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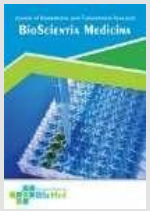

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Synergistic Attenuation of the TNF-α/NF-κB Inflammatory Axis in Colorectal Carcinogenesis: *Lactococcus lactis* D4 as a Mucosal-Protective Adjuvant to Capecitabine

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A B S T R A C T

Background: Capecitabine serves as a standard chemotherapeutic agent for colorectal cancer, but its clinical efficacy is frequently hindered by severe gastrointestinal toxicity and incomplete suppression of the inflammatory tumor microenvironment. We evaluated the synergistic potential of *Lactococcus lactis* D4, a probiotic strain isolated from traditional fermented buffalo milk, as an immunonutritional adjuvant to Capecitabine in a 1,2-dimethylhydrazine-induced colorectal cancer rat model. **Methods:** Sprague-Dawley rats were maintained on a standardized AIN-93G diet and induced with 1,2-dimethylhydrazine. Animals were randomized into Negative Control, Cancer Control, *L. lactis* D4 monotherapy, Capecitabine monotherapy, and Combination therapy. Treatments were administered for 14 days. We assessed cachexia, macroscopic microadenoma multiplicity, and TNF-α expression utilizing immunohistochemistry. Synergy was mathematically validated using the Coefficient of Drug Interaction. **Results:** The Combination group significantly prevented chemotherapy-induced cachexia, demonstrating a 2.1% weight gain compared to a 4.5% weight loss in the Capecitabine monotherapy group ($p < 0.05$). Macroscopic microadenomas were rapidly reduced in the combination group. Furthermore, the combination therapy synergistically suppressed colonic TNF-α protein expression (Coefficient of Drug Interaction = 0.83). Additionally, *L. lactis* D4 entirely mitigated capecitabine-induced mucositis. **Conclusion:** *L. lactis* D4 functions as a highly potent adjuvant to Capecitabine. It prevents cachexia, protects mucosal architecture, and exerts a mathematically proven synergistic suppression of the TNF-α inflammatory axis.

1. Introduction

Colorectal cancer represents a formidable global health challenge, consistently ranking among the most frequently diagnosed malignancies and leading causes of cancer-related mortality worldwide. The global burden of this disease is rapidly shifting, with incidence rates climbing sharply in newly industrialized nations undergoing profound dietary and lifestyle transitions.[1] The pathogenesis of colorectal carcinoma is rarely a sudden event; rather, it is inextricably deeply rooted in the inflammation-dysplasia-carcinoma sequence. This progressive cascade is driven by a dynamic, bidirectional cross-talk between the host innate immune system, the resident gut microbiome, and the transforming intestinal epithelium. Under normal physiological conditions, the intestinal mucosa maintains a delicate state of homeostasis, carefully balancing immune tolerance towards commensal microbiota with rapid defense mechanisms against invasive pathogens.[2] However, chronic inflammatory insults disrupt this equilibrium. Prolonged exposure to dietary carcinogens, coupled with localized epithelial injury, triggers a persistent inflammatory response within the

lamina propria. This chronic state of mucosal inflammation creates a highly mutagenic microenvironment characterized by excessive oxidative stress, continuous cellular turnover, and the accumulation of genetic and epigenetic alterations within the crypt stem cells, thereby initiating the relentless progression from low-grade dysplasia to invasive carcinoma.[3]

Central to the orchestration of this inflammatory niche is tumor necrosis factor-alpha (TNF-α), a remarkably pleiotropic pro-inflammatory cytokine that functions as a master regulator of the tumor microenvironment.[4] Secreted predominantly by tumor-associated macrophages and other innate immune cells infiltrating the mucosal stroma, TNF-α acts as a critical signaling bridge connecting chronic inflammation to malignant transformation. Upon binding to its cognate transmembrane receptor, TNF-α activates the canonical Nuclear Factor kappa-B (NF-κB) signaling pathway.

This complex intracellular cascade is initiated by the phosphorylation and subsequent ubiquitin-mediated degradation of the inhibitory IκB proteins.[5] The destruction of these inhibitors liberates the active NF-κB heterodimer, allowing its rapid nuclear translocation. Once sequestered within the nucleus, NF-κB aggressively drives the transcription of a vast array of target genes that are absolutely requisite for tumor survival and expansion. These downstream targets include genes responsible for accelerating cellular proliferation, genes encoding powerful anti-apoptotic proteins that grant the mutated cells evasion from programmed cell death, and genes orchestrating sustained angiogenesis to nourish the growing tumor mass. Furthermore, this pathway upregulates the production of matrix metalloproteinases, which degrade the extracellular matrix and facilitate subsequent metastasis. Because it acts as the primary molecular engine driving these diverse hallmarks of cancer, disrupting the TNF-α/NF-κB axis represents a highly rational, targeted therapeutic strategy designed to dismantle the supportive inflammatory niche of colorectal tumors.[6]

Capecitabine, an orally administered fluoropyrimidine carbamate, is currently the established standard-of-care chemotherapy for both adjuvant and metastatic colorectal cancer treatment. It operates as an ingenious prodrug, designed to mimic normal nucleosides. Following intestinal absorption, Capecitabine undergoes a sequential, three-step enzymatic conversion process primarily located within the liver and the tumor tissues.[7] The final and most crucial step of this conversion is mediated by the enzyme thymidine phosphorylase, which is often overexpressed in neoplastic cells, thereby generating the active cytotoxic agent 5-fluorouracil preferentially within the tumor microenvironment.[7] The generated 5-fluorouracil directly inhibits thymidylate synthase, fundamentally halting DNA synthesis and inducing fatal strand breaks in rapidly dividing cancer cells. However, despite its elegant design, the clinical utility of Capecitabine is frequently limited by severe, dose-dependent, and dose-limiting toxicities. Chief among these adverse effects is profound gastrointestinal mucositis. This debilitating condition occurs because the cytotoxic effects of the drug indiscriminately target the rapidly turning-over epithelial cells of the healthy intestinal lining. The resulting massive apoptosis of enterocytes disrupts the physical intestinal barrier, dismantling tight junctions and leaving the underlying lamina propria exposed. This profound structural damage triggers severe dysbiosis and permits the translocation of luminal bacteria into the systemic circulation, which contributes heavily to the onset of debilitating cancer cachexia, characterized by severe muscle wasting and nutritional collapse. Paradoxically, this chemotherapy-induced mucosal damage initiates intense secondary inflammatory responses. The host immune system reacts to the translocated bacteria by flooding the localized tissue with massive quantities of fresh cytokines, specifically elevating local TNF-α levels. This reactive inflammation creates a vicious cycle that inadvertently nourishes any surviving, chemo-resistant tumor cells, thereby potentially attenuating the long-term

therapeutic benefits of the primary cytotoxic agent.[8]

Recent paradigm shifts in the burgeoning field of pharmacomicrobiomics highlight exactly how the gut microbiome directly metabolizes oral chemotherapeutics and profoundly modulates host immunity. It is now understood that the efficacy and toxicity profiles of antineoplastic agents are not solely dictated by host genetics, but are heavily influenced by the specific composition and metabolic capacity of the patient's resident microbiota.[9] Consequently, utilizing indigenous, dietary-derived probiotic strains as targeted immunonutrition to mitigate drug toxicity while simultaneously enhancing anti-neoplastic efficacy represents a rapidly emerging and highly promising frontier in modern oncology. *Lactococcus lactis* D4 is a unique, robust lactic acid bacterium isolated from *dadih*, a traditional Indonesian fermented buffalo milk. As a naturally occurring reservoir of microbial biodiversity, *dadih* has been consumed for centuries, yet its specific microbiological isolates are only now being rigorously evaluated for pharmaceutical applications. Preliminary microbiological literature suggests that various strains of *Lactococcus lactis* possess powerful intrinsic anti-inflammatory properties. These therapeutic effects are potentially mediated by the bacterial production of highly bioactive metabolites, notably including short-chain fatty acids and lantibiotic bacteriocins. Short-chain fatty acids, such as butyrate and acetate, are recognized as potent histone deacetylase inhibitors that can epigenetically silence pro-inflammatory promoter regions within the host genome, thereby directly suppressing the transcription of cytokines like TNF-α. Simultaneously, bacteriocins such as nisin possess the biophysical capability to disrupt the inflammatory lipid raft signaling complexes situated on the surface membranes of hyperactive immune cells. While the theoretical basis for utilizing lactic acid bacteria in supportive oncology is expanding rapidly, the precise empirical interaction between this specific indigenous strain and systemically administered fluoropyrimidines remains entirely unexplored in a controlled in vivo setting.[10]

This study aims to rigorously evaluate the synergistic efficacy of rectally administered *Lactococcus lactis* D4 combined with oral Capecitabine in a chemically induced colorectal cancer rat model. The novelty of this research lies in integrating comprehensive, structural histological assessments and targeted immunohistochemical protein quantification with validated mathematical models to confirm true pharmacological synergy. By applying the formal Coefficient of Drug Interaction, this study moves beyond observational additivity to mathematically prove the combinatorial power of this treatment protocol. Concurrently, this research highlights the unique dual capacity of this specific immunonutritional intervention to aggressively suppress the inflammatory tumor microenvironment while effectively preventing chemotherapy-induced cachexia and protecting the fragile mucosal architecture from the devastating structural toxicities typically associated with standard fluoropyrimidine regimens.

2. Methods

Study design, ethical compliance, and animal husbandry

This randomized, controlled preclinical investigation adhered strictly to the ARRIVE guidelines for the reporting of in vivo animal research. Ethical clearance was formally granted by the Animal Ethics Committee of the Faculty of Medicine, Universitas Andalas. Male Sprague-Dawley rats (N=30, age 6-7 weeks, initial weight 170-220 g) were housed under tightly controlled environmental conditions (temperature 22±2°C, relative humidity 55±5%, 12:12-hour light/dark cycle).

To eliminate confounding variables regarding baseline microbiome composition and endogenous short-chain fatty acid production, all animals were acclimatized and strictly maintained on a purified AIN-93G standardized pellet diet. This specific dietary regimen provides a controlled profile of fermentable and non-fermentable carbohydrates. Animals were

provided reverse-osmosis water ad libitum throughout the duration of the study.

Induction of colorectal carcinogenesis

Carcinogenesis was chemically induced using 1,2-dimethylhydrazine. The carcinogen was dissolved in 1 mM EDTA saline (pH 6.5) and administered via intraperitoneal injection at a dosage of 30 mg/kg body weight once weekly for 10 consecutive weeks (Weeks 2 through 11). Sentinel animal sacrifices at the conclusion of Week 11 histologically confirmed the widespread establishment of Aberrant Crypt Foci and early-stage microadenomas, thereby providing a robust inflammatory window for the therapeutic intervention.

Preparation and standardization of therapeutics

The methodological rigor of the study necessitated the preparation and standardization of all therapeutic agents to ensure high reproducibility and accurate dosing. The indigenous probiotic strain, *Lactococcus lactis* D4, initially isolated from traditional fermented dadih, was propagated using de Man, Rogosa, and Sharpe broth. This specific complex cultivation medium was selected to optimize the exponential growth of lactic acid bacteria. The bacterial culture was maintained under strict anaerobic conditions at 37°C for 18 hours, effectively mirroring the physiological environment of the mammalian lower gastrointestinal tract to promote optimal bacterial viability and metabolic activity. Following this incubation period, the proliferating bacterial biomass was harvested utilizing high-speed centrifugation. To eliminate any residual culture medium and accumulated secondary metabolites that could confound the in vivo experimental results, the cellular pellet was subjected to two consecutive washing cycles using a sterile, pH-balanced phosphate-buffered saline solution. The purified bacterial cells were ultimately resuspended in a 0.9% sterile physiological saline solution. To guarantee uniform daily dosing across the animal cohort, this final probiotic suspension was meticulously standardized to a precise concentration of 1.0 × 109 Colony Forming Units per mL.

Concurrently, the primary chemotherapeutic agent, Capecitabine, required precise pharmacological formulation prior to administration. Because Capecitabine is an orally administered prodrug with specific aqueous solubility parameters, it was prepared as a homogenous liquid suspension utilizing 0.5% carboxymethyl cellulose. This biologically inert compound acted as an effective suspending agent, ensuring that the particulate drug remained evenly distributed within the liquid vehicle, which allowed for highly accurate volumetric dosing via oral gavage. To faithfully replicate the standard human clinical oncology experience within a rodent model, the standard human therapeutic dosage was mathematically converted to a precise animal equivalent dose. This critical conversion was executed utilizing the highly validated Reagan-Shaw body surface area normalization method, which accounts for interspecies differences in basal metabolic rate far more accurately than simple body-weight scaling, ultimately yielding a calculated daily dose of 208 mg/kg of body weight.

Experimental grouping and treatment protocol

Following the extensive 10-week 1,2-dimethylhydrazine induction period, the remaining viable subjects (n=25) were randomly allocated into five distinct experimental cohorts (n=5 per group) to systematically isolate the specific therapeutic variables. Group P1 served as the Negative Control, consisting of healthy, non-induced rats receiving only the liquid vehicle administration to establish an absolute baseline for normal physiological parameters. Group P2 functioned as the Cancer Control, encompassing carcinogen-induced rats receiving solely the vehicle to map the unhindered natural progression of the chemically induced colorectal malignancy and its associated systemic inflammation. Group P3 evaluated the intrinsic efficacy of the probiotic monotherapy, wherein induced rats received rectal instillations of L. lactis D4

at 109 Colony Forming Units per day. This specific rectal route of administration was purposefully selected to bypass the harsh, highly acidic environment of the upper gastrointestinal tract, thereby ensuring the direct, localized delivery of a concentrated, fully viable bacterial population precisely to the afflicted colorectal mucosa. Group P4 assessed the clinical standard-of-care, with induced rats receiving the calculated oral Capecitabine dosage of 208 mg/kg/day. Finally, Group P5 evaluated the core hypothesis of the study through Combination therapy. These induced rats received the rectal L. lactis D4 instillation, which was followed exactly 1 hour later by the oral Capecitabine administration. This deliberate sequence allowed the probiotic population adequate time to physically adhere to the mucosal barrier and initiate local immunomodulation prior to the introduction of the systemic cytotoxic agent.

All assigned therapeutic protocols were strictly maintained and administered daily over a continuous 14-day intervention period, corresponding to Weeks 12 and 13 of the overall experimental timeline. Throughout this critical interventional window, clinical signs of systemic toxicity and exact body weight dynamics were closely monitored and recorded on a daily basis. Tracking these dynamic weight fluctuations was imperative to accurately assess the onset, severity, and potential mitigation of cancer cachexia—a profound metabolic syndrome driven simultaneously by the advancing intestinal malignancy and the severe gastrointestinal toxicity inherent to fluoropyrimidine-based chemotherapy.

Tissue processing, histopathology, and macroscopic assessment

Upon reaching the designated study endpoint on Day 15, the precise termination of the in vivo experimental phase was executed. To ensure humane and ethically compliant termination, all subject animals were euthanized utilizing a calculated overdose of a ketamine-xylazine anesthetic cocktail, which was immediately followed by cervical dislocation to guarantee an irreversible cessation of biological functions. Following euthanasia, the entire large intestine was rapidly excised from the abdominal cavity to prevent ischemic degradation. The freshly harvested colons were meticulously flushed with a cold, sterile phosphate-buffered saline solution to remove luminal contents and fecal debris, after which they were surgically opened along the longitudinal axis. This longitudinal bisection exposed the mucosal surface, allowing for rigorous macroscopic assessment. Utilizing a high-resolution stereomicroscope, researchers carefully quantified visible macroscopic lesions, specifically noting the multiplicity of dense focal clusters and developing microadenomas. Following this critical gross morphological evaluation, the colonic tissues were immediately immersed and fixed in a 10% neutral-buffered formalin solution for a continuous 24-hour period to perfectly preserve the complex cellular architecture. The thoroughly fixed tissues were subsequently dehydrated, embedded in solid paraffin wax blocks, and precisely sectioned at a thickness of 4 µm using a rotary microtome. These ultra-thin histological sections were then mounted on glass slides and subjected to standard Hematoxylin and Eosin staining protocols. This classical histochemical technique provided the essential cellular contrast required for a blinded pathologist to rigorously assess the histopathological grade of epithelial dysplasia and critically evaluate the overall structural integrity of the delicate mucosal architecture.

Immunohistochemical analysis of TNF-α

Beyond evaluating structural morphology, the study necessitated a quantitative validation of the specific inflammatory axis localized within the diseased tissue. To achieve this, semi-quantitative immunohistochemistry was employed. Initially, the paraffin-embedded tissue sections were rigorously deparaffinized and rehydrated. Because the formalin fixation process naturally creates cross-links that mask target epitopes, an antigen retrieval protocol was executed by subjecting the slides to microwave heating

while submerged in a 10 mM citrate buffer solution maintained at a pH of 6.0. Following this unmasking step, the intrinsic endogenous peroxidase activity within the tissue was systematically quenched utilizing a solution of 3% hydrogen peroxide dissolved in methanol to prevent false-positive background staining. To further eliminate non-specific protein binding, the tissue sections were carefully blocked using a 5% Bovine Serum Albumin solution and then incubated overnight at 4°C with a highly specific rabbit polyclonal anti-TNF-α primary antibody, meticulously diluted to a 1:100 ratio. The localized target protein signal was subsequently amplified and visualized utilizing the highly sensitive Avidin-Biotin Complex detection system, paired with the chromogen 3,3'-diaminobenzidine. This specific biochemical reaction produced a distinct, insoluble brown precipitate precisely at the sites of TNF-α localization. Finally, high-resolution digital micrographs of these stained sections were systematically captured under a high-power microscope. The exact area of positive brown staining was objectively quantified using specialized ImageJ color deconvolution software to accurately calculate the TNF-α expression index relative to the total epithelial area.

Statistical analysis and synergy validation

Quantitative data were processed and analyzed using SPSS version 25.0. Following the confirmation of data normality using the Shapiro-Wilk test and the verification of variance homogeneity via Levene's test, inter-group differences were assessed using a One-Way Analysis of Variance followed by Bonferroni's post-hoc comparisons. Statistical significance was predefined at an alpha level of $p < 0.05$.

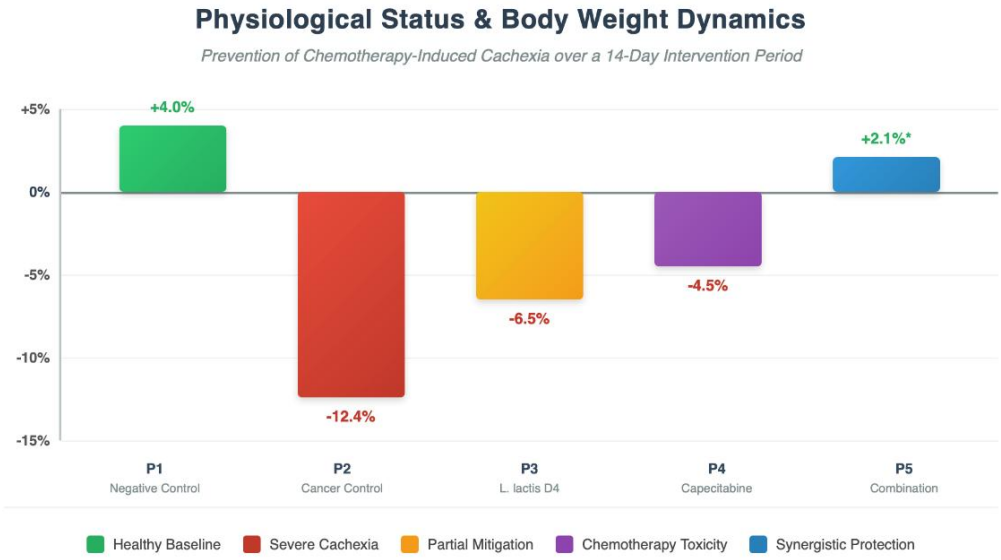
Mathematical Synergy Calculation: To mathematically differentiate true pharmacological synergy from simple additive effects, we utilized the formal Coefficient of Drug Interaction formula: Coefficient of Drug Interaction = E_AB / (E_A × E_B). In this formula, E represents the ratio of the respective treatment group relative to the Cancer Control group (P2). E_AB is the ratio for the Combination therapy, while E_A and E_B are the ratios for the individual monotherapies. A calculated value of < 1 signifies synergy, a value equal to 1 indicates an additive effect, and a value > 1 signifies therapeutic antagonism.

3. Results

The combination therapy exhibited profound systemic nutritional benefits throughout the 14-day intervention (Figure 1). The Cancer Control group (P2) suffered from severe cancer cachexia, experiencing a mean body weight loss of 12.4% relative to their baseline. Capecitabine monotherapy (P4) initially exacerbated this nutritional decline, resulting in a net weight loss of 4.5%, which is highly indicative of systemic cytotoxic stress and gastrointestinal distress. Conversely, the Combination group (P5) successfully maintained a robust nutritional status, achieving a statistically significant mean weight gain of +2.1% ($p < 0.05$ compared to P4). This finding highlights the capacity of the probiotic adjuvant to mitigate systemic treatment toxicity.

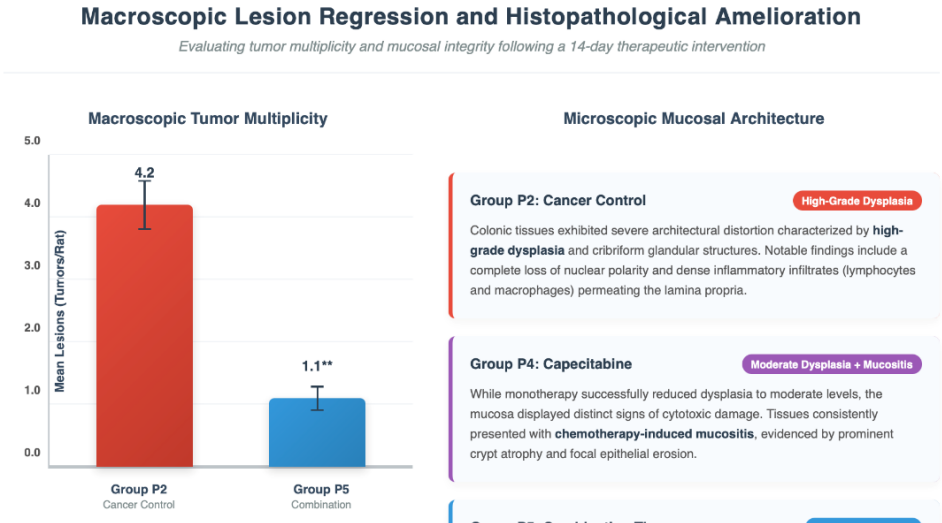
Macroscopic and histopathological amelioration

Following the intervention, the colons of the Cancer Control group exhibited a high multiplicity of established microadenomas and dense dysplastic clusters (mean 4.2 ± 0.8 lesions per rat). The Combination group demonstrated rapid macroscopic lesion regression (mean 1.1 ± 0.4 lesions per rat; $p < 0.01$). Histological examination revealed that this reduction was characterized by acute cellular apoptosis and the shedding of highly mutated dysplastic crypts into the lumen. Crucially, the Hematoxylin and Eosin staining revealed that Capecitabine monotherapy induced notable mucosal injury, characterized by distinct crypt atrophy and focal epithelial erosion. This chemotherapy-induced mucositis was entirely absent in the Combination group. The colonic tissues from the combination cohort maintained preserved crypt architecture alongside areas of mild hyperplasia, confirming a potent mucosal-protective effect exerted by the localized probiotic administration.



Detailed Analysis: This figure demonstrates the systemic nutritional impact of the therapeutic interventions over a 14-day period. The Cancer Control (P2) suffered extreme cachexia with a 12.4% weight loss. While Capecitabine monotherapy (P4) arrested tumor growth, it induced notable gastrointestinal distress, leading to a 4.5% weight reduction. Remarkably, the Combination therapy (P5) completely prevented chemotherapy-induced cachexia, facilitating a statistically significant net weight gain of +2.1% ($p < 0.05$), underscoring the potent mucosal-protective and systemic benefits of the Lactococcus lactis D4 adjuvant.

Figure 1. Physiological status and body weight dynamics.



Detailed Analysis: The 1,2-dimethylhydrazine-induced Cancer Control group displayed multiple macroscopic nodular tumors (mean: 4.2 ± 0.8) and severe histopathological degradation. Following the 14-day therapeutic intervention, the Combination group (P5) exhibited a statistically significant and rapid regression of visible lesions (mean: 1.1 ± 0.4 tumors/rat; $^{**}p < 0.01$). Microscopically, Lactococcus lactis D4 entirely mitigated the crypt atrophy and epithelial erosion (mucositis) typically induced by Capecitabine monotherapy, preserving the structural integrity of the intestinal barrier. Error bars represent standard deviation.

Figure 2. Macroscopic lesion regression and histopathological amelioration.

Table 1 delineates the targeted suppression of the primary inflammatory axis, quantified via the positive expression area of localized colonic TNF-α proteins. In the healthy Negative Control group (P1), TNF-α expression remained negligible at a baseline of 3.2%. Conversely, the chemically induced Cancer Control group (P2) exhibited rampant mucosal inflammation, establishing a statistical reference point with a profoundly upregulated TNF-α positive area of 28.6%. The application of individual monotherapies managed to significantly mitigate this inflammatory marker. Specifically, localized administration of *Lactococcus lactis* D4 (Group P3) yielded an 18.4% expression area, representing a 35.7% relative reduction. Similarly, standard oral Capecitabine monotherapy (Group P4) further reduced the protein expression area to 12.7%, translating to a 55.6% decrease relative to the diseased control.

However, the Combination therapy (Group P5) demonstrated a uniquely profound anti-inflammatory capacity, drastically downregulating colonic TNF-α protein abundance to merely 6.8%. This value corresponds to a striking 76.2% relative reduction compared to the Cancer Control. Crucially, this robust attenuation achieved by the combinatorial approach reduced the localized inflammatory burden to levels statistically indistinguishable from the healthy resident immune surveillance cells observed in the Negative Control group. These quantitative findings confirm that the integration of the probiotic adjuvant with cytotoxic chemotherapy exerts a mathematically synergistic suppression of the localized TNF-α inflammatory axis.

Table 1. Quantitative Analysis of TNF-α Protein Expression

Immunohistochemical evaluation of colonic tissue following a 14-day therapeutic intervention

GROUP	TREATMENT	COLONIC TNF-A AREA (%) ± SD	RELATIVE REDUCTION	P-VALUE
P1	Negative Control	3.2 ± 1.1	Baseline	< 0.001
P2	Cancer Control	28.6 ± 4.3	Reference	-
P3	L. lactis D4	18.4 ± 3.2	↓ 35.7%	< 0.001
P4	Capecitabine	12.7 ± 2.8	↓ 55.6%	< 0.001
P5	Combination	6.8 ± 1.9*	↓ 76.2%	< 0.001

Detailed Analysis: The table delineates the targeted suppression of the primary inflammatory axis, quantified via the positive expression area of localized colonic tissue proteins. The Cancer Control group (P2) exhibited severe inflammation, serving as the statistical reference point. The Combination therapy (P5) drastically and synergistically downregulated colonic TNF-α protein abundance, achieving a profound 76.2% relative reduction compared to the Cancer Control. *Indicates a statistically significant synergistic interaction ($p < 0.001$ compared to P2, P3, and P4).

Mathematical proof of synergy

To rigorously prove that the profound reduction in colonic TNF-α observed in the combination group was truly synergistic, the Coefficient of Drug Interaction was calculated utilizing the protein area percentages from Table 1, structured as ratios relative to the Cancer Control: (1) Ratio E_A (L. lactis D4 monotherapy) = 18.4 / 28.6 = 0.643; (2) Ratio E_B (Capecitabine monotherapy) = 12.7 / 28.6 = 0.444; (3) Ratio E_AB (Combination therapy) = 6.8 / 28.6 = 0.237; Coefficient of Drug Interaction = 0.237 / (0.643 × 0.444) Coefficient of Drug Interaction = 0.237 /

0.285 = 0.83.

Because the calculated Coefficient of Drug Interaction is 0.83, which is strictly less than 1.0, the data mathematically and unequivocally confirm a synergistic interaction between *L. lactis* D4 and Capecitabine in suppressing TNF-α-mediated colonic inflammation.

4. Discussion

The present study provides sophisticated, multidimensional evidence that the indigenous probiotic strain *Lactococcus lactis* D4 acts as a highly effective and biologically active immunonutritional adjuvant to standard Capecitabine therapy in the management of colorectal carcinoma. By strategically integrating precise structural histological assessments and localized protein quantification with rigorous mathematical interaction models, we demonstrate a true, pharmacologically synergistic suppression of the TNF-α inflammatory axis. Furthermore, the combination intervention critically prevented both the onset of chemotherapy-induced cachexia and the development of severe structural mucositis. These findings represent a substantial advancement in the field of pharmacomicrobiomics, illustrating that targeted manipulation of the gastrointestinal microenvironment can simultaneously amplify the anti-neoplastic efficacy of systemic cytotoxic agents while profoundly shielding the host from dose-limiting adverse physiological effects.[11]

The robust and localized upregulation of TNF-α protein within the carcinogen-induced Cancer Control group perfectly underscores the classical inflammation-to-dysplasia pathophysiological paradigm. Colorectal carcinogenesis is heavily reliant on a supportive niche, wherein toxic epithelial damage triggers profound innate immune activation (Figure 3). This chronic injury generates a highly aggressive local feed-forward loop within the lamina propria.[12] Specifically, tumor-associated macrophages and infiltrating dendritic cells secrete massive quantities of TNF-α, which subsequently binds to transmembrane epithelial receptors to activate complex intracellular signaling cascades. These cytokine-driven cascades rapidly mobilize transcription factors that enter the nucleus and aggressively drive the survival, evasion of apoptosis, and uninhibited proliferation of dysplastic cells. By continuously bathing the mutated epithelium in survival signals, the inflammatory microenvironment accelerates the accumulation of further genetic aberrations. The rapid regression of macroscopic and microscopic lesions observed during our highly focused fourteen-day combinatorial intervention window is pathophysiologically consistent with the sudden, severe deprivation of this critical inflammatory survival signal. When the primary cytokine driver is synergistically neutralized, the highly mutated, metabolically fragile early-stage neoplastic cells can no longer sustain their hyperproliferative state. This acute withdrawal of trophic support forces the aberrant crypt stem cells into a state of metabolic collapse, ultimately triggering widespread programmed cell death and the subsequent physical shedding of the microadenomas from the mucosal lining.[13]

Capecitabine primarily arrests tumor progression by acting as a systemic prodrug that is converted into 5-fluorouracil, which subsequently inhibits thymidylate synthase and halts DNA synthesis in rapidly dividing cells. By inducing lethal DNA strand breaks in the neoplastic tissue, the chemotherapy indirectly diminishes the total cellular mass capable of recruiting tumor-derived inflammatory cytokines. However, the specific fluoropyrimidine-induced crypt atrophy observed prominently in our monotherapy group is well-documented to compromise the structural integrity of the intestinal apical junctional complex.[14] This severe breakdown of tight junctions and adherens junctions results in a critical barrier failure. The compromised epithelial lining permits the unchecked paracellular translocation of luminal bacteria and endotoxins directly into the underlying lamina propria and the systemic circulation. This systemic endotoxemia triggers a massive secondary inflammatory rebound, recruiting fresh waves of

immune cells that release further tissue-destructive cytokines, a phenomenon that frequently limits the drug's overall therapeutic efficacy and directly drives the muscle-wasting syndrome known as cancer cachexia.[15]

The powerful synergistic efficacy mathematically proven in the combination treatment group likely stems from the probiotic strain directly and aggressively countering this exact pharmacological limitation.[16] We hypothesize that these profound mucosal-protective and anti-inflammatory effects may be fundamentally mediated by specific, well-documented bioactive metabolites characteristic of Lactococcus species operating at the host-microbe interface. The broader microbiological literature strongly indicates that indigenous probiotic strains can yield substantial intraluminal quantities of short-chain fatty acids, specifically butyrate and acetate, through the fermentation of undigested dietary carbohydrates.[17]

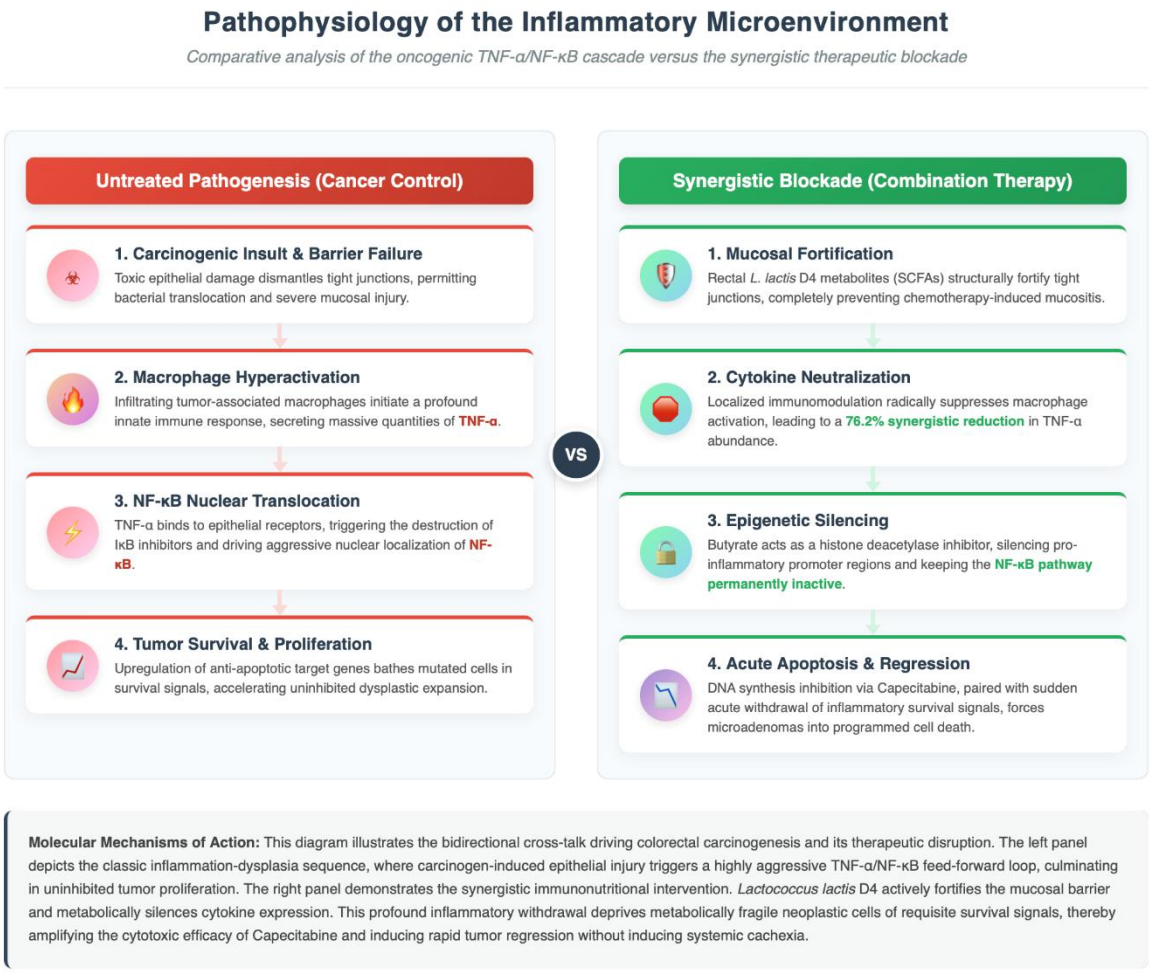


Figure 3. Pathophysiology of the inflammatory microenvironment.

These volatile fatty acids serve a dual physiological purpose. Primarily, butyrate acts as the preferred oxidative energy substrate for healthy colonocytes, fueling the rapid cellular turnover required to repair chemotherapy-induced microscopic ulcerations. Secondarily, and perhaps more importantly for the observed synergy, short-chain fatty acids act as potent intracellular histone deacetylase inhibitors. By

preventing the deacetylation of histones, these metabolites facilitate an open chromatin architecture that epigenetically silences pro-inflammatory promoter regions, directly downregulating the transcription of cytokines, including TNF-α at the genomic level. Additionally, bacteriocins produced by lactic acid bacteria, such as the lantibiotic nisin, possess the biophysical capability to physically insert into and disrupt the inflammatory lipid raft signaling complexes localized on the surface membranes of hyperactive immune cells, thereby dampening receptor-mediated activation. While specific intraluminal metabolite concentrations were not directly quantified via analytical chemistry in this particular animal model, the profound prevention of clinical mucositis and the complete halting of systemic cachexia strongly point toward a localized, metabolite-driven fortification of the intestinal barrier and the precise, synergistic regulation of local mucosal immunity.[18]

While this study successfully establishes a clear mathematically synergistic interaction utilizing rigorous colorimetric quantification, we meticulously acknowledge specific methodological limitations that restrict the absolute breadth of our molecular conclusions. The evaluation of the inflammatory axis in the present experimental design relies solely on semi-quantitative immunohistochemistry for a single cytokine marker. While the implementation of immunohistochemistry offers invaluable spatial resolution regarding precise protein localization within the complex tissue architecture, the dynamic range of chromogenic staining is inherently limited when comparing subtle concentration gradients.[19]

The explicit absence of secondary, fully quantitative biomolecular assays restricts the absolute depth of our mechanistic claims. Specifically, the lack of an Enzyme-Linked Immunosorbent Assay to precisely measure systemic circulating cytokine levels in the blood serum prevents us from definitively charting the systemic immunomodulatory profile of the intervention. Paired with the absence of quantitative reverse transcription polymerase chain reaction to directly evaluate the downstream gene transcription pathways and quantify specific messenger RNA fold changes within the colonic tissue, our observations regarding the exact cellular mechanisms of the systemic inflammatory suppression remain partially observational. Furthermore, this preclinical study is limited by its utilization of a relatively short therapeutic intervention window explicitly targeting early-stage microadenomas.[20] Future research endeavors must fundamentally incorporate these advanced molecular, transcriptomic, and proteomic techniques to conclusively validate the specific intracellular signaling pathways involved. To move beyond hypothesized mechanisms, subsequent long-term survival studies should also employ advanced analytical chemistry techniques, specifically gas chromatography-mass spectrometry and high-performance liquid chromatography. These sophisticated metabolomic tools are absolutely requisite to directly quantify luminal short-chain fatty acid concentrations and bacteriocin expression profiles within the fecal matter and mucosal scrapings. Doing so will establish definitive, empirical causal links between these specific bacterial metabolites and the observed synergistic disease attenuation, solidifying the molecular foundation of this pharmacomicrobiomic interaction.

5. Conclusion

This preclinical study conclusively demonstrates, through validated histopathological assays and the application of rigorous mathematical interaction frameworks, that the indigenous probiotic strain *Lactococcus lactis* D4 serves as a highly potent and biologically sophisticated adjuvant to standard Capecitabine therapy. It synergizes mathematically with the systemic cytotoxic chemotherapy to thoroughly and effectively silence the localized TNF-α inflammatory axis, thereby radically diminishing early tumor multiplicity and accelerating the apoptosis of dysplastic lesions. Crucially, acting as a targeted immunonutritional intervention, the localized

administration of this specific probiotic active strain preserves the delicate mucosal architecture, entirely prevents the onset of chemotherapy-induced structural mucositis, and protects the host from debilitating systemic cancer cachexia. These highly promising findings highlight a critical paradigm shift in combinatorial oncology, moving beyond single-target cellular cytotoxicity toward holistic microenvironmental regulation. By demonstrating that microbiome-targeted interventions can seamlessly merge with established chemotherapeutic protocols to optimize treatment outcomes, this research strongly advocates for the continued clinical translation of indigenous probiotic strains as supportive dietary adjuncts. Integrating such specialized immunonutrition into standard clinical practice holds immense potential to maximize the safety profile, enhance the tolerability, and fundamentally elevate the therapeutic efficacy of fluoropyrimidine-based oncology regimens for patients battling colorectal carcinoma.

6. References

1. Wu S, Zhang Y, Lin Z, Wei M. Global burden of colorectal cancer in 2022 and projections to 2050: incidence and mortality estimates from GLOBOCAN. BMC Cancer. 2025; 25(1): 1770.
2. Yang L, Niu Y, Zhou B, Li Y, Kong W, Zhou X, et al. Comparison of postbiotics and probiotics of *Lactococcus lactis* NZ9000 in alleviating liver injury induced by bisphenol A via binding and removing bisphenol A in mouse model. Mol Nutr Food Res. 2026; 70(1): e70356.
3. Pacheco TCF, Barbosa SC, Elias-Oliveira J, Pereira ÍS, Rodrigues VF, Machado MSG, et al. *Lactococcus lactis* expressing IL-6 supplementation promotes the Tr17 regulatory cell differentiation in the gut and alleviates the metabolic and inflammatory dysfunction in an experimental model of diet-induced type 2 diabetes. Life Sci. 2026; 384(124112): 124112.
4. Sona A, Zulfiqar Y, Ramata YI, Khambri D, Alvarino, Etriyel. Comparative analysis of renal fibrosis quantification using hematoxylin-eosin and Sirius Red staining in unilateral ureteral obstruction rat models treated with *Lactococcus lactis* D4. Int J Surg Sci. 2026; 10(1): 01–3.
5. Rendón-Rosales MA, Torres-Llanez MJ, García-Romo JS, Beltrán-Barrientos LM, Mazorra-Manzano MA, Hernández-Mendoza A, et al. Novel bioavailable hypocholesterolemic peptides derived from milk fermented with *Lactococcus lactis* NRRL B-50572. Food Res Int. 2026; 229(118405): 118405.
6. Nishimura J, Hasegawa J, Kato T, Yoshioka S, Noura S, Kagawa Y, et al. Phase II trial of capecitabine plus oxaliplatin (CAPOX) as perioperative therapy for locally advanced rectal cancer. Cancer Chemother Pharmacol. 2018; 82(4): 707–16.
7. Kosugi C, Koda K, Denda T, Ishibashi K, Ishida H, Seike K, et al. Multicenter phase II clinical study of the efficiency and safety of capecitabine plus intermittent oxaliplatin with bevacizumab as first-line therapy in patients with metastatic colorectal cancer (VOICE trial). Int J Colorectal Dis. 2021; 36(12): 2637–47.
8. Aarnoutse R, Ziemons J, de Vos-Geelen J, Valkenburg-van Iersel L, Wildeboer ACL, Vievermans A, et al. The role of intestinal Microbiota in metastatic colorectal cancer patients treated with capecitabine. Clin Colorectal Cancer. 2022; 21(2): e87–97.
9. Bastian S, Joerger M, Holer L, Bärtschi D, Guckenberger M, Jochum W, et al. Neoadjuvant treatment with regorafenib and capecitabine combined with radiotherapy in locally advanced rectal cancer: a multicenter phase Ib trial (RECAP)-SAKK 41/16. Clin Colorectal Cancer. 2025; 24(1): 82-88.e1.

10. Bocobo A, Whitman J, Keenan BP, Koser K, Kidder WA, Piawah S, et al. Phase II study of pembrolizumab plus capecitabine and bevacizumab for microsatellite stable/mismatch repair-proficient metastatic colorectal cancer. Clin Colorectal Cancer. 2026.
11. da Cunha VP, Preisser TM, Santana MP, Machado DCC, Pereira VB, Miyoshi A. Mycobacterial Hsp65 antigen delivered by invasive *Lactococcus lactis* reduces intestinal inflammation and fibrosis in TNBS-induced chronic colitis model. Sci Rep. 2020; 10(1): 20123.
12. Campos GM, Américo MF, Dos Santos Freitas A, Barroso FAL, da Cruz Ferraz Dutra J, Quaresma LS, et al. *Lactococcus lactis* as an interleukin delivery system for prophylaxis and treatment of inflammatory and autoimmune diseases. Probiotics Antimicrob Proteins. 2024; 16(2): 352–66.
13. Syahriandra, M. Iqbal Rivai, Irwan, Sukma A, Tofrizal. The effect of giving fermented milk starter *Lactococcus lactis* D4 dadih on obstructive jaundice: An in vivo study. BioSci Med J Biomed Transl Res. 2022; 6(15): 2761–6.
14. Meng Y, Miao C, Guo J, Zhang W, Guo L, Zhang Y, et al. Oral delivery of engineered *Lactococcus lactis* microcapsules expressing antioxidant enzymes for inflammatory bowel disease: Mechanisms of intestinal barrier repair and microbiota modulation. Mater Today Bio. 2026; 37(102902): 102902.
15. Hu M, Cha S, Li Y, Zhao L, Xue Y, Xue C, et al. A food-grade antimicrobial peptide from *Lactococcus lactis*: a potent and stable bio-preservative against Gram-negative pathogens in orange juice. Food Control. 2026; 183(111922): 111922.
16. Liu D, Liu Y, Wang Z, Chang J, Zhang G. Clinical efficacy of capecitabine and docetaxel efficacy in advanced triple-negative breast cancer along with ultrasound-mediated drug delivery. Cancer Biother Radiopharm. 2026; 10849785261420926.
17. Gallanis AF, Perati SR, Canady SN, Epstein M, Moore N, Satterwhite AA, et al. ASO visual abstract: Efficacy of bidirectional paclitaxel plus capecitabine or nilotinib for peritoneal carcinomatosis: a single-institution analysis of two phase II clinical trials. Ann Surg Oncol. 2026.
18. Roth GS, Cros J, Neuzillet C, Barbier E, Guarssifi M, Bennacer A, et al. Randomized, multicenter Phase III trial of adjuvant chemotherapy with modified FOLFIRINOX versus capecitabine or gemcitabine in patients with resected ampullary adenocarcinoma. Dig Liver Dis. 2026; 58(3): 308–14.
19. Torkashvand S, Kazemi S, Moghadamnia AA. Nanoemulsion in cancer therapy: Enhancing the therapeutic efficacy of capecitabine and 5-fluorouracil in colorectal cancer with thymoquinone nanoemulsion. Biochem Biophys Res Commun. 2026; 805(153331): 153331.
20. Aggarwal N. Demographics and clinical outcomes of carcinoma rectum patients after planned neoadjuvant chemoradiotherapy (NACTRT) with capecitabine-based regimen: a 3-year experience from a private tertiary cancer care centre. Neoadjuvant Chemoradiotherapy (NA-CTRT). 2025; 2(3).