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Differential protective effects of whole and skimmed whey on LPS-induced barrier dysfunction and inflammation in Caco-2/TC7 cells

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ABSTRACT

Background: Lipopolysaccharide (LPS) from Gram-negative bacteria disrupts intestinal epithelial integrity and promotes inflammation, contributing to the pathogenesis of gastrointestinal disorders. Dairy-based whey products are rich in bioactive compounds with known barrier-protective and immunomodulatory properties.

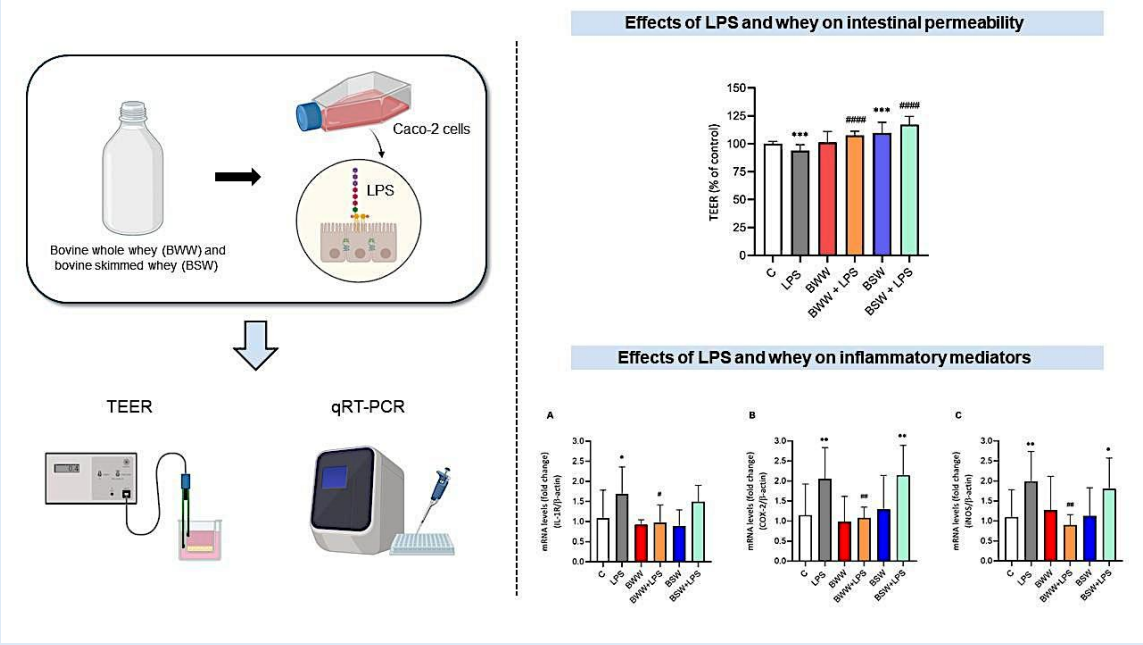
Objective: This study aimed to evaluate the effects of two types of bovine whey — bovine whole whey (BWW) and bovine skimmed whey (BSW) — on LPS-induced barrier dysfunction and inflammation in the intestinal *in vitro* model Caco-2/TC7 cells.

Methods: Caco-2/TC7 cell line was exposed to 20 µg/ml LPS for 24h to induce epithelial barrier disruption. Additionally, cells were treated with 5 mg/ml BWW or 5 mg/ml BSW alone, or in combination with 20 µg/ml LPS. Transepithelial electrical resistance (TEER), FITC-dextran permeability, and mRNA expression of tight junction (TJ) proteins (ZO-1, claudin-1, claudin-3, occludin) and inflammation-related genes (IL-1R, COX-2, iNOS) were assessed.

Results: LPS significantly reduced TEER without increasing macromolecular permeability or altering TJ gene expression. Both whey fractions restored TEER to control levels, with BSW also enhancing baseline barrier integrity. BWW and BSW upregulated ZO-1 and claudin-1 mRNA expression, though claudin-1 was downregulated when BSW was combined with LPS. Importantly, only BWW normalized LPS-induced overexpression of IL-1R, COX-2, and iNOS, indicating better anti-inflammatory properties likely due to its retained lipid components.

Conclusion: Both whey fractions reinforce epithelial barrier function under LPS challenge, but only whole whey (BWW) provides dual protection by also attenuating inflammatory responses. These findings support the potential of whole whey as a functional food ingredient for intestinal health by targeting both physical and immunological aspects of barrier dysfunction.

Keywords: bovine whey, LPS, intestinal permeability, tight junction proteins, inflammation



Graphical Abstract: Differential protective effects of whole and skimmed whey on LPS-induced barrier dysfunction and inflammation in Caco-2/TC7 cells

INTRODUCTION

Gastrointestinal infections represent a major cause of childhood morbidity and mortality worldwide, accounting for approximately 750,000 deaths annually in children under five, particularly in low and middle-income countries [1]. In this context, bacterial enteric pathogens represent one of the most relevant etiological agents, with *Escherichia coli, Shigella* spp., *Campylobacter* spp. and *Salmonella* spp. being the most commonly identified bacteria [2-3].

Lipopolysaccharide (LPS), the main component of Gram-negative outer membrane bacteria such as *E. coli,* acts as an endotoxin and plays a crucial role in the pathogenesis of bacterial gastroenteritis. LPS is recognized by Toll-like receptor 4 (TLR4) on intestinal epithelial and immune cells. This recognition initiates a potent inflammatory cascade, leading to the production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and interleukins (e.g., IL-6, IL-8), as well as enzymes like cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [4-5], which trigger mucosal inflammation and disrupt the integrity of the epithelial barrier [4]. This barrier, a selectively permeable monolayer essential for nutrient absorption and pathogen exclusion, is maintained by intercellular protein complexes known as tight junctions (TJs), which are composed of key proteins like occludin, claudins, and zonula occludens-1 (ZO-1) [6-7]. The molecular mechanisms underlying this LPS-induced barrier disruption are multifaceted. For instance, cytokines like TNF-α directly modulate TJ integrity and epithelial repair processes [8]. Furthermore, LPS can trigger the hyperactivation of ion channels, such as Ca^{2+}-activated Cl^- channels and epithelial Na^+ channels, causing apical membrane depolarization. This ultimately leads to the tyrosine phosphorylation of the key TJ protein ZO-1, destabilizing the junction and causing a pathological increase in intestinal permeability [9].

This impairment is often exacerbated by gut microbiota dysbiosis, an imbalance characterized by a reduction in beneficial commensal populations, such as *Lactobacillus* and *Bifidobacterium*, and an overgrowth of opportunistic pathogens [10]. In this context, targeted nutritional interventions using functional foods have emerged as a promising strategy to mitigate gut inflammation, restore microbiota homeostasis, and reinforce epithelial barrier function [11-12].

Among the functional foods proposed to counteract intestinal damage, dairy-derived products, particularly whey, have emerged as a promising source of bioactive compounds [13]. Whey, a by-product of the cheese industry, is a complex mixture composed primarily of lactose, minerals, vitamins, and a highly valuable protein fraction. The therapeutic potential of whey is largely attributed to these proteins, which collectively demonstrate a spectrum of beneficial properties, including immunomodulatory, anti-inflammatory, antioxidant, and antimicrobial effects [14-16].

The major bioactive proteins in whey include β-lactoglobulin and lactoferrin. Lactoferrin, an iron-binding glycoprotein, is particularly noteworthy for its ability to regulate the innate and adaptive immune system, regulate iron homeostasis, and modulate the gut microbiota [17-18]. Similarly, α-lactalbumin and β-lactoglobulin contribute significantly to some beneficial effects, exhibiting potent antioxidant and anti-inflammatory activities [19-20]. Collectively, these properties suggest that whey proteins could play a crucial role in maintaining intestinal homeostasis by strengthening the epithelial barrier, modulating local immune responses, and fostering a healthy gut microbiota.

Despite the promising bioactivity of whey proteins, their direct capacity to counteract LPS-induced intestinal barrier damage requires further elucidation in a controlled setting. The human epithelial Caco-2/TC7 cell line provides a well-established *in vitro* model for this purpose, as these cells spontaneously differentiate into polarized monolayers that mimic the key features of the intestinal barrier, including the formation of functional TJs [21]. Thus, this study aimed to evaluate the ability of two types of bovine whey —whole whey (BWW) and skimmed whey (BSW) to restore intestinal permeability

and enhance epithelial integrity in Caco-2/TC7 cells under an LPS-induced inflammatory challenge. Furthermore, we sought to investigate the modulatory effects of these whey fractions on key components of the intestinal immune response. We therefore hypothesized that the presence of the native lipid fraction in BWW conferred greater immunomodulatory properties compared with BSW, providing novel insights into the role of whey fractions in intestinal barrier function.

MATERIALS AND METHODS

Preparation of Whole and Skimmed Whey from Cow's Milk: Whey was obtained during the production of fresh cheese from pasteurized cow's milk supplied by Quesos La Pardina (Zaragoza, Spain), using enzymatic coagulation with bovine rennet. Whey was skimmed using an ARR-DES 125 model centrifuge (Suministros Químicos Arroyo, Santander, Spain). Both processes were conducted at the Food Science and Technology Pilot Plant of the University of Zaragoza (Zaragoza, Spain). Both whole and skimmed whey, were subjected to ultrafiltration using a 100 kDa membrane (Millipore, Burlington, MA, USA). The retentate volume was reduced to approximately one-tenth of the initial whey volume. This process yielded the whey fractions analyzed in this study: whole concentrated whey (BWW) and skimmed concentrated whey (BSW), with final pH values of 6.58 and 6.55, respectively. Both whey fractions were adjusted to a final protein concentration of 5 mg/ml and sterilized by filtration through 0.22 µm syringe filters (Millipore, Burlington, MA) prior to use in the assays.

Cell Culture and Treatments: The human intestinal epithelial Caco-2/TC7 cell line was kindly donated by Dr. Rousset from INSERM U178 (Villejuif, Francia) [22] and was used as an *in vitro* model of the intestinal epithelial barrier. Cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin solution (100 U/ml penicillin, 100 µg/ml streptomycin), 1% (v/v) non-essential amino acids, and 2 mM L-glutamine. Cells were maintained in 25 cm^2 cell culture flasks at an initial seeding density of 3×10^5 cells/flask. The culture medium was replaced every 48 h, and cells were passaged weekly upon reaching 80-90% confluency using 0.25% (w/v) trypsin-0.02% (w/v) EDTA solution. All cultures were maintained in a humidified incubator at 37°C with a 5% CO_2 atmosphere. To induce inflammatory conditions, differentiated Caco-2/TC7 monolayers (15 days post-seeding) were apically exposed to LPS from *Escherichia coli* O111:B4 (EMD Millipore, Burlington, MA, USA). Dose-response and time-course experiments were conducted with LPS concentrations of 1, 5, 10 and 20 µg/ml for 6, 24, and 48 h to establish an optimal model for barrier disruption. Based on these findings, a final LPS concentration of 20 µg/ml for 24 h was selected for subsequent experiments to test the effects of the two whey fractions. To evaluate the effects of the whey on LPS-induced changes, cells were exposed for 24 h to: (1) Control (medium only); (2) LPS (20 µg/ml); (3) BWW (5 mg protein/ml); (4) BSW (5 mg protein/ml); (5) LPS + BWW; (6) LPS + BSW. All treatments were prepared in FBS-free DMEM to avoid potential interference from serum components.

Transepithelial Electrical Resistance (TEER): Caco-2/TC7 cells were seeded onto 1.0 µm pore size polycarbonate Transwell® permeable supports (12-well plate format, 1.12 cm^2 growth area; Corning, NY, USA) at a density of 4×10^4 cells/insert. Cells were cultured for 15 days post-confluence to allow full differentiation and polarization, with medium changes every 48 h in both apical (0.5 ml) and basolateral (1.5 ml) compartments. For LPS dose-

response experiments, differentiated monolayers were treated apically with LPS (1, 5, 10 or 20 µg/ml) or medium control, and TEER was measured at 6, 24, and 48 h post-treatment. For whey evaluations, differentiated monolayers were treated apically with BWW (5 mg/ml) or BSW (5 mg/ml) alone, LPS (20 µg/ml) alone, or combinations of whey fractions and LPS for 24 h. An untreated control group was included. TEER was measured using an EVOM2™ Epithelial Voltohmmeter equipped with STX2 chopstick electrodes (World Precision Instruments, Sarasota, FL, USA). Baseline TEER (T_0) was recorded prior to the addition of treatments. TEER values (Ω) were multiplied by the surface area of the insert (1.12 cm^2) to obtain $Ω·cm^2$. Values were normalized to their respective T_0 readings and expressed as a percentage relative to the untreated control group at each time point.

Paracellular Permeability to FITC-Dextran: Following the final TEER measurement at 24 h, paracellular permeability was assessed using 4 kDa fluorescein isothiocyanate-dextran (FD-4; Sigma-Aldrich, St. Louis, MO, USA). The apical medium containing treatments was removed, monolayers were gently washed once with pre-warmed FBS-free DMEM, and then 0.5 ml of FD-4 solution (1 mg/ml in FBS-free DMEM) was added to the apical compartment. The basolateral compartment received 1.5 ml of fresh FBS-free DMEM. After 2 h of incubation at 37°C in the dark, 100 µL aliquots were collected from the apical compartment and transferred to a black 96-well microplate. Fluorescence intensity was measured using a FLUOstar® Omega microplate reader (BMG Labtech, Ortenberg, Germany) with excitation and emission wavelengths set at 485 nm and 535 nm, respectively. The amount of FD-4 (ng/ml) that permeated to the basolateral side was quantified using a standard curve generated from serial dilutions of a known concentration of FD-4.

Gene Expression Analysis: For gene expression analysis, Caco-2/TC7 cells were seeded in 6-well plates at a density of 5×10^5 cells/well and cultured for 15 days post-confluence to achieve differentiation. Cells were then subjected to the same treatment conditions with a 24-hour incubation period as described for the permeability assays. Following treatment, cells were washed twice with ice-cold PBS. Total RNA was extracted using the NZY Total RNA Isolation Kit (NZYtech, Lisbon, Portugal) according to the manufacturer's instructions. Briefly, cell monolayers were lysed directly in the culture wells, and lysates were homogenized by passing them five times through a 21-gauge needle fitted to a syringe. RNA was purified using silica-membrane spin columns. RNA concentration and purity were determined using a SPECTROstar Nano microvolume plate reader (BMG Labtech). First-strand complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the NZY First-Strand cDNA Synthesis Kit (NZYtech) following the manufacturer's protocol. Real-time qPCR (RT-qPCR) was performed using the NZYSupreme qPCR Green Master Mix (2x, ROX plus; NZYtech) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Specific primers for human tight junction protein genes (ZO-1, occludin, claudin-1, claudin-3) and immune-related genes (IL-1 receptor type 1, COX-2, iNOS) were selected from published literature and sequences are provided in Table 1. Gene expression levels were normalized to β-actin as the reference gene. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method, with results expressed as fold change relative to the untreated control group.

Table 1. Primer sequences used for RT-PCR analysis of gene expression**.**

Protein	Forward primer sequence	Reverse primer sequence	Reference
ZO-1	ATCCCTCAAGGAGCCATTC	CACTTGTTTTGCCAGGTTTTA	[23]
Occludin	GAGTTGTATCTGTTGTTGT	TTCGTGGTATAGCATTCT	[24]
Claudin-1	AAGTGCTTGGAAGACGATGA	CTTGGTGTTGGGTAAGAGGTT	[23]
Claudin-3	CTGCTCTGCTGCTCGTGTC	CGTAGTCCTTGCGGTCGTAG	[25]
IL1R1	GACAGGGCCTAGCTTTCATTT	TGGCCAATTTTGTCACTAACC	[26]
COX-2	TGGCTACAAAAGCTGGGAAG	GCTGCTTTTTACCTTTGACACC	[26]
iNOS	CCTTACGAGGCGAAGAAGGACAG	CAGTTTGAGAGAGGAGGCTCCG	[24]
β-actin	AAAGACCTGTACGCCAACACAGTGCTGTCTGG	CGTCATACTCCTGCTTGCTGATCCACATCTGC	[23]

Statistical Analysis: All experiments were performed with a minimum of three independent biological experiments. Statistical analysis was performed using GraphPad Prism 9.0.0 (GraphPad Software, San Diego, CA, USA). Data normality was assessed using the Shapiro-Wilk test and results were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA), following Dunnett's post-hoc multiple comparison test was performed to evaluate differences between treatment groups. A p-value < 0.05 was considered statistically significant.

RESULTS

TEER measurement: To assess the effect of LPS on epithelial barrier integrity, TEER was measured following LPS treatment with increasing concentrations (1, 5, 10, and 20 µg/ml) for 6, 24 and 48 h. At 6 h, no significant changes were observed at any concentration (Figure 1A). However, 20 µg/ml LPS after 24 h, promoted a significant reduction in TEER compared to control (Figure 1B). At 48 h, both 10 and 20 µg/ml LPS concentration led to a significant decrease in TEER, suggesting a time-dose dependent effect (Figure 1C).

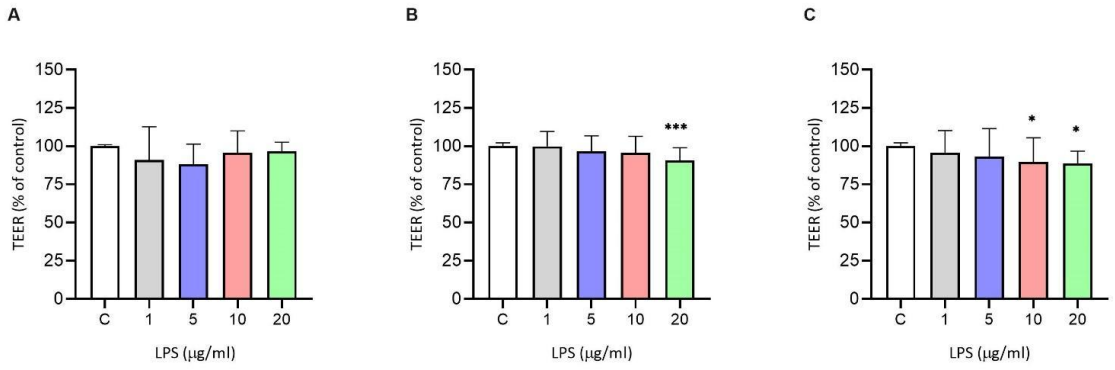


Figure 1. TEER values of Caco-2/TC7 cells treated with different LPS concentrations (1, 5, 10, 20 µg/ml), after **(A)** 6 h, **(B)** 24 h and **(C)** 48 h. Values are expressed as % of control (mean ± SD, n ≥ 3 independent experiments). **p* < 0.05, ****p* < 0.001 vs Control (C).

Based on these results, 24-h treatment with 20 µg/ml of LPS was selected as the treatment condition for subsequent assays. To further investigate the impact of whey fractions and LPS on intestinal permeability, an additional TEER experiment was conducted comparing individual whey fractions (BSW and BWW) and their combination with LPS. (Figure 2). LPS significantly reduced TEER values compared to control. Co-treatment

of 20 µg/ml LPS with either BWW or BSW (5 mg protein/ml) for 24 h restored TEER values significantly to control values. Notably, treatment with BSW alone also showed a significant increase in TEER relative to the control, suggesting a potential to enhance intestinal barrier integrity.

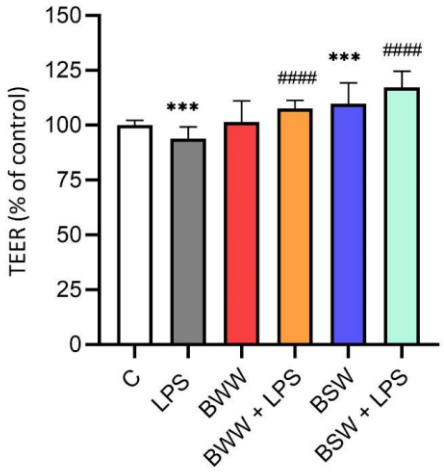


Figure 2. TEER values of Caco-2/TC7 cells treated with LPS (20 µg/ml), whey fractions BWW and BSW (each 5 mg protein/ml) or their combination for 24 h. Values are expressed as % of control (mean ± SD, n ≥ 3 independent experiments). ***$p < 0.001$ vs Control (C). #### $p < 0.0001$ vs LPS.

FITC-Dextran Assays: Paracellular permeability was further evaluated by FITC-dextran assay. Thus, Caco-2/TC7 cells were treated with increasing LPS concentrations (5, 10, 20 µg/ml) for 24 h. Nevertheless, no significant differences were observed in FITC-dextran flux to the basolateral compartment compared to the control group (Figure 3A). Similarly, treatment with either whey fractions alone or their combination with LPS (20 µg/ml) did not result in statistically significant differences compared to the control group (Figure 3B).

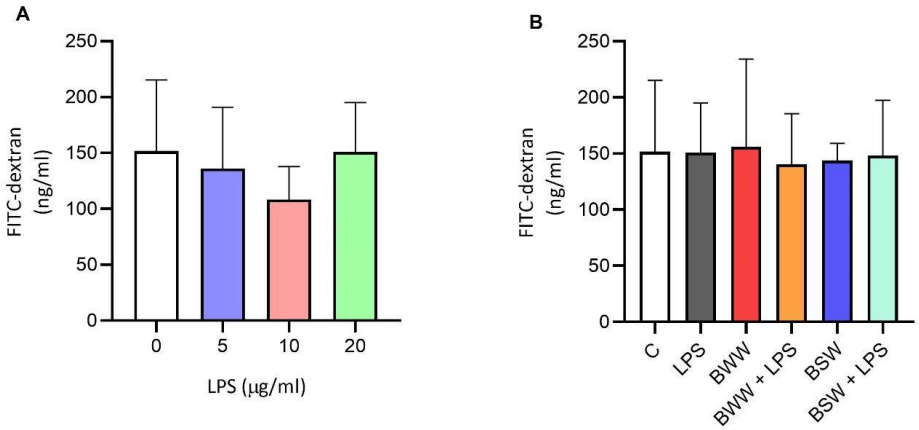


Figure 3. FITC-dextran flux in Caco-2/TC7 cells after 24 h of treatment. **(A)** Treatment with increasing LPS concentrations (5, 10 and 20 µg/ml). **(B)** Treatment with whey fractions BWW and BSW alone (5 mg/ml each) or their combination with LPS (20 µg/ml). Values are expressed as ng/ml of FITC-dextran in the basolateral compartment (mean ± SD, n ≥ 3 independent experiments).

Gene Expression of Tight Junction Proteins: To determine the impact of LPS on the expression of TJ proteins, RT-PCR was performed after 24 h of treatment with increasing LPS concentrations (5,10, and 20 µg/ml). Nevertheless, no significant changes were observed in the mRNA expression of any TJ-related genes at any concentration (Figure 4).

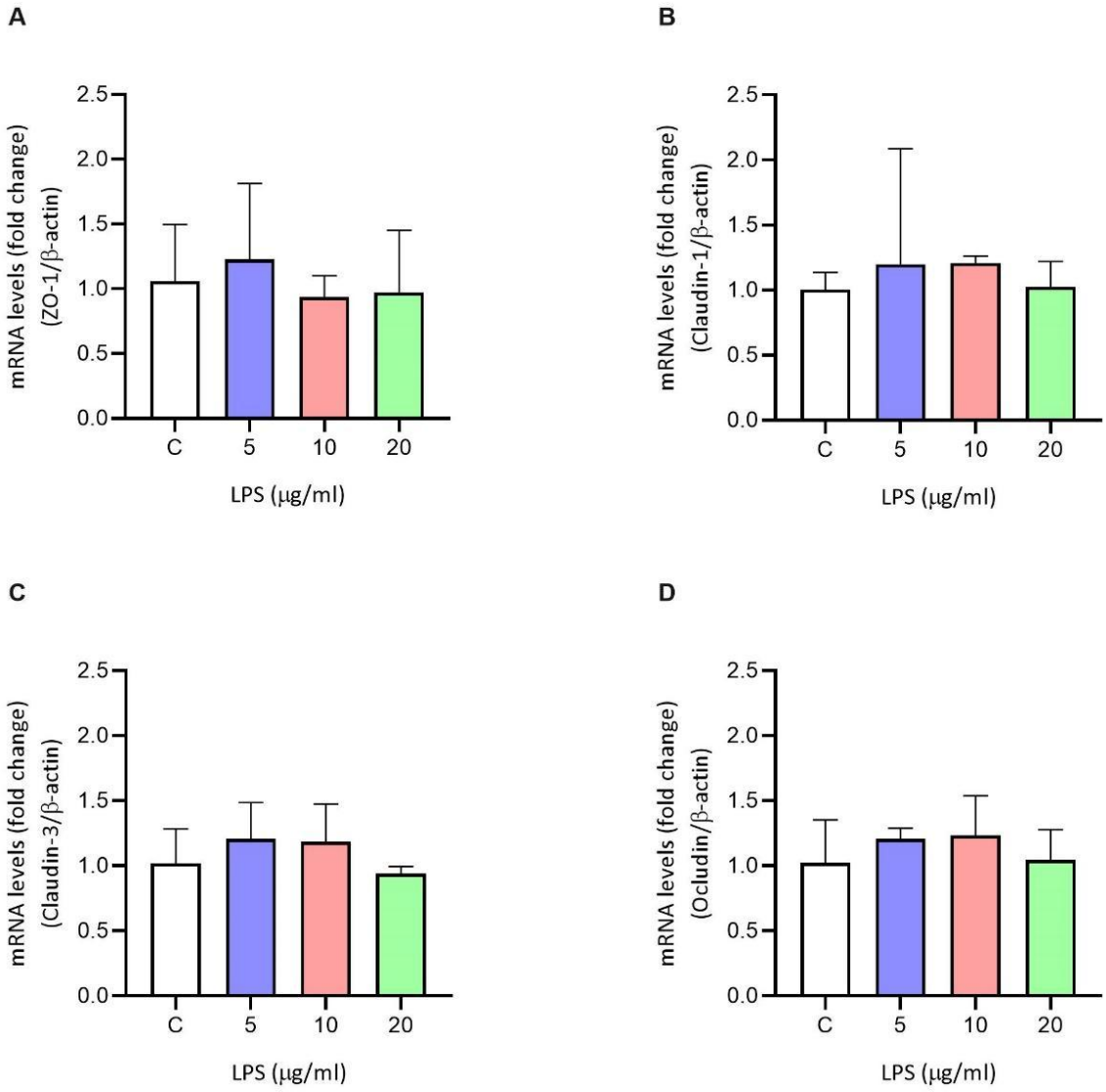


Figure 4. Levels of mRNA expression (fold change) of TJ-related genes **(A)** ZO-1, **(B)** Claudin-1, **(C)** Claudin-3 and **(D)** Occludin in Caco-2/TC7 cells after 24 h of treatment with increasing LPS concentrations (5, 10 and 20 µg/ml). Values are normalized to β-actin and expressed as fold change compared to control group (mean ± SD, n ≥ 3 independent experiments).

TJ gene expression was also assessed after 24-h treatment with BWW and BSW fractions (5 mg protein/ml), alone or in combination with 20 µg/ml of LPS (Figure 5). Both whey fractions, BWW and BSW, significantly upregulated ZO-1 and claudin-1 expression. However, co-treatment of BSW with LPS significantly downregulated claudin-1 expression.

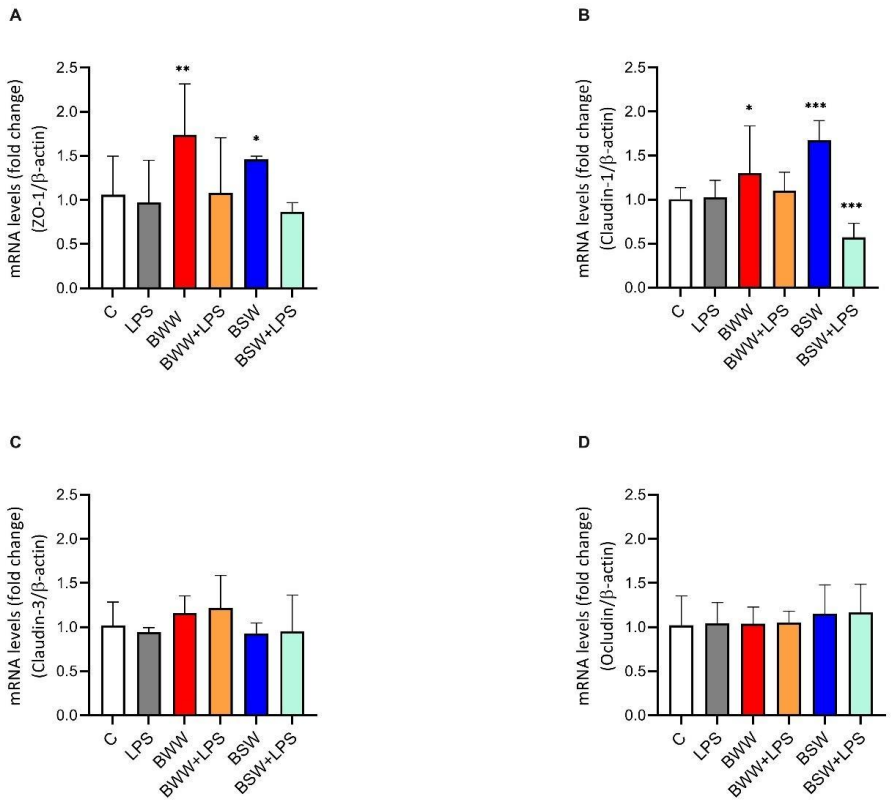


Figure 5. Levels of mRNA expression of TJ-related genes **(A)** ZO-1, **(B)** Claudin-1, **(C)** Claudin-3 and **(D)** Occludin in Caco-2/TC7 cells after 24 h of treatment with either BWW or BSW (5 mg protein/ml) alone or in combination with 20 µg/ml of LPS. Values are normalized to β-actin and expressed as fold change compared to control group (mean ± SD, n ≥ 3 independent experiments). **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs Control (C).

Inflammatory Gene Expression: Expression of inflammation-related genes was evaluated in response to increasing LPS concentrations (5, 10 and 20 µg/ml) (Figure 6). The genes IL-1R, COX-2 and iNOS showed a dose-dependent trend, with a significant increase in expression at the highest LPS concentration (20 µg/ml).

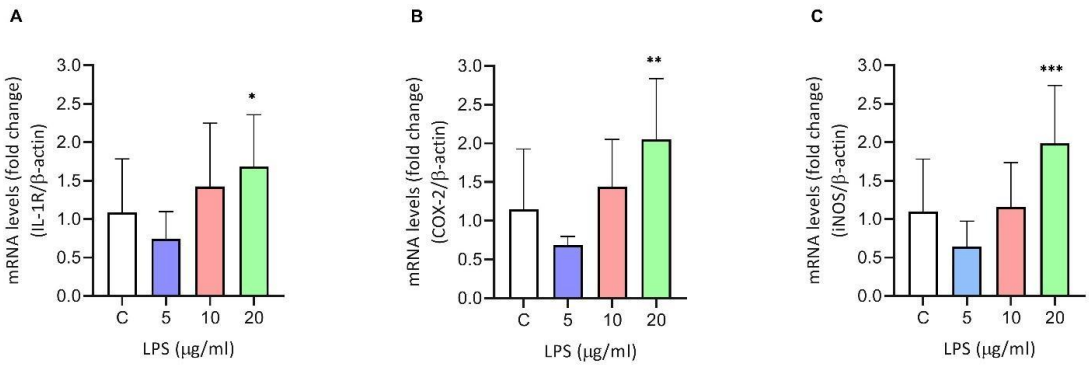


Figure 6. Levels of mRNA expression of inflammation-related genes **(A)** IL-1R, **(B)** COX-2 and **(C)** iNOS in Caco-2/TC7 cells after 24 h of treatment with increasing LPS concentrations (5, 10 and 20 µg/ml). Values are normalized to β-actin and expressed as fold change compared to control group (mean ± SD, n ≥ 3 independent experiments). **p* < 0.05, ***p* < 0.01, ****p* < 0.001 vs Control.

Subsequently, the anti-inflammatory potential of whey fractions was evaluated by adding either BWW or BSW (5 mg protein/ml) alone or in combination with 20 µg/ml of LPS for 24 h (Figure 7). Treatment with BWW significantly reversed the LPS-induced overexpression of IL-1R, COX-2 and iNOS, restoring their expression to similar levels as the control group. In contrast, the BSW did not mitigate LPS-induced overexpression of IL-1R, COX-2 and iNOS.

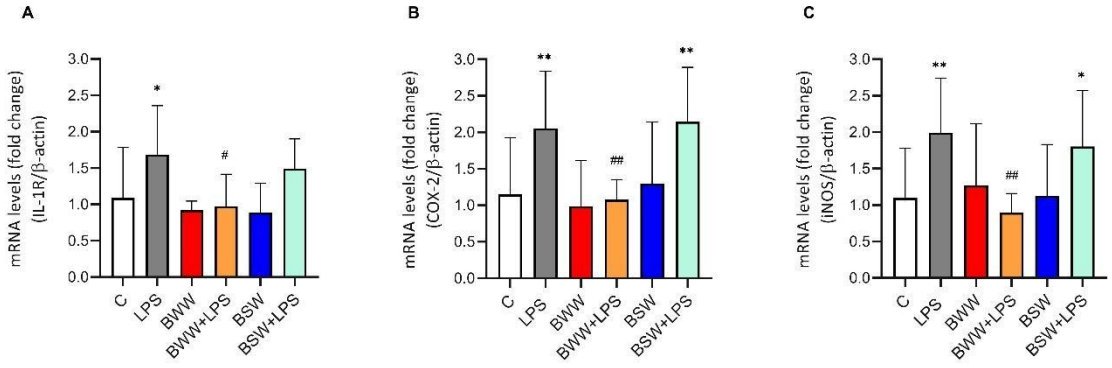


Figure 7. Levels of mRNA expression of inflammation-related genes **(A)** IL-1R, **(B)** COX-2 and **(C)** iNOS in Caco-2/TC7 cells after 24 h of treatment with either BWW or BSW (5 mg protein/ml) alone or in combination with 20 µg/ml of LPS. Values are normalized to β-actin and expressed as fold change compared to control group (mean ± SD, n ≥ 3 independent experiments). **p* < 0.05, ***p* < 0.01 vs Control. #*p* < 0.05, ##*p* < 0.01 vs LPS.

DISCUSSION

Intestinal epithelium plays a pivotal role in maintaining gut homeostasis, acting as both a physical and immunological barrier between the host and microbiota [10]. Disruption of this barrier, often triggered by bacterial endotoxins such as LPS, leads to increased permeability and induces local and systemic inflammatory responses [27]. Consequently, nutritional strategies employing bioactive compounds to reinforce the epithelial barrier and to modulate inflammatory responses are of significant scientific interest [28]. This study investigated the impact of LPS on a Caco-2/TC7 intestinal epithelial model and assessed the protective potential of two distinct bovine wheys, BWW and BSW, to mitigate the LPS-induced intestinal epithelial barrier impairment.

Our initial objective was to establish and characterize an *in vitro* model of LPS-induced intestinal barrier dysfunction. The results confirmed that apical exposure to high concentrations of LPS (10-20 µg/ml) for 24 to 48 h compromised epithelial barrier integrity, as evidenced by a significant and time-dose dependent reduction in TEER. This is in line with previous studies that describe a loss of barrier function after exposure to LPS at similar concentrations in intestinal cell models [29,30]. However, a noteworthy discrepancy emerged from our permeability assays. Despite the clear reduction in TEER, we observed no significant increase in the paracellular flux of 4-kDa FITC-dextran. These findings indicate that LPS may modulate the composition or localization of tight junction (TJ) proteins, leading to a finely tuned functional alteration of the epithelial barrier. Specifically, the barrier becomes more permeable to ions while retaining its integrity against high-molecular-weight solutes. This observation underscores the higher sensitivity of TEER measurements for detecting early or moderate barrier dysfunction compared to macromolecular flux assays [31].

Furthermore, the LPS-induced drop in TEER was not accompanied by a significant downregulation in the mRNA expression of key TJ proteins (ZO-1, claudin-1, claudin-3, and occludin). This lack of transcriptional change suggests that the initial loss of barrier function may be mediated by post-translational mechanisms, such as the phosphorylation and subsequent delocalization of TJ proteins from the junctional complex, rather than a reduction in their synthesis [32]. Concurrently, LPS treatment triggered a robust pro-inflammatory response, characterized by a significant upregulation of IL-1R, COX-2, and iNOS mRNA expression. This confirms the activation of the LPS-TLR4 signaling pathway, which is known to drive inflammatory cascades in intestinal epithelial cells. In fact, this is a primary mechanism behind the barrier loss induced by inflammation [33,34].

A central finding of this study is the potent protective effect of the whey fractions against the barrier damage induced by LPS. Co-treatment with either BWW or BSW (5 mg protein/ml) completely reverted the LPS-induced reduction in TEER, restoring barrier integrity to control levels. Interestingly, BSW administered alone significantly increased TEER values above those of the untreated control, indicating an intrinsic capacity to enhance baseline barrier function. This barrier-strengthening effect is likely attributable to the observed upregulation of ZO-1 and claudin-1 gene expression by both whey fractions. These proteins are fundamental to the architecture and stability of the TJ complex. This effect is plausibly mediated by bioactive proteins present in whey, such as lactoferrin, which has previously been shown to enhance TJ protein expression and fortify the epithelial barrier [35,36]. On the other hand, it has to be considered that during enzymatic coagulation of casein, chymosin, which is the main enzyme of rennet, cleaves the Phe105–Met106 bond in κ-casein, producing para-κ-casein and macropeptides, among them the casein macropeptide [37-38]. A fraction of casein macropeptide molecules is extensively glycosylated and is referred to as glycomacropeptide (GMP). GMP has attracted considerable interest due to its reported bioactive properties, particularly its ability to enhance anti-inflammatory activity and strengthen intestinal barrier function [39,40]. However, the co-treatment of BSW with LPS resulted in a paradoxical downregulation of claudin-1. This suggests a complex interaction where the inflammatory environment induced by LPS may alter the cellular response to BSW's bioactive components, a phenomenon that encourages further investigation.

The most striking difference between the two whey fractions was observed in their immunomodulatory capacities. While both fractions were effective at restoring TEER, only BWW significantly counteracted the LPS-induced overexpression of IL-1R, COX-2, and iNOS, effectively normalizing the inflammatory gene profile. In contrast, BSW failed to mitigate the pro-inflammatory response. This functional divergence strongly points to the crucial role of the lipid components retained in the whole whey. Native whey contains remnants of the milk fat globule membrane (MFGM), which is rich in bioactive proteins and in complex lipids such as phospholipids, sphingolipids (e.g., sphingomyelin), and gangliosides [41-42]. These lipid species, along with certain fatty acids like conjugated linoleic acid, have well-documented anti-inflammatory properties. They are known to modulate key inflammatory signaling pathways, such as NF-κβ, and to inhibit the expression of enzymes like COX-2 and iNOS [43-45]. Therefore, the superior bioactivity of BWW is likely attributable to a dual mechanism: a structural reinforcement of the intestinal barrier mediated by GMP and whey proteins (shared with BSW) complemented by a unique, lipid-mediated anti-inflammatory action that counteracts the primary cause of barrier damage.

The ability of whey fractions to restore intestinal permeability and regulate the expression of TJ proteins emphasizes the role of milk-derived bioactive

components as modulators of epithelial barrier integrity and intestinal homeostasis [46]. Furthermore, the enhanced anti-inflammatory properties displayed by BWW underscore the capacity of functional lipids within the milk fat globule membrane to regulate immune signaling pathways [47-48]. Collectively, these data position whey fractions as functional foods ingredients capable of supporting intestinal barrier function and provide a translational framework for intestinal health interventions [49].

While these findings are promising, the study has limitations inherent to its *in vitro* design, including the use of a single cell line and the absence of other crucial gut components like a mucus layer, immune cells, and microbiota. Future research should therefore aim to validate these findings in more physiologically relevant systems, such as co-culture models (e.g., absorptive Caco-2 cells with mucus-producing HT-29 cells or macrophages) or in animal models of intestinal inflammation.

CONCLUSION

In summary, this study demonstrates that while high concentrations of LPS impair epithelial barrier function and induce a potent inflammatory response in Caco-2/TC7 cells, this initial damage primarily affects ion permeability without altering macromolecular flux or TJ gene transcription. Remarkably, BWW and BSW fractions effectively protected against LPS-induced barrier dysfunction, likely by upregulating the expression of key TJ proteins. However, only the BWW fraction, which retains its native lipid content, was capable of mitigating the underlying pro-inflammatory gene expression. This suggests that the lipid-associated components of whole whey, such as MFGM fragments, provide a crucial anti-inflammatory function that complements the barrier-enhancing effects of whey proteins and GMP released from casein by rennet action. These findings highlight the potential of whole whey from cheese manufacture as a superior functional food ingredient for promoting intestinal health, offering a dual-action strategy that both reinforces the physical barrier and dampens inflammatory responses.

List of Abbreviations: LPS, Lipopolysaccharide; BWW, bovine whole whey; BSW, bovine skimmed whey; TEER, Transepithelial electrical resistance; TJ, tight junction; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-alpha; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; ZO-1, zonula occludens-1; GMP, glycomacropeptide; MFGM, milk fat globule membrane

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