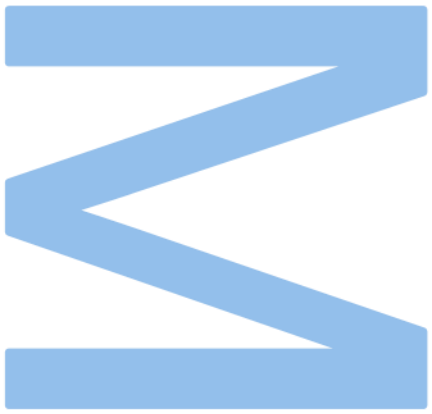
Exploring the interplay of celiac disease, gluten, and redox disturbance in intestinal epithelium: insights from cell models

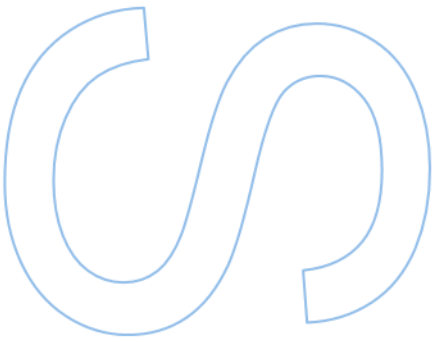
Sara Isabel Alves da Silva
Master in Food Science and Technology
Department of Chemistry and Biochemistry
2023

Supervisor
Ricardo Jorge Correia Dias, Researcher, Faculty of Sciences of the University of Porto

Co-supervisor
Victor Armando Pereira de Freitas, Full Professor, Faculty of Sciences of the University of Porto

Universidade do Minho





"Nothing in life is to be feared, it is only to be understood." - Marie Curie

Acknowledgements

Firstly, I want to thank the Food Polyphenol Lab (LAQV-REQUIMTE) for receiving me in their laboratories to perform all experimental studies during my dissertation project. Some special words of appreciation for Professor Victor Freitas, my co-supervisor, for giving me the opportunity to start my investigation journey early in this group, during my Bachelors degree in Biochemistry and allowing me to continue to work with him during my master's dissertation project.

I would also like to express my gratitude to my supervisor, Dr. Ricardo Dias, for everything he did for me, for allowing me to work alongside him on this research project, and for believing in my capacities even when I most doubted of myself. For the continuous support in the laboratory and for demonstrating me the beauty of working with cell cultures in Celiac Disease. In addition of overseeing my work, he constantly showed concern for me and my wellbeing, and I will always be grateful for that. In this moment, I know for sure that I am in the right research project with the best supervisor I could ever have.

I also acknowledge all the Food Phenol Lab researchers and students that shared with me the laboratory, for bringing a good-working environment and being always ready to help me when necessary. A special mention to Sofia and Catarina for their kind and supportive words when I most needed them and to Bernardo, for being my after 6 p.m. "babysitter". I would also like to thank Sílvia, from CEMUP, for all the encouraging words and support during this year.

I could not leave out all my friends that supported me during not only this challenging year, but also my all journey in college. To Andreia, for being my lab partner since the day 1 (literally) and helping me calm down during experimental lessons. To Dias, Beans and Zé, for helping me during my hardest moments, for the unlimited sessions of gossip and organized "prés". To Lenita and her beautiful humble soul that always surprises me. You are truly my college family and I would not switch you for anyone else. In this line, I also want to thank to my (Re)Seita crew, for always being there for me with their experience (in both academic and social worlds), to Ricky and to all the friends that share their lunch time with me in MATEC. To Jess, for being my master's friend, for all the night sessions preparing reports and presentations. In last, a much deserved thank you to all the directive board members of NEBQUP – Inesita, Cisca, Mariana and Castanho – for

understanding the difficulty of conciliate the thesis with the associative work and for always supporting my crazy ideas. We started as a team and we finished the year as a family. Without all of you, I could not “survive” this year.

Por último, gostava de agradecer todo o apoio que obtive por parte da minha família, durante este ano difícil. Aos meus pais, por perceberem o quão importante para mim está a ser esta jornada, que está apenas a começar; por todos os sacrifícios que fizeram para permitir que a menina Sara cumprisse o seu sonho de 12 anos de ser “cientista”. Sei que não percebem bem a minha área de investigação, mas reconheço o orgulho com que falam do meu percurso e é isso que me motiva a continuar o meu percurso na academia. A vocês, devo tudo o que sou.

Resumo

A doença celíaca (DC) é uma enteropatia crónica que consiste numa resposta imunológica das células a um estímulo externo – o glúten – provocando alterações na mucosa intestinal e uma perturbação do estado homeostático das células. Existem vários estudos que relatam a resposta imune associada à DC, no entanto pouco se sabe acerca dos fatores que promovem o desencadear da vertente inata desta doença em pacientes geneticamente suscetíveis.

Anteriormente avaliamos as perturbações induzidas pelo glúten em dois modelos de células do epitélio intestinal (monocultura de Caco-2 e Caco-2/HT29-MTX em co-cultura). Neste projeto, avaliamos os mesmos parâmetros, mas otimizando os modelos celulares utilizados anteriormente, quer adicionando citocinas pro-inflamatórias ou usando células associadas à resposta imune, com o principal objetivo de alcançar um modelo biologicamente mais representativo para uso em estudos relacionados à DC.

Primeiro, extraímos e digerimos gliadinas obtidas a partir de uma farinha de trigo comercial, para posterior uso como tratamento nas células. De seguida, iniciamos os ensaios celulares avaliando as perturbações do estado redox das células não só quando estas eram incubadas com gliadinas, mas também fazendo uma pré-incubação dessas células com diferentes citocinas e fatores de estimulação; com estes ensaios observamos que a resposta das células ao estímulo promovido pelas gliadinas não era dependente do tratamento externo com citocinas, uma vez que estes não foram suficientes para promover alterações significativas na resposta celular às gliadinas (via mobilização de iROS), enquanto uma inflamação aguda das células (causada por PMA) promoveu uma maior sensibilidade das células à presença de gliadinas. De seguida, realizamos ensaios em modelos celulares de Caco-2 e Caco-2/HT29-MTX em co-cultura, com pré-tratamento externo de citocinas (*i.e.* IFNγ); estes resultados sugerem que a sinalização da via inflamatória por IL-15 em células epiteliais é coordenada pela resposta imune adaptativa (Th1). Implementamos então células envolvidas na resposta imune ao nosso modelo celular, quer num estado não diferenciado (células THP-1 em suspensão), quer diferenciadas em macrófagos. As alterações observadas no potencial quimiotático da IL-8 indicam que o uso de células relacionadas com a resposta imune inata em modelos celulares pode ser uma importante adição aos modelos celulares utilizados em estudos relacionados à DC, uma vez que este aumento de IL-8 pode promover um efeito inflamatório no epitélio intestinal que pode vir a sensibilizar as

células para as gliadinas. No geral, a incubação com gliadinas resultou em distúrbios transientes no estado redox das células, no entanto a rutura da barreira epitelial causada pelas gliadinas é um aspeto importante a ter em conta, uma vez que permite a entrada de péptidos imunogénicos para a lâmina própria, onde estes causam uma resposta inflamatória nas células (quer pela via imune inata, quer pela via imune adaptativa).

Palavras-chave: doença celíaca; stress oxidativo; efeito pro-inflamatório; glúten; epitélio intestinal; modelos celulares.

Abstract

Celiac disease is a chronic enteropathy triggered by an immune response to an external stimulus – gluten – causing changes in the intestinal mucosa and a perturbation of the homeostatic state of cells. There are multiple studies unveiling the immune response associated to CD, however little is known about the onset of this disease and what triggers the gluten intolerance in genetically susceptible patients.

In previous work, the gluten-induced redox perturbations in two intestinal epithelial cell systems (Caco-2 monoculture and Caco-2/HT29-MTX co-culture) was evaluated and published. In this project, we evaluated the same parameters as before, but optimizing the cell systems used either through the addition of pro-inflammatory cytokines or use of immune cells, with the main goal of achieving a more biologically representative model to use in CD-related studies.

First, we extracted and digested gliadin proteins from a common commercial wheat flour for use as our gluten treatment in cells. Then, we started the cell experiments by accessing the redox perturbations observed in cells not only when gliadins were applied, but also pre-treating these cells with different cytokines and stimulant factors. From this, we observed that the cells response to a gliadin stimulus is not dependent of external treatment with cytokines, since this was not sufficient to promote changes in cell response to gliadins (through iROS), while a more significative inflammation of the cells (caused by PMA) induced an higher sensitivity of cells to gliadin peptides. Additionally, we performed assays in Caco-2 and Caco-2/HT29-MTX co-culture models with an external cytokine pre-treatment (IFNγ); these results suggested that IL-15 signaling in epithelial cells is coordinated by the adaptive immune response (Th1). We then implemented immune cells in our cell model, in a non-differentiated state (THP-1 cells in suspension) and differentiated into macrophage-like cells. The changes observed in the chemotactic potential of IL-8 indicate that adding innate immune-related cells in epithelial cell models can be an important upgrade for *in vitro* CD-related studies, as this IL-8 pool can promote an inflammatory effect in the epithelium that can sensibilize the cells to gliadins. The incubation with gliadins resulted in transient perturbations in the cell redox state, however the epithelial disruption caused by gliadins is a key aspect to account for, allowing immunogenic peptide to enter the lamina propria, which in turn propagates the inflammatory response in cells (through the innate and adaptive pathways).

Keywords: Celiac disease, oxidative stress, pro-inflammatory effect, wheat gluten, intestinal epithelium, cell models.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AcC	1x Cell Activation Cocktail
CAT	Catalase
CD	Celiac Disease
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
GST	Glutathione S-transferase
HLA	Human Leucocyte Antigen
IEC	Intestinal Epithelial Cell
IELs	Intraepithelial Lymphocytes
IFN	Interferon
IL	Interleukin
iROS	intracellular Reactive Oxygen Species
MIC	Major Histocompatibility complex class I chain-related molecules
NF	Transcription Nuclear Factor
Nfr2	Nuclear erythroid related factor 2
NK	Natural-kill-cell
PMA	Phorbol-12-myristate 13-acetate
ROS	Reactive oxygen Species

SOD Superoxide Dismutase

TEER Transepithelial Eletrical Resistance

Th T-helper cells

TNF Tumor Necrosis factor

tTG2 Tissue Transglutaminase 2

ZO-1 Zonula Occludens-1

1. Introduction

1.1. Celiac Disease

1.1.1. Definition and Epidemiology

Celiac Disease (CD) is an autoimmune enteropathy triggered by an external stimulus – dietary gluten, present in wheat, rye, barley and other cereals – in genetically predisposed individuals, *i.e.*, carriers of the human leucocyte antigen (HLA)-DQ2 and HLA-DQ8 haplotypes. This exposure can cause multiple lesions in the small intestinal mucosa, such as crypt hyperplasia and villous atrophy, boosted by an immune response that includes an increase of intraepithelial lymphocytes (IELs) (Green & Cellier, 2007).

In the past, CD was seen as an uncommon disease, affecting essentially children with European provenience. However, in most recent years, CD has begun to be seen as an relevant public health problem, with a worldwide increase in the number of diagnosed patients, independently of age. This increase can be explained not only by a global increase of gluten-rich foods consumption, ingestion of cereals with poorer quality, but also by other environmental conditions that caused changes in the human gut microbiome, susceptibility to infections, among others. The increase in CD prevalence can be, in fact, due to all this factors mentioned previously, however we need to account for the fact that there is an increased awareness to the disease and there are much more reliable diagnostic options available nowadays (Bradauskiene et al., 2023).

A study from 2021 revealed a biopsy-proven global CD prevalence of 0.77%, being Europe the continent with higher prevalence (1.07%), and a global prevalence of 1.25%, when CD was diagnosed by serological tests. It is estimated a biopsy-proven prevalence of 0.75% in Portugal (Bradauskiene et al., 2023); however, according to the Portuguese Celiac Association (APC, 2023), the number of diagnosed cases is much lower than the real CD prevalence in Portugal, estimating at least 85000 (0.85%) to 100000 (1.00%) undiagnosed cases.

1.1.2. Pathogenesis

CD pathogenesis is triggered by the influence of three major aspects: genetic predisposition, environmental factors and gluten ingestion (Green & Cellier, 2007).

1.1.2.1. Genetic predisposition

As previously mentioned, the predisposing genes among CD patients are two HLA class II genes: HLA-DQ2 and HLA-DQ8. There are, in fact, other non-HLA genes involved that might contribute to the development of CD: TAGAP, LPP, CCR3, IL12A, IL18RAP, among others (Huang et al., 2017; Hunt et al., 2008; Sharma et al., 2016); however HLA-DQ2 and/or HLA-DQ8 are present and almost all CD patients. Despite the fact that these two alleles are a strong diagnosis factor, it can't be solely used to diagnose a CD patient, since the fact that 30 to 40% of the population are carriers of at least one of these alleles, without being necessarily suffering from CD (Green et al., 2015).

1.1.2.2. Gluten

Gluten is the most representative protein fraction present in cereals such as wheat, rye and barley, being the ingestion of gluten the main cause of CD. In wheat – from the total of protein content present in the grain (8 to 15%) – we have around 85 to 90% of gluten, being the remaining 10 to 15% albumin and globulins; in wheat, the gluten fraction consists of gliadins and glutenins, being the gliadins the most toxic fraction and responsible for triggering CD (Biesiekierski, 2017).

Gliadin peptides are rich in glutamine and proline, which promotes high resistance to degradation – caused by gastric and pancreatic enzymes digestion during the gastrointestinal digestion process; this inability to digest these peptides leads to their accumulation in the intestinal lumen, causing their interaction with epithelial cells, translocation to the lumen and consequent interaction with immune cells (Green & Cellier, 2007).

Wheat plays an important role in CD and different species present different immunogenicity, being this crucial to a better understanding of CD onset. In this project, the studies were performed with gliadins extracted from type 65 common wheat flour.

Common commercial flour is obtained from an hexaploid wheat (*T. aestivum L.*), and is originally a result of hybridization of tetraploid wheat *T. turgidum subsp. dicoccon* with *Ae. Tauschii* (Dvořák, 2001); this wheat type corresponds to 95% of the total wheat global production (Mohan Kumar et al., 2017).

The 33-amino acid fragment of α-gliadin (commonly referred to as 33-mer gliadin peptide) – LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF – is considered the most immunodominant fragment present in gluten and one of the most toxic peptides, being considered the main responsible for triggering CD and other gluten-related disorders (Amundarain et al., 2019).

1.1.2.3. Environmental factors

It is important to understand that genetic predisposition and gluten ingestion are necessary, but not enough conditions to trigger CD onset. We need to assess other environmental factors, such as other intestinal-related pathologies, infections, vitamin D deficiency, changes in the intestinal microbiota and even the timing of introduction of gluten in children diet (Freire et al., 2019; Green & Cellier, 2007; Pérez et al., 2017).

1.1.3. The journey of gluten: from ingestion to absorption into the lamina propria

As referred previously, gliadin peptides are rich in glutamine and proline, resulting in an increased resistance to degradation by gastric and pancreatic enzymes. Therefore the ingested gluten cannot be fully digested, being the gastrointestinal system only capable of broking down gliadins to oligopeptides of 20 to 50 amino acids; this leads to an accumulation of undigested toxic gliadin fragments in the intestinal lumen, that then gain assess to the lamina propria trough two distinct routes: paracellular and transcellular (Ménard et al., 2012; Schumann et al., 2017).

In CD individuals, were HLA-DQ2 or HLA-DQ8 genes are present, these gliadin peptides can bind to a specific chemokine receptor, CXCR3, causing an overexpression of zonulin, a protein with immunomodulatory properties responsible for opening the tight junctions (via protease activated receptor 2/ EGFR pathway), resulting in an increased

intestinal permeability. The rupture of these junctions between the cells allows the translocation of the gliadin peptides to the lamina propria. Gliadin peptides can also assess to the lamina propria via transcellular route, trough IgA/CD71 system, (Ménard et al., 2012; Schumann et al., 2017). After entering the lamina propria, these peptides suffer deamidation by the enzyme tissue transglutaminase 2 (tTG2), causing the conversion of glutamine residues into glutamic acid (Vader et al., 2002), resulting in a negative charge in these gliadin peptides. Under inflammatory conditions, this negative charge facilitates the binding of the gliadin epitope with the HLA complex (Schumann et al., 2017), triggering the proliferation of gluten-associated CD4+ T lymphocyte cells, which are responsible for immune response (Jianyuan, 2021).

1.1.4. The innate and adaptive immune responses in CD

As said before, there is an increase in CD4+ cells and these cells are able of producing pro-inflammatory cytokines, eespecially Interferon (IFN) gamma. This overexpression of IFNγ promotes the polarization of these CD4+ cells along T-helper cells (Th) 1 and Th17, being these the most common T-helper adaptive immune response pathways cell phenotypes associated with inflammatory responses. During Th1/Th17 response, several pro-inflammatory cytokines – such as INFg, tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, IL-8, IL-15, IL-21, IL-17A, IL-6, among others – are released, resulting in increase of IELs, villous atrophy, crypt hyperplasia and other damage to the intestinal epithelium (Cukrowska et al., 2017; Gianfrani et al., 2005; Green & Cellier, 2007).

The mucosal inflammation (a characteristic in CD patients) is a result of a direct interaction between gliadin peptides and epithelial cells, resulting in an innate immune response, characterized by increased IEL levels and increased expression of IL-15 in both IELs and erythrocytes. When gliadin peptides start to accumulate in the gut lumen, there are an increase in cytokines, especially IL-15, leading to an increase in major histocompatibility complex class I chain-related molecules (MIC)-A and MIC-B expression (being these antigens located in the epithelium cell surface and known for becoming activated when stressed). Simultaneously, IL-15 will also promote the activation of IELs that can express NK-G2D receptors (natural-kill-cell markers), resulting in destruction of the epithelial cells that express MIC-A and MIC-B, causing an increased cell permeability (Cukrowska et al., 2017). In **Figure 1** is represented a simplified scheme

of both innate and adaptive immune responses observed in a gluten-challenged intestinal epithelium.

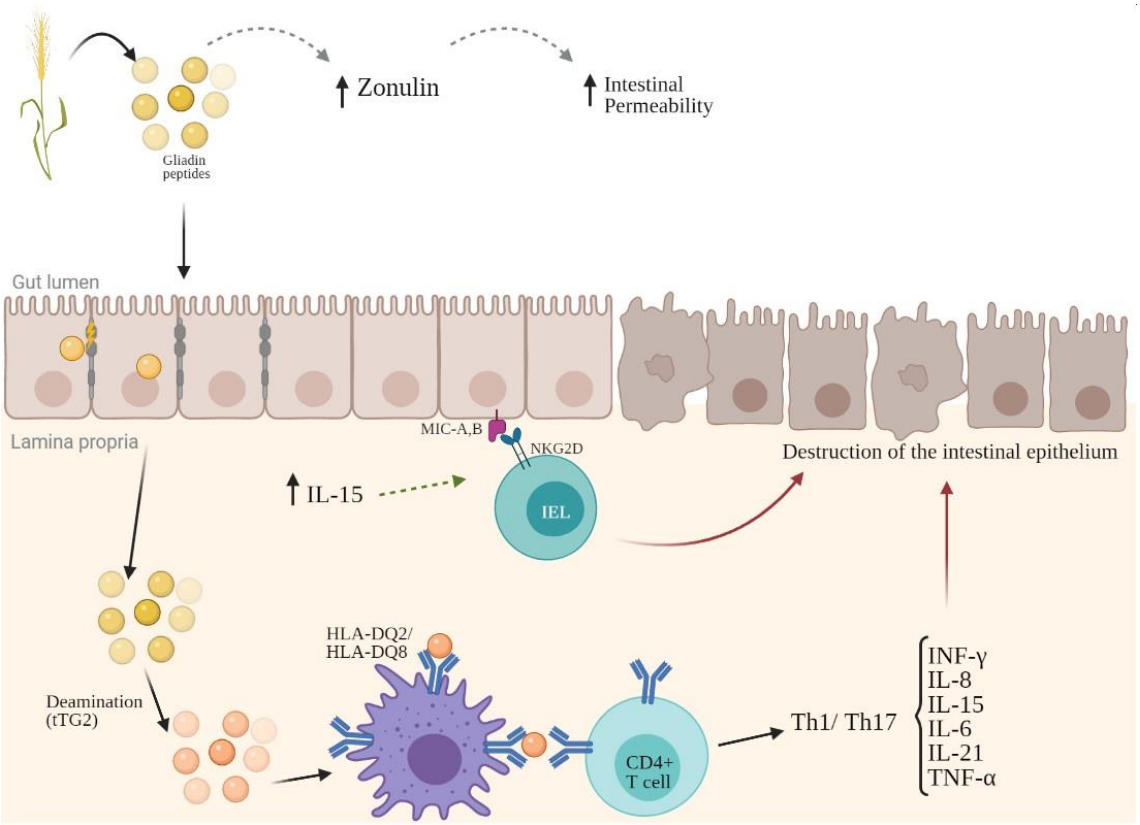


Figure 1 - Adaptive and immune responses observed in gliadin-challenged intestinal epithelium. Figure adapted from (Cukrowska et al., 2017).

The immune response observed in CD patients in response to gluten ingestion is known, as well as the mechanisms behind it. However, the causes behind the unpredictability of appearance of symptoms and consequent diagnostic at any age, even after years of regular gluten consumption remains unclear.

1.1.5. Oxidative stress in CD

Besides the epithelial damage caused by gliadin peptides in the intestinal epithelium, cell oxidative stress is also an important biomarker in CD pathogenesis.

In normal conditions, reactive oxygen species (ROS) are produced because of cell's regular metabolism (being important cell messengers) and it is necessary to control the presence of these compounds in cells, due to their oxidative power and dangerous

reactivity. Therefore, cells developed their own antioxidant defense mechanisms against ROS, using antioxidant enzymes – such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione-S-transferase (GST) and glutathione peroxidase (GPx) – to prevent cell oxidative stress. Additionally, other nonenzymatic compounds present antioxidant properties against ROS: vitamins A and C, β-carotenes, and even phenolic compounds such as flavonoids and polyphenols (Di Sabatino et al., 2015).

Despite all antioxidant cell defenses, when cells are challenged by a toxic external stimulus that cause them stress, the cell antioxidant capacity may not be enough to alleviate the increased harmful levels of ROS, leading them to an oxidative stage thus becoming stressed (Moretti et al., 2018).

Gliadins promote that redox instability in cells, as a result of excessive ROS accumulation. These ROS species, such as H_2O_2, can promote NF-κB activation and inhibition of the Nrf2 pathway, – both being transcription factors involved in cells antioxidant and immune responses – causing an upregulation in pro-inflammatory cytokines, glutathione deficiency, increased permeability in the intestinal barrier and even chronic inflammation of the intestinal mucosa. The transcription factor nuclear factor κB (NF-κB) is involved in expression of genes related to cell proliferation, apoptosis, inflammatory and innate immune responses (Lingappan, 2018; Pérez et al., 2017). The nuclear erythroid related factor 2 (Nrf2) is crucial in oxidative stress control, increasing redox transportation and reducing factors (GSH and NADPH) synthesis, as well as regenerating oxidized proteins (GSSG) and promoting SOD-mediated superoxide and peroxide catabolism (SOD, GPx) (Ma, 2013; Pérez et al., 2017).

1.2. *In vitro/ In vivo* models used in CD-related studies

A gluten-free diet is the only known treatment for CD symptomatology and the discovery of alternative therapeutic options for CD patients is necessary. There has been an increase in CD-focused research and the development of CD-relevant *in vivo* and *in vitro* models as a result of the desire to learn more about the pathophysiology of CD, as well as the understanding of the disruptive mechanisms connected to the disturbance of the intestinal homeostatic balance (caused by gliadins). These models can be very useful for unveiling the mechanisms behind the CD-onset, allowing future research on the modulation of these mechanisms trough, for instance, food bioactive compounds.

1.2.1. Mucosal biopsies

The oldest model used in CD (and in general gastrointestinal-related studies) are intestinal biopsy cultures (extracted in this case from CD patients). This model allows the conservation of all aspects observed in the small intestine and the studies can be performed considering genetic predisposition. This model allows the analysis of gluten-induced innate and adaptive immune responses, gluten translocation across intestinal epithelium, wheat immunogenicity, among other aspects (Lindfors et al., 2012; Stoven et al., 2013). However, this model has some disadvantages:

- the biopsies can only be kept in culture for 24 to 48h – after that period, certain mucosal characteristics and morphological aspects start to deteriorate;
- this is an extremely invasive procedure for CD patients that should be used in last resource to diagnostic and use in *in vitro* studies.

As a result, less invasive methods are used more frequently in CD studies.

1.2.2. Cell culture models and CD-derived T-cell lines

It is known that gluten can induce an inflammatory response in the first physical barrier of the intestinal mucosa – the epithelium – and it is crucial to understand these epithelial changes, to a better understanding of CD. Increase in zonulin release, disruption of tight junctions (and consequent transepithelial resistance (TEER) reduction and increased monolayer permeability), as well as different expression of inflammatory cytokines are key aspects that cell models need to fulfill, in order to be used in CD-related studies. Therefore, the most used cell lines in CD *in vitro* models are Caco-2 (derived from human colon carcinoma and being the most used human epithelial cell line in CD (Lea, 2015)), T84 (derived from a lung metastasis of a colon cancer (Lindfors et al., 2012)) and IEC-6 (small intestinal epithelial cell line found in rats, with epithelial crypt cells characteristics (Yi et al., 1995)) cells. In addition, Caco-2 cells can also be used to study the gut microbiota, in terms of inflammatory response promoted by gliadins and the interaction of this external stimulus with the intestinal microbiome (Lea, 2015; Stoven et al., 2013).

However, we cannot assume that a cell model consisting in solely epithelial cells is an accurate model to study CD. Our intestinal mucosa is very complex and is crucial the development of cell models biologically more accurate. Therefore, cell co-culture models

are used, consisting in the use of Caco-2 cells with monocytes (U937), fibroblasts (L929) or human cancer colon cell lines (HT-29) (Truzzi et al., 2020), providing more accurate cell models and allowing the study of a wider range of cell responses to gluten. The HT-29 cell line can also be treated with methotrexate (MTX), resulting in a more stable sub-population of mucus-secreting cells with a differentiated cell phenotype that can secrete small amounts of MUC2 mucins - heavily 25 glycosylated proteins secreted typically by the small and large intestine, forming the intestinal mucus layer (Huet et al., 1995). A study performed by Gagnon et al. between the Caco-2, HT-29 and HT29-MTX cells revealed that these mucin-secreting cells are more suitable for studying the interactions in the intestinal epithelial mucosa, promoting a much physiologically relevant cell model (Gagnon et al., 2013).

Monocytes and dendritic cells are also used in CD studies, since their ability to interact with T-cells can provide more information about gluten-induced increase in inflammatory cytokines (Stoven et al., 2013). Studies performed by Jelínková et al. revealed that both monocytes and dendritic cells have increased IL-8 and TNFα levels after direct gliadin stimulation (Jelínková et al., 2004; Palová-Jelínková et al., 2005).

Altogether, these cells provide a simple, yet effective, model to study gluten-induced effects and testing novel therapeutics; however, cell lines used in these models do not present an important feature associated to CD, *i.e.*, genetic predisposition.

To overcome that obstacle, some T-cell and antigen presenting cell models are used in CD studies, especially to evaluate the adaptive immune response to gluten and test new treatment options. Nevertheless, the T-cell response to different gluten peptides varies from patient to patient, revealing limitations when studying the general toxicity of gluten peptides, being necessary a large sample obtained from different patients (Lindfors et al., 2012; Stoven et al., 2013).

1.2.3. *In vivo* models

In vitro models allow a good insight into CD pathogenesis, however these models do not replicate the neurologic and hormone signaling from other organs or cells, as observed *in vivo*. Therefore, animal models are used in different ways: directly (without inducing CD in the animal), CD-induced (mainly in rats) and transgenic mice expressing HLA-DQ2 and DQ8 genes (Stoven et al., 2013).

1.2.4. New approaches

There has been a search for new models that can give to researchers more control in terms of morphological, metabolic and genetic CD-related aspects, without the need of invasive approaches. As a result, recent research has focused on using new approaches, such as human gut-derived organoids (cell cultures obtained from CD stem cells) (Freire et al., 2019; Kim et al., 2020), CD-on-chip (systems made from sophisticated human induced pluripotent stem cell technology and organ-on-chip devices) (Moerkens et al., 2019), and even nanotechnology (Domsa et al., 2020).

2. Material and Methods

2.1. Reagents

Unless otherwise specified, all reagents used in this study were purchased to Sigma-Aldrich or Thermo Fisher Scientific.

2.2. Extraction of gliadin proteins

Gliadin proteins were extracted as described elsewhere (Mazzarella et al., 2014), with some modifications. Briefly, 10 g of type 65 wheat flour (obtained from Espiga, Fábricas Lusitana Produtos Alimentares, SA) were defatted twice with petroleum ether (50 mL) for 45 min, filtered and left overnight for complete petroleum ether evaporation. Albumin and globulin proteins were extracted by resuspending the defatted flour in 0.4 M NaCl, 0.067 M NaH_2PO_4 buffer at pH 7.5 (50 mL). The suspensions were shaken for 10 min at room temperature (RT) and centrifuged at 15000 g for 15 min. This process was repeated three times. Proline and glutamine-rich gliadin proteins were then extracted from the pellet by adding a 70% (v/v) ethanol solution (50 mL). The suspension was, again, shaken for 45 min at RT and centrifuged at 15000 g for 10 min.

The pellets were combined and extracted two additional times following the same procedure. Between albumin/ globulin and gliadin extractions, the pellet was washed twice with ultrapure water, with 10 min shake and then centrifugation for 5 min at 15000g.

The gliadin-enriched supernatant underwent an ethanol removal procedure on a rotary evaporator – set to 37°C – freeze-dried and stored at -20°C.

2.3. Gastrointestinal protein digestion

The *in vitro* gastrointestinal digestion of gliadin proteins was optimized according to a recently standardized protocol – INFOGEST – that uses artificial fluids simulating the exact composition of digestive juices (Brodkorb et al., 2019), with some modifications.

In brief, 200 mg of previously extracted gliadins were dissolved in 500 µL of Ultrapure Water and was added 560 µL of 1.25x simulated salivary fluid (SSF) (at pH 7.0) and 140 µL of a stock solution containing 75 U/mL of α-amylase (from Porcine Pancreas, Sigma-Aldrich) diluted in Ultrapure Water. The resulting mixture was incubated for 2 min. At this bolus was added 770 µL of 1.25x simulated gastric fluid (SGF) (pH 3.0) and 350 µL of a stock solution containing 2.000 U/mL of pepsin diluted in 1.25x SGF. Before incubation, the pH was adjusted to 3.0 by adding 5 M HCl and Ultrapure water was added to fulfill the final gastric phase volume of 2,8 mL. Then, this mixture was incubated for 120 min. Subsequently, 434 µL of 1.25x simulated intestinal fluid (SIF) (pH 7.0) and 824 µL of a stock solution containing 10 mg/mL of Bile diluted in 1.25x SIF were supplemented to our gastric digestion and incubated for 30 min. Upon this period, the addition of 982 µL of a 100 U/mL of pancreatin stock solution in 1.25x SIF, the establishment of the final pH of 7.0 – using 5 M NaOH – and volume adjustment to 5,6mL with Ultrapure Water close the final intestinal fluid composition, that was incubated for 120 min.

The whole digestion protocol was performed under constant mixing on a temperature controlled 37ºC bath. Digestion process was interrupted by heating samples at 95 °C for 5 minutes, for enzyme inactivation.

The resulting peptide digestion mixtures were subjected to solid phase extraction on 20 cc Oasis HLB cartridges (Waters) according to manufacturer instructions. The tubes were pre-conditioned with 10 mL methanol and equilibrated with 20 mL ultrapure water. After loading the peptide mixtures – diluted to a final volume of 20 mL – the tubes were washed with 10 mL ultrapure water and the peptides eluted with 20 mL of methanol:acetonitrile (50:50, v/v). The peptide mixture and wash eluate was again applied to the column, on a second extraction. Finally, the organic solvent was removed from the eluates on a rotatory evaporator (37°C), reconstituted in ultrapure water, freeze-dried and stored at -20ºC

2.4. Cell culture conditions

2.4.1. Caco-2 and HT29-MTX cells

Caco-2 and HT29-MTX cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L glucose and 4 mM L-glutamine (from Cell Line Services,

CLS), supplemented with 10% of inactivated fetal bovine serum (FBS), 1% sodium pyruvate, 1% Pen-Strep antibiotics (10000 Units/ml penicillin and 10000 µg/ ml streptomycin) and 1% (m/v) human transferrin. Cells were sub-cultured twice a week and seeded at a density of 3.0×10^6 cells (Caco-2) and 2.0×10^6 cells (HT29-MTX) per 75 cm^2 flask. The culture medium was replaced every two days. Cells were maintained in an incubator at 37°C with 5% CO_2.

2.4.2. Caco-2/ HT29-MTX cell co-culture

Caco-2 cells and Caco-2/HT29-MTX co-cultures - in a proportion of 9:1 - were seeded on the apical chamber of 6-well, 12-well or 24-well Transwell® permeable supports (Corning Inc.) or 96-well regular plates (Thermo Fisher Scientific), at a cell density of 1.0×10^5 cells/cm^2 in each insert/ well. After seeding, cells grew for 21 days and differentiated to confluent monolayers. Twelve hours before experiments (overnight), co-cultures were serum-deprived in DMEM Medium.

2.4.3. THP-1 cells

THP-1 cells were cultured in RPMI-1640 Medium containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate (from ATCC), supplemented with 10% FBS, 1% antibiotic-antimycotic (AA) and 0.05 mM β-mercaptoethanol. Cells were sub-cultured twice a week at a density of 4.0×10^5 cells/ mL. Cells were maintained in an incubator at 37°C with 5% CO_2.

2.4.4. THP-1 cell differentiation process

THP-1 cells were differentiated with two different treatments, all performed in complete RPMI medium: (a) 1x Cell Activation Cocktail with phorbol-12-myristate 13-acetate (PMA) at 40.5 µM (25 ug/mL) and ionomycin at 669.3 µM (500 ug/mL) (without Brefeldin A) (from Biolegend®) for 48h and (b) PMA at 1 ng/mL, 5 ng/mL and 50 ng/mL for 36h. Twelve hours before the experiment the cells were washed with DPBS and serum-deprived in RPMI Medium (supplemented with 1% AA and 0.05 mM β-

mercaptoethanol). All assays were performed in RPMI Medium with 1% AA and without β-mercaptoethanol.

2.5. Cell Assays

2.5.1. Caco-2/HT29-MTX Transwell

In Transwell experiments, cells were treated with various concentrations of digested gliadins dissolved in Hanks Balanced Salt Solution (HBSS) or DMEM Medium 0 or 1% FBS, for 6h or 24h. Transepithelial Electric Resistance (TEER) was measured - using a Millicel-ERS-2 volt-ohmmeter (Millipore) - before the application of the gliadin treatments and after the stipulated incubation time. The TEER values obtained for the 12-well transwells were used as control between assays and the values measured were between 350 and 400 $\Omega.cm^{-2}$ before treatment, with a decrease in TEER after treatment of approximately 30 to 50 $\Omega.cm^{-2}$.

2.5.2. Transwell experiments with adjuvant factors - IFNγ

When IFNγ was used, cells were incubated for 24h with 50 ng/mL IFNγ in DMEM Medium 0% FBS. After this incubation period, cells were washed and the gliadin treatment was added, for 6 or 24h.

2.5.3. Transwell experiments with adjuvant factors – THP-1 cells

THP-1 cells were used in two stages of differentiation: monocytes (non-differentiated) and macrophages (differentiated as indicated in section 2.4.4.):

- When non-differentiated cells were used, the co-culture transwell was differentiated for 21 days and the day prior to the experiment both co-culture and THP-1 cells in suspension were serum-reduced to 1% FBS. At the beginning of the experiment, THP-1 cells were moved to the transwell's bottom well, and gliadins were applied to the transwell's upper chamber.

- The differentiation of THP-1 cells was performed separately from the Caco-2/HT29-MTX co-culture. Briefly, Caco-2/HT29-MTX cells were differentiated for 21 days, as usual; in the 19th day of differentiation, the THP-1 cells in suspension

were treated with differentiation factors for 48h, in a separate plate. When both differentiation processes were achieved, cells were washed and combined, *i.e.*, the upper chamber of Caco-2/HT29-MTX is inserted into the wells with THP-1 cells and upon a brief acclimating time (20-30 min.) gliadins were applied in the upper chamber.

2.5.4. Caco-2/HT29-MTX Intracellular ROS (iROS) assessment

96-well iROS assays were performed using an adjuvant stimulus (in addition to gliadins): different cytokines (CIT) or 1x Cell Activation Cocktail (without Brefeldin A) (from Biolegend®) (AcC). In these situations, cells were serum-deprived overnight an then incubated for 24h (with CIT) or 6h (with AcC) before experiment.

iROS production was determined with 2',7'-dichlorofluorescein diacetate (H_2DCFDA) as probe. Before the application of gliadin samples, cells were washed twice with HBSS buffer and incubated with 25 µM H_2DCFDA for 30 min at 37°C (protected from light). All gliadin concentrations used were diluted from freshly prepared stock solution (1.0 mg/mL). After probe incubation time, cells were washed twice with HBSS and incubated for 6h or 24h with different concentrations of gliadin digests. Fluorescence was monitored at time 1 h, 2 h, 4 h and 6 h (or 24h, depending on the assay conditions), with temperature maintained at 37°C. The fluorescence was captured on a FlexStation™ 3 Multi-Mode Microplate Reader (Molecular Devices), with the excitation filter set to 485 nm and the emission filter to 530 nm. ROS production is expressed on fluorescence arbitrary units.

2.5.5. Immunofluorescence staining of Caco-2 and Caco-2/HT29-MTX cells

Differentiated Caco-2 and Caco-2/HT29-MTX (seeded in a four-compartment cell culture dish with high optical quality) were pre-incubated with either a cocktail of cytokines containing 50 ng/mL IFNγ, 20 ng/mL TNFα and 10 ng/mL IL1b) (2) or 5 ng/mL PMA (3) for 24h in 1% DMEM; compartments (1) and (2) did not have any pre-stimulation. After this first stimulus, all compartments (except (1)) were subjected to a 24h period of incubation with 1 mg/mL gliadins.

After challenging our cells with different conditions, we performed an immunofluorescence protocol for adherent cells adapted from a standard protocol offered by Thermo Fisher Scientific. Firstly, we did the fixation of the cells, incubating the plates at 37ºC for 10 minutes with 4% paraformaldehyde (pH 7.4); secondly, the permeabilization was achieved by incubating the cells for 15 minutes at 37ºC with 0.1% Triton-X in PBS; then, a blocking buffer is used for 1h at room temperature – in this case, 2% BSA in PBS. After preparing our plates, the immunostaining procedure starts by incubating the cells with a primary antibody (in this case, ZO-1 antibody was used at 5 µg/mL in blocking buffer and this was incubated overnight at 4ºC). Until this phase, the cells were washed three times with PBS between treatments. Finally, cells were incubated with a secondary antibody (in this case, Hoechst dye DAPI (for the nuclei)) at 1:10000 dilution in blocking buffer for 45 minutes at room temperature, protected from light. The cells can be storage at 4ºC until the imaging; to do so, it is necessary to wash them three times with 0.1% Tween-20 in PBS and apply a mounting medium containing an antifade agent. The immunofluorescence staining was visualized trough a fluorescence inverted microscope Leica DMi8 using 100x magnification. The images were treated using the software FIJI.

2.6. Cytosolic cell extract preparation

Confluent Caco-2/HT29-MTX cells were washed twice with ice-cold DPBS, collected with trypsin-EDTA at 37ºC, homogenized by agitation for 45 min in ice-cold extraction buffer (100 mM phosphate buffer pH 7.4, 1 mM EDTA (KPE buffer) with 0.6% sulfosalicylic acid, 0.1% Triton X-100 and protease inhibitors (from Roche)). After homogenization, the cell extract was centrifuged at 3.000 g for 4 min at 4 °C (Rahman et al., 2006). The resulting cytosolic extracts were used for protein determinations and enzymatic assays.

THP-1 differentiated cells were washed twice with ice-cold DPBS and harvested with 1 mL of DPBS, using a cell scraper.

2.7. Antioxidant and detoxifying defenses evaluation

Reduced (GSH) and oxidized (GSSG) glutathione levels were quantified by using the 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB) recycling assay as described elsewhere (Rahman et al., 2006). Glutathione reductase (GR) and glutathione-S-transferase (GST) activities were spectrophotometrically assayed by standard protocols (Mannervik, 1999; Vontas et al., 2000) and, upon normalization to the protein content, they were expressed as ΔAbs 340 $nm.min^{-1}.mg^{-1}$

2.8. Cytotoxic activity of gliadin digests

IL-8 and IL-15 cytokine production was quantified in Transwell recovered supernatants, using enzyme-linked immunosorbent assay (ELISA) kits, obtained from Invitrogen® (IL-15) and Biolegend® (IL-8).

2.9. Statistical analysis

Differences between different treatment conditions and between replicates were determined by ordinary one-way ANOVA followed by Tukey's multiple-comparison test, with a single pooled variance, on GraphPad Prism 8 software. $P < 0.05$ was considered significantly relevant.

3. Results and Discussion

As previously referred, little is known about the onset of CD. Although considerable research has been dedicated to understanding the adaptive immune response in CD patients – mostly concerning the role of gluten-specific T-cells (Jabri & Sollid, 2017), tTG (Caputo et al., 2009), among other antigen-presenting cells associated with the immune response (Qiao et al., 2012) – there is a need to investigate the innate immune response associated to CD, to achieve a better understanding of the role of macrophages and other immune-related cells in the detection and onset of immune response. In our research, our focus is to understand the early stages of the disease, concerning the first interaction of gliadins with the intestinal epithelium, and the consequent immune response.

We recently published an article – Attachment 1, in *Food Research International* (*https://doi.org/10.1016/j.foodres.2023.113317*) – where we extracted gliadin proteins from a commercial wheat flour and then these gliadins underwent a digestive process, where they either suffered a complete gastrointestinal digestion or an incomplete digestive process (without the gastric phase); afterwards, both gliadin preparations where characterized in terms of their peptide composition and potential differences in toxicity among fragments; then, we studied the oxidative and inflammatory potential of these gliadin peptides in cell culture models (Caco-2 and Caco-2/HT29-MTX co-culture). From this, we concluded that:

- the digestibility of gliadin proteins is affected by the digestive process, being significantly dependent of pepsin activity (gastric phase);
- at an intestinal level, gliadins without gastric digestion appear to be more toxic;
- when applied to different cell models, gliadins promoted different pro-oxidative and pro-inflammatory effects; these results highlight the importance of using a differentiated co-culture cell model of Caco-2/HT29-MTX, in terms of physiologically resemblance with the human intestinal barrier.

With this project, we aimed to continue the progress made by then, not only by confirming the previous results obtained on Caco-2/HT29-MTX, but also studying both pro-oxidative and pro-inflammatory potential of wheat gluten in more complex and biologically representative epithelial cell models – either by supplementing cells with external adjuvant factors, or incorporating other relevant cells – *i.e.* immune cells.

In terms of cell assays, we can divide it in four stages:

- at first, we wanted to assess different combinations of cytokines and pro-inflammatory stimulants in our already established co-culture cell model; to do so, reactive oxygen species (ROS) quantification assays were performed with increasing gliadin concentrations, to evaluate their influence in cell inflammation process and oxidative response.
- secondly, we aimed to understand if by pre-treating our co-culture cell model with IFNγ before challenging with gliadins, we would have a much more significative change in our cells before and after gliadin incubation or if we would have different results of the ones previously reported.
- in third, we wanted to continue with the optimization of this cell model, shortening the difference between this model and the *in vivo* intestinal barrier, thus assuring an even more representative response in terms of redox cell signaling upon gluten-challenge; to achieve that goal, we implemented THP-1 cells, a cell line originated from monocytic human leukemia, used as *in vitro* cell model to explore several characteristics, including those relating to a monocyte stage as well as a differentiated stage, where cells displayed macrophage-like properties.
- Lastly, we performed immunostaining of Caco-2 and Caco-2/HT29-MTX cells to understand the differences in terms of ZO-1, a representative protein involved in intestinal barrier integrity.

3.1. ROS determination of Caco-2/HT29-MTX co-culture

In previous work, the intracellular reactive oxygen species (iROS) production in both Caco-2 and Caco-2/HT29-MTX co-culture models was accessed and changes in iROS production were detected with increasing gliadin concentrations in the Caco-2/HT29-MTX co-culture. However, we were using solely gliadins as stimulus and the results observed therein were in the borderline of statistical significance. Therefore, it was important to understand if by pre-stimulating epithelial cells with external factors we would cause changes in pro-inflammatory and redox perturbation onset.

iROS production was evaluated in differentiated Caco-2/HT29-MTX cells, using 2',7'-dichlorofluorescein diacetate (H_2DCFDA) as fluorescent probe. As presented in **Table 1**, various pro-inflammatory pre-stimuli were applied, for an incubation period of either 6 or 24 h. Upon stimulation, cells were challenged with gliadins, for a period of 4 h, followed by an MTT assay to assess cell viability.

Table 1 - iROS production was evaluated in differentiated Caco-2/HT29-MTX cells, co-cultured for 21 days in 96-well plates, using as fluorescent probe 2',7'-dichlorofluorescein diacetate (H_2DCFDA). Various pro-inflammatory stimulus were applied, for an incubation period of either 6h or 24h. Upon stimulation, cells were challenged with gliadins, for a period of 4h (at different concentrations), followed by an MTT assay to assess cell viability.

	Treatment	Concentration (ng/mL)	Pre-treatment incubation time (h)	Gliadins concentration range (mg/mL)
(1)	1x Activation Cocktail w/PMA	-	6	0,05 - 1,0
(2)	IL-21	50	24	0,05 - 1,0
	IFNγ	50		
(3)	IL-21	50	24	0,5 - 1,0
	IFNγ	50		
	TNFα	20		
	IL-15	10		

Our control group consisted in differentiated Caco-2/HT29-MTX cells without pre-treatment, were concentrations of gliadin digests ranging from 0.05 to 1.0 mg/mL were applied. Results of fluorescence monitorization after 2h and 4h are presented in **Figure 2**. There, we can observe a consistent tendency to increasing iROS levels when higher gliadin concentrations were applied. From these results, iROS percentual change after 4h of gliadins incubation is presented in both **Figure 2B** and *Figure 7* of the published article (Attachment 1, page 84 of this document).

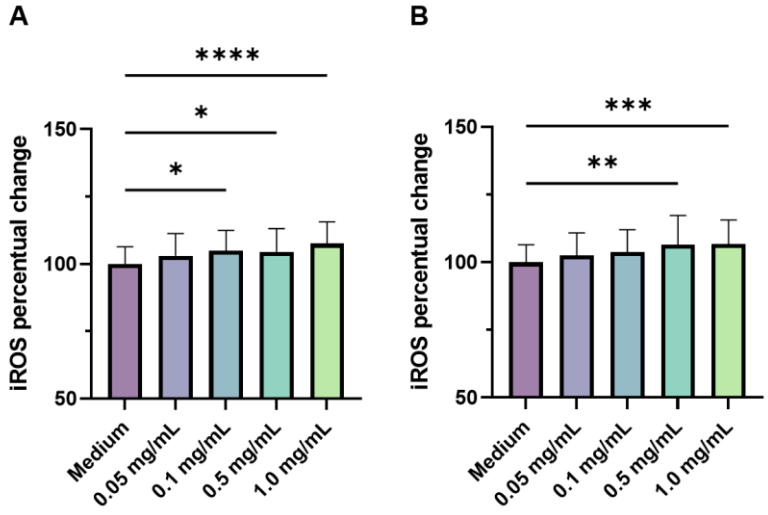


Figure 2 - Assessment of differentiated Caco-2/HT29-MTX iROS levels, using H_2DCFDA as a probe. Four different concentrations of gliadin peptides (0.05, 0.1, 0.5 and 1.0 mg/mL) were applied and fluorescence was monitored during 4h of incubation. Figure show the percentual change observed in fluorescence after 2h (A) and 4h (B) of incubation with gliadins. Assays were performed in 3 independent days, n=48. *p<0.05, **p<0.005, ***p<0.001, ****p<0.0001.

To start with, cells were treated with 1x Activation Cocktail (with 0.081 µM PMA and 1.339 µM ionomycin) (AcC), used with the goal of promoting a strong perturbation of cell redox homeostasis and pro-inflammatory state. This cocktail is commonly used to stimulate T-cells, causing activation of transcription factors such as NF-kB, inducing cytokine production (Ai et al., 2013). In Caco-2 cells, it acts as an *in vitro* pro-inflammatory stimulus (Mazzei et al., 2023), trough the complementary activity of both PMA and ionomycin.

PMA activates protein kinase C, inhibiting Na^+-dependent phosphate transportation (Boneh et al., 1989); in T84 human intestinal epithelial cell lines (similar to Caco-2 cells, being both obtained from colorectal adenocarcinomas (Devriese et al., 2017), PMA was confirmed as an intestinal barrier disruptive compound, inducing a decrease in TEER measurements after incubation (Vogt et al., 2014). In addition, ionomycin enables calcium transportation trough cell membranes, increasing the intracellular levels of calcium (Yoshida et al., 2010); in normal levels, calcium is crucial to the intestinal barrier integrity, improving barrier functionality (McClintock et al., 2020). However, when oxidative stress in triggered at the intestinal epithelium (which occurs in active CD), certain molecules involved in calcium paracellular and intracellular pathways are affected, resulting in increase of intracellular levels of calcium that can modulate the inflammatory response in cells (Diaz de Barboza et al., 2015); when excessive, this calcium accumulation (especially at a mitochondrial level) might cause cell apoptosis

(Patergnani et al., 2020). Both PMA and ionomycin also lead cells to an stimulated state were cytokine production increases, especially Th1 type cytokines (Ai et al., 2013), being this response similar to the immune response when cells are gluten-challenged.

Therefore, it is important to access the effects of this activation cocktail in our model, to understand how an already sensitized epithelium perceives gliadin peptides.

As expected, when compared with the control group, the cells pre-treated with AcC for 6h revealed a much more significative increase in iROS with higher concentration of gliadins applied and, therefore, a much more significative perturbation in cell redox homeostasis. Results are shown in **Figure 3**:

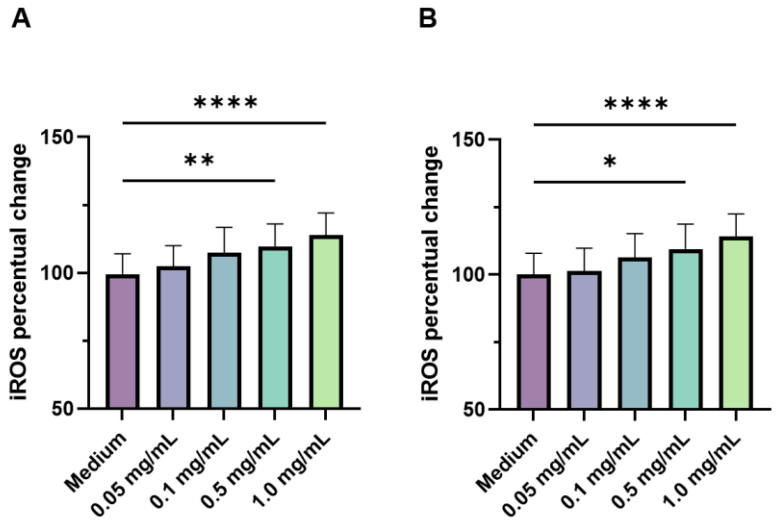


Figure 3 - Assessment of differentiated Caco-2/HT29-MTX iROS levels, using H_2DCFDA as a probe. Cells were incubated with AcC for 6h before gliadin treatment. Four different concentrations of gliadin peptides (0.05, 0.1, 0.5 and 1.0 mg/mL) were applied and fluorescence was monitored during 4h of incubation. Figure show the percentual change observed in fluorescence after 2h (A) and 4h (B) of incubation with gliadins. Assays were performed in 2 independent days, n=16. *p<0.05, **p<0.005, ****p<0.0001.

Having the confirmation that in fact an epithelial stimulant cocktail would induce changes in iROS levels, we wanted to study specific cytokines as external stimulus to evaluate the influence of this cytokines in the inflammatory response of our cell model.

To do so, we treated our cells with IL-21 + IFNγ for 24h. Interleukin-21 is one of the most relevant cytokines associated with inflammatory response in CD, being a positive regulator of Th1 cell response, stimulating chemokines secretion by epithelial cells, activating other cytotoxic cells, among many other activities (De Nitto et al., 2009). IFNγ is a pro-inflammatory cytokine with a major role in CD, involved in T-cell activation and

being the principal cytokine released during active CD, affecting the permeability of epithelial cells (Bethune et al., 2009; Wapenaar et al., 2004). Additionally, there are indications that gut epithelial cells express receptors to both cytokines (Caruso et al., 2007; Chavez et al., 1999).

To understand the involvement of these two cytokines in modulating a CD innate response, we evaluated the effects of these in our cell model. According to **Figure 4** there is a tendency to an increase in iROS levels, though in a very similar fashion with our control assay, where the cells were not pre-treated with cytokines, but only incubated with gliadins (**Figure 2**).

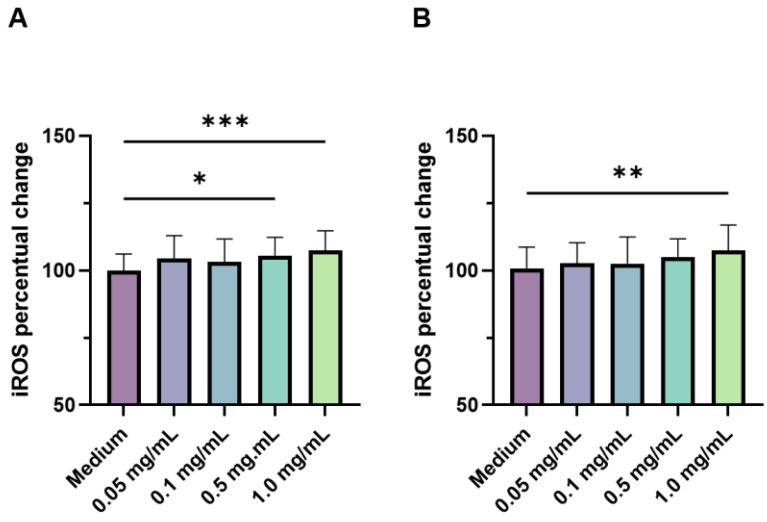


Figure 4 - Assessment of differentiated Caco-2/HT29-MTX iROS levels, using H_2DCFDA as a probe. Cells were incubated with IL-21 and IFNγ for 24h before gliadin treatment. Four different concentrations of gliadin peptides (0.05, 0.1, 0.5 and 1.0 mg/mL) were applied and fluorescence was monitored during 4h of incubation. Figure show the percentual change observed in fluorescence after 2h (A) and 4h (B) of incubation with gliadins. Assays were performed in 2 independent days, n=16. *p<0.05, **p<0.005, ***p<0.001.

To IL-21 and IFNγ, we then decided to add two more cytokines involved in CD: IL-15 and TNFα. IL-15 is a cytokine whose overexpression is associated with autoimmune disorders including CD (Ferretti et al., 2012); in terms of pro-oxidative response, IL-15 stimulates the increase of local intraepithelial lymphocytes and is one of the principal mediators of the innate immune response in CD. In terms of intestinal epithelial changes, IL-15 also promotes the proliferation of crypt enterocytes, causing crypt hyperplasia (Ferretti et al., 2012; Green et al., 2015). Tumor necrosis factor alpha (TNFα) is a cytokine with an important role in CD, mediating the damage in the intestinal mucosa, through the increase in proliferation of intraepithelial lymphocytes, activation of

chemokinesis in cells and even the regulation in transcription of inflammatory molecules in early stages of the inflammation (Cherñavsky et al., 2008). For this assay, we only assessed two gliadin concentrations: 0.5 and 1.0 mg/mL. In **Figure 5** we can observe that between the two gliadin concentrations there is a small, non-significant, decrease in iROS percentual change.

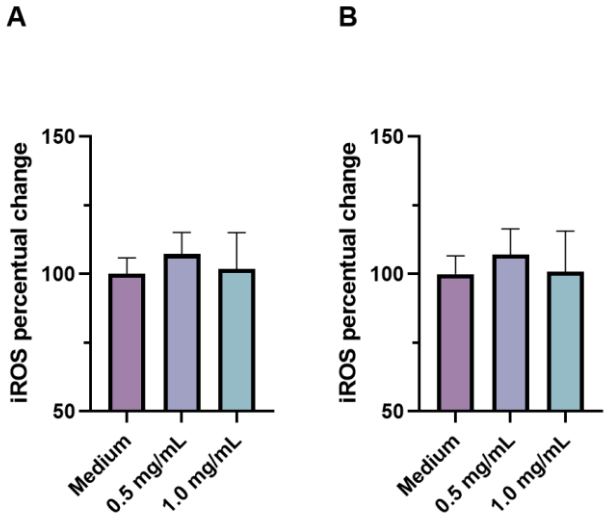


Figure 5 - Assessment of differentiated Caco-2/HT29-MTX iROS levels, using H_2DCFDA as a probe. Cells were incubated with IL-21, IFNγ, TNFα and IL-15 for 24h before gliadin treatment. Two different concentrations of gliadin peptides (0.5 and 1.0 mg/mL) were applied and fluorescence was monitored during 4h of incubation. Figure show the percentual change observed in fluorescence after 2h (A) and 4h (B) of incubation with gliadins. Assays were performed in 2 independent days, n=16.

These iROS results showed that the cells mobilize ROS when treated with gliadins, having a tendency of increasing iROS levels with higher gliadins concentrations, and this effect can be observed in cells without pre-treatment with cytokines and cells treated with different cytokines; this can indicate us that the stimulant effect promoted by cytokines was not sufficient to promote a pro-inflammatory state in cells that would cause them to be more sensibilized to gliadins. On the other hand, when non-specific epithelial barrier stimulants like PMA and ionomycin are added, cells are more sensitive to concentration increases of gliadin peptides. Such behavior indicates that when “efficiently” inflamed, epithelial cells become more responsive to gliadin peptides, which may be critical in the propagation phase of CD.

The fact of having a small pre-stimulant effect by the later cytokines (at least at ROS level) might also be explained by the fact of our cell model only be constituted by epithelial cells which are not, per se, inflammatory cells nor CD-specific. In CD, the

epithelial cells are the first cells that come into contact with gliadins. This first interaction results in a cytokine release, which contributes to the establishment of positive feedback loops that can act directly in the epithelial layer (for example, studies using TNFα and IFNγ as pro-inflammatory stimulation factors revealed a downregulation of ocludins, a membrane-linker protein that affects membrane permeability (Onyiah & Colgan, 2016)), and also induce intestinal epithelial cells and other immune-related cells to increase cytokine production, continuing the inflammatory response (Mahapatro et al., 2021). Additionally, it is important to notice that when gliadin peptides pass through this epithelial barrier, they induce an inflammatory response in dendritic cells, increasing cytokine production in Th1 cells and promoting the migration of dendritic cells in the lamina propria (Chladkova et al., 2011). Therefore, the lack of immune cells in our cell model is restricting the full potential of the cytokines, confirming that this is one of the aspects that need to be improved in our cell model, to achieve a much more representative *in vitro* model to use in CD studies.

3.2. Optimization of the intestinal epithelial cell model with external adjuvant factors - INFγ

In the previous work (Attachment 1) we observed that Caco-2 and Caco-2/HT29-MTX co-cultures grown on transwell supports, under certain conditions, suffer a perturbation of redox homeostasis when exposed to gliadin peptides – characterized by a reduction of GSH levels and antioxidant enzymes, increased cytokines levels and higher ROS production with the increase in gliadins concentration (*Figures 5 and 7* of Attachment 1, pages 83 and 84 of this document).

As observed in iROS experiments (**Figures 4** and **5**) the stimulant effect of using different combinations of cytokines was not as effective as when we used a pro-inflammatory stimulant (AcC). Therefore, we decide to simplify our approach (reducing the number of cytokines used) and understand if only using one specific Th1 cytokine we would have some changes in cell behavior before and after incubating with gliadins. Therefore, we assessed the impact of IFNγ, a common cytokine implicated in CD inflammatory response, in Caco-2 and HT29-MTX cells in order to understand the potential pro-oxidative and pro-inflammatory responses of these cells to that external adjuvant stimuli. IFNγ was the cytokine chosen for these experiments, not only because of the key role of this cytokine in CD, – being one of the cytokines responsible for the

damages in the intestinal mucosa of CD patients and being the major cytokine involved in Th1 immune response (Wapenaar et al., 2004) – but also accounting with the fact that this cytokine is able to induce changes in the intestinal epithelium directly or through the adaptive immune pathway, activating immune cells (Nilsen et al., 1998). The ability of interacting directly with the epithelial barrier is an aspect very important to us, due to the lack (at this point) of immune cells in our cell model (Caco-2/HT29-MTX).

Additionally, IFNγ is commonly used in cell experiments to evaluate changes in intestinal epithelial barrier: IFNγ was used in various studies performed in the T84 human intestinal epithelial cell line to assess barrier disruption (Adams et al., 1993; Bruewer et al., 2003; Sugi et al., 2001) and was already used in some experiments with both Caco-2 and HT29-MTX cells as an external stimuli: in 1999, Chavez et al. showed that IFNγ stimulation of Caco-2 cells can increase inducible nitric oxide synthase (iNOS) mRNA levels (related to gut barrier dysfunction) and express specific IFNγ receptors (Chavez et al., 1999); Juuti-Uusitalo et al. evaluated Caco-2 cells morphology and barrier integrity after IFNγ incubation (Juuti-Uusitalo et al., 2011) and Johnson et al. revealed that treating HT29-MTX cells with IFNγ promoted a degradation of the monolayer mucus barrier. Knowing that *in vivo* gliadins enter the lamina propria trough epithelium gaps caused by disrupted tight junctions, by pre-incubating our cells with IFNγ we might promote a barrier perturbation that could increase the effects of gliadins in our cells.

First, transwell monocultures of differentiated Caco-2 cells were incubated with 50 ng/mL of IFNγ for 24h and then treated with 1 mg/mL gliadins for 6h. After that, the same assays were performed in Caco-2/HT29-MTX co-culture, with the same 24h IFNγ incubation period and a gliadin treatment of 6h or 24h. Total GSH (tGSH) levels, GR and GST activity were quantified in both Caco-2 (**Figure 6**) and Caco-2/HT29-MTX co-culture assays (**Figures 8** and **9**).

Additionally, the cytokines IL-8 and IL-15 were selected, in resemblance of previous studies, to understand the cytotoxic activity of gliadin digests. IL-8 is a cytokine with proinflammatory characteristics, being produced in oxidative response; this is also a chemokine with a high chemoattraction to the infection site, contributing for the immune response trough neutrophils activation (Lammers et al., 2011). IL-15 is a pleiotropic cytokine that is associated with several autoimmune diseases when overexpressed – including CD (Sestak et al., 2018); IL-15 is also a pro-oxidative cytokine, inducing the increase of local intraepithelial lymphocytes during oxidative response, being one of the principal cytokines associated with the innate immune response in CD (Ferretti et al.,

2012; Green & Cellier, 2007). IL-8 and IL-15 cytokine production was quantified for Caco-2 cells (incubated with gliadins for 6h – **Figure 7**) and for Caco-2/HT29-MTX cells (incubated with gliadins for 24h – **Figure 10**). Unfortunately, we were not able to determine the cytokine levels for the experiment of 6h of gliadin incubation in our co-culture model.

3.2.1. Caco-2 cell model pre-treated with IFNγ

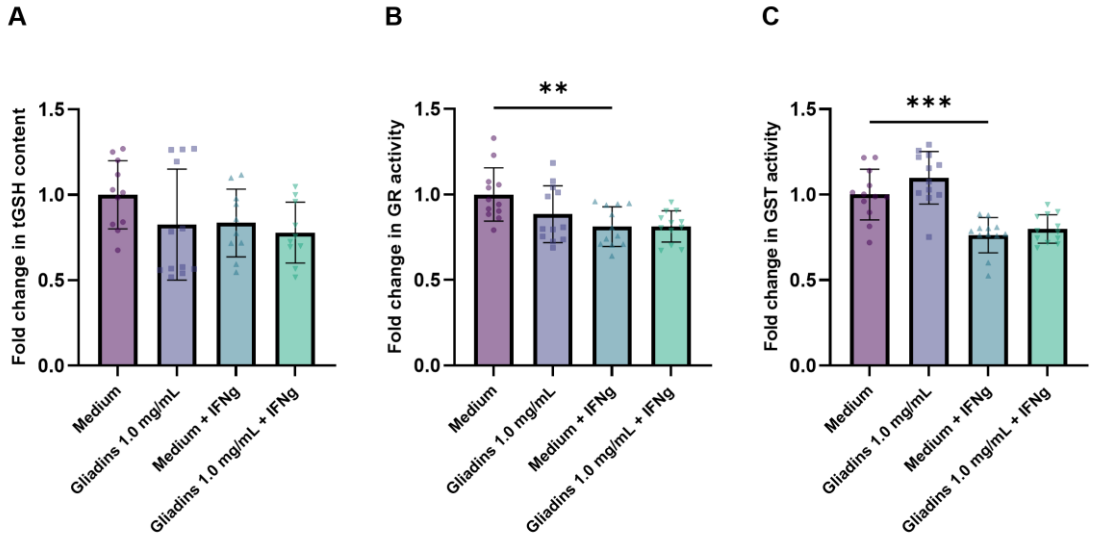


Figure 6 - Quantification of tGSH levels (A) and enzymatic activity of GR (B) and GST (C) in Caco-2 cells, treated with IFNγ for 24h. Cells were incubated with 1.0 mg/mL gliadins for 6h. Data presented in terms of fold change, normalizing the treatments with the control mean. Assays were performed in two independent days, n=6. **p<0.005, ***p<0.001.

From the results obtained for the experiments performed in Caco-2 cells (**Figure 6**) we can observe a non-significative decrease in tGSH levels when gliadins were applied (in both treated and non-treated cells). Unfortunately, we could not quantify GSSG values. In terms of GR and GST activities there are some fluctuations when cells were pre-treated with IFNγ and a reduction of both GR and GST levels occurs when IFNγ is used. Interpretating these results, we can conclude that a pre-incubation with IFNγ caused a subtle perturbation in cells redox homeostasis, and the stimulation with gliadins did not promote any additional changes in cells.

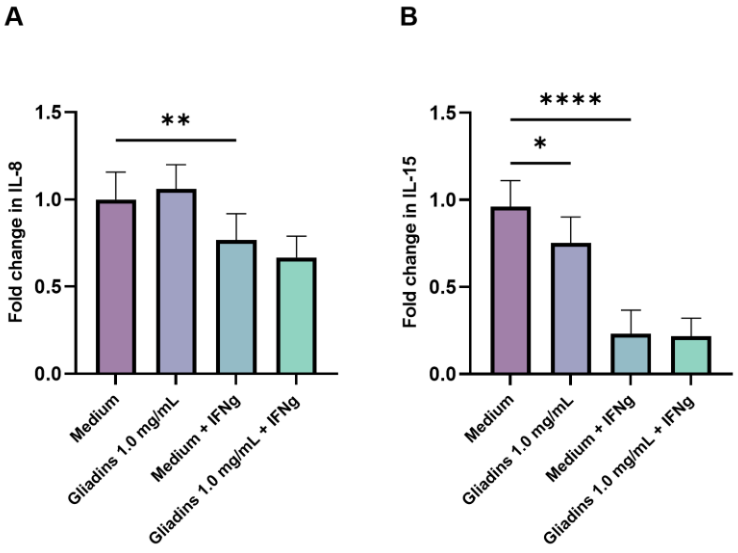


Figure 7 - Quantification of IL-8 (A) and IL-15 (B) cytokines in Caco-2 cells, treated with IFNγ for 24h. Cells were incubated with 1.0 mg/mL gliadins for 6h. Data presented in terms of fold change, normalizing the treatments with the control mean. Assays were performed in two independent days, n=6. *p<0.05, **p<0.005, ****p<0.0001.

In terms of cytokine release (**figure 7**), the IL-8 levels were low (around 3 to 4 pg/mL) and there was a decrease from the non-treated to the IFNγ-treated cells. In terms of gliadin incubation, there is a tendency of IL-8 to increase in the non-treated cells and a decrease in IFNγ treated cells, however the differences are non-significative. Regarding IL-15, we have IL-15 levels in the control without IFNγ (between 90 and 110 pg/mL) approximately 4 times higher than the levels observed when cells when treated with IFNγ. Observing the fold change in IL-15 levels, we can observe a significative reduction when cells non-incubated with IFNγ are treated with gliadins. With IFNγ, there are no differences with and without gliadins. From the decrease in both IL-8 and IL-15 values we can observe that a pre-incubation with IFNγ promoted a certain perturbation in the metabolism of cells, that needed to readjust their basal metabolic state; additionally, the incubation with gliadins was not sufficient to induce changes in cells.

In these Caco-2 assays, IFNγ caused a disturbance in cells metabolic state (in terms of glutathione metabolism - by the decrease in GR and GST activities - and inflammatory response - IL-8 and IL-15), and the addition of gliadins did not promote an additional perturbation. However, the Caco-2 model has its limitations (as explained in Attachment 1), therefore we needed to understand if these results would be the same if we used a more complex cell model – in this case, the Caco-2/HT29-MTX co-culture.

3.2.2. Caco-2/HT29-MTX co-culture cell model pre-treated with IFNγ

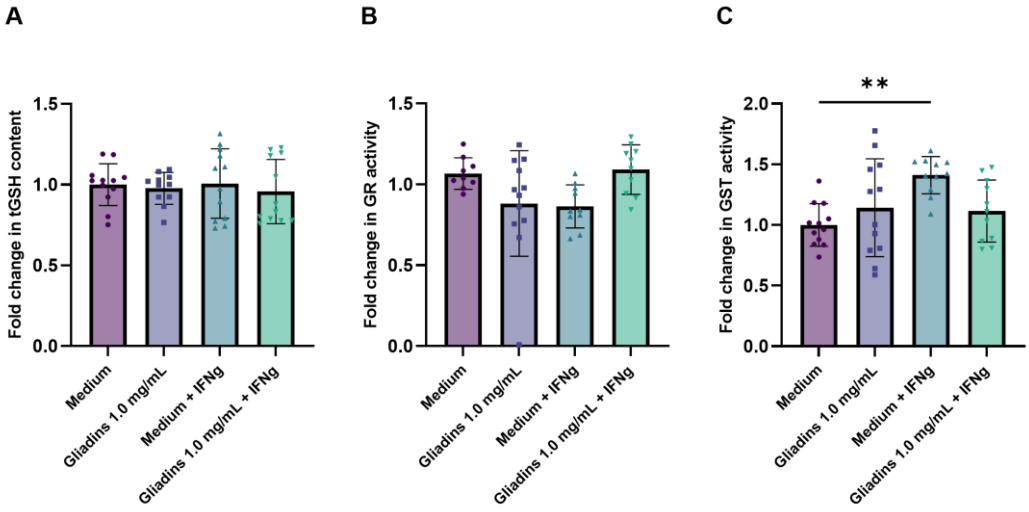


Figure 8 - Quantification of tGSH levels (A) and enzymatic activity of GR (B) and GST (C) in Caco-2/HT29-MTX co-culture cells, treated with IFNγ for 24h. Cells were incubated with 1.0 mg/mL gliadins for 6h. Data presented in terms of fold change, normalizing the treatments with the control mean. Assays were performed in two independent days, n=6. **p<0.005.

Figure 8 shows the results of the experiments performed in our co-culture model, when treated with gliadins for 6h. In terms of tGSH, there is no significative differences between treatments, only a variance in the values obtained. The GR fold change presented a higher value dispersion when cells non-treated with IFNγ were incubated with gliadins, however there are no significative differences; when pre-incubated with IFNγ, the GR rate increased from the medium to the gliadin group. In GST rates, there is a significative increase in basal levels of GST with the IFNγ incubation and comparing the influence of the gliadin treatment, a non-significative decrease from the medium to gliadin treated cells in the IFNγ-treated group is observed; from this information, we can conclude that the incubation with IFNγ caused a certain perturbation in glutathione metabolism, triggering changes in enzyme levels that were partially restored when gliadins were applied, as we can observe comparing the *Medium* and *Gliadins 1.0 mg/mL + IFNγ* columns in **Figure 8 (B and C)**.

Comparing both Caco-2 and Caco-2/HT29-MTX cell models we can conclude that in the Caco-2 model, the pre-incubation of cells with IFNγ cause them to readjust their basal metabolic state, decreasing glutathione metabolism and cytokine release. On the other

hand, the co-culture model revealed a different behavior as if the IFNγ treatment promoted a more significative perturbation in the glutathione metabolism, enabling the cells to respond to a second mild stimulation (gliadins), thus promoting adjustments in terms of glutathione-related enzymes to maintain GSH values.

To understand if this response would be similar in prolonged gluten exposure conditions, Caco-2/HT29-MTX cells experiments were performed as explained before, only changing the gliadin exposure time from 6 to 24h (**Figures 9** and **10**).

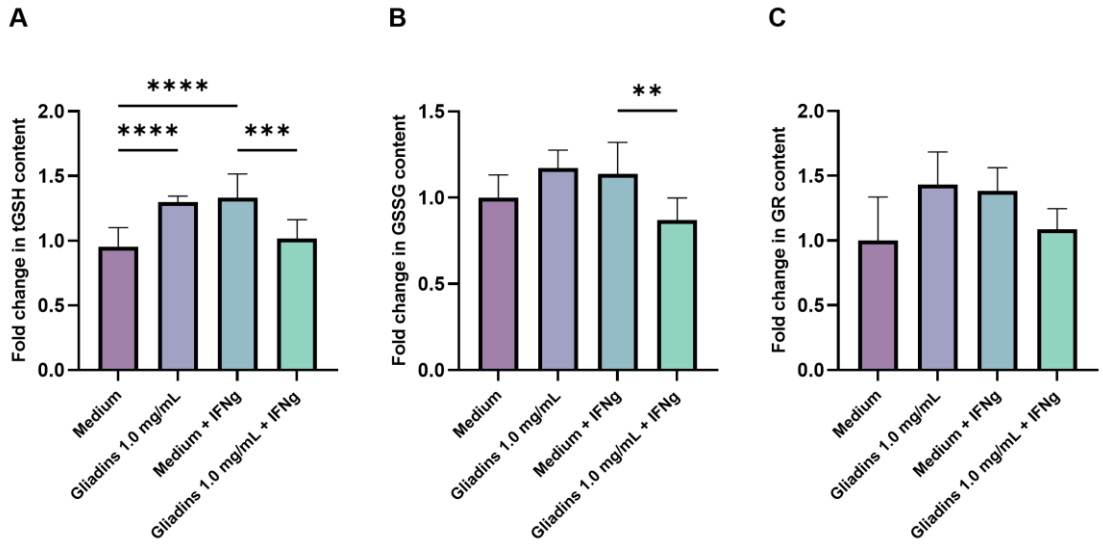


Figure 9 - Quantification of tGSH (A) and GSSG (B) levels and enzymatic activity of GR (C) in Caco-2/HT29-MTX co-culture cells, treated with IFNγ for 24h. Cells were incubated with 1.0 mg/mL gliadins for 24h. Data presented in terms of fold change, normalizing the treatments with the control mean. Assay performed once, n=3. **p<0.005, ***p<0.001, ****p<0.0001.

In **Figure 9** are presented the tGSH, GSSG and GR levels. Concerning the tGSH and GSSG levels, without the treatment with IFNγ we observe an increase in both tGSH (significative) and in GSSG fold change when incubated with gliadins for 24h; in the IFNγ treated conditions we observed a significative decrease in both tGSH and GSSG values. In terms of GR activity change, we can observe the same tendency presented in glutathione levels. Comparing all treatments, we can observe that pre-treating the cells with IFNγ and incubating non-treated cells with gliadins for 24h caused a similar reaction, in terms of their nucleophilic tone. When gliadins were applied to IFNγ-treated cells, we observe the opposite tendency as observed before. This might indicate that when a gliadin treatment is applied to cells already stimulated with IFNγ, the cells used this mild stimulus (gliadins) as a trigger for them to attempt to restore their homeostatic redox equilibrium.

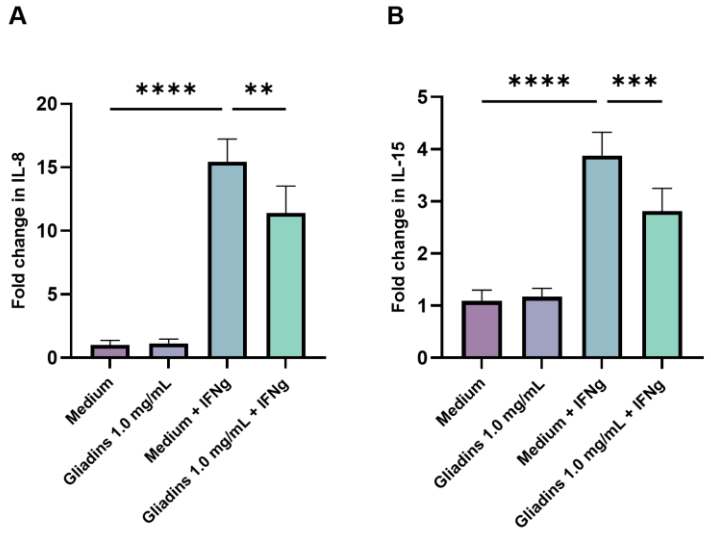


Figure 10 - Quantification of IL-8 and IL-15 cytokines fold change in Caco-2/HT29-MTX co-culture cells, treated with IFNγ. Cells were incubated with 1.0 mg/mL gliadins for 24h. Data presented in terms of fold change, normalizing the treatments with the control values mean. Assay performed once, n=3. **p<0.005, ***p<0.001, ****p<0.0001.

When we observe the cytokine levels (**Figure 10**), we have an increase in IL-8 when IFNγ-treated (from approximately 10 pg/mL to 150 pg/mL), indicating that cells have, in fact, a much more significative perturbation of their inflammatory state when IFNγ treated. When IFNγ-treated cells were incubated with gliadins, there was a reduction in cytokine levels, as if the gliadins had an hormetic effect in cells. In conclusion, a higher gliadin incubation time (24h) might have caused a similar reaction to the IFNγ stimulus in terms of glutathione metabolism, however the IFNγ caused a higher inflammatory response in cells.

In IL-8, a general cytokine with pro-inflammatory characteristics, this increase is easily understandable; however in IL-15's case, a cytokine highly specific to CD, involved in many pathways, interacting with different cell types and involved in the innate immune response (Abadie & Jabri, 2014), this increase (from approximately 70 pg/mL to 180-240 pg/mL) needs to be more carefully evaluated. In this experiment, the cells were incubated with IFNγ for 24h and then this stimulus was removed before incubation with gliadins. According to our results, the incubation with IFNγ promoted an increase in IL-15 basal levels that could be explained if the IFNγ caused a direct increase of IL-15 levels. Kim et al. had proved that IFNγ induces the production of IL-15 via IRF1 in epithelial cells (Kim et al., 2022) and in biopsy samples of pediatric CD patients, Pietz et al. observed an up-regulation of IRF1-related genes, being IFNγ is one of the causes for its up-regulation (Pietz et al., 2017). To confirm that IFNγ is directly involved in IL-15 increase in cells, we

need to perform additional replicates of this experiment; additionally, we could not determine the cytokine levels of the experiments performed with Caco-2/HT29-MTX cells, incubated 24h with IFNγ and treated 6h with gliadins. Therefore, without these replicates, we could not confirm this hypothesis.

These first experiments with IFNγ were made with the main goal of understanding how cells would react to gliadins after an CD-like adaptive cytokine stimulus. The fact that in these experiments the presence of cytokine and gliadins promoted differences (in comparison to the control) could indicate that we might have differences when we upgraded our cell model by incorporating immune cells (not only due to the capability of these cells producing cytokines, but also because of these cells involved in the innate immune response). From our results, the external stimulus promoted differences in terms of glutathione levels, glutathione associated enzymes activities and cytokine production, specially in co-cultured cells with a longer period of exposure to gliadins, indicating that the incorporation of immune cells is a necessary step into the development of a more biologically accurate cell model.

3.3. Optimization of the intestinal epithelial cell model - incorporation of THP-1 cells in Caco-2/HT29-MTX

To continue with the improvement of the cell model used in CD studies we wanted to incorporate another cell type, to approximate the cell model used with a biologically more representative one. Therefore, we included innate immune-related cells into our Caco-2/HT29-MTX co-culture model. The study of these innate immune cells is important, since these cells have a key role in immune response, specially when challenged by an external stimulus. Some of the characteristics are: detection of pathogens and other external harmful molecules; production of cytokines and other factors associated to the inflammatory response; recruitment of other cells to sites of infection, among others (Chanput et al., 2014).

Having all aspects presented before, it was crucial to choose an adequate cell line that could be versatile and accurate. The selected cell line was THP-1, a cell line derived from the monocytic human leukemia, established by Tsuchiya et al. in 1980 (Tsuchiya et al., 1980), being already used as an *in vitro* cell model to study various characteristics concerning monocyte differentiation. In intestinal cell models, THP-1 cells are already being used in co-culture with Caco-2 cells (Ishimoto et al., 2011; Kämpfer et al., 2017;

Kordulewska et al., 2021; Marzorati et al., 2023) and in studies concerning membrane integrity performed by Watanabe et al. they revealed a decrease in TEER values, due to cytokine release of THP-1 macrophage-like differentiated cells (Watanabe et al., 2004).

After choosing the immune cells we wanted to use, it was important to access the best route for optimization of this *in vitro* cell model. THP-1 monocyte cells have the ability to differentiate in either macrophage or dendritic cells, only changing the differentiation factors: THP-1 cells can be differentiated to macrophage-like cells using phorbol-12-myristate-13-acetate (PMA) (Starr et al., 2018); to differentiate these into dendritic cells, a cocktail of different factors need to be applied, such as IL-4, GM-CSF, ionomycin and TNFα (Hölken & Teusch, 2023). For CD, both cell types are crucial for the immune response: dendritic cells have a key role in CD, acting as antigen presenting cells and interacting with T-cells during the inflammatory response (Kheiri et al., 2022); macrophages are considered as key cells in the innate immune response, being the first immune cells to act when in presence of a pathogen (or other external toxic stimulus), maintaining the intestinal homeostasis (Molaaghaee-Rouzbahani et al., 2022). Being the adaptive immune response to gliadins thoroughly unveiled and given the necessity to understand the innate immune response pathways involved in CD, we opted to start our studies with macrophage-like cells, having in mind that in future works dendritic cells should be also explored.

At first, we wanted to know if non-differentiated THP-1 cells (at an immature stage) would promote any changes concerning the redox homeostatic state of cells, in terms of glutathione and cytokine levels. After that, we applied to THP-1 cells the same treatment we applied to Caco-2/HT29-MTX cells in iROS experiments – *i.e.* 1x Activation Cocktail with PMA (AcC) for 6 and 24h – since PMA is commonly used to differentiate THP-1 cells (Starr et al., 2018) and ionomycin works together with PMA as an enhancer of protein kinase C (Chatila et al., 1989).

In **Figure 11** we have a schematic presentation of the final cell model used. Briefly, it consists in an Caco-2/HT29-MTX co-culture differentiated for 21 days in the upper-chamber of a transwell plate, combined with differentiated THP-1 cells placed in the bottom wells of the plate.

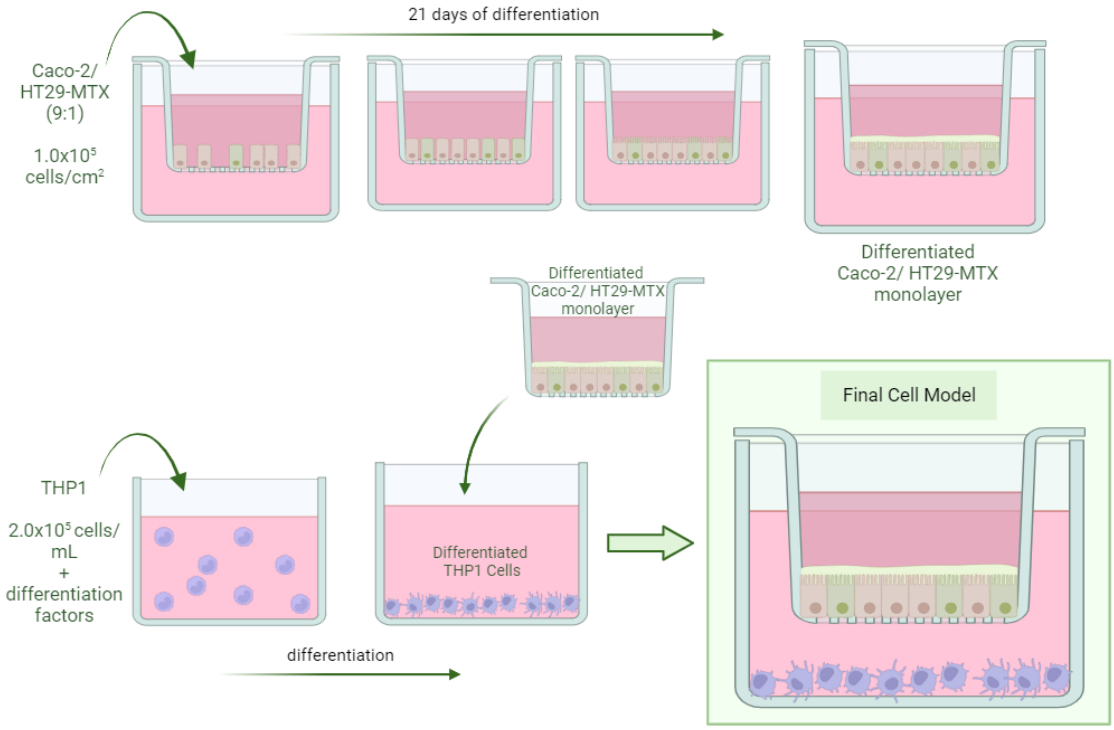


Figure 11 - Schematic representation of the final cell model developed during this project. It consists in an Caco-2/HT29-MTX co-culture seeded in the upper-chamber of an Transwell® 12–well plate at an initial 1.0x10^5 cells/ cm^2 concentration. Upon seeding, the cells were incubated for 21 days for differentiation purposes. After differentiation of the upper-chamber cells, THP-1 cells were supplemented with different differentiation factors and incubated for 48h separately until becoming differentiated THP-1 cells. Finally, both THP-1 cells and Caco-2/HT29-MTX cells were combined at the assay time.

We evaluate the same parameters as for the experiments presented in section 3.2., *i.e.* tGSH, GSSG, GR, GST and the cytokines IL-8 and IL-15 production, with a gliadin incubation of 6h. THP-1 cells were treated with different differentiation factors in 1% FBS RPMI while gliadins were applied in 1% FBS DMEM. Results are presented in **Figures 12 to 14**.

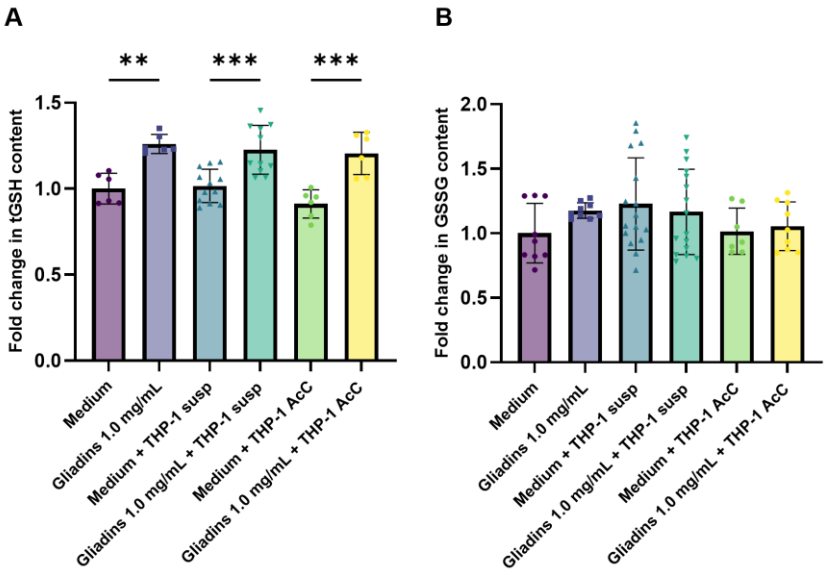


Figure 12 - Quantification of tGSH (A) and GSSG (B) levels in Caco-2/HT29-MTX co-culture cell model with THP-1 cells non-differentiated (susp) and differentiated with 1x Activation Cocktail with PMA (AcC) for 6h. Cells were incubated with 1.0 mg/mL gliadins for 6h. Data presented in terms of fold change, normalizing the treatments with the control values mean. Assay performed only once, n=3. **p<0.005, ***p<0.001.

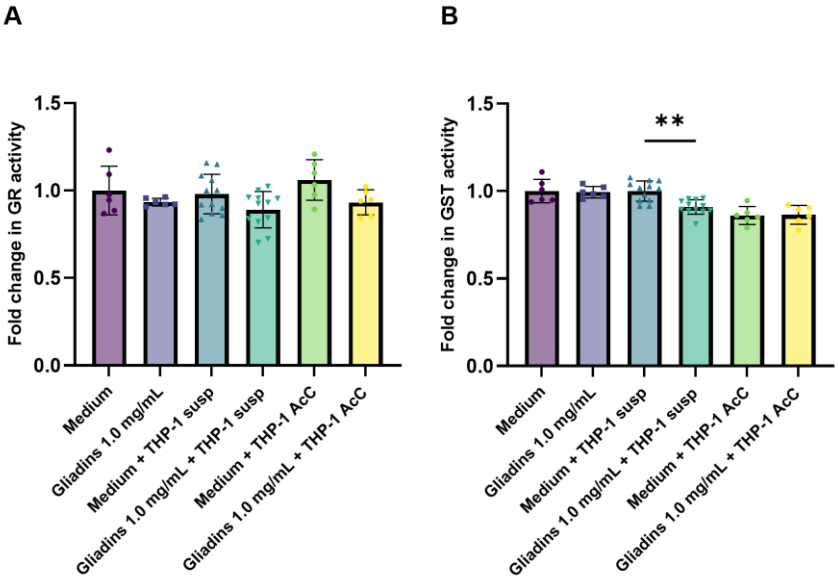


Figure 13 - Quantification of GR (A) and GST (B) activity in Caco-2/HT29-MTX co-culture cell model with THP-1 cells non-differentiated (susp) and differentiated with 1x Activation Cocktail with PMA (AcC) for 6h. Cells were incubated with 1.0 mg/mL gliadins for 6h. Data presented in terms of fold change, normalizing the treatments with the control values mean. Assay performed only once, n=3. **p<0.005.

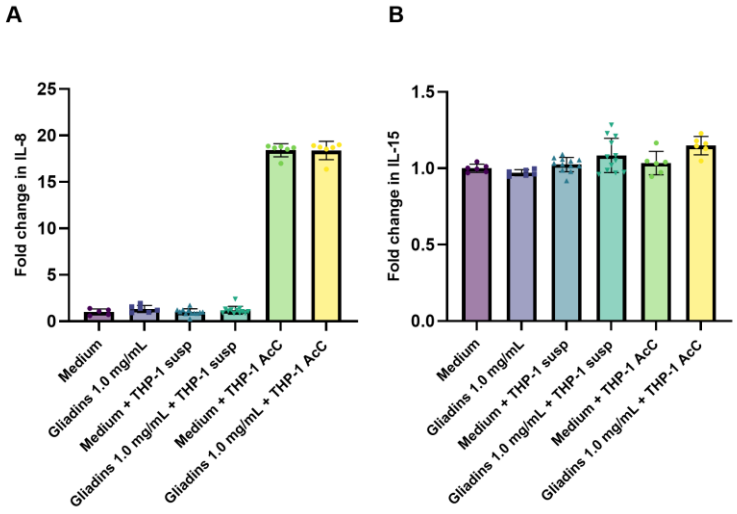


Figure 14 - Quantification of IL-8 and IL-15 levels in Caco-2/HT29-MTX co-culture cell model with THP-1 cells non-differentiated (susp) and differentiated with 1x Activation Cocktail with PMA (AcC) for 6h. Cells were incubated with 1.0 mg/mL gliadins for 6h. Data presented in terms of fold change, normalizing the treatments with the control values mean.Assay performed only once, n=3.

The control group without THP-1 cells show a significative increase in tGSH levels when gliadins are used and a tendency to increase in GSSG levels (**Figure 12**); GR and GST values did not reveal significative changes when gliadin stimulated (**Figure 13**). In terms of IL-8 and IL-15 cytokine levels (**Figure 14**), there are no differences observed in the control group. Experiments with THP-1 cells in suspension reveal significative changes in glutathione levels, presenting similar results in terms of tGSH and a higher value dispersion in terms of GSSG (**Figure 12**). Both GR and GST revealed a significative decrease when gliadins were used (**Figure 13**). In terms of cytokine release, there are no differences in terms of IL-8 fold change (around when treated with gliadins (**Figure 14A**); in IL-15 we have a non-significative increase when treated with gliadins (**Figure 14B**). When AcC was used, we observe a statistically significant increase in tGSH levels (**Figure 12A**) and GSSG levels did not change (consequently increasing GSH/GSSG ratio) (**Figure 12B**), as cells were incubated with gliadins; in terms enzymes activity (**Figure 13**), we have a decrease in GR and no changes in GST levels are observed. Having in consideration the cytokine levels, there is a significative increase from the control and non-differentiated cells to these AcC differentiated ones in IL-8 levels (from 60-80 pg/mL up to 1440 pg/mL), not revealing differences when gliadin-treated; IL15 revealed a similar tendency as observed in experiments with THP-1 cells in suspension (values between 60 and 80 pg/mL).

From all these results, we can observed that, even though we do not have significative differences in terms of glutathione, when THP-1 cells were differentiated with AcC, the co-culture cells were affected, as observed by the boost of IL-8; this indicates that the adaptive response observed was promoted by cells incubation with AcC and this response was more at an inflammatory level rather than a redox perturbation. On the other hand, the fact of not having differences in the IL-15 levels - being this the opposite of what we observed in IFNγ stimulated cells, where IL-15 levels increased when cells were treated with IFNγ, indicate that these immune cells have the ability of triggering an inflammatory response in cells, however it is not as specific to IL-15 as the IFNg stimulus; to understand better this difference in IL-15, we would need to quantify the IFNg levels in the THP-1 cells. When cells were treated with gliadins, even though we have an increase in tGSH, we did not observe any other significative changes in cell response, indicating that the cells responded to the gliadin stimulus, without significant perturbation in cell redox homeostasis.

To confirm if the cells would keep their response when subjected to a longer period of incubation with gliadins, we performed a single experiment were we evaluated the same parameters, only changing the gliadins incubation period from 6 to 24h. Results are presented in **Figures 15** to **17**:

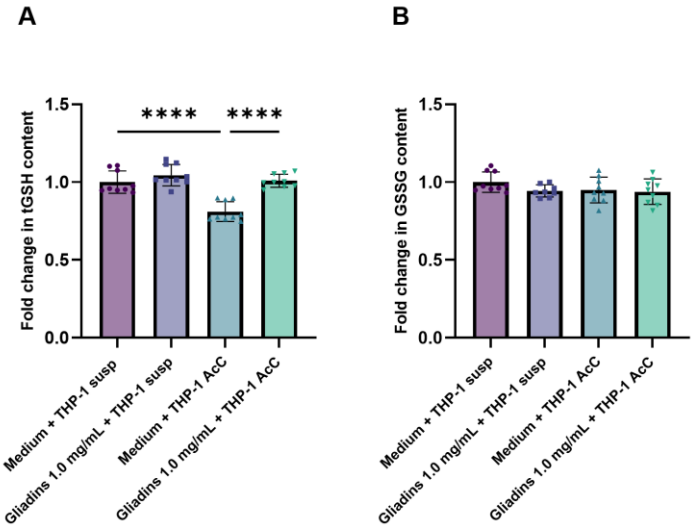


Figure 15 - Quantification of tGSH (A) and GSSG (B) levels in Caco-2/HT29-MTX co-culture cell model with THP-1 cells non-differentiated (susp) and differentiated with 1x Activation Cocktail with PMA (AcC) for 6h. Cells were incubated with 1.0 mg/mL gliadins for 24h. Data presented in terms of fold change, normalizing the treatments with the control mean. Assay performed only once, n=3. ****p<0.0001.

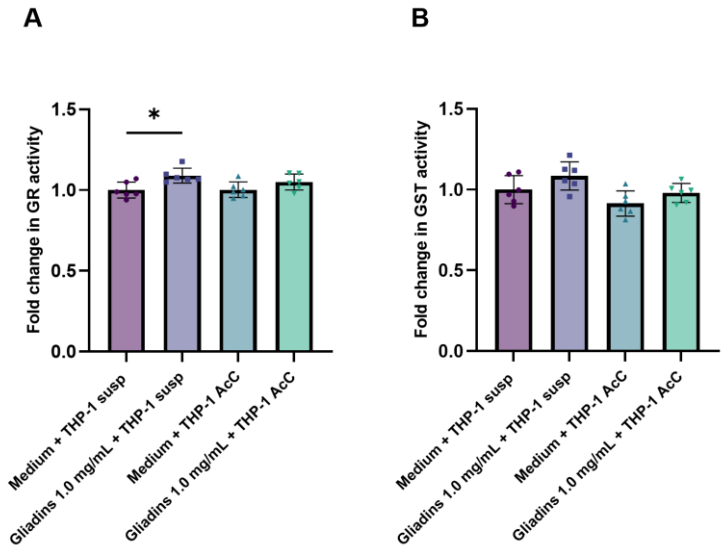


Figure 16 - Quantification of GR (A) and GST (B) activity levels in Caco-2/HT29-MTX co-culture cell model with THP-1 cells non-differentiated (susp) and differentiated with 1x Activation Cocktail with PMA (AcC) for 6h. Cells were incubated with 1.0 mg/mL gliadins for 24h. Data presented in terms of fold change, normalizing the treatments with the control mean. Assay performed only once, n=3. *p<0.05.

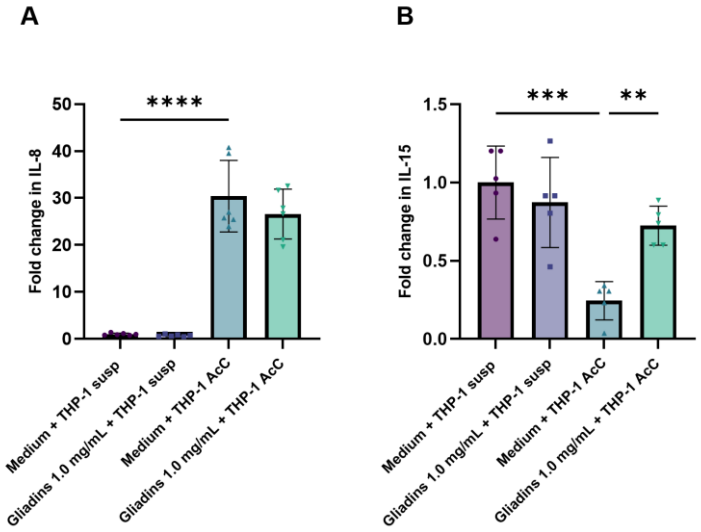


Figure 17 - Quantification of IL-8 (A) and IL-15 (B) levels in Caco-2/HT29- MTX co-culture cell model with THP-1 cells non-differentiated (susp) and differentiated with 1x Activation Cocktail with PMA (AcC) for 6h. Cells were incubated with 1.0 mg/mL gliadins for 24h. Data presented in terms of fold change, normalizing the treatments with the control mean. Assay performed only once, n=3. **p<0.005, ***p<0.001, ****p<0.0001.

As observed, when gliadins were applied for 24h there was an increase in tGSH (**Figure 15A)** when cells were differentiated with AcC. Additionally, the tendency observed in IL-8 (**Figure 17**) is maintained. These results confirmed that we needed to

refine the differentiation conditions that we applied to our THP-1 cells, in order to reduce any background activity that these differentiation factors might have that can overshadow the disturbing effect that gliadins induce to cells.

For a better control of the differentiation conditions that THP-1 cells were subjected to when differentiated with AcC, we decided to use PMA solely as differentiation factor, to refine the differentiation process and understand how PMA concentration might affect our data.

PMA is vastly used as differentiation factor in THP-1 cells and it is used in concentrations ranging from 10 ng/mL up to 400 ng/mL; however, when high concentrations of PMA are used, this compound can be too toxic, causing an up-regulation of certain genes that can mask the cell response to other external toxic stimulants (Park et al., 2007). Understanding this, we wanted to assess which PMA concentration would be the most adequate to differentiate THP-1 cells without causing such bias.

In a first assessment of which PMA concentration we should use, we performed the cytotoxicity evaluation through IL-8 and IL-15 quantification in 24-well transwell co-culture with PMA-differentiated cells, at three different concentrations. The first chosen concentration was 50 ng/mL, to use the equivalent dosage of PMA as when we treated the cells with AcC – the final concentration of PMA applied to the cells was 0.081 µM, which translates to a PMA mass concentration of 49.96 ng/mL. Then, we chose two other concentrations: 1 ng/mL and 5 ng/mL. Park et al. performed a study were they found 5 ng/mL as the minimal PMA concentration which THP-1 cells would differentiate and respond to secondary stimulus without overexpressing genes (Park et al., 2007). With these experiments, we can understand if this concentration would be effective in terms of THP-1 differentiation, without interfering directly with redox and inflammatory response when applying gliadins to this co-culture model.

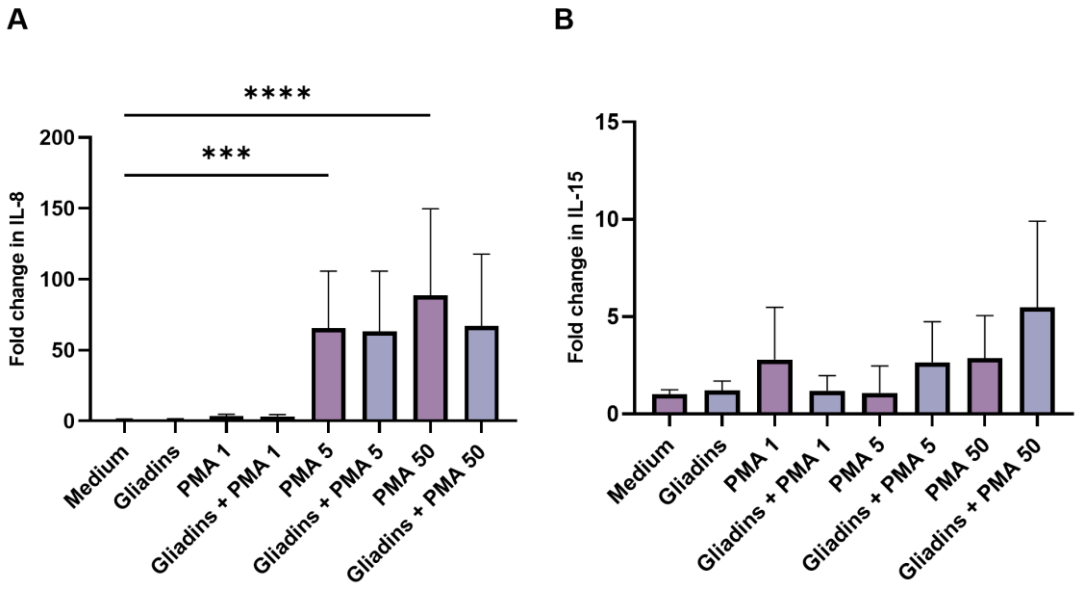


Figure 18 - Quantification of IL-8 (A) and IL-15 (B) levels in Caco-2/HT29-MTX co-culture cell model with THP-1 cells differentiated with PMA at 1, 5 and 50 ng/mL for 24h. Gliadin peptides were applied at 1.0 mg/mL for 6h. Data presented in terms of fold change, normalizing the treatments with the control mean. Assays were performed in two independent days, n=6. ***p<0.001, ****p<0.0001.

From the results obtained for IL-8 fold change – **Figure 18** – we can observe that we have a clear difference between the control and the 50 ng/mL PMA-stimulated THP-1 cells (from 8 to 500 pg/mL), giving the indication that this stimulus is extremely strong and induce an inflammatory response in cells, explaining the different results observed previously in cells. Between 1 and 5 ng/mL we can observe significative differences between the two PMA concentrations in terms of IL-8. In IL-15 we can observe that there is a shift in cytokine production from 1 to 5 ng/mL, when gliadin-treated. However, the IL-15 values obtained present high variability and need to be refined. From these results, we can understand that THP-1 differentiated with PMA present a quimiotatic potencial (explained by the IL-8 values) when PMA is used in 5 ng/mL or higher. Therefore, this behavior of THP-1 cells can promote the signaling of the inflammatory response in cells, that can sensibilize the epithelium to the presence of gliadins, being these results are a strong indicator that merging THP-1 cells (with the adequate differentiation factors) with our co-culture model is a positive move into a much more biologically accurate cell model.

A more detailed evaluation of Caco-2/HT29-MTX cells with THP-1 cells (differentiated with 1 and 5 ng/mL PMA) needed to be performed. Therefore, glutathione levels, glutathione-related enzymes and cytokines were assessed, in 12-well transwell plates. The results are presented from **Figures 19** to **21**:

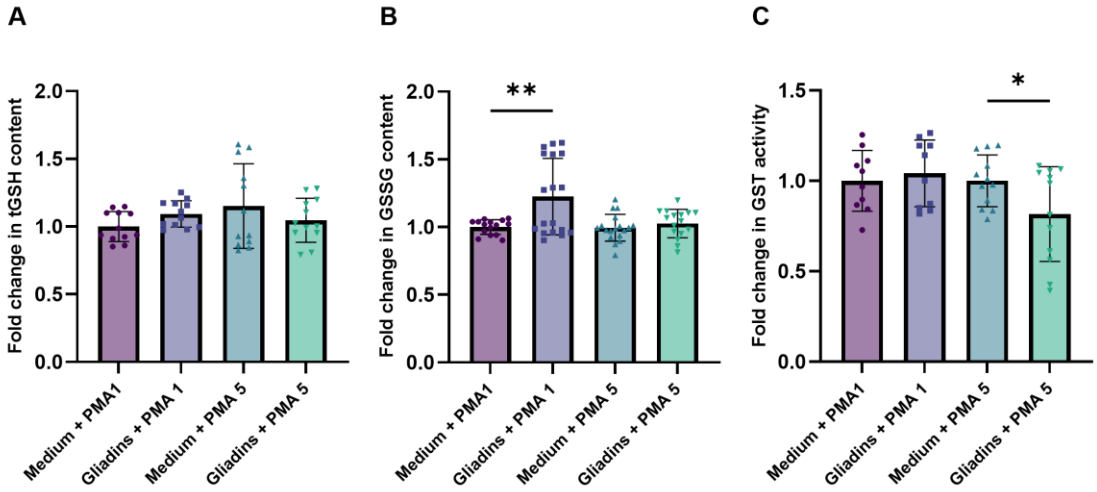


Figure 19 - Quantification of tGSH and GSSG levels and enzymatic activity of GST in Caco-2/HT29-MTX co-culture cell model with THP-1 cells differentiated with PMA at 1 and 5 ng/mL for 24h. Cells were incubated with 1.0 mg/mL gliadins for 6h. Data presented in terms of fold change, normalizing the treatments with the control values mean. Assays were performed in two independent days, n=6. *p<0.05, **p<0.005.

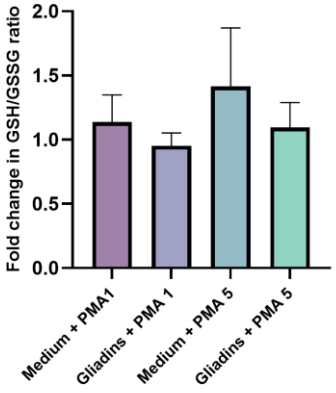


Figure 20 - GSH/GSSG ratio in Caco-2/HT29-MTX co-culture cell model with THP-1 cells differentiated with PMA at 1 and 5 ng/mL for 24h. Cells were incubated with 1.0 mg/mL gliadins for 6h. Data presented in terms of fold change, normalizing the treatments with the control values mean. Assays were performed in two independent days, n=6.

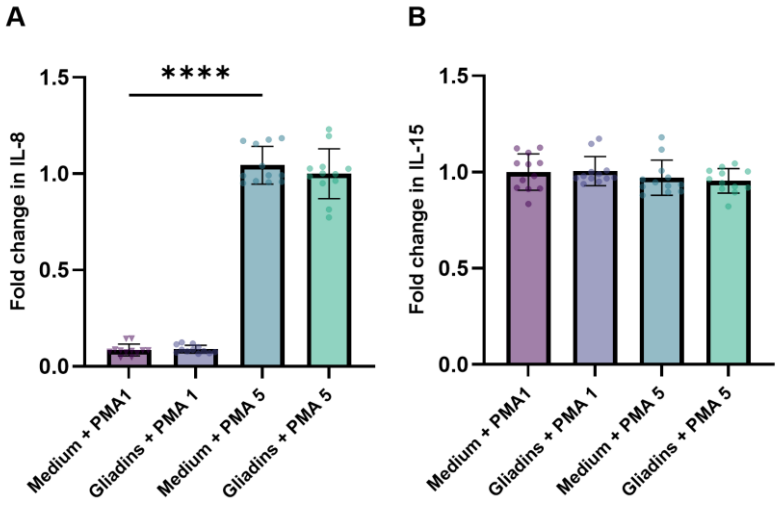


Figure 21 - Quantification of IL-8 (A) and IL-15 (B) levels in Caco-2/HT29- MTX co-culture cell model with THP-1 cells differentiated with PMA at 1 and 5 ng/mL for 24h. Cells were incubated with 1.0 mg/mL gliadins for 6h. Data presented in terms of fold change, normalizing the treatments with the control values mean. Assays performed in two independent days, n=6. ****p<0.0001.

Despite differences in tGSH and GSSG distribution between conditions, both PMA-treated groups we have a decrease in GSH/GSSG ratio (**Figure 20**), when cells were gliadin-treated; we can observe that there is a reduction in tGSH levels (**Figure 19A**) when gliadins were applied in the cells treated with 5 ng/mL PMA, and in terms of GST activity (**Figure 19C**) there is a decrease. However, the large distribution of values and the lack of GR activity determination requires further replicates to minimize values dispersion. When it comes to cytokine concentration, we can confirm the previous statistically significative increase in IL-8 (**Figure 21**) from PMA 1 to 5 ng/mL (IL-8 concentration values increased from 80-90 pg/mL (PMA1) up to 900-1100 pg/mL). In IL-15 levels we did not observe differences.

With this results, we can understand that using innate immune related cells (such as THP-1 cells, specially when differentiated with PMA) we have an increase of the inflammatory response in cells, that can affect the intestinal epithelium integrity and cause the cells to increase their sensibility to an external stimulant, such as gliadins.

3.4. Immunofluorescence staining of Caco-2 and Caco-2/HT29-MTX cells – qualitative evaluation of ZO-1

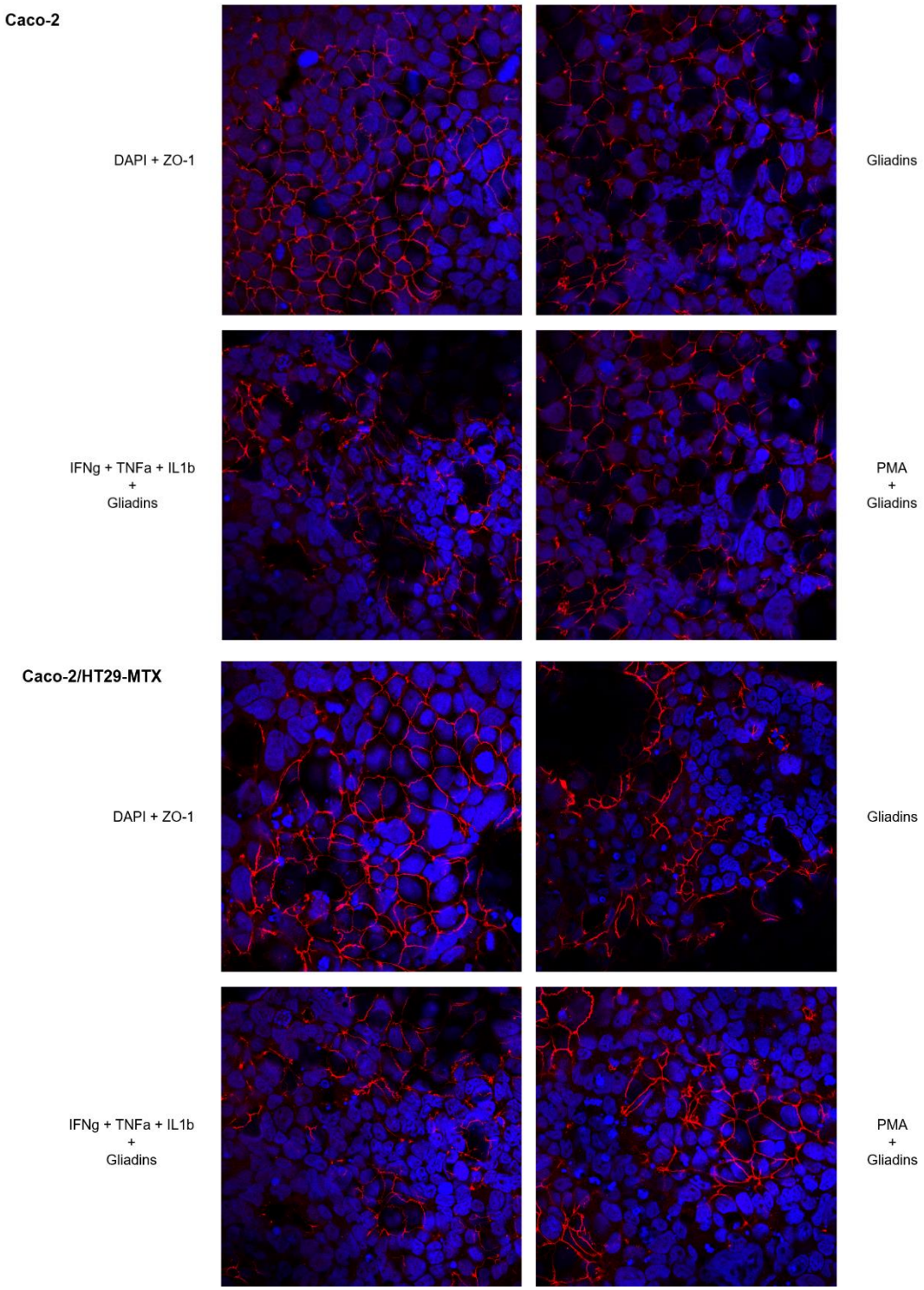


Figure 22 - Immunofluorescence staining for ZO-1 (red) in Caco-2 cells and Caco-2/HT29-MTX co-culture, both differentiated for 21 days. Cells were stained with DAPI (blue) to reveal the position of the nuclei. Cells were incubated with cytokines (IFNγ, TNFα and IL1b) and PMA for 24h and gliadins at 1 mg/mL were applied for 24h.

The integrity of the intestinal epithelial layer is crucial to maintain gastrointestinal homeostasis, and its disruption is a common feature observed in gastrointestinal disorders, including CD. In CD, gliadins can enter trough a paracellular via, due to the disruption of tight junctions (Ménard et al., 2012). Therefore, it is important to assess the integrity of the epithelial monolayer in our *in vitro* studies, to evaluate if gliadins are, in fact, causing a disruption of the barrier. Usually, TEER is a sufficient measurement of the membrane integrity, however we can observe variations in TEER values due to changes in temperature, pH, medium used in cell experiment, among other conditions (Srinivasan et al., 2015). Furthermore, in our Caco-2/HT-29MTX, we have some heterogenicity in our monolayer caused by the presence of two different cell types and a mucus layer, indicating that the local were we measure TEER might not be as representative as we wanted. Therefore, we performed the measurement of TEER in our cell assays as a control, rather than using the values obtained as an indicator of the disruption of the monolayer.

We wanted to observe if our Caco-2 and Caco-2/HT29-MTX cell models presented changes in terms of epithelial integrity. The presence of Zonula Occludens-1 (ZO-1), an integral structural component of the tight junctions, was the condition chosen for evaluating the barrier integrity of our cell models, not only because ZO-1 plays an important role in maintaining intestinal barrier integrity (Alizadeh et al., 2022), but also due to the fact of a decrease in ZO-1 levels being reported in the intestinal barrier of CD patients (Drago et al., 2006). To do so, we seeded both Caco-2 and Caco-2/HT29-MTX in a four-compartment cell culture dish and these cells were maintained in culture for 21 days (as all our cell assays performed before). After this differentiation period, cells were incubated with different cytokines and PMA, as well as were treated with gliadins. The conditions used in each well are presented in **Table 2**:

Table 2 – Resume of the conditions used in both Caco-2 and Caco-2 cells experiments. We use as control DAPI and ZO-1 in cells without any previous treatment.

	Pre- incubation conditions	Gliadins 1 mg/mL
DAPI + ZO-1	-	-
Gliadins	-	+
IFNγ + TNFα + IL1β + Gliadins	IFNγ 50 ng/mL TNFα 20 ng/mL IL1β 10 ng/mL	+
PMA + Gliadins	PMA 5 ng/mL	+

After treating the cells, the immunofluorescence staining was visualized trough a fluorescence inverted microscope and the images obtained are presented in **Figure 22**.

From these images, we can qualitatively observe that in Caco-2 cells there are differences between the control image (with DAPI and ZO-1 marker) and the other images where cells were incubated with gliadins. Therefore, we can conclude that in fact, gliadins promote perturbation in intestinal barrier integrity. For a quantitative determination of presence of ZO-1, we needed to calculate the fluorescence values.

Observing the Caco-2/HT29-MTX images, we do not have a cell layer as regular as the Caco-2, due to the fact that we have two different types of cells and additionally, we have a mucus layer, that could compromise the staining of both cells and ZO-1. In terms of the ZO-1 presence (in red) we can not see with clarity the differences in the mesh, as observed before in Caco-2 cells. Additionally, we can observe a certain cell overlay, that might be a result of the 21-day differentiation time, that might have promoted the cells to proliferate vertically. Therefore, we need to optimize this immunostaining protocol, specially to Caco-2/HT29-MTX cells, to perform a much more accurate comparison between treatments.

Conclusion

The assessment of the homeostatic changes triggered by the interaction of gliadins and different cell models is crucial to a better knowledge of the redox and inflammatory cell response observed in CD. The development of more complex and biologically accurate cell models is a necessity that we need to fulfill, in order to achieve a better knowledge of the changes that gluten cause in CD patients, from the first interaction of gliadin peptides with intestinal cells up to the stress caused in the cells.

From this work, we could understand that in iROS experiments, the cells tendency of increasing iROS levels with increasing gliadins concentration is not dependent of the incubation with cytokines; however, when we use a stimulant factor as PMA, that promotes an increased perturbation in cells homeostatic state, the cells have higher sensibility to respond to gliadins, indicating that a chronic inflammation of the cells epithelium increases the sensitivity to gliadins.

When we use an external factor as IFNγ (a representative of the adaptive branch of CD) in simple cell models (as Caco-2 monoculture and Caco-2/HT29-MTX co-cultures) we can conclude that IFNγ can promote a pro-inflammatory stimulus in the epithelial cells, not only due to the increase in IL-8 pool, but specially trough IL-15 signaling. The addition of THP-1 cells, particularly when these were differentiated with PMA (into macrophage-like cells, mediators of the innate immune response) it appears to cause an increase in the chemotactic potential (as observed by the higher IL-8 levels), that could indicate that using innate immune-related cells in a epithelial co-culture model can promote a higher pro-inflammatory effect in the epithelium, confirming that the incorporation of immune cells – such as THP-1 – is an important step to implement a better study model for CD and the studies need to continue to improve these cell models.

In terms of gliadins, we observed that these can induce transient perturbations in the cell redox state, in terms of the cells nucleophilic tone, causing a readjustment of GSH metabolism. Despite the fact of gliadins did not promote a permanent damage to the cells, we need to understand that gliadins cause a disturbance in cells (as revealed by the ROS mobilization observed) and that we have, in fact, a perturbation of the epithelial barrier when gliadins were present (as observed in immunofluorescence images); altogether, these results indicate a disruption of the intestinal barrier, that allows the access of immunogenic peptides to the lamina propria, promoting an inflammatory response.

With this work, we were able to evaluate the effects of gliadins in the intestinal epithelial integrity and study how IFNγ (representative of the adaptive immune response) and THP-1 (innate immune-related cells) interact with epithelial cells.

There is a long way to do from the simple monoculture models up to the genetically susceptible CD-organoid models, and this gradual process of cell model optimization and evaluation of multiple external factors involved in cell response and inflammatory signaling are the key to understand more about how gluten really interacts and signals IEC for CD.

Ongoing work and future perspectives

In addition to the work presented in this dissertation (and in the published article), during this dissertation we were able to initiate the characterization of other wheat varieties – in this case, five novel wheats were assessed: Bohemia (red grain), Bona Vita (yellow endosperm grain), Oxana (blue aleurone grain), Zora (purple pericarp grain) and Jumiko (a black grain that comprises both blue aleurone and purple pericarp characteristics). In flours obtained by grinding these wheat grains, we are currently characterizing both gliadins and free phenolic compounds present.

Having in consideration all the work presented, as well as the ongoing research concerning novel colored wheat alternatives, we are on the right path to continue studying both pro-oxidative and pro-inflammatory potential of these different wheat gluten sources in biologically epithelial cell models. In the future, we aimed:

- Perform additional studies in Caco-2/HT29-MTX cells with THP1 differentiated cells, to validate the results presented and consequently have a better understanding of the inflammatory response presented in these cells, when incubated with gliadins.
- Quantify the IFNγ levels in THP-1 cells (in different stages of differentiation), to understand how this important cytokine is expressed by immune-related cells, connecting our experiments;
- To finish the characterization of both gliadins and phenolic compounds presented in colored wheat flours. After these flours being fully characterized, we want to study the effects of the gliadins extracted from these colored flours in cells, to evaluate the true potential of incorporating these novel wheat grain alternatives into CD patient's diet.
- To continue the increase in complexity of the cell model used to achieve an even more biologically representative model to study gluten interaction with cells; to do so, we need to implement other immune cells (such as THP-1 differentiated into dendritic cells).
- In the future, the main goal is to perform these experiments in organoids, solving the main problematic that we have with these *in vitro* cell models: the lack of genetic predisposition.

References

Abadie, V., & Jabri, B. (2014). IL-15: a central regulator of celiac disease immunopathology. *Immunol Rev*, *260*(1), 221-234. https://doi.org/10.1111/imr.12191

Adams, R. B., Planchon, S. M., & Roche, J. K. (1993). IFN-gamma modulation of epithelial barrier function. Time course, reversibility, and site of cytokine binding. *J Immunol*, *150*(6), 2356-2363.

Ai, W., Li, H., Song, N., Li, L., & Chen, H. (2013). Optimal Method to Stimulate Cytokine Production and Its Use in Immunotoxicity Assessment. *IJERPH*, *10*(9), 3834-3842. https://www.mdpi.com/1660-4601/10/9/3834

Alizadeh, A., Akbari, P., Garssen, J., Fink-Gremmels, J., & Braber, S. (2022). Epithelial integrity, junctional complexes, and biomarkers associated with intestinal functions. *Tissue Barriers*, *10*(3), 1996830. https://doi.org/10.1080/21688370.2021.1996830

Amundarain, M. J., Herrera, M. G., Zamarreño, F., Viso, J. F., Costabel, M. D., & Dodero, V. I. (2019). Molecular mechanisms of 33-mer gliadin peptide oligomerisation. *Phys. Chem. Chem. Phys.*, *21*(40), 22539-22552. https://doi.org/10.1039/C9CP02338K

APC. (2023). Retrieved 31st august 2023 from https://www.celiacos.org.pt/

Bethune, M. T., Siegel, M., Howles-Banerji, S., & Khosla, C. (2009). Interferon-γ Released by Gluten-Stimulated Celiac Disease-Specific Intestinal T Cells Enhances the Transepithelial Flux of Gluten Peptides. *J. Pharmacol. Exp. Ther.*, *329*(2), 657-668. https://doi.org/10.1124/jpet.108.148007

Biesiekierski, J. R. (2017). What is gluten? *J. Gastroenterol. Hepatol.*, *32*(S1), 78-81. https://doi.org/https://doi.org/10.1111/jgh.13703

Boneh, A., Mandla, S., & Tenenhouse, H. S. (1989). Phorbol myristate acetate activates protein kinase C, stimulates the phosphorylation of endogenous proteins and inhibits phosphate transport in mouse renal tubules. *Biochim Biophys Acta*, *1012*(3), 308-316. https://doi.org/10.1016/0167-4889(89)90113-4

Bradauskiene, V., Vaiciulyte-Funk, L., Martinaitiene, D., Andruskiene, J., Verma, A. K., Lima, J. P. M., Serin, Y., & Catassi, C. (2023). Wheat consumption and prevalence of celiac disease: Correlation from a multilevel analysis. *Crit Rev Food Sci Nutr*, *63*(1), 18-32. https://doi.org/10.1080/10408398.2021.1939650

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., . . . Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat. Protoc.*, *14*(4), 991-1014. https://doi.org/10.1038/s41596-018-0119-1

Bruewer, M., Luegering, A., Kucharzik, T., Parkos, C. A., Madara, J. L., Hopkins, A. M., & Nusrat, A. (2003). Proinflammatory cytokines disrupt epithelial barrier function by apoptosis-independent mechanisms. *J Immunol*, *171*(11), 6164-6172. https://doi.org/10.4049/jimmunol.171.11.6164

Caputo, I., Barone, M. V., Martucciello, S., Lepretti, M., & Esposito, C. (2009). Tissue transglutaminase in celiac disease: role of autoantibodies. *Amino Acids*, *36*(4), 693-699. https://doi.org/10.1007/s00726-008-0120-z

Caruso, R., Fina, D., Peluso, I., Stolfi, C., Fantini, M. C., Gioia, V., Caprioli, F., Del Vecchio Blanco, G., Paoluzi, O. A., Macdonald, T. T., Pallone, F., & Monteleone, G. (2007). A functional role for interleukin-21 in promoting the synthesis of the T-cell chemoattractant, MIP-3alpha, by gut epithelial cells. *Gastroenterology*, *132*(1), 166-175. https://doi.org/10.1053/j.gastro.2006.09.053

Chanput, W., Mes, J. J., & Wichers, H. J. (2014). THP-1 cell line: An in vitro cell model for immune modulation approach. *Int. Immunopharmacol.*, *23*(1), 37-45. https://doi.org/https://doi.org/10.1016/j.intimp.2014.08.002

Chatila, T., Silverman, L., Miller, R., & Geha, R. (1989). Mechanisms of T cell activation by the calcium ionophore ionomycin. *J Immunol*, *143*(4), 1283-1289.

Chavez, A. M., Morin, M. J., Unno, N., Fink, M. P., & Hodin, R. A. (1999). Acquired interferon γ responsiveness during Caco-2 cell differentiation: effects on iNOS gene expression. *Gut*, *44*(5), 659-665. https://doi.org/10.1136/gut.44.5.659

Cherñavsky, A. C., Páez, M. C., Periolo, N., Correa, P., Guillén, L., Niveloni, S. I., Mauriño, E., Bai, J. C., & Anaya, J. M. (2008). The simultaneous presence of IL-1B and TNFA two-positions risk haplotypes enhances the susceptibility for celiac disease. *Cytokine*, *42*(1), 48-54. https://doi.org/10.1016/j.cyto.2008.01.015

Chladkova, B., Kamanova, J., Palova-Jelinkova, L., Cinova, J., Sebo, P., & Tuckova, L. (2011). Gliadin fragments promote migration of dendritic cells. *J Cell Mol Med*, *15*(4), 938-948. https://doi.org/10.1111/j.1582-4934.2010.01066.x

Cukrowska, B., Sowińska, A., Bierła, J. B., Czarnowska, E., Rybak, A., & Grzybowska-Chlebowczyk, U. (2017). Intestinal epithelium, intraepithelial lymphocytes and the gut microbiota - Key players in the pathogenesis of celiac disease. *World J Gastroenterol*, *23*(42), 7505-7518. https://doi.org/10.3748/wjg.v23.i42.7505

De Nitto, D., Monteleone, I., Franzè, E., Pallone, F., & Monteleone, G. (2009). Involvement of interleukin-15 and interleukin-21, two gamma-chain-related cytokines, in celiac disease. *World J Gastroenterol*, *15*(37), 4609-4614. https://doi.org/10.3748/wjg.15.4609

Devriese, S., Van den Bossche, L., Van Welden, S., Holvoet, T., Pinheiro, I., Hindryckx, P., De Vos, M., & Laukens, D. (2017). T84 monolayers are superior to Caco-2 as a model system of colonocytes. *Histochem. Cell Biol.*, *148*(1), 85-93. https://doi.org/10.1007/s00418-017-1539-7

Di Sabatino, A., Lenti, M. V., Giuffrida, P., Vanoli, A., & Corazza, G. R. (2015). New insights into immune mechanisms underlying autoimmune diseases of the gastrointestinal tract. *Autoimmun Rev*, *14*(12), 1161-1169. https://doi.org/10.1016/j.autrev.2015.08.004

Diaz de Barboza, G., Guizzardi, S., & Tolosa de Talamoni, N. (2015). Molecular aspects of intestinal calcium absorption. *World J Gastroenterol*, *21*(23), 7142-7154. https://doi.org/10.3748/wjg.v21.i23.7142

Domsa, E. M., Filip, G. A., Olteanu, D., Baldea, I., Clichici, S., Muresan, A., David, L., Moldovan, B., Para, I., Suciu, M., Achim, M., Negrean, V., & Andreica, V. (2020). Gold nanoparticles phytoreduced with Cornus mas extract mitigate some of gliadin effects on Caco-2 cells. *J. Physiol. Pharmacol.*, *71*(2). https://doi.org/10.26402/jpp.2020.2.04

Drago, S., El Asmar, R., Di Pierro, M., Grazia Clemente, M., Tripathi, A., Sapone, A., Thakar, M., Iacono, G., Carroccio, A., D'Agate, C., Not, T., Zampini, L., Catassi, C., & Fasano, A. (2006). Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scand J Gastroenterol*, *41*(4), 408-419. https://doi.org/10.1080/00365520500235334

Dvořák, J. (2001). Triticum Species (Wheat). In S. Brenner & J. H. Miller (Eds.), *Encyclopedia of Genetics* (pp. 2060-2068). Academic Press. https://doi.org/https://doi.org/10.1006/rwgn.2001.1672

Ferretti, G., Bacchetti, T., Masciangelo, S., & Saturni, L. (2012). Celiac disease, inflammation and oxidative damage: a nutrigenetic approach. *Nutrients*, *4*(4), 243-257. https://doi.org/10.3390/nu4040243

Freire, R., Ingano, L., Serena, G., Cetinbas, M., Anselmo, A., Sapone, A., Sadreyev, R. I., Fasano, A., & Senger, S. (2019). Human gut derived-organoids provide model to study gluten response and effects of microbiota-derived molecules in celiac disease. *Sci Rep*, *9*(1), 7029. https://doi.org/10.1038/s41598-019-43426-w

Gagnon, M., Zihler Berner, A., Chervet, N., Chassard, C., & Lacroix, C. (2013). Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-MTX intestinal cell models to investigate Salmonella adhesion and invasion. *J Microbiol Methods*, *94*(3), 274-279. https://doi.org/10.1016/j.mimet.2013.06.027

Gianfrani, C., Auricchio, S., & Troncone, R. (2005). Adaptive and innate immune responses in celiac disease. *Immunol Lett*, *99*(2), 141-145. https://doi.org/10.1016/j.imlet.2005.02.017

Green, P. H., & Cellier, C. (2007). Celiac disease. *N Engl J Med*, *357*(17), 1731-1743. https://doi.org/10.1056/NEJMra071600

Green, P. H., Lebwohl, B., & Greywoode, R. (2015). Celiac disease. *J Allergy Clin Immunol*, *135*(5), 1099-1106; quiz 1107. https://doi.org/10.1016/j.jaci.2015.01.044

Hölken, J. M., & Teusch, N. (2023). The Monocytic Cell Line THP-1 as a Validated and Robust Surrogate Model for Human Dendritic Cells. *Int. J. Mol. Sci.*, *24*(2), 1452. https://www.mdpi.com/1422-0067/24/2/1452

Huang, S. Q., Zhang, N., Zhou, Z. X., Huang, C. C., Zeng, C. L., Xiao, D., Guo, C. C., Han, Y. J., Ye, X. H., Ye, X. G., Ou, M. L., Zhang, B. H., Liu, Y., Zeng, E. Y., Yang, G., & Jing, C. X. (2017). Association of LPP and TAGAP Polymorphisms with Celiac Disease Risk: A Meta-Analysis. *Int J Environ Res Public Health*, *14*(2). https://doi.org/10.3390/ijerph14020171

Huet, G., Kim, I., de Bolos, C., Lo-Guidice, J. M., Moreau, O., Hemon, B., Richet, C., Delannoy, P., Real, F. X., & Degand, P. (1995). Characterization of mucins and proteoglycans synthesized by a mucin-secreting HT-29 cell subpopulation. *J Cell Sci*, *108 (Pt 3)*, 1275-1285. https://doi.org/10.1242/jcs.108.3.1275

Hunt, K. A., Zhernakova, A., Turner, G., Heap, G. A. R., Franke, L., Bruinenberg, M., Romanos, J., Dinesen, L. C., Ryan, A. W., Panesar, D., Gwilliam, R., Takeuchi, F., McLaren, W. M., Holmes, G. K. T., Howdle, P. D., Walters, J. R. F., Sanders, D. S., Playford, R. J., Trynka, G., . . . van Heel, D. A. (2008). Newly identified genetic risk variants for celiac disease related to the immune response. *Nature Genetics*, *40*(4), 395-402. https://doi.org/10.1038/ng.102

Ishimoto, Y., Satsu, H., Totsuka, M., & Shimizu, M. (2011). IEX-1 suppresses apoptotic damage in human intestinal epithelial Caco-2 cells induced by co-culturing with macrophage-like THP-1 cells. *Biosci Rep*, *31*(5), 345-351. https://doi.org/10.1042/bsr20100083

Jabri, B., & Sollid, L. M. (2017). T Cells in Celiac Disease. *J Immunol*, *198*(8), 3005-3014. https://doi.org/10.4049/jimmunol.1601693

Jelínková, L., Tucková, L., Cinová, J., Flegelová, Z., & Tlaskalová-Hogenová, H. (2004). Gliadin stimulates human monocytes to production of IL-8 and TNF-alpha through a mechanism involving NF-kappaB. *FEBS Lett*, *571*(1-3), 81-85. https://doi.org/10.1016/j.febslet.2004.06.057

Jianyuan, C. (2021). Introductory Chapter: Celiac Disease - Now and Then. In C. Jianyuan (Ed.), *Celiac Disease* (pp. Ch. 1). IntechOpen. https://doi.org/10.5772/intechopen.97238

Juuti-Uusitalo, K., Klunder, L. J., Sjollema, K. A., Mackovicova, K., Ohgaki, R., Hoekstra, D., Dekker, J., & van Ijzendoorn, S. C. D. (2011). Differential Effects of TNF (TNFSF2) and IFN-γ on Intestinal Epithelial Cell Morphogenesis and Barrier

Function in Three-Dimensional Culture. *PLOS ONE*, *6*(8), e22967. https://doi.org/10.1371/journal.pone.0022967

Kämpfer, A. A. M., Urbán, P., Gioria, S., Kanase, N., Stone, V., & Kinsner-Ovaskainen, A. (2017). Development of an in vitro co-culture model to mimic the human intestine in healthy and diseased state. *Toxicology in Vitro*, *45*, 31-43. https://doi.org/https://doi.org/10.1016/j.tiv.2017.08.011

Kheiri, F., Rostami-Nejad, M., Amani, D., Sadeghi, A., Moradi, A., Aghamohammadi, E., Sahebkar, A., & Zali, M. R. (2022). Expression of tolerogenic dendritic cells in the small intestinal tissue of patients with celiac disease. *Heliyon*, *8*(12), e12273. https://doi.org/https://doi.org/10.1016/j.heliyon.2022.e12273

Kim, J., Koo, B.-K., & Knoblich, J. A. (2020). Human organoids: model systems for human biology and medicine. *Nat Rev Mol Cell Biol*, *21*(10), 571-584. https://doi.org/10.1038/s41580-020-0259-3

Kim, T.-S., Rha, M.-S., & Shin, E.-C. (2022). IFN-γ Induces IL-15 Trans-Presentation by Epithelial Cells via IRF1. *J. Immunol.*, *208*(2), 338-346. https://doi.org/10.4049/jimmunol.2100057

Kordulewska, N. K., Topa, J., Tańska, M., Cieślińska, A., Fiedorowicz, E., Savelkoul, H. F. J., & Jarmołowska, B. (2021). Modulatory Effects of Osthole on Lipopolysaccharides-Induced Inflammation in Caco-2 Cell Monolayer and Co-Cultures with THP-1 and THP-1-Derived Macrophages. *Nutrients*, *13*(1), 123. https://www.mdpi.com/2072-6643/13/1/123

Lammers, K. M., Khandelwal, S., Chaudhry, F., Kryszak, D., Puppa, E. L., Casolaro, V., & Fasano, A. (2011). Identification of a novel immunomodulatory gliadin peptide that causes interleukin-8 release in a chemokine receptor CXCR3-dependent manner only in patients with coeliac disease. *Immunology*, *132*(3), 432-440. https://doi.org/10.1111/j.1365-2567.2010.03378.x

Lea, T. (2015). Caco-2 Cell Line. In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.), *The Impact of Food Bioactives on Health: in vitro and ex vivo models* (pp. 103-111). Springer International Publishing. https://doi.org/10.1007/978-3-319-16104-4_10

Lindfors, K., Rauhavirta, T., Stenman, S., Mäki, M., & Kaukinen, K. (2012). In vitro models for gluten toxicity: relevance for celiac disease pathogenesis and development of novel treatment options. *EBM*, *237*(2), 119-125. https://doi.org/10.1258/ebm.2011.011294

Lingappan, K. (2018). NF-κB in Oxidative Stress. *Curr Opin Toxicol*, *7*, 81-86. https://doi.org/10.1016/j.cotox.2017.11.002

Ma, Q. (2013). Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol*, *53*, 401-426. https://doi.org/10.1146/annurev-pharmtox-011112-140320

Mahapatro, M., Erkert, L., & Becker, C. (2021). Cytokine-Mediated Crosstalk between Immune Cells and Epithelial Cells in the Gut. *Cells*, *10*(1). https://doi.org/10.3390/cells10010111

Mannervik, B. (1999). Measurement of Glutathione Reductase Activity. *Curr. Protoc. Toxicol.*, *00*(1), 7.2.1-7.2.4. https://doi.org/https://doi.org/10.1002/0471140856.tx0702s00

Marzorati, M., Van den Abbeele, P., Verstrepen, L., De Medts, J., & Ekmay, R. D. (2023). The Response of a Leaky Gut Cell Culture Model (Caco-2/THP-1 Co-Culture) to Administration of Alternative Protein Sources. *Nutraceuticals*, *3*(1), 175-184. https://www.mdpi.com/1661-3821/3/1/13

Mazzarella, G., Bergamo, P., Maurano, F., Luongo, D., Aufiero, V. R., Bozzella, G., Palmieri, G., Troncone, R., Auricchio, S., David, C., & Rossi, M. (2014). Gliadin intake alters the small intestinal mucosa in indomethacin-treated HLA-DQ8 transgenic mice. *Am. J. Physiol.-Gastrointest. Liver Physiol.*, *307*(3), G302-G312. https://doi.org/10.1152/ajpgi.00002.2014

Mazzei, A., Pagliara, P., Del Vecchio, G., Giampetruzzi, L., Croce, F., Schiavone, R., Verri, T., & Barca, A. (2023). Cytoskeletal Responses and Aif-1 Expression in Caco-2 Monolayers Exposed to Phorbol-12-Myristate-13-Acetate and Carnosine. *Biology*, *12*(1), 36. https://www.mdpi.com/2079-7737/12/1/36

McClintock, S. D., Attili, D., Dame, M. K., Richter, A., Silvestri, S. S., Berner, M. M., Bohm, M. S., Karpoff, K., McCarthy, C. L., Spence, J. R., Varani, J., & Aslam, M. N. (2020). Differentiation of human colon tissue in culture: Effects of calcium on

trans-epithelial electrical resistance and tissue cohesive properties. *PLOS ONE*, *15*(3), e0222058. https://doi.org/10.1371/journal.pone.0222058

Ménard, S., Lebreton, C., Schumann, M., Matysiak-Budnik, T., Dugave, C., Bouhnik, Y., Malamut, G., Cellier, C., Allez, M., Crenn, P., Schulzke, J. D., Cerf-Bensussan, N., & Heyman, M. (2012). Paracellular versus transcellular intestinal permeability to gliadin peptides in active celiac disease. *Am J Pathol*, *180*(2), 608-615. https://doi.org/10.1016/j.ajpath.2011.10.019

Moerkens, R., Mooiweer, J., Withoff, S., & Wijmenga, C. (2019). Celiac disease-on-chip: Modeling a multifactorial disease in vitro. *United European Gastroenterol J*, *7*(4), 467-476. https://doi.org/10.1177/2050640619836057

Mohan Kumar, B. V., Prasada Rao, U. J. S., & Prabhasankar, P. (2017). Immunogenicity characterization of hexaploid and tetraploid wheat varieties related to celiac disease and wheat allergy. *Food Agric Immunol.*, *28*(5), 888-903. https://doi.org/10.1080/09540105.2017.1319342

Molaaghaee-Rouzbahani, S., Asri, N., Jahani-Sherafat, S., Amani, D., Masotti, A., Baghaei, K., Yadegar, A., Mirjalali, H., & Rostami-Nejad, M. (2022). The modulation of macrophage subsets in celiac disease pathogenesis. *Immun Inflamm Dis*, *10*(12), e741. https://doi.org/10.1002/iid3.741

Moretti, S., Mrakic-Sposta, S., Roncoroni, L., Vezzoli, A., Dellanoce, C., Monguzzi, E., Branchi, F., Ferretti, F., Lombardo, V., Doneda, L., Scricciolo, A., & Elli, L. (2018). Oxidative stress as a biomarker for monitoring treated celiac disease. *Clin Transl Gastroenterol*, *9*(6), 157. https://doi.org/10.1038/s41424-018-0031-6

Nilsen, E. M., Jahnsen, F. L., Lundin, K. E. A., Johansen, F. E., Fausa, O., Sollid, L. M., Jahnsen, J., Scott, H., & Brandtzaeg, P. (1998). Gluten induces an intestinal cytokine response strongly dominated by interferon gamma in patients with celiac disease. *Gastroenterology*, *115*(3), 551-563. https://doi.org/10.1016/S0016-5085(98)70134-9

Onyiah, J. C., & Colgan, S. P. (2016). Cytokine responses and epithelial function in the intestinal mucosa. *Cell Mol Life Sci*, *73*(22), 4203-4212. https://doi.org/10.1007/s00018-016-2289-8

Palová-Jelínková, L., Rozková, D., Pecharová, B., Bártová, J., Sedivá, A., Tlaskalová-Hogenová, H., Spísek, R., & Tucková, L. (2005). Gliadin fragments induce phenotypic and functional maturation of human dendritic cells. *J Immunol*, *175*(10), 7038-7045. https://doi.org/10.4049/jimmunol.175.10.7038

Park, E. K., Jung, H. S., Yang, H. I., Yoo, M. C., Kim, C., & Kim, K. S. (2007). Optimized THP-1 differentiation is required for the detection of responses to weak stimuli. *Inflamm. Res.*, *56*(1), 45-50. https://doi.org/10.1007/s00011-007-6115-5

Patergnani, S., Danese, A., Bouhamida, E., Aguiari, G., Previati, M., Pinton, P., & Giorgi, C. (2020). Various Aspects of Calcium Signaling in the Regulation of Apoptosis, Autophagy, Cell Proliferation, and Cancer. *Int.l J. Mol. Sci.*, *21*(21), 8323. https://www.mdpi.com/1422-0067/21/21/8323

Pérez, S., Taléns-Visconti, R., Rius-Pérez, S., Finamor, I., & Sastre, J. (2017). Redox signaling in the gastrointestinal tract. *Free Radic Biol Med*, *104*, 75-103. https://doi.org/10.1016/j.freeradbiomed.2016.12.048

Pietz, G., De, R., Hedberg, M., Sjöberg, V., Sandström, O., Hernell, O., Hammarström, S., & Hammarström, M.-L. (2017). Immunopathology of childhood celiac disease—Key role of intestinal epithelial cells. *PLOS ONE*, *12*(9), e0185025. https://doi.org/10.1371/journal.pone.0185025

Qiao, S. W., Iversen, R., Ráki, M., & Sollid, L. M. (2012). The adaptive immune response in celiac disease. *Semin Immunopathol*, *34*(4), 523-540. https://doi.org/10.1007/s00281-012-0314-z

Rahman, I., Kode, A., & Biswas, S. K. (2006). Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nat Protoc*, *1*(6), 3159-3165. https://doi.org/10.1038/nprot.2006.378

Schumann, M., Siegmund, B., Schulzke, J. D., & Fromm, M. (2017). Celiac Disease: Role of the Epithelial Barrier. *Cell Mol Gastroenterol Hepatol*, *3*(2), 150-162. https://doi.org/10.1016/j.jcmgh.2016.12.006

Sestak, K., Dufour, J. P., Liu, D. X., Rout, N., Alvarez, X., Blanchard, J., Faldas, A., Laine, D. J., Clarke, A. W., & Doyle, A. G. (2018). Beneficial Effects of Human Anti-Interleukin-15 Antibody in Gluten-Sensitive Rhesus Macaques with Celiac

Disease [Original Research]. *Front. Immunol.*, *9*. https://doi.org/10.3389/fimmu.2018.01603

Sharma, A., Liu, X., Hadley, D., Hagopian, W., Liu, E., Chen, W.-M., Onengut-Gumuscu, S., Simell, V., Rewers, M., Ziegler, A.-G., Lernmark, Å., Simell, O., Toppari, J., Krischer, J. P., Akolkar, B., Rich, S. S., Agardh, D., She, J.-X., & Group, T. S. (2016). Identification of Non-HLA Genes Associated with Celiac Disease and Country-Specific Differences in a Large, International Pediatric Cohort. *PLOS ONE*, *11*(3), e0152476. https://doi.org/10.1371/journal.pone.0152476

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L., & Hickman, J. J. (2015). TEER Measurement Techniques for In Vitro Barrier Model Systems. *J. Lab. Autom.*, *20*(2), 107-126. https://doi.org/10.1177/2211068214561025

Starr, T., Bauler, T. J., Malik-Kale, P., & Steele-Mortimer, O. (2018). The phorbol 12-myristate-13-acetate differentiation protocol is critical to the interaction of THP-1 macrophages with Salmonella Typhimurium. *PLOS ONE*, *13*(3), e0193601. https://doi.org/10.1371/journal.pone.0193601

Stoven, S., Murray, J. A., & Marietta, E. V. (2013). Latest in vitro and in vivo models of celiac disease. *Expert Opin Drug Discov*, *8*(4), 445-457. https://doi.org/10.1517/17460441.2013.761203

Sugi, K., Musch, M. W., Field, M., & Chang, E. B. (2001). Inhibition of Na+,K+-ATPase by interferon gamma down-regulates intestinal epithelial transport and barrier function. *Gastroenterology*, *120*(6), 1393-1403. https://doi.org/10.1053/gast.2001.24045

Truzzi, F., Tibaldi, C., Whittaker, A., Dilloo, S., Spisni, E., & Dinelli, G. (2020). Pro-Inflammatory Effect of Gliadins and Glutenins Extracted from Different Wheat Cultivars on an In Vitro 3D Intestinal Epithelium Model. *Int J Mol Sci*, *22*(1). https://doi.org/10.3390/ijms22010172

Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., & Tada, K. (1980). Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer*, *26*(2), 171-176. https://doi.org/https://doi.org/10.1002/ijc.2910260208

Vader, L. W., de Ru, A., van der Wal, Y., Kooy, Y. M., Benckhuijsen, W., Mearin, M. L., Drijfhout, J. W., van Veelen, P., & Koning, F. (2002). Specificity of tissue transglutaminase explains cereal toxicity in celiac disease. *J Exp Med*, *195*(5), 643-649. https://doi.org/10.1084/jem.20012028

Vogt, L. M., Meyer, D., Pullens, G., Faas, M. M., Venema, K., Ramasamy, U., Schols, H. A., & de Vos, P. (2014). Toll-Like Receptor 2 Activation by β2→1-Fructans Protects Barrier Function of T84 Human Intestinal Epithelial Cells in a Chain Length–Dependent Manner. *J. Nutr.*, *144*(7), 1002-1008. https://doi.org/https://doi.org/10.3945/jn.114.191643

Vontas, J. G., Enayati, A. A., Small, G. J., & Hemingway, J. (2000). A Simple Biochemical Assay for Glutathione S-Transferase Activity and Its Possible Field Application for Screening Glutathione S-Transferase-Based Insecticide Resistance. *Pestic Biochem Physiol*, *68*(3), 184-192. https://doi.org/https://doi.org/10.1006/pest.2000.2512

Wapenaar, M. C., van Belzen, M. J., Fransen, J. H., Fariña Sarasqueta, A., Houwen, R. H. J., Meijer, J. W. R., Mulder, C. J. J., & Wijmenga, C. (2004). The interferon gamma gene in celiac disease: augmented expression correlates with tissue damage but no evidence for genetic susceptibility. *J Autoimmun*, *23*(2), 183-190. https://doi.org/https://doi.org/10.1016/j.jaut.2004.05.004

Watanabe, F., Satsu, H., Mochizuki, T., Nakano, T., & Shimizu, M. (2004). Development of the method for evaluating protective effect of food factors on THP-1-induced damage to human intestinal Caco-2 monolayers. *BioFactors*, *21*(1-4), 145-147. https://doi.org/https://doi.org/10.1002/biof.552210129

Yi, H.-K., Hachimura, S., Ametani, A., & Kaminogawa, S. (1995). Induction of Apoptosis in an Intestinal Epithelial Cell Line, IEC-6. In E. C. Beuvery, J. B. Griffiths, & W. P. Zeijlemaker (Eds.), *Animal Cell Technology: Developments Towards the 21st Century* (pp. 1183-1187). Springer Netherlands. https://doi.org/10.1007/978-94-011-0437-1_185

Yoshida, H., Hirono, C., Shimamoto, C., Daikoku, E., Kubota, T., Sugita, M., Shiba, Y., & Nakahari, T. (2010). Membrane potential modulation of ionomycin-stimulated Ca2+ entry via Ca2+/H+ exchange and SOC in rat submandibular acinar cells. *J Physiol Sci*, *60*(5), 363-371. https://doi.org/10.1007/s12576-010-0098-7

Attachment 1

Food Research International 173 (2023) 113317

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Evidence of increased gluten-induced perturbations in the nucleophilic tone and detoxifying defences of intestinal epithelial cells impaired by gastric disfunction

Sara da Silva [a], Rosa Pérez-Gregorio [a,b], Nuno Mateus [a], Victor Freitas [a], Ricardo Dias [a,*]

[a] LAQV-REQUIMTE, Department of Chemistry and Biochemistry, Faculty of Sciences of the University of Porto, Porto, Portugal
[b] Department of Analytical and Food Chemistry, Nutrition and Bromatology Area, Faculty of Sciences of the University of Vigo, Ourense, Spain

ARTICLE INFO

Keywords:
Celiac disease
Oxidative stress
Gluten peptides
Gluten digestion

ABSTRACT

It has been increasingly demonstrated over the past few years that some proteolytically resistant gluten peptides may directly affect intestinal cell structure and functions by modulating pro-inflammatory gene expression and oxidative stress. The relationship between oxidative cell damage and Celiac Disease (CD) is supported by several studies on human intestinal epithelial cell lines, such as the Caco-2 cell model, already shown to be particularly sensitive to the pro-oxidative and pro-apoptotic properties of gluten protein digests. Through providing valuable evidence concerning some of the pathophysiological mechanisms that may be at play in gluten-related disorders, most of these *in vitro* studies have been employing simplified digestion schemes and intestinal cell systems that do not fully resemble mature enterocytes in terms of their characteristic tight junctions, microvilli and membrane transporters. Herein the peptide profile and pro-oxidative effect of two different gastrointestinal gliadin digestions was thoroughly characterized and comprehensively compared: one following the complete INFOGEST workflow and a second one by-passing gastric processing, to assess the dependence of gliadin-triggered downstream cell effects on pepsin activity. In both matrices, gluten-derived immunogenic peptide sequences were identified by non-targeted LC-MS/MS. Altogether, this study provides first-hand data concerning the still unexplored peptide composition, gastric-dependence and immunogenicity of physiologically representative gliadin protein digests as well as foundational clues stressing the need for more complex and integrated *in vitro* cell systems when modelling and exploiting gluten-induced perturbations in the nucleophilic tone and inflammatory status of intestinal epithelial cells.

1. Introduction

Along the human gastrointestinal tract, the gastrointestinal mucosa and the gut microbiota interact to enable an efficient digestive process (Verhoeckx et al., 2015). This symbiotic relationship is also crucial to prevent gastrointestinal disorders such as intestinal barrier dysfunctions, inflammation, and bacterial overgrowth, all of which could compromise food digestion (Okumura & Takeda, 2017). Additionally, each digestive phase have different enzymes, pH, and physical aspects to account for, and changes in the digestion process leads to differences in peptide sequence constitution and size, with significantly different immunogenicity and toxicity to the intestinal epithelium (Pali-Schöll, Untersmayr, Klems, & Jensen-Jarolim, 2018).

There are many food components with high resistance to the digestive process, resulting in an increased allergenicity/ immunogenicity (Freitas, Gómez-Mascaraque, & Brodkorb, 2022). When food allergenics are discussed, gluten is one of the most referred, due to the increased global prevalence of gluten-related disorders. The term "gluten" describes the main protein component found in wheat, rye, and barley. In wheat, gluten consists mainly on alcohol-soluble gliadins and alcohol-insoluble glutenins, being the gliadin fraction the most toxic and responsible for triggering Celiac Disease (CD) (Biesiekierski, 2017). CD is a food-related autoimmune disease caused by dietary gluten exposure in genetically susceptible individuals, resulting on a variable range of lesions in the intestinal mucosa. According to the literature, gluten proteins are resistant to human digestive enzymes as a result of their high glutamine and proline contents; this incomplete digestion leads to the production of unusually large peptides (such as the 33 amino acid

* Corresponding author.
E-mail address: ricardo.dias@fc.up.pt (R. Dias).

https://doi.org/10.1016/j.foodres.2023.113317
Received 18 May 2023; Received in revised form 21 July 2023; Accepted 22 July 2023
Available online 27 July 2023

fragment of α-gliadin, the 33-mer), that can cross the intestinal mucosa and trigger an inflammatory immune response associated to CD (Freire et al., 2019; Green & Cellier, 2007).

In addition to all-known gluten-related disorders, there are many case reports of patients that developed CD symptoms after upper digestive tract surgeries, such as gastrectomy due to gastric cancer (Iwamoto et al., 2022), among others (Bai et al., 1991). At the moment, it is still unclear how upper gastrointestinal surgery unmasks CD; the key might be in understanding the true dependence of gliadin downstream cell effects on each digestive phase as well as what triggers the change between tolerance and reactivity to gliadin peptides in non-genetically susceptible individuals. Importantly, over the past years, many studies performed in intestinal cell models aiming the evaluation of deleterious effects of gluten digests *in vitro* have been using one simplified peptic-tryptic/chymotryptic digestion method. Nevertheless, this method is not physiologically accurate, since the peptides generated could be substantially different to the ones generated after human gastrointestinal digestion, thus promoting different responses in terms of toxicity and pathogenicity and less accurate results in CD-related studies (Prandi et al., 2014; Wanyera & Owuoche, 2017). Therefore, it is important to establish a protocol that is much more accurate and similar to biological conditions, for the results achieved in CD assays to be as close as possible to the effects seen in CD patients.

Another important factor in CD research is the cell model used. In food science, the Caco-2 cell model has been widely used, for example, to predict food components toxicity or the beneficial/ deleterious effects of diverse agents on the intestinal monolayer integrity. Caco-2 is human epithelial cell line that spontaneously differentiate into a monolayer that structurally resembles the enterocytes of the small intestine (Hilgers, Conradi, & Burton, 1990; Verhoeckx et al., 2015). The majority of CD-related studies using Caco-2 cells are performed in undifferentiated cells, which is not physiologically relevant, since the cells are at a development state were they are still not fully epithelial cells and are metabolically unstable, presenting different metabolic profiles during the differentiation process (Hundsberger et al., 2017; Lee, Hom, Bai, & Shapiro, 2009; Rivabene, Mancini, & De Vincenzi, 1999; Stenman et al., 2009; Stricker, de Laffolie, Zimmer, & Rudloff, 2023; Truzzi et al., 2020). Therefore, the results obtained in these studies might be conditioned by a reposition of cell homeostasis (characteristic of a specific stage of differentiation of the cell), not truly representing the effects of gluten in the intestinal mucosa. Moreover, in the natural gut monolayer, a few goblet cells are also present; these secrete mucins, which form a protective mucous layer. In this regard, the epithelial colon-derived cell line, HT29-MTX is often co-cultured with Caco-2 cells. HT29-MTX cells, when treated with methotrexate (MTX) it results in a more stable sub-population of mucus-secreting cells that exhibits a differentiated cell phenotype capable of secreting low amounts of MUC2 mucins, forming the intestinal mucus layer (Gagnon, Zihler Berner, Chervet, Chassard, & Lacroix, 2013; Huet et al., 1995).

Taking into account these observations - particularly the still unclear gastric dependence of gliadin digestion on its downstream cell effects - the aim of this work was to (1) evaluate the differences between two gliadin digestions (one following the INFOGEST protocol *vs* an incomplete one neglecting gastric processing) in terms of sequence similarity, and immunogenicity of gliadin peptides, and (2) assess both gliadin-induced perturbations in the nucleophilic tone and detoxifying defenses of Caco-2 and Caco-2/HT29-MTX intestinal epithelial cell systems.

2. Material and methods

2.1. Reagents

All chemicals used in this study were purchased to Sigma-Aldrich with the exception of: *β-Nicotinamide Adenine Dinucleotide Phosphate Tetrasodium Salt* - NADPH - (from *PanReac AppliChem*), and *Acrylamide/bis-acrylamide* (from *NZYtech*).

2.2. Extraction of gliadin proteins

Proline and glutamine-rich gliadin proteins were extracted from a commercial Portuguese wheat flour (*Espiga, Produtos Alimentares, SA*) as described elsewhere (Mazzarella et al., 2014). Briefly, 10 g of a type 65 wheat flour was defatted twice with diethyl ether (50 mL) for 30 min. Albumin and globulin proteins were extracted three times by resuspending the defatted flour in 0.4 mol/L NaCl, 0.067 mol/L NaH_2PO_4 buffer at pH 7.5 (50 mL). The suspensions were shaken for 10 min at room temperature (RT) and centrifuged at 15 000 g for 15 min. Gliadins were then extracted from the pellet by adding a water/ethanol mixture (50 mL) to a final concentration of 70% (v/v) ethanol. The suspension was, again, shaken for 45 min at RT and centrifuged at 15 000 g for 10 min. The pellets were combined and extracted two additional times following the same procedure. Between albumin/globulin and gliadin extractions, the pellet was washed twice with ultrapure water. Upon ethanol removal on a rotary evaporator - set to 37 °C - the supernatants were freeze-dried and stored at -20 °C.

2.3. SDS-PAGE of gliadin proteins

The reproducibility and complexity of the previously extracted gliadin fractions were first assessed by *Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis* (SDS-PAGE) by comparing three equivalent protein samples extracted on three different, independent, days. SDS-PAGE was carried out on a 12% polyacrylamide gel using a Tris-glycine running buffer containing 1% of SDS. Gliadin samples (1 mg/mL) were dissolved in 0.5 mol/L Tris-HCl pH 6.8, 6 mol/L urea under reducing conditions (DTT, 20 mmol/L), diluted in 5 x sample loading buffer (from *NZYtech*) and heated to 95 °C for 10 min while shaking. *Per* sample, 10 μL were applied to the slots. A mixture of 10 standard proteins (*Mw* 10——250 000 Da) was used as marker (Precision Plus Protein™ Unstained Protein Standards, from *Biorad*). The running time was 60 min at 120 V. After the run, the proteins were stained for 10 min with Coomassie Brilliant Blue R-250 (*Biorad*) and de-stained twice with a solution containing 20% methanol, 10% acetic acid (in water).

2.4. Western blot

The separated proteins were transferred to a PVDF membrane, using a wet tank blotter (*BioRad*), according to manufacturer protocol. The blot was blocked overnight at 4 °C in 5% w/v nonfat dry milk (prepared in TBS-T: 20 mmol/L Tris-HCl, 0.5 mol/L NaCl buffer with 0.05% Tween-20). The blot was then incubated with the gliadin polyclonal antibody from *Invitrogen*, diluted 1:5 000, in 3% nonfat dry milk for 1.5 h. The blots were then washed 4 times with TBS-T, and incubated with anti-rabbit IgG-HRP-linked antibody (*Cell Signaling*), diluted 1:5 000, in 3% nonfat dry milk for 1 h. Finally, the blot was washed six times in TBS-T and incubated for 3 min with *ECL Western Blotting Substrate* (*Thermo Scientific*), and then revealed on a *ChemiDocTM XRS+ System* with *Image LabTM Software*.

2.5. Reduction, alkylation and tryptic digestion of gliadin proteins

The gliadin composition of each isolated protein fraction was determined by untargeted LC-MS/MS analysis. Gliadins were first dissolved in 0.5 mol/L Tris-HCl, 6 mol/L urea, 20 mmol/L DTT pH 6.8 to a final concentration of 2 mg/mL. Ten microliters of each gliadin solution were then transferred to 0.5 mL microcentrifuge tubes and added with 15 μL of 50 mmol/L ammonium bicarbonate and 7 μL of ultrapure water. Mixtures were incubated at 95 °C for 5 min and allowed to cool at RT. For protein alkylation, 3 μL of iodoacetamide at 100 mmol/L was added to each tube, incubated in the dark for 20 min and added with 1 μL of a 1 mg/mL trypsin solution from bovine pancreas (greater than10 000 BAEE

units/mg protein). Reactions were left stirring overnight at 30 °C. Prior to MS analyses, peptide samples were desalted and concentrated on C18 GL-Tips SDB (*GL Sciences Inc*), according to manufacturer instructions. The latter were then injected into an *UltiMate 3000 RSLCnano* system (*Thermo Fisher Scientific*) coupled to an LTQ-Orbitrap XL mass spectrometer through an EASY-nano spray source (*Thermo Fisher Scientific*). The peptides were separated on a *PepMap* C18 column (3 μm, 15 cm x 150 μm, *Thermo Fisher Scientific*) and water/FA (99:1, v/v) (A) and acetonitrile/FA (99:1, v/v) (B) as solvents with a flow rate of 0.200 μL/min, an injection volume of 20 μL and a linear gradient: 0——3 min 10 % B, 60 min 30% B, 80 min 40 % B, 100 min 65 % B, 130 min 95 % B, 135——140 min 10% B. The ESI interface was operated using the following parameters: positive mode, capillary voltage: 9 V, capillary temperature: 275.0 °C, tube lens: 100 V, spray voltage: 1.9 kV. The MS instrument settings were: scan: standard enhanced, resolution: 60.000, *m/z* range: 300——2.000, MS/MS setting: auto-MS^n, collision gas: helium, normalized collision energy: 35.0, activation time: 30.0, isolation width (*m/z*): 2. All MS and MS/MS spectra were acquired in a data dependent mode. The instrument executed one MS scan followed by a MS/MS scan of each of the seven most intense peaks. Dynamic exclusion parameters were as follows: repeat count: 1, repeat duration: 30.0 s, exclusion list size: 500, exclusion duration: 80.0, exclusion mass width: 1.5 (high and low). Charge states of 1 as well as unassigned ones were rejected. Data analysis was carried out with the *Xcalibur*™ software from *Thermo Fisher Scientific*. The RAW files generated by MS were uploaded into *Proteome Discoverer 2.5* (*Thermo Fisher Scientific*) and analysed using the *SequestHT* search engine with the Percolator algorithm for validation of protein and peptide identification. The maximum *Delta Cn* parameter for Peptide Spectrum Matches (PSMs) was set to 0.05 and their minimum score to 1.0. Proteins were searched within a recently curated database compiling 630 gluten sequences (Bromilow et al., 2017). Trypsin was selected as enzyme and up to 2 missed cleavages were allowed. Precursor ion mass tolerance was set at 10 ppm and 0.02 Da for-product ions. Carbamidomethylcysteine was selected as a fixed modification, while oxidation of methionine and of C-terminals were defined as variable modifications. High confidence identifications were ordered in relation to their protein scores and examined for common molecular signatures.

2.6. Gastrointestinal protein digestion

The *in vitro* gastrointestinal digestion of gliadin proteins was based on a recently standardized protocol making use of artificial fluids simulating the exact composition of digestive juices (Brodkorb et al., 2019). In brief, 0.5 g of protein were dissolved in 3 mL of simulated salivary fluid (pH 7.0) for 2 min at 37 °C. Then, 3 mL of simulated gastric juice (pH 3.0), containing pepsin (2 000 U/mL of digesta), were added and incubated for 120 min at 37 °C. Subsequently, 6 mL of simulated intestinal fluid (pH 7.0), containing 100 U/mL of pancreatin and 10 mmol/L bile were added and incubated for 120 min at 37 °C. The whole digestion protocol was performed under constant mixing on a rotating wheel. Digestions were stopped by heating samples at 95 °C for 5 min. The resulting peptide mixtures were subjected to solid phase extraction (for desalting) on 35 cc Oasis HLB cartridges (from *Waters*) according to manufacturer instructions. The tubes were pre-conditioned with methanol and equilibrated with ultrapure water. After loading the peptide mixtures, the tubes were washed with 50–100 mL water and the peptides eluted with 250 mL of methanol:acetonitrile (50:50, v/v). The organic solvent was removed from the eluate on a rotatory evaporator (37 °C), reconstituted in ultrapure water, and freeze-dried. Proteolytically-resistant gluten peptides were identified by mass spectrometry, following the same chromatographic, MS/MS and search parameters as before (with the exception that no enzyme or peptide modifications were specified).

2.7. Cell culture conditions

Culture medium was composed of *Dulbecco's Modified Eagle Medium* (DMEM) containing 4.5 g/L glucose, L-glutamine, 10% of inactivated fetal bovine serum, 1% sodium pyruvate, 1% antibiotics (100 UI/ml streptomycin and penicillin) and 0.01 mg/mL human transferrin. Cells were sub-cultured once a week in 75 cm^2 culture flasks, and the medium was replaced every two to three days. Cells were maintained in an incubator at 37 °C and 5% CO_2. Caco-2 and Caco-2/HT29-MTX co-cultures - in a proportion of 9:1 - were seeded on the apical chamber of either 6 or 12-well transwell permeable supports (*Corning Inc.*) at a cell density of 1.0×10^5 cells/cm^2 in each insert. Cells were allowed to grow and differentiate to confluent monolayers for 21 days after the initial seeding. Twenty-four hours before experiments, cells were washed with phosphate buffered saline (PBS). Gliadins were added the day after in *Hanks Balanced Salt Solution* (HBSS). Unless otherwise specified, cells were treated with 1.0 mg/mL for 6 h.

2.8. Cytosolic cell extract preparation

Caco-2 and Caco-2/HT29-MTX cells were harvested with trypsin-EDTA, homogenized in ice-cold extraction buffer composed of 100 mM phosphate buffer pH 7.4, 1 mM EDTA, 0.6% sulfosalicylic acid, 0.1% Triton X-100 and protease inhibitors (from *Roche*) for 45 min and centrifuged at 3.000 g for 4 min at 4 °C. The resulting cytosolic extracts were used for protein determinations (bicinchoninic acid assay, *Thermo Fisher Scientific*) and enzymatic assays.

2.9. Anti-oxidant and detoxifying defences evaluation

The basal levels of cytoprotective defences were examined in Caco-2/HT29-MTX co-cultures at both confluency and differentiated stage (about 16 days after their visual confluence). Total glutathione level (and GSSG concentration when quantifiable) was quantified by using the 5,5′-dithio-bis-(2-nitrobenzoic) acid (DTNB) recycling assay as described elsewhere (Rahman, Kode, & Biswas, 2006). Glutathione reductase (GR) and glutathione-S-transferase (GST) activities were spectrophotometrically assayed by standard protocols and, upon normalization to the protein content, they were expressed as ΔAbs@340 $nm.min^{-1}.mg^{-1}$ (Mannervik, 1999; Vontas, Enayati, Small, & Hemingway, 2000).

2.10. Intracellular ROS determination

Intracellular reactive oxygen species (ROS) production was accessed by using 2',7'-dichlorofluorescein diacetate (*H_2DCFDA*) as probe. Caco-2 and Caco-2/HT29-MTX co-cultures were plated into black 96-well plates (*Thermo Fisher Scientific*), 3 days before experiments (~ 80% confluency). Twelve hours before the assay, cells were serum-deprived while on the experiment day, following medium removal, cells were washed with HBSS buffer and incubated with 25 μM *H_2DCFDA* for 30 min at 37 °C. From this step on, plates were protected from light. Cells were washed twice with HBSS and incubated for 4 h with different concentrations of gliadin digests, ranging from 0.05 to 1.0 mg/mL. Fluorescence was monitored at time 1 h, 2 h, and 4 h, with temperature maintained at 37 °C. The excitation filter was set at 485 nm and the emission filter to 530 nm. The fluorescence from each well was captured on a *FlexStation*™ *3 Multi-Mode Microplate Reader* (*Molecular Devices*). ROS production is expressed as fluorescence arbitrary units.

2.11. Statistical analysis

Differences among various treatment groups were determined by ordinary one-way ANOVA followed by Tukey's multiple-comparison

Table 1
Proteomic and physical chemical characterization (i.e. molecular weight, MW and isoelectric point, IP) of the previous hydroalcoholic gliadin extracts. A, B and C represent three independent extractions from the same wheat flour.

	MED. SCORE	COVERAGE	α/β	γ	ω	LMW-G	HMW-G	MEAN MW (Da)	MEAN IP
A	531.5	87.8 %	89	60	4	36	14	37 709.7	7.99
B	515.2	86.0 %	100	68	5	41	17	37 089.4	7.87
C	527.5	85.8 %	88	49	5	44	9	36 887.8	8.00

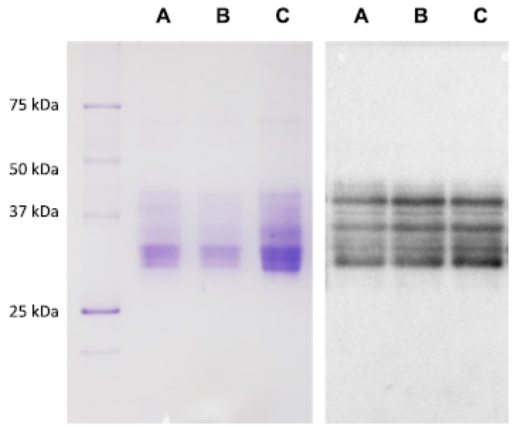


Fig. 1. SDS-PAGE (left) and Western Blot (right) protein profile of the hydroalcoholic gliadin extract. A, B and C represent three independent extractions from the same wheat flour.

test, with a single pooled variance, on GraphPad Prism 8 software. $P < 0.05$ was considered significant. All experiments were performed at least 3 times, in independent days.

3. Results

3.1. Gliadin protein characterization

Gliadin proteins were isolated from a type 65 wheat flour, as described in section 2.2., three different gliadin pools, extracted in three independent days, were characterized by untargeted LC-MS/MS analysis (Table 1). Results show that α/β-gliadins are the most representative protein subtype in wheat flour (44.1± 0.9 %), followed by γ-gliadins (28.0 ± 2.5 %), LMW-G (19.3 ± 2.8 %), HMW-G (6.3 ± 1.5 %) and ω-gliadins (2.3 ± 0.3 %). This distribution is in agreement with the typical values for each gluten protein subtype in hexaploidy wheat cultivars (relative to the total amount of gluten), as determined by modified Osborne fractionation and reversed-phase high-performance liquid chromatography (HPLC) by Schalk et al. (2017) (Schalk, Lexhaller, Koehler, & Scherf, 2017). Within each protein group identified herein, differences arise from small changes of short amino acid sequences and protein lengths (*data not shown*), mostly caused by allelic variations (including pseudogenization) in their coding regions (Wang et al., 2017). The presence of both high and low molecular weight glutenin subunits (HMW-GS and LMW-GS respectively), though atypical in the gliadin fraction (particularly the HMW ones), probably results from they being in a native monomeric/oligomeric state and therefore co-extractable with α/β-, γ- and ω-gliadins (Wieser, Koehler, & Scherf, 2023). The concentration of HMW-GS, however, - having molecular weights ranging from 69.7 to 88.4 kDa - should be lower than 5 $\mu g.mg^{-1}$ of protein extract as they were nearly undetectable by SDS-PAGE (Fig. 1). According to Table 1, the score values, coverage percentage and number of different peptide sequences found on *SEQUEST HT* were quite high for most proteins. As such, one may argue that there is a high degree of confidence associated with the identification of the gluten proteins in this hydroalcoholic extract.

To assess the immunogenicity of the previous gliadin extract, and to further corroborate the already anticipated CD T-cell stimulatory effect based on the protein sequences identified by LC-MS/MS (especially the high proportion of α/β-gliadins, the most CD toxic protein elements of wheat gluten (Ludvig M Sollid, Qiao, Anderson, Gianfrani, & Koning, 2012)), a Western Blot analysis was performed (Fig. 1). According to the obtained protein distribution pattern and signal intensity of the blot, this analysis not only did highlight the immunoreactive potential of the latter gliadin preparation as it also demonstrated the high predominance of gliadin proteins in the mixture.

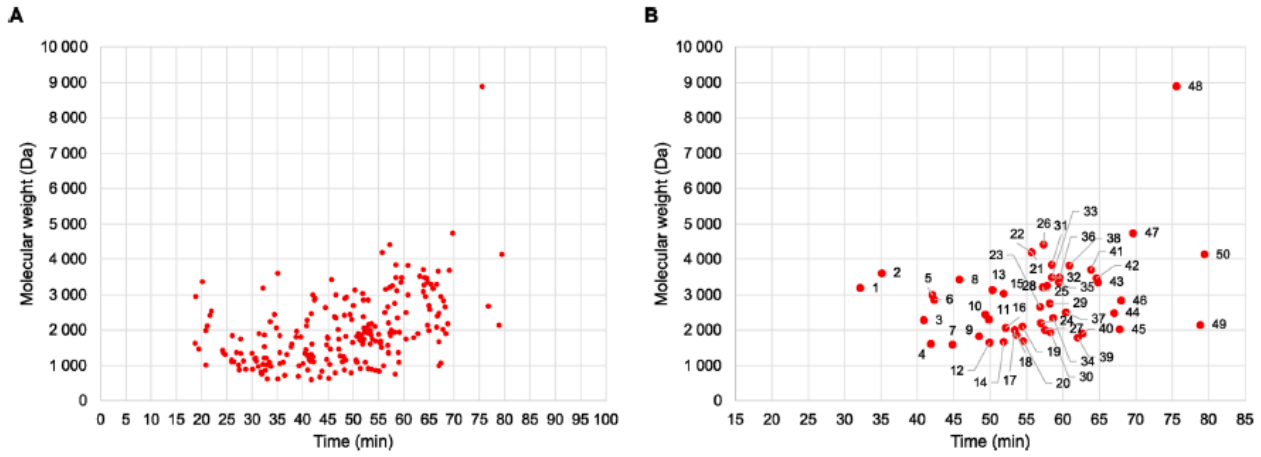


Fig. 2. Peptide mapping of the gastrointestinal gliadin digest by LC-MS/MS. A) Chromatographic representation of all peptides assigned by Proteome Discoverer. The peptides identified with high confidence (percolator q-values ≤ 0.01) are highlighted in red. B) High-score immunoreactive peptide sequences based on the presence of at least one already identified CD T-cell epitope.

Table 2
Listing of high-score immunoreactive peptide sequences in Glia$^{+/+}$ containing at least one CD T-cell epitope.

	SEQUENCE	# PSMS	# PROTEINS	# PROTEIN GROUPS	PROTEIN GROUP ACCESSIONS	XCORR	MH+ [Da]	ΔM [ppm]	RT [min]	Shared in Glia $^{-/+}$?
1	QSGQGQQQGYYPTSPQQSGQGQQPGQGQQPR	1	12	1	G3FLC5	3.88	3 187.5	0.73	32.16	
2	QQPGQGQQPEQGQQPGQGQQGYYPTSPQQPGQGK	1	4	1	Q0Q5D8	3.29	3 621.7	3.25	35.10	
3	QQLPQPQQPQQSFPQQQRP	6	7	1	K7X1S8	4.82	2 288.2	1.81	40.86	●
4	QPQQSFPQQQRPF	5	9	1	K7X1S8	4.12	1 615.8	0.85	41.80	●
5	sLQQPGQGQQQIGQGQQGYYPTSPQHPGQ	1	7	1	Q0Q5D8	3.64	2 994.4	2.34	42.06	
6	sLQQPGQGQQQIGQGQQGYYPTSPQHPG	1	7	1	Q0Q5D8	2.63	2 866.4	1.14	42.31	●
7	QQQIPQQPQQPFQ	1	1	1	Q4QIJ5	3.43	1 594.8	1.23	44.83	
8	SQQPEQTISQPQQPFPQQPHQPQQPYPQ	4	5	2	U5U6L8;R9XT67	6.06	3 424.6	1.96	45.77	
9	TQQPQQPFPQPQQPH	3	1	1	R9XU56	3.40	1 835.9	1.00	48.46	
10	QQLPQPQQPQQSFPQQQRPF	20	7	1	K7X1S8	5.38	2 435.2	2.42	49.30	●
11	QLPQPQQPQQSFPQQQRPF	1	7	1	K7X1S8	3.90	2 307.2	-0.49	49.78	
12	GIIQPQQPAQLEGIR	3	11	3	K7X1Q2;R9XWA6;M9TLK0	3.85	1 647.9	0.63	49.93	●
13	QTFPHQPQQQPFPQPQQPFPQQPQ	1	2	1	R9XWC4	6.60	3 139.5	0.28	50.29	
14	GIIQPQQPAQLEAIR	3	25	5	K7X1S8;R9XV78;R9XU99;B6UKN7;B6DQB9	3.45	1 661.9	0.06	51.81	
15	IQPQQPFPQQPQQTYPQRPQQPFPQ	1	6	2	D0ES82;D0ES84	3.64	3 031.5	0.71	51.83	●
16	VQGQGIIQPQQPAQLEAIR	3	25	5	K7X1S8;R9XV78;R9XU99;B6UKN7;B6DQB9	2.87	2 074.1	3.40	52.15	
17	QGQGIIQPQQPAQLEVIR	3	22	8	P06659;D0ES82;R9XWC4;D0ES81;D0ES84; B6UKQ7;D0E580;B6URM8	3.48	2 003.1	0.99	53.34	●
18	GQGIIQPQQPAQLEVIR	1	22	8	P06659;D0ES82;R9XWC4;D0ES81;D0ES84; B6UKQ7;D0E580;B6URM8	2.70	1 875.0	2.13	53.57	
19	VQGQGIIQPQQPAQLEVIR	4	22	8	P06659;D0ES82;R9XWC4;D0ES81;D0ES84; B6UKQ7;D0E580;B6URM8	4.58	2 102.2	1.06	54.40	●
20	GIIQPQQPAQLEVIR	5	22	8	P06659;D0ES82;R9XWC4;D0ES81;D0ES84; B6UKQ7;D0E580;B6URM8	3.62	1 690.0	-0.19	54.52	●
21	QQFPQPQQPQQPFPQPQQPQQFPQPQQPQQPFPQPQ	4	2	1	R9XWC4	6.76	4 205.1	4.75	55.73	●
22	QQFPQPQQPQQPFPQPQQPQQFPQPQQPQQPFPQQP	4	2	1	R9XWC4	6.76	4 205.1	4.75	55.73	●
23	TQQPQQPFPQPQPHQPFPQPQ	6	1	1	R9XU56	5.05	2 658.3	1.96	56.89	●
24	LVQGQGIIQPQQPAQLEVIR	1	22	8	P06659;D0ES82;R9XWC4;D0ES81;D0ES84; B6UKQ7;D0E580;B6URM8	3.27	2 215.3	0.85	56.96	●
25	SQQQQVGQGSLVQGQGIIQPQQPAQLEAIR	2	18	3	K7X1S8;B6UKN7;B6DQB9	4.55	3 214.7	2.09	57.22	●
26	QQPQQQYPSGQGSFQPSQQNPQAQGSVQPQQLPQFEEIR	1	25	9	J7HWD3;K7X0I8;K7X1G2;K7XR49;J7I026; X2K561;A5J3B5;A5J6A6;R9XW75	6.45	4 423.1	1.38	57.28	●
27	LQPQQAFPQQPQQPFPQ	2	3	2	R9XU99;R9XU56	3.81	2 007.0	1.45	57.53	
28	QQFPQPQQPQQQFPQPQQPQQPFPQPQ	5	23	3	A7XDG7;U5U7C0;B6UKQ8	6.81	3 254.6	2.17	57.76	
29	SQQQPFPSQQQQQPVIPQQPSFSQQ	8	19	1	B2Y2Q7	4.34	2 767.3	1.42	58.24	
30	QGLGIIQPQQPAQLEGIR	1	11	3	K7X1Q2;R9XWA6;M9TLK0	2.59	1 946.1	0.94	58.29	●
31	LQQPQQPFPQPQQQLPQPQQPQQSFPQQQRPF	5	6	1	K7X1S8	6.45	3 851.9	1.72	58.47	●
32	VLPQQPFPSQQQQQPVLPPQQSFPQQQQQHQ	1	6	1	B6DVG3	4.08	3 489.8	3.81	58.50	●
33	VLPQQPFPSQQQQQPVLPPQQSFPPQQQQQH	1	12	0		4.08	3 489.8	3.81	58.50	●
34	QLVQGQGIIQPQQPAQLEVIR	3	8	5	R9XWC4;D0ES81;B6UKQ7;D0ES80; B6UKM8	6.03	2 343.3	0.68	58.71	●
35	VLPQQPFPSQQQQQPVLPPQQSFPPQQQQH	4	6	1	B6DVG3	5.06	3 361.7	1.70	59.42	●
36	TQPQQPTPIQPQQPFPQQPQQPQQPFPQPQ	2	8	3	U5U6M9;R9XT67;U5U6N3	4.43	3 488.7	0.88	59.57	●
37	SQQQPFPSQQQQQPVIPQQPSFS	4	19	1	B2Y2Q7	3.43	2 511.2	1.50	60.37	
38	PFPQPQQAQLPFPQPQQPFPQPQQPQQPFPQS	1	25	5	A7XDG7;U5U7C0;B6UKQ6;R7XEG8; R9XUC7	2.27	3 835.9	1.51	60.90	
39	LQFPQPQLPYPQPH	3	4	3	J7I026;X2K561;A5J6A7	2.87	1 786.9	2.58	62.01	

(continued on next page)

Table 2 (*continued*)

	SEQUENCE	# PSMS	# PROTEINS	# PROTEIN GROUPS	PROTEIN GROUP ACCESSIONS	XCORR	MH+ [Da]	ΔM [ppm]	RT [min]	Shared in Glia $^{-/+}$?
40	QLQPFPQPQLPYPQPH	6	4	3	J7I026;X2K561;A5JSA7	4.06	1 915.0	1.14	62.75	●
41	SQQQPPFSQQQPPFSQQQPVIPQQPSFSQQ	4	18	1	B2Y2Q7	4.94	3 707.8	-1.07	63.84	
42	FPQPQQPFPQTQPQQPFPQQPQQPFPQ	1	18	5	K7X1S8;R9XU99;H9BFB6;B6DQB9;R9XUS6	5.66	3 471.7	0.62	64.61	
43	QQPFPSQQQPFPSQQQQPVLPQQPSFSQQ	2	19	1	B2Y2Q7	4.22	3 364.6	0.36	64.80	
44	FQLVQGGQIIQPQQPAQLEVIR	2	8	5	R9XWC4;D0ES81;B6UKQ7;D0ES80;B6UKM8	1.84	2 490.4	-0.29	67.00	●
45	LQLQPFPQPQLPYPQPH	9	4	3	J7I026;X2K561;A5JSA7	4.10	2 028.1	0.48	67.82	●
46	VLPQQPPFSQQQQPVLPPQQSPFPQ	6	20	1	B6DVS3	3.69	2 840.5	1.19	67.98	●
47	QQPFPLQPQQPFPQPQQPFPQPQQSFPQQPQQPFPQQP	2	1	1	R9XT67	3.56	4 746.3	1.21	69.66	
48	QTQQPQQPFPQPQQPFPQTQQPQQPFPQQPQQPFPQTQQPQQPF	1	13	3	K7X1S8;H9BFB6;B6DQB9	1.52	8 891.4	6.35	75.59	
49	PQLQQPQQPFPQPQQPLQPQQPQQSFPQQ QLQPFPQPQLPYPQPQPF	5	12	7	A5JSB5;K7X0J8;A5JSA8;Q3S4V8;J7HZV4;K7X1G2;Q3S4V7	5.26	2 150.1	1.80	78.86	●
50	TQPQQPTPLQPQQPFPQQPQQPQQPFPQPQQPFPW	2	8	3	U5U6N3;U5U6M9;R9XT67	1.64	4 144.0	0.81	79.41	

3.2. Gliadin digestion and peptide mapping

As mentioned earlier, the COST INFOGEST network has recently standardized a static *in vitro* digestion protocol of both solid and liquid food matrices, developed so that the *in vitro* digestion methods used in research could be normalized and therefore comparable between laboratories. Herein, the same approach has been employed to digest the previously obtained gliadin extract to fully define the identity and biological effects of gliadin peptides at the intestinal level. Following *in vitro* digestion, untargeted proteomic analysis was conducted by LC-MS/MS in time dependent acquisition mode and data processed using SEQUEST HT against a curated gluten FASTA library. Accordingly, a total of 13 364 gluten peptides were identified with precursor MH^+ masses ranging from 601.3 (seq. PFLPQ) to 8,891.4 Da (seq. $(QTQQPQQPFPQQPQQPFP)_2$QTQQPQQPFPQLQQPQQPFPQPQQQLPQ $(PQQ)_2$SFPQQ) (mean: 2 4381.3 Da; median: 2 322.1 Da) (Fig. 2***A***) and percolator PEP scores varying between 0.025 (high confidence peptide-spectrum match, PSM) to 1.0 (low confidence PSM). For simplicity purposes, only unambiguous assignments (percolator q-values ≤ 0.01) are presented in Supplementary Table 1 and further discussed.

For CD pathophysiological mechanisms to be triggered, gluten peptides must be partially resistant to gastrointestinal digestion and contain at least one of the more than 35 wheat epitopes already known to be recognized by HLA-DQ2/8 receptors (L. M. Sollid et al., 2020). A gluten peptide containing at least one of these epitopes is described as an immunogenic gluten peptide (Ogilvie et al., 2020). Various approaches have been used throughout the literature to ascertain the immunogenic peptide content and distribution in bread wheat and related species. Nowadays, all known T-cell and B-cell immunogenic sequences are deposited in curated databases such as prolamin peptide epitope database *ProPepper*, a curated database for identification and analysis of peptide and immune-responsive epitope composition of cereal grain protein families (Juhasz, Haraszi, & Maulis, 2015). Among the top 225 high-score sequences selected, a total of 50 gluten peptides were found to have a strong immunoreactivity potential due to the presence of at least one copy of 13 different epitope hits (Fig. 2***B***), including the DQ2.5-glia-γ2a (FPQQPQQPF), DQ2-glia-γ30 (IIQPQQPAQ), DQ2.5-glia-α1a (PFPQPQLPY), DQ2.5-gliadin-ω1 (PFPQPQQPF), DQ2.2-glut-L1 (PFSQQQQPV), DQ2.5-glia-α2 (PQPQLPYPQ), DQ2-*sec*-γ1 (PQQSFPQQP), DQ2.5-glia-γ1 (PQQSFPQQQ), DQ8-glia-α1 (QGSFQPSQQ), DQ8-glut-H1 (QGYYPTSPQ), DQ2.5-glia-γ5 (QQPFPQQPQ), DQ8-glia-γ1a (QQPQQPFPQ) and DQ2.5-glu-5 (QIPQQPQQF) sequences (Table 2).

Next, the peptide identity, molecular weight distribution and immunogenicity of the gliadin digest bypassing gastric processing was evaluated. Compared to the complete gastrointestinal digestion of gliadins (henceforth defined as glia$^{+/+}$), the absence any pepsin activity (glia$^{-/+}$) resulted in the generation of approximately 15 097 different peptides with molecular weights ranging between 611.4 to 9 908.0 Da (mean: 3 339.0 Da; median: 2 881.5 Da). The sequences identified with high confidence in glia$^{-/+}$ (a total of 257 peptides) can be found in Supplementary Table 2.

From early experiments, pepsin is known to have much less specificity than that of trypsin and other proteases. Pepsin preferentially cleaves after phenylalanine and leucine residues whereas it rarely cleaves after histidine and lysine unless they are adjacent to leucine, phenylalanine, and a few others (Ahn, Cao, Yu, & Engen, 2013). Being leucine and phenylalanine the two most abundant essential amino acids of both common and *Durum* wheat varieties (Mickowska, Socha, Urminská, Cieslik, & Address, 2012), the digestibility of gliadin proteins happens to be significantly dependent on the activity of pepsin at the gastric level, reason why differences ($p < 0.0001$) were observed in the molecular weight distribution of gluten peptides in glia$^{+/+}$ and glia$^{-/+}$ samples (Fig. 3).

Next, the extent by which both mixtures were similar or different in their peptide content was determined. To do so, a set of comparisons

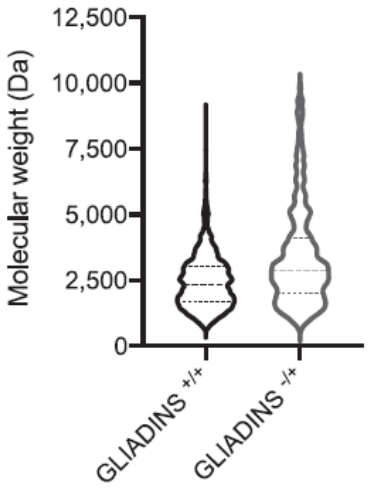


Fig. 3. Molecular weight distribution of peptides in gliadin samples subjected to complete (GLIADINS +/+) and incomplete (GLIADINS -/+) INFOGEST gastrointestinal digestion.

were made between the high-score peptide matches in each gliadin sample against all other peptide assignments in their counterpart mixture i.e. high score peptides in glia$^{+/+}$ were compared with all peptide assignments (be they low, medium or high-scored) in glia$^{-/+}$ and vice-versa. From the top-225 high-score peptide sequences of glia$^{+/+}$, 116 were found to be featured as high score peptide matches in glia$^{-/+}$ (q-value < 0.01), 28 as medium score (q-value < 0.05), 25 as low score peptide assignments (q-value ≥ 0.05) and 56 as unique peptide sequences. This results in a sequence similarity around 75%. Looking at the glia$^{-/+}$ digest, from the 255 high-score peptide matches, 117 were also found to be high-score assignments in glia$^{+/+}$, 22 were identified as medium score, 35 as low score and 81 as unique sequences. Their similarity - taking only into account high score peptide matches in glia$^{-/+}$ as reference - was 68%. Overall, however, the similarity between both samples was found to be much lower - around 30% - with a total of 4 263 peptide sequences identified in both matrices (*data not shown*).

3.3. Assessment of gliadin-induced redox perturbations

In the intestine, the intracellular and extracellular thiol redox status modulates the proliferative, self-renewal and differentiative potential of epithelial cells. Not surprisingly, the loss of redox homeostasis is involved in the pathogenesis and development of a wide diversity of gastrointestinal disorders, including CD, in which oxidative stress mediates most of the cytotoxic effects induced by gluten peptides (Pérez, Taléns-Visconti, Rius-Pérez, Finamor, & Sastre, 2017). Herein, the intracellular redox, detoxifying and inflammatory status of Caco-2 and Caco-2/HT29-MTX co-cultures treated to 1 mg.mL^{-1} of glia$^{+/+}$ and glia$^{-/+}$ were evaluated and thoroughly compared. The chosen timepoint was 6 h and the cellular readouts were: total glutathione levels (reduced, GSH, + oxidised, GSSG), glutathione reductase activity (GR), glutathione S-transferase activity (GSR), interleukin 8 (IL-8) and interleukin 15 (IL-15, the central regulator of CD).

On differentiated Caco-2 cell cultures, no statistically significant differences were found in the intracellular activity of both GR and GST, both of which remained similar to the control medium (Fig. 4). On the opposite, glia$^{-/+}$ treatment induced a significant decrease in the total levels of glutathione, coupled to a non-statistical increase of GSSG (*data not shown*). Regarding the production of IL-8 and IL-15, at the end of 6 h, their concentration remained below the detection threshold of ELISA in both cases (*data not shown*).

Looking at the effects induced by gliadin treatment on Caco-2/HT29-MTX co-cultures, the trend was somewhat different from the ones observed on Caco-2 cells alone. Indeed, though glia$^{+/+}$ did not appear to produce any kind of redox or inflammatory perturbation on co-cultures, glia$^{-/+}$ did disturb Caco-2:HT29-MTX cells, possibly reflecting increased oxidative stress. Accordingly, glia$^{-/+}$ induced a statistically significant decrease in total GSH levels (Fig. 5***A***), GR (Fig. 5***B***) and GST enzyme activities (Fig. 5***C***), while increasing IL-8 concentration at the basolateral compartment (Fig. 5***D***). No differences were seen in IL-15 production (Fig. 5***E***), being this result consistent with the lack of genetic predisposition of both Caco-2 and HT29-MTX cells for CD. Overall, in view of the presented data, one may conclude that gastric processing of gliadin proteins does influence gliadin cytotoxic properties at the intestinal level, as by by-passing it, gliadin peptides become able to significantly impair both antioxidant and detoxifying functions of Caco-2:HT29-MTX cells. Such outcome is not seen following a complete gliadin digestion (glia$^{+/+}$) - nor at the end of 24 h of incubation (Fig. 6) -, whose effect was limited to a dose-dependent increase in intracellular ROS (iROS) levels, as measured by the DCFDA assay (Fig. 7).

Therefore, in a physiologically relevant timeframe - and notwithstanding the limitations that this cellular model has *in vivo* -, glia$^{+/+}$ stimulation of Caco-2:HT29-MTX cells appear to provide nothing more than a mild oxidative stimulus, and not a pro-inflammatory one, to which cells can promptly respond and neutralize. Such outcome is in line with previous studies confirming that intestinal epithelial cells do become less sensitive to inflammatory stimulation upon differentiation, and that mature adult as compared to young proliferating cells could contribute differentially to the inflammatory response in the gut (Van De

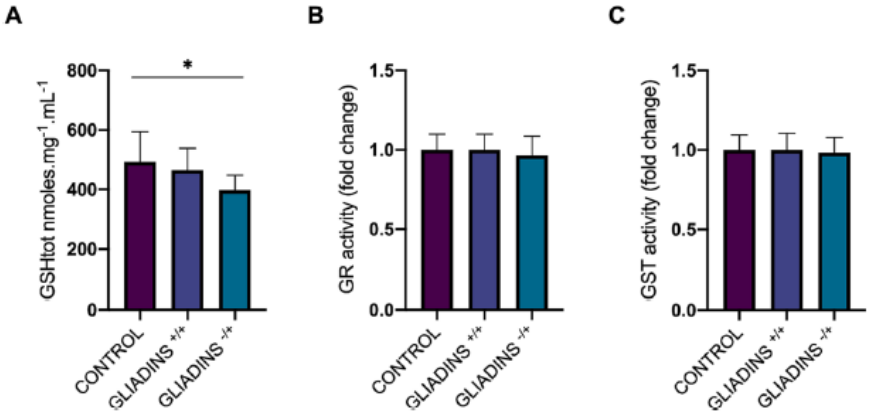


Fig. 4. Effect of gliadin peptide treatments on the total GSH content (A), GSR (B) and GST (C) enzyme activities on differentiated Caco-2 cells. Bars represent mean values ± standard deviation. *p<0.05.

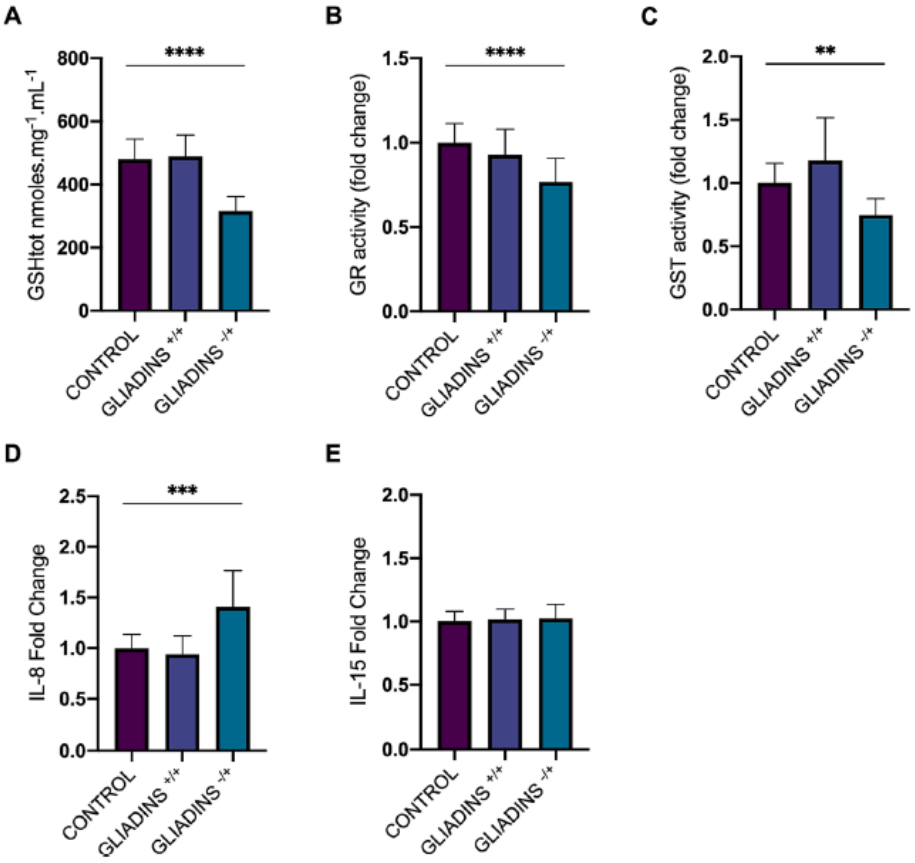


Fig. 5. Effect of gliadin peptide treatments on the total GSH content (A), GSR enzyme activity (B), GST (C), IL-8 production (D) and IL-15 secretion on Caco-2:HT29-MTX co-cultures. Bars represent mean values ± standard deviation. **p<0.005, ***p<0.001, ****p<0.0001.

Walle, Hendrickx, Romier, Larondelle, & Schneider, 2010). This is most probably the result of a differential expression of receptors and modified intracellular signalling pathways occurring with cell differentiation (Buhrke, Lengler, & Lampen, 2011). Concerning the antioxidant defence mechanisms of Caco-2 cells, it has been found that over time (after confluence), with the exception of GPx, enzymes such catalase, SOD and GSR have a small, but statistically significant increase in their activity (Baker & Baker, 1992). Additionally, the gene and protein expression levels of GST also increase during differentiation (Scharmach, Hessel, Niemann, & Lampen, 2009). Future fine-tuning of these experimental conditions, through the employment of more complex and integrated *in vitro* cell systems (including immune cells and microbiota populations) and use of human gut-derived CD organoids, will be useful in modelling and exploiting gluten-induced perturbations in the nucleophilic tone

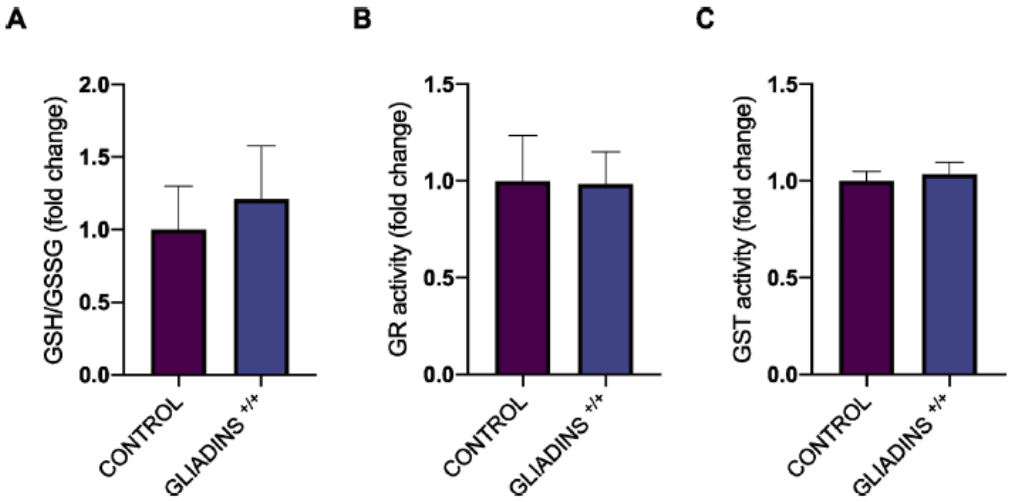


Fig. 6. Effect of glia$^{+/+}$ peptide treatments on GSH:GSSG ratio (A), GSR (B) and GST (C) enzyme activities on Caco-2:HT29-MTX co-cultures at the end of 24 h of incubation at 37 °C. Bars represent mean values ± standard deviation.

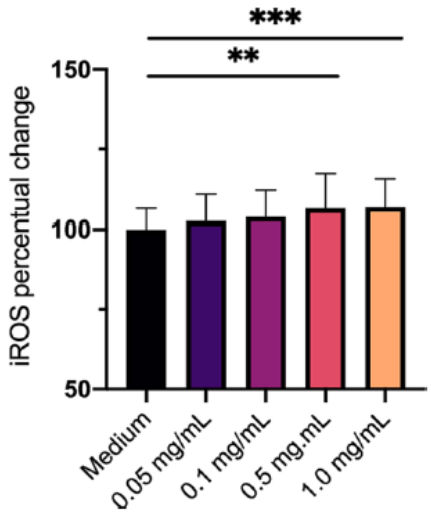


Fig. 7. Intracellular ROS (iROS) levels measured using the DCFDA assay in Caco-2:HT29-MTX co-cultures treated with different concentrations of glia$^{+/+}$ for 4 h at 37 °C. Bars represent mean values ± standard deviation. **p<0.01, ***p<0.001.

and inflammatory status of intestinal cells *in vitro* and ways of modelling it through nutrition.

4. Conclusion

Ever since the INFOGEST protocol for the *in vitro* simulation of gastrointestinal food digestion was established, the number of scientific records employing it to study protein hydrolysis from different dietary sources has grown exponentially. The number of studies exploiting their peptide release profile and biological effects at the cellular level, however, - be they focused on gastric or intestinal bioactivities - remain rather limited.

Early on, it has been shown that the matrix and proteolytic system used in different *in vitro* digestion models of gluten proteins has a strong influence in the type of peptides produced, both qualitatively as quantitatively. Although the simplified peptic-tryptic/chymotryptic method can still be used for varietal screening, at the end, it is not representative of the peptides that are truly generated at the gastrointestinal tract after physiological human digestion. Therefore, depending on the protocol followed, the content of proteolytic resistant peptides could be significantly different, which could have dramatic implications on their pathogenicity on a CD context and the cause of conflicting results in many CD-related studies. Herein, it has been shown that digestion of gliadin proteins through the harmonized INFOGEST method results in the generation of many different gliadin peptides, some of which potentially immunoreactive *in vivo*. When tested *in vitro* on Caco-2 and Caco-2:HT29-MTX co-cultures, however, their pro-oxidative and pro-inflammatory effects were found to be negligible in a 6-hour period, contrarily what was seen when the gastric phase of gliadin processing was by-passed. With the growing number of people undergoing gastrectomy for gastric carcinoma over the last few years, these results may shed light into a still unknown "dark face" of gluten proteins in modern and future nutrition, whose dietary inclusion may need to be reassessed in certain clinical contexts.

Funding

This work was financially supported through the project UIDB/50006/2020, funded by FCT/MCTES through national funds.

CRediT authorship contribution statement

Sara Silva: Investigation, Writing – original draft. **Rosa Pérez-Gregorio:** Writing – review & editing. **Nuno Mateus:** . **Victor Freitas:** Funding acquisition, Project administration, Writing – review & editing. **Ricardo Dias:** Conceptualization, Supervision, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to acknowledge the AGRIFOOD XXI project (NORTE-01-0145-FEDER-000041), co-financed by European Regional Development Fund (ERDF), through the NORTE 2020 (*Programa Operacional Regional do Norte 2014/2020*) and FCT for the Scientific Employment Stimulus grant (*2021.01830.CEECIND*).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.113317.

References

Ahn, J., Cao, M. J., Yu, Y. Q., & Engen, J. R. (2013). Accessing the reproducibility and specificity of pepsin and other aspartic proteases. *Biochimica et Biophysica Acta, 1834*(6), 1222–1229.

Bai, J., Moran, C., Martinez, C., Niveloni, S., Crosetti, E., Sambuelli, A., & Boerr, L. (1991). Celiac sprue after surgery of the upper gastrointestinal tract. Report of 10 patients with special attention to diagnosis, clinical behavior, and follow-up. *Journal of Clinical Gastroenterology, 13*(5), 521–524.

Baker, S. S., & Baker, R. D. (1992). Antioxidant enzymes in the differentiated Caco-2 cell line. *In Vitro Cellular & Developmental Biology-Animal, 28*(9–10), 643–647.

Biesiekierski, J. R. (2017). What is gluten? *Journal of Gastroenterology and Hepatology, 32*(Suppl 1), 78–81.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., … Carrière, F. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014.

Bromilow, S., Gethings, L. A., Buckley, M., Bromley, M., Shewry, P. R., Langridge, J. I., & Mills, E. C. (2017). A curated gluten protein sequence database to support development of proteomics methods for determination of gluten in gluten-free foods. *Journal of Proteomics, 163*, 67–75.

Buhrke, T., Lengler, I., & Lampen, A. (2011). Analysis of proteomic changes induced upon cellular differentiation of the human intestinal cell line Caco-2. *Development, Growth & Differentiation, 53*(3), 411–426.

Freire, R., Ingano, L., Serena, G., Cetinbas, M., Anselmo, A., Sapone, A., … Senger, S. (2019). Human gut derived-organoids provide model to study gluten response and effects of microbiota-derived molecules in celiac disease. *Scientific Reports, 9*(1), 7029.

Freitas, D., Gómez-Mascaraque, L. G., & Brodkorb, A. (2022). Digestion of protein and toxic gluten peptides in wheat bread, pasta and cereal and the effect of a supplemental enzyme mix. *Frontiers Nutrition, 9*.

Gagnon, M., Zihler Berner, A., Chervet, N., Chassard, C., & Lacroix, C. (2013). Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-MTX intestinal cell models to investigate Salmonella adhesion and invasion. *Journal of Microbiological Methods, 94*(3), 274–279.

Green, P. H., & Cellier, C. (2007). Celiac disease. *The New England Journal of Medicine, 357*(17), 1731–1743.

Hilgers, A. R., Conradi, R. A., & Burton, P. S. (1990). Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa. *Pharmaceutical Research, 7*(9), 902–910.

Huet, G., Kim, I., de Bolos, C., Lo-Guidice, J. M., Moreau, O., Hemon, B., … Degand, P. (1995). Characterization of mucins and proteoglycans synthesized by a mucin-secreting HT-29 cell subpopulation. *Journal of Cell Science, 108*(Pt 3), 1275–1285.

Hundsberger, H., Koppensteiner, A., Hofmann, E., Ripper, D., Pflüger, M., Stadlmann, V., … Eger, A. (2017). A screening approach for identifying Gliadin neutralizing

antibodies on epithelial intestinal Caco-2 cells. *SLAS DISCOVERY: Advancing Life Sciences R&D, 22*(8), 1035–1043.
Iwamoto, M., Kato, K., Kusumi, Y., Masuda, S., Nakayama, T., & Moriyama, M. (2022). Celiac Disease Diagnosed after Gastrectomy for Gastric Cancer. *Internal Medicine, 61*(3), 323–328.
Juhasz, A., Haraszi, R., & Maulis, C. (2015). ProPepper: a curated database for identification and analysis of peptide and immune-responsive epitope composition of cereal grain protein families. *Database (Oxford), 2015*.
Lee, I. J., Hom, K., Bai, G., & Shapiro, M. (2009). NMR metabolomic analysis of caco-2 cell differentiation. *Journal of proteome research, 8*(8), 4104–4108.
Mannervik, B. (1999). Measurement of Glutathione Reductase Activity. *Current Protocols in Toxicology, 00* (1), 7.2.1-7.2.4.
Mazzarella, G., Bergamo, P., Maurano, F., Luongo, D., Rotondi Aufiero, V., Bozzella, G., Palmieri, G., Troncone, R., Auricchio, S., David, C., & Rossi, M. (2014). Gliadin intake alters the small intestinal mucosa in indomethacin-treated HLA-DQ8 transgenic mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 307* (3), G302–312.
Mickowska, B., Socha, P., Urminská, D., & Cieslik Address, E. (2012). The comparison of prolamins extracted from different varieties of wheat, barley, rye, and triticale species: Amino acid compostion, electrophoresis, and immunodetection. *Journal of Microbiology, Biotechnology and Food Sciences, 1*, 742–752.
Ogilvie, O., Roberts, S., Sutton, K., Domigan, L., Larsen, N., Gerrard, J., & Demarais, N. (2020). Proteomic modelling of gluten digestion from a physiologically relevant food system: A focus on the digestion of immunogenic peptides from wheat implicated in celiac disease. *Food Chemistry, 333*, Article 127466.
Okumura, R., & Takeda, K. (2017). Roles of intestinal epithelial cells in the maintenance of gut homeostasis. *Experimental & Molecular Medicine, 49*(5), e338–e.
Pali-Schöll, I., Untersmayr, E., Klems, M., & Jensen-Jarolim, E. (2018). The Effect of Digestion and Digestibility on Allergenicity of Food. *Nutrients, 10*(9), 1129.
Pérez, S., Taléns-Visconti, R., Rius-Pérez, S., Finamor, I., & Sastre, J. (2017). Redox signaling in the gastrointestinal tract. *Free Radical Biology and Medicine, 104*, 75–103.
Prandi, B., Faccini, A., Tedeschi, T., Cammerata, A., Sgrulletta, D., D'Egidio, M. G., … Sforza, S. (2014). Qualitative and quantitative determination of peptides related to celiac disease in mixtures derived from different methods of simulated gastrointestinal digestion of wheat products. *Analytical and Bioanalytical Chemistry, 406*(19), 4765–4775.
Rahman, I., Kode, A., & Biswas, S. K. (2006). Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nature Protocols, 1*(6), 3159–3165.
Rivabene, R., Mancini, E., & De Vincenzi, M. (1999). In vitro cytotoxic effect of wheat gliadin-derived peptides on the Caco-2 intestinal cell line is associated with intracellular oxidative imbalance: Implications for coeliac disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 1453*(1), 152–160.
Schalk, K., Lexhaller, B., Koehler, P., & Scherf, K. A. (2017). Isolation and characterization of gluten protein types from wheat, rye, barley and oats for use as reference materials. *PLoS One1, 12*(2), e0172819.
Scharmach, E., Hessel, S., Niemann, B., & Lampen, A. (2009). Glutathione S-transferase expression and isoenzyme composition during cell differentiation of Caco-2 cells. *Toxicology, 265*(3), 122–126.
Sollid, L. M., Qiao, S.-W., Anderson, R. P., Gianfrani, C., & Koning, F. (2012). Nomenclature and listing of celiac disease relevant gluten T-cell epitopes restricted by HLA-DQ molecules. *Immunogenetics, 64*(6), 455–460.
Sollid, L. M., Tye-Din, J. A., Qiao, S. W., Anderson, R. P., Gianfrani, C., & Koning, F. (2020). Update 2020: Nomenclature and listing of celiac disease-relevant gluten epitopes recognized by CD4(+) T cells. *Immunogenetics, 72*(1–2), 85–88.
Stenman, S. M., Venäläinen, J. I., Lindfors, K., Auriola, S., Mauriala, T., Kaukovirta-Norja, A., … Sollid, L. M. (2009). Enzymatic detoxification of gluten by germinating wheat proteases: Implications for new treatment of celiac disease. *Annals of Medicine, 41*(5), 390–400.
Stricker, S., de Laffolie, J., Zimmer, K.-P., & Rudloff, S. (2023). Inhibition of Transglutaminase 2 as a Therapeutic Strategy in Celiac Disease—In Vitro Studies in Intestinal Cells and Duodenal Biopsies. *International Journal of Molecular Sciences, 24*(5), 4795.
Truzzi, F., Tibaldi, C., Whittaker, A., Dilloo, S., Spisni, E., & Dinelli, G. (2020). Pro-Inflammatory Effect of Gliadins and Glutenins Extracted from Different Wheat Cultivars on an In Vitro 3D Intestinal Epithelium Model. *International Journal of Molecular Sciences, 22*(1), 172.
Van De Walle, J., Hendrickx, A., Romier, B., Larondelle, Y., & Schneider, Y.-J. (2010). Inflammatory parameters in Caco-2 cells: Effect of stimuli nature, concentration, combination and cell differentiation. *Toxicology in Vitro, 24*(5), 1441–1449.
Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland, C., Lea, T., Mackie, A., … Wichers, H. (2015). *The Impact of Food Bioactives on Health in vitro and ex vivo models* ((1st ed.):). Springer Open.
Vontas, J. G., Enayati, A. A., Small, G. J., & Hemingway, J. (2000). A Simple Biochemical Assay for Glutathione S-Transferase Activity and Its Possible Field Application for Screening Glutathione S-Transferase-Based Insecticide Resistance. *Pesticide Biochemistry and Physiology, 68*(3), 184–192.
Wang, D.-W., Li, D., Wang, J., Zhao, Y., Wang, Z., Yue, G., … Dong, L. (2017). Genome-wide analysis of complex wheat gliadins, the dominant carriers of celiac disease epitopes. *Scientific Reports, 7*(1), 1–14.
Wanyera, R., & Owuoche, J. (2017). *Wheat improvement, management and utilisation*. BoD–Books on Demand.
Wieser, H., Koehler, P., & Scherf, K. A. (2023). Chemistry of wheat gluten proteins: Qualitative composition. *Cereal Chemistry, 100*(1), 23–35.