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Research Article

Temporal shifts in mineral composition and microbial diversity of Chamliyal shrine clay

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Abstract

The clay from Chamliyal shrine has a long history of its use to treat various skin diseases particularly psoriasis, with its effectiveness linked to its mineral composition and microbial diversity. This study investigated the mineral composition and microbial diversity of the Chamliyal clay using two different media at two different time points i.e. 2008 and 2017. The results revealed a decline in mineral composition, bacterial load and diversity over the period of nine years. Molecular identification through 16S rRNA gene sequencing and subsequent BLAST analysis revealed 48 bacterial isolates in 2008 and 24 isolates in 2017 with 5 common isolates present in both the years. *Firmicutes* constituted the dominant phylum in both sampling years, largely due to the prevalence of *Bacillus* species. Proportion of isolates having antibacterial activity decreased from 12.5% in 2008 to 9.5% in 2017. The bacteria that were common in both the years did not show antibacterial activity in both the years. It was observed that not only the mineral composition and microbial diversity of the Chamliyal clay, but also the antimicrobial potential of the isolates, had decreased. Therefore, evaluating the actual impact of Chamliyal clay on healing necessitates further investigation through human applications.

Keywords: Chamliyal shrine, healing clay, antimicrobial, mineral composition

Introduction

Complementary and alternative medicines (CAMs) are being widely used in the healthcare system, reflecting the growing interest in traditional practices along with conventional medicine (1). In CAMs, various skin disorders are being treated using clay /mud therapy, also known as pelotherapy. This therapy involves the use of peloids natural products consisting of uniform mixture of finely divided organic and inorganic matter (2). Healing properties of the clay have been used by man since ancient times. The benefits of clay have been documented in various reports (3, 4, 5, 7, 9, 10, 11). Some of the clays that have been used for pelotherapy are Jordan red soil, Jordan (14), Dead Sea mud, Dead Sea (15), French green clay, France (16), Kisameet clay, Canada (17), Nubian Earth, Ancient Egypt (6), Chamliyal clay, Jammu & Kashmir, India (18, 19), etc.

The effect of clay therapy has mainly been attributed to two factors: the mineral composition of clays and their interaction with microorganisms (12, 13). Clay minerals, formed from the weathering of silicate possess physicochemical properties crucial for their therapeutic potential (20). Clay and its minerals enter the body through ingestion, inhalation and skin absorption. The primary minerals found in clay are bentonite, kaolinite, halloysite, talc, and smectites (montmorillonite, sepiolite, saponite, and hectorite), sepiolite and palygorskite (13). Minerals of the group kaolin are known to remove secretions, oils and toxins from the skin (21). Properties such as high surface area, cation exchange capacity, electrical charge, water retention capacity and the ability to absorb & adsorb substances help the clay molecules, to interact with the microorganisms (22, 23). These microorganisms, in turn, play a significant role in the healing properties of the clay. For instance, studies have linked the presence of specific microorganisms in peloids to their therapeutic properties, attributing this to the production of various organic compounds such as galactolipids, diacylgylcerolipids, which possess anti-inflammatory properties (8).

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Chamliyal is a holy shrine located in the district Samba of Jammu & Kashmir, and it is well known for its healing clay for skin diseases. Clay from this location is mixed with the water from the well located at the same location and the paste is applied on the affected part of the skin of the patients with symptoms of psoriasis for about 21 days on daily basis (24). Our laboratory is also engaged with the scientific validation of the healing properties of this clay and catalogued microbes present in the clay (25, 18, 19). It was proposed that the healing properties of clay may be due to the presence of different microbes in the clay and their genes involved in mineral metabolism like that of iron and Sulphur (18). Furthermore, studies have reported the presence of specific bacterial phyla in Chamliyal clay that are also prevalent in the healthy human skin microbiome, potentially contributing to homeostatic balance (19).

In the present study attempts have been made to compare the cultivable bacteria, screen them for antimicrobial activity and mineral composition of Chamliyal soil at two time point, 2008 and 2017 to document the change in these and correlation with skin healing, if any.

Materials and Methods

Sample collection:

The clay samples were collected from the shrine located at the India–Pakistan border in the village Chamliyal (32.4745° N, 74.8934° E) of Samba district of Jammu and Kashmir, India twice, in the year 2008 and 2017 during the month of November. The clay samples were collected from the depth of 20 cm from the surface and were packed in sterile bags, to ensure that most of the microbial load was not disturbed and retained in its natural form (27). The samples were transported to laboratory for further experimentation at 4°C.

Physiochemical analysis of the clay:

The clay samples were analyzed for pH, electrical conductivity (EC) and other minerals such as iron, zinc and potassium. For the clay samples collected in the year 2008, the pH value was measured in a 1:5 (w/w) soil water suspension using electric digital pH meter and the salinity of a soil was determined by measuring the electrical conductance (EC) of soil water saturation extract with the help of a conductivity meter (28). Whereas, chemical profiling of the soil was done by following the method developed by Raman and Sathiyanarayanan, 2009 (29).

The clay samples collected in the year 2017 were outsourced to Yara fertilizer India Pvt. Ltd for the analysis of physiochemical properties of clay. The outcomes were generated using Yara's Megalab^{TM} software.

Isolation and purification of cultivable bacteria

The diversity of cultivable bacteria was determined using two culture media R_2A (Himedia) and Nutrient agar (Himedia). 1 gm of clay was dissolved in 10 ml sterile saline and was serially diluted up to 10 times. 100 µL aliquot of 10^{-6} dilution was plated on media plates and incubated at 35±2°C for 24 hours in Orbiteck incubator. The number of CFU (colony forming unit) per gram of clay was calculated using the formula:

$$\text{CFU/gm} = \text{Number of colonies} \times \text{dilution factor} / \text{volume spread}$$

Morphologically different colonies from every plate were selected and purified using streak plate method (30) and purified colonies were stored in 50% glycerol at -80°C till further screening.

Preliminary characterization and Molecular identification of the isolates:

Prior to further analysis, preliminary characterization of isolates was done using Gram's staining kit (HiMedia) (31). Further, the molecular identification was done based on 16S rRNA sequencing. Bacterial DNA was isolated using GES Method (32) and DNA was analysed on gel electrophoresis using 0.8% agarose. The 16S rRNA gene for bacterial isolates was amplified from the genomic DNA, using universal 16S rRNA gene primers (8F- AGAGTTTGATCCTGGCTCAG and 16S R 1522R- AAGGAGGTGATCCAGCCACA) (33). The PCR was performed following protocol developed by Fierer et al., (2007) with some modifications, 100pM primer concentration instead of 0.5µM, 30 cycles PCR cycles instead of 25 cycles). The 50 ng DNA was taken as template, denatured initially at 95°C for 5 min. This was followed by 30 cycles of 95°C for 60s (denaturation), 54°C for 30s (annealing), and 72°C for 90s (extension), with a final extension at 72°C for 10 min (34).

Amplicons from bacterial samples isolated from Chamylial clay in 2008 were custom sequenced at

SciGenom Labs Private Ltd., Cochin, Kerala, India. Whereas, the amplicons from samples isolated in 2017 were outsourced to Biologia Research India Pvt Ltd's Karnal, Haryana, India for sequencing. The resulting nucleotide sequences were assigned bacterial taxonomic affiliations based on the closest match to sequences available at the NCBI database (http://www.ncbi.nlm.nih.gov/) using the BLAST (ww.ncbi.nlm.nih.gov/BLAST). Sequences of bacteria thus obtained were deposited in the GenBank nucleotide sequence database.

Screening of clay and bacterial isolates for antimicrobial activity:

Eleven bacterial pathogens namely Bacillus subtilis MTCC-121, Bacillus cereus MTCC-430, Staphylococcus aureus MTCC-1430, Enterococcus feacalis MTCC-439, Klebsiella pneumonia MTCC-432, Escherichia coli MTCC-2127, Pseudomonas aeruginosa MTCC-1934, Pseudomonas alcaligenes MTCC-493, Micrococcus luteus MTCC-4821, Alcaligenes denitrificans MTCC-299, Campylbacter coli MTCC-1126) and three fungal pathogens (Aspergillus niger MTCC-1344, Aspergillus flavus MTCC-2798, Penicillium MTCC-2192, were used for the screening of isolates for inhibiting their growth. All the pathogenic strains were procured from IMTECH, Chandigarh, India.

For the antimicrobial activity of clay, leachate of the clay collected in 2017 were prepared using the protocol mentioned in Azmi et al., (2021) (35). The purified bacterial strains were used separately to screen for antimicrobial activity. The isolates were grown in Luria-Bertani (LB) broth and incubated at 37ºC for 24 hours. After incubation, one mL of each grown bacterial culture was taken in eppendorf tubes and centrifuged at 12000 rpm for 5 min at 4°C to obtain supernatant. Seventy microlitres of the supernatant from each tube were loaded in the 0.6 cm well bored with a cork borer on the MHA (Muellor Hinton Agar) plates pre-seeded with the known bacterial and fungal pathogens. Plates were incubated overnight at 37ºC for bacterial pathogens and at 28ºC for 3 days for fungal pathogens and zone of inhibition in mm was recorded.

Results

Physico-chemical characteristics of Chamliyal clay in 2008 and 2017:

Chamliyal clay exhibited distinct physical characteristics in 2008 compared to 2017. In 2008, the clay was alkaline (pH 9±0.2) with an electric conductivity of 4.55±0.4 Sm^{-1}, whereas in 2017, the pH was 7.1±0.11 and the electric conductivity was 0.016±0.05 Sm^{-1} (Table 1). The concentration of iron, zinc and potassium was 12623±0.9 ppm, 64.2±0.7 ppm & 1013±0.5 ppm respectively in 2008 and 25.2±0.7 ppm, 0.37±0.01 ppm & 43.56±0.2 ppm respectively in 2017 (Table 1).

Comparison of bacterial load in the Chamliyal clay:

The significant difference in the bacterial load was observed between 2008 and 2017. The highest bacterial load i.e. $2\pm0.5x10^8$ CFU/g was observed on R2A media in 2008 and lowest in 2017 on R2A media i.e. $0.92\pm0.21x10^7$ CFU/g. The highest load in 2017 was observed on NA media (Table 2). The bacterial load in 2008 was approximately 7.57 times and 21.74 times higher on NA and R2A media respectively compared to 2017. In 2008, around a total of 48 different morphotypes were isolated, 34 from R2A media and 14 from NA media based on colony color, shape, margins and elevation, ensuring that different bacterial types were represented. Similarly in 2017, 24 bacterial strains were isolated following same strategy, 12 from R2A media and 16 from NA (Table 2). The isolates were preliminary characterized using Gram staining. The number of Gram positive and negative bacteria has been represented in the fig. 1.

Molecular identification of strains based on 16S rRNA sequencing and diversity analysis

Analysis of 16S rRNA gene sequences in 2008 classified 48 bacterial isolates into four major phyla namely: *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Bacteriodetes*. *Firmicutes* were dominant with 54% of the total bacterial isolates followed by *Actinobacteria* (25%), *Proteobacteria* (20%) and *Bacteriodetes* (2%) (Fig. 2a). In contrast, isolates from 2017 grouped into three phyla namely: *Firmicutes*, *Actinobacteria* and *Proteobacteria*. Similar to 2008, in 2017 also *Firmicutes* was the dominant phylum with 79% abundance followed by *Actionabacteria* (13%) and *Proteobacteria* (8%) (Fig. 2b). However, in both the years, no similarity in the members of phylum *Actinoabacteria* was reported.

At the genus level, Bacillus was the dominant genus in both the samples as 41.6% and 54.17% of the strains belongs to genus Bacillus in 2008 and 2017 respectively. The relative abundance of Bacillus has increased 12.57% in the period of 9 years. The other dominant genus was

Pseudomonas in both the years. However, Arthobacter was the third dominant bacteria in 2008 and Microbacterium in 2017 (Table 3). At the species level, 48 different bacterial species were identified in 2008, compared to 24 in 2017 (Table 3).

Common and unique bacteria in the Chamlyial clay in the year 2008 and year 2017

Five bacteria namely Bacillus subtilis, Bacillus safensis, Bacillus sonorensis, Bacillus tequilensis and Pseudomonas aeruginosa were found common in both the years. Sequence similarity analysis revealed that sequences of Bacillus safensis showed 100% identity followed by Pseudomonas aeruginosa (99.4%), Bacillus tequilensis (99.25%). However, Bacillus subtilis and Bacillus sonorensis showed less percentage identity i.e. 98.5% and 93.76% (Table 4). There were 43 and 19 bacterial species specific to Chamliyal clay of 2008 and 2017 respectively (Fig. 3).

Antimicrobial activity of the clay and isolated bacterial strains:

Initially, the antimicrobial activity of the clay collected in 2008 was not determined. However, the clay collected in 2017 was investigated for antimicrobial activity and showed only antibacterial activity against *Bacillus cereus* MTCC-430 (2 mm inhibition zone) and *Micrococcus luteus* MTCC-4821 (4 mm inhibition zone).

Antimicrobial screening of bacterial isolates revealed that only 5 out of 48 identified species displayed antibacterial activity against the tested Gram-positive and Gram-negative bacteria (Table 5). Notably, *Pseudomonas aeruginosa* strain JU-29 demonstrated broad-spectrum antibacterial and antifungal activity against the tested pathogens except *Pseudomonas aeruginosa* MTCC-1934 (Table 5). In 2017, only two species of *Bacillus* namely *Bacillus pumilus* CH-3 and *Bacillus subtilis* CH-4 inhibited the growth of *Bacillus cereus* MTCC-430 and *Micrococcus luteus* MTCC-4821 respectively (Table 5). Surprisingly, none of the common bacteria has shown antimicrobial activity. Out of 5 common bacteria, three has shown antimicrobial activity against different pathogens (Table 6).

Discussion

The practice of using clays for skin healing dates back to ancient times and are prevalent in medical and cosmetic formulations even today (13). The clay from the shrine located on the border of India–Pakistan in the village Chamliyal of Samba district of Jammu and Kashmir, India, has a history of use for the treatment of various skin ailment (18, 19, 24, 25). In continuation to the work being done on this clay in our laboratory, this study was undertaken to assess and compare the mineral composition and microbial diversity of clay from the Chamliyal shrine at different time intervals. Healing clays have been earlier reported to posses' antimicrobial properties due to the presence of certain minerals and their microbial composition (18, 20, 36, 37).

Not all clays can be used for clay therapy, as the suitability of the clay depends on its physico-chemical properties and mineralogical composition. Soil investigation revealed that the transition of pH from alkaline conditions with pH 9±0.2 in 2008 to a neutral with pH 7.1±0.11 in 2017. This observation is in contrast to existing literature, where acidic pH in clays is often associated with high antimicrobial activity (38, 39). The probable reason for this change could be the leaching of basic cations from the clay over the period of time, as evidenced by the significant decline in potassium ion concentration from 2008 to 2017 (Table 1). The concentration of other ions such as iron and zinc also showed significant declined from 2008 to 2017. Umeaku et al., (2019) have analyzed the mineral composition of four types of clay sampled from Anambra State in Nigeria and found iron concentration in the range 0.44-21.7 ppm and zinc ranged from 1.4-8.2 ppm, both of which are lower than the concentration observed in Chamlayal clay in the present study even after its reduction in last 9 years (40).

Electrical conductivity (EC) that measures the charge and conductivity of the ions present in the clay has direct correlation with the anti-microbial activity of the clay. In the present study, the concentration of EC in 2017 was very low compared to EC in 2008 (Table 1). It has been reported that the clay with low electric conductivity has high antimicrobial activity compared to clay with high electric conductivity (41) but the trend was not seen in present case as with decrease in electric conductivity, load, diversity and antimicrobial potential decreased. The antimicrobial activity of the clay was analyzed only in 2017 and showed antibacterial activity against *Micrococcus luteus* and *Bacillus cereus.* Similar to present study, Jordan's Red soil has been reported to possess antibacterial activity against *Micrococcus luteus* (42). Behroozian et al., (2016) reported that Kisameet clay

from British Columbia, Canada have antibacterial activity against multidrug-resistant bacteria with positive results observed for *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species (43). Another study reported the antibacterial activity of clay samples of Mamu Formation and Imo Formation origins collected from the Department of Geology, University of Benin, Benin City, Nigeria against *Pseudomonas aeruginosa*, antibiotic-susceptible *Pseudomonas aeruginosa*, methicillin resistant *Staphylococcus aureus* and antibiotic-susceptible *Staphylococcus aureus* (44).

It has been reported that in addition to mineral composition the microbes also contribute to the antimicrobial activity of the clay. The microbial diversity of the Chamylial clay has been reported earlier by our group using culture independent approach (18, 19, 25). Though it is an established fact that cultivation based methods does not assess more than 1% of microbial diversity, but in present study it was used to retrieve the cultivable bacteria to further screen them for the antimicrobial potential. The bacterial load and diversity were determined using two media; Nutrient agar (NA) and Reasoner's 2A (R2A). NA is a general purpose medium commonly used to grow wide range of non-fastidious bacteria, on the other hand R2A is a low nutrient medium used to grow broader spectrum of heterotrophic bacteria with different nutritional requirements. In the present study, in 2008, the highest bacterial load and diversity was reported on R2A medium compared to NA medium but in 2017, contrasting results were obtained with highest load and diversity on NA medium (Table 2). Tiago et al., (2004) reported the highest CFU count 8.0 x 10^3 for R2A medium buffered at pH 9.5 compared to 3x 10^3 on ANA medium (Alkaline nutrient agar) (45). Similarly, Pham and Kim, (2016) isolated 13 different strains using R2A medium and 7 using NB from rhizospheric soil (46).

On comparison of clay for bacterial load over the period of time, significant difference in the bacterial load and diversity was observed, both were decreased. In 2008, 48 bacterial species belonging to four phyla were reported whereas in 2017, 24 species belonging to three phyla were reported (Fig. 2). However, in both the years, *Firmicutes* were the dominant genera. Interestingly, the metagenomics studies (16S rRNA gene targeted and whole metagenomics) of Chamliyal clay conducted by our group identified *Proteobacteria* as the dominant phylum followed by *Actinobacteria* and *Firmicutes* (18, 25) but a recent metatranscriptomics study of the same clay (19) revealed *Firmicutes* as the dominant phylum followed by *Proteobacteria* and *Actinobacteria.* The contrasting results between DNA based analysis and RNA based analysis strongly suggests that the actively microbial community within the Chamliyal clay is dominated by the *Firmicutes* which is further supported by the findings of the present study. In both 2008 and 2017, all the bacteria classified as *Proteobacteria* belonged to class Gammaproteobacteria. Similar findings have been reported by other researchers where Gammaproteobacteria was the most abundant class of *Proteobacteria* in the healing clays (19, 47, 48). Gammaproteobacteria play a direct role in maintaining healthy human skin. Studies indicate that a healthy skin microbiome is characterized by a higher abundance and greater generic diversity of Gammaproteobacteria. In contrast, inflammatory and autoimmune skin conditions such as psoriasis and atopic dermatitis are associated with a reduction in both the abundance and generic diversity of this bacterial class (49, 50).

At the genus level, *Bacillus* with 45.83% abundance in 2008 and 51.6% abundance in 2017 was the most abundant genus in the present study (Table 3). Sharma et al., (2023) also reported *Bacillus* (80%) as the most active genus in the Chamlyial clay by metatranscriptomics studies. The increase in *Bacillus* that belongs to *Firmicutes* over the period of time can be correlated to the increase in temperature as temperature has been reported to regulate the bacterial diversity (19). A study was conducted where the bacterial diversity of hot spring was studied and found that with increase in temperature the bacterial diversity shifts from *Proteobacteria* to *Firmicutes* (51). A recent study also reported shift in the microbial diversity with rise in temperature and concluded that *Firmicutes* and *Actinobacteria* thrive at high temperatures (52). In soils, *Bacillus* species are recognized as the second most significant group of antibiotic producers. *Bacillus* spores have also been detected in various healing clays, including southern African medicinal clay (53), bentonite (54) and Dead Sea clay (48, 55).

Further, all the strains were tested for antimicrobial activity and in 2008; only six bacteria namely *Pseudomonas aeruginosa*, *Bacillus sonorensis*,

Streptomyces sp., Nocardia sp., and *Streptomyces peutius* had antibacterial activity against selected pathogens mentioned in material and methods section (Table 5). *Pseudomonas aeruginosa* showed both antibacterial and antifungal activity. Soil is rich source of antibiotic producing bacteria such as *Streptomyces*, *Nocardia* and *Bacillus* (56). *Streptomyces* is the producer of various secondary metabolites with clinical use, for example, amphotericin, erythromycin, streptomycin, tetracycline, Chlortetracycline, Oxytetracycline and rifamycin (57, 58). *Streptomyces peucetius* have been reported to produce a macrolide compound known as peucemycin that has broad spectrum antibacterial activity (59, 60). Moreover, around 90% strains of the *Pseudomonas aeruginosa* have been reported to produce antimicrobial compounds. They are known to produce bacteriocins like peptide, pyocins that has antibacterial activity against Gram negative bacteria (61) and phenazine-1-carboxamide (PCN), an anti-fungal compound (62).

In 2017, only two bacteria *Bacillus pumilus* CH4 and *Bacillus subtilis* CH6 showed antibacterial activity (Table 5). *Bacillus subtilis* is widely recognized for its antimicrobial potential and has reported to play role in wound healing and scar inhibition (63). Another study reported the antimicrobial and wound healing properties of cellulose based material containing *B. subtilis* cells (64). Various antibacterial metabolites has been isolated and characterized from *Bacillus pumilus* and reported to posses antibacterial activity against *Staphylococcus aureus*, *Micrococcus luteus* and other pathogens (65). In the present study, *Bacillus Pumilus* showed antibacterial activity against *Micrococcus luteus* (Table 5).

Interestingly, only the 2008 isolates (*Pseudomonas aeruginosa* JU-29 and *Bacillus tequilensis* JU-13) exhibited antibacterial activity, even though their 2017 counterparts (*P. aeruginosa* CH-24 and *B. tequilensis* CH-9) showed a close genetic relationship based on 16S rRNA similarity (Table 4 and 6). The reasons behind this observed decline in antibacterial activity over the nine-year period require further investigation.

Conclusion

A comparative analysis of Chamliyal clay collected in 2008 and 2017 revealed a decrease in pH, electric conductivity, and mineral ion concentration and bacterial diversity over the period of time. However, five bacterial species were consistently present in both the years of the study. The proportion of isolates exhibiting antibacterial activity was reduced and the ones that exhibited antimicrobial potential were not common, but different in different years. The *Pseudomonas aeruginosa* JU-29 isolated in 2008 and *Pseudomonas aeruginosa* CH-24 isolated in 2017 displayed close genetic relationship based on 16S rRNA gene sequence similarity with each other. However, they differed in their antibacterial activity. A similar observation was made for *Bacillus tequilensis* isolates from 2008 and 2017, necessitating further investigation into this behavior. The observed decline in bacterial diversity over the decade suggests a potential shift in the clay's ecological balance, which may contribute to the diminished antimicrobial properties. To gain a more comprehensive understanding of the microbial diversity and functional dynamics within Chamliyal clay over time, future temporal metagenomic and metatranscriptomic studies would be highly valuable.

Declarations

Conflict of interest

All authors declare that there is no conflict of interest.

Author contributions

Jyoti Vakhlu conceptualized the research, secured funding, and reviewed the manuscript. Monika Kumari and Simmi Grewal designed and performed the experiments. Nancy Bhagat, Nitika Sharma, and Ayushi Verma drafted the manuscript. All authors read and approved the final version.

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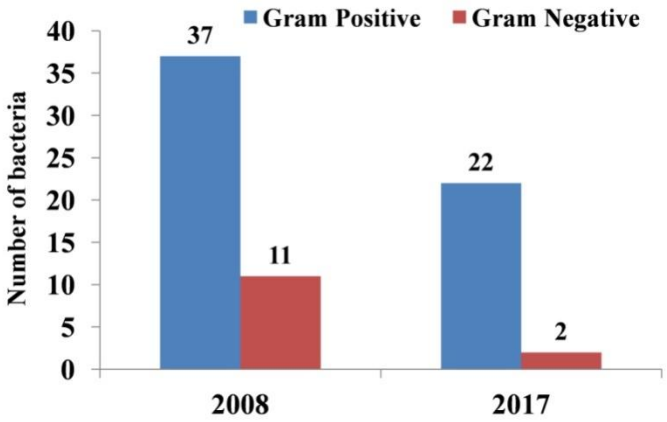

Figure 1: Bar graph representing the distribution of Gram positive and Gram negative bacteria.

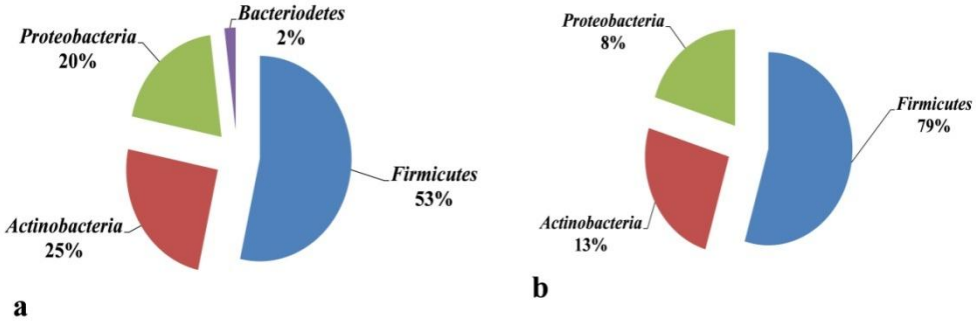

Figure 2: Comparison of relative abundance of major phyla present in Chamliyal clay sample collected in 2008 (a) and 2017 (b).

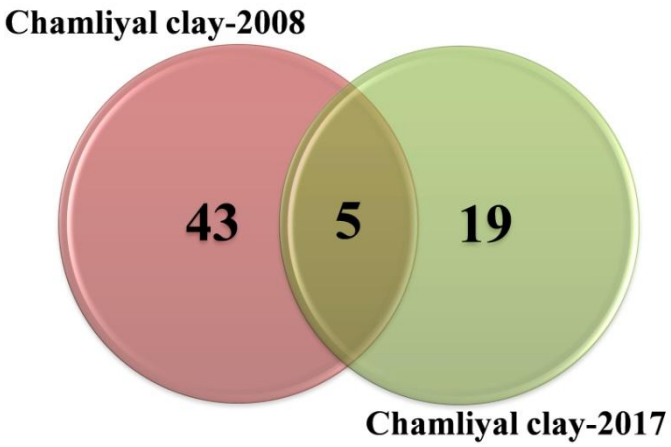

Figure 3: Venn diagram representing the number of common and unique bacteria present in both the years.

Table 1: Physical and chemical analysis of Chamliyal clay

S.no.	Parameters	2008	2017
1	**pH**	9±0.2	7.1 ± 0.11
2	**Electric Conductivity (**Sm^{-1}**)**	4.55±0.4	0.016±0.05
3	**Potassium (ppm)**	1013±0.5	43.56±0.2
4	**Iron (ppm)**	12623±0.9	25.2±0.7
5	**Zinc (ppm)**	64.2±0.6	0.37±0.01

Results are expressed as mean ± standard deviation. The mean value is the average of three biological replicates. Concentration of metallic ion is taken in ppm (parts per million). Sm^{-1}- Siemens per meter.

Table 2: Comparison of bacterial loads across the two growth media and years

S.No.	Growth media	Bacterial load (CFU/gm of clay)		No. of different strains	
		2008	**2017**	**2008**	**2017**
1	**Nutrient agar media**	1.06±0.15 x10^8	1.4±0.23 x10^7	14	16
2	**R2A media**	2±0.5 x10^8	0.92±0.21 x10^7	34	12

Table 3: Molecular identification of bacterial isolates based on 16S rRNA gene sequencing

S.no.	Soil Isolate code	Accession	Identified as	Max. Identity	Phylum
	2008				
1	JU- 1	KJ541496	*Bacillus Brevibacterium*	99	***Firmicutes***
2	JU- 2	KJ541497	*Bacillus atrophaeus*	99	
3	JU- 3	KJ541498	*Bacillus subtilis*	99	
4	JU-4	KJ541499	*Bacillus anthracis*	99	
5	JU-5	KJ541500	*Bacillus megaterium*	99	
6	JU-6	KJ563105	*Bacillus sp*	99	
7	JU-7	KJ563107	*Bacillus inaquosorum*	99	
8	JU-8	KJ563108	*Bacillus amyloliquifaciens*	97	
9	JU-9	KJ563112	*Bacillus cereus*	99	
10	JU-10	KJ590513	*Exiguobacterium sibiricum*	97	
11	JU-11	KJ563112	*Bacillus indicus*	99	
12	JU-12	KJ563110	*Bacillus safensis*	99	
13	JU-50	AF302118	*Bacillus sonorensis*	95	
14	JU-13	KJ541501	*Bacillus tequilensis*	99	
15	JU-14	KJ541502	*Bacillus sibi*	99	

16	JU-15	KJ563109	*Bacillus firmus*	99	
17	JU-16	KJ590516	*Bacillus thuringensis*	99	
18	JU-17	KJ590517	*Bacillus samanii*	99	
19	JU-18	KJ590525	*Clostridium beijirinckii*	99	
20	JU-19	KJ590537	*Bacillus niacin*	99	
21	JU-20	KJ620976	*Bacillus asahii*	99	
22	JU-21	KJ590531	*Bacillus simplex*	99	
23	JU-22	KJ590532	*Bacillus bacterium*	99	
24	JU-23	KJ590534	*Planomicrobium sp*	97	
25	JU-24	KJ590535	*Heliobacillus sp*	99	
26	AC-1	KJ563114	*Arthobacter aqilis*	99	***Actinobacteria***
27	AC-2	KJ563115	*Dermabacter hominis*	99	
28	AC-3	KF911110	*Arthobacter sulfonivorans*	99	
29	AC-4	KJ590515	*Arthobacter koreensis*	99	
30	AC-5	KJ590518	*Streptomyces sp*	99	
31	AC-6	KJ590520	*Agromyces albus*	99	
32	AC-7	KJ590521	*Nocardiodes luteus*	99	
33	AC-8	KJ590522	*Solirubrobacter soil*	92	
34	AC-9	KJ590536	*Streptomyces peucetius*	99	
35	AC-10	KJ590529	*Rhodococcus sp*	99	
36	AC-11	KJ590533	*Actinobacterium*	99	
37	AC-12	KJ590523	*Nocardia sp*	99	
38	JU-25	KJ563113	*Acinetobacter radioresistens*	99	***Proteobacteria***
39	JU-26	KJ590512	*Pseudomonas frederiksbergensis*	99	
40	JU-27	KJ590514	*Pantoea vagans*	99	
41	JU-28	KJ590519	*Klebsiella oxytoca*	99	
42	JU-29	KF453965.1	*Pseudomonas aeruginosa*	99	
43	JU-30	KJ590524	*Enterobacter aerogenes*	99	
44	JU-31	KJ590526	*Pseudomonas sp*	99	
45	JU-32	KJ590527	*Acinetobacter sp*	99	
46	JU-33	KJ590528	*Pseudomonas vranovensis*	99	
47	JU-34	KJ590531	*Acinetobacter strain*	99	
48	BAC-1	KF911109	*Sediminibacterium Salmoneum*	95	***Bacteriodetes***
	2017				
1	CH-1	PV476532	*Bacillus sonorensis*	95.71	***Firmicutes***
2	CH-2	PV477722	*Bacillus aerius*	99.35	
3	CH-3	PQ358727	*Bacillus pumilus*	100	
4	CH-4	PQ358619	*Bacillus subtilis*	100	

5	CH-5	PV477847	*Bacillus zhangzhouensis*	100	
6	CH-6	PV478027	*Bacillus infantis*	99.37	
7	CH-7	PV478097	*Bacillus zhangzhouensis*	99.84	
8	CH-8	PV489959	*Bacillus altitudinis*	92.98	
9	CH-9	PV477803	*Bacillus tequilensis*	99.71	
10	CH-10	PV478019	*Bacillus stratosphericus*	99.04	
11	CH-11	PV478066	*Bacillus safensis*	100	
12	CH-12	PV563557	*Bacillus licheniformis*	96.31	
13	CH-13	PV478034	*Bacillus aerophilus*	99.77	
14	CH-14	PV477842	*Bacillus pumilus*	100	
15	CH-15	PV477823	*Lysinibacillus sphaericus*	98.55	
16	CH-16	PV478007	*Priestia megaterium*	100	
17	CH-17	PV477802	*Cytobacillus horneckiae*	98.47	
18	CH-18	PV484508	*Oceanobacillus kimchii*	**100**	
19	CH-19	PV477804	*Paenibacillus lautus*	99.64	
20	CH-20	PV478015	*Microbacterium hydrothermale*	99.84	***Actinobacteria***
21	CH-21	PV477963	*Microbacterium hydrothermale*	99.04	
22	CH-22	PV477981	*Microbacterium testaceum*	99.61	
23	CH-23	PV478014	*Pseudomonas putida*	100	***Proteobacteria***
24	CH-24	PV477797	*Pseudomonas aeruginosa*	99.58	

Table 4: Comparison of 16S rRNA sequence identity of the common bacteria in 2008 and 2017

S.no.	Common bacteria	Accession number 2008	Accession number 2017	Percentage identity (%)
1	*Bacillus subtilis*	KJ541498	PQ358619	**98.5**
2	*Bacillus safensis*	KJ563110	PV478066	**100**
3	*Bacillus sonorensis*	AF302118	PV476532	**93.76**
4	*Bacillus tequilensis*	KJ541501	PV477803	**99.25**
5	*Pseudomonas aeruginosa*	KF453965.1	PV477797	**99.4**

Table 5: Antimicrobial activity of bacterial species against selected pathogens

S.no.	Pathogens	*Pseudomonas aeruginosa*	*Bacillus sonorensis*	*Streptomyces sp*	*Nocardia sp*	*Streptomyces peutius*	*Arthobacter koreensis*	*Bacillus pumilus*	*Bacillus subtilis*
		Zone of inhibition (mm)							
1	***Staphylococcus aureus***	10	10	9.5	8	5	7.5	-	-
2	***Micrococus leuteus***	9	5	-	-	-	-	-	6
3	***Enterococcus feacalis***	6	-	8	8.5	9	6	-	-

4	***Bacillus subtilis***	14	-	-	-	-	-	-	-
5	***Bacillus cereus***	13	5	-	10	6	7	4	-
	Gram -ve								
6	***Escherichia coli***	7.8	7	-	-	-	-	-	-
7	***Pseudomonas aeruginosa***	-	-	4	-	-	4.5	-	-
8	***Pseudomonas alkaligenes***	9	-	6	8	5	7.9	-	-
9	***Alkaligenes denitrificans***	10	-	-	-	-	-	-	-
10	***Klebsiella***	11		5	7	8	6	-	-
11	***Campylobacter coli***	14	-	-	-	-	-	-	-
	Fungal pathogens								
12	***Penicilium***	14	-	-	-	-	-	-	-
13	***Aspergillus niger***	11	-	-	-	-	-	-	-
14	***Aspergillus flavus***	15	-	-	-	-	-	-	-

*****Zone of Inhibition measured in millimeters (mm

Table 6: Comparison of the common bacteria of 2008 and 2017 for the antimicrobial activity

S.no.	**Common bacteria**	**Antibacterial**		**Antifungal**	
		2008	**2017**	**2008**	**2017**
		Zone of inhibition (mm)			
1	*Bacillus subtilis*	**-**	**+**	**-**	**-**
2	*Bacillus safensis*	**-**	**-**	**-**	**-**
3	*Bacillus sonorensis*	**+**	**-**	**-**	**-**
4	*Bacillus tequilensis*	**-**	**-**	**-**	**-**
5	*Pseudomonas aeruginosa*	**+**	**-**	**+**	**-**

*Zone of Inhibition measured in millimeters (mm).

(+) indicates the presence of a Zone of Inhibition, (-) indicates the absence of a Zone of Inhibition.