

Faculdade de Engenharia da Universidade do Porto



**Effects of fermented products from human
microbiota in intestinal *in vitro* models**

Verônica Techmeier Morato

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Supervisor: Victor Calero Martin

Co-Supervisor: Maria Pia de Melo Alvim Ferraz Tavares

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Resumo

Uma comunidade microbiana intestinal equilibrada e diversificada é crucial para manter a saúde humana, uma vez que perturbações e alterações em sua composição têm sido associadas a diversas doenças gastrointestinais. Entre as contribuições das bactérias comensais, destaca-se a produção de ácidos graxos de cadeia curta (AGCCs) por meio da fermentação de fibras alimentares. Os AGCCs contribuem significativamente para a manutenção da homeostase intestinal, fornecendo energia que sustenta o crescimento das células epiteliais do intestino, modulando a resposta imune e aprimorando a integridade da barreira. Assim, este estudo explora os potenciais benefícios de produtos fermentados contendo AGCCs derivados da microbiota humana, utilizando um modelo celular *in vitro* composto por uma monocamada de células Caco-2 e HT29-MTX co-cultivadas. Amostras fecais foram obtidas de doadores saudáveis, misturadas, fermentadas e diluídas. Diferentes concentrações (1:5, 1:6, 1:10 e 1:20) dos produtos fermentados foram testadas, uma vez que elevadas quantidades de AGCCs podem ser tóxicas para as células. Para avaliar a influência desses produtos na integridade do epitélio saudável e inflamado, foram mensuradas a permeabilidade e a resistência elétrica transepitelial (TEER) em poços estáticos Transwell. Além disso, a fim de mimetizar um ambiente intestinal mais realista, um modelo de intestino em chip microfluídico dinâmico também foi empregado para comparar a diferenciação celular e a resposta à permeabilidade dos produtos fermentados com o modelo estático. A imagem confocal foi utilizada para examinar a morfologia do epitélio e a avaliação qualitativa das exposições. No modelo estático saudável, os resultados demonstraram uma TEER mais elevada nas diluições 1:6, 1:10 e 1:20, enquanto a menor permeabilidade foi significativa apenas na diluição 1:20. A diluição 1:5 apresentou uma TEER mais baixa em comparação com os controles. Além disso, a monocamada na diluição 1:6 não é plana como nos controles. Assim, a diluição 1:10 foi escolhida devido à sua sustentada TEER mais elevada, o que não foi observado na diluição 1:20, e ausência de alteração na monocamada em comparação com o controle, para avaliar o efeito pelos AGCCs antes e depois da introdução do estímulo inflamatório pelo TNF- α . Não foram observadas alterações relevantes na TEER após a exposição aos produtos fermentados antes da inflamação. No entanto, uma TEER significativamente mais elevada foi observada quando foram introduzidos após a remoção do estímulo inflamatório. Nos chips intestinais, a monocamada exibe projeções semelhantes a vilosidades presentes no epitélio do intestino delgado humano e que são ausentes na cultura estática. A permeabilidade no modelo dinâmico é maior em comparação com o modelo estático e a diluição 1:20 também possui valores de permeabilidade significativamente mais baixos. Em conclusão, o estudo do papel da microbiota e de seus produtos é de grande importância para compreender a homeostase intestinal. O uso de modelos *in vitro* permitiu a avaliação fácil da barreira epitelial do intestino. Os resultados apresentados aqui demonstraram que produtos fermentados derivados da microbiota humana podem de fato ter fortes implicações na modulação da integridade do epitélio intestinal e nas respostas imunes. Trabalhos futuros se concentrarão em induzir a inflamação no modelo dinâmico e comparar o perfil imunológico das células com o modelo estático.

Abstract

A balanced and diverse gut microbial community is crucial to maintaining human optimal health, as disruptions and alterations on its composition have been implicated in various gastrointestinal diseases. Among the contribution of the commensal bacteria, they produce short-chain fatty acids (SCFAs) through the fermentation of dietary fibers. SCFAs contribute significantly to the maintenance of gut homeostasis by providing energy that supports gut epithelial cell growth, modulating the immune response and enhancing barrier integrity. Thus, this study explores the potential benefits of fermented products containing SCFAs derived from human microbiota, utilizing an *in vitro* cell model composed of a co-culture of Caco-2 and HT29-MTX cells monolayer. Fecal samples were obtained from health donors, pooled, fermented, and diluted. Different concentrations, 1:5, 1:6, 1:10 and 1:20, of fermented products were tested as high quantity of SCFAs can be toxic to the cells. To assess the influence of these fermented products on health and inflamed epithelium, permeability and transepithelial electrical resistance (TEER) were measured in static Transwell inserts. Moreover, in order to mimic a more realistic gut environment, a dynamic microfluidic gut-on-chip model was also employed to compare the health cells differentiation and permeability response to fermented products with the static model. Confocal imaging was used to examine morphology of the epithelium and qualitative assessment of the exposures. In static health model, the results demonstrated higher TEER on 1:6, 1:10, and 1:20, whereas lower permeability was only significant in the 1:20 dilution. The 1:5 dilution presented lower TEER when compared to the controls. Also, the 1:6 dilution monolayer is not flat as the controls. Thus, the dilution 1:10 was chosen due to its sustained higher TEER, which was not observed in the 1:20 dilution, and no alteration in the monolayer compared to control to evaluate the effect before and after inflammation of the SCFAs. No relevant changes were observed in TEER after exposure to the fermented products prior to inflammation. However, a significant higher TEER was observed when they were introduced after the removal of the inflammatory stimuli. On gut-chips, the monolayer display villi-like projections absent in the flat static culture. The permeability in the dynamic model is higher compared to the static model and the dilution 1:20 has also significantly lower permeability values. In conclusion, the study of the role of the microbiota and its products is of strong importance to understand gut homeostasis. The use of *in vitro* models enabled for the easy assessment gut epithelial barrier. The results presented here demonstrated that human microbiota derived fermented products can indeed have strong implications in modulating gut epithelial integrity and immune responses. Future work will focus in introducing inflammation in the dynamic model and comparing the cells immunoprofile with static model.

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Abbreviations and Symbols

P _{app}	Apparent permeability
⁵¹ Cr	Chromium-51
CXCL	C-X-C Motif Chemokine Ligand
BM	Basement membrane
BSA	Bovine serum albumin
DAPI	4',6-Diamidino-2-Phenylindole Dihydrochloride
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic acid
E-selectin	Endothelial selectin
ECM	Extracellular matrix
FBS	Fetal bovine serum
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HBSS	Hanks' Balanced Salt Solution
IBD	Inflammatory bowel disease
ICAM-1	Intercellular Adhesion Molecule-1
IFN	Interferon
IL	Interleukin
ILs	Interleukins
IM	Interstitial matrix
IP-10	Interferon-gamma-inducible protein 10
JAMs	Junctional adhesion molecules
LPS	Lipopolysaccharide
M cells	Microfold cells
MCP-1	Monocyte Chemoattractant Protein-1
MEM	Minimum Essential Medium
MIP-1	Macrophage Inflammatory Protein-1
MLCKs	Myosin light-chain kinases
NF-κB	Nuclear factor kappa B
PBS	Phosphate-buffered saline
PDMS	Polydimethylsiloxane
PFA	Paraformaldehyde
P-selectin	Platelet selectin
RT	Room temperature
SCFAs	Short-chain fatty acids
Sulfo-SANPAH	Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate)
T75	Tissue culture flasks of 75 cm ² surface growth area
TEER	Transepithelial electrical resistance
TNF	Tumor Necrosis Factor
TRITC	Tetramethylrhodamine B isothiocyanate
TSA	Tryptone soy agar
UV	Ultraviolet
ZO-1	Zonula occludens-1
α	Alpha
β	Beta
γ	Gamma
κ	Kappa

1. Introduction

1.1. The small intestine

The small intestine is the longest part of the human digestive system, it participates in the breakdown of food, and is the main route of nutrient absorption. It has a complex structure with a highly specialized epithelial lining, a rich vascular network, and host a diverse population of microorganisms (1).

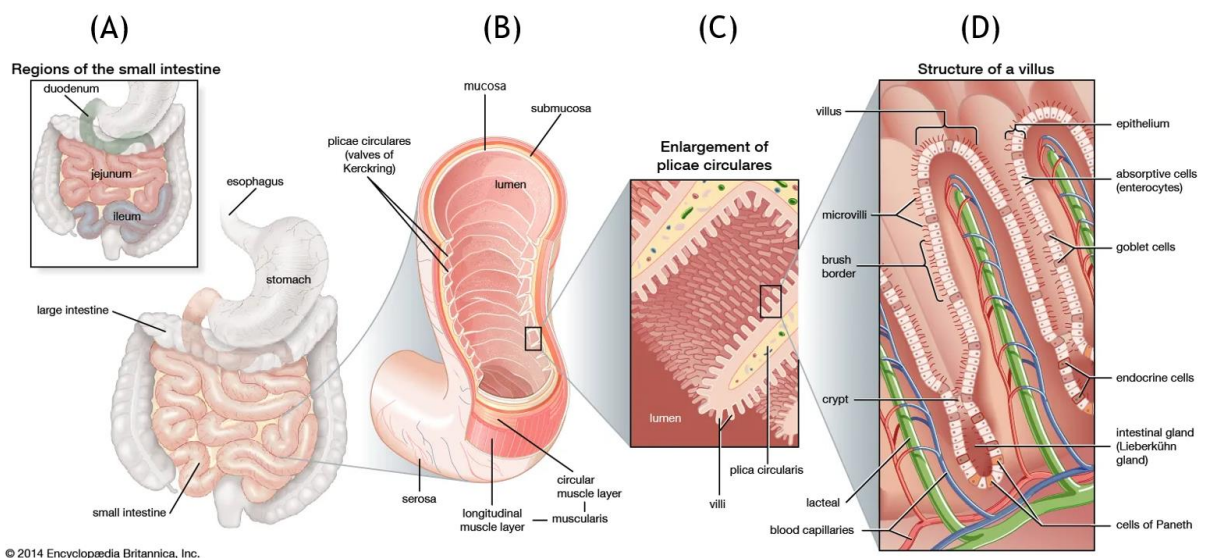


Figure 1. (A) Diagram of the human digestive tract and, in color, the small intestine, which is divided into three distinct sections: duodenum, jejunum, and ileum. (B) Structure of the small intestine walls showing the mucosa, submucosa, muscularis and serosa layers. Plicae circulares (valves of Kerckring) form structural folds. (C) Plicae circulares are covered with villi. (D) Structure of a villus with microscopic cellular microvilli arrangements, which collectively delineate the brush border, at the edges of the absorptive enterocytes in the epithelium. A villus epithelium is also composed of goblet cells, endocrine cells, and cells of Paneth. Internally, it has lacteal, a lymphatic capillary, and blood capillaries. Image adapted from Encyclopædia Britannica, 2014 (2).

Anatomically, the small intestine is divided into different sections (Figure 1 A). The duodenum is the shortest section, and primarily receives partially digested food from the stomach, being a key site for the secretion of digestive enzymes. The jejunum is the middle portion, where most nutrient absorption takes place, owing an extensive surface area. The ileum further absorbs nutrients and also plays a role in reabsorbing bile salts produced by the liver (3).

The complex structure of the small intestine walls is composed by the presence of several layers, each playing an important role for its correct functioning (Figure 1 B). The serosa layer acts as a protective covering barrier, reducing friction during intestinal movement. The muscularis layer is composed by two layers of smooth muscle, one of them that generates segmental contractions, and another that generates longitudinal contractions. Together, the coordinated contractions of these muscle layers enable peristalsis, the wave-like motion that propels food through the intestine. Covering the muscularis layer, the submucosa layer functions as structural support and conduct nutrient and waste transportation, housing blood and lymphatic vessels. Above, the mucosa layer is

subdivided into three sublayers. The muscularis mucosae layer aids movement of the mucosa, enhancing its efficiency in absorbing nutrients from digested food. The lamina propria contains blood vessels, lymphatic vessels, and immune cells that support the epithelium. A single layer of specialized cells responsible for absorbing nutrients and secreting digestive enzymes and mucus forms the intestinal epithelium. Increasing the surface area available for absorption and digestion are the permanent irregularly arranged circular folds, termed plicae circulares, originating in the mucosa and submucosa layers, covered by finger-like microstructures projections, the villi (Figure 1 C and 1 D)(3,4).

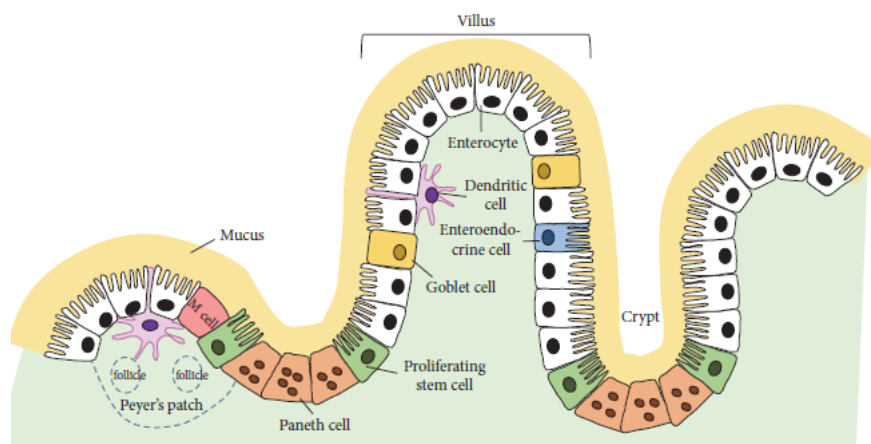


Figure 2. The epithelial lining of the intestinal tract comprises a monolayer of specialized cells, featuring enterocytes, enteroendocrine cells, M cells, Paneth cells, goblet cells, among others. Mucus that covers the epithelial cells are produced by goblet cells. Villus is a finger like-projection and crypts are invaginations in the intestinal epithelium. Peyer's patch consists of aggregated lymphoid follicles. Figure adapted from Kong et al., 2018 (4).

The intestinal epithelium regulates the absorption of nutrients from digested food and its transport to the bloodstream. Multi-potent stem cells engage in a regulated interplay of signals, culminating in the differentiation into epithelial cells. Enterocytes, the prevailing cell category within the intestinal lining, are in charge of absorption. Moreover, the epithelium accommodates additional cellular variants, notably secretory cells, such as goblet cells, Paneth cells, and enteroendocrine cells (Figure 2) (5).

Enterocytes have numerous microscopic projections called microvilli on their apical surface, producing a brush border that also greatly increases the surface area available for nutrient absorption (Figure 1 D and Figure 2). Additionally, these cells also express various transport proteins and digestive enzymes on their apical and basolateral surfaces, allowing for the breakdown of complex molecules and efficient uptake of nutrients from the intestinal lumen. Goblet cells secrete mucus, providing a protective barrier against mechanical damage, pathogens, and harmful substances. Paneth cells secrete antimicrobial peptides, defending against intestinal infections. Enteroendocrine cells produce hormones that coordinate digestive processes, regulate bile secretion, modulate gut motility, and regulate appetite (6). Tuft and Microfold (M) cells can also be found in the intestinal epithelium but in a considerably smaller proportion. Tuft cells function as chemosensory cells, detecting chemical

signals in the gut and participating in immune responses. M cells sample antigens and transport pathogens and particles from the gut lumen to initiate immune responses (7,8).

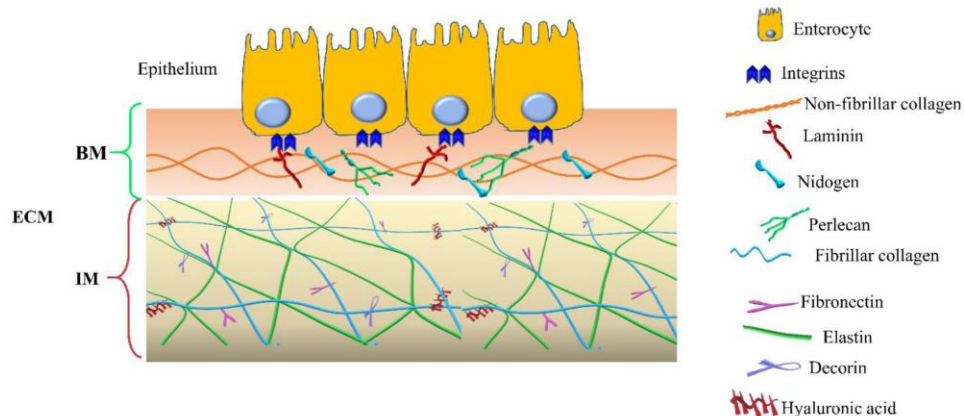


Figure 3. Diagram of the sections of the extracellular matrix (ECM) showing the basement membrane (BM) and the interstitial matrix (IM) component molecules. Integrins are cell receptors which bind to the extracellular matrix. Figure from Pompili et al., 2021 (10).

The intestinal epithelium is supported by an extracellular matrix (ECM), which is composed of a complex network of collagen, laminin, nidogen, perlecan, fibronectin, elastin, decorin, and hyaluronic acid (Figure 3). The ECM is divided in two sections, the basement membrane (BM) and an interstitial matrix (IM) (9). The BM is located underneath epithelial cells, while the IM is present in the lamina propria, submucosa, muscular, and serosa layers (10,11). They together facilitate cell-ECM adhesion, stabilize the basement membrane, provides elasticity and, are involved in cell differentiation and proliferation (10,12).

1.2. Epithelial barrier and intestinal permeability

The integrity of the gut epithelium is crucial for a correct functioning of the intestine and overall health. This barrier effectively delineates the luminal contents, exerting control over the selective transport of nutrients, ions, and signaling molecules. The impairment of this barrier can lead to the entry of microorganisms and substances into the bloodstream, activating pathological processes (13).

The maintenance of a functional intestinal barrier depends on cell-cell adhesion structures, including tight junctions, adherens junctions, and desmosomes (Figure 4 A). Tight junctions are specialized protein complexes that form a physical barrier, controlling the passage of molecules and ions between adjacent cells and maintaining the cell polarity (14). The proteins involved in tight junctions include occludins, claudins, zonula occludens-1 (ZO-1), myosin light-chain kinases (MLCKs), and junctional adhesion molecules (JAMs). This creates a tight seal between cells and prevent the entry of harmful substances into the bloodstream (15). Adherent junctions link actin filaments (F-actin) of adjacent cells using a complex of cadherin-catenin proteins, forming a continuous belt-like

structure, which encircles the cell and provides essential mechanical strength to the surrounding tissue (16). Desmosomes act as anchoring points that establish strong connections between neighboring cells, and rely on the presence of desmogleins, desmocollins, and desmoplakins (17,18).

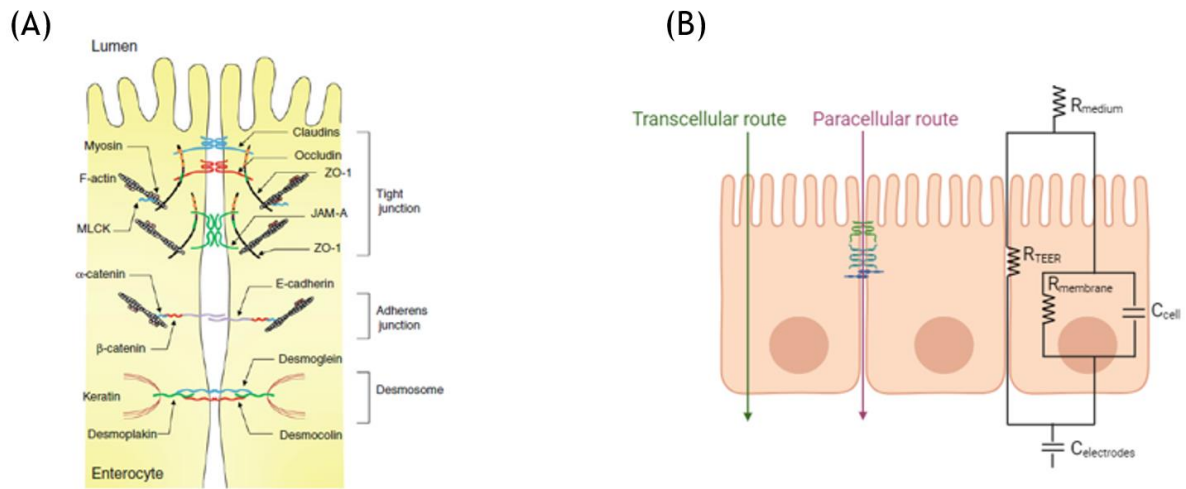


Figure 4. (A) Diagram showing intercellular junctions: tight junctions, adherens junctions, and desmosomes, along with the associated proteins. Figure from Suzuki et al., 2013 (14). (B) Methods of evaluation of intestinal barrier integrity. In green, transcellular route which molecules pass through cell membrane. In red, the paracellular route, via the tight junctions and intercellular space. In black, the cell equivalent circuit. R_{medium} is the resistance of the culture media. R_{TEER} is the resistance of tight junction proteins in the paracellular route. R_{membrane} is the lipid cell bilayer resistance in the transcellular route. C_{cell} is the cell capacitance (stores energy). $C_{\text{electrodes}}$ is the capacitance of the electrodes for resistance measurement. Created with BioRender.com.

There are two types of transport through the intestinal epithelium: paracellular and transcellular routes (Figure 4 B). Paracellular transport involves the movement of molecules between adjacent epithelial cells via passive diffusion. It depends on the local concentration gradient of solutes, allowing them to flow from areas with higher concentrations to those with lower concentrations (19). In contrast, transcellular transport is crucial for intestinal epithelial cell absorption by enterocytes and includes the transport of solutes through the cell membrane, which can occur either through transcellular diffusion or active carrier-mediated transport (20). It was observed that the majority of water-soluble compounds are unable to pass the brush-border lipid-cell membrane of enterocytes and are transported across the epithelium via paracellular tight junctions. On the contrary, lipid soluble and small hydrophilic compounds can passively diffuse through the cells, binding to protein carriers or to transporters channels and cross the transcellular route (21).

One way to assess intestinal barrier permeability is through the passage of markers across the epithelial passage routes (Figure 4 B). For instance, ethylenediaminetetraacetic acid (EDTA) binds strongly to radioactively labeled ^{51}Cr and can cross epithelial cells via the paracellular route, making possible assess colonic permeability by being detectable in urine (22). A substitute for radiolabeled probes involves markers that are linked to fluorochromes, such as fluorescein, which provides equal results of paracellular permeability compared to ^{51}Cr -EDTA. Other fluorescent compound, lucifer yellow, is used for paracellular permeability studies in both *in vitro* and *in vivo* contexts, particularly in drug absorption investigations. In addition, polyethylene glycols also can be

used and come in various sizes, offering insights into size-dependent permeability changes. Moreover, mannitol, a quickly absorbed small sugar, serves as a marker for transcellular uptake, while lactulose, a bigger sugar and a less absorbed, indicates mucosal integrity, with an increased lactulose to mannitol ratio revealing intestinal barrier dysfunction (23,24).

Epithelial barrier can also be evaluated in a well-established and widely used method based on electrical measurements. This technique is known as transepithelial electrical resistance (TEER), more frequently conducted in *in vitro* models. Measurements involve the placement of two electrodes on opposite sides of a cellular monolayer. An alternate current electrical signal with low frequency, usually 12.5 Hz, flows between these electrodes, enabling the assessment of epithelial resistance, which reflects the ionic conductance of the paracellular pathway (25,26). The method provides information about the tightness and integrity of the cell layer's barrier, allows monitor changes in barrier function over time, assess the effects of different experimental conditions or treatments, and compare the barrier properties of different cell types or models (27,28).

1.2.1. Implications of disrupted gut epithelial barrier integrity and inflammatory bowel disease (IBD)

The "leaky gut hypothesis" proposes that when the integrity of the intestinal barrier is compromised, it instigates chronic, subclinical inflammation in various target organs by the presence of microbial-derived substances (23). Dysfunction of the intestinal epithelial barrier and increased permeability have been observed in various human disorders such as alcoholic liver disease, inflammatory bowel disease (IBD), liver cirrhosis, severe acute pancreatitis, obesity and even depression (13,29).

Specifically related to the gastrointestinal tract, IBD is a chronic disease that comprises myriad conditions, with Crohn's disease and ulcerative colitis representing the main subtypes (30,31). The exact causes of IBD and its progression are not entirely understood but different factors that can impact the intestinal barrier appear to be involved, including genetic defects in tight junctions' proteins production, dietary patterns, bacterial infections, and medication interaction such as non-steroidal anti-inflammatory drugs (32-36).

Crohn's disease is known for its propensity to affect any segment of the digestive tract often caused by inflammation that can lead to complications like strictures and fistulas. Ulcerative colitis, on the other hand, predominantly impacts the colon and rectum, causing continuous mucosal inflammation (37). Symptoms of IBD can include abdominal pain, diarrhea, rectal bleeding, weight loss, and fatigue (27). As the prevalence of IBD continues to rise, with approximately 4.9 million cases estimated worldwide in 2019, understanding the disease etiology, its progression, and potential therapies becomes imperative due to the significant impact of these symptoms on patients' quality of life (38,39).

Also, due to its unknown pathogenesis, IBD therapy focuses mainly on tackling and controlling the inflammatory processes. The process of gut inflammation involves a coordinated series of immune responses and several inflammatory signaling molecules, known as cytokines, have been identified influencing the epithelial barrier and causing alterations in its permeability. Pro-inflammatory cytokines as tumor necrosis factor alpha (TNF- α), interleukins (ILs) 17, and 18 are reported to be upregulate during the active state of the disease (40,41). For instance, TNF- α can directly disrupt tight junction proteins, and anti-TNF- α antibodies emerged as a solution in mitigating the inflammatory process associated with chronic inflammatory conditions in IBD, enabling long-term remission of the disease (42,43). However, primary non-response, loss of response, and adverse effects are still observed, demanding novel approaches and treatment alternatives, whose efficacy must be tested prior to being used in clinical practice (44).

1.2.2. Microbiota and gut inflammation

The diverse community of microorganisms inhabiting an organism is known as microbiota (45). In the gut, it establishes a symbiotic relationship with the mucosa, playing crucial role in maintaining the health of individuals. This microbial community contributes significantly to metabolic, immunological, and protective functions within the gastrointestinal tract. Notably, it takes on the essential task of synthesizing vitamin K and B (46). Moreover, when carbohydrates undergo fermentation by the gut microbiota, it leads to the biosynthesis of short-chain fatty acids (SCFAs), which serve as substantial sources of energy for epithelial cells (47).

Imbalance in the gut microbial community, commonly known as dysbiosis, often leads to an increased intestinal permeability (48). It can result from the loss of beneficial microorganisms, excessive growth of potentially pathogenic microorganisms, and reduction in microbiota diversity (49). Although dysbiosis can be promoted by various factors (infection, diet, exercise, antibiotic use), it is not known whether intestinal dysbiosis in IBD is a cause or consequence of the immune response (50,51).

In recent years, a strong relationship has been found between the gut microbial community and intestinal inflammation, with certain bacterial species associated with both pro-inflammatory and anti-inflammatory effects (52,53). Bacteria of the genera *Lactobacillus* and *Bifidobacterium* have demonstrated beneficial effects in the presence of inflammatory conditions. On contrary, enteropathogenic bacteria can release toxins and possess molecules as lipopolysaccharides (LPS), peptidoglycans, and flagellin, that can activate immune cells and stimulate inflammation (52,54,55). A disruption in the gut epithelial barrier may also facilitate these harmful bacteria and bacterial products to translocate into bloodstream and provoke inflammation through immune response (56). In situations where dietary fiber is lacking, mucophilic bacteria engage in the degradation of mucus, resulting in diminished colonic mucus layer thickness and compromised integrity of the intestinal

barrier, increasing the risk of IBD (57). Also, disturbed mucus production has been associated with bacterial translocation and development of colitis in mouse model (58).

1.2.3. Role of short-chain fatty acids (SCFAs) on intestinal health

Gut commensal bacteria metabolize indigestible carbohydrates forming SCFAs as their primary byproducts, with propionate, butyrate, and acetate being the most predominant ones (59). SCFAs contribute to the regulation of the immune system and inflammatory response by influencing cytokine production, including the stimulation of IL-18, which helps maintain and repair epithelial integrity (48). Moreover, they reduce local inflammation, protect against pathogen infiltration, and maintain barrier integrity by regulating host immune cells (60).

Butyrate can enhance gut barrier function and mitigate bacterial translocation by influencing the assembly of tight junctions and mucin synthesis (61-63). A decreasing gradient of butyrate from the gut lumen to the crypt has been proposed to regulate intestinal epithelial turnover and homeostasis, stimulating proliferation at the bottom of crypts while promoting apoptosis and shedding cells closer to the lumen (64). Propionate exerts beneficial effects on B-cell function in humans and modulates reward-based eating behavior via striatal pathways (65,66). Acetate has been found to mediate anti-apoptotic and anti-inflammatory responses in the colonic epithelium of mice (67).

To elucidate the beneficial effects of bacteria and the production of SCFAs on human health, a combination of techniques from microbiology, genomics, metabolomics, and clinical research has been employed (68,69). In particular, fecal fermentation is a microbiological approach used to simulate the bacterial breakdown of food components within the gastrointestinal tract. In this process, a mixture of fecal material, representing the complex community of microorganisms present in the gut, is combined with fermentation medium specifically formulated to support the growth and metabolism of microorganisms during fermentation (70,71). The result are fermented products rich in SCFAs that allow to assess the role of bacterial metabolism on epithelial intestinal cells (72).

1.3. Current models for studying the gut

Studying the gut is essential due to its significance in human health. To investigate the organ and its components, researchers employ either *in vivo* or *in vitro* approaches. These strategies are essential for gaining insights into the foundational mechanisms that underlie conditions capable of impacting the organ, exemplified by the previously mentioned IBD (73).

Clinical human trials offer the advantage of studying the organ in the actual target species, being more directly relevant. They also allow for exploring complex interactions and interindividual

differences. However, this variability lead by genetics, age, sex, diet, and lifestyle choices makes it challenging to generalize or draw conclusions that apply to everyone, underscoring the importance of carefully considering the specific objectives of the study (74,75). This type of study also presents challenges related to the recruitment and retention of participants, particularly among special populations such as children, pregnant women, and individuals with rare diseases, adherence to protocols, sample size, cost, regulatory compliance (76-79). Additionally, ethical concerns arise regarding privacy, scientific integrity, consent, protection of vulnerable populations, and harm minimization (80).

Using animals facilitates the variable control, establishment of standardized conditions, and execution of experiments that would be ethically impractical or impossible to perform on humans. However, ethical concerns are still present, requiring careful justification for their use, for example, in invasive procedures (81). Furthermore, animals possess anatomical structures, physiological processes, and metabolic rates distinct from humans. As a result, although animal studies provide valuable insights, they often fail recapitulating the human condition due to interspecies variability (82,83).

Cell-based *in vitro* models are often chosen not only for their speed, cost-effectiveness, and reproducibility but also for their ethical advantages compared to *in vivo* studies. Although there are limitations in fully capturing the complexity of the human digestive system, notable advancements have been made in the development of new *in vitro* models that incorporate elements to enhance their physiological relevance (84,85). Indeed, researchers and regulatory entities are planning a future without lab animals, raising interest in alternative non-animal models (86).

1.3.1. *In vitro* cell-based models and cell source

The selection of an appropriate cell source for a given model is intricately linked to the specific goals and objectives of the study. Different cell types possess unique physiological properties and functionalities, which can be leveraged to mimic specific aspects of the biological system under investigation (85).

Intestinal stem cells possess the potential to self-renew and differentiate into various specialized cells of the gut, including enterocytes, goblet cells, and enteroendocrine cells. Despite of that, achieving proper differentiation and maturation of stem cells into functional intestinal cell types can be challenging and time-consuming. Also, inherent variability among stem cell populations can impact the reproducibility and consistency of experimental outcomes (87).

Unlike stem cells, primary cells have undergone the process of differentiation *in vivo* and display the specialized features of their respective cell types as they are extracted from tissues during biopsies or surgeries. Although they maintain the characteristics and functionality of the tissue, senescence after a certain number of cell divisions makes it necessary to employ methods to extend

their limited lifespan for long-term culture (88). Additionally, being organism-derived, the supply is limited and requires ethical approval and patient consent when obtained from humans (89).

Despite immortalized cell lines have different protein expression compared to the native or primary cells, they are frequently preferred over primary cells in research because they offer cost-efficiency, extended culture lifespan, and ease of use (90,91).

The Caco-2 cell line, an immortalized cell line originally derived from colon carcinoma, has gained widespread popularity as a model for studying the intestinal epithelial barrier. One of its most notable attributes is its inherent capacity to undergo spontaneous differentiation, forming a monolayer of cells that closely resemble enterocytes with a brush border layer as found in the small intestine (92). Although enterocytes represent the predominant cell type in the intestinal lining, utilizing a monoculture model falls short of replicating the complexity of the human gut (8,93). Consequently, alternative models incorporating multiple cell lineages have gained prominence (94). An illustrative example includes the incorporation of the HT29-MTX cell line to emulate mucin production, a function primarily associated with goblet cells – the second most prevalent cell type in the intestinal epithelium (8,95). This cell line, also derived from human colorectal adenocarcinoma, underwent an adaptation after exposed to increasing concentrations of methotrexate (MTX), leading to the development of differentiated mucus-secreting cells (96). Although Caco-2 cells exhibit distinct transporters expression patterns and HT-29 cells display higher glucose consumption when compared to normal human intestinal cells, they have still proven to be valuable in studying intestinal transport, absorption, toxin effects, and disease modeling, with recommended 9:1 ratio of Caco-2 to HT29-MTX cells to closely emulate physiological conditions (97-99).

The typical co-cultivation of Caco-2 and HT29-MTX cells involves utilizing cell culture inserts equipped with semi-permeable membranes, creating separate luminal and basolateral compartments (Figure 5 A). This feature, offering an advantage over standard cell well plates, not only provides access to both sides of the cell barrier, allowing cells' contact to diverse environments on each side of the membrane but also facilitates TEER measurements and permeability assays (100). The physiological relevance of this tool also can be increased by ECM coating the membrane, enabling enhanced cell attachment, induction of cell differentiation, recreation of 3D realistic tissue-like structures, establishment of natural barrier function, and added support (101).

1.3.2. Microfluidics and gut-on-a-chip

Conventional two-dimensional (2D) cell culture models, exemplified by traditional cell culture inserts, have undoubtedly contributed significantly to biomedical research. These models have provided valuable insights into basic cellular behavior although they fail to reproduce differentiated functions of many cell types and to accurately predict the *in vivo* functions of tissues. These limitations have led to research for development of more advanced models. Among them,

microfluidics, a research field that involves the manipulation of small volumes of fluids through micro or nanoscale channels, have emerged as a promising avenue (102,103). This offers numerous advantages, including reduced reagent usage, device design flexibility, continuous flow analysis, and real time analysis. Nevertheless, it also presents certain challenges, such as non-standardized protocols since it is an emerging technology, the need of chemical compatibility studies when implementing new materials, and microfluidic channels can be prone to flow interruption by bubbles (104-106).

Organ-on-a-chip uses microfabricated microfluidic channels or structures to host living cells or tissues to replicate the functionality of living organs (107). The creation of simplified versions of organ anatomy can be made given that microfluidic devices offer the ability to be designed in various patterns. This, in turn, allows for the precise replication of *in vivo* physical conditions, while also granting control over fluid flow dynamics (103).

Gut-on-chip is a subtype of organ-on-a-chip which incorporate intestinal cell. It usually consists of two parallel microfluidic channels separated by a semiporous membrane (Figure 5 B). As mentioned above, intestinal epithelial cells are typically cultivated in cell culture inserts with semi-permeable membranes, creating a barrier between the apical and basolateral compartments in static conditions, e.g., without fluid flow (Figure 5 A). Gut microdevices, instead, incorporate dynamic features such as fluid flow and peristalsis-like movements that provide a more accurate platform for studying gut biology (108,109).

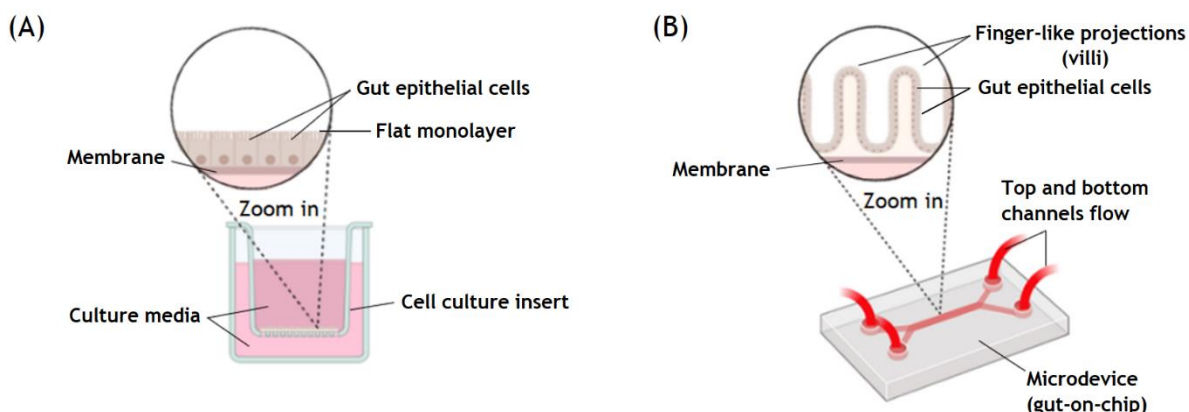


Figure 5. Schematic representation of *in vitro* models used for intestinal cell culture. (A) Transwell insert membrane submerged in culture medium, showcasing a flattened gut cells monolayer in zoom. (B) Gut-on-a-chip model consisting of upper and lower chambers where the medium can flow continuously. The differentiated gut epithelial cells with villi-like projections are shown in zoom. Created with BioRender.com.

It has been shown that the constant shear stress induced by the fluid flow leads to the development of polarized epithelial cells with villi-like projections, differentiated functions and morphology similar to those found in a normal human small intestine (Figure 5B). This opposes to common cultures in transwell inserts where the intestinal cells show flatten monolayer (Figure 5 A) (110-112).

Also, multi-organ-chip platforms allow for comprehensive inflammation studies that consider the interplay between different organs. Jeon et al. and Kim et al. used gut-liver and gut-brain axis platforms, respectively, to study inflammation pathways triggered by LPS (113,114). The first have demonstrated the anti-inflammatory effect of luteolin, reflected by a reduced production of nitric oxide by RAW264.7 cells, line of murine macrophages, following absorption by the gut epithelium and metabolism in the liver (HepG2 cells) (113). The latter showed that butyrate improved the barrier integrity of both Caco-2 and brain microvascular endothelial cells following administration in the intestinal lumen (114).

1.3.3. *In vitro* gut inflammation and microbiota

Several cell-based *in vitro* models of intestinal inflammation have been employed, varying in complexity and methodology to induce inflammation, yet a deficiency in standardized protocols persists. This is not due to scarce studies within the field. On the contrary, the abundant literature is typically inconsistent regarding critical parameters, including the number and type of different cells used, the inflammatory stimuli (type, concentration, site) applied, and even the measured outputs (115-117). Early *in vitro* models of gut inflammation were simplistic and normally based on single cultures on a dish or microplate (118). Monocultures, despite their simplicity and the absence of numerous human intestinal features, serve as valuable tools for investigating how various cell types respond to inflammatory stimuli. They provided essential insights before advancing to more complex co-culture systems which enhance the physiological relevance of the models. Later, the use of cell culture inserts allowed the evaluation of barrier function and intestinal permeability by granting access to both apical and basolateral sides of polarized cell monolayers (119). Data obtained from these simple approaches revolutionized some physiological concepts and paved the way for the development of more sophisticated models (120).

In this context, the microbiota is normally absent in static cell-based *in vitro* models due to bacterial overgrowth, which makes long-term cultures hard to attain (121,122). Calatayud et al. developed a model with Caco-2, HT29-MTX, THP-1-differentiated macrophages, and eight strains of commensal bacteria (123). The authors observed that adding microbiota did not affect the TEER, but slightly increased the permeability of lucifer yellow and increased TNF- α , IL-8, and chemokine (C-X-C motif) ligand 16 (CXCL16) production. Mohebbi et al. added the intestinal commensal strains *Faecalibacterium prausnitzii*, *Roseburia intestinalis*, and *Bacteroides faecis* alone or in combination to inflamed Caco-2 and HT29-MTX cells and observed an anti-inflammatory effect: all strains increased TEER, decreased paracellular permeability, and decreased IL-8 and monocyte chemoattractant protein-1 (MCP-1) release (124). To avoid bacterial overgrowth, in both studies the microbiota was co-cultured with cells for short periods of time (2h and 6h) (123,124). Thus, organ-on-chips offer a promising alternative because they allow for continuous flow, oxygen gradients, and mechanical

deformation. This approach helps prevent bacterial overgrowth, promotes cell differentiation, and maintains cellular viability (125).

2. Project goal

The intestinal microbiota has demonstrated to play a major role in the digestive process and has a significant impact on numerous aspects of human health. This dynamic community of microorganisms releases byproducts, mainly derived from bacterial metabolic fermentation process, into the intestinal lumen. Most of them are SCFAs that not only influence the composition of the intestinal microbiota but also can affect the balance between health and disease. Therefore, understanding the implications and effects of these compounds is crucial for comprehending the role of microbiota in host health.

Including these compounds in intestinal *in vitro* models enable the development of a more accurate environment for studying the gut in both healthy and inflamed states. Thus, the main goal of this project was to study the effects of fermented products in a co-culture model of Caco-2 and HT29-MTX cells. This was achieved by incorporating different dilutions of fermented products (rich in SCFAs) into the co-culture. Additionally, the static model was employed to evaluate the anti-inflammatory activity of fermented products on inflamed cells. Furthermore, the study included a comparative analysis of cellular differentiation under static and dynamic conditions and the effects of the fermented products in the co-culture on both models. Ultimately, these insights empowered a deeper understanding of how fermented products affect gut dynamics, fostering the development of a more reliable gut model.

3. Materials and Methods

3.1. Cell culture

Caco-2 cells were routinely maintained in Minimum Essential Media (MEM) supplemented with 20% fetal bovine serum (FBS), and an antibiotic solution of 1% penicillin (100 UI/mL) and streptomycin (100 µg/mL). HT29-MTX cells, on the other hand, were maintained in Dulbecco's Modified Eagle Medium (DMEM) media supplemented with 20% FBS, 1% penicillin and streptomycin, and 1% nonessential amino acids. Both cell lines were cultured in separate tissue culture flasks of 75 cm² surface growth area (T75) within a humidified incubator at 37 °C with a 5% CO₂ atmosphere. The culture media were replenished every 2 or 3 days, and cells were sub-cultured when reaching a confluence of between 70% and 80%.

Before initiating a seeding or performing subculture (passaging), old media was removed, and adherent cells were washed twice with phosphate-buffered saline (PBS) 1x to dilute serum proteins. This allows the trypsin, a proteolytic enzyme employed to facilitate the detachment of cells from the culture flask, and EDTA, utilized to dissociate cell-to-cell adhesion, to effectively act (126). After adding trypsin 0.25% and EDTA 2.21 mM, the culture flask was then placed in the incubator for 5 minutes to trypsin-EDTA take effect. Following this, the trypsin activity was stopped by adding the regular culture medium to each cell, at three times the volume of trypsin, due to the protease inhibitors present in FBS. The cell suspension was transferred to a Falcon flask to perform centrifugation for 5 minutes at 150 g. The supernatant was carefully discarded, and the cells were resuspended again in the respective culture media. A sample of this was used to cell counting, carried out using a Neubauer chamber with the aid of trypan blue staining in 1:1 dilution. An amount of 4×10^3 cells/cm² Caco-2 cells and 10^4 cells/cm² of HT29-MTX were passed to a new T75 flask to continue the culture.

3.2. Microfluidic devices

3.2.1. Device design

A diagram of the microfluidic devices used for the dynamic cultures can be found in Figure 6A (assembled device). The device design consisted of two parallel channels separated by a semiporous polydimethylsiloxane (PDMS) membrane. The dimensions of the channels in the culture area were 10 mm long, and 1x1mm cross section. The total length of each channel was 19.5 mm to incorporate inlets and outlets for fluidic connections. The membrane was between 35 µm and 40 µm

thick. Devices were assembled with both channels aligned in parallel with the membrane placed in between.

3.2.2. Device fabrication

The steps of assembling the gut chip devices are shown in Figure 6 A. The membranes were fabricated in PDMS using a spin-coating protocol. First, silicon wafers master molds with etched micropillars (8 μm diameter (Figure 6 B) and 50 μm tall (Figure 6 C) disposed in a hexagonal array (40 μm center to center (Figure 6 D)) were used for membrane fabrication by spin-coating. A glass slide with 10 μL of Trichloro(1H,1H,2H,2H-perfluorooctyl)silane surrounded by the wafers was placed in a desiccator that was vacuumed and left overnight at 65 °C to promote vapor deposition of the silane (127). This enable easy peeling of the membrane material, a cured PDMS, as the process of silanization is known to form self-assembled monolayers, a spontaneous adsorption of silane molecules onto the wafers, which have anti-adhesive properties (128).

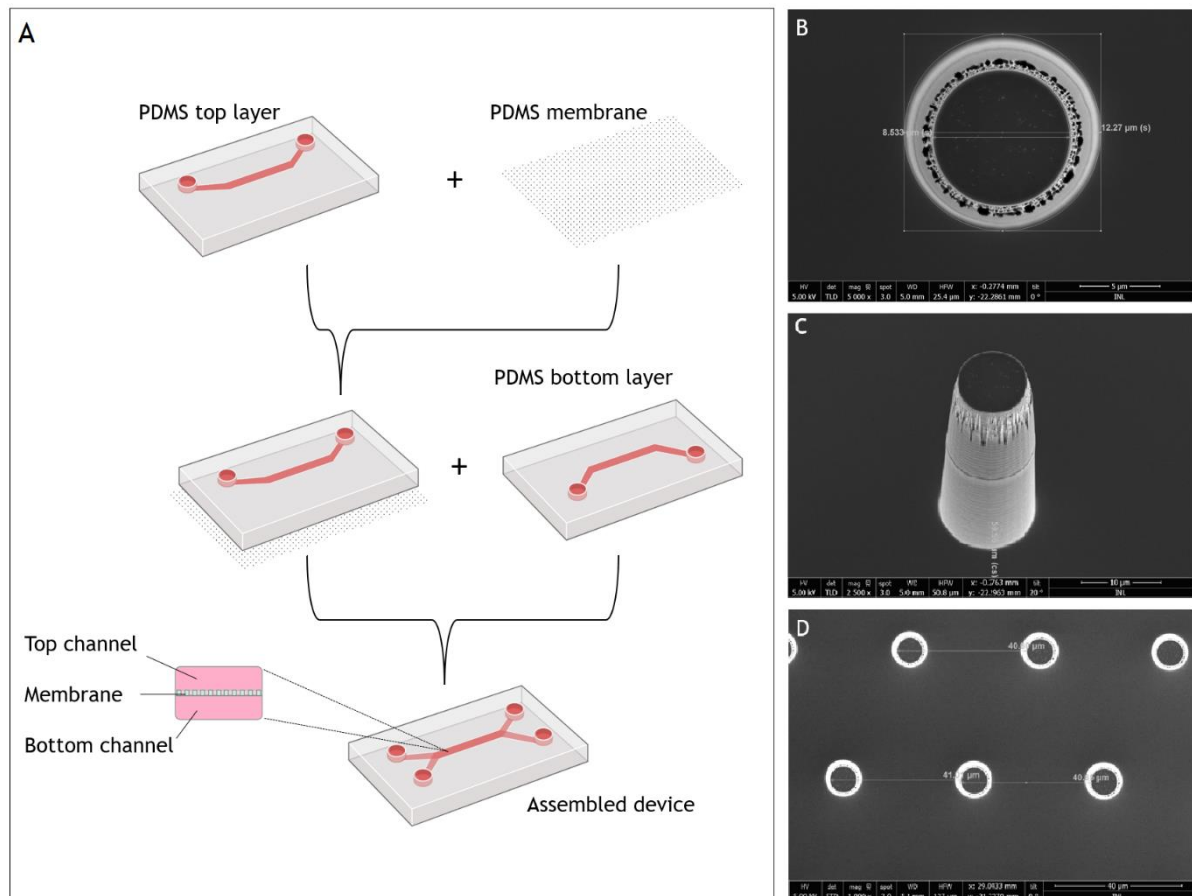


Figure 6. (A) In the assembly process for the gut-chip device, the first step involves attaching the top layer to a membrane. This membrane will later serve as the connection point between the top and bottom layers, ultimately completing the assembly of the final device. Top and bottom channels divided by the membrane are shown in zoom in. Created with BioRender.com. (B), (C), and (D) are scanning electron microscopy (SEM) images of a silicon wafer showing (B) dimensions of the smaller and larger base of the pyramidal pillar (scale 5 μm) (C) pillar height (scale 10 μm) (D) distance between each pillar (scale 40 μm).

Membranes were fabricated by PDMS casting using SYLGARD™ 184 Silicone Elastomer Kit. The ratio of 15:1 of base and curing agent, respectively, was employed instead of the standard 10:1 to reduce PDMS viscosity allowing for a better spreading on a membrane-forming silicon wafer. The mixture was stirred homogeneously and then left degas for 3 hours in a desiccator to achieve the desired viscosity as the final thickness depends on the viscosity of the material and spinning speed. Approximately 1 mL of PDMS was deposited on the silanized wafer surface and let to self-spread for 2 minutes. A SPS Spin150 spin coater was used to spread the PDMS, and a four steps program was performed: (1) 20 seconds at 500 rpm for speed up, (2) 300 seconds at 2400 rpm for spreading and achieving membrane thickness around 30 μm , (3) 10 seconds at 100 rpm for spin down, and (4) end. After spinning, the membranes were left to set for about 5 minutes to allow the edge bead to retract. The spin-coated silicon master molds were placed on a flat surface and cured at 65°C in the oven for at least 2 hours.

The top and bottom layers were made using polymethyl methacrylate negative molds. A PDMS mixture in 1:15 ratio was prepared, degassed in a vacuum chamber, and poured to fill the molds. The curing process was performed at 65°C for a least 2 hours. The bottom and top parts were removed from the molds and four holes for fluidic tube connection were made using a 1.5 mm biopsy punch.

The device assembly was performed by standard oxygen plasma bonding using a Harrick Plasma Cleaner PDC-002-CE (129,130). Oxidizing PDMS substrates in a plasma discharge converts methyl groups at the surface to silanol groups. The reaction between the two layers of oxidized PDMS forms covalent siloxane bonds and releases water molecule (131). Therefore, the top part was first bonded to the membrane, placed in the silicon master mold. They were placed in the oven for at least 30 minutes at 65°C to facilitate water evaporation, enabling the membrane to be properly peeled from the wafer. Following this, the bottom part was bonded to the other side of the membrane. Finally, a glass slide was bonded to the bottom part to promote device stability.

After this, the silicon master molds cleaning was done by immersing them in a solution of one part of tetramethylammonium fluoride and three parts of 1-methyl-2-pyrrolidinone. The solution was prepared fresh every time and left overnight under an agitation of 150 rpm. The process is finished by rising with acetone and propan-2-ol followed by drying with pressurized nitrogen.

Prior to introducing cells into the devices, they underwent a process of functionalization, as polydimethylsiloxane (PDMS) inherently exhibits inadequate cell adhesion properties owing to its hydrophobic nature (132). In a sterile laminar flow hood, the channels of the devices were sterilized with 70% ethanol for 30 minutes. The ethanol was removed and washed with deionized water filtered in a 0.2 μm membrane. Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (Sulfo-SANPAH) diluted in deionized water (0.5 $\mu\text{g/mL}$) was added into the devices and exposed to UV radiation (365 nm) in a distance of 5 cm during 30 minutes. Sulfo-SANPAH is a compound that under UV light exposure initiates beneficial reactions for crosslinking cell-surface proteins (133).

Non-polymerized Sulfo-SANPAH was then aspirated and washed away with deionized water. A solution containing rat tail collagen type I 30 $\mu\text{g/mL}$ and Corning® Matrigel® Matrix 100 $\mu\text{g/mL}$ diluted in free serum DMEM was added and left to slow polymerization at 4 °C overnight. The combination of Sulfo-SANPAH, collagen type I and Matrigel® provided a surface with ECM compounds to allow cell

adhesion on PDMS. Previously the seeding, the devices were placed in an incubator at 37 °C with a 5% CO₂ atmosphere for 60 minutes to reduce thermal shock upon cell placement.

In the co-culture seeding, a final cell amount required of 10⁵ cells/cm² was calculated based on the surface area of the devices (0.195 cm²) to achieve the desired cell density for subsequent experiments. The proportion of Caco-2 and HT29-MTX (Caco-2/HT29-MTX) cells was 9 to 1, respectively, due to its physiological relevance in barrier assays (134).

The cells were introduced into the upper microchannel and incubated overnight at 37 °C to allow their attachment to the ECM-coated PDMS surface. The following day, the devices were connected to a syringe pump using 1.6 mm outer diameter polytetrafluoroethylene tubing to perfuse culture medium continuously through the upper and lower microchannels. A constant flow rate of 120 µL/h, demonstrated in prior experiments to be capable of inducing cellular differentiation, was set (127). The syringe pump was connected to the Arduino software which regulated the flow rate based on the diameter of the syringe available. The media in the syringes was filled up every 2 or 3 days until completing 7 days of culture.

3.3. Cell culture inserts

Cell culture inserts, trademark as Transwell®, were used to perform the static culture. Each of these inserts had a diameter of 12 mm and was made for placement within a 12-well plate. The semipermeable membrane, made of polyester with a nominal thickness of 10 µm, offers a cell growth area of 1.12 cm² and features 1 µm pores at a pore density of 1.6 x 10⁶ pores/cm². The prior manufacturer's treatment for tissue culture makes the membrane ready to receive the cells (135).

The final cell amount of 10⁵ cells/cm² was calculated based on the insert surface area of 1.12 cm² and Caco-2/HT29-MTX cells were seeded at a 9:1 ratio. The medium was changed every 2 or 3 days until completing 21 days of culture.

3.3.1. Trans-epithelial electrical resistance monitoring

The integrity of the epithelial barrier was assessed using the TEER measurement technique with the assistance of the Millicell ERS-2 Volt-Ohmmeter

The well plates were removed from the incubator 15 minutes prior to TEER measurement to allow for the stabilization of the culture medium's temperature and to reduce measurement variations. Throughout the growth phase, the resistances, measured in units of Ω, of the blank membrane (R_{blank}), inserts without cells, and the co-culture (R_{cells}) were then annotated every 2 or 3 days, and the medium was subsequently replaced. The actual resistance (R_{real}) is obtained using the equation 1 (26).

$$R_{real} = R_{cells} - R_{blank} \quad \text{(Equation 1)}$$

The actual resistance (R_{real}) is inversely proportional to the membrane area (M_{area}) in cm^2 , and the final TEER value ($TEER_{final}$) is normalized by area using the following equation (26).

$$TEER_{final} = R_{real} \times M_{area}$$

(Equation 2)

During the assays, the TEER was consistently measured before and after solution changes.

3.4. Fermented products exposure

The acquisition of samples and the production of fermented products were conducted in collaboration with Teagasc, the Agriculture and Food Development Authority in Ireland. Although the characterization of the fermented products is pending, they are known to be rich in SCFAs (136).

The fermentation protocol was based on Lawrence et al. (70). A pool of fecal material from human healthy donors, was mixed with fermentation medium for 24 hours at 37 °C and 150 rpm. Following this, the fecal fermentation mixture was collected and then centrifuged at 4.600 rpm for 20 minutes. The supernatant was collected and frozen in Falcon tubes at -80 °C until needed. Prior to the assays, the tubes were defrosted and subsequently centrifuged at 16.000 x g for 5 minutes. The resulting supernatant was filtered using 0.22 µm syringe filters. The removal of bacteria was confirmed by plating the fermented products in tryptone soy agar (TSA).

The dilutions of fermented products used in this study were prepared using Hanks' Balanced Salt Solution (HBSS). The proportions were 1 part of fermented product to 5, 6, 10 and 20 of HBSS.

In transwell, after 21 days of culture, the inserts were washed three times with prewarmed HBSS at 37°C and placed into a new plate to eliminate the medium components. These fermented products dilutions were added in the apical side, while only HBSS was introduced to the basolateral side.

On chips, following 7 days of culture, medium syringes were unplugged, and HBSS was flushed to clear remaining medium. Syringes containing fermented products diluted in HBSS and HBSS alone were connected to the upper channels and bottom channels of the chips, respectively. The flow of the pump was reduced to 20 µL/h for 1 mL syringes with an internal diameter of 4.78 mm.

3.5. Inflammatory assay

TNF-α was diluted in MEM and used to induce inflammation in the cells at concentration of 50 ng/mL. Budesonide, a known anti-inflammatory compound, at concentration of 3 µM diluted in chloroform was employed as positive control (137).

In transwell, the TNF-α solution was placed in the basolateral side and MEM in the apical side after reaching the differentiation period (21 days). To assess the protective effect of fermented

products and budesonide, the exposure on apical side was conducted for 4 hours followed by 48 hours of TNF- α exposure. In the assay, to evaluate the reparative effect, the cells were inflamed for 48 hours followed by a 4-hour exposure to fermented products and budesonide. Between every change of media, the cells were washed with HBSS to remove remaining fermented products or TNF- α . The media was collected, both apical and basolateral, and stored in -20 °C for posterior evaluation of cytokine profile.

3.6. Permeability assay

The permeability assay was conducted following exposure to fermented products alone, exposure combined with inflammation, or inflammation only. Lucifer yellow was diluted in HBSS at a final concentration of 50 μ M to assess the apparent permeability (P_{app}) of the co-culture. P_{app} in cm/s is determined from the amount of compound transported per time by the (Equation 3, where dQ/dt is the flux of the substance (μ M /s), A is the surface area of the membrane (cm^2), and C_0 is the initial concentration in the apical or upper chamber (μ M) (138).

$$P_{app} = \frac{dQ}{dt \times A \times C_0}$$

(Equation 3)

The procedure for Lucifer Yellow solution insertion was similar to that of the fermented products in both transwell and chip systems. In transwell, at each hour, a sample was collected from the basolateral side and replaced with the same amount of pre-warmed HBSS. The assay has a duration of four time points. On chips, the samples were also collected from the basolateral side *via* a pipette tip attached to the chip outlets after three hours, spanning a three-hour duration with one time point per hour.

A 12-point calibration curve was previously prepared through serial dilution of the stock solution (50 μ M). The initial concentration was 6.25 μ M, and the final point contained only HBSS. Fluorometric readings (excitation 428 nm and emission 540 nm) were performed in triplicate using a microplate reader (BioTek Synergy H1). The unknown concentration of the collected samples was determined through a linear correlation of the slope from the equation of the curve.

3.7. Immunocytochemistry

After the permeability assay, the media in transwell and chips was removed and cells were rinsed using prewarmed PBS, a process repeated three times. Subsequently, PBS was removed, and cells were fixed using 4% paraformaldehyde (PFA) in PBS for 15 minutes at room temperature (RT). Formaldehyde forms chemical bonds (crosslinks) between lysine residues. This crosslinking process immobilizes the proteins and prevents them from changing or degrading. After fixing, PFA was

removed, and the cells were rinsed three times with cold PBS. A solution of Triton-X-100 0.2%, diluted in PBS, was added, and the cells were incubated for 10 minutes at 4 °C to permeabilize the cells by disrupting the cell membranes, thereby allowing the subsequent reagents to enter the cells and bind to their targets. The Triton-X-100 solution was then removed, and the cells were rinsed three times with cold PBS. Following this, a blocking buffer composed of 2% bovine serum albumin (BSA) in PBS was added to prevent non-specific binding of antibodies, and the cells were incubated for 30 minutes at RT. Subsequently, the blocking buffer was removed.

For immunostaining, without washing, a fluorophore solution containing Occludin Monoclonal Antibody (OC-3F10) Alexa Fluor 488 at a concentration of 10 µg/mL and Phalloidin-Tetramethylrhodamine B isothiocyanate (TRITC) at a concentration of 0.1 µg/mL, both diluted in antibody diluting buffer (0.5% BSA in PBS), was added. Phalloidin binds actin filaments and reveals the structure of the epithelial cells conjugated with TRITC, an orange-fluorescent dye with wavelength excitation (λ_{ex}) between 540 nm and 545 nm and wavelength emission between 570 nm and 573 nm (139,140). Occludin monoclonal antibody binds to Occludin protein and displays a green-fluorescent color conjugated with Alexa 488 λ_{ex} 499 λ_{em} 520 nm (141). The cells were incubated overnight at 4 °C with this solution.

After incubation, the solution was removed, and the cells were washed three times with PBS. DAPI (4',6-Diamidino-2-Phenylindole Dihydrochloride), a blue-fluorescent dye with λ_{ex} 461 nm and λ_{em} 358 nm that binds to adenine-thiamine regions of DNA, at a concentration of 0.2 µg/mL, was added and incubated for 10 minutes at RT (142). Subsequently, DAPI was removed, and the cells were washed three times with PBS. Finally, PBS was added and kept without removal.

3.8. Confocal imaging

The image acquisition was done using a Laser Scanning Microscopy (LSM) 780 system by Carl Zeiss equipped with 405 nm and 561 nm diode lasers and a 488 nm argon laser. The best signal approach was applied, where each fluorophore signal was obtained separately with the maximum emission signal possible at slowest speed. Each channel (TRITC, DAPI, and Alexa 488) was optimized in terms of gain and intensity by using the saturation detection tool. The size of the pinhole was adjusted to the minimum 1 air unit (AU). A smaller pinhole enhances the signal-to-noise ratio in the confocal image by blocking out-of-focus light. Also, enhance the spatial resolution, which quantifies how closely two distinct points or structures can be positioned while still being recognized as separate entities in the image (143).

The images were analyzed using Zen 2010 and ImageJ software.

3.9. Immunoassay

The ProcartaPlex Human Inflammation Panel 20plex Immunoassay detect Endothelial Selectin CD62E (E-selectin), Platelet Selectin CD62P (P-Selectin), Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Intercellular Adhesion Molecule-1 (ICAM-1), Interferon Alpha (IFN α), Interferon Gamma (IFN γ), Interleukin (IL) IL-1 α , IL-1 β , IL-4, IL-6, IL-8 (C-X-C Motif Chemokine Ligand 8, CXCL8), IL-10, IL-12p70, IL-13, IL-17A (CTLA-8 protein), Interferon-gamma-inducible protein 10 (IP-10, CXCL10), MCP-1 (CCL2), Macrophage Inflammatory Protein-1 (MIP-1 α (CCL3)), MIP-1 β (CCL4), and TNF α . It was used to assess the adhesion molecules and cytokines produced by the co-culture.

The frozen samples were defrosted gradually on ice, then vortexed and centrifuged at 10,000 x g for 5 minutes to remove particulates. Immune-coated fluorescent magnetic beads were diluted, vortexed for 30 seconds, and added to each well of a 96-well plate. The dilution liquid was removed by turning the magnetic plate upside down. The antigen standard, 4-fold serially diluted, and the samples were added to the wells containing the beads. The plate was sealed and incubated with shaking at 500 rpm for 60 minutes at RT. The beads were washed twice. Next, the detection antibody mix was added, and the plate was incubated again at the same speed and temperature for 30 minutes. Following this, Streptavidin-Phycoerythrin, a conjugated fluorescent protein, was added and incubated under the same previous conditions. The beads were resuspended by adding a reading buffer. The data were acquired using the Luminex™ 200 system (144).

3.10. Statistical analysis

Shapiro-Wilk test was used to analyze normality of the samples ($n < 50$). ANOVA (Mixed Model) with no correction for multiple comparisons was performed to statistical evaluation with post hoc test Fisher's Least Significant Difference (LSD) when data was parametric and Dunn's test with non-parametric data. Differences were considered statistically significant when $p < 0.05$. Data collected with a sample size smaller than 3 had only the mean calculated. All data analysis and graphs were done using GraphPad Prism version 9.2.0 (GraphPad Software Inc., San Diego CA).

4. Results and discussion

4.1. Effects of fermented products on static co-culture

The gut microbiota plays a vital role in our health. Among their many contributions, they produce SCFAs through the fermentation of dietary fibers (145). SCFAs, including acetate, propionate, and butyrate, serve as important signaling molecules that influence various aspects of our physiology. They support gut health, modulate the immune system, and play a crucial role in metabolic processes (146,147). Studying SCFAs themselves, rather than just the bacteria producing them, allows for a more direct and focused investigation into their specific effects on human health (148).

SCFAs are primarily absorbed by gut epithelial cells and can be used as an essential energy source, supporting their growth and maintenance (149). Moreover, SCFAs contribute significantly to the maintenance of gut health. They enhance the integrity of the gut barrier by promoting the production of mucins, which are crucial components of the mucus layer that lines the intestinal tract (149,150). This mucus layer acts as a protective barrier against harmful pathogens and toxins (150). Additionally, SCFAs stimulate the production of tight junction proteins, which help to seal the gaps between epithelial cells, preventing the entry of harmful substances into the bloodstream (151).

Although SCFAs confer numerous benefits in appropriate concentrations, it is important to note that they can potentially become detrimental in high amounts (152). At elevated concentrations, SCFAs can disrupt the delicate balance of the gut environment. This can potentially lead to damage to gut epithelial cells and compromise the integrity of the intestinal barrier (153).

Here, different concentrations of fermented products containing SCFAs were tested to evaluate their effects on an *in vitro* static gut model using a co-culture of Caco-2/HT29-MTX cells.

4.1.1. Health cells under fermented products exposure

As cells grow and multiply, they develop a more continuous and tighter monolayer, leading, therefore, to an increase of the TEER (154). Figure 7 A shows consistent increase of the TEER values measured during the Caco-2/HT29-MTX cells growth for 21 days. The reported TEER values for 90% Caco-2 and 10% HT29-MTX co-culture after 21 days vary widely in the literature. Vincentini et al. reported around 400 $\Omega \cdot \text{cm}^2$, Hilgendorf et al. around 1800 $\Omega \cdot \text{cm}^2$, and Pan et al. around 250 $\Omega \cdot \text{cm}^2$ (93,155,156). It is important to note that the TEER is influenced by various factors, including medium composition, passage number, and temperature (157). However, the obtained values align with measurements previously obtained in other experiments in the laboratory, corroborating the suitability of these cells for investigating the effects of fermented products. Although TEER may vary depending on factors such as temperature or cell passage number, deviations below the typically reported values in the laboratory suggest compromised cell viability. Additionally, while no assay for

metabolic activity was conducted, the cells were examined under an optical microscope to verify epithelial integrity.

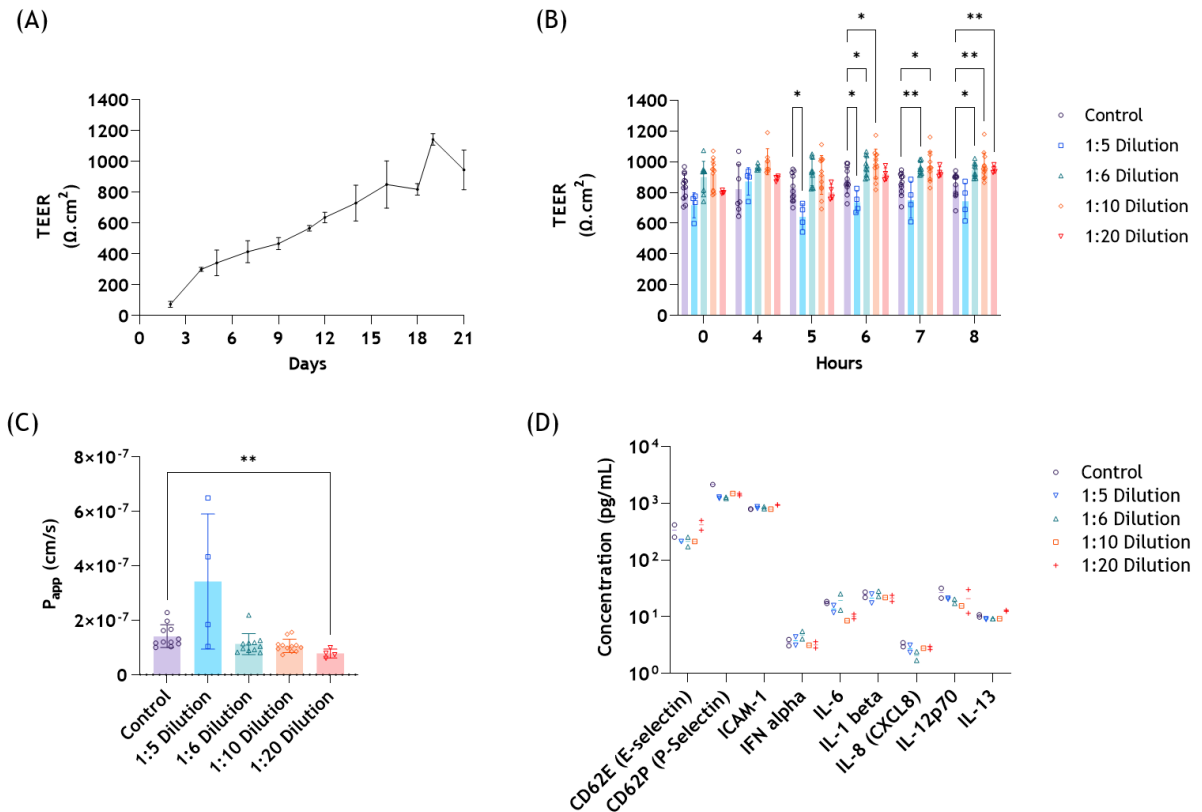


Figure 7. (A) TEER growth curve of Caco-2/HT29-MTX cells co-culture for 21 days. (B) TEER values immediately after fermented products exposure (0h) and at the end of 4 h followed by permeability assay (5h to 8h). (C) Apparent permeability (P_{app}) of the co-culture after exposure to different dilutions of fermented products. (D) Adhesion molecules and cytokines profile of the healthy co-culture. The average is represented by a dash. (B), (C) and (D) Control are cells in HBSS (purple circle). Fermented products are diluted in HBSS at a ratio of 5 (blue square), 6 (green triangle), 10 (orange lozenge) and 20 (red inverted triangle). Statistical significance is reported as * (p<0.05) and ** (p<0.005).

TEER at time zero (Figure 7 B) was measured on the 21st day following the medium change from MEM to fermented products diluted in HBSS, being HBSS alone used as control. Transitioning from MEM to HBSS leads to a slightly decrease in TEER observed at time zero (Figure 7 B) compared to previous measurements in MEM (Figure 7 A, 21st day). This decrease in TEER is attributed to the differing electrolyte composition and nutrient content between the two solutions (158). MEM contains a broader spectrum of nutrients and growth factors tailored for cell culture maintenance. In contrast, HBSS is a simpler, balanced salt solution without the same level of nutritional support. This shift in environment can temporarily disrupt the electrical resistance across the cell monolayer, resulting in the observed lower TEER (159).

After 4 hours of exposure to fermented products, cells demonstrate an average increase, although not statistically significant, in TEER compared to the control group (Figure 7B). A decrease in TEER was expected at 5 hours upon removal of fermented products and the introduction of a lucifer yellow solution for permeability assessment. There is a significant decrease in 1:5 dilution compared to controls that remains until 6 hours, suggesting that the integrity of the epithelial barrier appears to be compromised with high concentrations of the fermented products. Meanwhile, the 1:6 and 1:10

dilutions exhibit a significant TEER increase from 6 hours to 8 hours, following medium stabilization. Additionally, in the 1:20 dilution, the significant effect is only observed after 8 hours.

The apparent permeability values of the co-culture facing different fermented products dilutions are shown in Figure 7 C. The 1:5 dilution exhibits a notably high standard deviation with elevated permeability values, indicating a disruption in the tight junctions. In contrast, the 1:20 dilution, which has the same number of samples ($n=4$) as the 1:5 dilution, exhibits a considerably lower standard deviation and furthermore demonstrates a significant reduction in permeability compared to the control. The 1:6 and 1:10 dilutions do not show statistical differences in permeability compared to the control, although this is observed in the TEER measurements. Despite high TEER values do not necessarily reflect lower apparent permeability, in the 1:20 dilution the TEER increase combined with permeability decrease may be attributed to the promotion of gene expression related to the tight junctions, which enhances the mechanical barrier, reduces paracellular transport, and consequently, increases TEER (160,161).

The evaluation of the adhesion molecules and cytokines profile within the co-culture is depicted in Figure 7 D. The analytes GM-CSF, IFN γ , IL-1 α , IL-4, IL-10, IL-17A (CTLA-8), IP-10 (CXCL10), MCP-1 (CCL2), MIP-1 α (CCL3), MIP-1 β (CCL4), and TNF α were below the detection limit and are not shown in the graph. P-selectin and E-selectin are adhesive molecular receptors expressed on activated platelets, endothelial cells, and leukocytes that participates in cell rolling (162). Here, it is observed that Caco-2/HT29-MTX, epithelial cells, also exhibit expression of these molecules. Köhler et al. have demonstrated the presence of both E- and P-selectin binding sites on HT 29 cells cultured *in vitro*, emphasizing the critical role of these selectins in spontaneous metastasis formation (163). Mannori et al. have reported minimal expression of these selectins in Caco-2 cells (164). ICAM-1 is a cell surface glycoprotein and an adhesion receptor expressed on various cell types, including endothelial cells, leukocytes, and epithelial cells. It regulates barrier function and is important to immune response facilitating the adhesion and migration of immune cells (165). Almost all types of cells can produce small amounts of type IFN- α molecule which enhance immune response and is linked to the development of autoimmune diseases (166,167). Suzuki et al. reported that IL-6 increase the tight junction permeability by modulating the expression of claudin-2 (168). It is possible to see a decreasing concentration of IL-6 in the 1:10 and 1:20 dilutions compared to the controls, which would partially explain the significant decrease in permeability by the 1:20 dilution in the permeability assay. IL-1 β and IL-12p70 are immunoregulatory cytokines present at low levels in the healthy co-culture (169,170). This observation is consistent with Miao et al. and Álvarez-Mercado findings of similar cytokine levels in a Caco-2 monoculture (171,172). IL-8 is a chemokine important in cellular migration and inflammation that is also produced in low levels in health Caco-2 and HT29-MTX cells (173-176). On the other hand, IL-13 is another immunoregulatory cytokine that inhibits the production of cytokines such as IL-1 β , INF- α , and IL-8 (177).

Figure 8 shows confocal images of Caco-2/HT29-MTX co-culture from controls, and fermented products 1:6 and 1:10 dilutions on Transwell inserts. The nuclei, tight junctions and actin filaments are marked by DAPI in blue, Occludin-Alexa 488 in green and Phalloidin-TRITC in red, respectively. These pictures show a flat epithelium after 21 days of differentiation in static culture, as observed in

the orthogonal views on the sides of the images. A direct comparison shows no visible difference between the control and the epitheliums exposed to fermented products in 1:10 dilutions. They display preserved cellular structures after 8 hours of assay and do not exhibit notorious alterations in the monolayer, although the TEER values become significantly different starting from 5 hours of the assay, as shown in Figure 7B. When comparing condition 1:6 with both the control and the 1:10 dilution, the monolayer is irregular, yet it still maintains similar cellular structures. Diao et al. observed morphological alterations in the small intestine of pigs following SCFAs infusion, which they attributed to the stimulation of cell proliferation and discussed in terms of a possible dose-dependent effect of SCFAs (178).

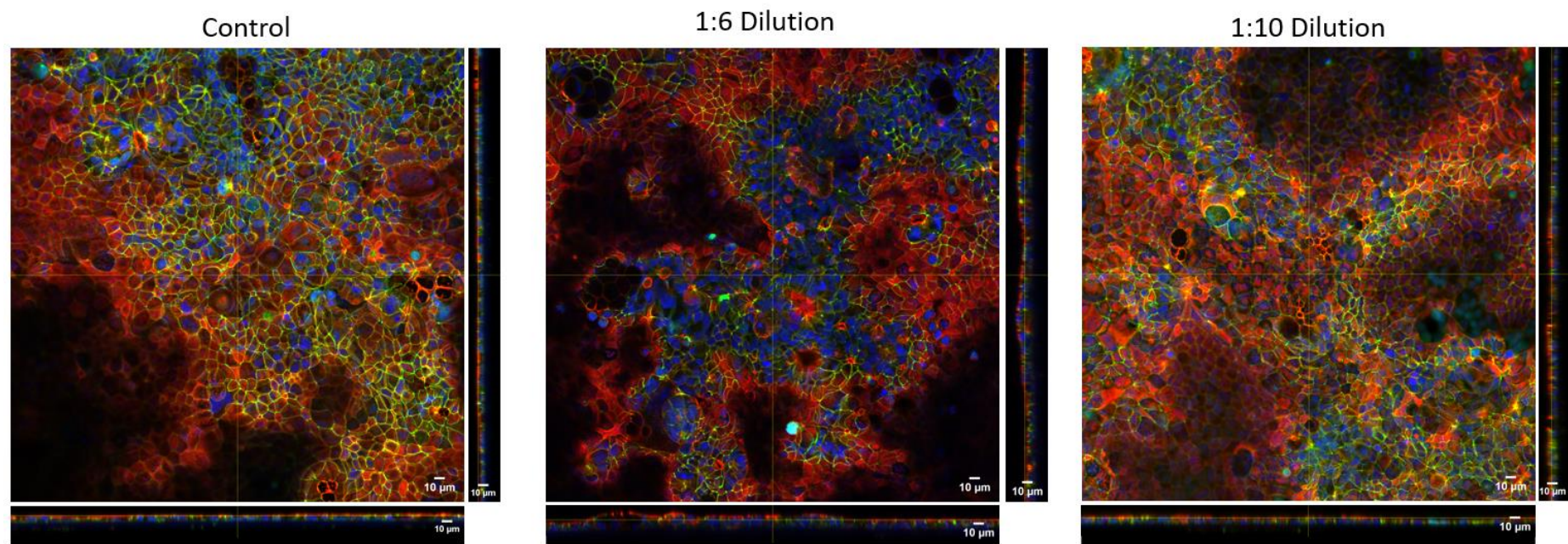


Figure 8. Confocal images at 20x magnification of Caco-2/HT29-MTX cells growth on 1 μm pore size transwell® inserts. The nuclei are stained in blue (DAPI), tight junctions in green (Occludin-Alexa 488), and actin filaments in red (Phalloidin-TRITC). Control (left) and fermented products 1:6 and 1:10 dilutions (center and right). Scale of 10 μm.

4.1.2. Effects of fermented products on inflamed Caco-2/HT29-MTX cells

TNF- α acting as pro-inflammatory cytokine serve as trigger in the initiation and progression of inflammation, including in the pathogenesis of IBD (179,180). Consequently, there is a keen interest in identifying compounds for prevention and treatment of the disease (181). Protective agents may inhibit TNF- α binding to receptors or suppress downstream signaling, thus tempering the inflammatory response (182,183). Restorative compounds, on the other hand, aid in tissue repair and regeneration caused by inflammation (184).

To assess the protective and restorative effects of the fermented products, two distinct exposure sequences were implemented. In the first scenario, cells were initially exposed to the fermented products for 4 hours, followed by a subsequent 48-hour induction of inflammation via TNF- α . This sequence facilitated an assessment of the potential protective influence of the fermented products against inflammation. Conversely, in the second scenario, cells underwent a 48-hour exposure to TNF- α -induced inflammation, succeeded by a subsequent 4-hour exposure to the fermented products, enabling an evaluation of their potential restorative properties.

Based on the results obtained in healthy cells, the 1:10 dilution was chosen to assess either the protective or restorative effects of the fermented products on inflammation. This dilution exhibited a lower standard deviation in the permeability assay compared to the 1:6 dilution (Figure 7 C) and also demonstrated a significant increase in TEER values compared to the control and (Figure 7 B). The 1:20 dilution was not selected due to the significant TEER increase observed in cells only after 8 hours (Figure 7 B), despite the permeability being significantly lower compared to the control (Figure 7 C).

The growth of Caco-2/HT29-MTX cells was monitored for 21 days until a polarized monolayer was achieved (Figure 9 (A.1) and (A.2.)). Although the curves appear similar, the data obtained are from different culture plates. Figure 9 A.1 represents the condition where the cells were exposed to fermented products before inflammation, while Figure 9 B.1 represents the scenario where the fermented products were introduced after inflammation. The TEER values obtained align with those measured in the experiment with healthy cells (Figure 7 A).

Budesonide, a glucocorticoid, was selected as a positive control due to its established efficacy as an anti-inflammatory agent in the treatment of IBD (185). As a glucocorticoid, this compound exerts its therapeutic effects through the suppression of pro-inflammatory cytokines and has shown some evidence of effects on tight junctions, resulting in enhanced TEER and lower permeability *in vitro* in basal conditions (186). The addition of fermented products to budesonide was to evaluate if they could interfere with the drug action, as in the normal gut, there is production of them (187).

Prior laboratory investigations defined the optimal conditions for inducing inflammation, establishing 48 hours as the sufficient duration to elicit a cellular inflammatory response. Additionally,

inflammation on the basolateral side shows better effects than apical exposure. The TNF- α receptor is situated on the basolateral side, making it inaccessible to TNF- α administered to the apical surface (117).

Figure 9 A.2 shows the TEER measurements on prevention approach. The decrease in TEER at time 0 hours (Figure 9 A.2), regarding to the last measurement before the experiment (Figure 9 A.1, day 21st), is related to the change of medium (from MEM to HBSS). A significant increase in TEER was observed in the condition of the 1:10 dilution when combined with the anti-inflammatory budesonide after 4 hours of exposure. However, there was no statistical difference compared to the effects of fermented products or budesonide alone, suggesting that when both are combined, they have an additive effect that leads to significant increase of TEER.

After 48 hours of inflammation, marked as 52 hours in the graph of Figure 9 A.2, a reduction in TEER is noticeable for both the control and the 1:10 dilution alone compared to samples with budesonide. Although a significant increase in TEER of the 1:10 dilution compared to the control after inflammation is observed, this is only seen at 52 hours. The previous experiment with healthy cells showed sustained TEER increase from time 6 to 8 hours at Figure 7 B, but here, after inflammation, the measurement was only taken after 48 hours. It is not possible to determine the behavior of the cells during the 48 hours due to the lack of measurements during that period, but it is indicative that the compound's effects continue up to 48 hours even after the removal of fermented products. Pearce et al. exposed enteroids to butyrate and acetate for 24 and 48 hours before subjecting the cells to hypoxia. They noticed that when the cells were exposed to the compounds for 48 hours the TEER remained constant for 48 hours in hypoxia but decreased after this period (188). This may explain the non-observation of increased TEER for more than 48 hours.

On the other hand, budesonide alone or with the 1:10 dilution appears to have a sustained protective effect on the cells against inflammation. It is reported that budesonide has the ability to be stored within cells, which could explain its continuous action even after drug removal and the onset of inflammatory stimulus (189). However, significance is higher ($p < 0.005$ and $p < 0.001$) when budesonide is administered alone, as shown in the statistical analyses for hours 52, 53, 54, and 55 at Figure 9 A.2. This indicates that the fermented products may negatively interfere with the drug's action in this case (187).

Permeability in none of the conditions seems to be altered compared to the control (Figure 9 A.3). Nevertheless, a decrease in budesonide permeability would be expected due to its significant sustained higher TEER after inflammation. It is hypothesized that the paracellular space did not undergo sufficient alteration in size to allow more passage of lucifer yellow and significantly increase permeability in the controls and in the 1:10 dilution, where a significantly lower TEER is observed. An assay that measures the size-dependent permeability of the tight junctions, as conducted by Itallie et al., could elucidate whether this indeed occurred in the experiment (190).

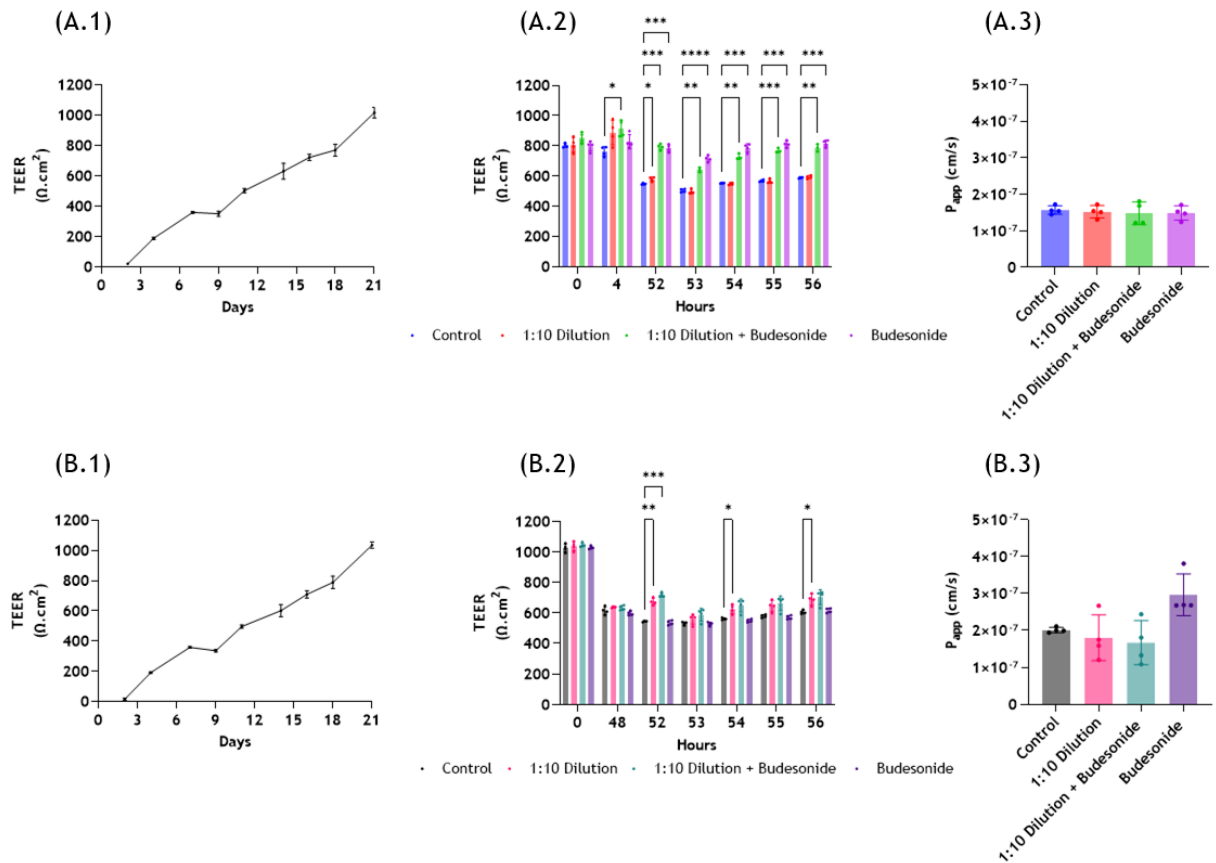


Figure 9. (A) Fermented products exposure before inflammation testing for protective effects. (B) Fermented products exposure after inflammation testing for restorative effects. (A.1) and (B.1) are TEER curves of Caco-2/HT29-MTX cells growth from distinct well plates which these experiments were conducted. (A.2) TEER values of exposure to fermented products from 0 hours to 4 hours followed by inflammation of 48 hours (finished at 52 hours). (B.2) TEER values of inflammation of 48 hours starting from 0 hours to 48 hours followed by exposure of 4 hours to fermented products (finished at 52 hours). Permeability assay TEER was measured from 53 hours to 56 hours in (A.2.) and (B.2). Apparent permeability (Papp) of the protective (A.3) and restorative (B.3) effects of tested conditions. Statistical significance is reported as * ($p < 0.05$), ** ($p < 0.005$), and *** ($p < 0.001$).

There is no alteration of TEER at time 0 hours (Figure 9 B.2) compared to the last measurement on 21st day (Figure 9 B.1) because TNF- α is diluted in the same medium used for culture. After inflammation, a decrease in TEER is observed across all samples. Subsequently, following a 4-hour exposure to fermented products, TEER values exhibit a significant increase in the 1:10 dilution alone or with budesonide. However, this significance is not sustained upon the removal of fermented products, being evident only at 54 and 56 hours for the 1:10 dilution alone. In the same way that cells could benefit from a more extended exposure before inflammation, as reported by Pearce et al., prolonged exposure after inflammation could also be beneficial. Further investigation is needed to determine whether this increased indeed leads to sustained increase in cellular resistance (188).

Also, here, budesonide appears unable to increase TEER with a single 4-hour exposure, despite being reported otherwise by Leonard et al. (191). After a 2-day exposure to IL-1 β as an inflammatory stimulus, followed by 4 hours of budesonide exposure, Caco-2 cells showed an increase in TEER. Finally, in Figure 9 B3, there is no significant difference in apparent permeability in any of the conditions compared to the control, where this observation aligns with the discussion presented in Figure 9 A.3.

The confocal images at Figure 10 show inflamed cells with TNF- α in the control (left), exposure to the 1:10 dilution before (right) and after inflammation (center). Both control and fermented products exposure before inflammation show a continuous monolayer (Figure 10 left and right). In the middle image (Figure 10 center), there is a loss of actin filaments marked in red by Phalloidin-TRITC. The precise etiology underlying the observed morphological alterations remains presently unclear as the TEER in this condition were significantly higher than controls when compared to the cells exposed to fermented products before the inflammation (Figure 9 A.2 and B.2).

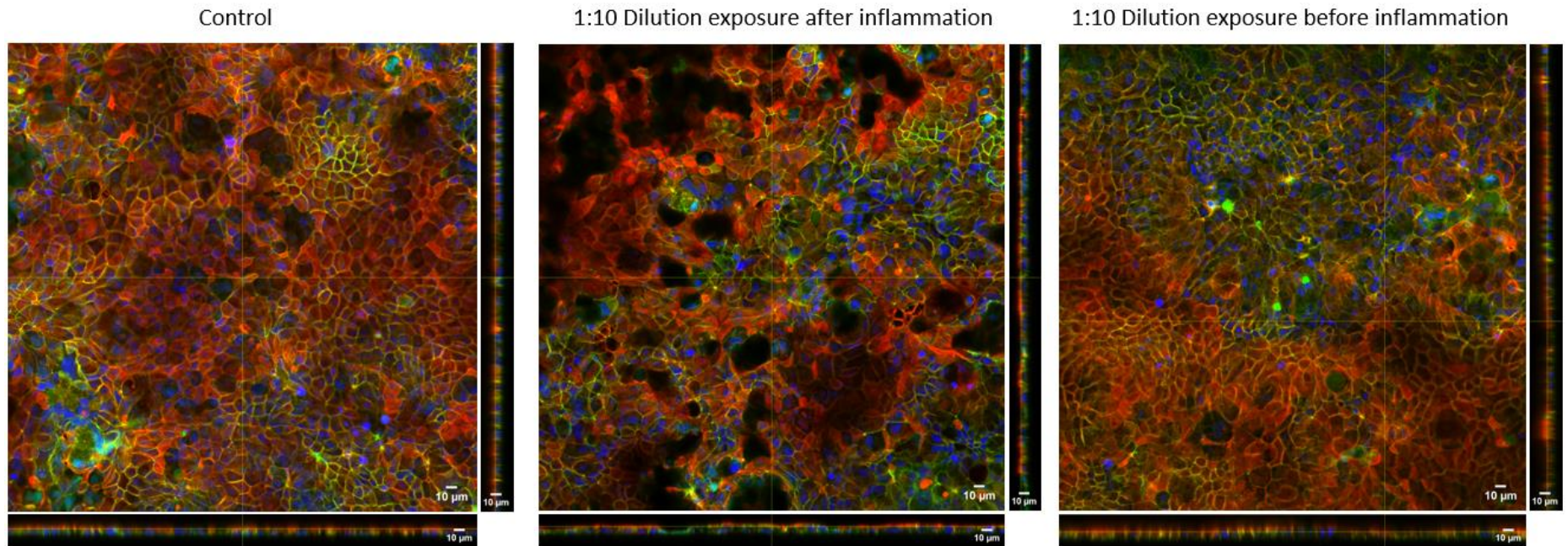


Figure 10. Confocal images at 20x magnification and orthogonal views of inflamed Caco-2/HT29-MTX cells grown on 1 μm pore size transwell® inserts. The nuclei are stained in blue (DAPI), tight junctions in green (Occludin-Alexa 488), and actin filaments in red (Phalloidin-TRITC). Control (left) and fermented product 1:10 dilution exposure after (center) and before inflammation (right). The center image shows regions of actin filaments absence in the brush border in orthogonal view. The monolayer of control and exposure before inflammation do not seem to be altered. Scale of 10 μm .

4.1.3. Immunoprofile of Caco-2/HT29-MTX cells under inflammation

The cytokines and adhesion molecules produced by the co-culture in a healthy state and under the influence of a 1:10 dilution of fermented products or budesonide are shown in Figure 11 A. The analytes GM-CSF, IL-1 α , IP-10, MCP-1, MIP-1 α , and MIP-1 β were below the detection limit in healthy cells (Figure 11 A) when compared to the inflamed cells (Figure 11 B and C).

After removal of TNF- α , IL-6 was detectable (Figure 11 C) and, in TNF- α presence, GM-CSF and IP-10 (Figure 11 B), whereas the reverse was not observed. In contrast, MCP-1 were detectable both during and after the TNF- α exposure, indicating its expression dependence on cell inflammation in this context, but without statistical difference. Budesonide are known to decrease IL-6, IP-10, and MCP-1 production (192,193), although a statistical difference was only seen to IP-10. In Figure 11 B, a significant decrease in the concentration of IP-10 is seen as well with the 1:10 dilution alone. This finding aligns with Korsten et. al results, which found that SCFAs inhibit the release of IP-10, also known as CXCL 10, in inflamed HT29 cells (194). Moreover, further investigation into IP-10, GM-CSF, and IL-6 different expression mechanisms with and without TNF- α in Caco-2/HT29-MTX cells is needed.

Intriguingly, IL-1 α is present during inflammation induction (Figure 11 B), whereas IL-1 β is expressed in the healthy condition and post TNF- α stimulus removal (Figure 11 A and C). Despite their structural similarity, IL-1 β , when bound to its exclusive receptor IL-1R2, exerts an anti-inflammatory effect (195). However, both IL-1 α and IL-1 β , upon binding to the IL-1R1 beta receptor, exert pro-inflammatory actions (169). It is hypothesized that, in order to ensure clarity in signaling that the cell is undergoing an inflammatory process, the secretion of IL-1 α appears to be preferential. Additionally, there is a significant reduction in the concentration of IL-1 α with budesonide alone or in combination with fermented products.

One of the mechanisms of glucocorticoids involves inhibiting the activity of nuclear factor kappa B (NF- κ B), a crucial transcription factor in the activation of various genes, including those related to cellular adhesion molecules such as E-selectin, P-selectin, and ICAM-1 (182,192,196,197). It is noteworthy that, indeed, in the presence of budesonide, either alone or with fermented products 1:10 dilution, the concentration of E-selectin, P-selectin, and ICAM-1 significantly decreases (Figure B and C). Additionally, glucocorticoids are known to inhibit the transcription of IL-1 α and MIP-1 α , although in this case, a significant reduction in concentration was observed for MIP-1 β , a homologue of MIP-1 α (198,199).

In lung cells, SCFAs inhibited the expression of E-selectin and ICAM-1 (200). Here, it is shown that the 1:10 dilution alone significantly reduced the concentration of E-selectin and, with budesonide, appear to have a synergic effect on inhibition of ICAM-1 (Figure 11 B).

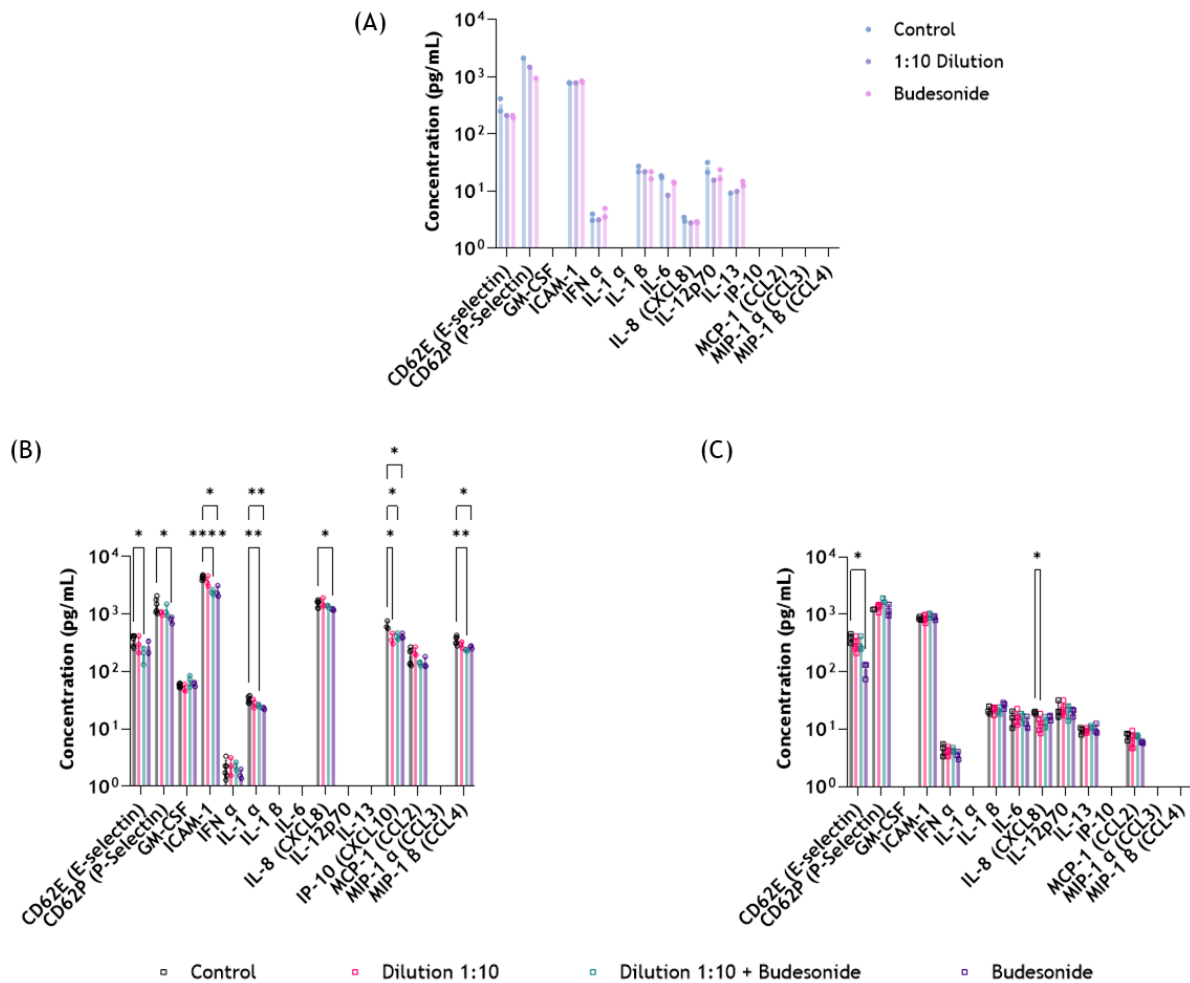


Figure 11. Cytokines and adhesion molecules profile production of Caco-2/HT-29MTX cells. (A) Healthy cells alone (control) or under fermented products 1:10 dilution and budesonide. (B) Inflammation before and (C) after exposure of fermented products 1:10 dilution alone or in combination with budesonide, and budesonide alone. Statistical significance is reported as * ($p < 0.05$), ** ($p < 0.005$), and **** ($p < 0.0001$).

Fusunyan et al. observed an increase in IL-8 secretion in Caco-2 intestinal epithelial cells in the presence of butyrate while Asarat et al. tested SCFAs on intestinal epithelial cell lines T4056 and HT-29 and noted a marked decrease in IL-8 production induced by butyrate (201,202). Huang et al. demonstrated a dose-dependent reduction in IL-8 mRNA levels in Caco-2 with increasing concentrations of sodium butyrate (203). Weng et al. reported that in a Caco-2 infection model, butyrate could both decrease and increase IL-8 production, dependent on the duration of exposure. In a short exposure (6 hours), butyrate decreased IL-8 production, but significantly enhanced it in extended exposure (9 and 24 hours). However, they did not find a beneficial effect when cells were incubated with butyrate prior to infection simulation (204). Despite the conflicting reports on butyrate's action on IL-8 production, this study demonstrates a reduction in IL-8 production after inflammation in the presence of fermented products, with no alteration when exposed prior to inflammation.

During TNF- α stimulus, IL-8 reduction is specifically observed with budesonide alone. Although no effect was observed after TNF-alpha removal, perhaps due to the short exposure as already

discussed, the action of budesonide is well-documented in IL-8 production inhibition via the NF- κ B pathway (205).

IL-12p70 and IL-13 are present in the healthy condition and after the removal of TNF- α (Figure 11 A and C), but not in its presence (Figure 11 B). IL-13 is an anti-inflammatory cytokine that negatively regulates the production of TNF-alpha (206). Although the reverse mechanism has not been described, here, the presence of TNF- α inhibit the production of IL-13. In contrast, TNF- α exerts a regulatory influence on the duration of the inflammatory response by modulating and restricting the production of the pro-inflammatory cytokine IL-12, as is evident from the observed results (207). Also, TNF- α can cross-regulate the production of IFN- α , which is present in both healthy and inflamed cells, although it does not show statistical difference in any of the inflammatory conditions (208).

4.2. Comparison between static and dynamic models

The role of fermented products was also examined dynamically in a gut-on-chip model, providing a basis for comparing the outcomes with those obtained from the static Transwell insert model. Dynamic models are demonstrated to replicate the structures and physiology of the studied organ more accurately, which allow for a better assessment of how fermented products affect cellular responses and barrier function (209).

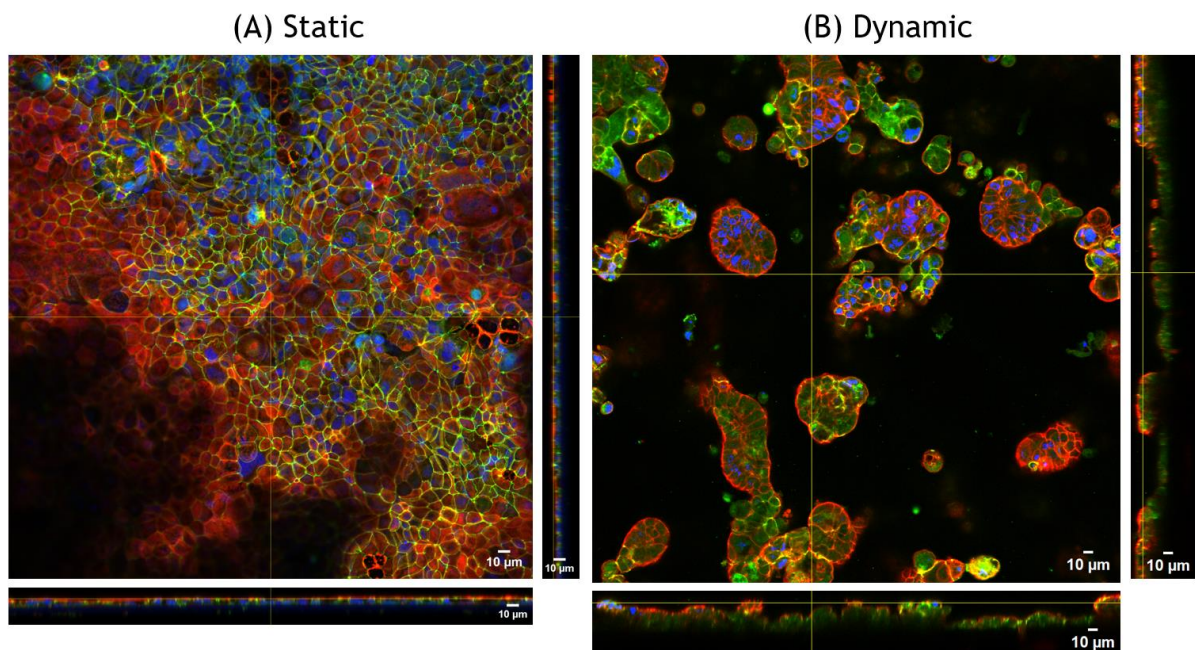


Figure 12. (A) Confocal image at 20x magnification of Caco-2/HT29-MTX cells differentiation after 21 days in static culture model. The orthogonal views reveal a flat monolayer. (B) Confocal image at 40x of Caco-2/HT29-MTX cell differentiation after 7 days, with orthogonal views highlighting the presence of villi-like structures in the dynamic model. In both images, tight junctions are marked by Alexa 488-Occludin (green), nuclei by DAPI (blue), and actin filaments by Phalloidin-TRITC (red).

Figure 12 shows a comparison of the morphology of Caco-2/HT29-MTX cells between 21 days in static culture (Figure 12 A) and 7 days in dynamic culture (Figure 12 B), with a flow rate of 120

$\mu\text{L/h}$ and a shear stress of 0.0025 dyn/cm^2 . Variations observed in the distribution patterns of actin filaments (in red) and occludin (in green) are evident between Figure 12 A and B. These differences can be attributed to the impact of constant shear stress on the expression of tight junctions and the formation of microvilli, as documented by Delon et al. (210). Shear stress has also been shown to promote faster cell polarization and the formation of 3D folds, demonstrated in the orthogonal views of Figure 12 B, that recapitulate the structure of villi in the small intestine (211,212).

The apparent permeability values of the static and dynamic models under fermented products exposure are displayed in Figure 13. In controls, the dynamic model has significant higher permeability than in static model, probably due a higher surface area created by the villi-like structure (213). These values are closer to the lucifer yellow *ex vivo* small intestine apparent permeability values of $4.02 \pm 2.20 \times 10^{-6} \text{ cm/s}$ found by Rozehnal et al., compared to the static model (214). Thus, the significance of the dynamic model is heightened by these findings, as it demonstrates a closer alignment with physiological conditions compared to the static model.

Regarding the effects of fermented products, the dilution 1:5 display higher standard deviation in both static and dynamic model. This variability in the results is also observed for the 1:6 dilution in chips, though not in transwell. Although the precise cause of these variations within a model is not currently understood, Kulthong et al. demonstrated that the expression profiles of Caco-2 genes diverged significantly between static and dynamic conditions, and here, this may influence how cells behave facing the different dilutions (215).

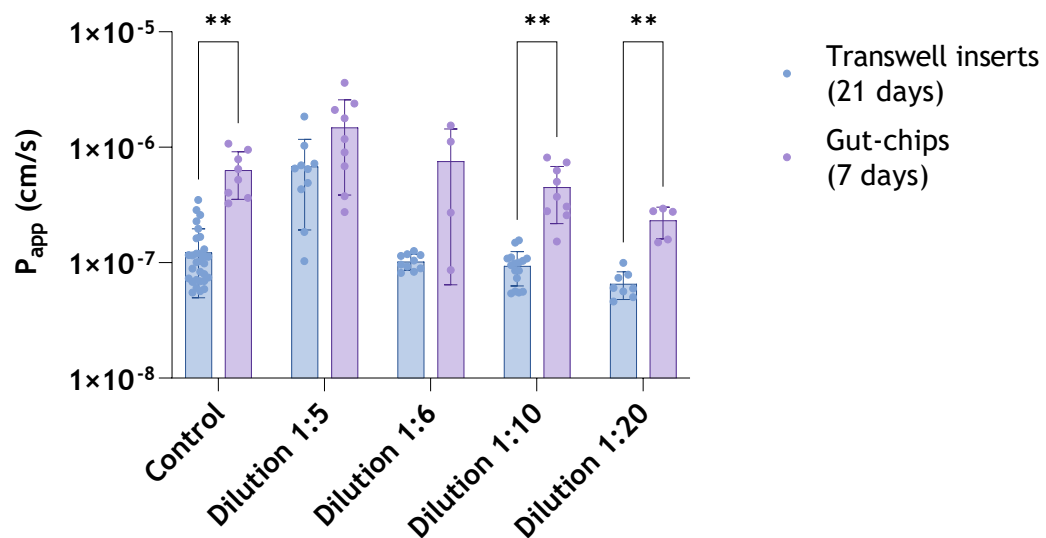


Figure 13. Apparent permeability of Caco-2/HT29-MTX cells cultured in static (transwell insert) and dynamic (gut-chip) models. Fermented product dilutions (1:5, 1:6, 1:10, and 1:20) were teste in both models. Statistical significance is reported as ** ($p < 0.005$).

Lucifer yellow was employed to assess the gut paracellular permeability, which is regulated by the tight junctions (24,216). In this study, a decreasing trend in permeability average is observed with increasing dilutions, where a statistically significant difference is noted between the control and the 1:20 dilution, in both models (see Annex 1). Lowering the concentration of SCFAs compounds in fermented products potentially enhance their known beneficial effects on tight junctions, particularly considering the potential cytotoxicity associated with higher levels of SCFAs, as discussed previously (61,152). In future research, an assessment of the expressions of tight junction proteins would elucidate whether the lower values of lucifer yellow permeability could be correlated with enhanced tight junction function (217,218).

Conclusions

The human microbiota, through anaerobic fermentation, produces byproducts rich in SCFAs. These compounds have demonstrated the ability to regulate epithelial barrier integrity and immune responses. However, at high concentrations, they can be toxic to cells. Therefore, this study utilized the Caco-2/HT29-MTX co-culture as an *in vitro* gut barrier model to evaluate the impact of dilutions of fermented products containing SCFAs derived from human microbiota fermentation on both healthy and inflamed epithelium. Additionally, in order to emulate a more realistic gut environment, a dynamic microfluidic *in vitro* gut-on-chip model was employed to compare cell differentiation and permeability responses to fermented products against the static model. On gut-chips the epithelium is exposed to constant flow and shear stress, inducing different genes expressions that lead to morphological changes in the epithelial barrier.

The assessment in the healthy static model demonstrated higher TEER in the 1:6, 1:10, and 1:20 dilutions, while lower permeability was only significant in the 1:20 dilution. The 1:5 dilution presented lower TEER when compared to the controls. Additionally, the 1:6 dilution monolayer exhibited non-planar characteristics unlike the controls. Therefore, the 1:10 dilution was chosen due to its sustained higher TEER, which was not observed in the 1:20 dilution, and no alteration in the monolayer compared to control to evaluate the effects of SCFAs before and after inflammation. No relevant changes were observed in TEER after exposure to the fermented products prior to inflammation. However, a significant increase in TEER was observed when they were introduced after the removal of the inflammatory stimuli. The permeability was not statically different between controls and dilutions, in neither the two approaches of introduction of fermented products in inflammation.

On the gut-chips, the monolayer displayed villi-like projections similar to those observed *in vivo* and which are absent in the flat static culture. The permeability in the dynamic model is higher compared to the static model and closer assemble to findings *ex vivo*. The 1:20 dilution also exhibited significantly lower permeability values as in the static model. Thus, by the morphological and permeability similarities of *in vivo* and *ex vivo*, respectively, the dynamic model has proven to provide more relevant scenario to study gut permeability and morphological structures.

The co-culture expressed detectable levels of E-selectin, P-selectin, ICAM-1, IFN- α , IL-1 β , IL-8, IL-12p70, and IL-13 under healthy conditions. Cytokines GM-CSG, IL-1 α , IP-10, MCP-1, IL-6, and MIP-1 β were exclusively expressed under inflammatory conditions. Following inflammation, exposure to a 1:10 dilution of fermented products significantly reduced IL-8, a crucial cytokine highly expressed during cellular inflammation. Budesonide, a glucocorticoid used as a positive anti-inflammatory control, exhibited significant decreases alone or together with 1:10 dilution in P-selectin, GM-CSF, IL-1 α , IL-8, and MIP-1 β when introduced prior to inflammation. After the removal of the

inflammatory stimulus, budesonide only decreased P-selectin concentration. The 1:10 dilution alone did not significantly decrease concentration prior to inflammation.

It was possible to assess the effects of intestinal microbiota byproducts, the fermented products, without having to use bacteria in the co-culture. Future work will focus on introducing inflammation in the dynamic model and comparing the cells immunoprofile with static model. Moreover, enhancing the complexity of the dynamic model will enable to test the epithelial cells interaction in the presence of fermented products with bioactive compounds, as budesonide, under healthy and inflamed states. In static culture, as the conditions of inflammation are already set, the immunoprofile of anti-inflammatory compounds can be compared having fermented products in the buffer. Finally, in both models, a protocol involving food for bacteria to produce fermented products, rather than a fermentation medium, could be implemented to assess the effect of dietary choices on intestinal epithelial cells.

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Annex 1

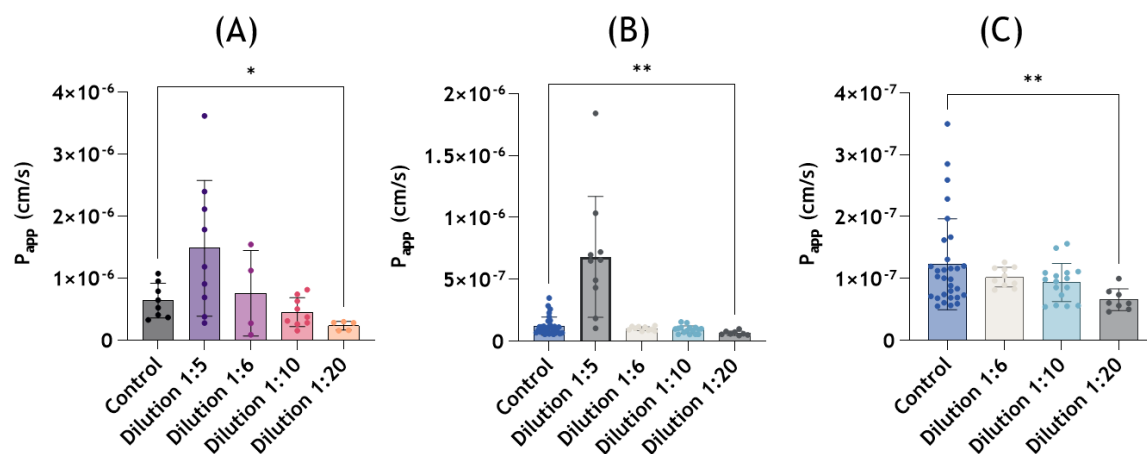


Figure 14. (A) Apparent permeability (P_{app}) of the dynamic model. (B) and (C) Apparent permeability (P_{app}) of the static model. A decreasing trend in permeability average is observed with increasing dilutions in both models, although only statistically significant in the 1:20 dilution. In (C) the 1:5 dilution was removed show to a more representative scale.