



Gut Feeling or Good Science: The Impact of Probiotics on Inflammation

Arianna Theleman¹, S. Thomas¹, and JF Trembl^{1†}, M. Daggett¹, B. Mattingly¹

Probiotic use has been promoted for numerous health benefits, however, there is limited research supporting the claims for reduced inflammation. We set out to determine if there was a correlation between probiotic exposure and inflammatory signaling in immune cells, comparing the changes in pro-inflammatory and anti-inflammatory cytokine production. Pre-inflamed macrophages were treated with the probiotic strain *Lactococcus lactis*, isolated from a probiotic yogurt culture and identified using 16S rRNA sequencing, to determine the immune response produced when introduced to the probiotic. RNA was isolated from macrophages and cDNA was generated to detect a change in cytokine transcription through RT-qPCR, and ELISA was used to measure secreted cytokines associated with inflammation to assess the relative abundance in response to the addition of a probiotic. Results were analyzed to determine if there was a change in the inflammatory response of immune cells when presented with a probiotic finding that there may be time dependent modulatory changes in inflammatory cytokine signaling.

Pseudoscience has been of great interest to researchers and has captivated individuals through highly desirable claims of healing and relief for hundreds of years. However, some of these claims come with exaggerated benefits and false scientific legitimacy, and these ‘quick-fix’ products undermine the initiatives of long-term behavioral health. Products that have been widely popular in recent past include ear candles, DMSO, and feet pads, to name a few. These products were marketed for their abilities to remove toxins from the body, help relieve pain, and even possibly cure cancer, although there was never any solid scientific evidence to back these claims. It is easy to look at these products and trends and laugh, but what about products that may be more difficult to distinguish. They aren’t obvious scams, but there is also not a lot of evidence to support them either.

Microbiome health has been a focus of many studies in recent years, and researchers have found that healthy and balanced microbiomes can lead to improved digestion and nutrient absorption, help reduce inflammation, support immune systems in identifying and fighting off harmful pathogens, and even has associations with men-

tal health issues when unbalanced.^{1,2} One product that has been highly popularized on the market for its potential benefits to gut health, reduced inflammation, improved immunity and mental health boosts are probiotics.^{3,4} Probiotics are live microorganisms that provide benefits to their host.^{5,6} Probiotics claim to help with restoring microbiomes to normal levels, improve symptoms of inflammation by lowering/improving cytokine signaling, *etc.*^{7,8} They are found in so many of the products available for us to consume; yogurt, supplements, kombucha, kefir, and many more.^{9,10} While science does support the importance of a healthy microbiome, how much does the consumption of probiotics actually help? Are these benefits due to the probiotic, or an already healthy microbiome?¹¹

One of the more popular claims for probiotic use is to reduce inflammation. In circumstances of chronic inflammation, serious damage can occur due to high levels of cytokine signaling and probiotics are said to help reduce these pro-inflammatory signals.¹² However, minimal research provides a lack of supporting evidence to these claims of reduced inflammation. The results that these studies have published are inconsistent and lack statistical significance.^{13,14,15} The goal for this research project was to look at the modulatory effects probiotics have on inflammatory signals released by immune cells when

interacting with each other in the same environment by measuring the production of inflammatory cytokines by macrophages. By culturing macrophages, immune cells that contribute substantially to inflammatory responses in the gut, and measuring their cytokine release after exposure to a probiotic under inflammatory conditions, we aim to determine whether the probiotic produces measurable anti-inflammatory effects. If the claims are true, it is expected to see a statistically significant reduction of pro-inflammatory cytokines, as well as an upregulation of anti-inflammatory cytokine signaling reflecting a reduced state of inflammation. Further testing will be conducted to test the cells’ response to the probiotic introduced in different forms, i.e. live, heat killed, or an acellular culture supernatant. It is of interest to determine if there is a change in the benefit received when the probiotic is dead versus alive, and to understand if this impacts the immune response produced by our macrophages.

Materials and Methods

Growth of Probiotic from Serial Dilutions of yogurt

Growth plates were made using Lactobacilli MRS Agar (Becton, Dickinson, Lot 5293921) or LB agar media (Fisher Scientific, Lot 242143). For the MRS plates

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ultrapure water was added and media was autoclaved before plates were poured. The LB plates were made with LB agar and ultrapure water, adjusted to pH 7.2, then autoclaved using the liquid 15 setting on the autoclave. Once media finished in the autoclave, it was removed, allowed to cool slightly, then plates were poured aseptically to about 20 mLs of media per plate, cooled overnight and left to sit for 48 hours before use. Probiotic yogurt samples were diluted by a 10-fold dilution series from 1:10 to 1:100000. 100 μ L of each dilution was added to an agar plate and aseptically spread across surface with glass spreader. These plates were then incubated at 37 $^{\circ}$ C for 48 hours. Colonies were picked and streak plates made to isolate bacteria strains. Repeat process of making streak plates 2-3 times to be sure that isolated colonies only contain one bacterial strain.

PCR Amplification of 16S rRNA gene for probiotic identification

Using colonies from streak plates, isolate DNA using a DNA Isolation protocol. Pick a colony of bacteria and resuspend in 1 mL of dH₂O. Centrifuge for one minute at 12000x g, saving pellet and discarding the supernatant. Add 200 μ L InstaGene Matrix (BioRad, batch# 64573327) to the pellet and incubate at 56 for 30 minutes. Vortex cells at high speed for 10 seconds, and incubate at 100 $^{\circ}$ C for 8 minutes. Vortex again at high speed for 10 seconds and then centrifuge for 3 minutes at 12000x g. DNA is in the supernatant.

Once DNA is purified run PCR using 10 μ L of GoTaq Green Mastermix (Promega, Lot 0000127680), 1 μ L of 27FHT & 1942RHT primers (amplifies the 16S rRNA gene), 6 μ L of DNA in InstaGene supernatant, 3 μ L of dH₂O. The thermocycling reaction utilized a 56 $^{\circ}$ C annealing temperature to amplify product.

Gel electrophoresis to verify amplification of 16S rRNA PCR product and cytokine amplification

Electrophoresis was performed using a 1.2% agarose gel (Fisher Scientific, Lot 188373) in TBE (prepared by Dr Daggett) against a 1 kb plus DNA ladder. Samples were run for one hour at 100V and visualized with UV.

Growth and differentiation of THP-1

Monocytes

Sterile 1640 media (Gibco, Lot 3267238)

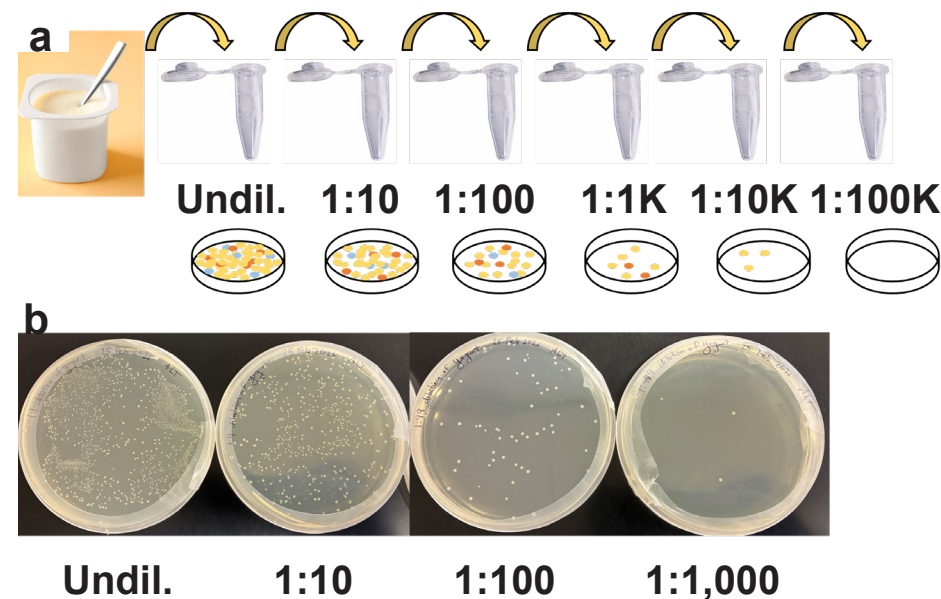


Figure 1 | Fishing experiment serial dilution. Probiotic candidates were isolated from a yogurt sample to be tested and later identified. **a**, A serial dilution from a sample of yogurt was performed and samples were plated for colony growth **b**, Plates depicted are LB media grown for 48 hours.

with 10% Fetal Bovine Serum, and pen strep (Gibco, Lot 268394) was used to grow THP-1 monocytes in T-25 or T-75 flasks, starting at $\sim 8 \times 10^5$ cells/mL (passing density) at 37 $^{\circ}$ C in 5% CO₂, and were passed every 2-3 days into a new flask with fresh media.

To differentiate the THP-1 monocytes to M0 macrophages, 100ng/mL PMA (Sigma, Lot MKCL1145) was added to cells at 0.5×10^6 cells/mL complete RPMI media. Cells were allowed to rest for 4 hours at 37 $^{\circ}$ C to allow for adherence to the flask. Stimulating media was removed and cells were gently washed in room temp or slightly warmed PBS pH7.4 (Gibco, lot 2412443). Cells were then provided fresh RPMI complete media and incubated for 24 hours. To further differentiate the M0s to M1 macrophages, media was removed and fresh media with LPS was added to a final concentration of 250 ng/mL. Cells were allowed to grow an additional 48 hours to differentiate.

RNA Isolation and purification

RNA was isolated using the Monarch Total RNA Miniprep kit (Monarch, #T2010S, Lot 10214460). Cells were pelleted and RNA isolated for cDNA generation. Nanodrop RNA to test for purity and to determine concentration.

cDNA generation

cDNA was generated using iScript super

mix (BioRad, Batch 64622404). A no-RT control was included to test for the presence of DNA. Reverse transcription was performed using the cDNA thermocycler program: Priming 25 $^{\circ}$ C x 5' > Reverse transcription 46 $^{\circ}$ C x 20' > RT inactivation 95 $^{\circ}$ C x 1'

qPCR

qPCR was performed using SYBR Green qPCR Master mix (Fisherbrand, Lot 2794602 and Lot 3058486), 1 μ L of the corresponding primers (F/R), 1 μ L of cDNA and 8 μ L deionized H₂O to a total of 20 μ L per well. Using the cDNA generated previously, qPCR was performed for beta actin (as a housekeeping gene), TNF- α , IL-1 β , IL-6 (pro-inflammatory cytokines) and IL-10 (anti-inflammatory cytokine). Cytokine primers were designed on Benchling, purchased from Integrated DNA Technologies, and reconstituted to contain both forward and reverse primers at a final concentration of 200 pmol/ μ L. Reaction was run following the qPCR program:

Initial denaturation (1 cycle)	95 $^{\circ}$ C x 5'
Amplification (30 cycles)	denaturing 94 $^{\circ}$ C x 30"
	annealing 55 $^{\circ}$ C x 30"
	extension 72 $^{\circ}$ C x 90"

Plate Read
Final extension (1 cycle)
Melt Analysis

Culturing M1 macrophages with *Lactococcus lactis*

M1 macrophages were grown with the *L. lactis* by seeding M1 cell into wells at a minimum density of 0.5×10^5 cells per mL in RPMI media (no additives). For each condition, probiotic cells were added in a 10:1 [probiotic:M1] ratio. For the heat killed condition, *L. lactis* was boiled for 8 minutes in the thermocycler prior to adding to M1 cells. The supernatant condition contained probiotic conditioned media spun down for 3 minutes at 14000x g to pellet bacteria, only supernatant was collected. All conditions were incubated at 37 °C for 4 hours before removing cells for the initial RT-qPCR, or for 24 hours before pulling media for ELISA and cells for RT-qPCR.

Quantibody ELISA Array

The ELISA array was completed following the protocol as written in the RayBiotech Quantibody Human Inflammation Array

Manual, Catalog #: QAH-INF-1 (RayBiotech, Lot 03102610051). Starting protein concentrations were calculated after running a BCA Protein Assay for normalizing protein concentrations. Samples were incubated for three hours at concentrations of 1:10 and 1:10000 dilutions. Detection antibody cocktail was allowed to incubate for two hours, and Cy3 dye-conjugated streptavidin was incubated for 1 hour. All wash steps were performed as instructed in the manual. ELISA slide was shipped back to RayBiotech for imaging and fluorescence results were sent back for analysis.

Results

16S Sequence data confirms isolated probiotic as *Lactococcus lactis*

A goal of this study was to identify one or more probiotic strain(s) present in the probiotic yogurt product to test with macrophages and measure the changes in immune response signaling of cytokines. To do so, a 16S PCR and sequencing reaction was performed. Samples were initially isolated following the serial dilution

scheme as depicted in Fig. 1, and were further isolated using a streak plate method to confirm each candidate colony was a single strain of bacteria. These colonies were then grown and DNA isolated and amplified using PCR to amplify the 16S rRNA gene (~1500 bp). The amplification products were run on a 1.2% gel (Fig. 2a) to verify amplification of the product, and select samples were sent off for sequencing. The returned sequencing results were then analyzed and identified. The colony assayed in lane B resulted in sequencing results (Fig. 2b) and the sequence was run through NCBI Blast, resulting in a match to *Lactococcus lactis*. This colony was utilized for further testing with the macrophages.

Addition of LPS differentiates M0 macrophages to M1s

In keeping with the inflamed environment expected of the gut of an individual suffering from chronic inflammation, macrophages were successfully differentiated from the THP-1 monocytes utilizing Phorbol 12-myristate 13-acetate (PMA), which was determined visually as non-adherent THP-1 cells differentiate into adherent M0s, LPS was added to the cell media to further differentiation into M1s, which are macrophages that have increased pro-inflammatory signaling. To verify that the addition of LPS did cause the M0s to shift to M1s, an RT-qPCR analysis was run to compare the production of mRNA template for the cytokines TNF- α , IL-1 β , IL-6 and IL-10. All cytokines except IL-6 (due to lack of amplification) were seen to be up-regulated in the M1 macrophage condition (Fig. 3c) compared to M0 controls. This provided confidence to adopt the differentiation protocol and test the M1 cells with the probiotic strain.

Culturing M1s with *L. lactis* appears to create a time dependent modulatory effect on cytokine mRNA transcription.

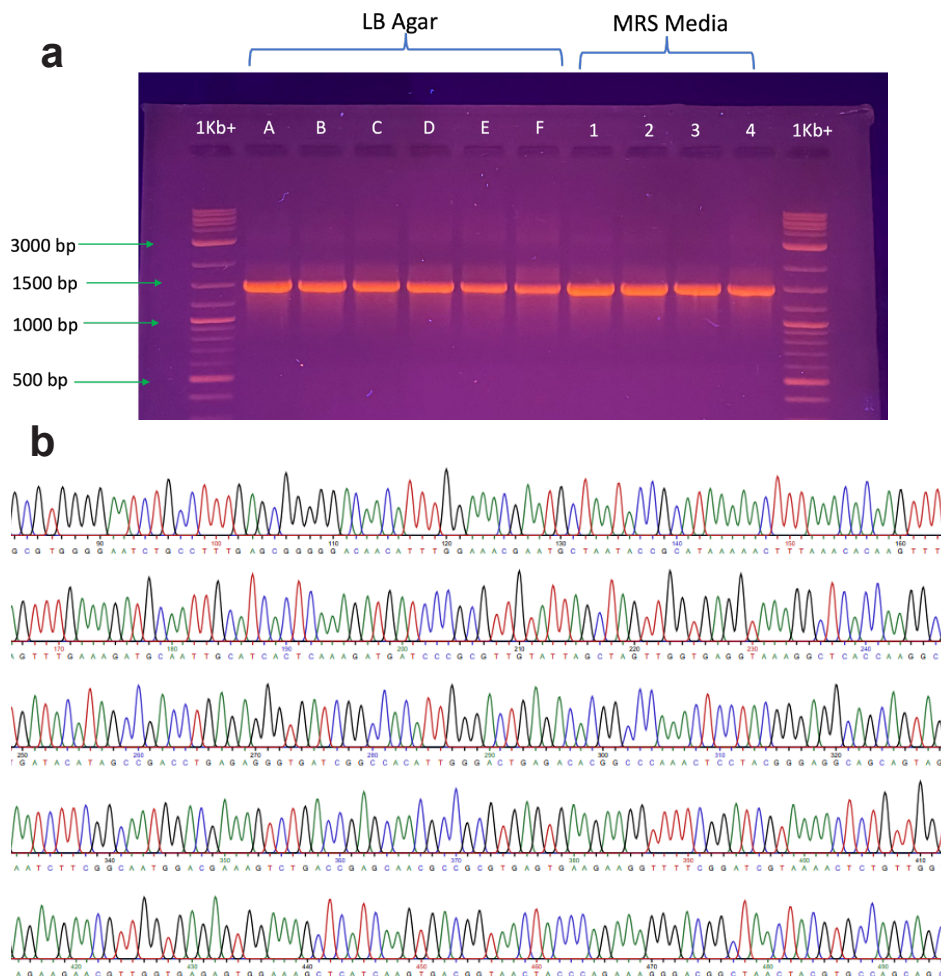


Figure 2 | DNA Sequencing of 16S rRNA. DNA amplification of 16S rRNA was run to identify probiotic strain. **a**, Gel of PCR amplification product showing samples from both LB agar and MRS media plates to verify amplification. **b**, Part of a sequencing readout received from sample B of the gel.

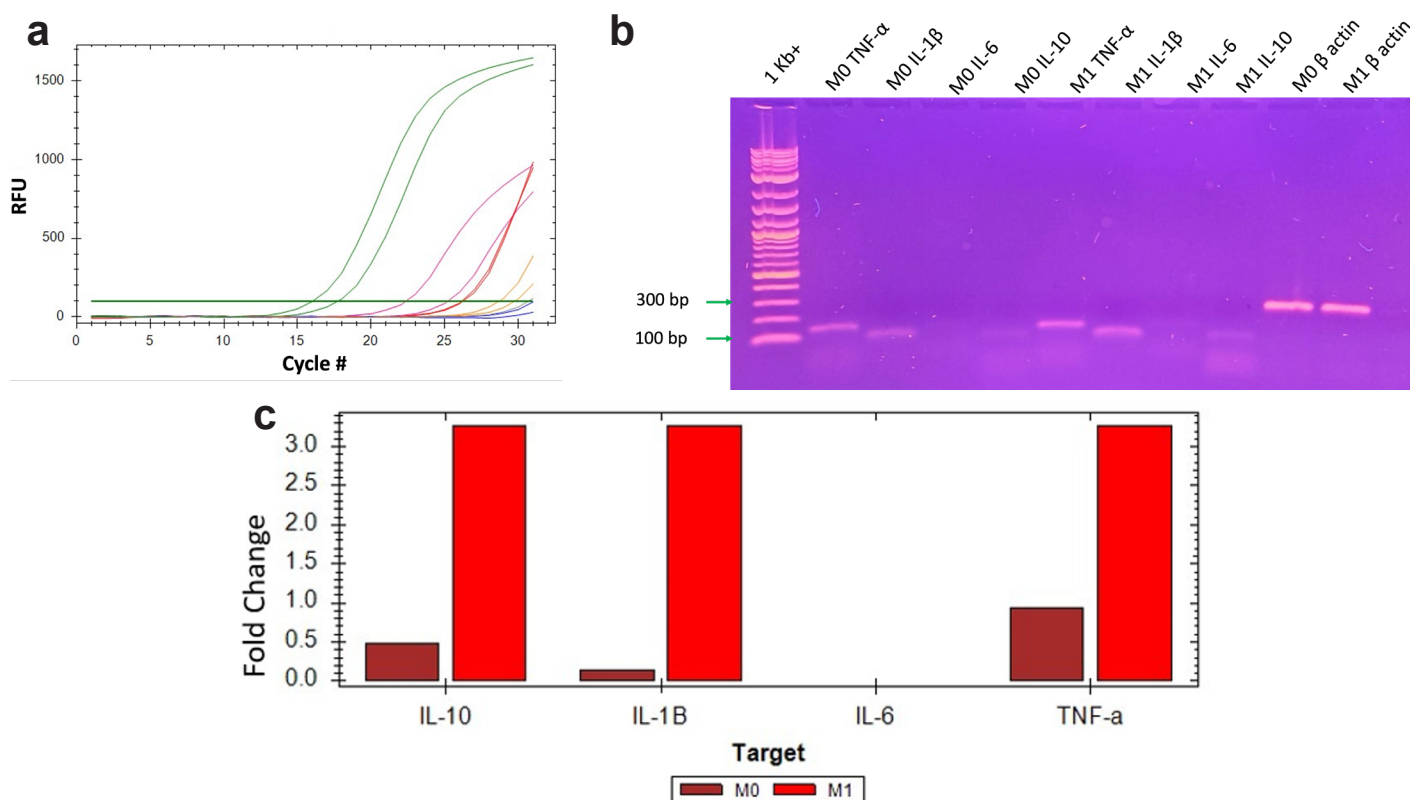


Figure 3 | qPCR validation for M0 differentiation to M1 Determination of cytokine mRNA transcript production change between M0s and M1s to verify that the differentiation protocol was successful. **a**, Amplification curve of the M0s vs M1 macrophages testing β Actin, IL-1 β , IL-6, IL-10 and TNF- α . **b**, Gel of the amplification products in A to visualize bands made in amplification. (C) Fold change analysis of M0s to M1s using a $\Delta\Delta CT$ analysis to compare the expression of cytokine mRNA.

Once it was confirmed that the macrophages were successfully differentiated to M1s, modeling an inflamed environment, we cocultured these cells with the probiotic *L. lactis*. Several conditions were tested to determine if signaling changed based on how the probiotic was presented to the immune cells. Various conditions in which the probiotic was presented alive, in which the probiotic had been heat killed, and using only the supernatant of media that *L. lactis* had grown in were assessed. The supernatant-treated condition, however, was determined to be contaminated, and the results are not considered in Fig. 4a, and are more accurately considered as a condition of unknown probiotic concentration. After incubating for a total of 4 and 24 hours, M1 cells were harvested and the RNA purified and RT-qPCR was run. The cytokine mRNA transcription results suggested a time-dependent modulatory effect of the co-culture. Based on the fold change analysis seen in Fig. 4a, there appears to be a down regulation of IL-1 β after 24 hours compared to the 4-hour time point in both the live and heat killed conditions, and an upregulation of IL-10 at 24 hours in the live probiotic condition. Fig. 4b shows that transcript numbers of IL-10

and IL-1 β change in response to the bacteria compared to the M1 control with no probiotic added. Transcripts for both cytokines exhibit up regulation compared to the condition with no probiotic added. however, in the live probiotic condition, the fold change seen in IL-10 appears to have a significant up regulation of anti-inflammatory signaling, but further replicates need to be run for statistical significance to be determined.

Discussion

Probiotics have been promoted to provide various health benefits to their host, especially in helping to modulate inflammatory signals, reducing inflammation. Through the preliminary findings of this research probiotics are seen to have possible modulatory effects on the cytokine signaling of macrophage cells changing the production and output of mRNA template for several cytokines involved in inflammatory signaling. This change in the production of cytokines may also be time dependent as seen in the RT-qPCR data showing that as incubation time increases, macrophages exhibit reduced transcription of the pro-in-

flammatory signaling of IL-1 β and increase the transcription the anti-inflammatory cytokine, of IL-10. The results received from the ELISA Quantibody Array were inconclusive, and will need to be repeated to see if the cytokine protein levels change in accordance with the transcriptional changes. While not obtaining enough data to establish statistical significance, the preliminary findings are encouraging. Seeing a potentially beneficial change in inflammatory signaling due to the probiotic helps strengthen further research on probiotic impacts to inflammation.

Future Directions

This study suggests that there may be beneficial modulatory effects seen in the inflammatory signaling of macrophage cells when exposed to *L. lactis*, however, further testing with increased sample replicates will need to be completed to demonstrate whether these findings are supported by statistical analysis. Further testing of this strain and other probiotic strains, as well as dosing requirements, will help in determining the true benefits probiotic products may have for their consumers and may establish confidence in the use of probiotics to improve health in ways that don't re-

quire medications with potentially harmful side effects. Future work to determine the specific modulatory effects of probiotics will help physicians better understand and prescribe probiotics as potential behavior health changes in diet that can help to treat various conditions in which inflammation is a chronic issue.

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Author Biography

Arianna Theleman is a graduating senior from the University of Kansas, earning her Bachelors of applied science in biotechnology. She plans to continue her education at KUMC in their IGPBS program. Arianna is passionate about discovery, family and her faith, and is motivated by the opportunity to participate in innovative research helping to impact and improve people's lives.

References

- Rodriguez, T., MA, & LPC. Probiotics, Depression, and the Role of Inflammation. *Psychiatry Advisor* <https://www.psychiatryadvisor.com/features/probiotics-depression-and-the-role-of-inflammation/> (2018).
- PhD, J. D., MD & MD, L. R. Can probiotics help calm inflammatory bowel disease? *Harvard Health* <https://www.health.harvard.edu/blog/can-probiotics-help-calm-inflammatory-bowel-disease-202411133081> (2024).
- Ciorba, M. A. A Gastroenterologist's Guide to Probiotics. *Clin. Gastroenterol. Hepatol. Off. Clin. Pract. J. Am. Gastroenterol. Assoc.* 10, 960–968 (2012).
- Cristofori, F. et al. Anti-Inflammatory and Immunomodulatory Effects of Probiotics in Gut Inflammation: A Door to the Body. *Front. Immunol.* 12, 578386 (2021).
- Probiotics: What They Are, Benefits & Side Effects. <https://my.clevelandclinic.org/health/treatments/14598-probiotics>.
- Plaza-Diaz, J., Gomez-Llorente, C., Fontana, L. & Gil, A. Modulation of immunity and inflammatory gene expression in the gut, in inflammatory diseases of the gut and in the liver by probiotics. *World J. Gastroenterol.* 20, 15632–15649 (2014).
- Virk, M. S. et al. The Anti-Inflammatory and Curative Exponent of Probiotics: A Comprehensive and Authentic Ingredient for the Sustained Functioning of Major Human Organs. *Nutrients* 16, 546 (2024).
- Park, C. et al. Probiotics for the treatment of depressive symptoms: An anti-inflammatory mechanism? *Brain. Behav. Immun.* 73, 115–124 (2018).
- Gorin, A., MS, & RDN. 12 Probiotics Brands Nutritionists Trust the Most. *The Healthy* <https://www.thehealthy.com/nutrition/probiotics/nutritionist-reviewed-probiotics/>.
- I'm A Dietitian, And I Recommend This Probiotic For Stomach Issues. *Women's Health* <https://www.womenshealthmag.com/health/g63171908/best-probiotic-for-gut-health/> (2025).
- Liu, Y., Alookaran, J. J. & Rhoads, J. M. Probiotics in Autoimmune and Inflammatory Disorders. *Nutrients* 10, 1537 (2018).
- Inflammation and Cytokine Storm ELISA Kits and Multiplex Immunoassays - US. <https://www.thermofisher.com/us/en/home/life-science/antibodies/immunoassays/immunoassay-research-areas/inflammation-cytokine-storm-immunoassays.html>.
- Wallace, C., Gordon, M., Sinopoulou, V. & Akobeng, A. K. Probiotics for management of functional abdominal pain disorders in children. *Cochrane Database Syst. Rev.* 2, CD012849 (2023).
- Więcek, J. et al. Evaluating the Impact of Probiotic Therapy on the Endocannabinoid System, Pain, Sleep and Fatigue: A Randomized, Double-Blind, Placebo-Controlled Trial in Dancers. *Int. J. Mol. Sci.* 25, 5611 (2024).
- Newlove-Delgado, T. V. et al. Dietary interventions for recurrent abdominal pain in childhood. *Cochrane Database Syst. Rev.* 3, CD010972 (2017).

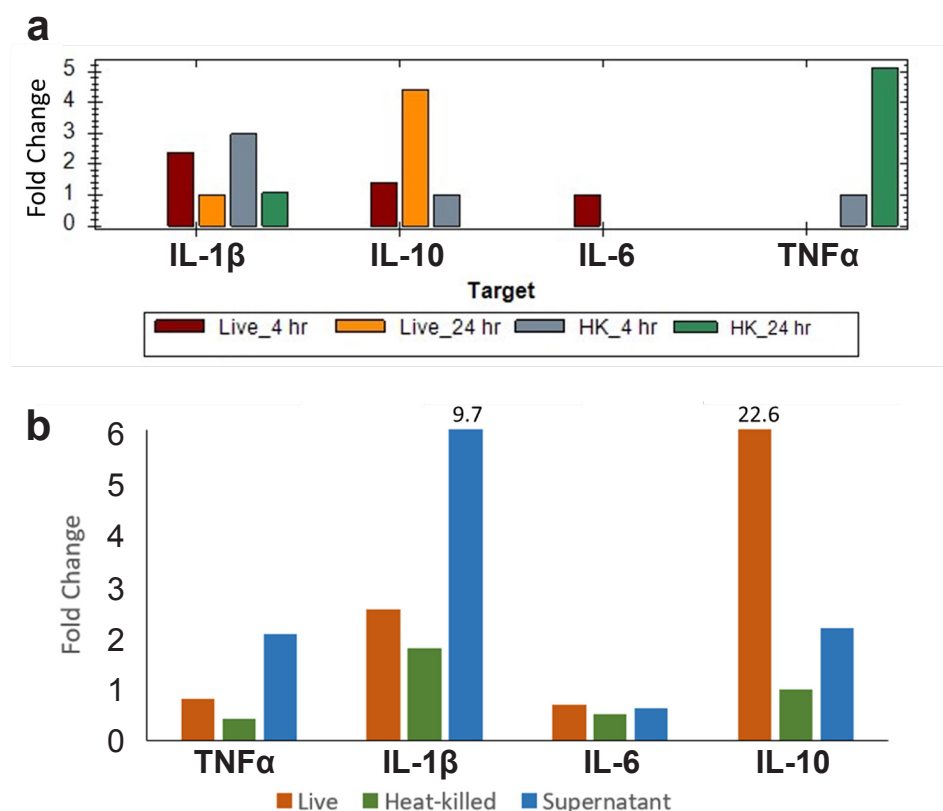


Figure 4 | RT-qPCR Fold Change Analysis. RT-qPCR was run on the M1 conditions containing probiotics and the mRNA expressions were compared against M1 + no probiotic added. **a**, Fold change analysis of 10:1 [probiotic: macrophage] coculture at time points of 4 and 24 hours to compare the expression of cytokine mRNA template produced by the cells. **b**, Fold change analysis of mRNA expression at time point 24 hours, repeated to compare expression levels as seen in **a**.



Adipose Tissue-Derived Factors Promote Raji B-Cell Lymphoma Survival during Doxorubicin Treatment

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Obesity is associated with increased risk and poorer outcomes in several cancers, but its effects on lymphoma responses to chemotherapy remain incompletely understood. This study examined whether soluble factors derived from adipose tissue influence the survival of Raji B-cell lymphoma cells during doxorubicin treatment. Raji cells were exposed to adipose tissue-derived medium with or without doxorubicin, and viability was assessed using LIVE/DEAD Fixable Red staining and flow cytometry. Addition of the adipose-derived preparation increased cell viability during doxorubicin exposure, with significant increases observed in the 20-, 30-, and 40- μ L treatment groups relative to doxorubicin alone. Exploratory RT-qPCR analysis suggested treatment-associated changes in TNF- α and GLUT1 expression; however, these measurements were performed in a single experiment and require replication. These findings suggest that soluble adipose-derived factors may partially protect Raji lymphoma cells from doxorubicin-induced cytotoxicity and support further investigation of the relationship between adipose tissue and chemotherapy resistance.

Cancer remains one of the leading causes of mortality worldwide, with chemotherapy serving as a cornerstone of treatment for many malignancies, including non-Hodgkin B cell lymphoma.^{1,2} Among commonly used chemotherapeutic agents, doxorubicin, an anthracycline antibiotic, is widely utilized due to its ability to induce cancer cell death through DNA intercalation, inhibition of topoisomerase II, and generation of reactive oxygen species.^{3,4} Despite its effectiveness, resistance to doxorubicin remains a major clinical challenge, limiting treatment success and contributing to disease relapse. Emerging evidence suggests that the tumor microenvironment plays a critical role in modulating chemotherapy response.⁵ Particularly, adipose tissue has gained attention as an active endocrine organ that can influence cancer progression and therapeutic outcomes. Adipocytes can secrete a wide range of cytokines, chemokines, and adipokines which can impact tumor cell behavior.⁶ These secreted factors are increasingly recognized as key contributors to cancer cell survival, proliferation, and drug resistance.

Adipose tissue is now understood to be metabolically active and highly involved in systemic and local signaling. Adipocytes release signaling molecules such as tumor

necrosis factor- α (TNF- α), which regulate inflammation, metabolism, and cellular survival pathways.^{7,8} These adipocyte-derived factors can alter the tumor microenvironment and promote tumor progression. Adipocytes located near tumors, often referred to as cancer-associated adipocytes, undergo phenotypic changes that enhance their ability to support tumor growth.⁹ These cells exhibit increased secretion of inflammatory mediators and metabolic substrates, such as free fatty acids, which can be utilized by cancer cells for energy production and survival. This metabolic crosstalk is a key mechanism contributing to therapy resistance.

In addition to secreting protective factors, adipocytes can also alter drug availability. Evidence suggests that adipocytes can sequester and metabolize chemotherapeutic agents, effectively reducing the concentration of active drug reaching cancer cells.¹⁰ This phenomenon further contributes to decreased drug efficacy.

Cytokines play a central role in mediating communication between adipocytes and cancer cells. TNF- α , a pro-inflammatory cytokine produced by adipose tissue, has been implicated in cancer progression and treatment resistance.¹¹ While TNF- α can induce apoptosis under certain conditions, it can also activate survival pathways such as NF- κ B signaling, promoting cell proliferation and resistance to chemotherapy.

In addition to inflammatory signaling, metabolic adaptations are critical for can-

cer cell survival. Glucose transporter 1 (GLUT1) is a key regulator of glucose uptake and is often upregulated in cancer cells to support increased metabolic demands.^{12,13} Enhanced GLUT1 expression allows cancer cells to maintain energy production under stress conditions, including exposure to chemotherapy.

While previous studies suggest that adipocytes contribute to chemotherapy resistance, the specific role of adipocyte-derived cytokines in lymphoma cells during doxorubicin treatment remains unclear.¹⁴ In particular, the effects of these cytokines on inflammatory signaling and metabolic adaptation require further investigation. This study aims to examine whether modelled adipocyte-conditioned media influences the survival of lymphoma cells treated with doxorubicin. In addition, the study evaluates changes in gene expression of TNF- α and GLUT1 to assess the impact of adipocyte-derived factors on inflammatory and metabolic pathways.

Understanding how adipocyte-derived cytokines influence cancer cell survival during chemotherapy is important for improving treatment outcomes. As obesity rates continue to rise, the interaction between adipose tissue and cancer cells becomes increasingly relevant in clinical settings. Insights from this study may help explain variability in chemotherapy response and contribute to the development of strategies targeting the tumor microenvironment.

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Materials and Methods

Raji B-Cells Culture

Raji B-cell lymphoma cells were cultured in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Cytiva HyClone) and 1% penicillin-streptomycin (Gibco). Cells were maintained in a humidified incubator at 37°C with 5% CO₂. Cell concentration and viability were monitored every two days using a hemocytometer and trypan blue (Gibco) exclusion assay at a 1:1 ratio of cells to trypan blue.

Modelled Adipocyte Conditioned Media Preparation

To model adipocyte conditioned media, approximately 800 mg of porcine adipose tissue was trimmed and homogenized. A total of 3.5 mL of sterile PBS-Tween (0.1%) solution was added to the tissue sample. The mixture was vortexed for 2 minutes and centrifuged at 5000 rpm for 1 minute. Following centrifugation, the upper lipid layer was removed, and the adipocyte-conditioned supernatant was collected. The collected supernatant was filtered twice using a sterile 20 mm filter before use in cell culture experiments.

Cell Seeding Assay

Raji cells were seeded into 24-well plates at different concentrations including 5×10^3 , 1×10^4 , 1×10^5 , 5×10^5 , and 1×10^6 cells/mL. Each condition was prepared

in triplicate. Cells were incubated for 24 hours before viability assessment.

Cell viability was measured using the LIVE/DEAD™ Fixable Red Dye assay (ThermoFisher, L23102). Cells were washed with sterile PBS and stained with 1 µL of LIVE/DEAD™ Fixable Red Dye for 30 minutes at room temperature in the dark. Samples were analyzed using flow cytometry. The optimal seeding density was selected based on the highest percentage of viable cells.

Lethal Dose 50 Determination

Raji cells were seeded at a concentration of 1×10^5 cells/mL in 24-well plates. Cells were treated with different concentrations of doxorubicin hydrochloride (MP Bio-medicals, 25316-40-9), including 1, 2, 4, 8, 16, and 20 µg/mL, for 24 hours. Following treatment, cell viability was assessed using the LIVE/DEAD™ Fixable Red Dye assay and flow cytometry. The lethal dose 50 (LD₅₀) concentration was determined from the resulting viability curve.

Treatment with Modelled Adipocyte-Cytokine Conditioned Media and Doxorubicin

Raji cells were cultured with modelled adipocyte-conditioned media at concentrations of 10, 20, 30, and 40 µL. Cells were incubated with modelled adipocyte-conditioned media for 12 hours prior to treatment with the LD₅₀ concentration of doxorubi-

cin. Following doxorubicin addition, cells were incubated for an additional 24 hours. Cell viability was assessed using the LIVE/DEAD™ Fixable Red Dye assay and analyzed by flow cytometry. All treatment conditions were performed in triplicate. Counts of live Raji cells and concentrations of drugs were controlled consistently to Raji cells with doxorubicin in adipocyte-cytokine conditioned media. Two-tailed, unpaired Student's t-tests assuming equal variances were used to determine statistical significance with a threshold of $p \leq 0.05$.

RT-qPCR

Total RNA was extracted from each treatment condition using the Monarch Total RNA Miniprep Kit (New England Biolabs) according to the manufacturer's instructions. RNA concentration and purity were measured using a NanoDrop spectrophotometer. Complementary DNA (cDNA) was synthesized in a 20 µL reaction containing 4 µL of 5X iScript Supermix, 500 ng RNA template, and nuclease-free water. Reverse transcription was performed using the following thermal conditions: priming at 25°C for 5 minutes, reverse transcription at 46°C for 20 minutes, and RT inactivation at 95°C for 1 minute.

Quantitative PCR (qPCR) reactions were prepared in a total volume of 20 µL containing 10 µL SYBR Green qPCR Master Mix, 1 µL forward/reverse primer mixture, 1 µL cDNA template, and 8 µL nu-

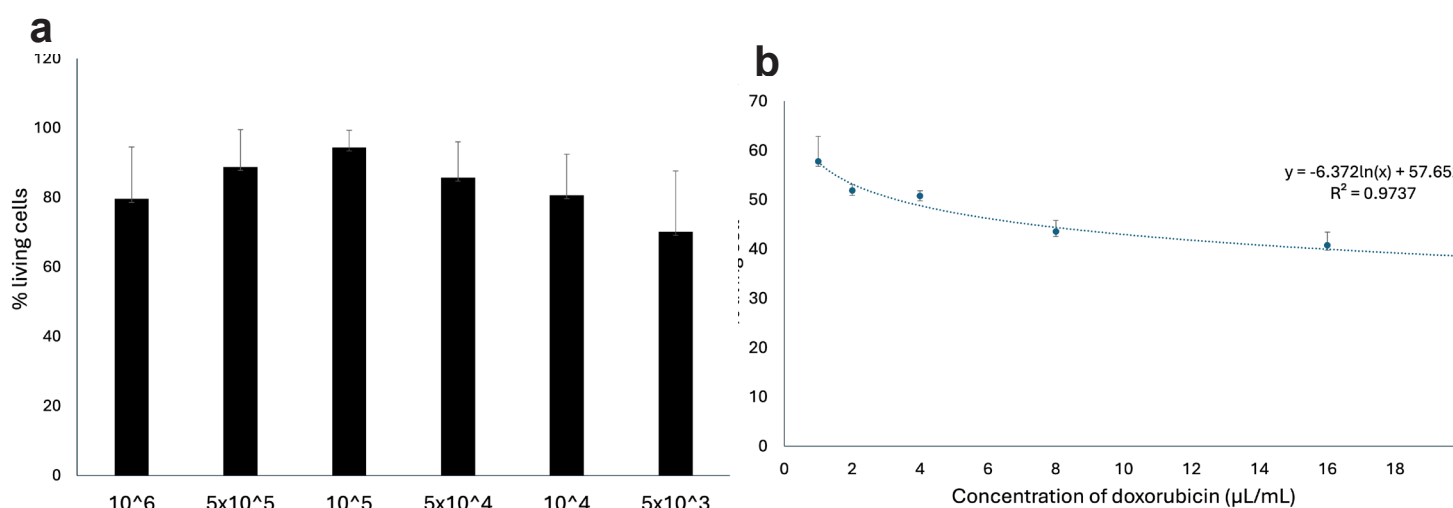


Figure 1 | Optimization of RAJI cell culture conditions and determination of doxorubicin LD₅₀. a, RAJI cell viability at different seeding densities after 24 hours. b, Dose-response curve of RAJI cells treated with increasing concentrations of doxorubicin. Cell viability decreased as doxorubicin concentration increased. The regression curve used to estimate the LD₅₀ concentration is shown. Data are presented as mean ± SD (n=3).