

ORIGINAL ARTICLE

Evolutionary changes of an intestinal *Lactobacillus reuteri* during probiotic manufacture

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Abstract

Probiotic bacteria are frequently used to treat intestinal diseases or to improve health; however, little is known about the evolutionary changes of these bacteria during probiotic manufacture and the bacterial ability to colonize the intestine. It has been observed that when bacteria adapt to a new environment, they lose some traits required to thrive in the original niche. In this study, a strain of *Lactobacillus reuteri* was isolated from mouse duodenum and subjected to 150 serial passes in milk to simulate the industrial propagation of probiotic bacteria. The strains adapted to milk outperformed their ancestor when grown in milk; we also showed evidence of reduced intestinal colonization of milk-adapted strains. Whole-genome sequencing showed that bacterial adaptation to milk selects mutants with altered metabolic functions.

KEYWORDS

antagonistic pleiotropy, experimental evolution, *Lactobacillus reuteri*, probiotics

1 | INTRODUCTION

The International Scientific Association for Probiotics and Prebiotics describes probiotics as “live microorganisms that, when administered in adequate amounts, confer a benefit to the health of the host” (Hill et al., 2014). Probiotics are among the most consumed dietary supplements (Hoy-Schulz et al., 2015; Jones, Martoni, Pietro, Simon, & Prakash, 2012; Mangalat et al., 2012; Valeur, Engel, Carbajal, Connolly, & Ladefoged, 2004; Weizman & Alsheikh, 2006; Wolf, Wheeler, Ataya, & Garleb, 1998). In the USA, around 3.9 million people consume prebiotic or probiotic supplements (Draper, Ley, & Parsonnet, 2017). There are many questions about the efficacy of these products in the medical field (Senok, Ismael, & Botta, 2005); one of the crucial uncertainties is how long the probiotic bacteria can linger in the intestine (Islam, 2016).

Bacterial adaptation to new environmental conditions is associated with the loss of adaptation to the original one (Bono, Smith, Pfenning, & Burch, 2017; Cooper, 2014; Hottes et al., 2013; Kawecki

et al., 2012; Sane, Miranda, & Agashe, 2018). This adaptation occurs through the selection of mutants displaying greater proficiency to thrive in new substrates, temperatures, pH values, etc. (Hamilton & Buckley, 1991; Hottes et al., 2013). Entering the intestinal microbiota requires ability to compete for nutrients and defend from antimicrobial factors produced by resident commensal bacteria (Bäumler & Sperandio, 2016; Ferreira et al., 2011; García-Bayona & Comstock, 2018; Sassone-Corsi & Raffatellu, 2015).

Many probiotic formulations contain different species of *Lactobacillus* (*L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. bulgaricus*, and *L. reuteri*), some of which are commonly found in the human intestine (7, 8). *Lactobacillus reuteri* is especially relevant as a probiotic bacterium because it has coevolved with different animal hosts (including humans) (46) and secretes antimicrobial compounds that inhibit other bacteria (Jacobsen et al., 1999; Valeur et al., 2004). We studied the evolutionary changes that occurred in an intestinal isolate of *L. reuteri* during 150 passages in milk.

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2 | MATERIALS AND METHODS

2.1 | Isolation and selection of *Lactobacillus reuteri* strains

The strains used in this study were isolated from the duodenum of a female CD1 mouse (all procedures with mice were previously approved by the Ethics Committee on the Use of Animals in Research and Teaching at the San Francisco University of Quito). A CD1 mouse was euthanized using chloroform; the duodenum was extracted; contents were inoculated onto Man Rogosa Sharpe (MRS) agar and incubated at 37°C for 48 hr under microaerophilic conditions (Rojas & Conway, 1996). Isolates were analyzed by Gram stain, catalase and oxidase tests (Appendix 1: Table A1). Six *Lactobacillus* spp. isolates were selected, and 16S rRNA gene sequence was used to determine bacterial species (Barros, Andreatti Filho, Oliveira, Lima, & Crocci, 2009); PCR products were sequenced at Functional Biosciences, Inc., Madison, WI, and sequences were compared to those in GenBank (Kumar, Stecher, & Tamura, 2016).

2.2 | Selection of rifampicin-resistant mutants

Rifampicin mutants were selected following the protocols previously described (Miller, 1992). One *L. reuteri* strain (Lr.1) was inoculated in 10 ml of MRS broth at 37°C under microaerophilic conditions for 24 hr; the bacterial culture received an additional 10 ml of MRS broth with rifampicin (200 µg/ml), for a final concentration of 100 µg/ml, and the tube was incubated for another 24 hr. The bacterial culture was streaked on MRS agar with 100 µg/ml of rifampicin and incubated for 24 hr. Colonies formed by rifampicin-resistant mutants were confirmed with Gram stain, catalase, oxidase, and antibiotic sensitivity tests (Boone, Castenholz, & Garrity, 2001). Confirmed strains were stored at -80°C in BHI medium + glycerol 20%. Fitness loss, due to the mutation causing rifampicin resistance, was evaluated by culturing together progenitor strains and rifampicin-resistant descendants as previously described (Lenski et al., 2015) (Appendix 1: Table A1). The strain LrRR1.2 showed the least fitness loss in MRS broth, and it was selected to carry out the rest of the experiments (Appendix 1: Table A1).

2.3 | Adaptation to milk and MRS

Two lineages of *L. reuteri* were created by inoculating one milliliter of LrRR1.2 culture (in MRS broth) in 2 tubes containing 9 ml of sterile (autoclaved) cow's milk and incubated at 37°C for 24 hr. Each of the lineages was subjected to 150 serial passes in milk, which corresponds to approximately 510 generations (Kram et al., 2017). The strain adapted to MRS was obtained by 150 passes in this medium, and the same procedures used for milk. To rule out potential *Lactobacillus* contamination, PCR amplification and sequencing of *leuS* gene (Oh et al., 2010) was carried out at passes: 55, 92, and 120

in each population. After 150 passes, in milk or MRS, we isolated a colony from each lineage; passed in milk strains: LrRR1.21M150 and LrRR1.22M150 and passed in MRS strain: LrRR1.2MRS150. All the strains were kept frozen at -80°C until used.

2.4 | Intestinal colonization

Male and female CD1 mice were donated by Laboratorios Agrocalidad, Quito. Animals were fed for 7 days with 100 µl of MRS medium containing 2×10^7 *L. reuteri* cells (as determined by Petroff-Hausser chamber count and confirmed by colony count in MRS + rifampicin). The animals were separated into three groups: Group 1 was fed with LrRR1.2 (nonadapted to milk), group 2 was fed with LrRR1.21M150 (strain better adapted to milk), and the group 3 or control group was fed with 100 µl of sterile MRS medium. The mice received the inocula once a day for 7 days and left untreated for 15 days (to ensure colonization) after which they were euthanized. A 10 mm duodenum piece from each mouse was ground with MRS medium, and the resulting menstruum was diluted serially (10-fold), plated in MRS plus rifampicin (Pavan, Desreumaux, & Mercenier, 2003), and rifampicin-resistant colonies were counted after 24 hr incubation.

2.5 | Relative fitness in milk

We compared the growth rate (in milk) of the adapted strains (LrRR1.21M150, LrRR1.22 M150, and LrRR1.2MRS150) with that of the parental strain (LrRR1.2). We inoculated 1ml of a 24 hr culture (in MRS) of each of the strains (LrRR1.21M150, LrRR1.22M150, LrRR1.2MRS150, and LrRR1.2) into 5 tubes containing sterile milk (5 replicates per strain), and colony counts were obtained at 0 hr and 24 hr of incubation; we used the spread plate method in duplicate. The relative fitness was calculated using a previously described formula (Wiser & Lenski, 2015) $W = \ln [A_{24 \text{ hr}}/A_0 \text{ hr}]/\ln [B_{24 \text{ hr}}/B_0 \text{ hr}]$, where W is the relative fitness; A_{24} corresponded to the colony count of each of the adapted strains (LrRR1.21M150, LrRR1.22M150, and LrRR1.2MRS150) at 24 hr of culture; A_0 corresponded to the colony count of each of these strains at 0 hr of culture. B_{24} hr corresponded to the average colony count of the 5 replicates of the ancestral strain after 24 hr incubation, and B_0 hr was the colony count average of the 5 replicates at 0 hr.

2.6 | DNA extraction and whole-genome sequencing

DNA was extracted from the 3 strains: Lr.1, LrRR1.2, and LrRR1.21M150 (the strain showing greater adaptation to milk) using DNAzol™ (Invitrogen) following the manufacturer's protocol (Ausubel, 1987) and sent to Macrogen Inc., Seoul, the Republic of Korea for genome sequencing with Illumina HiSeq 2500, 100 bp

PE. The complete genome sequences of the 3 strains were assembled using SPAdes (version 3.13.0). The raw data of the sequences were preprocessed before running the hashing. We used paired-end reads from SPAdes using Mauve version 2.4.0 for the reordering of the contigs (Darling, Mau, Blattner, & Perna, 2004), based on the complete genome sequence of *L. reuteri* DSM 20016 on GenBank (NC_009513.1). The gene annotation of the completed genomic sequences was accomplished in Prokka (version 1.13.3) (Seemann, 2014). ProgressiveMauve was used for the alignment of the three reordered genomes (Darling, Mau, & Perna, 2010). A comprehensive genome analysis for the characterization of the genome of the three strains, in Patrick version 3.5.22 (Wattam et al., 2014). For the identification of SNPs variants between the milk-adapted bacterial genome (LrRR1.2M150) and its progenitor (LrRR1.2) genome, we used the fast bacterial variant calling from NGS reads (Snippy; Seemann, 2015).

3 | RESULTS

3.1 | Relative fitness in milk

Both milk-adapted strains outperformed the ancestral one; however, only one strain showed a relative fitness that was statistically significant (values above 1): LrRR1.21M150 showed a $W = 2.92$; 99% CI [2.62–3.22], whereas the strain LrRR1.22M150 had $W = 1.35$; 95% CI [0.99–1.71]. The relative fitness of the strain adapted to the MRS medium, LrRR1.2MRS150, was negative $W = 0.98$, 95% CI [0.63–1.33] (Figure 1).

3.2 | Intestinal colonization

The duodenal samples of mice receiving different strains of *L. reuteri* were subjected to bacterial culture, but we obtain colonies of *L. reuteri* from two mice only; one from the mouse receiving the parental strains and the other from a mouse receiving the milk-adapted strains. Nevertheless, the mouse receiving the parental strain LrRR1.2 had 35 times higher colony counts than the animal receiving the milk-adapted strain LrRR1.21M150 (993 CFU/ml vs. 28 CFU/ml).

3.3 | Whole-genome sequencing

The alignment made in Mauve (Appendix 1: Figure A1) showed that all the contigs were correctly aligned among the three analyzed genomes. Comprehensive genome analysis in Patrick showed that the three genomes have 2,320 Mb, 2,303 Mb, and 2,296 Mb, respectively, distributed in 310, 264, and 315 contigs (Appendix 1: Figure A1). Genes annotation allowed us to compare 1,618 proteins with functional assignments and 647 hypothetical proteins from LrRR1.2 milk-adapted strain with the LrRR1.2 strain. Of the coding-sequence genes analyzed, 13 (0,6%) had mutations (substitutions, insertions,

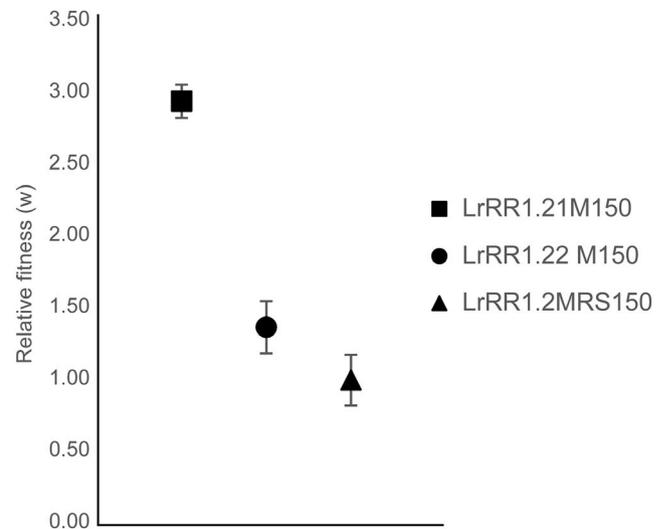


FIGURE 1 Relative fitness in milk of *Lactobacillus reuteri* strain isolated from the murine intestine, subjected to 150 passes in milk (LrRR1.21M150 and LrRR1.22M150) and subjected 150 passes in MRS medium (LrRR1.2M150MRS). The bacterial growth was compared to the parental strain LrRR1.2 (1 pass in MRS). Bars indicate standard error

or deletions) compared to the progenitor (Table 1; Appendix 1: Figure A1). Of the 13 mutations found, 8 were nonsynonymous; 5 of them were in genes associated with metabolism: *yhdG*, an amino acid permease; *ybiV*, a sugar phosphatase; *murG*, undecaprenol N-acetylglucosamine transferase; *amt*, an ammonium transport protein; and *perR*, a peroxide-responsive repressor.

4 | DISCUSSION

We showed that an intestinal *L. reuteri* isolate subjected to 150 passes in milk gained proficiency to grow in this substrate. We also showed some evidence that the adaptation to milk reduced the aptitude to colonize murine intestines. Our results are in agreement with previous studies showing that adaptation to a new ecosystem reduces the fitness in the original niche, a phenomenon is known as antagonistic pleiotropy (Bono et al., 2017; MacLean, Bell, & Rainey, 2004; Sane, Miranda, & Agashe, 2018). This finding is also in agreement with previous reports indicating that commercial probiotic bacteria (originated from intestines) failed to colonize mammalian intestines (McNulty et al., 2011).

A total of 13 mutations were observed in bacteria subjected to milk passes; some of these mutations occurred in genes associated with metabolic processes which have been observed in bacteria forced to grow in challenging environments (Tenaillon et al., 2016). The gene coding for the permease *yhdG* (an exporter for branched-chain amino acids; Li et al., 2018) showed a nonsynonymous mutation; mutations in this gene have been observed in an acid-tolerant mutant of *Streptococcus mutans* (Hamilton & Buckley, 1991). *Lactobacilli* growing in milk have to endure high concentrations of autogenous

TABLE 1 Substitutions of *Lactobacillus reuteri* rifampicin-resistant and milk-adapted strain

Gene	Function	Mutation type	Sequence change	Protein change
IS66 family transposase ISDeal1	Mobile genetic element	Nonsynonymous substitution	580649A > G	289Q > R
Putative amino acid permease yhdG	Metabolism	Nonsynonymous substitution	626849T > C	48F > L
Sugar phosphatase ybiV	Metabolism	Nonsynonymous substitution	1024578A > G	213Y > C
UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase (<i>murG</i>)	Metabolism	Nonsynonymous substitution	1063654A > G	342T > A
Group II intron reverse transcriptase/maturase (<i>ItrA</i>)	Membrane protein	Nonsynonymous substitution	1504134A > T	36H > L
Putative cell wall-anchored protein (LPXTG motif)	Cell wall anchor	Nonsynonymous substitution	1576481C > T	533R > stop codon
Transposase IS3	Mobile genetic element	Nonsynonymous substitution	1949560A > G	155N > S
DNA-directed RNA polymerase subunit beta (<i>rpoC</i>)	RNA β' ribosomal subunit Processing, stress response, defense and virulence	Nonsynonymous substitution	2045457C > T	736A > V
Hypothetical Protein_1	-	Deletion	1383389_1383391delGG	208Stop codon > G
Hypothetical Protein_2	-	Deletion	1748564_1748566delC	27Stop codon > H
Peroxide-responsive repressor PerR	Stress Response, Defense and Virulence	Deletion	1843469_1843488delGAATCGTTAATAAAAAATTCCCCGA AAACTCGGG	150_157delFL-CFL
Ammonia channel (<i>amt</i>)	Membrane Protein	Insertion	1997092_1997114insTCGACGGAAAGATGTTTCATTC	188_197insRRKDVHSQ
tRNA Synthase	Transduction	Insertion	2114543_2114545insA	44_46insN

organic acids (lactic acid) which can be deleterious. Acid-resistant mutants occur frequently in bacterial populations subjected to these types of acids (Azcarate-Peril, Tallon, & Klaenhammer, 2009). A mutation in the *amt* gene (an insertion of 21 nucleotides) was observed in *L. reuteri* adapted to milk; the product of this gene is involved in ammonium transmembrane transporter activity (Thomas, Mullins, & Merrick, 2000). This gene is overexpressed in *L. acidophilus* adapted to milk (Azcarate-Peril et al., 2009). The *ybiV* gene codes for a sugar phosphatase involved in bacterial protection from cytoplasmic phosphosugar accumulation (Papenfort, Sun, Miyakoshi, Vanderpool, & Vogel, 2013). This mutation may have some association with the high availability of sugars in milk. Peroxide-responsive repressor *perR* gene has a deletion of 34 nucleotides in the strain adapted to milk. PerR is a ferric uptake repressor that downregulates genes involved in oxidative stress responses, iron homeostasis and is essential for responses to peroxide (Wang et al., 2013). A deletion in this repressor gene may cause constitutive expression of these functions in the strain adapted to the milk. This trait may allow bacterial growth in milk, a substrate in which iron is sequestered by lactoferrin and transferrin (Jensen, 1995).

A nonsense mutation (arginine changed to stop codon) was observed in a putative cell wall-anchored protein (LPXTG motif). LPXTG-anchored proteins are covalently attached to the peptidoglycan; they are some of the outermost structures in *Lactobacillus* which may play an important part in interactions with intestinal cells (Sengupta et al., 2013); these proteins may not be necessary when growing in artificial media. Reductive evolution was also observed in *L. bulgaricus* adapted to milk (Fernandez et al., 2008).

Our failure to recover *Lactobacillus* from murine intestines may be associated with rifampicin resistance which is associated with mutations in the *rpoB* gene. These mutations affect bacterial fitness (Appendix 1: Table A1) (Maughan, Galeano, & Nicholson, 2004; Riva, Villani, Mastromei, & Mazza, 1976). This subunit is involved in the binding of the sigma 70 factor which may affect the expression of some genes (Naryshkina, Mustae, Darst, & Severinov, 2001). Although, mutations in this protein may also cause greater adaptation to grow in minimal medium (Conrad et al., 2010).

In this study, we showed that few passages in an artificial substrate can select mutants that can grow better in this novel substrate but unable to compete with ancestral strains in the original niche.

However, our results should be confirmed by a larger experiment using more replicates. Our results suggest that more effective probiotics may be produced with low passage strains. Finally, bacterial antagonistic pleiotropy is a phenomenon that has serious implications that go beyond probiotics such as antibiotic resistance and the evolution of environmental opportunistic pathogens.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

L.L., P.C., and G.T. conceived and designed experiments and contribute to the writing of the manuscript; L.L. and D.C. conducted experiments; L.L., D.C., C.V. and G.T. involved in statistical, mathematical, and computational analysis; L.L. and G.T. involved in acquisition of the financial support for the project leading to this publication.

ETHICAL APPROVAL

All the procedures performed with the mice were approved by the "Ethics Committee on the Use of Animals in Research and Teaching" at the San Francisco University of Quito.

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DATA AVAILABILITY STATEMENT

Data are provided in the results section of this paper, Appendix 1 and GenBank (accession numbers: PRJNA580514: strain Lr.1 (SAMN13161586, SUB6483510; strain LrRR1.2 (SAMN13161644, SUB6483581); strain LrRR1.21M150 (SAMN13161645, SUB6483589).

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APPENDIX 1

TABLE A1 Competitive assay of the *Lactobacillus reuteri* strain rifampicin-resistant mutant strain with rifampicin-sensible ancestor (denominator)

Rifampicin mutant-resistant strain	Rifampicin-sensible ancestor	Growth ratio
LrRR1.1	C1.01	0.86
LrRR1.2	C1.02	0.96
LrRR1.3	C1.03	0.93
LrRR2.1	A6.01	0.80
LrRR2.2a	A6.02	0.80
LrRR3.2	A7.2.00	0.68

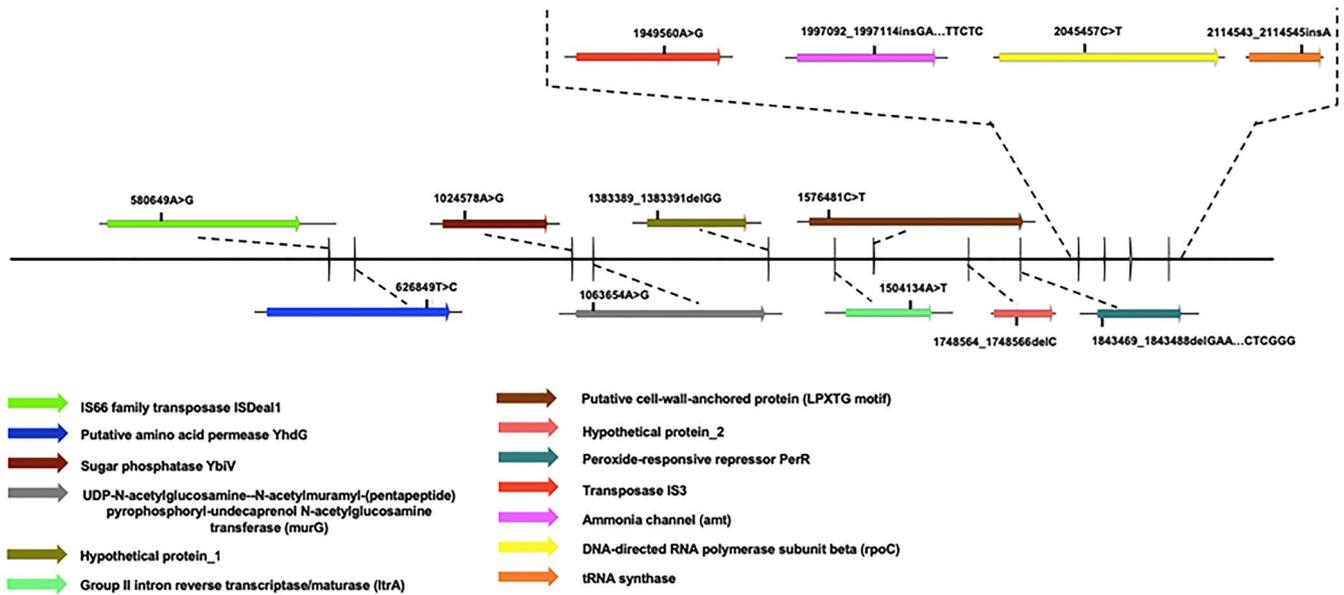


FIGURE A1 Schematic diagram of the SNPs in *Lactobacillus reuteri* rifampicin mutant-resistant strain after 150 passes in milk and *Lactobacillus reuteri* rifampicin-resistant mutant prior passes in milk. The colored arrows show the genes where mutations were found