World Journal of Microbiology and Biotechnology (2019) 35:102
https://doi.org/10.1007/s11274-019-2677-y

ORIGINAL PAPER

# Bacterial diversity and metabolite profiles of curd prepared by natural fermentation of raw milk and back sloping of boiled milk

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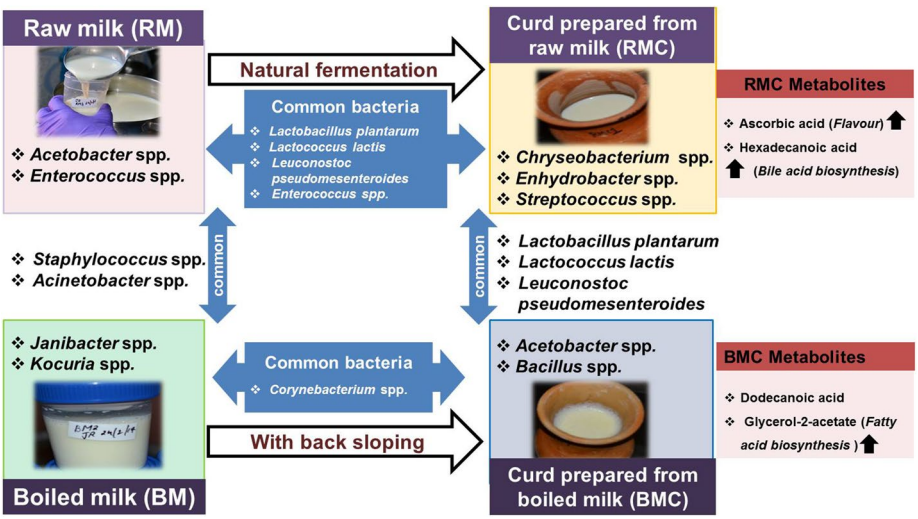
Received: 18 February 2019 / Accepted: 15 June 2019 / Published online: 24 June 2019


## Abstract

Preparation of curd vary worldwide due to which its taste, texture and impact on human health also differ. In Assam, curd prepared from raw milk (RMC) is preferred over curd prepared from boiled milk (BMC), a tradition believed to have originated from the Mongoloid customs. Microbial diversity of raw milk (RM), boiled milk (BM), RMC and BMC collected from three farms were investigated by culture dependent and independent techniques. Additionally, metabolite profiles of RMC and BMC were studied by gas chromatography and mass spectroscopy. A total of 59 bacterial isolates were identified from the four different dairy products. In RM, lactic acid bacteria such as *Lactococcus*, *Enterococcus*, *Lactobacillus* and *Leuconostoc* were obtained along with the environmental bacteria like *Bacillus*, *Staphylococcus*, *Acetobacter*, *Chryseobacterium*, *Streptococcus*, *Acinetobacter*, *Kocuria*, *Klebsiella* and *Macrococcus*. Additionally, *Prevotella*, *Oscillospira*, *Phascolarctobacterium* and *Akkermansia* were also detected in BM by culture independent technique. In RMC and BMC, *Lactococcus*, *Leuconostoc* and *Lactobacillus* were prevalent. RM and RMC shared *Enterococcus*, *Lactococcus*, *Streptococcus* and *Acinetobacter* as common bacterial genera. However, no bacterial genus was common in BM and BMC. The correlation analysis revealed that *Lactobacillus* was negatively correlated to other bacterial genera. Oligotyping analysis revealed that *Lactobacillus brevis* and *L.fermentum* were abundant in RMC and BMC, respectively. In metabolomic study, ascorbic acid, dodecanoic acid and hexadecanoic acid were found to be significantly higher in RMC. Presence of different types of probiotics in these curds samples opens a new avenue to understand their effects on human health.

### Graphic abstract




**Electronic supplementary material** The online version of this article (https://doi.org/10.1007/s11274-019-2677-y) contains supplementary material, which is available to authorized users.

Extended author information available on the last page of the article

**Keywords** Milk · Curd · Mongoloid · Lactic acid bacteria · Next generation sequencing · Metabolites

## Introduction

Dairy products are important components of a healthy diet. Fermented dairy products are preferred worldwide and are source of health beneficial microbes too. The process of making fermented dairy products has evolved with the human civilisations and therefore, varies across the world. Naturally fermented milk (NFM), a component of the Mongolian diet is believed to have health beneficial role and offer palatability to consumers due to rich microbial diversity (Zhong et al. 2016). In the north-eastern state of Assam, India few communities who are known to be of Mongoloid origin; traditionally prefer to take curds prepared from raw milk (RMC) over those prepared from boiled milk (BMC). RMC is prepared by allowing raw milk to undergo natural fermentation without adding any inoculum, whereas BMC is prepared by back sloping technique in which an inoculum from previous batch is added to boiled milk for fermentation.

Fermented dairy products are produced by specific group of microorganisms resulting decrease in pH that leads to coagulation of milk proteins (Dewan and Tamang 2007; Kabak and Dobson 2011). The microbiota of raw milk is complex and is reported to harbour thermoresistant, thermophilic, mesophilic, adventitious and psychrotrophic bacteria whose dominance decreases in semi cooked curd (47 °C) used for the production of Fontina (an Italian cheese) (Giannino et al. 2009). Microbial composition in some of the fermented dairy products, such as curd and *churpi* widely preferred in Sikkim, the north-eastern state of India included different species of *Lactobacillus*, and *Lactococcus* along with *Enterococcus faecium* and *Leuconostoc mesenteroides* (Dewan and Tamang 2007; Tamang et al. 2000). However, there is no report on the microbial composition of naturally fermented curd which is highly preferred in Assam over curd prepared from boiled milk. The traditional method of isolation of microbes has limitations as it cannot simulate the exact environment for their growth due to which a majority of them remains undetected (Zhong et al. 2016). To overcome this limitation, the culture independent techniques such as next generation sequencing (NGS) analysis of 16S rRNA amplicons have been widely used to detect the microbial diversity in various dairy products such as raw milk (Li et al. 2018), cheese (Wolfe et al. 2014), Koumisses (Zhong et al. 2016), curd and *churpi* (Tamang et al. 2000). However, the NGS analysis should be followed by oligotyping to investigate the bacterial diversity at sub-genus level (Eren et al. 2013).

The aim of this research was to compare the bacterial diversity of raw milk, boiled milk, curd prepared from raw and boiled milk. Bacterial diversity of both milk and curd samples collected from three farms of Assam were studied by both culture dependent and independent techniques (NGS analysis of 16S rRNA amplicons). The NGS data was further explored to determine the core bacterial genera and their interacting partners. Oligotyping was performed to identify the diversity within common core bacterial genera of RMC and BMC. A gas chromatography-mass spectrometry (GC–MS) based metabolomics profiling was also carried out to compare the metabolite profiles of the two types of curd samples.

## Materials and methods

### Sample collection

Dairy samples including raw milk (RM), boiled milk (BM) (boiled at 100 °C for 10 min) and curd prepared from RM (RMC) and BM (BMC) were collected in triplicates from three farms located at Aanthmile, Jagiroad and Pathgaon, Assam, India. RMC was prepared by allowing raw milk to undergo natural fermentation at room temperature (25–30 °C) without adding any starter culture, while BMC was prepared from boiled milk using back sloping technique where boiled milk was allowed to ferment after adding an inoculum from a previous batch for 48 h. For isolation of culturable microbes, samples were processed within 3–4 h after collection. Samples were stored at − 80 °C for culture independent and metabolomics studies.

## Bacterial isolation and characterisation

A 1 ml each of the milk and curd samples were homogenised in sterile 0.9% (w/v) NaCl solution (9 ml) for 5 min and a tenfold serial dilutions were carried out. A 100 µl of each of the dilutions of both milk ($10^{-2}$ to $10^{-4}$) and curd ($10^{-5}$ to $10^{-7}$) samples were spreaded onto plates containing different nutrient media. Isolation of *Lactobacilli* and *Enterobacteria* were carried out on deMan Rogosa Sharpe agar (MRSA, HiMedia, Mumbai, India) using both aerobic and anaerobic conditions (Anaerobic chamber, Don Whitely Scientific Limited, West Yorkshire, UK) at 30 °C and 37 °C, respectively for 48 h. M17 media (HiMedia, Mumbai, India) was used for isolation of *Lactococci* under aerobic and anaerobic conditions at 30 °C for 24 h. In addition, MRSA supplemented with 0.2% (w/v) cysteine (Merck, New Jersey, USA) was used for isolation of *Bifidobacteria* by incubating in an anaerobic chamber at 30 °C for 48–72 h. Pure cultures were maintained for

each isolated colonies and stored at − 80 °C in 15% (v/v) glycerol. In order to obtain genomic DNA, the isolates were cultured overnight in liquid media (MRS or M17) and DNA extraction was carried out following the protocol described by Sambrook et al. (1989). The quality of DNA was assessed by 0.8% (w/v) agarose gel electrophoresis. Quantification was performed using a Nano Drop™ ND-1000 (Thermo Fisher Scientific, Wilmington, USA). All the DNA samples were stored at − 20 °C for future use. PCR amplification of the 16S rRNA region of the bacterial DNA was carried out using the primer pair, 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and L1401(5′-CGG TGTGTACAAGACCC-3′). PCR reaction was performed in a 20 µl volume in a thermal cycler. Each PCR reaction contained a final concentration of 1 × standard buffer, 1.75 mM of $MgCl_2$, 200 µM of dNTPs, 0.2 µM of each primer, 1U of Taq DNA polymerase and 30 ng of template DNA. The PCR conditions were, initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 7 min. The PCR products (1400 bp) were visualised in a 1.2% (w/v) agarose gel under BioDoc-It Imaging System (UVP, California, US). Sequencing of the PCR amplicons covering the 16S rRNA region was carried out with Xcelris Genomics (Gujarat, India). The DNA sequences were compared against the GenBank database of NCBI using the BLAST program (Camacho et al. 2009).

## Metagenomic DNA extraction from milk and curd samples

Metagenomic DNA was extracted from milk samples (both RM & BM) by following the method earlier described by Sambrook et al. (1989). Briefly, aliquots (40 ml) of milk samples were centrifuged initially at 1000×*g* for 30 min and the pellets were resuspended in 1 ml of lysis buffer (Tris–EDTA buffer). A final concentration of 1 mg/ ml of proteinase K (Sigma) and 0.3% sodium dodecyl sulphate (SDS) were added to each sample and incubated at 42 °C overnight in a water bath. Similarly, the curd samples were initially centrifuged at 8000 rpm to reduce the fat content. The pellets were then resuspended in the lysis buffer (Tris–EDTA buffer) and incubated at 55 °C overnight in a water bath. Equal volume of Phenol and chloroform (1:1) was added and then DNA was precipitated by addition of sodium acetate (3 M, pH 5.2) and 100% (v/v) ethanol. The pellets were finally resuspended in 25 µl sterile Tris–EDTA buffer. Quantification was performed using a Nano Drop™ ND-1000 (Thermo Fisher Scientific, Wilmington, USA).

## Next generation sequencing (NGS) analysis

Metagenomic DNA of both milk and curd samples were subjected to NGS analysis with Macrogen (Seoul, Korea). Quantification of the double stranded DNA was performed using QuantiFluor dsDNA System (Promega, Wisconsin, USA). Bacterial diversity in the sample was analyzed using V3-V4 region of 16S rRNA amplicon sequencing on Illumina MiSeq platform. A 2 × 300 bp of MiSeq amplicon library was prepared using the Nextera XT Index Kit (Illumina Inc, California, USA). Amplicons were then ligated with Illumina adaptors and were amplified and gel purified as per the standard Illumina protocols.

## NGS data analysis

The NGS data analyses were performed using QIIME (version 1.9.1) pipeline (Caporaso et al. 2010) on the extracted high-quality sequences as described earlier (Zhang et al. 2016). Representative sequences were classified as an operational taxonomic unit (OTU) with a 97% threshold identity and taxonomic assignment were performed using SILVA database 128. For beta diversity, both weighted and unweighted UniFrac principal coordinate analyses (PCoA) were performed. All the sequences were uploaded in the Metagenomic RAST server (MG-RAST) and the details of each sample and IDs are tabulated in the Supplementary Table S1.

## Oligotyping analysis

Oligotyping of the three core bacteria, *Lactobacillus*, *Leuconostoc* and *Lactococcus* obtained in both the types of curd samples was performed using the oligotyping pipeline version 2.2 (available from https://oligotyping.org) (Eren et al. 2013). Following the initial entropy analysis, oligotyping was performed considering entropy values greater than 0.5 positions (C) were different for all the three bacteria and therefore, a minimum substantive abundance criterion (M) as 50 for all the three genera was used. Oligotypes not meeting the minimum substantive abundance criterion were discarded as noise. The matrix percent count generated during analysis was used for further analysis. We performed BLAST search for dominant oligotypes in the NCBI database to assign taxonomy.

## Metabolite study

Both the dairy samples (RMC & BMC) were extracted in hexane in 1:1 ratio for 12 h using a rotospin (Tarsons, Kolkata, India). After mixing, the samples were centrifuged at 7000×*g* for 15 min at room temperature. Supernatant was collected and were filtered with 0.45 µm syringe filter

(Sartorius, Gottingen, Germany). A 200 µl of the filtrate was transferred into GC–MS vials containing inserts and further analysis was carried out using Shimadzu Plus-triple quadrupole (TP-8030) GC–MS/MS system fitted with EB-5MS column (length-30 m, thickness-0.25 µm and ID-0.25 mm). The oven program started at 80 °C, subsequently ramped at a rate of 10 °C per min to 300 °C and then held for 10 min. A 1 µl sample was injected at 250 °C using He as carrier gas (1 ml per min) in split mode (1:10). The mass spectrometer was operated at a source temperature of 230 °C and a continuous scan from 45 to 800 m/z. The peaks were identified by matching the mass spectra with the National Institute of Standards and Technology (NIST) library, USA. The noisy peaks and the column bleeds (siloxanes and silane) were removed from the metabolite list before further analysis.

### Statistical analysis

The differences in microbial diversities across the milk and curd samples were investigated by Mann–Whitney *U* test within SPSS (SPSS Inc., Chicago IL, USA). The interactions among the microbes in both milk and curd samples were studied by bivariate correlation analysis {Spearman correlation (non-parametric) within SPSS (IBM SPSS, statistics 20)} and the networks were generated using Cytoscape (version 3.6.0) (Shannon et al. 2003) as described by Dehingia et al. (2015). Metabolite analysis was performed in MetaboAnalyst 4.0 package customized for metabolomics study (Xia et al. 2015). The metabolite data were normalized by sum method followed by log transformation and pareto scaling as described earlier (Dehingia et al. 2017; Dutta et al. 2012). Normalized data were subjected to multivariate analyses such as partial least square discriminant analysis (PLS-DA) and *t*-test in Metaboanalyst software.

## Results

### Culture dependent bacterial diversity

A total of 59 bacterial isolates were isolated from the four types of dairy products {RM (n = 19), BM (n = 15), RMC (n = 10) and BMC (n = 15)} collected from Aanthmile (AM), Jagiroad (JR) and Pathgaon (PG). The bacterial isolates were identified by 16S rRNA sequencing (Supplementary Table S2 and Table S3). The bacterial profiles of the RM, BM, RMC and BMC samples were farm specific. In the RM samples, species of *Lactobacillus* (ARM5, JRM8, PGRM9, ARC3, PGRC6 and PGRC20), *Lactococcus* (PGRM2, JRM6, PGRM10, PGRM3, ARC2, JRC2 and PGRC1) and *Leuconostoc* (ARM2, JRM9, ARC4, JRC1 and PGRC3) were found, which were also detected in the respective curd (RMC) samples (Supplementary Table S2). The species of *Enterococcus* (PGRM4 and PGBM4) were found in both the RM and BM samples of Pathgaon along with RM samples of Aanthmile (ARM3). In Aanthmile region, the species of *Staphylococcus* which was isolated from BM (*Staphylococcus sciuri* ABM4) was also obtained from its respective curd sample (BMC; *Staphylococcus sciuri* ABC4). In Pathgaon region, different species of *Corynebacterium* were isolated from BM (*Corynebacterium ureicelerivorans* PGBM2) and BMC (*Corynebacterium nuruki* PGBC1) (Supplementary Table S3). The diversity of lactic acid bacteria (LAB) such as *Lactobacillus* (ABC4, PGBC3, and JBC2), *Lactococcus* (PGBC6 and JBC8), *Leuconostoc* (JBC9 and PGBC5) and *Acetobacter* (ABC1, ABC3 and JBC6) in addition to non-lactic acid bacteria (n-LAB) such as *Staphylococcus* (ABC7), *Corynebacterium* (PGBC1), *Moraxella* (ABC5), *Rothia* (ABC6), *Chryseobacterium* (ABC8) were abundant in BMC samples in comparison to RMC samples (Supplementary Table S3).

## Culture-independent bacterial diversity

### NGS based analysis of metagenomic DNA of the dairy products

Amplicon sequencing produced a total of 1,790,112 high quality 16S rRNA gene reads. To compare the samples without biasness, the minimal read number of 1,22,614 was used as eigen value for calculation of diversity indices. Slopes of the rarefaction curves indicated sufficient coverage of bacterial diversity of the dairy samples (Supplementary Fig. S1). An unweighted Principal Coordinate Analysis (PCoA) plot based on the NGS data of 16S rRNA amplicons of the metagenomic DNA samples indicated distinct clustering of the samples RM, BM, RMC and BMC (Fig. 1).

The phylum level distributions of bacteria in the dairy products have been presented in the Fig. 2. The major bacterial phyla detected in the milk samples were *Firmicutes, Proteobacteria,Bacteroidetes* and *Actinobacteria,* while boiled milk had less *Proteobacteria* and more *Firmicutes* in comparison to raw milk. However, in the curd samples major bacterial phyla were *Firmicutes* and *Proteobacteria* (Fig. 2a–d). The major bacterial families found in raw milk of all the three regions were *Micrococcaceae*, *Staphylococcaceae*, *Enterococcaceae*, *Streptococcacea* and *Enterobacteriaceae*. However, a region wise variation was observed in which *Leuconostocaceae* was specific to Jagiroad, *Weeksellaceae* was found in both Aanthmile and Jagiroad, while *Moraxellaceae* was detected in Aanthmile and Pathgaon. The bacterial family detected in the boiled milk samples of all the three regions were *Staphylococcaceae* and *Moraxellacea, while Micrococcaceae*, *Enterococcaceae* and *Streptococcaceae* were found in Aanthmile and

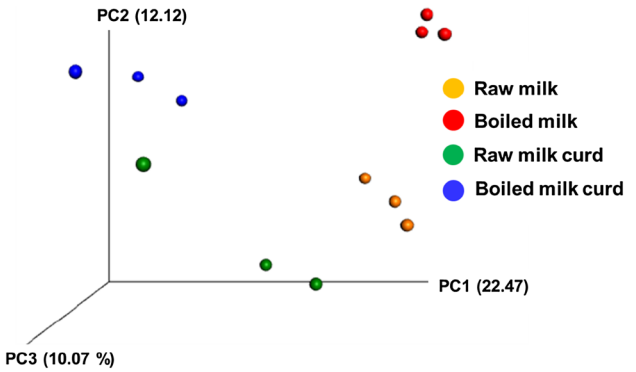



**Fig. 1** Unweighted Principal Coordinate Analysis (PCoA) plot based on the bacterial diversity obtained from Next Generation Sequencing (NGS) data of the dairy products (raw milk, boiled milk and curd prepared from raw milk and boiled milk)

Pathgaon and *Enterobacteriaceae* was found in Jagiroad and Pathgaon. There were few families which were region specific, such as *Weeksellaceae*, *Bacillaceae*, *Planococcaceae* and *Aerococcaceae* in Aanthmile, *Xanthomonadaceae* in Jagiroad and *Comamonadaceae* in Pathgaon. Curds prepared from raw milk of all the three regions had dominance of *Lactobacillaceae*, *Leuconostocaceae*, *Streptococcaceae*, *Enterobacteriaceae* and *Moraxellaceae*. However, dominance of *Acetobacteraceae* was specific to Aanthmile, *Pseudomonaceae* to Jagiroad and *Xanthomanadaceae* and *Staphylococcaceae* to Pathgaon. Curd prepared from boiled milk from all the three regions had dominance of *Lactobacillaceae*, *Leuconostocaceae*, *Streptococcaceae* and *Acetobacteraceae*. However, *Enterobacteriaceae* was specific to Aanthmile, *Bifidobacteriaceae* to Jagiroad and *Clostridiaceae* to Pathgaon (Fig. 2e).

At the genus level, *Georgenia*, *Xylanimicrobium*, *Paludibacter*, *Prevotella*, *Jeotgalicus*, *Dorea*, *Oscillospira*, *Phascolarctobacterium*, *Hylemonella*, *Acrobacter*, *Akkermansia* and *Sphingobacterium* were found to be significantly higher in BM in comparison to RM ($p < 0.05$), while *Escherichia* was higher in RM in comparison to BM ($p < 0.05$) (Fig. 2f). RMC had significantly higher abundance of *Corynebacterium*, *Kocuria*, *Sphingobacterium*, *Salinicoccus*, *Sphingomonas*, *Comamonas*, *Enhydrobacter* and *Pseudomonas* in comparison to BMC ($p < 0.05$). The RMC samples prepared from RM had higher abundance of *Sphingobacterium*, *Aerococcus*, *Lactobacillus*, *Pediococcus* and *Acetobacter* in comparison to the respective RM samples ($p < 0.05$). However, *Aerococcus*, *Ruminococcus* and *Paracoccus* were significantly higher in RM in comparison to RMC ($p < 0.05$).

Distribution of the LAB in the dairy samples was farm specific. *Lactobacillus* was dominant in both RMC and BMC of Aanthmile and *Lactococcus* in both RMC and BMC of Jagiroad. However, *Enterococcus*, *Leuconostoc*, *Streptococcus* and *Lactococcus* were dominant in RMC, while *Bacillus*, *Leuconostoc*, *Streptococcus* and *Acetobacteraceae* were dominant in the BMC samples of Pathgaon (Fig. 2f).

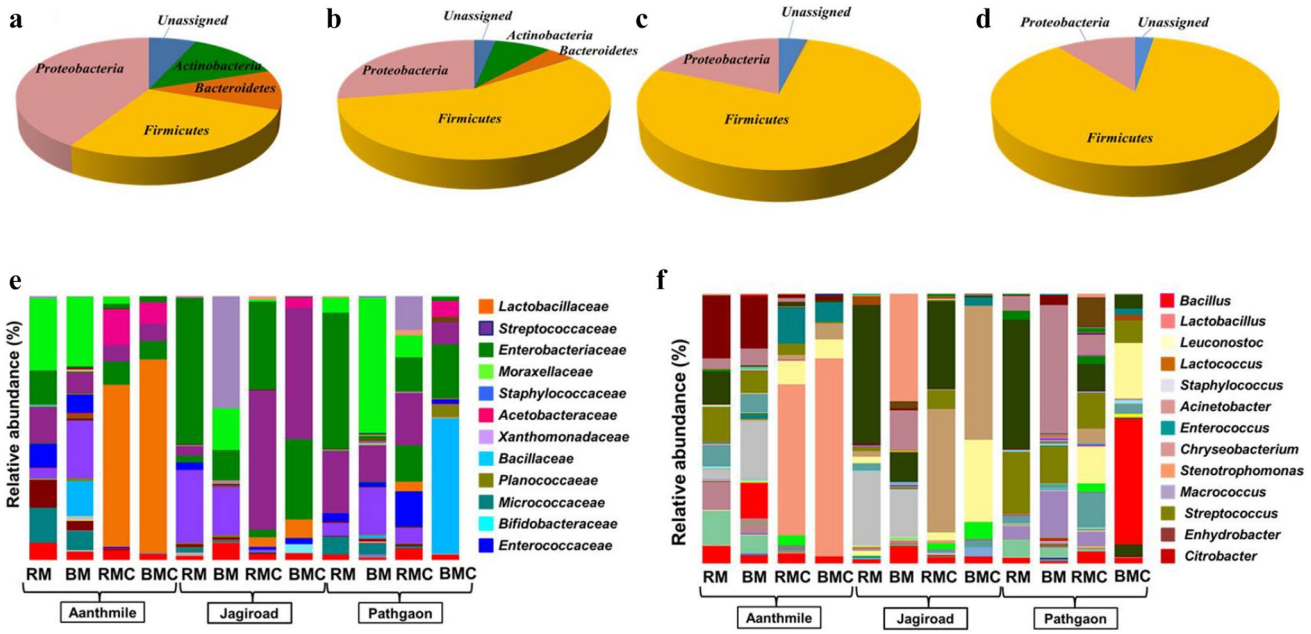



**Fig. 2** Relative abundance of the bacterial taxa found in the dairy products. Pie charts showing phylum level distribution of bacterial relative abundance (%) in raw milk (RM) (**a**), boiled milk (BM) (**b**), raw milk curd (RMC) (**c**) and boiled milk curd (BMC) (**d**). Bar charts show relative abundance (%) of bacterial families (**e**) and genera (**f**) in the dairy samples

## Core bacterial genera in the milk and curd samples

Among the 340 bacterial genera detected, bacteria which were present in all the replicates with more than 0.1% relative abundance were considered as the core bacteria (Table 1). The core bacteria in RM were *Staphylococcus*, *Enterococcus*, *Lactococcus*, *Streptococcus* and *Acinetobacter*. In BM, the core bacteria were found to be *Chryseobacterium*, *Staphylococcus*, *Streptococcus*, *Acinetobacter*, *Enhydrobacter*, *Jeotgalicus*, *Salinicoccus* and *Aerococcus.* In BMC, the core bacterial genera were found to be *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Acetobacter,* while in RMC the core bacterial genera were *Chryseobacterium*, *Enterococcus*, *Lactobacillus, Leuconostoc,Lactococcus*, *Streptococcus*, *Klebsiella*, *Acinetobacter, Pseudomonas* and *Enhydrobacter*. The core bacteria common in both RM as well as RMC were *Enterococcus*, *Lactococcus*, *Streptococcus* and *Acinetobacter*. However, no core bacterial genus was common to BM and BMC. The core bacterial genera of RMC and BMC were found to be *Lactobacillus*, *Leuconostoc* and *Lactococcus*.

Networks of co-occurrence of core bacteria found in the RM, BM, RMC and BMC sample were constructed based on the significant correlations between them {r = (−)1.00 to (+)1.00 and $p < 0.01$} (Fig. 4). In the RM samples, significant correlations were (i) positive correlation of *Staphylococcus* with *Lactococcus* (ii) negative correlations of *Staphylococcus* with *Acinetobacter* and *Streptococcus* (iii) negative correlations of *Lactococcus* with *Streptococcus* and *Acinetobacter* and (iv) positive correlation of *Streptococcus* with *Acinetobacter* (Fig. 3a). In BM, significant correlations were (i) positive correlations of *Chryseobacterium* with *Jeotgalicus* and *Staphylococcus* (ii) negative correlation of *Chryseobacterium* with *Acinetobacter* (iii) positive correlation of *Jeotgalicus* with *Staphylococcus* (iv) negative correlation of *Jeotgalicus* with *Acinetobacter* (v) negative correlation of *Salinicoccus* with *Streptococcus* (vi) negative correlation of *Staphylococcus* with *Acinetobacter* and (vii) positive correlation of *Aerococcus* with *Enhydrobacter* (Fig. 3c). In RMC, major correlations were (i) positive correlations of *Chryseobacterium* with *Enterococcus*, *Streptococcus*, *Klebsiella* and *Pseudomonas* (ii) negative correlation of *Chryseobacterium* with *Lactobacillus* (iii) positive correlations of *Enterococcus* with *Streptococcus*, *Klebsiella* and *Pseudomonas* (iv) negative correlation of *Enterococcus* with *Lactobacillus* (v) negative correlations of *Lactobacillus* with *Streptococcus*, *Klebsiella* and *Pseudomonas* (vi) positive correlation of *Leuconostoc* with *Acinetobacter* (vii) negative correlation of *Lactococcus* with *Enhydrobacter* (viii) positive correlations of *Streptococcus* with *Klebsiella* and *Pseudomonas* and (ix) positive correlation of *Klebsiella* with *Pseudomonas* (Fig. 3b). In BMC, a negative correlation of *Lactobacillus* was observed with *Leuconostoc* (Fig. 3d).

**Table 1** Percentage abundance of core bacteria in raw milk (RM), boiled milk (BM), curds prepared from raw milk (RMC) and boiled milk (BMC)

| Sl. No. | Core bacterial genera | RM | | | RMC | | | BM | | | BMC | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AM | JR | PG | AM | JR | PG | AM | JR | PG | AM | JR | PG |
| 1. | *Chryseobacterium* | 10.3 | 0.9 | 0.0 | 0.2 | 0.2 | 0.8 | 3.6 | 1.1 | 0.6 | 0.0 | 0.0 | 0.0 |
| 2. | *Staphylococcus* | 3.8 | 27.4 | 0.4 | 0.0 | 0.6 | 0.3 | 21.5 | 17.9 | 0.5 | 0.0 | 0.0 | 0.0 |
| 3. | *Enterococcus* | 8.1 | 2.8 | 2.9 | 0.6 | 1.1 | 12.6 | 6.7 | 0.0 | 1.7 | 0.0 | 2.1 | 0.3 |
| 4. | *Lactobacillus* | 0.0 | 0.0 | 0.0 | 55.9 | 1.0 | 0.1 | 0.0 | 0.0 | 0.0 | 73.5 | 0.1 | 0.4 |
| 5. | *Leuconostoc* | 0.0 | 0.0 | 0.0 | 8.9 | 2.9 | 13.7 | 0.0 | 0.0 | 0.0 | 7.0 | 30.5 | 20.4 |
| 6. | *Lactococcus* | 0.2 | 2.1 | 0.1 | 1.7 | 45.7 | 5.6 | 0.0 | 0.0 | 0.0 | 6.1 | 49.8 | 0.1 |
| 7. | *Streptococcus* | 13.3 | 1.5 | 23.4 | 4.3 | 7.1 | 13.2 | 7.6 | 0.3 | 13.8 | 0.5 | 0.0 | 8.1 |
| 8. | *Klebsiella* | 0.9 | 0.0 | 3.3 | 0.2 | 0.3 | 2.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 9. | *Acinetobacter* | 3.9 | 0.1 | 5.4 | 1.2 | 0.5 | 7.6 | 6.3 | 14.9 | 47.6 | 0.0 | 0.0 | 0.0 |
| 10. | *Enhydrobacter* | 23.3 | 0.0 | 0.1 | 1.4 | 0.2 | 0.3 | 19.4 | 0.6 | 3.5 | 0.0 | 0.0 | 0.0 |
| 11. | *Pseudomonas* | 0.0 | 0.2 | 0.4 | 0.1 | 1.1 | 1.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 12. | *Acetobacter* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.7 | 0.8 | 3.6 |
| 13. | *Corynebacterium* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.3 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 |
| 14. | *Brachybacterium* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 |
| 15. | *Micrococcus* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 |
| 16. | *Bacillus* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 13.2 | 0.0 | 1.2 | 0.0 | 0.0 | 0.0 |
| 17. | *Jeotgalicoccus* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.2 | 0.2 | 0.1 | 0.0 | 0.0 | 0.0 |
| 18. | *Salinicoccus* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 0.2 | 0.1 | 0.0 | 0.0 | 0.0 |
| 19. | *Aerococcus* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.5 | 0.1 | 0.3 | 0.0 | 0.0 | 0.0 |
| 20. | *Stenotrophomonas* | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.1 | 39.8 | 0.0 | 0.0 | 0.0 | 0.0 |

*AM* Aanthmile, *JR* Jagiroad, and *PG* Pathgaon

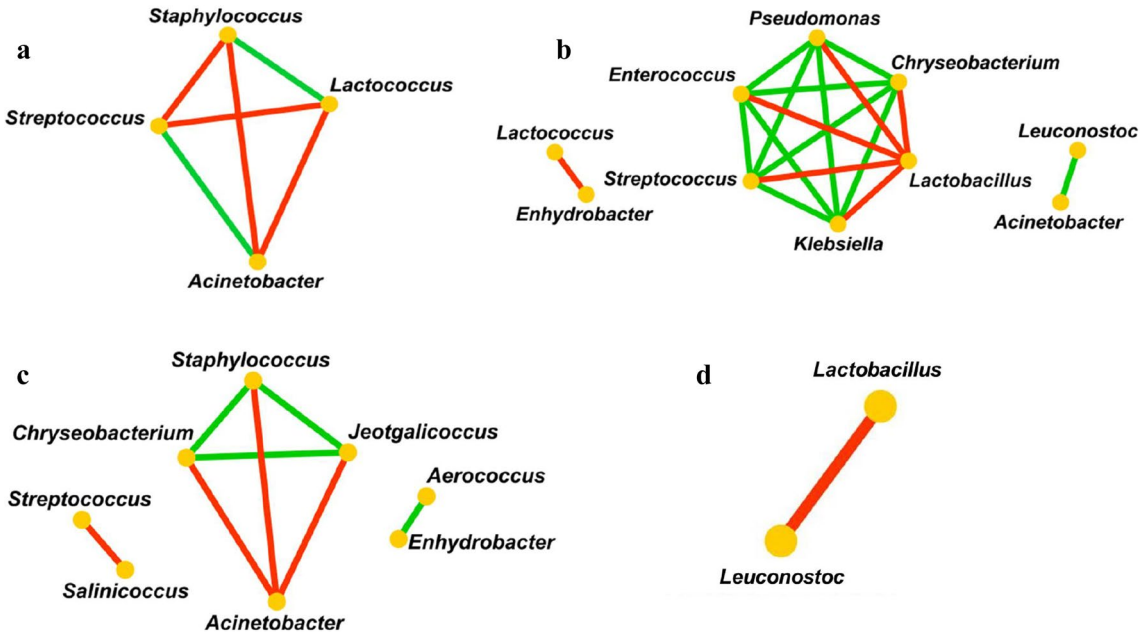



**Fig. 3** Networks of co-occurring core bacterial genera found in the dairy samples. Networks were visualized using prefuse force directed layout where the nodes represent the core bacterial genera of RM (**a**), RMC (**b**), BM (**c**) and BMC (**d**) and the edges represent the correlation (Negative-red; Positive-green)

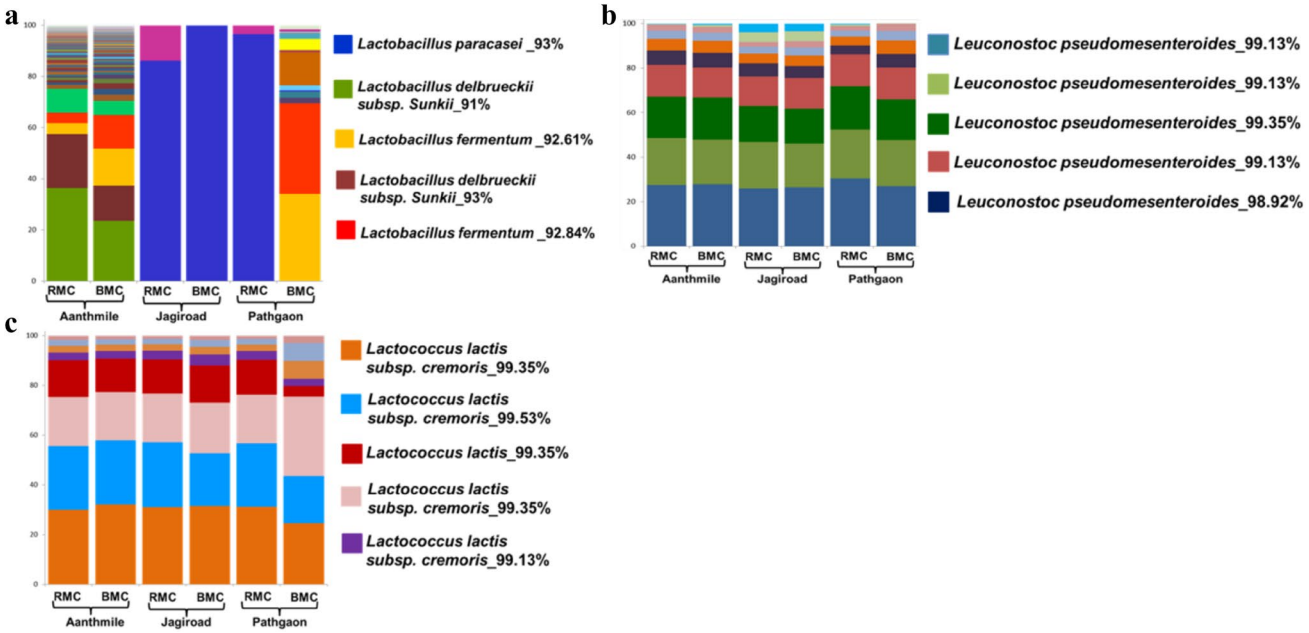



**Fig. 4** Distribution of bacterial oligotypes under the genera *Lactobacillus* (**a**), *Lactococcus* (**b**) and *Leuconostoc* (**c**)

## Oligotypes of the core bacterial genera

We analyzed the distribution of oligotypes of the core bacterial genera viz., *Lactobacillus*, *Leuconostoc* and *Lactococcus* found in the curd samples. Oligotyping of *Lactobacillus* revealed that the *Lactobacillus delbrueckii* was dominant in the samples of Aanthmile. However, *Lactobacillus paracasei* was found to be dominant in the RMC samples of both Jagiroad and Pathgaon along with the BMC samples of Jagiroad (Fig. 4a). *Lactobacillus brevis* was found to be dominant in the RMC samples of

both Jagiroad and Pathgaon. Oligotyping of *Leuconostoc* revealed that three different strains of *Leuconostoc pseudomesenteroides* were dominant in both the RMC and BMC samples of all the three farms (Fig. 4b). Similarly, it was observed that four different oligotypes of *Lactococcus* were dominant in both the RMC and BMC samples of all the three farms (Fig. 4c). An oligotype of the member species *Lactococcuslactis* subsp. *cremoris* was the most dominant oligotype in both the RMC and BMC samples of Aanthmile, Jagiroad and Pathgaon.

## Metabolomics

A GC–MS based analysis detected a total of 121 metabolites of which 88 had ≥ 0.1% peak area percentage and were selected for further analysis. Multivariate analysis and partial least squares discriminant analysis (PLS-DA) was performed based on the metabolite data among the RMC and BMC samples. PLS-DA was performed for maximizing the group separation and Leave-one-out cross validation (LOOCV) gave Q2 and R2 values which represent predictive capability and variance, respectively. The PLS-DA plot based on the curd metabolites had Q2 = 0.36 and R2 = 0.96. PLS-DA plot shows farm specific clustering of the RMC and BMC samples (Fig. 5). It was observed that few metabolites such as 10-methyl dodecanoic-5-olide, ascorbic acid and 2,2,4-Trimethyl-1,3-pentanediol disobutyrate were significantly higher in the RMC samples in comparison to the BMC samples of Aanthmile ($p < 0.01$). However, dodecanoic acid and glycerol 2-acetate were significantly higher in the BMC samples in comparison to the RMC samples of Aanthmile (Fig. 6a). It was observed that n-hexadecanoic acid was significantly higher in the RMC samples of Jagiroad in comparison to the BMC samples where glycerol -2-acetate was higher ($p < 0.01$) (Fig. 6b). 6-octadecanoic acid was found to be significantly higher in the RMC samples of Pathgaon in comparison to the BMC samples ($p < 0.01$) (Fig. 6c).

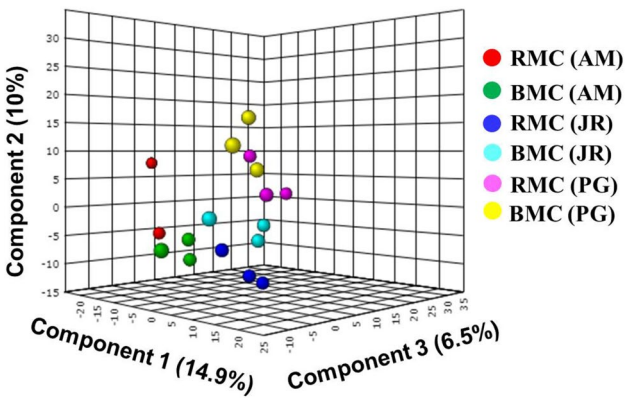



**Fig. 5** Partial list square discrimination analysis (PLS-DA) plot based on the metabolite data of curd prepared from raw milk (RMC) and boiled milk (BMC) depicting farm wise {Aanthmile (AM), Jagiroad (JR) and Pathgaon (PG)} clustering

## Discussions

In this study, a combination of both culture dependent and independent approaches were applied to explore the microbial communities in RM, BM, RMC and BMC coupled with metabolomic study to reveal their metabolite profiles. Previous reports suggested that the NFM products are vital components of regular diets in ethnic communities of the Mongolian population (Zhong et al. 2016). Many ethnic populations of Assam are of southern Mongolian origin who are referred to as *Kiratas* in the Sanskrit literature (Bose 1989). Similar to the Mongolians, many ethnic communities in the north-east of India still practice and prefer curd prepared from raw milk (RMC) by natural fermentation. Microbial diversities of the RM, BM, RMC and BMC collected from the three farms of Assam were found to be highly diverse indicating the crucial role of environmental factors along with indigenous milk bacteria in shaping the bacterial composition.

In culture dependent studies, the most important LAB detected in the RM samples of the three farms were *Lactococcus*, *Enterococcus*, *Lactobacillus* and *Leuconostoc*. These are the most common LAB genera found in bovine milk prior to pasteurisation (Quigley et al. 2013b). Fermentation is facilitated by the autochthonous microorganisms present in raw milk (Motato et al. 2017) due to which few bacterial genera such as *Lactococcus*, *Leuconostoc*, *Lactobacillus* were found to be common in both RM and RMC. Additionally, *Bacillus*, *Staphylococcus*, *Acteobacter*, *Chryseobacterium*, *Streptococcus*, *Acinetobacter*, *Kocuria*, *Klebsiella* and *Macrococcus* were also observed in the RM samples which were earlier reported to have originated from the environment, teat surface and milking equipment (Vacheyrou et al. 2011). All these environmental bacteria, *Enterococcus* and *Corynebacterium* were detected in BM. It was reported that *Lactococcus* have low tolerance to heat in comparison to *Enterococcus* which therefore was not detected in BM (Delgado et al. 2013). The BMC samples had abundance of LAB (*Lactococcus*, *Lactobacillus* and *Leuconostocs*) along with few n-LAB (*Corynebacterium* and *Staphylococcus*) genera which were farm specific. Both *Staphylococcus* and *Corynebacterium* were common among BM and BMC of Aanthmile and Pathgaon farm respectively. Few species of *Staphylococcus* can withstand pasteurisation by the production of heat-stable enterotoxins (Balaban and Rasooly 2000). However, both these bacterial genera are also involved in aroma, flavour and colour development in cheese

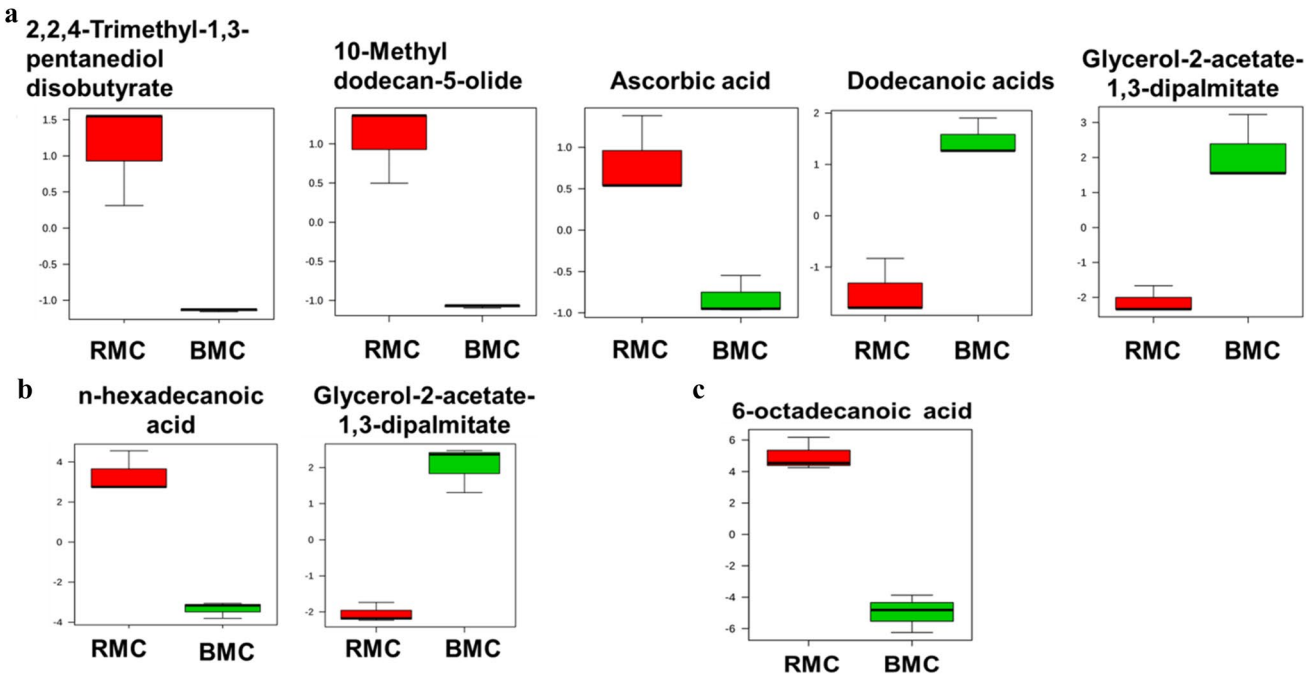



**Fig. 6** Box plots generated in the MetaboAnalyst 4.0 of two different types of curd samples prepared from raw milk (RMC) and boiled milk (BMC) of Aanthmile (**a**), Jagiroad (**b**) and Pathgaon (**c**). The Y-axis represents normalised peak area of metabolites obtained in GC–MS analysis

(Verdier-Metz et al. 2009). The success of such fermentation depends on the particular blend of microorganisms present in the previous batch along with the surrounding (Parente and Cogan 2004). Therefore, the primeval milk microbiota together with those from the environment directly impact microbiome of curd.

As conventional culture dependent methods cannot detect uncultivable bacteria, NGS analysis with the metagenomics DNA was performed (Jany and Barbier 2008). Previous reports suggest that *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were the most prevalent phyla in naturally fermented dairy products (Zhong et al. 2016). Interestingly, in our study, the curd prepared in either way had prevalence of *Firmicutes* and *Proteobacteria* in comparison to milk samples where *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* were prevalent. *Firmicutes* and *Proteobacteria* can survive high temperature and humidity respectively, while *Actinobacteria* and *Bacteroidetes* cannot (Li et al. 2018). Additionaly, in few important gut bacterial genera such as *Prevotella*, *Oscillospira*, *Phascolarctobacterium* and *Akkermansia* were also identified in BM. *Prevotella* is a commensal genus of rumen as well as human and is involved in metabolizing protein and carbohydrates (Quigley et al. 2013a). *Akkermansia* is regarded as a next generation beneficial microbe and is inversely associated with certain metabolic disorders (Cani and de Vos 2017; Everard et al. 2013). *Oscillospira* belongs to *Ruminococcaceae* family and is abundant in human faecal microbes and was reported to be positively associated with leanness (Gophna et al. 2017). *Phascolarctobacterium* is also a gut bacteria which produces short chain fatty acids (Wu et al. 2017).

The dominance of *Lactobacillus*, a common LAB involved in milk fermentation (Van de Casteele et al. 2006) was observed in the RMC and BMC samples of Aanthmile. However, dominance of *Lactococcus* over *Lactobacillus* was observed in RMC and BMC of Jagiroad. This might be due to the fact that few LAB are negatively correlated to each other which were also reflected in the correlation studies. *Lactobacillus* were found to be negatively correlated with other LAB such as *Enterococcus*, *Streptococcus* and *Leuconostoc* along with few other environmental microbes present in these curd samples which suggested that there was a competition within LAB for the ecological niches. The RMC samples of Pathgaon had co-occurrence of *Lactococcus*, *Leuconostoc*, *Streptococcus* and *Enterococcus*. In dairy industries, *Streptococcus thermophillus* is widely used as a starter culture (Hols et al. 2005) and are also bacteriocins producers which protect the dairy products from microbial spoilage (Kabuki et al. 2009). *Leuconostoc* strains are reported to be in synergestic functional relationship with acid producing *Lactococcus* (Hemme and Foucaud-Scheunemann 2004). *Leuconostoc* initiates citrate metabolism and aroma production in acidic conditions (Hemme and Foucaud-Scheunemann 2004). *Enterococcus* has proteolytic and hydrolytic activities that contribute to milk fermentation along with aroma production as reported earlier (Franz et al.

1999). *Bacillus* detected in the BM and BMC samples of Pathgaon might have originated from the farms including teat surfaces, dust, hay air and milking parlours (Vacheyrou et al. 2011).

Zhong et al. (2016) reported *Lactobacillus*, *Streptococcus* and *Lactococcus* as core bacteria in NFM products of the Mongolian diet. Interestingly, RMC prepared by natural fermentation in Assam had prevalence of *Lactobacillus*, *Lactococcus*, *Leuconostoc, Chryseobacterium*, *Enterococcus*, *Streptococcus*, *Klebsiella*, *Acinetobacter*, *Enhydrobacter* and *Pseudomonas* as core bacterial genera. Studies on the traditionally fermented milk products of Inner Mongolia in China have reported that *Enterococcus* sp., *Lactococcus lactis*, *Leuconostoc* sp. and *Saccharomyces cerevisae* predominated in the fermented cow's milk (Naer and Kitamoto 1995; Watabe 1998). A variety of genera such as *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Leuconostoc* were reported to be dominant in naturally fermented cow's milk (NFCM) of Russia which is also a traditional Mongolian fermented dairy product (Liu et al. 2015). Previous studies reported that *Lactobacillus* species was dominant in curd prepared with back sloping technique which is widely preferred in daily diet in the southern part of India (Balamurugan et al. 2014). Interestingly, in our study the bacterial genera in the curd prepared from boiled milk using back sloping technique which is generally preferred by few other communities of Assam were *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Acetobacter*. The culture independent technique also depicted that common bacterial genera found in both RMC and BMC were *Lactobacillus*, *Leuconostoc* and *Lactococcus*. In oligotyping analysis previously unexplored diversity of the genera *Lactobacillus*, *Lactococcus* and *Leuconostoc* in the curd samples were revealed. Diverse strains of *Lactobacillus brevis* and *L. paracasei* were found in the RMC of Jagiroad and Pathgaon respectively. However, Aanthmile farm had prevalence of *L.delbrueckii* and *L. fermentum* in RMC as well as BMC. It was earlier reported that few strains of *L. brevis* is a nonstarter LAB species which are responsible for flavour and texture development in cheese (Smit et al. 2005) and also has probiotic properties that improves human bowel function (Nobuta et al. 2009). Previous reports suggests that *Lactobacillus* conferred health benefits such as increase in immune response, lactose tolerance and colon cancer (Maragkoudakis et al. 2006).

During fermentation, microorganisms increases nutritional value of the dairy products by improving the organoleptic attributes (Von Mollendorff et al. 2006). The results of metabolomics study indicated that RMC and BMC had significantly different metabolites which were farm specific, as in the case of microbes. Presence of ascorbic acid in the RMC samples of Aanthmile might be responsible for enhancing flavour in the curd sample as microbial, enzymatic or chemical transformation of ascorbic acid found in milk produce flavour active compounds such as acetaldehyde, ethyl butyrate etc. (Boelrijk et al. 2003; McGorrin 2001; Tamime and Robinson 1999). Hexadecanoic acid and octadecanoic acid was found in the RMC samples of Jagiroad and Pathgaon, respectively. Hexadecanoic acid is involved in bile acid biosynthesis pathway and are a highly variable lipid component of human breast milk (Jensen et al. 1978). Unlike octadecanoic acid, hexadecanoic acid increases serum cholesterol (Serafeimidou et al. 2013). The *Streptococcus* and *Lactobacillus* bind to the free bile acids in the intestine for its removal from the human body (Pigeon et al. 2002). It is reported that milk fermented with *Lactobacilli* may have hypocholesteromic effect in human (Mann and Spoerry 1974).

## Conclusion

To our knowledge, this is the first report of microbial and metabolite profiles of curd prepared from raw milk (RMC) and boiled milk (BMC) using culture dependent, independent and metabolomic approaches. This study reveals that LAB was prevalent in raw milk (RM) and hence in respective RMC. Few strains of *Lactobacillus* were found to be farm specific. Additionally, metabolites detected in curd samples were also farm specific. These suggest that indigenous and environmental microbes present in dairy products might play an important role in metabolite production to increase its organoleptic and nutritional properties. Few important uncultivable bacterial genera were detected in boiled milk (BM) that might have some beneficial role in human gut health. Therefore the microbial and metabolites compositions of the dairy products should be a focus of research to know their probable implications on health.

**Acknowledgements** We thank the Institutional level Biotech Hub (DBT, Govt. of India) and Central instrumentation facility of IASST for providing the facilities. The author T.K.J. (IF150782) is thankful to Department of Science and Technology, Ministry of Science and Technology, Govt. of India, for supporting with DST-INSPIRE fellowship. This work was funded by Unit of Excellence project (BT/550/NE/U-Excel/2014) (DBT, Govt. of India). We thank all the households for providing the dairy samples.

### Compliance with ethical standards

**Conflict of interest** There is no conflict of interest.

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