Pedro Miguel Clamote Marques

Licenciatura em Biologia

Bioactivity of chicory byproducts and their colonic metabolites in intestinal inflammation

Dissertação para obtenção do Grau de Mestre em

Bioquímica para a Saúde

Setembro, 2025

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Acknowledgments

I would like to thank my supervisor Dr Teresa Serra, for her help, support and guidance throughout the realization of my thesis project. Dra Teresa, as my supervisor, showed encouragement and availability, while always transmitting confidence and a sense of comfort. She revealed patience and expertise, which was fundamental to my progress. I want to thank Teresa for accepting me to work on this project, sharing the knowledge and allowing me to gain practical experience in this research field. I am also grateful to the iBET, for providing me the opportunity to work in the Natural Bioactives and Nutraceuticals area and receiving quality facilities and resources that made this work possible.

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Resumo

A inflamação intestinal é uma resposta do intestino a lesões e infeções e constitui a base de doenças inflamatórias intestinais (DII), como a Doença de Crohn e a Colite Ulcerativa, que resultam de fatores genéticos e ambientais e cujo tratamento envolve fármacos e alterações alimentares. Alterações na barreira intestinal podem levar a um aumento da permeabilidade, sendo a sua disfunção uma característica chave da DII. Compostos bioativos de origem vegetal, como as lactonas sesquiterpénicas (SLs) da chicória, têm sido estudados pelo seu potencial na modulação de respostas inflamatórias.

O objetivo deste trabalho foi avaliar o potencial anti-inflamatório de um extrato supercrítico rico em SLs obtido a partir das raízes de chicória (SFE) e dos seus metabolitos derivados da fermentação colónica durante 6 horas com microbiota fecal, utilizando um modelo *in vitro* de inflamação intestinal baseado numa co-cultura tripla de Caco-2:HT29-MTX:Raji-B, inflamada com LPS (10 µg/mL) no lado apical e IL-1β (25 ng/mL) e TNF-α (50 ng/mL) no lado basolateral. Foram realizados ensaios de viabilidade celular, quantificação de IL-8 por ELISA, medições de TEER para avaliar a integridade da barreira, e análise de expressão génica por RT-qPCR (PTGS2, NOS2, IL-6 e IL-1β). Ensaios de Western blot também foram efetuados, mas com resultados limitados.

Os extratos mostraram ser não citotóxicos até 780 µg/mL. O controlo inflamatório validou a robustez do modelo, comprometendo a integridade da barreira (TEER), aumentando significativamente a secreção apical e basolateral de IL-8 e a expressão dos genes estudados. Todos as amostras reduziram a libertação de IL-8, com o maior efeito observado a 0 h, quando SLs intactos em combinação com microbiota reduziram 53% no lado apical e 51% no lado basolateral em comparação com o controlo estimulado. Após 6 h, os metabolitos derivados da microbiota em conjunto com SLs preservados ainda mostraram efeito relevante, com 43% de redução no lado basolateral, enquanto o SFE não fermentado demonstrou maior capacidade de preservar a integridade da barreira (TEER % change). A expressão de PTGS2 e NOS2 foi consistentemente reduzida, IL-6 mostrou alterações menos consistentes, mas mais evidentes nos extratos fermentados de 0 h e 6 h. IL-1β não apresentou alterações significativas.

Estes resultados sugerem que o SFE proveniente da raiz chicória e os seus metabolitos derivados da fermentação apresentam efeitos diferenciados ao longo da resposta inflamatória, modulando a resposta inflamatória intestinal e preservando a integridade da barreira. Em conclusão, este trabalho destaca o potencial de SLs e dos seus metabolitos como candidatos naturais na gestão da inflamação intestinal, embora a atividade da microbiota possa mascarar parte da contribuição específica do extrato.

Abstract

Intestinal inflammation is a response to tissue injury and infection and is a central feature of Inflammatory Bowel Disease (IBD), which includes Crohn's disease (CD) and Ulcerative Colitis (UC). These disorders arise from genetic and environmental factors, and current treatments involve pharmacological therapy and dietary interventions. Disruption of the intestinal barrier leads to increased permeability, and its dysfunction is a key hallmark of IBD. Plant-derived bioactive compounds, particularly sesquiterpene lactones (SLs) from chicory, have been investigated for their potential to modulate inflammatory responses.

This study aimed to evaluate the anti-inflammatory potential of a supercritical fluid extract rich in SL from chicory roots (SFE) and its colonic derived metabolites obtained from 6 hour fermentation with faecal microbiota using an *in vitro* intestinal inflammation cell model (triple co-culture of Caco-2:HT29-MTX:Raji-B cells inflamed with LPS (10 µg/mL) on the apical side and IL-1β (25 ng/mL) and TNF-α (50 ng/mL) on the basolateral side. Cytotoxic evaluation, IL-8 quantification by ELISA, TEER measurements, and gene expression analysis by RT-qPCR (PTGS2, NOS2, IL-6, and IL-1β) were performed. Western blot analysis was also attempted but yielded limited results.

The extracts were non-cytotoxic up to 780 µg/mL. The inflammatory control elicited a strong response, validating the robustness of the model by compromising barrier integrity (TEER), markedly increasing IL-8 secretion on both apical and basolateral sides, and upregulating the expression of target genes. All samples reduced IL-8 release, with the highest effect observed at 0 h when intact SLs combined with microbiota led to reductions of 53% on the apical side and 51% on the basolateral side compared to the stimulated control. After 6 h, microbiota-derived metabolites together with preserved SLs still showed a relevant effect, with a 43% reduction on basolateral compartment, while non-fermented SFE had the strongest impact on preserving barrier integrity (TEER % change). PTGS2 and NOS2 expression were consistently reduced, and IL-6 showed variable but clearer effects with 0h and 6h fermented extracts. IL-1β expression was not significantly affected.

These findings suggest that chicory root SFE and its fermentation derived metabolites exert differential effects during the inflammatory response. SFE modulated intestinal inflammation by preserving barrier integrity and suppressing inducible enzymes, while cytokine effects were more variable. In conclusion, this study highlights the potential of SLs and their metabolites as natural candidates for managing intestinal inflammation, although microbiota activity may mask specific extract contributions.

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List of Abbreviations

AMPK: AMP-activated protein kinase

BMS-345541: Selective inhibitor of IKK-1/IKK-2 and NF-κB signaling

BSA: Bovine serum albumin

cDNA: Complementary DNA

CD: Crohn's disease

COX-2: Cyclooxygenase-2

CT value: Cycle threshold value (qPCR)

DAD: Diode-array detector

DII : Doença inflamatória intestinal

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

DPBS: Dulbecco's phosphate-buffered saline

ELISA: Enzyme-linked immunosorbent assay

FAE: Follicle-associated epithelium

FBS: Fetal bovine serum

GALT: Gut-associated lymphoid tissue

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GI: Gastrointestinal

HPLC: High-performance liquid chromatography

HRP: Horseradish peroxidase

IBD: Inflammatory bowel disease

IBET: Instituto de Biologia Experimental e Tecnológica

IECs: Intestinal epithelial cells

IFN-γ: Interferon-gamma

Ig: Immunoglobulin

IKK: IκB kinase

IL: Interleukin

iNOS: Inducible nitric oxide synthase (encoded by NOS2)

LPS: Lipopolysaccharide(s)

MAPK: Mitogen-activated protein kinase

MLCK: Myosin light chain kinase

MUC: Mucins

NaN_3: Sodium azide

NEAA: Non-essential amino acids

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NO: Nitric oxide

NOS2: Pro-inflammatory gene encoding iNOS

PBS: Phosphate-buffered saline

PGE_2: Prostaglandin E_2

PG: Prostaglandin

PP: Peyer's patches

PTGS2: Prostaglandin-endoperoxide synthase 2 (gene encoding COX-2)

qRT-PCR: Quantitative real-time polymerase chain reaction

RhoA–ROCK: Rho-associated protein kinase signaling pathway

RIPA: Radioimmunoprecipitation assay buffer

RNA: Ribonucleic acid

ROS: Reactive oxygen species

SCFAs: Short-chain fatty acids

SD: Standard deviation

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SFE: Supercritical fluid extract

SLs: Sesquiterpene lactones

STAT3: Signal transducer and activator of transcription 3

TBST: Tris-buffered saline with Tween 20

TEER: Transepithelial electrical resistance

TJ: Tight junction

TNF-α: Tumor necrosis factor-alpha

UC: Ulcerative colitis

ZO-1: Zonula occludens-1

1. Introduction

1.1 Intestinal inflammation

The gastrointestinal (GI) system is composed of the GI tract and several accessory organs. The tract itself includes the oral cavity, pharynx, esophagus, stomach, small intestine, large intestine, and anal canal. Its primary functions are the ingestion and digestion of food, absorption of nutrients, secretion of water and digestive enzymes, and elimination of waste products. [1]. The GI tract is lined by an epithelial layer that is constantly exposed to many foreign antigens coming from commensal microbes, food, and occasional pathogens [2]. The intestinal mucosa creates a special immune environment where the body maintains tolerance to commensal bacteria and dietary antigens, while still producing a protective immune response against pathogens, keeping a delicate balance [2] . When this immune balance is disturbed, it can lead to uncontrolled inflammation and damage to the epithelial barrier [3]. The resulting intestinal inflammation is becoming increasingly relevant as one of the more commonly occurring diseases in developed countries [4].

Both acute and chronic gut inflammation are linked to a mix of genetic, environmental, and immune-related factors that affect normal host–microbe interactions [5]. While inflammation in the intestine is a natural defense mechanism that helps preserve tissue integrity and function, it depends on constant communication between the different cell types in the gut. This process involves the release of soluble mediators such as cytokines, eicosanoids, nitric oxide (NO), and growth factors. These molecules can either amplify or reduce the activity of other mediators in target cells, so their production needs to be tightly controlled to ensure a well-coordinated immune response [6].

1.2 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) (Fig. 1.1) is a chronic inflammatory disease of the gastrointestinal tract, and it is caused by an abnormal and exaggerated immune response to a normal stimulus, such as food or intestinal flora [7]. IBD affects millions worldwide, with the highest prevalence in Western countries, where more than 0.7% of the adult population is diagnosed, and incidence is rising globally across both young adults and children [138,139]. This disease is divided into Crohn disease and ulcerative

colitis. Crohn disease (CD) results in transmural ulceration of any portion of the gastrointestinal tract, affecting mostly the terminal ileum and colon. Ulcerative colitis (UC) involves the inflammation of the colonic mucosa, affecting the rectum and can also extend into the sigmoid [8].

The diagnosis of inflammatory bowel disease (IBD) requires a combination of clinical, laboratory, endoscopic, histological, and imaging tools, as no single standardized test exists. Laboratory analyses, such as blood tests, are often used to exclude infections, detect anemia, and evaluate systemic inflammation, liver function, or markers of ongoing infection. Endoscopic procedures are central in IBD diagnosis, both to establish the disease and to monitor its activity. Colonoscopy is the gold-standard technique, as it enables direct visualization of the colon and terminal ileum and allows the collection of biopsies. Histological examination of biopsy samples remains essential to confirm IBD and to distinguish between UC and CD. Imaging methods, including CT colonography, magnetic resonance enterography, or ultrasound, complement the diagnosis, particularly in Crohn's disease, as they provide information about the extent, severity, and transmural involvement of lesions [9].

The treatment of inflammatory bowel disease (IBD) is still challenging because it is a chronic condition with frequent relapses. Standard options include antibiotics and anti-inflammatory drugs such as aminosalicylates, corticosteroids, immunomodulators, and biologics, which help reduce inflammation and keep the disease under control. Biologics like anti-TNF or anti-integrin agents have improved treatment a lot, but many patients still do not respond or lose response over time [145]. In more severe or refractory cases, surgery may be needed, especially in ulcerative colitis. New strategies are also being explored, including stem cell therapy, faecal microbiota transplantation, helminth therapy, and natural bioactives, which may complement current treatments. Importantly, treatment goals have shifted from just controlling symptoms to reaching mucosal healing and following a treat-to-target approach for better long-term outcomes [10].

Both disorders (CD and UC) present a wide spectrum of intra- and extraintestinal manifestations and can typically be distinguished without difficulty. IBD substantially impacts quality of life, with common symptoms such as abdominal pain, rectal bleeding, diarrhea, fever, malnutrition, and weight loss [11]. There is a clear genetic predisposition for inflammatory bowel disease (IBD), and because it is a chronic inflammatory condition, patients carry a higher risk of developing colorectal cancer. Although no single cause has been identified for intestinal inflammation, several

environmental factors such as medication, stress, diet, and smoking, are known to influence both the onset and progression of the disease [12].

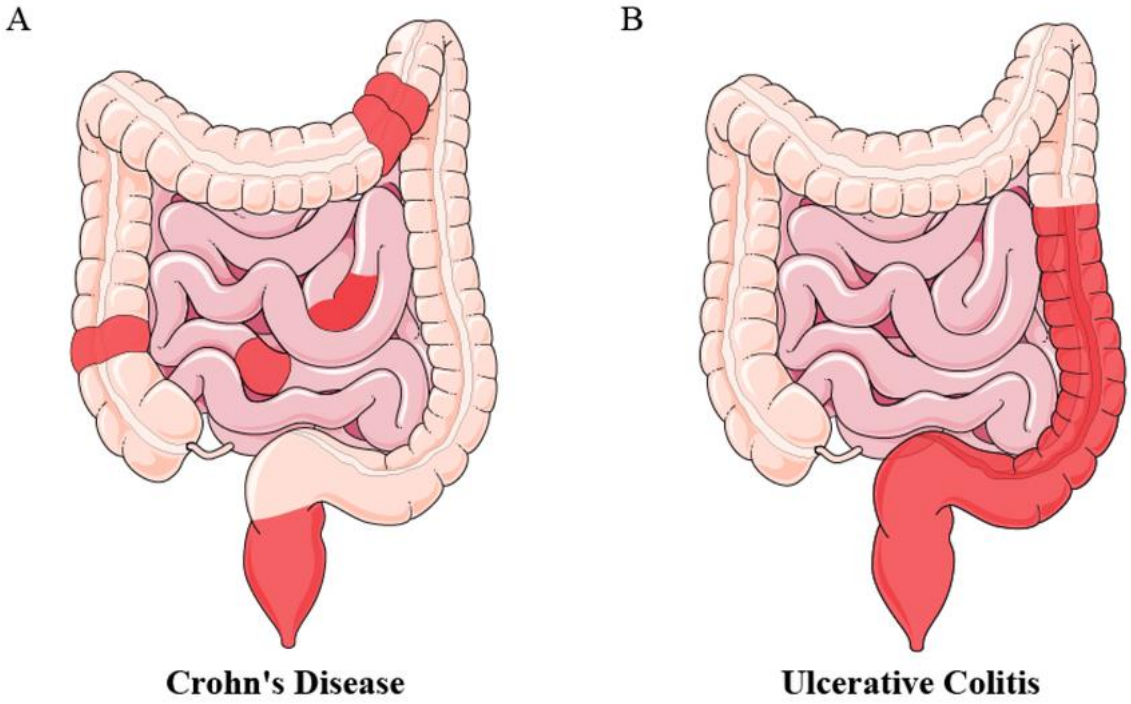

Figure 1.1. **Illustration of intestinal involvement in Crohn's disease and ulcerative colitis (**adapted from Microorganisms, "Illustration of intestinal involvement in Crohn's disease and ulcerative colitis")

1.3 Intestinal epithelial cells and *in vitro* models

Intestinal epithelial cells (IECs) line the intestinal surface, where they are essential for food digestion, nutrient absorption, and protection against microbial infections, while also regulating immune surveillance and host–microbe interactions. When their function is disrupted, this barrier becomes compromised, contributing to the onset of diseases such as inflammatory bowel disease (IBD) [13]. (IECs) have a strategic position at the interface between the antigenic luminal environment and the internal milieu. They actively contribute to the gut immune system, mediating processes as mucosal defense, tolerance to resident flora and barrier repair [14]. IECs establish bidirectional interactions with the underlying immune cells and participate in the mucosal inflammatory response [15]. IECs selectively regulate the permeability of the epithelial monolayer, controlling immune cell exposure to antigens, while also synthesizing and secreting inflammatory mediators that can activate immune cells and initiate inflammation [15]. In turn, they respond to mediators released by immune cells by

adjusting permeability and secretion, thereby amplifying or attenuating the inflammatory process [16].

In vitro intestinal models are widely used to study epithelial barrier function and inflammatory responses, providing insights into key features of intestinal diseases [140]. Epithelial cell models were established from gastrointestinal tumours to study mechanisms in cancer development and effects of cytotherapy [17]. Since the intestinal epithelium presents heterogeneity in terms of morphology and function, such as enterocytes, goblet cells, enteroendocrine cells, Paneth cells and M-cells [18], it is important to differentiate tumour cells into specialized cell types. One of them is the Caco-2 (Cancer coli-2) cell line, which can differentiate spontaneously when reaching confluence. Caco-2 was established from a human colorectal adenocarcinoma by Jorgen Fogh at the Sloan-Kettering Cancer Research Institute [19]. Caco-2 cells exhibit enterocyte-like morphology and functionality, with the ability to proliferate efficiently and remain stable in culture. They form polarized confluent monolayers that display characteristic features such as a brush-border with microvilli, tight junctions (TJs), hydrolytic enzymes, and carrier-mediated transport systems for various biomolecules and drugs [19]. However, these cells also overexpress P-glycoprotein (P-gp) efflux pumps, which enhance secretion and reduce absorptive permeability [20]. Consequently, Caco-2 monocultures have limitations, as they consist solely of absorptive enterocytes and fail to account for other intestinal cell types, including mucus-secreting goblet cells and M cells [21].

To present a better physiological representation of the intestinal epithelium, a co-culture of Caco-2 with mucus-producing HT29-MTX cell line [22] was described. HT29-MTX cells represent a methotrexate-adapted subpopulation of the parental HT29 adenocarcinoma line, characterized by increased expression of mucin genes such as MUC2 and MUC5AC [23]. These, resembling goblet cells, are the second most abundant intestinal cell type among the HT29-MTX cells, and provide a valuable complement to Caco-2. When combined in co-culture, they represent a more physiologically relevant intestinal epithelium model compared to Caco-2 alone. This is largely due to the establishment of a protective mucus layer, which constitutes the first line of defense against harmful agents [24]. The incorporation of HT29-MTX cells into the co-culture increases apical-to-basolateral paracellular permeability of hydrophilic compounds while reducing permeability in the secretory direction of molecules subject to P-gp-mediated secretion [25]. These effects arise from the fact that HT29-MTX cells display looser TJ and lack the expression of P-gp [26].

The mucosal surface of the mammalian gut is continuously exposed to a variety of foreign proteins and microorganisms, some of which are potentially harmful to the host [27]. To protect itself from these dangers, the host has evolved a specialized and organized lymphoid tissue, the gut-associated lymphoid tissue (GALT), which includes Peyer's patches (PPs) and isolated lymphoid follicles [28]. GALT is the inductive site for intestinal immunity and samples mucosal antigens across the epithelial barrier to initiate immune responses. In the case of harmful microbes, specialized intestinal epithelial microfold (M) cells residing in the follicle associated epithelium (FAE) of Peyer's patch covering the lymphoid follicles of GALT can sense toxic stimuli from the microbes, activate the barrier function and participate in the coordination of the appropriate immune response [28]. By engulfing antigens from the mucosal epithelium and transferring them to the underlying immune cells, M cells can facilitate further immune induction [29]. Raji B cells are lymphocytes that were derived from the B-lymphocytes of an 11-year-old Nigerian Burkitt lymphoma male patient in 1963 [31]. Raji is the first continuous human cell line of hematopoietic origin and is widely used as a transfection host. *In vitro*, M cells are typically generated by co-culturing human enterocyte Caco-2 cells with human B lymphocytes. When Caco-2 cells are cultured with Raji B cells, they can acquire an M-cell phenotype, which plays a key role in transporting antigens and pathogens from the luminal surface to the underlying sub-epithelium. This approach relies on the concept that lymphocytes introduced into the basolateral compartment migrate into the epithelial monolayer, inducing the differentiation of epithelial cells into phagocytic and transcytotic microfold (M) cells with immune-supporting functions [32]. A triple co-culture system combining Caco-2, HT29-MTX, and Raji B lymphocytes has been established as a valuable strategy to mimic the intestinal epithelium (Fig. 1.2).

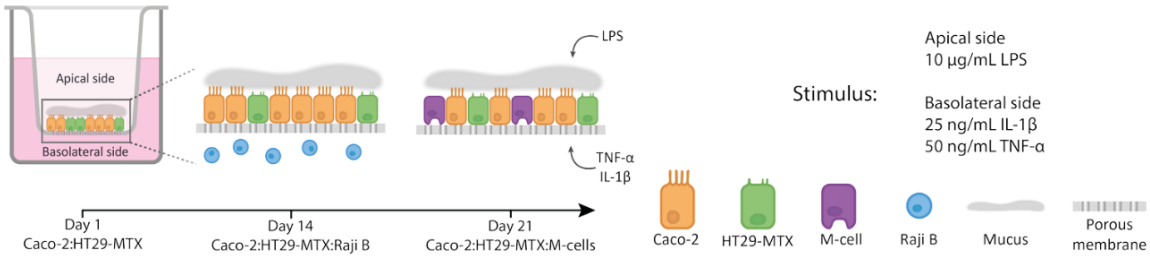

Figure 1.2. **Establishment of the pro-inflammatory stimulus in a triple co-culture composed of Caco 2:HT29-MTX:Raji B**. A) Triple cell co-culture experimental setup of the inflamed intestinal mucosa with the pro-inflammatory cocktail (10 µg/mL LPS on the apical side) (adapted from Matos, 2024 [PhD Thesis, NOVA University Lisbon]).

1.4 Biomarkers of Intestinal inflammation

Several inflammatory mediators are thought to be implicated in the development of IBDs. However, *in vitro* research on Intestinal Epithelial Cells (IECs) has mainly focused on the involvement of various major cytokines, such as Interleukin (IL)-1β and tumor necrosis factor (TNF)-α [33]. IL-1β is a multifunctional cytokine playing a major role both in the initiation and the amplification of many inflammatory conditions. It is released by various cell types including monocytes, macrophages, neutrophils and endothelial cells and has been found in increased concentrations in the intestinal tissue of IBD patients [34]. TNF-α is a cytokine that plays a key role in promoting inflammation and diverse immunological processes, modulating the recruitment and adhesion of leukocytes and the antigen presentation [35]. TNF-α is primarily produced by macrophages and monocytes and is one of the key cytokines driving the pathogenesis of IBD [35], with elevated levels consistently reported in the intestinal mucosa of affected patients [36]. The presence of bacterial components, such as lipopolysaccharides (LPS), is also considered as an important factor both in the initiation and in the reactivation of IBDs. LPS are a major cell wall component of Gram-negative bacteria and are released from the bacterial cell wall by shedding or through bacterial lysis [37]. LPS have been shown to disrupt intestinal tight junctions by increasing enterocyte membrane expression, ultimately enhancing epithelial permeability [38]. The intestinal tissue and circulating LPS levels are markedly elevated in IBD and play an important role in mediating inflammatory response [39]. LPS, is well known in every laboratory environment for use as a stimulant or co-stimulant in a variety of applications [40].

A dysregulation of IEC sensitivity to the common local microflora is believed to cause an initial inappropriate inflammatory stimulus, leading to an exaggerated cytokine presence and IBD development [41]. Moreover, the leaky IEC barrier observed during IBDs leads to increased permeation to bacteria during inflammation, which further enhances immune activity [42]. The *in vitro* effects of IL-1β, TNF-α and LPS on IECs consist in activation of intracellular cascades, leading to an increased transcriptional activity and the secretion of pro-inflammatory mediators, such as interleukin (IL)-8, IL-6, prostaglandin (PG)-E2 and/or nitric oxide (NO), as well as increase of the paracellular permeability through defects in tight junction functioning or assembly [43].

1.5 Gut Microbiome

In the GI tract, the interaction of commensal microbes with the host immune system helps to maintain the protective capacity of the epithelium [44]. These

microorganisms are known as gut microbiota and predominately encompasses species from the prokaryotic domain (comprising bacteria and other unicellular microbes lacking specialised organelles) and, to a lesser extent, fungi, parasites and archaea. Viruses are also constituents of this environment [45]. A vast pool of studies in animals and humans have indicated a critical interplay between the gut microbiota and inflammation that could inform therapeutic intervention for the treatment of these disorders. [46]. One important way in which the gut microbiota supports intestinal health is by preserving the barrier function, through reinforcement of tight junctions and stabilization of the mucus layer, thereby limiting direct contact between pathogens and the mucosa. [47]. The gut microbiota plays a central role in regulating host immunity through the production of metabolites that interact with epithelial and immune cells. Dysbiosis can be defined as a quantitative or qualitative imbalance in microbial composition [48]. Disruption of this microbial balance is strongly associated with increased levels of pro-inflammatory mediators such as IL-6, TNF-α, and IL-1β, as well as endotoxins like LPS, which together activate signalling cascades including NF-κB and MAPK pathways. These mechanisms drive the secretion of cytokines and amplify the inflammatory process, contributing to chronic conditions such as IBD and metabolic disorders. Clinical evidence shows that elevated inflammatory markers linked to microbial imbalance not only correlate with intestinal barrier dysfunction but also with systemic consequences, reinforcing the importance of targeting microbiota–immune interactions for therapeutic strategies [49]. The gut microbiota plays a central role in phytochemical metabolism, modifying their structures and potentially altering their stability, activity, or bioavailability [50]. Beyond this, the microbiome can also modulate host gene expression [51].

1.6 Chicory and Its bioactive compounds

Phytochemicals from medicinal plants, including flavonoids, terpenoids, polyphenols, alkaloids, saponins, tannins, and essential oils, have demonstrated strong anti-inflammatory potential [52]. They are present in crude extracts, purified compounds, and even in metal or metal oxide nanoparticles, and have been applied in medical, nutraceutical, and cosmeceutical contexts [53]. This bioactive diversity makes plant-derived extracts attractive as alternative or complementary strategies for managing inflammatory diseases [54]. Among them, dietary phytochemicals are non-nutrient secondary metabolites that display a wide range of bioactivities. For example, Sesquiterpene lactones (SLs) are known to be metabolized by the human gut microbiota (Fig. 1.9). Due to their low bioavailability, these compounds often remain in the

gastrointestinal tract for extended periods. Poor absorption in the upper intestine allows them to reach the colon, where they can modulate gut microbiota composition by selectively stimulating or inhibiting specific microbial communities [55]. Sesquiterpene lactones (SLs) have gained growing attention in recent years due to their diverse biological effects that support human health. Their anti-inflammatory action arises from the capacity to interfere with major pro-inflammatory mediators across multiple signaling pathways, helping to limit or prevent tissue damage during inflammation [30].

Chicory (*Cichorium intybus* L.) (Fig. 1.3) is a perennial herb of the Asteraceae family, native to Europe, North Africa, and Western Asia, and now naturalized in North America [56]. It is mainly cultivated for the commercial production of inulin from its roots and is also widely recognized as a popular coffee substitute [57]. Beyond its alimentary uses, chicory has a long history in traditional medicine, where both roots and leaves have been employed to treat conditions such as diabetes, tumors, tachycardia, hepatitis, and atherosclerosis [58]. The roots are particularly rich in inulin, sesquiterpene lactones (SLs), and phenolic compounds, supporting digestive, hepatoprotective, antidiabetic, and anti-inflammatory effects, while the leaves contain polyphenols, flavonoids, and vitamins, contributing to antioxidant, antibacterial, and hepatoprotective activities [58]. This diverse phytochemical profile highlights chicory as a valuable source of bioactive compounds, with SLs from the roots (Fig. 1.4) being especially relevant for their anti-inflammatory potential [59].

After inulin extraction, the remaining chicory material is often treated as waste, despite its evident potential as a reservoir of therapeutic compounds [60]. Terpenes such as SLs are typically removed during processing due to their bitter flavour. However, these compounds are valuable because of their multiple medicinal properties, including anti-inflammatory effects [61]. To efficiently recover bioactive phytochemicals, more sustainable and selective methods are required. Recently, supercritical CO_2 extraction has been optimized as a functional and eco-friendly strategy to valorize root chicory residues, showing greater efficiency and selectivity for isolating SLs compared to conventional extraction techniques [62]. Importantly, SLs continue to gain attention for their therapeutic benefits, particularly in the context of inflammation, since natural extracts containing a broad spectrum of these compounds may exert additive or synergistic bioactive effects [63,64].

Figure 1.3. **Chicory *(Cichorium intybus)***, whose roots are commonly used for extracts rich in bioactive phytochemicals (source: Gillco, 2025).

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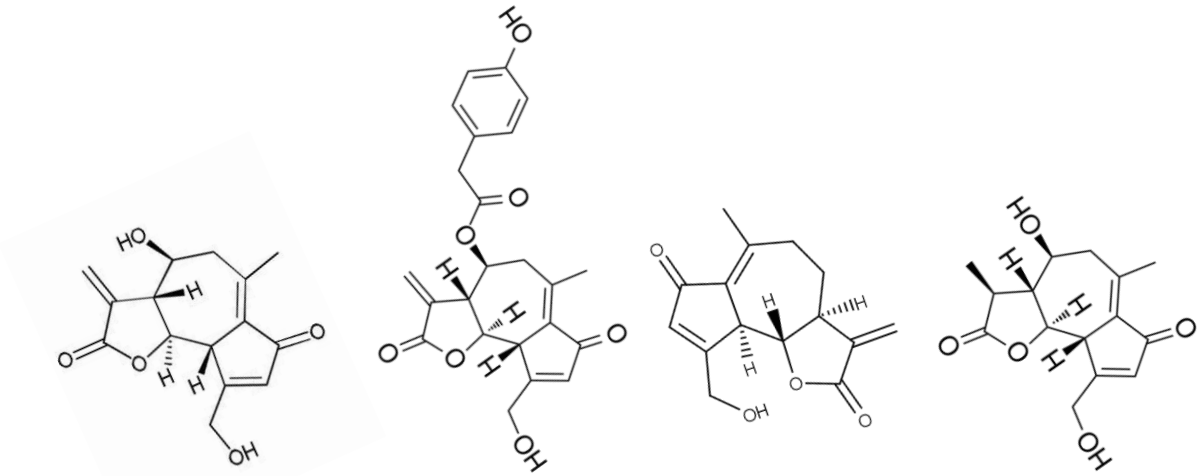

Figure 1.4. **Chemical structures of the main sesquiterpene lactones present in chicory**, compounds known for their anti-inflammatory activity: (A) lactucin, (B) lactucopicrin, (C) 8-deoxylactucin, and (D) 11β,13-dihydrolactucin.

1.7 Foundations of This Work

This work builds on a project carried out at the Instituto de Biologia Experimental e Tecnológica (iBET) within the context of EU Horizon 2020 CHIC project, which aimed to develop chicory varieties to produce dietary fibre with enhanced prebiotic properties to support gut health. More specifically, the project performed at iBET focused on the evaluation of the anti-inflammatory potential of chicory-derived terpenes for application in Inflammatory Bowel Disease (IBD).

In this project, an *in vitro* triple co-culture intestinal inflammation model representing the inflamed intestinal mucosa was implemented, comprising enterocytes (Caco-2), goblet-like cells (HT29-MTX), and microfold (M)-cells induced by Raji B lymphocytes (Fig. 1.2). Using this physiologically relevant system, an initial screening was conducted for the anti-inflammatory activity of pure sesquiterpene lactones (SLs) present in chicory (61). Among the tested compounds, 11β,13-dihydrolactucin and 11β,13-dihydrolactucopicrin demonstrated promising effects, significantly reducing IL-8 release (Fig. 1.5).

In addition, a broad-spectrum chicory root extract obtained by supercritical CO_2 fluid extraction (SFE) process (62) was characterized as being particularly rich in SLs such as lactucin and lactucopicrin (Table 2.1). When compared to SL-rich purified fractions, the whole SFE extract appeared more effective across a wider panel of inflammatory biomarkers, including IL-8 (Fig. 1.6), IL-6, IL.1β (Fig. 1.7), TNF, iNOS and COX-2 (Fig.1.8), suggesting a potential synergistic effect among SLs.

Finally, the project also evaluated the interaction between chicory supercritical fluid extract (SFE) and the human colonic microbiota. Using an *in vitro* fermentation model, it was shown that microbial metabolism rapidly modified the profile of sesquiterpene lactones (SLs), with early formation and subsequent decline of cysteine conjugates, along with deglycosylation (Fig. 6.3 in Annexes) leading to higher levels of compounds such as 11β,13-dihydrolactucin and 11β,13-dihydro-8-deoxylactucin (Fig. 1.9). Despite these structural changes, the SFE retained a dose dependent anti-inflammatory effect, reducing IL-8 secretion in a triple co-culture model (Fig. 6.2). Building on this work, the present thesis investigates the anti-inflammatory potential of fermented chicory SFE extracts, focusing on their effects in an in vitro intestinal inflammation model and aiming to clarify how fermentation time and metabolite formation may influence biological outcomes.

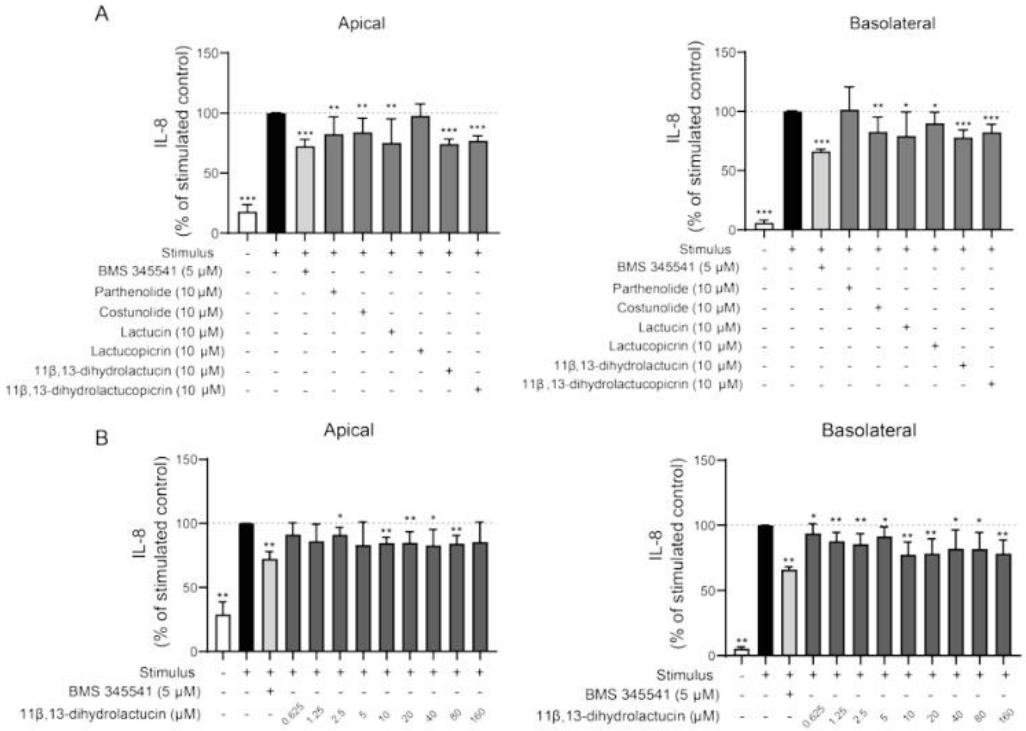

Figure 1.5. **11β,13-dihydrolactucin shows anti-inflammatory potential in intestinal triple co culture**. A) IL-8 release assessed by ELISA in both apical and basolateral cell media of cells treated with an IKK-1/IKK-2 inhibitor (BMS 345541 at 5 µM) or 10 µM of the in indicated pure SLs for 48h in co-incubation with the pro-inflammatory stimulus (10 µg/mL LPS in the apical compartment; 25 ng/mL IL-1β and 50 ng/mL TNF-α in the basolateral compartment). B) IL-8 release assessed by ELISA in both apical and basolateral cell media of cells treated with BMS 345541 at 5 µMor a range of concentrations of 11β,13-dihydrolactucin for 48h in co-incubation with the pro-inflammatory stimulus. The dotted line in the y-axis depicts the 100% IL-8 release in the stimulated control. Results are expressed as mean ± standard deviation (SD) from at least three independent assays. *p<0.05, **p<0.01, ***p<0.001 relative to the stimulated control. *Matos, M.S. (2024) PhD thesis, Molecular Biosciences, ITQB NOVA*

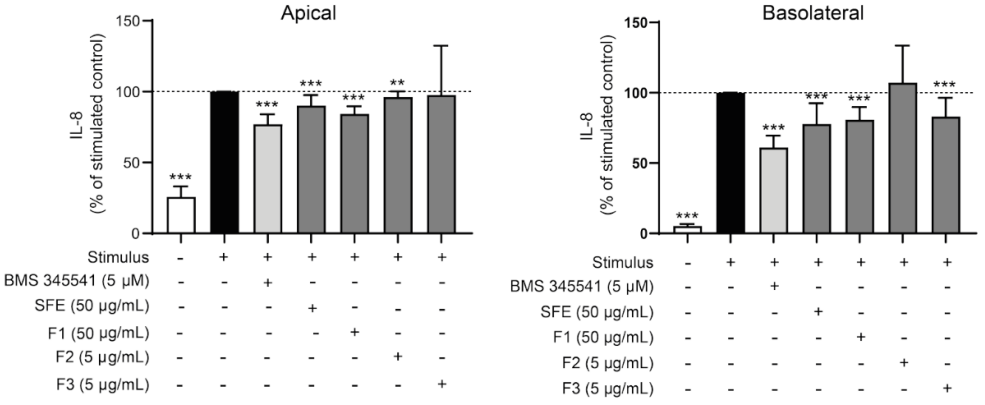

Figure 1.6. **Anti-inflammatory activity of the highest non-cytotoxic concentrations of SFE and its fractions in the intestinal triple co-culture**. IL-8 secretion was quantified by ELISA in apical and basolateral media after 48 h of treatment with BMS 345541 (5 µM), whole SFE, or SFE fractions, in co-incubation with the pro-inflammatory cocktail (10 µg/mL LPS apical; 25 ng/mL IL-1β and 50 ng/mL TNF-α basolateral). Data are mean ± SD from at least three independent experiments. The dotted line represents 100% IL-8 release in the stimulated control. **p < 0.01, ***p < 0.001 vs. stimulated control. *Matos, M.S. (2024) PhD thesis, Molecular Biosciences, ITQB NOVA*

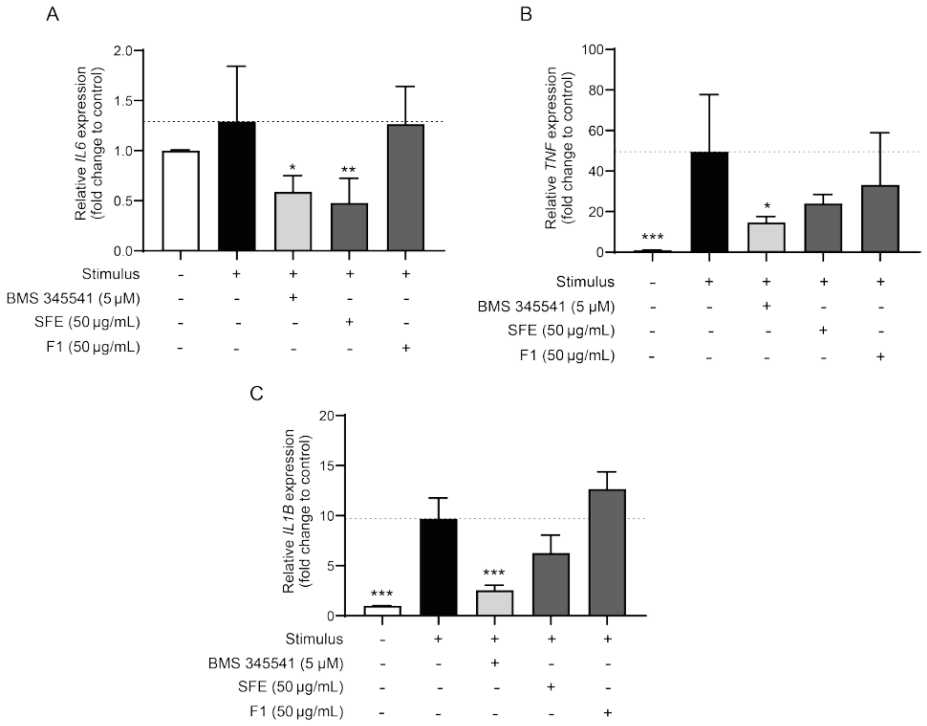

Figure 1.7. **SFE and fraction F1 reduced IL-6, TNF-α, and IL-1β expression in the intestinal triple co-culture.** Gene expression of IL-6 (A), TNF-α (B), and IL-1β (C) was measured by qRT-PCR after 3 h treatment with BMS 345541 (5 µM), SFE (50 µg/mL), or F1 (50 µg/mL) in the presence of the pro-inflammatory cocktail (10 µg/mL LPS apical; 25 ng/mL IL-1β and 50 ng/mL TNF-α basolateral). F1 was mainly composed of 8-deoxylactucin and 11β,13-dihydro-8-deoxylactucin, with 11β,13-dihydrolactucopicrin also detected. The dotted line represents expression in the stimulated control. Results are mean ± SD from at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 vs. stimulated control. *Matos, M.S. (2024) PhD thesis, Molecular Biosciences, ITQB NOVA*

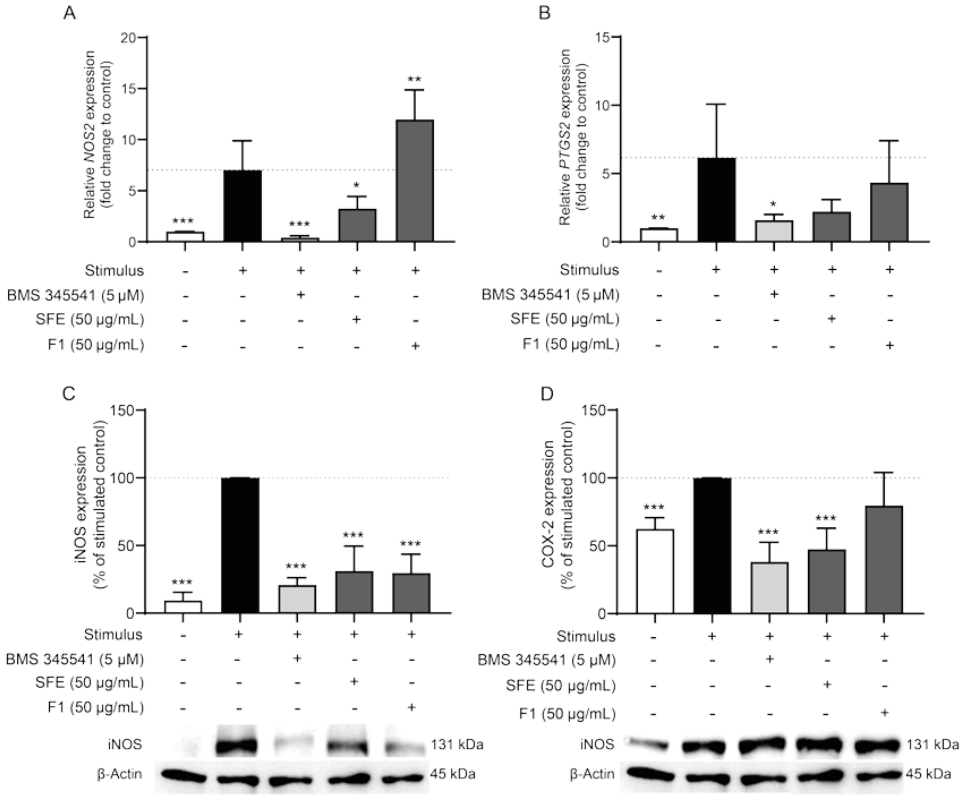

Figure 1.8. **SFE and fraction F1 reduced the expression of the inflammatory enzymes iNOS and COX-2 in the intestinal triple co-culture model**. Gene expression of A) iNOS and B) COX-2 was evaluated by qRT-PCR after 12 h of treatment with BMS 345541 (5 µM), SFE (50 µg/mL), or F1 (50 µg/mL) in the presence of the pro-inflammatory cocktail. Protein expression of C) iNOS and D) COX-2 was assessed by Western blot after 3 h of treatment under the same inflammatory conditions (10 µg/mL LPS on the apical side; 25 ng/mL IL-1β and 50 ng/mL TNF-α on the basolateral side). The dotted line on the y-axis represents expression levels in the stimulated control. Results are reported as mean ± standard deviation (SD) from at least three independent experiments. *p < 0.05, **p<0.01, ***p < 0.001 versus stimulated control. *Matos, M.S. (2024) PhD thesis, Molecular Biosciences, ITQB NOVA*

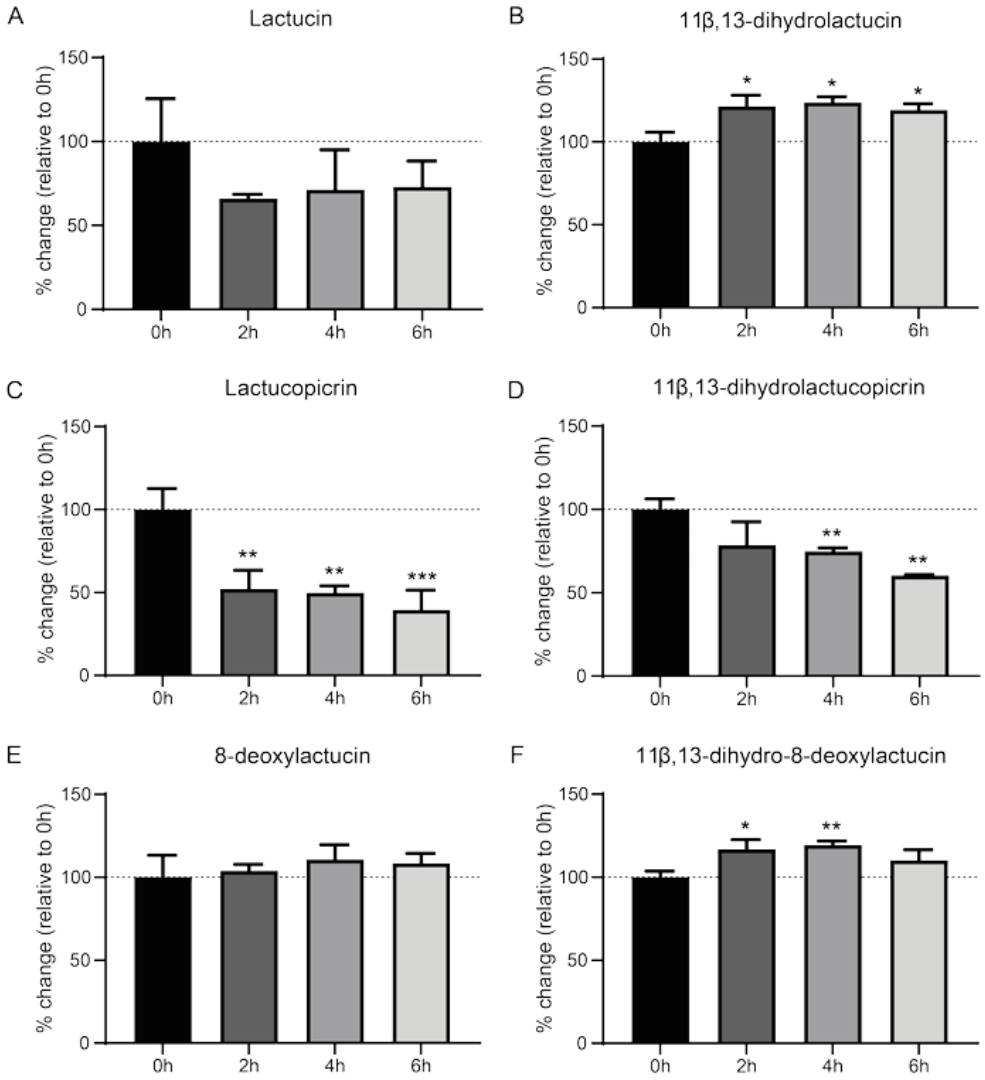

Figure 1.9. **Free sesquiterpene lactone (SL) composition of the chicory supercritical fluid extract (SFE) after microbiota metabolization over a 6-hour period**. Changes are shown for lactucin (A), 11β,13-dihydrolactucin (B), lactucopicrin (C), 11β,13-dihydrolactucopicrin (D), 8-deoxylactucin (E), and 11β,13-dihydro-8-deoxylactucin (F). Results are expressed as percentage mean ± SD relative to the initial timepoint (t = 0 h), with statistical significance indicated as *p < 0.05, **p < 0.01, and ***p < 0.001. The 0 h timepoint corresponds to the beginning of incubation with the colonic microbiota. *Matos, M.S. (2024) PhD thesis, Molecular Biosciences, ITQB NOVA*

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1.8 Objectives

This thesis aimed to evaluate the anti-inflammatory properties of fermented chicory extracts in intestinal inflammation. For this purpose, a human triple co-culture cell model of the inflamed intestinal mucosa, composed of enterocyte-like, mucus-secreting, and M-like cells was be used to analyse the impact of chicory (*Cichorium intybus L.*) supercritical fluid extracts in anti-inflammatory activity for application in IBD. Various biomarkers were assessed at different stages of inflammation, by measuring gene and protein expression.

To evaluate the anti-inflammatory potential of SFE using a human intestinal triple co-culture model the goal was to:

1. **Analyse the effects of chicory root SFE on intestinal inflammation**

The main objective of this work was to evaluate the anti-inflammatory potential of a chicory supercritical fluid extract (SFE) using an in vitro model of intestinal inflammation. To achieve this, the first step was to identify non-cytotoxic concentrations of the samples, ensuring that all subsequent experiments were performed at physiologically safe levels. These selected concentrations were then applied to an intestinal triple co-culture model exposed to a pro-inflammatory cocktail to screen for samples with the strongest effects on IL-8 secretion. The most promising conditions were further characterized by assessing their ability to modulate key inflammatory signalling pathways. Paracellular permeability, protein expression and phosphorylation of relevant markers were evaluated in parallel with gene expression analysis of pro-inflammatory cytokines and inducible enzymes, providing complementary evidence of the anti-inflammatory potential of the extract in the context of IBD.

2. **Assess whether colonic fermentation modifies the anti-inflammatory activity of SFE**

A second objective was to compare non-fermented and fermented SFE and assess the role of colonic microbiota metabolites in modulating the anti-inflammatory response. This involved analyzing results obtained at different microbiota exposure timepoints to identify temporal patterns in extract activity and to evaluate the contribution of individual SLs to the observed effects across all samples. The results were interpreted in the context of previously reported changes in sesquiterpene lactone (SL) composition during fermentation, including the loss of glycosides and cysteine conjugates. By integrating biological readouts with chemical evidence from earlier studies, this work

aimed to clarify whether microbiota driven metabolism enhances or diminishes the anti-inflammatory capacity of chicory extracts.

2. Materials and Methods

2.1 Caco-2 cell culture

Caco-2 cells were cultured in a 75cm^2 T-flask with Dulbecco's modified eagle medium (DMEM) with high glucose and high pyruvate, supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, 100 µg/mL streptomycin 1% (v/v) and 10 mM non-essential amino acids 1% (v/v). Cells were grown and incubated in a humidified atmosphere at 37ºC with 5% CO_2. The passage of cells was performed twice a week, with a trypsinization protocol that consisted of removing the old medium, adding trypsin, and incubating until detachment from the T-flask. This protocol was followed by the renewal of culture medium with a ratio of 1:4. The passage number did not exceed 20, and after this interval new frozen cell stocks were thawed for culture continuation.

2.2 HT29-MTX cell culture

HT29-MTX cells were cultured in a 75 cm^2 T-flask with Dulbecco's modified eagle medium (DMEM) with high glucose and high pyruvate, supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) 100 units/mL penicillin, 100 µg/mL streptomycin, and 10 mM nonessential amino acids 1% (v/v). Cell culture was kept in a humidified atmosphere at 37ºC with 5% CO2. The passage of cells was performed twice a week with a trypsinization protocol followed by the renewal of culture medium with a ratio of 1:6. The passage number did not exceed 20, and after this interval new frozen cell stocks were thawed for culture continuation.

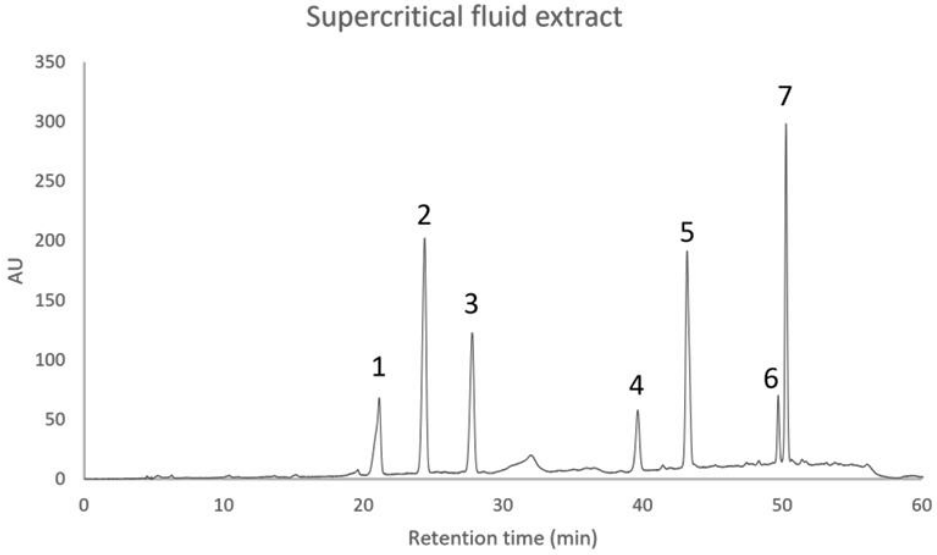
2.3 Raji-B cell culture

Raji-B cells were cultured in a 75 cm² T-flask with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin 1% (v/v) and 10 mM nonessential amino acids 1% (v/v). Cultures were maintained at 37 °C in a humidified atmosphere with 5% CO_2. Since Raji-B cells grow in suspension, no trypsinization was required. Instead, the culture medium was renewed at a 1:4 ratio twice a week to maintain cell viability. The passage number did not exceed 30, after which new frozen cell stocks were thawed and used for culture continuation.

2.4 Samples

Samples of (SL)-broad spectrum chicory extract were obtained through a supercritical fluid extraction (SFE) system as reported previously [141]. To simulate colonic fermentation, the new SFE batch was incubated with an *in vitro* model of colonic microbiota prepared from healthy human donors. These processes of fermented sample collection and chemical analyses were done by our CHIC partners (H2020) from the VTT institute in Finland (Dr. Suvi Hakkinen). Human faecal samples were processed, filtered (1 mm), and suspended in triplicate bottles, each containing 200 mg of extract per 30 mL of faecal suspension. Aliquots were collected at 0, 2, 4, and 6 hours for both SFE+microbiota and microbiota control samples. The SFE was dissolved in DMSO (also used in control conditions) and diluted to a final concentration of 78 mg/mL (171.03 µL DMSO per sample). Each condition was prepared in triplicates for SFE+microbiota, and for microbiota+DMSO controls. Table 2.1 presents the SL composition of a new batch of SFE, extracted prior to the start of this study and characterized by quantification using HPLC-DAD and identification of individual SLs by LC-MS.

A



B

Chicory SFE								
Compound	11β,13-dihydrolactucin-glucoside	11β,13-dihydrolactucin	Lactucin	11β,13-dihydro-8-deoxylactucin-glucoside	8-deoxylactucin	11β,13-dihydro-8-deoxylactucin	11β,13-dihydro-lactucopicrin	Lactucopicrin
Peak ID	1	2	3	4	5		6	7
Concentration (nmol SL/mg)	4.93 (lactucin equivalents)	6.22	7.28	2.76 (lactucin equivalents)	10.62 (lactucin equivalents)		2.05	9.73

Table 2.1 **Sesquiterpene lactone (SL) composition of the chicory supercritical fluid extract (SFE).** (A) HPLC-DAD chromatogram (280 nm) of the chicory SFE. Peaks containing identified SLs are labelled. B) Amount of each identified SL per dry mass of extract, quantified through a calibration curve of a standard mixture of pure compounds. *Matos, M.S. (2024) PhD thesis, Molecular Biosciences, ITQB NOVA*

2.5 Cytotoxicity evaluation in Caco-2 cells

Cytotoxicity was assessed in confluent Caco-2 monocultures. Cells were seeded in 96-well tissue culture-treated plates at a density of 2 × 10^5 cells/mL and allowed to grow until full confluence. Once confluence was achieved, the cells were exposed to different concentrations (50, 125, 315 and 780 µg/mL) of SFE, SFE combined with microbiota, and faecal control samples. Treatments were performed in culture medium supplemented with 0.5% (v/v) FBS and 10 mM non-essential amino acids, for 48 h at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was renewed once during the incubation period.

Possible cytotoxic effects of SFE extracts on human intestinal epithelial cells (Caco-2) were evaluated based on cell viability using the PrestoBlue®. PrestoBlue is a resazurin-based membrane permeable solution, which, upon reduction, forms a red fluorescent compound called resorufin via mitochondrial enzymes of viable cells in the tested systems. Consequently, the reagent exhibits a change in colour, as well as a shift in its fluorescence. The amount of resorufin produced is directly proportional to the number of viable cells. After those 48 hours, the treated cells were incubated with PrestoBlue (5% v/v) in culture medium (0.5% v/v FBS) for 2 hours at 37ºC with 5% CO_2. After this period, fluorescence was measured (Ex./Em. 560/590 nm) in a FLx800 fluorescence microplate reader, and cell viability was determined as a percentage of control, after blank subtraction. Fluorescence filters with an excitation wavelength of 560±20 nm and an emission wavelength of 590±20 nm were used.

2.6 Caco-2:HT29-MTX-E12: Raji B triple cell co-culture preparation

In this work, a triple co-culture comprising Caco-2, HT29-MTX and Raji B cells was prepared. To closely mimic the permeability features of the human intestinal barrier, the fraction of goblet cells in the co-culture needs to be adjusted. A co-culture system consisting of Caco-2 and HT29-MTX cells was seeded onto 12 mm Transwell® inserts (polyester membrane, 0.4 µm pore size) and 24 Transwell inserts (polyester membrane, 0.4 µm pore size), in a 9:1 proportion for all inserts, respectively, and at a total density of 1.0 × 10^5 cell/cm2. After a 14-day co-culture period, Raji B cells were introduced into the basolateral compartment at a concentration of 4.0 x 10^4 cell/mL. To induce the M-cell phenotype, the triple co-culture system was maintained for an additional 7 days, with the culture medium being renewed thrice weekly to ensure cell growth and viability.

2.7 Trans-Epithelial Electrical Resistance (TEER)

Throughout the entire culture period, Transepithelial Electrical Resistance (TEER) was monitored using an EVOM voltmeter to verify the establishment and maintenance of a confluent and differentiated epithelial monolayer. Measurements were performed with the ENDOHM-12G chamber for 12-well Transwell plates and with the STX2 chopstick electrode set for 24-well plates. TEER was recorded both before the addition of Raji-B cells and prior to each experiment. Only monolayers that reached a TEER value ≥ 400 Ω·cm² after 21 days of differentiation were selected for subsequent inflammation assays, ensuring epithelial integrity and barrier functionality. Importantly, TEER was also measured immediately after each inflammatory assay to determine TEER variation and assess barrier responses to inflammatory stimuli and treatment effects.

2.8 Induction of Inflammation

Triple co-cultures in Transwell plates with 21 days post-seeding were washed with DPBS before initiating the inflammation assay. The cells were then exposed to microbiota-metabolized SFE samples and colonic microbiota samples for 48 hours on the apical side, in co-incubation with the pro-inflammatory stimulus (10 µg/mL lipopolysaccharide (LPS) from Escherichia coli O55:B5 on the apical compartment of the Transwell®, and 25 ng/mL interleukin-1β (IL-1β) along with 50 ng/mL tumor necrosis factor-α (TNF-α) on the basolateral compartment) (0.5% v/v FBS and 1% v/v NEAA). These stimuli have been described frequently in literature to mimic the intestinal inflammation [78].

BMS-345541, a selective inhibitor of NF-κB signalling, has been previously used as an anti-inflammatory reference compound to validate intestinal inflammation models. In the present study, however, the aim was to analyse the inflammatory response to physiologically relevant stimuli. For this reason, the comparison was established between non-stimulated conditions and cells exposed to pro-inflammatory mediators, without the inclusion of a pharmacological anti-inflammatory control.

2.9 Quantification of IL-8 release by ELISA

To analyse the possible anti-inflammatory effects of the chicory extracts, a 48-hour inflammation assay was performed. After 2 days, the apical and basolateral supernatants were collected, immediately snap-frozen in liquid nitrogen, and stored at -

80°C until the day of analysis. The amount of the chemokine IL-8 in cellular supernatants was assessed using the Invitrogen™ Human IL-8 ELISA Development Kit (TMB), PeproTech®, following manufacturer's instructions. Absorbance was measured at 450 and 620 nm. For the analysis, supernatants were diluted with DPBS to attain IL-8 concentrations within the standard curve (0-200 pg/mL). Data are presented as mean ± standard deviation (SD) from at least three independent biological replicates.

2.10 Determination of COX-2 protein expression by Western blot

To evaluate COX-2 protein expression, the intestinal triple co-culture was exposed to the pro-inflammatory cocktail for 12 h. Cells were lysed in RIPA buffer (Thermo Scientific, ref. 10017003) supplemented with 1% (v/v) Halt™ Phosphatase and Protease inhibitor (Thermo Scientific, ref. 78441), and protein content was quantified using the Micro BCA Protein Assay Kit (Thermo Scientific, ref. 23235). Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in TBST (Tris-buffered saline with 0.05% Tween-20) for 1 h at room temperature and then incubated overnight at 4°C with rabbit anti-COX-2 (1:1000, Abcam, clone EP1978Y), or mouse anti-β-actin (1:1000, Cell Signaling Technology, clone 8H10D10), used as a loading control. After washing, membranes were incubated for 1 h at room temperature with HRP-conjugated secondary antibodies, namely sheep anti-rabbit (1:2000, CST, ref. 7074) or horse anti-mouse (1:3000, CST, ref. 7076). Protein detection was performed using the Clarity Western ECL kit (Bio-Rad) and visualized with the iBright FL1500 imaging system (Invitrogen, Thermo Fisher Scientific). COX-2 levels were normalized to β-actin in the same membrane.

2.11 RNA isolation and cDNA preparation

The intestinal triple co-culture was exposed to the pro-inflammatory cocktail for 3 h. Following stimulation, cells were harvested, pelleted, and stored at −80°C until RNA extraction. RNA isolation was performed using the High Pure RNA Isolation Kit (Roche Diagnostics) according to the manufacturer's protocol. For cDNA synthesis, RNA samples were reverse transcribed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) following the manufacturer's instructions. cDNA was stored at −80°C until further analysis.

2.12 Evaluation of IL-1β, IL-6, NOS2 and PTGS2 gene expression levels by ΔCtquantitative real-time PCR

RT-qPCR was performed to quantify the expression of pro-inflammatory cytokines (IL1B, IL6) and inducible enzymes (NOS2/iNOS, PTGS2/COX-2). The housekeeping gene GAPDH was used as an internal reference. Amplification was conducted in a LightCycler® 480 System (Roche Diagnostics) using the SYBR Green I Master mix (Roche Diagnostics).

Normalization of gene expression levels to the GAPDH reference gene for each condition was performed, and the fold change relative to the control was calculated using the comparative cycle threshold (Ct) method (Livak, $2^{-\Delta\Delta Ct}$) [144]. This method assumes similar amplification efficiencies between target and reference genes. Data are expressed as fold change (mean ± standard deviation, SD) from at least three independent biological replicates.

2.13 Statistical analysis

All results are presented as the averages of the three independent biological replicates ± standard deviation (SD). For each independent assay, each sample was analysed in triplicates. Statistical analyses were performed using the GraphPad Prism 10.3.1 software. To ascertain significant differences between means ($\alpha<0.05$) comparisons were made using either a one-way analysis of variance (ANOVA), followed by the normality and lognormality tests for multiple comparisons, or individual student t-tests for specific and more precise individual comparisons.

3. Results and Discussion

3.1 Cytotoxicity Evaluation of SFE samples

The cytotoxicity assay was performed to identify non-cytotoxic concentrations of the chicory SFE and fermented samples for subsequent inflammation assays. Samples corresponding to SFE incubated with colonic microbiota for 0, 4, and 6 hours were tested at SFE concentrations ranging from 50 to 780 µg/mL. The 4- and 6-hour fermentation samples were chosen because they represent stages in which the main sesquiterpene lactone (SL) profile is most altered (Fig. 1.9). Microbiota control samples (microbiota with DMSO) were also tested at equivalent dry weight concentrations.

Cytotoxicity was evaluated using the Presto Blue assay in confluent Caco-2 cells. Only the second and third replicates of the extracts and microbiota control samples were analysed in this work, since one replicate of each condition, as well as the non-fermented SFE, had been assessed previously (Fig. 6.1 in annex). Each sample was tested at four SFE concentrations: 50, 125, 315, and 780 µg/mL. The results (Fig. 3.1) show that all tested samples maintained cell viability above the 70% threshold [65], indicating minimal to no cytotoxic effects, even at the highest concentration tested (780 µg/mL). No dose-response effect was observed, as increasing concentrations did not reduce cell viability. These findings are consistent with previous studies reporting the absence of cytotoxicity for chicory SFE and its microbiota combined forms (Fig. 6.1 in annex).

Based on these results, all concentrations tested can be considered non-cytotoxic and suitable for use in inflammation assays. The highest concentration (780 µg/mL) was selected for subsequent experiments, as it represents the upper safe limit and maximizes the likelihood of detecting anti-inflammatory effects without interference of toxic effects.

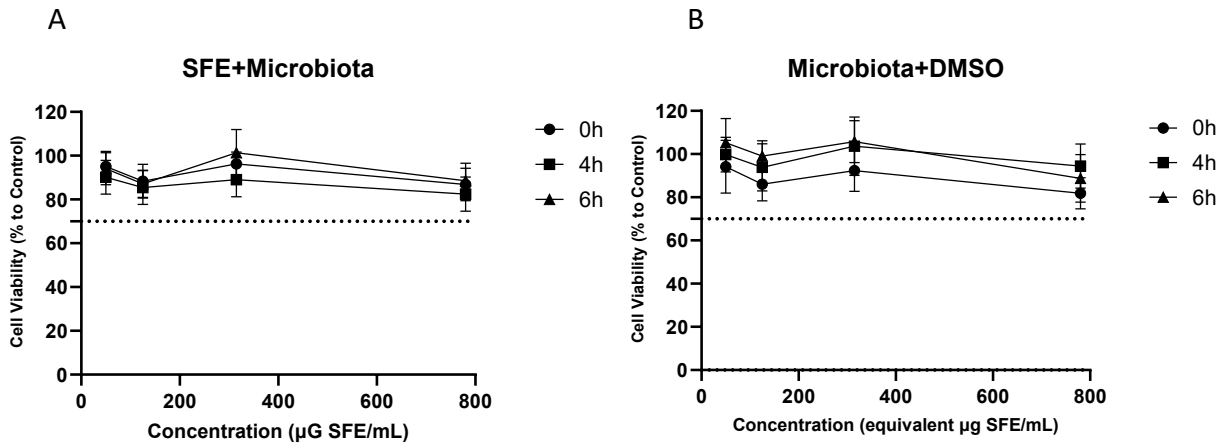


Figure 3.1. **Cytotoxicity evaluation of chicory SFE, fermented samples and microbiota controls after 0 h, 4 h, and 6 h of microbiota exposure in confluent Caco-2 cells.** (A) SFE + microbiota samples (fermented extracts) and (B) microbiota control samples (microbiota + DMSO). Results are expressed as mean ± SD from 9 replicates of 12 samples tested. Data represent cell viability relative to control for all four concentrations (50, 125, 315, and 780 µg/mL), including the respective errors. The dashed line indicates the 70% viability threshold.

3.2 TEER measurements before and after Raji-B addition

Transepithelial electrical resistance (TEER) is a well-established method to monitor the integrity of epithelial barriers, providing a quantitative measure of tight junction function [66]. By applying a small electrical current across the cell monolayer, TEER reflects the ability of tight junctions to restrict the passage of ions and macromolecules. High TEER values are therefore indicative of a mature and functional barrier, whereas low values suggest increased paracellular permeability [67]. In this work, TEER was used as a quality control parameter to confirm the health and differentiation of the co-culture both before and after the addition of Raji B cells, ensuring that the triple co-culture model was stable and suitable for subsequent inflammation assays.

The results (Fig. 3.2) show a significant increase in TEER between day 14 (prior to Raji B addition) and day 21 (before the inflammation experiments). This rise indicates that the epithelial barrier not only remained intact during this period but also continued to mature. The addition of Raji B cells to the basolateral compartment, which induces the differentiation of Caco-2 cells into M cell-like phenotypes, did not compromise barrier integrity. On the contrary, TEER values suggest that the co-culture further reinforced the tight junction network following the introduction of immune cells. These observations are relevant for two reasons. First, they confirm that the Caco-2 cells, the dominant cell

population in the model, had reached a mature enterocyte-like phenotype, characterized by the presence of strong tight junctions. Second, they validate the robustness of the triple co-culture system, meaning that, despite multicellular culture and M-cell induction, the epithelial barrier preserved its integrity [68]. This is consistent with previous studies reporting that TEER stabilizes or increases in differentiated Caco-2/HT29-MTX co-cultures over a 21-day culture period, reaching relevant resistance values [69].

A TEER value of ≥400 Ω·cm² after 21 days is generally considered adequate for intestinal *in vitro* models used in permeability and inflammation studies [24]. The fact that most cell layers in this study reached or exceeded this threshold demonstrates that the culture conditions were adequate to reproduce the intestinal mucosa, and that the model provides a reliable basis for the interpretation of subsequent assays. These findings are consistent with previous studies [21]. In summary, the observed significant increase in TEER over time highlights the successful maturation of the intestinal epithelium, validates the addition of Raji B cells as a strategy to enhance physiological relevance without compromising barrier function, and confirms that the triple co-culture system is suitable for downstream inflammatory assays.

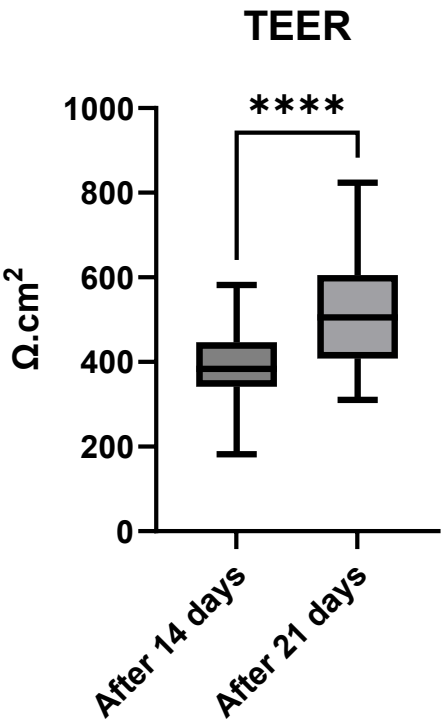
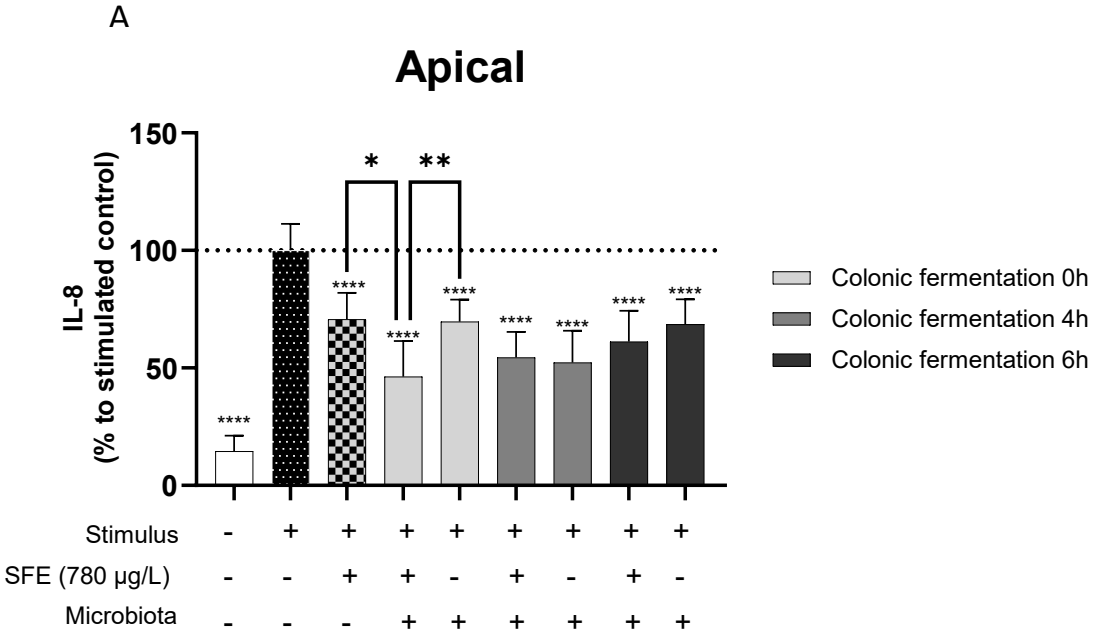


Figure 3.2**. Transepithelial electrical resistance (TEER) measurements of 12- and 24-well Transwell® inserts during the culture period.** Values are shown at day 14 (prior to the addition of Raji-B cells; median = 398 Ω·cm²) and at day 21 (prior to the inflammation assays; median = 518 Ω·cm²). Data are expressed in Ω·cm² as median with interquartile range (IQR) from all insert replicates. A significant increase in TEER was observed between day 14 and day 21 (****p < 0.0001), reflecting the maturation and stabilization of the intestinal epithelial barrier.

3.3 Modulation of IL-8 secretion by chicory SFE and microbiota fermentation in the intestinal triple co-culture model

To assess the impact of fermentation on the anti-inflammatory potential of SFE extracts, their effect on IL-8 release was quantified by ELISA at the selected concentration of 780 µg/mL, which had been previously shown to be non-cytotoxic (Fig. 3.1 & 6.1 in Annex). The assays were performed in the intestinal triple co-culture model, which combines Caco-2 absorptive enterocytes, HT29-MTX mucus-secreting cells and Raji B-induced M-like cells. This model mimics several physiological functions of the intestinal barrier, including absorption, mucus secretion, antigen sampling and barrier integrity [70]. The cells were exposed for 48 hours to an optimized pro-inflammatory cocktail consisting of LPS on the apical side, reflecting its exogenous luminal nature, and TNF-α and IL-1β on the basolateral side, mimicking endogenous cytokines in *the lamina propria*. The induction reproduced conditions of chronic intestinal inflammation, and IL-8 was chosen as biomarker given its well established role in IBD [71]. The results of the effect of the tested samples on IL-8 are shown in Figure 3.3.



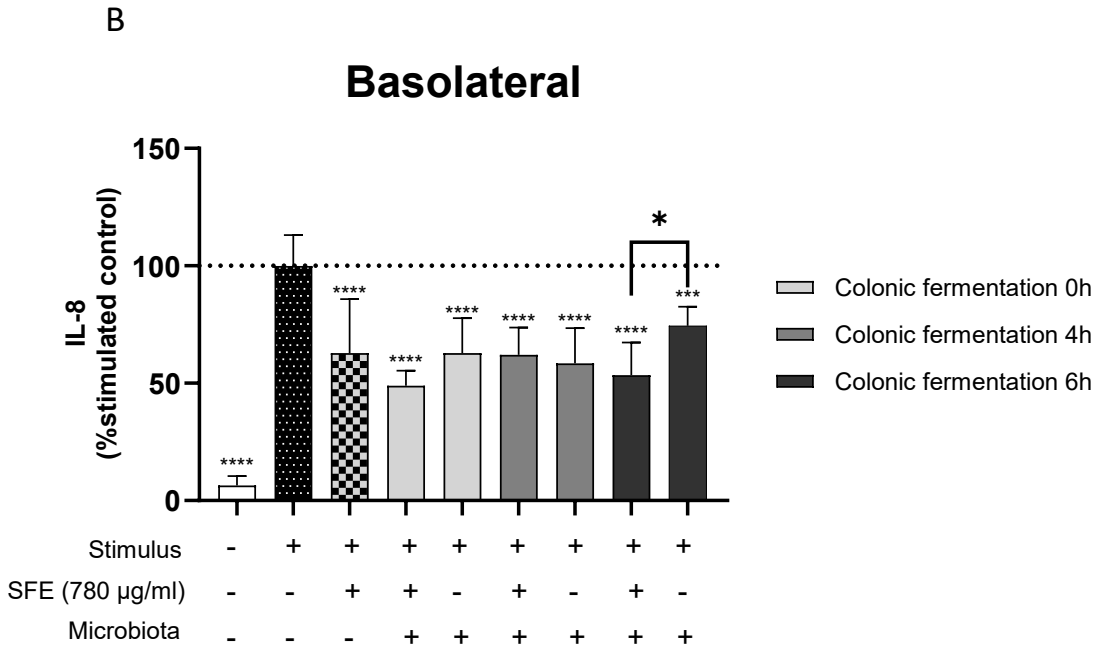


Figure 3.3**. Anti-inflammatory effect of chicory SFE and SFE after gut microbiota fermentation in the intestinal triple co-culture model following inflammation induction** (A) Apical and (B) basolateral IL-8 release were assessed by ELISA after treatment with SFE, fermented samples (0 h, 4 h, 6 h fermentation exposure), and microbiota controls, all at 780 µg/mL. Cells were co-incubated for 48 h with the pro-inflammatory cocktail (10 µg/mL LPS apically; 25 ng/mL IL-1β and 50 ng/mL TNF-α basolaterally). Results are expressed as mean ± SD from at least three independent assays. The dotted line on the y-axis indicates 100% IL-8 release in the stimulated control. *p < 0.05, **p < 0.01, ***p < 0.001, **p < 0.0001 versus stimulated control and as indicated by defined pairwise comparisons.

As expected, the non-stimulated control displayed low IL-8, while the cells submitted to a cocktail of inflammatory stimulus markedly increased IL-8 in both apical and basolateral compartments, confirming a robust inflammatory response and a good baseline for treatment comparisons. IL-8 secretion was strongly induced by the inflamed control in both compartments. On the apical side, IL-8 levels increased from 420 ± 234 pg/mL in the control to 2185 ± 550 pg/mL in the stimulated group, corresponding to a ~519% rise. On the basolateral side, levels increased from 294 ± 182 pg/mL to 2739 ± 1174 pg/mL, representing a ~932% increase, which was almost twice as high as the apical induction, which is in line with the literature [77]. This aligns with IL-8's relevant role in promoting neutrophil recruitment to systemic circulation [72], while its apical release mainly reflects epithelial signalling toward the lumen and the microbiota [77]. This relatively high variability observed for IL-8 concentrations likely reflects differences between independent Transwell inserts and experimental runs. Although all cultures were prepared under the same conditions, they originated from different plates, introducing inherent biological and technical variability typical of complex co-culture

models. To account for this, results were normalized to the stimulated control within each experiment, allowing a more reliable comparison of treatment effects across conditions.

Results show that all tested samples reduced IL-8 compared to the stimulated control, indicating that the non-fermented chicory extract (SFE), the microbiota controls, as well as the SFE combined with microbiota at different time points of exposure, exert anti-inflammatory activity. This suggests that chicory compounds, particularly SL compounds present in SFE before and after colonic fermentation, contribute to attenuate inflammatory responses [73,74], while microbiota metabolites such as short-chain fatty acids (SCFAs) may also play an important role [75]. Supporting this interpretation, gut microbes can stimulate regulatory B cells to produce IL-10 and TGF-β, which in turn reduce IL-8 release and enhance anti-inflammatory signalling [76].

On the apical side (3.3A), all samples showed efficacy in reducing IL-8 levels. The SFE extract combined with microbiota at 0h showed the greatest reduction, reaching 53% relative to the inflamed control, and was also the only condition that showed a significant difference from both the non-fermented SFE and the corresponding microbiota control ($p<0.05$). This result suggests that the native SLs present in SFE may act together with early metabolites released by the microbiota. These interactions can enhance anti-inflammatory activity, supporting the idea that combining plant-derived compounds with microbiota metabolites can rapidly strengthen epithelial protection, as reported by other authors [79-81]. The 4-hour fermented sample reduced IL-8 release by around 45% relative to the stimulated control on the apical side, while the microbiota control at this timepoint showed an even higher decrease. This suggests that microbial metabolites, probably SCFAs, may be the main drivers of the suppression of IL-8 release at this stage [82], consistent with studies reporting that SCFAs and other microbiota-derived metabolites help maintain gut immune balance and provide anti-inflammatory activity [83]. Across colonic fermentation times, there was a tendency for anti-inflammatory activity to decrease in the apical side, although not statistically significant. The 6-hour fermented extract showed the lowest reduction at 38%, which may be explained by compositional changes during fermentation. At early stages, the extract is richer in SL cysteine and glycoside conjugates (Fig. 6.3 in annex), as well as compounds such as lactucin, lactucopicrin, and 11β,13-dihydrolactucopicrin (Fig. 1.9), which may enhance IL-8 inhibition. Over time, cysteine conjugates and related derivatives decline due to microbial cleavage, releasing free SLs and cysteine into the colonic environment, while glycosides are progressively degraded and largely lost after 6 h (Fig. 6.3 in annex). These metabolic transformations may result in distinct bioactivities [84], including anti-inflammatory effect. In contrast, compounds such as 11β,13-dihydrolactucin and 11β,13-

dihydro-8-deoxylactucin accumulate over time (Fig. 1.9). Since 11β,13-dihydrolactucin is reported to exert strong anti-inflammatory effects [78], its increase over time may partially counterbalance the reduction of other SLs. Overall, all SFE extracts combined with microbiota showed efficacy in attenuating the inflammatory response at the intestinal lumen and the dynamic shifts in the SL profile likely explain the slight, though not statistically significant, differences observed in apical IL-8 inhibition across fermentation times.

Given the nature of IL-8 secretion in polarized epithelia, basolateral readouts capture systemic-facing responses to inflammatory stimulus [85]. On the basolateral side (3.3B), the microbiota combined SFE extract at 0 hours showed the strongest reduction in IL-8 release (51%) relative to the inflamed control, consistent with the apical trend (3.3A). Although it was not significantly different from the respective microbiota control, this suggests that both microbiota derived metabolites and native SFE compounds contribute to a clear reduction in IL-8, as also observed on the apical side. At 4 hours of fermentation, the extract showed the weakest effect (37% reduction to the stimulated control) and did not differ from controls, supporting the idea that this timepoint may reflect a metabolic transition in which key SL conjugates decline and microbial products may dominate the response. The 6-hour fermented extract still induced a higher reduction (43% relative to the inflamed control) and more importantly, was significantly different from its microbiota control ($p<0.05$), suggesting that fermentation-derived metabolites from SFE extract add to the anti-inflammatory effect. Although, no study has directly tested the impact of fermented chicory root extracts and relative compounds on intestinal inflammation to substantiate this effect, the additional effect at 6 hours of fermentation versus microbiota alone suggests that later-stage fermentation can generate new metabolites that modulate immune signalling at the *lamina propria*, implying a positive effect when SFE interacts with gut microbiota.

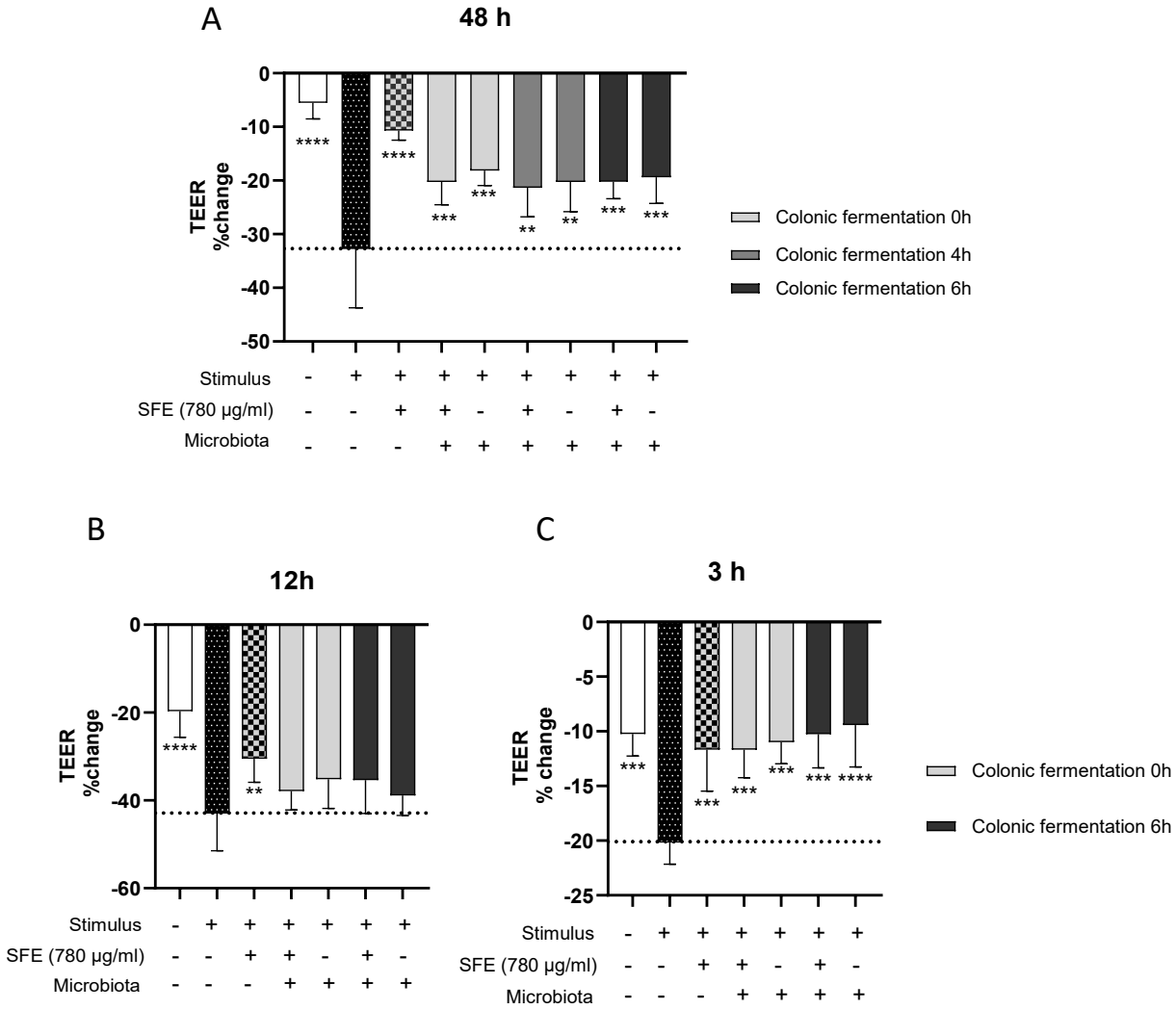
Some of the non-significant trends observed may reflect experimental variability, since assays were performed with both 12- and 24-well Transwell plates of different surface areas and in duplicates for each biological replicate. This setup increases variability and can obscure subtle differences. Even so, the consistent reduction of IL-8 is meaningful, since this pro-inflammatory cytokine plays a central role in recruiting neutrophils from the bloodstream to sites of inflammation. These findings suggest that SLs preserved in the SFE can help limit such responses (as shown in Fig. 1.5 and 1.6). Importantly, the metabolized SFE samples maintained an anti-inflammatory effect on IL-8 release, indicating that, although fermentation altered the extract's composition both quantitatively and qualitatively, these slight changes did not compromise its activity.

Instead, the combination of native SLs and microbiota derived metabolites continued to suppress IL-8 in the inflamed intestinal mucosa model. It is well established that plant-derived SLs are extensively metabolized by the colonic microbiota, meaning that once they reach the lumen, they are already structurally modified [86]. Sesquiterpene lactones (SLs) from chicory show low systemic bioavailability, with human studies indicating that only small amounts are detected in blood and urine, while a substantial fraction remains in the intestinal lumen [146]. This suggests that their biological activity occurs mainly at the intestinal level. The predominance of metabolites such as lactucin and 11β,13-dihydrolactucin in humans supports the relevance of studying SFE is consistent with the strong bioactivity observed for these compounds. At the same time, the microbiota control samples also showed an inherent anti-inflammatory effect, as reported in the literature [87,90,91]. Indeed, patients with colitis or Crohn's disease often display reduced levels of bacteria and its biodiversity in the colon [88,142,143], and dysbiosis is generally linked to inflammatory disorders [89]. Since the faecal material used here originated from healthy donors, this background activity most likely explains the anti-inflammatory effect seen in the controls, which may have partially masked SFE-specific contributions. This reinforces the need to identify and characterize fermentation-derived metabolites beyond the SLs already described, as these compounds could account for the time-dependent patterns observed in both apical and basolateral compartments.

Overall, the results confirm a robust anti-inflammatory effect of chicory SFE in combination with microbiota, consistent with previous studies showing that metabolites derived from healthy microbiota can reduce inflammation [110]. Nevertheless, a clear combined effect of SFE compounds and microbiota was observed at 0 hours on the apical side, while a distinct systemic-facing effect emerged at 6 hours of fermentation on the basolateral side. Based on these findings, the 0- and 6-hour SFE and microbiota combined samples were selected for further assays such as Western blot and RT-qPCR. In contrast, SFE combined with microbiota at 4 hours of fermentation showed no additional effect compared to each tested separately, and the microbiota control alone had a stronger impact than the fermented extract. This suggests that metabolites generated at 4 h may have lower anti-inflammatory activity, either due to the loss of compounds present at 0 h or because other bioactive metabolites only emerge after 6 h. Therefore, this 4-hour fermented extract was considered less relevant for further validation.

3.4 Effect of chicory extracts and microbiota fermentation on epithelial barrier integrity

TEER is a key parameter of epithelial physiology [24], since its reduction reflects increased paracellular permeability and disruption of tight junctions, which are hallmark features of intestinal inflammation and IBD [92,93]. In this work, TEER of the cell monolayer culture was measured at different timepoints to dynamically evaluate epithelial barrier integrity during inflammation and treatment. The timepoints were selected in a sequential manner, with 15 min used to assess NF-κB and MAPK signalling, 3 h for RT-qPCR analysis of gene expression, 12 h for inducible enzymes expression (COX-2 and iNOS), and 48 h as the main endpoint coupled to IL-8 ELISA screening. This sequential monitoring confirmed cell health both at baseline and after the assays, while also providing insight into how the epithelial barrier responded during different phases of inflammation. Monolayers showing abnormally low TEER values were excluded to ensure data reliability. The results are shown in Figure 3.4, expressed as the percentage of TEER change relative to the start of the inflammation assay across all four time points.



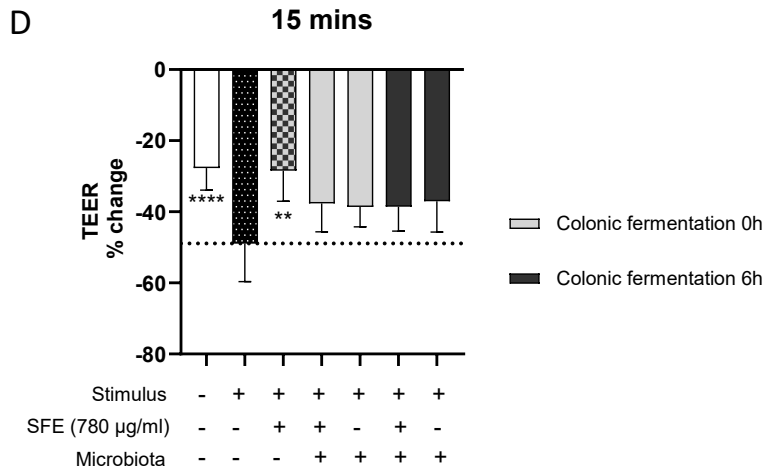


Figure 3.4. **Percentage of TEER change after exposure to the inflammatory cocktail at different timepoints**: (A) 48 h, (B) 12 h, (C) 3h, and (D) 15 mins. Treatments included chicory SFE (780 µg/mL), fermented SFE at 0 h and 6 h (780 µg/mL), and the respective microbiota controls (780 µg/mL), in co-incubation with the pro-inflammatory cocktail (10 µg/mL LPS apical; 25 ng/mL IL-1β and 50 ng/mL TNF-α basolateral). The dotted line on the y-axis depicts the stimulated control (TEER change %). Results are expressed as mean ± SD from at least three independent assays. **p < 0.01, ***p < 0.001, ****p < 0.0001 relative to the stimulated control and to the indicated pairwise comparisons.

At 15 min (3.4D), TEER decreased by 48% in the stimulated control, showing that the inflammatory cocktail induces an immediate and acute disruption of barrier function [94]. This is consistent with early cytoskeletal rearrangements triggered by LPS, TNF-α and IL-1β, which activate MLCK and RhoA–ROCK signalling, phosphorylate MLC, and contract the actomyosin ring, causing rapid disassembly and redistribution of TJ proteins such as occludin and ZO-1 [95]. However, technical factors such as short periods outside the incubator, changes in CO_2 and temperature, and the PBS wash before treatment may have also contributed to this decline. Interestingly, only the non-fermented SFE significantly improved TEER at this stage, suggesting that intact SLs in SFE may act rapidly to counteract the initial inflammatory disruption [61], whereas fermented extracts and microbiota samples showed no measurable protective effect in such an acute phase.

After 3 h (3.4C), TEER decline in the cocktail was less pronounced (−20%), reflecting a stabilization of the initial acute response. This stage coincides with the transcriptional phase of inflammation [96,97] , when NF-κB and MAPK activation drive cytokine gene expression but the downstream accumulation of effector proteins has not yet peaked [96]. All tested samples significantly preserved TEER at this point, suggesting that both intact compounds and fermentation-derived metabolites can modulate early inflammatory signalling and reduce barrier permeability. The partial recovery of TEER in the inflamed control itself also indicates a dynamic cellular adjustment, possibly linked to

temporary reorganization of claudins and oxidative stress defenses that attenuate the initial disruption [98].

At 12 h (3.4B), the inflammatory response intensified, with the stimulated control reducing TEER by about 42%. This reflects the establishment of a strong inflammatory peak characterized by high production of cytokines and inducible enzymes, oxidative stress, and extensive TJ protein alterations [99,100]. In this context, only the non-fermented SFE significantly improved TEER compared with the inflamed control. This supports the idea that intact bioactive compounds, particularly free SLs, have a stronger capacity to preserve barrier integrity and permeability [61], while fermentation products may lose these protective properties. The inability of fermented SFE and microbiota controls to significantly counteract the peak disruption suggests that once inflammation is fully established, their modulatory effect becomes limited, possibility shown by recent studies [101].

By 48 h (3.4A), the model had entered a prolonged inflammatory state, consolidating barrier loss and providing the main endpoint to evaluate treatment effects. As expected, the stimulated control reduced TEER by 33%, consistent with reports of TEER reduction under inflammatory stimulation [104] and tight junction disassembly following sustained exposure to LPS, TNF-α, and IL-1β [103]. At this stage, all tested samples significantly improved TEER relative to the inflamed control, with the non-fermented SFE showing the strongest protection (−11% reduction), almost indistinguishable from the non-stimulated control. Fermented SFE and microbiota control samples also attenuated TEER loss (−18% to −22%), but without significant differences among them, indicating no evident effect of fermentation. The microbiota controls reduced TEER, which is consistent with studies showing that microbial communities can modulate gut barrier permeability and integrity [106]. The stronger effect of the non-fermented SFE on TEER suggests that native compounds are particularly important for long-term barrier stabilization, whereas fermentation may degrade molecules relevant for tight junction preservation. Interestingly, this contrasts with the IL-8 results at 48 h (Fig. 3.3), where the non-fermented extract showed the weakest effect, indicating that IL-8 is not the only driver of barrier dysfunction and that other mechanisms also contribute to increased permeability, such as IL-6 and IL-1β expression, as discussed later.

Taken together, TEER analysis across all timepoints highlights both the dynamic progression of inflammation and the different capacities of treatments to intervene. The non-fermented SFE consistently showed the strongest barrier-protective effects, being

the only sample effective in the acute (15 min) and peak (12 h) phases, while at 3 h and 48 h, all samples were able to attenuate permeability increases. This suggests that intact bioactive compounds act rapidly and strongly, whereas fermentation products and microbiota metabolites can contribute gut barrier improvement [105], but with less potency. Importantly, studies have shown that in IBD patients, the gut microbiota differs from that of healthy individuals [107], and that dysbiosis can increase intestinal permeability and promote inflammation [108], highlighting the key role of the microbiota in regulating intestinal barrier function. Overall, these findings reinforce that SLs present in chicory byproducts can protect epithelial barrier integrity, most likely through modulation of NF-κB and MAPK signalling and stabilization of tight junctions [109]. This barrier-protective action complements the reduction of IL-8 release observed in parallel assays, reinforcing the anti-inflammatory potential of chicory SFE to preserve intestinal epithelial homeostasis in the context of colonic fermentation [111].

3.5 Protein Expression Analysis of Inflammatory Markers in Response to Chicory Extracts

One of the objectives of this work was to assess, through Western Blot analysis, the expression of iNOS and COX-2, as well as the activation of inflammatory signalling pathways NF-κB and MAPK, by determining phosphorylation ratios (phosphorolyzed/total) of p65 and p38, respectively. This approach is widely applied in intestinal inflammation models and has previously been employed in previous studies [78].

However, the performance of Western blotting proved to be particularly challenging in this project. Western blot is a demanding technique, known for its variability, and requires careful optimization and extensive practical experience. Despite the efforts undertaken, most membranes did not present clear or quantifiable bands for the targets of interest, which prevented analysis across replicates. As a result, only one assay produced usable results for COX-2 protein expression, which are presented in Figure 3.5, as percentage relative to the stimulated control. Since only one replicate was obtained for the condition, no statistical analysis could be performed. Also, it is also important to note that for the microbiota control at 6 hours, the COX-2 band appeared too faint and irregular to allow reliable quantification. For this reason, it was excluded from the graph, although the corresponding membrane image is provided for illustration purposes. Although no definitive discussion can be drawn, the observed reduction of IL-

8 release by the fermented extracts (Fig. 3.3), together with the known role of IL-8 in activating NF-κB and MAPK via modulation of the p65 and p38 subunits [136,137], suggests that these extracts may also reduce the expression of these inflammation-induced phosphorylated proteins. Moreover, previous studies have shown that chicory SFE and potential SLs are able to reduce COX-2 and iNOS (Fig. 1.8) expression and modulate inflammatory pathways such as NF-κB and MAPK p38 (Fig. 6.4 in annex). Nevertheless, the results obtained here do not allow any interpretation regarding the possible effect of fermented SFE on COX-2 expression, as reproducibility would be required for validation.

The technical challenges encountered may be explained by several factors. Firstly, the antibodies used had been stored at −20 °C for more than two years, making partial degradation a likely issue. Secondly, Western blotting involves several critical steps, from protein electrophoresis to membrane transfer and correct molecular weight cutting, where human error may have influenced the outcome, particularly given the demanding nature of the assay. In addition, the technique itself is known for its low reproducibility, further increasing the probability of failure.

Although the intended goal was not achieved, this work documents the experimental effort undertaken and highlights the main technical constraints encountered. The limited results obtained should not be interpreted as evidence against the biological activity of the extracts but rather as a reflection of methodological limitations. Despite these difficulties, the assay provided valuable practical training and contributed to the development of technical skills that will facilitate future improvements. For subsequent work, the use of fresh and validated antibodies, the performance of additional biological replicates, and the optimization of critical steps of the procedure are recommended. It would be important to repeat Western blot assays to further assess the phosphorylation of p65 and p38 subunits of the NF-κB and MAPK pathways, respectively, as well as the expression of the inducible enzyme iNOS, to gain a deeper understanding of how chicory SFE and its colonic fermentation influence these mechanisms relevant to IBD. However, due to limited time and sample availability, further repetitions could not be performed in this project and the work had to proceed with subsequent assays. Alternatively, complementary approaches for assessing protein expression and signalling pathways, such as ELISA, immunohistochemistry, or flow cytometry could be explored to overcome some of the limitations associated with Western blotting.

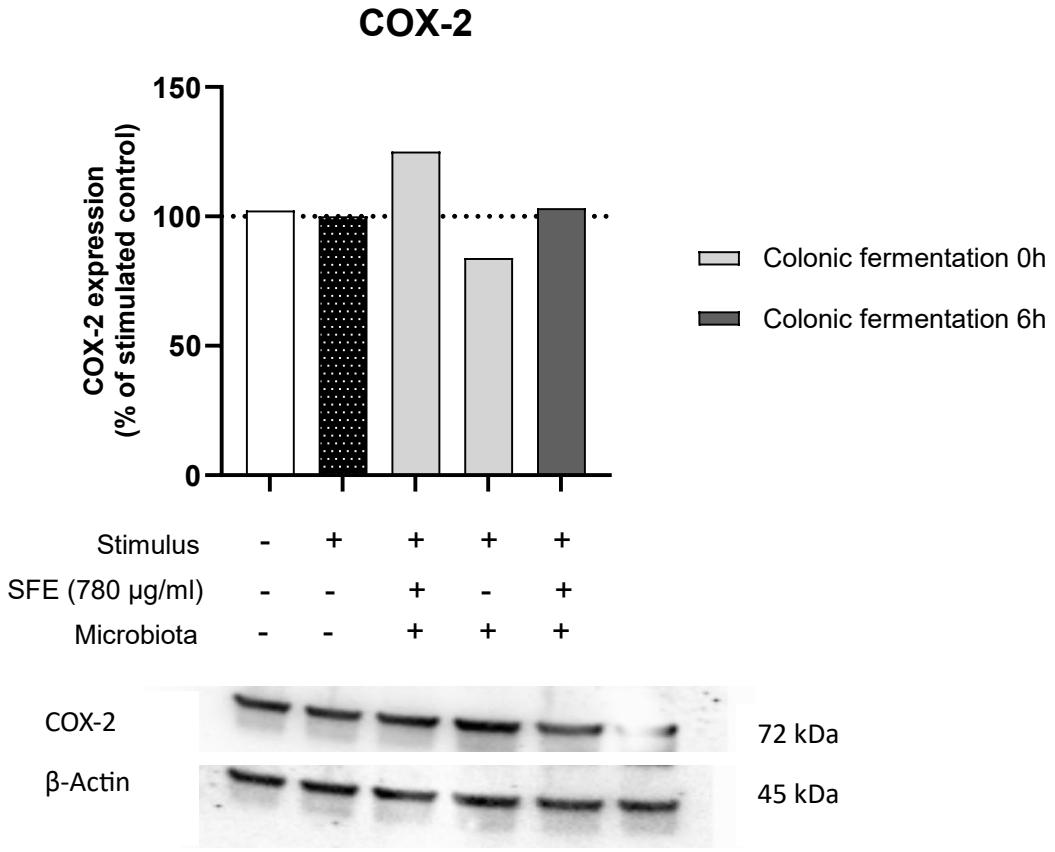


Figure 3.5. **COX-2 protein expression after 12 hours of inflammation induction in the intestinal triple co-culture.** Protein levels were assessed by Western blot following treatment with chicory SFE (780 µg/mL), fermented SFE at 0 h and 6 h of fermentation (780 µg/mL), and the microbiota control at 6 h (780 µg/mL), in co-incubation with the pro-inflammatory stimulus (10 µg/mL LPS on the apical side; 25 ng/mL IL-1β and 50 ng/mL TNF-α on the basolateral side). Protein expression is shown as percentage relative to the stimulated control (cocktail = 100%, indicated by the dotted line). Data were obtained from a single membrane (n = 1 per condition). The microbiota 6 h β-actin band was too weak for reliable quantification and was excluded. Protein bands are shown below the graph. No statistical analysis was performed due to n = 1.

3.6 Effect of Chicory Extracts on the Expression of Key Inflammatory Genes

After a 3-hour inflammatory induction, RT-qPCR was performed to assess the effects of chicory SFE and its fermented samples on the expression of key inflammatory genes. Four targets were selected: NOS2 and PTGS2, which encode the inducible enzymes iNOS and COX-2, as proximal effectors of the inflammatory cascade, and IL-6 and IL-1β, two cytokines central to the initiation and propagation of intestinal inflammation [112]. Fold-changes relative to the non-stimulated control were calculated

for each condition and results are presented in Figure 3.6. As expected, the inflamed control activated all four targets compared with the control, confirming the responsiveness of the model and providing a solid basis for evaluating treatment effects.

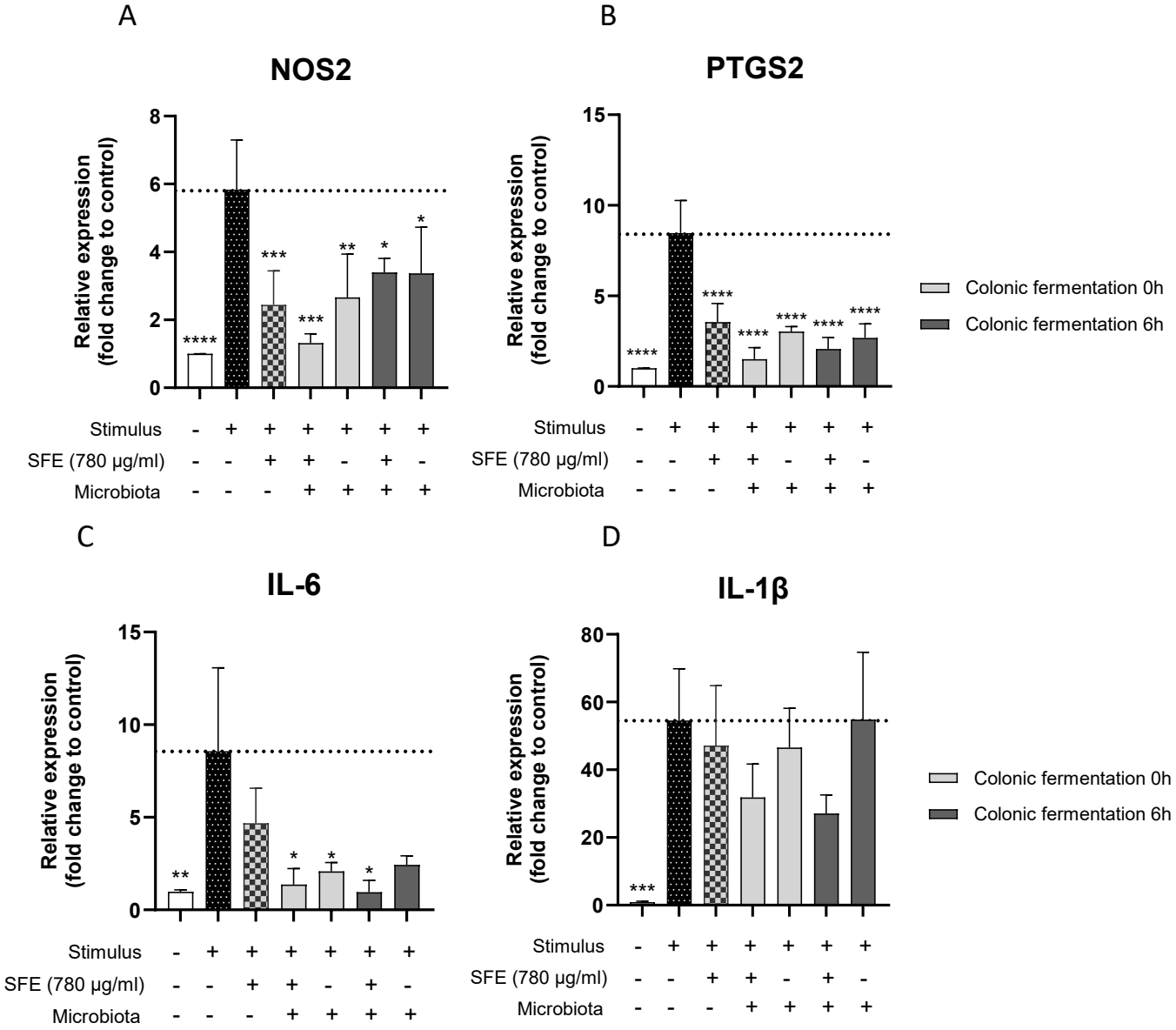


Figure 3.6. **Gene expression analysis of inflammatory markers in intestinal triple co-culture.** Gene expression levels of NOS (A), PTGS2 (B), IL-6 (C), and IL-1β (D) were assessed by qRT-PCR after 3 h of treatment with chicory SFE (780 µg/mL), fermented SFE extracts at 0 h and 6 h (780 µg/mL), and their respective microbiota controls, in co-incubation with the pro-inflammatory stimulus (10 µg/mL LPS on the apical side; 25 ng/mL IL-1β and 50 ng/mL TNF-α on the basolateral side). The dotted line in the y-axis represents the expression level of the stimulated control. Results are expressed as mean ± SD from at least three independent assays. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 relative to the stimulated control.

The inducible enzymes showed the clearest patterns of modulation. PTGS2 expression (3.6B) was strongly reduced by all treatments compared with the stimulated control, making it the most consistently affected target. COX-2, encoded by PTGS2, drives prostaglandin E2 (PGE2) synthesis from arachidonic acid, amplifying inflammatory signalling [113]. The lowest fold-change was observed for the 0-hour colonic combination with SFE, although pairwise differences among treatments were not significant. This suggests that native SFE compounds, and those still preserved during fermentation, retain high potency against this pathway [61,78]. Microbiota-derived metabolites also lowered PTGS2 expression, but without synergism when combined with SFE. These results are consistent with recent studies showing that SLs and SL-rich plants can decrease PGE2 levels by reducing COX-2 expression [114,116], and that microbiota metabolites can also regulate this activity [115]. NOS2 followed a similar pattern [3.6A). All samples significantly reduced expression relative to the stimulated control, with the microbiota combined SFE at 0 hours again showing the highest reduction. The 6-hour fermented extract retained activity but was less effective than the 0 hour combined sample and non-fermented SFE. NOS2 encodes iNOS, which produces large amounts of nitric oxide (NO) during inflammation, contributing to oxidative stress and epithelial injury [117]. Considering that microbiota metabolites also strongly affect NOS2 expression, as shown in literature [119], this explains why differences among treatments were not significant. Nevertheless, the stronger suppression by intact SFE suggests that native compounds (Fig. 1.9) may be especially efficient in limiting NO-mediated stress, as reported in previous studies [61,78,118]. This interpretation aligns with the 3-hour TEER data (Fig. 3.4), where SFE consistently showed protective effects. It is also consistent with evidence that PTGS2 and NOS2 are often co-regulated, reinforcing each other's expression [120], driven by cytokines such as IFN-γ and TNF-α (the latter included in the cocktail), reinforcing the importance of their suppression [121]. Supporting this, Costunolide, a sesquiterpene lactone known to be present in chicory though not abundant in the SFE [125] inhibited the production of TNF-α and IL-6 [122], two cytokines that regulate COX-2 and PGE2 expression [123,124]. Together, these studies provide a broader context for interpreting the results observed here.

Cytokine expression showed greater variability. For IL-6 (3.6C), significant reduction in gene expressions compared to the inflamed control was observed only for the 0- and 6-hour microbiota combined SFE samples and for the 0-hour microbiota control. In contrast, the non-fermented SFE and the 6-hour microbiota control did not reach significance, although fold-changes were lower than the stimulated control. Notably, fermented SFE consistently showed a more evident effect than the

corresponding microbiota controls, suggesting that fermentation may generate or preserve metabolites with the ability to suppress IL-6 transcription, especially at later stages. This observation is consistent with previous findings (Fig. 1.7), where SFE significantly reduced IL-6 expression, likely due to the presence of SLs already described as anti-inflammatory in the context of IL-6. Among them, 11β,13-dihydrolactucin has been shown to exert the strongest anti-inflammatory effect [61,78] and its levels increase quantitatively after 6 hours of colonic fermentation (Fig. 1.7). As mentioned before, costunolide has been shown to modulate IL-6 [122], but its role in this study is likely limited due to its low abundance in SFE (not detected in Fig. 2.1). Since IL-6 promotes intestinal permeability through claudin-2 upregulation [126], these results may also help explain the TEER improvements observed after 3 hours with fermented extracts. For IL-1β (3.6D), none of the treatments produced significant reductions compared with the cocktail. However, both microbiota combined SFE at 0 hours and fermented at 6 hours samples showed a trend towards lower expression, with the 6-hour fermented extract presenting the lowest fold-change. IL-1β is a key mediator of inflammation, produced from an inactive precursor by caspase-1 cleavage, and responds strongly to pathogens, tissue damage, and inflammatory stimuli [127]. It is a primary driver of acute inflammatory responses, often maintained by feed-forward loops, which increases variability and makes modest treatment effects more difficult to detect. IL-1β activates a cascade of intracellular signalling events, inducing the expression of various cytokines, such as IL-6 [134,135]. This is in line with previous studies showing that 11β,13-dihydrolactucin [78] and SFE itself did not significantly reduce IL-1β expression (Fig. 1.7), suggesting that SLs present in SFE and their colonic metabolites have minimal or no effects on this cytokine. Still, IL-1β is known to interact with IL-6. While both act synergistically to drive acute-phase protein production, studies on hepatic acute inflammation showed that IL-1β can antagonize certain IL-6 responses through NF-κB–STAT3 competition [128]. This dynamic may help explain why IL-6 suppression was more evident than IL-1β in this work. In addition, IL-1β is an upstream pro-inflammatory cytokine whose regulation depends not only on gene transcription but also on inflammasome assembly [148] and caspase-1 activation [147]. Since the SLs present in the SFE mainly modulate NF-κB and MAPK pathways, which are more directly involved in IL-6, iNOS and COX-2 regulation, their impact on IL-1β expression is likely limited in this model. In addition, IL-1β activity is largely controlled at the post-translational level through inflammasome activation, meaning that mRNA levels do not necessarily reflect its biological activity. Therefore, future studies could also include anti-inflammatory markers such as IL-10 or TGF-β to better assess immune regulation and tissue repair mechanisms [149].

Taken together, the most consistent effects were observed on PTGS2 and NOS2, which parallel the TEER protection and IL-8 reduction described elsewhere in this study (Fig. 3.3). Cytokine modulation was weaker and more variable, with clearer effects on IL-6 than IL-1β. These data suggest that chicory root SFE, particularly in its early fermented states, more effectively suppresses downstream effector enzymes than upstream cytokines. Despite the loss of some native SLs during fermentation (Fig. 6.3), the combination of key bioactive SLs (Fig. 1.9) with microbiota-derived metabolites was able to preserve the overall anti-inflammatory response. Interestingly, a study on chicory extract fermented by *Akkermansia muciniphila* in obesity-related inflammation [129] reported reduced IL-6 and IL-1β expression together with a decrease in reactive oxygen species (ROS), which are known to activate inflammatory pathways such as NF-κB [130]. This provides relevant perspectives for interpreting the metabolization of SFE by gut microbiota, since this extract is rich in SLs and microbial metabolism is known to cause compositional changes (Fig. 1.9) [131]. From a mucosal perspective, it would also be important to further examine MUC2 and MUC5AC gene expression, as microbiota interactions could reveal additive or synergistic effects on mucus barrier reinforcement, even though previous work suggested little effect of non-fermented SFE on these genes (Fig. 6.5 in Annex).

Several limitations should be acknowledged. The number of biological replicates was modest (n = 3 for test samples; n = 6 for control, inflamed control, and non-fermented SFE), which reduces the power to detect treatment differences and limits firm conclusions, especially for variable cytokines. Overall, the qPCR results reinforce that chicory SFE can attenuate key inflammatory targets, with the clearest suppression seen for PTGS2 and NOS2. Supporting this, another study on chicory leaf extract showed strong effects in modulating MAPK/NF-κB and reducing IL-6, IL-1β, TNF-α, NO/iNOS and COX-2 expression. Although these findings come from leaf extracts, their compounds are very similar to those found in roots, such as lactucin, lactucopicrin and 8-deoxylactucin [132,133]. In summary, these results support that intact chicory root extracts can modulate cytokine responses and inhibit effector enzyme induction, while fermentation modifies but does not abolish these effects. On the contrary, metabolites generated during colonic fermentation, including those derived from SLs, may further contribute to or even enhance the anti-inflammatory activity, as suggested by gene expression patterns. This highlights the potential of SLs from chicory to manage intestinal inflammation and support epithelial homeostasis.

4. Conclusions

The aim of this work was to evaluate the anti-inflammatory potential of chicory SFE and its fermented metabolites using an *in vitro* triple co-culture model of intestinal epithelial inflammation. The impact of a broad SL-enriched extract after colonic fermentation was analysed through its effects on signalling pathways, cytokine release, and inducible enzyme expression. The optimized epithelial model proved to be robust, as shown by the strong induction caused by the pro-inflammatory cocktail. Clear differences between apical and basolateral compartments highlighted the importance of evaluating both sides of the monolayer when testing potential modulators. Still, this model has limitations, since it lacks immune cells and only allows the assessment of cytokine, inducible enzyme, and mucin responses within the epithelial context of intestinal inflammation.

All tested extracts were non-cytotoxic up to 780 µg/mL. Both native and SFE combined with microbiota reduced IL-8 release on the apical and basolateral sides, confirming their role in modulating epithelial barrier responses. Native SLs from SFE and early microbial products had a significant impact on preserving barrier integrity, while later metabolites appeared to influence neutrophil recruitment more prominently. TEER protection reinforced the role of chicory extracts in stabilizing the epithelial barrier and maintaining tight junctions. Notably, when microbiota was combined with SFE native SLs at 0 hours or with early and late fermentation-derived metabolites, the effects slightly differed from each other and from microbiota alone. This suggests that, beyond SCFAs and native SLs, microbial conversion of SLs in the colonic lumen generates additional bioactive compounds that contribute to epithelial homeostasis. At the gene level, PTGS2 and NOS2 were consistently reduced by both non-fermented and fermented SFE. IL-6 modulation was more variable, but microbiota combined SFE extracts at 0- and 6-hours showed significant activity. In contrast, IL-1β expression was not significantly reduced. Together, these results suggest that chicory root SLs and their metabolites act downstream of cytokine signalling, mainly by targeting effector enzymes that drive barrier disruption in the intestinal epithelium. However, the absence of conclusive protein-level data from Western blot analysis and the limited number of replicates in RT-qPCR reduce statistical power and restrict firm conclusions. In addition, microbiota control samples showed strong anti-inflammatory activity, likely reflecting the use of faecal donors from healthy individuals, which may have partially masked the specific contribution of the SFE in the context of IBD, due to the inherent bioactivity of the microbiota.

Marker	SFE	SFE+M 0h	SFE+M 4h	SFE+M 6h
TEER loss	↓	↓	↓	↓
IL-8 secretion	↓	↓	↓	↓
Protein expression	-	-	-	-
PTGS2 gene expression	↓	↓	-	↓
NOS2 gene expression	↓	↓	-	↓
IL-6 gene expression	↔	↓	-	↓
IL-1β gene expression	↔	↔	-	↔

Table 4.1 **Summary of the effects of non-fermented SFE and SFE at 0h, 4h, and 6h combination with microbiota on intestinal inflammation markers in the triple co-culture model.** Results are shown for TEER change after 48 hours, IL-8 secretion, protein expression (Western blot, non-conclusive), and gene expression (IL-6, IL-1β, PTGS2, and NOS2). Downward arrows indicate significant reductions compared to the stimulated control, horizontal arrows indicate no significant changes, and dashes represent inconclusive or untested results.

4.1 Future Perspectives

Future work should include an increased number of technical and biological replicates to improve statistical robustness and account for variability between biological samples and differences in SFE extract composition. Further optimization of protein expression analyses, particularly Western blot assays, is also needed to overcome limitations related to low protein yield in Transwell-based co-culture models. For medium term future studies, it would be advisable to use microbiota from patients with inflammatory phenotypes such as IBD, to provide a more representative gut environment. Moreover, identifying and testing individually specific metabolites produced during SFE fermentation (e.g., by LC-MS/MS) would help clarify which compounds contribute most to the anti-inflammatory effects beyond the known native SLs. In addition to evaluating NF-κB p65, MAPK p38, and iNOS protein expression, it would also be relevant to analyse MUC2 and MUC5AC genes to better understand mucin production in the context of intestinal inflammation. Using other cell models that include immune

cells would be important to assess how SFE influences the interaction between the intestinal epithelium and the immune response. Finally, in the longer term, if the bioactivity of SFE remains consistent, in vivo studies of intestinal inflammation would be an important next step to evaluate its physiological relevance.

In conclusion, the results of this work support that intact SLs present SFE, such as 11β,13-dihydrolactucin, together with fermentation derived metabolites, act in complementary ways to preserve epithelial integrity and regulate inflammatory signalling pathways. This study showed that SFE [61,78] exert differential effects during the inflammatory response, a variability that may prove advantageous for long-term applications, supporting its potential use as dietary supplement for IBD management. Overall, SFE combined with colonic microbiota, was able to reduce IL-8 release, suppress inducible enzymes, and protect epithelial barrier integrity. This work emphasizes the therapeutic potential of chicory root byproducts as a sustainable source of bioactive compounds for intestinal health, while highlighting the crucial role of microbiota in shaping their anti-inflammatory activity.

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6. Annexes

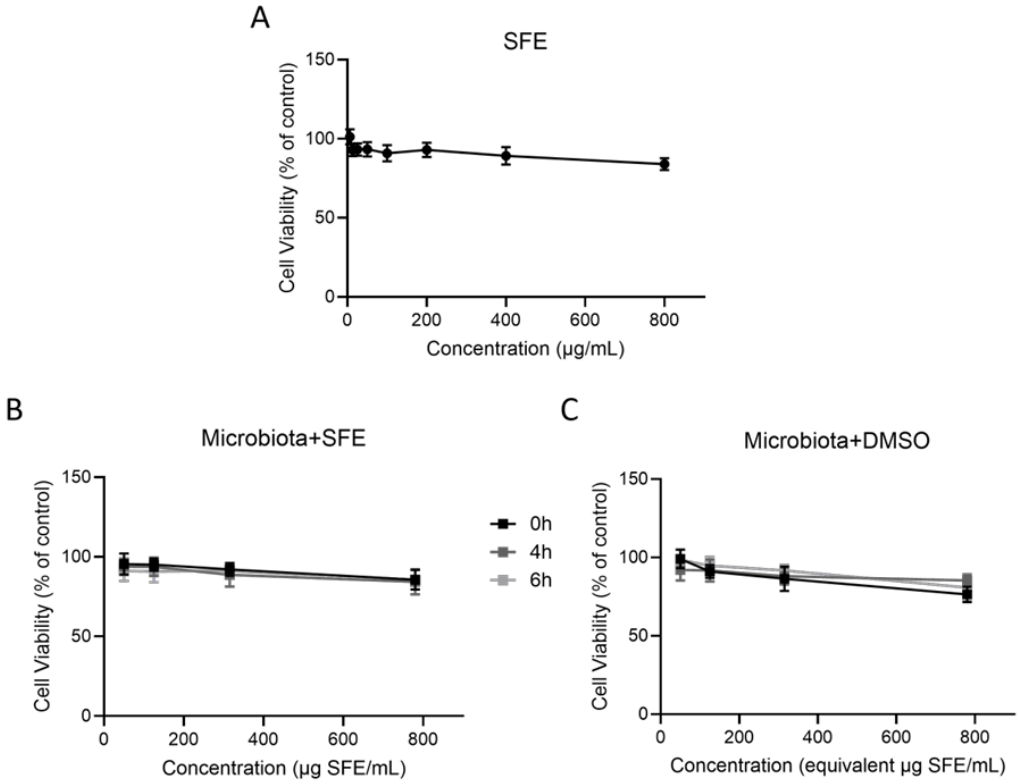

Figure 6.1. **Cytotoxicity testing in confluent Caco-2 cells.** The supercritical fluid extract (SFE) from chicory was tested across a concentration range of 0–800 µg/mL (A), the metabolized SFE after colonic microbiota biotransformation was assessed (B), and colonic microbiota controls in equivalent amounts to those tested for the SFE were also evaluated (C). Results are expressed as mean ± SD from at least three independent assays from the 1st biological replicate, with 0 h corresponding to the start of incubation with the colonic microbiota. *Matos, M.S. (2024) PhD thesis, Molecular Biosciences, ITQB NOVA*

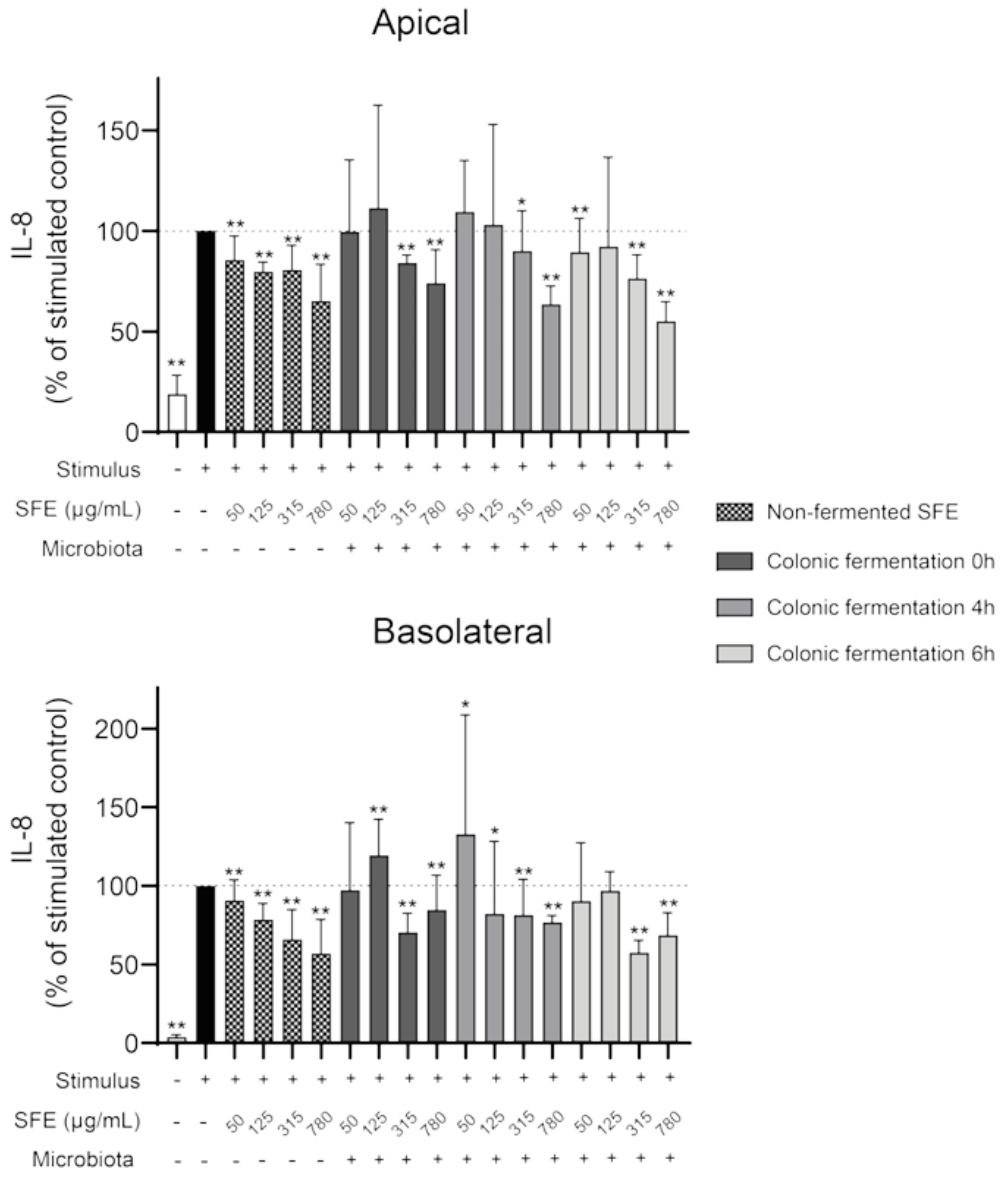

Figure 6.2 **Anti-inflammatory potential of chicory SFE before and after fermentation by the gut microbiota**. IL-8 release was quantified by ELISA in both apical and basolateral supernatants after treatment with different concentrations of SFE, either non-fermented or fermented for 0, 4, and 6 h, in co-incubation with the pro-inflammatory cocktail (10 µg/mL LPS apically; 25 ng/mL IL-1β and 50 ng/mL TNF-α basolaterally). The 0 h condition represents the start of incubation with colonic microbiota. The dotted line on the y-axis indicates 100% IL-8 release in the stimulated control. Results are presented as mean ± standard deviation (SD) from at least three independent assays. *p < 0.05, **p < 0.01 versus stimulated control. *Matos, M.S. (2024) PhD thesis, Molecular Biosciences, ITQB NOVA*

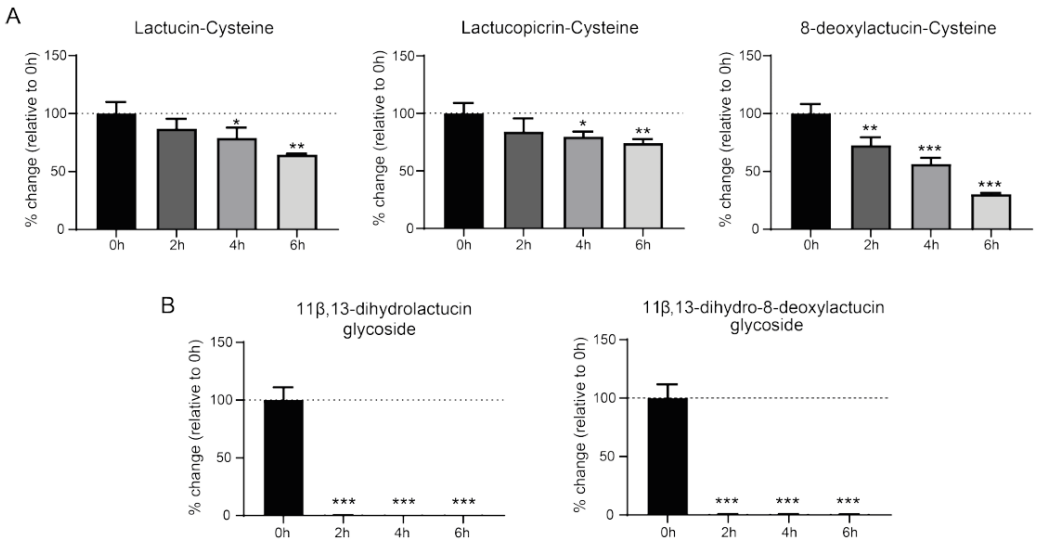

Figure 6.3 **Sesquiterpene lactone (SL) composition of the chicory supercritical fluid extract (SFE) after microbiota metabolization over a 6-hour period.** Changes are shown for (A) cysteine-SL conjugates of lactucin, lactucopicrin, and 8-deoxylactucin; and (B) SL-glycosides (B) 11β,13-dihydrolactucin glycoside ; and 11β,13-dihydro-8-deoxylactucin glycoside. Results are expressed as mean percentage ± standard deviation (SD) relative to the initial timepoint (t = 0 h). Statistical significance is indicated as *p < 0.05, **p < 0.01, ***p < 0.001. The 0 h timepoint corresponds to the start of incubation with colonic microbiota. *Matos, M.S. (2024) PhD thesis, Molecular Biosciences, ITQB NOVA*

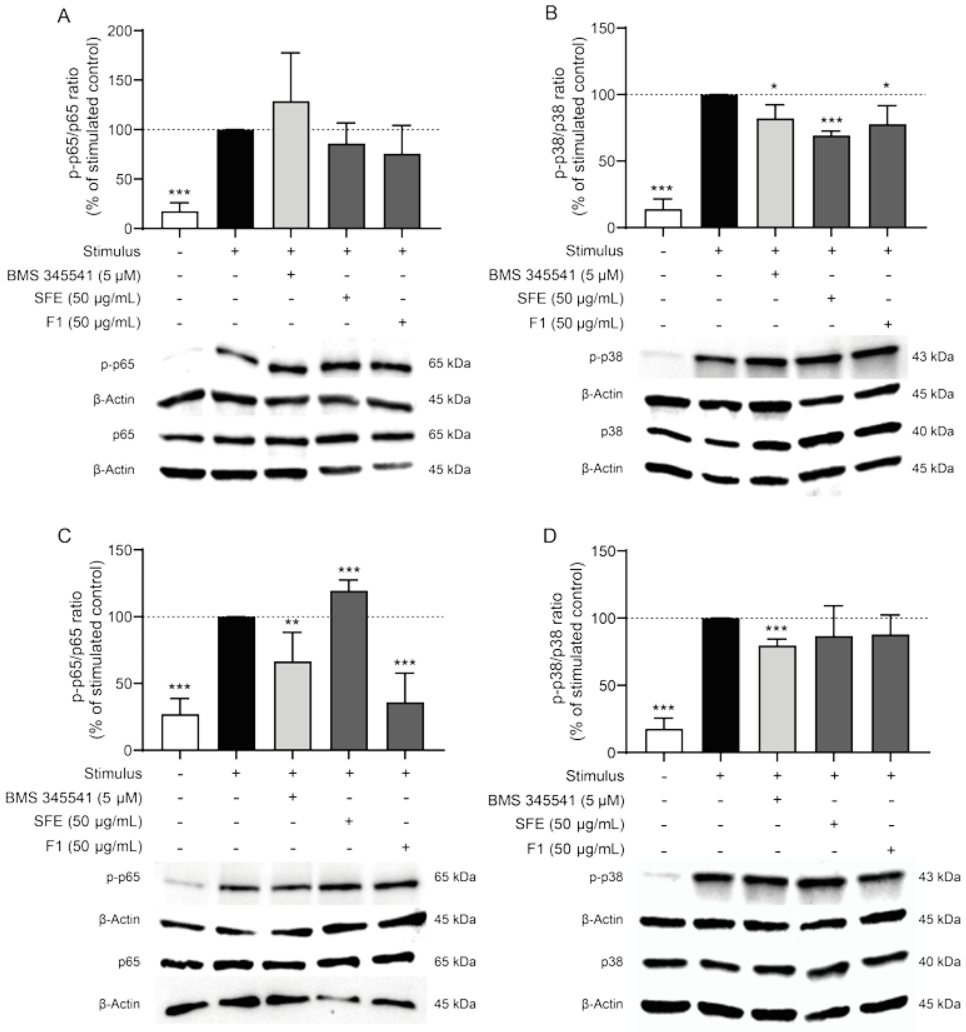

Figure 6.4 **Effect of SFE and F1 on NF-κB and MAPK signaling in the intestinal triple co-culture**. Phosphorylation ratios of NF-κB p65 (A, C) and MAPK p38 (B, D) were determined by Western blot. In (A, B), cells were co-incubated for 15 min with the pro-inflammatory stimulus (10 µg/mL LPS apical; 25 ng/mL IL-1β and 50 ng/mL TNF-α basolateral) in the presence of BMS-345541 (5 µM), SFE (50 µg/mL), or F1 (50 µg/mL). In (C, D), cells were pre-treated with BMS-345541, SFE, or F1 for 4 h prior to a 15 min exposure to the stimulus. The dotted line indicates the 100% phosphorylation ratio of the stimulated control. Data are presented as mean ± SD of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 vs. stimulated control. *Matos, M.S. (2024) PhD thesis, Molecular Biosciences, ITQB NOVA*

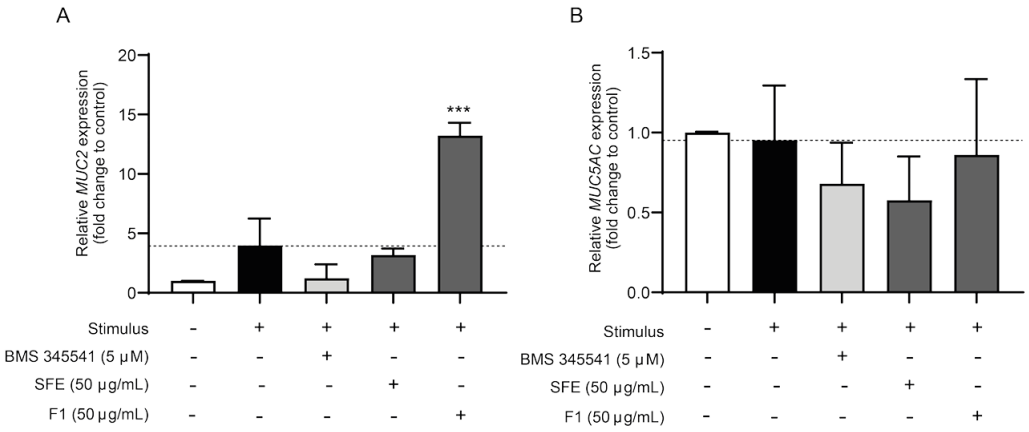

Figure 6.5 **Impact of the SFE on the gene expression of mucins MUC2 and MUC5AC in the intestinal triple co-culture**. Gene expression levels of (A) MUC2 and (B) MUC5AC were assessed by qRT-PCR after 3 h treatment with BMS 345541 (5 µM), SFE (50 µg/mL), or Fraction 1 (F1, 50 µg/mL; mainly composed of 8-deoxylactucin and 11β,13-dihydro-8-deoxylactucin, with 11β,13-dihydrolactucopicrin also detected), in co-incubation with the pro-inflammatory stimulus. The dotted line on the y-axis indicates the expression level in the stimulated control. Results are expressed as mean ± SD from at least three independent assays. ***p < 0.001 vs. stimulated control. *Matos, M.S. (2024) PhD thesis, Molecular Biosciences, ITQB NOVA*