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Comparative genomic analysis of *Periweissella* and the characterization of novel motile species

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ABSTRACT The genus *Periweissella* was proposed as a novel genus in the *Lactobacillaceae* in 2022. However, the phylogenetic relationship between *Periweissella* and other heterofermentative lactobacilli, and the genetic and physiological properties of this genus remain unclear. This study aimed to determine the phylogenetic relationship between *Periweissella* and the two closest genera, *Weissella* and *Furfurilactobacillus*, by the phylogenetic analysis and calculation of (core gene) pairwise average amino acid identity. Targeted genomic analysis showed that fructose bisphosphate aldolase was only present in the genome of *Pw. cryptocerci*. Mannitol dehydrogenase was found in genomes of *Pw. beninensis*, *Pw. fabaria*, and *Pw. fabalis*. Untargeted genomic analysis identified the presence of flagellar genes in *Periweissella* but not in other closely related genera. Phenotypes related to carbohydrate fermentation and motility matched the genotypes. Motility genes were organized in a single operon and the proteins shared a high amino acid similarity in the genus *Periweissella*. The relatively low similarity of motility operons between *Periweissella* and other motile lactobacilli indicated the acquisition of motility by the ancestral species. Our findings facilitate the phylogenetic, genetic, and phenotypic understanding of the genus *Periweissella*.

IMPORTANCE The genus *Periweissella* is a heterofermentative genus in the *Lactobacillaceae* which includes predominantly isolates from cocoa fermentations in tropical climates. Despite the relevance of the genus in food fermentations, genetic and physiological properties of the genus are poorly characterized and genome sequences became available only after 2020. This study characterized strains of the genus by functional genomic analysis, and by determination of metabolic and physiological traits. Phylogenetic analysis revealed that *Periweissella* is the evolutionary link between rod-shaped heterofermentative lactobacilli and the coccoid *Leuconostoc* clade with the genera *Weissella* and *Furfurilactobacillus* as closest relatives. *Periweissella* is the only heterofermentative genus in the *Lactobacillaceae* which comprises predominantly motile strains. The genomic, physiological, and metabolic characterization of *Periweissella* may facilitate the potential use of strains of the genus as starter culture in traditional or novel food fermentations.

KEYWORDS *Periweissella, Weissella*, *Furfurilactobacillus*, *Lactobacillus*, heterofermentative lactobacilli, phylogenetic relationship, carbohydrate fermentation, motility

Lactobacillaceae are significant members of human and animal gut microbiota, the plant phyllosphere and are dominant in most food fermentations (1). A taxonomic re-organization of the *Lactobacillaceae* revealed that the former *Leuconostocaceae* and *Lactobacillaceae* belong to the same family (2). However, genome sequence data that were available in August 2019 could not confidently establish the phylogenetic relationship of genera previously classified in the *Leuconostocaceae* to other lactobacilli

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(2). The availability of additional whole genome sequences in November 2021 revealed that all heterofermentative lactic acid bacteria form a monophyletic group (3). The inclusion of additional genome sequences also demonstrated that the genus *Weissella* was no longer monophyletic (3). The genus *Periweissella*, comprising strains previously classified as *Weissella* species, was proposed as a novel genus in 2022 (4). Five species, *Pw. beninensis*, *Pw. fabalis*, *Pw. fabaria*, *Pw. ghanensis*, and *Pw. cryptocerci* formed a single clade which was closely related to *Weissella*. *Periweissella* also shares common morphological characteristics with *Weissella*, i.e., cells are short rods or cocci. *Periweissella* spp. were isolated from spontaneously fermented cocoa beans (4), fermented cassava (5), and the gut of insects (6). *Weissella* species occur in many spontaneous plant fermentations where *Periweissella* have not been isolated, including fermented kimchi (7), carrot juice (8), and maize (9), spontaneous sourdoughs (10) or *daqu* (11).

Periweissella is also closely related to the genus *Furfurilactobacillus* and appeared to form the missing link between the former *Leuconostocaceae* and other (heterofermentative) *Lactobacillaceae*, based on a core genome phylogenetic tree of type strains in the family *Lactobacillaceae* (3). Moreover, one species in the genus *Periweissella*, *Pw. beninensis*, was described as motile (4). To date, motility has been characterized biochemically for strains in only seven of the more than 370 species of the *Lactobacillaceae* (3). Because motility is an exceptional physiological trait in the *Lactobacillaceae*, it is not routinely assessed when characterizing new strains or taxa, indicating the possibility of additional motile species in genus *Periweissella*.

The genus *Periweissella* may form the missing link between the coccoid and rod-shaped lactobacilli, but physiological and genetic traits that differentiate *Periweissella* from rod-shaped lactobacilli on the one hand and coccoid-shaped on the other are poorly described. It was, therefore, the aim of this study to investigate the genetic and physiological information on *Periweissella* which can putatively differentiate from closely related *Lactobacillaceae*. We aimed to determine the phylogenetic position of *Periweissella* relative to other heterofermentative lactobacilli and *Lactiplantibacillus plantarum,* the homofermentative type species that is most closely related to heterofermentative lactobacilli. Targeted and untargeted analysis of differentiating genetic traits were performed using genomes of type strains and relevant predicted metabolic or physiological traits were verified by analyses of the carbohydrate metabolism.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Pw. beninensis LMG 25373^T (DSM 22752), *Pw. fabalis* LMG 26217^T (DSM 28407), *Pw. fabaria* LMG 24289^T (DSM 21416), *Pw. ghanensis* LMG 24286^T (DSM 19935), *Pw. cryptocerci* LMG 32586^T (KACC 18423), and *Liquorilactobacillus vini* LMG 23202^T were cultured aerobically in de Man-Rogosa-Sharpe (MRS) medium at 30°C overnight. The selection of strains included all strains of *Periweissella* spp. for which genome sequence data were available in May 2022 when the analyses were completed.

To determine the carbohydrate fermentation profile, strains were cultured at 30°C in the carbohydrate-free medium with supplementation of different carbohydrates (glucose, fructose, arabinose, xylose, glycerol, mannitol, and 1,2-propanediol) at a concentration of 1% (wt/vol). The carbohydrate-free medium contained the following ingredients per liter: peptone, 10 g; Lab-lemco powder, 8 g; yeast extract, 4 g; Tween 80, 1 mL; tri-ammonium citrate, 2 g; sodium acetate, 5 g; $MgSO_4$, 0.2 g; $MnSO_4$, 0.05 g; and K_2HPO_4, 2 g. The final pH was adjusted to 6.2.

DNA extraction, whole genome sequencing, and assembly

DNA of strains *Pw. fabaria* LMG 24289^T and *Pw. ghanensis* LMG 24286^T was extracted using an automated Maxwell DNA preparation instrument (Promega, Madison, Wisconsin, USA). DNA extracts were treated with RNAse (2 mg/mL, 5 µL/100 µL of extract)

and incubated at 37°C for 1 hour. The DNA quality was checked using 1% agarose gel electrophoresis, and DNA quantification was performed using the QuantiFluor ONE dsDNA system and the Quantus fluorometer (Promega, USA). Draft genomes of both strains were sequenced at the MiGS center (Pittsburgh, Philadelphia, USA) using the Illumina NextSeq 2000 (PE150) platform. Quality reports were created with fastp version 0.20.0. Prior to assembly, reads were trimmed (Phred score >Q30) and filtered (length >50 bp) with fastp 0.20.0 (12) with correction option enabled. Assembly was performed with Shovill version 1.1.0, with SPAdes genome assembler 3.14.0 (13) at its core with read error correction disabled and default settings. Contigs shorter than 500 bp were removed from the final assembly. The quality of the final assembly was verified with The Quality Assessment Tool for Genome Analysis [QUAST (14)], which generates summary statistics such as the number of contigs, N50, L50, and the G + C content. Finally, the assemblies were checked for completeness and contamination using CheckM version 1.1.2 (15).

Phylogenetic analysis of type strains genomes

For the phylogenetic analysis of heterofermentative lactobacilli, genome sequences of 67 type strains of the genera *Furfurilactobacillus*, *Periweissella*, *Weissella*, *Oenococcus*, *Convivina*, *Fructobacillus*, and *Leuconostoc*, and type species of each of the heterofermentative genera in the *Lactobacillaceae* and the type strain of *Lp. plantarum* as provided on the List of Prokaryotic names with Standing in Nomenclature (https://lpsn.dsmz.de/) were downloaded from Genbank and re-annotated by Prokka (Table S1) (16). Protein sequences of all genomes were extracted, and an all-against-all blast was performed using BLASTp with the E-value as 10^{-10}. Then single-copy core gene sequences were extracted, trimmed, and concatenated into a new alignment using PGCGAP (17). Phylogenetic trees were computed by IQ-TREE (18) and were visualized by iTOL (19). Bootstrap values were calculated from 1,000 replicates. The final tree was rooted using the clade of the type strain of *Lp. plantarum* and type species of each of the genera of heterofermentative *Lactobacillaceae* as outgroup.

The pairwise average amino acid identity (AAI) between type strains was calculated by CompareM (https://github.com/dparks1134/CompareM). Sequences of soft-core genes, i.e., genes that are present in more than 90% of the genomes, were also used to determine the average amino acid identity of core proteins (cAAI) between type strains and genera. For determination of the inter-family/inter-genus relatedness, AAI or cAAI values are preferable over ANI values that are most commonly used to determine inter-genus relatedness (2).

In silico genome analysis and analysis of carbohydrate metabolism

The comparative genomic analysis of the genera *Weissella*, *Periweissella*, and *Furfurilactobacillus* was performed with all type strain genomes. Owing to the low number of type strain genomes for the genus *Furfurilactobacillus*, additional genomes of strains in the genus (Table S2) were included. Annotations from Prokka were used by Roary (20) to produce a gene presence/absence table of *Periweissella*, *Weissella,* and *Furfurilactobacillus* strains. Genome annotation by Prokka is based on curated databases including Swissprot/Uniprot to provide high quality annotations, which facilitates subsequent verification of gene function by wet-lab experimentation (21). The three genera were treated as different phenotype groups and were associated with gene presence/absence patterns using Scoary (Benjamini-Hochberg adjusted P value of < 0.05) (22). Duplicate hits to the same protein, as well as putative and hypothetical proteins were removed from the output results. Only the hits for which four or more *Periweissella* species were present were chosen. Key enzymes (23) of metabolic pathways for carbohydrate metabolism were downloaded from NCBI (Table S3) and were used as query sequences for BLAST analysis against type strain genomes of *Lactobacillaceae* (Tables S1 and S4) (3) with an amino acid identity of 40% and a query coverage of more than 70% as cut-offs. Heatmaps depicting the percentage of type species in each genus of the family

Lactobacillaceae that harbor genes for metabolic traits were drawn with R software (version 4.1.3, https://www.r-project.org/).

In silico comparison of flagellar operons in *Lactobacillaceae*

An initial assessment of whether motility operons in *Periweissella* are functional, the four motility operons in *Periweissella* were compared to the corresponding operons of seven genome-sequenced species of *Lactobacillaceae* for which motility was determined by reliable motility assays or by electronmicroscopic observation of flagellar (Table S5). Using the second contig of *Pw. ghanensis* LMG 24286^T as the template, contigs of other draft genomes were re-ordered by Mauve (24) and Benchling Biology Software. A built-in BLASTn with an E-value of 0.01 was performed to determine the % protein identity between homologous proteins in different genomes and multiple alignments of flagellar coding regions were visualized using EasyFig (25).

The amino acid sequences of 45 flagellar-related genes identified from the EasyFig analysis were downloaded from NCBI and were used as query sequences for BLAST analysis following the same procedures as for the analysis of carbohydrate metabolism. A heatmap depicting the presence and absence of genes in each strain was drawn with R software (version 4.1.3, https://www.r-project.org/).

Characterization of motility

Four *Periweissella* strains, *Pw. ghanensis*, *Pw. fabaria*, *Pw. fabalis*, and *Pw. cryptocerci*, were inoculated into the center of a tube containing semi-solid MRS medium by stabbing. The tube was incubated at 30°C overnight to observe the spreading growth of strains.

For transmission electron microscopy (TEM), bacterial cells of a one-day-old broth culture were centrifuged at 123 × *g* for 20min, washed in phosphate buffered saline (PBS) and fixed for 20min in 50 µL of 4% paraformaldehyde at pH 7.3. To prevent clumping of cells, 0.1% Triton-X 100 (Sigma) was added to the fixative. The cells were then adsorbed onto a formvar-coated copper single slot grid for 10 minutes and were rinsed twice in PBS and once in distilled water. Cells were negatively stained with a 2% aqueous solution of uranyl acetate for 10 seconds. The excess fluid was removed with a filter paper and the grid was then air-dried. TEM analysis of *Pw. beninensis* LMG 25373^T, *Pw. fabalis* LMG 26217^T, *Pw. fabaria* LMG 24289^T, *Pw. ghanensis* LMG 24286^T, and *Pw. cryptocerci* LMG 32586^T was performed using a Jeol JEM 1010 transmission electron microscope at 60 kV, equipped with a CCD side-mounted Veleta camera (Emsis, Münster, Germany).

For scanning electron microscopy (SEM) of *Pw. beninensis* LMG 25373^T, 10 µL of a 1-day-old broth culture was spotted on a polycarbonate Whatman Nuclepore Track-Etched Membrane with a pore size of 0.4 µM (Merck, Darmstadt, Germany), dried overnight, and after a short wash in 0.1M sodium cacodylate buffer at pH 7.4 (VWR International, Leuven, Belgium) fixed in freshly prepared fixative (2% paraformaldehyde, VWR), 2.5% glutaraldehyde (VWR) in 0.1 M cacodylate buffer, pH 7.4, at room temperature for 1 hour. The fixative was removed by washing 3 × 5 minutes in 0.1 M cacodylate buffer and samples were then incubated in 2% osmium tetroxide (VWR) in 0.1 M cacodylate buffer for 30 minutes at room temperature. After washing in water for 3 × 5 minutes, the sample was dehydrated using solutions of increasing ethanol concentration (50%, 70%, 85%, 95%, 2 × 100%), for 15 minutes each. Samples were then critical point dried (Leica EM CPD300) and coated with a thin layer of gold or platinum (Quorum Q150T ES) before imaging with a scanning electron microscope (Crossbeam 540, Zeiss, Jena, Germany). Images were taken using a SE2 detector at 1.5 kV.

Carbohydrate fermentation profile

Type strains of *Pw. beninensis, Pw. fabalis, Pw. fabaria,* and *Pw. ghanensis* were tested for their ability to metabolise glucose and fructose. *Liqorilactobacillus vini* LMG 23202^T was used as a control strain. Each carbohydrate was added to 10 mL of carbohydrate-free MRS medium at a concentration of 1% (wt/vol) in the case of arabinose, fructose, glucose,

and xylose, and of 10 mM in the case of glycerol, mannitol, and 1,2-propanediol. All media were prepared with and without 1% glucose, so that a total of 13 different fermentation conditions were tested. After 48 hours of incubation at 30°C, the cultures were centrifuged at 4,696 × *g* for 20 minutes and the supernatants were kept at −20°C until further analysis. All incubation tests were performed in triplicate. The concentrations of carbohydrates (arabinose, fructose, glucose, and xylose) and sugar alcohols (arabitol, erythritol, glycerol, mannitol, and sorbitol) were quantified by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). To this end, 100 µL of cell-free culture supernatant was mixed with 900 µL of deproteinization solution, consisting of 1 L of acetonitrile (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and 0.05 g of fucose as internal standard (IS). Samples were then vortexed for 2 minutes, centrifuged at 19,000 × *g* for 15 minutes, and filtered using 0.2 µM H-PTFE filters (Millex; Merck). Ethanol, acetic acid, and lactic acid concentrations were quantified using high-performance liquid chromatography with refractive index detection (HPLC-RI) as described previously (26). In this case, the deproteinization step was performed by adding 300 µL of Carrez A solution [36 g/L of $K_4Fe(CN)_6.3H_2O$] and 300 µL of Carrez B solution (72 g/L of $ZnSO_4.7H_2O$) to 600 µL of cell-free culture supernatant. The quantification of 1,2-propanediol was performed by liquid injection gas chromatography with tandem mass spectrometry (LI-GC-MS/MS). In this case, 100 µL of sample were mixed with 900 µL of deproteinization solution, consisting of 1 L of acetone (Merck) with 50 µg of deuterated 3-methyl-1-butanol, 2,3-butanedione, and ethyl decanoate (CDN Isotopes, Pointe-Claire, Quebec, Canada) as IS. The quantification of all compounds mentioned above was performed by external calibration.

RESULTS

Whole genome sequencing

The assembly of the Illumina NextSeq 150 bp paired-end reads resulted in assemblies of 19 (*Pw. fabaria* LMG 24289^T) and 66 (*Pw. ghanensis* LMG 24286^T) contigs with N50 values of 201 Kb and 59 Kb and a total genome size of 1.93 Mb and 1.99 Mb, respectively. Both genomes showed more than 98% completeness and less than 1% contamination as determined using CheckM.

Phylogenetic analysis of the genera *Furfurilactobacillus, Periweissella, Weissella, Oenococcus, Convivina, Fructobacillus,* and *Leuconostoc*

The description of the genus *Periweissella* was based on phylogenetic comparison with only the genera *Weissella*, *Oenococcus*, *Convivina*, *Fructobacillus* and *Leuconoctoc* (4); however, these genera form a monophyletic clade together with other heterofermentative *Lactobacillaceae* (3). The tree calculated in this study also clearly separated lactobacilli and the former *Leuconostocaceae* (Fig. 1). The *Lactobacillaceae,* however, are monophyletic only if the former *Leuconostocaceae* are included (Fig. 1). We, therefore, extended the phylogenetic analyses of *Periweissella* to include the genus *Furfurilactobacillus* and the type species of all 10 other heterofermentative genera using *Lactiplantibacillus* as outgroup (Fig. 1). *Periweissella* clustered between *Furfurilactobacillus* and *Weissella* and thus provided the phylogenetic link of the coccoid heterofermentative *Lactobacillaceae*, formerly *Leuconostocaceae,* to other heterofermentative *Lactobacillaceae*. The pairwise AAI and cAAI values for the type strains of heterofermentative *Lactobacillaceae* were also calculated (Fig. 2). The intra-genus AAI and cAAI values of *Periweissella* were higher than 68% and 74%, respectively (Tables S6 and S7). The genera *Periweissella* and *Weissella* were exclusive, i.e., the lowest intra-genus cAAI values were higher than the highest inter-genus cAAI of species in these genera (Fig. S1). The inter-genus AAI values for the genera *Periweissella* and *Furfurilactobacillus* were in a range of 57.53%–59.21% (average 58.30%) and the corresponding AAI values of the genera *Periweissella* and *Weissella* were in a range of 57.59%–60.00% (average 58.43%), again indicating that the genus *Periweissella* is about as closely related to *Furfurilactobacillus* as it is to *Weissella*.

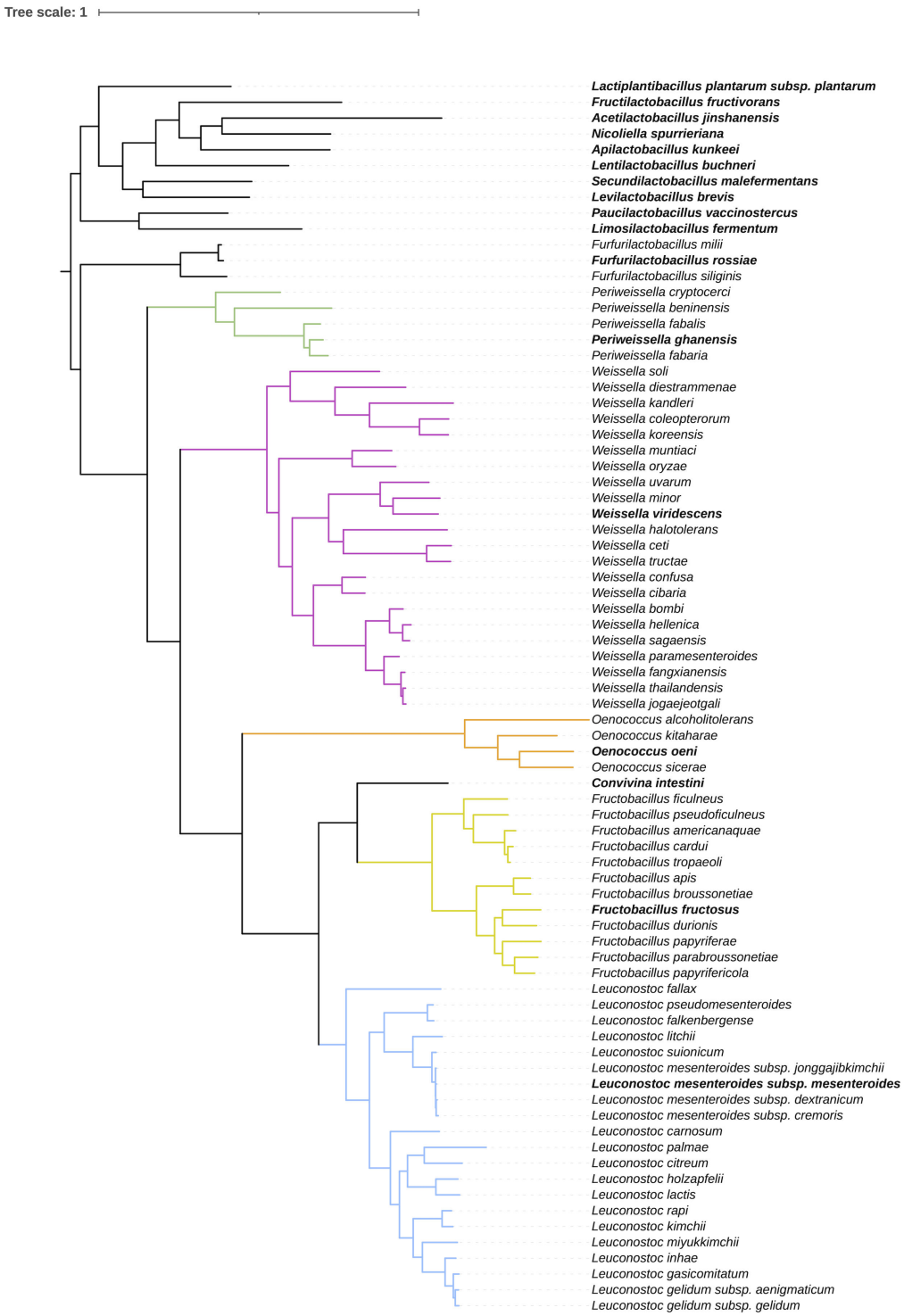

FIG 1 Core genome phylogenetic tree of 50 type strains of the genera *Furfurilactobacillus*, *Periweissella*, *Weissella*, *Oenococcus*, *Convivina*, *Fructobacillus*, and *Leuconostoc*. The type strain of *Lactiplantibacillus plantarum* and type species of each of the heterofermentative genera in the *Lactobacillaceae* were used as outgroups. The maximum likelihood tree is based on the concatenated alignment of protein sequences from single-copy core genes and was inferred by IQ-TREE as described in Zheng et al. (2). Species of the same genus are indicated by the same color code for branches and the type species of each genus is printed in bold. The genomes used for the graph are provided in the Table S1.

Targeted genomic analysis of the metabolic potential of *Periweissella*

To investigate the metabolic potential of *Periweissella*, key enzymes for carbohydrate metabolism (3, 27) in the genus *Periweissella* as well as in other genera of *Lactobacillaceae* were identified by protein BLAST (Fig. 3). Fructose bisphosphate aldolase was only present in the genome of *Pw. cryptocerci*. *Periweissella* was the only genus in heterofermentative lactobacilli other than *Weissella* in which mannitol phosphate dehydrogenase was identified (28). The gene coding for mannitol dehydrogenase, which enables the conversion of fructose to mannitol to regenerate reduced co-factors in heterofermentative lactobacilli (27) was found in the genomes of *Pw. beninensis*, *Pw. fabaria*, and *Pw. fabalis* but not in those of *Pw. ghanensis* and *Pw. cryptocerci* (Fig. 3). Glycerol dehydratase (28) was present in the genome of *Pw. cryptocerci*. *Pw. cryptocerci* does not produce acid from glycerol (29), but its ability to convert glycerol to 1,3 propanediol remains unknown. Glycerol dehydratase (29) was also present in the genomes of several *Furfurilactobacillus* species but absent in *Weissella, Oenococcus*, *Convivina*, *Fructobacillus,* and *Leuconoctoc*. Dextransucrases were frequently present in the genomes of *Weissella, Oenococcus*, *Convivina,* and *Leuconoctoc* but absent in *Periweissella*.

Untargeted genomic analysis of the metabolic potential of *Periweissella*

To identify other genes that contribute to the metabolic and ecological traits of *Periweissella*, Roary and Scoary were used to identify differentially distributed genes in the core genome of *Periweissella* and the two closest genera, *Furfurilactobacillus* and *Weissella*. Around 400 genes were differentially distributed among the three genera (Tables S8 and S9). Of these, 34 genes related to carbohydrate metabolism, drug resistance, and bacterial motility were further selected (Table 1). Motility-related genes were present in four out of five *Periweissella* strains, but not in *Furfurilactobacillus* or *Weissella*. Several ribose metabolism-related genes were present in *Periweissella* strains but absent from all *Weissella* strains. Genes coding for multidrug resistance, maltose phosphorylase, and glycerol dehydrogenase were found only in *Furfurilactobacillus* strains.

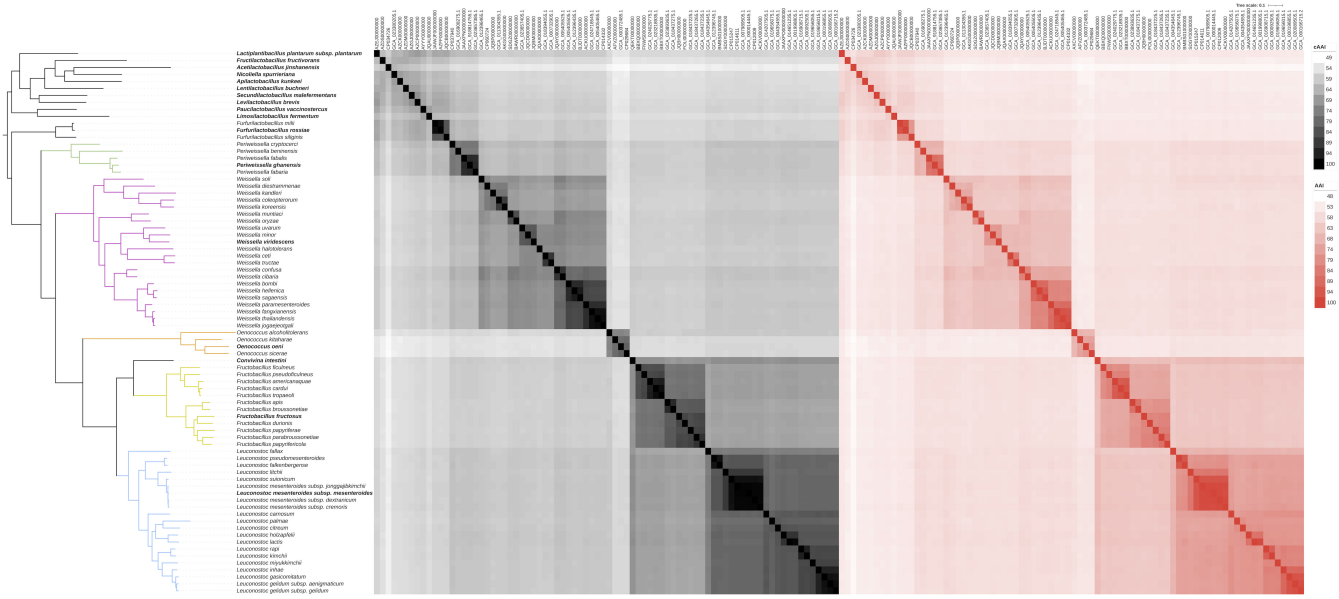

FIG 2 Heatmap of the average amino acid identity (AAI) and average amino acid identity of core proteins (cAAI) values between 50 type strains of the genera *Furfurilactobacillus*, *Periweissella*, *Weissella*, *Oenococcus*, *Convivina*, *Fructobacillus*, and *Leuconostoc*. The type strain of *Lactiplantibacillus plantarum* and type species of each of the heterofermentative genera in the *Lactobacillaceae* were used as outgroups. Rows and columns are clustered according to the phylogenetic tree. Values of AAI and cAAI are shown in black and red, respectively. The data used for the graph are provided in Tables S6 and S7.

FIG 3 Heatmap depicting the percentage of type species in each genus of the family *Lactobacillaceae* that harbor genes for metabolic traits. Red indicates the gene is present in all type strains of a genus and white indicates the gene is absent in all type strains. Genes used for the graph are provided in Table S3. Genes are indicated as follows: Fba, aldolase; Pfk, phosphofructokinase; Mpd, mannitol-phosphate-dehydrogenase; Pkl, phosphoketolase; Mdh, mannitol dehydrogenase; Adh2, two-domain alcohol dehydrogenase; Adh1, alcohol dehydrogenase; Pfl, pyruvate formate lyase; Tal, transaldolase; Tak, transketolase; LdhD, lactaldehyde dehydrogenase; PduE,; PduD, glycerol dehydratase subunit PduD; GapI, galactose-6-phosphate isomerase; GalT; galactose-1-phosphate-uridyltransferase; Udp, UDP-4-galactose-epimerase; OrdC, ornithine decarboxylase; AgdI, agmatine deiminase; Dex, dextransucrase; Lev, levansucrase.

Phenotypic carbohydrate fermentation profile of *Periweissella*

The carbohydrate fermentation profiles of *Pw. beninensis, Pw. fabalis, Pw. fabaria,* and *Pw. ghanensis* type strains were tested by detailed quantification of products and metabolites of carbohydrate metabolism. Glucose was metabolized to lactate and acetate or

TABLE 1 Gene presence and absence from untargeted genomic analysis of the genera *Furfurilactobacillus*, *Periweissella*, and *Weissella*[b]

Annotation[a]	Gene	Accession no. used for verification	Traits		
			Furfuri-lactobacillus	*Periweissella*	*Weissella*
Maltose phosphorylase	MalP	QJU50634.1	+	–	
Glycerol dehydrogenase	GldA	ARW49736.1	+	–	
Multidrug efflux system permease	MesP	CAH0417302.1	+	–	
Multidrug resistance protein MdtH	MdtH	GFI63498.1	+	–	
Multidrug resistance protein MdtL	MdtL	QRQ96649.1	+	–	
Multidrug transporter EmrE	EmrE	KRN47543.1	–	+	–
Chemoreceptor glutamine deamidase CheD	CheD	CAH0417903.1	–	+	–
Chemotaxis protein CheA	CheA	GFI63782.1	–	+	–
Chemotaxis protein CheW	CheW	WP_235719183.1	–	+	–
Chemotaxis protein CheY	CheY	KRN88084.1	–	+	–
Chemotaxis protein methyltransferase	CheM	CAH0417905.1	–	+	–
Chemotaxis protein PomA	PomA	CAH0417878.1	–	+	–
CheY *P* phosphatase CheC	CheC	CAH0417907.1	–	+	–
Flagellar basal body rod protein FlgB	FlgB	CAH0417879.1	–	+	–
Flagellar basal body rod protein FlgC	FlgC	WP_235315572.1	–	+	–
Flagellar basal body rod protein FlgG	FlgG	AUJ33046.1	–	+	–
Flagellar biosynthesis protein FlhA	FlhA	WP_057895838.1	–	+	–
Flagellar biosynthetic protein FlhB	FlhB	BBA81011.1	–	+	–
Flagellar biosynthetic protein FliP	FliP	CAH0417893.1	–	+	–
Flagellar biosynthetic protein FliR	FliR	WP_235315560.1	–	+	–
Flagellar hook basal body complex protein	FliE	WP_235807225.1	–	+	–
Flagellar motor switch protein FliG	FliG	WP_235315569.1	–	+	–
Flagellar motor switch protein FliM	FliM	WP_224288758.1	–	+	–
Flagellar M ring protein	FlmR	CAH0417882.1	–	+	–
Flagellar secretion chaperone FliS	FliS	CAH0417922.1	–	+	–
L asparaginase 1	Ans	GFI59533.1		–	+
Ribose import ATP-binding protein RbsA	RbsA	CAH0416239.1		+	–
Ribose import binding protein RbsB	RbsB	GFI19485.1		+	–
Ribose import permease protein RbsC	RbsC	CAH0416238.1		+	–
Multidrug export protein EmrB	EmrB	GFI63251.1		+	–
Multidrug export protein MepA	MepA	GFI60335.1	+	+	–
L fucose proton symporter	FucP	GFI59483.1	+	+	–

[a]Scoary additionally identified ribokinase and ribose-5-phosphate isomerase A as present in *Periweissella* but absent in *Weissella*; however, the corresponding genes are present in *Weissella* but with an amino acid identify of less than 50%.
[b]The presence and absence of genes is indicated by "+" and "–", respectively.Cells are blank if they are not differentiated between two of the three genera.

ethanol (Table 2), matching the pattern of metabolites in heterofermentative hexose metabolism via the phosphoketolase pathway (Fig. 3). Xylose and arabinose as the sole carbohydrate sources were not metabolized, in agreement with the absence of the gene for xylose isomerase (XylA) and the gene for L-arabinose isomerase (AraA) in the genomes of all type strains tested. However, these pentoses were metabolized when glucose was also present, especially xylose. None of the strains consumed 1,2-propanediol or glycerol, matching the absence of glycerol dehydratase, glycerol dehydrogenase or the aerobic α-glycerophosphate oxidase in the genomes of the four strains evaluated (Fig. 3). 1,2-Propanediol was not produced by any of the strains, matching the absence of lactaldehyde dehydrogenase. When glucose and fructose were present as substrates, fructose was reduced to mannitol with concomitant formation of acetate instead of ethanol (Table 2). The *Pw. fabalis* and *Pw. fabaria* type strains used fructose as carbon source; in these fermentations, fructose was partially reduced to mannitol and partially converted to lactate and acetic acid or ethanol. Despite the presence of mannitol phosphate dehydrogenase in the genomes of the *Pw. ghanensis and Pw. fabalis* type strains, none was able to use mannitol as carbon source when mannitol was present as the sole carbon source or in combination with glucose.

TABLE 2 Carbohydrate consumption, indicated by (–) and metabolite production, indicated by (+) after 48 hours fermentation in modified MRS media[a]

Substrates/ metabolites (mM)	Mean consumption/production ± standard deviation (mM)				
	Pw. beninensis LMG 25373^T	*Pw. fabalis* LMG 26217^T	*Pw. fabaria* LMG 24289^T	*Pw. ghanensis* LMG 24286^T	Lq. vini LMG 23202^T
Fructose and glucose					
Glucose (–)	69 ± 0	70 ± 2	57 ± 2	69 ± 1	21 ± 3
Fructose (–)	58 ± 0	33 ± 0	29 ± 7	58 ± 0	9 ± 4
Mannitol (+)	39 ± 10	39 ± 6	9 ± 1	46 ± 7	0*
Lactate (+)	64 ± 5	96 ± 2	85 ± 12	80 ± 11	44 ± 5
Acetate (+)	23 ± 4	34 ± 3	21 ± 6	38 ± 8	0
Ethanol (+)	11 ± 1	58 ± 12	38 ± 17	13 ± 6	0*
Fructose					
Fructose (–)	23 ± 6	53 ± 1	48 ± 4	19 ± 1	9 ± 5
Mannitol (+)	0	22 ± 3	16 ± 2	0	0
Lactate (+)	20 ± 3	37 ± 0	43 ± 4	22 ± 2	35 ± 3
Acetate (+)	0	10 ± 2	8 ± 2	0	0
Ethanol (+)	7 ± 3	8 ± 0	10 ± 4	9 ± 3	0

[a]Concentrations are expressed as the difference between the concentrations in the negative control (not inoculated) and in the respective sample ± standard deviation.

Comparison of flagellar operons of *Periweissella* as well as all reported motile strains of the family *Lactobacillaceae*

Prior to the comparison of the motility operons of four *Periweissella* type strains together with seven other motile lactobacilli (Table S5), the motility genes of the *Periweissella* species were investigated for their organization into single operons. In *Pw. ghanensis* LMG 24286^T, genes related to motility were located on only one contig, whereas they were distributed on several contigs in all other *Periweissella* genomes examined. Therefore, the operon of *Pw. ghanensis* LMG 24286^T was used to identify the presence and organization of motility operons in other genomes (Fig. 4). Among the five type strains of *Periweissella*, *Pw. beninensis* LMG 25373^T was the only strain for which motility was described before (5). The motility operon contained 45 genes involved in flagellar structure, function, and chemotaxis (Fig. 4). Of these 45 genes, 34 genes were also present in *Pw. fabalis* LMG 26217^T and *Pw. fabaria* LMG 24289^T, in a nearly identical organization (Fig. 4). The number and organization of motility genes in *Pw. beninensis* differs substantially from the other three strains and motility genes were absent in *Pw. cryptocerci* LMG 32586 (Fig. 4). Since May 2022, the genome of *Pw. beninensis* 716 became additionally available (Genbank accession number GCA_025211165.1). The genome of *Pw. beninensis* 716 also encodes for a motility operon that shares 98.96% nucleotide identity with the operon of the type strain and has an identical organization of motility genes (data not shown).

Phenotypic identification of flagellum-mediated motility of *Periweissella*

To confirm the motility of the *Periweissella* type strains, semi-solid agar assays, TEM, and SEM were conducted. The strains *Pw. ghanensis* LMG 24286^T, *Pw. fabaria* LMG 24289^T, and *Pw. fabalis* LMG 26217^T were motile in the semi-solid agar assay and TEM revealed flagellated cells, whereas *Pw. cryptocerci* LMG 32586^T was non-motile and no flagellar structures were detected (Fig. 5). *Pw. beninensis* LMG 25373^T failed to grow under the semi-solid agar conditions, but flagellar filaments of *Pw. beninensis* LMG 25373^T were observed by TEM (Fig. 5) and SEM (data not shown), which was consistent with the presence of motility operons in its genome. TEM analysis showed thin filaments which were positioned along the length of the cells, indicating the swimming motility of these bacteria by the rotation of flagellar filaments (30). Among four motile strains, *Pw. fabalis* LMG 26217^T was considered to have a highly motile ability from its moving at exponential phase in semi-solid MRS medium.

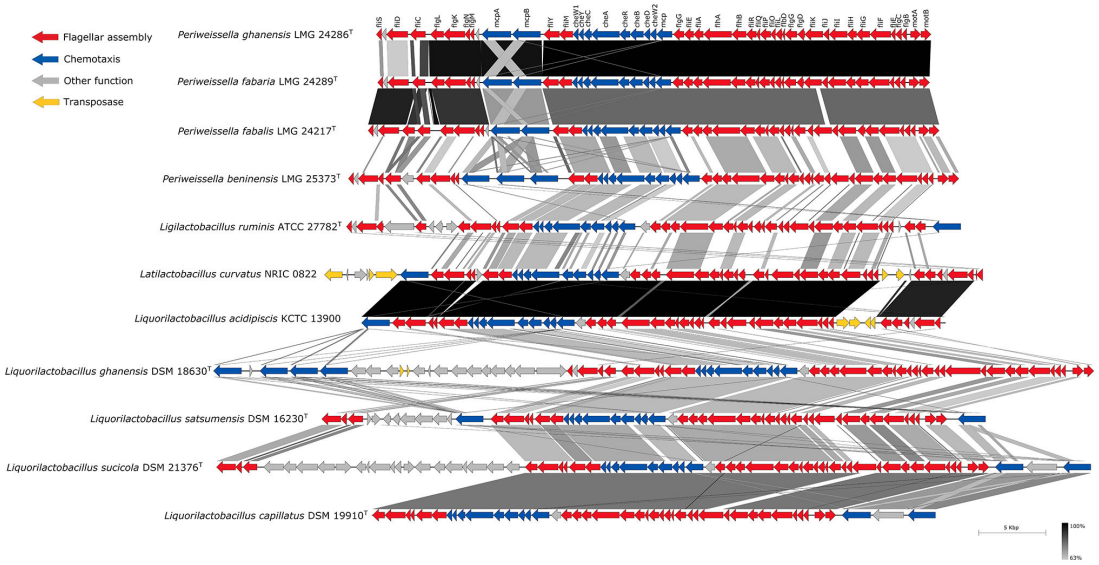

FIG 4 Comparison of genetic loci coding for flagellar-related proteins in type strains of four *Periwissella* species as well as seven reported motile lactobacilli strains. Shades of gray of connecting lines represent percent blast identity according to the scale on the right. *Periwissella cryptocerci* LMG 32586^T does not contain any flagellar-related proteins. *Pw. beninensis* LMG 25373^T was re-ordered, and *Pw. fabalis* LMG 26217^T was reversed along with the template of *Pw. fabaria* LMG 24289^T using Mauve (version 2.4.0) (24). Genomes used for the graph are provided in Table S5.

DISCUSSION

This study determined the genetic and physiological properties that may differentiate *Periweissella* from other heterofermentative *Lactobacillaceae* by genomic and

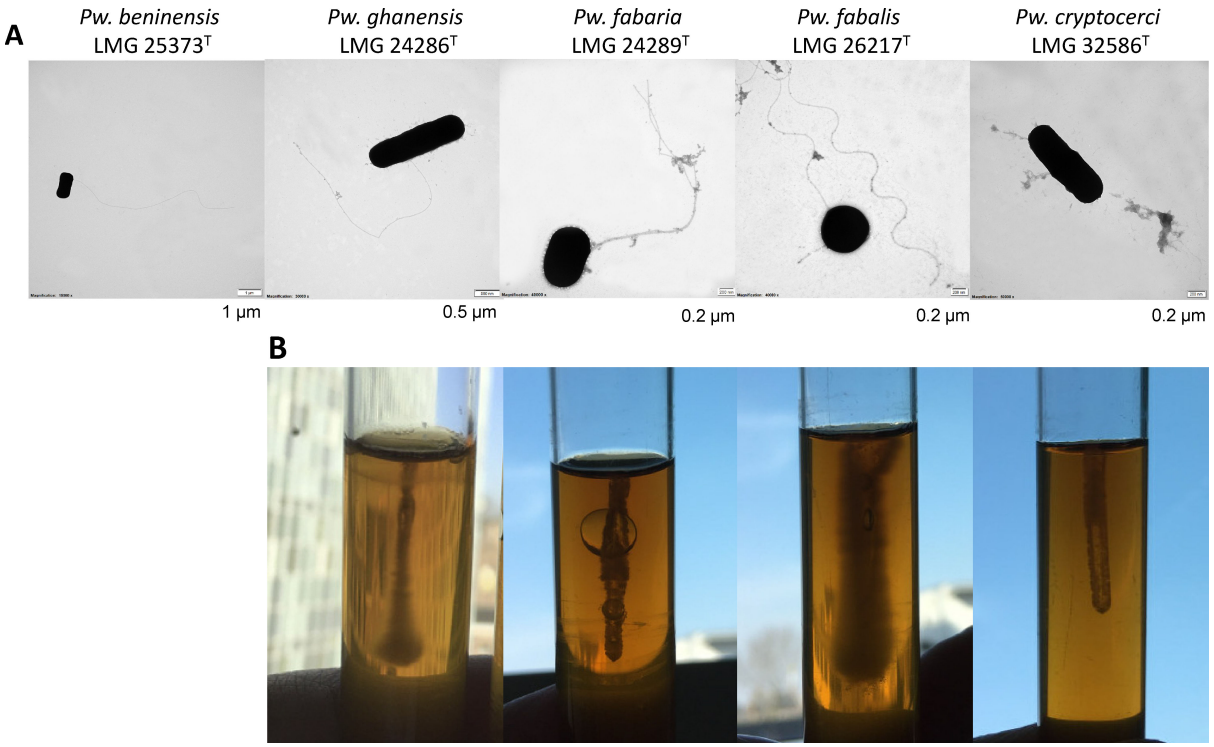

FIG 5 Flagellar and motility of strains in the genus *Periwissella*. (A) Transmission electron micrographs of five *Periwissella* strains. The size of the scale bar at the bottom right is indicated below the images. (B) Semisolid MRS medium assays in tubes for *Pw. fabalis* LMG 26217^T, *Pw. fabaria* LMG 24289^T, *Pw. ghanensis* LMG 24286^T and *Pw. cryptocerci* LMG 32586^T. *Pw. beninensis* did not grow in the semi-solid MRS medium.

physiological analyses. The phylogenetic position of *Periweissella* documents that the genus forms missing link between *Leuconostoc*, *Oenococcus*, *Convivina, Fructibacillus,* and *Weissella* and other heterofermentative *Lactobacillaceae*. The genus *Periweissella* was also found to be only the second predominantly motile genus in addition to *Liquorilactobacillus* (31–34), thus providing an important physiological trait that distinguishes most *Periweissella* species from all other heterofermentative lactic acid bacteria (4).

The proposal of the genus *Periweissella* was based on the phylogenetic analysis of coccoid lactobacilli (4). By including other heterofermentative lactobacilli in the analysis, the phylogenetic tree as well as cAAI and AAI values of the present study showed that the phylogenetic relationship of the new genus *Periweissella* to *Furfurilactobacillus* species is as close as the relationship to *Weissella* species. Addition of the genomes of *Periweissella* species to the core genome phylogenetic tree of the *Lactobacillaceae* also demonstrated that all heterofermentative lactic acid bacteria form a monophyletic clade among the *Lactobacillaceae* (3), indicating that the transition from homofermentation to heterofermentation was a unique and irreversible event in the evolution of lactic acid bacteria.

Periweissella have the shape of short, coccoid rods or of cocci. In the *Lactobacillaceae,* the coccoid or coccus-shaped genera *Leuconostoc, Oenococcus, Weissella,* and *Periweissella* each form a monophyletic clade. The genus *Pediococcus* and the species *Lapidilactobacillus dextrinicus, Loigolactobacillus coryniformis,* and *Limosilactobacillus equigenerosi* are also monophyletic and have been described as cocci or as short coccoid rods (2). Analyses of the orders *Bacilli* and *Erysipelotrichia* indicated that the transition of rod to coccus is essentially irreversible (35). This assumption is only partially congruent with cell morphology in the *Lactobacillaceae*. Most clades with coccoid morphology have a rod-shaped ancestor; however, the rod-shaped genera *Fructobacillus* and *Convivina* appear to have coccus-shaped ancestors (Fig. 1) (3), thus indicating the possibility of a coccus-to-rod transition.

The phylogenetic position of a genus in the *Lactobacillaceae* family often relates to its physiological and metabolic potential as well as its ecology (23, 36), but only limited information is available on differentiating ecological and metabolic properties of the genus *Periweissella* (4). This study analyzed the metabolic potential with a genome data set that was rarefied to include only type strains and additional genome sequences of furfurilactobacilli. With very few exceptions, genome sequences are available for the over 340 type strains in the *Lactobacillaceae,* but for most species, the type strain is the only strain with genome sequence data. Less than 30 species, e.g., *Lactiplantibacillus plantarum*, *Levilactobacillus brevis*, *W. cibaria,* and *W. confusa,* account for more than half of the 5,000 genomes of strains in the *Lactobacillaceae* that are available on NCBI. Rarefaction of the genome data set is necessary to have equal representation of each species (23). Rarefaction by type strains, however, also prevents elucidation of the full species-level metabolic diversity. *Periweissella* species are heterofermentative and follow the common genus-level trait proposed by Zheng et al. (23). The phosphoketolase gene is present in all *Periweissella* species, indicating carbohydrate metabolism via the phosphoketolase pathway (27), which matches the phenotype that all tested *Periweissella* strains can grow with glucose as the sole carbon source and produce lactate and ethanol.

Most heterofermentative *Lactobacillaceae* express mannitol dehydrogenase to use fructose as electron acceptor if other hexoses or disaccharides are present (3, 23). In contrast, mannitol phosphate dehydrogenase, which supports the use of mannitol as carbon source, was detected exclusively in homofermentative *Lactobacillaceae* (3, 23, 37). The presence of mannitol phosphate dehydrogenase in *Periweissella* is thus a feature that distinguishes *Periweissella* from all other heterofermentative *Lactobacillaceae,* including the genera *Weissella* and *Furfurilactobacillus*. Acid production from mannitol by resting cells has been described for *Pw. beninensis* and *Pw. fabaria*; however, genes coding for mannitol-phophate-dehydrogenase are present in *Pw. ghanensis* and *Pw. fabalis* (5, 38, 39). Our study demonstrates, however, that mannitol does not support growth of

these two species when offered as sole carbon source (Fig. 3; Table 2). The analysis of carbohydrate fermentation by lactobacilli can provide different results when conversion by resting cells or growth is compared (40). *Periweissella* and the closely related genera *Furfurilactobacillus* and *Weissella* are among the five exceptional genera of heterofermentative *Lactobacillaceae* that include type strains that lack mannitol dehydrogenase (3, 23) (Fig. 3). Accordingly, in the presence of fructose as the sole carbon source, only the type strains of *Pw. fabalis* and *Pw. fabaria* converted fructose into lactate, acetate, and mannitol via the phosphoketolase pathway and mannitol dehydrogenase (Table 2). The strain *Pw. beninensis* LMG 25373^T did not produce mannitol when fructose was present as the sole carbon source; however, mannitol was produced when both glucose and fructose were available as substrates. Remarkably, *Pw. ghanensis* LMG 24286^T also produced mannitol when glucose as well as fructose were present as substrate, although mannitol dehydrogenase was not identified in the genome of *Pw. ghanensis* LMG 24286^T (Fig. 3; Table 2) when using the biochemically characterized proteins from *Limosilactobacillus reuteri* (41) or *Fructilactobacillus sanfranciscensis* (42) as BLAST query sequences. *Pw. ghanensis* LMG 24286^T encodes for mannitol-phosphate dehydrogenase, which converts fructose-6-phosphate into mannitol 1-phosphate (Fig. 3). Mannitol 1-phosphate is converted into mannitol by a phosphatase without recovery of the chemical energy of the phosphate bond, which is energetically unfavorable (28, 43). Although the phenotype for carbohydrate fermentation in strains of *Periweissella* largely matched the genotype, the fate of fructose in those *Periweissella* strains that encode both mannitol dehydrogenase and mannitol 1-phosphate dehydrogenase (*Pw. fabalis* LMG 26217^T) or only mannitol 1-phosphate dehydrogenase (*Pw. ghanensis* LMG 24286^T) remains subject of future studies.

Pangenomes of *Periweissella* and the two closest genera, *Weissella* and *Furfurilactobacillus*, were compared with the aim to elucidate the genes that are differentially distributed between these genera. Flagellar-related genes are exclusive to *Periweissella*; the L-asparaginase gene is exclusive to *Weissella*. Genes coding for the multidrug export protein MepA and the L-fucose proton symporter are present in both *Furfurilactobacillus* and *Periweissella* but are absent in *Weissella*. Considering the mismatching of motility genes and the motile phenotype in *Ligilactobacillus ruminis* (44) and the disruption of transposase in the motility operon in *Lactobacillus hordei* (45), the motility of four strains of *Periweissella* were further investigated by the semi-soft agar and TEM analyses. Four *Periweissella* strains were shown to be motile and / or to express flagella, which is consistent with the presence of flagellar operons.

The high similarity of motility operons in *Latilactobacillus curvatus* and *Ligilactobacillus acidipiscis* as well as the presence of mobile genetic elements flanking the *Lt. curvatus* NRIC 0822 motility operon suggested recent acquisition of motility in these two species (45). In contrast, our analysis provided no indication for the presence of mobile genetic elements in motility operons in the genera *Periweissella* and *Liquorilactobacillus*, or differences between the G + C content of the motility operon and the entire genome. Bioinformatic analyses indicated that the loss of motility is much more likely than acquisition of motility (35). Taken together, motility was likely acquired independently by the ancestral species of the genera *Periweissella* to *Liquorilactobacillus* but lost again in some of the progeny.

The comparison of free-living and host-associated bacteria has demonstrated that loss of motility is associated with the transition from a free-living to a host-associated lifestyle (35). In addition, the expression of motility genes by intestinal microorganisms was reduced by mucosal anti-flagellin antibodies (46). To date, several species in four genera of the *Lactobacillaceae* have been shown to be motile. Motile strains are frequent in the genera *Periweissella* and *Liquorilactobacillus*, much less frequent in the genus *Ligilactobacillus* and only one strain of *Lt. curvatus* has been described as motile (5, 31–34, 38, 45, 47–52). Several of these references, however, relate to species new descriptions without providing detail on methods and results. This study in conjunction with past studies clearly establishes that motility of lactobacilli is a physiological trait

that relates to phylogeny and ecology of the organisms (5, 31–34, 38, 45, 47–52). The vertebrate gut adapted genera *Lactobacillus* and *Limosilactobacillus* are not known to include motile species or strains. Of the four motile genera, only *Ligilactobacillus* consists predominantly but not exclusively of vertebrate host-adapted species; *Latilactobacillus* and those species of *Liquorilactobacillus* for which sufficient information is available were designated as free-living organisms (36). The lifestyle of species in the genus *Periweissella* is unknown and most isolates were obtained from fermented plant foods, which suggests a free-living lifestyle in association with plants (5, 32, 38, 48, 53). The source of isolation of motile *Periweissella* species partially overlaps with the source of isolation of *Liquorilactobacillus* (31, 32, 34). Conversely, *Periweissella* does not share the source of isolation with closely related heterofermentative lactobacilli including *Leuconostoc*, *Weissella*, *Furfurilactobacillus,* or *Levilactobacillus*. The similarity of sources for motile *Periweissella* species and *Liquorilactobacillus* species is indicative of the importance of flagellum-mediated motility and the advantages that likely accompany this trait, such as niche colonization or biofilm formation (44).

In conclusion, this study provides a more comprehensive insight into the phylogenetic and physiological properties of *Periweissella* and builds a link to connect *Periweissella* and other heterofermentative lactobacilli. *Periweissella* is proposed as the second predominantly motile genus among lactobacilli. These findings will promote the understanding and industrial application of strains of this new genus.

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AUTHOR CONTRIBUTIONS

Nanzhen Qiao, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft | Julia Bechtner, Formal analysis, Methodology, Validation, Writing – review and editing | Margo Cnockaert, Formal analysis, Investigation, Methodology, Writing – review and editing | Eliza Depoorter, Formal analysis, Investigation, Methodology, Writing – review and editing | Christian Díaz-Muñoz, Formal analysis, Methodology, Visualization | Peter Vandamme, Investigation, Resources, Supervision, Validation, Writing – review and editing | Luc De Vuyst, Data curation, Funding acquisition, Methodology, Supervision, Writing – review and editing | Michael G. Gänzle, Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review and editing

DATA AVAILABILITY

The raw reads and annotated assemblies of Pw. fabaria LMG 24289^T and Pw. ghanensis LMG 24286^T have been publicly deposited under the BioProject accession number PRJEB48651 and the GenBank accession numbers CAKKNS000000000 and CAKKNT000000000, respectively.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Figures S1 and S2 (AEM01034-23-S0001.pdf). Figures S1 and S2 and supplemental table legends
Table S1 (AEM01034-23-S0002.xlsx). Table S1
Table S2 (AEM01034-23-S0003.xlsx). Table S2
Table S3 (AEM01034-23-S0004.xlsx). Table S3
Table S4 (AEM01034-23-S0005.xlsx). Table S4
Table S5 (AEM01034-23-S0006.xlsx). Table S5
Table S6 (AEM01034-23-S0007.xlsx). Table S6
Table S7 (AEM01034-23-S0008.xlsx). Table S7
Table S8 (AEM01034-23-S0009.xlsx). Table S8
Table S9 (AEM01034-23-S0010.xlsx). Table S9

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