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

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Abstract

Cereal-associated lactobacilli resist antimicrobial plant secondary metabolites. This study aimed to identify multi-drug-resistance (MDR) transporters in isolates from *mahewu*, a Zimbabwean fermented cereal beverage, and to determine whether these MDR-transporters relate to resistance against phenolic compounds and antibiotics. 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 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.

Key words: mahewu, millet, phenolic acid resistance, antimicrobial resistance, *Lactiplantibacillus plantarum, Limosilactobacillus fermentum, Lactobacillus*.

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 MATE or 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 glycosides, condensed tannins and 3-desoxyanthocyanidins (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.

2 Materials and Methods

2.1 Bacterial strains and growth conditions.

Bacterial strains used in this study and their origin are shown in Table 1. Strains were cultured from -80°C stock and grown in MRS5 medium (Meroth et al., 2003) at 25°C and 30°C under microaerophilic conditions.

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, Wisconsin, 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, Delaware, 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 (Lin and Gänzle, 2014) with group specific primers (Walter et al., 2001, Table 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).

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 S1). 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 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).

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 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 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.

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) (McArthur et al., 2013) 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.

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, Ontario, Canada) were used with a stock concentration of 20 gL^{-1}. (ii) The antibiotics acriflavine, erythromycin, chloramphenicol, norfloxacin, tetracycline, streptomycin, nisin (all Sigma-Aldrich, Oakville, Ontario, 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 (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, 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, 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. Principal Component Analysis was performed using METAGENassist (Arndt et al., 2012).

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

To determine which MDR transporters and genes coding for phenolic acid metabolism are expressed during growth in 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 S2).

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 (Pswarayi and Gänzle, 2019) 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 (Teixeira et al., 2014) and RNA was extracted using RNA protect Bacteria Reagent and RNeasy Minikit (Qiagen, USA) prior to DNAase treatment with RQ1 RNase-Free DNase Kit (Promega, Madison, USA) to eliminate residual DNA. RNeasy PowerClean Pro Cleanup Kit (Qiagen, USA) was used to clean isolated RNA from the sorghum and 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, Massachusetts, USA) prior to reverse transcription to cDNA using QuantiTect Reverse Transcription Kit (Qiagen, USA). Specific primers targeting *mpr* genes and phenolic acid enzymes (Table 2) were used for qPCR amplification, which was performed using the QuantiFast SYBR green master mixture (Qiagen) in a 7500 Fast Real Time-PCR System (Applied Biosystems, 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} 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% ($P<0.05$).

3 Results

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 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 3 and Table S3). 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 3 and Table S4). Genes coding for the same proteins in *Lp. plantarum* and *Lm. fermentum* were more than 95% identical (Table S5). Close homologues of *mprA* and *mprB* with more than 99% sequence identity were also identified in pediococci and in *Liquorilactobacillus mali* (Table 3). Some of these homologues in other genera of lactobacilli

219 are plasmid encoded (Table 3), however, *in silico* analysis indicated that *mpr* genes in *mahewu*
220 isolates were all chromosomally encoded. With the exception of *mprA* in *Lp. plantarum* FUA3584,
221 the G + C content of *mprA* in *Lm. fermentum* and *Lp. plantarum* is comparable to each other but
222 differs from the G + C content of the respective genomes (Figure 1). In strains of *Lm. fermentum*,
223 the *mprA* genes are flanked by mobile genetic elements. The mobile genetic elements in *mahewu*
224 isolates *Lm. fermentum* are more than 99% identical to each other and to mobile genetic elements
225 in *Lp. plantarum* and *Oenococcus oeni* (Table 4). Taken together, these results suggest recent
226 horizontal gene transfer of the *mpr* genes between *Lp. plantarum*, *Lm. fermentum* and other
227 lactobacilli.

228 In addition to the seven genome sequenced isolates from *mahewu*, six strains of *Lp. plantarum* and
229 *Lm. fermentum* were obtained from the same batches but not genome sequenced. The identification
230 of *mpr* genes in these strains was performed with PCR. PCR detection matched the detection by
231 genome analysis in all cases (Table 5). The genes coding for *mprA* or $mprA_D$ were identified in all
232 strains of *Lm. fermentum* and *Lp. plantarum* (Table 5).

233 **3.2 Inhibitory activity of antimicrobials and phenolic acids**

234 If MDR genes are shared between taxonomically diverse lactobacilli that occur in cereal
235 fermentations, the likely function is to increase the ecological fitness of the organisms but *in silico*
236 analyses did not provide an indication of the function of the Mpr proteins. To assess the inhibitory
237 activity of antimicrobial compounds, the MIC of different antibiotics, phenolic acids, an
238 isomerized hop extract and nisin against strains of *Lm. fermentum* and *Lp. plantarum* were
239 determined. In addition to the *mahewu* isolates, strains isolates from sourdough, ting, and
240 fermented wheat bran were included in the analyses. The MICs of the 12 antimicrobial compounds
241 against the 19 strains of *Lm. fermentum* and *Lp. plantarum* are shown in Table S6 of the online

supplementary material. PCA analysis of the data revealed that the resistance between *Lm. fermentum* and *Lp. plantarum* strains differed (Fig. 2A) with the *Lp. plantarum* strains having an overall higher resistance (Table S6). 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 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 S6). The two *mahewu* isolates *Lp. plantarum* FUA3590 and FUA3584 were most resistant to caffeic (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 S6). 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 S6). The loading plot indicated that resistance to antibiotics and the resistance to nisin, hops, ferulic and caffeic acid were highly correlated (Fig. S1), suggesting that similar mechanisms account for resistance to antibiotics, nisin, and plant secondary metabolites.

3.3 Expression of *mpr* genes during growth in 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% 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 genes *mpr* genes during growth in cereal substrates (Figure 3A); of the five, *mprB* and *mprD* were overexpressed in one or more of the cereal substrates (Figure 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 3B).

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 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 4A), while in *Lm. fermentum* FUA3582 *hcrF* was differentially overexpressed in both *mahewu* and sorghum sourdoughs (Figure 4B). The genes encoding for phenolic acid decarboxylase activity were not overexpressed in *Lp.*

plantarum FUA3590 or in *Lm. fermentum* FUA3582 (Figure 4). The gene for esterase *estF* in *Lm. fermentum* FUA3582 was overexpressed in *mahewu* (Figure 4B).

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 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 millet malt that is used in *mahewu* production, this was documented by strain-specific qPCR for two of the isolates (Pswarayi and Gänzle, 2019). We therefore considered the possibility that isolates from different cereals exhibit a different complement of genes coding for MDR transporters, or differ in their phenotypic resistance. 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, millet, or sorghum, did not differ in their resistance. Resistance and antimicrobial resistance genes are either shared by most or all strains of one species, or generally relates to adaptation of lactobacilli to cereals or plants.

Our 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 analysed 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 of 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^{-1} 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 mgL^{-1} 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, sorghum and millet 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 S6) (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 S7). 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 (Pswarayi and Gänzle, 2019) and the misuse of antibiotics is also considered 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 millet malt and the initial stages of *mahewu* fermentations also include *Enterococcus*, *Klebsiella* and *Cronobacter* species (Pswarayi and Gänzle, 2019), 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 of 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 S7). 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, our 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, our 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., 2015). (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.

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Figure legends

Figure 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.

Figure 2. Principle 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.

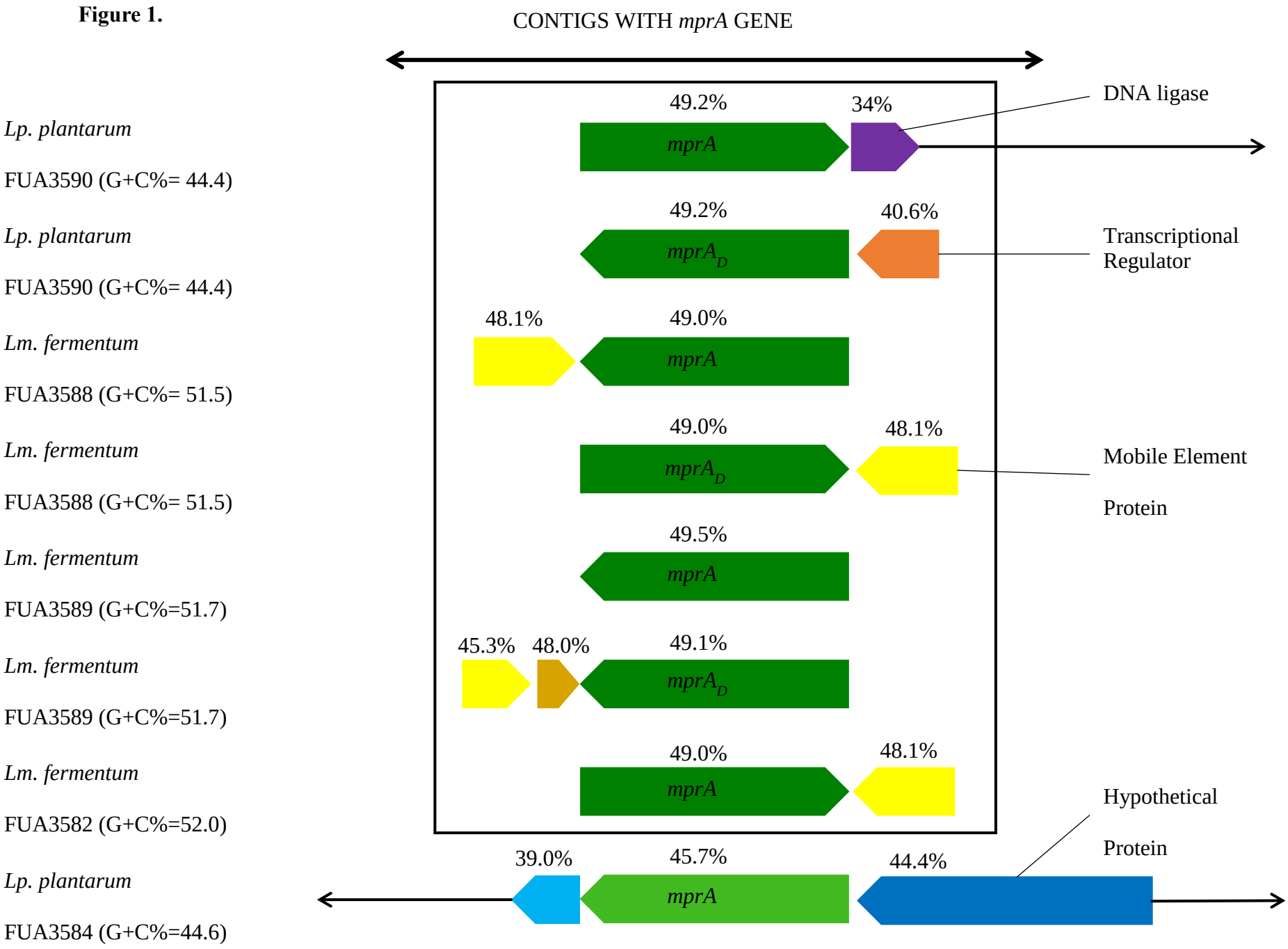
Figure 3. Expression of *mpr* genes during growth in sorghum, millet and wheat sourdoughs relative to the expression of the same genes during growth in MRS5 broth. **Panel A.** *Lp. plantarum* FUA3590; **Panel B.** *Lm. fermentum* FUA3582. Substrates and incubation conditions are color-coded as follows: 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.

764 Genes that were differentially expressed ($p < 0.05$) relative to expression by the same strain at
765 reference conditions are marked with an asterisk.

766 **Figure 4.** Expression of genes coding for enzymes of phenolic acid metabolism in *Lp. plantarum*
767 FUA3590 (**Panel A)** and *Lm. fermentum* FUA3582 (**Panel B)** during growth in sorghum and millet
768 sourdoughs relative to the expression of the same genes during growth in MRS5 broth. Substrates
769 and incubation conditions are color-coded as follows: Red bars, *mahewu* fermented at 25°C; green
770 bars, sorghum cultivar Town at 25°C. Results are shown as log 2 transformed means ± standard
771 error of triplicate biological repeats, each sample was analyzed in technical duplicates. An asterisk
772 indicates that a gene is significantly overexpressed ($P<0.05$) relative to its expression at the
773 reference conditions. Genes are as follows: *pad*, phenolic acid decarboxylase; *hcrB*, phenolic acid
774 reductase (*Lp. plantarum*); *hcrF*, phenolic acid reductase (*Lm. fermentum*); *estP*, carboxylesterase
775 (*Lp. plantarum*); *estF*, esterase (*Lm. fermentum*); *tanB*, tannase (*Lp. plantarum*).

776

Figure 1.


CONTIGS WITH *mprA* GENE
Lp. plantarum
FUA3590 (G+C%= 44.4)
Lp. plantarum
FUA3590 (G+C%= 44.4)
Lm. fermentum
FUA3588 (G+C%= 51.5)
Lm. fermentum
FUA3588 (G+C%= 51.5)
Lm. fermentum
FUA3589 (G+C%=51.7)
Lm. fermentum
FUA3589 (G+C%=51.7)
Lm. fermentum
FUA3582 (G+C%=52.0)
Lp. plantarum
FUA3584 (G+C%=44.6)
49.2%
34%
mprA
DNA ligase
49.2%
40.6%
mprA$_D$
Transcriptional Regulator
48.1%
49.0%
mprA
49.0%
48.1%
mprA$_D$
Mobile Element Protein
49.5%
mprA
45.3%
48.0%
49.1%
mprA$_D$
49.0%
48.1%
mprA
39.0%
45.7%
44.4%
mprA
Hypothetical Protein

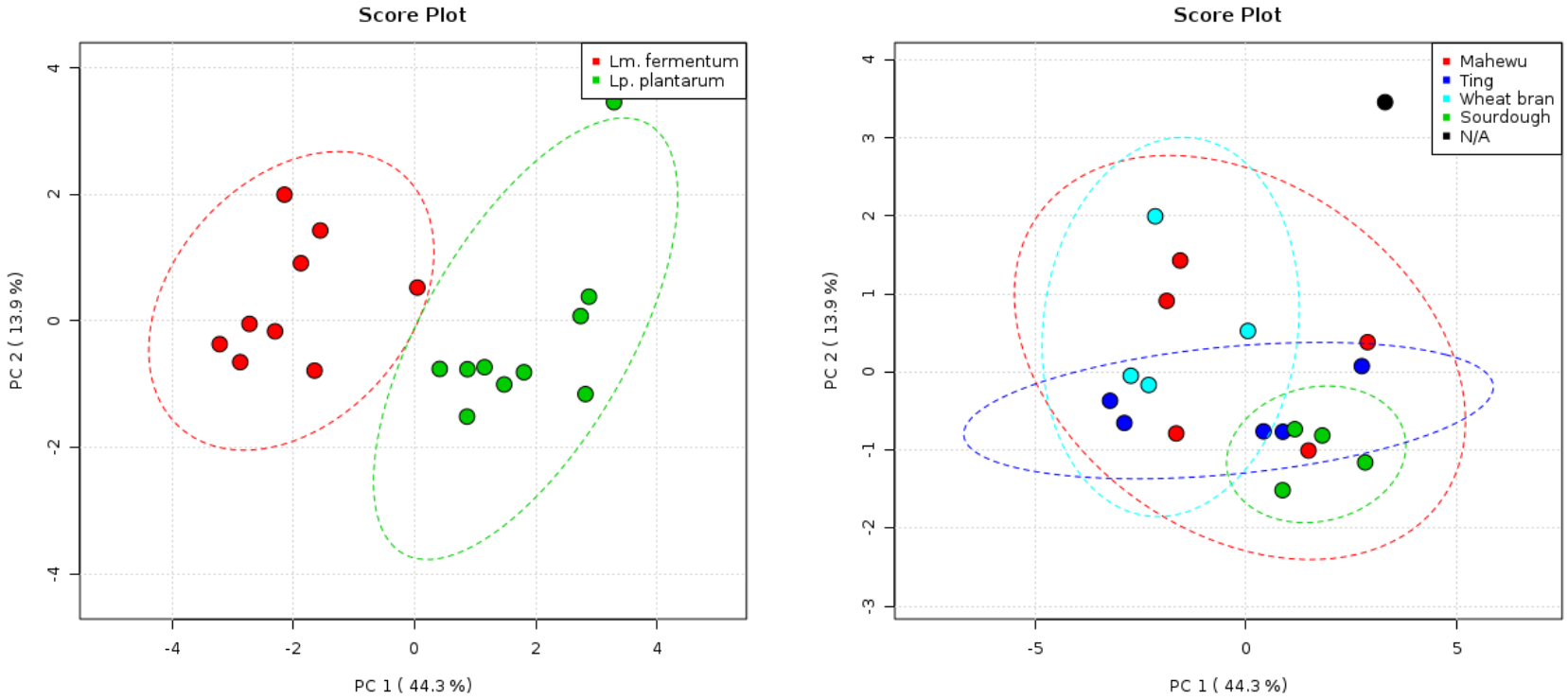

Score Plot
Lm. fermentum
Lp. plantarum
PC 2 (13.9 %)
PC 1 (44.3 %)
Score Plot
Mahewu
Ting
Wheat bran
Sourdough
N/A
PC 2 (13.9 %)
PC 1 (44.3 %)

Figure 2

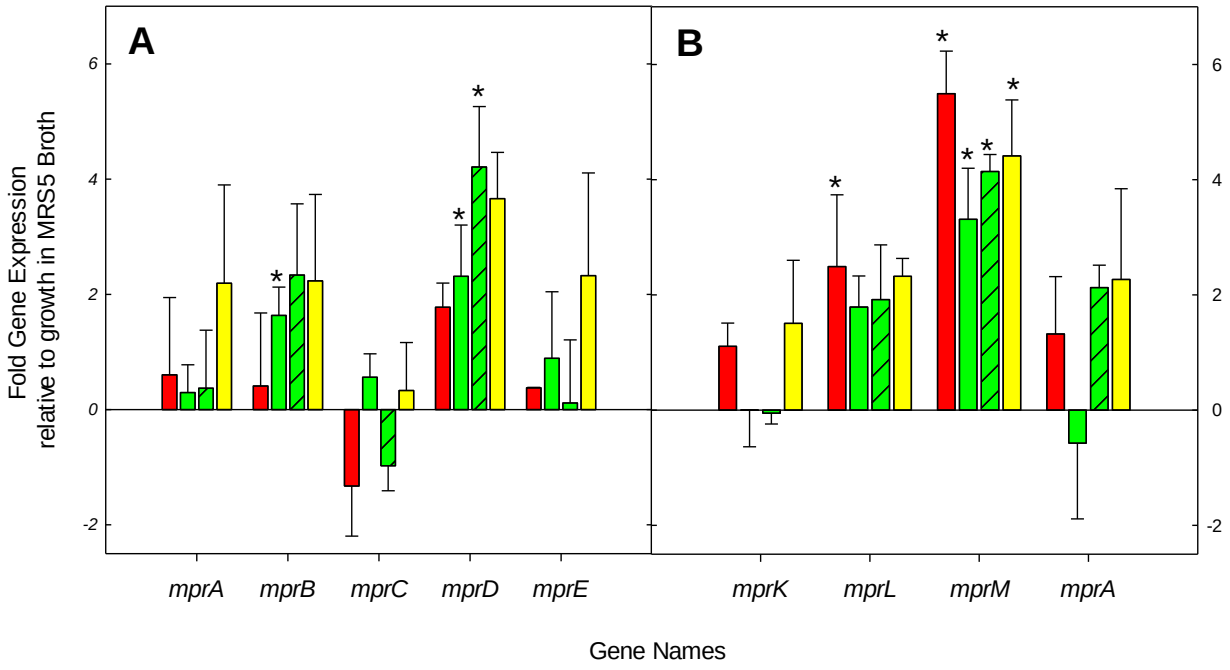

A
B
Fold Gene Expression
relative to growth in MRS5 Broth
6
4
2
0
-2
*
mprA
mprB
mprC
mprD
mprE
mprK
mprL
mprM
mprA
Gene Names

Figure 3

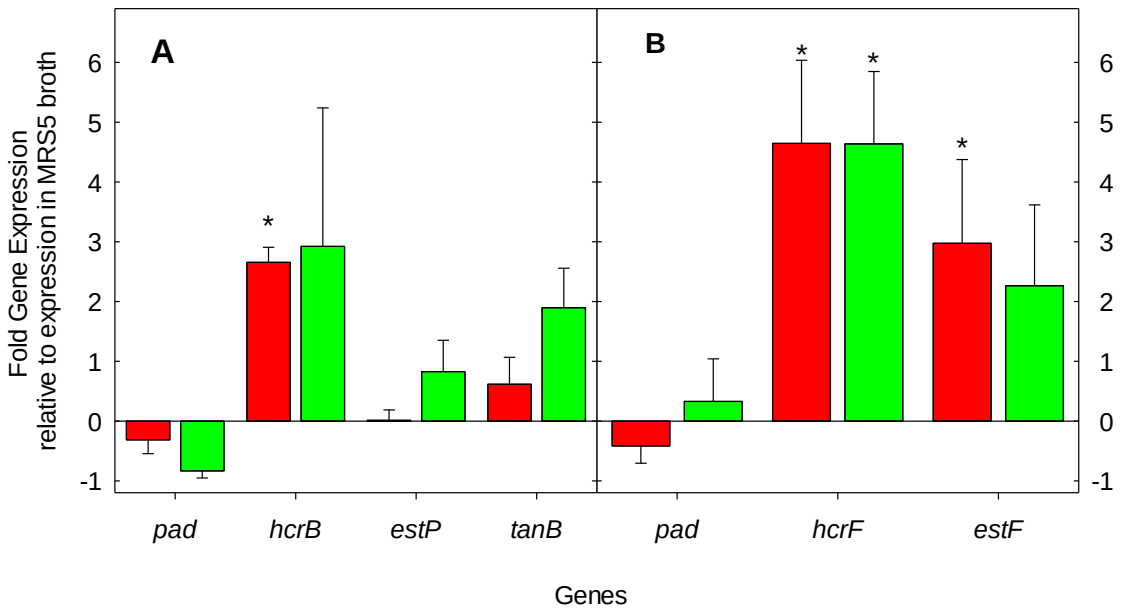

A
B
Fold Gene Expression
relative to expression in MRS5 broth
6
5
4
3
2
1
0
-1
*
*
*
*
pad
hcrB
estP
tanB
pad
hcrF
estF
Genes

Table 1. Bacterial strains used in this study and their origin

Species	Strain ID	Genome accession number
Mahewu (Pswarayi and Gänzle, 2019)		
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

n/a, genome sequence is not available.

Table 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		
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)

Table 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

Table 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 5. PCR detection of *mpr* genes in *mahewu* isolates

		Gene Name				
Species	**Strain ID**	***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.

Online supplementary material to

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

Felicitas Pswarayi, Nanzhen Qiao, Gautam Gaur and Michael Gänzle

University of Alberta, Department of Agricultural, Food and Nutritional Science, Edmonton, Canada.

Figure S1. 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 S3. Protein classification of the Mpr proteins from mahewu bacterial isolates

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

Table S5. Comparison of the nucleotide similarities between the *mpr* genes in mahewu bacterial isolates

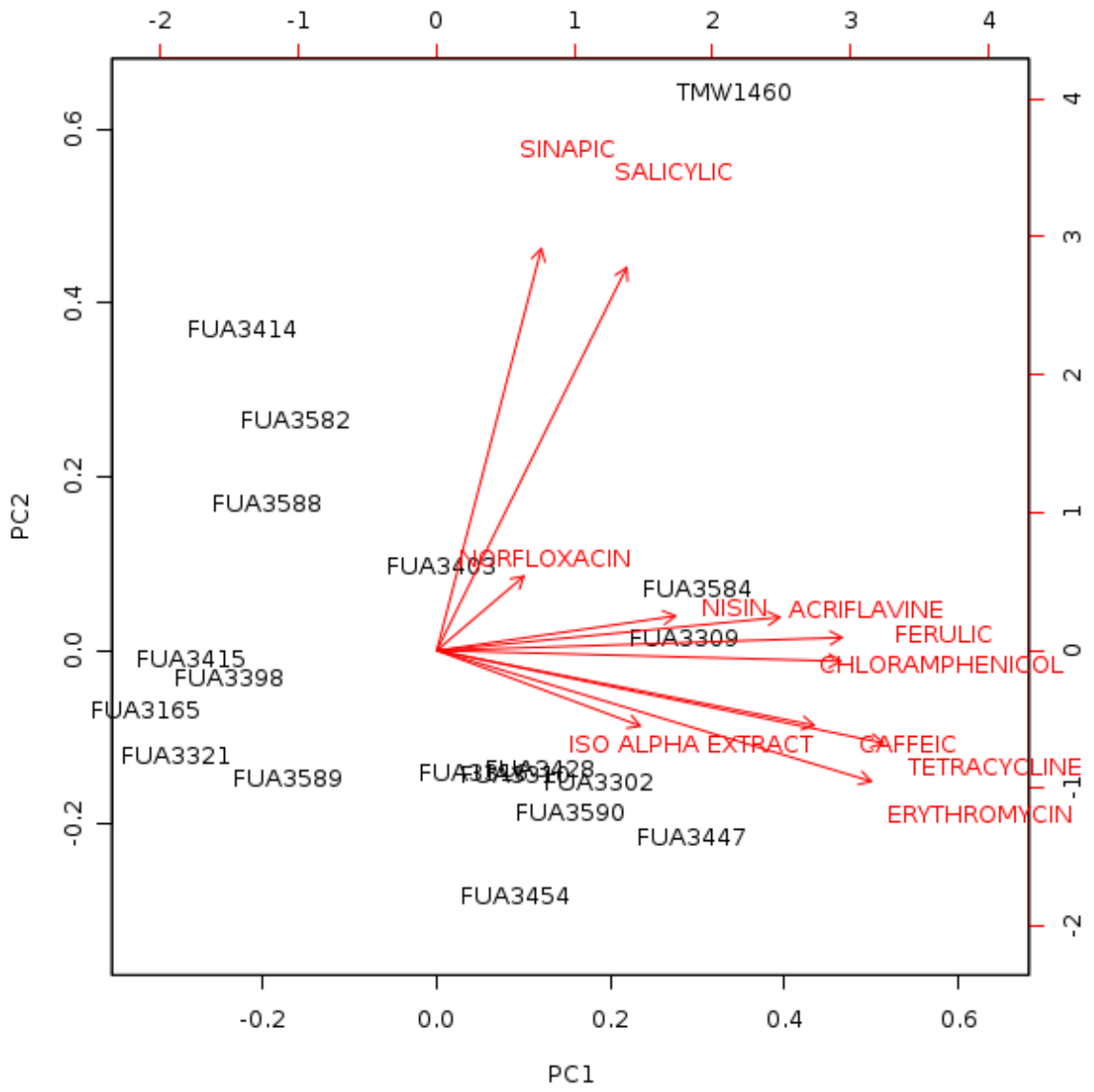


Figure S1. Loading plot of the MICs of strains of *Lp. plantarum* and *Lm. fermentum* with 12 antimicrobials. Results are shown as means of triplicate biological repeats.

Table S3. Protein classification of the Mpr proteins from mahewu bacterial isolates

			DOMAINS			
Mpr	**Nearest Homolog**	**Family**	**Non-Cytoplasmic**	**Transmembrane**	**Cytoplasmic**	**TMhelix**
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. 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 S5. 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