



World's Largest Mass Bathing Event Influences the Bacterial Communities of Godavari, a Holy River of India

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Abstract

Kumbh Mela is one of the largest religious mass gathering events (MGE) involving bathing in rivers. The exponential rise in the number of devotees, from around 0.4 million in 1903 to 120 million in 2013, bathing in small specified sites can have a dramatic impact on the river ecosystem. Here, we present the spatiotemporal profiling of bacterial communities in Godavari River, Nashik, India, comprising five sites during the Kumbh Mela, held in 2015. Assessment of environmental parameters indicated deterioration of water quality. Targeted amplicon sequencing demonstrates approximately 37.5% loss in microbial diversity because of anthropogenic activity during MGE. A significant decrease in phyla viz. *Actinobacteria*, *Chloroflexi*, *Proteobacteria*, and *Bacteroidetes* was observed, while we noted substantial increase in prevalence of the phylum *Firmicutes* (94.6%) during MGE. qPCR estimations suggested nearly 130-fold increase in bacterial load during the event. Bayesian mixing model accounted the source of enormous incorporation of bacterial load of human origin. Further, metagenomic imputations depicted increase in virulence and antibiotic resistance genes during the MGE. These observations suggest the striking impact of the mass bathing on river ecosystem. The subsequent increase in infectious diseases and drug-resistant microbes pose a critical public health concern.

Keywords 16S rRNA gene · Kumbh Mela · Antibiotic resistance · Disease outbreak · Public health · Godavari River

Introduction

The Kumbh Mela is considered to be the world's largest periodic religious mass gathering event (MGE) with as many as 120 million pilgrims recorded during the 2013 event at Allahabad, India [1–4]. It involves ritual bathing in the river on certain days, at one of four Indian cities in rotation every 3 years (Allahabad, Ujjain, Haridwar, and Nashik). The event is hosted by each city every 12 years based on the pre-

determined zodiac combinations [5]. There has been an exponential rise in the number of pilgrims attending Kumbh Mela, from around 0.4 million in 1903 to 120 million in 2013. Such massive participation imposes substantial difficulties for the accommodation and flow of people. Thus, a temporary “pop-up city” is created, which is generally built in the flood plains of the rivers, that demands houses with proper electricity, sanitation, and healthcare services [3, 6]. Unfortunately, these efforts and their implications are largely undefined. However, lately, there has been a growing interest to document the concurrent events underlying the planning and management of world's largest human gathering using multidisciplinary approach to minimize the impact of such events on public health. [4–7].

Kumbh Mela represents a periodic recreational use of water with religious paradigm, involving bathing in small specified sites that might drastically alter the biogeochemical balance of the river ecosystem [4, 8, 9]. Bacterial communities are sensitive to dynamic changes in their environments that could result in high spatiotemporal variations [10]. Bacteria are the key components of freshwater ecosystems and play a crucial role in primary production and nutrient cycling. However, the role

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of these bacterial communities in recreational waters is relatively unidentified. Hlavsa et al. [11] reported the relationship between various bacterial species such as *Escherichia coli* O157:H7, *Legionella*, *Pseudomonas*, and *Shigella* with waterborne illnesses in recreational freshwaters. Similarly, a recent study on recreational practices reported increased risk of waterborne infections during sea bathing [12]. Previous studies have also found dominance of unusual microorganisms under urban-impacted water bodies, which could thus elevate the risk to human health [13–15]. Therefore, perturbations of the microbial communities because of biotic and abiotic alterations under recreational usage could possibly have serious consequences on both the environment and public health. This could involve loss of autochthonous communities and/or elevated risk of gastrointestinal disorders and skin infections [4, 8, 16]. The magnitude of such waterborne infections is relative and might have long-term impact on human health.

The mechanisms underlying waterborne infections in response to the large recreational water use during MGE are largely unknown and have so far only been studied by conventional culture-based estimation of fecal indicator bacteria (FIB). The limitations associated with conventional techniques cannot be overlooked, and researchers have opted extensive investigation of bacterial communities using culture-independent methods, such as fluorescence in situ hybridization (FISH) [17, 18] and quantitative polymerase chain reaction (qPCR)-based detection [19]. Recent studies using next-generation sequencing (NGS) have already proved their efficiency in providing in-depth knowledge of microbial communities [20, 21]. Despite these technical advancements, understanding of complex microbial community dynamics in recreational freshwaters and its effects on ecological productivity and public health represents a major bottleneck. Thus, characterization of bacterial community structure under spatiotemporal variations would serve as a proxy to delineate the impact of MGE on freshwater microbial assemblage.

The current study attempts to elucidate alterations in bacterial community structure by deep sequencing and evaluates relationships among environmental factors, bacterial community composition, spread of infections, and drug resistance in the ecosystem. In this regard, we examined (1) the changes in bacterial communities of the Godavari River before and during the MGE and (2) the association between the environmental parameters and the river bacterial assemblage and (3) traced the source of invasive communities to the river ecosystem.

Materials and Methods

Sample Collection

The Nashik Kumbh Mela event takes places on the bank of the Godavari River, the largest river of southern India which

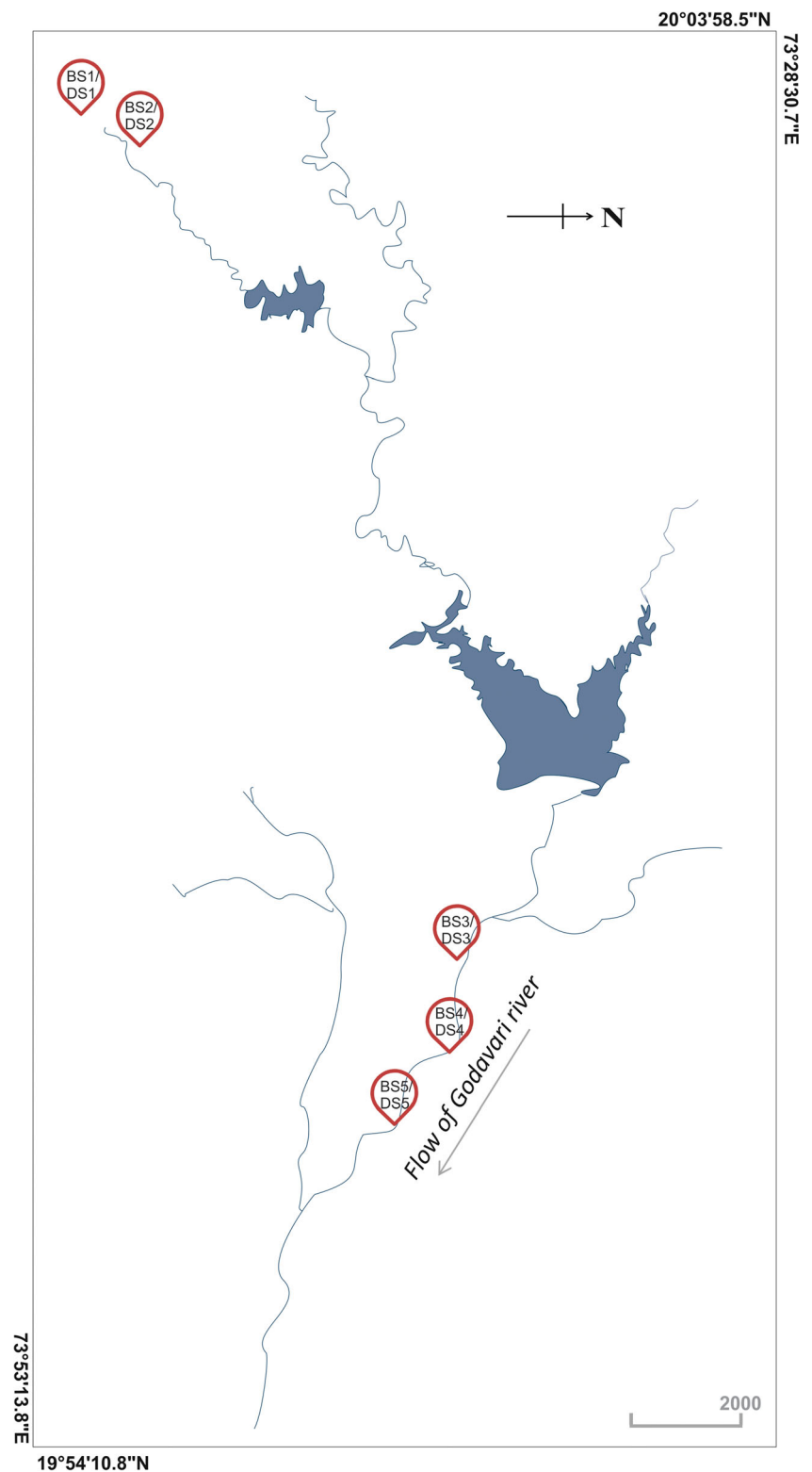
originates from “Brahmagiri” mountain ranges (located near Nashik, India) and travels approximately 1465 km southeast, before draining into the Bay of Bengal. The geographical location of sampling sites can be categorized by its altitudinal gradient. Thus, the earlier two sites located at higher altitude (mean 827.5 m a.s.l.) were named upstream (Gangadwar, Kushawart) while later three sites located at relatively lower altitude (mean 567 m a.s.l.) were named bathing sites (Anandvalli, Gharpure ghat, and Tapovan) (Fig. 1). The two upstream sites are situated in the mountain ranges and are relatively remote and in close vicinity of “Anjaneri” reserve forest which exhibits less civilization. Human activities at upstream sites may increase during the Kumbh Mela which is otherwise occasional. In contrast, the bathing sites are located within the Nashik city and are more prone to anthropogenic drivers during the MGE. Additionally, all the event sites have extended river banks which are built from concrete to accommodate the large number of pilgrims. Water samples were collected in triplicate from all the event sites immediately before (July 2015) and during (September 2015) the Kumbh Mela (hereafter, they will be referred to as before MGE and during MGE samples). Samples collected before the event are named as BS1, BS2, BS3, BS4, and BS5 while samples collected during the event are DS1, DS2, DS3, DS4, and DS5, and correspond to locations Gangadwar, Kushawart, Anandwalli, Gharpure ghat, and Tapovan, respectively.

Water samples were stored in sterile (gamma-irradiated) containers and transported immediately to the laboratory at 4 °C. Upon arrival, 100 ml of water samples from each site was filtered through 0.22- μ polyethersulfone filters (Merck Millipore, USA) and the filters were stored at –80 °C for further processing. Physicochemical parameters such as pH (Pocket digital pH meter, Milwaukee pH 600) and temperature (Vintage USA E8000) were measured at the time of sampling. Simultaneously, each sample aliquot was processed to determine the turbidity (OD), total dissolved solid (TDS), total solids (TS), total suspended solids (TSS), dissolved oxygen (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), fecal coliforms, and MPN of total coliforms at Department of Microbiology, KTHM College, Nashik, by following the protocols described by APHA (1998).

DNA Extraction and Amplicon Sequencing of Bacterial 16S rRNA Gene

DNA extraction was performed on the stored 0.22- μ polyethersulfone filter papers (Merck, Millipore, USA) using Power Water DNA extraction kit (Mobio, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality of DNA was assessed as described previously [22, 23] and stored at –20 °C until further processing. The DNA was subject to amplification of V4 hypervariable region of

Fig. 1 Map showing transect of Godavari River, Nashik, India, hosting mass bathing event (the Kumbh Mela). Points indicate locations used for sampling during this study. BS and DS represent before and during MGE samples. Samples were collected from two upstream sites Gangadwar (BS1/DS1) and Kushawart (BS2/DS2) and three bathing sites Anandvalli (BS3/DS3), Gharpure ghat (BS4/DS4), and Tapovan (BS5/DS5)



16S rRNA gene using forward primer 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3') [24]. Template and library preparation was carried out according to the manufacturer's protocol (Illumina, USA). 16S rRNA

amplicons libraries were sequenced on Illumina MiSeq platform using paired end 2×250 bp v4 chemistry. The sequences obtained were submitted to the NCBI Sequence Read Archive (SRA) and can be accessed under Bio Project ID PRJNA383664.

Amplicon Sequence Analysis

The raw paired end reads obtained from Illumina MiSeq platform were assembled using FLASH tool (Fast Length Adjustment of Short reads) described by Magočand Salzberg [25]. The resultant raw paired reads were processed in MOTHUR v1.32 [26], to remove the adaptor sequences, ambiguous reads, and low-quality sequences with an average quality score below 30. The quality filtered amplicon reads from all the samples were pooled into a single FASTA file for further analysis using QIIME v1.8 (quantitative insights into microbial ecology) [27]. Closed reference-based OTU picking approach against the Greengenes reference database [28] was adapted to cluster reads into operational taxonomic units (OTUs) by using UCLUST algorithm with 97% similarity threshold [29]. Taxonomic assignments of OTUs were performed using RDP naïve Bayesian classifier with Greengenes reference database [30]. The reads were rarefied to the least number of reads per sample (i.e., 105,246 reads in sample BS4) before performing further analysis. Alpha diversity estimation such as OTU richness, Shannon, Chao1, and Good's coverage associated with upstream and bathing sites was calculated using QIIME v1.8 based on sampling groups. The beta diversity analysis was performed using Bray-Curtis distances to microbial communities across the MGE.

Quantitative PCR Analysis

The total abundance of bacterial communities before and during the MGE was determined by absolute quantification of 16S rRNA gene copy number. The equimolar (50 ng/μl) template was subjected to qPCR amplification (in triplicate) with 341F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'-ATTACCGCGTCTGCTGG-3') in a 10 μl reaction of Power SYBR Green PCR Master Mix using 7300 Real time PCR system (Applied Biosystems Inc., USA). The qPCR assays involved initial denaturation at 95 °C for 10 min followed by 40 cycles with 95 °C for 15 s and 60 °C for 1 min. The standard curve was plotted by calculating the mean of tenfold dilutions of known concentration of PCR product which was later used to determine the 16S rRNA gene copy number [31]. The standard curve accounting for qPCR efficiency of > 90% and correlation coefficient of > 0.99 were considered as a threshold while calculating the copy number. We report all resultant data as copy number per liter of water sample.

Statistical Analysis

Statistical environment R (R Development Core Team, 2013), and R packages vegan, PhyloSeq, ggbiplot, ggplot2, corrplot, and psych were used to carry out multivariate and statistical analysis at different taxonomic levels for the upstream and bathing samples 2013 [32]. Determinants of physicochemical

factors from the water assessment were statistically validated and correlated with bacterial diversity by performing regression and principal component analysis to analyze the influence and the contribution of each environmental factor to the bacterial community structure. We also computed spearman correlation matrix at family level to dissect the complex microbial associations. Further, a function of PhyloSeq package was used to graphically visualize nonparametric alpha diversity parameter like OTU richness, Shannon, and Chao1, and further, it was used to perform nonmetric multidimensional scaling (NMDS) plots by calculating Bray-Curtis distances from the OTU table to illustrate differences between groups based on the overall bacterial community composition.

Source Tracker Analysis

Source Tracker is a computational model that uses a Bayesian approach to identify possible sources and proportions of bacterial contamination [33]. The model was applied to identify the potential source of microorganisms of human origin, into the river ecosystem during the MGE. The microbiome (human skin, stool and oral) data of Indian sub-population (unpublished study) was acquired and used as potential source of microbial communities and the data of MGE (before and during) was used as a sink.

Imputation of Microbial Functional Profiles

PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was used to categorize and compare the functional changes in the bacterial communities from the collection sites [34]. It involves the normalization of 16S rRNA gene copy number followed by prediction of the metagenome. The derived data-set was analyzed at KEGG (Kyoto Encyclopaedia of Genes and Genomes) level (L1 to L3). The LEfSe was performed to distinguish the functional elements of microbial communities before and during the MGE [35]. Furthermore, metagenomic contribution by each taxon was quantified by using normalized dataset.

Results

Assessment of Physico-chemical Parameters of the River Water

A significant increase in turbidity and optical density was observed in water samples during MGE (paired *t* test $t = 4.41$, $p = 0.01$). The elevated turbidity could be the consequence of rise in total dissolved solids, total suspended solids, and total solids (paired *t* test, $p < 0.05$) which reflects the introduction of inorganic and organic particles during MGE (supplementary Table 1). However, pH and temperature of

the water (paired t test $t=0.10$, $p=0.92$ and $t=0.92$, $p=0.4$, respectively) do not differ significantly over the course of MGE.

The biological and chemical oxygen demand (paired t test $t=3.42$, $p=0.02$ and $t=3.67$, $p=0.02$, respectively) increased during the MGE probably due to higher release of organic particles. The notion is further supported by substantial reduction in the concentration of dissolved oxygen (DO) (paired t test $t=10.44$, $p=0.0005$) during the MGE. The substantial decrease in DO while increase BOD and COD could be linked with increased bacterial load in the river. The qPCR-based estimation of absolute 16S rRNA gene copy number also showed approximately 130-fold increased bacterial abundance during the MGE. In addition, MPN and estimation of fecal coliforms increased nearly 157- and 3-fold, respectively, during the MGE when compared with before MGE.

Bacterial Diversity

High-throughput sequencing yielded ~5.3 million raw reads which were processed for quality trimming and an average of 490,863 (accounts for ~91.2%) high-quality sequences per sample were retained for subsequent analysis. Clustering of the sequences with reference database resulted in 6529 OTUs. The average Good's coverage for all the samples was found to be $99.31 \pm 0.30\%$ (mean \pm SD) which indicates that the majority of bacterial diversity was captured.

The assessment of alpha diversity indices revealed significant differences at bathing sites before and during the MGE (Fig. 2) whereas no significant differences were noted at upstream sites. The estimates of bacterial species richness and observed OTUs were found to decrease at bathing sites during MGE. Significant variation across MGE was also noticed in non-parametric Shannon index (paired t test $t=3.04$, $p<0.05$) suggesting dominance of fewer taxa during MGE. Interestingly, among all samples, BS5 featured highest values for species richness (4207.89) and Shannon index (7.17) followed by BS4 and BS3 indicating higher species richness in bathing site samples before MGE. Species richness was found to decrease significantly in bathing site samples during MGE. Shannon evenness exhibits order of $DS4 < DS5 < DS3 < BS3 < BS4 < BS5$ which indicates diverse microbial community pattern between the samples from bathing site. (Table 1).

Linear regression analysis was applied to explore the relationship of species richness and environmental factors during MGE (Suppl. Fig. 1). The environmental factors such as optical density of water, TDS, TS, BOD, and COD estimates were higher during the event and showed inverse relationship with diversity ($r=-0.787$, $r=-0.796$, $r=-0.793$, $r=-0.697$, $r=-0.708$, $p<0.05$), except the dissolved oxygen ($r=0.70$, $p=0.024$).

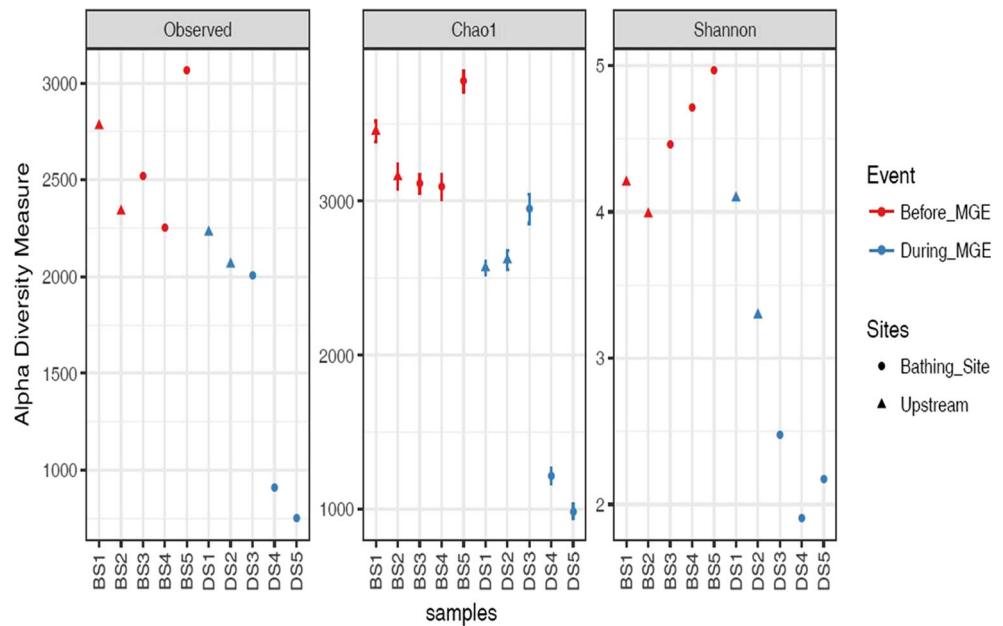
Therefore, it can be noted that physicochemical parameters render negative correlation with respect to event and further support the findings of the non-parametric alpha diversity estimation.

Bacterial Community Structure

A total of 25 bacterial phyla were detected of which the most abundant were *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Actinobacteria*, *Planctomycetes*, and *Cyanobacteria* contributing to 98.97% of the total bacterial diversity before MGE whereas *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* dominated during MGE and accounted for 98.49% of diversity (Fig. 3a). Bacteria belonging to phylum *Firmicutes* were ubiquitous and observed in higher abundance across all the bathing sites during MGE (DS4 98.88%, DS3 93.28%, and DS5 91.84%) as compared to the bathing sites before MGE (BS3 44.93%, BS5 40.95%, and BS4 36% respectively). In contrast, members of the *Proteobacteria* dominated bathing sites of before MGE samples (mean $33.67 \pm 6.97\%$) over bathing sites of during MGE (mean $3.89 \pm 3.79\%$). Likewise, *Bacteroidetes* were more abundant with mean of $9.79 \pm 4.36\%$ at the bathing sites before MGE and during MGE the mean of $0.87 \pm 1.49\%$ was noted (Suppl. Fig. 2). Bacterial taxa such as *Chlorobi*, *Gemmatimonadetes*, *Armatimonadetes*, and *Fusobacteria* and phyla like GN02 and OP3 were exclusively present before MGE. In contrast, *Tenericutes*, most of the members of candidate division like WS3, OD1, and SBR1093 were observed only during MGE.

Our investigation of spatiotemporal variation in the bacterial community composition showed differential abundance of OTUs (abundance $\geq 0.5\%$) corresponding to 15 bacterial families. It was observed that families such as *Comamonadaceae*, *Flavobacteriaceae*, *Verrucomicrobiaceae*, *Chitinophagaceae*, *Moraxellaceae*, *Oxalobacteraceae*, candidate family ACK-M1, *Microbacteriaceae*, *Methylophilaceae*, *Cytophagaceae*, and *Weeksellaceae* were prevalent before the MGE while *Planococcaceae*, *Sphingomonadaceae*, *Prevotellaceae*, and *Xanthobacteraceae* predominate during the MGE (Fig. 3b). Likewise, a comparison at genus level depicted 15 differentially abundant genera (abundance $\geq 0.5\%$) across MGE out of which *Flavobacterium*, *Sediminibacterium*, *Prostheco bacter*, candidate genus C39, *Hydrogenophaga*, *Acinetobacter*, *Polynucleobacter*, *Hylemonella*, *Limnohabitans*, *Rhodoferrax*, and *Cloacibacterium* were significantly abundant before the MGE. In contrast, *Planomicrobium*, *Novosphingobium*, *Prevotella*, *Akkermansia*, *Uliginosibacterium*, and *Dechloromonas* were highly prevalent during MGE. Collectively, these results suggested significant differences in the microbial community composition across the MGE.

Fig. 2 Elements of alpha diversity. Variations in alpha diversity indices among upstream and bathing sites of the event. Shapes denotes upstream (triangular) and bathing site (circle) while color code shows the two time points, i.e., before (red) and during (blue) the event



Determining the Impact of MGE on the Bacterial Community Composition

Beta diversity was analyzed to determine variations in bacterial community structure between two sampling points, i.e., before and during the MGE and to compare upstream and bathing sites before and during MGE. NMDS (Non-metric multidimensional scaling) plots based on Bray-Curtis dissimilarity metrics showed four distinct groups based on spatial (i.e., upstream and bathing sites) as well as temporal variation (i.e., before and during MGE) (Fig. 4). The inter-group variation between these groups was statistically validated. It was observed that spatial variation during the MGE was quite higher as compared to before the MGE.

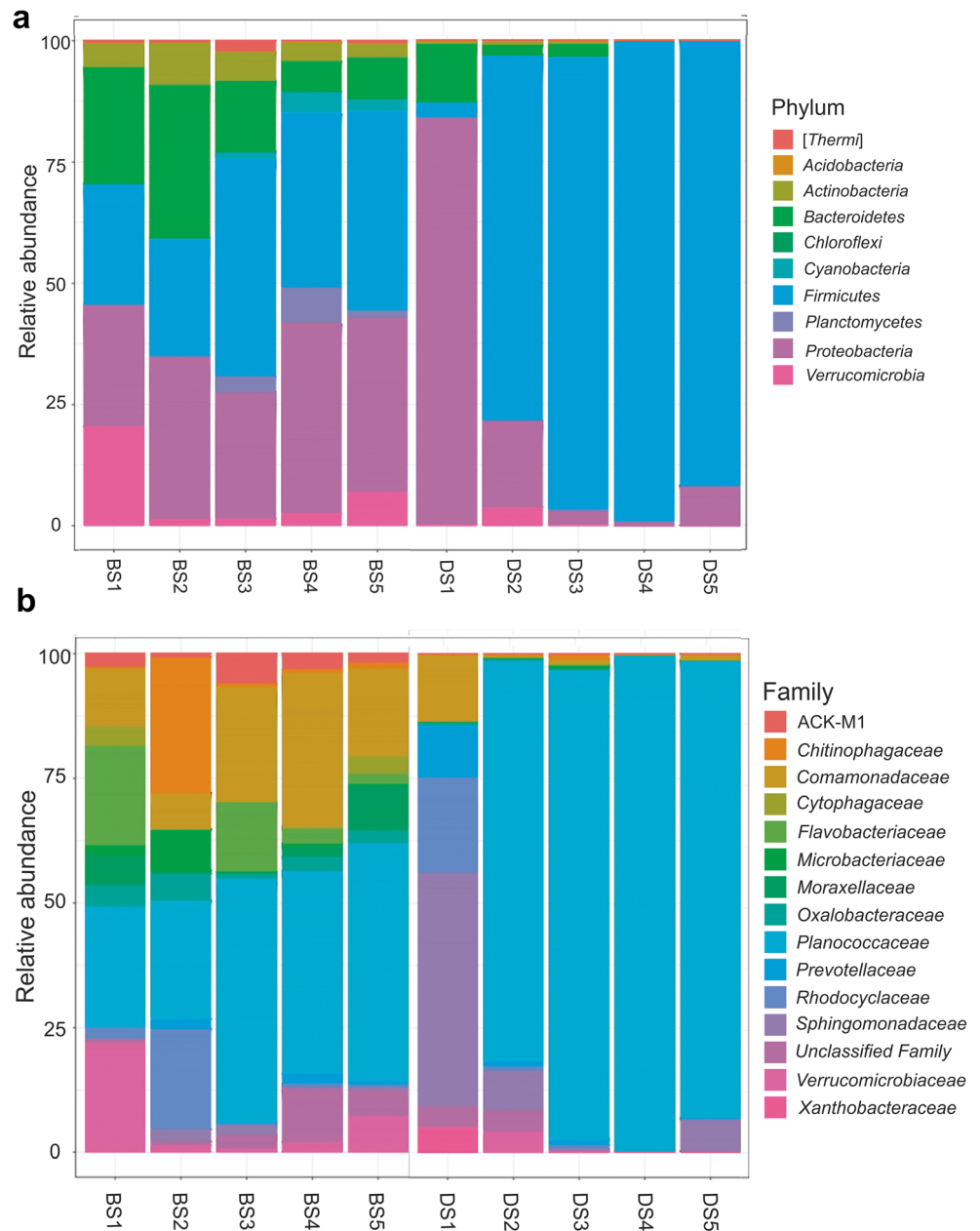
The assessment of complex microbial association network and their correlation with physiochemical parameters revealed that bacterial families like *Planococcaceae* and *Bacillaceae*

showed strong negative correlation with other bacterial families. Additionally, we found a positive correlation between the bacterial families *Planococcaceae* and *Bacillaceae* with the physiochemical parameters (OD, TS, TDS, TSS, BOD and COD, and MPN) during the MGE (Suppl. Fig. 3). In contrast, bacterial families like *Comamonadaceae*, *Oxalobacteraceae*, candidate family ACK-M1, *Chthoniobacteraceae*, *Rhodobacteraceae*, *Gemmataceae*, and *Pirellulaceae* showed a negative correlation with environmental factors such as OD, TS, TDS, TSS, BOD, and COD during the MGE. A positive correlation was noted between fecal coliform, MPN, and bacterial families viz. *Lachnospiraceae*, *Ruminococcaceae*, *Campylobacteraceae*, *Clostridiaceae*, and *Methylocystaceae* during the MGE. Similarly, principal component analysis of physiochemical parameters such as turbidity, solute concentration, and MPN was found to modulate bacterial communities during the MGE ($p < 0.05$). In contrast, microbial

Table 1 Sequence summary and alpha diversity estimation. The table shows the estimates of alpha diversity and sequencing depth for each sample

Samples Id	Sample title	Latitude/longitude	Sampling point	Sampling site	Chao1	Goods coverage	Observed species	Shannon
BS1	Gangadwar	19° 55' 40" N 73° 30' 59" E	Before_MGE	Upstream	3641.78	0.991	2251	6.082
BS2	Kushawart	19° 55' 57.5" N 73° 31' 39.2" E	Before_MGE	Upstream	3144.92	0.9921	1986	5.768
BS3	Anandvalli	20° 01' 03.9" N 73° 44' 46.2" E	Before_MGE	Mass bathing	3100.88	0.9928	2045	6.438
BS4	Gharpure ghat	20° 00' 33.7" N 73° 47' 04.9" E	Before_MGE	Mass bathing	3975.94	0.9904	2521	6.834
BS5	Tapovan	19° 59' 59.5" N 73° 48' 44.4" E	Before_MGE	Mass bathing	4207.89	0.9901	2710	7.176
DS1	Gangadwar	19° 55' 40" N 73° 30' 59" E	During_MGE	Upstream	2795.32	0.9932	1673	5.901
DS2	Kushawart	19° 55' 57.5" N 73° 31' 39.2" E	During_MGE	Upstream	3647.17	0.9903	2163	4.813
DS3	Anandvalli	20° 01' 03.9" N 73° 44' 46.2" E	During_MGE	Mass bathing	2212.92	0.9944	1275	3.555
DS4	Gharpure ghat	20° 00' 33.7" N 73° 47' 04.9" E	During_MGE	Mass bathing	826.61	0.9981	474	2.748
DS5	Tapovan	19° 59' 59.5" N 73° 48' 44.4" E	During_MGE	Mass bathing	618.05	0.9986	443	3.144

Fig. 3 Bacterial community composition across the mass gathering. **a** Phylum level distribution of dominant bacterial groups between before (BS) and during (DS) the event samples. **b** Family-level distribution of dominant bacterial groups between before (BS) and during (DS) the event samples



communities experience the influence of dissolved oxygen before the MGE ($p < 0.05$).

Tracking the Signature of Human Microbiota

The efforts of comparing human microbiota from healthy Indian subpopulation with MGE samples, by applying Bayesian mixing model (Source tracker), depicted increase in skin microbiota into the river upon mass bathing. We noted ~2.32-fold increase in skin-associated bacterial communities during the MGE (Fig. 5a). Similarly, the abundance of human fecal-associated bacterial communities showed a net change of ~2.89-fold which align with the results of MPN and fecal

coliform estimations. However, we could not detect microbial communities associated with oral microbiota. Comprehensive assessment of microbial source information would serve to mitigate the extent of pollution and ensure the human health under higher fecal contamination.

Imputed Metagenomics

We noted significant differences in major metabolic functions of the river microbial assemblage before and during MGE (Suppl. Fig. 4). Predominantly, gene families associated with glycanbiosynthesis and metabolism, genetic information processing, biosynthesis of secondary metabolites, lipid

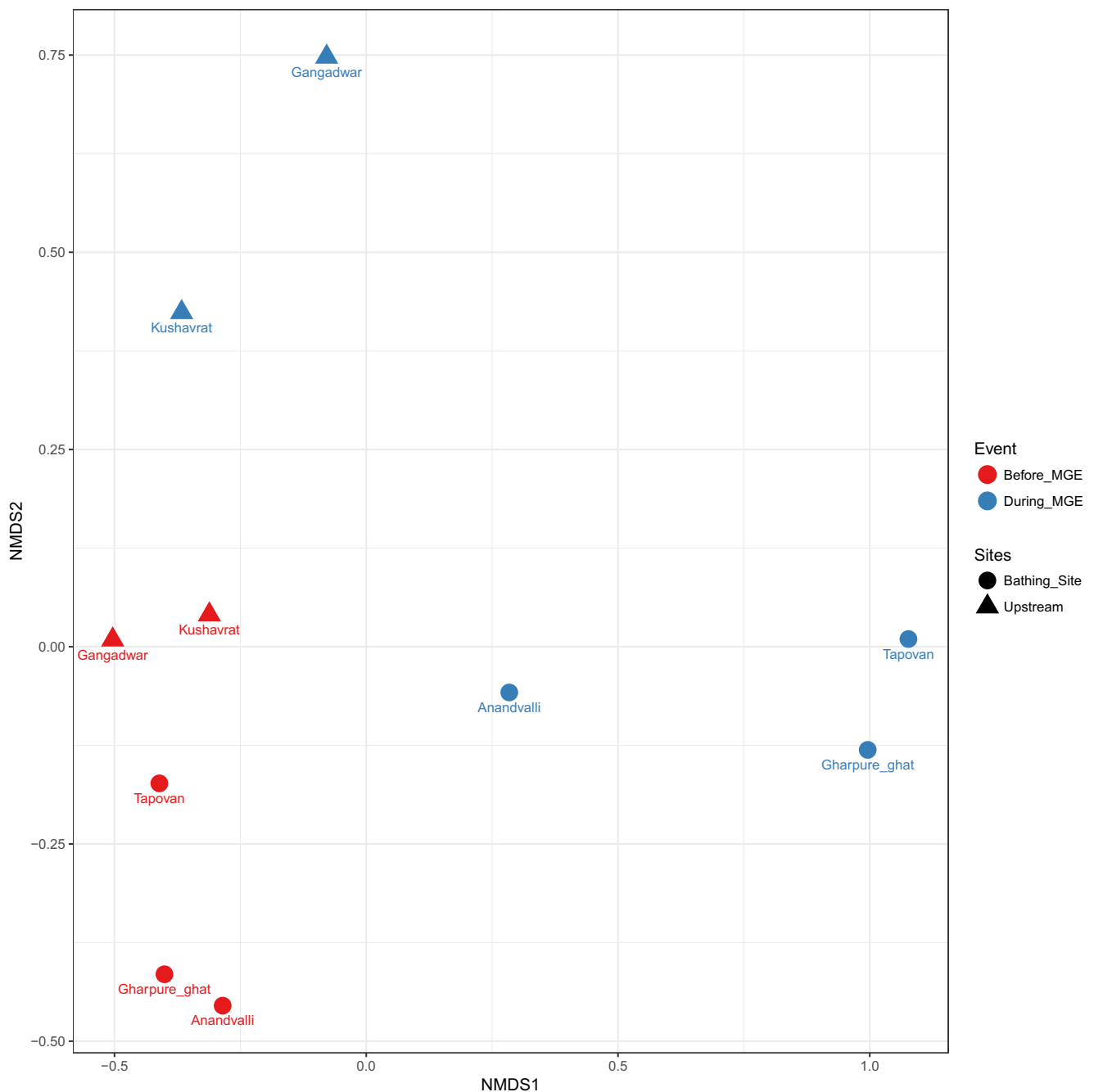


Fig. 4 Beta diversity analysis. Non-metric multidimensional scaling plot based on Bray-Curtis distance represents the spatiotemporal variation in the bacterial community structure. Spatial variation is denoted by shapes

upstream (triangular) and bathing site (circle). Color code defines the temporal divergence, before (red) and during (blue) the event

metabolism, and amino acid metabolism were significantly higher in before MGE samples. In contrast, gene families such as cellular processing, motility, bacterial chemotaxis, xenobiotic degradation, and infectious diseases were significantly higher during the MGE. From the functional profiles of microbial communities, we targeted gene families related to infectious diseases and drug resistance to investigate the impact of MGE on public health. The analysis revealed elevated levels of genes related to infections, described as fold change

for *Helicobacter pylori* infection (~ 0.469), *Legionellosis* (~ 0.58), pertussis (~ 0.157), *Salmonella* infection (~ 1.051), *Staphylococcus aureus* infection (~ 3.433), and tuberculosis (~ 0.092) during MGE (Fig. 5b). Similarly, increased drug resistant-related gene includes antifolate resistance (~ 0.25), beta-lactam resistance (~ 0.15), cationic antimicrobial peptide (~ 0.483), platinum drug resistance (~ 0.809), and vancomycin resistance (~ 0.346) during MGE (Fig. 5c). The above observations pose a critical challenge ensuring public health during

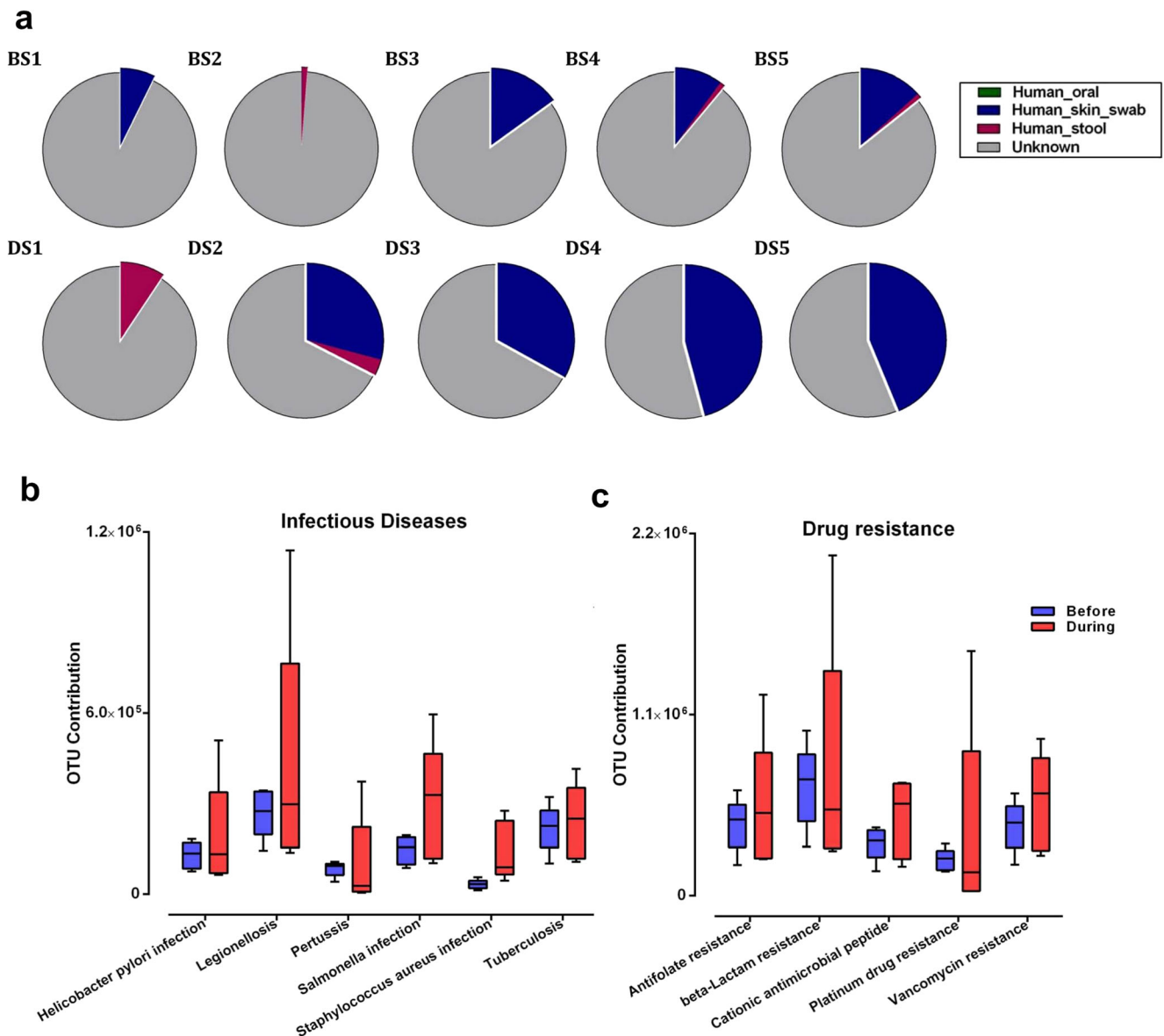


Fig. 5 **a** Pie chart depicts the signatures of human associated microbiota (skin, stool and oral) recovered during the mass bathing event. The color code denotes the source of bacterial communities associated with human-oral (green), skin (blue), and stool (red). Box plots represent functional

acquaintance of bacterial communities across the gathering. The differential abundance of gene families associated with **b** infectious diseases and **c** drug resistance. Before samples (blue) and during samples (red)

the MGE which could otherwise lead to unfavorable outcomes of epidemics.

Discussion

In the current spatiotemporal investigation, we observed significant alterations in the bacterial communities during the world's largest and unique mass bathing event. Our analysis depicted loss of diversity, with distinguished augmentation of bacterial taxa during the MGE. We further noted an increase in the drug resistance and waterborne infections which might pose serious threats to public health.

We evaluated the physicochemical parameters of river water which represent significant deterioration in the quality of water during the MGE and are previously reported to influence the bacterial diversity and their composition [36–42]. We assessed the turbidity, solute concentration, oxygen availability, and fecal contamination, which provided the primary leads about spatiotemporal differences in the bacterial communities. These results were not surprising given the numerous introductions of human waste being routinely discharge into the river over the course of MGE [4, 5]. Additionally, sediment re-suspension and release of the industrial and house hold waste have shown an influence on the bacterial community composition of freshwater ecosystems [18, 43, 44]. In particular, we

observed a significantly higher particle load and salinity (TS and TSS), whereas reduced oxygen availability (BOD, COD, and DO) during the event [45–48]. However, the concrete constructions along the bank of Godavari River minimize the accumulation and re-suspension of particulate matter into the ecosystem which otherwise reported to associated with potential risk of pollution and human health [49–53]. Instead, we believe that sources of nutrients and contaminants to the river are due to the impaired waste disposal at bathing sites [4, 5]. This is further proved by the linear regression analysis indicating the negative association between physiochemical parameters and bacterial communities along the temporal variations. Similarly, results of spearman correlation analysis demonstrated loss of common freshwater bacterial families under the relatively high salt and oxygen-limiting conditions. In contrast, we found augmentation of human fecal signature communities due to the practice of open defecation by the pilgrims and abrupt sewage management at the MGE, which could pose potential risk of gastrointestinal diseases [4, 54].

Our results of 16S rRNA gene-based microbial profiling demonstrated drastic differences in the bacterial diversity of MGE-impacted water bodies. Alpha diversity estimates a significant reduction in the microbial diversity during the MGE ($p < 0.05$) [55]. The water bodies before the MGE harbored bacterial groups such as *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* [56, 57], while *Firmicutes* were predominant at the bathing sites. The members of phylum *Proteobacteria* such as *Alphaproteobacteria* and *Betaproteobacteria* are known as the major player in the primary production of freshwater ecosystem, while reduced light availability could lead to decrease in these predominant freshwater communities [37, 57, 58]. Similarly, the members of phylum *Actinobacteria* were found to be depleted which otherwise are reported as autochthonous communities with potentials to degrade variety of complex compounds [56]. Moreover, the abundance of family *Planococcaceae* belonging to phylum *Firmicutes* was also found to be higher during the MGE. The members of this family have been isolated from the variety of halophilic environments, explaining its distinguished augmentation under high solute conditions reported in the current study [59]. Absolute quantification of 16S rRNA gene copy number using qPCR showed the influence of MGE under temporal variation with ~130-fold increase in the bacterial load which substantiates the fact of bathers being the potential source of non-point pollution [60]. Moreover, previous epidemiological studies have shown the correlation between the concentrations of bacteria in bathing water to the bather's health [61]. Interestingly, our results of spatiotemporal analysis also delineate extent of distinctness and diversity in bacterial communities before and during the MGE.

Looking at the number of pilgrims who attended the event, we attempted to differentiate the contribution of humans associated microbiota within the river which could deplete common freshwater bacterial diversity. The efforts of understanding the

sources of human microbiota using a signature approach reflected ~2.32- and ~2.89-fold rise in human skin and fecal microbiota, respectively, during the gathering. It also provides information about the differential distribution of the human microbiota at individual sites of the event. Therefore, the risk associated with use of such contaminated water remains uncertain, as it is difficult to differentiate the impact of these microorganisms in correlation to the gastrointestinal illness, skin, and eye infections [62–68]. The influence of the MGE was also reflected in predicted metabolic functions of river bacterial communities where we observed a higher abundance of genes responsible for infectious diseases and drug resistance during the MGE. A previous study on the Ganga pilgrimages by targeting *bla*_{NDM-1} gene has also suggested substantial increase in antimicrobial resistance [69]. Resistance towards cefotaxime shown by the recently described *Corynebacterium godavarianum* isolated from the Godavari River during the Kumbh Mela 2015 supports the presence of high drug-resistant bacterial communities observed in the samples collected during the event [70]. These observations collectively indicate that numerous influxes of human microbiota during MGE contribute to alteration in microbial community structure and modulate metabolic functions.

Alternatively, previous studies have indicated the association of inadequate planning and management of participants at MGEs with spread of infectious diseases such as 1817–1824 Asiatic cholera pandemic during the Kumbh Mela, W135 meningococcal infections at Hajj and influenza outbreak, Australia [71–75]. Further, David and Roy suggested that rising population and better connectivity increase the participation at such MGEs which may substantiate the risk to public health. Therefore, MGE organizers have adapted the preparatory guidelines amended by local health commission in conjugation with WHO [76–78] which have proven to maintain the harmony of the event by supplementing adequate sanitation, randomize clinical inspections, and vaccinations etc.

We conclude that this study is the first report addressing impact of Kumbh Mela, the world's largest MGE on river bacterial communities and its likely impact on human health. The significant changes in the bacterial diversity and composition with its potential impact on public health could be collectively attributed to the response of spatiotemporal variation of MGE. Also, our findings merit detailed investigation to evaluate long-term impact of such event on human health and river ecosystem.

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D.D., Y.S., V. R. and A.S interpretation of the data; K.J., D.D., Y.S., V. R. and A.S. were involved in the drafting of the manuscript; K.J., D.D., Y.S., V. R. and A.S. were involved in the critical revision of the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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