Title: Exploiting Endogenous Cas9-Based Genome Editing of *Lacticaseibacillus rhamnosus* GG (LGG) for Precision Food Fermentation and Live Biotherapeutics

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Abstract

Lacticaseibacillus rhamnosus GG (LGG) is one of the most studied probiotic strains and is widely used in both food and therapeutic industries. With its safe and health-beneficial properties, LGG is an ideal candidate for genome modification to enhance its functionalities for food, industrial and pharmaceutical applications. However, precise and efficient genome engineering tools for LGG are unavailable. In this study, we developed a novel genome engineering tool for LGG based on its endogenous type II-A CRISPR-Cas9 system. By employing a validated protospacer adjacent motif (PAM), a customized single guide RNA (sgRNA) expression cassette and a homologous repair template, we successfully reprogrammed the native CRISPR-Cas9 system for targeted genome modifications. Using this method, we successfully constructed a fucose-negative LGG incapable of growing on L-fucose and a lactose-positive LGG. The lactose-positive LGG exhibited significantly improved growth in milk compared to its parental strain LGG, which is unable to ferment lactose, attributed to its ability to metabolize lactose efficiently. Moreover, this strain showed robust growth during yogurt fermentation and maintained viability during cold storage for at least three weeks. These findings highlight the potential of the lactose-positive LGG as an improved culture for dairy industry and a functional probiotic for food and therapeutic applications.

Importance A precise and efficient genome editing tool is essential for unlocking the full potential of *Lacticaseibacillus rhamnosus* GG (LGG), the most extensively studied probiotic strain. This study established the first high-efficiency genome editing platform for LGG by harnessing the endogenous type II-A CRISPR-Cas9 system. Using this tool, we achieved targeted gene modifications, without introducing scars, heterologous DNA, or antibiotic resistance genes. The engineered lactose-positive LGG strain demonstrated improved growth in lactose-containing dairy products and robust performance during yogurt fermentation, highlighting its industrial and therapeutic value. This platform not only advances the development of enhanced probiotic strains but also establishes LGG as a versatile vehicle for synthetic biology and biotechnological applications.

43 **Introduction**

44 Lactic acid bacteria (LAB) are widely used in various industrial fields as starter
45 cultures and cell factories for producing fermented foods and value-added compounds (1,
46 2). Their primary application is as starter cultures in fermented foods, particularly dairy
47 products like yogurt and fermented milk, which serve as key vehicles for delivering
48 probiotics. Beyond their crucial role in food production, numerous LAB strains have been
49 investigated in clinical trials for their nutritional and therapeutic benefits, including
50 pathogen protection, immune modulation, antioxidant effects, and improved nutrient
51 bioavailability (3–6). These well-documented health benefits, along with their generally
52 recognized as safe (GRAS) status, make LAB ideal candidates for genome modification
53 to enhance their desirable properties for both food and therapeutic applications (7, 8).
54 Recently, many novel genome modification approaches have been introduced and applied
55 to LAB strains to improve their beneficial properties and to determine relevant
56 phenotypes (9).

57 The CRISPR-Cas (clustered regularly interspaced short palindromic repeats and
58 CRISPR-associated proteins) system, originally discovered as an adaptive immune
59 mechanism in bacteria, has emerged as a powerful tool for precise DNA modification in
60 various organisms (10, 11). The heterologous CRISPR-Cas system has been successfully
61 employed in many lactobacilli, such as *Limosilactobacillus reuteri*, *Lactiplantibacillus*
62 *plantarum*, *Lacticaseibacillus casei*, *Lactobacillus acidophilus*, *Lactobacillus gasseri*,
63 and *Lacticaseibacillus paracasei* (12–15). However, despite these successes,
64 heterologous CRISPR systems face challenges such as low transformation efficiency,
65 suboptimal activity of heterologous Cas proteins, and potential toxicity to host cells (16,
66 17) . To address these problems, researchers have exploited endogenous CRISPR-Cas
67 systems present in some lactobacilli to achieve genome modifications. These native
68 systems offer a more compatible genome-editing platform, providing higher precision
69 and lower toxicity. For instance, the endogenous subtype I-E CRISPR-Cas system has
70 been utilized for efficient genetic manipulation of *Lactobacillus crispatus* (18), while the
71 subtype II-A CRISPR-Cas9 system has been used to achieve genome engineering in *Lcb.*
72 *paracaseri* (19).

73 *Lacticaseibacillus rhamnosus* GG (LGG) is the world's most documented
74 probiotic strain, known for its health benefits and safety across all life stages, including
75 its ability to modulate the gut microbiome, promote oral health, and support the immune
76 system (20–23). Despite over 300 publications of human studies and well-established
77 health benefits, no precise genome editing tool has been developed for LGG, limiting the
78 ability to (1) elucidate the underlying mechanisms of LGG's probiotic effects and to (2)
79 exploit the long history of safety and efficacy of LGG by engineering it for therapeutic
80 applications.

81 LGG is commonly used in dietary supplements and dairy products to deliver these
82 health benefits. However, its inability to ferment lactose (24, 25), the primary
83 carbohydrate in milk, restricts its growth in milk and other dairy products, which are key
84 probiotic delivery systems. Developing a precise genome editing tool could offer a
85 potential solution to this challenge by restoring the ability of LGG to metabolize lactose,
86 thereby expanding LGG applications in the dairy industry and expanding its role as a
87 functional probiotic and a biotherapeutic vehicle.

88 In this study, we developed a novel genome editing tool in LGG by harnessing the
89 endogenous CRISPR-Cas9 system (Figure 1) and applied it for generating fucose-
90 negative LGG and lactose-positive LGG variants. The lactose-positive LGG
91 demonstrated the ability to metabolize lactose effectively, improving its growth in milk
92 and yogurt fermentation conditions. This advancement represents a significant step
93 toward creating functional probiotics and expanding LGG's applications in food and
94 therapeutic contexts.

95

96 **Results**

97 **Verification of the Activity of the Endogenous CRISPR-Cas9 System in LGG**

98 LGG contains a complete type II-A CRISPR-Cas9 system (Figure 2A) (26). This
99 system is composed of 4 Cas protein-encoding genes including *cas1*, *cas2*, *csn2*, and the
100 critical *cas9*. It also contains a CRISPR array with 24 different 30-base pair (bp) spacers,
101 each flanked by a 36-bp direct repeat sequence (Figure 2A). A protospacer adjacent motif

102 (PAM) is essential for target DNA recognition and binding (27, 28). Analysis of the
103 spacers and their corresponding protospacers predicted a PAM sequence, 5'-NGAAA-3'
104 (Figure 2B).

105 To assess the activity of the native CRISPR-Cas9 system in LGG, we conducted a
106 plasmid interference assay. An active CRISPR system can prevent the uptake of plasmids
107 carrying sequences complementary to native CRISPR spacers (Figure 2C). For this assay,
108 we designed interference plasmids (Table 1) containing a protospacer matching the first
109 spacer (the most recently acquired spacer) in the CRISPR array, either with or without an
110 adjacent PAM sequence (5'-NGAAA-3') (Figure 2D). Our results showed that the native
111 CRIPSR-Cas9 system effectively interfered with plasmids containing functional PAM
112 sequences (Figure 2E). Specifically, transformation efficiency reduced by 10^3-fold and
113 over 10^4-fold for plasmids with PAM sequences of 5'-GGAAA-3' and 5'-TGAAA-3',
114 respectively (Figure 2E). This demonstrated the high activity of the native CRISPR-Cas9
115 system. To further evaluate the PAM specificity, we constructed a new interference
116 plasmid, pPS1TC (Table 1), in which the second conserved nucleotide of the PAM
117 sequence was mutated from G to C (5'-TCAAA-3') (Figure 2D). As presented in Figure
118 2E, pPS1TC did not trigger the endogenous system to produce a detectable interference
119 response, reflecting the high specificity of the type II-A CRISPR-Cas9 system for its
120 PAM sequence. Overall, these findings validated the predicted 5'-NGAAA-3' as the
121 functional PAM and confirmed the robust activity and high specificity of the native
122 CRISPR-Cas9 system under standard laboratory conditions.

123 **Reprogramming Endogenous CRISPR-Cas9 System for Gene Deletion**

124 After confirming the activity of the native CRISPR-Cas9 system with the
125 predicted PAM sequence, we then explored its potential for genome editing in LGG.
126 Initially, we designed an editing plasmid with a homologous repair template and a
127 CRISPR RNA (crRNA) expression cassette controlled by the native leader sequence of
128 the CRISPR array. However, due to the inherent instability of rolling circle plasmids (29),
129 the space between the repeats was easily looped out from the plasmid during plasmid
130 replication, resulting in spacer-deficient plasmids and many wild-type (WT) survivors. To

131 address this, we shifted to using single-guide RNA (sgRNA) to target a specific sequence
132 in the genome of LGG.

133 Based on the predicted structure of the crRNA/ trans-activating CRISPR
134 RNA (tracrRNA) duplex and previous transcriptional sequencing results (26), we linked
135 crRNA and tracrRNA with an artificial AAUC loop to form an sgRNA (Figure 2F). As a
136 result, we constructed a targeting plasmid with a sgRNA expression cassette under the
137 strong constitutive promoter *Ppgm* (*L. acidophilus* NCFM) and an editing plasmid
138 containing a sgRNA expression cassette and a homologous repair template (HR1 and
139 HR2) with the desired mutations for genome editing (Figure 2G). With this design, we
140 proposed to achieve genome editing in LGG by reprogramming the endogenous CRISPR
141 system.

142 As a test, we first attempted to delete L-fucose isomerase, *fucI* (LGG_02685)
143 (Figure 3A), in LGG, as resulting mutants are expected to display a significantly lower
144 growth rate compared to the WT when fucose is used as the sole carbon source (30). A
145 30-bp spacer sequence complementary to *fucI* (Table 3) and two 1-kb homologous
146 regions (HR1 and HR2) were cloned into the plasmid pTRK870 to generate an editing
147 plasmid pfucI-sgRNA-RT (Table 1). Apart from this, pfucI-RT (repair template only) and
148 pfucI-sgRNA (sgRNA only) were also constructed as control plasmids (Table 1). Results
149 showed that no clone was obtained after the transformation of LGG with pfucI-sgRNA,
150 which confirmed the lethality of Cas9-mediated double-stranded DNA breaks when a
151 self-targeting sgRNA is introduced to repurpose the endogenous system and trigger lethal
152 cleavage (Figure 3B). When pfucI-RT was transformed into LGG, hundreds of
153 transformants were obtained (Figure 3B) and no sequenced clones displayed the deletion
154 genotype. In contrast, when pfucI-sgRNA-RT was transformed into LGG, substantially
155 fewer colonies (4 colonies) were observed. These four transformants were examined by
156 PCR, with one producing the desired 2.1-kb PCR product indicating a deletion, while the
157 other three displayed a WT genotype (2.7-kb PCR product) (Figure 3C). The 2.1-kb
158 amplicon was sequenced to confirm the mutant, which was subsequently named MJM568
159 (Figure 3D). Furthermore, phenotypic analysis revealed that the MJM568 strain could not
160 utilize fucose as the sole sugar source (Figure 3E). These results confirm that the

161 endogenous CRISPR system in LGG can be reprogrammed to achieve gene deletion, and
162 both the sgRNA-guided Cas9 targeting and the repair template are crucial for generating
163 the intended mutation.

164 **Construction of Lactose-Positive LGG**

165 After demonstrating the possibility of genome editing with the native CRISPR-
166 Cas9 system, we attempted to construct a lactose-positive LGG strain using genome
167 editing. The ability to metabolize lactose is an important feature commonly exploited in
168 dairy fermentation. However, this capability is lost with LGG. Compared with the closely
169 related lactose-positive strain *Lcb. rhamnosus* Lc705, LGG carries the genes *lacTEGF*
170 operon for the lactose phosphotransferase system, but two mutations in the operon make
171 LGG lactose-negative (incapable of assimilating lactose): a 4-bp deletion causing a
172 frameshift in the antiterminator gene (*lacT*, LGG_00648) and a point mutation (Q231*)
173 in the 6-phospho-β-galactosidase gene (*lacG*, LGG_00650) (Figure 4A) (24, 31). To
174 restore the function of the *lacTEGF* operon, both mutations need to be corrected.

175 A previous study demonstrated that disrupting the stem-loop structure of the *t1*
176 transcriptional terminator can result in unimpeded transcription of the *lacTEGF* operon
177 (31). Based on this, we designed and constructed an editing plasmid placT-sgRNA-RT to
178 create a 20-bp deletion, thus inactivating the transcriptional terminator t1 (Figure 4C).
179 Sequencing of the edited region in five transformants revealed that one colony carried the
180 desired 20-bp deletion mutation. This mutant was designated MJM569 (Figure 4B and
181 4C).

182 Next, 10^8 cells of the WT and MJM569 strains were plated on MRS agar plates
183 containing 2% lactose as the sole sugar source and incubated anaerobically for 2 days at
184 37°C. After the incubation, approximately 40 colonies per plate were observed from the
185 plating of the MJM569 strain (Figure 4D). In contrast, no colonies were observed from
186 the plating of the WT LGG strain (Figure 4D), likely due to the chances of obtaining cells
187 with simultaneous corrections in both *lacT* and *lacG* were extremely low. Next, fifteen
188 lactose-positive colonies derived from the MJM569 strain were randomly picked and
189 tested in MRS broth containing 2% glucose or lactose using a multi-mode microplate
190 reader. The results showed that all 15 colonies were lactose-positive and exhibited robust

growth in both glucose and lactose conditions (Figure 4E). These lactose-positive isolates obtained from this approach were likely to be revertants in *lacG*. To verify this, the *lacG* region of these 15 lactose-positive isolates was amplified by PCR and sequenced. The analysis revealed a point mutation at amino acid 231 of *lacG* in each strain, reverting the premature stop codon in *lacG* to a functional codon (Figure 4F). No other mutations were observed in the *lacG* gene. Specifically, in strain L12 and L13, the thymine at position 691 in *lacG* mutated to a cytosine, reverting the codon at this position from TAG (stop) back to CAG (glutamine, *232Q), as observed in *Lcb. rhamnosus* Lc705 (Figure 4F).

To prepare strain L13 for continued genome editing and food applications, we passed it twice in MRS broth without selective pressure, allowing these cells to naturally lose the plasmid placT-sgRNA-RT. The resulting strain, named MJM570, was confirmed to have lost the plasmid, as it could not grow under erythromycin selection. Its growth curves in both glucose and lactose conditions are shown in Figure 4G.

Growth of LGG and MJM570 Strains in Milk

Since MJM570 could utilize lactose, we wanted to determine whether this trait would improve its growth in milk compared to that of WT LGG strain. As shown in Figure 5A, the lactose-positive MJM570 exhibited robust growth in milk, whereas the WT LGG strain could not grow under the same condition. Notably, after 24 hours of incubation, the milk inoculated with MJM570 showed coagulation (Figure 5B), indicating a pH drop as a result of lactic acid production (Figure 5C). In contrast, the milk inoculated with the WT LGG strain remained in liquid form after 24 hours at 37°C (Figure 5B). In summary, the new ability to metabolize lactose improved the growth of MJM570 in milk.

Viability of Lactose-Positive LGG in Yogurt During Refrigerated Storage

To evaluate the growth and fermentation properties of the WT LGG and MJM570 strains in yogurt, we monitored pH levels and cell counts throughout both the fermentation process and refrigerated storage (5 ± 1 °C, 21 days). The MJM570 strain was compared with the WT LGG strain and a PBS control (Figure 6A).

As shown in Figure 6B, the initial pH of the milk mixture with a yogurt starter culture (*Streptococcus thermophilus* and *Lactobacillus delbureckii* subsp. *bulgaricus*) was approximately 6.5, with no significant differences between the groups ($p > 0.05$). After about 5 hours of fermentation, the pH reached the desired range of 4.3-4.4 due to lactose fermentation by *S. thermophilus* and *L. bulgaricus*. Although pH changes were not statistically significant, differences in the growth of LGG strains were observed. As shown in Figure 6B, the initial cell counts of the WT LGG strain and MJM570 in yogurts were approximately 1×10^8 CFU/g (colony-forming units per gram of the yogurt sample) and 5×10^7 CFU/g, respectively. During the fermentation, MJM570 exhibited robust growth, with cell counts significantly increasing throughout the fermentation period ($p < 0.05$), reaching about 8×10^8 CFU/g by the end. In contrast, the WT LGG strain didn't show significant growth ($p > 0.05$). By the end of fermentation, MJM570 had significantly higher cell counts than the WT LGG strain ($p < 0.05$), highlighting its superior ability to proliferate in a lactose-containing environment with lactose-fermenting strains *S. thermophilus* and *L. bulgaricus*.

After yogurt fermentation, pH levels and cell density were monitored for all three yogurt groups during 21 days of refrigerated storage. As shown in Figure 6C, all three groups experienced a significant pH drop during the first week of cold storage ($p < 0.05$). This decrease was likely due to the continued metabolic activity of the yogurt starter culture and LGG strains, which continue to break down carbohydrates into organic acids, resulting in a lower pH. After the first week, pH levels stabilized across all groups and remained consistent throughout the rest of the storage period (Figure 6C). Regarding sustained viability of LGG and MJM570 during cold storage (Figure 6C), both strains were viable during cold storage for at least 21 days, likely due to the inherent acid tolerance of LGG (32). Notably, MJM570 consistently exhibited significantly higher cell densities than the WT LGG strain throughout the storage period ($p < 0.05$), reflecting its enhanced performance in a fermented dairy product.

Discussion

248 LGG is the world's most documented probiotic strain and has been extensively
249 used in dietary supplements and fermented foods. However, the development of enhanced
250 probiotic LGG strains has been limited by the lack of precise and efficient genome
251 editing tools. Traditional homologous recombination has been the only established
252 genome modification method in LGG (33, 34). However, this method is time-consuming
253 and inefficient, requiring multiple rounds of subculture and screening, and relies on
254 antibiotic resistance genes for selection (9). Recently, CRISPR-Cas9 gene-editing
255 systems have been developed for engineering serval lactobacilli strains (35), with
256 endogenous CRIPSR-Cas9-based genome editing showing particularly high efficiency.
257 One major challenge in developing an endogenous CRISPR system for LGG has been the
258 lack of a replicable cloning vector that can stably replicate in LGG. To address this, we
259 identified and characterized the plasmid pTRK830 (29), which can replicate stably in
260 LGG and effectively overcomes this limitation.

261 Building on this foundation, in this study, we successfully demonstrated the
262 potential of using the endogenous type II-A CRISPR-Cas9 system for precise genome
263 editing in LGG. This native system not only enhanced the precise recognition of target
264 location but also effectively activated the native homologous recombination machinery to
265 yield the desired genetic modifications. This study represents the first successful
266 establishment of a highly efficient and convenient native CRISPR-Cas gene-editing
267 system in LGG. By harnessing the native Cas9 protein, we achieved targeted gene
268 deletions with high precision. Importantly, the genetically modified LGG strains
269 generated through this method do not contain any scars, heterologous DNA, or antibiotic-
270 resistant genes, making them suitable for food and therapeutic applications.

271 As a demonstration of this CRISPR-Cas9 editing tool's potential, we developed
272 the lactose-positive strain MJM570 by deleting a transcriptional terminator upstream of
273 the *lacT* gene and correcting a point mutation in the *lacG* gene. This lactose-positive
274 strain can offer numerous advantages in both industrial and therapeutic applications. The
275 lactose metabolism activity of MJM570 makes it highly suitable for use in a variety of
276 lactose-containing dairy products such as milk, yogurt, and cheese, where it can grow to
277 higher densities by utilizing the lactose in these environments. This trait can also make

278 MJM570 an ideal starter culture for the dairy industry. In yogurt production, MJM570
279 exhibited robust growth during fermentation and stably maintained during cold storage,
280 consistently achieving higher cell density compared to WT LGG, making it an ideal
281 candidate for dairy product formulations. In addition, compared to the commonly used
282 lactose-positive industrial strain *Lcb. rhamnosus* Lc705 (24), our new probiotic strain
283 MJM570 not only exhibits lactose metabolism activity but also presumably retains the
284 numerous health benefits of its parental strain LGG. Furthermore, MJM570 shows
285 potential therapeutic applications for addressing lactose intolerance, a common condition
286 where undigested lactose causes gastrointestinal discomfort, such as bloating and
287 diarrhea (36, 37). Dairy products or dietary supplements containing MJM570 may have
288 the ability to alleviate symptoms by digesting lactose *in situ*, reducing colonic
289 fermentation and preventing related symptoms. Therefore, MJM570 not only contributes
290 to better lactose tolerance but also retains the broader probiotic benefits associated with
291 its parental strain LGG, making it a valuable strain for both dairy production and health
292 applications.

293 In conclusion, this study establishes the first endogenous CRISPR-Cas system for
294 genome editing in LGG, significantly enhancing the available toolkit for probiotic
295 engineering. Our success in developing the lactose-positive strain MJM570 demonstrates
296 the utility of this system for creating novel probiotic strains with potential enhanced
297 industrial and therapeutic applications. In the future, this precise genome editing tool can
298 also hold promise for applications in heterologous gene expression, further broadening
299 LGG's role as a live therapeutic vehicle. This advancement paves the way for future
300 research and applications, offering a powerful tool for genetic manipulation in LGG and
301 other *Lactobacillus* species to improve probiotic functionalities and industrial use.

302

303 **Material and Methods**

304 **Strains and Culture Conditions**

305 LGG and its mutants (Table 1) were grown in de-Man-Rogosa-Sharpe (MRS;
306 Hardy Diagnostics, Santa Maria, CA, USA) broth or on 1.5% agar plates at 37 °C under

307 anaerobic conditions (90% N_2, 5% CO_2, and 5% H_2). *Escherichia coli* MC1061 was
308 cultured in Luria-Bertani (LB) medium at 37 °C with vigorous shaking. Antibiotic was
309 added to the growth medium when applicable: erythromycin (Em; Gold Biotechnology,
310 St. Louis, MO, USA) 5 µg/ml for LGG and 150 µg/ml for *E. coli*.

311 **CRISPR-Cas System Characterization *in silico***

312 The LGG genome is available at the National Center for Biotechnology
313 Information (NCBI). The CRISPR array and the Cas proteins were identified using
314 CRISPRCasFinder web server (38). Bioinformatic analyses were performed to predict the
315 PAM sequence based on spacer–protospacer match, using the spacers of LGG and the
316 web server CRISPRTarget (39). The WebLogo server was used to represent the PAM
317 sequence based on a frequency chart where the height of each nucleotide represents the
318 conservation of that nucleotide at each position (40). In type II systems, the tracrRNA has
319 a complementary region to the CRISPR repeat sequence of the crRNA. Therefore, the
320 repeat sequence was used to identify the tracrRNA in the CRISPR locus using the Basic
321 Local Alignment Search Tool (BLAST) (41), and the interaction between crRNA and
322 tracrRNA was then predicted using the NCPACK web server and depicted manually (42).

323 **DNA Manipulations and Molecular Cloning**

324 A Promega Wizard genomic DNA purification kit (Promega Corporation,
325 Madison, WI, USA) was used to extract genomic DNA. Plasmid DNA from *E. coli* cells
326 was extracted using the QIAGEN plasmid mini-prep kit (QIAGEN, Germantown, MD,
327 USA). DNA restriction enzymes and T4 DNA ligase were purchased from New England
328 Biolabs (NEB, Ipswich, MA, USA) and used following the instructions of the
329 manufacturers. All polymerase chain reactions (PCRs) were conducted according to
330 standard procedures using DreamTaq DNA polymerase (Thermo Fisher Scientific,
331 Waltham, MA, USA) or Phusion DNA polymerase (NEB, Ipswich, MA, USA). PCR
332 primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA)
333 and gene fragments were synthesized by Twist Bioscience (Twist Bioscience, San
334 Francisco, CA, USA). PCR products were electrophoresed in 1.0% agarose gel. DNA
335 fragments were purified using the QIAquick PCR purification kit (QIAGEN,
336 Germantown, MD, USA) before downstream application. DNA sequence analysis was

337 performed at the UIUC Core Sequencing Facility (Urbana, IL, USA) using the Sanger
338 sequencing method.

339 **Construction of Interference Plasmids**

340 pTRK830 is a replicating shuttle vector for *E. coli* and lactobacilli strains (29).
341 The multiple cloning site (MCS) from pBluescript KS (+) was ligated into the *StuI* site of
342 pTRK830, which generated pTRK870 (Table 1). Plasmid pTRK870 was used for all
343 following plasmid constructions. The interference plasmids were constructed as described
344 before (26). The double-stranded DNA protospacers, with or without the PAM, were
345 amplified by PCR, and then ligated into XbaI-HindIII-digested pTRK870 to construct
346 interference plasmids. The resulting interference plasmids were PCR-screened in *E. coli*
347 transformants using PS1 and PS2 primers for the presence of the insert and sequenced to
348 confirm sequence content.

349 **Construction of Editing Plasmids**

350 Each editing plasmid was commonly composed of a sgRNA expression cassette
351 and a repair template (Table 3). The sgRNA expression cassettes included a promoter
352 *Ppgm* of the phosphoglycerate mutase from *L. acidophilus* NCFM, a designed sgRNA
353 containing a 30-bp sequence targeting a specific genomic locus of LGG strain, and a
354 Rho-independent terminator. The sgRNA expression cassette sequence was synthesized
355 and amplified by primers sgRNA-XbaI and sgRNA-XmaI (Table 2) and then cloned into
356 XbaI-XmaI-digested pTRK870 to generate targeting plasmids, pfucI-sgRNA and placT-
357 sgRNA.

358 Next, the repair templates were amplified from the genomic DNA of LGG strain
359 or synthesized by Twist Bioscience. The repair template with two 1-kb homologous
360 regions for *fucI* gene editing was amplified from the genomic DNA of LGG strain. For
361 this, two 1-kb homologous regions were fused by overlap extension PCR to obtain the 2-
362 kb repair template to the *fucI* gene. This repair template was digested with SalI and ApaI,
363 then cloned into SalI-ApaI-digested plasmids pfucI-sgRNA and pTRK870 to generate the
364 plasmid pfucI-sgRNA-RT and pfucI-RT, respectively. For the transcriptional terminator

365 *t1* deletion, the repair template was synthesized and cloned into the SalI-ApaI-digested
366 placT-sgRNA generating the plasmid placT-sgRNA-RT.

367 All these final plasmid constructs were PCR-screened using PS1 and PS2 primers
368 and sequenced to confirm sequence content.

369 **Transformation of LGG**

370 The transformation method was optimized based on previous study (43). 100 µl of
371 an overnight culture was inoculated in 10 ml prewarmed MRS medium and was
372 incubated without agitation at 37 °C. After overnight growth, 2 ml of the culture was
373 inoculated into 100 ml of freshly prepared, prewarmed MRS medium and incubated at
374 37 °C until its optical density OD_{600} reached 0.5-0.6. Cells were harvested by
375 centrifugation at 4000 rpm for 15 min. The cells were washed three times with cold 3.5×
376 Sucrose Magnesium Electroporation Buffer (SMEB) containing 1 M sucrose, 3.5 mM
377 $MgCl_2$, pH 7.0. After washing, the cells were resuspended in 1 ml of the same buffer,
378 ready for electroporation. For each transformation, 1 µg of plasmid was combined with
379 100 µL of cells, and 2-mm cuvettes (Bio-Rad, Hercules, CA, USA) were used for
380 transformation under 2.5-kV, 25-µF, and 200-Ω conditions. DNA concentrations of
381 plasmids used for transformation were quantified using a spectrophotometer,
382 NanoDrop2000c (Thermo Fisher Scientific, Waltham, MA, USA). Then, 900 µl pre-
383 warmed MRS broth was added to the cuvette. The transformed cells were allowed to
384 recover by incubation at 37 °C overnight. After recovery, the cells were plated on MRS
385 agar plates with appropriate antibiotic and incubated at 37 °C in anaerobic chamber for
386 48 to 72 h.

387 The transformants obtained were PCR-screened and sequenced to confirm the
388 presence of desired mutations (Table 2). For the fucose isomerase gene *fucI*, the primers
389 fucI-check-F and fucI-check-R were used for the chromosomal PCR amplification and
390 sequencing (2.7-kb in the WT and 2.1-kb in deletion mutant). For the transcriptional
391 terminator t1, the primers lacT-check-F and lacT-check-R were used for the chromosomal
392 PCR amplification and sequencing. For the 6-phospho-β-galactosidase *lacG*, the primers
393 lacG-F and lacG-R were used for the chromosomal PCR amplification and sequencing.

394 **Growth Curve Measurement**

395 The growth of the LGG strain and its mutant *L. rhamnosus* MJM568 and
396 MJM570 was analyzed in sugar-free MRS media (US Biological, Swampscott, MA,
397 USA) containing 2% glucose, fucose, or lactose (Sigma, St. Louis, MO, USA). Strains
398 were picked and cultured in MRS broth overnight. Then the cultures were harvested and
399 washed three times with phosphate-buffer solution (PBS, pH 7.4) and resuspended in
400 same volume of PBS buffer. Cells were inoculated 2% into MRS containing different
401 sugar sources and transferred into a sterile 96-well plate. The plates were incubated at
402 37 °C without agitation for 24 h in FilterMax F5 Multi-Mode Microplate Reader
403 (Molecular Devices, LLC, Sunnyvale, CA, USA). The absorbance at 595nm (Abs_{595nm}) in
404 each well was recorded every 20 or 30 min. The maximum specific growth rate (μ_{max})
405 was defined as the slope of the tangent line at the inflection point of the logistic
406 regression curve and was reported in $hours^{-1}$.

407 **Growth in Milk**

408 After two passages of LGG and MJM570 in MRS broth, the cultures were
409 harvested and washed three times with PBS buffer and then resuspended in an equal
410 volume of PBS buffer. The cells were inoculated 2% into 10 mL of milk. The milk,
411 reconstituted to 10% (w/v) from skimmed milk powder (Sigma, St. Louis, MO, USA),
412 was heated at 85 °C for 30 min, and then cooled to room temperature before inoculation.
413 The inoculated milk samples were incubated at 37°C for 24 h, and samples were taken at
414 0, 2, 4, 6, 8, 10, 12, 14, 22, and 24 h to determine pH values and the number of colony-
415 forming units per milliliter (CFU/mL) by plate counting.

416 **Yogurt Fermentation**

417 Prior to yogurt production, both LGG and MJM570 were inoculated and cultured
418 anaerobically in MRS broth for 24 h at 37 °C for 2 consecutive passes. Freeze-dried
419 cultures of *Streptococcus thermophilus and Lactobacillus delbureckii subsp. bulgaricus*
420 (YF-L706, Chr. Hansen, Milwaukee, WI, USA) were stored at -80 °C and defrosted at
421 room temperature for 10 min before use.

422 Milk was reconstituted (10% w/v) from skimmed milk powder (Sigma, St. Louis,
423 MO, USA) and heated at 85 °C for 30 min. After cooling to 42 °C, the milk was
424 inoculated with 0.02% yogurt starter culture. One milliliter (1 mL) of overnight cultures
425 of LGG and MJM570 were centrifuged at 3,900 rpm for 5 min. The supernatant was
426 discarded, and the pellets were washed twice with PBS buffer. Each pellet was
427 resuspended in 1 mL PBS buffer and added individually to the yogurt mixture to obtain a
428 final concentration of approximately 10^7 CFU/mL. For the control group, 1 mL of PBS
429 buffer was added to the yogurt mixture. There were 3 replications for each treatment.

430 The yogurt mixtures were fermented at 42 °C until the pH reached 4.3. During
431 yogurt fermentation, pH values of the yogurt samples were measured at room
432 temperature using a digital pH meter (Accumet AR15, Fisher Scientific, Pittsburgh, PA,
433 USA) once an hour for a total of 5 hours. To assess the growth of LGG and MJM570
434 during fermentation, yogurt samples were collected at 0, 2, 4, and 5 h to determine the
435 colony-forming units per gram (CFU/g) of the yogurt sample. Each sample was from a
436 previously undisturbed yogurt.

437 After yogurt fermentation, the samples were cooled and stored in a refrigerator
438 (4 °C) for 21 days. Samples were collected the day following fermentation (day 1), and
439 then once weekly for 3 weeks (day 7, 14, and 21) to assess the viability of LGG and
440 MJM570 as well as pH levels. Each sample was from an undisturbed portion of yogurt.

441 **Viability of LGG and MJM570**

442 One gram (1 g) of yogurt was suspended in 1 mL of PBS buffer, and then 10-fold
443 serial dilutions were performed. Selective enumeration of LGG and MJM570 was carried
444 out using MRS vancomycin plates, which contained 50 ppm vancomycin (Gold
445 Biotechnology, St. Louis, MO, USA) (44). The plates were incubated at 37 °C for 48
446 hours under anaerobic conditions. Plates were counted and recorded as CFU per gram of
447 the yogurt product (CFU/g).

448 **Statistical Analysis**

449 The results were presented as the means of independent experiments ± standard
450 deviations. Statistical significance was determined using a threshold of $p < 0.05$ using

451 unpaired *t* test and one-way ANOVA test. Distinct letters were employed to denote
452 significant differences in all figures. Plots were generated using GraphPad Prism
453 (GraphPad Software, Boston, MA, USA).

454

455 **Acknowledgements**

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457 Illinois, Synthetic Biology for Food and Nutrition Innovation (SynFONI).

458

459 **Tables and Figures**

460 **Table 1 Strains and plasmids used in this study**

Strains or plasmids	Description	Sources or references
Strains		
E. coli MC1061	*E. coli* transformation host, Str^R.	Lab stock
Lcb. rhamnosus GG	*Lcb. rhamnosus* GG was originally isolated from fecal samples of a healthy human adult.	ATCC
Lcb. rhamnosus MJM568	*Lcb. rhamnosus* GG mutant with the deletion (640 bp) of the L-fucose isomerase *fucI* gene.	This study
Lcb. rhamnosus MJM569	*Lcb. rhamnosus* GG mutant with the partial deletion of the transcriptional terminator t1.	This study
Lcb. rhamnosus MJM570	*Lcb. rhamnosus* MJM569 mutant with the point mutation (*231Q) in 6-phospho-β-galactosidase *lacG* gene.	This study
Plasmids		
pTRK830	Low-copy number *E. coli* and Lactobacilli shuttle vector; Erm^R.	(29)
pTRK870	The multiple cloning site sequence cloned into pTRK830 at *StuI* site.	This study
pS1	Protospacer sequence of spacer 1 from LGG CRISPR array cloned into pTRK870 at *XbaI-HindIII* sites.	This study
pPS1TG	Protospacer sequence of spacer 1 and PAM sequence (5'-TGAAA-3') cloned into pTRK870 at *XbaI-HindIII* sites.	This study
pPS1GG	Protospacer sequence of spacer 1 and PAM sequence (5'-GGAAA-3') cloned into pTRK870 at *XbaI-HindIII* sites.	This study

pPS1TC	Protospacer sequence of spacer 1 and 5-bp sequence (5'-TCAAA-3') cloned into pTRK870 at *XbaI-HindIII* sites.	This study
pfucI-RT	Control plasmid containing the repair template for generating a knockout of the *fucI* gene, cloned into pTRK870 at *SalI-ApaI* sites.	This study
pfucI-sgRNA	Targeting plasmid for the *fucI* gene, constructed by cloning the *fucI* targeting sgRNA expression cassette gBlock into pTRK870 at *XbaI-XmaI* sites.	This study
pfucI-sgRNA-RT	Editing plasmid containing the repair template for generating a knockout of the *fucI* gene, cloned into pfucI-sgRNA at *SalI-ApaI* sites.	This study
placT-sgRNA	Targeting plasmid for the transcriptional terminator *t1*, constructed by cloning the *t1* targeting sgRNA expression cassette gBlock into pTRK870 at *XbaI-XmaI* sites.	This study
placT-sgRNA-RT	Editing plasmid containing the repair template for generating a deletion at the terminator *t1*, cloned into placT-sgRNA at *SalI-ApaI* sites.	This study

461

462

463 **Table 2 Primers used in this study**

Primer	Sequences (5'-3')
PS1	GCGAAAGGGGGATGTGCTGC
PS2	GCACCCCAGGCTTTACACTTTATGC
sgRNA-XbaI	CGTACCATCGTCTAGATGCGACAAGT
sgRNA-XmaI	CGATCCCGGGAAAAAAATGTATGGCC
Plasmid interference assay	
PS1XbaI	AGTCATCTAGAAAGAAACGATCCCGAGTTTCTGG
S1HindIII	AGTCAAAGCTTTAGTAAGCAATCCAGAAACTCGGGATC
PS1TGHindIII	AGTCAAAGCTTTAGTTTTCAAAGCAATCCAGAAACTCGGGATC
PS1GGHindIII	AGTCAAAGCTTTAGTTTTCCAAGCAATCCAGAAACTCGGGATC
PS1TCHindIII	AGTCAAAGCTTTAGTTTTGAAAGCAATCCAGAAACTCGGGATC
Construction of the editing plasmid for the deletion of *fucI* gene	
fucI-HR1-F	CAAATGCGCAAAGTCACCTGGAAC
fucI-HR1-R	CGCATCAAACCCGCCGTCAATTTTCTTCACAGTTCATCAAGCTTATTAG
fucI-HR2-F	TGACGGCGGGTTTGATGCGAAATAAGAGTTATC
fucI-HR2-R	CCCGGATCTGTTCGCTTGTTG
fucI-RT-SalI	TACCGTCGACCAAATGCGCAAAG
fucI-RT-ApaI	GTCAGGGCCCCCCGGATCTGTTC
fucI-check-F	GTCCCGTCAAAATTGGAACAAAGAATGTTG
fucI-check-R	CCCAGAGCTTTGTCGAGTCACC
Construction of the lactose positive LGG strain	
lacT-RT-SalI	GATACCGTCGACCTTCGAATCAACAG
lacT-RT-ApaI	GTACCGGGCCCTATTCGCC
lacT-check-F	GACAGGGCCTGACAGATGTAATTGG
lacT-check-R	CCATCACGGATTGCGGACACATAC

lacG-F	ATGCGTAAACAATTACCCAAGGACTTTGTAATC
lacG-R	TTATTCGGGAATCACATGCGTTTGGCTAAC

464 Introduced restriction sequences are underlined.

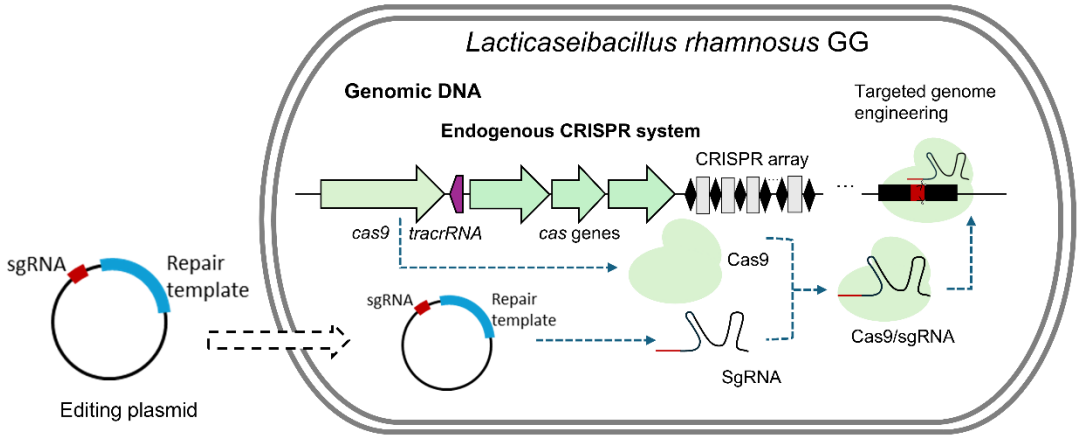
465 **Table 3 Sequences of sgRNA expression cassettes and repair templates**

gBlock	Sequences (5'-3')
fucI targeting sgRNA expression cassette	CGTACCATCGTCTAGATGCGACAAGTAATAAACTAAACAAAA CAACTACAAAATATTTCTTTTTGTTTTTCATGATTTTTACACTT CTCTTAGTATGCTTTTGTTATAAGTTAGCACAAAAAAGCAGAA AATAAAAAGTAGAAATAAAAAAAGATGTTTTTTTGCCCATATC TCTATGAAAAAAACTGTGAAATGTGTAAAATATGGATGAAAC ATTGAATTTAAATGCTTCTTTAACACCGCCAATGGTTGAATCG TCTCAGGTAGATGTCAGATCAATCTCTGACATCTACGAGTTGA GATCAAACAAAGCTTCAGCTGAGTTTCAATTTCTGAGCCCAT GTTGGGCCATACATTTTTTTCCCGGGATCG
Transcriptional terminator *t1* targeting sgRNA expression cassette	CGTACCATCGTCTAGATGCGACAAGTAATAAACTAAACAAAA CAACTACAAAATATTTCTTTTTGTTTTTCATGATTTTTACACTT CTCTTAGTATGCTTTTGTTATAAGTTAGCACAAAAAAGCAGAA AATAAAAAGTAGAAATAAAAAAAGATGTTTTTTTGCCCATATC TCTATGAAAAAAACTGTGAAATGTGTAAAATATGGATGAAAC ATTGAATTTAAACGGCATCTCATCATCTCCTCACCCAGACATG TCTCAGGTAGATGTCAGATCAATCTCTGACATCTACGAGTTGA GATCAAACAAAGCTTCAGCTGAGTTTCAATTTCTGAGCCCAT GTTGGGCCATACATTTTTTTCCCGGGATCG

Repair template for terminator *t1* deletion	GATACCGTCGACCTTCGAATCAACAGGTGGAAAATGGTCTTA ACCAACGGCCTGCTCAATTAATCACAGATGGTCTTCCTAATGC AAATTATGGGTATATTAAAGGTAATAAATGGTATTTTGCTCAAT ACATAAGTCCGAGTGATGTCAAAACAATTTCTGAGGCAGGTA ATCTCCCTGACTTTTTACTAGTAGATAATACGACTAAGTTTGGC ATAGACCTTTTTATCAATGACTTGATAAAAACACTGCATCTATC AAGTTACTTTAGTGGGTATATGTTATATAGTGCAATCTTCGCGG ATTATTTAACTCCATTCAAAAAGGCTGCTCAAGAAGGTCGAG GAATTTATATTGTTACAGAGGCAGATATGACCAACCCAACTGC TGGCTATAACTATGTTGAGTATAGGATAGCGAGTTACTAACCTT ATTTTGAGTAGAGTTGACAAGGGCTTATTACTGGCTAAAAGG GCAAATACTATGAAAAGACTTAGACTTATAACTGTTAAGAGA ATATTGATATTTGCCGTCATTGGCGCCATTGGTTGCATAGTAAC AGAGCTAGTAACCAAAATAGATCCTAATGTTGCCAGTTATATT TGGGTGATAGGCTTCTTTGTGACCTTTAAAGTTGCAGATAATG TCTCAATTAAAGCAACTGCAGGTAAAGGCAGCAAACGTTAAA GTATAATTACTTGACAGGGCCTGACAGATGTAATTGGAATGAC GAACGGAGAGTCTCCCTAACTGAATGATAAATAACGGCTGTG TCAACATAGTCGTTATTTTATTTCTATGAAACCTTGTTAGGCAA GCGTAGTTAATTTTAAAAACCATTATGAAAAGCATTGCGAGAA TCAAGTTTAAAGTAGGTCCTGAAGAAGTTCAAATAAAACGTT TACATTTTGTTTATATGTGGTACAATTTGTTTGTTGATAGGAAT ATTCATTGTGGATTGTGACTATTCAATTAGGCGAGACCACAAA TCATGACGCTGAGAGTAGCGCGTTCTGGGTGAGGAGATGATG AGATGCCGAAAATCGATCAGATCTTTAACAATAACGTGGCGTT GGTTGAGCTAGACAACCATAGTCAAGCGGTTGTTAAGGGACG GGGGATCGCGTTTAGAAGCGTGGCGATGTGATTCCGGCTAAA CAAATTGAAAAAATTTTCTACCTTGCAAGTGAAACTTCCCGC CAGAATCTGTACTTTTTACTTCGCAATATTCCGATCGACGTGG TGACGACCACTTATGAAATTATTGACGTTGCGCAAAAACAATT TCACCTTAAGGTACTTGACTATATTTACATTACGTTAAGCGATC

	ATATTTACGAAGCCTATAAACGTTATCAGAATGGAACGTATCA AGAGACGATGGTGCCGGATTTTCATATTCAGTATCCTGCAGAG TATGCCGTAGCTAACCAAGCGTTGCAGATCATTGCAGCTAACC TTGGCGTAGCATTTCCACCTTCCGAAGTGAAAAATCTTGCCTT ACATTTTATCAATGCCAGCGGTGAAGACGACAACGAGCAGGC CTTTGACAAGAATAATGAGGCGTCATTAAGTCAACTGGTGCA ACGTGTTTTGAAGCGGCATCGCATTACGAGATCGCAAACTAA CGGCAATTATTATGATCGCTTCATGATTCACTTACAGTATCTGA TTGACCGACTGCAGCGTGTTAATACGGATGCTGTTGCCATTGT GCCCGAGGTGGCCAATGAATTAGAGCTAAATTATCCGCGGTC TTATCAGATTGCTTCGGAAATTTTTGATGAGATTAAGGATCAG CTTTATCGCAACATGAGTGAAGACGAACGCTTATACTTTATCA TTCATATTCAACGGCTAATTAACGAAGCGCCTGCCCACGATCA AAAACAGGGCAAAAAATCATAACGCGTTTGCTGGCTTGGAG GGCAGCATACGCTCACGGGAGGAAAGTATCATGAATAAGGTT TTCGATAAGCTAAAGCCGATTTTTGAAGCGATTGCGGCGAATA GGGCCCGGTAC

Introduced restriction sequences are underlined and targeting spacers are double underlined.



468

469 **Figure 1 Schematic of the harnessing the endogenous CRISPR-Cas9 system for**

470 **genome engineering in LGG.**

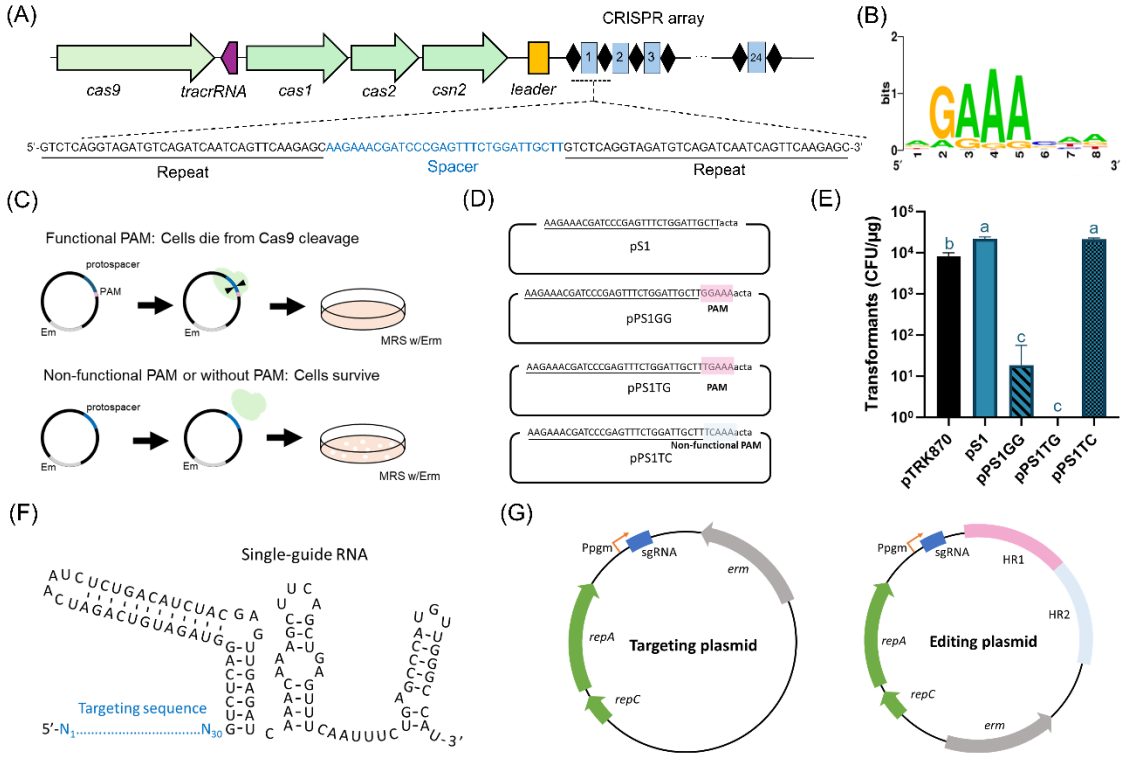
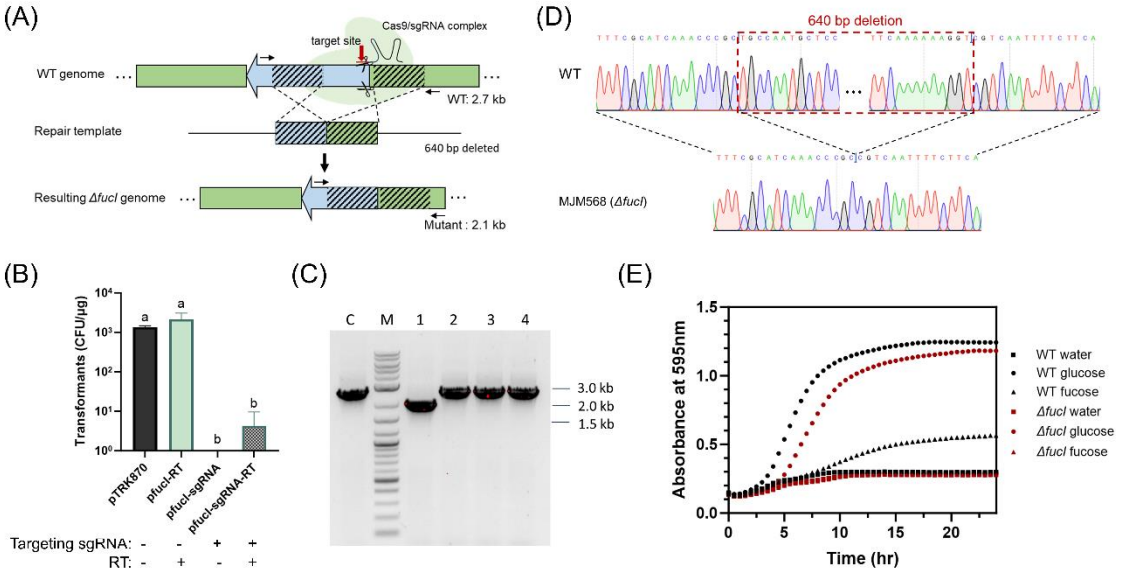


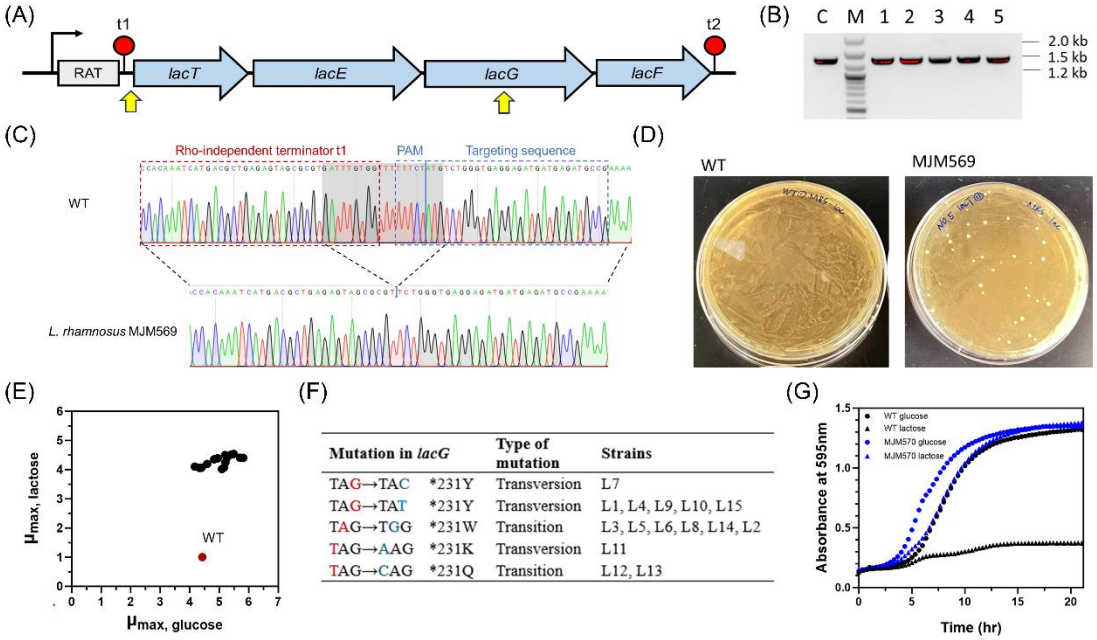
Figure 2 Characterization of the endogenous type II-A CRISPR-Cas9 system in LGG. (A) Architecture of the CRISPR-Cas locus in LGG. The type II-A CRISPR-Cas9 system contains four Cas protein-encoding genes (*cas1, cas2, csn2*, and the critical *cas9*). The CRISPR array includes 24 spacers (30 bp, blue rectangles) interspaced with repeats (black diamonds). (B) PAM prediction visualized using a frequency plot generated using WebLogo. (C) Schematic of the plasmid interference assay. (D) Interference plasmids with a protospacer (underlined) matching the first spacer of the native CRISPR array, with or without an adjacent PAM (5'-NGAAA-3'), were designed for plasmid interference assays. (E) Transformation efficiencies of interference plasmids compared to the control plasmid pTRK870. CFU: colony forming unit. Distinct letters were employed to denote significant differences ($p < 0.05$). (F) Structure of the SgRNA, designed by linking the crRNA and tracrRNA with an artificial AAUC loop. (G) Schematic of targeting and editing plasmids. *repA* and *repC*, plasmid replication gene; *erm*, erythromycin resistance gene; HR1 and HR2, the homologous repair templates; Ppgm, the promoter of phosphoglycerate mutase (*Ppgm*) from *L. acidophilus* NCFM.



487

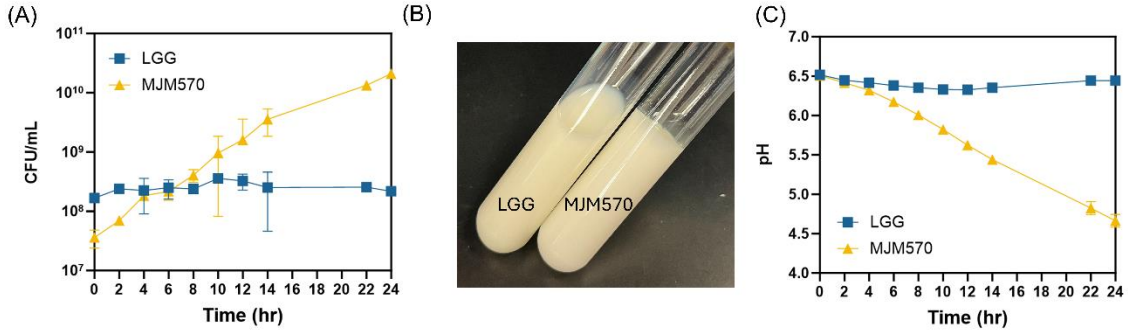
488 **Figure 3 Deletion of *fucI* by reprogramming the endogenous CRISPR-Cas9 system**
489 **in LGG.** (A) Diagram illustrating the genotype of the wild type (WT) and the *ΔfucI*
490 mutant. (B) Transformation efficiencies of the control plasmid pTRK870, the
491 homologous repair template containing plasmid pfucI-RT, the self-targeting plasmid
492 pfucI-sgRNA, and the editing plasmid pfucI-sgRNA-RT. Distinct letters were employed
493 to denote significant differences ($p < 0.05$). (C) Colony PCR screening of four surviving
494 transformants carrying the editing plasmid pfucI-sgRNA-RT to confirm the deletion
495 genotype. Expected products sizes corresponding to the WT and the *ΔfucI* strains are 2.7
496 kb and 2.1 kb, respectively. C: WT control; M: DNA marker; 1-4: surviving
497 transformants. (D) Sequencing results of the genotypes of the WT and MJM568 strains.
498 (E) Growth curve (absorbance at 595nm) of the WT (black) and MJM568 (red) in MRS
499 containing water (square), 2% glucose (circle) or 2% fucose (triangle).

500



Mutation in *lacG*		Type of mutation	Strains
TAG→TAC	*231Y	Transversion	L7
TAG→TAT	*231Y	Transversion	L1, L4, L9, L10, L15
TAG→TGG	*231W	Transition	L3, L5, L6, L8, L14, L2
TAG→AAG	*231K	Transversion	L11
TAG→CAG	*231Q	Transition	L12, L13

Figure 4 Construction of lactose-positive LGG strain by harnessing endogenous CRISPR-Cas9 system and spontaneous mutation. (A) Diagram illustrating two mutations in the *lacTEGF* operon in WT LGG strain (yellow arrows): a 4-bp deletion causing a frameshift in the antiterminator gene (*lacT*) and a point mutation (Q231*) in the 6-phospho-β-galactosidase gene (*lacG*). RAT: ribonucleic antiterminator sequence; t1 and t2: rho-independent terminator. (B) Colony PCR screening of five surviving transformants carrying the editing plasmid placT-sgRNA-RT. C: WT control; M: DNA marker; 1-5: surviving transformants. (C) Sequencing results of the genotypes of the WT and MJM569 strains. The 20-bp deletion area is shaded in grey. (D) Growth of the WT and MJM569 strains on MRS agar plates containing 2% lactose as the sole sugar source. (E) Maximum cell growth rate (h^{-1}) of 15 randomly selected colonies (from MJM569 plates, black dots) and the WT strain (red dot) under glucose and lactose conditions. (F) Point mutations within the premature stop codon in *lacG* identified in these 15 randomly selected colonies. (G) Growth curve (absorbance at 595nm) of the WT (black) and MJM570 (blue) in MRS with 2% glucose (circle) or 2% lactose (triangle).



518

519 **Figure 5 Growth of LGG and MJM570 strains in milk.** (A) Growth curve of the WT
520 (blue square) and MJM570 (yellow triangle) in milk. Error bars show standard deviations
521 of the means from two parallel cultures. (B) LGG-milk remained liquid after 24 h at
522 37 °C, while MJM570-milk was coagulated after 24 h of incubation. (C) pH variation of
523 the WT (blue square) and MJM570 (yellow triangle) strains in milk. Error bars show
524 standard deviations of the means from three parallel cultures.

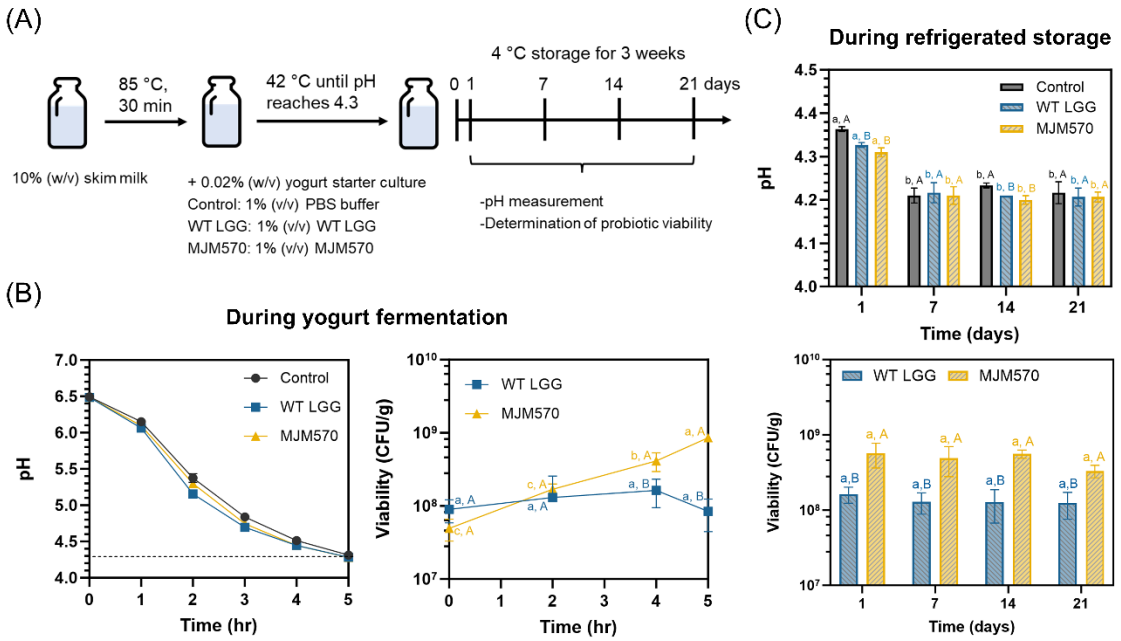


Figure 6 Yogurt fermentation with WT LGG and MJM570 strains. (A) Schematic of yogurt fermentation experiment. (B) pH variation of control, LGG, and MJM570 during yogurt fermentation (left), and the viability of LGG and MJM570 in yogurt during fermentation (right). (C) pH variation of control, LGG, and MJM570 during refrigerated storage (top), and the viability of LGG and MJM570 in yogurt during refrigerated storage (bottom). Distinct lowercase letters (e.g., abc) indicate statistically significant differences ($p < 0.05$) between different time points within the same group, while distinct uppercase letters (e.g., AB) indicate statistically significant differences ($p < 0.05$) between different groups at the same time points.

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