

Original Research Article

Evaluation of the Effect of *Haridra* (Turmeric) on Leech Gut Flora: A Metagenomics Study

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ABSTRACT

Sushruta has advocated blood letting (Raktamokshana) as one of the main treatment measures especially using leeches (Jalukavacharana), for the management of inflammation, abscess, cellulitis, wounds, ulcers, skin diseases etc. Various recent research studies have reported infection at the site of leech-bite wound, resulting in septicemia in untreated patients. Leech bite site was commonly infected with the bacterial species, which necessitated prophylactic antibiotic cover. But before Jalukavacharana, Sushruta has described a method of preparing or detoxifying the leeches in *Haridra* (turmeric) water for a period of 45 minutes before it is used on a patient. Four batches of gut samples from both *Haridra* treated and untreated leeches were subjected to metagenomics study. Total count of gut flora in *Haridra* treated leeches was found to be lower in comparison to the gut flora in untreated leeches in three of these batches. Count of bacterial species belonging to phyla Proteobacteria and Bacteroidetes, especially the members belonging to proteobacteria was lower in the gut samples obtained from treated leeches.

Key words: *Haridra*, Hirudotherapy, Jalukavacharana, Metagenomics, Proteobacteria

INTRODUCTION

In Ayurveda, Shalyatantra, the branch of surgery, has described medical management of diseases, surgical procedures, minimally invasive surgical procedures or parasurgical procedures and medical management of surgical diseases, and hence is a unique branch of Ayurveda. Among parasurgical procedures, Raktamokshana (blood letting) is an important procedure. Jalukavacharana (Hirudotherapy), a type of Raktamokshana, has been used since ages for the

management of various diseases like abscess, inflammation, cellulitis, non-healing ulcers, necrotizing fasciitis, thrombosed hemorrhoids, skin diseases etc.

The Western Science in the recent times has started adopting Hirudotherapy into its practice for various diseases. Hirudotherapy is seen to provide great therapeutic benefits during post-operative remediation, with studies demonstrating an associated decrease in the rate of graft failures and risk of amputation. Leeches are applied to the venous-congested sites ^[1] to

withdraw obstructive blood while simultaneously secreting an anti-coagulating agent and vasodilators to further reduce circulatory obstruction and facilitate blood flow through the area. In spite of various studies confirming its efficacy in the management of ulcers, reconstructive surgeries etc, there are many recent studies which have widely reported about the causation of leech-bit borne infections, which maybe minor incidences to major incidences like septicemia (when no timely treatment is done). Occurrence of *Aeromonas* species in the digestive tract of Leeches has been reported. [2] Its also been reported that prophylaxis with antimicrobial agents active against *Aeromonas* spp. is necessary to avoid opportunist infections caused by indigenous leech flora during medical leech therapy. [3] Biochemical and morphological tests indicated that *Aeromonas* spp. is the dominant culturable symbiont in leeches collected from north of Iran. Isolates were highly susceptible to tetracycline, gentamicin, cotrimoxazole and ceftriaxone. According to results, tetracycline was superior to other antibiotic to cleansing of bacteria from leeches. *Aeromonas* spp. was eliminated completely from all leeches and statistically significant cleaning was obtained with the use of tetracycline and gentamicin solution after 4 days for 5.5 h daily. [4]

Acharya Sushruta in Sushruta Samhita has explained that leeches have to be collected from water bodies which are not contaminated with sewage etc [5] and also has mentioned that the leeches have to be placed in Haridra (turmeric) before being used in the patients. [6] This was an important pre-operative procedure that had to be carried out so as to prevent the development of any form of complications during or after the procedure of Jalukavacharana.

In the previous work, in the gut of Indian cattle leech (*Hirudinaria granulosa*) members of the enterobacteriaceae family which belong to class Gammaproteo bacteria were found to predominate the gut flora.

Bacteria belonging to the genus *Morganella* were found to predominate. *Morganella* species are also known to be opportunistic pathogens capable of causing nosocomial infections. [7] Metagenomics is an approach which can provide an insight into these microbial communities.

When the leeches are kept in Haridra water, it is seen that they vomit out whatever blood is in their gut, thus it is likely that the microbial load in the gut (from oral cavity to the intestine) is thrown out and thus microbial load may be significantly reduced. Thus in the present work, the effect of Haridra treatment on the gut flora of the leeches (through Metagenomics) was studied.

MATERIALS AND METHODOLOGY

Collection of Leeches:

Leeches from Udupi and Goa regions were collected from fresh water lakes, washed well and stored in transparent glass containers with fresh water. Leeches collected from the same locality on the same day were maintained as one batch. The leeches were maintained for 7 days prior to subjecting them to microbial studies.

Haridra treatment:

Leeches were treated by keeping them in a solution of 10gm of fine powdered Haridra rhizome in 1 liter of clean water, for 45 minutes. The leeches were then subjected to microbial studies.

Microbial studies:

The leeches from the same batch were weighed and pooled approximating same weight ($\pm 10\%$). They were cleaned with sterile cotton dipped in normal saline. The selected leeches were kept in different conical flasks and about 5 ml of ether was poured on a cotton ball and the mouth of the flask was covered with it. Flasks were kept in situ till the leeches became motionless. They were then fixed on wax trays and dissected. The dissection board surrounding the leech was wiped dry with sterile cotton swabs. The guts were collected in sterile microfuge tubes.

The samples were subjected to microbiological studies and sent for metagenomic studies.

During this period patients undergoing Jalukavacharana in the OPD of Department of Shalyatantra, Muniyal Institute of Ayurveda Medical Science, Manipal, were observed for signs of infection at the site of bite for a period of one week. Only if any patient developed signs of infection, then the sample collected from the leech bite site would be sent for culture to identify the organism.

Microbial load analysis:

The gut samples collected in the microfuge tubes were teased with sterile surgical scalpels. The tissue pieces were transferred aseptically into 10ml of sterile saline and vortexed. Serial dilutions of each were prepared and 1ml of each dilution was inoculated into Soyabean casein digest agar. The plates were incubated at 37°C and colonies were counted after 24 hours. The count was reported as colony forming unit per gut.

16S Metagenome analysis:

The gut samples collected in the microfuge tube were stored at -20°C. The samples were packed and transported under cold conditions (enveloped in ice-pack) to Genotypic Technology (P) Ltd., Bangalore for metagenomic analyses. The study carried out at Genotypic Technology (P) Ltd as follows- Isolation of DNA from the sample was performed using QiagenDneasy Blood and tissue Kit (Qiagen, Catalog #69504) as per manufacturer recommended protocol. Purity and concentration of the DNA was assessed using ND1000 nanodrop spectrophotometer (Thermo Scientific, MA, USA). 16S V3-V4 metagenome libraries were prepared using 16S Bacteria V3-V4 region-specific targeting proprietary primers

at Genotypic Technology Pvt. Ltd., Bangalore, India. The pooled library was sequenced on Illumina Miseq for 300 bp paired end chemistry according to the manufacturer’s protocol. The generated illumina paired end reads (275*2) were quality checked using FastQC tool. The stitched reads were analyzed using QIIME v 1.9.0 software. The query sequences were mapped against the curated chimera free 16s rRNA database (Greengenes v 13.8) using UCLUST method and OTUs (Operational Taxonomic Unit) were identified at >=97% sequence similarity.

Representation of results: Amplicon of 16srRNA (V3-V4 region) was sequenced. Sample-wise reports were prepared which gave abundance values from phylum to species. Stacked bar plot were generated using the relative abundance of phylum and family. Heatmap was generated by representing high abundance values in different shades of blue and those with lower abundance were indicated in various shades of red. Color slab was generated based on the maximum and minimum values in the matrix. The heatmap was clustered row-wise and has been developed using R package NMF⁹.

RESULTS AND DISCUSSION

The leeches were subjected to Haridra treatment. Guts from two to four leeches were pooled and subjected to analysis. Weight of the gut collected from a leech weighed around 0.11 to 0.14g. The sets (treated and untreated of the same batch) which approximated in weight (± 10%) were selected. Microbial count on casein soyabean digest agar of various batches of leeches collected from Karnataka are given in Table 1.

Table 1: Viable count of leeches treated with Haridra

Sample ID		A	B	C	D	E	F
Cfu/gut	Untreated	78000	18250	575000	18000	14200	29600
	Treated	24250	25000	825000	12000	1800	1200
% change		-68.9	+36.9	+43.4	-33.33	-87.32	-95.94

Bacterial count in the samples was calculated per gut.

The microbial count per gut in these leeches under study ranged from 1.4×10^4 to 5.8×10^5 . Gut samples from set C when dispensed in sterile saline, the suspension attained dark red coloration due to presence of blood and was found to have high microbial load (5.8×10^5 cfu/ml). Guts collected from Haridra treated leeches of the same set too showed presence of heavy microbial load (8.3×10^5). Presence of blood in the gut may have affected the efficacy of Haridra treatment. Suspensions prepared from rest of the sets exhibited a tinge of red coloration. Samples A, B and C were collected in rainy season (august-October) and D, E and F were collected post rainy season in the months of November and December. The count of gut microbes in

leeches belonging to sets D, E and F was relatively lower. The guts harvested from the leeches collected in the month of December (from Untreated -GB5, GB9, LB5; from treated-GA4, GA5, GA9, LA5) and October (Untreated - LB14; Treated - LA14) were sent for metagenomics analyses.

The gut samples extracted from leeches after treatment are designated as LA and GA. L represented the gut from leeches collected in Karnataka and G for those collected from Goa.

The result for the gut samples from the leeches belonging to the same batch, namely GB5 (untreated) and Haridra treated samples GA4 and GA5 (same set) are shown in Table 2.

Table 2: Comparative analysis of metagenomics results of GB5 with GA4 and GA5

Phylum	Percentage population			Absolute count (n=2)*		
	GB5	GA4	GA5	GB5	GA4	GA5
Proteobacteria	79.4488	68.2949	42.3428	269433	41916	26897
Bacteroidetes	17.0818	5.8574	4.0285	57929	3595	2559
Actinobacteria	1.2172	11.1397	15.4545	4128	6837	9817
Acidobacteria	0.7322	6.5711	11.6841	2483	4033	7422
Chloroflexi	0.4355	3.0289	8.9764	1477	1859	5702
Cyanobacteria	0.2928	0.8228	0	993	505	0
Planctomycetes	0.258	1.6293	6.1947	875	1000	3935
Firmicutes	0.2082	0.9222	5.9098	706	566	3754
AD3	0.1141	0.3226	0.3951	387	198	251
Verrucomicrobia	0.1109	0.6126	3.0352	376	376	1928
WPS-2	0.0528	0.3715	0.9697	179	228	616
Gemmatimonadetes	0.0248	0.0049	0.4172	84	3	265
Elusimicrobia	0.0106		0.0929	36		59
GAL15	0.0077		0	26		0
Fusobacteria	0.0029		0	10		0
WS3	0.0018		0.0189	6		12
TM7	0	0.2868	0.4266	0	176	271
Synergistetes	0		0.0535	0		34
fbp		0.132			81	
Tenericutes		0.0033			2	
OD1						
Total	100	100	100	339128	61375	63522

*Gut samples from two leeches (2n) were pooled and subjected to metagenomic analysis.

Guts extracted from two untreated leeches gave an absolute total count of 3.39×10^5 . Guts obtained from Haridra treated leeches post Haridra treatment gave a total count of 6.14×10^4 and 6.35×10^4 respectively for GA4 and GA5. Bacterial count in the guts post Haridra treatment was 81-82% lower than the guts extracted from untreated leeches. Percentage of bacteria belonging to Proteobacteria and Bacteroidetes was significantly higher in untreated samples,

while in both the Haridra treated samples GA4 and GA5, it was significantly low. Absolute count of microbes classified under the phylum proteobacteria was 85-90% less in GA4 and GA5 in comparison to the gut samples extracted from untreated leeches.

Role played by the gastrointestinal microbial flora in human health and disease is well known. However, it is likely that the GIT microbiota of leeches may affect the health of patients undergoing leech therapy.

Complex community of microbiota exists in the gastro intestinal tract of animals.

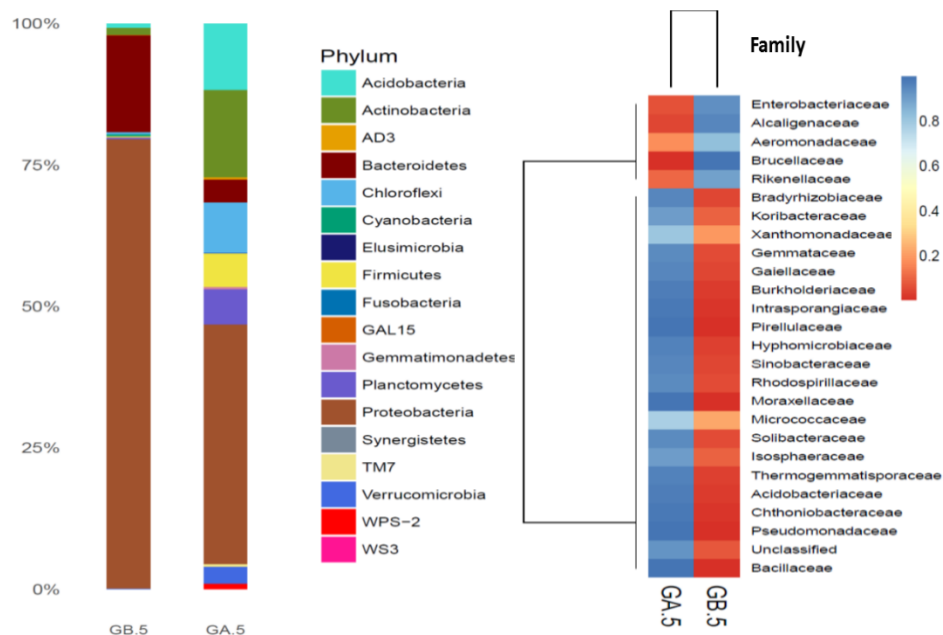


Figure 1: Phylum level bar plot and family level heat map for GB5 and GA5

Proteobacteria, Bacteroidetes, Actinobacteria forms the major phyla of microbiota. The phylum Proteobacteria includes wide genera of microbes. Proteobacterial load is often suggested as a potential diagnostic criterion for dysbiosis and disease. [7] Many pathogenic and opportunistic organisms such as Escherichia, Salmonella, Vibrio, Helicobacter etc. are included in this phylum. The group is defined primarily in terms of ribosomal RNA (rRNA) sequences. These gram negative microbes are categorized into six classes namely, Alphaproteobacteria, Beta proteobacteria, Gamma proteobacteria, Delta proteobacteria, Epsilon proteobacteria and Zeta proteobacteria. The group of pathogens, belonging to the order Rickettsiales is classified under Alphaproteobacteria. Epsilon proteobacteria consists the genera which comprises mainly the curved to spirilloid species such as Wolinella spp., Helicobacter spp., and Campylobacter spp. Most of these species inhabit the digestive tract of animals and serve as symbionts (Wolinella spp. in cows) or pathogens (Helicobacter spp. in the stomach, Campylobacter spp. in the

duodenum). The microbial symbionts are responsible for the digestion of the blood meal or the symbionts provide essential nutrients that the leech is unable to synthesize for itself and cannot derive in sufficient quantities from the blood meal directly. [8]

Three large classes of Gram-negative, non spore forming anaerobic/aerobic, rod-shaped bacteria which are widely distributed in the environment, including soil, sediments, and sea water are classified under the phylum Bacteroidetes. These are also found in the guts and on the skin of animals. By far, the microbes in the class Bacteroidia including the genus Bacteroides (an abundant organism in the feces of warm-blooded animals including humans), and Porphyromonas, a group of organisms inhabiting the human oral cavity are the most well-studied. Some members of the genus Bacteroides are opportunistic pathogens.

The members belonging to the phylum Chlamydiae are obligate intracellular pathogens. A common sexually transmitted infection (STI) in humans and psittacosis is caused by Chlamydia species. Actinobacteria are well-known as secondary

metabolite producers and are hence of high pharmacological and industrial interest. However a few species belonging to this class are known to inhabit plants and animals, including a few pathogens, such as Mycobacterium, Corynebacterium etc. Most microbes categorized under *Firmicutes* have Gram-positive cell wall structure and are a core group of related forms called the low-G+C group, in contrast to the Actinobacteria. The blue-green bacteria Cyanobacteria, are a phylum of bacteria which obtain their energy through photosynthesis.

The bacteria of medical significance such as the Enterobacteriaceae, Vibrionaceae and Pseudomonadaceae are categorized under the class Gamma proteobacteria. This class comprises of Salmonella spp., Yersinia pestis (plague), Vibrio cholerae, Pseudomonas aeruginosa and Escherichia coli. *Aeromonas hydrophila* and *Aeromonas veronii* biovarsobria, found in the gut contribute to the digestion of ingested blood. *A. hydrophila* is generally regarded to be more pathogenic in humans than *A. veronii*. [9]

Enterobacteriaceae members which contain group of major pathogenic organisms were thus found to be present in lower amounts in the guts of leeches treated

with Haridra. Significant difference in the abundance of species belonging to aeromonadaceae can be observed as per the bar plot and heatmap.

The results of analysis for GB9 and GA9 are presented in Table 3. Total count of bacteria in treated samples was 34% less than the count in untreated samples.

Phylum	GB9 (Untreated)*		GA9 (Treated)*	
	Absolute Count	Percent	Absolute Count	Percent
Proteobacteria	141331	87.6363	100233	94.4125
Bacteroidetes	10299	6.3862	5457	5.1401
Firmicutes	4234	2.6254	330	0.3108
Cyanobacteria	2846	1.7647	64	0.0603
Actinobacteria	1533	0.9506	51	0.048
Planctomycetes	948	0.5878		
Synergistetes	61	0.0378	2	0.0019
Chloroflexi	10	0.0062		
OD1	5	0.0031		
TM6	1	0.0006		
SR1	1	0.0006		
Euryarchaeota	1	0.0006		
Fusobacteria			21	0.0198
Elusimicrobia			7	0.0066
Total	161270	99.9999	106165	100

*n=2

Table 3: Comparative analysis of metagenomics results of GB9 and GA9

Although the percentage of proteobacteria post treatment was 94% of total population, count of microbes belonging to the phyla proteobacteria and bacteroidetes was significantly less than the guts of untreated leeches.

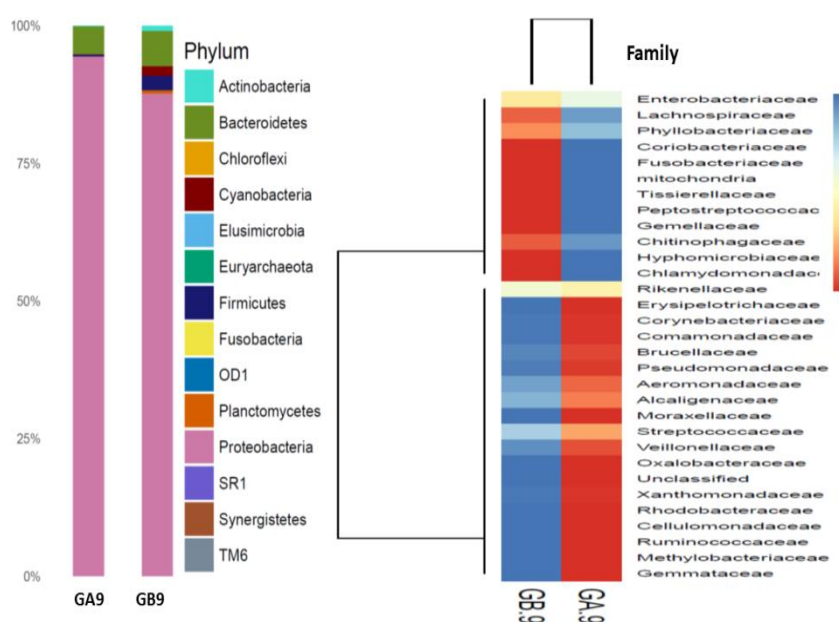


Figure 2: Phylum level bar plot and family level heatmap for GB9 and GA9

Gut samples extracted from two leeches collected in Karnataka showed a count of 1.02×10^5 (Table 4). Guts extracted from Haridra treated leeches gave a total count of 6.07×10^4 . Thus bacterial count in Haridra treated samples was 61% in comparison to the bacterial count picked from the guts of the untreated leeches. The gut samples collected from Haridra treated leeches showed significantly lower counts of bacteria belonging to the phyla Proteobacteria and Bacteroidetes.

Table 4: Comparative analysis of metagenomics results of LB5 and LA5

Phylum	LB5 (Untreated)*		LA5 (Treated)*	
	Absolute Count	Percent	Absolute Count	Percent
Proteobacteria	52455	51.3058	9100	14.99
Firmicutes	25954	25.3854	15601	25.6988
Bacteroidetes	15502	15.1624	2298	3.7854
SR1	4458	4.3603	17742	29.2256
TM7	1118	1.0935	1469	2.4198
Cyanobacteria	1107	1.0827		
Actinobacteria	1099	1.0749	1498	2.4676
Synergistetes	395	0.3863	12987	21.3929
Tenericutes	136	0.133		
OD1	16	0.0156		
Verrucomicrobia			10	0.0165
Planctomycetes			1	0.0016
Euryarchaeota			1	0.0016
Total				
	102240	100	60707	100

* n=2 per each set

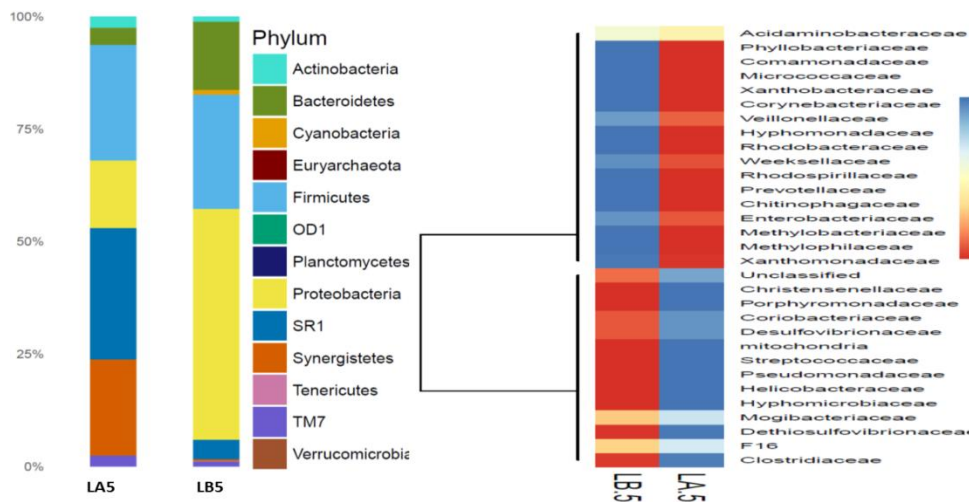


Figure 3: Bar plot and heatmap depicting distribution of bacterial phyla and families for LB5 and LA5

Presence of bacteria categorized under phylum SR1 was noted in the gut samples. This recently discovered phylum consists of bacteria found in marine environments, fresh water lakes and subsurface aquifers, terrestrial high temperature regions. These bacteria are yet to be cultivated in laboratory conditions. SR1 bacterial genomes have been identified in samples collected from animal guts and termites.

Although the count of proteobacterial members was significantly lower in treated samples, helicobacter species appeared to dominate in the phylum.

As the microbial count done by laboratory culture methods on soyabean casein digest agar showed that leeches

collected in the rainy season with significant amount of blood sucked in their gut had shown inefficacy of Haridra treatment, the leeches were collected during the rainy season (during the months of August) and subjected to treatment. The gut samples from four leeches were pooled for each set namely LB13, LB14, LA13 and LA14. LB13 when suspended in saline, released significant amount of blood and viable count showed presence of high microbial load 3.5×10^7 per gut. The results of metagenomics analyses are presented in Table 5. Leeches chosen for Haridra treatment appeared to harbor significant number of microbes with a bacterial count of 2.49×10^5 , while the untreated samples showed a count of 1.22×10^5 . However,

Haridra treated samples in spite of harboring higher load of microbes showed presence of significantly lower count of proteobacteria.

Firmicutes dominated the gut samples in LA14, followed by bacteroidetes.

Table 5: Comparative analysis of metagenomics results of LA14 and LB14

Phylum	LB14 (Untreated)*		LA14 (Treated) *	
	Absolute Count	Percent	Absolute Count	Percent
Proteobacteria	117063	95.5694	83818	33.6387
SR1	3655	2.9839		
Synergistetes	897	0.7323	92	0.0369
Firmicutes	558	0.4555	110544	44.3647
Bacteroidetes	154	0.1257	36633	14.702
Actinobacteria	114	0.0931	10360	4.1578
OD1	23	0.0188	118	0.0474
Planctomycetes	7	0.0057	1058	0.4246
Verrucomicrobia	6	0.0049	289	0.116
Cyanobacteria	5	0.0041	4588	1.8413
Acidobacteria	4	0.0033	1076	0.4318
TM7	1	0.0008		
[Thermi]	1	0.0008	21	0.0084
Tenericutes	1	0.0008	3	0.0012
Gemmatimonadetes	1	0.0008	48	0.0193
Chloroflexi			355	0.1425
Nitrospirae			84	0.0337
GAL15			84	0.0337
Total	122490	99.9999	249171	100

*n=4

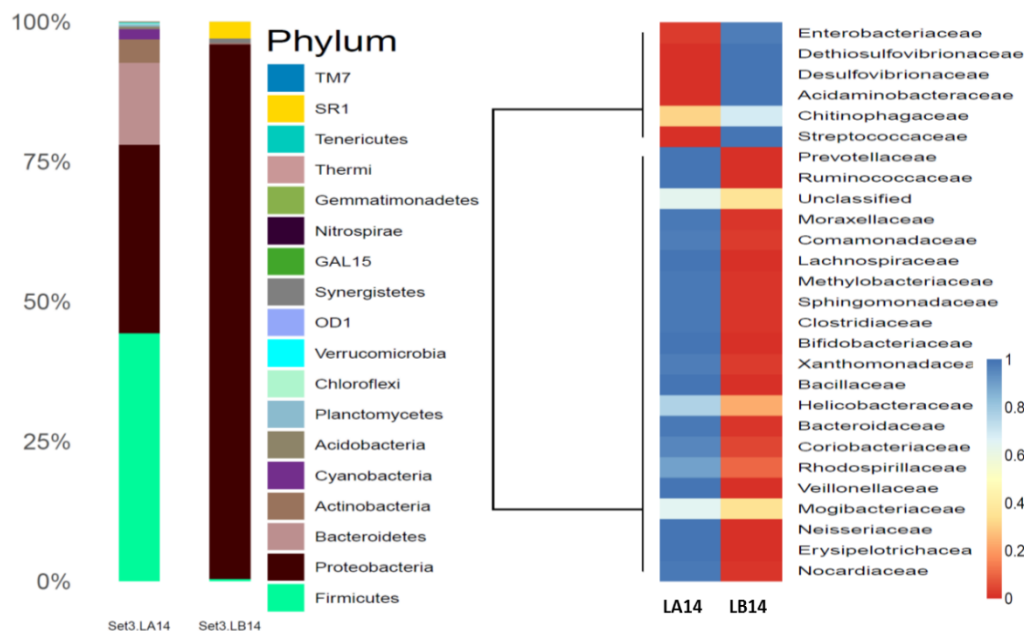


Figure 4: Phylum level bar plot and family level heat map for LB14 and LA14

Heatmap for the family level distribution shows that the abundance of family harbouring most of the pathogenic species i.e. enterobacteriaceae was significantly lower in treated samples while the abundance of helicobacteriaceae members was perhaps unaffected in treated samples. Similar results were observed for helicobacteriaceae family in batch of LA5/LB5. Interestingly, both

enterobacteriaceae and helicobacteriaceae belong to the phylum Proteobacteria.

As enterobacteriaceae was found to be one of the dominant family belonging to the phylum Proteobacteria comprising of many bacteria of clinical significance, microbial load in pooled gut samples (n=4) of treated and untreated samples from the same batch (sets GB13 and GA13) was

analysed using CSDA and, MacConkey's agar for enteric bacteria.

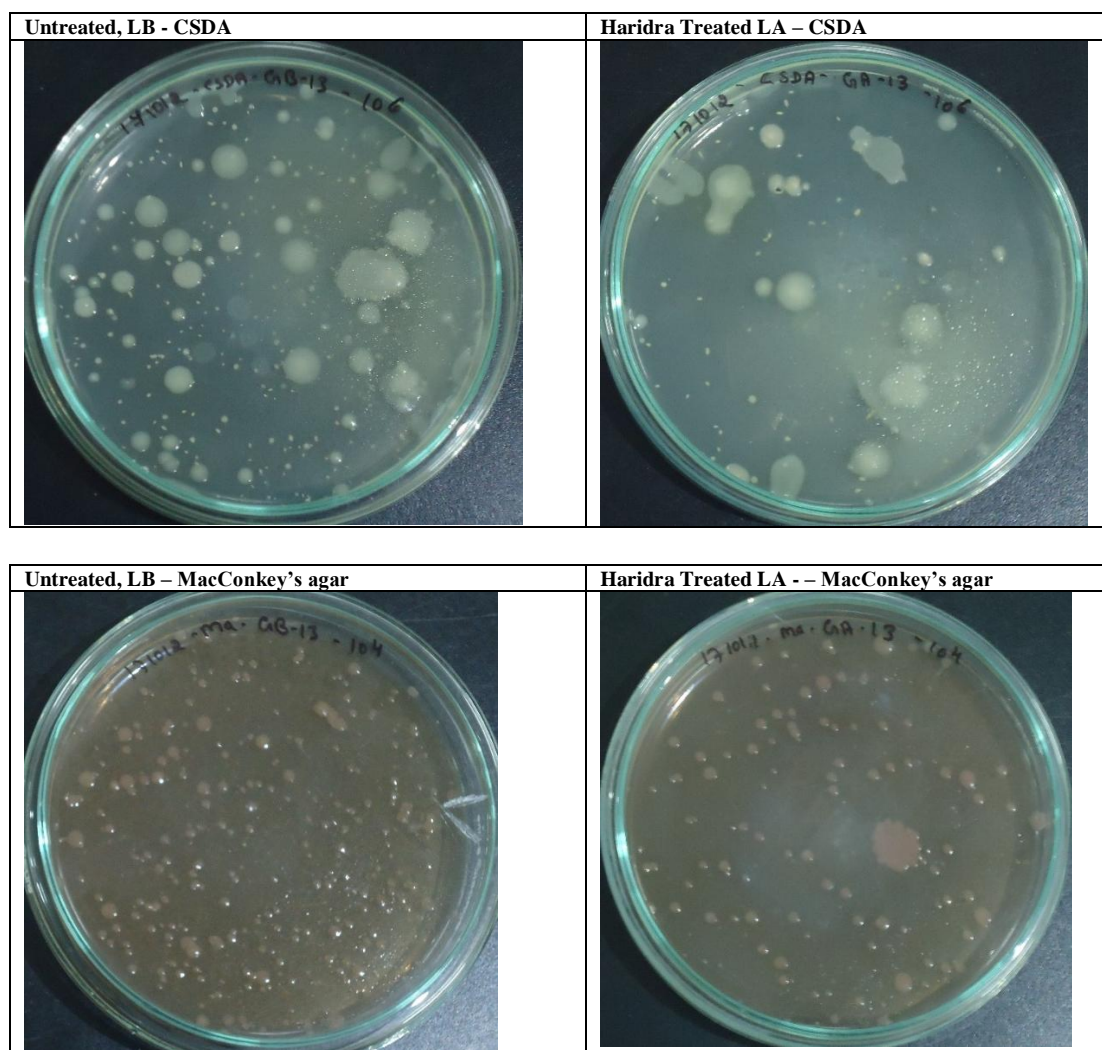


Figure 5: Bacterial count of samples in Casein soyabean digest agar and MacConkey's agar

As can be seen in the figures 5, there was significant reduction in the total microbial count and enteric count in Casein soyabean digest agar (CSDA) and MacConkey's medium respectively.

During the course of research work, it was observed that none of the patients undergoing Jalukavacharana showed any signs of infection secondary to Jalukavacharana.

Haridra by irritating the leech may induce vomiting due to which there is a possibility that loosely attached, planktonic and motile organisms may be easily dislodged and vomited out via the treatment must be considered. Curcumin and rhizome extract of *C. longa* has antimicrobial activity against different bacteria, viruses, fungi, and

parasites. [10] A wide variety of factors like the type of bacteria, their ability to associate with each other and to the host tissue and their sensitivity to Haridra may affect the efficacy of Haridra treatment at reducing the bacterial count.

CONCLUSION

Microbial culture studies showed great variation in the gut microbial load ranging from 1.4×10^4 to 5.8×10^5 (40 fold). However, in the leeches subjected to metagenomics analyses, the microbial load in the varied from 1×10^5 to 3.4×10^5 (3.4 fold). A common observation made in all the gut samples tested was the predominance of Proteobacteria (51.3-95.5%). With the exception of LA14 and

LB14, the Haridra treated samples showed lower microbial load. However, the significant variation in microbial load in the guts of untreated leeches must be taken into account before claiming efficacy of treatment in reduction of microbial load. The result of significance however is the lower number of the bacterial species belonging to phyla Proteobacteria and Bacteroidetes especially the members belonging to proteobacteria in gut samples obtained from treated leeches (including LA14) collected from both Goa and Karnataka. As the phylum Proteobacteria comprises of many disease causing bacteria, the efficacy of Haridra in inhibiting proteobacterial members and thus the safety of Jalukavacharna need to be further investigated in detail.

Conflicts of Interest: Nil

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