UNIVERSITÀ DI PARMA

UNIVERSITA' DEGLI STUDI DI PARMA

DOTTORATO DI RICERCA IN

Scienze Medico-Veterinarie

CICLO XXXV

Responsiveness of intestinal epithelial cells to eubiotics: IPEC-J2 as *in vitro* model in swine

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Anni Accademici 2019/2020 – 2021/2022

Summary

ABSTRACT

The effects of eubiotics are of considerable interest as they are used as additives in pig farming in response to current regulations aiming to avoid antibiotic resistance. Since 2013, World Health Organization banned antibiotics and zinc as growth promoters. In study 1, we characterized a co-culture model composed of intestinal epithelial cells (IPEC-J2) and peripheral blood immune cells (PBMC). Arginine is an essential amino acid among the various substances used to replace antibiotics. Using this model, we evaluated how arginine deprivation induced an increase in intestinal damage and the consequent activation of the intestinal immune response. The deprivation leads to the immunoregulation mediated by the expression of pro-inflammatory (Tumor Necrosis factor (TNF)α, Interleukin (IL)-8, Interleukin (IL)-1α, Interleukin (IL)-6) and anti-inflammatory (Transforming Growth Factor (TGF)-β) cytokines. Among other alternative molecules, Short-Chain Fatty Acids (SCFA), important sources for energy metabolism and anabolic processes in mammals, can regulate the inflammatory response and increase the integrity of the intestinal barrier. In Study 2, we evaluated the effects of SCFA supplementation (acetate, butyrate, propionate, and lactate) at different concentrations in IPEC-J2. SCFA–enhance the functions of tight junction proteins (TJp), which prevent the passage of antigens across the intestinal barrier into the paracellular space. The results show impacts on IPEC-J2, that depend on the type and specific concentration of SCFA. Acetate and propionate exhibit a greater protective action than lactate and butyrate, at the chosen concentrations. Moreover, SCFA supplementation stimulates the expression of TJp and β-defensin 1 (BD-1), which, in turn, may have been involved in the inhibition of Nuclear factor (NF)-κB and TNFα gene expression. Finally in study 3, based on the previous study, 5 mM acetate and 1 mM propionate were used as treatments for the co-culture upon lipopolysaccharides (LPS) challenge, miming the acute inflammatory state. Acetate and propionate have a protective effect by TJp regulation upon acute inflammation.

In summary, a co-culture model of IPEC-J2 and PBMC has been characterized. The model could be important to study and elucidate the role of nutritional conditions in the functionality and reactivity of the intestinal epithelial cells (IECs) in pigs. Arginine deprivation affects intestinal epithelial cells and stimulates their functional response; the amino acid acts as a modulator of the local anti-inflammatory response to adapt and react to a stressful stimulus. Moreover, the data could be translated to humans that there are similarities between pigs and humans in terms of intestinal microbiota and in the mechanisms of intestinal defense systems.

Finally, SCFA have protective roles at the level intestinal. We have highlighted the positive *in vitro* effect of SCFA, even in the presence of LPS-challenge, on intestinal viability and maintaining intestinal integrity. SCFA actions depend on the type and concentration of them. Responses activated by arginine and SCFA are promoted by regulating TJp and the secretion of pro- and anti-inflammatory cytokines. Therefore, it would be desirable for SCFA, widely used in pig diets, to be utilized based on the different actions of each SCFA.

1 INTRODUCTION

The pig gut microbial community is a very complex ecosystem where colonization begins at birth with the consumption of the sow's milk. The microbiome is initially "milk-oriented" during the suckling period, and the gut microbiota development is modulated and influenced by different factors (breed, environment, mother). Most illnesses are related to weaning, a very stressful time in a piglet's life. In this period, the diet changes induce a state of dysbiosis, characterized by a marked decrease in the obligate anaerobic bacteria (*Clostridia* and *Bacteroidia*), and an increase in facultative anaerobic bacteria (*Enterobacteriaceae*). In commercial pig farms, the weaning occurs earlier (3/4 weeks of age) than in nature (10/12 weeks of age) [1]. Low feed intake is the main consequence of weaning, which is responsible for intestinal morphological alterations such as villous atrophy, with consequent lower nutrient absorption and reduced available energy [2]. Stressors involved in early weaning include maternal separation, change in feed composition, transportation, mixing, struggles to establish a new social hierarchy and immunological stressors such as vaccination [3]. All these stresses lead to several widespread diseases in pig farms, among which diarrhea is a common clinical condition. The high prevalence of diarrhea in piglets is a significant problem resulting in increased preventive use of antibiotics and risk of developing bacterial resistance to the antibiotic, causing decreased animal welfare [4,5].

Antibiotic resistance develops when bacteria adapt and grow in the presence of antibiotics, resulting in fewer options for treating a disease. Drug-resistant bacteria can circulate in human and animal populations through food, water and the environment (Figure 1). The choice to avoid the use of antibiotics in farms is due to the possible risk of the presence of resistant bacteria, which could be found in feed and food products intended for human consumption [6].

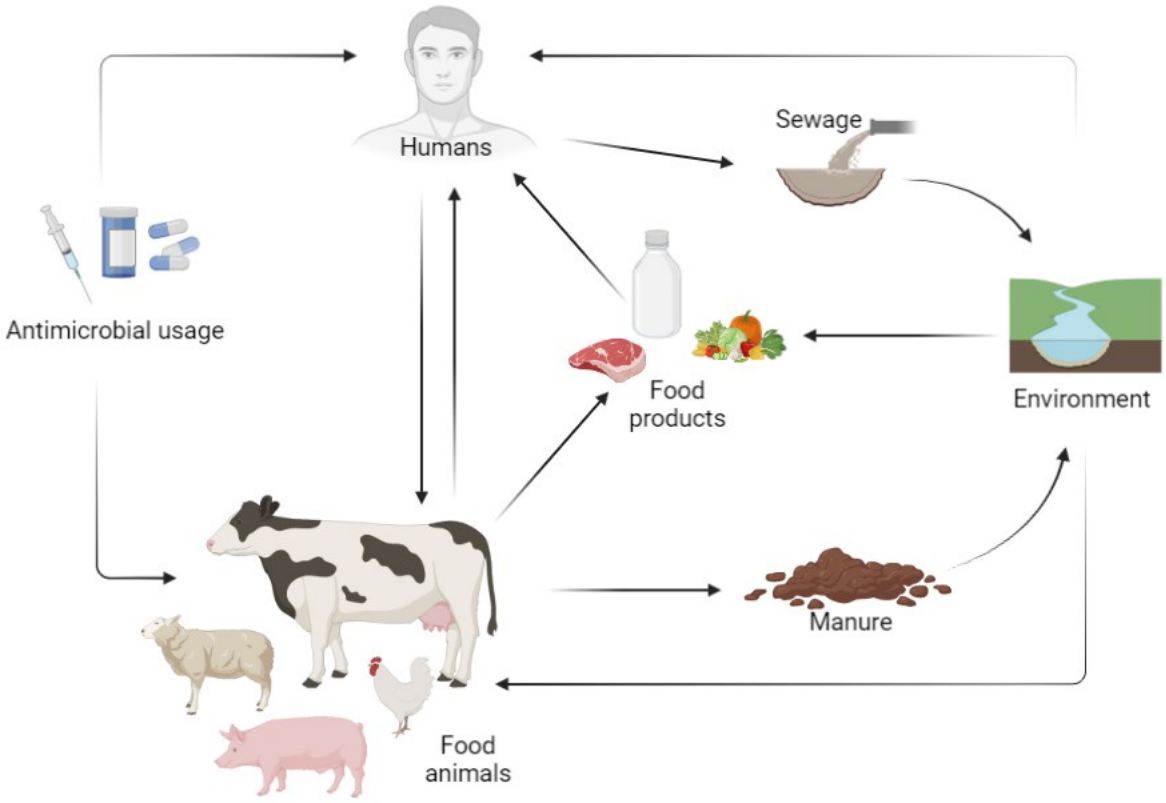


Figure 1 *Schematic representation of the transmission routes of antimicrobial resistance between farm animals, the environment in general and humans.*

Antibiotics also lead to reduce the microbial population, which has the function of locally protecting and activating the maturation proteins of the adaptive immune system [7]. Specific dietary, probiotic means or integrated alternative substances can be used to reduce the stresses induced by weaning, with a consequent improvement in the intestinal health of the piglets by positively influencing the gastrointestinal tract [8].

Phytobiotics, probiotics and prebiotics are found naturally in the environment and belong to the group of so-called "eubiotics" (from the Greek "eubiosis"), which refers to a healthy balance of the microbiota in the gastrointestinal tract. According to the Italian health ministry, probiotics are live or freeze-dried strains of various microorganisms ingested in adequate quantities that can benefit the host [9,10]. The main characteristics of probiotic bacteria: they should be harmless, have no antibiotic resistance, have high survival and resistance at low pH and good adhesion to the intestinal walls, reproduce rapidly and show antagonistic activity against pathogenic

microorganisms [11]. A prebiotic is a non-vital food constituent that confers a health benefit by modulating the microbiota [10]. Prebiotics include inulin, enzymes, protein lysates, fructo-oligosaccharides, organic fumaric, citric, butyric acids. Any dietary component that reaches the colon is a potential prebiotic, but most of the interest in developing prebiotics is in non-digestible oligosaccharides. They can be stable to heat or oxygen exposure, improving the composition of the flora. Using more than one eubiotic substance (multi-eubiotic) as a feed additive could be more efficient than using them separately [12].

1.1 One Health

Antibiotics have always represented a fundamental tool for controlling the health status of farms in the veterinary field. They represent an important means of guaranteeing the regular food production of animal origin. In addition to the containment of infectious diseases, their introduction has favoured the improvement of animal welfare. However, despite their efficiency, the massive use of these substances can lead to the onset of potentially undesirable effects related to the release of residues in original animal products for consumption the environment, with the onset of antibiotic resistance [13].

It is triggered and amplified by the selective pressure of antibiotics on various microorganisms. Resistance is achieved by genetic mutations or by acquiring pre-existing resistance genes from other organisms through vertical and horizontal gene transfer of resistant bacteria [14].

The situation has worsened owing to the need for more investment in developing new effective antibiotics, resulting in slower development of new molecules than the emergence and spread of resistance mechanisms among bacteria. Based on a global approach and in line with the "One Health" initiative, which aims to strengthen the prevention and control of antimicrobial resistance in the fields of medicine, nutrition and veterinary medicine, as well as to ensure the availability, intensity, and duration of action of antibiotics. Because of the global nature of this problem, the action plan emphasises the importance of international cooperation in countering antimicrobial resistance. The European Union (EU) actively collaborates with international organisations such as the World Health Organization, the Food and Agriculture Organization of the United Nations, the World Organization for Animal Health and the Codex Alimentarius Commission [6]. There are several provisions relating to the use of antimicrobials aimed at curbing the spread of antimicrobial resistance, defined in EU legislation and binding throughout the Union. The use of antimicrobials in animals must comply with national and EU standards.

In addition, the EU legislation on medicated feed regulates the conditions for production, market placement, and use of medicated feed. Italy, as a EU member precursor, adopts an electronic prescription for veterinary drugs. The new provision of the Ministry of Health, effective on January 1st 2019, provides for the traceability of medicines and therapies to which animals destined for the food chain are subjected.

In pigs, antimicrobials are mainly used to treat weaning diarrhea, intestinal infections and respiratory diseases often associated with transport and stress. That occurs when pigs from different farms are brought together or when animals are placed on farms with inadequate ventilation systems, unsuitable feeding methods, and/or insufficient biosecurity measures [15].

On farms, before animals are treated with antibiotics to fight an infection, it is advisable to evaluate the problem and take steps to limit the spread and prevent recurrence of the disease. Possible actions to be taken are: provide thorough cleaning and disinfection of production units when animals arrive and are moved on and off the farm; isolate the pathogen and evaluate a vaccination strategy where available; develop appropriate feeding strategies according to the age of the pigs, especially during weaning [16].

The search for alternative substances stimulating the immune system is among the actions to be applied for defending the body from pathogens through the implementation of a response. The response is made effective by the coexistence of two types of reactions: an early one, which does not require pre-exposure to the antigen and is known as innate immunity; the other, involves highly specialized cells, lymphocytes, and is known as adaptive (or cell-mediated) immunity [17].

1.2 Intestinal immunity

The immune system secretes inflammatory cytokines to protect the host against agents, eliminating the pathogen. The defence system against microbial consists of anatomical and physiological barriers, innate immunity and adaptive immunity. Failure in any of these systems will result in high susceptibility to infection.

1.2.1 Innate immunity

Innate immune cells include dendritic cells (DCs), macrophages, and neutrophils. Dendritic cells are specialized antigen-presenting cells (APCs) that capture, process, and present antigen (microbe-derived peptides) to T cells; they are highly specialized for activating the adaptive immune response and represent a unique system in recognition of the antigen.

In innate immunity there are PRRs (pattern recognition receptors). Several subgroups of PRRs are classified according to their ligand specificity, function, location (transmembrane and cytosolic), and/or evolutionary relationships (Figure 2). Transmembrane-bound PRRs include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs); cytoplasmic are NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs).

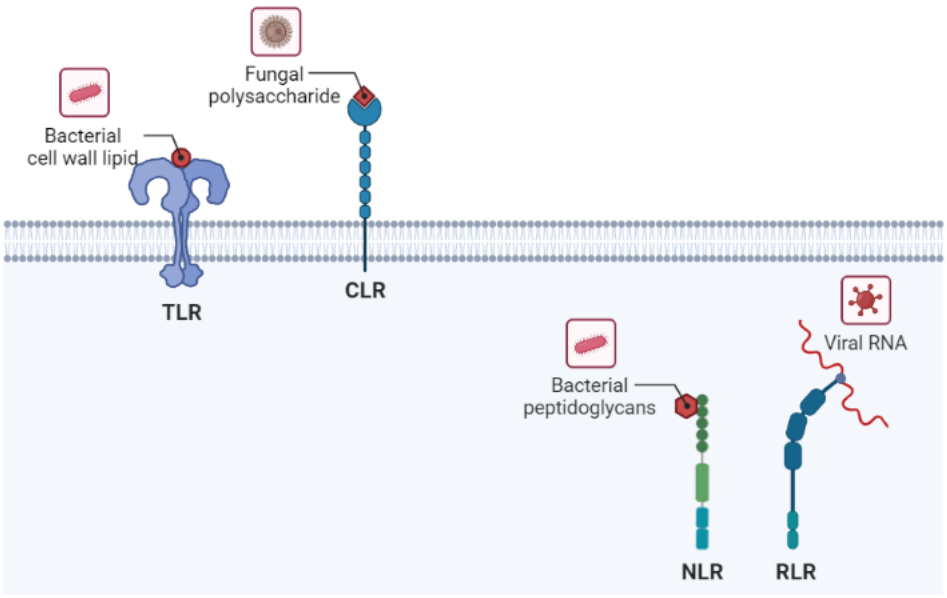


Figure 2 *Representation of different PRR types, transmembrane TLR and CLR, cytoplasmatic NLR and RLR.*

PRRs recognize molecular patterns associated with conserved pathogens such as LPS, peptidoglycan, zymosan, and isoleucine [18]. The secreted PRRs (such as collectins, phycolins and pentraxins) bind to microbial cell surfaces and lead to the activation of classical and lectin pathways of the complement system and opsonize pathogens for phagocytosis by macrophages and neutrophils.

TLRs, as trans-membrane PRRs, can recognize and respond to abnormal changes in the microbial landscape and maintain tissue integrity. Their activation induces the expression of chemotactic factors and cell surface molecules, which trigger the screening of cells at the site of infection to eliminate pathogens. Activation of basolateral TLR in enterocytes causes a cascade of inflammatory response that begins with activation of the NF-κβ pathway and produces chemokines and cytokines, followed by recruitment and activation of immune cells [19].

Other transmembrane receptors are C-type lectin receptors or CLRs. They play an important role in antimicrobial immunity, recognizing most types of pathogens, including bacteria, fungi, viruses and parasites. Membrane-bound CLRs are classified into two groups: type I CLRs, which include receptors belonging to the mannose

receptor family, and group II CLRs, which are part of the asialoglycoprotein receptor family. CLRs have multiple actions, including phagocytosis, activation of innate killing mechanisms by generating microbicidal compounds such as reactive oxygen species (ROS) and producing inflammatory mediators [20].

The cytosolic PRRs are RLRs, a small group of cytosolic receptors that act as sensors for viral RNA. Several immune and non-immune cells constitutively express RLRs. The activation of RLRs is important for the onset of antiviral responses and causes the upregulation of the expression of other PRRs, including TLRs [21].

Finally, the cytosolic NLRs are sensors of infection and stress in intracellular compartments. They can stimulate innate immunity and inflammation in response to harmful signals within the cell. NLRs detect the presence of pathogen-associated molecular patterns (PAMPs). They are activated following the loss of cell membrane integrity, excessive oxygen radical species, or detection of extracellular ATP (adenosine triphosphate) [22].

The PRRs initiate an appropriate antimicrobial response to contain the infection, which involves the regulation of specific antimicrobial peptides, including defensins. These are cationic cystine-rich peptides composed of three families: α-, β- and θ-defensins. They protect the host from a wide variety of bacteria, fungi, viruses, and parasites, so they are constitutively expressed or induced by bacterial products or pro-inflammatory cytokines [23].

1.2.2 Humoral immunity

Specific immunity represents the effector function performed by lymphocytes. Specific immune responses consist of two types: humoral immunity and cell-mediated immunity. Both responses rely on lymphoid cell functions, but humoral immunity is mainly driven by B cells and antibodies, which recognize microbial antigens and cause their destruction (Figure 3). Humoral immunity represents the main defence mechanism against extracellular microbes and their toxins.

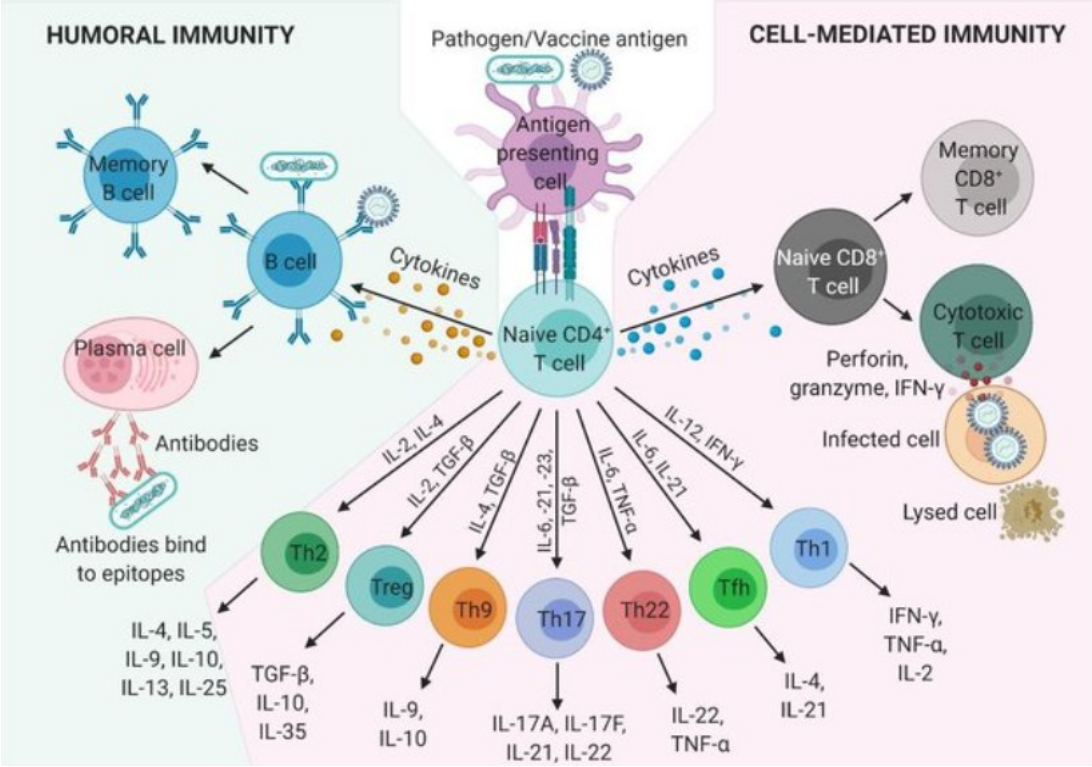


Figure 3 *Synthetic representation of humoral and cell-mediated adaptive immune responses. Pathogenic antigens, recognized by APCs, present antigenic peptides on the surface of MHC-II, which are recognized by T-cell receptors on naïve CD4+ T cells. The cells differentiate into subpopulations: T helper and T regulatory cells (Treg). B lymphocytes, following the regulation of the cytokines produced by CD4+ T lymphocytes, are transformed into plasma cells which secrete antibodies into the blood and extracellular fluids. A cell-mediated immune response involves CD8+ Ts recognizing and binding to intracellularly processed antigenic peptides via their TCR, which appears on MHC-I molecules on the surface of APCs and infected cells. Cytotoxic CD8+ T cells destroy the infected host cell via granzyme, perforin, and IFN-γ. From Schijn et al., 2021* [24].

Cell-mediated immunity is mainly driven by mature T cells, macrophages and by the regulation of cytokines in response to antigens. This immunity promotes the destruction of microbes found within the phagocytes or the lysis of infected cells.

T lymphocytes can be divided into functionally distinct subpopulations, of which the best characterized are Helper and cytotoxic (or cytolytic) T cells. T cells are unable to recognize soluble antigens, do not produce antibodies and have a narrow specificity for antigens, identifying only protein-bound non-self-antigens present in the major histocompatibility complex (MHC) and expressed on the APCs membrane. Therefore, the functional responses of T lymphocytes are triggered thanks to the recognition of the MHC-peptide complexes exposed on the APCs membrane.

Such responses require specific antigen recognition, stable adhesion between T lymphocytes and APCs, and the transmission of activating signals within cells. T lymphocytes recognize MHC-peptide complexes exposed on APCs thanks to clonally distributed specific receptors (TCRs). In addition to expressing the TCR complex, T lymphocytes also express many accessory molecules important for antigen-induced cell activation. Some of these molecules interact with ligands on APCs or target cells, thus stabilizing the adhesion between the two cell types. In contrast, others transmit activation signals within the T lymphocytes. CD4 and CD8 are co-receptors expressed mutually exclusively on different subpopulations of mature T lymphocytes; they bind non-polymorphic regions of MHC molecules. Adaptive responses are mediated by different populations of immune cells, such as lymphocytes found between epithelial cells and called intraepithelial lymphocytes (IELs). IELs contribute to barrier integrity and regulatory functions but can occasionally become pro-inflammatory [7].

1.2.3 Cytokines

Cytokines are peptides, proteins or glycoproteins produced by cells of both innate and specific immunity. Once secreted, they can regulate many of the functions performed by the producing cells. The production of cytokines, mediated by lymphocytes and macrophages, occurs in response to various stimuli, especially antigenic ones. Once the immune response is induced, cytokines stimulate the growth and differentiation of lymphocytes, while in the effector phase, they activate those cells responsible for eliminating pathogens. The action of cytokines occurs through the modulation of gene expression: different molecules can act with a cascade effect, have a synergistic action or act as antagonists [25].

The roles of some Cytokines of interest in our study have been specified below.

***Tumor necrosis factor alpha* (TNFα)**

TNFα is the main mediator of the acute inflammatory response, originally identified as a serum factor capable of inducing tumor necrosis. TNFα is mainly produced by activated mononuclear phagocytes, activated T lymphocytes, natural killer (NK) cells, and mast cells. Gene transcription of TNFα occurs following an infection, inflammatory response or cellular stress [26].

On infected or damaged cells, TNFα usually plays in an autocrine way, i.e. it acts on the same cell that produced it following the recognition of damage-associated molecular patterns (DAMPs) or PAMPs [27]. To limit infection at the site of pathogen entry, TNFα stimulates functional changes, allowing for isolation of the pathogen, including enhanced coagulation and stimulation of cytotoxic leukocyte activity and granuloma formation.

The most effective stimulus that induces the synthesis of TNFα by macrophages is an activation of the TLR by LPS or other microbial components [28]. Interaction of the ligand with members of the TNFα receptor family results in the association of the

cytoplasmic portion of the receptor with TNF-Receptor-Associated Factors, which induce the activation of transcription factors such as NF- κB and Activator protein-1 (AP-1).

TNFα is implicated in intestinal barrier dysfunction in various cell types, including intestinal epithelial cells. TNFα reduces protein expression of the TJp proteins claudin, occludin, and zonula occludens, reducing cytoskeletal rearrangement of actin [29]. It is rapidly induced in the intestinal mucosa upon initial activation of immune cells and appears important for the different inflammatory responses [30]. Consequently, the improvement in disease progression in several experimental models of IBD (Inflammatory bowel disease) is related to the absence or inhibition of TNFα activity. At the same time, in some autoimmune pathologies such as psoriasis, TNFα is employed for its anti-inflammatory properties since it strongly sensitizes T cells to the induction of apoptosis, resulting in an accelerated resolution of the inflammatory response [31].

***Transforming growth factor-β* (TGF-β)**

TGF-β is mainly expressed and activated by lymphocytes, monocytes/macrophages and intestinal epithelial cells. TGF-β functions in normal cell maintenance and the inflammatory response but also suppresses proliferation by inhibiting several key transcription factors, such as c-Myc, and induces several factors associated with cell proliferation and differentiation.

TGF-β inhibits T cell proliferation by inhibiting IL-2 production and, during bacterial infections, limits excessive cell expansion through up-regulation of the pro-apoptotic gene Bcl2. The TGF-β acts through binding with its receptor, consisting of four subunits, and the consequent recruitment of the transcription factor Smad. Several signalling pathways have been studied to be involved in TGF-β action, including Smad, MAPK/MEK, JNK/p38, NF-κB and AKT/PI3K pathways. Overall, TGF-β induces cellular apoptosis, MAPK/MEK pathway can antagonize cell cycle arrest.

TGF-β and PI3K/Akt pathways can antagonize each other to support the balance of cell growth, death and differentiation [32].

TGF-β and IL-6 induce Foxp3 in naïve CD4+ T cells, stimulating the differentiation of Th17 cells, producing IL-17 and IFN-γ, promoting intestinal inflammation, and activating mucosal defense against bacteria [33].

TGF-β reduces inflammatory responses in intestinal immunity, contributing to immune tolerance induction. TGF-β modulates the barrier function of the epithelium regulation of expression levels of tight junction proteins and adhesion molecules. TGF-β can stimulate the intestinal epithelial barrier function, inducing the production of Claudin-1, trying to limit the negative effects caused by pathogenic bacteria that reduce the levels of the TJp proteins Claudin-2, Occludin and ZO-1 [33].

***Interleukin-1* (IL-1)**

IL-1 is a pro-inflammatory cytokine that regulates the host inflammatory response to infection and other stimuli. IL-1 is produced by many cell types other than macrophages, such as endothelial cells and epithelial cells. In the IL-1 family, two isoforms are larger than the others, IL-1α and IL-1β. These have a pro-inflammatory action, and the natural antagonist is IL-1Ra (IL-1 receptor antagonist). IL-1Ra regulates the proinflammatory activity of IL-1α and IL-1β by competing with them for receptor binding sites [34]. The major source of IL-1 is activated mononuclear phagocytes. IL-1 production by cells is induced by LPS and/or TNFα. IL-1 induces its biological effects by interacting with a well-defined membrane receptor for IL-1 type I that is involved in the transduction of signals that activate NF-κB and AP-1 [35]. After the binding of IL-1 to its receptor, the adapter protein myeloid differentiation primary response-88 (MyD88), associates with the TIR domain of the IL-1 receptor, followed by two kinases IRAK and IRAK4, and by an adapter protein TRAF6. The signals expressed downstream of these events include the phosphorylation processes of several second messengers and the formation of further complexes between other kinases and adapter

proteins that work by inducing the activation of NF-κB [36]. The biological effects of IL-1 are similar to TNFα and depend on several cytokines produced. Mucosal inflammation is characterized by an infiltration of neutrophils and mononuclear cells, which at the time of activation, are important producers of proinflammatory IL-1α and IL-1β. These are produced early during inflammation and induce the production of other cytokines (IL-6, TNFα), amplifying their proinflammatory action [25].

Interleukin-6 **(IL-6)**

IL-6 is a cytokine involved in the regulation of innate and specific immunity. It is produced by mononuclear phagocytes, activated T lymphocytes, endothelial cells and fibroblasts in response to microorganisms and various cytokines IL-1 and TNFα influence [37]. IL-6 serves as an alarm signal, and its expression increases rapidly in response to local stressors. IL-6 can lead to responses with both pro- and anti-inflammatory effects. IL-6 induces anti-inflammatory signalling by binding to its membrane receptors, which are highly expressed, for example, in patients with intestinal bowel disease. In serum and tissue, IL-6 increased secretion and dysregulation; it was observed in subjects with IBD [38].

Conversely, binding of IL-6 to the soluble receptor induces proinflammatory trans-signalling (Nf-κB pathway). Once activated, the resident macrophages in the tissues produce IL-6 and TNFα; they induce a pro-inflammatory response that determines changes in the vascular permeability and the molecules expressed by the endothelial cells, facilitating the entry of neutrophils into the tissues [39]. IL-6 promotes the release of different cytokines depending on the type of infection (viral, bacterial or fungal). In the early stages of viral infection, however, the binding of TLR4, TLR8 and TLR9 to portions of viral nucleic acids stimulates dendritic cells, monocytes/macrophages and Natural Killer cells to produce IL-6, IL-12, IL-18, IL-15, interferon (IFN)-γ and TNFα [25]. IL-6, acting on Treg and Th17 lymphocytes, promotes cell-mediated immune reactions through the induction of the production of pro-inflammatory cytokines, IL-1, IL-17, IL-22, IL-23, which leads to protection against bacterial infection or fungal [40].

Interleukin-8 **(IL-8)**

IL-8 is known for its chemotactic role in neutrophil granulocytes [41]. In addition to being called interleukin, it is called chemokine (C-X-C motif) or CXC(L)-ligand 8 and is encoded by the CXCL-8 gene. IL-8 can be secreted by any cell with a Toll-type receptor (macrophages and smooth muscle cells), recognizing patterns of antigens, such as LPS, from gram-negative bacteria. IL-8 has pro-inflammatory activity and is secreted by macrophages, neutrophils, fibroblasts, endothelial cells, epithelial cells and intestinal epithelial cells in which the synthesis occurs thanks to the stimulation of IL-1β and TNFα.

Endothelial cells accumulate IL-8 in vesicles known as Weibel-Palade bodies [25]. The surface receptors capable of binding IL-8 are the G protein-coupled receptors: CXCR1 and CXCR2. Both receptors are involved in the activation of multiple transcription factors, including signal transducer and activator of transcription (STAT) 3, hypoxia-inducible factor (HIF)-1, AP-1, and NF-κB [41].

IECs have the immune function of providing the signal for the initial wave of leukocyte and neutrophil recruitment during inflammation. For this reason, one of the first chemokines produced by the IECs is IL-8, which has a chemotactic action on neutrophils [42]. IL-8 results in the activation of the TNFα-mediated pro-inflammatory response [43]. IL-8 stimulates chemotaxis, guiding immune cells towards target cells; it induces processes of migration (an increase of intracellular Ca^{2+} concentration) and phagocytosis (release of histamine and oxidative burst) [44].

1.3 Intestinal mucosal system

The mucosal immune system exists in various anatomical areas of the body, such as the respiratory, reproductive, or digestive tracts, and plays a complex role in host defense [45]. In the intestinal tract, the mucosal immune system plays an important role. It contributes to the digestion and absorption of nutrients and serves as an important barrier for the body to resist pathogenic insult [46]. The intestinal mucosal immune response is induced by local mucosal tissues, pathogens, and other antigens that stimulate immunoreactive cells. Mucosal immunity is characterized by producing secretory immunoglobulin A (sIgA) by plasma cells [47].

The mucosal immune system is identifiable as mucosa-associated lymphoid tissue (MALT). These formations are not organized to form lymphatic system organs but rather as lymphatic nodules (or even isolated cells). MALT organizes along the surfaces of the mucous membranes and ensures a complete humoral and cellular immune response following local antigenic stimuli. It locates at strategic points to allow the different populations of cells of the adaptive immune system (T and B lymphocytes) and antigen-presenting cells, and components of the innate immunity (macrophages) to immediately mount an immune response on the mucosal surface [47].

The components of MALT can be divided into 5 groups: GALT (gut-associated lymphoid tissue); BALT (bronchial-associated lymphoid tissue); NALT (nose-associated lymphoid tissue), SALT (skin-associated lymphoid tissue); VALT (vascular-associated lymphoid tissue); CALT (conjunctiva-associated lymphoid tissue in the human eye) [48].

1.3.1 Gut-Associated Lymphoid Tissue

Intestinal mucosal immune cells are organized in a specialized and compartmentalized system known as the GALT, which provides a cellular barrier against antigens and microorganisms [49] (Figure 5). GALT is the largest lymphoid organ and consists of Peyer's patches, isolated lymphoid follicles, intraepithelial lymphocytes and mesenteric lymph nodes [50].

Peyer's patches are well-organized lymphoid structures in the superficial layer of connective tissue of the intestine (the *lamina propria*). T lymphocytes and dendritic cells dominate this structure. The epithelium is constituted by microfold (M) cells, specialized epithelial cells responsible for the uptake of luminal antigens, and by APCs, which take up and present antigens to immune effector cells [51].

Some APCs include DCs, macrophages, and IECs [52]. The presentation of antigens by the APCs to these cells results in the production of antibodies and various cytokine immunomodulatory factors [46].

Furthermore, the B lymphocytes of the *lamina propria* also participate in this system by producing immunoglobulin A (IgA). IgA is taken up by enterocytes and secreted into the intestinal lumen. It can interact with antigens and microbes via 'immune exclusion' (series of events involving agglutination, trapping in mucus and/or clearance through peristalsis), thus performing its chemical barrier function [19].

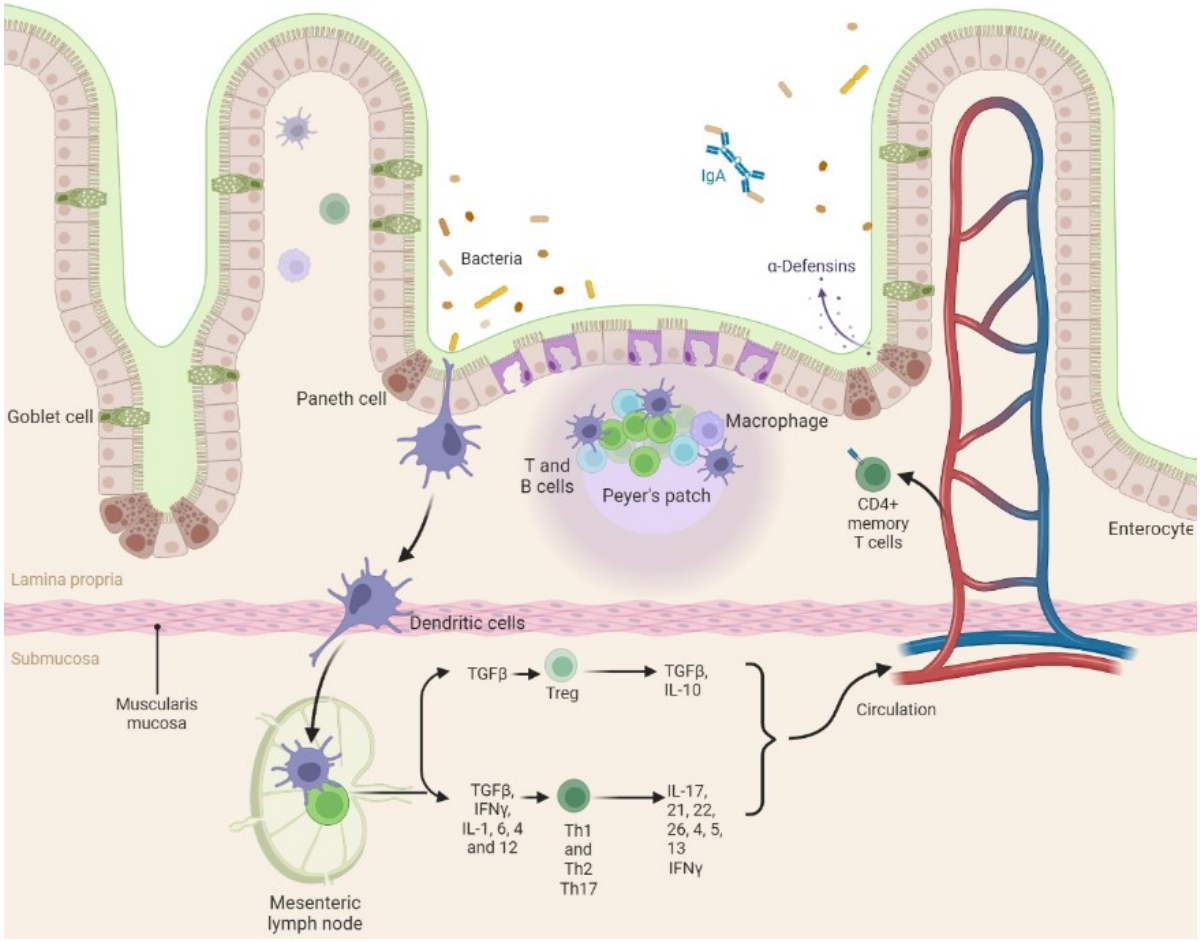


Figure 5 *Schematic representation of the intestinal immune response through the mucosa of the small intestine. The first level of the response is the barrier, which consists of IECs layers formed by villi and crypt structures. There are goblet cells secreting mucus, and Paneth cells, which secrete antimicrobial peptides (defensins). Furthermore, the innate response begins with the activation of immune cells (organized in Peyer's patch), including macrophages, dendritic cells, intraepithelial lymphocytes, and finally, the adaptive response with lamina propria effector T cells, plasma cells (B cells) secreting IgA.*

The intestinal luminal content is separated from the epithelial lining by inner and outer mucus layers. The outer layer (closest to the lumen) is loosely organized and populated by bacteria, while the inner layer is firmly attached and, under normal circumstances, free from bacteria. Mucus is made up of glycosylated mucins (especially mucin-2, MUC2) secreted by goblet cells [53], which act as adhesins that enable the binding of commensal and pathogenic bacteria. In the gut, bacteria colonise and proliferate, have to be able to adhere to the mucus layer, where surface-bound mucin-binding proteins are present [54]. In addition to constituting a physical barrier and acting as bacterial

binding sites, mucins are important energy substrates for commensal bacteria. Studies in pigs have confirmed that MUC2 genes are also expressed in the pig intestine [55].

1.4 The intestinal barrier

The intestinal barrier comprises the epithelial mucosal layer, the microbiota, the innate and adaptive immune system associated with the mucosa, the intestinal lymphatic system and the intestinal endocrine/neurenteric system [56]. The epithelium of the gastrointestinal tract is constantly attacked by foreign antigens derived from ingested material and gut microbiota [57]. This condition requires a complex defensive system that separates the intestinal contents from the host tissues and regulates the absorption of nutrients; this is allowed by the interactions between the resident microbiota and the intestinal immune system, called the "Intestinal Barrier" function [58].

The physiological barrier is made up of intestinal epithelium, in close contact with a layer of mucus, which acts as a barrier against both harmless commensal bacteria and dangerous pathogenic bacteria. Intestinal mucus is the first physical barrier bacteria encounter in the intestinal tract, and it contains antimicrobial proteins (AMPs, as defensins and cathelicidins) and sIgA [59].

The intestinal epithelium formed by enterocytes, cells essential for absorbing nutrients, collaborate with the mucus in the barrier activities [1]. Intestinal epithelial consists of absorptive enterocytes, antimicrobial peptide-producing Paneth cells (restricted to small intestine only), mucus-producing goblet cells, hormone-secreting enteroendocrine cells and M cells [60]. Through the junction proteins, IECs act as a protective barrier to prevent pathogens, arriving in the intestinal lumen, from overcoming the epithelial barrier and causing mucosa inflammation [61].

In *lamina propria* there are cells of the innate and adaptive immune system, e.g. macrophages, dendritic cells, mast cells, T cells, B cells, and innate lymphoid cells (ILC); on the contrary, the epithelium contains predominantly intraepithelial lymphocytes. IECs continuously interact with nearby immune cells and can secrete

molecules such as cytokines, chemokines and growth factors (TGF-β, IL-6, IL-8, TNFα, IL-1). They support epithelial barrier functions to eliminate infected or damaged IECs (cytotoxic activity) and induce IECs to produce antimicrobial peptides [62].

The intestinal epithelium is an important physical barrier against antigens and microbes; it is a monolayer made so by the presence of junctional proteins among enterocytes (Figure 6):

1) Desmosomes
2) Gap junction
3) Adherens junctions (AJ) in the basal-lateral part under the TJs
4) Tight junctions in apical part.

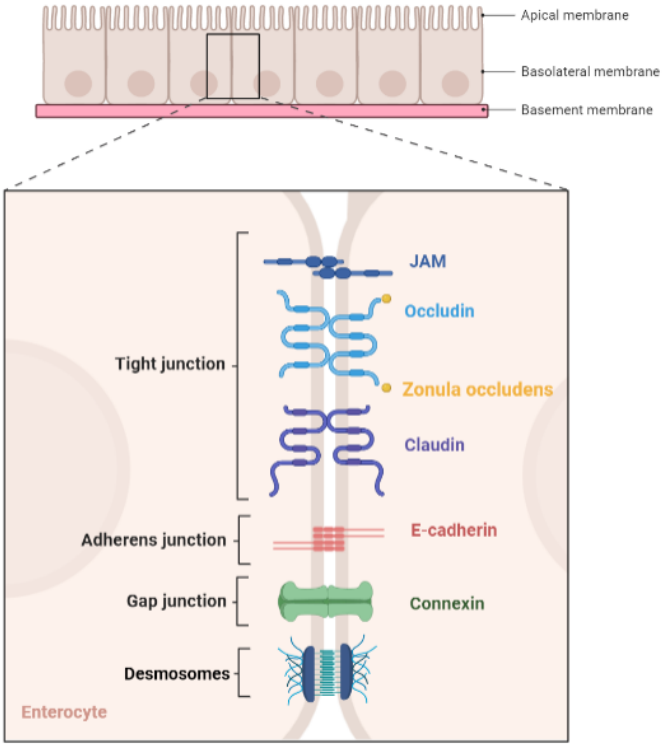


Figure 6 *Diagram Representing Types of cell Junctions.*

Tight and Adherent junctions are connected to the actin cytoskeleton. The cell-cell adhesion complex depends on tight junction structures which polarize the intestinal epithelium, permitting selective regulation of the ionic passage and creating a

potential difference on both sides of the tissue. The adherents offer structural support, anchoring the different types of epithelial cells.

Briefly, adherent junctions result from a complex association between multiple components and play a central role in forming contacts between neighbouring cells and stabilising adhesion [63]. A loss of the adherent junctions implies an interruption in cell-cell and cell-matrix contacts with consequences that lead to premature apoptosis. The main AJ components are Cadherins (120 kDa).

Gap junctions (innexins, connexins, and pannexins) are clusters of intercellular channels that facilitate a direct connection between the cytoplasm of two neighbouring cells for mediating intercellular communication. Gap junctions exhibit relatively low substrate specificity and are permeable to a wide variety of molecules with mass <1 kDa, such as small metabolites, ions, and intracellular signalling molecules (ions, ATP, ADP, cAMP, amino acids) [64].

Desmosomes in the basolateral membrane anchor the epithelial cells to the *lamina propria*. Their up-regulation contributed to the maintenance of barrier function [57].

1.4.1 Tight junction

The intestinal epithelial structure of the TJp plays a crucial role in physiological processes, being a dynamic and permeable barrier, depending on two types of pores. The first type is highly capacitive, selective and permeable to small ions and small uncharged molecules. The second type of pore is larger, with a low capacity and permeable to large ions and molecules regardless of the charge. Occludin and zonula occludins regulate it [65].

Junctional adhesion molecules (JAMs) are members of an immunoglobulin subfamily expressed by epithelial and endothelial cells. They associate with TJs promoting epithelial barrier function. The cross-talk between JAMs and claudin is tissue specific.

Indeed, in intestinal epithelial cells, JAM-A regulates the expression of claudin-10 and claudin-15 but not of claudin-2; the opposite occurs in the kidney [66].

Claudins (CLDN) (18-27 kDa) are proteins with 2 extracellular loops and a C-terminal cytoplasmic domain. The extracellular loops of claudin molecules make homophilic and heterophilic interactions with adjacent cells, and the interactions create either barriers against or pores for the passage of selective molecules [67]. They constitute a large gene family in which 24 isoforms have been identified that are expressed in a tissue-specific manner, and a mutation or deletion of one of the members of this family can have significant effects on organ function [68]. Claudins perform different functions, including involvement in barrier formation (decreasing paracellular permeability) and regulation of channel pores (increasing paracellular permeability) [69].

Occludins (OCLN) (65 kDa) are proteins with 4 transmembrane domains and 2 extracellular loops. They have an important role in the intestinal barrier, providing structural integrity, and being an integral component in the barrier function of the tight junction. The occludin expression was found to be correlated with the barrier properties *in vitro* and *in vivo* [70]. Inflammation affects epithelial barriers, increasing occludin leakiness and decreasing the barrier function of this protein. Occludin is not fundamental for the formation of TJ strands. However, occludin can interact directly or indirectly with claudins by being recruited to the long strands formed by the co-expression of claudin-1 and claudin-2 [71]. The redistribution of occludin from the tight junction to the cytoplasmic vesicles frequently occurs during the loss of barrier function [72].

Peripheral membrane proteins zonula occludens (ZO) (130-220 kDa) are crucial for the assembly and maintenance of TJs as they possess multiple domains for interaction with other proteins, including integral membrane proteins and actin. The protein that plays the central role is ZO-1 which directly and indirectly links the integral membrane proteins (occludins and claudins) to the other cytoplasmic proteins of TJ and the actin cytoskeleton [73].

Tight junctions are responsible for limiting the intercellular space; by regulating paracellular permeability, they allow electrolytes and substrates to bypass the epithelial lining and enter the submucosal layers. Increased intestinal permeability can be caused by a reduction in the expression of tight junction proteins leading to increased levels of inflammatory cytokines [74].

1.5 Alternative substance to antibiotics

Valid alternatives to antibiotics are probiotics; these are non-toxic, stable, poor sensitivity to additives, and acidifying capacity of the food bacteriostatic and/or bactericidal action. In Italy, the Ministry of Health [10] has defined probiotics as "microorganisms which, once ingested in adequate quantities, have beneficial effects on the organism". Currently, to reduce antimicrobials, some pig farmers are using probiotics which are perfect candidates as they stimulate the immune system, by increasing the production of antimicrobial peptides and cytokines in the intestinal tract. For example, administering lactic acid bacteria (*Pediococcus acidilactici*) or yeast (*Saccharomyces cerevisiae boulardii*) improves intestinal defences against microbial infections. It increases the performance of several species of monogastric animals [75]. In addition, *S. cerevisiae boulardii* induced weight gain in pigs and increased the number of macrophages in the mucosa.

Effects on the intestinal mucosa suggest that supplementing the probiotic in the diet can promote a "healthy" gut, encouraging an early restoration of the intestinal mucosal thinning that often occurs at weaning and, probably, improving local resistance to infection [76]. “Gut health” is a term that refers to animal health, and Bischoff [77] proposed five criteria for defining a healthy gastrointestinal tract :

1) effective digestion and absorption of food,
2) absence of gastrointestinal illness,
3) normal and stable intestinal microbiota,
4) effective immune status
5) status of well-being.

The height of the intestinal villi and the depth of the crypts are histomorphology markers of pig intestinal health. The reduction of the height of the villi and the deeper crypt is observed, especially during weaning, a critical period in piglet life [1].
Another important indicator of gut health is barrier integrity.

The regulation of gene expression of tight junction proteins is very important because the TJp modification leads to a change in paracellular permeability.

Increased epithelial paracellular permeability is associated with synthesising various reactive oxygen species that disrupt tight and adherens junctions. A reduction in viability is usually observed with an increase in oxidative products. Still, studies support the hypothesis that a low nitric oxide synthesis has a protective effect in maintaining barrier integrity [78]. The TJp barrier disruption stimulate the activation of the mucosal immune system, releasing inflammatory mediators [67].

The healthy pig gut contains a high number of bacteria but a low number of potentially pathogenic bacteria. Such potentially pathogenic bacteria are especially those belonging to the Enterobacteriaceae family, such as some *E. coli* and *Salmonella* [79].

Currently, the most used genera in swine production are *Bacillus, Lactobacillus, Bifidobacterium, Enterococcus*, and *Saccharomyces*; therefore, they can improve the immune response, reduce the load of pathogenic bacteria, stimulate colonization by beneficial microbiota, and stimulate digestion. Again, a nutritional strategy to promote intestinal health is using amino acids as feed supplements.

1.5.1 Amino acids

Amino acids are molecules that contain amino groups and acids. Amino acids constitute the primary structural units of proteins, forming polymer chains, peptides or polypeptides. Amino acids are nutritionally essential (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) and non-essential (alanine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, proline, serine and tyrosine). The essential amino acids are fundamental for growth and intestinal development, which are vital factors for the health of piglets, with a consequent reduction in the incidence of diseases and an increase in growth performance [80]. Specific amino acids (glutamine, tryptophan, arginine, cysteine, and threonine) can promote health by improving gut tissue anabolism, reducing the impact of stress, and modulating local immunology [81]. Amino acid requirements are based on assessing their effect on growth performance, but amino acids have several functional roles beyond muscle protein synthesis.

The National Research Council has defined the need for amino acids to be introduced into the diet of domestic pigs, the choice of amino acids changes in the various phases of the life cycle. Consequently, their specific introduction could be tailored to circumstances, such as during disease challenges or to stimulate developmental aspects affecting gastrointestinal health. In the post-weaning period, it is recommended to introduce foods such as corn, soybean meal, wheat and fishmeal, which bring essential amino acids such as lysine, methionine, threonine and tryptophan into the diet [82]. For example, the choice of supplementing with lysine derives from the fact that its deficiency or excess in the diet can decrease or increase nitrogen retention and protein turnover in the body, influencing the pig's production performance, nutrient digestibility, parameters serum metabolic rates and carcass characteristics. Lysine is absorbed directly from the intestine for protein synthesis and other metabolic processes, at the same time, participates in fat metabolism and regulates the metabolic and hormonal balance in animals [83].

On the other hand, glutamine serves as major energy substrate for enterocytes in the small intestine, providing ATP to pig enterocytes than glucose and fatty acids. Furthermore, glutamine acts as a precursor (Figure 7) for synthesising citrulline and arginine in the enterocytes of piglets in the first week after birth.

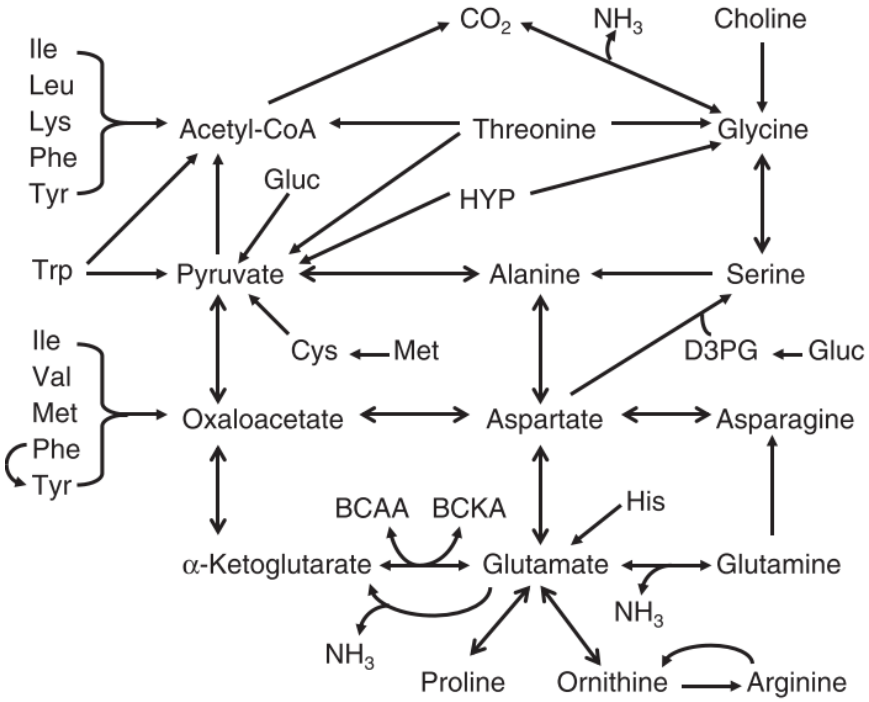


Figure 7 *Representative scheme of essential or non-essential amino acids in animals. The essential ones must be integrated into the diet in adequate quantities as they are not synthesized in vivo. For example, arginine is an essential amino acid that is produced by the degradation of aspartate, glutamate, or ornithine (all non-essential amino acids). The latter are synthesized in the body. From Rezaei et al., 2013* [84]

Their endogenous synthesis is important for the optimal growth and development of the animal, particularly in the neonatal period the arginine requirements are much higher than those supplied by milk [84].

Amino acids, probiotics, prebiotics and micronutrients influence the nature and intensity of systemic and intestinal immune responses, stimulating the immune system to fight infections [85]. Amino acids, such as threonine, arginine and glutamate, have a main role in maintaining functional and structural integrity by promoting cell renewal [81]. Glutamine and arginine strongly impact the immune system by regulating the secretion of pro-inflammatory cytokines, encouraging the proliferation of T lymphocytes and the secretion of IgA at the intestinal level [86].

1.5.1.1 Arginine

Arginine is traditionally classified as an essential basic amino acid in neonates, acts as a building block for proteins, and plays an essential role in immune system functions.

Arginine is transported to the cells by cationic transporters CAT1–4. CAT proteins are classified as members of the solute transporter family 7 (SLC7). The transport of l-arginine, l-lysine, and l-ornithine via CAT-1 (y+ system) is pH-independent, responsive to stimulation, and saturable at circulating plasma concentrations (~0.1–0.2 mM) [87]. CAT-1 is sensitive to membrane potential changes. Vasoactive agonists induce hyperpolarization, leading to an increase in the driving force for cationic amino acids transport into the endothelium and other cell types [88]. The activity of the CAT-1 transporter in cells depends on protein kinase C (PKC) α phosphorylation and activation of PKCα lead to the downregulation of CAT-1 at the cell surface [89].

Arginine is a physiological substrate for synthesising nitric oxide (NO), which is a key mediator of immune responses and multiple metabolic pathways [90]. Oxidative stress is considered one of the main actors of malabsorption and inflammation of the gastrointestinal tract, representing one of the main causes of barrier malfunction and lack of regeneration. Besides these alterations, oxidative stress also affects the mitosis and apoptosis of intestinal epithelial cells. However, basal nitric oxide production maintains adequate perfusion, regulates intestinal permeability, and minimizes mucosal barrier dysfunction [91]. Therefore, amino acids supplementation is necessary to maintain homeostasis and exert a regulatory role in signal transduction pathways. These involve protein kinases activated by specific mitogenic growth factors expressed in immunocytes, thus affecting immunity [92]. The activation of inducible nitric oxide synthase (iNOS) and/or arginase (which converts L-arginine into urea and ornithine) reflects the type of inflammatory response. In fact, during a particular pathological process, T lymphocytes are dependent on arginine for proliferation, T cell receptor expression and memory cell development [93]. Stimuli-inducing iNOS include the cytokines IL-1, TNFα, and IFN-γ and endotoxins. Several researchers have further

investigated the importance of arginine in specific cellular and molecular functions in T lymphocytes [94]. In myeloid cells, arginine deprivation activates transcriptional upregulation of arginine succinate synthase expression, which allows T lymphocytes to generate endogenous arginine from citrulline even in the absence of arginine or in the presence of a higher expression of arginase-1 [95]. A deficit of arginine causes a decrease in NK cell function and T cell proliferation, while high levels of this amino acid induce a shift in cellular metabolism from glycolysis to oxidative phosphorylation, promoting the proliferation of memory cells. L-arginine can modulate the LPS-induced inflammatory response by inhibiting the downregulation of tight junction proteins and suppressing LPS-induced IL-6, IL-8, IL-1β, and TNFα levels [96].

Furthermore, by inhibiting the activation of TLR4/NF-κB signalling and mitogen-activated protein kinase (MAPK) pathways, arginine regulates LPS-induced TLR4 expression. Arginine can strongly induce the expression of β-defensin-1 in porcine epithelium by activating mammalian target of rapamycin (mTOR) pathway in mammals, alleviating inflammation [97]. Arginine increased the numbers of IgA-secreting cells, CD8+ and CD4+ T cells, and decreased mast cell number and lymphocyte apoptosis of Peyer's patches in piglets challenged by LPS [98].

1.5.2 Short-chain fatty acids

Short-chain fatty acids, with a chain of less than six carbon atoms, are produced by the anaerobic fermentation of indigestible polysaccharides, such as dietary fiber and resistant starch produced by the microbiota in the large intestine.

The most abundant SCFA are acetate, propionate, and butyrate, but others such as formate, isobutyrate, valerate, and hexanoate are bacterial-produced metabolites of amino acids [99]. Acetate, propionate and butyrate are present in the colon in an approximate molar ratio of 3:1:1, respectively [100].

SCFA are produced by several routes (Figure 8), such as acetate is made from pyruvate via acetyl-CoA; propionate is produced from phosphoenolpyruvate via the acrylate and succinate pathways. Finally, butyrate is made from butyryl phosphate via the phospho-transbutyrylase/butyrate kinase pathways and also from acetate by the enzyme butyryl-CoA:acetyl-CoA transferase [101].

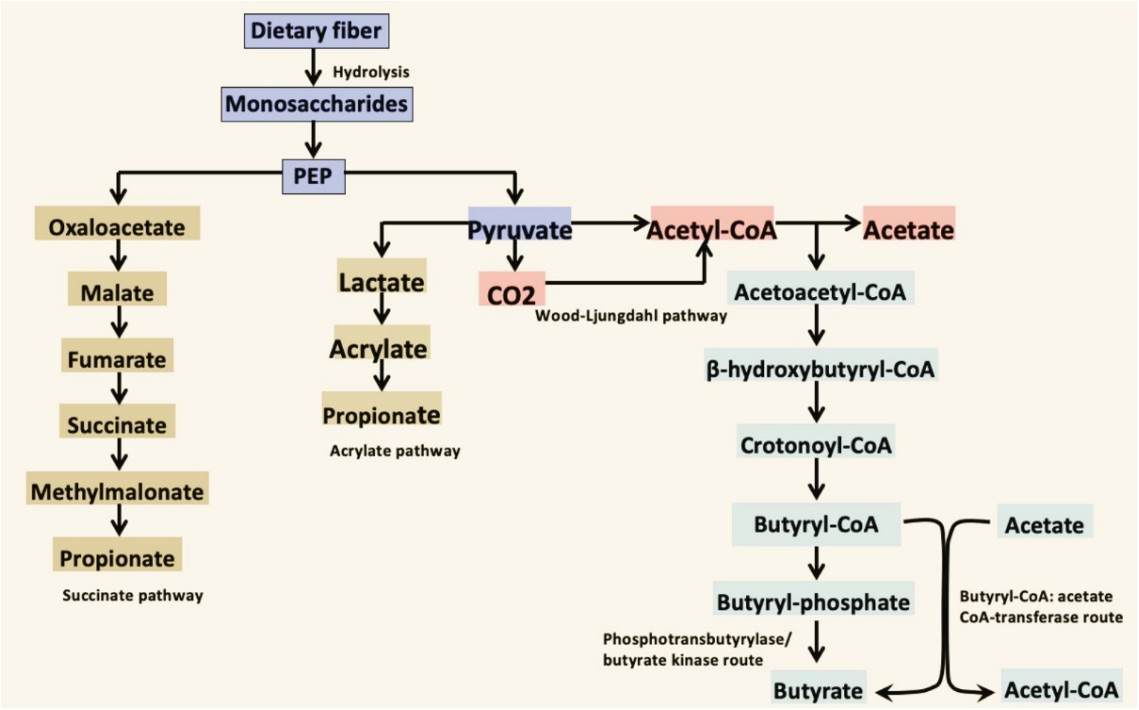


Figure 8 *Schematic representation of the production of acetate, propionate and butyrate. Acetate is produced from pyruvate via acetyl-CoA; propionate comes from PEP via the acrylate route and the succinate route; butyrate is produced from butyryl phosphate via the phosphor-transbutyrylase/butyrate kinase pathways and is also produced from acetate by the enzyme butyryl-CoA:acetyl-CoA transferase. From Liu P. et al., 2021* [101]

In the zootechnical field, to improve the quality of the product derived from pigs, ruminants and chickens, more fibers have begun to be introduced into the diet of the animals, which are an additional energy source [102]. The consumption of dietary fiber leads to the accumulation of fat mass, promotes insulin sensitivity, and regulate the feeling of satiety. From the degradation of fibers, SCFA are obtained, which regulate the balance between fatty acid synthesis, fatty acid oxidation and lipolysis in the body. Among other characteristics, there is the ability to positively influence intestinal conditions such as diarrhea caused by antibiotics and ulcerative colitis [103].

At the intestinal level, the main biological effects of SCFA (Figure 9) include maintaining mucosal immune cell activity and integrity of the epithelium to protect the intestinal epithelial barrier, reducing the pH of the colon to inhibit the growth of bacteria, and regulating energy metabolism and inflammation [99].

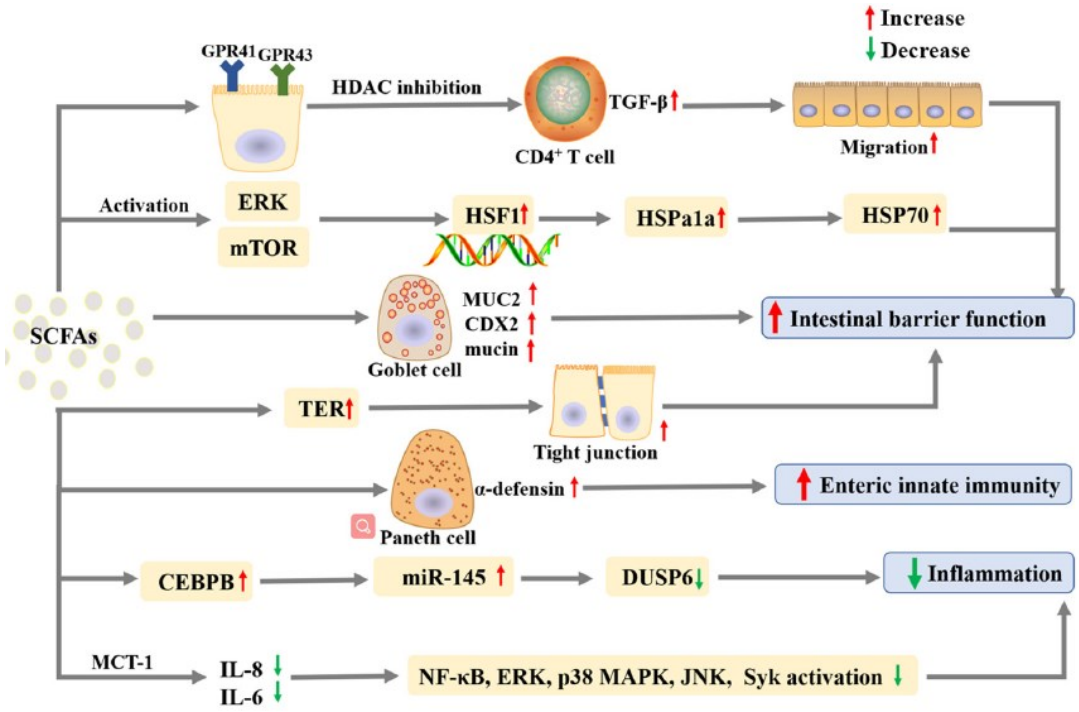


Figure 9 *The main effects and mechanisms of SCFA on inflammatory bowel diseases. SCFA promote 1) Intestinal barrier functions through migration of intestinal epithelial cells and promoting CD4+ T cell by GPR41 GPR43 and histone deacetylases (HDAC) inhibition. They influence the metabolism by phosphorylating the heat shock factor (HSP). Furthermore, they increase the expression of MUC2 and CDX2, and mucin production. And finally, they increase transepithelial electrical resistance (TER). 2) SCFA regulate the innate enteric immune response through the secretion of α-defensin by Paneth cells with microbicidal activity. 3) SCFA stimulate the downregulation of inflammation through the inhibition of dual specificity phosphatase 6 (DUSP6). In the monocarboxylate transporter (MCT) -1, various pathways such as NF-κB, ERK, p38-MAPK are inhibited. From Xiong R et al., 2022* [104]

SCFA cross cells either by passive diffusion, carrier-mediated transport (SMCT1/Slc5a8 and MCT1/Sl-c16a1) or by activating G protein-coupled receptors (GPRs). GPR41 is activated by propionate and butyrate, while GPR43 is activated by acetate and propionate. GPR41 and GPR43 are differentially expressed in intestinal cells, adipocytes and phagocytes. GPRs differ in their downstream signalling cascades: while GPR41 decreases intracellular cAMP levels, GPR43 promotes Ca2+ mobilization. [105]. The immunoregulatory action of SCFA is activated by binding to GPR on the cell surface and entering cells to regulate cellular metabolism. Binding promotes the production of IL-22 in CD4+ T cells, which regulates the expression of hypoxia-inducible factor, leading to histone modification [106].

SCFA are involved in several antibacterial processes with consequent cell lysis including disruption of the electron transport chain at the membrane level of the pathogen, suppression of enzyme activity, disorder of nutrient absorption, peroxidation and autoxidation [107].

SCFA act as epigenetic regulators of the local and systemic inflammatory response. They inhibit potent HDAC that increases histone and non-histone proteins acetylation from chromatin, modulating transcriptional factors (e,g NF-κB, MyoD) and gene expression [108]. Studies have shown that SCFA exert anti-inflammatory effects by inhibiting NF-κB activity and improving the production of anti-inflammatory mediators, such as IL-10 and TGF-β [104,109]. Another mechanism of the anti-inflammatory action is through the regulation of Th1/Th2 cytokines, by promoting an anti-inflammatory response (DC with tolerogenic profile, production of Foxp3+ cells (TREG), IL10 release, NF-κB suppression). Furthermore, butyrate can inhibit the production of inflammatory cytokines, such as TNFα, MCP-1 and IL-6, via the activation of GPR41 in macrophages [108].

In addition, SCFA play a trophic role on the intestinal mucosa, stimulate the proliferation of normal colonic cells and maintain the integrity of TJp. SCFA oppose bacterial and LPS translocations, reducing epithelial permeability via TJp [110], and stimulating the expression of OCLN and CLDN1 in the jejunum, duodenum and

ileum. After SCFA high-concentration infusion into the distal ileum, there is an increase in OCLN in the piglet jejunum. It suggests that SCFA can reduce weaning-induced damage to intestinal structural integrity, promoting TJp expression [111], so the effects of SCFA may be concentration-dependent.

1.6 IPEC-J2

IPEC-J2 is a cell line of enterocytes isolated from the pre-colostral piglets. This permanent, untransformed cell line was isolated in 1989 by Berschneider [112]. IPEC-J2 cells show fast growth and replication capabilities while retaining strong morphological and functional similarities with intestinal epithelial cells *in vivo* (Figure 10) [113].

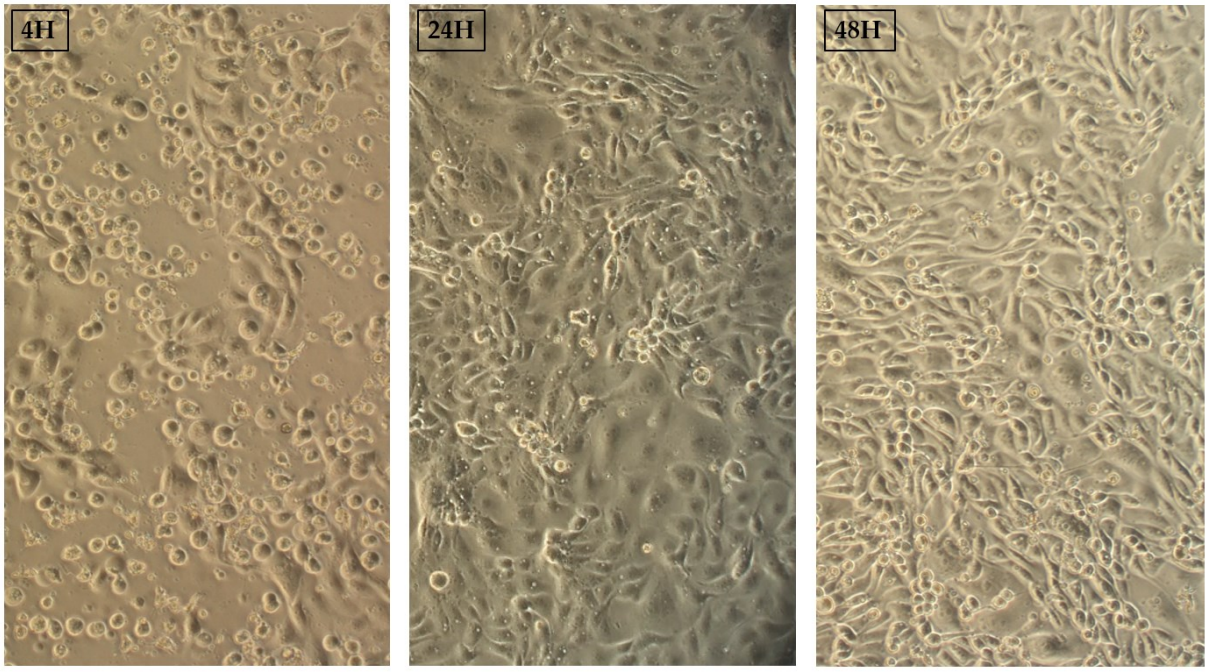


Figure 10 *Light microscopy of IPEC-J2 culture growth from 4, 24 to 48h in a plate 24-well (10× original magnification).*

The cells develop microvilli of different lengths and diameters on the apical side, whereas, on the basolateral side, cytoplasmic projections of variable length cross the intercellular space, anchoring adjacent cells to each other through occluding junctions [114]. Hence, the IPEC-J2 cell line consists exclusively of epithelial cells [79]. In fact, in this cell line goblet cells are absent, with consequent difficulty in determining the expression of mucins, which leads to low mucus production.

The cellular model has been largely characterized, defining which genes are expressed in standard conditions. First, IPEC-J2 express various TJp types, such as claudins,

including claudin-1, -2. -3, -4, -5, -7, -8 and sporadically claudin-12 and claudin-15. Claudins type 3 and 4 are among the most expressed within the tight junction, as are the proteins zonula occludens and [115].

IPEC-J2 cells can also express toll-like receptors, several cytokines, chemokines, growth factors, defensins and antimicrobial peptides that play critical roles in the innate immune response (Table 1) [115,116].

Cytokines	IPEC-J2 expression
IL-1α /IL-6/IL-7/IL-8/IL -18	+
IL-1β/IL-2/IL-4 /IL-10	-
IFN-γ	-
TNFα	+
TNFβ	-
CLDN3 - CLDN4	+
MYD88	+
NF-κB	+
OCLN	+
BD-1	+
TLR4	+
TLR5	-

Table 1 *Gene expression in IPEC-J2 cultivated in standard culture condition are summarized in this table. In second column, the presence and absence of gene expression for cell line are respectively represented by ''-'' and ''+''. Data are from Schierack et al., 2006* [79] *and Mariani V. et al., 2009* [115].

For food science studies, in addition to IPEC-J2, two porcine cell lines are also widely used IPEC-1 and IPI-2I. The latter was transformed with an SV40 plasmid, isolated from the ileum of the adult boar. In comparison, IPEC-1 is a non-transformed cell line derived from a mixture of ileal and jejunal tissue of the pre-colostral piglet [117]. Since the 1960s, the first isolated intestinal epithelial cells were human, such as Caco-2 (isolated in 1977), HT-29, HUTU-80, these cells are isolated from the colon, and most are carcinogenic. These cell lines are often used to study epithelial cell differentiation,

the location of enzymes within the brush border, and the expression of nutrient transporters and adhesion molecules [118]. However, cell lines derived from cancer, despite the epithelial-like structure and function, still retain properties derived from the original cancer source. For this reason, for the detailed study of epithelial functions to compare with physiological situations *in vivo*, it is ideal to choose other models [119]. IPEC-J2 represent a better model of normal intestinal epithelial cells than porcine transformed cell lines.

IPEC-J2 is an excellent model for studying infectious processes and interactions with pathogenic and commensal bacteria in the intestine and shows numerous advantages when compared to transformed intestinal epithelial cell lines:

- It maintains its characteristics and shows strong structural and functional similarities to primary intestinal epithelial cells [120];
- Provides more reliable information as transformed small intestinal cell lines are generally more resistant to stress or cytotoxic insults and may therefore provide ambiguous information that could underestimate cellular damage in response to insults [118]

They are excellent tools for characterizing epithelial interactions with enteric pathogens and viruses, as they are directly comparable to animal models used for *in vivo* experiments [118]. However, it is necessary to consider that cells and tissues *in vivo* respond differently to environmental stimuli, such as changes in the diet, which can vary their gene expression.

2. Aim of work

The characteristics of IPEC-J2 make this line a suitable model to evaluate and study the direct impact of different stimuli.

Our study was divided into three parts:

1) Co-culture model and arginine deprivation
2) Short-chain fatty acid treatment on IPEC-J2
3) Co-culture model with LPS-challenge and SCFA treatment.

In the first study, the main objective was to develop and characterize a co-culture model composed of IPEC-J2 and PBMC to obtain a model that would allow us to elaborate on the mechanisms involved in the communication between epithelial cells and cells of the immune system.

PBMC are derived from the peripheral blood of 9-10 months old, immunocompetent piglets. PBMC are all blood cells with a round nucleus (lymphocytes, monocytes, natural killer cells or dendritic cells). The advantage of PBMC is that they are an easily accessible source of immune cells, as they are isolated from whole blood or buffy coats. The disadvantages and limitations of this model system are the phenotypic differences between peripheral mononuclear cells and intestinal mucosal immune cells (B cells, natural killer T cells and innate lymphoid cells).

At the same time, in study 1 we evaluated the efficacy of arginine in modulating the expression of some cytokines related to the regulation of intestinal cell functions.

In the second study, we evaluated the effects of supplementation of SCFA (acetate, propionate, butyrate and lactate) in different concentrations on IPEC-J2. The decision to treat with SCFA depended on the fact that in recent years in the zootechnical field, these molecules have been considered interesting for various reasons: they stimulate cell proliferation, have antimicrobial capacity, modulate immune responses, influence body weight gain and feed conversion rate in diet-fed pigs. Investigating how SCFA

modulated the expression of certain cytokines and TJs related to the regulation of intestinal cell functions.

In the third study, we subjected the co-culture model to LPS -challenge. We evaluated whether SCFA could interfere in the cell response to an inflammatory stimulus caused by LPS-challenge. As this is a component of the cell walls of Gram-negative bacteria, it causes the activation of the acute inflammatory response in monocytes/macrophages. The response leads to the release of inflammatory cytokines, chemokines and an alteration of junction proteins. We evaluated the regulatory effects on the "intestinal barrier" of acetate and propionate. These two specific SCFA were chosen, as they proved to be the best in maintaining barrier integrity in the previous study.

3. Material and methods

The following techniques (Chapter 3.1) were used in all three studies. Specific treatments and gene expression evaluation, as well as statistical analysis, were added separately for each study (see Chapters 3.2 and 3.3).

3.1 Common methods

3.1.1 Cell culture

Initially, IPEC-J2 once thawed from -80°, are washed with Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/Ham's F-12) (Merck; Darmstadt, Germany) + 5% fetal bovine serum (FBS) (ThermoFisher; Carlsbad, CA, USA), supplemented with 5% penicillin/streptomycin/amphotericin B, glutamine (2mM) (Merck; Darmstadt, Germany). IPEC-J2 are centrifuged at 800g for 8min in order to eliminate the freezing medium (10% DMSO/dimethyl sulfoxide and 50% FBS). Subsequently, cells are cultured in a flask with fresh medium and incubated at 37 °C, 5% CO2. The number of viable cells (never less than 95%) is evaluated through the Burker chamber with the use of Trypan blue (0.1%) (Merck; Darmstadt, Germany).

PBMC were isolated from the blood of 9-10 months old adult pigs collected from a slaughterhouse certified by the Italian Ministry of Health according to Regulation (EC) 853/2004 (Sassi S.P.A., Parma, Italy; EC approval nr. -IT- 190M). PBMC were isolated from 4/5 mL of blood by density gradient centrifugation with Histopaque-1077® solution (Merck; Darmstadt, Germany). PBMC present in buffy coat, are subsequently collected and transferred to another tube, two washings are carried out with sterile PBS + 1% FBS and frozen at -80°C by inserting them in a Mr. Frosty® device (Sigma, St. Louis, MO, USA). Within 24 h they must be transferred to liquid nitrogen until use.

3.1.2 Cell viability

The viability was evaluated, using a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) colorimetric test. The assay measures the activity of dehydrogenase enzymes which, in the mitochondrion, reduce MTT (Merck; Darmstadt, Germany) to formazan, giving the substance a dark violet/blue colour. The mitochondrial succinate dehydrogenase enzyme is active only in living cells. Its function consists of cutting the tetrazolium ring of the MTT (yellow substance) with the formation, of formazan (a blue salt). Initially, IPEC-J2 were seeded at a density of 10^5 cells/well and PBMC at 6 x 10^6 cells/well in 24-well plates or transwells (monoculture or co-culture) for the time and with the specific treatment required by the experiment. MTT assays were performed by incubating IPEC-J2 or PBMC cells with 20 µL (5 mg/mL in sterile PBS) of MTT solution. After 4 h of incubation, medium and MTT solution were removed from the wells, and then IPEC-J2 cells and PBMC were lysed with 150 µL of DMSO (Merck; Darmstadt, Germany). After the experiment time, 200 µL of their media were transferred to a 96-well plate. After 15 minutes in the incubator at 37°, the violet solubilized formazan crystals were detected at 490 nm using a VICTOR® Nivo™ Multimode Microplate Reader (PerkinElmer, Waltham, MA, USA).

3.1.3 Griess test

Nitric oxide (NO) production was measured in the cell' culture medium by Griess reaction after the time required by the experiment of culture. The aim is to measure the nitrite level (NO^{2-}), a stable metabolic product of NO. Initially, a Nitrite Standard reference curve (50, 25, 12.5, 6.25, 3.13 and 1.56µM) is prepared in tubes with 10mM sodium nitrite diluted in distilled H_2O. The standard (100 µL) from tubes and the culture medium (the treatments at different times) were transferred to the plate, add to them 100 µL of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylenediamine-HCl] (Merck; Darmstadt, Germany) and incubate at room temperature (RT) for 15 min. The absorbance is at 540

nm is measured using a VICTOR® Nivo™ Multimode Microplate Reader. A standard curve is set up in the culture medium using serial dilutions of sodium nitrite (50 - 0.39 µM; linear regression: y = 0.0223x + 0.102; r = 0.99). The inter-assay variability was less than 5%.

3.1.4 RNA extraction

Total RNA is isolated from about 10^5 IPEC-J2 and $6x10^6$ PBMC. We recuperated the cells by scraping the surface of the plate and/or transwell with a scraper, after adding 1 mL of TRI-reagent (ThermoFisher; Carlsbad, CA, USA) for 5min. Subsequently, we added 10% of the initial volume of 1-Bromo-3-chloropropane (100 µl) (Merck; Darmstadt, Germany), vigorously stirred for about 15 seconds and incubated for 8 minutes at RT. After centrifuging at 12000 rpm for 12 minutes at 4°C, the colourless aqueous phase is transferred to a new tube (DNA and proteins remain in the interphase and the red-coloured lower organic phase). To the initial volume we added isopropanol (500 µl) (Merck; Darmstadt, Germany), vortexed and incubated 8 minutes at RT. We centrifuged and removed the supernatant taking care not to aspirate the pellet. The tubes are moved on ice to prevent RNA degradation. The pellet is washed with 75% ethanol (1 ml) (Merck; Darmstadt, Germany) and vortexed. Following centrifugation at 8000 rpm for 5 minutes at 4°C, the supernatant is removed and the pellet allowed to dry for 3 minutes. Finally, The RNA is resuspended in an appropriate amount of diethylpyrocarbonate (DEPC)(20-30µl). RNA purity and concentration (ng/µl) are assessed using a BioSpectrometer® (Eppendorf AG, Hamburg, Germany) at 260/280 ~ 2,0.

RNA samples were DNAse-treated, and 1µg/20 µL was reverse transcribed using a High-capacity cDNA Reverse Transcription kit (ThermoFisher; Carlsbad, CA, USA). The volume of components required to perform the procedure is calculated by referring to the Reverse transcription Master Mix with RNase Inhibitor components table 2. All tubes contain RNA (1µg) and 10 µl of RT Master Mix.

Reverse transcription Master Mix Components with RNase Inhibitor:	
10X Reverse transcription Buffer	2.0 µl
25X Deoxynucleotide triphosphates (dNTP) Mix (100mM)	0.8 µl
10X Reverse transcription Random Primers	2.0 µl
MultiScribe™ Reverse Transcriptase	1.0 µl
RNase Inhibitor	1.0 µl
Nuclease-free H_2O	3.2 µl
	10.0 µl

Table 2 *Master mix components for reverse transcription.*

Reverse transcription was performed using a StepOne thermocycler (Applied Biosystems, StepOne software v.2.3, Foster City, CA, USA) described in table 3.

	Step 1	**Step 2**	**Step 3**
Temperature	25 °C	37 °C	85 °C
Time	10 min	120 min	5 min

Table 3 *Temperature conditions and specific time for reverse transcription.*

3.1.5 Real-time polymerase chain reaction (PCR)

The cDNA samples were used as a template for real-time quantitative PCR (qPCR) performed using a StepOne thermocycler. The cDNA (20 ng/20 µL) was amplified in duplicate using Fast Power-Up™ SYBR® Green Master Mix (Applied Biosystems; Foster City, CA, USA) and (1.2 µl/well) specific primer sets (forward + reverse) (Eurofins Genomics; Ebersberg, Germany) + DEPC water to reach volume. Concentration was set for ZO-1 at 400 nM and at 300 nM for the other genes. Specifics of each primer set for identification of gene expression are reported in Table 4.

Samples were kept at 95 °C for 20 s (hold step) to allow DNA- polymerase activation and were then subjected to 40 cycles consisting of a denaturation step at 95 °C for 3 s followed by an annealing/extension step at 60 °C for 30 s.

Data were analysed according to the $2^{-\Delta\Delta Ct}$ method [21] in which the expression levels of each cytokine is normalized to the housekeeping gene(glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or hypoxanthine phosphoribosyltransferase-1 (HPRT1)). The cDNA amount, expressed as relative quantities (RQ), is calculated regarding the expression level in experimental conditions. A melting curve analysis for specific amplification control was performed (60–95 °C) at the end of the amplification cycles. No-RT controls and no-template controls (NTC) were included, and the latter were assumed to be negative and reliable if the quantification cycle (Cq) was ≥35.

Gene	Primer sequence	E (%)	S	r^2	A. L. (bp)	Study
CAT-1 [89]	F: 5′-AGACGGGCTGCTGTTTAAGT-3′ R: 5′-ACCGTTAAAATACCGGCGTG-3′	100.6	–3.30	0.99	131	1
IL-8 [121]	F: 5′-CCGTGTCAACATGACTTCCAA-3′ R: 5′-GCCTCACAGAGAGCTGCAGAA-3′	98.9	–3.29	0.99	75	1
IL-6 [122]	F: 5′-GGCAAAAGGGAAAGAATCCAG-3′ R: 5′-CGTTCTGTGACTGCAGCTTATCC-3′	99.6	–3.33	0.99	87	1
TGF-β [123]	F: 5′-AGGGCTACCATGCCAATTTCT-3′ R: 5′-CCGGGTTGTGCTGGTTGTACA-3′	106.3	–3.18	0.98	102	1
TNFα [122]	F: 5′-ACTGCACTTCGAGGTTATCGG-3′ R: 5′-GGCGACGGGCTTATCTGA-3′	98.5	–3.36	0.99	118	1 and 2
IL-1α [115]	F: 5′-GCTCAAAACGAAGACGAACC-3′ R: 5′-TGATGGTTTTGGGTGTCTCA-3′	99.4	–3.34	0.97	61	1
GAPDH [124]	F: 5′-GGTGAAGGTCGGAGTGAACG-3′ R: 5′-GCCAGAGTTAAAAGCAGCCCT-3′	102.0	–3.27	0.99	70	1
BD-1 [115]	F 5′-CAGCCACCAGCATGAGACT-3′ R 5′-CAGGTAACAGGACCATGAGGA-3′	101.5	–3.28	0.99	63	2
CLDN-4 [115]	F 5′-TATCATCCTGGCCGTGCTA-3′ R 5′-CATCATCCACGCAGTTGGT-3′	100.2	–3.29	0.99	71	2 and 3
OCLN [125]	F5′-GGAGTGATTCGGATTCTGTCTATGCT-3′ R 5′-CGCCTGGGCTGTTGGGTTGA-3′	103.8	–3.13	0.98	423	2 and 3
ZO-1 [125]	F 5′-GGCGCACGGCGAAGGTAATT-3′ R 5′-CTATCAAACTCAGGAGGCGGCACT-3′	103.7	–3.21	0.98	405	2 and 3
Nf-κB [115]	F 5′-GAAGGACCTCTAGAAGGCAAAA-3′ R 5′-GCTTTGGTTTATGCGGTGTT-3′	104.2	-3.22	0.99	63	2
***HPRT-1** [115]	F 5′-ACACTGGCAAAACAATGCAA-3′ R 5′-TGCAACCTTGACCATCTTTG-3′	102.0	-3.25	0.99	71	2 and 3

E= Efficiency S=Slope A.L. Amplicon Length

Table 4 *Target genes and details of the primer sequences used for SYBR Green qPCR. The GAPDH are HPRT-1 genes were used as endogenous reference genes. *The reference HPRT-1 gene was selected among other tested reference genes (i.e., GAPDH, β-2-microglobulin (β-2MG)*[126]*, and 18S rRNA*[127]*) as endogenous control according to minimal intra-/inter- assay variation and based on previous results.*

To evaluate the performance of a primer set, a serial dilution of the target were analyzed (10-fold dilution, for example, over 5 to 7 log). The sample can be any cDNA preparation in which the gene of interest is known to be present. R^2 is the coefficient of correlation obtained for the standard curve and should be >0.99.

Slope/Efficiency: a good reaction should have an efficiency between 90% and 110%, which corresponds to a slope between -3.58 and -3.10. The efficiency is calculated from the slope of the standard curve according to the following formulas E = 10(-1/slope)-1.

3.2 Study 1 - Co-culture model and arginine

3.2.1 Cell culture conditions

PBMC and IPEC-J2 were used for the co-culture assessment (Figure 11). One day before starting the experiments, PBMC were thawed and washed with DMEM/Ham F-12 to remove the freezing medium that could interfere with proper cell growth. Viability was determined by trypan blue exclusion (95%). PBMC were seeded in 24-well tissue culture plates at a density of $6x10^6$ cells/well. PHA/phytohemagglutinin (5 μg/mL final) was added to the wells in 1 mL of DMEM/Ham's F-12, in order to transform lymphocytes into lymphoblasts, stimulating cell division. At the end of PHA incubation (24h), the plates were centrifuged, the medium was discarded, and 1 mL of DMEM/Ham's F-12 or arginine-free DMEM/Ham's F-12 was added. After 24 h, IPEC-J2 were trypsinized and seeded on the upper surface of collagenized cell culture inserts (0.3 cm^2 polyethylene terephthalate membrane with 0.4 μm pore size; Costar, Corning Inc., Corning, NY, United States Spliced) at a density of 10^5 cells/cell culture insert. The inserts were inserted into 24-well plates where PBMC were seeded, and 0.5 mL of fresh medium with or without arginine was added to the respective wells.

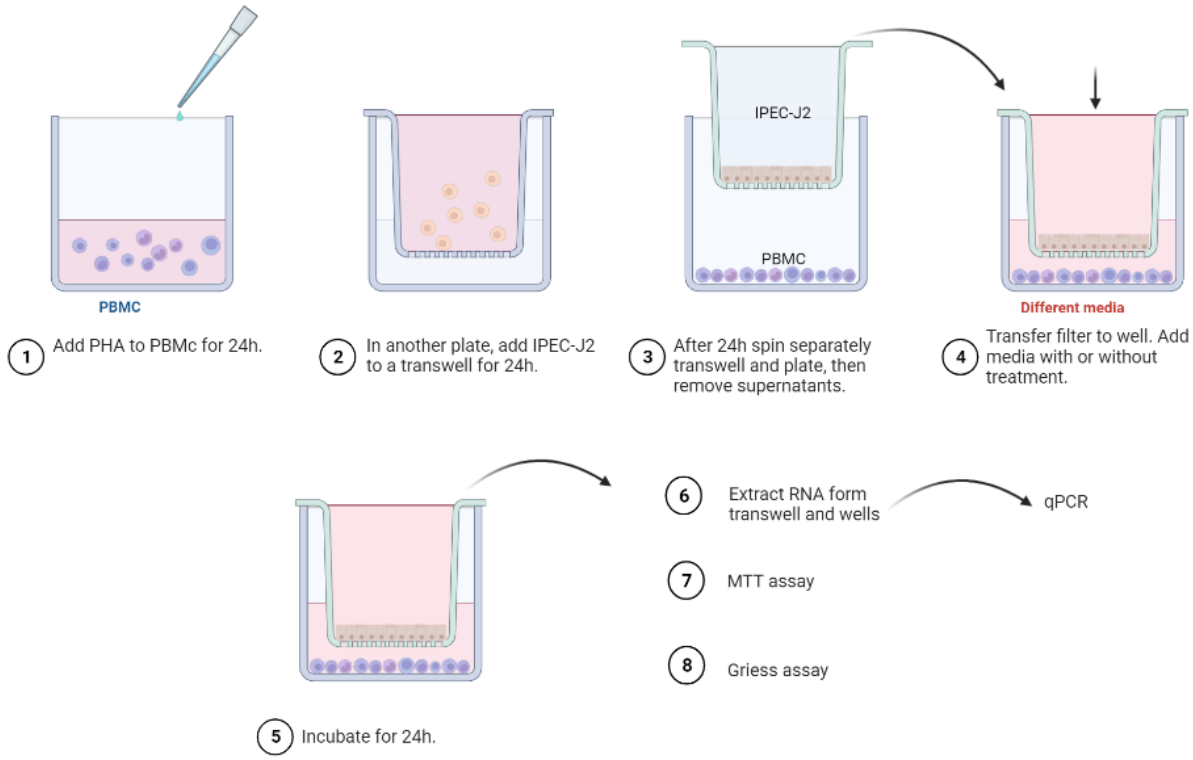


Figure 11 *Schematic representation of IPEC-J2+ PBMC co-culture system preparation.*

The experimental groups, incubated for 24 or 48 h, were the following:

1) IPEC-J2 or PBMC monoculture in DMEM/Ham's F-12 medium, with arginine: IPEC or PBMC group;

2) IPEC-J2 or PBMC monoculture in DMEM/Ham's F-12 without arginine: IPEC/–Arg or PBMC/–Arg group;

3) IPEC-J2 co-culture with PBMC in DMEM/Ham's F-12, with arginine: IPEC+PBMC group;

4) IPEC-J2 co-culture with PBMC in DMEM/Ham's F-12 without arginine IPEC+PBMC/–Arg group.

Viability in IPEC-J2 and PBMC was evaluated for monoculture vs co-culture under experimental conditions (presence/absence of arginine) at the end of both incubation times (24 and 48 h) by trypan blue exclusion.

There were no significant differences in IPEC-J2 or PBMC viability compared to the same culture and experimental condition at any time.

Viability and NO synthesis were determined for 24, 48 and 72h by MTT assay and Griess test, respectively. Gene primers chosen for the study are showed in (Table 4)

3.2.2 Statistical analysis

Each experiment was repeated six times, and each culture condition was performed in eight replicate wells. Data were analyzed by ANOVA (IBM® SPSS® Statistics v.28, NY, USA) using a model with groups and interaction between groups as fixed factors. The least significant difference (LSD) post-hoc test was used to compare means when significant differences ($P < 0.05$) were found. Experimental data are presented as the mean ± standard deviation. Differences among groups were considered significant at $P < 0.05$.

3.3 Study 2 - Short-chain fatty acids treatment

3.3.1 Cell culture conditions

The experimental groups were the following:

(1) IPEC-J2 in DMEM/Ham's F-12 medium (group: CONTROL);

(2) IPEC-J2 in DMEM/Ham's F-12 with 0.5, 1, 2.5, or 5 mM of sodium acetate (Merck; Darmstadt, Germany) (groups: ACE 0.5, 1, 2.5, 5);

(3) IPEC-J2 in DMEM/Ham's F-12 with 1, 2.5, 5, or 10 mM of sodium propionate (Merck; Darmstadt, Germany) (groups: PROP 1, 2.5, 5, 10);

(4) IPEC-J2 in DMEM/Ham's F-12 with 7.5, 15, 30, or 60 mM of sodium lactate (Merck; Darmstadt, Germany) (groups: LAC 7.5, 15, 30, 60);

(5) IPEC-J2 in DMEM/Ham's F-12 with 0.5, 1, 2.5, or 5 mM of sodium butyrate (Stemcell Technologies; Vancouver, Canada) (groups: BUT 0.5, 1, 2.5, 5).

The ranges of SCFA concentrations were chosen based on data reported in the literature. It was chosen to narrow acetate, butyrate, and propionate ranges as no benefits were observed at high concentrations [128,129]. For lactate, it was chosen to narrow the range and define an optimal concentration [130].

Viability and NO synthesis were determined for 24h by MTT assay and Griess test, respectively. The genes chosen in this specific study for evaluating gene expression are in Table 4.

3.3.2 Statistical analysis

Each experiment was repeated seven times, and each culture condition was performed in eight replicate wells. Data were analysed by ANOVA (IBM® SPSS ® Statistics v.28, NY, USA) using a model with the group and interaction between groups as fixed factors. The least significant difference (LSD) post-hoc test was used to compare means when significant differences ($P < 0.05$) were found. Furthermore, linear regression ($y = ax + b$) and quadratic regression ($y = ax^2 + bx + c$) were fitted by determining the linear and quadratic effects of SCFA concentrations on IPEC-J2 viability and NO release and considered significant if $P < 0.05$. Pearson's correlation analysis was carried out for all markers. Experimental data were presented as means ± standard deviation. Differences among groups were considered significant if $P < 0.05$.

3.4 Study 3 Co-culture with LPS-challenge and SCFA treatment

3.4.1 LPS stimulation in monoculture or co-culture cell conditions

The study was divided into two parts: 1) monocultures of IPEC-J2 or PBMC, grown and seeded as in the previous studies; 2) the co-culture system (set up as in the study N.1). To trigger an inflammatory response in the whole co-culture system, after having centrifuged the previously prepared plates, the medium was changed by adding LPS (1 μg/ml), and cells were incubated at 37 °C, 5% CO_2, for 24 h. At the same time, SCFA supplementations were added (5 mM acetate or 1 mM propionate), or cells were incubated without any supplementation, for 24 h. The experimental groups for IPEC-J2 or PBMC in monoculture were the IPEC-J2 or PBMC in DMEM/Ham's F-12 medium (groups: IPEC-J2 or PBMC) or with LPS (groups: IPEC-J2 + LPS or PBMC + LPS).

The experimental conditions for IPEC-J2 or PBMC in co-culture were the following:

1) IPEC-J2 co-culture with PBMC in DMEM/Ham's F-12 (control) or with LPS;
2) IPEC-J2 co-culture with PBMC in DMEM/Ham's F-12 with acetate or propionate;
3) IPEC-J2 co-culture with PBMC in DMEM/Ham's F-12 with LPS and acetate or propionate.

The ranges for SCFA concentrations were chosen based on data reported in our previous study [131].

Viability and NO synthesis were determined for 24h, by MTT assay and Griess test, respectively. Gene primers chosen for this study are shown in (Table 4)

3.4.2.Western blotting analysis

IPEC-J2 proteins were extracted by lysis of cells in cold buffer (Tris-HCl 10mM, pH 8; NaCl 10mM; MgCl2 3mM; SDS 0,1%; TRITON 0,1%; EDTA 0,5mM) containing protease inhibitor mix (APMSF 10μg/ml; Leupeptin 0.5 μg/ml; Pepstatin 0.7 μg/ml), and proteins concentration was measured using the Pierce BCA Protein assay Kit (Thermo Fisher Scientific, Rockford, Illinois, USA). Equal quantity of proteins (20μg) was heated at 100°C for 5 min and then added 5X Laemli buffer. A PageRuler prestained protein ladder (Thermo Fisher Scientific, Rockford, Illinois, USA) was used as the molecular weight standard. After electrophoresis, the proteins were transferred to a PVDF membrane (Hybond–P; Amersham, Buckinghamshire, UK), and the membrane was blocked in PBS+0,05% Tween (BST) with 5% skimmed milk for 1h at RT. Membrane were incubated with the same buffer containing a specific primary antibody [Claudin-4 1:500 (#32-9400; Invitrogen, Carlsbad, CA, USA); Occludin 1:2000 (#33-1500; Invitrogen, Carlsbad, CA, USA); β-Actin 1:2000 (#4970S; Euroclone)] overnight at 4°C. After repeated washing with PBST buffer, HRP-conjugated secondary antibody [HRP-conjugated goat anti-rabbit IgG 1:50000 (#31460 Thermo Fisher); HRP-conjugated goat anti-mouse IgG 1:50000 (#31430 Thermo Fisher)] was applied for 1h at RT. Visualization was performed using a chemiluminescent enzyme substrate (SuperSignal West Pico Plus #34580; Thermo Fisher Scientific, Rockford, Illinois, USA). The signal intensities of specific bands were detected using the ChemiDoc™ MP Imaging System (Bio-Rad) and quantified using Image J software (U.S. National Institutes of Health, Bethesda, MA). β-Actin was used as a control for equal loading, and the results are expressed as the optical density of the primary antibody/ β-actin.

3.4.3 Statistical analysis

Each experiment was repeated six times, and each culture condition was performed in eight replicate wells. Data were analysed by ANOVA (IBM® SPSS® Statistics v.28, NY, USA) using a model with groups and interaction between groups as fixed factors. The least significant difference (LSD) post-hoc test was used to compare means when significant differences ($P < 0.05$) were found. Experimental data were presented as means ± standard deviation. Differences among groups were considered significant if $P < 0.05$.

4 Results

4.1 Study 1

Cell viability

At 48 and 72h incubation, deprivation of arginine in the culture medium (IPEC/–Arg and PBMC/–Arg group) significantly reduced cell viability in IPEC-J2 cells compared to controls (IPEC and PBMC group) ($P < 0.05$; Figure 12). IPEC-J2s in complete medium showed a significant reduction in viability over time. On the contrary, in PBMC the viability remained unchanged.

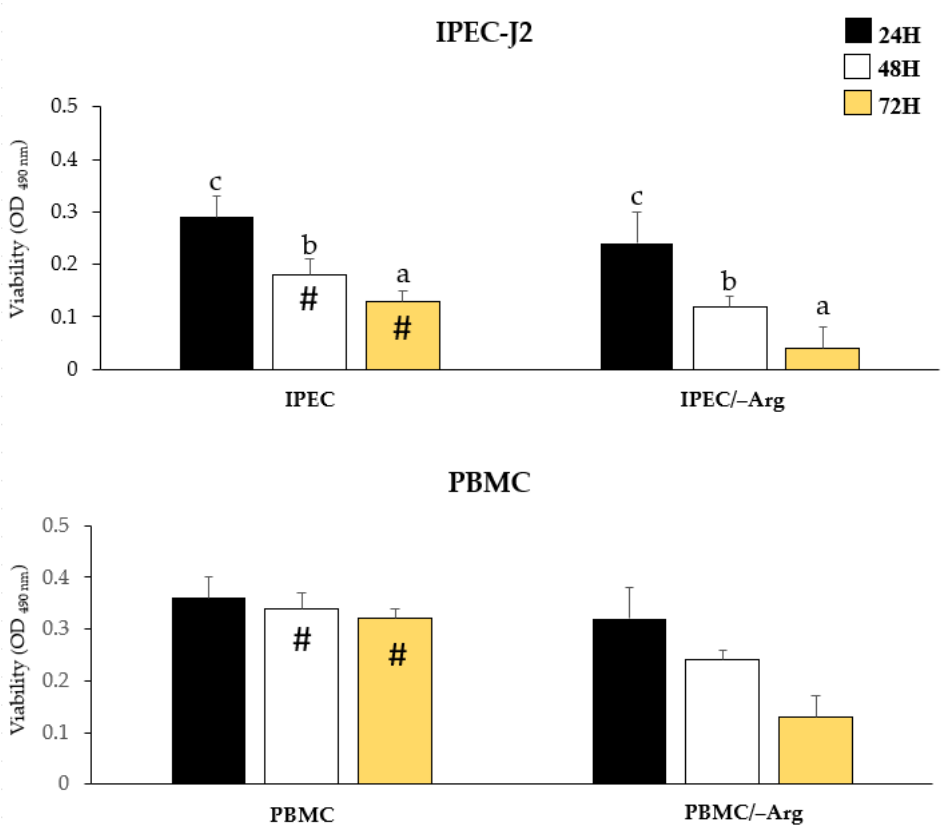


Figure 12 *Cell viability of IPEC-J2 and PBMC determined using an MTT assay at 24, 48 and 72 h of incubation. Significant differences ($P < 0.05$) at 24, 48 or 72 h in the same group are indicated with hashtags. Significant differences ($P < 0.05$) among groups at the same time-point are indicated with letters.*

Nitric oxide accumulation

Arginine deprivation induces a significant accumulation of NO in the culture medium at 24 h ($P < 0.05$; Figure 13). At 48 h of incubation, the amount of NO was significantly reduced in all groups ($P < 0.05$).

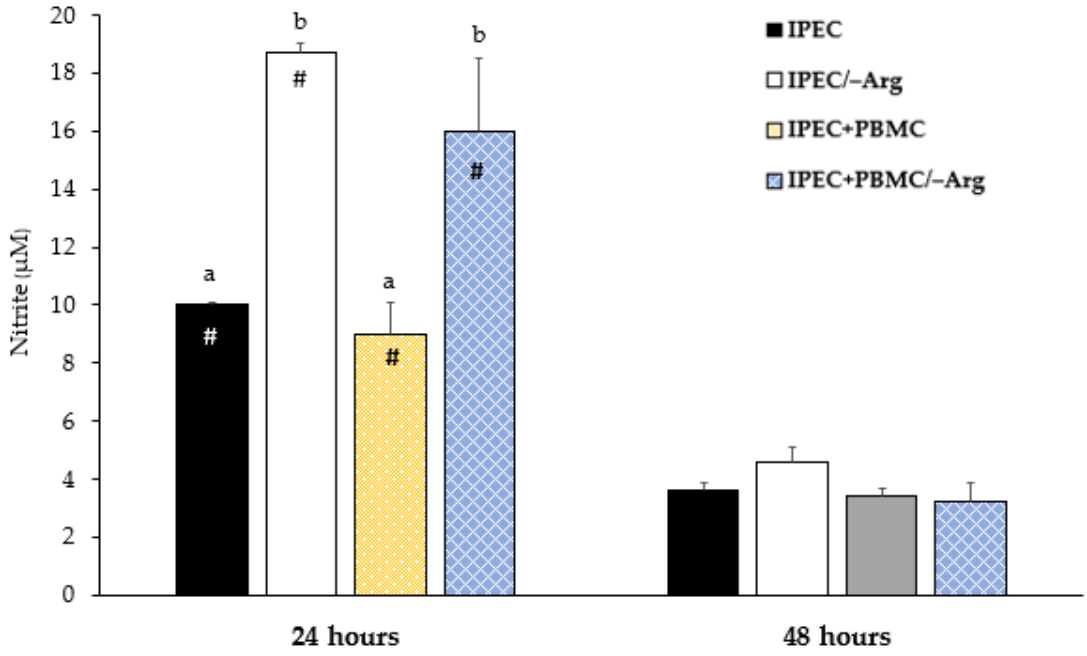


Figure 13 *Effects of the culture condition (absence of arginine in mono and co-culture) on nitrite release at 24 and 48 h of incubation. Each value represents the mean ± SE of 8 wells of 6 independent experiments. Significant differences ($P < 0.05$) among groups are labelled with letters. Significant differences ($P < 0.05$) between 24 and 48 h in the same group are indicated with hashtags.*

Gene expression

Gene expression was evaluated only at 24h and 48h, because after 72h we observed a reduction in cell viability and a reduction in the ability to express cytokines both in IPEC-J2 and in PBMC (data not shown).

Cationic Amino Acid Transporter-1 (*CAT-1*)

In IPEC-J2, CAT-1 expression of IPEC/–Arg and IPEC+PBMC/–Arg groups showed a significant increase ($P < 0.05$)compared to the control group (IPEC group). On the contrary, at 48 h of incubation, no significant differences were observed between the experimental groups, but a significant reduction was observed compared to 24h ($P < 0.05$). CAT-1 expression in PBMC/–Arg at 48h induced a strong reduction of expression compared to the control group (PBMC) ($P < 0.05$). In the 48h co-culture condition, in the IPEC+PBMC group (co-culture in complete medium), CAT-1 expression is strongly increased compared to the PBMC (control) and IPEC+PBMC/–Arg groups. At 48 h, CAT-1 expression in IPEC-J2 decreased in all groups, while it increased markedly in PBMC in all conditions except the PBMC/–Arg group compared with values at 24 h ($P < 0.05$). The data are shown in Figure 14.

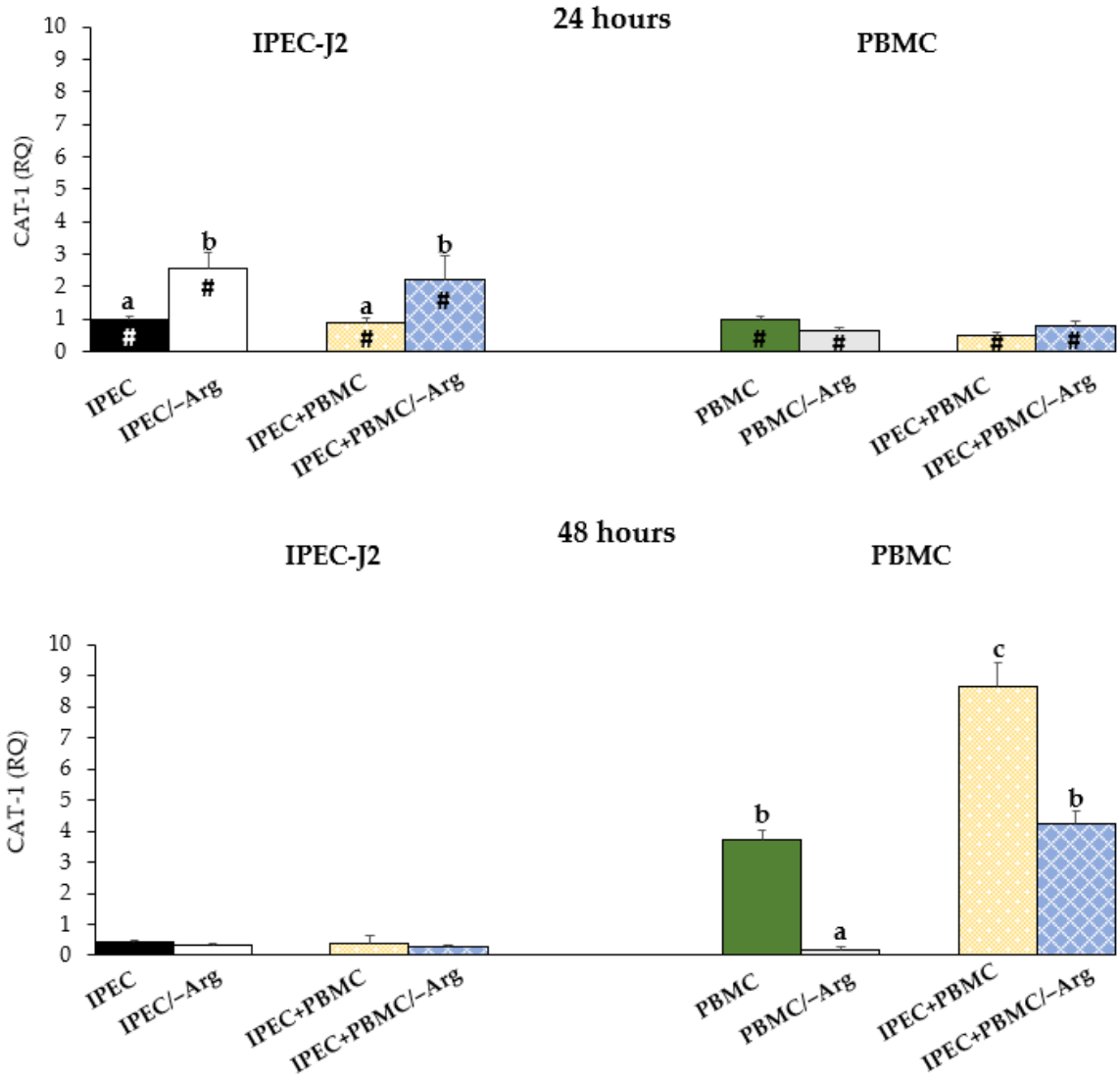


Figure 14 *CAT-1 gene expression in IPEC-J2 cells and in PBMC at 24 and 48 h of incubation. Each value represents the mean ± SE of 8 replicates of 6 independent experiments. Significant differences (P < 0.05) among groups (upon row comparisons within paired groups) are indicated with letters. Significant differences (P < 0.05) between 24 and 48 h in the same group (upon column comparisons vs respective control group) are indicated with hashtags. Data were analysed according to the* $2^{-\Delta\Delta Ct}$ *method, in which the expression levels of each transporter, normalized to the GAPDH cDNA amount, were expressed as relative quantities (RQ).*

Tumor Necrosis Factor α (*TNFα*)

At 24 h of incubation in IPEC-J2, in monoculture and coculture, TNFα expression was significantly increased by arginine deprivation (IPEC/–Arg group and IPEC+PBMC/–Arg group) compared to control (IPEC group) ($P < 0.05$), as shown in Figure 4. In all groups, expression decreased at 48 h of incubation. In PBMC at 24h, TNFα expression is reduced by arginine deprivation in PBMC/–Arg and IPEC+PBMC/–Arg groups compared with the control (PBMC group) ($P < 0.05$). In PBMC co-culture, expression at 48 h increased up to 1.7- and 2.4-fold in IPEC+PBMC and IPEC+PBMC/–Arg groups, respectively, compared to controls (PBMC group) ($P < 0.05$). The absence of arginine inhibited TNFα expression in the PBMC/–Arg group even at 48 h ($P < 0.05$). At 48 h, in IPEC-J2 the expression of TNFα is reduced in all groups, while in PBMC the co-culture values increased compared to 24 h ($P < 0.05$). The data are shown in Figure 15.

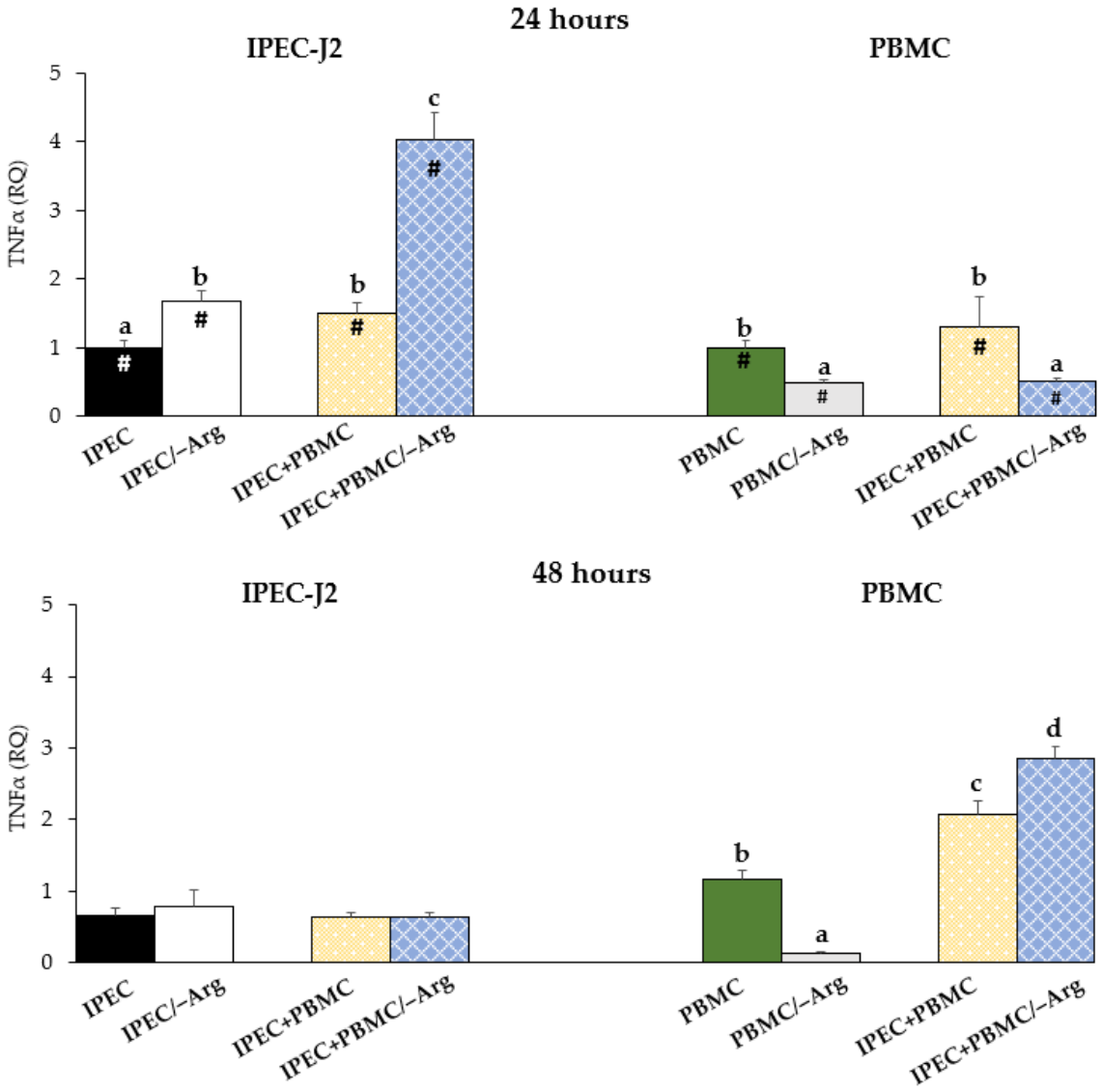


Figure 15 *TNFα gene expression in IPEC-J2 cells and in PBMC at 24 and 48 h of incubation. Each value represents the mean ± SE of 8 replicates of 6 independent experiments. Significant differences (P < 0.05) among groups (upon row comparisons within paired groups) are indicated with letters. Significant differences (P < 0.05) between 24 and 48 h in the same group (upon column comparisons vs respective control group) are indicated with hashtags. Data were analysed according to the* $2^{-\Delta\Delta Ct}$ *method, in which the expression levels of each cytokine, normalized to the GAPDH cDNA amount, were expressed as relative quantities (RQ).*

Interleukin-1α (*IL-1α*)

As shown in Figure 16, IL-1α expression in IPEC-J2 at 24 h does not show significant differences in all groups. In contrast, after 48 h of culture, IL-1α showed a significant increase in the IPEC/−Arg, IPEC+PBMC and IPEC+PBMC/−Arg groups compared to the control (IPEC group) ($P < 0.05$). In PBMC at 24 h, expression was increased in all experimental groups compared with the control (PBMC group) ($P < 0.05$). In particular, the absence of arginine induces the expression of IL-1α (PBMC/−Arg group), compared to IPEC+PBMC and IPEC+PBMC/−Arg groups. This condition at 48 h, however, reduced the expression in PBMC monoculture (PBMC/−Arg) compared to the PBMC group (control), as well as the IPEC+PBMC and IPEC+PBMC/−Arg groups). IL-1α expression at 48 h in IPEC-J2 was stimulated in all groups compared to 24 h ($P < 0.05$), while in PBMC there was a dramatic decline in all conditions ($P < 0.05$).

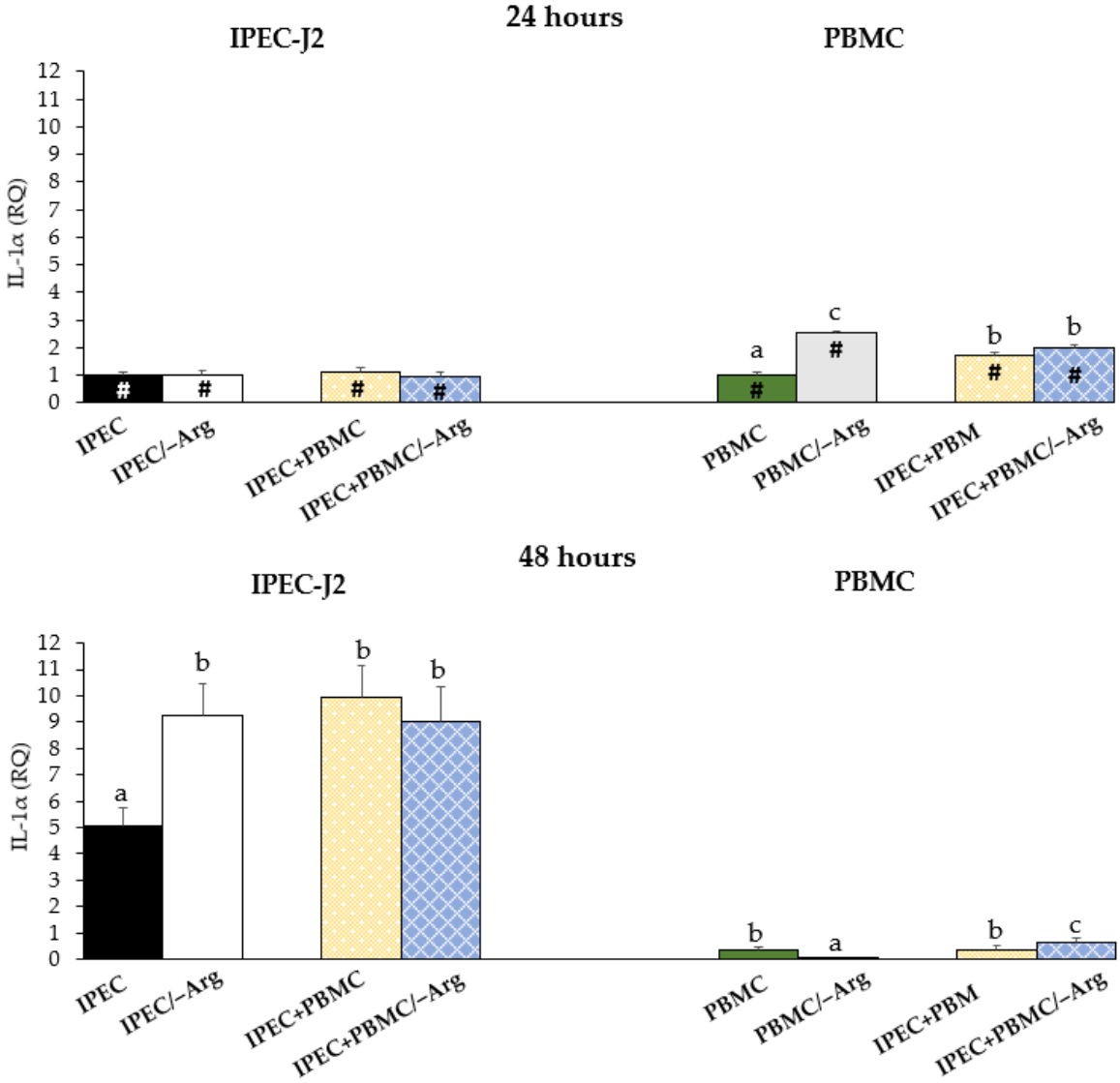


Figure 16 *IL-1α gene expression in IPEC-J2 cells and in PBMC at 24 and 48 h of incubation. Each value represents the mean ± SE of 8 replicates of 6 independent experiments. Significant differences (P < 0.05) among groups (upon row comparisons within paired groups) are indicated with letters. Significant differences (P < 0.05) between 24 and 48 h in the same group (upon column comparisons vs respective control group) are indicated with hashtags. Data were analysed according to the* $2^{-\Delta\Delta C}t$ *method, in which the expression levels of each cytokine, normalized to the GAPDH cDNA amount, were expressed as relative quantities (RQ).*

Interleukin-6 (*IL-6*)

In Figure 17, at 24 h IL-6 expression is significantly increased in the IPEC/–Arg, IPEC+PBMC and IPEC+PBMC/–Arg groups compared to the control (IPEC group) ($P < 0.05$). No significant differences were observed between the three groups. At 48 h, IL-6 decreased in IPEC-J2 in all conditions compared to values at 24 h ($P < 0.05$). At this time point in IPEC-J2, IL-6 is not affected by any of the culture conditions (presence/absence of arginine; mono-/co-culture). At 24h, in PBMC, no changes in expression in the presence or absence of arginine (PBMC/–Arg and IPEC+PBMC/–Arg groups) are observed compared to the control (PBMC). Conversely, at 48 h of incubation, IL-6 expression increased in all groups except for the PBMC/–Arg group, in which expression decreased dramatically ($P < 0.05$).

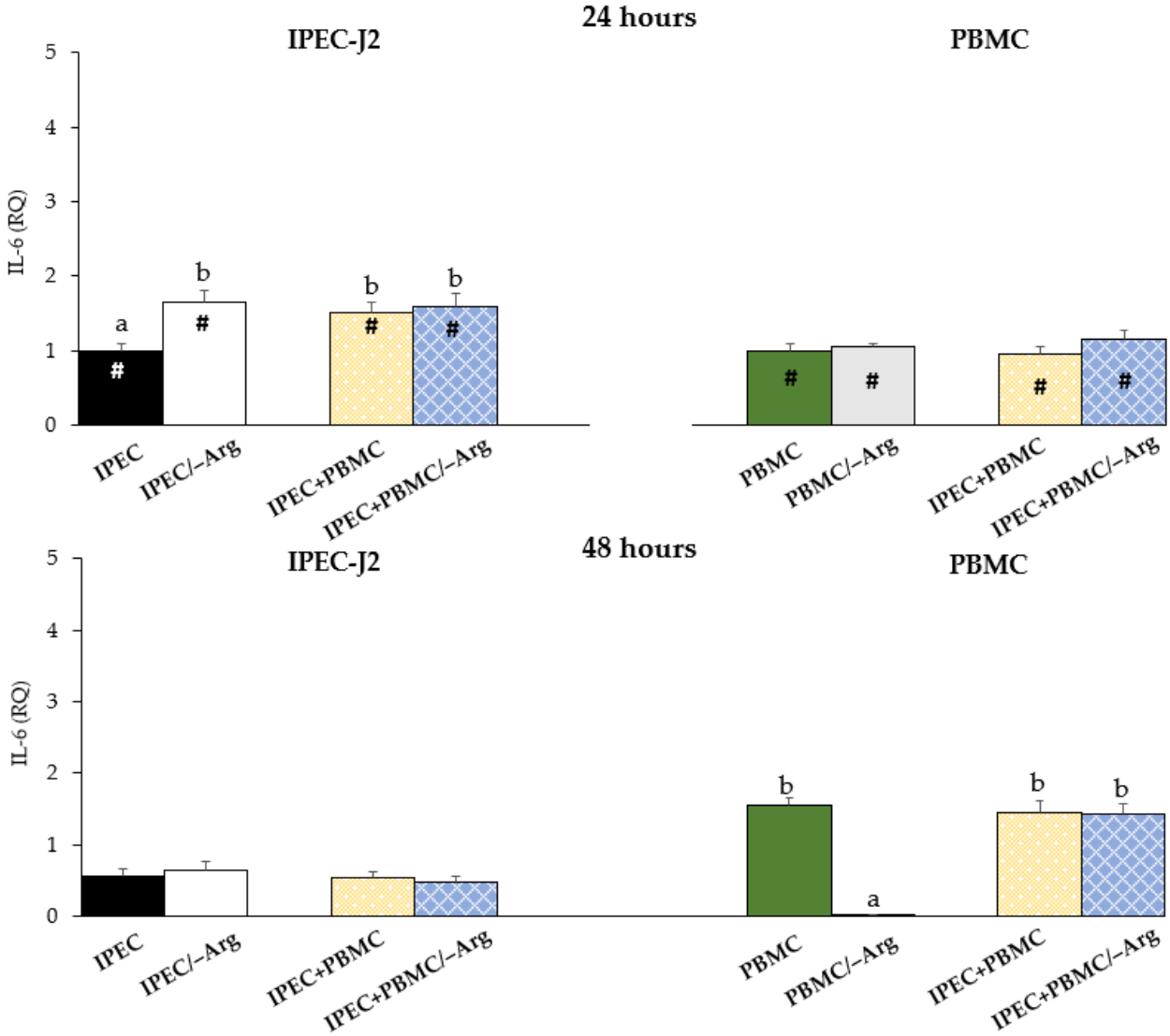


Figure 17 *IL-6 gene expression in IPEC-J2 cells and in PBMC at 24 and 48 h of incubation. Each value represents the mean ± SE of 8 replicates of 6 independent experiments. Significant differences (P < 0.05) among groups (upon row comparisons within paired groups) are indicated with letters. Significant differences (P < 0.05) between 24 and 48 h in the same group (upon column comparisons vs respective control group) are indicated with hashtags. Data were analysed according to the* $2^{-\Delta\Delta Ct}$ *method in which the expression levels of each cytokine, normalized to the GAPDH cDNA amount, were expressed as relative quantities (RQ).*

Interleukin-8 (*IL-8*)

In IPEC-J2 at 24 h of incubation, IL-8 expression was statistically increased in the IPEC/−Arg and IPEC+PBMC groups compared to the control (IPEC group), while the IPEC+PBMC/−Arg group showed a significant increase compared to the IPEC group ($P < 0.05$) and no difference compared to the IPEC/−Arg group. At 48 h of incubation, a significant decrease was shown in all groups ($P < 0.05$) compared to the control (IPEC) at 24 h, with no significant differences between groups. Regarding PBMC, after 24h of culture, arginine deprivation significantly induced IL-8 expression (PBMC/−Arg and IPEC+PBMC/−Arg groups) ($P < 0.05$), compared to control (PBMC). At 48 h, expression increased in the IPEC+PBMC and IPEC+PBMC/−Arg groups compared to the control (PBMC group) ($P < 0.05$). Instead, IL-8 way suppressed in PBMC seeded without arginine (PBMC/−Arg group) ($P < 0.05$). The data are shown in Figure 18.

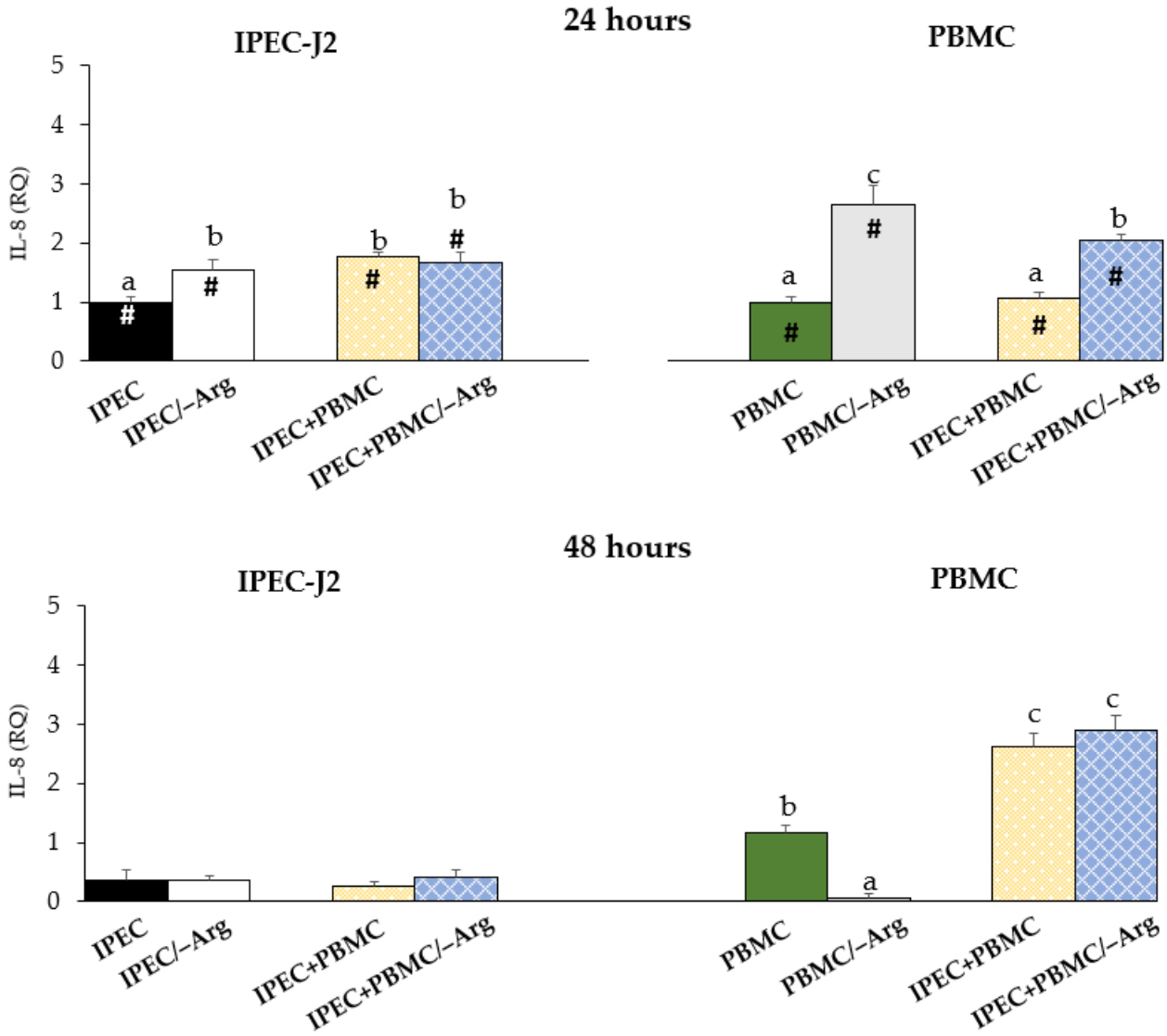


Figure 18 *IL-8 gene expression in IPEC-J2 cells and in PBMC at 24 and 48 h of incubation. Each value represents the mean ± SE of 8 replicates of 6 independent experiments. Significant differences (P < 0.05) among groups (upon row comparisons within paired groups) are indicated with letters. Significant differences (P < 0.05) between 24 and 48 h in the same group (upon column comparisons vs respective control group) are indicated with hashtags. Data were analysed according to the* $2^{-\Delta\Delta Ct}$ *method in which the expression levels of each cytokine, normalized to the GAPDH cDNA amount, were expressed as relative quantities (RQ).*

Transforming Growth Factor-ß (*TGF-ß*)

In IPEC-J2 at 24h, TGF-β expression significantly increased in the absence of arginine (IPEC/–Arg and IPEC+PBMC/–Arg) ($P < 0.05$), compared to control (IPEC). The IPEC+PBMC group was not different from the control group (IPEC). At 48 h of incubation, no significant differences were observed between the groups. Similarly, PBMC showed increased expression at 24 h of culture in the PBMC/–Arg and IPEC+PBMC/–Arg groups ($P < 0.05$), compared with the control (PBMC). At 48 h, TGF-ß expression was downregulated in the PBMC/–Arg group and IPEC+PBMC/–Arg group, compared with the control (PBMC group) ($P < 0.05$). In IPEC-J2, after arginine deprivation, TGF-β decreased significantly compared to 24 h ($P < 0.05$). Data are shown in Figure 19.

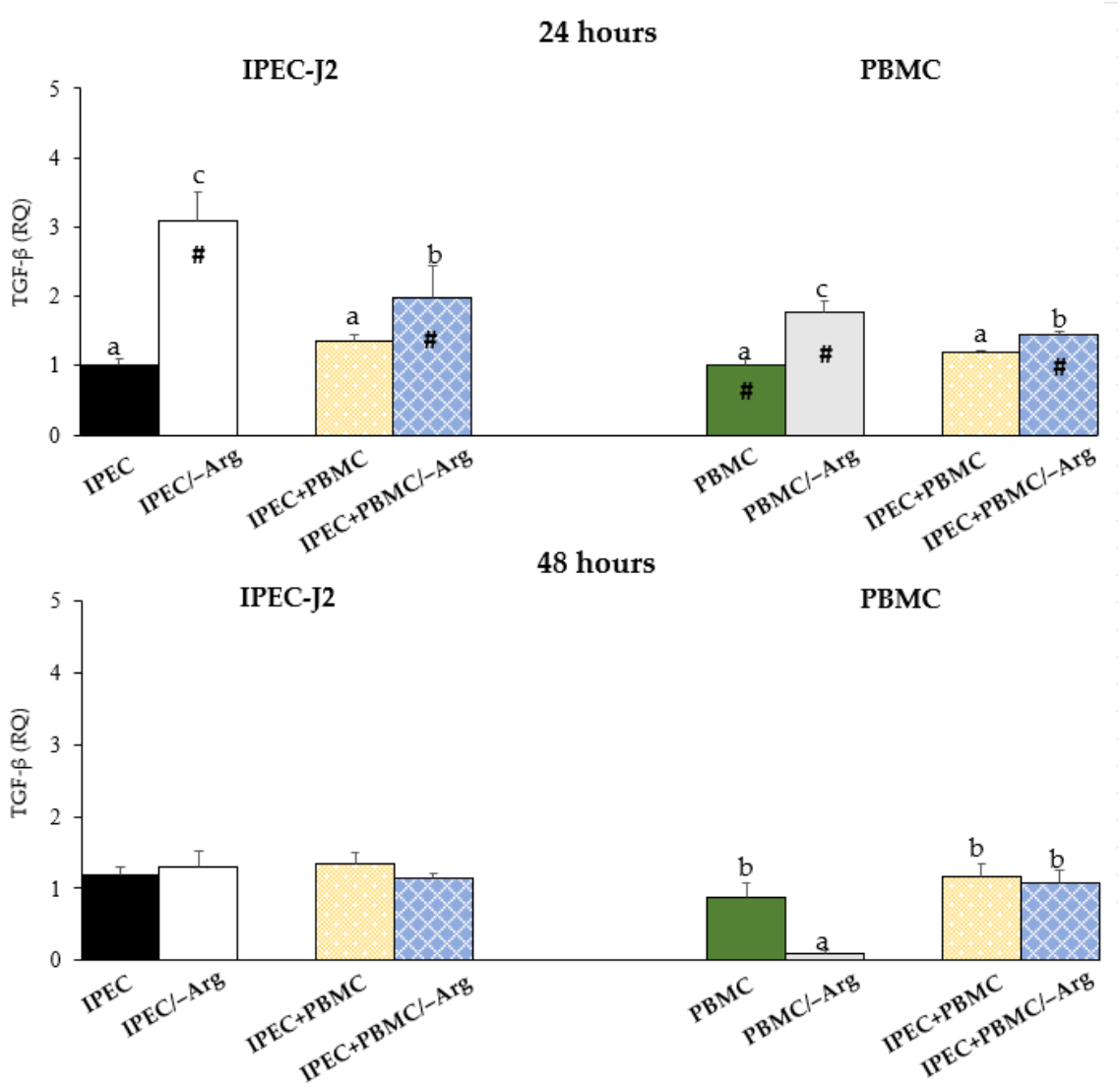


Figure 19 *TGF-β gene expression in IPEC-J2 cells and in PBMC at 24 and 48 h of incubation. Each value represents the mean ± SE of 8 replicates of 6 independent experiments. Significant differences (P < 0.05) among groups (upon row comparisons within paired groups) are indicated with letters. Significant differences (P < 0.05) between 24 and 48 h in the same group (upon column comparisons vs respective control group) are indicated with hashtags. Data were analysed according to the* $2^{-\Delta\Delta Ct}$ *method in which the expression levels of each cytokine, normalized to the GAPDH cDNA amount, were expressed as relative quantities (RQ).*

4.2 Study 2

Cell viability

The viability of IPEC-J2, in the presence of ACE 2.5 and ACE 5, significantly increases linearly ($y_L = 0.045x + 0.229$) and quadratic ($y_Q = 0.016x^2 - 0.053x + 0.344$) ($P < 0.05$) compared to the control group. PROP 1 and PROP 2.5 supplementation also stimulated viability with a significant linear ($y_L = -0.015x + 0.351$) and quadratic ($y_Q = -0.015x^2 + 0.076x + 0.245$) ($P < 0.05$) increase compared to the control. On the other hand, lactate and butyrate showed no significant differences compared to the control group (Figure 20).

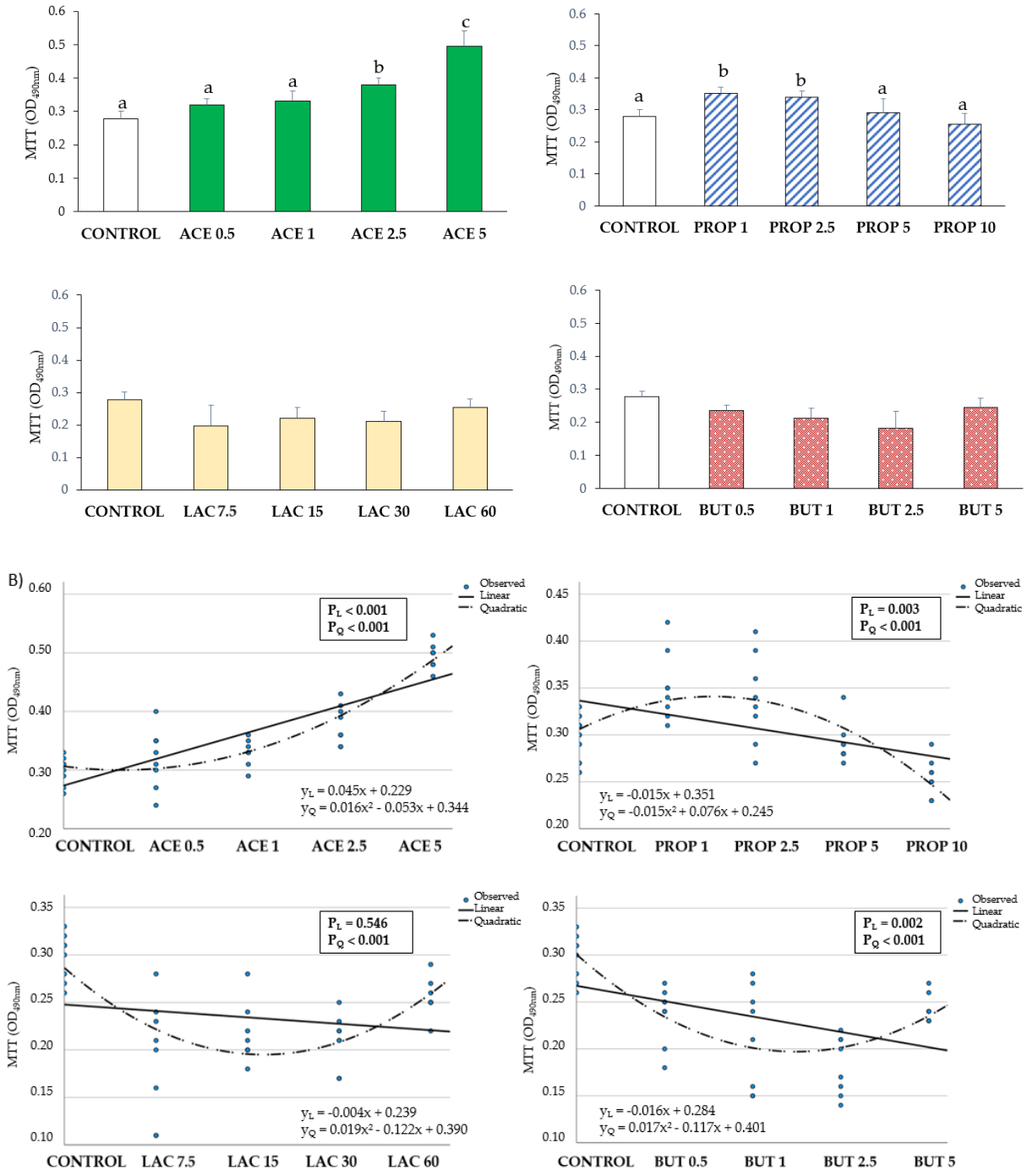


Figure 20 *A) Cell viability of IPEC-J2 upon medium supplementation with different concentrations of short-chain fatty acids (SCFA) at 24 h of incubation determined by an MTT assay. Each value represents the mean ± SD of 8 wells of 6 independent experiments. Different letters indicate significant (P < 0.05) differences among groups. B) Regression graphs and analysis showing the mean values of each independent experiment and equation.* y_L*: linear regression;* P_L*: P-value linear regression;* y_Q*: quadratic regression;* P_Q*: P-value quadratic regression. The numbers in all treatment group designations refer to an mM concentration.*

Nitric oxide accumulation

The data relating to the NO release are shown in Figure 21. NO release in the presence of acetate is significantly increased linearly ($y_L = 0.479x + 1.841$) and quadratic ($y_Q = 0.254x^2 - 1.047x + 3.622$) ($P < 0.05$), with statistical differences for ACE 2.5 and ACE 5 compared to control ($P < 0.05$). On the contrary, lactate supplementation induced a linear ($y_L = -0.131x + 3.596$) and quadratic ($y_Q = -0.178x^2 + 0.935x + 2.352$) variation of NO release, with statistical differences for LAC 7.5 and LAC 15 versus control ($P < 0.05$). Propionate caused a significant squared increase ($y_Q = -0.113x^2 + 0.625x + 2.437$) ($P < 0.05$), with statistical differences from PROP 1 to PROP 5 compared to control ($P < 0.05$). Butyrate stimulated a significant squared increase ($y_Q = -0.248x^2 + 1.452x + 1.610$) ($P < 0.05$), with statistical differences from BUT 0.5 to BUT 2.5 compared to control ($P < 0.05$).

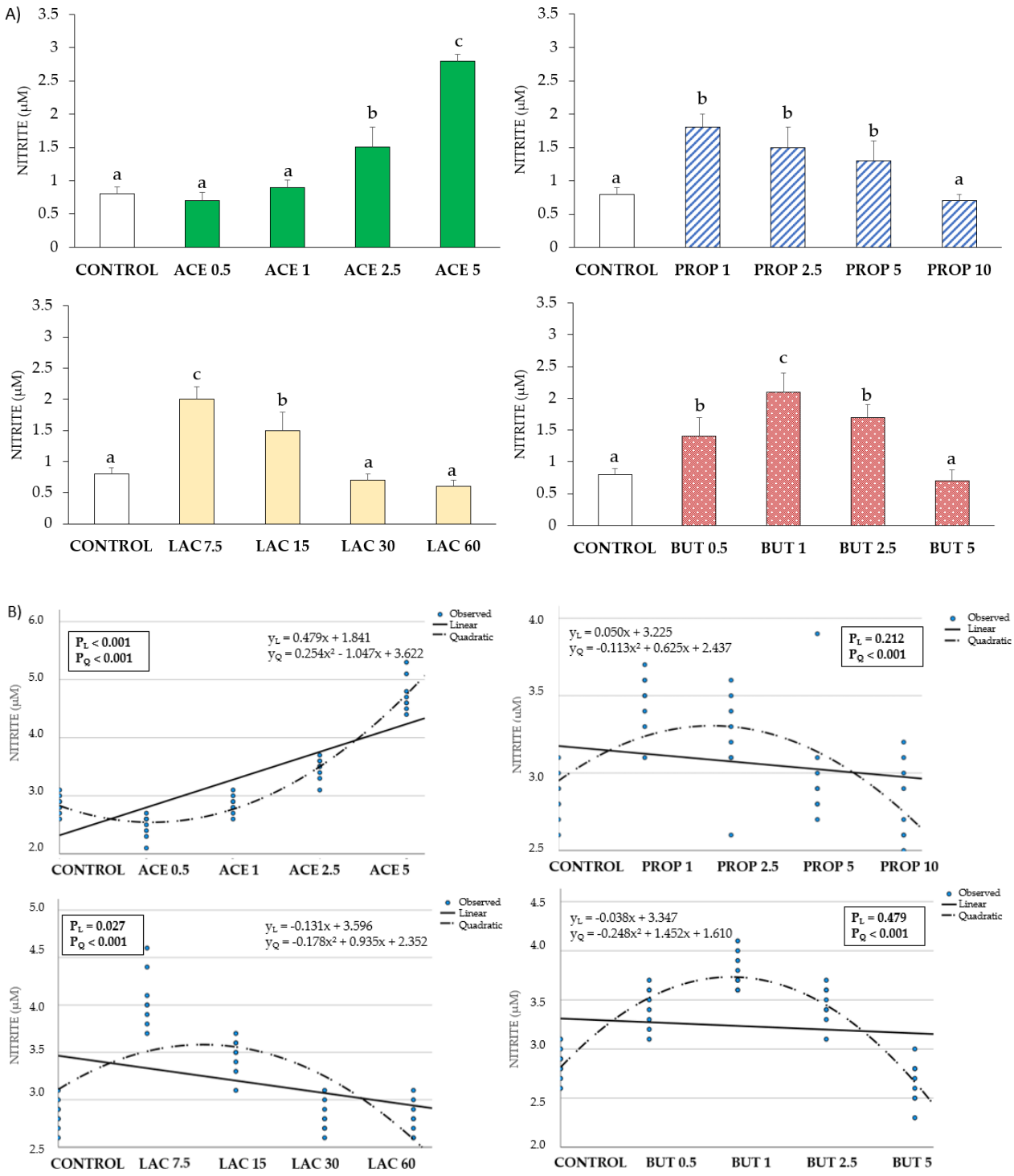


Figure 21 *A) Effect of the culture condition (different concentrations of SCFA) on nitrite release at 24 h of incubation. Each value represents the mean ± SD of 8 wells of 6 independent experiments. Different letters indicate significant (P < 0.05)differences among groups. B) Regression graphs and analysis showing the mean values of each independent experiment and equation. y_L: linear regression; P_L: P-value linear regression; y_Q: quadratic regression; P_Q: P-value quadratic regression. The numbers in all treatment group designations refer to an mM concentration.*

Gene expression

Zonula occludens-1 (ZO-1)

The expression of ZO-1 was significantly reduced in the presence of acetate, in all groups ($P < 0.05$). Even with high concentrations of propionate (2.5, 5 and 10 mM) ($P < 0.05$)a strong reduction of ZO-1 expression is observed compared to the control, instead ZO-1 was significantly increased in PROP 1. Lactate and butyrate supplementation significantly increased in ZO-1 expression in all groups compared with the control ($P < 0.05$). Data are shown in Figure 22.

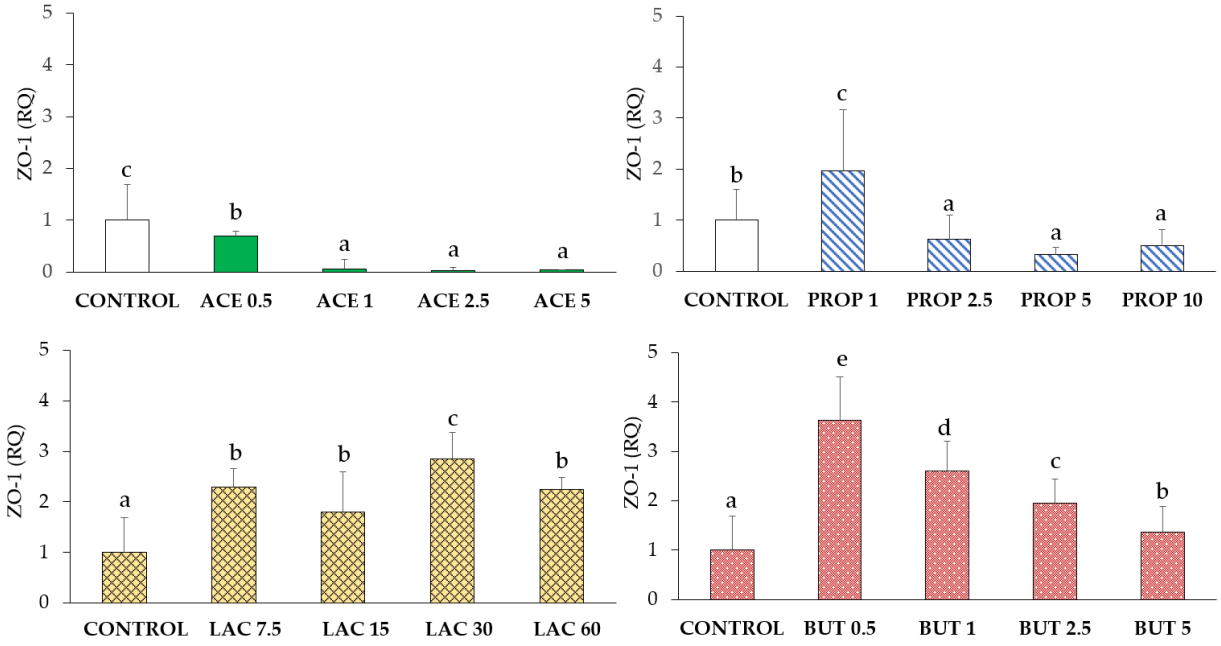


Figure 22 *ZO-1 gene expression in IPEC-J2 cells (control) and in IPEC-J2 with SCFA supplementation (acetate, propionate, lactate or butyrate). Each value represents the mean ± SD of 8 replicates of 6 independent experiments. Different letters indicate significant ($P < 0.05$) differences among groups. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method in which the expression levels of the gene, normalized to the expression of the reference gene HPRT1, were expressed as relative quantities (RQ). The values were then normalized to the expression in the control group. The numbers in all treatment group designations refer to an mM concentration.*

Occludin (OCLN)

OCLN expression was significantly increased in ACE 0.5 compared to the control (IPEC-J2) ($P < 0.05$), as shown in figure 23. Instead, compared with the control group, a significant decrease in OCLN expression was observed in the groups ACE 1, ACE 2.5 and ACE 5 ($P < 0.05$). Propionate caused maximal OCLN expression at concentrations of 1 and 10 mM ($P < 0.05$) compared to the control. OCLN expression was down-regulated in all groups in the presence of lactate compared with the control ($P < 0.05$). Butyrate stimulated the increase of OCLN at the lowest concentration tested (BUT 0.5) ($P < 0.05$), while no differences were detected in the BUT 5 group compared to the control.

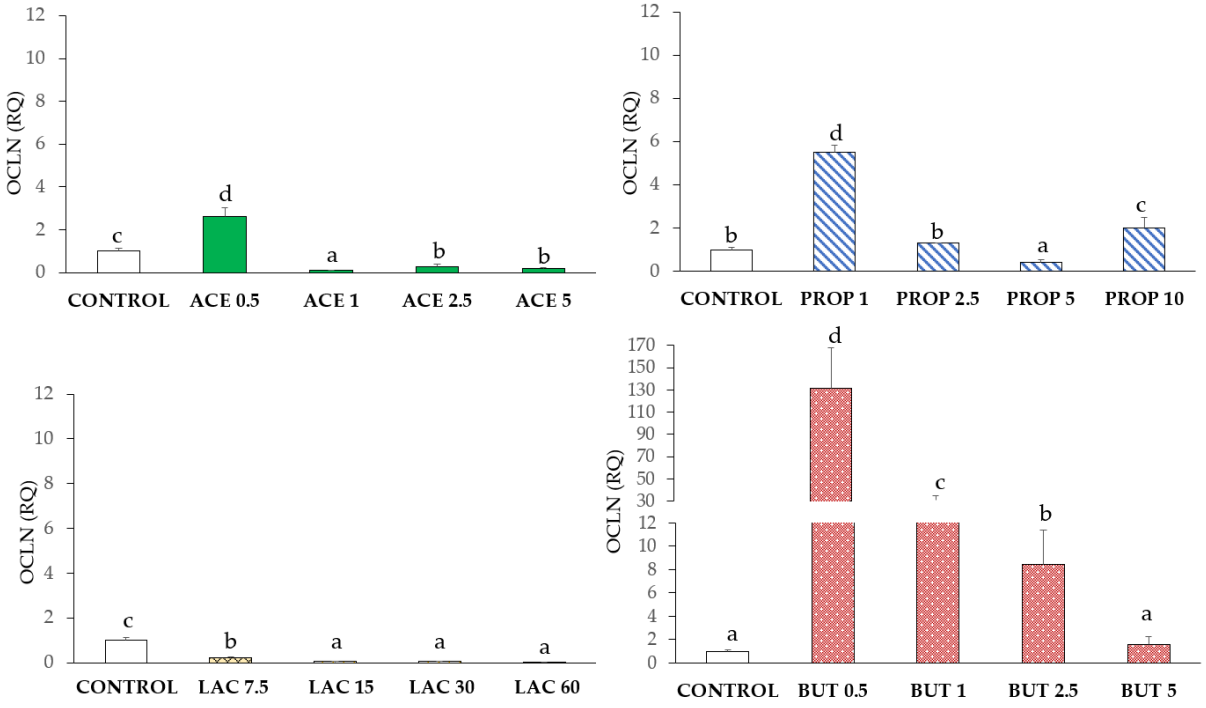


Figure 23 *OCLN gene expression in IPEC-J2 cells (control) and in IPEC-J2 with SCFA supplementation (acetate, propionate, lactate or butyrate). Each value represents the mean ± SD of 8 replicates of 6 independent experiments. Different letters indicate significant ($P < 0.05$) differences among groups. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method in which the expression levels of the gene, normalized to the expression of the reference gene HPRT1, were expressed as relative quantities (RQ). The values were then normalized to the expression in the control group. The numbers in all treatment group designations refer to an mM concentration.*

Claudin-4 (CLDN4)

As shown in Figure 24, CLDN4 expression was significantly increased in the presence of the acetate at different concentrations, particularly in ACE 5 ($P < 0.05$) compared to the control. Furthermore, propionate induced the increase in CLDN4 expression at all concentrations ($P < 0.05$) compared with the control, precisely, PROP 1 ($P < 0.05$). After supplementation with lactate and butyrate, CLDN4 expression was almost negligible and significant differences were observed at all concentrations ($P < 0.05$) compared to the control.

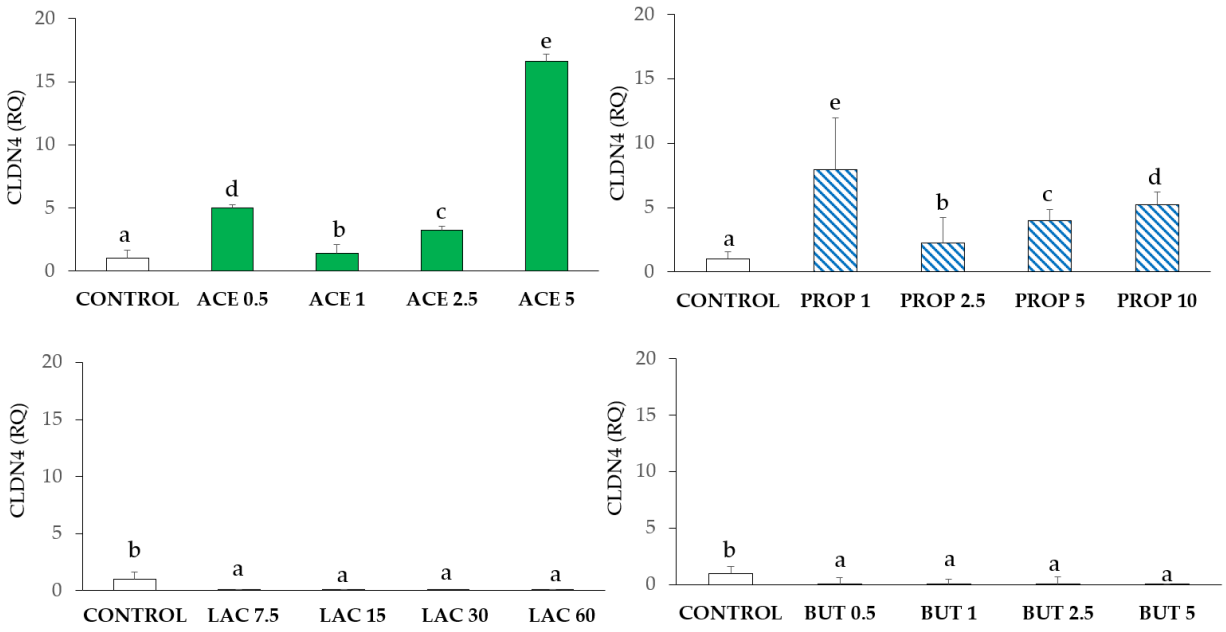


Figure 24 *CLDN4 gene expression in IPEC-J2 cells (control) and in IPEC-J2 with SCFA supplementation (acetate, propionate, lactate or butyrate). Each value represents the mean ± SD of 8 replicates of 6 independent experiments. Different letters indicate significant ($P < 0.05$) differences among groups. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method in which the expression levels of the gene, normalized to the expression of the reference gene HPRT1, were expressed as relative quantities (RQ). The values were then normalized to the expression in the control group. The numbers in all treatment group designations refer to an mM concentration.*

Nuclear factor-κB (NF-κB)

NF-κB expression was significantly reduced ($P < 0.05$) in the presence of all SCFA compared with the respective controls. The highest lactate doses tested (LAC 30 and LAC 60) showed higher values than the lowest doses (LAC 7.5 and LAC 15) ($P < 0.05$). Furthermore, butyrate supplementation caused the highest inhibition of NF-κB expression at the highest concentration tested (BUT 5) ($P < 0.05$). Data are shown in Figure 25.

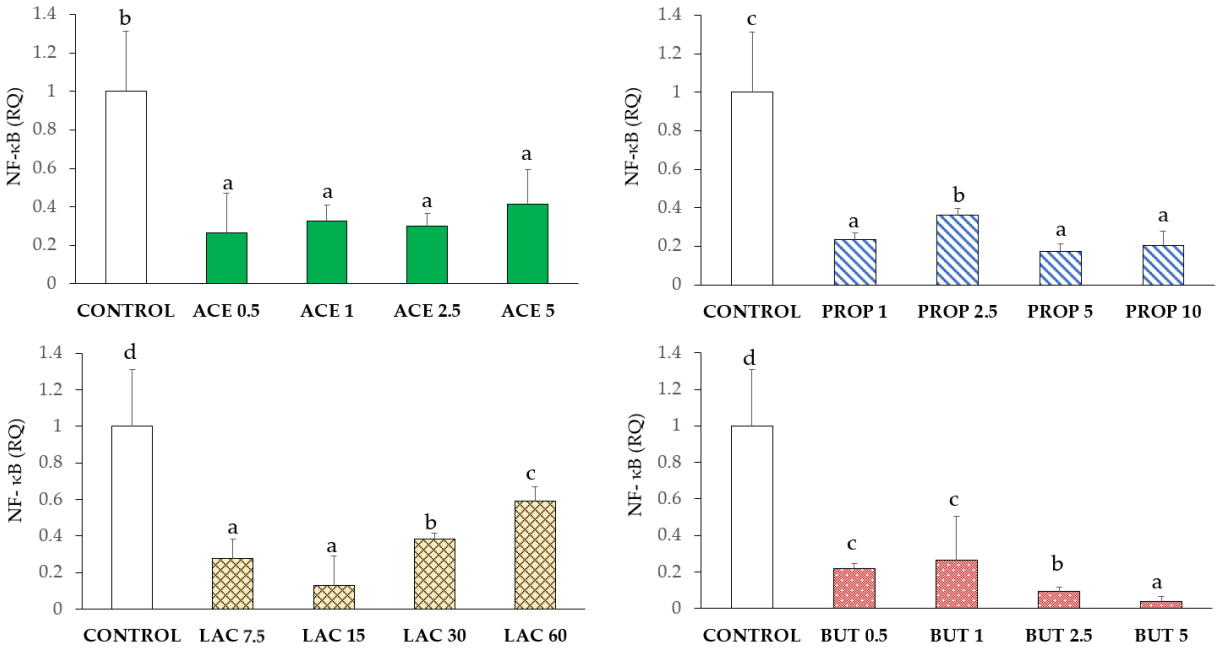


Figure 25 *NF-κB gene expression in IPEC-J2 cells (control) and in IPEC-J2 with SCFA supplementation (acetate, propionate, lactate or butyrate). Each value represents the mean ± SD of 8 replicates of 6 independent experiments. Different letters indicate significant ($P < 0.05$) differences among groups. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method in which the expression levels of the gene, normalized to the expression of the reference gene HPRT1, were expressed as relative quantities (RQ). The values were then normalized to the expression in the control group. The numbers in all treatment group designations refer to a mM concentration.*

Tumor necrosis factor alpha (TNFα)

As shown in Figure 26, the expression of TNFα, in the presence of acetate or lactate, was very strongly down-regulated ($P < 0.05$) in all groups compared to the control. Propionate induced a significant reduction ($P < 0.05$) of TNFα expression in PROP 1, PROP 2.5 and PROP 5, compared to the control. A minor decrease in TNFα expression was caused by the presence of butyrate in all groups compared with the control ($P < 0.05$).

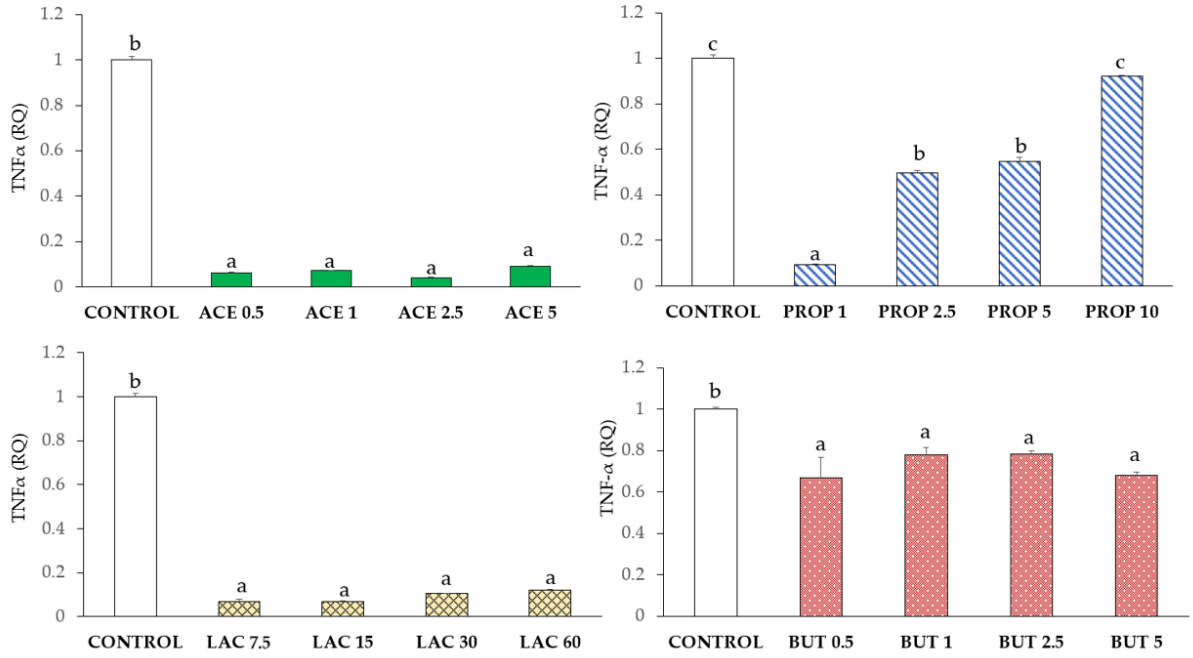


Figure 26 *TNFα gene expression in IPEC-J2 cells (control) and in IPEC-J2 with SCFA supplementation (acetate, propionate, lactate or butyrate). Each value represents the mean ± SD of 8 replicates of 6 independent experiments. Different letters indicate significant ($P < 0.05$) differences among groups. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method in which the expression levels of the gene, normalized to the expression of the reference gene HPRT1, were expressed as relative quantities (RQ). The values were then normalized to the expression in the control group. The numbers in all treatment group designations refer to an mM concentration.*

Beta-defensin 1 (BD-1)

Figure 27 shows the significant increase in BD-1 expression in all acetate and propionate groups ($P < 0.05$) compared to the respective controls with opposite courses. Lactate supplementation caused a strong up-regulation of BD-1 in LAC 30 and LAC 60 ($P < 0.05$) compared to the control. Furthermore, butyrate stimulated a significant increase in BD-1 expression in BUT 0.5 and BUT 1 compared with the control ($P < 0.05$).

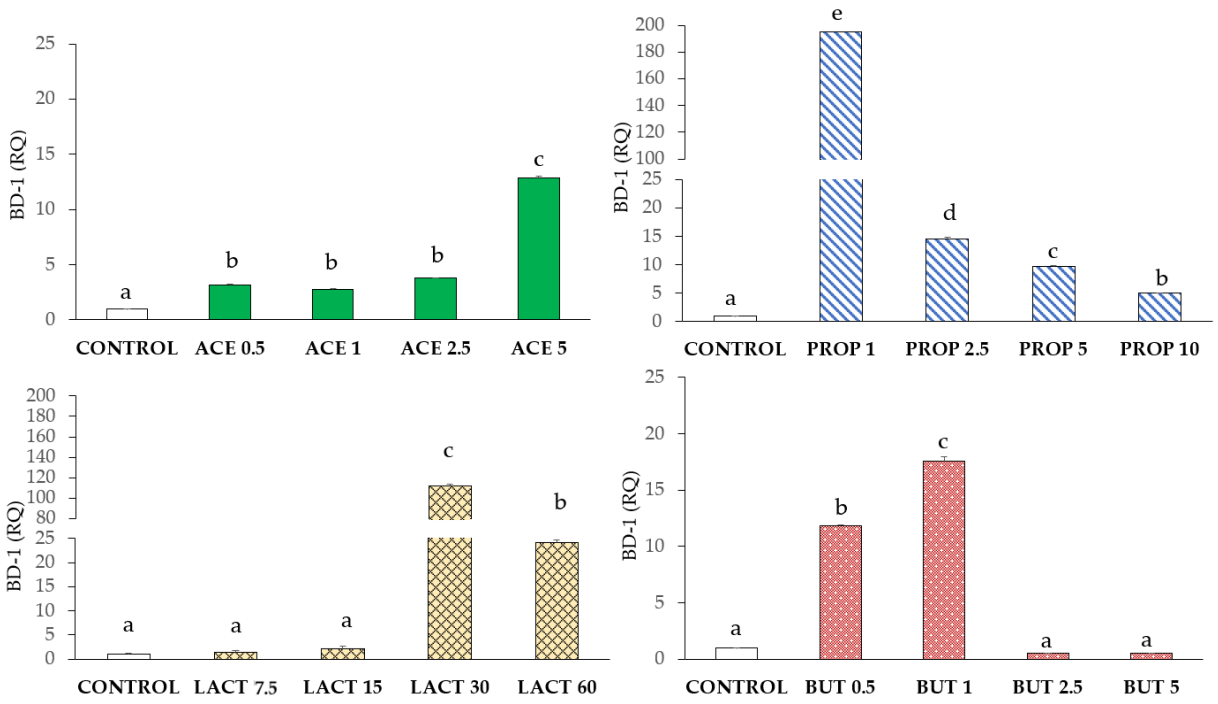


Figure 27 *BD-1 gene expression in IPEC-J2 cells (control) and in IPEC-J2 with SCFA supplementation (acetate, propionate, lactate or butyrate). Each value represents the mean ± SD of 8 replicates of 6 independent experiments. Different letters indicate significant ($P < 0.05$) differences among groups. Data were analyzed according to the $2^{-\Delta\Delta Ct}$ method in which the expression levels of the gene, normalized to the expression of the reference gene HPRT1, were expressed as relative quantities (RQ). The values were then normalized to the expression in the control group. The numbers in all treatment group designations refer to a mM concentration.*

4.3 Study 3

MTT assay

In Figure 28, monoculture control groups of IPEC-J2 and PBMC are tightly connected and fleshy, possess well-proportioned size and shape, and fill the entire field of view. Compared with the control group, upon exposure to LPS, IPEC-J2 was less elongated, and the rosettes in PBMC showed a decrease in cell number and size. Table 5 shows the differences and significant reductions in the viability of the IPEC-J2 and PBMC monoculture ($P < 0.05$) in the presence of LPS. In addition, IPEC-J2 viability in the co-culture (with/without LPS challenge) was significantly reduced ($P < 0.05$), while PBMC viability (with/without LPS challenge) remained unchanged. Cell viability, after acetate treatment [5mM], increased in IPEC-J2 cells ($P < 0.05$) compared with the control. However, no significant differences were found in all other IPEC-J2 and PBMC groups (with/without LPS and/or with propionate) compared to their respective control. Data are shown in Figure 29.

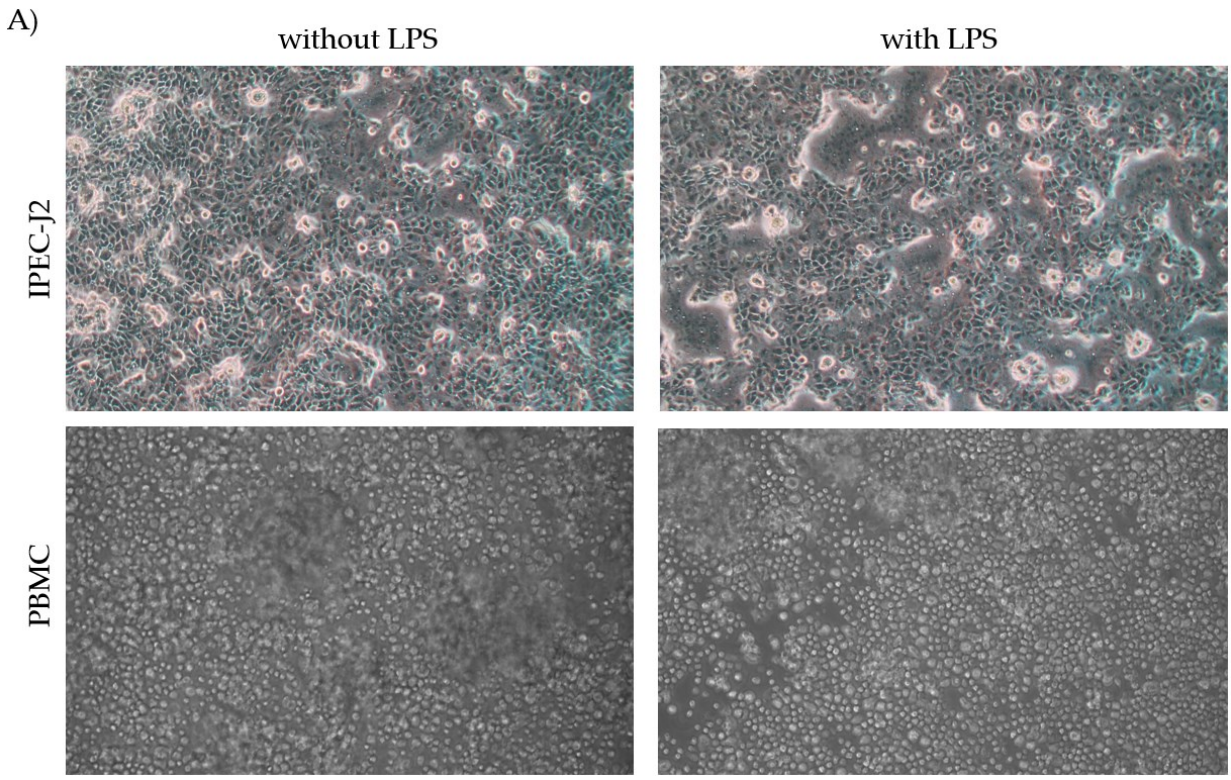


Figure 28 *Light microscopy of co-cultures: IPEC-J2 in a transwell and PBMC in a plate well, with or without LPS; 4× original magnification.*

IPEC-J2	IPEC-J2 + LPS	PBMC	PBMC + LPS
0.30 ± 0.04*	0.27 ± 0.03	0.33 ± 0.04*	0.30 ± 0.01

Table 5 *Viability in IPEC-J2 or PBMC monocultures and with without LPS challenge. Each value represents the mean ± SD of 8 replicates of 6 independent experiments. Asterisks indicate significant differences ($P < 0.05$)among the groups.*

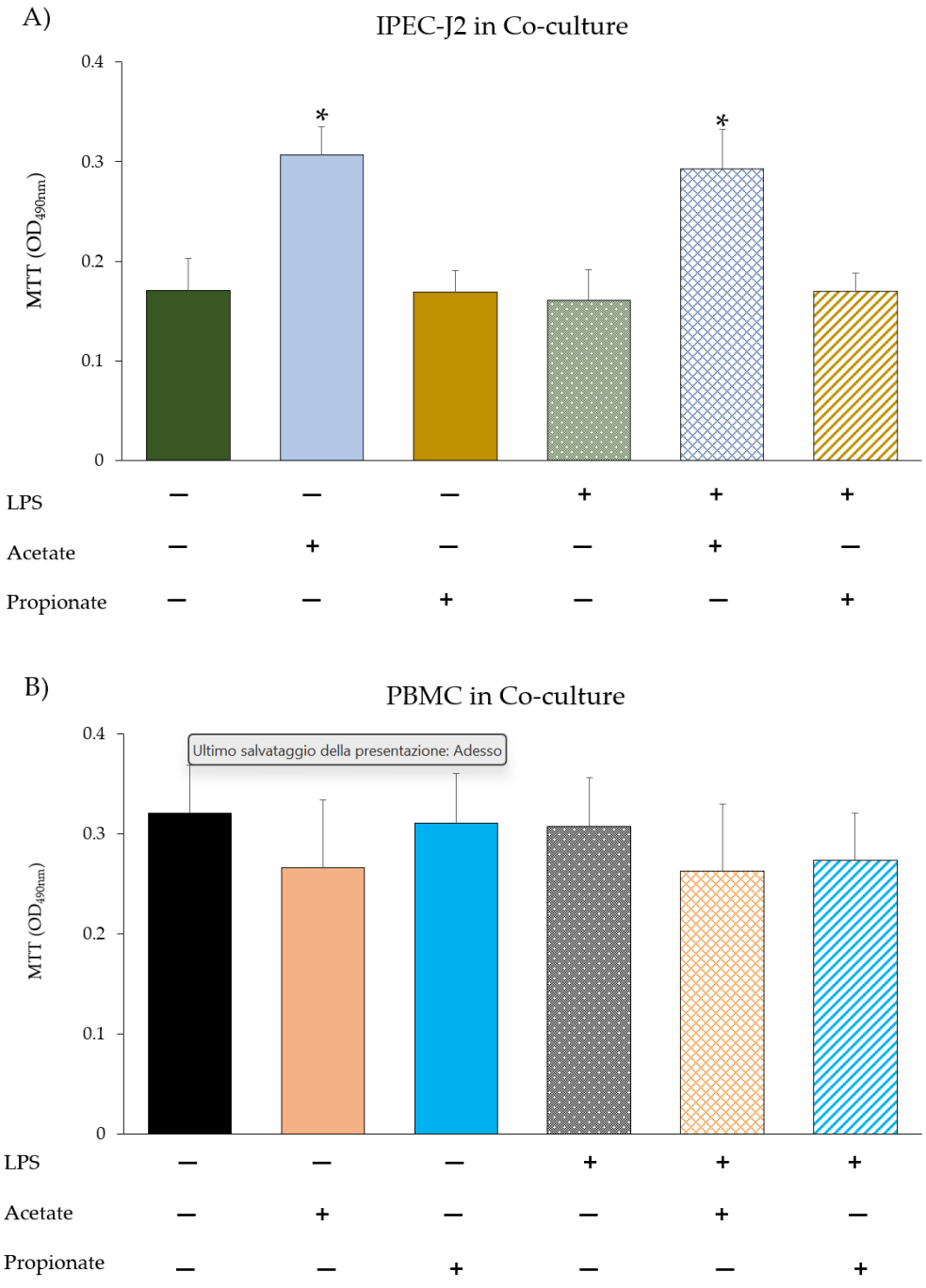


Figure 29 *Cell viability of IPEC-J2 (A) and PBMC (B) upon LPS challenge and medium supplementation with acetate (5 mM) or propionate (1 mM) at 24 h of incubation was determined using an MTT assay. Each value represents the mean ± SD of 8 wells of 6 independent experiments. Significant differences (P < 0.05)among the groups are indicated by asterisks. Values were normalized to the expression in the control group (IPEC-J2 in the left green first column and PBMC in the right orange first column).*

Nitric oxide accumulation

In Table 6, the release of NO is shown. LPS causes a significant increase in NO release in IPEC-J2 ($P < 0.05$) and PBMC ($P < 0.05$). In Figure 30, a significant increase in nitric oxide release was observed in co-culture ($P < 0.05$) compared to monoculture. Acetate treatment stimulated NO release, while propionate treatment reduced it. Conversely, in the presence of LPS-challenge, both treatments stimulated the reduction of nitrite release.

IPEC-J2	IPEC-J2 + LPS	PBMC	PBMC + LPS
0.84 ± 0.05*	1.21 ± 0.02	3.03 ± 0.04*	3.48 ± 0.01

Table 6 *Nitric oxide release in IPEC-J2 or PBMC monocultures and with or without LPS challenge. Each value represents the mean ± SD of eight replicates of six independent experiments. Asterisks indicate significant differences ($P < 0.05$) among the groups.*

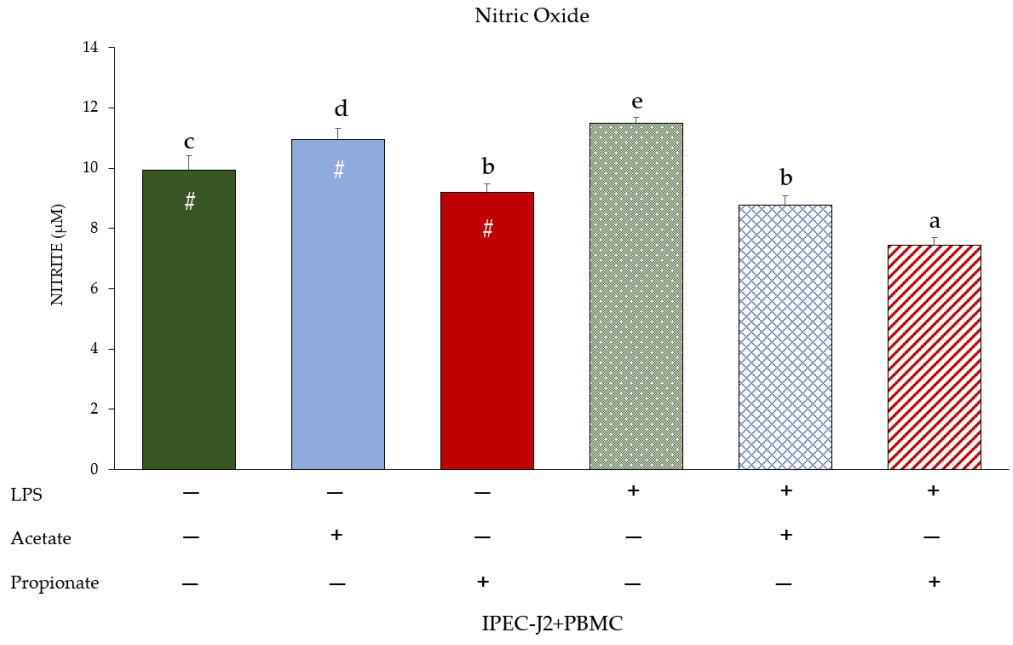


Figure 30 *Effect of co-culture conditions (acetate or propionate treatment) with or without LPS challenge on nitrite release after 24 h of incubation. Each value represents the mean ± SD of 8 wells of 6 independent experiments. Different letters indicate significant ($P < 0.05$) differences among groups. Values were normalized to the expression in the control group (IPEC-J2 in the green first column).*

Gene expression and protein level in monocultures

The data reported in Table 7 show the gene expression in IPEC-J2 monoculture. The expression of ZO-1, CLDN4 and OCLN was significantly reduced ($P < 0.05$) after LPS-challenge compared with that of the control (IPEC-J2). In Figure 31, the expression levels of CLDN4 and OCLN proteins were further determined by Western blotting. Similarly to the mRNA expression results, the expression level of occludin proteins decreased significantly with the LPS challenge.

	IPEC-J2	**IPEC-J2 + LPS**
ZO-1	1.00 ± 0.04 *	0.04 ± 0.09
CLDN4	1.00 ± 0.02	0.07 ± 0.04
OCLN	1.00 ± 0.01 *	0.08 ± 0.03

*p-value < 0.05.

Table 7 *Gene expression in IPEC-J2 monoculture with/without LPS challenge. Each value represents the mean ± SD of eight replicates of six independent experiments. Asterisks indicate significant differences ($P < 0.05$) among the groups. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method, in which the expression levels of the gene, normalized to the expression of the reference gene HPRT1, were expressed as relative quantities (RQ). The values were normalized to those in the control group (IPEC-J2).*

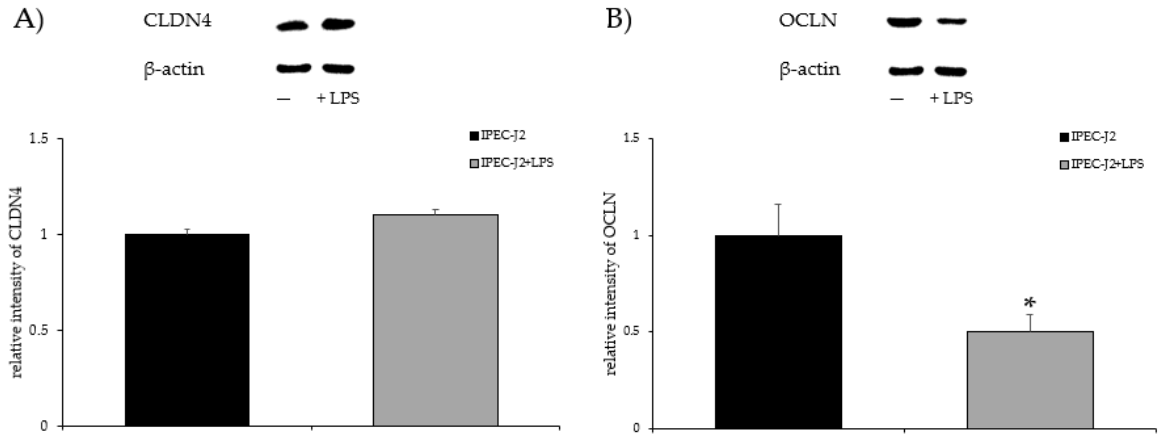
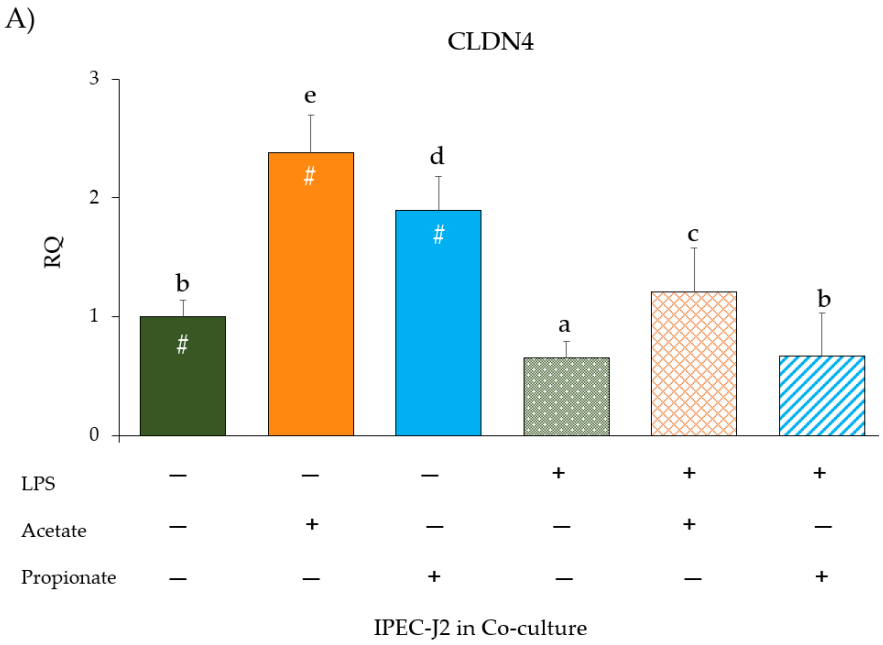


Figure 31 *Protein synthesis of CLDN4 (A) and OCLN (B) in IPEC-J2 monoculture and with or without LPS challenge. Each value represents the mean ± SD of eight replicates of six independent experiments. Asterisks indicate significant differences ($P < 0.05$) among the groups. Data were normalized to the synthesis of the reference protein β-actin as relative quantity (RQ). The values were normalized to synthesis in the control group (IPEC-J2).*

Tight junction protein (TJp) gene expression and protein level in IPEC-J2 co-cultured with PBMC and LPS

TJp gene expression was evaluated in IPEC-J2 cells but not in PBMC. The data reported in Figures 32A, B and C show the expression in the co-culture alone. After treatment with acetate and propionate the expression of CLDN4, OCLN and ZO-1 increased significantly compared to the co-culture control ($P < 0.05$). A significant increase of CLDN4, OCLN and ZO-1 expression was observed after acetate treatment and LPS challenge compared to IPEC-J2 after LPS challenge only ($P < 0.05$). Conversely, in the presence of propionate and LPS, ZO-1 expression was significantly reduced compared with the control (IPEC-J2+PBMC) ($P < 0.05$)(Figure 28C). Furthermore, as shown in Figure 33, the protein levels of CLDN4 and OCLN were significantly reduced in the presence of LPS. Acetate alone or with LPS stimulated an increase in CDLN4 and OCLN levels ($P < 0.05$). The same increase was shown with propionate supplementation compared with control (IPEC-J2 + PBMC) ($P < 0.05$; Fig 29B).



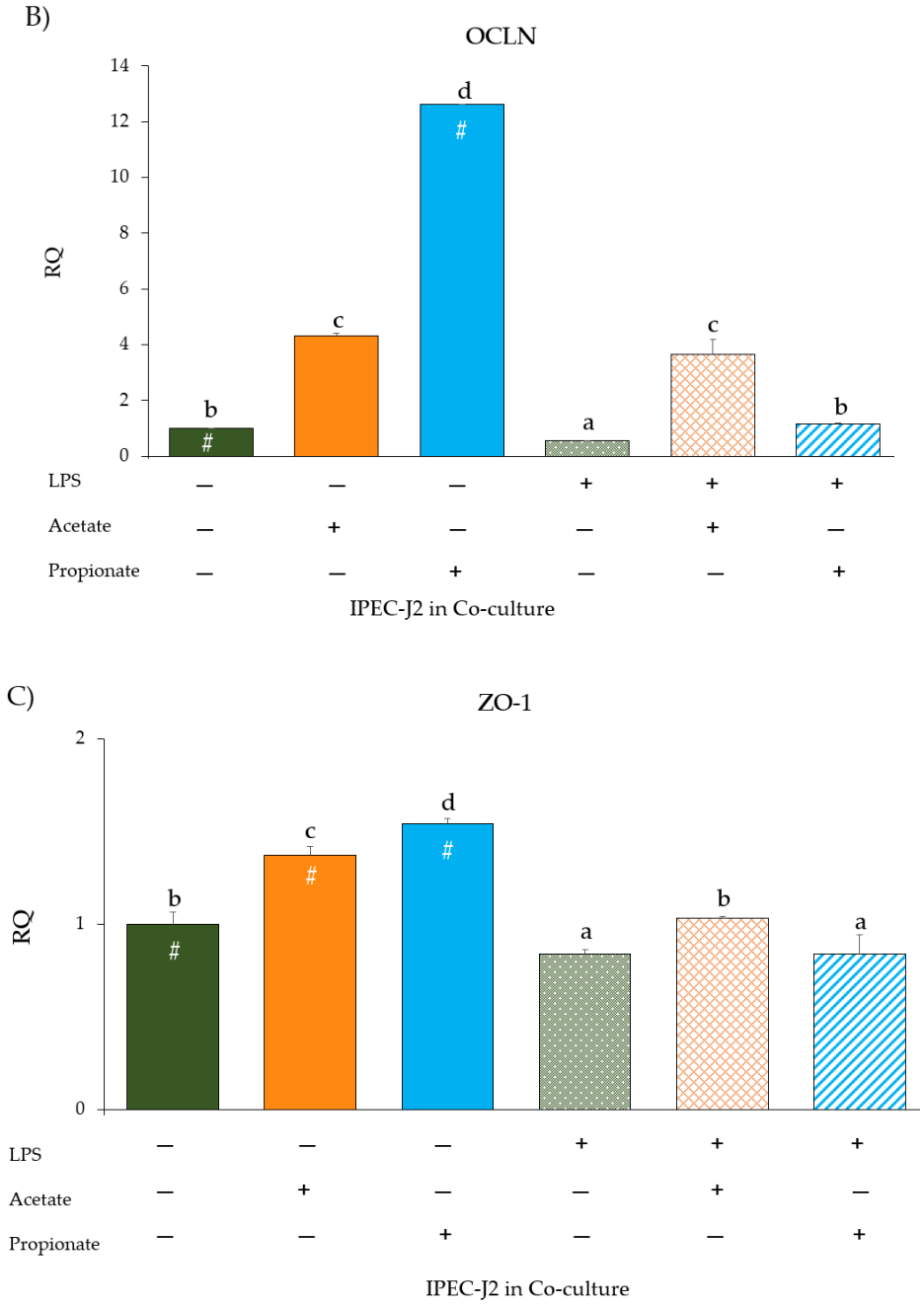


Figure 32 *CLDN4 (A), OCLN (B), and ZO-1 (C) gene expression in IPEC-J2 co-cultured with PBMC with/without LPS challenge and/or acetate or propionate treatment. Each value represents the mean ± SD of eight replicates of six independent experiments. Different letters indicate significant (P < 0.05)differences among groups. Data were analyzed using the* $2^{-\Delta\Delta Ct}$ *method, in which the expression levels of the gene, normalized to the expression of the reference gene HPRT1, were expressed as relative quantities (RQ). The values were normalized to the expression in the control group (IPEC-J2 in the green first column of each graph).*

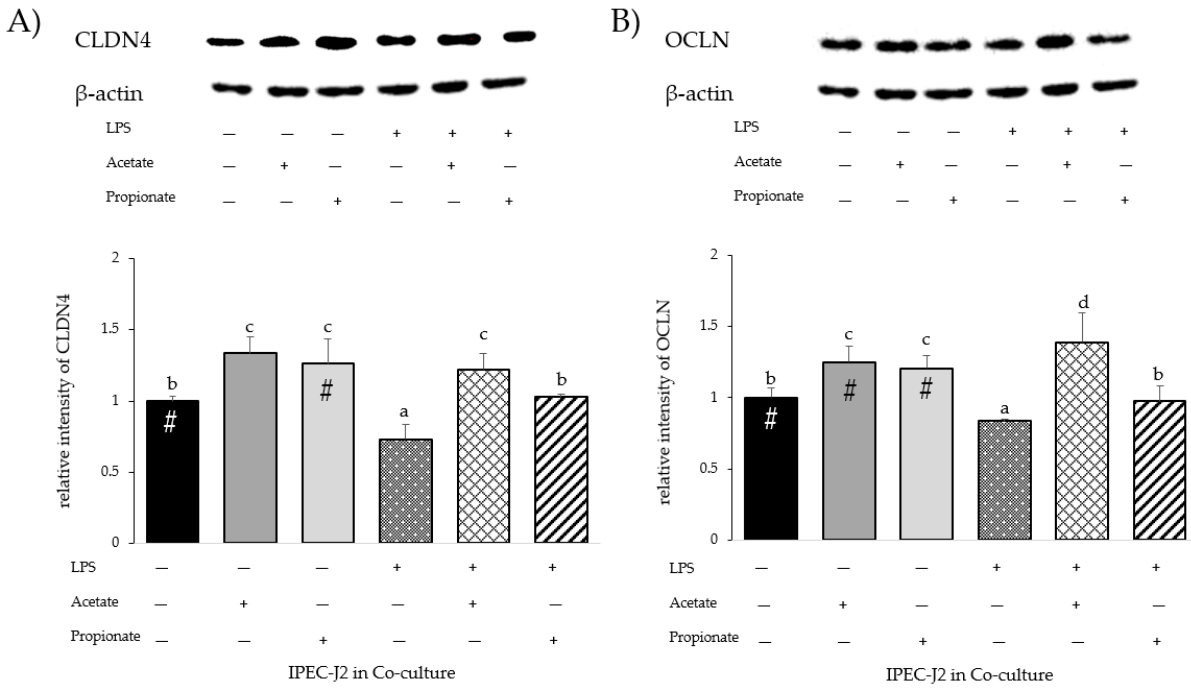


Figure 33 *Protein synthesis of CLDN4 (A) and OCLN (B) in IPEC-J2 co-culture with PBMC with/without LPS challenge and/or acetate or propionate treatment. Each value represents the mean ± SD of eight replicates of six independent experiments. Asterisks indicate significant differences (P < 0.05) among the groups. Data were normalized to the synthesis of the reference protein β-actin as relative quantity (RQ). The values were normalized to synthesis in the control group (IPEC-J2+ PBMC).*

5. Discussion

5.1 Study 1

In the first study, we set up an experimental swine co-culture model to study the interaction of intestinal epithelial cells with immune cells. In fact, our aim was to create a system mimicking the gut system *in vivo*. Considering the complexity of the intestinal system, an *in vitro* co-culture approach could be interesting and functional. The advantage of using PBMC is that they are an easily accessible source of pig immune cells, as the cells are isolated from peripheral blood and contains major B and T cell subpopulations as well as monocytes, NK cells and other subsets involved in adaptive and innate immune responses. The main limitation of this model system is that PBMC can display phenotypic differences and responsiveness when compared to immune cells involved in the mucosal immunity in the intestine (i.e., intraepithelial lymphocytes, Peyer's patches lymphocytes, and *lamina propria* immune cells). Once isolated, these immune resident cells can be employed in co-culture conditions with IPEC-J2 to better simulate *in vivo* conditions both spatially and functionally.

After setting up the co-culture model, we tested an essential amino acid in the piglet, arginine. Arginine is an essential amino acid in newborns and piglets [80]. In weaned piglet arginine supplementation reduces intestinal damage and improves intestinal immunity [132,133].

In the initial and stressful phase of a pig, dietary modifications are among the first actions considered to strengthen the capabilities of the immune system, for example, supplementing with probiotics, prebiotics, vitamins, amino acids and micronutrients. We evaluated on IPEC-J2 the direct or indirect effect of arginine deprivation as nutritional stress.

In our model, the MTT assay results showed that the absence of arginine influenced IPEC-J2 and PBMC viability at 24 and 48h. After 72 hours, we noticed a marked

reduction in cell viability. In order not to have non-functional data, we decided to consider gene expression only at 24h and 48h, in our experimental design.

As can infer, arginine is also necessary for cells to survive. It is interesting to underline the data on PBMC viability in absence of arginine: after 48 h of arginine deprivation, PBMC lose the ability to express some cytokines. In PBMC, arginine regulates survival, metabolic activity and all subsequent processes [134].

Arginine uptake, essential for cell viability, depends on the transporter CAT family [88]. In our study, we have chosen CAT-1, ubiquitous in most cells and the main arginine transporter. In IPEC group, CAT-1 expression at 24h reflected the arginine availability: in fact, the expression of CAT-1 decreased after 48h. This reduction could be related to the reduction of available arginine in the medium. On the other hand, the increase of CAT-1 in the absence of arginine was an unexpected result. Still, we hypothesized that arginine reduction in cells could activate a *de novo* synthesis from precursors such as glutamate and its derivatives, which are present in standard culture medium [135]. We know that glutamine and glutamate are the main precursors for the intestinal synthesis of arginine (Figure 7).

Furthermore, the conversion of glutamate leads to the synthesis of ornithine, another precursor of arginine [84]. The *de novo* synthesis could be considered a new arginine availability and thus could stimulate positive feedback on CAT-1 expression to overcome the intracellular arginine deficiency.

This hypothesis is also supported by the enhanced accumulation of nitrites in arginine-free mono/co-culture media since nitric oxide is produced by arginine [136]. After 48h, both CAT-1 expression in IPEC-J2 and nitrite release are significantly reduced. In co-culture, PBMC presence influenced the arginine availability and, consequently, the expression of CAT-1 in IPEC-J2.

In PBMC, CAT-1 expression was not influenced by arginine deprivation at 24h, regardless of the type of culture condition. Probably, PBMC likely still have availability of the arginine present in the medium used for the stimulation with PHA.

We may assume that the reduction in the intracellular levels of arginine occurs between 24 and 48 h after activation [134]. After 48 hours, CAT-1 expression increased in the presence of arginine in PBMC (mono- and co-culture), which could be due to cells heavily consuming arginine and rapidly converting it into metabolites, thus requiring an increased activation of the transporter [137]. In arginine-free co-culture conditions, the increased CAT-1 expression should lead to the recovery of arginine, which is still potentially available in the medium and essential for cell survival.

The effect of arginine is also evident by evaluating cytokine gene expression in IPEC-J2. Intestinal epithelial cells are a physical barrier and are also able to secrete pro-inflammatory cytokines and chemokines [138,139]. The IPEC-J2 model has previously been characterized in numerous studies, identifying which cytokines and how much they responded to it were subjected. For example, TNFα, IL-1α, IL-6 and IL-8 are expressed in IPEC-J2 and IL-2, IL-1β, IL-10 and IL-4 are not expressed (Table 1) [79,115].

In our study, in IPEC-J2 the expression of TNFα at 24 h was significantly higher in the IPEC/–Arg, IPEC+PBMC and IPEC+PBMC/–Arg groups compared to control. The presence of PBMC enhanced the cellular response to arginine deprivation. TNFα, released by IECs [140], plays a key role in the proliferative processes and in starting the inflammatory cascade [141,142], stimulating the release of other cytokines.

At 24h, IPEC-J2 in the absence of arginine (IPEC/–Arg group) showed the ability to respond to the stimulus, inducing a direct pro-inflammatory response [143]. On the other hand, the co-culture system underlines the collaboration and mutual influence between the two cell systems. The increase of TNFα expression in the IPEC+PBMC group (co-culture in complete medium), compared to monoculture, support the hypothesis of a reciprocal effect between PBMC and IPEC-J2. The expression in IPEC-J2 and PBMC (co-culture) is significantly higher than the control groups in monoculture. Furthermore, a sequential response over time is also observed; the increased TNFα expression in PBMC arginine-free is subsequent (48 h) to that of IPEC-J2 (24 h).

The IECs response may have led to the upregulation of PBMC expression, to support the cellular response driven by the negative metabolic stimulus. Moreover, this may be due to an autocrine action of TNFα through its receptors, caused by the TNFα cascade [144]. Furthermore, this effect can be noted at 48 h, when the cytokine expression drastically decreases, returning to the control values. At the same time, upon reduction of CAT-1 expression, a cell viability decrease was also observed in the control group (IPEC). As previously mentioned, TNFα controls the production of other inflammatory mediators, such as IL-1α and IL-8. IL-1α functions as an "alarm", once cell death signals are recognized [145]. These stimulate IL-1α transfer from the cytosol to the nucleus; once binding to chromatin, it is not available to initiate the inflammatory process. Following a necrosis signal, IL-1α moves from the nucleus to trigger the inflammatory response [146]. In IPEC-J2, IL-1α increased in the IPEC/−Arg, IPEC+PBMC and IPEC+PBMC/−Arg groups at 48h. The trend confirms the influence of TNFα in modulating it [145,147], also in association with signals of reduction of cell viability. At 24h, IL-1α was also expressed in PBMC, possibly related to the simultaneous increase of TNFα in IPEC-J2.

Several transcription factors and TNFα modulate IL-6 gene transcription [147]. IL-6 also plays the role as an alarm signal, and its expression increases rapidly in response to local stress factors. In our model, at 24h in IPEC-J2, an increased IL-6 expression was observed in the IPEC/−Arg, IPEC+PBMC and IPEC+PBMC/−Arg groups, and as TNFα, IL-6 decreased to 48 h. IL-6 is a pleiotropic cytokine; it is a mediator of cell communication, regulating both pro- and anti-inflammatory processes [148]. Two responses are observed following binding of IL-6 to its receptor α, which exists in soluble and transmembrane forms. Binding to its membrane receptor induces classical anti-inflammatory signalling, while binding to the soluble receptor induces pro-inflammatory trans-signalling [38]. The last pathway is probably activated in our model: the pro-inflammatory response is guaranteed in synergy with the other pro-inflammatory cytokines, as shown by the increase in IL-6 expression after 24h.

TNFα also regulates IL-8 production, which is a chemokine capable of recruiting neutrophils and immune cells to the site of infection [149]. In the differentiated human cell line Caco-2, TNFα in contact with its TNFα type 1 and 2 receptors stimulates the apical and basolateral secretion of IL-8 [150]. In our study, IL-8 and TNFα showed a similar expression pattern in IPEC/–Arg, IPEC+PBMC and IPEC+PBMC/–Arg groups. Furthermore, the same trend reappears with an increase in gene expression after 24 h and a decrease after 48 h. In our model, IL-8 expression in PBMC confirms this role, showing the same expression model as TNFα.

Arginine can probably increase the secretion of anti-inflammatory factors, which is critical in modulating pro-inflammatory cytokines [151]. TGF-β is abundant in the gut [152] and is also produced by epithelial cells, and its expression and production be induced by arginine supplementation. Considering the role of TGF-β in intestinal immune cell homeostasis [33], the evaluation of TGF-β expression allowed us to characterize better the response of IPEC-J2 and PBMC to stressful conditions. At 24h, the arginine absence increase of TGF-β, as an increase of CAT-1 and NO. TGF-β is directly involved in the modulation of NO and CAT-1 synthesis in the intestine [153]. On the contrary, after 48h all three parameters were reduced, which may be related to the reduction of available arginine in the medium.

In summary, the IPEC-J2/PBMC co-culture model is functional to elucidate the interaction between IECs and immune cells. To better characterize this model, it will be necessary to investigate how gene expression is associated/correlated with the secretion of these and other mediators involved in the cross-talk between intestinal cells and immune cells. We think that applying this experimental model could be important for studying and clarifying the role of nutritional conditions in IECs functionality and responsiveness in swine and humans based on the similarities between pigs and humans in terms of gut microbiota and the mechanisms of the intestinal defence systems. Together, this approach supports the feasibility of developing a system to investigate the impact of and the interaction between specific nutrients and the complex intestinal environment. With particular reference to swine,

our cell co-culture model can be used to evaluate feed additives to improve animal health and production, identifying the interplay between IECs and immune cells in the protective response to stimuli. In addition, arginine deficiency strongly influences IECs and stimulates their functional response as a modulator of the local anti-inflammatory response to adapt and react to a stress-inducing stimulus.

5.2 Study 2

This second study aimed to evaluate the effects of some SCFA (acetate, butyrate, propionate and lactate) at different concentrations on IPEC-J2; the concentrations were chosen based on available literature data. SCFA play a key role in preventing intestinal diseases by modulating multiple effects on immune cells during intestinal inflammation and providing energy to the IECs. IECs respond by redistributing TJ and activating mucosal immune cells that secrete cytokines and chemokines.

Dietary use of SCFA, with a view to reducing antibiotic intake, exert immunomodulatory effects on intestinal inflammation [154]. It was demonstrated that high concentrations of SCFA could damage intestinal functions; therefore, controversial results have been obtained *in vivo* and *in vitro* on cell viability [155,156]. Ranges of lower and more restrictive concentrations were chosen, which could positively affect intestinal epithelial cells *in vitro*.

For this reason, the first data we analyzed concerns cell viability increased with acetate supplementation while decreased upon propionate supplementation in a dose-dependent manner. On the other hand, butyrate and lactate did not affect viability. SCFA can exert their effects on proliferation through different mechanisms, such as altering mitochondrial function and/or energy. The mitochondrion is the site of oxidation of SCFA, and their concentration play an important role in the regulation and coordination of responses in epithelial cells, including signal transduction and gene expression. [157].

A change in cell viability, as evidenced by the MTT assay, was also compared with NO production. Indeed, NO release and viability were observed in the same modulation at the corresponding concentration of acetate and propionate. We can presume that the ability of SCFA to regulate NO signalling is likely attributable to its positive effect on survival at low concentrations [158,159]. Furthermore, SCFA modulate NO signalling independently of their ability to inhibit HDAC activity, like other HDAC inhibitors [160]. On the other hand, butyrate as an HDAC inhibitor has intestinal anti-

inflammatory effects [161]. This action would support why NO increases were observed without viability changes. HDAC inhibitors induce growth arrest, activation of extrinsic and/or intrinsic apoptotic pathways, autophagic cell death, cell death induced by ROS, mitotic cell death and senescence [162].

The same effect occurred with lactate supplementation at the lowest concentrations (7.5 and 15 mM). Therefore, out of all SCFA supplementation, low NO release could also contribute to the beneficial effect [159]. NO responses are context dependent and highly dependent on nitrite levels. Low NO concentrations activate cGMP-dependent signalling pathways, which promote pro-growth and anti-apoptotic. Instead, at higher levels of NO, an increase in AKT is observed, which induces phosphorylation of Bad and caspase-6, ultimately resulting in the activation of p53, favouring pathways that induce cell cycle arrest, senescence, or apoptosis [163].

Cell viability and NO production, controlled by the level of SCFA, could be related to the alteration of TJp [164]. The absence of barrier functions occurs if there is a lack of claudin expression, which prevents the passage of luminal molecules through the paracellular pathways [165]. Increased expression of CLND4 (the only claudin expressed in our study) and increased cell viability was observed in the presence of acetate and propionate supplementation. Upon lactate and butyrate supplementation, very low levels of CLDN4 were associated with unchanged viability at all concentrations.

Although claudins are critical for integrity, their reduction can be compensated by occludins forming long filaments. Although ZO-1 and OCLN were more expressed in lactate and/or butyrate, higher NO production and unchanged viability were observed. As explained above, OCLN has less influence than CLDN4 on barrier integrity [166]. The reduced expression of ZO-1 on acetate and propionate supplements may be caused by the action of TNFα, which can induce a redistribution of TJp [152]. TNFα is secreted, enters the NF-κB pathway, stimulates cytoplasmic-nuclear translocation of NF-κB, increases binding of NF-κB to the DNA binding site,

and in turn, downregulates the expression of the ZO-1 protein, resulting in disruption of the TJ barrier [167].

Our data confirm the ability of SCFA supplementation at different concentrations to enhance the barrier function in intestinal epithelial cells, which *in vivo* react to changes in the luminal environment. SCFA have a protective effect by maintaining the integrity of the intestinal mucosal barrier and regulating innate immunity [126].

NF-κB, as a transcription factor, plays an important role in regulating the expression of genes encoding cytokines in immune and inflammatory responses [168]. Our results show that acetate, propionate, lactate and butyrate supplementation can inhibit the expression of TNFα and NF-κB. Furthermore, an important mechanism of innate immune response is related to the release of antimicrobial peptides in the lumen of the gastrointestinal tract. It allows directing the killing of bacteria (by forming micropores in the phospholipid bilayer of bacterial membranes, causing the loss of structural integrity and collapse of the bacterial cell) and viral pathogens [169].

In our study, we considered BD-1, a member of the antimicrobial peptide family. The expression pattern of BD-1 also reflects the expression of membrane proteins (ZO-1, OCLN and CLDN4), which could confirm the role of defense in maintaining barrier integrity [170]. Although BD-1 has been shown to inhibit the pro-inflammatory cytokine cascade, involving TNFα secretion and downregulation of MAPK and NF-κB signalling pathways [169]. Our results do not support a correlation between BD-1, TNFα and NF-κB gene expression. In summary, each SCFA tested has a different effect depending on a specific concentration or range. Notably, acetate and propionate improved the viability and maintenance of barrier integrity at 2.5-5mM and 1mM, respectively. On the other hand, lactate and butyrate showed a predominant effect on barrier protection at 30 mM and 0.5-1 mM, respectively.

In summary, we tested some substances used in vivo, the SCFA. We noted the direct effects on the maintenance of intestinal barrier functions, dependent on the type and concentration of SCFA. The co-culture model could allow us to investigate the

molecular mechanisms of action in the presence of an inflammatory stimulus. It might be useful to optimize formulations with different substances, such as different SCFA to enhance the beneficial effects of each.

5.3 Study 3

The third study aims to evaluate the capacity of SCFA (acetate and propionate) to interfere with and modulate the response to the stimuli mimicked by the LPS-challenge.

To better understand the effects of SCFA in co-culture in the modulation of the inflammatory response, we observed only the effects of LPS on monocultures. LPS challenge had a significant effect on IPEC-J2 and PBMC monocultures, inducing a reduction of TJp synthesis, thus promoting intestinal epithelial permeability and resulting in disruption of the intestinal barrier, which led to reduced cell viability and increased NO release.

The first data to focus on was related to the acetate action. Although NO release is related to oxidative stress, representing a potential negative condition, acetate supplementation alone in co-culture induced an increase in NO production and a concomitant increase in viability. As already clarified in previous studies, evaluating the data based on the concentration released, there is an increase in NO production, but it is a low concentration (< 10 μM). Several studies argue that low NO synthesized from constitutional nitric oxide synthase has a protective role in maintaining the integrity of the intestinal barrier [78]. Furthermore, the increase in NO could be explained by the effect of acetate on a de novo synthesis of arginine, which is both an essential amino acid promoting cell viability and a precursor of NO [171]. Indeed, upon LPS-challenge, acetate promoted the protective effect, reducing NO release and inducing IPEC-J2 to increase viability; thus, NO release could depend on the PBMC response.

In PBMC, compared with acetate treatment alone, in the presence of LPS and acetate supplementation, we observed a reduction of NF-κB expression (data not shown), which is essential for the induction of iNOS gene expression [172]. Acetate was found to have positive effects on the modulation of inflammation, considering that NF-κB is the main regulatory factor, upregulating the response of iNOS, which produces NO

with important second messenger functions involving the mediation of inflammatory events [173].

In our model, intestinal epithelial cells and PBMC viability were not affected by treatment with propionate alone or with LPS. In a previous study [131], propionate (1 mM) stimulated cell viability of the IPEC-J2 monoculture. Still, in our co-culture model, it may not be sufficient to induce a proliferative effect on both co-cultured cell types.

The effect of SCFA on NO viability and production may be related to alterations in TJp [164]. After inflammation, the reduction of CLDN4 is associated with decreased cytoplasmic protein ZO-1 and the transmembrane protein OCLN. Their falling cause damage to the barrier with functional alterations, loss of polarity due to incorrect ion localization, dysregulation of the absorption/secretion of nutrients [138], changes in cell proliferation and differentiation, and gene transcription [174].

In our co-culture model, we demonstrated that acetate significantly enhanced the expression and synthesis of TJp, especially CLDN4, affecting endothelial barrier function, and this modulation was related to cell viability. Conversely, although propionate supplementation induced the increased expression of OCLN, this TJp has a minor influence of CLDN4 on barrier integrity [166]; in fact, no changes in cell proliferation were observed.

The presence of acetate or propionate had the same impact on the LPS challenge; we could consider their protective role on the intestinal epithelial barrier due to the stimulation of TJp synthesis. We can speculate that this protective effect is attributable to HDAC inhibition. Through this inhibition, SCFA increases the acetylation of histone and non-histone proteins from chromatin, thereby modulating transcription factors and gene expression [175].

In summary, our results suggest that propionate and acetate reduce LPS-induced NO production in intestinal epithelia and immune cells and strengthen cell-cell contacts of

cells by increasing membrane TJp, which could limit the negative effect of an acute inflammatory condition.

6. Conclusion

It would be interesting to use the co-culture model to study the effects of different SCFA in modulating the inflammatory response to a prolonged stimulus such as LPS-challenge or PHA or pathogens (bacteria, viruses), which could mimic chronic inflammation and infection *in vivo*. It could be evaluated the gene of structural proteins and inflammatory mediators such as cytokines, chemokine, growth factor, antimicrobial peptide can be couple with the protein evaluation by using different molecular biology (ELISA, western blotting, immunochemistry).

In fact, inflammatory diseases such as IBD are often linked to periods of acute activity induced by viruses, bacteria, and parasites, which leads to a dysregulation of the intestinal immune response. Inflammatory bowel disease can have a strong negative impact on animal health, welfare and farm costs. Despite the antibiotics tend to be used continuously, an ideal approach for the long-term treatment of IBD should take into account diet or a safe and well-tolerated therapy that reduce mucosal inflammation and maintain a correct balance of the intestinal microbiota. In particular, a combined therapy of antimicrobial agents to reduce the antigenic load and immune system modulators (i.e probiotics, prebiotics, short-chain fatty acid) to influence the dysregulated responses can be proposed.

7. References

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The researchers reported in this PhD thesis were published in international open access peer reviewed scientific journals:

Saleri, Roberta, Paolo Borghetti, Francesca Ravanetti, Melania Andrani, Valeria Cavalli, Elena De Angelis, Luca Ferrari, and Paolo Martelli. 2021. "**A Co-Culture Model of IPEC-J2 and Swine PBMC to Study the Responsiveness of Intestinal Epithelial Cells: The Regulatory Effect of Arginine Deprivation.**" *Animals* 11, 2756.

https://doi.org/10.3390/ani11092756.

Saleri, Roberta, Paolo Borghetti, Francesca Ravanetti, Valeria Cavalli, Luca Ferrari, Elena De Angelis, Melania Andrani, and Paolo Martelli. 2022. "**Effects of Different Short - Chain Fatty Acids (SCFA) on Gene Expression of Proteins Involved in Barrier Function in IPEC-J2.**" *Porcine Health Management*, 8:21, 1–13.

https://doi.org/10.1186/s40813-022-00264-z.