Beyond viability: comparative functional evaluation of live and inactivated industrial biomasses of probiotic lactobacilli

Introduction

Probiotics are widely acknowledged for their beneficial effects on gut health, including modulation of immune responses and reinforcement of the intestinal barrier. However, the use of live probiotics poses certain limitations, such as safety concerns in immunocompromised individuals and reduced stability during storage and transport. These challenges have driven increasing interest in inactivated probiotics, also referred to as *paraprobiotics*[1] or *postbiotics*[2], which aim at retaining health-promoting functions without the risks associated with viability.

This study investigates the strain-specific functional properties of different industrial lyophilized inactivated bacterial biomasses in vitro, focusing on their interactions with intestinal epithelial cells and immunomodulatory effects, and compares them to their viable forms.

Materials and Methods

The industrial biomass of four commercial probiotic strains were subjected to different thermal and mechanical inactivation methods at industrial level (Table 1).

Species	Strain designation	DSMZ accession nr.	Inactivation method	Short code
Lacticaseibacillus rhamnosus	LRH020	DSM 25568	None (lyophilized)	vLRH
			Heat treatment 1	LRH-T1
			Heat treatment 2	LRH-T2
			Heat treatment 3	LRH-T3
			Heat treatment 4	LRH-T4
			Mechanical	LRH-M
Lactobacillus acidophilus	PBS066	DSM 24936	None (lyophilized)	vLA
			Heat treatment	LA-T
			Mechanical	LA-M
Lactiplantibacillus plantarum	PBS067	DSM 24937	None (lyophilized)	vLP
			Heat treatment	LP-T
			Mechanical	LP-M
Lacticaseibacillus paracasei	LPC1114	DSM 34559	None (lyophilized)	vLPC
			Heat treatment	LPC-T
			Mechanical	LP-M

Table 1. Overview of the different probiotic strains used with their different types of inactivation and their respective code.

To confirm successful inactivation, resuspended lyophilized biomasses were plated on MRS agar for CFU enumeration. We further verified cell viability using a CytoFLEX S flow cytometer, employing a combination of fluorescent dyes: SYTO® 24 / Propidium Iodide (PI) to assess membrane integrity, 3,3'-Dihexyloxacarbocyanine iodide ($DiOC_2(3)$) to detect membrane potential and Carboxyfluorescein diacetate (cFDA)/Propidium Iodide (PI) to evaluate intracellular esterase activity.

Adhesion assays were conducted using differentiated Caco-2 monolayers. Approximately 2×10^8 inactivated bacterial cells were incubated with Caco-2 cells for 1 h at 37 °C. Post-incubation, a semi-quantitative measuring based on Adhesion Index (ADI: number of bacterial cells per 100 Caco-2 cells) was calculated by microscopy observations. *Bifidobacterium bifidum* MIMBb23sg was included as a positive control.

To investigate the immunomodulatory effects of inactivated strains, we used recombinant HEK293 expressing human Toll-like receptor 2 and an alkaline phosphatase (SEAP) reporter gene. Cells were seeded in 96-well plates, then stimulated with bacterial suspensions at MOIs 10, 50 and 100; Pam3CSK4 (50 ng/mL) served as the positive control. After 16 h, the supernatants were collected and the SEAP activity was quantified using the Quanti-Blue™ reagent.

NF-κB activation was evaluated in pNiFty2-SEAP Caco-2 cells stimulated with bacterial suspensions (MOIs 50, 100, 500) and IL-1β (2 ng/mL). After 24 h of incubation at 37 °C, SEAP levels in the supernatants were measured using Quanti-Blue™.

To evaluate the impact of inactivated strains on epithelial barrier integrity, we performed transepithelial electrical resistance (TEER) assays using fully differentiated Caco-2 monolayers. Cells were treated on the apical side with inactivated bacterial cells (MOI 100). After 24 h, TEER values were measured using a Millicell ERS-2 Voltohmmeter (Merck).

All assays described above were performed in duplicate and repeated in two independent experiments.

Results and discussion

First, we focused on *Lacticaseibacillus rhamnosus*, since it is a bacterial species widely used for probiotic applications, exploring the effects of five inactivation methods (Table 1). Plate counts on MRS agar confirmed the effectiveness of all treatments, as no colony growth was observed in any of the inactivated samples.

To further evaluate bacterial viability, we employed flow cytometry using SYTO-24 and PI staining. Viable lyophilized strain (vLRH) retained over 70% of cells with intact membranes, while all thermal inactivation methods resulted in more than 99,9% cells with damaged or disrupted membrane (Figure 1). The mechanical inactivation preserved membrane integrity in 22% of cells. Membrane potential, evaluated through $DiOC_2(3)$ staining, was retained in the vLRH, whereas it was no longer detectable in all inactivated biomasses (Figure 2, orange bars). These data are consistent with the metabolic activity assessment based on cFDA staining (Figure 2, blue bars), suggesting that all industrial inactivation protocols caused membrane damage and a concurrent loss of metabolic activity.

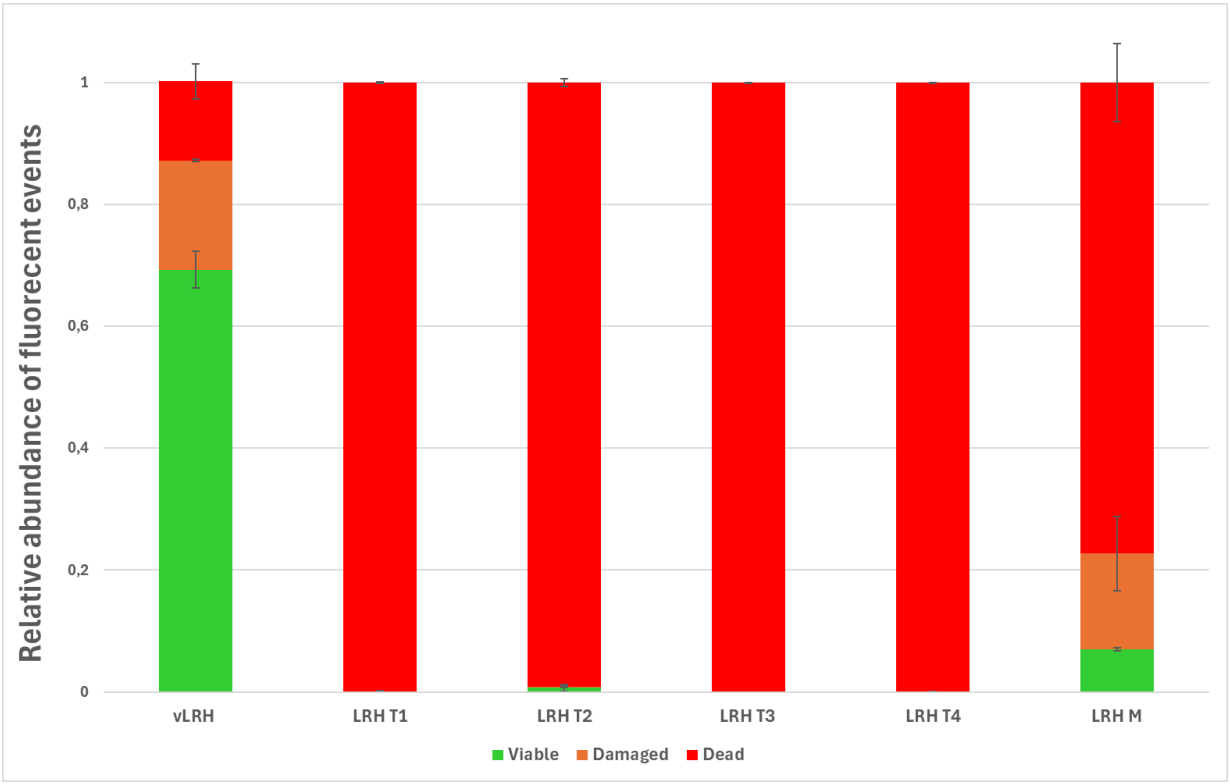


Figure 1. Cyfluorimetric measurement of the relative distribution of viable (green), damaged (orange), and dead (red) LRH cells after SYTO® 24/Propidium Iodide staining. Data are reported as mean ± standard deviation.

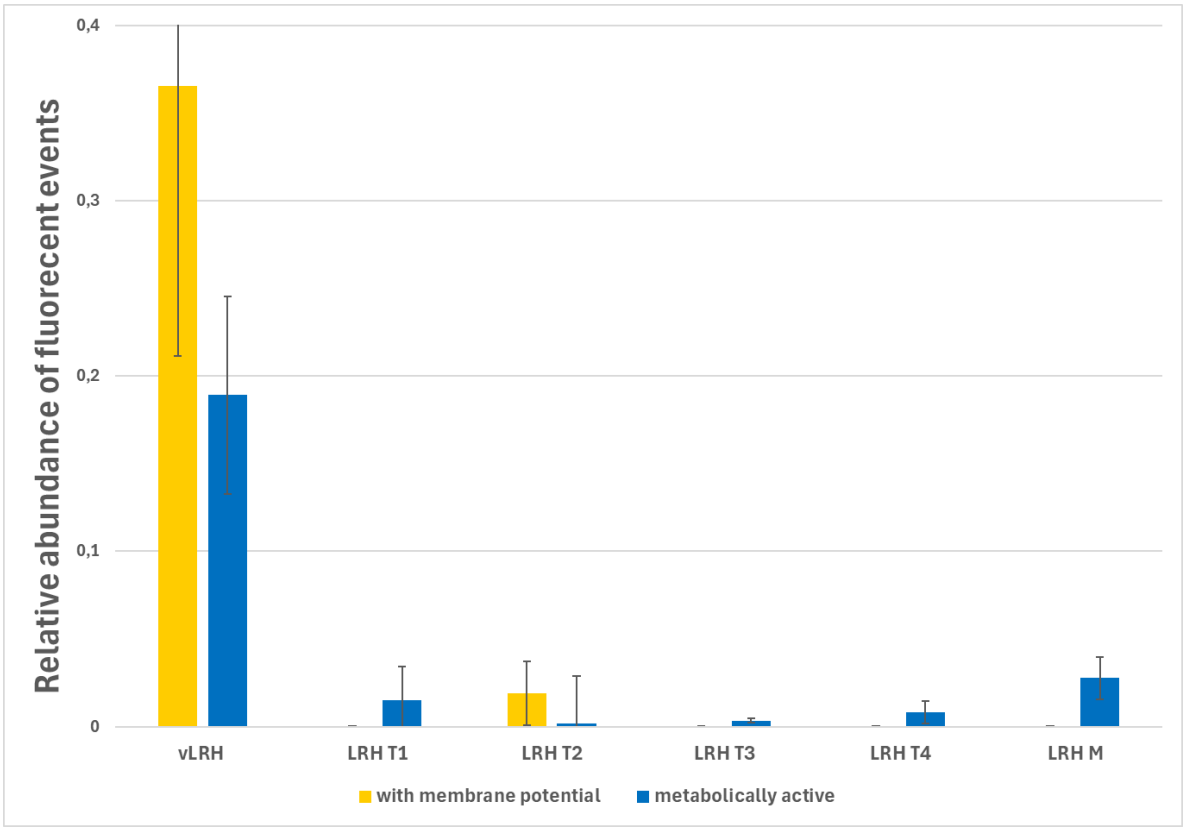


Figure 2. *Cyfluorimetric measurement of the relative fluorescent events indicating metabolic activity (blue, cFDA staining) and membrane potential [orange, $DiOC_2(3)$] in treated cells. Data are reported as mean ± standard deviation.*

Viable lyophilized strain (vLRH) showed a strong adhesion capacity, with an ADI value falling in the high adhesion range. Interestingly, the thermally treated sample LRH-T1 exhibited a comparable adhesion profile (Figure 3), suggesting that, differently from the others, this inactivation method may preserve surface structures involved in adhesion.

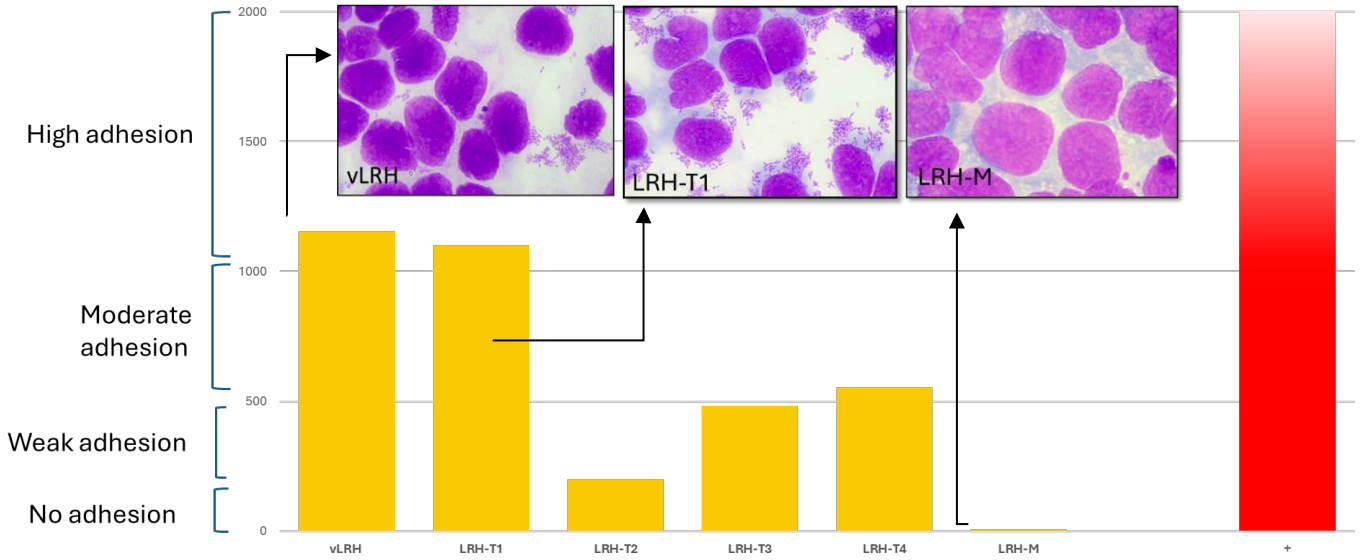


Figure 3 Adhesion Index (ADI) of LRH strains to Caco-2 cells. +, *B. bifidum* MIMBb23sg (positive control).

Subsequently, immunomodulatory characterization showed that the vLRH strain (LRH at MOI 10, 50, 100) induced significantly higher TLR2 activation compared to inactivated samples. This suggests that industrial inactivation processes may markedly reduce the strain's immunostimulatory potential on TLR2 signaling, likely due to damage to surface microbe-associated molecular patterns (MAMPs) caused by inactivation-related stress (Figure 4).

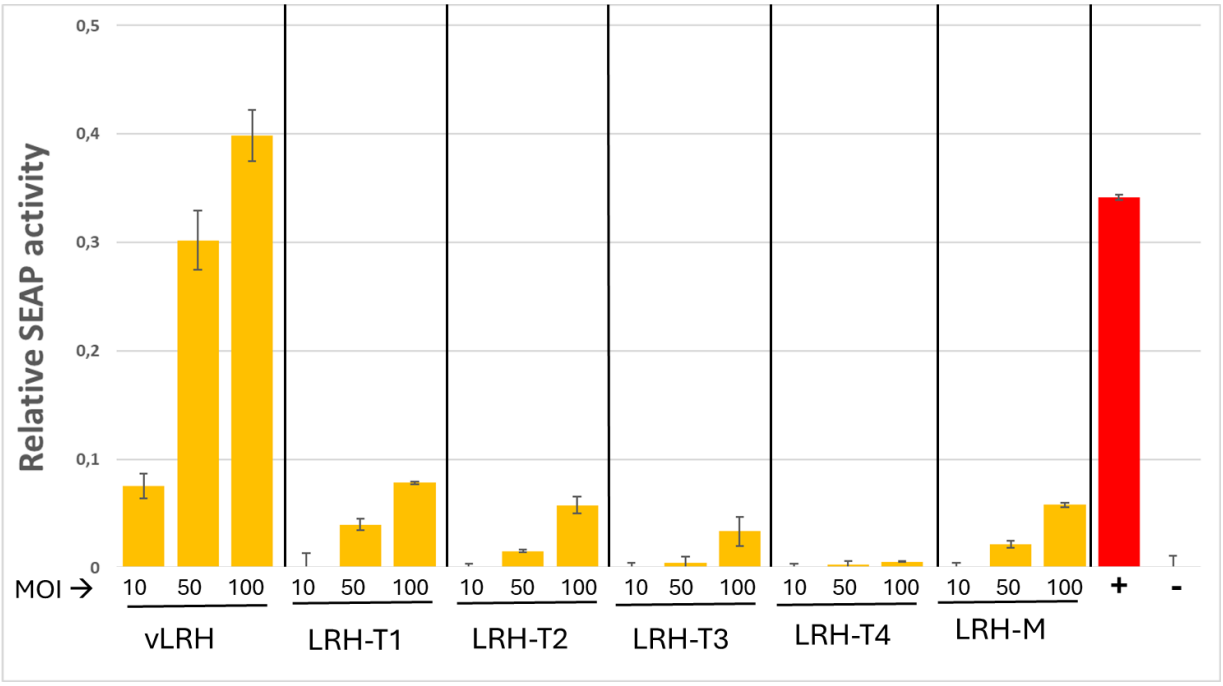


Figure 4: SEAP relative activity in HEK-TLR2 reporter cells stimulated with the LRH industrial preparations. SEAP activity was measured as absorbance at 600 nm and normalized to negative control (medium without bacterial cells). Data are reported as mean ± standard deviation.

The same set of functional characterization experiments previously performed on LRH was extended to three additional probiotic strains (Table 1) to assess whether the conclusions drawn from the LRH study could also apply to other probiotics.

Cytofluorimetric analysis of the industrial biomass revealed an almost complete loss of membrane integrity in inactivated cells, consistent with the observations made for LRH (Figures 5 and 6).

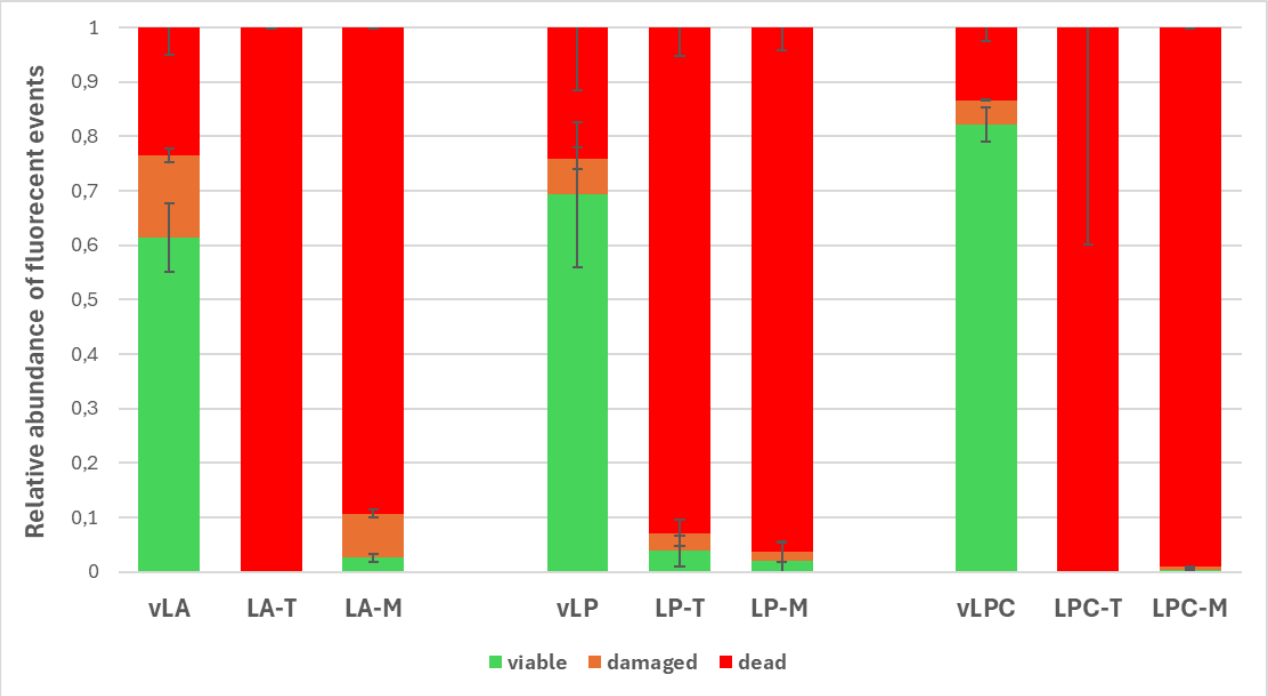


Figure. 5: *Cyfluorimetric measurement of the relative distribution of viable (green), damaged (orange), and dead (red) LA, LP and LPC cells after SYTO® 24/Propidium Iodide staining. Data are reported as mean ± standard deviation.*

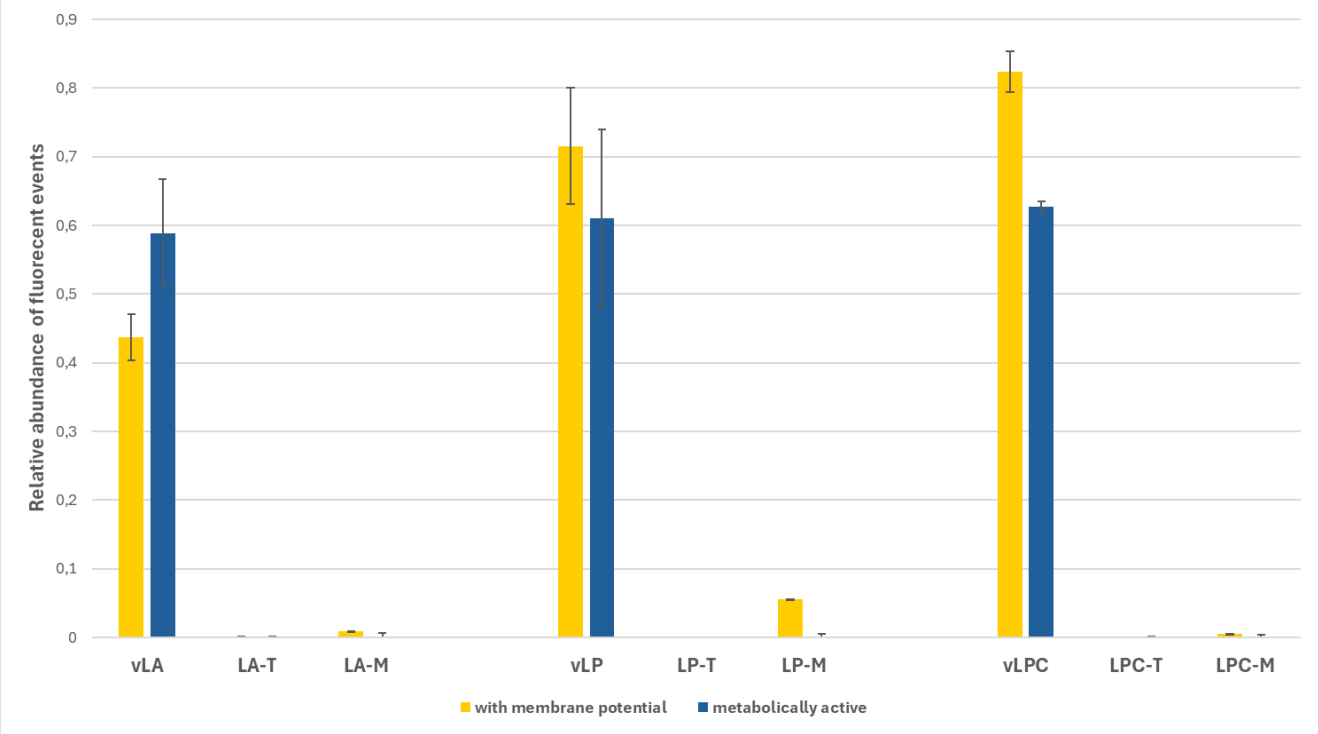


Figure. 6: Cyfluorimetric measurement of the relative fluorescent events indicating metabolic activity (blue, cFDA staining) and membrane potential [orange, $DiOC_2(3)$] in treated cells. Data are reported as mean ± standard deviation.

Regarding adhesive capacity, LA-T exhibited notably high adhesion, with an ADI substantially higher than vLA (weak adhesion) (Figure 7). Similarly, LPC-T reached the moderate adhesion range, far surpassing the no adhesion observed for vLPC. In contrast, LP-T showed a more moderate increase, compared to its viable form (vLP). These findings suggest that thermal inactivation may preserve, and in some cases even enhance, bacterial adhesion to epithelial cells.

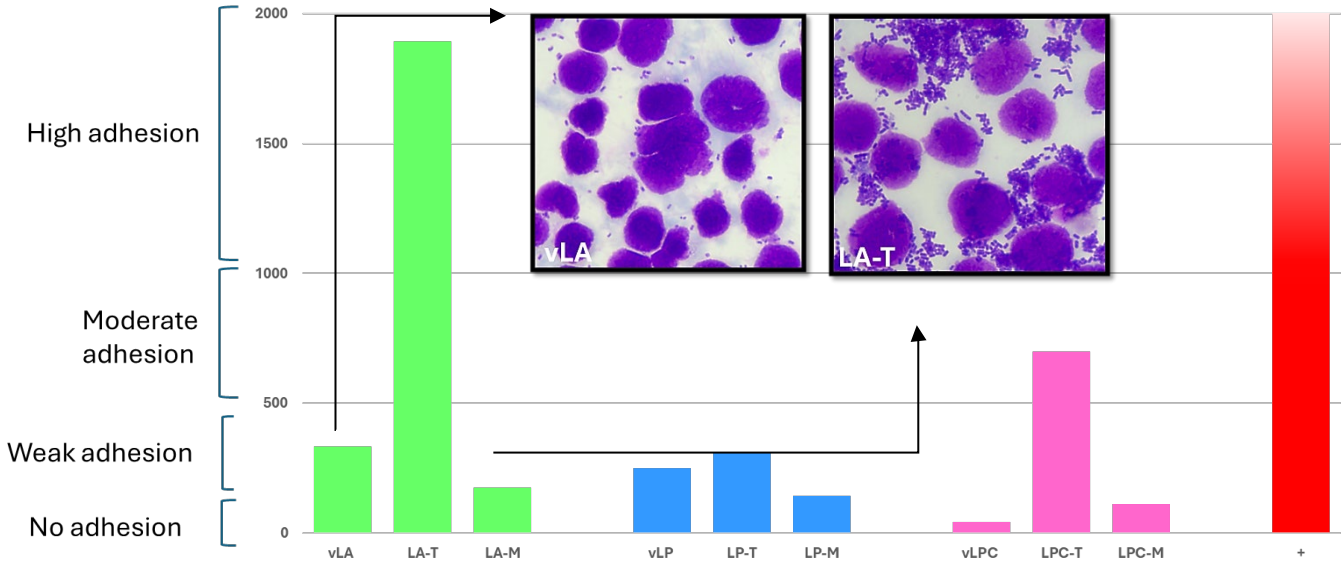


Figure. 7. Adhesion Index (ADI) of LA, LP and LPC strains to Caco-2 cells. +, *B. bifidum* MIMBb23sg (positive control).

Immune response analysis revealed that vLA and vLP elicited negligible responses, while both thermal and mechanical inactivation slightly increased TLR2 activity, suggesting partial unmasking of ligands. In contrast, vLPC showed a modest basal activation in the live state, which was slightly reduced following inactivation. Notably, LA-M and LP-M induced the strongest, dose-dependent responses, highlighting the potential of mechanical inactivation to enhance TLR2 ligand accessibility. (Figure 8).

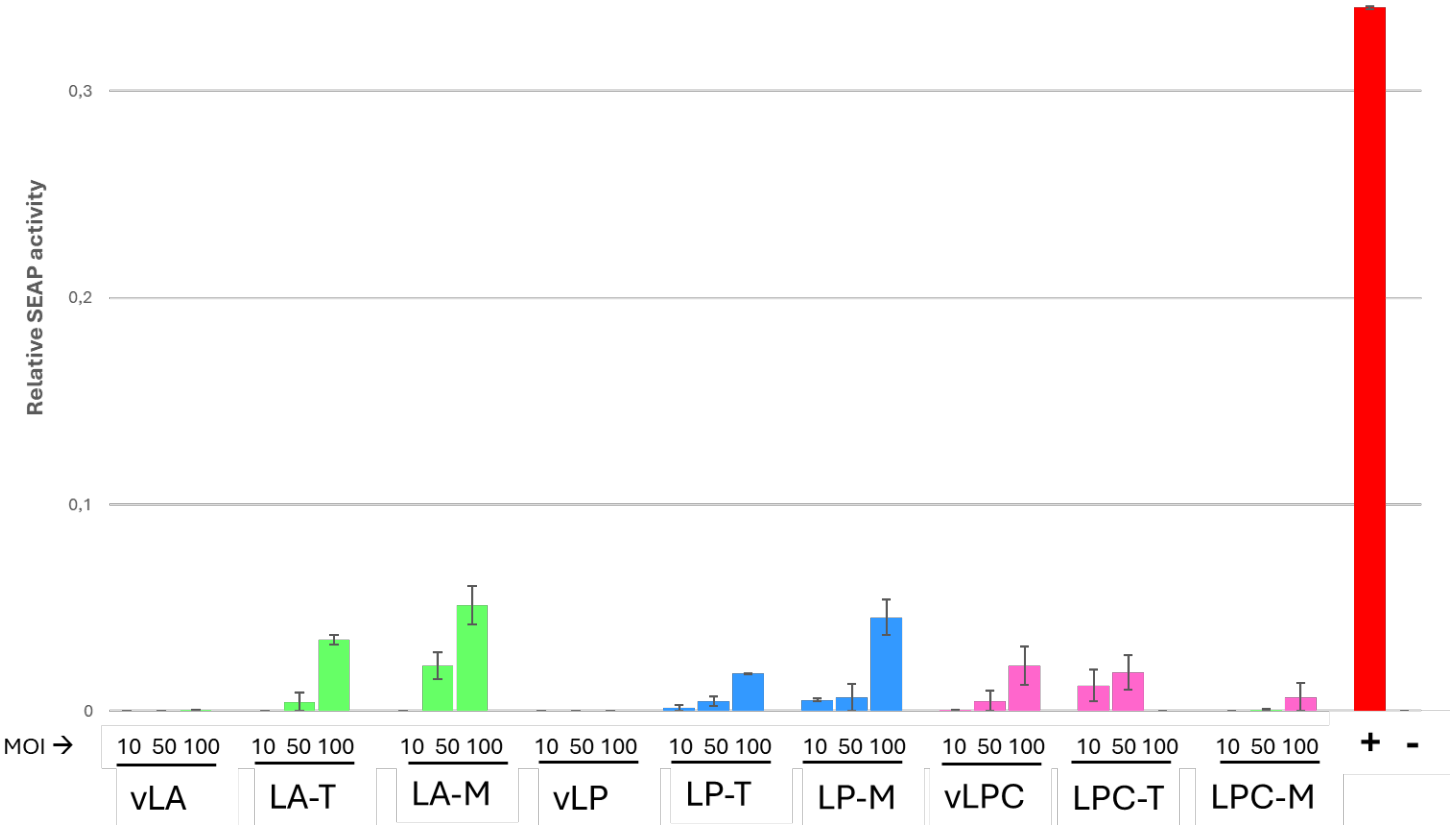


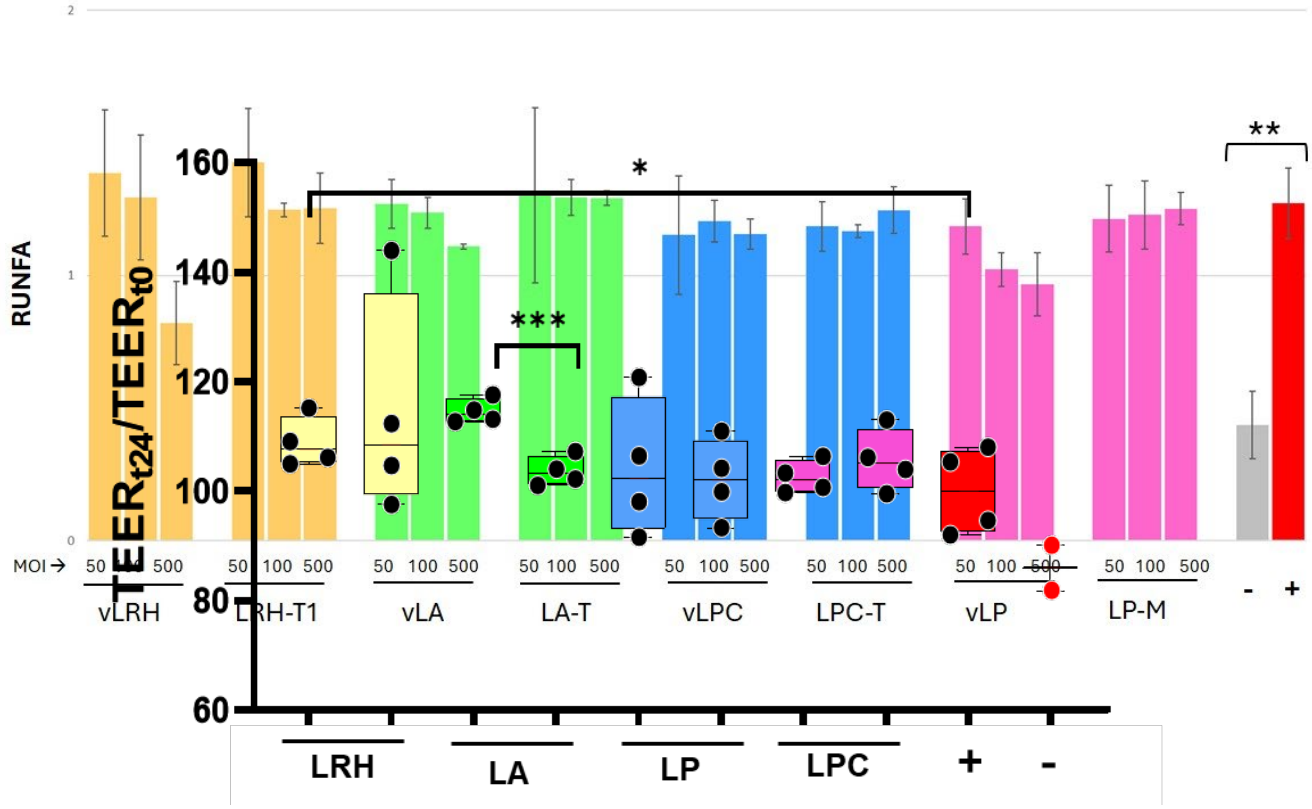
Figure. 8 SEAP relative activity in HEK-TLR2 reporter cells. Data are reported as mean ± standard deviation.

Then, we performed additional experiments with these three strains, also including LRH-T1, selected due to its strong adhesion capacity.

The NF-κB assay showed a strong activation by IL-1β compared to baseline. Only vLP exhibited a dose-dependent decrease in IL-1β-induced SEAP levels, though this trend did not reach statistical significance. All other bacterial stimuli had no effect on SEAP, demonstrating an absence of pro-inflammatory activity and indicating safety even in the presence of an inflammatory challenge.

Figure. 9 Activation of NF-κB in IL-1 β-stimulated Caco-2 cell layer by bacterial biomasses. Results are expressed as Relative Units of NF-κB Activation (RUNFA). Data are reported as mean ± standard deviation. **, P<0.01 according to unpaired Student's t test.

TEER measurements showed that, unlike IL-1β, none of the microbial preparations impaired Caco-2 monolayer permeability. Live and heat-inactivated forms of LP, LPC, and LRH had similar effects,



whereas only live LA significantly increased transepithelial resistance compared to its inactivated counterpart.

Figure. 10 Effect of industrial lyophilized bacterial preparations on TEER in Caco-2 cells. *, P < 0.05 vs. IL-1β control.

Conclusion

Industrial inactivation of microbial biomass can alter probiotic functions, often preserving or enhancing adhesion and immunomodulatory activity, without compromising safety. Because these effects are both strain- and process-dependent, preclinical characterization should employ actual industrial-scale preparations rather than laboratory-scale ones.

Candidate contribution

I personally performed all experiments described in the study, including sample preparation and washing, operation of all instruments listed in the Materials and Methods, cultivation and maintenance of the cell lines, as well as data analysis and figure generation. I also performed analysis of data and preparation of results. The project started in July 2024 and I worked on this project continuously since then.

References:

(1) doi:10.1007/s12263-011-0218-x

(2) doi:10.5281/zenodo.15133307