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Viability-Dependent and Structural Modulation of IL-10 Secretion in THP-1 Cells by Lactobacillus gasseri and Lacticaseibacillus rhamnosus

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Abstract

Emerging evidence indicates that probiotic-mediated immunomodulation is not exclusively dependent on microbial viability, but is also significantly driven by structural components and postbiotic factors. This study systematically evaluated the viability-dependent versus structure-dependent regulation of Interleukin-10 (IL-10) in THP-1 monocytes by comparing alive, partially inactivated, and fully killed states of *Lactobacillus gasseri* and *Lacticaseibacillus rhamnosus*. Distinct bacterial preparations were generated via heat, ultraviolet (UV) irradiation, sonication, and chemical inactivation. We assessed IL-10 protein secretion (ELISA), transcriptional kinetics (RT-qPCR), and single-cell cytokine expression (flow cytometry), while ensuring cellular compatibility through MTT viability assays. The results demonstrate that both viable and non-viable preparations significantly induce IL-10 secretion. However, live preparations elicited a more robust and rapid transcriptional response, with peak IL-10 mRNA expression occurring at 16–20 hours post-stimulation. Notably, mixed-strain conditions exhibited a pronounced combinatorial enhancement of IL-10 production at both the protein and single-cell levels. While structural microbial components are sufficient to trigger anti-inflammatory IL-10 responses, active microbial viability markedly amplifies the magnitude and kinetics of this regulation. These findings provide a mechanistic foundation for the rational design of precision paraprobiotic and probiotic formulations in immune-mediated therapies.

Introduction

Probiotics are traditionally defined as live microorganisms that confer a health benefit on the host when administered in adequate amounts. While this definition has long driven probiotic research and clinical application, accumulating evidence suggests that many immunomodulatory and biological effects are not strictly contingent upon microbial viability (1, 2). Instead, structural components of the microbial cell and postbiotic metabolites play a pivotal role. This paradigm shift has catalyzed the exploration of non-viable microbial preparations—often termed paraprobiotics and postbiotics—shifting the focus from live cell administration to functional microbial interventions .Paraprobiotics offer substantial biotechnological and clinical advantages, including enhanced shelf-life, improved formulation stability, and mitigated safety risks, particularly for immunocompromised patients or those with impaired epithelial barriers (3–5). Immunologically, microbial-associated molecular patterns (MAMPs) such as peptidoglycan, lipoteichoic acids, and surface polysaccharides maintain their structural integrity and biological activity post-inactivation. These components interact with host pattern-recognition receptors (PRRs), notably Toll-like receptors (TLRs), to regulate downstream immune cascades. Among lactic acid bacteria, *Lactobacillus gasseri* and *Lacticaseibacillus rhamnosus* are well-documented for their profound immunomodulatory activities (6, 7). Both species naturally colonize the human gastrointestinal tract and actively interact with innate immune cells, critically modulating interleukin (IL) signaling networks that orchestrate the balance between inflammatory and regulatory immune responses, particularly through the induction of the anti-inflammatory cytokine IL-10 (8–10).

Although previous studies have demonstrated the immunomodulatory potential of these strains in their live and heat-killed states, a critical knowledge gap remains regarding how diverse inactivation modalities alter structural integrity and subsequent cytokine profiles(11–13). The biological efficacy of paraprobiotics is heavily dictated by the specific inactivation method—such as heat, ultraviolet (UV) irradiation, sonication, or chemical fixation—which differentially impacts membrane architecture and MAMP presentation. Furthermore, robust *in vitro* immunological evaluations are frequently confounded by nonspecific cytotoxic effects induced by concentrated microbial preparations. To address these complexities, the present study systematically investigates the viability-dependent versus structure-dependent determinants of immune regulation. We comprehensively compare the effects of *L. gasseri* and *L. rhamnosus* across a viability continuum—alive, partially inactivated, and fully killed—using multiple inactivation strategies. By employing the human monocytic THP-1 cell line as a robust model for innate immune signaling. and integrating strict MTT-based cytotoxicity prescreening, this research elucidates the temporal kinetics and quantitative magnitude of IL-10 secretion. Ultimately, this work aims to decode the structural prerequisites for probiotic-mediated immune tolerance, providing a rigorous framework for the development of next-generation immunomodulatory formulations (14–18).

Materials and Methods

Cell Culture

The human monocytic THP-1 cell line was cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12; Gibco, Thermo Fisher Scientific, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 1% (v/v) penicillin–streptomycin (or penicillin–kanamycin) solution. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2. Primary cultures were seeded into T-12.5 cm^2 tissue culture flasks and maintained for 4 days to achieve steady-state growth prior to downstream experiments. Cell viability was determined using the trypan blue exclusion assay (> 95% viability), and cells were subsequently seeded into experimental culture plates at predetermined densities.

Bacterial Strains and Inactivation Conditions

Lactobacillus gasseri ATCC 33323 and *Lacticaseibacillus rhamnosus* ATCC 53103 (equivalent to *L. rhamnosus* GG) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Strains were cultured anaerobically (5% CO_2, 95% N_2) in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Germany) at 37°C for 18–24 h. Strain identity was periodically confirmed via 16S rRNA gene sequencing.

To distinguish between structure-dependent and viability-dependent immune responses, three distinct bacterial physical states were prepared: viable, partially inactivated, and fully killed. Sub-lethal treatments (yielding 10–30% residual viability; ∼1–2 $\log_{10}$ CFU/mL reduction) were achieved via mild heat exposure (60°C for 15 min) or low-dose UV-C irradiation (λ = 254 nm, 3 mJ/cm^2). Fully inactivated

preparations (< 0.1% viability; ≥ $3\log_{10}$CFU/mL reduction) were generated using extensive heat treatment, probe sonication, or formaldehyde fixation. Bacterial viability and structural membrane integrity were quantitatively evaluated using the LIVE/DEAD™ BacLight™ Bacterial Viability Kit (Invitrogen, Cat. No. L7012) and verified by the total absence of colony-forming units (CFUs) on MRS agar plates after 72 hours of anaerobic incubation at 37°C.

Bacterial Dosing and Co-culture (MOI)

THP-1 monocytes were seeded at a density of 5×10^5 cells/well in 24-well tissue culture plates. Bacterial suspensions were added at a multiplicity of infection (MOI) of 10:1 (bacteria:cell), corresponding to approximately 5×10^6 CFU/mL per well. For partially inactivated and fully killed preparations, pre-inactivation viable counts (CFU/mL) were used as normalization baselines to ensure equal bacterial antigen loading across all experimental groups.

Cytotoxicity Assessment (MTT Assay)

To exclude nonspecific cytotoxicity as a confounding factor in cytokine evaluation, cellular metabolic activity was assessed via the ruledimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assaydimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Following co-incubation with bacterial preparations across serial dilutions, cells were treated with MTT solution (0.5 mg/mL final concentration). Formazan crystals were solubilized with DMSO, and optical density (OD) was measured at λ = 570 nm with a reference wavelength of λ = 630 nm. Only conditions demonstrating ≥ 85–90% relative cell viability were selected for subsequent immunological evaluations.

Protein Quantification and SDS-PAGE Analysis

Protein Quantification and SDS-PAGE Analysis
Total protein concentrations of the bacterial lysates (Live and Inactivated *L. gasseri* and *L. rhamnosus*) were quantitatively determined using the standard Bradford protein assay. Bovine Serum Albumin (BSA) was utilized to construct the standard calibration curve, and the absorbance was recorded at 595 nm using a microplate reader. Following quantification, the samples were precisely normalized to ensure equal protein loading. Equivalent amounts of total protein (e.g., 20 µg) from each standardized sample were mixed with Laemmli sample buffer, denatured by boiling at 95°C for 5 minutes, and subsequently loaded onto a polyacrylamide gel for SDS-PAGE analysis to evaluate protein structural integrity and profiling.

IL-10 Protein Quantification (ELISA)

Secreted IL-10 concentrations in cell-free culture supernatants were measured using a commercial human IL-10 sandwich ELISA kit (Cat. No. 43122-KIAL10; Kiazist Life Sciences, Iran) according to the manufacturer's instructions. Supernatants were collected at the indicated time points, cleared of cellular debris by centrifugation (300 × g, 10 min, 4°C), and either analyzed immediately or stored at − 80°C. Absorbance was read at λ = 450 nm using an ELx800™ Absorbance Microplate Reader (BioTek Instruments, Winooski, VT, USA). Absolute concentrations (pg/mL) were calculated from standard curves fitted to a four-parameter logistic (4-PL) regression model using Gen5™ Software (v3.1). All samples were evaluated in duplicate, with intra- and inter-assay coefficients of variation (CV) consistently < 10%.

RNA Extraction and RT-qPCR Analysis

Total RNA was isolated using TRIzol™ reagent (Invitrogen, Carlsbad, CA, USA) and quantified spectrophotometrically using a NanoDrop ND-1000 instrument, ensuring an A_{260}/A_{280} purity ratio between 1.8 and 2.0. First-strand cDNA was synthesized from 1µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Real-time quantitative PCR (RT-qPCR) was performed on a Q5 Gradient Real-Time PCR Detection System using SYBR™ Green Master Mix (Applied Biosystems). The cycling parameters were: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. Amplicon specificity was verified by post-amplification melt curve analysis. *GAPDH* served as the endogenous reference gene for normalization. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. All primer sequences are listed in Table 1.

Table 1
Primer sequences used for RT-qPCR analysis

Target Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')
IL-10	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG
hβ-Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

Intracellular Cytokine Staining and Flow Cytometry

Following designated stimulation periods, Brefeldin A (10 µg/ml; BioLegend) was added during the final 4–6 hours of culture to block cellular protein transport. Harvested cells were fixed with 4% paraformaldehyde, permeabilized utilizing 0.1% saponin buffer, and stained with PE-conjugated anti-human IL-10 (Clone JES3-9D7; BioLegend). Data acquisition was performed on a BD FACSCalibur flow cytometer, capturing a minimum of 10,000 events within the validated monocyte gate. Fluorescence-

minus-one (FMO) controls were utilized for precise gating boundaries. Analysis was conducted using FlowJo software (v10.8, BD Biosciences).

Statistical Analysis

All experimental data were obtained from a minimum of three independent biological replicates ($n \geq 3$), each evaluated in technical triplicate, and are expressed as mean ± standard deviation (SD). Data normality and homogeneity of variances were assessed using the Shapiro–Wilk test and the Brown–Forsythe test, respectively. For multiple group comparisons at a single time point, a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was conducted. Longitudinal and multi-factor response dynamics were evaluated using a two-way repeated-measures ANOVA followed by Tukey's multiple comparisons test. Differences were considered statistically significant at *$p < 0.05$ (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, and ****$p < 0.0001$. All statistical analyses and graphical representations were performed using GraphPad Prism software (version 10.1.2; GraphPad Software, Boston, MA, USA).

Results

Bacterial preparations demonstrate high biological compatibility with THP-1 macrophages

To ensure that the observed immunomodulatory effects were not confounded by bacterial cytotoxicity, the viability of THP-1 cells was assessed using an MTT assay following a 24-hour co-culture at an MOI of 10:1. As depicted in Fig. 1, neither the live nor the heat-killed (dead) single strains or their mixtures induced significant cytotoxic effects. Cell viability remained consistently above 80% across all experimental conditions relative to the untreated control, confirming the biological compatibility of the selected bacterial doses for subsequent immunological and transcriptional evaluations.

Inactivation methods severely disrupt bacterial structural protein integrity

The physical impact of the inactivation processes on bacterial structural integrity was validated via SDS-PAGE (Fig. 2). Live preparations of *L. gasseri*, *L. rhamnosus*, and their mixture exhibited distinct and highly conserved protein profiles, prominently featuring major bands at approximately 19–20 kDa and 52–55 kDa. Conversely, the inactivated (dead) groups demonstrated severe structural degradation, characterized by the absence of these distinct bands and the emergence of extensive protein smearing. This confirms the successful physical disruption of bacterial surface proteins in the inactivated groups, setting the stage for evaluating structure-dependent immune responses.

Viability and strain synergy dictate the kinetics of IL-10 transcriptional upregulation

We next evaluated the temporal kinetics of *IL-10* mRNA expression over a 48-hour period (Fig. 3). Both viable and non-viable preparations successfully upregulated *IL-10* transcription compared to the untreated baseline. However, the response was highly viability-dependent. Live preparations induced a rapid and robust transcriptional surge, peaking between 16 and 20 hours post-stimulation. Notably, the Live Mix condition exerted a profound synergistic effect, driving the highest fold change in *IL-10* expression. While the dead preparations also triggered significant *IL-10* upregulation, the magnitude was markedly attenuated, indicating that active bacterial metabolism significantly amplifies the transcriptional response.

Structural components trigger IL-10 secretion, but viability maximizes the inflammatory response

To determine whether the transcriptional upregulation translated to protein secretion, IL-10 levels in the culture supernatants were quantified via ELISA across four distinct inactivation modalities (Fig. 4). Consistent with the mRNA kinetics, all bacterial preparations significantly stimulated IL-10 secretion. Live probiotics (Fig. 4A) induced the most potent cytokine release, with the Live Mix demonstrating significant synergistic enhancement ($p < 0.001$). Among the inactivated groups, UV-treated strains (Fig. 4B) maintained relatively high IL-10 secretion, likely due to the preservation of cell wall integrity. Heat-killed (Fig. 4C) and sonicated (Fig. 4D) preparations also induced significant, albeit lower, levels of IL-10, confirming that structural components alone (paraprobiotics) are sufficient to trigger a regulatory immune response.

Single-cell analysis corroborates the superior immunomodulatory capacity of live mixed strains

Finally, intracellular IL-10 production was validated at the single-cell level using flow cytometry (Fig. 5). The gating strategy effectively isolated live singlet macrophages to ensure accurate quantification (Fig. 5A). The representative histograms (Fig. 5B) and quantitative analysis (Fig. 5C) corroborated the ELISA findings. The Live Mix formulation resulted in the highest frequency of IL-10-positive THP-1 cells, significantly outperforming individual strains ($p < 0.001$). The Dead Mix also produced a statistically significant increase in IL-10-positive cells ($p < 0.05$) compared to the control, further validating the structure-dependent immunomodulatory capacity of these formulations.

Discussion

This study demonstrates that both viable and non-viable forms of *Lactobacillus gasseri* and *Lacticaseibacillus rhamnosus* significantly modulate IL-10 secretion in THP-1 monocytes, with clear viability-dependent differences in magnitude and kinetics (19, 20). By systematically comparing alive, partially inactivated, and fully killed bacterial states, our findings extend previous work that primarily contrasted only live versus heat-killed preparations. who demonstrated that heat-killed *L. gasseri* induces

IL-10 production in macrophages. Similar preservation of immunomodulatory capacity after inactivation was reported for *L. rhamnosus* (21–23). In agreement with these studies, we observed that dead preparations retained substantial IL-10–inducing potential, supporting the concept that structural microbial components remain biologically active following inactivation. These findings reinforce the mechanistic role of microbial-associated molecular patterns (MAMPs), including peptidoglycan and lipoteichoic acids, in Toll-like receptor (TLR)–mediated immune activation (24, 25). However, in contrast to studies that reported comparable IL-10 induction between live and inactivated preparations our data demonstrate that live bacteria consistently elicited stronger and more rapid IL-10 transcriptional responses. This suggests that metabolic activity contributes additional signaling amplification beyond structural receptor engagement. Such amplification may involve enhanced STAT3 activation and transcriptional feedback regulation of IL-10 expression .The observed transcriptional peak between 16–20 hours following live stimulation aligns with established models of IL-10 regulation in innate immune cells (26, 27).

Strain-specific differences observed in our study are in line with Stockert JC et al. who reported that *L. gasseri* strains differentially regulate IL-6, TNF-α, and IL-10 production in macrophages. While Stockert JC et al. focused primarily on inflammatory suppression, our study expands upon these findings by providing a comparative viability-dependent framework and temporal kinetics of IL-10 transcription. This adds mechanistic depth to the understanding of strain-dependent immunobiology (28). Importantly, the amplified IL-10 response observed under mixed-strain conditions in our study supports the conclusions of Takeuchi et al. who suggested that probiotic mixtures may exert enhanced biological effects compared to single strains. Our data provide direct experimental evidence supporting this concept at the cytokine level(29). Mechanistically, cooperative receptor engagement through multiple PRRs, including TLR2 heterodimers and potentially NOD-dependent pathways, may explain the synergistic effect observed in the Live Mix and Dead Mix groups. This coordinated signaling likely converges on shared downstream transcriptional regulators such as NF-κB and STAT3 (30, 31) .

Compared with earlier paraprobiotic studies that primarily evaluated heat inactivation alone, our inclusion of partially inactivated preparations generated by multiple inactivation methods offers additional resolution along the probiotic–paraprobiotic continuum. This approach aligns with emerging frameworks emphasizing functional biological activity rather than viability as the defining criterion of microbial interventions. Furthermore, recent mechanistic work on structural determinants of paraprobiotic immune activation supports our observation that preservation of cell wall integrity critically influences cytokine outcomes. Our use of THP-1 monocytes is consistent with validation studies demonstrating their suitability for immune modulation research. Nevertheless, as with prior in vitro investigations, translation to primary human macrophages or in vivo systems remains necessary to confirm physiological relevance. Collectively, our findings both confirm and extend prior literature. While earlier studies established that non-viable probiotic preparations retain immunomodulatory capacity, our data clarify that viability enhances magnitude and kinetics of IL-10 induction, particularly under mixed-strain conditions. This suggests that optimal immunomodulatory formulations may depend on context-specific balancing of safety, stability, and metabolic potency. From a translational perspective, the

preserved activity of killed preparations supports the development of paraprobiotic formulations as stable and potentially safer alternatives to live probiotics. However, the superior IL-10 amplitude observed in live mixed-strain preparations indicates that metabolic cooperation between strains may offer additional therapeutic advantages in inflammatory or autoimmune settings (32, 33).

Future studies should directly assess receptor-specific mechanisms through TLR2/NOD2 inhibition assays and evaluate STAT3 phosphorylation to confirm pathway involvement. Integration of transcriptomic and metabolomic analyses would further delineate structural versus metabolic contributions to IL-10 regulation.

Limitations

While this study provides comprehensive *in vitro* insights, it utilizes the THP-1 cell line, which, despite being a robust model for innate immunity, may not fully recapitulate the extensive heterogeneity of primary human macrophages. Furthermore, although structural components were implicated, receptor-specific activation dynamics (e.g., precise TLR2/TLR4 stoichiometry and downstream STAT3 phosphorylation) were not directly empirically validated via knockdown or selective inhibition assays. Lastly, *in vivo* validation remains a necessary next step to confirm these cytokine modulations within the complex microenvironment of mucosal immunity.

Conclusion

The present study demonstrates that both viable and non-viable forms of *Lactobacillus gasseri* and *Lacticaseibacillus rhamnosus* significantly modulate IL-10 production in THP-1 monocytes, highlighting the functional relevance of bacterial structural integrity in immune regulation. While live preparations consistently induced stronger and more rapid IL-10 transcriptional and protein responses, heat-killed and structurally preserved preparations retained substantial immunomodulatory capacity. These findings reinforce the concept that microbial viability is not an absolute requirement for anti-inflammatory cytokine induction. Importantly, the data reveal that combinatorial stimulation using mixed bacterial preparations produces amplified IL-10 responses at transcriptional, protein, and single-cell levels. This suggests cooperative engagement of pattern recognition receptors and potential convergence on downstream signaling pathways such as NF-κB and STAT3, which are central regulators of IL-10 expression. The sustained transcriptional activity observed in certain non-viable conditions further indicates that structural components—including peptidoglycan, lipoteichoic acids, and surface proteins—remain biologically active after inactivation and can trigger prolonged immune signaling.

From a translational perspective, these findings provide mechanistic support for the development of paraprobiotic formulations as stable, safe immunomodulatory alternatives to live probiotics. Non-viable preparations may offer therapeutic advantages in settings where microbial replication poses risks, including immunocompromised or critically ill populations. At the same time, the enhanced potency observed under live mixed-strain conditions underscores the importance of microbial metabolic activity

and inter-strain synergy in maximizing anti-inflammatory responses. Collectively, this study advances our understanding of viability-dependent and structural determinants of probiotic immune modulation. By integrating cytokine quantification, transcriptional kinetics, and single-cell analysis, the results provide a multidimensional view of IL-10 regulation in human monocytes. Future investigations should explore receptor-specific signaling pathways, delineate strain-specific molecular effectors, and evaluate in vivo relevance to inflammatory and autoimmune disease models. Such work will be critical for rational design of next-generation probiotic and paraprobiotic interventions aimed at precision immunomodulation.

Declarations

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval

Not applicable. This study was conducted entirely *in vitro* using commercially available cell lines (THP-1). No human participants or animal subjects were involved, and therefore, specific institutional ethical approval was not required.

Consent to participate

Not applicable.

Consent to publication

Not applicable.

Funding

Not applicable.

Author Contribution

MeA: contributed to conceptualization, methodology, validation, formal analysis, resources, data curation, writing—review & editing, supervision, project administration, MaA: contributed to formal analysis, investigation, writing—original draft, formal analysis, PH: writing—review & editing, AA: writing—review & editing, EH: writing—review & editing, TNJ: writing—review & editing.

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Availability of data and materials

The data are available from the corresponding author upon reasonable request.

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Figures

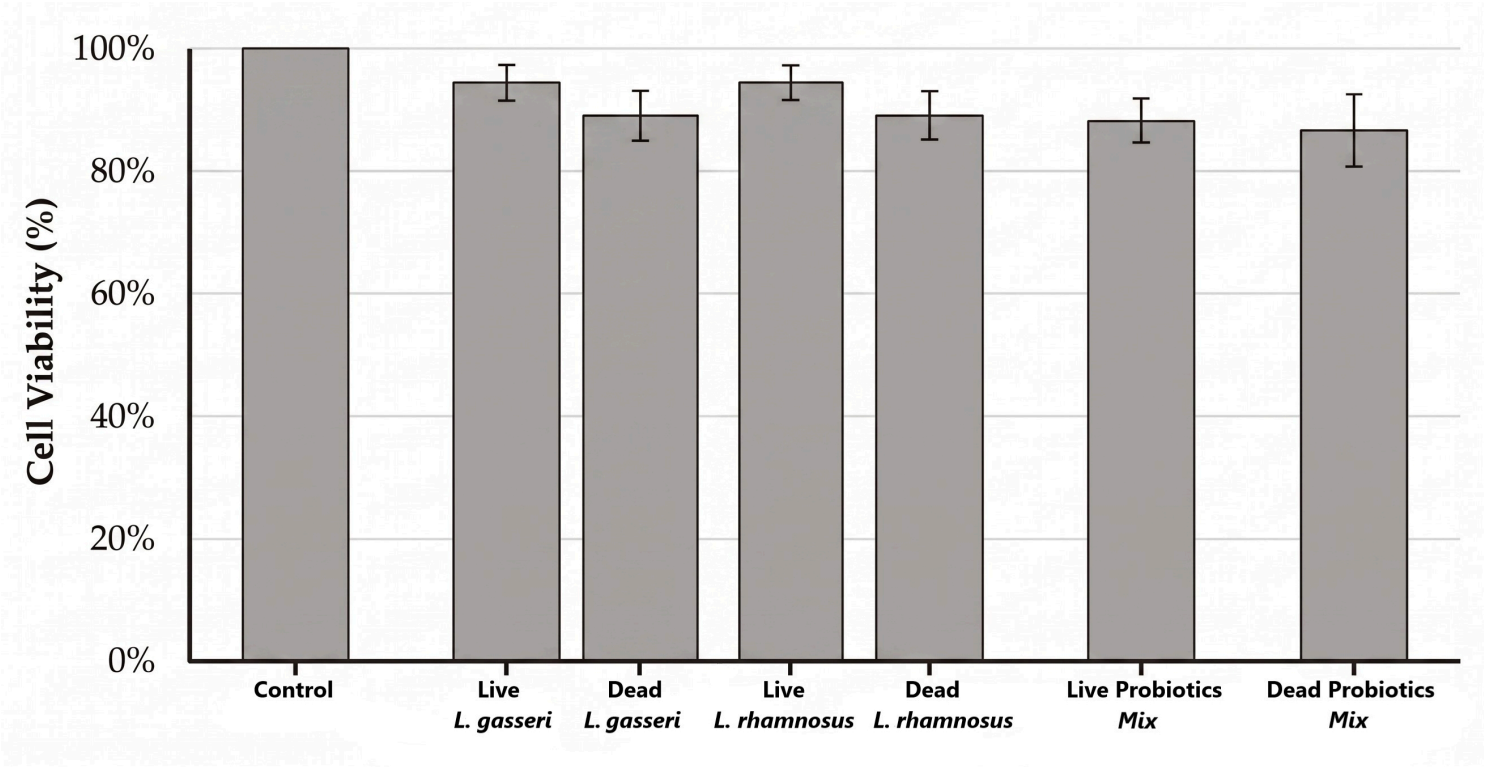


Figure 1

Cytotoxicity assessment of *Lactobacillus* strains on THP-1 macrophages. Cell viability was evaluated using the MTT assay after 24 hours of co-culture. THP-1 cells (at ~60-70% confluence) were treated with Live and Heat-Killed (Dead) forms of *L. gasseri*, *L. rhamnosus*, and their mixture at a multiplicity of infection (MOI) of 10:1. The viability of all treated groups remained above 80%, indicating no significant cytotoxic effects compared to the untreated control. Data are presented as mean ± SD of three independent experiments.

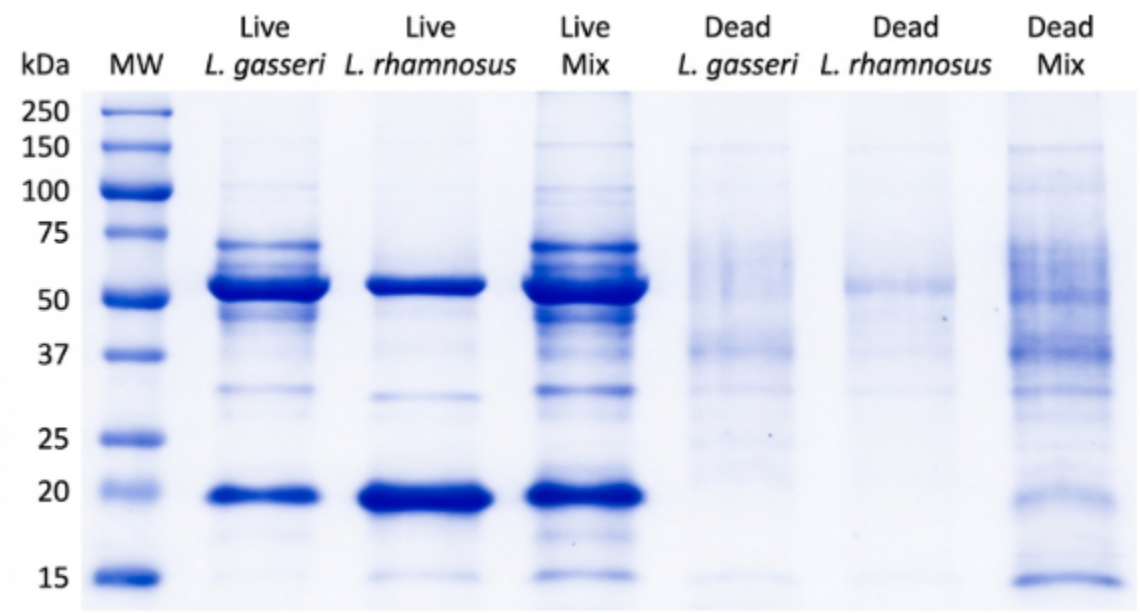


Figure 2

SDS-PAGE protein profiling and structural integrity of Live versus Inactivated (Dead) probiotic strains. The molecular weight (MW) marker indicates protein sizes in kDa. Live *L. gasseri*, *L. rhamnosus*, and the Live Mix display distinct, intact protein profiles characterized by major dominant bands at ~52–55 kDa and ~19–20 kDa (potentially representing critical surface layer proteins or structural PAMPs), along with minor bands at 65–70 kDa and 30–32 kDa. In contrast, the Inactivated (Dead) groups exhibit severe protein denaturation and degradation. This is evidenced by the disappearance of the major structural bands and the emergence of protein smearing, particularly notable in the Dead Mix lane. The physical degradation of these proteins and the subsequent loss of three-dimensional surface epitopes mechanistically explain the altered TLR-mediated recognition and the differential IL-10 immunomodulatory responses observed in THP-1 macrophages.

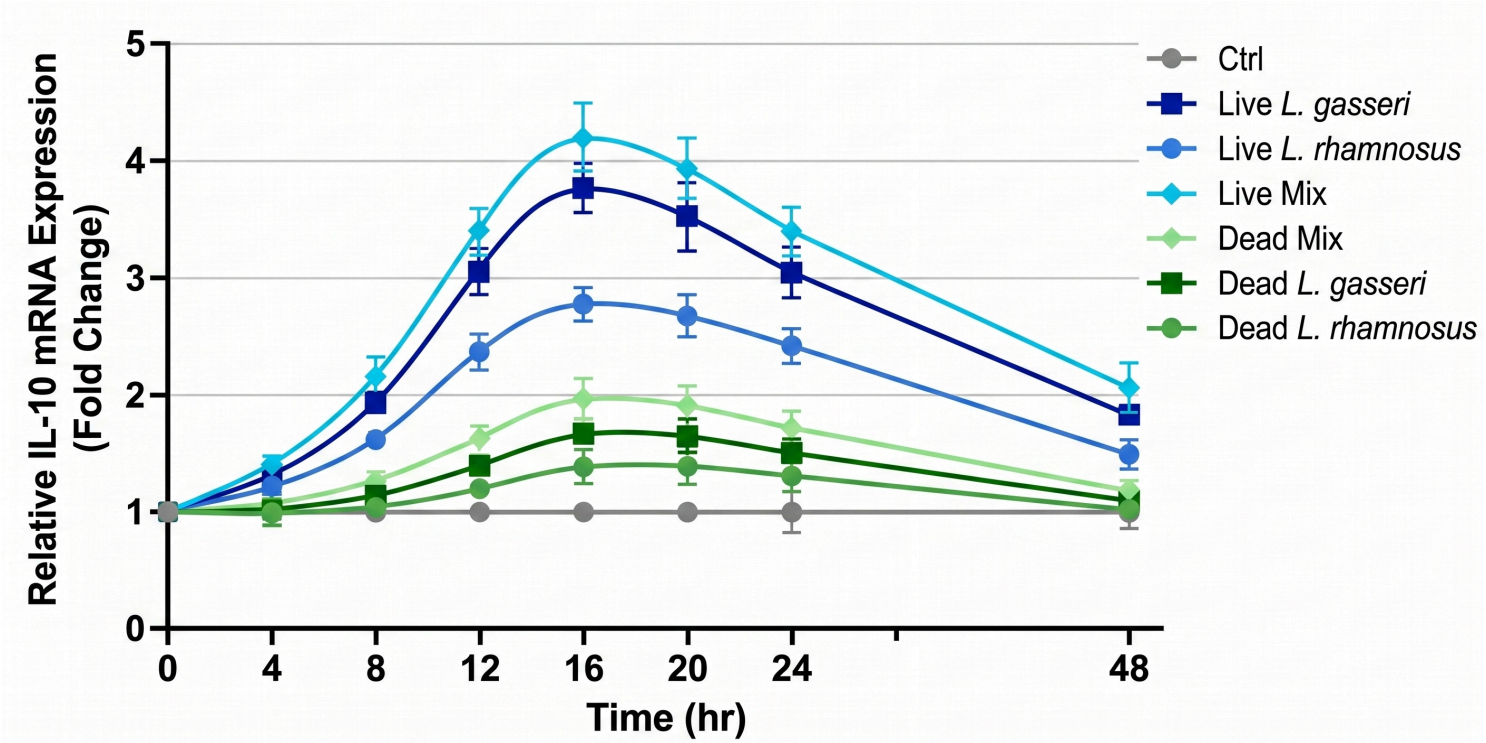


Figure 3

Kinetics of *IL-10* mRNA expression in THP-1 macrophages over time. Cells were co-cultured with Live and Heat-Killed (Dead) probiotic strains (MOI 10:1) over a 48-hour period. Relative gene expression (Fold Change) was calculated using the $2^{-\Delta\Delta Ct}$ method, normalized to the internal control (e.g., *GAPDH*), and calibrated against the untreated baseline (Ctrl = 1). The peak of *IL-10* transcriptional activity was consistently observed at 16 hours post-treatment, with the Live Mix group demonstrating the most potent upregulation.

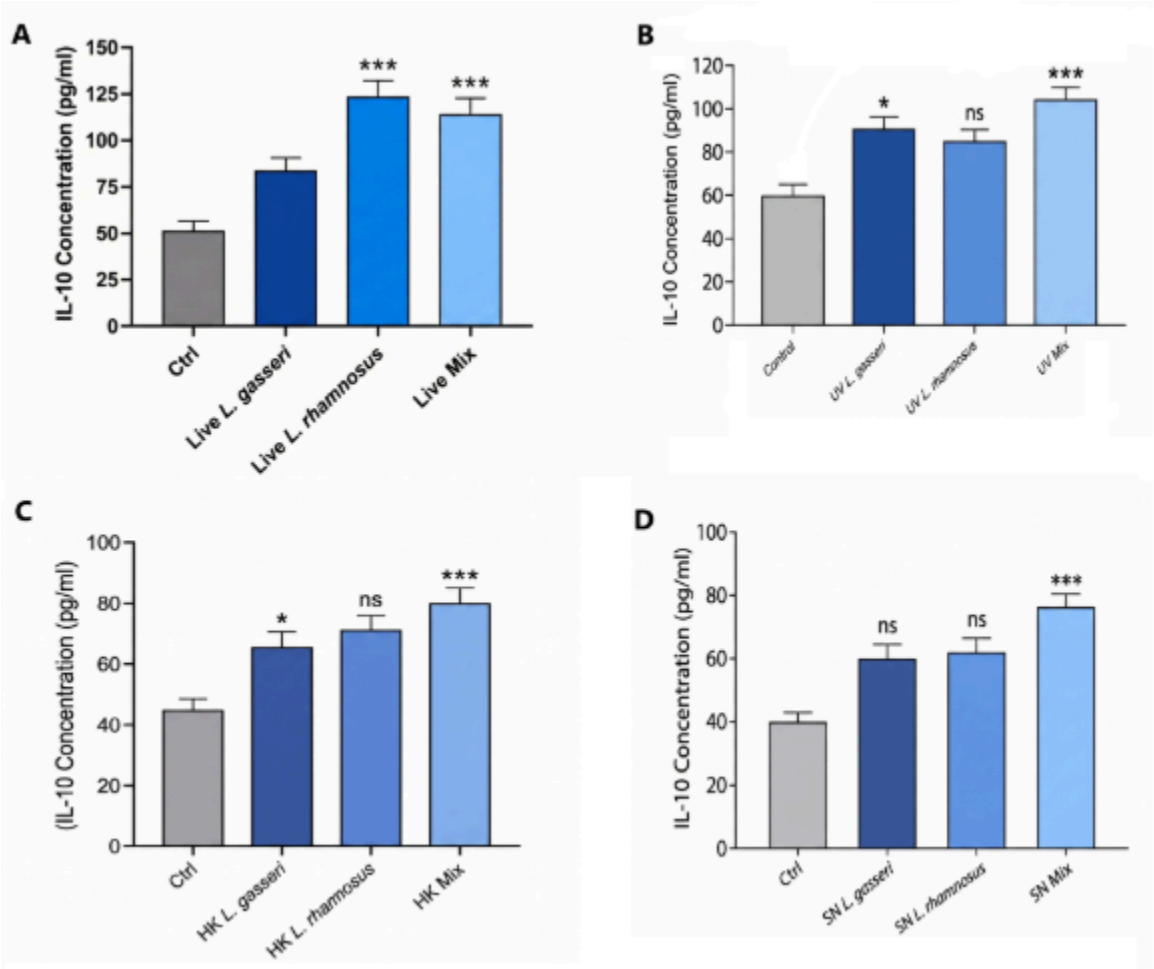


Figure 4

Quantitative analysis of IL-10 cytokine secretion in response to various bacterial inactivation methods. ELISA was performed on THP-1 cell culture supernatants 24 hours post-treatment (MOI 10:1). The panels demonstrate the immunomodulatory potential of: **(A)** Live probiotics, exhibiting the highest IL-10 induction; **(B)** UV-inactivated strains, maintaining high secretion levels due to intact cell wall structures; **(C)** Heat-Killed (HK) strains; and **(D)**Sonicated (SN) lysates. The Live Mix group consistently acted as the most potent inducer of anti-inflammatory responses. Data are shown as mean ± SD (*p < 0.05, ***p < 0.001, ns = non-significant compared to the control group).

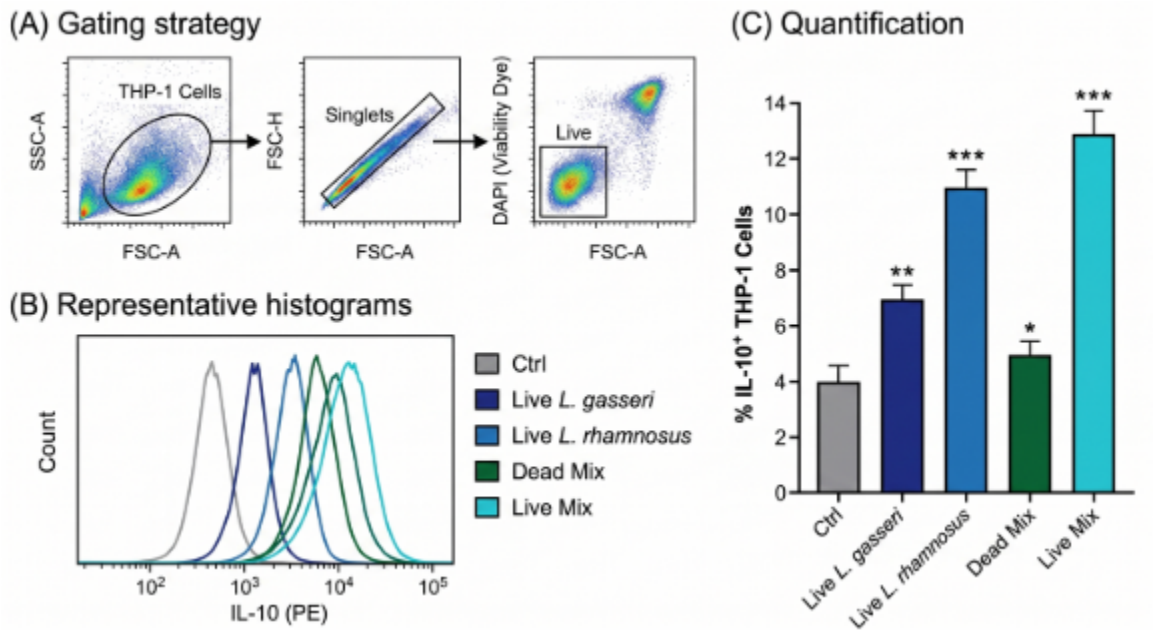


Figure 5

Flow cytometric analysis of intracellular IL-10 production in THP-1 cells. (A) The gating strategy employed to identify live singlet macrophages, utilizing DAPI for live/dead discrimination. **(B)** Representative staggered histograms displaying IL-10 (PE) fluorescence intensity across different treatments. **(C)** Quantitative bar graph indicating the percentage of IL-10-positive THP-1 cells. The Live Mix formulation induced the highest frequency of IL-10 expressing cells. Statistical significance was determined compared to the untreated control (*p < 0.05, p < 0.01, ***p < 0.001).