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Research Paper

Antibiotic Susceptibility Profiles of *Pediococcus pentosaceus* from Various Origins and Their Implications for the Safety Assessment of Strains with Food-Technology Applications

NOAM SHANI,[1]* SIMONE OBERHAENSLI,[2] AND EMMANUELLE ARIAS-ROTH[3]

[1]*Competence Division Method Development and Analytics and* [3]*Research Division Food Microbial Systems, Agroscope, 3003 Bern, Switzerland (ORCID: https://orcid.org/0000-0002-3570-9947 [N.S.]); and* [2]*Interfaculty Bioinformatics Unit and SIB Swiss Institute of Bioinformatics, University of Bern, 3012 Bern, Switzerland*

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ABSTRACT

In the fight against the spread of antibiotic resistance, authorities usually require that strains "intentionally added into the food chain" be tested for their antibiotic susceptibility. This applies to strains used in starter or adjunct cultures for the production of fermented foods, such as many strains of *Pediococcus pentosaceus*. The European Food Safety Authority recommends testing strains for their antibiotic susceptibility based on both genomic and phenotypic approaches. Furthermore, it proposes a set of antibiotics to assess as well as a list of microbiological cutoffs (MCs), allowing classification of lactic acid bacteria as susceptible or resistant. Accurate MCs are essential not only to avoid false-negative strains, which may carry antibiotic resistance genes and remain unnoticed, but also to avoid false-positive strains, which may be discarded while screening potential candidates for food-technology applications. Because of relatively scarce data, MCs have been defined for the whole *Pediococcus* genus, although differences between species should be expected. In this study, we investigated the antibiotic susceptibility of 35 strains of *P. pentosaceus* isolated from various matrices in the past 70 yr. MICs were determined using a standard protocol, and MIC distributions were established. Phenotypic analyses were complemented with genome sequencing and by seeking known antibiotic resistance genes. The genomes of all the strains were free of known antibiotic resistance genes, but most displayed MICs above the currently defined MCs for chloramphenicol, and all showed excessive MICs for tetracycline. Based on the distributions, we calculated and proposed new MCs for chloramphenicol (16 instead of 4 mg/L) and tetracycline (256 instead of 8 mg/L).

HIGHLIGHTS

- None of the 35 tested *Pediococcus pentosaceus* strains displayed ABR genes.
- Calculated MCs were higher than recommended for five antibiotics.
- We propose to increase MCs for chloramphenicol and tetracycline.
- Accurate MCs are crucial for a valid selection of strains for food applications.

Key words: Antibiotics; Food; Microbiological cutoffs; *Pediococcus pentosaceus*

Pediococcus pentosaceus (37) is a gram-positive, homofermentative, facultative aerobic lactic acid bacterium with coccus-shaped cells that often form pairs or tetrads *(24)*. *Pediococcus* spp. are closely related to members of the heterofermentative lactobacilli and also to *Weissella* spp. and *Leuconostoc* spp. both phylogenetically *(46, 60)* and physiologically, reflecting their frequently shared habitats *(24)*. The type strain of *P. pentosaceus* has been isolated from dried American beer yeast, but the species has also been found in a wide variety of environments. For example, it is present on plants *(39),* in the gastrointestinal tracts of various organisms *(57),* in human saliva *(50),* and in fish *(35)*. More considerable populations have often been found in fermented feed and food, where they play an active role in fermentation. Industrial applications of the species as a component of starter cultures are as diverse as, for example, the production of silage *(5),* sauerkraut *(42),* olives *(26),* and fermented sausages *(1)*. *P. pentosaceus* also plays a role as a nonstarter lactic acid bacterium in the production of cheeses, where it may be involved in the formation of aromas *(6, 20)*. In view of the abilities of several strains to produce bacteriocins (pediocins), *P. pentosaceus* also finds applications as a biocontrol agent against spoilage microorganisms and foodborne pathogens *(43, 52)* as well as clinical applications as a probiotic *(43)*.

The application of *P. pentosaceus* strains for food and feed fermentation, such as in starter cultures, requires

* Author for correspondence. Tel: +41 (0)58 463 81 79; Fax: +41 (0)58 463 82 27; E-mail: noam.shani@agroscope.admin.ch.

relatively few controls due to the qualified presumption of safety status of the species *(16, 17)*. Nevertheless, in the frame of the global fight against the spread of antibiotic resistance (ABR), producers have the responsibility to ensure that the strains comprising the cultures brought to market and "intentionally introduced into the food chain" do not carry any acquired and transferable ABR determinants *(17)*.

The guidance of the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority (EFSA) describes how the ABR of strains entering the food chain should be tested *(14)* and is commonly used as a reference in evaluating strains intended to be used as starter or adjunct cultures for fermented products. This document states that ABR should be assessed by both genomic and phenotypic approaches. The genomic approach consists of seeking known ABR genes in the whole genome sequence of the investigated strains by using databases such as the Comprehensive Antibiotic Resistance Database (CARD) *(29, 36),* the Antibiotic Resistance Gene-Annotation (ARG-ANNOT) *(23),* and Resfinder *(59)*. The phenotypic approach "should be based on an internationally recognized and standardized method," such as the broth microdilution method described in the ISO Standard 10932|IDF 223:2010 *(27)*. Furthermore, the FEEDAP recommends a set of antibiotics of human and veterinary importance to evaluate and provides "microbiological" cutoffs (MCs) to distinguish strains with acquired ABR from susceptible strains. MCs are defined based on the distributions of MICs of given sets of antibiotics in a certain population of strains belonging to the investigated species. To determine MCs, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommends ECOFFinder, a Microsoft Excel macro for the statistical analysis of MIC distributions that implements the iterative statistical method of Turnidge et al. *(55)*.

Classifying strains of *P. pentosaceus* as resistant or susceptible by using phenotypic methods faces the challenge that MCs have been determined for the whole genus *Pediococcus,* which encompasses 11 species (https://lpsn.dsmz.de/, last accessed 07.12.2020) *(18)*. Furthermore, existing phenotypic data about ABR in *Pediococcus* spp. are scarce and have often been generated in nonstandardized ways, making them hard to compare. Therefore, the MCs defined for *Pediococcus* spp. might be inaccurate and lead to either over- or underestimation of the susceptibility of *P. pentosaceus* strains. Although this inaccuracy may be somewhat corrected by genomic analysis, this approach still relies on the search for known or putative ABR genes and might not detect genes related to undescribed ABR mechanisms. Therefore, there is a need to determine MCs specifically for *P. pentosaceus* to increase confidence in the outcomes of the phenotypic tests.

The objective of this study was to reevaluate existing MCs by analyzing the MIC distributions of 35 *P. pentosaceus* strains isolated from various types of samples by using a phenotypical method and searching for known ABR genes in whole genome sequences.

MATERIALS AND METHODS

Bacterial strains and culturing conditions. Thirty-five *P. pentosaceus* strains from various sources were selected for analysis (Supplemental Table S1). The strains originated in cheese or dairy environments ($n = 20$), fermented meat ($n = 5$), plant material ($n = 4$), a brewery environment ($n = 3$), mouse feces ($n = 1$), and unknown environments ($n = 2$).

The strains were stored at −80°C in de Man Rogosa Sharpe (MRS) *(10)* broth with Tween 80 (Biolife Italiana Srl, Milan, Italy) containing sterile low-fat milk as a cryoprotectant. They were routinely cultured in MRS broth with Tween 80 at 30°C under aerobic conditions.

The purity of the strains was assessed by plating 10-fold serial dilutions in 10 mL of 0.9% NaCl onto MRS agar plates and observing the colony morphologies and the cells under a microscope.

Before antibiotic susceptibility testing, the strains were propagated in MRS broth with Tween 80 at 30°C under aerobic conditions.

Bacterial identification. The identities of the strains were confirmed using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) on a MicroFlex LT/SH mass spectrometer (Bruker Daltonics, Bremen, Germany), as described previously *(34)*. Data were acquired with FlexControl v.3.4.105 software. The spectra were analyzed with MBT Compass software v.1.4 (Bruker Daltonics) and a Realtime Classification Biotyper MBT RUO 3.1 with the BDAL v.5.0 library.

DNA extraction. The DNA of the strains whose genome assemblies were not available at the National Center for Biotechnology Information (NCBI) was extracted as described previously *(12)*. In brief, bacterial pellets were harvested from 1 mL of the overnight cultures by centrifugation at 10,000 × *g* for 5 min at room temperature. In a prelysis treatment, the cells were incubated in 1 mL of 50 mM sodium hydroxide for 15 min at room temperature. The cells were then collected by centrifugation (10,000 × *g* for 5 min at room temperature) and treated with 50 μL of lysozyme solution (2.5 mg/mL dissolved in 100 mmol/L Tris, 10 mmol/L EDTA, and 25% [w/v] sucrose, pH 8.0) for 1 h at 37°C. After the pretreatment, the cells were collected by centrifugation (10,000 × *g* for 5 min at room temperature). Cell lysis and genomic DNA extraction were performed using the EZ1 DNA tissue kit and a BioRobot EZ1 workstation (Qiagen, Hilden, Germany) according to the manufacturer's instructions and eluted in a volume of 100 μL of the buffer EB provided in the kit.

Genome sequences of *P. pentosaceus* strains. When available, genome assemblies were downloaded from the NCBI. Strains with no available assemblies on open databases were de novo sequenced. In these cases, the quality-control assessment of the extracted DNA, generation of libraries, and sequencing runs were performed on the Next Generation Sequencing Platform, University of Bern, Switzerland. In brief, the libraries were prepared using the "TruSeq DNA PCR-free Library Prep" kit (20015963, Illumina) in combination with TruSeq DNA UD Indexes (20022370, Illumina) according to Illumina's guidelines. Pooled DNA libraries were sequenced paired-end (2 × 250 bp) by using a shared Illumina NovaSeq 6000 S Prime Reagent kit (500 cycles; 20029137, Illumina) on an Illumina NovaSeq 6000 instrument, generating an average of 4.3 million reads per library. ConFindr v.0.7.2 was used with raw reads to check for bacterial intraspecies contamination *(33)*. The quality control of raw data

was performed with FastQC v.0.11.7 *(2)*. Adaptor removal and trimming of raw data were done using fastp v.0.20.0 *(7)*. Trimmed reads were assembled with SPAdes v.3.14.0 *(3)* in –isolate mode, and contigs shorter than 200 bp were removed from the final assembly. QUAST v.4.6 *(22)* and BUSCO v.4.0.6 *(48)* in –auto-lineage-prok mode were used to assess the quality of the assemblies. The assemblies were then uploaded to NCBI with automatic annotation using PGAP build 4894 *(53)*.

Visualization of strain differences with a SNP-based phylogenetic tree. Assemblies of strains studied in this work were analyzed using PhaME v.1.0.2 *(49)*. PhaME extracts single-nucleotide polymorphisms (SNPs) from genomes and uses SNP multiple sequence alignment to construct a phylogenetic tree. The pipeline was run with the following parameters: reference = 2, cdsSNPs = 0, buildSNPdb = 1, SNPsfilter = 0.6, data = 0, aligner = bowtie, tree = 3, and bootstrap = 1 with $n = 100$. DSM 20281 was picked as reference (1,765,784-bp genome length), the core genome alignment consisted of 1,329,369 bp and included a total of 59,723 SNP positions. Out of these, IQ-TREE multicore v.2.0.3 *(38)* (type of analysis: modelFindernon-parametric bootstrap) identified 35,980 parsimony informative sites. IQ-TREE's model finder chose TVM+F+ASC+G4 as model of substitution based on the Bayesian information criterion, and the consensus tree was constructed from 100 bootstrap trees. The resulting tree was visualized using MEGA X *(32, 51)*.

Searching for ABR determinants. The genome assemblies were screened for known acquired ABR genes by using ABRicate *(47)* with the default filtering parameters (minimum DNA %identity = 75 and minimum DNA %coverage = 0) and the databases NCBI AMRFinderPlus *(19),* CARD 2017 *(29, 36),* ARG-ANNOT v.4 *(23),* and Resfinder v.3.0 *(59)*. The genome assembly of *P. acidilactici* FAM 13875 (GenBank accession GCA_009789085.1), which contains the ABR genes *tetM* and *ermA (34),* was included as a positive control.

Antibiotic susceptibility testing and calculation of MCs. The procedure for evaluating existing MCs and proposing new MCs was based on the Standard Operating Procedure 10.1 of the EUCAST *(13)*. The antibiotic susceptibility of the strains was tested at the Culture Collection of Switzerland (Wädenswil, Switzerland) with a broth microdilution susceptibility method following the standard procedure of the ISO 10932|IDF 223:2010 *(27)* recommended by the FEEDAP *(14)*. In brief, the strains were propagated in MRS broth with Tween 80 (Biolife Italiana Srl) at 30°C for 24 h. They were then transferred into microplates containing lactic acid bacterium susceptibility medium (LSM) *(30)* and different antibiotic concentrations: ampicillin, 0.5 to 32 μg/mL; vancomycin, 0.25 to 128 μg/mL; gentamycin, 0.5 to 256 μg/mL; kanamycin, 2 to 1,024 μg/mL; streptomycin, 0.5 to 256 μg/mL; erythromycin, 0.016 to 8 μg/mL; clindamycin, 0.03 to 16 μg/mL; tetracycline, 0.12 to 512 μg/mL; and chloramphenicol, 0.12 to 64 μg/mL. The microdilution plates were incubated at 30°C for 48 h under anaerobic conditions. Growth was assessed visually.

The MIC distributions of the antibiotics were processed in the ECOFFinder program v.2.1 (available at http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/MIC_distributions/ECOFFinder_XL_2010_v2.1_web_version.xlsm) *(55),* and the MCs were defined as the 99.0% ECOFF values. The MIC distributions and the MCs were visualized with the ggplot2 package version 3.2.1 *(58)* by using RStudio Pro v.1.2.50.33-1 *(45)* with the R software v.3.5.3 *(44)*.

RESULTS

Bacterial identities. The identities of all strains tested in this study were confirmed by MALDI-TOF before any further analyses, and taxonomic assignments based on the 16S rRNA gene retrieved from the genomic data confirmed this result.

Strain differentiation. Based on the SNP analysis of genome assemblies, most strains were clearly genetically different (Fig. 1). However, four groups of strains showed very high similarities in pairwise comparison of non-repetitive regions of the genomes (Table S2): (i) FAM 19169 and FAM 20746 (233 SNPs); (ii) FAM 19080 and FAM 23908 (852 SNPs); (iii) DSM 46292 and LMG 11487 (861 SNPs); and (iv) FAM 18048, FAM 18327, FAM 18523, LMG 10478, and LMG 9445 (0 to 16 SNPs).

ABR determinants in the genome assemblies. No ABR genes were detected in the genome assemblies of the 35 *P. pentosaceus* strains analyzed using NCBI AMRFinderPlus, CARD, ARG-ANNOT, and Resfinder. Both *tetM* and *ermA* were detected in the positive control (*P. acidilactici* FAM 13875) with all the databases.

MICs and MCs. All strains could grow under the defined testing conditions. Table 1 summarizes the results of the susceptibility testing, and Table 2 and Figure 2 show the MIC distributions measured for the 35 strains in this study for the antibiotics recommended by the FEEDAP. The MIC distribution of vancomycin was not included, because the MICs were all greater than 128 μg/mL for all the strains due to the intrinsic nature of the resistance to this antibiotic in *Pediococcus* spp.

MCs for *P. pentosaceus* calculated with ECOFFinder based on the determined MIC distributions (Table 1) were higher than the MCs recommended by the FEEDAP for (i) ampicillin (calculated MC = 8 mg/L, FEEDAP MC = 4 mg/L), (ii) kanamycin (calculated MC = 128 mg/L, FEEDAP MC = 64 mg/L), (iii) streptomycin (calculated MC = 128 mg/L, FEEDAP MC = 64 mg/L), and (iv) chloramphenicol (calculated MC = 16 mg/L, FEEDAP MC = 4 mg/L). The MC could not be calculated for clindamycin because most strains had a MIC ≤0.03 mg/L, which was the lowest concentration tested for this antibiotic. The MC for tetracycline could not be determined using ECOFFinder, because the MICs were not normally distributed. However, all the strains had MICs above the recommended MC for tetracycline (8 mg/L; Table 2): most (14 strains) had a MIC of 128 mg/L, 9 had a MIC of 64 mg/L, 11 had a MIC of 32 mg/L, and 1 strain had a MIC just above the recommended MC, at 16 mg/L. No strain displayed a MIC above 128 mg/L. After visual inspection of the MIC distribution for this antibiotic and because most strains displayed a MIC of 128 mg/L, the MC was arbitrarily set at 256 mg/L.

The calculated MCs were lower than the recommended MCs for gentamycin (calculated MC = 8 mg/L, FEEDAP MC = 16 mg/L) and erythromycin (calculated MC = 0.5 mg/L, FEEDAP MC = 1 mg/L).

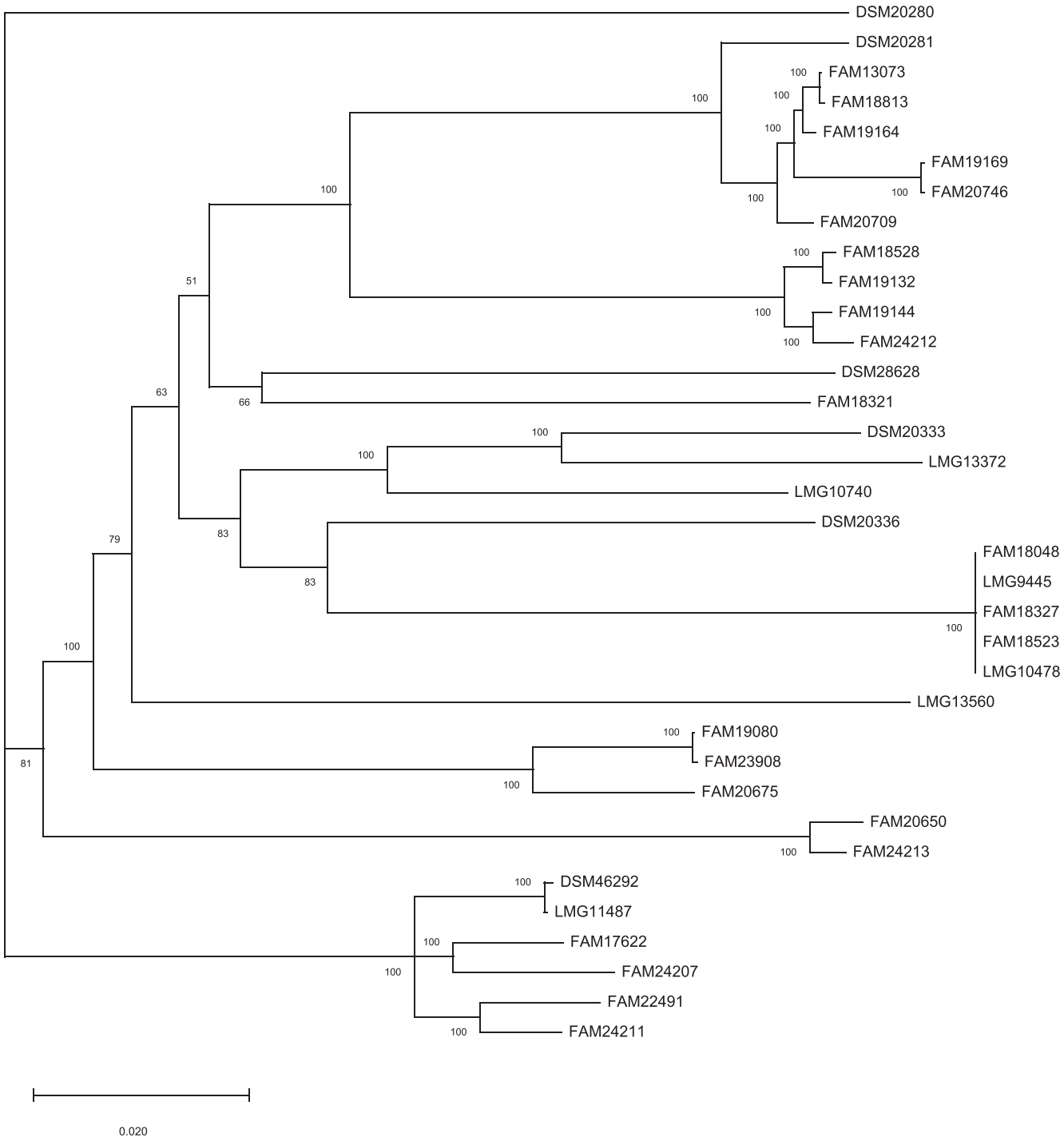

FIGURE 1. *Phylogenetic tree based on the analysis of single-nucleotide polymorphisms (SNPs) between 35* Pediococcus pentosaceus *genomes. The scale bar indicates the number of substitutions per site.*

DISCUSSION

Choice of strains. The determination of MCs for a given species relies on the analysis of the distribution of MICs of antibiotics in different strains of the investigated species. In this study, we determined the MICs of 35 different *P. pentosaceus* strains.

To avoid a potential bias related to the uneven dissemination of antibiotics and of their selective pressure in the environment, the strains in this study were selected in such a way that they covered several habitats of *P. pentosaceus*. Furthermore, the selected strains were isolated in a period of 70 yr or more. Although ABR mechanisms have already been reported as soon as in the 1920s with resistance to salvarsan *(28),* mainly in clinical isolates, the spread of ABR really gained momentum in the 1960s *(9)*. Thus, the strains analyzed here should partly cover the golden age of antibiotics and the subsequent dissemination of ABR mechanisms. One obvious prerequisite for

TABLE 1. *MICs of the* Pediococcus pentosaceus *strains tested in this study*[a]

Strain	Ampicillin (4/8)	Vancomycin (n.r.)	Gentamycin (16/8)	Kanamycin (64/128)	Streptomycin (64/128)	Erythromycin (1/0.5)	Clindamycin (1/n.a.)	Tetracycline (8/256)	Chloramphenicol (4/16)
DSM 20280	2	>128	<0.5	4	16	0.25	<0.03125	32	4
DSM 20281	4	>128	1	64	32	0.25	<0.03125	128	16
DSM 20333	8	>128	4	128	64	0.25	0.0625	64	8
DSM 20336^T	2	>128	1	32	16	0.25	<0.03125	32	8
DSM 28628	4	>128	1	32	64	0.125	0.0625	128	8
DSM 46292	4	>128	<0.5	16	16	0.125	<0.03125	32	4
LMG 9445	4	>128	8	64	64	0.125	<0.03125	128	8
LMG 10478	4	>128	4	64	32	0.25	<0.03125	128	8
LMG 10740	4	>128	2	128	64	0.25	<0.03125	64	8
LMG 11487	2	>128	1	32	32	0.125	<0.03125	32	4
LMG 13372	4	>128	2	64	32	0.25	0.0625	128	8
LMG 13560	4	>128	2	64	32	0.0625	<0.03125	16	4
FAM 13073	2	>128	1	32	16	0.25	<0.03125	32	8
FAM 17622	1	>128	1	32	16	0.0625	<0.03125	32	1
FAM 18048	2	>128	4	64	64	0.5	0.0625	64	8
FAM 18321	2	>128	4	32	32	0.25	<0.03125	32	4
FAM 18327	4	>128	4	64	32	0.25	<0.03125	128	8
FAM 18523	2	>128	4	128	64	0.5	<0.03125	64	8
FAM 18528	4	>128	2	64	32	0.125	<0.03125	128	8
FAM 18813	4	>128	1	64	32	0.125	<0.03125	128	8
FAM 19080	4	>128	2	64	32	0.25	<0.03125	64	8
FAM 19132	2	>128	2	32	32	0.25	<0.03125	64	8
FAM 19144	2	>128	<0.5	32	16	0.25	<0.03125	32	8
FAM 19164	2	>128	2	64	64	0.25	<0.03125	32	8
FAM 19169	4	>128	2	64	64	0.125	<0.03125	128	8
FAM 20650	4	>128	1	32	32	0.25	0.0625	128	4
FAM 20675	4	>128	2	64	32	0.125	<0.03125	32	4
FAM 20709	4	>128	2	64	32	0.125	0.0625	128	4
FAM 20746	2	>128	2	64	64	0.25	<0.03125	128	8
FAM 22491	2	>128	1	64	32	0.125	<0.03125	64	8
FAM 23908	2	>128	2	64	64	0.25	<0.03125	64	8
FAM 24207	2	>128	4	64	64	0.25	<0.03125	32	4
FAM 24211	2	>128	1	32	32	0.125	<0.03125	64	4
FAM 24212	4	>128	2	64	32	0.125	<0.03125	128	8
FAM 24213	4	>128	1	32	32	0.125	<0.03125	32	4

[a] Current FEEDAP MCs/calculated MCs based on data from this study are indicated in parentheses. All values are expressed in milligrams per liter.

calculating MCs is obtaining MIC distributions by using different strains. Although the debate around the definition of a "bacteriological strain" is not new (for a concise overview, see *(11)*), there remains room for interpretation. In this study, we adopted the definition of "strain in the taxonomic sense" *(4, 11)*. In other words, the strains were selected in light of their origins. Nevertheless, to gain more information about the similarity of the investigated strains, a phylogenetic analysis based on SNPs was performed and confirmed that most strains were clearly different. However,

TABLE 2. *MIC distributions of the strains analyzed in this study*

	MIC (mg/L)															
Antibiotic	<0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1,024
Ampicillin						1	14	19	1							
Gentamicin				3		11	13	7	1							
Kanamycin								1		1	11	19	3			
Streptomycin										5	18	12				
Erythromycin		2	13	18	2											
Clindamycin	29		6													
Tetracycline										1	11	9	14			
Chloramphenicol						1		11	22	1						

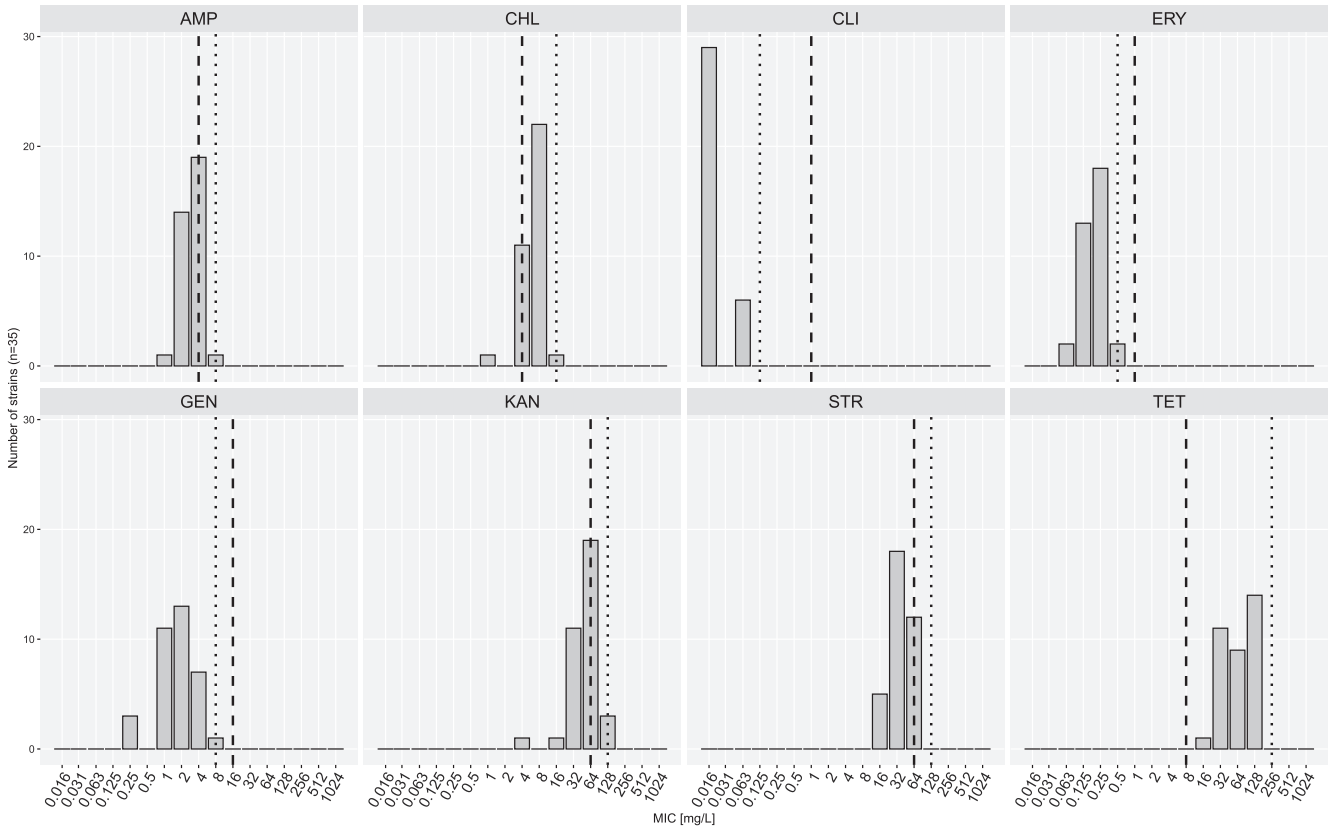

FIGURE 2. *Bar plots displaying the MIC distributions of the antibiotics tested in this study on 35 strains of* Pediococcus pentosaceus*. For each antibiotic, the actual FEEDAP MCs and the calculated MCs are displayed as a vertical dashed line and a dotted line, respectively. AMP, ampicillin; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; GEN, gentamycin; KAN, kanamycin; STR, streptomycin; TET, tetracycline.*

some pairs of strains displayed high similarities at the genomic level, which does not necessarily mean that they are identical. Indeed, the phylogenetic analysis based on SNPs relies on core genome regions and does not consider accessory genes *(49)*. Most of the strains described as phylogenetically highly similar have clearly different origins (original sample or date of isolation; Table S1). However, based on the available information, it could not be excluded that FAM 18048, FAM 18327, and FAM 18523 actually belong to the same strain. Indeed, in addition to the extremely low number of detected SNPs, FAM 18048 was isolated from a commercial culture for manufacturing fermented meat products, whereas FAM 18327 and FAM 18523 were isolated possibly approximately at the same period from different fermented sausages. Therefore, there is a chance, but no certainty, that FAM 18048 was used as a starter culture in the two sausages from which FAM 18327 and FAM 18523 were isolated. Conversely, no connection could be found between these three strains and LMG 10478 and LMG 9445, which were isolated from fermented cucumbers and cheese curd, respectively.

MICs and MCs. In our study, care was taken to determine MICs by using an internationally recognized standard procedure, as recommended by the FEEDAP *(14),* to enable further comparisons with data produced with the same standard procedure. Standard Operating Procedure 10.1 of the EUCAST *(13)* was used as a basis to determine the MCs. According to this document, epidemiological cutoffs (ECOFFs) are set by the EUCAST based on at least 100 MICs from aggregated MIC distributions obtained from several laboratories. Here, we describe MCs based solely on our own data due to the lack of data produced using the same standard conditions. Indeed, several studies have reported on MIC distributions of *P. pentosaceus* based on the broth microdilution test, but none of them fully agrees with the conditions described here, making comparisons risky. The ISO 10932|IDF 223:2010 *(27)* standard procedure neither explicitly mentions the incubation temperature and time nor the growth medium that should be used to test the ABR of *P. pentosaceus*. We chose to incubate the strains (i) in LSM because it is the recommended medium for all *Lactobacillus* spp., which are phylogenetically the closest relatives to *Pediococcus* spp. *(46, 60)* and because it has been shown that the medium is well adapted to the antimicrobial susceptibility testing of pediococci *(14, 31)*; (ii) at 30°C because the optimal growth temperature for this species lies between 28 and 32°C *(24)*; and (iii) over 48 h, the recommended incubation time defined for all species listed in the standard procedure. Among other studies investigating MIC distributions of *P. pentosaceus* by using the broth microdilution method, Cordeiro et al. *(8)* used LSM as a growth medium, but the incubation temperature was 35°C and the strains were grown overnight only.

Furthermore, they tested only two of the eight antibiotics required by the FEEDAP. Muñoz-Atienza et al. *(40)* analyzed the MIC distributions of the eight recommended antibiotics in LSM, but the incubation temperature (37°C) and time (18 h) were different from our conditions. Klare et al. *(31)* proposed tentative ECOFFs for *P. pentosaceus* based on MIC distributions obtained by the broth microdilution method by using LSM for six of the eight antibiotics recommended by the FEEDAP, but the incubation temperature and time were 37°C and 24 h, respectively.

Nevertheless, although the variety of conditions used in these studies is not suitable for an aggregation of MIC distributions, punctual comparisons can still be made.

The MCs calculated here were higher than the MCs recommended by the FEEDAP for five antibiotics: ampicillin, kanamycin, streptomycin, tetracycline, and chloramphenicol. The FEEDAP recommends an MC of 4 mg/L for ampicillin. Our MIC distribution for this antibiotic suggests that the MC should be increased to at least 8 mg/L. Klare et al. *(31)* proposed an MC of 4 mg/L based on 20 strains, with the MIC ranging from 1 to 2 mg/L. However, their higher incubation temperature (37°C) and lower incubation time (24 h) may explain why fewer strains could grow at higher concentrations. Furthermore, our data suggest that the MC for kanamycin should be increased from the current 64 to 128 mg/L. Muñoz-Atienza et al. *(40)* also suggested an MC of 64 mg/L based on a MIC distribution of 16 *P. pentosaceus* strains, with the majority displaying a MIC of 64 mg/L and only 2 having a MIC of 128 mg/L. However, in this case also, the higher incubation temperature and reduced incubation time may explain the discrepancy with our results. The calculated MC for streptomycin was also 128 mg/L (FEEDAP MC = 64 mg/L). In our case, no strain had a MIC above 64 mg/L, and most had a MIC of 32 mg/L, which is in the range observed by Muñoz-Atienza et al. *(40)* and by Klare et al. *(31),* who also proposed an MC of 128 mg/L. Tetracycline represented a unique case in our study, because ECOFFinder could not determine a fitting curve because no strain displayed a MIC above 128 mg/L, which was also the most frequent MIC. As most of our strains displayed a MIC of 128 mg/L when the FEEDAP MC was 8 mg/L; although none showed a higher MIC, we arbitrarily set the MC for tetracycline at 256 mg/L. In their study, Cordeiro et al. *(8)* found that all *P. pentosaceus* isolates had a MIC greater than 64 mg/L for this antibiotic. Similarly, previous work on the close relative *P. acidilactici* found that 83.3% of all tested strains were at or above the MC set by the FEEDAP for the *Pediococcus* genus, with no known ABR gene detected *(34)*. The relatively high tolerance toward tetracycline thus may be a feature of the *Pediococcus* genus. At this stage, the FEEDAP MC appears clearly underestimated. Finally, the MC calculated in our study for chloramphenicol (16 mg/L) was two twofold dilutions higher than the FEEDAP MC, with most strains displaying a MIC of 8 mg/L.

MCs are a good means of determining whether a particular bacterial strain is resistant or susceptible to a given antibiotic. In particular, it is a good complement to genomic tools searching for known ABR genes because it may detect resistance determinants, where yet-unknown genes or point mutations might go unnoticed. However, it is crucial that MCs are defined accurately based on robust data. Indeed, overestimated MCs may lead to false susceptible strains, that is, strains with undetected ABR determinants. In the case of feed or food production, these determinants can go unnoticed, be introduced to the food chain *(15, 41, 54),* and eventually be acquired by pathogenic bacteria through horizontal gene transfer *(56)* in the gastrointestinal tract *(25, 54)*. By contrast, underestimated MCs may strongly restrict the number of candidates for developing starter or adjunct cultures for manufacturing fermented products, potentially depriving the industry of strains bearing interesting technological or health properties. In the present case, all strains but one were above the FEEDAP MC for tetracycline by at least two twofold dilutions, and more than half were above the FEEDAP MC for chloramphenicol. Thus, solely on the basis of the phenotypic analysis, all investigated strains would have been dismissed in a screening for potential candidates for application in the food industry.

Based on our results, we propose to increase MCs for chloramphenicol from 4 to 16 mg/L and for tetracycline from 8 to 256 mg/L.

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SUPPLEMENTAL MATERIAL

Supplemental material associated with this article can be found online at: https://doi.org/10.4315/JFP-20-363.s1

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Supplemental material

TABLE S1. *Pediococcus pentosaceus* strains tested for antibiotic resistance.

Strain ID	Isolation source	Year of isolation	Original depositor; strain designation	Collection[a]	GenBank assembly accession[b]
DSM 20280	Brewery yeast	1990	243d	DSMZ	**GCA_015613315.1**
DSM 20281	Barley	Before 22.08.1990	W. Back; 260a	DSMZ	**GCA_015613305.1**
DSM 20333	Sake mash	Before 22.08.1990	K. Kitahara; P. lin	DSMZ	**GCA_015613245.1**
DSM 20336^T	Dried American beer yeast	Before 22.08.1990	C.B. van Niel	DSMZ	GCA_001437285.1
DSM 28628	Feces; wildtype C57BL/6 mouse (Germany)	2012	T. Clavel; JM-6755-f-WT, JM-80	DSMZ	**GCA_015613235.1**
DSM 46292	Cheddar cheese (New Zealand)	Before 27.02.1991	E. Coster; Coster R 3, SG 1468	DSMZ	**GCA_015613265.1**
LMG 9445	Cheese curd	1950 or before	Hiscox C30/6	BCCM	**GCA_015613205.1**
LMG 10478 (NCTC 8066)	Fermented cucumber pickle	1950 or before	C. Pederson; Pederson F166	BCCM	GCA_900454755.1
LMG 10740 (ATCC 25745)	Plants	Before 03.04.1969	Mundt 183-1w	BCCM	GCA_000014505.1
LMG 11487	Unknown	1954 or before	J. Dacre; Dacre A2	BCCM	**GCA_015613225.1**
LMG 13372	Grass samples (Belgium)	1991 or before	Radar P 5160 A	BCCM	**GCA_015613165.1**
LMG 13560	Unknown	1993 or before	EC-Target Strain 11; FBB 63; TNO-Voeding Zeist	BCCM	**GCA_015613145.1**
FAM 13073	Tilsit cheese (Switzerland)	1990	D. Isolini; PSP ZL21	ACC	GCA_009809665.1
FAM 17622	Gruyère cheese (Switzerland)	2001	9/1	ACC	GCA_009809195.1
FAM 18048	Culture for meat fermentation	2004 or before	LMP3	ACC	**GCA_015613155.1**
FAM 18321	Fermented sausage (Switzerland)	2005	7.13	ACC	GCA_009808645.1
FAM 18327	Fermented sausage (Switzerland)	2005	3.14	ACC	GCA_009808655.1
FAM 18523	Fermented sausage (Switzerland)	2006	12.04	ACC	**GCA_015613115.1**
FAM 18528	Fermented sausage (Switzerland)	2006	14.23	ACC	**GCA_015613105.1**
FAM 18813	Tête de Moine cheese (Switzerland)	2006	1.1	ACC	GCA_009808845.1
FAM 19080	Tête de Moine cheese (Switzerland)	2008	E. Marty; 80.1	ACC	GCA_009809425.1
FAM 19132	Milk (Switzerland)	2004	S. Irmler; 18157.1	ACC	GCA_009809595.1
FAM 19144	Sbrinz cheese (Switzerland)	2008	S. Pfister; 8.2	ACC	GCA_005864405.1
FAM 19164	Tilsit cheese (Switzerland)	2008	S. Pfister; 5408.1	ACC	**GCA_015613065.1**
FAM 19169	Tilsit cheese (Switzerland)	2008	S. Pfister; 5402.3	ACC	GCA_009808085.1

FAM 20650	Tilsit cheese (Switzerland)	1989–1990	D. Isolini; PSP BB21	ACC	GCA_009808815.1
FAM 20675	Tilsit cheese (Switzerland)	1989–1990	D. Isolini; PSP BS17	ACC	**GCA_015613055.1**
FAM 20709	Tilsit cheese (Switzerland)	1989–1990	D. Isolini; PSP KB12	ACC	**GCA_015613035.1**
FAM 20746	Tilsit cheese (Switzerland)	1989–1990	D. Isolini; PSP Mü20	ACC	**GCA_015613005.1**
FAM 22491	Whey (Switzerland)	1985	D. Marzohl; Div6.1.1	ACC	**GCA_015613015.1**
FAM 23908	Vacherin Fribourgeois cheese (Switzerland)	2015	P. Ascone; K3 (P3, Arg.)	ACC	**GCA_015612975.1**
FAM 24207	Tomme cheese (Switzerland)	2017	N. Shani; 35/11	ACC	**GCA_015612965.1**
FAM 24211	Milk (Switzerland)	2017	N. Shani; 73/11	ACC	**GCA_015613785.1**
FAM 24212	Milk (Switzerland)	2017	N. Shani; 107/9	ACC	**GCA_015613765.1**
FAM 24213	Wooden cheese container (Switzerland)	2017	N. Shani; 114/13	ACC	**GCA_015613745.1**

[a] ACC: Agroscope Culture Collection, Bern, Switzerland; DSMZ: German Collection of Microorganisms and Cell Cultures GmbH, Leibniz Institute, Braunschweig, Germany; BCCM: Belgian Co-ordinated Collections of Microorganisms, University of Gent, Belgium.

[b] Accession numbers in bold indicate the strains that have been sequenced in this study (description in the Material and Methods section).

[T] Type strain

TABLE S2. Number of SNPs found between the 35 strains of *Pediococcus pentosaceus* using PhaME

	DSM 20280	DSM 20281	DSM 20333	DSM 20336	DSM 28628	DSM 46292	FAM 13073	FAM 17622	FAM 18048	FAM 18321	FAM 18327	FAM 18523	FAM 18528	FAM 18813	FAM 19080	FAM 19132	FAM 19144	FAM 19164	FAM 19169	FAM 20650	FAM 20675	FAM 20709	FAM 20746	FAM 22491	FAM 23908	FAM 24207	FAM 24211	FAM 24212	FAM 24213	LMG 10478	LMG 10740	LMG 11487	LMG 13372	LMG 13560	LMG 9445
DSM 20280		19652	19101	20145	19741	21533	23056	21821	22395	22077	22427	22378	21977	22912	22901	21118	21080	24863	22382	24764	22469	20743	20575	22370	23377	24375	23800	19179	26256	22005	19659	20331	20505	26913	20749
DSM 20281	19654		14684	14801	14933	16968	5194	16392	15347	15313	15353	15342	13380	4565	15354	13737	12847	3828	6190	19022	16554	3512	6177	16984	15464	18398	16696	12684	20255	14951	15304	16698	15733	18223	15292
DSM 20333	19112	14684		16000	16348	19011	16178	18215	16107	14285	16108	16098	15563	16622	16070	15328	15663	14901	14660	19509	16160	15236	14807	18653	15914	19337	18101	14979	19363	15750	11246	18830	10350	18053	15749
DSM 20336	20106	14801	15997		15523	18317	16876	17431	14930	15440	14940	14835	14837	16812	16158	15032	14454	15736	15046	18932	16824	15481	14963	18052	16609	18869	17893	14686	19628	14701	13142	17711	14758	18561	14815
DSM 28628	19699	14969	16429	15522		17123	16726	17629	17675	15252	17673	17660	17208	17455	15624	15803	16392	16011	15778	18876	16101	15956	15831	17738	15678	18715	17547	15365	20014	17378	14956	16906	16679	18513	17485
DSM 46292	21529	16968	19011	18347	17123		19282	7899	20102	18749	20103	20071	17853	19374	18270	19057	17396	20005	19132	21687	19536	18354	18094	9187	19776	10299	8402	17050	23949	19681	17377	861	18971	22522	18495
FAM 13073	23063	5197	16133	16908	16725	19281		19135	18281	17113	18280	18270	14169	1615	17286	15177	15863	2672	5151	20509	18975	3950	4644	20251	17877	21876	18780	14777	23699	17867	16481	17820	16505	19723	17724
FAM 17622	21839	16453	18215	17493	17629	7899	19238		19445	18031	19445	19434	18118	19247	17739	18384	17817	19591	18973	21887	18590	17821	18182	8285	18184	6959	8121	17086	22425	18962	18252	7375	18998	21876	18262
FAM 18048	22395	15344	16106	14791	17758	20102	18197	19368		17716	3	2	16488	18288	17929	17772	16808	19493	18325	20992	18203	17139	17297	19860	18330	21296	19864	16690	21298	2	16512	19311	16164	19812	16
FAM 18321	22088	15369	14285	15523	15284	18857	17113	18064	17597		17604	17495	18409	17944	16996	16794	16440	18273	16483	21525	16923	15727	16270	20739	15695	22869	18322	15307	21424	17184	18026	17941	17248	21311	16743
FAM 18327	22436	15356	16053	14800	17672	20027	18280	19384	3	17624		1	16451	18274	17774	17855	16889	19480	18333	21013	18274	17146	17304	20030	18078	21498	19848	16777	21352	1	16513	19236	16111	19844	16
FAM 18523	22378	15339	16097	14837	17659	19995	18264	19353	2	17572	1		16556	18354	17769	17839	16874	19485	18315	20889	18270	17129	17287	19850	18170	21195	19770	16757	21313	0	16509	19163	16156	19812	15
FAM 18528	22051	13374	15563	14837	17205	17847	14169	18057	16570	18360	16451	16559		14779	18737	2410	4285	16949	14863	21635	18382	13157	13803	18946	17592	21720	18748	3443	21886	16356	16365	17475	15709	19452	16236
FAM 18813	22919	4565	16578	16843	17454	19373	1615	19144	18371	17941	18274	18360	14779		17152	14658	16409	2719	5225	20607	18458	3791	4296	20077	18522	21950	19234	14381	23981	17888	17387	17981	16051	19545	17808
FAM 19080	22910	15351	16070	16242	15623	18270	17283	17669	17781	17071	17775	17773	18737	17149		17368	16731	20057	17942	22174	8324	15947	16076	18801	852	20983	18766	15277	22237	17571	15094	17476	16346	22087	16233
FAM 19132	21192	13731	15325	15032	15795	19109	15177	18423	17831	16788	17831	17819	2410	14659	17505		3534	15550	15817	20646	18984	13320	14495	19076	17474	21180	18432	3406	21841	17547	16050	18565	15368	19994	16568
FAM 19144	21085	12847	15667	14351	16391	17393	15791	17758	16811	16440	16810	16798	4285	16264	16740	3551		18053	15708	20637	16760	14904	14905	17857	15455	20148	18532	2081	20161	16590	14586	16803	15700	20118	16394
FAM 19164	24867	3824	14820	15790	15971	19983	2654	19513	19493	18213	19395	19406	16840	2702	20057	15478	18033		5875	23590	19779	3406	3424	20570	19161	22466	20342	14839	23503	19166	16188	18872	15300	22092	17621
FAM 19169	22383	6190	14615	15001	15780	19132	5076	18883	18331	16423	18332	18320	14785	5151	17945	15741	15708	5865		21261	19058	4869	233	19937	17706	21220	19050	13541	23201	18009	16742	19405	16324	20734	16484
FAM 20650	24328	18976	19437	18959	19089	21815	20506	21821	20950	21558	20930	20849	21632	20604	22334	20646	20636	23625	21234		22785	19629	19345	21773	22042	24219	21320	18634	3437	20611	19876	21677	20219	26438	19557
FAM 20675	22484	16554	16159	16907	16101	19521	18885	18448	18203	16923	18202	18201	18358	18380	8346	18995	16738	19784	19058	22652		16656	17152	20211	7858	21016	20553	15639	23725	17912	16910	18478	16967	21590	17244
FAM 20709	20745	3511	15190	15490	15956	18354	3950	17722	17145	15670	17145	17134	13168	3790	15950	13348	14905	3280	4869	19589	16693		4947	18381	16478	19136	18142	13624	20338	16883	15580	17908	15451	18761	16880
FAM 20746	20574	6177	14804	14963	15790	18091	4641	18099	17298	16210	17298	17287	13805	4296	16077	14494	14981	3499	220	19388	17147	5073		18149	15840	19531	17405	13397	21018	16933	16285	17906	16246	18959	16632
FAM 22491	22442	16960	18528	17952	17714	9250	20228	8244	19860	20659	19890	19850	18982	20054	18704	19077	17889	20570	19866	21740	20144	18399	18149		19076	9896	5350	17308	22145	19420	20205	8204	18692	22752	18599
FAM 23908	23425	15464	15914	16775	15679	19693	17875	18103	17964	15768	17862	17956	17593	18520	852	17339	15447	19160	17704	21947	7546	16476	15840	19034		20808	20680	15534	23249	17880	15236	17277	16221	20031	16405
FAM 24207	24391	18308	19209	18705	18647	10402	21870	7006	21372	23018	21370	21276	21698	21945	20909	21185	20120	22460	21172	24115	21126	19169	19531	9928	20884		9858	18583	25408	20844	21316	8572	19778	24546	19860
FAM 24211	23755	16693	17949	17775	17631	8379	18782	8077	19864	18310	19769	19770	18810	19236	18914	18438	18593	20341	19047	21294	20597	18149	17405	5351	20903	9906		17477	24259	19354	18103	6795	18591	22361	18305
FAM 24212	19252	12678	14979	14686	15366	17044	14777	17025	16772	15259	16777	16760	3443	14381	15279	3392	2096	14911	13617	18680	15633	13614	13397	17270	15533	18605	17415		19739	16573	14017	16070	15167	17702	16785
FAM 24213	25871	20209	19363	19478	19946	23978	23618	22376	21255	21466	21268	21272	21883	23900	22154	21842	20161	23538	23198	3350	23773	20378	20975	22132	23249	25327	24287	19689		20968	21223	22056	21012	24750	19840
LMG 10478	22015	14951	15695	14561	17377	19606	17864	18962	2	17199	1	0	16354	17886	17452	17568	16666	19248	18007	20695	17985	16881	16936	19499	17711	20845	19433	16571	21053		16132	18894	15776	19392	14
LMG 10740	19669	15299	11247	13225	14946	17412	16476	18293	16515	18048	16682	16512	16402	17424	15134	16050	14584	16264	16738	19761	16924	15575	16283	20284	15277	21319	18139	14054	21065	16301		17604	13944	18685	15858
LMG 11487	20402	16695	18830	17713	17013	861	17817	7375	19235	17953	19236	19163	17533	17978	17477	18565	16857	18879	19402	21440	18554	18038	17906	8162	17277	8514	6899	16128	21818	18894	17604		18868	22197	17736
LMG 13372	20272	15733	10422	14758	16741	18904	16505	18823	16165	17266	16166	16157	15710	16051	16344	15498	15700	15435	16270	20059	16967	15505	16249	18802	16220	19827	18557	15168	21099	15832	13832	18870		18502	15889
LMG 13560	26913	18220	17916	18444	18455	22486	19691	21765	19812	21586	19817	19816	19386	19513	22088	19965	20012	22092	20702	26398	21598	18739	18964	22760	20031	24442	22388	17636	24744	19483	18618	22153	18393		18285
LMG 9445	20762	15289	15748	14762	17485	18495	17640	18263	16	16861	16	15	16154	17725	16381	16489	16391	17621	16478	19599	17244	16874	16631	18678	16771	19861	18384	16703	19883	14	15855	17813	15888	18253	