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Evolutionary changes in milk adaptation of a *Lactobacillus reuteri* intestinal strain

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DEDICATORIA

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RESUMEN

Las bacterias con características probióticas se utilizan tanto en la industria alimentaria como en las áreas de salud, sin embargo, poco se sabe sobre su evolución. Se ha observado que cuando las bacterias se adaptan a un nuevo entorno, pierden las habilidades necesarias para prosperar en el nicho original. En este estudio, se aisló una cepa de *Lactobacillus reuteri* del duodeno de un ratón y se realizaron pases en serie en la leche durante 150 días para definir los cambios evolutivos que afectan la capacidad de supervivencia y crecimiento en el intestino. Se llevaron a cabo experimentos de capacidad de crecimiento con las bacterias resultantes y su progenitor inicial (ancestro). La adaptación a la leche superó al antecesor resistente a la rifampicina en el 66% y el ancestro original (sensible a la rifampicina) en el 33%. La adaptación genética a la leche se confirmó por la presencia de mutaciones asociadas con el metabolismo y la transducción de señales. Las mutaciones no sinónimas fueron el tipo más frecuente de mutaciones y gran parte de ellas resultaron beneficiosas. Con estos resultados, se puede concluir que durante el proceso de adaptación a la leche por la cepa de *L. reuteri*, se seleccionaron cepas con nuevas habilidades beneficiosas para este nuevo entorno; pero esto llevó a la pérdida de varias de sus habilidades iniciales, como la capacidad de crecer en un entorno hostil como el intestino vertebrado.

Palabras clave: *Lactobacillus reuteri*; Evolución Experimental; Evolución bacteriana; Evolución Adaptativa; Pases en serie a largo plazo

ABSTRACT

Bacteria with probiotic characteristics are used both in the food industry and in health areas, however, little is known about their evolution. It has been observed that when bacteria adapt to a new environment, they lose the skills necessary to thrive in the original niche. In this study, a strain of *Lactobacillus reuteri* was isolated from the duodenum of a mouse and serial passes were made in milk for 150 days to define the evolutionary changes that affect the capacity for survival and growth in the intestine. Growth capacity experiments were carried out with the resulting bacteria and their initial progenitor (ancestor). The adapted to milk outperformed the rifampicin resistant ancestor in 66% and the original ancestor (rifampicin sensitive) in 33%. Genetic adaptation to milk was confirmed by the presence of mutations associated with metabolism and signal transduction. Non-synonymous mutations were the more frequent type of mutations and much of its where beneficial drivers. With these results, it can be concluded that during the process of adaptation to milk by the strain of *L. reuteri*, strains with new beneficial abilities were selected to this new environment; but this led to the loss of several of his initial skills such as the capacity to grow in a hostile environment like the vertebrate intestine.

Key words: *Lactobacillus reuteri*; Experimental Evolution; Bacterial Evolution; Adaptative evolution; Long- Term Serial Passage

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Probiotic bacteria

Centuries ago, people already knew of the possible beneficial effects of eating or drinking fermented products. In fact, fermented foods were used before the microorganisms were known to exist. Metchnikoff, a century ago, prescribed fermented products for medical conditions; he also mentioned that using "friendly" bacteria possibility improve health and retarded senility by modulating the intestinal microbiota of the patient (1).

Both FAO (United Nations Food and Agriculture Organization) and WHO (World Health Organization) have defined probiotics as live microorganisms that, when ingested in the correct amounts, provide a certain benefit to the health of the host. The ISAPP (International Scientific Association of Probiotics and Prebiotics) also uses this definition (2). Interestingly, the EFSA (European Food Safety Authority) and the FDA (Food and Drug Administration of the United States) do not use the above definition, they emphasize that this possible definition has been overwhelmed more by the commercial interest and that scientific justification still needs to be proven (3) . In some countries, such as the United States, there is no scientific approval for any probiotic by any governmental agency (4).

There is a large number of publications in recent decades about clinical benefits of ingesting probiotics (about 6,000 biomedical publications) which demonstrates a growing interest in the subject (3). The microorganisms that have been used most as potentially probiotics have been both bifidobacterias and lactic acid bacteria (LAB), including some yeasts (5). Something that is also worth noting is that the demonstration of the possible beneficial effects of a

probiotic can only be validated by *in vivo* experiments (6). But even so it is possible to use *in vitro* studies, but intended to demonstrate a particular effect of a probiotic, as well as to describe some mechanism of action or the safety of certain bacteria. That is, *in vitro* experiments should the first step in the evaluation of a potential probiotic; these are *in vitro* test that should be carried out like the bile and gastric acid resistance (7, 8), the possible side effects (9, 10), the range of antibiotic resistance and some metabolic capability (7, 9, 11). It is important to consider characteristics required for these strains such as resistance to both acidity and the bile, presenting antimicrobial activities mainly against intestinal pathogenic microorganisms (12–14) and intestinal commensals, ability to adhere to the mucus or epithelial cells (15).

Recent studies indicate that any microorganism entering the microbiota must cope with different bacterial toxins and antimicrobial compounds produced by the resident bacteria in the intestine (16–19).

Genus *Lactobacillus*

Lactobacillus spp. are the most common bacterial species present in probiotics (20). These bacteria belonging to the genus *Lactobacillus* share certain characteristics like absence of spores, Gram positive bacilli and microaerophilic. *Lactobacillus* species use hexose sugars and transform them into lactic acid, which leads to the generation of an acidic environment that usually inhibits the development of other commensals or pathogenic bacteria (21). In the case of *Lactobacillus* colonizing humans, these can be found both in the gastrointestinal tract and in the vagina (22). In fact, it is one of the first bacteria that colonize the intestine of newborns, along with Bifidobacteria (23). This genus include

species previously studied as possible probiotics like *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. bulgaricus*, and *L. reuteri* (24).

Lactobacillus reuteri

L. reuteri was isolated for the first time in 1962 and it has been described as a heterofermentative bacteria that do not need oxygen to grow and tolerate a range of pH environments. *L. reuteri* colonizes the gut of a variety of vertebrates during its evolution. It has coevolved with different hosts and diversified into distinct phylogenetic lineages (25, 26). And have the capability to inhibit other bacteria through the production of antimicrobial intermediates (27, 28). It has been shown that *L. reuteri* is truly indigenous to the human gut (29) and it is more typically found in the duodenum (30). The gastrointestinal tract of vertebrates is known to be a relatively hostile environment that presents several challenges for a microorganism to colonize (31, 32). The first thing that a colonizer microorganism needs to overcome is these adverse conditions of the intestine. Some strains *of L. reuteri* can colonize the intestine of vertebrates because they can resist the pH ranges of this environment as well as counteracting the action of bile salts. It has been seen that its capacity to form biofilms which may be responsible for its resistance (33).

This species can adhere to the mucus of the gut (34, 35). Several studies have investigated the possible mechanisms that allow *L. reuteri* to adhere to the mucus and of the gastrointestinal tract. The adhesion to the mucous is mediated by MUB proteins (mucus-binding proteins) which are encoded by a group of lactobacilar orthologues genes also known as adhesins (36–38).

Another characteristic of the bacteria that colonize the intestine is the ability to secrete antimicrobial factors and other compounds capable of modulating the immune response of the host. *L. reuteri* in birds and humans can synthesize and excrete an antimicrobial compound called reuterin (39–42). Reuterin inhibits a large group of microorganisms, especially Gram-negative bacteria (43). Other strains of *L. reuteri* are capable of producing other antimicrobial compounds such as: ethanol, lactic acid, reutericicline and acetic acid (44–47). *L. reuteri*, and other members of the genus, are able to synthesize vitamins such as vitamin B9 and B12, as well as other. It has also been found that they are capable of producing exopolysaccharide (EPS) which is important in the development of biofilms and in their ability to adhere to the epithelium (48). Additionally in *in vitro* experiments on pigs have shown that EPS prevents the colonization of *E. coli* to the epithelium, (49).

All these mechanisms allow *L. reuteri* to modulate the composition and diversity of the microbiota; these effects are specific to certain strains and not a generality of the species. (45, 50–52).

Lactobacillus spp. and probiotics

In USA, it is estimated that around 3.9 million people consume a prebiotic or probiotic supplement; probiotics are one of the most consumed dietary supplements nowadays (53). Nevertheless, there are very few studies correctly designed to understand the real impact of probiotic consumption in humans (54).

Many studies asses the safety of potentially probiotic bacteria in adults, children, infants, and even in an HIV-infected population (27, 55–59). For

example, Valeur *et al.* found that *L. reuteri* colonized the gastrointestinal tract of the healthy humans and modified the induction of CD4+ T cell in the ileum (27). Probiotic safety for children was demonstrated using milk-based formula with either *Lactobacillus reuteri* or *Bifidobacterium lactis* (58). Zmora *et al.* and Suez *et al.* demonstrated non-beneficial effects of probiotics in humans (43, 50) showing an individual and transitory effect in the microbiota and no effect in the intestinal transcriptome (53). Additionally, Suez *et al* (50) showed a delayed in the recovery of the intestinal microbiota dysbiosis induced for the probiotic use after antibiotics treatment (60).

Intestinal Microbiota

A colonizing bacteria needs to grow and survive the adverse conditions that this new environment entails (microbial toxins, stomach acid, antimicrobial peptides, bile salts, anoxic conditions and immune responses). Microbial toxins from native members of the intestinal microbiota such as hydrogen peroxide or bacterial peptides and proteins with antibacterial properties are important factors which deter the entry of a probiotic bacteria (19, 61, 62).

Previous studies of the human intestinal microbiota have shown a stability over time that can be maintained even for decades (63, 64) which seem contradictory if we consider the high prevalence of multiple antagonistic systems produced by members of any microbial community (19). Bacteria that make up the intestinal microbiota are very competitive for resource and produce antimicrobial compounds such as bacteriocins (65). Resistance to colonization, therefore, is a multifactorial phenomenon and is crucial in preventing the entry of pathogens. In general, resistance to colonization can be due to niche

occupation, modulation of virulence factors and host immunity, as well as competition for nutrient sources.

Resistance to colonization therefore is a multifactorial phenomenon and it is crucial in the prevention pathogen entry (66). For example, *Salmonella* expresses colicin Ib that stimulated the inflammation in the gut; this situation favors *Salmonella* over intestinal commensals such as *E. coli*. The expression of colicin Ib by *Salmonella* is triggered by the low concentrations of iron in the intestine (67). *Shigella sonnei* uses T6SS system to attack commensal *E. coli* and *Shigella flexneri* in the mouse intestines (68).

During the competition between intestinal bacteria antagonists, resulting dead cells could serve as source for competent organisms that can incorporate this foreign genetic material in their genomes ("bacterial evolution through antagonism"). Some cases of Streptococcal species such as *Streptococcus mutans* and *Streptococcus pneumoniae* can regulate the production system of bacteriocins with the machinery that allows them to incorporate genetic material from the environment (69).

Bacteria are thought to sense their surroundings to identify exogenous molecules (volatile compounds or secondary metabolites) that determine the presence of any microbial threat which is known as "danger sensing" and allows the bacteria respond accordingly (70). In *P. aeruginosa* bacterial cell lysis products induce the expression of T6SS as a defense mechanism (71).

Antagonistic pleiotropy and Probiotic bacteria evolution

Little is known about the evolutionary changes of probiotic bacteria as it adapts to new environments. It has been observed that when bacteria adapt to a new

environments, they lose aptitude to thrive in the original niche (72). During the adaptation to new environments, bacteria carrying mutations that increase the fitness for this novel environment are selected (73); however these mutations often reduce the aptitude to thrive in the original environment, a phenomenon known as antagonist pleiotropy (AP) (74)(75).

Another phenomenon of pleiotropy, usually uncommon, is synergistic pleiotropy (SP) that occurs when the selected mutations coincidentally increase or decrease fitness for two environmental conditions (75).

Experimental evolution

Experimental evolution has been used extensively to understand evolutionary processes (73). Numerous evolutionary studies have been designed to understand how populations are able to adapt to specific environmental conditions, like nutrients (76), temperature (73), parasites (77), competition (78, 79) and other environmental stressors (80). Among the evolution experiments in laboratory, the most notorious is carried out by *Lenski* et al., this is a long-term evolution experiment through 66,000 generations of 12 *E. coli* clones (81, 82). For more than 20 years this group has investigated frequency of mutations, changes in the fitness of bacteria during adaptation to a specific environment, convergence and divergence, parallel evolution, horizontal gene transfer and recombination (76-79).

Most studies of bacterial evolution in laboratory have focused on *E. coli* as a model microorganism. Addressing these studies, mainly to how *E. coli* adapts to artificial laboratory media (domestication) (84) and also how it responds to challenging conditions in new environments (81).

Most of the previous studies employ minimal means during serial passes and constant conditions, seeing this as a possible bias in evolutionary investigations Kram *et al.* (85) designed an experiment of serial passages *of E. coli* in relatively enriched medium (Luria-Bertani) allowing bacteria to develop throughout the five phases of bacterial growth in the laboratory. Obtaining that the adaptation to this new environment occurs in relatively fewer generations than the experiments previously performed in *E. coli* (81).

Previous studies with *Pseudomonas fluorescens* showed that a propagation in a static glass tube with nutrient-rich medium (classical experimental evolution in lab) produces a rapid diversification of an isogenic bacteria population (86). This rapid bacterial diversification generates multiple specialized genotypes in niches. But specific adaptation to any niche limits the ability of bacterial genotypes to diversify (87, 88). The authors propose that in environments where similar levels of diversity can be sustained, diversification will be more likely to occur immediately after the colonization of the rich medium and not through expansion into new niches within this new environment (86).

Evolution in *Lactobacillus* spp.

Previous studies of *L. reuteri* strains using Multilocus Sequence Analysis (MLSA) and Amplified Fragment Length Polymorphism (AFLP), identified a specific relationship between genotype and specific mammalian host (89, 90); studies carried out on *Lactobacillus*-free gnotobiotic mice showed that only *L. reuteri* strains isolated from rodents were able to colonize mice efficiently (90).

The current availability of LAB genomes has allowed a deeper understanding of they evolutionary path that this bacterium takes when cultured in milk; this

studies reveal a tendency to reduce the size of the genome (91) and gene loss (evidenced by pseudogene occurrence) is the result of adaptation of this bacterium to nutrient-rich environments such as milk (92). For example, it has been seen that *L. bulgaricus* in yogurt cultures has about 270 pseudogenes, which points to a recent adaptation to milk (93).

Evolutionary changes in milk adaptation of a *Lactobacillus reuteri* intestinal strain

Abstract

Bacteria with probiotic characteristics are used both in the food industry and in health areas, however, little is known about their evolution. It has been observed that when bacteria adapt to a new environment, they lose the skills necessary to thrive in the original niche. In this study, a strain of *Lactobacillus reuteri* was isolated from the duodenum of a mouse and serial passes were made in milk for 150 days to define the evolutionary changes that affect the capacity for survival and growth in the intestine. Growth capacity experiments were carried out with the resulting bacteria and their initial progenitor (ancestor). The adapted to milk outperformed the rifampicin resistant ancestor in 66% and the original ancestor (rifampicin sensitive) in 33%. Genetic adaptation to milk was confirmed by the presence of mutations associated with metabolism and signal transduction. Non-synonymous mutations were the more frequent type of mutations and much of its where beneficial drivers. With these results, it can be concluded that during the process of adaptation to milk by the strain of *L. reuteri*, strains with new beneficial abilities were selected to this new environment; but this led to the loss of several of his initial skills such as the capacity to grow in a hostile environment like the vertebrate intestine.

Key words: *Lactobacillus reuteri*; Experimental Evolution; Bacterial Evolution; Adaptative Evolution; Long- Term Serial Passage

Introduction

Bacteria with probiotic characteristics are used both in food and in health industries. 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” (2). Nevertheless, there are some principles that a bacteria potentially probiotic must achieve to considered effective; like the ability to perform clear benefits in the host, the nonexistence of any antibiotic resistance and the resistance to the aggressive environment during the digestion (15). Recent studies indicate that any microorganism entering the microbiota must cope with bacterial toxins and antimicrobial agents produced by the resident bacteria in the intestine (16–19).

Lactobacillus spp. includes, within the probiotic bacteria, very used species, that can be found in a wide range of food products (20). The genus *Lactobacillus* are Gram-positive bacteria, non-sporulated, that included species previously studied as possible probiotics like *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. bulgaricus*, and *L. reuteri* (24).

L. reuteri was isolated for the first time in 1962 and it has been described as a heterofermentative bacteria that do not need oxygen to grow and support a range of pH environments. *L. reuteri* colonizes the gut of a variety of vertebrates during its evolution. It has coevolved with different hosts and diversified into distinct phylogenetic lineages (25, 26). Furthermore, this microbiome have mechanisms that allow it to inhibit other bacteria, even its capable to secrete antimicrobial intermediates (27, 28).

It has been shown that *L. reuteri* is a bacteria truly indigenous of the human gut (29) and it is more typically found in the duodenum (30).

In USA, it is estimated that around 3.9 million people consume a prebiotic / probiotic supplement, which highlights probiotics as one of the most consumed dietary supplements nowadays (53). Nevertheless, there are very few studies, based on evidence, to understand the real impact of probiotic consumption in humans (54).

For a microorganism that enters the intestine through food to colonize, it needs not only to have its own characteristics that allow it to grow in this new environment, but also to give it the ability to survive the adverse conditions that this new environment entails. Like the pH during the passage through the stomach, the presence of antimicrobial peptides, the biliary salts discharged to the intestine, the anoxic conditions of the intestine, as well as the immune response of the host. Within the adverse conditions to be faced by a colonizing bacteria, one of the most important is the competition that is generated with the native members of the intestinal microbiota and with it the damages that can cause these inhabitants of the intestine such as: the production of molecules that can have an effect on the immune response of the host, others with antibiotic properties or antimicrobial peptides, the interference of cellular signals and the synthesis of metabolites such as hydrogen peroxide or peptides and proteins with toxic properties (19, 61, 62).

Many studies have been conducted to assess the safety of potentially probiotic bacteria in adults, children, infants, and even in an HIV-infected population (27, 55–59); but little is known about the evolution of these bacteria and almost nothing about the evolutionary changes that could lead to adaptation to new environments. It has been observed that when bacteria adapt to a new environment, they lose the skills necessary to thrive in the original niche (72).

Experimental evolution has been used extensively in evolution studies since it allows the study, in real time, of the evolutionary processes. (73). Numerous evolutionary studies have been designed to understand how populations are able to adapt to specific environmental conditions, like nutrients (76), temperature (73), parasites (77), competition (78, 79) and other environmental stressors (80).

In general, it is assumed that most adaptations to a condition are associated with the loss of adaptation to the original condition, which would mean that changes that increase the fitness in a given ecosystem would be detrimental in other different environments (73).

Adaptation occurs through the selection of mutants that have acquired biochemical or biophysical functions that adapt better to the new environment. Bacteria that find a new challenge in their ecosystem, such as a deficient nutrient source or an antibiotic treatment, will face a remarkable selective pressure that will result in the selection of mutants that have greater capacity for growth in the new conditions (72).

Adaptation may limit the ability of bacterial genotypes to genetically diversify. Studies in *Pseudomonas aeruginosa*, have shown that this limitation of the ability to diversify is not the result of the general evolution or the evolution of an intrinsic reduction in the capacity of evolution, but is caused by the specific adaptation to the environment (85).

The current availability of sequenced genomes of lactic acid bacteria (LAB) has allowed a deeper understanding of the evolutionary divergence of LABs revealing a tendency to reduce the size of the genome (91). It has been seen that most of the lost genes have been due to adaptation to new nutrient-rich

environments, especially bacteria that have adapted to milk and other food environments rich in carbohydrates and proteins (92).

Lactobacillus reuteri exhibits beneficial properties specific to the strain relevant to human health, which makes it a model organism to study host interactions-symbiont, as well as the coevolution of the microbe-host (94). We designed the present study to evaluate the evolutionary changes at the molecular and phenotypic level of a *Lactobacillus reuteri* strain, from the mouse duodenum, during adaptation to a new environment (artificial cow's milk).

Materials and Methods

Isolation and selection of strains of *Lactobacillus reuteri*

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 Bioethics Committee of the San Francisco University of Quito, Ecuador). A CD1 mouse was euthanized using chloroform, the duodenum was extracted and contents were inoculated onto Man Rogosa Sharpe (MRS) agar and incubated at 37°C for 48 hours under microaerophilic conditions (95). After incubation, they were analyzed morphologically (Gram stain) and biochemically (catalase and oxidase tests) (supplementary table 1) (96). Six *Lactobacillus* spp. isolates were selected and 16S rRNA gene analysis was used to determine bacterial species (97), PCR products were sequenced at Functional Biosciences, Inc., Madison, WI. (supplementary table 1) and sequences were compared to those in genbank (98).

Selection of Rifampicin resistant mutants

Four *Lactobacillus reuteri* strains were inoculated into 10 ml of MRS broth at 37°C under microaerophilic conditions for 24 hours, after which bacterial cultures received an additional 10 ml of MRS broth with rifampicin (200μg/ml), for a final concentration of 100μg/ml), incubated for another 24 hours and finally inoculated into MRS agar with rifampicin 100μg/ml) for a final 24-hour incubation. Colonies formed by rifampicin resistant mutants were confirmed with Gram strain and biochemical tests (catalase and oxidase) and antibiotic sensitivity tests (61). Confirmed strains were stored at -80C in BHI medium + glycerol 20%. Fitness loss due to rifampicin resistance was evaluated by

culturing together progenitor strains and rifampicin resistant descendants as previously described (83) (supplementary table 2). Finally, we selected a strain LrRR1.2 which showed the least fitness loss to carry out the rest of the experiments (supplementary table 2).

Lactobacillus reuteri Rifampicin-Resistant Strain Milk adaptation

The mutant strain LrRR1.2 was subjected to 150 serial 24h-passes in sterile (autoclaved) cow's milk incubated at 37°C under microaerobiosis; these number of passes corresponds to approximately 510 generations (85). To rule out potential *Lactobacillus* contamination, PCR amplification and sequencing of *LeuS* gene (26) was carried out at passes: 55, 92 and 120.

Intestinal colonization capacity assays in murine model

All the procedures performed with the mice were previously approved by the Ethics Committee on the Use of Animals in Research and Teaching at the San Francisco University of Quito. Male and female CD1 mice donated by "Laboratorios Agrocalidad", Tumbaco, Quito were used. Animals were fed for 7 days with 100 µl of *Lactobacillus reuteri* in culture medium at $2x10^8$ CFU/ml. The animals were separated into three groups: group 1 was fed with *Lactobacillus reuteri* rifampicin-resistant mutant strain (pass 0), group 2 was fed with *Lactobacillus reuteri* rifampicin-resistant mutant strain (pass 150) and the group 3 or control group that was fed only with the culture medium without *Lactobacillus reuteri*. After 15 post-administration days of the treatments, the animals were euthanized as previously described and contents from 10mm of duodenum were serially diluted and plated in MRS plus rifampicin (99).

Comparative Growth in MRS broth and Milk

The resulting strains from milk adaptation were taken (ancestor, mutant resistant to rifampicin without milk passes and mutant resistant to rifampicin adapted to the milk) and the individual culture of each one was carried out in both MRS and Milk. Following the methodology of Lenski *et al* (83) the initial population density was quantified and after 24 hours of culture in each medium and relative fitness was defined with the formula $W = \ln [A_{24h} / A_{0h}] / \ln [B_{24h} / B_{0h}]$. Obtaining the growth capacities of each strain in Milk and MRS comparing both the ancestor and the parent before adapting to milk (**Table 1 and Figure 1**).

DNA extraction and Whole Genome Sequencing

Lactobacillus reuteri strains sensitive to rifampicin (original ancestor), *Lactobacillus reuteri* rifampicin-resistant mutant strain without milk passes and *Lactobacillus reuteri* rifampicin-resistant mutant strain with 150 milk passes were selected for total DNA extraction using DNAzol™ Reagent, for isolation of genomic DNA from solid and liquid samples (Invitrogen™) following the manufacturer's protocol (100). The total DNA of the 3 samples was sent to Macrogen Inc., Seoul, Republic of Korea for genome sequencing, using Illumina Hiseq 2500, 100bp PE.

Molecular Analysis

The complete genome sequences of the 3 strains were assembled using Velvet version 1.2.10. The Raw Data of the sequences were pre-processed before running the hashing. We used Single Reads from Velvet to align contigs, Mauve

version 2.4.0 for the reordering of the contigs (101) and Progressive Mauve for the alignment (102). The 3 genomes were reordered based on the complete genome sequence of *Lactobacillus reuteri* DSM 20016 on GenBank (NC_009513.1).

For the analysis of the protein-coding genes, a Comprehensive Genome Analysis was made of the genome of the three strains, in Patrick version 3.5.22 (103) and from the sequences of the individual genes, alignments were made in Mega version 7.0 for the identification of possible mutations in the genes (98). The Codon-based Test of Positive Selection for analysis between sequences of MEGA7 was used for the analysis of Positive Selection and Purification Selection of the sequences of the genes that presented mutations using the Nei-Gojobori method (104).

Results

Competitions between Mutants Strains Resistant to Rifampicin and its Ancestor, in Milk

The rifampin-resistant mutant strain after 150 passes in sterile cow milk outperformed the rifampicin resistant ancestor in 66% (relative fitness = 1.66) and the original ancestor (rifampicin sensitive) in 33% (relative fitness = 1.33). While the rifampin-resistant mutant strain without passes in sterile cow milk had a fitness relative to the original ancestor of 0.80 **(Figure 1).**

Competitions between Mutant Strains Resistant to Rifampicin and its Ancestor, in MRS medium

Both mutant strains resistant to rifampicin lost fitness respect to their ancestor (*Lactobacillus reuteri* strain sensitive to rifampicin); but competition between both mutant strains shows that the strain without passes in milk (fitness relative to the ancestor = 0.95) exceeded the strain adapted to the milk (fitness relative to the ancestor = 0.84). Since the strain without passes in milk was 5% less efficient than the ancestor while strain adapted to the milk was developed 16% less than its ancestor (**Table 1**).

Intestinal colonization capacity assays in murine model

Bacterial growth from small intestine was possible to obtain in only 2 mice *in vivo* (supplementary figure 1), however in a mouse receiving the ancestral rifampicin resistant the count of *Lactobacillus* was 35 times larger than the animals receiving the strain passaged in milk (993 CFU/ml vs 28 CFU/ml).

Molecular Analysis

The alignment made in Mauve (**Figure 2**) showed that all the contigs were correctly aligned among the three analyzed genomes. Comprehensive Genome Analysis in Patrick showed that the three genomes have 2,262 Mb, 2,260 Mb and 2,240 Mb respectively distributed in 994, 899 and 1054 contigs. Gene annotation using Patrick allow us compare the sequences of 651 functional genes from *Lactobacillus reuteri* Rifampicin-resistant mutant Milk-adapted strain with the *Lactobacillus reuteri* Rifampicin-resistant mutant strain. Of the genes analyzed 4,61% had mutations (substitutions, insertions or deletions) compared to the progenitor. Of the 52 mutations found (distributed in 30 genes) 29 mutations were non-synonymous and 9 synonymous mutations (**Figure 3**). When we analyzed the observed mutations with Codon-based Test of Positive Selection in Mega7, it showed that one gene showed signs of positive selection: the gene coding for *Phosphate ABC transporter, permease protein PstA* (p-value 0.043) (**table 2**).

The greatest number of genes with non-synonymous mutations was observed in genes related to metabolism (12 mutated genes of 258 metabolism related genes) followed by genes related to protein processing (6 mutated genes of 131 protein processing related genes). Four genes: *Ribosome small subunit biogenesis RbfA-release protein RsgA* gen, *Translation initiation factor 1* gen, *Segregation and condensation protein B* gen and *dTDP-glucose 4,6-dehydratase* gen showed a return to the ancestor sequence (*Lactobacillus reuteri* Rifampicin-sensitive ancestor strain).

Discussion

A successful probiotic bacterium must resist digestion and antimicrobial compounds produced by the resident microbiota, environmental conditions that are very different from those found in dairy products. In this study, a strain of *Lactobacillus reuteri* (recently isolated from intestine) adapted to grow in milk after 150 passes (around 510 generations) in this substrate. Whole genome sequence comparison showed that the bacterial populations adapted to milk had evidence of positive selection in the *pstA* gene coding for *phosphate ABC transporter, permease protein*. Phosphate is one of the most abundant minerals in milk (105). It is an important mineral for bacterial growth, recently it was demonstrated that the accumulation of intracellular polyphosphate intervenes in the ability to respond to stress conditions in *Lactobacillus* spp. (106). PstA is a membrane protein involved in the transport of phosphorus in bacteria (107), so it is possible that a mutant with increased levels of phosphorous uptake may grow faster. Additionally, PstA has seems to be involved in maintenance of cell homeostasis interacting with Cyclic-di-AMP (c-di-AMP). c-di-AMP is a second messenger crucial for bacterial physiology (61) which may indicate that bacteria in milk were under constant adaptive pressure.

Genetic adaptation to milk was confirmed by the presence of mutations associated with metabolism and signal transduction. Previous studies have observed that genetic adaptation to new ecosystem reduces the fitness to the original ecosystem, a phenomenon known as antagonistic pleiotropy (61). Although we failed to show that *L. reutieri*, adapted to milk, was less able to colonize intestines, we did show that milk adapted strains have lost ability to grow in MRS broth (**Table 3**). The data suggest that *L. reuteri* after 150 passes

in milk may have also lost fitness for intestinal colonization. This finding is in agreement with previous reports indicating that commercial probiotics fail to colonize intestines; even low passage probiotics may have reduced ability to colonize intestines.

Previous reports indicate that bacterial adaptation to a new environment could be observed from 30 to thousands of generations (22, 44, 45-47) and at 300 generations, strains showed multiple non-synonymous mutations (85).

Differences in number of adaptive mutants in generations in different reports may be related to the bacterial species used, nutrients in culture media, stress level, type of bacterial passage, etc. Bacterial stress may increase the rates of mutation (108, 109) and growth in an enriched medium causes the cells to go through the five phases observed during typical in-vitro growth (including the lag and death phases) and therefore bacteria may experience more stress than experiments in minimal media with low glucose levels (110). Also in milk we obtained cell counts of ~10^{10} CFU/ml versus the 10^{7} CFU/ml observed in a low carbon source environment (85); larger population sizes increases the pool of potential adaptive mutants. Additionally, every pass involved a 1:10 dilution which is a population bottleneck, although it is not as narrow as other experiments (85), if it is in relation to others (111, 112).

Mutations in the rpoB gene sequence encoding the DNA subunit of the DNA-dependent RNA polymerase (RNAP) are the cause of resistance to rifampicin. What these mutations produce is the decrease in the affinity of rifampicin to RNAP (113). RNAP is a holoenzyme that costs a central enzyme, consisting of two α subunits, a β subunit, a β' subunit and a ω subunit, and a sigma factor (114). To initiate transcription, the central enzyme is associated with one of the

σ factors that are responsible for the recognition of the promoter sequences, allowing the specific binding of RNAP to the genetic promoters (115). The σ factors are divided into 2 families: the σ70 family (116) and the σ54 family (117). The σ70 family consists of 4 folded domains (σ1, σ2, σ3, σ4), the σ4 domain forms a helix-turn-helix motif that interacts with the β subunit of the central RNAP (118), while σ2 does contact with the central RNAP through the β' subunit (119). Mutations that affect RNAP can arise in any of the subunits (120). For example, mutation in α subunit (RpoA) can alter the cellular phenotype (121), mutations in β (RpoB) leads to the alteration of growth and the ability to compete (122). The loss of the growth capacity of our mutant strains in the intestine could be related to mutations in the beta subunit of the RNAP that gives them rifampicin resistance. As evidenced in other studies, such as that of Rothstein *et al*. (123) in which rifampin-resistant *B. subtilis* mutants showed a temperature-sensitive sporulation phenotype; and the studies by Maughan *et al.* (122) who identified mutations in rpoB that trigger specific alterations in the expression of global regulons, which control growth, competition, sporulation and germination.

Per Kram *et al.* (85) even in a complex and heterogeneous environment, there may be relatively few pathways leading to strong adaptive phenotypes. This could be related to our results in which only one gene: permease A of the transported ABC of phosphate was found under selective pressure (p-value of 0.045) during the adaptation to milk by the strain of *L. reuteri* resistant to rifampicin.

Analyzing the genes that code for proteins, a total of 52 mutations were observed in approximately 500 generations; if we take into account that other

experiments in *Escherichia coli* have found 76 mutations after 50,000 generations (100 times more generations than in our study) (124) we can affirm that the passes in milk generated a relatively high frequency of mutations. Most of these mutations were point mutations (73.1%) much higher than the results found in *E. coli* where point mutations represented 56% (124). These differences could be since the experiments in *E. coli* were performed in glucose-poor minimum media; while our experiments were performed on milk, a relatively enriched medium (124).

The highest number of genes with non-synonymous mutations was observed in genes related to metabolism (12/258) like Long Term Serial Passage experiments in *E. coli* where they showed a stronger convergence in genes that encode proteins with regulatory and metabolic functions basic (124).

Non-synonymous mutations were the more frequent type of mutations present in all the sequences genes analyzed. Tenaillon *et al.* (124) found in *E. coli* that non-synonymous mutations accumulated in more frequency and more faster than other type of mutations and they pointed out that these results suggests that the majority of these non-synonymous mutations were beneficial. We agree with this authors in the idea that the great majority of the mutations observed were beneficial drivers.

Pervious experiments in milk suggests that biosynthesis genes pointed out the adaptation to that environment rich principally in lactose and proteins (92). And this could be related to the genes Cardiolipin synthase, bacterial type *ClsA*, Cobalamin synthase, Phosphoribosylaminoimidazole-succinocarboxamide synthase, Asparagine synthetase [glutamine-hydrolyzing]/*AsnB*, Glycyl-tRNA synthetase alpha chain and Histidyl-tRNA synthetase that presented non-

synonymous mutations in our experiment. The absence of a statistically significant positive selection could be related to the presence of a single substitution, so a deeper study may be necessary to point out some of these genes in the process of adaptation to milk.

Ketoacyl synthases (KSs) (particularly 3-oxoacyl synthase) are condensing enzymes that play an important role in the synthesis of fatty acids. (125). The identification in the strain adapted to the milk of a deletion in the sequence of 3-oxoacyl- [acyl-carrier-protein] synthase, KASII (703delA) that causes a non-functional protein (reading frame shift and stop codons) it could be related to the loss of intestinal grow capacity or to some of its potentially probiotic characteristics, since it is known that the degradation of short-chain fatty acids is important in the bacteria that are part of the intestinal microbiota (126).

The presence of the *clsA* gene with several mutations [957T> C; 961_962insG] leads to a shift of the reading frame and stop codons that end in a non-functional protein or a deficiency of this protein in the strain adapted to milk. This gen was previously involved in the osmotic adaptation and membrane structure of *Bacillus subtilis* (127).This could be related to the high lipid content of milk (105, 128) that causes a change in the metabolism of membrane lipid synthesis in this bacterium that results in the loss of an initial characteristic that is no longer necessary in the new environment but that if it could cause effects on survival capacity in the mouse intestine.

The purine repressor, *purR*, represses the transcription of several genes with functions in the synthesis, transport and metabolism of purines (the pur operon) (129). The finding in the milk-adapted strain of this gene with an insert (798_799insT) that causes a stop codon shows us there is no inhibition or

negative control of the routes of synthesis of the purines in this strain, which could be related to the need of the de novo synthesis of purines in milk.

The Substrate-specific component RibU of riboflavin gene ECF transporter presents 13 non-synonymous substitutions in the strain adapted to milk, so it is to consider its involvement in the adaptation in this medium. RibU belongs to the prokaryotic riboflavin transporter family that is a member of the bile transporter/arsenite /riboflavin superfamily. The riboflavin (vitamin B2) is the precursor of the flavin mononucleotide coenzymes (FMN) and flavin adenine dinucleotide, two essential cofactors in some of the enzymes involved in redox reactions. Ribu is a secondary transporter that mediates the facilitated diffusion of riboflavin (130). In the milk, there is a moderate amount of riboflavin (128) so apparently these changes contribute, in theory, to a better use of this metabolite by bacteria.

The insertion identified in the Catabolite control protein A gene of the strain adapted to the milk causes a reading frame shift and consequently a different protein that could influence the use of lactose, the most representative sugar in milk. The presence in the medium of a new carbon source can cause the rapid metabolization of it by inhibiting pathways for the use of another type of substrate. Studies in *B. subtilis* have shown that CcpA is essential for the adjustment of lactose transport, the activity of beta-galactosidase (LacZ) and glycolysis, guiding the optimal glycolytic flow and an adequate growth rate (131). Other studies in *Streptococcus pneumoniae* have shown that CcpA is a transcriptional activator that in the presence of glucose in the medium modulates the synthesis of phosphofructokinase, pyruvate kinase and lactate dehydrogenase, thus modifying the glycolytic flow (132). CcpA can adjust both

the absorption of lactose and the ability of glycolysis to cause optimal glycolytic flow and growth rate in *Streptococcus thermophilus* (133). These previous evidences reaffirm the idea that the selection of mutations in this gene, during growth in milk, could be due to the need to use lactose as the primary source of carbon for our bacteria.

Conclusions

With the experiments of relative fitness in milk, we can have confirmed that the 500 generations, product of the 150 passes in milk, were enough to select adaptive phenotypes for this new environment. The largest number of mutations were non-synonymous and occurred more frequently in genes related to metabolism; what supposes an important paper in the modulation of the metabolic routes in the adaptation to a new environment.

Finally, during the adaptation to milk of *Lactobacillus reuteri*, bacteria were selected with new beneficial abilities to this environment, such as the use of phosphate, casein as a source of amino acids or lactose as a carbon source; but adaptation to this new environment resulted in the loss of several of his initial skills, such as the capacity to grow in a hostile environment like the vertebrate intestine.

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Figures and tables

Table 1. Competitions between Mutant Strains Resistant to Rifampicin and its Ancestor, in MRS medium.

Strain	Relative Fitness	Percentage
MRR(0)*	0.95	-5 %
MRR(150)**	0.84	-16%

* MRR (0) = *Lactobacillus reuteri* mutant strain resistant to rifampicin without passes in milk.
**MRR (150) = *Lactobacillus reuteri* mutant strain resistant to rifampin with 150 passes in milk.

Table 2. Synonymous and non-synonymous substitutions of *Lactobacillus reuteri* Rifampicin-resistant mutant Milk-adapted strain.

Gen	Function	Sequence Change	Type of Mutation	Protein Change
Translation initiation factor 1	Protein Processing, Cellular Processes	12G>C	synonymous	
dTDP-glucose 4,6-dehydratase	Cellular Envelope	[126T>A; 129T>C; 138T>C]	synonymous	
Glucose-6-phosphate 1-dehydrogenase	Metabolism, Energy	463G>T	nonsynonymous	154D>Y
Amidophosphoribosyltransferase	Metabolism	449G>T	nonsynonymous	150I>S
Aspartokinase	Metabolism	1002A>C	synonymous	
Cardiolipin synthase, bacterial type ClsA	Metabolism, Response to stress, Defense, Virulence	957T>C	synonymous	
Cobalamin synthase	Metabolism	334A>G	nonsynonymous	112S>G
Phosphate ABC transporter, permease protein PstA	Metabolism	[185T>G; 186G>T; 187T>G]	nonsynonymous	[62L>C; 63F>V]
Phosphate ABC transporter, permease protein PstC	Metabolism	549A>G	synonymous	
Phosphoribosylaminoimidazole-succinocarboxamide synthase	Metabolism	[536G>A; 545G>A]	nonsynonymous	[179G>D; 182S>N]
Substrate-specific component RibU of riboflavin ECF transporter	Metabolism	[494T>A; 495T>G; 496A>G; 497T>G; 498T>A; 499A>G; 501A>T; 502G>T; 505T>A; 506T>A; 507G>A;508G>A;511G>T]	nonsynonymous	[165I>K; 166I>A; 167K>D; 168G>C;169L>K;170V>I]
Asparagine synthetase [glutamine-hydrolyzing] / AsnB	Protein Processing	1319A>G	nonsynonymous	440Q>R
Glycyl-tRNA synthetase alpha chain	Protein Processing	103G >T	nonsynonymous	35A>S
Histidyl-tRNA synthetase	Protein Processing	976T>G	nonsynonymous	326F>V
LSU ribosomal protein L10p (P0)	Protein Processing	245C>T	nonsynonymous	82A>V
LSU ribosomal protein L2p (L8e)	Protein Processing	740T>G	nonsynonymous	247L>R
Translation initiation factor 2	Protein Processing	1476T>C	synonymous	
ATP-dependent helicase/nuclease AddAB, subunit A	DNA Processing	1340G>T	nonsynonymous	447R>L
DNA gyrase subunit A	DNA Processing, Response to stress, Defense, Virulence	[2271G>T; 2275G>T]	nonsynonymous	759V>F
Ribonuclease Y	RNA Processing	84A>G	synonymous	

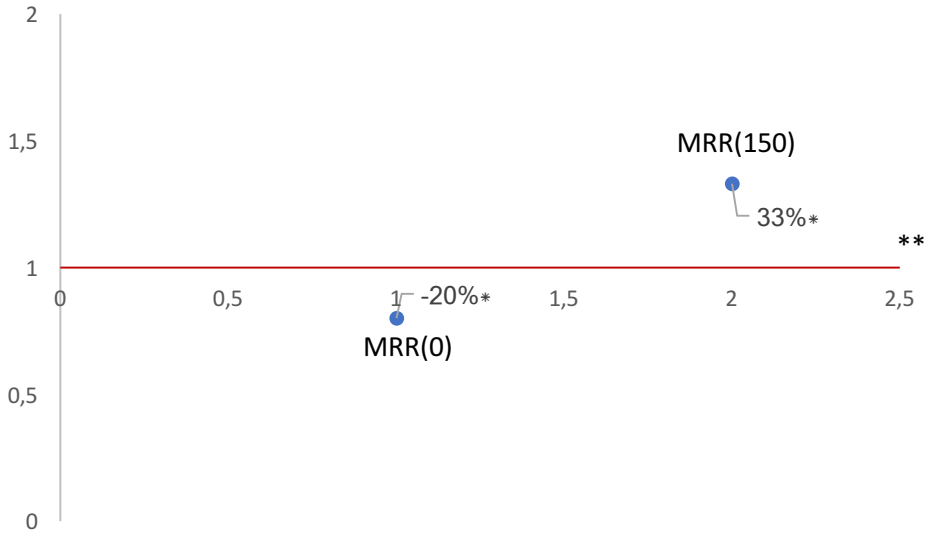


Figure 1. In milk competitions between *Lactobacillus reuteri* mutant strains resistant to rifampicin, without previous passes in milk (MRR (0)) and with 150 passes in milk (MRR (150)), against the original *Lactobacillus reuteri* strain (ancestor)

* Fitness relative to the ancestor expressed as a percentage
** Ancestor (red line)

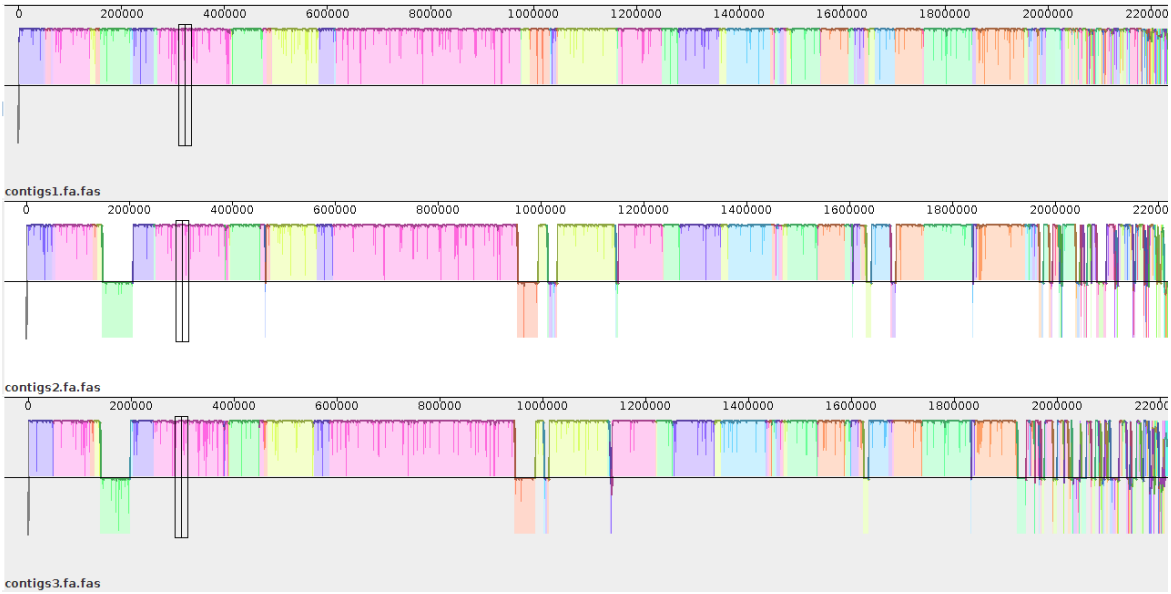


Figure 2. Progressive Mauve alignment of the three genomes: *Lactobacillus reuteri* strain sensitive to rifampicin (original ancestor), *Lactobacillus reuteri* rifampicin mutant resistant strain without previous passes in milk and *Lactobacillus reuteri* rifampicin mutant resistant strain with 150 passes in milk.

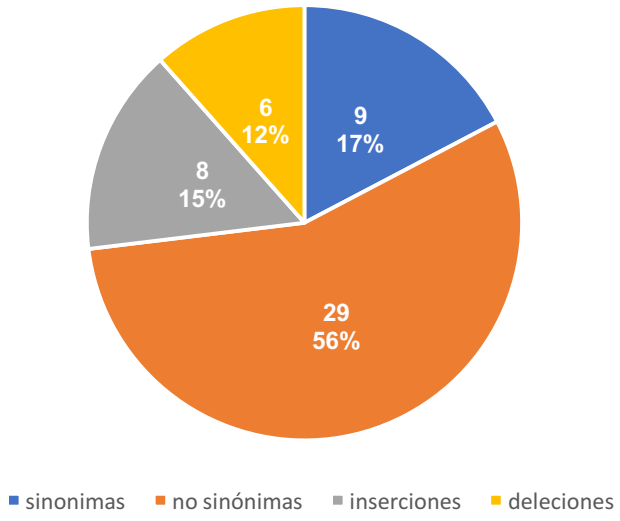


Figure 3. Types of Mutations present in the *Lactobacillus reuteri* Rifampicin-resistant mutant Milk-adapted strain.

Supplementary Materials

Supplementary table 1. Morphological and biochemical characteristics of the strains studied, and 16SrRNA gene species identification.

Strain	Gram Stain Morphology	Catalase Test	Oxidase Test	16S rRNA
A1.01	Bacillus Gram Positive	-	-	
A2.02	Bacillus Gram Positive	-	-	
A4.1.01	Bacillus Gram Positive	-	-	
A4.2.02	Bacillus Gram Positive	-	-	*Lactobacillus murinus*
A7.1.01	Bacillus Gram Positive	-	-	
A7.2.01	Bacillus Gram Positive	-	-	*Lactobacillus reuteri*
C1.01	Bacillus Gram Positive	-	-	*Lactobacillus reuteri*
C1.02	Bacillus Gram Positive	-	-	
AM1.01	Bacillus Gram Positive	-	-	
AM2.01	Bacillus Gram Positive	-	-	*Lactobacillus reuteri*
AM3.01	Bacillus Gram Positive	-	-	
A5.01	Bacillus Gram Positive	-	-	*Lactobacillus murinus*
A6.01	Bacillus Gram Positive	-	-	*Lactobacillus reuteri*

Supplementary table 2. Relative fitness of the *Lactobacillus reuteri* strain rifampicin-mutant resistant with its corresponding rifampicin sensible ancestor.

Rifampicin Mutant Resistant Strain	Rifampicin Sensible Ancestor	Relative Fitness
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

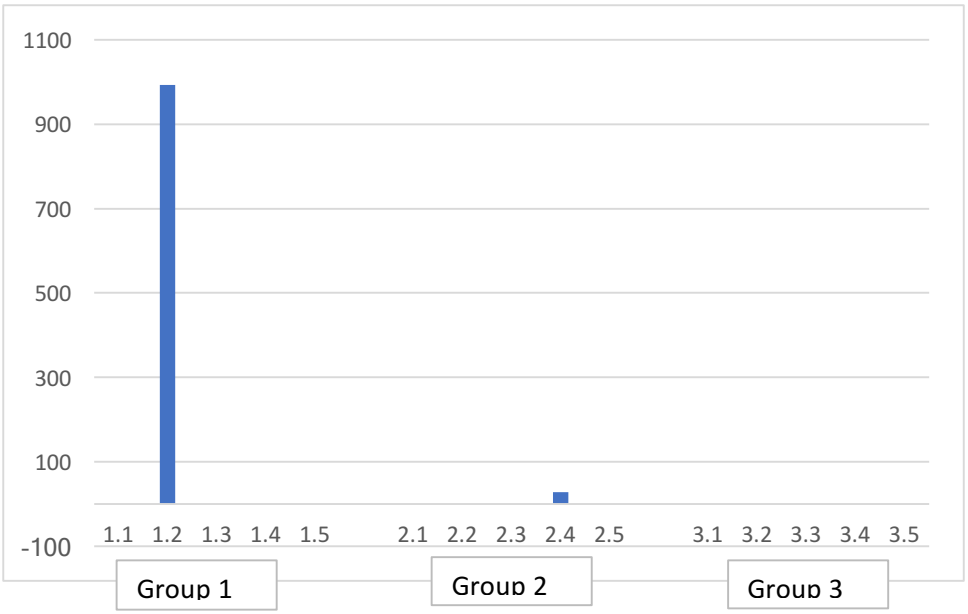
Supplementary table 3. Genome features of the complete genomes

	Genome 1*	Genome 2**	Genome 3***
contigs	994	899	1054
length (Mb)	2,262	2,260	2,238
GC Content	38,33	38,34	38,35
CDs	2613	2540	2660

*Genome 1: *Lactobacillus reuteri* Rifampicin-sensitive ancestor strain

**Genome 2: *Lactobacillus reuteri* Rifampicin-resistant mutant strain

***Genome 3: *Lactobacillus reuteri* Rifampicin-resistant mutant Milk-adapted strain



Supplementary figure 1. Intestinal colonization assay with mutant strains resistant to rifampicin, without previous passes in milk (Group 1), 150 passes in milk (Group 2) and a control group that did not receive any *Lactobacillus reuteri* strain (Group 3).