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Composition and Origin of the Fermentation Microbiota of Mahewu, a Zimbabwean Fermented Cereal Beverage

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ABSTRACT Mahewu is a fermented cereal beverage produced in Zimbabwe. This study determined the composition and origin of mahewu microbiota. The microbiota of mahewu samples consisted of 3 to 7 dominant strains of lactobacilli and two strains of yeasts. *Enterobacteriaceae* were not detected. *Candida glabrata* was present in high cell counts from samples collected in summer but not from samples collected in winter. Millet malt is the only raw ingredient used in the production of mahewu and is a likely source of fermentation microbiota; therefore, malt microbiota was also analyzed by culture-dependent and high-throughput 16S rRNA gene sequencing methodologies. Millet malt contained 8 to 19 strains of *Enterobacteriaceae*, lactobacilli, bacilli, and very few yeasts. Strain-specific quantitative PCR assays were established on the basis of the genome sequences of *Lactobacillus fermentum* FUA3588 and FUA3589 and *Lactobacillus plantarum* FUA3590 to obtain a direct assessment of the identity of strains from malt and mahewu. *L. fermentum* FUA3588 and FUA3589 were detected in millet malt, demonstrating that millet malt is a main source of mahewu microbiota. Strains which were detected in summer were not detected in samples produced at the same site in winter. Model mahewu fermentations conducted with a 5-strain inoculum consisting of lactobacilli, *Klebsiella pneumoniae,* and *Cronobacter sakazakii* demonstrated that lactobacilli outcompete *Enterobacteriaceae*, which sharply decreased in the first 24 h. In conclusion, mahewu microbiota is mainly derived from millet malt microbiota, but minor components of malt microbiota rapidly outcompete *Enterobacteriaceae* and *Bacillus* species during fermentation.

IMPORTANCE This study provides insight into the composition and origin of the microbiota of mahewu and the composition of millet malt microbiota. Fermentation microbiota are often hypothesized to be derived from the environment, but the evidence remains inconclusive. Our findings confirm that millet malt is the major source of mahewu microbiota. By complementing culture methods with high-throughput sequencing of 16S rRNA amplicons and strain-specific quantitative PCR, this study provides evidence about the source of mahewu microbiota, which can inform the development of starter cultures for mahewu production. The study also documents the fate of *Enterobacteriaceae* during the fermentation of mahewu. There are concerns regarding the safety of traditionally prepared mahewu, and this requires in-depth knowledge of the fermentation process. Therefore, this study elucidated millet malt microbiota and identified cultures that are able to control the high numbers of *Enterobacteriaceae* that are initially present in mahewu fermentations.

KEYWORDS *Cronobacter sakazakii*, *L. plantarum*, *Lactobacillus fermentum*, millet malt microbiota, cereal fermentation

Mahewu is a lactic fermented nonalcoholic cereal beverage produced in Zimbabwe. It is a refreshing drink and is also used as a complementary food for infants. Mahewu is prepared by fermenting cooked maize porridge with addition of millet or

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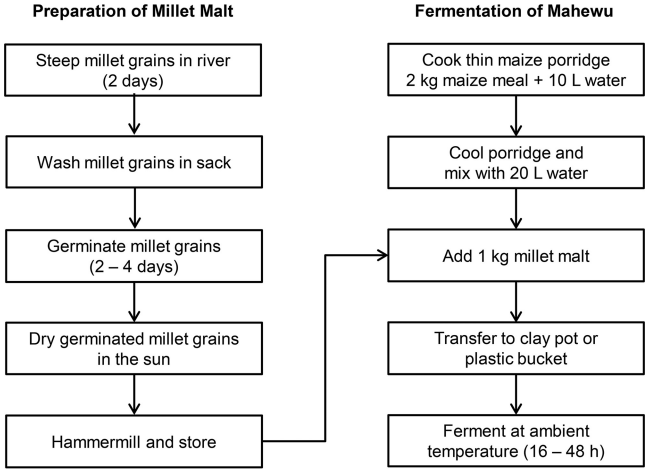

FIG 1 Traditional process for producing mahewu, a fermented maize beverage.

sorghum malt at the household level (1, 2) (Fig. 1). Mahewu is produced by spontaneous fermentation without control of microbiota by back-slopping or the addition of starter cultures (3–5). Cooking of the maize porridge inactivates microbiota from maize flour or water; however, millet or sorghum malt is used without a heating step to inactivate malt-associated microbiota (Fig. 1).

The microbiota of fermented cereal foods and nonalcoholic beverages generally consist of lactic acid bacteria and yeasts. Previous studies on mahewu enumerated lactic acid bacteria and yeasts in model fermentations but did not characterize fermentation microorganisms at the genus or species level (1, 2, 6). Data on the composition of fermentation microbiota is available for togwa, bushera, and obiolor, fermented cereal gruels or beverages that are produced in Tanzania, Uganda, and Nigeria, respectively, which are produced in a manner similar to that for mahewu from cooked maize or sorghum porridge with the addition of sorghum or millet malt (7, 8). The microbiota of togwa and bushera samples was composed predominantly of *Lactobacillus plantarum* as the most abundant organisms, along with *L. brevis*, *L. fermentum*, *Weissella confuse*, and *Pediococcus pentosaceus*. The thermophilic *Issatchenkia orientalis* was the most frequently isolated yeast in togwa (7).

Lactic acid bacteria identified in togwa are typical representatives of spontaneous cereal fermentations that are also present in other African cereal foods, such as ben-saalga from Burkina Faso and ogi and kunu-zaki from Nigeria; these are all prepared with spontaneously fermented millet or millet malt that is cooked into a thin porridge, gruel, and beverage, respectively (9–13). A comparable composition of microbiota is also observed in spontaneous wheat and rye sourdough fermentations (14). The assembly of microbiota in spontaneous fermentations is limited by dispersal (15). Spontaneously fermented cereals typically contain lactic acid bacteria with a nomadic or environmental lifestyle, particularly *L. plantarum*, *L. fermentum,* and *Weissella* spp. (16–18). Back-slopping eliminates dispersal limitation (15) and leads to dominance of host-adapted lactobacilli, including *L. sanfranciscensis* in type I sourdoughs or *L. pontis*, *L. amylovorus*, and *L. reuteri* in type II sourdoughs (14, 15).

Because mahewu is consumed without further heat treatment after fermentation, malt microbiota not only serves as inoculum for the lactic acid fermentation but also may transfer endophytic microbiota (18). Plant endophytes include the opportunistic pathogens *Enterobacter* spp. and *Cronobacter* spp. (19, 20); endophytic *Enterobacter* spp. were reported to protect finger millet against *Fusarium* infection (21). Low cell counts of *Enterobacteriaceae* were detected in weaning foods prepared from sorghum and millet (22), but the fate of these organisms during fermentation of malt-based cereal beverages is not documented.

The metabolic activity of mahewu microbiota in combination with the enzymatic

TABLE 1 Microbiological and biochemical characteristics of mahewu fermentations

Parameter	Site C		Site D		
	Feb 2016[a]	May 2016[b]	Feb 2016[a]	May 2016[b]	Site M, May 2016[b]
Fermentation vessel	Clay pot	Clay pot	Plastic bucket	Plastic bucket	Clay pot
pH	2.96	3.38	3.10	3.37	3.29
MRS5 agar and cycloheximide (CFU/ml)	9.9×10^8	7.7×10^8	1.1×10^9	4.7×10^8	2.6×10^8
MRS5 agar, erythromycin, and chloramphenicol (CFU/ml)	3.4×10^7	6.2×10^7	1.7×10^7	9.9×10^7	5.2×10^7
VRBG agar[c] (CFU/ml)	7.2×10^5	9.0×10^1	1.0×10^7	8.0×10^2	6.9×10^2
VRB agar[d] (CFU/ml)	<10	<10	<10	<10	<10
Quantification of mahewu metabolites[e] (mM)					
Lactic acid	48.5 ± 1.59	34.8 ± 0.66	43.1 ± 1.27	28.7 ± 0.60	31.8 ± 0.36
Glycerol	25.7 ± 0.27	14.1 ± 0.36	11.1 ± 1.93	11.6 ± 0.27	11.3 ± 0.23
Acetic acid	18.8 ± 0.64	11.4 ± 0.28	15.5 ± 0.52	4.15 ± 0.10	6.65 ± 0.13
Ethanol	308 ± 4.14	286 ± 1.83	148 ± 27.5	336 ± 1.68	280 ± 0.32

[a]Fermentation time of 16 to 24 h.
[b]Fermentation time of 36 to 48 h.
[c]Only *Candida glabrata* grew on violet red bile glucose (VRBG) agar.
[d]Cell counts on violet red bile (VRB) agar (fecal coliforms) were less than 10 CFU/ml in all samples.
[e]Means ± standard deviations of duplicate analyses are shown.

activity of the millet malt determines product quality (23) and safety; therefore, attaining mahewu with consistent quality and safety attributes requires control of the composition and activity of fermentation microbiota (15). Therefore, the aim of this study was to determine composition and origin of mahewu fermentation microbiota, and to establish the overlap between the mahewu and millet malt microbiota, by using strain-specific quantitative PCR (qPCR).

RESULTS

Microbial and biochemical characterization of mahewu. Microbial counts were obtained from five samples of mahewu that were obtained in summer and in winter from 3 production sites. All samples were prepared at the household level with traditional methods. Bacterial counts ranged from 2.6×10^8 to 1.1×10^9 CFU/ml (Table 1). Bacterial isolates were all identified as lactic acid bacteria (Table 2). Yeast cell counts ranged from 1.7×10^7 to 9.9×10^7 CFU/ml. The cell counts for fecal coliforms were below the detection limit of 10 CFU/ml in all samples. Cell counts on violet red bile glucose (VRBG) agar ranged from 9.0×10^1 to 1.0×10^7 CFU/ml; microscopic observation, however, demonstrated that all colonies on VRBG agar represented thermophilic yeasts. The pH of mahewu samples ranged between 3.0 and 3.4 (Table 1). Lactic and acetic acids, glycerol, and ethanol were identified as the major fermentation products (Table 1), indicating that microbial metabolism was mainly attributable to lactic acid bacteria and yeasts.

Microbial analysis of mahewu. The taxonomic identification of bacterial isolates was based on the elimination of clonal isolates by random amplification of polymorphic DNA (RAPD) typing, followed by sequencing of 16S rRNA genes (Table 2). Identification of isolates as *L. plantarum* additionally employed a *recA*-based multiplex PCR assay (24). Yeasts were identified by the sequencing of 28S rRNA genes. The microbiota of the mahewu samples typically consisted of 3 to 7 dominant strains of lactobacilli and two strains of yeasts (Table 2). *Saccharomyces cerevisiae* and *Candida glabrata* were present in all samples (Table 2). *C. glabrata* cell counts ranged from 10^5 to 10^7 CFU/g in samples from sites C and D obtained in summer (February 2016), but viable cell counts of the same species were below 10^3 CFU/ml in samples that were obtained in winter (May 2016). *S. cerevisiae* was the most abundant yeast in samples collected in winter. The microbiota of samples from the same site differed between sampling times (Table 2).

Microbiota of millet malt. In addition to the microbiota associated with the production environment, millet malt is a source of mahewu microbiota. We therefore determined cell counts of four millet malt samples and identified representative isolates at the species level. The viable bacterial cell counts of the 4 millet malt samples

TABLE 2 Identification and strain-specific quantification of isolates from mahewu

Strain ID and sample collection/analysis date	Species[a]	Cell count (CFU/ml)
Feb 2016		
Site C		
FUA3588	*L. fermentum*	9.9×10^7
FUA3589	***L. fermentum***[d]	9.9×10^7
FUA3590	*L. plantarum*[b,c]	7.9×10^8
FUA3568	*P. pentosaceus*[c]	
FUA4041	*S. cerevisiae*	2.4×10^7
FUA4042	*C. glabrata*	1.0×10^7
FUA4043	*C. glabrata*	7.2×10^5
Site D		
FUA3573	***L. fermentum***	9.4×10^8
FUA3574	*Weissella* spp.	5.5×10^7
FUA3575	*Weissella confusa*	1.1×10^8
FUA4046	*S. cerevisiae*	8.5×10^6
FUA4047	*C. glabrata*	8.5×10^6
FUA4048	*C. glabrata*	1.0×10^7
May 2016		
Site C		
FUA3569	*L. fermentum*	5.4×10^8
FUA3570	*L. fermentum*	3.9×10^7
FUA3571	*E. hermanniensis*[c]	1.5×10^8
FUA3572	*E. lactis*[c]	
FUA4044	*S. cerevisiae*	6.2×10^7
FUA4045	*C. glabrata*	9×10^1
Site D		
FUA3576	*Weissella cibaria*[c]	4.7×10^6
FUA3577	*P. pentosaceus*[c]	
FUA3578	*Leuconostoc holzapfelii*	1.9×10^8
FUA3579	*Lact. lactis*	1.9×10^8
FUA3580	*Leuconostoc pseudomesenteroides*[c]	8.9×10^7
FUA3581	*W. confusa*[c]	
FUA4049	*S. cerevisiae*	9.9×10^7
FUA4050	*C. glabrata*	8.0×10^2
Site M		
FUA3582	***L. fermentum***	1.0×10^8
FUA3583	*L. rossiae*	1.0×10^8
FUA3584	*L. plantarum*[b]	2.6×10^7
FUA3585	*Weissella cibaria*	2.6×10^7
FUA3586	*L. plantarum*[b]	7.8×10^6
FUA4051	*S. cerevisiae*	5.2×10^7
FUA4052	*C. glabrata*	6.9×10^2

[a]All isolates were identified with the sequence match tool of the ribosomal database project with >98% nucleotide identity of partial 16S rRNA (1,300 to 1,455 bp) to bacterial type strain or >98% nucleotide identify of more than 700 bp of the 28S rRNA genes.
[b]Differentiation of *L. plantarum* from *L. paraplantarum* and *L. pentosus* was carried out using a multiplex PCR assay using *recA* gene-based primers.
[c]Cell counts of two strains that could not be differentiated on the basis of their colony morphologies.
[d]PCR specific for *L. fermentum* FUA3589-generated amplicons with all strains printed in boldface.

ranged from 2.2×10^6 to 7.7×10^7 CFU/g (Table 3); fecal coliforms accounted for more than 20% of total cell counts in all samples (Table 3). The taxonomic identification of millet malt isolates was based on the procedure outlined above for mahewu microbiota. The microbiota of the millet malt samples typically consisted of 8 to 19 strains of plant-associated *Enterobacteriaceae*, lactic acid bacteria, bacilli, and a few yeasts (Table 4). Only the most abundant isolates could be quantified by differential enumeration on the basis of colony morphology. *Bacillus subtilis* and *Enterococcus* spp. were the most abundant organisms in the sample from site C in February 2016 and May 2016, respectively. *Cronobacter sakazakii* was the most abundant microorganism in both millet malt samples from site D.

Characterization of millet malt microbiota by 16S rRNA gene sequencing. Illumina sequencing of 16S rRNA gene amplicons was used to determine the relative

TABLE 3 Microbial characterization of millet malt

Malt sample and sampling date	Cell counts (CFU/g)			
	Bacteria[a]	Yeast[b]	*Enterobacteriaceae*[c]	Fecal coliforms[d]
C				
February 2016	7.7×10^7	$<10^3$	1.0×10^7	5.3×10^7
May 2016	1.3×10^7	$<10^3$	2.3×10^6	3.7×10^7
D				
February 2016	1.3×10^7	$<10^3$	2.7×10^6	3.0×10^6
May 2016	1.7×10^7	$<10^3$	4.7×10^6	1.9×10^7

[a]MRS5 agar and cycloheximide.
[b]Yeast cell counts were detected on MRS5 agar with addition of erythromycin and chloramphenicol. Plates inoculated with the lowest dilution (10^{-1}) were overgrown with molds; thus, the detection limit was 10^3 CFU/g for yeasts.
[c]Detected on violet red bile glucose (VRBG) agar.
[d]Detected on violet red bile agar with incubation at 44.5°C.

abundance of bacterial genera in 3 millet malt samples. The millet malt microbiota was characterized by the analysis of the relative abundance of bacterial taxa at the phylum and genus levels (Table 4) and by matching to the most closely related species (see Table S1S in the supplemental material). Of the total 135 operational taxonomic units (OTUs), 96 were classified to the genus level (Table 4). *Proteobacteria* was the most abundant phylum (32.1 to 44.4%), with *Cronobacter* (26.4 to 52.8%) as the most abundant genus in all 3 millet malt samples, followed by *Firmicutes* (22.6 to 30.2%), with *Weissella* (6.35 to 10.9%) as the most abundant genus. A comparison of the culture-dependent and sequencing results revealed that the major members of malt microbiota were detected by both methods. However, 4 genera of *Enterobacteriaceae* representing a total of 28% of isolates were not identified by sequencing. Furthermore, sequencing provided a lower relative abundance of *Bacillus* spp., 3.68% compared to 19.1%, as determined by culture-dependent analysis (Table 4).

Sequencing also revealed the presence of DNA from *Rhizobium*, a root symbiont, *Massilia* spp. and *Pseudomonas* spp., strict aerobic water- and soil-associated organisms, *Xanthomonas*, a plant pathogen, and *Bacteroidetes*, strict anaerobic members of intestinal microbiota. With the exception of *Bacteroidetes*, these taxa grow readily on the media that were employed for culture-dependent analysis of malt samples (Table 4). Short sequences of bacterial rRNA genes can be amplified more than 60 days after cell death (25), and the presence of rRNA genes from these taxa is unlikely to represent the presence of viable and metabolically active bacterial cells.

Determination of the origin of mahewu microbiota. RAPD patterns and the observation of the colony morphology of isolates suggested that different samples contained similar strains of *L. fermentum*. To obtain a more direct assessment of strain identity, a strain-specific quantitative PCR assay was established on the basis of the genome sequences of *L. fermentum* FUA3588 and FUA3589 and *L. plantarum* FUA3590. Genomes of *L. fermentum* FUA3588 and FUA3589 and *L. plantarum* were aligned against 20 reference genomes each of *L. fermentum* and *L. plantarum*, respectively. As an example, the genome alignment of *L. fermentum* FUA3588 against the most closely related genome-sequenced strain, *L. fermentum* NB-22 (NCBI accession no. GCA_000496435.1), is shown in Fig. 2 The primers CMA1F and CMA1R targeted a unique exopolysaccharide operon (EpsD gene cluster) with 3 hypothetical proteins and 4 glycosyltransferases only found on the *L. fermentum* FUA3588 genome (Fig. 2). The regions targeted by the CMB1F/CMB1R and CMC1F/CMC1R primers code for hypothetical proteins. Nucleotide BLAST on the NCBI database verified that the primer pairs do not bind to other sequences that were deposited in the NCBI database.

Strain-specific qPCR determined the origin of the mahewu microbiota (Table 5). *L. fermentum* FUA3588 and FUA3589 were detected by strain-specific qPCR in community DNA isolated from the millet malt that was used to produce the sample. However, *L.*

TABLE 4 Comparison of the relative abundance of bacterial genera in millet malt[a]

Genus	% of 16S rRNA amplicons	% of isolates	Species isolated
Proteobacteria	38.06 ± 5.04		
Aureimonas	0.34 ± 0.00	ND	
Rhizobium	2.16 ± 0.00	ND	
Sphingomonas	1.97 ± 0.01	ND	
Roseomonas	0.11 ± 0.00	ND	
Burkholderia-Paraburkholderia	0.22 ± 0.00	ND	
Massilia	2.06 ± 0.01	ND	
[F: *Comamonadaceae*]	1.90 ± 0.01	ND	
Aquitalea	2.21 ± 0.02	ND	
Vogesella	0.32 ± 0.00	ND	
Cronobacter	35.2 ± 0.12	23.27 ± 8.51	*sakazakii*, *dubliniensis*, *malonaticus*
Citrobacter	0.18 ± 0.00	2.94 ± 4.16	*fameri*, *amalonaticus*, *koseri*
Enterobacter	ND	8.26 ± 7.25	*asburiae*, *freundii*, *xiangifangensis*, *ludwigii*
Klebsiella	ND	11.3 ± 5.99	*pneumoniae*, *variicola*
Kosakonia	ND	7.28 ± 6.01	*cowanii*
Trabulsiella	ND	0.98 ± 1.39	*odontotermitis*
Acinetobacter	2.13 ± 0.01	ND	
Pseudomonas	2.29 ± 0.01	ND	
Xanthomonas	2.89 ± 0.00	ND	
Firmicutes	25.5 ± 3.31		
Bacillus	3.68 ± 0.05	19.05 ± 22.08	*subtilis*, *ginsengihumi*
Paenibacillus	2.04 ± 0.00	ND	
Saccharibacillus	1.34 ± 0.24	ND	
Enterococcus	3.77 ± 0.02	13.3 ± 6.37	*durans*, *lactis*, *camelliae*, *italicus*, *casseliflavus*, *pallens*
Lactobacillus	0.22 ± 0.00	2.58 ± 1.84	*fermentum*, *plantarum*
Pediococcus	0.38 ± 0.00	4.13 ± 3.62	*pentosaceus*
Leuconostoc	0.15 ± 0.00	ND	
Weissella	8.58 ± 1.86	3.57 ± 5.05	*beninensis*, *cibaria*, *confusa*, *paramesenteroides*
Lactococcus	2.51 ± 0.01	2.17 ± 1.56	*lactis*, *taiwanensis*
[F: *Peptostreptococcaceae*]	0.13 ± 0.00	ND	
Clostridium sensu stricto 1	1.46 ± 0.01	ND	
Clostridium sensu stricto 5	0.12 ± 0.00	ND	
Ruminococcaceae UCG-010	0.82 ± 0.01	ND	
Bacteroidetes	17.7 ± 5.77		
Siphonobacter	0.70 ± 0.00	ND	
Chryseobacterium	3.35 ± 2.40	ND	
Sphingobacterium	1.33 ± 0.01	ND	
Mucilaginibacter	0.31 ± 0.00	ND	
Pedobacter	0.23 ± 0.00	ND	
Actinobacteria	5.42 ± 3.07		
Cellulomonas	1.31 ± 0.00	ND	
Saccharibacteria	0.66 ± 0.47		
[P: *Saccharibacteria*]	0.14 ± 0.00	ND	
Planctomycetes	0.53 ± 0.75	ND	
Marinimicrobia (SAR406 clade)	0.61 ± 0.86	ND	
Fusobacteria	0.31 ± 0.43	ND	
Verrucomicrobia	0.31 ± 0.43	ND	
Chloroflexi	0.89 ± 0.66	ND	
Cyanobacteria	1.50 ± 0.32	ND	
Unassigned	5.13 ± 2.53	ND	
Total	100 ± 0.00	100 ± 0.00	

[a]The comparison of the relative abundance was determined by Illumina sequencing of 16S rRNA amplicons and 16S rRNA gene sequencing of bacterial isolates. Data are presented as means ± standard deviations from 3 millet malt samples. Unassigned genera are presented with upper-level family (F) or phylum (P) in brackets. Unassigned means a good hit to a particular sequence, but that sequence is rare and is not bacterial 16S rRNA. ND, not detected. OTUs of ≤0.1% are not shown but are included in the total.

plantarum FUA3590, which is the most abundant lactic acid bacteria in mahewu, was not detected in the millet malt samples. Strain-specific primers were also used to determine whether isolates obtained from different samples are identical. Amplicons were obtained with primers targeting *L. fermentum* FUA3589 and template DNA

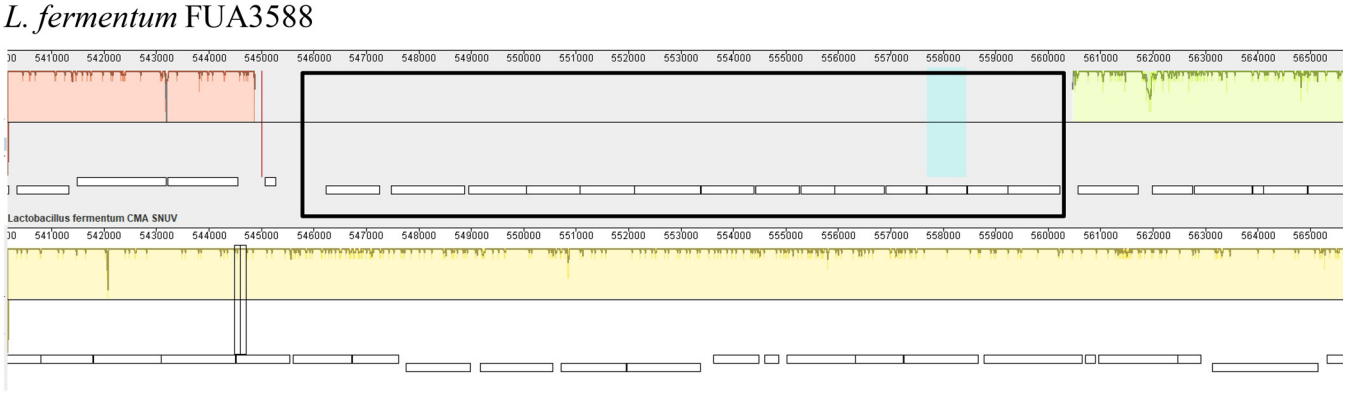

FIG 2 Genome alignment in MAUVE of *L. fermentum* FUA3588 against the most closely related genome-sequenced strain, *L. fermentum* NB-22 (NCBI accession no. GCA_000496435.1). Shown is a genomic region that is present in *L. fermentum* FUA3588 but absent from *L. fermentum* NB-22 (black box). The region encodes a putative exopolysaccharide synthesis cluster; BLAST analysis against the NCBI database revealed that the 7 genes at the 5′ end are unique to *L. fermentum* FUA3588 but absent from any other sequence in the NCBI database (red box). This region codes for 3 hypothetical proteins and 4 putative glycosyl transferases and was used for the design of strain-specific primers (Table 6).

isolated from *L. fermentum* FUA3573 and *L. fermentum* FUA3582 (Table 2). Primers targeting strain-specific regions in the genomes of *L. fermentum* FUA3588 and *L. plantarum* FUA3590 did not generate PCR amplicons in any other isolates, suggesting that these strains were present only in samples obtained from site C in summer.

Model mahewu fermentations. To investigate the discrepancy between malt and mahewu microbiota, particularly with respect to *Enterobacteriaceae*, and to determine the fate of *Enterobacteriaceae*, including *Cronobacter sakazakii*, model mahewu fermentations were conducted. The simulated mahewu fermentation was inoculated with a 5-strain cocktail comprised of 2 mahewu isolates, *L. fermentum* FUA3588 and *L. plantarum* FUA3590, and 3 isolates from millet malt, *C. sakazakii* FUA10024, *Enterococcus lactis* FUA3587, and *Klebsiella pneumoniae* FUA10025. The 5 strains were chosen for their diverse and distinct colony morphologies to enable differential cell counts without the use of selective media (Fig. 3). Yeasts were not included in the model mahewu fermentations, because yeasts were not detected in millet malt microbiota and because the growth of yeasts in spontaneous cereal fermentations is typically observed only at late fermentation stages (14, 26). Fermentations were characterized by differential cell counts, pH, and metabolite concentrations (Fig. 4). Lactic acid and ethanol were the major bacterial metabolites in model mahewu fermentations. The concentrations of the metabolites were 82.0 ± 3.1 mmol liter^{-1} lactic acid, 42.2 ± 6.2 mmol liter^{-1} ethanol, 8.6 ± 1.2 mmol liter^{-1} acetic acid, and 5.9 ± 0.8 mmol liter^{-1} glycerol. The pH decreased to 4.5 after 8 h; the final pH of 3.3 was reached after 24 h (Fig. 4). All five strains grew in the initial phase of fermentation. Cell counts of *C. sakazakii* and *K. pneumoniae* increased up to 8 h and 16 h, respectively, and sharply decreased within 24 h. Cell counts of *E. lactis* increased up to 16 h and then decreased gradually. Growth of *L. fermentum* stopped after 16 h and decreased slightly, whereas growth of *L. plantarum*

TABLE 5 Strain-specific qPCR detection of mahewu isolates in millet malt

Mahewu strain	Relative abundance (%) in:	
	Malt[a]	Mahewu[b]
L. fermentum FUA3588	0.07 ± 0.01	10
L. fermentum FUA3589	0.01 ± 0.01	10
L. plantarum FUA3590	Not detected	80

[a]Means ± standard deviations of duplicate independent experiments are shown. Quantitative PCR analysis comparing site C mahewu strain isolates with site C millet malt isolates.
[b]Relative abundance of 3 mahewu isolates in mahewu from site C.

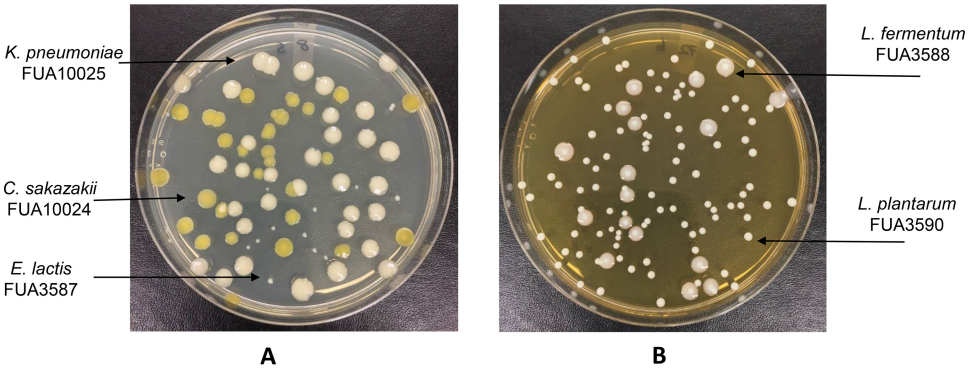

FIG 3 Strain isolated from model mahewu fermentations. Differential enumeration of 5 bacterial strains was determined on the basis of colony morphology. (A) LB agar plate after 8 h of incubation at 30°C. *E. lactis* FUA3587 (small white colonies), *C. sakazakii* FUA10024 (yellow colonies), and *K. pneumoniae* FUA10025 (gray large mucoid colonies) were used. (B) MRS5 agar plate after 72 h of incubation at 30°C. *L. fermentum* FUA3588 (large white mucoid colonies) and *L. plantarum* FUA3590 (off-white medium-sized colonies) were used.

stopped after 48 h, and cell counts were maintained over the remaining fermentation time (Fig. 4).

DISCUSSION

This study analyzed the composition and origin of mahewu microbiota to determine the overlap between the mahewu and millet malt microbiota and to evaluate the suitability of mahewu isolates to outcompete *Enterobacteriaceae* during fermentation.

Multiple studies in past decades consistently and unambiguously demonstrated that culture-dependent methodology on appropriate cultivation media identifies all relevant fermentation organisms in cereal fermentations, while DNA-based approaches, including amplified ribosomal DNA-restriction analysis (ARDRA) (5), denaturing gradient gel electrophoresis (4, 26, 27), quantitative PCR (28–30), and high-throughput sequencing of 16S RNA sequence tags (31), often fail to recover specific species and do not allow identification at the species level. In addition, the recovery of DNA from dead bacterial cells severely distorts the microbiota composition when assessed by sequence-based approaches, particularly in spontaneous plant fermentations that are

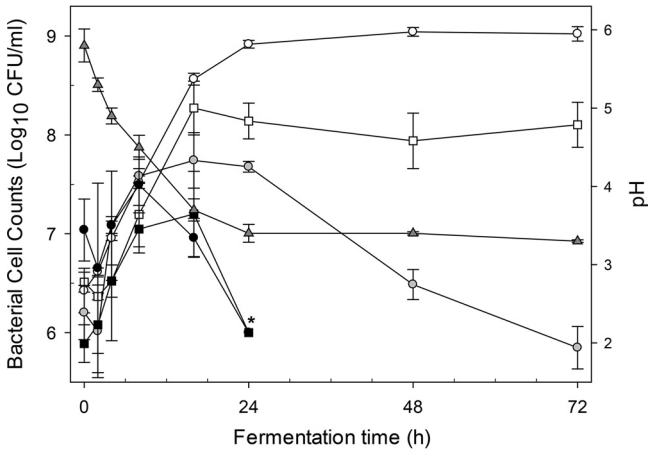

FIG 4 Differential cell counts in model mahewu fermentations. Model mahewu fermentations were inoculated with 10^6 CFU/ml each of five bacterial strains and incubated at 30°C for 72 h. Differential enumeration of 5 bacterial strains was determined on the basis of colony morphology. Symbols indicate *L. fermentum* FUA3588 (□), *L. plantarum* FUA3590 (○), *E. lactis* FUA3587 (●), *C. sakazakii* FUA10024 (●), and *K. pneumoniae* FUA10025 (■). Results are shown as means ± standard deviations from triplicate independent experiments analyzed in duplicate. The asterisk indicates values below the detection limit because the LB agar plates were overgrown with lactic acid bacteria and bacilli. □ and ○, enumerated on MRS5 agar; ● and ■, enumerated on LB agar.

characterized by a succession of fermentation microbiota (25, 26, 31). Sequence-based approaches thus achieve rapid monitoring of fermentation microbiota but are not suitable for an in-depth analysis of fermentation microbiota at the strain level, as was attempted in the present study.

Owing to the paucity of data on the composition of (millet) malt microbiota, malt samples were evaluated with culture-dependent methods and high-throughput amplicon sequencing. DNA- and culture-based methods were generally in agreement; however, sequencing results also indicated the presence of root symbionts, strict anaerobic intestinal microorganisms, and plant pathogens that are unlikely to remain viable after malting and dry storage. In addition, sequencing did not identify four genera of *Enterobacteriaceae*, likely because the close phylogenetic relationship of different genera in the *Enterobacteriaceae* makes even the genus-level identification with short rRNA gene sequences questionable (32).

Composition. Mahewu microbiota consisted of lactic acid bacteria and yeasts. *L. fermentum* was the most frequent and the most abundant organism in mahewu samples; *L. plantarum*, *L. rossiae*, *P. pentosaceus*, *Leuconostoc,* and *Weissella* spp., as well as enterococci and lactococci, additionally were present. Thus, the composition of mahewu microbiota is comparable to that of the microbiota of other spontaneous cereal fermentations, which also typically include *L. fermentum*, *L. plantarum*, and *P. pentosaceus* (3, 9, 11, 13, 33–37), species which are characterized by a nomadic or environmental lifestyle (17). The high abundance of *L. plantarum* at the late stages of spontaneous cereal fermentation (33) likely relates to its ability to acidify the fermentation substrate to a pH of 3.2, which is lethal to most other lactic acid bacteria (38).

Yeasts were identified as *Saccharomyces cerevisiae* and *Candida glabrata*; these yeasts also occur in other cereal fermentations (39, 40). Cell counts of the yeast *C. glabrata* were high in summer but low in winter, suggesting that its presence depends on the fermentation temperature. The thermotolerance also contributes to its ability to cause opportunistic infections (41). *C. glabrata* infections have high mortality in immunocompromised, at-risk, and hospitalized patients (42), and its presence may thus be of concern in countries, such as Zimbabwe, with a high prevalence of HIV (43).

Origin. The production environment and the raw material were related to the microbiota of spontaneous and back-slopped wheat sourdoughs (18, 30). Strain-specific qPCR was used to compare mahewu microbiota prepared in the same household between summer and winter. None of the three strains was identified at the same production site at two different sampling times. One of 3 strains, *L. fermentum* FUA3589, was identified in three different production sites in summer and in winter. This pattern suggests a common source of contamination rather than persistence of a single strain in the site of production. We subsequently characterized the microbiota of millet malt as a likely source of fermentation microbiota. Strain-specific qPCR demonstrated that the millet malt is a main source of mahewu microbiota. Strain-specific primers have previously been used for strain-specific quantification of bifidobacteria and lactobacilli in fecal samples (44, 45). Past studies on molecular source tracking of food microbiota used RAPD PCR or repetitive element sequence-based PCR to trace the origin of lactic acid bacterial strains in sourdoughs; however, the low specificity of these techniques did not provide conclusive evidence on strain identity (18, 46). In addition, the detection limit of strain-specific qPCR is lower than that of cultivation-based methods.

Millet malt microbiota consisted mainly of environmental *Enterobacteriaceae*, environmental lactic acid bacteria, bacilli, and a few yeasts. Barley malt microbiota also include *Enterobacteriaceae* and lactic acid bacteria as dominant representatives (47). Daqu is a spontaneously fermented saccharification starter prepared from sorghum and wheat or rice hull and includes a diverse microbial community, with bacilli, *Enterobacteriaceae*, lactic acid bacteria, and yeasts and molds as abundant representatives (27, 48, 49). *C. sakazakii* is ubiquitous in the environment and has been isolated from plant food, cereals, fruit and vegetables, herbs, and spices (50, 51). *C. sakazakii* has a high tolerance to desiccation (51, 52), which may contribute to its competitive advantage in

millet malt. *Cronobacter* spp. were found to endophytically and epiphytically colonize tomato and maize roots, which suggests that plants are the natural habitat of *Cronobacter* spp. (19). The presence of *C. sakazakii* in millet malt may be a health risk, as the organism is linked with life-threatening infections in neonates and with urinary tract infections in persons over 80 years of age (52–54). *Cronobacter dublinensis*, like *C. sakazakii*, has been recovered from the environment and food (55) and is also considered an opportunistic pathogen in neonates (56). *Klebsiella pneumoniae* is widely distributed in the environment, is a commensal bacteria in the mucosal surfaces of humans and animals, and is an opportunistic pathogen causing nosocomial infections (57). *Bacillus subtilis* has been isolated from diverse environments, particularly including soils and plants (58).

The model mahewu study was conducted to determine the fate of *C. sakazakii*, *K. pneumoniae,* and *E. lactis* during fermentation. The initial competition of lactic acid bacteria and plant-associated *Enterobacteriaceae* is common for many plant fermentations, including cereal fermentations, sauerkraut, kimchi, and carrot juice (25, 59, 60). Growth of *Enterobacteriaceae* in plant fermentations is inhibited by the low pH; extended fermentation at low pH reduced cell counts of *Enterobacteriaceae* in carrot juice, kimchi, and sorghum doughs (25, 37, 60). Plant-associated *Enterobacteriaceae* were detected in several spontaneously fermented cereal foods (39), suggesting that fermentation conditions or fermentation time does not always suffice to eliminate these organisms. The model mahewu fermentations showed a rapid decrease in *Enterobacteriaceae* as soon as the pH was reduced to less than 4.5.

In conclusion, this study describes the composition and origin of mahewu microbiota and elucidates the role of millet malt in the fermentation of mahewu. Mahewu is consumed by infants and immunocompromised individuals without inactivation of the fermentation microbiota. Thus, viable probiotic fermentation organisms and viable opportunistic pathogens may positively and negatively affect the health of consumers (61). While *L. fermentum* organisms were very minor components of millet malt microbiota and *L. plantarum* was not detectable in the raw material, they became dominant members of mahewu microbiota after 16 to 24 h of fermentation. Conversely, *C. sakazakii*, *K. pneumoniae*, and enterococci, which were abundant representatives of millet malt microbiota, were eliminated or reduced after 24 to 48 h of fermentation. Comparative genomic analyses of mahewu lactic acid bacterial isolates may further elucidate their role in product quality and safety.

MATERIALS AND METHODS

Sampling of mahewu and millet malt. Five mahewu samples and four millet malt samples were collected from three different sites in Chekure village, Gutu, in Masvingo province of Zimbabwe in February and May 2016. The mahewu samples from three different sites are described as C, D, and M. Two samples from sites C and D were collected from the same households in February and May 2016, respectively, and one sample from site M was collected in May 2016. Prior to sample collection, the fermentation process of mahewu from sites C and D was observed. The maize and finger millet used in the production of mahewu were grown by the households; water was obtained from a well near the homesteads. No commercial ingredients were used. Mahewu samples from sites C and M were prepared in clay pots, whereas samples from site D were prepared in plastic buckets. The samples were collected aseptically into sterile 50-ml tubes, which were placed on ice in a cooler box and transported to the Department of Agriculture Food and Nutritional Science at the University of Alberta, AB, Canada. Samples were maintained between 0 and 20°C during transport, analyzed at 76 $\pm$ 2 h after collection in Zimbabwe with respect to pH and total cell counts, and stored at -20°C for subsequent biochemical analyses and DNA isolation. Time and temperature profile of transport match the storage life of mahewu.

Isolation and enumeration of microorganisms in mahewu and millet malt. Total cell counts for bacteria and yeasts were determined by surface plating of 10-fold serial dilutions of mahewu samples in sterile peptone saline water (10 g/liter peptone, 9 g/liter NaCl) on MRS5 agar (4). The composition of MRS5 per liter contains 20 g bacteriological agar, 10 g tryptone, 5 g beef extract, 5 g yeast extract, 10 g maltose, 5 g fructose, 5 g glucose, 5 g sodium acetate, 3 g ammonium chloride, 2.6 g potassium phosphate dibasic, 4 g potassium phosphate monobasic, 0.5 g L-cysteine, 0.1 g magnesium sulfate, 0.05 g manganese sulfate, 1 ml Tween 80, and 0.1 ml vitamin mix (final pH 5.9). The vitamin mixture containing 250 mg cobalamin, 200 mg folic acid, 200 mg nicotinic acid, 250 mg pantothenic acid, 200 mg pyridoxal phosphate, and 200 mg thiamine in 100 ml was sterilized by filtration at 0.22 μm. For the enumeration and isolation of lactic acid bacteria, MRS5 containing 100 mg/liter of cycloheximide (Sigma, Oakville, Canada) was used, and the plates were incubated under modified conditions (10% CO_2, 90% N_2) at 30°C

TABLE 6 Primer sequences used in the study

Target or primer	Primer		T_m[a] (°C)	Amplicon length (bp)	Reference or source
	Name	Sequence (5′–3′)			
Bacteria	27F	AGAGTTTGATCMTGGCTCAG	53.2	1,500	64
	1492R	TACGGYTACCTTGTTACGACTT	54.6		
16S rRNA	784F	RGGATTAGATACCC		300	64
	1064R	CGACRRCCATGCANCACCT			
Yeasts	P1F	ATCAATAAGCGGAGGAAAAG	50.2	700	65
	P2R	CTCTGGCTTCACCCTATTC	52.4		
recA gene-based primers					
L. paraplantarum	ParaF	GTCACAGGCATTACGAAAAC	51.9	107	24
L. pentosus	PentF	CAGTGGCGCGGTTGATATC	55.6	218	24
L. plantarum	planF	CCGTTTATGCGGAACACCTA	55.0	318	24
	pREV	TCGGGATTACCAAACATCAC	52.5		24
Primers for HRM-qPCR					
Lactobacillus, *Pediococcus*, *Weissella*, *Oenococcus*	LabF	AGCAGTAGGGAATCTTCCA	63	341	69
	LabR	CACCGCTACACATGGAG			
Strain-specific primers for quantification of mahewu isolates in millet malt					
L. fermentum	CMA1F	CACTAACAGGCACCACTATCTT	62	119	This study
	CMA1R	CGCAGTCTTATTCTCATGCTTTAC			
L. fermentum	CMB1F	AACGCCTAGCCTGATTTATCTC	62	103	This study
	CMB1R	CAACAGGATCGTGCCATAGT			
L. plantarum	CMC1F	AGTTTGCCACATATTAGGAAGAGA	62	112	This study
	CMC1R	AGGCTCTAAGGGCTACCTATAC			

[a]T_m, melting temperature.

for 72 h. Yeasts were enumerated and isolated on MRS5 agar containing 100 mg/liter each of chloramphenicol and erythromycin (Sigma), and the plates were incubated aerobically at 30°C for 72 h. Fecal coliform bacteria and *Enterobacteriaceae* were enumerated and isolated on violet red bile agar (Difco) and violet red bile glucose agar (Oxoid), respectively, and plates were incubated aerobically at 44.5°C for 24 h and 72 h, respectively. The colony morphologies of the microorganisms were recorded, and three or four representative colonies of each morphotype, corresponding to at least 50 colonies per sample and more than 300 colonies in total, were purified by repetitive dilution streaks for further identification. Purified cultures were stored in 30% glycerol at −80°C. Cultivation of stock cultures for subsequent experiments was done on MRS5 agar at 30°C for 48 h, followed by overnight incubation in MRS5 broth at 30°C.

Identification of mahewu and millet malt isolates. Genomic DNA was isolated from overnight cultures in 10 ml MRS5 broth using the DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. Random amplification of polymorphic DNA (RAPD) analysis was performed as previously described (62) with the oligonucleotide primer M13V. PCR was performed with genomic DNA as the template in a reaction volume of 25 μl containing 1 μl genomic DNA, 2.5 μl 10× PCR buffer, 0.75 mM $MgCl_2$, 200 nM each deoxynucleotide, 1.5 U *Taq* DNA polymerase, and 150 pmol primer M13V (5′-GTT TTC CCA GTC ACG AC-3′) (63) (all reagents were from Invitrogen Corporation, Carlsbad, CA, USA). The mixtures were subjected to 1 min of incubation at 96°C; 3 cycles of 3 min at 96°C, 5 min at 35°C, and 5 min at 75°C; and 32 cycles of 1 min at 96°C, 2 min at 55°C, 3 min at 75°C, and 2 min at 75°C. RAPD PCR products were separated on 1.5% agarose gel electrophoresis and were visualized by UV transillumination after staining with SYBR Safe. Isolates differing in their RAPD patterns were identified to species level based on the partial sequences of their 16S rRNA genes. The primers 27F and 1492R were used for the amplification of 16S rRNA genes (64). Amplification of the 28S rRNA genes of yeasts was performed using primers P1 and P2 (65). Details of the primers are given in Table 6. The PCR products were sequenced by Macrogen (Rockville, MD, USA) and analyzed by the Ribosomal Database Project (https://rdp.cme.msu.edu/).

Differentiation of *L. plantarum*, *L. pentosus,* and *L. paraplantarum* was conducted with a multiplex PCR assay (24) targeting *recA* with the *recA*-based primers paraF (5´-GTC ACA GGC ATT ACG AAA AC-3´), pentF (5´-CAG TGG CGC GGT TGA TAT C-3´), planF (5´-CCG TTT ATG CGG AAC ACC TA-3´), and pREV (5´-TCG GGA TTA CCA AAC ATC AC-3´) (Table 6). The PCR mixture was composed of 1.5 mM $MgCl_2$, the primers paraF, pentF, and pREV (0.25 μM each), 0.12 μM primer planF, 0.2 mM deoxynucleotide triphosphates (0.05 mM each), 0.025 U of *Taq* DNA polymerase/μl (Invitrogen Corporation, Carlsbad, CA, USA), 1× PCR buffer (Invitrogen), and 5 μl of DNA template. PCR conditions were an initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C (30 s), annealing at 56°C (10 s), and elongation at 72°C (30 s), and a final extension at 72°C for 5 min. The PCR products were separated on 2% agarose gel electrophoresis and visualized by UV transillumination after staining with SYBR Safe.

Extraction of bacterial DNA from millet malt. In brief, approximately 0.2 g of millet malt was weighed into a sterile 1.5-ml Eppendorf tube, followed by the addition of 1 ml sterile saline (0.85% NaCl) to the tube. This mixture was then homogenized at full-strength vortex for 10 min, followed by centrifuging at a slow speed of 500 rpm for 7 min. The supernatant was transferred into a new sterile 1.5-ml Eppendorf tube and centrifuged at 700 rpm for 7 min. Subsequently, the supernatant was transferred into a new sterile 1.5-ml Eppendorf tube. Cells were harvested by centrifugation, and DNA was extracted from harvested cells by using a DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA, USA). The quantity and quality of DNA was checked on a NanoDrop One spectrophotometer system (Thermo Fisher Scientific, Inc., Wilmington, DE).

Characterization of millet malt microbiota by 16S rRNA gene sequencing. To determine the relative abundance of bacterial DNA in 3 millet malt samples, high-throughput sequencing of 16S rRNA gene amplicons using Illumina MiSeq producing 300-bp paired-end sequences was performed by the University of Minnesota Genomics Center (Minneapolis, MN, USA). The V5-V6 regions of the 16S rRNA gene was amplified using the primer pair 784F (5′-RGGATTAGATACCC-3′) and 1064R (5′-CGACRRCCATGCANCAC CT-3′) (64). The total number of sequences generated was 112,992, corresponding to an average of 37,664 ± 5,406 sequences per sample. Subsequently, these sequences were analyzed with fewer OTU scripts (LotuS v. 1.565) in the pipeline, which includes a simultaneous demultiplexer and quality filter C++ program and a simple demultiplexer (sdm) to calculate denoised, chimera-checked, operational taxonomic units (66). Filtered sequences were clustered into OTUs with UPARSE (67), and taxonomic assignment was performed using BLAST against the SILVA v128 reference database (68). After processing and quality control, a total of 79,514 sequences corresponding to an average of 26,504 ± 4,149 sequences per sample was obtained. The relative abundance was calculated as the percentage of OTUs representing specific bacterial taxa relative to the total abundance of bacterial 16S rRNA genes.

Physicochemical analysis of traditionally prepared and laboratory-scale prepared mahewu. The pH of the mahewu samples was measured with a glass electrode. For the determination of organic acids and alcohols, samples were prepared for high-performance liquid chromatography (HPLC) analyses by the removal of solids by centrifugation. The supernatant was mixed 1:1 with 7% perchloric acid. Proteins were precipitated overnight at 4°C and subsequently removed by centrifugation. Organic acids and alcohols were quantified by HPLC using an Aminex HPX-87H column (300 mm by 7.8 mm; Bio-Rad, Torrance, CA, USA) at a temperature of 70°C (62). The eluent, 5 mM H_2SO_4, was used at a flow rate of 0.4 ml/min. Quantification of the analytes was based on refractive index detection. Concentrations of lactate, acetate, glycerol, and ethanol were determined using external standards (all Sigma-Aldrich).

Genomic DNA isolation, genome sequencing, assembly, and annotation. Genomic DNA for whole-genome sequencing was isolated from overnight cultures of *L. fermentum* FUA3588 and FUA3589 and *L. plantarum* FUA3590 grown in 10 ml of MRS5 broth. Genomic DNA was isolated using the Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) by following the manufacturer's guidelines. The quality and quantity of each sample was assessed using a NanoDrop One spectrophotometer system (Thermo Fisher Scientific, Inc., Wilmington, DE, USA), gel electrophoresis, and high-resolution melting-qPCR (HRM-qPCR) as described previously (29), with primers targeting 16S rRNA genes of lactic acid bacteria (69).

Genomic DNA samples were sequenced by Macrogen (Rockville, MD, USA) using Illumina HiSeq2500 Rapid Mode with an insert size of 350 bp to generate 100-bp paired-end reads. Assemblies were obtained using ABySS 1.3.4 (Assembly By Short Sequence) (70) with the most optimal k-mer value for each genome. After assembly, the fasta files were compared to those for species in the NCBI genome database. The genomes assemblies of *L. fermentum* strains FUA3588 and FUA3589 were improved by resorting the contigs using *L. fermentum* SNUV175 (GenBank accession no. NZ_CP019030.1) as the reference genome. For *L. plantarum* FUA3590, the genome assembly was resorted using *L. plantarum* subsp. *plantarum* SRCM100434 (accession no. NZ_CP021528.1) as the reference genome. Genomes were annotated automatically by the RAST server (http://rast.nmpdr.org/rast.cgi).

Multiple-genome alignment and strain-specific primer design. Strain-specific primer design was based on the unique nucleotide sequences of the target strains compared to sequences of other strains from the same species. Multiple-genome alignments were conducted to search for unique sequences in the genomes of *L. fermentum* FUA3588 and FUA3589 and *L. plantarum* FUA3590. Twenty of the most closely related genomes of *L. fermentum* FUA3588 and FUA3589 and 20 of the most closely related genomes of *L. plantarum* FUA3590 were downloaded from the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov) FTP site and used as reference genomes. Progressive Mauve algorithm (the Darling laboratory at the University of Technology, Sydney, Australia) was used for the comparative sequence analysis. The alignment results were displayed as horizontal panels for all of the input genomes (Fig. 2). Strain-specific primers CMA1F and CMA1R, CMB1F and CMB1R, and CMC1F and CMC1R were designed from the unique sequences from the genomes of *L. fermentum* FUA3588 and FUA3589 and *L. plantarum* FUA3590 strains using the PrimerQuest tool (Integrated DNA Technologies, Coralville, IA, USA). Details of the strain-specific primers are given in Table 6.

To evaluate primer specificity *in silico*, the designed strain-specific primers were confirmed using nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) of the nucleotide collection (nr/nt). Strain-specific primers were synthesized at Integrated DNA Technologies (Coralville, IA, USA), tested in PCRs with the template DNA of *L. fermentum* FUA3588 and FUA3589 and *L. plantarum* FUA3590 for validation, and confirmed by gel electrophoresis. PCRs with strain-specific primers resulted in positive amplicons from the genomic DNA of the respective strains.

qPCR for quantification of mahewu strains in millet malt. The presence of mahewu strains *L. fermentum* FUA3588 and FUA3589 and *L. plantarum* FUA3590 in the millet malt samples was detected

by using the 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The qPCR reaction mixtures with a total volume of 25 μl contained 12.5 μl of QuantiFast SYBR green master mix (Applied Biosystems), 1 μM (each) the forward and reverse strain-specific primers, and 1 μl of DNA template. The annealing temperature for the universal primers 27F and 1492R used was 55°C. Negative controls contained no template DNA, and positive controls contained genomic DNA isolated from the respective bacterial cultures. The specificity of each primer pair (Table 6) was verified in qPCR reactions with template DNA from *L. fermentum* FUA3588 and FUA3589 and *L. plantarum* FUA3590, as well as with qPCR-generated melting curves (data not shown). The qPCR amplification program was operated with a predenaturation stage at 95°C for 5 min; 40 cycles of three steps of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, and elongation at 75°C for 30 s; followed by a melting stage with default settings. Melting curves and agarose gel separation of amplicons were used to verify the specific amplification of target genes. Quantification of strain-specific amplicons relative to amplicons obtained with universal 16S rRNA primers was done with the following equation: relative abundance (%) = $100 \times 2^{(CT\text{universal primers} - CT\text{strain-specific primers})}$, where C_T is threshold cycle.

Duplicate independent experiments with duplicate technical repeats were conducted for all qPCR reactions. Melting curves and agarose gel separation of amplicons were used to verify the specific amplification of target genes.

Model mahewu fermentations. Five different strains with distinct colony morphologies isolated from mahewu and millet malt from site C were selected for model mahewu fermentation: *Lactobacillus fermentum* FUA3588, *Lactobacillus plantarum* FUA3590, *Enterococcus lactis* FUA3587, *Cronobacter sakazakii* FUA10024, and *Klebsiella pneumoniae* FUA10025. The inoculum was prepared by growing the strains on MRS5 agar at 30°C for 48 h, followed by overnight incubation in 10 ml MRS5 broth at 30°C. Cells were washed twice with sterile peptone saline and resuspended in 10 ml sterile tap water for use as the inoculum.

Two grams of maize meal (National Foods Limited, Harare, Zimbabwe) was mixed with 10 ml sterile water in a sterile conical flask and cooked into a thin porridge by boiling for 10 min. The porridge was then cooled with 19.85 ml sterile tap water and inoculated with each of the selected strains to obtain an initial concentration of 10^6 CFU/ml. Finger millet malt from site C (1 g) was then added to the flask and mixed. The flasks were sealed with sterile aluminum foil and incubated at 25°C. The slurry was sampled for analysis at 0, 2, 4, 8, 16, 24, 48, and 72 h of fermentation to determine the microbial counts and pH. Microbial counts for *L. plantarum* FUA3590 and *L. fermentum* FUA3588 were enumerated on MRS5 agar, and those for *E. lactis* FUA3587, *C. sakazakii* FUA10024, and *K. pneumoniae* FUA10025 were enumerated on LB agar. The colony morphology of *L. plantarum* FUA3590 was off-white with smooth raised colonies. *L. fermentum* FUA3588 appeared as white, large, flat, and mucoid colonies on MRS5 agar. Colonies of *K. pneumoniae* FUA10025 were gray, round, shiny, and mucoid. *C. sakazakii* FUA10024 appeared as yellow colonies, and *E. lactis* FUA3587 appeared as small white colonies on LB agar. The colony morphologies of the 5-strain bacterial cocktail used are shown in Fig. 3. The experiment was performed in triplicate. The organic acid concentrations were determined in the 72-h model mahewu.

Data availability. The genome sequence data for *L. fermentum* FUA3588 and FUA3589 and *L. plantarum* FUA3590 have been deposited in GenBank under accession numbers SMZI00000000, SMZH00000000, and SMZG00000000, respectively. The 16S rRNA gene sequences obtained from 3 millet malt samples were deposited under BioSample accession no. SAMN11129559.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.03130-18.

SUPPLEMENTAL FILE 1, PDF file, 0.02 MB.

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