Characterization of *Mahewu*, a Traditional Fermented Cereal Beverage from Zimbabwe, as a Source of Functional Lactobacilli

by

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Abstract

Mahewu is a non-alcoholic fermented maize and finger millet malt beverage produced in Zimbabwe. Africa has a rich tradition of cereal fermentations to produce diverse products including baked goods, non-alcoholic and alcoholic beverages with live microbiota and the widespread use of fermented porridges based on maize, millets or sorghum. The overview on the diversity of African fermented cereal foods suggests that the composition of fermentation microbiota and thus the impact of fermentation on product quality is determined by the choice of fermentation conditions. Despite the rich diversity of traditional fermented foods in Africa, there is a paucity of information on fully characterized and documented fermentation microbiota, and starter cultures developed from African traditional fermented cereal foods are scarcely available, if at all. Therefore, the aim of this research program was to characterize lactic acid bacteria isolated from *mahewu* and to determine their potential as functional lactobacilli in fermented cereal foods to counteract the poor sanitation endemic in rural communities in sub-Saharan Africa.

The composition and origin of *mahewu* microbiota were elucidated. The microbiota of *mahewu* samples consisted of 3 to 7 dominant strains of lactobacilli and 2 strains of yeasts. *Enterobacteriaceae* were not detected. Finger millet malt contained 8 to 19 strains of *Enterobacteriaceae*, lactobacilli, bacilli, and very few yeasts. Strain-specific quantitative PCR assays provided a direct assessment of the identity of strains from finger millet malt and *mahewu*. *Lm. fermentum* FUA3588 and FUA3589 were detected in finger millet malt, demonstrating that finger millet malt is a main source of mahewu microbiota. 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.

Comparative genomic analyses indicated that *mahewu* isolates harbor multiple multidrug resistance (MDR) proteins of the multidrug and toxin extrusion (MATE) family and the major facilitator superfamily (MFS). Strains of *Lactiplantibacillus plantarum* and *Limosilactobacillus fermentum* encoded for the same gene, termed *mahewu* phenolics resistance gene *mprA,* with more than 99% nucleotide identity, suggesting horizontal gene transfer. Strains of *Lp. plantarum* were more resistant than strains of *Lm. fermentum* to phenolic acids, other antimicrobials and antibiotics but the origins of strains were not related to resistance. The resistance of several strains exceeded EFSA thresholds for several antibiotics. Analysis of gene expression in one strain each of *Lp. plantarum* and *Lm. fermentum* revealed that at least one MDR gene in each strain was over-expressed during growth in wheat, sorghum and millet relative to growth in MRS5 broth. In addition, both strains over-expressed a phenolic acid reductase. The results suggest that diverse lactobacilli in *mahewu* share MDR transporters acquired by lateral gene transfer, and that these transporters mediate resistance to secondary plant metabolites and antibiotics.

The findings presented in this thesis provide comprehensive knowledge of the microbiotas of *mahewu* and finger millet malt and a better understanding of spontaneous cereal fermentations. This can lead to the selection and development of functional and probiotic starter cultures that may be used to mitigate the risks associated with uncontrolled cereal fermentation processes. What is demonstrated for mahewu is likely also true for other African fermented cereal foods that are produced in a comparable way.

Preface

This thesis is an original work by Felicitas Pswarayi.

Chapter 2 of this thesis is a literature review and is in preparation for submission as Felicitas Pswarayi and Michael G. Gänzle, "African cereal fermentations: A review on bioprocesses, fermentation organisms and product quality." *International Journal of Food Microbiology.* I was responsible for concept formation, data collection, analysis as well as the manuscript composition. M.G. Gänzle was the supervisory author and was involved with concept formation and manuscript composition.

Chapter 3 of this thesis has been published as Felicitas Pswarayi and Michael G. Gänzle (2019), in the Journal of Applied and Environmental Microbiology. "Composition and Origin of the Fermentation Microbiota of *Mahewu*, a Zimbabwean Fermented Cereal Beverage". *Applied and Environmental Microbiology* 85: e03130-18. I was responsible for sample collection, concept formation, data collection, analysis as well as the manuscript composition. Kirill Krivushin performed the bioinformatic analysis of finger millet malt microbiota by 16S rRNA gene sequencing. M.G. Gänzle was the supervisory author and was involved with concept formation and manuscript composition.

Chapter 4 of this thesis has been published as Felicitas Pswarayi, Nanzhen Qiao, Gautam Gaur and Michael Gänzle (2022), "Antimicrobial plant secondary metabolites, MDR transporters and antimicrobial resistance in cereal-associated lactobacilli: is there a connection?" *Food Microbiology*. 102, 103917. Nanzhen Qiao and Gautam Gaur performed the Comprehensive Antimicrobial Resistance Database (CARD) analysis. I was responsible for concept formation, data collection, analysis as well as the manuscript composition. All authors reviewed and approved

the final version of the manuscript. M.G. Gänzle was the supervisory author and was involved with concept formation and manuscript composition.

Dedication

To My Mother, Florence Pswarayi, For Always Standing in the Gap.

To El Roi

The God Who Sees Me!

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List of Abbreviations

AMR	Antimicrobial Resistance
BLASTn	Nucleotide Basic Local Alignment Search Tool
BLASTp	Protein Basic Local Alignment Search Tool
CARD	Comprehensive Antibiotic Resistance Database
CFU	Colony Forming Unit
EFSA	European Food Safety Authority
EPS	Exopolysaccharide
EstF	Esterase (*Lm. fermentum*)
EstP	Esterase (*Lp. plantarum*)
HcrB	Phenolic acid reductase (*Lp. plantarum*)
HcrF	Phenolic acid reductase (*Lm. fermentum*)
HGT	Horizontal gene transfer
HPLC	High Performance Liquid Chromatography
LAB	Lactic acid bacteria
LB	Luria-Bertani
MATE	Multidrug and Toxin Extrusion
MDR	Multidrug Resistance
MIC	Minimum Inhibitory Concentration
MeDuSa	Multi-draft based Scaffolder

MFS	Major Facilitator Superfamily
*mp*r	Millet phenolics resistance
MRS5	Modified deMan-Rogosa-Sharpe
NCBI	National Center for Biotechnology Information
OTU	Operational taxonomic units
Pad	Phenolic acid decarboxylase
PCR	Polymerase Chain Reaction
qPCR	quantitative Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RAST	Rapid annotations using subsystems technology
SPAdes	St. Petersburg genome assembler
$TanB_{LP}$	Tannase (*Lp. plantarum*)
VRB	Violet Red Bile
VRBG	Violet Red Bile Glucose

Chapter 1 General Introduction and Thesis Objectives

In an African village, fermented foods, non-alcoholic and alcoholic beverages are the very soul of life, for sustenance and indeed, for traditional ceremonies. Fermented foods have been defined as "foods made through desired microbial growth and enzymatic conversions of food components" (Marco et al., 2021). Fermented cereal foods are ancient and were the likely prerequisite for the Neolithic revolution (also called the agricultural revolution) about 14,000 years ago (Arranz-Otaegui et al., 2018; Hayden et al., 2013). It has been hypothesized that cereals were domesticated to enable fermentation of alcoholic beverages for rituals or festivities (Hayden et al., 2013). Similarly, the oldest breads have been dated to the same time and location as the origin of brewing (Arranz-Otaegui et al., 2018). The earliest production of fermented foods were spontaneous fermentations due to the development of the microflora naturally present on the raw materials (Leroy and De Vuyst, 2004). Notably, the revelation that fermentation enhances food preservation, quality, and functionality occurred independently on every continent and nearly at the same time in human history (Tamang et al., 2020). Consequently, because of their unique sensory properties, fermented foods are often deeply rooted in local culture, and have remained a staple in the human diet after the shift from artisanal to industrial food production in the last 150 years (Gänzle, 2020). In rural communities in developing countries, the production of traditional fermented foods and beverages is mainly through spontaneous fermentation and back-slopping, the practice of inoculating with material from a preceding fermentation, represents a cheap processing method, whereas in developed countries, the large-scale production of fermented foods with defined starter cultures is an integral branch of the food industry (Leroy and De Vuyst, 2004).

Notwithstanding the common features of fermented foods, many differences exist with respect to the substrates, products and microorganisms involved in the manufacture of fermented foods and

beverages produced globally. Beer and bread are the most characterized fermented foods, with more than 1400 publications for sourdoughs from Europe, Asia and North America (De Vuyst et al., 2014). African cereal fermentations differ from those in other places in the world because: (i) different cereals mainly maize, sorghum and millet are used; (ii) most common foods are porridges and non-alcoholic beverages and; (iii) many contain live fermentation microbes in the final product whereas most Asian and European products do not.

Despite the rich diversity of traditional fermented foods in Africa, there is a paucity of information on fully characterized and documented fermentation microbiota. In rural communities in Africa, cereals undergo uncontrolled spontaneous fermentations in order to enhance their flavor and digestibility. Therefore, the quality of the fermented products is determined by the microbial diversity and load on the raw materials and processing parameters. There are many risks associated with spontaneous cereal fermentations as they are poorly controlled, have inadequate storage and maturation conditions and are consumed without prior cooking which may reduce the safety of fermented foods (Nout, 1994). However, in rural communities, the traditional non-alcoholic and alcoholic fermented cereal beverages can provide a safe and transportable source of liquids in the absence of safe drinking water (Steinkraus, 1979). *Mahewu* is a non-alcoholic fermented beverage, produced by fermenting maize porridge with finger millet malt in Zimbabwe (Chapter 3, this thesis). *Mahewu* is produced at the artisanal or household level by spontaneous fermentation without control of the microbiota by back-slopping or the addition of starter cultures, and is a refreshing drink for children and adults and a weaning food for infants (Gadaga et al., 1999). Previous studies on *mahewu* produced in Zimbabwe, did not characterize the fermentation microorganisms to genus and species level (Simango and Rukure, 1992).

The characterization of the microbiotas of *mahewu* and finger millet malt allows for the selection and development of starter cultures that can shorten the fermentation process and reduce the risk of fermentation failure (Leroy and De Vuyst, 2004). These need to be evaluated for antimicrobial resistance (AMR), as antimicrobial resistance, especially transferable resistances, are a safety concern and a decision criterion for determining a microorganism's qualified presumption of safety (Koutsoumanis et al., 2020). The qualified presumption of safety assessment considers the following: 1) taxonomic aspects; 2) body of knowledge; 3) safety concerns in relation to virulence/pathogenicity, and antimicrobial resistance; and 4) safety for the environment (Koutsoumanis et al., 2020).

Several factors including use of antimicrobials, heavy metals or biocides can contribute to the occurrence and persistence of antimicrobial resistant bacteria and antimicrobial resistant genes in the environment (Koutsoumanis et al., 2021). (FAO/WHO, 2019) concluded that "there is clear scientific evidence that foods of plant origin may serve as vehicles of foodborne exposure to antimicrobial-resistant bacteria", and that plants harvested from manured soil can carry an additional burden of AMR in the form of resistant enteric and or environmental bacteria. One of the mechanisms of bacterial resistance to antimicrobials is the active extrusion of antimicrobial compounds by membrane efflux pumps which reduce the cellular concentrations of antimicrobial compounds to levels which are insufficient to kill or retard the growth of bacteria (Davies, 1996). Therefore, the presence of antimicrobial resistance in food fermenting bacteria which can be transmitted to pathogens presents a food safety risk in fermented foods (Koutsoumanis et al., 2021; Neu, 1992).

Functional starter cultures have been defined as "starter cultures that possess at least one inherent functional property", such as contributing to food safety, and or offers organoleptic, technological,

nutritional advantages through the production of ethanol, organic acids, exopolysaccharides, bacteriocins and enzymes (Leroy and De Vuyst, 2004). Contrastingly, probiotic starter cultures are "microbial species that have been shown in properly controlled studies to confer benefits to health" of the host (Hill et al., 2014). Traditional fermented cereal foods have enormous potential as vehicles to deliver beneficial bacteria but have received little attention for prophylactic and therapeutic use in resource poor countries (Franz et al., 2014). Therefore, the aim of this research was to characterize lactic acid bacteria isolated from *mahewu* and to determine their potential as functional lactobacilli in fermented cereal foods to counteract the poor sanitation endemic in developing countries.

1.1 Hypotheses

Improved control of the fermentation of *mahewu* and related African fermented cereal foods reduces hygienic risks and enriches health-beneficial microbes.

Acidification of the fermentation substrate with lactic and acetic acids is the predominant factor that results in elimination of *Enterobacteriaceae* in *mahewu*.

Genomes of plant-associated lactobacilli in *mahewu* encode for antimicrobial resistance that aid in defense against plant-secondary metabolites.

1.2 Objectives

1. To review the scientific literature on fermented cereal foods and fermentation microorganisms and to explore commonalities and differences between different foods, especially with respect to African fermented cereal foods and foods produced in other parts of the world (Chapter 2).

2. To characterize microbiota of traditionally prepared *mahewu*. To determine the origin(s) of the microbiota of traditionally prepared *mahewu* (Chapter 3).

3. To evaluate *mahewu* lactic acid bacterial isolates as starter cultures and their possible role in reducing counts of enteric pathogens in cereal fermentations (Chapter 3).

4. To use comparative genomics to identify novel characteristics of *mahewu* microbiota including resistance to phytochemicals and the production of exopolysaccharides (Chapter 3).

5. To explore possible connections between plant secondary metabolites with antimicrobial activity, phenolic acid resistance genes and antibiotic resistance in cereal isolates of lactobacilli (Chapter 4).

Chapter 2 African cereal fermentations: A review on bioprocesses, fermentation organisms and product quality

2.1 Introduction

Africa has a rich tradition of fermenting cereals to obtain a large diversity of products including non-alcoholic and alcoholic beverages, porridges and baked goods. With more than 2,000 distinct languages and cultures in Africa, it is conceivable thousands of different fermented foods are produced with many variations in the production processes and ingredients. Most of this diversity is found in sub-Saharan Africa, the area that lies south of the Sahara Desert and has not been strongly influenced by Arabic traditions. Fermented cereal foods and beverages have been produced using traditional fermentation methods at the household level; this tradition continues in rural communities across Africa. The body of knowledge and skills to produce traditional fermented foods is referred to as indigenous knowledge systems and is specific to each community and country.

Despite the rich diversity of traditional fermented foods in Africa, fermentation microbiota of less than 200 fermented cereal products have been characterized, documented and described in detail. The assembly of microbiota in spontaneous fermentations is limited by dispersal (Gänzle and Ripari, 2016), therefore, traditional fermentation products rely on spontaneous fermentation by microbiota autochthonous to the raw material used (Chapter 3, this thesis). Back-slopping, the practice of inoculating with a previous batch, eliminates dispersal limitation (Gänzle and Ripari, 2016), which often leads to dominance of host-adapted lactobacilli in cereal fermentations (Gänzle, 2019). The types of fermentation containers used, the different cereals as well as the environmental conditions, contribute to the selection of specific microorganisms and are responsible for the different flavor characteristics of the products. Currently, calabashes, very large

fruits that have been dried and hollowed out, clay pots, and metal or plastic buckets are used as fermentation vessels. Clay pots and calabashes are continuously reused and are the preferred fermentation vessels for traditional fermented foods. Micropores in the container walls retain fermented product from the previous batch and thus transfers microorganisms from previous fermentations which act as inoculum (Schoustra et al., 2013; Zvauya et al., 1997) (Chapter 3, this thesis).

The process of spontaneous cereal fermentations leads to very similar fermentation microbiota in comparable products and processes globally (Gänzle, 2019) and has been termed “the usual suspects". Fermentation is initiated by plant-associated *Enterobacteriaceae*, which are among the most abundant facultative anaerobes in cereal grains, followed by growth of enterococci, lactococci, *Leuconostoc* and *Weissella* spp., lactobacilli, particularly *Lactiplantibacillus plantarum, Limosilactobacillus fermentum* or *Pediococcus* spp. are typically the last organisms in this succession, which is attributable to their high acid resistance when compared to earlier fermentation organisms. This sequence closely resembles the succession of microbiota in spontaneous vegetable fermentations (Lee et al., 2005; Wuyts et al., 2018).

The composition of fermentation organisms in sourdough fermentations is strongly dependent on the fermentation conditions and processes that are employed, irrespective of the specific region of where the fermentation is carried out (Gänzle and Zheng, 2019; Van Kerrebroeck et al., 2017). While past reviews on African fermented food products provide an overview of African fermented foods and fermentation microbiota (Franz et al., 2014; Nout, 2009; Todorov and Holzapfel, 2015), past communications did not differentiate the products by process or region. The aim of this review is to relate available data on the fermentation process to data on microbial composition of non-alcoholic fermented cereal foods and beverages in sub-Saharan Africa.

Publications were selected that (i) provide a description of the processes that are used to produce the products, preferably also with reference to the social context of food production and consumption and (ii) provide a reliable quantification and characterization of fermentation organisms.

A state-of-the-art characterization of fermentation microbiota in fermented cereal foods requires culture-dependent methods that are often complemented by culture-independent methods (Comasio et al., 2020). It was consistently shown that all dominant microorganisms in cereal fermentations are isolated using culture-dependent methods (Bessmeltseva et al., 2014; Meroth et al., 2003; Van der Meulen et al., 2007; Wuyts et al., 2018)**.** Species level identification of isolates requires sequencing of full length rRNA genes while lactic acid bacteria (LAB) and yeast identification is not reliable if it is based only on morphological, physiological and biochemical characteristics. Use of only sequence-based characterization is inadequate because culture independent data do not account for viability. While this shortfall generally does not confound the characterization of back-slopped fermentations, which are characterized by stable microbial communities, it distorts the analysis of spontaneous fermentations, where different microbial communities occur in succession (see above). One of the most comprehensive analyses of spontaneous plant fermentations is available for carrot juice (Wuyts et al., 2018). Comparison of culture, rRNA quantification and rDNA quantification convincingly demonstrated that DNA-based 16S rRNA gene sequencing also includes DNA from dead bacterial cells originating from the raw material or earlier fermentation steps (Wuyts et al., 2018). The adhesion of bacterial cells to insoluble cereal proteins or starch granules, which are removed during DNA isolation, additionally distorts the composition of microbiota when analyzed by DNA-based methodology only (Meroth et al., 2003; Van der Meulen et al., 2007; Zheng et al., 2015b). Moreover, analyses

of fragments of rRNA genes including amplified ribosomal DNA restriction analysis (ARDRA) (Vogel et al., 1999), denaturing gradient gel electrophoresis (Meroth et al., 2003; Van der Meulen et al., 2007; Zheng et al., 2012), quantitative PCR (Lin and Gänzle, 2014a; Scheirlinck et al., 2009; Sekwati-Monang et al., 2012), and high-throughput sequencing of 16S RNA sequence tags (Bessmeltseva et al., 2014) do not reliably inform on species level taxonomy although this shortcoming can be addressed by full shotgun metagenomics sequencing (Comasio et al., 2020).

2.2 Processing steps matched to fermentation microbiota

In the following sections, fermented cereal foods of sub-Saharan Africa are grouped on the basis of comparable production processes. The term sub-Saharan Africa is not precisely defined; for the purpose of this communication, Sudan was included as it lies at the intersection of North Africa, which is heavily influenced by Mediterranean and Arabic traditions, and sub-Saharan Africa. Flow charts depicting the key processing steps of representative fermented cereal food products are matched to the corresponding tables which list the fermentation microbiota of the fermented cereal food products.

2.2.1 Finger millet and sorghum malts

The fermented cereal products which use malted cereals in their production are listed in Table 2.1. The flow chart for the production of malted cereals is shown in Figure 2.1, with household, community, or country-specific variations in the length of the time of the various stages in the malting process. Germinated finger millet or sorghum grains are sometimes used without sun drying and are wet milled or used as malted whole grains (Tables 2.1). The malt acts as both a source of multiple hydrolytic enzymes and as source of starch, proteins and other nutrients. Malt is used in conjunction with cooked cereals as a source of amylases and proteases, and of fermentation organisms. Other products use malt as sole substrate and as a source of amylases and

proteases where it is typically cooked or heated above 60 °C prior to the fermentation step (Table 2.1). The choice of sorghum and finger millet malts is mainly based on regional preferences (Nout and Davies, 1982). Malting generally improves the nutritional quality of the foods by making nutrients more bioavailable and reducing some antinutrients, and impacts phytochemicals (Taylor, 2017). The levels of α-amylase in sorghum malt are similar to those of barley malt, but the β-amylase content of sorghum malt is very low compared with that of barley malt (Beta et al., 1995). The low β-amylase levels means that there are low concentrations of maltose that are insufficient to sustain fermentation which selects against lactobacilli that use maltose as the preferred carbon source (Sekwati-Monang et al., 2012).

Like barley malt, finger millet and sorghum malt microbiota consists mainly of environmental *Enterobacteriaceae*, *Enterococcaceae*, environmental lactic acid bacteria, bacilli, and a few yeasts and is the likely source of fermentation microbiota of fermented cereal beverages (Mukisa et al., 2012; Noots et al., 1999; Sawadogo-Lingani et al., 2010) (Chapter 3, this thesis).

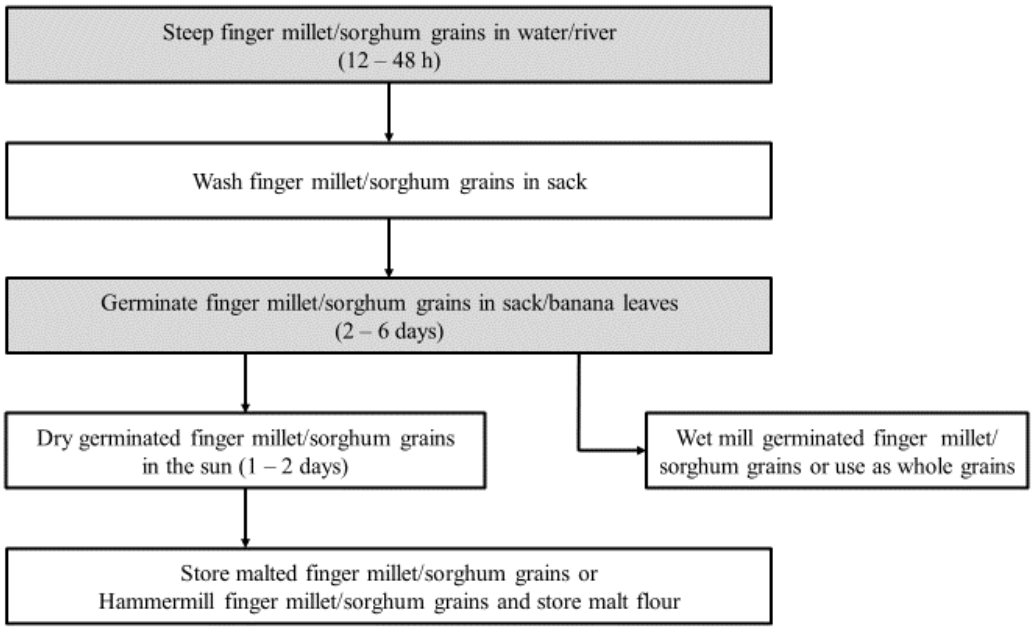

Figure 2.1 Preparation of finger millet and sorghum malts**.**
The grey shading indicates a fermentation step.

Table 2. 1 Malted cereals used in the preparation of fermented cereal foods

Product (country)	Malted Substrate	Malted Adjunct	Source of Enzymes	Source of Microbiota	References
Mahewu Zimbabwe		Finger millet/ Sorghum	+	+	(Chapter 3, this thesis), (Gadaga et al., 1999)
Togwa Tanzania		Finger millet/ Sorghum	+	+	(Kitabatake et al., 2003; Mugula et al., 2003)
Munkoyo Zambia		Finger millet	+	+	(Phiri et al., 2019)
Obushera Uganda		Finger millet/ Sorghum	+	+	(Mukisa et al., 2010)
Obutoko Uganda	Sorghum	Sorghum	+	+	(Mukisa et al., 2012, 2010)
Obuteire Uganda	Finger millet	Finger millet	+	+	(Mukisa et al., 2012, 2010)
Mangisi Zimbabwe	Finger millet		+	-	(Zvauya et al., 1997), (Gadaga et al., 1999)
Leting Lesotho	Sorghum		+	-	(Gadaga et al., 2013)
Obiolor Nigeria	Sorghum + Millet		+	-	(Achi, 1990)
Oshikundu Namibia	Sorghum	Sorghum	+	+	(Embashu et al., 2013)
Bushera Uganda	Finger millet/ Sorghum	Finger millet/ Sorghum	+	+	(Muyanja et al., 2003)
Gowé Benin	Sorghum		+	+	(Vieira-Dalodé et al., 2007)
Kwete Uganda		Finger millet	+	+	(Namugumya and Muyanja, 2009)
Malwa Uganda	Finger millet	Finger millet	+	+	(Muyanja et al., 2010)

Product (country)	Malted Substrate	Malted Adjunct	Source of Enzymes	Source of Microbiota	References
Hussuwa Sudan	Sorghum	Sorghum	+	+	(Yousif et al., 2010)
Hussuwa Sudan	Sorghum	Sorghum	+	+	(Yousif et al., 2005)
Hulu mur Sudan	Sorghum		+	+	(Mahgoub et al., 1999)
Sorghum malt Burkina Faso	Sorghum	Sorghum	+	+	(Sawadogo-Lingani et al., 2010)

2.2.2 Non-alcoholic fermented cereal beverages prepared from cooked porridge

The flow charts and fermentation organisms for production of non-alcoholic fermented beverages that are produced from cooked porridge are shown in Figure 2.2 and the fermentation microbiota are listed in Table 2.2 The lactic fermented non-alcoholic cereal beverages are a common feature in the diet of rural communities in East, Central and Southern Africa (Table 2.2). These beverages are processed in a similar manner which involves cooking of a thin maize porridge but differ with respect to the adjunct material that is added after cooking. Most beverages in the different countries are produced from maize but the porridges can also be made from finger millet or sorghum meal or varying combinations of the three cereals with the addition of different adjuncts (Table 2.2). These fermented beverages contain actively fermenting lactobacilli and yeasts, are colloidal, coarse and gritty. The beverages like *togwa* are opaque and reddish to brownish in color depending on the finger millet variety used and contain solid particles from the pericarp of finger millet and maize grains, which give a slightly floury flavor and a gritty mouthfeel (Kitabatake et al., 2003). These beverages are part of the staple diet and are refreshing drinks in the fields or at social gatherings and are also used as complementary food for infants and for the sick (Gadaga et al., 1999; Kitabatake et al., 2003; Schoustra et al., 2013).(Chapter 3, this thesis).

Since most LAB lack amylolytic enzymes and are therefore unable to utilize starch directly, the addition of malt to degrade starch has the advantage of releasing soluble sugars, which impart a sweet taste but also promote lactic fermentation and rapid acidification of the porridge, resulting in lower viscosity beverage products (Steinkraus, 2004; Taylor and Duodu, 2019). In the Democratic Republic of Congo and Zambia, *Rhynchosia* roots are used in the production of *munkoyo* and *chimbwantu* beverages (Foma et al., 2012; Schoustra et al., 2013). *Rhynchosia* roots contain exceptionally high levels of α- and β-amylases and rapidly liquefy the porridge gel

(Mulkay et al., 1985 as cited by (Zulu et al., 1997). In a few products, e.g. *tobwa, emahewu* and *ekitiribita*, no adjunct is used in the fermentation process which takes up to five days to ferment and is therefore, susceptible to proliferation of harmful microorganisms (Gadaga et al., 1999; Mukisa et al., 2012; Simatende et al., 2015). In South Africa, wheat flour acts a source of bacteria and hydrolytic enzymes in the preparation of *mageu* which is also known as *amahewu* (Taylor, 2016).

Reliable data on the composition of fermentation microbiota is available for *ekitiribita*, *obuteire*, and *mahewu* fermented cereal beverages that are produced in Uganda and Zimbabwe, respectively (Mukisa et al., 2012) (Chapter 3, this thesis). The microbiota of *ekitiribita*, *obuteire* and *mahewu* samples consisted of three to seven dominant strains of lactobacilli and 1 or 2 yeasts (Table 2.2). Isolates that were obtained from *obushera; obutoko, enturire* (an alcoholic variant of *obutuku*), *ekitiribita and obuteire* (Tables 2.2 and 2.3) include strains of *Lact. lactis, Lp. plantarum, Enterococcus* spp*., W. confusa, P. pentosaceus, Lm. fermentum, L. delbrueckii, Leuc. lactis, Strep. infantarius (S. lutetiensis), Cyberlindnera fabianii (Pichia fabianii) S. cerevisiae, Clavispora lusitaniae, Issatchenkia orientalis* (Mukisa et al., 2012). Data for other products *chimbwantu* and *munkoyo* based on amplicon sequencing without culture (Schoustra et al., 2013) confirm the assumption that fermentation microbiota are the "usual suspects". Variation in sensory attributes is due to the different raw materials and adjuncts used, stage of fermentation and to the effect of different household or producers' preferences among others (Foma et al., 2012; Gadaga et al., 1999; Mukisa et al., 2012; Phiri et al., 2020).

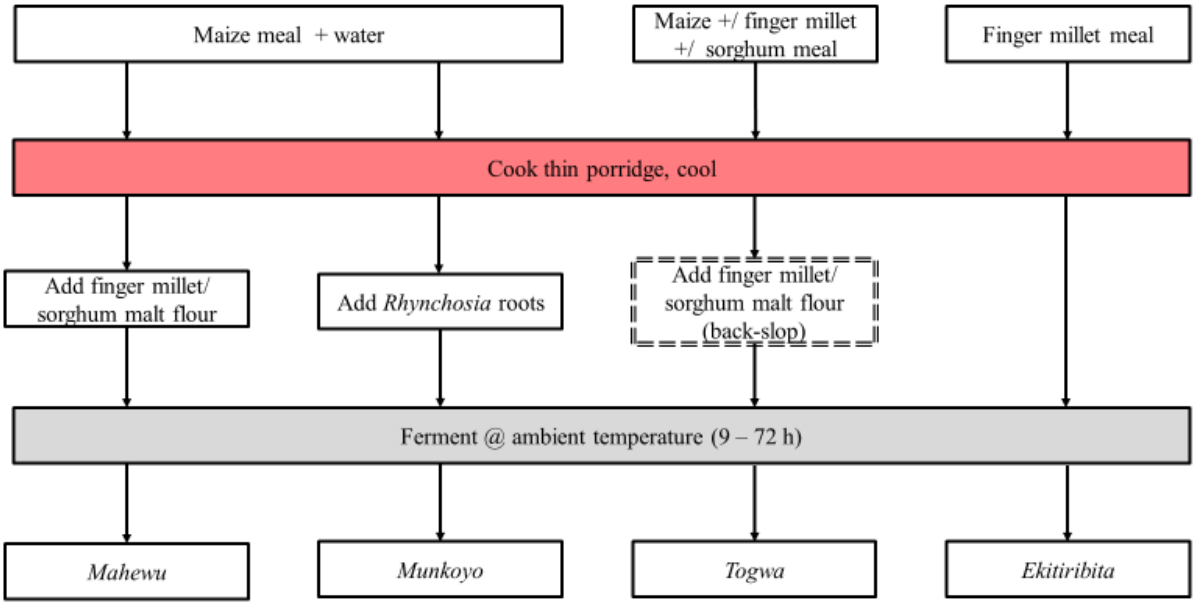

Figure 2.2 Preparation of non-alcoholic fermented cereal beverages from cooked porridge.

Shown are the names of the non-alcoholic beverages produced from the fermentation of cooked porridges. The steps in the flow chart are colour-coded as follows: grey shading indicates fermentation; dashed double lines indicate optional back-slopping; red shading indicates a heating step that inactivates vegetative bacterial cells.

Table 2.2 Non-alcoholic fermented cereal beverages prepared from cooked porridge

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Mahewu	Zimbabwe	Maize meal	Finger millet malt (Sorghum malt)	*Lm. fermentum, Lp. plantarum, P. pentosaceus, Ff. rossiae Candida glabrata, S. cerevisiae, W. cibaria, W. confusa, Weissella spp., Leuconostoc holzapfelii, Lactococcus lactis, Leuc. pseudomesenteroides, P. pentosaceus, Saccharomyces cerevisiae*	(Chapter 3, this thesis)
Ekitiribita (Obushera)	Uganda	Finger millet meal		*Leuc. lactis, Streptococcus gallolyticus, Bacillus* spp., *Enterobacteriaceae, Sc. infantarius subsp.infantarius/Sc. equinus, Lc .lactis, Sc. thermophilus* (DGGE)	(Mukisa et al., 2012, 2010)
Obuteire (Obushera)	Uganda	Finger millet meal	Finger millet malt	*Sc. gallolyticus, L. delbrueckii, S. cerevisiae* (DGGE)	(Mukisa et al., 2012, 2010)
Related products for which information on fermentation microorganisms is unavailable					
Munkoyo	Zambia	Maize meal (finger millet/ sorghum)	*Rhynchosia* root,	*Lactobacillaceae*	(Schoustra et al., 2013)
Chimbwantu	Zambia	Maize meal (finger millet/ sorghum)	*Rhynchosia* root,	*Lactobacillaceae*	(Schoustra et al., 2013)
Munkoyo	Zambia (Choma)	Maize grits	*Rynchosia* root extract	*Streptococcaceae, Lactobacillaceae,*	(Phiri et al., 2020)
Munkoyo	Zambia (Nyimba)	Maize meal	*Rhynchosia* root	*Enterobacteriaceae, Streptococcaceae, Moraxellaceae, Lactobacillaceae*	(Phiri et al., 2020)
Munkoyo	Zambia (Kitwe)	Maize meal (caramelized porridge)	*Rhynchosia* root	Unknown *Lactobacillaceae*	(Phiri et al., 2020)

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Munkoyo	Zambia	Maize meal	*Rynchosia* root (finger millet malt/ cowpea root/sweet potato peels)	Unknown *Streptococcaceae, Lactobacillaceae, Enterobacteriaceae, Bacillaceae Aeromonadaceae*	(Phiri et al., 2019)
Munkoyo	Democratic Republic of Congo	Maize meal	*Rynchosia* root	Unknown	(Foma et al., 2012)
Togwa	Tanzania	Maize+/finger millet+/ Sorghum+/meals	Sorghum/finger millet malt flour +/ back slopping	Unknown	(Mugula et al., 2003)
Togwa	Tanzania	Maize	Finger millet malt flour	Unknown	(Kitabatake et al., 2003)
Maxau	Namibia	Maize meal	Wheat flour + sugar + back slopping	Unknown	(Misihairabgwi and Cheikhyoussef, 2017)
Tobwa	Zimbabwe	Maize meal		Unknown	(Gadaga et al., 1999)
Amahewu	South Africa	Maize meal	Wheat flour	Unknown	(Chelule et al., 2010)
Emahewu	Swaziland	Maize meal	Maize bran, or potato or sugar, or sorghum malt grains/no adjunct	Unknown	(Simatende et al., 2015)

2.2.3 Non-alcoholic fermented malted cereal beverages

The process flow charts for the production of non-alcoholic beverages that are produced from malted grains rather than a cooked porridge are shown in Figure 2.3 and the fermentation microbiota are listed in Table 2.3. The preparation of beverages involves the spontaneous fermentation of the mash produced by heated or boiled malted finger millet or sorghum flour or grains or a mixture: mashing of malt serves as starch saccharification (Table 2.3). The fermentations are predominantly spontaneous and not controlled with back slopping (Table 2.3). *Oshikundu* is colloidal because it is not filtered whereas *mangisi* and *leting* are light brown liquids because the production process involves a filtration step (Embashu, 2014; Gadaga et al., 2013; Zvauya et al., 1997).

Amylases remain active in the initial stages of heating, until a temperature of greater than 60°C is reached, before the heat inactivates the amylase, a good part of the starch is converted to maltose or glucose (Zvauya et al., 1997). This roughly matches mashing of barley malt in beer production (Zvauya et al., 1997). *Mangisi* is a sweet and sour beverage because after the mashing process it is naturally sweet and then becomes sour after the fermentation of the mash that is known as *masvusvu* (Zvauya et al., 1997). The fermented malted cereal beverages are sweet and sour refreshing drinks for the whole household, for the sick and the elderly, as well as weaning foods for infants, and are consumed at social gatherings and important traditional functions (Embashu et al., 2013; Gadaga et al., 1999; Zvauya et al., 1997).

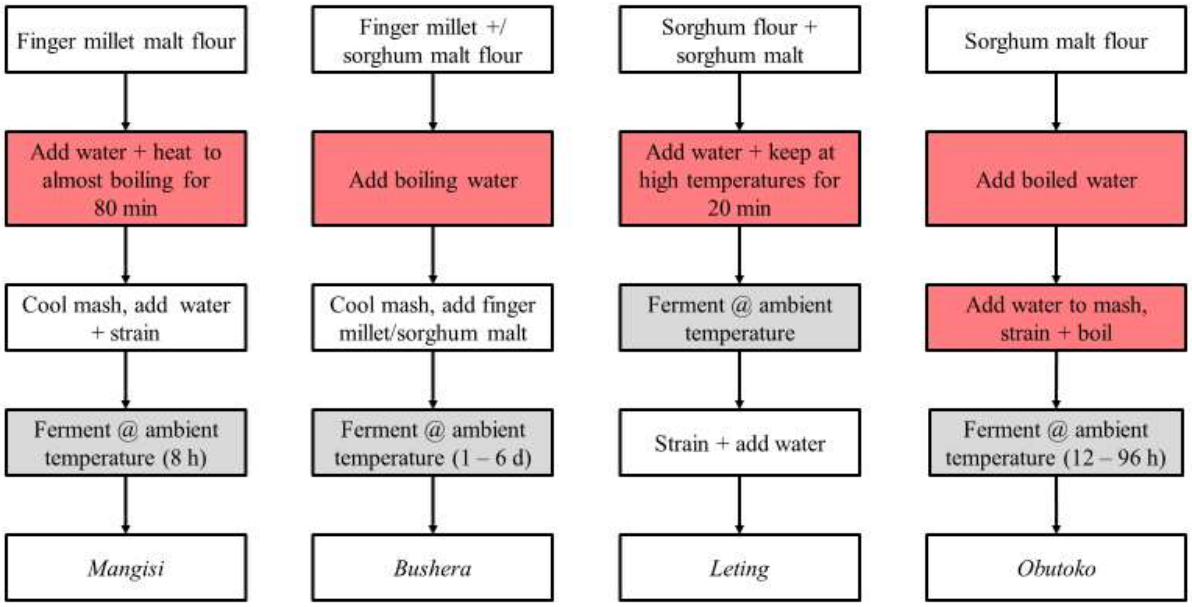

Figure 2.3 Preparation of non-alcoholic fermented malted cereal beverages.

Shown are the names of the non-alcoholic beverages produced from fermented malted beverages. The steps in the flow chart are colour-coded as follows: grey shading indicates fermentation; dashed double lines indicate optional back-slopping; red shading indicates a heating step that inactivates vegetative bacterial cells.

Table 2.3 Non-alcoholic fermented malted cereal beverages

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Obutoko (Obushera)	Uganda	Sorghum malt flour		*Sc. gallolyticus, W. confusa/W. cibaria, Lm. fermentum, L. delbrueckii, Streptococcus* spp. *S. cerevisiae, Pichia* spp. (DGGE)	(Mukisa et al., 2012, 2010)
Bushera	Uganda	Finger millet/Sorghum malt flour	Finger millet +/ sorghum malt flours	*Lp. plantarum, Lb. paracasei, Lm. fermentum, Lv. brevis L. delbrueckii; Sc. thermophilus* (API)	(Muyanja et al., 2003)
Oshikundu	Namibia	Pearl millet (boiling water)/ Pearl millet flour + sorghum malt (warm water)	Sorghum malt flour Pearl millet bran + back slopping +	*Lp. plantarum, Lc. lactis, L. delbrueckii, Lm. fermentum, Lp. pentosus, Lt. curvatus, Ent. cloacae, C. sakazakii, P. luteola, P. aeruginosa, Serratia ficaria* (API)	(Embashu et al., 2013) (Embashu, 2014)
Related products for which information on fermentation microorganisms is unavailable					
Mangisi	Zimbabwe	Finger millet malt		Unknown	(Simango, 1997; Zvauya et al., 1997) (Gadaga et al., 1999)
Leting	Lesotho	Sorghum + sorghum malt		Unknown	(Gadaga et al., 2013)
Obiolor	Nigeria	Millet +Sorghum malt flours		Unknown Bacilli, lactobacilli, lactococci	(Achi, 1990)

2.2.4 Non-alcoholic fermented malted cereal beverages with two fermentation steps

The flow chart for the production of non-alcoholic fermented malted cereal beverages with two fermentation steps is shown in Figure 2.4 and the fermentation microbiota are listed in Table 2.4. *Gowé* is a non-alcoholic beverage produced in Benin and involves the primary fermentation of sorghum malt and a secondary fermentation after the addition of sorghum flour to form a paste that is cooked and further diluted with cold water or milk (Vieira-Dalodé et al., 2007) (Table 2.4). Maize meal can also be used instead of sorghum flour to prepare *gowé*. Unlike other African fermented cereal beverages, the malted cereal is the substrate for the primary fermentation and therefore *gowé* has a natural sweet taste and a soft texture loved by children and adults (Vieira-Dalodé et al., 2007). *Gowé* processing is characterized by a mixed fermentation microbiota consisting of six lactobacilli and four yeasts (Table 2.4).

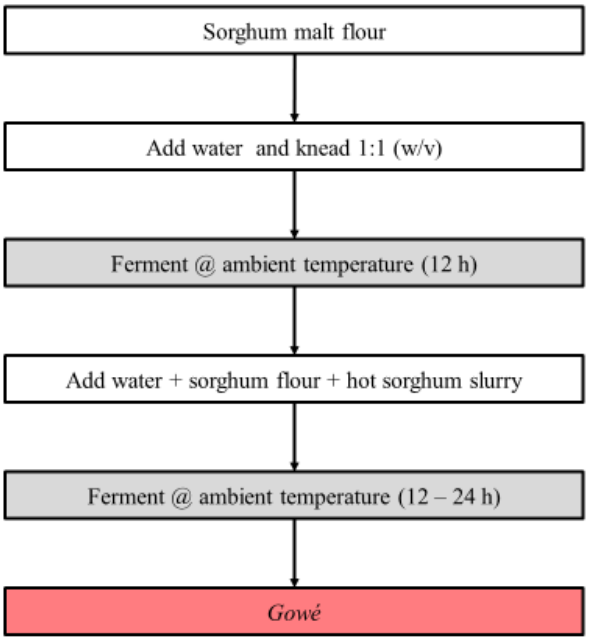

Figure 2.4 Preparation of non-alcoholic fermented malted cereal beverage with two fermentation steps.

Shown is the name of the intermediate product which is used for the preparation of *gowé*. The steps in the flow chart are colour-coded as follows: grey shading indicates fermentation; dashed double lines indicate optional back-slopping; red shading indicates a heating step that inactivates vegetative bacterial cells.

Table 2.4 Non-alcoholic fermented malted cereal beverage with two fermentation steps

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Gowé	Benin	Sorghum malt flour	Sorghum flour + hot sorghum slurry	*Lm. fermentum, Lm. mucosae, W. confusa, W. kimchii, P. acidilactici, P. pentosaceus, Kluyveromyces marxianus, Pichia anomala, C. krusei, C. tropicalis*	(Vieira-Dalodé et al., 2007)
Gowé	Benin	Maize + sorghum malt		*C. krusei, Cl. lusitaniae, C.tropicalis K. marxianus. (Culture independent: Dekkera bruxellensis, Debaryomyces hansenii)*	(Greppi et al., 2013b)

2.2.5 Non-alcoholic fermented cereal beverages prepared from baked fermented cereals

The flow charts for the production of non-alcoholic fermented cereal beverages that are produced from baked fermented cereals are shown in Figure 2.5 and the products are listed in Table 2.5. In Sudan, non-alcoholic beverages are made from fermented sorghum sourdoughs that are baked into thin sheets before being soaked in water (Odunfa and Oyewole, 1998). The baked thin transparent flakes are dissolved in water to make *abreh* (Table 2.5). The baked brown flat sheets are broken down into flakes and soaked in water for a few hours and the brownish supernatant becomes the beverage *hulu mur* (Table 2.5). Enzymes are from the malted sorghum, which is also a source of fermentation microbiota (Table 2.1). Baking generates color and flavor from Maillard reactions or nonenzymatic browning reactions (Ames, 1990). *Hulu mur* and *abreh* are thirst quenching drinks, with *hulu mur* being especially popular during the Muslims' Ramadan month of fasting (Mahgoub et al., 1999).

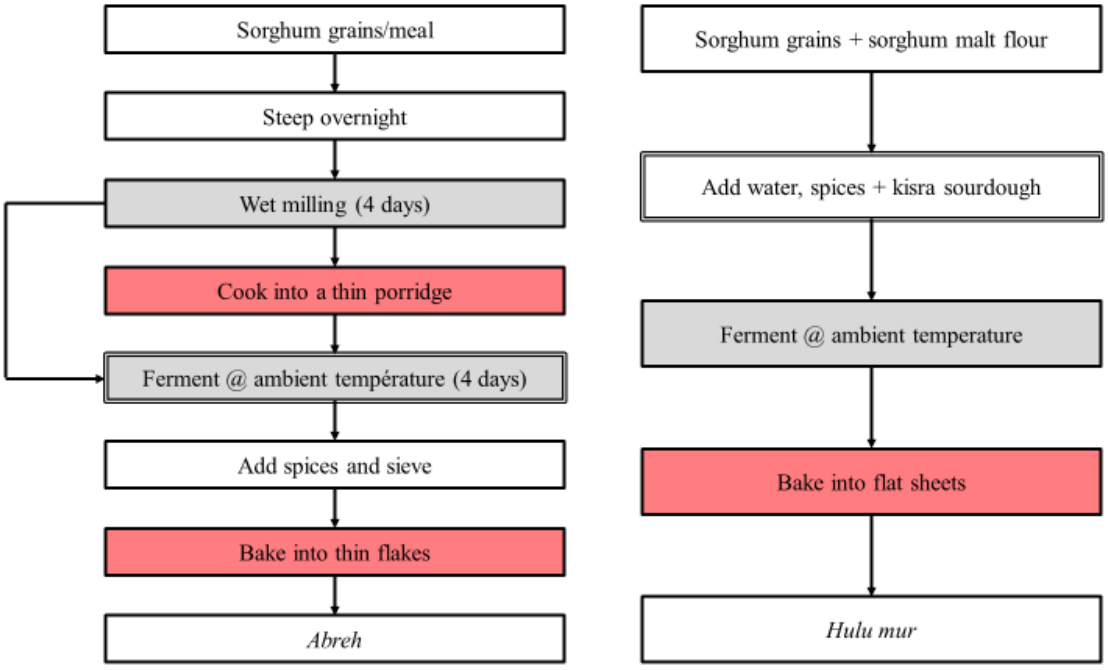

Figure 2.5 Preparation of non-alcoholic fermented beverages from baked fermented cereals.

Shown are the names of the products produced from baked fermented cereal products. The steps in the flow chart are colour-coded as follows: grey shading indicates fermentation; double lines indicate back-slopping; red shading indicates a heating step that inactivates vegetative bacterial cells.

Table 2.5 Non-alcoholic cereal beverages prepared from baked fermented cereals

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Abreh	Sudan	Sorghum grains	Mother dough (from 1st fermentation)	Unknown	(Odunfa and Oyewole, 1998)
Hulumur	Sudan	Sorghum malt flour + sorghum grains	Fermented *kisra* dough	Unknown	(Mahgoub et al., 1999)

2.2.6 Non-alcoholic fermented beverage prepared from fermented roasted sourdough

The flow chart for the production of a non-alcoholic fermented beverage that is prepared from fermented and roasted sourdough which is fermented again is shown in Figure 2.6 and the product is listed in Table 2.6. *Kwete* is a traditional fermented beverage produced in Uganda whereby maize sourdough is fermented, roasted, and then fermented again with the addition of finger millet malt and water and strained (Figure 2.6 and Table 2.6). Roasting generates color and flavor in the Maillard reaction, and facilitates the gelatinization of starch which is crucial for the activity of amylases during the mashing process. *Kwete* is a thirst quenching beverage for the whole family (Namugumya and Muyanja, 2009). The enzymes are from the finger millet malt which is a source of hydrolytic enzymes and fermentation microbiota (Table 2.1).

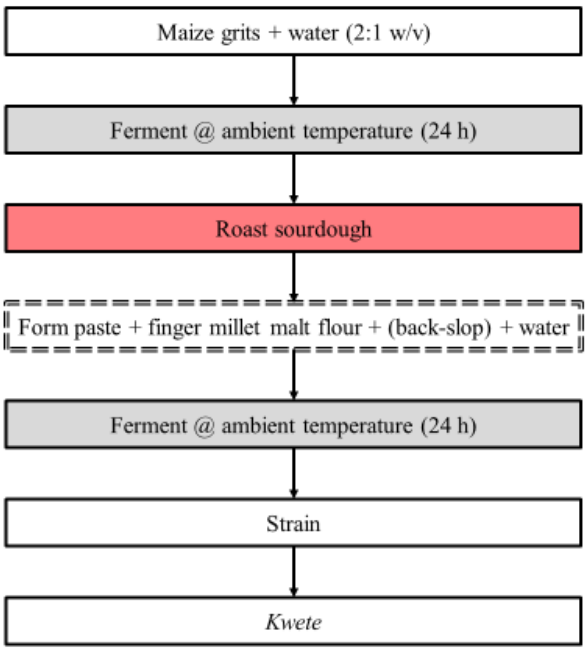

Figure 2.6 Preparation of non-alcoholic fermented beverage from fermented roasted sourdough.

Shown is the non-alcoholic beverage produced from fermented roasted sourdough. The steps in the flow chart are colour-coded as follows: grey shading indicates fermentation; dashed double lines indicate optional back-slopping; red shading indicates a heating step that inactivates vegetative bacterial cells.

Table 2.6 Non-alcoholic fermented beverage prepared from fermented cereal roasted sourdough

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Kwete	Uganda	Maize grits +/ finger millet grains	Finger millet malt flour +/ back slop	Unknown	(Namugumya and Muyanja, 2009)

2.2.7 Fermented sour porridges

The flow chart for the production of fermented cereal slurries that are produced as intermediate products in the preparation of sour porridges is shown in Figure 2.7 and the fermentation microbiota are listed in Table 2.7. Traditional fermented slurries from maize, millets and sorghum meals singly or a mixture thereof, back-slopped or not, are produced as intermediate products for the preparation of thin and thick porridges, which are an important staple of the African diet (Figure 2.7 and Table 2.7). The thin porridge is eaten at breakfast while the thick porridge forms the main part of the meal at lunch and dinner and is known by different names in different countries. Sour porridges are important weaning foods for infants and children (Graham et al., 1986; Madoroba et al., 2011; Masha et al., 1998; Simango, 1997). The porridges are produced with unmalted cereals. Fermentation is very important, as it improves the protein digestibility of cooked sorghum porridges like *ting* (Taylor and Taylor, 2002). As with other fermented cereal foods throughout Africa, the preparations are similar within the country and beyond but the differences in household preferences account for slight variations with respect to the level of souring and whether the cereals are mixed together or used singly. The microbiota of fermented cereal slurries consisted of two – five dominant strains of lactobacilli (Table 2.7).

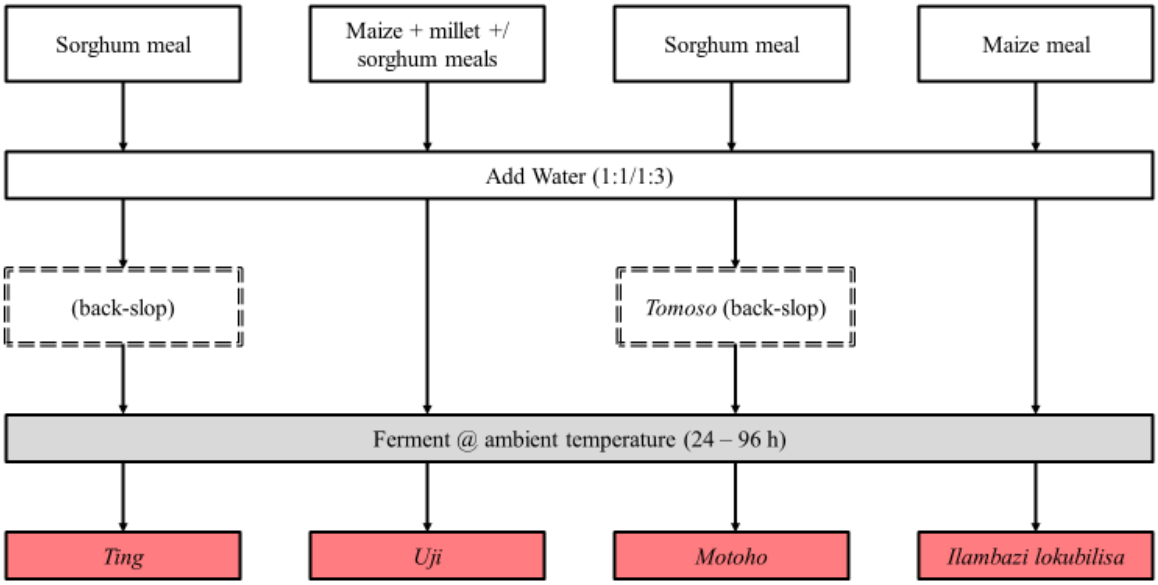

Figure 2.7 Preparation of fermented cereal slurries used to produce sour porridges.

Shown are the names of the intermediate products which are used for the preparation of sour porridges. The steps in the flow chart are colour-coded as follows: grey shading indicates fermentation; dashed double lines indicate optional back-slopping; red shading indicates a heating step that inactivates vegetative bacterial cells.

Table 2.7 Fermented cereal slurries used to produce sour porridges

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Ting	Botswana	Sorghum meal	Back-slop or not	*Lm. reuteri, Lm. fermentum, Schleiferilactobacillus harbinensis, Lp. plantarum, Ln. parabuchneri, Lb. casei, Lo. coryniformis*	(Sekwati-Monang and Gänzle, 2011)
Ting	South Africa	Sorghum meal (Lab preparation)		*Lc. lactis, Lm. fermentum, Lp. plantarum, Lc. rhamnosus, W. cibaria, E. faecalis, E. mundtii, Enterobacteriaceae* (DGGE)	(Madoroba et al., 2011)
Related products for which information on fermentation microorganisms is unavailable					
Motoho	Lesotho	Sorghum meal Back-slop	Tomoso (sorghum starter culture) +/ back slop	Unknown	(Gadaga et al., 2013)
Uji	Kenya	Maize + millet/ maize + sorghum meals		Unknown	(Masha et al., 1998)
Nasha	Sudan	Sorghum meal	Starter culture derived from wild yeasts	Unknown	(Graham et al., 1986)
Ilambazi lokubilisa	Zimbabwe	Maize meal		Unknown	(Simango, 1997)

2.2.8 Wet milled fermented cereal doughs and slurries

The flow chart for the production of wet milled fermented cereal slurries and doughs that are produced as multi-purpose intermediate products for the preparation of diverse foods is shown in Figure 2.8 and Table 2.8. The wide variety of products obtained from either fermented cereal slurries or doughs can be categorized into the following groups: non-alcoholic beverages, thin and thick porridges (gruels), dumplings and baked flatbreads (Table 2.8). Steeping is essentially the

first fermentation step and serves to facilitate the milling step. However, if the cereal grains are only steeped for six hours this is not a fermentation step, but if the grains are steeped for more than 12 hours fermentation has taken place and after 24 hours the "usual suspects" are present. Maize is the most common cereal used in the southern parts of West Africa, while sorghum and pearl millet are mainly used in the northern parts of West Africa where it is drier. The fermented cereal doughs and the cooked products are known by different names in different localities and countries with small variations in the preparation and cooking steps. These cereals doughs are cooked after the fermentation process and do not contain live microbiota. However, the beverage *kunun-zaki* contains live microbiota because a second fermentation step is included after cooking (Figure 2.8) (Efiuvwevwere and Akona, 1995). Unlike *mahewu* and *togwa, kunun-zaki* has a smooth, milky and creamy appearance because the preparation step includes a filtration step (Efiuvwevwere and Akona, 1995; Kitabatake et al., 2003). Another beverage that contains live microbiota is *koko* sour water, which is the top-layer of the fermenting *koko* slurry, that is consumed uncooked as a treatment for upset stomachs or as a refreshing drink (Lei and Jakobsen, 2004). These fermented cereal doughs are as a result of spontaneous fermentations prepared at the household level in rural communities and are also produced at small-scale informal micro-enterprises for sale at the local markets in urban communities using the locally available cereals because the processes are lengthy and laborious (Halm et al., 2004; Mouquet-Rivier et al., 2008). Fermented foods constitute a significant component of the West African diet, and some are part of the main course, others are beverages and porridges which are also used as weaning foods for infants (Table 2.8). *Lm. fermentum* is the predominant microorganism in many of the West African fermented cereal foods and the microbiota consists of two – five lactobacilli and yeasts (Table 2.8).

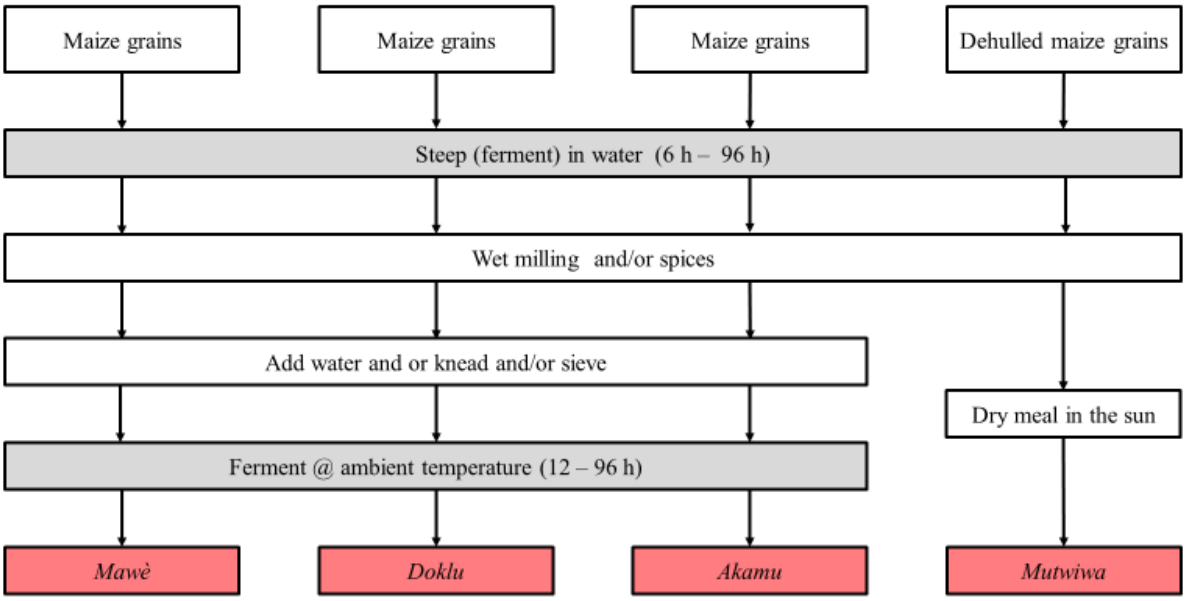

Figure 2.8 Preparation of wet milled fermented cereal doughs and slurries.

Shown are the names of the intermediate products which are used for the preparation of a variety of fermented foods that are always cooked. The steps in the flow chart are colour-coded as follows: grey shading indicates fermentation; dashed double lines indicate optional back-slopping; red shading indicates a heating step that inactivates vegetative bacterial cells.

Table 2.8 Wet milled fermented cereal doughs and slurries

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Akamu	Nigeria	Maize (millet/ sorghum)		*Lm. fermentum, Lp. plantarum, L. helveticus, Lc. lactis ssp. cremoris, L. acidophilus, Lb. casei, Lb. rhamnosus, C. tropicalis, C. albicans, Clavispora lusitaniae, Saccharomyces paradoxus*	(Obinna-Echem et al., 2014)
Mawè	Benin	Maize (sorghum)		*Lm. fermentum, Lp. plantarum, P. acidilactici, W. confusa, P. pentosaceus, Pichia kudriavzevii, Kluyveromyces marxianus, S. cerevisiae, Ogataea polymorpha, C. glabrata, Wickerhamomyces anomalus*	(Houngbédji et al., 2018)
Ogi	Nigeria	Maize		*Lm. fermentum, Lp. plantarum, Bacillus pumilus, B. cereus, B. subtilis, St. hominis,*	(Oguntoyinbo and Narbad, 2012)
Kunu-zaki	Nigeria	Pearl millet		*Lm. fermentum, Lp. plantarum, Sc. gallolyticus, P. pentosaceus Bacillus pumilus, B. cereus, B. subtilis, St. hominis*	(Oguntoyinbo and Narbad, 2012)
Ogi	Nigeria	Maize		*L. delbrueckii, L. helveticus, Lm. fermentum, Lp. plantarum, Lb. pantheris, Paucilactobacillus vaccinostercus, Lo. bifermentans, Companilactobacillus nantensis* (16S rRNA gene clone libraries)	(Oguntoyinbo et al., 2011)
Kunu-zaki	Nigeria	Pearl millet		*Sc. lutetiensis, Lm. fermentum, L. delbrueckii, W. confusa, Sc. gallolyticus, Sc. bovis, B. cereus, C. perfringens* (16S rRNA gene clone libraries)	(Oguntoyinbo et al., 2011)
Doklu	Cote d'Ivoire	Maize		*Lp. plantarum, Lm. fermentum, P. acidilactici, P. pentosaceus, W. cibaria*	(Assohoun-Djeni et al., 2016)

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Koko sour water (KSW)	Ghana	Pearl millet (Maize, sorghum)		W. *confusa, Ligilactobacillus salivarius, P. pentosaceus, P. acidilactici, Lp. plantarum*	(Adimpong et al., 2012)
Koko	Ghana	Pearl millet (Maize, sorghum)		Koko & KSW: W. *confusa, Lm. fermentum* KSW: *Lg. salivarius, P. pentosaceus, P. acidilactici, Lp. paraplantarum* (API)	(Lei and Jakobsen, 2004)
Ben-saalga	Burkina Faso	Pearl millet		*Weissella, Pediococcus, Lactobacillus, Streptococcus, Lactococcus, Leuconostoc, Enterococcus, Enterobacteriaceae* (Pyrosequencing 16S rRNA Gene Amplicons)	(Humblot and Guyot, 2009)
Dèguè	Benin	Maize, pearl millet, sorghum (lab preparation)		*Lm. fermentum, Lp. plantarum, Lp. pentosus, Cyberlyndnera fabianii, K. marxianus Raw materials: Lm. fermentum, P. acidilactici, W. paramesenteroides, Ent. mundtii, Cyberlindnera fabianii, C. glabrata, Meyerozyma caribbica*	(Angelov et al., 2017)
Dèguè	Burkina Faso	Pearl millet		*Lm. fermentum L. gasseri, Lv. brevis, Lb. casei, Enterococcus sp., E. coli* (TTGE) Yeasts not characterized	(Abriouel et al., 2006)
Poto poto	The Republic of the Congo	Maize		*L. gasseri, Lp. plantarum/paraplantarum, L. acidophilus, L. delbrueckii, Lm. reuteri, Lc. casei, Bacillus sp., Enterococcus sp., E. coli* (TTGE) Yeasts not characterized	(Abriouel et al., 2006)
Poto poto	Republic of the Congo	Maize		*L. delbrueckii* (DGGE) Yeasts not characterized	(Ampe and Miambi, 2000)
Fura	Ghana	Pearl millet		*Lm. fermentum, P. acidilactici, W. confusa, Lm. reuteri, Lg. salivarius, Lp. paraplantarum*	(Owusu-Kwarteng et al., 2012)

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Mawè	Benin	Maize		*C. krusei, Cl. lusitaniae and S. cerevisiae.* *(Culture independent: Dekkera bruxellensis, Debaryomyces hansenii)* *Bacteria not characterized*	(Greppi et al., 2013b)
Mawe	Benin	Maize		*Saccharomyces cerevisiae, C. krusei, K. marxianus*	(Greppi et al., 2013a)
Ogi	Benin	Maize		*C. krusei, C. lusitaniae, S. cerevisiae*. (Culture independent: Dekkera bruxellensis, *Debaryomyces hansenii)* Bacteria not characterized	(Greppi et al., 2013b)
Ogi	Benin	Maize		*Lp. plantarum, L. delbrueckii, Lm. fermentum* (DGGE) Yeasts not characterized	(Ampe and Miambi, 2000)
Related products for which information on fermentation microorganisms is unavailable					
Kenkey	Ghana	Maize		(Before fermentation) *Candida*, *Saccharomyces, Trichosporon, Kluyveromyces, Debaryomycesg* and early phases of fermentation. (After fermentation) *C. krusei,d S. cerevisiae*(API)	(Halm et al., 1993) (Amoa-Awua et al., 2007)
Kunun-zaki	Nigeria	Pearl millet (maize/sorghum)	Uncooked fermented dough	lactobacilli, bacilli, *Enterobacteriaceae*	(Efiuvwevwere and Akona, 1995)
Mutwiwa/ mudzvurwa	Zimbabwe	Dehulled maize		Unknown	(Simango, 1997)
Kirario	Kenya	Green maize +millet/ sorghum meals		Unknown	(Kunyanga et al., 2009)
Ben-saalga	Burkina Faso	Pearl millet		Unknown	(Tou et al., 2006)

2.2.9 Fermented cereal flatbreads

The flow chart for the production of fermented cereal sourdoughs used in the preparation of flatbreads is shown in Figure 2.9 and the fermentation microbiota are listed in Table 2.9. *Injera*, a fermented pancake-like bread with a slightly spongy texture, traditionally made of fermented dough from teff flour is a staple in Ethiopia (Figure 2.9) (Tamene et al., 2019). At the household level, the process of making *injera* is continuous, as it is baked and consumed while the next batch of dough is being fermented (Abraha et al., 2013). *Injera* can also be made from wheat, barley, maize, sorghum, singly or as a mixture of the cereals but teff*,* a tiny millet-like grain is preferred due to its softer texture and taste (Yetneberk et al., 2005, 2004). *Injera* is central to the dining process, like bread or rice elsewhere and is served with a variety of stews or sauces made from vegetables, pulses, meat, or their combinations (Abraha et al., 2013). *Injera* batter fermentation microbiota consisted of seven lactobacilli (Table 2.9) (Fischer et al., 2014). In Sudan, *kisra* can be made from either sorghum or millet flour, and is produced at the household level from spontaneously fermented sourdough or from a long-term sourdough produced in a Sudanese household by consecutive re-inoculations (Hamad et al., 1997). *Kisra* is a popular fermented sorghum flatbread with a very sour taste and constitutes a major part of the staple diet for the people in Sudan (Mohammed et al., 1991; Odunfa and Oyewole, 1998). The main microorganisms of *kisra* consisted of two – four lactobacilli depending on whether the sourdough was back slopped or not (Table 2.9).

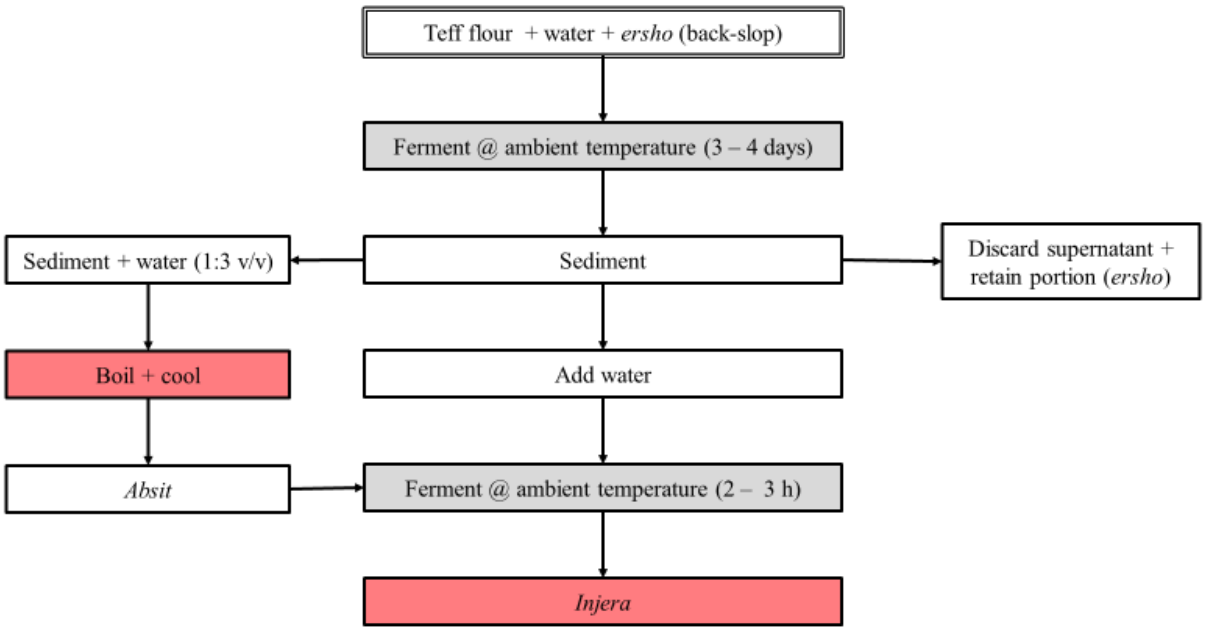

Figure 2.9 Preparation of fermented cereal flatbreads**.**

Shown is the name of the intermediate product which is baked prior to consumption. The steps in the flow chart are colour-coded as follows: grey shading indicates fermentation; double lines indicate back-slopping; red shading indicates a heating step that inactivates vegetative bacterial cells.

Table 2.9 Fermented cereal flatbreads

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Kisra	Sudan	Sorghum (pearl millet)	Back-slopped	*E. faecalis, Lc. lactis, Lm. fermentum, Lm. reuteri. Lm. vaginalis, L. helveticus*	(Hamad et al., 1997)
Kisra	Sudan	Sorghum (pearl millet)	Spontaneous	*P. pentosaceus, W. confusa, Lv. brevis, Lactobacillus sp., Erwinia ananas, K. pneumoniae, Ent. cloacae, C. intermedia, D. hansenii, Aspergillus sp., Penicillium sp., Fusarium sp. Rhizopus sp.,* (AIPI.)	(Mohammed et al., 1991)
Injera	Ethiopia	Teff	*Ersho* (back slop)	*Lp. plantarum, Lp. pentosus, Lm. fermentum, P. pentosaceus, Companilactobacillus crustorum, Lb. casei, Ln. buchneri, Lv. brevis/ Schleiferilactobacillus harbinensis*	(Fischer et al., 2014)

2.2.10 Solid pit fermented cereal products

The flow charts for the production of solid pit fermented cereal sourdoughs that are produced as intermediate products in the preparation of non-alcoholic cereal beverages and a dough-like fermented food are shown in Figure 2.10 and the fermentation microbiota are listed in table 2.10. Solid pit fermented food products include either the primary or secondary fermentation under the fire pit and are cooked before or after the solid pit fermentation (Table 2.10). *Hussuwa* is a semi-solid, sweet sour dough-like fermented food made in Sudan from sorghum or millet unmalted and malted flours and undergoes a secondary solid pit fermentation (Yousif et al., 2010). *Malwa* is drunk when diluted with hot water and is a sweet and sour beverage drunk with straws in Uganda (Muyanja et al., 2010). Both lactic and ethanolic fermentations take place during the production of these products but the yeasts were not characterized and the microbiota of *hussuwa* is dominated by strains of *Lm. fermentum* and *P. acidilactici* (Yousif et al., 2010) and another study only characterized the enterocooci (Table 2.10). As with other African cereal fermentations there are other variations on the production of *hussuwa,* where the solid pit fermentation is a major part of the process (Yousif et al., 2005). The enzymes are derived from the sorghum or finger millet malt (Figure 2.1, Table 2.1).

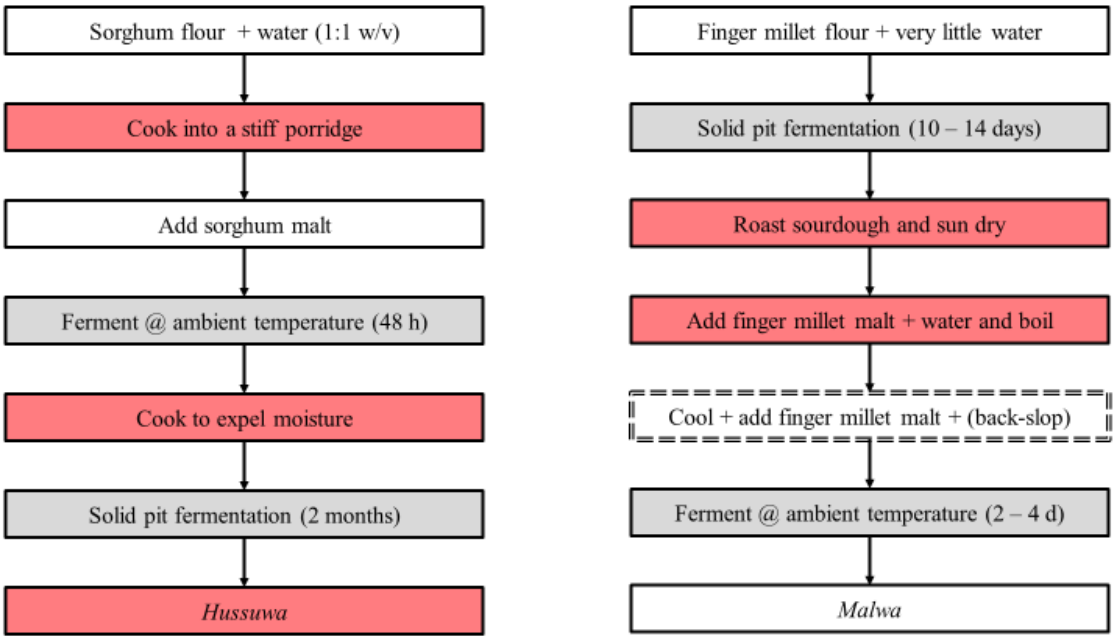

Figure 2.10 Preparation of solid pit fermented cereal sourdoughs.

Shown are the names of the intermediate products used for the preparation of a fermented food which is cooked and a non-alcoholic beverage which is not cooked. The steps in the flow chart are colour-coded as follows: grey shading indicates fermentation; dashed double lines indicate optional back-slopping; red shading indicates a heating step that inactivates vegetative bacterial cells.

Table 2.10 Solid pit fermented cereal sourdoughs

Product	Country	Substrate	Adjunct	Microorganisms	Reference
Hussuwa	Sudan	Sorghum	Sorghum malt	*Lm. fermentum, P. acidilactici, P. pentosaceus,* enterococci	(Yousif et al., 2010) (Yousif et al., 2005)
Related products for which information on fermentation microorganisms is unavailable					
Malwa	Uganda	Finger millet flour	Finger millet malt +/ back slopping	Unknown	(Muyanja et al., 2010)

2.3 Discussion

Traditions of sub-Saharan Africa include a world of knowledge on cereal fermentations that remains unexplored and undocumented. Moreover, the study of food fermentations and fermentation microbiota requires research resources and capacity which are not available in sub-Saharan Africa therefore, production processes and fermentation microorganisms of many fermented food products are not well represented in the scientific literature and in this review. This review does thus not reflect the entirety of the diversity of fermented cereal foods in sub-Saharan Africa, but current information suffices to outline differences between the major groups of products and between countries in East, West, Central and Southern Africa, and to compare African traditions with other regions in the world.

Most fermented foods are based on tradition and artisanal fermentation processes and are deeply rooted in the culture which is governed by climatic conditions that determine the availability of specific crops which require fermentation. In general, in North America and Europe bread and beer based on wheat and barley, respectively, are the major fermented cereal foods. South East Asia and East Asia steamed bread, in South Asia, Persia, Arabic countries including North Africa, and in North East Africa including Ethiopia, flatbreads based on wheat and sorghum are the major fermented cereal. In the remainder of sub-Saharan Africa fermented porridges and non-alcoholic beverages based on maize, millets or sorghum are the major fermented cereals. Regional differences within Africa with regards to the fermented food products are shown in Table 2.11.

African traditions differ from other areas of the world by the diversity of non-alcoholic cereal beverages and the widespread use of fermented porridges. Outside of Africa, there are a few examples of non-alcoholic fermented cereal beverages, namely *boza* produced in Bulgaria, Albania, Turkey, and Romania (Todorov and Dicks, 2006) and *kvass* produced in Eastern Europe

(Dlusskaya et al., 2008). A second difference relates to the site of production. African traditional fermentations are predominantly carried out at the household level in rural communities by women; this small scale of production adds to the diversity of fermented food products. In contrast,

Table 2.11 Comparison of fermented cereal beverages and porridges in Africa

East, Central and Southern Africa	West Africa
Porridges are coarse and gritty	Fine paste
Most beverages are coarse, gritty, colloidal	Smooth
Coarse maize particles	Fine pasty maize particles
Live microbiota in beverages	Few beverages have live microbiota (e.g. *Kunun-zaki,* KSW)
Cooked before fermentation	Cooked after fermentation
Initial wild fermentation by fungi, etc. is eliminated by boiling the maize meal	Initial wild fermentation by fungi, etc. is not eliminated as there is no initial cooking step
Nothing is discarded	Loss of nutrients and minerals during steeping and sieving
Few grains are wet milled	Wet milling of grains with spices is common
Finger millet used most	Pearl millet used most
Fermented without spices	Fermented with spices
Maize is the most commonly used cereal	Maize is the most commonly used cereal

traditional production of bread and beer in Europe was carried out by trades. In Europe, malted grains are used mainly for production of beer and distilled grain liquor, whereas in Africa malt is used for non-alcoholic beverages as well. Whereas fermented foods produced in Europe and North America usually depend on back-slopping or defined starter cultures, fermentations in sub-Saharan Africa predominantly but not exclusively rely on spontaneous fermentations with occasional use of back-slopping or fermentation vessels with porous walls that retain microorganisms from the previous batch and thus support the evolution of a beneficial fermentation microbiota (Tamang et al., 2020).

Overall, the organisms identified in African fermented cereal foods conform to prior observations that spontaneous cereal fermentations are dominated by the "usual suspects" which begin with plant-associated *Enterobacteriaceae*, followed by the growth of enterococci, lactococci, *Leuconostoc* and *Weissella* spp., and finally by the growth of pediococci, *Lp. plantarum* and *Lm. fermentum*. However, 'back-slopping" by re-use of fermentation vessels without sanitation generates somewhat of a hybrid of the "usual suspects" and host-adapted lactobacilli that has not been described elsewhere (Table 2.2).

Enterobacteriaceae, C. glabrata and C. sakazakii are opportunistic pathogens. Although infections with these organisms are rarely, if ever, food-borne, these organisms may represent a risk in spontaneous fermentations (Hamad et al., 1997; Holzapfel, 2002; Mukisa et al., 2012) (Chapter 3, this thesis). Interestingly, consumers prefer sour *malwa* to the sweet one (1-2 days old), because the sweet *malwa* causes upset stomachs (Muyanja et al., 2010). This may relate to the high bacterial load of *Enterobacteriaceae* at the beginning of the spontaneous fermentations (Wuyts et al., 2018) (Chapter 3, this thesis). Similar findings were also reported for *bushera* (2-day old) (Muyanja et al., 2003). Likewise, *ekitiribita,* a thin porridge prepared from un-malted millet is consumed within 1–2 days, the laboratory preparation however, revealed that it took four days for the pH to drop below 4.6 (Mukisa et al., 2012). Acidification of the fermentation substrate with lactic and acetic acids is the predominant factor that results in the elimination of *Enterobacteriaceae* in cereal fermentations (Dinardo et al., 2019) (Chapter 3, this thesis).

There are a few organisms that are rather unexpected for cereal fermentations, namely staphylococci in *ogi* and *kunu-zaki* (Oguntoyinbo and Narbad, 2012) (Table 2.8) and several culture-dependent as well as culture-independent reports of *L. delbrueckii* in *ekitiribita, obuteire, obutoku, ogi, kunu-zaki poto-poto* (Abriouel et al., 2006; Ampe and Miambi, 2000; Mukisa et al.,

2012; Oguntoyinbo et al., 2011) (Tables 2.2, 2.3, 2.8). Staphylococci and *L. delbrueckii* have been rarely, if ever, reported in other cereal fermentations; reasons for their repeated occurrence in African cereal fermentations remain to be elucidated.

In Africa the diversity of non-alcoholic fermented cereal beverages with live microbiota is much larger than anywhere else in the world, and there are many reviews on the probiotic potential of these products (Franz et al., 2014; Waters et al., 2015). Specifically, *Lm. fermentum* and *Lp. plantarum* are two bacterial species that are abundant in many African fermented foods but also include strains with well-documented probiotic properties (Hill et al., 2014). Even though probiotic activity is not documented at the strain level, live dietary microbes are increasingly recognized as health beneficial (Marco et al., 2021; Wastyk et al., 2021). Having well characterized and documented traditional fermentation processes and microbiota allows for the improvement of the fermentation process to decrease hygienic risks and to increase the abundance of health-beneficial microbes. Among the health benefits that may be provided by probiotics are reductions in the severity and duration of childhood diarrhea (Allen et al., 2010; Guandalini, 2011; Niel et al., 2002). The estimated number of deaths annually due to diarrheal disease of children under 5 years globally are 525,000, and mostly result from contaminated food and water sources (WHO, 2017). Many traditional fermented cereal beverages are consumed by infants and immunocompromised individuals without inactivation of the fermentation microbiota (Chapter 3, this thesis). Thus, viable probiotic fermentation organisms and viable opportunistic pathogens may positively and negatively affect the health of consumers (Marco et al., 2017)**.**

Many *Enterobacteriaceae* including *Klebsiella* and *Enterobacter* are part of plant microbial communities and occur as seed endophytes in grains (Ko et al., 2002; Kucerova et al., 2010; Podschun and Ullmann, 1998), and were thus also identified as initial fermentation microbes in

African spontaneous cereal fermentations. *Klebsiella* and *Enterobacter* are also notorious members of the "ESKAPE" pathogens that are leading causes of nosocomial infections with antibiotic resistant bacteria (Pendleton et al., 2013). Best example is *Enterococcus faecalis*, which is also a notorious opportunistic pathogen and member of the "ESKAPE" club (Franz et al., 2003) but also occurs in high cell counts in fermented dairy and meat products. The organism virtually never causes disease upon ingestion.

This review links the fermentation microbiota to the process which shows the necessity and potential to shift from household to industrial production of traditional cereal fermented beverages. However, in most of the sub-Saharan countries, there is a clear distinction between the urban population that consumes standardized, industrially produced fermented beverages, and rural communities which do not have the resources to buy and store manufactured beverages and produce traditional fermented foods at the household level. Traditional food fermentation represents an extremely valuable resource and harbors a huge potential of valuable but hitherto undiscovered probiotic strains. Looking at trends in Europe and North America there are two huge market opportunities where African traditional fermented cereal beverages can be extremely useful; in the gluten-free market and growth in the functional non-alcoholic beverage market.

Newly developed fermented cereal-based beverages must address markets globally including, high-nutrition markets (developing countries), lifestyle choice consumers (vegetarian, vegan, low-fat, low-salt, low-calorie), food-related non-communicable disease sufferers (cardiovascular disease, diabetes), and green label consumers (Western countries) (Waters et al., 2015). To fulfil these recommendations, suitable LAB starter cultures from traditional fermented cereal products which are already conditioned for growth in cereal-based raw materials like maize, sorghum and millets may potentially be developed to provide the ideal functional beverages to fulfil modern

consumer needs and preferences. Novel metabolites from novel lactic acid bacterial strains isolated from African traditional fermented cereal beverages may allow for novel applications of these starter cultures. This represents an untapped source for novel fermented cereal foods including functional food products with live probiotic bacteria that are produced based on templates out of Africa.

Chapter 3 Composition and origin of the fermentation microbiota of *mahewu*, a Zimbabwean fermented cereal beverage

3.1 Introduction

Mahewu is a lactic fermented non-alcoholic 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 finger millet or sorghum malt at the household level (Gadaga et al., 1999; Simango and Rukure, 1992) (Figure 3.1). *Mahewu* is produced by spontaneous fermentation without control of microbiota by back-slopping or the addition of starter cultures (Meroth et al., 2003; Sekwati-Monang and Gänzle, 2011; Vogel et al., 1999). Cooking of the maize porridge inactivates microbiota from maize flour or water, however, finger millet or sorghum malt are used without a heating step to inactivate malt-associated microbiota (Figure 3.1).

The microbiota of fermented cereal foods and non-alcoholic 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 (Gabaza et al., 2019; Gadaga et al., 1999; Simango and Rukure, 1992). 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 similar manner as *mahewu* from cooked maize or sorghum porridge with the addition of sorghum or finger millet malt (Mugula et al., 2003; Muyanja et al., 2003). The microbiota of *togwa* and *bushera* samples was composed predominantly of *Lactiplantibacillus plantarum* as the most abundant organisms with *Levilactobacillus brevis, Limosilactobacillus fermentum, Weissella confusa* and *Pediococcus pentosaceus*. The thermophilic *Issatchenkia orientalis* was the most frequently isolated yeast in *togwa* (Mugula et al., 2003).

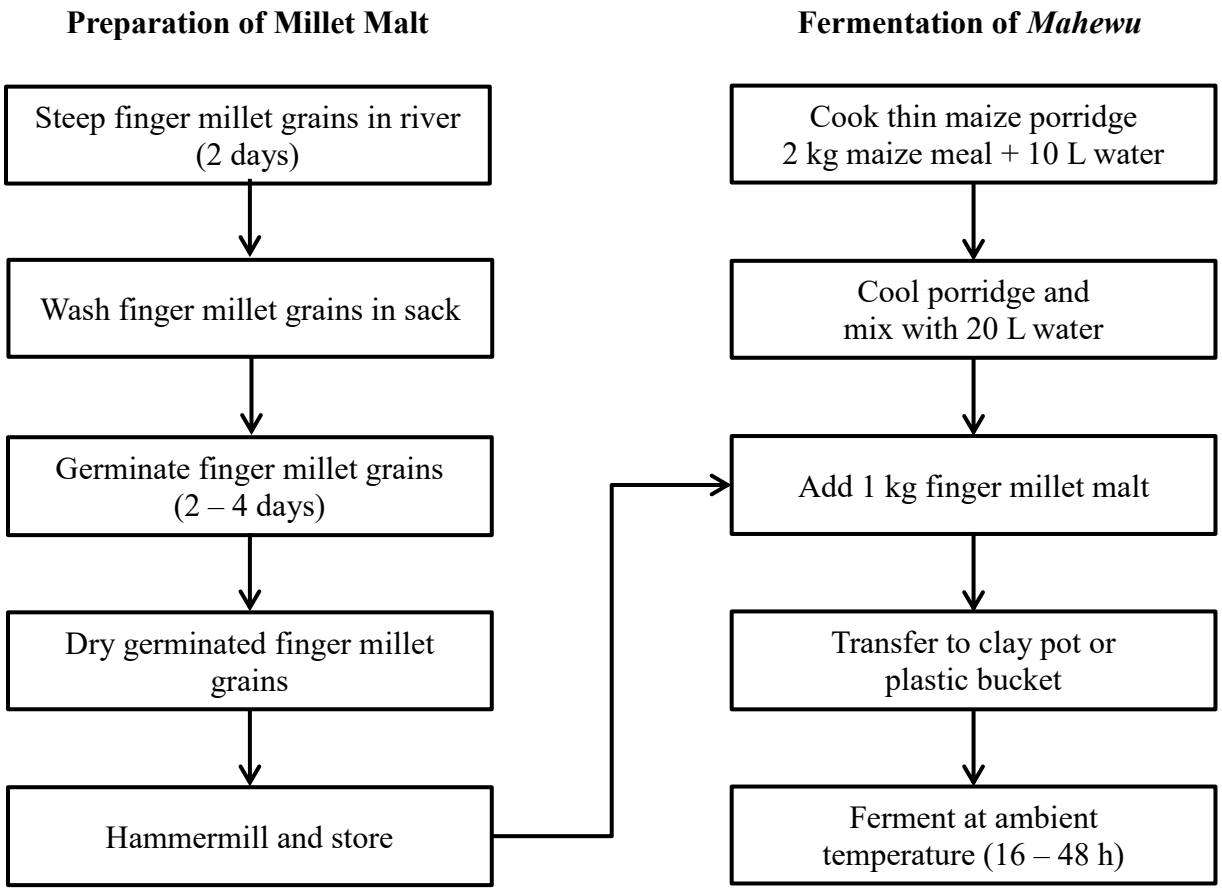

Figure 3.1 Traditional process for producing *mahewu*, a fermented maize and finger millet beverage

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-saal*ga from Burkina Faso, *ogi* and *kunu-zaki* from Nigeria - these are all prepared with spontaneously fermented millets or finger millet malt that is cooked into a thin porridge, gruel and beverage, respectively (Gaffa et al., 2002; Nout, 2009; Oguntoyinbo et al., 2011; Teniola et al., 2005; Tou et al., 2007). A comparable composition of microbiota is also observed in spontaneous wheat and rye sourdough fermentations (Gobbetti et al., 2016). The assembly of microbiota in spontaneous fermentations is limited by dispersal (Gänzle and Ripari, 2016). Spontaneously fermented cereals typically contain

lactic acid bacteria with a nomadic or environmental lifestyle, particularly *Lp. plantarum*, *Lm. fermentum* and *Weissella* spp. (Duar et al., 2017; Gänzle and Zheng, 2019; Minervini et al., 2015). Back-slopping eliminates dispersal limitation (Gänzle and Ripari, 2016) and leads to dominance of host-adapted lactobacilli including *Fructilactobacillus sanfranciscensis* in type I sourdoughs or *Lm. pontis, L. amylovorus*, and *Lm. reuteri* in type II sourdoughs (Gänzle and Ripari, 2016; Gobbetti et al., 2016).

Because *mahewu* is consumed without further heat treatment after fermentation, malt microbiota not only serves as inoculum for the lactic acid fermentation but may also transfer endophytic microbiota (Minervini et al., 2015). Plant endophytes include the opportunistic pathogens *Enterobacter* spp. and *Cronobacter* spp. (Schmid et al., 2009; Torres et al., 2008), endophytic *Enterobacter* spp. were reported to protect finger millet against *Fusarium* infection (Mousa et al., 2016). Low cell counts of *Enterobacteriaceae* were detected in weaning foods prepared from sorghum and finger millet (Badau et al., 2005) 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 activity of the finger millet malt determines product quality (Gänzle, 2014) and safety; therefore, attaining *mahewu* with consistent quality and safety attributes requires control of the composition and activity of fermentation microbiota (Gänzle and Ripari, 2016). It was therefore the aim of this study to determine composition and origin of *mahewu* fermentation microbiota, and to establish the overlap between the *mahewu* and finger millet malt microbiota, by using strain specific quantitative PCR.

3.2 Materials and Methods

3.2.1 Sampling of *mahewu* and finger millet malt

Five *mahewu* samples and four finger 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 Agricultural, Food and Nutritional Science at the University of Alberta, Edmonton, AB, Canada. Samples were maintained between 0 and 20°C during transport and analyzed at 76 ± 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.

3.2.2 Isolation and enumeration of microorganisms in *mahewu* and finger millet malt

Total cell counts for bacteria and yeasts were determined by surface plating of tenfold serial dilutions of *mahewu* samples in sterile peptone saline water (10 g/L peptone, 9 g/L NaCl) on MRS5 agar (Meroth et al., 2003). 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 sulphate, 0.05 g manganese sulphate, 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 using a 0.22 µm filter. For the enumeration and isolation of lactic acid bacteria, MRS5 containing 100 mg/L of cycloheximide (Sigma-Aldrich, Oakville, ON, Canada) was used, and the plates were incubated under modified conditions (10% CO_2, 90% N_2) at 30ºC for 72 h. Yeasts were enumerated and isolated on MRS5 agar containing 100 mg/L each of chloramphenicol and erythromycin (Sigma-Aldrich, Oakville, ON, Canada), and the plates were incubated aerobically at 30ºC for 72 h. Fecal coliform bacteria and *Enterobacteriaceae* were enumerated and isolated on Violet Red Bile Agar (Difco, Franklin Lakes, NJ, USA) and Violet Red Bile Glucose Agar (Oxoid, Basingstoke, Hampshire, UK), respectively, plates were incubated aerobically at 44.5ºC 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 – 80ºC. Cultivation of stock cultures for subsequent experiments was done on MRS5 agar at 30ºC for 48 h followed by overnight incubation in MRS5 broth at 30ºC.

3.2.3 Identification of *mahewu* and finger 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 (Dlusskaya et al., 2008) with the oligonucleotide primer M13V. PCR was performed with genomic DNA as template in a reaction volume of 25 µL containing 1 µL genomic DNA; 2.5 µL 10X PCR buffer, 0.75 mM, $MgCl_2$, 200 nM of each deoxynucleotide, 1.5 U Taq DNA Polymerase, and 150 pmol primer M13V (5′-GTT TTC CCA GTC ACG AC-3′ (Müller et

al., 2001), (all reagents from Invitrogen Corporation, Carlsbad, CA, USA). The mixtures were subjected to 1 min at 96 °C; 3 cycles of 3 min at 96 °C, 5 min at 35 °C, 5 min at 75 °C; 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 a 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 (Weisburg et al., 1991). Amplification of the 28S rRNA genes of yeasts was performed using primers P1 and P2 (Sandhu et al., 1995). Details of the primers are given in Table 3.1. The PCR products were sequenced by Macrogen (Rockville, MD, USA) and analyzed by the Ribosomal Database Project (https://rdp.cme.msu.edu/).

Differentiation of *Lp. plantarum*, *Lp. pentosus* and *Lp. paraplantarum* was conducted with a multiplex PCR assay (Torriani et al., 2001) 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 ATCAC-3′) (Table 3.1). 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), 1X PCR buffer (Invitrogen), and 5 μL of DNA template. PCR conditions were as follows; 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 final extension at 72°C for 5 min. The PCR products were separated on a 2% agarose gel electrophoresis and visualized by UV transillumination after staining with SYBR Safe.

3.2.4 Extraction of bacterial DNA from finger millet malt

In brief, approximately 0.2 g of finger 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 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, USA).

Table 3.1 Primer sequences used in the study

Target organism	Primer name and sequence (5′ – 3′)		[a]*Tm* (°C)	Amplicon Length (bp)	Ref.
Bacteria	27F 1492R	AGAGTTTGATCMTGGCTCAG TACGGYTACCTTGTTACGACTT	53.2 54.6	1500	(Weisburg et al., 1991)
16S rRNA	784F 1064R	RGGATTAGATACCC CGACRRCCATGCANCACCT		300	(Weisburg et al., 1991)
Yeasts	P1F P2R	ATCAATAAGCGGAGGAAAAG CTCTGGCTTCACCCTATTC	50.2 52.4	700	(Sandhu et al., 1995)
		recA gene-based primers			
Lp. paraplantarum	ParaF	GTCACAGGCATTACGAAAAC	51.9	107	(Torriani et al., 2001)
Lp. pentosus	PentF	CAGTGGCGCGGTTGATATC	55.6	218	(Torriani et al., 2001)
Lp. plantarum	planF	CCGTTTATGCGGAACACCTA	55.0	318	(Torriani et al., 2001)
	pREV	TCGGGATTACCAAACATCAC	52.5		(Torriani et al., 2001)
		Primers for HRM-qPCR			
Lactobacillus, Pediococcus, Weissella, Oenococcus	LabF LabR	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	63	341	(Walter et al., 2001)

Target organism	Primer name and sequence (5′ – 3′)		[a]*Tm* (°C)	Amplicon Length (bp)	Ref.
Strain specific primers for quantification of *mahewu* isolates in finger millet malt					
Lm. fermentum	CMA1F CMA1R	CACTAACAGGCACCACTATCTT CGCAGTCTTATTCTCATGCTTTAC	62	119	(Chapter 3, this thesis)
Lm. fermentum	CMB1F CMB1R	AACGCCTAGCCTGATTTATCTC CAACAGGATCGTGCCATAGT	62	103	(Chapter 3, this thesis)
Lp. plantarum	CMC1F CMC1R	AGTTTGCCACATATTAGGAAGAG A AGGCTCTAAGGGCTACCTATAC	62	112	(Chapter 3, this thesis)

[a] *Tm* melting temperature

3.2.5 Characterization of finger millet malt microbiota by 16S rRNA gene sequencing

To determine the relative abundance of bacterial DNA in 3 finger 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′-CGACRRCCATGCANCACCT-3′) (Weisburg et al., 1991). 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 less operational taxonomic units scripts (LotuS v. 1.565) pipeline which includes a simultaneous demultiplexer and quality filter C++ program, simple demultiplexer (sdm) to calculate denoised, chimera-checked, operational taxonomic units (Hildebrand et al., 2014). Filtered sequences were clustered into OTUs with UPARSE (Edgar, 2013), and taxonomic assignment was performed using BLAST against SILVA v128 reference database (Pruesse et al., 2007). 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 a percentage of OTUs representing specific bacterial taxa relative to the total abundance of bacterial 16S rRNA genes.

3.2.6 Physico-chemical analysis of traditionally prepared and laboratory scale prepared *mahewu*

The pH of the *mahewu* samples were measured with a glass electrode. For the determination of organic acids and alcohols, samples were prepared for 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 (300mm×7.8 mm, BioRad, Torrance, CA, USA), at a temperature of 70 °C (Dlusskaya et al., 2008). The eluent, 5 mM H_2SO4 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, Oakville, ON, Canada).

3.2.7 Genomic DNA isolation, genome sequencing, assembly, and annotation

Genomic DNA for whole genome sequencing was isolated from overnight cultures of *Lm. fermentum* FUA3588 and FUA3589, and *Lp. plantarum* FUA3590 grown in 10 mL of MRS5 broth. Genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Madisson, WI, USA) 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 (HRM)-qPCR (HRM-qPCR) as described by (Lin and Gänzle, 2014a) with primers targeting 16S rRNA genes of lactic acid bacteria (Walter et al., 2001).

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) (Simpson et al., 2009) with the most optimal k-mer value for each genome. After assembly, the fasta files were compared to species in the NCBI genome database. The genomes assemblies of *Lm. fermentum* strains FUA3588 and FUA3589 were improved by re-sorting the contigs using *Lm. fermentum* SNUV175 (Acc. No. NZ_CP019030.1) as the reference genome. For *Lp. plantarum* FUA3590 the genome assembly was re-sorted using *Lp. plantarum subsp. plantarum* SRCM100434 (Acc. no. NZ_CP021528.1) as the reference genome. Genomes were annotated automatically by the RAST server (http://rast.nmpdr.org/rast.cgi).

3.2.8 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 other strains from the same species. Multiple genome alignments were conducted to search for unique sequences in the genomes of *Lm. fermentum* FUA3588 and FUA3589 and *Lp. plantarum* FUA3590. Twenty of the most closely related genomes of *Lm. fermentum* FUA3588 and FUA3589 and 20 of the most closely related genomes of *Lp. plantarum* FUA 3590 were downloaded from National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov) FTP site and used as reference genomes. Progressive Mauve algorithm (The Darling lab at the University of Technology Sydney, Australia) was used for the comparative sequence analysis. The alignment results were displayed as horizontal panels for all the input genomes (Figure 3.2). Strain specific primers, CMA1F and CMA 1R; CMB1F and CMB1R; and CMC1F and CMC1R were designed from the unique sequences from the genomes of *Lm. fermentum* FUA3588 and FUA3589 and *Lp. plantarum* FUA3590 strains using the PrimerQuest Tool (Integrated DNA Technologies, Coralville, IA, USA). Details of the strain-specific primers are given in Table 3.1.

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

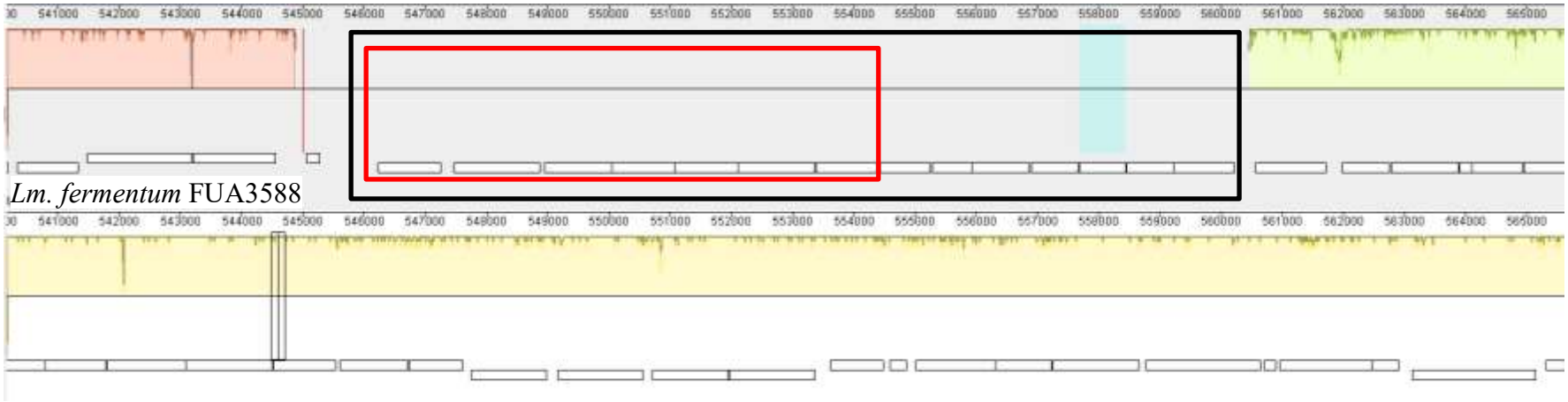

Figure 3.2 Genome alignment in MAUVE of *Lm. fermentum* FUA3588 against the most closely related genome-sequenced strain, *Lm. fermentum* NB-22 (NCBI Accession No. GCA_000496435.1).
Shown is a genomic region that is present in *Lm. fermentum* FUA3588 but absent in *Lm. fermentum* NB-22 (black box). The region encodes for a putative exopolysaccharide synthesis cluster; BLAST analysis against the NCBI database revealed that the 7 genes at the 5′ end are unique to *Lm. fermentum* FUA3588 but absent in 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 3.1).

3.2.9 Quantitative PCR for quantification of *mahewu* strains in finger millet malt

The presence of *mahewu* strains *Lm. fermentum* FUA3588 and FUA3589 and *Lp. plantarum* FUA3590 in the finger millet malt samples were 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 of the forward and reverse strain specific primers, 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, positive controls contained genomic DNA isolated from the respective bacterial cultures. The specificity of each primer pair (Table 3.1) was verified in qPCR reactions with template DNA from *Lm. fermentum* FUA3588 and FUA3589 and *Lp. plantarum* FUA3590, as well as with qPCR generated melting curves (data not shown). The qPCR amplification program was operated as follows, pre-denaturation stage at 95ºC for 5 min; 40 cycles of three steps: denaturation at 95ºC for 30 s, annealing stage at 62ºC for 30 s, elongation at 75ºC for 30 s; followed by 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 as follows:

$$relative\ abundance\ (\%) = 100 * 2^{(Ct_{universal\ primers} - Ct_{strain\ specific\ primers})}$$

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.

3.2.10 Model *mahewu* fermentations

Five different strains with distinct colony morphologies isolated from *mahewu* and finger millet malt from site C were selected for model *mahewu* fermentation: *Lm. fermentum* FUA3588, *Lp.*

plantarum FUA3590, *Enterococcus lactis* FUA3587, *Cronobacter sakazaki*i FUA10024, and *Klebsiella pneumoniae* FUA10025. The inoculum was prepared by growing the strains on MRS5 agar at 30ºC for 48 h followed by overnight incubation in 10 mL MRS5 broth at 30ºC. Cells were washed twice with sterile peptone saline and re-suspended in 10 mL sterile tap water for use as the inoculum.

Two g maize meal (National Foods Limited, Harare, Zimbabwe) were 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 aluminium 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 *Lp. plantarum* FUA3590 and *Lm. 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 *Lp. plantarum* FUA3590 was off white with smooth raised colonies. Colonies of *Lm. fermentum* FUA3588 appeared as white, large, flat and mucoid colonies on MRS5 agar. Colonies of *K. pneumoniae* FUA10025 were grey, 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 Figure 3.3. The experiment was performed in triplicate. The organic acids concentrations were determined in the 72 h model *mahewu*.

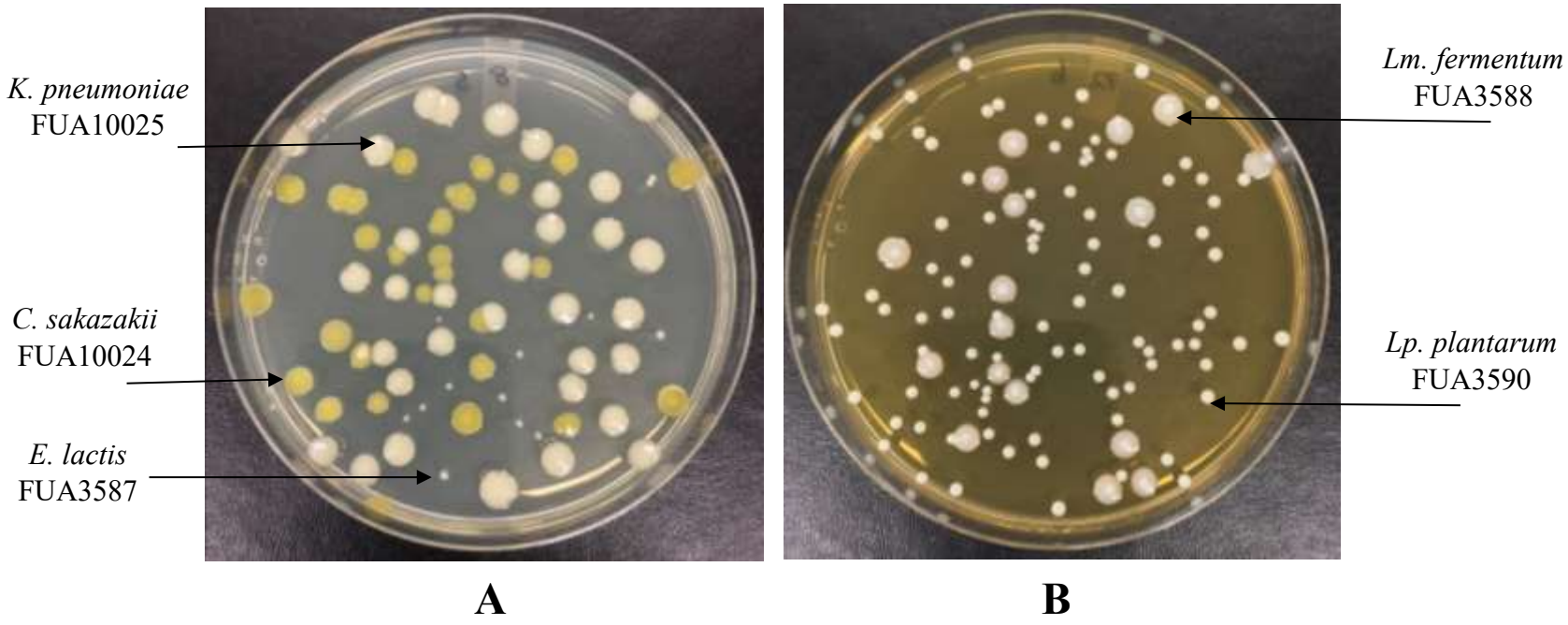

Figure 3.3 Strains isolated from model *mahewu* fermentations.

Differential enumeration of 5 bacterial strains was determined on the basis of the colony morphology. **PANEL A**: LB agar plate after 8 h incubation at 30ºC. *E. lactis* FUA3587 (small white colonies), and *C. sakazakii* FUA10024 (yellow colonies), *K. pneumoniae* FUA10025 (grey large mucoid colonies). **PANEL B:** MRS5 agar plate after 72 h incubation at 30ºC. *Lm. fermentum* FUA3588 (large white mucoid colonies), *Lp. plantarum* FUA3590 (off white medium colonies)

3.3 Results

3.3.1 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 x 10^8 to 1.1×10^9 cfu/mL (Table 3.2). Bacterial isolates were all identified as lactic acid bacteria (Table 3.2). Yeast cell counts ranged from 1.7 x 10^7 to 9.9 x 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 VRBG agar ranged from 9.0 x 10^1 to 1.0 x 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 3.2). Lactic and acetic acids, glycerol and ethanol were identified as the major fermentation products (Table 3.2), indicating that microbial metabolism was mainly attributable to lactic acid bacteria and yeasts.

Table 3.2 Microbiological and biochemical characteristics of *mahewu* fermentations

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

[a] Fermentation time 16 – 24 h

[b] Fermentation time 36 – 48 h

[c] Only *Candida glabrata* grew on VRBG agar

[d] Cell counts on VRB agar (fecal coliforms) were less than 10 cfu/mL in all samples

[e] Means ± standard deviations of duplicate analyses are shown.

3.3.2 Microbial analysis of *mahewu*

The taxonomic identification of bacterial isolates was based on the elimination of clonal isolates by RAPD typing, followed by sequencing of 16S rRNA genes (Table 3.3). Identification of isolates as *Lp. plantarum* additionally employed a *recA*-based multiplex PCR assay (Torriani et al., 2001). Yeasts were identified by the sequencing of 28S rRNA genes. The microbiota of the *mahewu* samples typically consisted of 3 – 7 dominant strains of lactobacilli and two strains of yeasts (Table 3.3). *Saccharomyces cerevisiae* and *Candida glabrata* were present in all samples (Table 3.3). *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 the sampling times (Table 3.3).

Table 3.3 Identification and strain specific quantification of isolates from *mahewu*

Site C			Site D			Site M		
Sample collection and analysis in February 2016								
Strain ID	Species[a]	Cell count (CFU/ml)	Strain ID	Species[a]	Cell count (CFU/ml)	Strain ID	Species[a]	Cell count (CFU/ml)
FUA3588	*Lm. fermentum*	$9.9x10^7$	**FUA3573**	***Lm. fermentum***	$9.4x10^8$			
FUA3589	***Lm. fermentum***[d]	$9.9x10^7$	FUA3574	*Weissella spp.*	$5.5x10^7$			
FUA3590	*Lp. plantarum*[bc]	$7.9x10^8$	FUA3575	*W. confusa*	$1.1x10^8$			
FUA3568	*P. pentosaceus*[c]		FUA4046	*S. cerevisiae*	$8.5x10^6$			
FUA4041	*S. cerevisiae*	$2.4x10^7$	FUA4047	*C. glabrata*	$8.5x10^6$			
FUA4042	*C. glabrata*	$1.0x10^7$	FUA4048	*C. glabrata*	$1.0x10^7$			
FUA4043	*C. glabrata*	$7.2x10^5$						
Sample collection and analysis in May 2016								
FUA3569	*Lm. fermentum*	$5.4x10^8$	FUA3576	*W. cibaria*[c]	$4.7x10^6$	**FUA3582**	***Lm. fermentum***	$1.0x10^8$
FUA3570	*Lm. fermentum*	$3.9x10^7$	FUA3577	*P. pentosaceus*[c]		FUA3583	*Ff. rossiae*	$1.0x10^8$
FUA3571	*E. hermanniensis*[c]	$1.5x10^8$	FUA3578	*Leuc. holzapfelii*	$1.9x10^8$	FUA3584	*Lp. plantarum*[b]	$2.6x10^7$
FUA3572	*E. lactis*[c]		FUA3579	*Lc. lactis*	$1.9x10^8$	FUA3585	*W. cibaria*	$2.6x10^7$
FUA4044	*S. cerevisiae*	$6.2x10^7$	FUA3580	*Leuc. pseudomesenteroides*[c]	$8.9x10^7$	FUA3586	*Lp. plantarum*[b]	$7.8x10^6$
FUA4045	*C. glabrata*	$9x10^1$	FUA3581	*W. confusa*[c]		FUA4051	*S. cerevisiae*	$5.2x10^7$
			FUA4049	*S. cerevisiae*	$9.9x10^7$	FUA4052	*C. glabrata*	$6.9x10^2$
			FUA4050	*C. glabrata*	$8.0x10^2$			

[a] All isolates were identified with the sequence match tool of the ribosomal database project with a > 98% nucleotide identity of partial 16S rRNA (1300 to 1455 bp) to bacterial type strain; or a > 98% nucleotide identify of more than 700 bp of the 28S rRNA genes.

[b] Differentiation of *Lp. plantarum* from *Lp. paraplantarum* and *Lp. 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 *Lm. fermentum* FUA3589 generated amplicons with all strains printed in bold.

3.3.3 Microbiota of finger millet malt

In addition to the microbiota associated with the production environment, finger millet malt is a source of *mahewu* microbiota. Therefore, cell counts of four finger millet malt samples were determined, and representative isolates were identified at the species level. The viable bacterial cell counts of the 4 finger millet malt samples ranged from 2.2 x 10^6 to 7.7 x 10^7 cfu/g (Table 3.4); fecal coliforms accounted for more than 20% of total cell counts in all samples (Table 3.4). The taxonomic identification of finger millet malt isolates was based on the same procedure that was outlined above for *mahewu* microbiota. The microbiota of the finger millet malt samples typically consisted of 8 – 19 strains of plant associated *Enterobacteriaceae*, lactic acid bacteria, bacilli, and few yeasts (Table 3.5). Only the most abundant isolates could be quantified by differential enumeration on the basis of the colony morphology. *Bacillus subtilis* and *Enterococcus* spp. were the most abundant organisms in the sample from site C in Feb 2016 and May 2016, respectively. *Cronobacter sakazakii* was the most abundant microorganism in both finger millet malt samples from site D.

3.3.4 Characterization of finger millet malt microbiota by 16S rRNA gene sequencing

Illumina sequencing of 16S rRNA gene amplicons was used to determine the relative abundance of bacterial genera in 3 finger millet malt samples. The finger millet malt microbiota was characterized by the analysis of the relative abundance of bacterial taxa at the phylum and genus level (Table 3.5) and by matching to the most closely related species (Table S3.1 – Appendix A). Of the total 135 OTUs, 96 were classified to the genus level (Table 3.5). *Proteobacteria* was the most abundant phylum (32.1 – 44.4%) with *Cronobacter* (26.4 – 52.8%) as the most abundant genus in all 3 finger millet malt samples, followed by Firmicutes (22.6 – 30.2 %) with *Weissella* (6.35 – 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.

Table 3.4 Microbial characterization of millet malt

Samples / Cell counts (cfu/g)	Malt C		Malt D	
	February 2016	May 2016	February 2016	May 2016
Bacterial cell counts[a)]	7.7×10^7	1.3×10^7	1.3×10^7	1.7×10^7
Yeast cell counts[b)]	$< 10^3$	$< 10^3$	$< 10^3$	$< 10^3$
Enterobacteriaceae[c)]	1.0×10^7	2.3×10^6	2.7×10^6	4.7×10^6
Fecal coliforms[d)]	5.3×10^7	3.7×10^7	3.0×10^6	1.9×10^7

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

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 % when compared to 19.1% determined by culture-dependent analysis 19.1 % (Table 3.5).

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 3.5). Short sequences of bacterial rDNA can be amplified more

than 60 days after cell death (Wuyts et al., 2018) and the presence of rDNA from these taxa is unlikely to represent the presence of bacterial cells.

Table 3.5 Comparison of the relative abundance (%) of bacterial genera in finger millet malt, determined by Illumina sequencing of 16S rRNA amplicons and 16S rRNA gene sequencing of bacterial isolates. Data presented as means ± standard deviation of 3 millet malt samples.

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, dublinensis, 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	

Genus	% of 16S rRNA amplicons	% of isolates	Species Isolated
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	

Unassigned genera are presented with upper level family (F) or phylum (P) in square 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 ≤ 0.1 % are not shown but are included in the total. Grey shading indicates phyla.

3.3.5 Determination of the origin of *mahewu* microbiota

RADP patterns and the observation of the colony morphology of isolates suggested that different samples contained similar strains of *Lm. 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 *Lm. fermentum* FUA3588, FUA3589 and *Lp. plantarum* FUA3590. Genomes of *Lm. fermentum* FUA3588 and FUA3589 and *Lp. plantarum* were aligned against 20 reference genomes each of *Lm. fermentum* and *Lp. plantarum*, respectively. As an example, the genome alignment of *Lm. fermentum* FUA3588 against the most closely related genome-sequenced strain, *Lm. fermentum* NB-22 (NCBI Accession No. GCA_000496435.1) is shown in Figure 3.2. The primers

CMA1F and CMA1R targeted a unique exopolysaccharide operon (EpsD gene cluster) with 3 hypothetical proteins and 4 glycosyltransferases only found on the *Lm. fermentum* FUA3588 genome (Figure 3.2). 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 3.6). *Lm. 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, *Lp. 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 *Lm. fermentum* FUA3589 and template DNA isolated from *Lm. fermentum* FUA3573, and *Lm. fermentum* FUA3582 (Table 3.1). Primers targeting strain-specific regions in the genomes of *Lm. fermentum* FUA3588 and *Lp. 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.

3.3.6 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 finger 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 (Figure 3.3). 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 (Gobbetti et al., 2016; Van der Meulen et al., 2007). Fermentations were characterized by differential cell counts, pH, and metabolite concentrations (Figure 3.4). Lactic acid and ethanol were the major bacterial metabolites in model *mahewu* fermentations. The concentrations of the metabolites were 82. 0 ± 3.1 mmol L^{-1} lactic acid; 42.2 ± 6.2 mmol L^{-1} ethanol; 8.6 ± 1.2 mmol L^{-1} acetic acid and 5.9 ± 0.8 mmol L^{-1} glycerol. The pH decreased to 4.5 after 8 h; the final pH of 3.3 was reached after 24 h (Figure 3.4). 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 *Lm. fermentum* stopped after 16 h and decreased slightly, whereas growth of *Lp. plantarum* stopped after 48 h, and cell counts were maintained over the remaining fermentation time (Figure 3.4).

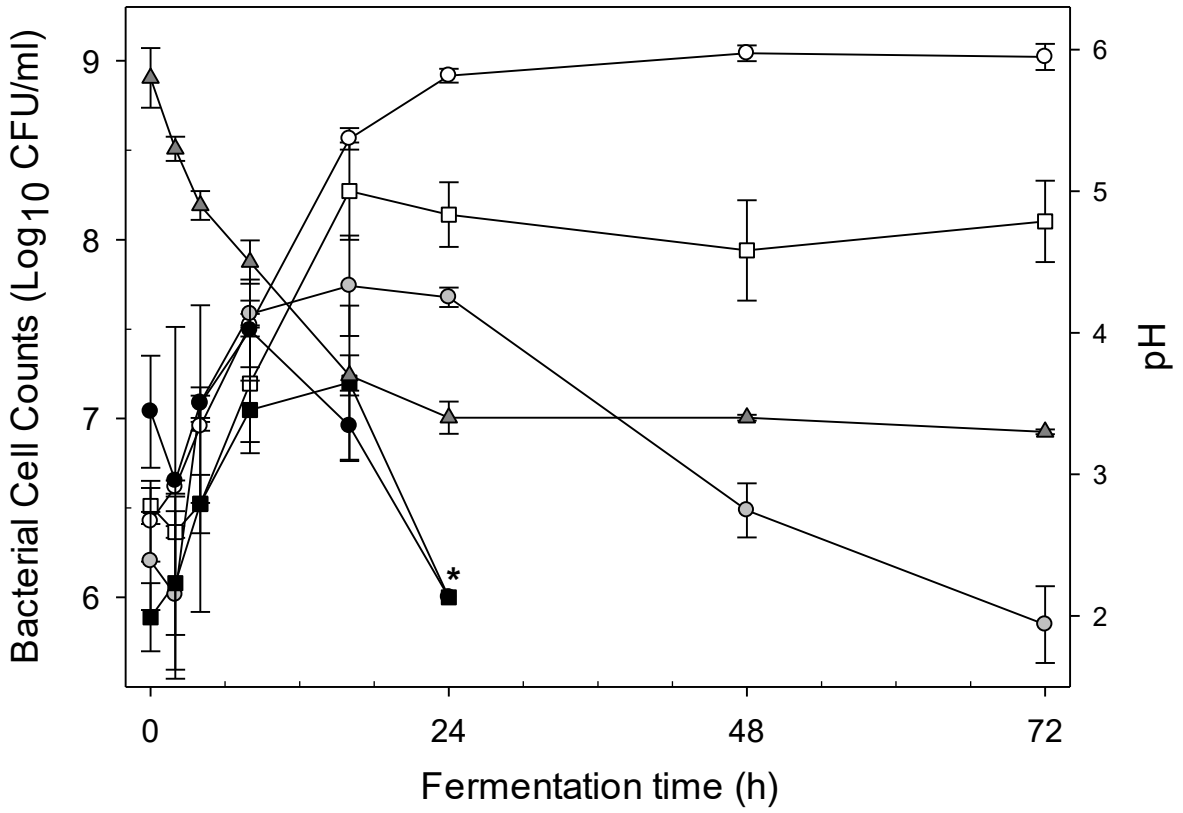

Figure 3.4 Differential cell counts in model *mahewu* fermentations.

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 the colony morphology. Symbols indicate *Lm. fermentum* FUA3588 (□), *Lp. plantarum* FUA3590 (○), *E. lactis* FUA3587 (●), and *C. sakazakii* FUA10024 (●), *K. pneumoniae* FUA10025 (■); pH (▲). Results are shown as means ± standard deviation of triplicate independent experiments analysed in duplicate. (*) indicates values below the detection limit because the LB agar plates were overgrown with lactic acid bacteria and bacilli. (□ ○) enumerated on MRS5 agar, (● ■ ●) enumerated on LB agar.

Table 3.6 Strain-specific qPCR detection of *mahewu* isolates in finger millet malt.

Mahewu strain	Relative Abundance %	
	Malt[ab]	*Mahewu*[c]
Lm. fermentum FUA3588	0.07 ± 0.01	10
Lm. fermentum FUA3589	0.01 ± 0.01	10
Lp. plantarum FUA3590	Not detected	80

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

3.4 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 the 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) (Vogel et al., 1999), denaturing gradient gel electrophoresis (Meroth et al., 2003; Van der Meulen et al., 2007; Zheng et al., 2012), quantitative PCR (Lin and Gänzle, 2014a; Scheirlinck et al., 2009; Sekwati-Monang et al., 2012) and high throughput sequencing of 16S RNA sequence tags (Bessmeltseva et al., 2014) 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 characterized by a succession

of fermentation microbiota (Bessmeltseva et al., 2014; Van der Meulen et al., 2007; Wuyts et al., 2018). 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 (finger 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 *Enterobacteriaciae* makes even the genus level identification with short rRNA gene sequences questionable (Alnajar and Gupta, 2017).

3.4.1 Composition

Mahewu microbiota consisted of lactic acid bacteria and yeasts. *Lm. fermentum* was the most frequent and the most abundant organism in *mahewu* samples; *Lp. plantarum*, *Furfurilactobacillus rossiae*, *P. pentosaceus*, *Leuconostoc* and *Weissella* spp. as well as enterococci and lactococci were additionally present. The composition of *mahewu* microbiota is thus comparable to the microbiota of other spontaneous cereal fermentations, which also typically include *Lm. fermentum, Lp. plantarum*, and *P. pentosaceus* (Hamad et al., 1997; Hounhouigan et al., 1994; Madoroba et al., 2011; Moroni et al., 2011; Nout, 2009; Oguntoyinbo et al., 2011; Sanni et al., 2013; Sekwati-Monang and Gänzle, 2011; Tou et al., 2007), species which are characterized by a nomadic or environmental lifestyle (Duar et al., 2017). The high abundance of *Lp. plantarum* at the late stages of spontaneous cereal fermentation (Sanni et al., 2013) 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 (G-Alegría et al., 2004).

Yeasts were identified as *Saccharomyces cerevisiae* and *Candida glabrata,* these yeasts also occur in other cereal fermentations (De Vuyst et al., 2016; Todorov and Holzapfel, 2015). 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 (Chen et al., 2012). *C. glabrata* infections have a high mortality in immuno-compromised, at risk and hospitalized patients (Fidel et al., 1999) and its presence may thus be of concern in countries such as Zimbabwe with a high prevalence of HIV (Mahomva et al., 2006).

3.4.2 Origin

The production environment and the raw material were related to the microbiota of spontaneous and back-slopped wheat sourdoughs (Minervini et al., 2015; Scheirlinck et al., 2009). 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, *Lm. 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. The microbiota of millet malt was subsequently characterized as a likely source of fermentation microbiota. Strain specific qPCR demonstrated that the finger 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 (Maldonado-Gómez et al., 2016; Zhao et al., 2019). 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 (Minervini et al., 2015; Scheirlinck et al., 2008). In addition, the detection limit of strain specific qPCR is lower than that of cultivation-based methods.

Finger millet malt microbiota consisted mainly of environmental *Enterobacteriaceae*, environmental lactic acid bacteria, bacilli and few yeasts. Barley malt microbiota also include *Enterobacteriaceae* and lactic acid bacteria as dominant representatives (Noots et al., 1999). *Daqu,* which is a spontaneously fermented saccharification starter prepared from sorghum and wheat or rice hull also includes a diverse microbial community with bacilli, *Enterobacteriaceae*, lactic acid bacteria, and yeasts and moulds as abundant representatives (Li et al., 2015; Xiao et al., 2017; Zheng et al., 2012). *C. sakazakii* is ubiquitous in the environment and has been isolated from plant food, cereals, fruit and vegetables, herbs and spices (Breeuwer et al., 2003; Friedemann, 2007). *C. sakazakii* and has a high tolerance to desiccation (Beuchat et al., 2009; Friedemann, 2007), which may contribute to its competitive advantage in finger millet malt. *Cronobacter* spp. were found to endophytically and epiphytically colonize tomato and maize roots which suggests that plants may be the natural habitat of *Cronobacter* spp. (Schmid et al., 2009). 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 (Beuchat et al., 2009; Lai, 2001; Patrick et al., 2014). *Cronobacter dublinensis*, like *C. sakazakii,* has been recovered from the environment and food (Cruz et al., 2011) and is also considered an opportunistic pathogen in neonates (Kucerova et al., 2010). *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 (Podschun and Ullmann, 1998). *Bacillus*

subtilis has been isolated from diverse environments, particularly including soils and plants (Earl et al., 2008).

The model *mahewu* study was conducted to determine the fate of *C. sakazakii, K. pneumoniae* and *Ec. lactis* during fermentation. The initial competition of lactic acid bacteria and plant-associated *Enterobacteriaceae* is common for many plant fermentations including cereal fermentations, sauerkraut or kimchi, or carrot juice (Ercolini et al., 2013; Lee et al., 2005; Wuyts et al., 2018). 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, or sorghum doughs (Hamad et al., 1997; Lee et al., 2005; Wuyts et al., 2018). Plant-associated *Enterobacteriaceae* were detected in several spontaneously fermented cereal foods (Todorov and Holzapfel, 2015), suggesting that fermentation conditions or fermentation time may 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 finger millet malt in the fermentation of *mahewu*. *Mahewu* is consumed by infants and immunocompromised individuals without inactivation of the fermentation microbiota. Viable probiotic fermentation organisms and viable opportunistic pathogens thus may positively and negatively affect the health of consumers (Marco et al., 2017). While *Lm. fermentum* were very minor components of finger millet malt microbiota and *Lp. plantarum* were not detectable in the raw material they became dominant members of *mahewu* microbiota after 16 – 24 h of fermentation. Conversely, *C. sakazakii*, *K. pneumoniae* and enterococci which were abundant representatives of finger millet malt microbiota, were eliminated or reduced after 24 – 48 h of

fermentation. Comparative genomic analyses of *mahewu* lactic acid bacterial isolates may further elucidate their role in product quality and safety.

Chapter 4 Antimicrobial plant secondary metabolites, MDR transporters and antimicrobial resistance in cereal-associated lactobacilli: is there a connection?

4.1 Introduction

Antimicrobial resistance in bacteria impacts public health globally, and affects human health and animal health (WHO, 2015). A "One Health" approach is used to counteract the threat to public health by integration of global, national and regional level action plans to mitigate antimicrobial resistance (FAO, 2021). Environmental sources and paths of transmission of resistant bacteria are a critical element in a One Health approach to antimicrobial resistance (Koutsoumanis et al., 2021).

Antimicrobial resistance in food fermenting bacteria can be transmitted to pathogens (Koutsoumanis et al., 2021; Neu, 1992). To prevent that food and feed cultures increase the pool of antimicrobial resistance genes, the European Food Safety Authority (EFSA) provided guidance related to the antibiotic resistance of starter cultures in food or feed (EFSA, 2012; Rychen et al., 2018). Intrinsic resistance presents a minimal risk for horizontal transmission but acquired resistance that is present on mobile genetic elements presents a higher risk for spread by horizontal gene transfer (Devirgiliis et al., 2011; Van Reenen and Dicks, 2011).

Food fermenting lactobacilli are of environmental or intestinal origin (Duar et al., 2017; Li and Gänzle, 2020). Intestinal organisms may be exposed to antibiotics in the intestines of production animals that are fed antimicrobial growth promotors. Some genes coding for antimicrobial resistance, e.g. *tetW*, are virtually exclusively found in those genera of lactobacilli that adapted to the vertebrate intestinal tract, *Lactobacillus*, *Ligilactobacillus* and *Limosilactobacillus* (Rozman et al., 2020). Plant-associated lactobacilli are less exposed to antibiotics in their natural habitat, however, these organisms encounter plant secondary metabolites with antimicrobial activity including essential oils, hop bitter compounds, and phenolic compounds. Lactobacilli have

evolved diverse mechanisms to resist plant secondary metabolites with antimicrobial activity (Behr et al., 2006; Rao et al., 2018). Hop resistance of lactobacilli is mediated by HorA, an ABC-family multidrug transporter which mediates the extrusion of structurally unrelated compounds including antibiotics and hop iso-α-acids (Sakamoto et al., 2001). The structurally and functionally related ABC-type MDR transporter LmrA also mediates antibiotic resistance, particularly to macrolide antibiotics and to tetracyclines (Poelarends et al., 2002). Bacterial Multidrug and Toxic Compound Extrusion (MATE) family or Major Facilitator Superfamily (MFS) transporters use transmembrane H^+ and/or Na^+ gradients to drive the efflux of polyaromatic and cationic compounds and also relate to antibiotic resistance of lactic acid bacteria (Du et al., 2018; Poelarends et al., 2002). Tannase activity and conversion of phenolic acids to metabolites with reduced antimicrobial activity increases the resistance of lactic acid bacteria to phenolic compounds (Gaur et al., 2020; Iwamoto et al., 2008; Sánchez-Maldonado et al., 2011).

Multidrug resistance (MDR) efflux pumps found in lactic acid bacteria are often encoded on plasmids (Paulsen et al., 1996; Putman et al., 2000; Sakamoto et al., 2001) and are therefore readily transmissible whereas other MDR efflux pumps are encoded on the chromosome (Schindler and Kaatz, 2016). Drug resistance due to chromosomally-encoded MDR pumps may also relate to increased gene expression (Grkovic et al., 2002; Schindler and Kaatz, 2016).

In cereals, the major class of phenolic compounds are phenolic acids, flavonoids and flavonoid glycoside and condensed tannins (Awika and Rooney, 2004; Ragaee et al., 2006; Shewry et al., 2010). Sorghum contains a higher level of phenolic compounds when compared to other cereals (Awika and Rooney, 2004; Svensson et al., 2010) and the antimicrobial activity of phenolic compounds in cereals was shown to select for fermentation organisms that are resistant to their antimicrobial activity (Dinardo et al., 2019; Sekwati-Monang et al., 2012). This study aimed to

determine whether multi-drug-resistance genes are present in bacterial isolates from *mahewu*, a Zimbabwean fermented cereal beverage, and to explore possible connections between plant secondary metabolites with antimicrobial activity, the presence and expression of *mahewu* phenolics resistance genes (*mpr*), and antibiotic resistance in cereal isolates of lactobacilli.

4.2 Materials and Methods

4.2.1 Bacterial strains and growth conditions.

Bacterial strains used in this study and their origin are shown in Table 4.1. Strains were cultured from ‑80°C stock and grown in MRS5 medium (Meroth et al., 2003) at 25 and 30°C under microaerophilic conditions.

4.2.2 Genomic DNA isolation, genome sequencing, assembly, and annotation

Genomic DNA for whole genome sequencing was isolated from overnight cultures of *Lm. fermentum* FUA3582 and *W. cibaria* FUA3585 grown in 10 mL of MRS5 broth. Genomic DNA was isolated using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA) 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) and gel electrophoresis. Prior to genome sequencing, the identity and purity of the DNA was verified with high-resolution melting (HRM)-qPCR as described by (Lin and Gänzle, 2014b) with group specific primers (Walter et al., 2001, Table 4.2).

Sequencing was performed using the Illumina HiSeq2500 platform Genome Quebec (Montreal, QC, Canada). The quality check of 125-bp paired-end reads was done using the FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequence assembly was performed using SPAdes (Bankevich et al., 2012) and MeDuSa (Bosi et al., 2015). Genomes were annotated automatically by the RAST server (Aziz et al., 2008).

Table 4.1 Bacterial strains used in this study and their origin

Species	Strain ID	Genome accession number
Mahewu (Chapter 3, this thesis)		
Lp. plantarum	FUA3590	SMZG00000000
	FUA3584	WEZU00000000
	FUA3586	n/a
Lm. fermentum	FUA3588	SMZI00000000
	FUA3589	SMZH00000000
	FUA3582	JAIRBV000000000
	FUA3569	n/a
	FUA3570	n/a
	FUA3573	n/a
P. pentosaceus	FUA3568	n/a
	FUA3577	n/a
Ff. rossiae	FUA3583	WEZT00000000
W. cibaria	FUA3585	JAIRBW000000000
Ting (Sekwati-Monang and Gänzle, 2011)		
Lm. fermentum	FUA3165	n/a
	FUA3321	n/a
Lp. plantarum	FUA3309	JAIRBY000000000
	FUA3310	n/a
	FUA3316	n/a
Household sourdoughs (unpublished; Gänzle and Zheng, 2019)		
Lp. plantarum	FUA3302	JAIRBX000000000
	FUA3428	JAIRBZ000000000
	FUA3447	n/a
	FUA3454	n/a
Wheat bran (unpublished)		
Lm. fermentum	FUA3414	n/a
	FUA3415	n/a
	FUA3398	n/a
	FUA3403	n/a
Spoiled beer (Ulmer et al., 2000)		
Lp. plantarum	TMW1.460	WEZR00000000

Table 4.2 Primers used in this study

Primer name	Primer Sequence (5′ – 3′)	[a]Tm °C, Amplicon length (bp)	
PCR primers for *mpr* genes *Lp. plantarum*			
MDR1F	GCAGACGCCAACGGATATTA	62	624
MDR1R	AGACCAGCAACGACACTAAAG		
MPRB_F	ACCAGTGGCTCGCCCTATTTTCTTTACTTAATAAGTCTAATTAAATTAG	62	610
MPRB_R	ACTGGTTTTGCTGTAGTACATTACGATGCACTTGAATAAAAC		
MDR4_F	CCTTCACTTCCGACCAAACT	62	228
MDR4_R	GTGATAGTCGCACGCCTTTA		
MDR5_F	CCCTACATTGCGGACTTCTATC	62	839
MDR5_R	CCAAAGAACTGTGCCAGAATAAC		
MDR7_F	TTCTGCGACCGTGTTTGT	62	323
MDR7_R	ATCAGGACATGGCGGTATTG		
qPCR PRIMERS for *mpr* genes in *Lm. fermentum*			
PHO_M_F	TGGCTGCTTCATGGTTCTC	62	112
PHO_M_R	CGGGAAAGGATAGTTGGGTTAG		
QMA_MDR2_F	GCGAGTCGAGCACTTGTTTAG	63	89
QMA_MDR2_R	GGGTGGCAAAGAGGTTGATTAG		
QMA_MDR3_F	GAAGAAGTGGGCGAGAATGA	62	101
QMA_MDR3_R	TCTTCCAGTCAATGGTCAAGG		
QMA_MDR4_F	CAGTCCGAAGATGTCACCAA	62	137
QMA_MDR4_R	TGGCCGTCACCCTAATTTAC		
QMA_MDR5_F	CCTGATGTGCGTCGTGTATATC	62	96
QMA_MDR5_R	AAATGTGCCCGTACTTCTACC		
qPCR PRIMERS for *mpr* genes in *Lp. plantarum*			
QMDR1_F	GCAGACGCCAACGGATATTA	62	112
QMDR1_R	GAGTGCGCGAATGATGTTTG		
QMDR2_F	GAACCGATTGTGCCTTGATTG	62	86
QMDR2_R	GGAATCGGTGGTGGCTATTT		
QMDR4_F	GCTTAGCCTTCCTGCGAATA	62	100
QMDR4_R	AGCGGCACTGAATAGTCTTG		
QMDR5_F	CCCTACATTGCGGACTTCTATC	62	95
QMDR5_R	AGACCCTCCGTTCGGATAA		
QMDR_6F	GAGTGCGCGAATGATGTTTG	62	112
QMDR6_R	GCAGACGCCAACGGATATTA		
QMDR7_F	CTGCAAACACCCGCATAAAG	62	127
QMDR7_R	GTCATCGGGAGCACGTATATC		

Primer name	Primer Sequence (5′ – 3′)	[a]Tm °C, Amplicon length (bp)	
qPCR primers for phenolic acid enzymes in *Lm. fermentum*			
MMA_PCA_F	GCTGACTGAAGGAGTATACAAGG	62	106
MMA_PCA_R	AAAGAAGATCGTCCCGTTGAG		
MMA_RED_F	CGGGCTAAATCCACCTTCTT	62	92
MMA_RED_R	TCGTCAATGTGCTCCCAATAG		
MMA_EST_F	GTAAGTCCGACGGTCAGTTTAG	62	118
MMA_EST_R	TGGCCAACCAGGATGATTT		
qPCR primers for phenolic acid enzymes in *Lp. plantarum*			
CMC_PDA_F	CGTACCGTGTAGTTTCTTCTCAT	62	100
CMC_PDA_R	CATGTTGACCGAAGGCATTTAC		
CMC_RED_F	CGCATACCTGACTGCCAATA	62	95
CMC_RED_R	CAGTCCGTTGACCACCTAAA		
CMC_EST_F	CAGGGTGGGCAAGATGAATTA	62	103
CMC_EST_R	GTCCAGCATCAGCATACCAA		
CMC_TANB_F	GAGTGGCGATTCGGCTTATT	62	118
CMC_TANB_R	GTCTGCGTGTTCCAGATTATGA		
[b]HRM-qPCR primers for lactic acid bacteria			
LabF	AGCAGTAGGGAATCTTCCA	63	341
LabR	CACCGCTACACATGGAG		

[a]Tm °C melting temperature

[b]HRM-qPCR primers (Walter et al., 2001)

4.2.3 Identification of genes coding for multi-drug-resistance transporters in genome-sequenced *mahewu* isolates

Closely related genomes were identified by BLAST with the largest contig of each genome as the query sequence. Up to 20 closely related genomes were downloaded from the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov) FTP site available in September 2017 and September 2018 (Table S4.1 – Appendix C). The genomes were aligned in MAUVE with the progressive Mauve algorithm (Darling et al., 2004) to identify sequences that were present in *mahewu* isolates but absent in closely related reference genomes. Multiple MDR genes of MATE and MFS families were present in all *mahewu* isolates but absent in the up to 20 most closely related genomes. To identify all the Multidrug and Toxic Compound Extrusion (MATE) families and the Major Facilitator Superfamily (MFS), 6 sequences in *Lp. plantarum* FUA3590 were used as query sequences for nucleotide BLAST and protein BLAST (Altschul et al., 1997) against all genomes of *mahewu* isolates with threshold of 30% protein identity and 75% coverage. Identification of all proteins as MDR proteins was verified by BLASTp analysis against the Swissprot / Uniprot database. Protein classification was performed using InterProScan (Mitchell et al., 2014) and InterPro tools (Mitchell et al., 2019). The MDR genes were subsequently renamed as *mahewu* phenolics resistance genes (*mpr*). Determination of whether the *mpr* genes are located on plasmids was performed in silico using PlasmidFinder (Carattoli et al., 2014).

4.2.4 PCR detection of genes coding for MDR-Transporters in other *mahewu* isolates

Primers for the *mpr* genes, *mprA*, *mprB*, *mprC*, *mprD* and *mprE* were designed from sequences from the genome of *Lp. plantarum* FUA3590 strain using the PrimerQuest Tool (Integrated DNA Technologies, Coralville, IA, USA). Details of the primers are given in Table 4.2. Primers were synthesized at Integrated DNA Technologies (Coralville, IA, USA), and were tested in PCR reactions with the genomic DNA of those strains of *Lm. fermentum, Lp. plantarum*, *Pediococcus*

pentosaceus, Fufurilactobacillus rossiae and *Weissella cibaria* for which genome sequences are not available (Table 4.1). PCR reactions with the *mpr* gene primers were validated with *Lp. plantarum* FUA3590 and positive or negative amplicons from the genomic DNA of respective strains were confirmed by gel electrophoresis.

4.2.5 Identification of genes coding for antibiotic resistance in genome-sequenced cereal isolates

Six *Lp. plantarum* and three *Lm. fermentum* genome sequences of cereal isolates used in this study were annotated using Prokka (Seemann, 2014) with default settings. Antibiotic resistance genes from the Comprehensive Antibiotic Resistance Database (CARD) (Alcock et al., 2020) were downloaded and used as query sequences for protein BLAST (Altschul et al., 1997) with cut-off values of 40% amino acid identity and 70% coverage. If multiple query sequences were similar to the same gene in a specific genome, only the protein with the highest amino acid identity was retained.

4.2.6 Determination of inhibitory activity of different antimicrobial compounds against *Lm. fermentum* and *Lp. plantarum*

The inhibitory effects of the following antimicrobial compounds against *Lm. fermentum* and *Lp. plantarum* were determined: (i) The phenolic acids caffeic acid, ferulic acid (Extrasynthese, Genay, France), sinapic acid and salicylic acid (Sigma-Aldrich, Oakville, ON, Canada) were used with a stock concentration of 20 gL^{-1}. (ii) The antibiotics acriflavine, erythromycin, chloramphenicol, norfloxacin, tetracycline, streptomycin and nisin (all Sigma-Aldrich, Oakville, ON, Canada) were used with stock concentrations of 0.2 gL^{-1}. Iso alpha extract (HopTech, Dublin, CA, USA) was used with a starting concentration of 10 International Bitterness Units (IBU). The minimum inhibitory concentrations (MIC) of antimicrobials were determined by a critical dilution assay as described by (Gänzle et al., 1999) with modifications. In brief, two-fold serial dilutions

of antimicrobials and phenolic acids were prepared with MRS5 broth in 96-well microtiter plates (Corning, NY, USA). *Lm. fermentum* and *Lp. plantarum* were sub-cultured twice in MRS5 broth and incubated at 30 °C for 10 h and 12 h, respectively. The cultures were diluted ten-fold with MRS5, and 50 µL of these diluted cultures were added to the microtitre plates. The plates were incubated for 16–20 h at 30 °C, the optical density was measured at 600 nm using a microtiter reader (Varioskan Flash, Thermo Electron Corporation, Burlington, ON, Canada), and the MICs of antimicrobials and phenolic acids were assessed as concentration in mgL^{-1} or gL^{-1} and iso alpha extract as IBU. All data were expressed as means ± standard deviation of triplicate independent experiments. To obtain a preliminary indication of which factors relate to antimicrobial resistance (AMR), the MIC of 10 strains of *Lp. plantarum* and nine strains of *Lm. fermentum,* isolated from mahewu, ting, wheat bran, wheat sourdough and spoiled beer, were analyzed using Principal Component Analysis (PCA). METAGENassist (Arndt et al., 2012), a web-based analytical pipeline, was used to generate PCA score plots to visualize differences in AMR in relation to the bacterial species and the isolation source.

4.2.7 Quantification of gene expression during growth of *Lp. plantarum* and *Lm. fermentum* in finger millet, sorghum and wheat sourdoughs

To determine which MDR transporters and genes coding for phenolic acid metabolism are expressed during growth in finger millet, sorghum and wheat sourdoughs, mRNA was quantified by reverse transcription-quantitative PCR (RT-qPCR). The identification of MDR transporters is described below; genes encoding for phenolic acid metabolism were identified by using esterases, tannases, phenolic acid reductases and phenolic acid decarboxylases that are known to contribute to the conversion of phenolic acids by lactobacilli (Cavin et al., 1997; Esteban-Torres et al., 2013; Gaur et al., 2020; Iwamoto et al., 2008; Lai et al., 2009; Reverón et al., 2017; Santamaría et al., 2018) (Table S4.2 – Appendix C).

Sorghum and wheat sourdoughs were prepared by mixing 10 g of flour and 10 mL sterile tap water with a cell suspension of *Lp*. *plantarum* FUA3590, or *Lm*. *fermentum* FUA3582 to achieve an initial cell count of about 10^7 cfu g^{-1} (Teixeira et al., 2014). Model *mahewu* fermentations were prepared as described in (Chapter 3, this thesis) with the addition of a cell suspension of *Lp. plantarum* FUA3590 or *Lm. fermentum* FUA3582. The doughs were fermented at 25° and 30 °C until the pH was reduced to 4.5 - 5.25, corresponding to the exponential phase of growth. Cells were isolated from sourdoughs as described by (Teixeira et al., 2014) and RNA was extracted using RNA protect Bacteria Reagent and RNeasy Minikit (Qiagen, Germantown, MD, USA) prior to DNAase treatment with RQ1 RNase-Free DNase Kit (Promega, Madison, WI, USA) to eliminate residual DNA. RNeasy PowerClean Pro Cleanup Kit (Qiagen, Germantown, MD, USA) was used to clean isolated RNA from the sorghum and finger millet sourdoughs which removes color as well as other PCR-inhibiting substances, such as polyphenols. RNA quality and quantity were assessed spectroscopically using a NanoDrop One spectrophotometer system (Thermo Fisher Scientific, Waltham, MA, USA), prior to reverse transcription to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Germantown, MD, USA). Specific primers targeting *mpr* genes and phenolic acid enzymes (Table 4.2) were used for qPCR amplification, which was performed using the QuantiFast SYBR green master mixture (Qiagen, Germantown, MD, USA) in a 7500 Fast Real Time-PCR System (Applied Biosystems, Waltham, MA, USA). Primers were designed based on the genome sequences of *Lm. fermentum* (FUA3582) and *Lp. plantarum (*FUA3590). DNase-treated RNA samples served as negative controls. The relative gene expression was calculated as:

$$relative\ gene\ expression = \frac{2^{[\Delta C_{T,\ target\ gene}(reference-sample)]}}{2^{[\Delta C_{T,\ housekeeping\ gene}(reference-sample)]}}$$

where $E_{target\ gene}$ is the PCR efficiency for the target gene, $E_{housekeeping\ gene}$ is the PCR efficiency for the housekeeping gene, and ΔC_T is the threshold cycle for samples obtained at sample and reference conditions (Pfaffl, 2001). Fructose-bisphosphate aldolase and phosphoketolase were used as housekeeping genes for *Lp. plantarum* FUA3590 and *Lm. fermentum* FUA3582, respectively. Exponentially growing cultures of *Lp. plantarum* FUA3590 and *Lm. fermentum* FUA3582 in MRS5 broth (OD_{600nm} 0.4–0.6) were used as reference conditions. The experiment was performed in triplicate independent experiments, each analyzed in duplicate qPCR reactions. Statistical analysis was performed using one-way analysis of variance (ANOVA) with the Holm-Sidak post hoc analysis. Significance was assessed at an error probability of 5% ($P<0.05$).

4.3 Results

4.3.1 Identification and comparison of genes coding for MDR transporters in genome sequenced strains

Comparative genomic analyses of *mahewu* isolates with closely related strains identified genes coding for putative MDR proteins of the multidrug and toxin extrusion (MATE) family or the major facilitator superfamily (MFS) that were present in *mahewu* isolates but absent in closely related strains. These genes were termed "*mahewu* phenolics resistance" genes or *mpr*. Genes that were confirmed to encode MDR proteins by BLASTp search against the Swissprot / Uniprot database on NCBI are shown in Table 4.3. Functional analysis of MDR proteins revealed that Mpr proteins are transmembrane transporters with antiporter activity against xenobiotics belonging to the MATE family or permeases of the MFS family (Table 4.3 and Table S4.3 – Appendix B). Genomes of strains of *Lp. plantarum* encoded up to six MDR proteins; the two genomes of strains of *Lm. fermentum* encoded up to five MDR proteins; two MDR proteins each were identified in the genome of *Ff. rossiae* and *W. cibaria*. Several strains of *Lm. fermentum* and *Lp. plantarum* encoded for two copies of highly similar proteins (Table 4.3 and Table S4.4 - Appendix B). Genes

coding for the same proteins in *Lp. plantarum* and *Lm. fermentum* were more than 95% identical (Table S4.5 – Appendix B). Close homologues of *mprA* and *mprB* with more than 99% sequence identity were also identified in pediococci and in *Liquorilactobacillus mali* (Table 4.3). Some of these homologues in other genera of lactobacilli are plasmid encoded (Table 4.3), however, *in silico* analysis indicated that *mpr* genes in *mahewu* isolates were all chromosomally encoded. With the exception of *mprA* in *Lp. plantarum* FUA3584, the G + C content of *mprA* in *Lm. fermentum* and *Lp. plantarum* is comparable to each other but differs from the G + C content of the respective genomes (Figure 4.1). In strains of *Lm. fermentum*, the *mprA* genes are flanked by mobile genetic elements. The mobile genetic elements in *mahewu* isolates *Lm. fermentum* are more than 99% identical to each other and to mobile genetic elements in *Lp. plantarum* and *Oenococcus oeni* (Table 4.4). Taken together, these results suggest recent horizontal gene transfer of the *mpr* genes between *Lp. plantarum*, *Lm. fermentum* and other lactobacilli.

In addition to the seven genome sequenced isolates from *mahewu*, six strains of *Lp. plantarum* and *Lm. fermentum* were obtained from the same batches but their genomes were not sequenced. The identification of *mpr* genes in these strains was performed with PCR. PCR detection matched the detection by genome analysis in all cases (Table 4.5). The genes coding for *mprA* or $mprA_D$ were identified in all strains of *Lm. fermentum* and *Lp. plantarum* (Table 4.5).

Table 4.3 Identification of genes coding for Multi-Drug-Resistance transporters in bacterial isolates. Shown are the closest homologues to MDR proteins as identified by BLASTp with the Swissprot database, and the closest homologues identified by BLASTn. More than one result is shown for the BLASTn analysis if other results were highly homologous and plasmid encoded or from a different bacterial species.

Organism	*mpr*	Closest Homolog (SWISSPROT)	ID %	Closest Homolog (BLASTn)	ID %	Other homologues (BLASTn)	ID %
Lp. plantarum	*A*	YpnP	31	*P. pentosaceus* SRCM 102734	99	*Lp. plantarum* SRCM103297 plasmid	99
FUA3590	*B**	NS (MFS)		*Lm. fermentum* SRCM103290	99	*P. parvulus* 2.6 plasmid pPP1	99
	C	YpnP	34	*Lp. plantarum* 83-18	100		
	D	MepA	30	*Lp. plantarum* TC1507	100		
	A_D	YpnP	31	*Ped. pentosaceus* SRCM 102734	99	*Lp. plantarum* SRCM103297 plasmid	99
	E	NS		*Lp. plantarum* 83-18	100		
Lm. fermentum	*F*	MepA	28	*Lm. fermentum* SRCM103290	100		
FUA3588	*G*	MepA	29	*Lm. fermentum* LTDM7301	97		
	H	YpnP	28	*Lm. fermentum* IMDO130101	100		
	A	YpnP	32	*Lq. mali* LM596 plasmid	99		
	A_D	YpnP	32	*Lq. mali* LM596 plasmid	99		
Lm. fermentum	*I*	MepA	28	*Lm. fermentum* SRCM103290	100		
FUA3589	*A*	YpnP	30	*Lm. fermentum* SRCM103290	99	*Lq. mali* LM596 plasmid	99
	A_D	YpnP	30	*Lm. fermentum* SRCM103290	99	*Lq. mali* LM596 plasmid	99
	J	MepA	29	*Lm. fermentum* USM 8633	99		
Lm. fermentum	*K*	MepA	28	*Lm. fermentum* SRCM103290	100		
FUA3582	*L*	YpnP	22	*Lm. fermentum* USM 8633	99		
	M	MepA	31	*Lm. fermentum* SRCM 103285	98		
	A	YpnP	32	*Lq. mali* LM596 plasmid	99		
Lp. plantarum	*A*	YpnP	31	*Lp. plantarum* SRCM100442	100		
FUA3584	*N*	MepA	29	*Lp. plantarum* G1	100		
Ff. rossiae	*O*	YpnP	33	*F. rossiae* L3	97		
FUA3583	*P*	Stp	31	*F. rossiae* L2	100		
W cibaria	*Q*	RiBZ	26	*W. cibaria* SRCM103448	99		
FUA 3585	*R*	YpnP	27	*W. cibaria* CMS1	99		

mprA: *mahewu* phenolic resistance gene; $mprA_{D:}$ duplicate *mprA* gene; **mprB*: putative MDR permease, possible multidrug efflux pump; YpnP: Probable multidrug resistance protein YpnP [*Bacillus subtilis* 168]; MepA: Multidrug export protein MepA [*Staphylococcus saprophyticus* ATCC 15305 and *Staphylococcus haemolyticus* JCSC1435]; Stp: Multidrug resistance protein Stp; RibZ: Riboflavin transporter RibZ [*Clostridioides difficile* 630]; NS: No significant similarity found

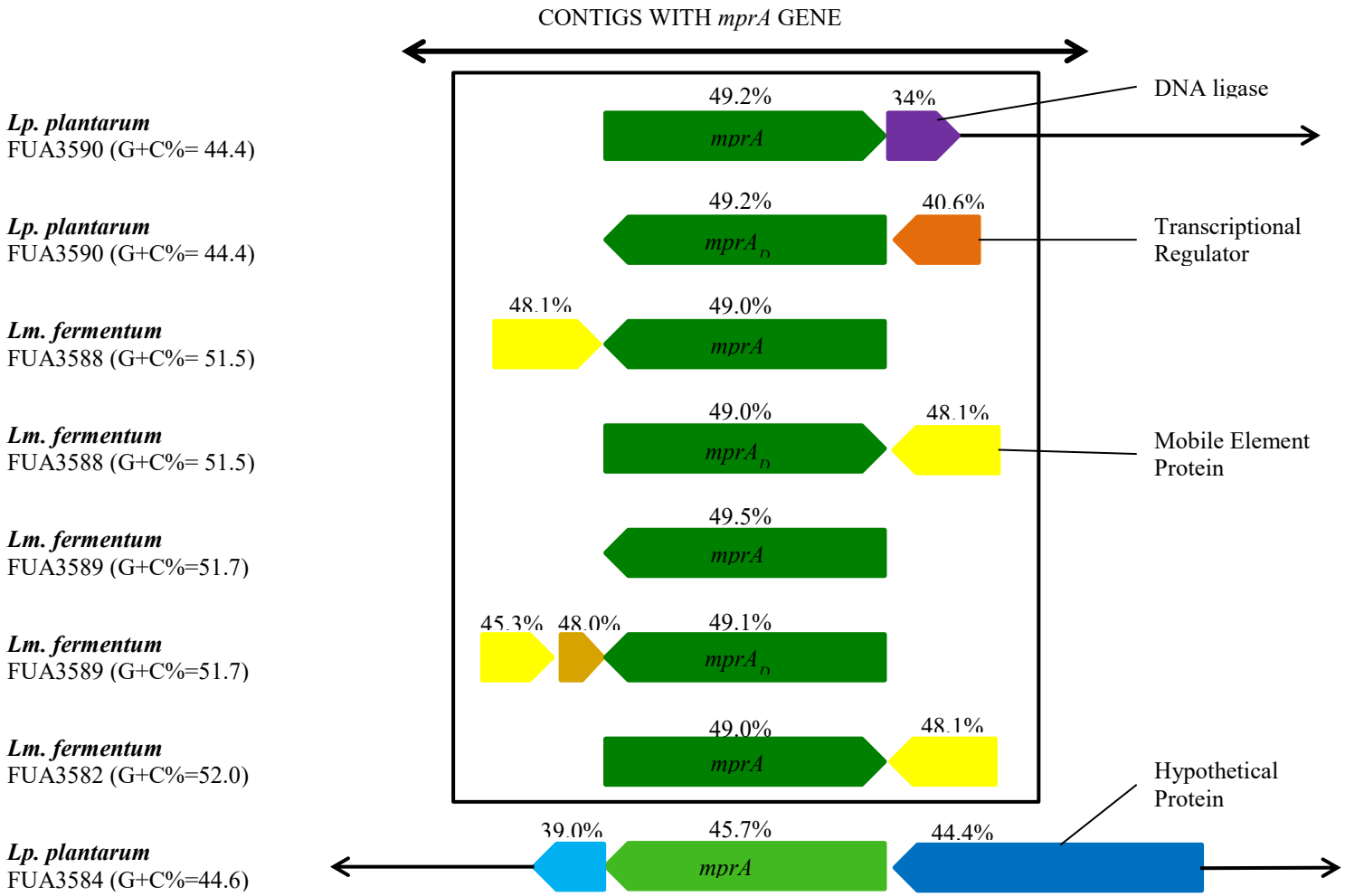

Figure 4.1 Comparison of the contigs containing one or two mprA genes found in *Lp. plantarum* and *Lm. fermentum* strains isolated from *mahewu*. The same color (green) of the *mprA* gene indicates that the sequence is 99 – 100 % homologous in the different strains. The mobile element protein genes found in the *Lm. fermentum* strains with the same color (yellow) indicates that the sequence is homologous. Different shades of the same color denote non-homologous sequences.

Table 4.4. Comparison of the nucleotide sequence identify genes coding for mobile protein elements **found in contigs with *mprA* genes *Lm. fermentum* genomes**

Organism	*mep*	Closest Homolog	ID %
Lm. fermentum FUA3588	*A*	*Oenococcus oeni* SD-2a	99
	A_D	*Oenococcus oeni* SD-2a	99
Lm. fermentum FUA3589	*B*	*Lp. plantarum* SPC-SNU 72-2 plasmid pLBP752	99
	C	*Oenococcus oeni* OE37	98
Lm. fermentum FUA3582	*A*	*Oenococcus oeni* SD-2a	99

mepA is mobile element protein gene

$mepA_D$ is a duplicate *mepA* gene

Table 4.5 PCR detection of *mpr* genes in *mahewu* isolates

Species	Strain ID	Gene Name				
		mprA[a)]	***mprB***	***mprC***	***mprD***	***mprE***
Lp. plantarum	**FUA3590**	+	+	+	+	+
	FUA3584	+	-	+	+	+
	FUA3586	+	-	+	+	+
Lm. fermentum	**FUA3588**	+	-	-	-	-
	FUA3589	+	-	-	-	-
	FUA3582	+	-	-	-	-
	FUA3569	+	-	-	-	-
	FUA3570	+	-	-	-	-
	FUA3573	+	-	-	-	-
P. pentosaceus	FUA3568	+	-	-	-	-
	FUA3577	+	-	-	-	-
Ff. rossiae	**FUA3583**	-	-	-	-	-
W. cibaria	**FUA3585**	-	-	-	-	-

A plus sign indicates the presence of *mpr* genes in *mahewu* bacterial strains as confirmed by PCR and gel electrophoresis. A minus sign indicates the absence of *mpr* genes. Shaded and unshaded boxes represent presence (gray) and absence (no shading) of the respective *mpr* genes in the genome sequenced strains (bold strain number shaded in gray).

[a)] Owing to the high nucleotide identity of *mprA* and $mprA_D$, primers did not distinguish between these two genes.

4.3.2 Inhibitory activity of antimicrobials and phenolic acids

If MDR genes are shared between taxonomically diverse lactobacilli that occur in cereal fermentations, the likely function is to increase the ecological fitness of the organisms but *in silico* analyses did not provide an indication of the function of the Mpr proteins. To assess the inhibitory activity of antimicrobial compounds, the MIC of different antibiotics, phenolic acids, an isomerized hop extract and nisin against strains of *Lm. fermentum* and *Lp. plantarum* were determined. In addition to the *mahewu* isolates, strains isolated from sourdough, ting, and fermented wheat bran were included in the analyses. The MICs of the 12 antimicrobial compounds against the 19 strains of *Lm. fermentum* and *Lp. plantarum* are shown in (Table S4.6 - Appendix

C). PCA analysis of the data revealed that the resistance between *Lm*. *fermentum* and *Lp*. *plantarum* strains differed (Figure 4.2A) with the *Lp*. *plantarum* strains having an overall higher resistance (Table S4.6 - Appendix C). The PCA score plot also identified the beer isolate *Lp*. *plantarum* TMW1.460 as an outlier with higher resistance compared to other strains of *Lp. plantarum*. With the exception of the single beer-spoiling isolate of *Lp. plantarum* TMW1.460, PCA did not differentiate the isolates by source (Figure 4.2B). The higher overall resistance of strains of *Lp*. *plantarum* was reflected in the MICs against antibiotics as well as the MICs against phenolic acids (Table S4.6 - Appendix C). The two *mahewu* isolates *Lp*. *plantarum* FUA3590 and FUA3584 were most resistant to caffeic acid (MIC of 4.4 gL^{-1}) while *Lp. plantarum* TMW 1.460 was most resistant to sinapic acid (MIC 5.6 gL^{-1}) but not to isomerized hop extract (Table S4.6 - Appendix C). All strains resisted more than 50 mgL^{-1} of streptomycin, norfloxacin and nisin and the resistance of several strains against erythromycin, chloramphenicol, tetracycline, and streptomycin were higher than the breakpoints that were established by EFSA for food and feed cultures of *Lp. plantarum* and *Lm. fermentum* (Table S4.6 - Appendix C). The PCA loading plot indicated that resistance to antibiotics and the resistance to nisin, hops, ferulic and caffeic acids were highly correlated (Figure S4.1 - Appendix B), suggesting that similar mechanisms account for resistance to antibiotics, nisin, and plant secondary metabolites.

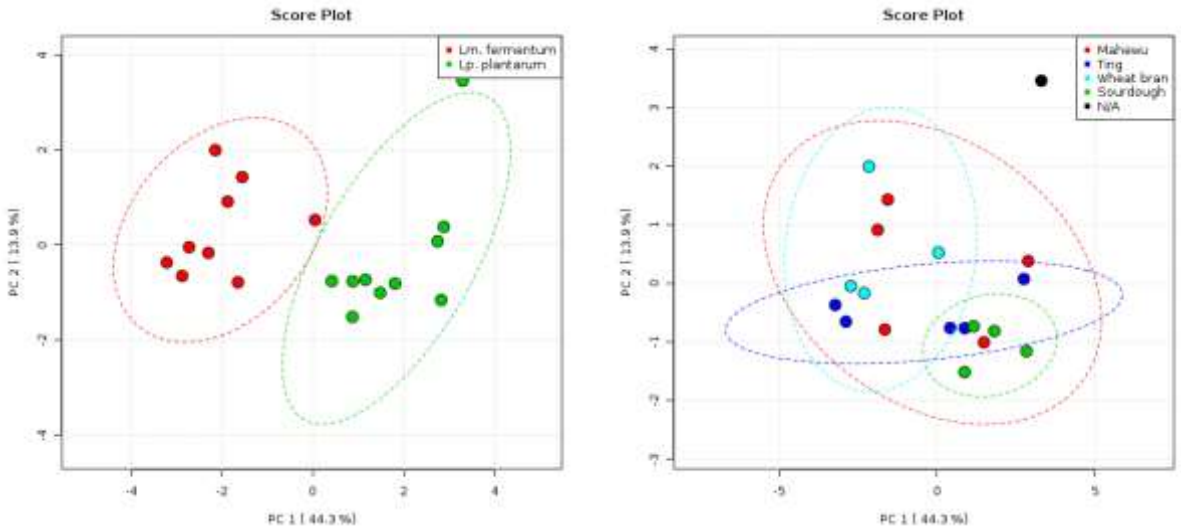

Figure 4.2 Principal component analysis of the MICs of strains of *Lp. plantarum* and *Lm. fermentum* with 12 antimicrobials. **Panel A** shows the score plot with a 95% confidence region to differentiate *Lp. plantarum* and *Lm. fermentum* irrespective of their origin. **Panel B** shows the score plot with a 95% confidence region to differentiate strains of *Lp. plantarum* and *Lm. fermentum* with respect to the source of isolation. Results are shown as means of triplicate biological repeats.

4.3.3 Expression of *mpr* genes during growth in finger millet, sorghum and wheat sourdoughs

To further explore a possible contribution of *mpr* genes to the resistance of lactobacilli to plant secondary metabolites, the expression of these genes in two strains was quantified during growth in cereal substrates. The expression during growth in sorghum sourdough, a model *mahewu* prepared with 3% finger millet malt and 6% maize flour (balance water), and in wheat sourdoughs was quantified relative to the expression in MRS5 broth which does not contain plant ingredients with phenolic compounds. The quantification of mRNA demonstrated that *Lp. plantarum* FUA3590 expressed all five *mpr* genes during growth in cereal substrates (Figure 4.3A); of the

five, *mprB* and *mprD* were overexpressed in one or more of the cereal substrates (Figure 4.3A). In *Lm*. *fermentum* FUA3582, all four genes were expressed during growth in cereal substrates and two of the four, *mprL* and *mprM*, were overexpressed during growth in at least one of the substrates (Figure 4.3B).

4.3.4 Expression of genes coding for enzymes of phenolic acid metabolism

Phenolic acid metabolism by lactobacilli is mediated by esterases or tannases, phenolic acid reductases, and phenolic acid decarboxylases. Phenolic acid esterases release active phenolic acids with antimicrobial activity from inactive pre-cursors while reductases and decarboxylases decrease the antimicrobial activity of phenolic acids (Sánchez-Maldonado et al., 2011). The genome of *Lp. plantarum* FUA3590 encoded for the phenolic acid decarboxylase Pad, the phenolic acid reductase HcrB, the esterase Lp_0796 (EstP), and the tannase $TanB_{LP}$, formerly called TanLp1 (Cavin et al., 1997; Esteban-Torres et al., 2013; Iwamoto et al., 2008; Santamaría et al., 2018). The genome of *Lm*. *fermentum* FUA3582 encoded for the phenolic acid decarboxylase Pad (Cavin et al., 1997), the phenolic acid reductase HcrF (Gaur et al., 2020) and an the esterase EstF that is 52% identical to the feruloyl-esterase Lp_2953 in *Lp. plantarum* (Lai et al., 2009; Reverón et al., 2017).

To elucidate a potential role of enzymes of phenolic acid metabolism in finger millet and sorghum sourdoughs, gene expression during growth in cereal substrates was quantified relative to the expression in mMRS5 broth. In *Lp. plantarum* FUA3590, the phenolic acid reductase *hcrB* was differentially overexpressed ($P<0.05$) in *mahewu* only (Figure 4.4A), while in *Lm*. *fermentum* FUA3582 *hcrF* was differentially overexpressed in both *mahewu* and sorghum sourdoughs (Figure 4.4B). The genes encoding for phenolic acid decarboxylase activity were not overexpressed in *Lp*. *plantarum* FUA3590 or in *Lm*. *fermentum* FUA3582 (Figure 4.4). The gene for esterase *estF* in *Lm*. *fermentum* FUA3582 was overexpressed in *mahewu* (Figure 4.4B).

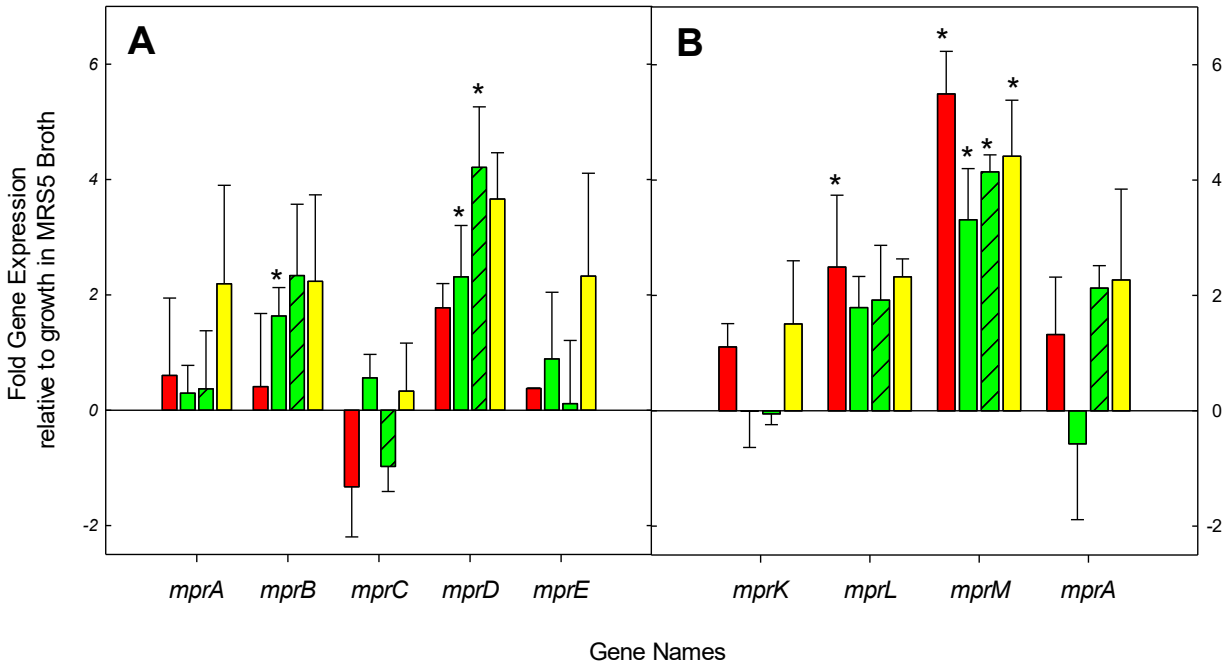

Figure 4.3 Expression of *mpr* genes during growth in sorghum, finger millet and wheat sourdoughs relative to the expression of the same genes during growth in MRS5 broth. Panel A. *Lp. plantarum* FUA3590; Panel B. *Lm. fermentum* FUA3582. Substrates and incubation conditions are color-coded as follows: Red bars, *mahewu* fermented at 25°C; green bars, sorghum cultivar Town at 25°C; green hatched bars, sorghum cultivar Town fermented at 30°C; yellow bars, wheat fermented at 30°C. Sourdoughs were incubated until the dough pH reached a value of 4.5 – 5.2, corresponding to the exponential phase of growth; cultures in MRS5 broth were incubated until an $OD_{600\ nm}$ of 0.5 was reached. The horizontal line represents unity (gene expression equivalent to gene expression at the reference conditions). Results are shown as log 2 transformed means ± standard error of triplicate biological repeats, each sample was analyzed in technical duplicates. Genes that were differentially expressed ($p < 0.05$) relative to expression by the same strain at reference conditions are marked with an asterisk.

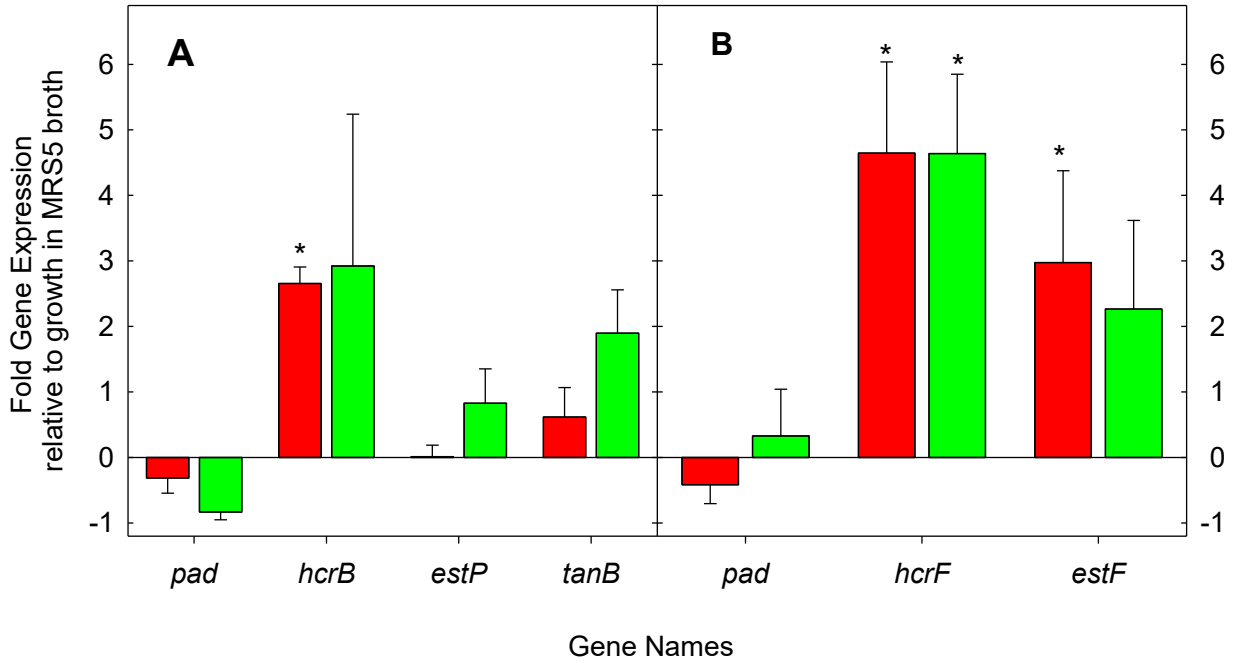

Figure 4.4 Expression of genes coding for enzymes of phenolic acid metabolism in *Lp. plantarum* FUA3590 (Panel A) and *Lm. fermentum* FUA3582 (Panel B) during growth in sorghum and finger millet sourdoughs relative to the expression of the same genes during growth in MRS5 broth. Substrates and incubation conditions are color-coded as follows: Red bars, *mahewu* fermented at 25°C; green bars, sorghum cultivar Town at 25°C. Results are shown as log 2 transformed means ± standard error of triplicate biological repeats, each sample was analyzed in technical duplicates. An asterisk indicates that a gene is significantly overexpressed ($P<0.05$) relative to its expression at the reference conditions. Genes are as follows: *pad*, phenolic acid decarboxylase; *hcrB*, phenolic acid reductase (*Lp. plantarum*); *hcrF*, phenolic acid reductase (*Lm. fermentum*); *estP,* carboxylesterase (*Lp. plantarum*); *estF*, esterase (*Lm. fermentum*); *tanB*, tannase (*Lp. plantarum*).

4.4 Discussion

Cereal-associated lactobacilli have evolved diverse mechanisms to resist plant secondary metabolites with antimicrobial activity. This study aimed to determine the presence of multi-drug-resistance transport genes in isolates of *Lactiplantibacillus plantarum* and *Limosilactobacillus*

fermentum from *mahewu*, a Zimbabwean fermented cereal beverage, by comparative genomic analyses. All seven strains harbored multiple genes coding for MDR transporters, termed *mahewu* phenolics resistance *mpr* genes. Several strains of *Lp. plantarum* and *Lm. fermentum* encoded for duplicate copies of the same gene *mprA*. *Mahewu* phenolic resistance genes with high (99%) nucleotide identity are shared between strains of different species, moreover, several of the genes are virtually identical to plasmid-encoded genes of other genera of the *Lactobacillaceae,* indicating that these genes were acquired by horizontal gene transfer. Horizontal gene transfer (HGT) is mediated by plasmids, prophages, transposons and natural transformation (Frost et al., 2005). *Lm. fermentum* include mobile genetic element proteins adjacent to *mpr* genes, which implies transposons are involved in HGT. Lactic acid bacteria that inhabit the same ecological niche share plasmid-encoded genes that are absent in strains of the same species that occupy different habitats; specifically, this was demonstrated for beer-spoiling strains of *Levilactobacillus brevis* (Fraunhofer et al., 2019) and for dairy isolates of *Lactococcus lactis* (Malesevic et al., 2021). The plasmidome of lactic acid bacteria also contributes to the spread of antibiotic resistance in lactic acid bacteria (Lanza et al., 2015). The present study extends these previous findings by documenting that phylogenetically diverse lactobacilli from cereal fermentations share plasmid-encoded MDR transporters with putative function in resistance to antimicrobials.

To determine the resistance of strains of *Lp. plantarum* and *Lm. fermentum* to antimicrobial compounds, their resistance to phenolic acids, hops, nisin and antibiotics was determined. Antimicrobial phenolic compounds in sorghum selected for strains with resistance to phenolics (Sekwati-Monang et al., 2012). The concentration of phenolic compounds in different grains as well as in different cultivars of the same grain species differs substantially (Awika and Rooney, 2004; Shahidi and Chandrasekara, 2013; Shewry et al., 2010). Fermentation organisms in *mahewu*

originate from the finger millet malt that is used in *mahewu* production, this was documented by strain-specific qPCR for two of the isolates (Chapter 3, this thesis). Therefore, the possibility that isolates from different cereals exhibit a different complement of genes coding for MDR transporters, or differ in their phenotypic resistance was considered. Although the resistance of lactobacilli to antimicrobials clearly differentiated *Lp. plantarum* and *Lm. fermentum,* strains of the same species but of different origin, wheat, wheat bran, finger millet, or sorghum, did not differ in their resistance. Resistance is either shared by most or all strains of one species, or generally relates to adaptation of lactobacilli to cereals or plants.

The data on the phenolic acid resistance generally conform to previous reports for *Lp*. *plantarum* (Campos et al., 2003; Cueva et al., 2010; Merkl et al., 2010; Sánchez-Maldonado et al., 2011; Taguri et al., 2006). Metabolism of phenolic acid through reduction and decarboxylation decreases their antimicrobial activities (Sánchez-Maldonado et al., 2011). In lactobacilli, hydroxycinnamic acid metabolism has been considered strain specific (Filannino et al., 2015; Ripari et al., 2019) but all strains that were analyzed in this study included hydroxycinnamic acid reductase and decarboxylase activities. This study extends previous reports on the overexpression of hydroxycinnamic acid reductases and decarboxylase in response to the presence of phenolic acids in laboratory culture (Gaur et al., 2020) by documenting that phenolic acid reductase genes *hcrB* and *hcrF* are overexpressed *in situ* and thus likely contribute to resistance against plant secondary metabolites. In addition, the phenolic acid metabolism in cereal fermentations was recently shown to also depend on interactions between lactobacilli and yeasts (Boudaoud et al., 2021). Feruloyl esterases were also overexpressed during growth of *Lm. fermentum* and *Lp. plantarum* in cereals. These enzymes release phenolic acids including ferulic, *p*-coumaric, caffeic, and sinapic acids

from plant cell walls (Benoit et al., 2008) but the contribution of specific genes to conversion of phenolic acid esters in cereal fermentations remains to be documented (Svensson et al., 2010).

Hop resistance of *Lp. plantarum* TMW1.460 was previously attributed to HorA (Ulmer et al., 2000), an ATP-binding cassette (ABC) family multidrug transporter which extrudes structurally unrelated compounds including iso-α-acids from the cytoplasmic membrane (Sakamoto et al., 2001). HorA also mediates resistance to antibiotics (Suzuki et al., 2002). Most strains investigated in this study tolerated more than 50 mg/L nisin, a bacteriocin that is used as food preservative (Delves-Broughton, 1996). Patterns of nisin resistance in lactic acid bacteria also contribute to bacterial resistance to other antibiotics, thereby increasing the risk of multidrug-resistant variants of pathogens (Kramer et al., 2006; Zhou et al., 2014). Strains of *Lp. plantarum* and *Lm. fermentum* isolated from fermented cereal products used in this study are fairly resistant to nisin with MICs over 50 mg/L which exceeded those reported in a previous study (Breuer and Radler, 1996; Rojo-Bezares et al., 2007).

The fermentation of cereals detoxifies and eliminates phenolic compounds other than phenolic acids that are inherently present in grains and have antinutritive properties (Gänzle, 2020). In particular, different sorghum and millet varieties contain tannins, which have a bitter taste and inhibit human digestive enzymes (Awika and Rooney, 2004; Dlamini et al., 2007). The different tannin content of red and white sorghum cultivars relates to the overall antimicrobial activity of sorghum extracts against lactobacilli (Sekwati-Monang et al., 2012) but information on the active compounds or the bacterial resistance mechanisms is currently unavailable.

The antibiotic resistance of strains of *Lp. plantarum* and *Lm. fermentum* used in this study exceed the threshold levels recommended by EFSA for food and feed cultures. Specifically, threshold levels were met or exceeded for erythromycin, chloramphenicol, tetracycline and streptomycin in

Lm. *fermentum* and for erythromycin and chloramphenicol in *Lp*. *plantarum* (Table S4.6 – Appendix C) (EFSA, 2012; Rychen et al., 2018). EFSA considers antibiotic resistance exceeding the threshold values a hazard if resistance relates to the presence of a known AMR gene. Analysis of the genomes with the Comprehensive Antibiotic Resistance Database identified 32 AMR genes and each of the strains encoded for at least one gene that is predicted to confer resistance to erythromycin, chloramphenicol, tetracycline and / or streptomycin (Table S4.7 – Appendix C). In *Lp. plantarum* and *Lm*. *fermentum*, several multiple drug resistance genes were present in addition to the *mpr* genes and the contribution of individual genes to the overall AMR can thus not be assessed on the basis of current information. Because some genes coding for MDR transporters are located on mobile genetic elements, isolates from *mahewu* as well as all other isolates from cereal fermentations, however, may be categorized as a potential hazard on the basis of current EFSA guidance.

Use or abuse of antibiotics is considered to be a major contributor to the spread of antimicrobial resistance (WHO, 2019). The *mahewu* isolates were obtained in 2016 in rural Zimbabwe (Chapter 3, this thesis) and the misuse of antibiotics is also considered a major driver of antimicrobial resistance in Zimbabwe (Caudell et al., 2020). In Zimbabwe, antibiotics are over-prescribed in human medicine (Center for Disease Dynamics, 2017). Farmers in rural Zimbabwe have limited access to animal health professionals; these gaps are filled by individuals with limited formal training on AMR and prudent antimicrobial use (Caudell et al., 2020). Tetracycline is the most commonly prescribed antimicrobial in animals, followed by penicillins. These antibiotics are mainly used in disease prevention (Caudell et al., 2020; Center for Disease Dynamics, 2017). Strains of *Escherichia coli* isolated in Zimbabwe harboured genes mediating resistance to tetracycline as well as to amoxicillin and trimethoprim, which are mainly used in humans (Mercat

et al., 2016). Because cow manure is used as a soil amendment and fertilizer in rural Zimbabwe, even plant associated bacteria including lactobacilli may be exposed to antibiotics or to antibiotic resistance genes. The microbiota of finger millet malt and the initial stages of *mahewu* fermentations also include *Enterococcus*, *Klebsiella* and *Cronobacter* species (Chapter 3, this thesis), thus providing opportunity for gene transfer between lactobacilli and opportunistic pathogens.

Bacterial antibiotic resistance, however, predates the human use of antibiotics (D'Costa et al., 2011). The presence of antibiotic resistance genes in human or animal associated microbiota in environments without exposure to antibiotic indicates that selective pressure for antibiotic resistance is also provided by compounds that are unrelated to human use of antibiotics (Boon and Cattanach, 1999; Clemente et al., 2015; Martinez, 2009). For example, AR genes were identified in uncontacted Amerindians, antibiotic resistance genes are likely poised for mobilization and enrichment upon exposure to antibiotics (Clemente et al., 2015). Selective pressure may be provided by microorganisms that produce antibiotics in soil or in plant-associated habitats (Simpson et al., 2004; Thomas et al., 2010). In addition, multi-drug efflux pumps were hypothesized to relate to bacteria-plant interactions to aid the plants' symbionts in defense against antimicrobial plant secondary metabolites (Blanco et al., 2016; Du et al., 2018). *Lp. plantarum* and *Lm. fermentum* are both known to occur in plant-associated habitats but the connection of MDR transporters in plant-microbe interaction has not been established for lactobacilli. Of the strains for which genome sequences were available, all genomes of *Lp. plantarum* and *Lm. fermentum* encoded for 16 and 12, respectively, antibiotic resistance genes that were identified by the CARD database (Table S4.7 Appendix - C). This indicates that these genes are part of the core genome of

these species rather than the accessory genome which is maintained only in the presence of specific selective pressure.

Likewise, the data supports the evidence provided by a previous study for the revision of the regulatory guidelines for safety assessment of lactobacilli entering the food chain as starter cultures, food preservatives or probiotics in light of the genetic basis for resistance (Campedelli et al., 2019). The *mprA* genes in lactobacilli from *mahewu* were likely acquired by horizontal gene transfer. To qualify for the Qualified Presumed Safety status regulated by EFSA, only strains which do not have acquired ARGs contributing to resistance to antimicrobials of clinical importance can be used as probiotics or starter cultures (Rychen et al., 2018).

In conclusion, this study falls short of providing conclusive evidence for a connection between antimicrobial plant secondary metabolites, MDR transporters, and antimicrobial resistance in cereal-associated lactobacilli. However, such a connection is supported by several lines of evidence: (i) multiple MDR transport genes are part of the core genome of *Lp. plantarum* and *Lm. fermentum,* lactobacilli that are adapted to plants and thus encounter phenolic compounds in their habitat (Zheng et al., 2015a). (ii) *Lp. plantarum* and *Lm. fermentum* are resistant to multiple natural antimicrobial compounds and antibiotics. (iii) Genes encoding for MDR transporters are over-expressed during growth in cereal substrates. The connection between antimicrobial plant secondary metabolites, MDR transporters, and antimicrobial resistance in lactobacilli certainly warrants further investigation.

Chapter 5 General Discussion

Despite the rich diversity of traditional fermented foods in Africa, there is a paucity of information on fully characterized and documented fermentation microbiota. Traditional fermented cereal beverages like *mahewu*, have enormous potential as vehicles to deliver beneficial bacteria but have received little attention for prophylactic and therapeutic use to counteract the poor sanitation endemic in rural communities in sub-Saharan Africa. This research study demonstrates the correlation between the microbiotas of finger millet malt and *mahewu,* by tracing the origin of the *mahewu* microbiota to the raw material. This study supports the hypothesis that improved control of the fermentation of *mahewu* reduces hygienic risks and enriches health-beneficial microbes. Furthermore, this analysis supports the theory that rapid acidification of the fermentation substrate with lactic and acetic acids is the predominant factor that results in the elimination of *Enterobacteriaceae* in *mahewu* fermentations. Lastly, the data suggests that genomes of plant-associated lactobacilli in mahewu encode for antimicrobial resistance that aid in the defense against plant-secondary metabolites.

5.1 Detailed characterization of community assembly and evolution of fermentation microbiota in *mahewu*

This study provides a new insight into the relationship between the microbiotas of finger millet malt and *mahewu,* by tracing the origin of the *mahewu* microbiota strains to the raw material. The microbiota of *mahewu* samples consisted of 3 to 7 dominant strains of lactobacilli and two strains of yeasts. Finger 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*. *Lm. fermentum* FUA3588 and FUA3589 were detected in finger millet malt, demonstrating that

finger millet malt is a main source of *mahewu* microbiota (Chapter 3, this thesis). These results support the theory that finger millet malt is a major source of *mahewu* microbiota because other than water finger millet malt is the only other raw ingredient that is used in the production of *mahewu* (Gänzle and Ripari, 2016).

This study supports the hypothesis that improved control of the fermentation of *mahewu* and related African fermented cereal foods reduces hygienic risks and enriches health-beneficial microbes. This is in line with the hypothesis that the rapid acidification of the fermentation substrate with lactic and acetic acids is the predominant factor that results in the elimination of *Enterobacteriaceae* in cereal fermentations, which is the link that relates to hygiene and safety (Dinardo et al., 2019) (Chapters 2 & 3, this thesis). Model *mahewu* fermentations conducted with a 5-strain inoculum consisting of *Lp. plantarum* FUA3590, *Lm. fermentum* FUA3588, *Klebsiella pneumoniae* FUA10025*, Cronobacter sakazakii* FUA10024 and *Enterococcus lactis* FUA3587 demonstrated that lactobacilli outcompete *Enterobacteriaceae*, which sharply decreased in the first 24 h. Despite the fact that *mahewu* microbiota is mainly derived from finger millet malt microbiota, minor components of malt microbiota rapidly outcompete *Enterobacteriaceae* and *Bacillus* species during fermentation (Chapter 3, this thesis). Therefore, these results build on existing evidence of the succession of microbial communities in cereal and vegetable fermentations that *Enterobacteriaceae*, *Leuconostoc* and *Weissella* species initiate the spontaneous cereal fermentation, and that lactic acid bacteria become dominant at later stages of the fermentation, with acid-tolerant lactobacilli *Lactiplantibacillus plantarum, Limosilactobacillus fermentum* and *Pediococcus pentosaceus* dominating at the end (Hamad et al., 1997; Lee et al., 2005; Wuyts et al., 2018). Therefore, viable *Enterobacteriaceae* at the end of the fermentation process indicates a presumed failure of the spontaneous fermentation process and is undesirable (Todorov and

Holzapfel, 2015; Wuyts et al., 2018). While, several studies reported the presence of *Enterobacteriaceae* in traditional fermented cereal beverages (Mukisa et al., 2012; Phiri et al., 2020, 2019), these may have been mainly due to the presence of persistent DNA derived from dead bacteria that were alive in the early stages of the fermentation (Wuyts et al., 2018).

Comparison with other fermented foods produced using similar methods and raw materials supports the hypothesis that a similar sequence occurs in many other African fermented cereal foods (Franz et al., 2014; Todorov and Holzapfel, 2015) (Chapters 2 & 3, this thesis). Like vegetable fermentations, the succession of fermentation microbiota in spontaneous cereal fermentations is highly reproducible, whereby the assembly of fermentation microbiota is limited by dispersal, and reflects the stable association of these organisms with the raw materials (Marco et al., 2021; Wuyts et al., 2018). Lactic acid bacterial strains which were detected in *mahewu* collected in summer were not detected in samples produced at the same site in winter. Likewise, *Candida glabrata* was present in high cell counts from samples collected in summer but not from samples collected in winter (Chapter 3, this thesis). This demonstrates that the assembly of the communities of fermentation microbes in spontaneous fermentations depends on the substrate and the fermentation conditions (Gänzle and Ripari, 2016).

Africa has a rich tradition of cereal fermentations to produce diverse products such as baked goods, porridges, non-alcoholic beverages and alcoholic beverages. Diversity also relates to the choice of the fermentation substrates, which include wheat, maize, teff, sorghum and millets, and the fermentation processes that are used in food production. For fermentation processes that are used in baking and brewing, it is well established that the composition of fermentation microbiota and thus the impact of fermentation on product quality is determined by the choice of fermentation conditions, however, this link has not been systematically explored for African cereal

fermentations. This thesis research therefore, included an overview on the diversity of African fermented cereal products, and interrogated currently available literature data with respect to the links between fermentation substrate, fermentation processes, and product quality (Chapter 2, this thesis). In spontaneously fermented cereals, lactic acid bacteria that dominate are typically species with a nomadic or environmental lifestyle (Duar et al., 2017). Generally, plant-associated *Lp. plantarum* and *Lm. fermentum* dominate fully fermented products obtained by spontaneous cereal fermentation (Chapter 2, this thesis). Whereas, in back-slopped fermentations, practiced for the production of *ting* and *kisra*, host-adapted lactobacilli, including *Lm. reuteri* and *Lm. vaginalis*, *Schleiferilactobacillus harbinensis* occur (Hamad et al., 1997; Sekwati-Monang and Gänzle, 2011). Furthermore, back-slopping may also occur through fermentation vessels when there is sufficient product residue to overwhelm plant associated lactobacilli (Sekwati-Monang and Gänzle, 2011). Selection is the major determinant for community assembly of back-slopped cereal fermentations; therefore, microbiotas of back-slopped cereal fermentations differ substantially from the microbiotas of the raw materials from which the fermentation organisms originated (Gänzle, 2019; Gänzle and Ripari, 2016). Based on the findings in this thesis research (Chapters 2 & 3) and the large body of knowledge of spontaneous cereal fermentations (Franz et al., 2014; Gänzle, 2019; Todorov and Holzapfel, 2015), it can be hypothesized that what is demonstrated for *mahewu* is likely also true for other African fermented cereal foods that are produced in a comparable way.

5.2 Fermentation can deliver health-beneficial microbes

This study demonstrates a correlation between the elimination of opportunistic pathogens and the increase of beneficial bacteria (Chapter 3, this chapter). Thus, the results are comparable with other studies which show that fermentation microbiota usually out-compete potential pathogenic and

spoilage organisms, and thus further enhance food safety and stability (Hamad et al., 1997; Lee et al., 2005; Wuyts et al., 2018) (Chapter 3, this thesis). The improvement of flavor, texture, digestibility and nutritional properties, removal of noxious plant compounds by fermentation serve a critical purpose in sub-Saharan African countries that have low food security, no access to electricity, refrigeration or clean water (Marco et al., 2021; Nout, 2009; Pswarayi et al., 2014; Soetan and Oyewole, 2009). *Mahewu*, like some fermented cereal foods and beverages contain live undefined microbial consortia, usually at variable levels, whose potential health benefits have generally not been demonstrated (Dimidi et al., 2019; Marco et al., 2017) (Chapter 3, this thesis). Fermented foods are believed to benefit human health through nutritive alteration of raw ingredients and the biosynthesis of bioactive compounds, modification of the human gut microbiota, and development and modification of the immune system (Gänzle, 2020; Marco et al., 2021). Community assembly in spontaneous fermentations are often reproducible at the species level *(e.g. Lp. plantarum* and *Lm. fermentum*) and may include strains for which some jurisdictions, Canada and Italy allow species-specific probiotic claims in fermented food products (Hill et al., 2014). Consequently, based on this definition, in Canada, *mahewu* could be defined as "contains probiotics" as the microbiological analysis shows that large numbers of *Lp. plantarum* and *Lm. fermentum* remain viable until the time of consumption (Chapter 3, this thesis). Outside these jurisdictions, the terms "fermented food" and "probiotics" cannot be used interchangeably, and although *mahewu* and other fermented food products may contain probiotics they can only be labelled as "containing probiotics" when there is evidence that their live microbial components provide health benefits and the specific microbiological strains are defined (Marco et al., 2021). Even though probiotic activity is not documented at the strain level in *mahewu*, live dietary microbes are increasingly recognized as beneficial to health (Marco et al., 2021; Wastyk et al.,

2021) (Chapter3 this thesis). Probiotic fermentation microbiota offer an approach to reducing the incidence of foodborne diseases (FAO/WHO, 2006). Probiotics have been defined as "live microorganisms that, when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014). Probiotics when consumed at appropriate levels reduce the severity and duration of childhood diarrhea (McFarland et al., 2021; Merenstein et al., 2020) and travellers' diarrhea (McFarland and Goh, 2019). Pediatric acute gastroenteritis is a leading cause of morbidity and mortality in children under 5 years old with an annual death toll of 525,000 globally, most cases occurring in developing countries (GBD 2016 Diarrhoeal Disease Collaborators, 2018; WHO, 2017). The factors which influence the incidence and severity of pediatric acute gastroenteritis in different geographic areas include contaminated food and water sources, degree of malnutrition, diet, lifestyle factors, and the type of diarrhea etiologies (GBD 2016 Diarrhoeal Disease Collaborators, 2018; Pswarayi et al., 2014). Bacterial causes of childhood diarrhea include enterotoxigenic *Escherichia coli* (ETEC) and *Shigella* spp. (Nhampossa et al., 2015; Qadri et al., 2005). Similarly, travellers' diarrhea affects 20–40 million travellers per year, and many of these develop when visiting developing countries (Giddings et al., 2016; Siikamäki et al., 2017). Many of the causes for travellers' diarrhea are similar to those causing acute diarrhea in young children in developing countries, and enterotoxigenic *Escherichia coli* (ETEC) and enteroaggregative *E coli* (EAEC) are the most common bacterial pathogens (Shah et al., 2009).

5.3 Functional or Probiotic Starter Cultures – Practical implications for rural Africa

Mahewu bacterial isolates, *Lm. fermentum* FUA3588 and *Lp. plantarum* FUA3590 were found to be suitable for selection as potential starter cultures as they outcompeted *Enterobacteriaceae* during the model *mahewu* fermentations (Chapter 3, this thesis). This study demonstrated that the use of starter cultures results in better process control, enhanced food safety due to the reduction

of hygienic risks. The functional properties of these two lactobacilli strains need to be determined. However, they could be used with functional starter cultures as co-cultures in fermentation processes to help to achieve *in situ* expression of the desired property, maintaining a perfectly natural and healthy product (Leroy and De Vuyst, 2004). Functional starter cultures with health-promoting properties derived from traditional fermented beverages offer a possible way to contribute to the development of small and medium sized enterprises in rural communities in developing countries on the one hand, and may also offer product diversification of large companies in developed countries on the other hand.

Traditional fermented cereal beverages may allow delivery of probiotics to rural communities in developing countries and help to prevent the incidence and severity of childhood diarrhea. Yoba, a probiotic formulation containing *Lacticaseibacillus rhamnosus* GG and *Streptococcus thermophilus* has been successfully validated as a means to produce fermented milk products in a controlled manner in rural areas in Uganda (Kort et al., 2015) and Zimbabwe (Mpofu et al., 2014). Apart from the improved quality, the use of starter cultures for controlled fermentation prevents spoilage and results in reduced food losses. The generic probiotic *Lb rhamnosus* yoba 2012 could be used to facilitate local and low-cost production of a wide range of fermented foods that subsequently act as delivery vehicles for beneficial bacteria to communities in Africa (Kort et al., 2015). The introduction of the starter cultures to produce small scale industrial *mahewu* in rural trading centers will present healthier alternatives of beverages to buy and consume. However, traditional fermented cereal foods will continue to be produced at the household level for subsistence farmers as it is a cheaper alternative without any cost to them. The freeze-dried strains stored in moisture-proof sachets remain active over a period of at least 2 years (Mpofu et al., 2016). and can be stored in adjacent commercial areas for disbursement to rural communities or they can

be stored at room temperature for three months (unpublished data Mpofu 2016) This initiative is a practical and alternate way to provide highly nutritious, health-promoting fermented food to people in developing countries who have no access to probiotics.

Mutandabota is traditionally made by mixing raw cow's or goat's milk with dried baobab fruit pulp and at a low pH of 3.4 still contained *L. monocytogenes, E. coli O157:H7 and Salmonella* spp. whereas in yoba *mutandabota* fermented with *Lb rhamnosus* yoba no pathogens were detected (Mpofu et al., 2016). This demonstrates the importance of fermentation which improves the food safety characteristics of traditional fermented beverages in rural communities. The use of starter cultures reduces the fermentation time through rapid acidification and growth of the inoculum (Sekwati-Monang and Gänzle, 2011). Furthermore, the nutritional properties of traditional milk fermented with *Lb. rhamnosus* yoba were improved with a three-fold increase in thiamine (Kort et al., 2015). Another application uses probiotic strains in the fermentation of cooked oatmeal and oat bran for the production of Yosa, a yogurt-type alternative to dairy- and soy-based yogurts (Gänzle and Salovaara, 2019; Tuorila et al., 1998).

5.4 Unique exopolysaccharide operon

A putative exopolysaccharide (EPS) gene cluster was identified in the genome of *mahewu* lactic acid bacterial isolate *Lm. fermentum* FUA3588. BLAST analysis against the NCBI database revealed that 7 genes at the 5′ end of the cluster are unique to *Lm. fermentum* FUA3588. This region codes for 3 hypothetical proteins and 4 putative glycosyltransferases. Characterization of the exopolysaccharide produced by *Lm. fermentum* FUA3588 and the role of the EPS gene cluster with novel genes is required to determine the probiotic potential of this strain. Therefore, it is possible that the exopolysaccharide may have probiotic properties that may confer potential health benefits to the consumer (Hill et al., 2014).

Diarrheal diseases are a major public health problem in Zimbabwe, primarily because of the lack infrastructure for the delivery of safe water and food (Mason, 2009; Pswarayi et al., 2014). Sanitary intervention strategies which are known to be potentially effective are difficult to implement in economically challenged societies (Guandalini, 2011). Therefore, the use of probiotics is a possible complementary approach. In randomized clinical trials to determine the efficacy of probiotics against diarrhea, the most important outcome is the duration of diarrhea for therapeutic studies and the incidence of episodes of diarrhea for prevention studies (Guarino et al., 2015). Irrespective of the definition of diarrhea used, there is evidence that probiotics reduce both clinical outcomes. The two common general benefits often associated with probiotics are supporting a healthy digestive tract or a healthy gut microbiota and a healthy immune system (Hill et al., 2014). The mechanisms of the reduction of diarrhea by probiotics are poorly understood but are likely to involve competitive exclusion of pathogens and interaction of the probiotic strains with the host immune systems (Ryan et al., 2015). In addition, the synthesis of exopolysaccharides (EPS) during fermentation by lactobacilli may offer specific protection against diarrheal pathogens (Chen et al., 2014). It has been hypothesized that EPS support biofilm formation, have immunomodulatory activity and protect against environmental stress (Kitazawa et al., 1998; Nagai et al., 2011).

5.5 Safety considerations of starter cultures isolated from *mahewu*

The hypothesis that genomes of plant-associated lactobacilli in *mahewu* encode for antimicrobial resistance that aid in defense against plant-secondary metabolites was not conclusively supported (Chapter 4, this thesis). Comparative genomic analyses indicated that all seven *mahewu* isolates harbored multiple MATE and MFS MDR proteins. Strains of *Lactiplantibacillus plantarum* and *Limosilactobacillus fermentum* encoded for the same gene, termed *mahewu* phenolics resistance gene *mprA,* with more than 99% nucleotide identity, suggesting horizontal gene transfer. Strains of *Lp. plantarum* were more resistant than strains of *Lm. fermentum* to phenolic acids, other

antimicrobials and antibiotics but the origins of strains were not related to resistance. The resistance of several strains exceeded EFSA thresholds for several antibiotics. Analysis of gene expression in one strain each of *Lp. plantarum* and *Lm. fermentum* revealed that at least one MDR gene in each strain was over-expressed during growth in wheat, sorghum and finger millet sourdoughs relative to growth in MRS5 broth. In addition, both strains over-expressed a phenolic acid reductase. The results suggest that diverse lactobacilli in *mahewu* share MDR transporters acquired by lateral gene transfer, and that these transporters mediate resistance to secondary plant metabolites and antibiotics (Chapter 4, this thesis).

The occurrence of *Enterococcus* species in the microbiotas of finger millet malt and the initial stages of *mahewu* fermentations (Chapter 3, this thesis), provide an opportunity for gene transfer between lactobacilli and opportunistic pathogens. Although enterococci occur in high numbers in fermented dairy and meat products, they are not added as starter cultures because some strains are resistant to many antibiotics and contain virulence factors (Franz et al., 2011). Lactobacilli however, are generally considered to be safe and nonpathogenic and are used in a wide variety of foods and products for humans and animals. Lactic acid bacteria are among the predominant microbial species in African traditional fermented cereal foods therefore, there is a lot of potential for their applications in the food and biotechnology industries (Franz et al., 2014; Todorov and Holzapfel, 2015). However, there are very few studies on the antimicrobial resistance of lactobacilli from African fermented cereal foods, although numerous studies have described antimicrobial susceptibility profiles of bacteria from the developed world (Rychen et al., 2018).

Most of the strains of *Lm. fermentum* and *Lp. plantarum* isolated from cereal sourdoughs examined in this study (Chapter 4, this thesis) had antibiotic resistance levels which were equal to or exceeded the breakpoints recommended by the EFSA (EFSA, 2012; Rychen et al., 2018). Isolates

from sorghum and finger millet sourdoughs were similar in terms of antimicrobial resistance (AMR) as European or North American sourdough isolates. There was no link between country of origin and MICs of the strains of *Lm*. *fermentum* and *Lp*. *plantarum*. Likewise, the minimum inhibitory concentrations (MICs) of strains of *Lp. plantarum* and *Lm. fermentum* from several African fermented foods were similar and the differences were most likely due to the different protocols and media that were used (Adimpong et al., 2012). It is very difficult to compare MIC data from this study (Chapter 4, this thesis) with EFSA break points because there is no standardized methodology and reference strains for which literature data is available. It has been suggested that these cutoff values should be reexamined in light of the genetic basis for resistance of lactobacilli entering the food chain as starter cultures, food preservatives, or probiotics (Campedelli et al., 2019). It is also conceivable that the existence of unknown resistance genes would make verification of the observed phenotypic resistance at the genetic level difficult (Hummel et al., 2007). Nonetheless, the EFSA guidelines are very important because it is the only jurisdiction which provides guidance for AMR, with threshold values that have been published by a regulatory agency.

5.6 Do food fermenting lactobacilli contribute to the spread of antimicrobial resistance?

The food production sector is linked with human, animal and environmental sources of antimicrobial resistance in a cyclical manner. These antibiotic resistant bacteria and antibiotic resistant genes and antimicrobial substances are introduced to animal- and plant-based food production environments, mostly through fecal waste of both human and animal origin (Koutsoumanis et al., 2021). A decrease in sanitation and hygiene standards increases the risk of transmission between the environment, animals and humans, which is a driver of AMR prevalence in human infections (Koutsoumanis et al., 2021). Moreover, in low- and middle-income countries,

antimicrobial use is not the main driver of AMR, instead contagion (i.e. transmission) is regarded as the most important factor (Collignon et al., 2018). In rural communities in Zimbabwe, manure is used to fertilize the crops; however, studies on manure did not evaluate the AMR of the pathogens that were isolated (Center for Disease Dynamics, 2017). According to expert knowledge, manure, irrigation and surface water are major sources and transmission routes of contamination for plant-based foods (Koutsoumanis et al., 2021). The One Health challenges of antimicrobial resistance are particularly striking in Zimbabwe as in other low- and middle-income countries, because of the disproportionately high burdens of infectious disease, alongside rural livelihoods where there are frequent interactions between people and livestock (Caudell et al., 2020; Center for Disease Dynamics, 2017). Transmission of AMR across people, animals and the environment has been demonstrated by genotypic studies. In Tanzania for example, genotypic similarity was observed between resistant enteric bacteria in people, cattle and wild animals and the environment (i.e., waters sources) (Katakweba et al., 2015).

Multivariate studies have shown that intervention efforts to limit AMR should be tailored to regional, country and local realities. The comparison of AMR levels across countries globally allowed for the estimation of the importance of contagion because low income is typically associated with poor water quality and sanitation, overcrowded housing, and inadequate practices to prevent infection (Collignon et al., 2018). Multivariate analysis also showed that better infrastructure and governance were significantly associated with lower measures of antimicrobial resistance, but that antibiotic consumption was not significantly associated with higher antimicrobial resistance. In Europe, poor governance and corruption were shown to be as closely associated with differences in antimicrobial resistance levels between countries as were antibiotic consumption patterns (Collignon et al., 2015).

Reduction of antimicrobial usage may not be sufficient to control antimicrobial resistance because contagion - the spread of resistant strains and resistance genes - seems to be the dominant factor (Collignon et al., 2018). There was a correlation between usage of antimicrobials and AMR in human populations only when there are high levels of sanitation, as is the case in high-income countries and therefore, low levels of environmental transmission occur (Collignon et al., 2018). They also report that inversely, as opportunities for transmission increase the significance of antimicrobial use for AMR decreases as is the case for low- and middle-income countries. Nonetheless, there are many other factors that probably contribute to AMR in different countries, regions and communities. Therefore, concurrent measures to improve sanitation, infection control and prevention, access to clean water, governance, and public expenditure on health-care need to be addressed to reduce global antimicrobial resistance (Collignon et al., 2018).

Nevertheless, it can be concluded that lactobacilli starter cultures isolated from *mahewu* are presumed safe to use, for the following reasons: (i) most antimicrobial resistance genes are in the core genome, and they are therefore not transmissible. (ii) The contribution of plasmid-encoded and transmissible *mpr* genes and their contribution to AMR remains to be demonstrated. This study did not establish a connection between *mpr* genes and AMR. (iii) No *tetW* resistance genes were detected in all six genome-sequenced strains that were used in the study. (iv) These species have a history of safe use for thousands of years. The origins and transfer routes of AMR in food-producing environments are complex. Therefore, it can be hypothesized that it is probable that food fermenting lactobacilli do not contribute to the spread of AMR.

5.7 Future Trends – Fermentation control vs diversity

It has been reported that the microbial species diversity of the sourdoughs was influenced by the house microbiota of the producer, such that when the producer used different flours, the

sourdoughs harbored similar microbial communities, independent of the flour used (Comasio et al., 2020). Therefore, since traditional fermented foods are produced at the household level, it can be theorized that there is a limitless amount of potential probiotic and functional lactobacilli starter cultures that have not yet been discovered and that could have enormous potential for prophylactic and therapeutic use in resource poor countries. The presence of opportunistic pathogens in finger millet malt and potentially in *mahewu* emphasizes the importance of using functional starter cultures to prevent pathogen propagation, however, the diversity of fermentation microbiota would be reduced. Thus, the loss of diversity may potentially result in the loss of hitherto undiscovered functional and probiotic strains of lactobacilli in traditional fermented cereal foods.

The production of a functional fermented cereal beverage that reduces the incidence and severity of childhood diarrhea would be the ultimate application of functional or probiotic starter cultures from African traditional fermented cereal beverages. Novel insights into the metabolism of lactic acid bacteria isolated from *mahewu* offer perspectives for the application of functional starter cultures that may offer several health, marketing, and technological advantages. Novel starter cultures are continually in demand for the development of ever-expanding personal options required by markets globally including new gluten-free fermented beverages, beverages with high nutrition and health benefits, and beverages without any additives (Waters et al., 2015)

This thesis describes factors promoting the exploration of locally sourced functional or probiotic lactobacilli from *mahewu*, targeting local populations to address local dietary needs and market opportunities. This would be particularly beneficial for rural communities in Zimbabwe and other developing countries, where people generally lack access to affordable probiotics and are often exposed to poor hygienic conditions, unsafe water, malnutrition, and chronic diarrheal enteric infections.

5.8 Limitations and recommendations

Although this study provided significant insight into the microbiological and biochemical characterization of *mahewu* fermentations, there are several limitations that it is hoped can be addressed in future studies. The study included a modest number of samples; 5 *mahewu* samples and 4 finger millet samples, due to logistical challenges of sample collection in a rural village in Zimbabwe, which limits statistical power and presents challenges with regard to generalizability of the results. Furthermore, on account of the huge distance between rural Zimbabwe and Canada, the microbiological and biochemical characterization of the *mahewu* samples was performed after 72 h, which is beyond the product shelf life. This, therefore, resulted in greater concentrations of ethanol, acetic acid and glycerol in the *mahewu* samples due to the greater concentration of yeasts, which are typically observed only at the late stages of spontaneous cereal fermentations indicating late spoilage by yeasts or acetic acid bacteria (Gänzle, 2015; Gobbetti et al., 2016; Van der Meulen et al., 2007). Further research is required to establish the microbiological and biochemical characterization of *mahewu* fermentations at the point of consumption.

The model *mahewu* study was conducted to determine the fate of *C. sakazakii*, *K. pneumoniae,* and *E. lactis* during fermentation using two strains of lactobacilli, *Lm. fermentum* FUA3588 and *Lp. plantarum* FUA3590. The limitations of this study were that the fate of *Candida glabrata*, an opportunistic pathogen isolated from *mahewu* was not determined in this study. Future studies should assess the role and evolution of *C. glabrata* throughout the competition experiments to evaluate more comprehensively the efficacy of the lactobacilli starter cultures. Likewise, this study did not monitor the presence of *Saccharomyces cerevisiae* throughout the model *mahewu* fermentations. Heterofermentative lactobacilli and *S. cerevisiae* are responsible for ethanol and CO_2 formation in food fermentations. The metabolic activity of yeasts is also important in the

production of diverse flavor compounds, nutritional and safety advantages (Dzialo et al., 2017). Therefore, the evolution and role of *S. cerevisiae* in *mahewu* fermentations should be considered for future studies.

Characterization of the exopolysaccharide (EPS) produced by *Lm. fermentum* FUA3588 and the role of the EPS gene cluster with novel genes is required to determine the probiotic potential of *Lm fermentum* FUA3588. Isogenic deletion mutants without the EPS gene cluster is an essential verification step to determine their roles and functionality. Further research is needed to determine whether this strain has probiotic potential by evaluating the potential of the exopolysaccharide to prevent adhesion of pathogens to intestinal mucosal cells (Chen et al., 2014). Furthermore, while probiotic immunomodulatory effects are strain-specific (Hill et al., 2014) the exopolysaccharides synthesized by lactobacilli during fermentation can interact with the gut microbiota, the intestinal epithelium or the host immune system and have a beneficial effect (Marco et al., 2021).

All of the *mahewu* phenolics resistance (*mpr*) genes except *mprB* genes were found in the core genome of strains of *Lp. plantarum* (data not shown) and, therefore, these genes are not transmissible. However, plasmid-encoded *mpr* genes and their contribution to antimicrobial resistance remains to be demonstrated. The connection between antimicrobial plant secondary metabolites, MDR transporters, and antimicrobial resistance in lactobacilli was not demonstrated in this study and would need to be confirmed in future studies. Further research is needed to establish the role of *mpr* genes in antimicrobial resistance by constructing insertion mutants which only contain the *mahewu* phenolics resistance (*mpr)* genes.

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Appendices

Appendix A Supplemental material for Chapter 3

Table S3.1 Identification of bacterial species in finger millet malt determined by Illumina sequencing of 16S rRNA gene amplicons

OTU	Genus	Species
OTU_1	*Cronobacter*	*sakazakii/ dublinensis/ muytjensii/ universalis/ malonaticus/ turicensis/ condimenti*
OTU_2	*?*	*?*
OTU_3	*Weissella*	*confusa/ cibaria*
OTU_4	*Enterococcus*	*moraviensis/ italicus/ sulfureus/ termitis/ camelliae/ quebecensis/ plantarum*
OTU_5	*Bacillus*	*subtilis/ mojavensis/ vallismortis/ amyloliquefaciens/ methylotrophicus/ tequilensis*
OTU_6	*Xanthomonas*	*vesicatoria/ codiaei/ arboricola/ cucurbitae/ bromi/ cassavae/ pisi/ vasicola/ hortorum/ fragaria/ oryzae/ populi/ cynarae/ campestris/ phaseoli/ euvesicatoria/ perforans/ gardneri/ citri*
OTU_7	*Chryseobacterium*	*formosense/ molle/ zeae*
OTU_8	*Lactococcus*	*lactis/ taiwanensis*
OTU_9	*Pseudomonas*	*putida/ oryzihabitans/ plecoglossicida/ monteilii/ parafulva/ fulva/ remoricolorata/ entomophila/ mosselii/ taiwanensis/ guariconensis/ soli*
OTU_10	*Aquitalea*	*magnusonii*
OTU_11	*Rhizobium*	*pusense*
OTU_12	*Massilia*	*phenanthrene-degrading bacterium 70-2/ niastensis/ kyonggiensis*
OTU_13	*Acinetobacter*	*parvus/ soli/ puyangensis*
OTU_14	*Paenibacillus*	*Paenibacillus sp. 75H/ hunanensis/ shenyangensis*
OTU_15	*Sphingomonas*	*parapaucimobilis/ pseudosanguinis*
OTU_16	*?*	*Rhodoferax ferrireducens/ Acidovorax temperans*
OTU_17	*Weissella*	*beninensis / ghanensis / fabalis/ fabaria*
OTU_18	*?*	*?*
OTU_19	*Clostridium sensu stricto 1*	*Clostridium sp. FA2/18/ roseum/ diolis*
OTU_20	*Sphingobacterium*	*thalpophilum/ canadense/ pakistanense/ ginsenosidimutans*
OTU_21	*Cellulomonas*	*hominis/ humilata/ denverensis/ terrae/ akistanensis*
OTU_22	*Saccharibacillus*	*macerans/ thermophilus*
OTU_23	*Ruminococcaceae UCG-010*	*?*
OTU_24	*Siphonobacter*	*Siphonobacter sp. MC618/ Siphonobacter aquaeclarae*
OTU_25	*Chryseobacterium*	*daeguense/ gwangjuense/ camelliae/geocarposphaerae*

OTU	Genus	Species
OTU_26	*?*	*?*
OTU_27	*?*	*?*
OTU_28	*Pediococcus*	*pentosaceus*
OTU_29	*Aureimonas*	*Aureimonas ureilytica*
OTU_30	*Vogesella*	*perlucida/ lacus*
OTU_31	*Mucilaginibacter*	*daejeonensis/ soli/ jinjuensis/ polytrichastri*
OTU_32	*Lactobacillus*	*paraplantarum/ pentosus/ plantarum*
OTU_33	*?*	*Glycine max/ Lotus japonicus/ Phaseolus vulgaris*
OTU_34	*Burkholderia-Paraburkholderia*	*ambifaria/ anthina*
OTU_35	*Pedobacter*	*sandarakinus/ rhizosphaerae/ kyungheensis*
OTU_36	*Citrobacter*	*Enterobacter cloacae/ Klebsiella pneumoniae/ Salmonella enterica/ Kosakonia sacchari*
OTU_37	*Saccharibacillus*	*Paenibacillus sp./ Cohnella panacarvi*
OTU_38	*?*	*?*
OTU_39	*Leuconostoc*	*mesenteroides/ pseudomesenteroides/ kimchii/ gelidum/ palmae/ miyukkimchii*
OTU_40	*Clostridium sensu stricto 5*	*amylolyticum*
OTU_41	*?*	*?*
OTU_42	*Roseomonas*	*aerophila/ ludipueritiae*

? means unassigned, a good hit to a particular sequence, but that sequence is rare and is not bacterial 16S rRNA.

Appendix B Supplemental material for Chapter 4

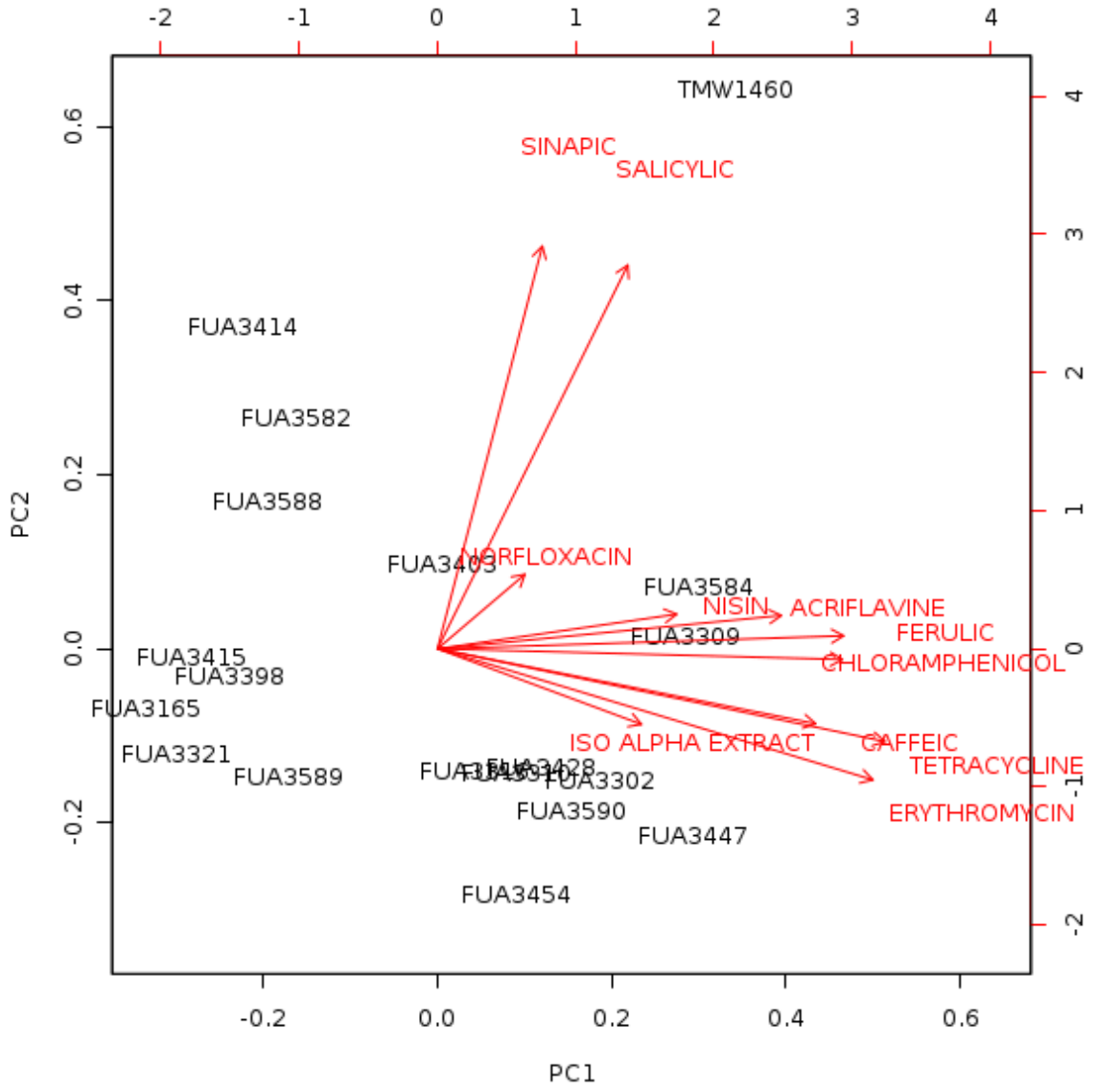

Figure S4.1 PCA Loading plot of the MICs of strains of *Lp. plantarum* and *Lm. fermentum* with 12 antimicrobials.
Results are shown as means of triplicate biological repeats.

Table S4.3 Protein classification of the Mpr proteins from *mahewu* bacterial isolates

			DOMAINS			
Mpr	**Nearest Homolog**	**Family**	**Non-Cytoplasmic**	**Transmembrane**	**Cytoplasmic**	**TM helix**
A	YpnP	MATE	+	+	+	12
B	NS	MFS	+	+	+	2
C	YpnP	MATE	+	+	+	7
D	MepA	MATE	+	+	+	11
E	NS	MATE	+	+	+	4

A plus sign (+) denotes presence

MATE Multi antimicrobial extrusion protein

MFS Major Facilitator Superfamily

TMhelix Transmembrane helix

NS No significant similarity found

Table S4.4 Comparison of the amino acid similarities between the Mpr proteins in *mahewu* bacterial isolates

STRAIN ID	Mpr	MprA	MprB	MprC	MprD	Mpr A_D	MprE
		ID %	ID %	ID %	ID %	ID %	ID %
Lp. plantarum	MprA	Q[a)]		93		100	89
FUA3590	MprB		Q				
	MprC	93		Q		93	
	MprD				Q		
	$MprA_D$	100		93		Q	89
	MprE						Q
Lm. fermentum	MprF						
FUA3588	MprG				46		
	MprH	35		36		35	33
	MprA	98		96		98	87
	$MprA_D$	98		96		98	87
Lm. fermentum	MprI						
FUA3589	MprA	97		95		97	86
	$MprA_D$	96		95		96	85
	MprJ				46		
Lm. fermentum	MprK						
FUA3582	MprL				47		
	MprM						
	MprA	97		96		97	87
Lp. plantarum	MprA	93		97		93	99
FUA3584	MprN				99		
Ff. rossiae	MprO	62		63		62	57
FUA3583	MprP						
W. cibaria	MprQ						
FUA3585	MprR	50		52.5		50	44

[a)] Query sequence.

mprA is *mahewu* phenolics resistance gene

$mprA_D$ is a duplicate *mprA* gene

**mprB* is putative MDR permease, possible multidrug efflux pump

Shown are the amino acid comparisons with > 75% query cover

Table S4.5 Comparison of the nucleotide similarities between the *mpr* genes in *mahewu* bacterial isolates

Organism	*mpr*	*mprA* ID %	*mprB* ID %	*mprC* ID %	*mprD* ID %	*mprA*$_D$ ID %	*mprE* ID %
Lp. plantarum FUA3590	*A*						
	*B**						
	C						
	D						
	A$_D$						
	E						
Lm. fermentum FUA3588	*F*						
	G						
	H						
	A						
	A$_D$						
Lm. fermentum FUA3589	*I*						
	A						
	A$_D$						
	J						
Lm. fermentum FUA3582	*K*						
	L						
	M						
	A						
Lp. plantarum FUA3584	*A*						
	N						
Ff. rossiae FUA3583	*O*						
	P						
W. cibaria FUA 3585	*Q*						
	R						

	Query
	100 %
	95 – 99 %
	80 – 94 %
	No significant similarity found

mprA is *mahewu* phenolics resistance gene; *mprA*$_D$ is a duplicate *mprA* gene; **mprB* is putative MDR permease, possible multidrug efflux pump

Appendix C List of online supplemental material for Chapter 4

Table S4.1: Genome Accession Numbers

https://doi.org/10.1016/j.fm.2021.103917

Table S4.2 Phenolic Acid Enzymes

https://doi.org/10.1016/j.fm.2021.103917

Table S4.6 MIC Comparisons

https://doi.org/10.1016/j.fm.2021.103917

Table S4.7 Comparison of CARD Genes

https://doi.org/10.1016/j.fm.2021.103917