

Supplementary material

Lactic Acid Bacteria as microbial cell factories for the in vivo delivery of therapeutic proteins as secretable TAT fusion products

Giorgio Medici, Giulia Candini, Nicola Mottolese, Beatrice Uguagliati, Federica Trebbi, Manuela Loi, Angelica Marina Bove, Spase Stojanov, Erika Esposito, Rosalba Vitagliano, Federica D'Amico, Silvia Turrone, Jessica Fiori, Aleš Berlec, Stefania Trazzi, Elisabetta Ciani

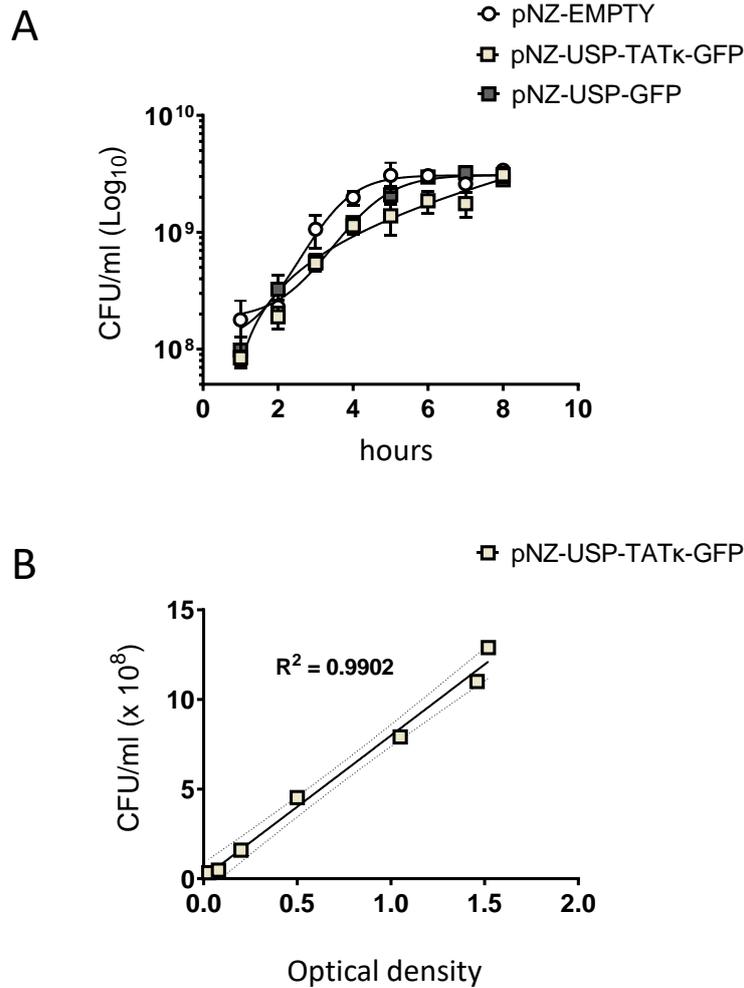
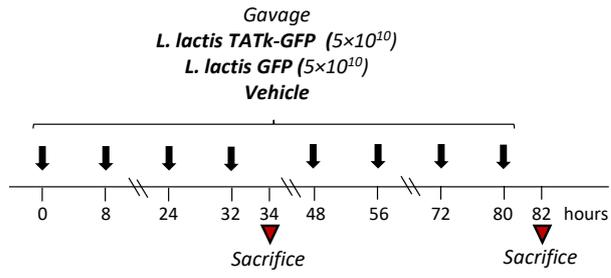


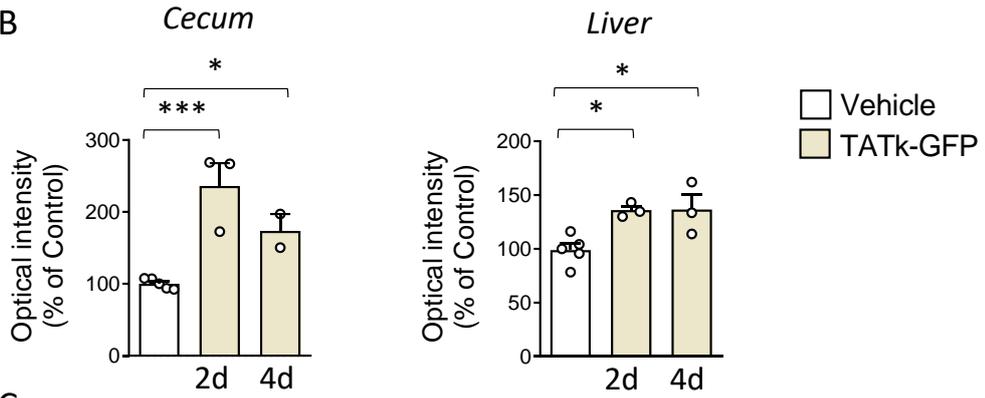
Figure S1. Effect of GFP expression on *L. lactis* growth and viability.

A: Overnight cultures of *L. lactis* harboring the pNZ-USP-TAT κ -GFP, pNZ-USP-GFP, or pNZ8148 (pNZ-EMPTY) plasmids were grown in GM-17 medium for 8 hours at 30°C. The number of live bacteria was evaluated through colony formation assay. Graph shows the number of live bacteria in different *L. lactis* strains with time, expressed as colony forming units per millilitre of growth medium in logarithmic scale, at time intervals of 1 hour. Values are represented as means \pm SEM, Tukey test after two-way ANOVA. **B:** Plot of CFU/mL versus OD₆₀₀ for *L. lactis* pNZ-USP-TAT κ -GFP transformed strain showing the linear regression line.

A



B



C

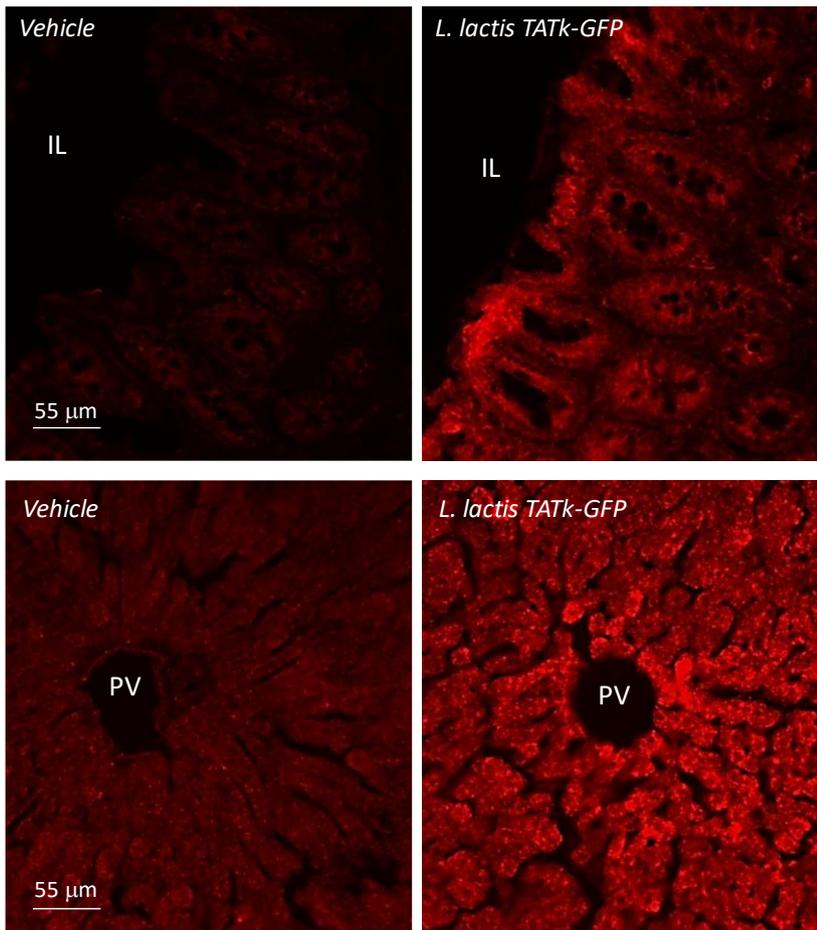


Figure S2. TATκ-GFP protein biodistribution in intestinal and hepatic tissues of treated mice.

A: Schematic representation of the experimental plan. Adult (6-month-old) mice were orally administered with 5×10^{10} cells of recombinant *L. lactis* harboring the pNZ-USP-TATκ-GFP plasmid or vehicle (PBS, as a negative control), twice per day (black arrows), with a 8-hour interval, for 2 or 4 consecutive days. Mice were sacrificed on day 2 or on day 4 (red arrowheads) 2 hours after the last administration. **B:** Comparative analysis of the TATκ-GFP protein biodistribution in intestinal and hepatic tissues of mice after two or four days of treatment with *L. lactis* strains expressing TATκ-GFP. Quantification of the mean intensity of GFP immunoreactivity per area in the cecum (TATκ-GFP 2d, n = 3; TATκ-GFP 4d, n = 2; vehicle, n = 5) and liver (TATκ-GFP 2d, n = 3; TATκ-GFP 4d, n = 3; vehicle n = 5) of mice after 2 (2d) or 4 (4d) days of treatment with recombinant *L. lactis* or vehicle. Data are expressed as percentage of fluorescence signals relative to vehicle-treated *L. lactis* strain. Values are represented as means \pm SEM. * $p < 0.05$, *** $p < 0.001$, Fisher's LSD test after one-way ANOVA. **C:** Representative images of GFP-stained cecum and liver sections of a mouse administered with recombinant *L. lactis* or vehicle for 4 days. Note the higher TATκ-GFP immunoreactivity in cells close to the border delimiting the portal veins (PV), suggesting diffusion of TATκ-GFP protein from the intestinal-derived bloodstream. Scale bar = 55 μm . Abbreviations: IL = intestinal lumen; PV = portal vein.

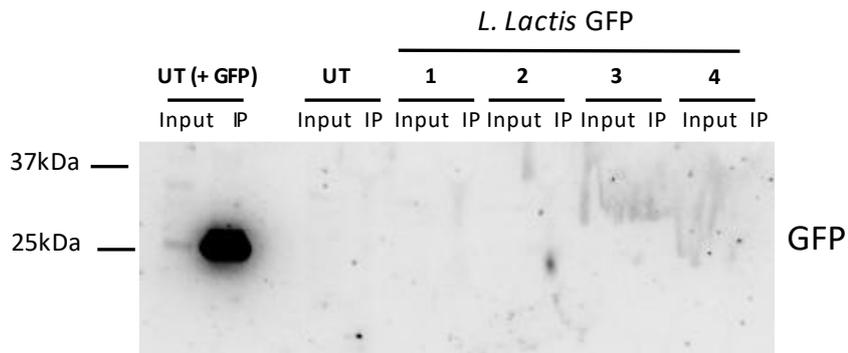


Figure S3. Immunoprecipitation analysis of GFP in intestinal tissues following oral administration of recombinant.

Lactococcus lactis. Cecal tissues were collected from four mice (1–4) orally administered recombinant *L. lactis* carrying the pNZ-USP-GFP plasmid for four consecutive days, and from two untreated mice (UT). Tissue lysates (Input) were immunoprecipitated with anti-GFP antibodies (IP) and analyzed by Western blot using an anti-GFP antibody. As a positive control, a known amount (2 ng) of purified GFP protein was added into the cecal homogenate of one untreated mouse prior to immunoprecipitation.

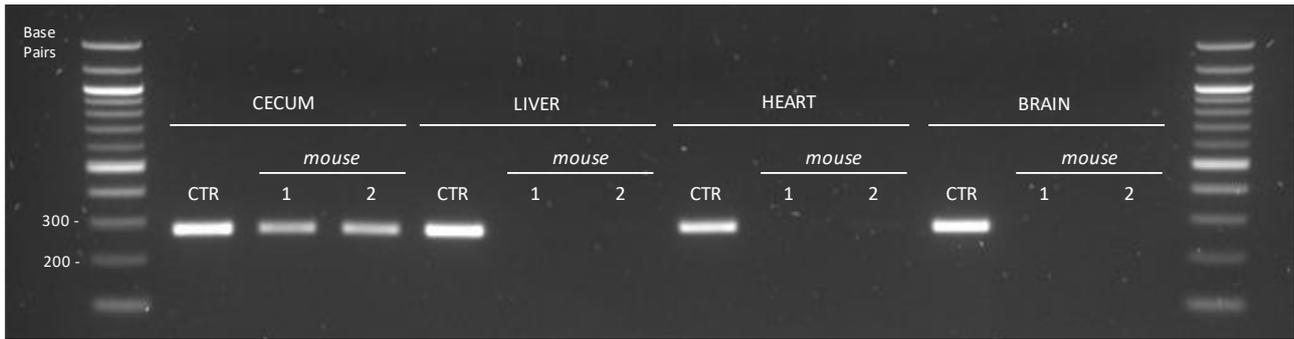
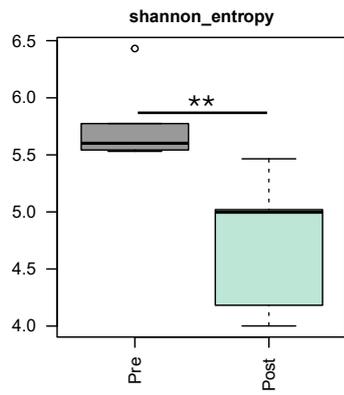


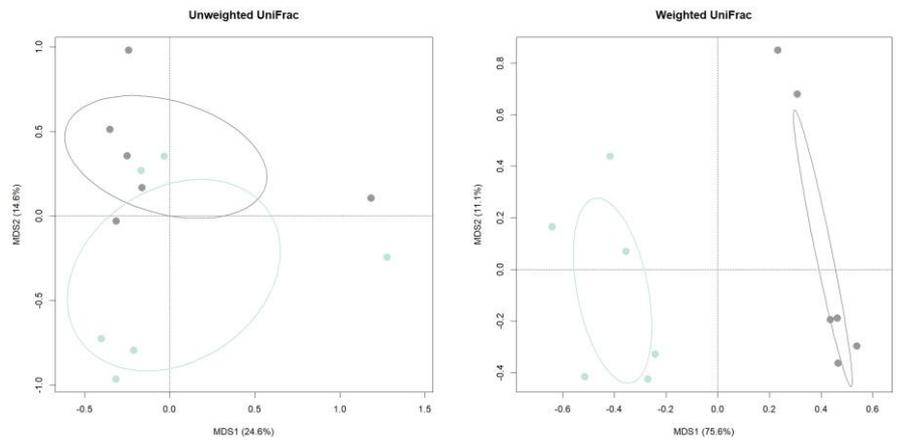
Figure S4. Detection of *L. lactis* DNA in tissue homogenates by PCR.

Agarose gel image showing PCR amplification products from homogenates of intestine, liver, heart, and brain tissues collected from two mice (1 and 2) treated by oral gavage for four consecutive days with *L. lactis* expressing TATκ-GFP. Tissue homogenates spiked with *L. lactis* expressing TATκ-GFP (7.5×10^8) were used as amplification controls (CTR).

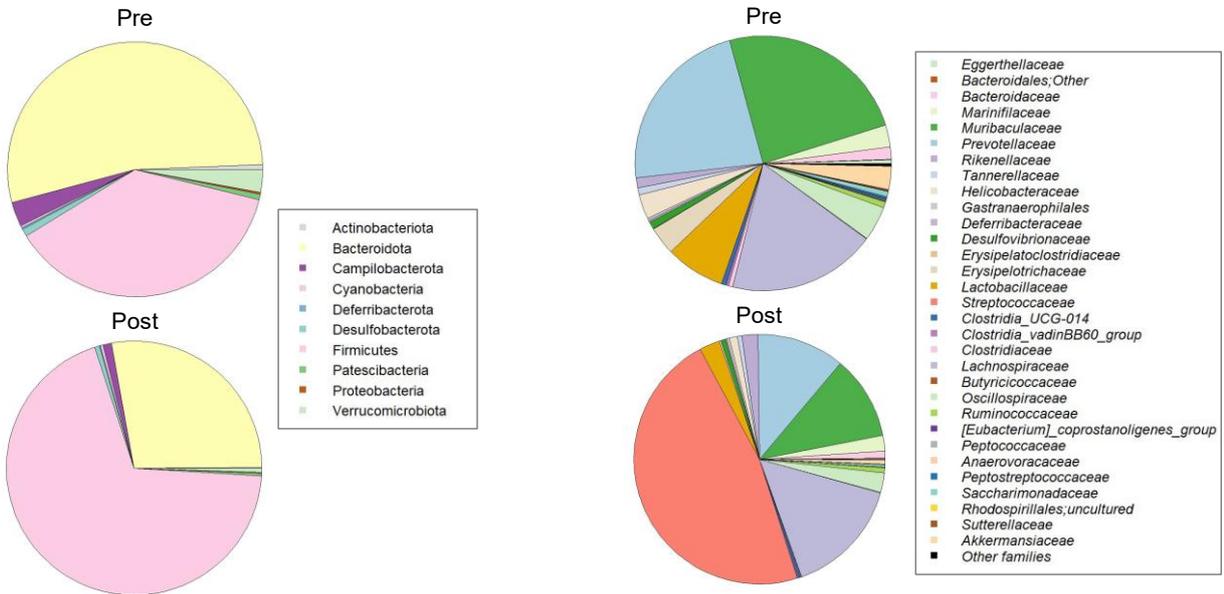
A



B



C



D

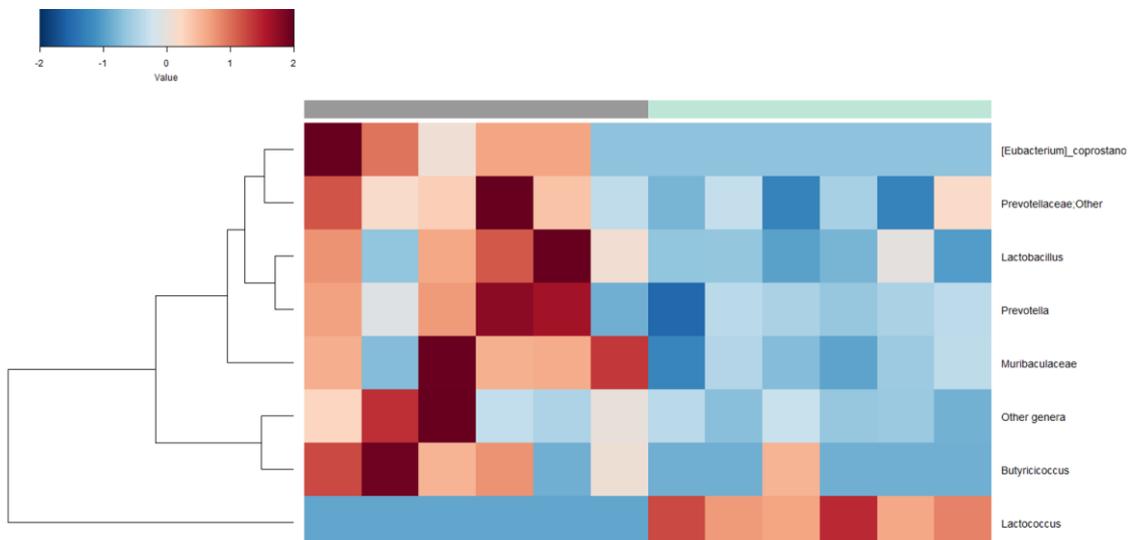


Figure S5. Impact of *Lactococcus lactis* administration on the gut microbiota.

A: Boxplots showing the distribution of alpha diversity, estimated by Shannon entropy, in the gut microbiota of six mice before and after oral administration of *L. lactis* for 4 days (Wilcoxon test, $p < 0.01$). **B:** Principal Coordinates Analysis (PCoA) plots of beta diversity estimated using unweighted (left) and weighted (right) UniFrac distances between study groups (same color code as in A). Ellipses represent the 95% confidence area based on the standard error of the weighted average of sample coordinates. Significant segregation was observed in the weighted UniFrac-based PCoA (PERMANOVA, $p = 0.003$). **C:** Pie charts showing the average relative abundance profiles at the phylum (left) and family (right) levels. Only taxa with relative abundance $>0.5\%$ in at least one sample are shown. **D:** Heatmap showing Ward linkage clustering based on Pearson's correlation coefficients of the relative abundance of differentially represented genera between study groups (Wilcoxon test, $p < 0.05$).

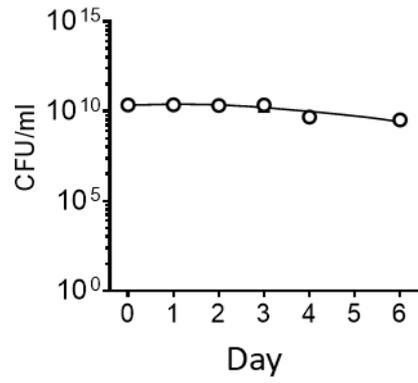


Figure S6. Survival of *Lactococcus lactis* in drinking water over time.

Quantification of *L. lactis* in drinking water stored at room temperature for 1 to 6 days. Results are expressed as the number of *L. lactis* colony-forming units (CFU) per milliliter o