Generation of lactose and protease positive probiotic *Lacticaseibacillus rhamnosus* GG by conjugation with *Lactococcus lactis* NCDO 712

Running title: Improvement of *L. rhamnosus* GG for growth in milk

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Abstract

Lacticaseibacillus rhamnosus GG (LGG) is the most studied probiotic bacterium in the world. It is used as a probiotic supplement in many foods, including various dairy products. However, LGG grows poorly in milk, as it neither metabolizes the main milk carbohydrate lactose, nor degrades the major milk protein casein effectively. In this study, we made *L. rhamnosus* GG lactose and protease positive by conjugation with the dairy *Lactococcus lactis* strain NCDO 712 carrying the lactose-protease plasmid pLP712. A lactose hydrolyzing transconjugant colony was obtained on agar containing lactose as the sole source of carbohydrates. By microscopic analysis and PCR with LGG- and pLP712-specific primers, the transconjugant was confirmed to be originated from LGG, and to carry the plasmid pLP712. The transconjugant was named *L. rhamnosus* LAB49. Isolation of plasmids revealed that not only pLP712, but also other plasmids had been transferred from *L. lactis* into LGG during conjugation. With plasmid-specific PCR primers, four additional lactococcal plasmids were detected in LAB49. Proteolytic activity assay and SDS-PAGE analysis verified that *L. rhamnosus* LAB49 effectively degraded β-casein. In contrast to its parental strain LGG, the ability of LAB49 to metabolize lactose and degrade casein enabled strong and fast growth in milk. As strains with new properties made by conjugation are not regarded as GMOs, *L. rhamnosus* LAB49 could be beneficial in dairy fermentations as a probiotic starter culture.

Importance

Probiotic strain *Lacticaseibacillus rhamnosus* GG (LGG) is widely sold on market as a probiotic or added as supplement in dairy foods because of its benefits in human health. However, due to the deficiency of lactose and casein utilization, LGG does not grow well in milk. On the other hand, lactose intolerance and cow's milk protein allergy are the two major problems related to milk consumption. One option to help with these two conditions is the use of probiotic or lactose and casein hydrolyzing bacteria in dairy products. The purpose of this study was to equip LGG with lactose/casein hydrolyzing ability by bacterial conjugation. As a result, we generated a non-GMO LGG derivative with improved properties and better growth in milk.

INTRODUCTION

Lacticaseibacillus rhamnosus GG (LGG; ATCC 53103) is a well characterized probiotic strain widely used in various foods (1, 2). LGG was originally isolated from feces of healthy human (3), and over the last three decades, it has been extensively studied for its probiotic properties and impact on human health (4, 5). The strain has strong adhesion ability to the intestinal mucosa, and it has been used for prevention and treatment of diarrhea, gastrointestinal infections, and to boost up the host immune responses against allergic reactions (6). LGG is documented to maintain a good microbial balance of bacteria in the human gut by preventing the growth of harmful bacteria (7).

Lactococcus lactis subsp. *cremoris* NCDO 712 is a dairy strain originally isolated from cheese starter culture. The strain carries six plasmids, namely pSH71 (2.1 kb), pSH72 (3.6 kb), pSH73 (8.7 kb), pSH74 (15.5 kb), pNZ712 (49.8 kb) and pLP712 (55.4 kb) (8). Plasmid pLP712 provides *L. lactis* NCDO 712 the ability to grow in milk, as it contains the gene encoding serine protease PrtP for casein degradation, as well as the genes for lactose catabolism (Fig. 1) (9, 10).

Bovine milk contains about 4.8% lactose, while human milk contains up to 7% lactose (11). Lactose is an important energy source for breast-fed human infants, constituting around 40% of the total daily energy intake (12). After weaning, around 70% of the world population experiences a decline in lactose hydrolyzing enzyme activity in small intestine due to genetic factors, leading to lactose intolerance (13-15). Besides lactose intolerance, cow's milk protein allergy (CMPA) is a common food allergy with an estimated prevalence of 2%–3% worldwide (16). One option to reduce the symptoms of lactose intolerance and CMPA is the use of probiotic or lactose and/or casein hydrolyzing bacteria in dairy products (11, 17). These bacteria increase the overall hydrolytic capacity of the small intestine that promotes the lactose digestion (18). In addition,

bacteria in fermented products can decrease the lactose concentration and increase active lactase enzyme entering the intestine (19). LGG is used as a probiotic supplement in various dairy products, such as fermented and pasteurized milk, cheese and yogurt. However, LGG does not grow well in milk (20, 21), due to the lack of its capacity to hydrolyze lactose and casein, the main nutrients in milk (22-24). The inability to grow in milk limits the possible uses of LGG as a starter strain.

The properties of probiotics can be modified and improved by genetic engineering. LGG has been made lactose positive by fixing the two mutations in its lactose operon (25). Even though the resulting lactose hydrolyzing LGG did not carry foreign genetic material, the strain is regarded as genetically modified organism (GMO) in the EU, because it was made by using recombinant DNA techniques. The EU legislation considers relevant "the way" the modification was performed. Use of GMOs in food is heavily regulated and not popular among consumers in the EU (26-28). Therefore, modifications and improvements of food bacteria should be performed by non-GMO transformation methods, which include e.g., transduction and conjugation of natural phages, plasmids, or transposons (28). Regarding lactic acid bacteria, the lactose-protease plasmid pLP712 in *L. lactis* NCDO 712 has been transferred into lactose deficient strains by conjugation (29). Even though conjugation can occur between different bacterial species and even between different genera, pLP712 has only been transferred within the species *L. lactis*. Regarding LGG, the only published study about conjugation is the unsuccessful transfer of vancomycin resistance between LGG and enterococci (30). There are no reports about effective conjugation in LGG.

In this study, we generated proteolytic and lactose positive LGG strain by conjugation of the lactose-protease plasmid pLP712 from *L. lactis*. The resulting transconjugant strain *L. rhamnosus*

91 LAB49 effectively hydrolyzed lactose and β-casein, and it grew well in milk. Hence, *L. rhamnosus*

92 LAB49 can be regarded as an upgraded food-grade and non-GMO derivative of LGG.

MATERIALS AND METHODS

Bacterial strains and growth media

Lactococcus lactis subsp. *cremoris* strain NCDO 712 carrying the lactose-protease plasmid pLP712 (Fig. 1) (9) was used as the plasmid donor strain, and the lactose negative *Lacticaseibacillus rhamnosus* strain GG (LGG; ATCC 53103) (3) was used as the recipient strain in mating experiment. LGG was routinely cultured in Man, Rogosa, Sharpe (MRS; Merck, Darmstadt, Germany) media at 37 °C. *L. lactis* NCDO 712 (9) was grown at 30 °C in M17 media supplemented with 0.5% (w/v) glucose.

Conjugation

L. lactis NCDO 712 and LGG were mated for the transfer of the conjugative plasmid pLP712. Prior to conjugation, both strains were grown in their respective media overnight. From each culture 1.5 ml were centrifuged (4300×*g*), the cells were washed with 1.5 ml of 0.85% NaCl, and resuspended in 1.5 ml MRS broth. Donor NCDO 712 (75 µl) and recipient LGG (750 µl) suspensions were added into 3 ml MRS broth. The mixture was incubated for 4 h at 37 °C. Then, 200 µl of broth was spread in triplicate onto transconjugant selective agar made of modified MRS (mMRS; MRS without glucose and meat extract) supplemented with 1% lactose, 100 µg/ml vancomycin, and 50 µg/ml bromocresol purple (BCP). Vancomycin prevents the growth of *Lactococcus*, whereas LGG does not utilize lactose. Fermenting of lactose produces lactic acid, which lowers the pH of the media and thus changes the color of BCP from purple to yellow. The plates were incubated for 24-48 h at 37 °C in an anaerobic jar. Obtained colonies were streaked onto new selective agar plates to obtain pure cultures, and further cultured and maintained in mMRS broth media with 1% lactose. Transconjugant strain was preserved in respective media containing 20% (v/v) glycerol and stored in cryovials at -80 °C.

Confirmation of conjugation: Strain and plasmid-specific PCR analysis

Transconjugant strain was confirmed by colony PCR using primers specific to *L. rhamnosus* GG (31, 32) or pLP712 (Table 1). The LGG-specific *spaC* primers amplify an 801 bp fragment from the pilus gene *spaC*. The other LGG-specific primer pair (A and B) amplify a 757 bp fragment containing the hypothetical conserved protein gene LGG_00154. Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) was used for PCR amplification. The reaction mixture (25 µl) contained 20 pmol of each primer, Phusion HF Buffer, 0.2 mM dNTPs, 0.5 U Phusion DNA polymerase, and cells from single colony as template. PCR amplifications were performed in Eppendorf Mastercycler (Hamburg, Germany) using the following thermal cycling profile: initial denaturation 98 °C for 30 s, 35 cycles of 98 °C for 10 s, 60 °C for 30 s, and 72 °C for either 30 s (LGG specific PCR) or 60 s (pLP712 specific PCR), and a final extension step of 72 °C for 5 min. PCR products (5 µl) were separated by electrophoresis in 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide, and the DNA was visualized by ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA). The size markers used were 100 bp and 1 kb GeneRuler DNA ladder (Thermo Fisher Scientific).

Plasmid analyses and identifications

Plasmid DNA was isolated from *Lacticaseibacillus* strains according to the method by Anderson and McKay (1983) (33) with modifications described by Wan et al. (34). Isolated DNA was run in agarose gel to analyze the plasmid profile. The size marker used was 1 kb GeneRuler DNA ladder (Thermo Fisher Scientific). PCR primers specific to different *L. lactis* NCDO 712 plasmids were used to identify the conjugated plasmids (Table 1). The primer sequences were obtained from the study by Tarazanova et al. (8). As the PCR with the published primers to detect pSH74 did not work, new pSH74 specific primers were designed. PCR conditions and cycles were the same as

above; the extension time at 72 °C was 1 min for amplicons from plasmids pNZ712 and pSH71 (primers *repAC*-FW/RV), 30 s for pSH72 and pSH73, and 20 s for pSH74.

Plasmid stability

To evaluate the segregational stability of pLP712 in *L. rhamnosus* LAB49 without selection pressure by lactose, fresh culture was sequentially inoculated in MRS broth at 0.1% inoculum and incubated at 37 °C for 16 h. After every passage, up to 10 passages (approximately 100 generations) in the absence of lactose, the cultures were serially diluted and plated onto MRS agar. Next day, on average 80 single colonies were randomly picked and transferred onto mMRS-lactose-BCP agar plates and checked for their lactose fermentation ability after incubation for 24 h at 37 °C.

Protease activity assays

Proteolytic activity was determined by using two different assays. Lactose negative LAB49 colonies from the plasmid stability test were examined for protease activity by an agar halo method based on Vandenberg et al (35). First, white Lac^- colonies from mMRS-lactose-BCP plates were transferred into MRS broth and incubated o/n at 37 °C. Then, three µl of the cultures were spotted on the indicator agar made of mMRS without triammonium citrate but supplemented with 2% skim milk. The indicator plates were incubated at 37 °C for 24 h. Hydrolysis of milk casein can be seen as a clear halo around the bacterial spot.

To show the actual casein degradation, another protease activity assay was performed according to the method described by Vukotic et al. with slight modifications (36). Protease production was induced by culturing the cells on milk citrate agar plates [containing 4.4% reconstituted milk, 0.1% yeast extract, 0.8% sodium citrate, 0.5% glucose, and 1.5% agar (w/v)] for 48 hours at 30 °C. The

cells were resuspended in 0.1 M sodium phosphate buffer (pH 7.0), and adjusted to approximately 10^9 CFU/ml. β-casein (~24 kDa) (Sigma-Aldrich, St. Louis, MO, USA) solution (5 mg/ml) was prepared by dissolving in same buffer. Cell suspension was mixed with β-casein solution at a ratio 1:1 (v/v) and incubated for 4 h at 30 °C, after which the cells were removed by centrifugation at 13 000×*g* for 10 min. Clear supernatant was used for SDS-PAGE analysis. The supernatant was mixed with sample loading buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 0.01 mM EDTA, 5% 2-mercaptoethanol, 25% glycerol, and 0.07% bromophenol blue) at a ratio 1:1 (v/v) and heated at 100 °C for 5 minutes. Heated samples were loaded 10 µl/well and the gel (15%) was run for 20 h at 20 mA. Same concentration of β-casein solution without cell treatment was used as a reference to compare the bands. To confirm that β-casein was degraded by the activity of a protease, phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor (2 mM), was added in β-casein solution and cell suspension mixture before overnight incubation in a separate experiment.

Growth in milk

To perceive the difference between the growth of the two strains in milk, LAB49 and LGG were pre-cultured for 20 h in milk (4.8% lactose, Valio Ltd., Helsinki, Finland), or milk with 2% glucose, respectively. After that, 10 ml of milk was seeded (1.5% inoculum) with the pre-cultures. Inoculated milks were incubated at 37 °C for 24 h, during which samples were taken every hour from 1 to 12 h, and at 14, 16, 18, 20, 22, and 24 h to determine the CFU/ml by plate counting. Growth rate was expressed as generation time (GT), which was derived by using the equation GT = t/n, where t = time interval in minutes, and n = number of generations, calculated with the formula n = (logb – logB)/log2, where B = number of bacteria at the beginning of the time interval, and b = number of bacteria at the end of the time interval (37). Milk coagulation was observed visually. Sterilized milk without bacterial inoculums was used as negative control.

RESULTS

Conjugation of pLP712 into *L. rhamnosus* GG

Lacticaseibacillus rhamnosus GG is a lactose negative strain. The first aim in this work was to make it lactose positive by conjugal transfer of the lactose-protease plasmid pLP712 from *Lactococcus lactis* NCDO 712. As LGG is endogenously resistant to vancomycin, whereas NCDO 712 is sensitive, transconjugants were selected on agar plates containing vancomycin and lactose as selective agents, supplemented with BCP indicating acid production from lactose. Approximately 15 vancomycin resistant, lactose hydrolyzing yellow colonies appeared on each selective agar plates, giving a transfer frequency of approximately 4×10^{-8} progeny per recipient. One colony was chosen for closer analyses. By phase contrast microscopy it was seen that the chosen strain was rod-shaped bacteria forming long chains, typical to *L. rhamnosus* GG. The strain was streaked on selective agar plate to confirm its lactose utilization capacity (Fig. 2). Wild-type LGG did not change the purple color of the agar by acid production, due to the lack of lactose digesting ability. The transconjugant changed the color from purple to yellow, due to lactic acid production by lactose fermentation. The putative transconjugant was named *L. rhamnosus* LAB49.

Molecular techniques were applied to confirm that LAB49 was a derivative of *L. rhamnosus* GG and that it contained pLP712. Two pairs of previously published strain-specific PCR primers for identifying LGG were used. Both primer pairs gave amplifications of expected size (801 bp and 757 bp) from LGG and the transconjugant, but not from *L. lactis* (Fig. 3). The presence of the lactose-protease plasmid pLP712 in LAB49 was then examined with lactococcal *prtP*-specific primers. Correct 2.5 kb bands were amplified from LAB49 and *L. lactis* NCDO 712, but not from

LGG (Fig. 3). These results confirmed that the transconjugant undoubtedly originated from LGG, and that the plasmid pLP712 had successfully been conjugated from its native carrier *L. lactis* NCDO 712 into LGG.

Identification of plasmids in *L. rhamnosus* LAB49

To visualize the plasmid pLP712 in the transconjugant, plasmids of *L. rhamnosus* LAB49 were isolated and compared to the plasmid profiles of LGG and *L. lactis* NCDO 712. In the results of plasmid electrophoresis, there were no plasmid bands from the wild type LGG, as anticipated, and only one band representing the chromosomal DNA could be seen in gel (Fig. 4, lane 3). As also expected based on previous studies (9) plasmid isolation from NCDO 712 resulted in several plasmid bands of different sizes, representing the six plasmids of the strain (Fig. 4, lane 2). Interestingly, the transconjugant strain LAB49 also carried many plasmids, obviously both large and small, as many bands were seen in plasmid gel (Fig. 4, lane 1). Apparently, conjugation of pLP712 had mobilized other plasmids, which were co-transferred to *L. rhamnosus* GG without any selection pressure. To identify, which plasmids had been transferred, PCR with plasmid specific primers was conducted. Four additional lactococcal plasmids were found and identified to be pSH71 (2062 bp), pSH72 (3597 bp), pSH73 (8663 bp), and pNZ712 (49.83 kb) (Fig. 5). Hence, of all six plasmids present in NCDO 712, five were transferred by conjugation, and only the plasmid pSH74 (15.52 kb) could not be detected in LAB49.

Stability of the plasmid pLP712 in LAB49

The stability of pLP712 in *L. rhamnosus* LAB49 under non-selective conditions was determined. LAB49 was grown in MRS broth containing 2% glucose for about 100 generations, and the lactose utilization was tested at about every 10 generations. After 90 generations only 2% of the cells retained the lactose fermenting ability, and after 100 generations all tested colonies (n=80) were

lactose negative (Fig. 6A). The results indicated that the plasmid pLP712 is either easily lost in *L. rhamnosus* LAB49 without selection pressure by lactose, or that the plasmid easily makes rearrangements causing mutations or deletion of lactose genes. To verify this, 20 lactose negative colonies were examined for protease activity on 2% milk agar. All the tested colonies showed clear proteolytic activity, indicating that the LAB49 cells still carried the gene *prtP* and hence the plasmid pLP712, but the lactose genes in the plasmid had been mutated or deleted (Fig. 6B).

Proteolytic activity

As *L. rhamnosus* GG does not effectively degrade β-casein, we tested whether the lactococcal PrtP protease encoded by the gene *prtP* in the plasmid pLP712 would make LGG proteolytically more active. To test the phenotype, proteolytic activity assay was performed for digestion of β-casein. The protease production was first induced on milk citrate agar, after which the cells were incubated with β-casein. SDS-PAGE analysis confirmed that β-casein was fully digested in four hours by LAB49 and NCDO 712, but not at all by LGG (Fig. 7). When the protease inhibitor PMSF was added to cell-casein mixture, β-casein was not degraded by any strain even after 16 h incubation. This verified that the observed casein degradation was indeed a result of a protease activity, obviously by the lactococcal protease PrtP. In conclusion, the ability of *L. rhamnosus* LAB49 to hydrolyze casein showed that the plasmid conjugation had made the strain proteolytic, and that LAB49 could probably degrade proteins in milk, thus improving its growth.

Growth in milk

Next, as LAB49 utilized lactose and casein, we wanted to determine whether these properties improve the growth in milk, compared to LGG. As shown in Fig. 8, the transconjugant *L.*

rhamnosus LAB49 grew well in milk, reaching stationary phase (approximately 10^9 CFU/ml) in 11-12 hours after inoculation. Its strongest exponential growth phase took place about 2-4 hours after inoculation, and a generation time calculated at that 2-h time period was about 40 minutes. During the first 4 h time period from the inoculation, there were 4.6 generations, giving a single generation time of 52 minutes. As expected, the parental strain LGG could not properly grow in milk, and thus its generation time was not calculated. Apparent coagulation of milk with LAB49 started in a few hours after inoculation, and the milk was fully coagulated after 12 h incubation. Quite the opposite, LGG-milk was still liquid after 24 h at 37 °C (results not shown). To sum up, the new abilities to metabolize lactose and degrade casein enabled good growth of LAB49 in milk.

Discussion

Lactose and casein are the main nutrients in milk. The most studied probiotic lactic acid bacterium in the world, *L. rhamnosus* GG, grows poorly in milk, as it is not able to hydrolyze lactose or casein. In this study, we have given LGG the ability to thrive in milk by transferring the lactose-protease plasmid pLP712 from *L. lactis* NCDO 712 via conjugation. With the aid of pLP712, the LGG transconjugant strain LAB49 could hydrolyze both lactose and casein, and thus the transconjugant grew considerably better in milk than the parental LGG.

The plasmid pLP712 has earlier been transferred by conjugation only into other strains of *L. lactis* (39, 40). Here we present the intergeneric conjugation of pLP712. Transfer frequency (4×10^{-8} progeny per recipient) from *L. lactis* to *L. rhamnosus* was somewhat lower than what has been described with conjugation of pLP712 between *L. lactis* strains (about 10^{-7} progeny per recipient) (9, 38). Successful conjugation of LGG has not been reported before, as the only published trial has been the unsuccessful transfer of vancomycin resistance from LGG to *Enterococcus* (30). This result cannot be regarded as a disappointment, as it emphasizes the safety of LGG. Vancomycin resistance in LGG is a chromosomally encoded property, and thus it is not easily transferred horizontally. However, mobile DNA elements, such as conjugative plasmids, may also mobilize chromosomal genes (41). Consequently, it is possible that the plasmids in LAB49 could mobilize the chromosomal D-alanine-D-alanine ligase gene *ddlA*, presumably behind the intrinsic vancomycin resistance. Still, most if not all of the naturally vancomycin resistant lactobacilli carry endogenous mobile DNA elements, transposases, insertion sequences and conjugative plasmids (42). Even though it has been suggested that probiotics may spread vancomycin resistance (43), their mobile DNA elements have not been shown to trigger mobilization of the *ddlA* gene and transfer vancomycin resistance, and thus such an incident could be considered unlikely.

Instead, properties encoded by plasmids, including those in non-conjugative plasmids, are much more likely to be transferred. In our work here, three non-conjugative plasmids, namely pSH71, pSH72, and pSH73 were mobilized with the conjugative plasmids pLP712 and pNZ712. Transfer of pNZ712 can, however, also be considered as co-conjugation, as there was no selection pressure for its transfer. Non-conjugative plasmids without selection pressure for their transfer have been shown to co-transfer easily with conjugative plasmids. For instance, in the study by Barry et al. (40), non-conjugative plasmids were transferred along with the conjugative ones in all 143 conjugation trials made. Thus, mobilization of non-transmissible plasmids seems to be a common phenomenon. Interestingly, the only plasmid not transferred from *L. lactis* NCDO 712 into LGG, was pSH74 carrying the *spa* genes for a pilus structure (8). Both NCDO 712 and LGG carry similar pili (44). Even though it has been shown that the NCDO 712 pilus contributes the conjugation efficiency, its precise function in DNA exchange is not known. Regarding LGG, its pili are associated to its probiotic nature, e.g. adhesive interactions with intestinal epithelial cells (45). The fact that the conjugated plasmids have provided several new features to LAB49 compared to LGG, may raise a concern about the probiotic capacity of LAB49. Hence, it would be good to investigate whether LAB49 still holds the probiotic activity of LGG, e.g. the adherence to epithelial cells.

Many studies have reported the structural instability of the plasmid pLP712, particularly occurring during conjugation (10, 46). The deletions and DNA rearrangements in pLP712 seem to happen spontaneously, often resulting in lactose or protease deficient transconjugants. Apparently, the deletions are caused by the numerous insertion sequence elements and transposase genes present in the plasmid (Fig. 1). Different ways for lactose gene deletions by transposases in pLP712 have been described previously (10). Despite the recognized structural instability, pLP712 has been reported to be segregationally stable in *L. lactis*. As shown in the study by O'Sullivan et al. (39),

after 75 generations in non-selective medium, no loss of pLP712 was observed in *L. lactis* transconjugant, as confirmed by plasmid isolations. In the present study here, the lactose metabolism of the transconjugant LAB49 was stable when the strain was grown with lactose, but without the selection pressure by lactose, the capacity to utilize lactose was completely gone in about 100 generations. However, when examining the proteolytic activity of the lactose negative colonies after 100 generations in non-selective conditions, we found that all of them exhibited protease activity, indicating the presence of pLP712. The proteolytic halo observed in Fig. 6B showed that the loss of lactose metabolism was not caused by plasmid disappearance, but rather the plasmid underwent structural changes resulting in mutations or deletion of lactose genes, possibly in the way shown by Wegmann et al. (10). Our results hence confirmed the previous findings that pLP712 is segregationally stable, but structurally unstable. Nevertheless, as long as the strain is cultured with lactose, the selection pressure will ensure that the lactose genes are functional.

For an unknown reason, LGG does not hydrolyze casein, which affects its growth in milk (44). The casein deficiency of LGG has been proposed to be caused by the lack of the gene LC705_02680 encoding a subtilisin-like serine protease containing an LPXTG motif, present in the proteolytic *L. rhamnosus* strain LC705 (44). However, LGG carries intact *prtP* and *prtM* genes encoding subtilisin-like cell envelope serine protease and its maturation protein, respectively. The proteins are identical (99% identity) with those of *L. rhamnosus* LC705, but only 74-78% identical with those encoded by pLP712. Thus, hypothetically, PrtP may be the responsible enzyme for casein degradation in *L. lactis*, but not in *L. rhamnosus*, and in the latter the additional protease encoded by the gene LC705_02680 is needed for casein hydrolysis. On the other hand, it is not known whether *prtP* or *prtM* are expressed in LGG. A possible reason for the LGG's inability to

329 degrade casein can be a fatal mutation in the intergenic *prt* promoter region. The *prt* promoters in
330 *L. lactis* and *L. rhamnosus* LC705 have been shown to be inducible in media with low peptide
331 concentrations and rich in partially hydrolyzed casein (44, 47). The DNA sequences of the *prtMP*
332 genes in LC705 and LGG are 98% identical, whereas the intergenic 284/285-bp regulatory region
333 is less identical, differing by 16 base pairs (94% identical). This suggests that the *prt* promoters in
334 LGG have mutated and may not be fully functional. It would require further studies to confirm
335 whether the casein deficiency of LGG is due to the lack of additional protease or a mutation in the
336 promoter region. Nevertheless, as shown in this study, heterologous lactococcal PrtP produced in
337 LGG restores its casein hydrolyzing phenotype.

338 LGG carries the genes *lacTEGF* for the lactose phosphotransferase system (44). Two mutations in
339 the Lac-PTS operon make LGG lactose deficient: a nonsense mutation in the phospho-β-
340 galactosidase gene *lacG*, and a 4-bp deletion creating a frameshift in the antiterminator gene *lacT*.
341 These mutations have been fixed by spontaneous mutagenesis with the help of a *lacT* expression
342 plasmid, resulting in lactose positive LGG (25). Because the strain was created by using a
343 recombinant plasmid, even though absent in the resulting strain, it is regarded as a GMO in the
344 EU, and thus its use in food is strongly regulated and restricted as well as widely disliked by
345 consumers (26, 27). On the contrary, the lactose positive LGG generated in our work here is not
346 regarded as a GMO in the EU, because conjugation is an accepted non-GMO method for bacterial
347 strain modifications (28). This is because conjugation is not recombinant DNA technique, but it
348 happens in nature between bacteria (48). Of course, to be regarded as a safe genetic modification,
349 the DNA to be conjugated must be endogenous DNA from food-grade bacteria, and it should not
350 carry antibiotic resistance genes (49). All the six plasmids in *L. lactis* NCDO 712 fulfill these
351 criteria.

L. rhamnosus LAB49 grew well in milk and it also coagulated milk thoroughly, apparently due to proteolytic digestion of casein and lactic acid production from lactose. The growth of LAB49 in milk was also expressed as generation time, calculated to be about 40 minutes at its lowest in early log-phase, and 52 minutes during the first 4-h period. Similar generation time of LGG has previously been determined in MRS (3), being 45 minutes during the first four hours after inoculation. In this work, it was not meaningful to calculate generation time for LGG, as it did not grow properly in milk.

The phenotype of *L. rhamnosus* LAB49 showed that the properties of probiotic *L. rhamnosus* GG can be modified by conjugation, as the transconjugant *L. rhamnosus* LAB49 could utilize lactose, degrade casein and grow better in milk than the parental strain. Therefore, compared to LGG, *L. rhamnosus* LAB49 has new alternative possibilities in dairy applications, for instance in helping with the symptoms of lactose intolerance or CMPA. Probiotics, particularly lactobacilli and bifidobacteria, have been used for prevention or treatment of CMPA among infants. The use of wild type *L. rhamnosus* GG for CMPA in animal models has provided positive effects and alleviated allergic reactions (50, 51). As shown in this paper, LAB49 hydrolyzes casein, and thus it would be interesting to investigate the efficacy of *L. rhamnosus* LAB49 in reducing the symptoms of CMPA.

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Tables

TABLE 1 PCR primers used in this study

Primers	Sequence 5' end to 3' end	Reference	Size	Use
***spaC*-FW**	CCAAATTGGCAACAGACCTT	(31)	801 bp	LGG-specific
***spaC*-RV**	GCCATCTGGTGCTTTTGTTT			
GG-spec-A	CGCCCTTAACAGCAGTCTTC	(32)	757 bp	LGG-specific
GG-spec-B	GCCCTCCGTATGCTTAAACC			
***prtP*-FW**	CTCGAGGCTAGCTCGTTTGATTTAATTGTG	This study	2550 bp	pLP712 specific
***prtP*-RV**	CCAGAATTCGGGCCCTATTCTTCACGTTGTTTCCG			
***repAC*-FW**	CGTTTCTGAGACGTTTTAGCG	This study	1680 bp	pSH71 specific
***repAC*-RV**	AAATAAAAGCCCCCTTCGACT			
pSH72-FW	GCTTTTTCGTTGGTTTGCTC	(8)	466 bp	pSH72 specific
pSH72-RV	GCCCAAAATAGTGGGTTAGTG			
pSH73-FW	TTTCAGTAGAAGGCCAAACAAC	(8)	803 bp	pSH73 specific
pSH73-RV	TGCAAATTTATCTACAAAGGCTTG			
pSH74-newFW	GTGGATGAACAAACAAAATACG	This study	195 bp	pSH74 specific
pSH74-newRV	GGATTGTGTCGATTTGCTTTACGC			
pNZ712-FW	CACTCTAGTTTCCTACCTTCGTTGCAAGC	(8)	1120 bp	pNZ712 specific
pNZ712-RV	GCTATACTTATACGGAGGATTAGCACTGG			

Figures

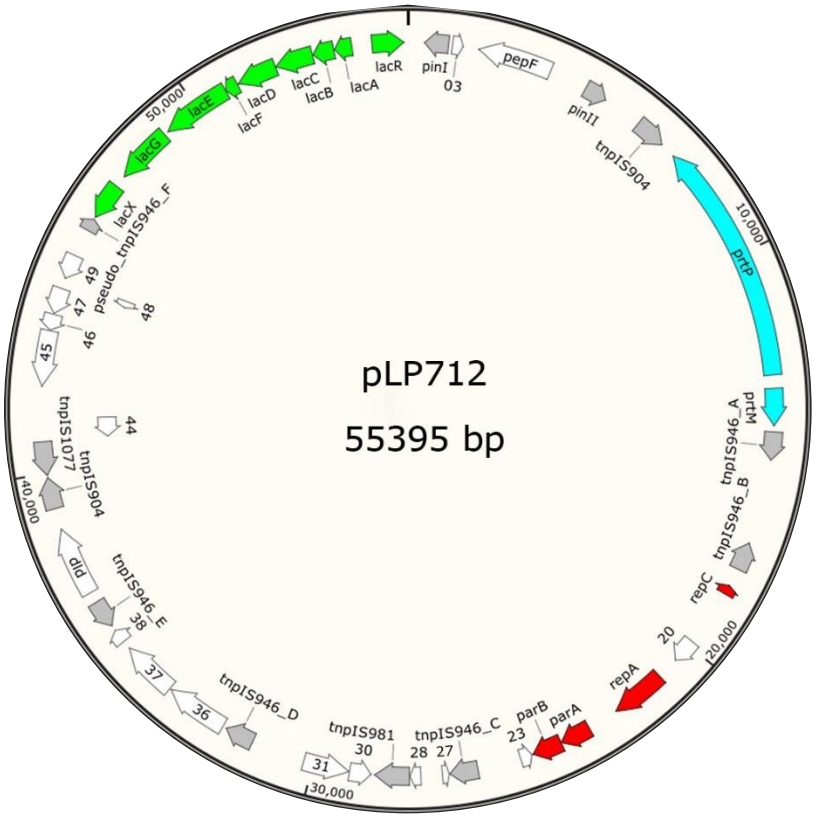


FIG 1 Plasmid pLP712. Genes relevant in this study are shown as arrows with different colours: lactose genes *lacR* and *lacABCDFEGX* are marked in green, protease and maturation genes *prtP* and *prtM* are marked in blue, replication and plasmid partitioning genes *repA*, *repC* and *parAB* are marked in red, and transposases are marked in grey. Other ORFs are marked as empty arrows with locus numbers or known gene abbreviations. The plasmid map was created by SnapGene® (Insightful Science, snapgene.com; GSL Biotech LLC, San Diego, CA, USA), and the sequence was extracted from NCBI GenBank database (accession number: FJ649478) (10).

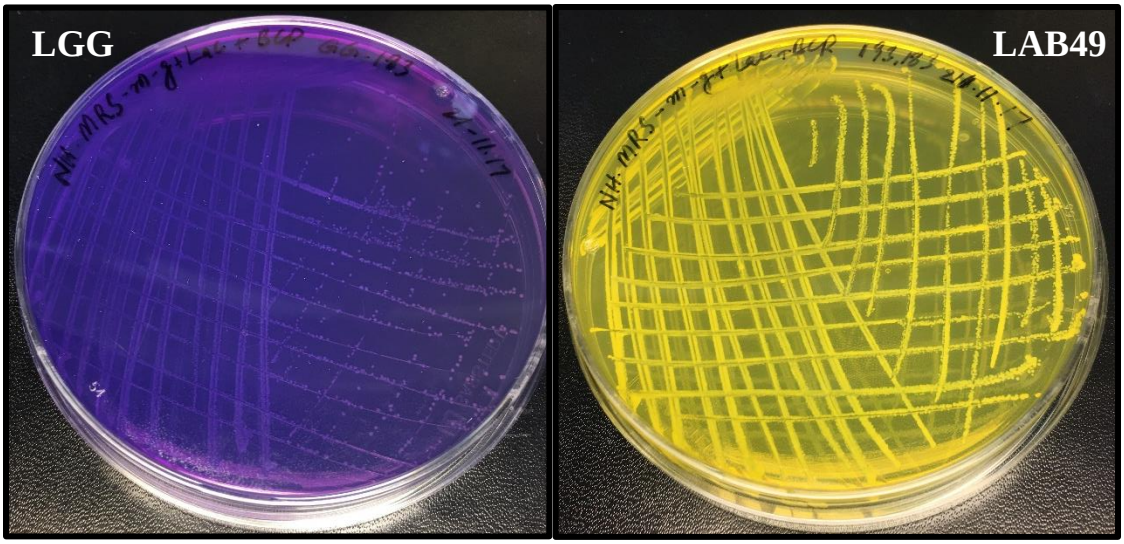


FIG 2 Growth of LGG and LAB49 on mMRS-lactose-BCP plates. LGG, *L. rhamnosus* GG with no acid production and colour change. LAB49, the transconjugant *L. rhamnosus* LAB49 has produced acid from lactose, causing colour change from purple to yellow.

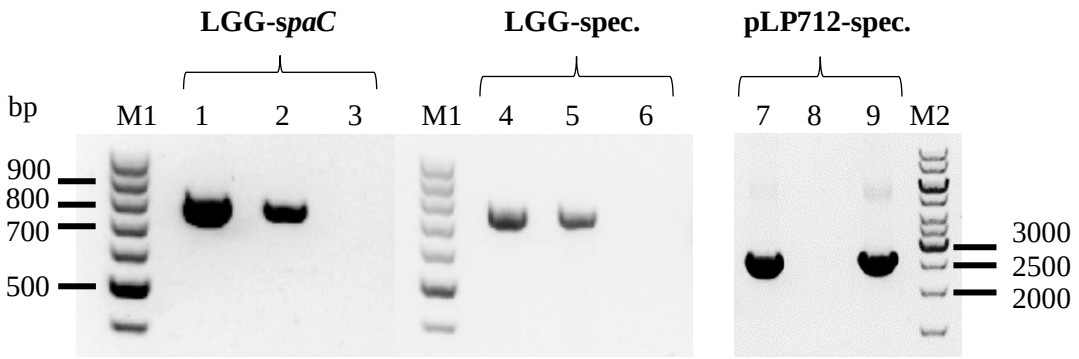


FIG 3 Verification of *L. rhamnosus* LAB49 as a derivative of *L. rhamnosus* GG, and the presence of pLP712 in LAB49 by PCR with *spaC*-, LGG- and pLP712-specific primers. Lanes 1, 4, 7: *L. rhamnosus* LAB49; lanes 2, 5, 8: *L. rhamnosus* GG; lanes 3, 6, 9: *L. lactis* NCDO 712. Lanes 1-3, LGG-specific *spaC* primers amplifying 801-bp PCR product; lanes 4-6, LGG-specific primers amplifying 757-bp PCR product; lanes 7-9, pLP712-specific primers amplifying 2.5 kb fragment of *L. lactis prtP* gene. M1, 100 bp GeneRuler DNA ladder. M2, 1 kb GeneRuler DNA ladder.

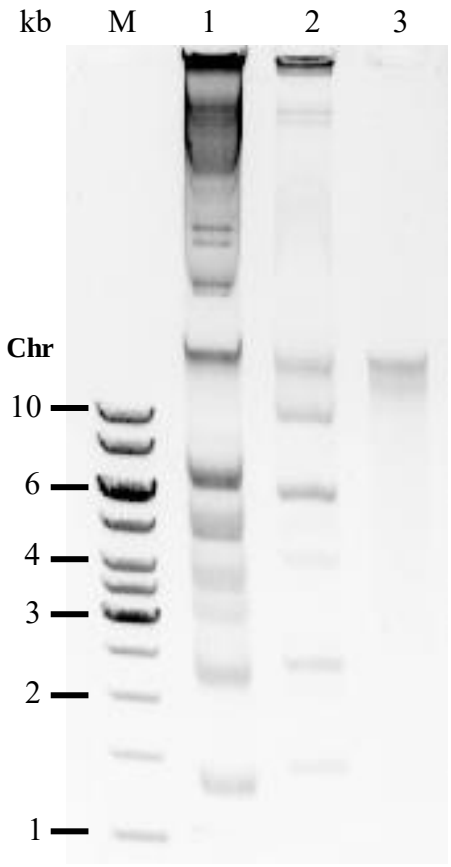
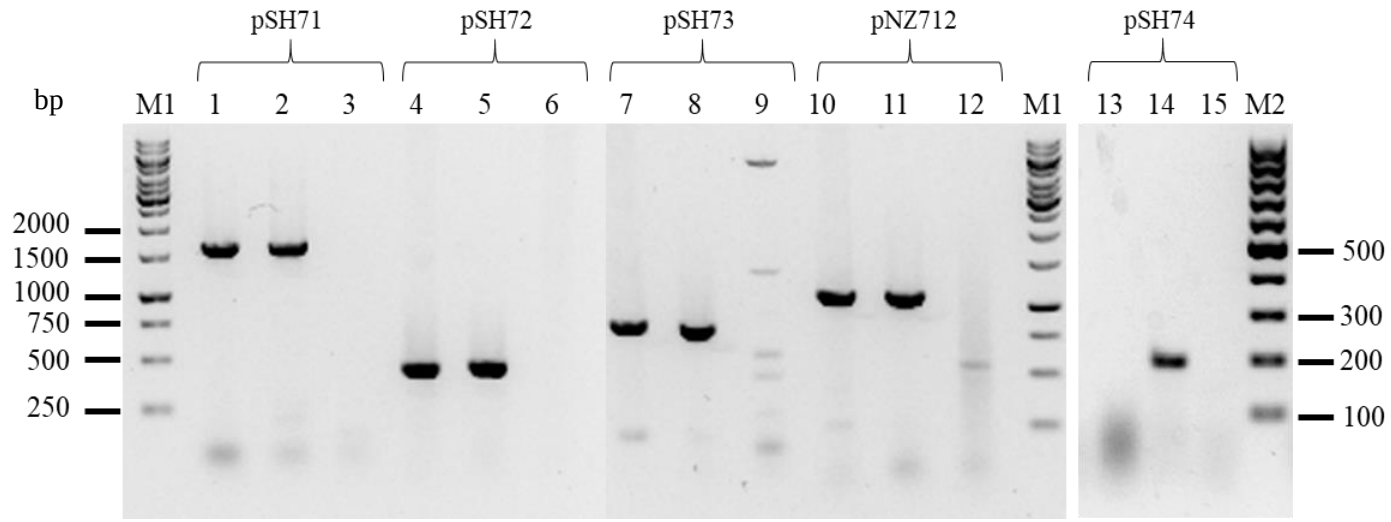


FIG 4 Plasmid profiles of *L. rhamnosus* LAB49, *L. lactis* NCDO 712, and *L. rhamnosus* GG. Lanes: 1, *L. rhamnosus* LAB49; 2, *L. lactis* NCDO 712; 3, *L. rhamnosus* GG. Chr, chromosomal DNA band; LAB49 and NCDO 712 carry several plasmids, whereas no plasmids can be seen in *L. rhamnosus* GG. M, 1 kb GeneRuler DNA ladder.



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578 **FIG 5** Identification of plasmids by PCR from isolated DNA of *L. rhamnosus* LAB49, *L. lactis* NCDO
579 712, and *L. rhamnosus* GG. Lanes 1, 4, 7, 10, 13: *L. rhamnosus* LAB49 as PCR template; lanes 2, 5, 8, 11,
580 14: *L. lactis* NCDO 712 as PCR template; lanes 3, 6, 9, 12, 15: *L. rhamnosus* GG as PCR template. Plasmid
581 specific primers were used to identify pSH71 (lanes 1-3), pSH72 (lanes 4-6), pSH73 (lanes 7-9), pNZ712
582 (lanes 10-12), and pSH74 (lanes 13-15). All plasmids were detected from NCDO 712, whereas pSH74 was
583 missing in LAB49. No plasmids were detected from LGG. M1, 1 kb GeneRuler DNA ladder. M2, 100 bp
584 GeneRuler DNA ladder.

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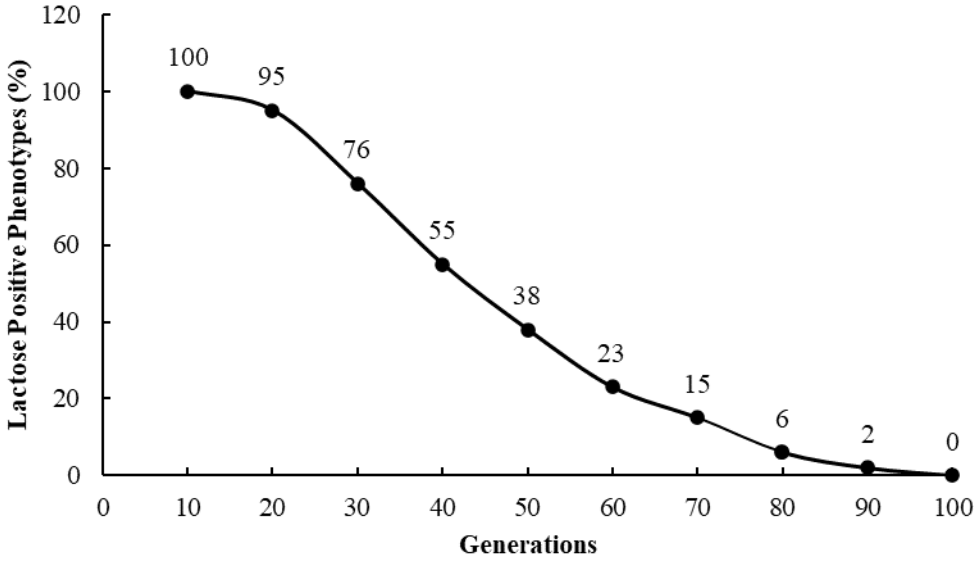
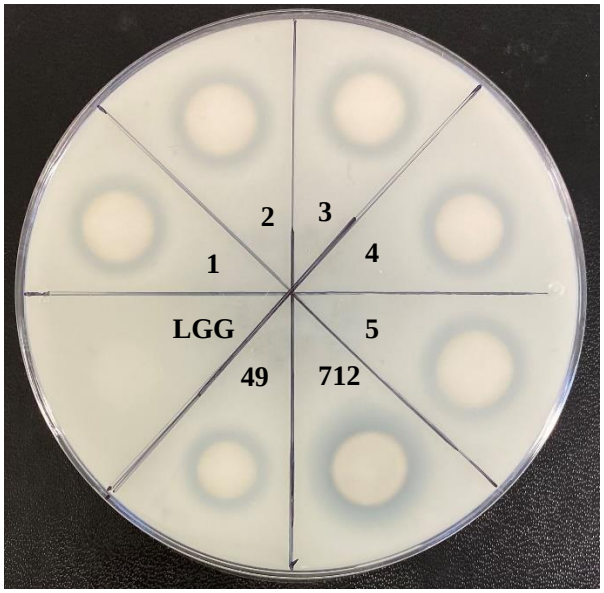
590 **A**



591

592 **B**



593

594 **FIG 6** A, Percentage of lactose positive *L. rhamnosus* LAB49 colonies as a function of generations grown
595 in non-selective conditions. LAB49 was cultured in MRS without lactose for 100 generations. Every 10
596 generations, colonies were tested for lactose utilization. LAB49 completely loses the capacity to ferment

lactose in 100 generations in non-selective conditions. B, Protease activity test for the lactose negative LAB49 colonies. 1-5: lactose negative *L. rhamnosus* LAB49 grown for 100 generations in non-selective media; 712: *L. lactis* NCDO712; 49: *L. rhamnosus* LAB49; LGG: *L. rhamnosus* GG. The clear zone around spotted bacteria demonstrate proteolytic activity. The protease positive but lactose negative phenotype of LAB49 indicates that the plasmid pLP712 is still present in the cells, and that pLP712 is segregationally stable but structurally unstable.

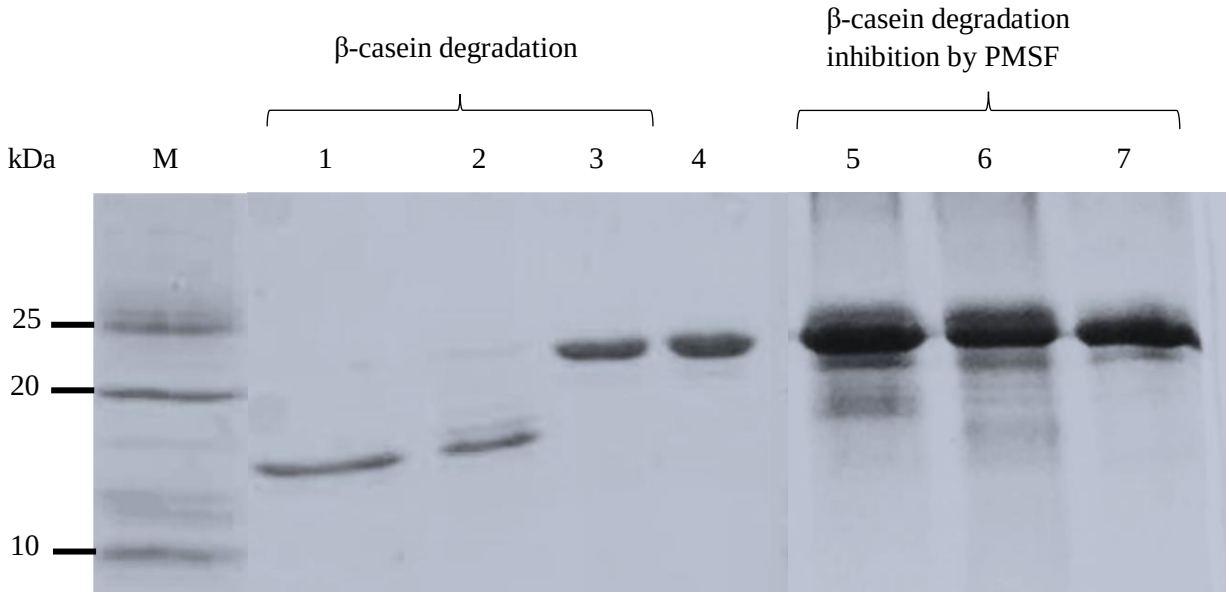


FIG 7 Proteolytic activity assay (β-casein degradation). Lane 1, 2 and 3: β-casein incubated with *L. lactis* NCDO 712, *L. rhamnosus* LAB49, and *L. rhamnosus* GG, respectively; Lane 4: β-casein without bacterial incubation; lanes 5, 6, and 7: β-casein and PMSF incubated with *L. lactis* NCDO 712, *L. rhamnosus* LAB49, and *L. rhamnosus* GG, respectively; M: Protein Ladder-Mid-range molecular weight (10-180 kDa)

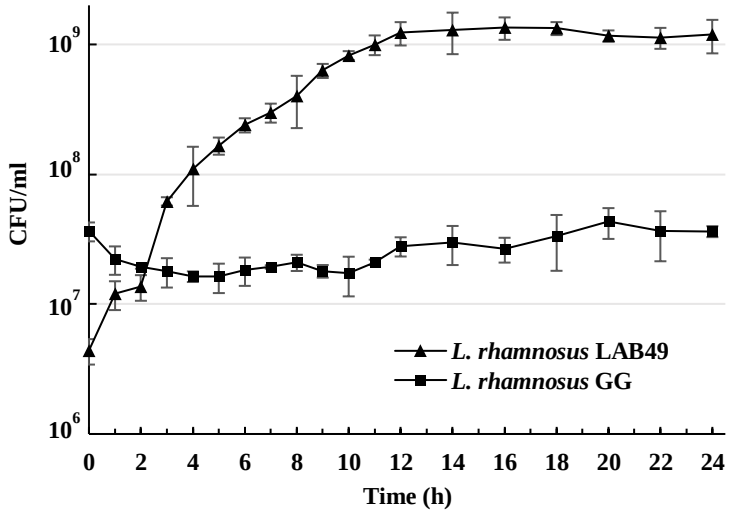


FIG 8 Growth of *L. rhamnosus* LAB49 and LGG in milk. LAB49 grows well and reaches stationary phase in about 12 hours. The growth of LGG is poor. Error bars show standard deviation of the mean from three parallel cultures.