Intestinal epithelial co-culture sensitivity to pro-inflammatory stimuli and polyphenols is medium independent

[*Sensibilidad del co-cultivo epitelial intestinal a estímulos pro-inflamatorios y el efecto de polifenoles independientes del medio*]

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List Of Abbreviations

Abreviation	*Term*
ATCC	American Type Culture Collection
cDNA	Complementary Deoxyribonucleic Acid
CASP3	CASPASE-3
CASP9	CASPASE-9
CAT	Catalase
CD	Crohn disease
COX	Cyclooxygenase
CXCL-8	C-X-C Motif Chemokine Ligand 8
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
ECACC	The European Collection Of Authenticated Cell Cultures
E. coli	*Escherichia Coli*
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
GADPH	Glyceraldehyde-3-phosphate Dehydrogenase
GBD	Global Burden Of Disease
GSH	Glutathione Peroxidase
HD	High Density
HEPES	(4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid)
IBD	Inflammatory Bowel Diseases
IDEALISS	Interactions Of The Environment And Of Diet On Animal And Human Health
IFN-γ	Interferon-gamma
IL-1β	Interleukin-1 Beta
IL-8	Interleukin-8
iNOS	Inducible Nitric Oxide Synthase
LPS	Lipopolysaccharide
NADPH	Oxidase-nicotinamide Adenine Dinucleotide Phosphate Oxidase
NF-κB	Nuclear Factor Kappa B
PABA	Parabenzoic Acid
PBS	Phosphate-buffered Saline
PPIA	Peptidylprolyl Isomerase A
PRRS	Pattern Recognition Receptors
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPMI 1640	Roswell Park Memorial Institute Medium
SEM	Standard Error Of The Mean
SOD	Superoxide Dismutase

TEER	Transepithelial Electrical Resistance
TJ	Tight Junctions
TLRs	Toll-like Receptors
TLR4	Toll-like Receptor 4
TNF-α	Tumor Necrosis Factor-alpha
UC	Ulcerative Colitis

Glossary

Apical medium: medium above the cell culture that represents the intestinal lumen.
Apoptotic pathways: biochemical pathways leading to cell death (Elmore, 2007).
Basal medium: medium below the cell culture that represents the blood circulation.
Catechin: natural phenolic antioxidant, belonging to the flavonoid family, more specifically Flavan-3-ols (Isemura, 2019).
Crohn disease: is a type of inflammatory bowel diseases**,** in can cause inflammation in all the digestive tract.
Co-culture: cultivation of 2 or more different cell types together in the same environment, allowing the study of these interactions (Bonati & Tang, 2021).
Cytokine: signaling proteins produced by immune cells and key modulators of immune responses (Bonati & Tang, 2021).
Cytokine cocktail: mixture of various cytokines. Is used in research stimulate or modulate immune responses in cell cultures.
Dysbiosis: imbalance in the bacterial composition, changes of the metabolic activities or changes in the distribution of the bacteria's in the gut (Degruttola et al., 2016).
Enterocytes: main cell type in the intestinal epithelium.
Flavan-3-ols: is a type of flavonoid.
Free radicals: any molecular species that contains an unpaired electron in an atomic orbital, resulting in instability and having the power to generate cellular damage(Lobo et al., 2010).
Immunomodulatory effect: The ability of a substance to modify or regulate one or more functions of the immune system
Immunonutrition: is an emerging, multidisciplinary field that studies the interactions between nutrition, the immune system, infection, inflammation and tissue damage (Zapatera et al., 2015).
Incidence: the number of new cases per year.
Inflammatory bowel disease: chronic inflammatory condition of the gastrointestinal tract, the main two types are chron disease and ulcerative colitis.
Intestinal epithelium: single cell layer that works as intestinal barrier.
***In vitro*:** experiments conducted outside a living organism, commonly in a controlled laboratory environment.
***In vivo**:* experiments conducted within a living organism.
Lysis: rupture or destruction.
Microbiota: group of microorganisms like bacteria, fungi, viruses and *archae* that co-exist in the same space such as the gut.
Reactive oxygen species (ROS): reactive molecules and free radicals from molecular oxygen (Bardaweel et al., 2018).
Polyphenols: organic compound characterized by their multiple phenol group, they are classified in different classes depending on their chemical structure, the two main classes are flavonoids and phenolic acids.
Pathogenesis: process by which a disease or disorder develops. It can include factors which contribute not only to the onset of the disease or disorder, but also to its progression and maintenance source of a disease.

Prevalence: portion of the population that has a certain disease at a certain time.
Pathology: scientific study of a disease with their causes, processes and effects.
Transwell®: permeable supports with inserts that provide independent access to both sides of the monolayer *in vitro* (Transwell_Instruction Manual, n.d.).
Ulcerative colitis: chronic inflammatory bowel disease that causes inflammation and ulcers.

Abstract

The use of *in vitro* models to evaluate nutritional components provides a controlled environment, allowing to evaluate the effect of these nutrients and the level of their cellular absorption, metabolism and fate in cells. The complexification of *in vitro* models requires compatibility of cells with the same medium. Since immune cells are the most sensitive to growth conditions, the necessity to grow intestinal epithelial cells in their usual medium seems to be necessary. This work was aimed at comparing the sensitivity of these epithelial cells to pro-inflammatory stimuli (LPS from *E. coli* and a cocktail of pro-inflammatory cytokines) but also to dietary polyphenols, specifically catechins in both DMEM (regular epithelial cells medium) and RPMI-1640 (immune cells preferred medium) media. Co-cultures of Caco-2 & HT29-MTX cells were grown 21 days in the two media before their stimulation with a cocktail of TNF-α (20 ng/ml), IL-1β (1 ng/ml) and IFN-γ (10 ng/ml) or with LPS (10 ng/ml) from *E. coli* (O111:B4). The role of catechins (15 µM), a dietary polyphenol, was evaluated after its incubation with the cells before their stimulation for 6h. The RPMI-1640 medium did not alter the intensity of the inflammatory response observed with the cytokines. By contrast, LPS failed to stimulate the co-culture in inserts whichever medium used. At last, catechins were unable to prevent the pro-inflammatory response observed with the cytokines in the two media. Preservation of the response of this model of intestinal epithelium in RPMI-1640 medium is promising when considering its complexification to evaluate the complex cellular crosstalk leading to intestinal homeostasis.

Keywords: Intestinal epithelium; *in vitro* co-culture systems; RPMI-1640; LPS; cytokine stimulation; catechins; inflammatory and apoptotic pathways.

Introduction

Inflammatory Bowel Disease (IBD) is a complex disorder, characterized by recurrent episodes of inflammation interspersed with periods of remission leading to a severe alteration of the quality of life of patients. These diseases are of unknown origin, but the scientific community agrees on the fact that they are associated with an imbalance of the gut microbiota and an uncontrolled immune response, both leading to intestinal epithelial disruption in genetically predisposed individuals (Kaser et al., 2010). The two main types of IBD are Ulcerative colitis (UC) and Crohn disease (CD). Due to their multifactorial origins, no effective treatment has been found so far. Although mortality rates are low, IBD is associated with high morbidity, and patients commonly report an increased risk of depression, indicative of the unpleasant nature of the condition (Atanasova et al., 2022).

One of the challenges of the gastroenterological field is to understand the complex mechanisms leading to this inflammatory condition to prevent as much as possible the onset of the disease but also to find the most appropriate treatment conditions to limit the progression of the disease among which is dietary therapy.

The Research Unit IDEALISS “interactions of the environment and of diet on animal and human health”, of the Institut Polytechnique UniLaSalle in Beauvais, France, has been studying for several years the early origins of human chronic diseases, among which IBD, and the role played by diet to prevent or contribute to therapeutical approaches. The project presented here is part of a PhD scientific program set up between IDEALISS and a private biotechnology company aiming to set up a complex human *in vitro* model to study the multiple interactions described *in vivo* between the intestinal epithelium, the intestinal immune system and the microbiota to find therapeutical and dietary approaches to limit and/or prevent intestinal homeostasis disruption leading to chronic gut inflammation. Inflammatory responses in diseases such as IBD may be activated by various stimuli activating receptors at the surface of enterocytes

among which Pattern Recognition Receptors (PRRs). This family of receptors includes Toll-like receptors (TLRs) recognizing bacterial fragments (e.g. lipopolysaccharide from *E. coli*) that stimulate several pro-inflammatory signaling pathways among which nuclear factor kappa-B (NF-κB), leading to an increase in inflammatory cytokines secretion (Kim & Heo, 2022). These past 20 years, numerous natural functional ingredients in food have been studied for their so-called health benefits, diseases prevention properties and their ability to limit inflammatory stimulus as mentioned before. Some of the most studied ingredients currently are polyphenols. They belong to a wide family of molecules, naturally produced by plants to protect them from multiple aggressions. The main polyphenols are flavonoids, lignans, phenolic acids, and stilbenes (Rana et al., 2022), they help to modulate inflammatory pathways by reducing the production of pro-inflammatory stimulus and oxidative stress (Farzaei et al., 2015; Grootaert et al., 2015). Flavonoids are naturally abundant in many food including berries, dark chocolate and tea. They have powerful antioxidant properties by neutralizing free radicals and reducing inflammation. Flavonoids include catechins that belong to the group of flavan-3-ols. They have been studied for their ability to modulate inflammatory pathways by interfering on pro-inflammatory signaling cascades. As a strategy to prevent chronic inflammatory diseases like diabetes, cardiovascular diseases and IDB, the incorporation of polyphenols in the diet takes on a leading role, since they have the properties to modulate gut microbiota (Farzaei et al., 2015; Grootaert et al., 2015). However, there is still a long way to go before fully understanding the pathways and bioactivity of the compounds and their real efficacy to limit the inflammatory responses (Bae et al., 2020; Isemura, 2019; Kim & Heo, 2022).

The results of this research are expected to contribute in the development of a more complexified functional and representative *in vitro* model to study intestinal pro-inflammatory response observed during IBD and to explore the efficacy of polyphenols to limit the progression of IBD.

Problem Statement

The understanding of the causes of IBD has been under investigation for several years, with the use of multiple techniques ranging from simple culture models to animal approaches and clinical observations. The use of animal models to understand the chronology of the induction of the inflammatory response and barrier disruption at the intestinal level is less and less accepted mainly for ethical reasons making it necessary to migrate to other options such as *in vitro* models but also due to theoretical use of a large number of animals needed to evaluate this chronology. Hence, multiple attempts to reproduce *in vitro* the interactions between the intestinal mucosa, the intestinal immune system and the microbiota have been made and described in the scientific literature, but, due to the inadequacy of existing *in vitro* models the actual understanding of intestinal inflammation, specifically IBD, is limited. Because of their simplistic conception, these models are, for the moment, unable to mimic the complex interactions between the different types of cells at the mucosal level and more specifically of intestinal epithelial cells and of immune cells. Most of them are based on 2D models (cells grown on a flat surface), which may use transformed cell lines (cancer or immortalized cells) or cells coming from resected tissues (primary cultures of short life span). More recently, some 3D approaches (cells grown on a three-dimensional gel-like matrices) have been published. More recently, some 3D approaches (cells grown on a three-dimensional gel-like matrices) have been published attempting to reproduce intestine–microbiota interactions (Dosh et al., 2019; García-Díaz et al., 2022). However, the close communication between the immune and epithelial cells is still poorly studied for methodological reasons primarily linked to the kinetics of proliferation and cell growth medium compatibility. According to the literature most studies use the Caco-2/HT29-MTX co-culture model in DMEM (Cheng et al., 2023; Hoffmann et al., 2021; Reale et al., 2021). However, while being adapted for these two cell lines, this medium is not adapted for the growth of immune cells such as the THP-1 monocytes that and more sensitive to the medium and require a more specific one such as RPMI-1640. As thus, to

consider there merging in a single environment (same medium), it is important to observe the behavior of the co-culture Caco-2/HT29-MTX in RPMI-1640 medium by comparing its growth, functionality and response to pro-inflammatory stimuli. *In vitro* stimulation of epithelial cells to induce an inflammatory response is, most of the time, reproduced by the application of a cocktail of pro-inflammatory cytokines applied at the apical surface of the epithelium. Physiologically these cytokines are released by stimulated immune cells laying underneath the epithelium. Furthermore, epithelial cells are also sensitive to pathogenic signals such as bacterial toxins released (eg, lipopolysaccharide from *E. coli)* via the receptors they have at their apical surface.

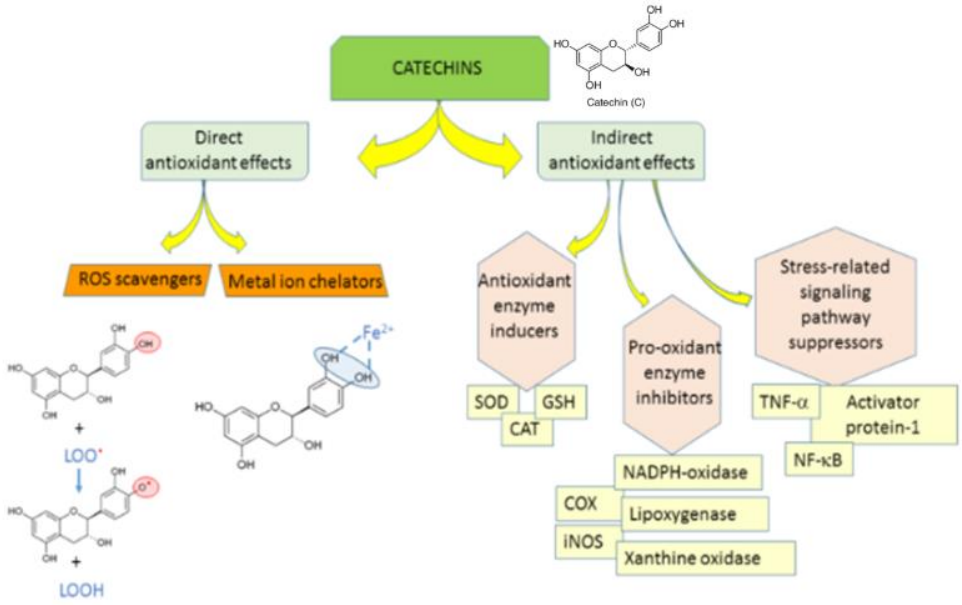
In addition, we needed to understand how a functional ingredient specifically behaves in this type of cell culture and whether it was possible to confirm through this approach its ability to prevent or limit the pro-inflammatory response to serve as a model for the evaluation of functional foods. Thus, we started this project by evaluating whether it is possible to propose a functional ingredient extracted from a food source for the limitation of the appearance of inflammatory phases observed in IBD patients to strengthen the dietary approach in the treatment of such diseases.

Justification

Environmental factors, among which dietary habits, play a crucial role in shaping the gut microbiota's composition. Some diet habits, such as western diet, can lead to dysbiosis, an imbalance of the beneficial gut microbiota (such as Firmicutes and *Bacteroidetes*) which triggers an inflammatory reaction (Rinninella et al., 2019). The intestinal immune system may perceive the altered microbial composition as a threat, resulting in the release of inflammatory mediators, activation and orientation of intestinal immune cells into the gut mucosa (Yoo et al., 2020). Despite advances in therapeutic treatments, there is currently no cure for IBD. Emerging evidence suggests that diet plays a crucial role in mitigating inflammation and reducing symptoms, highlighting the importance of personalized dietary strategies (Knight-Sepulveda et al., 2015). Each patient requires specific dietary interventions tailored to their unique condition and response to treatment (Levine et al., 2020). This underlines the relevance and importance of our study, where one of its objectives is to explore the anti-inflammatory potential of dietary components, in particular polyphenols. Within the theoretical context of gut health and IBD treatments, polyphenols have gained significant attention for their anti-inflammatory properties. Polyphenols are phenolic compounds widely present in our food. These natural substances extracted from plants possess anti-inflammatory and antioxidant properties that could contribute to the prevention of an inflammatory reaction at the epithelial level (Boonyong et al., 2020). Flavan-3-ols, a rich category of polyphenols, are particularly noteworthy as the review by Manach, et al. (2005) mentioned. These molecules are absorbed mainly in the small intestine and have been studied for their potential to prevent the formation of reactive oxygen species (ROS) which are responsible for cell membrane lysis, DNA disruption, and mitochondria dysfunction leading to cell death (Fan et al., 2017; Przystupski et al., 2019). Catechins, one of the most abundant flavan-3-ols in our diet as it is present in several foods such as chocolate, tea, red wine and some fruits such as cherries and apricots, have been also studied for their antioxidant, antibiotic, and anti-

inflammatory properties (Bae et al., 2020; Isemura, 2019).The antioxidant properties are due to the numerous hydroxyl groups, since they have the capacity of stabilizing free radicals (see Figure 1). Additionally, according to some studies, polyphenols could play a vital role in limiting the inflammatory response and improving gut health. Among their mechanisms of action, their immunomodulatory effect has also been suggested (Katayama et al., 2013).

Figure 1: *Antioxidant properties of catechins.*



Source: (bernatoniene & kopustinskiene, 2018).
Note: Reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH), oxidase-nicotinamide adenine dinucleotide phosphate oxidase (NADPH), cyclooxygenase (COX), inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), nuclear factor kappa B (NF-κB), light-chain-enhancer of activated B cell.

The study of literature to select the most appropriate polyphenol to use in the *in vitro* epithelial model was focused on the ability of this compound to be tested *in vitro* and to display antioxidant properties that allow neutralizing ROS (Hussain et al., 2016). Another point that was

important is the fact that catechins could act on Caco-2 cells. A study published a couple of years ago, described the property of this polyphenol to improve the integrity of the intestinal epithelial barrier by influencing on the expression of tight junctions' proteins (TJ) (Bernatoniene & Kopustinskiene, 2018).

The evaluation of benefits of dietary compounds for IBD patients is based on experimental approaches among which the use of *in vitro* models mimicking the intestinal epithelial response to nutrients or toxicants. The most widely used experimental procedure is a co-culture of Caco-2/HT29-MTX cells exposed to a cocktail of pro-inflammatory cytokines or to pro-inflammatory bacterial compounds such as lipopolysaccharide (LPS) from *E. coli*. This model aims to reproduce *in vivo* conditions and gives the chance to study the potential effects of catechins on epithelial cells. However, its traditional medium is DMEM which is not compatible to immune cell lines only growing in RPMI-1640. We intended to evaluate if this model of intestinal epithelium grew and differentiated optimally in this medium and if its response to the 2 types of pro-inflammatory stimuli and the protective effect of catechins would be preserved in switching to the RPMI medium. The choice of the Caco-2/HT29-MTX co-culture is based on the idea of replicating *in vivo* conditions in the small intestine, the major site of nutrient absorption (Kiela & Ghishan, 2016). Caco-2 cells are human colon adenocarcinoma-derived enterocytes that, after differentiation, mimic the morphological and functional characteristics of small intestinal enterocytes. HT29-MTX cells are derived from HT29 cells treated with methotrexate, to express a goblet cell phenotype able to produce mucus. Thus, the co-culture provides a more adequate model of the intestinal epithelium.

The expected accomplishments and benefits of this work are multifaceted. First, to be able to acquire knowledge about how the Caco-2/HT29-MTX co-culture behaves in a medium such as RPMI 1640, which is not the one in which they are normally cultured, to know if switching to such a medium could be a possibility to complexity the model with immune cells. Secondly, it will contribute to the growing research being done on diet-mediated IBD management, particularly emphasizing the role of polyphenols and specifically catechins, abundant in our diet and further

evaluate their anti-inflammatory power. At last, it may help to understand the interaction between dietary compounds and preservation or reinforcement of intestinal health.

Objectives

General Objective

Compare the sensitivity of epithelial cells to pro-inflammatory stimuli (LPS from *E. coli* and a cocktail of pro-inflammatory cytokines) and dietary polyphenols, specifically catechins in both DMEM (a standard epithelial cell medium) and RPMI-1640 (a medium preferred for immune cells).

Specific objectives

- Assess whether the change of growth medium from DMEM to RPMI-1640 could modify the functionality of the Caco-2/HT29-MTX co-culture.
- Evaluate whether the change of growth medium could alter the response to pro-inflammatory cytokines or LPS stimuli.
- Determine whether the use of a new growth medium could change the response of cells pre-incubated with catechins, as a model of functional food evaluation to pro-inflammatory stimuli.

Theoretical foundation

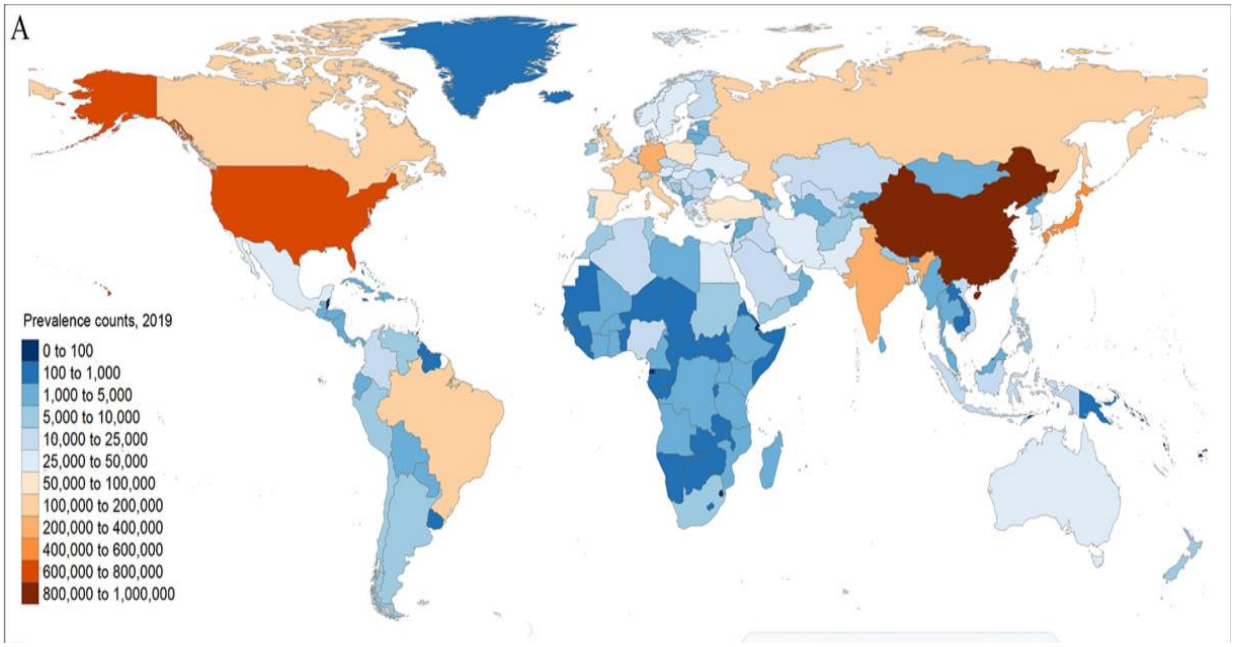
Inflammatory Bowel Disease (IBD)

Inflammatory Bowel Disease (IBD) encompasses chronic inflammatory conditions of the gastrointestinal tract, especially Crohn's disease (CD) and ulcerative colitis (UC). The exact etiology of IBD is unknown, but it is believed to involve a combination of genetic, environmental, and immunological factors with an impact on the gut microbiota (Jarmakiewicz-Czaja et al., 2022). It is also marked by recurrent episodes of gastrointestinal tract inflammation due to an atypical immune reaction to the gut microbiota. The study by Gevers, et al. (2014) demonstrated that genetic factors associated with IBD, influence immune responses and epithelial barrier integrity. In addition, this study explored the role of the gut microbiota in IBD, demonstrating that dysbiosis contributes significantly to the pathogenesis of the disease.

Incidence And Prevalence Of IBD

The incidence and prevalence of IBD has been increasing worldwide. A study from the global burden of diseases (GBD) 2019 shows data about the geographic prevalence of IBD from 1990 to 2019 in 204 countries (Figure 2).

Figure 2 *The global prevalence of IBD in 204 countries*



Source: (Wang et al., 2023).

By 2019, there were approximately 4.9 million cases of IBD worldwide with an increase of approximately 50% since 1990, with China and the United States being the countries with the largest number of cases. Interestingly, incidence rates are the highest in North America and Europe, while Asia and Africa are lower but growing rapidly (Wang et al., 2023). This difference may be explained by lifestyle and diet differences between the countries, as several studies have shown that Western diet, wherever it is customary to have a high consumption of refined sugars, fats, and ultra-processed foods may tend to increase the risk of developing IBD (Clemente-Suárez et al., 2023; Shon et al., 2023).

A review made by Zhao, et al. (2021) evidences the prevalence and incidence of IBD in Europe. The increase of prevalence observed in Northern France reflects trends observed in Northern and Western Europe. In France, and more specifically at the North of the country, an increase in the prevalence and incidence of IBD mainly in children has been observed lately. A study conducted from 1988 to 2011 in Northern France showed an increase for both CD and UC

in the pediatric population with an increase of the incidence of CD from 2.1 to 4.7 per 100,000 children/year and an increase of the incidence of UC from 0.8 to 2.1 per 100,000 children/year (Barnes & Kappelman, 2018).

The incidence and prevalence of IBD in the pediatric population is also increasing in Latin America and more especially in Colombia, according to a systematic review conducted by Kotze, et al. (2020) with an incidence of CD of 7.3 per 100,000 children/year and an incidence of UC of 4.9 per 100,000 children/year. A study from Juliao-Baños, et al. (2021) conducted in Medellín, Colombia reported an increase in IBD cases during a ten-year period, showing UC more prevalent than CD. This aligns with findings from other Latin American countries, such as Mexico where lifestyle changes and urbanization have been said to contribute to the increase of IBD incidence (Ciapponi et al., 2020). Another study from Fernández-Ávila, et al. (2020) using data from SISPRO, a tool adapted from the Colombian Ministry of Health, reported 42,647 patients diagnosed with IBD with a prevalence of 87 per 100,000 person-years, and with a predominance of women. A study compared the impact of the type of diet consumed by Colombians on their microbiota composition which was very different from Americans, Europeans and Asians. Authors suggested that the diet of this individuals (Colombians) has a high impact on the microbial population and that this variation could impact the prevalence and management of IBD. This emphasizes the need for more research to address the increasing prevalence of IBD in the region (Escobar et al., 2015).

IBD pathology

IBD is characterized by frequent episodes of inflammation in the gastrointestinal tract due to an atypical immune response against the intestinal microbiota. This inflammatory response involves the activation of mucosal immune cells, which leads to the release of chemokines, such as CXCL-8, into the bloodstream to attract circulating immune cells, such as neutrophils, into the

site of inflammation. The CXCL-8 is said to be one of the primary mediators of the inflammatory response; additionally, the expression of this gene is highly stimulated by IL-1β, TNF-α and LPS. Furthermore, the expression of CXCL-8 is involved in the activation of other immune cells such as neutrophils and the transcription of its gene, which is NF-κB-dependent, perpetuates the inflammatory response which contributes to tissue damage (Saez et al., 2023; Yeshi et al., 2020).

Another mechanism that can contribute to the inflammatory process is the binding of LPS to its preferred receptor: the Toll-like receptor 4 (TLR4) present at the surface of enterocytes. This binding activates signal transduction leading to the release and translocation of nuclear factor-kappa B (NF-κB), a transcription factor, to the nucleus to induce the production of proinflammatory mediators among which CXCL-8, thereby amplifying the inflammatory response (Neurath, 2019). A previous study by Friedrich, et al (2019) found evidence that CXCL-8 has a crucial role in the mediation of the inflammatory response in the IBD; The authors also showed the complex crosstalk between microbial components, immune cell activation and cytokines signaling in the pathogenesis of IBD offering insights about potential therapeutic interventions to mitigate chronic inflammation and tissue damage in the affected population.

Nutritional approaches to limit the inflammatory response in IBD patients

Immunonutrition and IBD

As introduced above, diet is a component of the modulation of host microbiota interactions and dietary habits may contribute to the alterations of the intestinal barrier characteristic of IBD. Based on this, immunonutrition, an emerging, multidisciplinary field that studies the interactions between nutrition, the immune system, infection, inflammation and tissue damage, is focusing on how specific nutrients such as fatty acids, vitamins, minerals and antioxidants can modulate the immune response or reduce the inflammation (Zapatera et al., 2015).

Immunonutrition could offer a promising complementary approach to limit the inflammatory phases observed in IBD, since it seeks to regulate the immune system and improve intestinal health using specific nutrients such as omega-3 fatty acids and vitamins C or E that can decrease the production of pro-inflammatory cytokines and prebiotics or probiotics that can be used to reduce inflammation, improve intestinal barrier function and enhance the immune response (Mariette, 2015; Shokryazdan et al., 2017; Simopoulos, 2002; Wu et al., 2024). In order to evaluate those benefits, *in vitro* studies become essential to elucidate the effects of these types of functional ingredients on cells. Furthermore, by testing nutrients in cellular models, cell proliferation, barrier integrity and cytokine release can also be assessed.

Oxidative stress in IBD and the effect on food compounds

Oxidative stress is defined as the inability of the cellular antioxidant system to neutralize an excessive production of reactive oxygen species (ROS) in cells and tissues. This imbalance damages some molecules such as membrane phospholipids or DNA at the origin of cellular and tissular lesions (Hussain et al., 2016).

Intestinal inflammation is usually associated to an increased production of reactive oxygen species (ROS), which leads to oxidative stress. This worsens tissue damage and prolongs the inflammatory response. *In vitro* cultures are also a useful tool to evaluate the benefits of antioxidant nutrients and their absorption. This tool was used to evaluate the health benefits of polyphenols, helping to understand how they are transported across the intestinal barrier and how they interact with cellular mechanisms. Researchers Rodríguez-Ramiro, et al. (2013) investigated the anti-inflammatory effects of cocoa polyphenols on Caco-2 cells stimulated with the pro-inflammatory cytokine TNF-α. Authors showed that cocoa polyphenols significantly inhibited NF-κB signaling and reduced the expression of proinflammatory enzymes. This indicates that cocoa polyphenols can effectively suppress intestinal inflammation by inhibiting NF-κB activation which

could contribute to a significant improvement of IBD symptoms. However, the amount, repetition of consumption and type of molecules among the cocoa polyphenols need to be investigated further before drawing conclusions. Another study conducted by Grgić, et al. (2020) using the co-culture of Caco-2/HT29-MTX cells also have demonstrated that polyphenols can prevent oxidative stress, reduce inflammation, and may even inhibit cancer cell proliferation. In Colombia, there has been no focus on this type of studies and therefore there is few information on the subject in university databases. However, there is a study elaborated by Carmona-Hernandez, et al. (2019) in which Caco-2 cells were used to evaluate the effects of a polyphenol extract from three Colombian passion fruits. Authors concluded that these extracts help to improve the function of the intestinal barrier and pointed out the benefits of this fruit, typical of the region. In another work investigating the effect of *Calendula officinalis*, a medicinal plant widely used in Colombia, on cell lines such as Caco-2 and HT29, it was found that the plant polyphenols were directly related to the antioxidant capacity of the extract (Hernández-Rosas et al., 2018). Based on their nutritional value, catechins, a type of polyphenol found in green tea and cocoa, have been studied for their anti-inflammatory power and their ability to decrease oxidative stress (Bae et al., 2020; Kim & Heo, 2022; Musial et al., 2020). These properties make catechins promising candidates for the prevention and treatment of inflammatory conditions among which IBD.

Modelisation of intestinal epithelial disruption

In vitro approaches

As previously indicated, one of the ways to study IBD is through *in vitro* models. They allow the study of biological processes outside living organisms, using cells or tissues cultured under controlled conditions and avoiding animal testing (Joshi et al., 2022). These models are essential for understanding cellular mechanisms and testing hypotheses in a controlled

environment. In terms of intestinal research, *in vitro* models enable the study of epithelial cell behavior, barrier function, and responses to various stimuli (Fedi et al., 2021). The Caco-2 cell line, derived from a human colon adenocarcinoma, is widely used to model the intestinal epithelial barrier. When cultured in DMEM medium, Caco-2 cells differentiate into enterocyte-like cells (with a brush border, nutrient transporters, and cell-to-cell junctions), resembling the absorptive cells of the small intestine. DMEM is used because it provides essential nutrients, vitamins, and minerals that support the growth and differentiation of Caco-2 cells. This makes them a good model for studying nutrient absorption and drug permeability. The HT29-MTX cell line, is also derived from a human colorectal adenocarcinoma. By contrast to the traditional HT29 cell line, HT29-MTX, which also grows in DMEM medium, have been orientated by methotrexate (MTX) to produce intestinal mucus. Consequently, the co-culture of Caco-2 and HT29-MTX cells creates a more accurate model of the intestinal epithelium, incorporating both absorptive and mucus-secreting cells (Haddad et al., 2023).

This thesis project was part of a larger PhD program aiming to develop a complex cellular model to study interactions between molecules such as drugs or functional ingredients and the gut. The research project aims to develop tri-cultures that include epithelial and immune cells, creating a more complex *in vitro* model that can more closely recreate what occurs *in vivo*. This approach would allow the study of the interactions between the intestinal epithelium and the immune system, which are crucial for understanding the pathogenesis of IBD. By contrast, immune cell lines, are typically cultured in RPMI 1640 medium, which is specifically formulated to support the growth and differentiation of these cells, providing the appropriate environment for maintaining their functionality. To do this, it is then necessary to understand the behavior of epithelial cells, such as Caco-2 and HT29-MTX, in both DMEM and RPMI-1640 media and this is where the relevance of this study comes in.

To recreate *in vitro* inflammatory conditions, epithelial cell cultures have been stimulated with pro-inflammatory stimuli. They can be cytokines such as interleukin-1β (IL-1β), tumor

necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) that are naturally released by immune cells from the intestinal mucosa, and then mimicking the inflammatory environment observed in diseases such as IBD. In the literature, authors may also use LPS, a component of the outer membrane of Gram-negative bacteria, mimicking the release by enteropathogenic bacteria of enterotoxins and triggering pathways that lead to inflammation and epithelial barrier disruption (Kuo et al., 2015; Wei et al., 2022).

Methods to measure permeability at the intestinal barrier

Intestinal permeability is tightly linked to the pathogenesis of IBD. Compromised intestinal barrier also called “leaky gut” increases the permeability which allows the entrance in the blood circulation of foreign bodies, bacteria and toxins from the intestinal lumen and may cause inflammation or disease, contributing to the exacerbation of the IBD. Intestinal permeability is evaluated by different means among which the Transepithelial Electrical Resistance (TEER), a technique measuring the loss of integrity of tight junctions in cell cultures leading to a reduction of electric difference between the apical (representing the intestinal lumen) and the basal medium (representing the blood circulation) (Srinivasan et al., 2015). This cell culture must be grown onto semi-permeable microporous membranes bathing into the wells and named Transwell® chambers. One of the disadvantages of using this method is that it involves taking several measurements at different time points, which means a lot of time invested; another difficulty is that the TEER measurement provides information about the permeability of the membrane and its integrity but does not provide specific information. Finally, one of its main limitations is that it is not very reproducible. The other methods allowing the measure of intestinal barrier permeability are based on the administration of a fluorescent molecule (Lucifer yellow or FITC-dextran, 4 kDa), normally not absorbed, in the apical medium and their assay in the basal medium at the end of the study.

In conclusion, *in vitro* models, in particular co-cultures of Caco-2/HT29-MTX cells, are essential tools for investigating the intestinal epithelial barrier and its response to inflammatory stimuli such as cytokines and LPS. This model provides insights into the mechanisms underlying IBD and the potential protective effects of dietary components such as polyphenols. Research on catechins has demonstrated their significant anti-inflammatory and antioxidant properties (Yan et al., 2020), making them promising candidates for the prevention and treatment of IBD.

Methodology

The work presented here is an applied experimental study based on an *in vitro* model with Caco-2/HT29-MTX co-cultures at a ratio of 9:1. The research is guided by two main hypotheses: **(1)** whether the change of growth medium from DMEM to RPMI-1640 will alter the response of the co-culture to stimulation with a pro-inflammatory cytokine cocktail, and **(2)** whether the switch of growth medium from DMEM to RPMI-1640 will also affect the response of the co-culture when stimulated with a cocktail of pro-inflammatory cytokines, lipopolysaccharide (LPS), and catechins as a dietary compound. For each experiment N=4 was performed, with control groups consisting of unstimulated cells containing only either DMEM or RPMI medium (Figure 3 and Figure 5). Since the study involved the use of commercial cell lines as an *in vitro* model, no ethical considerations were necessary. To assess the expression levels of CXCL-8 and NF-κB genes, quantitative polymerase chain reaction (qPCR) was performed on samples taken in duplicate.

Cell Culture

The experiments were run using 2 intestinal epithelial cell lines both coming from human colorectal adenocarcinoma: the Caco-2 cells (ATCC – American Type Culture Collection) and the HT29-MTX (ECACC - European Collection of Authenticated Cell Cultures). The first one shows characteristics of enterocytes upon full differentiation after 21 days. These cells were used between passages 56 and 61. The second one, by contrast, express a goblet cell phenotype and were used between passages 65 to 71. Cells were grown as a co-culture of Caco-2 and HT29-MTX to mimic the intestinal epithelial lining. The two cell lines were routinely grown in an atmosphere of 5% carbon dioxide at 37°C either in DMEM GlutaMAX (Dulbecco's Modified Eagle Medium) (Fisher Scientific SAS, Illkirch, France) or with RPMI-1640 (Roswell Park Memorial Institute), 1% (v/v), L-Glutamine (Eurobio Scientific, Les Ulis, France) 1% (v/v) Sodium pyruvate and 1% (v/v) HEPES buffer solution (Dutcher, Issy-les-Moulineaux, France). Both media were

added with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Dominique Dutcher, Issy-les-Moulineaux, France), 1% (v/v) non-essential amino acids and 1% (v/v) penicillin/streptomycin (Dutcher, Issy-les-Moulineaux, France). Cells were split when they reached an 80% confluency using a trypsin solution (0.25% Trypsin EDTA—Thermofisher Scientific, Illkirch, France).

Viability of Caco2 and HT29MTX monoculture in RPMI-1640

Mono-cultures of Caco-2 and HT29-MTX cells were seeded on a 24-well plate polycarbonate plate at a density of 1×10^5 cells/well (CytoOne, Starlab, Orsay, France), by adding 1 mL of cell suspension and culture medium per well. To assess growth and viability, the cells were grown for 21 days in DMEM and RPMI 1640 media, with the objective of observing the behavior of each cell line in the different culture medium (Figure 17). Since RPMI 1640 is not their usual growth medium. Cell counting was performed using the cellometer (Nexcelom Bioscience LLC). The Results can be seen in Appendix 1.

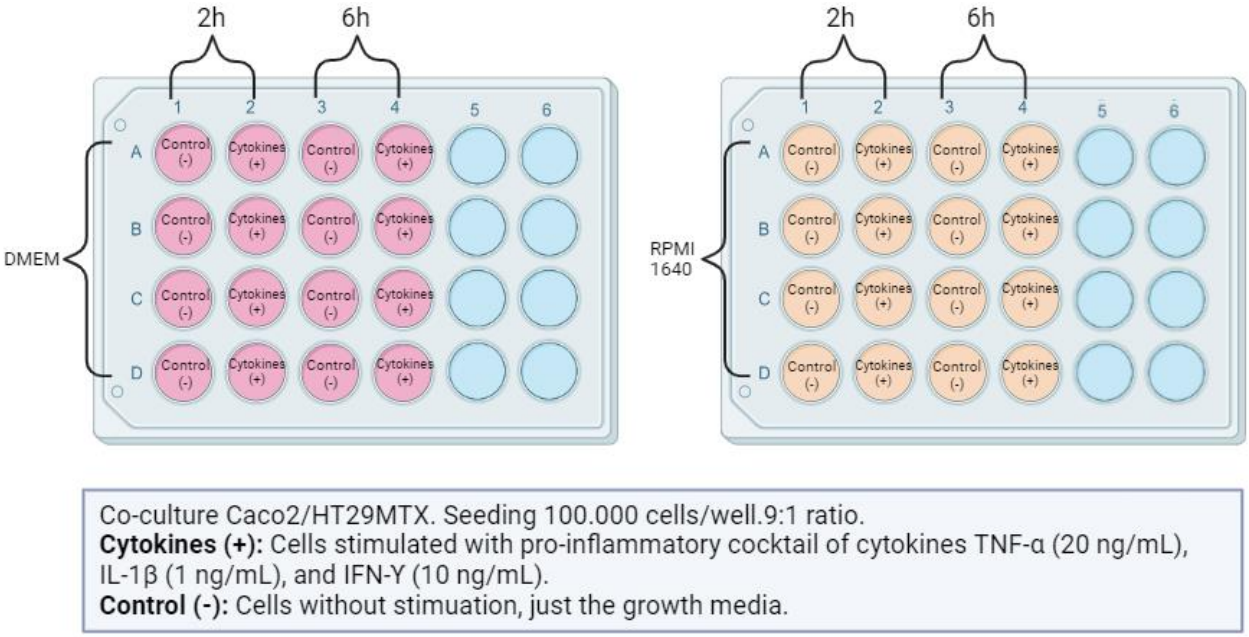
Co-culture stimulation

Time-dependent effect of the growth medium on the co-culture response to the pro-inflammatory cytokines cocktail

Co-culture of Caco-2 and HT29-MTX were seeded on a 24-well plate polycarbonate plate at a density of 1×10^5 cells/well (CytoOne, Starlab, Orsay, France) at a ratio 9:1 (Guibourdenche et al. 2021) by adding 1 mL of cell suspension and culture medium per well. Cells were grown for 21 days until they reached full differentiation. Two culture media were used: DMEM and RPMI-1640. They were changed every other day during the first two weeks and every day the last week. On day 20, cells were rinsed with phosphate-buffered saline (PBS) and put into their respective serum-free medium for 24h. Cells were then stimulated with a cocktail of pro-inflammatory

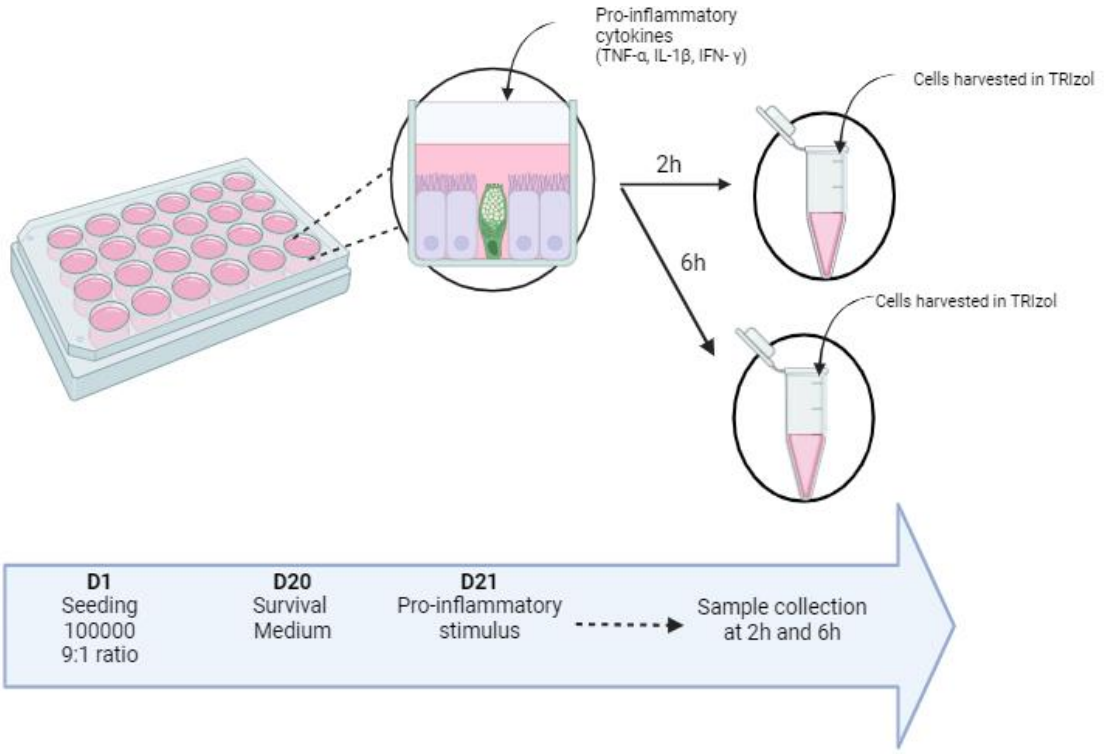
cytokines: TNF-α (20 ng/mL), IL-1β (1 ng/mL), and IFN-γ (10 ng/mL) (BioTechne, Lille, France) for 2 and 6h in order to compare the time-dependent response of the co-culture to this pro-inflammatory stimulus (Figure 3 and Figure 4). At the end of the stimulation, media were collected, and cells harvested in TRIzol (Thermofisher Scientific, Illkirch, France). Samples were frozen at -80°C until further use.

Figure 3: *Plate plan of the pro-inflammatory stimulation with cocktail of cytokines in the Caco-2/HT29-MTX co-culture, replicas, sample size, time points and media used*



Source: (Personal creation elaborated with Biorender.com).

Figure 4: *Experimental procedure of the pro-inflammatory stimulation with the cocktail of cytokines in the Caco-2/HT29-MTX co-culture materials and methods used*



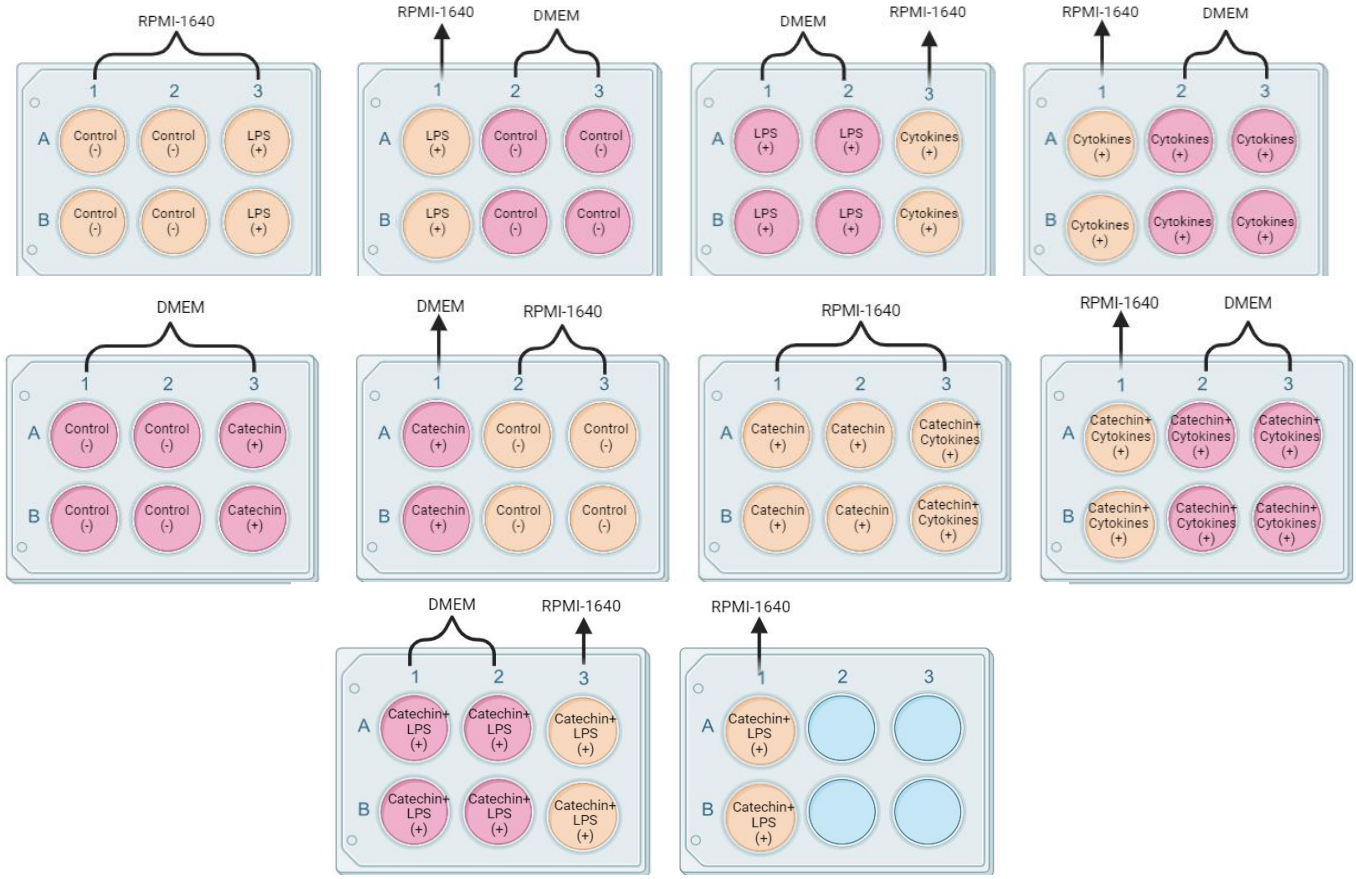
Source: (Personal creation elaborated with Biorender.com).

Incidence of medium on the co-culture response to pro- and anti-inflammatory stimuli

Coculture of Caco-2 and HT29-MTX were seeded on 6-well polycarbonate membrane cell culture inserts with HD (high density) 0.4 µm pores at a density of 4 x 10^5 cells (Corning, Dutcher, Issy-les-Moulineaux, France) at a ratio 9:1. The cells were grown for 21 days until they reached a total differentiation. Every condition was tested in RPMI-1640 and DMEM to compare the response of the cells to 2 pro-inflammatory stimuli: LPS from *E. coli* and the cocktail of pro-inflammatory cytokines and the protective effect of the catechins (Figure 5 and Figure 6). The

culture medium was changed every other day the first two weeks and every day during the last week. The day before stimulation, cells were rinsed with PBS and put into their respective serum free medium. Twenty-four hours later, cells were stimulated with a solution of catechins (Sigma-Aldrich Co, Darmstadt, Germany) (15 µM in ethanol), 1h before exposing the cells with either the cocktail of above-mentioned cytokines or a solution of LPS (10 ng/ml) serotype O111:B4 from *E. coli* during 6h. At the end of the stimulation, the insert media (called “apical medium”) were harvested and stored at -80°C until further measurement of markers of intestinal inflammation. The cell layers were harvested in TRIzol and stored at -80°C before performing RNA extraction.

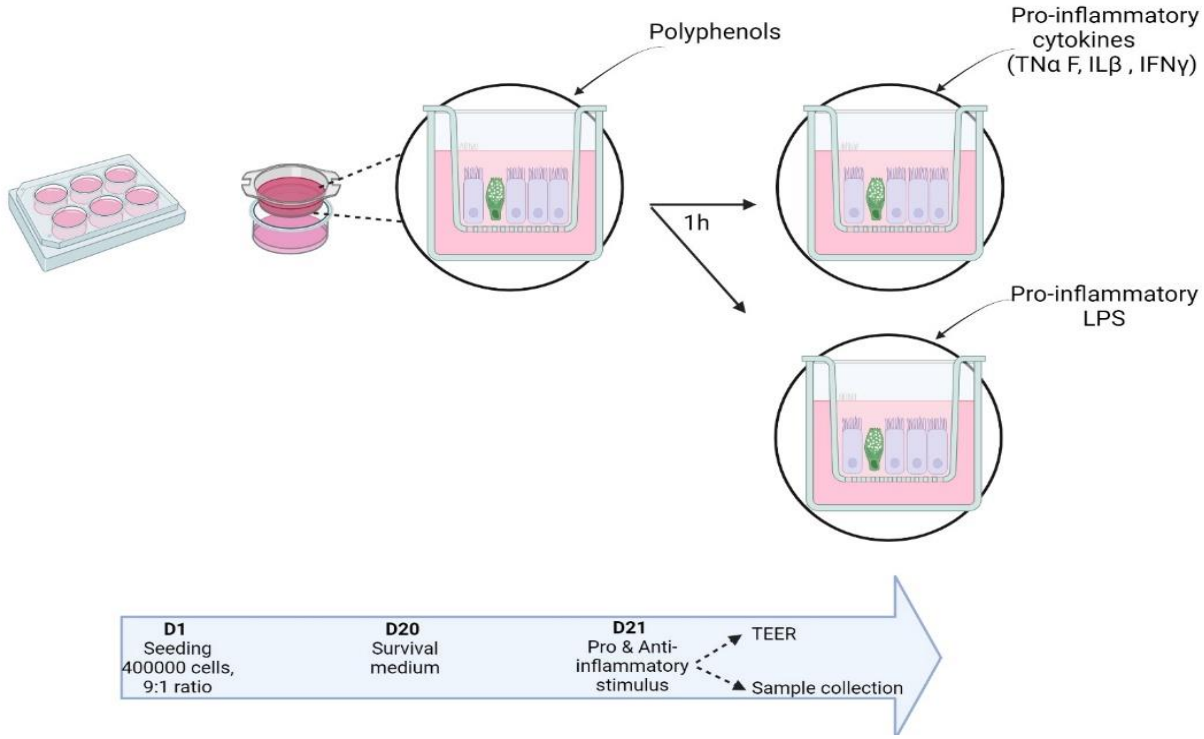
Figure 5: *Plate plan of the pro-and anti-inflammatory stimulation in the Caco-2/HT29-MTX co-culture, replicas, sample size, time points and media used.*



Co-culture Caco2/HT29MTX. Seeding 400.000 cells/well. 9:1 ratio.
Time point: 6h
LPS(+): Cells stimulated with pro-inflammatory lipopolysaccharide (10ng/ml) serotype O111:B4 from *E.coli.*
Cytokines(+): Cells stimulated with pro-inflammatory cocktail of cytokines TNF-α (20 ng/mL), IL-1β (1 ng/mL), and IFN-Y (10 ng/mL).
Catechin(+): Cells stimulated with catechin 15 μM in ethanol
Control(-): Cells without stimulation, just growth media.

Source: (Personal creation elaborated with Biorender.com).

Figure 6: *Experimental procedure of the pro- and anti-inflammatory stimulation in the Caco-2/HT29-MTX co-culture materials and methods used.*



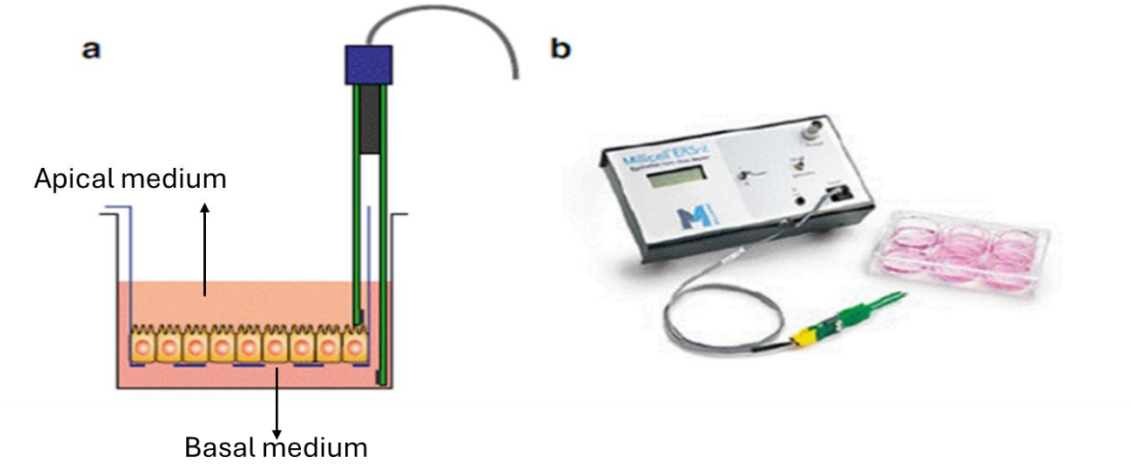
Source: (Personal creation elaborated with Biorender.com).

Measurement of membrane permeability alteration by TEER

In the second series of experiments, Trans-Epithelial Electrical Resistance (TEER) between the apical and the basal medium was measured using an ohmmeter (Millicell, Merck, Darmstadt, Germany) (Figure 7) to assess the integrity of the co-culture monolayers before and after the addition of the various stimuli applied. The changes in the TEER were calculated using the following equation 1 (Eq. 1). A well without cells, only with PBS, was used as a reference.

$$\Delta TEER = [(\text{mean of final sample TEER - final control of TEER}) \times 4.2] - [(\text{mean of initial sample TEER} - \text{initial control of TEER}) \times 4.2]. \quad \textbf{(Eq. 1)}$$

Figure 7: *Representation of the TEER (transepithelial electrical resistance) and the epithelial voltmeter*.



Source: (a) Personal creation elaborated with Biorender.com (b) Millicel, Merck, Darmstadt, Germany.
Note: (a) Schematic representation of the measure of TEER of an epithelial cell culture (personal creation elaborated with Biorender.com) (b) used to assess the integrity and the barrier function of epithelial cell layers from the apical chamber in a co-culture of Caco2/HT29MTX before adding catechin, a cocktail of cytokine and LPS and after the stimulus.

Real Time PCR for gene expression of cell mediators

Total RNA was isolated from cells using TRIzol (Fisher Scientific SAS, Illkirch, France). Absorbance ratios at 260/280 nm and at 260/230 nm were measured using spectrophotometry (NanoDrop 2000, ThermoScientific, Illkirch, France) to assess the purity and the concentration of the RNA samples. cDNA was obtained from 5 µg RNA using the Kit GoScript RT (Promega, Charbonnières, France) following the manufacturer's instructions. Absorbance ratios at 260/280 nm and at 260/230 nm were measured using spectrophotometry (NanoDrop 2000, ThermoScientific, Illkirch, France) to assess the purity and the concentration of the DNA samples. Primers and SYBR Green Polymerase Chain Reaction (PCR) master mix were, purchased from

Eurofins Scientific France (Nantes, France) and Qiagen (Courtaboeuf, France) for primers, Promega (Charbonnières, France) for RT-QPCR (Table 1).

qPCRs were run on a StepOnePlus Real-Time PCR System (Applied Biosystem, Foster City, CA, USA) and the data were processed with the StepOnePlus software v2.3. Reactions were performed in duplicate. The levels of amplified cDNA were calculated using the −ΔΔCT method (−ΔΔCt = mean ΔCT control − ΔCtexposed).After testing 2 traditionnally recommended housekeeping genes: glyceraldehyde-3- phosphate dehydrogenase (GADPH) with a mean of 27,65 and a CT range (26-28) and Peptidylprolyl Isomerase A (PPIA) with a mean of 25,10 and a CT range (24-25), only PPIA was kept since its expression was found to be the most stable one whichever stimulated conditions applied. The regulation of the expression of genes coding for the C-X-C motif chemokine ligand 8 (CXCL-8), for the p65 subunit of the nuclear factor-kappa β (NF-κB), and for CASPASE 3 and CASPASE 9 was then assessed.

Table 1: Primers used in the project (Guibourdenche, et al., 2021)

Function	Gene	Name	Sequences 5'-> 3' or Reference	Supplier
Housekeeping gene	PPIA	Peptidylprolyl Isomerase A	CCTATCCTAGAGGTGGCGGA TCATCGCAGAAGGAACCAGAC	Eurofins
Inflammatory Genes	CXCL8	Chemokine ligand 8	AGAGTGATTGAGAGTGGACC ACTTCTCCACAACCCTCTG	Eurofins
	NF-κB	Nuclear Factor κB p65 subunit	GGGGGCATCAAACCTGAAGA GGAGAGAAGTCCCCAAAGGC	Eurofins
Apoptosis Genes	CASP3	CASPASE 3	QT00029162	QIAGEN
	CASP9	CASPASE 9	QT00036267	QIAGEN

Source: *P*ersonal creation

Evaluation of Il-8 secretion in media by ELISA

The levels of IL-8 secretion in both the basal and apical media of the co-cultures were measured after the various stimulations. The measurement of IL-8 in the media harvested at the end of stimulation was performed using an ELISA kit according to the manufacturer's instructions (Human IL-8/CXCL-8 DuoSet ELISA, BioTechne, Lille, France).

Statistics

Data were expressed as the mean ± Standard Error of the Mean (SEM) and were analyzed using the GraphPad Prism software (GraphPad Prism version 5 for Windows, GraphPad Software, San Diego, CA, USA). Student's t-test was performed for comparisons of the co-culture sensitivity to pro-inflammatory cytokine stimulation. For all other experiments, a nonparametric Kruskal–Wallis analysis of variance was performed and, when the difference was statistically significant, a Dunn's post hoc test was then applied to analyze the effects. The threshold for statistical significance was set to $p < 0.05$. For all the experiments N=4 was performed (Figure 3 and Figure 5).

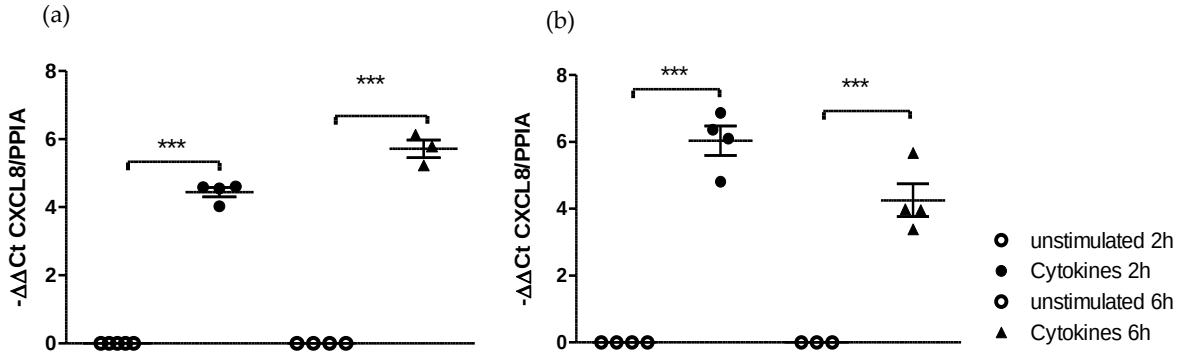
Results

Time-dependent effects of the pro-Inflammatory Cytokine cocktail stimulation on the Caco-2/HT29-MTX Co-Culture is medium dependent

Effect Of Medium On Kinetics Of Pro-Inflammatory Genes Stimulation

In DMEM medium, the incubation of the Caco-2/HT29-MTX cells for 2 and 6h with the cocktail of cytokines led to a significant ($p < 0.05$) increase in CXCL-8 gene expression after 2h (4.45 ± 0.14) as well as after 6h (5.72 ± 0.26) (Figure 8a). In RPMI-1640 medium, the incubation of the Caco-2/HT29-MTX cells for 2 and 6h with the cocktail of cytokines also resulted in a significantly upregulated ($p < 0.05$) expression of CXCL-8 after 2h (6.03 ± 0.44) as well as after 6h (4.25 ± 0.49) (Figure 8b).

Figure 8: *Time-dependent regulation of CXCL-8 gene expression after incubation with a cocktail of pro-inflammatory cytokines in DMEM and RPMI-1640 media.*



Source: Personal creation elaborated with GraphPad Prism software.
Note: (a) CXCL-8 expression in DMEM. (b) CXCL-8 expression in RPMI-1640. *** Significantly different ($p<0.001$) from unstimulated cells. Student's t-test was performed for comparisons of the co-culture sensitivity to pro-inflammatory cytokine stimulation (Unstimulated vs Cytokines). N=4.

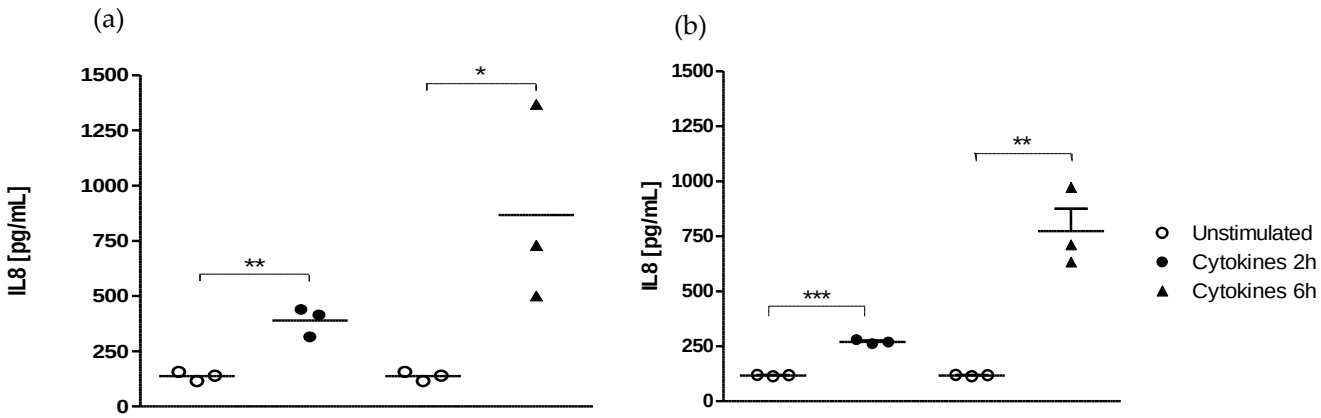
The incubation of the co-culture with the RPMI-1640 medium does not modify the kinetics of expression of CXCL-8 following its stimulation with a cocktail of pro-inflammatory cytokines.

Effect of medium on the kinetics of IL-8 secretion

The incubation of Caco-2/HT29-MTX cells during 2 or 6h with a cocktail of pro-inflammatory cytokines led to a time-dependent significant ($p < 0.05$) increase in IL-8 secretion after 2h (389.1 ± 37.9 vs. 136.5 ± 12.4 pg/mL in the unstimulated cell group) and 6h (866.0 ± 135.5 vs. 136.5 ± 12.4 pg/mL in the unstimulated cell group) of exposure in DMEM medium (Figure 9a).

The incubation of Caco-2/HT29-MTX cells during 2 or 6h with a cocktail of pro-inflammatory cytokines also resulted in a time-dependent significant ($p < 0.05$) increase in IL-8 secretion after 2h (269.6 ± 5.3 vs. 116.7 ± 1.6 pg/mL in the unstimulated cell group) and 6h (772.4 ± 102.4 vs. 116.7 ± 1.6 pg/mL in the unstimulated cell group) of exposure in RPMI-1640 medium (Figure 9b).

Figure 9: *Time-dependent regulation of IL-8 secretion after incubation with a cocktail of pro-inflammatory cytokines in DMEM and RPMI-1640 media*



Source: Personal creation elaborated with GraphPad Prism software
Note: (a) IL-8 secretion in DMEM. (b) IL-8 secretion in RPMI-1640. *, **, *** Significantly different (p 0.05; p < 0.01; p < 0.001) from unstimulated cells. Student's t-test was performed for comparisons of the co-culture sensitivity to pro-inflammatory cytokine stimulation (Unstimulated vs Cytokines). N=4

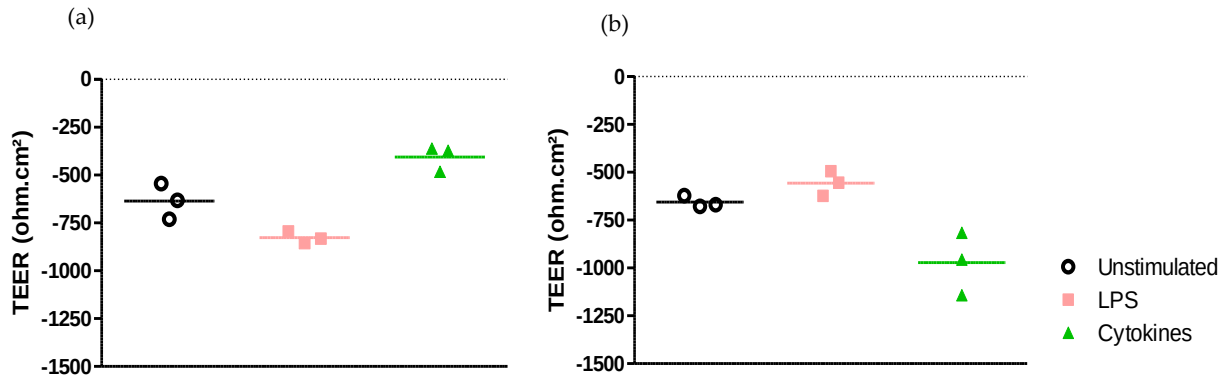
The stimulation of the co-culture in 24-well plates by the cocktail of pro-inflammatory cytokines resulted in the upregulation of CXCL-8 expression. It was followed by an upregulation of IL-8 secretion regardless of the timepoint considered and growth medium used.

Epithelial cell response is dependent of the stimulus but not on the medium

Medium-independent sensitivity of epithelial cell in response to pro-inflammatory stimuli

Effect On Epithelial Permeability. Exposing Caco-2/HT29-MTX cells grown in Transwell® chambers to LPS from *E. coli* or to a cocktail of pro-inflammatory cytokines for 6h did not alter the intestinal epithelial cell permeability measured by TEER either in DMEM medium (Figure 10a) or in RPMI-1640 medium (Figure 10b).

Figure 10: *Effect of medium on permeability modulation after stimulation with LPS from E. coli or a cocktail of pro-inflammatory cytokines.*



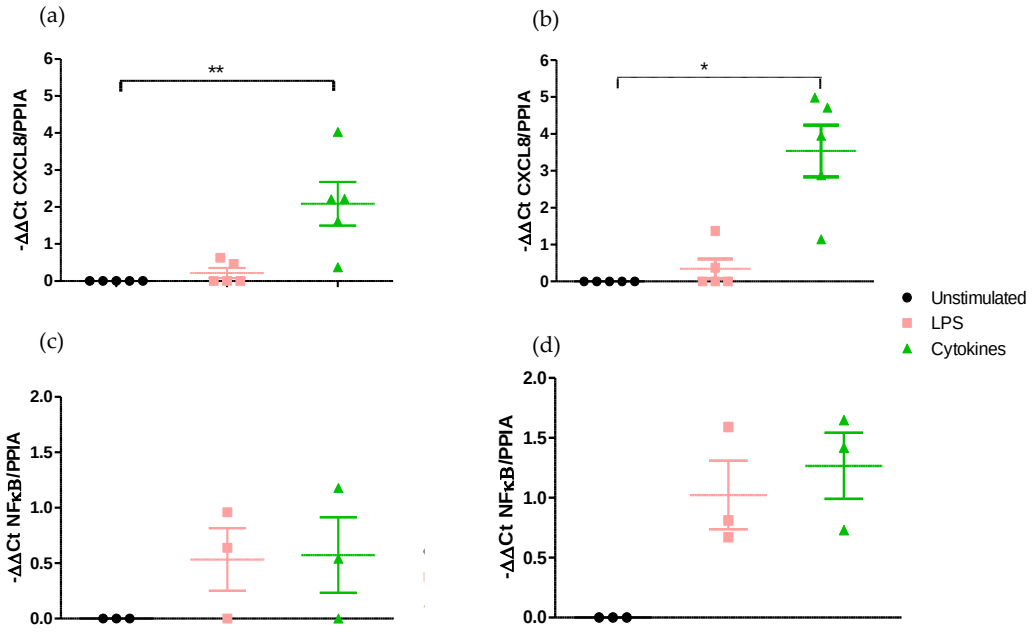
Source: Personal creation elaborated with GraphPad Prism software
Note: (a) Alteration of permeability in DMEM medium. (b) Alteration of permeability in RPMI-1640 medium. A nonparametric Kruskal-Wallis analysis of variance was performed. N=4

The exposure of the model of intestinal epithelium in Transwell® chambers to either LPS from *E. coli* or the cocktail of cytokines did not result in any modification of TEER in the two media.

Effect on CXCL-8 and NF-kB pro-inflammatory gene expressions. Exposing Caco-2/HT29-MTX cells in Transwell® chambers to LPS from *E. coli* for 6h did not stimulate gene expressions of CXCL-8 and of NF-κB either in DMEM medium (Figure 11 a,c) or in RPMI-1640 medium (Figure 11 b,d).

By contrast, the stimulation of the co-culture to the cocktail of pro-inflammatory cytokines during 6h significantly increased ($p < 0.05$) CXCL-8 gene expression both in DMEM medium (2.09 ± 0.59) (Figure 11a) and in RPMI-1640 medium (16.61 ± 5.56) (Figure 11b). However, neither the LPS of *E. coli* nor the cocktail of cytokines were able to modulate NF-κB gene expression in DMEM medium or in RPMI-1640 medium (Figure 11 c,d).

Figure 11: *Effect of medium on modulation of pro-inflammatory NF-κB and CXCL-8 gene expression after 6h exposure to LPS or a cocktail of pro-inflammatory cytokines.*



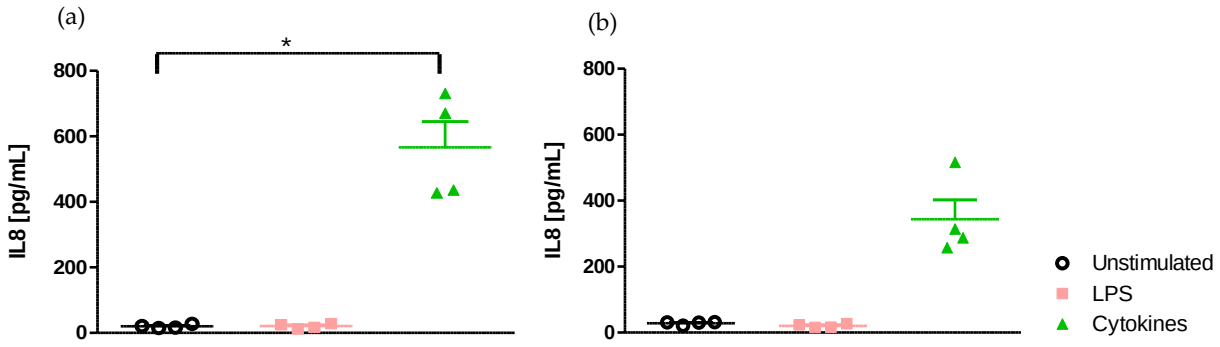
Source: Personal creation elaborated with GraphPad Prism software
Note: (a) modulation of CXCL-8 expression in DMEM medium. (b) Modulation of CXCL-8 expression in RPMI-1640 medium. (c) Modulation of NF-κB expression in DMEM medium. (d) Modulation of NF-κB expression in RPMI-1640 medium. *, ** Significantly different ($p<0.05$; $p<0.01$) from unstimulated cells. A nonparametric Kruskal-Wallis analysis of variance was performed, when the difference was statistically significant, a Dunn's post hoc test was then applied to analyze the effects. N=4.

The stimulation of the epithelium in Transwell® chambers to the cocktail of cytokines resulted in the upregulation of the expression of the CXCL-8 gene but not the of NF-κB gene in both the DMEM and the RPMI-1640 media.

Effect of medium on IL-8-induced secretion. Exposing Caco-2/HT29-MTX cells in Transwell® chambers to LPS from *E. coli* for 6h did not stimulate the secretion of IL-8 either in DMEM medium (Figure 12a) or in RPMI-1640 medium (Figure 12b).

By contrast, the exposure of the co-culture to the cocktail of pro-inflammatory cytokines resulted in a significant ($p < 0.05$) release of IL-8 in the apical DMEM medium (566.5 ± 78.7 vs. 20.0 ± 2.9 pg/mL in unstimulated cells) (Figure 12a). However, this increase was not significant in RPMI-1640 medium (343.9 ± 58.7 vs. 28.5 ± 2.6 pg/mL in unstimulated cells) (Figure 12b).

Figure 12: *Effect of medium on IL-8 secretion in the apical medium after 6h of exposure with LPS or the cocktail of pro-inflammatory cytokines.*



Source: Personal creation elaborated with GraphPad Prism software
Note: (a) Secretion of IL-8 in DMEM medium. (b) Secretion of IL-8 in RPMI-1640 medium * Significantly different ($p<0.05$) from unstimulated cells. A nonparametric Kruskal-Wallis analysis of variance was performed, when the difference was statistically significant, a Dunn's post hoc test was then applied to analyze the effects. N=4

The stimulation of the co-culture in Transwell® chambers only resulted in a significant secretion of IL-8 in DMEM medium.

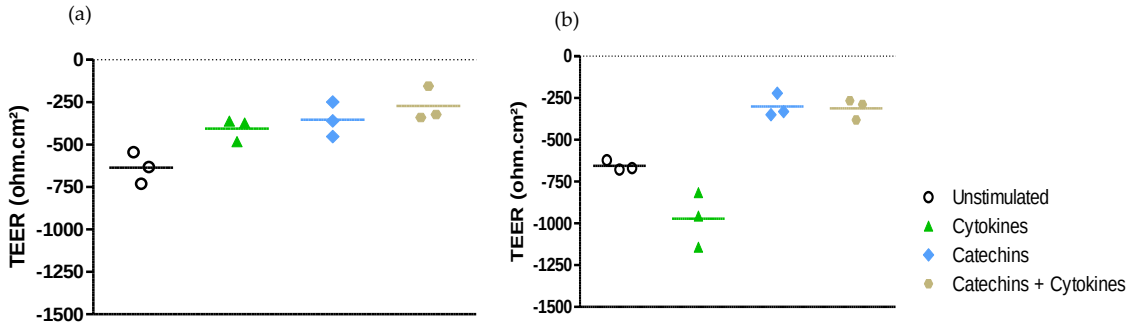
Medium-independent sensitivity of epithelial cell exposed to polyphenols before their stimulation by a pro-inflammatory cytokine

Effect on epithelial permeability. Exposing Caco-2/HT29-MTX cells grown in Transwell® chambers to the cocktail of pro-inflammatory cytokines for 6h did not alter the

intestinal epithelial cell permeability measured by TEER either in DMEM medium (Figure 13a) or in RPMI-1640 medium (Figure 13b).

Moreover, neither the exposure for 6h to catechins alone, nor the exposure to catechins with the cocktail of cytokines was able to modify the epithelial permeability in DMEM medium (Figure 13a) or in RPMI-1640 medium (Figure 13b).

Figure 13: *Effect of medium on transepithelial permeability modulation by catechins after stimulation with a cocktail of pro-inflammatory cytokines.*



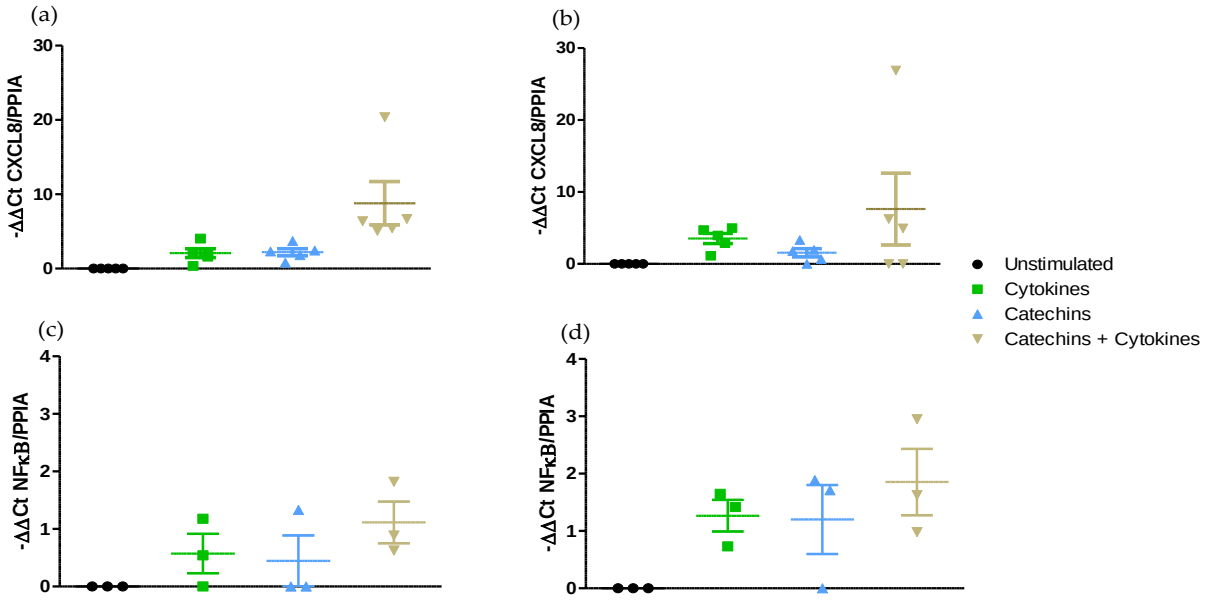
Source: Personal creation elaborated with GraphPad Prism software.
Note: (a) Permeability modulation in DMEM medium. (b) Permeability modulation in RPMI-1640 medium. A nonparametric Kruskal-Wallis analysis of variance was performed N=4.

Catechins did not change the TEER in the two media, regardless of whether the cells were stimulated or not.

Effect on pro-inflammatory NF-κB and CXCL-8 gene expressions. The stimulation of Caco-2/HT29-MTX grown in Transwell® chambers by the cocktail of pro-inflammatory cytokines for 6h did not result in a significant increase in the expression of CXCL-8 or NF-κB genes either in the DMEM medium (Figure 14 a,c) or in the RPMI-1640 (Figure 14 b,d).

Furthermore, the incubation of the co-culture with catechins (15 µM) did not result in any modification of CXCL-8 and NF-κB gene expressions in either the DMEM medium (Figure 14 a,c) or the RPMI-1640 medium (Figure 14 b,d).

Figure 14: *Effect of medium on NF-κB and CXCL-8 expression modulation by catechins after stimulation with a cocktail of pro-inflammatory cytokines.*



Source: Personal creation elaborated with GraphPad Prism software.
Note: (a) CXCL-8 gene expression in DMEM medium. (b) CXCL-8 gene expression in RPMI-1640 medium. (c) NF-κB gene expression in DMEM medium. (d) NF-κB gene expression in RPMI-1640 medium. A nonparametric Kruskal-Wallis analysis of variance was performed N=4.

Lastly, the incubation of the co-culture with catechins before their stimulation with the cocktail of pro-inflammatory cytokines did not modify the expression of CXCL-8 and NF-κB genes in either the DMEM (Figure 14 a,c) or the RPMI-1640 (Figure 14 b,d) medium.

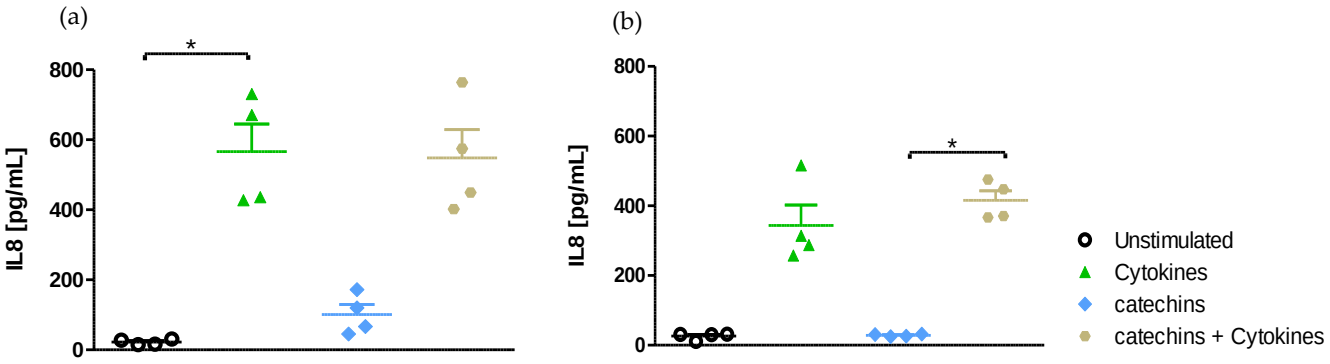
Catechins did not prevent the upregulation of CXCL-8 observed with the cocktail of cytokines regardless of the medium used.

Effect of medium on IL-8-induced secretion. The exposure of Caco-2/HT29-MTX cells grown in Transwell® chambers to the cocktail of pro-inflammatory cytokines for 6h in the absence or presence of catechins significantly ($p < 0.05$) stimulated the secretion of IL-8 in DMEM medium (566.5 ± 78.7 vs. 20.0 ± 2.9 pg/mL in unstimulated cells) (Figure 15a) but not in RPMI-1640 medium (343.9 ± 58.7 vs. 28.5 ± 2.6 pg/mL in unstimulated cells) (Figure 15b).

The exposure of the co-culture to catechins alone did not stimulate the secretion of IL-8 either in the apical DMEM medium (Figure 15a) or the apical RPMI-1640 medium (Figure 15b).

We did not observe any reduction in IL-8 secretion in cells pre-incubated with catechins before their stimulation with the cocktail of cytokines in either medium (Figure 15 a,b).

Figure 15: *Effect of medium on IL-8 secretion modulation after exposure to catechins and a cocktail of pro-inflammatory cytokines.*



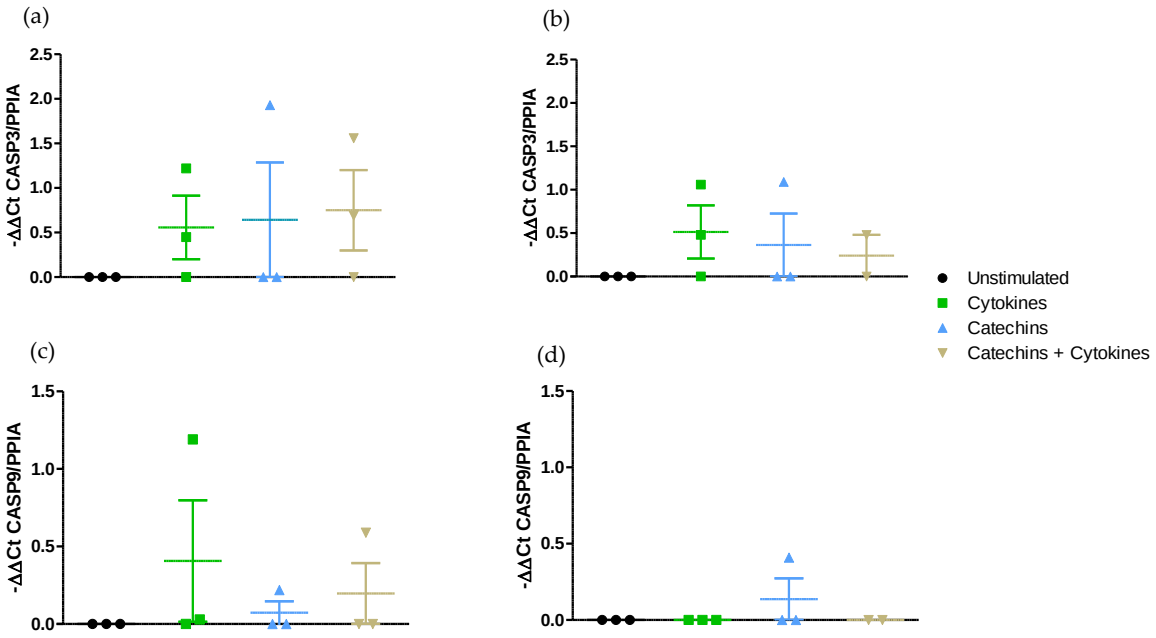
Source: Personal creation elaborated with GraphPad Prism software.
Note: (a) IL-8 secretion modulation in DMEM medium. (b) IL-8 secretion modulation in RPMI-1640 medium. * Significantly different ($p<0.05$; $p<0.01$) from unstimulated cells. + Significantly different ($p<0.05$) from catechins stimulated cells. A nonparametric Kruskal-Wallis analysis of variance was performed, when the difference was statistically significant, a Dunn's post hoc test was then applied to analyze the effects. N=4.

The upregulation of IL-8 secretion by the cocktail of cytokines was only slightly limited by catechins in DMEM medium.

Effect pro-apoptotic pathways CASP3 and CASP9 gene expressions. The exposure of Caco-2/HT29-MTX cells to the cocktail of pro-inflammatory cytokines did not activate the expression of CASP3 either in the DMEM medium or in the RPMI-1640 medium (Figure 16 a,b). Furthermore, this stimulation did not upregulate the expression of CASP9 gene in either medium (Figure 16 c,d).

The incubation of the co-culture with catechins did not modify the level of expression of CASP3 or CASP9 in the two media (Figure 16). Lastly, the catechins did not change the level of expression of either CASP3 or CASP9 in the presence of the cocktail of pro-inflammatory cytokines (Figure 16).

Figure 16: *Effect of medium on CASP3 and CASP9 expression modulation by catechins after stimulation with a cocktail of pro-inflammatory cytokines.*



Source: Personal creation elaborated with GraphPad Prism software.
Note: (a) CASP3 gene expression in DMEM medium. (b) CASP3 gene expression in RPMI-1640 medium. (c) CASP9 gene expression in DMEM medium. (d) CASP9 gene expression in RPMI-1640 medium. A nonparametric Kruskal-Wallis analysis of variance was performed N=4.

The apoptotic CASP3 and CASP9 were not activated by the cocktail of pro-inflammatory cytokines either in DMEM medium or in RPMI-1640 medium, and catechins did not act on these pathways.

Discussion

The first objective of this study was to compare the response of the Caco-2/HT29-MTX co-culture, as a model of intestinal epithelium, in their conventional medium, DMEM, and in the RPMI-1640 medium, usually dedicated to immune cell culture for the complexification of the cell model. The seeding was operated at a ratio of 9:1 for all the experiments realized in this study. It has been previously evidenced in the lab that this ratio was the most appropriate one to evaluate the effects of molecules on the intestinal epithelium in Transwell® chambers (Guibourdenche et al., 2021).

To answer this question, we first assessed whether the response of the co-culture to the cocktail of pro-inflammatory cytokines (Guibourdenche et al., 2021) could be modified by the culture medium. It is indeed well established that an inappropriate culture medium could be a source of stress for cells and could change their sensitivity to exogenous stimuli or promote spontaneous pro-inflammatory pathways not seen under standard conditions (Vis et al., 2020). The first challenge was to determine whether this new cell culture condition would be suitable to work with a fully differentiated epithelium. We observed that the RPMI-1640 medium did not alter either the expression of CXCL-8 or the secretion of IL-8 in the apical medium. These results confirm that RPMI-1640 medium did not alter the growth and differentiation of either the Caco-2 or the HT29-MTX cell lines since the amplitude of responses in RPMI-1640 and in DMEM as a control medium were quite similar at both 2 and 6h. Furthermore, the response observed after stimulating the co-culture with the cocktail of cytokines is consistent with our previous results (Guibourdenche et al., 2021).

Based on this first set of data, we decided to move to the evaluation of the response of cells in Transwell® chambers. This technique is more representative of the physiological exchanges happening at the level of the intestinal epithelium. The second aim of this study was to evaluate the response of the model of intestinal epithelium to LPS from *E. coli* and compare

this response to the one observed with the cocktail of cytokines in both DMEM and RPMI-1640 media. Under physiological conditions, the intestinal epithelium is in permanent contact with the microbiota and possesses at its surface pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) which are able to bind to bacterial toxins and stimulate the pro-inflammatory response. The present study was conducted with a 6h stimulation by LPS at a concentration of 10 ng/mL (Guo et al., 2013). We did not observe any modification of the epithelial permeability with either LPS or the cocktail of cytokines in the two media. By contrast, while LPS failed to stimulate CXCL-8 gene expression, the expression of this gene was upregulated in the presence of the cytokines in both DMEM and RPMI-1640 media. This was associated with a clear secretion of IL-8 which was only significant in the DMEM medium. A previous study indicated that 1 µg/mL LPS induced an inflammatory response of Caco-2 cells without causing cell death (Calvello et al., 2016). Furthermore, the proinflammatory effect of LPS at this concentration on Caco-2 cells was significantly limited by *Lactobacillus plantarum* (Wei et al., 2022). Another work evidenced that unstimulated Caco-2 cells did not express TLR4, the well-known receptor for *E. coli* LPS, while the expression of the gene and its protein were clearly upregulated by the exposure of Caco-2 to LPS (O26:B6) for 24h at concentrations ranging from 5 to 50 µg/mL (Kuo et al., 2015). This was associated with an increase in DNA fragmentation and caspase-3 activation. Those data are contradictory to our results, but the experimental conditions may explain such discrepancies. In contrast with all the previous experiments conducted with this toxin, the stimulus applied here was on a co-culture of Caco-2/HT29-MTX cells grown on Transwell® chambers for 21 days. We cannot exclude here that an incubation of the co-culture with LPS for 24h could have resulted in a stimulation of pro-inflammatory pathways. However, the Transwell® Caco-2 model is not responding properly to pro-inflammatory stimuli for such a length of time because after a 24h nutrient deprivation (i.e., in a serum free medium), Caco-2 cell intracellular activity is altered and transepithelial permeability is increased (Le Bacquer et al., 2003). Another explanation could be that published studies were run most of the time on Caco-2 cells grown for 48–72h (Wei et al.,

2022) and not always on Transwell® chambers (Kuo et al., 2015). At this time point of cell culture, the epithelium is not fully functional, cell junctions are not established (Zeller et al., 2015), and cells may be more vulnerable to noxious stimuli. Furthermore, with this model of co-culture on Transwell® chambers, the secretion of mucus by HT29-MTX may have partly shielded the Caco-2 cells. They may have prevented the anchorage of LPS on TLR4 receptors and consequently the activation of pro-inflammatory pathway signaling at the origin of the upregulation of CXCL-8 gene expression and the secretion of the chemokine. Lastly, in our study, we worked with the O111:B4 serotype of LPS from *E. coli.* In other studies, authors applied on a monoculture of Caco-2 cells, grown for 72h in DMEM medium, the O127:B8 or the O26:B6 serotypes of LPS from *E. coli* (Meng & Lowell, 1997; Stephens & von der Weid, 2020). The difference in experimental conditions could modify the reactivity of the cells. In other words, the response of Caco-2 cells seems to be tightly linked to (a) the environment in which they are grown, i.e., simple wells vs. Transwell® systems, (b) the fact of being coupled or not with a goblet cell line, (c) the growth length (in days), and (d) the concentration and duration of the stimulation with LPS but not of the medium. It is noteworthy that our stimulatory conditions were the lowest in terms of concentration and duration. They were realized on fully differentiated cells. Altogether, these conditions certainly contribute to the weak answer observed. Between all the stimulating conditions used, ours get closer to *in vivo* conditions. Those results are of peculiar interest when considering multiple cultures with immune cells such as THP-1 differentiated in macrophages. Since LPS is able to stimulate macrophage activation and their subsequent release of pro-inflammatory signals such as TNF-α or IL-1β (Meng & Lowell, 1997), its application on a co-culture of macrophages and epithelial cells could activate the pro-inflammatory response of the epithelial cells as observed *in vivo*. Such an approach was recently conducted on a 3D flipwell model using *B. subtilis* (Beamer et al., 2023).

This set of experiments also provided evidence on the suitability of RPMI-1640 to grow the co-culture although the upregulation of the CXCL-8 gene and the secretion of the protein in

the apical medium was lower than in their usual DMEM medium. Both the DMEM and the RPMI-1640 medium contain a balanced mixture of essential nutrients including amino acids and vitamins and a sodium bicarbonate buffer to help to maintain a physiological pH at a 5–10% CO2 environment. None of them contain growth factors which were brought by the same FBS. However, the main difference between the two media comes from the fact that RPMI-1640 contains glutathione (a reducing agent) and high concentrations of vitamins such as biotin, vitamin B12, and parabenzoic acid (PABA). Such compounds contribute to oxidative stress protection and prevent the impairment of DNA synthesis and methylation which are of prime importance for adequate cell growth (Labarrere & Kassab, 2022; Peterson et al., 2020).

The absence of upregulation of NF-κB under those conditions is intriguing. However, it could be due to the final timepoint chosen (6h). The literature clearly indicates that TNF-α is responsible for a quick activation of this transcription factor at the origin of the upregulation of the promoter of the CXCL-8 gene which is fully consistent with our data (Vallee et al., 2004). However, we did not observe any change in NF-κB gene expression contrary to a previous study undertaken with similar conditions (Guibourdenche et al., 2021).. This may be explained by the fact that, in that work, the stimulation was applied to the Caco-2/TC7 clone but not the traditional Caco-2 cell line, and these data confirm that these two cell types do not quite behave similarly.

The very last part of the study was dedicated to the evaluation of the so-called protective effects of dietary polyphenols on the prevention of the pro-inflammatory response associated with the cocktail of TNF-α, IL-1β, and IFN-γ. The pro-inflammatory response on the intestinal epithelium is associated with the loss of its integrity mainly due to the loss of tight junction (TJ) integrity. As such, the scientific community has been looking for the identification of dietary chemo-protectors of the epithelial barrier as an alternative pharmaceutical approach especially when considering nutritional factors in IBD patients (Amasheh et al., 2009; Gentile & Weir, 2018; Kaulmann et al., 2016). In this respect, dietary polyphenols have long been studied in an attempt

to reduce gut permeability by improving TJ function but also limiting the activation of proinflammatory signaling pathways (Yan et al., 2020). Several reviews have been dedicated to the evaluation of polyphenols in the management of IBD (Farzaei et al., 2015). Among the many different polyphenols, we opted for the evaluation of flavan-3-ol protective effects. They belong to the most representative classes of dietary polyphenols (Mena et al., 2014). Among them, catechins are the most present ones. They are quite abundant in our diet ranging from legumes to fruits such as grapes, litchis, or apples, or cocoa beans and tea leaves for the most concentrated food (Zamora-Ros et al., 2016). Their health benefits in inflammatory bowel diseases have been largely studied (Fan et al., 2017). Among the catechin forms available for *in vitro* studies, we used an antioxidant flavonoid of plant origin, (+)-Cyanidol-3, a catechin hydrate, which preserves its properties at 37 °C and pH = 7 (Curiel et al., 2009). It also is the main flavan-3-ol monomer in the human diet. When applied on human epithelial cell lines, flavan-3-ols usually promote antioxidant responses by preventing reactive oxygen species production and apoptotic pathway activation (Rodríguez-Ramiro et al., 2011). The fully differentiated co-culture of Caco-2/HT29-MTX was incubated with these catechins for 1h before exposing the epithelium to the cocktail of proinflammatory cytokines. None of the parameters studied were modified, in contrast to unstimulated cells in the presence of the polyphenol alone. Furthermore, the catechin was unable to prevent the pro-inflammatory response induced by the cocktail since we did not observe any limitation of IL-8 secretion in the apical medium, regardless of the medium used. These results are similar to those observed in a previous study using (+)-catechins on a model of Caco-2 and HT29-MTX co-culture stimulated with a conditioned medium of activated macrophages (Bianchi et al., 2019). In their work, the authors pointed out the fact that (+)-catechins were unable to limit the alteration of gut permeability of the Caco-2/HT29-MTX co-culture and the prooxidant effect of the medium of activated macrophages (Bianchi et al., 2019). The authors nevertheless pointed out that the polyphenol markedly increased the expression of claudin-7, one of the most common TJ proteins present in the intestinal epithelium. The concentration to which catechins are used is

of prime interest since their overdosage may damage the intestinal epithelium as already evidenced in animal models (Guo et al., 2023). The literature indeed describes possible adverse effects of polyphenols including pro-oxidant effects, the perturbation of transporters, and the modulation of the activity of a couple of phase I/II enzymes (Kaulmann et al., 2016). Interestingly, in this study, the concentration of the catechins was three times higher than the one used here (50 µM vs. 15 µM). As such, the concentration used here could not be considered as too high. Taken altogether, this information rather works in favor of the fact that polyphenols may not act directly on the epithelium to protect it but rather interact, *in vivo*, with the microbiota to improve intestinal health (Guo et al., 2023). The microbiota is able to metabolize polyphenols and generate catabolites promoting health (Kawabata et al., 2019). Furthermore, only 10% of the dietary polyphenols are absorbed in the small intestine. They mainly enter the colon where they are transformed into phenolic acids by the gut microbiota (Scalbert & Williamson, 2000). To confirm this hypothesis, it would then be more appropriate to apply a medium coming from the gut microbiota incubated with the (+)-catechins. One cannot exclude, however, that (+)-catechins have difficulties penetrating the cell membrane because of their poor stability and bioavailability due to their high hydrophobicity (Baba et al., 2001; Henning et al., 2008.). A previous work evidenced that, by encapsulating this polyphenol, its uptake by a monoculture of Caco-2 cells could be improved (Song et al., 2014). Another study found that encapsulating epigallocatechin-3-gallate (EGCG) (from catechin's family), with liposomes and testing it in Caco-2 cells, showed an improvement in the absorption levels (Luo et al., 2014). The encapsulation being of lipidic composition, it also allows the capsule to merge with the cell membrane and release directly its content into the cell. Nevertheless, this conclusion remains to be tested in the actual experimental conditions, i.e., a pro-inflammatory stimulus of the co-culture in Transwell® chambers.

We also were able to demonstrate the importance of catechins throughout the research of this work, but it seems relevant to highlight that other functional ingredients could be evaluated in this type of cell models. Curcumin a polyphenol from the ginger family was evaluated for the anti-

inflammatory effect on a co-culture of Caco-2/THP-1 cells evaluating by western blot the expression of TJ (ZO-1 and Claudin-1) and obtaining an upregulation of the TJ, which means the curcumin enhances the intestinal epithelial barrier integrity (Zhou et al., 2021). Nevertheless, polyphenols are not the only functional ingredients that can be tested *in vitro*. Probiotics, prebiotics and postbiotics are currently increasing their importance worldwide in market products, improving intestinal health, increasing the function of the intestinal barrier and improving the intestinal microbiota composition (Maftei et al., 2024). A study tested the probiotic and immunomodulatory properties of *Lactobacillus rhamnosus GG, L. rhamnosus KLSD, L. helveticus IMAU70129, and L. casei IMAU60214* on Caco-2 cells. In this work, authors showed that the *Lactobacillus* strains evaluated possess probiotic properties and the ability to modulate the inflammatory response, making them essential candidates for therapeutic applications in intestinal disorders such as IBD (Rocha-Ramírez et al., 2021). These pieces of literature reinforce the potential interest of developing immunonutrition approaches to limit or protect the patients suffering from chronic inflammatory disorders.

Since polyphenols are also considered as potent molecules to induce apoptosis and cell cycle arrest, particularly in the context of prevention of tumorigenesis, we were also interested in evaluating the apoptosis pathways in this study. The regulation of CASP3 and CASP9 gene expressions as, respectively, markers of the mitochondrial and intrinsic apoptotic pathways was assessed (Beroske et al., 2021). In contrast to what was previously evidenced in our lab (Guibourdenche et al., 2021), we were not able to see the upregulation of CASP3 gene expression in this study. The main difference comes from the use of Caco-2 cells instead of Caco-2/TC7 cells. Furthermore, (+)-catechins did not, by themselves, influence the expression of the two genes, indicating that, under physiological conditions, this pathway is not modulated by this polyphenol.

Conclusions and recommendations

In conclusion, the co-culture of Caco-2 & HT29-MTX cells in RPMI-1640 medium does not seem to influence neither the response of both the two cell lines to a pro-inflammatory stimulus, nor the response to dietary components confirming the same level of differentiation of the two cell lines and the suitability this medium for the settlement of more complexified co-culture models. Furthermore, while the response to a cocktail of pro-inflammatory cytokines remains consistent, the activation of an inflammatory response by LPS from *E. coli* seems to be more difficult to stimulate not because of a medium effect but rather due to the mucus production by goblet cells. As thus, the investigation of pro-inflammatory stimuli and intracellular signaling responses in this fully differentiated co-culture shall be adjusted either in duration or in intensity to reproduce pathophysiological stimuli observed in IBD without neglecting the sensitivity of cells to stressful conditions of medium modifications. To avoid variable responses of the culture to LPS from *E. coli*, it is also of importance to thoroughly choose the most appropriate serotype.

However, while the health benefits of polyphenols are widely acknowledged, the demonstration of the protective effect of catechins *in vitro* is still debatable certainly because of the physicochemical properties of these molecules and more especially their hydrophobicity. The lack of efficiency of catechins in this study cannot be generalized to other polyphenols and shall be tested after a longer period of incubation and other concentrations, since this could have influenced the results obtained. One cannot exclude the fact that the concentration used in this work may be too low for our experimental conditions. We can suggest to evaluate higher concentrations of catechin (higher than 15 µM) to generate a protective effect in the epithelial cells, but not exceeding 50 µM since at this concentration the polyphenol does not limit any more intestinal permeability alteration (Bianchi et al., 2019). In fact, polyphenols efficiency is largely dose dependent and each model responds differently to them.

In the future to ascertain the antioxidant effect we propose to use the TBARS (Thiobarbituric Acid Reactive Substances) technique to evaluate oxidative stress of membrane phospholipid oxidation at the origin of permeability alteration. We also recommend measuring the expression of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), since they are involved in the antioxidant effect of catechins (Figure 1). As a matter of fact too, since the use of TEER to evaluate the permeability alteration was not the most appropriate approach and it would be interesting to measure the paracellular passage of FITC dextran in the co-culture model for the coming studies.

This work nevertheless contributed to a better understanding of the complexity of *in vitro* cellular response and points out the need of pursuing the investigation on the characterization of cellular response. Furthermore, while being widely used by the scientific community, this co-culture model is of carcinogenic origin with different cellular response. It would be necessary to move onto non-carcinogenic human cell lines to better simulate what is observed in the human intestinal epithelium. At last, although several attempts have been made to complexify the actual *in vitro* models, none are still able to reproduce the complex crosstalk existing between the gut microbiota, the epithelium, and the immune system to regulate and maintain intestinal homeostasis. There remains a long work to run in line with the one presented here to move onto a satisfactory *in vitro* model to evaluate the alterations observed in IBD patients.

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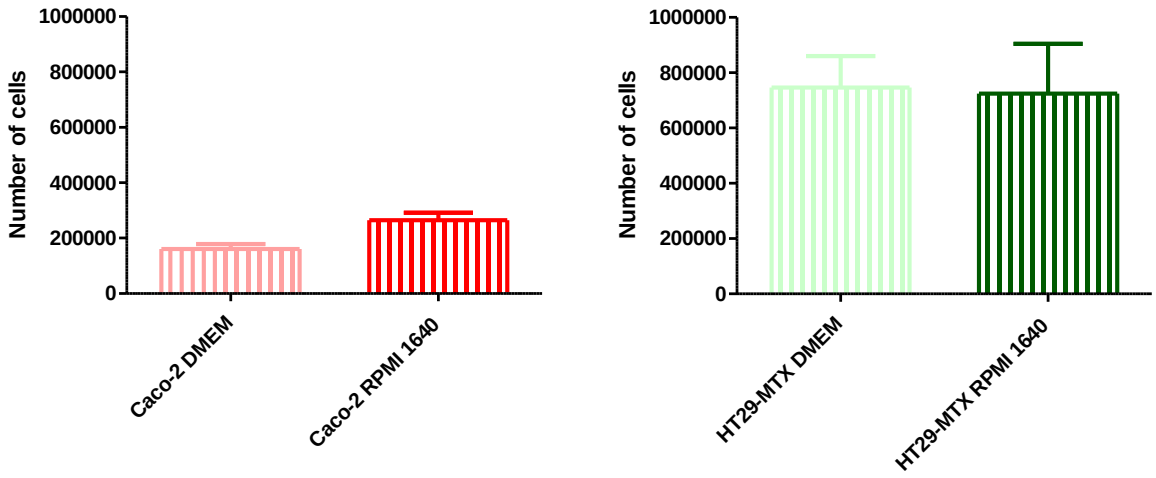
APPENDIX 1: Viability of Caco-2 and HT29MTX mono-cultures in RPMI1640 medium.

Monocultures of Caco-2 and HT29-MTX cells were seeded on a 24-well plate polycarbonate plate at a density of 1×10^5 cells/well (CytoOne, Starlab, Orsay, France), by adding 1 mL of cell suspension and culture medium per well. To assess growth and viability, the cells were grown for 21 days in RPMI 1640 and DMEM media, to compare the growth of each cell line in the different media (Figure 17 a,b), since RPMI 1640 is not their usual growth medium. Cell counting was performed using the cellometer (Nexcelom Bioscience LLC).

Figure 17:*Numbering of Caco2 and HT29MTX cells in DMEM and RPMI1640 medium after 21 days of differentiation.*



Source: Personal creation elaborated with GraphPad Prism software.
Note*:.* (a) Cell numbering of Caco2 cells in DMEM and RPMI 1640 media. (b) Cell numbering of HT29MTX cells in DMEM and RPMI 1640 media.

The number of Caco2 cells at D21 was similar between the DMEM and RPMI 1640 media (Figure 17a); This number was twice as much as the number of cells seeded at D1 and is consistent with previous studies run in the lab. Similarly, the number of HT29-MTX cells was 8-times higher the number of cells seeded at D21 in the two media (Figure 17b). As thus, the growth of Caco-2 and HT29-MTX cells was not altered in RPMI1640 medium. Furthermore, and still consistent with previous work run in the lab, the growth of HT29-MTX cells was faster than Caco-2, probably due to their respective phenotypic characteristics, although never acknowledged in the literature.