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# Physiological and molecular characterization of novel gut strains isolated from the fecal pellets of *Nilgiritragus hylocrius*

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## Abstract

The Nilgiri Tahr are the high-altitude dwelling mountain ungulates, endemic to the Southern Western Ghats of India. These endangered, hoofed mammals inhabit the unique shola grassland biome. These herbivores feed on a diversity of plants including grasses, herbs, shrubs etc. They play an important ecological role in the maintenance of montane grassland ecosystems. Grazing by Nilgiri Tahrs in the montane grasslands supports the hydrology of these areas. In the present study, four novel gut bacterial species were isolated from the fecal pellets of Nilgiri Tahr. The four isolated strains from the gut of Nilgiri Tahr were characterized through an integrated approach involving morphology, physiology, and phylogeny. Molecular characterization was conducted using universal bacterial primers, followed by sequencing. Homology analysis of the sequences confirmed that the isolates were *Bacillus safensis, B. aerius, B. subtilis* and *B. australimaris.* These four *Bacillus* isolates are of viable importance in maintaining the gut health of Nilgiri Tahr and improves digestion in ruminants. The present study prompts that more intense research could be done on *B. safensis* to augment its use as biofertilizer , whereas *B. aerius, B. subtilis and B. australimaris* could have a great potential for enzyme production and bioprospecting studies. However, in depth research is needed and this current research is only a basic study for characterization of gut microbial isolates of Nilgiri Tahr.

**Keywords:** Nilgiri Tahr; Gut microflora; 16S rRNA sequencing; Phylogeny; *Bacillus*

## 1. Introduction

The *Nilgiritragus hylocrius* (Ogilby, 1838) which are the only species of the genus *Nilgiritragus,* thrives in the lush green sub-alpine grasslands of Western Ghats in Tamil Nadu & Kerala. These endemic mountain goats are social animals living in herds composed of adult males, females and young ones (Abraham et al., 2006). These sure-footed ungulates are listed as Endangered in the IUCN Red List of Threatened Species and is protected under Schedule I of the Wildlife (Protection) Act of India, 1972 (Alempath and Rice, 2008). The genomic studies revealed that the *Arabitragus jayakari* and *Hemitragus jemlahicus* are closely related to the genus Capra, whereas *Nilgiritragus hylocrius* are related to the genus Ovis (Ropiquet and Hassanin, 2005). The Nilgiri Tahr comes under the family Bovidae and subfamily Caprinae of the order Atriodactyla. They feed on more than 120 plant species including grasses, herbs and some shrubs which undeniably remarks the nutrient recycling and the survival of mountain ecosystem in high altitude grasslands (Rice, 1984). *Chrysopogon zeylanicus, Themeda triandra, Themeda tremula, Commelina benghalensis* are some of the important fodder species of Nilgiri Tahr.

The characterization and delineation of the microbial diversity inhabiting particular mucosal surfaces or other body sites has been an interesting topic of research in recent years. In most of the herbivores, the high microbial diversity in

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the gastrointestinal tract and rumen has the potential to provide metabolic activities that the host lacks. Specifically, the colonization of gut microflora in most mammals especially ruminants has been proven to confer health benefits to the host through the synthesis of digestive enzymes, inhibition and prevention of colonization by pathogens, which directly interferes with the host immune response.

The present study revealed the isolation and characterization of gut microbiota from the gastrointestinal tract of a high-altitude dwelling mammal Nilgiri Tahr. Early gut colonization starts from the birth, which is directly influenced by both morphological and immunological development of the gastrointestinal tract. During growth, the microbiome changes exhibiting increased diversity, a relevant indicator of gut health. Once established, the microbiota ultimately reaches an equilibrated community composition that is essential in maintaining the health of the animal. Moreover, there are other several important factors like age, diet, phylogeny and environment which has a direct influence on the rumen and gut microbiome composition.

## 2. Materials and methods

### 2.1. Study area & Sample collection

The Mukurthi National Park, located in the Western Nilgiris plateau, is one of the pristine habitat of Nilgiri Tahr. It is also, a UNESCO World Heritage site, which harbors many endemic flora and fauna. The fresh fecal pellets of Nilgiri Tahr were collected from Devils gap of Mukurthi National Park using sterile containers. The Geo-coordinates of the site of sample collection are 11.34714 $^{o}$N and 76.55466 $^{o}$E at an elevation of 2563 m MSL. The fresh pellets were processed to isolate the gut microbes.

### 2.2. Isolation of bacteria & pure culture

The fecal pellet samples collected from Western catchment were analyzed within 24 hours after defecation. The samples were aliquoted into 0.5g each and four bacterial cultures were isolated using the spread plate technique. The single colony of the four bacterial isolates were inoculated in the selective and differential growth medium namely, Macconkey agar, Skim milk agar, starch agar, Nutrient agar and LB medium (Himedia) (Figure 1). The pure cultures of four bacterial isolates namely MTR 01, MTR 02, MTR 03 and MTR 04 were periodically subcultured and glycerol stocks were maintained. The optimum temperature of the isolates were maintained as 30- 34 $^{o}$C and pH at 8.2.

### 2.3. Morphological Characterization

The four isolates were morphologically and physiologically characterized. Gram staining and spore staining was performed as per standard protocols and the cell morphometrics were also analyzed using the optical microscope (Olympus) under oil immersion (100X) (Figure 2). Capsular staining was also tested for the four isolates namely MTR 01, MTR 02, MTR 03 and MTR 04.

**Figure 1** Pure culture of bacterial isolates

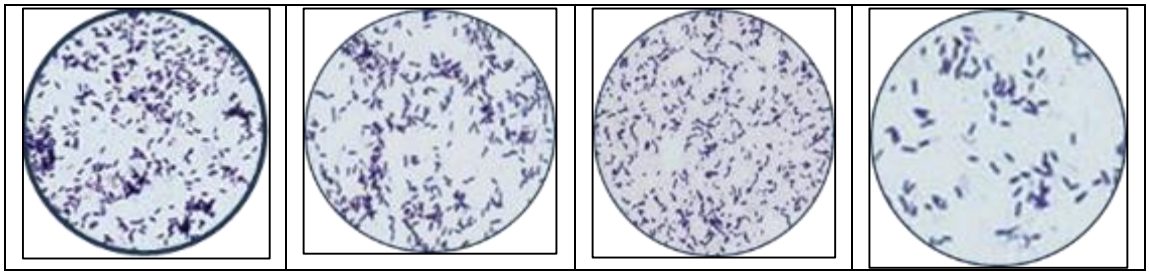

**Figure 2** Microscopic image showing Gram Positive rods of four bacterial isolates

### 2.4. Biochemical characterization

Important biochemical tests like Voges Proskauer, methyl red, oxidase, citrate utilization tests were carried out for the four bacterial strains. Similarly, nitrate reduction, starch hydrolysis and carbohydrate fermentation etc were also performed to delineate the biochemical characterization of the bacterial isolates.

### 2.5. Molecular characterization

To reveal the molecular characterization, genomic DNA isolation was carried out for the four strains. Colony PCR was performed and the 16S rRNA region was amplified and sequenced to understand the phylogeny.

#### *2.5.1. Genomic DNA isolation:*

Genomic DNA was isolated from bacterial samples using the Biobee Spin EXpure Microbial DNA isolation kit (Bogar Bio Bee stores Pvt Ltd). The cells were lysed by suspending 1-3 colonies aseptically and mixed with 500 µl of lysis buffer in a 2 ml microcentrifuge tube. 4 µl of RNAse and 500 µl of neutralization buffer was added and the contents were vortexed and incubated for 30 minutes at 65˚C in a water bath. The tubes were centrifuged for 10 minutes at 10,000 rpm and the resulting viscous supernatant was transferred into a fresh 2 ml micro centrifuge tube. 600 µl of binding buffer was added to the content and mixed thoroughly by pipetting and the tubes were incubated at room temperature for 5 minutes. About 600 µl of the contents were transferred to a spin column placed in 2 ml collection tube and centrifuged for 2 minutes at 10,000 rpm. The spin column was reassembled and the contents were mixed with lysate and centrifuged. The process was repeated using 500 µL washing buffer I followed by 500µl of washing buffer II. The spin tube was dried for 5 minutes at 10,000 rpm and the spin column was transferred to a sterile 1.5 ml micro centrifuge tube to which 100 µl of Elution buffer was added at the middle of spin column. The tubes were incubated for 2 minutes at room temperature and centrifuged at 10,000 rpm for 2 minutes. 600 µl of Chloroform Isoamyl Alcohol was added and centrifuged for 10 minutes at 10,000 rpm. A 600 µl of aqueous phase was carefully transferred into a fresh 2ml micro centrifuge tube and the mixture was resuspended.

#### *2.5.2. Quantification of DNA*

The Concentration and quantification of the isolated DNA was measured using a nanophotometer P330 (Implen®, München, Germany). The extracted DNA was quantified using 1% Agarose Gel Electrophoresis (Bio-Rad, San Francisco, CA, U.S.A.)

#### *2.5.3. PCR Protocol*

Genotypic characterization of bacteria is advantageous when compared to phenotypic methods. Polymerase Chain Reaction (PCR) is a process that uses primers to amplify specific cloned or genomic DNA sequences with the help of a very unique enzyme, DNA polymerase that directs the synthesis of DNA from deoxynucleotide substrates on a single-stranded DNA template.

The highly conserved 16S rRNA regions of the four bacterial isolates were amplified using universal bacterial primers 27F -5' AGAGTTTGATCTGGCTCAG 3' and 1492R- 5' TACGGTACCTTGTTACGACTT 3', holding 20 number of bases. The PCR reaction mixture contained Taq DNA polymerase (Qiagen, Germany) suspended in 2X Taq buffer and 0.4mM dNTPs and 3.2mM $MgCl_2$ along with 0.02% bromophenol blue. 5 µl of isolated DNA was mixed in 25 µL of PCR reaction solution (1.5 µL of forward primer and reverse primer, 5 µL of deionized water, and 12 µL of Taq Master Mix).

During the PCR protocol, for initial denaturation, the DNA template was heated to 95°C, which breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate, creating single stranded DNA. The mixture is then cooled to allow the primers to bind (anneal) to their complementary sequence in the template

DNA. The reaction is then heated to 72°C, which is the optimal temperature for DNA polymerase to act. The DNA polymerase extends the primers, adding nucleotides onto the primer in a sequential manner, using the target DNA as a template. The Polymerase Chain Reaction was performed using the following thermal cycling conditions.: Initial denaturation 95˚C 2 minutes followed by denaturation 95˚C 30 seconds and the cycle is repeated 25 times, annealing at 50˚C for 30 seconds, extension at 72˚C for 2 minutes followed by final extension at 72˚C for 10 minutes.

*2.5.4. Purification of PCR Products*

The unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR Clean up kit (Millipore). After PCR amplification, 5 µl products was analyzed by 1.7% agarose gel electrophoresis at 110 V for 30 min along with a molecular weight standard DL 1000 Marker. The products were visualized on the gel imaging system and images captured (Figure 3). The PCR product of the four strains namely MTR 01, MTR 02, MTR 03 and MTR 04 were sequenced using the 16s rRNA universal primers (Edwards et al., 1989).

### 2.6. Sequencing protocol

Single-pass sequencing was performed on each template using 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

### 2.7. Phylogenetic tree

The 16s rRNA sequence was blasted using NCBI blast similarity search tool (Castresana, 2000). The phylogeny analysis of query sequence with the closely related sequence of blast result was performed followed by multiple sequence alignment (Altschul et al., 1990). The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. These Gblocks eliminate poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007). The blastn algorithm was used to align the sequence with the GenBank database for homology analysis to identify the most similar sequences (Justo Arevalo et al., 2022). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as a Substitution model. The program Tree Dyn 198.3 was used for tree rendering (Dereeper *et al*., 2008).

## 3. Results and discussion

### 3.1. Morphological and biochemical characterization of the isolates

The raised, irregular-edged colonies of the strain MTR 01, were opaque, and appeared nearly circular in Nutrient agar medium. They were slightly yellowish to pale white in color and the cells were motile with a length of 1–2.2 µm and a width of 0.7–0.9 µm (Mukhtar et al., 2022). Microscopic observations revealed Gram-positive and spore -forming rods. The strains were positive for catalase, oxidase and gelatin hydrolysis.

The MTR02 colonies were white and opaque, rounded with smooth edges in Nutrient agar medium. The Gram-positive rods measured 0.9-1.5 µm in length and hydrolyzed starch and gelatin. They utilized citrate and were positive for urease test. The isolates were endospore formers.

The MTR03 isolates were medium-sized, gray-white, rounded flat and opaque colonies in skim milk agar medium. Biochemical analysis was further employed to examine the characteristics of the isolate. The bacterium fermented glucose for acid production, but failed to utilize lactose. The strains were VP-positive, while methyl red and indole tests were negative.

The MTR04 isolate had feathery edges in starch agar medium. Microscopic examination revealed that they were rod shaped, Gram positive and motile. The cells were 2- 2.2 µm in length with a cell width of 0.6 to 0.8 µm. They produced spores but capsule staining was negative. The strains exhibited anaerobic growth and decomposed starch. They were positive for nitrate reduction and oxidase activity. The strains showed negative results for utilization of mannitol, citrate, lactose and raffinose. The isolates were also negative for methyl red and indole tests.

### 3.2. Molecular identification

The PCR product yielded an amplicon size of approximately 1500 bp size and the electropherogram (EPG) was displayed in Figure 3. The phylogenetic analysis revealed that the MTR01 and MTR02 strains were homologous (95%) to *Bacillus*

*safensis* and *Bacillus aerius*. Similarly, the MTR03 strains were identified as *Bacillus subtilis.* The phylogenetic tree displaying the relationship between the *Bacillus* isolates and other strains, were constructed. The 16S rRNA sequence analysis and homology tests revealed that the MTR03 isolates showed 99% similarity to *B. subtilis*. The isolate MTR04 was identified as *Bacillus australimaris* with a homology of 96%. The phylogenetic tree of four *Bacillus* isolates namely *B. safensis, B. aerius, B. subtilis* and *B. australimaris* were created using the program Tree Dyn 198.3 was displayed in Figure 4,5,6 and 7 respectively.

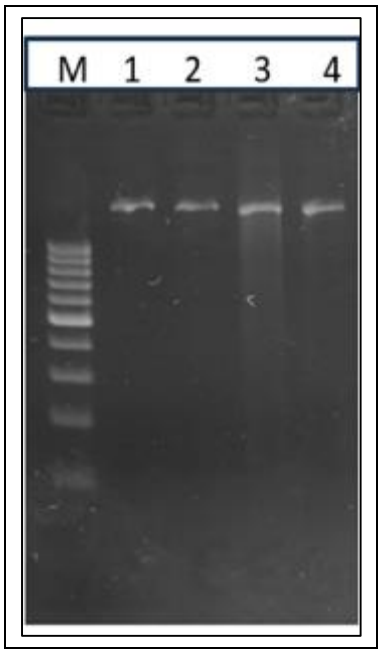


**Figure 3** Electropherogram of PCR amplicon of four bacterial isolates (M- DNA ladder, 1- MTR01, 2-MTR02, 3-MTR03, 4-MTR04)

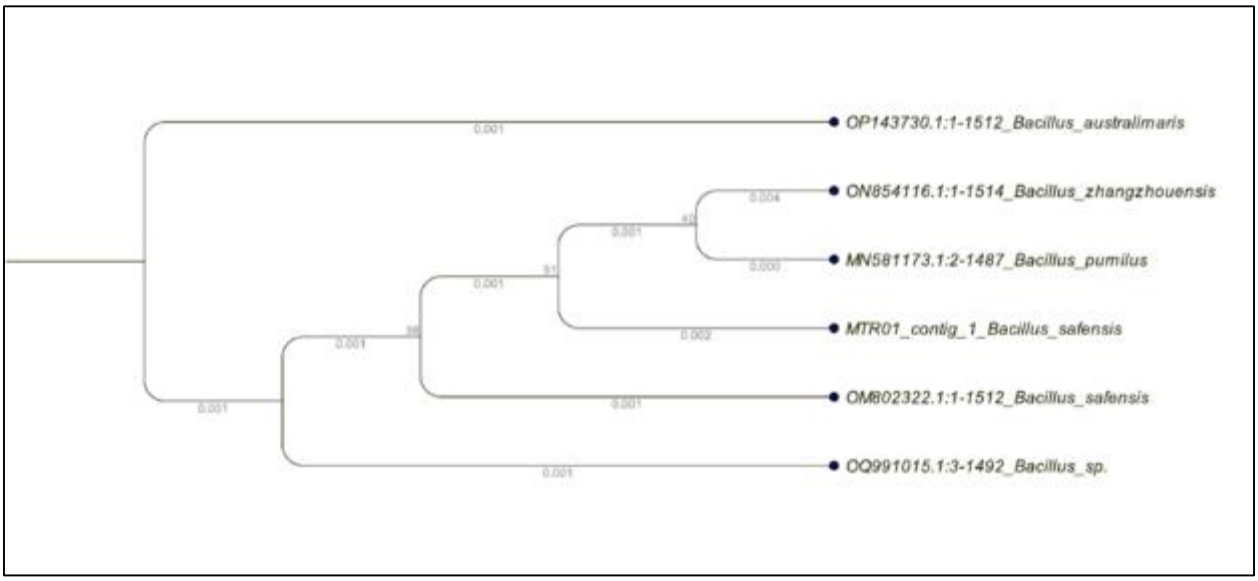


**Figure 4** Phylogenetic tree of the bacterial isolate MTR01-*Bacillus Safensis*

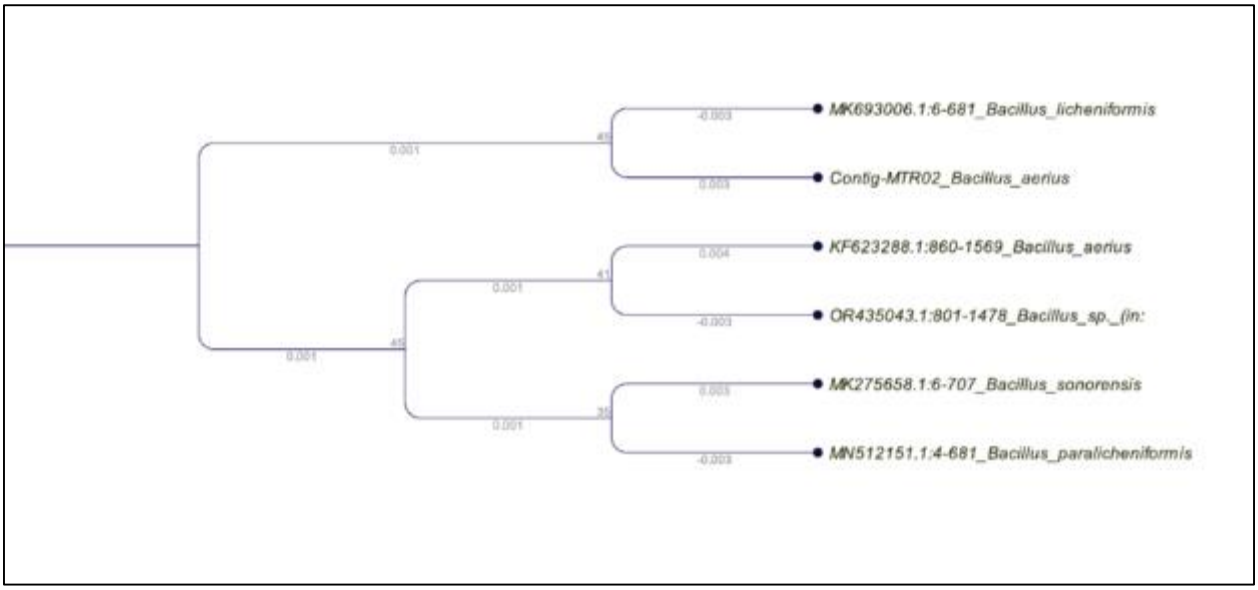


**Figure 5** Phylogenetic tree of the bacterial isolate MTR02-*Bacillus aerius*

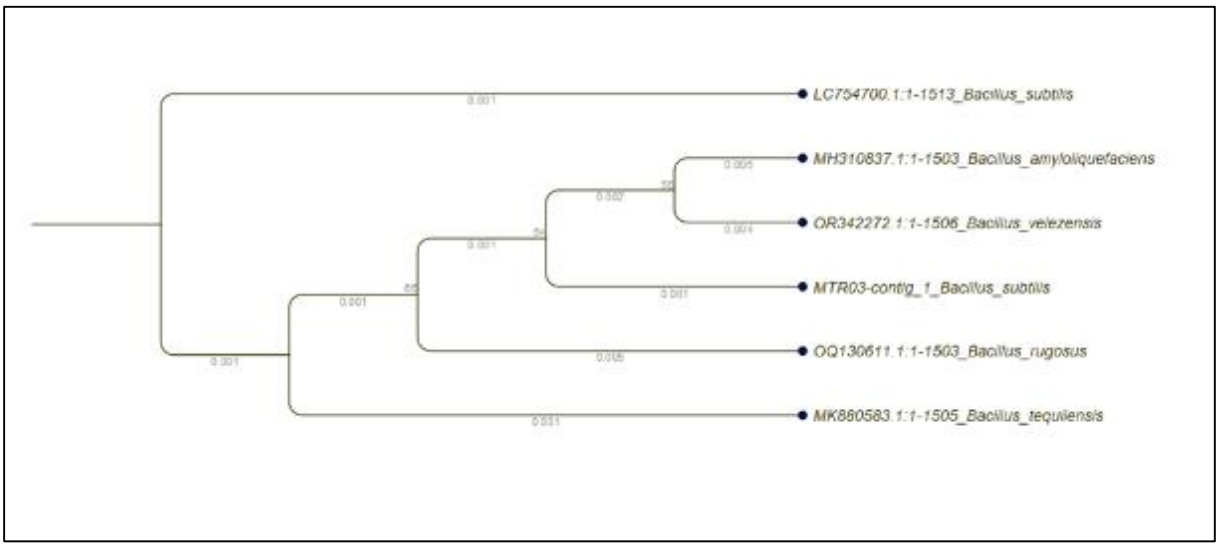


**Figure 6** Phylogenetic tree of the bacterial isolate MTR03-*Bacillus subtilis*

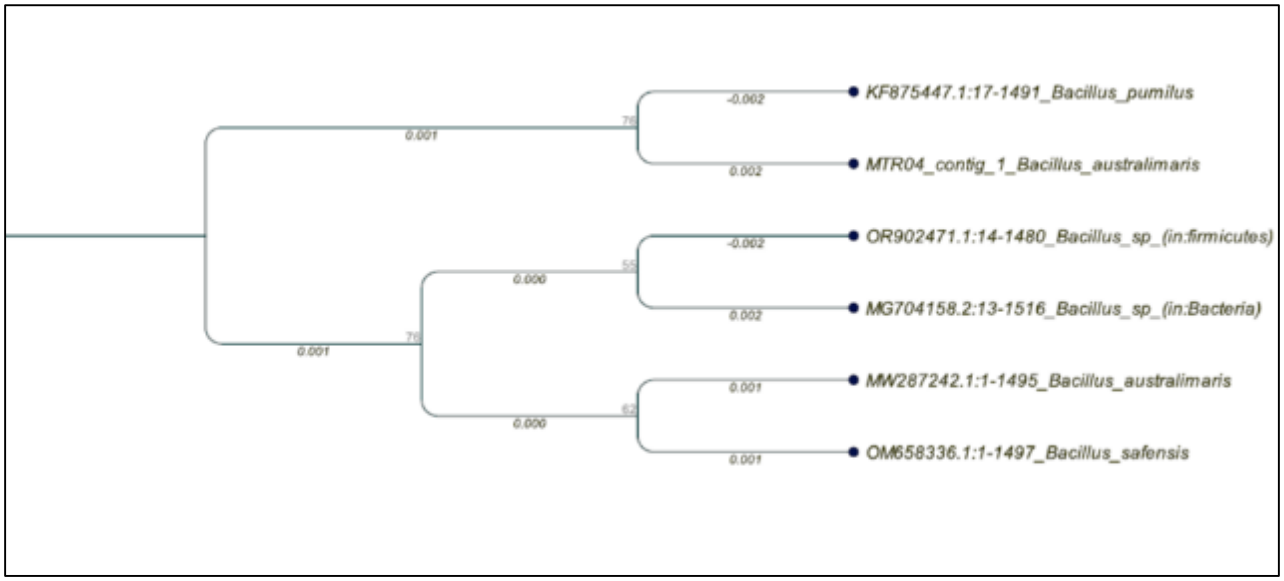


**Figure 7** Phylogenetic tree of the bacterial isolate MTR04-*Bacillus australimaris*

With the advancements of the nucleic acid sequence analysis techniques, the conserved regions of bacterial genome are amplified and sequenced and compared with the GenBank sequences. Thus, homology analysis is the most preferred approach for rapid identification of bacteria due to its promptness. The 16S rRNA sequence of four *Bacillus* isolates were deposited in the GenBank nucleotide sequence data library and the Genbank accession numbers were obtained as follows: *Bacillus safensis* -PQ192592, *Bacillus aerius* -PQ192625, *Bacillus subtilis* -PQ192637 and *Bacillus australimaris*-PQ192629.

### 3.3. Significance of gut bacteria

The aerobic chemoheterotroph *B. safensis* is a plant growth-promoting rhizobacteria that stimulates plant growth by production of growth hormones and colonizes the roots and rhizosphere (Kothari et al., 2013). Some strains were found to synthesize lipase, which is an important enzyme for fat digestion (Kumar et al., 2014). They are widely used in the biotechnological industry for their augmented role in the biocatalysis and bioremediation activities. Sharma et al (2024) studied the bioprospecting activity of *B. safensis* and *B. australimaris* in degradation of synthetic polymer, the high-density polyethylene. Owing to its plant growth promoting activity, *B. safensis* released from the defeaction of Nilgiri Tahr, colonizes the rhizosphere and enhances the growth of native floral species.Hence, feces of Nilgiri Tahr could be a potent organic manure for the vegetation in the high altitude grassland ecosystem, which reflects the importance of conservation of these mountain dwelling endemic ungulates. Several researchers documented the probiotic activity of *B. aerius* and are known to promote gut health and innate immunity. These strains are also known as high-altitude bacterium. These species were isolated from the gut of catfishes and play a major role in the feed utilization and improved metabolism rate.

The probiotic *B. subtilis* are producers of gut-healthy metabolites and postbiotics, including butyrate. Sarmikasoglou et al (2024) studied the role of *B. subtilis* in decreasing the production of acetate, propionate and methane in ruminal fermentation in cows. Hence, these bacteria are economically important as methane emissions from ruminants pose a major threat to the environment, being a major pollutant. Jia et al (2022), documented that *B. subtilis* stimulates synthesis of proteolytic and amylolytic bacterial growth and improves digestion and milk production in cattle. Similarly, Rhayat et al (2019) studied the synthesis of enzymes such as proteases, amylases, and cellulases by this species. Various researchers documented that cellulase production in *B. subtilis*, which aids in the digestion of cellulose, which is the predominant component of herbivores like Nilgiri Tahr (Fouda et al., 2024). Also, Lucey et al (2021), studied the role of *B. subtilis* in suppression of fecal pathogens in Holstein heifer calves. Along with other beneficial bacterial consortia, *B. subtilis* are also fed as probiotic to diary live stocks (Sarmikasoglou et al 2024). The obtained results for the characterization of *B. subtilis* were similar to those obtained by Lu et al (2018). The *B. subtilis* isolated from fecal pellets of Nilgiri Tahr has potent enzyme production activity thus aids in digestion, especially cellulose degradation and thus plays an important role in the gut health of Nilgiri Tahr.

Hama Cisse et al (2019) documented the probiotic activity and lactic acid and acetic acid production of *B. australimaris,* thus improving the overall gut health and fermentation. The physiological and biochemical characterization of the *B. australimaris* strain presented in this study was similar to those reported by Sharma et al (2024).

The present study revealed that the four bacterial species namely *B. safensis, B. aerius, B. subtilis* and *B. australimaris* isolated from facel pellets of Nilgiri Tahr are of vital importance in improving digestion by synthesis of digestive enzymes and are antagonistic towards gut-dwelling pathogens. These bacterial strains have a potent role in maintaining the overall gut health of Nilgiri Tahr, thus showing a vast probiotic potential. The isolate *B. safensis* (MTR01) could be a potent plant growth promotor and more research on these species will result in the advancement of biotechnological application.

## 4. Conclusion

The current study identifies four *Bacillus* isolates that play a crucial role in maintaining gut health in the Nilgiri Tahr and enhancing digestion in ruminants. Among the four strains, *Bacillus safensis* shows potential for development as a biofertilizer, while *Bacillus aerius*, *Bacillus subtilis*, and *Bacillus australimaris* have great potential for enzyme production and bioprospecting. Future studies focused on the development of consortia for phytoremediation, probiotics and enzyme synthesis. However, this research represents a foundational study, indicating the need for further in-depth investigations to explore various applications in the field of industrial biotechnology.

## Compliance with ethical standards

### *Acknowledgement*

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### *Disclosure of conflict of interest*

No conflict of interest to be disclosed.

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