Diversity and Succession of Microbiota during Fermentation of the Traditional Indian Food Idli

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ABSTRACT Idli, a naturally fermented Indian food, is prepared from a mixture of rice and black gram (lentil). To understand its microbial community during fermentation, detailed analysis of the structural and functional dynamics of the idli microbiome was performed by culture-dependent and -independent approaches. The bacterial diversity and microbial succession were assessed at different times of fermentation by 16S rRNA amplicon sequencing. Results highlighted that most microbiota belonged to phylum *Firmicutes* (70%) and *Proteobacteria* (22%). Denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR) analysis confirmed the diversity and succession involved therein. A culture-dependent approach revealed that the microbially diverse populations were conserved across different geographical locations. The fermentation was primarily driven by lactic acid bacteria as they constitute 86% of the total bacterial population, and genus *Weissella* emerged as the most important organism in fermentation. The natural microbiota of the grains mainly drives the fermentation, as surface sterilized grains did not show any fermentation. Growth kinetics of idli microbiota and physicochemical parameters corroborated the changes in microbial dynamics, acid production, and leavening occurring during fermentation. Using a metagenomic prediction tool, we found that the major metabolic activities of these microbial fermenters were augmented during the important phase of fermentation. The involvement of the heterofermentative hexose monophosphate (HMP) pathway in batter leavening was substantiated by radiolabeled carbon dioxide generated from D-[1-¹⁴C]-glucose. Hydrolases degrading starch and phytins and the production of B vitamins were reported. Moreover, culturable isolates showing beneficial attributes, such as acid and bile tolerance, hydrophobicity, antibiotic sensitivity, and antimicrobial activity, suggest idli to be a potential dietary supplement.

IMPORTANCE This is a comprehensive analysis of idli fermentation employing modern molecular tools which provided valuable information about the bacterial diversity enabling its fermentation. The study has demonstrated the relationship between the bacterial population and its functional role in the process. The nature of idli fermentation was found to be more complex than other food fermentations due to the succession of the bacterial population. Further studies using metatranscriptomics and metabolomics may enhance the understanding of this complex fermentation process. Moreover, the presence of microorganisms with beneficial properties plausibly makes idli a suitable functional food.

KEYWORDS idli, lactic acid bacteria, *Weissella*, fermentation, metagenomics, succession

Cereals and pulses (edible dry seeds of leguminous plants) are major dietary constituents of individuals in developing countries. The presence of complex proteins, insoluble fibers, and antinutritional factors makes a cereal diet less salutary. These difficult-to-digest cereals and pulses are converted to nutrition-rich food by simple household fermentations, and this practice is an important part of provincial

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legacy (1). During the process, fermentative microorganisms play important roles in the preparation and preservation of food and also contribute to the taste and flavor of the final product (2). A small acid-leavened steamed cake, idli, is a naturally fermented Indian food. It is popular for its excellent organoleptic properties, soft and spongy texture, and subtle aroma. Idli is prepared from dehulled cotyledons of black gram (lentil; *Vigna mungo* [L.] Heppel) and parboiled rice (*Oryza sativa* [L.]). Constituents are soaked separately, pulverized, and mixed to obtain a coarse batter. The batter is allowed to ferment overnight at ambient temperature, without any starter culture. After fermentation, the batter is steamed to obtain the final product in a cake form (3). Lowering of batter pH due to acid production and leavening leading to dough rising are two major changes that occur during idli batter fermentation. The only fundamental work describing the role of microorganisms in the fermentation of idli was reported using laboratory fermented batter (4), which highlighted that the acid and gas required for batter leavening are generated solely by the heterofermentative bacteria *Leuconostoc mesenteroides*, *Enterococcus faecalis*, and *Pediococcus cerevisiae*. Low-acid producers *E. faecalis* and *L. mesenteroides* were present during the early part of fermentation, followed by the high-acid-producing *P. cerevisiae* (4). Idli fermentation closely resembles sourdough, in which the leavening is carried out by bacteria rather than yeast (5). Although few yeast species have been isolated from idli batter, their role in fermentation is not well defined (6). Idli fermentation has been the subject of quite a few research investigations involving different aspects, such as ingredient optimization (7), physicochemical changes (8–10), and nutritional improvement due to fermentation (11, 12).

Along with the isolation of cultivable organisms, the role and behavior of microorganisms in food fermentation have been elucidated using advanced techniques involving genomics, transcriptomics, proteomics, and metabolomics (13–17). The recently developed high-throughput DNA sequencing methods have been employed to find out the different microorganisms and their proportion and succession in complex food fermentations. 16S rRNA gene profiling provides more rapid and accurate identification of bacteria and is extensively used to decipher the microbial community involved in fermented foods. However, a detailed analysis of the structure and function of the microbial community involved in the fermentation of idli batter using modern methods has received little attention. In light of this, it was aimed to explore the diversity of the batter by culture-dependent and -independent methods. Eubacterial diversity changes were indicated during fermentation by 16S rRNA amplicon sequencing, PCR-denaturing gradient gel electrophoresis (DGGE), and culturable studies. This study also helped to decipher the bacterial succession during batter fermentation. Moreover, metagenomic predictions using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) provided a better understanding of the functional composition of the microbiota in idli fermentation.

RESULTS

Microbial composition of idli batter by culture-independent methods. DGGE was employed to compare and find out the similarity of the microbiota in 10 different idli samples obtained from different vendors. The representative DGGE fingerprint demonstrated in Fig. 1a showed that each sample had 13 to 17 major PCR bands, indicating wide bacterial diversity exists in idli samples. The DGGE profiles of 10 samples of idli obtained from different vendors were similar, indicating the reproducibility of DNA extraction, PCR amplification, and DGGE analysis and the presence of similar bacterial populations. The similarity among the various samples can be compared by cluster analysis of the digitized profiles of the gel. Cluster analysis for this DGGE gel showed that 10 samples can be grouped into 3 distinct clades. Members of each group demonstrated approximately 90% similarity with each other (see Fig. S1a in the supplemental material). A DGGE gel was also done for different times of laboratory fermented batter to see the diversity changes during the fermentation process (Fig. 1b). Across the 9 time points, 5 identical bands and 12 dissimilar bands were found.

FIG 1 DGGE analysis of PCR-amplified bacterial 16S rRNA gene fragments from (a) 10 different steamed idli cakes obtained from different vendors (S1 to S10) and (b) 9 time points of laboratory fermented idli batter from 00 to 24 hours. Bands common to all the lanes are marked by an asterisk (*) and those peculiar to few lanes are marked by a triangle (▲).

The high-intensity PCR bands from both DGGE gels were selected for identification by DNA sequencing. Of the 117 bands analyzed, DNA sequences of 76 bands corresponded to order *Lactobacillales*, revealing that lactic acid bacteria (LAB) were the most dominant bacterial group in idli fermentation. The remaining bands belonged to order *Bacillales* and *Enterobacteriales*. At the genus level, most of the bands were identified as *Weissella* upon sequencing (see Table S1 in the supplemental material).

DGGE is a semiquantitative technique and, for a single species, can yield multiple bands and heteroduplex formation, causing biases in analysis. Thus, to obtain detailed quantitative and reproducible taxonomic information about idli batter microbiota, amplicon sequencing of the V3 region of 16S rRNA gene using the MiSeq platform was performed. Sequencing was performed for PCR products obtained from amplification of DNA isolated from batter samples collected at nine time points of idli fermentation carried out in the laboratory.

A total of 4.4 million reads were obtained which, after assembly and quality filtering of the paired-end reads, yielded a total of 3.5 million reads, accounting to 80% good-quality usable reads. Using reference database SILVA 123 (release July 2015), a total of 1,477 observed operational taxonomic units (OTUs) were obtained (Table 1). From the final biological observation matrix (BIOM) table, cyanobacteria were filtered out under the assumption that they were contributed by the grains used in fermentation (18, 19).

TABLE 1 Summary of MiSeq amplicon sequencing and α -diversity indices

Time point (h)	No. of raw reads	No. of quality reads	Observed OTUs	Good's coverage	Chao 1 index	Shannon index	Simpson index
00	825,301	566,690	97	0.57	239.06	6.18	0.98
03	632,036	447,771	90	0.65	183.88	6.13	0.98
06	464,707	378,315	90	0.64	261.91	6.02	0.98
09	351,613	273,432	211	0.96	481.93	3.75	0.83
12	490,651	417,657	438	0.99	917.72	2.81	0.76
15	526,607	437,404	643	0.99	981.75	3.63	0.83
18	394,368	337,349	641	0.99	1110.45	3.73	0.81
21	380,868	351,494	706	0.99	1130.46	3.62	0.78
24	347,656	322,186	579	0.99	1055.28	3.74	0.84

FIG 2 Diversity dynamics during idli batter fermentation as determined by 16S rRNA gene amplicon sequencing. (a) Family-level and (b) genus-level distribution of the bacterial communities at different time points of the idli batter fermentation. (c) Correlation matrix showing the Pearson's rank correlation among the top 10 core genera. The Pearson's rank correlation coefficient ranges from 1 to -1 , corresponding to a strongly positive to a strongly negative correlation. (d) Weighted Unifrac PCoA plot highlighting a potential difference in community structure at different intervals of the fermentation process.

A total of 19 bacterial phyla were present at various times of the fermentation process (see Fig. S2 in the supplemental material). The first five abundant phyla constitute up to 99.4% of the entire bacterial diversity. An exploration of the complete bacterial community revealed that phylum *Firmicutes* was the most dominant, and its contribution increased from an average 35% in the first 6 hours to 87% in the remaining duration. Abundance-wise, *Firmicutes* was followed by *Proteobacteria* (22%) and *Actinobacteria* (4%); the latter two phyla were abundant in the first 6 hours and then decreased thereafter. Among the first five abundant families, *Leuconostaceae*, *Enterococcaceae*, *Streptococcaceae*, and *Bacillaceae* belong to phylum *Firmicutes*, whereas *Enterobacteriaceae* belongs to *Proteobacteria* (Fig. 2a). *Leuconostaceae* constituted 71% to 85% during the 9th and 12th hour of fermentation. At the genus level (Fig. 2b), almost 48% of the OTUs corresponded to genus *Weissella*, confirming its dominance in the fermentation of idli. Further analysis showed the presence of different *Weissella* species, such as *W. cibaria*, *W. confusa*, *W. korensis*, *W. viridescens*, *W. oryzae*, *W. beninensis*, *W. thailandensis*, and several uncultured *Weissella* species. The average abundances of other important genera, such as *Alteromonas*, *Bacillus*, *Cronobacter*, *Enterobacter*, *Enterococcus*, *Escherichia-Shigella*, *Halomonas*, *Lactobacillus*, *Pantoea*, *Propionibacterium*, *Pseudomonas*, *Shewanella*, and *Streptococcus*, all exceeded 1%. Throughout fermentation, LAB constituted more than 85% of the total population. Of these, the major LAB enabling idli fermentation were found to be *Weissella*, *Enterococ-*

cus, and *Streptococcus*, among others, such as *Carnobacterium*, *Desemzia*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Vagococcus* (see Table S2 in the supplemental material).

Correlations among the above predominant genera were assessed based on Pearson's rank correlation (Fig. 2c), which showed that *Weissella* negatively correlates with most core organisms, such as *Alteromonas*, *Bacillus*, *Halomonas*, *Lactobacillus*, *Propionibacterium*, *Pseudomonas*, *Shewanella*, and *Streptococcus*. *Enterococcus* spp. correlated positively with *Streptococcus* and *Pantoea* and negatively with most other organisms. *Bacillus*, *Halomonas*, *Pseudomonas*, *Shewanella*, *Alteromonas*, *Lactobacillus*, *Enterobacter*, and *Propionibacterium* correlated positively with each other. The Good's coverage ratio ranged between 57% and 99%, indicating identified diversity to be present during the fermentation process (Table 1). The community diversity indices Shannon and Simpson index varied over a broad range (2.81 to 6.18 and 0.76 to 0.98, respectively). Chao1, the richness estimator, seemed to increase after 6 hours of the process as the richness of the abundant genera increased. The number of the observed OTUs also increased as the fermentation progressed (Table 1).

Quantification of predominant genera by qPCR. Employing genus-specific primers, the predominant microbiota were quantified in the idli batter by real-time quantitative PCR (qPCR), further substantiating the results obtained from amplicon data. *Weissella* growth increased significantly from 0th to 15th hour (4.3 to 7.9) and declined slightly thereafter, highlighting its abundance throughout the process (Fig. 3). Other bacteria, such as *Lactobacillus* (2.9 to 6.9), *Lactococcus* (1.4 to 5.1), and *Enterococcus* (3.9 to 6.4), also increased in significant numbers, whereas *Streptococcus* (2.3 to 3.1) and *Pantoea* (3.3 to 4) varied little during the fermentation process (Fig. 3). All the values have been expressed as log-transformed 16S rRNA gene copy numbers per gram of idli batter.

Microbial composition of idli batter by culture-dependent method. The bacterial diversity as assessed by a culture-independent approach was confirmed by a culture-dependent method. Using a spread plate technique and based on colony characters, a total of 354 bacterial isolates were obtained from 3 fully fermented idli batter samples, including 2 collected from different geographical locations (Bangalore and Pune) and 1 from laboratory fermented batter. All these isolates were identified up to the species level by morphological and biochemical properties and a partial 16S rRNA gene sequence. The Bangalore sample showed maximum diversity with 26 different bacterial species belonging to 12 genera (Fig. 4a), whereas in the Pune sample, 13 bacterial species representing 13 genera were identified (Fig. 4b). In the laboratory fermented idli batter, 14 genera and 26 different species were identified (Fig. 4c).

The identification of bacteria from these three samples revealed that the microbial community of all samples was similar with most isolates belonging to orders *Lactobacillales* and *Bacillales* (phylum *Firmicutes*) and *Enterobacteriales* (phylum *Proteobacteria*). The Pearson's coefficient showed that the correlation between the Bangalore sample and laboratory fermented batter was 97% and 95% at the phylum and genus level, respectively, while that between the Pune sample and laboratory fermented batter was 35% and 92% at the phylum and genus level, respectively. The common isolates from these three samples belonged to genera *Citrobacter*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus*, *Pantoea*, *Pediococcus*, *Staphylococcus*, and *Weissella* (see Table S3 in the supplemental material). These results showed that there exists wide bacterial diversity in all the ready-to-steam idli batters compared with that reported earlier (4). The lactic acid bacteria constituted nearly 56%, 19%, and 63% of the Bangalore, Pune, and laboratory fermented sample, respectively, confirming that LAB play an important role in the fermentation of idli. From all three samples, different *Weissella* species were isolated which had the capacity to ferment glucose and maltose, producing acid and gas. However, few variations in the diversity of these samples were noted, for example

FIG 3 Absolute quantification of important bacterial genera (as indicated) using quantitative real-time PCR. The radar plots elucidate the abundance distribution of predominant organisms (expressed as $\log_{10}$) essential during the fermentation of idli batter.

the Pune sample showed less diversity at the species level and its LAB content was only 19%, one-third of the other two samples. Nonetheless, organisms belonging to genera *Weissella*, *Enterococcus*, *Lactobacillus*, and *Pediococcus* could be cultured from the Pune sample, but their relative abundance was less than the Bangalore and laboratory fermented samples.

Microbial succession during idli fermentation. Idli batter fermentation is a result of natural cereal fermentation, independent of the back-slopping process. It normally takes 12 to 15 hours to obtain the fermented batter at ambient temperature. The substrates involved are complex proteins and carbohydrates which are finally metab-

FIG 4 Donut plots representing the bacterial population in fully fermented idli batter samples from (a) Bangalore, (b) Pune, and (c) laboratory fermented batter by the culture-dependent method. The inner and outer circles show the percentage distribution at the phylum and genus levels, respectively.

olized to acids and gas. Therefore, it is likely that there would be a succession of microbiota enabling this complex metabolic process, as reported earlier (4).

The succession pattern was first studied by a DGGE profile obtained for 9 time points of the fermentation spanning from 0 to 24 hours (Fig. 1b). A peculiar succession pattern in the DGGE gel highlighted the presence of few bands in the early time points which later disappeared and some new bands appeared in the later stages of fermentation. The dendrogram showed a progressive clustering of samples from the nine time points, indicating succession in the bacterial diversity as the fermentation progresses (Fig. S1b).

The 16S rRNA amplicon sequencing also highlighted the presence of succession in the bacterial diversity. Bacterial richness at the phylum level was found to be higher at earlier time points of the process, indicating a lot of microbiota to be contributed by the grains. Phylum *Firmicutes* increased, whereas phyla *Proteobacteria* and *Actinobacteria* decreased after the first 6 hours of the process, suggestive of microbial succession (Fig. S2). Family *Leuconostaceae* showed a gradient which gradually starts increasing from 0th to 12th hour and then decreases thereafter (Fig. 2a). Genera such as *Altero-*

TABLE 2 Summary of the physicochemical parameters during the 24-h fermentation of idli batter

Time (h)	Bulk density (g/cm ³)	Volume (ml)	pH	CO ₂ evolution (cpm) ^a	CFU g ⁻¹ (×10 ⁶) (MRS)	CFU g ⁻¹ (×10 ⁶) (NA)
0	1.2	0	6.3	40	0.06	0.82
6	0.8	1	6.0	216	33.6	57.6
12	0.6	34	4.6	250	37.6	109.6
18	0.5	52	4.2	1600	51.1	119
24	0.5	62	4.5	15,708	77.2	100.2

^acpm, counts per minute; CFU counts were taken per g of idli batter on MRS and nutrient agar (NA).

monas, *Bacillus*, *Halomonas*, *Lactobacillus*, *Propionibacterium*, *Pseudomonas*, and *Shewanella* were found to be abundant during the first 6 hours which then declined and were taken over by *Weissella*, as its abundance increased drastically from 9% to 71% from the 6th to 9th hour of fermentation (Fig. 2b). This is also clear from the correlation matrix, where the genus *Weissella* showed a negative correlation with these organisms (Fig. 2c). The abundance of *Weissella* gradually increased from time 0 to the 6th hour, exponentially increased in the 9th and 12th hour, and finally declined thereafter, highlighting the essential role of this heterofermentative organism in acid production and leavening of the idli batter during the major duration of the fermentation process. Genera *Enterococcus* and *Streptococcus* succeeded *Weissella* after the 12 hours of fermentation (Fig. 2b). This finding was also confirmed by quantitative real-time PCR showing the abundance pattern of important genera during the fermentation process (Fig. 3).

The diversity indices calculated across the 24-hour fermentation process revealed that the overall bacterial diversity was greater in the early stages, i.e., up to 6 hours of the process, while it decreased from then onward (Table 1). To explore the changes in the microbiota structure across different time points of the fermentation process, the weighted Unifrac distances were computed based on the OTU data. As is evident from Fig. 2d, the Unifrac distance distributions at different time points were wide. Thus, the fermentation process appears to be divided into three time clusters, i.e., 0 to 6th hours constitute the prefermentation phase, 9th and 12th hour constitute the fermentative phase, and postfermentative phase comprises the remaining time points of the process. This result indicates that different sets of organisms are active at different times of fermentation. This difference in the microbial structure at different times of the process, therefore, proves the presence of a microbial succession during the process.

Physicochemical properties and growth kinetics during idli fermentation. In the laboratory, it was observed that with the progression of fermentation, the pH decreased from 6.3 at the 0th hour to 4.5 after 24 hours, which correlated well with the previous reports (4). Simultaneously, gas production had taken place, with an increase in batter volume and reduction in bulk density of the batter (Table 2). This leavening effect due to the action of heterofermentative organisms was evident from the radiolabeled carbon dioxide generated from D-[1-¹⁴C]glucose (Table 2). There was a concomitant increase in the total viable count (TVC) as the fermentation progressed (Table 2). The TVC from de Man Rogosa and Sharpe (MRS) agar and nutrient agar showed nearly logarithmic growth up to the first 12 hours of fermentation, which corroborated well with the physicochemical parameters.

Structure-function relationship of the components and factors resulting in the fermentation of idli batter. As idli fermentation is spontaneous and natural, it occurs without any external microbial inoculum. Therefore, it was interesting to study the contribution of the raw materials, namely, rice and lentil, in idli fermentation. This was assessed by individually fermenting rice and lentil and comparing them with the normal batter prepared from rice and lentil [2:1]. In the normal batter, at the end of 15 hours, the pH decreased from 6.3 to 4.5 and volume increased by 100% due to the leavening activity of the microbiota. Batter prepared from rice alone did not ferment, as there were minimal changes in pH and volume compared with the normal batter

FIG 5 Structure-function relationship of the components and factors involved in idli batter fermentation as assessed by decrease in pH, increase in volume, and total viable counts per gram of idli batter. (CFU is expressed as log₂ values.)

fermentation. However, in the case of batter prepared from lentil alone, fermentation occurred, resulting in leavening as assessed by the increase in volume (Fig. 5). This leavening was probably due to the presence of gas entrapping polysaccharide arabinogalactan and surface-active globulin fractions in lentils. The pH in this case was not very acidic, as the protein-rich fractions in lentils serve as buffers. Furthermore, the bacterial growth was comparable in all three batters, i.e., rice alone, lentil alone, and normal batter prepared from the mixture of rice and lentil (Fig. 5). No fermentation occurred in the sample kept at low temperature, i.e., 4°C, which indicated that an ambient temperature (26 to 30°C) is a prerequisite for microbial metabolism which enables fermentation.

Moreover, the essentiality of the microbiota in initiating the fermentation process was established by surface sterilizing the grains with isopropanol and, thereby, devoiding the grains of the surface microbiota. This sterile batter showed no fermentation after 15 hours, as there was no change in volume and a slight decrease in pH (6.3 to 5.8), confirming that the grain microbiota is essential for fermentation to ensue. In another set, the addition of 10% of fermented normal batter to the sterile batter at the beginning of fermentation restored the fermentation to a level comparable to that of normal batter (Fig. 5).

Weissella emerged as the major genus in the fermentation of idli batter from our culture-dependent and -independent studies. Whether *Weissella* alone can ferment the batter was assessed by adding 5-ml culture (0.5 optical density [OD]) from two different *Weissella* isolates into two different surface-sterilized batters at the beginning of fermentation. Maximum fermentation occurred in the cylinders with *Weissella* isolates as the inoculum, as evident from the pH and volume (Fig. 5). This finding indicated that *Weissella* alone is capable of facilitating the fermentation of idli batter.

Functional role of the idli microbiota by PICRUSt analysis. For enhanced understanding of the metabolic role played by the idli microbiota during fermentation, the phylogenetic investigation of communities by reconstruction of unobserved states

(PICRUSt) program was used. It predicts the functional composition of the microbiota using data obtained from a 16S rRNA amplicon profile. The data were further analyzed in the context of the KEGG database to obtain a microbial KEGG profile. The PICRUSt analysis suggested that the major metabolic processes, such as carbohydrate, amino acid, lipid, nucleotide and energy metabolism, glycan biosynthesis, and metabolism of cofactors and vitamins, were slow during the first 9 hours of fermentation, which then progressed rapidly and remained vigorous until the end of the process (Fig. 6a).

Employing the metagenomic contributions from the PICRUSt imputation, a correlation matrix was generated between the core microbiota and the important metabolic processes (Fig. 6b). From the correlation matrix, *Weissella*, *Enterococcus*, *Streptococcus*, *Bacillus*, and *Lactobacillus* (phylum *Firmicutes*) and *Pantoea*, *Enterobacter*, and *Pseudomonas* (phylum *Proteobacteria*) appeared to correlate positively with the major metabolisms. The overall reconstruction from the PICRUSt analysis showed increased starch and sugar (glycolysis, pyruvate metabolism, and pentose phosphate pathway) metabolism and an enhanced amino acid and nucleotide metabolism, indicating rapid proliferation of the microbiota to enable the fermentation of idli batter (Fig. 6b). PICRUSt prediction also revealed the synthesis of short-chain fatty acids, butanoate and propanoate, and B-group vitamins by the idli microbiota. A plate assay for vitamin B₁₂ production revealed significant synthesis of B₁₂ during the fermentation of idli batter (Fig. 7). A closer inspection into the third level of the KEGG pathway also shed light on reputed fermentation-specific functions, such as the production of flavoring and aroma compounds (arginine, proline, alanine, aspartate, and glutamate).

Degradation of the starch content of grains is an early essential step in fermentation. PICRUSt analysis predicted that the large amounts of amylase contributed by *Enterococcus*, *Klebsiella*, *Streptococcus*, *Enterobacter*, *Erwinia*, *Citrobacter*, *Bacillus*, *Lactococcus*, and *Pediococcus* are responsible for the amylolytic activity in the batter. High amylolytic activity during the major duration of the process was demonstrated by starch agar assay (Fig. 7). Phytase, a major enzyme involved in metabolism of calcium phytate, was detected in the idli batter during all the phases of fermentation, as seen in Fig. 7. Several complex galactosidases which hydrolyze indigestible oligosaccharides have also been found from PICRUSt imputation. Additionally, PICRUSt prediction indicated that glycolytic enzymes, such as hexokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase, and phosphoketolase from the pentose phosphoketolase pathway, were initially absent and increased several folds after the 6th hour. The release of radioactive carbon dioxide from D-[1-¹⁴C]-glucose confirmed the presence of the active heterofermentative phosphoketolase pathway during idli fermentation. These enzymes and the radioactivity assay, thereby, signify the heterolactic mode of idli fermentation (see Fig. S3 in the supplemental material).

Functional traits of the idli batter isolates. Fermented foods are considered to be imparting several beneficial properties, owing to the presence of viable microbiota. With an interest to assay these traits, the idli batter was spread on MRS agar plates and the isolates so obtained were assessed for several properties, such as acid and bile tolerance (important for gastrointestinal survival), hydrophobicity and cell adherence ability (for retention in gastrointestinal tract), hemolytic activity (for pathogenicity), antibiotic sensitivity, and antimicrobial activity. A total of 72 isolates from MRS agar were tested for tolerance to acidic pH 2.0. Of these, 62 MRS isolates were found to be acid tolerant. All these isolates were also tolerant to 2% bile concentration. Also, none of the acid- and bile-tolerant isolates were found to be hemolytic. To assess the hydrophobic nature of the isolates, an assay was performed using nonpolar solvents toluene and xylene. This resulted in eight positive isolates which did not show adherence to the HT-29 cells in culture. All these isolates were identified as *Weissella confusa* by 16S rRNA sequencing. Furthermore, these isolates showed antimicrobial activity against common pathogens, such as *Escherichia coli*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, and *Klebsiella* spp. (see Fig. S4 in the supplemental material). These isolates were also found to be sensitive to several antibiotics, such

FIG 6 Functional analysis of the metabolic composition of idli batter microbiota by PICRUSt. (a) Stacked bar plots emphasizing the contribution of important metabolisms over the 24-hour fermentation of idli batter. (b) Correlation matrix showing the correlation between the important metabolic processes and the core microbiota.

FIG 7 Functional assays showing amylase and phytase activity and vitamin B₁₂ production during the time course of fermentation of idli batter.

as cephalosporins, azithromycin, erythromycin, clarithromycin, and ciprofloxacin. However, some of them were resistant to ampicillin, penicillin G, and a combination of amoxicillin and clavulanate (see Table S4 in the supplemental material).

***Weissella*, key player in the fermentation of idli batter.** Genus *Weissella* emerged as the most important organism from both the culture-dependent and -independent studies. The [1-¹⁴C]glucose assay showed maximum carbon dioxide generation from the *Weissella* isolates 1 and 2 (Fig. 8). This finding highlighted that genus *Weissella* is the major heterofermentative organism contributing significantly to leavening action during the important part of idli fermentation. Moreover, the addition of only *Weissella* isolates to the batter obtained from surface-sterilized grains led to significant fermentation, as judged by the decrease in pH and increase in volume and microbial content that were comparable to the normal batter fermentation (Fig. 8). This result revealed that members of the genus *Weissella* can independently ferment the idli batter.

These *Weissella confusa* isolates also showed beneficial phytase- and B₁₂-producing activities. (Fig. 8). These results, therefore, signify the abundance and role of this essential organism in the fermentation of idli batter.

DISCUSSION

The 16S rRNA gene is the most common target sequence for bacterial phylogenetic analysis and is widely used in microbial community profiling of different food fermentations. With newer and advanced culture-independent molecular techniques, it is easier to study microbial community dynamics and functionality of fermented foods (15, 20, 21). On similar lines, in this study, a combination of culture-dependent and

FIG 8 *Weissella*, key player in the fermentation of idli batter. (a) Table indicating changes in pH, volume, and CFU counts after inoculation of two *W. confusa* isolates separately in sterile batter. (b) Phytase activity and vitamin B₁₂ synthesis by two isolates of *W. confusa*.

-independent strategies was employed to investigate the microbial community of idli batter. This study also deciphers a detailed account of the natural microbial succession and functional capabilities of the microbiota enabling the fermentation of idli.

An analysis of the 16S rRNA amplicon data for the relative abundance at phylum level showed a dominance of *Firmicutes* and *Cyanobacteria* in the fermented batter. As rice and lentils are the main components of idli, it was assumed that cyanobacterial sequences correspond to cereal chloroplasts and, therefore, they were not included in the analysis (18, 19). Similarly, cyanobacterial sequences were not considered for further analysis in the case of chicha, a traditional fermented maize-based beverage from Argentina (22). 16S rRNA amplicon sequencing confirmed that the idli microbiome mainly consists of *Lactobacillales* (LAB), as a large number of OTUs (86%) belong to this order. The improved taste, aroma, texture, shelf life, and nutritional value of fermented foods can be attributed to the metabolic activities of LAB. The production of lactic, acetic, and other acids by LAB during fermentation is known to enhance the food flavor (3). These acids also prolong the shelf life of the fermented food by lowering the pH, which restricts the growth and survival of spoilage and some pathogenic organisms (23, 24). During idli batter fermentation, the gradual decrease of pH due to an increase in acid production correlated well with the increase in abundance of LAB, predominantly *Weissella*, *Enterococcus*, and *Streptococcus*. These genera are abundantly present and involved in many other fermented foods prepared from different raw materials, such as cereals, milk products, and animal and vegetable sources (see Table S5 in the supplemental material). A culture-dependent study of three batter samples showed that the bacterial diversity is conserved during fermentation, as seen by the variety of bacterial isolates. However, the Pune sample showed less diversity than the two other samples. Further investigations using culture-independent approach are needed to unravel the reasons for the variation in diversity of this sample.

Antinutritional factors, such as phytic acid found in cereals and legumes, lead to poor protein digestibility and mineral bioavailability (25). Fermentation probably provides an optimum pH for the enzymatic degradation of phytic acid, leading to the bioavailability of iron, zinc, and calcium (26, 27). Significant phytase activity has been detected in idli batter. Many LAB produce the phytase enzyme, and it is one of the desired characteristics of a potential probiotic. *Weissella kimchii* R-3 isolated from poultry gut has been shown to exhibit a substantial phytase-producing ability (28). Indigestible oligosaccharides, such as stachyose, verbascose, and raffinose present in cereals and legumes, cause flatulence, indigestion, and diarrhea (29). An *in silico* PICRUSt evaluation suggested the presence of complex galactosidases which can hydrolyze such oligosaccharides in idli batter. LAB possess metabolic pathways for the synthesis of B-group vitamins. PICRUSt analysis indicated that the bacterial population of the batter has a gene pool essential for the synthesis of B-group vitamins. An improved B-vitamin content has been reported in cereal-based products, such as ogi, mahewu, and kenkey, thereby improving their nutritional value (30). Vitamin B₁₂ production in the batter has been observed during fermentation of idli, which corroborated earlier reports (12, 31, 32). PICRUSt imputation showed that the idli microbiota also contributes to the production of short-chain fatty acids propanoate and butanoate, which help to lower the pH, enhance bioavailability of minerals, and inhibit harmful bacteria in the gut (33, 34).

In the initial phase of batter fermentation, large gene pools of hydrolytic enzymes were found by PICRUSt analysis. The substrate for carbon metabolism in batter is mainly starch found in legume and rice, and thus, high amylolytic activity is essential for fermentation (1). Higher amylolytic activity has been detected during the earlier time points of batter fermentation by starch agar assay. The monosaccharides and disaccharides produced from amylase action are metabolized further by homofermentative and heterofermentative LAB to acids and gas by Embden-Meyerhof and phosphoketolase pathways (3). A radioactivity assay using D-[1-¹⁴C]-glucose illustrated the employment of these pathways for carbon dioxide generation by heterofermentative

organisms. PICRUSt also predicted the presence of heterofermentative enzymes glucose-6-phosphate-dehydrogenase and phosphoketolase in idli batter.

The amplicon sequencing data revealed the abundance of members of the genus *Weissella* to be more than 70% during the important part of idli fermentation. The culturable, DGGE, and qPCR studies also highlight *Weissella* as the most abundant genus during the process. Nearly 45% of the identified organisms were *Weissella* spp. from DGGE analysis. The culturable studies revealed that *Weissella* spp. constituted more than 30% of the diversity of the batter samples. Thus, among LAB, *Weissella* emerged as the predominant genus in idli fermentation by both culture-dependent and -independent methods, with its different species present in significant numbers. The [1-¹⁴C]glucose assay showed that it is the major heterofermentative organism contributing significantly to leavening during idli fermentation. PICRUSt analysis showed a positive correlation between the major metabolic processes and *Weissella* spp., suggesting their active participation in the fermentation process, both metabolically and functionally. Moreover, it was found that *Weissella* spp. can independently ferment the idli batter, highlighting the importance and, therefore, abundance of that genus in this process. Genera *Leuconostoc* and *Weissella* belong to the family *Leuconostocaceae*. The taxonomic positioning of genus *Weissella* results from restructuring of the genus *Leuconostoc* and some atypical heterofermentative *Lactobacillus* species. *Leuconostoc* species have coccoid to ovoid morphology, whereas *Weissella* species vary from ovoid cells to irregular rods, making it difficult to distinguish between them based on morphology and colony characters. It is likely that *L. mesenteroides* identified in an earlier study (4) was in actuality a member of the genus *Weissella*, as proposed by new taxonomic classification and 16S rRNA sequence analysis (35). This finding suggests that classical phenotypic criteria may be insufficient, thereby making the use of molecular approaches necessary for correct identification.

Microbial succession has been reported in different fermented foods, including idli (4, 36, 37). In idli batter, there was an initial growth of aerobic bacteria followed by microaerophilic LAB during succession (4). Our results corroborate the earlier report, as the LAB gradually increase during this fermentation. Amplicon sequencing highlighted the abundance of a few genera in the earlier time points of fermentation, such as *Alteromonas*, *Bacillus*, *Halomonas*, *Lactobacillus*, *Pseudomonas*, *Propionibacterium*, and *Shewanella*, which probably make conditions favorable for their successors. Genus *Weissella* was found to be abundant and contributing the most during the valuable phase of fermentation, as observed from amplicon and DGGE analysis. The dominance of *Weissella* in this fermentative process was also evident from the correlation matrix wherein *Weissella* spp. showed a negative correlation with most other organisms. The significant increase in *Weissella* abundance during the important phase of this fermentation can be explained from its ability to produce acid and generate carbon dioxide, as established by the radioactivity assay. Hence, the leavening action occurring during this period can be largely attributed to the presence of *Weissella*. The postfermentation phase (i.e., after 12 h) consists of increased numbers of *Enterococcus* and *Streptococcus* spp. The presence of these organisms has been reported in many fermented foods (Table S5). However, this increased abundance of *Enterococcus* and its role during the late phase of fermentation need to be further investigated. The difference in the microbial structure at different times of fermentation, as observed in the β -diversity plot, confirms the microbial succession during the process. Moreover, the increase in the microbial metabolism and expression of important enzymes during the crucial period of fermentation, as observed from PICRUSt analysis, corroborate the microbial succession. To add further, the physicochemical changes, such as pH and volume, also coincided with the succession pattern. The microbial content showed a progressive buildup, with the fermentation time supporting the succession study.

Recently, the consumption of fermented foods has emerged as an important dietary strategy for improving human health (38). This concept stems from the presence of viable organisms which impart nutritional and functional properties to these foods by the transformation of their substrates and formation of bioactive end products. Specific

strains of genera such as *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Leuconostoc*, *Weissella*, *Pediococcus*, *Enterococcus*, and *Bacillus* are known to possess several beneficial properties and, thereby, impart health benefits by different mechanisms (38–40). Different species of LAB, such as *Lactobacillus acidophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus fermentum*, *Lactococcus lactis*, *Enterococcus durans*, *Enterococcus faecium*, *Bacillus subtilis*, *Bacillus cereus*, *Streptococcus thermophilus*, and *Weissella cibaria*, *W. confusa*, *W. koreensis*, and *Weissella hellenica* have been found in the idli batter by 16S rRNA amplicon sequencing. However, none of these bacteria have been identified up to strain level and, thus, their specific beneficial properties need to be evaluated. The culturable isolates obtained from idli batter were found to be nonhemolytic and acid and bile tolerant, and eight isolates identified as *Weissella confusa* also produced antimicrobial substances against major bacterial pathogens and showed sensitivity to important antibiotics (40, 41). Many studies have evaluated the functional role of several *Weissella* species and proposed members of the genus to be potential probiotic organisms (42–44). However, more *in vitro* and *in vivo* studies are needed to establish their safety for potential probiotic applications.

This is a comprehensive analysis of idli batter fermentation employing modern molecular tools which gives a detailed account of the bacterial diversity enabling its fermentation. The study has provided valuable information about its microbiota and its succession during fermentation. The nature of idli fermentation is more complex than other food fermentations due to the succession of the bacterial population. This may be a single reason why starter cultures or the back-slopping procedure has not been applied in idli fermentation. However, the use of a single *Weissella* culture as the inoculum (as starter culture) for successful batter fermentation as seen in this study, needs further characterization. Further studies using advanced techniques, such as metatranscriptomics and metabolomics, will add to the information on the role of these microorganisms in this complex fermentation process. Moreover, the presence of microorganisms with beneficial properties makes idli cakes a suitable food for the delivery and supplementation of beneficial probiotic organisms.

MATERIALS AND METHODS

Idli batter fermentation and sampling in the laboratory. For preparation of idli batter in the laboratory, thoroughly washed parboiled rice and black gram (2:1 ratio) were soaked in water separately for 4 hours and then pulverized to obtain coarse dough. The mixture was allowed to ferment overnight at ambient temperature (about 30°C) (3). Samples were withdrawn every 3 hours during the fermentation process of 24 hours. The samples for the nine time points were then processed for different experiments.

The decrease in pH due to acid production, the increase in volume due to leavening, and increase in CFUs are parameters studied to assess the progress of fermentation. To assay these at different time points of fermentation, the pH decrease was measured with a pH meter and an increase in volume of the batter was measured using graduated cylinders. For growth kinetics studies, 1 g of idli batter from each time point was suspended in 9 ml of normal saline and vortexed vigorously, and serial dilutions were plated onto de Man Rogosa and Sharpe (MRS) agar and nutrient agar plates and incubated for 48 h at 30°C and 37°C, respectively. The CFU counts were calculated by measuring the colonies so obtained.

In order to assess the contribution of the components of idli batter, i.e., rice and lentil, in the fermentation process, individual components were fermented and compared with the normal batter prepared from rice and lentil (2:1 ratio). Moreover, the role of temperature in enabling the fermentation was studied by fermenting the normal batter at low temperature, i.e., 4°C. To obtain sterile batter, grains were surface sterilized with isopropanol for 30 min and then soaked, pulverized, and fermented for 15 hours. The decrease in pH, increase in volume, and CFU counts for these samples were monitored.

DGGE analysis. DNA from idli samples obtained from 10 different vendors and samples for laboratory fermented batter collected at 9 time points was isolated with a QIAamp DNA stool minikit, as described by the vendor. DNA amplification of the 16S rRNA gene sequences was performed with 341F (with GC clamp) and 518R primers (45) (Table 3). The PCR was set up using AmpliTaq Gold PCR master mix (Thermo Fisher Scientific) with following conditions for touchdown PCR: initial denaturation at 95°C for 10 min, followed by 10 cycles each of 95°C denaturation, 65 to 56°C for annealing (reduction of 1°C in annealing temperature per cycle), and extension at 72°C with each step for 30 s. The initial touchdown PCR was followed by 32 cycles of PCR with an annealing temperature of 56°C. The temperature cycle for this PCR was 30 s of denaturation at 95°C, 30 s of annealing at 56°C, and 30 s of extension at 72°C. The final extension was carried out at 72°C for 10 min. The PCR products were purified by polyethylene glycol (PEG)-NaCl precipitation and then subjected to DGGE in a 12% acrylamide gel with a gradient of 30% to 50% of denaturants, namely urea and formamide. The electrophoresis was performed using the DCode universal mutation detection system (Bio-Rad, USA) in Tris-acetate-EDTA (TAE) buffer (pH 8; 40 mM Tris-HCl, 20 mM sodium acetate, and 1 mM EDTA) at 80 V and 60°C for 18 h. The gel was stained with

TABLE 3 List of primers used in this study and their sequences

Sequence no.	Name of primer	Primer sequence	No. of bases
1	16S rRNA-8F	GGATCCAGACTTTGATYMTGGCTCAG	26
2	16S rRNA-907R	CCGTCAATTCMTTGTGAGTTT	20
3	16S rRNA 341F	CCTACGGGAGGCAGCAG	17
4	16S rRNA-341F-GC	CGCCCGCCGCGCGGGCGGGCGGGCGGG GGCACGGGGGGCTACGGGAGGCAGCAG	57
5	16S rRNA 518R	ATTACCGGGCTGCTGG	17
6	<i>Weissella</i> F	CTGAGGAATTGCTTGGAACTGGATG	27
7	<i>Weissella</i> R	AAACCTCAAACACCTAGCACTCATCG	27
8	<i>Streptococcus</i> F	CTGAAGTTAAAGGCTGTGGCTCAACC	26
9	<i>Streptococcus</i> R	GGATCCAACACCTAGCACTCATCGTT	26
10	<i>Enterococcus</i> F	TCTAGAGATAGAGCTTCCCTTCGGG	26
11	<i>Enterococcus</i> R	GACTTCGCGACTCGTTGTACTCCC	25
12	<i>Lactococcus</i> F	GGAAGTTCCTTCGGGACACGGG	22
13	<i>Lactococcus</i> R	ATTAGCTAAACATCACTGTCTCGCGACTC	29
14	<i>Lactobacillus</i> F	AGCAGTAGGGAATCTTCCA	19
15	<i>Lactobacillus</i> R	CGCCACTGGTGTTCYTCATATA	23
16	<i>Pantoea</i> F	CCGATAGAGGGGATAACCACTGG	24
17	<i>Pantoea</i> R	CCGCACCGCCTTCTCCC	18

SYBR gold (Invitrogen) for 20 min, visualized using a gel documentation system, and analyzed using Gene Tools software (SynGene, UK). Well-separated bands were excised, allowed to diffuse passively in 10 μ l distilled water at 4°C for 8 h, and subsequently PCR amplified and sequenced using 341F (without GC clamp) and 518R primers, an ABI BigDye terminator version 3.1 sequencing kit, and ABI 3730 XL DNA analyzer. Generated sequences were analyzed using BLAST (www.ncbi.nlm.nih.gov/BLAST) and EzTaxon (www.eztaxon.org), and bacterial identity for each band was recorded. Using GeneTools software, a dendrogram was prepared using the unweighted pair group method using average linkages (UPGMA) linkage rule based on similarity between clusters of similar tracks.

DNA sequencing of 16S rRNA gene amplicons. Total DNA extracted from nine time points of laboratory fermented idli batter was checked for its quality and concentration. Universal bacterial primers specific for the V3 region of the 16S rRNA gene, namely 341F and 518R, were used for PCR amplification (46) (Table 3). Template and library preparation using amplified DNA was carried out according to the manufacturer's protocol (Illumina, USA). The sequencing of multiplexed 16S rRNA gene amplicon libraries was performed using paired-end 2 $\times$ 150-bp chemistry on the Illumina MiSeq platform.

Absolute quantification of specific bacterial taxa. The abundance of important bacterial taxa during fermentation was confirmed by quantification of the total and specific bacterial population using quantitative real-time PCR in terms of copy numbers of 16S rRNA genes per gram of idli batter. Targeted groups of genera, genus-specific primer sequences, and amplicon size are summarized in Table 3. Absolute quantification PCR assays were performed as described previously (47) wherein, briefly, for each genus under consideration, three biological replicates with duplicate technical replicates of each were set up (10 μ l each) containing an appropriate pair of primers, 50 μ g of metagenomic DNA, and SYBR green master mix. The reactions were performed using the 7300 real-time PCR system (Applied Biosystems, USA) using the following PCR conditions: initial denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 10 s and 60°C for 1 min. Genus-specific standard curves were generated from serial dilutions of a known concentration of PCR products. Additionally, melting curve analysis was performed at the end of qPCR cycles to check the amplification specificity. Average values of the samples were used for enumerations of tested gene copy numbers for each genus using standard curves generated under similar conditions (48). For all the assays, PCR efficiency was maintained above 90% with a correlation coefficient of >0.99. The results have been expressed per gram of idli batter as 16S rRNA gene copy numbers transformed by log to the base 10.

Isolation and identification of culturable organisms from idli batter. Multiple samples of naturally fermented ready-to-steam idli batters from two popular idli vendors (from Pune and Bangalore) were obtained in sterile plastic containers and transported in a cool box to the laboratory. Samples were received within 12 h, i.e., within their shelf life, and subjected immediately to microbial analyses. Bacteria were isolated using a serial dilution method. Appropriate aliquots from each dilution were spread onto different complex media, such as nutrient agar, de Man Rogosa and Sharpe (MRS) agar, and Luria Bertani agar, and incubated at 37°C for 48 hours. Primary identification of the isolates was performed by Gram staining and biochemical reactions. The idli batter was prepared in laboratory as described earlier. The laboratory fermentation essentially had a similar fermentation process to samples collected from two vendors. Laboratory fermented batter as well as fermentation by vendors was carried out at ambient temperature (26 to 30°C) without any external microbial inoculum for a period of 12 hours. Genomic DNA was isolated from a well-isolated colony of each morphotype, after purification by the phenol-chloroform extraction method (49). The concentration and quality of the genomic DNA were estimated using the Nanodrop 1000 instrument (Thermo Scientific, USA). The identity of the isolated organisms was confirmed by Sanger sequencing of the partial 16S rRNA PCR product amplified using conserved primers, namely, 8F and 907R (Table 3). Generated sequences were analyzed using BLAST, and the bacterial identity for each isolate was recorded.

Bioinformatic analyses. The sequences obtained from 16S rRNA amplicon sequencing had a mean read length of 150 bases and Q score of >30. The raw sequences were trimmed by Cutadapt to remove the low-quality sequences (50). The sequences were assembled and separated into fasta and qual files using FLASH and mothur, respectively (51, 52). Bioinformatic analyses were performed using quantitative insights into microbial ecology (QIIME) on the high-quality sequences (53). Sequence reads were assigned to operational taxonomic units (OTUs) in QIIME v1.7 by using a closed reference-based OTU-picking approach with the SILVA 123 database (released on July 2015) (54). OTU picking was carried out using the UCLUST method with a similarity threshold of 97% (55). To evaluate α -diversity, data were normalized according to the least number of reads per sample and diversity indices, such as Shannon, Simpson's, Good's coverage, and Chao1, were calculated. Unifrac metrics were calculated to estimate the beta diversity (56). Both weighted and unweighted calculations were performed prior to a principal-coordinate analysis (PCoA).

Correlations among the core genera at different time points of fermentation were determined based on Pearson's correlation coefficient. In addition, the metabolic capabilities of the bacterial community were inferred by utilizing a computational approach, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) (57). Briefly, reference-based OTU picking was performed in QIIME, the OTU table was imported to an online PICRUSt tool (<http://huttenhower.sph.harvard.edu/galaxy>), and functional predictions were made using the KEGG orthology database. The metagenomic contributions for each KEGG ortholog were calculated. Also, the correlation between the core microbiota and the metabolic functional features was explored using the Pearson's correlation coefficient.

Study of leavening action using D-[1-¹⁴C]glucose. In order to understand whether the carbon dioxide generated during the fermentation of idli batter was processed through the hexose monophosphate pathway, [1-¹⁴C]glucose was used (58). In 15-ml glass vials, 5 g of idli batter was taken to which 1 μ Ci of D-[1-¹⁴C]glucose was added. Radiolabeled carbon dioxide generated from the oxidation of glucose was trapped in 5% potassium hydroxide-saturated filter paper, and the radioactivity was quantified using a Beckman scintillation counter (59). The experiment was performed three times, and results from one representative experiment were mentioned.

Plate assay for amylase and phytase activity. To assess the amylolytic and phytase activity in the batter, Tris buffer (10 mM Tris, 0.15 M NaCl, and 1 mM EDTA; pH 7.4) was added to 1 gram of batter to make a final volume of 5 ml, which was mixed thoroughly and centrifuged at 4,900 rpm for 30 min. The supernatant was filtered using a 0.45- μ m-pore-size syringe filter and was added in the wells made in the starch agar plates for amylase activity (www.asmscience.org/content/education/imagegallery/image.3172) and modified Chalmer's agar containing 2% sodium phytate for phytase activity (60) and incubated for 48 hours at 37°C. For phytase activity, a zone of clearance surrounding the well was observed. Assays were repeated thrice, and images from one experiment are shown.

Bioassay for vitamin B₁₂ production. This assay was performed on plates using a vitamin B₁₂-requiring auxotroph of *Escherichia coli*, namely, Davis A 113-3 strain (ATCC 11105) (61). B₁₂ assay agar (HiMedia) was seeded with *E. coli* strain 113-3D (OD at 600 nm, 0.5), and supernatant obtained as described above from batter samples was seeded in the wells and incubated for 48 hours at 37°C. Growth of the auxotroph around the well indicates the presence of vitamin B₁₂ in the batter. The assay was performed thrice and results from one assay are shown.

Assay of the idli batter isolates for some functional traits. To assay the acid and bile tolerance ability, isolates obtained from MRS agar plates were inoculated into MRS broth having a pH of 2 (as adjusted by 1 N HCl) or containing 2% bile salts (62). The production of hemolysin was tested using blood agar containing 5% sheep blood (63). The hydrophobic nature of the isolates was assessed using nonpolar solvents xylene and toluene (64). The cell adhesion assay was performed using the HT-29 human colon adenocarcinoma cell line (65). Cells were stained with DAPI (4',6-diamidino-2-phenylindole) and were viewed under a fluorescence microscope. The antimicrobial activity of these isolates against common pathogens such as *Escherichia coli*, *Klebsiella* spp. (clinical isolate), *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus* was determined by the well diffusion method (66). Antibiotic disks (HiMedia) were used to test the antibiotic sensitivity of these isolates by the disk diffusion method on MRS agar plates (67). All the assays were done three times, and results from one representative experiment are shown.

Data availability. Raw sequences generated by Illumina MiSeq sequencing in the present study have been deposited to NCBI with BioProject accession number [PRJNA415908](https://doi.org/10.1016/j.fmf.2019.07.002) and SRA accession number [SRP122484](https://doi.org/10.1016/j.fmf.2019.07.002).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00368-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

REFERENCES

1. Nout M. 2009. Rich nutrition from the poorest—cereal fermentations in Africa and Asia. *Food Microbiol* 26:685–692. <https://doi.org/10.1016/j.fmf.2009.07.002>.
2. Steinkraus KH. 2002. Fermentations in world food processing. *Comp Rev Food Sci Food Safety* 1:23–32. <https://doi.org/10.1111/j.1541-4337.2002.tb00004.x>.

3. Blandino A, Al-Aseeri ME, Pandiella SS, Cantero D, Webb C. 2003. Cereal-based fermented foods and beverages. *Food Res Int* 36:527–543. [https://doi.org/10.1016/S0963-9969\(03\)00009-7](https://doi.org/10.1016/S0963-9969(03)00009-7).
4. Mukherjee SK, Albury MN, Pederson CS, Van Veen AG, Steinkraus KH. 1965. Role of *Leuconostoc mesenteroides* in leavening the batter of idli, a fermented food of India. *Appl Microbiol* 13:227–231.
5. Wood BJ. 2012. *Microbiology of fermented foods*. Springer Science & Business Media, Berlin, Germany.
6. Aidoo KE, Rob Nout MJ, Sarkar PK. 2006. Occurrence and function of yeasts in Asian indigenous fermented foods. *FEMS Yeast Res* 6:30–39. <https://doi.org/10.1111/j.1567-1364.2005.00015.x>.
7. Steinkraus KH, Van Veen AG, Thiebaut DB. 1967. Studies on idli—an Indian fermented black gram-rice food. *Food Technol* 21:916–919.
8. Bhattacharya S, Bhat KK. 1997. Steady shear rheology of rice-blackgram suspensions and suitability of rheological models. *J Food Eng* 32: 241–250. [https://doi.org/10.1016/S0260-8774\(97\)00027-7](https://doi.org/10.1016/S0260-8774(97)00027-7).
9. Venkatasubbaiah P, Dwarakanath CT, Sreenivasamurthy V. 1984. Microbiological and physico-chemical changes in idli batter during fermentation. *J Food Sci Technol* 21:59–62.
10. Durgadevi M, Shetty PH. 2014. Effect of ingredients on sensory profile of idli. *J Food Sci Technol* 51:1773–1783. <https://doi.org/10.1007/s13197-012-0686-z>.
11. Iyer BK, Singhal RS, Ananthanarayan L. 2013. Characterization and in vitro probiotic evaluation of lactic acid bacteria isolated from idli batter. *J Food Sci Technol* 50:1114–1121. <https://doi.org/10.1007/s13197-011-0445-6>.
12. Rajalakshmi R, Vanaja K. 1967. Chemical and biological evaluation of the effects of fermentation on the nutritive value of foods prepared from rice and grams. *Br J Nutr* 21:467–473. <https://doi.org/10.1079/BJN19670048>.
13. van Hijum S, Vaughan EE, Vogel RF. 2013. Application of state-of-art sequencing technologies to indigenous food fermentations. *Curr Opin Biotechnol* 24:178–186. <https://doi.org/10.1016/j.copbio.2012.08.004>.
14. Gibbons JG, Rinker DC. 2015. The genomics of microbial domestication in the fermented food environment. *Curr Opin Genet Dev* 35:1–8. <https://doi.org/10.1016/j.gde.2015.07.003>.
15. Kergourlay G, Taminiau B, Daube G, Champomier Vergès MC. 2015. Metagenomic insights into the dynamics of microbial communities in food. *Int J Food Microbiol* 213:31–39. <https://doi.org/10.1016/j.ijfoodmicro.2015.09.010>.
16. Stefanovic E, Fitzgerald G, McAuliffe O. 2017. Advances in the genomics and metabolomics of dairy lactobacilli: a review. *Food Microbiol* 61: 33–49. <https://doi.org/10.1016/j.fm.2016.08.009>.
17. Cao Y, Fanning S, Proos S, Jordan K, Srikumar S. 2017. A review on the applications of next generation sequencing technologies as applied to food-related microbiome studies. *Front Microbiol* 8:1–16. <https://doi.org/10.3389/fmicb.2017.01829>.
18. Giovannoni SJ, Turner S, Olsen GJ, Barns S, Lane DJ, Pace NR. 1988. Evolutionary relationships among Cyanobacteria and green chloroplasts. *J Bacteriol* 170:3584–3592. <https://doi.org/10.1128/jb.170.8.3584-3592.1988>.
19. Hanshaw A, Mason C, Raffa K, Currie C. 2013. Minimization of chloroplast contamination in 16S rRNA gene pyrosequencing of insect herbivore bacterial communities. *J Microbiol Methods* 95:149–155. <https://doi.org/10.1016/j.mimet.2013.08.007>.
20. Oguntuyinbo FA, Tourlomousis P, Gasson MJ, Narbad A. 2011. Analysis of bacterial communities of traditional fermented West African cereal foods using culture independent methods. *Int J Food Microbiol* 145: 205–210. <https://doi.org/10.1016/j.ijfoodmicro.2010.12.025>.
21. Zheng XW, Yan Z, Han BZ, Zwietering MH, Samson R, Boekhout T, Robert Nout MJ. 2012. Complex microbiota of a Chinese “Fen” liquor fermentation starter (Fen-Daqu), revealed by culture-dependent and culture-independent methods. *Food Microbiol* 31:293–300. <https://doi.org/10.1016/j.fm.2012.03.008>.
22. Elizaquível P, Aristimuño C, Cocconcelli PS, Yépez A, Vignolo G, Pérez-Cataluña A, Aznar R, Jiménez E. 2015. Pyrosequencing vs. culture-dependent approaches to analyze lactic acid bacteria associated to chicha, a traditional maize-based fermented beverage from Northwestern Argentina. *Int J Food Microbiol* 198:9–18. <https://doi.org/10.1016/j.ijfoodmicro.2014.12.027>.
23. Oyewole OB. 1997. Lactic fermented foods in Africa and their benefits. *Food Control* 8:289–297. [https://doi.org/10.1016/S0956-7135\(97\)00075-3](https://doi.org/10.1016/S0956-7135(97)00075-3).
24. Nout MJR, Rombouts FM. 1992. Fermentative preservation of plant foods. *J Appl Bacteriol* 73:136s–147s. <https://doi.org/10.1111/j.1365-2672.1992.tb03633.x>.
25. Anderson RL, Wolf WJ. 1995. Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *J Nutr* 125:581S–588S. https://doi.org/10.1093/jn/125.3_Suppl.581S.
26. Chavan JK, Kadam SS, Beuchat LR. 1989. Nutritional improvement of cereals by fermentation. *Crit Rev Food Sci Nutr* 28:349–400. <https://doi.org/10.1080/10408398909527507>.
27. Gupta RK, Gangoliya SS, Singh NK. 2015. Reduction of phytic acid and enhancement of bioavailable micronutrients in food grains. *J Food Sci Technol* 52:676–684. <https://doi.org/10.1007/s13197-013-0978-y>.
28. Andrabi ST, Bhat B, Gupta M, Bajaj BK. 2016. Phytase-producing potential and other functional attributes of lactic acid bacteria isolates for prospective probiotic applications. *Probiotics Antimicrob Proteins* 8:121–129. <https://doi.org/10.1007/s12602-016-9220-3>.
29. Holzapfel WH. 2002. Appropriate starter culture technologies for small-scale fermentation in developing countries. *Int J Food Microbiol* 75: 197–212. [https://doi.org/10.1016/S0168-1605\(01\)00707-3](https://doi.org/10.1016/S0168-1605(01)00707-3).
30. Campbell-Platt G. 1994. Fermented foods—a world perspective. *Food Res Int* 27:253–257. [https://doi.org/10.1016/0963-9969\(94\)90093-0](https://doi.org/10.1016/0963-9969(94)90093-0).
31. Rao MR. 1961. Some observations on fermented foods. *Meet Needs Infants Child Natl Acad Sci Natl Res Counc* 843:291–293.
32. Chandrasekar Rajendran SC, Chamlagain B, Kariluoto S, Piironen V, Saris PEJ. 2017. Biofortification of riboflavin and folate in idli batter, based on fermented cereal and pulse, by *Lactococcus lactis* N8 and *Saccharomyces boulardii* SAA655. *J Appl Microbiol* 122:1663–1671. <https://doi.org/10.1111/jam.13453>.
33. Teitelbaum JE, Walker WA. 2002. Nutritional impact of pre- and probiotics as protective gastrointestinal organisms. *Annu Rev Nutr* 22: 107–138. <https://doi.org/10.1146/annurev.nutr.22.110901.145412>.
34. Wong JM, de Souza R, Kendall CW, Emam A, Jenkins DJ. 2006. Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol* 40:235–243. <https://doi.org/10.1097/00004836-200603000-00015>.
35. Collins MD, Samelis J, Metaxopoulos J, Wallbanks S. 1993. Taxonomic studies on some *leuconostoc*-like organisms from fermented sausages: description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. *J Appl Microbiol* 75:595–603. <https://doi.org/10.1111/j.1365-2672.1993.tb01600.x>.
36. Sulaiman J, Gan HM, Yin WF, Chan KG. 2014. Microbial succession and the functional potential during the fermentation of Chinese soy sauce brine. *Front Microbiol* 5:1–9. <https://doi.org/10.3389/fmicb.2014.00556>.
37. Walsh AM, Crispie F, Kilcawley K, Sullivan OO. 2016. Microbial succession and flavor production in the fermented dairy beverage kefir. *mSystems* 11:1–16. <https://doi.org/10.1128/mSystems.00052-16>.
38. Marco ML, Heeney D, Binda S, Cifelli CJ, Cotter PD, Foligné B, Gänzle M, Korr R, Pasin G, Pihlanto A, Smid EJ, Hutkins R. 2017. Health benefits of fermented foods: microbiota and beyond. *Curr Opin Biotechnol* 44: 94–102. <https://doi.org/10.1016/j.copbio.2016.11.010>.
39. Fijan S. 2014. Microorganisms with claimed probiotic properties: an overview of recent literature. *Int J Environ Res Public Health* 11: 4745–4767. <https://doi.org/10.3390/ijerph110504745>.
40. Wedajo B. 2015. Lactic acid bacteria: benefits, selection criteria and probiotic potential in fermented food. *J Probiotics Heal* 3:129. <https://doi.org/10.4172/2329-8901.1000129>.
41. Morelli L. 2000. In vitro selection of probiotic lactobacilli: a critical appraisal. *Curr Issues Intest Microbiol* 1:59–67.
42. Ayeni FA, Sánchez B, Adeniyi BA, de los Reyes-Gavilán CG, Margolles A, Ruas-Madiedo P. 2011. Evaluation of the functional potential of *Weissella* and *Lactobacillus* isolates obtained from Nigerian traditional fermented foods and cow’s intestine. *Int J Food Microbiol* 147:97–104. <https://doi.org/10.1016/j.ijfoodmicro.2011.03.014>.
43. Sharma S, Kandasamy S, Kavitha D, Shetty PH. 2018. Probiotic characterization and antioxidant properties of *Weissella confusa* KR780676, isolated from an Indian fermented food. *LWT* 97:53–60. <https://doi.org/10.1016/j.lwt.2018.06.033>.
44. Xu X, Luo D, Bao Y, Liao X, Wu J. 2018. Characterization of diversity and probiotic efficiency of the autochthonous lactic acid bacteria in the fermentation of selected raw fruit and vegetable juices. *Front Microbiol* 9:1–16. <https://doi.org/10.3389/fmicb.2018.02539>.
45. Muyzer G, de Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59:695–700.
46. Bartram AK, Lynch MD, Stearns JC, Moreno-Hagelsieb G, Neufeld JD.

2011. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. *Appl Environ Microbiol* 77:3846–3852. <https://doi.org/10.1128/AEM.02772-10>.
47. Marathe N, Shetty S, Lanjekar V, Ranade D, Shouche Y. 2012. Changes in human gut flora with age: an Indian familial study. *BMC Microbiol* 12:222. <https://doi.org/10.1186/1471-2180-12-222>.
48. Lyons SR, Griffen AL, Leys EJ. 2000. Quantitative real-time PCR for *Porphyromonas gingivalis* and total bacteria. *J Clin Microbiol* 38:2362–2365.
49. Sambrook JF, Russell DW. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. CSHL Press, New York, NY.
50. Martin M. 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. *Embnet J* 17:10–12. <https://doi.org/10.14806/ej.17.1.200>.
51. Magoc T, Salzberg SL. 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27:2957–2963. <https://doi.org/10.1093/bioinformatics/btr507>.
52. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol* 75:7537–7541. <https://doi.org/10.1128/AEM.01541-09>.
53. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JL, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. QIIME allows analysis of high-throughput community sequencing data intensity normalization improves color calling in SOLiD sequencing. *Nat Methods* 7:335–336. <https://doi.org/10.1038/nmeth.f.303>.
54. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res* 41:D590–D596. <https://doi.org/10.1093/nar/gks1219>.
55. Edgar RC. 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26:2460–2461. <https://doi.org/10.1093/bioinformatics/btq461>.
56. Lozupone C, Knight R. 2005. UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* 71:8228–8235. <https://doi.org/10.1128/AEM.71.12.8228-8235.2005>.
57. Langille MG, Zaneveld J, Caporaso JG, McDonald D, Knights D, Reyes JA, Clemente JC, Burkepile DE, Thurber RV, Knight R, Beiko RG, Huttenhower C. 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nat Biotechnol* 31:814–821. <https://doi.org/10.1038/nbt.2676>.
58. Maugeri DA, Cazzulo JJ. 2004. The pentose phosphate pathway in *Trypanosoma cruzi*. *FEMS Microbiol Lett* 234:117–123. <https://doi.org/10.1016/j.femsle.2004.03.018>.
59. Bland ML. 2016. Measurement of carbon dioxide production from radio-labeled substrates in *Drosophila melanogaster*. *J Vis Exp* 112:1–6. <https://doi.org/10.3791/54045>.
60. Anastasio M, Pepe O, Cirillo T, Palomba S, Blaiotta G, Villani F. 2010. Selection and use of phytate-degrading LAB to improve cereal-based products by mineral solubilization during dough fermentation. *J Food Sci* 75:M28–M35. <https://doi.org/10.1111/j.1750-3841.2009.01402.x>.
61. Chiao JS, Peterson WH. 1953. Microbiological assay of vitamin B12 with a mutant strain of *Escherichia coli*. *Appl Microbiol* 1:42–46.
62. Boonkumkiao P, Kongtong P, Assavanig A. 2006. Acid and bile tolerance of *Lactobacillus thermotolerans*, a novel species isolated from chicken feces. *Kasetsart J (Nat Sci)* 17:409. http://kasetsartjournal.ku.ac.th/kuj_files/2008/A080403093459.pdf.
63. Hosseini SV, Arlindo S, Böhme K, Fernández-No C, Calo-Mata P, Barros-Velázquez J. 2009. Molecular and probiotic characterization of bacteriocin-producing *Enterococcus faecium* strains isolated from non-fermented animal foods. *J Appl Microbiol* 107:1392–1403. <https://doi.org/10.1111/j.1365-2672.2009.04327.x>.
64. Saran S, Bisht MS, Singh K, Teotia UVS. 2012. Comparing adhesion attributes of two isolates of *Lactobacillus acidophilus* for assessment of prebiotics, honey and inulin. *Int J Sci Res Publ* 2:2250–3153. <https://pdfs.semanticscholar.org/72a0/0e939894c9e05b9d0f158f0a0c96f6e5bc6f.pdf>.
65. Duany RK, Rajput YS, Batish VK, Grover S. 2011. Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. *Indian J Med Res* 134:664–671. <https://doi.org/10.4103/0971-5916.90992>.
66. Dasari S, Shouri RND, Wudayagiri R, Valluru L. 2014. Antimicrobial activity of *Lactobacillus* against microbial flora of cervicovaginal infections. *Asian Pacific J Trop Dis* 4:18–24. [https://doi.org/10.1016/S2222-1808\(14\)60307-8](https://doi.org/10.1016/S2222-1808(14)60307-8).
67. Verdenelli MC, Ghelfi F, Silvi S, Orpianesi C, Cecchini C, Cresci A. 2009. Probiotic properties of *Lactobacillus rhamnosus* and *Lactobacillus paracasei* isolated from human faeces. *Eur J Nutr* 48:355–363. <https://doi.org/10.1007/s00394-009-0021-2>.