Synergy between probiotic *Lactobacillus casei* and milk to maintain barrier integrity of intestinal epithelial cells

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Abstract

We hypothesized that *Lactobacillus casei* BL23 and milk work synergistically to prevent damage to epithelial barrier integrity induced by pro-inflammatory cytokines. To test this, barrier disruption was induced in polarized Caco-2 monolayers by sequential, basolateral treatment with IFN-γ and TNF-α. Apical application of either 25% v/v reconstituted skim milk (RSM) or Ultra High Temperature (UHT) milk (2% fat) prior to cytokine exposure reduced losses to transepithelial electrical resistance (TER). Permeability to fluorescein isothiocyanate-dextran (FD-4; 4 kDa) was also significantly reduced in the presence of 25% v/v UHT milk ($P < 0.05$), but not RSM. Protection against increases in paracellular permeability was even greater when cell-free preparations of *L. casei* BL23 fermented UHT milk or fermented RSM were applied. The permeability coefficients of cells incubated with BL23 fermented UHT milk were equivalent to the untreated controls ($P = 0.12$) and those cells also produced 247.6 ± 35.5 pg/mL IL-8, which were significantly lower than found for cytokine-treated controls (353.9 ± 40.0 pg/mL). The benefits of the fermented milk were also confirmed by the reduced expression of TNF receptor 2 (TNFR2), myosin light-chain kinase (MLCK), and claudin-encoding genes relative to the controls. By comparison, apical application of viable *L. casei* onto the Caco-2 cells did not result in protection from the barrier-disruptive actions of IFN-γ and TNF-α. These results indicate that milk can maintain intestinal barrier integrity during pro-inflammatory cytokine exposure and that this is enhanced by modifications to milk matrix caused by prior incubation with *L. casei* BL23.

Keywords: *Lactobacillus casei*, milk, intestinal barrier, inflammation, *TNFR2*

Introduction

Intestinal epithelial cells (IECs) maintain intestinal homeostasis by providing a physical and biochemical barrier which segregates host tissues from luminal contents containing digestive enzymes, degraded food products, and microorganisms[1-2]. Disruptions to the intestinal barrier that result in the induction of inappropriate inflammatory responses are thought to occur in inflammatory bowel diseases (IBD)[3-4]. Although there are presently no cures for IBDs or other intestinal inflammatory conditions, anti-inflammatory drugs, steroids, or biologics (e.g. anti-tumor necrosis factor [anti-TNF-α]) help to maintain the epithelial barrier and reduce symptoms [5-6]. Pharmacological therapies provide sustained benefits, however, those therapies are costly and can result in severe side-effects. Therefore, alternative approaches such as nutrition-based strategies to managing inflammatory flare-ups could complement and be used to support current clinical practices.

Probiotics are useful for the prevention and treatment of a variety of acute and chronic human diseases[7-8]. While some attention has been given to the potential mechanisms by which probiotics improve health by modulating the intestinal microbiota[9-10], direct interactions between probiotics and the intestinal epithelium and immune system have also been shown[11-12]. In tests using human IEC cultures, probiotics were observed to dampen pro-inflammatory responses, and prevent deficits to intestinal barrier function[13-15]. Strains of *Lactobacillus casei* have shown promise for the prevention or mitigation of intestinal inflammation in mice[16-18] and IECs[19-20]. However, studies addressing the direct protective effects of *L. casei* on barrier integrity are limited to *L. casei* DN114 001. This strain was shown to improve the epithelial barrier by down-regulating the NF-kB signaling pathway, increasing the production of the tight junction protein ZO-1, and inhibiting ZO-1 redistribution from the membrane to the cytoplasm[21-23].

Dairy products are the most common delivery matrices of probiotic bacteria in foods and beverages. Bovine milk is a rich source of proteins, lipids, and carbohydrates as well as other bioactive compounds including but not limited to oligosaccharides, glycosphingolipids, lactoferrin, and immunoglobulins. Some bioactive compounds from milk such as beta-lactoglobulin[24], lactoferrin[25] and casein-derived peptide[26] were reported to improve the epithelial barrier function *in vitro* and *in vivo*. Probiotic-containing, fermented dairy products (mainly yogurts) were found to improve symptoms of IBD in dietary recall surveys[27]. In mice, fermented skim milk containing *L. paracasei* CBA L74 ameliorated dextran sulfate sodium (DSS)-induced colitis and elicited anti-inflammatory responses in human dendritic cells[28].

We previously observed that *L. casei* BL23 protected against the development of DSS-induced colitis in mice when ingested in the presence of Ultra High Temperature (UHT)-processed milk (2% fat) but not when ingested in a nutrient-free buffer[29]. Consumption of UHT milk alone also provided some protection against weight loss and intestinal inflammation, but was not as effective as the *L. casei*-fermented milk combination. Therefore, it is expected that combining *L. casei* BL23 and milk can confer advantages beyond the ingestion of either *L. casei* or milk alone.

In this study, we hypothesized that *L. casei* and milk work synergistically to prevent disruptions to the intestinal epithelial barrier caused by pro-inflammatory cytokines. We employed an IEC Caco-2 Transwell model to investigate the effects of milk, *L. casei* BL23, and cell-free preparations of *L. casei* fermented milk on preventing epithelial barrier disruption induced by IFN-γ and TNF-α. In addition to testing UHT milk, reconstituted skim milk (RSM) was also included because it is commonly used in preclinical probiotic studies[30-32] and affords a more standardized format for investigating probiotic-milk interactions. Transepithelial electrical

resistance (TER) and paracellular permeability were measured as well as changes in the expression of IEC signaling pathways and tight junction proteins.

Materials and Methods

Chemicals. Difco Skim Milk and de Man Rogosa and Sharpe (MRS) broth were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Ultra high temperature (UHT)-processed 2% reduced fat milk was purchased from Gossner Foods (Logan, UT, USA). Cell culture reagents, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, sodium pyruvate, nonessential amino acids, GlutaMax, penicillin/streptomycin and sterile phosphate buffer saline (PBS, pH 7.4) were obtained from Gibco (Life Technologies, Carlsbad, CA, USA). Cytokines IFN-γ and TNF-α were purchased from R&D Systems (Minneapolis, MN, USA). Fluorescein isothiocyanate-dextran (4 kDa; FD-4) was purchased from Sigma-Aldrich (St Louis, MO, USA). ELISA kits used for quantification of IL-8 and TGF-β were obtained from Life Technologies (Carlsbad, CA, USA). TRIzol reagent for RNA extraction was purchased from Invitrogen (Carlsbad, CA, USA). TURBO DNA-free for RNA purification and RETROscript for first-strand cDNA synthesis were purchased from Ambion (Austin, TX, USA). SsoFast Eva Green Supermix used for real-time PCR assay was obtained from Bio-Rad Company (Hercules, CA, USA).

***Lactobacillus casei* and milk sample preparation.** *L. casei* BL23 was grown under static conditions at 37 °C in MRS broth for 24 h before inoculation into reconstituted skim milk medium (RSM) at 1% (v/v) and incubation at 37 °C for another 24 h to stimulate adaptations for growth in milk. *L. casei* BL23 cells were then collected by centrifugation at 10,000 × g for 10 min at 4 °C, and washed twice with PBS. BL23 cells were either suspended in DMEM and applied directly in the Transwells at a density of 3×10^3 (low), 3×10^5 (moderate), and 3×10^7

(high) cells/cm^2, respectively or suspended in DMEM, RSM, or UHT (2% fat) milk at a number of approximately 1×10^8 cells/mL and then incubated at 37 °C for 6 h. Next, the cultures were diluted to 25% (v/v) in DMEM and the cells were removed by centrifugation at 10,000 × g for 10 min at 4 °C. The supernatant was collected, adjusted to pH 7.4, and then filtered through 0.22 µm filters (SLGP033RB; Millipore, Bedford, MA, US). Supernatants from non-fermented UHT and RSM milk samples diluted to 25% (v/v) in DMEM were prepared following same procedure and included as controls. To test if diluted DMEM affected the Caco-2 cell integrity, DMEM diluted with sterile water was tested.

Epithelial cell culture and cytokine stimulation. Human intestinal Caco-2 cells ATCC HTB-37, purchased from American Type Cell Culture (Rockville, MD, USA), were grown in DMEM as previously described[33]. Briefly, the Caco-2 cells were incubated in DMEM, 20% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2 mM GlutaMax and 1% v/v penicillin/streptomycin at 37 °C in 10% CO_2. To form a monolayer, the cells were seeded onto polycarbonate membranes in Transwell inserts (6.5 mm, 0.4 µm pore size, 24 wells; Corning, NY, USA) at a density of 1×10^4 cells/cm^2. After incubation for at least 21 days, the cells were fully differentiated and formed polarized monolayers with intercellular tight junction structures. TER was measured at 37 °C with an epithelial voltohmmeter (World Precision Instruments, Sarasota, FL, USA) equipped with STX-2 “chopstick” electrodes. Caco-2 monolayers with an initial TER of more than 250 Ohms·cm^2 were used for the subsequent experiments.

Initial TER values were measured for each monolayer before adding fresh DMEM or DMEM containing milk or bacteria to the apical surface. Consistent with previous studies,[14,34] the cells were apically exposed to the different treatments at 37 °C for 3 h prior to basolateral

addition of 100 ng/mL IFN-γ. The DMEM of the basal compartment wells was replaced 20 h later and supplemented with 10 ng/mL TNF-α.

Paracellular permeability assay. Paracellular permeability was determined by the flux of FD-4 through differentiated Caco-2 monolayers as previously described with minor modifications[33,35]. After 48 h stimulation with TNF-α, the apical side contents were replaced with DMEM containing FD-4 (2 mg/mL). After 30 min, FD-4 content on the basolateral side was quantified by measuring the fluorescence intensity 485 nm (excitation) and 530 nm (emission) in a microplate reader (Synergy-2; BioTek, Winooski, VT, USA). The concentration of FD-4 was calculated using a standard curve of FD-4 in DMEM. The apparent permeability coefficient, P_{app} (cm/s), was determined according to the following equation: $P_{app} = \frac{dQ}{dt}\frac{1}{A \times C_0}$, where d$Q$/d$t$ is the quantity of FD-4 transported per second (ng/s), A is the surface area of the filter (cm^2), and C_0 is the initial FD-4 concentration in DMEM on the apical side (ng/mL).

ELISA assays for IL-8 and TGF-β detection. After 48 h stimulation with TNF-α, DMEM collected from the basolateral side was used for quantification of interleukin 8 (IL-8) and transforming growth factor beta (TGF-β) by ELISA. All assays were performed in triplicate according to the manufacturer's instructions.

RNA extraction and quantitative reverse-transcription PCR. After 48 h stimulation with TNF-α, total RNA from the Caco-2 monolayers was isolated using TRIzol reagent and purified using the TURBO DNA-free Kit. RNA was quantified on the NanoDrop 2000c (Thermo-Fisher). Quality was assessed on the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and found to be intact (RIN 9.4 to 9.8). RNA (1 μg) was reverse transcribed to cDNA using the RETROscript kit, following the manufacturer's protocol. Quantitative reverse-transcription PCR (RT-qPCR) was performed using SsoFast Eva Green Supermix on a 7500 Fast

Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). The primers used for RT-qPCR are listed in **Table S1**. Amplification was initiated at 95 °C for 20 s, and was followed by 40 cycles of 95 °C for 10 s (denaturation), and 60 °C for 30 s (annealing extension). Primer specificity was assessed by examination of the melting curve at the end of amplification. All reactions were performed in triplicate. Data were analysed using the $2^{-\Delta\Delta Ct}$ method corrected for primer efficiencies using the untreated cells as the reference condition[36]. The housekeeping gene *GAPDH* was used for transcript normalization.

Statistical analysis. Data were analyzed using GraphPad Prism 6 software for Windows (GraphPad Software, Inc., La Jolla, CA, USA). All data are reported as mean ± standard deviation (SD) and analyzed by ANOVA with post-hoc Tukey HSD (Honestly Significant Difference) test for comparing multiple treatments.

Results

RSM and UHT (2% fat) milk protect against IFN-γ and TNF-α induced losses to Caco-2 barrier integrity. To develop conditions that permit assessments of the effects of milk on intestinal permeability, DMEM was adjusted to contain either 6.25%, 12.5%, 25% v/v RSM or UHT (2% fat) milk. The TER of Caco-2 cell monolayers did not change in response to any of these milk additions relative to the untreated controls (DMEM, no milk) (**Figure 1A**). Additionally, the dilution of DMEM caused by the inclusion of RSM or UHT milk did not result in barrier deficits as shown by testing DMEM diluted up to 50% with water (**Figure S1**). Therefore, DMEM containing 25% v/v RSM or UHT milk was used in subsequent experiments.

Caco-2 cell monolayers were next exposed to IFN-γ and TNF-α in succession. After TNF-α application, the TER values were reduced by up to 30% within 48 h incubation, indicating that

the cytokines caused disruptions to barrier integrity (**Figure 1B,** $P < 0.0001$ relative to the untreated controls). By comparison, incubating the monolayers in the presence of DMEM containing 25% v/v RSM or UHT milk for 3 h prior to cytokine addition resulted in average reductions in TER by only 2.3% and 7.7%, respectively (**Figure 1B,** $P = 0.0004$ and $P < 0.0001$ relative to the untreated controls). Notably, the response of the Caco-2 cells to the milk was dose dependent, as shown by applications 6.25%, 12.5%, or 25% v/v RSM milk (**Figure 1C**).

To further verify the beneficial effects of milk on the Caco-2 cell barrier, paracellular permeability to uncharged macromolecules was measured using FD-4. IFN-γ and TNF-α exposure resulted in significant increases in the transfer of FD-4 from the apical to the basolateral side of the Transwells, leading to a permeability coefficient (P_{app}) 2-fold higher than found for untreated controls (**Figure 1D,** $P < 0.0001$). Caco-2 cells exposed to DMEM containing 25% v/v RSM were not protected from IFN-γ and TNF-α according to this measurement (**Figure 1D**). By comparison, incubation with 25% v/v UHT milk partially prevented the cytokine-mediated, permeability increase (**Figure 1D**). The P_{app} with 25% v/v UHT milk was significantly lower than found for Caco-2 cells exposed to IFN-γ and TNF-α in the absence of milk ($P = 0.003$) and only slightly, albeit still significantly, higher than the untreated controls ($P = 0.045$).

***L. casei* BL23 cells do not prevent cytokine-induced intestinal barrier disruption.** To investigate whether viable *L. casei* BL23 can influence IEC barrier integrity, the apical surfaces of the Caco- 2 monolayers were exposed to low (3×10^3), moderate (3×10^5), and high (3×10^7) numbers of *L. casei* BL23 cells/cm^2. Low and moderate numbers of *L. casei* BL23 did not impact the monolayers, whereas high *L. casei* cell quantities resulted in significant losses to TER within 72 h incubation (**Figure S2**). Therefore, the moderate level (3×10^5 cells/cm^2) of *L. casei*

BL23 cells was tested for the capacity to prevent impairments caused by IFN-γ and TNF-α. However, no protection was found when viable BL23 were applied according to measurements of TER and permeability to FD-4 (**Figure 2**).

Attenuation of barrier dysfunction by *L. casei* BL23 fermented milk. In order to avoid cytotoxic effects caused by *L. casei* BL23 on the Caco-2 cells, and because direct contact with the epithelium might not be essential for probiotic-induced health benefits, we tested whether metabolites secreted by *L. casei* BL23 have the capacity to alter intestinal barrier integrity. To measure this, *L. casei* BL23 was incubated in DMEM, RSM, or UHT milk at a level of 1×10^8 cells/mL for 6 h prior to application of cell-free preparations of the fermented media (DMEM or milk) at a 25% v/v ratio onto the Caco-2 monolayers. *L. casei* BL23 was metabolically active in DMEM, RSM and UHT milk as indicated by the reductions in pH from approximately pH 7 to pH 5 by the end of the 6 h incubation period (data not shown). When cell-free preparations of the fermented DMEM or milk were applied at a 25% v/v ratio in DMEM onto the Caco-2 monolayers, no losses in barrier integrity were found over 72 h incubation (data not shown).

When *L. casei* fermented RSM and UHT milk was tested on monolayers exposed to IFN-γ and TNF-α, the TER remained comparable to the native (non-fermented) milk counterpart (**Figure 3A**). However, the fermented UHT milk resulted in significantly reduced FD-4 permeability coefficients compared to the native UHT milk ($P = 0.006$) and untreated Caco-2 cell controls ($P = 0.12$, **Figure 3B and S4**). The *L. casei* fermented RSM did not protect the Caco-2 monolayers against increases in paracellular permeability caused by IFN-γ and TNF-α (**Figure 3B**). *L. caesi* fermented DMEM also did not prevent cytokine-induced losses to TER or increases in translocation of FD-4 (**Figure S3)**.

Inhibition of IL-8 production by UHT milk exposed to *L. casei* BL23. Quantities of IL-8, an indicator of inflammatory responses in epithelial cells[14,37], were elevated in the basolateral medium of Caco-2 cells exposed to IFN-γ and TNF-α (353.9 ± 40.0 pg/mL). These levels were significantly lower in cells exposed to the *L. casei* fermented UHT milk, which was 247.6 ± 35.5 pg/mL (**Figure 4,** $P < 0.05$). By comparison, intermediate, non-significant reductions in this cytokine were found for the other (fermented) milk types. None of the (fermented) milk treatments prevented IFN-γ and TNF-α-induced reductions in the anti-inflammatory cytokine TGF-β (**Figure S5**).

Down-regulation of *TNFR2, MLCK,* and tight junction gene expresssion by *L. casei* BL23 fermented UHT milk. The expression of tumor necrosis factor receptor 2 (*TNFR2*) in Caco-2 cells was previously shown to be up-regulated in response to IFN-γ[37]. Similarly, we found that monolayers exposed to IFN-γ and TNF-α contained, on average, 4.5-fold higher quantities of *TNFR2* transcripts compared to the untreated Caco-2 controls (**Figure 5**, average of 2.2-fold on a $\log_2$ scale). These levels were similarly high when the monolayers were exposed to either RSM or UHT milk. By comparison, incubation with either *L. casei* BL23 fermented RSM or UHT milk resulted in reduced *TNFR2* expression relative to the cytokine-treated controls (**Figure 5**, $P < 0.05$).

It was previously shown that the major effector responsible for TNF-α induced intestinal barrier disruption is the myosin light chain kinase (MLCK)[38]. Although there was no change in *MLCK* transcript abundance between cytokine-treated and untreated controls, *MLCK* expression was slightly, but significantly, reduced in the Caco-2 monolayers exposed to *L. casei* BL23 fermented RSM as well as native and fermented UHT milk relative to both the untreated Caco-2 cells and cytokine-treated controls (**Figure 5**, $P < 0.05$).

Transcript levels of *CLDN2*, coding for the pore-forming tight junction protein Claudin-2, were 1.5-fold lower in Caco-2 cell monolayers incubated with *L. casei* fermented RSM or native or fermented UHT milk compared to the cytokine-treated controls (**Figure 6**, $P < 0.05$). For cells incubated with *L. casei* BL23 fermented UHT milk, *CLDN1* (Claudin-1) expression was also reduced (**Figure 6**, $P < 0.05$). Moreover, both *L. casei* fermented milks resulted in reduced quantities of *CLDN3* and *CLDN4* transcripts, coding for barrier-forming, tight junction proteins Claudin-3 and Claudin-4, compared to the cytokine-treated controls (**Figure 6**, $P < 0.05$). Lastly, occludin (*OCLN*) and ZO-1 (*ZO-1*) expression levels were unchanged (**Figure 6,** $P > 0.05$).

Discussion

Both RSM and UHT milk reduced the impact of IFN-γ and TNF-α on epithelial barrier integrity of Caco-2 cells. However, the effects of these milk types were significantly augmented by the application of cell-free preparations of the RSM and UHT milk that had been incubated in the presence of *L. casei* BL23. These distinctions were evident in the improved paracellular permeability, lower IL-8 levels, and reduced expression of *TNFR2*, *MLCK*, and altered tight junction proteins in response to the *L. casei* fermented milk. The findings are also notable because the direct application of viable BL23 cells onto the monolayers was insufficient to prevent reductions in barrier integrity. These results are consistent with our prior work showing that *L. casei* BL23 in milk prevented the development of DSS-induced colitis and this response was specific to the *L. casei* and milk combination[29].

Bovine milk as well as individual milk components were previously reported to improve the intestinal barrier and have anti-inflammatory properties in the digestive tract [26,39-40]. To this regard, both RSM and UHT milk significantly reduced the damaging effects of IFN-γ and TNF-α

on Caco-2 cells according to assessments of TER. This was achieved even when a low quantity of milk (6.5% v/v RSM in DMEM) was applied. The UHT milk, but not RSM, conferred additional changes indicated by the reduced paracellular permeability to FD-4 relative to the DMEM controls. To this regard, 2% fat UHT milk appears to be more protective than RSM. Although the two milk types have obvious differences in fat content, there are also numerous other compositional differences, such as variations in protein composition that could affect these outcomes [41-42].

Incubation of intact, viable *L. casei* BL23 cells with Caco-2 cells did not alleviate cytokine-induced barrier disruption, and the application of higher *L. casei* numbers even resulted in cytotoxic effects. These findings differ from other studies showing that some *Lactobacillus* strains, applied in similar or higher numbers (up to approximately 1×10^8 cells/cm^2), improved epithelial barrier function *in vitro*[14,43-44]. Such differences support the premise that *Lactobacillus* interactions with IECs are strain and species specific. Because the application of a cell-free preparation of *L. casei* BL23 fermented DMEM did not prevent declines in TER or increased translocation of FD-4, the findings here also strongly indicate that probiotic activity is dependent on the incubation conditions (e.g. milk or DMEM) to which the probiotics are exposed.

L. casei fermented RSM and UHT milk attenuated cytokine-induced reductions in paracellular permeability according to FD-4 translocation from the apical to basolateral compartments in the Caco-2 Transwells. This finding is notable because TNF-α is known to increase paracellular permeability, as opposed to only cause more general impairments measured by TER. Moreover, only the *L. casei* fermented UHT milk reduced IL-8 production levels to the extent that quantities were significantly lower compared to the cytokine-treated controls. These outcomes indicate that *L. casei* was able to alter the milk in such a way that the beneficial

properties of the milk matrix were preserved while also providing additional components or modifications that caused reinforcements to the paracellular tight junctions.

The impacts of the *L. casei* fermented milk on the Caco-2 cells were also shown by the changes in *TNFR2, MLCK,* and tight junction protein gene expression levels. As expected, *TNFR2* was up-regulated in cells exposed to IFN-γ and TNF-α. However, exposure of the monolayers to the *L. casei* fermented milk resulted in significantly lower *TNFR2* transcript levels than both the cytokine-treated controls and cells exposed to either RSM or UHT milk. Similarly, application of *L. casei* BL23 fermented RSM and UHT milk also resulted in reduced expression of *MLCK* and several claudin genes. Among the claudin genes, *CLDN2* was reported to be up-regulated in inflammatory bowel diseases and under inflamed conditions in the gut [45-46]. The other claudin genes *CLDN1, CLDN3* and *CLDN4* form a charge barrier[47]. The changes in gene expression observed here indicate that (fermented) milk application modifies tight junction architecture caused by exposure to IFN-γ and TNF-α.

Ultimately, these results should be verified and further investigated in studies quantifying IEC protein levels and localization. The studies should also be supported by efforts to identify the specific bioactive metabolites modified or produced by *L. casei* in milk. For example, we previously observed that the quantities of several *L. casei* BL23 proteins involved in proteolysis (PepO and PepF1) were elevated when the strain was incubated in milk[29]. These enzymes might be used to hydrolyze milk proteins and generate bioactive peptides. Alternatively, short chain fatty acids, such as lactate and acetate produced by lactobacilli in food matrixes are known to directly stimulate epithelium growth and improve intestinal barrier function[48-49]. Knowledge on these specific *L. casei*-elicited modifications to the milk matrix combined with identification of the predominant bioactive compounds in milk[50] is needed in order to identify the precise

receptors and signal transduction pathways activated in Caco-2 cells to maintain barrier integrity in the presence of pro-inflammatory cytokines and inflammatory insults. Similarly, this information will assist in designing experiments to determine the strain specificity and numbers of *L. casei* cells required to sufficiently augment the barrier protective properties of milk. Lastly, the stability and activity of those components should be studied *in vivo* to determine the impact of digestive processes on *L. casei* – milk interactions.

In conclusion, this study provides new evidence of synergistic probiotic-milk matrix interactions. At least for *L. casei* BL23 studied here, the effects on IECs were not dependent on cell to cell contact but rather secreted compounds or modifications to the milk matrix caused by *L. casei.* The baseline benefit of milk, and the 2% fat UHT milk in particular, together with the increased responses caused by incubating *L. casei* BL23 in the milk prior to application, provides opportunities for pairing whole foods such as dairy products with different probiotic strains to improve health outcomes.

Abbreviations

IEC, Intestinal epithelial cell;

IBD, inflammatory bowel disease;

TNF-α, tumor necrosis factor alpha;

DSS, dextran sulfate sodium;

IFN-γ, interferon gamma;

FD-4, fluorescein isothiocyanate-dextran (4 kDa);

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells;

TER, Transepithelial electrical resistance;

RSM, sterile reconstituted skim milk;

UHT, ultra-high temperature treated;

DMEM, Dulbecco's Modified Eagle Medium;

IL-8, interleukin-8;

MLCK, myosin light chain kinase;

OCLN, occludin;

ZO-1, zonula occludens-1;

CLDN, claudin;

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Primers used for RT-qPCR.

Figure S1. Caco-2 monolayers are unaffected by the presence of 50% v/v DMEM.

Figure S2. High numbers of *L. casei* BL23 cells impair Caco-2 cell TER.

Figure S3. Lack of protection by *L. casei* BL23 fermented DMEM.

Figure S4. Change of the permeability to FD-4 in Caco-2 monolayers over time.

Figure S5. TGF-β in the basal medium after pro-inflammatory cytokine stimulation.

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Tables

Table 1. Nutritional composition of 2% fat UHT milk[a] and reconstituted skim milk[b].

Nutritional composition (100 mL)	2% fat UHT milk	Skim milk
Energy	50.8 kcal	36.6±2.3 kcal
Protein	3.4 g	3.5±0.2 g
Total fat	2.1 g	0 g
Total carbohydrates	4.7 g	5.2±0.4 g
Cholesterol	8.5 mg	1.8±0.6 mg
Sodium	42.4 mg	52.2±2.0 mg

[a] Data was obtained from product label provided by Gossner Foods (Logan, UT, USA).
[b] Collected from USDA Food Composition Databases.

Figure legends

Figure 1. The effects of milk on Caco-2 monolayers. (A) Transepithelial electrical resistance (TER) of Caco-2 monolayers 72 h after incubation in DMEM containing 6.25%, 12.5%, 25% v/v reconstituted skim milk (RSM) or Ultra High Temperature (UHT) 2% fat milk. (B) TER after exposure to IFN-γ and TNF-α. Caco-2 monolayers were either untreated (black bars) or basally stimulated with 100 ng/mL IFN-γ for 20 h and then 10 ng/mL TNF-α (white bars). DMEM was refreshed in the controls or modified to contain 25% v/v RSM (slashed bars) or UHT milk (checkered bars) 3 h prior to IFN-γ supplementation. TER was measured 24, 36, and 48 h after TNF-α addition. (C) TER after exposure to IFN-γ and TNF-α in the presence of different ratios of 6.25%, 12.5%, 25% v/v RSM. Milk and cytokine additions were carried out as described for (B). (D) Paracellular permeability was determined by the flux of fluorescein isothiocyanate-dextran (4 kDa; FD-4) through differentiated Caco-2 monolayers 48 h after stimulation with TNF-α. Cells were incubated in the presence of FD-4 for 30 min before measuring for the compound in the basal medium. One representative experiment of at least two independent experiments is shown. Bars with different letters are statistically significant compared to each group ($n = 6$, $P < 0.05$).

Figure 2. *L. casei* BL23 in DMEM did not alleviate intestinal barrier dysfunction. (A) Caco-2 monolayers were either untreated (black bars) or basally stimulated with 100 ng/mL IFN-γ for 20 h and then 10 ng/mL TNF-α (white bars). A total of 3×10^5 *L. casei* BL23 cells/cm^2 in DMEM were added to the apical surface 3 h before the addition of IFN-γ. TER was measured 24, 36, and 48 h after TNF-α addition. (B) Paracellular permeability was determined by the flux of FD-4 through differentiated Caco-2 monolayers 48 h after stimulation with TNF-α. Cells were

incubated in the presence of FD-4 for 30 min before measuring for the compound in the basal medium. Bars with different letters are statistically significant compared to each group (n = 6, $P < 0.05$).

Figure 3. *L. casei* BL23 combined with milk alleviated the effects of pro-inflammatory cytokines on epithelial barrier integrity. BL23 fermented milk cultures (pH 5) were diluted to 25% (v/v) with DMEM and then centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was collected, adjusted to pH 7.4 and then filtered through 0.22 μm filters. Fresh DMEM with or without 25% v/v BL23 fermented RSM (FRSM) or UHT milk (FUHT) was added 3 h before cytokine exposure. TER was measured 24, 36, and 48 h after TNF-α addition. (B) Paracellular permeability was determined by the flux of FD-4 through differentiated Caco-2 monolayers after 48 h stimulation with TNF-α. The permeability coefficient (P_{app}, unit: cm/s) was determined from the amount of compound transported per time. Bars with different letters are statistically significant compared to each group (n = 6, $P < 0.05$).

Figure 4. BL23 fermented UHT milk alleviates the effects of pro-inflammatory cytokines on IL-8 production in Caco-2 cells. Basolateral concentrations of IL-8 were measured 48 h after TNF-α addition. FRSM- fermented RSM; FUHT- fermented UHT milk. The coefficient of variation between the three replicate monolayers was <15% for each treatment group. Bars with different letters are statistically significant compared to each group (n = 3, $P < 0.05$).

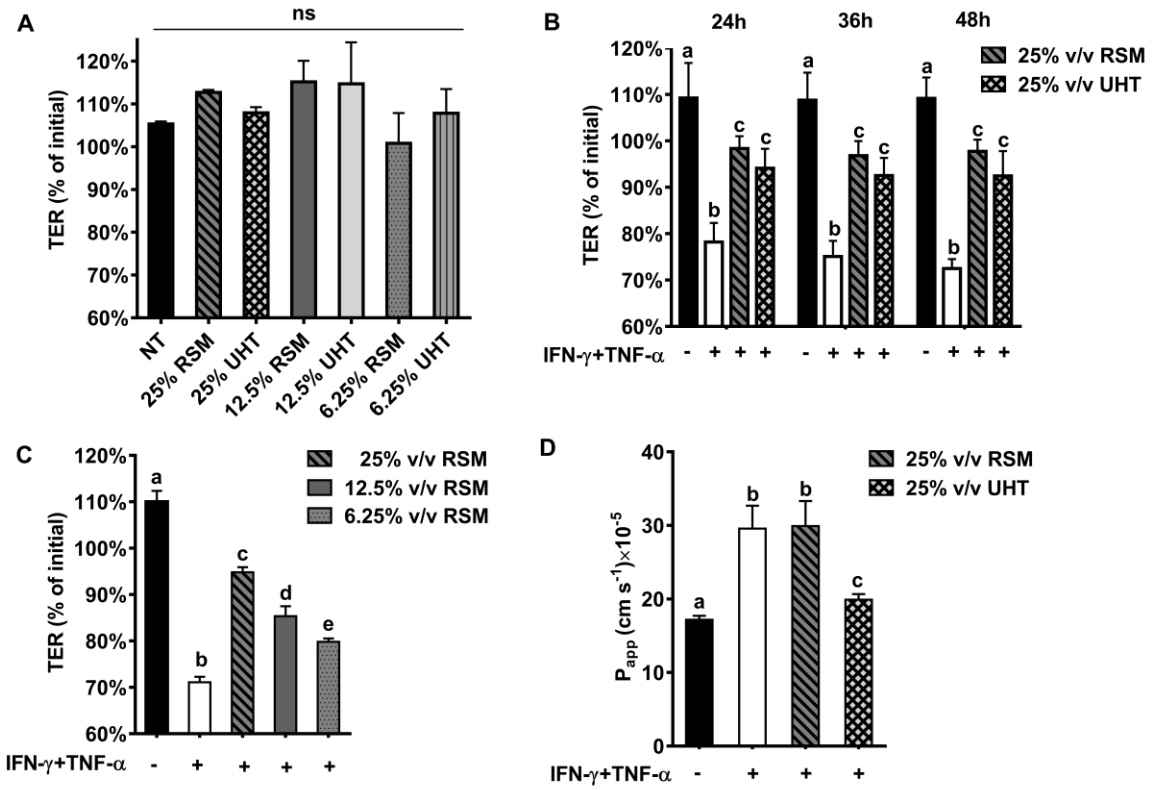
Figure 5. The expression of *TNFR2* and *MLCK* was down-regulated after BL23 fermented milk treatment. Data were normalized using the $2^{-\Delta\Delta Ct}$ method with the untreated cells as the

reference condition. CK- cytokine treated control; FRSM- fermented RSM; FUHT- fermented UHT milk. Bars with different letters are statistically significant compared to each group (n = 6, $P < 0.05$).

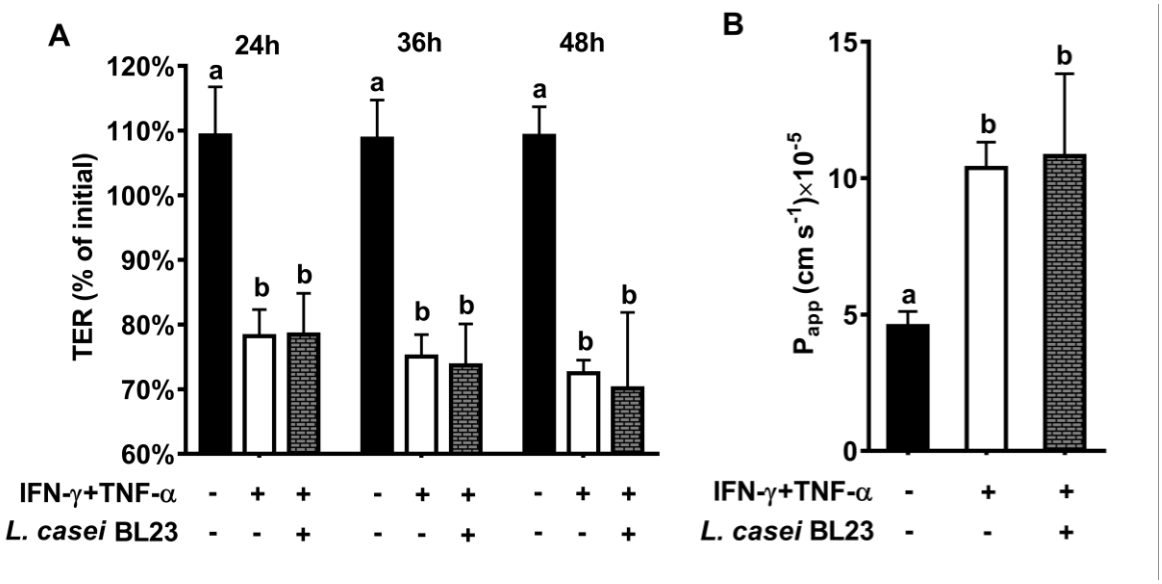
Figure 6. Expression of tight junction genes in Caco-2 cells. CLDN1: claudin-1; CLDN2: claudin-2; CLDN3: claudin-3; CLDN4: claudin-4; OCLN: occludin; ZO-1: zonula occludens-1. Data were normalized using the $2^{-\Delta\Delta Ct}$ method with the untreated cells as the reference condition. CK- cytokines treated control; FRSM- fermented RSM; FUHT- fermented UHT milk. Bars with different letters are statistically significant compared to each group (n = 6, $P < 0.05$).

Figures

Figure 1



572 **Figure 2**



573

Figure 3

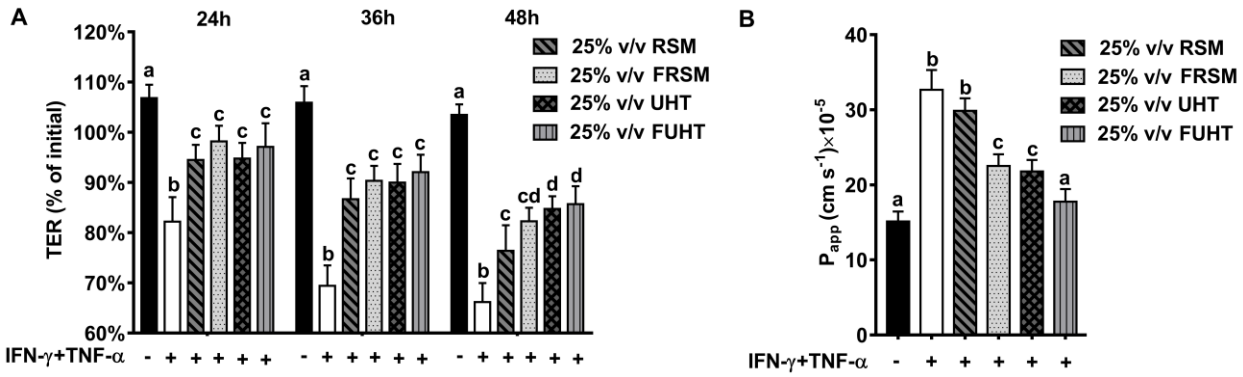

A
24h
36h
48h
TER (% of initial)
120%
110%
100%
90%
80%
70%
60%
IFN-γ+TNF-α
25% v/v RSM
25% v/v FRSM
25% v/v UHT
25% v/v FUHT
B
P_{app} (cm s^{-1})×10^{-5}
40
30
20
10
0
IFN-γ+TNF-α
25% v/v RSM
25% v/v FRSM
25% v/v UHT
25% v/v FUHT

Figure 4

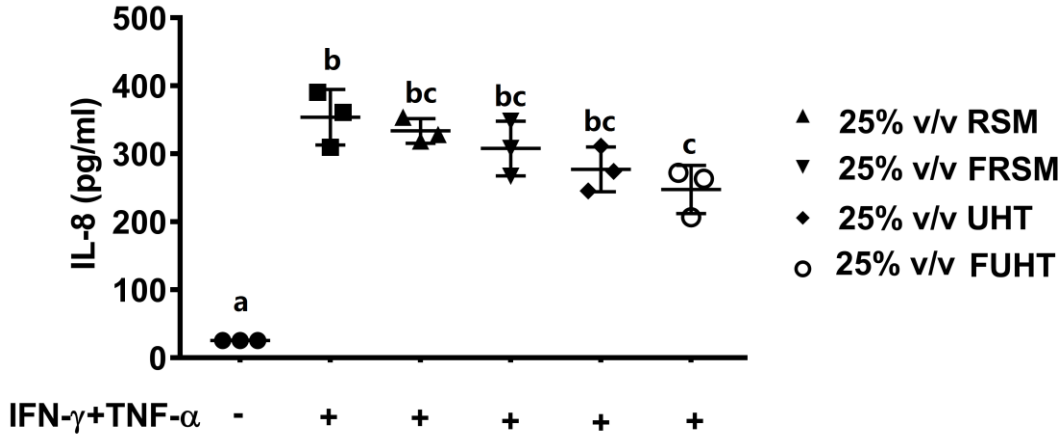

500
400
300
200
100
0
IL-8 (pg/ml)
a
b
bc
bc
bc
c
IFN-γ+TNF-α
-
+
+
+
+
+
25% v/v RSM
25% v/v FRSM
25% v/v UHT
25% v/v FUHT

Figure 5

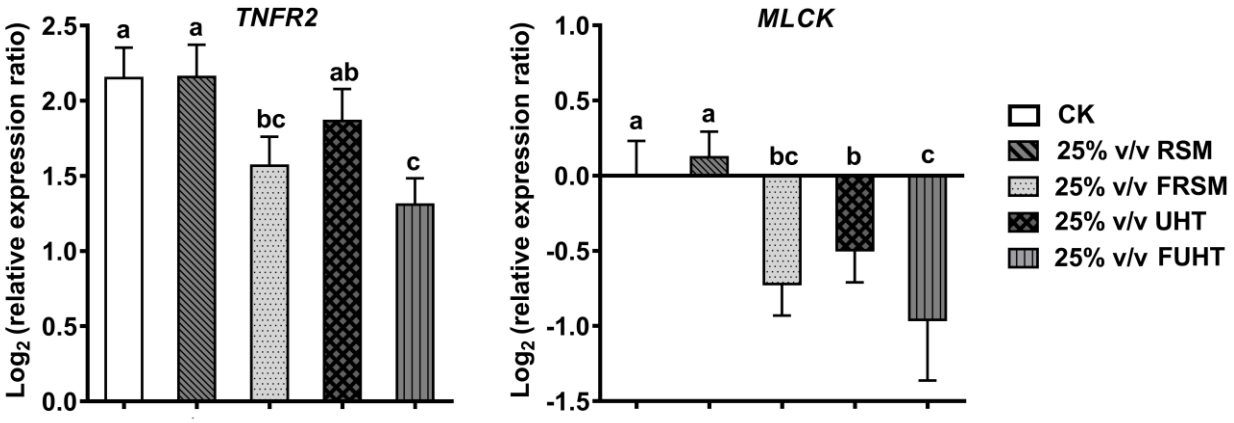

TNFR2
Log2 (relative expression ratio)
2.5
2.0
1.5
1.0
0.5
0.0
a
a
bc
ab
c
MLCK
Log2 (relative expression ratio)
1.0
0.5
0.0
-0.5
-1.0
-1.5
a
a
bc
b
c
CK
25% v/v RSM
25% v/v FRSM
25% v/v UHT
25% v/v FUHT

Figure 6

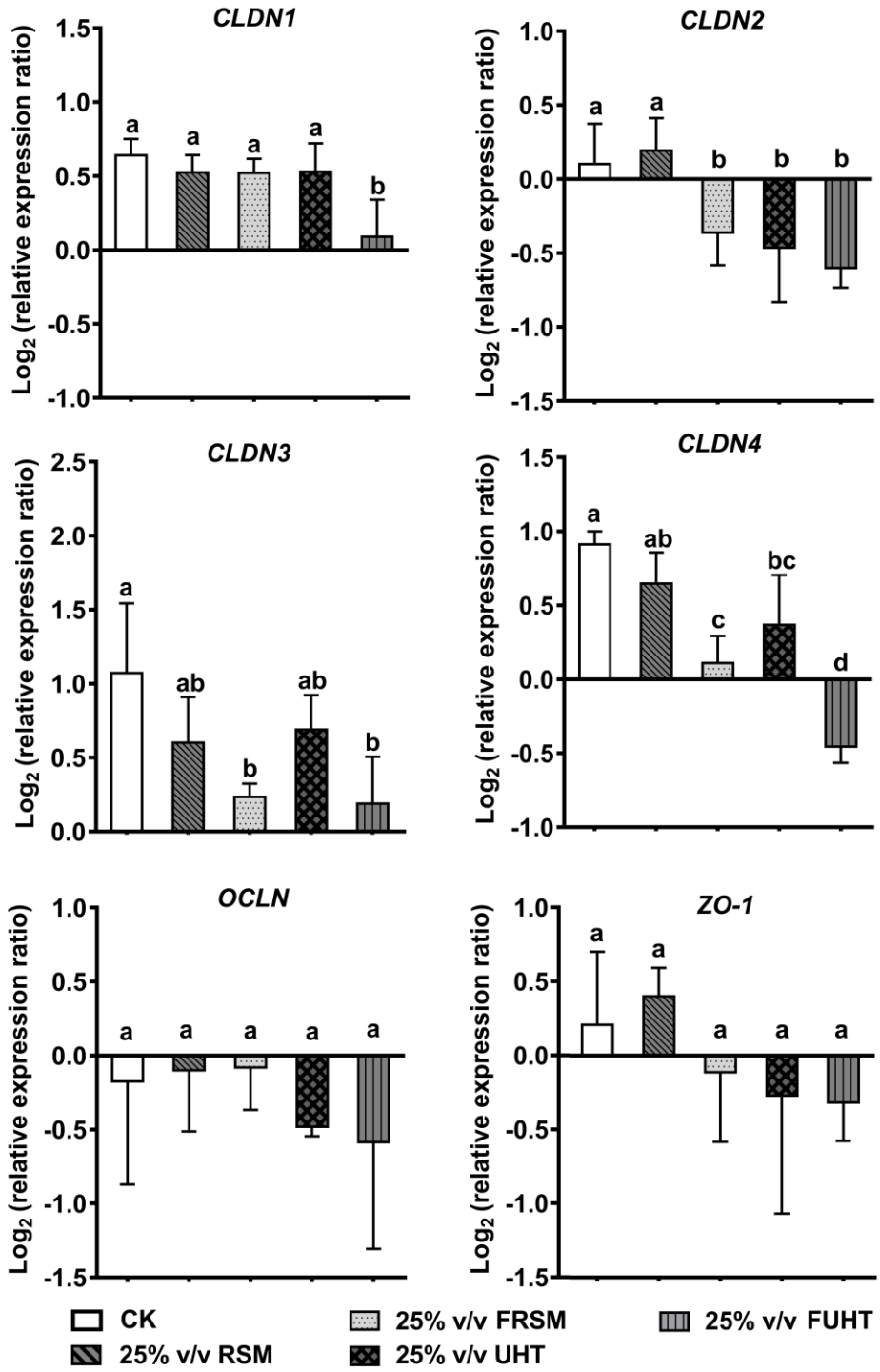

CLDN1
CLDN2
CLDN3
CLDN4
OCLN
ZO-1
Log2 (relative expression ratio)
CK
25% v/v RSM
25% v/v FRSM
25% v/v UHT
25% v/v FUHT

Table of contents (TOC)