



Microbial Profiling of Ethnic Fermented Beverages of Himachal Pradesh Using Phenotypic and Molecular Approaches

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KEYWORDS

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Indigenous fermentation,
Probiotic potential.

ABSTRACT:

Traditional fermented beverages of the Himalayan region represent an important repository of indigenous knowledge, microbial diversity, and functional nutrition. The present study investigates the bacterial diversity associated with selected ethnic fermented beverages of Upper Himachal Pradesh, India, with emphasis on their molecular identification and potential health relevance. Samples of Angoori, Chaas, Sattu, Chol, and Brandy were collected from different districts and subjected to microbial isolation using serial dilution and spread plate techniques on skim milk agar. Selected bacterial isolates were characterized based on colony morphology, Gram staining, and standard biochemical assays, including catalase, oxidase, methyl red, Voges–Proskauer, and hydrogen sulphide production. Isolates exhibiting desirable biochemical traits and negative H₂S production were further analyzed through 16S rRNA gene amplification and sequencing. Phylogenetic analysis revealed that the dominant isolates belonged to the genus *Bacillus*, including *Bacillus anthracis*, *Bacillus arachidis*, *Bacillus velezensis*, *Bacillus amyloliquefaciens*, and closely related *Bacillus* spp. The findings highlight the microbial richness of these traditional beverages and underline the limitations of 16S rRNA sequencing in resolving closely related species. Overall, this study provides scientific validation of the microbial composition of Himalayan fermented beverages and supports their potential as functional foods with probiotic and health-promoting attributes, while emphasizing the need to conserve traditional fermentation practices and associated microbial resources.

1. Introduction

Fermented beverages have long been an integral part of human culture and tradition, offering not only a distinctive array of flavors but also potential health benefits due to their complex microbial communities¹. In Himachal Pradesh, a state located in the northern part of India, ethnic fermented beverages hold deep cultural and social significance. These beverages, including varieties like *Chhaang*, *Sidhu*, and *Raksi*, are consumed during festive occasions, rituals, and social gatherings, reflecting the region's rich cultural heritage². The fermentation of these beverages primarily involves microorganisms such as yeasts, bacteria, and molds, which play a crucial role in determining their unique characteristics, including flavor, aroma, texture, and nutritional properties³. The tradition of producing

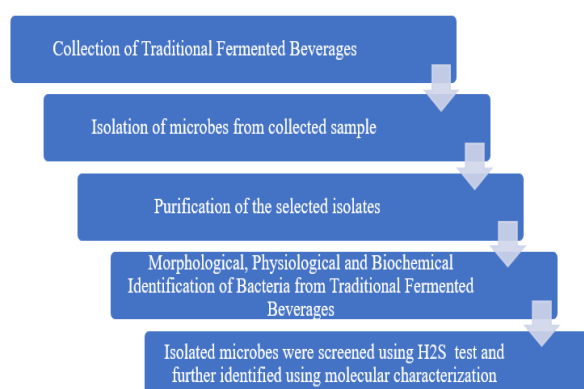
fermented beverages has been an integral part of human civilization for thousands of years, long before the scientific understanding of microbes and fermentation⁴. In India, the practice dates back to pre-Vedic times, with references in texts such as the Ramayana. Tribal communities across the country have preserved unique fermentation techniques, with over 350 types of ethnic beverages documented. These drinks are often prepared using locally available cereals, fruits, and indigenous starter cultures, holding great cultural and nutritional significance⁵. Himachal Pradesh, particularly the tribal regions of Lahaul, Spiti, Kinnaur, and Kullu, is known for its distinctive fermented beverages such as sura, chhaang, lugri, angoori, chulli, and daru. These drinks are deeply woven into the social, religious, and daily life of the local communities and are often consumed during



festivals, marriages, and religious ceremonies⁶. Traditional methods using natural fermentation and ingredients like rice, barley, jaggery, apples, and wild apricots are still widely practiced. Despite their importance, these beverages have received limited scientific attention⁷. The continued preparation and consumption of these ethnic drinks reflect the close connection between food, culture, and heritage in the Himalayan region, emphasizing the value of preserving this traditional knowledge for future generations⁸. The primary aim of this study is to profile the microbial diversity of ethnic fermented beverages from Himachal Pradesh using both phenotypic and molecular methods⁹. By combining these approaches, the study seeks to provide a comprehensive understanding of the microorganisms involved in fermentation, their functional roles, and their potential health benefits¹⁰. This research will not only contribute to the scientific understanding of traditional fermentation processes but also pave the way for the sustainable utilization and improvement of these beverages, ensuring their continued relevance in both cultural and commercial contexts¹¹. Furthermore, it will highlight the potential of these indigenous microbial communities for applications in food science, biotechnology, and medicine.

2. Materials and Methods

Workflow of the Research Methodology



The Traditional Fermented Beverages sample used for isolation and Molecular Characterization was collected from 7 Traditional Fermented Beverages (Chaas and Sattu from Kullu District, Lugri from Lahaul Spiti District, Chol and Brandy from Shimla District, Angoori from Kinnaur District, Chulli from Chamba District of Upper Himachal Pradesh, India). Sampling was

conducted in February & March 2025. The sample was stored in glass sterile bottles.

Isolation of bacteria from Traditional Fermented Beverages Samples

A microorganism was isolated from a Traditional Fermented Beverages sample by serial dilution and the spread plate method using skim milk agar¹². The bacterial isolates were screened by using a tenfold serial dilution method and were spread onto skim-milk agar medium plates. These spread Skim-Milk plates were incubated for 24-48 hours at 30°C for the growth of bacteria.

Colony morphology and pigment production

Colony morphology (form, elevation, margin, shape & surface) and the production of pigment were checked on skim milk agar¹³.

Physiological and Biochemical characterization of selected isolates

Isolates were scanned by morphological and biochemical. These characterizations of isolates were done microscopically through Gram's staining. These isolates were additionally assessed for catalase, oxidase, indole, methyl red, and Voges-Proskauer tests¹⁴ and Hydrogen Sulphide.

Hydrogen Sulphide Test

The Hydrogen Sulphide (H₂S) test assesses a microbe's capacity to release hydrogen sulphide during growth. On media like bismuth sulphite agar, H₂S production causes darkening of colonies. It is valuable for identifying and selecting strains with low H₂S output, helping reduce undesirable odors in fermented foods and probiotics.

Qualitative analysis and sequencing

The results of the PCRs samples of the 16S rRNA region were subjected to 1.5% agarose gel with 1.0% TBE (Tris-borate-EDTA) buffer. Purification of the products was then done using a commercially available purification kit and sequencing was done using the BigDye Terminator Cycle Sequencing Ready Reaction Kit on an Applied Biosystems ABI3500 DNA Sequencer¹⁵.

3. Results and Discussion

Collection of Traditional Fermented Beverages sample



Traditional Fermented Beverages samples were collected from 7 samples across 5 districts. In Kullu District, Sattu and Chaas; Lahaul Spiti District, Lugri; Shimla District, Chol; and Kinnaur District, Angoori and Brandy; and Chamba District,

Chulli, are shown in Figure 2. And samples were collected in the morning in a sterile container/bottle and stored in the laboratory refrigerator.

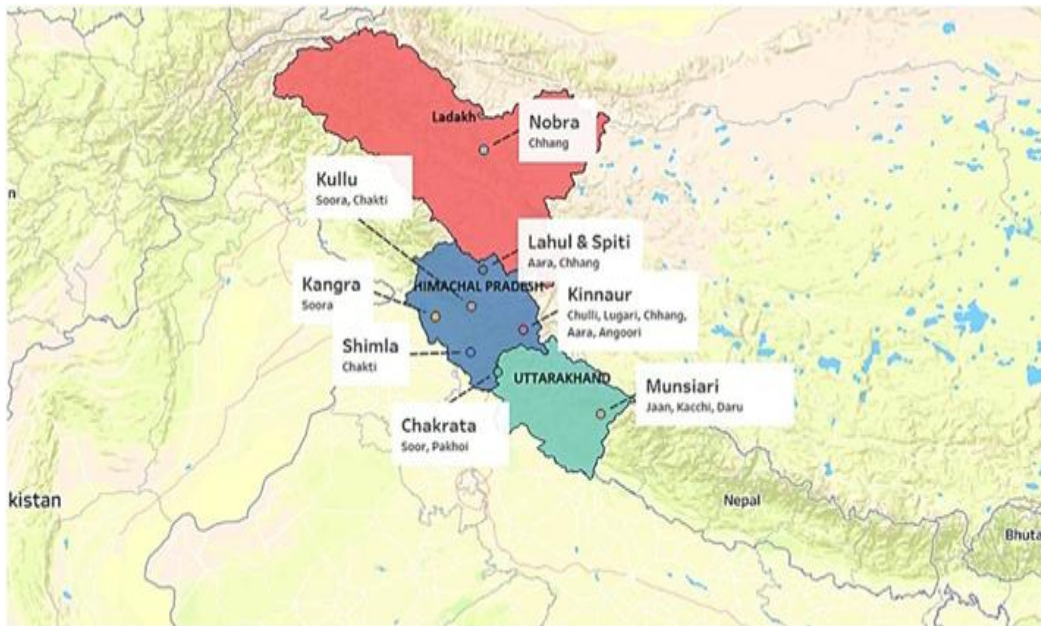


Fig. 1. Location map of the study area

Source: Tomar, S., Pant, K., Sharma, P., Sinha, S., & Mitra, D. (2023). Unravelling the hidden ethnic fermented treasure of the Himalayas-A review on the

traditionally fermented beverages of the Northwest Indian Himalayan Region. Food Chemistry Advances, 2, 10025 ¹⁶.

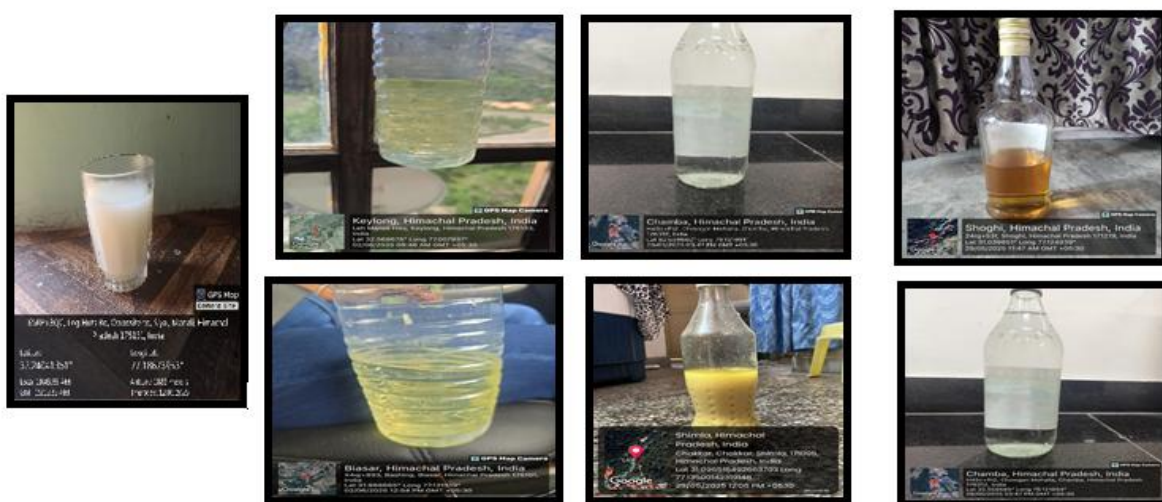


Fig 2: Collection of Traditional Fermented Beverages Samples from Kullu (Chaas and Sattu), Lahaul Spiti (Lugri), Shimla (Chol and Brandy), Kinnaur (Angoori), Chamba (Chulli) H.P.



We have selected 5 samples of Traditional Fermented Beverages for isolation and further identification i.e. Angoori from Kinnaur, Chaas and Sattu from Kullu, Chol and Brandy from Shimla on the bases of Hydrogen Sulphide Test.

Isolation of bacteria from Traditional Fermented Beverages Sample

Sample 1: Angoori Sample from Kinnaur District

Microorganisms were isolated from the Angoori sample by serial dilution and the spread plate method using skim

milk agar at 37°C for 24 to 48 hours. In Kinnaur District, number of colonies in the following dilution factor 10⁻², 10⁻⁸ are as 45 and 13 respectively, presented in Fig.3 and table 2. i.e. Gram's staining presented in fig.5 and table 1 pigmentation, form, elevation, margin, shape and gram-reaction were noted down and presented in table 2. One isolate (A1) was screened through biochemical tests showing in table 2, H₂S Test presented in fig.6 and table 2. And we select this isolate on the bases of H₂S Test i.e. A1 for molecular identification and phylogenetic tree presented in fig.26

Table 1: Morphological characterization of selected isolates

Sr. No.	Isolates	Colony Shape	Color Pigmentation	Colony Elevation	Colony Margin
1.	A ₁	Round / Punctiform	White/ Creamish	Flat	Entire

Table 2: Biochemical Characterization of selected isolates from Angoori Sample

Sr. No.	Isolates	Gram Reaction	Catalase Test	Oxidase Test	Indole Test	MR Test	VP Test	Hydrogen Sulphide
1.	A ₁	Positive	Positive	Negative	Negative	Positive	Negative	Negative

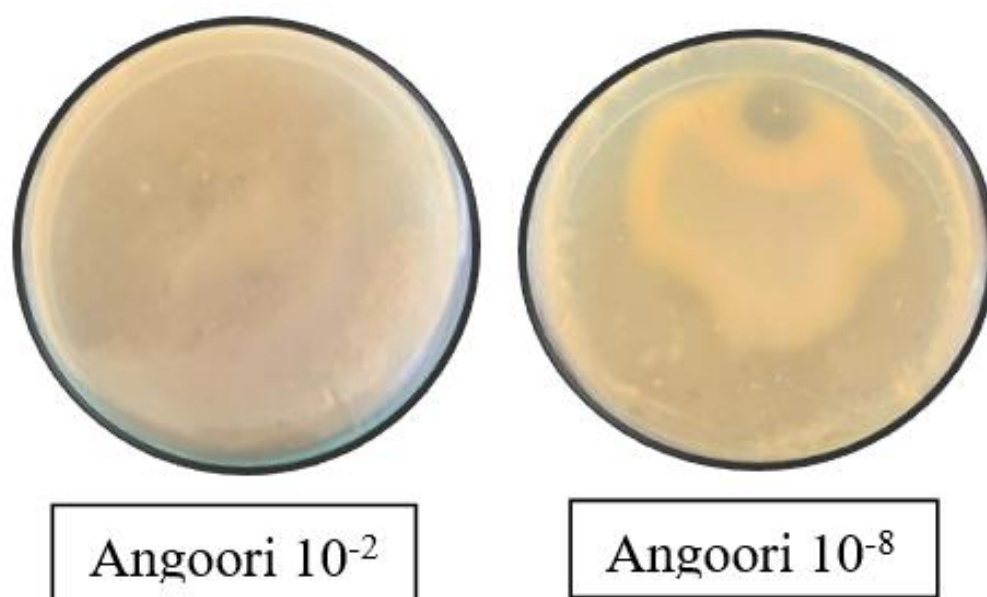


Fig.3: Total viable count of bacteria isolates from Angoori Sample on skim milk agar

A₁-10²

Fig.4: Selected purified isolates

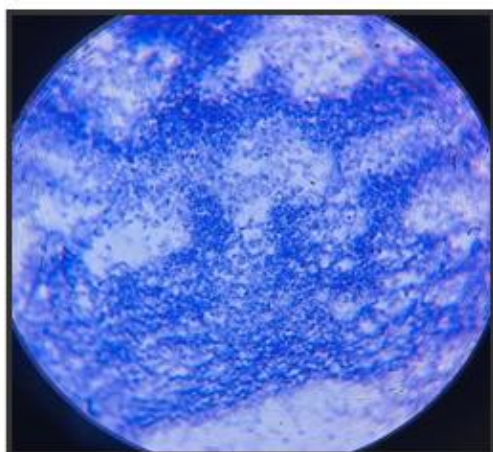
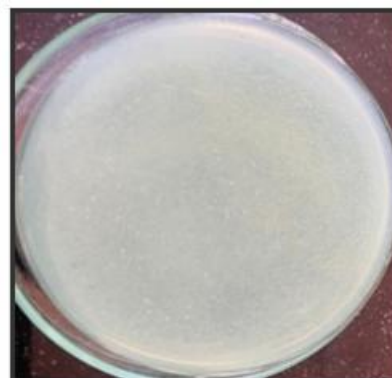


Fig.5: Gram stain of A1-10-2. This showing purple colour bacteria and positive reaction for Gram's staining.

Fig.6: This showing Hydrogen Sulphide (H₂S) A1-10-2 Negative.**Sample2: Chaas Sample from Kullu District.**

The Chaas sample was subjected to microbial isolation using skim milk agar, the serial dilution and spread plate technique, and the plates were incubated at 37 °C for 24-48 hours. In Kullu District, number of colonies in the following dilution factor 10⁻⁶, 10⁻⁸ are as 300 and 189, respectively presented in Fig.7. i.e. gram's staining presented in fig.9 and fig.10, and table3 represents pigmentation, form, elevation, margin, shape and gram-reaction were noted down and presented in table 4. Two isolates (C₁&C₂) were screened through biochemical tests shown in table 4, H₂S Test presented in fig.11 and table 4. And we further select one isolate on the basis of H₂S Test i.e.C₁ for molecular identification and phylogenetic tree presented in fig.27

Table 3: Morphological characterization of selected isolates

Sr. No.	Isolates	Colony Shape	Color Pigmentation	Colony Elevation	Colony Margin
1.	C ₁	Circular	White/ Creamish	Flat	Entire
2.	C ₂	Circular	White/ Creamish	Flat/ Raised	Entire

Table 4: Biochemical Characterization of selected isolates from Chaas Sample

Sr. No.	Isolates	Gram Reaction	Catalase Test	Oxidase Test	Indol Test	MR Test	VP Test	Hydrogen Sulphide
1.	C ₁	Positive	Positive	Negative	Negative	Positive	Negative	Negative
2.	C ₂	Positive	Positive	Negative	Negative	Negative	Negative	Negative

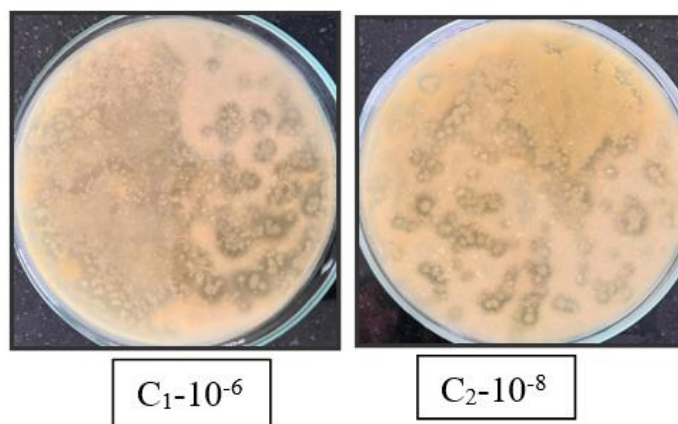


Fig.7: Total viable count of bacteria isolates from Chaas Sample on skim milk agar

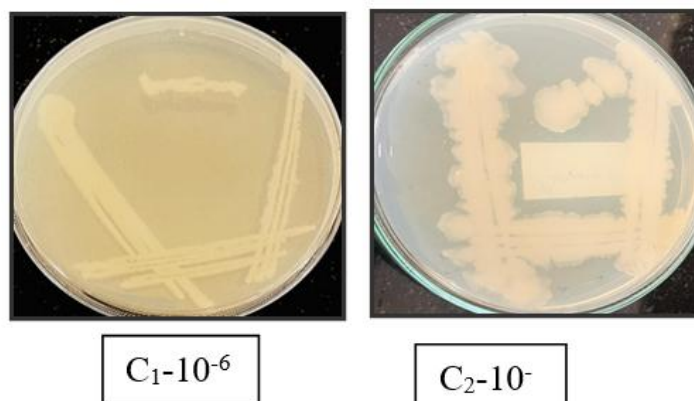


Fig.8: Selected purified isolates



Fig.9: Gram stain of C_1-10^{-6} . This showing purple colour bacteria with rod shape and positive reaction for Gram's staining.

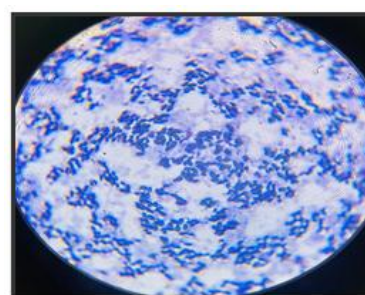


Fig.10: Gram stain of C_2-10^{-8} . This showing purple colour bacteria with rod shape and positive reaction for Gram's staining.

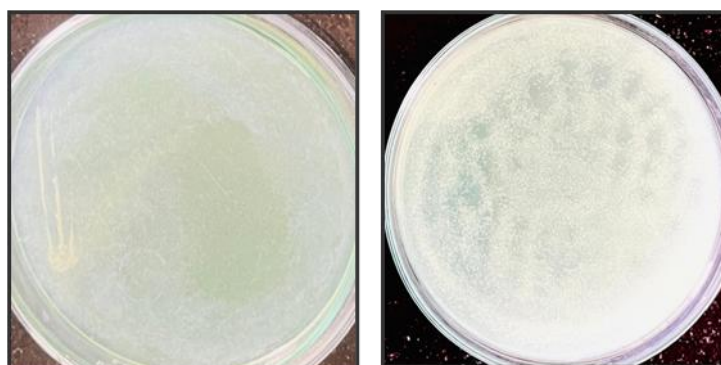


Fig.11: This showing Hydrogen Sulphide (H₂S)_{C₁-10⁻⁶} Negative and _{C₂-10⁻⁸}Negative.

Sample 3: Chol Sample from Shimla District

Microorganisms were isolated from the Chol sample by serial dilution and the spread plate method using skim milk agar at 37°C for 24 to 48 hours. In Shimla District, number of colonies in the following dilution factor 10⁻², 10⁻⁴, 10⁻⁸ are as 300,48 and 42 respectively, presented in Fig.12. i.e. Gram's staining presented in fig.14 and

fig.15, and table 5 represents pigmentation, form, elevation, margin, shape, and gram-reaction were noted down and presented in table 6. Two isolates (CH₂&CH₃) were screened through biochemical tests shown in table 6, H₂S Test presented in fig.16 and table 6. And we further select one isolate on the basis of H₂S Test i.e. CH₂ for molecular identification and phylogenetic tree presented in fig.28

Table 5: Morphological characterization of selected isolates

Sr. No.	Isolates	Colony Shape	Color Pigmentation	Colony Elevation	Colony Margin
1.	CH ₂	Circular/ puntiform	White	Flat	Entire
2.	CH ₃	Rhizoid/circular	White	Flat	Entire

Table 6: Biochemical Characterization of selected isolates from Sattu Sample

Sr. No.	Isolates	Gram Reaction	Catalase Test	Oxidase Test	Indole Test	MR Test	VP Test	Hydrogen Sulphide
1.	CH ₂	Positive	Positive	Negative	Negative	Positive	Negative	Negative
2.	CH ₃	Positive	Positive	Negative	Negative	Positive	Negative	Negative

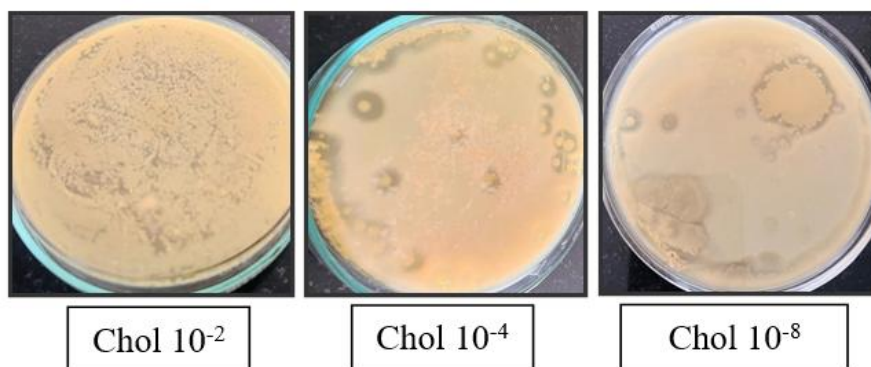


Fig 12: Total viable count of bacteria isolates from Chol Sample on skim milk agar

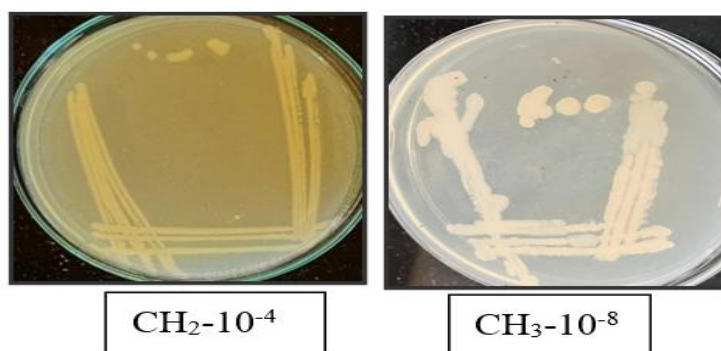


Fig 13: Selected purified isolates

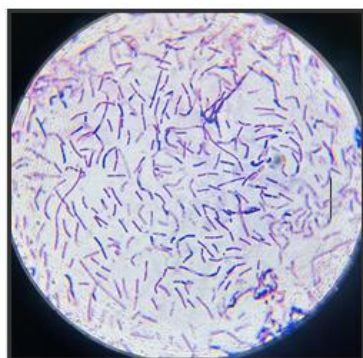


Fig. 14: Gram stain of CH₂-10⁻⁴. This showing purple colour bacteria with Bacillus Chain Form and positive reaction for Gram's staining.

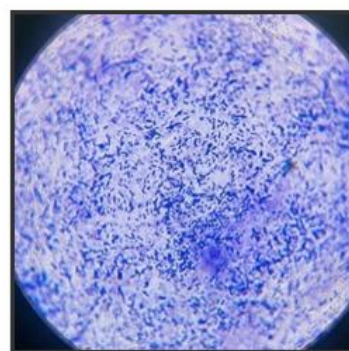


Fig.15: Gram stain of CH₃-10⁻⁸. This showing purple colour bacteria with Bacillus Form and positive reaction for Gram's staining.

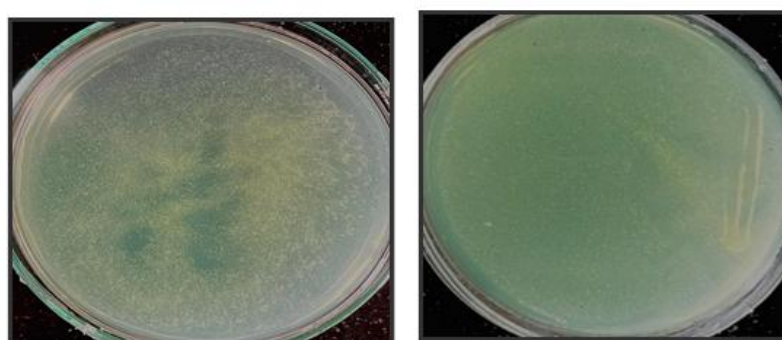


Fig.16: This showing Hydrogen Sulphide (H₂S) CH₂-10⁻⁴ Negative and CH₃-10⁻⁸ Negative.

Sample 4: Brandy Sample from Shimla District

Microorganisms were isolated from the Brandy sample by serial dilution and the spread plate method using skim milk agar at 37°C for 24 to 48 hours. In Shimla District, the number of colonies in the following dilution factors 10⁻², 10⁻⁴ are 10 and 4, respectively, as presented in Fig.14. i.e. gram's staining presented in fig.19 and table

7 represents pigmentation, form, elevation, margin, shape and gram-reaction were noted down and presented in table 8. One isolate (B₂) was screened through biochemical tests shown in table 8, H₂S Test presented in fig.20 and table 8. And we further select this isolate on the bases of H₂S Test i.e. B₂ for molecular identification and phylogenetic tree presented in fig.29



Table 7: Morphological characterization of selected isolates

Sr. No.	Isolates	Colony Shape	Color Pigmentation	Colony Elevation	Colony Margin
1.	B ₂	Round	White/ Creamish	Raised / Convex	Entire

Table 8: Biochemical Characterization of selected isolates from Brandy Sample

Sr. No.	Isolates	Gram Reaction	Catalase Test	Oxidase Test	Indole Test	MR Test	VP Test	Hydrogen Sulphide
1.	B ₂	Positive	Positive	Negative	Negative	Positive	Negative	Negative



Fig.17: Total viable count of bacteria isolates from Brandy Sample on skim milk agar



B₂-10⁻⁴

Fig.18: Selected purified isolates

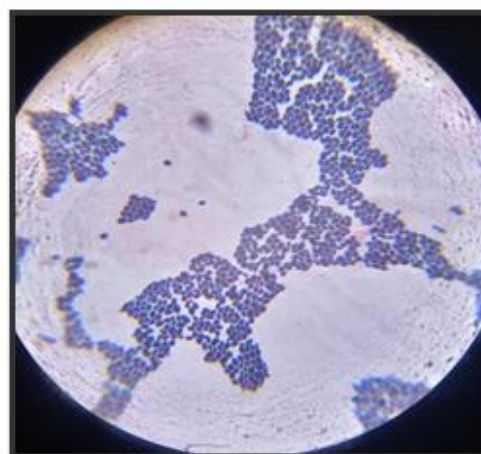


Fig.19: Gram stain of B₂-10⁻⁴. This showing purple colour bacteria with Bacillus Form and positive reaction for Gram's staining.

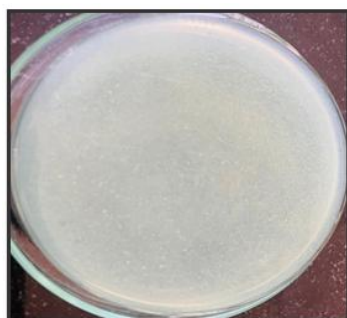


Fig.20: This showing Hydrogen Sulphide (H₂S) B₂-10⁻⁴

Sample 5: Sattu Sample from Kullu District.

Sattu sample was inoculated with microorganisms by serial dilution and spread plate technique on skim milk agar at 37°C for 24-48 hours. In Kullu District, number of colonies in the following dilution factor 10⁻⁶, 10⁻⁸ are as 11 and 6, respectively presented in Fig.17. i.e. Staining of Gram in fig. 23 and fig. 24, table 9, which was presented by Gram, showed pigmentation, form, elevation, margin, shape, and Gram reaction, which were recorded and appear in table 4. Two isolates (S₁&S₂) were screened through biochemical tests shown in table 10, H₂S Test presented in fig.25and table 10. And we further select one isolate based on the H₂S Test, i.e., S₂, for molecular identification and the phylogenetic tree presented in Fig. 30¹⁷.

Table 9: Morphological characterization of selected isolates

Sr. No.	Isolates	Colony Shape	Color Pigmentation	Colony Elevation	Colony Margin
1.	S ₁	Circular/Punctiform	White/ Creamish	Flat/ Raised	Entire
2.	S ₂	Circular	White/ Creamish	Flat	Entire

Table 10: Biochemical Characterization of selected isolates from Sattu Sample

Sr.No.	Isolates	Gram Reaction	Catalase Test	Oxidase Test	Indol Test	MRTTest	VP Test	Hydrogen Sulphide
1.	S ₁	Positive	Positive	Negative	Negative	Positive	Negative	Negative
2.	S ₂	Positive	Positive	Negative	Negative	Positive	Negative	Negative

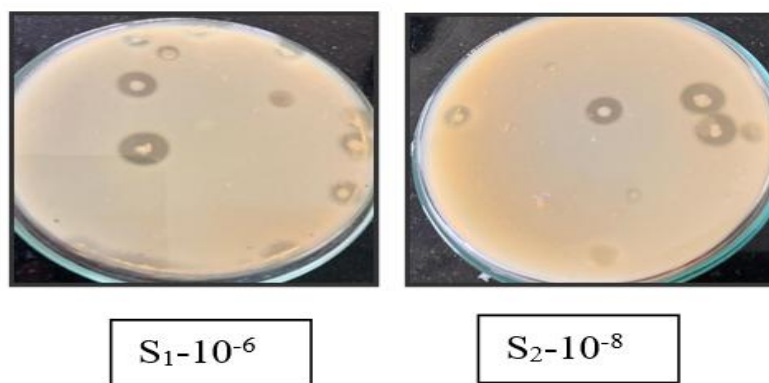


Fig.21: Total viable count of bacteria isolates from Sattu Sample on skim milk agar

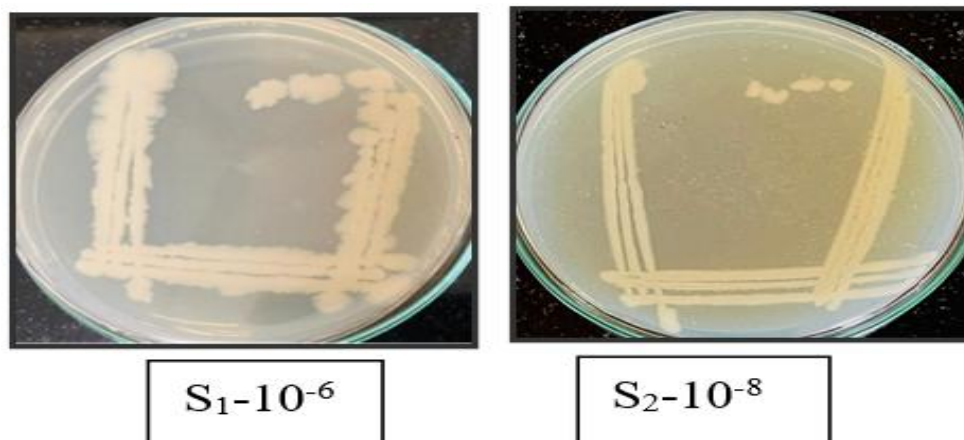


Fig.22: Selected purified isolates

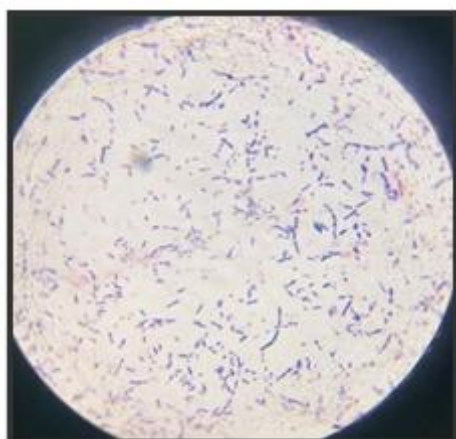


Fig.23: Gram stain of S_1-10^{-6} . This showing purple colour bacteria with Bacillus and positive reaction for Gram's staining.

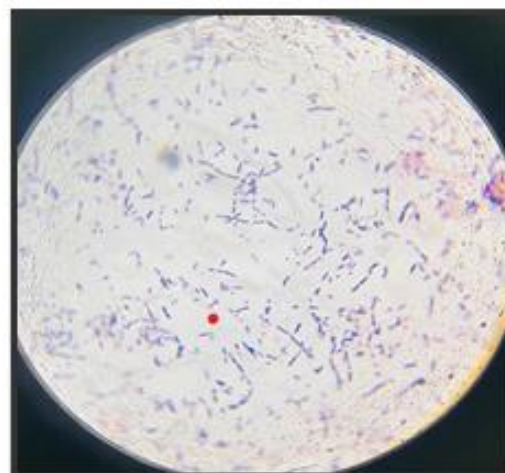


Fig.24: Gram stain of S_2-10^{-8} . This showing purple pink colour bacteria with Rod Bacillus and positive reaction for Gram's staining.

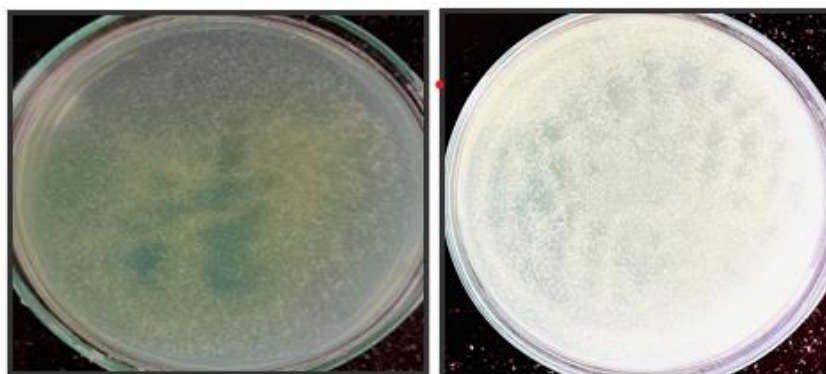


Fig.25: This showing Hydrogen Sulphide (H_2S) S_1-10^{-6} Negative and S_2-10^{-8} Negative



Sequence and Phylogenetic Analysis of Representative Isolates by 16s rRNA Analysis

The bacterial endophyte A1, C1, CH2, B2 & S2 isolated from Angoori Chass, Chol, Brandy & Sattu was subjected to 16S rRNA amplification and sequencing. The amplified product (~1400 bp) was sequenced and manually curated to remove unreadable or ambiguous bases. BLAST analysis of the high-quality sequence revealed a 100% identity with *Bacillus anthracis* strain Angoori (A1), *Bacillus arachidis* strain Chass (C1), *Bacillus* sp. Ts-116 strain Chol (CH2), *Bacillus velezensis*/*Bacillus amyloliquefaciens* strain Brandy (B2)

& *Bacillus anthracis*/*Bacillus cereus* strain Sattu (S2). The sequence has been submitted to GenBank under the accession number PQ814080.1 for Angoori (A1), PQ814105.1 for Chass (C1), GU190368.1 for Chol (CH2), OR056169.1 & MH210860.1 for Brandy (B2), KC790242.1 & MK859958.1 for Sattu (S2)¹⁸. To be even more sure of its phylogenetic position, the Neighbour-Joining method with UPGMA clustering and 1000 bootstrap replicates was used to construct a tree. This observation highlights the limitations of 16S rRNA in resolving species-level taxonomy among closely related species.

Sample 1: Angoori (A₁)

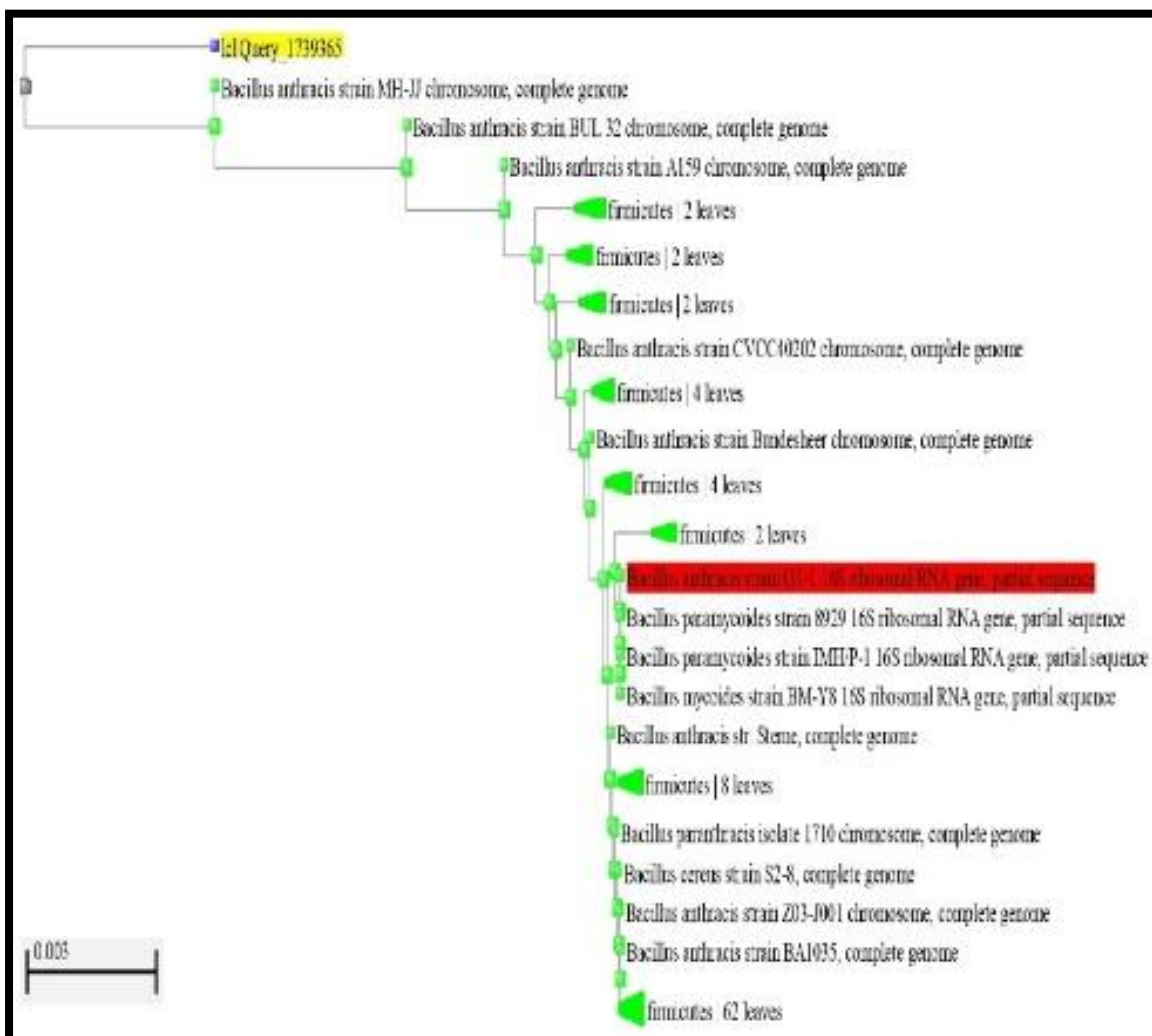


Fig.26. Neighbour-joining tree based on 16S rRNA sequence showing the phylogenetic relationship of bacterial isolates, isolated from A₁(Angoori) and similar other sequences deposited in NCBI GenBank



Sample 2: Chass(C₁)

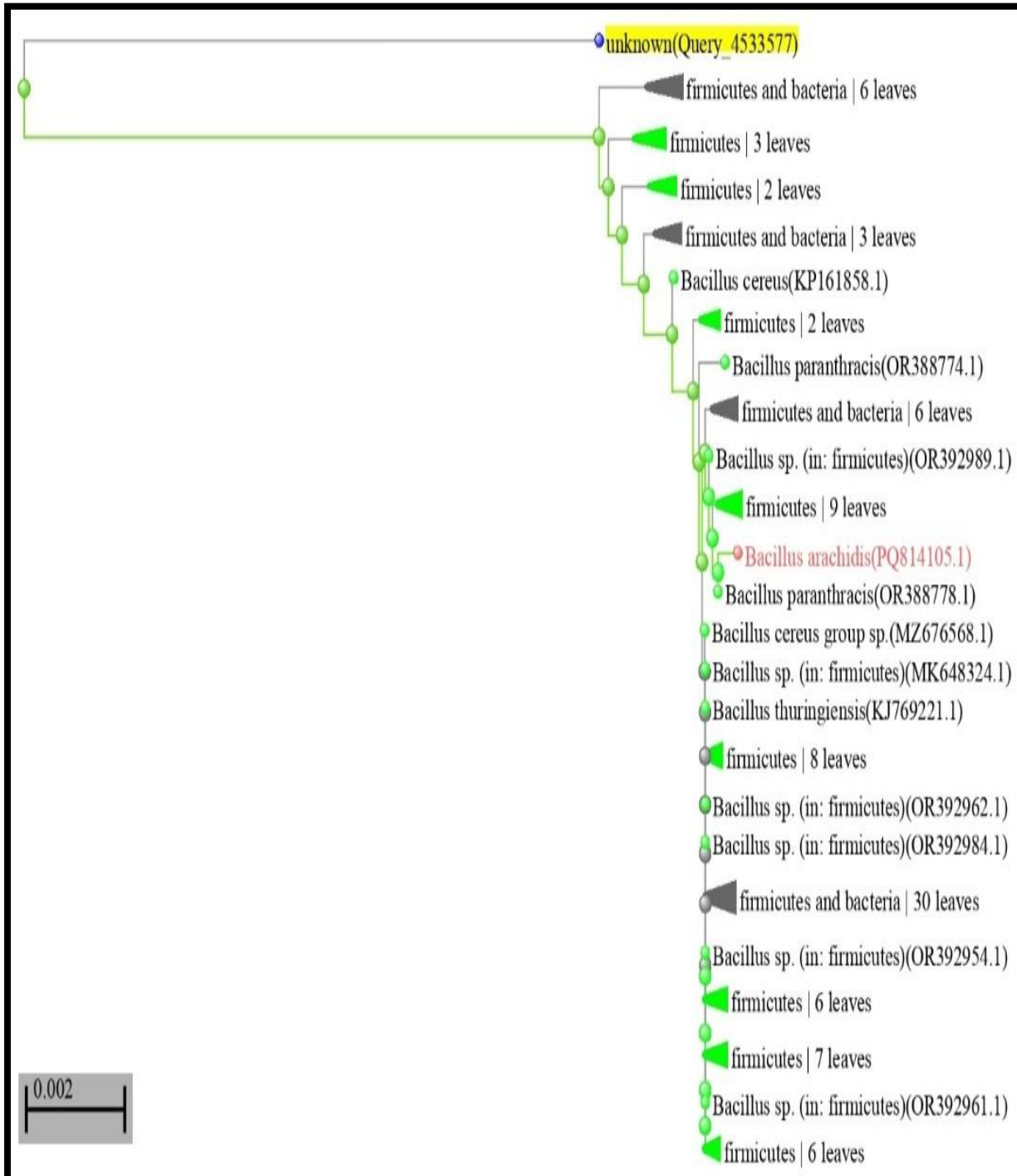


Fig.27.Neighbour-joining tree based on 16S rRNA sequence showing the phylogenetic relationship of bacterial isolates, isolated from C₁(Chass)and similar other sequences deposited in NCBI GenBank.

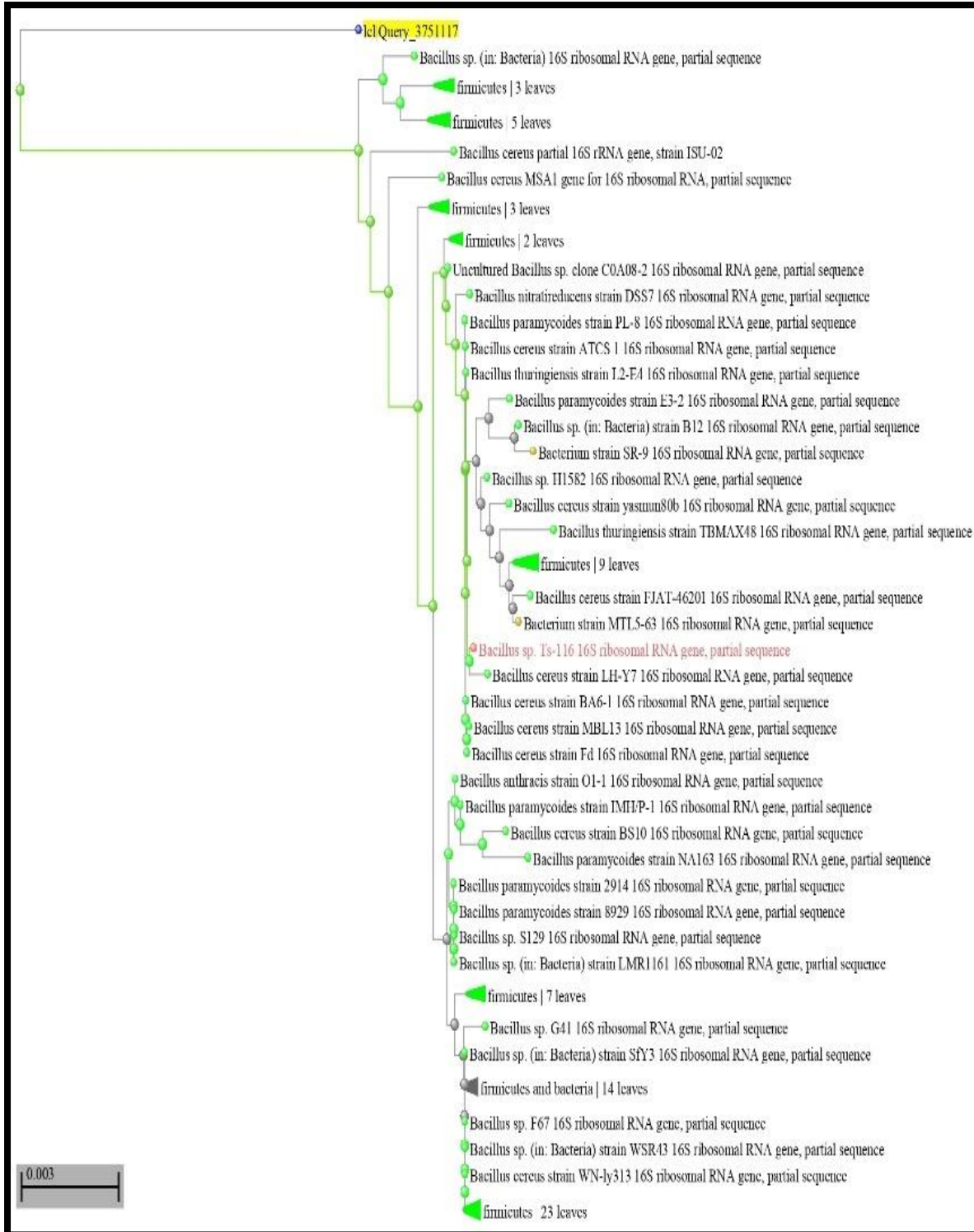
Sample 3: Chol(CH₂)

Fig.28. Neighbour-joining tree based on 16S rRNA sequence showing the phylogenetic relationship of bacterial isolates, isolated from CH₂(Chol) and similar other sequences deposited in NCBI GenBank.



Sample 4: Brandy (B₁)

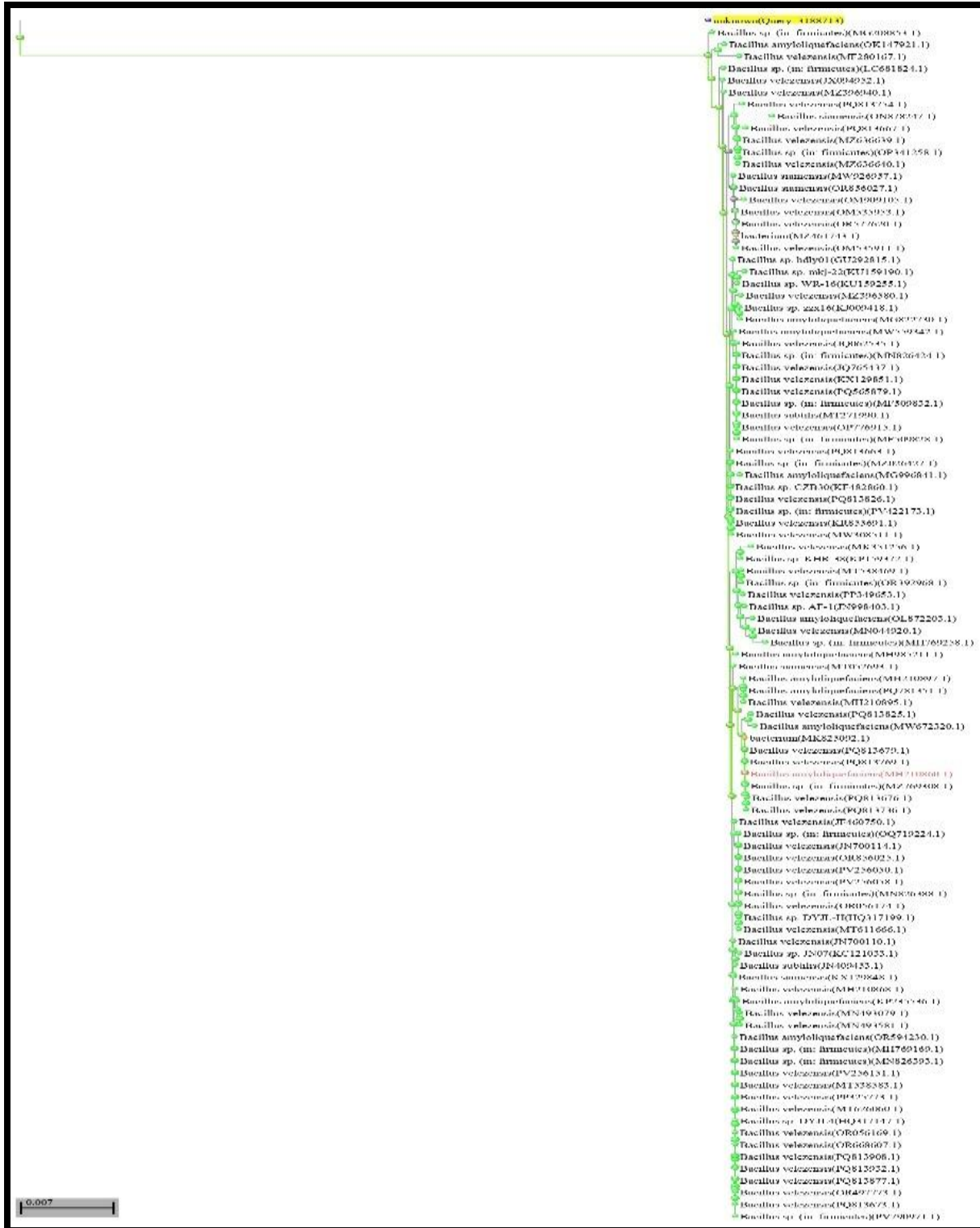


Fig.29.Neighbour–joining tree based on 16S rRNA sequence showing the phylogenetic relationship of bacterial isolates, isolated from B₁(Brandy)and similar other sequences deposited in NCBI GenBank.



Sample 5: Sattu (S₂)

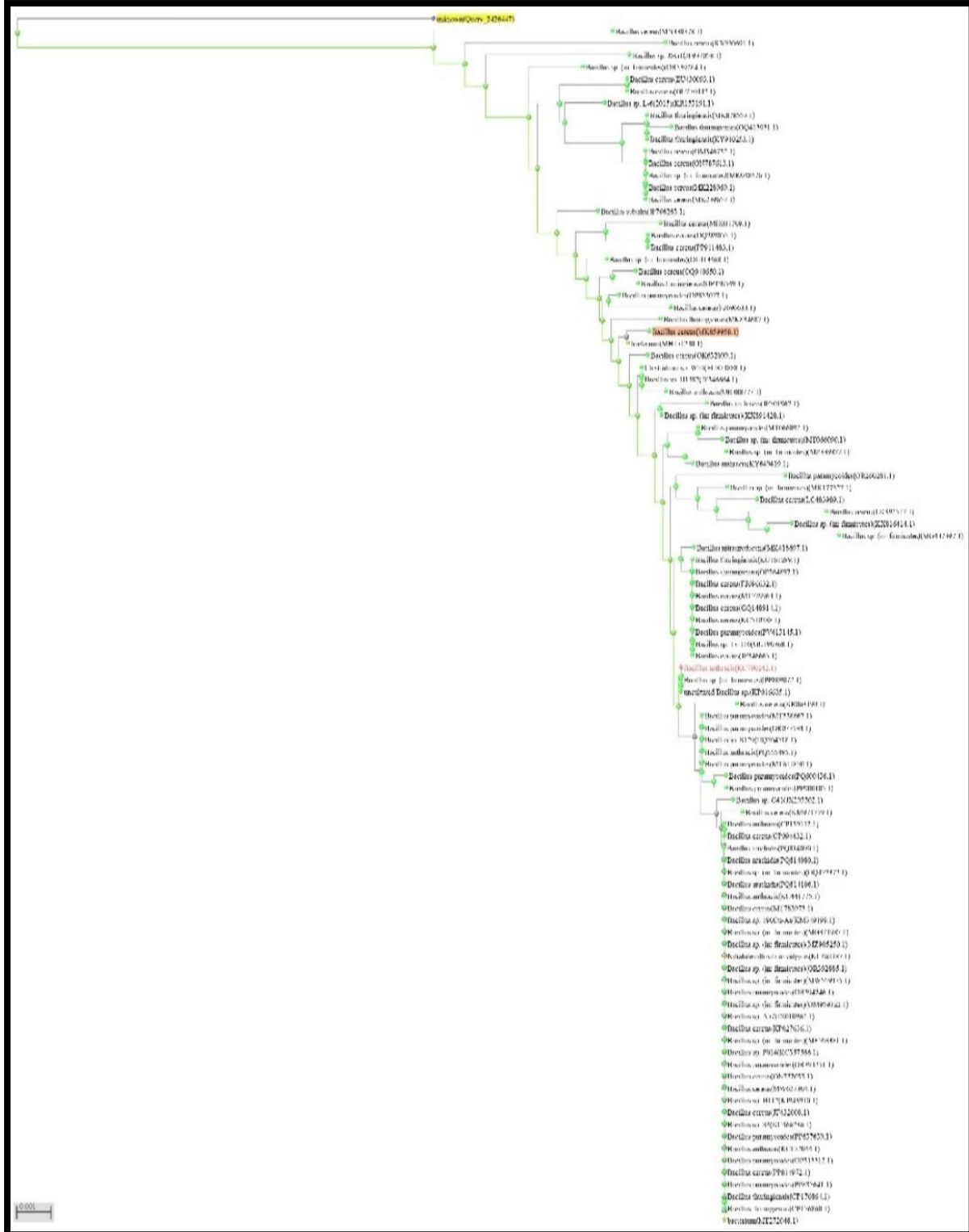


Fig.30.Neighbour-joining tree based on 16S rRNA sequence showing the phylogenetic relationship of bacterial isolates, isolated from S₂(Sattu)and similar other sequences deposited in NCBI GenBank.



Conclusion: Currently, traditional fermented beverages from Upper Himachal, such as Chhang, Sura, Angoori, and Lugri, are widely recognized for their potential health benefits. These naturally fermented drinks are known to aid digestion, support heart health, and contribute to overall wellness. Their nutritional value is enhanced by the presence of bioactive compounds, such as antioxidants (e.g., carotenoids, polyphenols, and betalains), which help reduce oxidative stress and inflammation. Additionally, these beverages may support antidiabetic effects by regulating glucose levels and improving insulin sensitivity. The presence of probiotics further promotes gut health and boosts the immune system. As local communities continue to consume these drinks, they not only benefit from their health-promoting properties but also sustain the cultural and ecological heritage of the Himalayan region. Fermented drinks like Chhang, Sura, Angoori, and Lugri from Upper Himachal support digestion, heart health, and overall wellness.

Fruit and vegetable-based beverages are excellent sources of antioxidants like carotenoids, polyphenols, betalains, and chlorophylls. These bioactive compounds, present in colorful produce, help combat free radicals, enhance immunity, and reduce inflammation, promoting overall health and lowering the risk of chronic diseases. These beverages may help manage diabetes due to their rich bioactive compounds, antioxidants, vitamins, and fiber. They can improve insulin sensitivity, regulate glucose levels, reduce inflammation, support pancreatic function, and enhance gut health, offering natural benefits for diabetes management.

Probiotics are beneficial microorganisms that support digestive health, inhibit harmful bacteria, and strengthen the immune system. Present in fermented beverages, they produce vitamins, bioactive compounds, and antimicrobial substances, improve enzyme activity, lower cholesterol absorption, and promote overall gut health and well-being.

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