MICROBIOLOGY OF ETHNIC FERMENTED FOODS AND ALCOHOLIC BEVERAGES OF THE WORLD

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MICROBIOLOGY OF ETHNIC FERMENTED FOODS AND ALCOHOLIC BEVERAGES OF THE WORLD

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EDITORIAL
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Editorial: Microbiology of Ethnic Fermented Foods and Alcoholic Beverages of the World

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Keywords: editorial, fermented foods, food microbiology, next generation sequence, global

Editorial on the Research Topic

Microbiology of Ethnic Fermented Foods and Alcoholic Beverages of the World

Approximately there may be around 5,000 varieties of common and uncommon fermented foods and alcoholic beverages in the world. Global fermented foods are classified into 9 major groups on the basis of substrates (raw materials) used from plant/animal sources: fermented cereals, fermented vegetables and bamboo shoots, fermented legumes, fermented roots/tubers, fermented milk products, fermented and preserved meat products, fermented, dried, and smoked fish products, miscellaneous fermented products, and alcoholic beverages. Fermented foods are the hubs of consortia of microorganisms, which transform the chemical constituents of raw materials of plant/animal sources during *in situ*/*ex situ* fermentation, thereby enhance the nutritional value with health-promoting bioactive compounds to consumers.

Common genera of the lactic acid bacteria isolated from various fermented foods globally are *Alkalibacterium, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella*. Species of Bacillus are reported for alkaline-fermented foods of Asia and Africa. The association of several species of *Kocuria, Micrococcus* (members of the Actinobacteria), and *Staphylococcus* (belonging to the Firmicutes) has been reported for fermented milk, fermented meat, and fish products. Species of *Bifidobacterium, Brachybacterium, Brevibacterium*, and *Propionibacterium* have been isolated from cheese and species of *Arthrobacter* and *Hafnia* from meat fermentation. *Enterobacter cloacae, Klebsiella pneumoniae, K. pneumoniae* subsp. *ozaenae*, *Haloanaerobium, Halobacterium, Halococcus, Propionibacterium*, and *Pseudomonas*, are also present in numerous fermented foods.

Genera of yeasts reported for fermented foods, alcoholic beverages, and non-food mixed amylolytic starters are *Brettanomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Dekkera*, *Galactomyces*, *Geotrichum*, *Hansenula*, *Hanseniaspora*, *Hyphopichia*, *Issatchenkia*, *Kazachstania*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Rhodosporidium*, *Saccharomyces*, *Saccharomycodes*, *Saccharomycopsis*, *Schizosaccharomyces*, *Sporobolomyces*, *Torulaspora*, *Torulopsis*, *Trichosporon*, *Yarrowia,* and *Zygosaccharomyces*. Major roles of filamentous fungi in fermented foods and alcoholic beverages are mainly production of enzymes and also degradation of anti-nutritive factors. Species of *Actinomucor*, *Amylomyces*, *Aspergillus*, *Monascus*, *Mucor*, *Neurospora*, *Parcilomyces*, *Penicillium*, *Rhizopus,* and *Ustilago* are reported for many fermented foods, Asian non-food amylolytic starters and alcoholic beverages.

Direct DNA extraction from samples of fermented foods, commonly called culture-independent methods, is nowadays frequently used in food microbiology to profile both cultivable and

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uncultivable microbial populations from fermented foods. Amplified ribosomal DNA restriction analysis (ARDRA) and denaturing gradient gel electrophoresis (DGGE) techniques developed to profile microbial communities directly from fermented foods, and are based on sequence-specific distinctions of 16S rDNA or 26S rDNA amplicons produced by PCR.

Application of next generation sequencing (NGS) such as metagenomic approaches by using parallel pyrosequencing of tagged 16S rRNA gene amplicons provide information on microbial communities as profiled in kimchi, a naturally fermented vegetable product of Korea, nukadoko, a fermented rice bran of Japan, narezushi, a fermented salted fish and cooked rice of Japan, and ben-saalga, a traditional gruel of pearl millet of Burkina Faso. A proteomics identification method based on protein profiling using matrix-assisted laser desorption ionizing-time of flight mass spectrometry (MALDI-TOF MS) is used to identify bacteria in fermented foods. NGS has revealed the new dimension of microbial ecology comprising both cultivable and uncultivable microorganisms in many ethnic fermented foods and beverages of the world.

This e-book is a compilation of 15 originals and reviews papers written by 94 authors. We tried to represent the main Asian, African, European, and American fermented foods and alcoholic beverages.

AUTHOR CONTRIBUTIONS

JT prepared a draft concept on Resource Topic of the present e-book and list of authors for papers, supplemented and corrected by WH, DS, and GF. JT was the main corresponding Editor, however, WH, DS, and GF also helped to provide the names of referrers, etc.

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ORIGINAL RESEARCH
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Comparative mRNA Expression Profiles of Riboflavin Biosynthesis Genes in Lactobacilli Isolated from Human Feces and Fermented Bamboo Shoots

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With the aim to bioprospect potent riboflavin producing lactobacilli, the present study was carried out to evaluate the relative mRNA expression of riboflavin biosynthesis genes namely *Rib* 1, *Rib* 2, *Rib* 3, and *Rib* 4 from potent riboflavin producers obtained from our previous studies. All the four genes were successfully cloned and sequenced for further analysis by *in silico* procedures. As studied by non-denaturing Polyacrylamide gel electrophoresis, no difference in size of all the four genes among those of various lactobacilli was observed. The relative fold increase in mRNA expression in *Rib* 1, *Rib* 2, *Rib* 3, and *Rib* 4 genes has been observed to be 10-, 1-, 0.7-, and 8.5-fold, respectively. Due to increase in relative mRNA expression for all the *Rib* genes as well as phenotypic production attribute, KTLF1 strain was used further for expression studies in milk and whey. The fold increase in mRNA expression for all the four *Rib* genes was higher at 12 and 18 h in milk and whey respectively. After exposure to roseoflavin, resistant variant of KTLF1 showed considerable increase in expression of all the targets genes. This is the first ever study to compare the mRNA expression of riboflavin biosynthesis pathway genes in lactobacilli and it also under lines the effect of media and harvesting time which significantly affect the expression of *rib* genes. The use of roseoflavin-resistant strains capable of synthesizing riboflavin in milk and whey paves a way for an exciting and economically viable biotechnological approach to develop novel riboflavin bio-enriched functional foods.

Keywords: riboflavin, lactobacilli, fermented bamboo shoots, milk, whey, mRNA, roseoflavin

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INTRODUCTION

In the recent years, many researchers have shown burgeoning interest in riboflavin which has now regarded as an essential component of cellular biochemistry (Thakur et al., 2016a). Several bacteria have the trait to synthesize riboflavin and its pathway has been studied in bacteria whereas humans lack its biosynthesis ability (Perkins and Pero, 2002). The microorganisms harbor the genetic structure to synthesize B vitamins particularly riboflavin to obtain bio-enriched food (Capozzi et al., 2011). Due to adaptability to fermentation processes, lactic acid bacteria (LAB) act an ideal candidates for *in situ* riboflavin production in food (Arena et al., 2014). Though, ability for riboflavin biosynthesis is strain specific (Capozzi et al., 2012). An alternative RNA structure involving the RFN element serves a model for regulation of riboflavin biosynthesis (Gelfand et al., 1999; Vitreschak et al., 2002). Riboflavin metabolism and transport genes are regulated at transcription attenuation and translation initiation level in Gram-positive bacteria and Gram-negative bacteria respectively (Vitreschak et al., 2002). Four genes (*rib1, rib2, rib3,* and *rib4*) are required for biosynthesis of riboflavin from guanosine triphosphate (GTP) and ribulose-5- phosphate (Perkins et al., 1999). According to these authors, these genes are located in an operon and their order differs from that of enzymatic reactions (Richter et al., 1992, 1993, 1997). There are mainly two promoters responsible for transcription of riboflavin genes where all the four genes are controlled are primarily controlled by the *rib*P1 promoter (Perkins et al., 1999). The *rib*3 and *rib*4, are regulated from a second promoter (*rib*P2) and regulatory region RFN (Perkins et al., 1999).

There are number of reports where overexpression in riboflavin production was observed after exposure to range of roseoflavin (a chemical analog to riboflavin) (Burgess et al., 2004, 2006; del Valle et al., 2014). In these studies, riboflavin overproduction directly correlated with the spontaneous roseoflavin resistant strains (Burgess et al., 2006; Capozzi et al., 2011). The tolerance to the toxic roseoflavin signifies the mutations in the regulatory region of the rib operon which ultimately give rise to riboflavin over producing phenotype. Lately, *in situ* bacterial overproduction of the B group vitamins, including riboflavin is of significant interest (Burgess et al., 2009; Capozzi et al., 2012). In particular for riboflavin, promising results have been reported for the production of yogurt (Burgess et al., 2006) or pasta and bread (Capozzi et al., 2011; Arena et al., 2014) and Soymilk (del Valle et al., 2014). Many researchers (Jayashree et al., 2011; Guru and Viswanathan, 2013; del Valle et al., 2014; Thakur and Tomar, 2015a; Thakur et al., 2016c) have studied the riboflavin production in LAB in MRS, Riboflavin free media, milk and whey but no one has ever reported the expression levels of riboflavin biosynthesis genes. The Lactobacilli used for present study were previously isolated and identified from various niches (human feces, fermented bamboo shoots, and curd) (Thakur and Tomar, 2015a; Thakur et al., 2015a, 2016c). Among them Lactobacilli isolated from fermented bamboo shoots (Manipur, India) have shown highest riboflavin producing properties as well as displayed probiotic and appreciable techno-functional properties (Thakur et al., 2015a). In the continuance of our previous reports, the present study reveals the first ever profile of mRNA expression of four *Rib* genes (molecular determinants for riboflavin biosynthesis which form a complete functional *rib* operon) in four different media by harvesting the test isolates at different intervals of time. There are few reports where the regulatory mechanism of riboflavin biosynthesis has been studied in roseoflavin resistant variants in LAB. However, there exists *per se* no such report for *Lactobacillus* species till date.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The *Lactobacillus* strains (**Table 1**) used in this work were confirmed for riboflavin production by an array of analytical methods viz. Polymerase chain reaction (PCR) based method (presence of riboflavin biosynthesis genes), Spectrophotometric method, Microbiological assay method, and High Performance Liquid Chromatography in our previous studies (Thakur and Tomar, 2015a; Thakur et al., 2016b). All the strains stored previously at −80°C in MRS supplemented with glycerol (20% v/v) were routinely cultured on de Man-Rogosa -Sharp (MRS) medium (Sigma- Aldrich, St. Louis, MO, USA) for this study.

Cloning, Transformation, and Sequencing

Purified PCR products (HiPuraA^{TM} purification kit, Himedia, India) were used for cloning of all the four genes. The cloning vector used in this study was pTZ57R/T clone vector amp (InstClone PCR cloning kit, Stratagene, USA). The clones were transformed into competent cells of *Escherichia coli* (*E. coli*) (XL1 blue). The successful clones were picked from Luria broth+ ampicillin plates and amplified for target genes by colony PCR method followed by plasmid isolation. The positive clones were identified by PCR analysis of plasmid DNA by using primers used in our previous study (Thakur et al., 2016b). The nucleotide sequencing was performed by sequencing services provided by Xcelris Labs, Ltd, Ahmedabad, India. The chromatograms of sequences obtained were analyzed and converted to Fasta using Bio-Edit Software. Nucleotide sequence similarity searches were performed for the obtained sequences by matching with previously published complete genome of *Lactobacillus* species of interest.

Size Variation in Rib Genes by Polyacrylamide Gel Electrophoresis (PAGE)

Non-denaturing PAGE was used to detect the difference is size of all the four *Rib* genes amplified in different lactobacilli. Silver

TABLE 1 | Isolates used in this study.

Sr. No.	Genus	Species	Given name	Source
1	*Lactobacillus*	*fermentum*	KTLF1	Our previous studies
2	*Lactobacillus*	*fermentum*	KTLF3	
3	*Lactobacillus*	*plantarum*	KTP13	
4	*Lactobacillus*	*mucosae*	KT2	
5 (Standard)	*Lactobacillus*	*fermentum*	MTCC8711	MTCC, Chandigarh, India

TABLE 2 | Real-time (RT-PCR) primers designed for this study.

Gene name	Primer sequence	Melting temperature	Product size	Reference
Rib1	F′ GGCAGTCATTCGGGGTGCAACCG R′ CTTAAAGCCAGCGCGATCCATAGCTTGTTC	62 63	157 bp	Present study
Rib2	F′ CCGGCGACGGTCAACTTCATGACCAA R′ GTCGACTTGTGGTCTAGGGAAACCGTAAAAGC	63 64	158 bp	
Rib3	F′ CCGTCAACGGAACCTGCCTGACGGT R′ TTGAAGGTGGTCAGGTTGTAAGTCTGCGGCAT	64 64	94 bp	
Rib4	F′ CTAACTGTGCGGCAACGTACTTGCC R′ GGAGGTTGGTTCCCACTCACCTATG	67 61	168 bp	
REC	F′ CACGTGCCGAAATTGAAGGTGAAATGGGTG R′ CACCAGGAGTCGTTTCAGGATTACCAAACAT	63 62	110 bp	
TUF	F′ GGTCCGATGCCACAAACTCGTGAACACAT R′ CGGACAGAAGGTCACGAACTTCCATTTCAAC	63 63	130 bp	

staining was used to view the band pattern in the PAGE after the final gel run.

Growth in MRS, RAM, Milk and Whey Based Media

The test isolates were washed thrice with saline solution (0.85% m/v NaCl), resuspended in this solution and used to inoculate at 2% (v/v) riboflavin-free culture medium (Riboflavin Assay Medium, Difco, Becton, Dickinson, and Co., Sparks, MD, USA), reconstituted skim milk and whey based medium and then incubated without agitation at 37°C for 18 h. The optical density of selected isolates was observed before harvesting them for RNA isolation. The log count/ml was checked at lag, log, and stationary phases of growth.

Designing of Primers

The primers (**Table 2**) were designed by aligning sequences of riboflavin operon using CLUSTALW program. The *Lactobacillus fermentum* IFO3956 strain was considered for primer selection: GenBank accession number NC_010610. The house keeping genes for normalizing real time reaction were synthesized for *REC* gene essential for the repair and maintenance of DNA and *TUF* gene encoding elongation factor from the database of genome in NCBI.

RNA Extraction, cDNA Synthesis, and RT-PCR

Selected Lactobacilli were grown in 10 ml of MRS, Riboflavin Assay Medium (devoid of riboflavin) (RAM), Skim milk and Whey based medium. The RNA was extracted after 6, 12, 18, and 24 h of incubation using TRIzol reagent followed by cell lysis by lysozyme (10 mg/100 ml) (Sigma, USA) (Ramiah et al., 2007). The quality of isolated RNA was checked (Sambrook and Russell, 2001). RNA was quantified and its purity of RNA was judged and used for reverse transcription. The cDNA was prepared with cDNA kits (RevertAid^{TM} First strand c-DNA synthesis kit, Fermentas, India), according to the manufacturer's instructions. SYBR Green I Master mix (Roche) on 2 μL of diluted cDNA (1:1) using exon-spanning primers, 5 μL of SYBR green buffer 2X (Roche) and 2.5 pmol of each primer (**Table 2**) for a total volume reaction of 10 μL were used for qualitative PCR. The amplification was run in Lightcycler® 480 II, and the results were analyzed using Lightcycler® 480 II software release 1.50 SP3. The PCR conditions were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of amplification at 63°C for 30 s and 72°C for 30 s. At the end of the each run a melting curve was achieved from 70 to 95°C and continuous fluorescence measurement was taken. A melting curve analysis was performed in order to verify the specificity of real-time PCR (RT-PCR) and finally, a cooling step to 4°C was achieved. Fluorescence was measured once every cycle after the extension step using filters for SYBR Green I (excitation at 465 nm and emission at 510 nm). To calculate the threshold cycle value, the normalized fluorescence data was converted to a log scale and threshold value was determined. The quantitative data of RT-PCR is generated on the basis of number of cycles required for optimal amplification generated fluorescence to reach a specific threshold of detection (the quantification cycle:Cq values) (Bustin et al., 2009). The comparative critical threshold (ΔΔCT) method was used to calculate the relative expression ratios in which the amount of target RNA is adjusted to a reference (internal target RNA) (Livak and Schmittgen, 2001). The Prism 7.00 was used to analyze the RT-PCR data sets.

$$\Delta CT = CT\ \textit{of internal control} - CT\ \textit{of gene of interest}. \quad (1)$$
$$\Delta\Delta CT = \Delta CT\ \textit{of sample} - \Delta \textit{CT of reference}. \quad (2)$$
$$\textit{Relative expression level} = 2^{\Delta\Delta}CT. \quad (3)$$

Isolation of Roseoflavin-Resistant Strains

The roseoflavin-resistant strains of KTLF1 was performed (according to Burgess et al., 2004, 2006) by exposing wild strain to increasing concentrations of roseoflavin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in RAM. Further experiments were carried out by using subsequent inoculum in 1 mL of RAM supplemented with roseoflavin. From the culture grown at the maximum range of roseoflavin, 15 separated colonies were randomly isolated after spreading onto MRS agar plates, and those stocks were stored at −80°C in CDM roseoflavin-free supplemented with 20% of glycerol (Russo et al., 2014).

Principal Component Analysis

To discriminate the riboflavin producing isolates and riboflavin biosynthesis genes on the basis of mRNA expression levels in different media and at different time and principal component analysis (PCA) treatment, IBM SPSS Statistics 21.0 software program (IBM, Armonk, NY, USA) was used (ANOVA followed by a Tukey's *post hoc* test). $P < 0.05$ was considered as statistically significant.

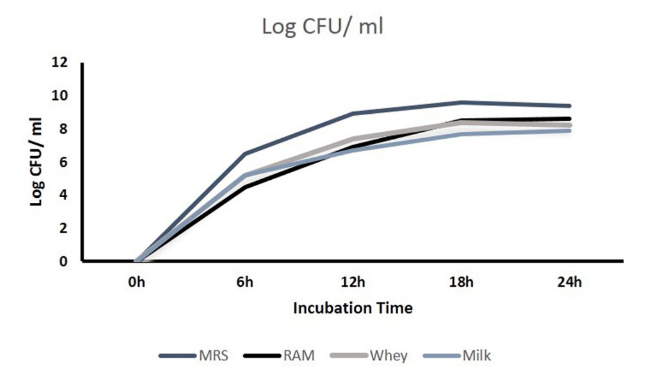

FIGURE 1 | Growth characteristics of KTLF1 in four different media.

RESULTS

Cloning Transformation and Sequencing

Purified amplicons were ligated into PTZ57R/T cloning vector. The ligates were transformed into competent cells of *E. coli* XL1 blue strain. The recombinant clones showed white (recombinant) colonies on LB agar plates supplemented with ampicillin (100 μg/ml). Ten randomly selected recombinant clones were analyzed by colony PCR for *Rib* genes. Consequently, positive clones were used for plasmid DNA analysis and isolated plasmids were further confirmed for their size by PCR and subsequently sequenced (Supplementary File). The sequences obtained from the isolates were compared by BLAST analysis for similarity check with three reference strains of *Lactobacillus* submitted to NCBI GenBank (Supplementary File). No size variation was found in the studied genes in different lactobacilli after staining the PAGE with silver staining (Supplementary File).

Growth of Isolates in MRS, RAM, Milk and Whey for RNA Isolation and Gene Expression

The CFU/ml was observed for KTLF1 at different growth phases (**Figure 1**) and the mRNA and the cDNA were prepared as described above. During the early growth, expression levels remain low and get elevated at later phase. The expression levels of *Rib* 1, *Rib* 4 were significantly higher in all the media used at four time intervals (**Figure 2**), whereas *Rib*2 and *Rib* 3 have shown almost constant regulation with different variables (Isolates, Media, and Time) (**Figure 2**). After incubation in different media at different time, the level of mRNAs expression was found to be changed in all the tested isolates. Particularly in KTLF1 strain, the mRNA expression level increased significantly in MRS at 12–18 h, in RAM at 6–12 h, in Milk at 6 h and in whey the upregulation was observed at 12 h (**Figure 3**). Among all the media used, RAM has shown increase in relative expression followed by MRS, Milk and Whey (**Figure 4**). Overall, there was no statistical difference in expression levels of *Rib*2 and *Rib*3 at different variables but a gradual decrease in the mean expression with time was recorded. **Figure 5** revealed the marked difference of increase in the intensity of RT-PCR products at different variables in KTLF1 in MRS and RAM which correspond significantly to change in mRNA expression profile. In order to

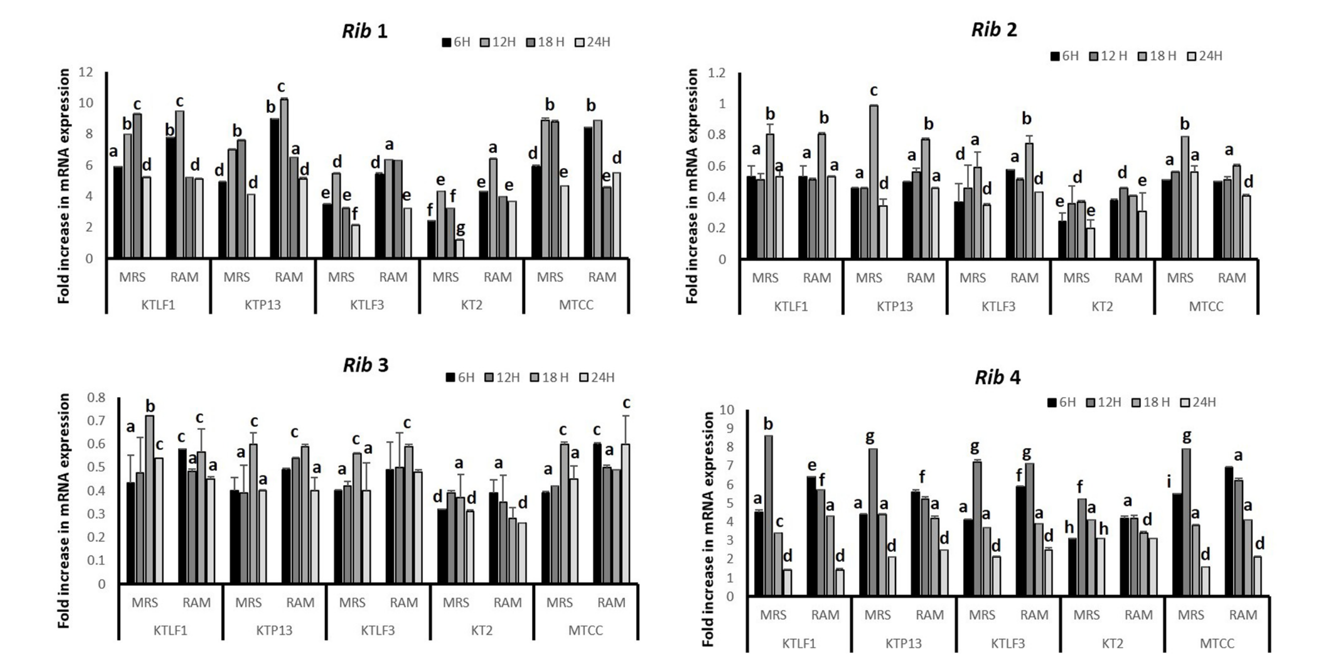

FIGURE 2 | Comparative mRNA expression profile (fold increase) of four test isolates with respect to reference culture (relative expression is considered as 1) in MRS and RAM. Values are mean ± SD ($n = 3$) and different letters denote the significant difference at $P < 0.05$ (abcd refer to statistical differences with respect to MRS and RAM. Strain MTCC8711 is taken as control.

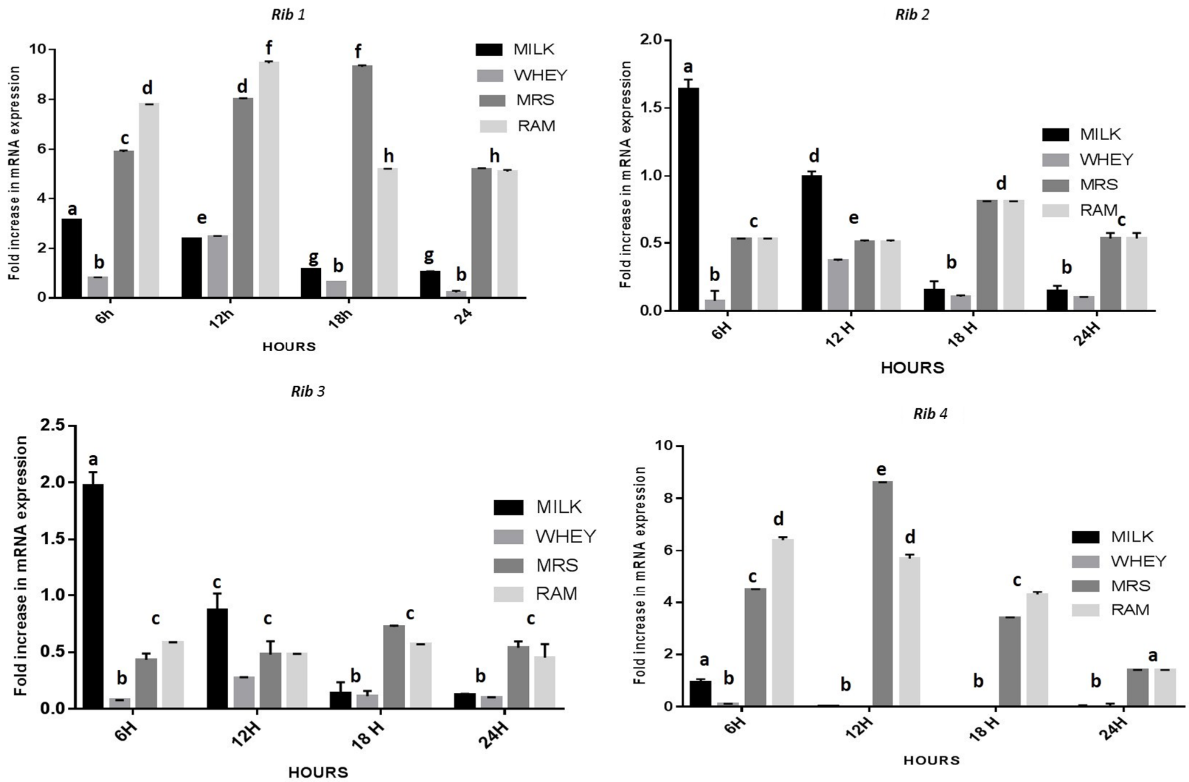

FIGURE 3 | Comparative mRNA expression profile (fold increase) of KTLF1 in MRS, RAM, Whey and Milk. Values are mean ± SD ($n = 3$) and different letters denote show the significant difference at $P < 0.05$.

study the effect of different parameters mentioned on riboflavin production, relative expression levels of the *Rib* 1, *Rib* 2, *Rib* 3, and *Rib* 4 genes were calculated in comparison with lowest riboflavin producing strain obtained from our previous study. The gene expression profiles for each strain grown under these conditions were different (**Figure 2**). Among the five tested strains, KT2 has shown significant variation in gene expression profile across the different incubation time intervals. The *Rib* 3 and *Rib*4 genes had a steady transcript level (fold change equal to one) in all the tested strains except in KT2 strain. The most significant difference was found at 6 and 24 h. This showed that expression of these genes was not much affected with the strain in two different media. Whereas, in KTLF1 (**Figure 3**), effect of media on different genes was significant in Milk and whey however, the relative expression levels of these genes was steady in MRS and RAM across different incubation. In particular, the expression levels of these genes were found to be lowered with increased incubation time in all the four media. In KTLF1, all the genes have shown significant variation in their expression in milk, Whey and MRS (**Figure 4**). In milk and whey, the fold increase was found in descending order with the increased incubation times, whereas, in whey media, except *Rib* 1, remaining three genes have shown steady expression over different incubation periods. In MRS and RAM, except *Rib* 1 and *Rib* 4, remaining two genes have shown significant change in expression profile.

Principal Component Analysis

The multivariate analysis was used for comparison of experimental data obtained for five lactobacilli for mRNA expression of four *Rib* genes (PCA). Two dimensional plots are drawn in **Figure 6**. In plot 1 (**Figure 6A**), contribution of the media with respect to variance is shown. The first two components present the total variance of 53.7%. The discrimination of isolates along PC1 is mainly due to RAM and Whey. Whereas along PC2, the isolate KT2 is discriminated vis-à-vis other isolates. In plot 2 (**Figure 6B**), the first two components present the total variance of 73.7%. The discrimination along samples along PC1 is mainly due to *Rib* 1 and *Rib* 4, whereas *Rib* 2 and *Rib* 3 are responsible for discrimination along PC2.

mRNA Expression of *Rib* Genes in Roseflavin Resistant Variant KTLF1 (4) and Riboflavin Overproduction

By following the procedure described by Burgess et al. (2006), KTLF1 was exposed to roseoflavin, a structural analog of riboflavin, which induces mutations in riboflavin-producing strains leading to a novel producer phenotype of the vitamin. Roseoflavin resistant variants were isolated and inoculated in the riboflavin-free medium and incubated for 16 h at 30°C. Both wild type and variant strains were re-inoculated in MRS, RAM, Milk and Whey based media at 37°C, 24 h and harvested

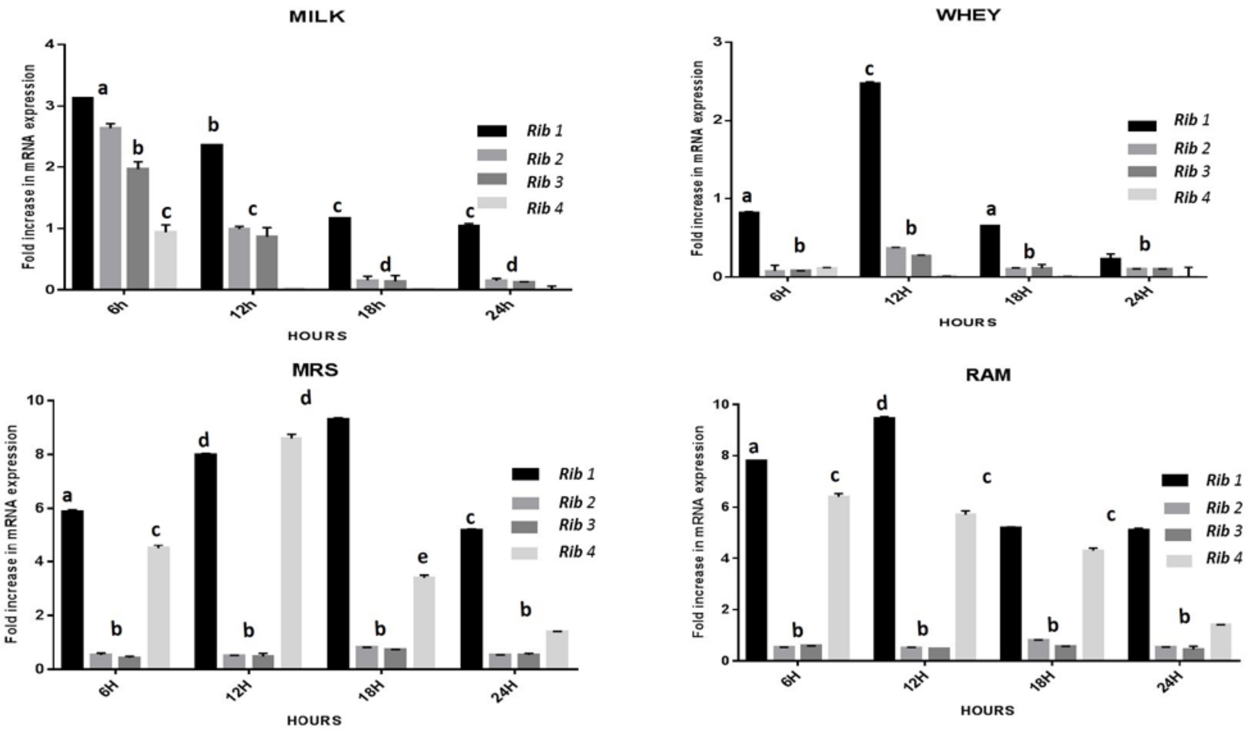

FIGURE 4 | Overall fold increase in relative mRNA expression of riboflavin structural genes in KTLF1 with respect to media and time intervals over control (MTCC8711). Values are mean ± SD ($n = 3$) and different letters denote show the significant difference at $P < 0.05$.

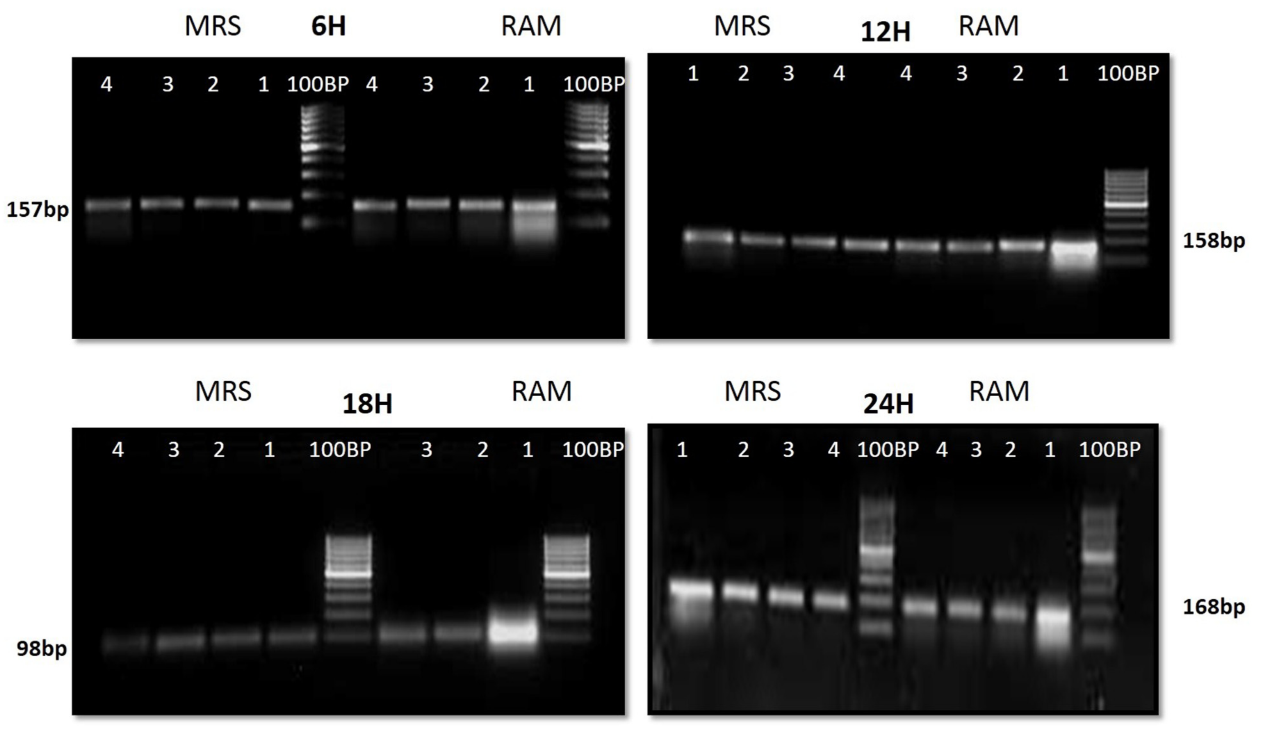

FIGURE 5 | 1.5% agrose gel electrophoresis to discriminate the band intensity after RT-PCR in KTLF1 where 1, 2, 3, and 4 denote *rib* 1, *rib* 2, *rib* 3, and *rib* 4 genes.

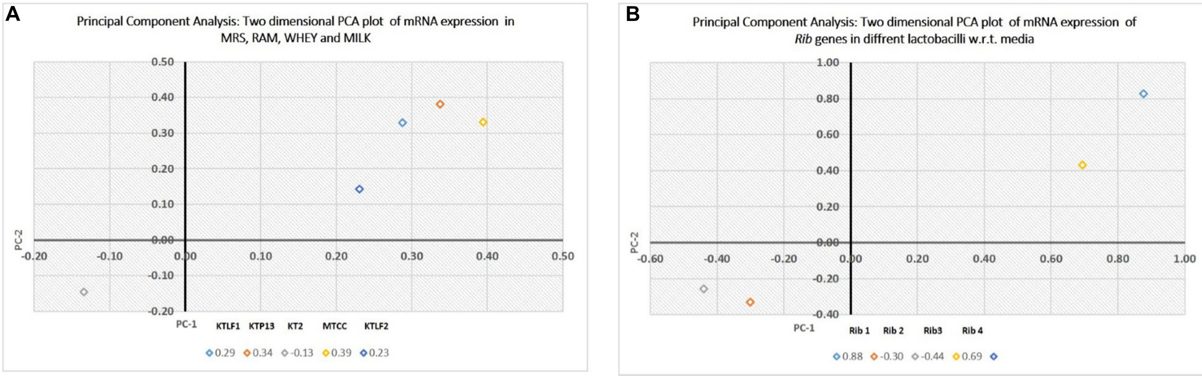

FIGURE 6 | (A) Principal component analysis (PCA), expressed as two dimensional plot with respect to media as variables for the first two principal components. **(B)** PCA, expressed as two dimensional plot with respect to *Rib* genes for the first two principal components.

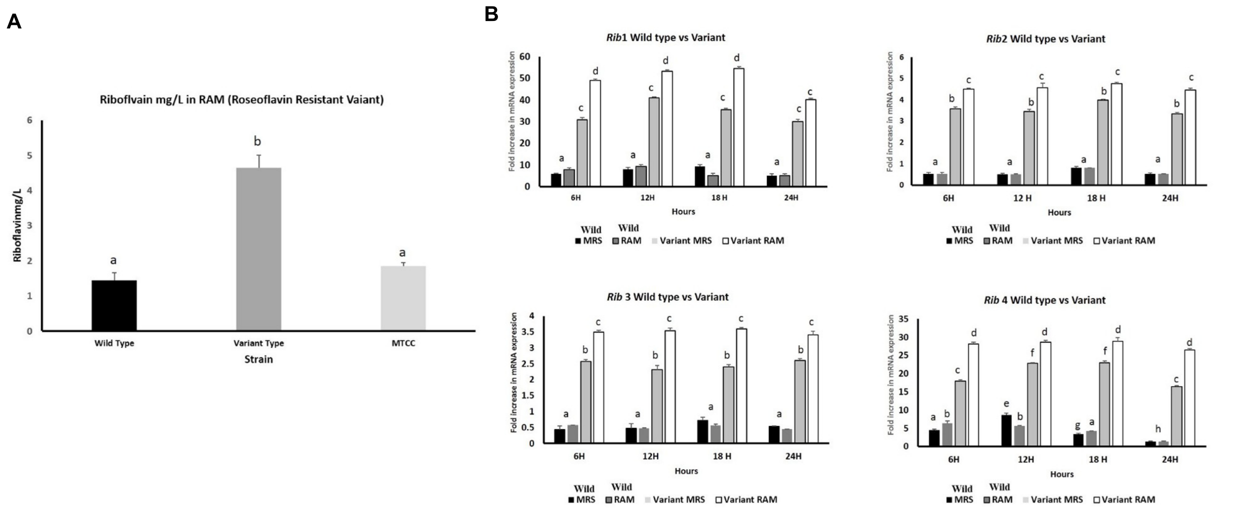

FIGURE 7 | (A) Riboflavin production by roseoflavin-resistant KTLF (4) and wild strain in RAM. The line above the bar represents the SD of the mean and different letters denote show the significant difference at $P < 0.05$. **(B)** Over expression of *Rib* genes after exposure to roseoflavin in KTLF (4). Values are mean ± SD ($n = 3$) and different letters denote show the significant difference at $P < 0.05$.

for RNA isolation and riboflavin production. From five isolated variants, only KTLF1 variant [KTLF1 (4)] was able to up regulate mRNA expression of *Rib* genes and the increase in riboflavin production was more than 3.5-fold (4.5 mg/L) as compared with wild type strain in a culture medium without riboflavin (**Figure 7A**). The spike in mRNA expression level was observed after the exposure of roseoflavin in all the four genes across the media at different times of incubation (**Figure 7B**). The maximum up regulation was observed in *Rib*1 followed by *Rib*4. The fold increase in expression was lower as to that of other wild type strains (**Figure 7B**).

DISCUSSION

Consumers are increasingly becoming conscious for their nutritional requirements, thus, vitamins produced *in situ* by microbes may suit their needs and expectations (LeBlanc et al., 2013; Thakur et al., 2015b). Since little information is available on what factors affect riboflavin production in LAB, the aim of this study was to investigate the influence of incubation time and difference strains on the expression of the rib genes by a wild type strains. Also, the vitamin production by the roseoflavin resistant strain under these conditions was also evaluated in mutant strain. As it is known that riboflavin operon is inducible, it is essential to evaluate the mRNA levels *rib* genes in different strains in different media (MRS, RAM, Milk and Whey) at different interval of growth time (6, 12, 18, and 24 h). The isolates used in this study are prospected from dairy, non- dairy sources (fermented bamboo shoots, human feces) (Thakur and Tomar, 2015a). The isolate KTLF3 was isolated from fermented bamboo shoots collected from Manipur, North East Region (Ethnic) of India. The riboflavin producing isolates used in this study are well-characterized by *in vitro* methods for their functional probiotic (Thakur and Tomar, 2015b) as well as technological properties (Thakur et al., 2016c). Arena et al. (2014) have also reported the probiotic lactobacilli for riboflavin production as well as its overproduction by using roseoflavin. In our study, riboflavin-producing strains were selected on the basis of mRNA expression of riboflavin biosynthesis genes. Out of four strains, three strains were able to show change in relative expression in the targeted genes with different incubation and media variables. Thus, KTLF1 was confirmed as prolific riboflavin producer and hence was selected to screen the mRNA profile of these genes in milk and whey based media. Milk and whey enriched in riboflavin have shown the increase in relative expression after the riboflavin in the media was utilized by the bacteria for its growth. The riboflavin may be required by bacteria in small amounts, but it constitutes a vital growth factor for *Enterococcus faecalis*, *Streptococcus pyogenes*, *Listeria monocytogenes,* and some lactobacilli (Koser, 1968). The biosynthetic deficiency correlates well with the absence of riboflavin in the growth media as the riboflavin operon is an inducible one where the quantity of riboflavin inhibits its production by bacteria. Unlike, other media, the RAM has shown higher expression levels which is in accordance with the aforementioned hypothesis.

KTLF1 was further selected to observe the overexpression of *Rib* genes after exposure to certain levels of roseoflavin. These results clearly indicated the roseoflavin exposure led to 3.5-fold increase in riboflavin production besides increase in the expression of all the rib genes by the mutant strain compared with those obtained with wild type strains, being more marked this difference at **Figures 7A,B**. The tolerance to the toxic roseoflavin signifies the mutations in the regulatory region of the rib operon which ultimately give rise to riboflavin overproducing phenotype. Till date, riboflavin overproduction is led either by employing genetic engineering (Burgess et al., 2004, 2006). The increase in expression levels of *Rib*1 and *Rib*4 followed by *Rib*2 and *Rib*3 is due to increased transcription of riboflavin biosynthesis genes because riboflavin metabolism and transport genes are being regulated at transcription attenuation. Further, threefold (4.5 mg/L) increase in riboflavin production in a culture medium without riboflavin was in agreement with the all these reports. Overexpression of all the four genes contributes to enhanced riboflavin production (Burgess et al., 2004) which was also observed in our study. Roseoflavin-resistant strains of *Leu. mesenteroides* over produced up to 0.5 mg l^{-1} of riboflavin, whereas riboflavin-overproducing *Lactobacillus plantarum* and *Propionibacterium freudenreichii* were able to synthesize up to around 0.6 and 3 mg l^{-1} respectively (Burgess et al., 2006). According to del Valle et al. (2014) roseoflavin resistant strains increased six times (1860 $\pm$ 20 ng/mL) the initial riboflavin levels of soy milk.

The PCA plots have well-discriminated the isolates on the basis of their levels of mRNA expression. The *Rib* genes are also put into two different groups due to their high and low expression with respect to variables. Overall, this study reveals that isolates showed variations for expressing their *Rib* genes which qualifies riboflavin production as strain specific attribute.

CONCLUSION

The expression profile as well as phenotypic production of riboflavin have revealed that both the genotypic and phenotypic traits are dependent on riboflavin in media used for growth. Though the genotypic as well as phenotypic expression of riboflavin in milk and whey is lower as compared to riboflavin free media but it is better to use riboflavin producing bacteria than riboflavin consuming ones. From this study, it is clear that milk and whey can be used for development of riboflavin enriched fermented products. To the best of our knowledge, the present study reports for the first time the mRNA expression profile of riboflavin biosynthesis genes in lactobacilli. Furthermore, exposure of roseoflavin led to over expression of *Rib* genes in the variant of KTLF1 as compared to wild strains facilitates the enhanced riboflavin content in the final product. Lactobacilli isolated from fermented bamboo shoots have shown highest riboflavin producing properties as well as displayed probiotic and appreciable techno-functional properties which can be further explored to develop functional bamboo shoot foods. The riboflavin enriched products could be introduced by using these isolates as starters to prevent or treat riboflavin deficiencies which are still to be addressed.

AUTHOR CONTRIBUTIONS

KT is the first author and has carried out the research work as a part of her Ph.D. program. The data analysis and manuscript writing was done by KT. ST has corrected the manuscript and helped in experimental work. Z-JW has helped revising the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00427/full#supplementary-material

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ORIGINAL RESEARCH
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Some Technological Properties of Lactic Acid Bacteria Isolated from *Dahi* and *Datshi*, Naturally Fermented Milk Products of Bhutan

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Dahi and *datshi* are common naturally fermented milk (NFM) products of Bhutan. Population of lactic acid bacteria (LAB) in *dahi* (pH 3.7) and *datshi* (pH 5.2) was 1.4×10^7 and 3.9×10^8 cfu/ml, respectively. Based on 16S rRNA gene sequencing isolates of LAB from *dahi* and *datshi* were identified as *Enterococcus faecalis, E. faecium, Lactococcus lactis* subsp. *lactis.* LAB strains were tested for some technological properties. All LAB strains except *E. faecalis* CH2:17 caused coagulation of milk at both 30°C for 48 h. Only *E. faecium* DH4:05 strain was resistant to pH 3. No significant difference ($P > 0.05$) of viable counts was observed in MRS broth with and without lysozyme. All LAB strains grew well in 0.3% bile showing their ability to tolerate bile salt. None of the LAB strains showed >70% hydrophobicity. This study, being the first of its microbiological analysis of the NFM of Bhutan, has opened up to an extent of research work that gives a new insight to the products.

Keywords: technological properties, lactic acid bacteria, *dahi, datshi*, naturally fermented milk products

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INTRODUCTION

Naturally fermented milk (NFM) products are prepared by the practice of one of the oldest techniques of milk fermentation known as the ‘back-sloping’ method in which a previous batch of a fermented product is used to inoculate the new batch (Josephsen and Jespersen, 2004; Tamang et al., 2016b). NFM products are prepared and consumed daily in Bhutan. Some NFM products of Bhutan are *dahi*, *datshi*, *mohi*, *gheu*, hard-*chhurpi* (*chugo/churkam*) and *hitpa*. *Dahi* (**Figure 1A**) is a yogurt-like NFM product of Bhutan, which is traditionally prepared by allowing the boiled milk to undergo spontaneous fermentation at room temperature for 2–3 days with the inoculation of the previous *dahi* sample. *Dahi* is drunk as a refreshing non-alcoholic beverage in Bhutan. *Datshi* (**Figure 1B**) is a cottage cheese like product, which is prepared by churning *dahi* for 10–15 min until a clumping product; butter (locally called *gheu*) is extracted. The butter is collected in another vessel and the buttermilk, locally called *mohi* is then heated for 15–20 min for the curdling of the product, called *datshi,* which is made into round small balls. It is consumed as curry in main meals in Bhutan. Most of these NFM products are occasionally used for religious ceremonies in Bhutan. Some people are economically dependent upon these NFM products where they sell at local markets. Some NFM products of other countries were well studied such as *dahi, misti dahi, shrikhand, chhu, chhurpi, philu* and *somar* of India, Nepal, Pakistan, and Bangladesh (Tamang et al., 2000; Dewan and Tamang, 2006, 2007; Harun-ur-Rashid et al., 2007; Sarkar, 2008; Patil et al., 2010; Tamang, 2010), *kurut* of China (Sun et al., 2010), *aaruul, airag, byasulag, chigee, tarag,* and *khoormog* of Mongolia (Watanabe et al., 2008; Takeda et al., 2011; Oki et al., 2014), *ergo* of Ethiopia, *lben, rayeb, zabady,* and *zeer* of Morocco and Northern African and Middle East

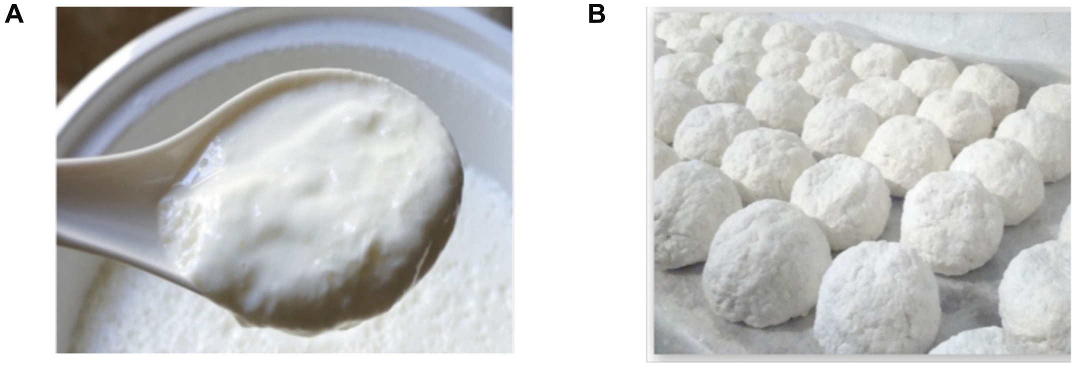

FIGURE 1 | (A) *Dahi* and **(B)** *datshi*.

countries, *rob* (from camel milk), *biruni*, *mish* (cow/camel milk) of Sudan, *amasi* (*hodzeko, mukaka wakakora*) of Zimbabwe, *nunu* of Ghana (Akabanda et al., 2013), *filmjölk* and *långfil* of Sweden (Mayo et al., 2010), and *koumiss* or *kumis* or *kumys* or *kymys* of the Caucasian area (Wu et al., 2009). Among species of lactic acid bacteria (LAB), *Lactococcus lactis* subsp. *cremoris,* and *Lc. lactis* subsp. *lactis* are the dominant microbiota along with other mesophilic lactobacilli (*Lactobacillus casei/Lb. paracasei, Lb. fermentum, Lb. helveticus*, *Lb. plantarum,* and/or *Lb. acidophilus*), *Enterococcus faecium,* species of *Leuconostoc* and *Pediococcus* in NFMs (Tamang et al., 2000, 2016b; Mathara et al., 2004; Dewan and Tamang, 2006, 2007; Patrignani et al., 2006; Watanabe et al., 2008; Wu et al., 2009; Hao et al., 2010; Yu et al., 2011; Akabanda et al., 2013; Oki et al., 2014). Technological properties including probiotics characters have been extensively studied in some NFM products of the world (Patrignani et al., 2006; Dewan and Tamang, 2007; Harun-ur-Rashid et al., 2007; Wu et al., 2009; Tamang et al., 2016a). Till date, there has been no report on the microbiological analysis and technological properties of the NFM from Bhutan, making this research the first of this kind. This paper is aimed to determine some technological properties of the LAB isolates from two popular NFM products of Bhutan- *dahi* and *datshi* such as acidification and coagulation, resistance to low pH, tolerance against bile, lysozyme tolerance and hydrophobicity assay, and also to isolate and identify LAB species by 16S rRNA sequencing.

MATERIALS AND METHODS

Samples

A total number of eight fresh samples of *dahi* (4) and *datshi* (4) were collected from Tabthangbu village, Bhutan in pre-sterilized sampling bags and were transported to the laboratory in an icebox carrier, stored at 4°C and analyzed within a week.

Microbiological Analysis

Samples (10 ml) were homogenized with sterile physiological saline (90 ml) in a stomacher lab-blender (400, Seward, London, UK) for 1 min, and were serially diluted in the same diluent. LAB were enumerated on MRS agar (M641, HiMedia, Mumbai, India) plates under anaerobic conditions in an anaerobic gas-pack system (LE002, HiMedia, Mumbai, India) and incubated at 30°C for 48–72 h (Dewan and Tamang, 2007). Colonies were selected randomly from the plates which contained less than 10 colonies, according to Leisner et al. (1997). Purity of the isolates was checked by streaking again and sub-culturing on fresh agar plates of the isolation media, followed by microscopic examinations. LAB isolates were preserved at −20°C in MRS broth (M369, HiMedia, Mumbai, India) mixed with 20% (v/v) glycerol.

Determination of pH

The pH of samples was determined using a pH meter (Crison basic 20, Barcelona, Spain) calibrated with standard buffers.

Phenotypic Characterization

Cell morphology of all isolates and their motility was determined using a phase contrast microscope (Olympus CH3-BH-PC, Japan). Isolates were Gram-stained and tested for catalase production, and were preliminarily identified based on the phenotypic properties including sugar fermentations, following the methods of Schillinger and Lücke (1987) and Dykes et al. (1994).

Molecular Identification

DNA Extraction

Based on similar sugar fermentation and other phenotypic characteristics criteria, six representative strains of LAB were randomly selected from 44 strains of LAB. Total genomic DNA of six representative strains of LAB was extracted from 2-ml samples of overnight cultures grown in MRS broth at 30°C according to the methods of Martín-Platero et al. (2007). DNA was quantified using fluorometer (Qubitol® 3.0, Fisher Scientific, USA).

16S rRNA Gene Sequencing

The 16S rRNA gene was amplified by PCR mixtures (25 μL) contained approximately 30–50 ng template DNA, 1 μM forward primer 27F and 1 μM reverse primer 1492R (Lane, 1991)

TABLE 1 | Phenotypic characteristics of the lactic acid bacteria (LAB) isolated from *dahi* and *datshi* of Bhutan.

Representative Isolates (no. of grouped strains)	Growth at 45°C	Sugar fermentation											Tentative genera
		Arabinose	Fructose	Galactose	Melibiose	Ribose	Xylose	Raffinose	Aesculin	Melezitose	Salicin	Rhamnose	
*DH4:05 (12)	10/2	7/5	+	+	+	9/3	−	+	+	−	−	−	*Enterococcus*
**CH1:14 (3)	+	+	+	+	+	2/1	2/1	+	+	+	+	−	*Enterococcus*
CH2:02 (10)	9/1	+	+	+	−	−	−	+	6/4	5/5	+	−	*Enterococcus*
CH2:17 (4)	2/2	+	−	+	3/1	−	+	2/2	+	+	+	−	*Enterococcus*
CH3:03 (7)	+	+	+	+	3/4	+	+	6/1	+	−	+	+	*Enterococcus*
CH4:01 (8)	6/2	+	−	+	4/4	−	−	−	+	−	−	+	*Lactococcus*

**DH, denotes isolates from dahi samples; **CH, denotes isolates from datshi samples. All strains were Gram-positive, catalase negative, coccoids, non-motile and non-sporing; +, all strains positive; -, all strains negative; (../..), number of positive/negative strains. All strains grew at 10 and 15°C. All strains fermented cellobiose, mannose and maltose.*

using a PCR Master Mix (Promega, Canada) performed under the standard PCR amplification procedure in a SimpliAmp^{TM} Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA). The PCR amplicons were checked for their purity on 1% agarose gel electrophoresis in the presence of ethidium bromide (10 mg/mL), which was later analyzed by the Gel Doc System (Ultra-Violet Products Ltd, UK). Sequencing service was outsourced.

Phylogenetic Analysis

The BLAST (Basic Phylogenetic Local Alignment Search Tool) program was used for comparing DNA databases for sequence similarities available in the NCBI database. Five different strains/species from each BLAST results were chosen for phylogenetic analysis using Molecular Evolutionary genetics Analysis software (MEGA version 6).

Technological Properties

Activation of LAB Strains

Enterococcus faecalis CH1:14, *E. faecalis* CH2:02, *E. faecalis* CH2:17, *E. durans* CH3:03, *Lactococcus lactis* subsp. *cremoris* CH4:01 and *E. faecium* DH4:05, isolated from *dahi* and *datshi*, were grown in MRS broth for 16-24 h at 30°C, and were used for determinations of acidification and coagulation, tolerance against bile, and lysozyme tolerance. Activation of LAB strains for resistance to pH 3 and hydrophobicity were mentioned below.

Acidification and Coagulation

Acidification and coagulation ability of LAB strains were assayed by inoculating 10% skim milk (RM1254, HiMedia, Mumbai, India) at 1% level and incubated at 30°C for 72 h. Observation was made for commencement of clotting, followed by pH measurement (Olasupo et al., 2001).

Tolerance against Bile

MRS broth containing 0.3% bile was inoculated with active cultures for 4 h (Prasad et al., 1998) and viable cells were enumerated in MRS agar plates after 24 h incubation and growth was recorded.

Lysozyme Tolerance

10 mL of MRS broth with lysozyme (MB098-1G, HiMedia, India) and without lysozyme, respectively, was inoculated with 1 mL of both culture suspensions of 10^8 cfu/ml cell concentration and incubated at 30°C for 24 h and viable cells were enumerated in MRS agar plates after 24 h incubation (Brennan et al., 1986).

Resistance to Low pH

Active cultures were harvested by centrifugation and pellets were washed once in phosphate-saline buffer (PBS, pH 7.2), re-suspended in PBS (pH 3) and incubated in MRS agar plates at 30°C for 24 h, and growth was recorded (Prasad et al., 1998).

Hydrophobicity Assay

Bacterial affinity to hydrocarbons was determined and results were expressed according to Perez et al. (1998), modified by Tamang et al. (2009) as follows. Fresh cultures were grown in MRS broth at 30°C for 24 h and centrifuged at 8,000 *g* for

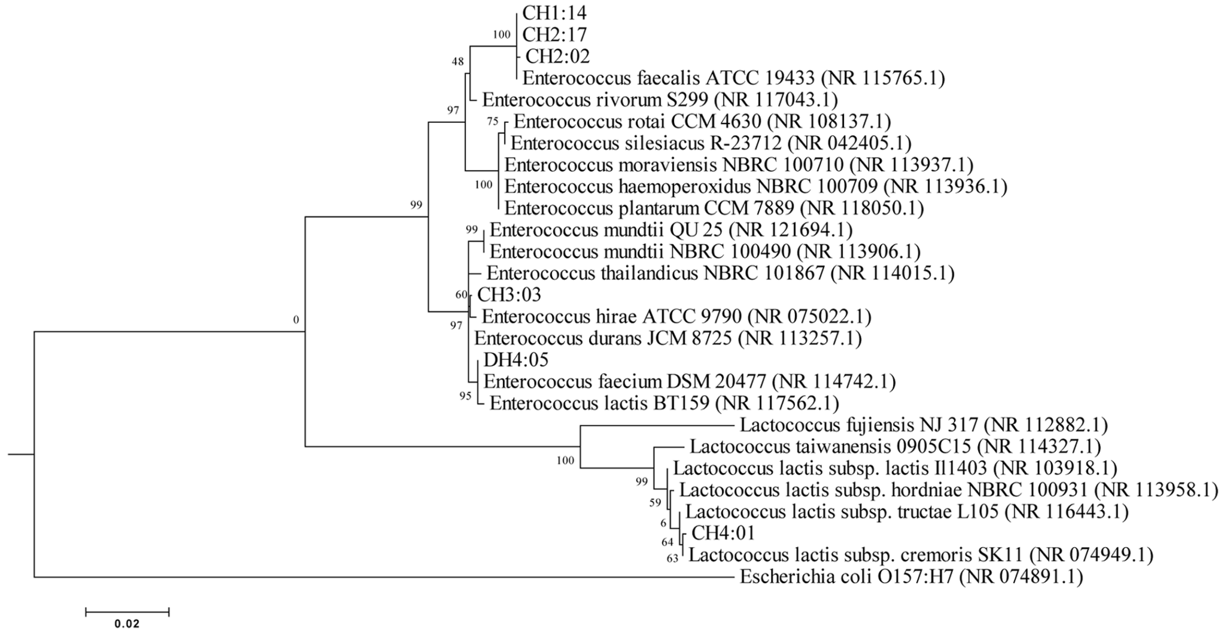

FIGURE 2 | Phylogenetic tree based upon the Neighbor-Joining of 16S rDNA sequences (*E. coli* 84 to 1437) derived by PCR with the primer 27F and 1492R.

TABLE 2 | Identification table based on NCBI-BLAST.

Isolates	Length (bp)	Max Score	Query coverage (%)	*E*-value	% Identification	Closest Known Relative (Strain No., GenBank Accession No.)
CH1:14	1406	2591	100	0.0	99	*Enterococcus faecalis* (ATCC 19433, NR 115765.1)
CH2:02	1370	2525	100	0.0	99	*Enterococcus faecalis* (ATCC 19433, NR 115765.1)
CH2:17	1386	2556	100	0.0	99	*Enterococcus faecalis* (ATCC 19433, NR 115765.1)
CH3:03	1384	2536	99	0.0	99	*Enterococcus durans* (JCM 8725, NR 113257.1)
CH4:01	1361	2508	100	0.0	99	*Lactococcus lactis* subsp. *cremoris* (SK11, NR 074949.1)
DH4:05	1378	2542	100	0.0	99	*Enterococcus faecium* (DSM 20477, NR 114742.1)

TABLE 3 | Technological properties of the LAB isolates from *dahi* and *datshi* of Bhutan.

Isolates	pH at Commencement of clotting	Coagulation (hours)		Resistance to pH 3	[a]Lysozyme tolerance	[b]Bile tolerance	(%) Hydrophobicity
		24	48				
E. faecium DH4:05	5.54	-	+	+	+	+	17.53
E. faecium CH1:14	5.24	-	+	-	+	+	56.58
E. faecalis CH2:02	5.52	-	+	-	+	+	8.91
E. faecalis CH2:17	5.50	-	-	-	+	+	5.99
E. faecium CH3:03	5.00	+	+	-	+	+	1.3
Lc. lactis subsp. *lactis* CH4:01	4.70	+	+	-	+	+	3.02

Data represent an average of three sets of experiments. +, indicates growth (>10^6 cfu/ml) of LAB strains; [a]no significant difference ($P > 0.05$) of viable LAB counts in MRS broth with and without lysozyme after incubation (30°C/24 h) was considered as a strain resistant to lysozyme.; [b]MRS broth with 0.3% bile.

5 min. The pellet was washed with 9 ml of Ringer solution (Merck, Germany) and thoroughly mixed. Suspension (1 ml) was taken and the absorbance at 580 nm was measured. Then, 1.5 ml of suspension was mixed with equal volume of n-hexadecane (RM 2238, HiMedia, Mumbai, India) in duplicates and mixed thoroughly. Phases were allowed to separate for 30 min at room temperature, after which aqueous phase was carefully transferred to a new tube and absorbance at 580 nm was measured. The percentage hydrophobicity was expressed as follows:

$$\text{hydrophobicity } \% = \left[A_0 - A/A\right] \times 100,$$

where A_0 and A are the absorbance values of the aqueous phase before and after contact with n-hexadecane.

RESULTS AND DISCUSSION

Dahi and *datshi* are acidic fermented milk products showing an average pH of 3.7 ± 0.17 and 5.2 ± 0.12, respectively. Isolation of LAB was performed on the classical media i.e., *Lactobacillus* MRS Agar media under anaerobic conditions at 30°C incubation for 48 h. The microbial load of LAB in *dahi* was 1.4×10^7 cfu/ml and in *datshi* was 3.9×10^8 cfu/mL, respectively. A total of 44 LAB isolates were isolated from *dahi* and *datshi* and phenotypically characterized and were randomly grouped into six representative strains based on similar sugar fermentation and other phenotypic characteristics (**Table 1**). These isolates were tentatively identified as *Enterococcus* and *Lactococcus* (**Table 1**).

Total genomic DNA of 6 representative strains of LAB was extracted and amplified and were identified by partial 16S rRNA gene sequencing which were compared to the NCBI database for their phylogenetic relationship by using the software MEGA 6 (**Figure 2**). On the basis of molecular identification, the following species of LAB were identified from *dahi* and *datshi* of Bhutan with percentage similarity of LAB: *E. faecalis* CH1:14 (99%), *E. faecalis* CH2:02 (99%), *E. faecalis* CH2:17 (99%), *E. durans* CH3:03 (99%), *Lactococcus lactis* subsp. *cremoris* CH4:01 (99%), and *E. faecium* DH4:05 (99%; **Table 2**).

Lactococcus lactis subsp. *lactis, Lc. lactis* subsp. *cremoris, E. faecium, E. faecalis, Leuconostoc mesenteroides* and *Pediococcus* and lactobacilli (*Lactobacillus casei, Lb. fermentum, Lb. helveticus, Lb. plantarum,* and/or *Lb. acidophilus*), were reported from many NFM products of different countries (Tamang et al., 2000; Mathara et al., 2004; Dewan and Tamang, 2006, 2007; Patrignani et al., 2006; Watanabe et al., 2008; Wu et al., 2009; Hao et al., 2010; Yu et al., 2011; Akabanda et al., 2013).

Lactic acid bacteria strains were tested for some technological properties (**Table 3**). All LAB strains except *E. faecalis* CH2:17 caused coagulation of milk at both 30°C for 48 h with a significant drop in pH (**Table 3**). Coagulation of milk by LAB strains reveals their potential as starters or adjunct cultures in the production of NFM of Bhutan. Only *E. faecium* DH4:05 strain showed positive result indicating its resistance to pH 3 in applied method (**Table 3**). Resistance to pH 3 is often used *in vitro* assays to determine the resistance to stomach pH (Prasad et al., 1998). Resistances to the lysozyme by all six strains of LAB were evaluated in MRS broth with and without lysosome at 30°C for 24 h (**Table 3**). Lysozyme is capable of lysing bacteria, but it doesn't impair activities of LAB (Saran et al., 2012). Tolerance against bile was also tested and found that all LAB strains grew well in 0.3% bile showing their ability to tolerate bile salt. The mean intestinal bile concentration is 0.3% (w/v) and the staying time of food in small intestine is suggested to be 4 h (Prasad et al., 1998). The probiotic bacteria survival in the gastrointestinal transit is primordial, and implies in the ability of microorganisms to survive at the stomach acidity and bile, so that they can exert their beneficial effects on the host (Pozza et al., 2011).

Bacterial affinity to hydrocarbons, such as hexadecane, proved to be a simple method to determine cell surface hydrophobicity (van Loosdrecht et al., 1987). None of the LAB strains showed >70% hydrophobicity (**Table 3**). A percent hydrophobic index greater than 70% was classified as hydrophobic (Nostro et al., 2004). Hence, LAB strains from *dahi* and *datshi* do not show hydrophobic character in the applied method. However, these limited technological properties are not enough to validate the potential probiotic uses of these isolates.

CONCLUSION

Based on 16S rRNA gene sequencing isolates of LAB, isolated from *dahi* and *datshi* of Bhutan, were identified as *E. faecalis, E. faecium, Lactococcus lactis* subsp. *lactis* and some strains showed promising technological properties. This is the first report on NFM of Bhutan, which may be used as baseline data for further research on NFM products.

AUTHOR CONTRIBUTIONS

HS: Molecular analysis of LAB isolates. SS: Isolation and phenotypic characterization. RR: Determination of technological properties of isolates. JT: Compilation of data and preparation of manuscript.

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Isolation, Identification and Characterization of Yeasts from Fermented Goat Milk of the Yaghnob Valley in Tajikistan

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The geographically isolated region of the Yaghnob Valley, Tajikistan, has allowed its inhabitants to maintain a unique culture and lifestyle. Their fermented goat milk constitutes one of the staple foods for the Yaghnob population, and is produced by backslopping, i.e., using the previous fermentation batch to inoculate the new one. This study addresses the yeast composition of the fermented milk, assessing genotypic, and phenotypic properties. The 52 isolates included in this study revealed small species diversity, belonging to *Kluyveromyces marxianus*, *Pichia fermentans*, *Saccharomyces cerevisiae*, and one *Kazachstania unispora*. The *K. marxianus* strains showed two different genotypes, one of which never described previously. The two genetically different groups also differed significantly in several phenotypic characteristics, such as tolerance toward high temperatures, low pH, and presence of acid. Microsatellite analysis of the *S. cerevisiae* strains from this study, compared to 350 previously described strains, attributed the Yaghnobi *S. cerevisiae* to two different ancestry origins, both distinct from the wine and beer strains, and similar to strains isolated from human and insects feces, suggesting a peculiar origin of these strains, and the existence of a gut reservoir for *S. cerevisiae*. Our work constitutes a foundation for strain selection for future applications as starter cultures in food fermentations. This work is the first ever on yeast diversity from fermented milk of the previously unexplored area of the Yaghnob Valley.

Keywords: yeast, fermented goat milk, Yaghnob Valley Tajikistan, identification, phenotyping, genotyping

INTRODUCTION

The history of fermented beverages and dairies dates back to more than 3500 years (Cavalieri et al., 2003) and possibly occurred with the first neolitic settlements, fermentation likely evolved to preserve crops and dairies as fermented foods, by creating an environment less favorable for spoilage microorganisms. In many rural areas, spontaneous food fermentations are still the main method for food processing, often using back-slopping to inoculate the new batch by transferring an aliquot of the previous food batch. This method allows for microbial adaptation and natural selection of strains thriving in the food matrix. There are several players involved in spontaneous fermentations, and previous studies have reported isolation of various yeasts and/or bacteria from natural fermentations of e.g., cereal based foods (Hellström et al., 2010; Ogunremi et al., 2015; Todorov and Holzapfel, 2015), or from various milk (Gadaga et al., 2001; Mathara et al., 2004; Bai et al., 2010; Yun Li and Guoqing, 2015), or cheese (Fasoli et al., 2015) fermentations. The analyses of the microbiota associated to spontaneous fermentations allows the isolation of microorganisms possessing properties desirable for implementation in industrial food or feed processes. Furthermore, the microbiota of a traditional food fermentation will likely also reflect the microbiota of the geographical area where it has been produced, as there is a continuous transfer of microbes between the close-by environment and the food fermentation. Those natural fermentations are conducted without pasteurization/sterilization of the substrate, and without applying particular hygienic protocols. Thus, selection of the environmental microbial population may occur only through the fermentative process, by chemico-physical modifications of the substrate induced by microbes themselves.

Both yeasts and bacteria are frequently isolated from fermentations (Tamang et al., 2016) and can possess traits that gives beneficial effects on the food product itself and for the consumer. Probiotic bacteria have been long studied, and lately also commercialized, as health promoting food ingredients, for example in some brands of yogurt (Sen et al., 2002). Recently the use of yeasts as probiotic agents in food has received increased attention. One example is the lactic yeast species *Kluyveromyces marxianus*, frequently isolated from dairy food fermentations. The strain *K. marxianus* B0399®, for example, was shown to have probiotic properties such as the modulation of the immune response in CaCo-2 cell line (Maccaferri et al., 2012) and further showed a positive effect on patients with irritable bowel syndrome (IBS) (Lisotti et al., 2013). Other studies on yeast strains with probiotic properties have investigated their lipolytic and proteolytic properties (Psomas et al., 2001) and the positive effects on the expression of pro-inflammatory cytokine IL-1α (van der Aa Kühle et al., 2005), as well as production of several vitamins, bioactive peptides, and more (Czerucka et al., 2007; Fernandez et al., 2015). Other beneficial effects of introducing selected yeast strains in food processes are for example the ability of such strains to metabolize lactose as a way of producing low lactose dairy products for lactose intolerant consumers (Gadaga et al., 2001; Mathara et al., 2004; Bai et al., 2010; Yun Li and Guoqing, 2015) and yeast strains acting as antagonists toward spoilage or pathogenic microorganisms (Mufandaedza et al., 2006) to mention a few examples. However, for a microorganism to be considered as a probiotic, the ability to survive/pass through the harsh conditions of the gastrointestinal tract (low pH), in presence of ox bile and at a temperature of 37°C with maintained viability is often applied as a first assessment.

The fermented milk of the Yaghnob Valley represents a precious resource for studying spontaneous fermentations for several reasons. First of all, it is one of the few still untapped traditional fermented productions yet to be investigated, hence both the yeast community and their phenotypic properties are unknown. As the use of health promoting microorganisms is of increasing interest, isolation, and phenotyping of strains from a previously unexplored fermented food may yield fruitful information of potentially new probiotic strains for future application in food industries. Further, isolation and identification of yeasts from this geographically unexplored area will add information to the body of knowledge on yeast species distribution and prevalence, and also about the genetic variations of strains evolved in an isolated area such as the Yaghnob Valley. The Yaghnob people are a Tajikistan ethnic minority living through their natural economy in areas remote from the "modern civilization" and avoiding exchanges with it. The long lasting isolation of this population has largely prevented mixing with other populations, thus preventing at the same time the eventual contamination of microbes among fermentative processes. This cultural-economic settings have thus prevented the flux of microorganisms supposed to have homogenized the worldwide populations of some fermentative microbes (Fay and Benavides, 2005).

The aim of this work was to investigate the yeast biodiversity of the Yaghnob populations traditionally fermented goat milk and to perform genotypic and phenotypic characterization of the isolated yeasts in order to contribute to the body of knowledge of yeasts in traditional food fermentations, and to add new information for a previously unexplored geographical area.

MATERIALS AND METHODS

Yeast Isolation

From original Yaghnob yogurt, isolations were done on different common agar lab media under aerobic conditions and at 30°C. Colonies were firstly selected based on colony morphology, aiming at selecting colonies of varying morphology, and thereafter additional colonies were randomly selected. Five isolates were obtained from M17 medium (annotated AL 1-5), seven isolates on deMan, Rosa and Sharp (MRS) medium (annotated CL 1-7), 14 isolates from MRS pH 5.4 medium (annotated DL 1-12), 12 isolates on Wallerstein Laboratory (WL) medium (annotated BL3-14), and two isolates on Yeast extract, Peptone, Dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% dextrose) (annotated BL1-2). The yogurt was further maintained in-house by regular backslopping into pasteurized cow milk. Isolation from in-house maintained yogurt was done on YPD agar supplemented with chloramphenicol (100 μg/ml) (YPD+Cam). The original sample had been maintained in-house by repeated backslopping according to the procedure by the Yaghnob population, but using pasteurized cow milk instead of goat milk, for a total time period of 3 years. Twelve colonies of varying morphology were selected from the maintained sample, annotated TJY50-61. Purity was checked by streaking all isolates on YPD agar and pure cultures were maintained on agar of the same medium at 4°C for short term storage, and in YPD broth supplemented with glycerol (15% v/v) at −80°C for long term storage.

Genotypic Characterization

ITS1-4 Sequencing

Yeast genomic DNA was extracted from isolated colonies as previously described (Hoffman and Winston, 1987). Strains were identified by amplification and sequencing of the ribosomal Internal Transcribed Spacer (ITS) region, using ITS1 (5′-GTTTCCGTAGGTGAACTTGC-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers, as previously described (Sebastiani et al., 2002). Species attribution was obtained by using the Basic Logarithmic Alignment Search Tool (BLAST) algorithm in the National Centre for Biotechnology Information (NCBI) database (minimum 97% sequence similarity and 95% coverage). All ITS1-4 sequences were submitted to GenBank and the accession numbers are presented in **Table 1**. Multiple alignments were performed using online tool ClustalW2.

PCR-RFLP Analysis

Restriction fragment length polymorphism (RFLP) analyses of the amplified ITS1-4 region were performed as described by Esteve-Zarzoso et al. (1999), using *HaeIII* or *HinfI*.

Microsatellite Analysis

In this work, microsatellite analysis was performed only for *Saccharomyces cerevisiae* isolates. The genomic DNA was extracted by phenol-chloroform-isoamyl alcohol method to be used for $(GTG)_5$ Rep PCR. The PCR mixture consisted of 1.25 μL buffer (10x), 1 μL $MgCl_2$ (25 mM), 2.5 μL dNTP (5 mM), 0.4 μL forward primers (10 mM), 0.4 μL reverse primer (10 mM), 0.05 μL AmpliTaq Gold® DNA polymerase (5 U/μL), 4.4 μL H_2O and 2.5 μL DNA template (10 ng/μL). The investigated loci were C3, C4, C5, C6, C8, C11, SCYOR267c, YKL172w, SCAAT1, SCAAT3, SCAAT5, and YPL3 (Legras et al., 2005). The PCR program consisted of an initial step at 95°C for 5 min, followed by 35 cycles of 95°C for 0.5 min, 57°C for 2 min, and 72°C for 1 min, before a final elongation step at 60°C for 30 min. Thereafter samples were cooled down to 8°C until further use. The PCR products were checked by gel electrophoresis. The chord distances (Dc) were calculated among each couple of strains with a laboratory-made R script. The phylogenetic tree was obtained from the distance matrices with the Phylip Neighbor 3.67 package and drawn up using Figtree. The tree was rooted using the midpoint method.

Strains ancestry was estimated by using the model-based program Structure (Pritchard et al., 2000). $K = 7$ was chosen as the most representative of the population structure for the microsatellite sequences. The results of 10 independent Structure chains were combined with CLUMPP (Jakobsson and Rosenberg, 2007).

Phenotypic Characterization

Phytate Utilization

The strains from the Yaghnob yogurt were screened for their ability to degrade phytate in a nutrient deficient medium, consisting of phytate (3 g/L) and glucose (20 g/L) in succinate buffer at pH 5.5. A volume of 195 μL of the medium was dispensed in each well of a micro plate, and inoculated in duplicate using 5 μL from overnight precultures in YPD. Incubation was done at 30°C with 150 rpm orbital shaking for 48 h. After 48 h of incubation, 22 μL of 5 M HCl was added to each well to stop the phytate degradation. Cells were allowed to sediment, and thereafter 150 μL cell-free sample was mixed with 200 μL of 0.5 M HCl before analyzing the phytate (IP_6) concentration by High Pressure Ion Chromatography (HPIC). The HPIC analysis method has been previously described by Carlsson et al. (2001).

The isolates were further assessed for their ability to release extracellular non-cell-bound phytase to the surrounding medium. Inoculations were done in 4 mL volumes of Yeast Nitrogen Base plus Yeast Extract (YNB+YE) (6.5 g/L YNB w/o phosphate, 10 g/L yeast extract and 20 g/L glucose in succinate buffer at pH 5.5) to a starting optical density at 600 nm (OD_{600}) of about 0.1. The YNB+YE medium has previously shown to trigger release of phytase enzymes to the surrounding medium (Hellström et al., 2015). The incubation was performed for 24 h at 30°C with stirring. After incubation, cells were pelleted by centrifugation at 5000 × g, and the cell-free supernatant was used for assay of phytase activity as previously described (Qvirist et al., 2015). The assay samples were analyzed for IP_6 concentration using HPIC as previously described (Carlsson et al., 2001), and compared with the phytase positive reference strain *Pichia kudriavzevii* TY13 from previous work (Qvirist et al., 2015).

Growth on Different Carbon Sources, pH, Temperatures, and Ox Bile Concentrations

To investigate the strains ability to grow on different carbon sources, 6.7 g/L YNB without carbon source (with amino acids) in succinate buffer (pH 5.5) was supplemented with 20 g/L of one of the following carbon sources; glucose, sucrose, lactose, maltose, mannitol, arabinose, xylose, and galactose. The strains were also tested for growth in 8 different media based on 1% yeast extract, 2% peptone; supplemented with either glucose at 50 or 60% (w/v), or ethanol at 1, 6, or 12% or lactic acid at 1, 6, or 12% (v/v). All isolates were also investigated for their ability to grow in YPD broth at different temperatures (4, 27, 37, 40, 42, 46, and 48°C), at different pH (4.8, 3, and 2), and at different levels of added ox bile (0.5, 1, and 2% v/v).

Cultures were done for each strain by adding 5 μL preculture (from overnight incubation in YPD) into 195 μL of the experimental media, giving a starting OD (630 nm) between 0.08–0.1. Incubations were done at 150 rpm orbital shaking for 3 days at 30°C for all tests except the pH and ox bile tests which were done at 37°C. For strain TJY51, 27°C was used due to its poor growth at higher temperatures. The optical density was read at 630 nm, and values below 0.2 are considered as negative, from 0.2 to 0.4 as positive but inhibited growth and above 0.4 as positive growth.

Further, the viability of strains after incubation at (i) 48°C in YPD broth for 24 h, and (ii) in YPD broth of pH 2 for 2 h at 30°C was investigated. To assess the viability, 10 μL of the cell suspensions were spotted in duplicates onto YPD agar, together with a negative control from cultivation in normal YPD at 30°C. The YPD agar plates were incubated at 30°C overnight and then visual evaluation of the growth was done.

All tests were conducted in triplicates.

Invasiveness of Isolates

All isolates were investigated for invasiveness on YPD agar in triplicates. Volumes of 2.5 μL liquid yeast suspensions were

TABLE 1 | The 52 isolates and their respectively species identity, isolation medium, fermentation origin, and the GenBank accession number is indicated in the table.

Isolate	Species	Isolation medium	Fermentation sample	GenBank accession number
AL1	*Kluyveromyces marxianus*	M17	Original	KX905245
AL2	*Kluyveromyces marxianus*	M17	Original	KX905246
AL3	*Kluyveromyces marxianus*	M17	Original	KX905247
AL4	*Kluyveromyces marxianus*	M17	Original	KX905248
AL5	*Kluyveromyces marxianus*	M17	Original	KX905249
BL1	*Kluyveromyces marxianus*	YPD	Original	KX905250
BL3	*Kluyveromyces marxianus*	WL	Original	KX905251
BL4	*Kluyveromyces marxianus*	WL	Original	KX905252
BL5	*Kluyveromyces marxianus*	WL	Original	KX905253
BL6	*Kluyveromyces marxianus*	WL	Original	KX905254
BL7	*Kluyveromyces marxianus*	WL	Original	KX905255
BL8	*Kluyveromyces marxianus*	WL	Original	KX905256
BL12	*Kluyveromyces marxianus*	WL	Original	KX905257
BL13	*Kluyveromyces marxianus*	WL	Original	KX905258
BL14	*Kluyveromyces marxianus*	WL	Original	KX905259
CL5	*Kluyveromyces marxianus*	MRS	Original	KX905260
CL6	*Kluyveromyces marxianus*	MRS	Original	KX905261
DL2	*Kluyveromyces marxianus*	MRS pH 5.4	Original	KX905262
DL4	*Kluyveromyces marxianus*	MRS pH 5.4	Original	KX905263
DL5	*Kluyveromyces marxianus*	MRS pH 5.4	Original	KX905264
DL6	*Kluyveromyces marxianus*	MRS pH 5.4	Original	KX905265
DL10a	*Kluyveromyces marxianus*	MRS pH 5.4	Original	KX905266
DL10b	*Kluyveromyces marxianus*	MRS pH 5.4	Original	KX905267
DL11	*Kluyveromyces marxianus*	MRS pH 5.4	Original	KX905268
DL12	*Kluyveromyces marxianus*	MRS pH 5.4	Original	KX905269
TJY52	*Kluyveromyces marxianus*	YPD+Cam	Maintained	KX905270
TJY54	*Kluyveromyces marxianus*	YPD+Cam	Maintained	KX905271
TJY59	*Kluyveromyces marxianus*	YPD+Cam	Maintained	KX905272
TJY60	*Kluyveromyces marxianus*	YPD+Cam	Maintained	KX905273
BL9	*Saccharomyces cerevisiae*	WL	Original	KX905274
BL10	*Saccharomyces cerevisiae*	WL	Original	KX905275
BL11	*Saccharomyces cerevisiae*	WL	Original	KX905276
CL2	*Saccharomyces cerevisiae*	MRS	Original	KX905277
CL3	*Saccharomyces cerevisiae*	MRS	Original	KX905278
CL4	*Saccharomyces cerevisiae*	MRS	Original	KX905279
DL3	*Saccharomyces cerevisiae*	MRS pH 5.4	Original	KX905280
DL7	*Saccharomyces cerevisiae*	MRS pH 5.4	Original	KX905281
TJY58	*Saccharomyces cerevisiae*	YPD+Cam	Maintained	KX905282
TJY61	*Saccharomyces cerevisiae*	YPD+Cam	Maintained	KX905283
BL2	*Pichia fermentans*	YPD	Original	KX905284
CL1	*Pichia fermentans*	MRS	Original	KX905285
CL7	*Pichia fermentans*	MRS	Original	KX905286
DL1	*Pichia fermentans*	MRS pH 5.4	Original	KX905287
DL8a	*Pichia fermentans*	MRS pH 5.4	Original	KX905288
DL8b	*Pichia fermentans*	MRS pH 5.4	Original	KX905289
DL9	*Pichia fermentans*	MRS pH 5.4	Original	KX905290
TJY50	*Pichia fermentans*	YPD+Cam	Maintained	KX905291
TJY53	*Pichia fermentans*	YPD+Cam	Maintained	KX905292
TJY55	*Pichia fermentans*	YPD+Cam	Maintained	KX905293
TJY56	*Pichia fermentans*	YPD+Cam	Maintained	KX905294
TJY57	*Pichia fermentans*	YPD+Cam	Maintained	KX905295
TJY51	*Kazachstania unispora*	YPD+Cam	Maintained	KX905296

spotted onto the surface of YPD agar and incubated at 27°C for 5 days. Thereafter cells were removed and plates were carefully washed with deionized water before being stained as described by Vopálenská et al. (2005). The invasiveness was graded from 0 (not invasive) to 4 (highly invasive).

Resistance toward Oxidative Stress

All isolates were investigated for resistance toward oxidative stress. Cell suspensions from each strain was spread on YPD agar and allowed to absorb, thereafter a paper disk soaked in hydrogen peroxide (H_2O_2) was placed in the center of the agar plate. The resistance toward the oxidative stress was determined by measuring the radius from the border of the growing yeast to the H_2O_2-disk after 2 days of incubation at 27°C.

Hyphae Formation

To investigate the isolates ability to produce hyphae and pseudo hyphae, 5 μL preculture was inoculated into 195 μL of YPD, YNB (without carbon source or ammonium sulfate) and RPMI (Roswell Memorial Park Institute) media, and incubated at 27 and 37°C (only 37°C was used for RPMI) for a total of 7 days, with microscopic investigation at 2 and 7 days.

Antifungal Tolerance

The antifungal tests were carried out according to the Eucast protocol (Eucast, 2012) with minor adaptations. Selected strains were cultivated in YPD based medium containing the antifungals fluconazole (32–128 mg/L), clotrimazole (0.06–0.5 mg/L) or amphotericin B (0.06–0.5 mg/L) respectively to determine their minimum inhibitory concentration (MIC). The strains used were *Pichia fermentans* CL1 and BL2, *Kluyveromyces marxianus* BL3, BL8, DL4, and TJY52, *S. cerevisiae* CL2 and BL9, and the *Kazachstania unispora* TJY51. Precultures were prepared overnight and the biomass was then washed and resuspended in sterile saline before inoculation into a final volume of 200 μL in the test plates, yielding a starting concentration of about $0.5–2.5*10^5$ CFU/mL. Positive controls were made by inoculation into YPD without antifungal drug, and negative controls were made by using the test media without inoculation. Incubations were done in duplicates at 30°C with 170 rpm in micro well plates. After 24 h of incubation, microbial growth was evaluated by optical density at 530 nm by using a spectrophotometer (Multiskan EX, Thermo Scientific). The MIC was defined as the lowest concentration in absence of visible growth and confirmed by OD analysis. OD data above 0.2 was considered as positive growth, while for wells having growth below OD 0.2, re-inoculation was done and the plates were incubated for another 24 h to ensure the result as true negative.

Statistical Analyses

The growth data from the phenotypic characterizations were subjected to statistical evaluation. For each strain, the mean value of duplicate cultures were used. Principal component analysis (PCA) was performed on the OD measurement after standardization (zero mean, unit deviation), and permANOVA (using the vegan R package Jari Oksanen et al., 2015) for statistical analysis. For the two genotypes within the *K. marxianus* species, Wilcoxon rank-sum tests were performed using the stats R package (version 3.1.2).

RESULTS

Yeast Strain Identification

A total of 52 strains were isolated from either original (40 isolates) or maintained (12 isolates) Yaghnob yoghurt. The isolated yeasts belonged to the species *Kluyveromyces marxianus* (29 isolates), *S. cerevisiae* (10 isolates), *P. fermentans* (12 isolates), and *K. unispora* (1 isolate) (**Table 1**). Strain characterization was firstly assessed by PCR-RFLP analysis after digestion of the amplified ITS1-4 region using the enzymes *HinfI* or *HaeIII* (**Table 2**).

The PCR-RFLP analysis revealed that within the *K. marxianus* species there are two groups corresponding to different band patterns after digestion with *HinfI* (**Figure 1**). All the *K. marxianus* isolates have bands length at 240, 185, and 80 bp, but only 12 out of the 29 strains show the frequently reported *K. marxianus* profile (Esteve-Zarzoso et al., 1999; Bockelmann et al., 2008; Pham et al., 2011) with a band at 120 bp (from now on referred to as Group I), while the other 17 strains show a larger band, approximately of 140 bp (from now on referred to Group II).

To further assess the genetic differences between Group I and Group II of *K. marxianus* isolates, the ITS1-4 sequences were aligned. The alignment revealed that the two groups are separated by having a G (Group I) or an A (Group II) in one of the nucleotide positions marked in **Figure 2**. To note, all the *K. marxianus* strains isolated in MRS (pH 5.4) medium possess the A allele (8 strains, DL series), whereas the strains isolated on YPD medium both before and after yogurt in-house maintenance bore the G allele (5 strains, TJY series and strain BL1). This indicates that the two *K. marxianus* sub-populations are characterized genetically by two alleles in the ITS1-5.8S-ITS2 region. The combination of genetic and phenotypic differences between the two groups of *K. marxianus* strains may indicate a substantial genomic difference, possibly influencing different phenotypic traits such as tolerance to different environmental (chemico-physical) characteristics.

Microsatellites

Among all the yeast species involved in fermentative processes coupled to food and beverage production, a particular interest has been given to the budding yeast *S. cerevisiae*, known to be the principal player in wine, beer and bread fermentations. We thus analyzed the microsatellite profiles of our *S. cerevisiae* isolates from the Yaghnob fermentation together with the microsatellite data obtained from 350 *S. cerevisiae* strains isolated worldwide from a vast plethora of sources. The phylogenetic analysis (**Figure 3**) revealed that the *S. cerevisiae* strains found in the Yaghnob yogurt cluster apart from the worldwide strains. The Yaghnob strains clustered close to strains isolated from a wide variety of sources, most interestingly insect intestines (red), human feces (blue), bread fermentations (yellow), and wild sources such as tree barks or soils (brown). It is noteworthy that the Yaghnobi strains appear isolated from the wine strains. In

TABLE 2 | Sizes in base pairs (bp) of PCR products from alpifications fo the ITS1-4 region after restriction digestion using enzymes *Hae*III and *Hin*fI respectively for each strain.

Species	Strain	Restriction fragments (bp)[a]	
		***Hae*III**	***Hin*fI**
Kluyveromyces marxianus	AL1	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	AL2	655, 80	240, 185, 120, 80
Kluyveromyces marxianus	AL3	655, 80	240, 185, 120, 80
Kluyveromyces marxianus	AL4	655, 80	240, 185, 120, 80
Kluyveromyces marxianus	AL5	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	BL1	655, 80	240, 185, 120, 80
Kluyveromyces marxianus	BL3	655, 80	240, 185, 120, 80
Kluyveromyces marxianus	BL4	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	BL5	655, 80	240, 185, 120, 80
Kluyveromyces marxianus	BL6	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	BL7	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	BL8	655, 80	240, 185, 120, 80
Kluyveromyces marxianus	BL12	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	BL13	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	BL14	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	CL5	655, 80	240, 185, 120, 80
Kluyveromyces marxianus	CL6	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	DL2	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	DL4	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	DL5	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	DL6	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	DL10a	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	DL10b	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	DL11	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	DL12	655, 80	240, 185, 140, 80
Kluyveromyces marxianus	TJY52	655, 80	240, 185, 120, 80
Kluyveromyces marxianus	TJY54	655, 80	240, 185, 120, 80
Kluyveromyces marxianus	TJY59	655, 80	240, 185, 120, 80
Kluyveromyces marxianus	TJY60	655, 80	240, 185, 120, 80
Saccharomyces cerevisiae	BL9	320, 230, 180, 150	365, 155
Saccharomyces cerevisiae	BL10	320, 230, 180, 150	365, 155
Saccharomyces cerevisiae	BL11	320, 230, 180, 150	365, 155
Saccharomyces cerevisiae	CL2	320, 230, 180, 150	365, 155
Saccharomyces cerevisiae	CL3	320, 230, 180, 150	365, 155
Saccharomyces cerevisiae	CL4	320, 230, 180, 150	365, 155
Saccharomyces cerevisiae	DL3	320, 230, 180, 150	365, 155
Saccharomyces cerevisiae	DL7	320, 230, 180, 150	365, 155
Saccharomyces cerevisiae	TJY58	320, 230, 180, 150	365, 155
Saccharomyces cerevisiae	TJY61	320, 230, 180, 150	365, 155
Pichia fermentans	BL2	340, 80	250, 200
Pichia fermentans	CL1	340, 80	250, 200
Pichia fermentans	CL7	340, 80	250, 200
Pichia fermentans	DL1	340, 80	250, 200
Pichia fermentans	DL8a	340, 80	250, 200
Pichia fermentans	DL8b	340, 80	250, 200
Pichia fermentans	DL9	340, 80	250, 200
Pichia fermentans	TJY50	340, 80	250, 200
Pichia fermentans	TJY53	340, 80	250, 200

(Continued)

TABLE 2 | Continued

Species	Strain	Restriction fragments (bp)[a]	
		*Hae*III	*Hin*fI
Pichia fermentans	TJY55	340, 80	250, 200
Pichia fermentans	TJY56	340, 80	250, 200
Pichia fermentans	TJY57	340, 80	250, 200
Kazachstania unispora	TJY51	550, 150	370

[a]*Fragments smaller than 80 bp could not be distinguished, but probably bands exists also at 80 and 65 bp for K. marxianus after digestion with HinfI, and at 30 bp for P. fermentans after digestions with HaeIII, as reported by Esteve-Zarzoso et al. (1999).*

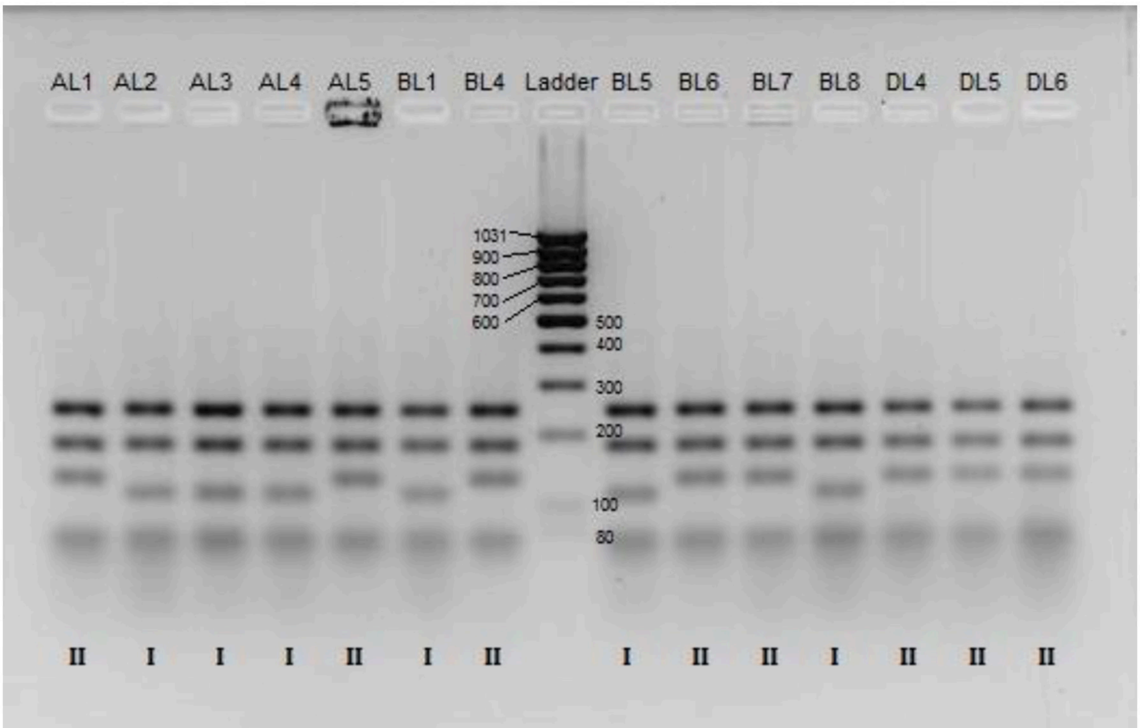

FIGURE 1 | RFLP patterns for a selected set of *K. marxianus* strains after digestion of the ITS1-4 region by *HinfI* and separation on agarose gel. The lanes contain, from left to right, samples of strain; AL1, AL2, AL3, AL4, AL5, BL1, BL4, Low Range DNA ladder, BL5, BL6, BL7, BL8, DL4, DL5, and DL6. The genotypic groups, Group I or Group II, is indicated for each strain below each respectively lane.

previous studies, several of these strains were shown to have a mosaic genome as a common feature (Legras et al., 2005).

The mosaic nature of the genome of these strains was also confirmed by means of ancestry analysis. The analysis revealed that the *S. cerevisiae* strains isolated from Yaghnob yogurt fell in two groups, both having mosaic ancestry (**Figure 3**), but originating from different sets of ancestors. Both groups were inferred to descend from a common ancestor (orange), from which directly originated a set of strains isolated from human feces (blue strains, i.e., YP4_40D, YA5-28C). The larger ancestry group contained the strains CL3, CL4, DL3, BL9, BL10, TJY58, and TJY61, originating from an ancestor (red) shared with strains isolated from wild sources, and from a third ancestor (light green) shared with the meiotic segregants of a strain isolated from the intestine of social wasps (F31x). The second ancestry group consisted of the strains CL2, BL11, and DL7 originating from two of the three ancestors inferred for the other group (orange and red).

Furthermore, we did not identify any genotypic differences among the *S. cerevisiae* strains isolated with different isolation media, as we did for the *K. marxianus* strains. This could be ascribed to the fact that the *S. cerevisiae* strains were not affected by the same selective pressures as *K. marxianus*.

Phenotypic Characterization

The results of phenotypic characterizations are shown in **Figure 4** for growth in media based on different carbon sources or growth at different cultivation temperatures, and in **Figure 5**

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AL3_fw_A10.ab1       TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
TJY60_fw_B11.ab1     TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
TJY59_fw_B10.ab1     TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
BL5_fw_D07.ab1       TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
CL5_fw_A05.ab1       TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
CL6_fw_A06.ab1       TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
AL5_fw_A12.ab1       TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
AL1_fw_A08.ab1       TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
AL2_fw_A09.ab1       TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
AL4_fw_A11.ab1       TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
BL8_fw_D10.ab1       TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
DL10A_fw_C11.ab1     TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 119
DL10B_fw_C12.ab1     TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
BL6_fw_D08.ab1       TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 116
BL4_fw_D06.ab1       TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
BL7_fw_D09.ab1       TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
DL6_fw_C06.ab1       TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 119
DL5_fw_C05.ab1       TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 118
BL14_fw_E04.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 116
TJY54_fw_B05.ab1     TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 119
DL11_fw_D01.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 114
DL12_fw_D02.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 113
DL4_fw_C04.ab1       TGCGCGGC-AATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 112
TJY52_fw_B03.ab1     TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 113
BL1_fw_D03.ab1       TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 115
BL3_fw_D05.ab1       TGCGCGGCCAGTTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 117
DL2_fw_C02.ab1       TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 112
BL13_fw_E03.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 114
BL12_fw_E02.ab1      TGCGCGGCCAATTCTTGATTCTCTGCTATCAGTTTTCTATTTCTCATCCTAAACACAATG 115
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FIGURE 2 | Multiple sequence alignment of the ITS1-4 sequences from *K. marxianus* strains. The location of nucleotide variation is indicated by the box. The two groups of *K. marxianus* are marked by bold text (Group I) or normal text (Group II).

for growth in presence of ox bile, at low pH, in presence of ethanol or lactic acid and in osmotic stress inducing media.

The *K. marxianus* strains showed remarkably broad substrate utilization and high tolerance to elevated incubation temperatures. Comparison between the two genotype groups I and II were also done and is presented in **Figure 7**.

The *S. cerevisiae* strains grew well up to 37°C, and two strains (BL9 and BL10) grew even at 46°C. All strains except BL11 and TJY61 also grew well at pH 3. Our data show that all strains could utilize glucose, galactose and to some extent also lactose. All strains except BL11 and TJY58 also grew on maltose, and all strains except BL11 and DL7 showed some growth on sucrose. Strains TJY58 and TJY61 seemed able to grow in the mannitol and the xylose based medium as well. All strains could grow in ethanol at 6%, and three strains (DL7, TJY58, and CL2) showed positive but impaired growth at 12% concentration, and only one strain (TJY61) grew well also at 12%. All strains showed high resistance to osmotic stress.

Within the *P. fermentans* species all strains grew well at 37°C, and could utilize all carbon sources tested, with exception of strain DL1 (being negative for arabinose and xylose). Large variations in pH tolerance was observed in this species. The growth in the ethanol and lactic acid media were high within this species, having 8 strains growing at 12% lactic acid and 6 strains growing at 12% ethanol.

The *K. unispora* strain appears rather fastidious and showed to be sensitive to most stresses tested, except for the osmotic stress (induced by 60% glucose) where it together with the genetically close species *S. cerevisiae* show high growth.

It should be pointed out that all strains in this study showed fully recovered growth after incubation at pH 2 for 2 h, which indicates that they could survive through the stomach passage to the intestinal tract.

The data obtained from the phenotypic characterizations presented in **Figures 4**, **5** were further used for a PCA where the isolates belonging to the three species, *K. marxianus*, *P. fermentans,* and *S. cerevisiae* could be clustered separately ($p < 0.001$), showing also that *K. unispora* phenotypically cluster together with the *S. cerevisiae* strains (**Figure 6**). In addition, the two genetically different groups within the *K. marxianus* isolates clustered in function of their phenotypic traits, revealing that the two genetically different groups also are phenotypically different.

Further investigation of the strains among the *K. marxianus* species, revealed that there are significant differences between the two genetically different groups ($p < 0.05$, Wilcoxon rank sum test) (**Figure 7**).

Group I (G-nucleotide group) showed significantly better growth at elevated temperatures (42 and 46°C), at pH 3, on xylose and on phytic acid than Group II (A-nucleotide group). Group I also showed remarkably higher growth in presence of lactic acid, using a medium of yeast extract (1%), peptone (2%) and

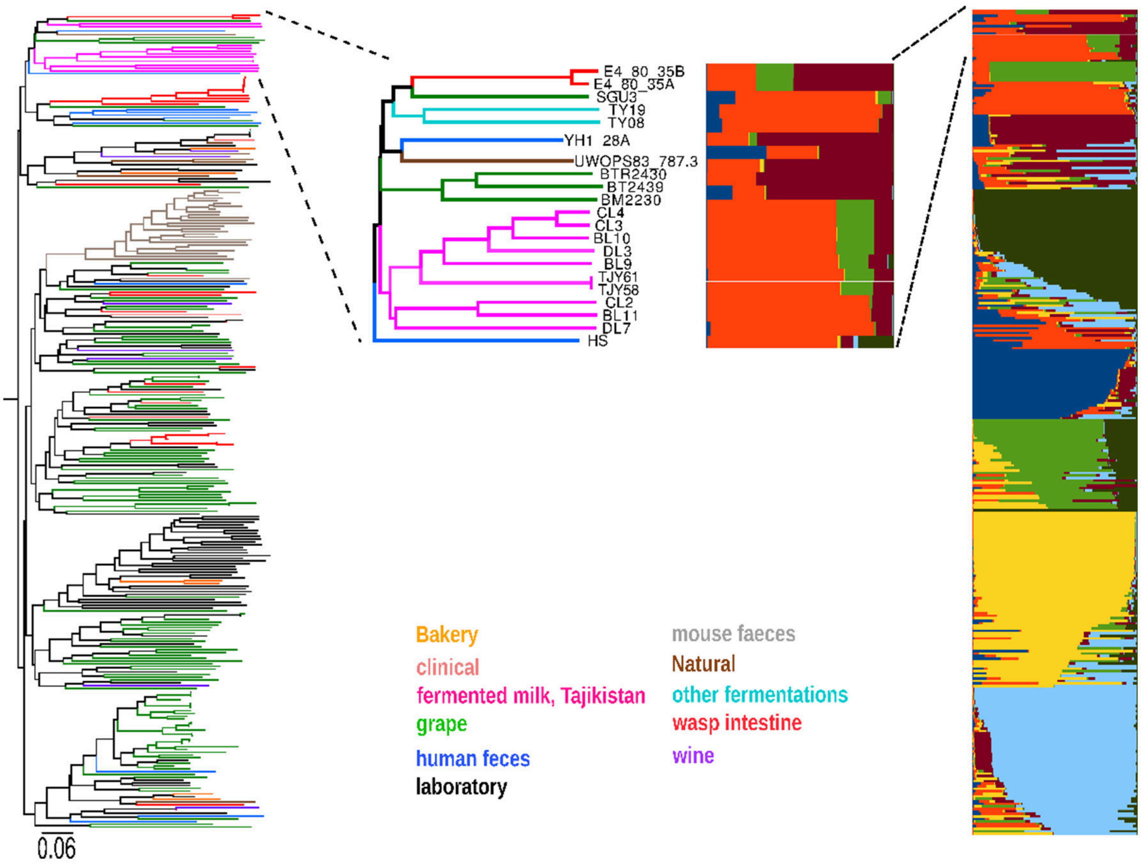

FIGURE 3 | The *Saccharomyces cerevisiae* strains isolated in the Yaghnob yoghurt (in pink color) were compared to a set of around 350 previous *S. cerevisiae* isolates from various worldwide origins (left part of figure), revealing that the Yaghnob strains cluster separately and apart from the previous isolates. The ancestry analysis of those strains (right part of figure) further show that the *S. cerevisiae* from the Yaghnob yoghurt work are all of mosaic ancestry, and constitute two genetically different groups, originating from two ancestors (red and orange) or three ancestors (red, orange, and light green).

lactic acid at 1 and 6%. Group I showed higher tolerance toward osmotic stress compared to Group II. Group II on the other hand showed stronger growth on the sucrose and lactose based media, compared to Group I. Among the other two species (*S. cerevisiae* and *P. fermentans*) there were no significant differences in phenotypes found.

All isolates were also investigated for (i) invasiveness on YPD agar, (ii) resistance toward oxidative stress induced by H_2O_2 and (iii) formation of hyphae in different media (**Table 3**). The *K. unispora* showed low resistance toward oxidative stress, no invasiveness and no formation of hyphae. Within the three other species there were several strains (31% of *K. marxianus* strains, 40% of *S. cerevisiae* strains, and 33% of *P. fermentans* strains) showing hyphae formation in at least one of the tested media. The invasiveness was generally low in *K. marxianus* with only 10% of strains showing higher grading than 1 in invasiveness. The strains of the *S. cerevisiae* species were especially interesting by having either no invasiveness (70% of isolates) or very high invasiveness (30% of isolates). One strain of *S. cerevisiae* (CL2) and three strains of *K. marxianus* (AL1, AL4, CL5) showed high resistance toward hydrogen peroxide by having a distance of 5 mm or lower from the H_2O_2 disk and growth boarder.

The screening for phytate degradation after 48 h of incubation in a nutrient deficient medium revealed that only few isolates were able to degrade phytate under this condition, in particular isolates AL3 (43% IP_6 degraded), BL8 (30% IP_6 degraded), and BL1 (29% IP_6 degraded). Isolates AL1, BL3, BL6, BL7, BL12, BL13, CL6, DL2, DL5, DL6, DL10b, DL11, and DL12 showed between 15 and 20% IP_6 degradation. The remaining isolates showed no detectable levels of IP_6 degradation. The reference strain *Pichia kudriavzevii* TY13 (Hellström et al., 2015) showed 93% IP_6 degradation in this condition. The analysis of the isolates ability to release extracellular non-cell-bound phytase in an YNB+YE medium revealed no phytase activity in the supernatant from any of the investigated strains under tested conditions, phytase activity was however seen in the supernatant of the positive control references strain.

Selected strains were then subjected to determination of minimum inhibitory concentration (MIC) of selected antifungal agents (**Table 4**).

The antifungal tolerance was varying between both species and strains. *P. fermentans* BL2 and *K. unispora* TJY51 showed resistance toward fluconazole (up to 128 mg/L), and *K. marxianus* DL4 showed resistance toward amphotericin B (up

	Glucose	Lactose	Galactose	Maltose	Arabinose	Sucrose	Mannitol	Xylose	IP_6	27°C	37°C	40°C	42°C	46°C
AL2*	0.57	0.51	0.73	0.65	0.42	0.43	0.76	0.67	0.73	1.52	1.54	1.47	1.33	1.05
AL3*	0.37	0.42	0.82	0.59	0.61	0.32	0.82	0.99	0.78	1.35	1.53	1.48	1.28	1.38
AL4*	0.35	0.65	0.76	0.40	0.62	0.28	0.76	0.87	0.72	1.54	1.52	1.50	1.32	1.45
BL1*	0.38	0.39	0.80	0.86	0.42	0.35	0.88	0.97	0.80	1.46	0.92	0.23	0.15	0.12
BL3*	0.26	0.65	0.84	0.38	0.84	0.27	0.74	1.01	0.97	1.46	1.49	0.30	0.08	0.15
BL5*	0.31	0.37	0.74	0.38	0.39	0.37	0.40	0.47	0.95	1.51	1.52	1.49	1.27	1.22
BL8*	0.35	0.42	0.84	0.57	0.52	0.44	0.24	0.76	0.99	1.50	1.57	1.48	1.38	1.24
CL5*	0.49	0.69	0.79	0.55	0.34	0.23	0.76	0.77	0.81	1.56	1.56	1.40	1.22	0.21
TJY52*	0.31	0.32	0.76	0.40	0.72	0.28	0.82	0.85	0.87	1.49	1.48	1.47	1.34	1.50
TJY54*	0.30	0.34	0.78	0.56	0.67	0.29	0.88	0.87	0.96	1.55	1.47	1.47	1.33	1.53
TJY59*	0.28	0.37	0.75	0.20	0.28	0.13	0.30	0.28	0.39	1.40	1.50	1.49	1.35	1.33
TJY60*	0.44	0.22	1.11	0.18	0.07	0.09	0.24	0.19	0.50	1.53	1.44	1.43	1.25	1.20
AL1	1.14	0.68	0.88	0.69	0.35	1.05	0.40	0.41	0.73	1.48	1.48	1.45	1.29	0.21
AL5	0.48	0.70	0.76	0.56	0.33	0.71	0.51	0.42	0.75	1.47	1.41	1.48	1.19	0.23
BL4	0.76	0.72	0.79	0.42	0.25	0.69	0.34	0.34	0.86	1.45	1.45	1.20	0.10	0.10
BL6	0.63	0.56	0.79	0.66	0.42	0.38	0.69	0.46	0.91	1.46	1.46	1.46	1.00	0.09
BL7	0.71	0.67	0.84	0.35	0.29	0.65	0.37	0.47	0.49	1.32	1.30	0.14	0.14	0.05
BL12	0.97	0.93	0.73	0.62	0.21	1.03	0.65	0.39	0.48	1.50	1.51	1.47	1.19	0.83
BL13	0.25	0.93	0.74	0.69	0.20	1.02	0.65	0.45	0.48	1.54	1.37	1.44	1.08	0.17
BL14	1.18	0.83	0.82	0.52	0.97	0.91	0.89	0.97	0.23	1.42	1.37	1.40	0.98	0.14
CL6	0.61	0.65	0.85	0.44	0.28	0.66	0.38	0.46	0.65	1.54	1.53	1.29	1.11	0.17
DL2	0.33	0.72	0.75	0.64	0.12	1.00	0.59	0.35	0.52	1.41	1.36	1.16	0.82	0.15
DL4	0.23	0.84	0.78	0.58	0.11	0.98	0.38	0.32	0.54	1.46	1.45	1.50	0.84	0.15
DL5	0.59	0.87	0.74	0.57	0.19	1.04	0.39	0.40	0.62	1.42	1.39	1.47	1.06	0.15
DL6	0.95	0.75	0.71	0.37	0.24	0.21	0.39	0.30	0.43	1.52	1.36	1.38	0.86	0.51
DL10A	0.24	0.65	0.80	0.47	0.63	0.70	0.29	0.34	0.66	1.24	1.43	1.37	1.16	0.08
DL10B	0.24	0.63	0.73	0.35	0.74	0.59	0.30	0.48	0.63	1.27	1.54	1.41	0.91	0.13
DL11	0.39	0.65	0.78	0.25	0.25	0.62	0.63	0.60	0.63	1.35	1.50	1.46	1.19	0.18
DL12	0.71	0.45	0.74	0.27	0.40	0.45	0.34	0.47	0.53	1.46	1.48	1.47	1.10	0.13
BL2	0.57	0.71	0.94	0.76	0.29	0.72	0.67	0.95	0.78	1.30	1.47	0.79	0.13	0.12
CL1	1.01	0.99	1.18	0.87	0.53	0.93	0.73	0.99	0.84	1.46	1.44	1.45	1.20	1.36
CL7	1.18	0.70	0.91	1.01	0.34	0.57	0.64	1.04	0.83	1.34	1.59	1.33	1.01	0.09
DL1	0.11	0.32	0.25	0.63	0.12	0.70	0.39	0.55	0.55	1.42	1.57	1.41	0.94	0.08
DL8A	1.05	0.83	1.09	0.92	0.29	0.52	0.72	1.04	1.08	1.41	0.77	0.12	0.12	0.10
DL8B	0.63	0.70	0.84	0.91	0.24	0.61	0.68	1.00	1.04	1.37	0.74	0.12	0.11	0.10
DL9	0.94	0.79	1.09	0.77	0.35	0.56	0.55	0.69	1.05	1.36	1.34	0.15	0.19	0.09
TJY50	1.09	0.82	1.00	0.78	0.37	0.57	0.74	0.85	0.86	1.38	0.17	0.14	0.12	0.05
TJY53	1.12	0.72	0.92	0.69	0.30	0.77	0.71	0.87	0.78	1.43	0.29	0.13	0.11	0.10
TJY55	0.47	0.57	1.00	0.50	0.33	0.53	0.55	0.62	0.96	1.35	1.24	0.12	0.13	0.10
TJY56	0.51	0.56	0.69	0.51	0.34	0.51	0.58	0.62	1.02	1.45	1.14	0.12	0.12	0.09
TJY57	0.95	0.73	0.82	0.62	0.32	0.64	0.56	0.77	0.83	1.35	1.27	0.11	0.12	0.15
BL9	0.47	0.53	0.72	0.63	0.06	0.48	0.17	0.14	0.09	1.54	1.54	1.23	0.96	0.25
BL10	0.40	0.47	0.74	0.43	0.06	0.34	0.12	0.10	0.09	1.51	1.51	1.46	1.29	1.47
BL11	0.12	0.46	0.77	0.17	0.07	0.12	0.13	0.10	0.10	1.56	0.59	0.21	0.13	0.09
CL2	0.17	0.38	0.75	0.22	0.08	0.20	0.10	0.12	0.09	1.51	1.26	0.62	0.10	0.10
CL3	0.34	0.28	0.75	0.35	0.07	0.30	0.10	0.10	0.08	1.53	1.18	0.58	0.10	0.10
CL4	0.38	0.31	0.79	0.33	0.08	0.38	0.13	0.14	0.09	1.53	1.40	1.03	0.18	0.10
DL3	0.25	0.49	0.74	0.48	0.07	0.24	0.10	0.11	0.08	1.52	1.43	0.65	0.11	0.10
DL7	0.09	0.25	0.71	0.26	0.08	0.08	0.09	0.11	0.09	1.33	1.33	0.45	0.13	0.13
TJY58	0.52	0.28	0.86	0.13	0.09	0.20	0.28	0.28	0.09	1.77	1.49	0.50	0.16	0.11
TJY61	0.66	0.55	0.76	0.23	0.09	0.41	0.79	0.31	0.08	1.03	0.91	0.87	0.23	0.09
TJY51	0.63	0.20	0.87	0.19	0.11	0.24	0.09	0.11	0.09	1.44	0.17	0.10	0.10	0.12

FIGURE 4 | Growth data after 3 days of incubation, measured as optical density (at 630 nm) for each strain when grown on different carbon sources and at different cultivation temperatures. Growth below 0.2 is considered as negative (red), growth between about 0.2–0.4 is considered as positive but repressed (yellow) and above circa 0.4 is positive growth (green). *Indicates the *K. marxianus* strains belonging to genotype group I.

	Ox bile 0.5%	Ox bile 1%	Ox bile 2%	pH 2	pH 3	pH 4.8	EtOH 1%	EtOH 6%	EtOH 12%	Lact.acid 1%	Lact.acid 6%	Lact.acid 12%	Glucose 50%	Glucose 60%
AL2*	1.56	1.52	1.36	0.12	1.78	1.10	0.42	1.43	0.13	1.19	1.94	0.09	1.07	0.69
AL3*	1.60	1.50	1.47	0.09	1.78	1.14	0.43	1.13	0.08	1.27	1.75	0.11	1.36	1.04
AL4*	1.59	1.64	1.60	0.08	1.76	1.03	0.46	1.44	0.19	1.19	1.64	0.12	1.34	0.86
BL1*	0.60	0.67	0.54	0.07	0.54	1.17	0.47	1.25	0.15	1.33	1.85	0.11	1.38	1.01
BL3*	0.78	0.47	0.52	0.06	1.18	0.76	0.45	1.23	0.08	1.23	1.55	0.16	1.06	0.46
BL5*	1.64	1.69	1.56	0.09	1.81	1.05	0.40	1.33	0.20	1.04	1.87	0.13	1.12	0.45
BL8*	1.60	1.29	1.14	0.08	1.92	1.45	0.54	1.22	0.08	1.38	1.47	0.11	1.13	0.11
CL5*	1.55	1.50	1.34	0.10	1.68	0.98	0.53	1.54	0.06	1.26	1.91	0.06	1.09	0.57
TJY52*	1.61	0.96	0.81	0.07	1.81	1.06	0.49	1.51	0.06	1.28	1.79	0.14	1.43	0.97
TJY54*	1.64	1.61	1.44	0.06	1.82	1.18	0.49	1.49	0.08	1.23	1.71	0.23	1.43	1.15
TJY59*	1.68	1.59	1.45	0.14	1.84	1.10	0.49	0.78	0.15	1.29	1.89	0.22	1.18	1.35
TJY60*	1.59	1.51	1.38	0.12	1.85	0.68	1.78	0.99	0.26	2.10	1.96	0.45	1.18	1.69
AL1	1.26	1.26	0.95	0.10	1.32	1.19	0.68	1.18	0.11	0.83	1.11	0.13	0.56	0.10
AL5	1.59	1.52	1.37	0.12	1.41	1.08	0.59	1.13	0.21	0.73	1.35	0.12	1.08	0.51
BL4	0.99	0.14	0.09	0.10	1.15	0.87	0.52	1.19	0.16	0.58	1.04	0.13	0.40	0.22
BL6	1.46	1.38	1.26	0.10	1.39	0.98	0.54	1.21	0.14	0.64	1.18	0.10	0.95	0.29
BL7	0.55	0.44	0.56	0.06	1.63	1.07	0.63	1.25	0.19	0.81	0.72	0.15	0.39	0.11
BL12	1.53	1.45	1.53	0.11	1.47	0.94	0.57	1.08	0.08	0.61	1.06	0.09	1.16	0.93
BL13	1.59	1.45	1.29	0.07	1.22	0.95	0.56	1.06	0.14	0.66	1.12	0.08	1.25	0.90
BL14	1.59	1.36	1.23	0.07	1.21	0.77	0.41	0.79	0.10	0.49	0.68	0.08	0.36	0.65
CL6	1.33	1.17	0.97	0.10	1.49	1.01	0.56	0.87	0.08	0.68	0.25	0.08	0.44	0.21
DL2	1.37	1.36	1.26	0.07	1.18	0.42	0.52	0.99	0.09	0.59	0.85	0.06	1.09	0.92
DL4	1.51	1.42	1.25	0.09	1.04	1.01	0.48	0.99	0.11	0.53	0.56	0.07	0.73	0.69
DL5	1.56	1.53	1.29	0.07	1.30	1.01	0.52	1.07	0.08	0.61	1.04	0.10	1.20	0.96
DL6	1.58	1.51	1.31	0.07	1.23	0.95	0.47	0.96	0.14	0.49	0.66	0.08	0.84	0.13
DL10A	1.20	0.96	0.08	0.07	1.62	0.82	0.56	0.84	0.06	0.69	0.91	0.11	0.06	0.05
DL10B	1.44	1.38	1.33	0.08	1.43	1.18	0.52	1.04	0.12	0.51	0.82	0.06	0.09	0.14
DL11	1.58	1.54	1.36	0.09	1.46	1.12	0.65	1.19	0.08	0.74	0.79	0.06	1.05	0.46
DL12	1.49	1.49	1.29	0.07	1.24	1.02	0.56	1.06	0.12	0.61	0.93	0.05	0.19	0.17
BL2	0.30	0.14	0.13	0.06	0.94	0.82	1.86	2.22	0.35	1.09	0.67	0.44	0.06	0.09
CL1	1.51	1.33	1.29	0.08	1.70	0.97	1.22	2.32	0.72	1.65	0.93	0.67	0.05	0.19
CL7	1.24	0.95	0.52	0.09	1.52	0.98	1.65	1.97	0.28	1.47	2.10	0.14	0.76	0.10
DL1	1.02	0.78	0.70	0.06	1.19	0.92	0.39	1.59	0.13	0.65	1.43	0.18	0.65	0.12
DL8A	0.59	0.57	0.56	0.06	0.86	0.82	1.70	2.10	0.53	1.54	1.82	0.29	0.05	0.14
DL8B	0.53	0.62	0.45	0.06	0.77	0.94	1.27	2.03	0.30	1.05	1.91	0.12	0.05	0.15
DL9	0.61	0.98	0.51	0.07	0.57	0.23	1.67	1.76	0.06	1.41	2.00	0.06	0.06	0.06
TJY50	0.41	0.24	0.14	0.04	0.08	0.05	1.39	2.08	0.09	1.21	1.99	0.28	0.06	1.10
TJY53	0.84	0.72	0.50	0.05	0.62	0.16	1.34	2.17	0.55	1.19	1.97	0.20	0.05	0.17
TJY55	0.52	0.48	0.47	0.05	0.29	0.15	1.55	2.10	0.49	1.50	1.82	0.33	0.79	0.15
TJY56	0.41	0.51	0.58	0.05	0.86	0.06	1.50	1.94	0.18	1.08	1.82	0.12	0.28	0.10
TJY57	0.58	0.56	0.77	0.05	0.56	0.20	1.55	1.48	0.06	1.43	1.94	0.05	0.11	0.07
BL9	1.40	1.42	1.23	0.10	1.32	0.88	0.84	1.36	0.11	0.72	0.05	0.11	1.42	1.30
BL10	1.62	1.55	1.56	0.11	1.60	1.03	1.13	1.61	0.13	1.68	0.05	0.18	1.38	1.25
BL11	0.50	1.08	0.50	0.07	0.14	0.17	0.34	1.32	0.10	0.76	0.05	0.12	1.48	1.19
CL2	1.04	1.08	0.98	0.11	0.86	0.60	0.31	1.39	0.18	0.64	0.15	0.22	1.46	1.40
CL3	1.17	1.17	0.79	0.06	1.07	0.73	0.29	1.73	0.23	0.54	0.08	0.18	1.32	1.33
CL4	1.04	0.30	0.15	0.06	1.15	0.82	0.68	1.79	0.19	0.51	0.07	0.18	1.48	1.27
DL3	1.15	1.22	1.21	0.09	0.93	0.99	1.14	1.69	0.06	0.79	0.20	0.18	1.35	1.27
DL7	0.93	1.13	0.63	0.08	0.77	0.68	0.55	1.63	0.18	0.41	0.22	0.28	1.48	1.75
TJY58	0.10	0.11	0.12	0.20	1.37	0.81	0.40	0.71	0.23	0.66	0.15	0.13	1.32	1.14
TJY61	0.07	0.10	0.10	0.07	0.36	0.04	0.30	1.10	0.30	0.51	0.26	0.17	1.33	1.25
TJY51	0.13	0.15	0.40	0.09	0.22	0.25	0.31	0.39	0.06	0.32	0.29	0.05	1.34	1.23

FIGURE 5 | Growth data after 3 days of incubation, measured as optical density (at 630 nm) for each strain when grown in cultivation media containing either ox bile, ethanol, or lactic acid, or with modified pH, or in high-glucose media to induce osmotic stress respectively. Growth below 0.2 is considered as negative (red), growth between about 0.2–0.4 is considered as positive but repressed (yellow) and above circa 0.4 is positive growth (green). *Indicates the *K. marxianus* strains belonging to genotype group I.

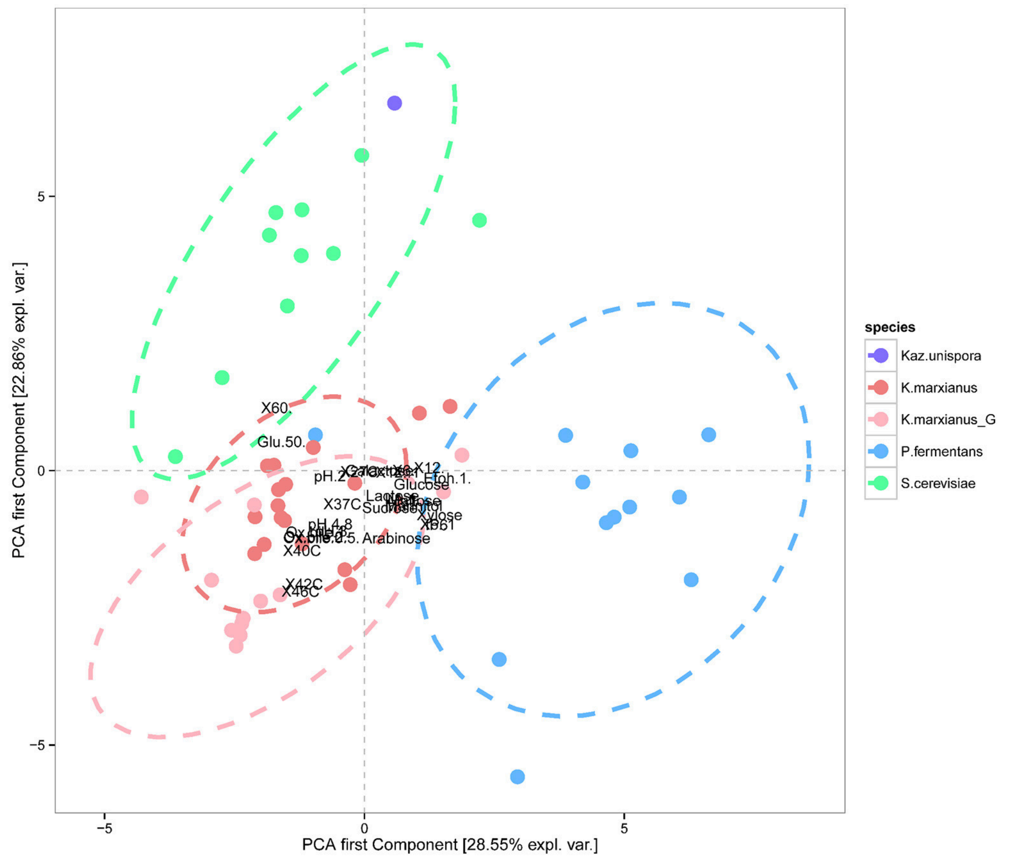

FIGURE 6 | Principal component analysis (PCA) revealing that the three different species *S. cerevisiae* (green), *P. fermentans* (blue), and *K. marxianus* (pink for Group II and light pink for Group I) could be phenotypically separated from each other ($p < 0.001$), and that the strain of *Kazachstania unispora* (purple) cluster together with the *S. cerevisiae* strains. The analysis also showed that the two genetically different groups of *K. marxianus*, Group II (A-nucleotide) in pink and Group I (G-nucleotide) in light pink, could be separated by means of phenotypic characterization. Ellipses were drawn to indicate the data grouping at 95% confidence assuming a multivariate t-distribution of data.

to 32 mg/L). Strains *K. marxianus* TJY52, *P. fermentans* CL1 and BL2, and *S. cerevisiae* CL2 were all inhibited already at the lowest tested concentration of clotrimazole (0.03 mg/L). Neither of the tested strains show resistance toward all antifungals, which is an important feature in order to allow external control of unwanted growth.

DISCUSSION

This is the first report on isolation, identification and characterization of yeast isolates from fermented goat milk of the Yaghnob Valley in Tajikistan. The yogurt contained a small variation of yeast species, dominated by the three species *K. marxianus*, *S. cerevisiae,* and *Pichia fermentans*. We isolated also one strain belonging to the species *K. unispora*. The same species that we identified in this work has also been isolated in other studies on fermented milk. In particular we observed that the yeast species composition of the Yaghnob fermentation is quite similar to that found in fermented Yak milk from the Tibetan plateau in China (Bai et al., 2010). However in other previous studies on traditional milk fermentations, a larger species diversity has generally been found as compared to our results. For example in *Amasi* made of cow milk (Gadaga et al., 2000) (20 different species, with the most predominant being *S. cerevisiae, Candida lusitaniae, C. colliculosa, S. dairensis*), in *Sameel* made of cow, goat, camel or sheep milk (Al-Otaibi, 2012) (36 different species, with the most prominent being *Candida lusitania, Cryptococcus laurentii, S. cerevisiae*), or in *Chal* made from camel milk (Yam et al., 2015) (35 species, with the most predominant being *Kluyveromyces lactis* and *K. marxianus* at 9% each). It seems as the predominant species, and the species variety, differs between different milk fermentations, probably both due to differences in raw material, physical factors (temperature, humidity etc.) and processing (the microflora of the people handling the fermentations, cleaning procedures, etc.).

Within the yeast species of the Yaghnob yogurt, the phenotypic analyses showed broad strain variation. All species presented remarkable differences in temperature tolerance, invasiveness, resistance to oxidative stress, hyphae formation, and inhibition by tested antifungals. It may be speculated that the broad strain variation within the three yeast species in the

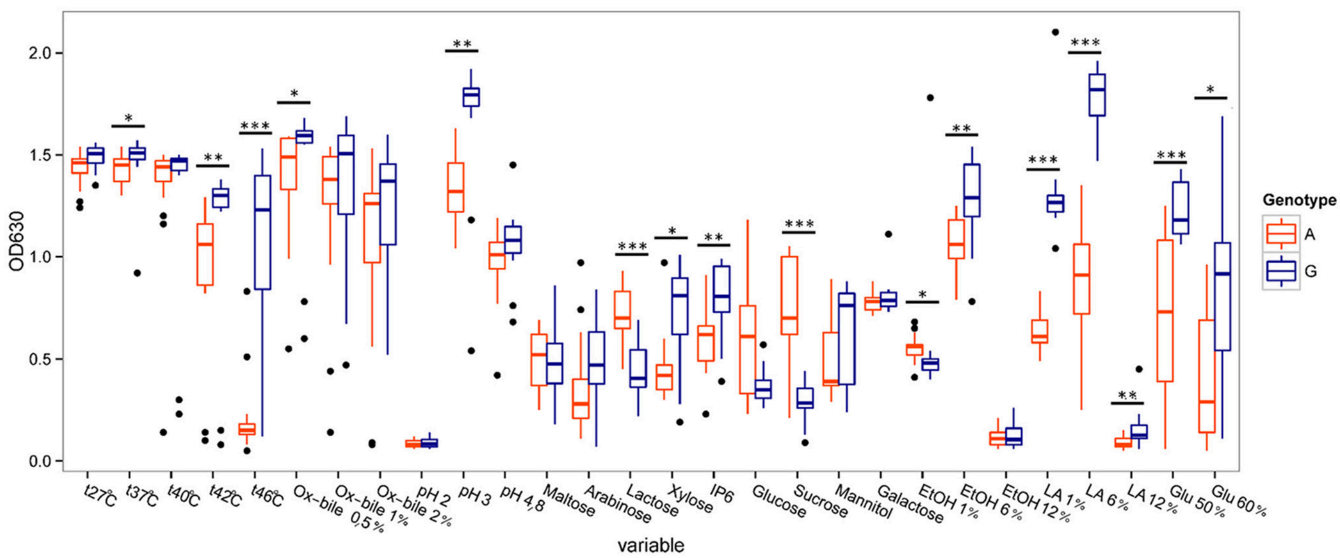

FIGURE 7 | Comparison of phenotypic characteristics between *Kluyveromyces marxianus* genotype Group II (A-nucleotide, orange color) and Group I (G-nucleotide, blue color). Significant differences are marked by *($p < 0.05$), **($p < 0.01$) or ***($p < 0.001$). Filled dots represent outliers (1.5 times the interquartile range above the upper quartile and below the lower quartile). The variables are temperatures (27°C–46°C), ox bile concentrations of 0.5%–2%, pH 2–4.8, different carbon sources, ethanol concentrations 1, 6, and 12%, lactic acid concentrations 1, 6, and 12%, and glucose at 50 and 60%.

Yaghnob yogurt fermentation could be a phenotypic, and perhaps genotypic, adaptation restricted to the few species isolated in this fermentation niche.

Broad phenotypic strain variations within the *K. marxianus* species have previously been reported by, among others, Lane et al. (2011), where investigation of 13 strains from two European strain collections revealed variations in thermotolerance, tolerance to osmotic stress and to cell wall stress. The RFLP fingerprinting performed in this study revealed the presence of two groups (Group I and Group II) within the *K. marxianus* species. The strains belonging to Group II showed an RFLP pattern which to our knowledge has not been previously reported, and with a unique single nucleotide polymorphism in the ITS1-4 region compared to Group I. Since the ITS1-4 region is known to be well preserved, the nucleotide difference found in Group II may indicate other genetic variations between the two groups. From the phenotypic characterization of the strains, it became evident that there are also significant phenotypic differences between the two genetically different groups. Furthermore, Group I isolates, showing an RFLP pattern in accordance with those previously reported for *K. marxianus*, were isolated both from the original and maintained sample, while strains belonging to Group II, showing the novel RFLP pattern, was only isolated form the original sample. It may be speculated whether the strains of Group II constitutes a new species, and the genetic differences in those *K. marxianus* strains will be further investigated in future studies.

The strains of *S. cerevisiae* were in addition to ITS1-5.8S-ITS4 analyses, also assessed by microsatellites and used for creating a phylogenetic tree. Microsatellites are tandem repetitive DNA sequences of up to 10 nucleotides, which are spread throughout the genome and are inherited in a codominant matter (Pérez et al., 2001). Yeast microsatellite loci are reported to have a high degree of variability (Field et al., 1996). Previous articles described a set of microsatellite loci as successful in the discrimination between different *S. cerevisiae* strains (Field and Wills, 1998; Gallego et al., 1998; Pérez et al., 2001) enabling to discriminate beer, wine and bread strains from strains from other sources (Legras et al., 2005). Interestingly, all isolates from the Yaghnob Valley fermented milk clustered apart from previous isolates of *S. cerevisiae* collected from a wide variety of ecological niches, indicating that a separate evolution may have occurred in the geographically isolated area of the Yaghnob Valley. As observed for the *K. marxianus* strains, also the *S. cerevisiae* strains showed two different genetic backgrounds, based on ancestry analysis. One group, containing strains CL2, BL11, and DL7, originated from two ancestors, while the other group, consisting of strains CL3, DL3, CL4, BL10, BL9, TJY58, and TJY61, originated from three ancestors, two of them being common with the ancestor of the first group. The majority of *S. cerevisiae* strains could be the result of either convergent selection or, more likely, of clonal expansion. Still, as previously shown for strains isolated from fermenting beers and breads (Liti et al., 2009), all these strains bear a mosaic genome and were inferred to descend from two shared ancestors. Several strains in addition showed to descend from a third ancestor shared with strains isolated from wasp intestine. Furthermore, the phenotypic assessment of the *S. cerevisiae* strains revealed some variations in tolerances to low pH and high temperatures. In a study by Edwards-Ingram and co-workers (Edwards-Ingram et al., 2007), the comparison of the probiotic *S. boulardii* strains

TABLE 3 | Invasiveness of each isolate in YPD agar was assessed and graded from 0 (not invasive) to 4 (highly invasive), where "b" indicates more intense invasiveness at the colony border.

Species	Strain	H_2O_2 resistance (mm) 27°C	Invasive (0–4) 27°C	Hyphae (168 h)				
				YPD 27°C	YPD 37°C	YNB 27°C	YNB 37°C	RPMI 37°C
K. marxianus	AL1	4	0	–	–	–	–	–
K. marxianus	AL2	9	0	–	–	–	–	–
K. marxianus	AL3	11	0	–	–	–	–	–
K. marxianus	AL4	4	0	–	–	–	–	–
K. marxianus	AL5	11	0	–	–	–	–	–
K. marxianus	BL1	10	1	–	–	–	–	–
K. marxianus	BL3	10	1	+	+	+	–	+
K. marxianus	BL4	14	0	–	–	–	+	–
K. marxianus	BL5	9	1	+	–	+	+	–
K. marxianus	BL6	10	0	–	+	–	–	–
K. marxianus	BL7	11	0	–	–	–	–	–
K. marxianus	BL8	11	0	–	–	–	–	–
K. marxianus	BL12	10	0	–	–	–	–	–
K. marxianus	BL13	12	0	–	–	–	–	–
K. marxianus	BL14	13	3	–	–	–	–	–
K. marxianus	CL5	4	2	+	+	+	+	+
K. marxianus	CL6	13	0	–	–	–	–	–
K. marxianus	DL2	13	0	–	–	–	–	–
K. marxianus	DL4	12	2 b	–	–	–	–	–
K. marxianus	DL5	11	0	–	–	–	–	–
K. marxianus	DL6	12	0	–	–	–	–	–
K. marxianus	DL10a	11	0	–	–	–	–	–
K. marxianus	DL10b	13	0	–	–	–	–	–
K. marxianus	DL11	13	1	–	–	–	–	–
K. marxianus	DL12	10	0	–	–	–	–	–
K. marxianus	TJY52	9	1	+	+	+	+	–
K. marxianus	TJY54	9	1	+	+	+	+	+
K. marxianus	TJY59	9	1	+	+	+	+	–
K. marxianus	TJY60	10	1	+	+	+	+	+
S. cerevisiae	BL9	17	3	+	–	+	–	+
S. cerevisiae	BL10	16	3	–	–	–	–	–
S. cerevisiae	BL11	9	0	–	–	–	–	–
S. cerevisiae	CL2	5	0	–	–	–	–	–
S. cerevisiae	CL3	14	0	–	–	–	–	–
S. cerevisiae	CL4	13	0	+	+	+	–	–
S. cerevisiae	DL3	17	0	–	+	–	+	+
S. cerevisiae	DL7	15	3 b	–	–	–	–	–
S. cerevisiae	TJY58	16	0	+	–	+	–	+
S. cerevisiae	TJY61	12	0	–	–	–	–	–
P. fermentans	BL2	11	2	+	+	+	–	+
P. fermentans	CL1	13	3	+	+	+	+	+
P. fermentans	CL7	11	1	–	–	–	–	–
P. fermentans	DL1	14	3	–	+	–	–	–
P. fermentans	DL8a	13	0	–	–	–	–	–
P. fermentans	DL8b	14	0	–	–	–	–	–
P. fermentans	DL9	15	1	–	–	–	–	–
P. fermentans	TJY50	9	1	–	–	–	–	–
P. fermentans	TJY53	14	1	–	–	–	–	–

(Continued)

TABLE 3 | Continued

Species	Strain	H_2O_2 resistance (mm) 27°C	Invasive (0–4) 27°C	Hyphae (168 h)				
				YPD 27°C	YPD 37°C	YNB 27°C	YNB 37°C	RPMI 37°C
P. fermentans	TJY55	13	1	–	–	–	–	–
P. fermentans	TJY56	13	1	+	+	+	+	+
P. fermentans	TJY57	14	1	–	–	–	–	–
Kaz. unispora	TJY51	12	0	–	n.d	–	n.d	n.d

The hyphae formation, given as positive (+) or negative (–) was determined based on microscopic investigation after cultivation in the media YPD, YNB and RPMI respectively and at two different incubation temperatures. All experiments were carried out in duplicates and presented is the mean value. n.d indicates that no data was obtained, due to no growth at this temperature.

TABLE 4 | The minimum inhibitory concentration (MIC) of the antifungals fluconazole, clotrimazole, and amphotericine B are presented as mg/L needed for full inhibition.

Strain	Fluconazle (mg/L)	Clotrimazole (mg/L)	Amphotericin B (mg/L)
BL3 (*K. marxianus*)	4	0.12	2
BL8 (*K. marxianus*)	8	0.5	8
DL4 (*K. marxianus*)	8	0.5	32*
TJY52 (*K. marxianus*)	8	0.03**	4
CL1 (*P. fermentans*)	64	0.03**	8
BL2 (*P. fermentans*)	128*	0.03**	16
CL2 (*S. cerevisiae*)	16	0.03**	2
BL9 (*S. cerevisiae*)	16	0.5	2
TJY51 (*K. unispora*)	128 *	0.25	4

Additionally, a single asterisk (*) indicates that growth was observed at the highest tested concentration (i.e., MIC not determined), and double asterisk (**) indicates that no growth was observed even at the lowest tested concentration (i.e., MIC may be lower than the tested concentration).

and other *S. cerevisiae* was done, and one of the phenotypic traits that appeared to separate the *S. boulardii* strains was the increased tolerance toward high temperatures and low pH. This led us to suggest that some of our isolated *S. cerevisiae* strains could in fact be *S. cerevisiae var. boulardii*. Especially the two strains BL9 and BL10, which show temperature tolerance up to 46°C and good growth at pH3, could according to the work by Edwards-Ingram et al. potentially belong to *S. cerevisiae var. boulardii*. As *S. boulardii* is a subtype of *S. cerevisiae* (Edwards-Ingram et al., 2004) they are difficult to separate based on the genomic work we performed in this study, hence further investigation of those strains genetic and phenotypic variations, as well as their potential probiotic effects need to be evaluated.

The fermented milk from the Yaghnob Valley is consumed without any prior sterilization step, meaning it contains viable cells when consumed. As several strains in this study show the ability to survive the conditions occurring in the intestinal tract (low pH, temperatures of 37°C and presence of ox bile), possible beneficial traits of those strains may be carried into the host. Other groups have investigated the probiotic potential of a strain of *K. marxianus* (BO399), presenting for example a positive effect on the immune response in CaCo-2 cell line (Maccaferri et al., 2012) and a positive effect on patients with irritable bowel syndrome (IBS) (Lisotti et al., 2013). The strains of this species isolated from the Yaghnob yogurt are therefore especially interesting for further studies of their possible probiotic properties.

One well-studied effect of yeast fermentation in cereal based foods is the degradation of the anti-nutrient phytate (IP_6) and subsequent release of minerals (Fredrikson et al., 2002; Hellström et al., 2010) by phytase enzymes originating from the present microorganisms (Lopez et al., 2001; Reale et al., 2004; Nielsen et al., 2007). Although the strains in this work were isolated from a dairy fermentation, all strains were tested for the ability to degrade phytate under nutrient starved conditions. Several strains showed the ability to degrade phytate, although further investigations are needed in order to identify the optimal cultivation condition for an improved phytate degradation. Since degradation of phytic acid has shown to increase the mineral availability from cereal based foods (Sandberg et al., 1999; Lopez et al., 2001; Hurrell et al., 2002; Schlemmer et al., 2009), phytase positive strains may be industrially interesting not only in dairy fermentations, but also in cereal based fermentations. It may further be hypothesized that consuming viable phytase active yeasts, e.g., from the fermented Yaghnob milk, together with a cereal based meal may aid phytic acid degradation and subsequent mineral release inside the intestinal tract. Traits such as phytase activity, ethanol tolerance and lactic acid tolerance further indicate potential for use also in e.g., sourdough fermentations, where co-fermentation between yeast and lactic acid bacteria (LAB) occurs (Di Cagno et al., 2014). It is widely known that co-fermentation between yeasts and LAB takes place in many natural food fermentations, which is further supported by several previous studies (Narvhus and Gadaga, 2003; Al-Otaibi, 2012; Nyambane et al., 2014) where isolation of both of them has been done from the same fermentation sample. One interesting study by Plessas et al. (2008) investigated sourdough fermentations with *K. marxianus* together with the two LAB, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus*, revealing promising results such as prolonged shelf life, improved resistance to spoilage moulds and improved sensory qualities of the bread product. This indicates another interesting potential application for some of the strains isolated from the Yaghnob yogurt, especially since bacterial isolation from this same yogurt resulted in isolation of

Lactobacillus delbrueckii and *Lactobacillus helveticus* as the two main species (data not published).

CONCLUSIONS

This study present the first ever yeast isolation from fermented goat milk of the geographically isolated Yaghnob Valley. Genetic and phenotypic differences among strains were observed; (i) a single-nucleotide difference separating *K. marxianus* strains into two groups, (ii) *S. cerevisiae* strains phylogenetically clustering apart from a large set of previously isolated strains—the mosaic nature of these strains, together with the role of wasps gut as favoring sporulation and mating of *S. cerevisiae* (Stefanini et al., 2016)—suggests the gut as an unexplored niche for *S. cerevisiae*, (iii) phenotypic intra-species variations, e.g., ability to resist high temperatures, low pH and presence of ox bile, indicating their potential to survive the human gastro-intestinal tract.

AUTHOR CONTRIBUTIONS

LQ was responsible for performing the experiments, analysing most of the data and for writing the manuscript. LQ, FS, and PM planned most of the experiments. IS and MS were responsible for handling and analysing the microsatellite data. GF was responsible for yeast isolations from original sample. TA, CDF, and DC were involved in supervision and discussions of the work. All authors were involved in revising the manuscript.

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Microbial Diversity and Biochemical Analysis of Suanzhou: A Traditional Chinese Fermented Cereal Gruel

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Suanzhou as a traditional Chinese gruel is fermented from proso millet and millet. The biochemical analysis showed Suanzhou had relatively high concentrations of lactic acid, acetic acid, and free amino acids. The metagenomics of Suanzhou were studied, with the analysis of the V4 region of 16S rRNA gene, the genera *Lactobacillus* and *Acetobacter* were found dominant with the average abundance of 58.2 and 24.4%, respectively; and with the analysis of the ITS1 region between 18S and 5.8S rRNA genes, 97.3% of the fungal community was found belonging to the genus *Pichia* and 2.7% belonging to five other genera. Moreover, the isolates recovered from 59 Suanzhou samples with various media were identified with the 16S rRNA or 18S rRNA gene analyses. *Lactobacillus fermentum* (26.9%), *L. pentosus* (19.4%), *L. casei* (17.9%), and *L. brevis* (16.4%) were the four dominant *Lactobacillus* species; *Acetobacter lovaniensis* (38.1%), *A. syzygii* (16.7%), *A. okinawensis* (16.7%), and *A. indonesiensis* (11.9%) were the four dominant *Acetobacter* species; and *Pichia kudriavzevii* (55.8%) and *Galactomyces geotrichum* (23.1%) were the two dominant fungal species. Additionally, *L. pentosus* p28-c and *L. casei* h28-c1 were selected for the fermentations mimicking the natural process. Collectively, our data demonstrate that Suanzhou is a nutritional food high in free amino acids and organic acids. Diverse *Lactobacillus*, *Acetobacter*, and yeast species are identified as the dominant microorganisms in Suanzhou. The isolated strains can be further characterized and used as starters for the industrial production of Suanzhou safely.

Keywords: Suanzhou, metagenomic analysis, lactic acid bacteria, acetic acid bacteria, yeast, free amino acid

INTRODUCTION

Many types of ethnic fermented cereal foods are widely consumed across the world. Compared with foods cooked directly from raw materials, fermented cereal foods are generally more tasteful, easily digested, and richer in various nutrients, such as vitamins, organic acids, and free amino acids (Blandino et al., 2003). Almost all types of cereals have been prepared into many kinds of foods in various natural fermented processes. Diverse microorganisms, mainly comprised of a number of bacteria and yeast species originated from the cereal grains and local environments, have been identified with diverse techniques (Tamang et al., 2016a,b).

A number of indigenous fermented foods have been made of rice or rice as the main material, such as Idli, dominant with *Leuconostoc lactis* (Saravanan and Shetty, 2016); Ang-kak also named Chinese red rice, dominant with *Monascus* strains (Lotong and Suwanarit, 1990); Selroti, dominant with multiple LAB and yeast species (Das et al., 2012); and Jiuniang or Laozao, dominant with *Rhizopus*, *Mucor*, *Monilia*, *Aspergillus*, and yeast species (Li and Hsieh, 2004). Wheat is an important source of diet proteins. However, wheat-based foods may contain a certain level of gluten which may cause allergic reactions in some individuals. Fermented wheat flour foods can greatly reduce the gluten content to safe levels, such as Sourdough, dominant with *Lactobacillus* species, and *Saccharomyces cerevisiae* (Settanni et al., 2005); Bhatooru, dominant with *S. cerevisiae*, *Lactobacillus plantarum*, and *Bacillus* sp. (Savitri and Bhalla, 2013); and Miso, dominant with *Pediococcus acidilactici* (Asahara et al., 1992). Maize based fermented foods mainly include doklu, dominant with *Lactobacillus fermentum*, *L. plantarum*, and *Pediococcus pentosaceus* (Assohoun-Djeni et al., 2016); and Ogi, dominant with *P. acidilactici* and *Lactobacillus paraplantarum* (Okeke et al., 2015). Sorghum based fermented foods are consumed in a number of African countries, such as Injera (Fischer et al., 2014), Kisra (Mohammed et al., 1991), and Hussuwa (Yousif et al., 2010), and all of them are rich in lactic acid bacteria (LAB). Millet is another important cereal grain and consumed as a staple food throughout the world (Saleh et al., 2013). Dosa (Palanisamy et al., 2012) and Ben-saalga (Tou et al., 2006) are two types of fermented millet foods which are also rich in LAB.

Proso millet and millet are highly drought-resistant crops with low demanding to environments. In northwestern China, proso millet and millet are commonly fermented to make Suanzhou, a sour gruel food easily prepared in local individual households. Until now, no studies of Suanzhou have been conducted in terms of its nutrients and microbial populations. In this work, totally 59 Suanzhou samples were collected for metagenomic DNA analysis and detection of free amino acids and organic acids concentration. Additionally, the dominant microorganisms in Suanzhou were isolated, identified, and characterized for possible applications in industrial production of Suanzhou.

MATERIALS AND METHODS

Preparation of Suanzhou

Suanzhou is a gruel made of fermented cereals prepared in individual households. Briefly, four types of raw materials were used in fermentations, group A containing samples fermented from millet with a small amount of rice (<10%), group B from millet, group C from white proso millet, and group D from red proso millet (**Table S1**). About 100 g grains were soaked in the fermentation soup or supernatant from the previous fermentation and kept at room temperature for 24 h in a jar sealed with a lid. Fermented grains are taken away for cooking. Raw materials are again added and soaked in the acidic soup for future fermentation and consumption. The water loss is supplemented with boiled water. Thirty samples (h1-30) were from Hequ county, in which different proso millet were used (**Table S1**). Twenty-nine samples (p1-30, the sample p25 was contaminated and removed from the analyses) from Pianguan county, in which millet was used as main raw material (**Table S1**). Both counties are located in Shanxi Province, China. All the Suanzhou samples were collected after 24 h incubation. Two 50-ml sour soup samples were obtained from each jar-fermentor and stored at 4°C for assays.

Metagenomic Analysis of Suanzhou Samples

The samples for metagenomic analysis were randomly selected on the basis of raw materials used for fermentation from two regions, Pianguan County and Hequ County. Suanzhou samples were centrifuged and the pellets were subjected to the extraction of genomic DNA by using Qiagen DNA blood and tissue kit (Qiagen, Dutch). To investigate the bacterial communities, the hypervariable V4 region (∼207 bp) of the 16S rRNA gene was analyzed with the primers 520-F (5′ AYTGGGYDTAAA GNG 3′) and 802-R (5′ TACNVGGGTATCTAATCC 3′) (Cole et al., 2005). To investigate the fungal communities, the ITS1 region between 18S and 5.8S rRNA genes was analyzed with the primers ITS1-F (5′ CTTGGTCATTTAGAGGAAGTAA 3′) and ITS2 (5′ GCTGCGTTCTTCATCGATGC 3′) (Schnabel et al., 1999). Metagenomic sequencing was performed on an Illumina MiSeq system by Shanghai Personal Biotechnology Co., Ltd., China (http://www.personalbio.cn). The obtained sequences were assigned to the operational taxonomic units (OTUs) with a threshold of 97% pairwise identity using the BLASTN tool in the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The species diversity, richness, and abundance were estimated by the Shannon, Chao1, and ACE indices (http://www.mothur.org/wiki/). Totally, 1,046,828 clean sequencing reads with length around 225 bp were obtained from the libraries of the 24 Suanzhou samples. Venn diagram was used to group the samples on basis of the genus level (http://bioinformatics.psb.ugent.be/webtools/Venn/). The possible correlations of the microbial communities were analyzed by the online software Cytoscape (www.mothur.org/wiki/Otu.association).

Determination of pH, Lactic Acid, Acetic Acid, and Free Amino Acids

The pH was measured by using a Sartorius pH indicator. Lactic acid, acetic acid, and free amino acids were determined by using ACQUITY UPLC M-Class System with BEH C18 Column (2.1 × 50 mm × 1.7 µm) and PDA detector (Waters Corporation, Milford, MA, USA). The supernatants of Suanzhou samples were subjected to filtration by the syringe filter (0.2 µm pore size). The filtrate was directly used for the lactic acid and acetic acid analysis. The mobile phase used was prepared by mixing 0.01 mol/l KH_2PO_4 (pH 3.0) and CH_3CN in a ratio of 98:2. Free amino acids in the supernatant samples were determined according to the manual from Waters and the method described previously (Fiechter et al., 2011). The reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC, Waters) was used to derivatize amino acids. Amino acids AAS18 and A9906 (Sigma-Aldrich) were used as analytical standards. All experiments were repeated three times. The values for pH, organic acids, and free amino acids were subjected to one-way analysis of variance (ANOVA) by Tukey's method of the statistical software Statistica 7.0.

Isolation and Identification of the Microorganisms from Suanzhou

Serial dilutions of Suanzhou samples with 0.9% NaCl (normal saline) were prepared. The dilutions were spread on the plates with the selective media, including *Lactobacilli* MRS agar (pH 5.0) for the isolation of LAB (De Man et al., 1960), GYC agar with $CaCO_3$ for the isolation of acetic acid bacteria (AAB; Raspor and Goranovic, 2008), and YEPD agar for the isolation of yeast species (Treco and Lundblad, 2001). The plates were incubated at 30°C for 48 h to enumerate the colonies. All experiments were repeated three times. Average and standard deviation (STDEV) were calculated using Excel.

Bacterial genomic DNA was extracted with the E.Z.N.A.® Bacterial DNA Kit (Omega Bio-tek Inc., USA) from a wide variety of gram positive and negative bacterial species. Primers 27f (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492r (5′ GGT TACCTTGTTACGACTT 3′) were used for amplification of the 16S rRNA gene (Lane, 1991). Fungal genomic DNA was extracted with the method as described previously (Löffler et al., 1997). Primers NS1 (5′ GTAGTCATATGCTTGTCTC 3′) and NS4 (5′ CTTCCGTCAATTCCTTTAAG 3′) were used to amplify the 18S rRNA gene (White et al., 1990). The amplicons were sequenced directly and the obtained sequences were deposited in GenBank. The accession number of 16S rRNA gene sequences was KX150543 through KX150609 for the LAB isolates and KX150610 through KX150652 for the AAB isolates. The accession number of 18S rRNA gene sequences was KX150653 through KX150704 for the yeast isolates. Sequence similarity was analyzed by using the online tool BLAST in the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were assigned to species level when similarities were at 97% or higher. The phylogenetic tree was constructed with the software MEGA6 (Tamura et al., 2013).

Growth Curve of LAB Strains

The growth curve of the isolated LAB isolates was determined in MRS medium (pH 6.0). The single colony was inoculated in 3 ml fresh MRS medium for static cultivation at 30°C for 12 h. The overnight precultures were diluted in fresh MRS medium to OD_{600} <0.2. The mixtures were dispensed into the 96-well plates with 250 μl per well. The culture was then grown for static cultivation at 30°C for 48 h. The OD_{600} was recorded at the 10-min intervals by the Synergy H1 Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT, USA). All experiments were repeated three times. Average and STDEV were calculated using Excel.

In-lab Fermentation of Suanzhou

Proso millet (100 g) was weighed and cleaned with water. After drying, proso millet was put in a 1-l bottle and filled with 900 ml distilled water. The mixture was pasteurized at 65°C for 30 min and ready for in-lab fermentation of Suanzhou. Two of the isolated LAB strains were selected and used as starter separately. Overnight culture of each strain was transferred in the sterilized proso millet suspension with 5% inoculation for static cultivation at 30°C. The fermented grains were replaced with fresh raw proso millet daily. As aforementioned, Suanzhou samples were analyzed in terms of pH, lactic acid, and free amino acids. LAB cells were enumerated by the standard plating method. The experiment was repeated three times. Average and STDEV were calculated using Excel.

RESULTS

Biochemical Characteristics of Suanzhou Samples

A collection of 59 Suanzhou samples were subjected to the analysis of acidity, lactic acid, and acetic acid. The pH value ranged from 3.22 ± 0.01 to 5.15 ± 0.02 in all samples (**Table S1**). The lactic acid concentration was from 0.74 ± 0.02 to 6.20 ± 0.04 mg/ml in the samples fermented from proso millet and 2.93 ± 0.00 to 17.00 ± 0.00 mg/ml in the samples from millet (**Table S1**). The acetic acid concentration was from 0.33 ± 0.00 to 7.66 ± 0.05 mg/ml in the samples fermented from proso millet and 0.28 ± 0.04 to 3.80 ± 0.00 mg/ml in the samples from millet (**Table S1**). With statistical analysis, the significant differences were observed in the comparison pairs, including groups A and C, A and D, B and C, and B and D, suggesting the raw materials were associated with the organic acid levels in Suanzhou.

Analysis of Free Amino Acids Content

The content of free amino acids was measured, ranging from 81.93 ± 5.01 to 665.47 ± 2.19 μ g/ml in the samples fermented from proso millet and 67.91 ± 0.41 to 1257.30 ± 0.93 μ g/ml in the samples from millet (**Table S1**). Of the total amino acids, essential amino acids accounted for 13.27 ± 0.29 to 48.02 ± 0.48% in the samples fermented from proso millet and 27.50 ± 0.09 to 51.81 ± 0.02% in the samples from millet (**Table S1**). With statistical analysis, Suanzhou fermented from the different cereals displayed the significant difference in the content of free amino acids among the comparison pairs, including groups A and C, A and D, B and C, and B and D. However, rice had no effects on the amino acids levels, possibly due to the low ratio (<10%) in the raw material.

Analysis of the V4 Region of 16S rRNA Gene

The species diversity, richness, and evenness in the 24 Suanzhou samples were estimated by the collector rarefaction curves of the observed species, chao1, and Shannon indices (**Figure S1**). The results showed that the libraries were relatively well-sampled and constructed. The bacterial diversity was mainly analyzed at the genus level. In the 24 Suanzhou samples, the top 4 dominant species groups were the genus *Lactobacillus*, the genus *Acetobacter*, the family *Acetobacteraceae*, and the order *Lactobacillales*, with an average abundance of 58.20 ± 0.28, 24.40 ± 0.23, 9.00 ± 0.15, and 2.00 ± 0.04%, respectively (**Figure 1**). The sample h25 had 55 OTUs, the maximum of all tested samples; while only 21 OTUs were found in the sample h19,

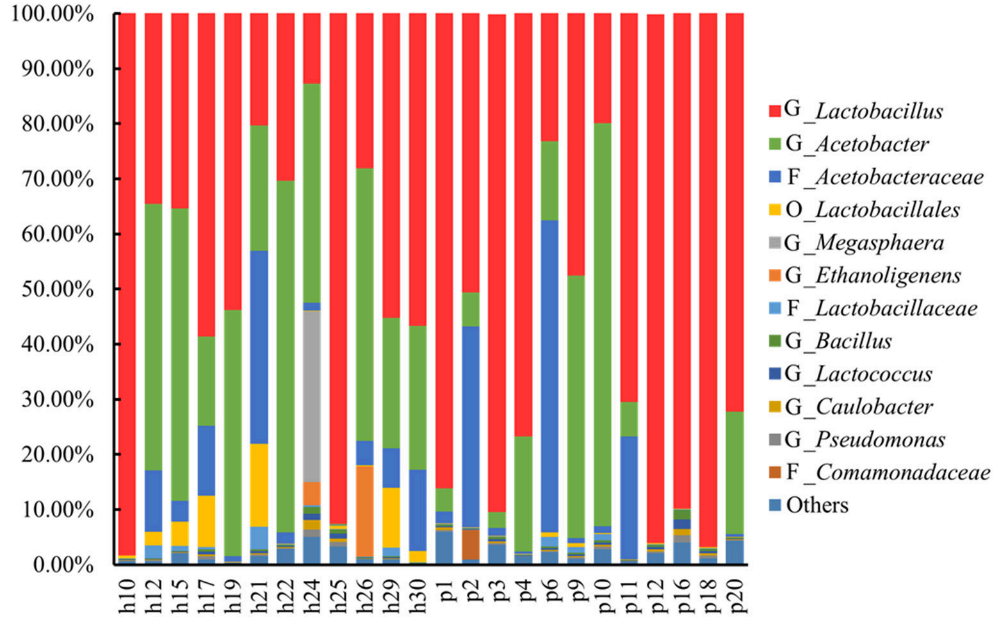

FIGURE 1 | Abundance of the top 12 abundant operational taxonomic units (OTUs) among the Suanzhou samples. OTUs with the abundance <0.10% were not included in this diagram. G: OTUs at the genus level. F: OTUs at the family level. O: OTUs at the order level. Others: all OTUs with the abundance ≥0.10% were included. In summary, h10 with 0.10% *Streptophyta* (order); h12 with 0.10% *Psychrobacter* and 0.10% *Clostridium*; h15 with 0.10% *Psychrobacter* and 0.3% *Streptophyta* (order); h17 with 0.30% *Psychrobacter*, 0.10% *Micrococcaceae*, 0.10% *Phenylobacterium*, and 0.10% *Methylobacteriaceae* (family); h21 with 0.20% *Psychrobacter*, 0.10% *Micrococcaceae* (family), 0.10% *Phenylobacterium*, 0.10% *Bacteroides*, and 0.30% Bacteroidales (order); h22 with 0.10% *Psychrobacter*, and 2.60% *Gluconacetobacter*; h24 with 1.30% *Psychrobacter*, 0.20% Micrococcaceae (family); 0.5% *Phenylobacterium*, 0.40% *Methylobacteriaceae* (family), 0.10% *Bacteroides*, 0.10% *Bacteroidales* (order), 0.10% *Brochothrix*, 0.10% *Caulobacteraceae* (family), 0.10% *Balneimonas*, 0.90% *Methylobacterium*, 0.10% *Halomonas*, 0.30% *Acinetobacter*, 0.10% *Enhydrobacter*, and 0.10% *Stenotrophomonas*; h25 with 0.40% *Psychrobacter*, 0.30% *Streptophyta* (order), 0.20% *Phenylobacterium*, 0.10% *Methylobacteriaceae* (family); 0.10% *Bacteroides*, 0.20% *Myroides*, 0.20% *Sphingobacterium*, 0.10% *Bacteroidales* (order), 0.10% *Brochothrix*, 0.20% *Staphylococcus*, 0.10% *Gemellales* (order), 0.10% *Kuenenia*, 0.20% *Enterobacteriaceae* (family), 0.10% *Klebsiella*, 0.20% *Acinetobacter*, 0.10% *Xanthomonadaceae* (family); h26 with 0.10% *Streptophyta* (order); h29 with 0.10% *Psychrobacter*, and 0.50% *Pediococcus*; p1 with 0.40% *Psychrobacter*, 0.10% *Micrococcaceae* (family), 0.40% *Streptophyta* (order), 0.10% *Phenylobacterium*, and 0.10% *Methylobacteriaceae* (family); p2 with 0.10% *Comamonas*, 0.20% *Enterobacteriaceae* (family), 0.10% *Klebsiella*, 0.10% *Moraxellaceae* (family), 0.10% *Acinetobacter*, and 0.10% *Xanthomonadaceae* (family); p3 with 0.20% *Psychrobacter*, 0.10% *Streptophyta*, 0.10% *Phenylobacterium*, 0.10% *Methylobacteriaceae* (family), 0.60% *Bacteroides*, 0.20% *Bacteroidales* (order), 0.30% *Bacteroidia* (class), 0.30% *Barnesiellaceae* (family), 0.10% *Veillonellaceae* (family), 0.10% *Desulfovibri*, and 0.10% *Akkermansia*; p4 with 0.20% *Streptophyta* (order); p6 with 0.20% *Psychrobacter*, 0.10% *Micrococcaceae* (family), 0.10% *Streptophyta* (order), 0.10% *Phenylobacterium*, 0.10% *Methylobacteriaceae* (family), and 0.10% *Bifidobacteriaceae* (family); p9 with 0.20% *Psychrobacter*, 0.10% *Phenylobacterium*, and 0.10% *Methylobacteriaceae* (family); p10 with 0.20% *Psychrobacter*, 0.10% *Micrococcaceae* (family), 0.10% *Phenylobacterium*, 0.10% *Actinomyces*, 1.10% *Chryseobacterium*, 0.20% *Klebsiella*, and 0.30% *Acinetobacter*; p12 with 0.20% *Psychrobacter*, 0.10% *Micrococcaceae* (family), 0.10% *Streptophyta* (order), 0.10% *Phenylobacterium*, 0.20% *Methylobacteriaceae* (family), 0.10% *Bacteroidales* (order); p16 with 0.80% *Psychrobacter*, 0.30% *Micrococcaceae* (family), 0.30% *Streptophyta* (order), 0.30% *Phenylobacterium*, 0.30% *Methylobacteriaceae* (family), 0.10% *Nocardioidaceae* (family), 0.10% *Myroides*, 0.10% *Brochothrix*, 0.10% *Paenibacillus*; p18 with 0.30% *Psychrobacter*, 0.10% *Micrococcaceae* (family), 0.10% *Phenylobacterium*, 0.10% *Methylobacteriaceae* (family), 0.10% *Halomonas*; and p20 with 0.10% *Psychrobacter*, 0.10% *Streptophyta* (order), 0.40% *Halomonas*, and 0.20% *Thermus.*

mainly including *Lactobacillus* (53.80%) and *Acetobacter* (44.7%) (**Figure S2**).

There were many microorganisms commonly present in the Suanzhou samples with low abundance. *Psychrobacter* species were detected in 17 Suanzhou samples with the average abundance from 0.10 to 1.30%. *Phenylobacterium* species were detected in 12 Suanzhou samples with the average abundance from 0.10 to 0.50%. *Streptophyta* (order) species were detected in 11 Suanzhou samples with the average abundance from 0.10 to 0.40%. *Methylobacteriaceae* (family) species were detected in 11 Suanzhou samples with the average abundance from 0.10 to 0.40%. *Micrococcaceae* (family) species were detected in 9 Suanzhou samples with the average abundance from 0.10 to 0.30% (**Figure 1**).

Analysis of the ITS1 Region

The sample h21 was analyzed for its fungal communities by the Miseq system. In the obtained sequences, 29.40% showed no blast hits. Of the remaining sequences, at the genus level, the abundance of *Pichia*, *Xeromyces*, *Candida*, *Issatchenkia*, *Cryptococcus*, and *Trichosporon* accounted for 97.30, 1.50, 0.81, 0.35, 0.02, and 0.01%, respectively (**Figure 2**). *Pichia* was the prominent OTUs in the sample h21.

In contrast to the fungal population analysis, the bacterial diversity was also determined in the sample h21. The dominant OTUs included *Acetobacteraceae* (family) 35.0%, *Lactobacillales* (order) 15.0%, *Acetobacter* 22.8%, *Lactobacillus* 20.3%, and *Lactobacillaceae* (family) 4.1% (**Figure 1**).

Enumeration and Identification of LAB Isolates

White transparent or opaque colonies on the MRS agar plates were counted. Totally 67 presumptive LAB strains were isolated and the corresponding bacteria concentrations ranged from 4.58 ± 0.03 lg cfu/ml (sample h30) to 8.69 ± 0.05 lg cfu/ml (sample h20) except that no colonies were observed with the samples h18, h22, p5, p7, and p22 (**Figure 3**). The 16S rRNA gene sequence of the LAB isolates was analyzed. All isolates were identified as the members of the genus *Lactobacillus*, including *L. brevis* (11), *L. casei* (12), *L. coryniformis* (1), *L. fermentum* (18), *L. harbinensis* (4), *L. helveticus* (2), *L. parafarraginis* (2), *L. pentosus* (13), *L. reuteri* (3), and *L. rossiae* (1). Fourteen samples had two *Lactobacillus* species coexisted in the same gruel fermentor (**Figure 4**, **Table 1**).

Enumeration and Identification of AAB Isolates

Yellow transparent colonies surrounded with clear zones were counted on the GYC-agar plates. Forty-two isolates were recovered from Suanzhou samples with the concentrations ranging from 4.26 ± 0.24 lg cfu/ml (sample p24) to 8.41 ± 0.17 lg cfu/ml (sample h28) (**Figure 5**). The 16S rRNA gene sequence of the AAB isolates was analyzed. All isolates were identified as the members of the genus *Acetobacter*, including *A. cibinongensis* (1), *A. indonesiensis* (5), *A. lovaniensis* (16), *A. okinawensis* (7), *A. orientalis* (1), *A. papaya* (2), *A. senegalensis* (1), *A. syzygii* (7), *A. malorum* (1), and *A. fabarum* (1) (**Figure 6**). *Acetobacter* species were absent in the remaining 20 Suanzhou samples (**Table 1**). Twenty Suanzhou samples were found absent of *Acetobacter* species. Seventeen of them had no colonies on the GYC plates and the remaining three samples recovered the bacteria other than *Acetobacter* spp., including *Gluconobacter oxydans* in the sample h18, *Pseudomonas psychrophila* and *Sphingobacterium mizutaii* in the sample h28, and *Microbacterium schleiferi* in the sample p14.

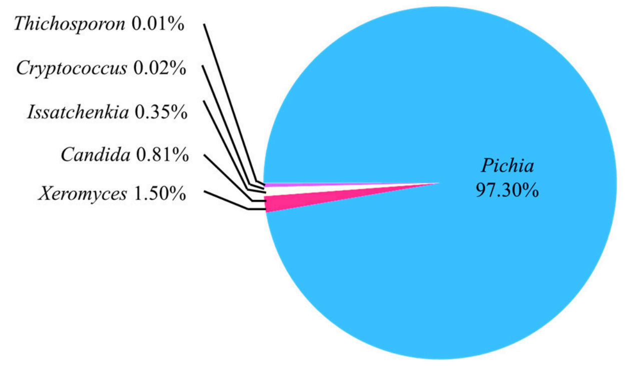

FIGURE 2 | Abundance of the operational taxonomic units (OTUs) on the basis of IST1 region analysis. All OTUs were at the genus level. The sequences with no blast hits (29.43% of the total clean reads) weren't included in this pie-chart.

Enumeration and Identification of Yeast Isolates

On the YEPD-agar plates, three types of colonies were counted, large yellow colonies, large white colonies, and small white colonies with hyphae. The concentrations of the isolates ranged from 4.10 ± 0.17 lg cfu/ml (sample p27) to 8.35 ± 0.07 lg cfu/ml (sample h28), and no isolates were found in 13 Suanzhou samples (**Figure 7**). The 18S rRNA gene sequence of the yeast isolates was analyzed. All isolates were classified into 3 genera, including *Pichia fermentans* (2), *P. kudriavzevii* (29), *P. membranifaciens* (5), *P. occidentalis* (3), *S. cerevisiae* (1), and *Galactomyces geotrichum* (12) (**Figure 8**, **Table 1**).

Growth Curves of the LAB Strains

L. fermentum, *L. pentosus*, *L. casei*, and *L. brevis* were the most frequently isolated strains from Suanzhou and 31 of them were selected for the growth study (**Table 1**). As inferred from the growth curves, all strains were divided into three groups based on their growth rates. Group I strains had the lag phase shorter than 1 h and their highest cell density was at 1.3–1.6 of OD_{600}, including 10 *L. pentosus* strains and *L. casei* h6-c, h16-c, h17-c1, and h28-c1. Group II strains had the lag phase of 2–3 h and their highest cell density was at 1.2–1.4 of OD_{600}, including 10 *L. brevis* strains and *L. casei* h3-c, h25-c, h27-c2, and p27-c2.

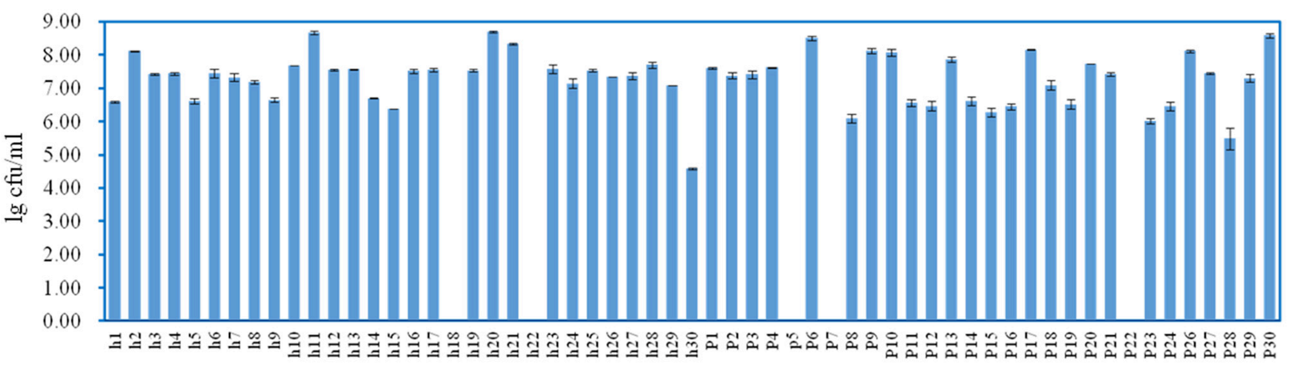

FIGURE 3 | Enumeration of lactic acid bacteria (LAB) in the Suanzhou samples.

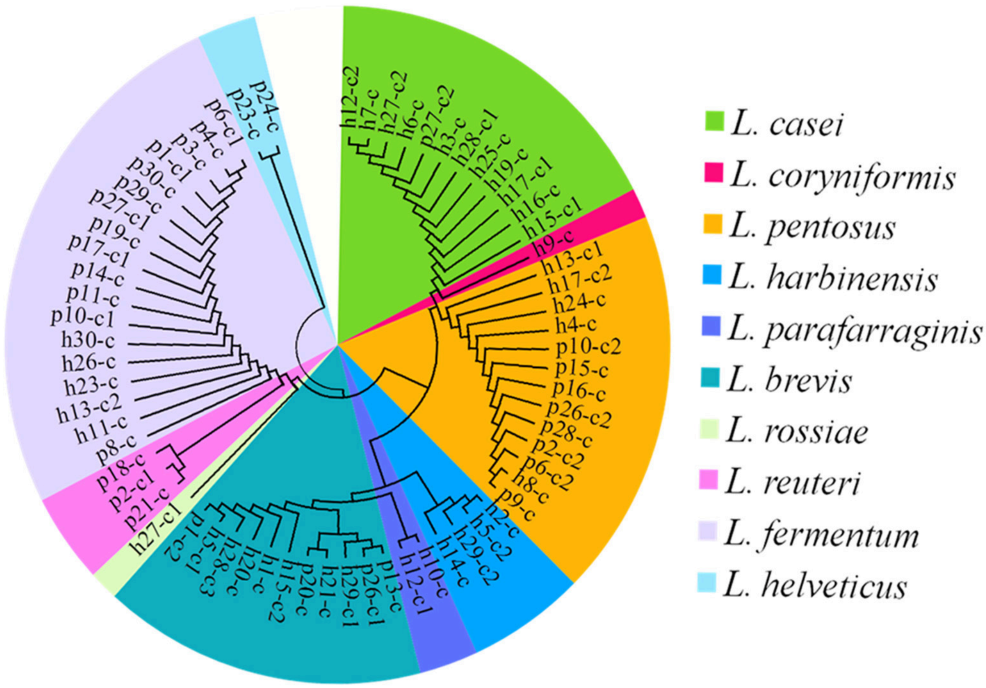

FIGURE 4 | The cladogram tree of the lactic acid bacteria (LAB). The partial 16S rRNA gene sequences (921–925 bp) were analyzed using the Neighbor-Joining method. L, *Lactobacillus*.

Group III strains grew very slowly without perceptible lag phases and their highest cell density was at 0.2–0.4 of OD_{600}, including *L. fermentum* p17-c1, *L. fermentum* p27-c1, and *L. casei* h12-c2 (**Figure S3**).

In-lab Fermentation of Suanzhou

Two fast growing strains *L. pentosus* p28-c and *L. casei* h28-c1 were selected for a 5 d-fermentation mimicking the natural process for Suanzhou preparation. Proso millet was used as raw material and the relevant parameters were analyzed daily. With 5% innoculum, the population of *L. casei* h28-c1 decreased one log unit after 5 days of fermentation, while the population of *L. pentosus* p28-c increased from 7.16 ± 0.16 to 8.44 ± 0.11 lg cfu/ml after 2 d cultivation (**Figure 9**). The pH value of the two cultures decreased from about 6.6 to 3.5 after 1-day fermentation and remained constant, consistent with the elevated lactic acid content in the cultures (**Figure 9**). The amount of the total amino acids, essential amino acids, and alanine increased significantly during the fermentation. Strain *L. casei* h28-c1 produced higher amount of free amino acids and essential amino acids than strain *L. pentosus* p28-c (**Figure 9**).

DISCUSSION

In our work, totally 69 OTUs at the genus level were detected in Suanzhou samples with the metagenomic analysis. The possible logic relationship of the OTUs identified among the samples was analyzed with the Venn diagram (**Figure 10**). The samples were divided into group A, B, C, and D based on the cereals, which were made of millet with a small amount of rice, millet, white proso millet, and red proso millet, respectively (**Table S1**). Fifty-four of them were found in all groups and 67 OTUs found in the samples from both counties, indicating no significant difference existed. The associations among the microbial populations in the samples were predicted. Of the top 20 abundant OTUs found in the metagenomic analysis, the genera *Lactobacillus*, *Acetobacter*, and *Gluconacetobacter* had no correlations with the remaining 17 OTUs. However, the abundance of *Lactobacillus* was inversely correlated with *Acetobacter* and *Gluconacetobacter*, respectively (**Figure 11**). Could the antagonistic effect between the genera *Lactobacillus*, *Acetobacter*, and *Gluconacetobacter* lead to the dying off of any strains was still a question remained be answer by in-lab fermentations using the isolated microbes.

The majority of environmental microorganisms are inculturable with the available methods. In Suanzhou samples, only a few species including LAB, AAB, and yeasts were found with the culture-dependent methods, much less than the OTUs identified with the metagenomic analysis. Furthermore, with the metagenomic analysis, microbial structure was found not unique among Suanzhou samples and some special OTUs were detected in the individual samples (**Figure 1**). *Gluconacetobacter* was detected in the sample h22 with the abundance of 2.6%, which was commonly the dominant bacterium found in the traditional vinegar production (Hommel, 2014). *Akkermansia* was detected in the sample p3 with a low abundance 0.10%. *A. muciniphila* is the type species of this genus and related with the diet-induced obesity (Everard et al., 2013). *Megasphaera* species was dominant in the sample h24 with an abundance of 31.00%. It's a strictly

TABLE 1 | Phylogenetic affiliations of the isolates.

No.	Closest sequence of LAB	Closest sequence of AAB	Closest sequence of yeast
h27	*L. rossiae*, *L. casei*	*A. lovaniensis*	*P. membranifaciens*
p2	*L. reuteri*, *L. pentosus*	*A. papayae*	*G. geotrichum*
p18	*L. reuteri*		*P. kudriavzevii*
p21	*L. reuteri*		
h13	*L. pentosus*, *L. fermentum*	*A. okinawensis*	*P. kudriavzevii*
p15	*L. pentosus*		*P. kudriavzevii*
h4	*L. pentosus*	*A. lovaniensis*	*P. kudriavzevii*, *G. geotrichum*
h8	*L. pentosus*	*A. lovaniensis*	*P. kudriavzevii*
h24	*L. pentosus*	*A. lovaniensis*	*P. kudriavzevii*
p9	*L. pentosus*	*A. lovaniensis*	
p16	*L. pentosus*		*P. kudriavzevii*
p28	*L. pentosus*		*G. geotrichum*
h12	*L. parafarraginis*, *L. casei*	*A. syzygii*	*P. kudriavzevii*
h10	*L. parafarraginis*		*P. kudriavzevii*
p24	*L. helveticus*	*A. lovaniensis*	*P. kudriavzevii*
p23	*L. helveticus*	*A. lovaniensis*	
h14	*L. harbinensis*	*A. fabarum*	
h2	*L. harbinensis*		*P. kudriavzevii*
p10	*L. fermentum*, *L. pentosus*	*A. orientalis*	*G. geotrichum*
p6	*L. fermentum*, *L. pentosus*	*A. malorum*	
p27	*L. fermentum*, *L. casei*		*P. kudriavzevii*
p1	*L. fermentum*, *L. brevis*		*S. cerevisiae*
p17	*L. fermentum*		
p4	*L. fermentum*	*A. lovaniensis*	*P. kudriavzevii*
p14	*L. fermentum*		*P. kudriavzevii*
p11	*L. fermentum*	*A. okinawensis*, *A. papayae*	
h11	*L. fermentum*	*A. okinawensis*	*G. geotrichum*
h23	*L. fermentum*	*A. lovaniensis, A. okinawensis*	*P. kudriavzevii*, *G. geotrichum*
h26	*L. fermentum*	*A. lovaniensis*	*P. kudriavzevii*
h30	*L. fermentum*	*A. lovaniensis*	*P. kudriavzevii*
p30	*L. fermentum*	*A. indonesiensis*	*P. kudriavzevii, G. geotrichum*
p3	*L. fermentum*	*A. indonesiensis*	*P. kudriavzevii*
p19	*L. fermentum*		*G. geotrichum*
p29	*L. fermentum*		*G. geotrichum*
p8	*L. fermentum*		
h9	*L. coryniformis*	*A. lovaniensis*	*P. kudriavzevii*
h17	*L. casei*, *L. pentosus*	*A. indonesiensis*	*P. fermentans*,
h15	*L. casei*, *L. brevis*	*A. okinawensis*	
h28	*L. casei*, *L. brevis*		*P. membranifaciens*
h6	*L. casei*	*A. syzygii*	*P. occidentalis*
h19	*L. casei*	*A. syzygii*	*P. kudriavzevii*
h25	*L. casei*	*A. senegalensis*	*P. kudriavzevii, P. membranifaciens*
h7	*L. casei*	*A. okinawensis*	*P. kudriavzevii, G. geotrichum*
h16	*L. casei*	*A. lovaniensis*	*P. membranifaciens*
h3	*L. casei*	*A. lovaniensis*	
p26	*L. brevis*, *L. pentosus*	*A. indonesiensis*	*P. kudriavzevii*
h5	*L. brevis*, *L. harbinensis*	*A. lovaniensis*	*P. occidentalis*
h29	*L. brevis*, *L. harbinensis*		*P. fermentans*
h21	*L. brevis*	*A. syzygii*	*P. membranifaciens*
p13	*L. brevis*	*A. okinawensis*	

(Continued)

TABLE 1 | Continued

No.	Closest sequence of LAB	Closest sequence of AAB	Closest sequence of yeast
p20	*L. brevis*	*A. cibinongensis*	*P. kudriavzevii*, *G. geotrichum*
h20	*L. brevis*		*G. geotrichum*
h1	*L. brevis*		
h22		*A. syzygii*, *A. lovaniensis*	*P. kudriavzevii*
p22		*A. lovaniensis*	*P. kudriavzevii*
p5		*A. syzygii*	*P. kudriavzevii*
p7		*A. indonesiensis*	
h18			*P. occidentalis*
p12			*P. kudriavzevii*

All isolates display 98–100% identity to the closest sequences. The isolates were arranged according to alphabetical order of LAB species names. L, Lactobacillus; A, Acetobacter; G, Galactomyces; P, Pichia; S, Saccharomyces; LAB, lactic acid bacteria; AAB, acetic acid bacteria. Gluconobacter oxydans was isolated in the sample h18. Pseudomonas psychrophila and Sphingobacterium mizutaii were isolated in the sample h28. Microbacterium schleiferi was isolated in the sample p14.

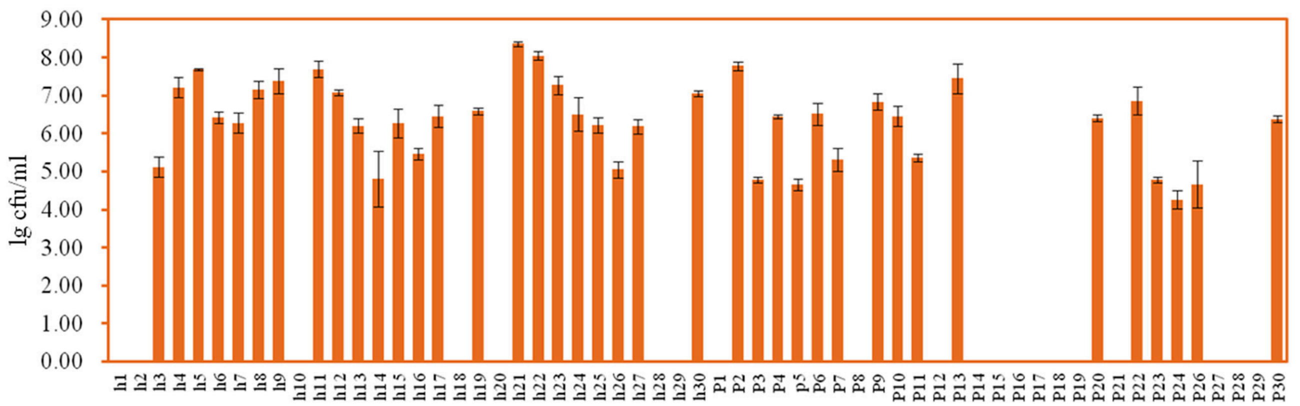

FIGURE 5 | Enumeration of acetic acid bacteria (AAB) in the Suanzhou samples.

anaerobic microorganism and the members of this genus were usually recovered from the gastrointestinal tracts of animals (Stanton and Humphrey, 2011; Shetty et al., 2013). *Megasphaera paucivorans* sp. nov. and *Megasphaera sueciensis* sp. nov. were the two new species isolated from brewery samples, probably causing beer spoilage (Juvonen and Suihko, 2006). *Ethanoligenens* was present in two samples h24 and h26 with an abundance of 4.2 and 16.4%, respectively (**Figure 1**). *Ethanoligenens harbinense* was the only known species relevant to the biomass conversion and biofuels production (Hemme et al., 2010; Liu et al., 2015). The unexpected microorganisms were also found in cultivation process in our work, such as *G. oxydans* (De Muynck et al., 2007), *P. psychrophila* (Abraham and Thomas, 2015), *S. mizutaii* (Wauters et al., 2012), and *M. schleiferi* (Gneiding et al., 2008). These microorganisms were coexisted in Suanzhou at relatively high abundance and some of them may pose potential risks to food safety and human health. It's an innate disadvantage for household-scale fermentation and can be overcome by industrial production using well-characterized starter strains with good manufacturing practice (GMP).

All Suanzhou samples were collected from the household fermentors which had been operating for at least 2 months. In combined with the culture-dependent methods and sequence analyses, totally 10 *Lactobacillus* species, 10 *Acetobacter* species, 1 *G. oxydans* strain, 4 *Pichia* species, 12 *G. geotrichum* strains, and 1 *S. cerevisiae* strain were found in Suanzhou. The results are consistent with the previous findings in a variety of the fermented foods and beverages (Goerges et al., 2008; Eida et al., 2013). Twenty Suanzhou samples had no *Acetobacter* species identified and it may be due to the existence of bacteriocins synthesized by LAB strains, which will inhibit the growth of many bacteria (Heng et al., 2007; Gabrielsen et al., 2014). No LAB were isolated in the 5 Suanzhou samples, 4 of them with the presence of *Acetobacter* spp. and h18 with *G. oxydans* (**Table 1**), indicating LAB strains may not be the sole organism for Suanzhou production.

Tiny LAB colonies were observed on the MRS plates spread with the sample p12, however, the colonies failed in growth with the subsequent cultivations. The metagenomic analysis showed that p12 was rich in LAB with the total OTUs abundance of 96.20%, including *Lactobacillus*, *Lactobacillaceae*, and *Lactobacillales* (**Figure 1**). The growth failure of the pure culture was possibly due to the special nutrient requirements of the LAB isolate, whereas the yeast strain *P. kudriavzevii* in

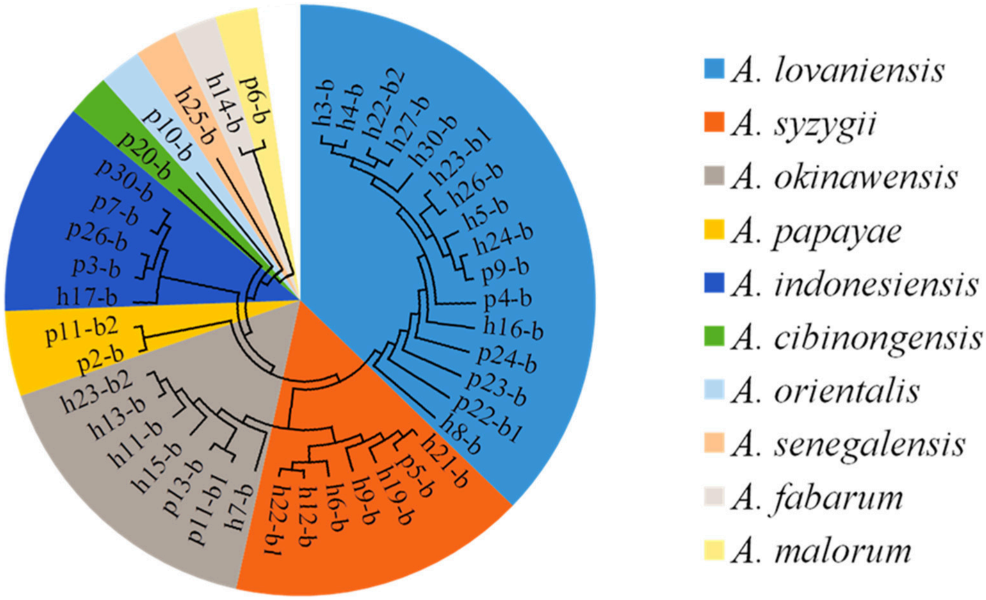

FIGURE 6 | The cladogram tree of the acetic acid bacteria (AAB). The partial 16S rRNA gene sequences (1005–1026 bp) were analyzed using the Neighbor-Joining method. A, *Acetobacter*.

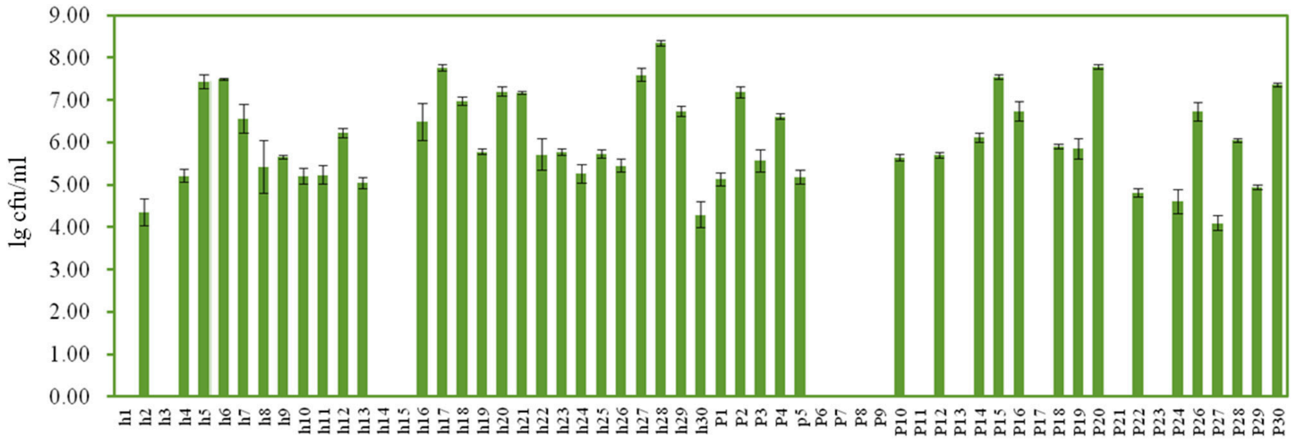

FIGURE 7 | Enumeration of yeasts in the Suanzhou samples.

Suanzhou may provide the nutrients essential for the growth of the isolate (Assohoun-Djeni et al., 2016). Strain *L. fermentum* p17-c1, *L. fermentum* p27-c1, and *L. casei* h12-c2 also exhibited low growth rates in the pure cultures, significantly lower than the relevant cell numbers in the corresponding Suanzhou samples (**Figure 3** and **Figure S3**). The results indicated that the Suanzhou micro-ecosystems possibly provided necessary nutrients for the growth of microorganisms either by degradation of the cereals or by the biosynthesis.

In our work, several yeasts were identified. *Pichia* species were commonly isolated in the most samples of Suanzhou without detecting other fungi. The result is consistent with the previous findings that *Pichia* species can antagonize and decrease the abundance of a number of yeast and mold pathogens in various niches (Golubev, 2006; Mukherjee et al., 2014). *G. geotrichum* was coexisted with *Pichia* species in 5 Suanzhou samples, showing that its growth wasn't affected by *Pichia*, also consistent with the previous findings (Viljoen, 2006). *G. geotrichum* or its anamorph *Geotrichum candidum* was widely present in the early stages of ripening on soft cheeses (Marcellino et al., 2001) and some strains were the starter organisms for fermented foods and beverages (Goerges et al., 2008; Tamang et al., 2016a). Only one *S. cerevisiae* strain was isolated from the sample p1 which was absent of *Acetobacter* strains (**Table 1**). The data is consistent with the fact that acetic acid can suppress the growth of *S. cerevisiae* in sour dough (Suihko and Mäkinen, 1984).

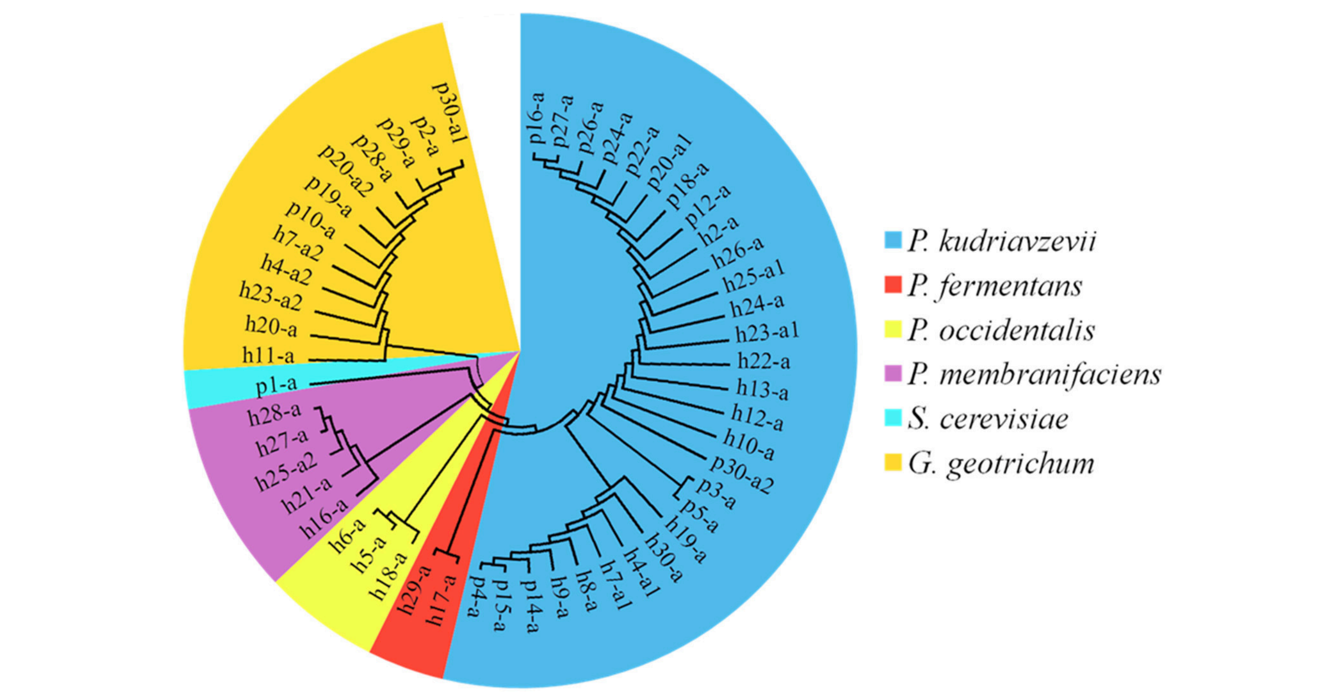

FIGURE 8 | The cladogram tree of the yeasts. The partial 18S rRNA gene sequences (928–944 bp) were analyzed using the Neighbor-Joining method. P, *Pichia*. S, *Saccharomyces*. G, *Galactomyces*.

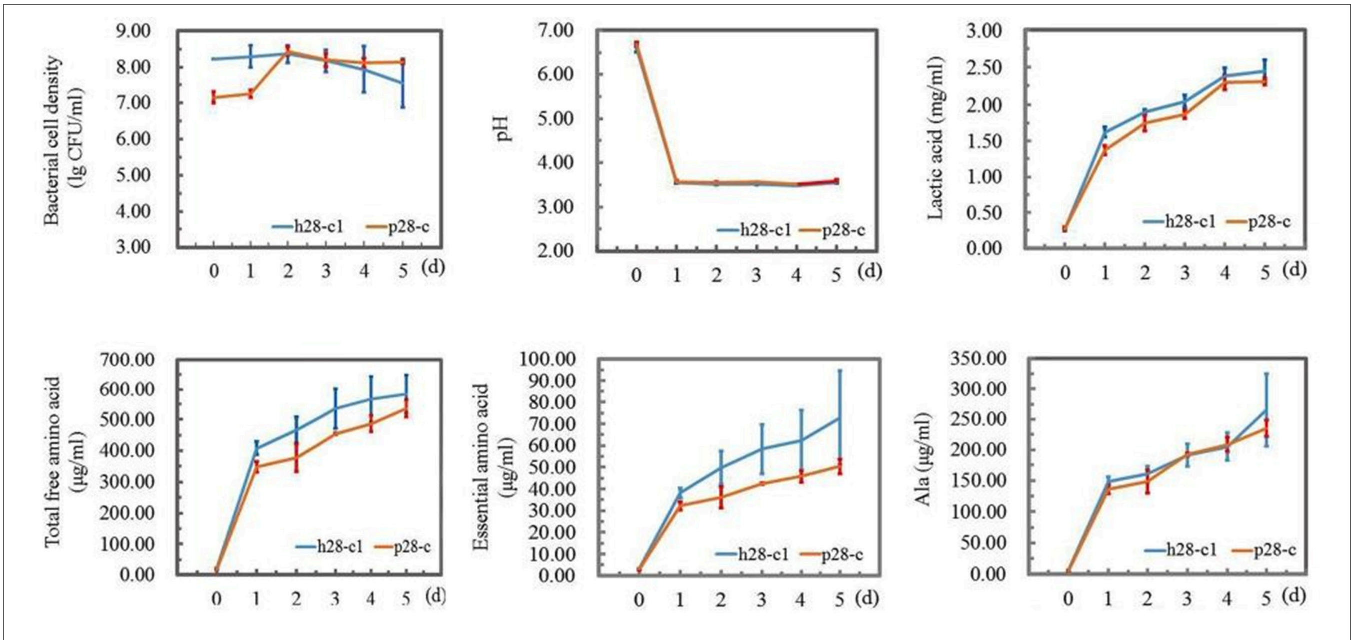

FIGURE 9 | Preparation of Suanzhou with in-lab fermentations. Strain *L. casei* h28-c1 and *L. pentosus* p28-c were used as starters. Bacterial cell density (lg cfu/ml), pH, lactic acid, total free amino acid, essential amino acid, and alanine content were evaluated, respectively.

Taken together, the yeast strains could play an inhibitory role against food-borne pathogens and also provide some nutrients for the growth of other microbes (Goerges et al., 2006; Viljoen, 2006).

LAB strains have been widely used in fermented food industry as starters (Giraffa et al., 2010). In our wok, 2 *lactobacillus* strains were used for in-lab fermentations separately. The results showed that the in-lab products were similar to traditional gruel in terms

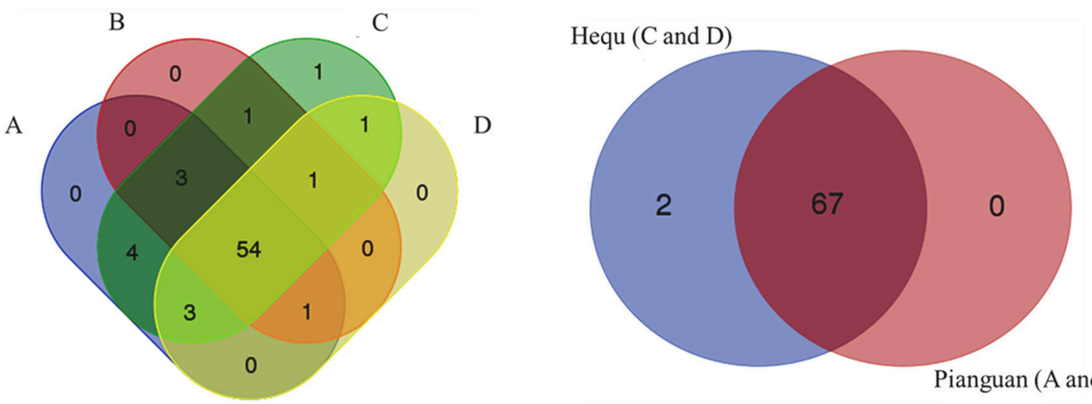

FIGURE 10 | Venn diagram analysis of the logic relationships of the OTUs identified in the different samples. The left panel represented the OTUs from the samples fermented with different cereal grains, A: millet and a small amount of rice; B: millet; C: white proso millet; and D: red proso millet. The right panel represented the OTUs from the samples from two different counties: group A and B from Pianguan county, and group C and D from Hequ county.

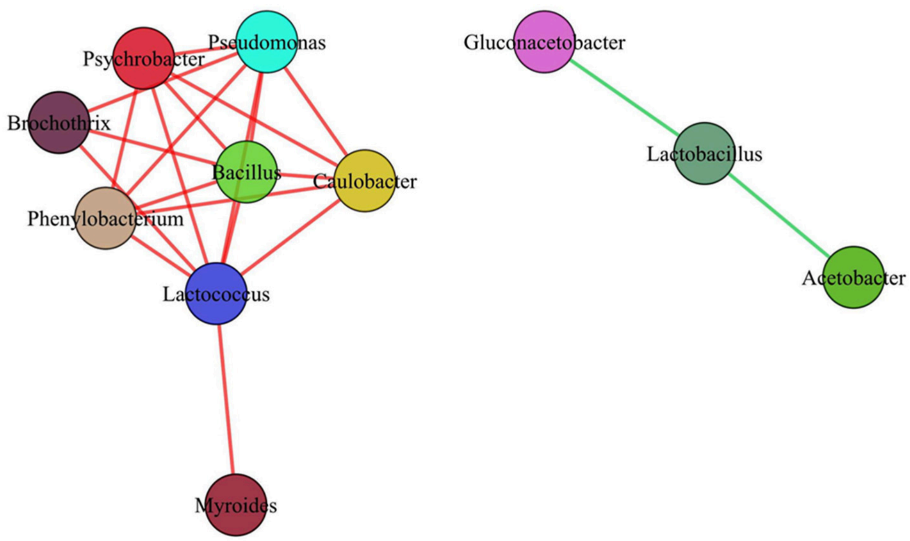

FIGURE 11 | Implications of the antagonistic or synergistic interactions between the microbial populations. Green lines represented the antagonistic relationship between the two microbial populations. Red lines represented the synergistic relationship between the two microbial populations.

of pH value, organic acids, and free amino acids, indicating that *Lactobacillus* species might be one of the key microorganisms for Suanzhou fermentation. In future, co-fermentation with the isolated bacteria and yeast strains will be conducted to select the appropriate candidate microorganisms for possible applications.

Millet is seldom reported as raw material for preparing fermented foods. *Ben-saalga* is a traditional fermented food in Burkina Faso of Africa. It's made from pearl millet by two fermentations, one happens in the step of grains soaking and the other happens in the settlement step of wet flour. Indian *dosa* is another traditional fermented food made from co-fermentation of finger millet and horse gram flour. Both foods are prepared from flour, while Suanzhou is prepared from intact grains. All the three foods are rich in amino acids and can provide diet proteins to infants and young children. With culture-dependent methods, LAB and yeasts were found dominant in both the fermented foods, similar to our findings. However, no molecular identification of the isolates was conducted in both studies.

In conclusion, Suanzhou gruel displayed low acidity and relatively high-level of free amino acids and organic acids. The microbiota in Suanzhou was rich in *Lactobacillus*, *Acetobacter*, *Pichia*, and *G. geotrichum* strains. The characteristics of Suanzhou can assure the fermented food to be devoid of many environmental molds, yeasts, and bacteria pathogens and keep the Suanzhou food safely for consumption. The isolated strains may be further characterized and used as starters in the industrial production of Suanzhou food and other applications.

AUTHOR CONTRIBUTIONS

HQ carried out the bioinformatic analysis and the experiments and wrote the manuscript. QS and XP performed the bioinformatic analysis. ZQ designed the experiments. HY designed the experiments and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01311

Table S1 | Biochemical analysis of the acid-gruel samples.

Figure S1 | The collector retraction curves of the observed species, Chao1, and Shannon indice of the metagenomic libraries of the 24 Suanzhou samples.

Figure S2 | The OTUs distributions in the 24 Suanzhou samples.

Figure S3 | Growth curves of the isolated lactic acid bacteria.

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REVIEW
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The Biotechnology of *Ugba*, a Nigerian Traditional Fermented Food Condiment

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Legumes and oil bean seeds used for the production of condiments in Africa are inedible in their natural state; they contain some anti-nutritional factors especially undigestible oligosaccharides and phytate. Fermentation impact desirable changes by reducing anti-nutritional factors and increasing digestibility. *Ugba* is an alkaline fermented African oil bean cotyledon (*Pentaclethra macrophylla*) produced by the Ibos and other ethnic groups in southern Nigeria. Seen as a family business in many homes, its preparation is in accordance with handed-down tradition from previous generations and serves as a cheap source of plant protein. Its consumption as a native salad is made possible by fermentation of the cotyledon for 2–5 days, but could also serve as a soup flavoring agent when fermentation last for 6–10 days. The fermentation process involved is usually natural with an attendant issue of product safety, quality and inconsistency. The production of this condiment is on a small scale and the equipment used are very rudimentary, devoid of good manufacturing procedures that call to question the issue of microbial safety. This paper therefore reviews the production process and the spectrum of microbial composition involved during fermentation. In addition, potential spoilage agents, nutritional and biochemical changes during production are examined. Furthermore, information that can support development of starter cultures for controlled fermentation process in order to guarantee microbiological safety, quality and improved shelf life are also discussed.

Keywords: microbiology, *Ugba*, fermentation, condiment

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INTRODUCTION

Ugba, a product of alkaline fermentation of oil bean seeds (*Pentaclethra macrophylla*) is very popular among the Ibos and other ethnic groups in southern Nigeria. The product serves both as a delicacy and a food flavoring agent. As an important nutritional item, *ugba* is very rich in protein. It similarly plays an economic, social and cultural role among the Ibos in the eastern part of Nigeria. The production of *ugba* is usually pursued as a family business that has become an art that is handed over from one generation to another.

The processing of these large brown glossy seeds of the African oil bean (**Figure 1**) to obtain *ugba* is usually by natural fermentation, a process that involves microbiological and biochemical changes, caused by hydrolysis and desirable changes. This process is usually influenced by the raw materials and the processing method with variations observed from one production batch or producer to another (Steinkraus, 1983).

FIGURE 1 | African oil bean seed (A), Dehulled seeds of African oil bean (B) and Processed slices of the African oil bean cotyledon (C). (Okorie and Olasupo, 2013a).

Most studies on African fermented foods have focused on isolation and identification of desirable microorganisms involved in the fermentation process. The general consensus from these studies is that fermented African oil bean seeds during *ugba* fermentation is predominantly brought about by bacteria identified as *Bacillus* species (Odunfa, 1981; Obeta, 1983; Isu and Ofuya, 2000; Okorie and Olasupo, 2013a; Eze et al., 2014). Other groups of bacteria have also been implicated in the fermentation of this product and they include species of *Escherichia, Proteus, Micrococcus, Staphylococcus, Streptococcus, Alcaligenes, Pseudomonas, Corynebacterium,* and *Enterococcus* (Oyeyiola, 1981; Odunfa, 1986; Sanni et al., 2002; Okorie and Olasupo, 2013a). No fungi or yeast species have been implicated in the fermentation of *ugba*.

There is very little information on the occurrence and growth of pathogens in African fermented foods. The natural fermentation process used routinely for *ugba* production allows participation of diverse microorganisms. The involvement of pathogenic and spoilage microorganisms during production cannot be totally ruled out, especially if fermentation takes place under very poor hygiene conditions and sanitation, which is a very common occurrence in West Africa. Product inconsistency as a result of mixed-culture processing and post-fermentation contamination constitutes a major challenge to microbial safety and quality of this product.

Production of *ugba* in Nigeria is still on a small scale industrial process involving production at the household level where there is little or no consideration for good manufacturing practices (GMP) and sanitation (Olasupo et al., 2002; Gadaga et al., 2004). Consequently, microbiota responsible for fermentation is often unpredictable and equipment used is rudimentary. Similarly poor hygiene of handlers, lack of portable water and other raw materials often introduce spoilage and pathogenic microorganisms. All these factors affect the quality of the final product and ultimately the health of the consumers. Fermentation period is chosen according to human judgment and varies from one manufacturer to the other. The lack of standardization in the production process often results in product inconsistency and quality variation.

Lactic fermentation is noted to be a major mode of food processing used to achieve preservation and improve shelf life of foods especially in the West African sub-region, where cereals and tubers are processed to variety of foods. This practice has been very reliable in terms of maintaining quality and safety of food especially at the household level where many of the traditional foods are produced (Steinkraus, 1983). Unfortunately, alkaline fermentation of legumes is about hydrolysis of proteins and release of amino acids and ammonia responsible for the pungent smell as well as characteristic flavor. This preservative influence of condiments after fermentation appears to be limited;

similar observation has been reported during the processing of fermented African oil bean seeds. The unfermented seeds are much more stable with longer shelf life than the fermented products. Fermentation thus leads to flavor enhancement, complex molecules reduction (oligosaccharides and proteins) but reduces the shelf life of the seeds and exposes the product to post fermentation contamination (Mbajunwa et al., 1998; Oguntoyinbo et al., 2007). Post processing techniques proposed for condiment production in Africa include drying and salting of final product (Achi, 2005; Eman, 2009). However, while these methods could increase shelf life considerably, it is characterized with inherent disadvantages such as loss of volatile compounds and vitamins. Also, the consumption of salt in diet has been identified as having deleterious effects on human health, responsible for cardiovascular diseases in the West African sub-region (Brown et al., 2009; He and MacGregor, 2009; Strazzullo et al., 2009).

Since fermentation of African oil bean seeds increases pH toward alkalinity (pH 8) (Odunfa, 1985a; Sanni and Oguntoyinbo, 2014), the anti-microbial effect often associated with most fermented food due to lowering of pH to acidity is lacking in this product. It is therefore possible that some organisms that are of public health concern could survive the fermentation process. Whether the presence of these organisms is as a result of post-fermentation contamination or they survive the fermentation process, their presence in the product portends great danger to the consuming public. The risk is particularly high also because the product can be eaten without pre-heating. The alkaline pH selects and encourages the dominance of *Bacillus* species. This has been consistently reported to be due to production of peptides, amino acids and ammonia during the hydrolysis of the cotyledons.

Recently, Oguntoyinbo (2014) reported that very little attention is placed on the type of packaging used for many traditional foods in West Africa. Unhygienic and substandard packaging materials can engender easy contamination by hazardous materials, including biological, physical, and chemical hazard of well-prepared foods during preservation. *Ugba* is usually wrapped in leaves (in most cases banana leaf), and nylon bags and sold to the public. These packaging materials could be the source of contamination of the product.

Many of the agricultural raw materials used for the preparation of traditional W. African food products contain endogenous toxins (Kar and Okechukwu, 1978; Okorie and Olasupo, 2014). However, studies have shown that fermentation drastically reduces anti-nutritional factors in many fermented legumes-based foods (Oboh et al., 1998; Khan et al., 2012; Okorie and Olasupo, 2014). It is well known that these foods contain naturally occurring toxins and anti-nutritional compounds. The removal of anti-nutrients from Nigerian fermented food is an important step in ensuring toxicological safety and quality. Fermentation plays significant roles in detoxification of substrates; for instance, removal of toxins during *kawal* production, through the fermentation of the leaves of *Cassia obtusitfolia* in Sudan has been shown to improve safety quality and acceptability (Egwim et al., 2013; Taylor and Duodu, 2015).

Most of the legumes and oil seeds used for the production of condiments are inedible in their unfermented state because they suffer from one drawback or the other. For instance, legumes are a particularly rich source of natural toxicants, including proteinase inhibitors, amylase inhibitors, metal chelates, flatus factors, hemagglutinins, saponins, cyanogens, lathyrogens, tannins, allergens, acetylenic furans, and isoflavonoid phytoalexins (Issoufou et al., 2013; Oguntoyinbo, 2014). The unfermented African oil bean seeds contain a number of anti-nutritional and /or toxic factors including saponins, alkaloids (alkaloid paucine), sterols, glycosides, and growth depressant caffeolyputrescine, but no hemagglutinins (Kar and Okechukwu, 1978).

Understanding the biotechnological principles during fermentation of African oil bean seeds is a crucial strategy for the process optimization of fermented condiments in West Africa. The understanding of the microbiological dynamics, biochemical kinetics and toxicology during fermentation will significantly impact product quality, safety and acceptability. The foregoing has been a review of the different scientific literatures relevant to biotechnology of *ugba* production in Nigeria and highlighted relevant strategies toward process improvement. In addition, current condiment food safety issues are discussed.

PRODUCTION PROCESS

The production process of *ugba* is shown in **Figure 2**. It has been previously described as alkaline fermentation of the seeds of the African oil bean tree (Ikenebomeh et al., 1986; Sanni et al., 2002; Ogueke et al., 2010). Although the production method varies from one community to the other and from one processor to another, a similar end-product, which usually comes with pungent ammonia-like smell is commonly produced across South Eastern Nigeria (Nwokeleme and Ugwuanyi, 2015). There is variation in boiling time and the procedure that aided dehulling of the seeds. Obeta (1983) reported 16–18 h of boiling, Odunfa and Oyeyiola (1985) and Odunfa (1986), reported initial 12 h boiling time, while Njoku and Okemadu (1989), boiled the seeds for 5–8 h. However, Sokari and Wachukwu (1997) used toasting of the bean seeds in hot (ca. 100°C) sand and holding for a further 30 min at 100°C to dehull the seeds. After dehulling, cotyledons are either sliced or cooked for 30 min or longer. Odunfa and Oyeyiola (1985) reported overnight boiling before soaking and slicing. In the fermentation process, varied methods are used. Odunfa and Oyeyiola (1985) reported that the cotyledons are mixed with salt (sodium chloride ca.1–2 w/w), put in a clean pot, covered and fermented for up to 5 days at room temperature, with or without salt. On the other hand, Sokari and Wachukwu (1997) reported that sliced cotyledons were washed and allowed to drain for ½-1 h, in a basket lined with banana leaves (*Musa sapientum Linn*.) and later wrapped (about 40–50 g of slices per wrap) using another leaf (*Mallotus oppositifolius*) and incubated for 72 h at room temperature.

However, the essential steps in the production of this product are similar and as shown in **Figure 2**. The differences in the various processing methods described could be responsible for the variations in the products quality observed from one community to the other. The fermented bean slices at the end of the fermentation process are kept near smoldering firewood to develop the characteristic *ugba* flavor and the product is

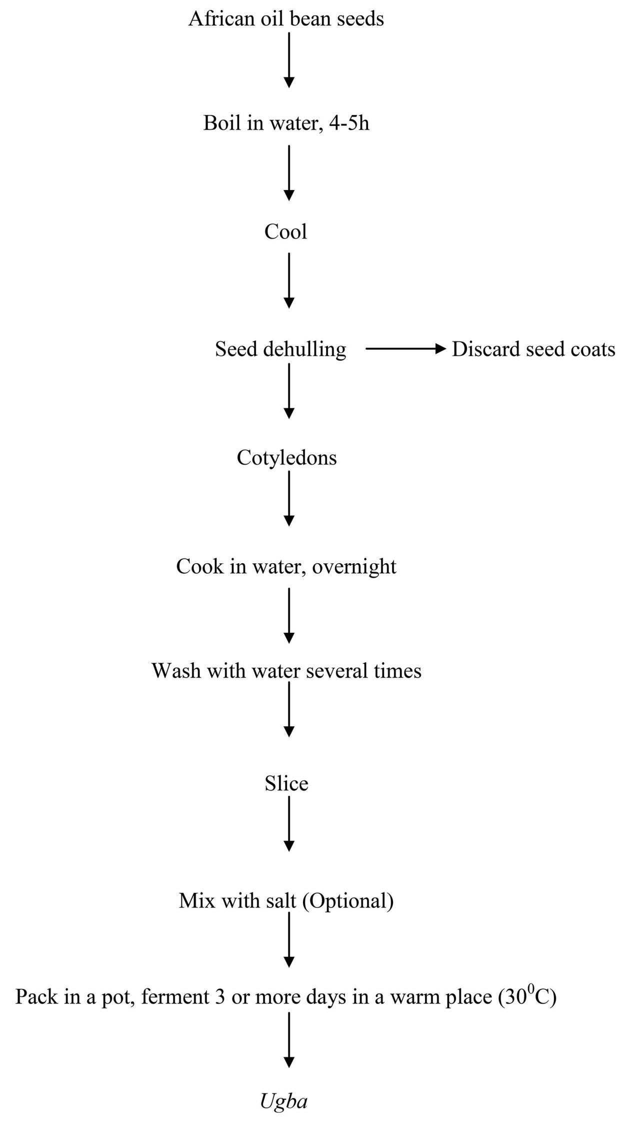

FIGURE 2 | Flow chart for the preparation of *ugba* (Odunfa and Oyeyiola, 1985).

consumed as native salad. However, fermentation for a longer period of time (6–10 days) produces very soft *ugba* which is used as soup flavoring (Odunfa and Oyeyiola, 1985; Sanni et al., 2002). Irrespective of which method is employed in the processing, one major drawback observed is the drudgery involved in the slicing process.

MICROBIOLOGICAL CHANGES DURING FERMENTATION

The microbiota in fermenting food matrix is a function of the hygienic status of the production environment, the utensil and raw materials used and the handlers. The traditional fermentation method employed in the processing of *ugba* is by chanced inoculation. The microbial interaction in its production is therefore determined by the microbiological status of the raw materials, utensils, handlers and the production environment. Daeschel et al. (1987) and Ling et al. (2013) noted that the dynamics of fermentation in any food matrix is a complex microbiological process involving interactions between different microorganisms. During fermentation of African oil bean seeds, dominant microorganisms capable of enzymatic hydrolysis are responsible for the biochemical and nutritional changes which constitute the observable changes especially in the chemical composition and taste of the final product.

Several works have been carried out on the microbiological changes during fermentation of African oil bean seed for *ugba* production (Obeta, 1983; Odunfa and Oyeyiola, 1985; Ejiofor et al., 1987; Ogueke and Aririatu, 2004; Enujiugha and Akanbi, 2008; Nwagu et al., 2010; Okorie and Olasupo, 2013a). The major fermenting microorganisms involved in the fermentation process have been identified to be proteolytic *Bacillus* species identified as *B. subtilis* (which is the most predominant), *B. licheniformis*, *B. megaterium*, *B. macerans*, and *B. circulans* (Obeta, 1983; Sanni, 1993; Isu and Ofuya, 2000; Sanni et al., 2002). The endospores of these bacilli must have been associated with the cotyledons from the beginning of the fermentation. Due to high level of hydrolytic enzyme production by *Bacillus* species, all the species have been reported to have one or more enzymatic hydrolytic properties during legume fermentation (Aderibigbe et al., 1990; Sanni et al., 1999; Oguntoyinbo et al., 2007). However, it appears that *B. subtilis* is the most adapted and dominant with properties such as higher protease and amylase production, production of poly glutamic acid (responsible for mucilage production), pyrazine and antimicrobial such as subtiliosin production (Oguntoyinbo et al., 2007).

Protein has been identified as one of the major components of African oil bean cotyledon (Obeta, 1983). Metabolic and enzymatic hydrolysis of *Bacillus* species serves to break down the protein into amino acids (Isu and Njoku, 1997). Odunfa and Oyewole (1986) and Ghosh et al. (2013) observed that all the *Bacillus* species that have been associated with the fermentation of the oil bean seeds are mainly proteolytic, and 97.3% of these *Bacillus* species are also lipolytic. Proteolysis is therefore the major biochemical activity during the fermentation and has been found to increase constantly during the fermentation of *ugba* and the other food condiments (Odunfa, 1986; Wang and Fung, 1996; Oguntoyinbo et al., 2007). Also, a corresponding increase in the population of *Bacillus* species is reported from the *beginning* of the fermentation process till the end (Ogueke and Aririatu, 2004).

Other groups of organisms that have been found to be associated with the fermentation of this condiment include *Escherichia* species, *Proteus, Pediococcus, Micrococcus, Staphylococcus, Streptococcus, Alcaligenes, Pseudomonas, Corynebacterium, Enterococcus* (Odunfa, 1981; Antai and Ibrahim, 1986; Ogbadu and Okagbue, 1988; Njoku and Okemadu, 1989; Suberu and Akinyanju, 1996; Ogbonna et al., 2001; Okorie and Olasupo, 2013a).

Staphylococcus spp. and *Micrococcus* spp. are very active at the early stage of the fermentation process. They rapidly multiply within 24 h of fermentation and then decrease as fermentation progresses. *Escherichia* species, *Proteus* and

Pediococcus are generally observed to play a minor role in the fermentation process (Odunfa, 1985a) while *Staphylococcus* sp. and *Micrococcus* sp. play a subsidiary role in the production process (Obeta, 1983; Odunfa and Komolafe, 1989).

Apart from proteolysis, other important biochemical changes mediated by microorganisms during the production of this condiment include production of flavor enhancing compounds, production of vitamins and essential fatty acids, and degradation of indigestible oligosaccharides responsible for flatus factors. A reduction in the contents of stachyose, raffinose, and melibiose in fermented soy bean cotyledon during *kinema* production was previously reported (Sarker et al., 1997). Significant increases in thiamine and riboflavin have been observed in *ugba*, and these have been ascribed to riboflavin synthase associated with *Bacillus subtilis* (Odunfa, 1986). These reductions are ascribed to sucrase activities of the *Bacillus* group Aderibigbe and Odunfa (1990) and possibly by the alpha galactosidase activities of the other microorganisms in the fermenting mash, especially *Staphylococcus* sp. and LAB among which alpha galactosidase activities are common (Odunfa and Oyewole, 1998).

Members of the *Enterobacteriaceae* have also been associated with the ecology of fermenting plant proteins (*ugba* inclusive) especially at the early stages of production (Mulyowidarso et al., 1989; Achi, 1992; Okorie and Olasupo, 2013a). These species do not survive until the end of the fermentation, presumably because of the modified environment. It is evident that production of this fermented condiment is initially mediated by a diverse microbial flora, which eventually becomes Gram-positive flora (a reflection of many African fermented foods; Odunfa, 1985b).

NUTRITIONAL CHANGES ASSOCIATED WITH FERMENTATION OF AFRICAN OIL BEAN SEED

Fermentation has been generally observed to improve the nutritional quality of the products obtained. The protein content, essential amino acids, vitamins and mineral contents of most fermented foods have been shown to increase during fermentation.

Fermented foods and beverages harbor diverse microorganisms from the environment, including mycelia molds, yeasts, and bacteria, mostly lactic acid bacteria and micrococci. These microorganisms transform the chemical constituents of raw materials during fermentation and enhance the nutritional value of the products. The activities of these microorganisms are noted to enrich bland diets with improved flavor and texture; preserve perishable foods; fortify products with essential amino acids, bioactive compounds, vitamins, and minerals for healthy living. They also bring about degradation of undesirable compounds and anti- nutritive factors; imparts antioxidant and antimicrobial properties; improve digestibility; and stimulate probiotic functions. While fermentation results in a lower proportion of dry matter in the food product, the concentration of the vitamins, minerals, and protein appear to increase when measured on dry weight basis (Adams, 1990; Chung et al., 2010; Shil et al., 2010; Savadogo et al., 2011; Makanjuola and Ajayi, 2012; Okechukwu et al., 2012; Olakunle and Adebayo, 2012; Tofalo et al., 2012).

African oil bean seeds support diet and improve nutritional availability. Proximate analysis of raw oil bean seed reveals that it is mainly composed of proteins (36–42%), lipids (43–47%) and carbohydrates (4–17%; Odunfa and Oyeyiola, 1985; Njoku and Okemadu, 1989; Ogueke and Aririatu, 2004).

Slight increases in the crude protein and ash contents of the fermented beans have been reported. Enujiugha (2003) reported a steady increase in the level of amino nitrogen from 1.23 mg/Ng-1 DM at the start of fermentation to 13.68 mg/Ng-1 DM after 72 h. The amino acid component of the fermented seed has been shown to contain the 20 essential amino acids (**Table 1**). The high content of the essential amino acids makes the seed a potential source of protein (Achinewhu, 1982).

Glutamic acid appears to be the largest amino acid contained in the seed and its fermented product. This may be responsible for its use as a flavoring agent for soups in south eastern Nigeria. Aspartic acid, lysine and phenylalanine are also present in appreciable amounts in the fermented seeds. In their study of compositional changes in oil bean seeds observed during thermal treatment, Enujiugha and Akanbi (2005) reported a reduction of the protein content from 22.32% dry wt. in the raw seeds to 19.00% dry wt. in the canned product (**Table 2**). Each processing step brought about a decrease in levels of anti-nutritional factors analyzed. Oxalates, tannins and phytic acid were reduced from 2.79 mg/g, 0.38 g/100 g, and 2.11 g/100 g in the raw seeds to 0.81 mg/g, 0.22 g/100 g, and 1.16 g/100 g in the canned product, respectively.

The oil component of the seed contains about 75% of saturated fatty acids and 25% of unsaturated fatty acids (Kar

TABLE 1 | Amino acid content (g/100 g protein) of African oil bean seeds.

Amino acids	Content
Aspartic acid	7.95–10.30
Threonine	3.27–4.17
Serine	4.80–5.54
Glutamic acid	9.32–11.60
Proline	2.90–5.77
Glycine	3.84–4.62
Alanine	3.81–4.70
Cysteine	1.10–4.80
Valine	4.90–6.60
Methionine	0.90–1.80
Isoleucine	3.30–4.88
Leucine	5.30–6.68
Tyrosine	1.80–5.58
Phenylalanine	5.01–7.00
Lysine	5.46–6.97
Histidine	1.53–2.44
Arginine	4.70–6.53
Tryptophan	1.15–1.78

Source: Mba et al. (1974) and Achinewhu (1982).

TABLE 2 | Effect of processing on the proximate chemical composition of African oil bean seeds (mean ± s.d.).

Sample	Components (% dry wt)				
	Crude protein	Oil	Crude fiber	Ash	Carbohydrate
Raw	22.32 ± 0.37	53.98 ± 0.99	2.13 ± 0.55	2.40 ± 0.11	19.16 ± 0.76
Cooked	19.15 ± 0.13	58.95 ± 0.46	3.26 ± 0.04	1.43 ± 0.13	17.49 ± 0.46
Fermented	17.13 ± 0.21	61.35 ± 1.21	2.93 ± 0.11	1.11 ± 0.04	17.48 ± 1.07
Canned	19.00 ± 0.19	60.11 ± 0.86	3.27 ± 0.12	2.37 ± 0.17	15.26 ± 1.04

Source: Enujiugha and Akanbi (2005).

TABLE 3 | Fatty acid composition of African oil bean seeds*.

Composition	Values
Yield of oil (%)	43.3
SATURATED FATTY ACIDS	
Palmiitic acid	3.4
Behenic acid	5.2
Lignoceric acid	12.0
UNSATURATED FATTY ACIDS	
Oleic acid	29.0
Linoleic acid	42.8
Linolenic acid	3.2
Gadoleic acid	0.28

**As percentage of total oil.*
Source: Achinewhu (1982).

TABLE 4 | Mineral and vitamin content of unfermented and fermented *ugba*.

Component (mg/100 g)	Unfermented ugba	Fermented ugba
MINERALS		
Phosphorus	172	–
Calcium	192	110
Iron	16	3.3
VITAMINS		
Thiamin	0.07	0.07
Riboflavin	0.32	0.30
Niacin	0.90	0.30

Source: Duke (1981).

TABLE 5 | Changes in mineral contents of African oil bean seeds during processing (mg/kg dry wt).

Mineral	Raw	Cooked	Fermented	Canned
P	351.89 ± 2.58	317.92 ± 2.24	291.02 ± 0.53	176.06 ± 12.69
K	127.19 ± 7.99	175.80 ± 12.46	110.39 ± 6.18	156.67 ± 11.49
Na	184.98 ± 12.31	113.49 ± 2.17	172.06 ± 9.42	168.57 ± 7.30
Ca	314.30 ± 11.32	329.29 ± 11.35	208.92 ± 14.37	404.54 ± 13.34
Mg	292.05 ± 9.86	479.37 ± 5.61	334.98 ± 11.07	397.03 ± 2.02
Zn	9.78 ± 0.61	13.47 ± 0.28	9.23 ± 0.78	15.41 ± 1.98
Fe	56.28 ± 5.42	56.80 ± 1.39	42.46 ± 1.02	42.48 ± 3.19
Mn	23.99 ± 3.06	27.71 ± 1.69	26.87 ± 0.36	15.60 ± 2.75

Source: Enujiugha and Akanbi (2005).

and Okechukwu, 1978; **Table 3**). For the saturated fatty acids, lignoceric acid appears to be present in the largest amount constituting about 12% of the total fatty acid concentration, while palmitic acid is the least with 3.4%. The major unsaturated fatty acid in the seeds is linoleic acid constituting 42.8%. Oleic acid is also present in appreciable amounts (29.0%). Linolenic and gadoleic acids are present in very small amounts (3.2 and 0.28%, respectively). The presence of appreciable amounts of behenic and lignoceric acids is not desirable for edible oils (Odunfa, 1986). However, Odoemelam (2005) noted that the high degree of unsaturation makes it suitable for cooking purposes and for use as a drying oil for cosmetics, paints and varnishes.

Fermentation has been found to have minimal effect on the fatty acid content of the oil bean seed. (Onwuliri et al., 2004) reported that fatty acid concentrations did not change appreciably with processing and fermentation. Enujiugha and Akanbi (2005) however observed an increase in the oil content from 53.98 to 60.11%. Information available shows that fatty acid content of the oil bean seeds is not qualitatively affected by fermentation. The principal fatty acid linoleic acid however has been shown to increase from 60.68 to 67.57% of the total fatty acids while oleic acid decreased from 26.95 to 22.59% during fermentation. Palmitic acid and other saturated fatty acids in the seed oil are also slightly affected by fermentation.

Available information shows that the vitamin content of the seeds is low while they are a poor source of calcium and phosphorus (Duke, 1981). The mineral and vitamin contents are observed to decrease during fermentation (**Table 4**). The niacin and riboflavin of the seeds have been found to decrease during fermentation. Enujiugha and Akanbi (2005) noted that fermentation and canning significantly ($P < 0.05$) reduced the phosphorus and iron contents of the seeds while processing generally raised the calcium and magnesium contents (**Table 5**).

CHEMICAL AND BIOCHEMICAL CHANGES ASSOCIATED WITH FERMENTATION OF AFRICAN OIL BEAN SEEDS

The major biochemical changes that take place during the fermentation of African oil bean seeds have been shown to be proteolysis. During the process, the protein component of the cotyledons is hydrolyzed to amino acids. *Bacillus* species are the predominant bacteria during fermentation. Protease activity has been shown to rapidly increase from the start of the fermentation period till the end (Odunfa, 1985a).

Another biochemical change that has been shown to occur during the fermentation of oil bean seeds is lipid hydrolysis. Lipids are usually hydrolyzed to fatty acids by lipases. However, though lipids are one of the major components of the oil bean seeds (43–47%), lipolytic activity is reported to be low during the fermentation of the oil bean seeds (Achinewhu, 1986; Njoku and Okemadu, 1989; Onwuliri et al., 2004). Enujiugha (2003) found out that the principal fatty acid of the seeds, linoleic acid, increased from 60.68 to 67.57% of the total fatty acids while oleic acid decreased from 26.95 to 22.59% during fermentation.

Carbohydrates constitute about 4–17% of the total components of the oil bean seed and the major sugars identified in the bean are oligosaccharides hydrolyzed by amylases (Achinewhu, 1982). These are oligosaccharides that are hydrolyzed by amylases to simple sugars during the fermentation process. Monago et al. (2004) observed that the content of this carbohydrate decreased significantly as fermentation time increased.

Obeta (1983) found out that pH increased from 6.5 at 0 h to 9.0 at 48 h and declined to 7.1 at 72 h. The rise in pH has been attributed to the abundant production of ammonia during the fermentation due to protein hydrolysis and deaminase activity.

Also, moisture content has been found to increase throughout the period of fermentation (52–56.90% to 71.20–73%; Odunfa and Oyeyiola, 1985; Njoku and Okemadu, 1989; Ogueke and Aririatu, 2004). The increase in moisture is believed to be due to the hydrolytic activities of the microorganisms. However, Odunfa and Oyeyiola (1985) and Ogueke and Aririatu (2004) believe that the high moisture level brought about by fermentation predisposes the product to rapid spoilage.

ANTI-NUTRITIONAL CONTENT OF *Ugba*

The African oil bean seeds are inedible in its unfermented state because it suffers from some drawbacks. Little is known about anti-nutritional factors in the raw and fermented African oil bean seeds. Although, Kar and Okechukwu (1978) and Enujiugha and Agbede (2000) reported the presence of a number of anti-nutritional and /or toxic factors, our recent studies (**Table 6**), have revealed the detection of tannins, saponins, alkaloids, steroids, glycosides, flavonoids, and phytate in the unfermented African oil bean seed (Okorie and Olasupo, 2014). This study also showed that processing and fermentation drastically reduced the content of these toxic factors in the fermented product (**Table 7**) (Okorie and Olasupo, 2014), mainly due to soaking of the seeds overnight and washing in water before fermentation. This had a significant effect on all the phytochemicals/anti-nutritional factors identified. Tannin was reduced from 12.58 to 3. 65 mg/100 g, saponin from 52.00 to 22.00 mg/100 g, phytate from 25.63 to 14.47 mg/100 g, glycosides from 34.76 to 11.33 mg/100 g, alkaloids from 2.52 to 0.14 mg/100 g, flavonoids from 4.66 to 2.49 mg/100 g and steroids from 26.48 to 5.43 mg/100 g. Alkaloids and tannins were completely removed from the samples after 24 and 48 h of fermentation respectively.

TABLE 6 | Preliminary assay for anti-nutritional factors and phytochemicals in African oil bean seed (Okorie and Olasupo, 2014).

Phytochemical	Processing method		Fermentation period (h)		
	Unsoaked	Soaked	24	48	72
Tannin	+++	+	−	−	−
Saponin	+++	++	+	+	+
Flavonoid	+++	+	+	+	+
Alkaloid	++	−	−	−	−
Steroid	++	+	+	+	+
Glycoside	+++	+	++	+	+

+++, very high; ++, high; +, low; −, absent.

MICROBIOLOGICAL SAFETY OF FERMENTED AFRICAN OIL BEAN SEEDS

Most works on African fermented foods (*ugba* inclusive) have centered on the isolation and characterizations of organisms involved in the fermentation processes. Not much effort seems to have been made toward the occurrence and growth of possible pathogens in the product. However, Adewunmi et al. (2014) used a combination of genome-based culture dependent and independent techniques to examine *iru* microbiota and reported bacterial species with both spoilage and pathogenic history. In addition, genome typing of *Bacillus* species isolated from *okpehe* and *soumbala* identified species of *Bacillus cereus* with enterotoxin production potential (Ouaba et al., 2008; Oguntoyinbo et al., 2010). It is therefore very important to use genotypic method in combination with phenotypic data to assess microbial quality of fermenting *ugba*, in order to guarantee its microbial safety. Furthermore, because of the stress associated with the food processing, it would be important to use culture dependent and independent methods in order to find/detect non-culturable or not yet cultured microorganisms. Available information in literature shows that organisms such as *E. coli, Staphylococcus aureus* and other members of the *Enterobacteriaceae* have been isolated from condiments in West Africa (Isu and Njoku, 1997; Okorie and Olasupo, 2013a).

SELECTION OF STARTER CULTURES FOR CONTROLLED FERMENTATION OF *Ugba*

The traditional method of production of *ugba* involves natural solid state fermentation of the African oil bean seeds. This chanced inoculation method has the inherent drawback of possible growth and occurrence of pathogens in the final product. Although, microbiota that best adapted brings about the final product, variation in final product due to fermentation time and unhygienic handling does affect the product and its consistency.

Selection and application of starter cultures in the production process has been identified as critical to the elimination of

TABLE 7 | Effect of soaking and fermentation period on the anti-nutritional/phytochemical contents of African oil bean seed.

Phytochemical (mg/100 g)	Soaking period (h)					Fermentation period (h)			
	0	6	12	18	24	0	24	48	72
Tannin	12.58	10.26	7.02	4.63	3.65	3.65	1.79	0.46	0.00
Saponin	52.00	49.56	40.23	34.29	22.00	22.00	16.06	8.00	2.00
Flavonoid	4.66	4.02	3.46	2.96	2.49	2.49	1.96	1.10	0.43
Alkaloid	2.52	1.94	1.03	0.76	0.14	0.14	0.06	0.00	0.00
Steroid	26.48	12.06	8.68	6.97	5.43	5.43	3.68	2.96	2.07
Glycoside	34.76	30.54	22.09	17.78	11.33	11.33	8.64	5.71	0.78
Phytate	25.63	22.06	18.34	15.69	14.47	14.47	8.67	1.26	0.15

Source: Okorie and Olasupo (2014).

pathogens and spoilage microbes (Holzapfel, 2002). Several efforts have been made on the selection and application of starter cultures in a controlled fermentation of some fermented condiments including *ugba* in Nigeria. Oguntoyinbo et al. (2007) used a combination of highly proteolytic and bacteriocin producing starter cultures for the production of *okpehe*, a fermented *Prosopis africana* cotyledon. Isu and Ofuya (2000) studied the use of pure cultures of *Bacillus subtilis* attached to cowpea and maize granules in the fermentation process of *ugba*. They monitored changes in pH, amino-nitrogen and protease activity as fermentation indicators, carried out with the immobilized cells. Protease activity increased from 4.5 to 27.65 mg N/min for the immobilized cells with respect to 10.5 mg N/min produced by the natural fermentation, and there was a reduction in the fermentation time to 48 h as compared to 96 h for the natural fermentation process.

Okorie and Olasupo (2013b) developed controlled fermentation of *ugba* using *B. subtilis* and *B. lichenformis* singly and as mixed cultures fermentation. The process fermentation time was reduced from 96 to 48 h. *Ugba* produced with the starters were similar in terms of color, taste and nutritional content to those produced by natural fermentation.

Several other attempts have been made to control the fermentation of this product with similar results as stated above (Ogueke and Aririatu, 2004; Eze et al., 2014). There, however, still exists a need for more field application and extension of starter cultures to small and cottage processors of condiments in Nigeria.

CONCLUSION

Ugba is an important part of the diet of the Ibos and other ethnic groups in the eastern and southeastern parts of Nigeria. It is produced through a natural solid state fermentation of the oil bean seeds. The major microorganisms involved in the process are *Bacillus* species. These microorganisms metabolize the protein content of the seeds into free amino acids and ammonia, having undergone a biochemical reaction during the fermentation process known as proteolysis.

Fermentation of the oil bean seeds leads to increase in the nutritional values of the product. The natural process of its production, and the subsistent level at which the condiment is being produced leaves the safety of this product in doubt and makes its quality inconsistent. Efforts at controlled fermentation of the product have shown that some of these observed drawbacks could be overcome by the application of starter cultures in the production process. There is therefore a need to make the local processors of this product realize the potential benefits derivable from the application of starter cultures in their process line.

AUTHOR CONTRIBUTIONS

NO: Participated in conception, literature search, design and write up. CO: Participate in literature search, design and write up. FO: Participated in literature search, design and write up.

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Exploring the Bacterial Microbiota of Colombian Fermented Maize Dough "Masa Agria" (Maiz Añejo)

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Masa Agria is a naturally fermented maize dough produced in Colombia, very common in the traditional gastronomy. In this study we used culture-dependent and RNA-based pyrosequencing to investigate the bacterial community structure of *Masa Agria* samples produced in the south west of Colombia. The mean value of cell density was 7.6 log CFU/g of presumptive lactic acid bacteria, 5.4 log cfu/g for presumptive acetic bacteria and 5.6 og CFU/g for yeasts. The abundance of these microorganisms is also responsible for the low pH (3.1–3.7) registered. Although the 16S rRNA pyrosequencing revealed that the analyzed samples were different in bacteria richness and diversity, the genera *Lactobacillus*, *Weissella,* and *Acetobacter* were predominant. In particular, the most common species were *Lactobacillus plantarum* and *Acetobacter fabarum*, followed by *L. fermentum, L. vaccinostercus,* and *Pediococcus argentinicus.* Several microorganisms of environmental origin, such as *Dechloromonas* and most of all *Sphingobium* spp., revealed in each sample, were detected, and also bacteria related to maize, such as *Phytoplasma.* In conclusion, our results elucidated for the first time the structures of the bacterial communities of *Masa Agria* samples obtained from different producers, identifying the specific dominant species and revealing a complete picture of the bacterial consortium in this specific niche. The selective pressure of tropical environments may favor microbial biodiversity characterized by a useful technological potential.

Keywords: fermented maize, pyrosequencing, *Lactobacillus*, *Acetobacter*, dough

INTRODUCTION

Maize or corn (*Zea mays*), the "gift of the goddess" of the ancient Amerindians, is a cereal crop that is produced in a wide range of agro-ecological environments worldwide. Archeological evidence from the central Andes indicates that the role of maize changed between A.D. 500 and 1500, shifting from a culinary item, simply prepared by boiling, to a more complex symbolic food with elaborated political meanings, transformed by grinding and chewing into beer (Hastorf and Johannessen, 1993). Today maize plays an extremely important role in the Andean culture and is the main cereal grain in Colombia, as measured by production of 260.700 Ha[1] (2015).

[1] www.dane.gov.co

Fermented foods, besides being more than a pleasure and a satisfaction of nutritional needs, are a rich source of insight into many aspects of cultural life (Hastorf and Johannessen, 1993). Since pre-Hispanic times, fermented products from maize have been consumed widely in Colombia (Chaves-López et al., 2014a), where their manufacturing remains a traditional art in houses, villages, and small-scale industries. In Colombian fermented foods and beverages, microbial interactions have been demonstrated to be of paramount importance in the development of the particular traits of the final product (Chaves-López et al., 2014b).

Masa Agria, also known as *Maiz añejo*, is a traditional fermented maize dough, produced in Colombia, which is still prepared by natural fermentation. In the traditional method of preparation, described by Chaves-López et al. (2014a), yellow maize kernels are peeled, covered with water, and stored in a hot place (35 to 40°C) to favor spontaneous fermentation. The period of fermentation varies from 3 to 5 days and determines the degree of sourness with a final pH from 4.4 to 3.8. After fermentation, the grains are washed with water, drained and milled, and then a dough is formed and allowed to stand for 3 or 5 days. During this period, dehydration occurs, and a_w is reduced at different levels depending on the producers. As reported by the same authors, fermentation is triggered mainly by lactic acid bacteria (LAB) and yeasts that are able to reach values of about 8.8 and 6.8 log CFU/g, respectively. *Masa Agria* is a very common product in Colombian gastronomy, as it is commonly used to prepare soups, tamales (dough filled with meat and vegetables and cooked in a banana leaf), empanadas (cooked dough filled with meat and potatoes and then fried), carantanta (the leftover material in the bottom of the pot that is peeled off after cooked Masa Agria for empanadas) and envueltos (dough steamed in a corn husk).

In the last years, metagenomic analyses have been broadly used to investigate microbial communities of fermented foods (Sakamoto et al., 2011; Chao et al., 2013; Elizaquível et al., 2015; Minervini et al., 2015). In general, these studies revealed a widely unknown microbial biodiversity, underreported by conventional cultivation-based methods. Although several studies have been conducted to explore the bacterial comunities in fermented maize doughs from different countries, to the best of our knowledge no information is available on the microbial communities of the Colombian *Masa Agria*. Therefore, the aim of this study is to explore the microbial diversity of Colombian *Masa Agria* by pyrosequencing of 16S rRNA of the bacterial microbiota, to improve the knowledge on microbial communities that can be essential for product characterisation and process optimization (Oguntoyinbo et al., 2011).

MATERIALS AND METHODS

Masa Agria Samples

Six distinct samples of *Masa Agria* from different producers of Valle del Cauca (Colombia) were acquired. Producers were located in the north (producer 1), north-east (2 and 6), and south (3, 4 and 5) of the region. Samples were immediately refrigerated and transferred to the laboratory for the following analyses.

Microbiological Analyses

Ten grams of each sample were mixed with 90 mL of 0.85% (w/v) sterile physiological saline, and homogenized in a Stomacher Lab-blender 400 Circulator (Seward, Worthing, UK) for 2 min. Serial dilutions were prepared in the same diluent. Total viable count was determined on Plate Count Agar, after incubation at 30°C for 48 h. Sabouraud Dextrose Agar added with Chloramphenicol (Sigma–Aldrich), incubated at 25°C for 72 h, was used for the enumeration of yeasts, while fungi were detected on Czapek Dox Agar after 5 days of incubation at 25°C.

Presumptive LAB were enumerated on Man, Rogosa and Sharpe (MRS) agar and presumptive enterococci on Slanetz and Bartley, both incubated anaerobically by means of anaerobic jars and BBL GasPak anaerobic system envelopes (Becton Dickinson, Cockeysville, MD, USA) at 37°C for 48 h. Micrococci and staphylococci were determined on Mannitol Salt Agar, incubated at 35°C for 48 h. Presumptive *Pseudomonas* spp. were counted on *Pseudomonas* Agar Base added with CFC supplement after 48 h at 25°C. Total coliforms were detected on Violet Red Bile Agar (VRBA) incubated at 37°C for 24 h. Finally acetic bacteria were enumerated on GYC Agar (Conda, Pronadisa, Madrid, Spain) supplemented with 3.0 g/L $CaCO_3$ (Sigma–Aldrich, Milan, Italy).

All media were from Oxoid-Thermofisher (Rodano, Italy), except where differently specified. Three repetitions for each sample were performed.

Physical-Chemical Analyses

The pH value was determined using a pH meter (Mettler Toledo MP 220, Novate Milanese, Italy). Aliquots of 10 g of *Masa Agria* were homogenized thoroughly with 10 mL of distilled water and the homogenate was used for pH determination.

The water activity (a_w) of the samples was determined by means of Aqualab instrument model Series 3 (Decagon Devices, Pullman, WA, US).

Three replicates were analyzed for each sample.

RNA Extraction from *Masa Agria* Samples and Pyrosequencing Analysis

An aliquot of about 200 mg of each *Masa Agria* sample was diluted in RNAlater (Sigma–Aldrich) and was then used for RNA extraction by Stool total RNA purification kit (Norgen Biotech Corporation, Thorold, ON, Canada). Total RNA was treated with RNase-free DNase I (Roche, Almere, Netherlands; 10 U of DNase for 20 μg of RNA) for 20 min at room temperature. Quality and concentration of RNA extracts were determined by using 1% agarose-0.5X Tris borate EDTA (TBE) gels and by spectrophotometric measurements performed at 260, 280, and 230 nm by using the NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific- Wilmington, DE, USA). Random examers and the Tetro cDNA synthesis kit (Bioline, Taunton, US) were used to transcribe the extracted RNA (about 2.5 μg) to cDNA, according to the manufacturer's instructions (Gowen and Fong, 2010).

For each dough, three cDNA samples were used for bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), which was performed by Research and Testing Laboratories (RTL, Lubbock, TX, USA), using a 454 FLX Sequencer (454 Life Sciences, Branford, CT, USA). The bTEFAP procedures were performed on the basis of RTL protocols http://www.researchandtesting.com (Research and Testing Laboratories, Lubbock, TX, USA). cDNA was analyzed by bTEFAP, using primers forward 28F:GAGTTTGATCNTGGCTCAG and reverse 519R: GTNTTACNGCGGCKGCTG based upon the V1–V3 region of the 16S rRNA gene (*Escherichia coli* position 27–519; Suchodolski et al., 2012). Following sequencing, the QIIME pipeline version[2] 1.4.0, with default settings, was used to screen, trim, and filter raw sequence data. B2C2[3]) was used to exclude chimeras, according to Gontcharova et al. (2010). Sequences lower than 250 bp were removed. FASTA sequences for each sample, without chimeras, were evaluated.

Bioinformatics and Data Analysis

The sequences were first clustered into OTUs (operational taxonomic units) clusters with 97% identity (3% divergence) using USEARCH sequence analysis tool[4]. To determine the microbial identities, sequences were first queried using a distributed BLASTn algorithm (Dowd et al., 2005) against a database of high-quality 16S bacterial sequences derived from NCBI. Database sequences were characterized as high quality based on the similar criteria originally described by Ribosomal Database Project (RDP, v10.28; Cole et al., 2009).

Operational taxonomic units were identified using the appropriate taxonomic levels using a database of high quality sequences derived from NCBI.

First, overall richness (i.e., number of distinct organisms present within the microbiome; alpha diversity) was expressed as the number of OTUs, and was quantified using the Chao1 richness estimator:

$$S_{chaol} = S_{obs} + \frac{n1(n1-1)}{2(n2+1)}$$

where n_i is the number of OTU with abundance i.

Second, overall diversity (which is determined by both richness and evenness, the distribution of abundance among distinct taxa) was expressed as Shannon Diversity. Shannon diversity (H') is calculated using:

$$H' = -\sum_{i=1}^{R} piln\,(\mathrm{pi})$$

where R is richness and pi is the relative abundance of the ith OTU.

Venn diagrams were realized on the bases of the OTUs obtained for the different species, according to Heberle et al. (2015).

Statistical Analysis

All values are shown as means with the standard deviation. The data on microbial population were analyzed by ANOVA. Differences among means were studied using the Tukey's test at a p-value of <0.05, using statistical software STATISTICA 7.0 (Statsoft, Tulsa, OK, USA) for Windows.

As regards pyrosequencing results, the relative abundance of each OTU was determined for each sample and the differences between the samples were calculated using Student's t-test.

Principal Component Analysis was performed to analyze dissimilarities among the samples regarding their bacterial species, using statistical software STATISTICA 7.0 (Statsoft, Tulsa, OK, USA) for Windows.

RESULTS

Physical-Chemical Parameters

Table 1 shows the values of pH and water activity measured in the *Masa Agria* samples. The analyses revealed some differences among samples from different producers. In particular, samples from producer 3 showed the lowest a_w values, with significant ($p < 0.05$) differences with respect to the other samples (except for sample 6), probably suggesting a more advanced age, thus implying a higher dehydration. In general, pH values of the analyzed *Masa Agria* samples were lower of equal than 3.76, as a consequence of the metabolic activity of LAB and acetic bacteria. Samples obtained by producer 5 were characterized by the lowest pH values, around 3.12.

Microbiological Profile of *Masa Agria* Samples

Microbial counts (**Table 2**) revealed a mesophilic aerobic population comprised between 7.4 and 7.9 log CFU/g, with samples 1 and 4 showing the highest counts.

Plate counts confirmed that the viable microbiota was dominated by the association of presumptive LAB and yeasts, as already observed by Chaves-López et al. (2014b), together with acetic bacteria, particularly in samples from producers 3, 4, and 5. Counts comprised between 2.0 and 2.9 log CFU/g were observed for presumptive enterococci. The presence of micro-staphylococci was instead variable: while counts of 3.4 and 3.8 were observed in samples 3 and 5, respectively, they were undetectable (<2.0 log CFU/g) in samples 2, 4, and 6.

Significantly different numbers ($p < 0.05$) were counted for presumptive *Pseudomonas* spp. in the different samples, with

TABLE 1 | Physical-chemical parameters of Masa Agria samples obtained from six different producers.

Producer	pH	a_w
1	3.76 ± 0.01	0.994 ± 0.001
2	3.56 ± 0.02	0.992 ± 0.002
3	3.56 ± 0.01	0.989 ± 0.002
4	3.63 ± 0.02	0.997 ± 0.001
5	3.12 ± 0.02	0.997 ± 0.001
6	3.58 ± 0.03	0.991 ± 0.002

Data are expressed as mean of three repetitions ± standard deviation.

[2]http://qiime.sourceforge.net
[3]http://www.researchandtesting.com/B2C2.html
[4]http://drive5.com/usearch

TABLE 2 | Microbial counts detected in Masa Agria samples obtained from 6 different producers.

Microbial group	Producer 1	Producer 2	Producer 3	Producer 4	Producer 5	Producer 6
Mesophilic aerobic count	7.87 ± 0.00	7.43 ± 0.05	7.54 ± 0.03	7.90 ± 0.02	7.38 ± 0.02	7.63 ± 0.04
Lactic acid bacteria	8.15 ± 0.03	7.93 ± 0.02	7.56 ± 0.04	7.95 ± 0.02	7.91 ± 0.03	7.93 ± 0.01
Enterococci	2.60 ± 0.02	2.50 ± 0.01	2.76 ± 0.02	2.88 ± 0.02	2.04 ± 0.03	2.01 ± 0.02
Staphylococci	2.03 ± 0.01	<2.00	3.40 ± 0.03	<2.00	3.82 ± 0.03	<2.00
Acetic bacteria	2.48 ± 0.03	3.50 ± 0.04	7.40 ± 0.04	7.80 ± 0.03	7.80 ± 0.05	3.72 ± 0.02
Pseudomonas	<2.00	<2.00	2.46 ± 0.01	3.42 ± 0.02	3.75 ± 0.03	2.23 ± 0.03
Coliforms	<1.00	2.48 ± 0.01	<1.00	<1.00	<1.00	2.30 ± 0.04
Yeasts	5.60 ± 0.04	6.60 ± 0.05	5.16 ± 0.03	5.47 ± 0.02	5.13 ± 0.03	5.78 ± 0.03
Molds	2.36 ± 0.02	2.50 ± 0.01	2.76 ± 0.03	2.88 ± 0.04	2.04 ± 0.02	2.43 ± 0.01

Data are expressed as Log CFU/g (mean of three repetitions ± Standard Deviation).

TABLE 3 | Comparison of estimates OTUs, richness and diversity indices of the 16S rRNA gene libraries as obtained from the pyrosequencing analysis.

	Number of Reads	Number of OTUs	Chao 1	Shannon
Producer 1	34076	15	15	1.48
Producer 2	34001	28	25	2.27
Producer 3	33947	18	9	1.45
Producer 4	34006	18	8	1.23
Producer 5	34105	15	9	1.38
Producer 6	33862	33	25	2.21

counts below 2.0 log CFU/g only in samples 1 and 2. Coliforms were detected only in samples 2 and 6.

Sequencing Statistics and Diversity Estimate

Pyrosequencing of the bacterial 16S rRNA genes generated more of 33.800 reads for each samples. The optimized data at 97% sequence similarity cut-off are shown in **Table 3**. The bacterial community was analyzed by richness estimator (Chao 1), and diversity index (Shannon). In general samples from producers 2 and 6 harbored higher bacterial diversity (Shannon index) than the other samples and similar patterns of richness (Chao 1), as shown in **Table 3**.

Microbial Community Structure

Taxonomy-based analysis showed a total of seven bacteria Phyla (*Bacterioidetes, Actinobacteria, Cyanobacteria, Firmicutes, Proteobacteria, Planctomycetes,* and *Tenericutes).*

Firmicutes was the major Phylum in the different fermented doughs, containing for more than 47% of all the bacterial community (**Figure 1**). The relative abundance of *Proteobacteria*, detected in each sample, was lower than *Firmicutes,* in particular in the samples from producers 1, 2, and 3, while Phylum *Tenericutes* was found only in samples of producers 1, 2, and 6. *Bacteriodetes* and *Planctomycetes* were present only in samples from producers 3 and 6, respectively.

In **Table 4** the relative abundance of microbial components at Family level in *Masa Agria* doughs is reported. *Firmicutes* were divided in three families (*Lactobacillaceae, Leuconostocaceae,* and *Streptococcaceae*), with *Lactobacillaceae* as the most abundant, accounting for a minimum of 28.5% (producer 3) to 89% (producer 1), followed by the sequences attributed to *Leuconostocaceae,* up to 18.2% (producer 2), and 20.1% (producer 6). *Streptococcaceae* were present only in samples from producers 2 (3.7%), 4 (0.04%), and 6 (6.2%).

Proteobacteria Phylum was represented by nine families: *Acetobacteriaceae, Sphingomonadaceae, Rhodocyclaceae, Comamonadaceae, Enterobacteriaceae, Moraxellaceae, Xanthomonadaceae, Thiotrichaceae,* and *Pseudomonadaceae*. In particular *Acetobacteriaceae* family was present in all the samples and was the most abundant family, accounting for 100% of *Proteobacteria* in sample from producer 4. Except for samples 4 and 1, in which only two families where represented (*Acetobacteraceae* and *Sphingomonadaceae*), OTUs of at least five families were present.

Planctomycetes Phylum, that was only a smaller proportion of the samples profile, was represented only by *Planctomycetaceae* family, with a incidence of 0.09% in sample from producer 3. *Tenericutes* Phylum was represented by the *Acholeplasmataceae* family that accounted for 9.91, 40.35, and 23.5% in samples from producers 1, 2, and 6, respectively.

At genus level, the number of identified OTUs of the samples varied depending on the producer: the samples from producers 2 and 6 were characterized by the major presence of OTUs, with 14 and 21 different genera, respectively, while in the other samples 6, 11, 6, and 8 OTUs were revealed (in samples from producers 1, 3, 4, and 5, respectively), as described in **Table 5**. The top 9 most abundant OTU demonstrated that *Lactobacillus,* belonging to *Firmicutes* Phylum (ranging from 17.3 to 89.3%), dominated in all the samples followed by *Acetobacter* (*Proteobacteria* phylum; from 0.36 to 69.9%) and *Weissella* (from 0.03 to19.1%) (**Table 5**).

Pediococcus was present in all samples with the exception of sample from producer 5. Several sub-dominants genera (less than 4%), belonging to Proteobacteria (*Comamonas, Enterobacter, Escherichia, Serratia,* and *Acinetobacter*), were identified only in samples from producers 2 and 6. *Sphingobium* was detected in all samples excepted for sample 5. *Dechloromonas,* and *Thiothrix* were found in the samples from producers 3 and 5, while *Pseudomonas* was also found in samples from producer 6. *Gemmata* was found only in sample 5.

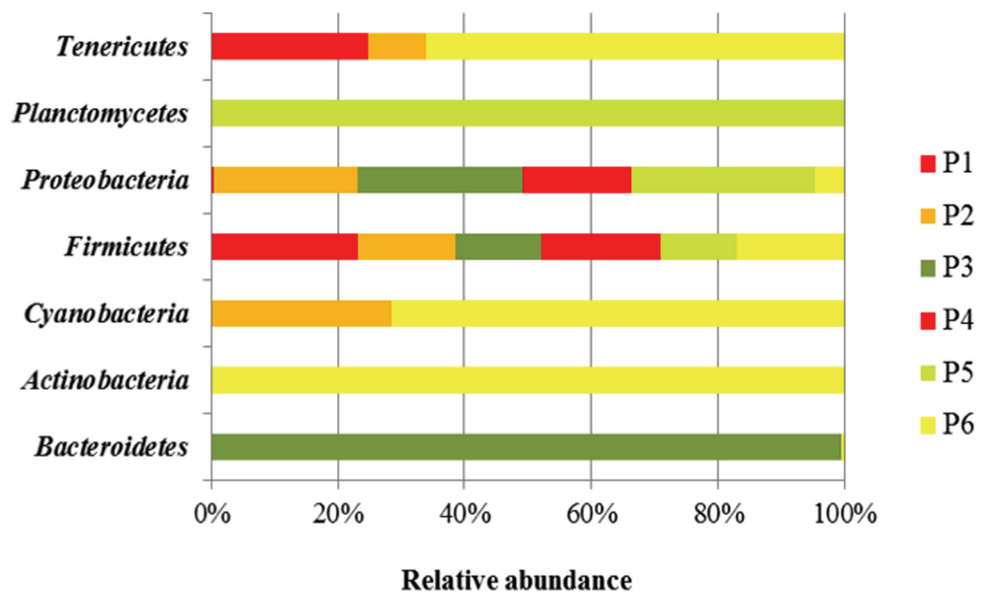

FIGURE 1 | Bacterial community diversity in six *Masa agria* samples from different producers (P, producers). The relative abundance of bacterial 16S rRNA genes was estimated through classification at the Phyla level.

TABLE 4 | Bacterial community diversity in six samples of *Masa Agria* from different producers (*P*, producer).

Family	P1	P2	P3	P4	P5	P6
Flavobacteriaceae	–	–	0.20	–	–	0.03
Streptomycetaceae	–	–	–	–	–	2.15
Bacteroidaceae	–	–	0.09	–	–	–
Lactobacillaceae	89.38	29.64	28.52	29.69	33.04	39.14
Leuconostocaceae	0.10	18.17	0.45	0.27	0.03	20.11
Streptococcaceae		3.70	–	0.04	–	6.27
Acetobacteraceae	0.36	4.50	69.94	69.49	65.54	5.82
Sphingomonadaceae	0.20	0.50	0.22	–	0.41	1.0
Rhodocyclaceae	–	–	0.02	–	0.03	–
Comamonadaceae	–	0.08	–	–	–	0.17
Moraxellaceae	–	0.08	–	–	–	0.07
Enterobacteriaceae	–	0.26	–	–	–	0.41
Pseudomonadaceae	–	–	0.07	–	0.47	0.06
Thiotricaceae	–	–	0.33	–	0.31	–
Xanthomonadaceae	–	0.043	–	–	–	0.07
Planctomycetaceae	–	–	–	–	0.09	–
Acholeplasmataceae	9.91	40.35	–	–	–	23.49
Unknown	–	0.04	–	–	–	0.06
Unclassified	–	–	–	–	–	0.1

The relative abundance of bacterial 16S rRNA genes was estimated through classification at the Family level.

The analysis on the species level showed that a certain percentage of the readers could not be classified to any existant group, which is common in pyrosequencing, because many uncultured bacteria can be detected or because they represent new species. The total number of OTUs at species level was 56, of which 15, 27, 18, 18, 15, and 33 were detected in the samples from producers 1 to 6.

Venn diagrams were plotted in order to evidence similarities among different microbial communities in the different samples, in terms of OTUs overlapping (**Figure 2**). As evidenced, only 2 OTUs were shared by all maize dough samples. The number of shared OTUs among the dough producers was low; for example, 11 between doughs from producer 1 and producer 2, while 8 between doughs from producer 2 and producer 3. Doughs from

TABLE 5 | Bacterial community diversity in *Masa Agria*.

Genus	P1	P2	P3	P4	P5	P6
Bacteroides	–	–	0.08	–	–	–
Streptomyces	–	–	–	–	–	0.07
Chryseobacterium	–	–	0.20	–	–	0.03
Planktothricoides	–	–	–	–	–	0.10
Lactobacillus	89.33	26.86	17.35	26.80	33.03	37.30
Pediococcus	0.051	2.78	11.17	2.87	–	3.83
Leuconostoc	–	0.91	0.22	–	–	0.97
Weissella	0.10	17.28	0.22	0.27	0.03	19.13
Lactococcus	–	3.78	–	0.03	–	6.20
Streptococcus	–	–	–	–	–	0.06
Gemmata	–	–	–	–	0.09	–
Acetobacter	0.36	4.57	69.93	69.33	65.54	6.92
Sphingomonas	–	–	–	–	–	0.03
Comamonas	–	0.08	–	–	–	0.03
Enterobacter	–	0.17	–	–	–	0.12
Delftia	–	–	–	–	–	0.14
Escherichia	–	0.04	–	–	–	0.27
Serratia	–	0.04	–	–	–	0.03
Acinetobacter	–	0.08	–	–	–	0.06
Frateuria	–	0.04	–	–	–	–
Gluconobacter	–	–	–	0.015	–	–
Sphingobium	0.23	0.52	0.223	–	0.41	1.01
Dechloromonas	–	–	0.22	–	0.03	–
Pseudomonas	–	–	0.06	–	0.47	0.06
Thiothrix	–	–	0.33	–	0.03	–
Stenotrophomonas	–	–	–	–	–	0.07
Candidatus Phytoplasma	9.91	40.35	–	–	–	23.49

The relative abundance of bacterial 16S rRNA genes was estimated through classification at the genera level.

producers 2 and 6 shared the greatest number of OTUs, with 24 common species. Number and abundance of OTUs from rRNA samples are reported in Supplementary Table S1, with taxonomic details up to species level when such assignment was possible.

To analyze bacterial community at species level among the different producers, a heat-map plot was generated using the relative abundance of the OTUs (**Figure 3**). As evidenced, the dominant OTUs in doughs from producers 1, 2, and 6 were different from those of the producers 3, 4, and 5. In fact in dough from producer 1, *L. gallinarum* (62%) was dominant, *Sugarcane phytoplasma* (40%) in dough from producer 2, and *L. fermentum* in dough 6 (19%), while doughs from producers 3, 4, and 5 were dominated by species of the genus *Acetobacter*, such as *Acetobacter cibinongensis, A. fabarum, A. lovaniensis,* and *A. orientalis*. It has to be underlined that the producers 1, 2, and 6 are located in the north/north-east of the region, while the others were located in the south, thus suggesting differences in the environmental conditions (i.e., temperature, water, and maize provenance).

Lactobacillus plantarum and *A. fabarum* were the most common species in all the samples, independently on the producers.

In further analyses, aimed at highlighting the microbiota similarity among the different producers, a PCA on the realtive abundance of bacterial species was performed (**Figure 4**). The PC1 accounted for the 67.67% of the variance while PC2 for 18.48%. Samples were separated in three cluster: cluster I containing sample from producer 1, was distinguished by the relative high percentages in OTUs of *L. pontis, L. panis, L. gallinarum, L. coleohominis,* and *Lactobacillus* spp. Cluster II was formed by samples from producers 3, 4, and 5, that were characterized by a relative high abundance of *L. plantarum* and *Acetobacter* spp. The samples from producers 2 and 6 were included in cluster III, featuring a high relative abundance of OTUs of *Lactobacillus* species, *Weissella fabaria* and *S. phytoplasma,* and contained also unique species, probably due to different geographical origin of maize and producers.

DISCUSSION

In this preliminar study, we used the pyrosequencing of tagged 16S rRNA gene amplicons to explore the bacterial microbiota in Colombian maize fermented dough *Masa Agria*. The data here presented provide a detailed insight of the bacterial profile of this product, as in our knowledge this is the first report of the bacterial community and structure of this Colombian fermented dough.

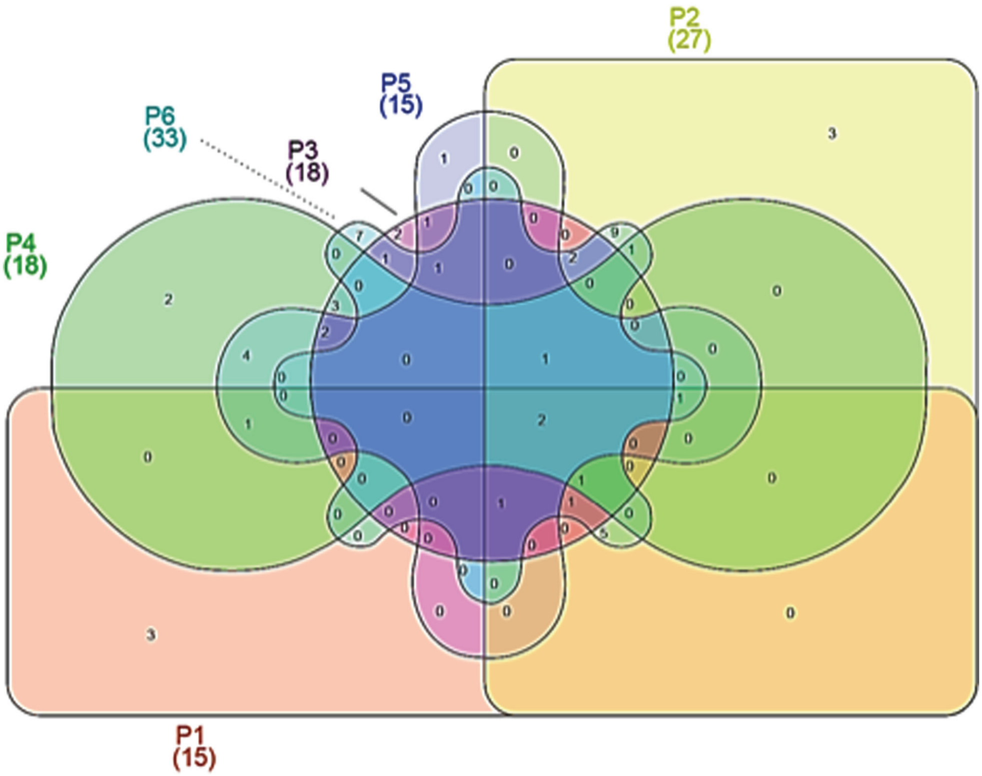

FIGURE 2 | Venn diagrams showing operational taxonomic units (OTUs) at species level, depicting similarities and differences among in the microbial community in six *Masa agria* samples from different producers (P, producers).

Microbial culture revealed growth on the plate counts media used. While the presence of presumptive *Lactococcus, Lactobacillus*, acetic bacteria and *Pseudomonas* was confirmed by 16S rRNA pyrosequencing, micro-staphylococci and enterococci were not confirmed. Enterococci may derive from raw materials, such as maize kernels or water, from the environment, and also from the tools used during grain milling and dough manufacturing (Elizaquível et al., 2015). Being able to grow over a wide range of temperature and easily supporting acid pH (Giraffa, 2002; Serio et al., 2010), their presence in the fermented *Masa Agria* would be unsurprising. Nevertheless our *Masa Agria* samples were market samples, analyzed at the end of the fermentations process, therefore enterococci deriving from raw materials and environment could have been overgrown by LAB species, as it happens in sourdough, where they are usually found in the first days (De Vuyst et al., 2014). Some authors reported that Slanetz and Bartley Agar lacks of selectivity when used for food samples, as it could over-estimate the actual number of enterococci, being able to support the growth also of lactococci and some *Lactobacillus* strains (Klein and Reuter, 2012). On the other side, also Mannitol Salt Agar was demonstrated to give false positive results as regards staphylococci, therefore lacking of specificity (Kircher et al., 2002). *Pseudomonas* spp. were present at extremely low sequence reads; indicating that the counts of bacteria that grew on the medium used (PSB + CFC) were unlikely to be all *Pseudomonas.* In fact, although the used medium is selective for this genus, the growth of other Gram-negative bacteria such as *Serratia marcescens, Aeromonas* spp., *Xanthomonas* spp., *Alcaligenes* spp., and *Acinetobacter* spp. is possible (Krueger and Sheikh, 1987).

The presence of lactobacilli, acetic bacteria and yeasts explains the low pH values profiles of the analized doughs from six different producers. The pH values observed in *Masa Agria* samples, are compatible with those reported in literature for other maize doughs (Annan et al., 2003). Chaves-López et al. (2014b) reported the presence of lactic and acetic acids in *Masa Agria* samples obtained from the same geographic area in Colombia. These acids not only are responsible for the sour taste, but their effect in pH reduction play a key role in the activation of endogenous and bacterial phytases, increasing dough nutritional value. In fact, phytic acid complexes amino acids and minerals, therefore acting as anti-nutritional factor. High LAB abundance and low pH are also responsible for coliforms decrease (Elizaquível et al., 2015).

Our findings showed that complex microbiota is associated to natural fermented maize doughs and that community membership and structure considerably differed depending on the producer. In fact, some OTUs were detected only in samples from one producer; in addition, the bacterial composition changed in terms either of species and of their relative abundance. This is not surprising, because the bacterial composition of fermented doughs differed on the bases of ecological parameters

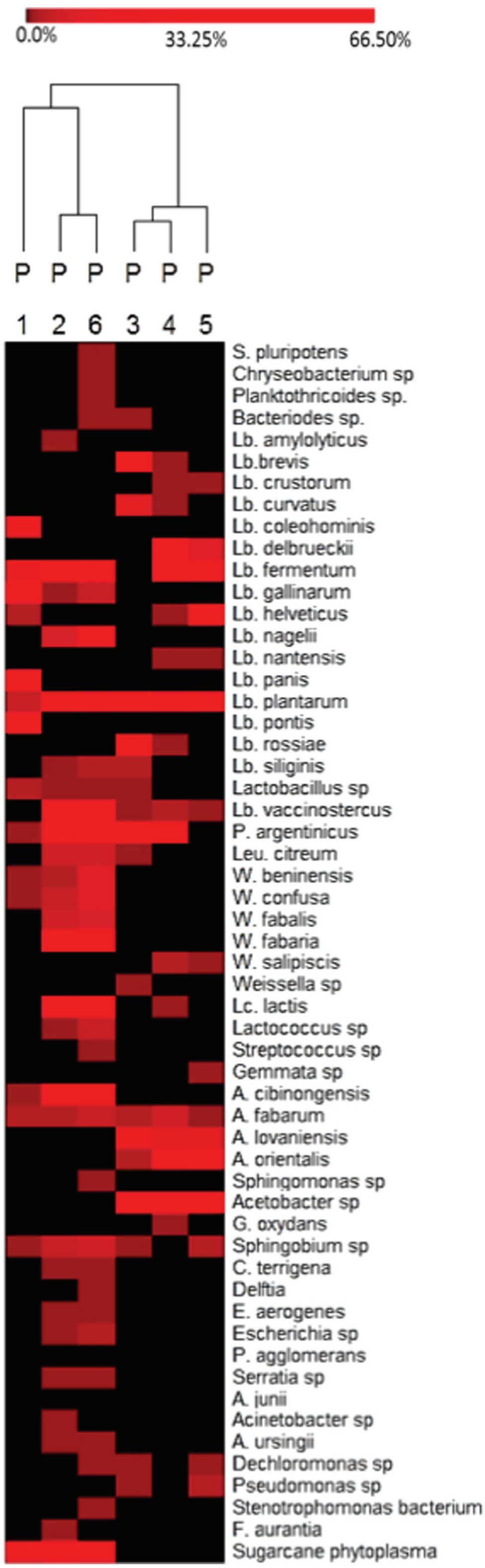

FIGURE 3 | Heat-map depicting bacterial diversity and relative abundance of species in six *Masa agria* samples from different producers (P, producers), as revealed by 16S rRNA pyrosequencing. The color key defines the percentages of OTUs in the samples.

such as type of maize used, temperature, time of fermentation, water activity, etc. Another possible explanation for differences among the samples could derive from phytochemical treatments that the maize was subjected to, during the cultivation. In fact, the total microbial population and the relative species proportion on maize grains can be affected by many factors, mainly temperature and rainfall, physical damage due to insects and application of phytopharmaceuticals. Although the producers of the analyzed samples of *Masa Agria* in this study did not know the provenience of the maize, in Colombia it is cultivated from 350 to 2400 meters above sea level. Thus it is evident that bacteria from this natural environment are different and their growth during the fermentation process lead to particular treats of each *Masa Agria*.

Interestingly, 4% of the readings could not be associated with a known species suggesing that *Masa Agria* may be an unexplored reservoir of unknown bacterial species.

In different *Masa Agria* many bacterial species have been found, and some of them have been commonly reported in indigenous Mexican or African fermented maize dough, considerably contributing to the development of the final characteristics of the products (Ben-Omar and Ampe, 2000; Escalante et al., 2001; Abriouel et al., 2006; Assohoun-Djenia et al., 2016). However, the predominant bacterial consortium depends on its source of production with mixtures of maltose and non-maltose fermenting species. As regards the bacterial active population in *Masa Agria*, evidenced by 16S rRNA pyrosequencing, it is immediately clear that LAB, and particularly *Lactobacillus* spp. and *Weissella* spp., together with *Acetobacter* spp., were dominant in all the samples. In particular, while several differences were observed among samples from the different producers, the two species *L. plantarum* and *A. fabarum* were common in all the analyzed doughs. The sequences obtained in the samples from the different producers exhibited high similarities and the species *L. fermentum, L. vaccinostercus, P. argentinicus,* and *Sphingobium* spp. were repeatedly found. This observation suggests that these particular species could play a specific role in *Masa Agria* production and that they could be a typical microbiota of this type of maize fermented product, although the variability should be deeply investigated.

In Mexican fermented maize dough Pozol, Escalante et al. (2001) reported the dominance of *Lc. lactis* followed by *L. alimentarius*, *L. plantarum,* and *Streptococcus suis.* In addition, Ben-Omar and Ampe (2000), studying the bacterial succession during its production, at the end of the fermentation detected *L. plantarum*, *L. fermentum*, *L. casei*, *S. bovis*, *Bifidobacterium minimum*, or *Exiguobacterium aurantiacum*. Halm et al. (1993) concluded that a homogenous group of obligatively heterofermentative lactobacilli related to *L. fermentum* and *L. reuteri* played a dominating role during the production of Ghanaian maize dough *Kenkey*. On the other hand, the analysis of bacterial community composition of maize dough samples from the Congo Republic, by 16S rRNA gene temporal temperature gradient gel electrophoresis (TTGE), revealed that the most intense band corresponded to *L. plantarum/paraplantarum;* moreover, although other bacteria such as *L. gasseri*, *Enterococcus* spp., *L. delbrueckii*, *L. reuteri*, *L. casei, L. acidophilus*, *L. delbrueckii*, *E. coli,* and *Bacillus* spp.

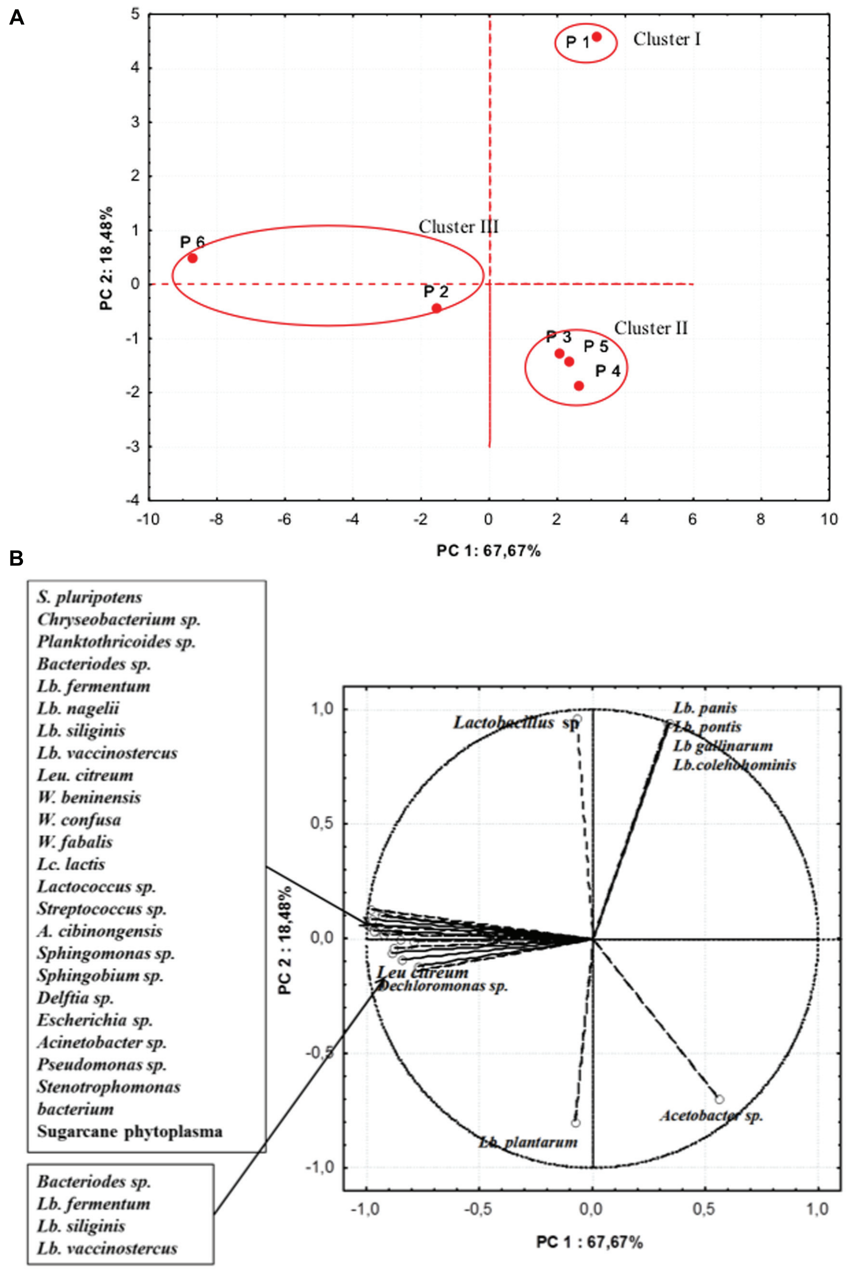

FIGURE 4 | Principal component analysis (PCA) on the relative abundance of bacterial species. (A) Scores **(B)** Loadings.

were detected, they were represented by DNA bands of lower intensities (Abriouel et al., 2006). Recently Assohoun-Djenia et al. (2016) reported the predominance of *L. fermentum, L. plantarum, P. pentosaceus, P. acidilactici,* and *W. cibaria* species in *Doklu* from Côte d'Ivoire.

The selective pressure of tropical environments may favor microbial biodiversity and highlights a useful technological potential (Chaves-López et al., 2009), and the geographical isolation among the fermented maize dough products leads to great divergent microbial communities, agreeing with the fact that each maize fermented dough can be considered as unique. Nevertheless it is evident that *L. plantarum* and *L. fermentum* are important species in fermented maize dough. Kunene et al. (2000) suggested that *L. plantarum* and *L. fermentum* associate in spontaneous fermentation of cereals-based foods.

The importance of *L. fermentum* in maize fermentation has been confirmed by previous researches in Ghanan, Benin, Mexican fermented maiz doughs (Agati et al., 1998; Ampe et al., 1999; Hayford et al., 1999; Oguntoyinbo et al., 2011; Obinna-Echem et al., 2014) and the high amylolitic activities found in different strains suggest that *L. fermentum* may be a key organism for fermentation of maize, making the large amounts of starch available to the overall community. In addition, the fermentation products (lactate, formate, and ethanol) may also serve as carbon sources for organisms, such as yeasts (Ben-Omar and Ampe, 2000). Interestingly, Assohoun-Djenia et al. (2016) reported a high prevalence of bacteriocin-producing *L. fermentum* strains, and their detection in different stages of *Doklu* production indicates a high potential of these strains to grow and dominate the microbial population in the fermented maize dough.

The amylolytic activity of some strains of *L. plantarum, L. lactis, Streptococcus* spp. and *Leuconostoc mesenteroides* had been also reported (Sanni et al., 2002; Díaz-Ruiz et al., 2003). In particular the presence of amylolytic *L. plantarum* in cereal fermented products is associated to (i) increasing the availability of energy sources for other associated non-amylolytic LAB (ii) contributing to a rapid pH decrease, and (iii) imparting favorable rheological properties to the dough (Sanni et al., 2002). *L. plantarum* is considered a highly acid-tolerant LAB that dominates in fermentation processes with vegetables and cereals, due to its metabolic flexibility and low pH adaptation (Vrancken et al., 2011). Also several strains of this species have been reported to display a broad spectrum of anti-fungal activity (Schnürer and Magnusson, 2005). Thus, it is possible that these features (amylolytic activity, acid tolerance, and bacteriocin production) contributed to the consolidated presence of both *L. plantarum* and *L. fermentum* in maize fermented dough.

Acetobacter, which was the second most represented genus in *Masa Agria*, varied in abundance among the samples, and was particularly abundant in those from producers 3, 4, and 5. Members of this genera are obligately aerobic bacteria that oxidize ethanol to acetic acid, although some species are also able to further oxidize acetic acid completely to CO_2 and water (Hutkins, 2006). Different species of the genus *Acetobacter* have been associated with whole crop maize silage (Oude Elferinck et al., 2001), where they dominate the first step of fermentation (Sträuber et al., 2012), together with LAB. While the presence of acetic acid bacteria in naturally fermented wheat dough, such as sourdough, is uncommon, on the contrary *Gluconobacter oxydans* and *A. xylinum,* together with *L. saccharolyticum* and *Saccharomyces cerevisiae* have been demonstrated to be important in the fermentation of sorghum grains to produce Hussuwa (El Nour et al., 1999). *Acetobacter* genus has been also associated with maize doughs (Ampe et al., 1999).

Dough fermentation leads to selective environmental conditions, due to sugar consumption and to the progressive pH reduction. Being acid-tolerant, and utilizing also molecules other than sugars for their energetic needs, *Acetobacter* spp. could be selected in the later stage of fermentation. In a polyphasic study on spatial distribution of microorganisms in *Pozol* from Mexico, Ampe et al. (1999) reported the presence of yeasts, fungi, EPS producers (including members of the genus *Leuconostoc*), and enterobacteria, as well as other non LAB, such as members of the genus *Acetobacter,* at the periphery of a pozol ball, in the outer part. Thus it can be hypothesized that samples of producers 3, 4, and 5 were collected overall from the surface of the dough, where oxygen should not have been a limiting factor for the growth of this genus, while the low presence found in samples from producers 1, 2, and 6 could probably be related to the poor presence of *Acetobacter* inside the dough. Further studies on spatial distribution of microorganisms in *Masa Agria* should be performed to confirm this hypothesis.

The presence of *Proteobacteria* observed in *Masa Agria* samples, such as *Comamonas, Sphingomonas, Acinetobacter,* and *Pseudomonas* spp., has been also recognized in the first step of rye and durum wheat sourdough fermentation (Ercolini et al., 2013), as flour and environment contaminants. In sourdough these genera usually become dominated by LAB such as *Lactobacillus* and *Weissella* in the following fermentation steps. However, it has to be underlined that *Masa Agria* production does not imply refreshments as in the case of sourdough, but the fermentation first of maize kernels and then of maize dough, therefore determining different dynamics of microbial succession.

Girma et al. (1989) observed that *Pseudomonas aeruginosa* inoculated in the Ethiopian fermented bread Tef injera, grew well until dough pH was reduced to 5.5, and thereafter the population decreased until only few viable cells were isolated at pH 4.0. Nevertheless, *Pseudomonas* species are characterized by a wide metabolic adaptability to substrates and stressing conditions, thus peculiar species could be selected by the *Masa Agria* environment. On the other hand, *Enterobacteriaceae* such as *Escherichia, Serratia* spp., and *Enterobacter aerogenes* which could derive from the maize kernels, but more probably from the water used for the dough, were only recognized in samples from producers 2 and 6. It is noteworthy the absence of enteropathogenic species.

Several species of environmental origin were recognized in the different samples and particularly notable are *Sphingobium* spp. and *Dechloromonas* spp., playing a role in soil and water bioremediation (Young et al., 2007), and *Gemmata* spp. (sample 5), a freshwater bacteria originally isolated from Queensland. The presence of *Sphingobium* spp. is correlated with the water used to wet the maize during *Masa Agria* production, as it has the capacity to survive in chlorinated waters, allegedly due to the oligotrophic character and the production of biofilms (Vaz-Moreira et al., 2011). This species has been involved in the degradation of chloroacetamide herbicide butachlor (Kim et al., 2013). On the other hand, the relative abundance of *Phytoplasma,* a plant pathogen able to cause maize bushy stunt that is among the most widespread diseases in herbaceous hosts, causing severe yield losses, may be assumed by the fact that the dough samples from producers 1, 2, and 6 were presumptively obtained from maize of low quality.

The complex microbiota of *Masa Agria* included also some less-abundant species such as some *Lactobacillus* (*curvatus, rossiae,* and *silingis*), and *L. citrineum* that migh play an important role in effectiveness and stability of the microbial community, as their microbial metabolism provides molecules able to affect this food ecosystem.

The combined application of culture-dependent and culture-independent analytical strategies allowed us to obtain an insight on the richness of the microbiota of *Masa Agria,* providing information on the diversity and on the relative abundance of microbial species.

CONCLUSION

For the first time, this study explored the microbial diversity of *Masa Agria,* by pyrosequencing of 16S rRNA gene amplicons. Our results elucidated the structures of the bacterial communities of six samples obtained from different producers, identified specific dominant species, and suggested the presence of possibly unknown microorganisms.

In particular, this research was focused on bacterial characterisation at the end of fermentation in commercial samples. Further investigations are needed to evaluate the microbial dynamics throughout the manufacturing process and to investigate the role of the different bacterial groups during fermentation.

AUTHOR CONTRIBUTIONS

CL: Devised and drafted the manuscript-statistical analyses. AS: drafted the manuscript. JO: molecular analysis. CR: culture dependent analyses. CT: statistical analyses. AP: manuscript revision.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01168

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Fermented Foods: Are They Tasty Medicines for *Helicobacter pylori* Associated Peptic Ulcer and Gastric Cancer?

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More than a million people die every year due to gastric cancer and peptic ulcer. *Helicobacter pylori* infection in stomach is the most important reason for these diseases. Interestingly, only 10–20% of the *H. pylori* infected individuals suffer from these gastric diseases and rest of the infected individuals remain asymptomatic. The genotypes of *H. pylori*, host genetic background, lifestyle including smoking and diet may determine clinical outcomes. People from different geographical regions have different food habits, which also include several unique fermented products of plant and animal origins. When consumed raw, the fermented foods bring in fresh inocula of microbes to gastrointestinal tract and several strains of these microbes, like *Lactobacillus* and *Saccharomyces* are known probiotics. *In vitro* and *in vivo* experiments as well as clinical trials suggest that several probiotics have anti-*H. pylori* effects. Here we discuss the possibility of using natural probiotics present in traditional fermented food and beverages to obtain protection against *H. pylori* induced gastric diseases.

Keywords: fermented food, probiotics, prevention of *H. pylori* infection, peptic ulcer, gastric cancer

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INTRODUCTION

"Let food be thy medicine and medicine be thy food."

Hippocrates (460 – 370 BC)

Every year peptic ulcer and gastric cancer takes approximately 301,000 and 740,000 lives, respectively (Piazuelo and Correa, 2013; Naghavi et al., 2015). Although both diseases have multiple etiologies like stress, diet, smoking and host genetic background, *Helicobacter pylori* infection is perhaps the most critical among them (Malfertheiner et al., 2014). However, every *H. pylori* infected individual does not develop peptic ulcer or gastric cancer. More than half of the world population is infected by *H. pylori*, but 10–20% of the infected people suffer from these diseases (Dorer et al., 2009). Why ∼80% of the *H. pylori* infected people in any given population never suffer from gastric disorders is unknown at present. Also, the clinical outcomes among the *H. pylori* infected population suffering from gastric disorders vary tremendously with geography (Covacci et al., 1999). For example, gastric cancer is fairly common in East-Asian countries like Japan and Korea, but in most African countries and India, the incidence of gastric cancer is low in spite of having high prevalence of *H. pylori* infection (Holcombe, 1992; Singh and Ghoshal, 2006; Shiota et al., 2013). Variations in bacterial and human genetic factors have been linked to explain the differences in clinical outcome, but our understanding of *H. pylori* infection and related diseases are really incomplete.

Microbiota is the ecological community of commensal, symbiotic and pathogenic microorganisms that literally share our body space. Microbiome is the combined genomes of the microbiota (Lederberg, 2001). Recent metagenomic analyses of DNA isolated from gastric tissue specimens show that human stomach is the niche of many bacterial species (Maldonado-Contreras et al., 2011). While the exact significance of the microbes that co-exist in highly acidic gastric milieu is not understood till date, it seems apparent that *H. pylori* infection can alter the dynamics of gastric microbiota (Andersson et al., 2008). However, microbiota can also be modulated by several other factors like alteration in immunity due to other infections and change in lifestyle including food and beverage consumptions (De Filippo et al., 2010). Interestingly, almost every geographical location has unique tradition of consuming fermented foods and beverages (Campbell-Platt, 1987). These fermented foods are rich source of bacteria, yeasts and molds and many of these microbes provide benefits to hosts and act as probiotics (Tamang et al., 2016a,b). More intriguingly, adding purified probiotics to therapy against *H. pylori* gives better eradication rate and reduces the side effects of antibiotics (Zhang et al., 2015). Unfortunately, however, the significances of the natural probiotics in traditional fermented foods and beverages are less studied in the context of *H. pylori* associated diseases. In this mini-review, we will discuss how probiotics present in different fermented foods and beverages may have a role in preventing *H. pylori* related gastric diseases.

H. pylori INFECTION AND GASTRIC DISEASES

Presence of spiral bacilli in stomach have been reported several times during the past century, but the culture of this slow growing species remained unsuccessful until a serendipitous prolonged incubation of human gastric specimens in microaerophillic conditions during Easter holidays by Barry James Marshall and John Robin Warren (Doenges, 1938; Freedberg and Barron, 1940; Warren and Marshall, 1983; Marshall and Warren, 1984). To prove Koch's postulate Barry Marshall drank pure culture of *H. pylori*, which resulted in hypochlorhydric vomiting and gastritis before he was treated with antibiotics (Marshall et al., 1985). Subsequently, a huge number of studies confirmed the role of *H. pylori* virulence factors in peptic ulcer and gastric cancer and *H. pylori* was classified as a type I carcinogen by WHO (Malfertheiner et al., 2014).

H. pylori expresses many virulence factors, but two multitasking proteins, the vacuolating cytotoxin (VacA) and the cytotoxin-associated gene A (CagA), seem to play the most crucial role in developing the gastro-duodenal diseases. The VacA is a secreted toxin, which forms large cytoplasmic vacuoles inside the host cells (Leunk et al., 1988). The VacA is also involved in reducing mitochondrial transmembrane potential, releasing cytochrome c, inducing cell death, activating MAP-kinases and inhibiting T-cell activation (Galmiche et al., 2000; Gebert et al., 2003; Willhite and Blanke, 2004; Yamasaki et al., 2006; Torres et al., 2007). The *vacA* gene has mosaic structures viz. s1/s2 alleles (encoding signal peptides), m1/m2 alleles (encoding mid-regions) and i1/i2/i3 (encoding intermediate regions) (Cover et al., 1994; Atherton et al., 1995, 1999; Rhead et al., 2007). The s1 and the i1 alleles of *vacA* are associated with aggressive clinical outcomes (Rhead et al., 2007; Yamaoka, 2010). The *H. pylori* strains carrying *vacA* s1 usually carry *cagA* gene, which is located in the *cag*-pathogenicity island (Blaser et al., 1995; Xiang et al., 1995; Yamaoka, 2010). The *cagA*$^+$ strains are associated with more severe diseases in most regions (Blaser et al., 1995). The *cagA* gene shows length polymorphism at the 3′ end and this variable region encodes EPIYA motifs that undergo phosphorylation once the CagA protein is translocated into the host cells (Yamaoka et al., 1998; Higashi et al., 2002). The phospho-CagA interacts and deregulates the SHP-2 protein, which leads to cancer, but the CagA can hijack cellular pathways also by phosphorylation independent manner (Higashi et al., 2002; Hatakeyama, 2014).

Polymorphisms in host immune genes also contribute to determine the clinical status of the host (Datta De and Roychoudhury, 2015). For example, polymorphisms in interleukin-1 (IL1) and tumour necrosis factor (TNF) genes have been shown to play important roles in progression of gastric diseases among Scottish, Japanese, American, and Indian populations (El-Omar, 2001; Datta De and Roychoudhury, 2015). Moreover, every geographic region has unique lifestyle including food and beverage intakes, which are known to have effects on gut microbiota (De Filippo et al., 2010).

It is now appreciated that human stomach microbiota consists of 44 bacterial phyla, dominated by four phyla: Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes (Maldonado-Contreras et al., 2011). A study using Swedish patients showed that the presence of *H. pylori* in stomach may significantly alter the relative abundance of other bacteria (Andersson et al., 2008). Colonization by specific groups of bacteria seems to correlate with *H. pylori* infection status. *H. pylori* colonization dramatically reduced the diversity and increased the colonization of Proteobacteria. Positive *H. pylori* status in America is also associated with increased abundance of Proteobacteria, Spirochetes and Acidobacteria, and with decreased abundance of Actinobacteria, Bacteroidetes and Firmicutes (Maldonado-Contreras et al., 2011). Recently, in mouse, it has been shown that *H. pylori* colonization can influence both gastric and intestinal microbiota (Kienesberger et al., 2016). While it appears that the stomach and intestinal microbiota in the presence and in the absence of *H. pylori* infection may have a role in gastric diseases the mechanism is not known.

Treatment for all *H. pylori* infections has been recommended for several geographical locations (Shiota et al., 2013; Malnick et al., 2014). The usual treatment regimen for *H. pylori* is a short course of two antibiotics (mostly clarithromycin and amoxicillin) along with proton pump inhibitors (e.g., omeprazole or lansoprazole). The treatment, however, is complicated by several factors like bacterial resistance to antibiotics, re-infection, side effects (bloating, diarrhea and taste disturbances) and alteration of healthy gut microbiota (Malnick et al., 2014;

Zhang et al., 2015). The destruction of the commensal flora may lead to increased prevalence of opportunistic pathobionts, like *Clostridium difficile* (Malnick et al., 2014). Hence, the treatment of *H. pylori* using antibiotics has the risk for microbiota imbalance or dysbiosis, which may lead to other diseases. Also, eradication of *H. pylori* may lead to esophageal cancer (Blaser, 2008; Blaser and Falkow, 2009). Therefore, alternative approach that can eradicate or prevent *H. pylori* infection without affecting gut microbiota is needed.

USE OF PROBIOTICS FOR THE ERADICATION OF *H. pylori*

Probiotics (means 'for life') are live microorganisms which provide beneficial effects when taken in sufficient quantity. Examples include several species of *Lactobacillus*, *Bifidobacterium*, *Enterococcus, Lactococcus, Streptococcus* as well as *Saccharomyces* (Reid, 1999; Fijan, 2014). Probiotics are known to have beneficial roles in curing antibiotic associated diarrhea, constipation, traveler's diarrhea, food allergies and cancer (McFarland, 2007; Chmielewska and Szajewska, 2010; Hempel et al., 2012; Isolauri et al., 2012; Russo et al., 2014).

Lactobacillus is normally present in human intestinal tract including stomach and it is tolerant to acid and bile (Ruiz et al., 2013). Therefore, *Lactobacillus* is an attractive candidate for probiotic for the treatment of *H. pylori* related gastric diseases. Bhatia et al. (1989) showed that the culture supernatant of *Lactobacillus acidophilus* inhibits *H. pylori in vitro* due to an extracellular secretory product. Direct application of *L. acidophilus* on blood agar plate can also inhibit *H. pylori* (Vilaichone et al., 2002). Subsequently, it was found that both *L. acidophilus* and *L. casei* subsp. *rhamnosus* can inhibit *H. pylori* due to the production of lactic acid (Midolo et al., 1995; Enany and Abdalla, 2015). The lactic acid produced by *L. casei* strain Shirota inhibits 70% of urease activity *in vitro* and significantly reduces the levels of *H. pylori* colonization in mouse model (Sgouras et al., 2004). Lorca et al. (2001) studied antibacterial activity of 17 *Lactobacillus* strains on 10 *H. pylori* strains and concluded that the inhibition was due to acid production. They also found that autolysis of *L. acidophilus* after 24 h of culture releases a proteinaceous compound and this event is related to the bactericidal effect (Lorca et al., 2001). Furthermore, *H. pylori* colonized mice when treated with a commercial mixture of live probiotics (*L. rhamnosus*, strain R0011, and *L. acidophilus*, strain R0052) they suppressed colonization of *H. pylori* strain SS1 (Johnson-Henry et al., 2004).

The sulfatide-binding protein of the *L. reuteri* competes and binds to the gangliotetraosylceramide (asialo-GM1) and sulfatide, which are putative receptors of *H. pylori* (Mukai et al., 2002). *Weissella confusa* can inhibit *H. pylori* adherence to human gastric cell line by 90%. (Nam et al., 2002).

H. pylori infected MKN45 cells showed increased expression of Smad7 and NFkB, and induced pro-inflammatory cytokines IL-8 and TNF-α *in vitro*. Probiotic *L. acidophilus* pre-treatment, however, inactivate the Smad7 and NFkB pathways and reduces the *H. pylori* induced inflammation (Yang et al., 2012). Using gnotobiotic murine model, it was shown that *L. salivarius* infection also inhibits the colonization of *H. pylori* and associated inflammatory responses like IL-8 release (Kabir et al., 1997; Avía et al., 1998).

Since *in vitro* experiments and *in vivo* mouse studies showed promising results, a significant number of clinical trials have been performed in the recent past (**Table 1**). Several meta-analyses published in 2013 revealed that addition of probiotics in triple therapy against *H. pylori* improves overall efficacy and reduces the side effects of therapy like nausea, diarrhea metallic taste, abdominal/epigastric pain (Ruggiero, 2014). However, it needs further improvement since the benefits conferred by the probiotics are often not too remarkable. For example, a meta-analysis based on literature search strategy suggest that use of probiotics (mostly *Lactobacillus*, *Bifidobacterium* and *Streptococcus* and in few trials *Enterococcus*, *Clostridium*, *Saccharomyces* etc) in triple therapy improve eradication rate of *H. pylori* by ∼10% and reduce adverse effects of therapy by ∼15% (Zhang et al., 2015).

ROLE OF FERMENTED FOODS AND BEVERAGES AGAINST *H. pylori* ASSOCIATED DISEASES

Fermentation of food dates back to the early ages of human evolution and provides an effective way of preserving food for longer durations (McGovern et al., 2004). Many of the bacteria, yeasts and molds that are present in fermented foods and beverages are known probiotics and probably provide health benefits when consumed raw (Stanton et al., 2005). The significance of the microbes present in fermented food in maintaining human health was first noticed by Elie Metchnikoff (Mackowiak, 2013). He hypothesized that the long and healthy lives of Bulgarian peasants were due to the regular consumption of sour milk and yogurt containing the necessary beneficial microbes (Mackowiak, 2013).

Many of the probiotic that are isolated directly from the fermented foods, particularly fermented dairy products, have anti *H. pylori* effects. Based on dietary interviews it was found that yogurt, but not unfermented dairy products, when consumed one serving per week or more has protective effect against *H. pylori* infection in Mexican population (Ornelas et al., 2007). Several strains of *Lactobacilli* and two strains of yeast directly isolated from yogurt were found to have inhibitory effect on *H. pylori* (Oh et al., 2002). A meta-analysis of randomized controlled trials shows that there is ∼10% improvement in eradication rates when using fermented milk based probiotics, which seems to be better than capsule/sachet-based bacteria-only preparations (Sachdeva and Nagpal, 2009). Similarly, 4-week treatment with *L. gasseri*-containing yogurt improved the efficacy of triple therapy in patients with *H. pylori* infection (Deguchi et al., 2012). Another study showed that *H. pylori* infected children have a lower number of *Bifidobacterium* in their gut, but intake of probiotics-containing yogurt had multiple effects like, restoration of *Bifidobacterium*, reduction of *H. pylori* load, increase in IgA and decrease in IL-6 (Yang and Sheu, 2012). Three

TABLE 1 | Some of the anti-*Helicobacter pylori* clinical trials and meta-analyses that used probiotics.

Study	Species	Results	Reference
Meta-analysis	*Lactobacillus* strains	Improvement in eradication rates	Zheng et al., 2013
Randomized open label clinical study	*Bifidus infantis*	Used as adjuvant improves cure rate	Dajani et al., 2013
Meta-analysis	*Lactobacillus* and *Bifidobacterium* species	Beneficial effects on eradication rate and incidence of side effect	Wang et al., 2013
Meta-analysis	*Lactobacillus acidophilus, Lactobacillus casei* DN-114001*, Lactobacillus gasseri, Bifidobacterium infantis* 2036.	Increases eradication rates	Dang et al., 2014
Clinical trials	*Lactobacillus gasseri* OLL2716(LG21)	Supression of *H. pylori,* reduction in gastric mucosal inflammation	Sakamoto et al., 2001
Double blind randomized placebo-controlled crossover clinical study	*Lactobacillus reuteri* strain SD2112	Suppression of urease activity and *H. pylori* density	Imase et al., 2007
Double blind placebo-controlled study	*Lactobacillus reuteri* ATCC 55730	Suppresses *H. pylori* infection, decreases the occurrence of dyspeptic symptoms	Francavilla et al., 2008
Double blind placebo-controlled study	*Lactobacillus reuteri* ATCC 55730	*H. pylori* eradicated in half of the patients by omeprazole plus *L. reuteri*	Saggioro et al., 2005
Double blind randomized placebo-controlled study	*Lactobacillus reuteri* DSM 17938, *Lactobacillus reuteri* ATCC PTA 6475	Combination of both strains alone exert an inhibitory effect and when used with eradication therapy reduces side effects	Francavilla et al., 2014
Open label single center study	*Lactobacillus reuteri* DSM 17938	Reduction of urease activity	Dore et al., 2014
Single center, double-blind, prospective, randomized, placebo-controlled trial	*Lactobacillus GG*	Reduced side effects and overall treatment tolerability	Armuzzi et al., 2001

strains of lactic acid bacteria, LY1, LY5 AND IF22, which are from the spent culture supernatant of fermented milk, showed anti-*H. pylori* effect (Lin et al., 2011). In china, several probiotics from traditional fermented foods were isolated and two strains of *Lactobacillus- L. plantarum 18* and *L. gasseri* showed potential anti-*H. pylori* activity (Chen et al., 2010). Kefir, a fermented milk product was found to be effective in eradication and reducing side effects when used along with triple therapy (Bekar et al., 2011). An *in vitro* study proved that *L. plantarum* (MLBPL1) isolated from sauerkraut (fermented cabbage) had an anti-*Helicobacter* activity (Rokka et al., 2006). Interestingly, the main inhibitory activity is mostly associated with cell wall.

Unfortunately, however, anti-*H. pylori* activity alone does not ensure protection from gastric diseases and gastric cancer may sometimes develop even after eradication of *H. pylori* since some of the *H. pylori* proteins like CagA may act by 'hit and run' mechanism (Shiota et al., 2013; Hatakeyama, 2014). More interestingly, prevalence of *H. pylori* and incidence of gastric diseases does not match in some countries. In Africa and India, the prevalence of *H. pylori* infection and associated gastritis is high, but the incidence of gastric cancer is very low (Holcombe, 1992; Singh and Ghoshal, 2006). On the other hand, East-Asian countries like Japan and Korea have high rates of gastric cancer (Singh and Ghoshal, 2006). Genotype alone cannot be responsible to explain the clinical outcome since nearly all *H. pylori* strains isolated from East-Asia are virulent (Shiota et al., 2013). Therefore, it is intriguing to compare the microbes that are present in traditional fermented foods and beverages of Japan or Korea and African countries (**Table 2**). Apparently, fermented foods in African countries are based on milk, beans, grains and roots. They are dominated by *Lactobacillus* and other lactic acid bacteria. Conversely, Japanese fermented foods are primarily based on rice, soy and fish and these foods have varieties of bacteria and fungi. Interestingly, the soy foods may reduce the risk for gastric cancer, while high salt containing foods might be a risk factor in Japan and Korea (Hirayama, 1981; Woo et al., 2013).

A similar comparison would be exiting between the fermented foods of ethnic populations in North-Eastern India (e.g., ethnic populations in Sikkim state like Bhutias) and Mid-Eastern India (e.g., ethnic population of Jharkhand and West Bengal states like Santhals). North-Eastern states have highest incidence of gastric cancer in India (Pradhan et al., 2003–2004). This high prevalence has been thought to be due to smoking and high salt consumption that possibly come from fermented and pickled foods including fish and meat (Phukan et al., 2005; Verma et al., 2012). Recent analyses of some of the fermented foods showed presence of huge microbial variety but their significances in gastric diseases have not been studied (Tamang and Sarkar, 1996; Tamang et al., 2016a,b). Unfortunately, not much is known about the microbes that are present in fermented foods consumed by the Santhals. But interestingly, they regularly consume intoxicating alcoholic beverages like Handia and Mahua fermented in traditional way and these beverages are not common elsewhere (Kumar and Rao, 2007). Among Santhals, infections with virulent *H. pylori* strains are extremely common without any manifestation of gastric diseases (Datta et al., 2003).

Our current understandings of microbes present in the ethnic fermented foods are incomplete at present, but with modern methodologies like metagenomic analysis using Next-generation sequencing the microbial species are now easy to identify (Mozzi et al., 2013). However, to prove or disprove the hypothesis—whether or not microbes present in the ethnic fermented food can protect certain population from peptic ulcer or gastric cancer is very tricky, particularly when *H. pylori* infection is not the only determinant in precipitating the

TABLE 2 | Microbes present in traditional fermented foods and beverages in Japan and Africa.

Fermented food	Ingredients	Microorganism	Known probiotics or anti-*H. pylori* activity	Country	Reference
Fermented food of Japan and Korea					
Sake	Rice	*Aspergillus sojae, Bacillus subtilis* and lactic acid bacteria	Lactic acid bacteria and *Bacillus subtilis*	Japan	Sakaguchi, 1958a,b
Narezushi	Fish, salt and cooked rice	*L. plantarum* and *L. brevis*	*L. plantarum*	Japan	Kiyohara et al., 2012
Takju	Rice	*Lb. harbinensis*, *Lb. parabuchneri Lactobacillus (Lb.) paracasei, Lb. plantarum*, and *Leuconostoc pseudomesenteroides*	*L. plantarum*	Korea	Kim et al., 2010
Vinegar	Rice	*Aspergillus oryzae*, *Lactobacillus acetotolerance*, *Acetobacter pasteurianus*, *Saccharomyces* sp. and lactic acid bacteria	Lactic acid bacteria and *Saccharomyces* sp.	Japan	Haruta et al., 2006
Natto	Soybean	*Bacillus subtilis*	*Bacillus subtilis*	Japan	Kubo et al., 2011
Starch Noodle	Starch from sweet potato, mung bean etc	*L. casei, L. cellobiosus, L. fermenti*	*L. casei*	Korea, Japan	Rhee et al., 2011
Kimchi	Korean cabbage, radish, various vegetables, salt	*L. mesenteroides, L. brevis, L. plantarum*	*L. plantarum*	Korea	Rhee et al., 2011
Miso	Soybean and sometime rice or barley	*Aspergillus oryzae Saccharomyces cerevisiae and lactic acid bacteria*	Lactic acid bacteria and *Saccharomyces* sp.	Japan	Hirayama, 1981
Komesu and kurosu	Rice	*Aspergillus oryzae*, *Saccharomyces cerevisiae* and acetic acid bacteria	*Saccharomyces* sp.	Japan	Nanda et al., 2001
Tempeh	Soybean	*Rhizopus* sp.	?	Japan	Aoki et al., 2003
Fermented food of Africa					
Rigouta	Milk	*Lactococcus lactis and Enterococcus faecalis*	*Enterococcus faecalis*	Tunisia	Ghrairi et al., 2004
Wara	Cow milk	*Lactobacillus plantarum* and other lactic acid bacteria	*Lactobacillus plantarum* and other lactic acid bacteria	Nigeria	Olasupo et al., 1997
Ugba	Oil bean seed	*Bacillus subtilis*	*Bacillus subtilis*	Nigeria	Olasupo et al., 1997
Fufu	Cassava	*Lactobacillus plantarum* and other lactic acid bacteria	*Lactobacillus plantarum* and other lactic acid bacteria	Nigeria	Olasupo et al., 1997
Ogi	Maize	*Lactobacillus plantarum* and other lactic acid bacteria	*Lactobacillus plantarum* and other lactic acid bacteria	Nigeria	Olasupo et al., 1997
Kunu-zarki	Millet	*Lactobacillus plantarum* and other lactic acid bacteria	*Lactobacillus plantarum* and other lactic acid bacteria	Nigeria	Olasupo et al., 1997
Kenkey	Maize	*Lactobacillus plantarum* and other lactic acid bacteria	*Lactobacillus plantarum* and other lactic acid bacteria	Nigeria	Olasupo et al., 1997
Iru	African locust bean	*Lactobacillus plantarum* and other lactic acid bacteria	*Lactobacillus plantarum* and other lactic acid bacteria	Nigeria	Olasupo et al., 1997
Garri	Cassava	Yeast, *Lactobacillus plantarum, Leuconostoc fallax*, *Lactobacillus fermentum* and other lactic acid bacteria	*Lactobacillus plantarum* and other lactic acid bacteria	Nigeria and other part of Africa	Kostinek et al., 2005
Kule naoto	Milk	*Lactobacillus plantarum* and other lactic acid bacteria	*Lactobacillus plantarum* and other lactic acid bacteria	Maasai in Kenya	Mathara et al., 2004
Poto Poto	Maize dough	*Lactobacillus plantarum, Bacillus* sp., *Lactobacillus reuteri, Lactobacillus casei* and other lactic acid bacteria	*Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus casei*	Congo	Abriouel et al., 2006
Degue	Pearl millet dough	*Lactobacillus plantarum, Bacillus* sp., *Lactobacillus reuteri, Lactobacillus casei,* other lactic acid bacteria and yeast and molds	*Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus casei*	Burkina Faso	Abriouel et al., 2006

gastric diseases (Parekh et al., 2014; De and Roychoudhury, 2015). How the microbes present in the ethnic fermented food can alter the pathogenicity of *H. pylori* in combination with gastric and duodenal microbiome as well as host immunity for different population is perhaps the key question at present.

CONCLUSION

H. pylori infection is the major risk factor for peptic ulcer and gastric cancer and the eradication of this bacterium using antibiotics is often unsuccessful. Several microbes with known probiotic activities are shown to have inhibitory effects against *H. pylori in vitro* and *in vivo*. Inclusion of probiotics in triple therapy leads to improved efficacy and reduced side effects. Most traditional fermented foods and beverages are natural sources of probiotic microbes. Microbes directly isolated from the fermented products are shown to have anti-*H. pylori* activity. Few studies showed that consumption of probiotics containing yogurt and kefir are somewhat beneficial in the context of *H. pylori* infection. Many ethnic populations have significantly low incidences of peptic ulcer and gastric cancer in spite of having very high prevalence of *H. pylori* infection. Incidentally, each ethnic population also has unique tradition of consuming fermented food and beverages that contain probiotics. It is intriguing to hypothesize that regular consumptions of these probiotics may have protective effect against peptic ulcer and gastric cancer for some populations. Analyzing these traditional fermented foods and beverages using modern techniques is needed to understand these microbes and their significances.

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MN and DC equally contributed 60% of the mini-review works. SG contributed 15% and SC contributed 25% in the mini-review works.

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REVIEW
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Produce from Africa's Gardens: Potential for Leafy Vegetable and Fruit Fermentations

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A rich variety of indigenous fruits and vegetables grow in Africa, which contribute to the nutrition and health of Africa's populations. Fruits and vegetables have high moisture and are thus inherently prone to accelerated spoilage. Food fermentation still plays a major role in combating food spoilage and foodborne diseases that are prevalent in many of Africa's resource disadvantaged regions. Lactic acid fermentation is probably the oldest and best-accepted food processing method among the African people, and is largely a home-based process. Fermentation of leafy vegetables and fruits is, however, underutilized in Africa, although such fermented products could contribute toward improving nutrition and food security in this continent, where many are still malnourished and suffer from hidden hunger. Fermentation of leafy vegetables and fruits may not only improve safety and prolong shelf life, but may also enhance the availability of some trace minerals, vitamins and anti-oxidants. Cassava, cow-peas, amaranth, African nightshade, and spider plant leaves have a potential for fermentation, as do various fruits for the production of vinegars or fruit beers and wines. What is needed to accelerate efforts for production of fermented leaves and vegetables is the development of fermentation protocols, training of personnel and scale-up of production methods. Furthermore, suitable starter cultures need to be developed and produced to guarantee the success of the fermentations.

Keywords: horticulture, postharvest, fermentation, food security

INTRODUCTION

Statistics show that hunger is still a dramatic problem facing humanity and that nearly 795 million people do not have enough food[1] (Burchi et al., 2011). Hunger as based on caloric deficits is, however, only part of the story, as many of the hungry have access to the minimal required amount of calories, but are deficient in one or more micronutrients. Micronutrient deficiencies are the so-called 'hidden-hunger' and affect approximately 2 billion people worldwide (Burchi et al., 2011), with the majority of people occurring on the African continent and the Indian subcontinent

[1]https://www.wfp.org/hunger/stats

(Muthayya et al., 2013). Worldwide, malnutrition is estimated to contribute to more than one third of all child deaths, although it is rarely listed as the direct cause (Bain et al., 2013). In 2013, an estimated 6.3 million children under the age of five died, 2.9 million of these in the WHO Africa region (WHO, 2013).

Dietary micronutrient deficiencies include calcium, copper, iron, iodine, magnesium, selenium, zinc, and/or vitamin A deficiency (Bain et al., 2013; Joy et al., 2014). Micronutrient deficiencies have detrimental effects on children growth and development, and the most common and clinically significant micronutrient deficiencies in children and childbearing women include deficiencies in iron iodine, zinc and vitamin A (Bain et al., 2013). Joy et al. (2014) estimated the micronutrient deficiency risks due to inadequate intakes of seven minerals in Africa. They showed that deficiency risks were highest for calcium (54% of the population), followed by zinc (40%), selenium (28%), and iodine (19% after accounting for iodized salt consumption), while the risks for copper (1%) and magnesium (<1%) deficiencies were low (Joy et al., 2014). The deficiency risk for iron was lower than expected (5%), and multiple micronutrient deficiency risks were high in many countries (Joy et al., 2014).

While the world human population drastically increases, there is a corresponding reduction in availability of land for farming. To worsen this scenario, global warming has a deleterious impact on the agricultural productivity, with dire consequences on the food supply for both developed and developing countries (Rosenzweig and Parry, 1994). Africa, on the other hand, is a world region where a high diversity of food crops is grown. Vegetables and fruits are produced throughout the continent and are sources of much needed micronutrients. However, there is limited industrial scale processing of most of the agricultural products in the continent, leading to large economic losses of up to 40% and, as a consequence, to poverty and hunger (Gustavsson et al., 2011).

Africa is rich in the provision of traditional fermented foods, particularly those based on plant materials as substrates. These are often produced using minimal technology and inputs (Odunfa, 1985). Despite this, many people in sub-Saharan Africa are malnourished and this is due to agronomic constraints, as well as a lack of appropriate local food processing techniques. Accordingly, a huge proportion (ca. 30–50 %) of harvest is lost at the postharvest stage (Shiundu and Oniang'o, 2007). The main causes for this are inadequate production conditions (Abukutsa-Onyango, 2007), as well as rapid product decay during transport, storage, and marketing (Muchoki et al., 2007). Therefore, effective postharvest strategies based on sound scientific principles need to be developed for an efficient crop utilization. These should be applicable and adaptable to different situations in African countries, where there are varying levels of infrastructure and technology.

Traditional methods of processing and value addition to vegetables and fruits have a long history throughout Africa (Steinkraus, 1985). Odunfa (1985) identified food processing that involved fermentation as an important method to facilitate the availability of food and support food security throughout the continent. Cereals and tubers, as well as legumes, fruits, and vegetables are produced in large quantities in many parts of Africa, and because of their mostly perishable nature, these would be targets for optimized postharvest processing. Postharvest processing based on fermentation has been used to produce and increase the shelf life of a variety of foods at either household or small scale, cottage-type business in Africa for decades (Odunfa, 1985; Steinkraus, 1985). The many advantages of fermenting agricultural produce must have been recognized throughout the continent as important strategy for increasing micronutrient supply, improving palatability and detoxification, as well as shelf life and digestibility. The significance of food fermentation as a sustainable postharvest technology, especially for developing countries, has become well-recognized by FAO which published global perspectives (Battcock and Azam-Ali, 1998; Haard et al., 1999; Deshpande et al., 2000). Apart from contributing to the dietary intake of the people at both the macro- and micronutrient levels, it improves safety, quality and availability of foods and generates income for the food processors.

The aim of this review is to describe different lactic fermented fruit and vegetable fermentations that are currently utilized in Africa and to identify possible novel production processes. The involvement of the different microorganisms associated with the fermentations will be assessed. The beneficial roles that traditional fermented foods may play in the diet and health of African consumers will also be addressed, as well as the development of concepts that could facilitate development of new products or process optimization which may lead to products with improved safety, quality or added value.

Fruits and vegetables produced in the different regions of Africa are classified in this chapter as foods that include leafy vegetables, fruits, and protein-oil seeds. The starchy vegetables are not considered in this review. Very high percentages of fruits and vegetables are consumed after harvest in Africa. In many countries, traditional processing of fruits and vegetables play important roles in the food supply, especially during off seasons and harvest.

ROLES OF FRUITS AND VEGETABLES IN NUTRITION AND HEALTH OF AFRICAN CONSUMERS

Plant products including fruit and vegetables, cereals, legumes, seeds, roots, and tubers are an important source of fiber, carbohydrate, protein (**Table 1**), as well as source of amino acid, fatty acids, minerals (**Table 2**), and vitamins (**Table 3**). African leafy vegetables (ALVs) are a good source of vitamin A, being able to provide >75% of the recommended daily allowance (RDA; van Jaarsveld et al., 2014). Especially black nightshade, pigweed, cowpea and spider flower were found to have higher β-carotene content than conventional leafy vegetables. ALVs also have much higher mineral concentrations (>1% of plant dry weight) than conventional leafy vegetables, thus making them a superior source of mineral supplements (Odhav et al., 2007). Apart from this, they may also be an important source

TABLE 1 | Proximate composition of some raw leafy African vegetables per 100 g fresh material.

	Moisture (g)	Protein (g)	Fat (g)	Total ash (g)	Dietary fiber (g)	Carbohydrates (g)
Cucurbita maxima (pumpkin leaves)[a]	87.3	4.24	0.12	3.23		
Amaranthus tricolor (misbredie)[a]	89.9	3.49	0.15	2.12		
Corchorus tridens (wild jute)[a]	81	5.19	0.25	3		
Solanum retroflexum (black nightshade)[b]	89.5	0.5	0.4	1.32	2.5	8.2
Amaranthus cruentus (pigweed)[b]	82	4.2	0.3	2.38	6.7	11.2
Corchorus olitorius (jew's mallow)[b]	79.6	3.2	0.1	1.81	10.8	15.3
Vigna unguiculata (cowpea)[b]	82.4	4.7	0.6	1.76	5.8	10.5
Cucurbita maxima (pumpkin leaves)[b]	85.6	2.9	0.2	1.51	3	9.8
Citrullus lanatus (tsamma melon leaves)[b]	81.3	3.5	0.4	1.66	3.8	13.1
Cleome gynandra (spider flower)[b]	87.5	5	0.3	1.46	3.1	5.7
Amaranthus hybridus (cockscomb)[c]	85	6	0.5	4.91	2.81	6.09
Bidens pilosa (black jack)[c]	88	5	0.6	2.82	2.92	3.72

[a]*Schoenfeldt and Pretorius, 2011;* [b]*van Jaarsveld et al., 2014;* [c]*Odhav et al., 2007.*

TABLE 2 | Mineral composition of some raw leafy African vegetables per 100 g fresh material.

	K (mg)	P (mg)	Ca (mg)	Mg (mg)	Mn (μg)	Fe (mg)	Cu (mg)	Zn (mg)
Cucurbita maxima (pumpkin leaves)[a]		119	383	142		15.9		0.9
Amaranthus tricolor (misbredie)[a]		70.6	232	141		16.2		0.8
Corchorus tridens (wild jute)[a]		136	585	80,9		6.3		0.8
Solanum retroflexum (black nightshade)[b]	257	36	199	92	2080	7.2	0.16	0.56
Amaranthus cruentus (pigweed)[b]	459	81	443	242	2340	5.1	0.17	0.7
Corchorus olitorius (jew's mallow)[b]	407	118	310	87	790	3.6	0.19	0.57
Vigna unguiculata (cowpea)[b]	238	51	398	62	2690	4.7	0.14	0.42
Cucurbita maxima (pumpkin leaves)[b]	351	102	177	67	540	9.2	0.21	0.75
Citrullus lanatus (tsamma melon leaves)[b]	260	119	212	59	760	6.4	0.2	0.74
Cleome gynandra (spider flower)[b]	374	138	232	76	580	2.1	0.25	1.04
Amaranthus hybridus (cockscomb)[c]		106	401	224	4.1	4	0.3	3.1
Bidens pilosa (black jack)[c]		60	162	79	2.5	2	1.2	2.6

[a]*Schoenfeldt and Pretorius, 2011;* [b]*van Jaarsveld et al., 2014;* [c]*Odhav et al., 2007.*

TABLE 3 | Selected vitamins of some raw leafy African vegetables per 100 g fresh material.

	Carotene (mg)	Vitamin A (μg) RAE	Ascorbic acid (mg)	B1 (mg)	B2 (mg)
Cucurbita maxima (pumpkin leaves)[a]	1.7				0.12
Amaranthus tricolor (misbredie)[a]	1.6				0.03
Corchorus tridens (wild jute)[a]	3.67				0.07
Solanum retroflexum (black nightshade)[b]	5.57	422	5	0.08	0.17
Amaranthus cruentus (pigweed)[b]	7.14	537	2	0.04	0.05
Corchorus olitorius (jew's mallow)[b]	4.3	329	1	0.02	0.03
Vigna unguiculata (cowpea)[b]	7.03	537	9	0.07	0.08
Cucurbita maxima (pumpkin leaves)[b]	4.25	325	2	0.04	0.1
Citrullus lanatus (tsamma melon leaves)[b]	4.96	375	10	0.01	0.1
Cleome gynandra (spider flower)[b]	5.94	434	2	0.06	0.21

[a]*Schoenfeldt and Pretorius, 2011;* [b]*van Jaarsveld et al., 2014.*

of antioxidants (Willcox et al., 2003). A shift in the oxidative potential in the human body has been recognized to be due to the limitation of antioxidants, which leads to oxidative stress and cellular oxidative damage. Antioxidants from fruits and vegetables were identified to be essential for the balancing of oxidative stress (Rautenbach et al., 2010) by way of supplying antioxidants such as vitamin C, carotenoids, tocopherols, and polyphenols, all which are important to human health.

Antioxidants play a role also in the prevention of development of chronic diseases such as cancer, cardio vascular disease (hypertension) and pathogenesis of immune deficiency virus (Willcox et al., 2003). Some fermented plant products have been shown to possess higher vitamin contents than the unfermented foods. This was the case for instance for fermented vegetable proteins occurring in fermentations for the production of *iru* or *dawadawa*. These contain higher levels of riboflavin than the unfermented seeds (Odunfa, 1986). Methionine- and lysine- producing lactobacilli strains have also been isolated from traditional fermented *ogi* (Odunfa et al., 2001). A novel *Lactobacillus rossiae* DSM15814^{T} species was shown to possess a complete *de novo* biosynthetic pathway for synthesis of riboflavin, vitamin B12 and other B vitamins (De Angelis et al., 2014), and an *in situ* study showed the relevance of such strains in cereal fermentations (Capozzi et al., 2012). Thus, in the fermentations the microorganisms or their products can contribute to the micronutrient supply and may thus contribute to prevention of malnutrition.

FOOD FERMENTATION AS A POSTHARVEST STRATEGY FOR FOOD SECURITY IN AFRICA

Fermentation used as a traditional food processing technique, contributes to human energy food requirement, protein intake, fatty acids, and micronutrient intake. It has been well reported, that especially lactic acid fermentations used as traditional food processing techniques are based on general methods such as mechanical de-hulling of seeds, peeling of tubers, grating, boiling, soaking, and pressing the starting material in order to prepare the substrate for fermentation. This is followed then by the common fermentation stage, where microbial biochemical changes are brought about by wild-type lactic acid bacteria (LAB) that originate from the raw materials (Leroy and De Vuyst, 2004). These biochemical changes are based on the LAB sugar metabolism and result in product acidification, as well as a concomitant flavor enhancement and aroma development (Leroy and De Vuyst, 2004). Traditional processes that involve fermentation of agricultural products are common practice throughout Africa, with a long history of household and small scale, cottage-type level production (Kimaryo et al., 2000; Holzapfel, 2002). Many of the methods were developed based on a need for food preservation and for attaining an adequate nutrition (Nout and Motarjemi, 1997; Galati et al., 2014). Furthermore, fermentation processes resulted in acceptable developments of flavor and aromas, and/or in detoxification of product, which improve either the raw material sensory characteristics or render them edible (Holzapfel, 1997; Nout and Motarjemi, 1997).

Cereals (Nout, 2009; Franz et al., 2014; Galati et al., 2014) and starchy roots (Franz et al., 2014) are important substrates for probably the majority of African fermented plant products. This review, however, specifically addresses the fruit and vegetable fermentations in Africa, which are relatively less practiced and for which relatively less information is available. The major types of fruit and vegetable fermentations identified in different regions of Africa are classified here on the basis of LAB either dominating or occurring in co-metabolism with other microbes, thereby impacting biochemical transformation of different vegetal components. These include (i) lactic fermented leafy vegetables (ii) alkaline fermented vegetable proteins containing LAB (iii) fermented fruits. These will be discussed with different examples in the sections below. It should be noted that the classification of the bacteria associated with fermentations described in some of the older studies mentioned below were based on phenotypic and biochemical data only and may thus not be according to current classification.

AFRICAN FERMENTED VEGETABLES AND FRUITS

Lactic Acid Fermented Leafy Vegetables

The tropical climate and agricultural land in Africa supports the growth of different leafy vegetables. Some ALV plants that are traditional to Africa and only successfully grow in this continent are listed in **Table 4**. Leafy vegetables have a short shelf life and are highly perishable, and different ALVs are indigenous to different regions of the continent (Shiundu and Oniang'o, 2007) (**Table 4**). Processing of ALVs immediately after harvest includes washing, shredding and drying. Sun-drying and fermentation are the two most important processing techniques used for processing of ALVs (Ayua and Omware, 2013). Some ALVs are also fermented after shredding, an example for this is the production of *kawal* in the Sudan, where the fresh leaves of the leguminous plant *Cassia obtusifolia* L. are fermented and they are consumed as meat or fish protein substitutes in soups and sauces (Suliman et al., 1987). The leaves are abundantly available and serve as cheap source of proteins and amino acids, with a high composition of oxalate (Dirar et al., 1985). Production of *kawal* involves a solid state fermentation of the leguminous leaves by bacterial species such as *Bacillus subtilis*, *Propionibacterium,* and *Staphylococcus sciuri,* with participation of LAB such as *L. plantarum* (Dirar et al., 1985).

In the Congo, *ntoba mbodi* is a fermented leafy vegetable consumed as condiment (Sanni and Oguntoyinbo, 2014). Kobawila et al. (2005) produced a flow diagram describing the fermentation processing of *ntoba mbadi*. The processing involves sun-drying cassava leaves for 2–3 h to wilt the leaves, which allows easier removal of stalks and petioles. The lamina are cut into fragments, washed with water, packed and wrapped in papaya (*Carica papaya* L.) leaves, and are then left to ferment for 2–4 days in a basket. The fermentation is a semi-solid process, alkaline fermentation, which leads to a steady increase in pH to 8.5. The bacteria reported to be involved include the *Bacillus* spp., *B. macerans*, *B. subtilis,* and *B. pumilus.* Other bacteria, such as *Staphylococcus xylosus* and *Erwinia* spp., as well as LAB such as *Enterococcus faecium*, *E. hirae*, *E. casseliflavus*, *Weissella confusa*, *Weisella cibaria,* and *Pediococcus* spp., have also been reported to co-occur in the fermentation (Ouoba et al., 2010; Sanni and Oguntoyinbo, 2014).

TABLE 4 | Distribution of some regional and common African leafy vegetables.

All over the sub-continent	West/East and Central Africa	West and Southern Africa	East/Central and Southern Africa
Abelmoschus esculentus (ladies' fingers)	*Basella alba* (vine spinach)	*Amaranthus caudatus* (Aluma)	*Solanum nigrum* (black nightshade)
Amaranthus cruentus (amaranth)	*Citrullus lanatus* (watermelons)	*Amaranthus hybridus* (amaranth)	*Bidens pilosa* (black-jack)
Corchorus olitorius (jute mallow)	*Colocasia esculenta* (cocoyam)	*Portulaca oleracea* (purslane)	*Cleome gynandra* (African cabbage)
Cucurbita maxima (pumpkins)	*Hibiscus sabdariffa* (zobo)		
Vigna unguiculata (cow-pea)	*Ipomea batatas* (sweet potato)		
Solanum macrocarpon (African eggplant)	*Manihot esculenta* (cassava)		
	Solanum aethiopicum (mock tomato)		
	Solanum scabrum (garden huckleberry)		
	Talinum triangulare (waterleaf)		
	Vernonia amygdalina (ewuro)		
	Moringa oleifera (moringa or drumstick tree)		
	Solanecio biafrae (Worowo)		

Adapted from Smith and Eyzaguirre (2007).

It should be noted, that some of the bacteria mentioned above which occur in leafy vegetable fermentations are regarded as potentially pathogenic, as is the case for *Enterococcus* spp. such as *E. faecalis* and *E. faecium*, and for some toxinogenic *Bacillus* spp.

Apart from the effect of lactic preservative influence, reduction of cyanogenic acid in the leaves and mineralization, further beneficial changes are brought about by the fermentation process (Ouoba et al., 2010; Sanni and Oguntoyinbo, 2014). In Kenya, cowpea leaves (*Vigna unguiculata* syn. *Vigna sinensis*) are part of the diet, and a recent study showed that natural fermentation can improve the keeping quality, retaining β-carotene by 91% and ascorbic acid by 15%, while a sensory evaluation showed a good consumer acceptance of the fermented cowpeas (Muchoki et al., 2007). This study, as well as the study by Wafula et al. (2015), showed that cowpeas leaves do not contain sufficient levels of sugar to support the fermentation by autochthonous bacteria, and that sugar and preferentially also starter cultures should be added to obtain a reliable fermentation of this product.

In Kenya, African kale leaves are also processed in a fermentation-like manner, by soaking the vegetables in milk for a few days to achieve the removal of the bitter taste. However, little is known about the fermentation of kale and studies on which bacteria are important for the fermentation and on the dynamics of the fermentation are required.

Alkaline Fermented Vegetable Proteins Involving Lactic Acid Bacteria in the Fermentation

A significant proportion of the protein intake in African countries is vegetal-plant-protein sources, notably the proteinaceous seeds (oil seeds), many of which are consumed in form of fermented vegetable proteins (Odunfa, 1988). The seeds bearing the cotyledon used in production of condiments are produced in large quantity in Africa, especially from members of the *Malvaceae* family plants, such as *Adansonia digitata, Parkia biglobosa*, *Prosopis africana, Hibiscus sabdariffa,* and from the *Fabaceae*, leguminous-bean producing plants, e.g., cowpeas (*Vigna unguiculata*) and soy beans (*Glycine max*; Parkouda et al., 2009). Some of African fermented vegetable proteinaceous seeds and the corresponding condiments produced and consumed from these in different regions of Africa are shown in **Table 5**.

The climatic condition in Africa favors a wide diversity and distribution of plants of the family *Malvaceae* across the continent. The seeds are, however, not directly consumed without processing, because of their anti-nutritional compounds such as proteinase inhibitors, amylase inhibitors, metal chelators, flatus factors, haemagglutinins, saponins, cyanogens, lathyrogens, tannins, allergens, acetylenic furan, and isoflavonoid phytoalexins (Pariza, 1996). *Parkia biglobosa* and soybean typically contain trypsin inhibitors, which reduce the digestibility of proteins (Collins and Sanders, 1976) and carbohydrate fractions that are responsible for flatulence after ingestion (Fleming, 1981). Soybean contains high levels (120–150 gkg^{-1} dry wt) of α-galactosides of sucrose, causing gastrointestinal gas production in humans (Sarkar et al., 1997). Kawamura (1954) observed that over 90% of the sugars present in ripe soybeans comprise sucrose and the indigestible (but fermentable) sugars raffinose and stachyose. Cottonseed also contains gossypol, an antinutritional factor, while mesquite seeds *Prosopis africana* can cause fetal abortion in domestic animals. However, there is long history of consumption of these seeds in Africa (Odunfa, 1985). Processing and fermentation must therefore have contributed significantly to the extensive hydrolysis of the seeds and concomitant detoxification. Different communities have developed strategies for processing of the seeds for food, especially through the use of natural fermentation, to produce foods which are rich in vegetable proteins and which are used as seasoning agents or as meat or fish substitutes (Odunfa, 1985; Steinkraus, 1996).

Traditional processing of these seeds includes wet de-hulling, boiling and fermentation. There are similar fermented vegetables proteins bearing different names in Africa, also the processing techniques often follow a similar methodology. The common examples of fermented vegetable proteins reported in Africa are shown in **Table 6**. The fermentation process during production

TABLE 5 | African fermented vegetable proteins with reported microorganisms involved.

Fermented food product	Country	Vegetal Substrate	Microorganisms	Reference
Iru or Dawadawa	Nigeria	*Pakia biglobosa*	*B. subtilis, B. amyloliquefaciens,* LAB	Adewumi et al., 2013
Okpehe	Nigeria	*Prosopis africana*	*B. subtilis, B. amyloliquefaciens, B. cereus,* and *B. licheniformis, Enterococcus* spp.	Oguntoyinbo et al., 2010
Maari	Burkina Faso	*Adansonia digitata*	*B. subtilis, E. faecium, E. casseliflavus, Pediococcus acidilactici*	Parkouda et al., 2009; Sanni and Oguntoyinbo, 2014
Bikalga	Burkina Faso		*B. subtilis, B. licheniformis, B. cereus, B. pumilus, B. badius, Weissella confusa, Weissella cibaria, L. plantarum, Pediococcus pentosaceus, Enterococcus casseliflavus, E. faecium, E. faecalis, E. avium, E. hirae, Brevibacillus bortelensis, B. Sphaericus,* and *B. fusiformis*.	Ouoba et al., 2008, 2010
Ugba	Nigeria	*Pentaclethra macrophylla*	*B. subtilis, B. licheniformis, B. megaterium, B. pumilus*	Anyanwu et al., 2016

has been described as an alkaline fermentation, due to the microbial enzymatic changes that involve hydrolysis of proteins to polypeptides, peptides, amino acids, and ammonia, thereby bringing about the increase in the pH value from 6.8 to 8.0. Fermented vegetable proteins have been described to be very rich in polyglutamic acid as a result of *Bacillus* metabolism, with compounds such as 3-hydroxybutanone (acetoin) and derivatives [butanedione (diacetyl) and 2,3-butanediol], acids (acetic, propanoic, 2-methylpropanoic, 2-methylbutanoic, and 3-methylbutanoic), as well as pyrazine also being produced.

The ecology of microbes predominantly responsible for the important biochemical changes occurring during traditional fermentation of vegetal proteins was shown to involve diverse bacterial species. Starter cultures are generally not used, and natural fermentation is dominated by different bacteria with enzymatic activities, including *B. subtilis*-group bacteria such as *B. subtilis senso stricto*, *B. licheniformis, B. amyloliquefaciens,* and *B. pumilus*. In some alkaline fermentations of vegetal proteins, potentially pathogenic *B. cereus* strains were also described to occur (Oguntoyinbo et al., 2010). Studies indicated high proteolytic and amylolytic microbial activities, occurring from the onset of the fermentation for up to 48 h. Different species of LAB were also isolated during fermentation of vegetal proteins for condiment production in Africa. Ouoba et al. (2010) reported *Enterococcus faecium*, *E. hirae* and *Pediococcus acidilactici* to occur in *bikalga* and *soumbala.* Oguntoyinbo et al. (2007) isolated

TABLE 6 | Examples of mixed lactic, acetic acid and alcoholic fermented vegetal starch beverages in Africa.

Fermented food product	Country	Vegetal Substrate	Microorganisms	Reference
Tella	Ethiopia	Sorghum	Yeast and LAB	Faparusi, 1973
Burukutu	Ethiopia Nigeria, Ghana	Guinea corn and cassava	*Saccharomyces cerevisiae*, *Lactobacillus plantarum* and *L. fermentum*	Faparusi, 1973
Pito	Nigeria, Ghana	Guinea corn and maize	*L. fermentum, L. delbrueckii, P. acidilactici*, *S. cerevisiae, C. tropicalis, K. apiculata, H. anomala, S. pombe, K. africanus*	Sefa-Dedeh et al., 1999; Sawadogo-Lingani et al., 2007
Kaffir beer	South Africa	Kaffir corn or maize	*Saccharomyces cerevisiae, Lactobacillus, Acetobacter*	Hesseltine, 1979; Odunfa and Oyewole, 1998
Busaa	East Africa	Maize	*Saccharomyces cerevisiae, Candida krusei, Lactobacillus plantarum, L. helveticus, L. salivarius, L. brevis, Weissella viridescens, Pediococcus damnosus, P. parvulus.*	Nout, 1980;
Malawa beer	Uganda	Maize	Unknown	–
Zambian opaque beer	Zambia	Maize	Unknown	–
Merissa	Sudan	Sorghum	LAB, yeast	Dirar, 1978
Sekete	Nigeria (south)	Maize	*A. aceti, A. pasteurianus, L. brevis, L. buchneri, L. plantarum, Lactobacillus* spp., *S. cerevisiae, Saccharomyces* spp., *Flavobacterium* spp., *Micrococcus varians, B. licheniformis*	Sanni et al., 1999
Bouza	Egypt	Wheat and maize	Unknown	
Kishk	Egypt	Wheat and milk	*Lactobacillus*, yeast, and *B. subtilis*	Morcos et al., 1973
Tchoukoutou	Benin	Sorghum	Yeast and LAB	Greppi et al., 2013

Enterococcus spp. from *okpehe* which led to a cheese-like aroma development during model fermentations, demonstrating that these bacteria could also affect the product characteristics in a negative way. As mentioned before, enterococci are not always regarded as favorable microorganisms because of the association of specific strains with infections in hospitals (Franz et al., 2011). A recent study showed that LAB could also play a positive role in the flavor development during fermentation of vegetable proteins of other legumes. An *in vitro* determination of volatile compound development during starter culture-controlled fermentation of *Cucurbitaceae* cotyledons showed that a mixed culture of *L. plantarum, Torulaspora delbrueckii,* and *Pediococcus acidilactici* could contribute to development of volatile compounds such as esters and low concentrations of aldehydes and ketones during fermentation (Kamda et al., 2015).

Fermented Fruits

Alcoholic Beverages from Fruits Involving Lactic Acid Bacteria in the Fermentation

Different fruits are grown in Africa and are harvested annually in different regions of the continent. Fruits used in Africa include banana, papaya, *marula*, mango, tomato, the sausage tree (*Kigelia Africana)* fruit and the *Ziziphus mauritiana* (*masau* or *jujube*) fruits. Because of the low pH and high acidity of fruits, microbial deterioration is very slow, and usually only osmophilic and acetotolerant microorganisms or yeasts are responsible for the major biochemical changes. Fruits are processed into different products that include juices, pickles, alcoholic beverages and vinegar. The fermentation aspect thus relies mostly on the production of alcoholic beverages or vinegars from fruit juices. A typical method of processing and fermentation of African fruit during *muratina* production from fruit of the 'sausage tree' (*Kigelia africana*) is shown in **Figure 1**. Other examples of fermented fruits in Africa are shown in **Table 7**. In Nigeria, information is available on *agadagidi*, an effervescent drink produced from ripe plantain (*Musa paradisiaca*) pulp. It is a popular drink in South Western Nigeria during ceremonies (Sanni and Oso, 1988; Sanni, 1989). Similarly, *uruaga* is a fermented banana in Uganda, while cashew and cocoa wine are also popular in Nigeria.

Banana beer is a beverage popular throughout Africa and is made by fermenting banana juice with cereal flour, often sorghum flour (Marshall and Mejia, 2012). It is sweet and slightly hazy with a shelf life of several days. Ripe bananas (*Musa* spp.) are used, as these have high sugar content. Preparation involves extracting the juice from peeled bananas and the juice is diluted with clean, boiled water. Grinded cereal (sorghum or millet) is roasted over an open fire, added to the diluted banana juice in a bucket and left to ferment 18–24 h. The naturally occurring yeasts on the banana are responsible for fermentation. The ground cereal improves the color and flavor of the beer. After fermentation, the beer is filtered through a cotton cloth (Marshall and Mejia, 2012). In Rwanda, the banana beer '*urugwa*' is produced by crushing and squeezing peeled ripe bananas to obtain juice that is then mixed with water to a desired proportion. Crushed roasted sorghum grains are added, and the mixture is then allowed to ferment for even 2–4 days in a warm pit covered with banana leaves (Shale et al., 2014). As banana beer is made from raw materials which are not boiled, the beer has only a short shelf life and should be kept as cool as possible, as it is an excellent substrate for microbial growth. Thus it is essential, that attention is paid to using clean equipment and processing area, as well as personal hygiene for the production of this beverage (Marshall and Mejia, 2012).

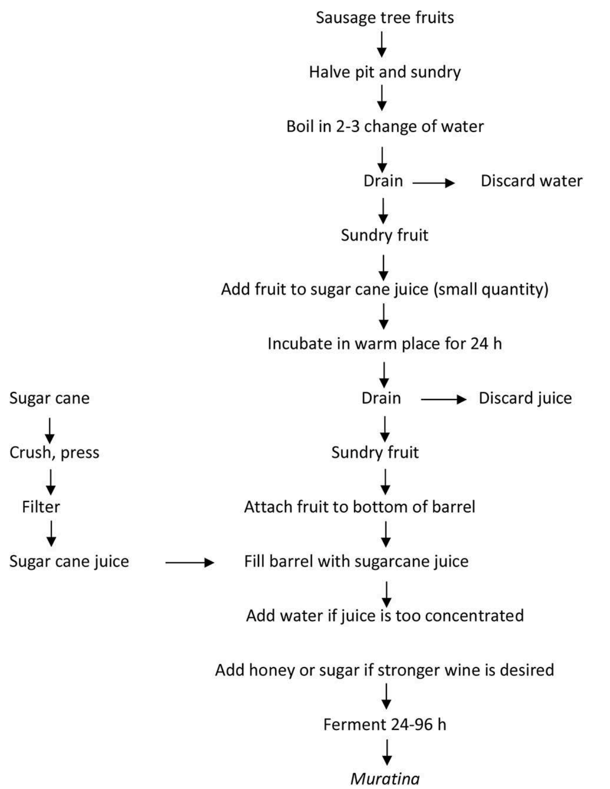

FIGURE 1 | Flow diagram of fermented sausage tree fruit for *muratina* production. Adapted from Harkishor (1977).

In Zimbabwe, wild fruits from the buffalo thorn (*Ziziphus mauritiana, masau)* are usually processed into porridge, traditional cakes, *mahewu* and jam (Nyanga et al., 2007). Moreover, they are also fermented to produce alcoholic beverages such as *kachasu*. They are crushed, soaked for some hours and then allowed to ferment (Gadaga et al., 1999). Nyanga et al. (2008) reported that *masau* is rich in citric-, tartaric-, malic-, succinic- and oxalic acids, as well as in minerals, fiber, crude protein and vitamin C (Nyanga et al., 2013). *Lactobacillus agilis, L. plantarum, W. minor, W. divergens, W. confusa, L. hilgardii, L. fermentum,* and *Streptococcus* spp. were isolated from *masau* fruit products and were identified as bacteria that could be developed as starter cultures for fermentation of the fruit products (Nyanga et al., 2007).

In Zimbabwe, an alcoholic beverage called *mudetemwa* is produced from the fruits of the sand apple (*Parinari curatellifolia*). The fruits are pounded and the juice is extracted

TABLE 7 | Examples of mixed lactic, acetic acid and alcoholic fermented fruit beverages in Africa.

Fermented food product	Country	Fruit and vegetable	Fermentation	Microorganisms	Reference
Agadagidi	Nigeria	Plantain	Alcoholic	*Saccharomyces, Leuconostoc*, and *Streptococcus. Bacillus* and *Micrococcus*	Sanni, 1989
Cashew wine	Nigeria	Cashew	Alcoholic	Unknown	–
Cocoa wine	Nigeria	Cocoa	Alcoholic	Unknown	–
Palm wine *Emu* or *oguro*	Africa	Palm sap	Lactic, later alcoholic and acetic acid	*Lactobacillus plantarum, Leuconostoc mesenteroides, Fructobacillus durionis,* and *Streptococcus mitis.* Acetic acid bacteria. *Saccharomyces cerevisiae, Arthroascus, Issatchenkia, Candida, Trichosporon, Hanseniaspora, Kodamaea, Schizosaccharomyces, Trigonopsis*, and *Galactomyces.*	Faparusi and Bassir, 1972; Ehrmann et al., 2009; Ouoba et al., 2012
Uruaga	Kenya	Banana	Alcoholic and lactic	Unknown	–
Ulansi	East and South Africa	Bamboo	Alcholic and lactic	Unknown	–
Muratina	Kenya	*Sausage tree fruit (Kigelia africana)*	Alcholic and lactic	Uknown	–

by hand and boiled. After allowing to ferment overnight, the juice is again boiled, then allowed to cool and is drunk as beer (Gadaga et al., 1999). The fruit of the sugar plum tree (*Uapaca kirkiani*) are also used for production of alcoholic beverages in Zimbabwe. For this purpose, the fruits are pounded to break the skins and the seeds are extracted. The pulp is mixed with cold water and left to ferment into a sweet wine called *mutandavira* (Gadaga et al., 1999).

Recently, a fortified lactic acid fermented probiotic dairy product with a 14% (wt/vol) concentrated baobab fruit pulp, *mutandabota*, was developed in Zimbabwe. *Lactobacillus rhamnosus* (yoba) was used as starter culture for the fermented dairy drink, leading to a product with pH value of 3.5, which was rich in protein and vitamin C, with potential for improvement of intestinal health (Mpofu et al., 2014). The need for development of lactic fermented beverages that could support a healthy living and contribute to the dietary intake has been strongly suggested for the African population recently (Franz et al., 2014).

The fermented juice from palm sap of both *Rafia guineensis* and *Borassus akeassii*, popularly known as palm wine, is consumed widely in many African countries. During the fermentation process, *Saccharomyces cerevisiae* ferments the glucose as well as other plant derived carbohydrates such as sucrose, maltose and raffinose to produce alcohol. Apart from the yeasts, bacteria such as strains of *Leuconostoc, Lactobacillus*, and acetic acid bacteria have been described to play a role in the fermentation, and these were isolated at the initial and later stages of the fermentation, respectively (Amoa-Awua et al., 2007; Ouoba et al., 2012).

Wine is also produced from the fruits of the *marula* (*Sclerocarya birrea*) tree. A potent wine made from *marula* is *buganu,* which is produced in Swaziland (Simatende et al., 2015). For *buganu* production, fresh ripe fruits (10 kg) are washed and pounded or pressed to remove the juice. The juice, pulp and seeds are transferred to plastic buckets and water (10 L) is added, followed by the addition of 2 kg of sugar. The slurry is then fermented for 3 days at 25–30°C and additional water (10 L) is added. The mixture is then stirred and sieved with a traditional grass sieve or metal mash. Sugar (2 kg) is again added and the juice is fermented for a further 12 h at 25–30°C to obtain the *marula* wine *buganu* (Simatende et al., 2015). Both fermentative and non-fermentative yeasts were isolated from *marula* fruits, but the role of these in the production of *marula* wine has not been studied (Okagbue, 1995; Gadaga et al., 1999). Prouction of *marula* at a commercial scale has been achieved in South Africa, with the liquor Amarula, which is internationally available.

Fruit processing into wine is well developed at an industrial scale in South Africa. Grapes are commonly used for wine production, and LAB play an important role for instance in the malolactic fermentation important for biological de-acidification of wine. This is a decarboxylation process by which malic acid, a dicarboxylic acid naturally present in grape, is converted to lactic acid with concurrent liberation of carbon dioxide. This fermentation plays an important role in de-acidification and aroma development of specific wines. LAB such as *Oenococcus oeni*, and various species of *Lactobacillus* and *Pediococcus* have been reported to occur in wine or to play a role in malolactic fermentation during South African wine production (Du Toit et al., 2011; Miller et al., 2011). Recently, a bacteriocin-producing *Enterococcus faecium* was isolated from South African wine production, (Ndlovu et al., 2015), but whether such bacteria play a beneficial or detrimental role is currently not known.

Production of Vinegar from Fruit Juices

In Africa, different indigenous fruits are also processed into vinegar, however, at a very small scale. Fruit vinegars are made from fruit wines that are processed from fruits such as plum, mango, apple cider, *marula*, coconut and grapefruit (Ndoye et al., 2007). Ameyapoh et al. (2010) investigated the potential for vinegar production from mango (*Mangifera indica* var. Linn) in Togo. Vinegar was produced by a successive fermentation with *Saccharomyces cerevisiae* and acetic acid bacteria. For this, mangos were washed and peeled and mango juice was extracted by mechanical pressure. The juice was pasteurized and concentrated to obtain sugar content of 20° Brix. Yeasts were inoculated (2 mL, total no. of yeasts amounting to 10^6 CFU) and the alcoholic fermentation was done at 30°C for 144 h (Ameyapoh et al., 2010). After this, acetic acid bacteria (2 mL, 10^6 CFU total number bacteria) were added for the acetic acid fermentation at 30°C for 15 days. The successful fermentation in two stages led to a vinegar containing 4.7° acetic acid (mass in gram acetic acid in 100 g vinegar; Ameyapoh et al., 2010). This method for mango vinegar production may thus aid in avoiding postharvest losses, and can provide additional cash income for small-scale producers.

Effect of Fermentation on Detoxification and Nutrient Bioavailability

Fermentation is accompanied by a decomposition of macromolecules. Proteases are active during the alkaline fermentation of vegetable proteins, while amylases and pectinases are important in the macromolecule degradative processes of starchy vegetables. The enzymatic degradative processes result in the breakdown of proteins, carbohydrates and oligosaccharides and thus contribute to the release of important compounds essential to human nutritional requirements (Motarjemi and Nout, 1996). Processing by traditional fermentation thus relies on enzymes produced during germination or from bacteria during fermentation, and these contribute significantly to the bio-availability of macro- and micronutrients of fermented products. Microbial phytase activities may also contribute to the reduction of the antinutritive factor phytate, which occurs in various cereals and legumes (Kayode et al., 2007; Adeyemo and Onilude, 2014). The enzymatic activity of β-glucosidase enzymes of certain LAB or yeasts are important for the breakdown of cyanogenic glucosides such as linamarin and lotaustraline, which is present in maniok (*Manihot esculenta* var. Crantz; Okafor and Ejiofor, 1986; Kostinek et al., 2007) and this, combined with utilization of cyanhydric acid by certain *Bacillus* strains (Kobawila et al., 2005) significantly contribute to the detoxification of the final fermented products (Kostinek et al., 2007; Lambri et al., 2013).

From a health point of view, African vegetables and fruits contain significant levels of micronutrients, as well as high concentrations of bioactive compounds such as carotenoids, flavonoids, phenolic constituents, alkylresorcinols, glucosinolates and saponins which are present in many fruits and vegetables consumed in Africa and may contribute to the consumer's health. Furthermore, the dietary fiber and vitamins in African fruits and vegetables, whose levels vary with cultivar, pre- and post-harvesting, processing and storage conditions (Nout, 2009; Uusiku et al., 2010; Medoua and Oldewage-Theron, 2011; Ogbuanu et al., 2014) are also relevant to consumer health. Microorganisms may play a pivotal role during fermentation in transforming chemical constituents, thereby enhancing the overall nutrition value of the final products via formation of health-promoting bioactive compounds, increased availability of vitamins and minerals, production of antimicrobial and antioxidant compounds or by stimulation of probiotic functions (Đorđević et al., 2010; Shahidi and Chandrasekara, 2013; Wang et al., 2014).

Yeast activity in the fermentation may also increase the vitamin content of vegetables and fruits, such as the availability of riboflavin, vitamin B12 and niacin. Riboflavin and niacin concentrations increased in alcoholic fermented vegetal starch products such as sorghum beer, a popular drink in South Africa, which has been shown to significantly reduce incidences of pellagra (Steinkraus, 2002). Palm wine is also a very rich source of ascorbic acid, thiamine and pyridoxine as well as vitamin B12 and other B vitamins (Steinkraus, 1997). Also, fermented foods are a rich source of folate, this compound is present in various green leafy vegetables, cereals, legumes and they have been linked to the prevention of heart disease, cancer and neuropsychiatric disorders (Brouwer et al., 1999). Group B vitamins (e.g., folic acid, riboflavin, thiamine, and cobalamin) are furthermore synthesized by a variety of LAB (LeBlanc et al., 2011). Vegetables and fruit products can become fortified with these vitamins, present in the biomass of LAB, as a result of fermentation. An increased content of niacin, thiamine, and riboflavin has thus been achieved through the fermentation of fluted pumpkin seeds (Achinewhu, 1986a), oil beans (Achinewhu, 1986b), and of melon seeds (Achinewhu and Ryley, 1987), to produce the condiments *iru* and *ogiri*. *Dawadawa*, which is also known as *uru*, *kpalugu*, *netetou*, or *soumbara*, is an African fermented food used as food condiment and meat substitute. It is obtained by fermentation of the African locust beans, which after fermentation have a higher digestibility than the unfermented beans, due to the enzymatic activity of the microbiota involved (Eka, 1980). *Dawadawa* contains a higher amount of riboflavin and thiamine as a result of fermentation, as well as a lower amount of flatus-forming oligosaccharides, the latter mainly due to the α- and β-galactosidase activities of the microbiota (Odunfa, 1983, 1985; Oboh et al., 2008).

RESEARCH AND DEVELOPMENT POTENTIAL AND RECOMMENDATIONS

The multiple problems that are still rampantly occurring on the African continent include problems of infrastructure, water supply, sanitation, and hygiene during processing. These, however, often still compromise the safety and quality of many traditional lactic fermented foods. Home and cottage sized, small-scale food processing endeavors, using crude techniques and rudimentary utensils, are mainly adopted and these are relatively uncontrolled processes, thereby exposing many of these foods to inconsistent quality or to different pathogenic microbes (Oguntoyinbo, 2014).

Research and Development Potential

Processing using fermentation for value addition to fruits and vegetables is still majorly done in small scale and at household levels. Apart from supporting family nutritional intake, it also contributes to the economic activities, especially by increasing the income of women, who are the major processors and traders. Many of these fermented vegetal foods face safety or quality challenges and the strategies to ameliorate these challenges for sustainable industrial processing is further discussed.

Microbial Safety Challenges

Fermented vegetables and fruit face different microbial deterioration and safety issues. This is mainly a result of contamination during handling or post processing and cross contamination. Inadequate sanitation, inadequate and uninterrupted water supply and lack of good manufacturing practices are challenges to processors in developing countries. As mentioned above, potentially pathogenic bacteria such as *B. cereus* strains or *E. faecium* and *E. faecalis* strains have been described to occur as part of the microbiota of many vegetal protein or leafy vegetable fermentations. Different efforts and strategies have been suggested for the production of traditional vegetables and fruits in Africa, in order to guarantee microbial and chemical safety quality (Motarjemi and Nout, 1996; Holzapfel, 1997; Holzapfel, 2002). Development of Hazard Analysis and Critical Control (HACCP) is promising; it has been designed as base-line intervention strategy for some of the fermented vegetable protein such as *dawadawa* (Oguntoyinbo, 2012) and the fermented cassava product *fufu* (Obadina et al., 2009). Another strategy that has been proposed is the improvement on the back-slopping technique during fermentation. Back slopping refers to adding a small portion of a previous successful fermentation to a new fermentation, without knowing which microorganisms actually where present and responsible for the fermentation. For this improvement, an undefined mixture of starter cultures with known ability to dominate fermentations and more importantly, to inhibit pathogens is used as starting material to start fermentations. Starter cultures with such ability abilities have been selected in some pilot, as well as field studies for improvement of fermentation. Small portions of successful fermentation batches are kept and re-used for subsequent fermentation batches (Holzapfel, 2002). The fast growth of the starters and their success to establish themselves as dominant microorganisms in the fermentation leads to fast acidification and prevents growth of potential pathogens (Motarjemi and Nout, 1996; Holzapfel, 2002). An attractive alternative to back-slopping is the development of suitable starter cultures for fast growth and acidification in the fermentation medium (Holzapfel, 1997, 2002; Leroy and De Vuyst, 2004; Huch et al., 2008). However, for this a suitable industrial starter culture producer would need to be present locally, unless starter cultures are produced also at a household level using low-level technology (Holzapfel, 2002).

Process Optimization

Small scale traditional processing of vegetable and fruits is improving in term of scale-up technology. The processes now utilize specialized, mechanical equipment for grating and milling as well as fermentation tanks, cookers, and hydraulic presses. This has improved processing time and has aided in process scale-up. However, there is still a need for the development of techniques for larger scale industrialization, including peeling and de-hulling systems for seeds and tubers, pressure cookers and boilers, as well as industrial dryers. Optimized packaging and storage of fermented vegetables and fruits may also affect keeping quality and may improve attractiveness. There is ample opportunity for small business development in this sector, but this will depend on a close collaboration of small scale-producers with academic institutions who can provide the training in fermentation technology and who can develop and provide starter cultures. Food microbiologists and food technologists could work hand with women's groups and local entrepreneurs, while local stakeholders and financial institutions could help to initiate small startup initiatives.

Nutritional Improvement

Nutritional value addition to fermented vegetal and fruits would contribute to the dietary status of consumers and thus toward a healthy population, and would also improve product acceptability. Such value addition may arise from the use of multifunctional starter cultures with high potential to increase the bio-availability of especially minerals, different vitamins and antioxidants. Thus, lactic fermentation could play an important role in the improvement of not only shelf life, but also the nutrient availability of fermented vegetal products. An open question which needs to be addressed is that of consumer acceptability of the local population. While lactic fermented foods are common in Africa, the fermentation of leafy vegetables is not common and studies would be required on the sensory acceptability of these products to local consumers.

Recommendations

The research and marketing potential for ALV fermentation should be given high priority. A high variety of indigenous vegetables rich in micronutrients occur in Africa and these should be utilized in order to minimize post-harvest losses. Fermentation is a likely post-harvest processing method that can prevent losses and which contributes to food security and safety. Fermentation of indigenous ALVs with selected starter cultures may lead to improved bio-availability and preservation of trace elements, vitamins and anti-oxidants. Advanced techniques for the production of locally fermented vegetables should be encouraged by local communities, local academic institutions, non-governmental organizations and other stakeholders. Age-old traditions of vegetable fermentation are typical for Europe (e.g., *sauerkraut*) and Asia (*kimchi* for Korea, and diverse vegetable fermentations on a household scale in China). These experiences may serve as valuable guidance for introducing similar (mainly lactic) and well-controlled, small-scale fermentations throughout the African continent, wherever leafy raw materials are available. Africa is rich in different leafy vegetables containing high amounts of nutrients and micronutrients (**Tables 1–4**). It is conceivable, therefore, that efforts for the fermentation of, e.g., cowpea, sorghum, spider plant, or kale leaves are intensified, in order to preserve the nutrients and prevent postharvest losses of such highly perishable products. What needs to be established, however, is whether leaves of these plants contain sufficient amounts of fermentable sugars to lend themselves for fermentation, or whether novel fermentation processes, based on selected starter cultures and on added fermentable sugars, need to be devised and tested. Lastly, it urgently needs to be established, if the local consumers agree to the taste of such fermented leafy vegetables. Sauerkraut may off course be a regional European food which appeals to people in the production region, but possibly not to the African taste. On the other hand, fermented products such as sorghum, cow pea or kale leaves probably don't really taste like Sauerkraut and thus could be incorporated into local foods to agree to local tastes.

In addition, the potential for production of wines and vinegars from fruits should be intensified. Africa has a rich diversity of fruits in its gardens, which could be microbiologically enhanced to high quality vinegars or wines, as to obtain high value products. There certainly could be a good market in Africa or elsewhere for high quality, new juice products and vinegar products for example from indigenous fruits such as cactus pears, marula, Mobola plum (*Parinari curatellifolia*), wild loquat (*Uapaca kirkiana*), Dika tree fruit (*Irvingia barteri*), or wild orange (*Strychnos cocculoides*). There is much potential for fermentation of fruits and vegetables in Africa, what is needed is for universities and research institutes to work together with local producers and possibly NGO's to help develop starter cultures, establish appropriate fermentation technologies, develop innovative and sustainable packaging and improve marketing of these local products.

AUTHOR CONTRIBUTIONS

FO, VF, CF, WH, AG, and HA wrote the main text regarding malnutrition, hidden hunger, and food processing in Africa. G-SC, WB, LF, and BT wrote the parts on nutrition contents and antioxidant activities of the vegetables. BB, JK, HN, NB, and MH wrote the parts on existing fermentations and improving the safety by fermentation, as well as the microbiology of the fermentations.

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Pulque, a Traditional Mexican Alcoholic Fermented Beverage: Historical, Microbiological, and Technical Aspects

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Pulque is a traditional Mexican alcoholic beverage produced from the fermentation of the fresh sap known as *aguamiel* (mead) extracted from several species of *Agave* (maguey) plants that grow in the Central Mexico plateau. Currently, *pulque* is produced, sold and consumed in popular districts of Mexico City and rural areas. The fermented product is a milky white, viscous, and slightly acidic liquid beverage with an alcohol content between 4 and 7° GL and history of consumption that dates back to pre-Hispanic times. In this contribution, we review the traditional *pulque* production process, including the microbiota involved in the biochemical changes that take place during *aguamiel* fermentation. We discuss the historical relevance and the benefits of *pulque* consumption, its chemical and nutritional properties, including the health benefits associated with diverse lactic acid bacteria with probiotic potential isolated from the beverage. Finally, we describe the actual status of *pulque* production as well as the social, scientific and technological challenges faced to preserve and improve the production of this ancestral beverage and Mexican cultural heritage.

Keywords: ***pulque***, ***aguamiel***, **maguey, lactic acid bacteria,** ***Saccharomyces cerevisiae***, **dextran, fructans, probiotics**

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INTRODUCTION

The role of maize in the origin of humans as described in the *Popol Vuh*, the sacred Maya book, together with the betrayal of the Toltec god *Quetzalcoatl* by *Tezcatlipoca* -the omnipresent god of the night who sees everything- are the two favorite stories of Mesoamerican mythology. *Quetzalcoatl* was ruined and had to exile after a ridicule behavior due to an excess of *pulque* intake. Both maize and *pulque* were key in the cosmological vision in Mesoamerica: while maize was linked to their origins, *pulque* was associated to their destiny, the *Temoanchan*, or lost Paradise, inhabited by several gods, where humans were created and *pulque* invented. Both Quetzalcoatl and *Mayahuel* -the Mexican nurturing mother- came to Earth to sing and dance to escape from paradise and to adopt the form of tree branches. However, they were punished by *Mayahuel*'s grandmother who was a *tzitzimitl* -a darkness being- who, together with other *tzitzimime* destroyed the branch where *Mayahuel* was hiding. *Quetzalcoatl*, whose branch was not destroyed, buried *Mayahuel* with great sadness. The first agave plant grew in the place where *Mayahuel* was buried (Gonçalves de Lima, 1956; Anawalt, 1998; Ramírez, 2002).

However, the *Agavaceae* Family is very much older than pre-hispanic mythology, its origin dating back to about 10 million years ago (Good-Avila et al., 2006). Agave is a proliferous Family with nine known genera, comprising 300 species, most of them still present in Mexico. Agaves belong to the Amarilidaceas order and are endemic to Mexico. A restricted number of species are devoted to *pulque* including *A. atrovirens, A. americana, A. salmiana,* and *A. mapisaga* (**Table 1**; Alfaro Rojas et al., 2007; Mora-López et al., 2011).

The ancient Aztecs knew pulque as m*etoctli* (from nahuatl language *metl* = agave or maguey, and *octli* = wine) agave wine, or *iztacoctlli* (from *izac* = white and *octli* = wine) white wine, or *poliuhquioctli* (from *poliuhqui* = spoiled or rotted and *octli* = wine) the spoiled beverage with unpleasant odor and flavor. It is probably from *poliuhquioctli*, that the Spanish conquerors designated as *pulque*, the freshly fermented agave beverage (Gonçalves de Lima, 1956; Sahagún, 1999). *Pulque* is a milky white, viscous, and slightly acidic beverage whit an alcoholic content which depends on several factors but usually between 4 and 7° GL, produced by spontaneous fermentation of *aguamiel*, the sugary sap extracted from the *Agave* species mentioned above (Secretaría de Economía, 1972b). According to Fray Bernardino de Sahagún, in his "Historia General de las Cosas de Nueva España," numerous gods were involved in the *Mayahuel*'s gift to humanity. Among others, he mentions *Ometochtli* who for the Aztecs was also the god of drunkenness, also associated with plant fertility and the wind. He ruled over the 400 *Centzontochtli*, or God rabbits of drunkenness, such as *Patecatl*, who knew how to mix *aguamiel* with plant roots, *Cuatlapanqui* (the "head-opener") or *Papaztac* (the "nervous one"), among many others to whom the drunken and intoxicated were sacrificed (Gonçalves de Lima, 1956; Anawalt, 1998; Sahagún, 1999; Ramírez, 2002).

While most documents place the most probable origin of *pulque* in the ancient Otomi civilization toward the year 2000 BC, archeological evidence indicates that hunters and gatherers used maguey thousands of years ago (Jennings et al., 2005; Valadez-Blanco et al., 2012). Recent organic evidence shed new light on *pulque* history. In effect, although chemical components of this alcoholic beverage are water-soluble, limiting their conservation, hydrophobic lipids of food residues are more stable, Correa-Ascencio et al. (2014), applied a novel lipid biomarker approach to detect bacterial hopanoids derived from the widely recognized *pulque* fermenting bacteria *Zymomonas mobilis* as a *pulque* marker in more than 300 potsherds. The authors using this methodology were able to demonstrate for the first time the use of ceramic vessels to contain *pulque* in the locality of La Ventilla around 200–550 AD, at the height of Teotihuacan's culture. The presence of hopanes as bacterial markers of *pulque,* demonstrate that this beverage was produced in the ancient city of Teotihuacan and opens a new avenue of research for a systematic analysis to establish the level and intensity of *pulque* production and consumption in this culture (Correa-Ascencio et al., 2014).

During the height of the Aztec culture, *pulque* was produced and consumed preponderantly in religious and sacred rituals. It was restricted to the common citizens, with strict rules limiting its consumption. Excessive consumption was severely punished, in some cases including the capital punishment, even for priests. Upon the fall of the Aztec empire, *pulque* lost its religious significance gradually and became a food beverage and a popular intoxicant (Gonçalves de Lima, 1956; Ramírez et al., 2004; Ramírez Rodríguez, 2004). During the Spaniard Colony (1521–1821), *pulque* production was one of the main economic activities, and the most popular alcoholic beverage, resulting in the flourishment of *Haciendas pulqueras* (large farms dedicated to the cultivation of agave, *pulque* production, and commercialization), mainly in the central Mexican Plateau including the actual states of Hidalgo, Tlaxcala, Puebla, Morelos, Michoacán, and Querétaro. Interestingly, the production process remained practically unchanged since the Spaniard conquest and during Colony (Crist, 1939; Wilson and Pineda, 1963; Ramírez Rancaño, 2000). By 1629–1786, before the Mexican Independence War, *pulque* production and consumption was forbidden as it became a major health and social problems

TABLE 1 | Agave species used for *aguamiel* extraction and *pulque* production.

Name	Accepted name according to the Plant List web site[a]	Comments	References
A. atrovirens Kraw ex Salm-Dyck	Accepted	Cultured mainly in the states of Mexico, Tlaxcala, Hidalgo y Puebla	Alfaro Rojas et al., 2007
A. atrovirens var. *salmiana* (Otto ex Salm-Dyck) Maire and Weiller	Synonym *A. salmiana* Otto ex Salm-Dyck	Cultured mainly in the states of Mexico, Tlaxcala, Hidalgo y Puebla	Alfaro Rojas et al., 2007
A. americana L	Accepted	Cultured mainly in the states of Mexico, Tlaxcala, Hidalgo y Puebla	Alfaro Rojas et al., 2007
A. *mapisaga* Trel	Accepted	Include 13 variants. Cultured mainly in the states of Mexico, Tlaxcala, Hidalgo y Puebla	Alfaro Rojas et al., 2007; Mora-López et al., 2011
A. salmiana var *angustifolia* A. Berger	Accepted	Cultured mainly in the states of Mexico, Tlaxcala, Hidalgo y Puebla	Alfaro Rojas et al., 2007; Mora-López et al., 2011
A. salmiana var *ferox* (K. Koch) Gentry|	Accepted	Include three variants	Mora-López et al., 2011
A. salamina var *salmiana*	Unresolved name	The most diverse group including 31 variants	Mora-López et al., 2011

[a] *The Plant List (2010). Version 1.*

among the Indians. However, the economic relevance of maguey during the Spaniard Colony forced the authorities in 1786 to end the prohibition period as, despite the ban, *pulque* production competed with European wines and sugar cane liquor controlled by Spaniards (Lorenzo Monterrubio, 2007).

At the end of the Independence War (1810–1821), the production of *pulque* by the *Haciendas pulqueras* recovered its economic relevance, particularly by the introduction of the railway for the transport of thousands of liters of the fermented beverage directly from the *Haciendas pulqueras* to the main cities including Mexico City. By the beginning of the twentieth-century *pulque* production reached about 500 million L/year. By 1905, it is estimated that 350,000 L of *pulque* were consumed only in Mexico City. After the Revolution Civil War (1910–1920), the production structure of the *Haciendas pulqueras* was destroyed as *pulque* and its associated economic activity were owned by *hacendados*, an important part of to the upper class. By the period between 1920 to mid-1930s, the fresh *pulque* production and transport to Mexico City flourished again. However, by 1935–1940, the production and consumption of *pulque* was seriously affected again by an official anti-alcoholic policy, a severe devastation of agave plantations and the consolidation of the beer as a popular alcoholic beverage (Gonçalves de Lima, 1956; Ramírez Rancaño, 2000; Jácome, 2003; Ramírez et al., 2004; Ramírez Rodríguez, 2004; Lappe-Oliveras et al., 2008; Escalante et al., 2012).

Pulque had its major success in the last decades of the nineteenth century when rich fortunes derived from its successful production in *haciendas* and transport by train to the central Mexico urban centers. Significant efforts to preserve *pulque* and to face the increasing demand for *beer* failed. This effort, as well as the diversification of the agave industry, were led in particular by Ignacio Torres Adalid, known as "El Rey del *Pulque*" ("The King of *Pulque*" (Ramírez Rancaño, 2000). A campaign against *pulque* after the Mexican Revolution during the Venustiano Carranza government since 1914 until 1920, forced the *hacendados* to leave the country. *Pulque* consumption was associated with "criminality and degradation of the Mexican race." That was the beginning of the *pulque* agroindustrial twentieth century debacle. Nevertheless, by 1882 *pulque* was the main alcoholic beverage consumed in the country and one of the most important Mexican agroindustries by the end of the nineteenth century. A train transported daily hundreds of wood barrels containing *pulque* from more than 300 *haciendas* and *tinacales* mainly from the Eastern states of Hidalgo and Tlaxcala, then rich region thanks to their "crops of the century" (maguey) and "white gold" (*pulque*) productivity (Parsons and Darling, 2000; Ramírez Rancaño, 2000; Jennings et al., 2005). Several factors have been mentioned to explain *pulque*'s decline, among others the fact that *pulque* could not cope with the introduction of a competing alcoholic beverage: *beer*.

Despite the substantial differences in composition and organoleptic properties, probably the fact that *pulque* consumption dropped dramatically during the first decades of the twentieth century, besides the already mentioned campaign against consumption, was the lack of investment in science and technology. Interestingly, while consumers are now favoring traditional beers over the industrialized product, *pulque* consumers have no choice other than the traditional product which, in the context of the actual consumption trends, is now paradoxically, an advantage. The number of *pulquerías* offering *pulque* in Mexico City has considerably increased with more than 100 places offered to the consumer in internet pages, most of them of high quality (Ramírez Rancaño, 2000). The main production in Mexico is still the central state of Hidalgo where more than 260 million liters of *pulque* were produced in 2010, equivalent to 82% of the national production, followed by Tlaxcala with 13.3% and the State of Mexico with 2.68%, according to unofficial sources. As far as the National Institute of Statistics (INEGI), beer is described as responsible in 2014 of 1.2% of the total bulk manufacturing, while *pulque* was 0.0022% (INEGI, 2016). Other sources such as the "Encuesta Nacional de Adicciones 2011" (Instituto Nacional de Salud Pública, 2011) estimates that beer is consumed by 50 and 30% of the male and female population respectively, while other fermented beverages like *pulque* are consumed by only 4.4% of the population.

TRADITIONAL PRODUCTION OF *PULQUE*

The main process of *aguamiel* extraction and *pulque* fermentation remains practically unchanged since pre-Hispanic times (Parsons and Darling, 2000; Jennings et al., 2005). Agave plants are relatively easy to cultivate as propagation is mainly carried by transplanting young off springs (called *matecuates* or *hijuelos*) from adult plants after a 7–25 years maturation cycle. Nevertheless, agave seeds cultivation has been an alternative for maguey propagation since pre-Hispanic times (Parsons and Darling, 2000). Agave plants are grown in specific agave plantations known as *magueyeras* where the trasplanted young *matecuates* are arrayed in parallel rows known as *melgas* or *metepnatle* (maguey wall) (Parsons and Darling, 2000; Ramírez Rancaño, 2000; Jácome, 2003). Agave plantations are located away from tall trees to avoid plant competence for light, water, and soil nutrients. Natural fertilization of agave plantations is self-provided by recycling naturally degraded agave plants or by the addition of agave ashes dispersed around the growing plants.

Aguamiel extraction and *pulque* elaboration are performed traditionally by the *tlachiquero*, who has a deep knowledge of the biology and care of the maguey species used for production. The process starts with the selection of mature plants from 6 to 15 years old and comprises four common steps with slight variations across producing zones (Crist, 1939; Wilson and Pineda, 1963; García-Garibay and López-Munguía, 1993; Parsons and Darling, 2000; Jennings et al., 2005): (1) castration, (2) pit scraping and *aguamiel* extraction, (3) seed preparation, and (4) fermentation (**Figure 1**).

Maguey Castration

For this operation, selected mature plants are castrated by destroying the embryonic floral peduncle that surrounds the floral bud (*quiote*). During this operation, the central leaves of the plant (*meloyote* or heart), from which the flower rises are eliminated using a pointed and sharp instrument, leaving a cavity

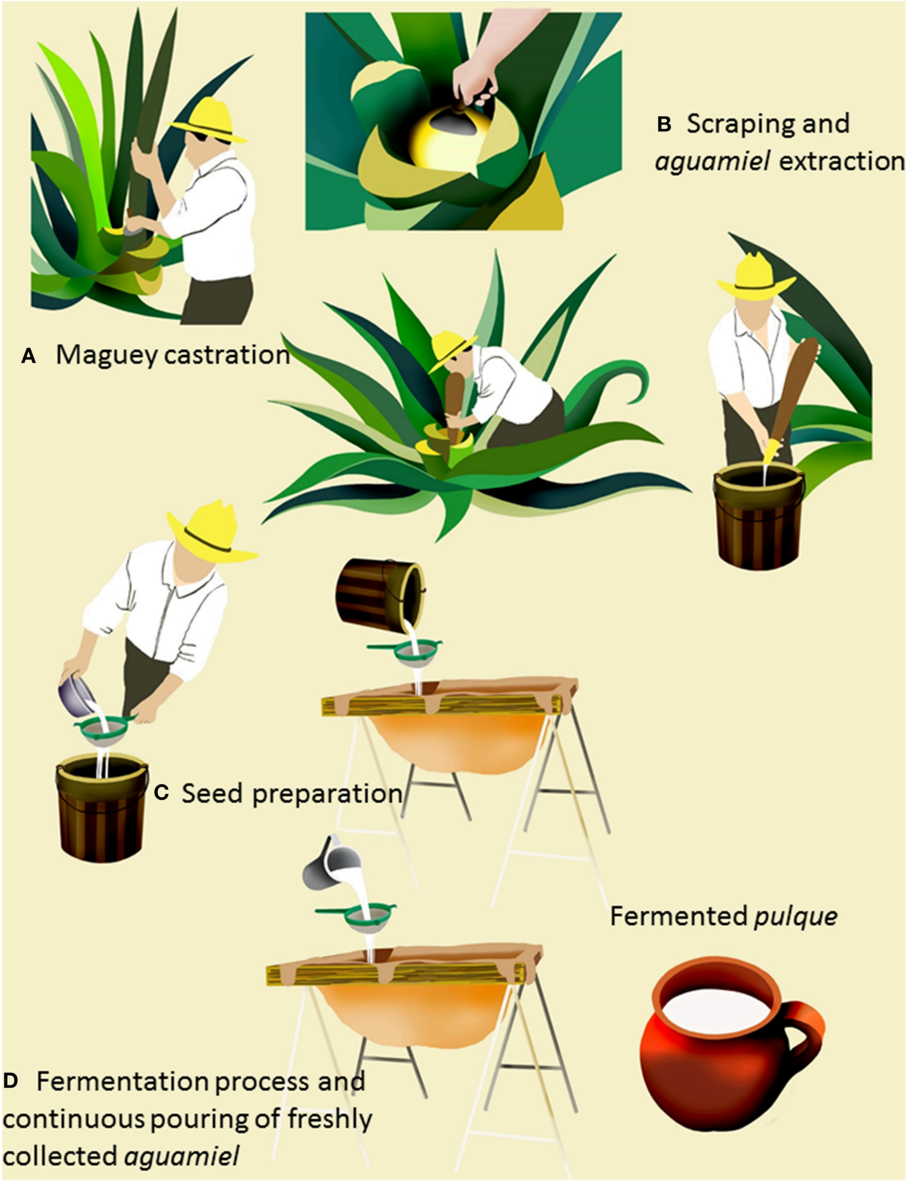

FIGURE 1 | Traditional *pulque* elaboration process. The traditional process involves four common steps: **(A)** Castration of the mature plant by cutting the floral bud and make the pit (*cajete*). **(B)** Pit scraping to promote *aguamiel* accumulation and sap extraction. **(C)** Seed preparation. **(D)** Fermentation. For details of the castration process see **Supplementary Files 1**, **2**.

(known as *cajete*) in the center of the plant (Jennings et al., 2005). The cavity is covered with a large stone or with agave leaves to protect it from animals and the environmental conditions. A maturation period follows castration and varies from 3 months to 1 year (Crist, 1939; Wilson and Pineda, 1963; García-Garibay and López-Munguía, 1993; Parsons and Darling, 2000; Jennings et al., 2005).

The castration process varies among producing regions: in the production region of Huitzilac (Morelos state), the cavity is digged without eliminating the central leaves, and the floral bud is cut off after the maturation process. The precise moment for castration is the *thachiquero* responsibility to avoid floral budding. If the inflorescence grows, the plant will never produce *aguamiel.* Moreover, early castration will result in a reduced volume of poor quality *aguamiel* production. Traditionally, some hints used by the *tlachiquero* to select mature plants are the abundance of leaves, the thinness of *meloyote*, and the surrounding leaves, which are also spikeless and adopt a lighter green tone. A detailed video showing the castration process and the instruments used is available in **Supplementary Files 1**, **2** (Crist, 1939; Wilson and Pineda, 1963; García-Garibay and López-Munguía, 1993; Parsons and Darling, 2000; Jennings et al., 2005).

Scraping and *Aguamiel* Extraction

Fresh *aguamiel* is a lightly cloudy, thick, very sweet, fresh-plant flavored and neutral to slightly acid sap. By scraping the *cajete*'s wall the sap outflow is induced, so *aguamiel* flows and accumulates in the cavity. This operation is performed by the *tlachiquero* using a scraping tool (Crist, 1939; Wilson and Pineda, 1963; García-Garibay and López-Munguía, 1993; Parsons and Darling, 2000; Jennings et al., 2005). The accumulated sap is extracted twice a day (usually at daybreak and dusk) by oral suction using a dried gourd (*Lagenaria siceraria*) known as *acocote*. After each *aguamiel* collection, the walls of the cavity are scraped again to maintain the sap flow induction. Freshly collected *aguamiel* is stored in plastic containers and transported to specific vats where the main fermentation takes place (**Figure 2**). A mature agave plant may produce *aguamiel* from 3 to 6 months until the plant dies, depending on the frequency of the scraping process. On a daily basis, the plant yields 4–6 L of *aguamiel* with a maximum average production of around 1000 L in its production lifetime (Crist, 1939; Wilson and Pineda, 1963; García-Garibay and López-Munguía, 1993; Parsons and Darling, 2000; Ramírez Rancaño, 2000; Jennings et al., 2005).

Seed Preparation

This operation refers to the production of starting material (inoculum) for the fermentation of freshly collected sap in a new container. For this purpose, around 2 L of fermented *pulque* are placed in a ~20 L vat made of clay, glass, wood, plastic or fiberglass, were fresh, high-quality *aguamiel* is poured. A spontaneous fermentation starts at room temperature until a characteristic alcoholic, and acetic taste develops or until a white

FIGURE 2 | *Aguamiel* extraction from producing maguey, transportation to the *tinacal* and fermentation process. (A) *Tlachiquero* extracting freshly *aguamiel* with an *acocote* (Hidalgo state). **(B)** *Aguamiel* is transferred into a plastic container for transportation to the *tinacal* (Morelos state). **(C)** Freshly collected *aguamiel* appearance (Morelos state). **(D)** *Aguamiel* accumulated in *cajete* previous to the twice-daily extraction (Hidalgo state). **(E)** *Aguamiel* pouring into a plastic vat for seed preparation (Hidalgo state). **(F)** Fermented *pulque* in a plastic vat (Hidalgo state). **(G)** Fermented *pulque* in a traditional leather vat (Hidalgo state). **(H)** Serving *pulque* for direct consumption from the fermentation vat (Tlaxcala state). Note the characteristic filament associated to final product viscosity.

layer -called *zurrón*- is formed on the surface, a process that usually takes from 1 to 4 weeks, depending on the season). Finally, the *tlachiquero* transfers the fermented product (seed) to one or more clean vats where *pulque* fermentation will takes place once freshly collected *aguamiel* is added (Crist, 1939; García-Garibay and López-Munguía, 1993; Parsons and Darling, 2000; Jennings et al., 2005; Escalante et al., 2012).

Pulque Fermentation

Fermentation takes place in vats usually made of cow-leather, glass-fiber, plastic or wood barrels located either in closed rooms known as *tinacal* or in specific open spaces (**Figure 2**). Freshly collected *aguamiel* is filtered to separate insects or any large object and poured into the vat, where the seed was previously transferred. The fermentation time varies strongly depending on *aguamiel* quality, seed maturity, season and producing region, among other factors. It usually lasts from 3 to 6 h, but overnight or even extended periods of time (e.g., 3–12 days) are not uncommon (Crist, 1939; Parsons and Darling, 2000; Ramírez Rancaño, 2000; Jennings et al., 2005).

Mexican norm NMX-V-022.1972 defines the sensorial properties required for the fresh collected sap or *aguamiel* used for *pulque* fermentation as a translucent, light amber-colored, sweet, fresh-flavored and lightly acid liquid with characteristic flavor and odor. Based on their physicochemical properties this norm defines two types of *aguamiel*. Type I or high-quality *aguamiel* and Type II, poor quality or slightly acid *aguamiel*. As for the alcohol content, Mexican norm NMX-V-037-1972 defines the alcoholic content of *pulque*. According to this norm, *pulque* is a beverage with low alcoholic content, not-clarified, of white color, acid, and viscous texture. The norm defines two types of *pulque*, Type I or *pulque* for seed (Section Biochemistry of the Fermentation) and "*puntas*" and Type II or commercial *pulque*. The requirements specified for *aguamiel* and *pulque* in norms NMX-V-022.1972 and NMX-V-037-1972 are presented in Table (Secretaría de Economía, 1972a,b).

Despite the Mexican norm NMX-V-037-1972 defined the desirable physicochemical properties of bulk *pulque* for direct consumption, particularly for density, pH (3.5–4.2), and alcohol degree (4–9%) (**Table 2**; Secretaría de Economía, 1972b), during traditional production of *pulque* the degree of fermentation varies according to the producer and is considered adequate when a characteristic alcohol, acetic notes, and texture (viscosity) is reached. Fermented *pulque* is withdrawn from the vat and consumed either natural or *curado*, as it is known when mixed with macerated fruits, vegetables, nuts or spices (Parsons and Darling, 2000; Ramírez Rancaño, 2000; Jennings et al., 2005; Lappe-Oliveras et al., 2008; Escalante et al., 2012). Sometimes, particularly when the fermentation yields a low-quality *pulque* (e.g., with low viscosity or off flavors), the *tlachiquero* adds plant

TABLE 2 | Physicochemical characteristics of *aguamiel* and *pulque*.

Characteristic	*Aguamiel*			References
	Type I		Type II	
	Minimum	Maximum	Lower to	
pH	6.6	7.5	4.5	Secretaría de Economía, 1972a
Density (°Bé)	5	7	4.5	
Refractive index (immersion, 20°C)	59	100	27	
Total solids[a]	13	17	7	
Total reducing sugars[a] (as glucose)	8	12	6	
Direct reducing sugars[a] (as glucose)	2	3	3	
Gums[a] (as glucose)	2	6	0.2	
Proteins[a]	300	600	100	
Ashes[a]	300	430	100	
Total acidity[a] (as lactic acid)	0.9	1.03	4	

	Pulque				
	Type I		Type II		
	Minimum	Maximum	Minimum	Maximum	
Refractive index (immersion, 20°C)	32	35	25	ND	Secretaría de Economía, 1972b
Refractive index (Abbé, 20°C)	1.3390	1.3406	1.3365	1.3380	
pH	>3.7	4.2	3.5	4	
Total acidity[a] (as lactic acid)	0.4	0.75	0.4	0.7	
Total reducing sugars[a] (as glucose)	0.1	0.8	0.2	0.5	
Alcoholic degree (%/vol)	6	9	4	6	

[a]mg/100 mL, ND, non-defined. °Bé, Baumé degrees.

roots, herbs or pieces of agave plants, a practice known as *cardón*, to improve the fermentation process (Parsons and Darling, 2000; Jennings et al., 2005).

MICROBIOLOGY AND BIOCHEMISTRY OF THE FERMENTATION

Toward the Definition of an Essential Microbiota Responsible for *Pulque* Fermentation

Pulque fermentation is a batch non-stirred process, performed under non-aseptic conditions. The microorganisms involved in the fermentation are those naturally occurring during sap accumulation in the *cajete* cavity in maguey and those incorporated during collection, transport, seed preparation and manipulation (Lappe-Oliveras et al., 2008; Escalante et al., 2012). Earlier studies on the microbiology of *pulque* performed by Sanchéz-Marroquín by 1950's reported the presence of homo- and heterofermentative LAB identified as *Lactobacillus* sp., *Leuconostoc mesenteroides,* and *L. dextranicum,* the yeast *Saccharomyces cerevisiae* (identified as *S. carbajali*) and the α-Proteobacteria *Zymomonas mobilis* (identified as *Pseudomonas lindneri*) (Sánchez-Marroquín and Hope, 1953).

These microorganisms develop three distinctive metabolic products during *pulque* fermentation: lactic acid produced by *Lactobacillus* sp. and *Leuconostoc* sp. which conduct the acid fermentation, ethanol resulting from the alcoholic fermentation and synthesized mainly by *S. cerevisiae* and *Z. mobilis,* and the extracellular polysaccharides (EPS), which include dextrans and fructans produced from sucrose by glycosyltransferases from *Leuconostoc* sp. and *Z. mobilis* (Sánchez-Marroquín and Hope, 1953; Lappe-Oliveras et al., 2008; Escalante et al., 2012). Due to this complex fermentation process, *pulque* is considered an acid and viscous alcoholic beverage. Sánchez-Marroquín et al. (1957), used isolated strains of the species mentioned above in a mixed inoculum, as a starter for a controlled fermentation of *aguamiel.* The Sánchez-Marroquín group was able to obtain a fermented beverage with similar organoleptic and physicochemical characteristics of the fermented product regarding flavor, aroma, alcohol content, acidity, and viscosity, suggesting the essential role of these microorganisms in traditional *pulque* properties (Sánchez-Marroquín et al., 1957).

Further studies on the microbiology of *pulque,* allowed the identification of a wider bacterial and yeast diversity. This diversity has been classified according to the microorganisms' main metabolic traits as (i) acid producing bacteria, including LAB and acetic acid bacteria (AAB); (ii) alcohol-producing microorganisms, including *S. cerevisiae* and *Z. mobilis,* (iii) dextran-producing bacteria (*L. mesenteroides*), and (iv) putrefactive microorganisms (**Table 2**). Interestingly, microorganisms involved in the four fermentative processes of *pulque* fermentation have been systematically isolated in *pulque* samples of different regions around the central Mexican Plateau (Escalante et al., 2004; Lappe-Oliveras et al., 2008). Regarding yeast diversity in *pulque*, *Saccharomyces,* and non-*Saccharomyces* species have been identified and proposed as essential fermentative yeast responsible for the production of ethanol, amino acids, vitamins, and volatile flavor compounds participating in the sensorial properties of the beverage (Lappe-Oliveras et al., 2008). Additionally, diverse killer and killer-resistant yeasts were isolated from *aguamiel* and *pulque,* some of them with a remarkable alcohol tolerance (Estrada-Godina et al., 2001) (**Table 2**).

Analysis of the bacterial diversity of *pulque* samples of different geographical origins (Estado de Mexico, Hidalgo, and Morelos states) as determined by 16S rDNA clone libraries was reported by Escalante et al. (2004). These authors reported the identification of an even wider diversity including non-previously reported bacteria. Interestingly, this study allowed to conclude that the bacterial diversity present among *pulque* samples was dominated by LAB, particularly *Lactobacillus acidophilus* (homofermentative LAB), corresponding to ~60–85% of total 16S rDNA clones analyzed for each *pulque* sample. Other clones identified as *L. mesenteroides* ranging from ~0.5 to 25% of total clones analyzed for each sample. *Z. mobilis* was detected in low amounts only in two samples, and 16S rDNA clones identified as the AAB *Acetobacter pomorium* and *Gluconobacter oxydans* (~33% of detected clones) were detected only in one sample. These results allowed defining the common bacterial diversity in *pulque* samples of different geographical origin, as well as a bacterial diversity specific of a given region (Escalante et al., 2004).

Assessment of the Changes in the Bacterial Community during the Fermentation of *Pulque*

The dynamics of bacterial diversity was studied in the laboratory with fresh *aguamiel* and *pulque* collected from Huitzilac, Morelos state by Escalante et al. (2008), using a polyphasic approach, including the isolation of LAB, aerobic mesophiles, and 16S rDNA clone libraries from total DNA extracted from fresh collected *aguamiel* used as substrate, after inoculation with previously produced *pulque* and followed by 6-h fermentation. Freshly collected *aguamiel* contained a count of 1.3×10^7 CFU/mL of total aerobic mesophilic bacteria (AMB), 3.2×10^9 CFU/mL of total LAB, and 3.1×10^4 CFU/mL of total yeasts. These results revealed the presence of a major microbial content associated to the accumulated sap in the maguey cavity (Escalante et al., 2008).

These authors also reported that total microbial counts determined after mixing fermented *pulque* with freshly collected *aguamiel* (initial fermentation time = 0 h) resulted in an increase of yeasts to 8.8×10^6 CFU/ml. After three h of fermentation, total yeasts further rose to 1.4×10^7 CFU/mL and remained constant until the end of the fermentation (1.9×10^7 CFU/mL). Total counts of both bacterial groups at the beginning of the fermentation were 1.2×10^7 CFU/mL for total AMB and 1.5×10^8 CFU/mL for LAB. By the end of the fermentation, total counts of both bacterial groups remained relatively constant as reached 3.5×10^7 CFU/mL and 1.5×10^8 CFU/mL, respectively (Escalante et al., 2008).

The microbial diversity identified in *aguamiel* was composed mainly by LAB including *L. mesenteroides, L. kimchi, L.*

citreum and in minor proportion *Lactococcus lactis*. The γ-Proteobacteria *Erwinia rapontici, Enterobacter* sp., and *Acinetobacter radioresistens* were the second most abundant bacterial group detected in agave sap. As the identified γ-Proteobacteria are naturally distributed microorganisms in diverse environments such as freshwater, soil, and vegetable surfaces, it may be possible to suppose that these bacteria are a contaminant incorporated to the *sap* during its accumulation in the *cajete*, or during the extraction and handling procedures (Escalante et al., 2008). Although Escalante et al. (2008) did not report the detection of lactobacilli in *aguamiel*, the isolation of *Lactobacillus brevis* and *L. collinoides* from agave sap samples collected from Huitzilac, Morelos state, was described in a recent publication (Reyes-Naya et al., 2016).

The addition of freshly collected *aguamiel* to previously fermented *pulque* results in a considerable increase in the count of yeasts (∼155% on total CFU/mL respect *aguamiel*). *L. kimchi* and *A. radioresistens* decreased, and *L. mesenteroides* remained relatively constant respect *aguamiel* (Escalante et al., 2008). Interestingly, after mixing *aguamiel* with *pulque* (T0), the most abundant microorganism detected was the LAB identified as *Lactobacillus acidophilus*. The γ-Proteobacteria *Enterobacter agglomerans*, and the α-Proteobacteria *Z. mobilis* and *Acetobacter malorum* were also detected but in low proportions in T0. Important physicochemical changes were observed in T0. After mixing fresh *aguamiel* and fermented *pulque,* the pH decreased from 6.0 to 4.5 in the mixture. Total sugars in *aguamiel* decreased 53.9%, and total carbon in fermented products detected in T0 (mainly as ethanol) increased 942.5% when compared to *aguamiel* (Escalante et al., 2008; **Figure 3**).

Microbial diversity present at T0 includes microorganisms in *aguamiel* and those from fermented *pulque* resulting in a microbial diversity composed by homo- and heterofermentative LAB, EPS-producing LAB, AAB, AMB, ethanol producing *Z. mobilis*, and yeasts. After 3 h of fermentation, diverse changes in the microbial diversity occurred despite the relatively constant total CFU/ml observed for LAB and total AMB. *L. acidophilus, L. mesenteroides,* and *E. agglomerans* were the most abundant bacteria; some others (both LAB and Proteobacteria) decreased or disappeared while yeast increased 102.9%. Also after 3 h, total sugars measured in T0 decreased 56%, and total carbon in fermented products (mainly ethanol) increased 120.7%. Finally, after 6 h of fermentation, the final microbial diversity was composed mostly by the homofermentative *L. acidophilus, L. mesenteroides. L. lactis* subsp. *lactis* and the α-Proteobacteria *A. malorum.* As a consequence of the microbial activity, after 6 h of fermentation, the final pH further decreased to 4.3, while 63.3% of the total sugar present after inoculation was consumed. Final fermentative products corresponded to 939.5 mM C as ethanol, 106.2 mM C as acetic acid, and 108 mM as lactic acid (**Figure 3**; Escalante et al., 2008).

Biochemistry of the Fermentation

As already described, microbiological studies of *aguamiel* and *pulque* have revealed the presence of a complex bacterial and yeast diversity. The final sensorial properties of *pulque* are defined by the simultaneous development of the four fermentation types already described in Section Toward the Definition of an Essential Microbiota Responsible for Pulque Fermentation, which depend on the most abundant microorganisms present in *pulque,* also depending on its geographical origin (**Figure 4**):

i. An acid fermentation performed mainly by homo- and heterofermentative LAB such as *Lactobacillus* and *Leuconostoc* (Sánchez-Marroquín and Hope, 1953; Sánchez-Marroquín et al., 1957; Escalante et al., 2004, 2008; Lappe-Oliveras et al., 2008), species involving the catabolism of available glucose to pyruvate by the Embden-Meyerhoff pathway and its subsequent conversion to lactic acid and other metabolic products such acetic acid, CO_2, and ethanol (Carr et al., 2002).
ii. An alcoholic fermentation performed mainly by the yeast *S. cerevisiae* and in minor degree by the α-Proteobacteria *Z. mobilis* from sucrose, glucose, and fructose in *aguamiel*. *Z. mobilis* converts efficiently fermentable sugars to ethanol and CO_2 by the Entner-Doudoroff pathway (Lau et al., 2010; Xiong He et al., 2014).
iii. The synthesis of EPS performed by *Leuconostoc* species including *L. mesenteroides* and *L. kimch*i resulting in the production of dextran and fructan exopolysaccharides from sucrose by enzymes such as glucosyl- and fructosyl-transferases, respectively (Chellapandian et al., 1998; Torres-Rodríguez et al., 2014). *Z. mobilis* is also a levan producer (Xiong He et al., 2014).
iv. An acetic acid fermentation performed probably by AAB such *Acetobacter* and *Gluconobacter* species (Escalante et al., 2004, 2008). AAB produce acetic acid as the main product through the oxidation of sugars, sugar-alcohols, and ethanol by the sequential activity of alcohol dehydrogenase and aldehyde dehydrogenase located in the outer membrane. *G. oxydans* catabolizes preferentially sugars and *Acetobacter* sp. in a minor proportion. Additionally, these bacteria produce gluconic acid and oxidize several organic acids including lactic acid to CO_2 and water (Raspor and Goranovič, 2008).

The specific role of diverse microorganisms, particularly those identified as dominant in *aguamiel* and *pulque* fermentation in the production of essential amino acids, vitamins, and a variety of flavored volatile compounds remains a research subject (**Figure 4**).

FUNCTIONAL PROPERTIES OF *AGUAMIEL* AND *PULQUE*

Nutritional Benefits Associated with *Pulque* Consumption

According to the traditional pharmacopeia, *aguami*el and *pulque* consumption has been related to diverse nutritional and health-promoting benefits since Pre-Hispanic times despite the alcohol content of the fermented beverage (mild value ∼4.8% ethanol) (Secretaría de Economía, 1972b; Backstrand et al., 2002). However, the first study directly reporting the health benefits of *pulque* consumption, is the successful treatment of scurvy

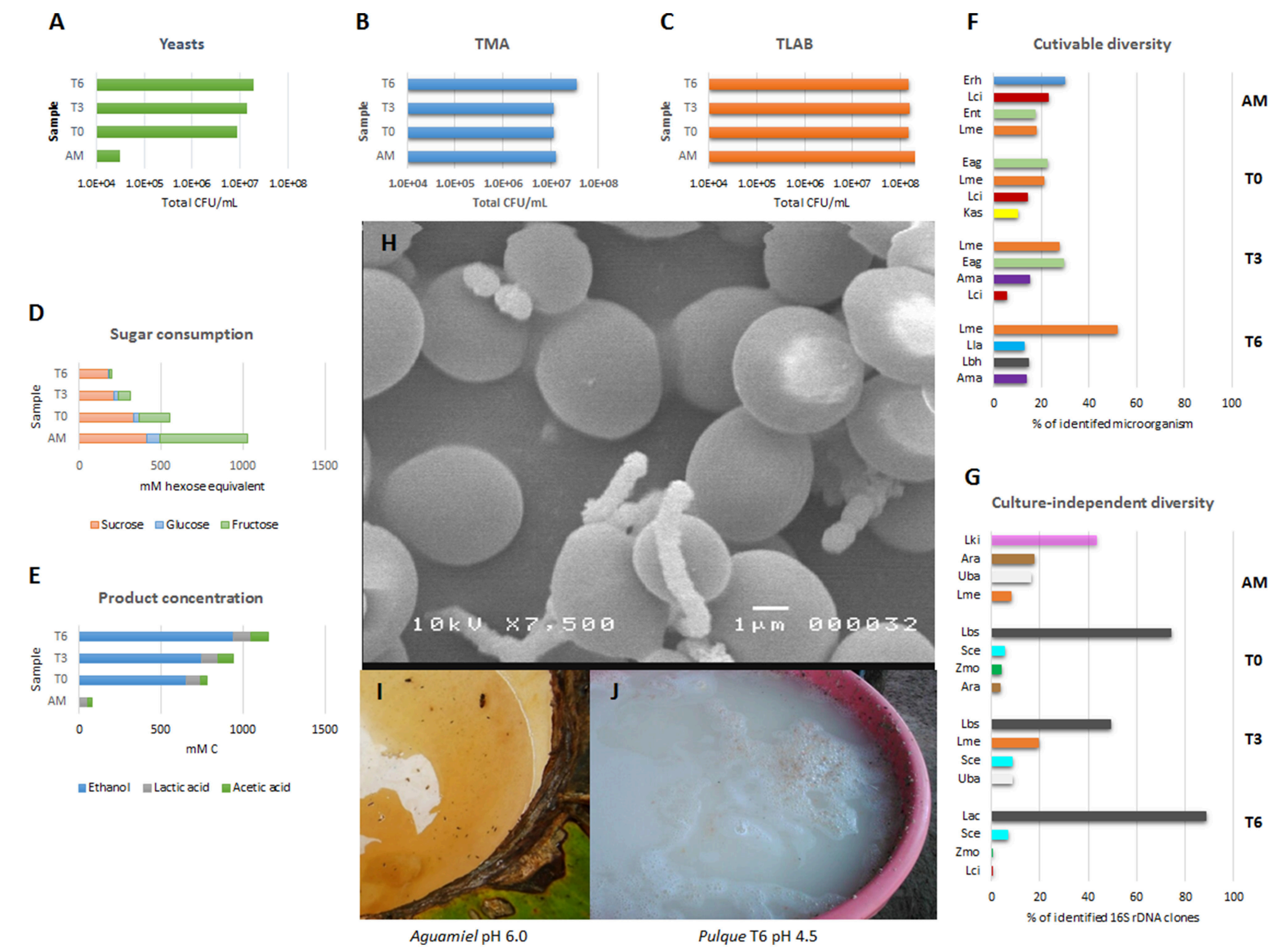

FIGURE 3 | Microbial, metabolic and physicochemical changes during *pulque* fermentation. Proposed microbial, physicochemical and metabolic changes during *pulque* fermentation as described by Escalante et al. (2008). **(A)** Total CFU/mL counts for yeasts; **(B)** Total mesophilic aerobes (TMA); **(C)** LAB determined during 6 h fermentation in laboratory; **(D)** Sugar consumption expressed as mM hexose equivalent; **(E)** Fermentation products (ethanol, lactic acid, and acetic acid) expressed as mM C; **(F)** Cultivable diversity (% of four most abundant isolates); **(G)** Culture-independent diversity (% of four most abundant 16S rDNA clones); **(H)** Scanning electron micrograph corresponding to *pulque* fermentation after 6 h showing some yeast and short cocci chains (T6) (non-previously published photograph); **(I)** *Aguamiel* accumulated in *cajete*; **(J)** Fermented *pulque*. AM, *aguamiel*, T0, T3, and T6, the start of the fermentation, 3 and 6 h of cultivation, respectively. Ama, *Acetobacter malorum*; Ara, *Acinetobacter radioresistens*; Eag, *Enterobacter aglomerans*; Erh, *Erwinia rhapontici*; Ent, *Enterobacter* sp.; Kas, *Kluyvera ascorbata*; Lbh, homofermentative *Lactobacillus* sp.; Lbs. *Lactobacillus* sp.; Lac, *L. acidophilus*; Lla, *Lactococcus lactis*; Lme, *Leuconostoc mesenteroides*; Lci, *L. citreum*; Lki, *L. kimchi;* Sce, *Saccharomyces cerevisiae*; Zmo, *Zymomonas mobilis;* Uba, Uncultured bacterial clone.

in penitentiary inmates in 1887 in Puebla state, well before the discovery of vitamin C (Ramírez Rancaño, 2000). The first systematic study on the nutritional benefits of *pulque* consumption associated with a regular intake was carried out in the indigenous Otomí population of theValle del Mezquital (Hidalgo state) was performed by Anderson et al. (1946). Results obtained from the analysis to 100 adult consumers, under a 7 days' based diet, conclude that daily intake of *pulque* (up to 2 L) provides calories (12%), total protein (6%), thiamin (10%), riboflavin (24%), niacin (23%), vitamin C (48%), calcium (8%), and iron (51%). These results indicate that for this ethnic group, *pulque* consumption constitutes the second most important "food" in the diet after tortilla. Authors concluded that these results are relevant considering the marginal character of this indigenous population diet, highlighting the daily contribution of vitamin C trough *pulque* (Anderson et al., 1946).

Sánchez-Marroquín and Hope (1953), determined the main content of some vitamins in *pulque* (µg/100 mL of *pulque*) and found: 65.2 of pantothenic acid, 30.7 of thiamine, 21.6 of ρ-amino benzoic acid, 23 of pyridoxine, including also 19.6 (ng/100 mL of *pulque*) of biotin (Sánchez-Marroquín and Hope, 1953). Further studies on the nutritional benefits of *pulque* intake demonstrated that after maize tortillas and legumes, *pulque* was the third most important source of iron (non-heme form), ascorbic acid, riboflavin, and other B-vitamins. Additionally, *pulque* provides significant amounts of folate, steroidal saponins, many of them

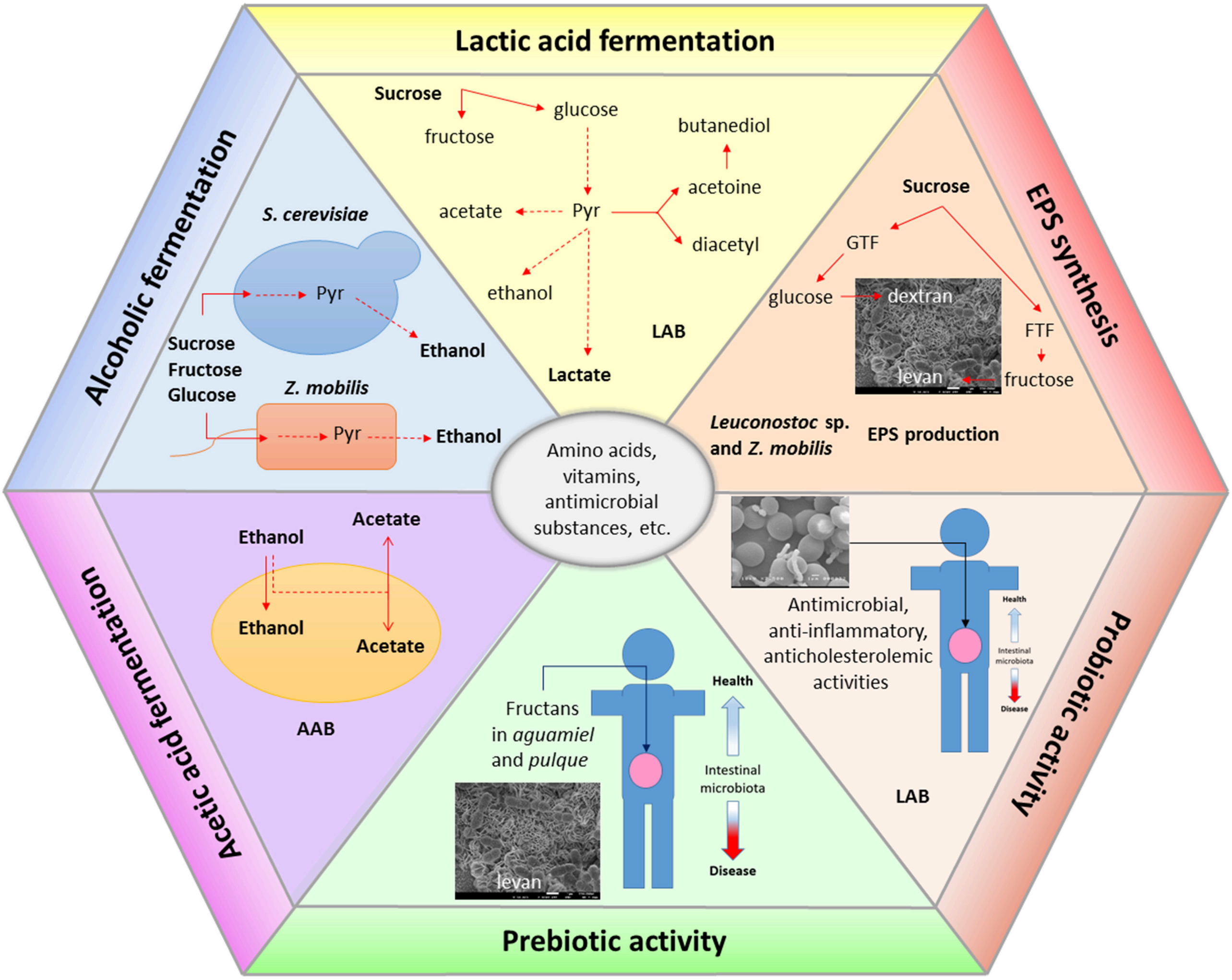

FIGURE 4 | Metabolic traits of main microbial groups present in *aguamiel* and during *pulque* fermentation. Main metabolic traits comprise homo- and heterofermentative lactic acid metabolism by LAB. Production of ethanol by *Saccharomyces*, non-*Saccharomyces* yeasts, and *Z. mobilis.* Acetic acid metabolism. Extracellular polysaccharide synthesis resulting in the synthesis of dextran and levan polymers by *Leuconostoc* sp. and *Z. mobilis* (levan). Microorganisms and metabolic pathways involved in the amino acid production, vitamins, and some antimicrobial compounds remain to be determined. Functional properties such as prebiotic and probiotic activities are related to fructooligosaccharide content in *aguamiel* and *pulque* or produced by LAB such as *Leuconostoc* sp. Probiotic properties are related to diverse LAB identified as *Lactobacillus* sp. and *Leuconostoc* sp.

bioactive (Backstrand et al., 2002). Furthermore, *pulque* is a source of phytase which has been proposed to be produced by *Lactobacillus* species and *S. cerevisiae* present in *pulque*, resulting in an increased bioavailability of iron and zinc present in maize (Tovar et al., 2008). Regarding the amino acids content, it was found that *pulque* contains 0.27 g/100 *pulque* of crude protein. Detected amino acids (g/16 g of N), included Ile (4.04), Leu (8.65), Lys (1.76), Cys (1.59), Phe (6.45), Tyr (2.76), Thr (4.21), Trp (2.35), Val (5.12), and His (2.01) (Morales de León et al., 2005). The total content of protein and amino acids is substantially less than what the common myth in rural areas propose, which is that "*pulque* lacks one degree to have the benefits of meat."

Studies on the relationship of iron status in a rural population from central Mexico highlands (Valle de Solís), performed in 125 non-pregnant women aged between 16 and 44 years old, assessed food intake during 12 months. Iron status determined after blood analysis showed higher plasma ferritin concentrations associated with significant intakes of non-heme iron and ascorbic acid. This study showed that better iron status correlated with significant *pulque* intake, an important source of non-heme iron and ascorbic acid, influencing the iron status of women from this rural zone. In this study, daily ethanol intake by *pulque* consumption was calculated using an average content of 47 g ethanol/L *pulque*; wich corresponds to the mean between 29 and 65 g/L (Backstrand et al., 2002).

The study of *pulque* intake in 70 expectant mothers from the Valle de Solís showed that 72.9% of women included in the study consumed *pulque* during pregnancy, and 75% continued consumption during the postpartum period as an important source of nutrients and energy. The consumption of 0.5 L of *pulque*, the amount commonly consumed by women in the research site supplied 24 g of ethanol, 9% of energy, 42.9% of ascorbic acid, 6.7 of thiamine, 5.9% of riboflavin, and 14.6% of iron of the Mexican Recommended Dietary Intake (RDI) during pregnancy. Results indicated that ascorbic acid intake from *pulque* was associated with a decrease in the risk of low ferritin and hemoglobin levels. The ethanol content in *pulque* was proposed to enhance iron absorption and to improve mother's daily iron intake. These authors showed the association between *pulque* intake during lactation and robust newborn growth, suggesting a beneficial effect of low *pulque* intake associated probably to the micronutrient content of the beverage. However, the study concludes that earlier intake of *pulque* during pregnancy and lactation was associated with poorer child height and weight (Backstrand et al., 2001, 2004).

Aguamiel Nutritional Content and Possible Functional Properties

Regarding *aguamiel,* the sap collected from *A. salmiana* 'Gentry' contains low amounts of crude fiber (0.57%), crude protein (0.69%) and a high level of nitrogen free extract (98.1%, corresponding to highly digestible carbohydrates). Mineral content analysis showed (in mg/L of *aguamiel*) 100 of N, 200 of Ca, 200 of P, 200 of Mg, 21.5 of Fe, 14.1 of Zn, 7.4 of Cu, and 19.9 of B. The consumption of 850 mL of *aguamiel* satisfy the daily human requirements of Fe and Zn, according to the Recommended Dietary Allowances or Adequate Intake (Silos-Espino et al., 2007).

The sap collected from *A. mapisaga* 'Blanco' contains (wt % in dry matter) 11.5% composed mainly of 75% of sugars (sucrose, fructose, glucose, and fructooligosaccharides), 0.3% of free amino acids (essential amino acids with exception of methionine), 3% of proteins, and 3% of ashes. Besides essential amino acid 26 mg/L of *aguamiel* of γ-aminobutyric acid (GABA) were identified (Ortiz-Basurto et al., 2008). These authors determined that *aguamiel* composition remain relatively stable throughout the production period (5 months), suggesting that the sap produced by *A. mapisaga* could be a stable substrate for a standardized *pulque* production processes.

Agave plants possess branched fructans (graminan) and graminan neoseries with two branches. One branch is attached to the fructosyl residue while the other is attached to the glucosyl unit of the sucrose molecule. These fructans have been designated as *agavins*, which are inulins with a complex mixture of structures and different degree of polymerization (DP) (Velázquez-Martínez et al., 2014). Due to the high fructan and fructooligosaccharide (FOS) content, agave extracts as well as the sap (consumed directly or concentrated) from different species, have been considered as an alternative source for prebiotic FOS syrups. This type of food additives has received increased attention due to its low glycemic index and, their demonstrated beneficial health effects such as improving calcium absorption in postmenopausal women, iron absorption, and, colon cancer prevention (García-Aguirre et al., 2009; Santos-Zea et al., 2016). *Aguamiel* from *A. mapisaga* "Blanco" contains inuline-type fructans (10.2% wt in dry matter) and glucooligosaccharides. The fructooligosaccharides identified up to now are highly branched, containing β-fructosyl units linked mainly by $\beta 1 \rightarrow 2$, but also $\beta 2 \rightarrow 6$ linkages (Ortiz-Basurto et al., 2008). Different extracts of *A. angustifolia* "Haw" agave have high molecular weight and branched fructans with the same structure regarding fructan linkages but different DP: high (3–60 fructose units), medium (2–40), and low (2–22) (Velázquez-Martínez et al., 2014).

Agave fructooligosaccharides have a demonstrated prebiotic function. In effect, several reports have demonstrated the *in vitro* growth promoting effects of diverse lactobacilli and bifidobacteria and well-known probiotic strains including *L. acidophilus, B. lactis, B. infantis, B. animals,* and *B. adolescentis*, some of them considered as predominant in human intestinal microbiota (Tripathi and Giri, 2014; Velázquez-Martínez et al., 2014; Castro-Zavala et al., 2015). As discussed above, *aguamiel* and *pulque* possess diverse well-documented nutritional traits; the main disadvantage of *pulque* remains its alcoholic content, which limits and restricts its promotion and consumption (Narro-Robles and Gutiérrez-Avila, 1997; Backstrand et al., 2001, 2004).

Assessment of the Probiotic Potential of LAB Isolated from *Aguamiel* and Fermented *Pulque*

The isolation and assessment of the probiotic potential of LAB from non-dairy products for the formulation of health-promoting functional foods have been a trending activity (Tripathi and Giri, 2014). This type of products containing probiotic bacterial strains but based on juices, fruits, and cereals, offer significant advantages as an alternative to dairy-based functional products such as low cholesterol and the absence of dairy-allergenic substances (Soccol et al., 2012).

LAB detected as the most abundant bacteria in *pulque* such as *Lactobacillus acidophilus* and *L. plantarum* (**Table 3**), are proposed to play an important role also due to their antimicrobial activities. The natural resistance of these LAB to the final *pulque* pH and alcohol content, their abundance at the end of fermentation (Escalante et al., 2008), and the traditional application of *pulque* for the treatment of gastrointestinal diseases suggest that LAB involved in *pulque* fermentation are potential probiotic candidates.

The successful screening of the *aguaniel* and *pulque* for the isolation of diverse *Leuconostoc* and *Lactobacillus* species showing some *in vitro* and *in vivo* probiotic properties have been the subject of several reports (**Table 4**). These properties include:

i. Resistance to antimicrobial barriers in the gastrointestinal tract such as lysozyme dilution by saliva, acid pH, gastric solution, and bile salt (Castro-Rodríguez et al., 2015; González-Vázquez et al., 2015; Giles-Gómez et al., 2016; Reyes-Naya et al., 2016; Torres-Maravilla et al., 2016).

TABLE 3 | Microbial diversity detected in *aguamiel* and during *pulque* fermentation.

Bacteria	Yeasts/Fungi	Remarkable metabolic traits defining sensorial properties of *aguamiel* or *pulque*	References
Lactobacillus sp. *Leuconostoc mesenteroides*, *L. dextranicum* *Zymomonas mobilis*	*Saccharomyces cerevisiae*	Essential microorganisms responsible for acid (lactic acid), alcoholic and production of EPS	Sánchez-Marroquín and Hope, 1953; Sánchez-Marroquín et al., 1957
	Yeasts isolated from *aguamiel: Candida lusitaneae, Klyuveromyces marxianus* var *bulagricus* (+), *S. cerevisiae* Yeast isolated from *pulque*: *C. valida* (+), *S. cerevisiae* (*chevalieri*), *S. cerevisiae* (*capensis*), *K. marxianus* var *lactis* (+)	Several isolates of *C. valida, S. cerevisiase* (*chevalier*) isolated from *pulque* were able to resist to >10% of alcohol. Potential relevance in ethanol production during the fermentation and resistance to killer toxins	Estrada-Godina et al., 2001
Acetobacter aceti, A. aceti subsp. *xylinus, Bacillus simplex, B. subtilis, Cellulomonas* sp., *Escherichia* sp., *Kokuria rosea, Lactobacillus* sp., L. *delbrueckii, L. vermiforme, Leuconostoc* sp., *L. mesenteroides* subsp. *dextranicum*, L. *mesenteroides* subsp. *mesenteroides, Macrococcus caseolyticus, Micrococcus luteus*, *Sarcina* sp., *Z. mobilis* subsp. *mobilis*	*Cryptococcus* sp., *Candida parapsilosis, Clavispora lusitaniae, Debaryomyces carsonii, Hanseniaspora uvarum, Kluyveromyces lactis, K. marxianus,* *Geotrichum candidum, Pichia* sp., *P. guilliermondii, P. membranifaciens, Rhodotorula* sp., *R. mucilaginosa,* *Saccharomyces bayanus, S. cerevisiae, S. pastorianus, Torulaspora delbrueckii*	Essential microorganisms responsible for lactic and acetic fermentation (LAB and acetic acid bacteria), alcoholic fermentation (*Z. mobilis* and *S. cerevisase*), EPS production y (*Leucocnostoc* sp.) and putrefactive bacteria	Lappe-Oliveras et al., 2008
Analysis of 16S rDNA clone libraries allowed to identify *Lactobacillus acidophilus*, *L. kefir, L. acetotolerans, L. hilgardii, L. plantarum, Leuconostoc mesenteroides* subsp. *mesenteroides, L. pseudomesenteroides, Acetobacter pomorum, Gluconobacter oxydans, Zymomonas mobilis, Flavobacterium jhonsonae, Hafnia alvei*		Homofermentative *L. acidophilus* was identified as the most abundant microorganism in three analyzed samples from different geographical origin, suggesting a possible essential role in lactic acid fermentation. *L. mesenteroides* was present in low proportion respect lactobacilli. *Z. mobilis* and AAB were detected low percentage or absent. Presence of possible putrefactive or contaminant bacteria	Escalante et al., 2004
A combined culture dependent and 16S rDNA libraries approach allowed to identify those microorganisms present in freshly collected *aguamiel* and during a 6 h of fermentation. α-Proteobaceria: *Acetobacter malorum*[a], *A. orientalis*[b], *Z. mobilis* subsp. *pomaceae*[b], γ-Proteobacteria: *Citrobacter* sp., *Enterobacter* sp.[a], *E. agglomerans*[a], *Erwinia rhapontici*[a], *Kuyvera acorbata*[c], *K. cochleae*[a], *Providencia* sp.[a], *Serratia grimensii*[a], *Acinetobacter radioresistens*[b], *Sterotrophomonas* sp.[a], *Chryseobacterium* sp. Firmicutes: *Bacillus* sp.[a], *B. licheniformis*[a], *Lactobacillus* sp.[c], *L. acidophilus*[b], *L. hilgardii*[b], *L. paracollinoides*[b], *L. sanfranciscensis*[b], *Lactocoocus* sp.[a], *L. lactis*[a], *L. lactis* susp. *lactis*[a] *Leuconostoc kimchi*[c], *L. citreum*[c], *L. gasocomitatum*[b], *L. mesenteroides*[c], *L. pseudomesenteroides*[c], *Pediococcus urinaeequi*[a], *Streptococcus deviesei*[a]	*S. cerevisiae*[b]	*Leuconostoc citreum* and *L. kimchi* species were identified as the most abundant LAB in *aguamiel*. After mixing fresh *aguamiel* with previously fermented *pulque*, *L. acidophilus, L. mesenteroides* were the most abundant LAB during 6 h of fermentation. *E. agglomerans* was the most abundant non-LAB during the first 3 h of fermentation. *Z. mobilis* and AAB were absent in *aguamiel* but detected in low proportion during the fermentation process Total bacterial counts (CFU/mL) for LAB and total aerobic mesophilic bacteria were constant during 6 h of fermentation. Total yeast counts (CFU/mL) detected in *aguamiel* increased after mixing *aguamiel* with fermented *pulque*, increased until 3 h and maintained constant until the end of the fermentation	Escalante et al., 2008

(+) Indicates killer activity detected.
[a]Identified from a culture isolate.
[b]Identified from 16S rDNA clone library.
[c]Identified by culture and non-culture dependent approaches.

TABLE 4 | Probiotic assessment of LAB isolated from *aguamiel* and *pulque*.

Source and identity of studied LAB	Resistance to *in vitro* gastrointestinal exposition conditions	Other relevant *in vitro* or *in vivo* activity	References
Lactobacillus brevis isolated from *pulque*	This isolate strain showed 60% relative survival after acid exposition (pH 1.5), and 50–55% relative survival to simulated gastric acid exposition (pH 2.0). Bile tolerance to 0.3% taourocholic acid <80%. Incubation conditions assayed: 4 h, 37°	Resistance to cefepime antibiotic, higher activity of bile salt hydrolase in MRS supplemented with 0.5% of taourocholic acid (671.72 U/mg protein)	González-Vázquez et al., 2015
Leuconostoc mesenteroides subsp. *mesenteroides* isolated from *aguamiel* (four strains)	Isolates showed <50% survival to acid exposition (pH 2, 3 h, 37°C). Bile tolerance to 0.5% oxgall (4 h, 37°C)	All strains showed resistance to dicloxacillin, pefloxacin, trimethoprim, ceftazidime antibiotics. *In vitro* antimicrobial activity of cell-free supernatants against *Escherichia coli, Salmonella enterica* and *Listeria monocytogenes*. Bacterial adherence to mice intestinal mucosa	Castro-Rodríguez et al., 2015
Lactobacillus plantarum, L. paracasei subsp. *paracasei, L. brevis, L. composti, L. sanfranciscensis* isolated from *pulque* (14 isolates)	Two assayed strains showed >80% survival to lysozyme exposition. Three assayed strains showed > 80% survival to both acid pH (2.5) and 0.3% bile salts exposition. Exposition conditions assayed: 3 h, 37°C	Low binding capacity to HT-29 cells (∼0.3%, best result) and to HT-29-MTX cells (10.78%, best result). In both assays, the binding capacity of isolated LAB was higher than control strain (*L. casei* BL23). Isolate identified as *L. sanfranciscensis* improve mice health by reduction of weight loss, significant decreases in gut permeability and anti-inflammatory effect by blocking the secretion of cytokines	Torres-Maravilla et al., 2016
Lactobacillus brevis and *L. collinoides* isolated from *aguamiel* (14 isolates)	Resistant to an *in vitro* model simulating gastrointestinal conditions	Capable of dissociating conjugated bile salts by the presence of diverse bile salt hydrolases. Some isolates were resistant to dicloxacillin, pefloxacin and ceftazidime antibiotics. The isolated strain of *L. brevis* Lb9H showed *in vivo* protective effect of liver damage associated with the prevention of ALT[a] activity and preventing the intoxication by LPS+D-GalN[b], indicator of lipid peroxidation	Reyes-Naya et al., 2016
L. mesentreoides strain P45 isolated from *pulque*	Resistance to lysozyme exposition 70% (2 h, 37°C). 100% resistance to 0.3% and 1% bile salts exposition (4 h, 37°C). ∼75% resistance to acid exposition (pH 2.5, 5 h, 37°C). This strain showed remarkable resistance to combined acid (pH 2.5) and bile salt (0.3%) exposition for 24 h, 37°C	*In vitro* antimicrobial activity against enteropathogenic *E. coli*, *S. enterica* serovar Typimurium, *S. enterica* serovar Typhi and *L. monocytogenes* in cell-to-cell assays (LAB-pathogen), cell-free supernatants assays and EPS-producing cell-to-cell assays (LAB-pathogen). *In vivo* assays showed that administration of strain P45 is associated with an important decrement in *S. enterica* serovar Typhimurium infection in liver and spleen in BALB/c female and male mice	Giles-Gómez et al., 2016

[a]*Serum alanine transferase.*
[b]*Lipopolysaccharide + D-Galactosamine.*

ii. Antimicrobial activity against pathogenic bacteria such as enteropathogenic *Escherichia coli, Salmonella enterica* serovar Typhimurium, *S. enterica* serovar Typhi and *Listeria monocytogenes* (Castro-Rodríguez et al., 2015; González-Vázquez et al., 2015; Giles-Gómez et al., 2016; Torres-Maravilla et al., 2016).
iii. *In vivo* adherence to mice intestinal mucosa (Castro-Rodríguez et al., 2015).
iv. *In vivo* anti-inflammatory activity in a mouse model (Torres-Maravilla et al., 2016).
v. *In vivo* anticholesterolemic affect (Reyes-Naya et al., 2016).
vi. *In vivo* anti-infective effect against *S. enterica* serovar Typhymurium (Giles-Gómez et al., 2016).

This scientific evidence of LAB responsibility for health-promoting effects associated with *pulque* consumption makes these bacteria relevant probiotic candidates for the development of non-dairy based functional products.

Functional Properties of EPS Produced by LAB Detected in *Aguamiel* and *Pulque*

Some EPS produced by LAB isolated from *aguamiel* and *pulque* have been purified and characterized. Results include the identification of dextran with a linear backbone linked in α1→6 D-Glc*p* linkages with branching in α1→3 D-Glc*p* produced by a cell-associated glycosyltransferase (GTF) from *L. mesenteroides* isolated from *pulque* collected from the Apan region, in the state of Hidalgo (Chellapandian et al., 1998). In the same context, two EPS LAB identified as *L. kimchii* were isolated from *pulque* produced in Huitzilac, in the state of Morelos. One of the strains (EPSA) produced dextran with a linear backbone joined by α1→6 D-Glc*p* with α1→2 and α1→3 branching linkages through enzymes found in the soluble and the cell-associated fractions. The second strain (EPSB) produced a polymer mixture including a levan composed by linear chains containing β2→6 linked β-D-fructofuranosyl moieties and β2→1 branches (79%), as well as a dextran Type I polymer (21%) (Torres-Rodríguez et al., 2014).

EPS and hetero-oligosaccharides produced by diverse LAB species, including those found in *pulque*, have gained attention because of their use as food additives and potential natural functional ingredients. Their main applications include their use as prebiotic agents as well as soluble fiber (Patel et al., 2011; Harutoshi, 2013) such as those produced by *Lactobacillus reuteri, L. rhamnosus, L. acidophilus,* and *Bifidobacterium bifidum* (Helal et al., 2015). EPS produced by LAB with potential probiotic properties have been proposed to play a positive effect in the intestinal adhesion (García-Ruiz et al., 2014). *In vitro* antimicrobial assays with EPS-producing *L. mesenteroides* strain P45 isolated from *pulque* against EPEC *E. coli, S. enterica* serovar Typhimurium, *S. enterica* serovar Typhi, and *L. monocytogenes* showed an improved *in vitro* antimicrobial activity in EPS-producing cell-to-cell assays (Giles-Gómez et al., 2016). These results are preliminary, as the detailed mechanisms involved both *in vivo* and *in-vitro* potential functional properties of EPS produced by LAB, particularly those species assayed for potential probiotic activities remain to be determined.

PULQUE INDUSTRIALIZATION AND MAJOR TECHNOLOGICAL CHALLENGES

Science and Technology of *Pulque*

A simple look at research figures illustrates the lack of interest in *pulque* by the scientific community: A PubMed search under "beer" results in today in 17,929 hits while only 30 references come out under "*pulque*" most of them published in the twenty-first century. However, 8 of them were released in the last 2 years (2014 and 2015) as evidence of a renew interest.

It is worthwhile looking at this extremely low figure in more detail, as the earliest scientific publication dealing with the process, dates back to 1957 when Alfredo Sanchez Marroquin (Sánchez-Marroquín et al., 1957), first tried to industrialize *pulque* starting from the basic/minimum microbiological requirements to transform *aguamiel* into *pulque*. We, of course, acknowledge the initial efforts of Dr. Leopoldo Río de la Loza to elucidate the microbiology of *pulque* in 1864. He reported in the *Boletin de la Sociedad de Geografía y Estadística*, the isolation of *Termobacterium mobile* by Paul Lindner in 1924 (Weir, 2016), among others. *Pulque*'s microbiology, the isolation of strains, and more recently, its individual probiotic characterization, is probably the main research trend (Torres-Rodríguez et al., 2014; Castro-Rodríguez et al., 2015; González-Vázquez et al., 2015; Giles-Gómez et al., 2016; Torres-Maravilla et al., 2016). An additional research subject deals with the effect of *pulque* in the Mexican diet. The first reference given by PubMed is a document from 1897 in which Francisco Martínez Baca, a famous physician from the state of Puebla described the successful treatment with *pulque* of penitentiary inmates suffering scurvy (published in the Journal of the American Public Health Association) (Ramírez Rancaño, 2000). It was not until 1933 that vitamin C was finally discovered (Carpenter, 2012).

However, no references deal with *pulque* production technology, scaling up of the process, neither the definition of the main microbiota required to reproduce the beverage, as consumers know it. These concerns remain as technological challenges since last century when Sanchez Marroquin defined the four physiological processes involved in *pulque* production (Sánchez-Marroquín et al., 1957). Nevertheless, reducing the microbiota to three or four microorganisms would blindly eliminate possible bacteria contributing as probiotics to the claimed beneficial health effects, particularly to treat gastrointestinal problems and diarrhea. The simple decision between *S. cerevisiae* or *Z. mobilis* as the alcohol producer is not that evident. *S. cerevisiae* reaches higher ethanol concentrations without inhibition, while *Z. mobilis*, a faster ethanol producer, also contains two levansucrases, responsible for levan synthesis, part of the soluble fiber in which *pulque* is particularly rich (Lau et al., 2010; Xiong He et al., 2014; Weir, 2016). Up to now, *pulqu*e remains as a very heterogeneous beverage regarding its common final organoleptic properties (alcohol-acid taste and viscosity): while many drinkers prefer the fresh product, others prefer *pulque* after more than 24 h of fermentation combined with fruit juice (*curados*). Nevertheless, *pulque* does not stand large storage times without developing off flavors, and pasteurization not only affects flavor but also destroys one of its main properties: the microbiota.

It is probably to this aspect that the largest (but still minor) efforts in research have been devoted. The presence of prebiotic fructooligosaccharides from agave inulin present in *aguamiel*, as well as the soluble inulin-like agavin, levan and dextran polysaccharides have been described and characterized (Chellapandian et al., 1998; Ortiz-Basurto et al., 2008; Torres-Rodríguez et al., 2014). Some of this prebiotics have been evaluated both *in vitro* and *in vivo*, and we suggest that the beneficial effects observed among lactating mothers and their babies (Argote-Espinosa et al., 1992; Backstrand et al., 2001, 2004) is mainly due to its pre- and probiotic content. Unfortunately, most research is now devoted to the isolation and production of probiotic bacteria as alternative beverages, isolated from *pulque*, but out of the scope of the beverage. These efforts are similar to those carried out last century by Paul Lindner himself. He was convinced that *Pseudomonas lindneri* (that he had previously defined as *Thermobacterium mobile*) was responsible for the beneficial effects of *pulque* in the treatment of intestinal disorders and produced in Berlin from this single bacteria a "functional" fermented beverage (Gonçalves de Lima, 1956).

Challenges Associated with *Pulque* Production

Probably the main challenge associated with the industrialization of *pulque* is related to the natural substrate availability and the need for the introduction of a stabilization processes of the fermented product. *Aguamiel* differs from almost all other fermented beverages such as wine, beer or *tepache* (pineapple wine), in that agave, the raw material, takes 7 years to reach maturity. Furthermore, when ready for production, *aguamiel* has to be collected from the plant on a daily basis, and not produced by a single extraction, as it is usually the case for fermented beverages. Each agave plant is visited daily during several months and the accumulated *aguamiel* extracted, a labor-intensive activity, which also induce fermentation in the plant itself where *aguamiel* accumulates during the day. Therefore, the fermentation is already taking place when the substrate is collected. In contrast, the fermentation that leads to tequila or mezcal, also produced from agave sugars, does not require this process as sugars are extracted directly from the mature plant (*Agave tequilana*) in a single operation after the agave pine is cooked and mashed.

Several successful efforts for industrialization for the production of bottled/canned fermented *pulque* have been performed mainly by producers in the States of Puebla, Tlaxcala and Hidalgo (Ramírez et al., 2004; Jaurez Rosas, 2015). The producers include companies as Tecnología e Innovación en *Pulque* Industrial S.A. de C.V., comprising more than 300 *pulque* producers in Puebla state, Torre Grande in Hidalgo and Procesadora de *Pulque* S.A. de C.V and *Pulque* Hacienda 1881 in Tlaxcala. Both companies export canned *pulque* to Europe, Central America, and the United States, the latter being the largest market for canned *pulque* (mainly the cities of Los Angeles and Chicago where are the biggest settlement of Mexican immigrants) (Jaurez Rosas, 2015). However, the industrialization of *pulque* introduced fundamental changes in the public perception of traditional producers and consumers resulting in a product that the majority of traditional consumers never tasted before. Efforts to stabilize the fermented beverage by pasteurizing and/or filtrate *pulque* or by the addition of preservatives, antioxidants, colorants or texturizing agents will certainly improve stability and shelf life but could reduce the pre- and probiotic content of the fermented beverage (Ramírez et al., 2004; Escalante et al., 2012).

However, there is an increasing preference for local products and local markets (Jaurez Rosas, 2015). We believe that the main scientific and technological investment should come from the demonstration of the main nutritional, health-promoting and organoleptic attributes of *pulque* and its microbiota, introducing specific modifications in the traditional production *tinacales* that bring assurance to the consumer that *pulque* is produced hygienically, conserving its local characteristics and its regular strains, but safe to the consumer.

Functional Genomics of *Pulque* and Relevant Microorganisms Involved in the Fermentation Process

Application of a culture-independent approach such as 16S rDNA clone library to the study of bacterial diversity present in *aguamiel* and *pulque* allowed to determine a remarkable LAB diversity, suggesting an essential role of these microorganisms in the *fermentation* process (Escalante et al., 2004, 2008). Emerging research on the microbiology of *pulque* focuses on the isolation and *in vitro* as *in vivo* assessment of probiotic LAB with promising capabilities (Castro-Rodríguez et al., 2015; González-Vázquez et al., 2015; Giles-Gómez et al., 2016; Reyes-Naya et al., 2016; Torres-Maravilla et al., 2016; **Table 4**).

Functional genomics from available LAB genome information has provided new insights regarding the evolution of LAB, their metabolic profile and the interactions with other microorganisms and the environment, allowing to understand the role of these microorganisms in traditional or industrial food fermentations and their interactions with the human hosts (Douillard and de Vos, 2014). Genome sequencing of relevant LAB isolated from *pulque*, such as those recently identified with potential probiotic properties promises to provide valuable information on the genetic traits involved in the probiotic activity.

Complete genome analysis of potential probiotic *L. mesenteroides* strain P45 by Riveros-McKay et al. (2014), allowed the identification of diverse genes probably involve in the antimicrobial activity of this LAB such as those coding for diverse peptidoglycan hydrolases and a prebacteriocin (Giles-Gómez et al., 2016). This information provides new insights to focus further efforts on the characterization of the potential probiotic of this LAB from *pulque*. However, the next step in the study of *pulque* microbiology relies on the application of metagenomic approaches to study the entire microbial composition (including both bacteria and yeasts) in combination with other high-throughput omic methodologies such as transcriptomics, metabolomics or proteomics. These approaches applied to other regional traditional fermented foods and beverages (e.g., Korean *kimchi* Jung et al., 2011), could provide valuable insights into the complex microbial community involved in the fermentation process.

CONCLUDING REMARKS

All through Mexican history, from pre-hispanic times to our days, *pulque* has been a key reference regarding culture, tradition, and cuisine. Once the center of the cosmological vision of our ancestors, later a source of wealth through agro-industrial exploitation, abandoned and despised -described as a nutrient of underdevelopment and ignorance after the Revolution Civil War, and now the subject of wonder and scientific research. *Pulque* is now the center of research in many laboratories, not only due to its nutritional properties but also to the extremely complex microbial diversity responsible for its fermentation, a process that has resisted industrialization. No doubt, *pulque* is an essential element for the UNESCO decision in 2010 to include the traditional Mexican cuisine in the List of the Intangible Cultural Heritage of Humanity.

AUTHOR CONTRIBUTIONS

DL and JV collected the video and photographic material included in this contribution and prepared the information corresponding to the traditional process of *pulque* fermentation. AE, MG, FB, and AL wrote the manuscript and designed the graphic material. All the authors reviewed and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01026

Supplementary File 1 | Castration process of a mature maguey for *aguamiel* production 1. (Video in mp4 format, 2:27 min). *Pulque* producer or *tlachiquero* perform castration process. Once the plant has been selected the *tlachiquero* prepares the maguey by cutting off the central leaves of the plant surrounding the floral bud with a sharpened knife. With the floral peduncle exposed ("opening the door"), the *tlachiquero* cut off this part of the plant with a knife.

Supplementary File 2 | Castration process of a mature maguey for *aguamiel* production 2. (Video in mp4 format, 2:03 min). The remaining floral bud is destroyed to avoid the possible development of the embryonic floral peduncle. For this operation, the *tlachiquero* uses a pointed and sharpen metallic instrument (a jimmy bar) to make a pit in the residual floral bud (0:00–0:43 min). Finally, the *tlachiquero* uses a scraping tool to make the final shape of the cavity (*cajete*) and covers the pit with a maguey leaf (0:43–2:03 min).

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Fermentation of Apple Juice with a Selected Yeast Strain Isolated from the Fermented Foods of Himalayan Regions and Its Organoleptic Properties

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Twenty-three *Saccharomyces cerevisiae* strains isolated from different fermented foods of Western Himalayas have been studied for strain level and functional diversity in our department. Among these 23 strains, 10 *S. cerevisiae* strains on the basis of variation in their brewing traits were selected to study their organoleptic effect at gene level by targeting *ATF1* gene, which is responsible for ester synthesis during fermentation. Significant variation was observed in *ATF1* gene sequences, suggesting differences in aroma and flavor of their brewing products. Apple is a predominant fruit in Himachal Pradesh and apple cider is one of the most popular drinks all around the world hence, it was chosen for sensory evaluation of six selected yeast strains. Organoleptic studies and sensory analysis suggested Sc21 and Sc01 as best indigenous strains for soft and hard cider, respectively, indicating their potential in enriching the local products with enhanced quality.

Keywords: Western Himalayas, fermented foods, *Saccharomyces cerevisiae, ATF1* gene, apple cider

INTRODUCTION

Fermented food products are essential component of diet in a number of developing countries and are more common among people belonging to the rural areas, especially in hilly and tribal people, where the limited resources encourage the use of these products for the fulfillment of additional nutritional requirements (Kanwar et al., 2007). The knowhow of these traditional processes and technologies involved in the production of fermented products is being transferred from generation to generation as trade secrets. These fermented foods are made under primitive conditions, which result in low yield and poor quality and sometimes even in spoilage of the product. So there is a need to select the specific microflora associated with these products to maintain consistency in their production and quality. The most important organism associated with fermented food products is yeast and it has been observed that among several yeasts, *Saccharomyces cerevisiae* is the most common species associated with fermentation processes (Querol and Fleet, 2006). To preserve the typical organoleptic properties of the fermented product or beverage, it is essential to select a particular strain of yeast that imparts characteristic sensory and aromatic flavor to fermented product/beverage. Production of several wines from some tropical fruits using *S. cerevisiae* strains has already been reported (Ezeronye, 2004; Capece et al., 2012).

Apple is one of the prominent fruit of Western Himalayas and is highly perishable. Hence, it is required to be processed to preserve its nutritive value and to develop value added products.

Western Himalayan region is a rich repository of microbial genetic diversity. Forty-three indigenous isolates of yeasts had already been characterized in the Department of Microbiology, Himachal Pradesh Agricultural University, Palampur from various fermented foods of Western Himalayas. Twenty-three of them were identified as strains of *S. cerevisiae* by conventional and molecular marker techniques such as Randomly Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Universal Rice Primers (URP), and Delta markers (Pathania et al., 2010). These strains have already been studied for strain level diversity using internal transcribed spacer (ITS) region as a marker (Keshani et al., 2015). Further, on the basis of variation in brewing traits of these strains; they were further studied for their organoleptic effect at gene level. During fermentation processes, yeast cells produce a broad range of aroma-active substances especially volatile esters which greatly affect the complex flavor of fermented alcoholic beverages. While these secondary metabolites are often formed only in trace amounts, their concentration determines the distinct aroma of these beverages. The best-known enzymes involved in ester synthesis are alcohol acetyltransferases (AATases; EC 2.3.1.84). These AATases are encoded by *ATF1*, the *ATF1* homolog Lg-*ATF1*, and *ATF2* genes (Fujii et al., 1996; Yoshimoto et al., 1998; Yoshimoto et al., 1999). Verstrepen et al. (2003) demonstrated that overexpression of *ATF1* in a commercial brewer's strain led to significant increase in concentrations of isoamyl acetate and ethyl acetate in the product. These results indicate that the expression level of *ATF1* is an important limiting factor for ester synthesis under industrial conditions. The variation in *ATF1* gene could also be revealed by organoleptic studies and then comparing the profiles with variations observed at genetic level. This study will further help in comparison of the ester profiles encoded by *ATF1* gene sequence, of the selected strains for understanding and determining the range of flavor phenotypes (esters) that wine yeasts of Western Himalayas exhibit, and how this knowledge can been used to develop novel flavor-active yeasts or to incorporate these wild yeasts with great fermentation (flavor) potential in industrial sector for better utilization at commercial level.

MATERIALS AND METHODS

Yeast Isolates and Culture Maintenance

Out of 23 strains of *S. cerevisiae* available in the Department of Microbiology, HPAU, Palampur, India, 10 strains were used in the present investigation on the basis of variation in their brewing traits (**Table 1**) and were maintained on potato dextrose agar at 4°C and in 50% (v/v) glycerol at −80°C.

ATF1 Gene Studies

For DNA isolation, Yeast DNA isolation Kit was used (Biobasic Inc.). The DNA stock samples were quantified using Nanodrop. Quality and purity of DNA were checked by 0.8% agarose gel electrophoresis. For *ATF1* gene sequence, 293bp of upstream related to promoter and TATA box followed by 1578 bp of ORF and 217 bp of 3′UTR was used. For amplification and sequencing, this 2088 bp region was divided into three overlapping sequences. Three separate primer pairs were used to amplify these three overlapping sequences, i.e., ATF1FL (TGCACTCGATGGTCTTCTCA) and ATF1FR (GACAAATT AGCCGCCAACTC) for the first contig, ATF1SL (TGCAATGT TCTGCACGTTATT) and ATF1SR (TAGTTGTGAGCGGCAAT CTG) for the second contig and ATF1TL (GAACTTCGAATGG CTTACGG) and ATF1TR (TGCAATGTTCTGCACGTTATT) for the third contig. Polimerase chain reaction (PCR) amplification was carried out in the thermal cycler (BOECO, Germany) with an initial denaturation at 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 51°C for 30 s, and 72°C for 90 s with a final elongation step at 72°C for 10 min. The PCR product was analyzed on 1.2% agarose gel. For DNA sequencing, purified PCR products were freeze dried (CHRIST ALPHA I-2LD) and custom sequenced (ABI 3730xl automated sequencer) with both forward and reverse primers (Xcelris Labs Ltd., Ahmedabad, India). The overlapping regions of DNA sequences were aligned for retrieving complete gene sequence. The homology search for *ATF1* gene was carried out using NCBI BLASTN program http://www.ncbi.nih.gov/blast and phylogenetic analyses were conducted in MEGA 5.1 software program.

TABLE 1 | *Saccharomyces cerevisiae* strains used in the present investigation along with their source, place of collection, and GenBank accession numbers of *ATF1* gene.

S. No.	Strain code	Source	Place of collection	GenBank[a] accession number
1	Sc01	*Chhang*	Lahaul & Spiti	KF429732
2	Sc03	*Dhaeli*	Lahaul & Spiti	KF429733
3	Sc04	*Aara*	Lahaul & Spiti	KF429730
4	Sc05	*Chiang*	Lahaul & Spiti	KF429734
5	Sc 11	*Chuli*	Sangla	KF429737
6	Sc 12	Apple wine	Sangla	KF429739
7	Sc 15	Beverage	Bharmour	KF429736
8	Sc 19	Wine	Sangla	KF429735
9	Sc 21	Wine	Sangla	KF429738
10	Sc 24	Fermented product	Palampur	KF429731

[a]*GenBank, National Centre for Biotechnology Information (NCBI), USA.*

Organoleptic Studies

Royal Delicious apple variety was selected for conducting experiments. Healthy fruits were selected, washed in hot water, mixed with 0.1% of potassium metabisulphite and then used for the extraction of juice under hygienic conditions. The physico-chemical analysis of apple juice was carried out for different parameters which included estimation of total soluble solids (TSS), pH, titrable acidity, brix acid ratio, total sugars, reducing sugars, and ascorbic acid. Starter culture of six selected *S. cerevisiae* strains, viz., Sc01, Sc02, Sc05, Sc12, Sc21, and Sc24 was prepared by inoculating 2% of seed inoculum to pasteurized apple juice and incubated at 28°C for 24 h under shaking conditions. Pasteurized apple juice was inoculated by 1% inoculum supplemented with di-Ammonium hydrogen phosphate (DAHP) (300 mg w/v) and incubated at room temperature for fermentation. The periodic samples were taken, spun at 6000 rpm for 5 min and analyzed for TSS, pH and ethanol content till no further decrease in °Brix was noticed. After completion of fermentation, analysis of the final product was carried out for various parameters, i.e., Estimation of pH, total soluble solids, titrable acidity (Amerine et al., 1967), brix-acid ratio, ethanol content (Caputi et al., 1968), ascorbic acid content (Ranganna, 1976), reducing sugars (Miller, 1950), and total sugars (Dubois et al., 1956).

Sensory Evaluation

The organoleptic evaluation of cider was done on the basis of appearance, color, flavor, mouthfeel and overall acceptability by a panel of five judges. Consumer acceptance for the products was evaluated on a nine point "Hedonic scale" (Amerine et al., 1965).

Statistical Analysis

All experiments were performed in triplicate and the results were analyzed statistically by one-way ANOVA and are presented as mean values with the standard error calculated at the 95% confidence level.

RESULTS AND DISCUSSION

ATF1 Gene Studies

During fermentation processes, yeast cells produce a broad range of aroma-active substances which greatly affect the complex flavor of fermented alcoholic beverages. While these secondary metabolites are often formed only in trace amounts, their concentrations determine the distinct aroma of these beverages. Flavor-active substances produced by fermenting yeast cells can be divided into five main groups: sulfur-containing molecules, organic acids, higher alcohols, carbonyl compounds, and volatile esters (Nykanen and Suomalainen, 1983; Nykanen, 1986; Hammond, 1993; Lambrechts and Pretorius, 2000; Pisarnitskii, 2001). Of these, volatile esters represent the largest and most important group. They are responsible for the highly desired fruity character of beer and, to a lesser extent, other alcoholic beverages, such as wine. The major flavor-active esters in beer are acetate esters such as ethyl acetate (solvent-like aroma), isoamyl acetate (banana flavor), and phenylethyl acetate (flowery, rose aroma). In addition, C_6–C_{10} medium-chain fatty acid ethyl esters such as ethyl hexanoate (ethyl caproate) and ethyl octanoate (ethyl caprylate), which have "sour apple" aromas, are also important for the overall bouquet (Meilgaard, 2001).

The means of controlling ester synthesis during industrial beer fermentations are very limited (Verstrepen et al., 2001). It is well known that ester formation is highly dependent on the yeast strain used (Peddie, 1990) and on certain fermentation parameters. Alvarez et al. (1994) found a clear correlation between the concentrations of ethyl acetate and isoamyl acetate in beer, indicating that these esters may be synthesized by the same rate-limiting enzyme. The best-known enzymes involved in ester synthesis are the so-called alcohol acetyltransferases (AATases; EC 2.3.1.84), encoded by *ATF* genes (*ATF1, ATF2*, and *Lg-ATF1*). These enzymes catalyze the formation of acetate esters from the two substrates: alcohol and acetyl-CoA. It was shown that during fermentation, acetate ester production rates followed a pattern corresponding to the AATase activity (Malcorps et al., 1991). In one of the studies, overexpression of *ATF1* derived from an industrial lager brewer's yeast strain resulted in a 27-fold increase in isoamyl acetate production and a 9-fold increase in ethyl acetate production compared to empty-vector transformants (Fujii et al., 1994). These studies indicate that the expression level of *ATF1* is an important limiting factor for ester synthesis under industrial conditions.

In selected *S. cerevisiae* strains, the *ATF1* gene was found to consist of 1566 bp open reading frame that encodes 522

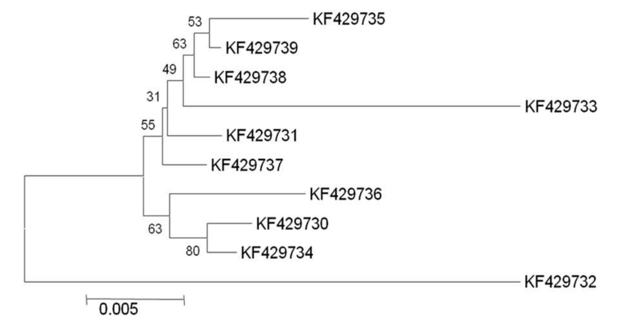

FIGURE 1 | Phylogenetic tree depicting variation in *ATF1* gene sequences with a scale of 0.005 substitutions per nucleotide position.

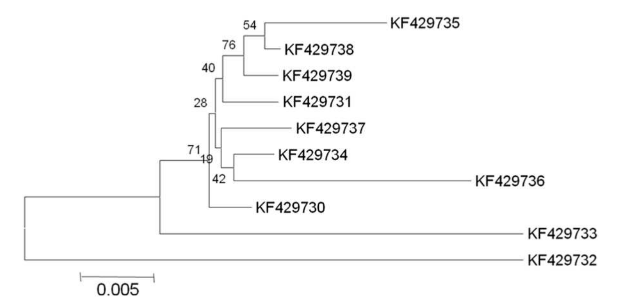

FIGURE 2 | Phylogenetic tree depicting variation in *ATF1* gene sequences with a scale of 0.005 substitutions per amino acid position.

TABLE 2 | Physicochemical characteristics of apple juice.

Parameters	Juice
TSS (°Brix)	9.7
pH	3.617
Reducing sugars (mg/100 mL)	151.2
Total sugars (mg/100 mL)	276.7
Titrable acidity (%)	0.48
Asorbic acid (mg/100 g)	10.256
Brix:acid ratio	20.208

TABLE 3 | Comparative physicochemical analysis of apple cider prepared by six *S. cerevisiae* strains after 15 days.

Strains	TSS (°Brix)	Alcohol content (%)	pH	Reducing sugars (mg/100 mL)	Total sugars (mg/100 mL)	Titrable acidity (%)	Ascorbic acid (mg/100 mL)	Brix:acid ratio	Log (CFU/mL)
Juice	18^{a}	0.01^{f}	3.617^{a}	263.783^{a}	660.743^{a}	0.2633^{e}	4.664^{a}	68.367^{a}	4.927^{e}
Sc01	7^{b}	5.433^{b}	3.133^{de}	126.193^{b}	231.873^{b}	0.4587^{d}	1.287^{b}	15.262^{b}	8.793^{a}
Sc03	5.767^{c}	4.633^{d}	3.143^{de}	94.579^{c}	195.013^{c}	0.507^{b}	0.641^{c}	11.98^{c}	8.623^{b}
Sc05	5.4^{d}	5.433^{b}	3.41^{b}	70.407^{f}	142.006^{d}	0.673^{a}	0.642^{c}	11.618^{d}	8.773^{a}
Sc12	5.63^{cd}	4.1^{e}	3.243^{d}	79.647^{e}	153.587^{e}	0.5047^{b}	0.6413^{c}	11.374^{c}	8.487^{c}
Sc21	5.567^{cd}	5.8^{a}	3.037^{e}	81.083^{e}	158.483^{f}	0.482^{c}	0.639^{c}	11.163^{c}	8.56^{b}
Sc24	5.766^{c}	5.03^{c}	3.35^{c}	85.38^{d}	173.867^{g}	0.4813^{c}	0.6413^{c}	8.024^{d}	8.333^{d}
CD (5%)	0.2506	0.2228	0.1105	2.4986	4.3467	0.0065	0.07	1.0623	0.04201

Results are shown as mean of three replications, different letters denote significant differences among values of various traits ($P < 0.05$).

TABLE 4 | Sensory evaluation of soft cider prepared by using six *S. cerevisiae* strains.

Sr. No.	Sample code	Sensory parameters				
		Appearance/color	Flavor	Mouthfeel	Taste	Overall acceptability
1	Sc01	8	5	5	4	5.5
2	Sc03	7	7	6	5	6.25
3	Sc05	6	6	6	5	5.75
4	Sc12	8	8	8	8	8
5	Sc21	8	9	9	9	8.75
6	Sc24	5	6	6	6	5.75

TABLE 5 | Sensory evaluation of hard cider prepared by using six *S. cerevisiae* strains.

Sr. No.	Sample code	Sensory parameters				
		Appearance/color	Flavor	Mouthfeel	Taste	Overall acceptability
1	Sc01	8	8	8	8	8
2	Sc03	8	7	6	6	6
3	Sc05	7.5	5	5	5.2	5.8
4	Sc12	8	5	5	6	4
5	Sc21	8	6	6	6	6
6	Sc24	7	7.5	7	7	7

amino acids. These results showed discrepancy from the earlier study reporting 1578 bp open reading frame of the structural gene encoding 525 amino acids in *S. cerevisiae* (Fujii et al., 1994). The sequences of the protein coding regions of *ATF1* gene showed a wide variation within these ten indigenous strains. Multiple sequence alignments revealed about 103 nucleotides substitutions at different locations without any deletions or insertions. Subsequent analysis of amino acid sequences of the *ATF1* genes revealed difference of about 47 amino acids among the indigenous yeast strains, suggesting great variations in aroma and flavor of the brewing products. Verstrepen et al. (2003) also showed that overexpression of different alleles of *ATF1* and *ATF2* leads to different ester production rates, indicating differences in the aroma profiles of yeast strains which may be partially due to mutations in their *ATF* genes. In phylogenetic trees (**Figures 1** and **2**) based on nucleotide and amino acid sequence analysis, the *ATF1* sequence of a strain, KF429732 (Sc01), was found to be highly dissimilar to other strains used in the study. This strain also had most desired organoleptic properties as evident from studies conducted with hard apple cider (**Table 5**). The phylogenetic tree obtained after amino acid sequence analysis of the *ATF1* gene (**Figure 2**) was almost similar to that obtained after analysis of nucleotide sequences. As evident from the results, *ATF1* gene can be used to reveal differences in ester formation among these indigenous yeast strains at genetic level.

Organoleptic Studies

Cider is one of the most popular drinks all around the world. In apple producing countries, the apple crop and its subsequent transformation in order to obtain derivatives (brandy, vinegar, apple juice, etc.), is of enormous commercial, economic as well as social relevance. Many different strains of yeast and methods of fermentation are used for producing cider. The interest for locally produced food is increasing due to consumer concern about the environment, distrust of industrial foods and a demand for high quality products. Apple is the predominant fruit crop of Himachal Pradesh and processing of apples into cider could significantly contribute towards the development of the market. The choice of yeast strain as starter culture can have a high impact on the flavor profile of fermented beverages (Nurgel et al., 2009). During fermentation of apple juice, the rate and content of ethanol, sugars, tannins, esters, methanol, and volatile acids are some of the quality characteristics that can be affected by the specific yeast strain (Joshi et al., 2002). The physicochemical analysis of apple juice was evaluated on the basis of chemical analysis and is presented in **Table 2**.

The fermentation conditions such as initial sugar concentration and temperature have been found to exert both positive and negative influence on the quality of beverage. The interaction between temperature and sugar concentration can determine the final quality of the beverage (Llaurado et al., 2002). Hence the sugar level of the pulp was adjusted to 18 °Brix using granulated sucrose. The pulp was inoculated with 1% of six selected yeast strains (Sc01, Sc02, Sc05, Sc12, Sc21, and Sc24) to evaluate the differences in their fermentation behavior. The samples were incubated at room temperature (25°C). Time course study of fermentation revealed 15 days optimum for hard cider preparation and 3 days for soft cider. The significant changes and differences up to 13 days were reported during fermentation of hard cider for every strain. Most of the parameters showed significantly different values after 15 days of fermentation (**Table 3**).

The apple cider samples were put to sensory analysis to find out the acceptability among the tasters. The soft and hard apple cider was subjected to evaluation by a panel of five judges on a 9 point 'Hedonic scale'. The soft cider prepared from Sc21 *S. cerevisiae* strain was found to be best among all other cider preparations (**Table 4**) and hard cider prepared by Sc01 strain was found to be of standard quality (**Table 5**) having 5.43% alcohol (v/v) and 7 °Brix of sugar.

CONCLUSION

ATF1 gene studies revealed wide variation within the 10 indigenous yeast strains, suggesting great variation in aroma and flavor of the brewing products. These findings signify that this gene can play role in revealing the differences in ester formation among indigenous *S. cerevisiae* strains. However, other gene groups associated with this trait are further needed to be studied as they are also important factors in deciding the aroma and flavor of brewing products. The *ATF1* gene sequence of Sc01 was found to be dissimilar to other strains used in the study and the organoleptic properties of this strain were most desirable among all the indigenous yeast strains. Sensory analysis suggested Sc21 and Sc01 as best strains for soft and hard apple cider, respectively, indicating their role in enhancing the quality of apple products.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Poly-γ-Glutamic Acid (PGA)-Producing *Bacillus* Species Isolated from *Kinema*, Indian Fermented Soybean Food

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Kinema, an ethnic fermented, non-salted and sticky soybean food is consumed in the eastern part of India. The stickiness is one of the best qualities of good *kinema* preferred by consumers, which is due to the production of poly-γ-glutamic acid (PGA). Average load of *Bacillus* in *kinema* was 10^7 cfu/g and of lactic acid bacteria was 10^3 cfu/g. *Bacillus* spp. were screened for PGA-production and isolates of lactic acid bacteria were also tested for degradation of PGA. Only *Bacillus* produced PGA, none of lactic acid bacteria produced PGA. PGA-producing *Bacillus* spp. were identified by phenotypic characterization and also by 16S rRNA gene sequencing as *Bacillus subtilis, B. licheniformis* and *B. sonorensis.*

Keywords: *Kinema*, *Bacillus*, fermented soybean, poly-glutamic acid

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INTRODUCTION

Poly-γ-polyglutamic acid (PGA), an amino acid polymer, is not synthesized by ribosomal proteins (Oppermann-Sanio and Steinbüchel, 2002); but is synthesized by Gram-positive bacteria (Yao et al., 2009) and few Gram-negative bacteria (Candela et al., 2009) produced as a polymer outside of the cell (Moraes et al., 2013). PGA-producing bacteria are mainly *Bacillus subtilis, B. anthracis, B. licheniformis, B. thuringensis, B. cereus, B. pumilus, B. amyloliquefaciens, B. mojavensis, B. atrophaeus, B. megaterium, Staphylococcus epidermidis, Natrialba aegyptiaca, Lysinibacillus sphaericus,* and *Fusobacterium nucleatum* (Kambourova et al., 2001; Cachat et al., 2008; Meerak et al., 2008; Candela et al., 2009; Cao et al., 2011). PGA is one of the functional properties of microorganisms present in fermented soybean foods (Tamang et al., 2016a). PGA is an anionic, biodegradable, water-soluble, non-toxic, and edible (Yoon et al., 2000; Zhang et al., 2011). Structurally there are two types of PGA: γ-PGA and α-PGA, which are composed of glutamic acids joined by γ or α linkages, respectively (Goto and Kunioka, 1992). γ-PGA has a structure of 5,000–10,000 units of D- and L-glutamic acids that generate a highly viscous solution when it accumulates in the culture medium (Ashiuchi et al., 2001; Tanimoto et al., 2001). PGA produced by *Bacillus* spp. has potential applications as thickener, cryoprotectant, humectant, drug carrier, biological adhesive, heavy metal absorbent, etc., with biodegradability in the fields of food, cosmetics, medicine, and water treatments (Bajaj and Singhal, 2011; Ogunleye et al., 2015).

Ethnic people of North East India consume spontaneously fermented soybean foods as side dish in meals, which include *kinema*, *tungrymbai*, *hawaijar*, *bekang*, *aakhone*, and *peruyaan* (Tamang, 2015). *Kinema* is a naturally fermented, sticky, mild-ammoniacal flavor and non-salted soybean food of Sikkim and Darjeeling in India, east Nepal and west Bhutan. It is similar to *natto* of Japan, and *chungkokjang* of Korea. PGA is produced by *Bacillus* spp. in many Asian fermented soybean products giving the characteristic of a sticky texture to the product (Urushibata et al., 2002; Nishito et al., 2010) such as *natto* of Japan (Nagai, 2012; Kada et al., 2013), *chungkokjang* of Korea (Lee et al., 2010), *tungrymbai* and *bekang* of India (Chettri and Tamang, 2014), and *thau nao* of Thailand (Chunhachart et al., 2006). One of the criteria for good quality of *kinema* is high stickiness of the product preferred by consumers (Tamang and Nikkuni, 1996). Relative viscosity and stickiness are probably due to production of PGA by *Bacillus* spp. (Nagai et al., 1994; Tamang and Nikkuni, 1996). *B. subtilis* KK3:B4, isolated from naturally fermented *kinema* of India, produced high amount of relative viscosity of 20.1 (Tamang and Nikkuni, 1996). PGA-producing *Bacillus* strain was isolated from *kinema* of Nepal (Hara et al., 1995). Though several species of *Bacillus* such as *B. subtilis, B. licheniformis, B. cereus, B. circulans, B. thuringiensis,* and *B. sphaericus* were previously isolated from *kinema* using phenotypic characterization (Sarkar et al., 1994, 2002; Tamang, 2003; Tamang et al., 2016b); however, there has been no further report on PGA-producing strains/species of *Bacillus*, isolated from *kinema* samples of India. Hence we conducted this experiment. The present study was to screen PGA-producing species of *Bacillus* from *kinema* and to identify species of *Bacillus* by 16S rRNA sequencing.

MATERIALS AND METHODS

Sample Collection

Fresh samples of *kinema* were collected from different markets of Sikkim in India. Samples were collected aseptically in pre-sterile bottles, sealed, labeled, kept in an ice-box and were transported immediately to the laboratory. Samples were stored at 4°C for further microbial and biochemical analyses.

Isolation of Microorganisms

Ten gram of sample was homogenized in 90 mL sterile physiological saline in a stomacher lab-blender (400, Seward, UK) for 1 min and a serial dilution was made. The diluents were heated at 100°C for 2 min for inactivation of vegetative cells of endospore bacteria (Tamang and Nikkuni, 1996), were isolated and enumerated on nutrient agar (MM012, HiMedia, India), and incubated for 24 h at 37°C. Lactic acid bacteria (LAB) were isolated on plates of MRS agar (M641, HiMedia, India) supplemented with 1% $CaCO_3$ and incubated at 30°C in an anaerobic gas-jar (LE002, HiMedia, India) for 48–72 h. Total viable counts were determined on plate count agar (M091A, HiMedia, India) incubated at 30°C for 48–72 h. Isolated colonies were purified and were preserved in 15% (v/v) glycerol at −20°C for further analysis.

Phenotypic Characterization

Cell morphology and motility of isolates were observed using a phase contrast microscope (Olympus CH3-BH-PC, Japan). Isolates were Gram-stained and tested for production of catalase, carbon dioxide from glucose, ammonia from arginine, growth at different temperatures, in different concentrations of NaCl and pH in nutrient broth (M002, HiMedia, India) following the method of Schillinger and Lücke (1987). Voges–Proskauer test, nitrate reduction, starch hydrolysis, casein hydrolysis, citrate utilization test, bile salt tolerance, anaerobic growth, and sugar fermentations were determined following the method of Duc et al. (2004). Taxonomic key of Slepecky

TABLE 1 | Screening of stickiness, and PGA production at different pH and temperatures.

Organisms	Strain code	Stickiness (cm)	PGA production	
			pH 7.5	30°C
Bacillus subtilis (*n* = 13)	KAS:B5	16	++	+++
	KAS:B6	18	++	+++
	KAS:B18	6	+	+
	KAS:B29	16	++	+++
	KAS:B36	4	+	+
	KAS:B39	15	++	+++
	KLM:B68	3	+	+
	KLM:B78	3	+	+
	KLM:B86	4	+	+
	KLM:B98	4	+	+
	KAS:B102	20	++	+++
	KLM:B112	23	++	+++
	KLM:B114	2	+	+
B. licheniformis (*n* = 4)	KAS:B46	4	+	+
	KAS:B56	20	++	+++
	KLM:B92	21	++	+++
	KLM:B108	2	+	+
B. pumulis (*n* = 5)	KAS:B15	3	+	+
	KAS:B48	5	+	+
	KLM:B73	5	+	+
	KLM:B93	6	+	+
	KLM:B106	4	+	+
B. sphaericus (*n* = 8)	KAS:B9	2	+	+
	KAS:B16	4	+	+
	KAS:B19	5	+	+
	KAS:B49	6	+	+
	KLM:B66	3	+	+
	KLM:B72	2	+	+
	KLM:B82	2	+	+
	KLM:B96	2	+	+
B. cereus (*n* = 9)	KAS:B8	2	–	–
	KAS:B10	1	–	–
	KAS:B38	2	–	–
	KAS:B58	2	–	–
	KLM:B74	2	–	–
	KLM:B84	2	–	–
	KLM:B85	2	–	–
	KLM:B88	3	–	–
	KLM:B104	1	–	–

n, number of isolates in parenthesis. +++, high clumping of insoluble precipitate; ++, more clumping of precipitate; +, moderate clumping of precipitate; –, no clumping of precipitate. No precipitate was observed in pH 5 and 9, and at 45°C.

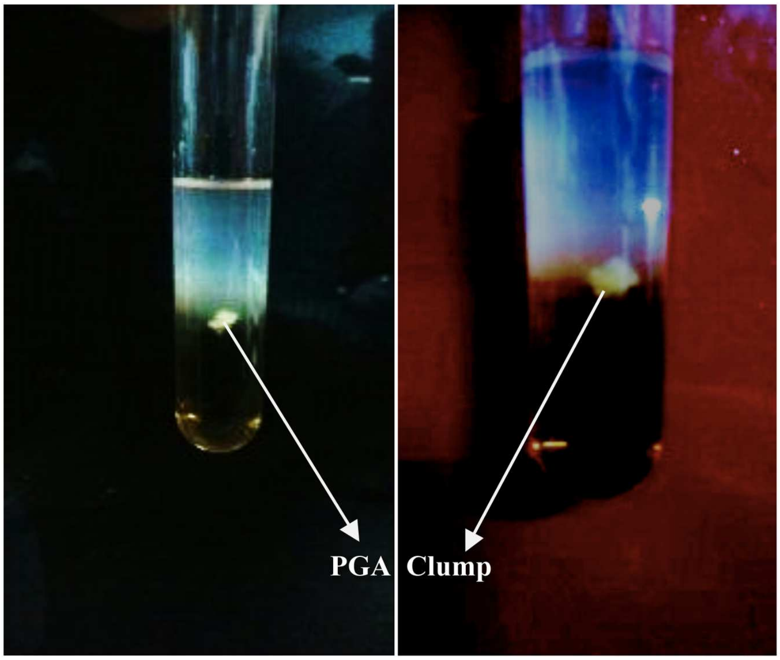

FIGURE 1 | Clumping of insoluble material presumably PGA biopolymer produced by *Bacillus subtilis* KAS:B5 after addition of ethanol into PGA medium.

and Hemphill (2006) was followed for identification of *Bacillus* spp.

Measurement of Stickiness

Cultures were grown on phytone agar (Nagai et al., 1994) at 37°C for 24 h were pulled by touching with an inoculating needle and the stickiness was measured by the length of the thread using scale in cm.

Screening of PGA

Screening of PGA by bacteria was done with a slightly modification of the method described by Nagai et al. (1997) and Meerak et al. (2007). *Bacillus* isolates were grown at 37°C for 24 h in a conical flask containing 100 ml of PGA medium that consisted of sodium glutamate 2.0%, glucose 2.0%, $(NH_4)_2SO_4$ 1.0%, Na_2HPO_4 0.1%, KH_2PO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 0.05%, $Mn(Cl_2)_4.H_2O$ 0.002%, $FeCl_3{\cdot}7H_2O$ 0.005% (Kunioka and Goto, 1994). The culture after incubation was centrifuged to obtain a supernatant that contained insoluble material. An equal volume of ethanol was added to the supernatant to get fibrous precipitate presumbly the PGA (Nagai et al., 1997).

Efficiency of PGA of the isolates were tested in different pH (5, 7.5, and 9) and temperature (30 and 45°C) following the method of Meerak et al. (2007).

Degradation of PGA

Screening of LAB for degradation of PGA was performed following the method described by Tanaka et al. (1993). Strains were grown in MRS broth (M369, HiMedia, India), for 18-24 h at 30°C. The isolates were streaked on MRS agar plates containing 0.5% pure PGA (Sigma) solution (pH 4.5), and incubated at 30°C for 2–3 days. The plates were flooded with 5 ml of 18 N H_2SO_4 and allowed to stand for 30 min at room temperature. The presence of halo around the colony determines the degradation of PGA.

Genomic DNA Isolation

Genomic DNA was isolated according to the method of Wilson (2001). Amplified 16S rDNA was obtained from each strain by polymerase chain reaction (PCR) with the universal primers; forward 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse 5′-AAGGAGGTGATCCAGCCGCA-3′ (Weisburg et al., 1991). The amplicons sizes ranged from 914 BP to 1814 BP.

Gel Electrophoresis

The amplified DNA fragments were separated through gel electrophoresis by applying 10 μL of each PCR product with 1.5 μL of loading dye [(6×), DV4371, Promega, USA] into the wells of 1.5% agarose (V3125, Promega) gel containing 1.5 μL/mL ethidium bromide (H5041, Promega). DNA size markers (RMBD135, Genei; G5711, Promega) were added as standard for the calculation of size of the DNA fragments. The gel was run and photographed using gel documentation system (GelDoc FQ, Biorad, USA).

16S rDNA Sequence Analysis

The sequencing reactions were performed using ABI PRISM 3100 Genettic Analyzers (Applied Biosystems) in both direction with universal primers used for amplification. The electrophenogram data for 16S rDNA sequence was validated using Chromas 2.33

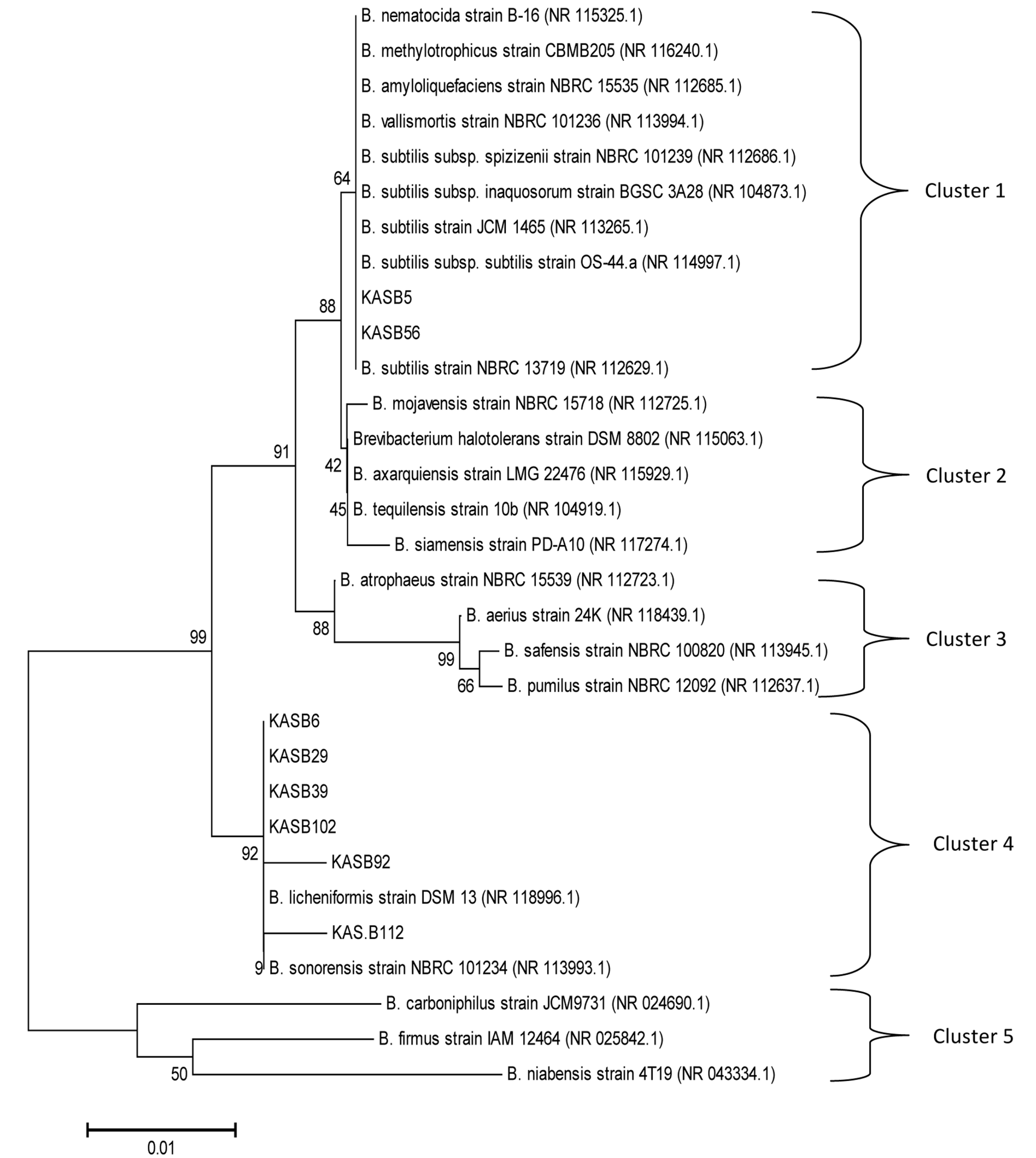

FIGURE 2 | Evolutionary relationships of the analyzed strains with their closest known taxa. The evolutionary history was inferred using the Neighbor-Joining method. The tree was constructed based on the evolutionary distance calculated from 16S rRNA gene sequences using Kimura 2-parameter method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances.

software.[1] Sequences obtained were matched with previously published bacterial 16S rDNA sequences available in the GenBank database using BLAST and the Ribosomal Database Project (RDP).

[1] www.technelysium.com.au

Phylogenetic Analysis

For phylogenetic analysis, 16S rDNA sequence of the isolates and reference sequence retrieved from NCBI-GenBank database were aligned with Clustal Omega. The resulting alignment were analysed with MEGA 6.0 to construct the phylogenetic tree. Phylogenetic tree was inferred with

TABLE 2 | Homogeny of PGA-producing *Bacillus* isolated from *kinema*.

Strain code	*Bacillus*	Accession number	Homogeny (% similarity)
KAS:B5	*Bacillus subtilis*	KX262911	96
KAS:B6	*B. licheniformis*	KX262910	98
KAS:B29	*B. licheniformis*	KX261423	94
KAS:B39	*B. licheniformis*	KX261424	97
KAS:B56	*B. subtilis*	KX262912	97
KAS:B92	*B. licheniformis*	KX261426	97
KAS:B102	*B. licheniformis*	KX261425	96
KAS:B112	*B. sonorensis*	KX262913	97

neighbor-joining (NJ) method (Saitou and Nei, 1987). Sequence divergence among the strain were quantified using Kimura-2-paramater distance model (Kimura, 1980). A total of 1,000 bootstrap replication were calculated for evaluation of the tree topology.

RESULTS AND DISCUSSION

Phenotypic Identification

The average population of *Bacillus* spp. in *kinema* was 10^7 cfu/g, LAB was 10^3 cfu/g and total viable counts were 10 cfu/g, respectively (data not shown). Thirty-nine isolates of *Bacillus* were isolated from 10 samples of *kinema*. Based on phenotypic characterization (data not shown) five species of *Bacillus* were identified from 10 samples of *kinema* as *B. subtilis, B. licheniformis, B. pumulis, B. sphaericus* and *B. cereus* (**Table 1**). About 90% of the total bacterial population found in *kinema* was *Bacillus*, indicating that *Bacillus* is the dominant bacterium in *kinema*. Sarkar and Tamang (1994) also reported that *Bacillus* is the predominant bacterium in *kinema*. *B. subtilis, B. licheniformis, B. cereus, B. circulans, B. thuringiensis,* and *B. sphaericus* were reported from *kinema* sample earlier (Sarkar et al., 1994, 2002; Nout et al., 1998; Tamang, 2003).

Screening of PGA Production

Stickiness of 39 isolates of *Bacillus* was measured (**Table 1**). The ability of 39 isolates of *Bacillus* were tested for production of PGA in PGA medium (Kunioka and Goto, 1994) in pH 5, 7.5, and 9, and at 30C and 45°C (**Table 1**). The isolates formed an insoluble material or fibrous precipitate after addition of equal volume of ethanol into the PGA medium (**Figure 1**) presumbly PGA biopolymer (Nagai et al., 1997; Ashiuchi et al., 2001). All species of *Bacillus* showed fibrous precipitate indicating the absence of PGA production except *B. cereus*.

We tested 25 isolates of LAB isolated from *kinema* for their ability to degrade poly-glutamic acid (PGA) to know whether LAB also produce PGA in *kinema* (data not shown). All LAB isolates were found to degrade PGA, indicating that they have no role in PGA production. Similar observations of degradation of PGA by LAB in fermented soybean were made earlier (Kimura and Fujimoto, 2010; Chettri and Tamang, 2014).

Molecular Characterization

On the basis of high (+++) fibrous precipitate at 30^o C and pH 7.5, and stickiness of >15 cm (**Table 1**), 8 strains of *Bacillus* viz. KAS:B5, KAS:B29, KAS:B39, KAS:B56, KAS:B102, KAS:B6, KAS:B92, and KAS:B112 were selected and were identified by 16S rRNA sequencing. Based on the similarity search with blastN and EzTaxon server the strain KAS:B5 was identified as *B. subtilis*, KAS:B6 as *B. licheniformis*, KAS:B29 as *B. licheniformis*, KAS:B39 as *B. licheniformis*, KAS:B56 as *B. subtilis*, KAS:B92 as *B. licheniformis*, KAS:B102 as *B. licheniformis* and KAS:B112 as *B. sonorensis*. Recovery of *B. sonorensis* from *kinema* is the first report.

Phylogenetic tree was constructed with neighbor joining method based on the evolutionary distance calculated from 1,000 replicates has showed 5 distinct clusters (**Figure 2**), which were separated on a scale of 0.01 nucleotide substitution. The homogeny similarity of *Bacillus* spp. and accession numbers were shown in **Table 2**. Out of 8 PGA-producing strains KAS:B5 and KAS:B56 showed similarities with *B. substilis* strain NBRC13719, *B. subtilis* subsp. *subtilis* strain OS44a and other strains of *subtilis* like JCM1465, NBRC 101236, NBRC 101239, and BGSC 3A28 with 64% of similarity percentage in cluster 1. KAS:B6, KAS:B29, KAS:B39, KAS:B102, and KAS:B92 were found in same clade of cluster 4 showing similarities with *B. licheniformis* DSM12 with 92% similarity and KAS:B112 showed similarities with *B. sonorensis* strain NBRC 101234 with 90% similarity. Strains KAS:B92 and KAS:B112 were found to show a distance gap between the other species of cluster 4 indicating the difference in nucleotide sequence and evolutionary lineage. In this paper, we could find that *B. subtilis* and *B. licheniformis* are PGA-producing bacteria in *kinema*. *B. subtilis* and *B. licheniformis* are the most widely used industrial producers of γ-PGA (Kambourova et al., 2001; Stanley and Lazazzera, 2005; Zhang et al., 2011).

CONCLUSION

Consumers prefer slimy texture of *kinema* as good quality product. Presumably slimy material in fermented soybean food is polyglutamic acid, which has been reported from several Asian fermented foods produced by *Bacillus* spp. PGA, has several applications as foods as well as non-foods. The present study revealed that some species of *Bacillus* produced PGA in *kinem*a. Further investigation is needed to characterize and purify PGA produced by *Bacillus* spp. during natural fermentation of *kinem*a.

AUTHOR CONTRIBUTIONS

RC: screening of PGA-producing *Bacillus* from *kinema*, molecular identification of *Bacillus*, screening go PGA, stickiness, and preparation of draft paper. MOB: phenotypic identification. JPT: analysis of data, compilation and finalization of paper.

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Functional Characterization of Bacterial Communities Responsible for Fermentation of *Doenjang*: A Traditional Korean Fermented Soybean Paste

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Doenjang samples were prepared in triplicate and their microbial abundance, bacterial communities, and metabolites throughout fermentation were analyzed to investigate the functional properties of microorganisms in *doenjang*. Viable bacterial cells were approximately three orders of magnitude higher than fungal cells, suggesting that bacteria are more responsible for *doenjang* fermentation. Pyrosequencing and proton nuclear magnetic resonance spectroscopy were applied for the analysis of bacterial communities and metabolites, respectively. Bacterial community analysis based on 16S rRNA gene sequences revealed that *doenjang* samples included *Bacillus*, *Enterococcus*, *Lactobacillus*, *Clostridium*, *Staphylococcus*, *Corynebacterium*, *Oceanobacillus*, and *Tetragenococcus*. These genera were found either in *doenjang-meju* or solar salts, but not in both, suggesting two separate sources of bacteria. *Bacillus* and *Enterococcus* were dominant genera during the fermentation, but their abundances were not associated with metabolite changes, suggesting that they may not be major players in *doenjang* fermentation. *Tetragenococcus* was dominant in 108 day-*doenjang* samples, when lactate, acetate, putrescine, and tyramine increased quickly as glucose and fructose decreased, indicating that *Tetragenococcus* might be primarily responsible for organic acid and biogenic amine production. *Lactobacillus* was identified as a dominant group from the 179-day samples, associated with the increase of γ-aminobutyric acid (GABA) and the decrease of galactose, indicating a potential role for this genus as a major GABA producer during fermentation. The results of this study clarified the functional properties of major bacterial communities in the *doenjang* fermentation process, contributing to the production of safe and high-quality *doenjang*.

Keywords: *doenjang*, soybean paste, bacterial community, metabolites, *Bacillus*, *Tetragenococcus*, biogenic amine, GABA

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INTRODUCTION

Doenjang is a Korean traditional soybean paste popularly consumed as a condiment for vegetables, fish, and meats or used as a seasoning ingredient in authentic Korean cuisine. The paste has received considerable attention because of numerous reported beneficial human health effects, including antioxidant, fibrinolytic, antimutagenic, and anticancer properties

(Kim, 2004; Yun, 2005; Jung et al., 2006; Park et al., 2008; Namgung et al., 2009; Kwon et al., 2010; Tamang et al., 2016a).

Culture-based approaches have been widely applied to bacterial community analysis of *doenjang* (Yoo et al., 1999; Jeong et al., 2014), but they have produced limited information because culturing is time-consuming and laborious, and because *doenjang* contains unculturable microbes. Recently, culture-independent methods, such as denaturing gradient gel electrophoresis (DGGE) and pyrosequencing, have been widely used to investigate bacterial communities in *doenjang* (Cho and Seo, 2007; Kim et al., 2009; Nam et al., 2012). However, previous studies using culture-independent methods have limited their analyses to snapshots of bacterial communities by focusing on short-time frames within the *doenjang* fermentation process. To the best of our knowledge, thus far, no study has been conducted to investigate microbial community fluctuation over the full *doenjang* fermentation period. In Korea, traditional *doenjang* is typically made by further fermentation of the solid parts from a fermented mixture of *doenjang-meju* (fermented soybean bricks) and brine. The additional fermenting procedure also suggests that the microbial community and indigenous enzymes in *doenjang-meju* are likely important in determining the microbial community and metabolite change during *doenjang* fermentation. However, no research exists on how *doenjang* microbial communities alter when *doenjang-meju* with known microbial community composition is used.

Traditional *doenjang* is produced by spontaneous fermentation without the use of starter cultures, leading to the growth of diverse microorganisms. In turn, quality variation of *doenjang* products tends to result, as well as the occasional production of undesirable metabolites, such as biogenic amines (BAs) or toxins (Cho and Seo, 2007; Shukla et al., 2010; Park et al., 2014). Most previous studies have focused on the analysis of either microbial communities or metabolites in *doenjang* (Cho and Seo, 2007; Kim et al., 2009; Rhyu and Kim, 2011; Nam et al., 2012), which makes it difficult to investigate microbial functional properties during *doenjang* fermentation. Instead, examining microbial successions and metabolite changes simultaneously is crucial for a better understanding of microbial community function in *doenjang*. However, such studies have not yet been performed.

Pyrosequencing based on 16S rRNA gene sequences has been broadly applied to analyze microbial communities in fermented foods, because it yields more detailed data compared with conventional microbiological methods, such as DGGE and culture-based approaches (Nam et al., 2012; Park et al., 2012). Additionally, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy is one of the easiest, yet most comprehensive, and powerful tools to analyze diverse metabolites simultaneously in fermented foods (Jeong et al., 2013; Jung et al., 2013; Lee et al., 2015). In this study, we used pyrosequencing and ^{1}H NMR techniques to investigate microbial succession and metabolite changes, respectively, across the full length of *doenjang* fermentation. The resultant data will increase our knowledge regarding the functional properties of major microbial communities involved in *doenjang* fermentation.

MATERIALS AND METHODS

Doenjang Preparation, Sampling, and Analysis

Doenjang was prepared in triplicate following a traditional manufacturing method. On January 25, 2013, 90 fermented *doenjang-meju* bricks from a previous study (Jung et al., 2014) were placed into a large porcelain pot (called jang-dok) filled with 180 L of approximately 20% (w/v) solar salt (salts made by exposing seawater to the sun; Shinan, Korea) solution (Jung et al., 2015). The mixture of *doenjang-meju* bricks and solar salt solution was stored for 42 days without temperature control in a temporary structure to avoid inclement weather, and then separated into liquid and solid portions. The solid parts (*doenjang*) were mashed well and equally dispensed into three small porcelain pots, marking the start (0 day) of *doenjang* fermentation. These pots containing *doenjang* were stored in the temporary structure without temperature control for 332 days. *Doenjang* samples were intermittently collected for analysis of viable cell numbers, pH, bacterial communities, and metabolites.

Total viable cells of bacteria and fungi were estimated using a standard counting method as described previously (Jung et al., 2014). *Doenjang* samples (2 g) were resuspended and serially diluted in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, and pH 7.2). The diluted supernatants were spread on agar media and incubated at 30°C for 3 days. Respectively, trypticase soy agar (TSA; BD, USA) and potato dextrose agar (PDA; BD, USA), each containing 3% (w/v) NaCl, were used for bacterial and fungal cell counts. Bacterial and fungal cell numbers were counted as colony forming units (CFU) per g-fresh weight of *doenjang*.

For pH measurements, 10 mL of distilled water was added to 2 g of *doenjang* samples and vortexed, after which pH values were obtained using a pH meter (Thermo Scientific, USA). For NaCl, concentrations were measured using the Mohr method (AOAC, 2000) and expressed as a percentage (w/w) in the *doenjang* water phase.

Barcoded Pyrosequencing for Bacterial Community Analysis

To analyze changes in the bacterial community during *doenjang* fermentation, 2 g each of *doenjang* samples were collected from the three porcelain pots and combined. Total genomic DNA was extracted from the combined *doenjang* samples using the FastDNA Spin kit (MP Biomedical, USA), following manufacturer protocol. The V1–V3 regions of bacterial 16S rRNA genes from total genomic DNA were amplified for barcoded pyrosequencing using Bac9F (5′-adaptor B-AC-GAG TTT GAT CMT GGC TCA G-3′) and Bac541R (5′-adaptor A-X-AC-WTT ACC GCG GCT GCT GG-3′) primers, as described previously (Lee et al., 2012). The "X" denotes 7–10 barcoded sequences for sorting mixed sequencing reads (Supplementary Table S1). The PCR products were purified using a PCR purification kit (Bioneer, Korea), and their concentrations were measured using an ELISA reader equipped with a Take3 multivolume plate (SynergyMx; BioTek). A pooled composite was prepared by mixing equal amounts of the purified PCR products and then sequenced using the 454 GS-FLX Titanium system (Roche, Germany) at Macrogen (Korea).

Processing and Analysis of Pyrosequencing Reads

Pyrosequencing reads were processed and analyzed using RDPipeline tools[1] (Cole et al., 2014). The reads were sorted into individual *doenjang* samples based on their unique barcodes, and then the barcodes were eliminated. Low-quality reads were excluded; these included sequences with more than two ambiguous base calls ("N"), shorter than 300 bp, or average quality scores below 25 (error rate, 0.005). Potential chimeric sequencing reads were also excluded using USEARCH 6.0 available in the RDPipeline (Edgar et al., 2011). The resultant high-quality reads were aligned using the fast, secondary-structure aware INFERNAL aligner (Nawrocki and Eddy, 2007). Their operational taxonomic units (OTUs) and rarefaction curves (Colwell and Coddington, 1994) were calculated at a 97% similarity level using the RDPipeline complete-linkage clustering tool. Shannon–Weaver (Shannon and Weaver, 1963), Chao1 richness (Chao and Bunge, 2002), and evenness indices were also calculated with the RDPipeline. Taxonomic classification of the reads was performed at the phylum and genus levels using the RDP Naïve Bayesian rRNA Classifier 2.5 trained on 16S rRNA training set 9 (Wang et al., 2007) with an 80% confidence threshold.

Metabolite Analysis using ^{1}H NMR Spectroscopy

We used ^{1}H NMR spectroscopy to analyze *doenjang* metabolites across the entire fermentation period. Metabolites included monosaccharides, organic acids, and nitrogen compounds such as amino acids and BAs. To minimize quantification errors due to large particles, 10 g of *doenjang* samples were dried in an oven at 80°C for 1 h and ground into a fine power using a pestle and mortar. For sufficient metabolite extraction, 0.2 g of *doenjang* powder was resuspended in 1.5 mL of 99.9% deuterium oxide (D_2O; Sigma–Aldrich, USA) containing 5 mM sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, 97%; Sigma–Aldrich) and incubated on ice with occasional shaking for 1 h. The *doenjang* powder solutions were centrifuged at 12,000 rpm and 4°C for 10 min, the 600 μL of the supernatant were transferred into NMR tubes. We obtained ^{1}H NMR spectra with a Varian Inova 600-MHz NMR spectrometer (Varian, USA); *doenjang* metabolites were identified and quantified using the Chenomx NMR Suite program (version 6.1; Chenomx, Canada). Metabolite concentrations were calculated as μmol per g-dry weight *doenjang*.

[1]http://pyro.cme.msu.edu/

Sequencing Data Accession Number

The sequence data of the 16S rRNA genes from this study are publicly available in the NCBI Short Read Archive under accession no. SRP072427 (NCBI BioProject PRJNA315598).

RESULTS

General Features of *Doenjang* Fermentation

The initial pH values of the *doenjang* samples were approximately 6.4 (**Figure 1**). During the early fermentation period (0–48 days), pH decreased relatively slowly to approximately 6.0; after 48 days, their drop in pH occurred more quickly. From 108 to 179 days of fermentation, pH remained at around 5.0, after which the samples gradually became more basic again, reaching approximately 6.0 during the late fermentation period (249–332 days of fermentation).

Bacterial and fungal viable cells in *doenjang* were counted on their representative growth agar media, TSA, and PDA, respectively, (**Figure 1**); bacteria and fungi grown on PDA and TSA, respectively, were excluded from the counting by their colony morphologies. The initial bacterial cells were approximately 2.7 × 10^8 CFU/g-fresh weight; as fermentation continued, bacterial cell numbers gradually decreased to approximately 4.1 × 10^7 CFU/g-fresh weight. Similarly, fungal cell counts also experienced a gradual decrease, from an initial number of 3.2 × 10^5 CFU/g-fresh weight to 7.9 × 10^4 CFU/g-fresh weight over the course of fermentation. The NaCl concentrations remained relatively constant at approximately 17.5 ± 0.5% (w/w) during the entire fermentation period.

Changes in Bacterial Diversity during *Doenjang* Fermentation

We generated 43,432 sequencing reads from eight *doenjang* samples using barcoded pyrosequencing. After cleaning, 23,031

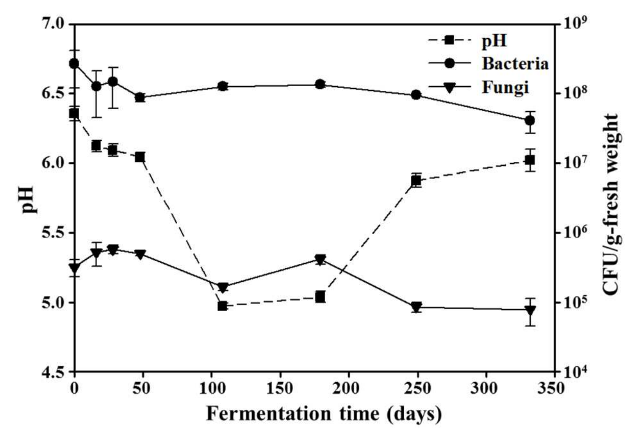

FIGURE 1 | Changes in pH, as well as bacterial and fungal abundances, during *doenjang* fermentation. Data for pH and colony forming units (CFU) are presented as the means ± SD of *doenjang* samples in triplicate.

TABLE 1 | Bacterial pyrosequencing data sets derived from the *doenjang* samples and their statistical diversity analysis.

Sample (day)	Total reads	High quality reads	OTUs[a]	Shannone–Weaver[a]	Chao1[a]	Evenness[a]
0	5296	3817	252	3.4	351.7	0.62
16	5877	4285	268	3.6	393.6	0.64
28	2783	2010	135	3.2	186.8	0.64
48	717	559	40	2.2	55.1	0.60
108	7520	1100	67	2.7	85.1	0.64
179	10816	3749	236	3.6	322.7	0.66
249	5704	4081	240	3.6	384.0	0.66
332	4719	3430	159	3.2	216.2	0.62

OTUs, operational taxonomic units. [a]*Diversity indices were calculated using the RDPipeline tools.*

high-quality reads, with an average 472-bp length and 2,878 reads per sample, were obtained for the analysis of bacterial diversity and community (**Table 1**). The rarefaction analysis showed that bacterial diversity fluctuated slightly over the entire *doenjang* fermentation period (**Figure 2**), potentially indicating the active occurrence of bacterial succession. Bacterial diversity decreased during the early fermentation period (28 and 48 days) and increase after 48 days until 179 days, only to decrease again during the late fermentation period (249–332 days). All calculated diversity indices (OTU, Shannon–Weaver, Chao1, and evenness) supported the results of the rarefaction curve analysis, although the number of reads obtained affected the bacterial diversity indices (**Table 1**).

Changes in Bacterial Community Composition during *Doenjang* Fermentation

Results from phylum- and genus-level classifications of high quality pyrosequencing reads are shown in **Figure 3**, demonstrating bacterial community fluctuations during *doenjang* fermentation. The phylum-level analysis revealed that only *Firmicutes* predominated during the entire fermentation period (**Figure 3A**). *Actinobacteria* was also detected as a minor group, with maximum relative abundance reaching approximately 6.5% at 48 days. The genus-level analysis revealed *Bacillus* to be predominant at a relative abundance of approximately 40%, without an evident fluctuation (**Figure 3B**). *Enterococcus* was also identified as a dominant group during early fermentation, but its relative abundance rapidly decreased with the sudden increase of *Tetragenococcus* at 108 day-*doenjang* samples. The latter genus was not observed initially but appeared as a dominant group from 108-day samples, and its high relative abundance lasted until the end of fermentation (day 332). Interestingly, *Lactobacillus* was identified as a dominant group in 179-day samples. Other minor bacterial genera detected in the *doenjang* samples included *Staphylococcus*, *Clostridium sensu stricto*, unclassified *Thermoactinomycetaceae* 1, and unclassified *Bacillales* from *Firmicutes*, as well as *Corynebacterium* from *Actinobacteria*; these groups did not exhibit dramatic fluctuations in their relative abundance during fermentation.

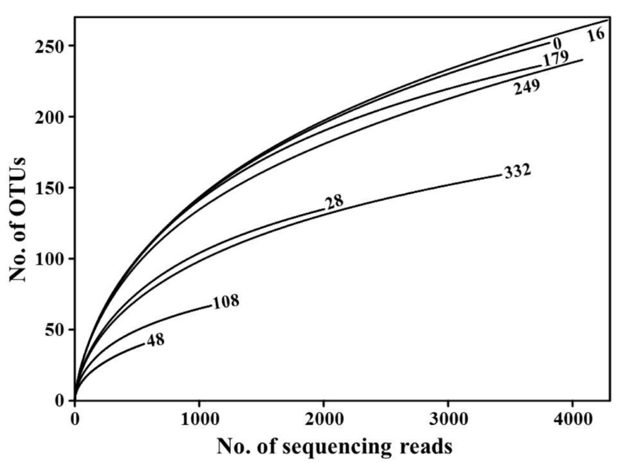

FIGURE 2 | Rarefaction curve analysis of 16S rRNA gene sequencing reads showing variation in bacterial diversity throughout the entire *doenjang* fermentation period. Rarefaction curves were generated with the RDPipeline, using a 97% OTU (operational taxonomic units) cutoff value. Numbers next to the curves indicate *doenjang* sampling time (days).

Metabolite Changes during *Doenjang* Fermentation

Results from the ^{1}H NMR analysis of metabolite content throughout *doenjang* fermentation are presented in **Figure 4**. Glucose, fructose, galactose, and glycerol were identified as the primary free organic compounds, and their levels increased quickly during the early fermentation period (**Figure 4A**). However, after approximately 16 days, glucose and fructose concentrations dropped by 108 days; they were almost entirely consumed. In contrast, galactose concentrations continued to increase until 108 days of fermentation, but then began to decrease from 179 days, finally approaching zero at 249 days. Glycerol concentrations reached maximum at 48 days and then experienced a gradual decrease until the end of fermentation.

Lactate and acetate were identified as the major organic acids during *doenjang* fermentation (**Figure 4B**). Both increased rapidly in 108-day samples and then exhibited fairly constant concentrations until the end of fermentation. Minor organic acids found to occur during *doenjang* fermentation were butyrate and propionate. Next, putrescine and tyramine were identified as the dominant BAs in *doenjang*; similar to lactate and acetate, these amines also increased rapidly in 108-day samples

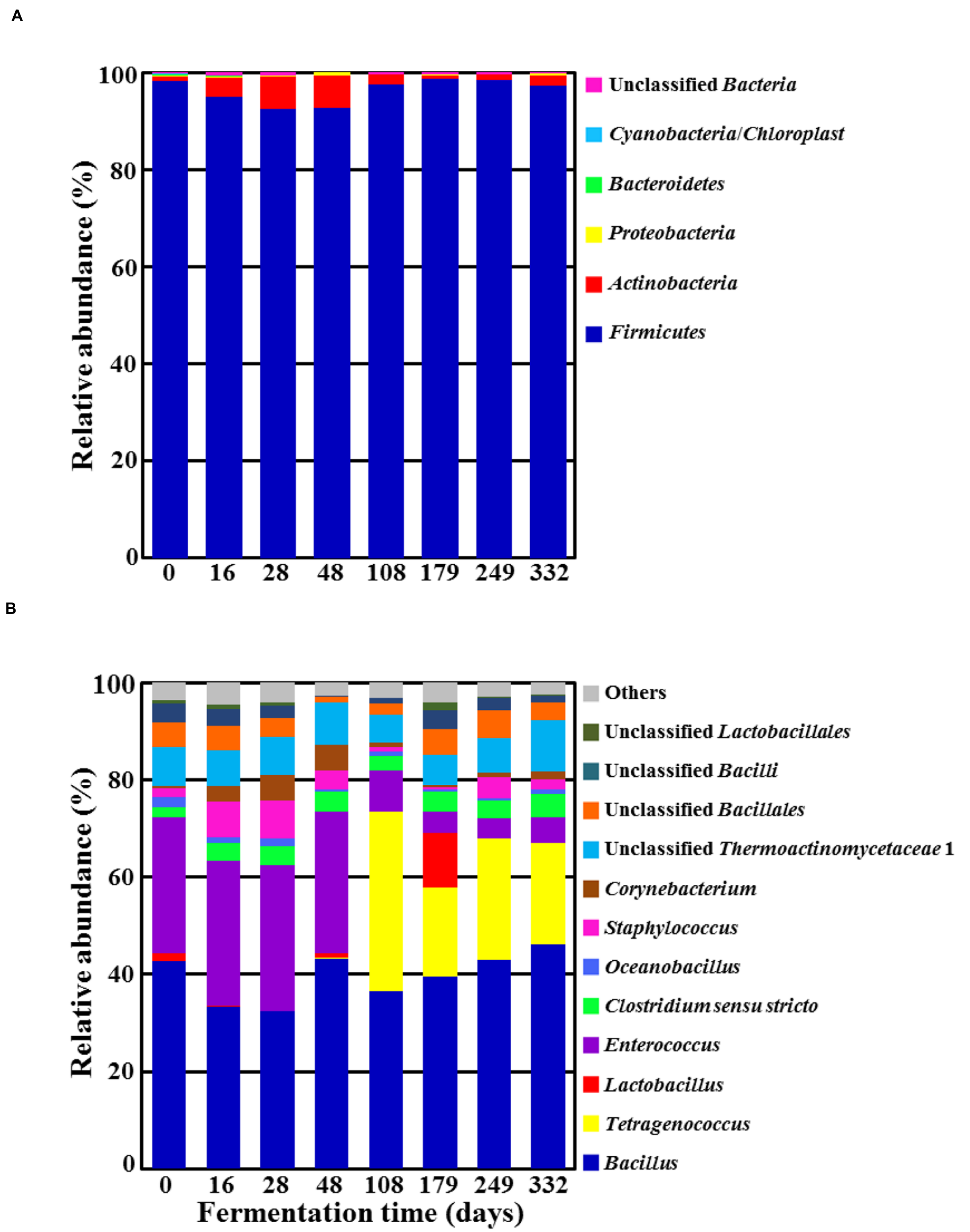

FIGURE 3 | Taxonomic classification at the phylum (A) and genus (B) levels showing bacterial community fluctuations during *doenjang* fermentation. "Others" in **(B)** refers to genera exhibiting a read percentage < 1.0% of the total reads in all *doenjang* samples.

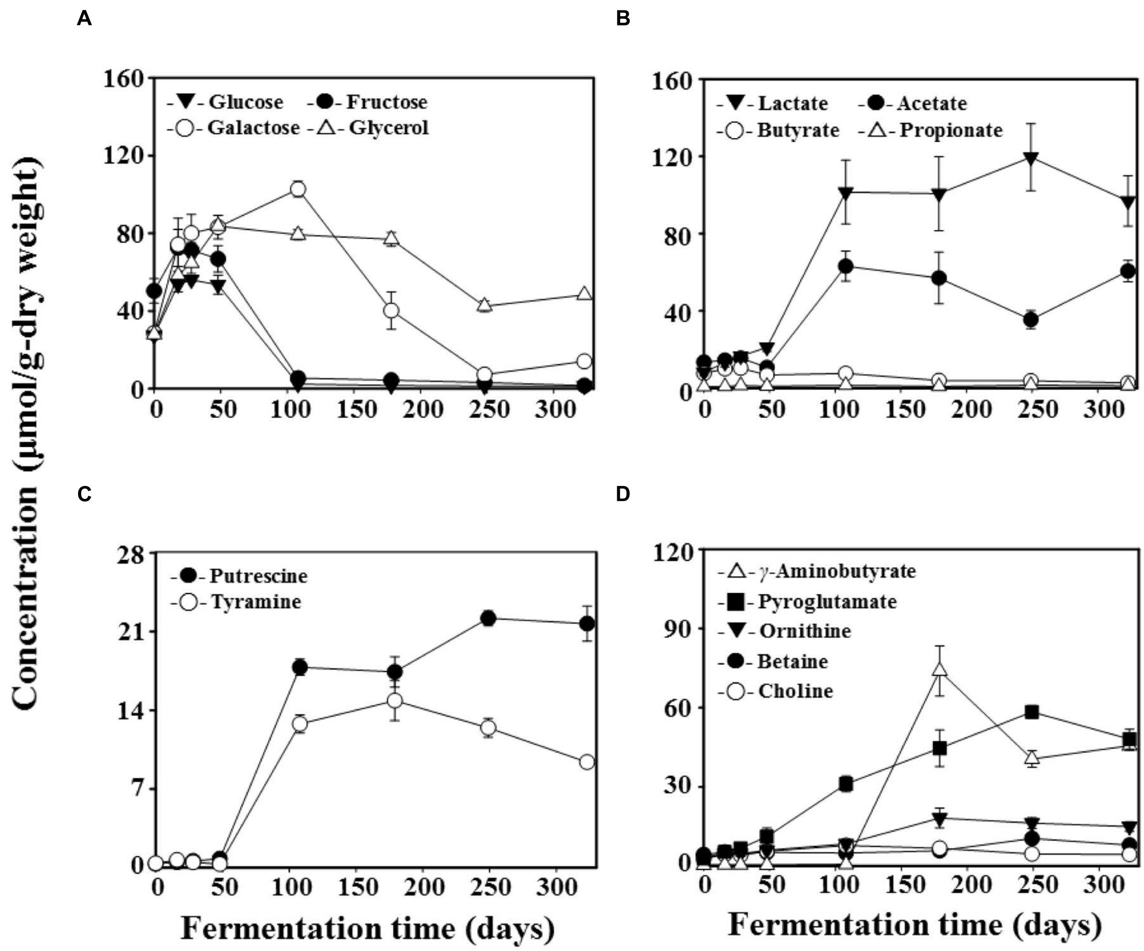

FIGURE 4 | Variation in the content of free organic compounds (A), organic acids (B), biogenic amines (C), and nitrogen compounds (D) during *doenjang* fermentation. Quantifications were performed in the Chenomx NMR suite program (version 6.1, Chenomx, Canada) with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, 97%) as the internal standard. Data are presented as means ± SD.

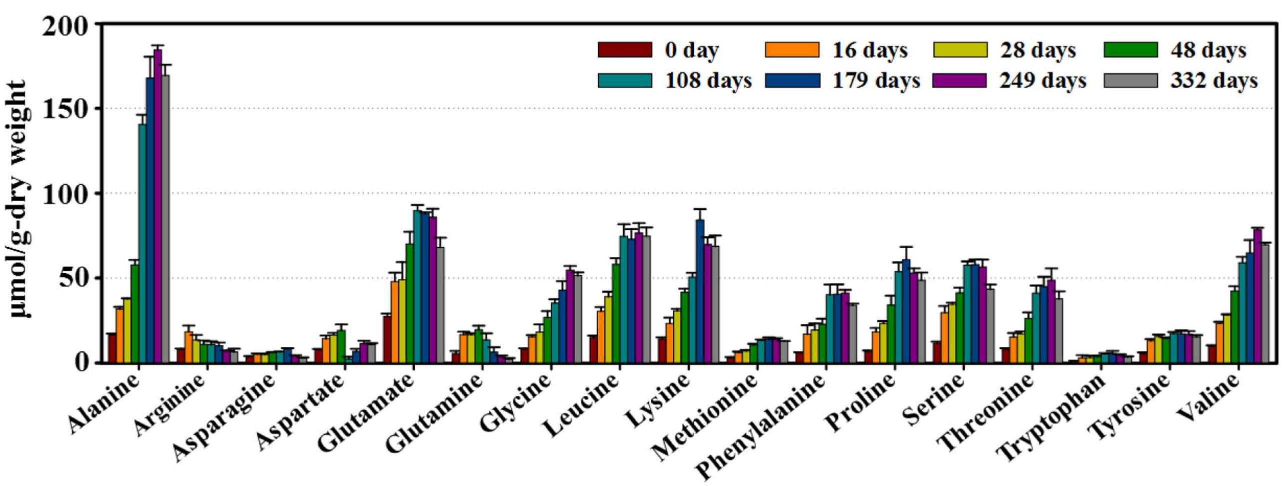

FIGURE 5 | Variation in major amino acids identified from *doenjang* samples during fermentation. Quantifications were performed in the Chenomx NMR Suite (version 6.1, Chenomx, Canada) with sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, 97%) as the internal standard. Data are presented as means ± SD.

(**Figure 4C**). Nitrogen compounds detected in *doenjang* were ornithine, betaine, choline, γ-aminobutyric acid (GABA), and pyroglutamate (**Figure 4D**). Pyroglutamate increased continually until 249 days of fermentation. In particular, GABA exhibited a very rapid increase at 179 days.

Finally, the amino acids well known as the main contributors to flavor and taste in *doenjang* products (Park et al., 2000, 2002; Kim and Lee, 2003) were also major metabolites in our samples (**Figure 5**). Amino acid concentrations rapidly increased during the early fermentation period. Most (alanine, glutamate, leucine, lysine, and phenylalanine) continued to increase until 108–249 days, and then gradually decreased throughout the remainder of the fermentation.

DISCUSSION

Traditional *doenjang* is produced by a long-term fermentation of solid parts obtained from a *doenjang-meju* and brine mixture, which is prepared by soaking the fermented *doenjang-meju* bricks in an 18–20% (w/v) solar salt solution for 40–60 days without the use of starter cultures. Because both bacteria and fungi are involved in *doenjang-meju* fermentation (Kim et al., 2010b; Lee J.H. et al., 2010; Jung et al., 2014) and have been detected in *doenjang* samples (Kim et al., 2009; Shukla et al., 2014), researchers have suggested that the two microbial groups may also play important roles in *doenjang* fermentation. We noted the presence of both bacteria and fungi in our *doenjang* samples (**Figure 1**). However, viable bacterial cell counts were approximately three orders of magnitude higher than fungal counts during fermentation. In addition, *doenjang* fermentation mostly occurs under anaerobic and high moisture conditions. Thus, fungi that prefer dry and aerobic conditions may exhibit lower metabolic activity compared with bacteria during this process. We therefore inferred that the observed fungal growth derived mostly from spores without metabolic activity that had originated in the *doenjang-meju*. This inference was supported by the lack of hyphae or mycelia in the *doenjang* samples (data not shown). These results suggest that bacteria are probably more responsible for *doenjang* fermentation than fungi. Hence, we chose to focus on bacteria in the microbial community analysis.

Although 16S rRNA gene sequencing is the most powerful tool in bacterial taxonomy, it is limitedly used at the species level classification due to the low phylogenetic resolution and poor discriminatory power of 16S rRNA gene sequence at the species level (Fox et al., 1992; Janda and Abbott, 2007). It has been generally accepted that it would be impossible to assign 16S rRNA gene sequences to the level of species because the taxonomies end at the level of genus and DNA relatedness studies are necessary to provide absolute resolution to these taxonomic problems (Schloss and Westcott, 2011). Therefore, in this study we taxonomically classified the 16S rRNA gene sequencing reads at the phylum and genus levels. The genera observed on day 0 of fermentation [*Bacillus*, *Enterococcus*, *Lactobacillus*, *Clostridium*, *Staphylococcus*, *Corynebacterium*, and *Oceanobacillus* (**Figure 3B**)] accorded well with genera detected in previous snapshot analyses of *doenjang* fermentation (Cho and Seo, 2007; Kim et al., 2009; Nam et al., 2012; Jeong et al., 2014). With the exception of *Oceanobacillus*, all genera had also been identified from *doenjang-meju* (Jung et al., 2014), despite the preparation step of soaking the bricks in solar salt solution for 42 days. These results suggest that *doenjang-meju* is a major source of bacteria in *doenjang* fermentation. In contrast, *Oceanobacillus* and *Tetragenococcus* (a dominant group at 108 days of fermentation) likely derived from solar salts because they were not found in *doenjang-meju* (Jung et al., 2014). Moreover, the two genera are halotolerant or halophilic groups that have been frequently isolated from high-saline environments, including solar salterns (Baati et al., 2010; Lee S.Y. et al., 2010). Thus, solar salts appear to be another important bacterial source in *doenjang* fermentation.

Bacillus was one of the predominant populations in *doenjang* during the entire fermentation period (**Figure 3B**). Due to the well-documented predominance of *Bacillus* in both culture-dependent and culture-independent studies on *doenjang*, researchers have generally assumed that this group is primarily responsible for the fermentation process (Yoo et al., 1999; Kim et al., 2010a; Jeon et al., 2016; Tamang et al., 2016b). However, members of *Bacillus* grow aerobically, and therefore, we consider it unlikely that they can be principal actors in *doenjang* fermentation, which occurs almost entirely under anaerobic conditions. Furthermore, most *Bacillus* members do not grow well under conditions more saline than 15% NaCl, although they have been detected from *doenjang* samples with ∼18% salt concentrations (Jung et al., 2014, 2016). Our data also showed that *Bacillus* abundance was relatively constant throughout fermentation and was unassociated with fluctuations in metabolite content (**Figure 4**). Therefore, we infer that *Bacillus* in *doenjang* are probably metabolically inactive or exist as spores. Although their relative abundance is high, we infer that this genus is not primarily responsible for *doenjang* fermentation.

Enterococcus are facultative anaerobic, Gram-positive, cocci-shaped lactic acid bacteria that occurred dominantly alongside *Bacillus* during the early fermentation period (**Figure 3B**). This finding corresponds to previous studies that have also reported *Enterococcus* as a dominant population in *doenjang* (Cho and Seo, 2007; Kim et al., 2009; Nam et al., 2012). Some members of *Enterococcus* are pathogenic and have caused infections in humans (Fisher and Phillips, 2009), but other species such as *E. faecalis* or *E. faecium* have been frequently isolated from fermented foods and even used as probiotics (M'hir et al., 2012; Todorov and Holzapfel, 2015). Therefore, the dominance of *Enterococcus* was expected and likely consisted of non-pathogenic species. The high counts of *Enterococcus* during the early fermentation period was associated with the increase of glucose, fructose, galactose, and glycerol (**Figures 3B** and **4A**), which were probably generated through the hydrolysis of polysaccharides, flavonoid glycosides, and lipids in *doenjang-meju*. However, most *Enterococcus* members do not show glyosidic and lipase activities under high saline conditions such as *doenjang* (Jeong et al., 2014), suggesting that their metabolism was too low during fermentation to hydrolyze the organic carbon compounds. Instead, we speculated that endogenous enzymes derived from *doenjang-meju* might be responsible for the observed increases in glucose, fructose, galactose, and glycerol. Moreover, despite the dominance of *Enterococcus*, we only observed slight increases in lactate and acetate during early fermentation, a finding that supports the hypothesis of weak metabolic activity in these lactic acid bacteria (**Figures 3B** and **4B**). However, additional studies will be necessary to investigate how organic carbon compounds increase in *doenjang* during the early fermentation period.

Bacterial community analysis showed that *Staphylococcus* increased to approximately 7.6% of the total bacterial abundance at 28 days of fermentation (**Figure 3B**). Similar to *Enterococcus*, some members of this genus are pathogenic, but other *Staphylococcus* species are considered starters for fermentation and accordingly, have been identified from several fermented foods, including *doenjang*, sausage, and fish sauce (Fontana et al., 2005; Yongsawatdigul et al., 2007; Kim et al., 2009; Guan et al., 2011; Jung et al., 2013; Lee et al., 2015). Therefore, the *Staphylococcus* species observed in our *doenjang* samples probably do not have pathogenic properties, but additional research is required to understand their exact role during fermentation, as the function of *Staphylococcus* was unclear in this study.

Bacterial community analysis demonstrated that *Tetragenococcus*, a genus of halophilic, Gram-positive lactic acid bacteria, was a dominant group in 108-day samples (**Figure 3B**). The dominance of *Tetragenococcus* corresponded well to the rapid increases in lactate and acetate concentrations, as well as the decreases in glucose and fructose, occurring around the same time (**Figures 3B** and **4A,B**). Additionally, the drop in pH at 108 days was in line with heightened lactate and acetate production (**Figure 1**). Together, these results suggest that *Tetragenococcus* may be primarily responsible for the production of organic acids during *doenjang* fermentation. BAs, including putrescine, cadaverine, spermidine, histamine, and tyramine, are low-molecular-weight nitrogenous organic compounds produced via microbial decarboxylation of amino acids and nitrogen compounds during food fermentation (Shalaby, 1996; Park et al., 2000; Moon et al., 2010; Costantini et al., 2013; Jung et al., 2015). These compounds are known to be produced in *doenjang* (Shukla et al., 2010; Kim and Ji, 2015), but the agents of their production are unknown. The increase of putrescine and tyramine, respectively, generated through ornithine and tyrosine decarboxylation, was also associated with *Tetragenococcus* dominance (**Figures 3B** and **4C**). These results suggest that *Tetragenococcus* may be a key agent in biogenic-amine production during *doenjang* fermentation. Previous studies have also shown that members of *Tetragenococcus* play important roles in the fermentation of salted goods, including fermented seafood; thus, this genus may be a good bacterial starter for flavor enhancement of fermented foods (Chen et al., 2006; Udomsil et al., 2011; Kim and Park, 2014; Jung et al., 2015, 2016). However, some *Tetragenococcus* species appear to produce BAs primarily via plasmid-encoded decarboxylation genes (Satomi et al., 2008, 2011, 2014), which increases the difficulty of using *Tetragenococcus* as starters for salted food fermentation. To address this problem, strains without the ability to produce these compounds have been applied to control biogenic-amine generation during fermentation (Udomsil et al., 2011; Kuda et al., 2012).

Interestingly, *Lactobacillus*, a group of Gram-positive, facultative anaerobic or microaerophilic, rod-shaped lactic acid bacteria, was identified as a dominant group in 179-day samples (**Figure 3B**). The non-protein amino acid GABA is a major inhibitory neurotransmitter and is produced through the irreversible α-decarboxylation of L-glutamate by glutamate decarboxylase. *Lactobacillus* species have been reported as major GABA-producing bacteria during fermentation (Makarova et al., 2006; Cho et al., 2007; Park and Oh, 2007; Siragusa et al., 2007; Komatsuzaki et al., 2008; Jeong et al., 2013). Our metabolite analysis supported these previous findings; galactose decreases and GABA increases were associated with the increase of *Lactobacillus* (**Figures 4A,D**), implying that members of *Lactobacillus* are responsible for GABA production during fermentation. Additionally, the ability of *Lactobacillus* to metabolize galactose is well-established and members of *Lactobacillus* have been detected in *doenjang* (Cho and Seo, 2007; Nam et al., 2012), indicating that lactate production from galactose and GABA synthesis by *Lactobacillus* are important processes during *doenjang* fermentation although no report showing their growth in 18% NaCl conditions exists.

To the best of our knowledge, this was the first study to investigate fluctuations in microbial communities and metabolite production simultaneously throughout the entire *doenjang* fermentation period. Ours was also the first to use *doenjang-meju* of known bacterial community composition in a study of *doenjang* fermentation. Here, we suggested that both *doenjang-meju* and solar salts are important bacterial sources in *doenjang* fermentation. Furthermore, we proposed that despite their overall abundance, *Bacillus* may be not as central to *doenjang* fermentation as previously assumed. Additionally, we showed that solar-salt-derived *Tetragenococcus* appears to be a primary producer of organic acids and BAs during *doenjang* fermentation, suggesting that *Tetragenococcus* strains without this ability are usable as starters, in order to reduce biogenic-amine concentrations. Finally, our results suggested that *Lactobacillus* is probably one of the major GABA producers during *doenjang* fermentation. In conclusion, this study contributed to an improved understanding of the biochemical processes underlying *doenjang* fermentation through exploring the functional properties of major *doenjang* microbial communities. The data generated should pave the way for additional research employing "omics" technologies, including metagenomics, metatranscriptomics, and metabolomics, which are certain to provide further insights into the production of safe and high-quality *doenjang*.

AUTHOR CONTRIBUTIONS

CJ conceived the ideas and supervised the work WJ and JJ developed the concepts and performed the experiments WJ and

HL analyzed the data and CJ and WJ wrote the manuscript. The manuscript has been reviewed and edited by all authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00827

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REVIEW
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The Microbiota and Health Promoting Characteristics of the Fermented Beverage Kefir

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Kefir is a complex fermented dairy product created through the symbiotic fermentation of milk by lactic acid bacteria and yeasts contained within an exopolysaccharide and protein complex called a kefir grain. As with other fermented dairy products, kefir has been associated with a range of health benefits such as cholesterol metabolism and angiotensin-converting enzyme (ACE) inhibition, antimicrobial activity, tumor suppression, increased speed of wound healing, and modulation of the immune system including the alleviation of allergy and asthma. These reports have led to increased interest in kefir as a focus of research and as a potential probiotic-containing product. Here, we review those studies with a particular emphasis on the microbial composition and the health benefits of the product, as well as discussing the further development of kefir as an important probiotic product.

Keywords: gut microbiota, fermented foods, immunomodulation, metabolic diseases, kefir

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INTRODUCTION

Fermented dairy products have long been associated with the ability to confer health benefits in those who regularly consume them, with Ellie Metchnikoff first theorizing that their impact on the bacterial microbiota in the gut contributed to health and long life (Metchnikoff, 1908). Indeed many reportedly probiotic-containing foods come in the form of fermented milk products, such as yogurt, koumis, and kefir, many of which have been consumed for 100s of years (Tamime, 2002; Parvez et al., 2006). Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). As is the case with the fermented dairy products referred to above, probiotics are consumed in foods containing these organisms in sufficiently large quantities to pass safely to the gastrointestinal tract but can also come in the form of supplements consisting of live organisms such as pills.

Although not as widely popular as other fermented dairy products, such as yogurt and cheese, kefir has been consumed and associated with health benefits for 100s of years; originally by communities in the Caucasian mountains. The beverage itself typically has a slightly viscous texture with tart and acidic flavor, low levels of alcohol, and in some cases slight carbonation. Kefir is traditionally made with cow's milk but it can be made with milk from other sources such as goat, sheep, buffalo, or soy milk (Ismail et al., 1983; Motaghi et al., 1997; Wszolek et al., 2001; Liu et al., 2006a). One of the features that distinguish kefir from many other fermented dairy products is the requirement for the presence of a kefir grain in fermentation and the presence and importance of a large population of yeasts (Tamime, 2002; Tamang et al., 2016). The aforementioned kefir grains are microbially derived protein and polysaccharide matrices that contain a community of bacterial and fungal species that are essential to kefir fermentation (Garrote et al., 2001; Marsh et al., 2013). Traditionally, fermentation was initiated through the addition of kefir grains, which originally formed during the fermentation of milk, to unfermented milk in a sheep or goat skin bag (Motaghi et al., 1997). Commercial, industrial-scale production rarely utilizes kefir grains for fermentation, but rather uses starter cultures of microbes that have been isolated from kefir or kefir grains in order to provide more consistent products (Assadi et al., 2000). While this industrially produced kefir may have health benefits of its own, research examining such benefits has either not been performed or is not published. Thus, any kefir referred to in this review has been produced in a traditional manner using kefir grains or grain fermented milk as the inoculum. In addition to the microbial population present in kefir, these beverages typically also contain an abundance of fermentation products such as organic acids and multiple volatile flavor compounds including ethanol, acetaldehyde, and diacetyl (Güzel-Seydim et al., 2000). As part of the fermentation process, an exopolysaccharide unique to kefir, kefiran, is produced. Kefiran makes up a large proportion of the kefir grain itself and is also found dissolved in the liquid phase, where it contributes to the rheology and texture of the finished product (La Rivière et al., 1967; Frengova et al., 2002; Rimada and Abraham, 2006).

In this review we will discuss the many health promoting effects that have been attributed to kefir, including tumor suppression and prevention, gastrointestinal immunity and allergy, wound healing, cholesterol assimilation and ACE inhibition, its antimicrobial properties, and the ability of kefir to modify the composition and activity of the gut microbiota (**Figure 1**).

BACTERIAL AND FUNGAL POPULATIONS OF KEFIR

Bacterial Populations

Since the first established use, 100s of years ago, the propagation of kefir has been performed by transferring kefir grains from one batch to fresh milk and incubating at ambient temperature. Over this period there has been substantial opportunity for the microbial component of kefir grains to evolve and diverge, resulting in the addition or loss of bacteria and yeasts as well as the addition and loss of genes. The bacterial genera most commonly found in kefir using culture dependent techniques are *Lactobacillus*, *Lactococcus*, *Streptococcus*, and *Leuconostoc* (Simova et al., 2002; Witthuhn et al., 2004; Chen et al., 2008). These genera tend to dominate the population present in both the kefir grain and milk, with *Lactococcus lactis* subsp. *lactis*, *Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus casei* subsp. *pseudoplantarum*, *Lactobacillus kefiri*, *Lactobacillus kefir*, and *Lactobacillus brevis* accounting for between 37 and 90% of the total microbial community present (Simova et al., 2002; Witthuhn et al., 2004; Miguel et al., 2010). While these species commonly make up the majority of the microbial population present in kefir grains, some grains are dominated by yeast species or other bacterial species such as *Leuconostoc mesenteroides* (Witthuhn et al., 2004). The proportions of species can also differ between the grain and milk (**Figure 2**). For example, *L. lactis* subsp. *lactis*, and *S. thermophilus* levels are generally much greater in the fermented kefir than in the kefir grains. The levels of these species increase further in kefir made from kefir as an inoculum. Indeed, the total increase observed

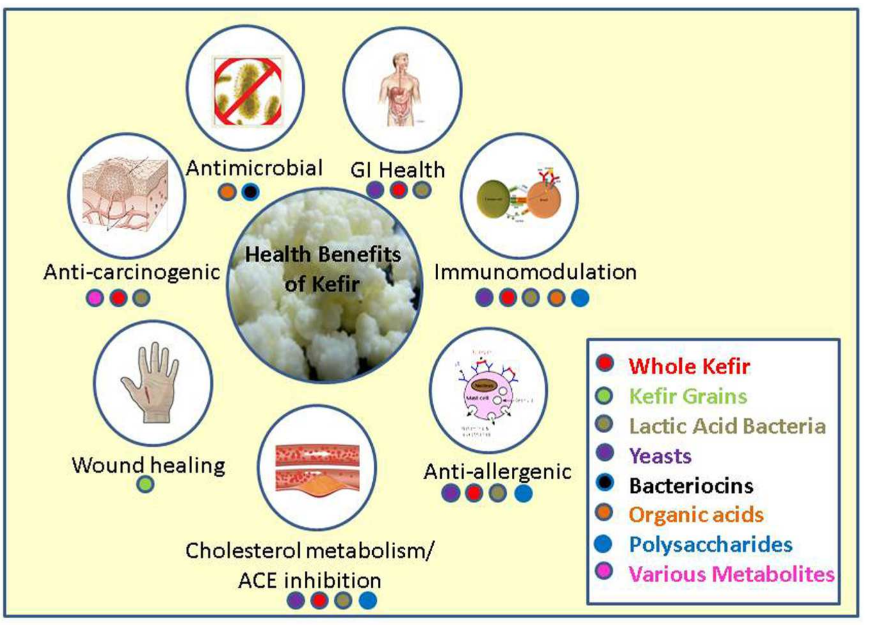

FIGURE 1 | Major health benefits associated with kefir and the fractions or parts of kefir responsible for these benefits.

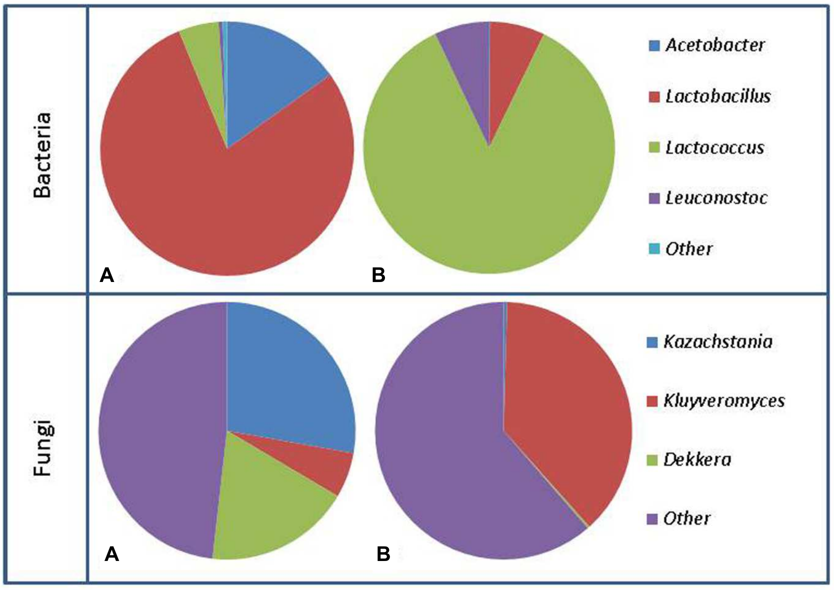

FIGURE 2 | Representation of bacterial population changes from kefir grain (A) to fermented milk (B) and fungal population changes from kefir grain (A) to fermented milk (B). Figure generated using data from Marsh et al. (2013).

has been as much as 30% in some cases (Simova et al., 2002). The reason for this increase during fermentation in the milk may be due to an increase in temperature created by the active fermentation or simply due to where these bacteria reside in the kefir grain, as organisms such as *Lactobacillus* may tend to reside deeper within the kefir grain, thus making it harder for them to escape in to the milk.

In agreement with the majority of culture base studies, investigation of the microbial composition of diverse kefir grains using culture independent techniques found that the overall bacterial populations were for the most part dominated by Firmicutes and Proteobacteria, and kefir milk contained a much higher level of representatives of the *Streptococcaceae* than any other family, (Dobson et al., 2011; Marsh et al., 2013). Based on high-throughput sequencing of 16S genes present in kefir grains and milk, it was established that kefir grains typically have 1 (*Lactobacillus*) or 2 (*Lactobacillus* and *Acetobacter*) dominant bacterial genera (Marsh et al., 2013; Nalbantoglu et al., 2014; Garofalo et al., 2015; Korsak et al., 2015). The most common species of *Lactobacillus* have been *L. kefiranofaciens*, *L. kefiri*, and *L. parakefiri* (Dobson et al., 2011; Leite et al., 2012; Hamet et al., 2013; Vardjan et al., 2013; Nalbantoglu et al., 2014; Garofalo et al., 2015; Korsak et al., 2015). There are many other genera present in these grains; however, they typically represent less than 10% of the community (Leite et al., 2012; Marsh et al., 2013; Nalbantoglu et al., 2014; Garofalo et al., 2015). When milk fermented by these same grains was examined, the relative abundance of the genera present vary much more than in the grain, with *Leuconostoc*, *Lactococcus*, *Lactobacillus*, and *Acetobacter* being the most abundant (Marsh et al., 2013; Korsak et al., 2015). As has previously been stated, bacteria found at lower abundance in the kefir grain can become dominant, as species such as *Lactococcus* are minimally represented in kefir grain, but regularly become the most abundant genus present in the kefir milk (Dobson et al., 2011; Marsh et al., 2013). This observation is consistent with past culture based work, where *Lactococcus* was found to increase through the fermentation process (Simova et al., 2002). At the species level, high throughput 16S analysis showed the number of OTUs vary from 24 to 56 in the kefir grain, and 22 to 61 in kefir milk, i.e., much higher than what has been observed utilizing culture dependent techniques (Marsh et al., 2013). These findings highlight the need for future studies to examine the kefir grain and fermented milk rather than the previous tendency to focus solely on the population of the grain.

With respect to the non-lactic acid bacteria (LAB) that have been associated with kefir, it is notable that culture independent methods have revealed *Acetobacter* as one of the dominant genera present in grains. This is of interest as *Acetobacter* is not commonly isolated from kefir *via* culture dependent techniques and, indeed, has been described as a non-essential contaminant of kefir (Angulo et al., 1993; Pintado et al., 1996; Rea et al., 1996; Witthuhn et al., 2004). While there are some studies that have found acetic acid bacteria in large quantities in kefir grains (Rea et al., 1996), many rely on isolation media that is not optimal for growth of acetic acid bacteria without further tests in order to gather an accurate identification (Witthuhn et al., 2005). *Bifidobacterium* species have also been identified through culture independent studies, however, *Bifidobacterium* has not been found in any culture based studies of the kefir microbiota (Dobson et al., 2011; Taş et al., 2012; Marsh et al., 2013). **Table 1** contains a complete list of bacterial species found in both culture dependent and culture independent studies, while **Figure 3** provides a breakdown of the distribution of species found in these studies.

TABLE 1 | List of bacterial and fungal species found in kefir grains and milk using both culture dependent and culture independent techniques.

Microbial species	Reference
Lactobacillus	
Lactobacillus kefir	Angulo et al., 1993; Pintado et al., 1996; Garrote et al., 2001; Santos et al., 2003; Mainville et al., 2006; Miguel et al., 2010
Lactobacillus kefiranofaciens	Santos et al., 2003; Mainville et al., 2006; Chen et al., 2008; Dobson et al., 2011; Hamet et al., 2013; Vardjan et al., 2013; Nalbantoglu et al., 2014; Garofalo et al., 2015; Korsak et al., 2015; Zanirati et al., 2015
Lactobacillus delbrueckii	Simova et al., 2002; Santos et al., 2003; Witthuhn et al., 2004; Nalbantoglu et al., 2014
Lactobacillus helveticus	Simova et al., 2002; Chen et al., 2008; Dobson et al., 2011; Nalbantoglu et al., 2014
Lactobacillus casei	Angulo et al., 1993; Simova et al., 2002; Nalbantoglu et al., 2014; Zanirati et al., 2015
Lactobacillus kefiri	Chen et al., 2008; Miguel et al., 2010; Dobson et al., 2011; Hamet et al., 2013; Vardjan et al., 2013; Nalbantoglu et al., 2014; Garofalo et al., 2015; Korsak et al., 2015; Zanirati et al., 2015
Lactobacillus brevis	Angulo et al., 1993; Simova et al., 2002; Santos et al., 2003; Witthuhn et al., 2005; Nalbantoglu et al., 2014
Lactobacillus paracasei	Santos et al., 2003; Miguel et al., 2010; Hamet et al., 2013; Nalbantoglu et al., 2014
Lactobacillus parakefir	Takizawa et al., 1994; Garrote et al., 2001; Miguel et al., 2010
Lactobacillus plantarum	Garrote et al., 2001; Santos et al., 2003; Miguel et al., 2010; Nalbantoglu et al., 2014
Lactobacillus satsumensis	Miguel et al., 2010; Zanirati et al., 2015
Lactobacillus curvatis	Witthuhn et al., 2004
Lactobacillus fermentum	Angulo et al., 1993; Witthuhn et al., 2004, 2005
Lactobacillus viridescens	Angulo et al., 1993
Lactobacillus acidophilus	Angulo et al., 1993; Santos et al., 2003; Dobson et al., 2011; Nalbantoglu et al., 2014
Lactobacillus gasseri	Angulo et al., 1993; Nalbantoglu et al., 2014
Lactobacillus kefirgranum	Takizawa et al., 1994; Vardjan et al., 2013
Lactobacillus parakefiri	Dobson et al., 2011; Hamet et al., 2013; Vardjan et al., 2013; Nalbantoglu et al., 2014; Korsak et al., 2015
Lactobacillus parabuchneri	Dobson et al., 2011; Nalbantoglu et al., 2014
Lactobacillus garvieae	Dobson et al., 2011
Lactobacillus buchneri	Nalbantoglu et al., 2014; Garofalo et al., 2015
Lactobacillus sunkii	Nalbantoglu et al., 2014; Garofalo et al., 2015
Lactobacillus crispatus	Nalbantoglu et al., 2014; Garofalo et al., 2015
Lactobacillus otakiensis	Nalbantoglu et al., 2014; Garofalo et al., 2015
Lactobacillus instestinalis	Garofalo et al., 2015
Lactobacillus amylovorus, *L. pentosus*, *L. salivarius*, *L. johnsonii*, *L. rhamnosus*, *L. rossiae*, *L. sakei*, *L. reuteri*, *L. kalixensis*, *L. rapi*, *L. diolivorans*, *L. parafarraginis*, *L. gallinarum*, *Pediococcus claussenii*, *P. damnosus*, *P. halophilus*, *P. pentosaceus*, *P. lolii*	Nalbantoglu et al., 2014
Lactococcus/Streptococcus	
Lactococcus lactis subsp. *lactis*	Angulo et al., 1993; Pintado et al., 1996; Garrote et al., 2001; Simova et al., 2002; Witthuhn et al., 2004, 2005; Yüksekdağ et al., 2004; Mainville et al., 2006; Chen et al., 2008; Garofalo et al., 2015; Zanirati et al., 2015
Lactococcus lactis subsp. *cremoris*	Yüksekdağ et al., 2004; Mainville et al., 2006; Korsak et al., 2015
Lactococcus lactis subsp. *lactis* biovar *diacetylactis*	Garrote et al., 2001
Lactococcus garvieae	Nalbantoglu et al., 2014
Streptococcus salivarius subsp. *thermophilus*	Angulo et al., 1993
Streptococcous thermophilus	Simova et al., 2002; Yüksekdağ et al., 2004; Mainville et al., 2006; Garofalo et al., 2015
Streptococcus durans	Yüksekdağ et al., 2004
Leuconostoc/Oenococcus	
Leuconostoc spp.	Angulo et al., 1993
Leuconostoc mesenteroides subsp. *mesenteroides*	Witthuhn et al., 2004; Mainville et al., 2006
Leuconostoc mesenteroides subsp. *cremoris*	Witthuhn et al., 2005; Mainville et al., 2006
Leuconostoc mesenteroides	Simova et al., 2002; Chen et al., 2008; Nalbantoglu et al., 2014; Korsak et al., 2015; Zanirati et al., 2015
Leuconostoc pseudomesenteroides	Mainville et al., 2006
Oenococcus oeni	Nalbantoglu et al., 2014

(Continued)

TABLE 1 | Continued

Microbial species	Reference
Acetobacter	
Acetobacter spp.	Angulo et al., 1993; Garrote et al., 2001; Marsh et al., 2013; Garofalo et al., 2015
Acetobacter sicerae	Li et al., 2014
Acetobacter orientalis, *Acetobacter lovaniensis*	Korsak et al., 2015
Bifidobacterium	
Bifidobacterium spp.	Marsh et al., 2013
Bifidobacterium breve, *B. choerinum*, *B. longum*, *B. pseudolongum*	Dobson et al., 2011
Yeast and fungal species	
Zygosaccharomyces spp.	Witthuhn et al., 2004, 2005
Candida kefyr	Angulo et al., 1993; Marquina et al., 2002; Witthuhn et al., 2004
Candida lipolytica	Witthuhn et al., 2004
Saccharomyces cerevisiae	Angulo et al., 1993; Marquina et al., 2002; Simova et al., 2002; Witthuhn et al., 2004; Latorre-García et al., 2007; Marsh et al., 2013; Vardjan et al., 2013; Diosma et al., 2014; Garofalo et al., 2015
Candida holmii	Angulo et al., 1993; Witthuhn et al., 2004; Latorre-García et al., 2007
Torulaspora delbrueckii	Angulo et al., 1993; Vardjan et al., 2013
Saccharomyces unisporus	Angulo et al., 1993; Pintado et al., 1996; Marquina et al., 2002; Latorre-García et al., 2007; Wang et al., 2008; Marsh et al., 2013; Vardjan et al., 2013; Diosma et al., 2014; Garofalo et al., 2015
Candida friedrichii	Angulo et al., 1993
Kluyveromyces lactis	Angulo et al., 1993; Marquina et al., 2002; Latorre-García et al., 2007
Pichia fermentans	Angulo et al., 1993; Wang et al., 2008; Marsh et al., 2013
Issatchenkia orientalis	Latorre-García et al., 2007; Marsh et al., 2013; Diosma et al., 2014
Kluyveromyces marxianus	Marquina et al., 2002; Wang et al., 2008; Marsh et al., 2013; Vardjan et al., 2013; Diosma et al., 2014; Korsak et al., 2015
Saccharomyces turicensis	Wang et al., 2008; Garofalo et al., 2015
Dekkera anomala	Marsh et al., 2013; Garofalo et al., 2015
Kazachstania exigua	Vardjan et al., 2013; Garofalo et al., 2015; Korsak et al., 2015
Naumovozyma spp.	Korsak et al., 2015
Cryptococcus humicolus, *Geotricum candidum*	Witthuhn et al., 2005
Kazachstania servazzii, *Ka. solicola*, *Ka. aerobia*, *Saccharomyces cariocanus*	Garofalo et al., 2015
Kluyveromyces marxianus var. *lactis*, *Candida inconspicua, C. maris*	Simova et al., 2002
Saccharomyces humaticus, *Candida sake, Yarrowia lipolytica, Dipodascus capitatus, Trichosporon coremiiforme*	Latorre-García et al., 2007
Ganoderma lucidum, *Dioszegia hungarica*, *Heterbasidion annosum*, *Peziza campestris*, *Cyberlindnera jadinii*, *Malassezia pachydermatis*, *Teratosphaeria knoxdaviesii*, *Cryptococcus* sp. *Vega* 039, *Microdochium nivale*, *Wallemia sebi*, *Zygosaccharomyces lentus*, *Eurotium amsteldami*, *Dekkera bruxellensis*, *Kazachstania barnettii*, *Naumovozyma castelli*, *Davidiella tassiana*, *Penicillium* sp. *Vega* 347	Marsh et al., 2013

Yeast Populations

In addition to the large and variable bacterial population in kefir grains, there is an abundant yeast population that exists in a symbiotic relationship with the bacteria (Simova et al., 2002; Witthuhn et al., 2004; Marsh et al., 2013). Three genera of yeasts are commonly isolated from kefir grains or milk, and typically make up the majority of the total yeast population; *Saccharomyces*, *Kluyveromyces*, and *Candida* (Angulo et al., 1993; Marquina et al., 2002; Simova et al., 2002; Diosma et al., 2014).

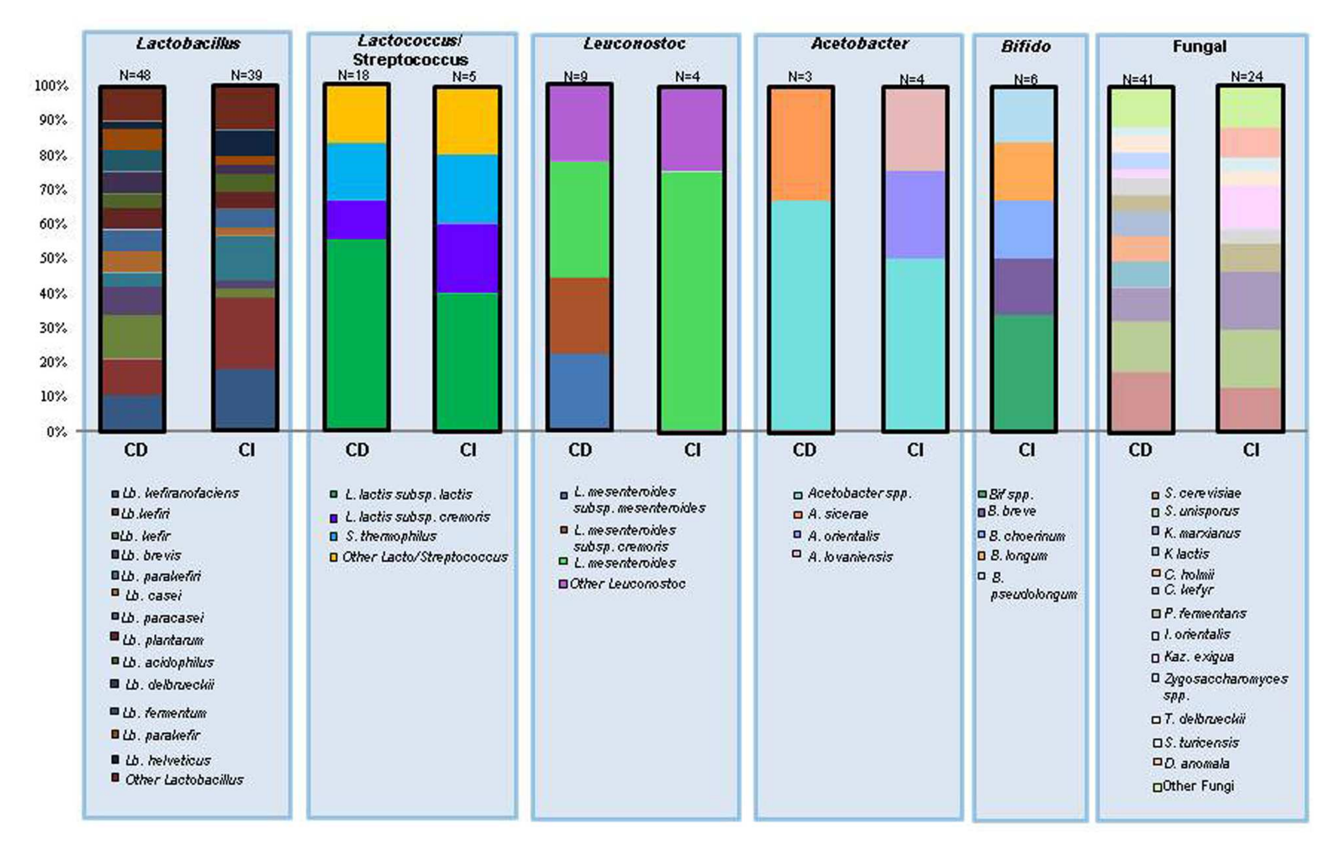

FIGURE 3 | The number of times an individual species has been identified in kefir expressed as a percentage of the total number of species in the same genera. CD, culture dependent identification; CI, culture independent identification; *N* values represent the total number of times a species within the genus has been identified.

Many different species of *Saccharomyces* have been isolated from kefir, however, *S. cerevisiae* and *S. unisporus* are the most common and present in many varieties (Angulo et al., 1993; Marquina et al., 2002; Latorre-García et al., 2007; Diosma et al., 2014). *Kluyveromyces* make up the majority or entirety of the lactose utilizing yeast population, with *K. marxianus* and *K. lactis* being the two most common species (Simova et al., 2002; Latorre-García et al., 2007; Diosma et al., 2014). The *Candida* population is made up of a wide range of species with *C. holmii* and *C. kefyr* being the most prevalent (Angulo et al., 1993; Marquina et al., 2002). Outside of these three genera, only *Pichia* has been identified with any regularity and in each case the species was identified as *Pichia fermentans* (Angulo et al., 1993; Wang et al., 2008). As fermentation progresses the proportions of some yeast species change with non-lactose fermenting yeasts, such as *Saccharomyces*, decreasing, whereas lactose utilizing *K. marxianus* and *K. lactis* show a similar distribution between grain and kefir (Simova et al., 2002).

Unlike the bacterial population in kefir grain, the yeast component of the grain fluctuates considerably between grains when analyzed using culture independent techniques. Despite this, a small number of yeasts such as *Kazachstania*, *Kluyveromyces*, and *Naumovozyma* tend to be the dominant genera present in both the grain and fermented milk (Zhou et al., 2009; Marsh et al., 2013; Vardjan et al., 2013; Garofalo et al., 2015; Korsak et al., 2015). Of these main genera, only *Naumovozyma* has not been isolated in culture based studies. *Kazachstania unispora*, the species of *Kazachstania* present is also known as *Saccharomyces unisporus* (Marsh et al., 2013). Sequencing based approaches have also identified over a dozen yeast species that had not previously been associated with kefir, such as *Dekkera anomala*, *Issatchenkia orientalis*, and *Pichia fermentans*, and have even shown that, in some grains, the yeast population is dominated by a mix of these other species (Marsh et al., 2013; Garofalo et al., 2015). **Table 1** contains a complete list of yeast species found in culture dependent and culture independent studies.

Culture Dependent vs. Culture Independent Methods

As expected, sequencing based methods often identify organisms that are not readily isolated by traditional culture based methods. This may be due to the presence of these organisms in extremely low numbers, or some of these organisms may be unable to grow on traditional media due to the complex symbiotic relationships present in kefir. Indeed, this may account for why certain *Lactobacillus* species have only been identified in sequencing based studies (Dobson et al., 2011). For example *L. kefiranofaciens* has not consistently been isolated in culture based methods but is regularly identified as a major part of the *Lactobacillus* population present in kefir when culture independent methods are used which may be due to the more strict anaerobic nature of this species when compared to other *Lactobacillus* species (Wang et al., 2012). While sequencing based methods have proven to be very valuable for identifying difficult to culture organisms, high throughput sequencing of 16S amplicons are limited with respect to their ability to consistently identify organisms at the species level (Marsh et al., 2013). Additionally, with metagenomic analyses there is the possibility that population dynamics may be skewed if there are dead cells present. While large numbers of dead cells from one species may indicate the importance of that species to kefir, the detection of these dead cells can still be problematic at later times during fermentation as they would not be actively involved in the community at these time points. Culture based methods remain essential as they allow organisms to be phenotypically tested. Regardless, the advent of sequence based technologies has increased the knowledge of which organisms are present in kefir grains and fermented milk and will allow for the development of new strategies to facilitate the isolation of organisms previously overlooked.

CHOLESTEROL METABOLISM AND ACE INHIBITION

Due to the highly complex microbiota of kefir, there is a multitude of organisms and metabolic products present in the fermented milk. This combination of live microbial organisms and metabolites contributes to a wide range of effects attributed to kefir many of which are health benefits. Cardiovascular disease (CVD) is one of the leading causes of death in the western world, with high levels of serum cholesterol being a major risk factor for the disease. Diet can play a major role in the management of serum cholesterol levels and thus, ones risk of contracting CVD (WHO, 1982). It has been shown that milk and especially fermented milks are able to reduce serum cholesterol levels in animal trials (Beena and Prasad, 1997; Sibel Akalin et al., 1997). Kefir grains are capable of reducing the cholesterol levels of milk through the fermentation process and have been shown to reduce the levels of cholesterol present by between 41 and 84% after 24 h fermentation and a further 48 h of storage (Vujičić, 1992). While cholesterol reduction varied from one grain to another, these differences did not reflect the country of origin of the grain; Yugoslavian grains had both the highest and lowest levels of cholesterol reduction. Single kefir isolates have also been shown to assimilate cholesterol, with *K. marxianus* being one of the more effective. When *K. marxianus* strains K1 and M3 were inoculated in broth supplemented with cholesterol for 20 h, cholesterol levels decreased 70–99% (Liu et al., 2012). These same strains of *K. marxianus* showed significant levels of bile salt hydrolase (BSH) activity which were proportional to the rate of cholesterol lowering (Liu et al., 2012). BSH deconjugates bile acids and, as deconjugated bile salts is less soluble and less efficiently reabsorbed from the intestinal lumen, this leads to increased bile salt excretion in the faces (Zhuang et al., 2012). BSH deconjugation contributes to cholesterol lowering abilities of kefir as cholesterol is utilized in bile acid synthesis.

Cholesterol lowering properties of kefir have been validated in animal models. In a study using male golden Syrian hamsters fed cholesterol free or cholesterol enriched diet, both milk kefir and soyamilk kefir reduced serum triacylglycerol and total cholesterol while improving the atherogenic index (i.e., ratio of non-HDL-cholesterol to HDL-cholesterol). The cholesterol lowering effect was independent of whether the hamsters were fed the cholesterol free or cholesterol enriched diet (Liu et al., 2006a) indicating that kefir feeding altered endogenous cholesterol metabolism. Concentrations of cholesterol in the liver were also observed to decrease in both milk kefir and soyamilk kefir fed hamsters, and the secretion levels of fecal bile acid and cholesterol significantly increased for both groups. The increase in fecal bile acid is likely a result of the deconjugation of bile acid by microbes present in the kefir, while the higher levels of cholesterol secretion were likely due to the inhibition of cholesterol absorption in the small intestine due to the binding and assimilation of cholesterol by these same microbes (Xiao et al., 2003).

Lactobacillus plantarum MA2 isolated from kefir has also shown hypocholesterolemic activity in male Sprague-Dawley (SD) rats fed a high cholesterol diet. Rats fed a diet supplemented with this organism had significantly lower total serum cholesterol, LDL-cholesterol, triglycerides, liver cholesterol and triglycerides in conjunction with increased fecal cholesterol secretion (Wang et al., 2009). A similar study that used a high cholesterol diet supplemented with *L. plantarum* strains Lp09 and Lp45 in SD rats found that these strains had the same effect (Huang et al., 2013a). Huang et al. (2013b) also found that *L. plantarum* Lp27 was able to decrease serum total cholesterol, LDL-cholesterol, and triglycerides in hypercholesterolemic SD rats that consumed a diet supplemented with Lp27. A proposed mechanism for decreased serum cholesterol is the inhibition of cholesterol absorption. The Niemann-Pick C1-like 1 (NPC1L1) gene, which plays a critical role in the absoption of cholesterol (Altmann et al., 2004), is down-regulated in rats fed Lp27 and in *in vitro* tests with Caco-2 cells (Huang et al., 2013b). Zheng et al. (2013) found that *L. acidophilus* LA15, *L. plantarum* B23, and *L. kefiri* D17 were all able to lower serum total cholesterol, LDL, and triglyceride levels in SD rats fed a high cholesterol diet. The three strains also increased fecal cholesterol and bile acid secretion (Zheng et al., 2013). *K. marxianus* YIT 8292 was also shown to decrease plasma and liver cholesterol levels in addition to increasing fecal sterol and bile acid excretion and the concentration of short chain fatty acids in the cecum (Yoshida et al., 2005), indicating that both bacteria and yeast can contribute to this characteristic. This effect was shown to be specific to α-mannan and β-glucan present in the cell wall of *K. marxianus* (Yoshida et al., 2005). In addition to individual microbes in kefir having an ability to reduce cholesterol, kefiran has also been shown to improve cholesterol and blood pressure levels.

In a study using spontaneously hypertensive and stroke prone (SHRSP/Hos) rats fed a high fat diet, kefiran supplementation reduced serum total cholesterol, serum LDL-cholesterol, serum triglycerides, liver cholesterol, and liver triglycerides (Maeda et al., 2004b), however, the concentrations used for kefiran supplementation were not discussed. Decreases in the blood pressure and angiotensin converting enzyme (ACE) activity were also observed. ACE inhibitory action has been attributed to commercial kefir made from caprine milk when tested *in vitro*, with the mode of action being attributed to two small peptides released from casein during the fermentation process (Quiros et al., 2005).

In contrast to these studies, St-Onge et al. (2002) found that when mildly hypercholesterolemic men consumed kefir as part of their diet for 4 weeks there was no significant change to total serum cholesterol, LDL-cholesterol, HDL-cholesterol, or triglyceride concentrations. They did note an increase in fecal bacterial counts and short chain fatty acid levels, including propionic acid. Additionally, a study examining Wistar rats fed a standard diet supplemented with kefir for 22 days found no significant differences in serum cholesterol when compared to rats on a control diet (Urdaneta et al., 2007). While these two studies seem to conflict with other findings, this may be in large part due to the fact that different kefir grains were used for each of these studies. Additionally, the aforementioned Liu et al. (2006a) study had a timeline of 8 weeks, while St-Onge et al. (2002) and Urdaneta et al. (2007) had timelines of 4 weeks and 22 days, respectively. It may be significant that, in the study of hypercholesterolemic men, an increase in fecal propionic acid was noted. Propionic acid has been shown to inhibit acetate incorporation in to triacylglycerol and plasma cholesterol (Wolever et al., 1995). Thus, a hypocholesterolemic effect may have been observed had the study continued for a longer time period.

EFFECTS ON THE HOST GUT AND GUT MICROBIOME

Pathogen Exclusion

One of the main ways through which probiotic-containing food products can exert beneficial effects is altering the gut microbiota. This can be done either through the introduction of new species or strains in to the gastrointestinal tract, or by promoting the growth of beneficial microbes which are already present. Some examples are presented here. In multiple studies, consumption of kefir or kefiran in an animal model has been associated with an increase in microbes thought of as beneficial, such as *Lactobacillus* and *Bifidobacterium*, while simultaneously decreasing harmful microbial species such as *Clostridium perfringens* (Liu et al., 2006b; Hamet et al., 2016). Kefir consumption was also able to reduce the severity of *Giardia intestinalis* infection in C57BL/6 mice, with the reported mechanism being through modulation of the immune system (Correa Franco et al., 2013). Furthermore, specific strains of *Lactobacillus* isolated from kefir have been shown to adhere to Caco-2 cells and inhibit the adherence of *Salmonella typhimurium* and *Escherichia coli* O157:H7 (Santos et al., 2003; Hugo et al., 2008; Huang et al., 2013a). The ability of these *Lactobacillus* species to bind to Caco-2 cells illustrates a likely mechanism for the increase in *Lactobacillus* species observed in the fecal microbiota of rats fed kefir (Liu et al., 2006b; Carasi et al., 2015). In an *in vivo* study where BALB/c mice were intragastrically challenged with *E. coli* O157:H7, mice receiving *L. kefiranofaciens* M1 prior to *E. coli* challenge showed reduced symptoms of infection, including intestinal and renal damage, bacterial translocation, and Shiga toxin penetration as well as increased EHEC-specific mucosal IgA responses (Chen et al., 2013)

Other *in vitro* work has also shown that lactobacilli isolated from kefir have the ability to protect Vero cells from type II Shiga toxin produced by *E. coli* O157:H7, leading to lower levels of cell death (Kakisu et al., 2013). Similar effects were apparent in another study where they observed that kefir fermented milk inhibited the ability of *Bacillus cereus* extracellular factors to cause damage to Caco-2 cells (Kakisu et al., 2007).

As well as regulating microbial composition, kefir can alter the activity of the microbiota. Certain *Bifidobacterium* strains have been shown to exhibit increases in growth rate when cultured in kefir and changes in gene expression have also been observed (Serafini et al., 2014). These changes in gene expression resulted in increased expression levels of multiple genes associated with *pil3*, a sortase dependent pilus that has been shown to be extremely important for interaction with the host endothelial cells and is especially important for adherence and modulation of the host inflammatory response (Turroni et al., 2013; Serafini et al., 2014). While this specific example shows the potential positive effects kefir can have on existing organisms within the gut microbiota, it is still unclear as to how this translates to the complex population of the whole microbiome.

Antibacterial and Antifungal Properties

Kefir, and kefir associated strains, has shown a multitude of antibacterial and antifungal activities (**Table 2**). Kefir fermented milk has been tested in disk diffusion experiments against a wide range of pathogenic bacterial and fungal species and found to have antimicrobial activity equal to ampicillin, azithromycin, ceftriaxone, amoxicillin, and ketoconazole against many of these species (Cevikbas et al., 1994; Yüksekdağ et al., 2004; Rodrigues et al., 2005; Huseini et al., 2012).

In addition to the antimicrobial effects of kefir fermented milk as a whole, there are also specific microbes which exert antimicrobial properties on their own. For instance, *L. plantarum* ST8KF produces the bacteriocin ST8KF which exhibits antimicrobial action against *Enterococcus mundtii* and *Listeria innocua* (Powell et al., 2007). Other kefir-derived *Lactobacillus* species such as *L. acidophilus* and *L. kefiranofaciens*, as well as some *S. thermophilus* strains have shown antimicrobial activity against a whole range of pathogenic organisms including *E. coli, L. monocytogenes, S. aureus, S. typhimurium, S. enteritidis, S. flexneri*, *P. aeruginosa*, and *Y. enterocolitica* when tested using an agar spot test (Santos et al., 2003;

TABLE 2 | List of pathogenic organisms that kefir or kefir-associated organisms have demonstrated antimicrobial effects against.

Microbial species	Reference
Bacteria	
Staphylococcus aureus	Cevikbas et al., 1994; Ryan et al., 1996; Yüksekdağ et al., 2004; Rodrigues et al., 2005; Miao et al., 2014; Leite et al., 2015; Zanirati et al., 2015
Pseudomonas aeruginosa	Cevikbas et al., 1994; Ryan et al., 1996; Yüksekdağ et al., 2004; Rodrigues et al., 2005; Huseini et al., 2012; Zanirati et al., 2015
Salmonella typhimurium	Santos et al., 2003; Rodrigues et al., 2005; Golowczyc et al., 2008; Zanirati et al., 2015
Escherichia coli	Ryan et al., 1996; Santos et al., 2003; Yüksekdağ et al., 2004; Rodrigues et al., 2005; Golowczyc et al., 2008; Leite et al., 2015; Zanirati et al., 2015
Salmonella enteritidis	Santos et al., 2003; Miao et al., 2014
Listeria monocytogenes	Ryan et al., 1996; Santos et al., 2003; Rodrigues et al., 2005; Likotrafiti et al., 2015; Leite et al., 2015; Zanirati et al., 2015
Bacillus subtilis	Cevikbas et al., 1994; Ryan et al., 1996
Salmonella enterica	Golowczyc et al., 2008; Miao et al., 2014; Leite et al., 2015
Enterococcus faecalis	Ryan et al., 1996; Zanirati et al., 2015
Shigella flexneri	Santos et al., 2003
Clostridium difficile	Rea et al., 2007
Klebsiella pneumonia, *Proteus vulgaris*	Cevikbas et al., 1994
Streptococcus pyogenes, *Staphylococcus salivarius*	Rodrigues et al., 2005
Bacillus cereus, *Clostridium sporogenes*, *C. tyrobutyricum*, *Enterococcus faecium*, *Listeria innocua*, *Salmonella typhi*	Ryan et al., 1996
Salmonella gallinarum, *Shigella sonnei*	Golowczyc et al., 2008
Bacillus thuringiensis, *Shigella dysenteriae*	Miao et al., 2014
Fungus	
Candida albicans	Rodrigues et al., 2005
Yersinia entocolitica	Santos et al., 2003
Aspergillus flavus, *A. niger*, *Rhizopus nigricans*, *Penicillium glaucum*	Miao et al., 2014
Staphylococcus epidermidis, *Candida stellatoidea*, *C. tropicalis*, *C. krusei*, *Saccharomyces cerevisiae*, *Rhodotorula glutinis*, *Torulopsis glabrata*	Cevikbas et al., 1994

Yüksekdağ et al., 2004; Golowczyc et al., 2008). Other kefir lactobacilli have also shown antimicrobial activity in *in vitro* tests against *S. typhimurium*, and *E. coli* that have already adhered to Caco-2 cells (Golowczyc et al., 2008). Lacticin 3147 is produced by a strain of *L. lactis* isolated from kefir and has an extremely broad range of antimicrobial activity, affecting *B. cereus*, *B. subtilis*, *C. sporogenes*, *C. tyrobutyricum*, *Enterococcus faecium*, *E. faecalis*, *L. innocua*, *L. monocytogenes*, *S. aureus*, and *C. difficile* (Ryan et al., 1996; Rea et al., 2007). Another bacteriocin of kefir origin is F1, which is produced by the *Lactobacillus paracasei* subsp. *tolerans* strain FX-6 source from a Tibetan kefir grain. F1 has been shown to inhibit a wide range of bacterial and fungal species including *S. aureus*, *Shigella dysenteriae*, and *Aspergillus niger* (Miao et al., 2014). *L. kefiri* B6 isolated from kefir was also capable of inhibiting and inactivating *L. monocytogenes* when in the presence of galactooligosaccharide *in vitro*, however, this effect was not observed with *E. coli* and, in this case, further investigation of the mechanism of this inactivation is needed (Likotrafiti et al., 2015). Similarly, Leite et al. (2015) isolated multiple strains of *L. lactis* and *Lb. paracasei* from kefir capable of producing bacteriocin-like substances that were inhibitory to *E. coli*, *S. enterica*, *S. aureus*, and *L. monocytogenes*, however, more work is needed in order to better characterize these substances and determine the range of their antimicrobial activity as well as their novelty. In a study examining LAB isolated from Brazilian kefir grains, *L. kefiranofaciens* 8U showed the ability to inhibit multiple pathogens including *P. aeruginosa*, *L. monocytogenes*, and *E. faecalis in vitro*, but again more work is needed in order to determine the mechanism behind this inhibition (Zanirati et al., 2015).

ANTITUMOR EFFECTS

Kefir also has significant antitumor activity against multiple cancer cell types. *L. kefiri* was shown to increase apoptosis of multiple drug resistant human myeloid leukemia cells *in vitro* through the activation of caspase 3 in a dose dependent manner (Ghoneum and Gimzewski, 2014). The cell free fraction of kefir has shown antitumor activity *in vitro* when it was observed to have a dose dependent anti-proliferative effect on the gastric cancer cell line SGC7901 (Gao et al., 2013). This study further demonstrated that cell free kefir was able to induce apoptosis in SGC7901 cells through up regulation of the *bax* gene, and apoptosis promoter and anti-oncogene, and down regulation of the *bcl-2* gene, which is an apoptosis inhibitor and known oncogene (Sorenson, 2004). In addition to the promotion of cell death in cancerous cells, antimutagenic effects have been demonstrated in studies with known carcinogens such as methylmethanosulphate, methy-lazoxymethanol, sodium azide, aflatoxin B1, and 2-aminoanthracene as indicated by the Ames test (Guzel-Seydim et al., 2006).

In mouse models of fusiform cell sarcomas, mice receiving intraperitoneal kefir had reduced tumor size, with some tumors completely disappearing over a 20 days treatment period (Cevikbas et al., 1994). While this is impressive, it has yet to be determined if these findings can be replicated in the case of oral consumption. A separate study utilizing a murine breast cancer model showed that kefir feeding prior to challenge with the tumor resulted in decreased size and increased apoptosis of the tumor, and that the levels of IgA+ cells and CD4+ T cells were also increased (de Moreno de LeBlanc et al., 2007). Mice with breast cancer tumors fed kefir also showed increased serum levels of Il-10 and IL-4 (de Moreno de LeBlanc et al., 2006). These studies both showed increases in immune cell populations and recruitment, pointing to a possible mechanism for the reduction of tumor size. These findings are consistent with other studies that have shown that kefir is able to modulate the immune system in the gut and show that the immunomodulatory abilities of kefir may not be limited to the gastrointestinal tract (Thoreux and Schmucker, 2001; Vinderola et al., 2005; Correa Franco et al., 2013).

WOUND HEALING

The antimicrobial properties of kefir may lead to its use for non-traditional applications. Indeed, when rats bearing open wounds inoculated with *S. aureus* were treated with a gel made from kefir grains, it was found that the wounds healed at a much faster rate than was observed in control rats that received no treatment or rats that received a traditional treatment of 5 mg/kg neomycin-clostebol emulsion (Rodrigues et al., 2005). Gels made from kefir and kefir grains were found to be more effective at reducing wound size in *P. aeruginosa* contaminated third degree burns than a traditional silver sulfadiazine treatment in a rat model of burn wounds (Huseini et al., 2012; Rahimzadeh et al., 2014). Furthermore, a study using a rabbit model for contaminated open wound also found that gel made from kefir grain resulted in quicker healing times and quicker clearing of infection (Atalan et al., 2003).

These decreased healing times are likely due to multiple factors. One such factor is the ability of kefir to inhibit the growth of bacterial and fungal cells, thus leading to a cleaner wound, as shown to be the case in some studies (Atalan et al., 2003; Huseini et al., 2012). Another possible factor is the ability to modulate the immune system and recruit immune cells to help with the healing process.

IMMUNOMODULATORY EFFECTS

One of the major ways probiotic products such as kefir are able to produce health benefits is through the modulation of the gastrointestinal immune system. When young rats inoculated intra-duodenally with cholera toxin (CT) were fed kefir, the levels of anti-CT IgA in the serum increased as did the secretion levels of anti-CT IgA in the Peyer's Patches, the mesenteric lymph nodes, the spleen, and the intestinal lamina propria compared to CT alone (Thoreux and Schmucker, 2001). This same effect, however, was not observed in older mice that underwent the same treatment, suggesting that whatever mechanism is responsible for the observed change in the young rats is either no longer present in the senescent mice or requires a much larger dosage of kefir in order to activate it. Additional studies in to the mechanism as well as investigations with middle aged mice are needed to provide further insight in to this phenomenon. In an infection of C57BL/6 mice with *G. intestinalis*, kefir consumption reduced intensity of infection by mitigating the ability of *G. intestinalis* to suppress the mounting of an inflammatory response. This impact was mediated through increases in the levels of TNF-α and IFN-γ expression and through higher levels of IgA positive and RcFcε positive cells (Correa Franco et al., 2013). There have also been studies showing increases in IgA and IgG+ cells in the small intestine of rats that were fed both regular and pasteurized kefir, as well as increases in the levels of IL-4, IL-10, IL-6, and IL-2 positive cells in the lamina propria of these same rats. Increases were also seen in anti-inflammatory cytokines such as IL-10, IL-4, and IL-6, all of which promote a Th2 response (Vinderola et al., 2005). Interestingly, increases in IFN-γ, TNFα, and IL-12 (all of which are pro-inflammatory and promote a Th1 response) were observed only in rats fed pasteurized kefir. The increase in pro-inflammatory cytokines in the pasteurized kefir groups was likely due to the reduced cell wall integrity of heat killed cells exposing more inflammatory microbial products. The fact that pasteurized kefir was able to elicit an effect shows that the mechanisms behind this immune modulation are not entirely dependent on live cells, and may be due to metabolites present in the kefir (Iraporda et al., 2014). However, it should be noted that in this study live cells had a generally more substantial impact as live kefir was able to confer a similar effect at 1/10 the concentration and without eliciting a pro-inflammatory immune response (Vinderola et al., 2005).

When fed to mice over 2–7 days, solid fractions of kefir that contained live bacteria have been shown to increase the levels of IFN-γ, TNF-α, and IL-6 in peritoneal macrophages as well as to increase the levels of IL-1α, IL-10, and IL-6 in adherent cells isolated from the Peyer's patch of mice (Vinderola et al., 2006b). IFN-γ and TNF-α increased early in feeding, however, they quickly decreased back to control levels by day 7 along with IL-1α while IL-6 and IL-10 levels remained high through the 7 days feeding period (Vinderola et al., 2006b). *In vitro* experiments with lactobacilli isolated from kefir have shown that they induce higher secretion levels of IL-1β, IL-6, TNF-α, IL-10, IL-8, and IL-12 in peripheral blood mononuclear cells and are able to decrease the ccl20 response in Caco-2 cells to TLR agonists such as bacterial flagella, with largely different effects being observed for different strains of lactobacilli tested (Carasi et al., 2015). In general, strains of *L. kefiri* that induced lower TNF-α/IL-10 and higher IL-10/IL-12 ratios showed a much greater decrease in the pro-inflammatory response of ccl20 to stimulation with bacterial flagella, indicating the importance of IL-10 in promoting a Th2 response while simultaneously inhibiting the pro-inflammatory Th1 response. Mice that were fed *L. kefiri* for a period of 21 days showed altered gene expression profiles in the ileum, colon, Peyer's Patches, and mesenteric lymph nodes, with proinflammatory cytokines such as IFN-γ and IL-23 being down regulated and IL-10 being up regulated (Carasi et al., 2015). This further indicates that lactobacilli isolated from kefir have the ability to supress the production of pro-inflammatory cytokines while promoting anti-inflammatory cytokine production. *L. kefiranofaciens* co-incubation with mouse macrophage cells decreased the levels of pro-inflammatory cytokines IL-1β, and IL-12 while simultaneously increasing the level of the anti-inflammatory cytokine IL-10, which acts to specifically inhibit the production of IL-12 and IL-1β (Hong et al., 2009). Additionally, *L. kefiranofaciens* was able to ameliorate colitis in a DSS induced mouse model and enhance Th1 responses to TLR agonists in germ free mice by increasing the production of IFN-γ and IL-12 upon stimulation (Chen and Chen, 2013). Further investigation into the mechanisms of protection against colitis showed that *L. kefiranofaciens* M1 decreased the production of pro-inflammatory cytokines IL-1β and TNF-α, while increasing the production of IL-10 *in vivo* (Chen et al., 2012). This effect was also TLR-2 dependent as *L. kefiranofaciens* M1 was unable to improve DSS colitis in TLR-2 knockout mice (Chen et al., 2012).

The cell free fraction of kefir is also capable of modulating the immune system, and has been shown to modulate innate immune responses *in vitro* by lowering the activation of Caco-2-ccl20:luc cells that had been stimulated by *Salmonella* flagellar protein FliC, IL-1β, or TNF-α (Iraporda et al., 2014). One of the likely mechanisms was revealed when it was found that a 100 mM lactic acid solution at pH 7 was able to elicit a comparable level of immune modulation in FliC stimulated cells when preincubated with the solution (Iraporda et al., 2014). The lactic acid solution was also found to lower the level of NFκ-B activation in Caco-2 cells stimulated with FliC and was even able to down regulate the expression of pro-inflammatory cytokines ccl20, IL-8, CXCL 2, and CXCL 10 without affecting genes involved in the normal function of enterocytes (Iraporda et al., 2014). These results indicate just how important the metabolites produced during fermentation are to the ability of kefir to elicit beneficial responses or effects in the host.

In general studies using whole kefir, kefir fractions, or organisms isolated from kefir found that whether tested *in vitro* or *in vivo*, the result was a shift from a Th1 immune response to a Th2 response as well as increases in the levels of IgA present (Thoreux and Schmucker, 2001; Vinderola et al., 2005, 2006b; Hong et al., 2009; Carasi et al., 2015). The only study which seems to show a consistently increased Th1 response was conducted with germ free mice, while all other studies used conventional mice or rats (Chen and Chen, 2013). This may account for the difference in findings as it is quite possible that the observations from the germ free mice had more to do with the introduction of a bacterial population to the gut than it did with the specific bacterial species that comprised that population. The fact that most studies also observed increases in some pro-inflammatory cytokines such as TNF-α, IFN-γ, or IL-12 may be explained by an initial reaction of the immune system to common TLR agonists present, which was ultimately supressed following further interaction with the immune cells of the GI tract.

ANTI-ALLERGENIC EFFECTS

Allergic diseases have been on the rise in the developed world for decades, leading to higher incidences of conditions such as asthma and food allergy (Yazdanbakhsh et al., 2002). Many allergies, especially those related to food, are developed early in life, with the majority of food allergies developing within the first 2 years of life (Wood, 2003). Although most food allergies developed early in life do not persist, some can become lifelong conditions (Wood, 2003). Recent work has shown that an increasingly important factor in determining if a child develops allergic disease, be it food allergy or asthma, is the level of complexity and the specific organisms present in the gut microbiota (Kirjavainen et al., 2002; Sjogren et al., 2009; Azad et al., 2013; West, 2014). Higher levels of *Bifidobacterium* and group 1 lactobacilli (obligate heterofermentative lactobacilli such as *L. acidophilus, L. delbrueckii, and L. helveticus*) in the gut of infants have been associated with a lower incidence of allergic disease later in life (Sjogren et al., 2009), and both kefir and kefiran have been observed to exert these effects on the gut microbiota in animal trials (Liu et al., 2006b; Hamet et al., 2016). Supplementation with *Bifidobacterium* has been shown to influence the intestinal microbiota of weaning infants by reducing levels of *Bacteroides* and has been associated with lower incidence of food allergy (Kirjavainen et al., 2002). Studies with antibiotics in the early life period have also highlighted the importance of appropriate microbial stimulation of the immune system for protection against asthma development (Russell et al., 2012).

One of the main mechanisms behind food allergy is an imbalance in the Th1/Th2 cell ratio, leading to a heightened IgE response (Tanabe, 2008). Studies of *in vitro* reactions of human monocytes with a probiotic made up of multiple LAB showed that exposure to these LAB resulted in a much higher IFN-γ/IL-4 ratio, similar to what would be seen during a Th1 response (Tsai et al., 2012). In addition to the *in vitro* studies carried out, Tsai et al. (2012) found that both total IgE and OVA-specific IgE were significantly lower in mice that had been sensitized to OVA (ovalbumin) and then fed a LAB mixture than in control mice which had also been sensitized to OVA but did not receive any LAB mixture. Studies such as this indicate that kefir may help relieve some allergy symptoms.

In a study utilizing an ovalbumin sensitization mouse asthma model, it was found that mice receiving intra-gastric kefir showed lower levels of airway hyper-responsiveness (AHR) than control mice, and, impressively, had lower levels of AHR than the positive control group receiving an anti-asthma drug (Lee et al., 2007). This same study found that mice receiving kefir exhibited significantly lower levels of eosinophil infiltration in the lung tissue as well as in the brochoalveolar lavage fluid (BALF). These mice also showed lower levels of IgE, IL-4, and IL-13 in the BALF, all of which are associated with the Th2 response which is responsible for allergic reaction (Lee et al., 2007). It has also been found that oral feeding of kefir in OVA sensitized mice resulted in significantly lower levels of anti-OVA serum IgE and IgG1 antibodies than those found in mice given water or unfermented milk (Liu et al., 2006b). Studies examining the *in vitro* effect of heat-killed lactobacilli isolated from kefir on mouse peritoneal macrophages showed that even after being heat-inactivated, the lactobacilli were able to induce the expression of Th1 cytokines such as IFN-γ, TNF-α, IL-12, and IL-1β (Hong et al., 2010). These same heat-inactivated lactobacilli also reduced the levels of anti-OVA IgE in the serum when fed orally to OVA sensitized mice, while increasing the expression of IL-12 and decreasing the expression of IL-5 in splenocytes. An increase in the levels of regulatory T-cells was also detected in these mice (Hong et al., 2010). In a study of OVA sensitized mice fed with heat-inactivated strain M1 of *L. kefiranofaciens*, the inactivated M1 was able to decrease levels of pro-inflammatory and Th2 cytokines such as IL-4, IL-6, IL13, and ccl20 in both the splenocytes and BALF of the mice while decreasing OVA-specific IgE and the Th17 associated cytokine IL-17, both of which are strongly associated with an asthmatic response. The M1 treatment was also able to increase the levels of regulatory T cells present (Hong et al., 2011).

While all of these studies reveal a consistent pattern, it is interesting to note that many of the cytokine profiles are in stark contrast to those found in studies without antigen sensitization or challenge. This highlights both the complexity of the immune system and the need for a balance between the different possible reactions such as the Th1 and Th2 responses. The fact that kefir can induce shifts in the immune system in both directions is promising as it may mean that the organisms in kefir are capable of regulating this balance in the immune system. This may be in part due to the increased number of regulatory T-cells observed in some of these studies, as regulatory T-cells play an important role in maintaining tolerance and supressing unnecessary inflammatory immune responses (Sakaguchi, 2011).

HEALTH BENEFITS OF YEAST IN KEFIR

As noted above, one unique characteristic of traditionally produced kefir relative to many other commercially produced fermented dairy products is the presence of a large population of yeast in both the kefir grain and in the fermented milk (Marsh et al., 2013). Although the majority of commercialized probiotic microbes are bacteria such as lactobacilli and bifidobacteria, there are some yeast species and strains that have been recognized to have probiotic properties, such as *Saccharomyces boulardii* (Corthier et al., 1986; Czerucka et al., 2007). *S. boulardii* has been shown to improve the symptoms of *Clostridium difficile* associated diarrhea as well as reduce inflammation and alter the immune state and reactions in the gut, leading to its adoption as a treatment for *C. difficile* diarrhea (Buts et al., 1994; Castagliuolo et al., 1999; Kotowska et al., 2005; Villarruel et al., 2007).

Some yeasts from kefir have also shown immunomodulatory activities. For example *K. marxianus* B0399 has been shown to have the ability to adhere to Caco-2 cells (Maccaferri et al., 2012). When co-incubated with lipopolysaccharide (LPS) stimulated Caco-2 cells, a significant decrease in the secretion of IL-10, IL-12, IL-8, and IFN- γ was observed (Maccaferri et al., 2012). Additionally, *K. marxianus* B0399 elicited a decrease in the secretion of pro-inflammatory cytokines TNF-α, IL-6, and MIP-1α when co-incubated with PBMCs that had been stimulated with LPS (Maccaferri et al., 2012). This same study showed that in an *in vitro* colonic model system, *K. marxianus* was able to stably form a population in the model while simultaneously enhancing the levels of *Bifidobacterium*. Increases in the levels of the short chain fatty acids acetate and propionate were also observed. Utilizing a Caco-2 cell line with a ccl20 reporter gene, Romanin et al. (2010) were able to show that multiple yeast strains of *S. cerevisiae* (CIDCA 81109, 81106, 8112, 9127, 9123, 9136, 9133, 9124, 81103, 9132, 81108, 81102, 8175, and 8111), *K. marxianus* (CIDCA 81111, 8116, 8118, 81105, 8153, 8154, 8113, 81104, and 9121), and *Issatchenkia* spp. (CIDCA 9131) were able to inhibit the expression of the ccl20 reporter when incubated with the cells prior to stimulation with *Salmonella* flagellar protein FliC. From these yeasts, *K. marxianus* CIDCA 8154 was selected for further testing and showed the ability to inhibit the levels of ccl20 expression in Caco-2 cells regardless of whether the stimulation came from FliC, IL-1β, or TNF-α. The strain also inhibited the expression of IL-8 and MIP-2α in HT-29 cells and inhibited ccl20 expression in a mouse ligated intestinal loop model when administered prior to stimulation with FliC (Romanin et al., 2010). Yeasts isolated from kefir have also shown the ability to improve the probiotic properties of bacterial species by improving the viability of these bacterial strains over time in simulated gastric and intestinal juice, and through improving the adhesion of LAB to Caco-2 cells in an *in vitro* model. This effect is likely due to the co-aggregation of the two microbial species (Xie et al., 2012).

KEFIRAN AND THE CELL FREE FRACTION OF KEFIR

In addition to the microbial populations present in kefir and other fermented probiotics, there are also fermentation products and other by-products of the metabolism of these microbes that possess bioactivity. Some of these by-products may have a profound effect on the host without the presence of the microbial population. Such a by-product is kefiran, the exopolysaccharide produced by *L. kefiranofaciens* during fermentation (Maeda et al., 2004b; Vinderola et al., 2006a). Mice fed kefiran dissolved in drinking water showed increases in the levels of IgA+ B cells, as well as increases in IL-6, IL-10, and IL-12 in the lamina propria of the small intestine after 7 days of feeding (Vinderola et al., 2006a). In a murine model of asthma using OVA sensitization, kefiran introduced intra-gastrically 1 h prior to challenge reduced levels of the Th2 cytokines IL-4 and IL-5 and lowered AHR when compared to OVA challenged mice that did not receive kefiran (Kwon et al., 2008). After the same period the study showed increases in serum levels of IL-4, IL-6, IL-10, and IFN-γ (Kwon et al., 2008). Addition of kefiran to a co-incubation of *B. cereus* culture supernatant and Caco-2 cell monolayer resulted in reduced cell detachment and greater mitochondrial activity, as well as negated the haemolytic effect of the *B. cereus* culture supernatant on human red blood cells (Medrano et al., 2008). Genetically diabetic (KKAy) mice fed kefiran were found to have decreasing levels of blood glucose throughout a 30 days examination while a control group was found to have constantly increasing and generally higher levels of blood glucose throughout the same timeline (Maeda et al., 2004a). Using SD rats as a model for constipation, it was also found that kefiran significantly improved the symptoms of constipation over the control group (Maeda et al., 2004a).

A water-soluble polysaccharide isolated from kefir grain (KGF-C) was shown to improve humoral immune response in mice against Sheep Red Blood Cells (SRBC). The levels of anti-SRBC cells isolated from the spleen of mice immunized with SRBC while being intubated with KGF-C was significantly higher than in control mice 4 days post immunization (Murofushi et al., 1986). However, this effect was not seen in nu/nu mice (no thymus or T cell population) immunized with SRBC, or in conventional mice immunized with thymus-independent antigens, indicating that the mechanism of action is likely through the T cell population (Murofushi et al., 1986). Sphingomyelin isolated from kefir has been shown to increase IFN-β secretion in human MG-63 cells when compared to commercial sphingomyelin and sphingosine (Osada et al., 1993).

Kefir cell-free supernatant (KCFS) has been shown to increase the levels of IFN-β, IL-6, IL-12, and TNF-α secreted by RAW 264.7 cells through a TLR2 dependent mechanism (Hong et al., 2009). Cell-free fractions of kefir have also been shown to increase the levels of these cytokines in peritoneal macrophages and adherent cells from the Peyer's patches of mice (Vinderola et al., 2006b). In addition, KCFSs were found to have a significant impact on tumor size, apoptosis, and immune recruitment in a murine breast cancer model, resulting in increased apoptosis of tumor cells and increases in the CD4+ T cell population (de Moreno de LeBlanc et al., 2007). In *in vitro* studies utilizing human T-lymphotropic virus 1 (HTLV-1) positive HuT-102 Malignant T lymphocytes as a model for T cell leukemia, the KCFS was found to inhibit proliferation by up to 98% while simultaneously decreasing the transcriptional levels of TGF-α. These effects have also been observed in HTLV-1 negative malignant T cells with the same decrease in TGF-α transcription being observed (Rizk et al., 2009; Maalouf et al., 2011). In addition to anti-proliferative effects, KCFS was found to induce apoptosis in both HTLV-1 positive and negative malignant T cells through the up regulation of *bax* and down regulation of *bcl-2* in a dose dependent manner (Rizk et al., 2013).

CONCLUSION

The purpose of this review has been to collate and summarize that which is known about the microbial composition of kefir and how this composition plays a role in the health benefits associated with kefir consumption. Kefir is a dynamic fermented dairy product with many different factors affecting the benefits associated with its consumption. These factors include the variable yeast and bacterial species present, as well as metabolites such as kefiran and other exopolysaccharides. While kefir has been associated with health benefits for 100s of years, the exact form of these benefits has, until recently, not been studied. The use of animal models and other *in vitro* analyses has allowed for the elucidation of how kefir positively impacts host health. Whole kefir, as well as specific fractions and individual organisms isolated from kefir, provide a multitude of positive effects when consumed. These range from improved cholesterol metabolism and wound healing, to the modulation of the immune system and microbiome, and even the potential alleviation of allergies and cancers. Further studies into the mechanisms behind these effects will allow scientists to better understand exactly how kefir and other fermented dairy products confer these benefits as well as how to harness these traits outside of kefir itself.

The wide range of potential health promoting effects of kefir could lead to a further expansion on the popularity of both traditional fermented kefir and products that are manufactured with kefir fractions or organisms. In order to fully exploit the beneficial characteristics of kefir, a more in-depth understanding of the composition of kefir is critical. With advances in metagenomic analysis through the development of high-throughput sequencing technology, this is a very realistic prospect. Armed with this knowledge, it should be possible to more readily isolate and examine the phenotypic characteristics of individual organisms present in a kefir blend while also providing a greater insight into the evolution of these organisms and how they became specialized to the kefir ecosystem. The additional knowledge gained can also provide crucial information relating to the mechanisms and exact agents responsible for beneficial effects that have been attributed to kefir (Atalan et al., 2003; Rodrigues et al., 2005; Huseini et al., 2012; Rahimzadeh et al., 2014).

The need for further research does not only apply to the mechanisms by which kefir consumption exerts these effects but also which organisms or parts of kefir are responsible for each benefit. By determining which organisms and metabolites are essential for each process, the possibility arises for the commercial manufacturing of kefir that is specifically designed to create the most profound effect in those that consume it. As it stands currently, the highly variable nature of the organisms and metabolites present in traditional kefir requires health claims to be verified individually in each grain and kefir beverage. The ability to combine the best possible strains of the best organisms from multiple sources of kefir would create the potential for greater benefits than have been previously observed, with a measure of control over these effects that has not been possible in traditional kefir.

AUTHOR CONTRIBUTIONS

BB wrote the review and compiled, figures, tables, and references. PC supervised, edited, and approved the review. BW supervised, edited, and approved the review.

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REVIEW
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Functional Properties of Microorganisms in Fermented Foods

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Fermented foods have unique functional properties imparting some health benefits to consumers due to presence of functional microorganisms, which possess probiotics properties, antimicrobial, antioxidant, peptide production, etc. Health benefits of some global fermented foods are synthesis of nutrients, prevention of cardiovascular disease, prevention of cancer, gastrointestinal disorders, allergic reactions, diabetes, among others. The present paper is aimed to review the information on some functional properties of the microorganisms associated with fermented foods and beverages, and their health-promoting benefits to consumers.

Keywords: fermented foods, microorganisms, functional properties, health benefits, bioactive compounds

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INTRODUCTION

Existing scientific data show many fermented foods have both nutritive and non-nutritive components in foods, which have the potential to modulate specific target functions in the body relevant to well-being and health of the consumers. However, 90% of naturally fermented foods and alcoholic beverages in different countries and regions of the world are still at home production under traditional conditions. Naturally fermented foods and beverages contain both functional and non-functional microorganisms (Tamang et al., 2016). Functional microorganisms transform the chemical constituents of raw materials of plant/animal sources during food fermentation thereby enhancing the bio-availability of nutrients, enriching sensory quality of the food, imparting bio-preservative effects and improvement of food safety, degrading toxic components and anti-nutritive factors, producing antioxidant and antimicrobial compounds, stimulating the probiotic functions, and fortifying with some health-promoting bioactive compounds (Tamang et al., 2009, 2016; Farhad et al., 2010; Bourdichon et al., 2012; Thapa and Tamang, 2015). Among bacteria associated with fermented foods and alcoholic beverages, lactic acid bacteria (LAB) mostly species of *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Weissella*, etc. are widely present in many fermented foods and beverages (Axelsson et al., 2012; Holzapfel and Wood, 2014). Species of *Bacillus* are also present in legume-based fermented foods (Kubo et al., 2011; Tamang, 2015). Species of *Bifidobacterium, Brachybacterium, Brevibacterium,* and *Propionibacterium* are isolated from cheese, and species of *Arthrobacter* and *Hafnia* from fermented meat products (Bourdichon et al., 2012). Several genera with hundred of species of yeasts have been isolated from fermented foods, alcoholic beverages and non-food mixed amylolytic starters which mostly include *Candida, Debaryomyces, Geotrichum, Hansenula, Kluyveromyces, Pichia, Rhodotorula, Saccharomyces, Saccharomycopsis, Schizosaccharomyces, Torulopsis, Wickerhamomyces,* and *Zygosaccharomyces* (Tamang and Fleet, 2009; Lv et al., 2013). Species of *Actinomucor, Amylomyces, Aspergillus, Monascus, Mucor, Neurospora, Penicillium, Rhizopus,* and *Ustilago* are reported for many fermented foods, Asian non-food amylolytic starters, and alcoholic beverages (Chen et al., 2014).

Functional properties of microorganisms in fermented foods include probiotics properties (Hill et al., 2014), antimicrobial properties (Meira et al., 2012), antioxidant (Perna et al., 2013), peptide production (De Mejia and Dia, 2010), fibrinolytic activity (Kotb, 2012), poly-glutamic acid (Chettri and Tamang, 2014), degradation of antinutritive compounds (Babalola, 2014), etc. which may be important criteria for selection of starter culture(s) to be used in the manufacture of functional foods (Badis et al., 2004). Some genera and species of microorganisms are used as commercial starters in food fermentation (**Table 1**), and some of products are commercialized and marketed globally as functional foods, health foods, therapeutic foods and nutraceuticals foods (Bernardeau et al., 2006; Bourdichon et al., 2012; Thapa and Tamang, 2015). The present paper is aimed to review the information on some functional properties of the microorganisms associated with fermented foods and beverages, and their health-promoting benefits to consumers.

Probiotic Microorganisms

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). Probiotic organisms used in foods must have the ability to resist gastric juices, exposure to bile, and be able to proliferate and colonize the digestive tract (Saad et al., 2013). The beneficial effects of probiotic foods on human health and nutrition are constantly increasing (de LeBlanc et al., 2007; Monteagudo-Mera et al., 2012), and probiotics are popularly using bio-ingredients in many functional fermented foods (Chávarri et al., 2010). The most commonly used probiotic bacteria belong to the heterogeneous group of LAB (*Lactobacillus, Enterococcus*) and to the genus *Bifidobacterium*, however, yeasts and other microbes have also been developed as potential probiotics during recent years (Ouwehand et al., 2002). Some popular commercial probiotic cultures which are available in global markets include *Bacillus coagulans* BC30 marketed by Ganeden Biotech, Inc., Cleveland, OH, USA; *Lactobacillus acidophilus* NCFM, *Lactobacillus rhamnosus* HN001 (DR20) and *Bifidobacterium lactis* HN019 (DR10) marketed by Danisco (Madison, WI, USA), *L. casei* strain Shirota and *B. breve* strain Yakult marketed by Yakult (Tokyo, Japan), *L. fermentum* VRI003 (PCC) marketed by Probiomics (Eveleigh, NSW, Australia), *L. rhamnosus* R0011 marketed by Institut Rosell (Montreal, QC, Canada), *Streptococcus oralis* KJ3 marketed by Oragenics, Inc. (Alachua, FL, USA), and *Saccharomyces cerevisiae* (boulardii) marketed by Biocodex (Creswell, OR, USA; US Probiotics Home, 2011).

Products containing probiotic bacteria generally include foods and supplements (Varankovich et al., 2015). Fermented milk products are the most traditional source of probiotic strains of lactobacilli (Bernardeau et al., 2006; Shah, 2015); however, commercial probiotic lactobacilli have also been added to meat products, snacks, fruit juice, etc. (Ranadheera et al., 2010). Probiotic properties of *Lactobacillus plantarum* isolated from *kimchi*, Korean fermented vegetable product, has been reported (Ji et al., 2013), and is also found to prevent the growth of *Helicobacter pylori* (Lim and Im, 2009). Probiotic strain *L. acidophilus* La-5 produces conjugated linoleic acid (CLA), an anti-carcinogenic agent (Macouzet et al., 2009). *Pediococcus pentosaceus* CIAL-86 isolated from wine shows anti-adhesion activity against *Escherichia coli* CIAL-153, indicating its probiotic potential in wine (García-Ruiz et al., 2014).

Antimicrobial Properties

Many species of LAB isolated from fermented vegetable and milk products have antimicrobial activities due to production of antimicrobial compounds such as bacteriocin and nisin (Tamang et al., 2009; Khan et al., 2010; Gaggia et al., 2011; Jiang et al., 2012; Grosu-Tudor and Zamfir, 2013). Many strains of LAB isolated from *kimchi* produce antimicrobial compounds such as bacteriocin by *L. lactis* BH5 (Hur et al., 2000) and *L. citreum* GJ7 (Chang et al., 2008), and pediocin by *P. pentosaceus* (Shin et al., 2008). Species of LAB isolated from *kimchi* show strong antimicrobial activity against *Listeria monocytogenes, Staphylococcus aureus, E. coli,* and *Salmonella typhimurium* (Lee et al., 2009). *Weissella cibaria* isolated from fermented cabbage product shows antimicrobial activity against Gram-positive and Gram-negative pathogens (Patel et al., 2014). *Lactococcus lactis* isolated from *dahi*, Indian curd, produces nisin Z that inhibits *L. monocytogenes* and *S. aureus* (Mitra et al., 2010). Several LAB species isolated from Romanian traditional fermented fruits and vegetables have antimicrobial activity against *L. monocytogenes*, *E. coli, Salmonella,* and *Bacillus* (Grosu-Tudor and Zamfir, 2013). Microorganisms as protective cultures, e.g., bacteriocin producers, may have several advantages, as they can contribute to the flavor, texture and nutritional value of the product besides the production of bacteriocin (Gaggia et al., 2011).

Antioxidant Activity

Antioxidant activities in fermented foods include 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, 2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid; ABTS) radical scavenging activity, total phenol content (TPC) estimation, and reducing power assay (Liu and Pan, 2010; Abubakr et al., 2012). Many Asian fermented soybean foods have antioxidant properties, e.g., *natto*, *Bacillus*-fermented soybean food of Japan (Ping et al., 2012), *chungkokjang* and *jang,* fermented soybean foods of Korea (Shon et al., 2007; Shin and Jeong, 2015), *douchi,* a fermented soybean food of China (Wang et al., 2007a), *kinema, Bacillus*-fermented soybean food of India and Nepal (Moktan et al., 2008; Tamang, 2015), *bekang* and *tungrymbai, Bacillus*-fermented soybean foods of India (Chettri and Tamang, 2014), *thua nao, Bacillus*-fermented soybean food of Thailand (Dajanta et al., 2013), and *tempe* mold-fermented soybean food of Indonesia (Nurrahman et al., 2013). Antioxidant activities have also been observed in *kimchi* (Park et al., 2011) and yogurt (Sabeena et al., 2010).

Peptide Production

Bioactive peptides are formed during food fermentation by proteolytic microorganisms (De Mejia and Dia, 2010). In fermented foods peptides have some functional properties such as immunomodulatory (Qian et al., 2011), antithrombic (Singh et al., 2014), and antihypertensive properties (Phelan and Kerins, 2011). Species of *Bacillus* are involved in enzymatic hydrolysis of

TABLE 1 | Some functional microorganisms used as commercial starters in food fermentation (amended and compiled from references: Mogensen et al., 2002; Bernardeau et al., 2006; Bourdichon et al., 2012; Thapa and Tamang, 2015).

Group	Genera/species	Product/application(s)
Bacteria		
	Acetobacter aceti subsp. *aceti*	Vinegar
	A. pasteurianus subsp. *pasteurianus*	Vinegar, cocoa
	Bacilllus acidopullululiticus	Pullulanases (food additive)
	B. coagulans	Cocoa; glucose isomerase (food additive), fermented soybeans
	B. licheniformis	Protease (food additive)
	B. subtilis	Fermented soybeans, protease, glycolipids, riboflavin-B_2 (food additive)
	Bifidobacterium animalis subsp. *lactis, B. breve*	Fermented milks with probiotic properties; common in European fermented milks
	Brachybacterium alimentarium	Gruyère and Beaufort cheese
	Brevibacterium flavum	Malic acid, glutamic acid, lysine, monosodium glutamate (food additives)
	Corynebacterium ammoniagenes	Cheese ripening
	Enterobacter aerogenes	Bread fermentation
	Enterococcus durans	Cheese and sourdough fermentation
	E. faecium	Soybean, dairy, meat, vegetables
	Klebsiella pneumoniae subsp. *ozaenae*	*Tempe;* production of vitamin B_{12}
	Lactobacillus acetotolerans	Ricotta cheese, vegetables
	L. acidophilus	Fermented milks, probiotics, vegetables
	L. alimentarius	Fermented sausages; ricotta; meat, fish
	L. brevis	Bread fermentation; wine; dairy
	L. buchneri	Malolactic fermentation in wine; sourdough
	L. casei subsp. *casei*	Dairy starter; cheese ripening; green table olives
	L. delbruecki subsp. *bulgaricus*	Yogurt and other fermented milks, mozarella
	L. fermentum	Fermented milks, sourdough, urease (food additive)
	L. ghanensis	Cocoa
	L. helveticus	Starter for cheese; cheese ripening, vegetables
	L. hilgardii	Malolactic fermentation of wine
	L. kefiri	Fermented milk (*kefir*), reduction of bitter taste in citrus juice
	L. kimchii	*Kimchi*
	L. oeni	Wine
	L. paracasei subsp. *paracasei*	Cheese fermentation, probiotic cheese, probiotics, wine, meat
	L. pentosus	Meat fermentation and biopreservation of meat; green table olives; dairy, fruits, wine
	L. plantarum subsp. *plantarum*	Fermentation of vegetables, malolactic fermentation, green table olives; dairy, meat
	L. sakei subsp. *sakei*	Fermentation of cheese and meat products; beverages
	L. salivarious subsp. *salivarius*	Cheese fermentation
	L. sanfranciscensis	Sourdough
	L. versmoldensis	Dry sausages
	Lactococcus lactis subsp. *lactis*	Dairy starter, Nisin (protective culture)
	L. lactis, L. mesenteroides subsp. *Cremoris, L. mesenteroides* subsp. *Dextranicum, L. mesenteroides* subsp. *mesenteroides*	Dairy starter
	Oenococcus oeni	Malolactic fermentation of wine
	Pediococcus acidilactici	Meat fermentation and biopreservation of meat; cheese starter
	P. pentosaceus	Meat fermentation and biopreservation of meat
	Propionibacterium acidipropionici	Meat fermentation and biopreservation of meat
	P. arabinosum	Cheese fermentation; probiotics

(Continued)

TABLE 1 | Continued

Group	Genera/species	Product/application(s)
	P. freudenreichii subsp. *freudenreichii*	Cheese fermentation (Emmental cheese starter)
	Streptococcus natalensis	Natamycin (food additive)
	Weisella ghanensis	Cocoa
	Zymomonas mobilis subsp. *mobilis*	Beverages
Yeasts		
	Candida famata	Fermentation of blue vein cheese and biopreservation of citrus; meat
	C. guilliermondii	Citric acid (food additive)
	C. krusei	*Kefir* fermentation; sourdough fermentation
	Debaryomyces hansenii	Ripening of smear cheeses; meat
	Geotrichum candidum	Ripening of soft and semisoft cheeses or fermented milks; meat
	Kluyveromyces marxianus	Cheese ripening; lactase (food additive)
	S. bayanus	*Kefir* fermentation; juice and wine fermentation
	S. cerevisiae	Beer, bread, invertase (food additive)
	S. cerevisiae subsp. *boulardii*	Used as probiotic culture
	S. florentius	*Kefir* fermentation
	S. pastorianus	Beer
	S. sake	*Sake* fermentation
	S. unisporus	*Kefir* fermentation
	Schizosaccharomyces pombe	Wine
	Zygosaccharomyces rouxii	Soy sauce
Filamentous moulds		
	Aspergillus flavus	α-amylases (food additive)
	A. niger	Beverages; industrial production of citric acid; amyloglucosidases, pectinase, cellulase, glucose oxidase, protease (food additives)
	A. oryzae, A. sojae	Soy sauce, beverages; α-amylases, amyloglucosidase, lipase (food additives)
	Penicillium camemberti	White mold cheeses (camembert type)
	P. notatum	Glucose oxidases (food additive)
	P. roqueforti	Blue mold cheeses
	Rhizopus oligosporus	*Tempe* fermentation
	R. oryzae	Soy sauce, *koji*

protein producing peptides and amino acids, which claim to have health benefits (Nagai and Tamang, 2010). Inhibitory properties of Angiotensin converting enzyme (ACE) have been studied in various fermented milk products such as *kefir* (Quiros et al., 2005), *koumiss* (Chen et al., 2010), yogurt (Papadimitriou et al., 2007), fermented camel milk (Moslehishad et al., 2013), cheese (Meyer et al., 2009), and fermented fish products (Ichimura et al., 2003).

Production of Enzymes by Microorganisms

Another important reason to ferment foods is to coax microorganisms into producing enzymes that also provide very useful services. During food fermentation microorganisms produce enzymes to break down complex compounds to simple bio-molecules for several biological activities such as proteinase, amylase, mannase, cellulase, and catalase in many Asian fermented soybean foods by *Bacillus* spp. (Tamang and Nikkuni, 1996; Chettri and Tamang, 2014). Common genera of mycelial fungi in fermented foods and beverages such as *Actinomucor, Amylomyces, Aspergillus, Monascus, Mucor, Neurospora,* and *Rhizopus* produce various carbohydrases such as α- amylase, amyloglucosidase, maltase, invertase, pectinase, ß-galactosidase, cellulase, hemi-cellulase; acid and alkaline proteases; and lipases (Nout and Aidoo, 2002). Taka-amylase A (TAA), an enzyme produced by *Aspergillus oryzae* in *koji* has many uses in industry (Suganuma et al., 2007). Dry, solid, cake-like mixed amylolytic starters used for alcohol production in the Himalayas have yeasts *Saccharomycopsis fibuligera, S. capsularis* and *Pichia burtonii* with high amylase activities (Tsuyoshi et al., 2005; Tamang et al., 2007).

Bacillus subtilis subsp. *natto* in *natto* produces nattokinase showing fibrinolytic activity (Mine et al., 2005; Kotb, 2012). Among bacteria isolated from fermented foods, *B. subtilis* and *B. amyloliquefaciens* (Chang et al., 2012; Zeng et al., 2013; Singh et al., 2014), *Vagococcus carniphilus*, *V. lutrae*, *Enterococcus faecalis*, *E. faecium*, *E. gallinarum,* and *P. acidilactici* (Singh et al., 2014), and *Virgibacillus halodenitrificans* SK1-3-7 isolated from fish sauce fermentation (Montriwong et al., 2012) produce fibrinolytic enzymes.

Increase in Isoflavones and Saponin and Production of PGA

Isoflavones are daidzein, genistein and glycitein, each of which exists in four chemical forms viz., aglycones, β-glucoside, acetylglucoside, and malonylglucoside in soybeans (Kudou et al., 1991). Isoflavone glucosides are hydrolyzed into their corresponding aglycones during fermentation of some Asian fermented soybean foods such as *sufu* and *douchi* of China (Wang et al., 2007b; Yin et al., 2007), *miso* and *natto* of Japan (Chiou and Cheng, 2001), *chungkokjang* and *doenjan*g of Korea (Lee et al., 2007), *tempe* of Indonesia (Lu et al., 2009), and *thua nao* of Thailand (Dajanta et al., 2009). During *tempe* fermentation, isoflavone particularly Factor-II and aglycone contents are found to increase (Nakajima et al., 2005). Isoflavones in *doenjan*g increase the activation of an LDL-C receptor, which is beneficial to prevent vascular diseases (Kwak et al., 2012).

Soybean saponins, which are oleanane triterpenoid glycosides, are again of two types viz., Group A and DDMP (2,3-dihydro-2,5-dihydroxy-6-metyl-4*H*-pyran-4-one; Paucar-Menacho et al., 2010). DDMP and their derivatives, Groups B and E saponins show health promoting benefits such as prevention of hypercholesterolemia (Murata et al., 2006), suppression of colon cancer cell proliferation (Ellington et al., 2006), and anti-peroxidation of lipids (Ishii and Tanizawa, 2006). Saponin contents are increased in *natto*, which are generated by *Bacillus natto* (Yanagisawa and Sumi, 2005). *Kinema* has high content of Group B saponin, which may indicate its health-promoting benefits to consumers (Omizu et al., 2011).

Poly-glutamic acid (PGA) is not synthesized by ribosomal proteins (Oppermann-Sanio and Steinbüchel, 2002), but is produced by some strains of *Bacillus* spp. in fermented soybean foods of Asia (Urushibata et al., 2002; Meerak et al., 2007; Nishito et al., 2010; Chettri and Tamang, 2014). *B. subtilis* and *B. licheniformis* are widely used industrial producers of γ-PGA (Stanley and Lazazzera, 2005). It is safe eating the viscous materials of Asian fermented soybean foods since PGA is completely biodegradable and water-soluble and non-toxic to human (Yoon et al., 2000).

Degradation of Anti-nutritive Compounds

Some microorganisms present in fermented foods may degrade anti-nutritive substances and thereby convert the substrates into consumable products (Nout, 1994; Tamang, 2015). Various steps employed during the processing of *gari* and *fufu*, fermented cassava products of Africa, such as peeling, washing, grating, fermentation, dewatering and roasting minimizes the residual cyanide contents of the product (Babalola, 2014). Bitter varieties of cassava tubers contain the cyanogenic glycoside linamarin and lotaustralin, which are detoxified by species of *Leuconostoc, Lactobacillus,* and *Streptococcus* during traditional method to *gari* and *fufu* productions to yield hydrocyanic acid (HCN) which has low boiling point and escapes from the dewatered pulp during toasting rendering the product safe for human consumption (Lambri et al., 2013; Babalola, 2014; Bamidele et al., 2015). In *tempe, Rhizopus oligosporus* eliminates the flatulence causing indigestible oligosaccharides such as stachyose and verbascose into the absorbable monosaccharides and disaccharides (Hesseltine, 1983; Sanchez, 2008). Degradation of anti-nutritive compounds by *B. subtilis* has been reported in *kinema* (Sarkar et al., 1997). Phytic acid is reduced during fermentation of *idli* (Reddy and Salunkhe, 1980) and *rabadi*, a fermented cereal food of India (Gupta et al., 1992).

HEALTH BENEFITS OF FERMENTED FOODS

Ethnic foods have in-built systems both as foods and medicine to meet up hungry and also curative (Shin and Jeong, 2015; Thapa and Tamang, 2015). The highest longevity observed among the people of Okinawa prefecture in Japan is mostly due to their traditional and cultural foods such as *natto, miso, tofu, shoyu,* fermented vegetables, cholesterol-free, low-fat, and high bioactive-compounded foods in addition to active physical activity, sound environment, happiness and other several factors (Willcox et al., 2004). Korean *kimchi* has been claimed to possess health-promoting benefits (Cheigh, 1999; Lee et al., 2011; Park et al., 2014; Han et al., 2015). *Kimchi* has also anti-aging effect (Kim et al., 2002). *Natto* has several health benefits such as high contents of nattokinase, isoflavones, saponins, vitamin K, unsaturated fatty acids, probiotics and immunomodulating activities mostly produced by *B. subtilis* (*natto*; Tsubura, 2012; Nagai, 2015). *Kinema* has also some health promoting benefits (Omizu et al., 2011; Tamang, 2015). Indian popular fermented milk *dahi* has anti-carcinogenic property (Arvind et al., 2010). Lactic acid produced in *kimchi* may prevent fat accumulation and to improve obesity-induced heart diseases (Park et al., 2008). Anti-obesity effects have been reported in *kimchi* (Kim et al., 2011; Park et al., 2012) and in *doenjang* (Kwak et al., 2012) based on clinical trials (Cha et al., 2012; Jung et al., 2014). Red wine has anti-aging property due to presence of melatonin that regulates the body clock (Corder et al., 2006; Walker, 2014).

Ethnic people have customary belief in medicinal values of some of their ethnic foods including fermented foods and beverages, however, clinical trials and validation of the health benefits claims of almost all naturally fermented foods and beverages of the world need to be studied. Some health benefits of fermented foods are listed in **Table 2**.

Synthesis of Nutrient

Enrichment of substrates with vitamins, essential amino acids, and bioactive compounds occur during food fermentation

(Holzapfel et al., 1995; Steinkraus, 1996; Thapa and Tamang, 2015). In *tempe*, mold-fermented soybean food of Indonesia, contents of folic acid, niacin, riboflavin, nicotinamide and pyridoxine are found to be increased by *Rhizopus oligosporus* (Astuti, 2015), whereas vitamin B_{12} is synthesized by non-pathogenic strains of *Klebsiella pneumoniae* and *Citrobacter freundii* (Liem et al., 1977; Okada, 1989; Keuth and Bisping, 1994). Contents of thiamine, riboflavin and methionine in *idli*, a rice-legume based fermented food of India and Sri Lanka enhance during fermentation (Ghosh and Chattopadhyay, 2011). Similarly, vitamins B complex and C, lysine and tryptophane, and iron contents have been found to increase during fermentation of *pulque*, an alcoholic drink of Mexico made from cactus plant (Ramirez et al., 2004). Riboflavin and niacin contents are increased in many *Bacillus*-fermented Asian fermented foods (Sarkar et al., 1998; Kim and Hahm, 2002; Nagai, 2015). Riboflavin and folic acid were found to be synthesized in *kimchi* by *L. mesenteroides* and *L. sakei* (Jung et al., 2013). Yeasts *Saccharomyces cerevisiae*, *Candida tropicalis*, *Aureobasidium* sp., and *Pichia manschuria* isolated from *idli* and *jalebi*, fermented cereal foods of India and Pakistan produce vitamin B_{12} (Syal and Vohra, 2013). Free amino acids are increased in fermented soybean foods (Nikkuni et al., 1995; Sarkar and Tamang, 1995; Tamang and Nikkuni, 1998; Kiers et al., 2000; Dajanta et al., 2011).

Prevention of Hypertension and Heart Disease

Antihypertensive properties of many fermented milk products have been validated using animal models and clinical trials (Seppo et al., 2002; Sipola et al., 2002). Consumption of fermented milks or probiotic bacteria (Agerholm-Larsen et al., 2000) and fermented soybean foods (Liu and Pan, 2010) lowers the risk of heart diseases. Fermented whole grain foods can lower the serum LDL-cholesterol values, hypertriacylglycerolaemia,

TABLE 2 | Some bioactive compounds in fermented foods and their health benefits.

Bioactive compounds	Synthesized in fermented foods	Health benefits	Reference
Genistein	*Doenjang*	Facilitates the β-oxidation of fatty acid, reducing body weight	Kwak et al., 2012
Lipoteichoic acid from *L. rhamnosus* GG	Fermented milk	Oral photoprotective agent against UV-induced carcinogenesis	Weill et al., 2013
Isocyanate and sulphide indole-3-carbinol	*Kimchi*	Prevention of cancer, detoxification of heavy metals in liver, kidney, and small intestine	Kwak et al., 2014
Ornithine		Anti-obesity efficacy	Park et al., 2012
Vitamin A, Vitamin C, fibers		Suppression of cancer cells	Han et al., 2015
Capsaicin, Allicin		Prevention of cancer, suppression of *Helicobacter pylori*	Lim and Im, 2009
Chlorophyll		Helps in prevention of absorbing carcinogen	Ferruzzi and Blakeslee, 2007
S-adenosyl-L-methionine (SAM)		Treatment of depression	Lee and Lee, 2009
HDMPPA (an antioxidant)		Therapeutic application in human atherosclerosis	Kim et al., 2007
Nattokinase, antibiotics, Vitamin K	*Natto*	Antitumor, immunomodulating	Nagai, 2015
Vitamin C	Sauerkraut	Scurvy	Peñas et al., 2013
Glucosinolates		Activation of natural antioxidant enzymes	Martinez-Villaluenga et al., 2012
Antioxidant genestein, daidzein, tocopherol, superoxide dismutase	*Tempe*	Prevents oxidative stress causing non-communicable disease such as hyperlipidemia, diabetes, cancer (breast and colon), prevents the damage of pancreatic beta cell	Astuti, 2015
Phenolics- resveratrol	*Wine (red)*	Anti inflammatory	Jeong et al., 2010
Phenolics, succinic acid		Digestive aid	Jackson, 2008
Phenolics, resveratrol, flavonoids – quercitin, Vitamins C and E, mineral selenium		Prevent cardiovascular diseases, reduce incidence of heart attacks and mortality rate	Walker, 2014
Melatonin, resveratrol		Antioxidant and anti-aging property	Fernández-Mar et al., 2012
Resveratol		Anti-diabetic	Ramadori et al., 2009

hypertension, coronary heart disease, insulin resistance, and hyperhomocysteinaemia (Anderson, 2003). Consumption of some fermented foods reduces the cholesterol level in *tempe* (Hermosilla et al., 1993), fermented soybean foods (Lee, 2004), and *kefir* (Otes and Cagindi, 2003). *Calpis*, the Japanese fermented sour milk containing two peptides VPP and IPP has shown hypotensive effect (Nakamura et al., 1996). *L. helvetius* in fermented milk reduces elevated blood pressure (Aihara et al., 2005; Shah, 2015). *Monascus purpureus* in fermented red-rice of China locally called *angkak*, prohibits creation of cholesterol by blocking a key enzyme, HMG-CoA reductase due to presence of mevinolin citrinin (Pattanagul et al., 2008).

Drinking of fermented tea of China prevents heart disease (Mo et al., 2008). Some Asian fermented soybean foods have antihypertensive properties as observed in *natto* (Nagai, 2015) and *tempe* (Astuti, 2015). Isoflavone in *doenjan*g, mold-fermented soybean food of Korea, plays an important role in preventing cardiovascular diseases (Kwak et al., 2012; Shin et al., 2015). Fermented whole-grain intake appears to protect from development of heart disease and diabetes (Anderson, 2003). Moderate consumption of wine is healthier (Walker, 2014). Polyphenols in red wine probably are synergists of the tocopherol (Vitamin E) and ascorbic acid (Vitamin C), thus they inhibit lipid peroxidation (Feher et al., 2007). Regular consumption of the Korean fermented soybean foods by hypertensive and Type 2 diabetic patients results in favorable changes in cardiovascular risk factors (Jung et al., 2014) and reduction of hypocholesterolemic effect (Lim et al., 2014). ACEs inhibitory peptides derived from food proteins are used for treating hypertension (Jakubczyk et al., 2013). Fermented foods, which are rich in fibrinolytic enzymes, are useful for thrombolytic therapy to prevent rapidly emerging heart diseases (Mine et al., 2005; Singh et al., 2014).

Prevention from Cancer

Some LAB-fermented foods have antimutagenic and anticarcinogeinc activities (Lee et al., 2004). *Kefir* is used for the treatment of cancer (Otes and Cagindi, 2003; Yanping et al., 2009). Sauerkraut, fermented vegetable of Germany, contains *s*-methylmethionine, which reduces tumourigenesis risk in the stomach (Kris-Etherton et al., 2002). Consumption of fermented milk products containing live cells of *L. acidophilus* decreases ß-glucuronidase, azoreductase, and nitroreductase (catalyze conversion of procarcinogens to carcinogens), probably removes procarcinogens, and activate the immune system of consumers (Goldin and Gorbach, 1984; Macouzet et al., 2009). Similarly, Indian *dahi* has anti-carcinogenic property (Mohania et al., 2013). Cancer preventive potential of *W. cibaria,* and *L. plantarum* has been reported in *kimchi* (Kwak et al., 2014). Consumption of yogurt can reduce bladder, colon and cervical cancer has been observed (Chandan and Kilara, 2013).

Protection against Gastrointestinal Disorders

Lactic acid bacteria present in fermented foods may decrease number of incidence, duration and severity of some gastrointestinal disorders (Verna and Lucak, 2010). Administration of some strains of *Lactobacillus* improves the inflammatory bowel disease, paucities and ulcerative colitis (Orel and Trop, 2014). *L. rhamnosus* GG is effective in the treatment of acute diarrhea (Szajewska et al., 2007) and administration of *L. helveticus*-fermented milk in healthy older adults produced improvements in cognition function (Chung et al., 2014). Consumption of fermented milk products containing live bacteria has immunomodulation capacity (Granier et al., 2013), and cures diarrhea (Balamurugan et al., 2014). Korean *kimchi* is suitable for control of inflammatory bowel diseases (Lim et al., 2011).

Anti-allergic Reactions

Lactobacillus kefiranofaciens M1 isolated from *kefir* grains has an anti-allergic effect (Hong et al., 2010). Digestion of caseins during maturation of fermented milk products has shown to facilitate loss of allergenic reactivity thus increases tolerance (Alessandri et al., 2012). *Chongkokjang* has anti-allergic effect such as dermis thickness, decreased ear thickness, auricular lymph node and infiltrating mast cells (Lee et al., 2014). *Lactobacillus* species isolated from *kimchi* are found to modulate Th1/Th2 balance by producing a large amount of IL-12 and IFN-γ with ability to alleviate atopic dermatitis and food allergy (Won et al., 2011). Fermented fish oil, which is rich with Omega-3 polyunsaturated fatty acids, can reduce sensitization of allergy (Han et al., 2012).

Protection from Diabetes and Osteoporosis

Intake of high fiber foods may decrease the insulin requirements in diabetic persons (Meyer et al., 2000), and may increase the sensitivity to insulin for non-diabetic persons (Fukagawa et al., 1990; Anderson, 2003). Probiotic *dahi*-supplemented diet significantly delays the glucose intolerance, hyperglycemia, hyperinsulinemia, oxidative stress and dyslipidemia indicating a lower risk of diabetes (Yadav et al., 2007). Daily consumption of *chungkokjang* may increase the insulin resistivity thus controls diabetics (Shin et al., 2011; Tolhurst et al., 2012).

Vitamin K2 present in *natto* stimulates the formation of bone, which may help to prevent osteoporosis in older women in Japan (Yanagisawa and Sumi, 2005). Mineral such magnesium, calcium, phosphorus, potassium, and also protein present in yogurt may function together to promote formation of healthy bones (Chandan and Kilara, 2013).

Alleviation of Lactose Malabsorption

Some people suffer from lactose malabsorption, a condition in which lactose, the principal carbohydrate of milk, is not completely digested into glucose and galactose due to lack of ß-D-galactosidase (Shah, 2015). *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* used in production of yogurt contain substantial quantities of ß-D-galactosidase which improve the symptoms of lactose malabsorption in lactose intolerant people (Shah et al., 2013). Consumption of fresh yogurt (with live yogurt cultures) has been demonstrated better lactose digestion and absorption than with the consumption of a pasteurized product

(Pedone et al., 2000). *Kefir* can minimize the symptoms of lactose intolerance by providing extra source of β-galactosidase (Hertzler and Clancy, 2003).

HEALTH RISK OF FERMENTED FOODS

One of the important health risks in fermented foods is presence of biogenic amines. Biogenic amines are low molecular weight organic compounds by microbial decarboxylation of their precursor amino acids or by transamination of aldehydes and ketones by amino acid transaminases (Zhai et al., 2012), which are are present in some fermented foods such as *sauerkrau*t, fish products, cheese, wine, beer, dry sausages, etc. (Halász et al., 1994; Suzzi and Gardini, 2003; Spano et al., 2010; Visciano et al., 2014). Enterobacteriaceae and enterococci are major biogenic amine producers in foods (Nout, 1994). Foods with high levels of biogenic amines could be considered as unhealthy (Latorre-Moratalla et al., 2010). High levels (>100 mg/kg) of histamine and tyramine can cause adverse effects to human health (Rauscher-Gabernig et al., 2009). Fermentation of cabbage with certain lactic starters such as *L. casei* subsp. *casei, L. plantarum* and *L. curvatus* could reduce the biogenic amine content of *sauerkraut* (Rabie et al., 2011). The ingestion of food containing small amounts of histamine has little effect in healthy individuals, but it can result in histamine intolerance in persons characterized by impairment of diamine oxidase activity, either due to genetic predisposition, gastrointestinal diseases, or medication with monoamine oxidase inhibitors (Maintz and Novak, 2007). A maximum limit of 100 mg/kg of histamine in food indicates a safe level for consumption (Halász et al., 1994).

CONCLUSION

Some fermented foods and beverages have health benefits due to presence of functional microorganisms. Although, some fermented foods and beverages are marketed globally as health foods, functional foods, therapeutic foods, nutraceutical foods, bio-foods, however, due to urbanization, changes in life-style, and the shifting from traditional food habits to commercial fast foods, the production and consumption of traditional fermented foods is in decline mostly in Asia and Africa. Reliance on fewer providers of fermented foods is also leading to a decline in the biodiversity of microorganisms. We recommend that validation of health claims by clinical trials and animal models of some common fermented foods of the world may be studied in details, and also introduction of new fermented food products containing well-validated functional microorganism(s) may emerge in global food market.

AUTHOR CONTRIBUTIONS

JPT (70% – data collection, analysis, writing), D-HS (10% – data collection), S-JJ (10% – data collection) and S-WC (10% – data collection).

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Review: Diversity of Microorganisms in Global Fermented Foods and Beverages

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Culturalable and non-culturable microorganisms naturally ferment majority of global fermented foods and beverages. Traditional food fermentation represents an extremely valuable cultural heritage in most regions, and harbors a huge genetic potential of valuable but hitherto undiscovered strains. Holistic approaches for identification and complete profiling of both culturalable and non-culturable microorganisms in global fermented foods are of interest to food microbiologists. The application of culture-independent technique has thrown new light on the diversity of a number of hitherto unknown and non-cultural microorganisms in naturally fermented foods. Functional bacterial groups ("phylotypes") may be reflected by their mRNA expression in a particular substrate and not by mere DNA-level detection. An attempt has been made to review the microbiology of some fermented foods and alcoholic beverages of the world.

Keywords: global fermented foods, LAB, *Bacillus*, yeasts, filamentous molds

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INTRODUCTION

Traditionally, boiled rice is a staple diet with fermented and non-fermented legume (mostly soybeans) products, vegetables, pickles, fish, and meat in Far-East Asia, South Asia, North Asia, and the Indian subcontinent excluding Western and Northern India; while wheat/barley-based breads/loaves comprise a staple diet followed by milk and fermented milk products, meat, and fermented meats (sausages) in the Western and Northern part of India, West Asian continent, Europe, North America, and even in Australia and New Zealand (Tamang and Samuel, 2010). Sorghum/maize porridges, on the other hand, are the main courses of diet with many fermented and non-fermented sorghum/maize/millets, cassava, wild legume seeds, meat, and milk products in Africa and South America. Fermented foods are the hub of consortia of microorganisms, since they are either present as natural indigenous microbiota in uncooked plant or animal substrates, utensils, containers, earthen pots, and the environment (Hesseltine, 1979; Franz et al., 2014), or add starter culture(s) containing functional microorganisms (Holzapfel, 1997; Stevens and Nabors, 2009) which modify the substrates biochemically, and organoleptically into edible products that are culturally and socially acceptable to the consumers (Campbell-Platt, 1994; Steinkraus, 1997; Tamang, 2010b). Microorganisms convert the chemical composition of raw materials during fermentation, which enrich the nutritional value in some fermented foods, and impart health-benefits to the consumers (Steinkraus, 2002; Farhad et al., 2010; Tamang, 2015a).

Several researchers have reviewed the microbiology, biochemistry, and nutrition of fermented foods and beverages from different countries of Asia (Hesseltine, 1983; Steinkraus, 1994, 1996; Nout and Aidoo, 2002; Tamang et al., 2015); Africa (Odunfa and Oyewole, 1997; Olasupo et al., 2010; Franz et al., 2014); Europe (Pederson, 1979; Campbell-Platt, 1987; Wood, 1998); South America (Chaves-López et al., 2014), and North America (Doyle and Beuchat, 2013). Many genera/species of microorganisms have been reported in relation to various fermented foods and beverages across the world; the usage of molecular tools in recent years have helped to clarify, at least in part, the nomenclatural confusion and generalization caused by conventional (phenotypic) taxonomic methods. The present paper is an attempt to collate and review the updated information on microbiology of some globally fermented foods and beverages.

Microorganisms in Fermented Foods

Lactic acid bacteria (LAB) are widely present in many fermented foods and beverages (Stiles and Holzapfel, 1997; Tamang, 2010b). Major genera of the LAB such as *Alkalibacterium*, *Carnobacterium, Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus,* and *Weissella* (Salminen et al., 2004; Axelsson et al., 2012; Holzapfel and Wood, 2014) have been isolated from various globally fermented foods and beverages.

Bacillus is present in alkaline-fermented foods of Asia and Africa (Parkouda et al., 2009; Tamang, 2015b). Species of *Bacillus* that are present, mostly in legume-based fermented foods, are *Bacillus amyloliquefaciens, Bacillus circulans, Bacillus coagulans, Bacillus firmus*, *Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus subtilis, Bacillus subtilis* variety *natto,* and *Bacillus thuringiensis* (Kiers et al., 2000; Kubo et al., 2011), while strains of *Bacillus cereus* have been isolated from the fermentation of *Prosopis africana* seeds for the production of *okpehe* in Nigeria (Oguntoyinbo et al., 2007). Some strains of *B. subtilis* produce λ-polyglutamic acid (PGA) which is an amino acid polymer commonly present in Asian fermented soybean foods, giving the characteristic of a sticky texture to the product (Urushibata et al., 2002; Nishito et al., 2010).

The association of several species of *Kocuria, Micrococcus* (members of the *Actinobacteria*), and *Staphylococcus* (belonging to the *Firmicutes*) has been reported for fermented milk products, fermented sausages, meat, and fish products (Martín et al., 2006; Coton et al., 2010). Species of *Bifidobacterium, Brachybacterium, Brevibacterium,* and *Propionibacterium* are isolated from cheese, and species of *Arthrobacter* and *Hafnia* from fermented meat products (Bourdichon et al., 2012). *Enterobacter cloacae*, *Klebsiella pneumoniae, K. pneumoniae* subsp. *ozaenae, Haloanaerobium, Halobacterium, Halococcus*, *Propionibacterium, Pseudomonas,* etc. are also present in many global fermented foods (Tamang, 2010b).

Genera of yeasts reported for fermented foods, alcoholic beverages and non-food mixed amylolytic starters are mostly *Brettanomyces*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Dekkera*, *Galactomyces*, *Geotrichum*, *Hansenula*, *Hanseniaspora*, *Hyphopichia*, *Issatchenkia*, *Kazachstania*, *Kluyveromyces*, *Metschnikowia*, *Pichia*, *Rhodotorula*, *Rhodosporidium*, *Saccharomyces*, *Saccharomycodes*, *Saccharomycopsis*, *Schizosaccharomyces*, *Sporobolomyces*, *Torulaspora*, *Torulopsis*, *Trichosporon*, *Yarrowia*, and *Zygosaccharomyces* (Watanabe et al., 2008; Tamang and Fleet, 2009; Lv et al., 2013).

Major role of filamentous molds in fermented foods and alcoholic beverages is the production of enzymes and the degradation of anti-nutritive factors (Aidoo and Nout, 2010). Species of *Actinomucor, Amylomyces, Aspergillus, Monascus, Mucor, Neurospora, Parcilomyces, Penicillium, Rhizopus,* and *Ustilago* are reported for many fermented foods, Asian non-food amylolytic starters and alcoholic beverages (Nout and Aidoo, 2002; Chen et al., 2014).

TAXONOMIC TOOLS FOR IDENTIFICATION OF MICROORGANISMS FROM FERMENTED FOODS

Use of culture media may ignore several unknown non-culturable microorganisms that may play major or minor functional roles in production of fermented foods. Direct DNA extraction from samples of fermented foods, commonly known as culture-independent methods, is nowadays frequently used in food microbiology to profile both culturable and non-culturable microbial populations from fermented foods (Cocolin and Ercolini, 2008; Alegría et al., 2011; Cocolin et al., 2013; Dolci et al., 2015), provided that the amplification efficiency is high enough. PCR-DGGE analysis is the most popular culture-independent technique used for detecting microorganisms in fermented foods and thereby profiling both bacterial populations (Cocolin et al., 2011; Tamang, 2014) and yeast populations in fermented foods (Cocolin et al., 2002; Jianzhonga et al., 2009). Both culturable and non-culturable microorganisms from any fermented food and beverage may be identified using culture-dependent and -independent methods to document a complete profile of microorganisms, and also to study both inter- and intra-species diversity within a particular genus or among genera (Ramos et al., 2010; Greppi et al., 2013a,b; Yan et al., 2013). A combination of Propidium MonoAzide (PMA) treatment on samples before DNA extraction and molecular quantifying method can be used to accurately enumerate the viable microorganisms in fermented foods (Desfossés-Foucault et al., 2012; Fujimoto and Watanabe, 2013).

Molecular identification is emerging as an accurate and reliable identification tool, and is widely used in identification of both culture-dependent and culture-independent microorganisms from fermented foods (Giraffa and Carminati, 2008; Dolci et al., 2015). Species-specific PCR primers are used for species level identification (Tamang et al., 2005); this technique is widely applied in the identification of LAB isolated from fermented foods (Robert et al., 2009). The application of real-time quantitative PCR (qPCR) with specific primers enables the specific detection and quantification of LAB species in fermented foods (Park et al., 2009).

Random amplification of polymorphic DNA (RAPD) is a typing method based on the genomic DNA fragment profiles amplified by PCR, and is commonly used for disintegration of LAB strains from fermented foods (Coppola et al., 2006; Chao et al., 2008). The repetitive extragenic palindromic sequence-based PCR (rep-PCR) technique permits typing at subspecies level and reveals significant genotypic differences among strains of the same bacterial species from fermented food samples (Tamang et al., 2008). Amplified fragment length polymorphism (AFLP) is a technique based on the selective amplification and separation of genomic restriction fragments, and its applicability in identification and to discriminate has been demonstrated for various LAB strains (Tanigawa and Watanabe, 2011).

Techniques of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) have been developed to profile microbial communities directly from fermented foods, and are based on sequence-specific distinctions of 16S rDNA and 26S rDNA amplicons produced by PCR (Ercolini, 2004; Flórez and Mayo, 2006; Alegría et al., 2011). However, DGGE has some disadvantages as well like it is time consuming, unable to determine the relative abundance of dominant species and distinguish between viable and nonviable cells, as well as it has difficulties in interpretation of multi-bands (Dolci et al., 2015). DGGE is also limited to detect specific species as it may only reveal some of the major bacterial species such as *B. licheniformis* and *Bacillus thermoamylovorans* in *chungkokjang* (sticky fermented soybean food of Korea) and not detect a large number of predominant or diverse rare bacterial species identified in pyrosequencing analysis (Nam et al., 2011).

The amplified ribosomal DNA restriction analysis (ARDRA) technique using restriction enzymes is also useful in identification of microorganisms from fermented foods (Jeyaram et al., 2010).

Multilocus sequence analysis (MLSA), using housekeeping genes as molecular markers alternative to the 16S rRNA genes, is used for LAB species identification: *rpoA* and *pheS* genes for *Enterococcus* and *Lactobacillus*, *atpA* and *pepN* for *Lactococcus* species, and *dnaA*, *gyrB*, and *rpoC* for species of *Leuconostoc, Oenococcus*, and *Weissella* (de Bruyne et al., 2007, 2008b, 2010; Diancourt et al., 2007; Picozzi et al., 2010; Tanigawa and Watanabe, 2011).

Effective tools of next generation sequencing (NGS) such as metagenomics, phylobiomics, and metatranscriptomics are nowadays applied for documentation of cultures in traditionally fermented products (Mozzi et al., 2013; van Hijum et al., 2013). However, NGS as a sophisticated tool needs well-trained hands and a well-equipped molecular laboratory, which may not always be available. Application of metagenomic approaches, by using parallel pyrosequencing of tagged 16S rRNA gene amplicons, provide information on microbial communities as profiled in *kimchi*, a naturally fermented vegetable product of Korea (Jung et al., 2011; Park et al., 2012), *nukadoko*, a fermented rice bran of Japan (Sakamoto et al., 2011), *narezushi*, a fermented salted fish and cooked rice of Japan (Kiyohara et al., 2012), and *ben-saalga*, a traditional gruel of pearl millet of Burkina Faso (Humblot and Guyot, 2009). Pyrosequencing has revealed the presence of numerous and even minor bacterial groups in fermented foods, but DNA-level detection does not distinguish between metabolically "active" and "passive" organisms. "Functionally relevant phylotypes" in an ecosystem may be specifically detected by, e.g., weighted UniFrac principal coordinate analysis based on 454 pyrosequencing of 16S rRNA genes, as applied in studies on gut microbiota (Wang et al., 2015). The 16S rRNA gene sequence based pyrosequencing method enables a comprehensive and high-throughput analysis of microbial ecology (Sakamoto et al., 2011), and this method has been applied to various traditionally fermented foods (Oki et al., 2014).

A proteomics identification method based on protein profiling using matrix-assisted laser desorption ionizing-time of flight mass spectrometry (MALDI-TOF MS) has been used to identify species of *Bacillus* in fermented foods of Africa (Savadogo et al., 2011), and species of LAB isolated from global fermented foods (Tanigawa et al., 2010; Dušková et al., 2012; Sato et al., 2012; Nguyen et al., 2013a; Kuda et al., 2014).

Global Fermented Foods

Campbell-Platt (1987) reported around 3500 global fermented foods and beverages, and had divided them into about 250 groups. There might be more than 5000 varieties of common and uncommon fermented foods and alcoholic beverages being consumed in the world today by billions of people, as staple and other food components (Tamang, 2010b). Global fermented foods are classified into nine major groups on the basis of substrates (raw materials) used from plant/animal sources: (1) fermented cereals, (2) fermented vegetables and bamboo shoots, (3) fermented legumes, (4) fermented roots/tubers, (5) fermented milk products, (6) fermented and preserved meat products, (7) fermented, dried and smoked fish products, (8) miscellaneous fermented products, and (9) alcoholic beverages (Steinkraus, 1997; Tamang, 2010b,c).

Fermented Milk Products

Fermented milk products (**Table 1**) are classified into two major groups on the basis of microorganisms: (A) lactic fermentation, dominated by species of LAB, comprising the "thermophilic" type (e.g., yogurt, Bulgarian buttermilk), probiotic type (e.g., acidophilus milk, bifidus milk), and the mesophilic type (e.g., natural fermented milk, cultured milk, cultured cream, cultured buttermilk); and (B) fungal-lactic fermentations, where LAB and yeasts cooperate to generate the final product, which include alcoholic milks (e.g., acidophilus-yeast milk, *kefir, koumiss*), and moldy milks (e.g., *viili*; Mayo et al., 2010). Natural fermentation is one of the oldest methods of milk processing using raw and boiled milk to ferment spontaneously, or of using the back-slopping method where a part of the previous batch of a fermented product is used to inoculate the new batch (Holzapfel, 2002; Josephsen and Jespersen, 2004). Cheese and cheese products derived from the fermentation of milk are of major nutritional and commercial importance throughout the world (de Ramesh et al., 2006). Starter cultures in milk fermentation are of two types: primary cultures that are mostly *Lactococcus lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis, Lactobacillus delbrueckii* subsp. *delbrueckii*, *Lb. delbrueckii* subsp. *lactis, Lb. helveticus, Leuconostoc* spp., and *Streptococcus thermophilus* to participate

TABLE 1 | Microorganisms isolated from some common and uncommon fermented milk products of the world.

Product	Substrate	Sensory property and nature	Microorganisms	Country	References
Airag	Mare or camel milk	Acidic, sour, mild alcoholic drink	*Lb. helveticus, Lb. kefiranofaciens, Bifidobacterium mongoliense*, *Kluyveromyces marxianus*	Mongolia	Watanabe et al., 2008, 2009b; Yu et al., 2011
Amasi	Cow milk	Acidic, sour, with thick consistency	*Lc. lactis* subsp. *lactis* (dominating), *Lc. lactis* subsp. *cremoris, Lactobacillus, Enterococcus,* and *Leuconostoc* spp. Several non-culturable strains	South Africa, Zimbabwe	Osvik et al., 2013
Cheese	Animal milk	Soft or hard, solid; side dish, salad, used in many cooked/baked dishes	*Lc. lactis* subsp. *cremoris, Lc. lactis* subsp. *lactis, Lb. delbrueckii* subsp. *delbrueckii*, *Lb. delbrueckii* subsp. *lactis, Lb. helveticus, Lb. casei, Lb. plantarum, Lb. salivarius*, *Leuconostoc* spp., *Strep. thermophilus*, *Ent. durans, Ent. faecium,* and *Staphylococcus* spp., *Brevibacterium linens, Propionibacterium freudenreichii, Debaryomyces hansenii, Geotrichum candidum, Penicillium camemberti, P. roqueforti*	Worldwide	Parente and Cogan, 2004; Quigley et al., 2011
Chhu	Yak/cow milk	Cheese like product, curry, soup	*Lb. farciminis, Lb. brevis, Lb. alimentarius, Lb. salivarius, Lact. lactis, Candida* sp. *Saccharomycopsis* sp.	India, Nepal, Bhutan, China (Tibet)	Dewan and Tamang, 2006
Chhurpi	Yak/cow milk	Cheese like product, soup, curry, pickle	*Lb. farciminis, Lb. paracasei, Lb. biofermentans, Lb. plantarum, Lb. curvatus, Lb. fermentum, Lb. alimentarius, Lb. kefir, Lb. hilgardii, W. confusa, Ent. faecium, Leuc. mesenteroides*	India, Nepal, Bhutan, China (Tibet)	Tamang et al., 2000
Dahi	Cow/buffalo milk, starter culture	Curd, savory	*Lb. bifermentans, Lb. alimentarius, Lb. paracasei, Lact. lactis, Strep. cremoris*, *Strep. lactis*, *Strep. thermophilus*, *Lb. bulgaricus*, *Lb. acidophilus*, *Lb. helveticus, Lb. cremoris, Ped. pentosaceous, P. acidilactici, W. cibaria, W. paramesenteroides, Lb. fermentum, Lb. delbrueckii* subsp. *indicus, Saccharomycopsis* sp., *Candida* sp.	India, Nepal, Sri Lanka, Bangladesh, Pakistan	Harun-ur-Rashid et al., 2007; Patil et al., 2010
Dadih	Buffalo milk	Curd, savory	*Leuc. mesenteroides*, *Ent. faecalis*, *Strep. lactis* supsp. *lactis*, *Strep. cremoris*, *Lb. casei* subsp. *casei,* and *Lb. casei* subsp. *rhamnosus*	Indonesia	Hosono et al., 1989
Kefir	Goat, sheep, cow	Alcoholic fermented milk, effervescent milk	*Lb. brevis, Lb. caucasicus, Strep. thermophilus, Lb. bulgaricus, Lb. plantarum, Lb. casei, Lb. brevis, Tor. holmii, Tor. delbruechii*	Russia	Bernardeau et al., 2006
Koumiss	Milk	Acid fermented milk, drink	*Lb. bulgaricus, Lb. salivarius, Lb. buchneri, Lb. heveticus, Lb. plantarum, Lb. acidophilus, Torula* sp.	Russia, Mongolia	Wu et al., 2009; Hao et al., 2010
Laban rayeb	Milk	Acid fermented milk, yogurt-like	*Lb. casei, Lb. plantarum, Lb. brevis, Lact. lactis, Leuconostoc* sp., *Sacch. kefir*	Egypt	Bernardeau et al., 2006
Leben / Lben	Cow milk	Sour milk	*Candida* sp., *Saccharomyces* sp., *Lactobacillus* sp., *Leuconostoc* sp.	North, East Central Africa	Odunfa and Oyewole, 1997
Misti dahi (mishti doi, lal dahi, payodhi)	Buffalo/cow milk	Mild-acidic, thick-gel, sweetened curd, savory	*Strep. Salivarius* subsp. *thermophilus*, *Lb. acidophilus*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lc. lactis* subsp. *lactis, Sacch. cerevisiae*	India, Bangladesh	Ghosh and Rajorhia, 1990; Gupta et al., 2000

(Continued)

TABLE 1 | Continued

Product	Substrate	Sensory property and nature	Microorganisms	Country	References
Nunu	Raw cow milk	Naturally fermented milk	*Lb. fermentum*, *Lb. plantarum, Lb. helveticus, Leuc. mesenteroides*, *Ent. faecium, Ent. italicus, Weissella confusa, Candida parapsilosis, C. rugosa, C. tropicalis, Galactomyces geotrichum, Pichia kudriavzevii*, *Sacch. cerevisiae*	Ghana	Akabanda et al., 2013
Philu	Cow/ yak milk	Cream like product, curry	*Lb. paracasei, Lb. bifermentans, Ent. faecium*	India, Nepal, Tibet (China)	Dewan and Tamang, 2007
Shrikhand	Cow, buffalo milk	Acidic, concentrated sweetened viscous, savory	*Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *diacetylactis, Lc. lactis* subsp. *cremoris, Strep. thermophilus, Lb. delbruecki* subsp. *bulgaricus*	India	Sarkar, 2008; Singh and Singh, 2014
Somar	Yak or cow milk	Buttermilk	*Lb. paracasei, Lact. lactis*	India, Nepal	Dewan and Tamang, 2007
Sua chua	Dried skim milk, starter, sugar	Acid fermented milk	*Lb. bulgaricus, Strep. thermophilus*	Vietnam	Alexandraki et al., 2013
Tarag	Cow/yak/goat milk	Acidic, sour, drink	*Lb. delbrueckii* subsp. *bulgaricus, Lb. helveticus*, *Strep. thermophilus, Sacch. cerevisiae, Issatchenkia orientalis*, *Kazachstania unispora*	Mongolia	Watanabe et al., 2008
Viili	Cow milk	Thick and sticky, sweet taste, breakfast	*Lc. lactis* subsp. *lactis, Lc. lactis* subsp. *cremoris*, *Lc. lactis* subsp. *lactis* biovar. *diacetylactis, Leuc. mesenteroides* subps. *cremoris, G. candidum*, *K. marxianus, P. fermentans*	Finland	Kahala et al., 2008
Yogurt	Animal milk	Acidic, thick-gel viscous, Curd-like product, savory	*Strep. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus, Lb. acidophilus, Lb. casei, Lb. rhamnosus, Lb. gasseri*, *Lb. johnsonii*, *Bifidobacterium* spp.	Europe, Australia, America	Tamime and Robinson, 2007; Angelakis et al., 2011

in the acidification (Parente and Cogan, 2004); and secondary cultures that are used in cheese-making are *Brevibacterium linens, Propionibacterium freudenreichii, Debaryomyces hansenii, Geotrichum candidum, Penicillium camemberti,* and *P. roqueforti* for development of flavor and texture during ripening of cheese (Coppola et al., 2006; Quigley et al., 2011). Some non-starter lactic acid bacteria (NSLAB) microbiota are usually present in high numbers in fermented milk, which include *Enterococcus durans, Ent. faecium, Lb. casei, Lb. plantarum, Lb. salivarius,* and *Staphylococcus* spp. (Briggiler-Marcó et al., 2007).

Fermented Cereal Foods

In most of the Asian countries, rice is fermented either by using mixed-culture(s) into alcoholic beverages or by using food beverages (Tamang, 2010c), whereas in Europe, America, and Australia, most cereals like wheat, rye, barley and maize are fermented by natural fermentation or by adding commercial baker's yeast into the batter for dough breads/loaves (Guyot, 2010). In Africa, fermented cereal foods are traditionally used as staples as well as complementary and weaning foods for infants and young children (Tou et al., 2007). In Europe, people still practice the old traditional method of preparation of breads or loaves without using any commercial strains of baker's yeast (Hammes and Ganzle, 1998). Yeasts and LAB conduct dough fermentation, mostly San Francisco sourdough, and the resultant products are generally called sourdough breads because they have higher contents of lactic acid and acetic acid due to the bacterial growth (Brandt, 2007; de Vuyst et al., 2009).

Cereal fermentation is mainly represented by species of LAB and yeasts (Corsetti and Settanni, 2007). *Enterococcus*, *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus,* and *Weissella* are common bacteria associated with cereal fermentations (**Table 2**; de Vuyst et al., 2009; Guyot, 2010; Moroni et al., 2011). Native strains of *Saccharomyces cerevisiae* are the principal yeast of most bread fermentations (Hammes et al., 2005), but other non-*Saccharomyces* yeasts are also significant in many cereal fermentations including *Candida, Debaryomyces, Hansenula, Kazachstania, Pichia, Trichosporon,* and *Yarrowia* (Iacumin et al., 2009; Weckx et al., 2010; Johnson and Echavarri-Erasun, 2011).

Fermented Vegetable Foods

Perishable and seasonal leafy vegetables, radish, cucumbers including young edible bamboo tender shoots are traditionally fermented into edible products (**Table 3**). Fermentation of vegetables is mostly dominated by species of *Lactobacillus* and *Pediococcus*, followed by *Leuconostoc, Weissella, Tetragenococcus*, and *Lactococcus* (Chang et al., 2008; Watanabe et al., 2009a). A complete microbial profile of LAB in *kimchi* has been characterized using different molecular identification tools (Shin

TABLE 2 | Microorganisms isolated from some common and uncommon fermented cereal foods of the world.

Product	Raw material/ Substrate	Sensory property and nature	Microorganisms	Country	References
Ang-kak	Red rice	Colorant	*Monascus purpureus*	China, Taiwan, Thailand, Philippines	Steinkraus, 1996
Boza	Cereals	Sour refreshing liquid	*Lactobacillus* sp., *Lactococcus* sp., *Pediococcus* sp., *Leuconostoc* sp., *Sacch. cerevisiae*	Bulgaria	Blandino et al., 2003
Busa	Maize, sorghum, millet	Submerged	*Sacch. cerevisiae*, *Schizosacchromyces pombe*, *Lb. plantarum, Lb. helveticus, Lb. salivarius, Lb. casei, Lb. brevis, Lb. buchneri, Leuc. mesenteroides, Ped. damnosus*	East Africa, Kenya	Odunfa and Oyewole, 1997
Ben-saalga	Pearl millet	Weaning food	*Lactobacillus* sp., *Pediococcus* sp., *Leuconostoc* sp., *Weissela* sp., yeasts	Burkina Faso, Ghana	Tou et al., 2007
Dosa	Rice and black gram	Thin, crisp pancake, Shallow-fried, staple	*Leuc. mesenteroides, Ent. faecalis, Tor. candida, Trichosporon pullulans*	India, Sri Lanka, Malaysia, Singapore	Soni et al., 1986
Enjera/ Injera	Tef flour, wheat	Acidic, sourdough, leavened, pancake-like bread, staple	*Lb. pontis, Lb. plantarum, Leuc. mesenteroides, Ped. cerevisiae, Sacch. cerevisiae, Cand. glabrata*	Ethiopia	Olasupo et al., 2010
Gowé	Maize	Intermediate product used to prepare beverages, porridges	*Lb. fermentum, Lb. reuteri, Lb. brevis, Lb. confusus, Lb. curvatus, Lb. buchneri, Lb. salivarius, Lact. lactis, Ped. pentosaceus, Ped. acidilactici, Leuc. mesenteroides; Candidatropicalis, C. krusei, Kluyveromyces marxianus*	Benin	Vieira-Dalodé et al., 2007; Greppi et al., 2013a
Hussuwa	Sorghum	Cooked dough	*Lb. fermentum, Ped. acidilactici, Ped. pentosaceus*, Yeasts	Sudan	Yousif et al., 2010
Idli	Rice, black gram or other dehusked pulses	Mild-acidic, soft, moist, spongy pudding; staple, breakfast	*Leuc. mesenteroides, Lb. delbrueckii, Lb. fermenti, Lb. coryniformis, Ped. acidilactis, Ped. cerevisae, Streptococcus* sp., *Ent. faecalis, Lact. lactis, B. amyloliquefaciens, Cand. cacaoi, Cand. fragicola, Cand. glabrata, Cand. kefyr, Cand. pseudotropicalis, Cand. sake, Cand. tropicalis, Deb. hansenii, Deb. tamarii, Issatchenkia terricola, Rhiz. graminis, Sacch. cerevisiae, Tor. candida, Tor. holmii*	India, Sri Lanka, Malaysia, Singapore	Steinkraus et al., 1967; Sridevi et al., 2010
Jalebi	Wheat flour	Crispy sweet, doughnut-like, deep-fried, snacks	*Sacch. Bayanus, Lb. fermentum, Lb. buchneri, Lact. lactis, Ent. faecalis, Sacch. cerevisiae*	India, Nepal, Pakistan	Batra and Millner, 1976
Kenkey	Maize	Acidic, solid, steamed dumpling, staple	*Lb. plantarum, Lb. brevis, Ent. cloacae, Acinetobacter* sp., *Sacch. cerevisiae, Cand. mycoderma*	Ghana	Oguntoyinbo et al., 2011
Khamak (Kao-mak)	Glutinous rice, *Look-pang* (starter)	Dessert	*Rhizopus* sp., *Mucor* sp., *Penicillum* sp., *Aspergillus* sp., *Endomycopsis* sp., *Hansenula* sp., *Saccharomyces* sp.	Thailand	Alexandraki et al., 2013
Kunu-zaki	Maize, sorghum, millet	Mild-acidic, viscous, porridge, staple	*Lb. plantarum, Lb. pantheris, Lb. vaccinostercus, Corynebacterium* sp., *Aerobacter sp., Cand. mycoderma, Sacch. cerevisiae, Rhodotorula* sp., *Cephalosporium* sp., *Fusarium* sp., *Aspergillus*sp., *Penicillium* sp.	Nigeria	Olasupo et al., 2010; Oguntoyinbo et al., 2011
Kisra	Sorghum	Thin pancake bread, staple	*Ped. pentosaceus, Lb. confusus, Lb. brevis, Erwinia ananas, Klebsiella pneumoniae, Ent. cloacae, Cand. intermedia, Deb. hansenii, Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Rhizopus* sp.	Sudan	Hamad et al., 1997
Koko	Maize	Porridge	*Ent. clocae, Acinetobacter* sp., *Lb. plantarum, Lb. brevis, Sacch. cerevisiae, Cand. mycoderma*	Ghana	Blandino et al., 2003
Lao-chao	Rice	Paste, soft, juicy, glutinous dessert	*Rhiz. oryzae, Rhiz. chinensis, Chlamydomucor oryzae, Sacchromycopsis* sp.	China	Blandino et al., 2003

(Continued)

TABLE 2 | Continued

Product	Raw material/ Substrate	Sensory property and nature	Microorganisms	Country	References
Mawè	Maize	Intermediate product used to prepare beverages, porridges	*Lb. fermentum, Lb. reuteri, Lb. brevis, Lb. confusus, Lb. curvatus, Lb. buchneri, Lb. salivarius, Lact. lactis, Ped. pentosaceus, Ped. acidilactici, Leuc. mesenteroides; Candida glabrata, Sacch. cerevisiae, Kluyveromyces marxianus, Clavispora lusitaniae*	Benin, Togo	Greppi et al., 2013a,b
Mbege	Maize, sorghum, millet	Submerged	*Sacch. cerevisiae*, *Schizosaccharomyces pombe*, *Lb. plantarum, Leuc. mesenteroides*	Tanzania	Odunfa and Oyewole, 1997
Ogi	Maize, sorghum, millet	Mild-acidic, viscous, porridge, staple	*Lb. plantarum, Lb. pantheris, Lb. vaccinostercus, Corynebacterium* sp., *Aerobacter* sp., *Candida krusei, Clavispora lusitaniae, Sacch. cerevisiae, Rhodotorula* sp., *Cephalosporium* sp., *Fusarium* sp., *Aspergillus* sp., *Penicillium* sp.	Nigeria	Greppi et al., 2013a
Pito	Maize, sorghum	Submerged	*Geotrichum candidum, Lactobacillus* sp., *Candida* sp.	West Africa	Odunfa and Oyewole, 1997
Poto poto	Maize	Slurry	*Lb. gasseri, Lb. plantarum/paraplantarum, Lb. acidophilus, Lb. delbrueckii, Lb. reuteri, Lb. casei, Bacillus* sp., *Enterococcus* sp., Yeasts	Congo	Abriouel et al., 2006
Pozol	Maize	Mild-acidic, thick viscous, porridge, staple	*Strep. bovis, Strep. macedonicus*, *Lc. lactis*, *Ent. sulfureus*	Mexico	Díaz-Ruiz et al., 2003
Puto	Rice	Steamed cake, breakfast	*Leuc. mesenteroides, Ent. faecalis, Ped. pentosaceus*, Yeasts	Philippines	Steinkraus, 2004
Rabadi	Buffalo or cow milk and cereals, pulses	Mild-acidic, thick slurry-like product	*Ped. acidilactici*, *Bacillus* sp., *Micrococcus* sp., yeasts	India, Pakistan	Gupta et al., 1992
Selroti	Rice-wheat flour-milk	Pretzel-like, deep fried bread, staple	*Leuc. mesenteroides, Ent. faecium, Ped. Pentosaceus* and *Lb. curvatus, Sacch. cerevisiae*, *Sacch. kluyveri*, *Deb. hansenii, P. burtonii, Zygosaccharomyces rouxii*	India, Nepal, Bhutan	Yonzan and Tamang, 2010, 2013
Sourdough	Rye, wheat	Mild-acidic, leavened bread	*Lb. sanfranciscensis, Lb. alimentarius, Lb. buchneri, Lb. casei, Lb. delbrueckii, Lb. fructivorans, Lb. plantarum, Lb. reuteri, Lb. johnsonii, Cand. humili, Issatchenkia orientalis*	America, Europe, Australia	Gänzle et al., 1998; de Vuyst et al., 2009
Tape Ketan	Glutinous rice, *Ragi*	Sweet, sour, mild alcoholic, dessert	*Thizopus* sp., *Chlamydomucor* sp., *Candida* sp., *Endomycopsis* sp., *Saccharomyces* sp.	Indonesia	Steinkraus, 1996
Togwa	Cassava, maize, sorghum, millet	Fermented gruel or beverage	*Lb. brevis, Lb. cellobiosus, Lb. fermentum, Lb. plantarum and Ped. pentosaceus, Candida pelliculosa, C. tropicalis, Issatchenkia orientalis, Sacch. cerevisiae*	Tanzania	Mugula et al., 2003
Tarhana	Sheep milk, wheat	Mild-acidic, sweet-sour, soup or biscuit	*Lb. bulgaricus, Strep. thermophilus*, yeasts	Cyprus, Greece, Turkey	Sengun et al., 2009
Uji	Maize, sorghum, millet, cassava flour	Acidic, sour, porridge, staple	*Leuc. mesenteroides, Lb. plantarum*	Kenya, Uganda, Tanzania	Odunfa and Oyewole, 1997

et al., 2008; Nam et al., 2009; Park et al., 2010; Jung et al., 2011, 2013a). Natural fermentations during production of *sauerkraut*, a fermented cabbage product of Germany, had been studied and a species of LAB were reported. (Johanningsmeier et al., 2007; Plengvidhya et al., 2007). Species of LAB constitute the native population in the Himalayan fermented vegetable products such as *gundruk, sinki, goyang, khalpi,* and *inziangsang* (Karki et al., 1983; Tamang et al., 2005, 2009; Tamang and Tamang, 2007, 2010) and in several naturally fermented bamboo products of India and Nepal (Tamang and Sarkar, 1996; Tamang et al., 2008; Tamang and Tamang, 2009; Jeyaram et al., 2010; Sonar and Halami, 2014).

Fermented Soybeans and Other Legumes

Two types of fermented soybean foods are produced: soybean foods fermented by *Bacillus* spp. (mostly *B. subtilis*) with the stickiness characteristic, and soybean foods fermented by filamentous molds, mostly *Aspergillus, Mucor, Rhizopus* (Tamang, 2010b). *Bacillus*-fermented, non-salty and sticky soybean foods are concentrated in an imaginary triangle with three vertices lying each on Japan (*natto*), east Nepal and north-east India (*kinema* and its similar products), and northern Thailand (*thua-nao*), named as "*natto* triangle" (Nakao, 1972) and renamed as "*kinema-natto-thuanao* (KNT)-triangle"

TABLE 3 | Microorganisms isolated from some common and uncommon fermented vegetable products of the world.

Product	Substrate/ Raw materials	Sensory property and nature	Microorganisms	Country	References
Burong mustala	Mustard	Acidic, wet	*Lb. brevis, Ped. cerevisiae*	Philippines	Rhee et al., 2011
Cucumbers (fermented*)*	Cucumbers	Acidic, wet, pickle	*Leuc. mesenteroides*, *Ped. cerevisiae, Ped. acidilactici, Lb. plantarum, Lb. brevis*	Europe, USA, Canada	Pederson, 1979
Dha muoi	Mustard and beet, eggplant	Acidic, wet	*Lb. fermentum*, *Lb. pentosus, Lb. plantarum, Ped. pentosaceus, Lb. brevis, Lb. paracasei*, *Lb. pantheris, Ped. acidilactici*	Vietnam	Nguyen et al., 2013a
Ekung	Bamboo shoot	Acidic, sour, soft, curry	*Lb. plantarum, Lb. brevis, Lb. casei*, *Tor. halophilus*	India	Tamang and Tamang, 2009
Eup	Bamboo shoot	Acidic, sour, dry, curry	*Lb. plantarum, Lb. fermentum, Lb. brevis, Lb. curvatus, Ped. pentosaceus, Leuc. mesenteroides, Leuc. fallax, Leuc. lactis, Leuc. citreum, Ent. durans*	India	Tamang and Tamang, 2009
Fu-tsai	Mustard	Acidic, sour	*Ent. faecalis, Lb. alimentarius, Lb. brevis, Lb. coryniformis, Lb. farciminis, Lb. plantarum, Lb. versmoldensis, Leuc. citreum, Leuc. mesenteroides, Leuc. pseudomesenteroides, Ped. pentosaceus, W. cibaria, W. paramesenteroides*	Taiwan	Chao et al., 2009, 2012
Goyang	Wild vegetable	Acidic, sour, wet, soup	*Lb. plantarum, L. brevis, Lc. lactis, Ent. faecium, Ped. pentosaceus, Candida* sp.	India, Nepal	Tamang and Tamang, 2007
Gundruk	Leafy vegetable	Acidic, sour, dry, soup, side-dish	*Lb. fermentum, Lb. plantarum, Lb. casei*, *Lb. casei* subsp. *pseudoplantarum, Ped. pentosaceus*	India, Nepal, Bhutan	Karki et al., 1983; Tamang et al., 2005
Hirring	Bamboo shoot tips	Acidic, sour, wet, pickle	*Lb. brevis, Lb. plantarum, Lb. curvatus, Ped. pentosaceus, Leuc. mesenteroides, Leuc. fallax, Leuc. lactis, Leuc. citreum, Ent. durans, Lc. lactis*	India	Tamang and Tamang, 2009
Hom-dong	Red onion	Fermented red onion	*Leuc. mesenteroides, Ped. cerevisiae, Lb. plantarum, Lb. fermentum, Lb. buchneri*	Thailand	Phithakpol et al., 1995
Jiang-gua	Cucumber	Fermented cucumber, pickle	*Ent. casseliflavus, Leuc. lactis, Leuc. mesenteroides, Lb. pentosus, Lb. plantarum, Lb. paraplantarum, Lc. lactis* subsp. *lactis, W. cibaria, W. hellenica*	Taiwan	Chen et al., 2012
Jiang-sun	Bamboo shoot, salt, sugar, *douchi* (fermented soybeans)	Fermented bamboo; side dish	*Lb. plantarum, Ent. faecium, Lc. lactis* subsp. *lactis*	Taiwan	Chen et al., 2010
Khalpi	Cucumber	Acidic, sour, wet, pickle	*Lb. brevis, Lb. plantarum, Ped. pentosaceus, Ped. acidilactici, Leuc. fallax*	India, Nepal	Tamang et al., 2005; Tamang and Tamang, 2010
Kimchi	Cabbage, green onion, hot pepper, ginger	Acidic, mild-sour, wet, side-dish	*Leuc. mesenteroides, Leuc. citreum, Leuc. gasicomitatum, Leuc. kimchii, Leuc. inhae, W. koreensis, W. kimchii, W. cibaria, Lb. plantarum, Lb. sakei, Lb. delbrueckii, Lb. buchneri, Lb. brevis, Lb. fermentum, Ped. acidilactici, Ped. pentosaceus, Lc. Lactis,* yeasts species of *Candida, Halococcus, Haloterrigena, Kluyveromyces, Lodderomyces, Natrialba, Natronococcus, Pichia, Saccharomyces, Sporisorium* and *Trichosporon*	Korea	Chang et al., 2008; Nam et al., 2009; Jung et al., 2011
Naw-mai-dong	Bamboo shoots	Acidic, wet	*Leuc. mesenteroides, Ped. cerevisiae, Lb. plantarum, Lb. brevis, Lb. fermentum, Lb. buchneri*	Thailand	Phithakpol et al., 1995
Mesu	Bamboo shoot	Acidic, sour, wet	*Lb. plantarum, Lb. brevis, Lb. curvatus, Leu, citreum, Ped. pentosaceus*	India, Nepal, Bhutan	Tamang et al., 2008
Oiji	Cucumber, salt, water	Fermented cucumber	*Leuc. mesenteroides, Lb. brevis, Lb. plantarum, Ped. cerevisiae*	Korea	Alexandraki et al., 2013

(Continued)

TABLE 3 | Continued

Product	Substrate/ Raw materials	Sensory property and nature	Microorganisms	Country	References
Olives (fermented)	Olive	Acidic, wet, Salad, side dish	*Leuc. mesenteroides, Ped. pentosaceus; Lb. plantarum Lb. pentosus/Lb. plantarum, Lb. paracollinoides*, *Lb. vaccinostercus/Lb. suebicus* and *Pediococcus* sp. non-lactics (*Gordonia* sp./*Pseudomonas* sp., *Halorubrum orientalis, Halosarcina pallid, Sphingomonas* sp./*Sphingobium* sp./*Sphingopyxis* sp., *Thalassomonas agarivorans*) and yeasts (*Candida* cf. *apicola, Pichia* sp., *Pic. manshurica/Pic. galeiformis*, *Sacch. cerevisiae*)	USA, Spain, Portugal, Peru, Chile	Abriouel et al., 2011
Pak-gard-dong	Leafy vegetable, salt, boiled rice	Acidic, wet, side dish	*Lb. plantarum, Lb. brevis, Ped. cerevisiae*	Thailand	Phithakpol et al., 1995
Pak-sian-dong	Leaves of *Gynandropis pentaphylla*	Acidic, wet, side dish	*Leuc. mesenteroides, Ped. cerevisiae, Lb. plantarum, Lb. germentum, Lb. buchneri*	Thailand	Phithakpol et al., 1995
Pao cai	Cabbage	Sweet and sour rather than spicy, Breakfast	*Lb. pentosus*, *Lb. plantarum*, *Lb. brevis*, *Lb. lactis*, *Lb. fermentum, and Leuc. mesenteroides,* and *Ped. pentosaceus*	China	Yan et al., 2008
Sauerkraut	Cabbage	Acidic, sour, wet, salad, side dish	*Leuc. mesenteroides, Ped. pentosaceus; Lb. brevis, Lb. plantarum, Lb. sakei*	Europe, USA, Canada, Australia	Johanningsmeier et al., 2007
Sayur asin	Mustard leaves, cabbage, salt, coconut	Acidic, sour, wet, salad	*Leuc. mesenteroides, Lb. plantarum, Lb. brevis, Lb. confuses*, *Ped. pentosaceus*.	Indonesia	Puspito and Fleet, 1985
Soibum	Bamboo shoot	Acidic, sour, soft, curry	*Lb. plantarum, Lb. brevis, Lb. coryniformis, Lb. delbrueckii, Leuc. fallax, Leuc. Lact. lactis, Leuc. mesenteroides, Ent. durans, Strep. lactis, B. subtilis, B. lichniformis, B. coagulans, B. cereus, B. pumilus, Pseudomonas fluorescens*, *Saccharomyces* sp., *Torulopsis* sp.	India	Tamang et al., 2008; Jeyaram et al., 2010
Soidon	Bamboo shoot tips	Acidic, sour, soft, curry	*Lb. brevis, Lb. plantarum*, uncultured *Lb. acetotolera, Leuc. fallax, Leuc. citreumns*, *Lc. lactis* subsp. *cremoris*, *Weissella cibaria*, uncultured *W. ghanensis*	India	Tamang et al., 2008; Romi et al., 2015
Sinki	Radish tap-root	Acidic, sour, dry, soup, pickle	*Lb. plantarum, Lb. brevis, Lb. casei*, *Leuc. fallax*	India, Nepal, Bhutan	Tamang and Sarkar, 1993; Tamang et al., 2005
Suan-cai	Vegetables	Acidic, sour, wet	*Ped. pentosaceus, Tetragenococcus halophilus*	China	Chen et al., 2006
Suan-tsai	Mustard	Acidic, sour, dry	*Ent. faecalis, Lb. alimentarius, Lb. brevis, Lb. coryniformis, Lb. farciminis, Lb. plantarum, Lb. versmoldensis, Leuc. citreum, Leuc. mesenteroides, Leuc. pseudomesenteroides, Ped. pentosaceus, W. cibaria, W. paramesenteroides*	Taiwan	Chao et al., 2009
Sunki	Turnip	Acidic, sour, wet	*Lb. plantarum, Lb. fermentum, Lb. delbrueckii, Lb. parabuchneri, Lb. kisonensis*, *Lb. otakiensis*, *Lb. rapi*, *Lb. sunkii*	Japan	Endo et al., 2008; Watanabe et al., 2009a
Takuanzuke	Japanese radish, salt, sugar, *Shochu*	Pickle radish	*Lb. plantarum, Lb. brevis, Leuc. mesenteroides, Streptococcus* sp., *Pediococcus* sp., yeasts	Japan	Alexandraki et al., 2013
Tuaithur	Bamboo shoot	Solid, wet, sour, curry	*Lb. plantarum, Lb. brevis, Ped. pentosaceou, Lc. lactis, Bacillus circulans, B. firmus, B. sphaericus, B. subtilis*	India	Chakrabarty et al., 2014

(Tamang, 2015b). Within the KNT-triangle-bound countries, *Bacillus*-fermented sticky non-salty soybean foods are consumed such as *natto* of Japan, *chungkokjang* of Korea, *kinema* of India, Nepal and Bhutan, *aakhune, bekang*, *hawaijar*, *peruyaan*, and *tungrymbai* of India, *thua nao* of Thailand, *pepok* of Myanmar, and *sieng* of Cambodia and Laos (Nagai and Tamang, 2010; Tamang, 2015b; **Table 4**). Although, the method of production and culinary practices vary from product to product, plasmids, and phylogenetic analysis of *B. subtilis* showed the similarity among the strains of *B. subtilis* isolated from common sticky

TABLE 4 | Microorganisms isolated from some common and uncommon fermented legume (soybeans and non-soybean) products of the world.

Product	Substrate/Raw material	Sensory features and nature	Microorganisms	Country	References
Bekang	Soybean	Alkaline, sticky, paste, curry	*B. subtilis, B. brevis, B. circulans, B. coagulans, B. licheniformis, B. pumilus, B. sphaericus,* and *Lysinibacillus fusiformis*	India	Chettri and Tamang, 2015
Bhallae	Black gram	Mild acidic, side dish	*B. subtilis, Candida curvata, C. famata, C. membraneafaciens, C. variovaarai, Cryptococcus humicoius, Deb. hansenii, Geotrichum candidum, Hansenula anomala, H. polymorpha, Kl. marxianus, Lb. fermentum, Leuc. mesenteroides, Ped. membranaefaciens, Rhiz. marina, Sacch. cerevisiae, Ent. faecalis, Trichosporon beigelii, Trichosporon pullulans, Wingea robertsii*	India	Rani and Soni, 2007
Bikalga	Roselle (*Hibiscus sabdariffa*)	Condiment	*B. subtilis, B. licheniformis, B. megaterium, B. pumilus*	Burkina Faso	Ouoba et al., 2008
Chungkokjang (or *jeonkukjang, cheonggukjang*	Soybean	Alkaline, sticky, soup	*B. subtilis, B. amyloliquefaciens, B. licheniformis, B. cereus, Pantoea agglomerans, Pantoega ananatis, Enterococcus* sp., *Pseudomonas* sp., *Rhodococcus* sp.	Korea	Hong et al., 2012; Nam et al., 2012
Dawadawa	Locust bean	Alkaline, sticky	*B. pumilus, B. licheniformis, B. subtilis, B. firmus, B. atrophaeus, B. amyloliquefaciens, B. mojavensis, Lysininbacillus sphaericus.*	Ghana, Nigeria	Amoa-Awua et al., 2006; Meerak et al., 2008
Dhokla	Bengal gram	Mild acidic, spongy, steamed, snack	*Leuc. mesenteroides, Lb. fermenti, Ent. faecalis, Tor. candida, Tor. pullulans*	India	Blandino et al., 2003
Douchi	Soybean	Alkaline, paste	*B. amyloliquefaciens, B. subtilis, Asp. oryzae*	China, Taiwan	Wang et al., 2006; Zhang et al., 2007
Doenjang	Soybean	Alkaline, paste, soup	*B. subtilis, B. licheniformis, B. pumilis, Mu. plumbeus, Asp. oryzae, Deb. hansenii, Leuc. mesenteroides, Tor. halophilus, Ent. faecium, Lactobacillus* sp.	Korea	Kim et al., 2009; Nam et al., 2011
Furu	Soybean curd	Mild acidic	*B. pumilus, B. megaterium, B. stearothermophilus, B. firmus, Staph. hominis*	China	Sumino et al., 2003
Gochujang	Soybean, red pepper	Hot-flavored seasoning	*B. velegensis, B. amyloliquefacious, B. subtilis, B. liqueformis,* spcecis of *Oceanobacillus, Zygosaccharomyses, Candida lactis, Zygorouxii, Aspergillus, Penicillium, Rhizopus*	Korea	Shin et al., 2012
Hawaijar	Soybean	Alkaline, sticky	*B. subtilis, B. licheniformis, B. amyloliquefaciens, B. cereus, Staph. aureus, Staph. sciuri, Alkaligenes* sp., *Providencia rettgers, Proteus mirabilis*	India	Jeyaram et al., 2008b; Singh et al., 2014
Iru	Locust bean	Alkaline, sticky	*B. subtilis, B. pumilus, B. licheniformis, B. megaterium, B. fumus, B. atrophaeus, B. amyloliquefaciens, B. mojavensis, Lysininbacillus sphaericus, Staph. saprophyticus*	Nigeria, Benin	Meerak et al., 2008
Kanjang	Soybean, *meju*, salt, water	Soya sauce	*Asp. oryzae, B. subtilis, B. pumillus, B. citreus, Sarcina mazima, Sacch. rouxii*	Korea	Shin et al., 2012
Kawal	Leaves of legume (*Cassia* sp.)	Alkaline, strong flavored, dried balls	*B. subilis, propionibacterium* sp., *Lb. plantarum, Staph. sciuri,* yeasts	Sudan	Dirar et al., 2006
Kecap	Soybean, wheat	Liquid	*Rhiz. oligosporus, Rhiz. oryzae, Asp. oryzae, Ped. halophilus, Staphylococcus* sp., *Candida* sp., *Debaromyces* sp., *Sterigmatomyces* sp.	Indonesia	Alexandraki et al., 2013
Ketjap	Soybean (black)	Syrup	*Asp. oryzae, Asp. flavus, Rhiz. oligosporus, Rhiz. arrhizus*	Indonesia	Alexandraki et al., 2013
Kinda	Locust bean	Alkaline, sticky	*B. pumilus, B. licheniformis, B. subtilis, B. atrophaeus, B. amyloliquefaciens, B. mojavensis, Lysininbacillus sphaericus*	Sierra Leone	Meerak et al., 2008

(Continued)

TABLE 4 | Continued

Product	Substrate/Raw material	Sensory features and nature	Microorganisms	Country	References
Kinema	Soybean	Alkaline, sticky; curry	*B. subtilis, B. licheniformis, B. cereus, B. circulans, B. thuringiensis, B.* sphaericus, *Ent. faecium, Cand. parapsilosis, Geotrichum candidum*	India, Nepal, Bhutan	Sarkar et al., 1994; Tamang, 2003
Maseura	Black gram	Dry, ball-like, brittle, condiment	*B. subtilis, B. mycoides, B. pumilus, B. laterosporus, Ped. acidilactici, Ped. pentosaceous, Ent. durans, Lb. fermentum, Lb. salivarius, Sacch. cerevisiae, Pic. burtonii, Cand. castellii*	Nepal, India	Chettri and Tamang, 2008
Meitauza	Soybean	Liquid	*B. subtilis, Asp. oryzae, Rhiz. oligosporus, Mu. meitauza, Actinomucor elegans*	China, Taiwan	Zhu et al., 2008
Meju	Soybean	Alkaline, paste	*Asp. flavus, Asp. fumigatus, Asp. niger, Asp. oryzae, Asp. retricus, Asp. spinosa, Asp. terreus, Asp. wentii, Botrytis cineara, Mu. adundans, Mu. circinelloides, Mu. griseocyanus, Mu. hiemalis, Mu. jasseni, Mu. racemosus, Pen. citrinum, Pen. griseopurpureum, Pen. griesotula, Pen. kaupscinskii, Pen. lanosum, Pen. thomii, Pen. turalense, Rhi. chinensis, Rhi. nigricans, Rhi. oryzae, Rhi. Sotronifer; Candida edax, Can. incommenis, Can. utilis Hansenula anomala, Han. capsulata, Han. holstii, Rhodotorula flava, Rho. glutinis, Sacch. exiguus, Sacch. cerevisiae, Sacch. kluyveri, Zygosaccharomyces japonicus, Zyg. rouxii, B. citreus, B. circulans, B. licheniformis, B. megaterium, B. mesentricus, B. subtilis, B. pumilis, Lactobacillus* sp., *Ped. acidilactici*	Korea	Choi et al., 1995
Miso	Soybean	Alkaline, paste	*Ped. acidilactici, Leuc. paramesenteroides, Micrococcus halobius, Ped. halophilus, Streptococcus* sp., *Sacch. rouxii, Zygosaccharomyces rouxii, Asp. oryzae*	Japan	Asahara et al., 2006; Sugawara, 2010
Natto	Soybean	Alkaline, sticky, breakfast	*B. subtilis (natto)*	Japan	Nagai and Tamang, 2010
Oncom Hitam (Black *Oncom*) and *Oncom Merah* (Orange *Oncom)*	Peanut press cake, tapioca, soybean curd starter	Fermented peanut press cake, roasted or fried	*Neurosporaintermedia, N. crassa, N. sitophila* (from red *oncom), Rhi. oligosporus* (from black *oncom)*	Indonesia	Ho, 1986
Ogiri / Ogili	Melon Seeds, castor oil seeds, pumpkin bean, sesame		*B. subtilis, B. pumilus, B. licheniformis, B. megaterium, B. rimus, Pediococcus* sp., *Staph. saprophyticus, Lb. plantarum*	West, East and Central Africa	Odunfa and Oyewole, 1997
Okpehe	Seeds from *Prosopis africana*	Alkaline, sticky	*B. subtilis, B. amyloliquefaciens, B. cereus*, *B. licheniformis*	Nigeria	Oguntoyinbo et al., 2010
Soumbala	Locust bean	Alkaline, sticky	*B. pumilus, B. atrophaeus, B. amyloliquefaciens, B. mojavensis, Lysininbacillus sphaericus. B. subtilis, B. thuringiensis, B. licheniformis, B. cereus, B. badius, B. firmus, B. megaterium, B. mycoides, B. sphaericus, Peanibacillus alvei, Peanibacillus larvae, Brevibacillus laterosporus*	Burkina Faso	Ouoba et al., 2004
Shoyu	Soybean	Alkaline, liquid, seasoning	*Asp. oryzae* or *Asp. sojae*, *Z. rouxii, C. versatilis*	Japan, Korea, China	Sugawara, 2010
Sufu	Soybean curd	Mild-acidic, soft	*Actinomucor elenans, Mu. silvatixus, Mu. corticolus, Mu. hiemalis, Mu. praini, Mu. racemosus, Mu. subtilissimus, Rhiz. chinensis*	China, Taiwan	Han et al., 2001; Chao et al., 2008

(Continued)

TABLE 4 | Continued

Product	Substrate/Raw material	Sensory features and nature	Microorganisms	Country	References
Tauco	Soybean	Alkaline, paste, use as flavoring agent	*Rhiz. oryzae, Rhiz. ologosporus, Asp. oryzae, Zygosaccharomyces soyae, Bacillus* sp., *Ent. hermanniensis, Lb. agilis, Lb. brevis, Lb. buchneri, Lb. crispatus, Lb. curvatus, Lb. delbrueckii, Lb. farciminis, Lb. fermentum, Lb. pantheris, Lb. salivarius, Lb. vaccinostercus, Lc. lactis, Lactococcus* sp., *Leuc. camosum, Leuc. citreum, Leuc. fallax, Leuc. lactis, Leuc. mesenteroides, Leuc. pseudomesenteroides, Ped. acidilactici, Strep. bovis, Strep. macedonicus, W. cibaria, W. confusa, W. paramesenteroides, W. soli*	Indonesia	Winarno et al., 1973
Tempe	Soybean	Alkaline, solid, fried cake, breakfast	*Rhiz. oligisporus, Rhiz. arrhizus, Rhiz. oryzae, Rhiz. stolonifer, Asp. niger, Citrobacter freundii, Enterobacter cloacae, K. pneumoniae, K. pneumoniae* subsp. *ozaenae, Pseudomas fluorescens* as vitamin B_{12}-producing bacteria, *Lb. fermentum*, *Lb. lactis, Lb. plantarum*, *Lb. reuteri*	Indonesia (Origin), The Netherlands, Japan, USA	Feng et al., 2005; Jennessen et al., 2008
Thua nao	Soybean	Alkaline, paste, dry, side dish	*B. subtilis, B. pumilus, Lactobacillus* sp.	Thailand	Chunhachart et al., 2006
Tungrymbai	Soybean	Alkaline, sticky, curry, soup	*B. subtilis, B. licheniformis*, *B. pumilus*	India	Chettri and Tamang, 2015
Ugba	African oil bean (*Pentaclethra macrophylla*)	Alkaline, flat, glossy, brown in color	*B. subtilis, B. pumilus, B. licheniformis, Staph. saprophyticus*	Nigeria	Ahaotu et al., 2013
Wari	Black gram	Ball-like, brittle, side dish	*B. subtilis, Cand. curvata, Cand. famata, Cand. krusei, Cand. parapsilosis, Cand. vartiovaarai, Cryptococcus humicolus, Deb. hansenii, Deb. tamarii, Geotrichum candidum, Hansenula anomala, Kl. marxianus, Sacch. cerevisiae, Rhiz. lactosa, Ent. faecalis, Wingea robetsii, Trichosporon beigelii*	India	Rani and Soni, 2007
Yandou	Soybean	Alkaline, sticky, salted, snack	*B. subtilis*	China	Qin et al., 2013

fermented soybean foods of Asia (Hara et al., 1986, 1995; Tamang et al., 2002; Meerak et al., 2007) suggesting the common stock of *Bacillus*. Mould-fermented soybean products are *miso* and *shoyu* of Japan, *tempe* of Indonesia, *douchi* and *sufu* of China, and *doenjang* of Korea (Sugawara, 2010). Some common non-soybean fermented legumes (e.g., locust beans) are *bikalga, dawadawa, iru, okpehe, soumbala,* and *dugba* of Africa (Ouoba et al., 2004, 2008, 2010; Amoa-Awua et al., 2006; Azokpota et al., 2006; Oguntoyinbo et al., 2007, 2010; Meerak et al., 2008; Parkouda et al., 2009; Ahaotu et al., 2013), fermented black-grams products such as *dhokla, papad,* and *wari* of India (Nagai and Tamang, 2010), and *maseura* of India and Nepal (Chettri and Tamang, 2008).

Species of *Bacillus* have been reported for several Asian fermented soybean foods (Sarkar et al., 2002; Tamang et al., 2002; Tamang, 2003; Park et al., 2005; Inatsu et al., 2006; Choi et al., 2007; Kimura and Itoh, 2007; Shon et al., 2007; Jeyaram et al., 2008b; Dajanta et al., 2009; Kwon et al., 2009; Kubo et al., 2011; Singh et al., 2014; Wongputtisin et al., 2014; Chettri and Tamang, 2015). However, *B. subtilis* is the dominant functional bacterium in Asian fermented soybean foods (Sarkar and Tamang, 1994; Tamang and Nikkuni, 1996; Dajanta et al., 2011). Japanese *natto* is the only *Bacillus*-fermented soybean food now produced by commercial monoculture starter *B. natto*, earlier isolated from naturally fermented *natt*o by Sawamura (Sawamura, 1906). *Ent. Faecium*, as a minor population group, is also present in *kinema* (Sarkar et al., 1994), in *okpehe* (Oguntoyinbo et al., 2007), and in *chungkukjang* (Yoon et al., 2008).

Fermented Root and Tuber Products

Cassava (*Manihot esculenta*) root is traditionally fermented into staple foods such as *gari* in Nigeria; *fufu* in Togo, Burkina Faso, Benin and Nigeria; *agbelima* in Ghana; *chikawgue* in Zaire; *kivunde* in Tanzania; *kocho* in Ethiopia; and *foo foo* in Nigeria, Benin, Togo, and Ghana, respectively (Franz et al., 2014; **Table 5**). The initial stage of cassava fermentation is dominated by *Corynebacterium manihot* (Oyewole et al., 2004) with LAB succession (*Lb. acidophilus*, *Lb. casei, Lb. fermentum, Lb. pentosus, Lb. plantarum,* Oguntoyinbo and Dodd, 2010)**.** Cassava root is also traditionally fermented into sweet dessert known as *tapé* in Indonesia (Tamang, 2010b).

Fermented Meat Products

Fermented meat products are divided into two categories: those made from whole meat pieces or slices such as dried meat and jerky; and those made by chopping or comminuting the meat,

TABLE 5 | Microorganisms isolated from some fermented root crop products of the world.

Product	Substrate/raw materials	Sensory property and nature	Microorganisms	Country	References
Chikwangue	Cassava	Solid state, staple	Species of *Corynebacterium, Bacillus, Lactobacillus, Micrococcus, Pseudomonas, Acinetobacter, Moraxella*	Central Africa, Zaire	Odunfa and Oyewole, 1997
Cingwada	Cassava	Solid state	Species of *Corynebacterium, Bacillus., Lactobacillus, Micrococcus*	East and Central Africa	Odunfa and Oyewole, 1997
Fufu	Cassava	Submerged, staple	*Bacillus* sp., *Lb. plantarum, Leuc. mesenteroides, Lb. cellobiosus, Lb. brevis; Lb. coprophilus, Lc. lactis; Leuc. lactis, Lb. bulgaricus, Klebsiella* sp., *Leuconostoc* sp., *Corynebacterium* sp., *Candida* sp.	West Africa	Odunfa and Oyewole, 1997
Gari	Cassava	Solid state, staple	*Corynebacterium mannihot, Geotrichum* sp., *Lb. plantarium, Lb. buchnerri, Leuconsostoc* sp., *Streptococcus* sp.	West and Central Africa	Oyewole et al., 2004
Lafun /Konkonte	Cassava	Submerged, staple	*Bacillus* sp., *Klebsiella* sp., *Candida* sp., *Aspergillus* sp., *Leuc. mesenteroides, Corynebacterium manihot, Lb. plantarum, Micrococcus luteus, Geotrichum candidum*	West Africa	Odunfa and Oyewole, 1997
Tapé	Cassava	Sweet dessert	*Streptococcus* sp., *Rhizopus* sp., *Saccharomycopsisfibuligera*	Indonesia	Suprianto Ohba et al., 1989
Tapai Ubi	Cassava, *Ragi*	Sweet dessert	*Saccharomycopsis fibuligera*, *Amylomyces rouxii*, *Mu. circinelloides*, *Mu. javanicus*, *Hansenula* spp, *Rhi. arrhizus, Rhi. oryzae*, *Rhi. chinensis*	Malaysia	Merican and Yeoh, 1989

usually called sausages (Adams, 2010). Traditionally fermented meat products of many countries have been well-documented (**Table 6**), such as fermented sausages (Lücke, 2015) and *salami* (Toldra, 2007) of Europe, jerky of America and Africa (Baruzzi et al., 2006), *nham* of Thailand (Chokesajjawatee et al., 2009), and *nem chua* of Vietnam (Nguyen et al., 2013b). The main microbial groups involved in meat fermentation are LAB (Albano et al., 2009; Cocolin et al., 2011; Khanh et al., 2011; Nguyen et al., 2013b), followed by coagulase-negative staphylococci, micrococci and *Enterobacteriaceae* (Cocolin et al., 2011; Marty et al., 2011), and depending on the product, some species of yeasts (Encinas et al., 2000; Tamang and Fleet, 2009), and molds, which may play a role in meat ripening (Lücke, 2015).

Fermented Fish Products

Preservation of fish through fermentation, sun/smoke drying and salting (**Table 7**) is traditionally practiced by people living nearby coastal regions, lakes, and rivers and is consumed as seasoning, condiments, and side dishes (Salampessy et al., 2010). Several species of bacteria and yeasts have been reported from fermented and traditionally preserved fish products of the world (Kobayashi et al., 2000a,b,c; Wu et al., 2000; Thapa et al., 2004, 2006, 2007; Saithong et al., 2010; Hwanhlem et al., 2011; Romi et al., 2015).

Miscellaneous Fermented Products

Vinegar is one of the most popular condiments in the world and is prepared from sugar or ethanol containing substrates and hydrolyzed starchy materials by aerobic conversion to acetic acid (Solieri and Giudici, 2008). *Acetobacter aceti* subsp. *aceti, Acetobacter pasteurianus, Acetobacter polyxygenes, Acetobacter xylinum, Acetobacter malorum*, *Acetobacter pomorum* dominate during vinegar production (Haruta et al., 2006), while yeast species such as *Candida lactis-condensi*, *Candida stellata*, *Hanseniaspora valbyensis*, *Hanseniaspora osmophila*, *Saccharomycodes ludwigii, Sacch. cerevisiae, Zygosaccharomyces bailii, Zygosaccharomyces bisporus, Zygosaccharomyces lentus*, *Zygosaccharomyces mellis*, *Zygosaccharomyces Pseudorouxii,* and *Zygosaccharomyces Rouxii* have also been reported (Sengun and Karabiyikli, 2011).

Though normal black tea is consumed everywhere, some ethnic Asian communities enjoy special fermented teas such as *miang* of Thailand (Tanasupawat et al., 2007) and *puer* tea, *fuzhuan brick*, and *kombucha* of China (Mo et al., 2008). *Aspergillus niger* is the predominant fungus in *puer* tea while *Blastobotrys adeninivorans, Asp. glaucus*, species of *Penicillium*, *Rhizopus,* and *Saccharomyces* and the bacterial species *Actinoplanes* and *Streptomyces* are isolated (Jeng et al., 2007; Abe et al., 2008). *Brettanomyces bruxellensis, Candida stellata*, *Rhodotorula mucilaginosa, Saccharomyces* spp., *Schizosaccharomyces pombe, Torulaspora delbrueckii, Zygosaccharomyces bailii, Zygosaccharomyces bisporus, Zygosaccharomyces kombuchaensis,* and *Zygosaccharomyces microellipsoides* are also isolated from *kombucha* (Kurtzman et al., 2001; Teoh et al., 2004). Major bacterial genera present in *kombucha* are *Gluconacetobacter*. However, Marsh et al. (2014) reported the predominance of *Lactobacillus*, *Acetobacter,* and *Zygosaccharomyces*. *Lb. thailandensis, Lb. camelliae*, *Lb. plantarum, Lb. pentosus, Lb. vaccinostercus*, *Lb. pantheris, Lb. fermentum, Lb. suebicus, Ped. siamensis, Ent. casseliflavus* and *Ent. camelliae* in the fermentation of *miang* production (Sukontasing et al., 2007; Tanasupawat et al., 2007). Species of *Aspergillus, Penicillium,* and *Eurotium* are major fungi for fermentation of *fuzhuan brick* tea (Mo et al., 2008).

TABLE 6 | Microorganisms isolated from some common and uncommon fermented meat products of the world.

Product	Substrate/Raw materials	Sensory property and nature	Microorganisms	Country	References
Alheira	Pork or beef, bread chopped fat, spices, salt	Dry/Semi-dry, sausage	*Lb. plantarum, Lb. paraplantarum, Lb. brevis, Lb. rhamnosus, Lb. sakei, Lb. zeae, Lb. paracasei, Ent. faecalis, Ent. faecium, Leuc. mesenteroides, Ped. pentosaceus, Ped. acidilactici, W. cibaria, W. viridescens*	Portugal	Albano et al., 2009
Androlla	Pork, coarse chopped, spices, salt	Dry, pork sausage	*Lb. sake, Lb. curvatus, Lb. plantarum*	Spain	Garcia-Fontan et al., 2007
Arjia	Large intestine of chevon	Sausage, curry	*Ent. faecalis*, *Ent. faecium*, *Ent. hirae*, *Leuc. citreum*, *Leuc. mesenteroides*, *Ped. pentosaceus*, *Weissella cibaria*	India, Nepal	Oki et al., 2011
Chartayshya	Chevon	Dried, smoked meat, curry	*Ent. faecalis*, *Ent. faecium*, *Ent. hirae*, *Leuc. citreum*, *Leuc. mesenteroides*, *Ped. pentosaceus*, *Weissella cibaria*	India	Oki et al., 2012
Chorizo	Pork	Dry, coarse chopped, spices, salt; sausage	*Lb. sake, Lb. curvatus, Lb. plantarum*	Spain	Garcia-Fontan et al., 2007
Kargyong	Yak, beef, pork, crushed garlic, ginger, salt	Sausage like meat product, curry	*Lb. sakei, Lb. divergens, Lb. carnis, Lb. sanfranciscensis, Lb. curvatus, Leuc. mesenteroides, Ent. faecium, B. subtilis, B. mycoides, B. thuringiensis, Staph. aureus, Micrococcus* sp., *Deb. hansenii, Pic. anomala*	India	Rai et al., 2010
Nham (Musom)	Pork meat, pork skin, salt, rice, garlic	Fermented pork	*Ped. cerevisiae, Lb. plantarum, Lb. brevis*	Thailand	Chokesajjawatee et al., 2009
Nem-chua	Pork, salt, cooked rice	Fermented sausage	*Lb. pentosus, Lb. plantarum, Lb. brevis*, *Lb. paracasei*, *Lb. fermentum, Lb. acidipiscis, Lb. farciminis*, *Lb. rossiae*, *Lb. fuchuensis*, *Lb. namurensis, Lc. lactis, Leuc. citreum*, *Leuc. fallax, Ped. acidilactici, Ped. pentosaceus*, *Ped. stilesii*, *Weissella cibaria, W. paramesenteroides*	Vietnam	Nguyen et al., 2011
Pastirma	Chopped beef meat with lamb fat, heavily seasoned	Dry/semi-dry, sausage	*Lb. plantarum, Lb. sake, Pediococcus, Micrococcus, Staph. xylosus, Staph. carnosus*	Turkey, Iraq	Aksu et al., 2005
Peperoni	Pork, beef	Dried meat, smoked, sausage	Species of LAB, *Micrococcus* spp.	Europe, America, Australia	Adams, 2010
Sai-krok-prieo	Pork, rice, garlic, salt	Fermented sausage	*Lb. plantarum, Lb. salivarius, Ped. pentosacuns*	Thailand	Adams, 2010
Salchichon	Pork or beef meat, fat, NaCl, spices	Dry, sausage	Species of LAB, *Staph*. spp., *Micrococcus* spp., enterobacteriaceae, molds	Spain	Fernandez-Lopez et al., 2008
Salsiccia	Chopped pork meat, spices, NaCl	Dry/ semi-dry, sausage	Species of LAB, *Staph*. spp., *Micrococcus* spp., enterobacteriaceae, yeast	Italy	Parente et al., 2001a,b
Soppressata	Chopped lean pork meat, NaCl and spices	Dry/ semi-dry, sausage	Species of LAB, *Staph*. spp., *Micrococcus* spp., enterobacteriaceae, yeast	Italy	Parente et al., 1994
Sucuk	Chopped meat, pork or beef, curing salts and various spices	Dry, sausage	Species of LAB, *Staph*. spp., *Micrococcus* spp., enterobacteriaceae	Turkey	Genccelep et al., 2008
Suka ko masu	Goat, buffalo meat, turmeric powder, mustard oil, salt	Dried or smoked meat, curry	*Lb. carnis, Ent. faecium, Lb. plantarum, B. subtilis, B. mycoides, B. thuringiensis, Staph. aureus, Micrococcus* sp., *Debaromyces hansenii, Pic. burtonii*	India	Rai et al., 2010
Tocino	Pork, salt, sugar, potassium nitrate	Fermented cured pork	*Ped. cerevisiae, Lb. brevis, Leuc. mesenteroides*	Philippines	Alexandraki et al., 2013

Nata or bacterial cellulose produced by *Acetobacter xylinum* is a delicacy of the Philippines, eaten as candy (Chinte-Sanchez, 2008; Jagannath et al., 2010; Adams, 2014). Two types of *nata* are well-known: *nata de piña,* produced on the juice from pineapple trimmings, and *nata de coco,* produced on coconut water or coconut skim milk (Adams, 2014). Bacterial cellulose has significant potential as a food ingredient in view of its high purity, *in situ* change of flavor and color, and having the ability to form various shapes and textures (Shi et al., 2014).

Chocolate is a product of cocoa bean fermentation where *Lb. fermentum* and *Acetobacter pasteurianus* are reported as the predominating bacterial species (Lefeber et al., 2010; Papalexandratou et al., 2011). Diverse LAB species appear to be typically associated with the fermentation of cocoa

TABLE 7 | Microorganisms isolated from some common and uncommon fermented fish products of the world.

Product	Substrate/raw materials	Sensory property and nature	Microorganisms	Country	References
Balao-balao (Burong Hipon Tagbilao)	Shrimp, rice, salt	Fermented rice shrimp, condiment	*Leuc. mesenteroides, Ped. cerevisiae, Lb. plantarum, Lb. brevis, Ent. faecalis*	Philippines	Alexandraki et al., 2013
Belacan (Blacan)	Shrimp, salt	Paste, condiment	*Bacillus*, *Pediococcus*, *Lactobacillus*, *Micrococcus*, *Sarcina*, *Clostridium*, *Brevibacterium*, *Flavobacterium*, *Corynebacteria*	Malaysia	Salampessy et al., 2010
Bakasang	Fish, shrimp	Paste, condiment	*Pseudomonas*, *Enterobacter*, *Moraxella*, *Micrococcus*, *Streptococcus, Lactobacillus*, *Pseudomonas*, *Moraxella*, *Staphylococcus*, *Pediococcus* spp.	Indonesia	Ijong and Ohta, 1996
Burong Bangus	Milkfish, rice, salt, vinegar	Fermented milkfish, sauce	*Leuc. mesenteroides, Lb. plantarum, W. confusus*	Philippines	Dalmacio et al., 2011
Burong Isda	Fish, rice, salt	Fermented fish, sauce	*Leuc. mesenteroides, Ped. cerevisiae, Lb. plantarum, Strep. faecalis, Micrococcus* sp.	Philippines	Sakai et al., 1983
Budu	Marine fishes, salt, sugar	Muslim sauce, fish sauce	*Ped. halophilus, Staph. aureus, Staph. epidermidis, B. subtilis, B. laterosporus, Proteus* sp.*, Micrococcus* sp.*, Sarcina* sp.*, Corynebacterium* sp.	Thailand, Malayasia	Phithakpol et al., 1995
Gnuchi	Fish (*Schizothorax richardsonii*), salt, turmeric powder	Eat as curry	*Lb. plantarum, Lact. lactis, Leuc. mesenteroides, Ent. faecium, Ent. faecalis, Ped. pentosaceus, Cand. chiropterorum, Cand. bombicola, Saccharomycopsis* sp.	India	Tamang et al., 2012
Gulbi	Shell-fish	Salted and dried, side dish	*Bacillus licheniformis, Staphylococcus* sp., *Aspergillus* sp., *Candida* sp.	Korea	Kim et al., 1993
Hentak	Finger sized fish (*Esomus danricus*)	Condiment	*Lact. lactis, Lb. plantarum, Lb. fructosus, Lb. amylophilus, Lb. coryniformis, Ent. faecium, B. subtilis, B. pumilus, Micrococcus* sp.*, Candida* sp.*, Saccharomycopsis* sp.	India	Thapa et al., 2004
Hoi-malaeng pu-dong	Mussel (*Mytilus smaragdinus*), salt	Fermented mussel	*Ped. halophilus, Staph. aureus, Staph. epidermidis*	Thailand	Phithakpol et al., 1995
Ika-Shiokara	Squid, salt	Fermented squid	*Micrococcus* sp.*, Staphylococcus* sp.*, Debaryomyces* sp.	Japan	Alexandraki et al., 2013
Jeotkal	Fish	High-salt fermented, staple	LAB, halophilicFirmicutes including *Staphylococcus, Salimicrobium,* and *Alkalibacillus.* Also *Halanaerobium* and halophilic archaea.	Korea	Guan et al., 2011; Jung et al., 2013b
Karati, Bordia, Lashim	Fish (*Gudushia chapra, Pseudeutropius atherinoides, Cirrhinus reba*), salt	Dried, salted, side dish	*Lact. lactis, Leuc. mesenteroides, Lb. plantarum, B. subtilis, B. pumilus, Candida* sp.	India	Thapa et al., 2007
Kusaya	Horse mackerel, salt	Fermented dried fish	*Corynebacterium kusaya, Spirillum* sp.*, C. bifermentans, Penicillium* sp.	Japan	Alexandraki et al., 2013
Myulchijeot	Small sardine, salt	Fermented sardine	*Ped. cerevisiae, Staphylococcus* sp.*, Bacillus* sp.*, Micrococcus* sp.	Korea	Alexandraki et al., 2013
Narezushi	Sea water fish, cooked millet, salt	Fermented fish-rice	*Leuc. mesenteroides, Lb. plantarum*	Japan	Alexandraki et al., 2013
Nam pla (Nampla-dee, Nampla-sod)	*Solephorus* sp., *Ristelliger* sp. *Cirrhinus* sp., water, salt	Fish sauce	Species of *Micrococcus*., *Pediococcus*, *Staphylococcus*., *Streptococcus*., *Sarcina*., *Bacillus*., *Lactobacillus, Corynebacterium, Pseudomonas, Halococcus, Halobacterium*	Thailand	Saisithi, 1987
Ngari	Fish (*Puntius sophore*), salt	Fermented fish	*Lact. lactis, Lb. plantarum, Lb. pobuzihii*, *Lb. fructosus, Lb. amylophilus, Lb. coryniformis, Ent. faecium, B. subtilis, B. pumilus, B indicu, s Micrococcus* sp.*, Staphy. cohnii* subsp. *cohnii*, *Staphy. carnosus, Tetragenococcus halophilus* subsp. *flandriensis*, *Clostridium irregular, Azorhizobium caulinodans, Candida* sp.*, Saccharomycopsis* sp.	India	Thapa et al., 2004; Devi et al., 2015

(Continued)

TABLE 7 | Continued

Product	Substrate/raw materials	Sensory property and nature	Microorganisms	Country	References
Nuoc mam	Marne fish	Fish sauce, condiment	*Bacillus* sp., *Pseudomonas* sp., *Micrococcus* sp., *Staphylococcus* sp., *Halococcus* sp., *Halobacterium salinarium*, *H. cutirubrum*	Vietnam	Lopetcharat et al., 2001
Patis	*Stolephorus* sp., *Clupea* sp., *Decapterus* sp., *Leionathus* sp., salt	Fish sauce	*Ped. halophilus, Micrococcus* sp., *Halobacterium* sp., *Halococcus* sp., *Bacillus* sp.	Philippines, Indonesia	Steinkraus, 1996
Pla-paeng-daeng	Marine fish, red molds rice (*Ang-kak*), salt	Red fermented fish	*Pediococcus* sp., *Ped. halophilus, Staph. aureus, Staph. epidermidis,*	Thailand	Phithakpol et al., 1995
Pla-som (Pla-khao-sug)	Marine fish, salt, boiled rice, garlic	Fermented fish, condiment	*Ped. cerevisiae, Lb. brevis, Staphylococcus* sp., *Bacillus* sp.	Thailand	Saithong et al., 2010
Saeoo Jeot (Jeotkal)	Shrimp (*Acetes chinensis*), salt	Fermented shrimp	*Halobacterium* sp., *Pediococcus* sp.	Korea	Guan et al., 2011
Shidal	*Puntis*	Semi-fermented, unsalted product; 4–6 months fermentation; curry/pickle	*Staphy. aureus, Micrococcus* spp., *Bacillus* spp., *E. coli*)	India, Bangladesh	Muzaddadi, 2015
Shottsuru	Anchovy, opossum shrimp, salt	Fish sauce, condiment	*Halobacterium* sp., *Aerococcus viridians (Ped. homari),* halotolerant and halophilic yeasts	Japan	Itoh et al., 1993
Sidra	Fish (*Punitus sarana*)	Dried fish, curry	*Lact. lactis, Lb. plantarum, Leuc. mesenteroides, Ent. faecium, Ent. facalis, Ped. pentosaceus, W. confusa, Cand. chiropterorum, Cand. bombicola, Saccharomycopsis* sp.	India	Thapa et al., 2006
Sikhae	Sea water fish, cooked millet, salt	Fermented fish-rice, sauce	*Leuc. mesenteroides, Lb. plantarum*	Korea	Lee, 1993
Suka ko maacha	River fish (*Schizothorax richardsoni*), salt, turmeric powder	Smoked, dried, curry	*Lact. lactis, Lb. plantarum, Leuc. mesenteroides, Ent. faecium, Ent. faecalis, Ped. pentosaceus, Cand. chiropterorum, Cand. bombicola, Saccharomycopsis* sp.	India	Thapa et al., 2006
Sukuti	Fish (*Harpodon nehereus*)	Pickle, soup and curry	*Lact. lactis, Lb. plantarum, Leuc. mesenteroides, Ent. faecium, Ent. faecalis, Ped. pentosaceus, Cand. chiropterorum, Cand. bombicola, Saccharomycopsis* sp.	India	Thapa et al., 2006
Surströmming	Fish	Fermented herrings	*Haloanaerobium praevalens*	Sweden	Kobayashi et al., 2000a
Tungtap	Fish	Fermented fish, paste, pickle	*Lc. lactis* subsp. *cremoris, Lc. plantarumEnt. faecium, Lb. fructosus, Lb. amylophilus, Lb. corynifomis* subsp. *torquens, Lb. plantarum*, *Lb. puhozi, B. subtilis*, *B. pumilus*, *Micrococcus,* yeasts-species of *Candida, Saccharomycopsis*	India	Thapa et al., 2004; Rapsang et al., 2011

beans in Ghana, which include *Lb. ghanensis* (Nielsen et al., 2007), *Weissella ghanensis* (de Bruyne et al., 2008a), *Lb. cacaonum,* and *Lb. fabifermentans* (de Bruyne et al., 2009), and *Weissella fabaria* (de Bruyne et al., 2010). *Fructobacillus pseudoficulneus*, *Lb. plantarum*, *Acetobacter senegalensis,* and the enterobacteria *Tatumella ptyseos* and *Tatumella citrea* are among the prevailing species during the initial phase of cocoa fermentations (Papalexandratou et al., 2011). Yeasts involved during spontaneous cocoa fermentation are *Hanseniaspora uvarum, Hanseniaspora quilliermundii*, *Issatchenkia orientalis* (*Candida krusei*), *Pichia membranifaciens*, *Sacch. Cerevisiae,* and *Kluyveromyces* species for flavor development (Schillinger et al., 2010).

Pidan is a preserved egg prepared from alkali-treated fresh duck eggs and is consumed by the Chinese, and has a strong hydrogen sulfide and ammonia smell (Ganasen and Benjakul, 2010). The main alkaline chemical reagent used for making *pidan* is sodium hydroxide, which is produced by the reaction of sodium carbonate, water, and calcium oxide of pickle or coating mud. *B. cereus, B. macerans, Staph. cohnii*, *Staph. epidermidis*, *Staph. Haemolyticus,* and *Staph. warneri* are predominant in *pidan* (Wang and Fung, 1996).

Amylolytic Starters

Traditional way of culturing the essential microorganisms (consortia of filamentous molds, amylolytic, and alcohol-producing yeasts and LAB) with rice or wheat as the base in the form of dry, flattened or round balls, for production of alcoholic beverages is a remarkable discovery in the food history of Asian people, which is exclusively practiced in South-East

Asia including the Himalayan regions of India, Nepal, Bhutan, and China (Tibet; Hesseltine, 1983; Tamang, 2010a). Around 1–2% of previously prepared amylolytic starters are inoculated into the dough, and mixed cultures are allowed to develop for a short time, then dried, and used to make either alcohol or fermented foods from starchy materials (Tamang et al., 1996). Asian amylolytic starters have different vernacular names such as *marcha* in India and Nepal; *hamei, humao, phab* in India; *mana* and *manapu* of Nepal; *men* in Vietnam; *ragi* in Indonesia; *bubod* in Philippines; *chiu/chu* in China and Taiwan; *loogpang* in Thailand; *mae/dombae/buh/puh* in Cambodia; and *nuruk* in Korea (Hesseltine and Kurtzman, 1990; Nikkuni et al., 1996; Sujaya et al., 2004; Thanh et al., 2008; Yamamoto and Matsumoto, 2011; Tamang et al., 2012).

Microbial profiles of amylolytic starters of India, Nepal, and Bhutan are filamentous molds like, *Mucor circinelloides* forma *circinelloides*, *Mucor hiemalis*, *Rhi. chinensis*, and *Rhi. stolonifer* variety *lyococcus* (Tamang et al., 1988); yeasts like *Sacch. cerevisiae, Sacch. bayanus*, *Saccharomycopsis* (*Sm.*) *fibuligera, Sm. capsularis, Pichia anomala, Pic. burtonii,* and *Candida glabrata*; (Tamang and Sarkar, 1995; Shrestha et al., 2002; Tsuyoshi et al., 2005; Tamang et al., 2007; Jeyaram et al., 2008a, 2011; Chakrabarty et al., 2014); and species of LAB namely *Ped. pentosaceus*, *Lb. bifermentans,* and *Lb. brevis* (Hesseltine and Ray, 1988; Tamang and Sarkar, 1995; Tamang et al., 2007; Chakrabarty et al., 2014). A diversity of yeasts (*Candida tropicalis*, *Clavispora lusitaniae, Pichia anomala*, *Pichia ranongensis, Saccharomycopsis fibuligera*, *Sacch. cerevisiae*, *Issatchenkia* sp.); filamentous molds (*Absidia corymbifera*, *Amylomyces rouxii, Botryobasidium subcoronatum, Rhizopus oryzae*, *Rhi. microsporus*, *Xeromyces bisporus*); LAB (*Ped. pentosaceus*, *Lb. plantarum*, *Lb. brevis*, *Weissella confusa*, *Weissella paramesenteroides*); amylase-producing bacilli (*Bacillus subtilis*, *B. circulans*, *B. amyloliquefaciens*, *B. sporothermodurans*); and acetic acid bacteria (*Acetobacter orientalis*, *A. pasteurianus*) is present in *men*, a starter culture of Vietnam (Dung et al., 2006, 2007; Thanh et al., 2008).

A combination of *Asp. oryzae* and *Asp. sojae* is used in *koji* in Japan to produce alcoholic beverages including *saké* (Zhu and Trampe, 2013). *Koji* (Chinese *chu, shi,* or *qu*) also produces amylases that convert starch to fermentable sugars, which are then used for the second stage yeast fermentation to make non-alcoholic fermented soybean *miso* and *shoyu* (Sugawara, 2010). *Asp. awamori, Asp. kawachii, Asp. oryzae, Asp. shirousamii,* and *Asp. sojae* have been widely used as the starter in preparation of *koji* for production of *miso, saké, shoyu, shochu* (Suganuma et al., 2007).

Alcoholic Beverages

Tamang (2010c) classified alcoholic beverages of the world into 10 types:

(1) Non-distilled and unfiltered alcoholic beverages produced by amylolytic starters e.g., kodo ko jaanr (fermented finger millets; Thapa and Tamang, 2004) and bhaati jaanr (fermented rice) of India and Nepal (Tamang and Thapa, 2006), makgeolli (fermented rice) of Korea (Jung et al., 2012).
(2) Non-distilled and filtered alcoholic beverages produced by amylolytic starters e.g., saké of Japan (Kotaka et al., 2008).
(3) Distilled alcoholic beverages produced by amylolytic starter e.g., shochu of Japan, and soju of Korea (Steinkraus, 1996).
(4) Alcoholic beverages produced by involvement of amylase in human saliva e.g., chicha of Peru (Vallejo et al., 2013).
(5) Alcoholic beverages produced by mono- (single-strain) fermentation e.g., beer (Kurtzman and Robnett, 2003).
(6) Alcoholic beverages produced from honey e.g., tej of Ethiopia (Bahiru et al., 2006).
(7) Alcoholic beverages produced from plant parts e.g., pulque of Mexico (Lappe-Oliveras et al., 2008), toddy of India (Shamala and Sreekantiah, 1988), and kanji of India (Kingston et al., 2010).
(8) Alcoholic beverages produced by malting (germination) e.g., sorghum ("Bantu") beer of South Africa (Kutyauripo et al., 2009), pito of Nigeria, and Ghana (Kolawole et al., 2013), and tchoukoutou of Benin (Greppi et al., 2013a).
(9) Alcoholic beverages prepared from fruits without distillation e.g., wine, cider.
(10) Distilled alcoholic beverages prepared from fruits and cereals e.g., whisky and brandy.

Non-distilled Mild-Alcoholic Food Beverages Produced by Amylolytic Starters

The biological process of liquefaction and saccharification of cereal starch by filamentous molds and yeasts, supplemented by amylolytic starters, under solid-state fermentation is one of the two major stages of production of alcoholic beverages in Asia (Tamang, 2010c). These alcoholic beverages are mostly considered as food beverage and eaten as staple food with high calorie in many parts of Asia, e.g., *kodo ko jaanr* of the Himalayan regions in India, Nepal, Bhutan, and China (Tibet) with 5% alcohol content (Thapa and Tamang, 2004). Saccharifying activities are mostly shown by *Rhizopus* spp. and *Sm. fibuligera* whereas, liquefying activities are shown by *Sm. fibuligera* and *Sacch. cerevisiae* (Thapa and Tamang, 2006). *Rhizopus*, *Amylomyces*, *Torulopsis*, and *Hansenula* are present in *lao-chao*, a popular ethnic fermented rice beverage of China (Wei and Jong, 1983). During fermentation of Korean *makgeolli* (prepared from rice by amylolytic starter *nuruk*), the proportion of the *Saccharomycetaceae* family increases significantly and the major bacterial phylum of the samples shifts from γ-*Proteobacteria* to *Firmicutes* (Jung et al., 2012).

Non-Distilled and Filtered Alcoholic Beverages Produced by Amylolytic Starters

Alcoholic beverages produced by amylolytic starter (*koji*) are not distilled but the extract of fermented cereals is filtered into clarified high alcohol-content liquor, like in *sake,* which is a national drink of Japan containing 15–20% alcohol (Tamang, 2010c). Improved strains of *Asp. oryzae* are used for *saké* production in industrial scale (Kotaka et al., 2008; Hirasawa et al., 2009).

Distilled Alcoholic Beverages Produced by Amylolytic Starters

This category of alcoholic drinks is the clear distillate of high alcohol content prepared as drink from fermented cereal beverages by using amylolytic starters. *Raksi* is an ethnic alcoholic (22–27% v/v) drink of the Himalayas with aromatic characteristic, and distilled from the traditionally fermented cereal beverages (Kozaki et al., 2000).

Alcoholic Beverages Produced by Human Saliva

Chicha is a unique ethnic fermented alcoholic (2–12% v/v) beverage of Andes Indian race of South America mostly in Peru, prepared from maize by human salivation process (Hayashida, 2008). *Sacch. cerevisiae, Sacch. apiculata, Sacch. pastorianus*, species of *Lactobacillus* and *Acetobacter* are present in *chicha* (Escobar et al., 1996). *Sacch. cerevisiae* was isolated from *chicha* and identified using MALDI-TOF (Vallejo et al., 2013). Species of *Lactobacillus, Bacillus, Leuconostoc, Enterococcus, Streptomyces, Enterobacter, Acinetobacter, Escherichia, Cronobacter, Klebsiella, Bifidobacterium,* and *Propioniobacterium* have been reported from *chicha* of Brazil (Puerari et al., 2015).

Alcoholic Beverages Produced from Honey

Some alcoholic beverages are produced from honey e.g., *tej* of Ethiopia. It is a yellow, sweet, effervescent and cloudy alcoholic (7–14% v/v) beverage (Steinkraus, 1996). *Sacch. cerevisiae, Kluyvermyces bulgaricus, Debaromyces phaffi*, and *Kl. veronae*, and LAB species of *Lactobacillus*, *Streptococcus*, *Leuconostoc,* and *Pediococcus* are responsible for *tej* fermentation (Bahiru et al., 2006).

Alcoholic Beverages Produced from Plant Parts

Pulque is one of the oldest alcoholic beverages prepared from juices of the cactus (*Agave*) plant of Mexico (Steinkraus, 2002). Bacteria present during the fermentation of *pulque* were LAB (*Lc. lactis* subsp. *lactis*, *Lb. acetotolerans*, *Lb. acidophilus, Lb. hilgardii*, *Lb. kefir*, *Lb. plantarum, Leuc. citreum*, *Leuc. kimchi*, *Leuc. mesenteroides*, *Leuc. pseudomesenteroides*), the γ-Proteobacteria (*Erwinia rhapontici*, *Enterobacter* spp., and *Acinetobacter radioresistens,* several α-Proteobacteria), *Zymomonas mobilis, Acetobacter malorum, A. pomorium*, *Microbacterium arborescens*, *Flavobacterium johnsoniae*, *Gluconobacter oxydans*, and *Hafnia alvei* (Escalante et al., 2004, 2008). Yeasts isolated from *pulque* are *Saccharomyces (Sacch. bayanus, Sacch. cerevisiae, Sacch. paradoxus*) and non-*Saccharomyces (Candida* spp.*, C. parapsilosis, Clavispora lusitaniae, Hanseniaspora uvarum, Kl. lactis, Kl. marxianus, Pichia membranifaciens, Pichia* spp.*, Torulaspora delbrueckii*; Lappe-Oliveras et al., 2008).

Depending on the region, traditional alcoholic drinks prepared from palm juice called "palm wine" are known by various names, e.g., *toddy* or *tari* in India, *mu, bandji, ogogoro, nsafufuo, nsamba, mnazi, yongo, taberna, tua,* or *tubak* in West Africa and South America (Ouoba et al., 2012). Microorganisms that are responsible for *toddy* fermentation are *Sacch. cerevisiae*, *Schizosaccharomyces pombe, Acetobacter aceti, A. rancens, A. suboxydans, Leuc. dextranicum (mesenteroides), Micrococcus* sp., *Pediococcus* sp., *Bacillus* sp., and *Sarcina* sp. (Shamala and Sreekantiah, 1988).

Kanji is an ethnic Indian strong-flavored mild alcoholic beverage prepared from beet-root and carrot by natural fermentation (Batra and Millner, 1974). *Hansenlu anomala, Candida guilliermondii, C. tropicalis, Geotrichium candidum, Leuc. mesenteroides, Pediococcus* sp., *Lb. paraplantarum,* and *Lb. pentosus* are present in *kanji* (Batra and Millner, 1976; Kingston et al., 2010).

Alcoholic Beverages Produced by Malting or Germination

Bantu beer or sorghum beer of Bantu tribes of South Africa is an alcoholic beverage produced by malting or germination process (Taylor, 2003). Malted beer is common in Africa with different names e.g., as *bushera* or *muramba* in Uganda, *chibuku* in Zimbabwe, *dolo, burkutu,* and *pito* in West Africa and *ikigage* in Rwanda (Myuanja et al., 2003; Sawadogo-Lingani et al., 2007; Lyumugabe et al., 2012). Sorghum (*Sorghum caffrorum* or *S. vulgare*) is malted (Kutyauripo et al., 2009), characterized by a two-stage (lactic followed by alcoholic) fermentation, with *Lb. fermentum* as the dominating LAB species (Sawadogo-Lingani et al., 2007).

Alcoholic Beverages Produced from Fruits without Distillation

The most common example of alcoholic beverages produced from fruits without distillation is wine, which is initiated by the growth of various species of *Saccharomyces* and non-*Saccharomyces* (so-called "wild") yeasts (e.g., *Candida colliculosa, C. stellata, Hanseniaspora uvarum, Kloeckera apiculata, Kl. thermotolerans, Torulaspora delbrueckii*, *Metschnikowia pulcherrima*; Pretorius, 2000; Moreira et al., 2005; Sun et al., 2014; Walker, 2014). *Candida* sp. and *Cladosporium* sp. were isolated from fermenting white wine using mCOLD-PCR-DGGE, but had not been detected by conventional PCR (Takahashi et al., 2014). *Sacch. cerevisiae* strains developed during wine fermentations play an active role in developing the characteristics of a wine (Capece et al., 2013). *Saccharomyces Genome Database* (SGD; www.yeastgenome.org) provides free of charge access or links to comprehensive datasets comprising genomic, transcriptomic, proteomic and metabolomic information (Pretorius et al., 2015).

CONCLUSIONS

Every community in the world has distinct food culture including fermented foods and alcoholic beverages, symbolizing the heritage and socio-cultural aspects of the ethnicity. The word "culture" denotes food habits of ethnicity; another meaning for the same word "culture" is a cluster of microbial cells or inoculum, an essential biota for fermentation, often used in the microbiology. The diversity of functional microorganisms ranges from filamentous molds to enzyme-producing and alcohol-producing yeasts, and from Gram-positive to a few Gram-negative bacteria, while even *Archaea* has been ascribed roles in some fermented foods and alcoholic beverages. However, consumption of lesser known and uncommon ethnic fermented foods is declining due to the change in lifestyles that is shifting from cultural food habits to commercial foodstuffs and fast foods, drastically affecting traditional culinary practices, and also due to the climate change in some environments such as the Sahel region in Africa and the vast areas adjacent to the Gobi desert in Asia.

AUTHOR CONTRIBUTIONS

JT: contributed 50% of review works. WH, contributed 25% of review. KW contributed 25% of review.

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