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Toxigenic and pathogenic potential of enteric bacterial pathogens prevalent in the traditional fermented foods marketed in the Northeast region of India

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ABSTRACT

The microbial risk involved with natural food fermentation is largely unknown. Here, we report the prevalence of enteric bacterial pathogens in the traditional fermented foods marketed in Northeast region of India. A total of 682 samples of 39 food types (broadly categorized into fermented soybean, bamboo shoot, fish, milk and pork products) collected over four different seasons from seven states of India were analyzed in this study. Cultivation-independent analysis by MiSeq amplicon sequencing of V4-V5 region of the 16S rRNA gene showed the bacterial community structure in the foods. Among the WHO prioritized foodborne bacterial pathogens, we detected the prevalence of phylotypes related to *Clostridium botulinum*, *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium perfringens*, *Listeria monocytogenes*, and *Escherichia coli* in these ethnic foods. We also observed the occurrence of other well known human enteric pathogens like *Proteus mirabilis*, *Clostridium difficile*, and *Yersinia enterocolitica*. Further pathogen-specific qPCR assays confirmed a higher population ($> 10^7$ cells/g) of *B. cereus*, *P. mirabilis*, and a *C. botulinum* related phylotype in the fermented soybean, fish, and pork products. We noticed a general trend of higher pathogen occurrence during the colder months without any seasonal variation of total bacterial load in the fermented foods. Further qPCR analysis on toxigenic and pathogenic potential, and toxins production by immunoassays showed that all the soybean samples and the isolated *B. cereus* cultures were positive for diarrheal toxins (Nhe and Hb1), and nearly half of the samples were positive for emetic toxin (cereulide). Similarly, the food samples and associated swarming *P. mirabilis* cultures were positive with the pathogenic factors like hemolysin (*hpm*), urease (*ure*) and multidrug resistance. However, we could not confirm the presence of botulinum neurotoxin (toxins A, B, E, and F) in the *C. botulinum* positive food samples. This is the first baseline data of the enteric bacterial pathogens prevalent in the traditional fermented foods of India, which will support the sustained effort of WHO to estimate the global foodborne disease burden. The unusual presence of *P. mirabilis* in the fermented foods marketed in the Indian region with high incidence of urolithiasis cases is a concern. Our study emphasizes the need of the hour to have a coordinated action to control and prevent the spread of enteric bacterial pathogens through fermented foods marketed in India. Moreover, replacing the indigenous process with a defined starter culture based controlled fermentation will enhance the safety of Indian fermented foods.

1. Introduction

Fermented foods are believed to promote health but the illness caused by the microbial hazards and the related disability-adjusted life years (DALYs) due to regular consumption are largely unknown (Cocolin et al., 2016). A large variety of fermented foods are consumed as a delicacy by different ethnic communities in India (Tamang, 2009). These traditional foods are produced by unhygienic, uncontrolled natural fermentations and are distributed through local markets. Although frequent incidences of outbreaks due to the consumption of these fermented foods are reported in Northeast region of India (Table S1, Supplementary material), no scientific investigation or interventions are documented, except few records of detecting pathogens in the food products (Jeyaram et al., 2008; Jeyaram et al., 2010; Nema et al., 2007; Singh et al., 2014). Moreover, the mildness in symptoms makes it difficult to go for proper surveillance, and the outbreaks are underreported. At the same time, population studies have linked the consumption of these traditional foods with a high prevalence of stomach cancer (Phukan et al., 2006), gastrointestinal diseases (Gajamer and Tiwari, 2014), and urolithiasis (Singh et al., 1986) in the North-eastern

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region of India.

Global burden of disease (GBD) study of 2015 estimated that foodborne diarrheal illness is the sixth leading cause of global disability-adjusted life years (DALYs) (Kassebaum et al., 2016). Every year, 1.9 billion cases of illness (Kirk et al., 2015) and 1.7 million deaths related to foodborne diarrhea are reported (Bryce et al., 2005). Moreover, recent WHO estimates of the global burden of foodborne diseases (2007–2015) stated a high burden of diarrheal and invasive enteric diseases in the Indian region, which is nearly double of the global average and 20 times higher than that of Europe (Kirk et al., 2015). GBD study 2015 also indicated a poor hygienic condition in India with "hygiene" indicator points of just 8 out of 100 (Lim et al., 2016). Moreover, this region is recognized as an epicenter for many emerging infectious disease agents (Jones et al., 2008) with multidrug-resistance (Kumarasamy et al., 2010; Liu et al., 2016). Globalization of food trade and growing tourism in India inevitably allow the transmission of foodborne pathogens rapidly across distant borders. Therefore, a routine surveillance of foodborne illness is required to alleviate the disease burden in the region.

WHO has prioritized 22 enteric foodborne pathogens for assessing the burden of foodborne illness (Kirk et al., 2015). Though several industrialized countries have estimated the DALYs for the foodborne diseases, the global estimate is incomplete because of the unavailability of data from resource-limited countries like India (Devleesschauwer et al., 2015). For example, the global disease burden of *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, and *Staphylococcus aureus* could not be estimated by GBD 2015 due to unavailability of data. These pathogens with 100% foodborne attributes are frequently reported in fermented foodborne outbreaks worldwide (World Health Organization, 2015).

As most of the fermented foods marketed in India are produced by spontaneous fermentation in an uncontrolled environment and frequent incidences of foodborne outbreaks are reported in the region, a baseline surveillance data is essential to monitor the fermented foodborne disease burden in India. The recent advancements in cultivation-independent molecular techniques, particularly metagenome sequencing by next-generation sequencing techniques followed by the rapid deciphering of the sequence data with advanced bioinformatics tools make the microbial hazard analysis easier than ever before. In this molecular surveillance study, we aim to analyze the prevalence, toxigenic and pathogenic potential of the bacterial pathogens in the traditional fermented foods marketed in North-eastern region of India by using next-generation sequencing techniques (MiSeq) in combination with qPCR and immunoassays.

2. Materials and methods

2.1. Sample collection

Samples of traditionally processed food products broadly categorized into fermented soybean, bamboo shoot, fish, milk and pork products (39 food types, four seasons, 682 samples) were collected under aseptic conditions from the markets of seven states of Northeast India (Table 1). The samples were transported to the lab using a cooler box. For the purpose of cultivation-independent metagenomic analysis, the samples were directly stored at −80 °C. For culture-based studies, the samples were suspended in 50% glycerol stock and preserved at −80 °C.

2.2. Microbial analysis

Forty grams of each food sample was homogenized in 360 ml of sterile 0.1 M phosphate buffer saline (pH 6.4) using Stomacher (Seward, UK) and the metagenomic DNA was extracted with the protocols optimised by us for each type of food as reported earlier (Keisam et al., 2016). DNA extraction by enzymatic lysis principle was used for the fermented milk and soybean products. Briefly, 1.5 ml of the homogenate was centrifuged and the pellet was treated with lysozyme (50KU) and mutanolysin (25 U). After incubation at 37 °C for 1 h, proteinase K was added and incubated at 65 °C for 1 h. Further, 500 µl of GES reagent (5 M guanidine thiocyanate, 100 mM EDTA, and 0.5% sarkosyl) was added and the solution was mixed with ice cold 7.5 M ammonium acetate. The DNA was finally precipitated with isopropanol and dissolved in TE buffer. In case of fermented fish and pork products, bead beating and chemical lysis principles were employed. Briefly, 1.5 ml of the homogenate was added to a sterile 2 ml screw-cap tube containing 0.5 g of zirconia/silica beads (0.1 mm) and 4 glass beads (2 mm). After centrifugation, the pellet was washed with 1 ml of petroleum ether:hexane (1:1) to remove the fat. The pellet was treated with 25 µl of proteinase K (25 mg/ml) in 150 µl buffer [50 mM Tris-Cl, 10 mM EDTA (pH 8), 0.5% (w/v) SDS] and incubated overnight at 65 °C. Further, 150 µl of 2× breaking buffer [4% Triton X-100 (v/v), 2% (w/v) SDS, 200 mM NaCl, 20 mM Tris (pH 8), 2 mM EDTA (pH 8)] was added and the DNA was purified with phenol:chloroform:isoamyl alcohol mixture (25:24:1) and the DNA from aqueous layer was precipitated with ethanol. In case of fermented bamboo shoots, the steps of enzymatic lysis followed by addition of 5 M NaCl and CTAB/NaCl solution, purification by phenol:chloroform:isoamyl alcohol (25:24:1) and precipitation with 3 M Na-acetate and isopropanol were followed. In all extraction methods, the laboratory prepared reagents were confirmed for the negative PCR amplification using microbial specific primers before use. The total eubacterial load in the samples was quantified with a SYBR Green (Invitrogen) based qPCR assay (Applied Biosystem 7500 standard qPCR platform) by targeting the SSU rRNA gene V3 region (Table 2). Illumina MiSeq amplicon sequencing of V4-V5 region of the 16S rRNA gene (using the primer pair F563–577 and R924–907, Table 2) was used for the in-depth detection of foodborne pathogens from the food metagenome. The PCR amplicons generated from the metagenomic DNA of food samples were multiplexed with barcodes and subjected to sequencing (Romi et al., 2015). The sequence reads were quality filtered, demultiplexed and analyzed by QIIME v1.8.0 bioinformatics pipeline (Caporaso et al., 2010) and the data were uploaded to MG-RAST with project ID 11495. The relative abundance of the pathogens was derived from the quality-filtered species-level OTUs generated by MiSeq sequencing. These OTUs were log transformed ($\log_{10}\ x_i + 1$) and analyzed using the compute_core_microbiome.py script in QIIME to observe the presence of a core eubacterial community.

From the MiSeq sequencing data, seven enteric pathogens with high occurrence and abundance in the fermented foods were selected to quantify the pathogen load using qPCR assay. The primer pairs targeting the species-specific housekeeping genes of the seven pathogens were designed using NCBI Primer-BLAST. This online tool employs Primer3 algorithm for searching candidate primer pairs and Needleman-Wunsch global alignment algorithm for checking the specificity of the generated oligonucleotides (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Ye et al., 2012). The specificity of the generated oligonucleotide pairs was evaluated *in-silico* by performing nucleotide similarity searches using the BLAST algorithm for nearly exact matches on the NCBI website (http://www.ncbi.nlm.nih.gov). Primers with a desired product size and high specificity were then analyzed for their melting temperature, likelihood to form self-complementarity and hairpin loop using an online tool OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) (Kibbe, 2007). As an additional confirmation of the primer specificity, the selected primer pairs were used to amplify target genes from the corresponding pathogen reference strains, other reference strains available in our collection, and randomly selected MiSeq-positive food metagenomic DNA. Based on the prevalence of WHO prioritized foodborne pathogens in the Indian fermented foods during MiSeq analysis and the availability of pathogenic cultures in our collection, the cultures of *B. cereus* MTCC 430, *S. aureus* ATCC 11632, *E. coli* ATCC 11229, *L. monocytogenes* ATCC 19118,

Table 1
List of the naturally fermented foods collected from Northeast India for this molecular surveillance study.

Group	Type of food	Local name	Number of producers	Number of samples[a]	Place of collection	
					Place	State
I	Fermented soybean products	*Hawaijar*	5	20	Imphal	Manipur
		Akhoni	5	20	Dimapur	Nagaland
		Smoked *akhoni* paste	3	15	Dimapur	Nagaland
		Bekang	5	20	Aizawl	Mizoram
		Peru	5	18	Itanagar	Arunachal Pradesh
		Smoked *peru* paste	5	18	Itanagar	Arunachal Pradesh
		Tungrymbai	4	16	Shillong	Meghalaya
		Cooked *tungrymbai*	5	20	Shillong	Meghalaya
II	Fermented bamboo shoot products	*Erring*	5	19	Itanagar	Arunachal Pradesh
		Eku	5	20	Itanagar	Arunachal Pradesh
		Eup	5	17	Ziro	Arunachal Pradesh
		Khorisa	5	17	Jorhat	Assam
		Sene	5	20	Dimapur	Nagaland
		Sele	5	18	Dimapur	Nagaland
		Dried *sele*	3	12	Dimapur	Nagaland
		Soibum	5	20	Imphal	Manipur
		Lung siej	3	12	Shillong	Meghalaya
		Serhe	1	2	Dimapur	Shillong
III	Fermented fish products	*Ngari*	5	20	Imphal	Manipur
		Hentak	5	20	Imphal	Manipur
		Shedal	13	37	Silchar	Assam
		Lona ilish	5	20	Agartala	Tripura
		Shedal	9	35	Agartala	Tripura
		Dang pui thu	2	8	Aizawl	Mizoram
		Ngawum	8	33	Aizawl	Mizoram
		Tungtap	5	19	Shillong	Meghalaya
		Shedal	5	19	Jorhat	Assam
		Khukha ilish	3	9	Guwahati	Assam
		Lona ilish	1	5	Guwahati	Assam
		Ango-ango	1	3	Ziro	Arunachal Pradesh
		Shedal	3	13	Jorhat	Assam
		Khukha ilish	2	6	Silchar	Assam
IV	Fermented milk products	*Sanggom afamba*	5	20	Imphal	Manipur
		Doi	7	26	Agartala	Tripura
		Doi/Dahi	5	19	Silchar	Assam
		Dahi	4	16	Shillong	Meghalaya
		Dahi	4	16	Guwahati	Assam
		Dahi/Dei	4	15	Jorhat	Assam
V	Fermented pork	*Sa-um*	5	19	Aizawl	Mizoram
		Total	180	682		

[a] Three to four seasonal samples were collected from the same producer.

Enterococcus faecium ATCC 35667, and *P. mirabilis* ASB11 were used for optimising the qPCR assays. The genomic DNA of these reference cultures was used as a standard series for positive control in the qPCR assay, along with other reference cultures available in our collection to validate the cross-amplification of the designed and adopted primers. In case of *C. botulinum* quantification by qPCR assay, the *rpoB* gene amplified from the MiSeq-positive food metagenome (with > 97% sequence similarity with *C. botulinum* during NCBI BLAST search) was used as internal control. This is due to the non-availability of *C. botulinum* reference strain in our collection and restriction in use due to safety regulations. We further validated the specificity of the qPCR assays by sequencing the PCR amplicons (ABI3730xl DNA Analyzer, Xcelris, Ahmadabad) and querying the nucleotide sequences against NCBI GenBank. Additionally, qPCR standard graph, melt curve analysis and a run at 2% agarose gel (w/v) electrophoresis were performed to confirm the target-specific amplification for all the primer pairs used in this study. The list of adopted and newly designed pathogen-specific primers is listed in the Table 2. All the samples were run in triplicates with eight-point standard graph (R^2 > 0·99) range of 10^1–10^8 gene copies with assay efficiencies in the range of 0.942–0.993. To establish the phylogenetic relationship of *C. botulinum* present in the food samples, the nucleotide sequences of amplified *rpoB* gene were aligned with the *rpoB* gene sequences of different phylotypes of *C. botulinum* and other closely related species by using ClustalW. The aligned sequences were then used to construct a neighbor-joining phylogenetic tree based on Kimura-2 parameter evolutionary distance matrix with 1000 bootstrap replications using MEGA6 software (Tamura et al., 2013).

2.3. Risk analysis

We performed qPCR assays to quantify the pathogenic and toxigenic genes of the major pathogens from the food metagenome for establishing the risk of food poisoning and enteric infection. The primers used for the risk analysis are listed in the Table 2. We also validated the specificity of the primers by sequencing the PCR products as described earlier. The pathogens detected during MiSeq sequencing but not detected by housekeeping gene qPCR assays were further subjected to pathogenic gene qPCR assays to confirm their absence decisively in the samples. The isolates of two major pathogens (*B. cereus* and *P. mirabilis*) were recovered from the food homogenate (serially diluted using physiological saline and spread plated over plate count agar (PCA), incubated at 30 °C for a period of 48 h). The presence of pathogenic and toxigenic genes in the cultures of *P. mirabilis* and *B. cereus* was also examined. For the qualitative detection of bacterial toxins, an immunoassay was performed with 150 µl of the food homogenates (1 g sample in 9 ml sterile ultrapure water). The presence of *B. cereus* diarrheal enterotoxins (Nhe and Hbl) and emetic toxin (cereulide) were analyzed by the Duopath Cereus Enterotoxins and Singlepath Emetic

Table 2
List of the primers used in this molecular surveillance study.

Target group	Target gene	Oligo name	Primer sequence (5′ to 3′)	Function/product	Annealing temperature (°C)	Amplicon size (base pair)	Reference
Eubacteria	SSU rRNA V3 region	338f	ACTCCTACGGGAGGCAGCAG	16S ribosomal RNA	65–55, touchdown PCR	190	Ampe et al. (1999)
		518r	ATTACCGCGGCTGCTGG				
Eubacteria	SSU rRNA V4-V5 region	F563-577	AYTGGGYDTAAAGNG	16S ribosomal RNA	55	354	Romi et al. (2015)
		MID-R5[a]	CCGTCAATTCMTTTRAGT				
B. cereus	*pheS*	BC_pheS _F1	GTGGACGCTCTTCTGCTGAT	phenylalanyl-tRNA synthetase subunit alpha	60	113	This study[b]
		BC_pheS_R1	AGCGAAAGAGCTGAAAGGCT				
	nheA	45c1	GAGGGGCAAACAGAAGTGAA	Non hemolytic enterotoxin	52	186	Moravek et al. (2004)
		45c2	TGCGAACTTTTGATGATTCG				
	nheB	39b1	CCGCTTCTGCAAAATCAAAT	Non hemolytic enterotoxin	55	281	Moravek et al. (2004)
		39b2	TGCGCAGTTGTAACTTGTCC				
	nheC	nheC F	GAGTAATGATATGGTCATTGC	Non hemolytic enterotoxin	55	163	Stenfors and Granum (2001)
		nheC R	CGACTTCTCTGCTTGTGCTCCTG				
	hblA	HA-F1	ATTAATACAGGGGATGGAGAAACTT	Hemolysin	55	237	Yang et al. (2005)
		HA-R1	TGATCCTAATACTTCTTCTAGACGCTT				
	hblD	mp3L1R1 for	AGTTATTGCAGCTATTGGAGG	Hemolysin	60	148	Wehrle et al. (2010)
		mp3L1R1 rev	GTCCATATGCTTAGATGCTGTGA				
	entFM	FM-F2	CAAAGACTTCGTAACAAAAGGTGGT	Enterotoxin/cell wall peptidase	60	290	Yang et al. (2005)
		FM-R2	TGTTTACTCCGCCTTTTACAAACTT				
P. mirabilis	*gyrB*	PM_gyrB F	CAGTGAACATGCCCCTGCTA	DNA gyrase B	60	200	This study
		PM_gyrB	TCACCAAGCCACTCATCCAC				
	hpmA	PM_hpmA F	ACGAGCCACCAAAATGCAAC	Hemolysin	65	105	This study
		PM_hpmA R	ACCCGTAACGCTATCAGCAG				
	hpmB	PM_hpmB F	GCGCAGAGGAAACACCCTAT	Hemolysin	60	159	This study
		PM_hpmB R	GGAGTGTGTCGTGGCTGTAT				
	ureB	PM_ureB F	ACAGGTGGCTAATCATGGCG	Urease	68	167	This study
		PM_ureB R	CAACAGTGCGGCTTTGACC				
	ureC	PM_ureC F	AGTCGGTAGGAGAGCCCAAT	Urease	65	234	This study
		PM_ureC R	TGGCTTGTGAGTCTGACGAC				
	zapA	PM_zapA F	AAGTGGGCGCCTGTTTCATA	IgA metalloprotease	65	106	This study
		PM_zapA R	ACGCAGGTCAGAATGTTCCA				
	pmfA	PM_pmfA F	CTGGCGCAGGTATTGGCTTA	Major fimbrial subunit	65	148	This study
		PM_pmfA R	GGCGTTACTTTAGCGTCAGC				
C. botulinum	*rpoB*	CB_rpoB_F	GGAAGAACCCCTGAATCCGT	RNA polymerase, beta subunit	60	132	This study
		CB_rpoB_R	AAATGATGATGCCAGCCGTG				
	BoNTA	CB_A_F2	ACGCGAAATGGTTATGGCTCTACTC	Toxin A	60	142	Satterfield et al. (2010)
		CB_A_R2	GTGCTAATGCTACCGCTGGATCTG				
	BoNTB	CBMLB1	CAGGAGAAGTGGAGCGAAAA	Toxin B	60	205	Lindstrom et al. (2001)
		CBMLB2	CTTGCGCCTTTGTTTTCTTG				
	BoNTE	CB_E1	CTATCCAAAATGATGCTTATATACCAAA	Toxin E	60	115	Fach et al. (2009)
		CB_E2	GGCACTTTCTGTGCATCTAAATA				
	BoNTF	BoNT_F_F1	CCGGATTCATTAAAGAACGGAAG	Toxin F	60	153	Kirchner et al. (2010)
		BoNT_F_R1	TGATATTTCTTGTAACAAAACTTCCCCTG				
L. monocytogenes	*gyrB*	LM-gyrB_F	GTGCACCATCAACATCAGCA	DNA gyrase B	65	150	This study
		LM_gyrB_R	AGAAAGCGCGTTTAGACCGA				
	hlyA	hlyA-146-F	AAATCTGTCTCAGGCGATGT	Listeriolysin	55	103	Barbau-Piednoir et al. (2013)
		hlyA-146-R	CGATGATTTGAACTTCATCTTTTGC				
	IAP	LM_IAP_F	ACAAGCTGCACCTGTTGCA	Invasion associated protein	55	131	Furrer et al. (1991)
		LM_IAP_R	TGACAGCGTGTGTAGTAGCA				
Y. enterocolitica	*pheS*	YE_pheS_F2	TGATACCACCCGACTGTTGC	phenylalanyl-tRNA synthetase subunit alpha	60	132	This study
		YE_pheS_R2	GGTGTGTGGGTTTGGTCGTA				
	ystB	YE_ystB_F	GTACATTAGGCCAAGAGACG	Heat stable enterotoxin	61	146	Thoerner et al. (2003)
		YE_ystB_R	GCAACATACCTCACAACACC				
	ail	YE_ail_F1	GGTCATGGTGATGTTGATTACTATTCA	Attachment invasion locus	58	91	Bhaduri et al. (2005)
		YE_ail_R1	CGGCCCCCAGTAATCCATA				
S. aureus	*gyrB*	SA_gyrB_F	AAGTCGCACGTACAGTGGTT	DNA gyrase B	68	82	This study
		SA_gyrB_R	CGTGTTACTTCACGCGCTTT				
	sea	SEA-3	CCTTTGGAAACGGTTAAAACG	Staphylococcal enterotoxin A	60	127	Pinto et al. (2005)
		SEA-4	TCTGAACCTTCCCATCAAAAAC				
	see	SA_see_F	AAAGATTTGCGAAAAAAGTCTGAATT	Staphylococcal enterotoxin E	60	106	Letertre et al. (2003)
		SA_see_R	CATCACTCTCTTTGTTTTCAGTTATAGC				
E. coli	*uidA*	uid AF	CAACGAACTGAACTGGCAGA	beta-D-glucuronidase	60	121	Chern et al. (2009)
		uid AR	CATTACGCTGCGATGGAT				

[a] 12 bp Golay barcodes were attached at the 5′ end of the primers to enable sample multiplexing during Illumina MiSeq amplicon sequencing.
[b] The evidence of specificity of the designed primers are shown in the Supplementary figures (Figs. S3 and S4).

Tox Mrk (Merck Millipore, Germany) kits respectively. The culture supernatant of *B. cereus* isolates from different soybean products were also analyzed for the toxin production after growing overnight in Luria Bertani broth at 37 °C. The culture supernatant of *Bacillus subtilis* ATCC 6051 and *B. cereus* MTCC 430 grown in similar condition were used as controls for this assay. Similarly, immunoassays for detection of botulinum toxin A and B in food was performed using BADD Botulinum Toxin Detection kit (AdVnt Biotechnologies, USA) as per the

manufacturer's instruction. The *C. botulinum* cultures were not recovered from the food for analyzing the toxin production due to the safety regulations. The antibiotic resistance of 16 isolates of *P. mirabilis* recovered from the fermented foods was determined by antimicrobial susceptibility test discs (Himedia). Based on the clearing zone formation against 16 antibiotics in a spread culture over Mueller Hinton Agar media incubated at 37 °C for 18 h, the isolates were determined to be resistant, intermediate and sensitive in accordance to the performance standards for antimicrobial disc susceptibility by Clinical and Laboratory Standard Institute (CLSI) guidelines.

2.4. Statistical analysis

Boxplots and histograms for comparing the pathogen load and its temporal variation were plotted using Dell Statistica software v13. Heat maps of pathogens occurrence, abundance, and substrate-specific core bacterial community analysis were visualized using RStudio v0.98.1091. The pathogen occurrence in different food types was calculated by positive samples detected during in-depth MiSeq amplicon sequencing. The log-transformed gene copy numbers with standard deviation were used for the expression of pathogen load. Any significance in the temporal variation of pathogen abundance was tested with analysis of variance (ANOVA) using PAST v3.08.

3. Results

3.1. High prevalence of enteric pathogens in the fermented soybean, fish and pork products

We confirmed the bacterial presence in high load (Fig. 1) in the ethnic fermented food products marketed in India by eubacterial specific qPCR assay, which showed a bacterial load of 10^{10}–10^{11} cells/g in soybean and milk products, 10^{8}–10^{9} cells/g in bamboo, fish, and pork products (Fig. 1). Further, in-depth bacterial community structure analysis by MiSeq amplicon sequencing of V4-V5 region of 16S rRNA gene resulted in a total of 3,666,876 quality filtered sequence reads with an average length of 354 bp. Taxonomic assignment of the sequences at 97% identity and hierarchical clustering based on the assigned operational taxonomic units (OTUs) showed the existence of food substrate-specific (fish, milk, soybean, bamboo, and pork) core microbiota (Fig. S1, Supplementary materials) with high diversity (Fig. 1). The bacterial genus predominated in these food products are as follows: *Bacillus* in soybean products; *Lactobacillus* and *Bacillus* in bamboo shoot products; *Lactobacillus*, *Bacillus*, *Staphylococcus* and *Clostridium* in fish products; *Lactobacillus*, *Leuconostoc* and *Gluconobacter* in milk products; and *Clostridium* in pork products (Fig. S2, Supplementary materials). Among the WHO recognized foodborne bacterial pathogens, phylotypes related to *Clostridium botulinum*, *Bacillus cereus*, *Staphylococcus aureus*, *Clostridium perfringens*, *Listeria monocytogenes*, and *Escherichia coli* were detected in the Indian fermented foods by MiSeq amplicon sequencing (Fig. 2). Also, the well known human enteric pathogens like *Proteus mirabilis*, *Clostridium difficile*, and *Yersinia enterocolitica* occurred in these ethnic foods. Fig. 3 shows the occurrence and relative abundance of nine enteric bacterial pathogens in five broadly categorized food types (soybean, milk, bamboo shoot, fish and pork products). Our data suggested an abundant presence of a bacterial phylotype related to *C. botulinum* in the fermented foods of Northeast India. Moreover, we noticed a common occurrence of *B. cereus*, *C. perfringens* and *S. aureus* in these traditionally processed food products. Specifically, the fermented soybean products and milk products had a high prevalence of *P. mirabilis* and *Y. enterocolitica* respectively.

3.2. Alarming pathogen load and their temporal variation

As inferring species-level taxonomic resolution from the short sequence of 16S rRNA gene is usually not reliable, we performed pathogen-specific qPCR assays for the MiSeq-positive samples (detected with the presence of a particular foodborne pathogen) by targeting the housekeeping genes for confirming the pathogen identity and its load. The specificity of the primer pairs targeting the species-specific housekeeping genes of the seven pathogens designed during this study and adapted from earlier studies were validated for its specificity (Figs. S3 and S4, Supplementary material). The qPCR assay also showed a high load of *C. botulinum* related phylotype in the fermented foods of Northeast India, particularly in soybean, fish and pork products (Table 3). Though we could not use the genomic DNA of *C. botulinum* as a control for the qPCR assay due to safety regulations, the *rpoB* gene of *C. botulinum* amplified from the MiSeq-positive food metagenome (showed with a sequence similarity of 97% with the *C. botulinum* Group-I phylotype, Fig. 4) was used as the internal amplification control. Further, we noticed a high load of *P. mirabilis* and *B. cereus* in all the soybean samples analyzed. Also, observed the presence of *P. mirabilis* in the MiSeq-positive pork samples. In addition, qPCR assay detected the presence of *E. coli* in the MiSeq-positive milk and few soybean products. Although there was no temporal variation in the total bacterial load, we noticed a general trend of higher prevalence of *B. cereus* (ANOVA, $p < 0.05$; $F = 5.79$) and *P. mirabilis* (ANOVA, $p < 0.05$; $F = 4.70$) in the soybean products during colder months (Fig. 5). A similar trend of temporal variation also noticed in *C. botulinum*. The results of species-specific qPCR assays confirmed the presence of an alarming load of *B. cereus* in fermented soybean products, *P. mirabilis in* soybean and pork products, and a phylotype of *C. botulinum* in soybean, pork and fish products.

3.3. Confirmation of potential risk by molecular and immunoassays

As the dominant presence of these pathogens alone does not always imply a real health threat, we further examined their pathogenic

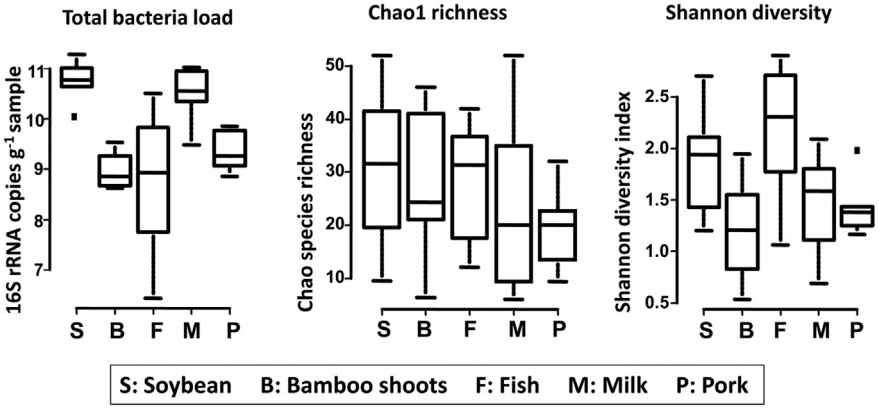

Fig. 1. Total eubacterial load (log_{10} 16S rRNA gene copies/g) quantified by qPCR assay and bacterial diversity (Chao species richness and Shannon diversity index) calculated from the MiSeq amplicon sequencing data of food metagenome in different types (soybean, bamboo shoot, fish, milk and pork) of naturally fermented foods.

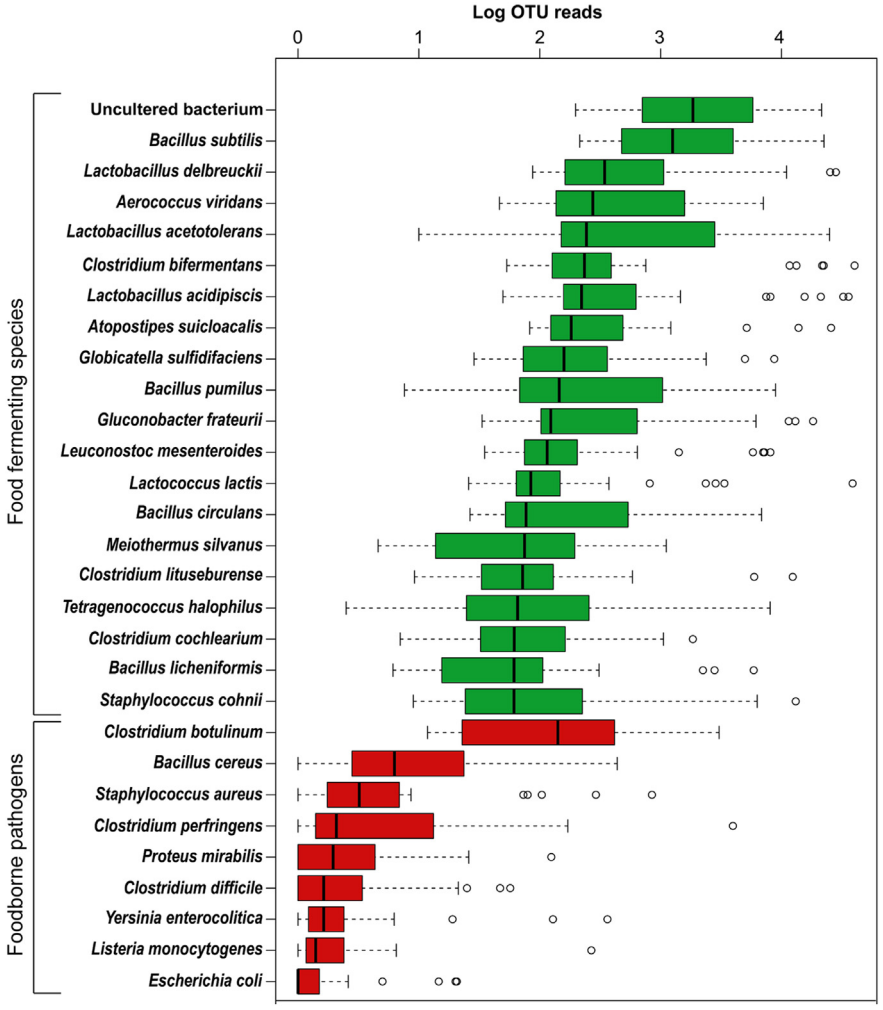

Fig. 2. Prevalence of enteric bacterial pathogens in the naturally fermented foods marketed in India assessed by Illumina MiSeq amplicon sequencing of V4-V5 region of the 16S rRNA gene. The box plot shows the level of dominance of nine enteric pathogens (red colour) among the core food fermenting bacteria (top 20, green colour) in all the food types. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

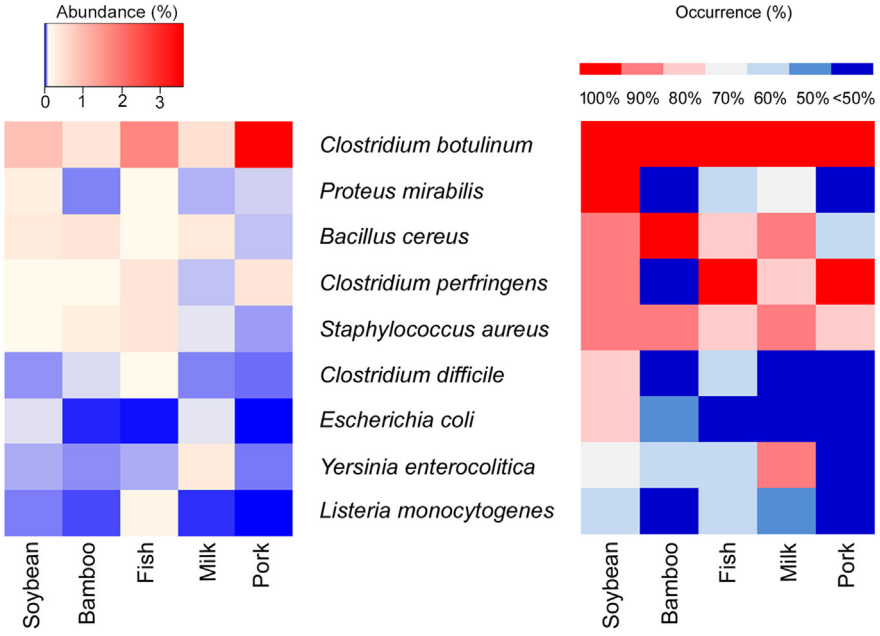

Fig. 3. The relative abundance and occurrence of nine predominant enteric bacterial pathogens detected in the naturally fermented foods of India by MiSeq amplicon sequencing. The abundance heat map shows the relative abundance (%) of the pathogen, while the occurrence heat map shows the % of samples positively detected with the presence of particular pathogen. The foods are broadly categorized into soybean, bamboo, fish, milk, and pork based products.

Table 3
Population load and occurrence of the major foodborne pathogens determined by species-specific qPCR assay in the naturally fermented foods (soybean, bamboo, fish, milk and pork products) marketed in India.

Target pathogens and genes	Occurrence (samples detected by qPCR assay/ MiSeq-positive samples)	Population load (log_{10} mean of gene copies/g ± SD, range)
Bacillus cereus (*pheS*)		
Soybean	147/147[a]	6.5 ± 0.2 (4.62–8.76)
Bamboo	20/157	3.82 ± 0.52 (3.21–4.61)
Proteus mirabilis (*gyrB*)		
Soybean	132/132	7.9 ± 0.2 (4.81–10.04)
Pork	9/9	5.19 ± 1.50 (5.22–7.52)
Clostridium botulinum (*rpoB*)		
Soybean	88/147	7.77 ± 0.69 (6.98–9.09)
Bamboo	24/157	3.55 ± 0.28 (3.08–3.86)
Fish	108/247	4.93 ± 1.73 (2.74–8.74)
Milk	5/112	5.31 ± 0.01 (5.29–5.31)
Pork	14/19	7.59 ± 1.05 (3.58–8.61)
Escherichia coli (*uidA*)		
Soybean	15/115	5.66 ± 1.7 (4.22–7.52)
Milk	17/17	4.70 ± 1.12(3.52–6.22)

[a] The pathogens prevalence in the MiSeq-positive samples was validated by species-specific qPCR assay.

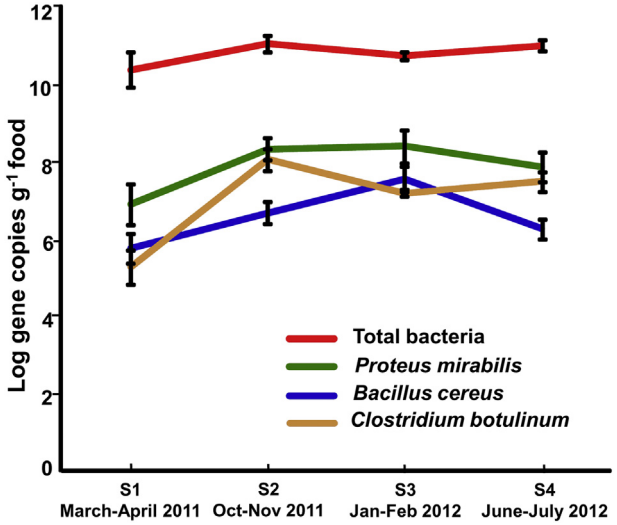

Fig. 5. Temporal variations in the population load of *Bacillus cereus*, *Proteus mirabilis* and *Clostridium botulinum* related phylotype in the naturally fermented soybean foods marketed in India. The changes during four seasonal points (S1–S4) are compared here with the baseline of the total bacterial load.

potential and toxin production in soybean, fish, and pork products by qPCR and immunoassays. Altogether 16 pathogenic genes of *B. cereus*, *P. mirabilis*, and *C. botulinum* were targeted to establish their toxigenic and pathogenic potential, which indicates the risk of food poisoning and enteric infection while consumption of these pathogens positive food products. The qPCR assays showed that most of the samples positive for *B. cereus* and *P. mirabilis* were also positive for their pathogenic and virulence factors (Table 4). The cultures of *P. mirabilis* and *B. cereus* isolated from these food samples were also positive for the pathogenic factors. On the other hand, immunoassays observed *B. cereus* diarrheal toxins (Nhe and Hbl) production in all the soybean samples analyzed while nearly half of the samples were positive for the rare emetic toxin (cereulide) (Fig. S5 and Table S2, Supplementary material). The *B. cereus* cultures isolated from the soybean products were also positive for diarrheal toxins but negative for the emetic toxin production. The *P. mirabilis* cultures (16 isolates from the fermented foods) showed highly swarming colonies with the presence of genes of major fimbrial subunit (*pmfA*), IgA metalloprotease (*ZapA*), urease (*ureB* and *ureC*), hemolysins (*hpmA*, *hpmB*) and intrinsic resistance to multiple antibiotics (Table S3) as an indicator of its virulence factor. Above results suggest a potential hazard in consumption of these naturally fermented foods.

We could not establish the toxin producing potential of *C. botulinum* related phylotype present in the fermented foods analyzed in this study, as the qPCR assays failed to detect the genes encoding the botulinum neurotoxins (toxin A, B, E, and F) in the *C. botulinum* positive food samples. Moreover, immunoassay failed to detect the presence of botulinum toxins A and B in the *C. botulinum* positive samples. We targeted the botulinum toxins A and B for the immunoassay as the *C. botulinum* phylotype detected in Indian fermented foods showed a close similarity with *C. botulinum* Group-I. We did not culture *C. botulinum* from Indian fermented foods because of the strict regulations required

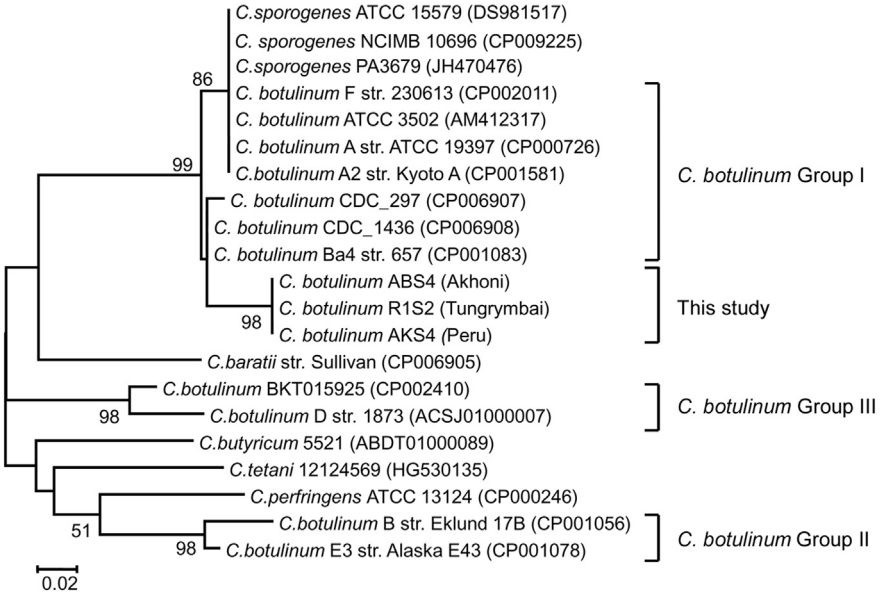

Fig. 4. The neighbor-joining phylogenetic tree generated based on the *rpoB* gene sequences of *C. botulinum* retrieved from the metagenome of Indian fermented foods shows its close relative.

Table 4
Toxigenic and pathogenic potential of the three major pathogens present in the naturally fermented soybean products marketed in India assessed by qPCR analysis of food metagenome by targeting the toxin and virulence genes.

Target pathogens and genes	Occurrence (samples detected by qPCR assay/MiSeq-positive samples)	Gene copies/g (log_{10} mean ± SD, range)
Bacillus cereus		
Hemolysin BL binding component gene (*hblA*)	117/145	5.66 ± 0.75 (4.05–7.2)
Hemolysin BL lytic component L1 gene (*hblD*)	109/145	5.10 ± 0.93 (3.58–7.05)
Nonhemolytic enterotoxin lytic component L2 gene (*nheA*)	135/145	6.24 ± 0.91 (4.34–8.02)
Nonhemolytic enterotoxin lytic component L1 gene (*nheB*)	113/145	6.72 ± 1.04 (4.17–8.35)
Nonhemolytic enterotoxin C gene (*nheC*)	145/145	5.68 ± 0.71 (3.54–7.07)
Enterotoxin and cell wall peptidase gene (*entFM*)	145/145	7.12 ± 1.29 (4.70–9.19)
Proteus mirabilis		
Hemolysin component A gene (*hpmA*)	147/147	7.39 ± 1.62 (3.85–10.26)
Hemolysin component B gene (*hpmB*)	147/147	7.55 ± 1.32 (5.19–10.06)
Urease gene (*ureB*)	142/147	7.49 ± 1.38 (3.71–10.01)
Urease gene (*ureC*)	147/147	8.14 ± 1.56 (4.78–10.73)
IgA metalloprotease gene (*zapA*)	142/147	7.67 ± 1.45 (4.04–10.09)
Fimbriae gene (*pmfA*)	147/147	7.52 ± 1.39 (4.68–10.08)
Clostridium botulinum		
Botulinum neurotoxin A (*botA*)	0/147	Not detected
Botulinum neurotoxin B (*botB*)	0/147	Not detected
Botulinum neurotoxin C (*botC*)	0/147	Not detected
Botulinum neurotoxin D (*botD*)	0/147	Not detected

to work with this biohazard.

Though MiSeq analysis detected several foodborne pathogens, pathogen-specific qPCR assays narrowed down to *B. cereus*, *P. mirabilis* and *C. botulinum* related phylotype as the prevalent pathogens in the fermented foods of Northeast India. Further, qPCR assays targeting toxigenic and pathogenic genes in combination with immunoassays showed the potential of *B. cereus* and *P. mirablis* positive food samples to cause food poisoning and infection. However, our results did not support the toxigenic potential of *C. botulinum* related phylotype detected in the Indian fermented foods.

4. Discussion

This molecular surveillance study showed a high prevalence of enteric bacterial pathogens with toxigenic and pathogenic potential in the traditional fermented foods marketed in Northeast region of India. We have noticed an unusual prevalence of *P. mirabilis* in the Indian fermented foods. Though *P. mirabilis* is not listed in WHO list of foodborne pathogens, it has been recently recognized as a potential emerging infectious agent of critical multidrug-resistant status (http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/). Though the presence of *P. mirabilis* is considered as a hygienic indicator of a spoiled food, there are reports of food outbreaks due to *P. mirabilis* consumption (Wang et al., 2010). The *P. mirabilis* isolates from the fermented foods also showed the pathogenic indicators: swarming colony, pathogenic genes and intrinsic resistance to multiple antibiotics. In addition, *P. mirabilis* is well known for urinary tract infection leading to urease-induced urolithiasis (Norsworthy and Pearson, 2017). The *P. mirabilis* isolates from the fermented foods showed resistance to Nitrofurantoin, the antibiotic commonly used to treat the urinary tract infection (Hirsch et al., 2016). Though there is no direct reference to support the *P. mirabilis* consumption to cause urolithiasis, the physical contact with the food (while production, marketing, and eating) and high swarming ability of *P. mirabilis* can cause an urirary tract infection. Our results support the report of high prevalence of urolithiasis cases (11.6% of all general surgeries) in Indian region (Singh et al., 1978). We suggest a detailed surveillance to estimate the disease burden and urinary tract infection by the *P. mirablis* spreading through naturally fermented foods marketed in India.

Soybean products which are frequently implicated in major food outbreaks in the region had a high prevalence of *B. cereus*, *P. mirabilis*, and *C. botulinum* related phylotype. Similarly, the pork product '*sa-um*' which has been linked with stomach cancer (Phukan et al., 2006) had a high load of *C. botulinum* phylotype and *P. mirabilis*. Fermented food outbreaks due to *B. cereus* (Zhou et al., 2014), *C. botulinum* (Wangroongsarb et al., 2013; Wangroongsarb et al., 2014) and *P. mirabilis* (Wang et al., 2010) have been reported worldwide. Usually, consumption of food containing 10^7 cells/g of these pathogens is sufficient to cause symptomatic illness, but no major illness was recorded while consuming these high-risk foods during our sampling and survey. Further studies on pathogen strain typing might give a better understanding of the issue.

There are reports of *B. cereus* food outbreaks (Tewari and Abdullah, 2015) and *Clostridium* food outbreaks (Rama et al., 1998) in India, but not related with naturally fermented food products. It is surprising to notice that all the fermented soybean products marketed in Northeast India have a risky load of *B. ceresus* with diarrheal toxins, and nearly half with emetic toxin. However, the cultures of *B. cereus* isolated from these samples were negative for emetic toxin indicating the possibility of horizontal transfer of this plasmid borne toxin (Vassileva et al., 2007) to another bacteria during high-density natural food fermentation, may lead to the emergence of new infectious pathogens. Though all the four phylotypes of *C. botulinum* are reported with neurotoxin production (Kenri et al., 2014), we could not confirm the presence of botulinum toxins in the *C. botulinum* positive food samples, As *Clostridium* spp. predominate in Indian soils (Sengupta et al., 2011), this soil-borne bacteria might have entered into the food during uncontrolled natural fermentation. Cultivating the *C. botulinum* related phylotype to fix its phylogenetic affiliation, and characterizing its toxins if any is the area of future research.

Among the other foodborne pathogens of WHO priority, we did not detect *Campylobacter* spp. and *Salmonella* spp. in the Indian fermented foods. While, these two pathogens are the major cause of foodborne illness in the industrialized countries (Hofreuter et al., 2006; Le Hello et al., 2013). Moreover, the qPCR assays not detected the presence of *S. aureus*, *Y. enterocolitica*, and *L. monocytogenes* in the MiSeq-positive samples of Indian fermented foods. Further cross-validation by qPCR assays with primers designed from earlier studies (Table 2) also negative for Staphylococcal enterotoxin A (*sea*) and Staphylococcal enterotoxin E (*see*) in the *S. aureus* MiSeq-positive qPCR-negative samples. Similarly, the *Y. enterocolitica* MiSeq-positive qPCR-negative samples also negative for the heat stable enterotoxin (*ystB*) and attachment invasion locus (*ail*) genes, and *L. monocytogenes* MiSeq-positive qPCR-negative samples are negative for listeriolysin (*hlyA*) and invasion

associated protein (IAP). Our results indicated a low risk of these foodborne pathogens detected during MiSeq sequencing. However, detection of *C. difficile* in some of the food samples suggest a potential risk as it is the leading cause of stomach infections worldwide (Burnham and Carroll, 2013) and its foodborne disease burden is still unrecognized (Hawkey et al., 2013).

Consumption of these high-risk foods with an adequate load of pathogens and toxins was not reported with illness during our sampling and survey. The remoteness of the study area, less access to health-care, and no willingness to report due to the traditional culture could be the possible reasons for underreporting the illness. Consequently, we raise some questions pertinent to the dietary habits and immune status of the people in the region. Are they asymptomatic because of the development of acquired immunity against these pathogens? Can it be attributed to their traditional dietary habits like consumption of a high amount of turmeric (Dey et al., 2015) and ginger? Does it explain the high prevalence of traveler's diarrhea in this region? Still, the prevalence of WHO prioritized foodborne pathogens in the naturally fermented soybean, pork, and fish products of India should be treated as a public health threat in the region. A complete surveillance all over the Southeast Asian Region with similar food habits can give a true picture of fermented foodborne disease burden in the region.

Most of the fermented foods of India are produced as a household art by age-old practice of uncontrolled natural fermentation or back slopping. Replacing the indigenous process with a defined starter culture consortium based controlled fermentation will enhance the food safety in the region. This can be achieved by selecting the predominant autochthonous microbes involved in the natural fermentation and optimising the fermentation conditions. Moreover, hygienic packaging and maintaining a proper storage condition will also improve the safety level.

5. Conclusion

This molecular surveillance study generated the first baseline data of the enteric bacterial pathogens prevalent in the traditional fermented foods of India. The toxigenic and pathogenic potential of *Bacillus cereus* and *Proteus mirabilis* present in these traditional foods showed the risk of food poisoning and infection. Our data will support the sustained effort of WHO to estimate the global foodborne disease burden. The unusual presence of *P. mirabilis* in the fermented foods marketed in the Indian region with high incidence of urolithiasis cases is a concern due to its uropathogenic potential and multidrug-resistant status. We emphasize an urgent need of coordinated action to limit the spread of enteric pathogens through marketing of ethnic fermented foods in India. Moreover, intervention measures to control the pathogen entry into the fermented food production chain and to replace the indigenous process with the defined starter culture based controlled fermentation processes will enhance the public health in the Indian region.

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Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2019.02.012.

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