Analysis of bacterial and fungal communities in Marcha and Thiat, traditionally prepared amylolytic starters of India

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Marcha and thiat are traditionally prepared amylolytic starters used for the production of various ethnic alcoholic beverages in Sikkim and Meghalaya states in India. In the present study, we have tried to investigate the bacterial and fungal community composition of marcha and thiat by using high throughput sequencing. Characterization of bacterial community depiction phyllum Proteobacteria is the most dominant in both marcha (91.4%) and thiat (53.8%), followed by Firmicutes, and Actinobacteria. Estimates of fungal community composition showed Ascomycota as the dominant phylum. Presence of Zygomycota in marcha distinguishes it from the thiat. The results of NGS analysis revealed dominance of yeasts in marcha whereas molds out numbers in case of thiat. This is the first report on microbial communities of traditionally prepared amylolytic starters of India using high throughput sequencing.

Traditional practice of sub-culturing by back-sloping and preservation of essential native microbiota consisting of consortia of yeasts, molds and bacteria in the form of dry, flattened, or round balls, for alcoholic beverages production in South-East Asia including the Himalayan regions of India, Nepal, Bhutan, and China is the wisdom of the ethnic people for centuries1. Some common and uncommon amylolytic starters in Asia are marcha of India, Nepal, and Bhutan, hamei, humao, thiat, phab of India, men of Vietnam, bubod of the Philippines, chi/chu of China and Taiwan, loong pang of Thailand, nusik of Korea, ma/dombae/buh/puhin Cambodia, etc.2–7 Traditionally prepared Asian amylolytic starters have consortia of mixed microbiota representing filamentous molds, yeast and bacteria1–7, hence many researchers have studied the fungal, yeast and bacterial populations in Asian starter cultures, commonly based on culture-dependent techniques including phenotypic and 16S rRNA sequencing, and isolated and identified filamentous molds Absidia corymbifera, Amylomyces rouxii, Botryobasidium subcoronatum, Mucor circinelloides forma circinelloides, Mucor hiemalis, Rhizopus oryzae, Rhi. microsporus, Rhi. chinensis, and Rhi. stolonifer, Xeromyces bisporus5,6,8; yeasts Candida glabrata, C. tropicalis, Clavispora lusitaniae, Issatchenkia sp., Pichia anomala, P. nanogenensis, P. burtonii, Saccharomyces cerevisiae, Sacch. Bayanus5,8–11, and bacteria Acetobacter orientalis, A. pasteurianus, Bacillus amyloliquefaciens, B. circulans, B. sporothermodurans, B. subtilis, Pedicoccus pentosaceus, Lactobacillus bifermantans, Lb. brevis, Lb. plantarum, Weissella confusa, W. paramesenteroides5,12–16.

Introduction of culture-independent methods and its applicability in food microbiology5,7–17, has been a motivation for few researchers to profile the microbial community structure of some Asian starter cultures using PCR-DGGE, pyrosequencing, etc. which is suggestive to provide more insight into the microbial diversity of ethnic starters5,6,18–22. Rapid evolution in next generation sequencing (NGS) technologies has enabled researchers to have increased accuracy, throughput, with reasonably low cost and in relatively short period of time17,23. However, there are still a limited number of studies, characterizing the microbial community composition of fermented foods such as cheese24–26, kefir grains27, some ethnic Asian fermented foods28–31. Furthermore, the information on the community composition of Asian starter culture is rudimentary and needs in depth exploration using cutting edge technologies5.

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In present study, we attempted to profile the microbial community composition of marcha and thiat, traditionally prepared ethnic starter cultures of India using targeted amplicon sequencing. We selected two different traditionally prepared amylolytic starter cultures from two regions in India, marcha (Fig. 1a) from Sikkim (www.sikkim.gov.in) and thiat (Fig. 1b) from Meghalaya (www.megtourism.gov.in). Marcha is prepared from soaked rice with some wild herbs (Plumbago zeylanica, Buddleja asiatica and Vernonia cinerea), ginger and red dry chili, 1–2 % of previously prepared marcha powder as an inoculum, crushed in a wooden mortal by wooden pestle, mixed and dough are made into round to flattened cakes of different size and shape. Cakes are covered with fern fronds (Glaphylopteriolopsis erubescens), fermented at room temperature for 24 h, sun dried for 3–5 days and are used as amylolytic starters for production of cereal-based ethnic fermented beverages such as kodo ko jaanr, bhaati jaanr, raksi, etc.2 During thiat preparation, soaked glutinous rice is grinded with leaves and roots of wild plant Amomum aromaticum, 1–2% of old thiat, mixed and made into a dough by adding water. Flat to round balls are made and fermented for 1–3 days. The freshly prepared thiat balls are sun dried for 3–5 days. It is used to ferment alcoholic beverage locally called kiaid in Meghalaya2. Fermentation process involved in preparation of these starters is unconditional and may harbor both bacterial and fungal communities as consortia. Therefore, we aimed to explore the bacterial and fungal (filamentous molds and yeasts) communities in marcha and thiat. This is the first report on complete microbial community profile of traditionally prepared amylolytic starters of India using NGS technique.

Results
Characterizing microbial diversity. High throughput sequencing and quality trimming of 16S rRNA and ITS gene yielded ~0.85 and ~0.29 million quality reads in both marcha and thiat, respectively, which was used for subsequent analysis. Taxonomic assignment of sequences with the reference database resulted into 5,015 operational taxonomic units (OTUs). The average Good’s coverage of both the samples of marcha and thiat for 16S rRNA amplicon sequencing was found to be 99.08% ± 0.1% (mean ± SD) whereas for ITS region was recorded as 87.53% ± 17.6% (mean ± SD) indicating majority of the diversity was captured.

The estimates of alpha diversity indices revealed significant differences between marcha and thiat when computed for both the bacterial and fungal diversity (Table 1a and b). The bacterial species richness was found to be higher in thiat (4256.83) than marcha (1520.92), in contrast, fungal species richness depicts higher in marcha (5.25) over thiat (5.0). Significant variations were also noticed in non-parametric shannon index for bacterial communities in thiat (5.48) and marcha (4.01). Shannon index for fungal communities follow the reverse trend with marcha (2.25) and thiat (1.80). This observation is suggestive of higher bacterial diversity in thiat while marcha showed higher fungal diversity.

Table 1. Alpha diversity estimation. Non parametric alpha diversity was calculated for ethnic amylolytic starter cultures marcha and thiat.

|       | Chao1 | Goods coverage | Shannon | Simpson |
|-------|-------|----------------|---------|---------|
| a) Bacterial |       |                |         |         |
| Marcha | 1520.925 | 0.998902539 | 4.01115959 | 0.866763863 |
| Thiat  | 4256.838  | 0.997475969  | 5.489325073 | 0.940199394 |
| b) Fungal   |       |                |         |         |
| Marcha | 5.25   | 0.75           | 2.25    | 0.78125 |
| Thiat  | 5      | 1              | 1.802366931 | 0.671398892 |

Figure 1. Traditionally prepared amylolytic starter cultures (a) Marcha and (b) Thiat.
Bacterial community profile of _thiat_ and _marcha_. 16S rRNA gene amplicon sequencing yielded 15 bacterial phyla in _thiat_ and _marcha_, respectively (Fig. 2a). In _thiat_ bacterial phyla distributions were Proteobacteria (91.4%), Actinobacteria (4%), Firmicutes (4%) and the rest (0.6%) constituted the minor phyla Cyanobacteria, Bacteroidetes, Verrucomicrobia, Fusobacteria, Planctomycetes, Deinococcus-Thermus, Chloroflexi, Synergistetes, Acidobacteria, Saccharibacteria, Gemmatimonadetes, Armatimonadetes. In _marcha_ the phyla distributions of bacteria were Proteobacteria (53.8%), Firmicutes (45.4%) and other minor phyla were 0.8% constituting Actinobacteria, Cyanobacteria, Bacteroidetes, Verrucomicrobia, Fusobacteria, Planctomycetes, Deinococcus-Thermus, Chloroflexi, Synergistetes, Acidobacteria, Saccharibacteria, Gemmatimonadetes, and Armatimonadetes. The abundance of thirteen minor phyla was very less hence percentage of composition was not shown in Fig. 2a. Bacterial phylum Proteobacteria was found to outnumber other bacterial phyla in _thiat_ whereas _marcha_ was found to constitute Proteobacteria and Firmicutes as major phyla.

At family level, OTUs with ≥1% abundance were filtered which differed quantitatively between _thiat_ and _marcha_ (Fig. 2b). The family level distributions of bacteria in _thiat_ were Enterobacteriaceae (84.6%), Microbacteriaceae (3.24%), Enterococcaceae (2.47%), Clostridiaceae (1.13%) and Oxalobacteraceae (0.59%) (Fig. 2b). Whereas the family level of bacterial distributions in _marcha_ were Acetobacteraceae (50.6%), Leuconostocaceae (25.5%), Streptococcaceae (10.5%), Lactobacillaceae (8.38%), Burkholderiaceae (2.13%) and Staphylococcaceae (0.54%) (Fig. 2b).

At the genus level, OTUs with ≥1% abundance were filtered (Fig. 3a,b), which retained 18 differentially abundant genera in both samples of _marcha_ and _thiat_. Distribution of bacterial genera in _marcha_ were Acetobacter (52.6%), Fructobacillus (21.1%), Lactococcus (10.3%), Lactobacillus (8.4%), Leuconostoc (4.0%) (Fig. 3a), Burkholderia (2.1%) and Gluconacetobacter (1.4%). Genera in _thiat_ were Pantoea (32.4%), Cronobacter (21.4%), Escherichia-Shigella (15.5%), Enterobacter (13.1%), Citrobacter (4.2%) (Fig. 3b), Salmonella (3.2%), Serratia (2.8%), Enterococcus (2.5%), Curtobacterium (2.2%), Kluyvera (1.6%) and Clostridium (1.1%). The composition percentage of bacterial genera which was less than 3.9% was not shown in Fig. 3a,b.

Fungal (filamentous molds and yeasts) composition in _thiat_ and _marcha_. Fungal ITS gene sequencing and taxonomic analysis demonstrated the predominance of yeast phylum Ascomycota (98.6%) in _thiat_, whereas the distribution of filamentous phyla Zygomycota was only 1.4% (Fig. 2c). However, in _marcha_ only yeast phylum Ascomycota constituted the fungal diversity (Fig. 2c). Filamentous mold phylum was not detected.

Figure 2. Taxa distributions of phylum and family at different phylogenetic level in _thiat_ and _marcha_. (a) bacterial phyla; (b) bacterial family; (c) fungal phyla and (d) fungal family.
in marcha. Distributions of fungi (filamentous molds and yeasts) at the family level in thiat were Trichocomaceae (15.7%), Dothioraceae (3.94%), Mucoraceae (2.63%) and unidentified fungi (77.73%). Whereas the distributions of yeasts at the order/family level in marcha were Saccharomycetales (50%), Saccharomycetaceae (37.5%) and Amphisphaeriaceae (12.5%). (Fig. 2d). Distributions of yeasts genera in marcha were Wickerhamomyces (25%), Candida (25%), Kazachstania (25%), Saccharomyces (12.5%) and Pestalotiopsis (12.5%) (Fig. 3c). The filamentous mold genera distribution in thiat were Aspergillus (15.7%), Aureobasidium (3.9%) and Mucor (2.7%) and unidentified genera (77.7%) (Fig. 3d). The unidentified genera represented the yeast phylum Ascomycota in thiat. The sequence reads showed the species of filamentous molds were Aspergillus penicillioides, Aureobasidium pullulans and Mucor circinelloides, whereas the yeasts species were Wickerhamomyces anomalus, Candida quercitrus and Kazachstania exigua (data not shown).

Discussion
Our study provides comprehensive microbial diversity analysis using deep sequencing approach of ethnic amylolytic starter from India. Quantitative differences were noted for the presence of bacterial and fungal taxa among marcha and thiat; which could be the consequence of differences in the preparation, incubation period and most importantly the type of preservations. Alpha diversity estimation of amylolytic starters marcha and thiat using species richness and non-parametric Shannon index suggested higher bacterial diversity in thiat while marcha shows the higher assemblage of fungal diversity with dominance of yeast phylum Ascomycota. Persistence of higher fungal diversity in marcha is determinant factor suggesting the higher acidic conditions of marcha; in contrast, higher bacterial diversity of thiat depicts the faster turnover from acidic to alkali with the presence of acid neutralizing bacterial taxa. Acetobacter, Fructobacillus, Lactococcus, Lactobacillus, Leuconostoc, Burkholderia, and Gluconacetobacter were the predominant bacterial genera in marcha. Higher proportion of Acetobacter was possibly due to its retention and enrichment during fermentation. We observed relatively lower proportion of Streptococcus and Lactococcus than Lactobacilli; as Lactobacilli have high acid tolerance over former two. Though some species of Lactococcus have low acid tolerance, however, they could be isolated from raw milk and were found flourishing during the early stage of fermentation. This supports the lower abundance of Lactococcus than Lactobacillus as seen in our samples. Another interesting observation was absence of Pediococcus in bacterial community profile which was otherwise present as a one of the dominant genus in earlier report by culture dependent methods in marcha. Enterobacter, Cronobacter, Escherichia, Shigella, Enterobacter, Citrobacter, Salmonella, Serratia, and Enterococcus depicts most dominant bacterial genera of thiat each comprised over 0.1% of total bacterial sequences. Significantly varied microbial composition among thiat and marcha is a clear indication of differences in amylolytic starters. Genus Enterobacter was also detected in Mexican alcoholic beverages speculated.
to originate from the bacterial contamination in raw milk and they subsequently decreased during the fermentation process.\(^3\). The lactic acid bacterium such as *Lb. plantarum* seemed to be one factor for the good quality of the alcoholic beverages, as it can perform malolactic fermentation to decrease wine acidity and also produces bacteriocins.\(^3\)

Exploration of fungal diversity of ethnic amylolytic starters suggested higher abundance of yeast in *marcha* and *thiat* constitutes for 32.33-fold yeast to the filamentous molds. This observation was in coherence with the earlier report of culture-dependent studies showing the dominance of *Mucor* and *Rhizopus* genera of *Mucorales* in *marcha*.\(^6\) Interestingly no filamentous molds were detected in *marcha* using the applied high throughout sequencing method; the exact reasons for the observed variation in the microbiota have not been identified. This may be due to lower abundance of molds, limited sample size and/or age of the sample and finally also due to inadequate cell lysis which may prevent the release of nucleases.\(^4\) Our study was in accordance to the previous reports describing the exposure of cheese to different external environments such as manufacturing process; geographical region, etc have varied impact on the microbial composition of the end products.\(^2\) Thus, we speculate that the factor of geographic environment including altitudes and climate play a more significant role over the manufacturing process in resulting in the different microbial compositions of the starter culture under study. Some other crucial factors that may affect the composition of microbial communities in fermented amylolytic starters are level of hygiene, quality of the glutinous rice, water, as well as the back slopping technique. In this study three dominant yeasts in *marcha* were *Wickerhamomyces anomalus*, *Candida queceritus* and *Kazachstania exigua*, followed by *Saccharomyces* and *Pestalotiopsis* which also accompany the findings of ref. 21 by PCR-DGGE method. ITS gene sequences analysis of the *thiat* revealed the existence of *Aspergillus penicillioides*, *Aureobasidium pullulans* and *Mucor circinelloides* as the most dominant filamentous molds in *thiat*. At family level *thiat* shows *Trichocomaceae*, *Dothiorraceae* and *Mucoraceae* the major constituents of fungal community composition emphasizing the significance differences between *thiat* and *marcha* viz differences in starters substrates, preparations, inoculums, consortia, geography, hygiene, preservation technique, caloric values etc.

In the present study *Ascomycota* was dominant in starter cultures of India like in Korean and Chinese starters cultures, which was also reported earlier, based on NGS tools, in Korean alcoholic beverages\(^1\) and in Chinese liquors\(^3\). We could also expect similar observation in case of *marcha* as it has higher abundance of lactic acid bacteria. *Aspergillus oryzae* has strong secretion of amylases including alpha-amylase, which may accelerate the degradation of grains and provide more nutrients for microbes in alcoholic fermentation.\(^3\)

Amylolytic starter culture-making technology reflects the traditional knowledge of the ethnic Indian people on sub-culturing desirable inocula from previous batch to new culture using rice as base substrates by back-slopping method. This technique preserves the consortia of microbial community ranging from filamentous molds, yeasts and bacteria which were co-existed in traditionally prepared amylolytic and alcohol producing starters, and also preserves vast biological genetic resources, otherwise, which may be forced to disappear. Fermented beverages produced by using amylolytic starters in India are generally mild-alcoholic (4–5%), sweet taste with several health benefits to the local consumers as high source of calories, some vitamins and minerals.\(^2\) Ethnic fermented beverages and alcoholic drinks have the potential to grow beverage industry if proper scientific and technical support are applied to the existing indigenous practices of home based alcoholic fermentation.

**Materials and Methods**

**Sample collection.** Samples of sun-dried amylolytic starters *marcha* and *thiat* were collected immediately after the preparation from local people of Gangtok and Shillong in Sikkim and Meghalaya states of India, respectively. Dry samples were transferred to sterile containers, sealed, and stored at desiccator at room temperature for the further analysis.

**Community DNA Extraction.** The total community DNA was extracted using ProMega DNA kit (ProMega). 1g of amylolytic starter culture sample was suspended in lysis solution and incubated at 65 °C for 15 min. Subsequently, the RNA was eliminated from the cellular lysate by administering the RNase solution following incubation at 35 °C for 15 min. The residual proteins were removed by adding protein precipitation solution and centrifuged at max speed. Finally, the DNA was precipitated by adding isopropanol, which was purified with two washes of 70% ethanol. The quality of DNA was checked on 0.8% agarose gel and concentration was measured using Nano-DropND-1000 spectrophotometer (Nano Drop technologies, Willington, USA) as described by ref. 39. The DNA was stored at −20°C until further processing.

**Amplicon sequencing.** Amongst the nine hypervariable regions of bacterial 16S rRNA gene, we have targeted V4 hyper-variable region\(^1\) to investigate bacterial diversity of *marcha* and *thiat*. The universal 16S rRNA gene primer sets F515 and 806R\(^1\) was used for the amplification of V4 hyper-variable region. Similarly, fungal Internal Transcribed Spacer (ITS) II region was targeted for taxonomic profiling amylolytic starters, which was subjected to amplification using ITS1 and ITS2 primers. The library preparation of both the 16S rRNA and ITS gene amplicons were in accordance with the protocols of Illumina (USA). These amplicon libraries were further processed for sequencing using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA). The resultant product was screened with the LabChip GX (Perkin Elmer, Waltham, MA, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA). Subsequently, the 16S rRNA and ITS gene library were sequenced on the Illumina MiSeq platform using 2x 250bp chemistry. The sequences obtained from high throughput sequencing effort were submitted to National Center for Biotechnology Information (NCBI) which are available under BioProject ID PRJNA376467.

**Bioinformatics analysis.** The raw sequences generated from MiSeq platform was assembled using FLASH tool (Fast Length Adjustment of Short reads) a Paired end assembler for DNA sequences.\(^2\) The assembled reads were subjected to quality filtering using via Quantitative Insights into Microbial Ecology (QIIME) 1.8.\(^2\) Sequence
reads were assigned to bacterial and fungal operational taxonomic units (OTUs) by a closed reference-based OTU picking approach by using SILVA and UNITE reference databases, respectively. The OTU picking was carried out using UCLUST method with similarity threshold of 97%\(^4\). Taxonomic assignments were performed using RDP naïve bayesian classifier\(^4\). Alpha diversity indices like Chao\(^4\), Shannon and Simpson were calculated via QIIME after rarefying all samples to the same sequencing depth\(^4,45\).

**Data availability.** The sequences obtained from high throughput sequencing effort, was submitted to NCBI which are available under Bio Project ID PRJNA376467.

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Acknowledgements

Authors gratefully acknowledged the financial support of Department of Biotechnology, New Delhi for research project and DAILAB (DBT-AIST International Laboratory for Advanced Biomedicine), Bioinformatics Centre of DBT.

Author Contributions

S.P.S., A.A. and P.P. contributed to this present work as co-authors that are a part of their research work. K.J. and A.S. helped and assisted in all the molecular work and NGS (Bioinformatics and statistical) analyses. A.S., Y.S. and J.P.T. have framed this research paper along with all the authors involved. All authors critically revised, read and approved the final manuscript with approval of J.P.T.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

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