



## Research article

## Arsenic disturbs the gut microbiome of individuals in a disadvantaged community in Nepal



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## ABSTRACT

Arsenic is ubiquitous in nature, highly toxic, and is particularly abundant in Southern Asia. While many studies have focused on areas like Bangladesh and West Bengal, India, disadvantaged regions within Nepal have also suffered from arsenic contamination levels, with wells and other water sources possessing arsenic contamination over the recommended WHO and EPA limit of 10 µg/L, some wells reporting levels as high as 500 µg/L. Despite the region's pronounced arsenic concentrations within community water sources, few investigations have been conducted to understand the impact of arsenic contamination on host gut microbiota health. This study aims to examine differential arsenic exposure on the gut microbiome structure within two disadvantaged communities in southern Nepal. Fecal samples (n = 42) were collected from members of the Mahuawa (n = 20) and Ghana-shyampur (n = 22) communities in southern Nepal. The 16S rRNA gene was amplified from fecal samples using Illumina-tag PCR and subject to high-throughput sequencing to generate the bacterial community structure of each sample. Bioinformatics analysis and multivariate statistics were conducted to identify if specific fecal bacterial assemblages and predicted functions were correlated with urine arsenic concentration. Our results revealed unique assemblages of arsenic volatilizing and pathogenic bacteria positively correlated with increased arsenic concentration in individuals within the two respective communities. Additionally, we observed that commensal gut bacteria negatively correlated with increased arsenic concentration in the two respective communities. Our study has revealed that arsenic poses a broader human health risk than was previously known. It is influential in shaping the gut microbiome through its enrichment of arsenic volatilizing and pathogenic bacteria and subsequent depletion of gut commensals. This aspect of arsenic has the potential to debilitate healthy humans by contributing to disorders like heart and liver cancers and diabetes, and it has already been shown to contribute to serious diseases and disorders, including skin lesions, gangrene and several types of skin, renal, lung, and liver cancers in disadvantaged areas of the world like Nepal.

## 1. Introduction

In South East Asia, an event that many are calling “the worst mass poisoning in history” is underway [1]. Millions of people living along the Mekong, Yangtze, Red, and Ganges rivers are exposed to arsenic

concentrations well over the WHO and EPA recommended limit of 10 µg/L (10 µg/L), with regions of India and Bangladesh reaching concentrations as high as 4730 µg/L [2, 3, 4, 28, 29, 30]. The high arsenic concentration can be mostly attributed to weathering of arsenic rich iron Fe (III) oxide sediments from the Himalayan Mountains, which has led to

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mass dispersal of arsenic rich sediments across South East Asia, much of it trapped in aquifer sands. Population growth in the past decade has led to increased demand for tube wells which expose trapped arsenic sulfides and pyrites to reductive dissolution and competitive replacement by stronger anions like phosphates and bicarbonates. While the arsenic crisis in Bangladesh and West Bengal, India has been well documented since the 1990s, it has only been in the last 18 years that countries like Vietnam and Cambodia have reported high arsenic concentration levels due to microbial contamination of surface water, installation of tubewell to prevent water borne diseases and reduce the children mortality [5].

Arsenic causes poisoning of the human body by disrupting glycolysis, namely by inhibiting acetyl-CoA and succinic dehydrogenase [6, 7]. Along with its metabolic disruption, arsenic exposure is known to increase the risk of hepatitis [8], several types of cancers, including lung and liver [9], and has been linked to the development of type II diabetes mellitus [10, 11]. Arsenic's influential role in the development of cancer is through binding to glutathione, a fundamental cellular antioxidant [12]. Once arsenic is bound, glutathione removes it from the cell. Higher cellular glutathione levels correspond with lower cellular arsenic levels. However, it has been reported that a low dietary intake of protein can lead to lower levels of intracellular glutathione in the liver [13, 14, 15]. Therefore, disadvantaged populations like those in South East Asia are particularly susceptible to the toxin.

In addition to the direct negative influence arsenic has on human health, its secondary effects through influence of the human gut microbiome are also well documented [16, 17, 18, 19]. Previous studies conducted by Dheer *et al.* [20] and Lu *et al.* [21] reported that adult mice exposed to arsenic display compositional and metabolic changes to their gastrointestinal tract microbiota. High concentrations of arsenic (10 µg/L) have also been shown to decrease levels bacteria commonly found within the human gut [20, 21, 22]. Arsenic can further alter the human gut microbiome by promoting the growth of arsenic-metabolizing bacteria like *Bilophila*, *Desulfovibrio*, and *Bacillus* [23, 24, 25, 26, 27]. These bacteria can metabolize arsenate (As<sup>V</sup>) and arsenite (As<sup>III</sup>), producing nitrogen metabolites toxic to the host [20].

While studies on the effects of arsenic on human health in Bangladesh and West Bengal, India in the past decade have been prolific, few studies have examined these same effects in Nepal and fewer have studied arsenic's effect on the human gut microbiome. Our study represents one of the first to examine arsenic's direct influence on the human gut microbiome. The goal of this study is to identify compositional changes to the gut microbiome induced by arsenic exposure that could be indicative of a pathogenic state. Further, this study will provide preliminary evidence to support supplementary studies that will examine the long-term effects of arsenic exposure on the microbiota in a disadvantaged population, such as that found in rural Nepal.

## 2. Methods

### 2.1. Sample collection

In this study, 44 low-income homes (Mahuawa (n = 20) and Ghanashyampur (n = 22)) in remote areas of Nepal, where arsenic is prevalent in potable well water were surveyed. This region of Nepal, Nawalparasi, has wells that report some of the highest levels of arsenic in the entire country, with some wells contaminated with as much as 500 µg/L arsenic [28]. This stands in contrast to wells in other districts of Nepal which generally report arsenic concentration levels that are below 10 µg/L [29]. Well arsenic concentrations are comparable to levels reported in Bangladesh of >200 µg/L, though falls short of one of the highest levels reported in that country of 4730 µg/L [28, 30]. The samples were collected from two different communities of Ghanashyampur and Mahuawa. Participants were recruited with the help of an NGO (non-governmental organization), the Environmental and Public Health Organization (ENPHO) based in Kathmandu, Nepal. Fecal and urine samples were collected from study participants for microbiome

sequencing and arsenic testing, respectively. Volunteers were requested to provide answers to a screening questionnaire consisting of questions designed to gather basic information on the health of the residents, medical histories, the number and age, and the history and condition of the home. All protocols and procedures were IRB (Institutional Review Board) approved.

For this study, information on arsenic concentration in the urine, age, sex, and the community individuals were from as well as their VDC (Village Development Committee) was collected to inform the multi-variate statistical analyses. For further analysis of the effects of arsenic on the gut microbiome, the arsenic concentration levels were categorically grouped by exposure level in the "Arsenic Exposure Level" metadata category. Individuals with urine arsenic concentrations of 0 µg/L were categorized as "Undetected", those with urine arsenic concentrations between 0 µg/L and 10 µg/L were categorized as "Moderate" and individuals with urine arsenic concentrations above 10 µg/L were categorized as having a "High" exposure level. Urine samples were measured using Hybrid Generation/Atomic Absorption Spectrometry method following Standard Methods 3114B (APHA 2012) [69].

### 2.2. PCR amplification methods

Illumina iTag Polymerase Chain Reactions (PCR) were performed at a total volume of 25 µL for each sample and contained final concentrations of 1X PCR buffer, 0.8 mM dNTP's, 0.625 U Taq, 0.2 µM 515F forward barcoded primer, 0.2 µM Illumina 806R reverse primer and ~10 ng of template DNA per reaction. PCR was carried out on a MJ Research PTC-200 thermocycler (Bio-Rad, Hercules, CA) using the following cycling conditions: 98 °C for 3 min; 35 cycles of 98 °C for 1 min, 55 °C for 40 s, and 72 °C for 1 min; 72 °C for 10 min; and kept at 4 °C. PCR products were visualized on a 1% SYBRsafe E-gel (ThermoFisher Scientific, Waltham, MA).

### 2.3. Library purification, verification and sequencing methods

Pooled PCR products were gel (2% Seakem LE agarose (Lonza Rockland, Inc, Rockland Maryland), stained with Gelstar (Lonza Rockland, Inc, Rockland Maryland) ethidium bromide) purified using the Qiagen Gel Purification Kit (Qiagen, Frederick, MD). Clean PCR products were quantified using the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA), and samples were combined in equimolar amounts. Prior to submission for sequencing, libraries were quality checked using the 2100 Bioanalyzer DNA 1000 chip (Agilent Technologies, Santa Clara, CA). Pooled libraries were stored at -20 °C until they were shipped on dry ice to Laragen (Culver City, CA) for sequencing. Library pools were size verified using the Fragment Analyzer CE (Advanced Analytical Technologies Inc., Ames IA) and quantified using the Qubit High Sensitivity dsDNA kit (Life Technologies, Carlsbad, CA). After dilution to a final concentration of 1 nM and a 10% spike of PhiX V3 library (Illumina, San Diego CA), pools were denatured for 5 min in an equal volume of 0.1 N NaOH then further diluted to 12 pM in Illumina's HT1 buffer. The denatured and PhiX-spiked 12 pM pool was loaded on an Illumina MiSeq V2 500 cycle kit cassette with 16S rRNA library sequencing primers and set for 250 base, paired-end reads. Arsenic levels in urine samples were measured using Atomic Absorption Spectroscopy at Environmental and Public Health Organization, Kathmandu, Nepal.

### 2.4. Quality filtering and OTU picking methods

Paired-end sequences were trimmed at a length of 251 bp and quality filtered at an expected error of less than 0.5% using USEARCH v7 [31]. After quality filtering, reads were analyzed using the QIIME 1.9.0 software package [32, 33]. Chimeric sequences were identified using USEARCH7 [34]. A total of 2,127,938 sequences were obtained after quality filtering and chimera checking. Open reference operational taxonomic units (OTUs) were picked using the USEARCH7 algorithm

[34], and taxonomy assignment was performed using the Greengenes 16S rRNA gene database (13-5 release, 97%) [35]. Assigned taxonomy were organized into a BIOM formatted OTU (operational taxonomic unit) table, which was summarized at the genus level (L6) within QIIME 1.9.0 [32].

## 2.5. Microbial community analysis

Three separate OTU tables were used for analysis. The first contained all samples and was used to compare phylogenetic differences between communities. The second and third were filtered to contain only samples within the Ghanashyampur and Mahuawa communities, respectively. Principal coordinates analysis (PCoA) plots and ADONIS tests for significance were calculated from a weighted UniFrac distance matrix generated from a CSS normalized OTU table within Qiime 1.9.0 [32]. Distance box plots were generated using the weighted UniFrac distances

to quantify the variation in sample composition observed in the PCoA plots. Adonis and ANOSIM tests for significance were calculated to determine significance of beta diversity clustering between experimental cohorts of interest grouped by arsenic concentration. ANOSIM and Adonis p-values under 0.05 were considered significant. Linear discriminant analysis effect size (LEfSe) biomarker identification analysis was conducted to identify significantly enriched (LDA >1, P < 0.05) bacterial taxa within each respective arsenic concentration cohort [36]. Genus-level relative abundances were multiplied by one million and were formatted as described in Segata et al. [36]. Initial comparisons were made with “Community” as the main categorical variable (Mahuawa or Ghanashyampur). Subsequent LEfSe comparisons were conducted with “Arsenic Exposure Level” (“Undetected”, “Moderate” and “High”) as the main categorical variable. Linear Discriminant Analysis scores for the enriched taxa within each class were then plotted. Features were plotted on a logarithmic scale according to the experimental group

**Table 1.** Summary of sample metadata.

Sample ID	Community	Arsenic Conc. in Urine	Arsenic Exposure Level	Age	Sex
A1	Ghanashyampur	0.026	High	49	M
A2	Ghanashyampur	0.026	High	33	M
A3	Ghanashyampur	0.014	High	45	F
A5	Ghanashyampur	0.045	High	44	F
A6	Ghanashyampur	0.012	High	45	F
A7	Ghanashyampur	0.011	High	49	M
A8	Ghanashyampur	0.013	High	45	M
A9	Ghanashyampur	0.009	Moderate	40	F
A10	Ghanashyampur	0.011	High	45	F
A11	Ghanashyampur	0.013	High	37	F
A13	Ghanashyampur	0.019	High	35	F
A14	Ghanashyampur	0.027	High	16	F
A15	Ghanashyampur	0.014	High	55	F
A16	Ghanashyampur	0	Undetected	28	M
A17	Ghanashyampur	0.012	High	17	F
A18	Ghanashyampur	0	Undetected	28	M
A19	Ghanashyampur	0.103	High	16	M
A20	Ghanashyampur	0.021	High	17	F
A21	Ghanashyampur	0.008	Moderate	17	M
A22	Ghanashyampur	0.013	High	16	F
A24	Ghanashyampur	0.009	Moderate	40	F
A25	Ghanashyampur	0	Undetected	48	F
B1	Mahuawa	0.011	High	60	M
B2	Mahuawa	0	Undetected	65	M
B3	Mahuawa	0.006	Moderate	71	F
B4	Mahuawa	0	Undetected	65	F
B5	Mahuawa	0	Undetected	29	M
B6	Mahuawa	0	Undetected	53	F
B7	Mahuawa	0.008	Moderate	45	F
B8	Mahuawa	0	Undetected	60	F
B9	Mahuawa	0	Undetected	61	M
B10	Mahuawa	0	Undetected	51	F
B11	Mahuawa	0.013	High	45	F
B12	Mahuawa	0	Undetected	45	F
B13	Mahuawa	0.008	Moderate	45	M
B14	Mahuawa	0.005	Moderate	51	F
B16	Mahuawa	0	Undetected	45	M
B17	Mahuawa	0.006	Moderate	65	M
B18	Mahuawa	0.05	High	57	M
B19	Mahuawa	0.035	High	35	F
B20	Mahuawa	0.031	High	57	M
B23	Mahuawa	0	Undetected	13	F

to which they were significantly associated. LEfSe explicitly requires all pairwise comparisons to reject the null hypothesis for detecting the biomarker taxa, thus, no multiple testing corrections were needed [36].

Alpha diversity indices, including Chao1, Heip's evenness, Observed Species, and PD Whole Tree, were generated from an unrarified OTU table with a max sampling depth of 1600, a step size of 140, and with 20 iterations at each step. A CSS normalized OTU and an accompanying continuous metadata table were formatted for RStudio [37]. Correlation plots were generated in RStudio using the "Hmisc" [38] and "corrplot" [39]. Heatmaps were generated in RStudio using the "pheatmap" [40], "phyloseq" [41], "ggplot2" [42], "vegan" [43], "dplyr" [44], "scales" [45], "grid" [46], and "reshape2" [47] packages. Input OTU tables for each individual community were filtered so that only known pathogenic and arsenic volatilizing bacteria were visualized within the final correlogram plot.

### 3. Results

#### 3.1. Sequencing results and description of study population

A total of 44 fecal samples were collected from the Mahuawa (n = 20) and Ghanashyampur (n = 22) communities within Nepal for 16S rRNA sequencing and analysis. The Illumina MiSeq yielded over 2,440,769 raw 16S rRNA sequences across all samples of which 2,127,938 sequences were maintained after quality filtering and chimera checking. The mean arsenic concentration for the Mahuawa community (n = 20) was 0.009 ( $\pm 0.014$ )  $\mu\text{g/L}$  and 0.018 ( $\pm 0.021$ )  $\mu\text{g/L}$  for the Ghanashyampur community (n = 22). A student's t-test (p = 0.085) revealed that the difference in arsenic concentration level was not significant. Table 1 shows a summary of the key characteristics of the samples within each community. In addition, Table 2 shows the Adonis R<sup>2</sup> value, the degree to which specific metadata items contribute to differences in bacterial community composition within samples. When comparing samples from both communities, "Arsenic Concentration in Urine" contributed the most to differences in bacterial community composition.

#### 3.2. Bacterial community structure comparisons

No significant differences in alpha diversity distance boxplots were observed between sampled communities or summarized ("Undetected", "Moderate", "High") arsenic concentration groupings when considering the observed species richness metric (Figure 1). No significant differences existed between the mean observed species of each categorical grouping (Mahuawa\_Undetected 73.505 observed species; Mahuawa\_Moderate 54.26 observed species; Mahuawa\_High 85.92 observed species; Ghanashyampur\_Undetected 67.13 observed species; Ghanashyampur\_Moderate 47.92 observed species; Ghanashyampur\_High 59.98 observed species) (Figure 1). Though there were no significant differences between arsenic exposure groupings, comparison of the two communities through

**Table 2.** Adonis R<sup>2</sup> value describes the degree to which specific metadata items contribute to differences in bacterial community composition within samples. When comparing samples from both communities, "Arsenic Concentration in Urine" contributed the most to differences in bacterial community composition.

	R <sup>2</sup>
Community	0.0245 (2.45%)
Arsenic Concentration in Urine	0.04365 (4.36%)
Age	0.03908 (3.91%)
Arsenic Concentration in Urine	
Ghanashyampur	0.0517 (5.17%)
Mahuawa	0.1407 (14.07%)
Age	
Ghanashyampur	0.08458 (8.46%)
Mahuawa	0.07093 (7.09%)

the species richness metric "observed species" revealed that Mahuawa, the community with lower urine arsenic concentrations, had greater species richness, or differential species present in a given category, than samples from Ghanashyampur which had greater urine arsenic concentrations. Figure 2 shows that the Mahuawa community had greater overall average species richness than the Ghanashyampur community. A smaller number of observed species is indicative of a more defined bacterial community structure.

Paired LDA effect size (LEfSe) analyses were performed to identify significant increases in the relative abundance (LDA cutoff score of >1.0 [P < 0.05]) of bacterial taxa in samples from the Mahuawa and, the more heavily contaminated, Ghanashyampur communities (Figure 3). Members of the order Methanobacteriales (p = 0.0247) such as *Methanobrevibacter* (p = 0.0247) and Methanobacteraceae (p = 0.0247) were among some of the most significantly enriched taxa within the Mahuawa community. Additionally, Desulfovibrionales (p = 0.019), Desulfovibrionaceae (p = 0.012) and *Desulfovibrio* (0.027) were also significantly enriched in the Mahuawa community along with  $\delta$ -Proteobacteria (p = 0.019) and Anaeroplasmataceae (p = 0.030), a member of the  $\alpha$ -Proteobacteria class. *Citrobacter* (p = 0.021), a member of the  $\gamma$ -Proteobacteria class was also enriched within the Mahuawa community. LEfSe analysis reported that the Ghanashyampur community had significant enrichment of the pathogens *Treponema* (p = 0.026), Spirochaetaceae (p = 0.026), Spirochaetales (p = 0.026) and Spirochaetes (p = 0.026).

Enriched taxonomy within categorical arsenic concentration groupings were identified within respective Mahuawa and Ghanashyampur communities (Figures 6 and 7; LDA <1, p < 0.05). Comparison of the "High" and "Undetected" categorical arsenic concentration groupings (Figure 6A) within the Mahuawa community revealed that Erysipelotrichales (p = 0.037) was significantly enriched within the "Undetected" category, and Bacillaceae (p = 0.038) was significantly enriched within the "High" category. A similar comparison of the "Moderate" and "Undetected" categorical arsenic concentration groupings within the Mahuawa community (Figure 6B) revealed the significant enrichment of the Erysipelotrichi class (p = 0.0033) within the "Undetected" category. Comparison of the "High" and "Undetected" categorical grouping in the Ghanashyampur only showed *Collinsella* (p = 0.048) as significantly enriched within "High" category (Figure 7A). Comparison of the "Moderate" and "Undetected" categories within the Ghanashyampur community revealed significant enrichment of the Erysipelotrichaceae (p = 0.049) and  $\gamma$ -Proteobacteria (p = 0.049) within the "Undetected" category (Figure 7B). *Lactobacillus* (p = 0.049) and Lactobacillaceae (p = 0.049) are the sole taxa enriched within the "Moderate" category of the Ghanashyampur community.

Principal coordinate analysis (PCoA) revealed significant differences in beta diversity between samples considering urine arsenic concentration ( $\mu\text{g/L}$ ) within the Mahuawa community (Adonis p = 0.009), but not within the Ghanashyampur community (Adonis p = 0.267) (Figure 4A, B).

Positive and negative correlations between bacterial taxa and arsenic concentration in urine were identified through QIIME 1.9.0 using a Spearman's Rank Order Correlation ( $\rho < -0.40$ ,  $\rho > 0.40$ ; p < 0.05) (Figure 5A, B). Within the Mahuawa community *Catenibacterium*, a member of the Erysipelotrichaceae family was negatively correlated ( $\rho = -0.48$ , p = 0.033) with arsenic concentration in urine, while the family Bacillaceae, was positively correlated ( $\rho = 0.52$ , p = 0.017) with arsenic concentration in urine. Additionally, *Ruminococcus* ( $\rho = -0.55$ , p = 0.009) and Clostridiaceae ( $\rho = -0.49$ , p = 0.024), both members of the Firmicutes phylum, were negatively correlated with arsenic concentration in urine. In the Ghanashyampur community *Haemophilus* ( $\rho = -0.48$ , p = 0.025) and *Luteimonas* ( $\rho = -0.47$ , p = 0.028), both members of the Proteobacteria phylum, negatively correlated with arsenic concentration in urine while additional members within Proteobacteria, Desulfovibrionaceae ( $\rho = 0.45$ , p = 0.043) and member genus *Bilophila* ( $\rho = 0.57$ , p = 0.0053) were negatively correlated with arsenic concentration in urine along with *Succinivibrio* ( $\rho = 0.43$ , p = 0.045).

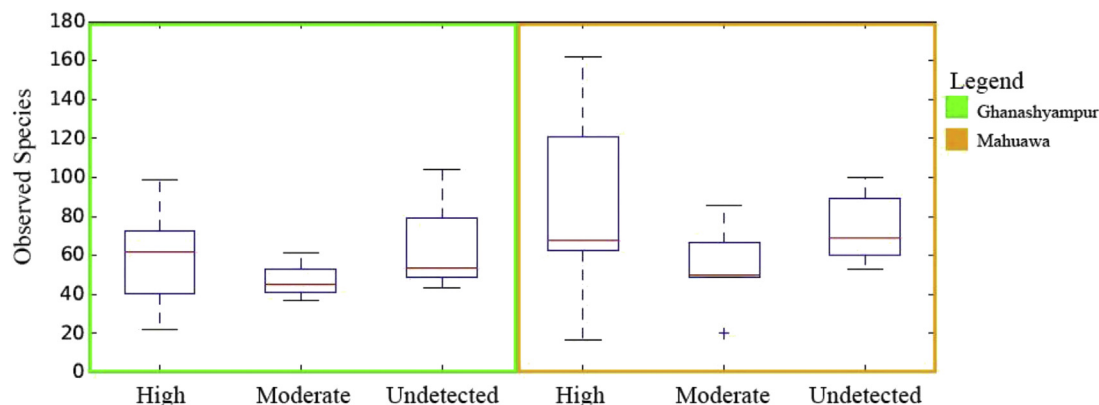


Figure 1. Average species richness measures.

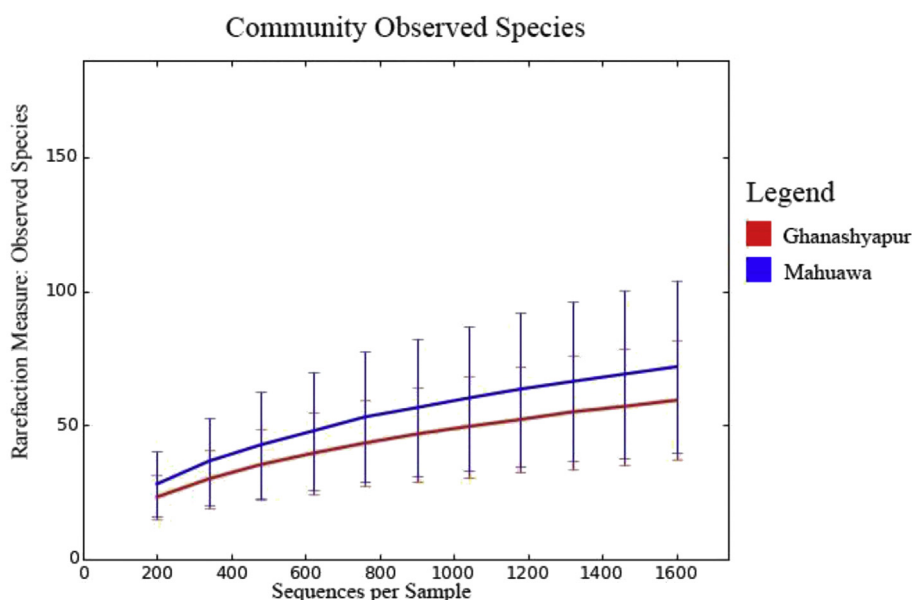


Figure 2. Observed species rarefaction curve of Community.

#### 4. Discussion

In this study, fecal samples obtained from members of two separate communities in southern Nepal that have been heavily affected by arsenic contaminated water, were used to better understand the influence of arsenic exposure on the human gut microbiome. High throughput sequencing of the 16S rRNA gene revealed significant bacterial community responses to elevated measures of arsenic within each respective community. We observed two distinctly defined taxonomic compositions between the two communities, shaped by geographic separation and differences in arsenic concentration. Our finding supports a previous study that identified similar spatial and concentration related differences in bacterial community response to a toxin [48]. Our study is the first of its kind to analyze changes to the gut microbiota of individuals in South East Asia, and lays the groundwork for future, more in-depth studies on the influence of arsenic on the health of the gut microbiome and overall human health.

Within the Ghanashyampur community, a selection for pathogenic bacteria was observed, including the *Treponema*, causal factor of syphilis [49]. While selection of this bacterial taxon has not yet been specifically linked to arsenic exposure, a study conducted in 1917, commented on the fact that after treatment with arsenic, a common syphilis remedy at that time, the *Treponema* became resistant to the arsenic compounds used to

treat the disease [50]. More recently, Shivani et al. [51] reported the presence of arsenic resistant genes in a related bacterium within the Spirochaetaceae family. The Spirochetes class, which includes the causal agents of Lyme's disease and syphilis, were also elevated within the Ghanashyampur community [49, 52, 53]. Further, the *Bilophila*, a member of the Desulfovibrionaceae family and candidate arsenic volatilizing bacterium yielded the strongest positive correlation with arsenic of all identified taxonomy within the Ghanashyampur community [54]. This specific genus is not very common in the gut microbiome but is known to be sulfate reducing [55], a possible contributor to colorectal carcinogenesis [56] and has been noted to have geno- and cytotoxic effects on gut epithelial cells [57]. Desulfovibrionaceae was also positively correlated with arsenic concentration in urine. This bacterial family possesses three-member genera including *Desulfovibrio*, *Bilophila* and *Lawsonia*. *Desulfovibrio* and *Bilophila* are arsenic volatilizing, or play some role in metabolizing and producing arsenic compounds in the gut [23, 54] and perform their arsenic reducing activity in the reductive environment of the human gut [58]. *Lawsonia* by comparison has no known arsenic volatilizing activity and has only been observed as pathogenic to animals [59, 60, 61]. Further, the genus *Clostridium* was positively correlated with arsenic concentration in the urine. This observation is congruent with Oremland and Stolz's [23] observation of the *Clostridium* species *OhilAS* as an arsenic volatilizing bacterium.

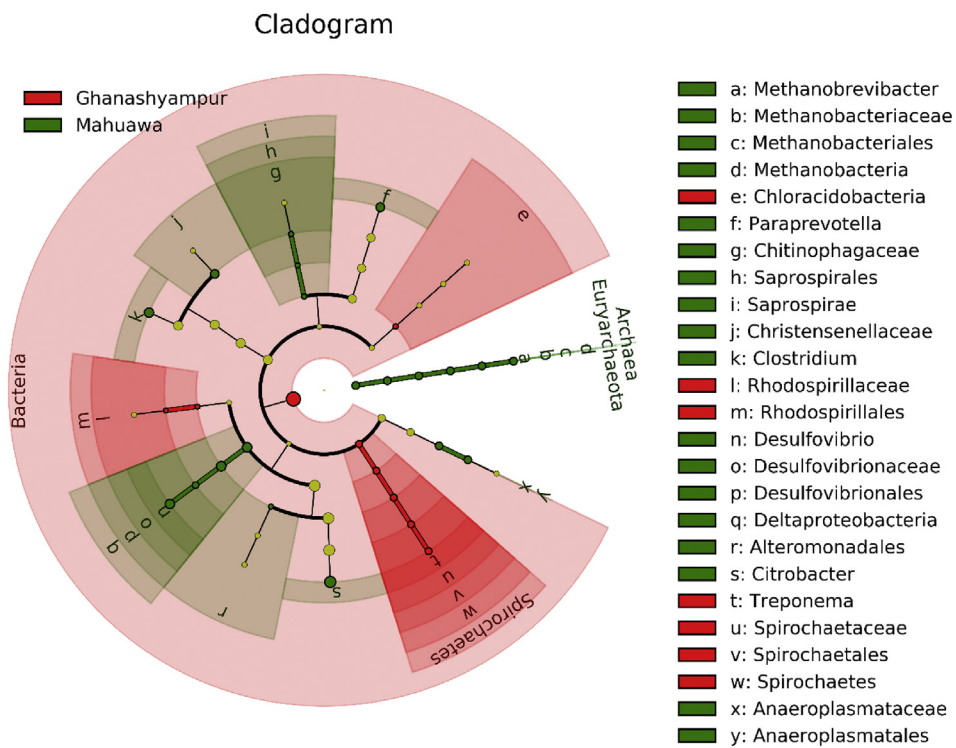


Figure 3. Cladogram comparing differences in significantly enriched taxa between the two communities.

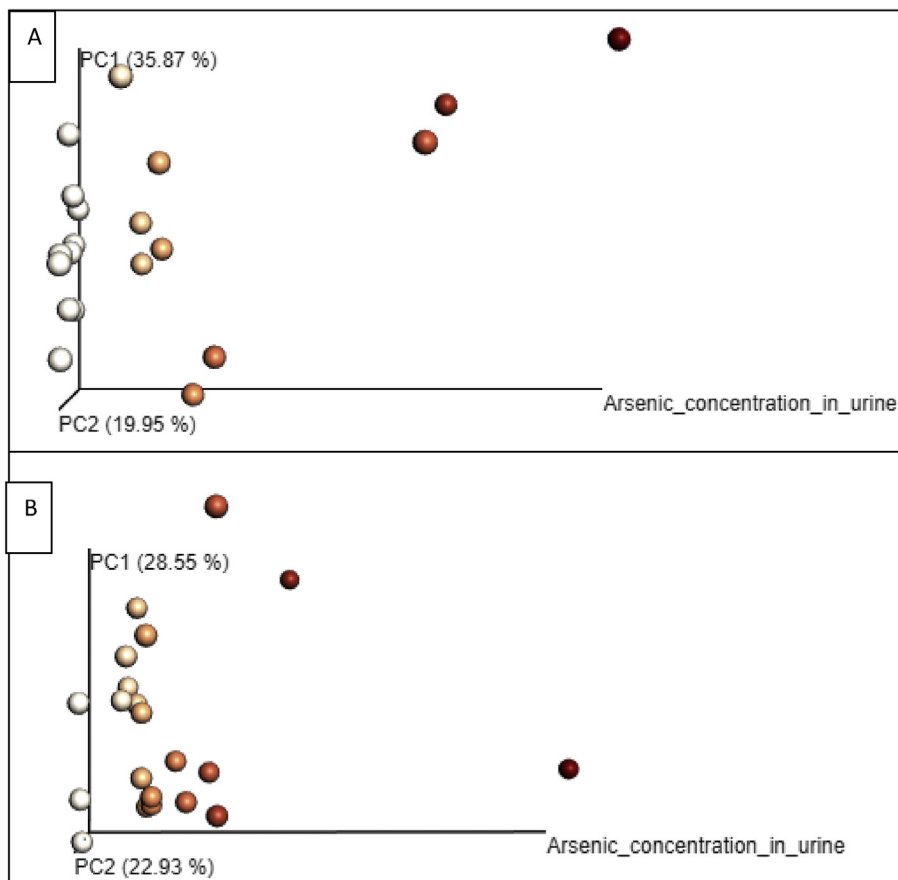
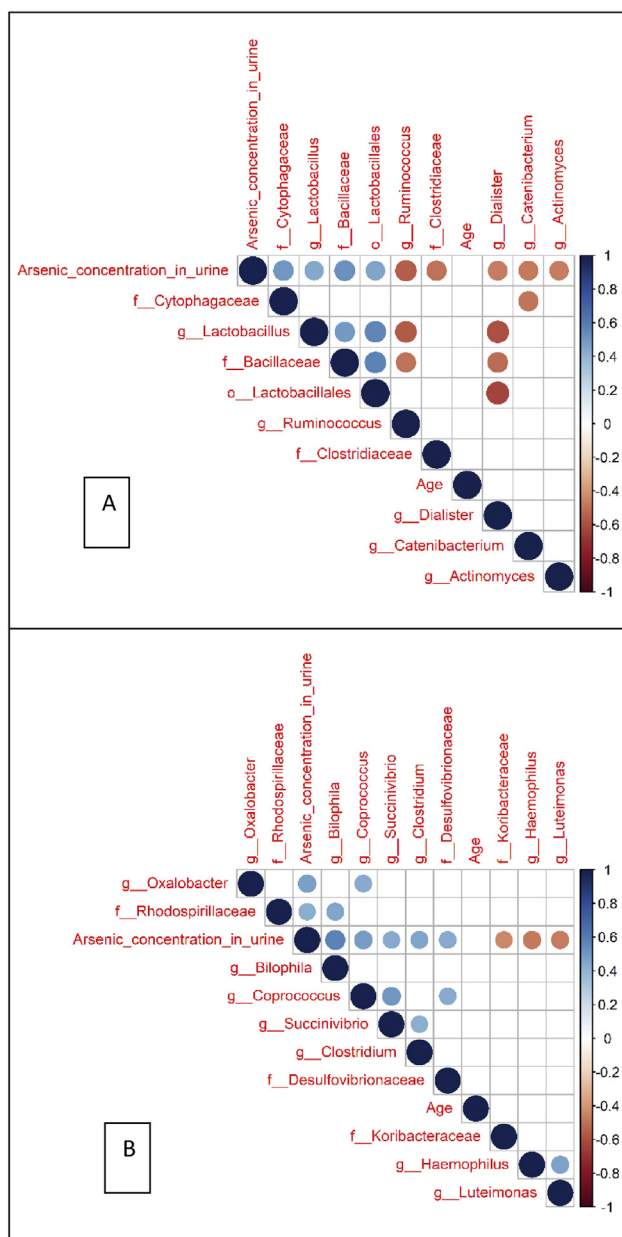


Figure 4. Comparison of differences in community composition between samples. A) Mahuawa and B) Ghanashyampur community.



**Figure 5.** Correlation plots analyzing bacterial correlations with arsenic concentration in urine. The size of the dot and the color indicate strength of the correlation. Dark blue indicates a strong positive correlation while dark red indicates a strong negative correlation. (A) Correlations between arsenic concentration in urine and bacterial taxa within the Mahuawa community. (B) Correlations between arsenic concentration and bacterial taxa within the Ghanashyampur community. Plotted correlations all have a p-value less than 0.05.

A defined structure of degradative bacteria was observed in the Mahuawa community as well, though with a noted absence of the *Treponema* and the other Spirochetes, but the presence of the *Citrobacter*. The *Citrobacter* have been observed to be opportunistic even in immunocompetent patients [62], and it has additionally been linked to arsenic volatilization [23]. The arsenic volatilizing bacteria *Desulfovibrio* and *Clostridium* were also selected for in the Mahuawa community [23]. An additional bacterium playing a degradative role in the Mahuawa community was the Bacillaceae family, which includes the genera *Bacillus* that is both pathogenic [63] and arsenic volatilizing [23]. The Mahuawa community, more so than the Ghanashyampur, was observed to have a depletion of specific gut commensal bacteria, *Ruminococcus* and *Clostridiaceae*. Both taxa fall within the Firmicutes phylum and interact

closely with host gut epithelial cells, are fundamental in maintaining a healthy gut microbe composition, and protect the host from opportunistic pathogens [64, 65]. In their 2015 study Dheer *et al.* [20] sought to observe structural and compositional changes to the gut microbiota of mice exposed to different arsenic concentrations (none, 10 µg/L and 250 µg/L) over the course of 10 weeks. They observed a reduction of Firmicutes, like Clostridiaceae and *Ruminococcus* and noted increased sporulation, a dormant state that bacteria enter when toxins are present or the surrounding environment becomes too inhospitable, over the course of the 10-week experiment.

Additional depletions of members of the less well documented bacterial order, Erysipelotrichales, were observed in both communities. This finding is supported by similar results from Lu *et al.* [21] who observed significant decrease in the relative abundance of Erysipelotrichaceae, a member family of Erysipelotrichales, in animals that had fed food containing high (10ppm) levels of arsenic. To our knowledge Lu *et al.* [21] and ourselves are the first to report original research that shows relative abundances of Erysipelotrichales decrease in response to arsenic exposure. There have been no definitive results on the role of Erysipelotrichales in the gut, but a 2015 review by Kaakoush [66] postulated that it may play a role in host lipid metabolism. Kaakoush [66] ultimately concluded that further research is necessary to determine this taxa's biological role in the body. Additionally, further research is necessary to learn more about the effects of arsenic on Erysipelotrichaceae.

Within the Ghanashyampur community, differential bacterial taxa were found to correlate with increased arsenic in comparison to the Mahuawa community. However, the identified taxonomic rankings in the two communities were found to carry out similar functions. Both communities shared selection for some arsenic volatilizing taxa like Desulfovibrionaceae, but each was possessive of its own unique assemblage of pathogenic bacteria and both showed higher selection for certain arsenic volatilizing bacteria that the other did not. As noted earlier, the observation of site specific compositional differences in response to a contaminant is not unique to this study [48].

Our study has shown that there are identifiable pathogens and arsenic volatilizing bacteria in the guts of individuals from both Nepalese communities. It has further determined that the specific levels of those bacteria differ between the two communities. Different arsenic volatilizing bacteria are dominant in the Ghanashyampur community than are dominant in the Mahuawa community. Future studies will aim to better understand this difference, and there are several methods that will help to refine and explain this difference. First, it will be necessary to revise the metric for determining arsenic exposure in an individual as arsenic concentration in the urine can't discern the full range of arsenic exposure someone has experienced over their lifetime. To that end, it would be better to use hair or fingernail samples, which retain the toxin much longer and would thus provide a better determination of arsenic exposure over the course of an individual's lifetime [67]. We would then be able to more accurately identify which individuals would be grouped into the "Undetected", "Moderate" and "High" categorical arsenic concentration groupings which would lead to better profiling of bacterial compositional shifts and enrichment of bacteria when arsenic is present. Metatranscriptomic analysis is also necessary to better identify enriched genes. Discerning the presence of pathogenic genes in a cohort will be important to better understand how arsenic is, in fact, affecting the overall all health of these individuals.

**4.1. Limitations**

At first, this study was sampling at only one time point which limited analysis of the effects of arsenic on the gut microbiome over an extended period. Secondly, no household water and diet rice samples were collected in this study due to limited funding. Finally, one of the most important additions to future studies in this area is a stronger control group. While there were individuals who registered 0 µg/L urine arsenic concentration there was no way to know whether individuals

Mahuawa Cladograms

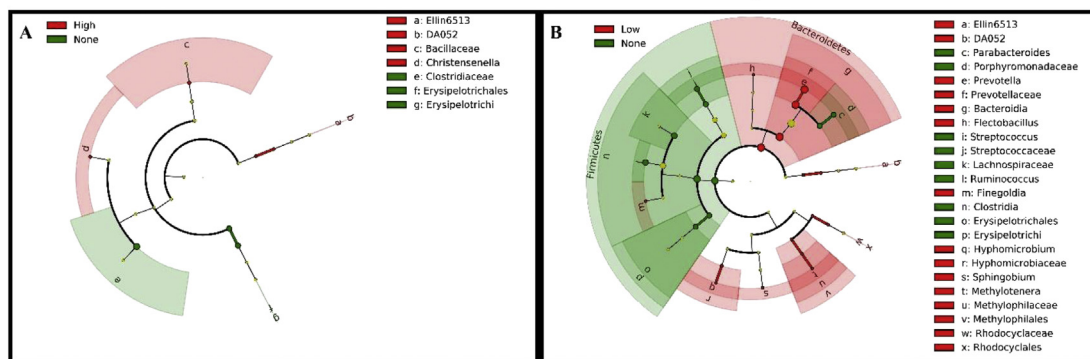


Figure 6. Cladograms comparing significantly enriched taxa within the Mahuawa community (A) High vs. Undetected Categories (B) Moderate Vs. Undetected Categories.

Ghanashyampur Cladograms

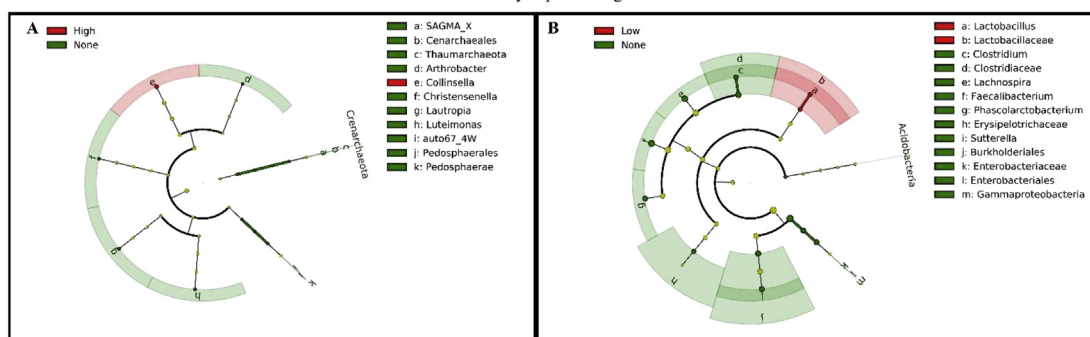


Figure 7. Cladograms comparing significantly enriched taxa within the Ghanashyampur community Arsenic Exposure Levels (A) High vs. Undetected (B) Moderate vs. Undetected.

classified in the “Undetected” arsenic concentration grouping, had never been exposed to the toxin, though the data suggest that they had all been exposed on some level due to the presence of indicator taxa and predicted gene families. The best control group would be from Nepal, but from an area that had better water quality control. Similar studies have used American samples [68], as there is more surety of limited to no arsenic contamination, but there are so many other confounding factors such as diet and influence of other environmental factors which makes the use of American samples as controls, highly biased. The hope is to better understand the role of arsenic in pathogenicity and gut dysbiosis, and how to remedy its effects.

Declarations

Author contribution statement

Jeffrey L Brabec: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Justin Wright: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Truc Ly, Vasily Tokarev: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Hoi Tong Wong, Christopher J McClimans: Analyzed and interpreted the data.

Regina Lamendella: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Shardulendra Sherchand, Dipendra Shrestha, Sital Uprety, Bipin Dangol, Sarmila Tandukar, Jeevan B. Sherchand: Performed the experiments.

Samendra P Sherchan: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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