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Assessing the Potential of Five Strains of Different Lactic Acid Bacterial Species as a Microbial Chassis for Oral Drug Delivery

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Abstract

Lactic acid bacteria (LAB) are promising oral drug delivery systems due to their probiotic properties, safety, and ability to stimulate mucosal immunity. In this study, we characterized five LAB strains, *Lacticaseibacillus casei* ATCC 393, *Lacticaseibacillus paracasei* UST1611, *Lactiplantibacillus plantarum* UST1611, *Limosilactobacillus reuteri* LR08, and *Lacticaseibacillus rhamnosus* GG, to assess their potential as a microbial chassis for oral drug delivery. We tested competence for transformation, plasmid stability, GFP expression under a constitutive promoter, survival under simulated gastrointestinal conditions, and survival in the gut of *Danio rerio* larvae. Successful transformation with the pTRKH3-ermGFP plasmid was achieved in *L. casei* ATCC 393, *L. paracasei* UST1611, and *L. reuteri* LR08. We also showed that the *L. casei* ATCC 393 strain had significantly higher protein expression than its *L. paracasei* UST1611 and *L. reuteri* LR08 counterparts. In the absence of selection, *L. casei* ATCC 393 and *L. paracasei* UST1611 retained their plasmid for 48 h. *L. reuteri* LR08 retained it for up to 72 h. The three transformed strains showed comparable tolerance to simulated gastrointestinal pH, though *L. casei* and *L. reuteri* were more resistant to 0.3% greater bile. In vivo testing showed the highest survival for *L. casei* ATCC 393 and *L. paracasei* UST1611 in the gut of zebrafish (*Danio rerio*) larvae. Overall, our results suggest that, among the five strains tested, *L. casei* ATCC 393 is the most promising candidate for an LAB-based microbial system for oral drug delivery.

Keywords: Lactic Acid Bacteria; *Lacticaseibacillus paracasei*; *Lacticaseibacillus casei*; *Lactiplantibacillus plantarum*; *Lacticaseibacillus rhamnosus*; *Limosilactobacillus reuteri*; oral drug delivery; pTRKH3 vector

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1. Introduction

Over the past decade, the use of genetically engineered microbes for drug delivery and protein expression has been extensively studied. The most commonly used recombinant microbial expression platform is *Escherichia coli* because of the many tools available to regulate gene expression [1]. Other microorganisms, including *Bacillus* sp., *Streptomyces*

sp., and *Pseudomonas* sp., have also been employed as microbial platforms for genetic engineering [2].

The use of Lactic Acid Bacteria (LAB) as a microbial platform for genetic engineering has also been studied because of their many advantages as a potential oral drug delivery system, including their ability to act as a probiotic, their capacity to influence the immune response, their ability to manage metabolism, and protection against specific diseases [3]. LAB species are generally recognized as safe (GRAS) and have been used as mucosal delivery systems for therapeutic molecules and as cell factories for various applications [4]. LAB-based microbial genetic engineering systems offer several benefits over other microbial vectors, particularly in vaccination and oral drug delivery. One of the main advantages of using LAB for drug delivery is its ability to modulate the production of secretory IgA, a crucial antibody class found in mucosal secretions [5]. Compared with other bacteria, LAB are much safer to use because they lack lipopolysaccharides (LPS) in their cell walls, a known cause of endotoxic shock [6].

LAB species that could potentially be used for oral drug delivery include the closely related *Lacticaseibacillus casei* (formerly *Lactobacillus casei*); *Lacticaseibacillus paracasei* (formerly *Lactobacillus paracasei*); *Lacticaseibacillus rhamnosus* (formerly *Lactobacillus rhamnosus*); *Limosilactobacillus reuteri*, and *Lactiplantibacillus plantarum*. These species have been recorded in the human and animal gut microbiota [7].

The *Lacticaseibacillus* group has been successfully engineered to express proteins that mitigate a lethal *L. monocytogenes* infection, prevent *Listeria monocytogenes* infection, and selectively inhibit *Helicobacter pylori* [8]. *L. plantarum* strains have also been used as vaccine delivery systems to SARS-CoV-2 proteins [9]. *L. reuteri* has been recognized as an obligatory heterofermentative, endogenous lactic acid bacterial species in the gastrointestinal tract of mammals and has been successfully engineered for quorum sensing [8]. *L. casei* has been used to develop a probiotic-based therapeutic that synergistically restores intestinal redox and microbiota homeostasis [10]. Finally, *L. plantarum* has recently been engineered to function as a vector for cerebral drug delivery through the olfactory epithelium [11]. When administered intranasally in a murine model of obesity prevention, the engineered *L. plantarum* significantly alleviated obesity-related symptoms. Despite numerous reports describing the genetic engineering of individual *Lactobacillus* sp. [9,12], direct comparative studies of their effectiveness as oral delivery systems, especially in vivo, remain scarce.

Danio rerio larvae have been extensively used to investigate the effects of various drugs, chemicals, and compounds on different organs, especially intestinal tissues [13,14]. Aside from their relatively small size and efficiency of use, the genes and organ functions are also well conserved between zebrafish and other higher vertebrates. The presence of ridges in the internal lining of the intestines can be observed in cross-sections of mammalian intestines as well. Thus, their high physiological and genetic homology with humans makes zebrafish a powerful model organism for studying mechanisms of oral drug delivery [15,16].

In this study, we characterized five selected LAB strains, *Lacticaseibacillus casei* ATCC 393, *Lacticaseibacillus paracasei* UST1611, *Lactiplantibacillus plantarum* UST1611, *Limosilactobacillus reuteri* LR08, and *Lacticaseibacillus rhamnosus* GG, to assess their potential as a microbial chassis for oral drug delivery. We wanted to identify the bacterial species that best fulfilled the following criteria: (1) it has the highest transformation efficiency with a commonly used LAB expression plasmid; (2) it has the highest expression levels of GFP driven by a commonly used LAB promoter; (3) it could survive best in simulated gastric conditions; (4) it could retain a commonly used LAB expression plasmid longest in the absence of selection; and (5) it could survive best in the gut of the *Danio rerio* larvae model. We believe these parameters could be used to identify a robust microbial chassis for drug

delivery. Based on our experiments, our data suggest that among the five LAB strains tested here, *Lacticaseibacillus casei* ATCC 393 is the most promising candidate as a molecular chassis for oral drug delivery.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, Primers, and Culture Conditions

Isolates of *L. casei* ATCC 393, *L. reuteri* LR08, and *L. rhamnosus* ATCC GG were obtained from the University of Santo Tomas Collection of Microbial Strains (Manila, Philippines), Natural Foundation Supplements (Whitney, Oxfordshire, UK), and Metagenics (Aliso Viejo, CA, USA), respectively. Previously undesignated strains of *L. paracasei* and *L. plantarum* were acquired from Spring Valley Probiotic (Bentonville, AR, USA) and Swanson Dietary Supplement (Fargo, ND, USA), respectively.

To confirm species identity, *L. casei*, *L. reuteri*, and *L. rhamnosus* were identified by polymerase chain reaction (PCR) using publicly available primer pairs [17]. Initially, *L. paracasei* and *L. plantarum* cells were harvested from overnight cultures, resuspended in 200 µL of cold Tris-EDTA (TE) buffer, and sent to Macrogen Inc. (Seoul, Republic of Korea) for 16S rRNA sequencing. Later, whole-genome sequencing was outsourced to Plasmidsaurus.com, and their assembled genomes were submitted to NCBI (BioProject PRJNA1497129). We designate these strains *L. paracasei* UST1611 (GenBank Accession No. SAMN61773077) and *L. plantarum* UST1611 (GenBank Accession No. SAMN62108866).

E. coli cells (HB101) were cultured at 37 °C on Luria Bertani (LB) medium. LAB isolates were cultured in MRS (de Man, Rogosa, and Sharpe) broth under microaerophilic conditions using a two-step combustion-modified candle-jar system at 37 °C [18]. The plasmids, pTRKH3-ermGFP and pTRKH3-slpGFP, were purchased from AddGene.org (plasmids #27169 and #27168, respectively). Table 1 shows the transformed strains, plasmids, and primers used in this study.

Table 1. Transformed bacterial strains, primers, and plasmids used in this study.

Strains or Plasmids	Features	References
Lacticaseibacillus casei-pTRKH3-ermGFP	*L. casei* with pTRKH3-ermGFP plasmid	This study
Limosilactobacillus reuteri-pTRKH3-ermGFP	*L. reuteri* with pTRKH3-ermGFP plasmid	This study
Lacticaseibacillus paracasei-pTRKH3-ermGFP	*L. paracasei* with pTRKH3-ermGFP plasmid	This study
pTRKH3-ermGFP	Emr, *Enterococcus faecalis* plasmid pAMβ1 origin, *Escherichia coli* plasmid p15A origin, *erm*B promoter with GFP	[19]
Escherichia coli-pTRKH3-ermGFP	*Escherichia coli* containing the pTRKH3-ermGFP plasmid	This study
Escherichia coli-pTRKH3-slpGFP	*Escherichia coli* containing the pTRKH3-*slp*GFP plasmid	This study
L. rhamnosus 16S–23S region primers	F-GCCGATCGTTGACGT TAGTTGG R-CAGCGGTTATGCGAT GCGAAT	[17]
L. reuteri 16S–23S region primers	F-GATTGACGATGGATC ACCAGT R-CATCCCAGAGTGATA GCCAA	[17]
L. casei Unique gene primers	F-CCA CAA TCC TTG GCT GTT CT R-GCT TGA GGC GAT TGT AAT CC	[17]

2.2. Plasmid Transformation of LAB Strains

Bacterial transformation involved preparing electrocompetent cells and then electroporating them. Electrocompetent cells were prepared by inoculating 1 mL of an overnight

culture into 5 mL of SGMRS (0.75 M Sorbitol and 1% glycine) broth [1] and then inoculating 2.5 mL of the culture into 50 mL of SGMRS broth. The cell culture was grown to an OD_{600} of 0.3–0.4 and centrifuged at 4000 g for 15 min. The cells were then washed with 50 mL of 0.5 M sucrose and 10% glycerol on ice and centrifuged at 4000 g for 15 min. The cells were resuspended in 25 mL of 0.5 M sucrose, 10% glycerol, and 50 mM EDTA. The cell suspension was kept on ice for 15 min and centrifuged. This process was repeated with 12.5 mL of 0.5 M sucrose and 10% glycerol. Afterward, the cells were resuspended in 500 µL of 0.5 M sucrose and 10% glycerol.

For the actual transformation, 50 µL of competent cells was thawed on ice, and 8 µg of plasmid DNA was added to the cell suspension [20]. Electroporation was performed using a Bio-Rad MicroPulser (Hercules, CA, USA) at 1.8 kV and 2.5 milliseconds in a pre-chilled cuvette with an inter-electrode distance of 0.2 cm. Following electroporation, 500 µL of MRS broth supplemented with 20 mM magnesium chloride ($MgCl_2$) and 2 mM calcium chloride ($CaCl_2$) was added to the cells, which were incubated for 3 h, then spread on MRS plates containing 30 µg/mL erythromycin (MRSe) to select for the plasmid. The plates were incubated in microaerophilic conditions for 36 h at 37 °C. Electroporation was repeated at least three times, along with successful controls, to determine whether a LAB strain could be efficiently transformed.

2.3. Flow Cytometry Analysis of GFP Expression in Transformed LAB Strains

For the flow cytometry analysis [21], LAB transformants were grown in MRSe broth at 37 °C with aeration. Untransformed LAB were grown in MRS broth at 37 °C. GFP fluorescence was monitored for 15 h by flow cytometry (CytoFLEX V0-B5-R0 Flow Cytometer, Beckman Coulter Life Sciences, Indianapolis, IN, USA). The culture pH was carefully monitored throughout the experiment.

Flow cytometry data were analyzed by first excluding debris and non-cellular events, then identifying fluorescent and non-fluorescent populations using FITC-H fluorescence intensity. The mean fluorescence intensity (MFI) of the FITC-positive population was determined from 10,000 acquired events per sample and normalized to the negative control.

2.4. Viability of Transformed LAB in Simulated Acidic Gastrointestinal Conditions

The survival of the LAB strains under simulated gastrointestinal conditions was evaluated in the presence of bile salts and at acidic pH as previously described [21]. Tolerance to acidic conditions was tested in MRSe broth adjusted to pH 4 with 1 M hydrochloric acid (HCl). Cells from an overnight culture in exponential phase (OD_{600} = 1.0) were harvested, added to 12.5 mL of pH-adjusted MRS or MRSe, and incubated for 4 h at 37 °C under microaerophilic conditions. Bile salt tolerance was tested in MRSe broth supplemented with 0.3% bile salts (Solarbio Technology Co., Ltd., Beijing, China), prepared in 12.5 mL of supplemented MRSe. Viability after 4 h was calculated by comparing CFUs with those of an untransformed strain, determined by colony counts on MRSe agar after incubation under microaerophilic conditions at 37 °C for 48 h.

2.5. Measurement of Plasmid Retention

A plasmid retention assay was conducted as previously described [22] with modifications specific to our LAB transformants. LAB cells were grown to an OD_{600} of 2.5 in MRSe medium. This was used as a starting inoculum in MRS broth without antibiotics. After an overnight incubation, the number of colonies on the antibiotic-free and antibiotic-containing plates was determined at different time points. Plasmid retention was expressed as the percentage of colonies tested that grew on the plates with selection over those without. This process continued until no growth was observed on antibiotic plates.

2.6. Zebrafish Husbandry

Adult wild-type zebrafish (*Danio rerio*) were obtained from a local supplier (Cartimar Pet Center, Manila, Philippines) and maintained at the University of Santo Tomas Zebrafish Biochemistry and Pharmacotoxicity Laboratory in accordance with established protocols [23]. Fish were quarantined for one month in a 10-gallon tank with filtered water supplemented with methylene blue (5 mL/10 gal) and 50% water changes every 3 days at 28.5 °C and pH 6.8–7.5 under a 12 h light/dark cycle [24] with Hang-On-Back filtration and 50% weekly water changes. The fish were cultured under conditions of ventilation and circulating water, and the brine shrimp were fed twice a day [15]. Then, suitable pairs of female and male fish were selected at a 3:5 ratio for mating, and the fertilized eggs were collected the next day.

Embryos were washed several times with a 0.5% aqueous methylene blue solution, then placed in incubators with the same illumination periods for light-controlled incubation at 28.5 °C; dead embryos were promptly removed during this period. Individual embryos were then allocated to 24-well plates (2 mL/well) in 1× E3 under standard laboratory conditions. Mild aeration was introduced from 4 days post-fertilization (dpf) onward to maintain adequate dissolved oxygen. For experimentation, only 4 dpf larvae meeting established viability criteria were used to ensure developmental uniformity [25]. The composition of E3 medium is NaCl 5 mmol/L, KCl 0.17 mmol/L, $CaCl_2 \cdot 2H_2O$ 0.4 mmol/L, and $MgSO_4 \cdot 7H_2O$.0.33 mmol/L.

2.7. LAB Immersion Test in the Zebrafish Model

Viability testing and colonization in the zebrafish model were performed as previously described [26,27]. Briefly, overnight cultures of transformed LAB cells, grown in MRSe broth under microaerophilic conditions, were used to visualize the bacteria in the zebrafish lumen. Larvae were immersed in LAB samples that had been concentrated to 4×10^9 CFU/mL and washed with PBS five times. Two negative control groups were used in our experiments: untreated zebrafish larvae and larvae immersed in solutions containing untransformed LAB cells.

Four-day post-fertilization (4 dpf) larvae (n = 6) were individually exposed to the specific strain in 24-well plates (1.5 mL) under standard static immersion conditions for 2 h to ensure appropriate consumption. Exposure was limited to 2 h at 28.5 °C to ensure sufficient contact while minimizing handling stress, as short larval immersions ($\leq$5 h) reliably elicit measurable responses without increased mortality or developmental disruption [28]. After the immersion period, the larvae were washed with E3 media for five passages and incubated at 28.5 °C for 4 h.

Larvae were then mounted and imaged using an EVOS FL Digital Inverted fluorescence microscope (Invitrogen, Carlsbad, CA, USA) to confirm GFP expression and gut localization. Mechanical homogenization of the larvae was then performed. The zebrafish homogenate was diluted 10-fold, and 100 µL of each sample was plated on MRSe agar and incubated for 48 h under microaerophilic conditions at 37 °C. The CFU/mL of each was calculated, and statistical significance was determined with a one-way ANOVA with a Dunn's post hoc test, applying a Bonferroni correction to lower the risk of false positives using PAST 4.0. All experiments were done in triplicate.

Our animal study protocol was approved by the University of Santo Tomas Institutional Animal Care and Use Committee (IACUC) with protocol number RC2025-290924 on 25 October 2025.

3. Results

3.1. Characterization of Competence and Growth of LAB Strains

To assess the transformation competence of our strains, we attempted to transform our five LAB species with two previously described plasmids, pTRKH3-slpGFP and pTRKH3-ermGFP [19]. Transformation with the pTRKH3-slpGFP plasmid was unsuccessful across all five LAB strains tested, even though it could be transformed into an *E. coli* HB101 control. Therefore, we did not continue working with this plasmid.

In contrast, transformation of the plasmid pTRKH3-ermGFP was successful with three of the five LAB strains: After a 48 h incubation period, colonies of *L. reuteri*-pTRKH3-ermGFP, *L. paracasei*-pTRKH3-ermGFP, and *L. casei*-pTRKH3-ermGFP were observed on plates supplemented with erythromycin. Transformation efficiencies were calculated as follows: 3.44×10^1 CFU/µg of plasmid DNA for *L. paracasei*, 2.56×10^1 CFU/µg for *L. reuteri*, and 1.25×10^2 CFU/µg, 1.25×10^2 CFU/µg for *L. casei*. Despite several repeated attempts and successful electroporation of the three LAB species described above, no transformed colonies were observed for *L. plantarum* and *L. rhamnosus*. Our findings remained unchanged despite our attempts to increase the DNA amount from 8 µg to 50 µg to improve transformation efficiency.

Observations of the growth rates of our *L. casei*, *L. paracasei*, and *L. reuteri* cells and their transformed counterparts revealed no significant differences between the untransformed and transformed strains. This suggests that the transformed pTRKH3-ermGFP plasmid does not affect bacterial growth.

3.2. Measurement of Protein Expression Driven by the ermB Promoter

To assess protein expression levels in our transformed strains, we monitored GFP expression for 12 h, driven by the *erm*B promoter, the time required for the strains to reach stationary phase. Though *L. paracasei*-pTRKH3-ermGFP had the highest levels of GFP expression at the outset of the experiment, it became clear that *L. casei*-pTRKH3-ermGFP showed a significantly higher fluorescence at later time points (Figure 1). In contrast, GFP fluorescence in *L. reuteri*-pTRKH3-ermGFP remained consistently lower than that in its two counterparts. All three transformed strains had higher fluorescent levels than negative untransformed controls.

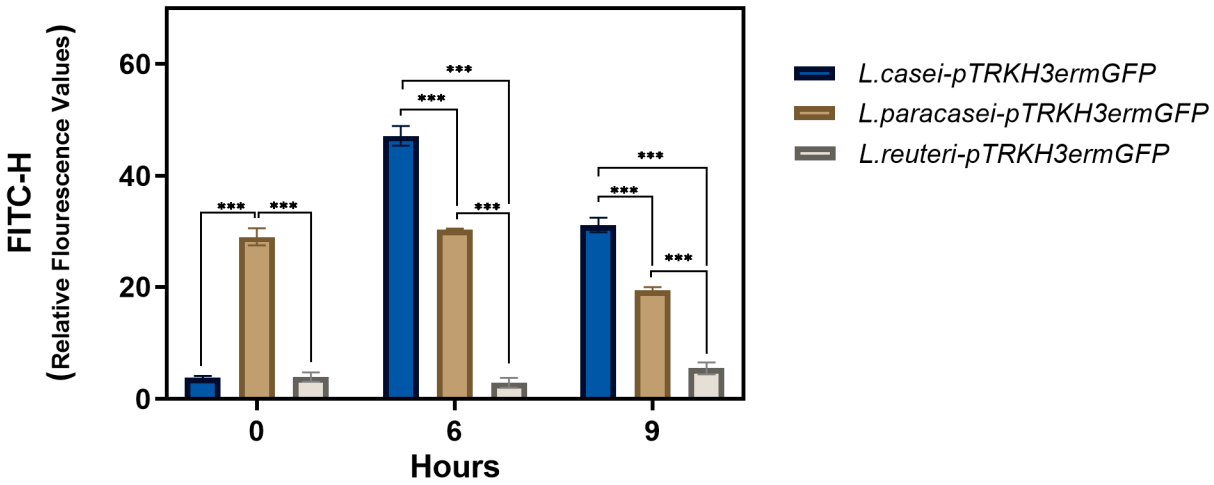


Figure 1. GFP Fluorescence in LAB Strains Transformed with pTRKH3-ermGFP (n = 3). Data is expressed as mean ± standard deviation. The graph is normalized to the negative control (plasmid-free LAB). Statistical analysis was performed with a two-way ANOVA followed by Tukey's Pairwise. *** $p < 0.001$.

Our results corroborate a previous study of the plasmid pTRKH3-ermGFP in *L. reuteri* DSM 20016, which reported relatively low GFP fluorescence when viewed under a blue LED (470 nm) through a green polyester filter [19]. We also note that the three transformed species exhibited significant fluorescence with both a fluorescent microscope and a flow cytometer. This fluorescence was sufficient to visualize the localization of the cells in the zebrafish gut.

3.3. Plasmid Retention in Non-Selective Conditions

To determine whether plasmid stability and retention differ among the three transformed LAB species, we conducted a plasmid retention assay: The three LAB strains retained their plasmids for 48 h in the absence of antibiotics. However, *L. reuteri*-pTRKH3-ermGFP retained its plasmid for up to 72 h (Figure 2, Table S1). None of the strains retained their plasmids at 120 h.

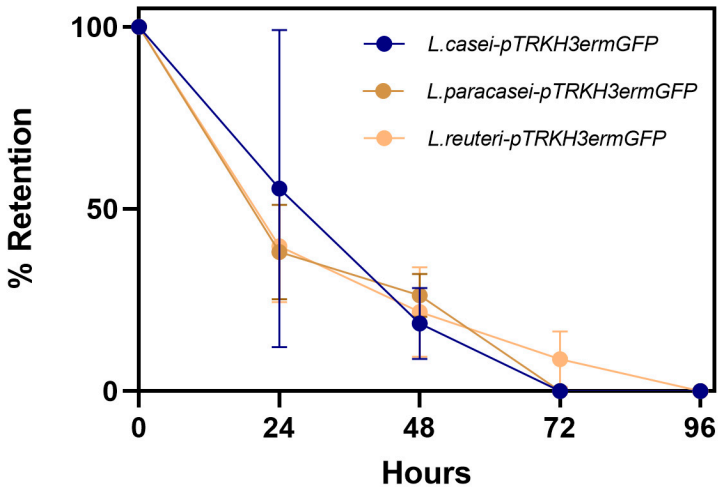


Figure 2. Plasmid retention of LAB strains transformed with pTRKH3-ermGFP. Plasmid retention was evaluated over four days by plating cultures on selective and non-selective plates. Each data point shows the mean of three trials with the standard deviation.

3.4. Survival of Transformed LAB Under Simulated Gastrointestinal Conditions

To determine the survivability of LAB strains under simulated gastrointestinal conditions, we tested their tolerance to low pH and bile. Acid tolerance was evaluated by measuring survival and the capacity to resume growth after exposure to a low-pH environment [29]. We chose simulated gastric juice at pH 4.0, a commonly used condition in probiotic survival assays [30]. Controls for the experiment included testing for possible degradation of erythromycin in MRSe adjusted to pH 4 by inoculating untransformed *L. casei*, which showed no growth after incubation, suggesting minimal erythromycin loss at pH 4.

As shown in Figure 3, the three transformed LAB species exhibited significantly higher survival at pH 6.5 compared to pH 4 ($p = 0.05$). The larger error bars at pH 6.5 indicate greater variability in survival among replicates. Nonetheless, our findings suggest that all transformed species can withstand the acidic environment of the gastrointestinal tract for at least 4 h.

The three transformed LAB strains were also exposed to 0.3% bile salts at pH 6.5 for 4 h (Figure 3). They were then cultured in microaerophilic conditions for 24 h at 37 °C [17]. Our results reveal that, following this protocol, *L. paracasei*-pTRKH3-ermGFP had significantly lower survival (3.0×10^6 CFU/mL) than the two other strains. In contrast, *L. casei*-pTRKH3-ermGFP (8.0×10^6 CFU/mL) and *L. reuteri*-pTRKH3-ermGFP (1.3×10^7 CFU/mL) were similarly effective in tolerating bile salt stress ($p = 0.06$).

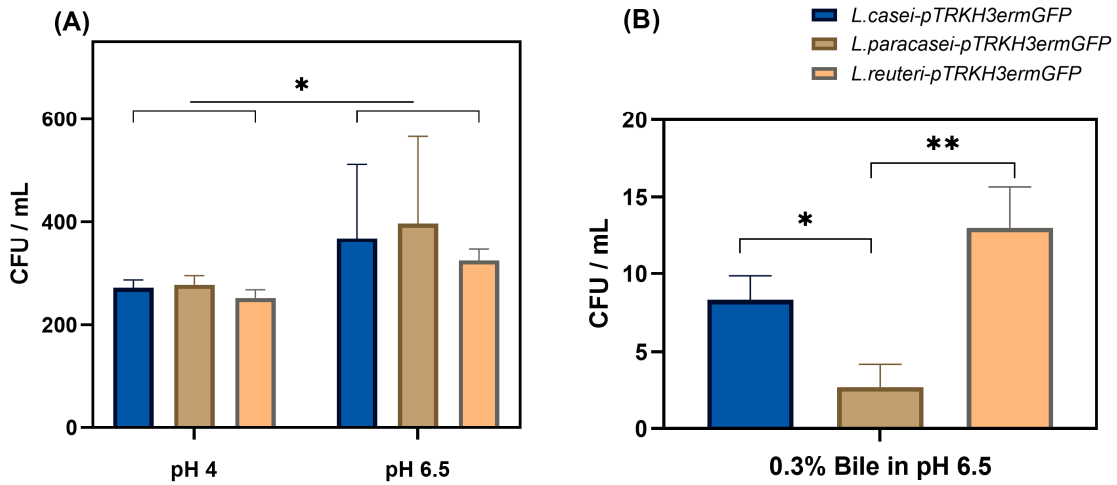


Figure 3. Survival of LAB strains transformed with pTRKH3-ermGFP after exposure to simulated gastrointestinal conditions. (**A**) Survival of LAB strains at different pH values. Survival of all transformed LAB species is significantly greater at pH 6.5 than at pH 4. No significant differences were observed among strains at each pH value. (**B**) Survival of LAB strains after exposure to 0.3% bile salts at pH 6.5 for 4 h. Data are expressed as mean ± standard deviation of three replicates. Asterisks indicate significant values: * $p < 0.05$; ** $p < 0.01$.

3.5. Survival of Transformed LAB in Zebrafish Larvae

Next, we wanted to determine if the transformed LAB strains could survive and persist in the zebrafish gut. After the larvae were immersed in cell suspensions of transformed LAB strains, we mounted the fish and imaged them with a fluorescence microscope to confirm GFP expression and gut localization. Our analysis revealed that GFP-expressing cells could be imaged in the lumen of zebrafish larvae (Figure 4).

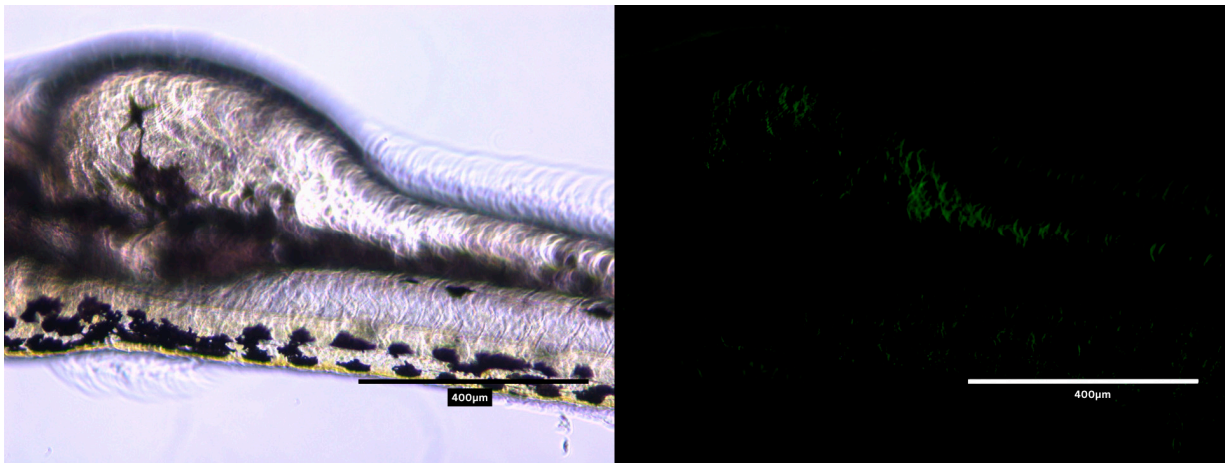


Figure 4. In-vivo imaging of *L. casei* ATCC 393 cells transformed with pTRKH3-ermGFP in the gut of a zebrafish (*Rerio danio*) larva. This representative image obtained with an EVOS FL Digital fluorescence scope shows *L. casei*-pTRKH3-ermGFP localized within the lumen of a zebrafish larva.

The viability of transformed LAB in zebrafish larvae was evaluated by immersing the larvae in a cell suspension for 2 h, washing them, and then mechanically homogenizing them. The zebrafish homogenate was then diluted, and a sample was plated on selective agar. As shown in Figure 5, *L. paracasei*-pTRKH3-ermGFP had the highest mean survivability at 2.0×10^3 CFU/mL, followed by *L. casei*-pTRKH3-ermGFP at 1.8×10^3 CFU/mL. *L. reuteri*-pTRKH3-ermGFP exhibited lower survivability, yielding 2.6×10^2 CFU/mL after 72 h. Our results showed gastrointestinal survivability across

all three tested LAB strains, with species belonging to the *Lactobacillus casei* group (LCG) exhibiting higher post-exposure viability than their *L. reuteri* counterpart.

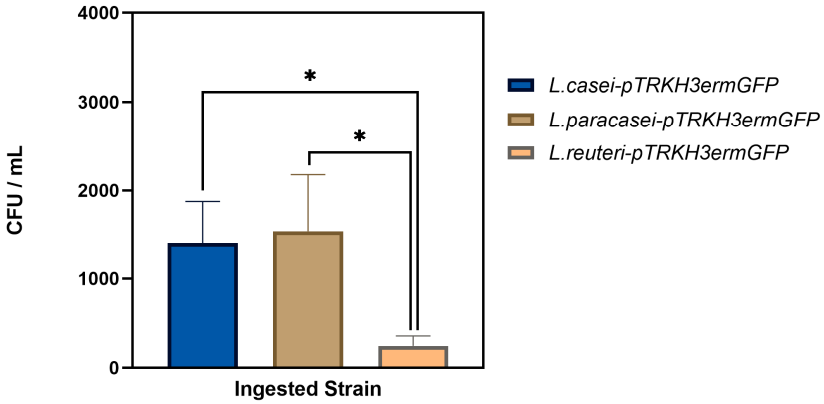


Figure 5. Viability of transformed LAB strains in Zebrafish (*Danio rerio*) Larvae. Data are expressed as mean ± standard deviation (n = 6). One-way ANOVA with Dunn's post hoc test applying a Bonferroni correction. An asterisk indicates significance values: $p = 0.0125$ for the difference between *L. casei* and *L. reuteri*, and $p = 0.01303$ for the difference between *L. paracasei* and *L. reuteri*.

4. Discussion

In this study, we characterized five LAB strains, *Lacticaseibacillus casei* ATCC 393, *Lacticaseibacillus paracasei* UST1611, *Lactiplantibacillus plantarum* UST1611, *Limosilactobacillus reuteri* LR08, and *Lacticaseibacillus rhamnosus* GG to assess their potential as a microbial chassis for oral drug delivery. We assessed their competence to be transformed with at least one of two plasmids, pTRKH3-slpGFP and pTRKH3-ermGFP; their capacity to express GFP with a commonly used constitutive LAB promoter; their ability to maintain a plasmid, pTRKH3-ermGFP, in the absence of selection; and their survivability in simulated gastrointestinal conditions and in zebrafish larvae. Based on our experiments, our data suggest that among the five LAB strains tested here, *Lacticaseibacillus casei* ATCC 393 is the most promising candidate as a molecular chassis for oral drug delivery.

Of the two plasmids included in our study, we were only able to transform pTRKH3-ermGFP into at least one of our LAB strains. It is unclear why we were unable to transform our isolates with pTRKH3-slpGFP. The plasmid was originally constructed and tested in *L. reuteri* strains [19]. There are also studies reporting the successful transformation of *Lactobacillus paracasei* with a modified version of this construct [31,32]. One possibility is that strain differences affect the competence of LAB with respect to this vector, but we did not pursue this hypothesis. Notably, we were able to transform *E. coli* HB101 cells with pTRKH3-slpGFP, suggesting that the plasmid was sufficiently intact for transformation into, at least, this alternative bacterial species.

We were able to transform three strains, *L. casei* ATCC 393, *L. paracasei* UST1611, and *L. reuteri* LR08, with plasmid pTRKH3-ermGFP. Once again, we do not know why we were unable to obtain transformed colonies of *L. plantarum* UST1611 and *L. rhamnosus* ATCC GG using this plasmid. In a relevant study, Laudete et al. [33] compared electrotransformation protocols across 12 LAB strains. They showed that transformation efficiency varied dramatically among the strains and that cell physiology, electroporation conditions, and host-specific properties strongly influence competence. A recent review identified several barriers to transformation among LAB strains, including their thick peptidoglycan cell walls, restriction-modification systems, strain-to-strain variability, replicon incompatibility, host-specific responses to electroporation, and the limited natural competence in most *Lactobacillus* species [34].

Our results suggest that the *erm*B promoter is relatively robust in our *L. casei* and *L. paracasei* strains. Both belong to the same genus, *Lacticaseibacillus*, and are closely related. Indeed, because of their similarities, the proper classification of these species remains ambiguous [35]. In contrast, the expression of this promoter was relatively low in our isolate of *L. reuteri*. Previous studies of GFP-expressing plasmids in *L. lactis* and *L. reuteri* showed a high level of fluorescence in *L. lactis* [19]. At the same time, this report showed that extremely specific conditions were required to achieve high fluorescence in *L. reuteri*. Our results appear to corroborate these findings since our *L. reuteri* strain also showed the lowest quantitative GFP fluorescence among all the strains we tested.

Regarding plasmid retention in our three transformed strains, we observed similar dynamics among the isolates. For the most part, the three strains were able to retain their plasmids for at least 72 h in the absence of antibiotic selection. A study of three *L. lactis* strains, U29, U52, and U70, in different growth environments revealed significant differences in plasmid retention among the strains [36]. Temperature influenced plasmid stability, and longer culture times (>72 h) dramatically increased plasmid loss. Given studies have shown that typical retention times for LAB species in the mammalian gut range from days to weeks, depending on the particular LAB species, with *L. lactis* disappearing after about 3 days and *L. casei* persisting from 4 to 7 days [37,38], our three transformed LAB strains should be able to be used to deliver molecular cargo for the duration of their retention times. Delivery protocols for LAB strains with short plasmid retention times could also be offset by repeated oral consumption of the recombinant bacteria.

The ability of a LAB strain to survive transit through the gastrointestinal tract is of critical importance if it is to be used as a molecular chassis to deliver a protein or antigen to the gut mucosa. Our three transformed strains, *L. casei*-pTRKH3-ermGFP, *L. paracasei*-pTRKH3-ermGFP, and *L. reuteri*-pTRKH3-ermGFP, survived exposure to an established in vitro model of gastric acid (pH 4 and 6.5) and bile salts (0.3%). The former condition is commonly used in probiotic survival assays, while the latter is the estimated average bile concentration in the human intestine [30,39].

Studies evaluating the survival of LAB at low pH (<pH 3) have shown that strains of these species are well adapted to survive at stomach pH [40,41]. Our study extends these findings: We have evaluated the viability of transformed LAB at pH 4 (upper stomach pH) and pH 6.5 (duodenum/ileum pH) for 4 h, the time required for gastric emptying [42,43]. Acid tolerance was evaluated by measuring survival and the capacity to resume growth after exposure to low pH [29]. Despite species-specific variability, all our transformants survived simulated gastric emptying.

To assess their ability to withstand bile salt exposure, our three transformed LAB strains were exposed to 0.3% bile salts for 4 h. A previous study has shown that *L. casei* exhibits significant bile resistance, surviving up to 4% bile [44]. Another report suggests that *L. reuteri* can adapt to bile concentrations of up to 5% through a two-phase response, with an initial bile shock followed by adaptive growth [45]. Our transformed *L. casei* ATCC 393 and *L. reuteri* LR08 cells had properties comparable to those of the counterparts described in the published work. In contrast, our transformed *L. paracasei*-pTRKH3-ermGFP isolate did not grow as well after bile salt exposure. One possibility for this is the absence of bile salt hydrolase (BSH) activity in this LAB species [46]. Nonetheless, compared to other bacterial genera being explored in preclinical and clinical treatment trials (*Salmonella*, *Listeria*, *Escherichia*, *Clostridium*, and *Streptococcus*), the ability of LAB to survive gastrointestinal conditions and their safety confer a significant advantage for their use as a molecular chassis for oral use [47].

Finally, our in vivo studies suggest that our three transformed strains can be observed in the gut of zebrafish (*Danio rerio*) larvae. A previous study with probiotic *L. fermentum*

11.5 and both *L. plantarum* 90 and *L. plantarum* B2 showed localization of the LAB in the intestinal tract of *Danio rerio* larvae at 6 h post-infection (hpi) [48]. Notably, this report showed notably weak fluorescence of *L. fermentum* 11.5 cells in zebrafish larvae. Our own results show a similar localization of our transformed LAB in the zebrafish intestinal tract 6 h post-immersion. This suggests that, like *L. fermentum* and *L. plantarum*, *L. casei*, *L. paracasei*, and *L. reuteri* can also localize to the zebrafish gut.

With regards to survivability, our three strains showed higher survivability at 6 h post-immersion than the published numbers for *L. plantarum* and *L. fermentum* [48].

Moreover, our findings reveal that our *L. casei* ATCC 393 and *L. paracasei* UST1611 strains survive better than our *L. reuteri* LR08 counterpart in the zebrafish gut. One study of *Lactobacillus* strains has demonstrated that improved survivability in the animal gut is linked to better adhesion of LAB strains to the host gastrointestinal tract [49].

Therefore, based on our experiments, we propose that among the five LAB strains tested here, *Lacticaseibacillus casei* ATCC 393 is the most promising candidate as a molecular chassis for oral drug delivery. It demonstrates relatively high transformation competence and significant GFP expression from the constitutive *erm*B promoter, and it has relatively robust survivability in in vitro and in vivo models for microbial probiotics. We believe these characteristics are associated with microbial strains that could serve as a robust chassis for the oral delivery of molecular cargo.

In recent years, *Lactococcus lactis* has become one of the best-characterized live microbial chassis for therapeutic protein delivery, and recombinant *L. lactis* has been used to express proteins intracellularly, to secrete proteins extracellularly, and to display proteins on the bacterial surface [8,50]. However, for oral probiotic applications, *L. casei* is generally more acid- and bile-resistant than *L. lactis* [51]. There is evidence that *L. casei* strains exhibit better growth at 0.3% bile than their *L. lactis* counterparts, for the most part, because the former often express bile salt hydrolase (BSH) and display a variety of proteins devoted to the efflux of bile salts [52]. A direct comparison of our *Lacticaseibacillus casei* strain with its *Lactococcus lactis* counterpart, especially *Lactococcus lactis* NZ9000 or its parental strain MG1363, especially in an in vivo animal model, would allow us to determine which of these two LAB strains would be the better probiotic microbial chassis.

Our study has several limitations. We sought to compare isolates of five different LAB species. However, it is becoming clearer that phenotypic differences between distinct strains of the same species can be as diverse as those between LAB isolates from different species. Therefore, it is difficult for us to propose that *Lacticaseibacillus casei* as a species in itself would be a more robust molecular chassis for the oral delivery of molecular cargo than all the strains of the four other LAB species that we tested. Nonetheless, our findings can still serve as a guide for future comparative studies of different LAB strains.

Next, we have shown that *Lacticaseibacillus casei* ATCC 393 can survive robustly in the zebrafish larvae gut. However, our results show only transient localization in the fish gut. Thus, further observations will be necessary to determine whether high survivability translates into stable, sustainable colonization of the zebrafish gut.

Third, our study has focused on the use of an erythromycin-resistance marker in our strains. Clearly, for therapeutic purposes in human patients, this would not be ideal. Instead, we would have to use alternatives, including food-grade selection systems and auxotrophic complementation for *L. casei* ATCC 393 to minimize the risk of horizontal transfer of antibiotic resistance genes [53,54].

Finally, our study relied solely on GFP expression as a readout for expression of molecular cargo. It is possible that different molecular cargoes would be expressed at levels that differ from those we observed with GFP. Moreover, we did not perform any experiments to assess production, secretion, immunogenicity, or drug-delivery efficacy

(depending on the molecular cargo) of a specific protein. Thus, a direct comparison among LAB species may have to be performed by particular laboratories using a particular LAB to deliver a particular cargo.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol6080088/s1, Table S1: Plasmid Retention of LAB Strains Transformed with pTRKH3-ermGFP.

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Institutional Review Board Statement: The animal study protocol was approved by the University of Santo Tomas Institutional Animal Care and Use Committee (IACUC) with protocol number RC2025-290924 on 25 October 2025.

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Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. The two novel microbial genomes for *L. paracasei* UST1611 and *L. plantarum* UST1611 have been submitted to the NCBI GenBank strains *L. paracasei* UST1611 (GenBank Accession No. SAMN61773077) and *L. plantarum* UST1611 (GenBank Accession No. SAMN62108866). Further inquiries can be directed to the corresponding author.

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Abbreviations

The following abbreviations are used in this manuscript:

LAB	Lactic Acid Bacteria
GFP	Green Fluorescent Protein

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