

Effect of Lineage-Specific Metabolic Traits of *Lactobacillus reuteri* on Sourdough Microbial Ecology

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This study determined the effects of specific metabolic traits of *Lactobacillus reuteri* on its competitiveness in sourdoughs. The competitiveness of lactobacilli in sourdough generally depends on their growth rate; acid resistance additionally contributes to competitiveness in sourdoughs with long fermentation times. Glycerol metabolism via glycerol dehydratase (*gupCDE*) accelerates growth by the regeneration of reduced cofactors; glutamate metabolism via glutamate decarboxylase (*gadB*) increases acid resistance by generating a proton motive force. Glycerol and glutamate metabolisms are lineage-specific traits in *L. reuteri*; therefore, this study employed glycerol dehydratase-positive sourdough isolates of human-adapted *L. reuteri* lineage I, glutamate decarboxylase-positive strains of rodent-adapted *L. reuteri* lineage II, as well as mutants with deletions in *gadB* or *gupCDE*. The competitivenesses of the strains were quantified by inoculation of wheat and sorghum sourdoughs with defined strains, followed by propagation of doughs with a 10% inoculum and 12-h or 72-h fermentation cycles. Lineage I *L. reuteri* strains dominated sourdoughs propagated with 12-h fermentation cycles; lineage II *L. reuteri* strains dominated sourdoughs propagated with 72-h fermentation cycles. *L. reuteri* 100-23 Δ *gadB* was outcompeted by its wild-type strain in sourdoughs fermented with 72-h fermentation cycles; *L. reuteri* FUA3400 Δ *gupCDE* was outcompeted by its wild-type strain in sourdoughs fermented with both 12-h and 72-h fermentation cycles. Competition experiments with isogenic pairs of strains resulted in a constant rate of strain displacement of the less competitive mutant strain. In conclusion, lineage-specific traits of *L. reuteri* determine the competitiveness of this species in sourdough fermentations.

Sourdough fermentation with lactic acid bacteria has been used as a leavening agent in artisanal baking, and sourdough or sourdough products are increasingly being applied as a baking improver in industrial baking (1). In artisanal as well as industrial practice, sourdoughs are generally maintained by continuous back-slopping. The formation of a stable sourdough microbiota consisting of lactic acid bacteria alone or in association with yeasts depends on complex interactions between the inoculum, the cereal substrate, processing parameters, and the environment (2). Type I doughs are propagated by frequent inoculation at ambient temperatures. The use of type I sourdoughs as leavening agents requires fermentation processes that maintain continuous metabolic activity and CO₂ formation by fermentation microbiota (1). Type I doughs propagated in temperate climates are generally dominated by *Lactobacillus sanfranciscensis* (3, 4), but type I doughs propagated in tropical climates harbor thermophilic lactobacilli, including *Lactobacillus reuteri* (5, 6). The frequent occurrence of *L. sanfranciscensis* has been attributed to the rapid growth of this organism, the efficient use of maltose as a carbon source, the use of fructose as an electron acceptor, and its small genome size (7–10). In contrast, type II doughs are used as baking improvers to modify flavor, texture, or shelf life in industrial processes in the form of active dough or dough that has been stabilized by drying or pasteurization (1). The elevated temperatures, long fermentation times, and high water content select for thermophilic and acid-resistant organisms (9, 11, 12). *L. reuteri* prevails in type I and type II sourdoughs fermented at elevated temperatures (2, 4, 5).

L. reuteri not only occurs in food fermentations but is known predominantly for its lifestyle as a vertebrate gut symbiont (13). *L. reuteri* is associated with humans, pigs, rodents, and different species of birds. Strains of *L. reuteri* have evolved into phylogenetically distinct and host-adapted lineages (13), with lineage-specific metabolic and genetic traits reflecting adaptation to different

hosts (14). Some rodent-lineage strains are acid resistant due to the presence of the glutamate decarboxylase GadB (12, 15). Human-lineage strains convert glycerol or 1,2-propanediol to regenerate NAD⁺ (14, 16, 17). Sourdough isolates of *L. reuteri* do not represent extraintestinal lineages but can be assigned to host-adapted lineages, reflecting their long-term association with intestinal ecosystems prior to adopting an alternative lifestyle as a fermentation organism in sourdough (18). The genetics, metabolism, and phylogeny of *L. reuteri* have been studied extensively (14, 19, 20), making it an excellent model organism to identify strain-specific metabolic traits that affect competitiveness in sourdough.

Previous studies suggested that specific metabolic traits of lactic acid bacteria influence their growth rate in cereal substrates and determine their competitiveness in artisanal and industrial sourdough fermentations (8, 10, 12). However, experimental validation of the effects of specific metabolic traits of lactobacilli on their competitiveness in sourdough is generally lacking. It was therefore the aim of this study to determine whether lineage-specific metabolic traits contribute to the competitiveness of *L. reuteri* in type I and type II sourdoughs. Experiments were focused on glycerol metabolism, which may influence the growth rate in cereal substrates, and glutamate metabolism, which influences acid resistance in sourdough. Experiments evaluated the competitiveness of sourdough isolates of *L. reuteri* representing different host-

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adapted lineages that differ in glycerol and glutamate metabolism in wheat and sorghum sourdoughs. Furthermore, the competitiveness of isogenic mutants with disruptions in glycerol and glutamate metabolism was compared to that of the cognate wild-type strains.

MATERIALS AND METHODS

Sourdough fermentation. Previously, seven strains of *L. reuteri* isolated from rye, wheat, and sorghum sourdoughs were assigned to three host lineages (18). To investigate the competitiveness of *L. reuteri*, this study used five strains: *L. reuteri* LTH5448 (rye/rodent-adapted lineage I), *L. reuteri* FUA3400 and FUA3401 (wheat/human-adapted lineage II), and *L. reuteri* FUA3168 and FUA3324 (sorghum/undefined lineage). Sourdough isolates of rodent-adapted lineage III were not suitable for use in competition experiments because they produce reutericyclin, an antimicrobial compound that inhibits other strains of *L. reuteri* (21). Strains were inoculated in modified MRS (mMRS) broth (8) and incubated at 37°C overnight. Two culture cocktails were prepared as biological repeats by mixing equal volumes of one strain from each origin to obtain approximately equal cell counts at the time of inoculation. Cocktail 1 contained *L. reuteri* FUA5448, FUA3400, and FUA3168, and cocktail 2 contained *L. reuteri* FUA5448, FUA3401, and FUA3324. Whole wheat and white sorghum sourdoughs were prepared with commercial flours and a dough yield ($100 \times \text{weight} [\text{flour} + \text{water}] / \text{weight} [\text{flour}]$) of 200; sourdoughs were propagated at 37°C by back-slopping every 12 or 72 h with 10% of the ripe sourdoughs. Sourdough samples were taken after inoculation and at the end of each fermentation cycle for analysis, as outlined below. Two independent experiments with each cocktail were performed.

To determine the roles of glycerol and glutamate metabolism in sourdough ecology, sourdough was inoculated with equal cell counts of *L. reuteri* FUA3400 and *L. reuteri* FUA3400 Δ *gupCDE* or *L. reuteri* 100-23 and *L. reuteri* 100-23 Δ *gadB*. *L. reuteri* FUA3400 Δ *gupCDE*, which lacks the ability to use glycerol as an electron acceptor, was generated in this study (see below); the generation of *L. reuteri* 100-23 Δ *gadB* with a disruption in the gene coding for glutamate decarboxylase was described previously (12). Competition experiments in sourdough were performed as described above, in triplicate independent experiments.

Generation of the *L. reuteri* FUA3400 Δ *gupCDE* mutant. The *gupCDE* gene (20) in *L. reuteri* FUA3400 was truncated by using pJRS233 (22) according to a deletion strategy described previously (12). The 5'-flanking fragment of *gupCDE* was amplified from genomic DNA of *L. reuteri* FUA3400 by using primers 5'-GGA GGT CGA CAG GCT TCA GTT GAT GCC GGA G-3' and 5'-ACC ATG CAT TGG GGT ACC TTA AAC AAA TGT ATC TTG ATG AAT TGG-3'. The 3'-flanking fragment was amplified by using primers 5'-CTG GTA CCT ATG AAA GTC GTA AGA AGC TAA AGG GCG ATA ACT AA-3' and 5'-CAA ATG CAT CGG ATC CCT TTC CTG TAA GAT CTG CCA TTG TTT-3'. The 5'-flanking fragment was ligated into the pGEMTeasy vector (Promega) to generate pGUP-A. Plasmid pGUP-A and the 3'-flanking fragment were digested with the restrictive enzymes KpnI and NsiI, purified, and ligated to create pGUP-AB. The DNA fragment in pGUP-AB was digested with SalI and BamHI and ligated into pJRS233. The resulting plasmid, pGUP-KO, was electrotransformed into competent *L. reuteri* FUA3400 cells. Transformants were grown in mMRS-erythromycin broth (5 mg/liter) at 42°C to 44°C for 80 generations to select for single-crossover mutants. *L. reuteri* with pGUP-KO AB integrated into the chromosome was cured by culturing in mMRS broth at 37°C for 100 generations. The culture was plated onto mMRS agar, and erythromycin-sensitive double-crossover mutants were identified by replica plating onto mMRS and mMRS-erythromycin agar. The truncation of *gupCDE* in *L. reuteri* FUA3400 Δ *gupCDE* was confirmed by PCR and sequencing. The phenotype was confirmed by the absence of 1,3-propanediol production, as determined by high-performance liquid chromatography (HPLC) (6), and the absence of 3-hydroxypropionaldehyde (reuterin) production, as determined by a colorimetric assay (23).

Culture-dependent quantification of sourdough microbiota. Sourdough samples were homogenized and diluted with a 0.8% (wt/vol) NaCl solution. *L. reuteri* cells were enumerated after surface plating onto mMRS agar. Plates were incubated anaerobically at 37°C for 24 h. When the individual strains used in the respective strain cocktails could be differentiated on the basis of their colony morphology, strains were differentially enumerated. The pH of sourdough was measured with a glass electrode; the sorghum sourdough pHs were 3.44 ± 0.03 and 3.38 ± 0.09 after 12 and 72 h of fermentation, respectively, and the wheat sourdough pHs were 3.40 ± 0.06 and 3.46 ± 0.09 after 12 and 72 h of fermentation, respectively.

Differentiation of *L. reuteri* strains by using a molecular beacon. Two grams of sourdough was homogenized with 80 ml of 0.8% (wt/vol) saline. The homogenate was centrifuged at 1,500 rpm for 5 min to remove solids. The cells were harvested by centrifugation at 5,000 rpm for 15 min. DNA was extracted with a DNeasy blood and tissue kit (Qiagen, USA) according to the manufacturer's instructions.

A molecular beacon was designed to distinguish strains from different lineages based on differences in melting temperatures (24). The primers used were forward (limiting) primer 5'-AAT ATG CAG AAG CCT TAG-3' and reverse (excess) primer 5'-TAT CAC CCA TAT CAC CAT-3'. The sequence of the molecular beacon was 5'-CGC GAT CAT GAT TAC GAA AAC AAG TTT GTG GAT GGA TCG CG-3' (the complementary sequences of the beacon stem are underlined). Each PCR mixture contained 8 μ l TaqMan Fast Universal PCR master mix (2 \times), No AmpErase UNG (Applied Biosystems, Canada), 2 μ M excess primer, 0.2 μ M limiting primer, 0.2 μ M molecular beacon, and bacterial DNA in a final volume of 16 μ l. PCR was done with an Applied Biosystems 7500 Fast real-time PCR system (Life Technologies, USA). The PCR conditions were as follows: denaturation for 5 min at 95°C, followed by 60 cycles of denaturation at 95°C for 10 s, annealing at 50°C for 15 s, and extension at 72°C for 20 s. At the melting-curve stage, the temperature was held at 95°C for 2 min and at 25°C for 60 min and was then increased from 25°C to 90°C at 1°C/step, with a 25-s holding time at each step. The melting temperatures were 47°C for human-lineage, 53°C for rodent-lineage, and 61°C for sorghum strains.

Relative quantification of *L. reuteri* wild-type and mutant bacteria by quantitative PCR. DNA was extracted from sourdough as described above, and cells of *L. reuteri* wild-type and mutant strains were quantified with strain-specific primers. The primers for wild types were *gad*-WT-F (5'-ATC TAG ATT ATC CTG CCA TAG ATA AAA-3'), *gad*-WT-R (3'-TAA AGC ACG AGC ATC ATT CG-3'), *gup*-WT-F (5'-GCA TTC GCA ACT GTT CTT GA-3'), and *gup*-WT-R (5'-ACT GTC GTC CCC TTT GAT TG-3'). The primers for mutant strains were *gad*-M-F (5'-AAA TTA ACC TAG GAG GTT TTA TCT ATG-3'), *gad*-M-R (5'-CAG GAC GCA GCA AAG AAG TA-3'), *gup*-M-F (5'-CTG TTA TGG CTG GAC GTG AA-3'), and *gup*-M-R (5'-TCG CCC TTT AGC TTC TTA CG-3'). Each PCR mixture contained 1 \times QuantiFast SYBR green PCR master mix (Qiagen, Canada), 1 μ M each primer, and 1 μ l bacterial DNA in a final volume of 20 μ l. PCR was done on an Applied Biosystems 7500 real-time PCR instrument (Life Technologies, Canada). The PCR conditions were as follows: denaturation for 5 min at 95°C, followed by 60 cycles of denaturation at 95°C for 10 s and annealing at 56°C for 30 s. Standard curves for absolute quantification of the strains in the sourdoughs were generated with purified PCR products obtained for the respective strains, as described previously (25).

In vitro growth rate of *L. reuteri* strains. The main sugars and electron acceptors for wheat and rye sourdoughs are maltose and sucrose; in contrast, glucose and glycerol are the most abundant substrates for use as a carbon source and as an electron acceptor, respectively, in sorghum sourdoughs (25). Modified MRS broth, which mimics sugar compositions of wheat and sorghum with and without an electron acceptor, was prepared with the following sugars as the sole carbon source: 10 g of maltose and 2 g of glucose (W1 medium); 10 g of maltose, 2 g of glucose, and 10 g of sucrose (W2 medium); 2 g of maltose and 10 g of glucose (S1

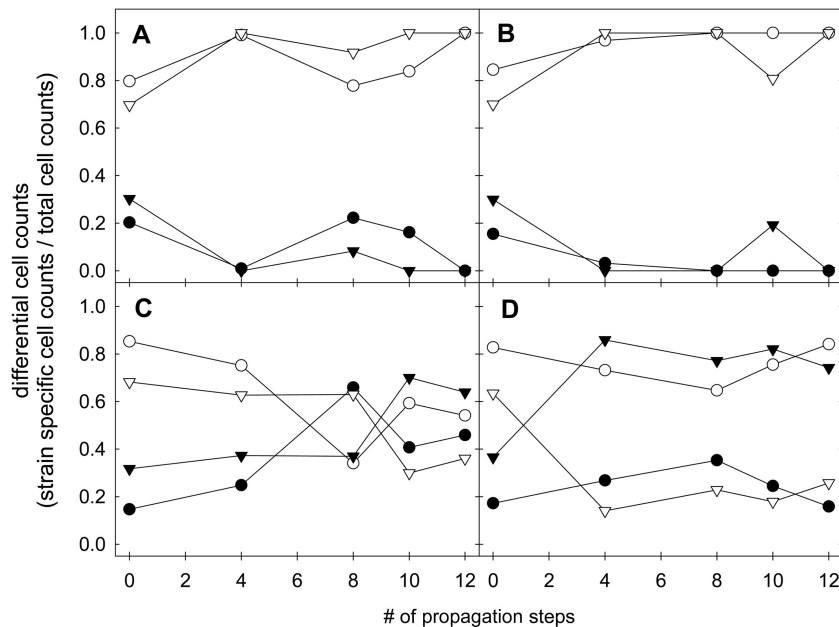


FIG 1 Microbial compositions of wheat sourdoughs (A and C) and sorghum sourdoughs (B and D) that were inoculated with three *L. reuteri* strains. Sourdoughs were propagated with 72-h fermentation cycles (A and B) or 12-h fermentation cycles (C and D). Symbols indicate human-lineage *L. reuteri* FUA3400 (●) and *L. reuteri* FUA5448 or FUA3168 (○) for cocktail 1 and human-lineage *L. reuteri* FUA3401 (▼) and *L. reuteri* FUA5448 or FUA3324 (▽) for cocktail 2. Representative data from two independent experiments are shown.

medium); and 2 g of maltose, 10 g of glucose, and 5 g of glycerol (S2 medium). mMRS-glucose medium, containing 10 g of glucose and lacking both maltose and electron acceptors, was used to prepare the precultures. Strains of *L. reuteri* were subcultured twice overnight in mMRS-glucose medium and grown to an optical density (at 600 nm) of 0.5, corresponding to the exponential phase of growth. mMRS medium and W1, W2, S1, or S2 medium were inoculated with a 10% inoculum of exponentially growing cultures and incubated in 96-well microtiter plates. The cultures were overlaid with 50 μ l paraffin oil to maintain an anaerobic environment. The plate was cultured at 37°C for 24 h, and the optical density was measured every 30 min, with 10 s of shaking before each measurement. Growth rates were calculated by fitting the optical density data to the logistic growth curve (26).

Quantification of 1,3-propanediol concentrations in bacterial cultures and sourdoughs. Bacterial cultures grown overnight or sourdough samples were homogenized in equal volumes of 7% HClO₄ and incubated at 4°C overnight to precipitate proteins. The sample was centrifuged at 14,000 rpm for 10 min. The supernatant was diluted three times with water and then used for HPLC. The 1,3-propanediol concentration was quantified by using an Aminex HPX-87 column (300 mm by 7.8 mm; Bio-Rad, USA) based on refractive index detection (6). Samples were eluted with 5 mmol/liter H₂SO₄ at 70°C with a flow rate of 0.4 ml/min.

Statistics. Data analysis was performed with the PROC MIXED procedure (SAS v.9.2; SAS Institute, USA), using two-way analysis of variance (ANOVA). A *P* value of ≤ 0.05 was considered statistically significant.

Nucleotide sequence accession numbers. The sequences of *gupCDE* and the truncated gene were deposited in GenBank under accession numbers KJ435307 to KJ435310.

RESULTS

Microbial ecology *L. reuteri* strains in wheat and sorghum sourdoughs. To determine the effects of substrate and fermentation time on sourdough microbial ecology, wheat and sorghum sourdoughs were propagated every 12 or 72 h over 12 fermentation cycles. Culture-dependent analysis and the characterization of

sourdough microbiota by high-resolution melting-curve quantitative PCR (HRM-qPCR) demonstrated that the *L. reuteri* strains used as inoculums accounted for >99% of the bacteria in all of the sourdoughs (Fig. 1 and Table 1). The rodent-lineage strains and sorghum isolates could not be distinguished from each other by colony morphology; they were enumerated together by culture-

TABLE 1 Lineage-specific detection of *L. reuteri* in wheat and sorghum sourdoughs propagated with 72-h and 12-h fermentation cycles^d

Fermentation time and sourdough mixture	Strain(s) of <i>L. reuteri</i> detected after indicated no. of fermentation cycles ^e				
	0	4	8	10	12
72 h					
Wheat, cocktail 1 ^a	HRS	RS	HRS	RS	RS
Wheat, cocktail 2 ^b	HRS	HRS	HRS	RS	RS
Sorghum, cocktail 1	HRS	RS	RS	R	R
Sorghum, cocktail 2	HRS	R	R	R	R
12 h					
Wheat, cocktail 1	HRS	HRS	HRS	HRS	HS
Wheat, cocktail 2	HRS	HRS	H	H	H
Sorghum, cocktail 1	HRS	HRS	HS	HS	HS
Sorghum, cocktail 2	HRS	H	H	H	H

^a Cocktail 1 was composed of *L. reuteri* FUA3400 (human lineage II) (H), *L. reuteri* FUA5448 (rodent lineage I) (R), and *L. reuteri* FUA3168 (undefined lineage) (S).

^b Cocktail 2 was composed of *L. reuteri* FUA3401 (human lineage II) (H), *L. reuteri* FUA5448 (rodent lineage I) (R), and *L. reuteri* FUA3324 (sorghum isolate, undefined lineage) (S).

^c Strains detected by observation of the lineage-specific melting peaks if they accounted for 1% or more of the total fermentation microbiota. Letters denote strains of the same host lineage.

^d Duplicate experiments were conducted with two strain cocktails. Lineage-specific detection of *L. reuteri* strains was achieved with HRM-qPCR.

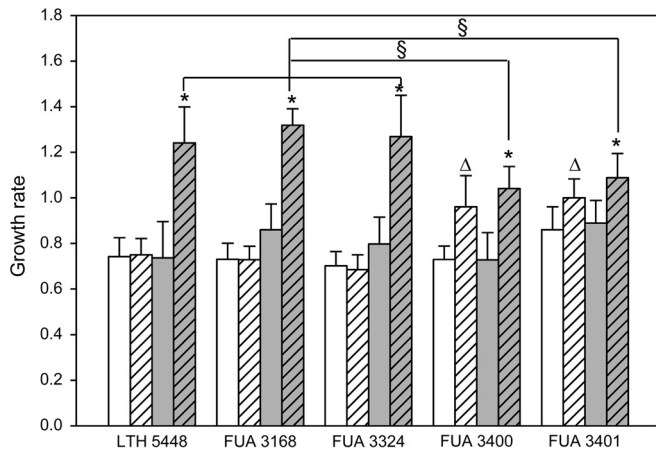


FIG 2 Growth rates of *L. reuteri* strains in mMRS broth. White bars indicate glucose as a carbon source (S1 and S2 media), gray bars indicate maltose as a carbon source (W1 and W2 media), and hatched bars indicate the addition of electron acceptors (10 mmol/liter glycerol in S2 medium and 10 mmol/liter sucrose in W2 medium), between S1 and S2 media (*), between W1 and W2 media (**), and between the wild-type strain FUA3400 and the mutant strain FUA3400 Δ gupCDE (Δ). Data shown are means \pm standard deviations from triplicate independent experiments.

dependent quantification (Fig. 1) but were readily differentiated by HRM-qPCR (Table 1). The total cell count after 12 h of fermentation was about 1 log higher than the cell counts after 72 h of fermentation (data not shown). After 12 fermentation cycles with a 72-h fermentation time in wheat or sorghum sourdoughs, rodent-lineage strains were identified in high cell counts in all sourdoughs; sorghum isolates were additionally identified in two of the four doughs. In wheat or sorghum sourdoughs fermented with 12-h fermentation times, human-lineage strains were identified in high cell counts after 12 fermentation cycles; sorghum isolates were additionally present in the two of the four doughs (Fig. 1 and Table 1). These results imply that different lineage-specific metabolic traits account for the competitiveness of *L. reuteri* in sourdoughs with short or long fermentation times.

In vitro growth rate of *L. reuteri* strains. In order to determine the effect of electron acceptors on growth of *L. reuteri*, media were prepared to represent the main carbon sources in wheat and sorghum with sucrose and glycerol as electron acceptors (W2 and S2 media) and without electron acceptors (W1 and S1 media). For all strains, the addition of sucrose as the electron acceptor increased the growth rate (Fig. 2). In contrast, glycerol as the electron acceptor increased the growth rate of human-lineage strains only. For human-lineage strains, the increased growth rate was less pronounced irrespective of whether sugar or glycerol was the electron acceptor.

Generation of the FUA3400 Δ gupCDE mutant. In human-lineage strains of *L. reuteri*, glycerol is converted to 1,3-propanediol by glycerol dehydratase and 1,3-propanediol dehydrogenase to regenerate NAD⁺ (16, 27). In this study, the glycerol dehydratase gene was truncated to disable glycerol metabolism in *L. reuteri* FUA3400 because of the possible presence of more than one 1,3-propanediol dehydrogenase in the genome (28). Truncation of the glycerol dehydratase avoids accumulation of the antimicrobially active intermediate reuterin; besides, the lack of propanediol dehydrogenase may be compensated for by other dehydrogenase

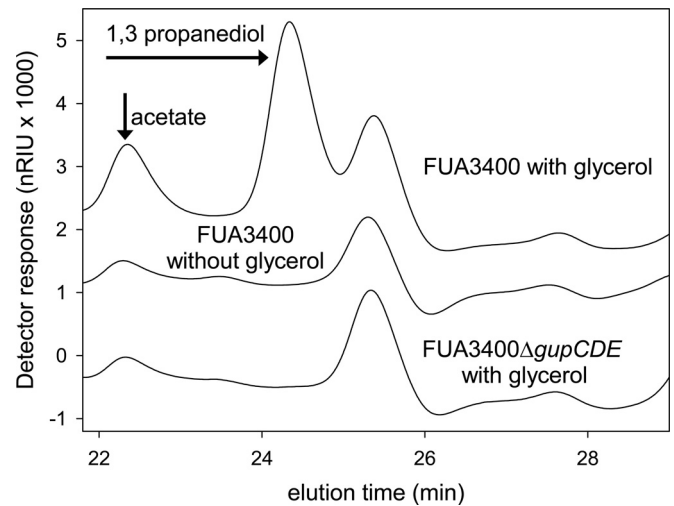


FIG 3 Separation of acetate and 1,3-propanediol in media fermented with *L. reuteri* FUA3400 or *L. reuteri* FUA3400 Δ gupCDE. Where indicated, media were supplemented with 10 mmol/liter glycerol as an electron acceptor. Chromatograms were offset by 1,500 nRIU (nano-refractive index units).

enzymes with broad substrate specificity (29). The candidate genes in *L. reuteri* FUA3400 show high sequence similarity to their counterparts in *L. reuteri* JCM1112 and were thus also designated gupCDE (17). The sequences of gupCDE and the truncated gene were deposited in GenBank (accession numbers KJ435307 to KJ435310). *L. reuteri* FUA3400 quantitatively converted glycerol to 1,3-propanediol (Fig. 3 and data not shown). *L. reuteri* FUA3400 Δ gupCDE did not convert glycerol to 1,3-propanediol, and glycerol supplementation of mMRS-glucose did not support acetate formation (Fig. 3). The colorimetric assay detected reuterin in cultures of *L. reuteri* FUA3400 but not in cultures of *L. reuteri* FUA3400 Δ gupCDE. The growth rates of *L. reuteri* FUA3400 and FUA3400 Δ gupCDE in media containing maltose, maltose and fructose, glucose, or glucose and glycerol (designated W1, W2, S1, and S2 media) were compared (Fig. 4). The addition of sucrose in W2 enhanced the growth rates of both strains, while the addition of glycerol in S2 increased the growth rate of *L. reuteri* FUA3400 but not the growth rate of *L. reuteri* FUA3400 Δ gupCDE, indicating that glycerol metabolism had a positive effect on growth without affecting other key aspects of microbial metabolism.

Role of glycerol and glutamate metabolism in short-term and long-term fermentations. To investigate the role of glycerol and glutamate metabolisms during sourdough fermentation, wheat and sorghum sourdoughs were fermented by using two pairs of wild-type strains and their mutants. As described above, the total cell counts of all doughs fermented with 72-h fermentation cycles were about 10-fold lower than those of the doughs fermented for 12 h (data not shown), indicating cell death during extended incubation under acidic conditions (30). Disruption of the gupCDE genes impaired the growth of the mutant in both 12-h and 72-h cycles, but the strain disappeared faster in doughs fermented with 72-h cycles (Fig. 5). Disruption of gadB in *L. reuteri* 100-23 resulted in reduced competitiveness in doughs propagated with 72-h fermentation cycles. The wild type outcompeted the GadB mutant faster in wheat than in sorghum; however, the com-

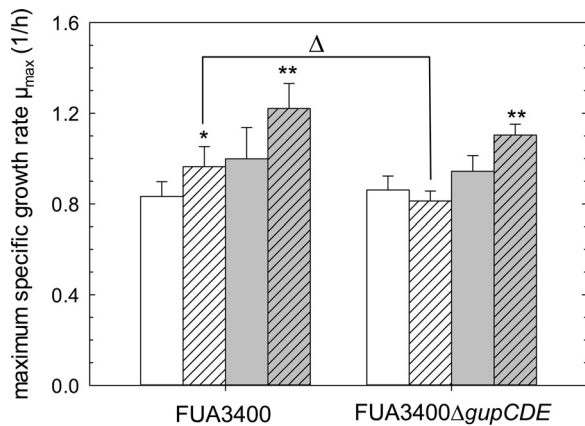


FIG 4 Growth rates of *L. reuteri* wild-type strain FUA3400 and *L. reuteri* mutant strain FUA3400 Δ *gupCDE* in mMRS broth. White bars indicate glucose as a carbon source, matching the major carbon source in sorghum doughs (S1 and S2 media); gray bars indicate maltose as a carbon source, matching the major carbon source in wheat doughs (W1 and W2 media); and hatched bars indicate the addition of electron acceptors (10 mmol/liter glycerol in S2 medium and 10 mmol/liter sucrose in W2 medium). Symbols indicate significant differences ($P < 0.05$) between S1 and S2 media (*), between W1 and W2 media (**), and between the wild-type strain FUA3400 and the mutant strain FUA3400 Δ *gupCDE* (Δ). Data shown are means \pm standard deviations from triplicate independent experiments.

petitiveness of the mutant was not affected in doughs propagated with 12-h fermentation cycles.

Quantification of 1,3-propanediol in wheat and sorghum.

Sorghum contains glycerol in the form of phenolic esters (31). Lacking any indication that glycerol is present as an electron acceptor in wheat sourdoughs, we expected that *L. reuteri* FUA3400 would outcompete FUA3400 Δ *gupCDE* in sorghum but not in wheat sourdoughs. Surprisingly, no difference between wheat and sorghum doughs was observed (Fig. 5). To determine the level of glycerol conversion in wheat, rye, and sorghum, glycerol and 1,3-propanediol were quantified in unfermented sourdoughs and in sourdoughs fermented with *L. reuteri* FUA3400. The concentration of 1,3-propanediol in all sourdoughs was equivalent to the glycerol concentration in unfermented sourdoughs, indicating quantitative conversion by bacterial metabolism (Fig. 6). Remarkably, glycerol and 1,3-propanediol concentrations in wheat demonstrate glycerol metabolism by *L. reuteri* in wheat and rye sourdoughs as well.

DISCUSSION

This study used microbial competition in back-slopped sourdough as a tool to study the impact of metabolic properties on the ecological competitiveness of closely related organisms. Sourdoughs that are propagated in the bakery are continuously contaminated by organisms from ingredients, pests, bakers, and the bakery environment (3, 4, 18). Continuous cycles of contamination and selection result in the establishment of highly competitive microbiota. The congruent evolution of microbiota in type I sourdoughs worldwide demonstrates that this process is highly selective and reproducible (3, 4, 7, 9, 32, 33).

Fermentations at a laboratory scale fail to reproduce this congruent selection process (34). Sourdough fermentations in bakeries are controlled to achieve a consistent technological function (e.g., fermentation kinetics, level of acidity, and leavening power),

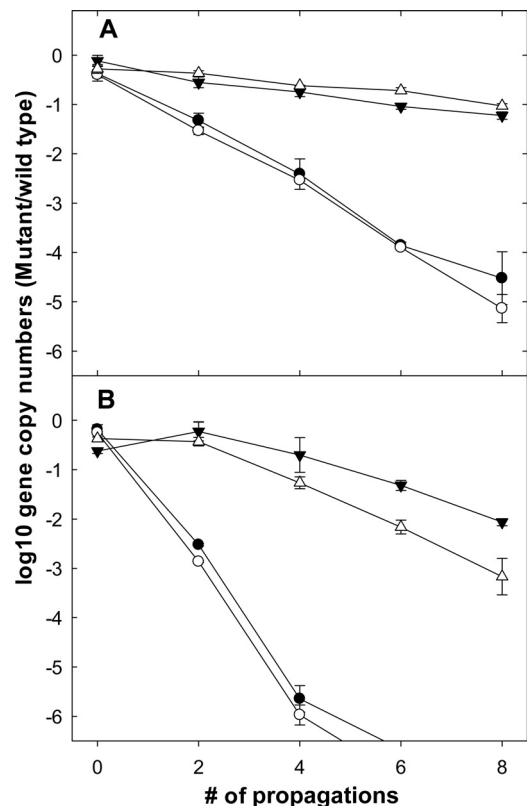


FIG 5 Microbial compositions in sorghum and wheat sourdough fermentations with 12-h and 72-h propagations. Symbols indicate log ratio of *L. reuteri* FUA3400/*L. reuteri* FUA3400 Δ *gupCDE* in sorghum (●) and wheat (○) sourdoughs and the log ratio of *L. reuteri* 100-23/*L. reuteri* 100-23 Δ *gadB* in sorghum (▼) and wheat (△) sourdoughs. The abundance of *L. reuteri* FUA3400 Δ *gupCDE* was below the detection limit after 6 propagations. Data shown are means \pm standard deviations from triplicate independent experiments.

while sourdough fermentations in the laboratory are controlled to achieve consistent fermentation conditions with respect to time and temperature (6, 11, 12, 34). Moreover, flour is the only source of bacterial contamination in laboratory-scale fermentations, ex-

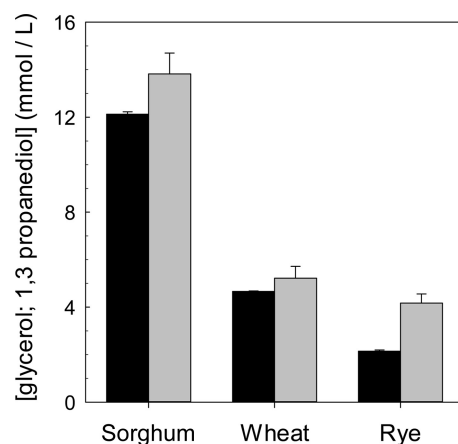


FIG 6 Concentration of glycerol (black bars) in unfermented wheat, rye, and sorghum sourdoughs and concentration of 1,3-propanediol in wheat, rye, and sorghum sourdoughs after 24 h of fermentation with *L. reuteri* FUA3400.

cluding other sources that are more relevant for sourdough ecology (18). Third, *L. sanfranciscensis*, the key organism in type I sourdoughs, was isolated only in sourdoughs with a long history—months or years—of continuous back-slopping (3, 4, 33), but laboratory-scale experiments are typically not conducted on that time scale. Competition experiments with back-slopped sourdoughs are nevertheless a sensitive tool to determine the competitivenesses of different strains or species that are inoculated into the same sourdough (11, 12, 25, 34). This study additionally demonstrates that competition experiments with knockout mutant strains provide a quantitative assessment of the contribution of individual metabolic traits to overall competitiveness. In sourdoughs inoculated with pairs of wild-type strains and knockdown mutants, the ratio of wild-type to mutant bacteria changed with a constant increment per fermentation cycle. For example, the log ratio of FUA3400 Δ *gupCDE* to FUA3400 bacteria changed by 0.30 ± 0.04 and 0.44 ± 0.05 per fermentation cycle in sorghum and wheat sourdoughs, respectively. Despite the use of different flour and slightly different fermentation conditions, the rate of strain displacement and, hence, the impact of *GadB* on competitiveness in wheat sourdough determined in this study were in excellent agreement with data from previous reports (12).

Short- and long-fermented sourdoughs select for different metabolic traits. Frequent propagation selects for fast growth. Long fermentation times select for acid resistance. The conversion of arginine to ornithine, which is species specific in lactobacilli, and glutamine metabolism, which is lineage specific in *L. reuteri* (14), contribute to acid resistance in lactobacilli (12, 15, 35). Arginine and glutamine conversions partially compensate for the lack of glutamate decarboxylation (15, 36, 37); glutamate decarboxylase-positive rodent-lineage strains of *L. reuteri* thus have only a modest advantage over other strains (12; this study). The difference in competitiveness between *L. reuteri* 100-23 and 100-23 Δ *gadB* was greater in wheat sourdoughs than in sorghum sourdoughs, possibly reflecting the higher content of glutamine plus glutamate in wheat proteins (31% [37]) than in sorghum proteins (19% [38]). *L. reuteri* 100-23 expressed *gadB* at the stationary stage but not at the exponential stage of growth (15); accordingly, *L. reuteri* 100-23 and 100-23 Δ *gadB* were equally competitive in sourdoughs maintained by 12-h fermentation cycles.

In *L. reuteri*, enzymes for glycerol utilization are encoded by the *pdu-cbi-cob-hem* cluster (16), which is conserved in human isolates but only in a small proportion of swine or rodent isolates (14). Previous studies on glycerol metabolism by *L. reuteri* focused on the production of reuterin, a metabolic intermediate with antimicrobial activity at millimolar concentrations. 1,3-Propanediol has no appreciable antimicrobial activity (39). The use of glycerol as an electron acceptor enhanced the growth of obligate heterofermentative lactobacilli, including *L. reuteri* (27, 40). This study demonstrates that glycerol conversion to 1,3-propanediol provides an ecological advantage during growth in cereal substrates. *L. reuteri* FUA3400 Δ *gupCDE* was outcompeted by the wild type in both short- and long-fermented doughs. The presence of glycerol as an electron acceptor increased the growth rate by approximately 20% (Fig. 4). A 10% inoculum in sourdoughs propagated with 12-h fermentation cycles corresponds to about 3 h of exponential growth (41). Because cell counts decreased by about 90% during the stationary phase of sourdoughs propagated with 72-h fermentation cycles, the exponential phase of growth upon back-slopping of 72-h-fermented doughs is extended, enhancing the

competitive advantage for the faster-growing wild-type strain. Because *L. reuteri* FUA3400 and FUA3400 Δ *gupCDE* did not differ in stationary-phase survival, this extended phase of exponential growth resulted in a more rapid decrease of the proportion of *L. reuteri* FUA3400 Δ *gupCDE* bacteria in long-term-fermented sourdoughs. However, in competitions of glycerol-metabolizing human-lineage wild-type strains with glutamate-decarboxylating rodent-lineage strains, the faster growth of the former was offset by the improved stationary-phase survival of the latter (Fig. 1 and Table 1) (12).

The conversion of glycerol to 1,3-propanediol was induced by 1,2-propanediol (42). Interestingly, both 1,2-propanediol- and 1,3-propanediol-producing lactobacilli have been isolated from traditional sorghum sourdoughs (6), suggesting a possible symbiotic relationship that might benefit glycerol-metabolizing organisms. Glycerol is present in sorghum flour in the form of glycerol esters of phenolic acids (31), whereas the presence of 1,3-propanediol from glycerol in wheat sourdough has not been described. However, results from this study showed that the amount of glycerol in wheat sourdough was sufficient to provide an ecological advantage for glycerol-metabolizing strains over nonmetabolizing strains. This study did not confirm the source of glycerol in wheat; wheat lipids that are concentrated in the outer layers of the grain are a potential source for the release of glycerol by esterases during fermentation (43).

In summary, glycerol metabolism enabled rapid growth and increased competitiveness of *L. reuteri* in type I sourdoughs, while glutamate metabolism provided acid resistance and increased competitiveness in type II sourdoughs. The use of glycerol as an electron acceptor affects bread quality through increased levels of acetate; glutamate accumulation may alter the taste of bread (19). The observation that the competitive advantage of glycerol-metabolizing *L. reuteri* in sourdough is dependent on the use of reuterin as an electron acceptor may also relate to intestinal ecosystems. Glutamate-mediated acid resistance may support the survival of probiotic lactobacilli during gastric transit (12, 15). In rodents, *L. reuteri* colonizes the forestomach, where sucrose is available to provide fructose as an electron acceptor (44). The conserved *pdu-cbi-cob-hem* gene cluster in human-associated *L. reuteri* strains indicates that glycerol metabolism contributes to the competitiveness of the organism in humans. In humans, *L. reuteri* colonizes the colon, where sucrose is not available but glycerol or 1,2-propanediol may be available through lipid hydrolysis or as a fermentation product of other bacteria (45, 46).

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