dilution; 0.1 ml of diluted sample was inoculated in MRS agar plates under anaerobic condition and incubated at 37 °C for 24 to 48 hours. The different morphological colonies were isolated and pure cultures were maintained in MRS agar slant at 4° temperature.



**Fig 3: Bacterial isolate from Horse gram**



### 3. Morphological characterization of isolates

For Morphological characterization, Cultural identification and microscopic observation was performed. Cultural characterization of LAB isolates was done on different agar plates. Cultural characteristics i.e., colony colour, margin, form, surface, elevation, and optical density were recorded. Microscopic observation was done by Gram's staining.

- **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% H<sub>2</sub>O<sub>2</sub> 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).

### 4. 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 Lactobacillus strain 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.
- **Methyl Red test:** Glucose phosphate broth was inoculated with LAB 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 LAB 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 LAB 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 lactic acid 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).
- **Urease test:** LAB isolates were tested for the urease test in urease broth. Inoculated a loopful culture of isolates on urease broth and incubated for 24 hours at 30 °C. Development of pink colour was a positive test for urease and development of yellow colour showed negative urease test (Aneja, 2003).
- **Sugar fermentation test:** Sugar fermentation broth with phenol red indicator was prepared using different sugar i.e., arabinose, fructose, galactose, glucose, lactose, maltose, mannitol, mannose, ribose and sucrose. After sterilization, the broth was inoculated with LAB isolates and incubated for 24 hrs. at 30 °C. Positive result was yellow after incubation and no colour change /remains reddish was negative fermentation test (Aneja, 2003)

### Probiotic attributes

Lactobacillus strains are commonly used as probiotics due to their beneficial effects on human health. Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host. Lactobacillus strains, being lactic acid bacteria, are known for their ability to ferment sugars and produce lactic acid, which contributes to their probiotic attributes (Sato *et al.*, 2021).

- **Low pH resistance:** The resistance of strains to low pH conditions and then incubated at 37° C. Resistance was assessed by counting bacterial colonies on MRS agar plate. MRS broth at pH 6.5 and the probiotic *Lactobacillus casei* were used as controls (Sato *et al.*, 2021)
- Isolates with survival rates expressed as log cycle reduction from 0.6 at pH 3 were selected for further experiments.

$$\text{Survival rate} = \frac{\log \text{CFUN1}}{\log \text{CFUN0}}$$

N1 represents the total viable strains count after 3- 5 hours at pH 3 and N0 represents the total viable strains at 0 hours.

- **Resistance to bile salts:** Cell viability was assisted by counting on MRS agar plate at 0 and 6 hrs after incubating for 37°C. MRS broth samples without bile salts and *L. casei* were used as controls (Sato *et al.*, 2021). Isolates with survival rates 0.6 expressed as:

$$\text{Survival rate} = \frac{\log \text{CFUN1}}{\log \text{CFUN0}}$$

The isolates with higher survival rate at 0.6 were selected.

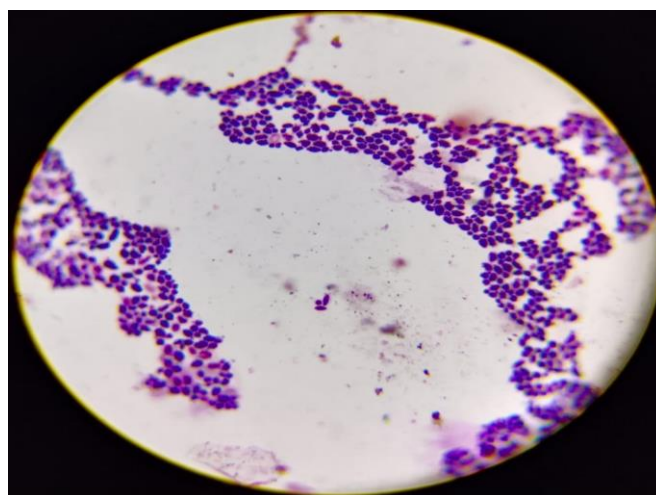
#### • Antimicrobial activity against enteropathogenic microbes

All strains were tested for antimicrobial activity against the pathogen *E. coli* and *Salmonella* isolated from the sewage sample of canteens at Sharda University. Brain Heart infusion culture medium (0.7%) of bacteriologic agar inoculated with 100 µl of the pathogenic indicator culture. Uninoculated MRS agar plates were used as a negative control and the probiotic strain *L. casei* used as a positive control. The zones of inhibition were measured (Sato *et al.*, 2021). The correspondence strains were (-) without inhibition.

### Results:

#### 1. Gram Staining:

- The Gram staining results revealed a predominance of Gram-positive strains among the isolated bacteria from Gahat Dal.



**Fig 4: Gram positive bacteria**

#### 2. Biochemical Analysis:

- Catalase and oxidase tests indicated the presence of catalase-negative and oxidase-negative strains, characteristic of lactic acid bacteria.
- Negative indole production and specific sugar fermentation patterns further supported the identification of potential probiotic strains.

#### 3. Functional Properties:

- The isolated strains demonstrated robust acid tolerance and bile resistance, essential for survival in the gastrointestinal environment.
- Significant adhesion to intestinal epithelial cells suggested a strong potential for colonization and persistence in the gut.

Biochemical Test	Lactic Acid Bacillus
Gram Staining	Mostly Gram positive (coccoid shape)
Catalase	Negative
Indole Red	Negative
Methyl Red	Negative
Voges-Proskauer	Negative
Citrate	Negative
Urease	Negative
Galactose	Positive
Fructose	Positive

Lactose	Positive
Glucose	Positive
Sucrose	Positive
Maltose	Positive

**Table 1 shows biochemical characterization results of the isolated Lactic Acid bacteria.**

#### Probiotic attributes results

- **Resistance to Low pH:** The viability of most assayed strains (95%) largely unchanged after 5 hours at pH 3.
- **Resistance to Bile salts:** In the present study 18 isolated strains that had a survival rate of more than 0.8 at pH 2.0 were tested and 8 of them remained stable and 10 strains were reduced for their ability to survive to the presence bile salts (0.3%) during 5 hours.
- **Antimicrobial activity against pathogen:** The results of the antimicrobial activity for 21 bacteria isolated from Horsegramme cereal against the pathogen *E. coli* and *Salmonella* by disc diffusion method. The 5 LAB strains showed different levels of inhibitory actions against the assayed pathogens.

ISOLATE	<i>E. coli</i>	<i>Salmonella</i>
LAB STRAIN	+++	+++

**Table 2 shows inhibition of pathogen growth by LAB strains from Horse Gram**

- (+) shows maximum inhibition zone

#### Conclusion

In the present study, a total number of 21 Lactic acid bacterial isolates were isolated from selected unconventional sources. The isolates were identified as *Lactobacillus* sp. by morphological, biochemical, and molecular characterization. The presence of Lactic Acid Bacteria in unconventional sources is proved as an alternative source of LAB and these LAB are being selected as probiotics for lactose intolerant people. Thus, further study is required to evaluate the probiotic potential and antimicrobial activity of isolated LAB for use as probiotics with different applications. The combination of Gram staining and biochemical analyses successfully identified potential probiotic strains from Gahat Dal. These strains exhibited favorable functional properties, including acid and bile tolerance, as well as strong adhesion to intestinal cells. The comprehensive characterization of these isolates positions them as promising candidates for nutraceutical applications, with potential benefits for human health. Further studies are warranted to explore their specific probiotic mechanisms and validate their suitability for incorporation into functional food products.

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