Effects of Soy and Milk Ferments on Measures of Innate Immunity: A Comparison of Effects in Normal and Microgravity Conditions

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ABSTRACT

Probiotics can influence intestinal responses and mucosal immunity either directly or indirectly through transient modulation of the endogenous microenvironment or the immune system. During space travel, astronauts experience various physiological stresses including putting them at risk for infections or inappropriate immune responses. Macrophages and monocytes are a key cell type involved in innate immunity. The effects of dairy milk or soy milk base fermented with *S. thermophilus* ST5 in combination with either *B. longum* R0175 or *L. helveticus* R0052 on the cell line U937 and all-trans retinoic acid differentiated U937 were examined under normal gravity and simulated microgravity conditions, in order to screen for effects on monocytes and macrophages. Soy and milk ferments demonstrated the ability to modulate certain aspects of the innate immune system, both in normal gravity and in simulated microgravity. These probiotics affected U937 cells differently depending on differentiation stage (monocyte or macrophage) and whether or not the cells were tested in regular gravity or in simulated microgravity conditions. These results provide insight into effects on this aspect of innate immunity and may provide guidelines to potential *in vivo* administration.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
ATRA	All-trans retinoic acid
BSA	Bovine serum albumin
CD	Cluster of Differentiation
CFU	Colony forming unit
DC	Dendritic cell
DPCSB	Double Protein Cell Staining Buffer
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbent Assay
FADD	FAS-Associated protein with Death Domain
FBS	Fetal bovine serum
GALT	Gut-associated lymphoid tissue
GI	Gastrointestinal
HRP	Horseradish peroxidase
IBS	Irritable Bowel Syndrome

IEC	Intestinal epithelial cell
Ig	Immunoglobulin
IGF	Insulin-like Growth Factor
IL-8	Interleukin-8
IL-10	Interleukin-10
IFN-γ	Interferon gamma
JAK-STAT	Janus Kinases and Signal Transducers and Activators of Transcription
LAB	Lactic acid bacteria
LF	Lactoferrin
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 antigen
MAPK	Mitogen-activated protein kinases
MB	Milk beverage ferment base
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MLN	Mesenteric lymph nodes
NASA	National Aeronautics and Space Administration

NF-κB	Nuclear factor kappa-light-chain-enhancer of B-cells
NLRs	Nucleotide-binding oligomerization domain-like receptors
PBS	Phosphate-buffered saline
PE	Phycoerythrin
PMA	Phorbol 12-myristate 13-acetate
PP	Peyer's patches
PRR	Pattern Recognition Receptors
R0052	*Lactobacillus helveticus*, strain R0052
R0175	*Bifidobacterium longum*, strain R0175
RCCS	Rotary Cell Culture System™
RPMI 1640	Roswell Park Memorial Institute (cell culture media)
RWV	Rotating wall vessel
SB	Soy beverage ferment base
SCFA	Short-chain fatty acid
SEM	Standard error of the mean
ST5	*Streptococcus thermophilus*, strain ST5
Strep-HRP	Streptavidin-Horseradish peroxidase

TGF-β1	Transforming growth factor beta 1
TMB	3,3',5,5' – tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
TNF-R	Tumour necrosis factor receptor
TRADD	TNFRSF1A-Associated via Death Domain
Vitamin D_3	1,25-dihydroxyvitamin D_3

INTRODUCTION

Probiotics and the Immune System

Probiotics are non-pathogenic, living microorganisms (bacteria or, less commonly, yeasts) that impart a health benefit to the host when ingested, particularly when present in the gastrointestinal tract and vagina (Douglas and Sanders, 2008; Pham *et al*, 2008; Elmer, 2001; Madsen, 2001; Marteau *et al*, 2001). They are often referred to as "living drugs". In a definition by the Food and Agricultural Organization and the World Health Organization, *probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host*. However, there is some discussion as to whether or not the bacteria must be viable to be beneficial and to be classified as a probiotic (Ostad *et al*, 2009; Kataria *et al*, 2009; Douglas and Sanders, 2008; Lopez *et al*, 2008; Simakachorn *et al*, 2000). Probiotics can influence intestinal physiology either directly or indirectly through transient modulation of the endogenous microenvironment or the immune system (Ng *et al*, 2010; Sanz and De Palma, 2009; Drakes *et al*, 2004; Marteau *et al*, 2001). Clinical studies suggest that probiotics might be useful in stimulation of the immune system, prevention of allergic diseases, control of gastrointestinal tract inflammatory diseases and potentially cancer prevention (Trafalska and Grzybowska, 2004). Numerous probiotic microorganisms (e.g. *Lactobacillus rhamnosus GG*, *L. reuteri*, *bifidobacteria spp.* and certain strains of *L. casei* or the *L. acidophilus* group) are used in probiotic or "functional" foods, particularly fermented milk products, or

have been investigated with regard to their medicinal use (de Vrese and Schrezenmeir, 2008). Probiotic microbial species such as those listed above may act by changing the composition of the gut microbiota (Trafalska and Grzybowska, 2004), although their fate in the gastrointestinal tract (GI tract) and overall effects differ among strains, with colonization often being transient (Douglas and Sanders, 2008; Marteau *et al*, 1993). The protective effects of probiotics against infections in the intestine have been shown in animal models (Corthier, 1997; Gibson *et al*, 1997; Elmer *et al*, 1996) and humans (Barbara *et al*, 2008; Vanderpool *et al*, 2008; Hickson *et al*, 2007) but the possible modes of actions vary, including production of short chain fatty acids (SCFA), hydrogen peroxide, or other antimicrobial substances, competition for nutrients or adhesion receptors, antitoxin actions, and stimulation of the immune system through direct interactions or cellular and metabolic products (Lebeer *et al*, 2010; Neish, 2004; Marteau *et al*, 2001). SCFA and their salts such as acetate, butyrate and lactate have recently been implicated in effects on nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB; a transcription factor for stress signals) expression induced by lactic acid bacteria (Kumar *et al*, 2009). In the gut, SCFA reach millimolar concentrations which are absorbed through passive and active transport (Tedelind *et al*, 2007) and are commonly associated with anti-inflammatory activity (Maslowski *et al*, 2009; Tedelind *et al*, 2007; Andoh *et al*, 1999).

The mucosal surfaces of the GI tract, reproductive tract and respiratory tract are in direct contact with the external environment and are therefore

susceptible to invasion by pathogens. The GI tract plays two roles in human physiology: digestion and uptake of nutrients, and the more daunting task of protecting the body from potentially harmful microbes, while inducing tolerogenic responses to innocuous food, commensals and self-antigens (Mason *et al*, 2008). A fairly large overall surface area allows the gut to house the largest number of immune cells in the body, in gut-associated lymphoid tissue (GALT) locations including Peyer's Patches (PPs) and mesenteric lymph nodes (MLNs). Key cell types participating in mucosal immunity include not only T and B lymphocytes, but also dendritic cells (DC), macrophages and intestinal epithelial cells (IECs), which are key players in innate immunity (See Appendix A) (Mason *et al*, 2008; Garside *et al*, 2004). Cell types involved in innate immunity have the ability to discriminate between harmful pathogens and the harmless members of the commensal flora by using cell surface Pattern Recognition Receptors (PRRs) such as the toll-like receptors (TLRs) (Albiger *et al*, 2007; Harris *et al*, 2006; Kelly and Conway, 2005).

Commensal microflora, or, normal indigenous microbiota, consists of those microorganisms which are present on body surfaces covered by epithelial cells and are exposed to the external environment (Tlaskalová-Hogenová *et al*, 2004). The presence of the commensal microbiota helps keeps pathogenic bacteria in check by out-competing those pathogens. At the interface between the contents of the gut and host tissues, the intestinal epithelium must incorporate pro- and anti-inflammatory signals to regulate immune responses (Clavel and Haller, 2007). An abnormal host response to the normal intestinal

microbiota can lead to chronic intestinal inflammation. Probiotic bacteria may moderate the intestinal microflora and the mucosal immune response and can be an effective therapy for such diseases as Irritable Bowel Syndrome (IBS), and potentially for ulcerative colitis and Crohn's disease (Heller and Duchmann, 2003). Regulation of commensal microbiota composition by probiotics also offers the possibility to influence the development of mucosal and systemic immunity (Tlaskalová-Hogenová *et al*, 2004).

One of the most complex systems in vertebrate species is that of the immune system. There is a need for an organism to detect material foreign from its own tissues. The immune system acts though a set of biological processes that protect an organism against disease by detecting and killing pathogens and tumour cells. There are two branches of the immune system in vertebrates: innate immunity and adaptive immunity. Innate immunity defends the host in a rapid and non-specific manner but confers no lasting protection (Alberts *et al*, 2002) and usually has common mechanistic elements. For example, a bacterial infection might set off a response of biochemical pathways that might also be utilized by other types of tissue damage that entail an immediate response, such as a skin burn (Ishii *et al*, 2008). The adaptive immune system is activated by the innate immune system (Janeway *et al*, 2001) and provides the host with the ability to remember and recognize individual pathogens and mount stronger attacks in subsequent exposures (Pancer and Cooper, 2006).

Monocytes, macrophages and IECs are among the participants at the frontline of the innate immune system. IECs provide a physical and immunological interface against microbes that pass through the intestinal lumen while monocytes and macrophages phagocytose cellular debris and pathogens and digest the pathogen with enzymes and toxic products of the oxidative burst reaction. Monocytes, macrophages and IECs produce cytokines upon intracellular infection with pathogenic bacteria (Lucas and Greaves, 2001; Kagnoff and Eckmann, 1997; Jung *et al*, 1995; Eckmann *et al*, 1993). Macrophages also function as antigen presenting cells (APCs) and are part of both the adaptive and innate immune systems (Thomson and Robbins, 2008). Instead of directly annihilating invading pathogens, APCs degrade pathogen proteins and, as their name suggests, present the fragments at their cell surface using Major Histocompatibility Complex (MHC) molecules. These fragments displayed by the APCs are recognized by helper T cells and cytotoxic T cells, leading to their activation through costimulatory molecule-dependent process. T cell activation in turn provides help for activated B cells, thus initiating antibody production and an immune response against the pathogen.

Two common pyrogens (a substance that induces fever and pro-inflammatory immune response) used in immunology research are Tumour Necrosis Factor Alpha (TNF-α; also a pro-inflammatory cytokine) and Lipopolysaccharide (LPS). TNF-α is manufactured by macrophages to induce apoptotic cell death and induce inflammation, and to prevent tumourigenesis and viral replication via cell lysis. TNF-α receptors (TNF-R) are associated with pro-

caspases through adapter proteins such as FAS-Associated protein with Death Domain (FADD) and TNFRSF1A-Associated via Death Domain (TRADD) that cleaves other inactive pro-caspases and trigger the caspase cascade, committing the cell to apoptosis. LPS is an endotoxin and a component of the outer-membrane of Gram-negative bacteria, and stimulates an immune response through a CD14/TLR4/MyD88/NF-κB pathway. LPS also induces TNF-α production by macrophages (Alciato *et al*, 2010; Allon *et al*, 2010; Tumurkhuu *et al*, 2009). Both TNF-α and LPS are often used in cell culture as a challenge to induce pro-inflammatory responses in target cells (Raabe *et al*, 1998; Drouet *et al*, 1991). Each toxin or challenge can act in a different way on individual cell lines. Both LPS and TNF-α have been utilized to challenge the human macrophage cell lines THP-1 and U937 (Mezayen *et al*, 2007; Steube *et al*, 2000; Vlahopoulos *et al*, 1999; Neale *et al*, 1988)

The outcome of these challenges on cells is, as stated previously, an innate response which includes production of certain key cytokines. Cytokines are a class of signaling molecule used by the body in cellular communication and are important immunomodulating agents. Many cytokines can be divided into two general categories: pro-inflammatory (including IL-8 and TNF-α) and anti-inflammatory (including IL-10 and TGF-β1). IL-8 is widely used as a gauge of inflammatory action in laboratory practice because it is one of the major mediators of the inflammatory response in the body (Vlahopoulos *et al*, 1999). TNF-α, in addition to being used as a challenge for cell lines, is itself a cytokine that through the TRADD pathway intensifies activation of NF-κB, increasing the

inflammatory response. Both are considered positive acute phase proteins where their plasma concentrations increase in response to inflammation. IL-10, on the other hand, blocks NF-κB activity and is involved in the regulation of the JAK-STAT (Janus Kinases and Signal Transducers and Activators of Transcription) pathway (Moore *et al*, 2001). Additionally, knockout studies in mice have shown that IL-10 is an essential immunoregulator in the intestinal tract (Ina *et al*, 2005). TGF-β1 is a regulatory cytokine with effects that vary depending on location or situation; TGF-β1 promotes regulatory T-cell development, down-regulates pro-inflammatory cytokine production and also acts as a class switching factor, promoting IgA production (Ouyang *et al*, 2010; Ghio *et al*, 2009; Watanabe *et al*, 2001). It is important to look at both pro- and anti-inflammatory responses to fully understand the range of immunomodulatory activity to a particular stimulant or product.

Fermented milk and soy products containing probiotics have recently become a “hot topic” among immunologists and gastroenterologists (Rekha and Vijayalakshmi, 2008; Farnworth *et al*, 2007; Saikali *et al*, 2004; Heller, 2001), and is a focus of research and product development for industry. However, probiotics are not a novel discovery. At the beginning of the 20th century, Russian Nobel laureate and Pasteur Institute professor Eli Metchnikoff first suggested that it is possible to modify the gut flora and replace harmful microbes with useful ones (Metchnikoff, 1907). Later, some strains of *L. acidophilus* were determined to be active when introduced into the human digestive tract (Rettger *et al*, 1935). In

1965, Lilly and Stillwell first coined the term “probiotic” (Lilly and Stillwell, 1965) meaning, “for life”.

The probiotic cultures themselves are believed to impart the majority of health benefits in fermented cow’s milk and soy products. Aside from providing the host gut with beneficial bacteria and increasing intestinal mobility (Seki *et al*, 1978) and potentially increasing vitamin content (Alm, 1982; Shahani and Chandan, 1979), the lactic acid bacteria (LAB) also secrete a variety of antimicrobial metabolites, compete for nutrients and adhesion sites in the gut against pathogenic bacteria (Sanz *et al*, 2007) and may provide immunomodulatory effects, among a list of other possible activities (Heyman, 2000). Today, lactobacilli and bifidobacteria (also known as LAB) are the primary probiotics used in fermented soy and dairy foods such as yogurt and dietary supplements (Cogan *et al*, 2007), and common starter cultures include *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (Farnworth *et al*, 2007). The probiotic cultures generally do not grow rapidly in cow’s milk, and in yogurt manufacture, do not reach as high numbers as starter cultures achieve (Wagar *et al*, 2009; Champagne *et al*, 2005). Some studies have previously shown that soy is a good substrate for the growth of probiotic bacteria (Champagne *et al*, 2009; Scalabrini *et al*, 1998; Mital *et al*, 1974; Angeles and Marth, 1971) but not for that of the starter culture *L. delbrueckii* subsp. *bulgaricus* (Wang *et al*, 2002; Karleskind *et al*, 1991; Mital *et al*, 1974). *S. thermophilus* on the other hand, has been shown to grow well in both cow’s milk and soy substrate (Champagne *et al*, 2009; Farnworth *et al*, 2007; Wang *et*

al, 2006; Hsieh and Chou, 2006). *S. thermophilus* has also previously been shown to stimulate innate immunity in mice models (Perdigon *et al*, 1995).

Bovine Milk, Soy Milk and the Human Immune System

Bovine milk and milk products, while containing a number of essential nutrients, have seen a decrease in consumption following claims of negative health effects (Haug *et al*, 2007; Insel *et al*, 2004). This critique arises especially since milk fats contain a large percentage (more than half) (Jensen and Newburg, 1995) of saturated fat, which has become associated with obesity, weight gain and heart disease. However, the common misconception that bovine milk causes an increase in mucus production and respiratory symptoms has never actually been confirmed (Wüthrich *et al*, 2005; Pinnock *et al*, 1990).

Aside from macronutrients, bovine milk contains a large number of biologically active components such as hormones, immunoglobulins (Ig), growth factors, enzymes and other peptides. Insulin-like Growth Factor (IGF) and Epidermal Growth Factor (EGF) regulate cell growth and development especially in the milk glands of lactating ruminants such as cows and sheep (Herbst, 2004; Forsyth 1996; Glimm *et al*, 1992), and the GI tract of bovine and ovine neonates (Blum and Baumrucker, 2008; Blum and Baumrucker, 2002; Odle *et al*, 1996). There may also be a human connection with these developmental changes (Purup *et al*, 2006; Gill *et al*, 2000). As bovine milk and its bioactive components are ingested, they survive the digestion process (Koldovsky, 1989), and affect the regions of the GI tract differently (Blum and Baumrucker, 2008). In addition,

IgG, IgA and IgM (Korhonen *et al*, 2000), hormones and hormone-like peptides are also potential factors found in bovine milk that are capable of modulating the immune system at the GI tract level (Clare and Swaisgood, 2000). However, bovine milk is a very complex solution and there may be components that have yet to be characterized.

Soy milk, on the other hand, is often touted as a healthy alternative to dairy milk, especially for those who are lactose intolerant or following a vegetarian or vegan diet. Soy milk has several advantages over dairy milk in terms of nutrition. Ounce for ounce, soy milk has less calories and fat than dairy milk, contains fiber, contains potentially bioactive molecules such as phenolic acids, saponins, isoflavones and phytosterols, lacks casein (an important aspect for those with milk allergies) and is a source of vitamin E and lecithin (USDA Nutrient Data Laboratory, 2010; Ishimi, 2009; Xu and Chang, 2009; Bolling and Parkin, 2008; Sakai and Kogiso 2008). A diet that includes soy-based products has been shown to modulate some aspects of the immune system (Maskarinec *et al*, 2009; Matsushita *et al*, 2008; Ryan-Borchers *et al*, 2006), possibly due to its isoflavone content. Soy milk fermentation has also been reported to improve soy isoflavone bioavailability (Tsangalis *et al*, 2005; reviewed in Uzzan and Labuza, 2004). Like dairy milk, soy milk is complex and there may be uncharacterized constituents.

Issues with Space Travel and Immunity

Experience with space exploration to date has raised more questions regarding nutritional requirements for astronauts than it has answered. As mission lengths continue to increase, nutrient imbalances due to alterations in food intake, dietary requirements, bioavailability, or excretion, may become more important (Seddon *et al*, 1994). Among the many physiological problems astronauts experience while in zero gravity are increase in stress, increased metabolic rate and increase in protein turnover. Changes in activity of the immune system have also been reported including reduced phagocytic and oxidative burst activity of monocytes (Kaur *et al*, 2005). It has also been shown that there is an increase in circulating IL-1, a pro-inflammatory cytokine and endogenous pyrogen (Huntoon *et al*, 1994; Stein *et al*, 1993; Leach *et al*, 1988; Leach *et al*, 1983). Decreases in lymphocyte blastogenesis, cytokine production, and natural killer cell activity have also been reported after space flight (Sonnenfeld, 1999). These changes start to occur after only a few days of space flight, and some changes continue throughout long-term space flight (Sonnenfeld, 1998) or even after cessation of microgravity conditions (Ritz *et al*, 2005). T cell activation is decreased and a detectable T_h2 cytokine shift also appears during and after spaceflight (Crucian *et al*, 2008). It is known that exposure of T cells in culture to actual or modeled low gravity is often accompanied by a major inhibitory effect, remarkably reducing their mitogenic activation process and inhibiting further signaling pathways such as the NF-κB and MAPK pathways (Boonyaratanakornkit *et al*, 2005; Cogoli *et al*, 1984).

Modeled microgravity has been reported to have varied effects including severely altered growth rate, gene expression, cytoskeletal structures, motility, and perhaps most importantly, cytokine production in cell culture (Cogoli-Greuter *et al*, 2004; Lewis, 2002; Cogoli, 1997; Cogoli and Cogoli-Greuter 1997; Cogoli, 1993). The cell cycle itself is influenced by gravity; during a 14-day space flight, an accumulation of p53 has been found in epidermis (keratinocytes) and muscle (myocytes), indicating that central regulatory molecules of nuclear signal transduction and cell cycle are influenced by gravity (Ohnishi *et al*, 1999). Meloni *et al*. (2006) has recently shown that simulated microgravity leads to substantial changes in the cytoskeleton of monocytes. This in turn influences motility, and as revealed during an International Space Station (ISS) experiment, a severe reduction in the locomotion ability of monocytic cells in microgravity (Meloni *et al*, 2008) possibly due to changes in cell-cell adhesion. However, little research has been completed on macrophage stage cells in actual or modeled microgravity.

The migration of immune cells is a crucial process during many physiological and pathophysiological conditions such as development, defense against infections and wound healing (Horwitz and Parsons, 1999; Lauffenburger and Horwitz, 1996). Leukocytes move through the body in order to keep the organism under immunological surveillance and to respond to pathogenic invading microorganisms, and microgravity may affect this motility (Li *et al*, 2009; Meloni *et al*, 2006; Buravkova and Romanov, 2001). Again, this is perhaps due to changes in the cytoskeleton (Crawford-Young, 2006; Meloni *et al*, 2006; Hughes-Fulford, 2003). However, this has received little attention. But it seems

that not all immune system cells are equally affected by the absence of “gravity”. Studies with natural killer cells in simulated weightlessness and in actual microgravity revealed that neither cytotoxic effects nor interferon production is altered in microgravity (Buravkova *et al*, 2004), although others have reported a decreased concentration of natural killer cells (Sonnenfeld and Shearer, 2002; Levine and Greenleaf, 1998).

In addition to direct effects, the intestinal microflora of the astronauts may also be affected, although there is little published. As early as two weeks into the confinement imposed by space flight, there is a significant reduction in the number of bacterial species that can be isolated from the intestinal tract as well as an interchange of intestinal bacteria between crew (Hales *et al*, 2002). Such large shifts in the composition of the commensal microflora may also provide opportunities for the dissemination and spread of antibiotic resistance genes. These effects might be circumvented, or at least ameliorated, by regular intake of probiotic foods (Taylor and Sommer, 2005).

The RCCS™ and Simulated Microgravity

Because actual microgravity is clearly difficult and expensive to work in, modeled microgravity conditions must be substituted on the ground. The term “microgravity” (or “zero gravity”) is actually somewhat of a misnomer. The weightlessness felt while in orbit around the earth in a shuttle or space station is not the result of the force of gravity being eliminated or even significantly reduced

(zero gravitational acceleration). Instead, it is that of the object or person essentially freefalling, falling out of the orbit and then returning to orbit by centripetal force, without colliding or experiencing any other forces. The difference between true freefall and the freefall of "zero gravity" is that gravitational acceleration causes a net change in the direction (around the earth), rather than the magnitude (falling faster).

Recently, a new cell culture technology has been developed by the National Aeronautics and Space Administration (NASA), and manufactured by Synthecon Incorporated (Houston, Texas) to study the effects of microgravity on cells. The Rotary Cell Culture System™ (RCCS) (Unsworth and Lelkes, 1998; Schwarz and Wolf, 1991), also called a bioreactor or rotating wall vessel (RWV), is a disk or drum shaped chamber which rotates along a horizontal axis, filled completely with cell culture medium (bubble free), allowing the cells to develop in an environment similar to the freefall of microgravity (See Appendix B). Cells are maintained in a gentle fluid orbit that creates a sustained low-shear force (less than 1 dyne/cm^2)[†], low-turbulence environment for cell growth (Hammond and Hammond, 2001). This design allows a constant rotation normal to the gravitational field, resulting in a culture condition in which the gravitational vectors are randomized over the surface of the cells (Klaus, 2001). Oxygen and carbon dioxide are allowed to reach the cells though a flat silicone rubber gas transfer membrane wall in the chamber. This innovation has allowed researchers to study the effects of microgravity on cells without entering

[†] 1 dyne = 10 µN, where 1 N = 1 $\frac{kg \cdot m}{s^2}$

microgravity themselves (Chen *et al*, 2007; Clement *et al*, 2007; Saxena *et al*, 2007; Wang *et al*, 2005).

The fluid mechanics affecting objects in a bioreactor have been studied in great detail (Hammond and Hammond, 2001; Klaus, 2001; Klaus *et al*, 1998; Gao *et al*, 1997; Wolf and Schwarz, 1991). The cells are in a state of constant freefall because of hydrodynamic forces counteracting the gravitational sedimentation in the bioreactor. Turbulence and shear forces are minimized by the solid-body rotation of the cell culture media, as indicated by Stokes' Law[‡] for flow around spherical objects (Hammond and Hammond, 2001; Gao *et al*, 1997; Tsao *et al*, 1994). Assuming that a cell acts similarly to the spherical particle mentioned in Stokes' Law, their microscopic size and relative low density suggests minimal shear force.

There are two different types of vessels the RCCS™ base accepts: the High Aspect Ratio Vessel (HARV) and the Slow Turning Lateral Vessel (STLV). The STLV comes in 55 to 500 mL sizes, are drum shaped, and are best used for batch cultures of adherent cells (See Appendix C). The HARV, available in 10 mL and 50 mL sizes, are disk shaped and come in both in a reusable, autoclavable version, and a disposable, pre-sterilized version (See Appendix D). The vessels used in this research were disposable 10 mL HARVs. Table 1 shows selected literature using the HARV to simulate microgravity.

[‡] $F_d = 6\pi\mu RV$ where F_d is friction (in N), μ is fluid viscosity (in Pa·s), R is the radius of the spherical particle (in m) and V is the particles velocity (in m/s)

Cell Culture Approaches

The most common system for modeling the immune system in the field of probiotics and immunological research are *in vitro* cell culture and *in situ* animal studies. Although relatively simple, cell lines provide a "first look" in evaluating effects of a growth factor, antigen or other stimulant before moving on to *in situ* models. The early stages of cell culture began in the 1900's and have since then presented the basis for hybridomas and monoclonal antibody production, two significant events in biotechnology history. In cell culture, standardized cell lines are established from a sample taken from transformed tissue. Because of the deregulation of the cell cycle, cell lines are easily cultured and can be permanently maintained.

The U937 monocytic cell line was established in 1974 and isolated from a 37 year old male patient with hematological malignancy (Sundstrom and Nilsson, 1976). This cell line can be further differentiated into a more mature macrophage stage using phorbol 12-myristate 13-acetate (PMA), all-trans retinoic acid (ATRA) or 1,25-dihydroxyvitamin D_3 (vitamin D_3; Kulseth *et al*, 1998; Kitamura *et al*, 2004). The THP-1 cell line is also monocytic, but is derived from a one year old male patient with acute monocytic leukemia (Tsuchiya *et al*, 1980). This cell line was established in 1980.

Many of the biomarkers presently used in immunological research have several roles which are often dissimilar between different tissues and cell types. The best example of this is TNF-α. In the context of this research, TNF-α acts as

a challenge and an inducer of NF-κB activation and cytokine production. Originally, TNF-α was discovered as possessing potent tumour cytotoxicity in earlier animal models (reviewed in Bradley, 2008). While typically a pro-inflammatory stimulus, the effects of TNF-α may vary on cells at different stages of differentiation. As mentioned previously, TNF-α is able to activate the NF-κB/TRADD and mitogen-activated protein kinase (MAPK) pathways and induce apoptosis through caspase activation and is commonly used to challenge cells in culture in order to induce an inflammatory response. TNF-α is an effective inducer of IL-8 production by the U937 cell line (Vlahopoulos *et al*, 1999).

Roswell Park Memorial Institute 1640 (RPMI 1640) cell culture media has been used to culture neoplastic and normal human leukocytes and was first developed by Moore *et al* (1967). It utilizes a bicarbonate buffering system to resist changes in pH to maintain optimal growth conditions for the cells. RPMI 1640 is often supplemented with serum (Bovine serum albumin (BSA), fetal bovine serum (FBS) etc) and phenol red (a pH indicator).

Immunological Cellular Outputs

The innate immune system defends the host in a rapid and non-specific manner but confers no lasting protection and usually has common mechanistic elements. Such elements include cytokine production, growth factor production and cell surface protein expression, and recognition of pathogens though PRRs

such as TLRs and nucleotide-binding oligomerization domain-like receptors (NLRs). Interleukins are a group of cytokines that were first seen to be produced by leukocytes (hence the name), although now it is known that they are expressed in a wide variety of cells (Smith and Humphries, 2009; Trevino *et al*, 2005; Sharma *et al*, 2000). Growth factors such as TGF-β1 are substances which are capable of altering cell growth and proliferation (Cheng *et al*, 2004; Matsuyama *et al*, 2003; Morrell *et al*, 2001), and also can be considered cytokines.

An important cytokine is Interleukin 8 (IL-8), one of the major players of inflammatory response in the innate immune system (Vlahopoulos *et al*, 1999). It is also known as CXCL8, and is often measured as an indicator of a pro-inflammatory response. The gene for the IL-8 protein (*IL8*) is found in chromosome 4 (Modi *et al*, 1990), and the protein is a dimer, each monomer 72 amino acids in length. The main function of IL-8 is to attract target cells such as neutrophil granulocytes to the site of infection via chemotaxis (DiVietro *et al*, 2001; Lane *et al*, 2001). These neutrophils phagocytose pathogens and can also be activated though TLRs and NLRs, promoting their phagocytic and respiratory burst activity (Clarke *et al*, 2010). As such, it can be said that IL-8 is the first domino to fall in a pro-inflammatory host response cascade.

Another important cytokine is Interleukin 10 (IL-10), which has pleiotropic effects in the immune system and is considered a key immunoregulatory cytokine. The gene *IL10* is found in chromosome 1 (Eskdale *et al*, 1997), and the protein itself is a homodimer, each subunit consisting of 178 amino acids

(Zdanov *et al*, 1995). IL-10 can down-regulate the expression of co-stimulatory molecules and MHC class II antigens and some cytokines such as Interferon gamma (IFN-γ) (Moore *et al*, 2001; Mosmann and Moore, 1991). In addition, IL-10 can improve B cell survival and block NF-κB activation induced by TNF-α or LPS (Dhingra *et al*, 2009; Park-min *et al*, 2009; Dagvadorj *et al*, 2008; Nachtwey and Spencer, 2008) as well as induce phagocytosis of apoptotic cells and bacteria in monocytes (Lingnau *et al*, 2007; Ogden *et al*, 2005).

Transforming Growth Factor beta 1 (TGF-β1) can influence cell proliferation and differentiation, as well as other functions depending on cell type, and is also considered an immunoregulatory cytokine. The *TGFB1* gene is found on chromosome 19 (Ghadami *et al*, 2000) and the protein is expressed as a 390 amino acid precursor protein which is proteolytically processed to produce a mature peptide of 112 amino acids (Derynck *et al*, 1985). Many cells produce TGF-β1 and have receptors for the protein, including immune system cells (Letterio and Roberts, 1998). In monocytes and macrophages, TGF-β1 has mainly suppressive effects; it can inhibit proliferation and prevent the formation of superoxide anions (O_2^-) and nitric oxide (NO) intermediates (Langermans *et al*, 2001; Ding *et al*, 1990; Tsunawaki *et al*, 1988). However, the effect may be caused though the suppression of production of other cytokines (Méndez-Samperio *et al*, 2002; Chantry *et al*, 1989).

Cell surface molecules on the surface of leukocytes are identified using a numbered cluster of differentiation (CD) system. Currently there are over 350 human CD molecules listed in the Human Cell Differentiation Molecules Council

website. CD molecules can act in various ways such as cell signaling, cell adhesion, and as receptors and ligands. When stimulated or differentiated, leukocytes usually vary their expression of various cell surface molecules.

CD54, also known as Intra-Cellular Adhesion Molecule 1 (ICAM-1), is important in adhesion between endothelial cells and leukocytes, allowing the leukocyte to transmigrate to site of injury or inflammatory response. It is usually expressed at a low level (van de Stolpe and van der Saag, 1996), however it is upregulated by cytokine stimulation, and may enhance this adhesion (Arkusz *et al*, 2010; Xie and Gu, 2008; Bella *et al*, 1998). CD58 or Lymphocyte Function-Associated Antigen 3 (LFA-3) is another cell adhesion molecule expressed on APCs, especially macrophages (Wallich *et al*, 1998; Barbosa *et al*, 1986), and is important in adhesion of T cells to APCs (Zhu *et al*, 2006; Wang *et al*, 1999). CD80 and CD86 (also called B7.1 and B7.2, respectively) are co-stimulatory molecules necessary to prime T cells responding to antigens presented by APCs (Slavik *et al*, 1999; Laniet *et al*, 1995). Both provide regulatory signals for T lymphocytes after binding to CD28 (stimulatory signal) and CTLA4 (cytotoxic T-lymphocyte-associated protein 4) (inhibitory signal) receptors (Magistrelli *et al*, 1999; Waterhouse *et al*, 1995). CD11b or Integrin alpha M (ITGAM), together with CD18, forms the heterodimeric integrin $\alpha_M\beta_2$, also known as macrophage-1 antigen (Mac-1) or complement receptor 3 (Solovjov *et al*, 2005). Mac-1 is found on natural killer cells, granulocytes and mononuclear phagocytes such as macrophages and increased CD11b expression can be used as a marker for U937 differentiation to the macrophage stage (Saeki *et al*, 2003). Genes for

CD54, CD58, CD80/CD86 and CD11b can be found on chromosomes 19, 1, 3 and 16, respectively.

Together these markers can provide a profile of the immunological effects of these ferments on the immune system and monocyte/macrophage activity.

Objectives

The key objective of this work was to examine the immunomodulatory effects of soy and milk ferments prepared with selected LAB strains on the activity of two key players in the innate immune system – the monocyte and macrophage. We hypothesized that the effects of soy and milk ferments on IL-8, IL-10 and TGF-β1 secretion, as well as CD54, CD58, CD80 and CD86 expression by U937 cells would not necessarily be the same on this cell type at different stages of differentiation. To explore this hypothesis, the U937 cell line able to differentiate from the monocyte to the macrophage stage was used, and the effects of soy and milk ferments at the monocyte and the ATRA-differentiated macrophage stage were determined. We also hypothesized that the responses of these cells to soy and milk ferment treatment under microgravity conditions would not necessarily be identical to those observed under regular gravity conditions. This aspect was examined using a HARV. Ultimately, a fermented product that could be utilized by astronauts to maintain and improve their immune system while in flight would be an advantageous addition to the astronaut diet. This work aimed to provide some initial steps toward development of such a fermented product.

MATERIALS AND METHODS

Ferment Preparation

Strains *Lactobacillus helveticus* R0052 (R0052), *Bifidobacterium longum* R0175 (R0175), and *Streptococcus thermophilus* ST5 (ST5) were used for the fermentation process. Soy beverage (SB) and milk beverage (MB) was prepared as reported previously (Champagne *et al*, 2009). Briefly, SB was prepared by soaking 250 g of dried soybeans in distilled water overnight, and 190 g of soaked beans plus 500 mL of fresh distilled water were ground with a commercial blender unit (Waring, New-Hartford, USA) for 3 min, filtered on Whatman #4 membranes and boiled for 5 min, cooled in an ice bath and stored at 4°C. MB was prepared by adding 61.9 g of skim milk powder (Crino, Agropur, Granby QC, Canada) to 715 mL of 3.25% milk fat commercial milk (Lactantia Pur Filtre, Parmalat, Victoraville, QC, Canada) and adding water to make 1 L. This milk blend was then boiled for 5 min, cooled in an ice bath and stored at 4°C. These lab-made beverages were inoculated with 1×10^7 CFU/mL ST5 and either 1×10^7 CFU/mL of R0175 or R0052. In addition to these ferment preparations, an unfermented SM and MB control was included to separate effects of dairy milk and soy milk alone, and a lactic acid acidified (pH of 4.70 ± 0.1) SM and MB control was included as well to determine if effects are due to lactic acid or other SCFAs.

Cultures and controls were incubated at 37°C until a pH of 4.70 ± 0.1 was reached or for a maximum of 24 hours. A total of 12 mL cultures and controls were placed into 15-mL screw-capped conical tubes (Starstedt, Newton, N.C., U.S.A.), cooled in an ice water bath for 30 minutes, and then frozen at -80°C in a cabinet air freezer (Thermo Forma model 995, Marietta, Ohio, U.S.A.) for 24 hours. The frozen cultures and controls were shipped in dry ice by rapid courier (less than 24 hours) and then maintained at -80°C until tested for bioactivity.

Since cultures were prepared in one site and tested in another, it was necessary to freeze and store samples. Preliminary studies determined the effects of fermentation substrate and storage on culture viability (Wagar *et al*, 2009). The 15 mL tubes were removed from -80°C, partially thawed and aseptically divided into aliquots, on ice to prevent a complete thaw, into 1.5 mL tubes (approximately 400 µL per tube) and immediately refrozen. For assays, aliquots were removed from -80°C and thawed at room temperature. Thawed ferments were aseptically homogenized using a Dremel® 4.8V MiniMite Cordless (Bosch Gear, Madison Heights, M.I., U.S.A.; model #750) power tool fitted with a conical blending attachment (See Appendix E), set to “HI” for approximately 5 to 10 seconds. Ferment dilutions were carried out in RPMI 1640.

Cell Culture

All reagents used for cell culture were supplied by Sigma-Aldrich Canada unless otherwise noted. All cells were grown in 250 mL, 75 cm^2 tissue culture flasks (Cellstar, Greiner Bio-One, Mississauga, Ontario, Canada). Cultures were maintained at 37°C, 5% CO_2 in a humidified incubator (Thermo Corporation, Toronto, Canada).

The THP-1 monocytic leukemia suspension cell line (ATCC# TIB-202) was obtained from the American Type Culture Collection (ATCC). Cultures of THP-1 were maintained in media consisting of RPMI 1640 medium supplemented with 10% FBS, 0.05 mM β-mercaptoethanol, and 0.05 mg/mL gentamycin sulphate. Sub-culturing of cells was carried out every 6-7 days by rinsing the bottom face of the flask with already-present growth media. Approximately 50% of the media in the flask was transferred to a new flask, at an inoculation of approximately 50% by volume. In preparation for bioassays, growth media was removed by centrifugation at 300 × *g* at 4°C for 10 minutes. Cell pellets were resuspended in RPMI 1640.

The U937 histiocytic lymphoma suspension cell line (ATCC# CRL-1593.2) was obtained from the ATCC. Cultures of U937 cells were maintained in media consisting of RPMI 1640 medium supplemented by 10% FBS, 1 mM sodium pyruvate, 0.05 mM β-mercaptoethanol, 2 mM L-glutamine and 0.05 mg/mL gentamycin sulphate. Sub-culturing of cells was carried out every 2-4 days by rinsing the bottom face of the flask with the already-present growth media.

Approximately 20-50% (depending on cell confluency) of the media in the flask was transferred to new flasks, at an inoculation of approximately 20-50% by volume. In preparation for bioassays, growth media was removed by centrifugation at 300 × *g* at 4°C for 10 minutes. Cell pellets were resuspended in RPMI 1640 media.

U937 TNF-α Challenge Kinetics

U937 monocytes were seeded in 96 well cell culture plate (Cellstar, Greiner Bio-One) at a concentration of 1×10^6 cells/mL and challenged at a final concentration of 5×10^5 cells/mL at initial viabilities greater than 80% (as determined by a Trypan-blue exclusion count (Sigma-Aldrich Canada)). A range of concentrations of TNF-α (PeproTech, Rocky Hill, N.J., U.S.A., Catalogue #300-01A) (1 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL) were tested to determine the optimal concentration for IL-8 induction. Cells were incubated with TNF-α at 37°C, 5% CO_2 for 4 hours. The lowest concentration of TNF-α which induced the greatest amount of IL-8 was used in all subsequent challenges. When an optimal concentration had been established, two additional time points (8 hours and 24 hours) was used to determine the time of optimal IL-8 production, using the established concentration of TNF-α. Plates were centrifuged at 300 × *g* for 10 minutes, supernatants aspirated, moved to a new plate and stored at -80°C until assayed. The time of incubation which produced the greatest amount of IL-8 was used in all subsequent challenges.

THP-1 LPS Challenge Kinetics

Monocytic THP-1 cells were seeded in 96 well cell culture plate (Cellstar, Greiner Bio-One) at a concentration of 1×10^6 cells/mL and challenged at a final concentration of 5×10^5 cells/mL at initial viabilities greater than 80% (as determined by a Trypan-blue exclusion count (Sigma-Aldrich Canada)). A range of concentrations of LPS serotype 026:B6 (Sigma-Aldrich Canada, Catalogue #L2654) (1 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL and 1000 ng/mL) were tested to determine the optimal concentration of IL-8 induction. Cells were incubated with LPS at 37°C, 5% CO_2 for 4 hours. The lowest concentration of LPS which induced the greatest amount of IL-8 was used in all subsequent challenges. When the optimal concentration had been established, two additional time points (8 hours and 24 hours) was used to determine the time of optimal IL-8 production, using the established concentration of LPS. Plates were centrifuged at 300 × *g* for 10 minutes, supernatants aspirated, moved to a new plate and stored at -80°C. The time of incubation which produced the greatest amount of IL-8 was used in all subsequent challenges.

Cytokine Kinetics

IL-8 protein had been previously shown to be optimally produced by TNF-α-treated U937 cells at 24 hours, so no time course was performed for this cytokine (See Results - Optimization of Cell Line and Stimulus Approaches for Testing Soy and Milk Ferment Bioactivity).

TNF-α challenged cells were seeded in 96 well cell culture plate (Cellstar, Greiner Bio-One) at a concentration of 1×10^6 cells/mL and challenged at a final concentration of 5×10^5 cells/mL at initial viabilities greater than 85% (as determined by Guava ViaCount Assay) and incubated for 24 hours, 48 hours and 72 hours. Plates were centrifuged at 300 × *g* for 10 minutes, supernatants aspirated then moved to a new plate and stored at -80°C. Supernatant designated for TGF-β1 ELISAs were placed in 96 well plates siliconized with SigmaCote® (Sigma-Aldrich Canada, Catalogue #SL2) to prevent TGF-β1 from adsorbing to the plates. Incubation times which produced optimal cytokine concentrations were used in all subsequent challenges.

U937 Differentiation Assay

Three different agents were compared for their effects on U937 differentiation: Phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich Canada, Catalogue #P1585), 1,25-dihydroxyvitamin D_3 (vitamin D_3; Sigma-Aldrich Canada, Catalogue #D1530) or all-trans retinoic acid (ATRA; Sigma-Aldrich Canada, Catalogue #R2625). As shown in Table 2, the most consistently used final concentrations of PMA, vitamin D_3 and ATRA for monocyte differentiation are 0.16 µM, 0.1 µM and 1 µM, respectively. 100x stock solutions were initially prepared in anhydrous ethyl alcohol and stored at -20°C. From this, 10x stocks were prepared in RPMI 1640 to a final volume of 1000 µL and stored at -20°C. A 1/10 dilution of the 10x stock was made by diluting with 1×10^6 cells/mL U937 cell culture suspension and incubated for 24 hours and 48 hours at 37°C,

5% CO_2 in a 24 well plate. Differentiation was confirmed by immunophenotyping for increased CD11b expression (see Cell Surface Marker Expression section), in order to determine which agent was most effective. Differentiated U937s were not passaged; new batches of cells were prepared each time as follows: 1000µL (one tube) of 10x stock was added to 9 mL of U937 cell suspension at a concentration of approximately 5×10^5 - 1×10^6 cells/mL and incubated in a new T-75 flask for the specified incubation time. In preparation for bioassays, growth media containing the differentiation agent was removed by centrifugation at 300 × *g* at 4°C for 10 minutes. Cell pellets were resuspended in RPMI 1640 media.

Normal Gravity Ferment Bioassays

TNF-α challenged non-differentiated monocytic or differentiated macrophage-like U937 cells were seeded in 96 well or 24 well cell culture plates (Cellstar, Greiner Bio-One) at concentration of 1×10^6 cells/mL (1×10^5 cells per well) at initial viabilities greater than 80% (as determined by a Trypan-blue exclusion count (Sigma-Aldrich Canada) or Guava ViaCount assay). 100 µL of diluted ferments and controls were added, for a final cell density of 5×10^5 cells/mL and ferment/control dilution of 1/100. Cells were incubated at 37°C, 5% CO_2 for previously determined lengths of time. Plates were centrifuged at 300 × *g* for 10 minutes, supernatants aspirated then moved to a new plate and stored at -80°C until assayed. Supernatant designated for TGF-β1 ELISAs were placed in 96 well plates siliconized with SigmaCote®.

Simulated Microgravity Ferment Bioassays

TNF-α challenged non-differentiated monocytic or differentiated macrophage-like U937 cells were diluted to a density of 5×10^5 cells/mL at initial viabilities greater than 80% (as determined by Guava ViaCount assay). An equal volume of diluted ferments and controls were added, for a final cell density of 2.5×10^5 cells/mL and ferment/control dilution of 1/100. This cell mixture was seeded into a disposable 10 mL HARV cell culture vessel, at a vessel rotation speed of ~15 rpm (personal communication, M. Hughes-Fulford), and incubated at 37°C, 5% CO_2 for the optimal time determined for each cytokine/cell surface marker. Cell suspensions were collected in a 15 mL conical tube and centrifuged at 300 × *g* for 10 minutes. Aliquots of supernatant were placed into 1.5 mL Eppendorf tubes and stored at -80°C until assayed. Supernatant designated for TGF-β1 ELISAs were placed in 1.5 mL Eppendorf tubes siliconized with SigmaCote®.

Quantification of IL-8, TGF-β1, and IL-10 protein by ELISA

All buffers and reagents were prepared and used in accordance with the manufacturer's protocol for IL-8 (BioSource, San Diego, California, Catalogue #CHC1303), TGF-β1 (R&D Systems, Minneapolis, Minnesota, Catalogue #DY240), and IL-10 (BioSource, Catalogue #CHC1323) ELISA (Enzyme-linked Immunosorbent Assay) kits. Polystyrene, high binding, Microlon® 600 ELISA

plates (Greiner bio-one, Catalogue #655061) were used for all ELISA assays. Table 3 compares buffer compositions and protocols for each ELISA kit.

For the IL-8 ELISA, capture antibody was used at a concentration of 1 µg/mL in coating buffer. The working concentration of biotinylated anti- Human IL-8 detection antibody was 0.04 µg/mL. Horseradish peroxidase (HRP)-conjugated streptavidin was used at a 1/2500 dilution. 1.8 N H_2SO_4 was used as a stop solution.

For the TGF-β1 ELISA, capture antibody was used at a concentration of 2.0 µg/mL in phosphate buffered saline (PBS). Samples were first acidified with one part 1 N HCl to 5 parts sample for 10 minutes, then neutralized with a volume equal to that of the acid of 1.2 N NaOH/0.5 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The working concentration of biotinylated chicken anti-human TGF-β1 was 300 ng/mL. HRP-conjugated streptavidin was used at 1/200 dilution. 2 N H_2SO_4 was used as a stop solution.

For the IL-10 ELISA, capture antibody was used at a concentration of 1 µg/mL in coating buffer. The working concentration of biotinylated anti- human IL-10 detection antibody was 0.16 µg/mL. HRP-conjugated streptavidin was used at a 1/1250 dilution. 1.8 N H_2SO_4 was used as a stop solution.

Human recombinant IL-8, TGF-β1 and IL-10 were used as standards in all ELISAs and all plates were read at a wavelength of 450 nm (reference absorbance: 650 nm) using a plate reader (Bio-Tek Instrumentation, Nepean,

Ontario), data collected with KC4 program for Windows (Bio-Tek) and compiled using Microsoft® Excel® (Microsoft Corp., Redmond, Wash., U.S.A.).

Cell Viability – Guava ViaCount Assay

A Millipore® Guava Personal Cell Analysis (PCA; Millipore, Billerica, MA, Catalogue #0500-1090) was used for all ViaCount assays. Cytosoft™ Software was used for data acquisition and analysis. The Guava PCA Instrument contains a laser, which operates at 532 nm and emits 7 mW visible laser radiation. ViaCount Reagent (Millipore, Catalogue #4000-0041) differentially stains viable and non-viable cells. Two dyes are used (a membrane-permeable dye that stains all nucleated cells, and another membrane-impermeable dye that stains damaged cells) to discriminate between viable and non-viable cells. The Guava PCA then detects forward angle light scatter to distinguish between free nuclei and cellular debris to give an accurate total and viable cell count. The Cytosoft™ software utilizes an "EasyFit" feature, which uses a computer-assisted population analysis method, in lieu of traditional cell gating. EasyFit allows better discrimination between viable cells, non-viable cells and debris when those populations overlap because the analysis is performed in three dimensions. This is useful when there is debris in the sample, such as ferment particulates and bacteria.

For viability assays, 560 µL of 1×10^6 cells/mL cell suspension was added to a 24 well plate. Dilutions of ferments and controls were added for a final

ferment/control dilution of 1/100 and a final cell concentration of 5×10^5 cells/mL. Plates were incubated for 24 hours at 37°C, 5% CO_2. Cells from HARV vessels were collected after centrifugation. Samples which contained large, visible debris were filtered though a BD Falcon™ 70 µm cell strainer (BD Biosciences, San Jose, C.A., U.S.A., Catalogue #352350). Cell samples were diluted 1/20 with ViaCount Reagent and incubated at room temperature for 5 minutes. The Guava PCA was calibrated before reading samples using the Guava Check software and Guava Check kit (Millipore, Catalogue #4500-0020). Data was collected (1000-2000 events collected) using the Guava ViaCount software, and exported to Microsoft® Excel® for analysis.

Cell Surface Marker Expression – Guava Express Assay

A Millipore® Guava PCA was used for all cell surface marker assays. Cytosoft™ Software was used for data acquisition and analysis. The Guava Express assay uses two fluorescence parameters along with forward scatter to identify cells expressing specific cell surface molecules. Fluorophore-conjugated antibodies are able to bind to specific surface molecules on the cells, labelling them, and the Guava PCA can detect the intensity of fluorescence labels on individual cells and the number of cells labelled.

560 µL of 1×10^6 cells/mL cell suspension was added to a 24 well plate. Dilutions of ferments and controls were added for a final ferment dilution of 1/100 and a final cell concentration of 5×10^5 cells/mL. Plates were incubated for 24

hours at 37°C, 5% CO_2. Samples which contained large, visible debris were filtered though a BD Falcon™ 70 µm cell strainer (BD Biosciences, Catalogue #352350). 1×10^6 pre-treated cells in a volume of 100 µL to 300 µL were added to a 1.5 mL tube and centrifuged at 300 × *g* for 10 minutes. Cell media was aspirated and cell pellet resuspended in 100 µL to 150 µL Double Protein Cell Staining Buffer (DPCSB; 5% FBS, 5% BSA in PBS, pH 7.4). 10-15 µL of 0.5 mg/mL human IgG1, κ (Sigma-Aldrich Canada, Catalogue #I5154) or human IgG2, κ (Sigma-Aldrich Canada, Catalogue #I5404), as appropriate (See Table 4), were added (to block non-specific binding though Fc receptors and reduce background) and incubated for 10 minutes. 20 µL of phycoerythrin (PE) labeled anti-CD54 (BioLegend, San Diego, CA, Catalogue #322708), PE labeled anti-CD58 (BioLegend, Catalogue #322506), PE labeled anti-CD80 (BioLegend, Catalogue #305208), PE labeled anti-CD86 (BioLegend, Catalogue #305406) or PE labeled anti-CD11b (BioLegend, Catalogue #301306) were added to the cell suspension, carried out in a darkened laminar flow hood. PE labeled mouse IgG1, κ (BioLegend, Catalogue #400114) and PE labeled mouse IgG2b, κ (BioLegend, Catalogue #400314) isotype control antibodies, as appropriate (See Table 4), were added to separate tubes as an internal control. Labeled cell suspensions were incubated on ice, in the dark, for 30 minutes. To remove non-specific binding, cells were washed twice in 1 mL DPCSB, then resuspended in 400 µL of DPCSB (or less if the cell pellet was particularly small), and incubated for 5 minutes on ice, in the dark. Data was collected using the Guava Express

software (2000 events collected), and exported to Microsoft® Excel® for analysis. Results are reported in mean fluorescence intensity (MFI).

Statistical Analysis

Data presented as means ± standard error of the mean (SEM) was analyzed using a one-way analysis of variance (ANOVA). Pre-treatment or negative control data (with the exception of viability results) was excluded from the ANOVA. Results indicating significance (values of $p < 0.05$) were then analyzed using Tukey-Kramer Multiple Range Test for further comparison of means. If a Tukey-Kramer Multiple Range Test was not able to delineate differences, a Dunnett's post test was used, with challenged cell controls as the comparison.

For ELISAs, each sample was tested in triplicate in each ELISA and assigned an average value. Therefore, a value represented as $n = 1$ is equivalent to a triplicate average for any single sample.

RESULTS

Optimization of Cell Line and Stimulus Approaches for Testing Soy and Milk Ferment Bioactivity

U937 monocytes required 20 ng/mL of TNF-α for optimal IL-8 production (Figure 1), with a 24 hour incubation time (Figure 2). THP-1 monocytes required 100 ng/mL LPS (Figure 3), also with a 24 hour incubation time (Figure 4).

A 48 hour incubation time was required for optimal TGF-β1 production. IL-10 concentrations produced by U937s or differentiated U937s at normal gravity were below the limit of detection by ELISA and although initially tested for, were not considered for further study.

Although initial results suggested that milk ferments prepared with ST5 + R0175 decreased IL-8 production in LPS-challenged THP-1 monocytes (Figure 5), it was noted that unfermented milk controls had marked effects suggesting THP-1 responses to milk controls. The THP-1 cell line also grows very slowly compared to U937s. In addition, the U937 cell line is noted for its ability to be differentiated to macrophage stage in response to several differentiation agents. For these reasons, the U937 line was selected for further study of the effects of soy and milk ferments at different stages of differentiation.

Effects of soy and milk ferments and controls were initially tested at a dilution range of 1/10, 1/100 and 1/1000 for their effects on TNF-α-induced IL-8 production by non-differentiated U937 cells. At a 1/10 dilution, soy and milk

controls had marked effects on IL-8 production, and at a 1/1000 dilution, ferment effects were diluted out (data not shown). Therefore the 1/100 dilution was selected for further study of the effects of soy and milk ferments.

U937 Cells Can Be Differentiated by All-Trans Retinoic Acid

U937 monocytic cells incubated with 1 µM ATRA for 24 hours showed the greatest increase in CD11b expression (Figure 6), and were chosen for future study. Treatment with 0.16 µM PMA for 24 and 48 hours did not increase CD11b expression, and treatment with 0.1 µM Vitamin D_3 for 24 and 48 hours did not increase CD11b expression to the extent of treatment with 1 µM ATRA. 48 hour treatment with PMA, ATRA or Vitamin D_3 did not increase CD11b expression compared to 24 hour treatment. For this reason, a 24 hour incubation with 1 µM ATRA was chosen as the optimal differentiation agent for U937 treatments.

Effects of Soy and Milk Ferments on Cytokine Production by Non-Differentiated and Differentiated U937 Cells

IL-8 production by non-differentiated U937s in response to TNF-α was not significantly affected by the presence of milk ferments or controls (Figure 7). However, soy ferment prepared with ST5 + R0175 did significantly decrease IL-8 production relative to TNF-α treated controls (Figure 8). Soy controls and soy ferment prepared with ST5 + R0052 also decreased IL-8 production to some extent but this did not reach statistical significance. In contrast, milk ferments significantly decreased IL-8 production by differentiated U937 cells (Figure 9)

relative to unfermented milk and acidified milk controls but not relative to TNF-α treated cell controls. Differentiated U937s treated with TNF-α and milk controls produced surprisingly high levels of IL-8, and this effect was also observed for differentiated U937s treated with soy controls. While milk ferments produced a decrease in IL-8 production relative to the milk controls, differentiated U937s treated with soy controls and soy ferments did not show this same decrease in TNF-α-induced IL-8 production (Figure 10).

Constitutive TGF-β1 production by differentiated U937 cells was higher than for non-differentiated U937 cells. While TNF-α treatment increased TGF-β1 production by non-differentiated U937s, it had the opposite effect on differentiated U937s (Figure 11).

TGF-β1 secretion by non-differentiated U937s was significantly decreased by milk ferments and milk controls relative to TNF-α treated control cells (Figure 12) but was not significantly decreased by soy ferments or soy controls (Figure 13). In contrast, soy ferments decreased TGF-β1 secretion by differentiated U937s relative to acidified soy controls but not relative to challenged controls (Figure14). Milk ferments or milk controls did significantly not affect TGF-β1 secretion by differentiated U937s (Figure 15).

Effects of Soy and Milk Ferments on Cell Viability by Non-Differentiated and Differentiated U937 Cells

Viability of non-differentiated U937s was significantly decreased by milk fermented with ST5 + R0052 (Figure 16) but not by soy fermented with these

strains (Figure 17). Viability of differentiated U937 cells was not significantly affected by the presence of milk ferments or controls (Figure 18). Minor effects were noted with soy ferments, as indicated by a significant ANOVA result ($p = 0.0092$) but no differences were detected by the Tukey-Kramer multiple range test (Figure 19). The ANOVA result may reflect slightly lower viability of ferments relative to soy controls, as the soy controls values appear slightly higher than the challenged control values. However, the ferments did not appear to decrease viability to any marked extent relative to TNF-α treated controls (Figure 19).

Effects of Soy and Milk Ferments on Cell Surface Marker Expression on Non-Differentiated and Differentiated U937 Cells

Soy controls and soy ferments significantly down-regulated CD54 expression on non-differentiated U937 (Figure 20). Milk ferments significantly down-regulated CD54 expression on non-differentiated U937s (Figure 21) (Dunnett's post test used to show why ANOVA was significant), while milk controls had no significant effect.

Overall, the MFI for CD58 was lower than the MFI for CD54, reflecting a lower level of expression of this cell surface molecule. Soy ferments significantly decreased CD58 expression on non-differentiated U937s relative to TNF-α treated controls (Figure 22), although some decrease was also observed with soy controls. There was a trend ($p = 0.0644$) towards milk ferments decreasing

CD58 expression on non-differentiated U937s (Figure 23) and the overall pattern of the response was similar to that observed for effects on CD54 expression.

On differentiated U937s, CD54 expression was significantly decreased by milk ferments and soy controls and ferments (Figure 24 and 25). CD58 expression was significantly decreased by soy ferments (Figure 26) although soy controls also decreased expression, and there was a trend ($p = 0.0788$) towards milk ferments decreasing CD58 expression on differentiated U937s (Figure 27).

CD80 expression was not affected by any ferments or controls at either differentiation stage (Figure 28, 29, 30 and 31) and levels of CD80 expression were essentially negative for all conditions tested.

There was some increase in CD86 expression by TNF-α treated controls for both non-differentiated U937s and differentiated U937s. The highest levels of CD86 expression were observed with TNF-α treated differentiated U937s, where the MFI was 132.3 (isotype background subtracted), relative to an MFI of 20.5 (isotype background subtracted). This increase was not observed when either milk or soy ferments or controls were added, suggesting some decrease in CD86 expression due to treatment. With respect to differentiated U937s, addition of soy ferments decreased the MFI, while milk ferment addition decreased the MFI, even though this was not statistically significant (Figure 32, 33, 34 and 35).

Effects of Soy and Milk Ferments on Cytokine Production by Non-Differentiated and Differentiated U937 Cells under Simulated Microgravity Conditions

Soy ferments did not affect IL-8 production by non-differentiated or differentiated U937s in simulated microgravity (Figure 36 and 37) but there was some decrease observed for non-differentiated U937s treated with milk controls and ferments (Figure 38). Milk fermented with ST5 + R0052 significantly decreased IL-8 production by differentiated U937s compared to treatment with acidified milk controls (Figure 39).

Conversely, milk controls and ferments did not affect TGF-β1 production by non-differentiated or differentiated U937s (Figure 40 and 41). There was a trend ($p = 0.0582$) towards soy ferments decreasing TGF-β1 by non-differentiated U937s (Figure 42), and a slight decrease in TGF-β1 production by differentiated U937s treated with unfermented soy and both ferments (Figure 43), although this was not statistically significant.

Effects of Soy and Milk Ferments on Cell Viability of Non-Differentiated and Differentiated U937 Cells under Simulated Microgravity Conditions

Soy controls and ferments did not significantly affect non-differentiated or differentiated U937 cell viability (Figure 44 and 45). Milk fermented with ST5 + R0175 significantly decreased cell viability compared to challenged cell control in differentiated U937s (Figure 46). Milk fermented with ST5 + R0052 decreased

non-differentiated U937 cell viability (Figure 47). As the Tukey-Kramer post test did not delineate the differences between conditions, even though the ANOVA was significant, a Dunnett's post test was used to determine the reason why the ANOVA was significant.

Effects of Soy and Milk Ferments on Cell Surface Marker Expression in Non-Differentiated and Differentiated U937 Cells under Simulated Microgravity Conditions

In non-differentiated U937s, soy controls and soy ferments showed a trend ($p = 0.0856$) to decreased CD54 expression (Figure 48). Both milk ferments decreased CD54 expression from 1058.1 MFI in TNF-α treated controls to between 268.5 for milk fermented with ST5 + R0175 to 282.2 for milk fermented with ST5 + R0052, although this was not statistically significant (Figure 49). In differentiated U937s, there is also a trend ($p = 0.0800$) towards soy controls and soy ferments decreasing CD54 expression (Figure 50) while acidified milk and both milk ferments showed a significant decrease in CD54 expression compared to TNF-α challenged controls (Figure 51).

Soy and milk controls and ferments all significantly decreased CD58 expression by non-differentiated U937s (Figure 52 and 53) although the lowest MFI levels were observed for cells treated with milk ferments (Figure 53). Differentiated U937s treated with acidified soy and both soy ferments had decreased CD58 expression (Figure 54) (Dunnett's post test used to show why ANOVA was significant). There was a significant decrease in CD58 expression

when treated with acidified milk and both milk ferments (Figure 55) while unfermented milk controls had no effect.

CD80 expression was not affected in non-differentiated or differentiated U937s by either milk or soy controls or ferments (Figure 56, 57, 58 and 59).

In non-differentiated U937s, there was a slight increase in CD86 expression in the TNF-α challenged control, but this was not observed for U937s treated with in soy or milk controls or ferments (Figure 60 and 61). CD86 expression by non-differentiated U937 cells treated with soy ferment prepared with ST5 + R0175 was low (MFI = 8.5, isotype subtracted), in contrast to challenged cell control MFI values (MFI = 37.9, isotype subtracted), even though this was not statistically significant. However for differentiated U937s, there was a significant decrease in CD86 expression by cells treated with soy and milk ferments and controls (Figure 62 and 63).

Table 1: Summary of selected current literature concerning use of HARV cell culture vessels to simulate microgravity including cell type and duration of culture.

Cell type	Duration of incubation	Bioreactor Vessel	Author(s)
Human IEC cell line Int-407	28-32 days	HARV	Nickerson *et al*, 2001
Murine myoblast cell line C2C12 cells	6+ days	10 ml HARV	Marquette *et al*, 2007
Peripheral Blood Mononuclear Cell (PBMC)	6 days	RWV	Bruno *et al*, 2006
Primary keratocytes from fresh bovine eyes	19 days	50 ml HARV	Chen *et al*, 2007
E. coli MG1655	"least ten generations"	50 ml HARV	Tucker *et al*, 2007
Human peripheral blood lymphocytes	up to 24h	50 ml/10 ml HARV	Mognato *et al*, 2009
PBMC	24h	RWV	Ward *et al*, 2006
PBMC	up to 96h	RWV	Sundaresan *et al*, 2004
PBMC	--	10 ml HARV	Ritz *et al* 2005
PBMC	24 or 72 h	(not clear)	Sundaresan and Pellis, 2009
T- and B-lymphocytes	4h, 72h, 7 d	Clinostat	Kumari *et al*, 2009
Primary Splenocytes	24, 48, and 72 h	HARV	Simons *et al*, 2005

Table 2: Summary of the current literature concerning commonly used U937 monocytic cell differentiation agents, concentrations used, duration, and cell surface marker used to confirm differentiation.

Differentiation Agent	Con'c	Duration	Macrophage Marker	Author(s)
ATRA	1 µM	24h	CD11b	Zhang *et al*, 2008
ATRA	1 µM	-	-	Witcher *et al*, 2008
PMA	50 ng/mL	48h	-	Tahan *et al*, 2008
PMA	100 ng/mL	48h	-	Sintiprungrat *et al*, 2010
VitD3	0.1 µM	4h	CD11b	Liu *et al*, 1996
VitD3	0.1 µM	24h	CD14	Liu *et al*, 2005
PMA	0.16 µM	> 24h	CD14	Kulseth *et al*, 1997
ATRA	1 µM	> 24h	CD14	Kulseth *et al*, 1997
VitD3	0.1 µM	> 24h	CD14	Kulseth *et al*, 1997
ATRA	1 µM	48h	-	Dimberg *et al*, 2006
VitD3	0.1 µM	48h	-	Dimberg *et al*, 2006
PMA	0.16 µM	-	-	Zanocco-Marani *et al*, 2009
ATRA	1 µM	< 96h	CD11b	Yang *et al*, 2003
ATRA	1 µM	< 48h	CD11b	Lee *et al*, 2002

Table 3: Composition of assay solutions for IL-8, TGF-β1 and IL-10 ELISA kits.

Solution	IL-8 (Mass Per Litre)	TGF-β1 (Molar)	IL-10 (Mass Per Litre)
Coating Buffer	8.0 g NaCl, 1.13g Na_2HPO_4, 0.2g KH_2PO_4, 0.2g KCl, pH to 7.4	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 0.2 µm filtered	8.0 g NaCl, 1.13g Na_2HPO_4, 0.2g KH_2PO_4, 0.2g KCl, pH to 7.4
Assay Buffer	8.0 g NaCl, 1.13 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, 5.0 g BSA, pH to 7.4	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.4% BSA, 0.05% Tween-20, pH 7.4	8.0 g NaCl, 1.13 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, 5.0 g BSA, pH to 7.4
Wash Buffer	0.2 g KH_2PO_4, 1.9 g $K_2HPO_4{\cdot}3H_2O$, 0.4 g EDTA, 0.5 mL Tween-20, pH to 7.4	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.05% Tween-20, pH 7.4	0.2 g KH_2PO_4, 1.9 g $K_2HPO_4{\cdot}3H_2O$, 0.4 g EDTA, 0.5 mL Tween-20, pH to 7.4
Substrate Solution	1:1 H_2O_2:TMB	1:1 H_2O_2:TMB	1:1 H_2O_2:TMB
Stop Solution	1.8M H_2SO_4 (Excess)	2N H_2SO_4 (Excess)	1.8M H_2SO_4 (Excess)

Table 4: Isotype controls used for immunofluorescence analysis of cell surface marker expression on U937 cells.

Cell Surface Marker	Isotype Control
CD11b	IgG1,κ
CD54	IgG2b,κ
CD58	IgG1,κ
CD80	IgG1,κ
CD86	IgG2b,κ

Table 5: Synopsis of effects on cytokine production, viability and cell surface molecule expression by non-differentiated and differentiated U937 cells in normal versus simulated microgravity conditions treated with milk and soy ferments.

		Normal Gravity			Simulated Microgravity	
		IL-8	**TGF-β1**		**IL-8**	**TGF-β1**
Non-differentiated U937	Milk	–	↓	Milk	↓	–
	Soy	↓	–	Soy	–	↓
Differentiated U937	Milk	↓	–	Milk	↓	–
	Soy	–	↓	Soy	–	↓
		Viability				
Non-differentiated U937	Milk	↓ by Milk ST5 + R0052		Milk	↓ by Milk ST5 + R0052	
	Soy	–		Soy	–	
Differentiated U937	Milk	–		Milk	↓ by Milk ST5 + R0175	
	Soy	–		Soy	–	
		CD54/ 58/86	**CD80**		**CD54/ 58/86**	**CD80**
Non-differentiated U937	Milk	↓	–	Milk	↓	–
	Soy	↓	–	Soy	↓	–
Differentiated U937	Milk	↓	–	Milk	↓	–
	Soy	↓	–	Soy	↓	–

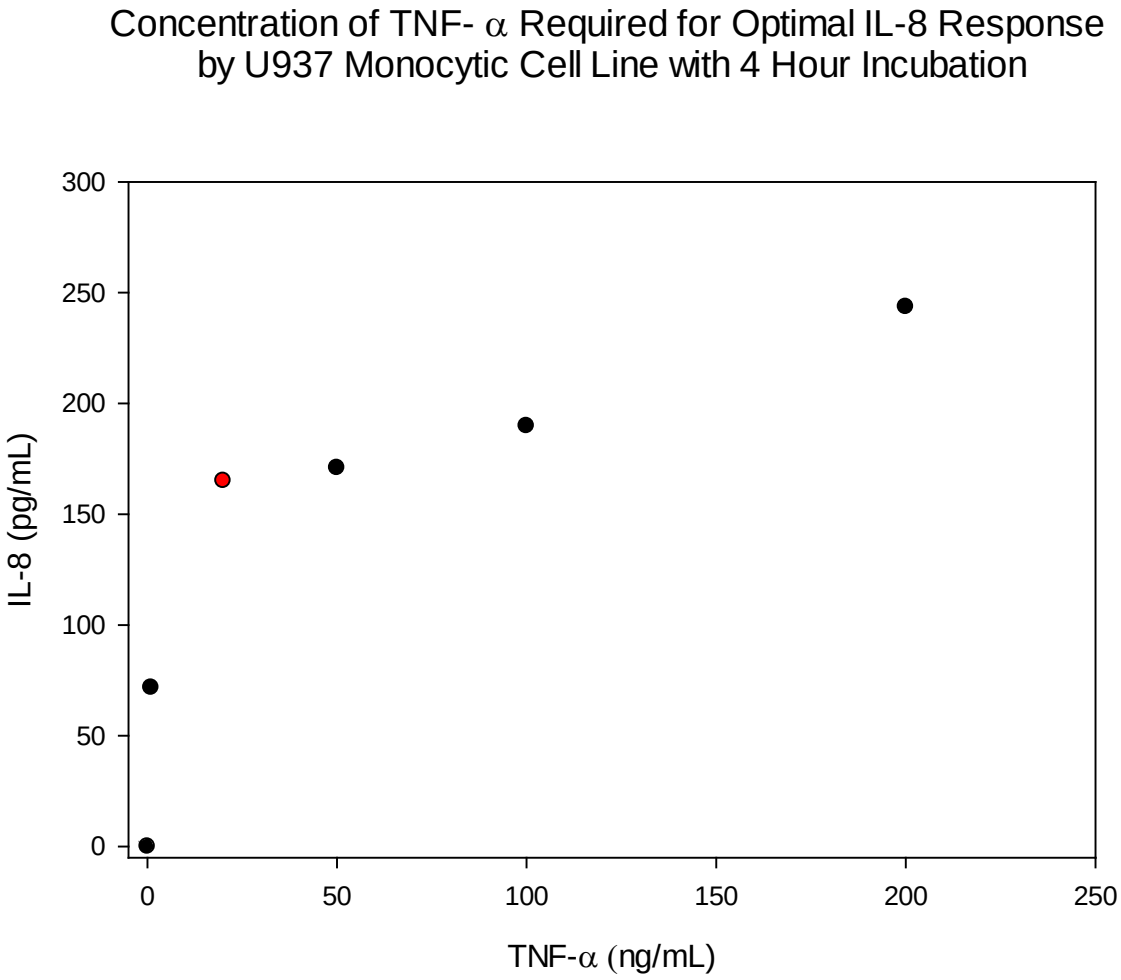

Figure 1. Effect of TNF-α concentration on IL-8 production by non-differentiated U937 monocytic cells (5×10^5 cells/mL) after a 4 hour challenge. (n = 1).

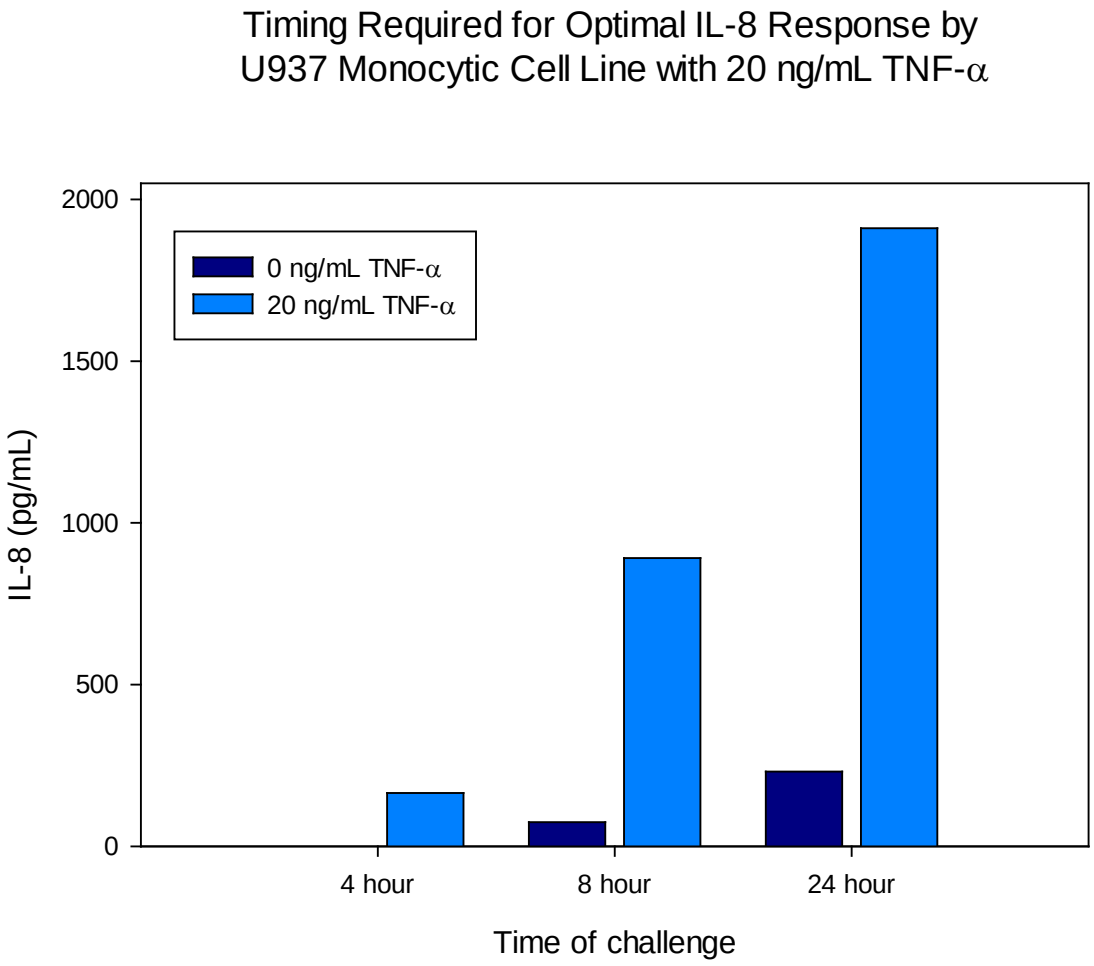

Figure 2. Time required for optimal IL-8 response by TNF-α challenged non-differentiated U937 monocytic cells (5×10^5 cells/mL). (n = 1).

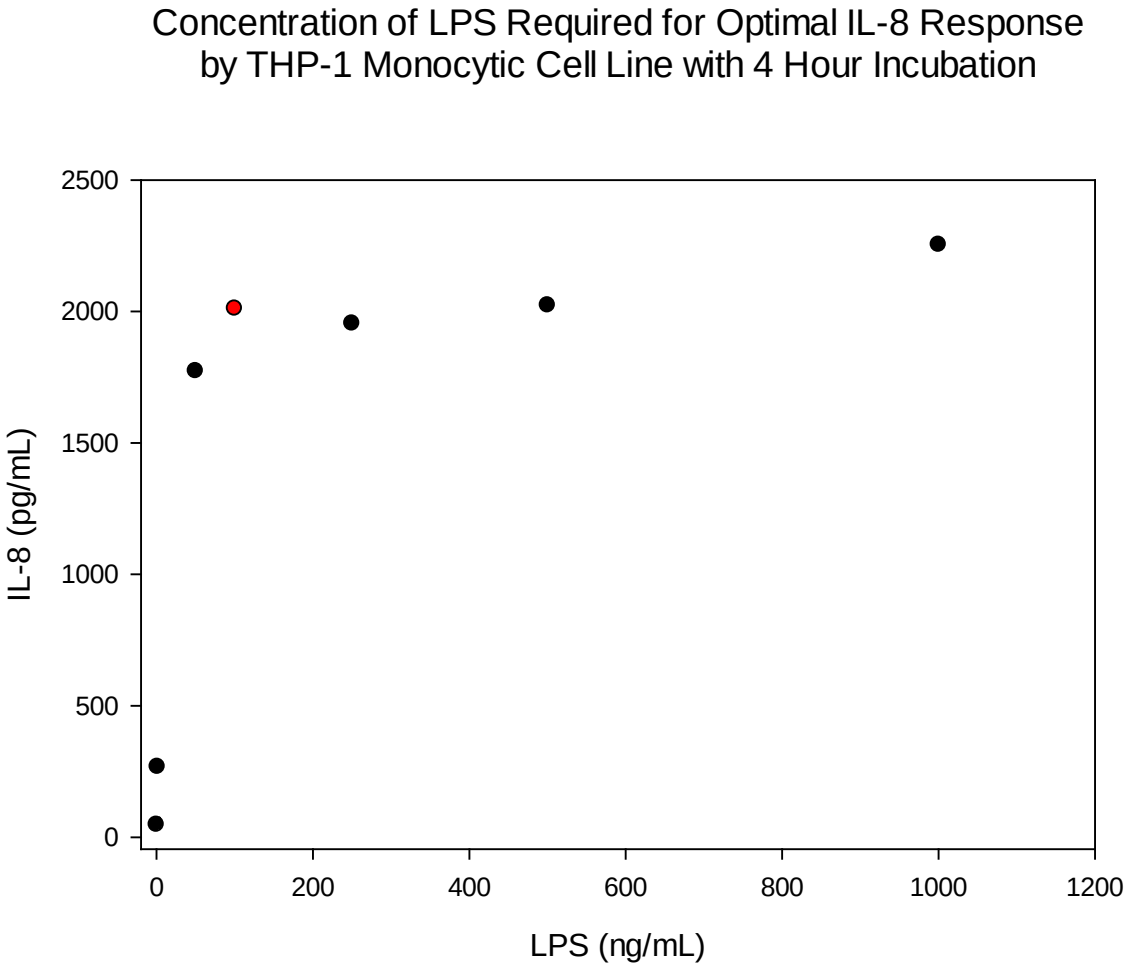

Figure 3. Effect of LPS concentrations on IL-8 production by THP-1 monocytic cells (5×10^5 cells/mL) after a 4 hour challenge. (n = 1).

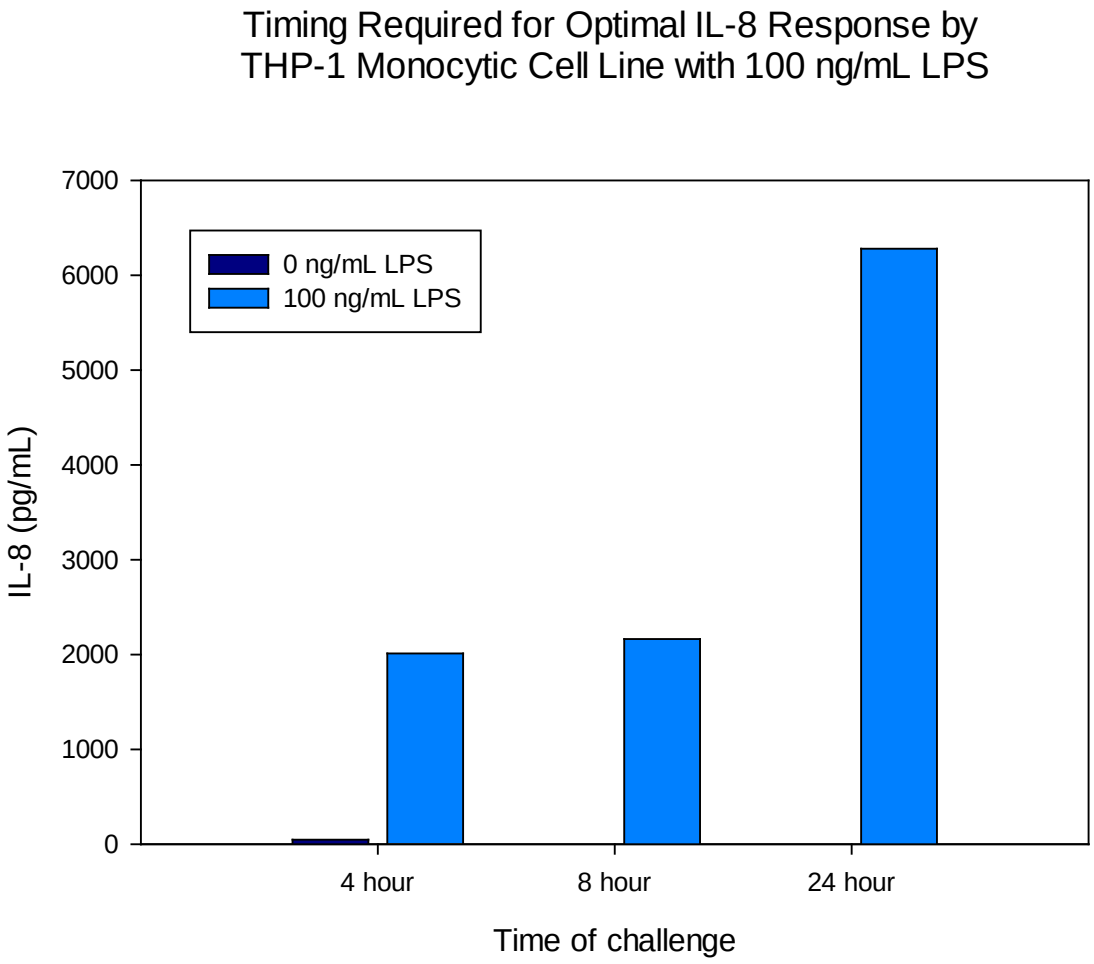

Figure 4. Time required for optimal IL-8 response to LPS challenged THP-1 monocytic cells (5×10^5 cells/mL). (n = 1).

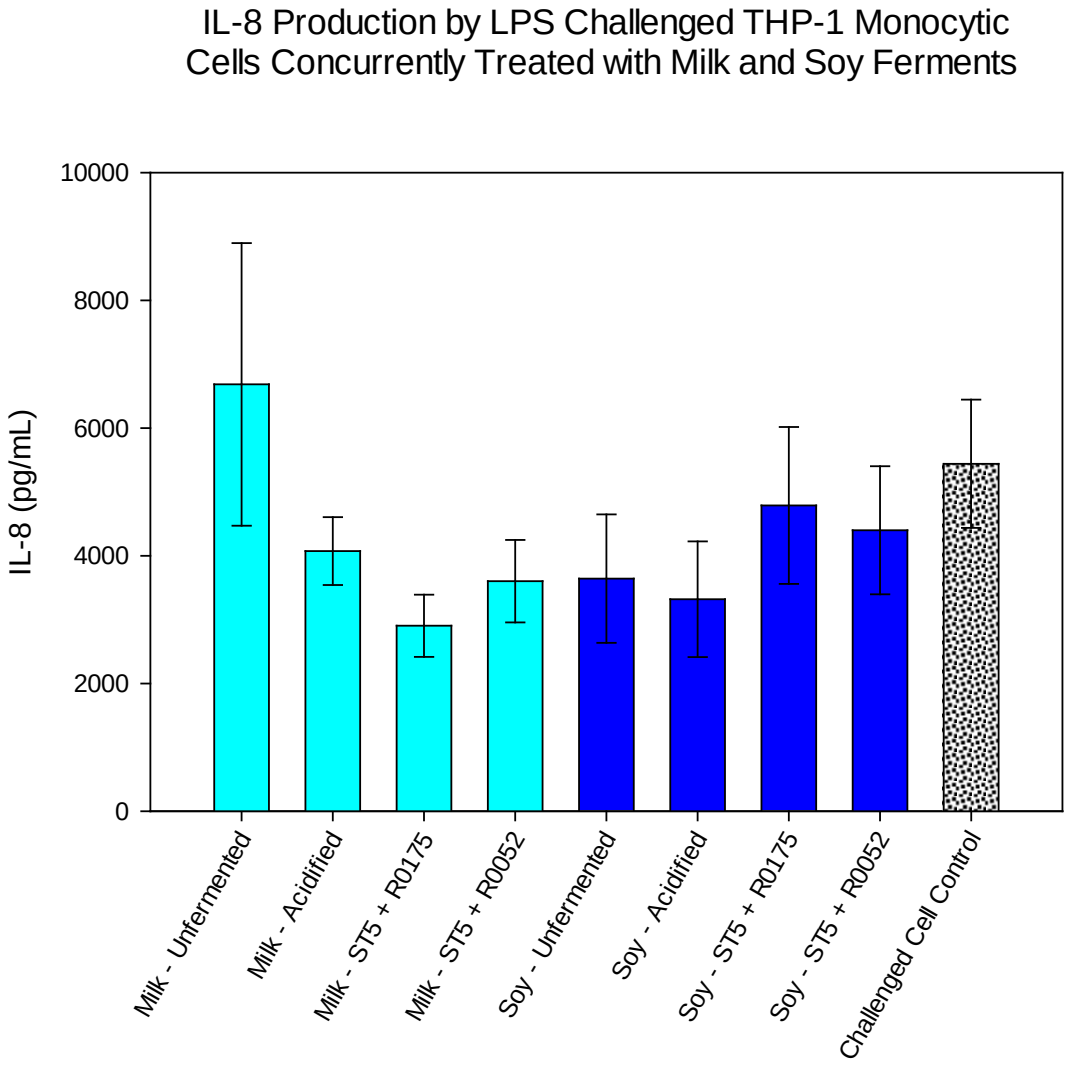

Figure 5: Effect of soy and milk ferments and controls (1/100 dilution in RPMI 1640) on IL-8 production by THP-1 monocytic cells (5×10^5 cells/mL) in the presence of 100 ng/mL LPS following concurrent incubation for 24 hours. Data shown as mean concentration in pg/mL ± SEM (n = 3). Unchallenged cell control produced 365.6 ± 220.5 pg/mL.

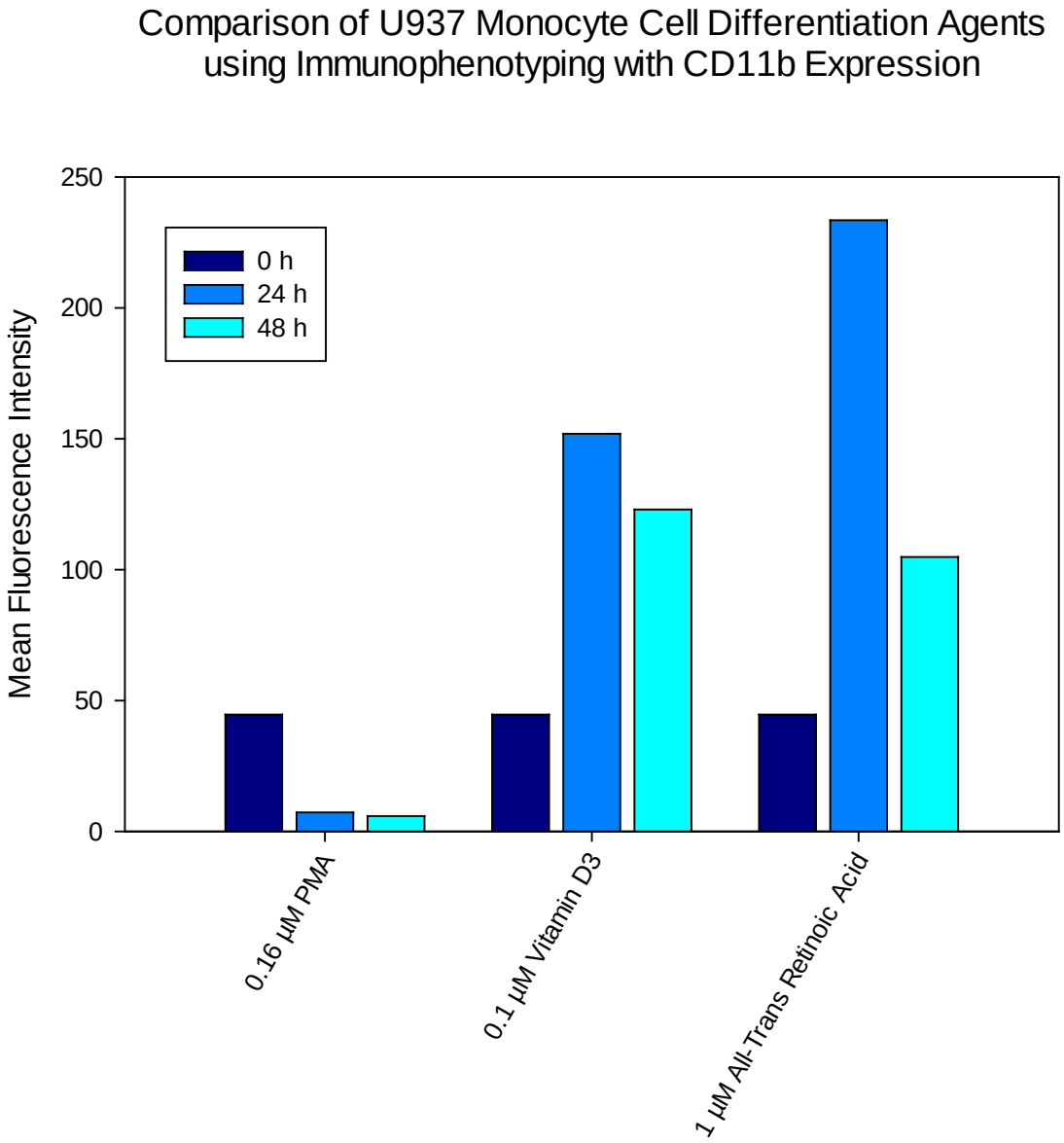

Figure 6: Effects of PMA, Vitamin D_3 and ATRA on U937 monocytic cell differentiation following incubation. U937 cells were collected after 24 hours and 48 hours and tagged with PE labeled anti-CD11b antibodies. Data shown as mean MFI (n = 1).

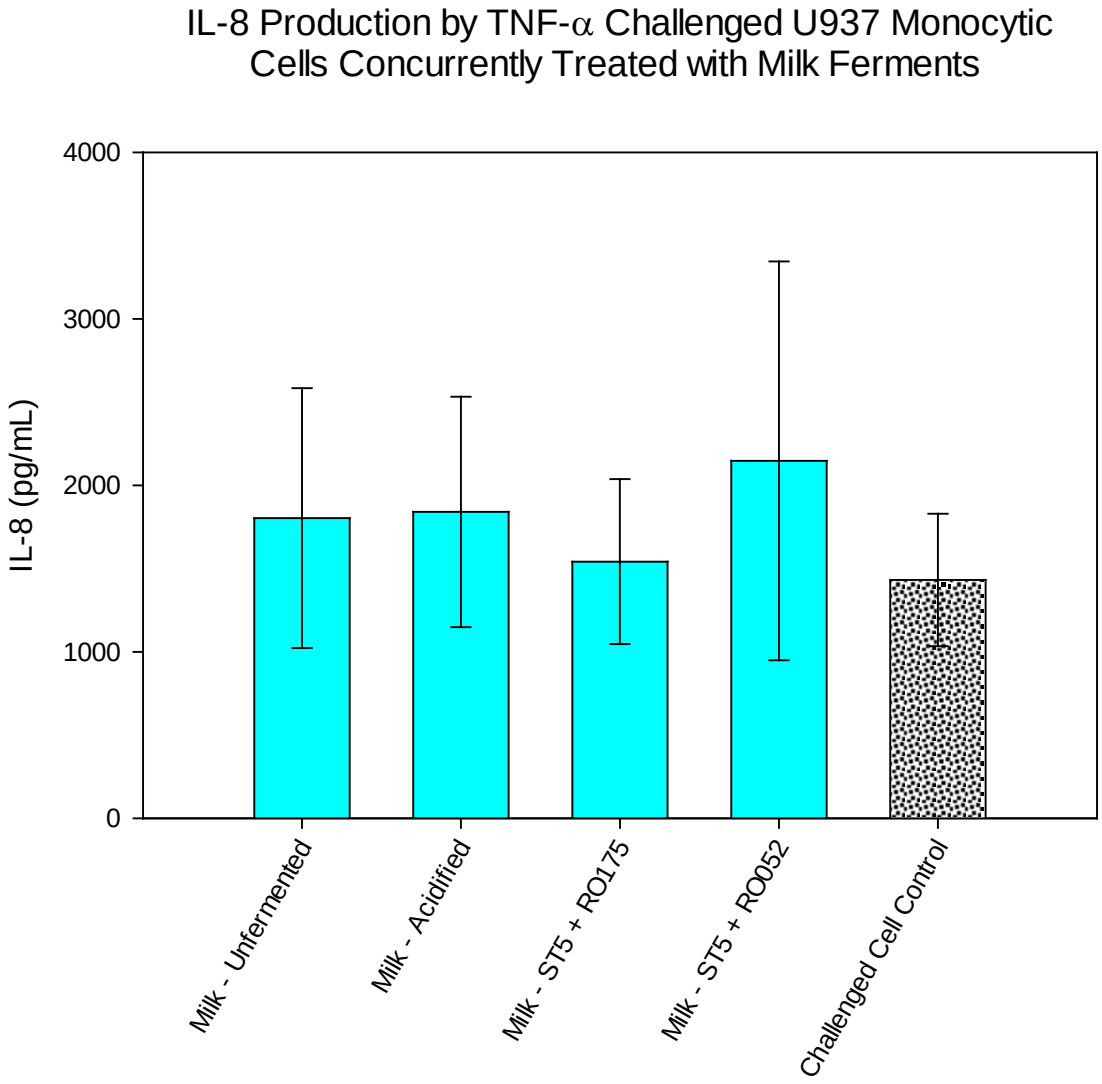

Figure 7: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on IL-8 production by non-differentiated U937 monocytic cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation of 24 hours. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; $p = 0.9663$; $n = 3$). Unchallenged cell control produced 86.7 ± 31.2 pg/mL.

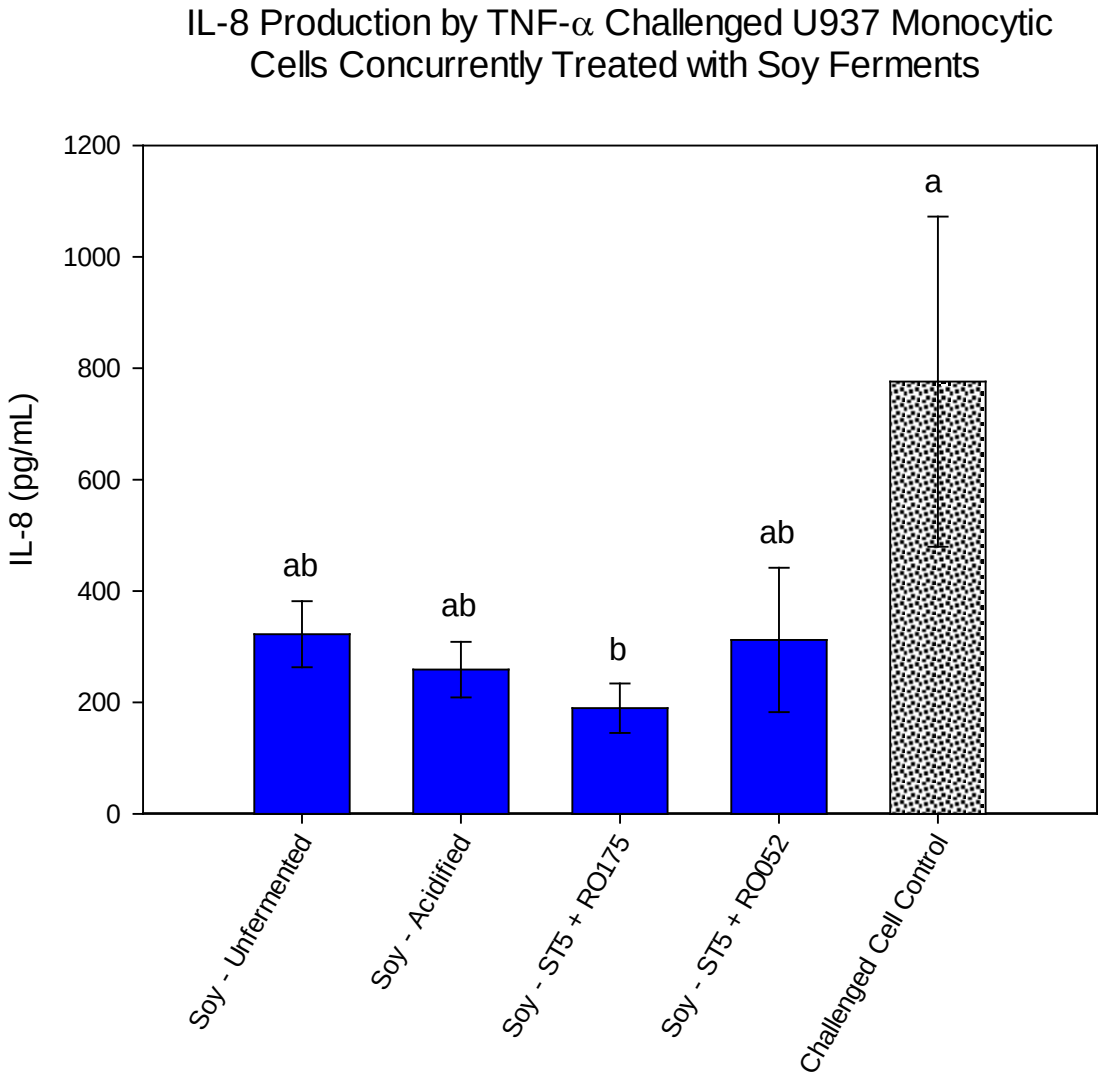

Figure 8: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on IL-8 production by non-differentiated U937 monocytic cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean concentration in pg/mL ± SEM) (One-way ANOVA; $p = 0.0390$; $n = 4$ for challenged cell controls and unchallenged cell controls, $n = 6$ for soy controls and soy ferments). Unchallenged cell control produced 49.5 ± 13.2 pg/mL.

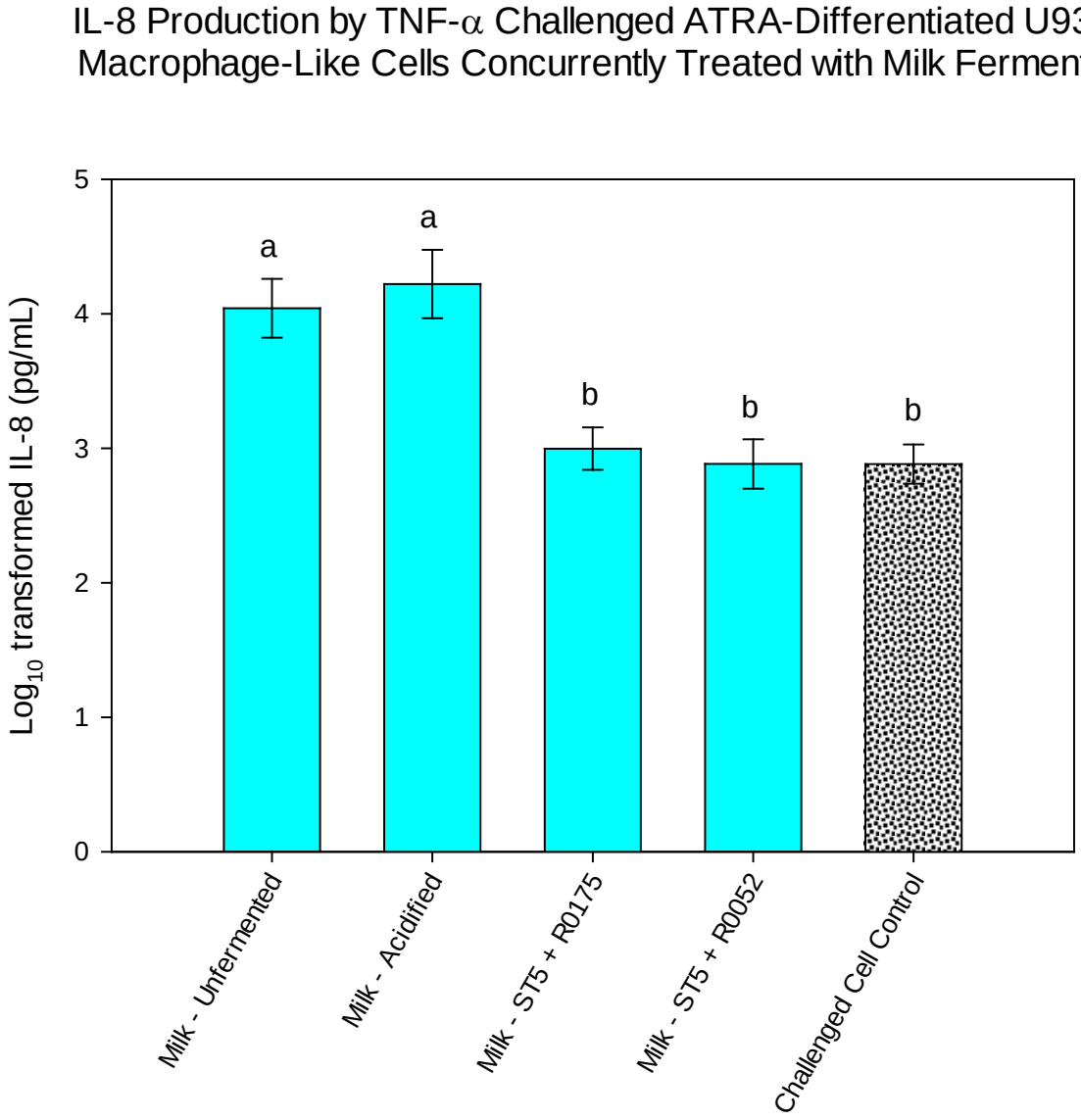

Figure 9: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on IL-8 production by ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as $\log_{10}$ concentration in pg/mL ± SEM. (One-way ANOVA; $p < 0.0001$; $n = 7$). Unchallenged cell control produced $\text{Log}_{10}1.1070 \pm \text{Log}_{10}0.21$ pg/mL.

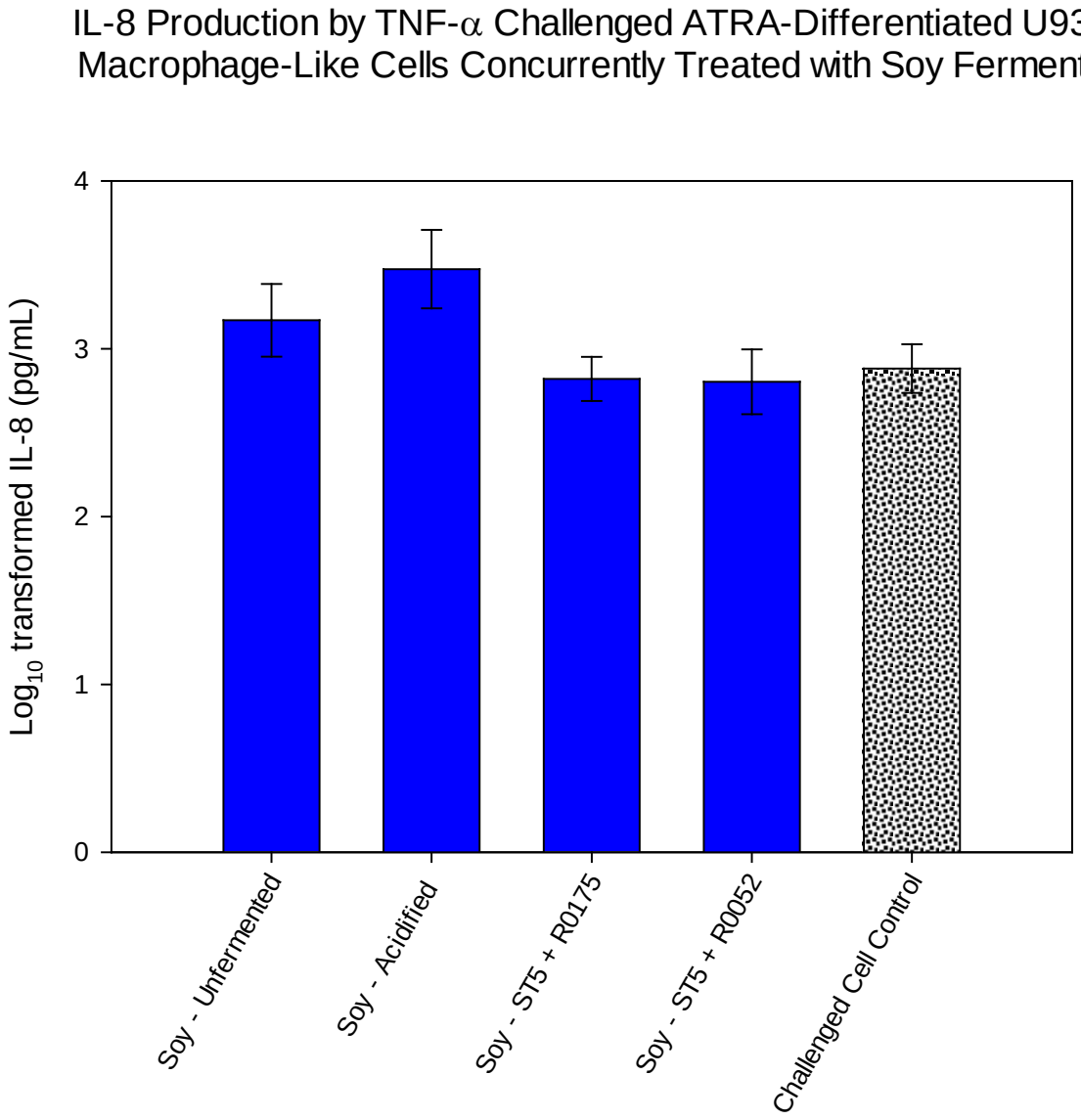

Figure 10: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on IL-8 production by ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as $\log_{10}$ concentration in pg/mL ± SEM. (One-way ANOVA; p 0.0767; n = 7). Unchallenged cell control produced $\text{Log}_{10}1.1070 \pm \text{Log}_{10}0.21$ pg/mL.

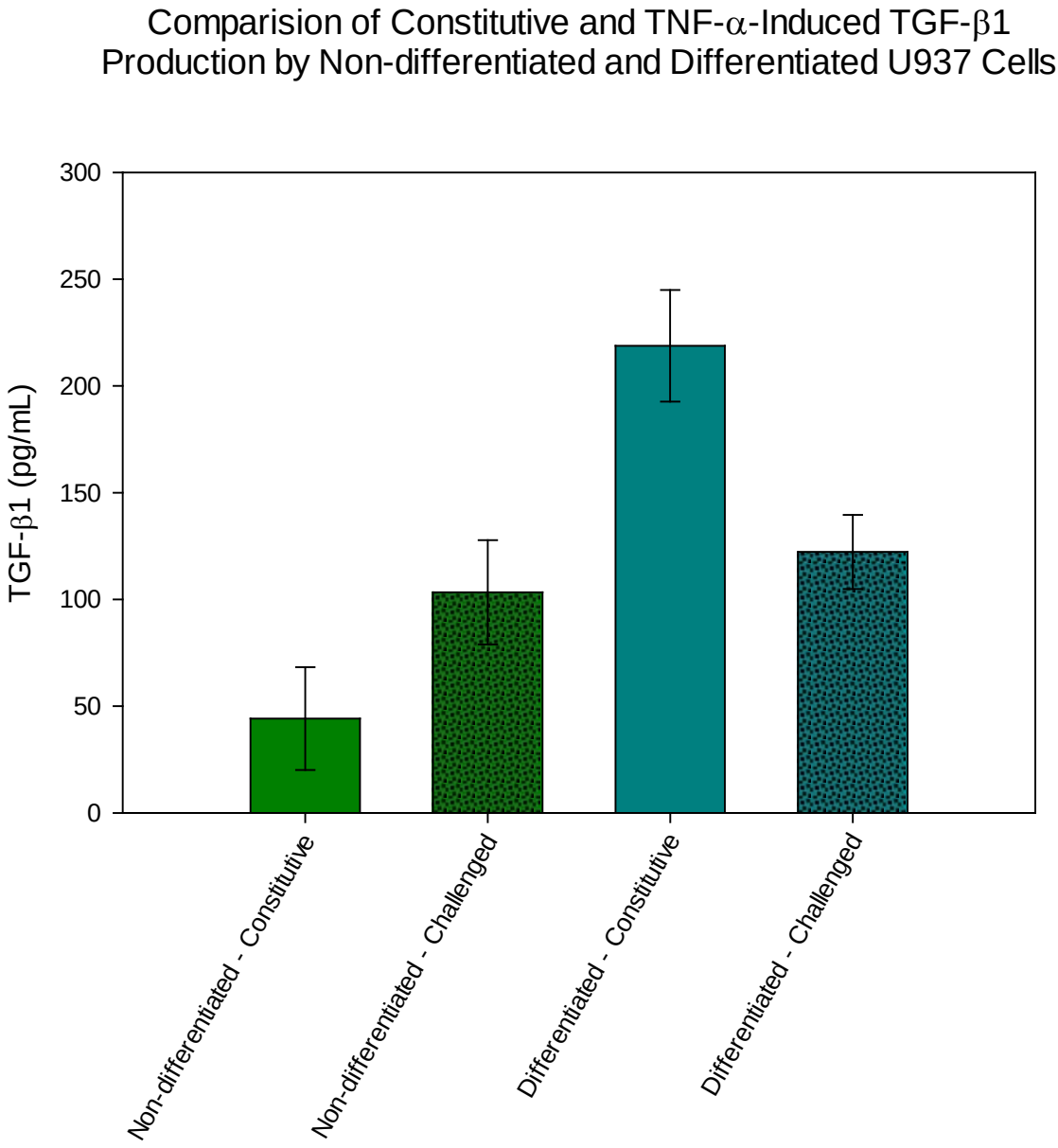

Figure 11: Comparison of TGF-β production by non-differentiated and differentiated U937 cells, unchallenged or challenged with 20 ng/mL TNF-α following concurrent incubation for 48 hours. Data shown as mean concentration in pg/mL ± SEM (n = 3).

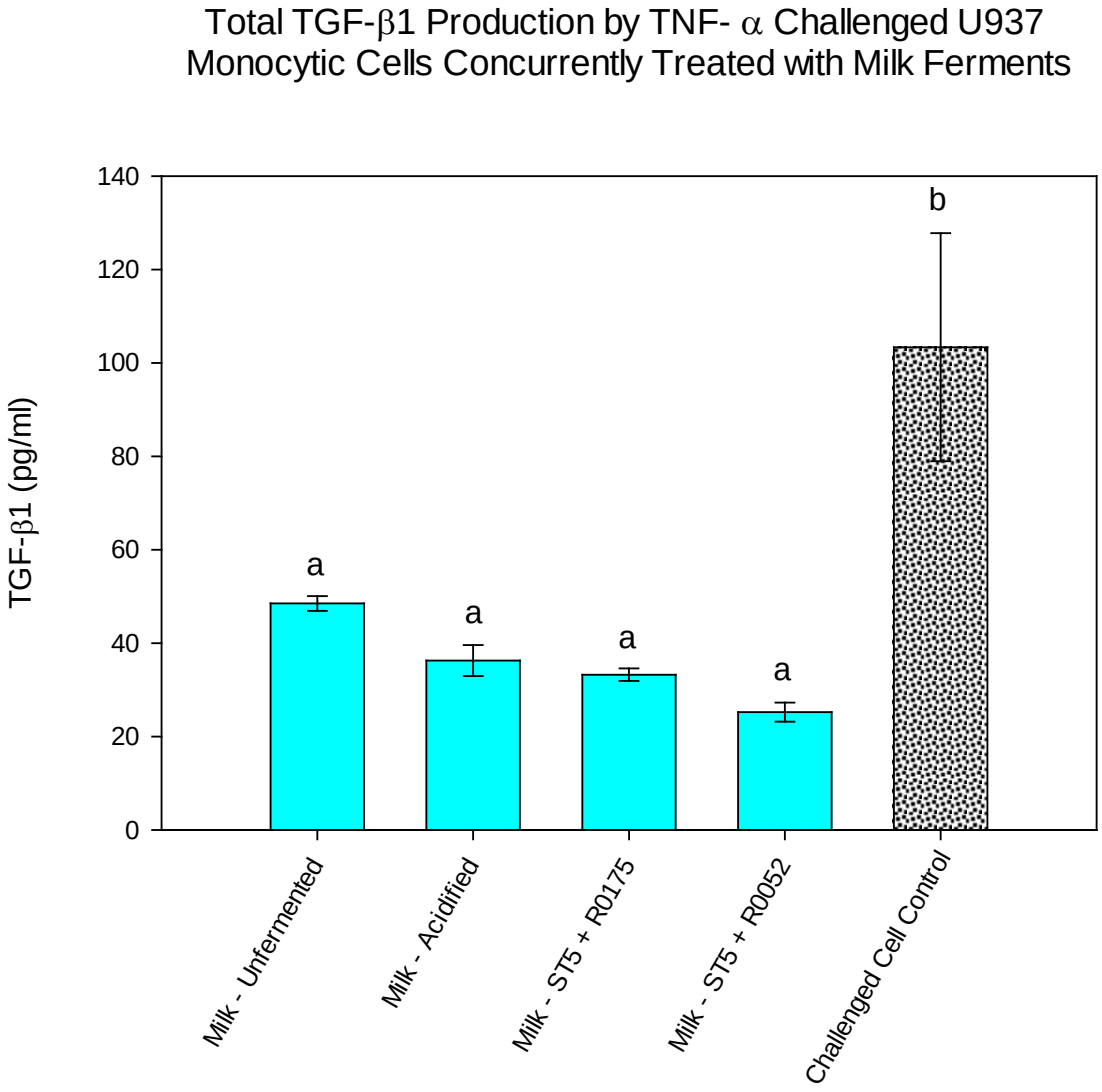

Figure 12: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on TGF-β1 production by non-differentiated U937 monocytic cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 48 hours. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; p = 0.0037; n = 3). Unchallenged cell control produced 44.2 ± 24.1 pg/mL.

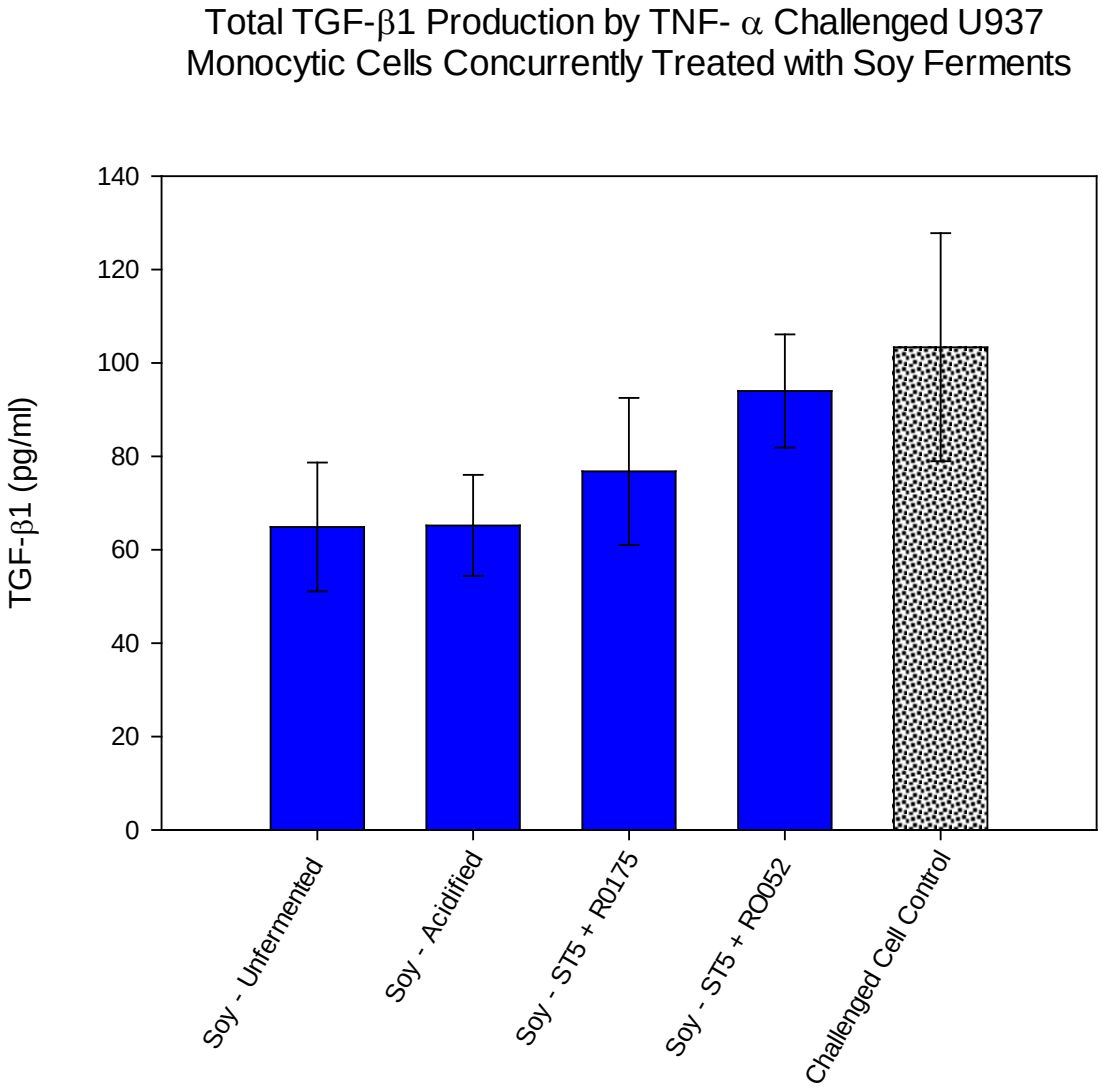

Figure 13: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on TGF-β1 production by non-differentiated U937 monocytic cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 48 hours. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; p = 0.3878; n = 3). Unchallenged cell control produced 44.2 ± 24.1 pg/mL.

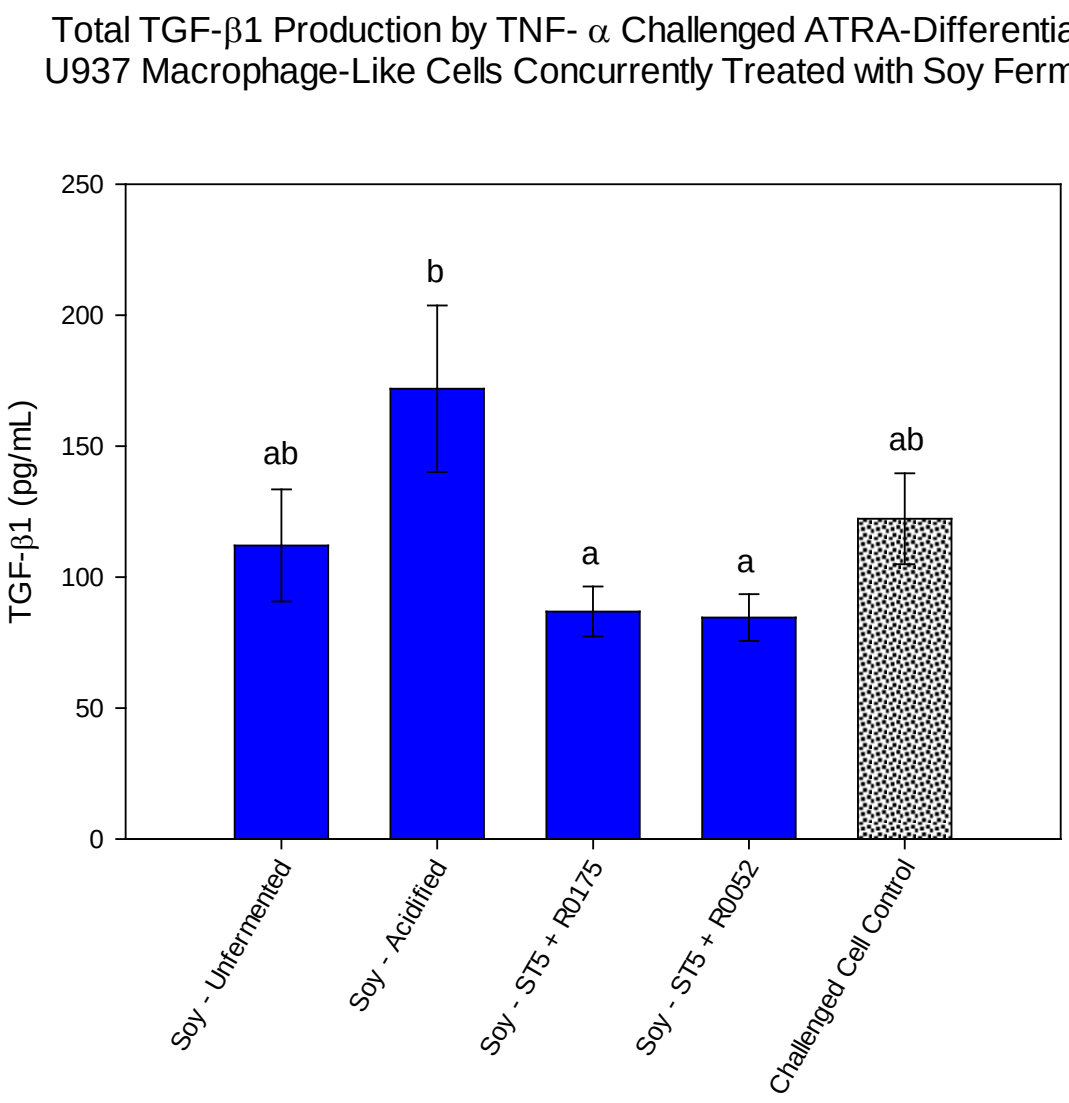

Figure 14: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on TGF-β1 production by ATRA-differentiated U937 macrophage-like cells (5 × 10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 48 hours. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; p = 0.0287; n = 6). Unchallenged cell control produced 218.8 ± 26.1 pg/mL.

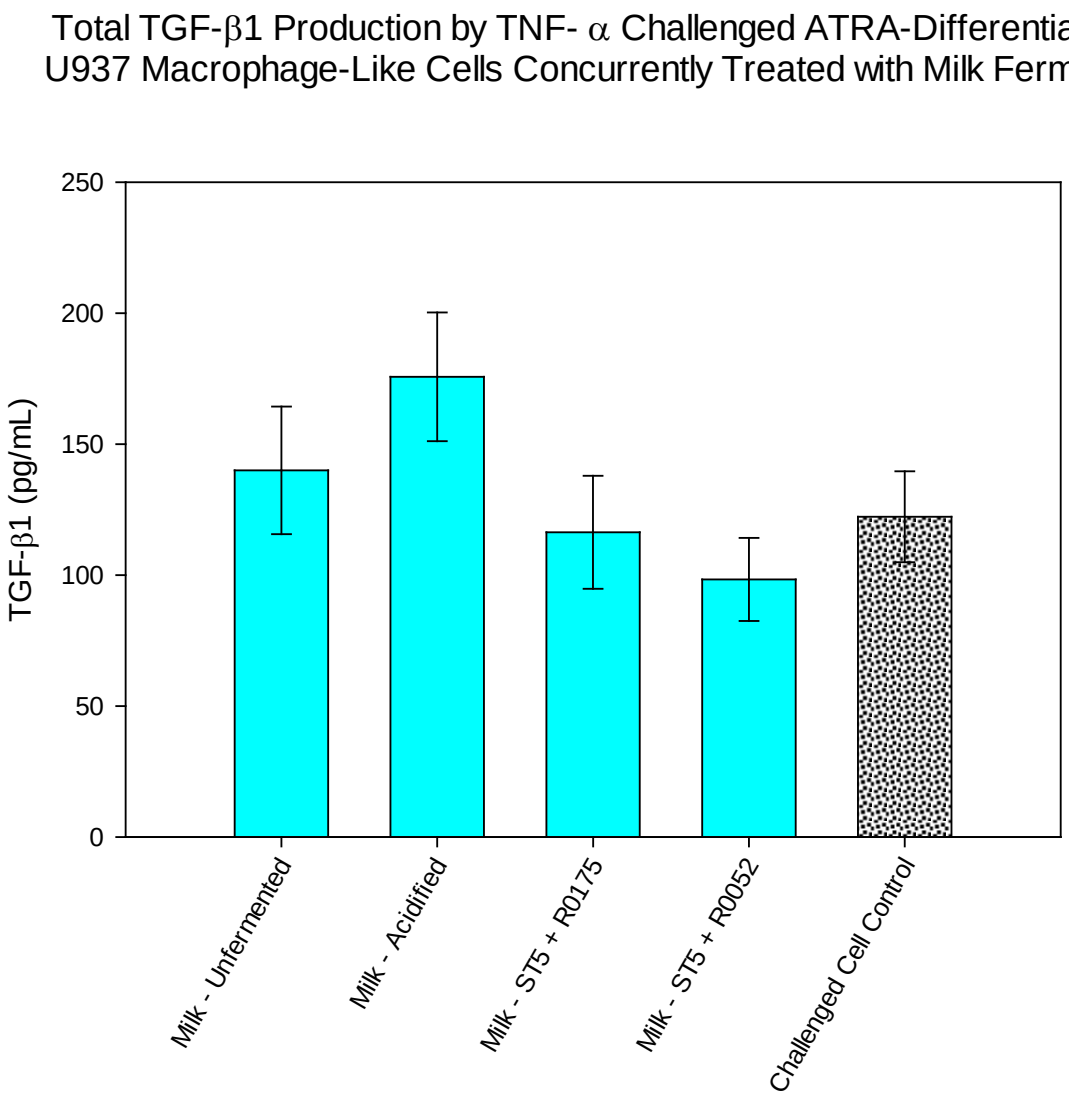

Figure 15: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on TGF-β1 production by ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 48 hours. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; p = 0.1349; n = 6). Unchallenged cell control produced 218.8 ± 26.1 pg/mL.

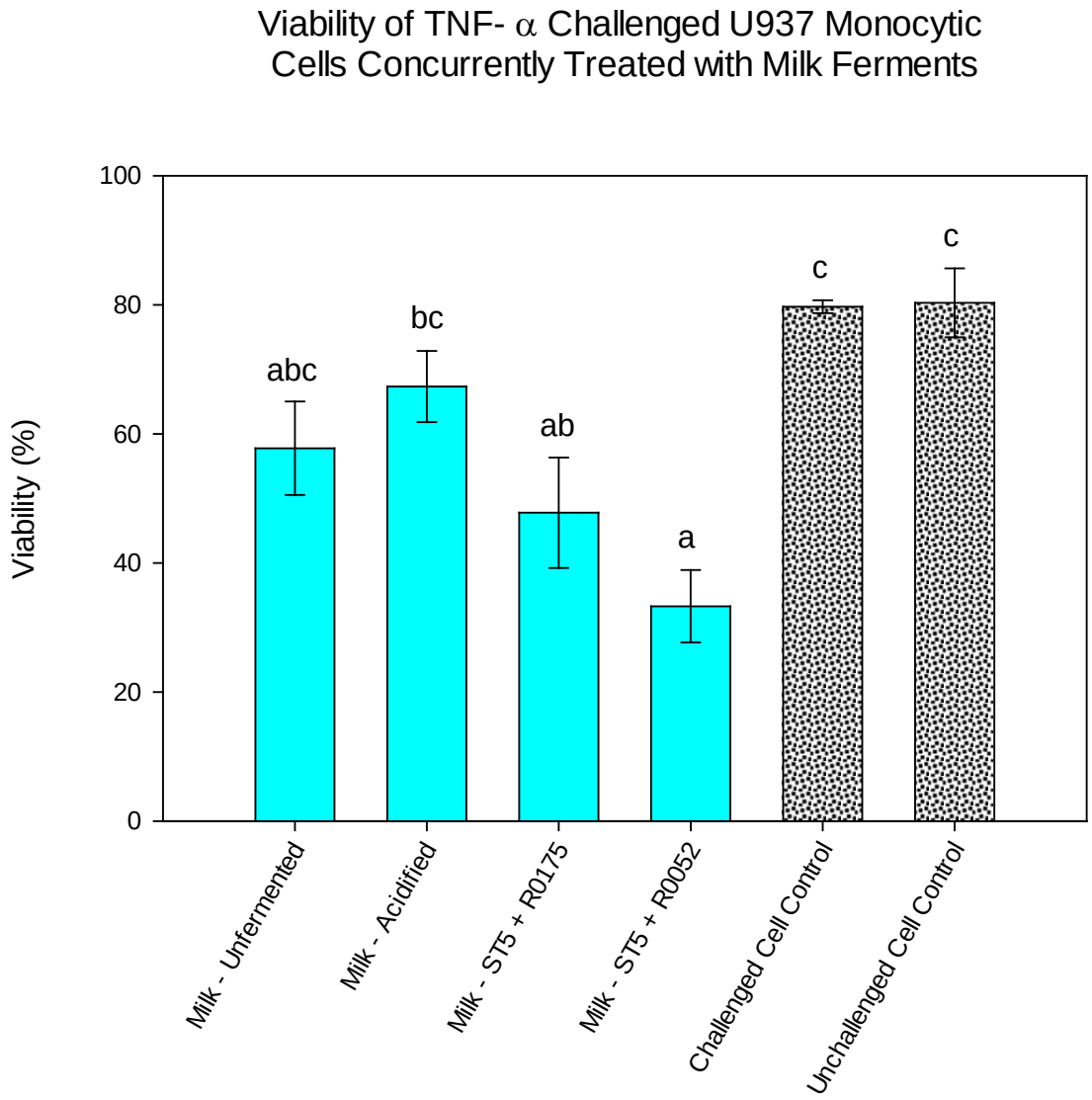

Figure 16: Viability of non-differentiated U937 monocytic cells (5×10^5 cells/mL) following concurrent incubation with milk ferments and milk controls (1/100 in RMPI 1640) and 20 ng/mL TNF-α for 24 hours. Data shown as mean percent viability ± SEM. (One-way ANOVA; $p = 0.0005$; $n = 6$ for acidified milk, milk ST5 + R0175 and milk ST5 + R0052, $n = 3$ for milk unfermented, challenged cell control and unchallenged cell control). Pre-treatment viability was 84.2 ± 2.6 %.

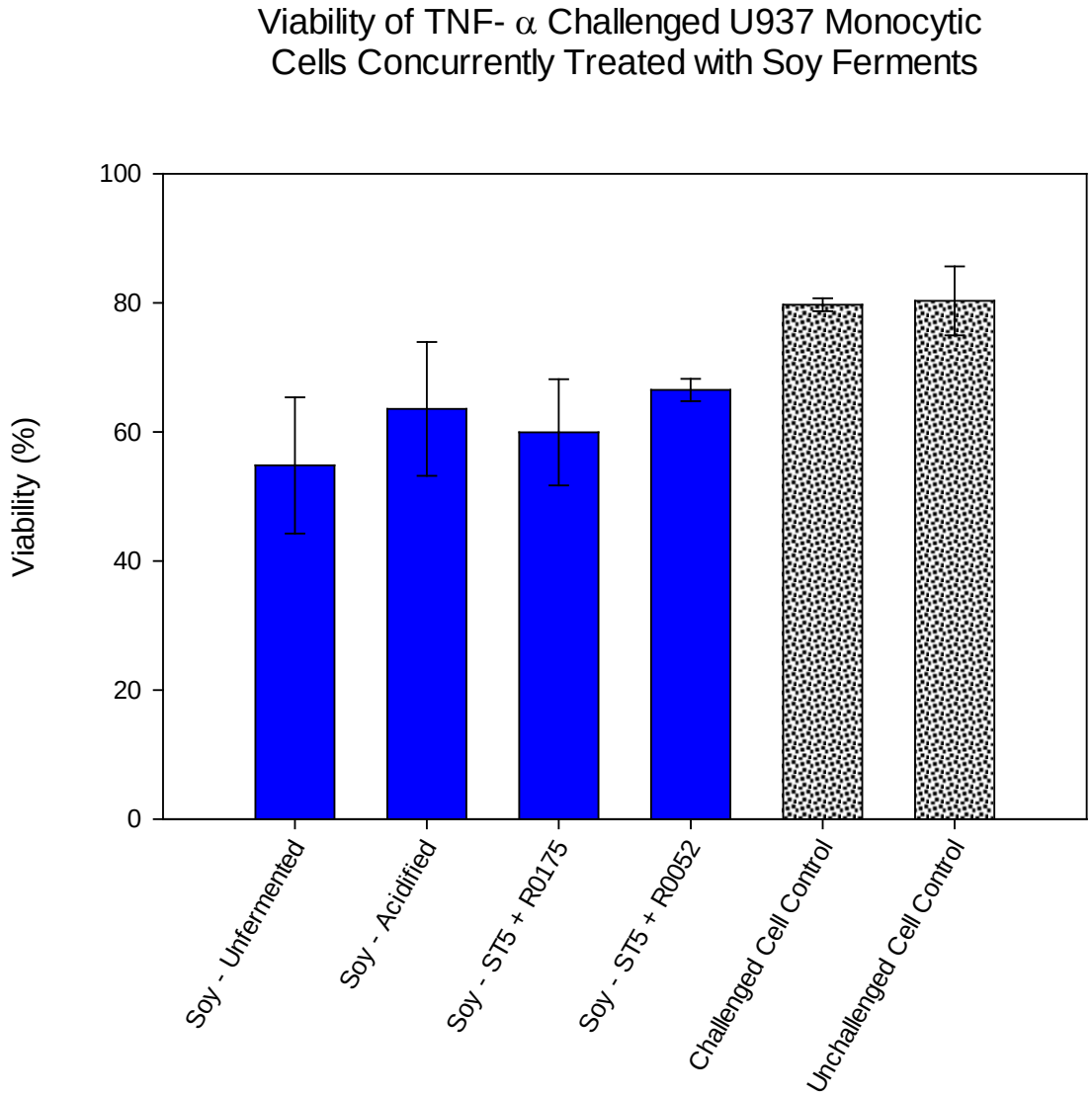

Figure 17: Viability of non-differentiated U937 monocytic cells (5×10^5 cells/mL) following concurrent incubation with soy ferments and soy controls (1/100 in RPMI 1640) and 20 ng/mL TNF-α for 24 hours. Data shown as mean percent viability ± SEM. (One-way ANOVA; $p = 0.3304$; $n = 6$ for acidified soy, soy ST5 + R0175 and soy ST5 + R0052, $n = 3$ for soy unfermented, challenged cell control and unchallenged cell control). Pre-treatment viability was 84.2 ± 2.6 %.

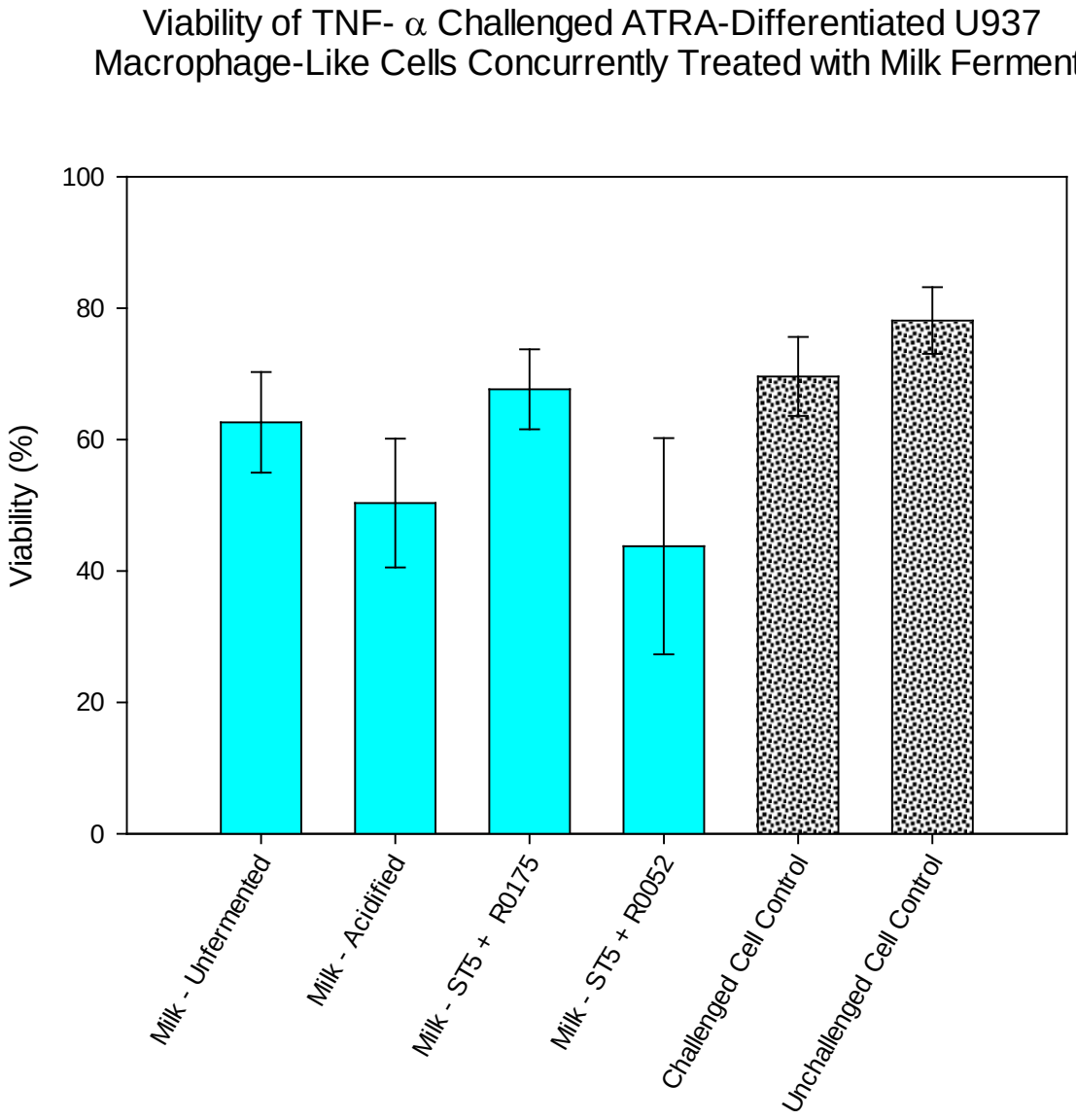

Figure 18: Viability of ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) following concurrent incubation with milk ferments and milk controls (1/100 in RPMI 1640) and 20 ng/mL TNF-α for 24 hours. Data shown as mean percent viability ± SEM. (One-way ANOVA; $p = 0.1290$; $n = 6$). Pre-treatment viability was 89.8 ± 2.9 %.

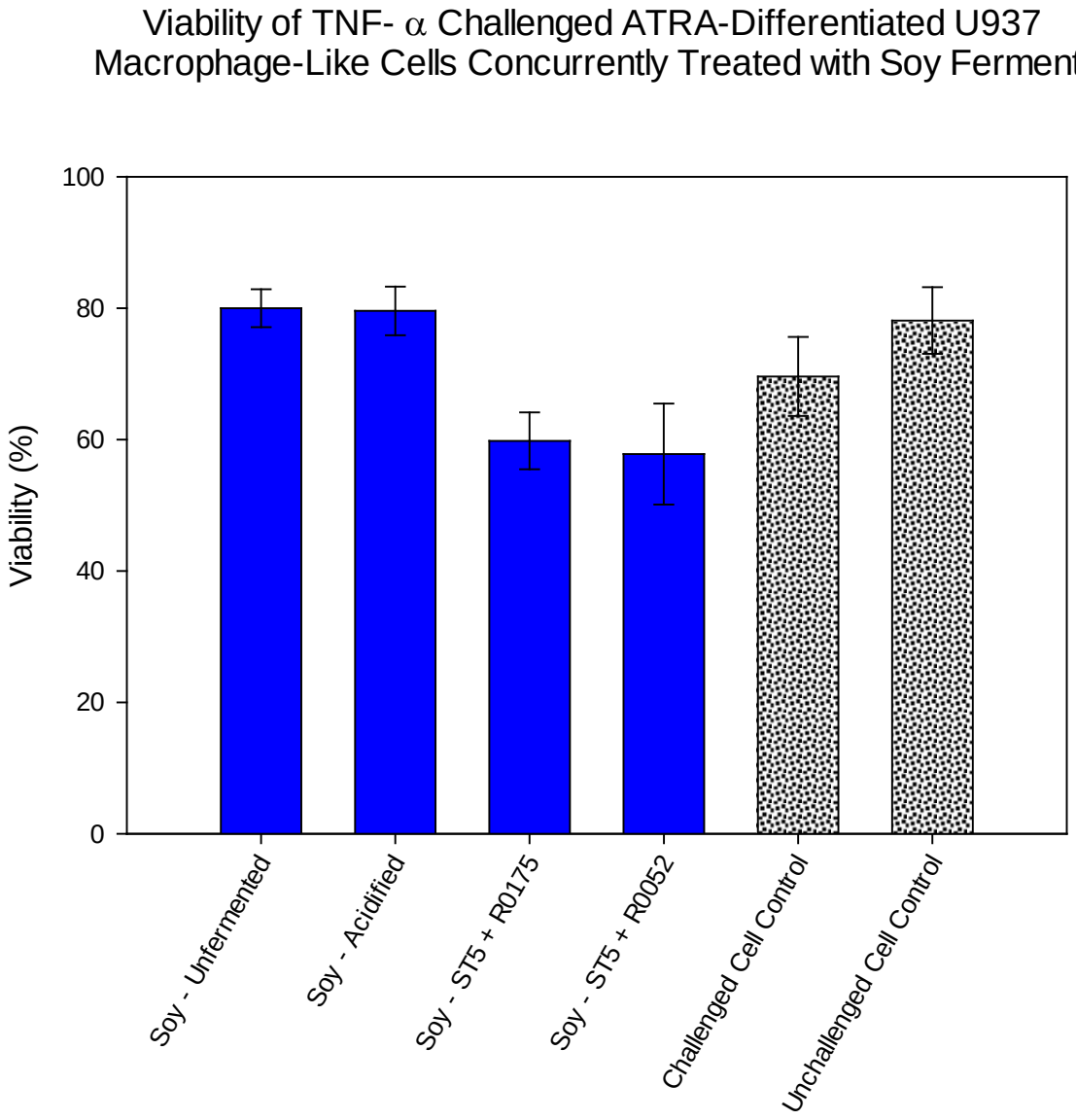

Figure 19: Viability of ATRA-differentiated U937s (5×10^5 cells/mL) following concurrent incubation with soy ferments and soy controls (1/100 in RPMI 1640) and 20 ng/mL TNF-α for 24 hours. Data shown as mean percent viability ± SEM. (One-way ANOVA; p = 0.0092; n = 6; multiple comparisons test did not indicate differences). Pre-treatment viability was 89.8 ± 2.9 %.

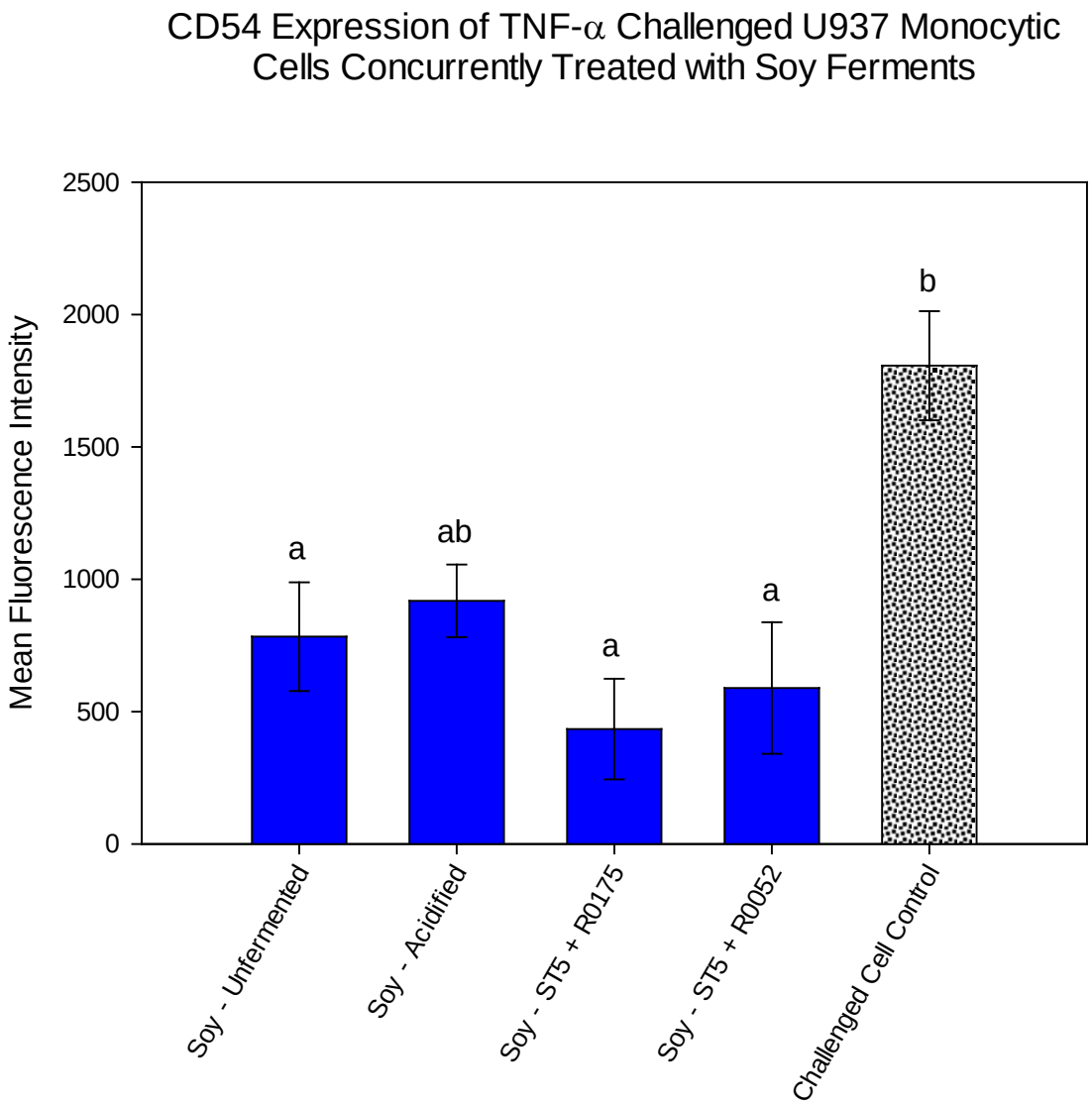

Figure 20: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD54 expression by non-differentiated U937 monocytic cells (5 × 10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM (One-way ANOVA, p = 0.0055, n = 3). Pre-treatment MFI was 1119.1 ± and 435.3 and unchallenged MFI was 725.1 ± 109.4.

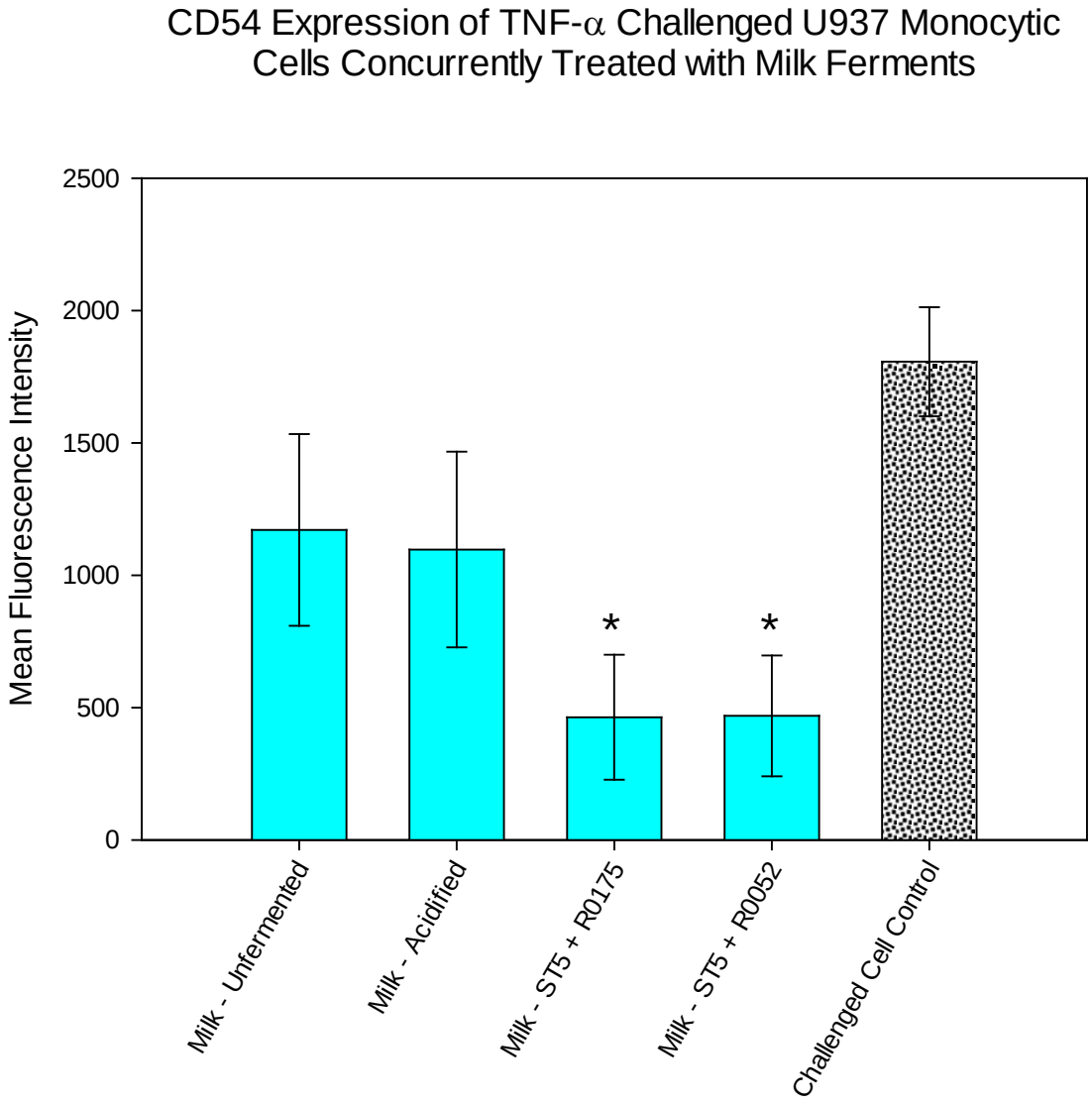

Figure 21: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD54 expression by non-differentiated U937 monocytic cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.0406$, $n = 3$). Pre-treatment MFI was 1119.1 ± and 435.3 and unchallenged cell control MFI was 725.1 ± 109.4. Dunnett's post test used (* shows significant difference from challenged cell control).

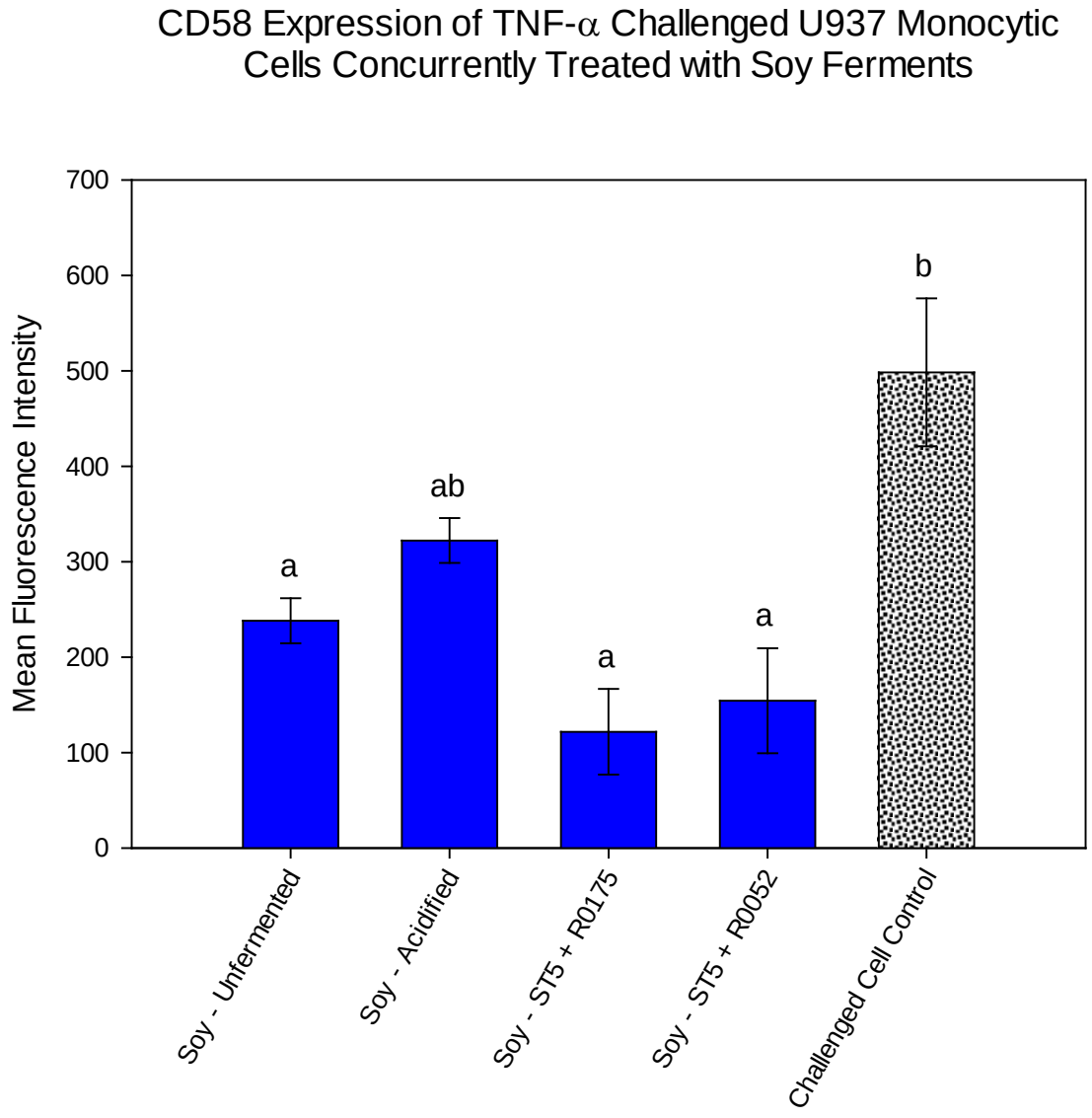

Figure 22: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD58 expression by non-differentiated U937 monocytic cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.0020$, $n = 3$). Pre-treatment MFI was 585.9 ± and 230.2 and unchallenged cell control MFI was 464.1 ± 61.3.

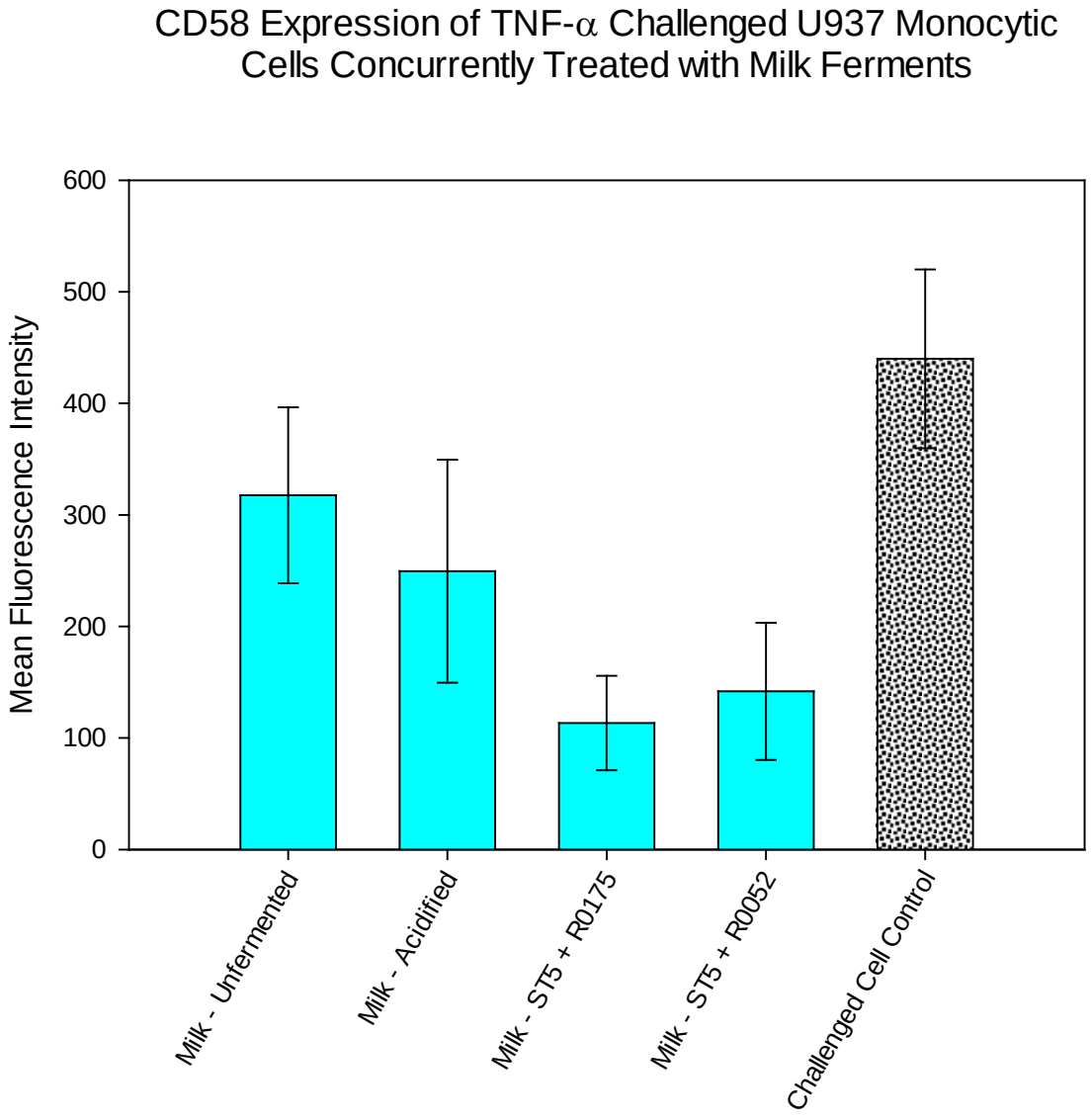

Figure 23: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD58 expression by non-differentiated U937 monocytic cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.0644$, $n = 4$). Pre-treatment MFI was 585.9 ± and 230.2 and unchallenged cell control MFI was 409.2 ± 69.9.

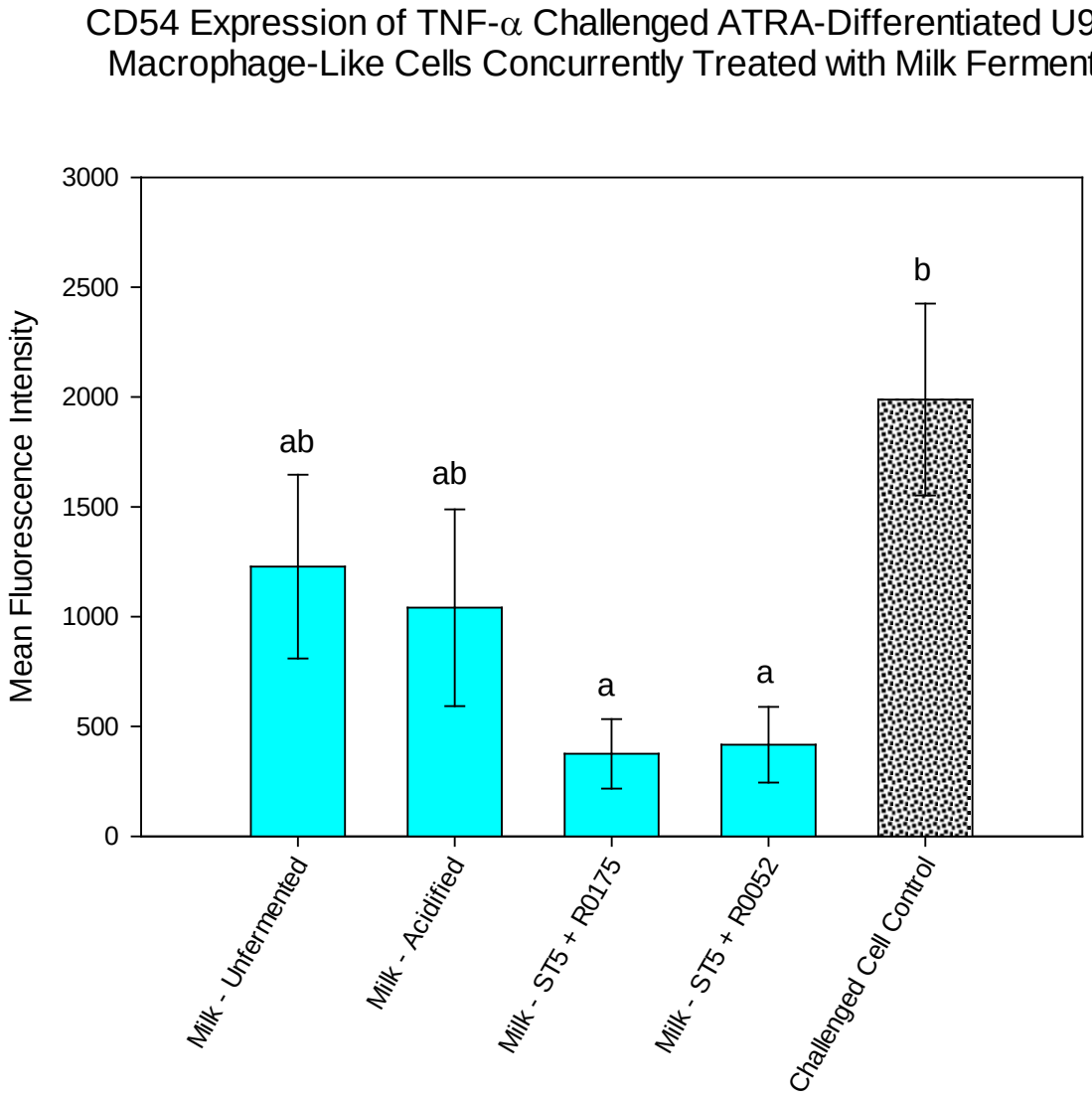

Figure 24: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD54 expression by ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.0298$, $n = 5$ for challenged cell control, $n = 4$ for milk controls and ferments). Pre-treatment MFI was 639.2 ± 304.8 and unchallenged cell control MFI was 719.5 ± 175.9.

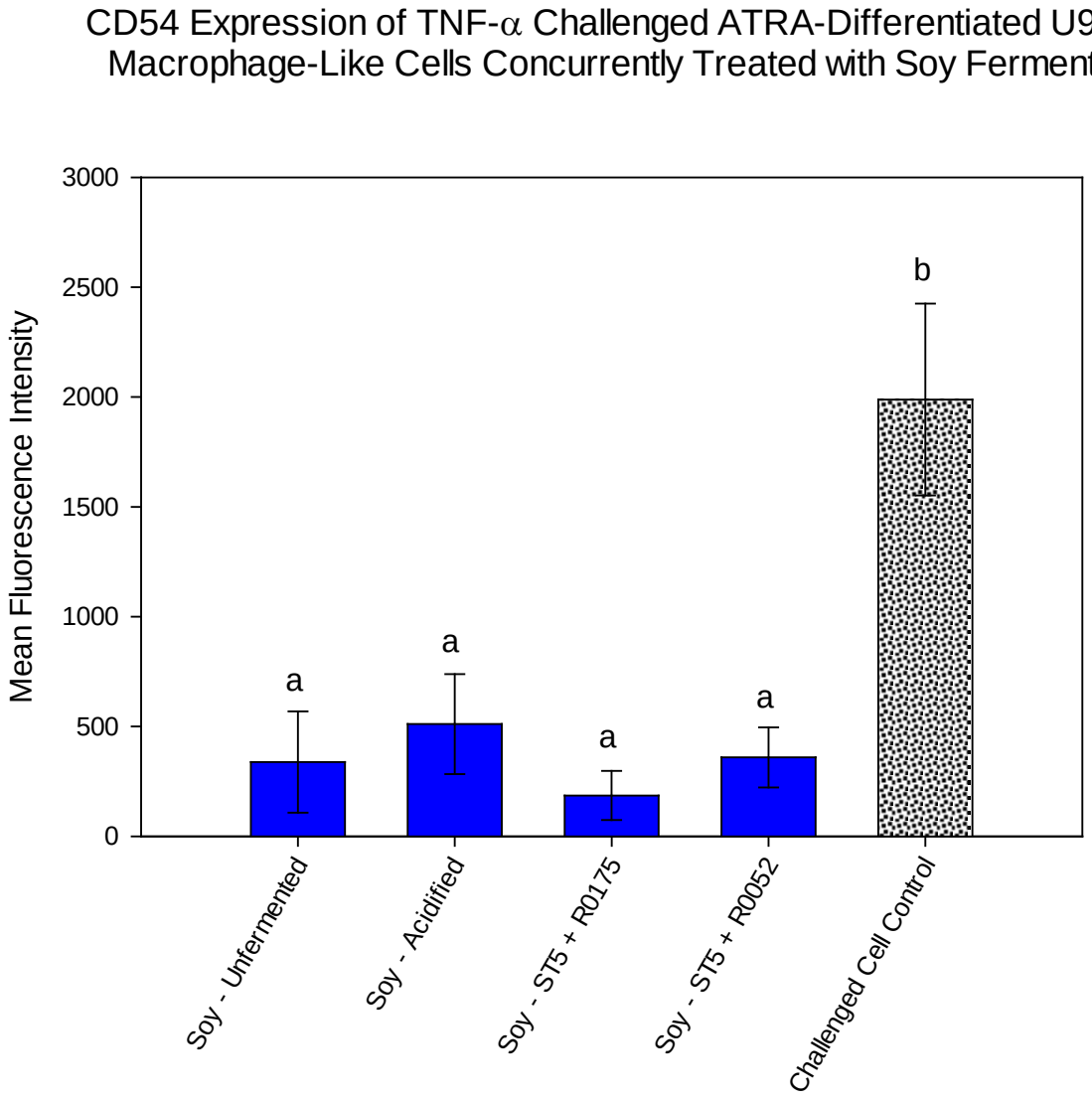

Figure 25: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD54 expression by ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.0012$, $n = 5$ for challenged cell control, $n = 4$ for soy controls and ferments). Pre-treatment MFI was 639.2 ± 304.8 and unchallenged cell control MFI was 719.5 ± 175.9.

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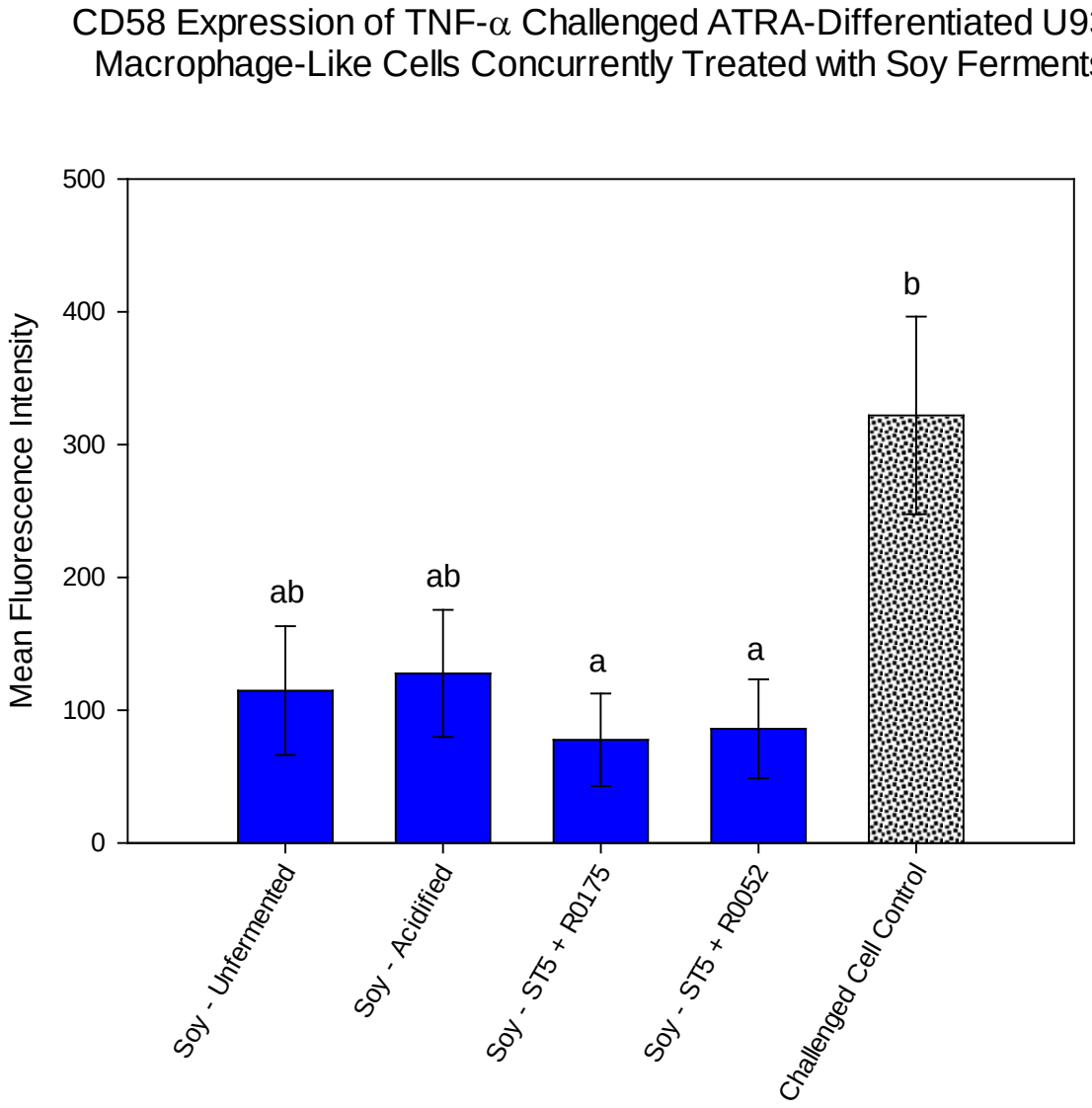

Figure 26: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD58 expression by ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.0218$, $n = 5$ for challenged cell control, $n = 4$ for soy controls and ferments). Pre-treatment MFI was 654.9 ± 52.4 and unchallenged cell control MFI was 349.2 ± 83.6.

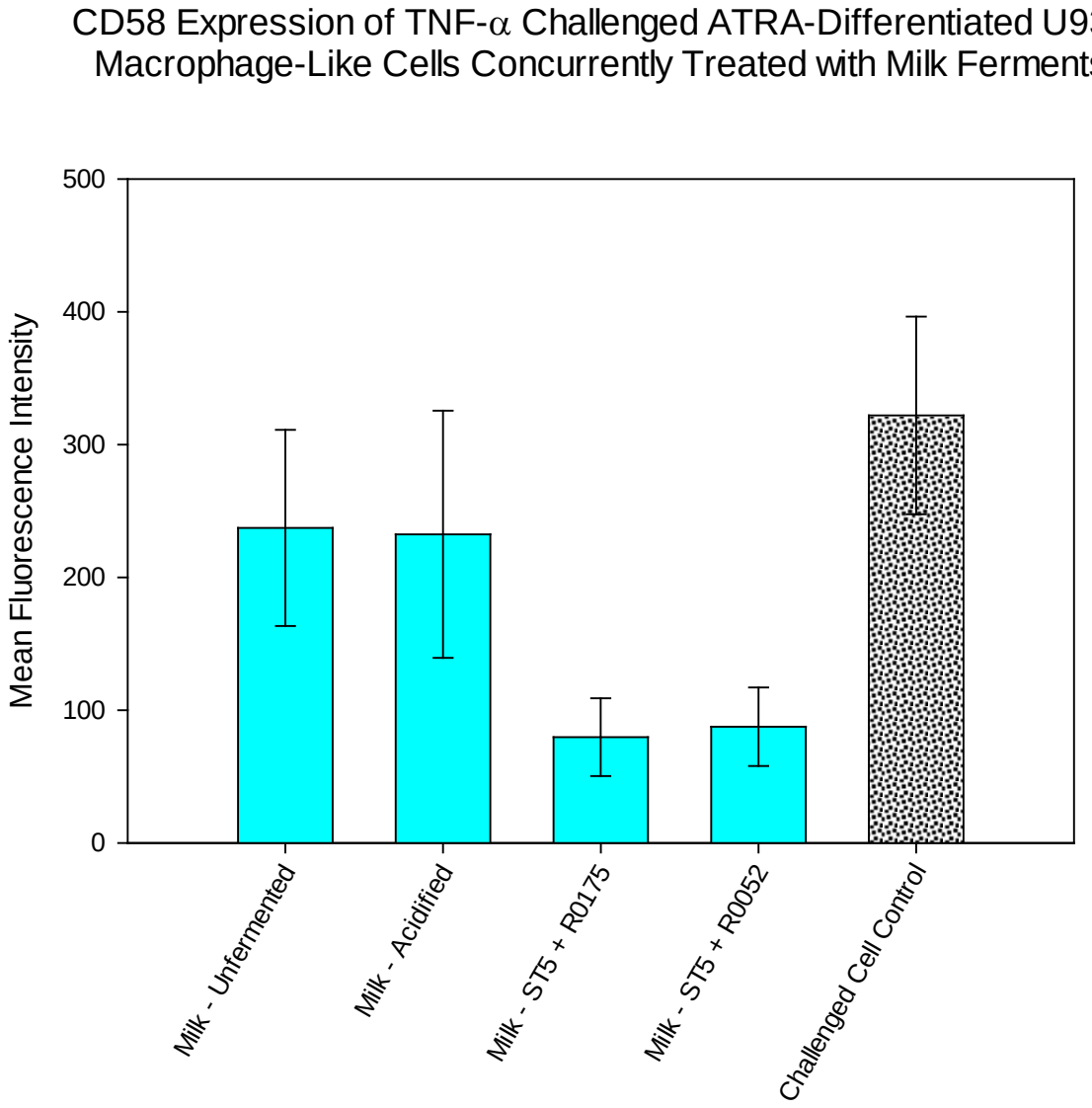

Figure 27: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD58 expression by ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.0788$, $n = 5$ for challenged cell control, $n = 4$ for milk controls and ferments). Pre-treatment MFI was 654.9 ± 52.4 and unchallenged cell control MFI was 349.2 ± 83.6.

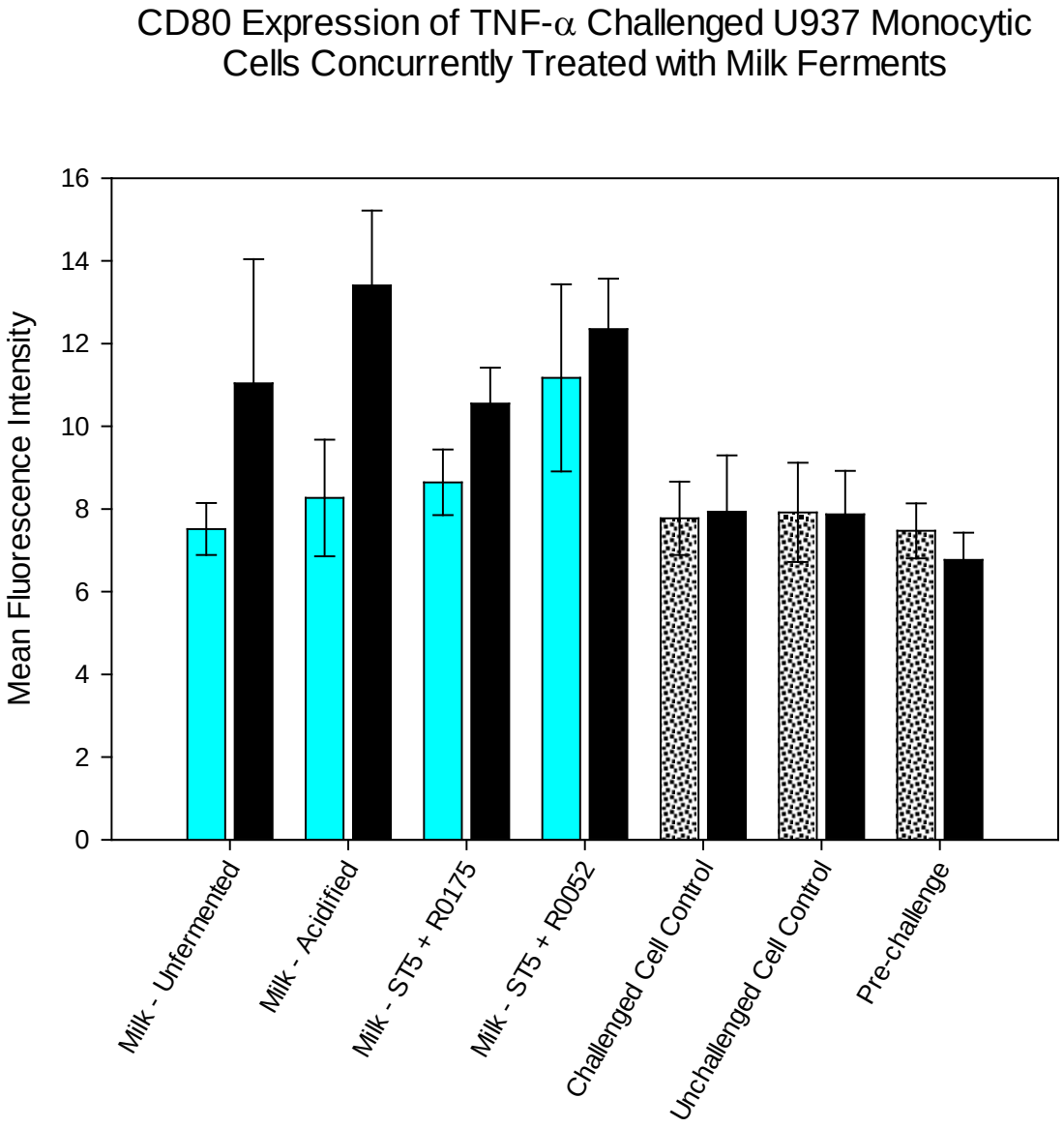

Figure 28: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD80 expression by non-differentiated U937 monocytic cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD80 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

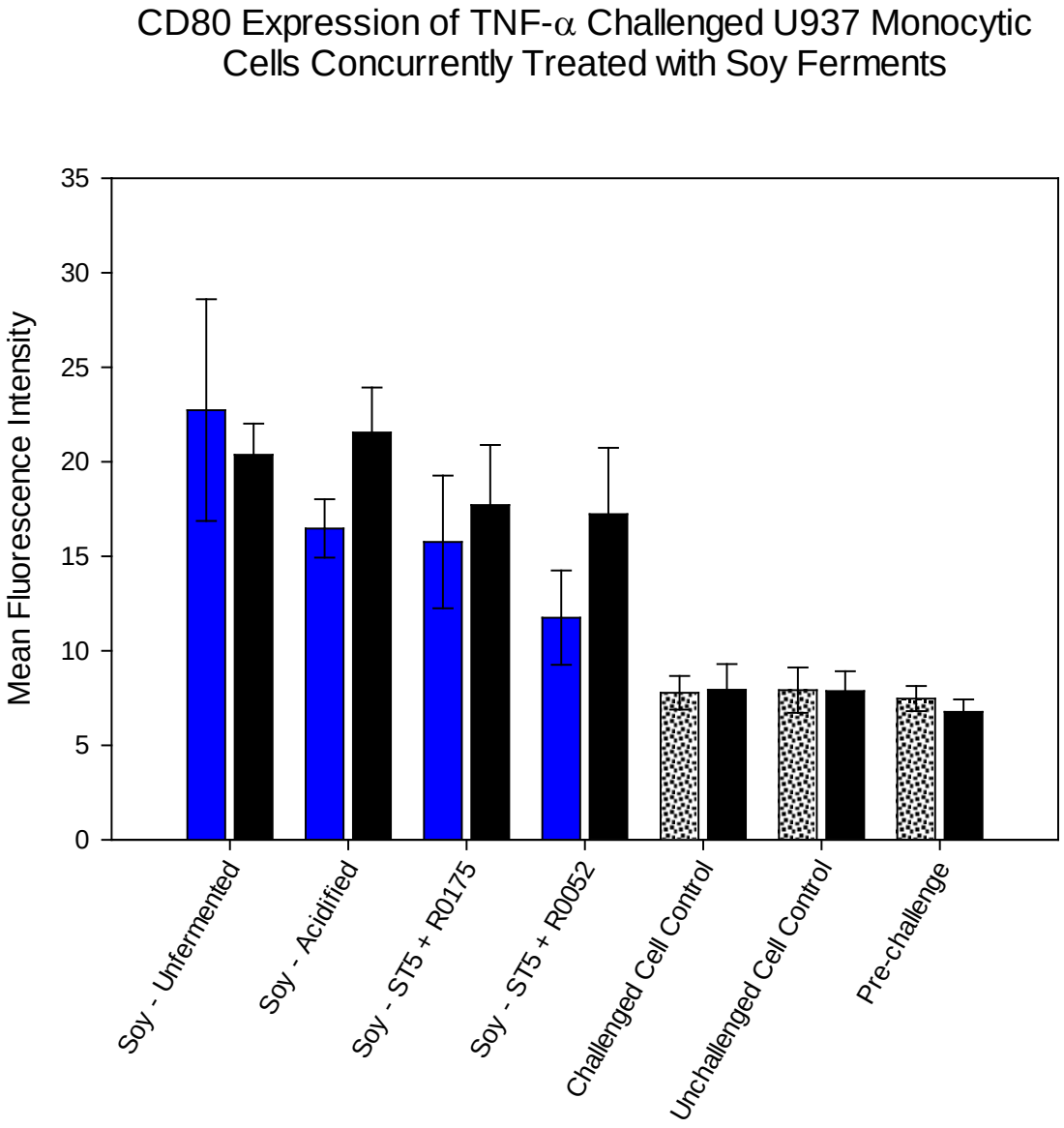

Figure 29: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD80 expression by non-differentiated U937 monocytic cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD80 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

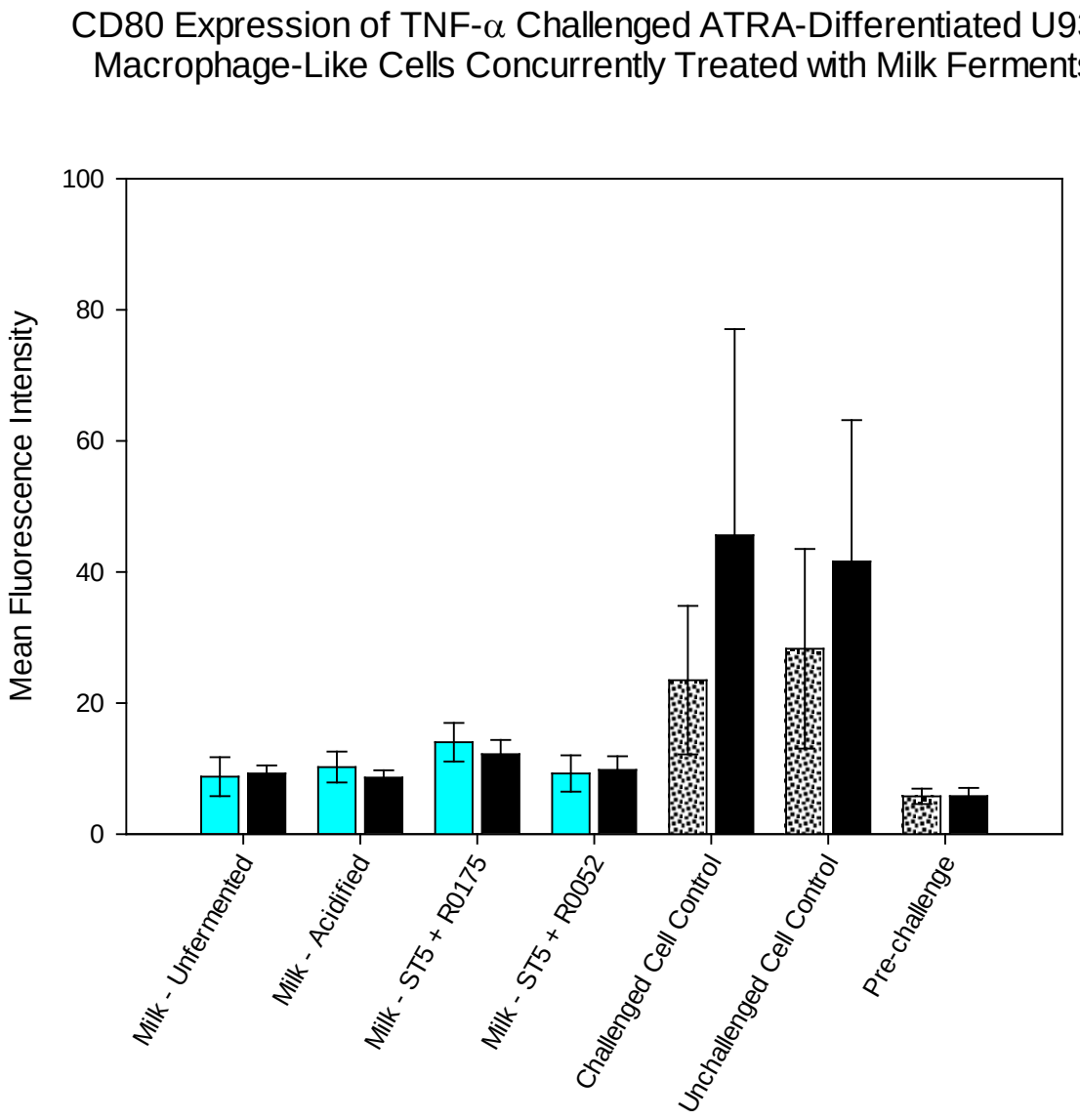

Figure 30: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD80 expression by ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD80 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

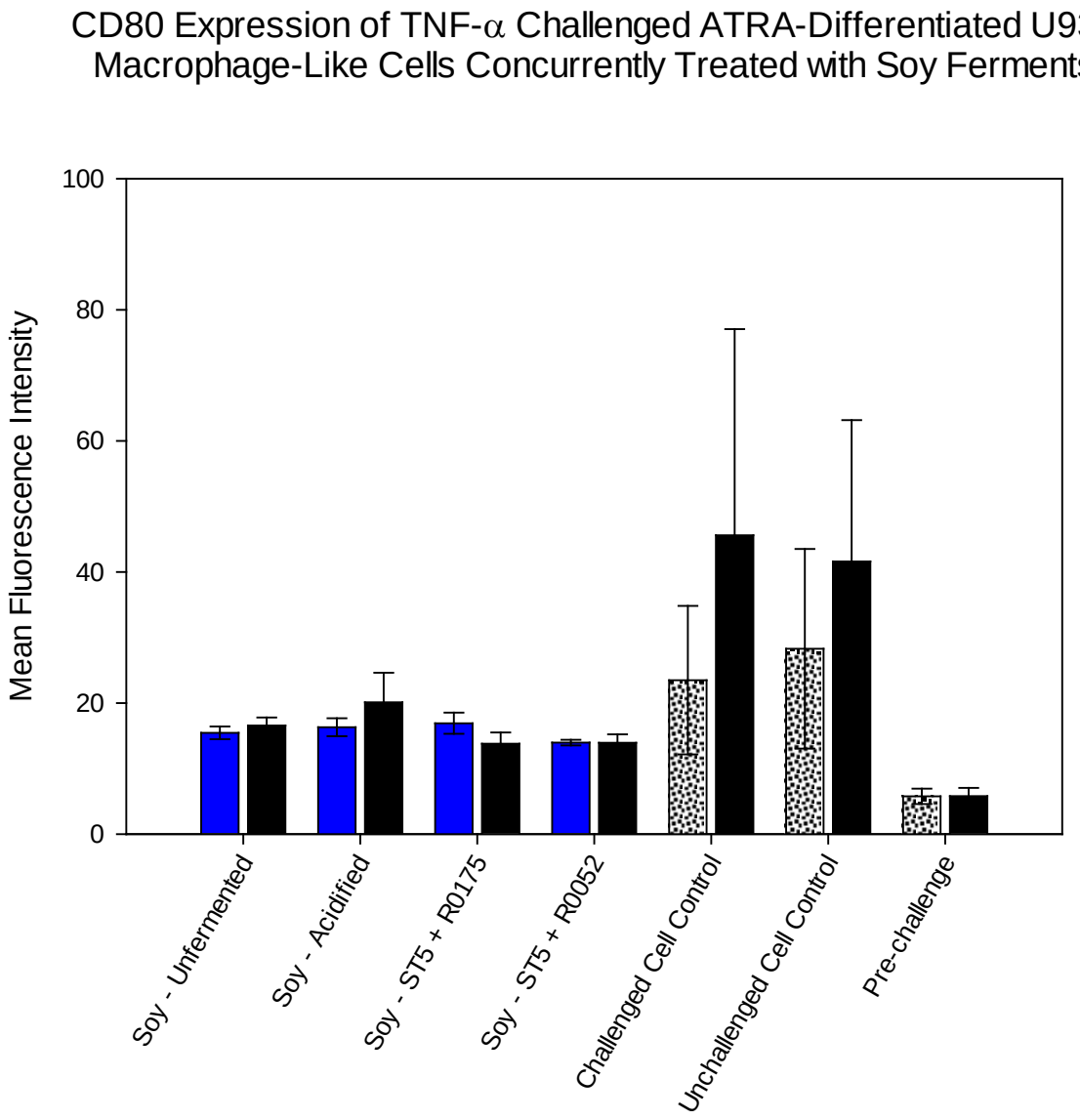

Figure 31: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD80 expression by ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD80 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

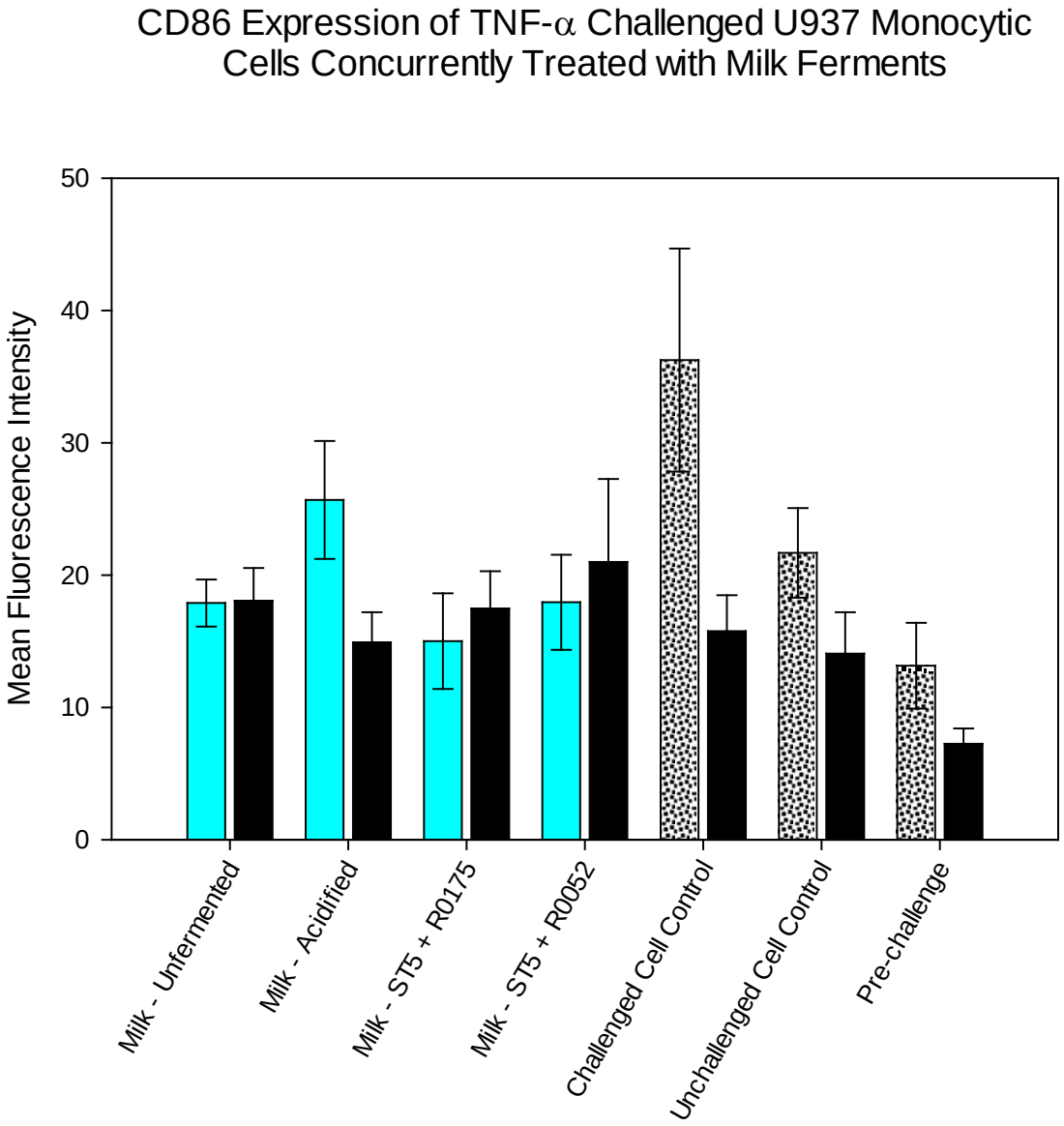

Figure 32: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD86 expression by non-differentiated U937 monocytic cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD86 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

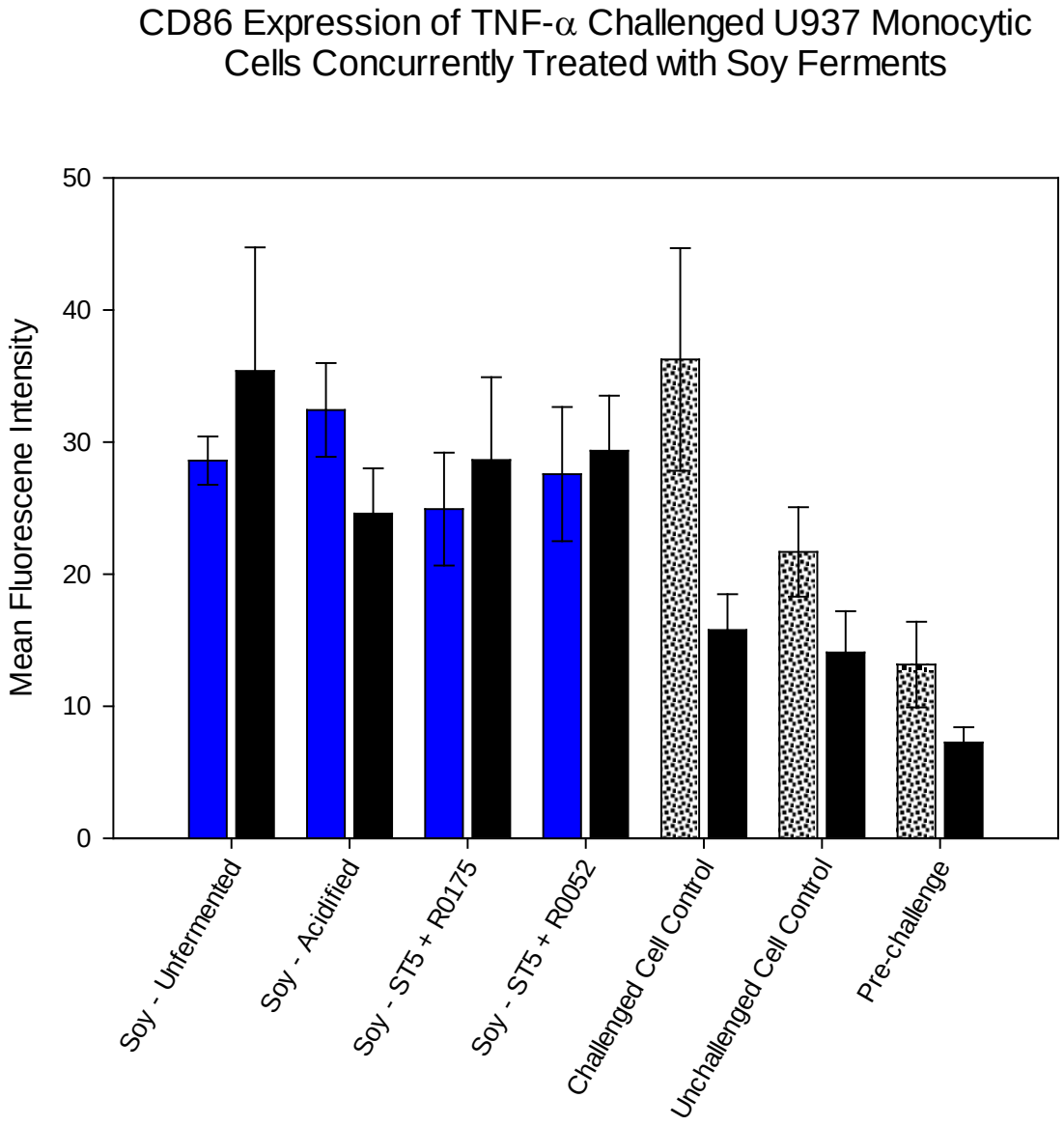

Figure 33: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD86 expression by non-differentiated U937 monocytic cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD86 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

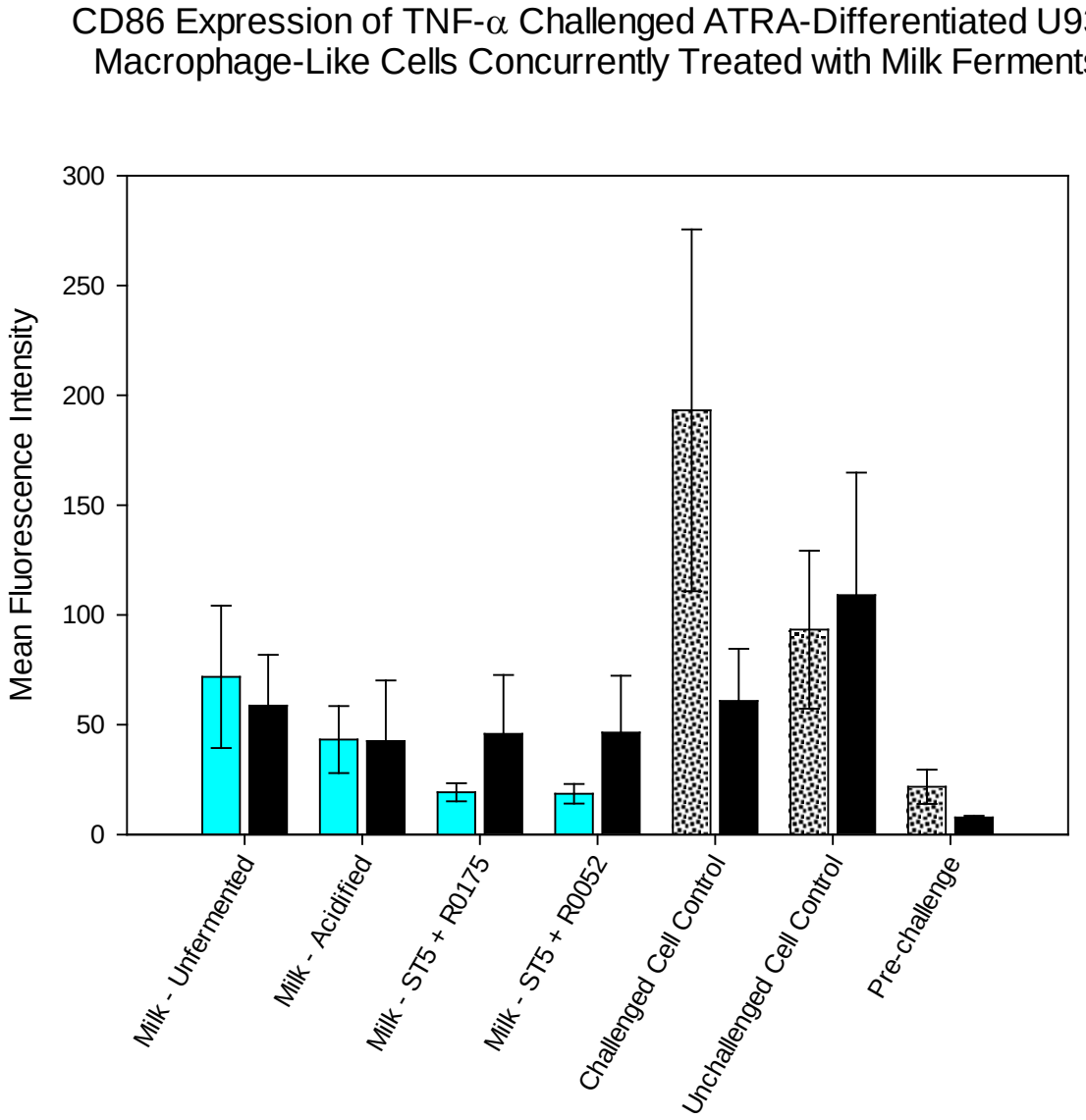

Figure 34: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD86 expression by ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD86 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

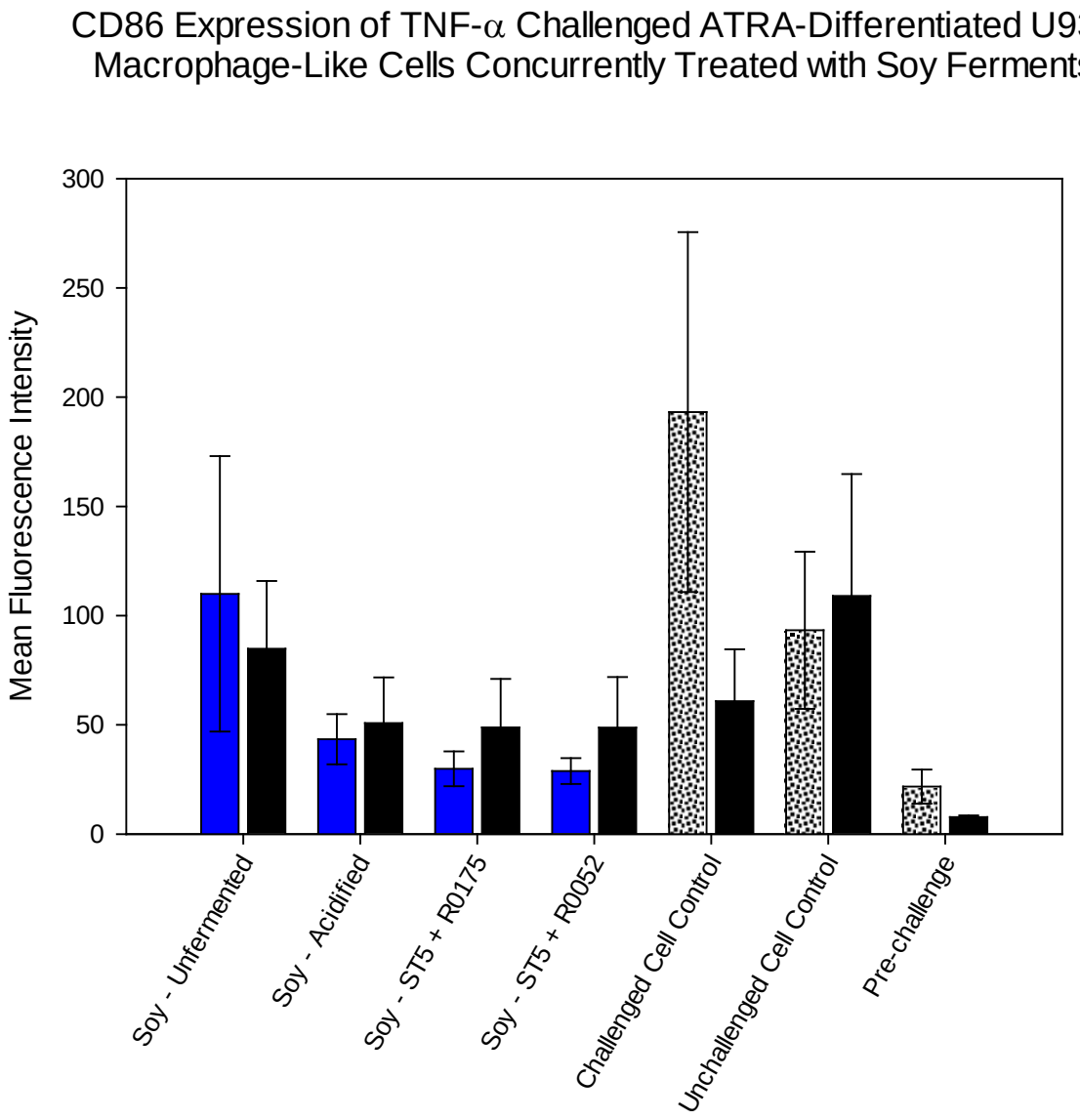

Figure 35: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD86 expression by ATRA-differentiated U937 macrophage-like cells (5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD86 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

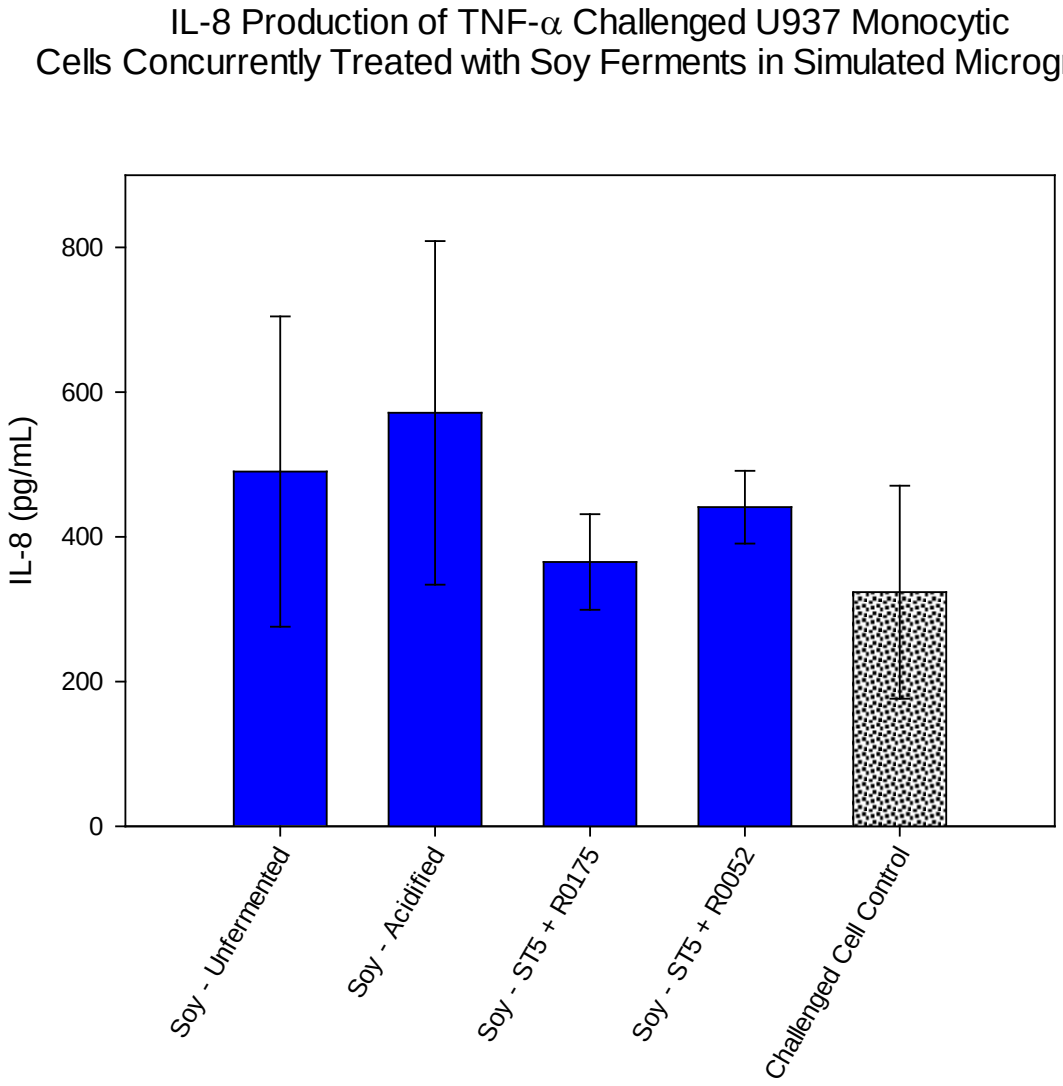

Figure 36: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on IL-8 production by non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; $p = 0.8237$; $n = 3$).

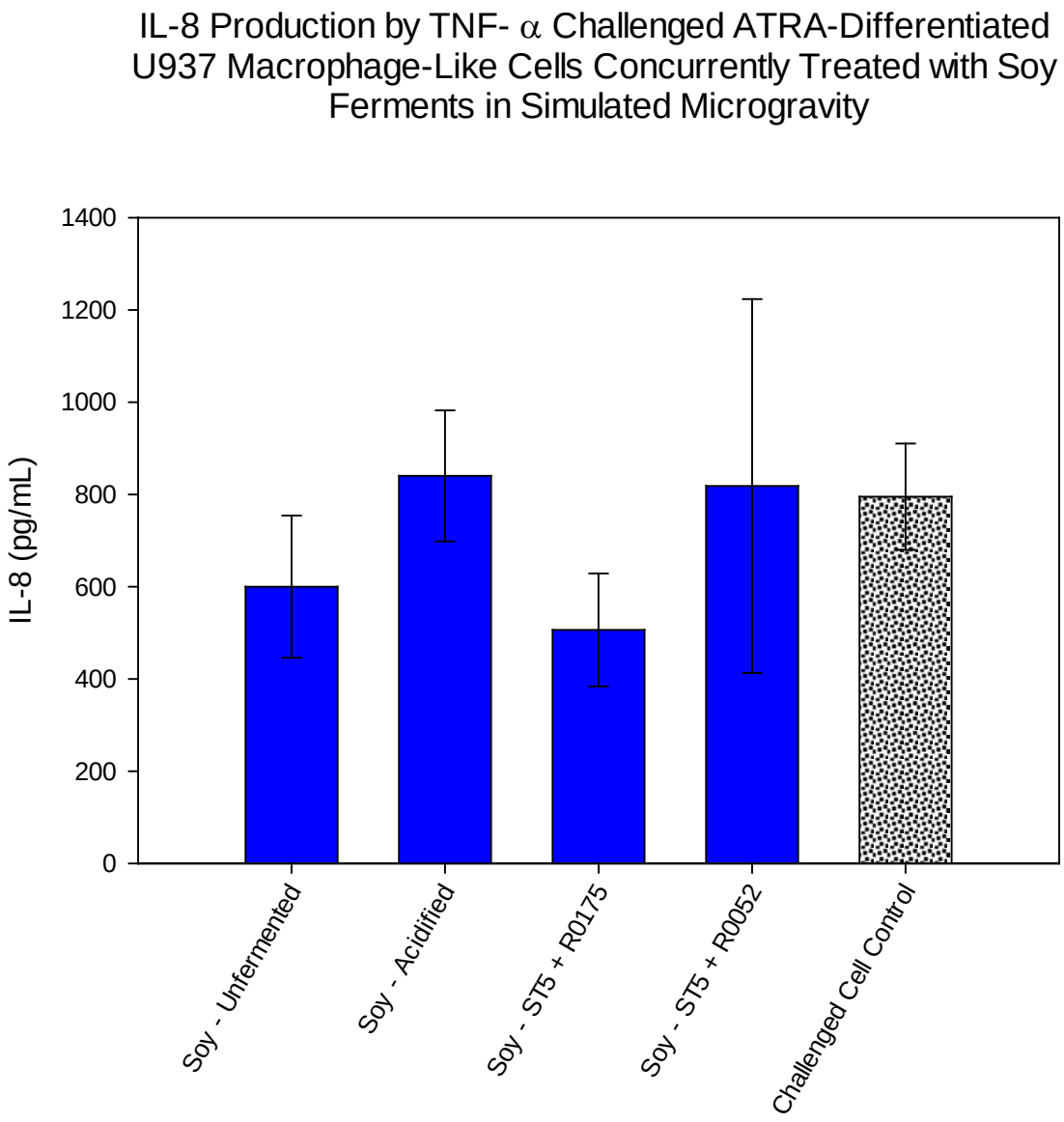

Figure 37: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on IL-8 production by ATRA-differentiated U937 macrophage-like cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; $p = 0.7548$; $n = 3$).

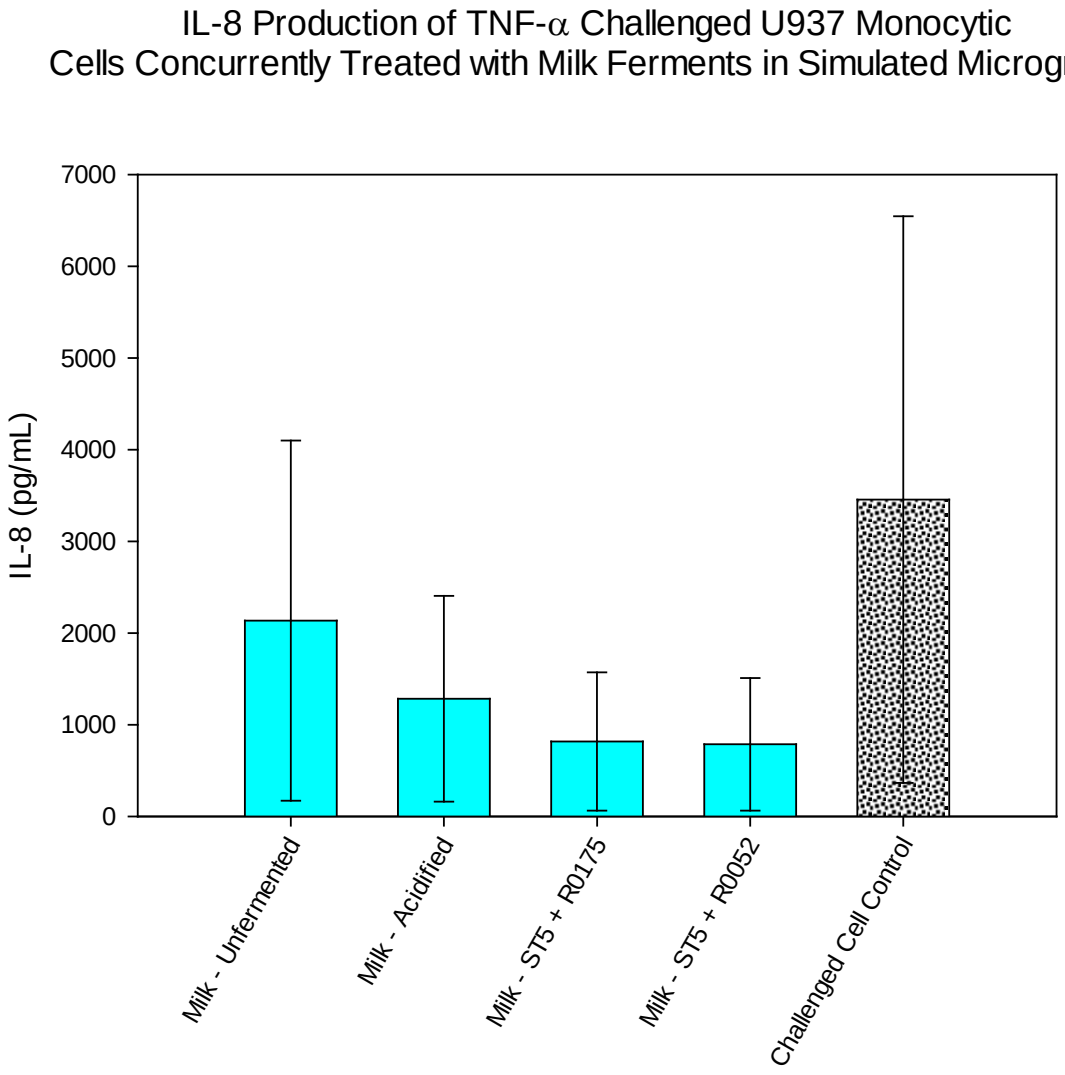

Figure 38: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on IL-8 production by non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; p = 0.8038; n = 3).

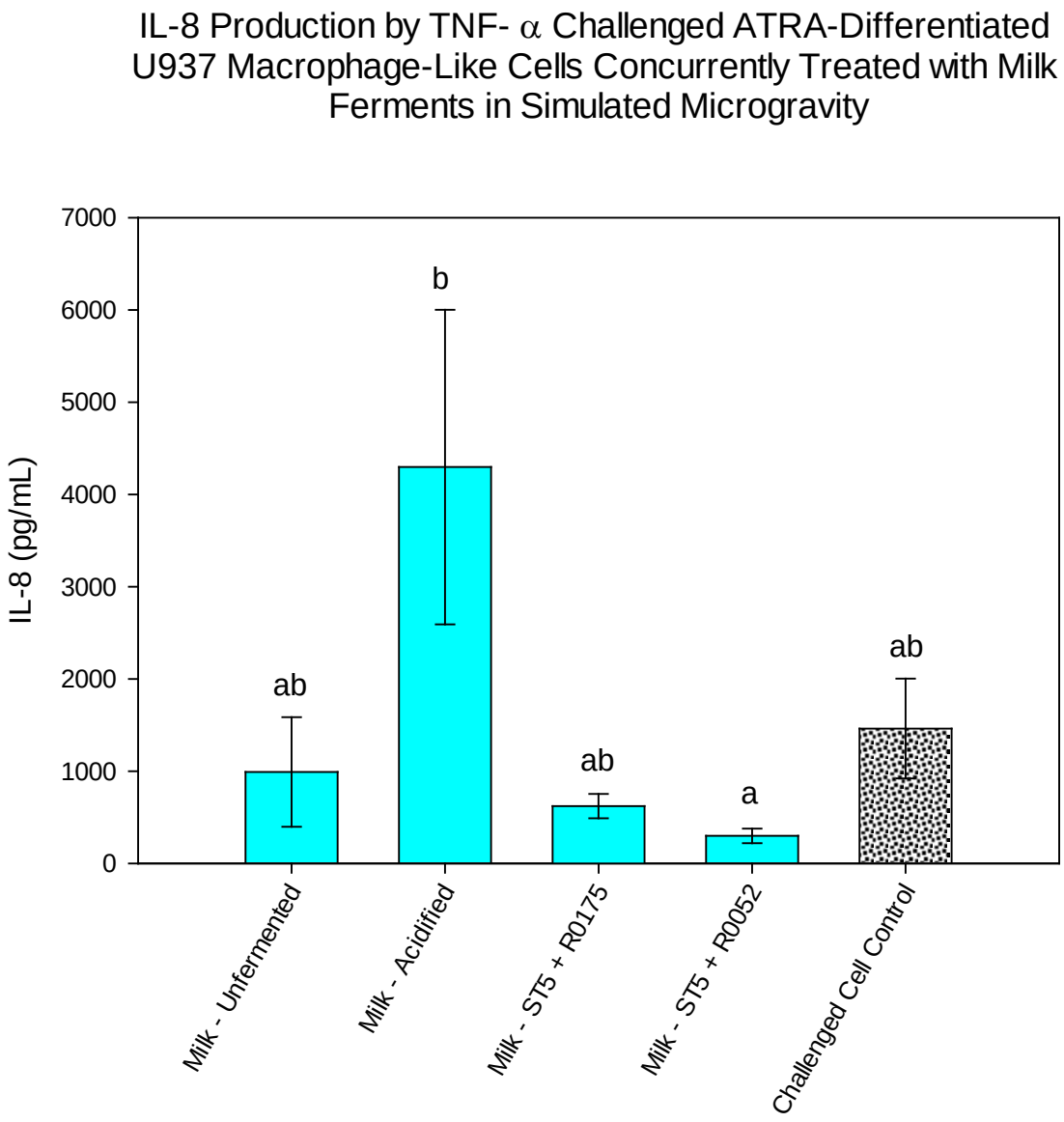

Figure 39: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on IL-8 production by ATRA-differentiated U937 macrophage-like cells (2.5 × 10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; p = 0.0458; n = 3).

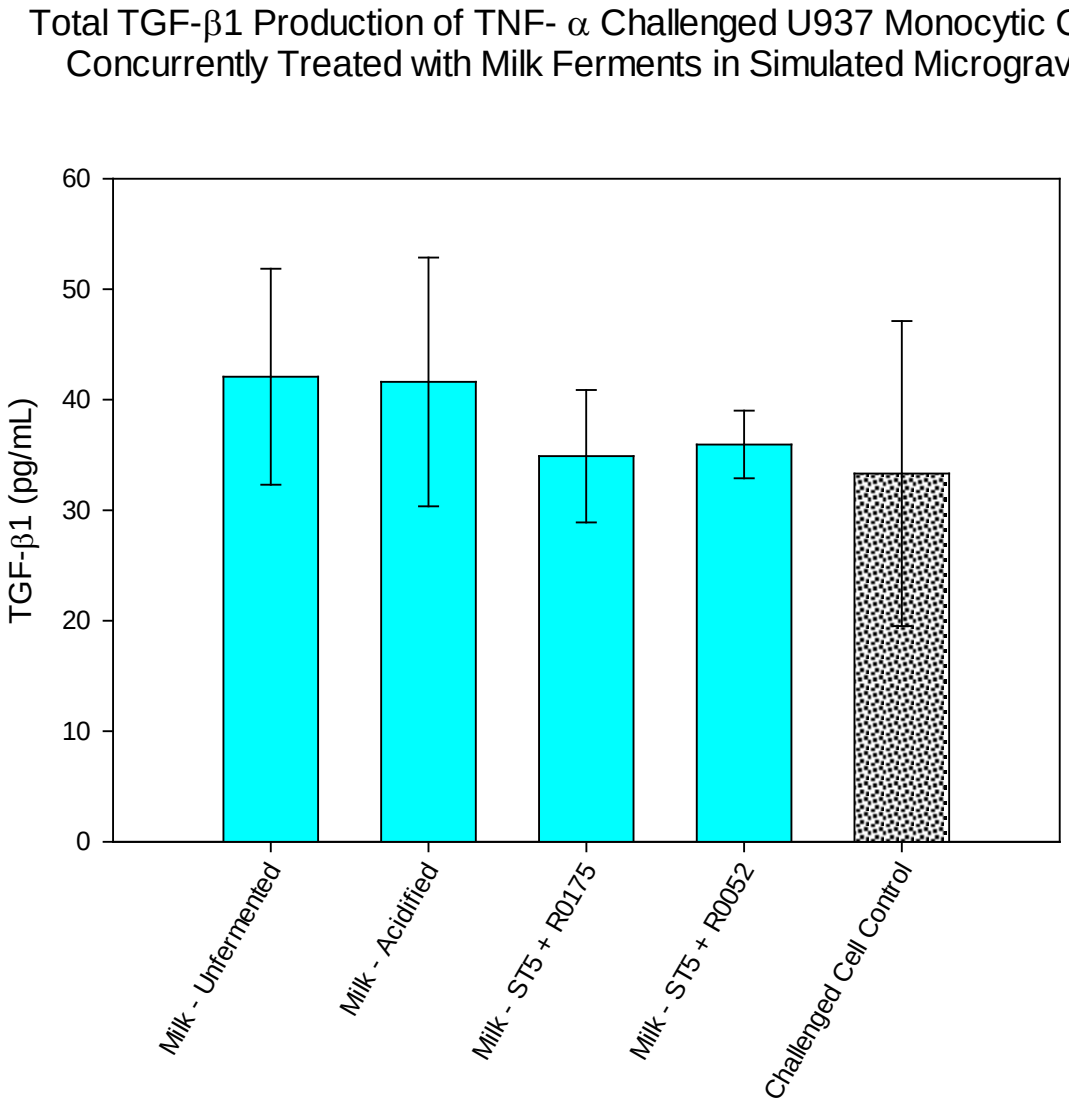

Figure 40: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on TGF-β1 production by non-differentiated U937 monocytic cells (2.5 × 10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 48 hours in simulated microgravity. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; p = 0.9455; n = 3).

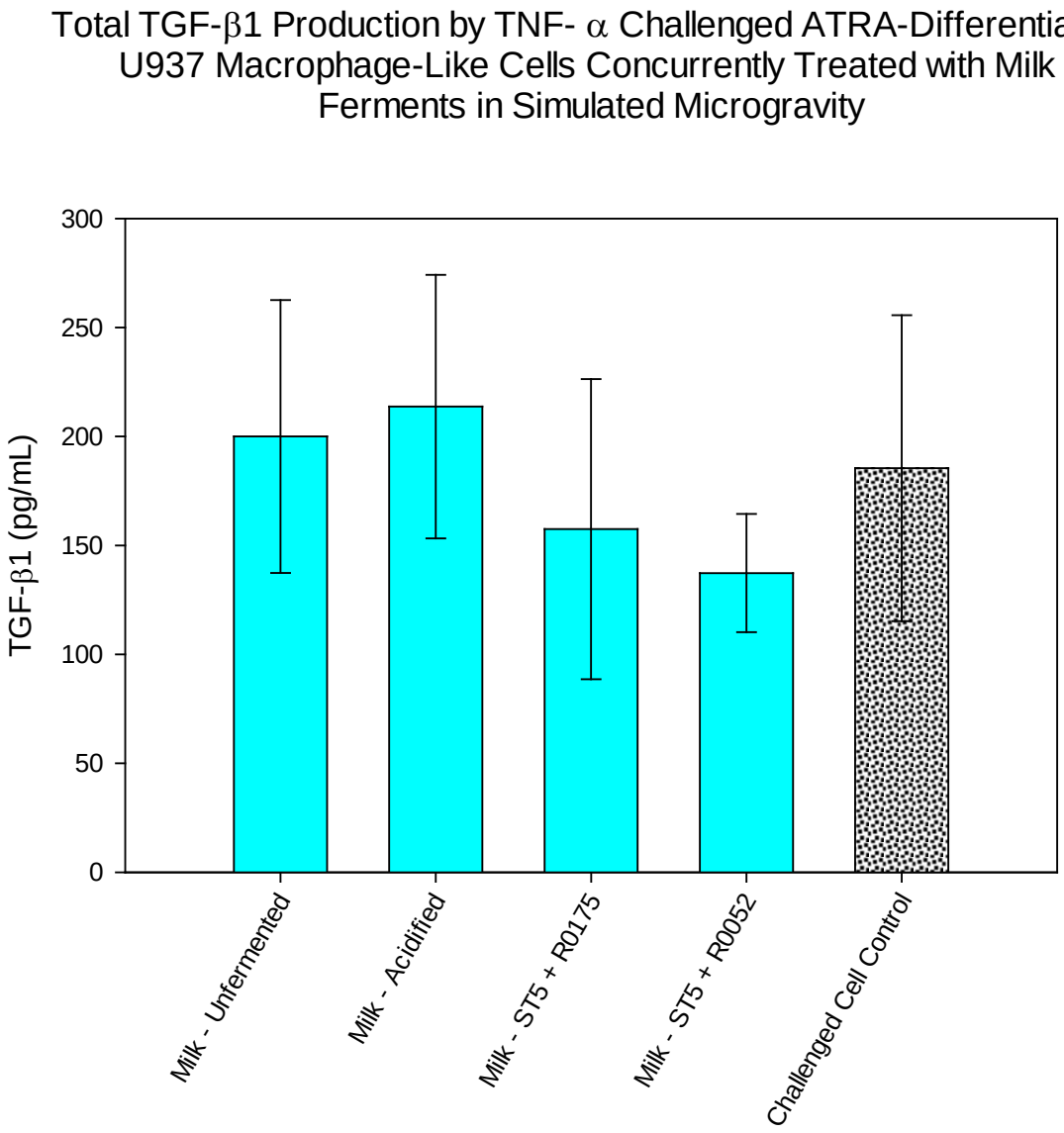

Figure 41: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on TGF-β1 production by ATRA-differentiated U937 macrophage-like cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 48 hours in simulated microgravity. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; p = 0.8905; n = 3).

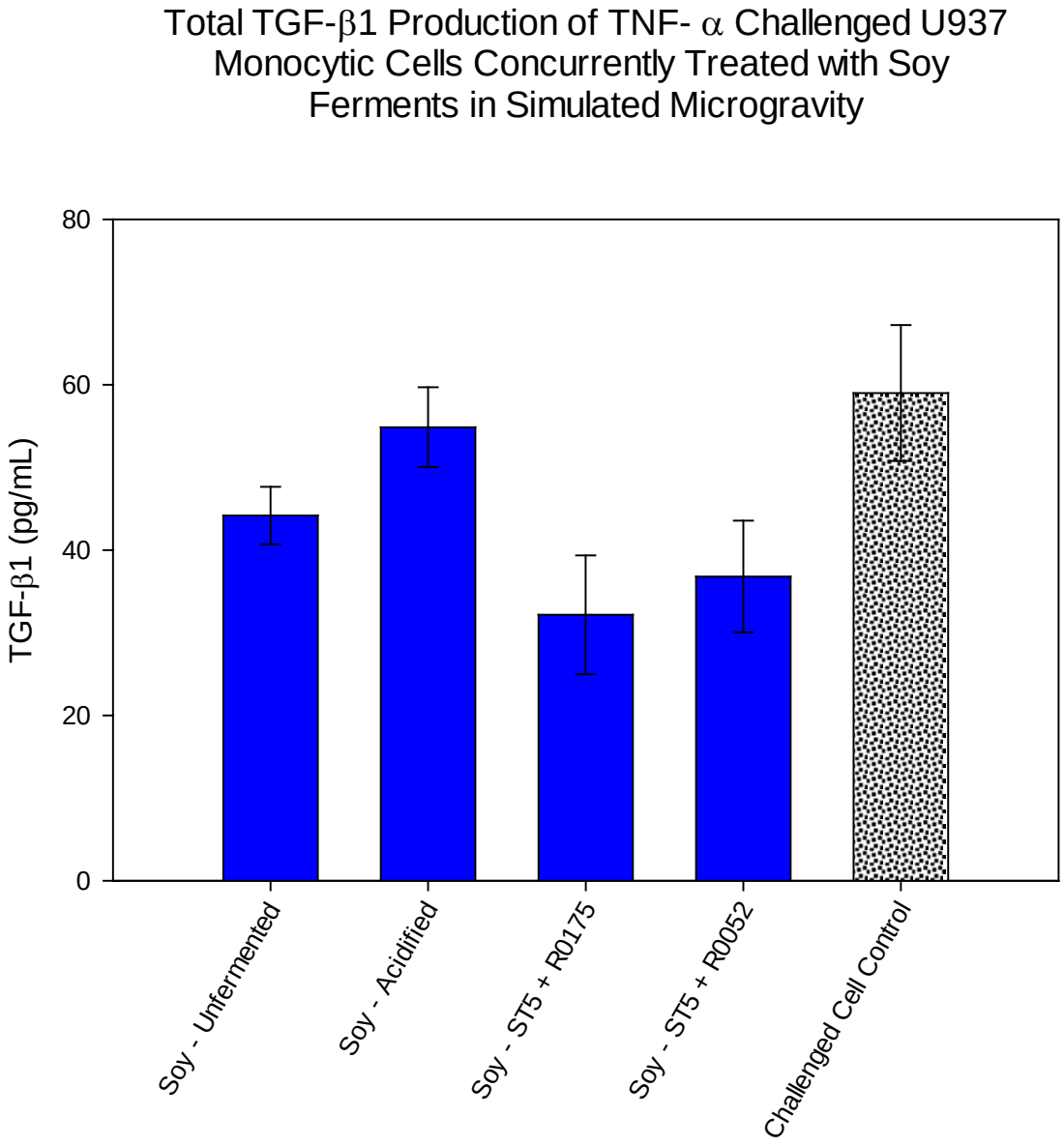

Figure 42: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on TGF-β1 production by non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 48 hours in simulated microgravity. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; $p = 0.0582$; $n = 3$).

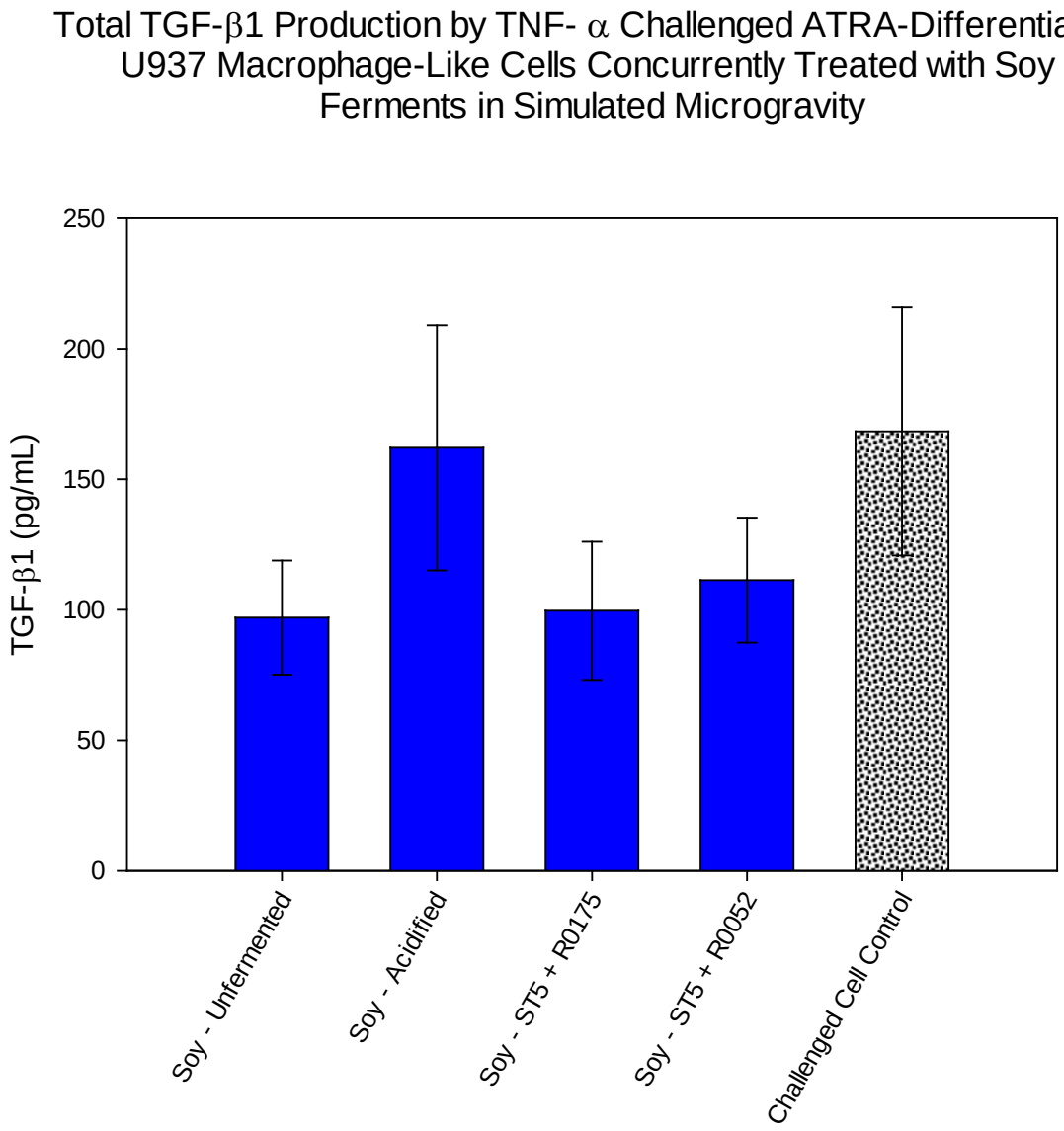

Figure 43: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on TGF-β1 production by ATRA-differentiated U937 macrophage-like cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 48 hours in simulated microgravity. Data shown as mean concentration in pg/mL ± SEM. (One-way ANOVA; $p = 0.4647$; $n = 3$).

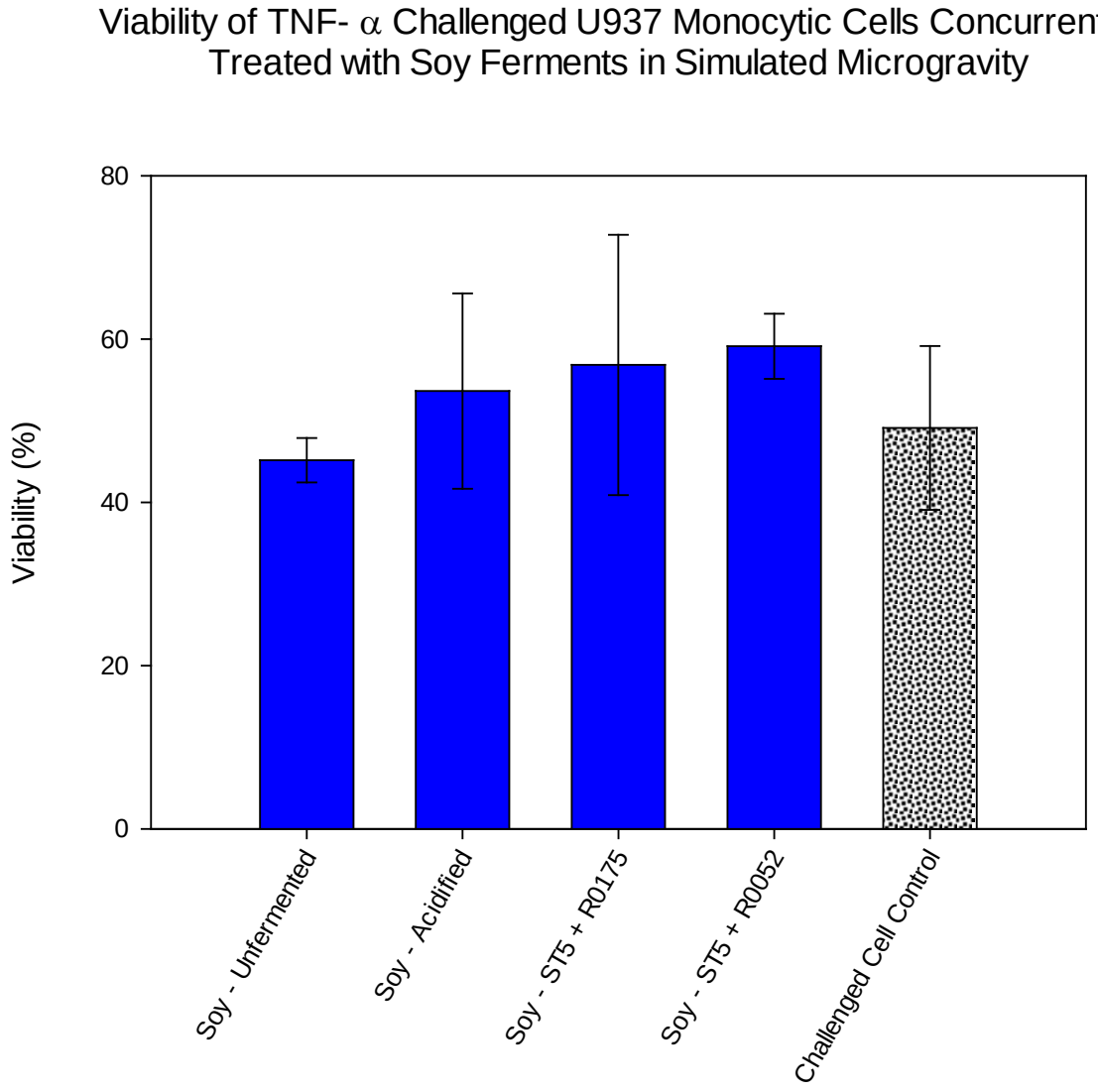

Figure 44: Viability of non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) following concurrent incubation with soy ferments and soy controls (1/100 in RPMI 1640) and 20 ng/mL TNF-α for 24 hours. Data shown as mean percent viability ± SEM. (One-way ANOVA; p = 0.8655; n = 3). Pre-treatment viability was 85.7 ± 0.03 %.

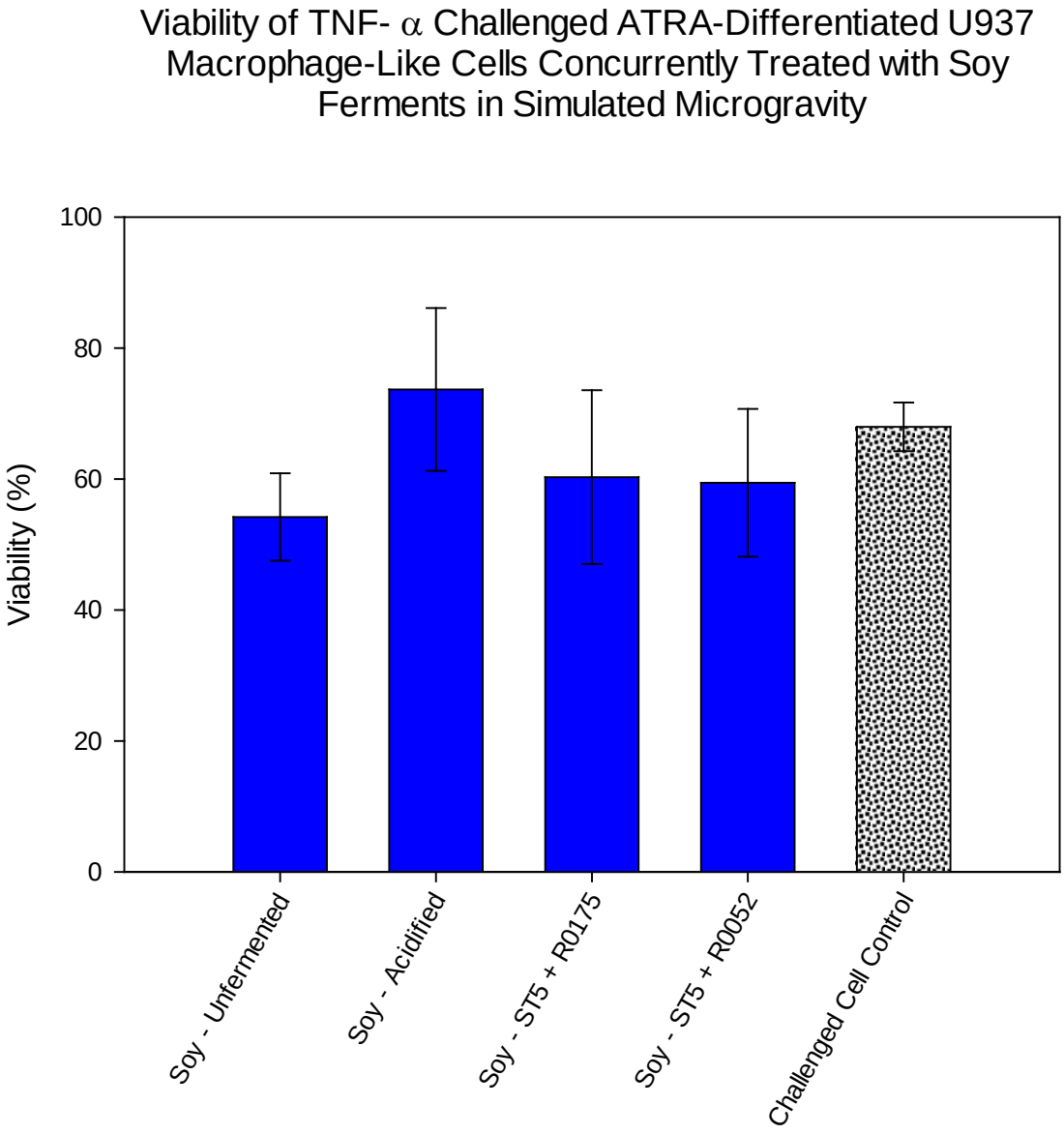

Figure 45: Viability of ATRA-differentiated U937 macrophage-like cells (2.5×10^5 cells/mL) following concurrent incubation with soy ferments and soy controls (1/100 in RPMI 1640) and 20 ng/mL TNF-α for 24 hours. Data shown as mean percent viability ± SEM. (One-way ANOVA; p = 0.6890; n = 3). Pre-treatment viability was 87.7 ± 1.7 %.

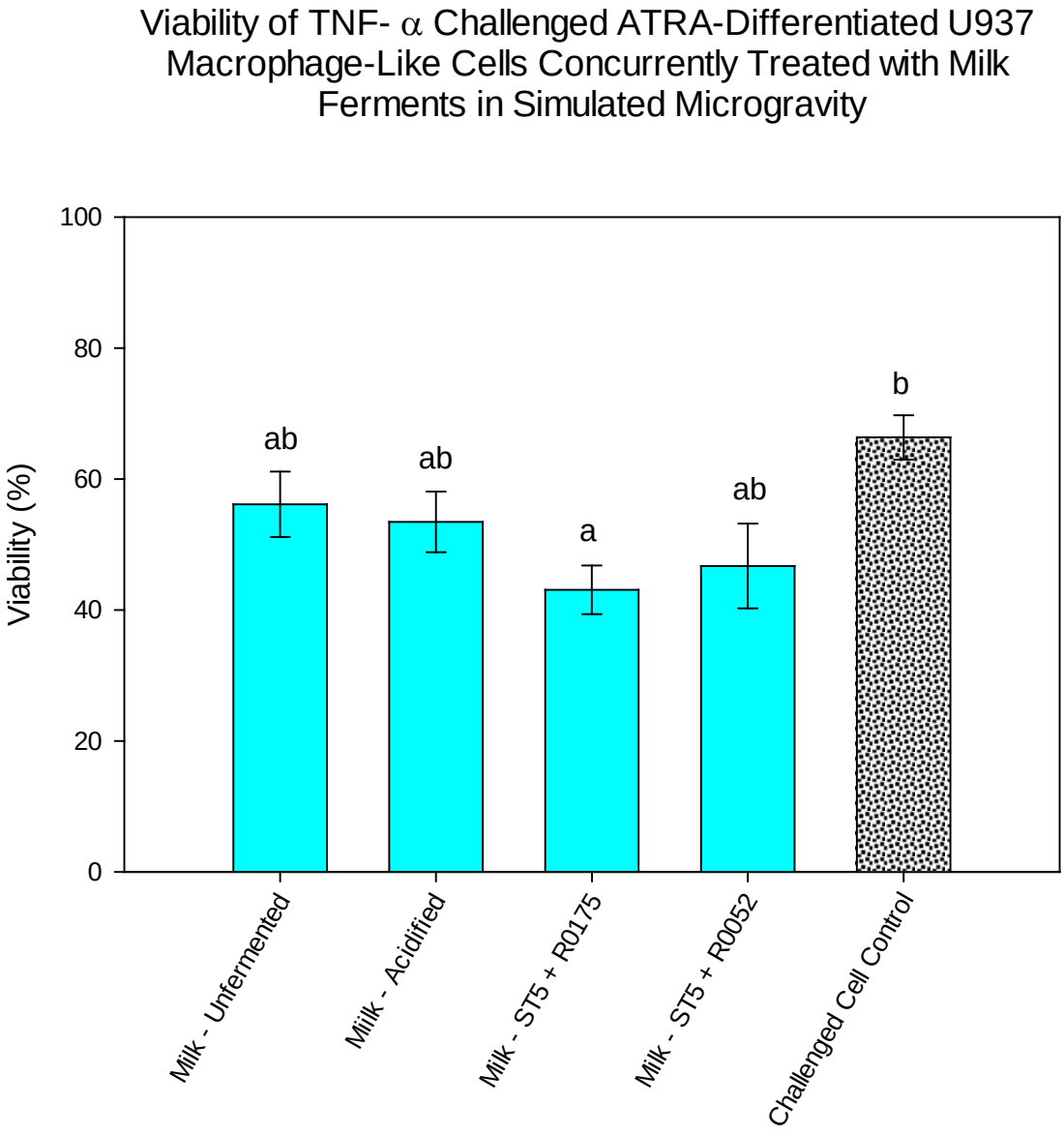

Figure 46: Viability of ATRA-differentiated U937 macrophage-like cells (2.5×10^5 cells/mL) following concurrent incubation with milk ferments and milk controls (1/100 in RPMI 1640) and 20 ng/mL TNF-α for 24 hours. Data shown as mean percent viability ± SEM. (One-way ANOVA; p = 0.0302; n = 4). Pre-treatment viability was 87.2 ± 2.0 %.

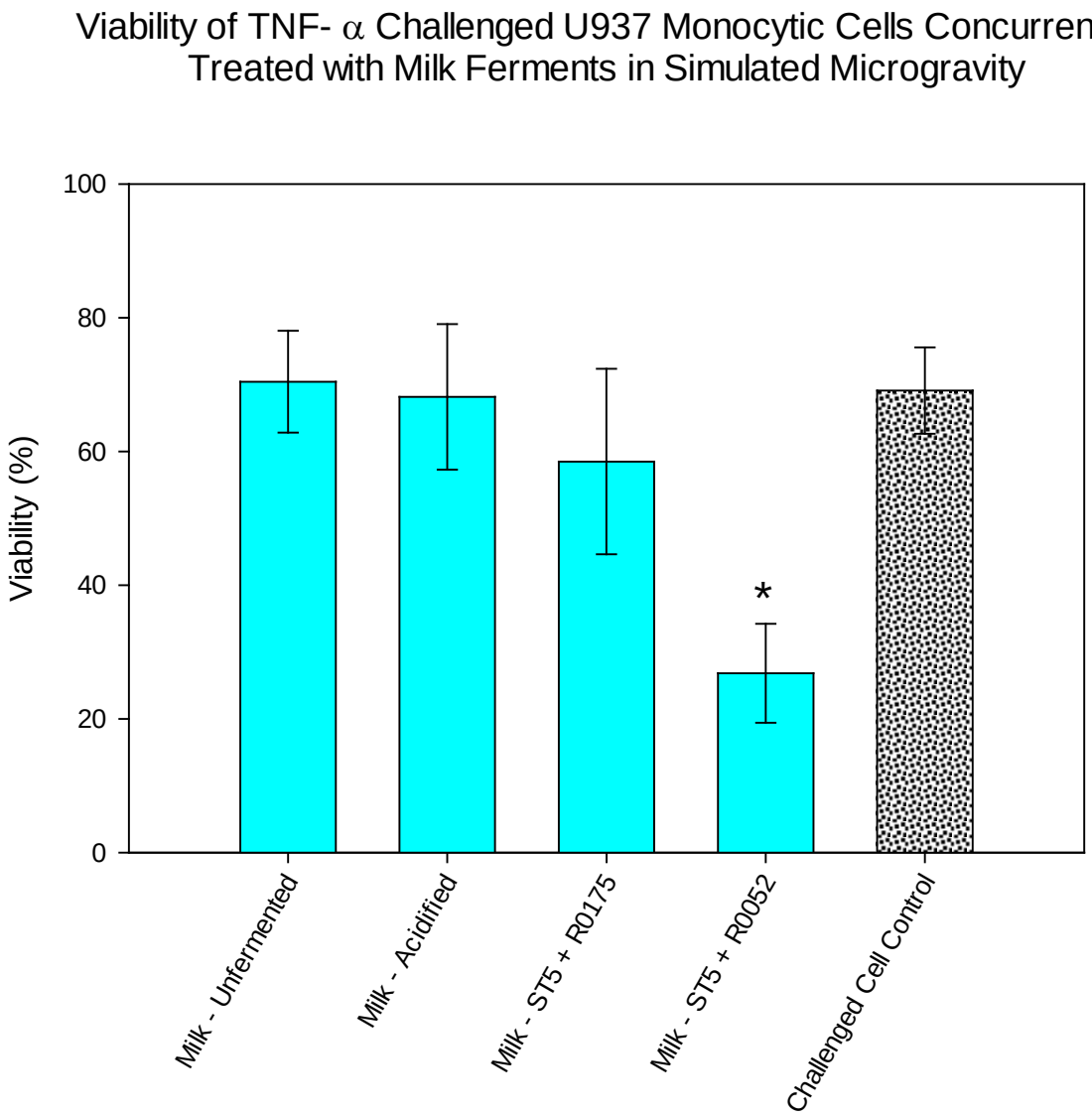

Figure 47: Viability of non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) following concurrent incubation with milk ferments and milk controls (1/100 in RPMI 1640) and 20 ng/mL TNF-α for 24 hours. Data shown as mean percent viability ± SEM. (One-way ANOVA; $p = 0.0447$; $n = 3$). Pre-treatment viability was 87.7 ± 5.2 %. Dunnett's post test used (* shows significant difference from challenged cell control).

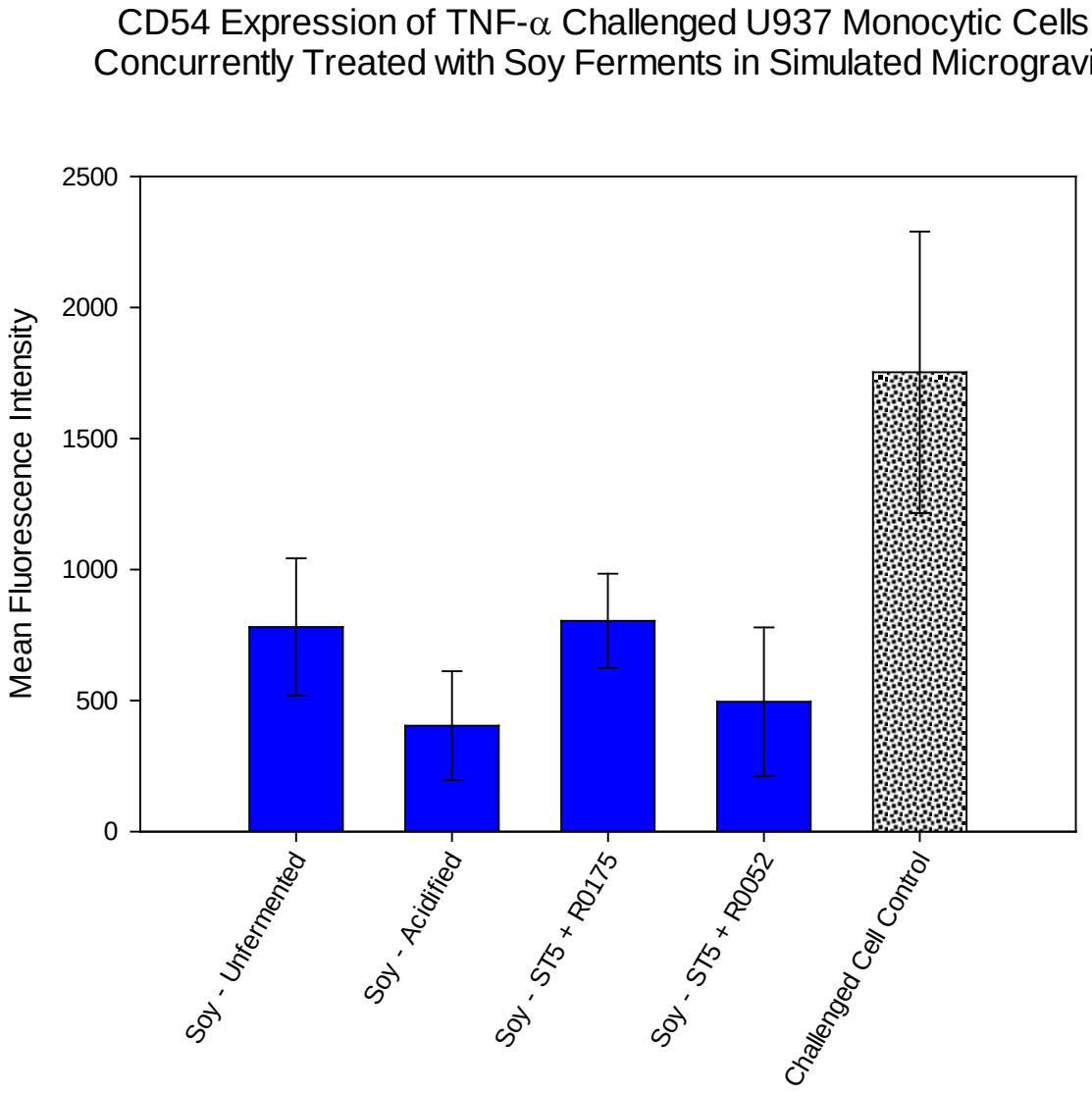

Figure 48: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD54 expression by non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.0856$, $n = 3$). Pre-treatment MFI was 1048.5 ± 347.4.

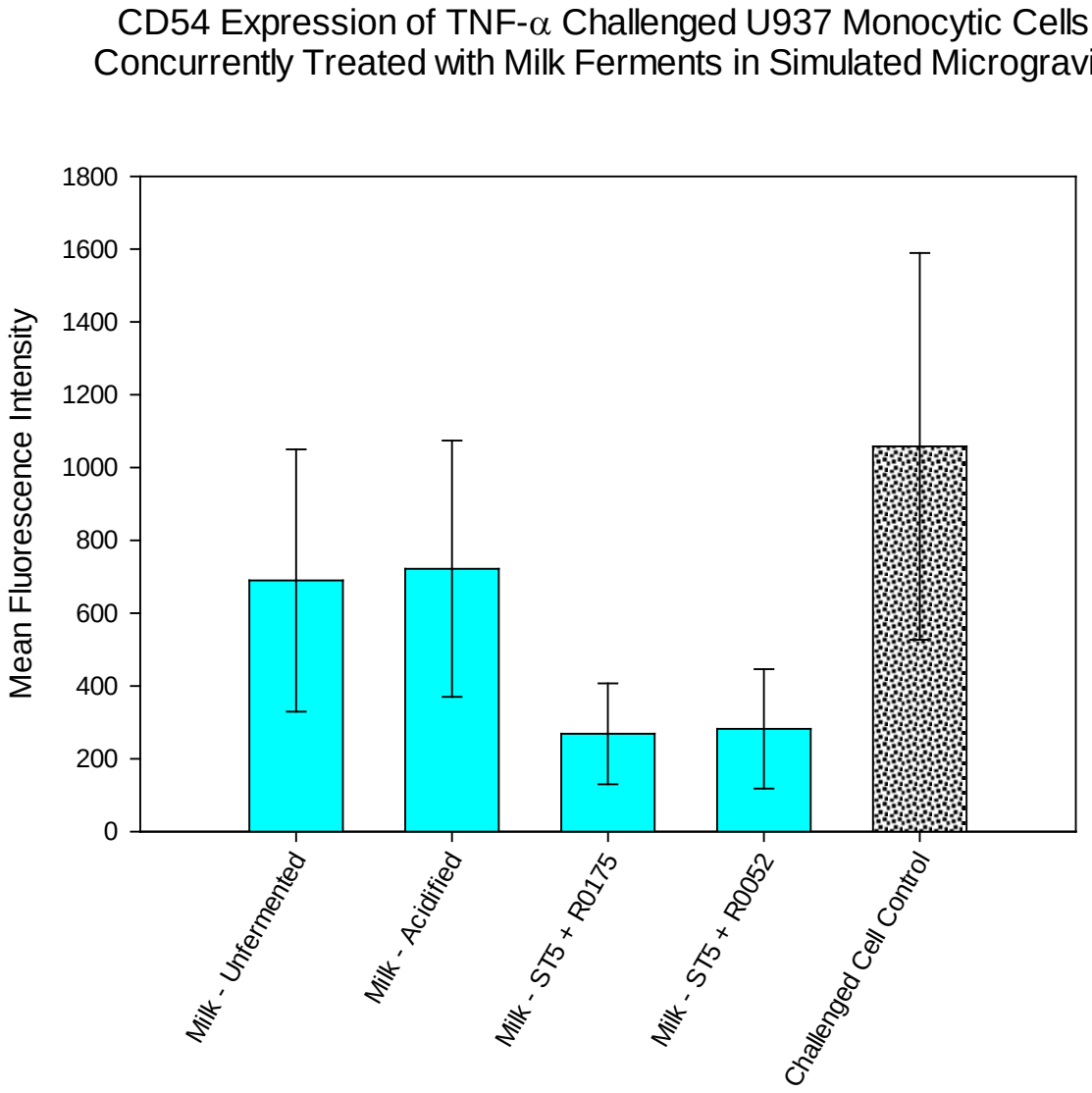

Figure 49: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD54 expression by non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.4737$, $n = 3$). Pre-treatment MFI was 1344.2 ± 87.6.

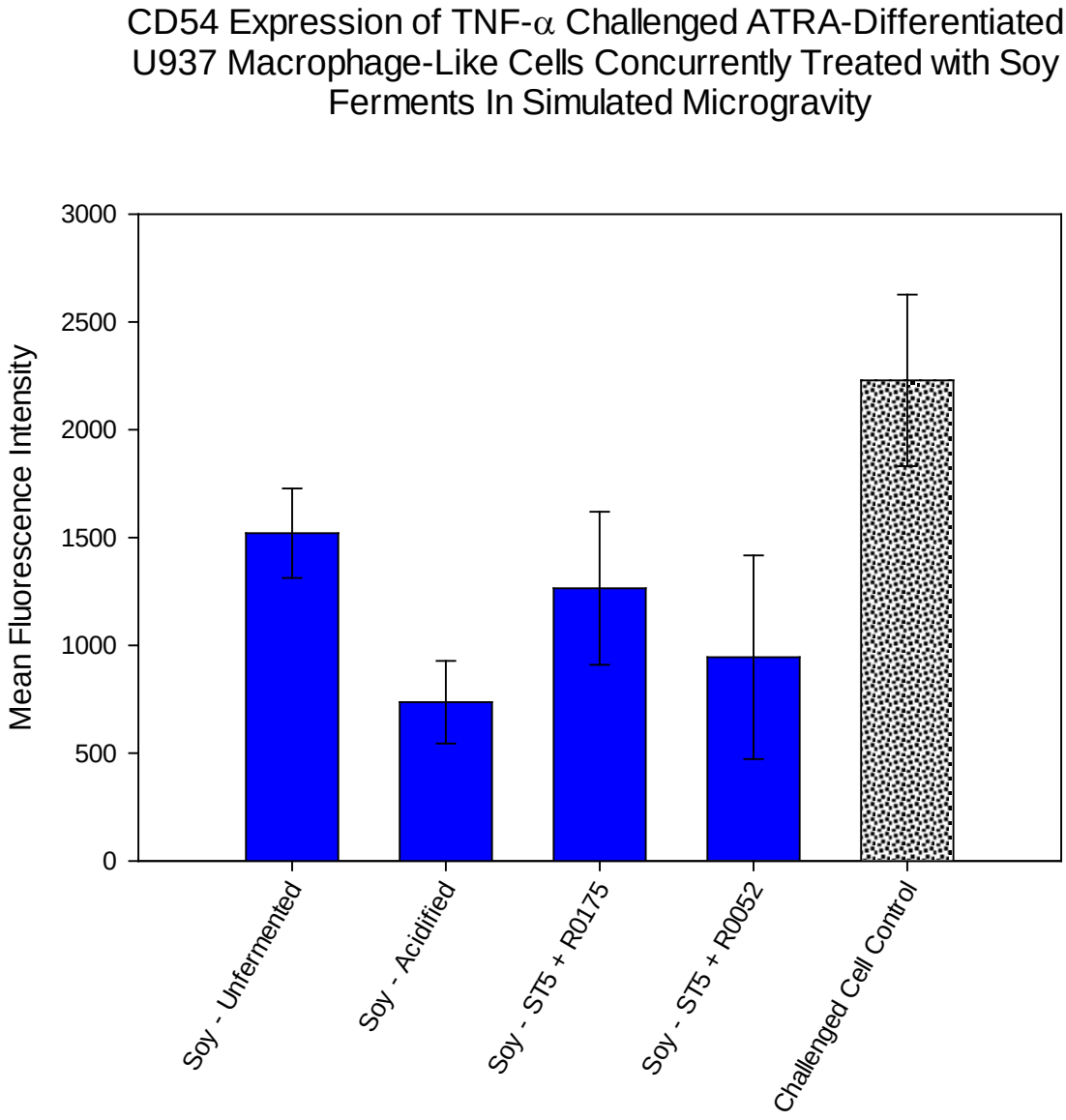

Figure 50: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD54 expression by ATRA-differentiated U937 macrophage-like cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.0800$, $n = 3$). Pre-treatment MFI was 1348.3 ± 117.6.

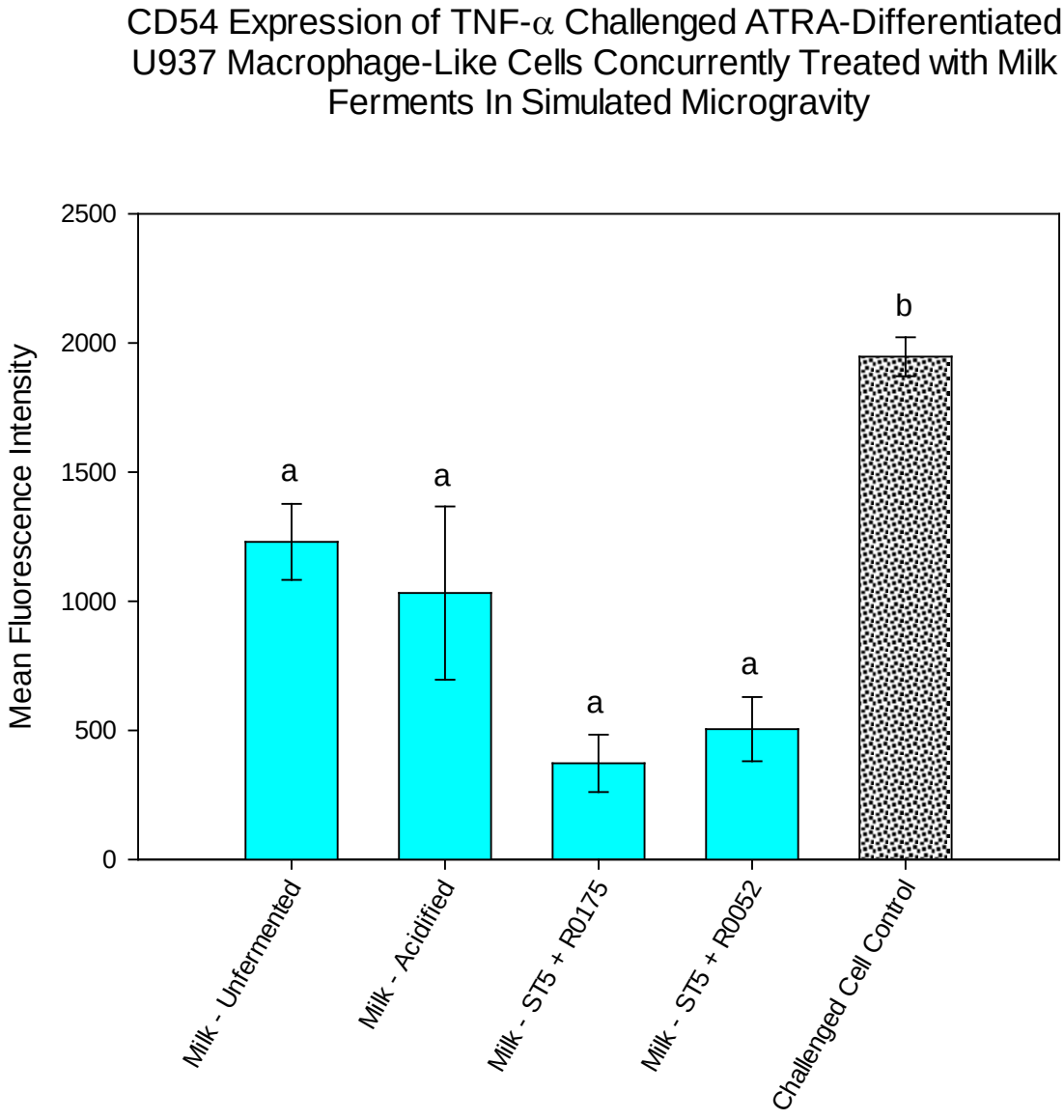

Figure 51: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD54 expression by ATRA-differentiated U937 macrophage-like cells (2.5 × 10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM (One-way ANOVA, p = 0.0008, n = 3). Pre-treatment MFI was 1540.3 ± 149.9.

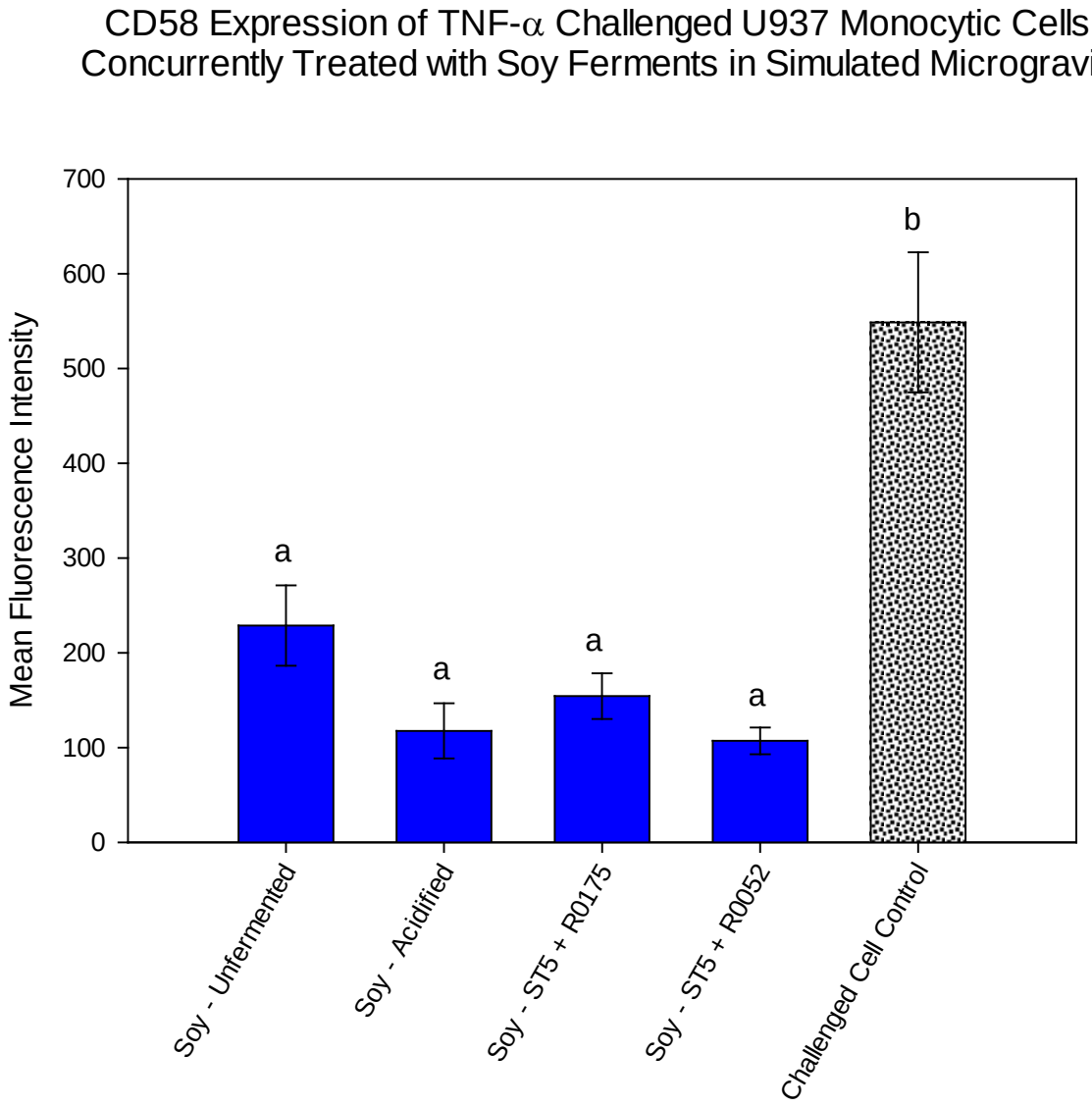

Figure 52: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD58 expression by non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.0001$, $n = 3$). Pre-treatment MFI was 400.2 ± 59.3.

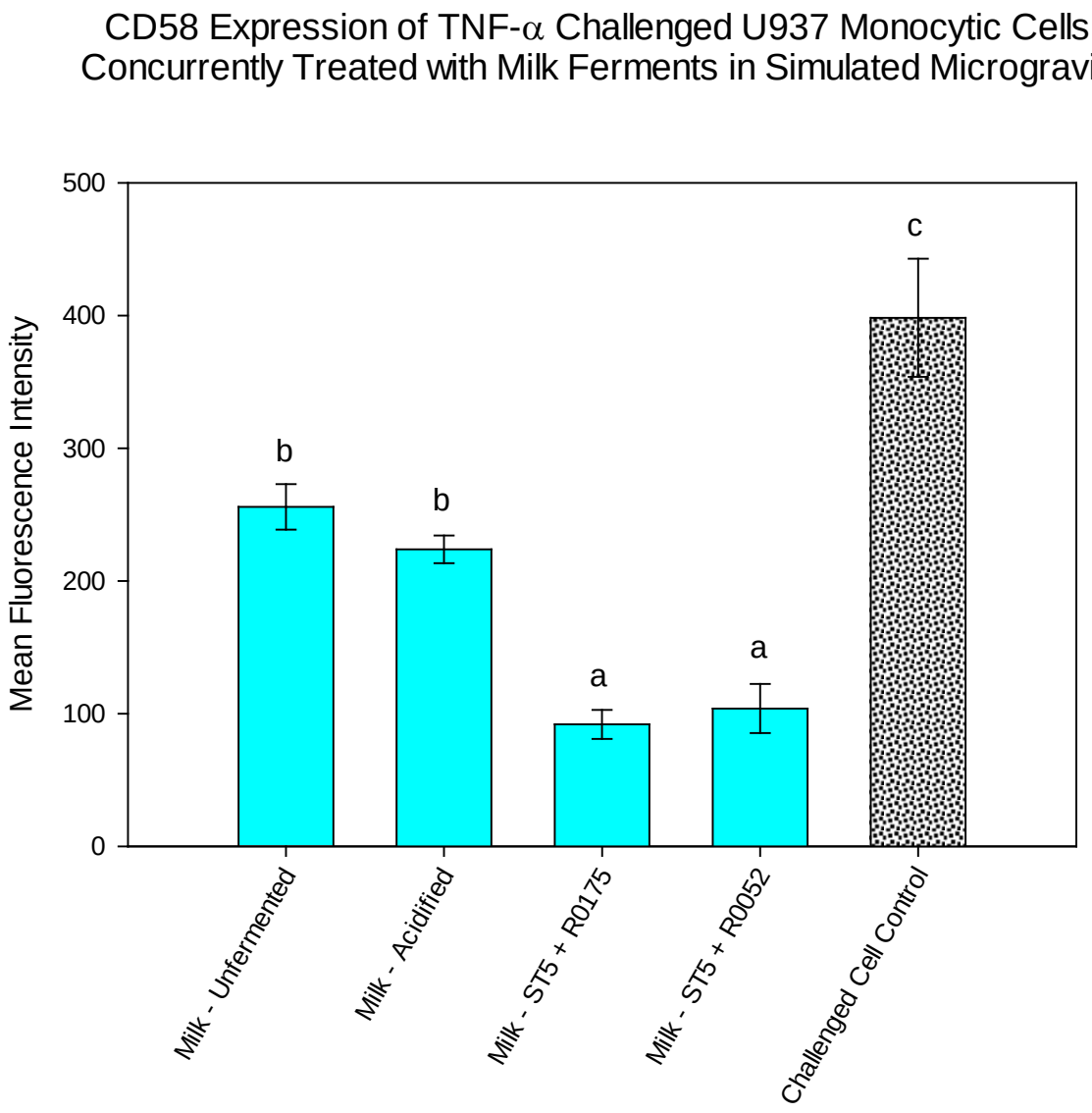

Figure 53: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD58 expression by non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM (One-way ANOVA, $p < 0.0001$, $n = 3$). Pre-treatment MFI was 459.0 ± 14.7.

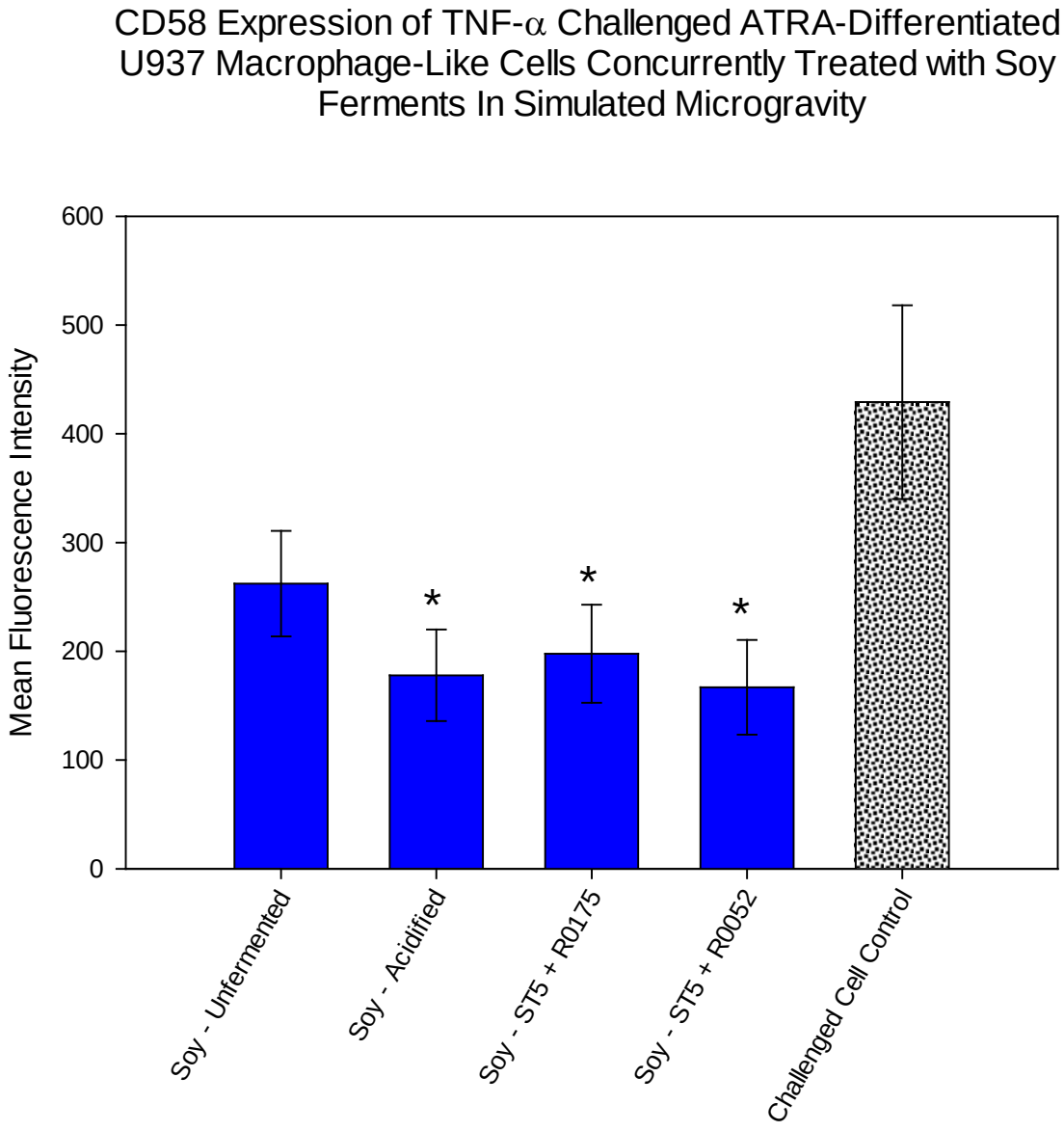

Figure 54: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD58 expression by ATRA-differentiated U937 macrophage-like cells (2.5 × 10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM (One-way ANOVA, p = 0.0430, n = 3). Pre-treatment MFI was 302.4 ± 143.2. Dunnett's post test used (* shows significant difference from challenged cell control).

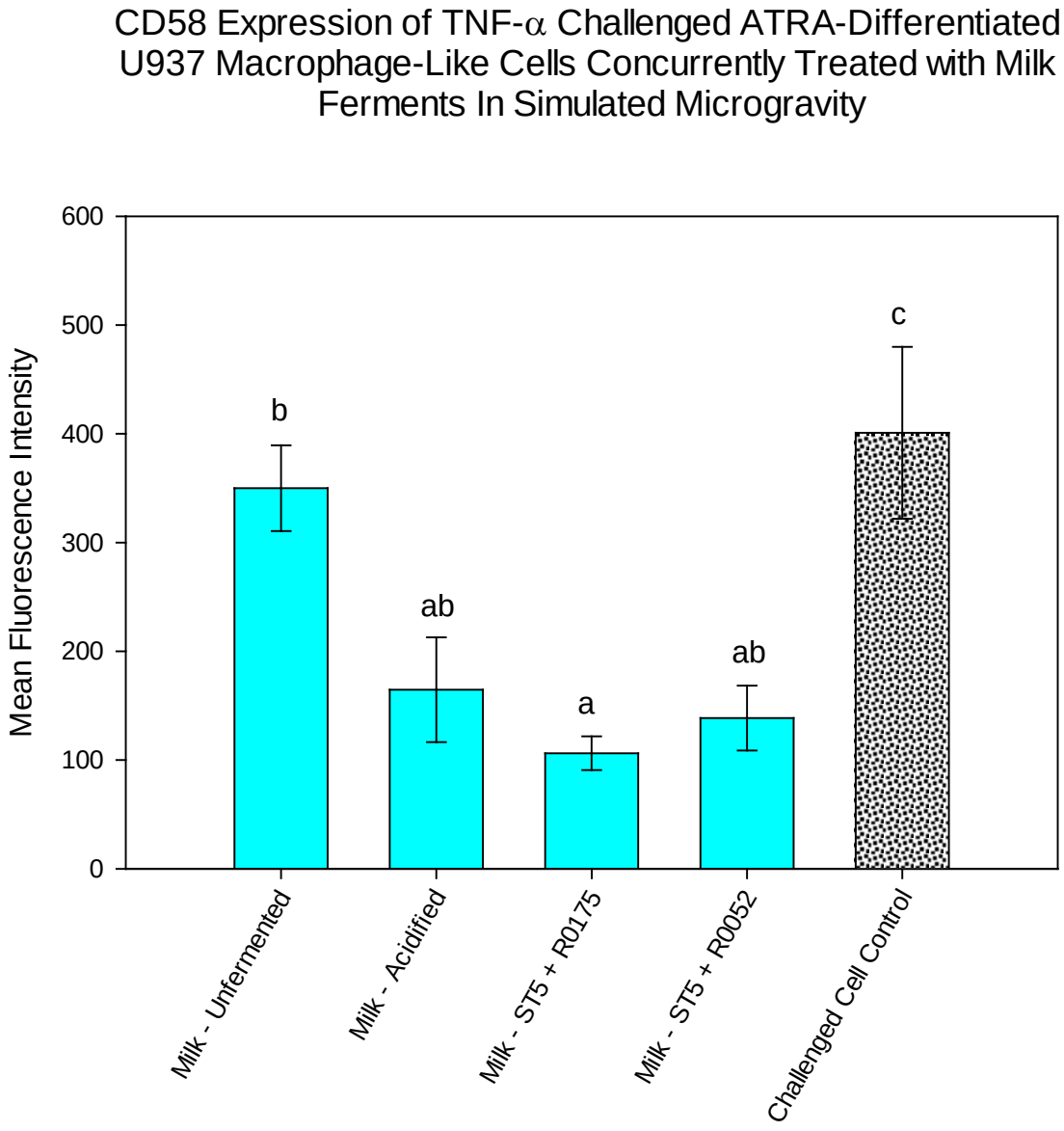

Figure 55: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD58 expression by ATRA-differentiated U937 macrophage-like cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM (One-way ANOVA, $p = 0.0038$, $n = 3$). Pre-treatment MFI was 521.2 ± 18.5.

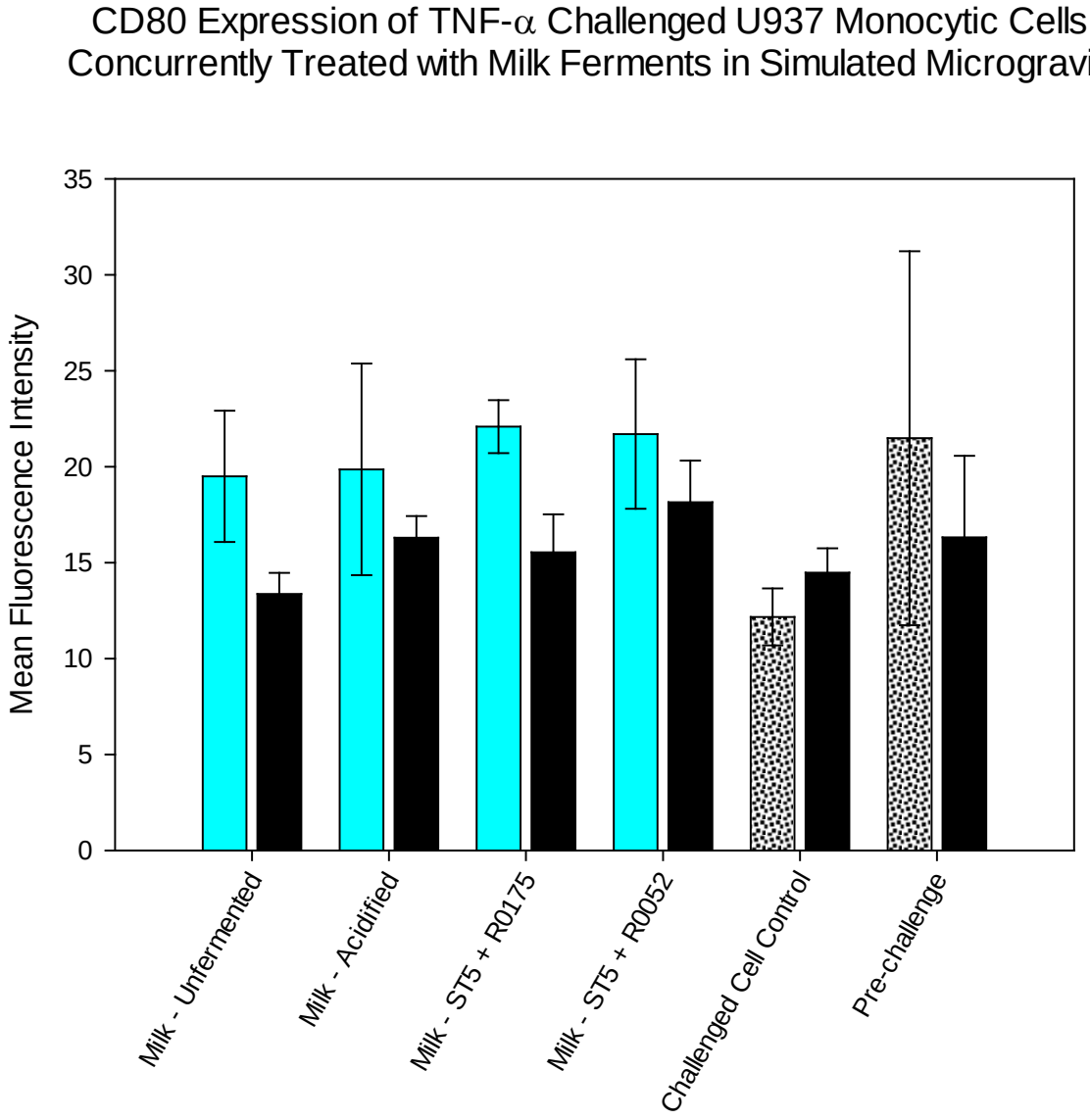

Figure 56: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD80 expression by non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD80 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

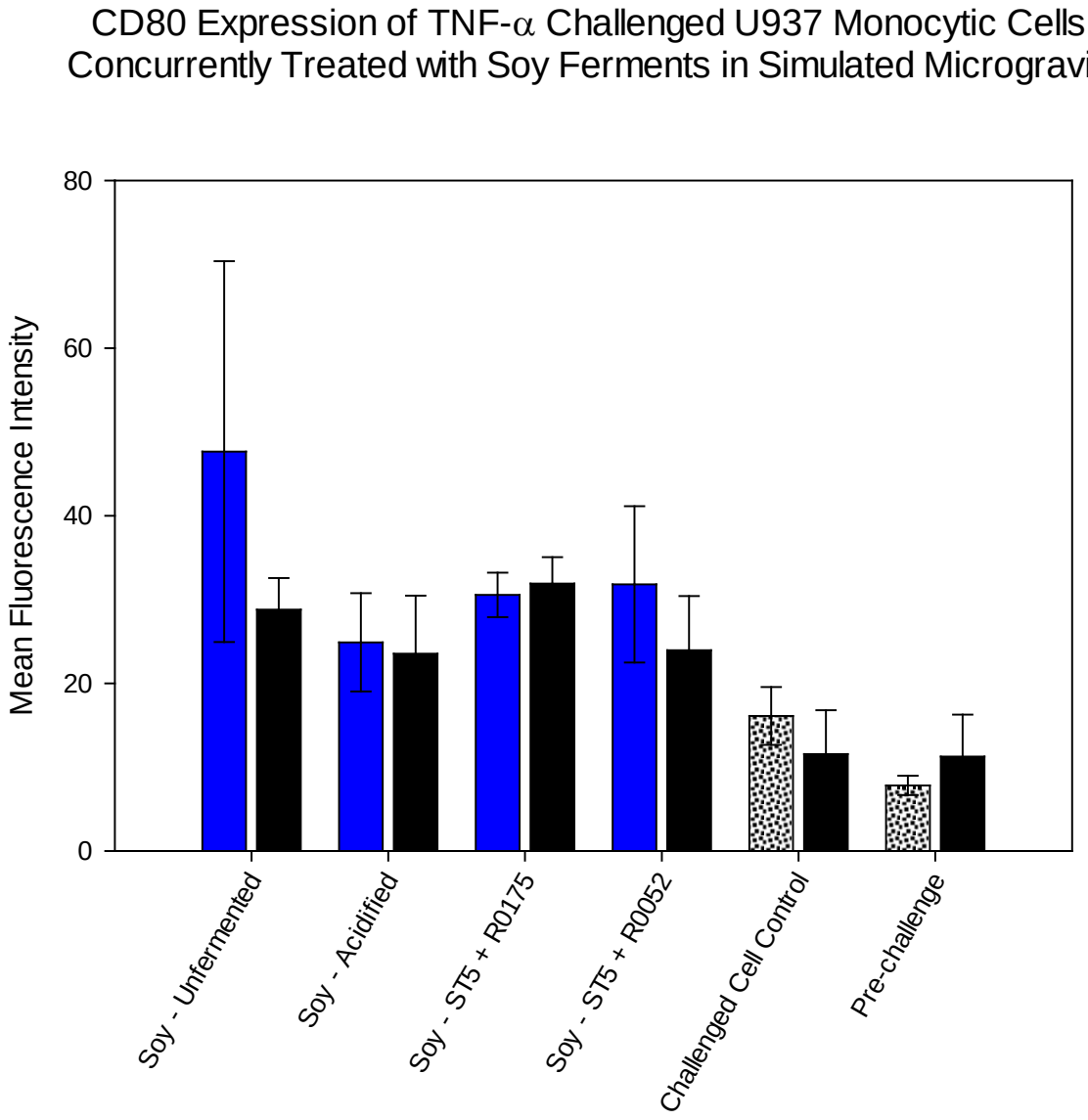

Figure 57: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD80 expression by non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD80 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

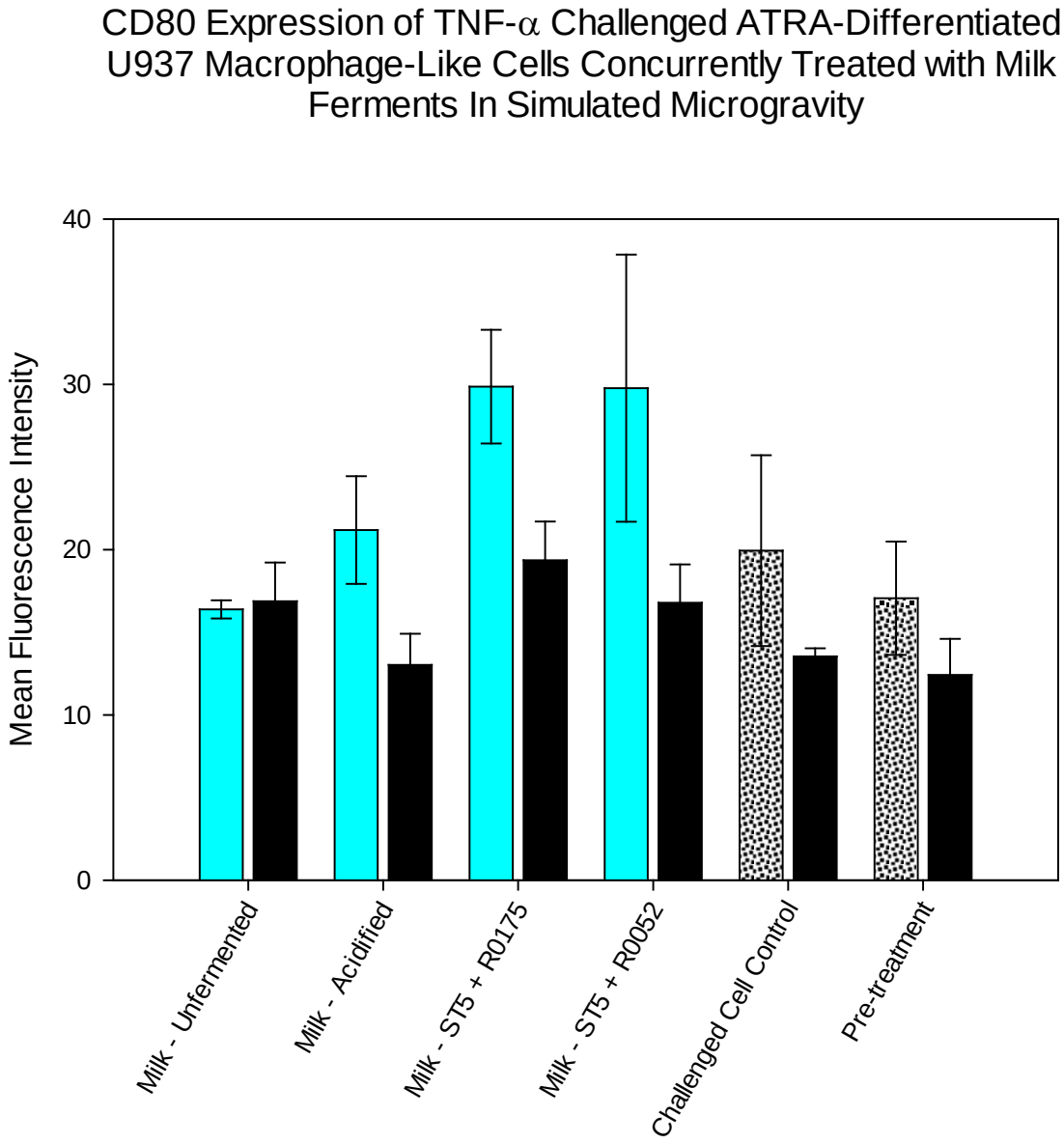

Figure 58: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD80 expression by ATRA-differentiated U937 macrophage-like cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD80 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

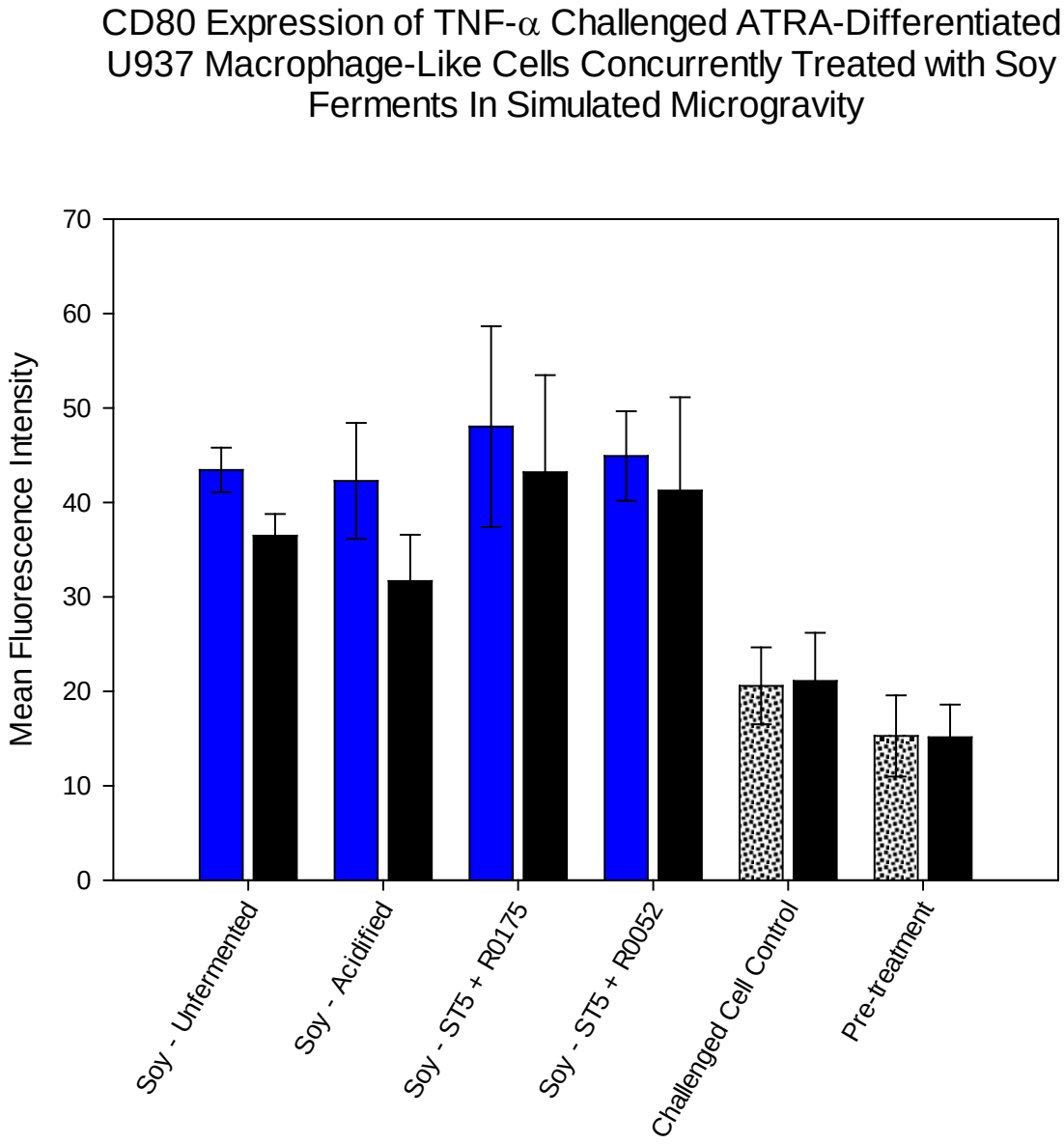

Figure 59: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD80 expression by ATRA-differentiated U937 macrophage-like cells (2.5 × 10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD80 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

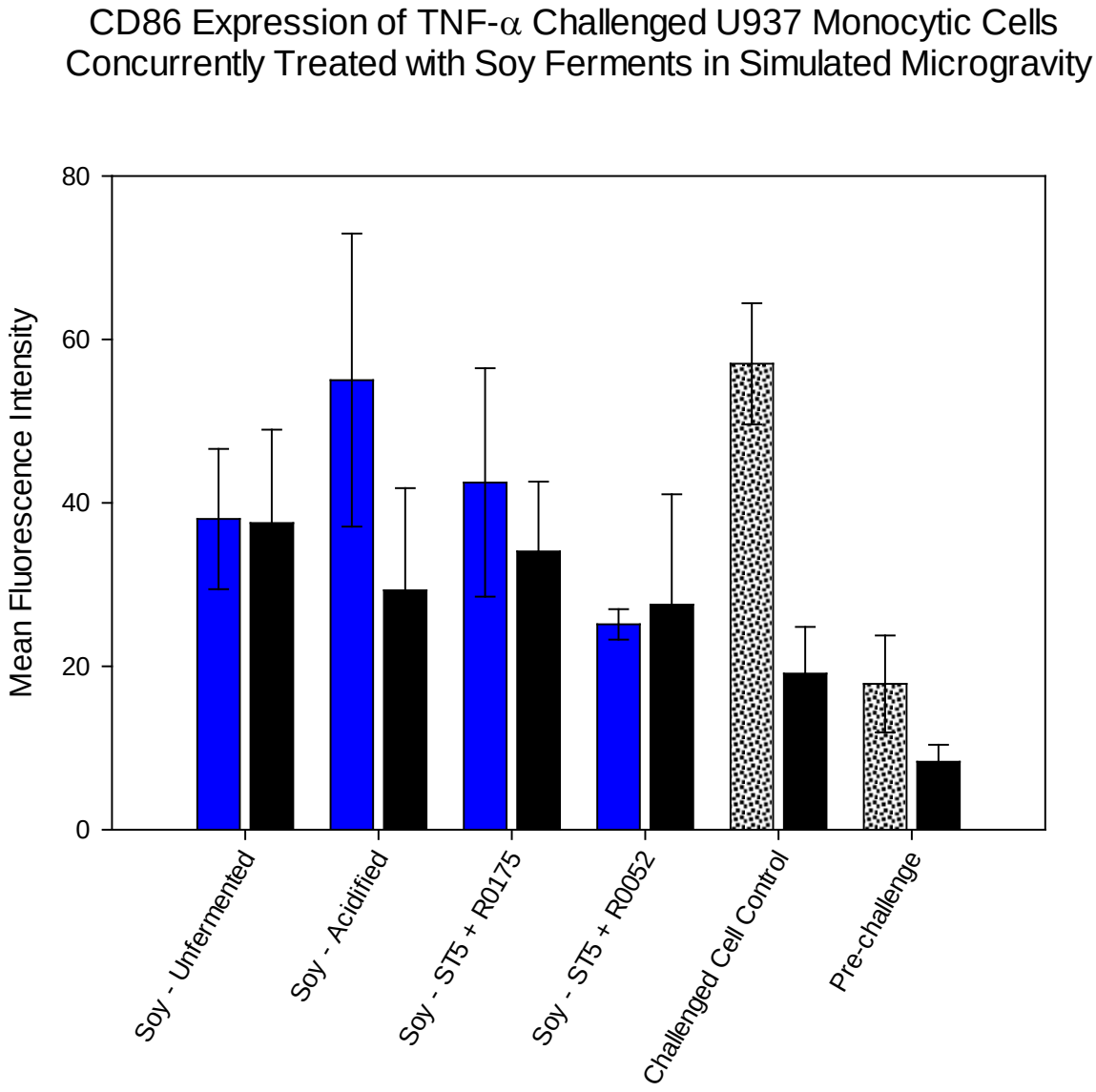

Figure 60: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD86 expression by non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD86 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

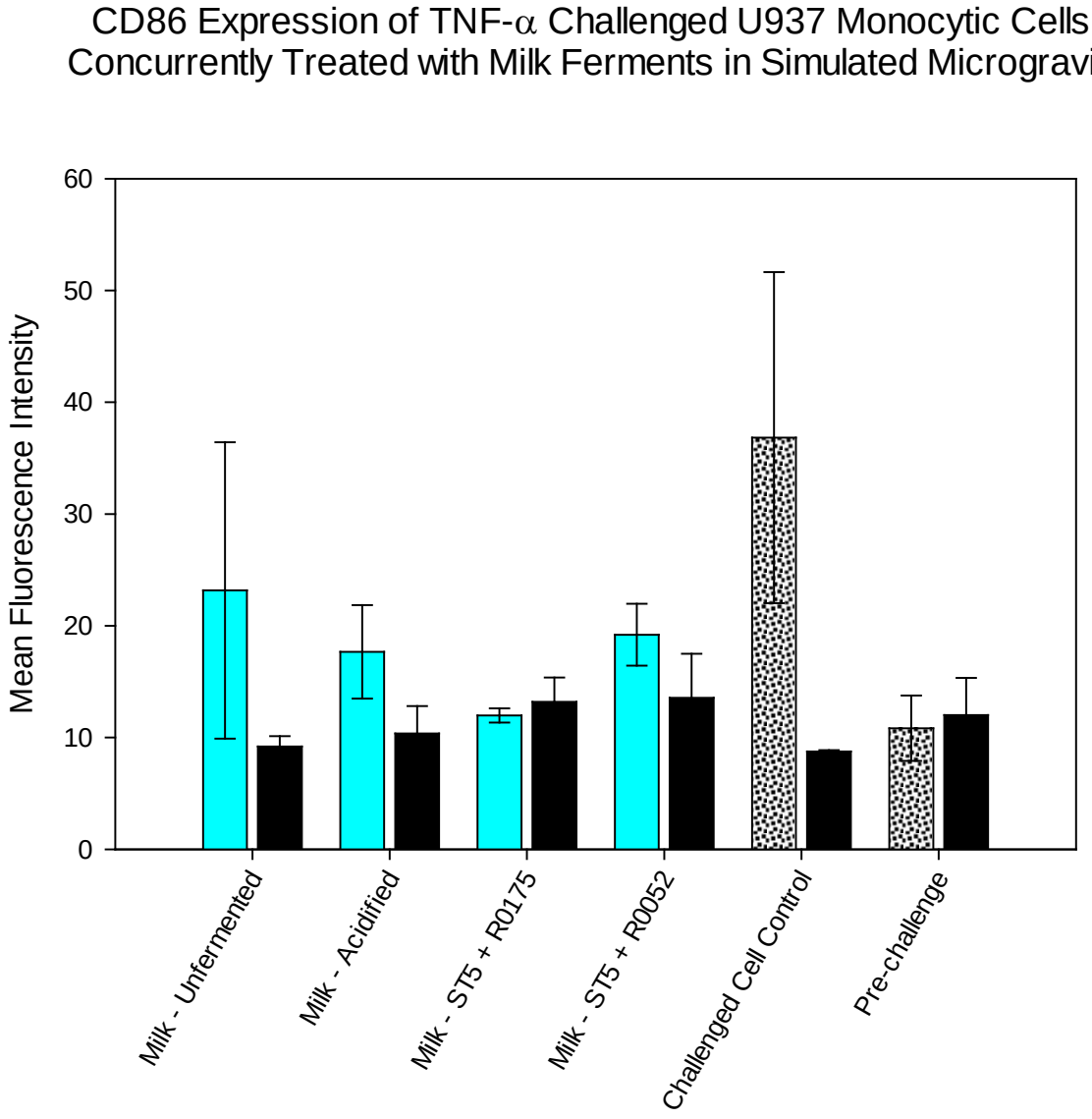

Figure 61: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD86 expression by non-differentiated U937 monocytic cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM. (n = 3). Coloured bars represent CD86 MFI, black bars represent isotype control MFI, indicating nonspecific binding.

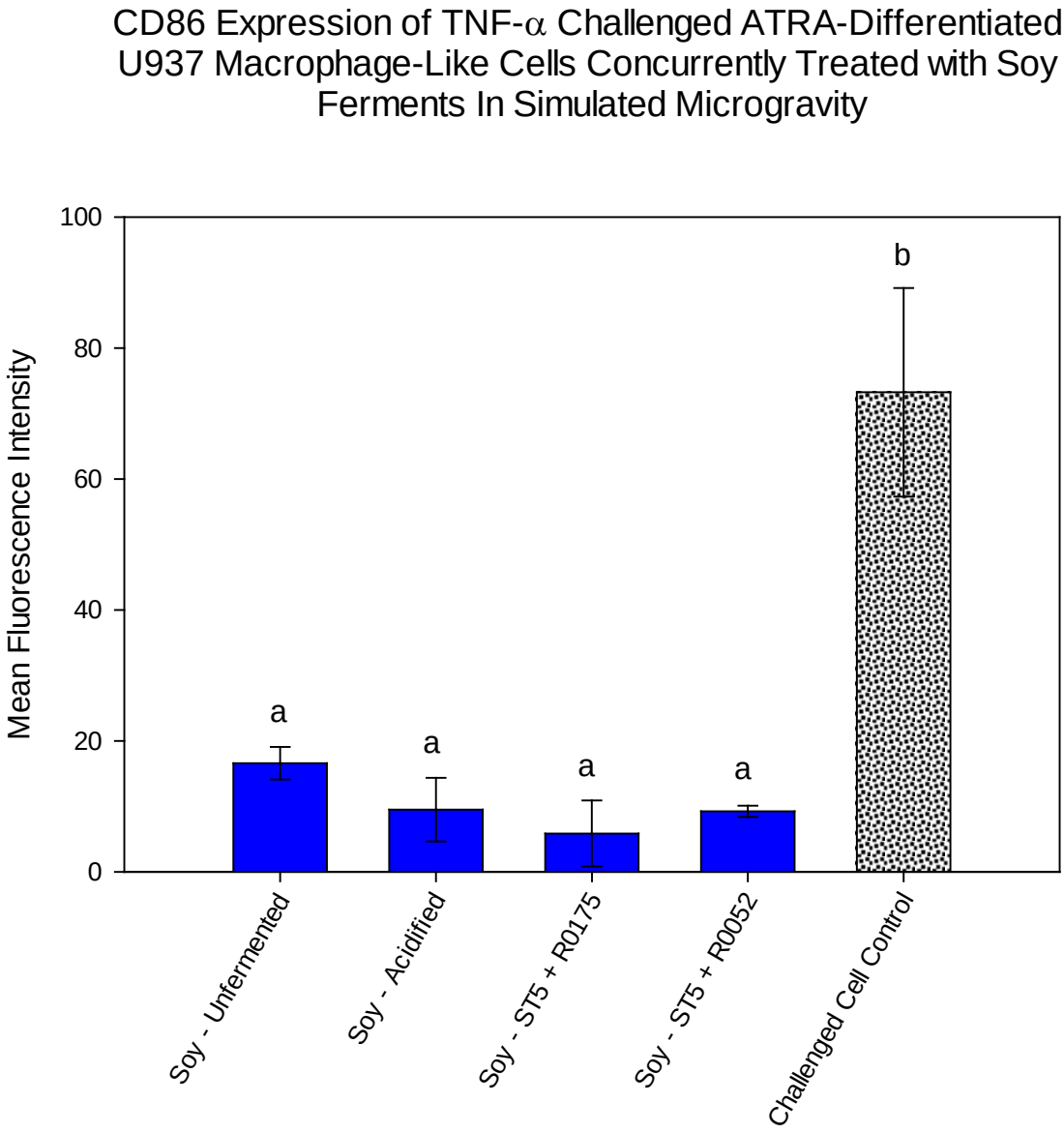

Figure 62: Effect of soy ferments and soy controls (1/100 dilution in RPMI 1640) on CD86 expression by ATRA-differentiated U937 macrophage-like cells (2.5 × 10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM. (One-way ANOVA; p = 0.0006, n = 3). Pre-treatment MFI was 0.9 ± 4.8.

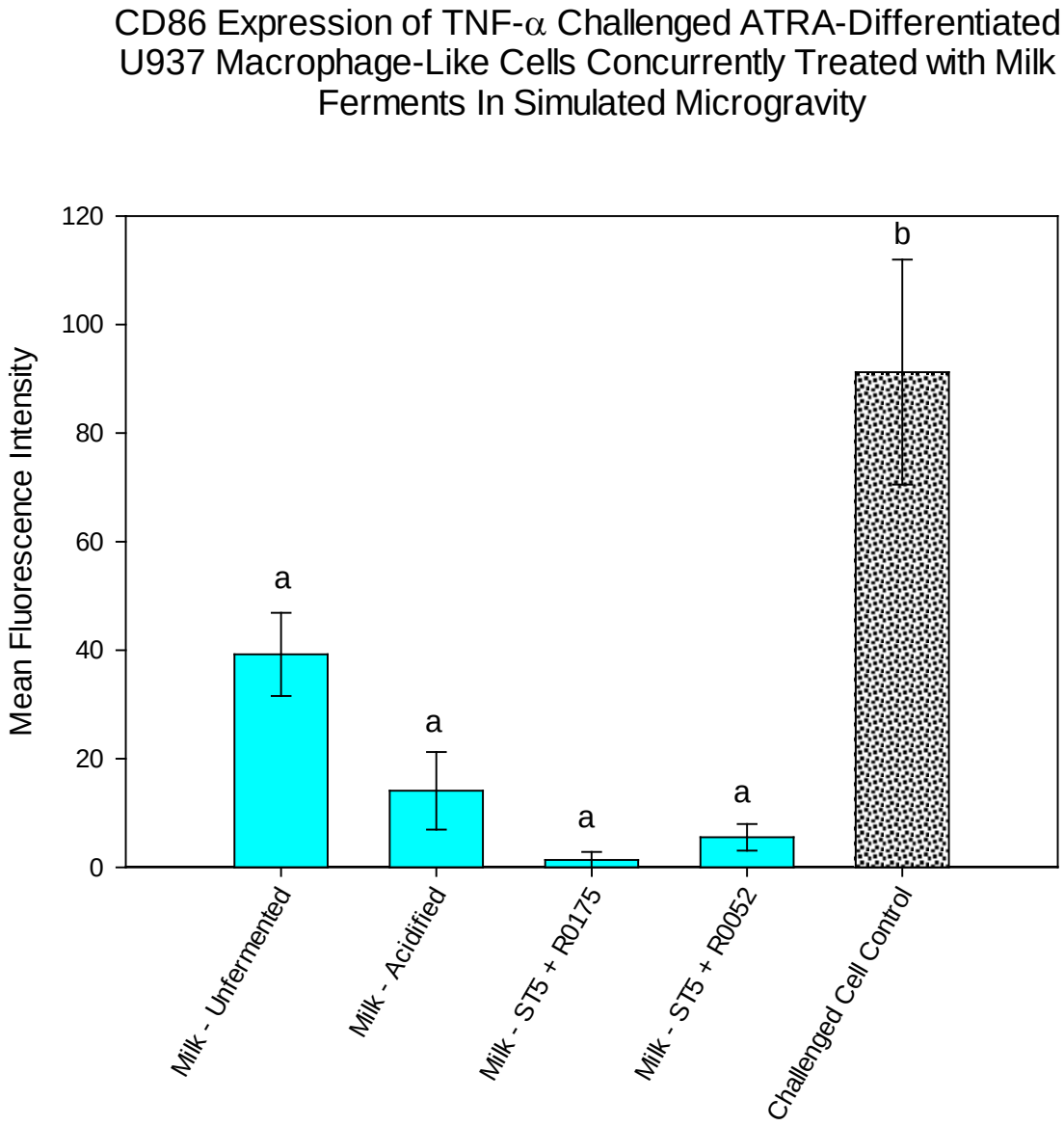

Figure 63: Effect of milk ferments and milk controls (1/100 dilution in RPMI 1640) on CD86 expression by ATRA-differentiated U937 macrophage-like cells (2.5×10^5 cells/mL) in the presence of 20 ng/mL TNF-α following concurrent incubation for 24 hours in simulated microgravity. Data shown as mean MFI ± SEM. (One-way ANOVA; $p = 0.0006$, $n = 3$). Pre-treatment MFI was 3.9 ± 2.7.

DISCUSSION

Generally, due to the need for astronauts to rebuild and maintain their immunity while in a mission, a probiotic soy or milk based ferment which decreases pro-inflammatory responses and/or increases anti-inflammatory responses would be of benefit. For example, IL-1, a pro-inflammatory cytokine, has been reported to be increased in astronauts during space flight (Huntoon *et al*, 1994; Stein *et al*, 1993; Leach *et al*, 1988; Leach *et al*, 1983). Such a product could also provide nutritional benefits such as calcium, B vitamins and antioxidants, which may help to address other health issues encountered in long-term space flight. With health risks such as bone loss, body fluid regulation, issues with the cardiovascular system and nutritional deficiencies due to a relatively small variety of food items available to astronauts (Heer *et al*, 2000), it is important to consider ways to supply nutrients to avoid or inhibit these issues. Lane *et al* (1998) has already noted that astronauts vary their nutritional intake while in flight compared to pre-flight, and that physiological changes due to dietary disruption can ultimately affect the immune system (Field *et al*, 2002; Sonnenfeld, 2001; Sonnenfeld, 1998).

The role of the GI tract microbiota in maintaining host health has recently been emphasized by numerous studies showing effects on the immune system (Clarke *et al*, 2010; Neish, 2004). In addition to effects on metabolism (Turnbaugh *et al*, 2009; Wikoff *et al*, 2009) and even behavior (Collins and

Bercik, 2009), it has been suggested that a key role of the gut microbiota is to maintain a homeostatic effect at the gut mucosa and prevent excessive pro-inflammatory responses that would otherwise result from the constant contact with antigenic, dietary and microbial stimuli. Disruptions in the astronaut microbiota could potentially result in a loss of this homeostatic effect (Hales *et al*, 2002). The ferments tested in this study could have the ability to regulate innate mucosal immune responses and possibly "restore the balance". While the effects of these ferments suggest anti-inflammatory activity, it is important to note that this is not equivalent to immunosuppression, and it would be anticipated that the astronauts' immune system would still able to ward off typical infections.

The LAB strains used for ferment preparation were selected for this research because they have previously shown the potential to modulate the immune system (Wood *et al*, 2007; Wallace *et al*, 2003; Easo *et al*, 2002), prevent infection (Johnson-Henry *et al*, 2005; Johnson-Henry *et al*, 2004), reduce symptoms of stress (Gareau *et al*, 2007; Zareie *et al*, 2006) and produce bioactive compounds (Fiander *et al*, 2005). Clinical trials have also been completed using these strains to reduce symptoms of inflammation in the gut (Benes *et al*, 2006; Haskey and Dahl, 2006; Kocián, 2004). Champagne *et al* (2009) screened potential strains for their ability to grow in both MB and SB. Unfortunately, at that point, one of the more effective strains, *Lactobacillus rhamnosus* R0011 was excluded because it did not efficiently grow in SB. Initial screening of pure (one probiotic strain alone per ferment) and mixed cultures (a starter culture plus one probiotic strain per ferment) was completed by Wagar

et al (2009) on HT-29 IECs and it was determined that mixed cultures were more effective than pure cultures.

The probiotic milk and soy ferments discussed herein have demonstrated the ability to modulate certain aspects of the innate immune system, both in normal gravity and in simulated microgravity. These ferments affected the U937 cells differently depending on the cellular differentiation stage (monocyte or macrophage) and whether or not the cells were in simulated microgravity. While these results are based on an *in vitro* monocyte/macrophage system, such an approach provides a useful initial step in testing for immunomodulatory activity, and determining which ferments, conditions and immune parameters warrant further testing, and may provide guidelines to potential *in vivo* administration.

The differences in TGF-β1 secretion between non-differentiated and ATRA-differentiated U937s in normal gravity (Figure 11) demonstrate why it was necessary to look at more than one cellular differentiation stage. This supports the initial hypothesis that soy and milk ferments would affect the two differentiation stages differently, and, on a broader scale, two related cell types (monocytes and macrophages). The same pro-inflammatory cytokine (TNF-α) can act differently depending on its target cells and their state. This was also reflected in the differences seen in CD58 expression: TNF-α challenged non-differentiated U937s (monocyte stage) express more CD58 than TNF-α challenged ATRA-differentiated U937s (macrophage stage) by about 150 mean fluorescence intensity (MFI). CD86 expression by TNF-α challenged non-differentiated U937s was about 36 MFI while expression by TNF-α challenged

ATRA-differentiated U937s was about 190 MFI. Even though these two differentiation stages arose from the same cell type, it is apparent that they act dissimilarly in their response to a pro-inflammatory challenge.

Aside from differences in effects of unchallenged and challenged cell controls, the ferments themselves act differently on non-differentiated and ATRA-differentiated U937s with respect to effects on cytokine production. Milk controls and ferments did not affect IL-8 production by non-differentiated U937s (Figure 7) but soy fermented with ST5 + R0175 did (Figure 8). Milk and soy controls greatly increased IL-8 production by ATRA-differentiated U937s (Figure 9 and 10) compared to non-differentiated U937s: mean IL-8 production was approximately 1800 pg/mL (both milk controls) for non-differentiated U937s versus 20000 pg/mL (unfermented milk treated) and 330000 pg/mL (acidified milk treated) by ATRA-differentiated U937s, producing a greater than 11 and 18 fold increase, respectively. Milk ferments and controls all significantly reduced TGF-β1 production by non-differentiated U937s (Figure 12) but soy ferments and controls did not (Figure 13). However, differentiated U937s secreted IL-8 similarly in response to treatment with controls and ferments regardless of ferment base (MB or SB) (Figure 9 and 10). Effects on cell surface molecules CD54, CD58 and CD86 expression showed similar patterns regardless of ferment base (Figure 20 to 27, 32 to 35) . This suggests the possibility that the cytokines used in this study (in contrast to cell surface molecules) may be influenced by by-product(s) generated by the probiotic bacteria that is determined by the base in which they are fermented (for example, product X is produced while fermenting in milk, but

product Y is produced while fermenting in soy). The presence of isoflavones in soy milk gives the probiotic bacteria a unique substrate for fermentation (Yeo and Liong, 2010; Marazza *et al*, 2009; Pham and Shah, 2009; Chun *et al*, 2008; Rekha and Vijayalakshmi, 2008) compared to dairy milk, which does not contain isoflavones but does contain other structurally similar animal-derived estrogen-like hormones. Examples of soy isoflavones include genistein and daidzein, which have been shown to be potent inhibitors of leukocyte activity including cytokine secretion (Sakai and Kogiso, 2008; Gredel *et al*, 2008). These effects are possibly mediated through suppression of NF-κB, although this effect has been reported to be independent of estrogenic activity (Vanden Berghe *et al*, 2006), suggesting other components of soy may also be responsible for bioactivity. Estrogen has been shown to play a regulatory role in T-cell dependent immune responses by inducing rapid signaling events (Adori *et al*, 2010).

Differences in responses between non-differentiated U937s and ATRA-differentiated U937s may be due to changes in cell surface receptors induced during differentiation. Aside from an increased expression of CD11b, other cell surface receptors may be up- or down-regulated in leukocytes treated with ATRA (Ozpolat *et al*, 2007; Rizzi *et al*, 2007; Xia *et al*, 2006; Li *et al*, 2003) such as CD52, Bfl-1/A1, programmed cell death-4 and death-associated protein kinase 2. With different receptors able to bind to possible ligands in fermented soy and milk, pathways may be activated that were quiescent in non-differentiated cells. Estrogen receptors may be upregulated, in which case isoflavones and their

metabolites found in SB might be increasing the response of differentiated U937s treated with soy controls and ferments. In differentiated cells treated with milk controls and ferments (Figure 9 and 10), it is possible that responses could be due to an increase in EGF and IGF receptors. EGF and IFG found in cow's milk (Purup *et al*, 2006; Herbst, 2004; Forsyth 1996; Glimm *et al*, 1992) are able to bind to their human receptors. If EGF Receptor (EGFR) and IGF Receptors 1 and 2 (IGF1R and IGF2R) that may have been upregulated during ATRA-mediated cellular differentiation, increased responsiveness of differentiated U937 cells would be anticipated, and this would be an interesting topic for further study. EGFR activates down-stream several signal-transduction cascades such as MAPK/ERK leading to DNA synthesis and cell proliferation (Oda *et al*, 2005) and leads to increased IL-6 concentrations in asthma models (Tsuchiya *et al*, 2010) and increased IL-8 in skin injury (Büchau, 2010). IGF receptors seem a less likely player in effects on monocytes and macrophages as most literature focuses on their role in T-cell activation (Baudler *et al*, 2005; Sharp *et al*, 2005; Stentz and Kitabchi, 2003).

Another factor in milk which may be causing this increase in IL-8 in differentiated U937s is lactoferrin (LF) which has antimicrobial activity and is part of mucosal innate defenses (Sánchez et al, 1992). Bournazou *et al* (2010) has described inhibition of eosinophil migration by LF regardless of source (milk or cell derived). LF has also been described to modulate APC migration, expression of cytokines, chemokines and other effector molecules (Puddu *et al*, 2009). LF concentrations can be found in increased concentrations locally in

areas of Inflammatory Bowel Syndrome (Uchida *et al*, 1994) and allergic inflammation (van de Graaf *et al*, 1991). Fermentation by R0175 and R0052 may be disrupting this normal pattern by modifying the LF content in milk itself or influencing its activity, or the effects of R0175 and R0052 themselves may cancel out any negative effects of LF. Milk estrogen acting through EGF receptors, if up-regulated, and LF provides possible candidates for the increased pro-inflammatory response to unfermented and acidified milk controls (Figure 9 and 10).

Referring to acidified controls specifically, differentiated U937s reacted differently, with respect to cytokine production, than non-differentiated U937s. SCFA have recently been implicated in effects on NF-kB expression induced by lactic acid bacteria (Kumar *et al*, 2009) and so lactic acid milk and soy controls were used throughout this study. Lactic-acid acidified soy and milk controls were generally not as effective at decreasing IL-8 (Figure 9, 10, 37 and 39) and TGF-β1 (Figure 14, 15, 41 and 43) production in differentiated U937s, suggesting SCFA are not as, or at all, effective in the macrophage stage, in normal gravity and simulated microgravity. In fact, Bailón *et al* (2010) suggest that macrophages treated with the SCFA butyrate produce inflammatory cytokines. Cell surface molecules, if significantly different in response to ferment treatments, were typically down-regulated equally by both unfermented and acidified controls, regardless of cell differentiation stage, suggesting that SCFA are not involved in effects on cell surface molecule expression

U937 viability was typically not affected by controls or ferments, except for milk fermented with ST5 + R0052, which decreased cell viability in both normal gravity and simulated microgravity conditions for non-differentiated U937s (Figure 16 and 47), and milk fermented with ST5 + R0175 in simulated microgravity conditions for ATRA-differentiated U937s (Figure 46). This again supports the idea that a ferment base-dependent by-product(s) is produced during fermentation, which in this case induces apoptosis. Although there is significant cell death, there may be enough viable cells left to produce the observed amounts of cytokines.

The decrease in CD54 expression seen on non-differentiated U937s and differentiated U937s in soy and milk controls and ferments and, in both normal gravity (Figure 20, 21, 24, 25) and simulated microgravity (Figure 48, 49, 50 and 51) may prevent both monocyte stage and macrophage stage cells from adhering efficiently to endothelial cells (Maio and Del Vecchio, 1992), impeding cell travel to the site of infection and therefore limiting the pro-inflammatory response. For soy treated cells, the soy isoflavone genistein may play a role in inhibiting monocyte adhesion (Nagarajan *et al*, 2006) which could explain why even unfermented and acidified soy controls down-regulate CD54 expression to the same extent as that observed with soy ferments. Another probiotic strain (*Enterococcus faecium* M-74) administered in an oral capsule was reported in Hlivak *et al* (2005) to decrease CD54 expression on peripheral blood monocytes. Roessler *et al* (2008) also describes two probiotic strains (*Lactobacillus paracasei* Lpc-37 and *Bifidobacterium lactis* 420) in a fermented milk drink that

also significantly decreased CD54 expression. These effects are similar to what was seen in this research with R0175 and R0052. The fact that this outcome is retained in simulated microgravity suggests potential for the use of these ferments for astronauts in flight.

CD58 down-regulation was also consistent in response to treatment with milk and soy ferments, on either non-differentiated or differentiated U937s, and in normal gravity (Figure 22, 23, 26 and 27) and simulated microgravity (Figure 52, 53, 54 and 55) conditions. With less CD58 expressed on the cell surface, adhesion between APCs, such as monocytes and macrophages, and T-cells is weakened. At time of writing, there is no literature linking the possible effects of probiotics or ferments to CD58 expression, so this is a novel finding. However, it would appear that strains R0175 and/or R0052 might use a common mechanism for this down-regulatory effect, as regardless of ferment base, these strains have down-regulated CD58. Up-regulation of CD58 expression by TNF-α challenged non-differentiated and differentiated U937s may be a response unique to leukocytes, as Kvale and Brandtzaeg (1993) report no up-regulation of CD58 on two hepatocytic cell lines (Hep-G2 and SK-Hep-1) after stimulation with TNF-α, and Kvale *et al* (1992) also report no up-regulation in human IECs of CD58 by response to TNF-α. After an antigen/MHC provides the first signal to a T-cell, a secondary signal is provided by interaction between CD2 (LFA-2) on the T-cells and CD58 from an APC (Wang *et al*, 1999; Wingren *et al*, 1995). Blocking these molecules inhibits this interaction (Crosby *et al*, 2004), and one can speculate

that even reducing their interaction may help regulate T-cell priming during a pro-inflammatory infection during space travel or otherwise.

Prior to challenging with TNF-α, non-differentiated and differentiated U937s did not express (or expressed very little) CD80 (Figure 28 to 31, 56 to 59) or CD86 (Figure 32 to 35, 60 to 63). This may be a feature unique to the U937 cell line as several recent papers report constitutive CD80/CD86 expression on other monocytes or monocyte-derivatives (Caicedo *et al*, 2010; Jin *et al*, 2010; Almerighi *et al*, 2009; Seipel *et al*, 2009; Cacere *et al*, 2008), particularly the THP-1 monocytic cell line or primary monocytes. Without CD80/CD86 expression, T-cells may not be primed efficiently. TNF-α did not induce expression of CD80 by either U937 differentiation stage, but CD86 expression was induced in response to TNF-α treatment, with the highest levels being seen on differentiated U937s. No ferment or control treatment increased expression of these surface molecules by TNF-α challenged U937s. Instead, CD86 expression appeared slightly lower on TNF-α challenged differentiated U937s treated with milk and soy ferments and acidified controls, although further testing would be required to verify this effect. *In vivo*, deletion or blockade of CD80/86 improves survival and attenuates pro-inflammatory cytokine production during microbial sepsis in rats (Nolan *et al*, 2008), illustrating the role these cell surface molecules play in pro-inflammatory activity, in addition to their co-stimulatory role in T cell activation. Thus interpreting the biological significance of potential effects on CD80/CD86 expression would depend on the context in which expression was occurring.

In summary, the effects of milk and soy ferments on non-differentiated or differentiated U937s varied if the cells were in a normal gravity or simulated microgravity situation. Fermented milk decreased IL-8 production in non-differentiated U937s in simulated microgravity but not in normal gravity. Soy had the opposite effect on non-differentiated U937s: soy ferments decreased IL-8 production in normal gravity but not in simulated microgravity. Effects of ferments on TGFβ1 production were opposite to those observed on IL-8 production. In non-differentiated U937s, milk ferments decreased TGF-β1 production in normal gravity but soy ferments decreased TGF-β1 production in simulated microgravity. Differentiated U937s responded to milk and soy ferments similarly in both normal gravity and simulated microgravity: milk ferments decreased IL-8 production, and soy ferments decreased TGF-β1 production (See Table 5). This means that, aside from non-differentiated U937 cytokine secretion, microgravity did not change effects of the ferments. The activity of the probiotics was retained in simulated microgravity.

Work in Progress and Future Directions

The work contained in this thesis could branch off into several endeavors. The first and maybe most important would be to build on the work of Wagar *et al* (2009) and use the HT-29 IEC line in the HARV to test the effects of these ferments on IECs in simulated microgravity. Since IECs are another important player of innate immunity, providing both a physical and immunological barrier in the GI tract, this would be an important compliment to this work. Combined with

the data from this thesis, a greater picture of how these ferments modulate innate immunity would be revealed.

With respect to monocytes and macrophages, other challenges such as IFN-γ, LPS, and IL-1 could be used to determine which cellular signal transduction pathway these ferments have an effect on and to better characterize the range of immunomodulatory activity they may have. The JAK-STAT pathway, the CD14/TLR4/MyD88/NF-κB pathway and the IL-1R pathway, respectively, would potentially be influenced in response to ferment treatment.

Although still ongoing at the time of writing, Western blots have revealed down-regulation of NF-κB activation induced by TNF-α challenge of non-differentiated U937s. This down-regulation was observed following pre-incubation (for 20 hours) with a 1/50 dilution of soy and milk ferments (in RPMI), but was not seen for cells pre-incubated with soy or milk controls or RPMI controls (personal communication, N. Treiselmann). Further elucidation of the kinetics and dynamics of effects on NF-κB activation will assist the current understanding of cellular responses to these ferments, and help to place these findings into the context of inflammatory, autoimmune and pathogenic challenges, where NF-κB activation is involved.

Using a filter to remove the probiotic bacteria from these ferments would enable us to discern if the observed effects are dependent on direct bacterial contact with the U937s cells, or are due to soluble factors secreted by the bacteria or produced from the soy and milk substrates during fermentation.

Earlier work from our laboratory with these strains suggested that soluble components produced by R0052 and *Lactobacillus rhamnosus* strain R0011 in milk ferments are involved in down-regulating IL-1β-induced prostaglandin E_2 production by HT-29 IEC (Fiander *et al*, 2005). Other recent studies have shown that indeed secreted factors can contribute to down-regulation of pro-inflammation based on testing of probiotic-conditioned media (Bayoumi and Griffiths, 2010; Heuvelin *et al*, 2010; Jones and Versalovic, 2009; Broekaert *et al*, 2007; Yan *et al*, 2007). Further study would be required to determine whether such soluble factors are responsible for the effects observed in this study, or whether direct contact between LAB in the ferments and the monocytes/macrophages is required.

The large increase in IL-8 in ATRA-differentiated U937s observed in response to combined treatment with TNF-α and milk or soy controls in normal gravity conditions also requires further investigation. Using antibodies to either block EGF and IGF receptors or to bind to EGF and IGF themselves, we may be able to elucidate whether or not it is indeed these factors inducing IL-8 production.

Conclusion

This work has characterized immunomodulatory effects of two strains of LAB, *Bifidobacterium longum* R0175 and *Lactobacillus helveticus* R0052, when used to prepare soy and dairy milk ferments. Both strains have the ability to down-regulate pro-inflammatory and regulatory cytokines in some aspect or

another, depending on the base (soy or milk) in which they were fermented. Expression of cell surface molecules CD54, CD58 and CD86 were also down-regulated compared to a TNF-α challenged cell control. Decreased cellular adhesion could inhibit the pro-inflammatory response. Experiments in normal gravity conditions were replicated in simulated microgravity using HARV cell culture vessels, and in most cases, similar effects were observed. Although the underlying mechanisms responsible for the observed effects require more investigation and observation, these results provide insight into effects on this aspect of innate immunity and may provide guidelines for potential *in vivo* administration, especially during space travel.

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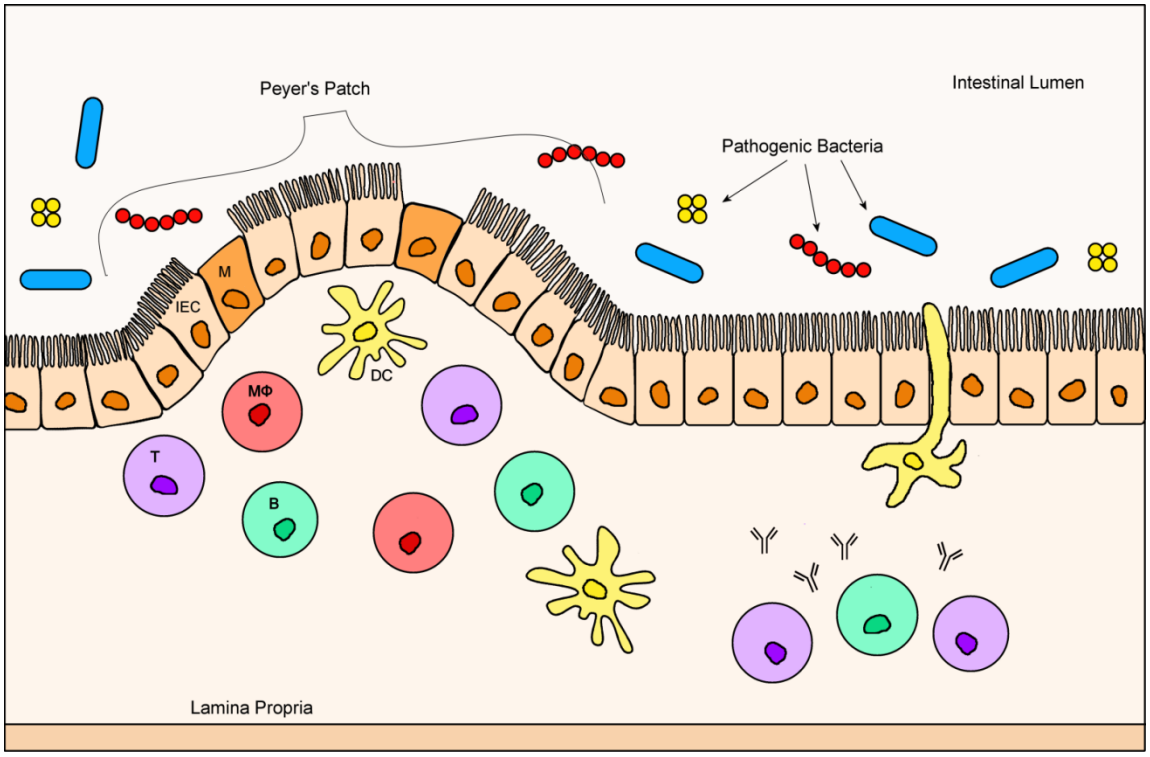
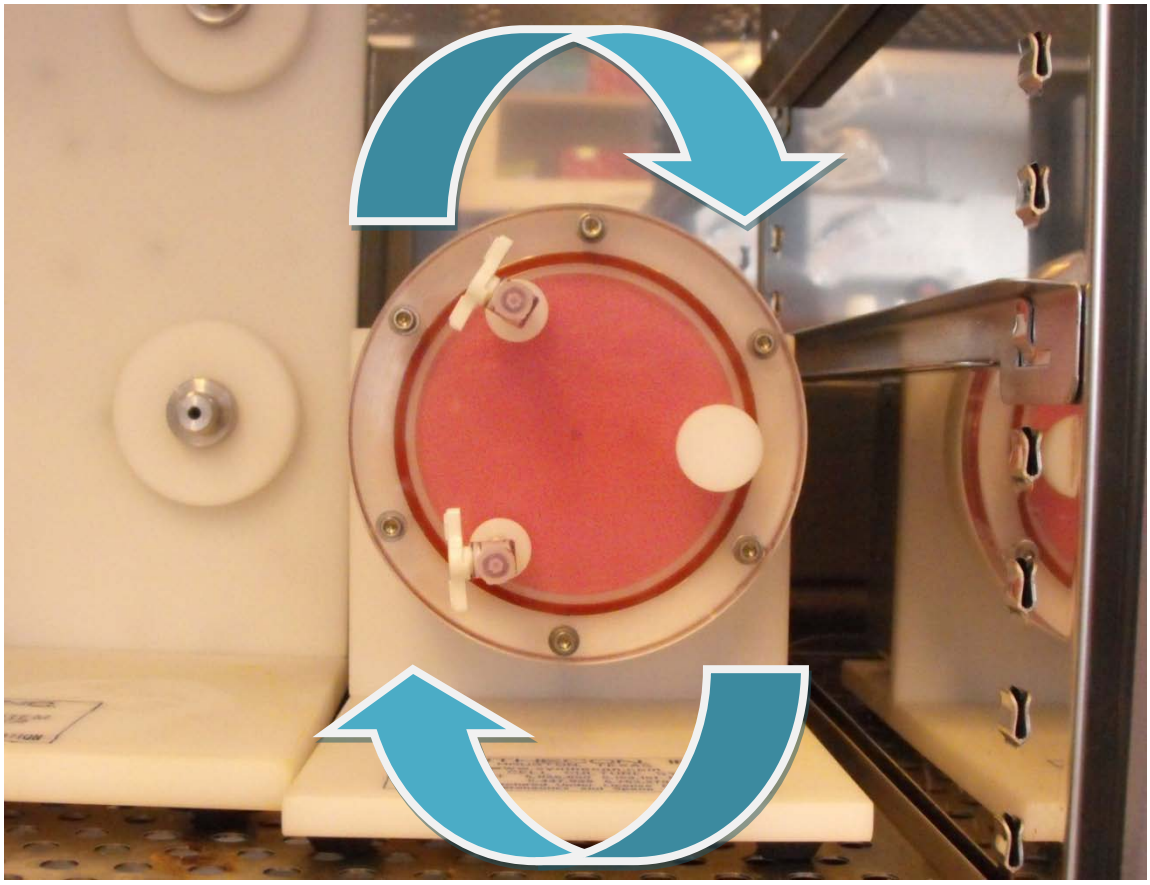
APPENDICES

Appendix A



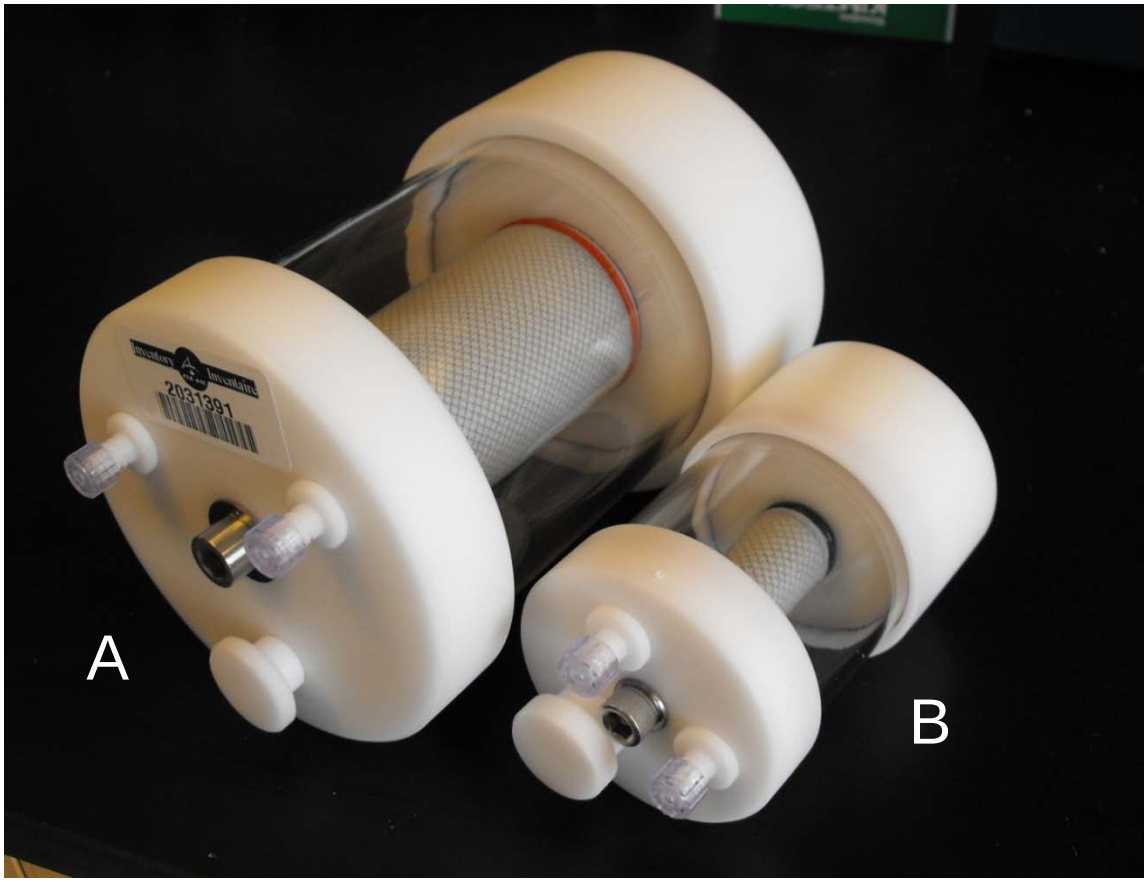
Cartoon diagram of GALT (gut-associated lymphoid tissue). Antigens and pathogenic bacteria entering the GI tract encounter macrophages (red, MΦ), dendritic cells (yellow, DC), B lymphocytes (green, B) and T lymphocytes (purple, T) found in the Peyer's Patch. Peyer's Patches contain specialized epithelium cells called microfold cells (orange, M) which sample antigens from the lumen to APCs such as macrophages. B and T cells are stimulated, and pass through the MLN were the immune response is amplified. Activated lymphocytes can then produce antibodies against the invading pathogenic bacteria.

Appendix B



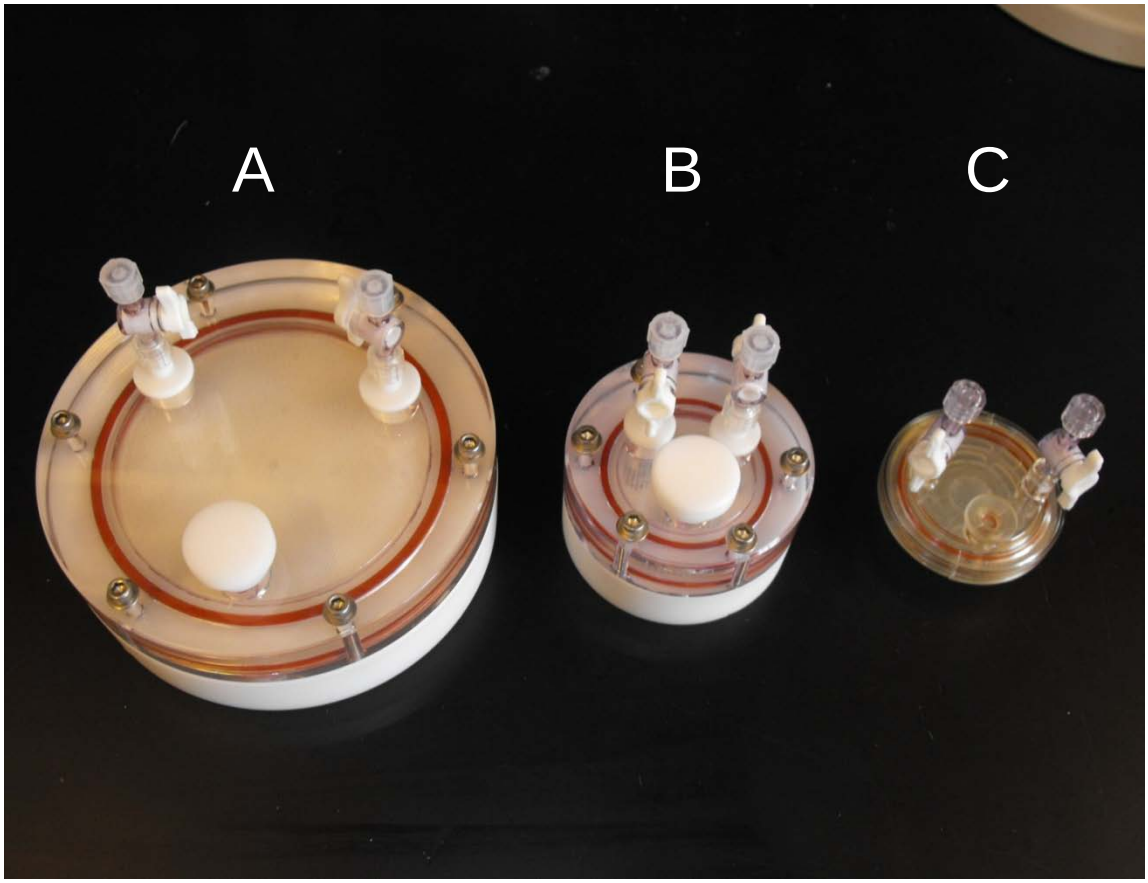
A 50 mL HARV vessel set up in a cell culture incubator. Arrows show how the vessel rotates to simulate microgravity.

Appendix C



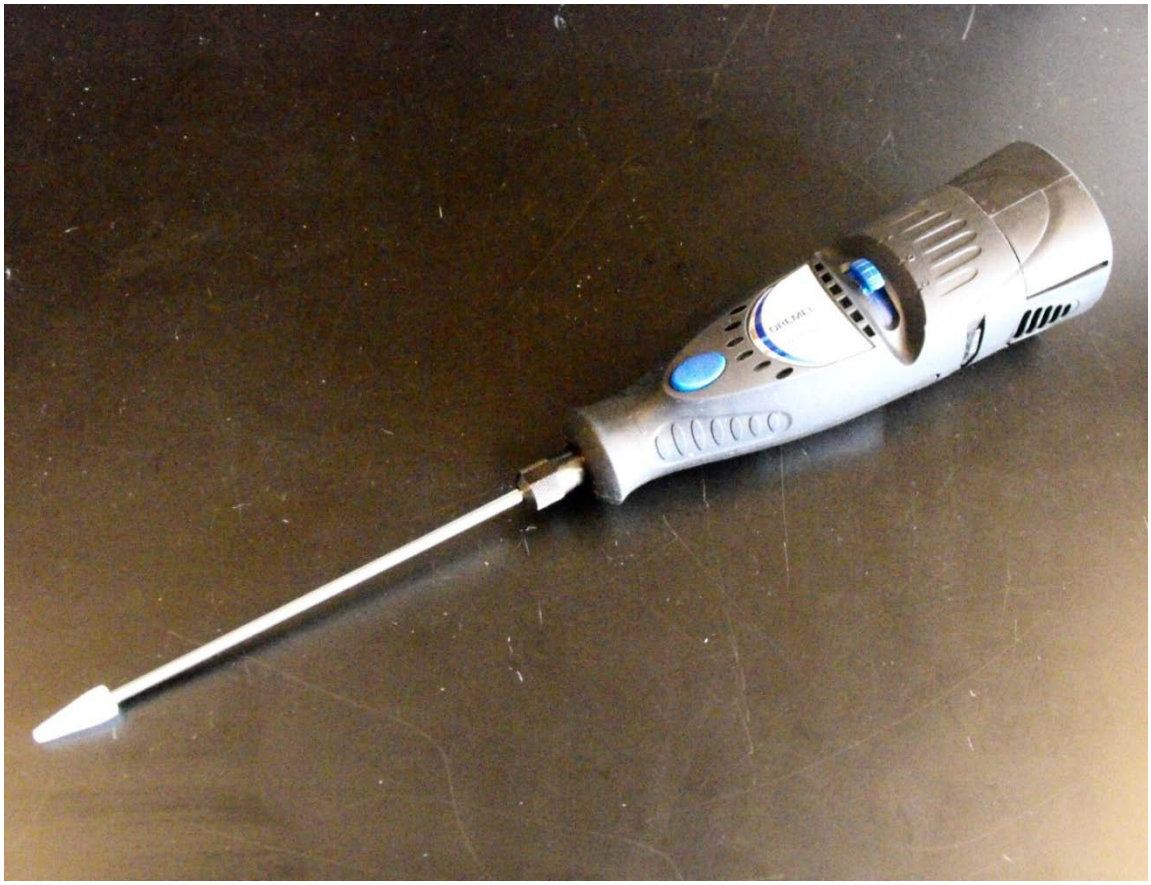
Two versions of the STLV cell culture vessel. A) shows a 250 mL version, B) shows a 55 mL version.

Appendix D



Three versions of the HARV cell culture vessel. A) shows a 50 mL reusable version, B) shows a 10 mL reusable version and C) shows a 10 mL disposable version. The vessels used in this research were disposable 10 mL HARVs.

Appendix E



A Dremel® 4.8V MiniMite Cordless with a custom conical blending attachment, used to homogenize ferments.