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Titolo della tesi

IN VITRO INTESTINAL EPITHELIAL CELL AND FIBROBLAST MODELS TO INVESTIGATE THE ANTI-INFLAMMATORY AND ANTI-FIBROTIC EFFECTS OF HIGH CONCENTRATION MULTI-STRAIN PROBIOTIC FORMULATIONS

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Abstract

The oral administration of probiotics has been shown to exert positive effects on chronic inflammatory disorders, such as inflammatory bowel disease (IBD). IBD is a recurrent inflammatory disease of the gastrointestinal tract. Several studies have demonstrated that hypoxia plays a key role in IBD. Hypoxia responses are modulated and affected by oxygen-sensitive transcription factors such as hypoxia-inducible factor (HIF). Hypoxia-inducible factor-1α (HIF-1α) plays a pivotal role in inducing adaptive mechanisms to hypoxia and is negatively regulated by prolyl hydroxylase 2 (PHD2). Moreover, HIF-1α is stabilized through PI3K/AKT signaling regardless of oxygen levels. Considering the crucial role of the HIF pathway in intestinal mucosal physiology and its relationships with gut microbiota, the first objective of this study aimed to evaluate the ability of the lysate from the multi-strain probiotic formulation SLAB51 to affect the HIF pathway and to counteract the inflammatory stimulus of lipopolysaccharide (LPS) in a model of in vitro human intestinal epithelium (intestinal epithelial cells, IECs). The exposure of IECs to SLAB51 lysate under normoxic conditions led to a dose-dependent increase in HIF-1α protein levels, associated with higher glycolytic metabolism and L-lactate production. Probiotic lysate significantly reduced PHD2 levels and HIF-1α hydroxylation, thus leading to HIF-1α stabilization. SLAB51 lysate ability to increase HIF-1α levels was also associated with the activation of PI3K/AKT pathway and with the inhibition of NF-κB, nitric oxide synthase 2 (NOS2), and IL-1β increase elicited by LPS treatment. Our results suggest that the probiotic treatment, by stabilizing HIF-1α, can protect from LPS-induced inflammatory response through a mechanism involving PI3K/AKT signaling.

An important complication associated to IBD is intestinal fibrosis. To date, no specific anti-fibrotic therapy exists to prevent or reverse intestinal fibrosis. Only few studies are available regarding the effects of probiotics on IBD-associated intestinal fibrosis. Therefore, the other aim of the present project has been to investigate the potential anti-fibrotic effect of two different high-concentration multi-strain probiotic formulations (VSL#3® and Vivomixx®) on CCD-18Co human intestinal fibroblasts activated by TGF-β1. CCD-18Co cells activated *in vitro* by TGF-β1 represent a model of intestinal fibrosis widely used in experimental studies. In particular, the cell growth and viability, Collagen-I, α-SMA, pSmad2/3 expression levels, and TGF-β1 levels were evaluated. The results suggested that Vivomixx® probiotic lysate, but not VSL#3® lysate, was able to counteract the pro-fibrotic effects of TGF-β1 on CCD-18Co cells. The mechanism underlying the observed effect appeared to be related to inhibition of the TGF-β1/Smad pathway. In conclusion, the results provide valuable evidence of Vivomixx®'s role as a promising therapeutic strategy against intestinal fibrosis in Crohn's patients.

1 Introduction

1.1. Probiotic

The word "probiotic" comes from Greek and means "for life". The concept of probiotic was developed in around 1900 by the Nobel Prize, Elie Metchnikoff, who discovered that the consumption of live bacteria (*Lactobacillus bulgaricus*) in yogurt or fermented milk improved some biological features of the gastrointestinal tract. Most probably, the term "probiotic" was used for the first time by Ferdinand Vergin in 1954 in his article entitled "Anti-und Probiotika", in which several microorganisms were studied to make a list of useful bacteria and to determine the detrimental effects of antibacterial agents and antibiotics on the intestinal microbiota. A few years later, Lilly and Stillwell described probiotics as beneficial microorganisms that exert growth-promoting factors for other microorganisms (Azad *et al*. 2018). Currently, the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) state probiotics as "*live microorganisms that, when administered in adequate amounts, confer a health benefit on the host*" (FAO/WHO 2002) . The most common microorganisms used as probiotics are lactic acid bacteria (LAB), *Bifidobacterium, Bacillus*, and yeasts (Pereira *et al*. 2018) (**Figure 1**). Among these, *Lactobacillus*, under the LAB group, was the first probiotic discovered. This genus of gram-positive bacteria comprises 183 recognized species, applied in several industrial processes such as those used to produce preservatives, acidulants, and food flavorings, drug and cosmetic manufacture. *Lactobacillus,* including *L. acidophilus, L. fermentum, L. plantarum, L. casei, L. paracasei, L. reuteri, L. rhamnosus, L. satsumensis*, and *L. johnsonii*, is the dominant LAB group in animal and human gastrointestinal and digestive systems, possessing proven action in the maintenance and recovery of health. Other LAB genera with demonstrated probiotic action include *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, and *Leuconostoc*. Metabolically, LAB are classified as homofermentative, which produce exclusively lactic acid through the Embden-Meyerhof-Parnas pathway, or heterofermentative, which, in addition to lactic acid, produce several other metabolites, including ethanol, acetic acid, and carbon dioxide via the pentose monophosphate pathway. LAB can also produce secondary metabolites, including bacteriocins, exopolysaccharides, and enzymes, which are used to increase the quality and shelf life of fermented foods (Pereira *et al*. 2018). The Frenchman Henry Tissier isolated *Bifidobacterium* at the end of the 19th century. These probiotics are heterofermentative, nonmotile, catalase-negative, and anaerobic bacteria with the ability to metabolize glucose, galactose, lactose, and fructose. Presently, the genus *Bifidobacterium* includes

30 species, where 10 are from humans, 17 from animals, two from residual waters, and one from fermented milk. *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum* have been reported for their various probiotic effects. *Bacillus* species (*Bacillus clausii* and *Bacillus subtilis*) are also utilized as probiotics in the food and pharmaceutical industry (Liu, Wu, *et al*. 2018). The main feature of this gram-positive, aerobic bacteria group is the formation of endospores that permit viability maintenance for long shelf periods. Yeasts are a large and heterogeneous group of eukaryotic microorganisms that are widespread in natural environments, including the gastrointestinal tract of humans, plants, airborne particles, and food products. The high content of proteins, vitamin B, traces of minerals, and various immune-stimulant compounds (proteases, β-glucans, and mannan oligosaccharides) has increased the interest in the use of yeasts as a probiotic (Arevalo-Villen *et al*. 2018; Fadda *et al*. 2017). Moreover, yeasts have the advantages of nonsusceptibility to antibiotics and good tolerance for industrial processing conditions. For example, *Saccharomyces boulardii*, according to The European Food Safety Authority (EFSA), has a Qualified Presumption of Safety (QPS) status (Hudson *et al*. 2016).

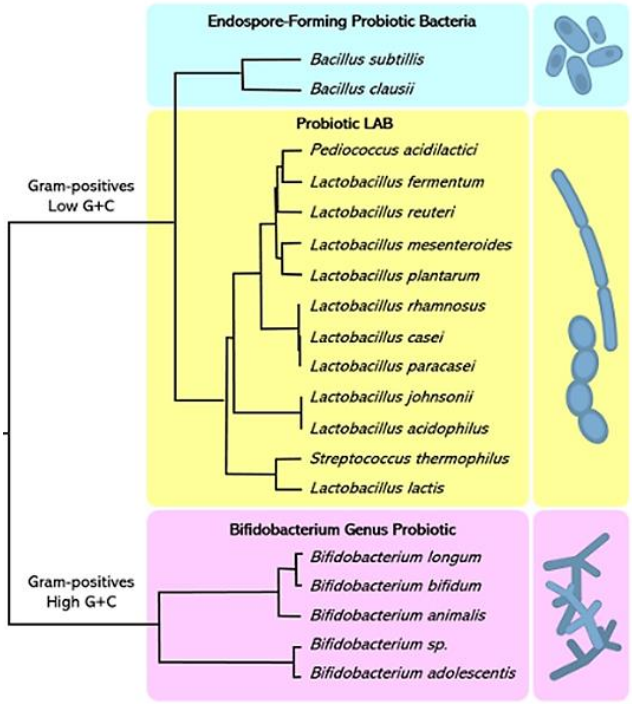


Figure 1: Probiotic microorganisms used in human nutrition (Pereira *et al*. 2018).

Additional exploration of probiotics has led to the development of *prebiotics*. The term prebiotics is defined as "*non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon for*

improving the host health" (Gibson and Roberfroid 1995). Recently, an expert panel of the International Scientific Association for Probiotics and Prebiotics (ISAPP) updated the definition of prebiotics in wider perspective as "*a substrate that is selectively utilized by host microorganisms conferring a health benefit*" (Gibson *et al*. 2017). These are generally short-chain carbohydrates which escape digestion but are used as substrates for the growth of probiotics in the upper gastrointestinal tract. Other compounds that are recommended to be prebiotics are cocoa-derived flavanols. *In vivo* and *in vitro* experiments demonstrate that flavanols can stimulate the growth of LAB.

High potential is attributed to the simultaneous use of probiotics and prebiotics. In 1995, Gibson and Roberfroid introduced the term "*synbiotic*" to describe a combination of synergistically acting probiotics and prebiotics. As the word "synbiotic" implies synergy, the term should be reserved for those products in which a prebiotic component selectively favours a probiotic microorganism. The principal aim of that type of combination is the improvement of the survival of probiotic microorganisms in the gastrointestinal tract (Markowiak and Slizewska 2017). Additionally, there is a growing interest in the application of *postbiotics* as a safer alternative to probiotics (Zolkiewicz *et al*. 2020). The term refers to all products obtained from non-viable probiotic microorganisms, including non-viable microbial cells, cell walls, lysates, fractions, secretions, components, and metabolites that, when received in adequate quantities (postbiotic supplements) confer benefit health on the host. Furthermore, the postbiotics used in a delivery system strengthen the endogenous probiotics of each host (Rad *et al*. 2020).

1.1.1 Selection criteria for probiotic strains

According to the suggestions of the WHO and FAO, in their selection process, probiotic strains must meet the following properties:

Stress tolerance ability

Strains of probiotics are evaluated to cope with the stress conditions that the human body imposes. The probiotic should be resistant to enzymes present in the oral cavity (amylase and lysozyme), and to antimicrobial factors in the stomach (low pH, gastric juice, and pepsin) and intestines (pancreatin and bile salts). Gastrointestinal resistance varies according to species; for example, *Lactobacilli* are broadly resistant, whereas *Bifidobacteria* are extremely sensitive to low pH, exhibiting low or no survival rates at pH 2 and pH 3. pH ranging from 2 to 5 and bile salt concentrations from 0.3 to 2% are considered critical for the selection of probiotic microorganisms (Pereira *et al*. 2018).

Adhesion ability

Microbial adhesion to the gastrointestinal tract epithelial cells is a complex process that depends on the chemical and physiochemical composition of the probiotic strain cell surface. Adhesion of microbes to epithelial cells is related to both the autoaggregation capacity and hydrophobic properties of the cell surface. Microbial cell's autoaggregation ability ensures that the probiotic reaches a high cell density in the gut, contributing to the adhesion mechanism, while cell surface hydrophobicity allows an improved interaction between microbe and human epithelial cells (Pereira *et al*. 2018).

Anti-pathogenic activity

Once adhered to the gut, probiotics produce extracellular antimicrobial components through the conversion of carbohydrates, proteins, and other compounds into substances that can kill pathogenic bacteria, such as organic acids, enzymes, hydrogen peroxide, bacteriocins, and low-molecular-mass peptides. Other mechanisms of antagonism by probiotics include competition for nutrients, coaggregation with pathogens, and immune system stimulation. These antagonist activities vary according to microbial strains (Vera-Pingitore *et al*. 2016; Veron *et al*. 2017). Coaggregation is another antagonistic probiotic activity that provides pathogen agglomeration with probiotic cells and facilitates its elimination through feces.

Safety assessment

Commercially available probiotic products can be divided into mono-strain (containing one strain of a defined microbial species) and multi-strain (containing more than one strain of the same species or genus). The treatment with probiotics may involve the consumption of large quantities of bacteria, so safety is a major concern. There are two aspects to safety: establishing the safety of the products per se and ensuring that marketed preparations meet stringent quality standards to make certain that the correct strains are present and that the product is free of contamination (Sanders *et al*. 2016). The main risks from probiotics are infection, effects of toxins produced either by the probiotic strains or contaminants and immunological effects. Safety assessments should take into account the nature of the probiotic microbe, administration method, exposure level, health status of the recipients, and the physiological functions the microbes are intended to perform. All common species of probiotics are considered safe for the population by EFSA, although this definition does not provide guidance on the increasing use of probiotics in people with disease (de Simone 2018). Most clinical probiotic studies reported in the literature did not raise significant safety concerns. However, some examples of serious adverse effects from probiotics have been documented

independently of the formulation, dosage, and daily intake. Those that have been reported include cases of bacterial sepsis linked to probiotic supplements containing *Lactobacillus* (Sanders *et al*. 2016). In patients with severe acute pancreatitis, treatment with a multi-strain probiotic preparation was associated with an increased risk of mortality. Thus, careful safety evaluation is required before the use of probiotics in vulnerable groups, including patients with damaged intestinal mucosa or immune dysregulation, such as can occur in patients with inflammatory bowel diseases, HIV, and other diseases (de Simone 2018).

1.1.2 Mechanisms of action of probiotics

The mechanisms of action of probiotics are complex, heterogeneous, and specific to probiotic strains. They include competitive exclusion of pathogens (Yan and Polk 2020), the ability to colonize the intestine (Wang *et al*. 2018), intestinal barrier function improvement (Gaspar *et al*. 2018)' and immune system modulation (**Figure 2**).

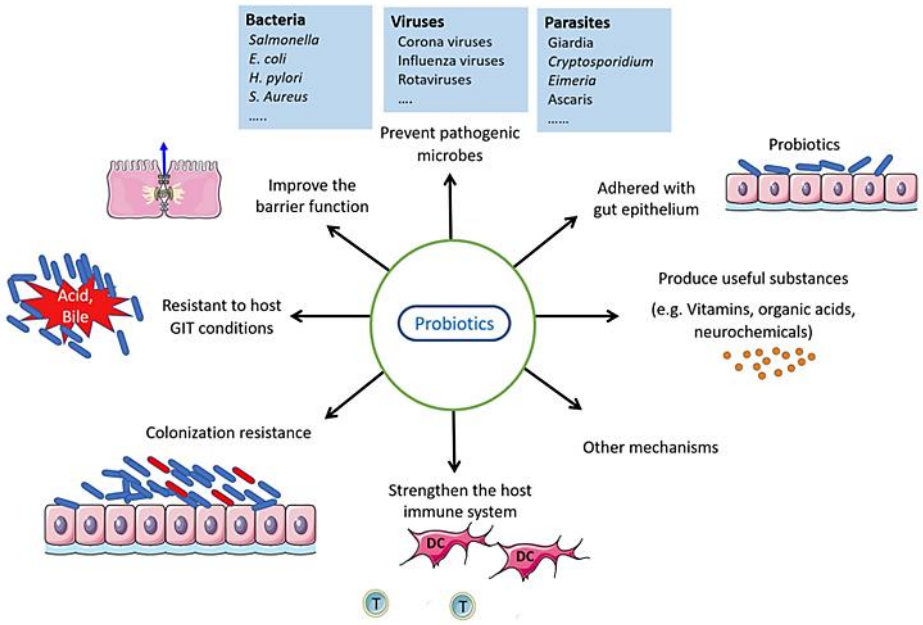


Figure 2: Probiotics properties and their mechanisms (Raheem *et al.* 2021).

Competitive pathogen exclusion

Competitive pathogen exclusion is a condition in which one bacterial species has a greater potential to attach the epithelia, through a receptor, than other species. Known competitive exclusion mechanisms mainly include lowering pH in the lumen, competition for available nutrients, and production of adenosine monophosphate (AMP) against competitors. Interaction between molecules

distributed in the gut epithelia and the surface of bacterial cells mediates the adhesion and colonization of bacteria. Commensal or probiotic bacteria produce adhesive surface molecules (enolases, glyceraldehyde-3-phosphate, and pyruvate dehydrogenase) allowing to counteract and prevent pathogenic bacterial attachment and colonization (Fukuda 2017). *Lactobacillus fermentum* competitively binds to collagen I of host epithelial cells and inhibits the binding of *Campylobacter jejuni*. Similarly, *Lactobacillus gasseri* expresses self-aggregation-promoting factors and its binding with the host extracellular matrix fibronectin. This facilitates the colonization of probiotics and the exclusion of pathogens from the gastrointestinal tract (Nishiyama *et al*. 2015). Competitive exclusion by probiotics exerts beneficial effects on the gastrointestinal tract and other organs of the host, increases the adhesion of probiotics, and performs protective actions against pathogens by competing for binding sites of the host. Furthermore, this adhesion of probiotics increases the opportunity for interaction with the host, which favors the immunostimulatory effects of probiotic surface molecules and their metabolites (Celebioglu *et al*. 2017; Monteagudo-Mera *et al*. 2019). Therefore, these properties of probiotics offer several benefits to host health, including the reduction of pathogenic attachment, colonization, the spread of the pathogen, and pathogenic load in hosts (Raheem *et al*. 2021).

Intestinal colonization ability

The potential of probiotics to colonize the intestine is one of the main properties recommended by WHO/FAO. The positive characteristics of probiotics, such as antagonism to harmful microbes or the modulation of the immune system, are linked to their intestinal colonization (Falah *et al*. 2019). Interaction between molecules distributed on gut epithelia and the surface of bacterial cells mediates the adhesion and colonization of bacteria and is highly variable between different bacterial strains.

Intestinal barrier function improvement

The intestinal epithelial barrier acts as a physical and biochemical barrier and is important to prevent the systemic entry of toxins, bacteria, and other foreign unwanted compounds. It has been reported in many studies that LAB can improve intestinal epithelial barrier damage induced by pathogenic infection (Heeney *et al*. 2019). Strains of *Lactobacillus, Bifidobacterium,* and *Streptococcus* upregulate tight junction proteins (occludin, claudin-1) resulting in enhanced barrier stability (Wang and Kasper 2014). *Lactobacillus plantarum* WCFS1 significantly increases occludin and zonulin-1 (ZO-1) expression by Toll-like receptor 2 (TLR2) dependent pathway and protects against tight junction disruption by toxins, pathogens, and cytokines (Karczewski *et al*.

2010). Qin *et al*. also showed that *L. plantarum* has protective effects on the intestinal barrier by rearranging tight junction proteins (occludin, claudin-1, and ZO-1) damaged by *Escherichia coli* and ameliorates barrier function (Qin *et al*. 2009). *E. coli* Nissle 1917 ameliorates *E. coli* induced intestinal epithelial barrier dysfunction by regulating the expression of occludin and claudin (Alvarez *et al*. 2019). *Lactobacillus rhamnosus* and *Lactobacilluus fermentum* significantly improve the *E. coli* disturbed tight junction proteins (occludin, ZO-1, cingulin-1, claudin-1) in Caco-2 cells (Bhat, Kapila, and Kapila 2020). Several studies on *Lactobacilli* have also shown that they ameliorate the intestinal barrier damage and pro-inflammatory cytokines production induced by *Salmonella* (Wang *et al*. 2019).

Bacteriocins production

Bacteriocins are antimicrobial peptides that differ in their size (2–10 kDa) and mechanisms of action. Production of bacteriocins by probiotics (usually LAB) is a key mechanism of action used to inhibit pathogens in the gastrointestinal tract (van Zyl, Deane, and Dicks 2020). Bacteriocins may have a bacteriostatic or direct bactericidal effect on pathogens, limiting the ability of the cells to colonize the gut. Bacteriocins adhere to microbial cells and penetrate phospholipid membranes because of the small size and variations in hydrophobic and hydrophilic properties. The general mechanisms of bacteriocin-mediated pathogen killing include the induction of cytoplasmic membrane permeabilization of sensitive bacteria, inhibition of DNA and RNA synthesis, and cell wall protein synthesis.

Bacteriocins possess immunomodulatory properties with pronounced anti-inflammatory effects during pathogenic infections. Several studies showed that certain kinds of probiotics inhibit many types of pathogenic bacteria by the action of their bacteriocins (Mandal *et al*. 2016). Bacteriocins from *Lactobacillus salivarius* inhibit bacteria, such as *Listeria monocytogenes*, many genera of *Staphylococcus*, *Neisseria gonorrhoeae*, *Bacillus*, and *Enterococcus*. *Lactobacillus plantarum* also exerts antimicrobial activities by producing many types of bacteriocins with antimicrobial effects against food spoilage bacteria, such as *Alicyclobacillus acidoterrestris* (Borrero *et al*. 2018), *Listeria monocytogenes*, *Staphylococcus aureus*, and *Escherichia coli* (Oleskin and Shenderov 2019). Apart from bacteria, some bacteriocins from *L. plantarum* are also effective against yeast and molds, such as *Fusarium*, *Candida*, and *Aspergillus*. Bacteriocins of other probiotic species markedly induce apoptosis and inhibit tumor formation, cancer cell proliferation, and membrane depolarization of cancer cells during treatment (Baindara, Korpole, and Grover 2018).

Immune system modulation

It is well known that probiotics can exert regulatory effects on host innate and adaptive immune responses. Probiotics have the ability to modulate the functions of dendritic cells (DCs), monocytes/macrophages, and T and B lymphocytes. By stimulating of the host immune responses, probiotics can displace pathogens in the gastrointestinal tract and prevent intestinal diseases. Probiotics are able to trigger an anti-inflammatory response from the innate immune system by signaling DCs to secrete anti-inflammatory cytokines such as interleukin 10 (IL-10). Moreover, they can also downregulate the secretion of pro-inflammatory cytokines during inflammation (van Zyl, Deane, and Dicks 2020). Downregulation of pro-inflammatory cytokine secretion from immune cells occurs as a result of inhibition of inflammatory signaling such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) pathways. Activation of these signaling pathways leads to the secretion of pro-inflammatory cytokines that can damage the intestinal epithelial barrier. NF-κB and MAPK pathways are activated by enteric pathogens to stimulate the secretion of pro-inflammatory cytokines, that lead to the recruitment of inflammatory immune cells to the infection sites resulting in severe inflammation, tissue damage, and disease (Llewellyn and Foey 2017). Several studies have identified probiotic strains with the ability to suppress the production of pro-inflammatory cytokines to avoid pathogen-induced inflammation at infection sites (van Zyl, Deane, and Dicks 2020). A study reported that *Lactobacillus amylovorus* DSM 16698 protected intestinal epithelial cells (IECs) against the pro-inflammatory response induced by the enterotoxigenic *E. coli* Pb176 (ETEC) through the suppression of pro-inflammatory cytokines IL-8 and IL-1β (Finamore *et al*. 2014). Another study showed the ability of *L. casei* OLL2768 to suppress ETEC-induced pro-inflammatory response by inhibition of NF-κB and MAPK pathways (Takanashi *et al*. 2013).

1.1.3 Probiotic applications

Recent preclinical and clinical trials have demonstrated the importance of probiotic intake in the prevention and treatment of several diseases as cancer (Vivarelli *et al*. 2019), neurodegenerative (Li *et al*. 2020), and metabolic disorders (Liu *et al*. 2021).

Cancer

Cancer is the second cause of death among non-communicable chronic diseases. Current cancer treatments are based on chemotherapy, radiotherapy, and immunotherapy. Clinical and preclinical studies have been conducted to evaluate the efficacy of probiotics administration to manage cancer

by improving intestinal eubiosis (Vivarelli *et al*. 2019). The anticancer activity of probiotics lies stabilizing of intestinal microflora, strengthening the gastrointestinal tract mucosal barrier, enhancing the anti-inflammatory response, and the delaying cancer cell proliferation and metastasis development (Eslami *et al*. 2019). Currently, *Lactobacillus rhamnosus* GG is one of the most widely investigated strains for improving oncology-therapy-based side effects. The antiproliferative or antimetastatic effects of *L. rhamnosus* GG have been extensively evaluated using *in vitro* models of breast, hepatic, ovary, and colorectal cancer. Besides this, *L. rhamnosus* GG modulates the immune system by promoting the inhibition of tumor cells' outgrowth (Behzadi, Hosseini, and Fooladi 2017; Mendes *et al*. 2018). The anti-inflammatory potential of probiotics can counteract cancer development under various discrete pathways, such as increased secretion of immunoglobulin A levels, regulation of cyclooxygenase-2, and modulation of NF-kB and interferon-γ (IFN-γ) (Shamekhi *et al*. 2020). Furthermore, the suppression of tumorigenesis by probiotics may be related to short-chain fatty acids (SCFAs) production, gut pathogen inhibition, intestinal enzyme activation, secondary bile acid reduction, and potential carcinogenic molecule trapping. Lagier *et al.* identified 11 human microbiota-based probiotic strains having the potential to trigger anticancer and anti-infectious immunity (Lagier *et al*. 2019).

Neurodegenerative disorders

Alzheimer's disease (AD) is the most prevalent neurodegenerative form of dementia characterized by a gradual decline in memory, thinking, and reasoning response. There are several factors involved in AD pathogenesis, which include increased intestine barrier permeability, higher pro-inflammatory cytokines production, and impaired mitochondrial functionality, leading to excessive amounts of reactive oxygen species (ROS) and oxidation (Ton *et al*. 2020). Extensive scientific research indicates that gut microbiota plays a crucial role in AD neurodegenerative mechanisms. Neuroactive compounds like dopamine, melatonin, serotonin, and γ-aminobutyric acid (GABA) are produced by gut microbiota (Szczechowiak, Diniz, and Leszek 2019). However, some gut bacteria synthesize detrimental lipopolysaccharides and amyloid peptides, inducing inflammation in patients with AD. Instability in gut-barrier function also contributes to neuroinflammation through contact of gut microbiota with lymphoid tissues (Jiang *et al*. 2017). Recently, *in vivo* studies have shown the positive influence of oral bacterio-therapy on gut microbiota modification, which indirectly improves neural functionality. The most common feature of AD is cognitive deficits that could be improved by administering *Lactobacillus* and *Bifidobacterium* species, as shown in a recent *in vivo* study (Mancuso and Santangelo 2018). However, Sun Z. *et al*. conducted an extensive *in vivo* investigation by using the probiotic *Clostridium butyricum* to retard neuroinflammation (Sun *et al*.

2020). The probiotic intervention considerably averted cognitive impairment, neurodegeneration, microglia stimulation, β-amyloid accumulation, and production of tumor biomarkers (IL-1β and tumor necrosis factor α (TNF-α)). The modulations of gut microbiota and metabolic butyrate are also key phenomena observed in this study. Abraham *et al.* suggested a combo strategy of exercise and probiotics supplementation, i.e., *Bifidobacterium longum*, *Lactobacillus acidophilus* lysates, vitamins, and omega 3 fatty acids against AD (Abraham *et al*. 2019). In such *in vivo* study, the cognitive deficit reduction was related to gut microbiota regulation, improved butyrogenesis, and decreased β-amyloid plaque level. Cerebral glucose uptake and metabolism impairment proved to be strongly associated with the pathogenesis of AD. This mechanism has been recently highlighted in mice. The oral administration of SLAB51 induced a gut microbiota prompt modification, with reference to energy metabolism and glycolysis cycle through modulation of glucose transporters and insulin growth factor-1 receptor β. Amelioration of cognitive impairment was supported by the modulation of the proteolytic cycle, reduction in β-amyloid plaques, and increase in concentrations of gut microbiota neuroprotective hormones (Abraham *et al*. 2019).

Parkinson's disease (PD) is the second most common neurodegenerative disorder, and it occurs because of the dysfunction of the motor system that triggers impairment of dopaminergic neurons in the substantia nigra. The principal symptomatic characteristics of PD are postural variability and muscle stiffness. However, non-motor manifestations were also identified, including gastrointestinal dysbiosis, olfactory impairment, and emotional and sensorial instability (Gazerani 2019). Preclinical and clinical studies toward beneficial aspects of probiotics are yet in the narrow spectrum. Magistrelli *et al.* conducted an *in vitro* intervention study by using *Bifidobacterium* and *Lactobacillus* probiotic strains on isolated peripheral blood mononuclear cells obtained from PD patients (Magistrelli *et al*. 2019). Promising and effective results were reported through a reduction of oxidative stress and inflammation, along with an improved gut barrier function. However, Hsieh *et al. in vivo* evaluated the neuroprotective efficacy of daily and long-term consumption of probiotics on dopaminergic neurons (Hsieh *et al*. 2020). Results showed that regular administration of probiotics reduced motor impairments in gait pattern, balance function, and motor coordination. Probiotics can protect dopaminergic neurons and prevent motor dysfunction. Analogously, the formulation SLAB51, a combination of eight different strains of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* species, was able to counteract the detrimental effect of 6-hydroxydopamine *in vitro* and *in vivo* models of PD (Castelli *et al*. 2020).

Metabolic disorders

Obesity is considered a global epidemic resulting in abnormal or excessive accumulation of fat that may be harmful to human health. Its etiology has been primarily associated with an energy imbalance along with other factors such as genetic, environmental, hormonal, and infectious agents. The imbalance of the intestinal microbiota directly influences metabolic mechanisms, which leads to several metabolic disorders, including obesity. *Bifidobacterium* and *Lactobacillus* spp. have been associated with improving barrier integrity. Krumbeck *et al*. described the symbiotic impact of galacto-oligosaccharides and *Bifidobacterium* strains on improving intestinal barrier function, but no synergistic effects were reported (Krumbeck *et al*. 2018). On the other hand, Ipar *et al*. demonstrated the symbiotic effect of *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, plus fructo-oligosaccharides in obese children (Ipar *et al*. 2015). After 4 weeks, probiotics-treated children experienced a significant reduction in total cholesterol, low-density lipoprotein cholesterol, and oxidative stress levels. According to Kobyliak *et al*. a combination of probiotics and omega-3-fatty acids proved to be effective in counteracting obesity in an *in vivo* trial (Kobyliak *et al*. 2018). A recent investigation demonstrated that the antiobesity impact of probiotics is strain specific. Various *in vitro* and *in vivo* studies demonstrated that probiotics, specifically *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus gasseri*, *Lactobacillus plantarum*, *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium animalis*, are widely associated with weight and fat mass index reduction (Ejtahed *et al*. 2019).

Type 2 diabetes mellitus (T2DM) is the fourth most common non-transmissible disease. T2DM has multifactorial roots, in particular physiological, psychological, genetic, and environmental factors. Recent studies reported that gut microbiota plays a critical role in the occurrence and management of T2DM (Vallianou, Stratigou, and Tsagarakis 2018). Probiotics can regulate insulin secretion and intestine hormonal secretion. The therapeutic potential of probiotics against T2DM is based on the improvement of the gut barrier function, the increase in insulin sensitivity and the reduction in serum lipopolysaccharides levels, *Lactobacillus acidophilus* KLDS1.0901 is a novel promising antidiabetic probiotic strain, as demonstrated by *in vitro* dipeptidyl peptidase IV inhibitory activity along with increased blood glucose level balance and retarded insulin resistance and oxidative stress (Yan and Polk 2020). An *in vivo* probiotic study with yeast *Saccharomyces boulardii* provided promising results in controlling the glycemic level and reducing inflammatory responses and cardiovascular disorders (Brandao *et al*. 2018).

1.2 Intestinal homeostasis

In mammals, the single-layered intestinal epithelium that lines the lumen of the small and large intestine performs important functions in food digestion and nutrient absorption. In addition, the intestinal epithelium serves as a front line of defense against pathogenic bacteria and toxins introduced into the lumen (**Figure 3A**). To maintain epithelial integrity, epithelial cells are continually replaced by multipotent intestinal stem cells (ISCs)-derived proliferating progenitors, with a turnover time of 3-5 days under normal conditions. The epithelium in the small intestine is organized into crypt-villus units. Villi, finger-like projections, are essential for nutrient absorption. At the base of each villus, invaginations form the crypt where ISCs reside (**Figure 3B**). Comparatively, the colon does not have villi but is comprised only of crypts (Zhu, Hu, and Xi 2021) (**Figure 3C**).

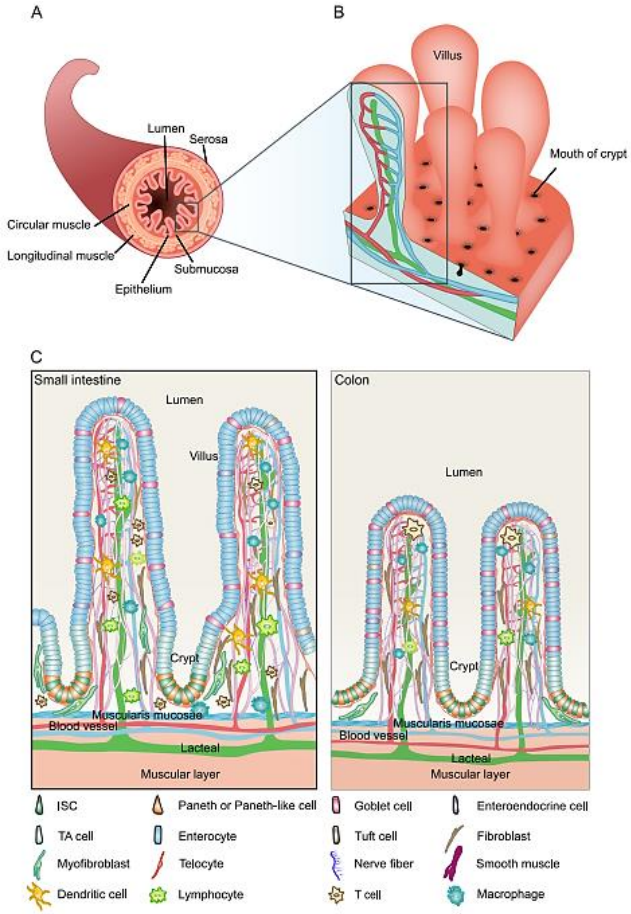


Figure 3: Anatomy of mammalian small intestine and colon. (**A**) Overall tissue structure of the small intestine. (**B**) Luminal surface of the small intestine. Each villus is surrounded by on average six crypts. (**C**) Cellular architecture of the small intestine (left) and colon (right) (Zhu, Hu, and Xi 2021).

Besides the epithelial layer, the intestine is composed of three additional layers:

1. Lamina propria: layer of loose connective tissue that contains a complex of cell populations, including fibroblasts, pericytes, and endothelial cells that constitute the vascular and lymphatic capillaries, and neural cell types, as well as scattered immune cells.
2. Circular and longitudinal smooth muscle layers.
3. Serosa: outermost layer enveloping the gut tube (**Figure 3C**).

The ISCs in the small intestine reside at the bottom of the crypt, and each crypt contains 12-16 ISCs. The ISCs are interspersed with a similar number of post-mitotic Paneth cells that occupies the bottom of the crypt. ISCs divide about once every 24 h. Once the ISCs leave away from the bottom of the crypt, they give rise to highly proliferative but short-lived progenitor cells in the transit amplifying (TA) region (Zhu, Hu, and Xi 2021) (**Figure 4A**). They pass through 4-5 cell divisions before terminal differentiation towards various lineages of mature epithelial cells including, the secretory Paneth cells, goblet cells, enteroendocrine cells and tuft cells, and the absorptive enterocytes. Most of them move upwards as differentiating to the flanks of intestinal villi to replenish the shedding epithelial cells at the top of villi, while the differentiated Paneth cells move back to the stem cell compartment to be interspersed with ISCs (van der Flier and Clevers 2009) (**Figure 4A** and **4B**).

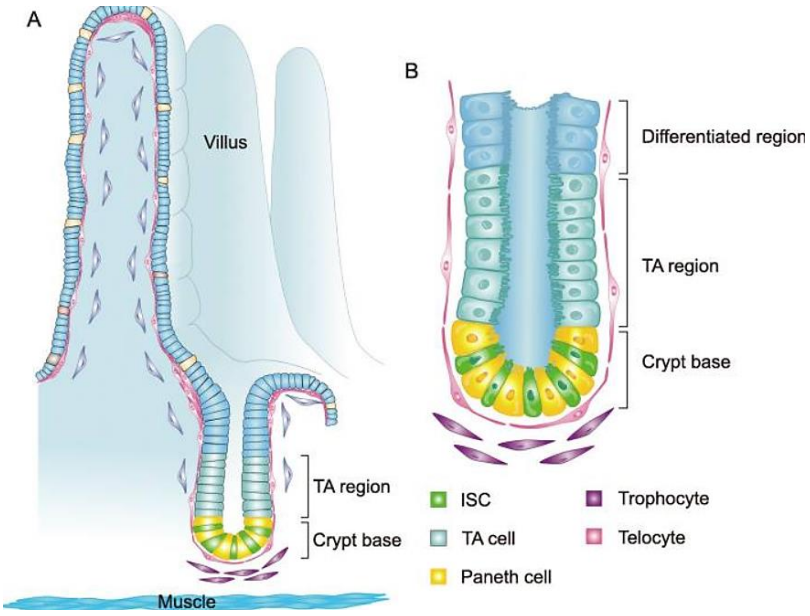


Figure 4: The ISC compartment and mesenchymal niche cells. (**A**) The cellular composition of the intestinal epithelium and the mesenchymal layer right beneath the epithelium. The ISCs are located at the bottom of crypt and are interspersed between Paneth cells. In the sub-epithelial compartment, the large flat telocytes form a sub-epithelial network to envelop the entire epithelium. The two clustered telocyte regions are found at the crypt-villus boundary and the villus tip. The trophocyte cluster is located in the pericryptal region. (**B**) The structure of the crypt (Zhu, Hu, and Xi 2021).

Oxygen (O_2) concentrations along the mammalian intestinal tract are lower than in other tissues and in the atmosphere (Taylor and Colgan 2017). A vertical O_2 gradient has been observed in more distal, colonic regions of the gastrointestinal tract, from the anaerobic lumen across the epithelium to the highly vascularized sub-epithelial mucosa (Colgan, Campbell, and Kominsky 2016). Intestinal O_2 concentrations are influenced by two processes such as oxygenation by the bloodstream and O_2 consumption by host cells and microbiota components (**Figure 5**).

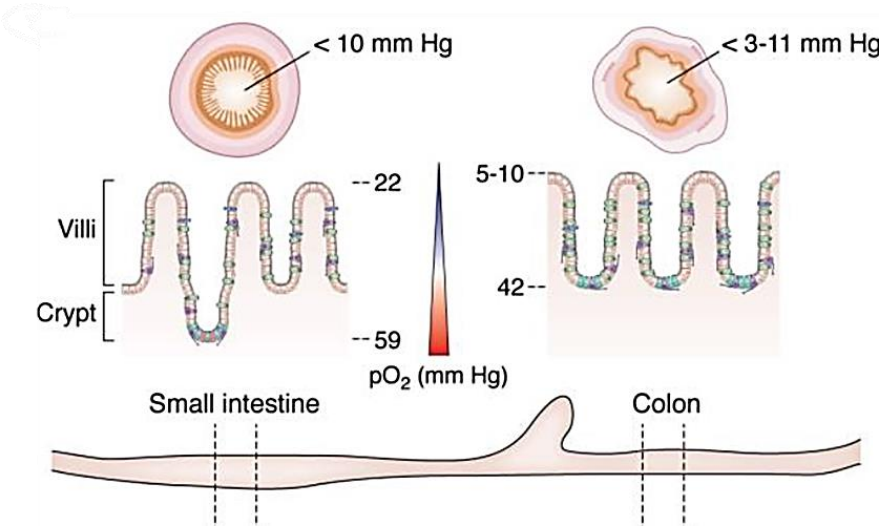


Figure 5: Intestinal O_2. Countercurrent blood flow reduces local pO_2 along the crypt-villus axis and the microbiome alters pO_2 from small intestine to the colon (Singhal and Shah 2020).

The microbiota is essential for establishing the hypoxic intestinal microenvironment. Newborn intestines are relatively better oxygenated compared with those of adults. Recent studies show that maternal microbiota seeds the offspring microbiota at birth. Microbiota diversity and density increase and stabilizes to adult-like by the first three years of life (Reyman *et al*. 2019). Studies demonstrate that the initial bacterial community is composed of aerobic and facultative anaerobic bacteria that consume O_2 in the intestine and thus provide an optimal niche for anaerobic bacteria. The number and diversity of commensals increase down the intestinal longitudinal axis from the small intestine to the colon (Donaldson, Lee, and Mazmanian 2016). Moreover, the utilization of O_2 by aerobic bacteria near the epithelium and the rise in tissue oxygenation led to an increase in the population of aerotolerant microbes at the epithelia-lumen interface and rendered the central portion of the lumen deficient in O_2. However, many gastrointestinal diseases are thought to have high

oxidative stress, which can influence luminal O_2 levels and lead to dysbiosis (Ramakrishnan and Shah 2016).

1.2.1 Role of HIF-1α in intestinal homeostasis

The adaptation to hypoxia at the cellular level in the intestine is mainly regulated by hypoxia-inducible factors (HIFs). In hypoxic conditions, HIF stabilization drives the expression of genes that aid in the adaptation to hypoxia, which primarily includes regulators of erythropoiesis (erythropoietin; EPO), angiogenesis (vascular endothelial growth factor; VEGF), and metabolism (glycolytic enzymes). HIFs are a heterodimer of an α- and β-subunit. The HIF-α subunit belongs to the basic helix-hoop-helix Per-Arnt-Sim (bHLH-PAS) family of transcription factors (Schito and Semenza 2016). Vertebrates have three α-subunits: HIF-1α, HIF-2α, and HIF-3α. The transcription factor HIF-1α (encoded by HIF1A) is constitutively produced at a high level in most cell types, while HIF-2α (encoded by EPAS1) is expressed in a more tissue-restricted manner (Taylor and Scholz 2022).

The N-terminal region of HIF-α subunit contains a domain that is required for DNA binding and heterodimerization. HIF-α subunit contains a highly conserved O_2-dependent degradation domain (ODD). The ODD domain contains two prolines that are hydroxylated, 402 and 562 on HIF-1α and prolines 405 and 531 on HIF-2α (Chan *et al*. 2005). Hydroxylation of HIF-α leads to proteasomal degradation. The enzymes that hydroxylate HIF-α, the prolyl hydroxylase domain proteins (PHDs), consisting of PHD1 (EGLN2), PHD2 (EGLN1), and PHD3 (EGLN3), are the principal O_2 sensors in a cell. PHDs are α-ketoglutarate–dependent dioxygenases. PHDs use O_2 to hydroxylate the HIF-α subunit on the prolines in the ODD. Hydroxylation enables the binding of the Von Hippel-Lindau (VHL) tumor suppressor protein, which is an E3 ubiquitin ligase. Moreover, hydroxylation by the 2-oxoglutarate-dependent factor inhibiting HIF (FIH, encoded by HIF1AN) renders the HIF α-subunits unable to bind to their transcriptional co-activators, the histone acetyl transferases CBP and p300, preventing the formation of a functional transcriptional complex and thereby repressing HIF activity in an O_2-dependent manner (Volkova *et al*. 2022). In low-O_2 conditions, the PHD and FIH enzymes can no longer utilize O_2 for HIF-α hydroxylation. HIF-α is stabilized and heterodimerizes with a constitutively expressed HIF-β subunit, also known as aryl hydrocarbon receptor nuclear translocator (ARNT) (**Figure 6**). This heterodimerization enables binding to HIF response elements (HREs) in the promoters of target genes.

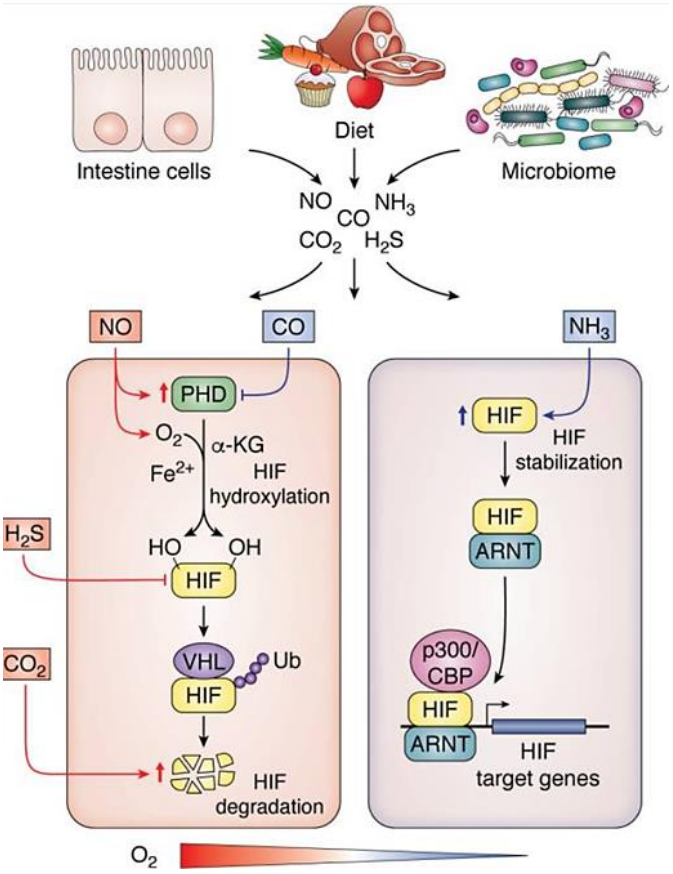


Figure 6 HIF pathway. In the presence of O_2, α-ketoglutarate (α-KG), and Fe^{2+}, PHD enzymes hydroxylate the two proline residues on HIF, allowing the binding of VHL to the HIF-α subunit. VHL binding is coupled with E3 ubiquitin ligase activity, which degrades HIF-α subunits under normoxic conditions. As O_2, Fe^{2+}, or α-KG levels decrease in a cell, PHD enzymes are no longer active. HIF-α is stabilized and binds to ARNT to activate transcription of target genes. The intestinal cells, diet, and microbiome generate numerous gaseous signaling mediators that interact with HIF pathway. Nitric oxide (NO), hydrogen sulphide (H_2S), and carbon dioxide (CO_2) inhibit HIF activity, whereas carbon monoxide (CO) and ammonia (NH_3) activate HIF (red lines indicate the inhibitory mechanism, and blue lines denote activation) (Singhal and Shah 2020).

The levels and activity of HIF can be regulated through several transcriptional, translational, and post-translational mechanisms beyond O_2-dependent regulation of HIF α-subunit stability (Taylor and Scholz 2022) (**Figure 7**).

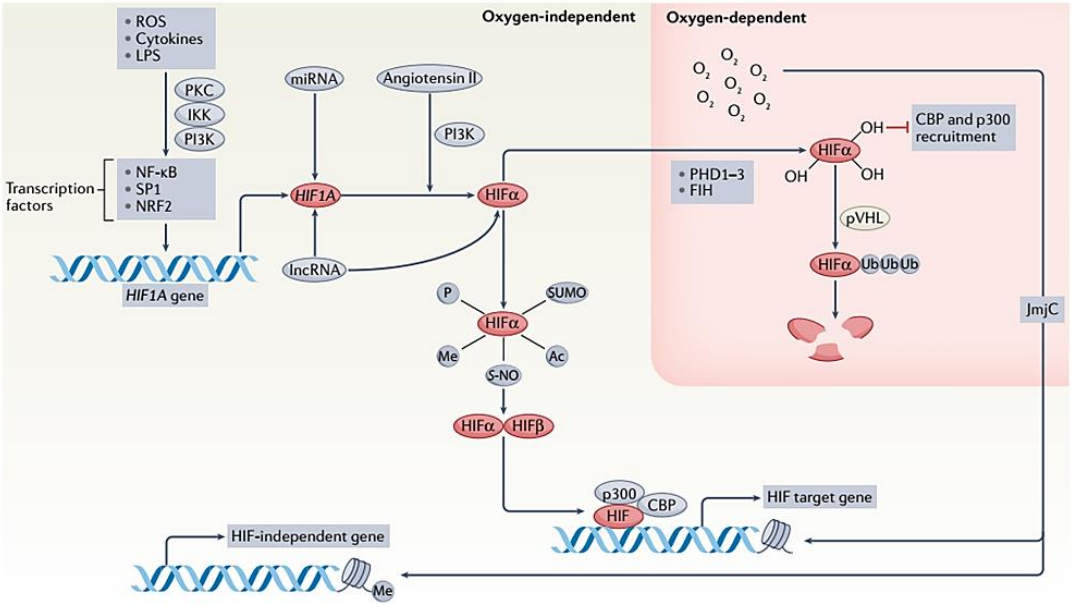


Figure 7: Mechanisms that regulate HIF. The activity of HIF is regulated by O_2-dependent and O_2-independent mechanisms directed towards the HIF α-subunit. O_2-independent mechanisms include the regulation of HIFA gene expression, through the actions of transcription factors such as NF-κB, specific protein 1 (SP1) and NF-E2-related factor 2 (NRF2), which in turn can be regulated by ROS, cytokine and lipopolysaccharide (LPS)-dependent pathways involving protein kinase C (PKC), inhibitor of NF-κB kinase (IKK) and phosphoinositide 3-kinase (PI3K). HIFA mRNA levels and translation can be regulated by microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and by angiotensin II-mediated signaling involving PI3K. The stability and activity of HIF-α can be modulated by a various of O_2-independent post-translational modifications, including phosphorylation, sumoylation (SUMO), acetylation (Ac), S-nitrosylation (S-NO) and methylation (Me). The best characterized mechanism of HIFα regulation occurs in an O_2-dependent manner through PHDs and FIH (Taylor and Scholz 2022).

Transcriptional regulation of HIF

At a transcriptional level, the production of HIF-1α mRNA is regulated by a number of transcription factors. Stimuli such as ROS can induce transcription of HIF-1α mRNA expression in a manner dependent on the activation of NF-κB pathway (Bonello *et al*. 2007) (**Figure 7**). Angiotensin II (ANGII) increases HIF1A mRNA expression in vascular smooth muscle cells via ROS-dependent activation of the phosphoinositide 3-kinase (PI3K) and protein kinase C (PKC) pathways (Page *et al*. 2002). To support this finding, ROS increased HIF-1α expression through PI3K and PKC-dependent mechanisms in hypoxic tumor cells under oxidative stress conditions. Inflammatory mediators and bacterial products such as lipopolysaccharide (LPS) can also stimulate HIF1A and EPAS1 transcription via NF-κB-dependent pathways. Moreover, the antioxidant transcription factor (NRF2) also regulates HIF-1α mRNA expression in normoxia and more markedly in the context of reoxygenation following hypoxia (Potteti *et al*. 2021) (**Figure 7**). Therefore, beyond the O_2-dependent regulation of HIFα protein stability in cells, the transcription of HIF genes is regulated through several mechanisms.

Translational regulation of HIF

Besides the regulation of HIF at the transcriptional level, the translation of HIF-α proteins can also be controlled. For example, ANGII increases ROS-PI3K-mediated translation of HIF-α. Several specific pharmacological inhibitors, including topoisomerase, mammalian target of rapamycin (mTOR), and PI3K inhibitors, have been demonstrated to interfere with HIF translation, implicating these pathways in the control of this process (Masoud and Li 2015).

Post-translational modifications

Hydroxylation and the resultant ubiquitination of HIF-1α via the VHL-recruited E3 ligase complex in the canonical pathway are the primary post-translational modifications responsible for the O_2-dependent regulation of HIF stability. Other post-translational modifications of HIF-1α, including phosphorylation, acetylation, *S*-nitrosylation, and methylation, can influence the stability and activity of HIF α-subunits (Albanese *et al*. 2021). Therefore, the stabilized HIF-1α protein is heavily decorated with post-translational modifications, which likely differ in nature and degree between cell types to enable fine-tuning of the HIF response in specific cell types and contexts.

Regulation of HIF by other biological signals

The level of HIF activity in a specific cell type or context can be modulated by several cellular signals, including physiological gases other than O_2 (for example, nitric oxide (NO), carbon dioxide (CO_2), and hydrogen sulphide (H_2S), ROS and the availability of HIF hydroxylase co-factors, including Fe^{2+} and 2-oxoglutarate (also known as α-ketoglutarate) (Taylor and Scholz 2022).

NO is a gas transmitter that is important for the control of vascular tone and thus, tissue perfusion and overall blood pressure. However, another important biological role for NO is in the regulation of mitochondrial O_2 consumption by controlling O_2 availability to HIF hydroxylases thereby affecting the overall HIF response. Under conditions of hypoxia, whereby PHDs are inhibited and HIF is activated, NO-mediated inhibition of cytochrome *c* oxidase (COX; also known as complex IV) of the mitochondrial respiratory chain leads to a redistribution of O_2 to the HIF hydroxylases, resulting in increased HIF hydroxylation and subsequent ubiquitination and degradation of HIF (Taylor and Moncada 2010).

CO_2 is another physiological gas that is generated in mitochondria as the primary gaseous product of respiration. It suppresses HIF-1α stability by reducing intracellular pH, which promotes HIF-1α degradation via an alternative lysosomal pathway. Other products of metabolism, including ROS, H_2S, iron, and tricarboxylic acid (TCA) cycle metabolites (including 2-oxoglutarate, succinate, and citrate) modulate the HIF response through the regulation of PHD or FIH activity (Bailey *et al*.

2020; Bailey and Nathan 2018). Thus, although the availability of O_2 provides the primary signal that determines levels of HIF activity in a cell (via HIF hydroxylase activity), multiple other biological signals can shape the quantitative, spatial, and temporal nature of the overall response.

HIF-1α has been involved in a broad range of cellular functions such as pH regulation (Sedlakova *et al*. 2014), increased glucose uptake (He *et al*. 2014)' erythropoiesis induction (Haase 2013), and lipid metabolism (Zhang 2015).

Recent studies highlight the potential role of HIF-1α in the maintenance of gut homeostasis. The intestinal epithelium is the main site of host-microbe interactions that modulate the physiologic outcome. To prevent microbial invasion, the barrier function needs to be tightly regulated (Kumar, Pandey, and Chauhan 2020).

The stabilization of HIF-1α upregulates the transcription of claudin-1, a tight junction membrane protein that maintains the tight junction structure and functions (Van Itallie and Anderson 2014). Enhancement of gut barrier function by hypoxia was confirmed by the growth of T84 cells in a hypoxic environment. An increase in transepithelial electrical resistance (TEER) along with the decrease in diffusion of fluorescein isothiocyanate (FITC) labeled dextran was observed in the cells grown under hypoxic conditions. In addition, junctional adhesion molecule-A (JAM-A) and occludin were also found upregulated under hypoxic conditions. Such examples of increased barrier functions were found to be HIF-1α-dependent, highlighting the direct role of HIF-1α in maintaining the integrity of intestinal epithelium (Muenchau *et al*. 2019). Moreover, dimethyloxalylglycine (DMOG), an inhibitor of PHDs and FIH has been shown to ameliorate dextran sulfate sodium (DSS)-induced colitis by stabilizing HIF-1α. On the other side of the physiological role of gut integrity, in the case of IBD, increased barrier permeability results in inflammation and altered microbial composition (Martini *et al*. 2017). Butyrate in Caco-2 cells was found to enhance O_2 consumption and stabilize HIF-1α which in turn reduced barrier permeability (Kelly *et al*. 2015). Similarly, butyrate was found to upregulate tight junction proteins in HIF-1α dependent manner to improve barrier integrity, reducing inflammation by inhibiting microbial translocation in mice (Fachi *et al*. 2019). These results highlight that HIF-1α upregulates tight junction proteins to improve the integrity of the epithelial barrier to counteract bowel inflammation.

In addition, HIF-1α induces mucin expression, facilitating the colonization of commensals. The mucous layer consists of a dense inner layer that restricts bacterial attachment and a loose outer layer, the residence of gut commensals. Intestinal mucus is mainly composed of glycoproteins O-glycosylated called mucins (MUC). The stomach mucous layer mainly consists of MUC1, MUC5AC, and MUC6, while MUC2 is a major component of the mucous layer of the small

intestine and colon. The carbohydrate-rich mucous layer acts as a growth substrate for the resident microbes. Further, commensals can induce MUC2 production to increase the thickness of the mucous layer, which not only provides a space to gut colonizers but also provides pathogen resistance to the host. Probiotics such as *Lactobacillus* can stimulate mucin production. Moreover, *Bacteroides thetaiotomicron* and *Faecalibacterium prausnitzi* were also found to accelerate goblet cell differentiation (Wrzosek *et al*. 2013). The mucous layer secreted by goblet cells in the gut is an essential part of the innate defense system. It forms a physical barrier to restrict direct access of pathogens to the gut epithelium. Mucins interact with trefoil factors to improve the mucosal barrier. It is also known that human gut commensals release SCFAs, which, in turn, stabilize HIF-1α by inducing metabolic hypoxia in colonocytes. Stabilized HIF-1α upregulates the expression of MUC2 (Dilly *et al*. 2016), MUC3 (Louis *et al*. 2006), and intestinal trefoil factor (ITF) (Glover, Lee, and Colgan 2016). Furthermore, it has been observed that mice lacking HIF-1α showed less organized and diffused mucin granules, suggesting HIF-1α expression is required for mucin processing (Whitney *et al*. 2016).

The immune system plays a crucial role in gut homeostasis. Exposures to microbes have been demonstrated to activate the host immune system, whilst a hyper-activated immune system could result in detrimental effects to the host itself (Kumar, Pandey, and Chauhan 2020).

Gut homeostasis involves extensive functional crosstalk between HIF-1α and NF-κB. The presence of a putative NF-κB binding site was found on the HIF-1α promoter with a potential role in transcriptional modulation. Hypoxia has been identified as a common activator of both HIF-1α and NF-κB in a kinase IκBI-transforming growth factor β-activated kinase 1 (IKK-TAK1)-dependent manner. Furthermore, the existence of an evolutionarily conserved negative feedback mechanism, through which HIF-1α can regulate NF-κB in an IKK-TAK1- and cell division protein kinase 6 (CDK6)-dependent manner, has emerged. On the other hand, NF-κB has been shown to be a direct modulator of HIF-1α expression in inflammation and hypoxia. Despite the relatively well-established ability of NF-κB to induce HIF, the knowledge about HIF-1α contributions to the NF-κB pathway is still mainly limited to HIF-1α. In fact, HIF-1α can restrict NF-κB transcriptional activity *in vivo* and *in vitro* under inflammatory conditions (D'Ignazio, Bandarra, and Rocha 2016).

Several studies have shown that HIF-1α contributes to the regulation of all cell types involved in the innate and adaptive immune response, including the regulatory T cells (Tregs) (McAnulty 2007). Tregs regulate immune system activation and act as a key component for the protection of the gut commensals against the host immune response. HIF-1α induces FoxP3 transcription that promotes the differentiation of naïve $CD4^+$ cells into Tregs under cellular hypoxia (Luu, Steinhoff, and

Visekruna 2017). Treg cells are known for their anti-inflammatory effects; they produce IL-10 (an anti-inflammatory cytokine) to downregulate the immune response. Hence, HIF-1α induces Treg differentiation that, in turn, reduces colonic inflammation and promotes immune tolerance for a hassle-free stay of gut residents (Cekic and Linden 2016).

Furthermore, FIH-1α indirectly interacts with ATP metabolites, contributing to maintaining intestinal homeostasis. Normally, ATP is located intracellularly, whereas, under stress conditions or infections, it is released extracellularly. Extracellular ATP functions as a signaling molecule binding P2 purinergic cell surface receptors to provoke a pro-inflammatory immune response (Allard *et al*. 2017). Extracellular ATP is further hydrolyzed to AMP by Nucleoside Triphosphate dephosphorylase or NTPDase (CD39) and finally to adenosine by Ecto-5'- Nucleotidase or 5-NT (CD73) to oppose the effects mediated by P2 receptors. The presence of a putative HIF-1α binding site was found on the CD73 promoter with a potential role in transcriptional modulation.

Adenosine has an immune-modulatory role and improves epithelial barrier function by A2B adenosine receptor-induced phosphorylation of vasodilator-stimulated phosphoprotein (VASP). VASP was found protective in DSS-induced colitis (Aherne *et al*. 2015). Adenosine A3 receptor activation was found to attenuate TNF-α induced NF-κB signaling, as well as to improve the epithelial barrier functioning. Improved barrier function was reflected as an increase in TEER and expression of tight junction protein ZO-1 (Ren and Zhou 2017). Adenosine promotes a tolerogenic phenotype in macrophages. This tolerogenic phenotype is characterized by low expression of pro-inflammatory cytokines such as IL-12 and TNF-α while an increase in anti-inflammatory cytokines (IL-10). Adenosine also inhibits the LPS-induced production of pro-inflammatory cytokines by macrophages. Adenosine thus complements the role of HIF-1α as a curator of gut homeostasis.

1.2.2 Role of HIF-1α in metabolic processes

HIFs transcriptionally regulate genes involved in energy metabolism under both physiological and pathological conditions. HIF activity has been associated with metabolic processes such as the Pasteur effect (Seagroves *et al*. 2001) and the Warburg effect (Semenza 2007). Under normoxic conditions, the majority of cellular ATP is produced by oxidative phosphorylation in the mitochondria, a process that uses approximately 90% of the available O_2. HIF-dependent regulation of cellular energy metabolism serves to adjust ATP production when O_2 is limited, thus allowing cell and tissue survival. HIF-1α is the main driver of metabolic adaptation to hypoxia. HIF-1α-dependent genes control cellular glucose utilization and decrease O_2 consumption to reduce metabolic dependence on O_2 while maintaining appropriate ATP concentrations (Taylor and Scholz

2022). Additionally, HIF-1α can reduce mitochondrial mass by increasing mitophagy and decreasing mitochondrial biogenesis. Therefore, HIF-1α is a major regulator of cellular metabolic strategy during periods of limited O_2 availability (**Figure 8**). Interestingly, upregulated HIF-1α expression and increased glycolysis are dependent on the PI3K/AKT (protein kinase B) signaling, a pathway playing a central role in regulating energy metabolism, through mechanisms that are independent of O_2 levels (Dong *et al*. 2022).

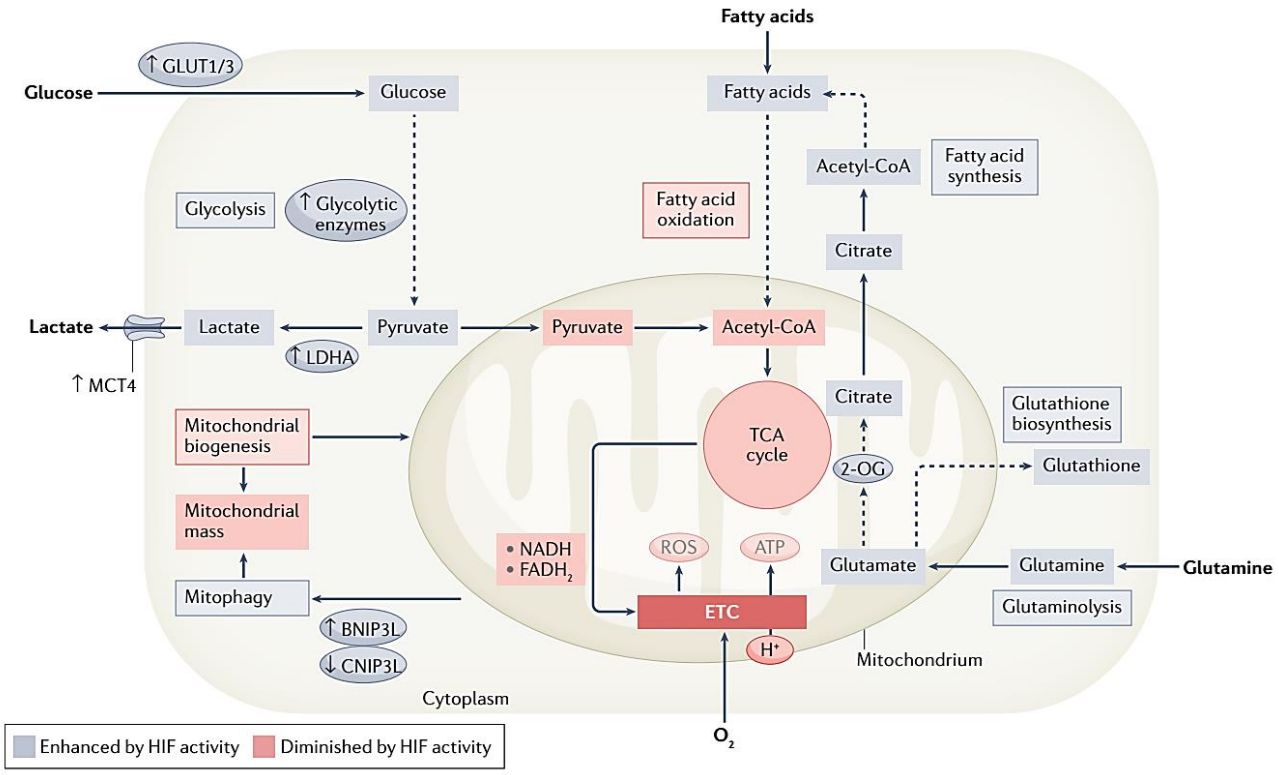


Figure 8: HIF-mediated adaptations of cellular metabolic pathways. HIF-1α activity has effects on cellular metabolism, adjusting cellular glucose, fatty acid and glutamine utilization to decrease metabolic O_2 consumption while maintaining appropriate concentrations of ATP and other necessary metabolic intermediates and products. Under hypoxic conditions, increased activity HIF-1α decreases mitochondrial O_2 consumption, and increases glycolysis, fatty acid synthesis and glutaminolysis. 2-OG, 2-oxoglutarate; BNIP3, BCL-2/adenovirus E1B 19-kDa interacting protein 3; ETC, electron transport chain; GLUT, glucose transporter; LDHA, lactate dehydrogenase A; MCT4, monocarboxylate transporter 4; ROS, reactive oxygen species; TCA, tricarboxylic acid cycle (Taylor and Scholz 2022).

Glycolysis

Under normoxic conditions, glycolysis provides the first step in the oxidative metabolism of glucose and converts glucose to pyruvate, which is transported into the mitochondria and used for the production of acetyl-CoA. Acetyl-CoA is then fed into the TCA cycle to generate electrons for the electron transport chain (ETC) in the form of NADH and FADH2. The ETC establishes a proton gradient across the inner mitochondrial membrane, which ATP synthase uses to produce ATP (**Figure 8**). In hypoxia, HIF-1α increases the expression of pyruvate dehydrogenase kinase 1 (PDK1) (Papandreou *et al*. 2006). This enzyme inhibits the mitochondrial enzyme pyruvate dehydrogenase (PDH) by phosphorylation, preventing the conversion of pyruvate into acetyl-CoA, thereby limiting its availability for the TCA cycle. This represses mitochondrial O_2 consumption and redirects pyruvate toward glycolysis. This switch from oxidative to glycolytic metabolism effectively reduces the availability of cellular O_2 required for ATP production. Moreover, the reduction of mitochondrial O_2 consumption leads to reduced mitochondrial ROS production, protecting the cell from ROS-induced damage (Samanta and Semenza 2018). Glycolysis is less efficient in producing ATP per glucose molecule than oxidative metabolism. To prevent a bioenergetic crisis, increased HIF activity, therefore, enhances glucose flux through glycolysis by increasing the expression of the glucose transporters GLUT. Moreover, HIF-1α increases the expression of all glycolytic enzymes (Samanta and Semenza 2018), including hexokinases (HK1 and HK2), phosphofructokinases (PFKL and PFKP), aldolases (ALDA and ALDC), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (PGK1), enolases (ENO1 and ENO2) and pyruvate kinase M (PKM). Finally, HIF-1α enhances the expression of lactate dehydrogenase A (LDHA) and monocarboxylate transporter 4 (MCT4). LDHA converts pyruvate to lactate and regenerates NAD^+, which is required by GAPDH for additional glycolytic cycles. MCT4 removes lactate from the cell, transporting it into the extracellular space. Therefore, HIF-1α drives a switch from oxidative to glycolytic metabolism during periods of hypoxia, an event that has implications not only for cellular bioenergetics but also for cell function and fate.

Oxidative phosphorylation

While enhancing glycolysis, HIF-1α activity also reduces oxidative phosphorylation. HIF-1α activity can decrease mitochondrial O_2 consumption by reducing mitochondrial mass through various mechanisms. For example, enhanced HIF-1α activity promotes mitophagy by increasing the expression of BCL-2/adenovirus E1B 19-kDa interacting protein 3 (BNIP3) and BNIP3-like (BNIP3L) (**Figure 8**). Moreover, HIF-1α reduces mitochondrial biogenesis by increasing the expression of MAX interactor 1 (MXI1). This transcriptional repressor negatively regulates the

expression of the transcription factor MYC, which decreases MYC-mediated expression of peroxisome proliferator-activated receptor-γ coactivator 1β (PGC1β). PGC1β regulates mitochondrial biogenesis, and its decrease reduces mitochondrial mass. HIF-1α also modifies the mitochondrial ECT, which transfers the electrons received from NADH and FADH2 to generate ATP through oxidative phosphorylation. COX is the final electron acceptor of the ECT and consumes the largest amount of O_2. Increases in hypoxia-induced HIF-1α activity reduce O_2 consumption by COX, which in turn probably reduces the production of mitochondrial ROS (Taylor and Scholz 2022).

1.3 Intestinal chronic diseases

The gut microbiota represents all the microbial species, including bacteria, fungi, archaea and viruses permanently present in the gastrointestinal tract of the host. It is made up of more than 100 trillion microorganisms that are essentially strict anaerobic bacteria (Markowiak and Slizewska 2017). Microbial colonization of the gastrointestinal tract begins in the newborn as soon as the fetal membrane breaks. Henceforth, it evolves and transforms over a lifetime, depending on a complex and dynamic interaction between the diet, genome, and lifestyle of the host, as well as the use of antibiotics. The microbiota carries out numerous activities, such as synthesizing vitamins, contributing to the digestion of cellulose, and promoting angiogenesis. In addition, the intestinal microflora plays a crucial role in protecting the host's health, as it covers metabolic, structural, and protective functions. Molecular analysis has shown that commensal bacteria modulate the expression of genes involved in a number of important intestinal and extra-intestinal functions, including xenobiotic metabolism, nutrient uptake, and mucosal barrier fortification. It is believed that the microbiota is responsible for more than 98% of the genetic activity of the organism, as a "second genome" for the human body.

Dysbiosis occurs when the balance of intestinal microbiota is disturbed and when potentially pathogenic microorganisms predominate at the expense of commensal bacteria, based on which a metabolic or immune response of the host may occur (Di Domenico *et al*. 2022). Gut dysbiosis is a key factor in the pathogenesis of intestinal chronic diseases. The principal clinical manifestations of intestinal chronic diseases are inflammatory bowel disease (IBD), necrotizing enterocolitis (NEC), and irritable bowel disease (IBS) (Markowiak and Slizewska 2017). The pathogenesis of intestinal chronic disease remains fundamentally indeterminate; however, it is believed to be a delicate interaction among a multitude of factors, including gastrointestinal immune dysregulation, genetic predisposition, gut dysbiosis, and environmental factors.

1.3.1 Inflammatory bowel disease (IBD)

IBD is a chronic, multifactorial disorder caused by inflammation of the gastrointestinal tract, and that includes Crohn's disease (CD) and ulcerative colitis (UC). IBD causes several clinical symptoms, including abdominal pain, diarrhea, rectal bleeding, fatigue, and weight loss. Additionally, it has been linked to other inevitable convolutions, such as fistulas, stenosis, and colorectal cancers (Spiller and Major 2016). CD and UC commonly appear in adulthood. However, the younger population is also affected, with more cases in children and in adolescent patients in the last two decades. IBD was initially described as pathology affecting mainly industrialized countries. Currently, their incidence is increasing worldwide, probably due to greater urbanization (Poddighe *et al*. 2020). CD and UC have some distinctive features that are key differences that affect treatments. The intestinal inflammation in CD is transmural, involving "skip areas" of any segment of the gastrointestinal tract. Whereas in UC, the inflammation is continuous and affects mainly the colon. The disturbance of the immune response in IBD is classically characterized by the predominance of a Th1 type immune response in CD promoted by the transcription factors, such as Signal Transducer and Activator of Transcription 4 (STAT-4) and T-box expressed in T cells (T-bet) and marked by high secretion of IFN-γ, IL-12, and TNF-α. UC is characterized by an atypical Th2 type immune response promoted by the expression of the transcription factors STAT-6 and GATA binding protein- 3 (Gata-3), which generates high levels of IL-5, IL-13, and IL-4. Moreover, abnormal activation of Th17 and deregulation of the balance between the different subsets of effector cells and Tregs characterize both CD and UC. Decreased number and function of Treg cells leads to insufficient regulation of the immune response during the active phase of the disease. Consequently, the intestinal mucosa in IBD is infiltrated by inflammatory cells that release cytokines, chemotactic molecules, ROS, and NO. These pro-inflammatory mediators trigger and perpetuate a chronic inflammatory response in the gut, leading to tissue damage and disease.

The quantitative or qualitative change in the composition of the gut microbiome is one of the most important factors regulating the intestinal immune system and thus may influence affecting the development and course of IBD. In IBD, a decrease in the α-diversity of gut microbiome such as *Faecalibacterium* spp. and *Roseburia* spp. was consistently observed. In patients with IBD, there is a decrease in the number of bacteria from the phylum *Firmicutes*, which includes Gram-positive bacteria that are mainly from the genera *Bacillus*, *Lactobacillus*, and *Enterococcus*, and an increase in Bacteroidetes, which includes about 7000 different types of Gram-negative bacteria that are mostly from the genera *Bacteroides*, *Parabacteroides* and *Prevotella*. Additionally, there is a

significant increase in the number of bacteria from the family *Enterobacteriaceae*, which includes *Escherichia coli*, *Shigella* spp. and, *Klebsiella* spp.(Santana *et al*. 2022) (**Figure 9)**. A study conducted by Pascal *et al.* also reported that loss of beneficial microbes was more abundant in CD patients, and such organisms are involved in butyrate production (Pascal *et al*. 2017). These bacterial metabolites are well-known for their role in reducing pro-inflammatory cytokines. The members of *Firmicutes* are the primary producers of butyrate and other SCFAs in the human gastrointestinal tract. Reduction in the SCFAs increases inflammation and affects the colonic barrier functions. The impaired gut barrier leads to the invasion of pathogenic microorganisms, which increases the IBD severity. Elevation of pathogenic microbes in the gastrointestinal environment, such as the species of *Enterobacteriaceae* and *Bacteroides fragilis*, was found to be a secondary inflammation state in the gastrointestinal tract of IBD.

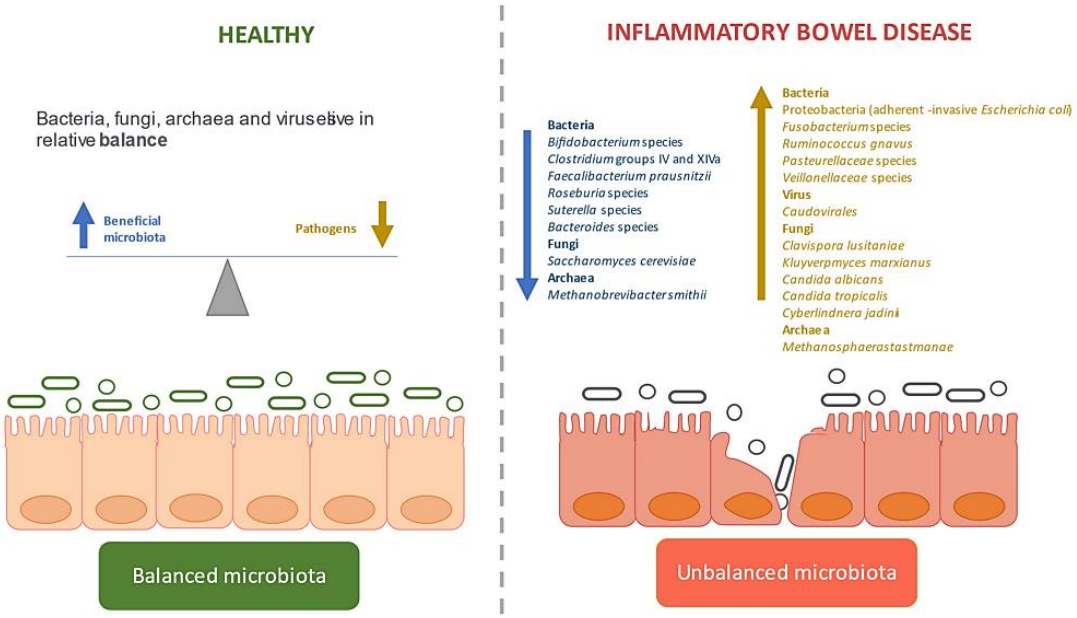


Figure 9: Gut microbiota disturbance in IBD compared to healthy individuals (Aldars-Garcia, Chaparro, and Gisbert 2021).

Both CD and UC are relapsing and remitting conditions alternating flare of active inflammation followed by periods of remission. Therefore, highlighting a new alternative and complementary therapeutic approach that targets both the deregulated immune response and the dysbiosis characterizing IBD is of great interest.

1.3.2 IBD and HIF-1α

HIF-1α is involved in maintaining intestinal homeostasis. Activation of HIF-1α contributes to the barrier function through the production of mucus, antimicrobial peptides, and tight-junction protein expression in humans and mice. In addition, gut commensals could lead to the stabilization of the HIF-1α in the gastrointestinal tract. *Bacteroidetes, Firmicutes,* and *Clostridial* clusters in the human gut convert dietary fibers and resistant starch into SCFAs (Venegas *et al*. 2019). SCFAs enter the nucleus of colonocytes and act as a histone deacetylase inhibitor. SCFAs stimulate PDK, that in turn, inactivates the pyruvate dehydrogenase complex. Inactivated pyruvate dehydrogenase complex cannot convert pyruvate to acetyl-CoA to meet cellular energy requirements. Thus, colonocytes start generating acetyl-CoA through β-oxidation of the SCFAs. Increased oxidative respiration induces physiologic hypoxia which in turn tends to stabilize HIF-1α (Kelly *et al*. 2015). In addition to the SCFAs-mediated HIF1α stabilization, human gut commensals also regulate the PHDs activity to stabilize HIF1α. PHDs require iron for their catalytic role to destabilize HIF1α and the absence of iron affects PHDs activity. Human gut commensals could secrete siderophores to quench iron from intestinal lumens, making it unavailable for PHDs and allowing HIF1α stabilization. These effects reduce bacterial translocation during an infection. In the absence of HIF-1, the production of mucus and AMPs by IECs is reduced, as well as the expression of tight junction proteins, compromising the epithelial barrier permeability. This dysfunctional epithelium is also associated with changes in the composition of the microbiota and lower production of SCFAs (Pral *et al*. 2021).

IBD involves the breakdown of the intestinal barrier with an unregulated inflammatory immune response. The leaky intestinal barrier allows exposure of microbial-associated molecular patterns (MAMPs) to the TLRs to activate the host immune response (Jones and Neish 2017). The host's innate immune system facilitates superoxide production. The generation of superoxides has been found to inhibit PHDs activity and promote the stabilization of HIF-1α. Stabilized HIF-1α exerts protective effects by downregulating the NF-κB pathway to reduce cellular inflammation. The anti-inflammatory effects of HIF-1α activation have been extensively demonstrated. Pharmacological activation of HIF-1α with PHD inhibitors (PHIs) has shown anti-inflammatory effects *in vivo* models. This finding is supported by results from clinical trial for the treatment of ulcerative colitis, in which protective effects of treatment with the PHI GB004 were reported. However, in colitis, HIF isoforms may exert opposing roles. For example, HIF-1α expressed in IECs is protective in colitis (Danese *et al*. 2022), whereas HIF-2α is detrimental (Singhal and Shah 2020). As such, the

role of HIF-1α in inflammation is complex, although evidence to date suggests that activation of the HIF pathway is largely anti-inflammatory.

1.3.3 IBD and probiotics

Probiotics have emerged as an interesting therapeutic approach for IBD. Recent studies have shown the potential mechanisms of action behind the therapeutic effect of probiotics in patients with IBD (Altun, Yildiz, and Akin 2019; Matsuoka *et al*. 2018).

Probiotics participate in intestinal homeostasis through their direct and indirect effects on the modulation of gut microbiota and the immune system. It has been demonstrated that certain probiotics are able to downregulate the expression of pro-inflammatory cytokines by interfering directly with the activation of NF-κB pathway or via the activation of the nuclear receptor peroxisome proliferator-activated receptor-gamma (PPAR-γ). Furthermore, numerous *in vitro* and *in vivo* studies have highlighted the ability of probiotics to induce the differentiation of tolerogenic DCs through downregulation of the expression of CD40 and CD80 co-stimulatory molecules, the production of IL-12, and the induction of IL-10 production. In addition to the modulation of the immune system, probiotics may compete with pathogens for nutrients and attachment sites on the surface of the epithelium. They are also capable of producing antimicrobial molecules or inducing the production of β-defensins by Paneth cells. They can reduce the severity of colitis by modulating the composition of the gut microbiota and by strengthening the barrier functions of the intestinal epithelium. Furthermore, probiotics can decrease epithelial permeability by enhancing tight junction stability and upregulating the expression of tight junction proteins.

In vivo studies

Animals

The anti-inflammatory effects of probiotics have been demonstrated in experimental *in vivo* models (**Table 1**). Probiotic supplementation provides protective effects during spontaneous and chemically induced colitis by downregulating the production of inflammatory cytokines or by inducing regulatory mechanisms in a strain-specific manner (Plaza-Diaz *et al*. 2017).

The anti-inflammatory effects of *Saccharomyces boulardii* on UC colitis have been investigated. Acute UC was induced in C57BL/6 mice using dextran sulfate sodium (DSS). Probiotic treatment improved clinical symptoms and histological alterations observed in the colitis group, reduced the expression of different inflammatory markers, and ameliorated the dysbiosis in DSS-treated

mice(Rodriguez-Nogales *et al*. 2018). Toumi *et al.* evaluated a mixture of *Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Lactobacillus plantarum*, and *Bifidobacterium breve* in an acute DSS-induced colitis mouse model (Toumi *et al*. 2014). The probiotic treatment ameliorated clinical symptoms and histological scores. Moreover, NO and IFN-γ production in plasma were decreased by probiotics. These results were associated with reduced TLR4, NOS2, and NF-κB expression in colonic tissue. Xia *et al.* studied the effect of *Lactobacillus plantarum* AR113 in a DSS-induced colitis mouse model (Xia *et al*. 2020). The probiotic treatment downregulated the expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) and upregulated that of the colonic anti-inflammatory cytokine (IL-10). Moreover, *L. plantarum* AR113 mitigated dysbiosis of gut microbiota induced by DSS treatment. The anti-inflammatory effects of a probiotic mixture of *Bifidobacterium infantis*, *Lactobacillus acidophilus*, *Enterococcus faecalis*, and *Enterococcus faecalis* on DSS-induced colitis mouse model have been evaluated. The probiotic treatment relieved the intestinal inflammation and improved (Toumi *et al*. 2014) the integrity of the mucous layer, enhancing transepithelial electrical resistance (Chen *et al*. 2021). Silveira *et al.* investigated the therapeutic effects of *Lactobacillus bulgaricus* on an experimental mouse model of colitis-associated colon cancer (CAC) (Silveira *et al*. 2020). The probiotic treatment reduced total tumor volume and mean size of tumors. Additionally, *L. bulgaricus* attenuated the clinical signs of intestinal inflammation, inducing a decrease in intestinal and tumor levels of IL-6, TNF-α, IL-17, IL-23, and IL-1β.

Type of Treatment	Animal Species	Type of Study	Main Outcome	Reference
Saccharomyces boulardii	C57BL/6J mice	*In vivo*, DSS-induced colitis	*S. boulardii* treatment improved gut inflammation	(Rodriguez-Nogales *et al*. 2018)
L. acidophilus, *B. lactis*, *L. plantarum* *B. breve*	C57BL/6J mice	*In vivo*, DSS-induced colitis	The probiotic mixture improved gut inflammation	(Toumi *et al*. 2014)
Lactobacillus plantarum AR113	C57BL/6J mice	*In vivo*, DSS-induced colitis	*L. plantarum* AR113 treatment ameliorated gut inflammation and dysbiosis	(Xia *et al*. 2020)
B. infantis, *L. acidophilus*, *Enterococcus faecalis* *Bacillus cereus*	C57BL/6J mice	*In vivo*, DSS-induced colitis	The probiotic mixture improved gut inflammation	(Chen *et al*. 2021)
L. bulgaricus	C57BL/6J mice	*In vivo* CRC-associated colitis model	*L. bulgaricus* treatment improved gut inflammation and prevented Colitis-associated cancer	(Silveira *et al*. 2020)

Table 1: Summary of probiotic anti-inflammatory effects in animal models. DSS, dextran sulfate sodium.

Human

Several studies have reported the efficacy of probiotic therapy in patients with UC. Tamaki *et al*. evaluated the usefulness and safety of probiotic treatment with *B. longum* 536 in Japanese patients with active UC using a randomized controlled trial (RCT) (Tamaki *et al*. 2016). The probiotic treatment was able to ameliorate clinical symptoms in patients with mild to moderately active UC, however, additional studies are needed to explain the efficacy and safety of *B. longum* 536 for UC. To evaluate the ability of probiotic treatment for one year, including a mixture of the strains *Streptococcus faecalis* T-110, *Clostridium butyricum* TO-A, and *Bacillus mesentericus* TO-A, to prevent the relapse of UC in patients who were already in remission, a single-center, randomized, double-blind, placebo-controlled study was performed. The treatment significantly enhanced the relapse rates at three and nine months, but no differences were observed at 12 months. Moreover, using the cluster analysis of fecal microbiota, which consists of the molecular method that compares the diversity and colony structure of microbial complexes, seven subjects were categorized in cluster I; 32 in cluster II, which represents the "appropriate intestinal microbiota"; and seven in cluster III. Consequently, probiotic treatment could be useful for UC, especially in cluster I patients (Yoshimatsu *et al*. 2015). Pilarczyk-Żurek *et al*. evaluated the clinical efficacy of a probiotic mixture containing *L. plantarum*, *L. rhamnosus,* and *B. longum*, given together with a standard treatment on patients with moderate-to-severe UC, through a single center, open-label, intention-to-treat study (Pilarczyk-Żurek *et al*. 2017). The probiotic mixture given together with mesalazine and ciprofloxacin to patients in the acute phase of UC significantly reduced their Mayo Clinic Index values. In addition, the mixture given to patients with UC in remission caused a decrease in their clinical scores, but a more prominent and significant decrease of the histopathological index values in biopsy samples was observed.

1.4 Intestinal fibrosis

Fibrosis is a pathophysiological mechanism of repair that leads to the deposition of connective tissue in the extracellular matrix (ECM) after an injury has occurred (Pakshir and Hinz 2018). This is a fundamental step of the wound healing process (**Figure 10**) that could be triggered in any human tissue by several noxious agents, including infections, autoimmune reactions, and physical, chemical, and mechanical injuries. Fibrogenesis may become a pathological process when it is uncontrolled and persistent or else when the acute injury is repeated or is followed by chronic damage (Lenti and Di Sabatino 2019).

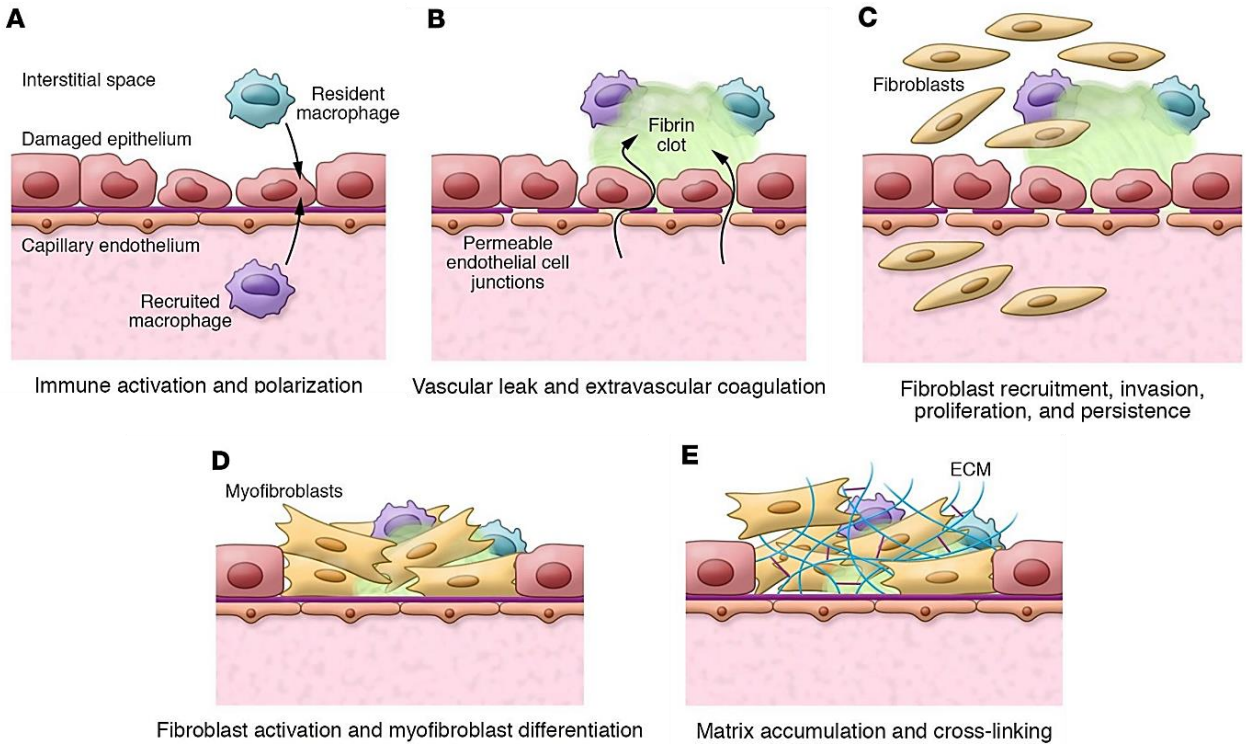


Figure 10: Representation of wound healing responses resulting in fibrosis. (**A**) Tissue injury occurs, resulting in cell death and influx of immune cells. Resident and recruited macrophages migrate to the area of injury. (**B**) Tissue injury also results in increased endothelial permeability, and activation of the coagulation cascade with formation of a fibrin clot. (**C**) Fibroblasts migrate to the area of injury. (**D**) Recruited fibroblasts become activated and differentiate into myofibroblasts. (**E**) Formation of a provisional extracellular matrix develops, and cross-linking occurs. In the setting of normal wound healing, tissue regeneration occurs. During fibrosis, excessive matrix accumulation occurs instead, resulting in organ damage (Montesi *et al*. 2019).

Fibrosis is a common outcome in the natural history of IBD and underlies most of its long-term complications, particularly in patients with CD, of whom almost 30% suffer from fibrosis-related morbidity. To a much lesser extent, some degree of fibrosis is also present in patients with UC.

Multiple mediators and effectors proved to have a role in the fibrogenic process affecting the gut, including cytokines, chemokines, and many cellular players. The essential multi-step process of fibrogenesis includes, among others, cellular damage, production of transforming growth factor (TGF)-β1, recruitment of inflammatory cells, release of ROS, activation of myofibroblasts, and collagen production (Kisseleva and Brenner 2008). Collagen deposition causes chronic hypoxia that stimulates neoangiogenesis through the upregulation of VEGF, favoring in turn, the deposition of further fibrotic tissue in a vicious circle. Inflammation is a powerful stimulus that triggers fibrosis. However, once fibrosis has been established, the mere control of inflammation is not enough to reverse the process. In a mouse model of IBD infected by *Salmonella typhimurium*, delayed

eradication of the pathogen was able to repress inflammation without preventing fibrosis, and early eradication significantly ameliorated but did not completely prevent fibrosis. This suggests that fibrosis is regulated by mechanisms other than inflammation. Actually, tissue damage in IBD is the result of a dysregulation of the whole wound healing process, with defective chronic wound healing occurring in the gut mucosa and submucosa, determining inflammation and ulceration. Instead, uncontrolled deposition of ECM occurring deeper in the intestinal wall, often with features of scar surrounding granulomatous inflammation, may have a significant role in confining inflammation and preventing tissue destruction with fistula formation. ECM accumulation can also be regulated by matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitors metalloproteinases (TIMPs). MMPs are proteolytic enzymes that cleave ECM components and break down the ECM, while TIMPs counteract their degrading activity, maintaining homeostasis. The imbalance between MMPs and TIMPs may lead to the accumulation of ECM and subsequent fibrosis (Yang *et al*. 2020). Moreover, tissue tension also plays a central role in the biomechanical development and progression of fibrosis. Mechanical forces may activate myofibroblasts, thus favoring the progressive accumulation of ECM and determining tissue remodeling.

1.4.1 Cellular mediators of intestinal fibrosis

Recurrent, or persistent, epithelial injury is crucial for initiating and sustaining intestinal inflammation and fibrogenesis. Epithelial and endothelial damage releases chemotactic factors promoting recruitment and activation of innate and adaptive inflammatory cells, as well as mesenchymal cell precursors of the activated myofibroblasts (Wang, Lin, *et al*. 2021) (**Figure 11**).

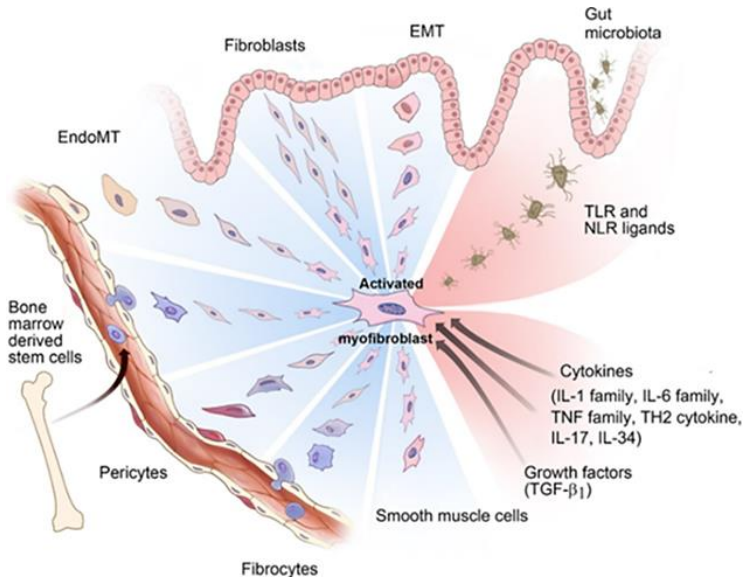


Figure 11: The main cell types and cell differentiation mechanisms involved in ECM secretion and fibrosis in intestine (Wang, Lin, *et al*. 2021).

- Innate and adaptive immune cells

The innate immune cells, monocytes, neutrophils, mast cells, eosinophils, and basophils, produce pro-inflammatory and pro-fibrotic molecules like the ILs, TNF-α, TGF-β1 and platelet-derived growth factor (PDGF). Innate immune signaling pathways, by provoking cellular activation and fibrosis, are also important drivers of myofibroblast transdifferentiation (Lawrance *et al*. 2017).

Monocyte-derived cells, including macrophages and dendritic cells, also impact both inflammation and fibrosis. These cells regulate the activated myofibroblast and their progenitors by direct effects on the matrix(Wynn, Chawla, and Pollard 2013). The recruitment of distinct functional subsets of macrophages, and their relative concentrations during injury, can also determine whether the inflammatory response leads to tissue repair or fibrosis. Classically activated M1 pro-inflammatory macrophages are induced by IFN-γ, TNF-α, or bacterial products, which activate MyD88 and NF-kB. M1 macrophages activate myofibroblasts and fibrosis by both cytokine-dependent and independent mechanisms, and ROS, by causing additional tissue injury and promoting myofibroblast resistance to apoptosis (Lawrance *et al*. 2017).

M2a macrophages are produced following exposure to IL-4 or IL-13, which signal through the common IL-4 receptor α and through STAT6 activation. M2a macrophages produce crucial pro-fibrotic factors including TGF-β1, connective tissue growth factor (CTGF), PDGF, fibroblast growth factor (FGF), and insulin-like growth factor (IGF). M2c/reg (regulatory) phenotype macrophages occur following IL-10 exposure, are anti-fibrotic, and act, in part, via STAT1 and NF-kB inhibition. Regulatory macrophages (M2c/reg) inactivate myofibroblasts and inhibit M1- and M2a-type macrophages through the local production of IL-10 and/or Arginase-1 (**Figure 12**). Macrophages can also change phenotype and function as tissue repair or fibrosis progresses, although the precise factors regulating these transitions *in vivo* remain poorly defined (Lawrance *et al*. 2017).

Adaptive cells include T helper cell subsets (Th1, Th2, Th17), regulatory T cells (Tregs), and B cells. The Th17-type immune response is pro-inflammatory and pro-fibrotic (Lawrance *et al*. 2017). Jian Li and colleagues investigated the level of pro-fibrotic molecules and collagen in the blood of Balb/c mice and found that the group treated with anti-IL-17 significantly decreased the quantity of collagen and expression of pro-fibrogenic molecules, leading to alleviated intestinal fibrosis (Li and Kuemmerle 2020).

At the same time, IL-17A is found to induce epithelial-mesenchymal transition (EMT) in IECs by reducing E-cadherin expression and increasing the expression of vimentin (Zhang 2015). Th2 is another T helper cell that produces IL-4, IL-5, and IL-13. IL-13 is able to inhibit MMP production,

causing elevated ECM deposition and inducing TGF-β function. By contrast, Th1-type immunity expressing IFN-γ may have anti-fibrotic activity (**Figure 12**). The role of Tregs in fibrogenesis is less clear, although they could suppress Th17- and Th2-driven fibrosis.

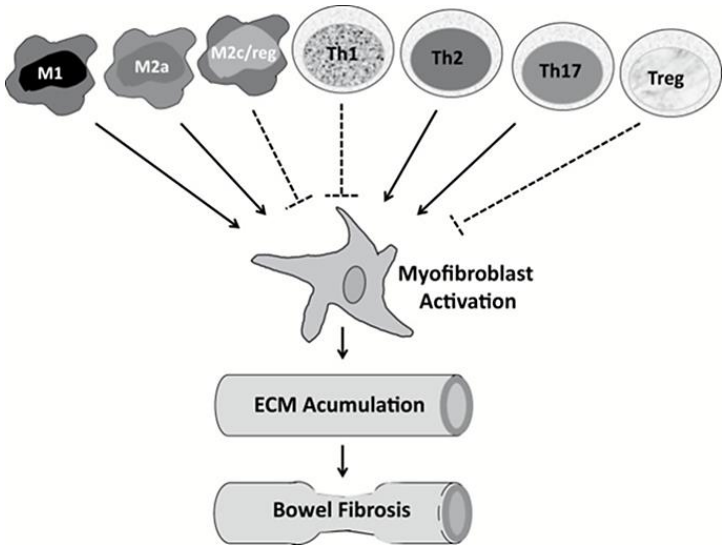


Figure 12: Intersection of immune response and fibrosis (Lawrance *et al*. 2017).

- Myofibroblasts and their mesenchymal cell precursors

Activated myofibroblasts secrete ECM proteins and promote an altered cytokine milieu that supports the fibrotic process. Myofibroblasts are derived from a variety of sources, not only resident mesenchymal cells, like fibroblasts, sub-epithelial myofibroblasts, and smooth muscle cells (SMCs), but also from dedifferentiation of epithelial cells by EMT (McAnulty 2007), and endothelial cells via endothelial-mesenchymal transition (EndoMT), stellate cells, pericytes, and bone marrow stem cells (Speca *et al*. 2012). Circulating fibrocytes derived from bone marrow enter tissues following injury and contribute to healing and scarring.

Fibroblasts, SMCs, and myofibroblasts

According to the current marker-based classification system, intestinal fibroblasts express the surface marker CD90 and vimentin but not desmin, whereas SMCs are CD90 (-), vimentin (low), α-smooth muscle actin (α-SMA) (+), and desmin (+). Myofibroblasts are CD90 (+), vimentin (+), α-SMA (+), and desmin (- or low), giving them an intermediate phenotype between fibroblasts and SMCs. In the inflamed gut, resident mesenchymal cells actively differentiate and dedifferentiate between these three interrelated mesenchymal cell phenotypes (Roulis and Flavell 2016). In case of

acute injury or inflammation, fibroblasts are activated, migrate to and proliferate at the site of injury and contribute to ECM production. Upon stimulation with specific growth factors such as TGF-β1, they start expressing α-SMA and differentiate into myofibroblast-like cells. Similarly, SMCs can also transdifferentiate into myofibroblasts. However, upon stimulation by pro-inflammatory cytokines, myofibroblasts can differentiate back into smooth muscle cells and induce SMC hyperplasia/hypertrophy, leading to thickened muscularis propria, stricture formation, and intestinal obstruction. In IBD, chronic inflammation fosters the production of inflammatory and pro-fibrotic mediators that sustain the activation and proliferation of myofibroblasts, leading to ECM overproduction and fibrosis (Wang, Lin, *et al*. 2021).

Fibrocytes

Fibrocytes are bone marrow-derived mesenchymal stem cells. In certain circumstances, fibrocytes circulating in the peripheral blood travel to affected tissues, where they differentiate into fibroblasts/myofibroblasts upon stimulation by inflammatory and pro-fibrotic growth factors. Fibrocytes may enhance inflammation by producing TNF-α and can directly augment fibrosis by producing collagen I (Wang, Lin, *et al*. 2021).

Epithelial and endothelial cell transformation

Intestinal fibrogenic cells may also be derived from non-mesenchymal cells, comprising epithelial and endothelial cells via transformation. EMT is a major pathophysiological process in the occurrence and development of many conditions, including intestinal fibrosis and cancer initiation, invasion, and metastasis (Pastushenko and Blanpain 2019). EMT is an important hallmark of intestinal fibrogenesis through which epithelial cells lose their polarity or their epithelial phenotype and transform into mesenchymal cells functionally and morphologically (Yu *et al*. 2021). Molecules associated with EMT were found in the fibrotic lesion of CD patients. It has been shown that some pro-inflammatory cytokines, such as IL-17A, have pro-fibrotic properties. IL-17A is found to participate in the initiation and development of intestinal fibrosis by inducing EMT. Other mechanisms involved in EMT induction that leads to intestinal fibrosis include TLR4 and succinate stimulation. The absence of the TLR4 gene attenuated chronic inflammation and colonic macrophage infiltration and suppressed intestinal fibrosis and collagen deposition. In addition, suppression of TLR4 transcription affected myofibroblasts' activity, collagen synthesis, and EMT in CCD-18Co cells, a human colon fibroblast cell line (Jun *et al*. 2020).

EndoMT can be viewed as EMT occurring in endothelial cells. Similarly, to epithelial cells, during the process of EndoMT, endothelial cells lose the expression of typical endothelial markers and

acquire fibroblast-like morphology and gene expression profile (Dejana, Hirschi, and Simons 2017). Evidence of *in vivo* EndoMT can be found in human colonic tissues from both CD and UC patients.

1.4.2 Major molecular mediators of intestinal fibrosis

In IBD, mucosal fibroblasts and myofibroblasts in the mucosa are exposed to a highly complex microenvironment consisting of multiple cytokines released by several different cell types, with immune cells in the lamina propria being the most important sources. Investigation in both human and animal models suggests that the release of cytokines during chronic inflammation could trigger the onset and directly promote intestinal fibrosis(Friedrich, Pohin, and Powrie 2019). However, it remains unclear whether the neutralization of a single cytokine or a combination of several cytokines will prevent or even reverse intestinal fibrosis.

TGF-β

TGF-β is considered the key driver of intestinal fibrosis in IBD. There are three subtypes of TGF-β: TGF-β1, TGF-β2, and TGF-β3. In 1990 it was reported that TGF-β1 could selectively augment collagen production by human intestinal smooth muscle cells *in vitro* (Graham, Bryson, and Diegelmann 1990). Following studies elucidated the overexpression of TGF-βs and their signaling receptors in tissue samples from intestinal CD resections, indicating a potential role of these regulatory molecules in the pathophysiology of CD. TGF-β is stored as a disulphide-bonded homodimer, non-covalently bound to a latency-associated protein (LAP), which keeps it inactive. TGF-β binding to its receptor requires dissociation of LAP, a process catalyzed by plasmin, urokinase-type and plasmin activators, tissue-type plasminogen activators, MMPs, cathepsins, and integrins. After binding to its receptor, TGF-β activates intracellular signaling through both the Smad-dependent (canonical TGF-β signaling) and Smad-independent (non-canonical TGF-β signaling) pathways. In the Smad-dependent pathway, the TGF-β receptor complex directly phosphorylates Smad2 and Smad3, which then forms a complex with Smad4 and enters the nucleus to regulate the transcription of target genes (Ihara, Hirata, and Koike 2017). Smad6 and Smad7 negatively regulate the TGF-β/Smad2-signaling pathway (Miyazawa and Miyazono 2017). Additionally, Smad7 was reported to have a lower expression level in the mucosa overlying strictured than non-strictured areas. In addition to the canonical TGF-β/Smad signaling pathway, TGF-β can activate other signaling pathways, including the extracellular regulated protein kinases (ERK) signaling pathway, phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) signaling pathway, and WNT signaling pathway (Wang *et al*. 2022) (**Figure 13**).

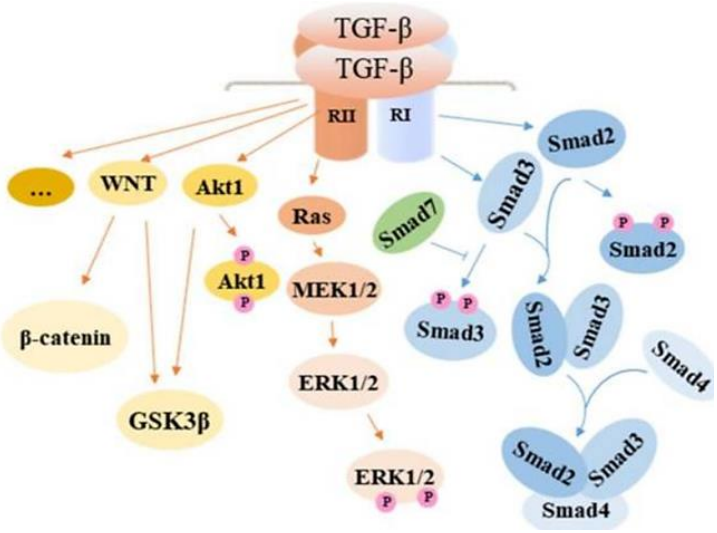


Figure 13: TGF-β activated signaling pathway in intestinal fibrosis. TGF-β promotes fibrosis by regulating related cells through the activation of the canonical Smad signaling pathway and non-canonical pathway including MAPK and WNT, which contribute to the development of EMT in epithelial cells, the proliferation of fibroblasts, and transformation of fibroblasts and SMCs to myofibroblasts. TGF-β, transforming growth factor β; Smad, drosophila mothers against decapentaplegic; Ras, rat sarcoma; MEK, methyl ethyl ketone; ERK, extracellular regulated protein kinases; GSK3β, glycogen synthase kinase3β (Wang, Lin, *et al*. 2021).

Activated TGF-β promotes the differentiation from fibroblasts to myofibroblasts, resulting in elevated expression of α-SMA, increased proliferation, and decreased apoptosis (Valatas *et al*. 2017). In addition, TGF-β treatment enhances the migration and collagen production of myofibroblasts. In human intestinal organoids, which contain both epithelial and mesenchymal cells, including myofibroblasts, TGF-β stimulation increased the production of a series of pro-fibrotic factors, such as collagen I, α-SMA, and fibronectin (Rodansky *et al*. 2015). Moreover, knockdown of Smad7, obtained with a specific antisense oligonucleotide, attenuated colitis, and colitis-driven colonic fibrosis in 2,4,6-trinitrobenzene sulfonic acid (TNBS) mice model (Izzo *et al*. 2018). TGF-β appears to be associated with other physiological changes in intestinal fibrosis. ROS can lead to the development of intestinal fibrosis on account of TGF-β dependency. NF-E2-Related Factor 2 (Nrf2) is a nuclear transcription factor that plays a role in defending the cells against oxidative stress. A study showed that Nrf2 could suppress intestinal fibrosis *in vivo* and *in vitro*. In this study, TNBS-induced-fibrosis mice were given an Nrf2 agonist, which resulted in a reduced degree of fibrosis compared with the no agonist group. Similarly, the application of siNrf2 inhibited the differentiation of TGF-β-induced CCD18Co cells (Guan *et al*. 2018).

In the fibrotic process, the interaction between TGF-β1, canonical WNT/β-catenin, and PPARγ has been well documented (Vallee and Lecarpentier 2019).

PPARγ is a transcription factor that belongs to the superfamily of nuclear hormone receptors. This factor heterodimerizes with the retinoid X receptor to bind PPAR response elements (PPRE).

PPARγ is present in many cells, including adipose tissue, muscles, brain, and cells of the immunity system, and stimulates the expression of numerous target genes and, thus, regulates glucose homeostasis, insulin sensitivity, lipid metabolism, the immune response and inflammation (Decara *et al*. 2020; Hong *et al*. 2019). PPARγ is also observed in fibroblasts and participates in the regulation of connective tissue, activation of mesenchymal cells, differentiation, and cell survival by linking with the metabolism and fibrogenesis (Chung *et al*. 2021). PPARγ plays a major role in decreasing the fibrotic process by antagonizing TGF-β1, and his agonists decrease the deposition of TGF-β induced collagen and myofibroblast differentiation.

Wnt/β-catenin signaling has a major role in metabolism, embryonic development, and EMT (El-Sahli *et al*. 2019; Salton, Volpe, and Confalonieri 2019). Stimulation of the canonical Wnt pathway leads to an increase in the nuclear and cytosolic levels of β-catenin. Ye *et al*. showed that TGF-β could upregulate the Wnt/β-catenin pathway through the inhibition of the kinase glycogen dynthase kinase 3 beta (GSK3β), thus enhancing the EMT process (Ye *et al*. 2013). Another study on alveolar cells has demonstrated that β-catenin is necessary for α-SMA transcription, and its induction by TGF-β depends on CREB-binding protein activity (Henderson *et al*. 2010). In fact, an inhibitor, ICG-00, of the β-catenin/CBP interaction reverted fibrosis and inhibited α-SMA and collagen induction. The Wnt/β-catenin pathway plays another role in EMT induction. In normal conditions, the inactive β-catenin forms a complex with E-cadherin located at the epithelial barrier. This complex is involved in maintaining epithelial stability and in keeping silent the β-catenin signaling. In pathological conditions, such as fibrosis, the Wnt signaling is active, the complex is disrupted, the β-catenin is free to translocate into the nucleus, and the E-cadherin is degraded, thus enhancing the loss of the epithelial phenotype. In fact, it has been shown that the EMT-mediated disruption of the E-cadherin/β-catenin complex leads to fibrosis. Moreover, by inhibiting the complex disruption, the epithelial phenotype is restored, suggesting an essential role for the Wnt-mediated EMT in fibrosis (Di Gregorio *et al*. 2020).

TGF-β1 can activate the canonical Wnt pathway and can decrease PPARγ expression. In contrast, PPARγ decreases the TGF-β1/WNT/β-catenin pathway. TGF-β1 is a major controller of fibrosis and an interesting target in fibrosis (Vallee and Lecarpentier 2019).

IL-1α and IL-1β

IL-1α is constitutively expressed in epithelial cells but also can be expressed by other cell types upon their activation, such as macrophages, monocytes, endothelial cells, and others. IL-1α released

from damaged IECs acts as a damage-associated molecular pattern (DAMP), contributing to the initiation and development of chronic intestinal inflammation (Valatas *et al*. 2017). In a DSS-induced colitis mouse model, IL-1α-deficient mice exhibited mild disease symptoms with improved recovery, and neutralization of IL-1α in control mice during acute colitis led to alleviation of clinical and histological manifestations.

IL-1β is primarily produced by mononuclear phagocytes, acting as a pro-inflammatory effector in intestinal inflammation (Friedrich, Pohin, and Powrie 2019). IL-1β stimulates the secretion of collagens I and IV, IL-8, monocyte chemoattractant protein (MCP)-1, and MMP-1 by colonic sub-epithelial myofibroblasts. However, another report suggested that IL-1β inhibited collagen synthesis and induced collagenase and TIMP-1 production in intestinal smooth muscle cells. The role of IL-1β in intestinal fibrosis remains unclear (Wang, Lin, *et al*. 2021).

IL-6

IL-6 can be expressed by intestinal mononuclear cells, epithelial cells, and mesenchymal cells (Friedrich, Pohin, and Powrie 2019). The role of IL-6 is well-established in IBD. Besides its known pro-inflammatory activity, IL-6 also contributes to fibrosis. Upon TGF-β activation, intestinal mesenchymal cells are activated and increase their autocrine production of various cytokines, including IL-6.

IL-33

IL-33 has been associated with Th2 immune responses and has been identified as a cytokine with pro-fibrotic function. In the gut, IL-33 is mainly expressed by fibroblasts, smooth muscle cells, endothelial cells, and adipocytes, as well as IECs and mononuclear cells infiltrating lamina propria in active UC (Wang, Lin, *et al*. 2021). Increased expression levels of IL-33 and its receptor, ST2, were found in IBD patients, particularly in UC.

IL-36

IL-36 consists of three agonists, IL-36α, IL-36β, IL-36γ, and IL-36 receptor antagonist (IL-36Ra). The IL-36 agonists have demonstrated pro-inflammatory effects, and recent evidence suggests additional roles in intestinal fibrosis(Buhl and Wenzel 2019). In the intestinal tract, IL-36 is generated by many different cell types, including fibroblasts, myofibroblasts, epithelial cells, goblet cells, macrophages, and glial cells (Nishida *et al*. 2017). In turn, many cell types can respond to IL-

36, including immune cells, epithelial cells, endothelial cells, and mesenchymal cells, and IL-36 induces distinct gene expression and functional changes (Elias *et al*. 2021). IL-36α and IL-36γ were upregulated in endoscopic biopsies of patients with active colonic CD compared to unaffected biopsies from the same patients. In addition, increased expression of IL-36α and IL-36γ, but not of IL-36β, was observed in the intestinal mucosa in UC patients rather than control or CD patients. IL-36R-deficient mice or mice injected with anti-IL-36R antibodies develop less severe colitis and fibrosis in DSS- or TNBS-induced colitis (Scheibe *et al*. 2019), suggesting that blockade of IL36R signaling has the potential to be an efficient mechanism for prevention and treatment of intestinal fibrosis in IBD patients.

IL-17

There is evidence demonstrating the regulatory role of IL-17 in the development of fibrosis, but its contribution to IBD and intestinal fibrosis remains controversial (Latella and Viscido 2020). IL-17A significantly inhibits myofibroblast migration and promotes the production of MMP-3, MMP-12, TIMP-1, and collagen by myofibroblasts from strictured CD tissues. Occurrence of EMT and high levels of IL-17A are observed in the intestinal mucosa of CD patients, and IL-17A may induce EMT in intestinal epithelial cells. Anti-IL-17A treatment alleviated intestinal fibrosis by reducing EMT in mouse intestines (Zhang 2015). Consistent with these data, in a TNBS-induced intestinal fibrosis mouse model, treatment with anti-IL-17 antibody significantly alleviated intestinal fibrosis and reduced both mRNA and protein levels of collagen 3, TNF-α, TIMP-1, and MMP-2, as well as the decreased levels of pro-fibrogenic cytokines IL-1β, TGF-β1, and TNF-α. However, secukinumab, a human anti-IL-17A monoclonal antibody, failed to show efficacy in CD and, in fact, led to worsening of intestinal inflammation in some patients, suggesting that baseline levels of IL-17 are necessary for intestinal homeostasis.

IFN-γ

IFN-γ suppresses fibrosis (Wang, Lin, *et al*. 2021). IFN-γ antagonizes TGF-β-induced phosphorylation of Smad3. Moreover, IFN-γ also directly inhibits fibroblast proliferation, TGF-β1-induced expression of genes encoding procollagens I and III, and collagen synthesis in activated myofibroblasts.

1.4.3 Involvement of the gut microbiome in intestinal fibrosis

The gut microbiome influences health and disease. Changes in the composition of the gut microflora, immune system, or intestinal barrier function can upset the host-microbiome interaction and lead to inflammation and fibrosis. For instance, adherent-invasive *Escherichia coli* (AIEC), a type of gut bacteria, is confirmed to be involved in IBD (O'Brien *et al*. 2017). A study discovered that AIEC could colonize the intestine when acute inflammation occurs, leading to fibrosis via increased expression of ST2, the receptor of IL-33, under the mediation of flagellin (Imai *et al*. 2019). The microbial infection triggers a disorder of the immune microenvironment, where the persistent infection of AIEC leads to an active T-helper 17 response and increases the fibrotic growth factors. Tumor necrosis factor-like cytokine 1A (TL1A) is associated with IBD, regulating the location and severity of intestinal inflammation and fibrosis. TL1A production is elevated in the inflamed gut mucosa, is linked with fibrostenosing CD, and is well dependent on the gut microbiome. Moreover, it was shown that pro-fibrotic and inflammatory phenotype resulting from TL1A-upregulation was abrogated in the absence of resident microbiota (Jacob *et al*. 2018). *Salmonella enterica* serovar *Typhimurium* also plays a key role in intestinal fibrogenesis. Ehrhardt *et al*. found that mice develop intestinal fibrosis under persistent infection with *Salmonella enterica* serovar Typhimurium through inducing protease expression in macrophages and epithelial cells (Ehrhardt *et al*. 2019). Microbial products such as cell wall components can be directly pro-fibrogenic, while the administration of fecal material or anaerobic bacteria into autologous animals could also trigger intestinal fibrosis. Generally, the CD is associated with shifts in the composition of the enteric microbiota, with overall reduced bacteria diversity and significantly depleted abundance of the phyla *Firmicutes* and *Bacteroidetes*, while *Proteobacteria* and *Actinobacteria* increase. However, the gut microbe does not only contribute to the induction but also the prevention of intestinal fibrosis. The gut microbe and related proteins can protect the intestine from fibrotic injury. For example, a study found that heat-shock-protein 56 (HSP56) secreted by invasive *Lactococcus lactis* can reduce the severity of fibrosis (da Cunha *et al*. 2020). When the microbiome leads to dysregulation of the immune response, the immune and non-immune cells sense the pathogen-associated molecular patterns (PAMPs) from microbe with the help of pattern recognition receptors (PRRs), which consist of TLRs and Nod-like receptors (NLRs). MyD88 is an adaptor molecule that helps all TLRs except TLR3 to release signals; hence, several researchers believe that MyD88 may have a role in intestinal fibrosis. Zhao *et al*. confirmed that intestinal fibrosis improves when the gene of MyD88 in α-SMA positive cells is deleted (Zhao *et al*. 2020). However, an earlier study from Lutz *et al*. indicated that MyD88 shows no or little effect on intestinal fibrosis (Lutz *et*

al. 2017). As a member of TLRs, TLR4 is reported to participate in intestinal fibrosis. Studies show that intestinal fibrosis and the deposition of collagen are suppressed under the silence of the TLR4 gene *in vivo* and *in vitro* (Jun *et al*. 2020).

1.4.4 Current therapies for intestinal fibrosis and stenosis

The therapeutic approach to intestinal strictures depends on the cause, localization, and extension of the stricture. No therapy targeting fibrosis is currently available in clinical practice. Therapeutic options include medical treatment, targeting the inflammatory component of the stricture, endoscopic treatment, and surgery (Lenti and Di Sabatino 2019) (**Table 2**). Strictures may benefit from anti-inflammatory therapy, which could reduce wall edema and, thus, intestinal wall thickness. Data on corticosteroids are limited and controversial. Although these drugs seem to provide short-term relief of symptoms, they do not decrease the need for surgery over time. Steroid-dependent or refractory patients may benefit from biological drugs. Currently, there are only a little data on anti-TNF agents. At first, some evidence led to concerns about this approach, given the apparent worsening of stenosis due to anti-TNF caused by the rapid healing of ulcers (Toy *et al*. 2000). However, more recent data have overcome this old assumption, demonstrating the efficacy of anti-TNF in stricturing CD in up to about two-thirds of the patients. A prospective multicentric observational cohort study (CREOLE) tested the effect of induction and maintenance therapy with adalimumab in patients with stenosing CD, demonstrating its safety and efficacy in maintaining surgery-free remission in half of the included patients over a 4-year follow-up (Bouhnik *et al*. 2018). However, the surgery-free interval is still short, and data on a direct anti-fibrotic effect of anti-TNF therapy are still lacking. About other biologic drugs, vedolizumab (anti-integrin α4β7) has been shown to be effective and safe in stricturing CD (Engel *et al*. 2018), whilst a recent multicentre study showed that ustekinumab (anti-IL12/23) is associated with a lower likelihood to achieve remission at six months in these patients (Ma *et al*. 2017). As for 5-aminosalicylic acid (5-ASA), it does not appear to have anti-fibrotic activity and fails to medically induce remission in CD (Gordon 2017). If anti-inflammatory therapy is not effective in relieving obstructive symptoms or if symptoms recur within a short period, endoscopic therapy, or bowel resection should be considered (Rieder, Fiocchi, and Rogler 2017). With regards to the endoscopic approach, endoscopic balloon dilatation (EBD) is considered the preferred technique for selected CD strictures, as it has proven a high rate of short-term technical and clinical efficacy, with substantial long-term efficacy and acceptable rates of complication (Hirai *et al*. 2018). When medical or endoscopic therapy fails or is contraindicated, surgery should be considered.

	Type	Treatment route	Effect
Drugs	Mesalazine	Oral	Lower endoscopic postoperative recurrences in Mesalazine group compared with placebo groups (Florent *et al*. 1996).
	Azathioprine	Oral	49% of patients are free of rehospitalization in 36 mouths (de Souza *et al*. 2013).
	Infliximab	Intravenous injection	No development of new small bowel stenosis; Part of stenosis completely regressed (Chen *et al*. 2021).
	Adalimumab	Intravenous injection	Effectively prevent the occurrence of small bowel stenosis (Bouhnik *et al*. 2018).
	Vedolizumab	Intravenous injection	The patient remains in clinical and endoscopic remission without need for surgical treatment (Urlep and Orel 2017).
	Thiopurines	Oral	Early use is associated with preventing surgery and the development of fibrostenosis (Rieder, Fiocchi, and Rogler 2017)
Endoscopic treatment	Endoscopic stricturotomy (ESt)	Endoscopic	Offers comparable surgery-free survival; Avoids a surgical resection for a stricture at a previous ileocolonic anastomosis (ICA).
	Stenting	Endoscopic	Effective treatment for strictures relapse; Risk of adherence of the stent to the mucus membrane of the bowel, perforation, and spontaneous distal migration of the stent.
	Endoscopic balloon dilation (EBD)	Endoscopic	Complete through gastrointestinal endoscopy; Reduce the need for surgery for resection.

Table 2: Present treatments in IBD-associated fibrosis.

1.4.5 Promising anti-fibrotic therapy

Increasing knowledge of the molecular mechanisms underlying intestinal fibrosis has enabled the identification of anti-fibrotic therapeutic targets. Currently, although there is no therapy to treat or reverse intestinal fibrosis into CD, several pre-clinical studies have been conducted *in vivo*, *ex vivo*, and *in vitro*, with encouraging results (Santacroce, Lenti, and Di Sabatino 2022).

Targeting TGF-β pathways

The most promising target for anti-fibrotic therapy is TGF-β and its signaling pathways. Several studies on fibrosis of other tissues have shown that TGF-β1 production was strongly stimulated by the local activation of angiotensin II (Warner *et al*. 2007; Uhal *et al*. 2007), the main effector of the renin-angiotensin system, whose activity is increased in the colonic mucosa of CD patients. For this reason, it was assumed that angiotensin conversing enzyme (ACE) inhibitors and sartans (angiotensin II receptor antagonists) could also play a role in the process of intestinal fibrogenesis. The first ACE inhibitor investigated was captopril, which showed to be effective in preventing colonic fibrosis in TNBS-induced colitis in rats. Its anti-fibrotic action has been assumed to derive from blocking TGF-β1 overexpression and/or from a direct downregulation of TGF-β1 transcripts.

More recently, losartan, an antagonist of the angiotensin II receptor, was investigated and exhibited a pleiotropic effect, reducing TGF-β1 concentration and significantly improving the macro- and microscopic scores of fibrosis in the colonic wall of rats (Wengrower *et al*. 2012).

Based on the known antagonistic relationship between the TGF-β/Smad pathway and PPARγ, the effect of a novel 5-ASA analog (named GED-0507-34 Levo), able to activate PPARγ, has been investigated. GED-0507-34 Levo showed improvement of intestinal fibrosis in DSS-induced chronic colitis in mice, reducing the activation of myofibroblasts and the expression of the main pro-fibrotic molecules including TGF-β, Smad3, IL-13, and CTGF (Speca *et al*. 2016). Similarly, it has been shown that other PPARγ agonists, such as troglitazone and rosiglitazone, may be useful in counteracting the fibrogenic process by suppressing TGF-β1-induced synthesis of collagen, fibronectin, and α-SMA in human primary intestinal myofibroblasts (Koo *et al*. 2017).

Other compounds have been shown to downregulate the TGF-β signaling. Anti-fibrotic intestinal efficacy has been proposed for two molecules approved for the treatment of idiopathic pulmonary fibrosis, namely pirfenidone and nintedanib (Ma *et al*. 2017). In particular, pirfenidone inhibited, both *in vivo* and *in vitro*, intestinal fibroblast proliferation and motility and reduced collagen production through different TGF-β1 signaling pathways (Cui *et al*. 2020).

Targeting TIMP/MMP balance

Intestinal fibrosis in CD is mainly due to the imbalance of deposition and degradation of ECM, regulated also by MMPs and TIMPs. Thalidomide, a molecule with anti-inflammatory activity and emerging as an alternative treatment for refractory CD, has been shown to inhibit *in vivo* intestinal fibrosis by regulating TIMP/MMP protein balance and degradation of ECM (Flier *et al*. 2010).

Targeting EMT

Another therapeutic target of recent interest is EMT. Recent data from animal models of renal, hepatic, and cardiac fibrosis have demonstrated the anti-fibrotic effect of recombinant human bone morphogenic protein-7 (rhBMP-7). BMP-7 is a member of the TGF-β superfamily with the ability to counteract the pro-fibrotic action of TGF-β1. *In vitro* and *in vivo* studies have shown the effect of rhBMP-7 in inhibiting TGF-β1-induced EMT associated with intestinal fibrosis (Santacroce, Lenti, and Di Sabatino 2022).

Targeting intestinal microbiota

Given the increasing emphasis on the pro-fibrotic impact of the gut microbiota, *in vitro* and *in vivo* studies have been carried out to assess the effect of probiotics and prebiotics on intestinal fibrosis (Liu *et al*. 2019; Park *et al*. 2018). Kashima *et al.* demonstrated that polyphosphate, a bioactive

molecule that is produced by *Lactobacillus brevis*, improved the intestinal inflammation and fibrosis in two murine colitis models and reduced IL-1β and TNF-α levels in colonic epithelial cells, but it did not influence collagen and CTGF expression levels in the CCD-18Co cells (Kashima *et al*. 2015). Deng *et al* studied the beneficial effect of a combination of probiotics (*Lactobacillus plantarum, L. acidophilus, L. rhamnosus,* and *Bifidobacterium animalis*) on abdominal adhesions in a rat model (Deng *et al*. 2020). The probiotic treatment significantly reduced the levels of pro-inflammatory factors IL-1β, IL-6, and TNF-α in serum and intestinal tissue, and markedly downregulated the inflammatory (TLR4/NF-κB) and fibrotic (TGF-β1/Smad) signalling pathways in intestinal tissue. Moreover, the probiotics significantly increased the relative abundance of probiotic *Bacteroidetes* (at the phylum level), *Bacteroidales* (at the order level), *Lactobacllales* (at the order level) and *Lactobacillus* (at the genus level), and markedly reduced the number of pathogenic *Proteobacteria* (at the phylum level), *Erysipelotrichales* (at the order level), *Verrucomicrobiales* (at the order level), *Klebsiella* (at the genus level) and *Serratia* (at the genus level).

2 Aim of the study

The doctoral project aimed to investigate the *in vitro* effects of high-concentration multi-strain probiotic formulations on two different intestinal cell models, to evaluate their anti-inflammatory and anti-fibrotic potential.

Chronic inflammatory bowel disease (IBD) is a recurrent inflammatory disease of the gastrointestinal tract. In this pathological condition, the hypoxia plays a decisive role. The hypoxia-inducible factor 1-alpha (HIF-1α), as a transcription factor, can regulate the cellular adaptation to low oxygen levels and support the development and function of the gut barrier. Moreover, a plethora of studies highlights the therapeutic potential of HIF-prolyl hydroxylase (PHD) inhibitors in several models of IBD, supporting HIF-1α as an anti-inflammatory agent. Despite the crucial role of the HIF pathway in intestinal mucosal physiology and its relationships with gut microbiota, the ability of probiotics to influence the HIF pathway has not been thoroughly investigated. Therefore, the first objective has been to evaluate the ability of the multi-strain probiotic formulation SLAB51, previously studied *in vitro* and in vivo, to influence the HIF-1α pathway and counteract the inflammatory stimulus of lipopolysaccharide (LPS) in a model of *in vitro* human intestinal epithelial cells (Caco-2 intestinal epithelial cells, IECs).

Thus, in the present study, the IECs were treated with SLAB51 lysate in presence or absence of LPS. HIF-1α, PHD2, and pAKT (phospho-protein kinase B) expression was evaluated; the glycolytic metabolism was studied by assessing the levels of L-lactate through colorimetric and real-time measurements of energetic metabolism using a Seahorse XFp Flux Analyzer. The inflammatory markers, such as NF-κB, nitric oxide synthase2 (NOS2), and IL-1β were also studied. The effect of specific inhibitors of PI3K (phosphatidylinositol 3 kinase) and AKT (protein kinase B) was investigated to analyze further the involvement of the PI3K/AKT pathway in the mechanisms underlying the effects induced by SLAB51 lysate. The PHD2 activity was evaluated by analyzing the HIF-1α hydroxylation levels following proteasome inhibition.

A severe complication associated to chronic inflammation is fibrosis, as often occurred in IBD. Despite the growing understanding around the mechanisms behind IBD-related inflammation and the possible solutions for its treatment, almost no progress has been made toward the development of anti-fibrotic therapies aimed at improving the life of patients with IBD. Only few studies are available regarding the effects of probiotics on IBD-associated intestinal fibrosis. Therefore, the other aim of the present project has been to explore the potential anti-fibrotic impact of the lysates from two different high-concentration multi-strain probiotic formulations (VSL#3® and Vivomixx®) on CCD-18Co human intestinal fibroblasts activated by TGF-β1. In particular, the

CCD-18Co cells were cultured in the presence of TGF-β1 to induce the development of a fibrotic phenotype and the cell growth and viability, Collagen-I, α-SMA, and pSmad2/3 expression levels, and TGF-β1 levels were assessed.

3 Materials and Methods

3.1 Preparation of bacterial lysates for cell treatments

The probiotic formulation SLAB51 (6 g sachet, 800 billion CFU*)* (sold as Sivomixx, Ormendes SA, Jouxtens-Mézery, Switzerland), according to the information on the boxes, contains eight different live bacterial strains: *Streptococcus thermophilus* DSM 32245, *Bifidobacterium lactis* DSM 32246, *Bifidobacterium lactis* DSM 32247, *Lactobacillus acidophilus* DSM 32241, *Lactobacillus helveticus* DSM 32242, *Lactobacillus paracasei* DSM 32243, *Lactobacillus plantarum* DSM 32244, and *Lactobacillus brevis* DSM 27961.

The probiotic formulation VSL#3® (0.658 g capsule, 112 billion CFU*)* (distributed by Ferring Pharmaceuticals S.p.A., Milan, Italy), according to the information on the boxes, contains eight different live bacterial strains: *Streptococcus thermophilus* BT01, *Bifidobacterium breve* BB02, *Bifidobacterium longum* BL03§, *Bifidobacterium infantis* BI04§, *Lactobacillus acidophilus* BA05, *Lactobacillus plantarum* BP06, *Lactobacillus paracasei* BP07, *Lactobacillus debrueckii* subsp. *bulgaricus* BD08§§ (§Reclassified as *Bifidobacterium lactis*; §§reclassified as *Lactobacillus helveticus*).

The probiotic formulation Vivomixx® (0.676 g capsule, 112 billion CFU*)* (trade name Vivomixx® in EU, Visbiome® in USA, De Simone Formulation® in Korea) (distributed by Biospaera Pharma S.r.L.), according to the information on the boxes, contains eight different live bacterial strains: *Streptococcus thermophiles* DSM 24731, *Bifidobacterium breve* DSM 24732, *Bifidobacterium longum* DSM 24736*, *Bifidobacterium infantis* DSM 24737*, *Lactobacillus acidophilus* DSM 24735, *Lactobacillus plantarum* DSM 24730, *Lactobacillus paracasei* DSM 24733, and L*actobacillus delbrueckii* subsp. *Bulgaricus* DSM 24734** (*reclassified as *Bifidobacterium lactis*; **reclassified as *Lactobacillus helveticus*).

For bacterial lysates preparation, SLAB51 formulation was suspended at the concentration of 133×10^9 CFU in 10 ml of phosphate buffered saline (PBS, Euro Clone, West York, UK) while the content of each capsule was suspended at the concentration of 102×10^9 CFU in 10 ml of PBS.

The probiotic formulations resuspended in PBS were centrifuged at 8600× g, washed twice, and sonicated (30 min, alternating 10 s of sonication and 10 s of pause) using a Vibracell sonicator (Sonic and Materials, Danbury, CT, USA). Bacterial cell disruption was verified by measuring the sample's absorbance at 590 nm (Eppendorf Hamburg, Germany) before and after every sonication step. The samples were then centrifuged at 17,949× g and the supernatants were filtered using a 0.22-µm-pore filter (Corning Incorporated, Corning, NY, USA) to remove any whole remaining

bacteria. Total protein content was determined by DC Protein Assay (Bio-Rad, Hercules, CA, USA), using bovine serum albumin (BSA, Sigma-Aldrich, Saint Louis, MO, USA) as the standard.

3.2 Cell lines and culture conditions

Model of in vitro human intestinal epithelium. The Caco-2 cell line purchased from Sigma-Aldrich (St. Louis, MO, USA) was grown in the DMEM supplemented with 10% Fetal Bovine Serum (FBS), 1% non-essential amino acid, 1 mM sodium pyruvate and 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Euro Clone, West York, UK), in a humidified incubator 5% CO_2, 95% air atmosphere at 37°C. After reaching 80% confluence, cells were seeded, into a sterile tissue culture 6-well plate (Becton, San Jose, CA, USA) or into Dickinson a 96 well-plate (Seahorse XF96 Cell Culture Micro-plate, Agilent, Santa Clara, CA, USA) at 60,000 cells/cm^2 and cell growth was monitored by microscopy. 14 days post-confluence, cells were incubated with or without the SLAB51 lysate at the indicated concentrations and with or without 10 µg/ml lipopolysaccharide (LPS, Sigma-Aldrich). Where indicated, the cells were also pre-treated for 30 min with 100 µM NOS2 inhibitor N-(3-(aminomethyl) benzyl) acetamidine (1400W) (Sigma-Aldrich) prior to LPS treatment. To evaluate the effect of the bacterial lysate and LPS on cell viability and proliferation, the cells were cultured in the 24-well plate and treated for 24 h, after which they were washed with PBS, centrifuged for 10 min at 400× g and the pellets incubated with a 0.04% Trypan blue (Euro Clone, West York, UK) solution for 5 min. Untreated cells were used as controls. Cells were transferred to a Bürker chamber and counted by microscopy (Eclipse 50i, Nikon Corporation, Tokyo, Japan). To inhibit the PI3K/AKT pathway, the cells were pre-treated for 30 min with 20 µM perifosine (AKT inhibitor, Cell Signaling Technology, Danvers, MA, USA) or 10 µM LY294002 (PI3K inhibitor, Cell Signaling Technology). 10 µM MG132 (Merck KGaA, Darmstadt, Germany) was used to inhibit proteasomal degradation of hydroxylated HIF-1α. The iron chelator deferoxamine (DFO, 150 µM) was used to inhibit PHD2 activity.

Model of in vitro human intestinal fibrosis. Human intestinal CCD-18Co fibroblast cell line was obtained from American Type Culture Collection (ATCC, Georgetown, DC, USA) and cultured in DMEM supplemented with 10% of FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine (Euro Clone). The cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For all the experiments, 5,000 cells/cm^2 CCD-18Co cells were plated and grown overnight, followed by 24 h serum-deprivation. This was followed by a 48 h stimulation with 10 ng/ml human transforming growth factor-β1 (hTGF-β1; Cell signaling Technology) to induce

the fibrotic phenotype. Stimulation was performed both in the presence or absence of VSL#3® or Vivomixx® lysates (50 μg protein/ml corresponding to 10^8 CFU/ml) in order to observe its effects. After treatment, cells were collected and centrifuged for 10 min at 400× g. To analyze their number and viability, pellets were incubated with 0.04% Trypan blue (Euro Clone) for 5 min., untreated cells were used as controls. Cells were then transferred to a Bürker chamber and counted by microscopy (Eclipse 50i, Nikon Corporation).

3.3 Western blot analysis

For protein expression analysis through western blot, cells were harvested, washed in PBS and then lysed in ice-cold RIPA buffer (Merck KGaA, Darmstadt, Germany) containing 100 mM protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The protein amount was evaluated with DC Protein Assay (Bio-Rad, Hercules, CA, USA) using BSA as standard. 25μg of protein lysates were mixed with sample buffer, samples were denatured (at 100°C for 5 min) and run on 10% SDS polyacrylamide gel. Proteins were transferred onto 0.45 μm nitrocellulose membrane sheets (Bio-Rad) for 1 h at 4°C at 70V using a Mini Trans-Blot Cell apparatus (Bio-Rad). Membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies. In **Table 3**, primary and secondary antibodies used for western blot analysis are shown. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech), according to the manufacturer's instructions. Band relative densities were determined using a chemiluminescence documentation system ALLIANCE (UVITEC, Cambridge UK), and values were given as relative units.

Antibodies	Donor	Dilution	Supplier
Primary antibodies			
Monoclonal antibody anti-HIF-1α	Rabbit	1:1,000	Cell Signaling Technology
Monoclonal antibody anti-Hydroxy-HIF-1α	Rabbit	1:1,000	Cell Signaling Technology
Monoclonal antibody anti-PHD2	Rabbit	1:1,000	Abcam
Monoclonal antibody anti-phospho-AKT	Rabbit	1:1,000	Cell Signaling Technology
Monoclonal antibody anti-AKT	Rabbit	1:1,000	Cell Signaling Technology
Polyclonal antibody anti-NOS2	Rabbit	1:500	Boster
Monoclonal antibody anti-phospho-NF-kB p65	Rabbit	1:1,000	Cell Signaling Technology
Monoclonal antibody anti-NF-kB p65	Rabbit	1:1,000	Cell Signaling Technology
Monoclonal antibody anti-β-actin	Mouse	1:1,000	OriGene
Monoclonal antibody anti-α-SMA	Mouse	1:1,000	OriGene
Polyclonal antibody anti-COL1A1	Rabbit	1:500	Boster
Monoclonal antibody anti- phospho-Smad2/3	Rabbit	1:100	R&D Systems Inc.
Monoclonal antibody anti-GAPDH	Mouse	1:1,000	OriGene
Secondary antibodies			
anti-rabbit IgG-HRP	Goat	1:2,000	Millipore
anti-mouse IgG-HRP	Goat	1:2,000	Bio-Rad

Table 3: Primary and secondary antibodies used for western blot analysis.

3.4 L-lactate assay

The levels of L-lactate were analyzed in the supernatants of Caco-2 cells using the L-lactate assay kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Accordingly, the supernatants were deproteinized with a 10 kDa NMWCO Centrifugal Filter Unit (Millipore EMD, Darmstadt, Germany), and the filtrate was added to reaction wells. The absorbance was measured by spectrophotometric reading at 570 nm. The L-lactate levels were determined by comparison to a standard curve.

3.5 Metabolic studies

Caco-2 cells treated as described above were evaluated for extracellular acidification rate (ECAR) and O_2 consumption rate (OCR) to calculate glycolysis rate (ECAR/OCR) using the Seahorse XFe96 Analyzer (Agilent) following the manufacturer's instructions. Briefly, on the day of the assay, the medium was changed for Seahorse XF DMEM Medium pH 7.4 supplemented with glucose (10 mmol/L), pyruvate (1 mmol/L), and glutamine (2 mmol/L) (Agilent), and the cells were

allowed to equilibrate in a non-CO_2 incubator for 1 h; OCR and ECAR were then measured. XFp Mito Stress Test Kit was used to test mitochondrial function. Injection of oligomycin (1 μM), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP, 1 μM), and the mixture of rotenone and antimycin A (1 μM) allows for determining the key bioenergetic parameters: basal respiration, ATP production-linked respiration (ATP production), maximal respiration, spare respiratory capacity, nonmitochondrial respiration, proton leak, and coupling efficiency.

3.6 IL-1β ELISA

The levels of released IL-1β were quantified in the Caco-2 cell supernatants using a human IL-1β enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich, Saint Louis, MO, USA), as described in the manufacturer's instructions. The collected media was cleared of cellular debris/dead cells by centrifugation at 1,000× g for 15 min. The absorbance was measured by spectrophotometric reading at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The IL-1β concentration was determined by comparison to a standard curve. Results are expressed as pg/ml.

3.7 Nitrite level assay

The enzymatic activity of NOS2 was evaluated by measuring nitrite levels using nitrate reductase and Griess reaction through a colorimetric assay (Nitrite Assay kit-Sigma-Aldrich, Saint Louis, MO, USA). Nitrite levels were assayed in the supernatants of Caco-2 cells, treated as above described, applied to microtiter plate well, according to the manufacturer's instructions. The absorbance was measured by spectrophotometric reading at 550 nm. The nitrite content of each sample was evaluated with a standard curve obtained by linear regression made with sodium nitrite and expressed as fold vs control.

3.8 Immunofluorescence staining for fibrotic markers

The CCD-18Co cells grown on coverslips in a 12-well plate (seeded at 5,000 cells/cm^2) were treated as reported above for the indicated time points. At the end of treatment, the coverslips were washed with PBS, fixed with 4% formaldehyde for 20 min, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, Saint Louis, MO, USA) for 5 min, and blocked with 3% BSA (Sigma-Aldrich) for 20 min at room temperature. CCD-18Co cells were incubated overnight at 4°C with rabbit monoclonal antibody anti-COL1A1 (Boster Biological Technology, Pleasanton, CA, USA) 1:250, or mouse monoclonal antibody anti-α-actin smooth muscle (ACTA2, α-SMA) (OriGene, Rockville, MA, USA) 1: 250. Next, the coverslips were stained using a FITC conjugated goat anti-rabbit

polyclonal IgG secondary antibody (Millipore EMD, Darmstadt, Germany) 1:1,000 or FITC conjugated goat anti-mouse polyclonal IgG secondary antibody (Bethyl Laboratories, Inc, Montgomery, TX, USA) 1:1,000 for 1 h at room temperature, washed, and then incubated with TRITC labelled phalloidin (Sigma-Aldrich) for 45 minutes at room temperature. Coverslips were mounted with VECTASHIELD® Antifade Mounting mounted Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and then examined at 40× magnification with the fluorescent microscope (Eclipse 50i, Nikon, Tokyo, Japan).

3.9 TGF-β1 ELISA

The levels of released TGF-β1 were quantified in the supernatants of CCD-18Co cells using a human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit (Sigma Aldrich), as described in the manufacturer's instructions. Briefly, CCD-18Co cells were plated at 5000 cells/cm^2, grown overnight, and serum-deprived for 24 h. After 48 h incubation in serum-free medium either with or without TGF-β1 (10 ng/ml), in the presence or absence of bacterial lysates from VSL#3® or Vivomixx® (50 µg protein/ml), media were collected and cleared of cellular debris/dead cells by centrifugation at 600× g for 10 min. The TGF-β1 concentration was determined using an ELISA kit in culture media. Results are expressed as pg/ml.

3.10 Statistical analysis

Statistical analysis of data was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). For comparison between two means, Student's unpaired t-test was used. For comparison of the mean values among the groups, a one-way ANOVA test followed by Dunnet or Tukey, or Bonferroni *post hoc* test, was used as specified below. The results were expressed as mean ± SEM, as specified in figure legends. Results were considered significant if $P<0.05$.

4 Results on model #1: *In vitro* intestinal epithelial cell model to evaluate the anti-inflammatory effect of SLAB51 probiotic lysate

4.1 Effect of SLAB51 lysate on HIF-1α stabilization on Caco-2 cells

First, we investigated the ability of SLAB51 probiotic lysate to modulate HIF-1α levels on Caco-2 cells treated with increasing concentrations of the probiotic lysate (10, 50, and 100 µg/ml) for 6 or 24 h. SLAB51 lysate treatment induced a dose-dependent increase in HIF-1α levels as evaluated by immunoblotting, being statistically significant after 6 h at 100 µg/ml (P<0.01) (**Figure 14**). After 24 h of treatment, HIF-1α levels were significantly higher at 50 and 100 µg/ml with respect to the control (P<0.05 and P<0.01, respectively, vs. untreated cells). Based on these initial results, SLAB51 lysate concentration of 100 µg/ml, being the most efficient in influencing HIF-1α levels, was selected for the following experiments.

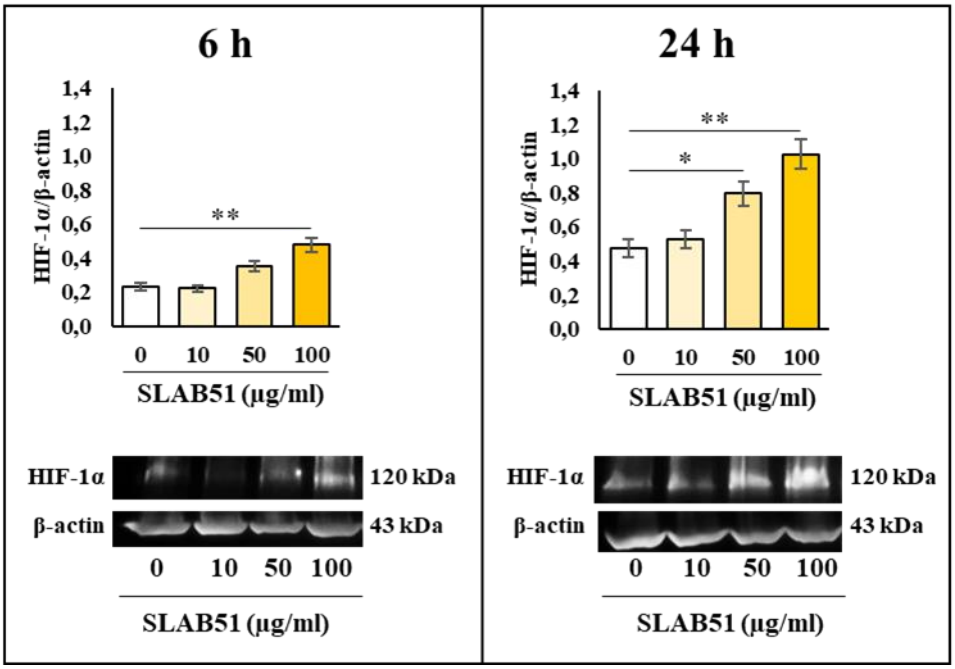


Figure 14. Effect of SLAB51 probiotic lysate on HIF-1α levels in Caco-2 cells. Caco-2 cells were incubated for 6 and 24 h with increasing concentrations of SLAB51 lysate; HIF-1α levels were then assessed by western blotting assay. Following densitometric analysis, obtained values were normalized *vs.* β-actin. Data are from three independent experiments, and values are expressed as mean ± SEM. For the comparative analysis of the data, a one-way analysis of variance (ANOVA) followed by the Dunnet test was used (*P<0.05, **P<0.01 vs. untreated cells). Representative immunoblots are also shown.

To elucidate whether SLAB51 probiotic lysate affected HIF-1α levels through regulation of PHD2 activity or expression under normoxia, the levels of HIF-1α and hydroxylated HIF-1α (HIF-1α-OH) were analyzed by western blotting. HIF-1α proline hydroxylation was determined using a specific

antibody for the proline-hydroxylation site (Pro564) following proteasomal degradation inhibition through MG132 incubation to allow the accumulation of HIF-1α-OH. The effect of MG132 on proteasomal degradation inhibition of HIF-1α-OH was demonstrated by the accumulation of HIF-1α (**Figure 15A**) as well as its hydroxylated form (**Figure 15B**). The treatment with deferoxamine (DFO) was used as a positive control, being an iron chelator known to attenuate the activity of PHD and, in turn, stabilize HIF-1α. As shown in **Figure 15A** and **Figure 15B**, DFO treatment reduced the accumulation of HIF-1α-OH under normoxia. Similarly, the treatment with SLAB51 lysate decreased HIF-1α-OH with respect to control under normoxia after 6 h of incubation, increasing HIF-1α levels. Subsequently, we evaluated the ability of the probiotic lysate to influence PHD2 expression by immunoblotting. SLAB51 lysate significantly reduced PHD2 expression compared with control at 6 h (**Figure 15C**).

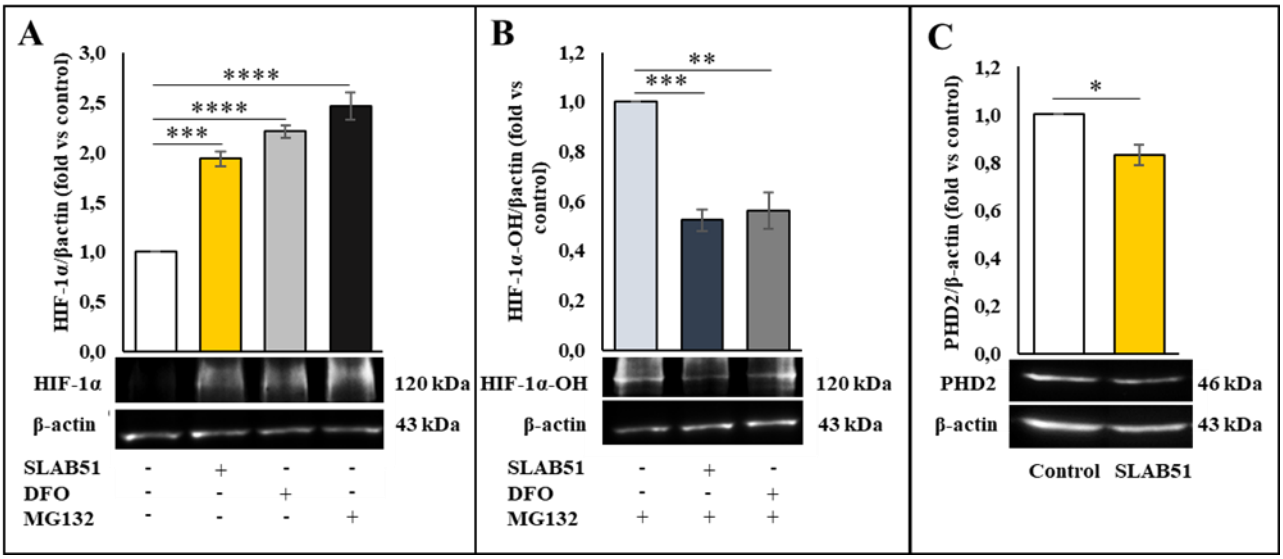


Figure 15. Effect of SLAB51 probiotic lysate on HIF-1α levels and PHD2 activity and expression in Caco-2 cells. Caco-2 cells were incubated for 6 h in normoxia with SLAB51 lysate (100 µg/ml) or DFO (150 µM, positive control) with or without proteasome inhibitor MG132 (10 µM). (**A**) HIF-1α, (**B**) HIF-1α-OH and (**C**) PHD2 levels were evaluated by western blotting assay. Following the densitometric analysis, obtained values were normalized *vs.* β-actin. Data are from three independent experiments, and values are expressed as mean ± SEM. For the comparative analysis of the data, a one-way analysis of variance (ANOVA) followed by the Dunnet test or Student's unpaired t-test was used, for the comparison of the mean values among the groups and for comparison between two means, respectively. (**P*<0.05, ***P*<0.01, ****P* <0.001, *****P* <0.0001 *vs.* untreated cells). Representative immunoblots are also shown.

4.2 Effect of SLAB51 lysate on cell metabolism and AKT pathway

Given the HIF-1α ability to induce genes encoding glycolytic enzymes (Nagao *et al*. 2019), the effect of SLAB51 probiotic lysate on glycolytic metabolism was investigated next. Levels of L-lactate, a key metabolite of the glycolysis pathway, were assessed in the supernatants of Caco-2 cells. The relative glycolysis rate was also analyzed as the ECAR/OCR ratio. ECAR is the

extracellular acidification rate of the media, a parameter that reflects glycolysis, while OCR stands for the O_2 consumption rate used to determine oxidative phosphorylation (TeSlaa and Teitell 2014). The levels of L-lactate (**Figure 16A**) and ECAR/OCR ratio were significantly increased in Caco-2 cells after 24 h treatment with probiotic lysate (**Figure 16B**). Upregulated HIF-1α expression and increased glycolysis are dependent on the activated PI3K/AKT pathway, which plays a central role in regulating energy metabolism (Dong *et al*. 2022). To verify the ability of the probiotic lysate to affect the AKT pathway, the analysis of AKT phosphorylation was performed. SLAB51 lysate significantly upregulated phospho-AKT (pAKT, Ser473) protein levels compared with control cells after 6 h of incubation (**Figure 16C).**

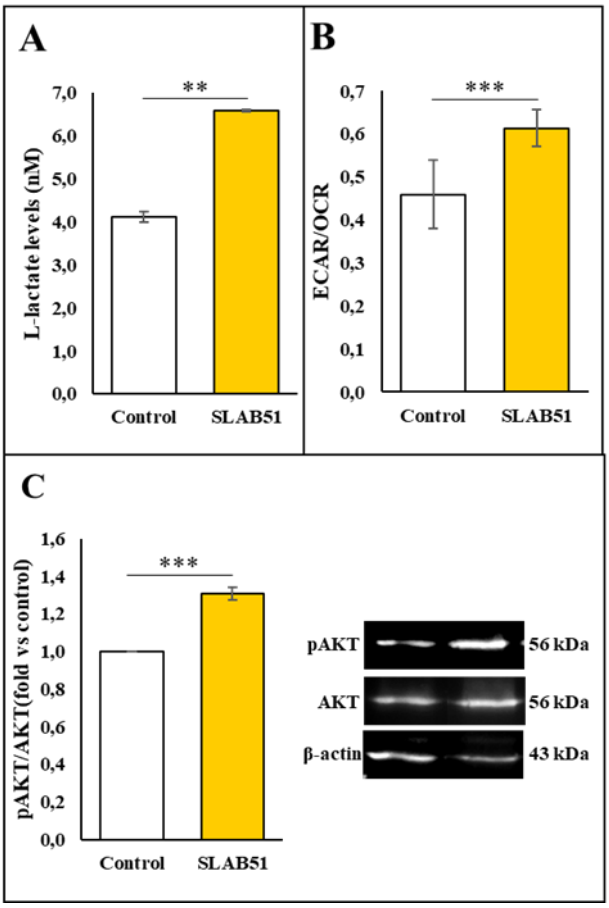


Figure 16. Effect of SLAB51 probiotic lysate on L-lactate production, glycolysis rate, and pAKT levels in Caco-2 cells. Cells were treated with or without SLAB51 lysate (100 µg/ml) for (**C**) 6 h or (**A** and **B**) 24 h. (**A**) Lactate levels were detected by a colorimetric assay on cell culture supernatants. Values are expressed as the means ± SEM of three independent experiments in triplicate. (**B**) The relative glycolysis rate ECAR/OCR was assessed using the Seahorse XF Analyzer. Values are expressed as the means ± SD of a representative experiment (N=9) out of three independent experiments. (**C**) pAKT levels were evaluated by western blotting assay. Following the densitometric analysis, obtained values were normalized *vs.* AKT. Values are expressed as the means ± SEM of three independent experiments. Representative immunoblots of pAKT, AKT, and β-actin are also shown. For the comparison between two means, the Student's unpaired t-test was used (***P*<0.01, ****P* <0.001).

The involvement of the PI3K/AKT pathway was further studied by investigating the influence of perifosine and LY294002, i.e., AKT and PI3K inhibitors, respectively, on the effect of SLAB51 lysate on HIF-1α levels of Caco-2 cells. The cells were pretreated with perifosine or LY294002 for 30 minutes and then incubated with the probiotic lysate for 6 h. As expected, both the inhibitors alone significantly decreased pAKT levels compared with the control; conversely no effect on HIF-1α levels was observed (data not shown). Pretreatment with each inhibitor counteracting the pAKT increase induced by SLAB51 lysate, inhibited its ability to increase HIF-1α, lowering the protein expression to levels comparable to those of the control (**Figure 17**). The obtained data suggest that the activation of the PI3K/AKT pathway is needed for the HIF-1α increase induced by SLAB51 treatment.

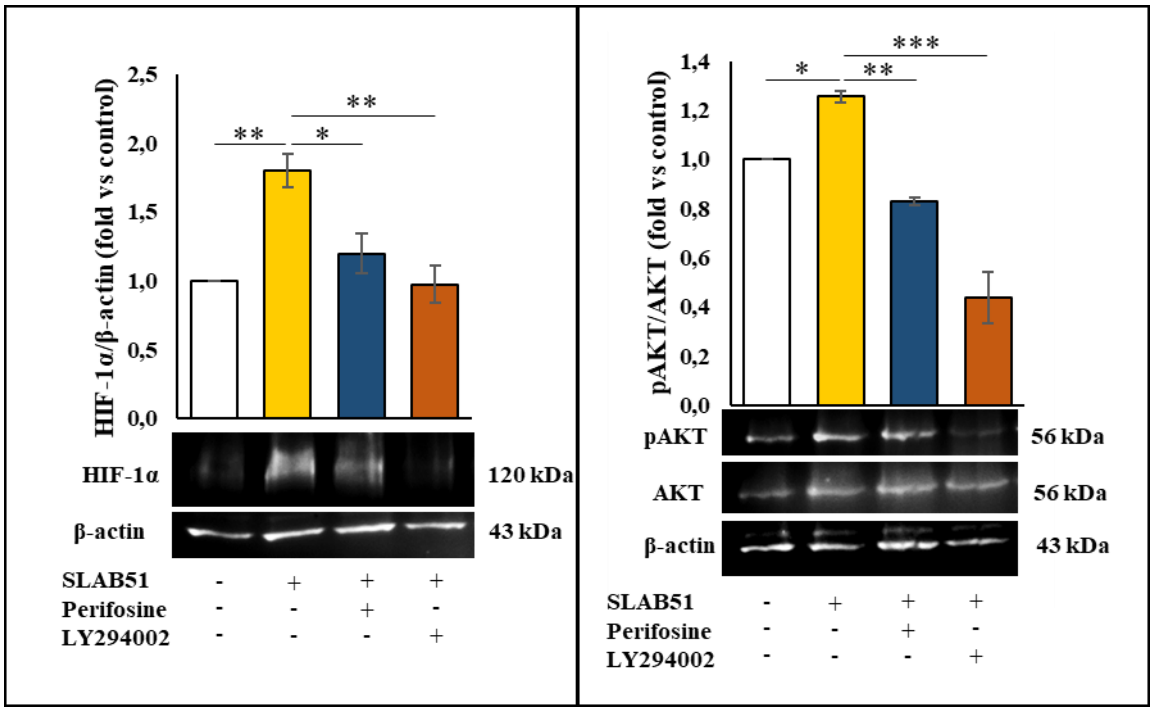


Figure 17. Effect of AKT and PI3K inhibition on SLAB51 probiotic lysate's ability to increase HIF-1α levels in Caco-2 cells. Cells were pretreated for 30 minutes with perifosine (20 μM) or LY294002 (10 μM) and then incubated with SLAB51 lysate (100 μg/ml) for 6 h. HIF-1α and pAKT levels were evaluated by western blotting assay. Following the densitometric analysis, obtained values were normalized *vs.* β-actin or AKT. Data are from three independent experiments, and values are expressed as mean ± SEM. For the comparative analysis of the data, a one-way analysis of variance (ANOVA) followed by the Tukey test was used (*P<0.05, **P<0.01, ****P<0.0001). Representative immunoblots are also shown.

4.3 Probiotic prevention of LPS-induced pro-inflammatory stress in Caco-2 cells through a mechanism involving HIF-1α stabilization

The effect of the probiotic lysate was then assessed on Caco-2 cells exposed to LPS, 10 µg/ml. In our study at 24 h, the treatment with LPS was not able to increase HIF-1α levels, which were not different with respect to the control. On the other hand, the ability of SLAB51 lysate to increase HIF-1α levels was not affected by the concomitant treatment with LPS (**Figure 18A**). Moreover, the effect on AKT activation was evaluated. The stimulation with LPS did not significantly influence the AKT pathway nor the ability of SLAB51 probiotic lysate to increase pAKT levels (**Figure 18B**).

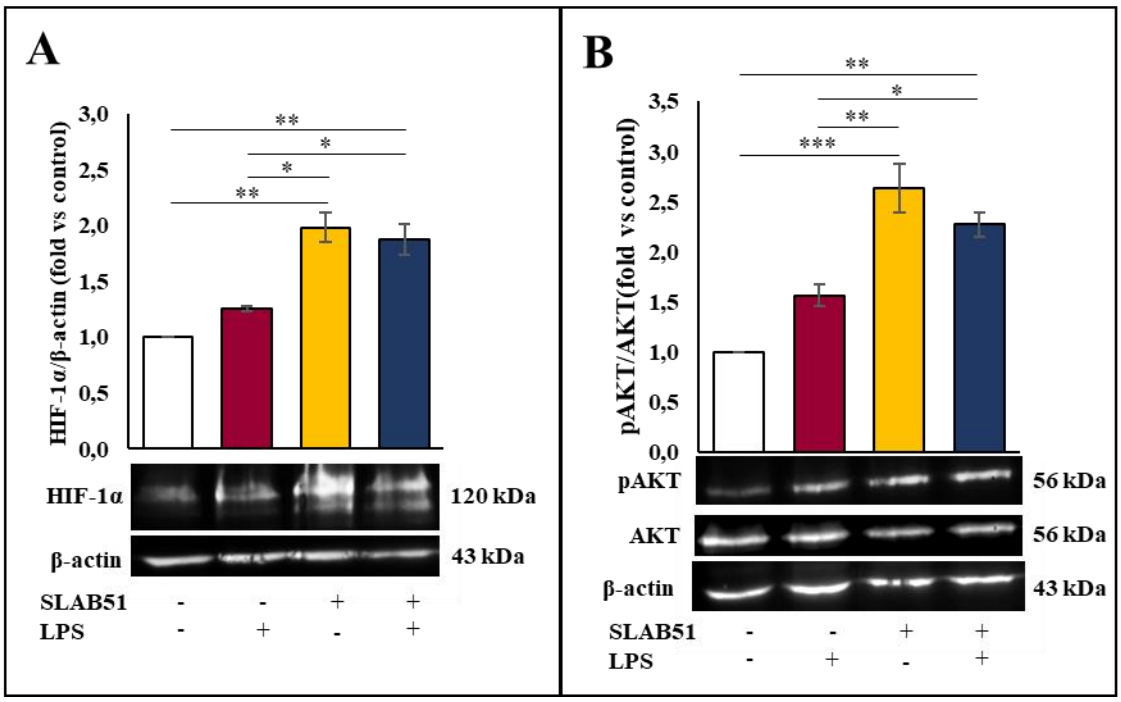


Figure 18. Effect of SLAB51 probiotic lysate on HIF-1α and pAKT levels in LPS-stimulated Caco-2 cells. Cells were treated for 24 h with or without LPS (10 µg/ml) and SLAB51 lysate (100 µg/ml). (**A**) HIF-1α and (**B**) pAKT levels were evaluated by western blotting assay. Following the densitometric analysis, obtained values were normalized *vs.* β-actin or AKT. Data are from three independent experiments, and values are expressed as mean ± SEM. For the comparative analysis of the data, one-way analysis of variance (ANOVA) followed by the Tukey test was used (**P*<0.05, ***P*<0.01, ***p<0.001, *****P*<0.0001). Representative immunoblots are also shown.

As known, the stimulation with LPS induces NF-κB signaling and upregulates the production of inflammatory cytokines at the intestinal level (Candelli *et al*. 2021). Also in our experiments, LPS treatment significantly enhanced phospho-NF-κB p65 (p- NF-κB p65) levels in Caco-2 cells and the secretion of the pro-inflammatory cytokine IL-1β into the culture medium. Of note, these effects were counteracted by SLAB51 lysate treatment (**Figure 19**).

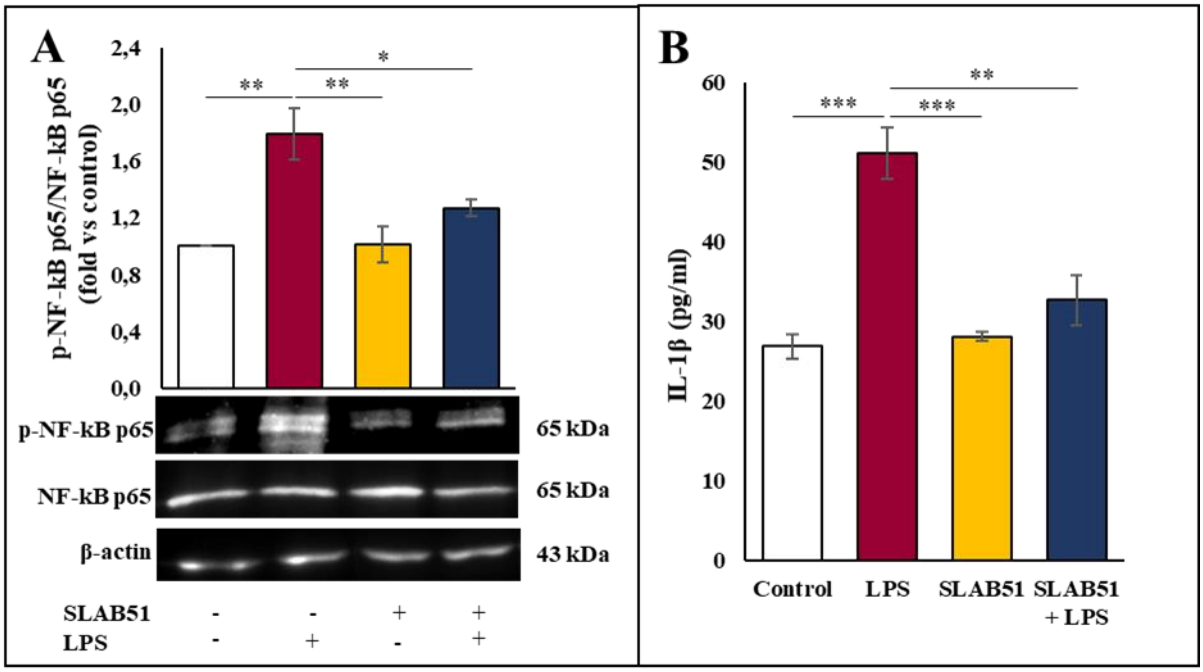


Figure 19. Effect of SLAB51 probiotic lysate treatment on inflammatory markers in LPS-stimulated Caco-2 cells. Cells were treated with or without LPS (10 µg/ml) and SLAB51 lysate (100 µg/ml) for 24 h. (**A**) p-NF-κB p65 and NF-κB p65 were analysed by western blotting assay. Following the densitometric analysis, obtained values for p-NF-κB p65 were normalized *vs.* NF-κB p65. Representative immunoblots of p-NF-κB p65, NF-κB p65, and β-actin are also shown. (**B**) IL-1β levels in the culture medium were assayed by ELISA kit. Data are from three independent experiments, and values are expressed as mean ± SEM. For the comparative analysis of the data, one-way analysis of variance (ANOVA) followed by Tuckey test was used (**P*<0.05, ***P*<0.01, ****P*<0.001).

The transcription factor NF-κB represents a key mediator of inflammatory responses as it is able to induce the expression of various pro-inflammatory genes, including NOS2 (Liu *et al*. 2017). Therefore, the effect of SLAB51 probiotic lysate was next evaluated on the expression and activity of NOS2 induced by LPS stimulation. Nitrite levels in the culture medium at 24 h after LPS treatment were increased with respect to control cells. The probiotic lysate abrogated the ability of LPS to promote NOS2 activity, bringing the nitrite levels back to those of the control (**Figure 20A**); the specificity of the assay was verified using the NOS2 inhibitor, 1400W. In **Figure 20B**, the results of immunoblotting of NOS2 protein are shown. As expected, LPS stimulation was able to induce the NOS2 expression. Of note, this effect was counteracted by the concomitant treatment with SLAB51, with the NOS2 expression not significantly different from untreated controls. No effect on NOS2 protein level was observed after treatment with the inhibitor of the NOS2 activity, 1400W.

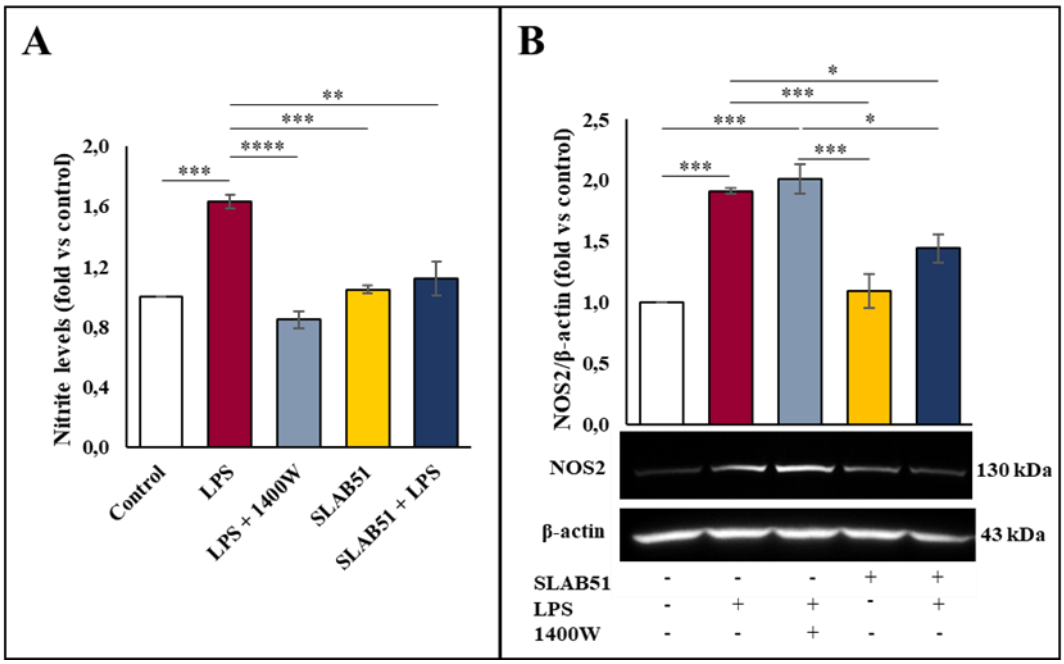


Figure 20. Effect of SLAB51 probiotic lysate on NOS2 expression and activity in LPS-stimulated Caco-2 cells. Cells were treated with or without LPS (10 µg/ml), NOS2 inhibitor 1400W (100 µM), SLAB51 lysate (100 µg/ml) for 24 h. (**A**) Nitrite levels in the culture medium were assayed by Griess reagent. Values are expressed as fold of nitrite levels *vs.* control. (**B**) Western blotting assay for NOS2 on cell lysates. Following the densitometric analysis, obtained values were normalized with respect *vs.* β-actin. Representative immunoblots are also shown. Data are from three independent experiments, and values are expressed as mean ± SEM. For the comparative analysis of the data, one-way analysis of variance (ANOVA) followed by Tuckey test was used (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001).

Finally, we verified the hypothesis that the anti-inflammatory effect of SLAB51 lysate observed in our model could be mediated by the ability of the probiotic to modulate the HIF-1α pathway. For this purpose, Caco-2 cells were incubated with AKT and PI3K inhibitors 30 minutes prior to the treatment with SLAB51 lysate and LPS, then the effect on NOS2 activity was evaluated after 24 h treatment. The results demonstrated that the two inhibitors were able to significantly interfere with the ability of probiotic lysate to reduce NOS2 activity (**Figure 21**). Indeed, nitrite levels in the culture medium after SLAB51+LPS+perifosine or LY24002 were significantly higher than SLAB51+LPS treatment (*P*<0.05). However, both inhibitors were unable to totally abrogate the effect of SLAB51 lysate since nitrite levels in the medium of Caco-2 cells also treated with perifosine or LY24002 were still significantly lower than stimulation with LPS (*P* <0.05).

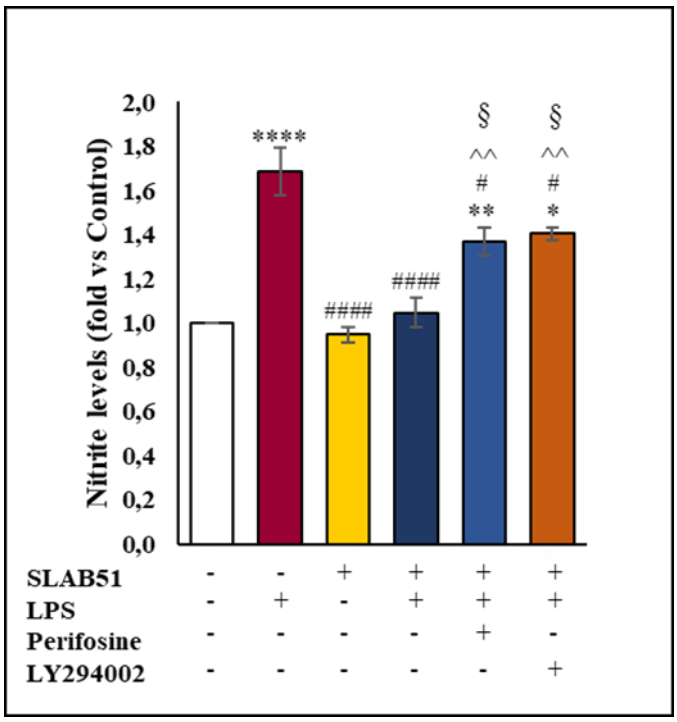


Figure 21. Effect of AKT and PI3K inhibition on SLAB51 lysate's ability to modulate NOS2 activity in LPS-stimulated Caco-2 cells. Cells were incubated in the presence or absence of perifosine or LY294002 for 30 minutes and then treated with or without LPS (10 µg/ml) and SLAB51 lysate (100 µg/ml) for 24 h. Nitrite levels in the culture medium were assayed by Griess reagent. Values are expressed as fold of nitrite levels *vs.* control. Data are from three independent experiments, and values are expressed as mean ± SEM. For the comparative analysis of the data, one-way analysis of variance (ANOVA) followed by Tukey test was used (**P*<0.05, ***P*<0.01, *****P*<0.0001 *vs.* control; #*P*<0.05, ####*P*<0.0001 *vs.* LPS; ^^*P*<0.01 *vs.* SLAB51; §*P*<0.05 *vs.* SLAB51 + LPS).

5 Results on model #2: *In vitro* fibroblast model to evaluate anti-fibrotic effect of high-concentration multi-strain probiotic formulations

5.1 Effects of the bacterial lysates from VSL#3® and Vivomixx® on TGF-β1-induced CCD-18Co cell proliferation

To evaluate the effects of VSL#3® and Vivomixx® formulations on the TGF-β1-induced fibrotic process in CCD-18Co fibroblasts, the bacterial lysates were prepared at a final concentration of 50 µg protein/ml, equivalent to 10^8 CFU/ml.

As expected from previous reports (Valatas *et al*. 2017), the treatment for 48 h with TGF-β1 at 10 ng/ml enhanced CCD-18Co cell proliferation (~50% more than the untreated control cells, $P<0.01$), as shown in **Figure 22A**. VSL#3® and Vivomixx® lysates alone in the culture medium did not affect the vitality and cell proliferation rate, which remained like the basal level seen in control cells. Of note, while VSL#3® lysate did not influence the TGF-β1-induced cell growth rate, the mean number of cells that were treated with Vivomixx® lysate plus TGF-β1 for 48 h was significantly lower either with respect to TGF-β1 alone ($P<0.01$) or TGF-β1 plus VSL#3® lysate ($P<0.05$) and was comparable to basal levels (**Figure 22A**). Representative images from phase-contrast microscopic observations confirmed the cell number counting results (**Figure 22B**).

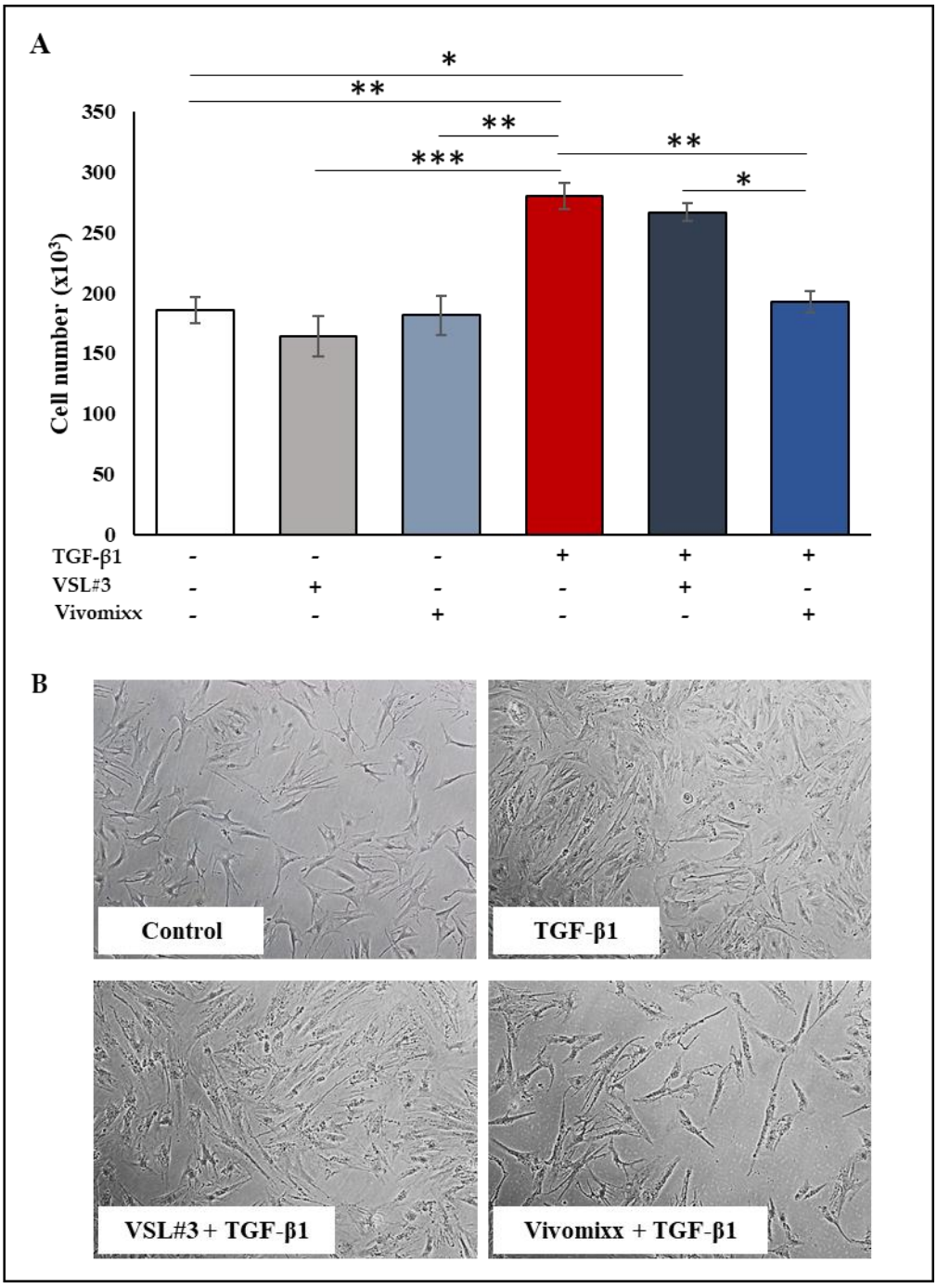


Figure 22. Effect of bacterial lysates from VSL#3 and Vivomixx formulations on CCD-18Co cell number. (**A**) CCD-18Co cells were starved for 24 h and then incubated with TGF-β1 (10 ng/ml) for 48 h in the presence or absence of VSL#3 or Vivomixx lysates (50 μg/ml), in serum-free medium. Data are from three independent experiments, and values are expressed as mean ± SEM. For comparative analysis of groups of data, a one-way analysis of variance (ANOVA) with post hoc Bonferroni test was used (**P*<0.05, ***P*<0.01, ****P*<0.001*)*. (**B**) Representative phase-contrast microscopic images from one out of three independent experiments are shown (20× magnification).

5.2 VSL#3® and Vivomixx® bacterial lysates differently affect fibrotic markers in TGF-β1-activated CCD-18Co cells

Differentiation of myofibroblast is a key event in the pathogenesis of intestinal fibrosis, characterized mainly by cytoskeleton organization and increased expression of fibrotic markers, such as α-SMA and collagen-I (Coll I). **Figure 23** shows the results of western blot analysis to assess the influence of VSL#3® and Vivomixx® lysates on Coll I expression levels. As expected, CCD-18Co cells, serum-deprived for 24 h and then stimulated with TGF-β1 for 48 h, underwent a change of phenotype into activated myofibroblast, which was characterized by a significant increase of Coll I (P<0.01) expression when compared to the control. Of note, the treatment with Vivomixx® lysate markedly reduced TGF-β1-induced Coll I expression (P< 0.001). Conversely, VSL#3® lysate was unable to affect the expression levels of Coll I.

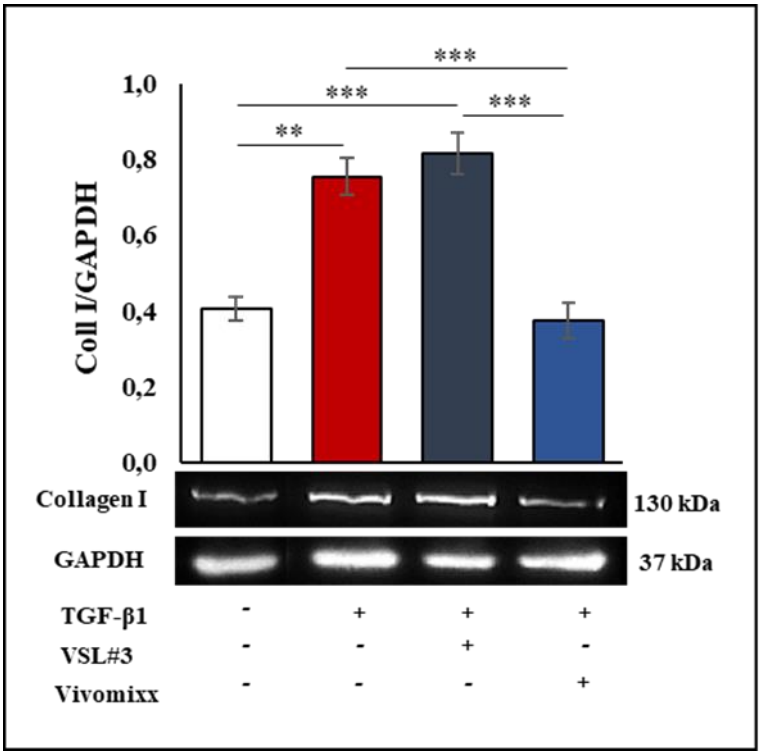


Figure 23. Influence of VSL#3 and Vivomixx bacterial lysates on TGB-β1-induced collagen I expression in CCD-18Co cells. Western blotting assay for collagen I was performed on CCD-18Co cells incubated with TGF-β1 (10 ng/ml) in the presence or absence of VSL#3 or Vivomixx lysates (50 µg/ml) for 48 h. Following densitometric analysis, obtained values were normalized *vs.* GAPDH. Data are from three independent experiments, and values are expressed as mean ± SEM. For comparative analysis of data, a one-way analysis of variance (ANOVA) with post hoc Bonferroni test was used (**P<0.01, ***P<0.001). Representative immunoblots are also shown.

Immunofluorescence analysis confirmed these results (**Figure 24**). The cells treated with TGF-β1 plus VSL#3® lysate showed more intense and widespread Coll I stain, similar to those observed with the cells treated with TGF-β1 alone. On the other hand, the Vivomixx® lysate induced a substantial reduction of staining for Coll I to the extent where the relative fluorescent signals appeared to be weak and diffuse similarly to the control untreated cells.

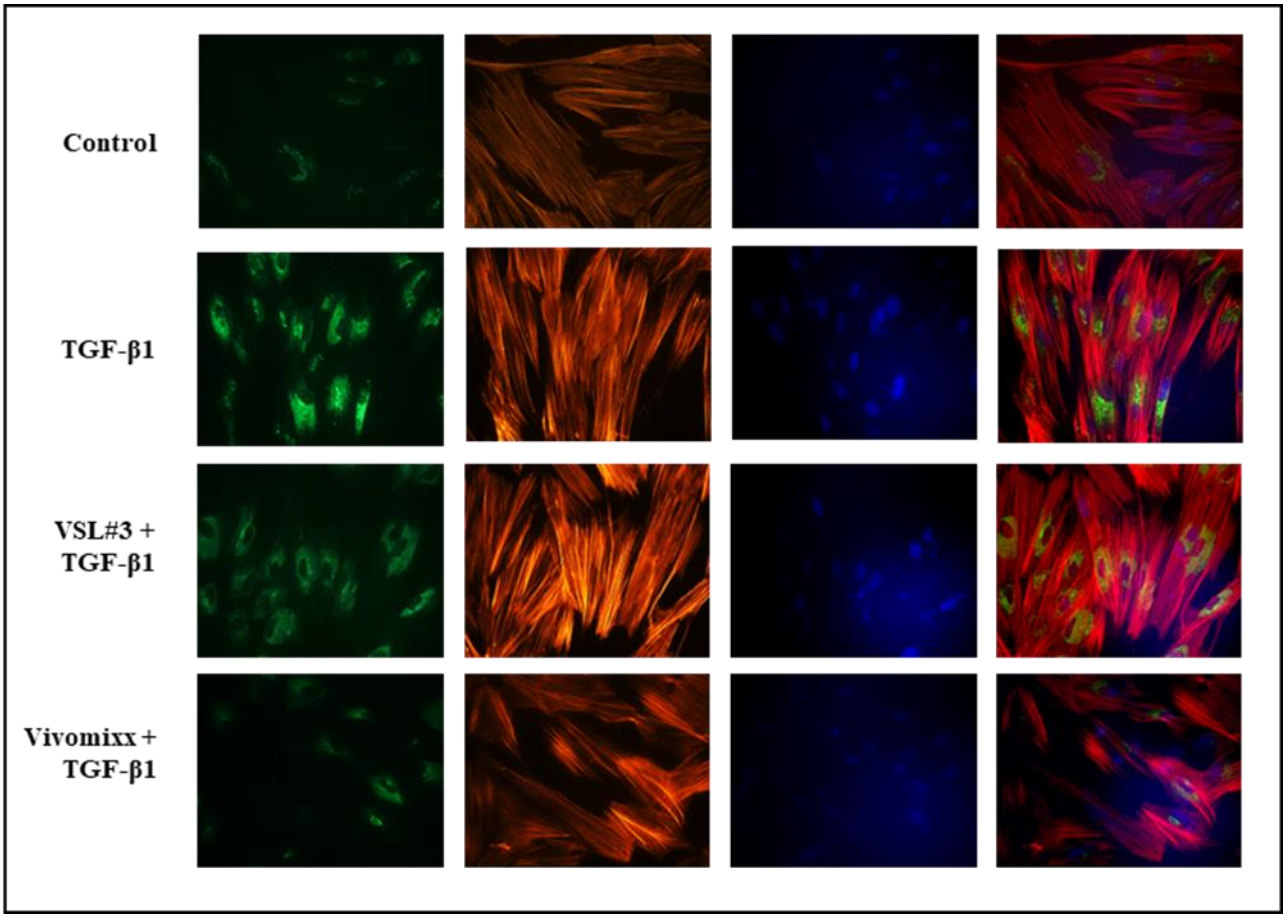


Figure 24. Influence of VSL#3 and Vivomixx bacterial lysates on TGB-β1-induced collagen I expression in CCD-18Co cells. Representative immunofluorescence images of CCD-18Co cells incubated for 48 h with TGF-β1 (10 ng/ml) in the presence or absence of VSL#3 or Vivomixx lysates (50 µg/ml) and stained with anti-collagen I antibody (green) and with TRITC-phalloidin (red) to reveal F-actin. Nuclei were counterstained with DAPI (blue) (magnification 40x). The images are representative of three independent experiments.

Of note, an important distinctive feature of the TGF-β1-induced fibrotic activation was represented by extensive cytoskeletal rearrangement and the formation of actin stress fibers, as indicated by phalloidin staining. The TGF-β1-activated morphology appeared strongly reduced in cells treated with Vivomixx® lysate, which showed a poorly organized cytoskeleton similar to control cells. Instead, when the VSL#3® bacterial lysate was added in the presence of TGF-β1, the CCD-18Co cells showed a more differentiated morphology with the formation of pronounced actin stress fibers. Similar results were obtained from the experiments analyzing the effects of VSL#3® and Vivomixx® lysates on α-SMA expression that was upregulated in TGF-β1-activated CCD-18Co

cells (**Figure 25** and **Figure 26**). Once more, the Vivomixx® lysate, but not the VSL#3® one, significantly inhibited ($P< 0.05$) the α-SMA expression that was induced by TGF-β1 in myofibroblasts, as evaluated either by western blot and immunofluorescence analysis (**Figure 25** and **Figure 26**).

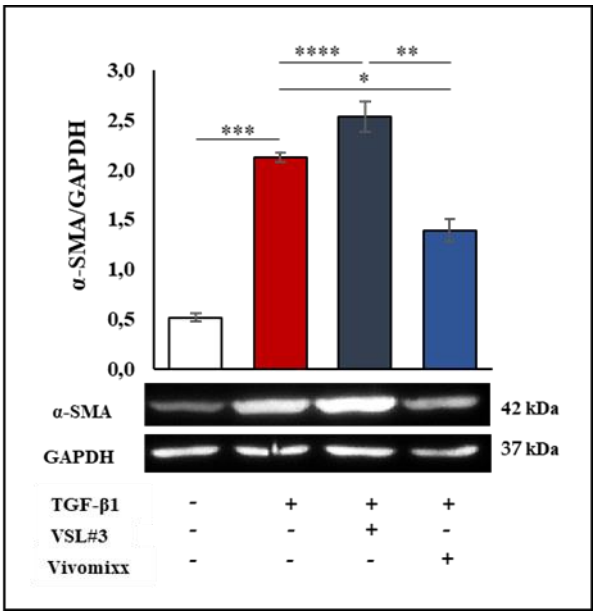


Figure 25. Influence of VSL#3 and Vivomixx lysates on TGB-β1-induced α-SMA expression in CCD-18Co cells. Western blotting assay for α-SMA was performed on CCD-18Co cells incubated with TGF-β1 (10 ng/ml) in the presence or absence of VSL#3 or Vivomixx lysates (50 µg/ml) for 48 h. Following densitometric analysis, obtained values were normalized *vs.* GAPDH. Data are from three independent experiments, and values are expressed as mean ± SEM. For comparative analysis of data, a one-way analysis of variance (ANOVA) with post hoc Bonferroni test was used (**P*<0.05, ***P*<0.01, ****P* <0.001). Representative immunoblots are also shown.

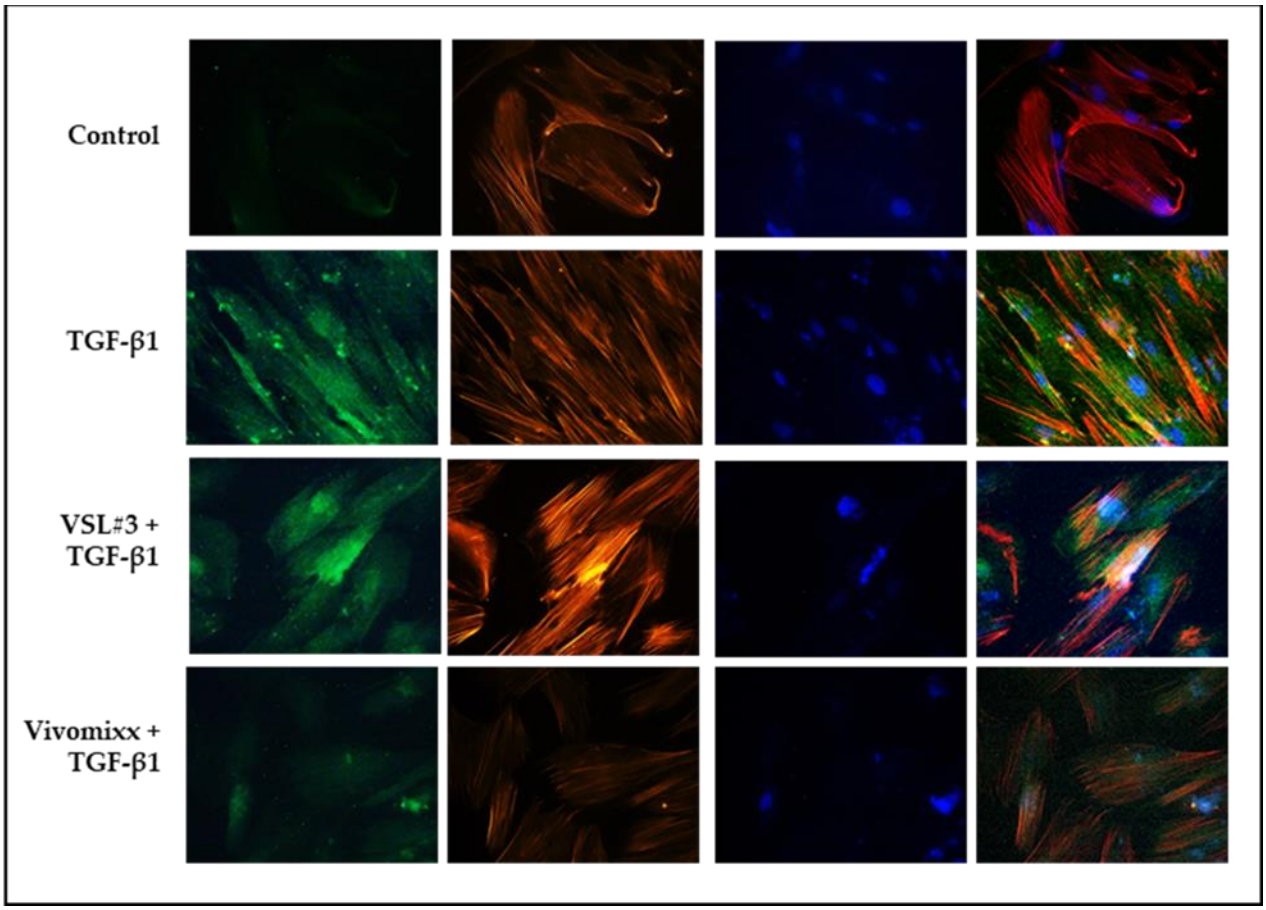


Figure 26. Influence of VSL#3 and Vivomixx lysates on TGB-β1-induced α-SMA expression in CCD-18Co cells. Representative immunofluorescence images of CCD-18Co cells incubated for 48 h with TGF-β1 (10 ng/ml) in the presence or absence of VSL#3 or Vivomixx lysates (50 µg/ml) and stained with anti-α-SMA antibody (green) and with TRITC-phalloidin (red) to reveal F-actin. Nuclei were counterstained with DAPI (blue) (magnification 40x). The images are representative of three independent experiments.

5.3 Effects of the probiotic lysates on TGF-β1-related signaling

In the canonical pathway, TGF-β1 induces Smad signaling responsible for fibrogenesis (Wang, Lin, *et al*. 2021; Walton, Johnson, and Harrison 2017). As shown in **Figure 27**, the treatment of CCD-18Co cells with TGF-β1 induced phosphorylation of Smad2/3 at 48 h. The Vivomixx® lysate counteracted the effect of TGF-β1, thus keeping Smad2/3 phosphorylation levels in line with those of the untreated control. Conversely, treatment with VSL#3® lysate failed to affect the Smad2/3 phosphorylation, confirming its lack of efficacy as an anti-fibrotic agent.

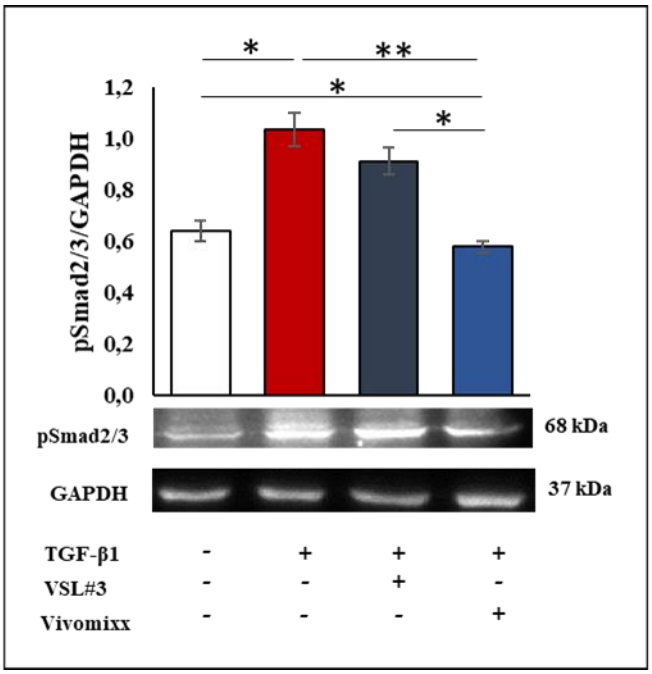


Figure 27. Effect of VSL#3 and Vivomixx lysates on TGF-β1-induced Smad activation. Western blotting assay for pSmad2/3 protein expression was performed on CCD-18Co cells incubated with TGF-β1 (10 ng/ml) in the presence or absence of VSL#3 or Vivomixx lysates (50 µg/ml) for 48 h. Following densitometric analysis, obtained values were normalized *vs.* GAPDH. Data are from two independent experiments in duplicate, and values are expressed as mean ± SEM. For comparative analysis of data, a one-way analysis of variance (ANOVA) with post hoc Bonferroni test was used (**P*<0.05, ***P*<0.01). Representative immunoblots are also shown.

Moreover, levels of TGF-β1 released in the extracellular medium by CCD-18Co cells increased significantly with the addition of TGF-β1 (*P*<0.01), possibly through an autocrine mechanism (**Figure 28**). Notably, the Vivomixx® lysate plus TGF-β1 led to a significantly lower TGF-β1 release (*P*< 0.01) when compared with those observed when the cells were treated with TGF-β1 alone or VSL#3® lysate plus TGF-β1.

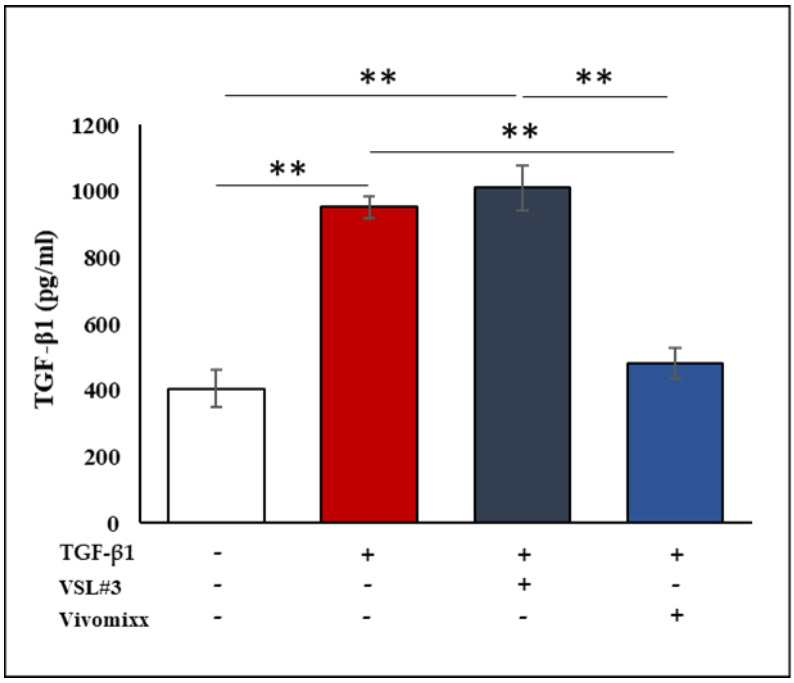


Figure 28. Effect of VSL#3 or Vivomixx lysates on TGF-β1 secretion in activated CCD-18Co cells. TGF-β1 levels were detected by ELISA in supernatants from CCD-18Co cells treated with TGF-β1 (10 ng/ml) in the presence or absence of VSL#3 or Vivomixx lysate (50 µg/ml) for 48 h. The results, relative to one representative out of two experiments performed in triplicate, are expressed as mean ± SD. For comparative analysis of groups of data, one-way ANOVA followed by post hoc test Bonferroni was used (**$P<0.01$).

6 Discussion

The oral administration of probiotics has been shown to exert positive effects on chronic inflammatory disorders, such as inflammatory bowel disease (IBD). Moreover, the anti-inflammatory action of various probiotic strains has been demonstrated in several *in vitro* and *in vivo* studies.

In the first part of the present study, the anti-inflammatory effect of SLAB51, a multi-strain probiotic formulation, in a model of *in vitro* human intestinal epithelium (intestinal epithelial cells, IECs) was investigated. Several studies emphasize the involvement of HIF pathway as the central hypoxia sensor in IBD (Brown and Taylor 2018). Despite the increasing attention on the role of HIF in regulating tissue barrier function, metabolism, and inflammatory and immune response in the gut, the ability of probiotics to influence the HIF pathway has not been thoroughly investigated. The existing studies evaluating probiotics' influence on HIF-1α stabilization in the intestinal environment are mainly limited to the effect of short-chain fatty acids, such as butyrate, a product of microbial fermentation of dietary fibers in the lower intestinal tract, able to directly and non-competitively inhibit PHD (Wang, Henen, *et al*. 2021). This effect has been linked to the colonization of the colon by the probiotic and not to its transit through the upper intestine.

Treatment with SLAB51 has recently been shown to induce HIF-1α stabilization, reduce PHD2 expression, and inhibit inducible nitric oxide synthase expression in the brain of the AD mouse model (Bonfili *et al*. 2021), indicating that the probiotic formulation can exert positive effects not only in the gastrointestinal tract but also in extraintestinal organs.

The effect of SLAB51 lysate on the HIF pathway in Caco-2 IECs was evaluated. The data obtained show that exposure of Caco-2 IECs to SLAB51 under normoxic conditions positively influenced the HIF pathway. Specifically, the upregulation of HIF-1α levels was dose-dependent and associated with an increase in L-lactate levels and the relative glycolysis rate in the treated cells. The ability of SLAB51 lysate to stabilize HIF-1α was accompanied with inhibition of PHD2 activity and expression. The involvement of the PI3K/AKT pathway underlying the ability of the SLAB51 probiotic lysate to induce the increase in HIF-1α levels was also evaluated, per previous reports supporting that upregulated HIF-1α expression and increased glycolysis are dependent on the activation of this pathway, regardless of oxygen levels (Dong *et al*. 2022). Moreover, PHD2 inhibition induced by SLAB51 probiotic lysate could also lead to AKT activation, thus indirectly contributing to the observed increase of HIF-1α levels (Guo *et al*. 2016; Liu *et al*. 2021). PHD2 can directly hydroxylate AKT on two major proline residues, thus leading to its inactivation (Guo *et al*.

2016). On the other hand, the deletion of PHD2 leads to AKT activation (Liu, Zhang, *et al*. 2018). Of note, the activation of PI3K/AKT pathway in melanoma cells has been reported to induce PHD2 inhibition, thereby promoting HIF-1α stability (Spinella *et al*. 2010).

LPS has been recognized as a promoter of different inflammatory pathways involved in the pathogenesis of both acute and chronic intestinal inflammation (Candelli *et al*. 2021). LPS-induced increase in intestinal epithelial TJ permeability is mediated by the activation of TLR-4. LPS binding to TLR4 leads to the activation of NF-κB signaling (Zhou *et al*. 2018). Increased activation of NF-κB has been found to be involved in the regulation of the inflammatory response in IBD (Liu *et al*. 2017). Moreover, the probiotics have been shown to suppress inflammation by inhibiting various signaling pathways, such as the NF-κB pathway (Cristofori *et al*. 2021). In this study, LPS failed to induce an increase in HIF-1α levels. Additionally, it was demonstrated that SLAB51 lysate was able to counteract the NF-κB activation and IL-1β secretion induced by LPS stimulation. The probiotic also counteracted NOS2 expression and activity induced by LPS treatment. Notably, it has been shown that while acute exposure to NO stabilizes HIF-1α, chronic exposure to NO destabilizes HIF-1α (Berchner-Pfannschmidt *et al*. 2010).

SLAB51 possesses high levels of arginine deiminase (ADI) (Ceccarelli *et al*. 2021). This prokaryotic enzyme, using the same substrate as NOS, L-arginine, indirectly inhibits the generation of NO due to substrate depletion (Riccia *et al*. 2007). The exposure of Caco-2 to SLAB51 induced a reduction in NOS2 activity (Ceccarelli *et al*. 2021). In these conditions, the inhibition of the PI3K/AKT pathway was able to counteract the effect of SLAB51 on NOS activity, demonstrating a link between the activation of the AKT/PHD2/HIF pathway and the anti-inflammatory effect of the probiotic. Moreover, the inability of AKT signaling inhibition to totally counteract the effects of SLAB51 on NOS activity could be due to the ADI present in the probiotic.

Overall, these results support our hypothesis that by increasing HIF-1α levels through the upregulation of the AKT pathway, the SLAB51 lysate can inhibit the NF-κB activation and its downstream signaling in IECs, counteracting the LPS-induced inflammatory response. To the best of our knowledge, this is the first report showing that a probiotic formulation led to HIF pathway activation in human IECs, by influencing PI3K/AKT signaling. Even though further studies are warranted to investigate the observed effects in further inflammatory models *in vitro* and *in vivo*, we can hypothesize that the SLAB51 formulation could act as a hypoxia mimetic agent, thus contributing to preserve intestinal homeostasis and protect IECs from the detrimental effects of chronic inflammation and pathologic hypoxia.

In the second part of the present study, the potential anti-fibrotic effect of two multi-strain probiotic formulations, VSL#3® and Vivomixx®, was explored on CCD-18Co human intestinal fibroblasts activated by TGF-β1.

Intestinal fibrosis is a common complication of IBD, and it is characterized by excessive extracellular matrix (ECM) protein deposition caused by abnormal activation of myofibroblasts, which are considered the crucial effector cells of the fibrotic process. Although several growth factors and inflammatory cytokines are involved in intestinal fibrosis, the TGF-β1-activated pathway is considered to play a crucial role in the fibrotic process by regulating numerous genes involved in the increased deposition of collagen and other extracellular matrix proteins with progressive tissue distortion, dysfunctional wound healing, and lumen narrowing (Speca *et al*. 2012).

To date, no specific intestinal anti-fibrotic therapy exists to prevent or reverse intestinal fibrosis. Indeed, the standard anti-fibrotic approach mainly focuses on the suppression of inflammation. However, once fibrosis has been established, the mere control of inflammation is not enough for reverting this process. It is important to note that the surgical procedure is currently the only choice to relieve intestinal fibrotic complications, but it often provides short-term benefits. Therefore, it is crucial to develop new alternative treatments to target the initiation and progression of intestinal fibrosis.

CCD-18Co cells activated *in vitro* by TGF-β1 represent a model of intestinal fibrosis widely used in experimental studies (Li *et al*. 2016). In our experimental condition, as evidenced by immunofluorescence and Western blot analysis, collagen I and α-SMA resulted significantly overexpressed in TGF-β1-activated CCD-18Co cells. TGF-β1 treatment also led to a reorganization of the actin cytoskeleton into thick actin bundles easily observed with phalloidin staining. Untreated cells, instead, did not show the features typical of activated myofibroblast with only a small network of parallel stress fibers. The upregulation of collagen-I and α-SMA and the cytoskeletal rearrangement were reverted by Vivomixx® probiotic lysates, which counteracted the TGF-β1-induced differentiation of CCD-18Co toward activated myofibroblast. On the contrary, VSL#3® lysate kept the TGF-β1-induced fibrotic phenotype stable, either in terms of collagen-I and α-SMA expression levels or the cytoskeleton's remodeling. Moreover, the activated form of Smad2/3 significantly increased in TGF-1-treated CCD-18Co cells, but the presence of Vivomixx® reduced the Smad2/3 phosphorylation. Another crucial characteristic of TGF-β1, which is worth noting, is its ability to sustain itself through an autocrine loop that induces its release from fibroblasts. The supplement of TGF-β1 in activated-CCD-18Co cells was also shown to improve its neo-synthesis,

an effect significantly suppressed by the addition of Vivomixx® lysate. The observed data suggest that the anti-fibrotic effect, observed only with Vivomixx exposure, could be associated with its ability to antagonize either the Smad2/3 phosphorylation or neo-synthesis of TGF-β1 by activated-CCD-18Co cells.

Despite proven effect of probiotics in favouring and maintaining intestinal homeostasis and modulating inflammatory processes, few studies have been made to show the contribution of probiotics in the treatment of IBD-associated intestinal fibrosis. For this reason, this study represents the first step in demonstrating their beneficial anti-fibrotic and anti-inflammatory effect in IBD patients. According to the current knowledge, probiotic bacteria administration has shown efficacy as adjuvant therapy in attenuating liver fibrosis as well as the respiratory and gastrointestinal outcomes in a stable cystic fibrosis clinic population. In the same populations, significant alterations in the gut microbiome structure have also been shown (Anderson, Miles, and Tierney 2017). These data suggest that probiotics might also play a beneficial role directly in fibrotic tissue that is associated with IBD.

The results of the present study suggest a beneficial effect of the Vivomixx® formulation. Its effect on CCD-18Co cells suggests the suppression of intestinal fibrosis, which may be associated with an inhibition of the TGF-β1/Smad signaling pathway. The next steps of our research should focus on the important task of identifying and purifying the Vivomixx®-derived factors which specifically reverse the intestinal fibrosis process.

In conclusion, the results provide valuable evidence of Vivomixx®'s role as a promising therapeutic strategy against intestinal fibrosis in Crohn's patients. It is important to note that this property is not a common trait in the other probiotic VSL#3 formulation. Despite its unquestionable beneficial effect, inhibition of the TGF-β1/Smad signaling pathway is most likely not the only anti-fibrotic strategy aimed at selectively blocking IBD-associated fibrosis. Therefore, Vivomixx®'s effect on other molecular mechanisms implicated in intestinal mucosal homeostasis, fibrosis, and related immune-regulatory pathways needs to be analyzed further.

7 References

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