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***Evaluation of new pharmacological and probiotic approaches  
for the treatment of fibrosis in inflammatory bowel disease***

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# TABLE OF CONTENTS

|                                                                                       |           |
|---------------------------------------------------------------------------------------|-----------|
| <b>1. INTRODUCTION</b>                                                                | <b>1</b>  |
| 1.1 INFLAMMATORY BOWEL DISEASE                                                        | 1         |
| 1.1.1 Ulcerative colitis                                                              | 2         |
| 1.1.2 Crohn's disease                                                                 | 2         |
| 1.1.3 Indeterminate colitis                                                           | 2         |
| 1.1.4 IBD-risk factors                                                                | 2         |
| 1.2 INTESTINAL FIBROSIS                                                               | 9         |
| 1.2.1 Components of intestinal fibrosis                                               | 11        |
| 1.2.2 Key molecules involved in intestinal fibrosis                                   | 17        |
| 1.2.3 TGF- $\beta$ 1 canonical and non-canonical pathways driving intestinal fibrosis | 24        |
| 1.2.4 Pathogenetic factors involved in intestinal fibrosis                            | 28        |
| 1.2.5 Current therapies for intestinal fibrosis                                       | 30        |
| 1.2.6 Probiotics approach in intestinal fibrosis                                      | 31        |
| <b>2. AIM OF THE STUDY</b>                                                            | <b>33</b> |
| <b>3. MATERIALS AND METHODS</b>                                                       | <b>34</b> |
| 3.1 CELL LINES                                                                        | 34        |
| 3.2. PREPARATION OF BACTERIAL LYSATE                                                  | 34        |
| 3.3 <i>IN VITRO</i> MODELS AND TREATMENTS                                             | 35        |
| 3.3.1 Intestinal Fibrosis Experimental Design                                         | 35        |
| 3.3.2 Epithelial to mesenchymal transition (EMT) experimental design                  | 35        |
| 3.4 ASSESSMENT OF CELL VIABILITY AND PROLIFERATION                                    | 35        |
| 3.3.1. Cell Proliferation Assay of CCD-18Co                                           | 35        |
| 3.3.2. Cell Viability Assay of Caco-2 IECs                                            | 35        |
| 3.5 WESTERN BLOT ANALYSIS                                                             | 36        |
| 3.6 IMMUNOFLUORESCENT STAINING                                                        | 37        |
| 3.7 TOTAL RNA EXTRACTION AND QUANTITATIVE REAL-TIME PCR (qPCR)                        | 37        |
| 3.8 STATISTICAL ANALYSIS                                                              | 38        |

|                                                                                                        |           |
|--------------------------------------------------------------------------------------------------------|-----------|
| <b>4. RESULTS .....</b>                                                                                | <b>38</b> |
| 4.1 MULTI-STRAIN PROBIOTIC LYSATE DECREASED PROLIFERATION IN CCD-18Co...                               | 38        |
| 4.2 <i>OXXYSLAB</i> LYSATE IMPACT ON FIBROTIC MARKER EXPRESSION ON TGF-B1-<br>STIMULATED CCD-18Co..... | 39        |
| 4.3 <i>OXXYSLAB</i> INTERFERES WITH THE ACTIVATION OF TGF-B1 PATHWAYS .....                            | 41        |
| 4.4. MULTI-STRAIN PROBIOTIC LYSATE MODULATES TGF-B1 GENE EXPRESSION .....                              | 43        |
| 4.5 CELL VIABILITY OF CACO-2-IECs WITH DIFFERENT CONCENTRATION OF <i>OXXYSLAB</i><br>.....             | 44        |
| 4.6 <i>OXXYSLAB</i> REVERTED TGB-B1-INDUCED EMT IN CACO-2 IECs.....                                    | 45        |
| <b>5. DISCUSSION .....</b>                                                                             | <b>49</b> |
| <b>6. REFERENCES .....</b>                                                                             | <b>55</b> |

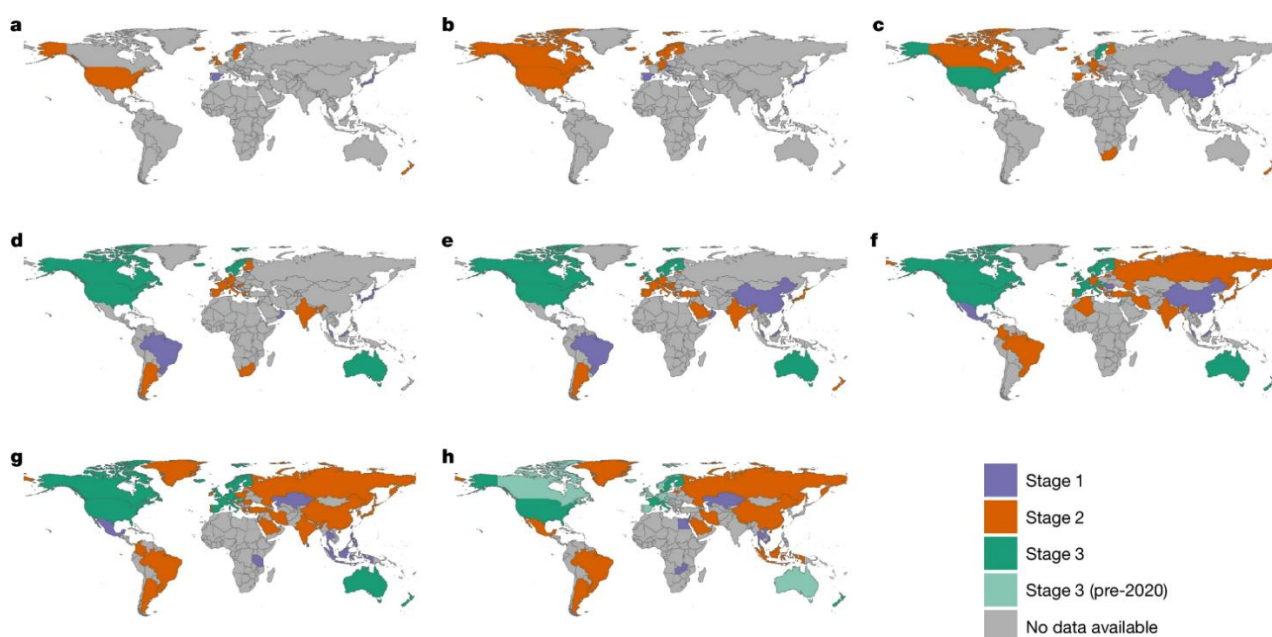
## ABSTRACT

Intestinal fibrosis is a common complication of Inflammatory bowel disease (IBD), and the underlying mechanism of its pathogenesis remains incompletely understood. Intestinal fibrosis develops due to ongoing tissue damage and is closely linked to chronic inflammation. It is, in fact, a frequent complication in patients with Crohn's Disease (CD), with more than 50% of patients experiencing complications such as stenosis and fistulae due to the progression of fibrosis. While understanding the mechanisms of IBD-related inflammation is moving forward and potential treatment options are emerging, effective and safe drugs for treating the fibrosis associated with IBD remain lacking, underscoring the urgent need to explore new strategies to address fibrosis and improve patient outcomes. Recent evidence suggests that probiotics may provide novel strategies to counteract fibrotic processes. In this study, the potential of a multi-strain probiotic formulation, *OxySlab*<sup>TM</sup>, to counteract key processes involved in intestinal fibrosis was evaluated using *in vitro* models of intestinal fibrosis and epithelial-mesenchymal transition (EMT). Specifically, the differentiation of intestinal fibroblasts into activated myofibroblasts was examined using CCD-18Co cells, while EMT was examined using Caco-2 cells differentiated into intestinal epithelial cells (IECs); both processes were induced by transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1). Treatment with the multi-strain probiotic formulation decreases cell proliferation and modulates fibrosis-related markers, as assessed by CCK-8 assay and MTT assay, Western blotting, and immunofluorescence. The multi-strain probiotic lysate inhibited both canonical and non-canonical TGF- $\beta$ 1 signalling pathways, and it also reduced TGF- $\beta$ 1 gene expression in activated myofibroblasts, as shown by RT-qPCR. Moreover, probiotic administration has been shown to reverse EMT by restoring epithelial markers and downregulating mesenchymal markers. These preliminary results highlight the beneficial effects of the *OxySlab* as an adjunctive therapeutic agent targeting key pathways involved in intestinal fibrosis.

# 1. INTRODUCTION

## 1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD), first described in the 19th century, is a complex chronic condition characterized by severe, recurrent inflammation and destruction of the mucosa of the gastrointestinal (GI) tract. IBD is classified into: Crohn's disease (CD), ulcerative colitis (UC), and indeterminate colitis (IC) (Silverberg *et al.*, 2005). IBD pathogenesis is multifactorial and still not fully understood, resulting from interactions among genetic susceptibility, compromised intestinal barrier function, alterations in the microbiota, immunological dysregulation, and environmental factors. The incidence of IBD has increased over the past decade to become a global problem, affecting millions of people worldwide. This is linked to environmental factors of Westernization (e.g., smoking, Western diet, improved hygiene) that alter mucosal immune responses to the gastrointestinal microbiome in genetically susceptible individuals. Since the 21st century, the incidence has stabilized in the first industrialized regions (except among children, where it has increased), but prevalence has increased across all ages. Meanwhile, in newly industrialized regions have reported an increases in the incidence of UC, which refutes calling IBD a "Western" disease (Kucharzik *et al.*, 2025). The evolution of IBD is described by Kaplan and Windsor in four distinct epidemiological stages that determine the epidemiological status of the last fifty years in the world: “Emergence, Acceleration of incidence, Compounding prevalence, Prevalence Equilibrium” (**Figure 1**).



**Figure 1.** Epidemiologic stages from 1950 to 2024 (Hracs *et al.*, 2025).

### **1.1.1 Ulcerative colitis**

UC is a chronic inflammatory condition that causes continuous mucosal inflammation of the colon, usually without granulomas on biopsy. It consistently involves the rectum and may extend to the colon in a continuous manner to a variable extent and is characterised by a relapsing and remitting course (Magro *et al.*, 2017). For decades, UC was considered exclusively a mucosal disease, but new histological and macroscopic studies have documented submucosal fibrosis, thickening of the muscularis mucosae, and other signs of involvement beyond the mucosa (Eggermont *et al.*, 2026).

### **1.1.2 Crohn's disease**

Crohn's disease (CD) was first described in 1932 by Crohn, Ginzburg, and Oppenheimer as regional ileitis, from that point forward medical community coined the name *Crohn's disease* (Crohn *et al.*, 1984). CD is characterized by discontinuous lesions that can involve any part of the GI tract, from the upper segments to the anus, the most common being the terminal ileum and colon. Inflammation is typically segmental, asymmetrical, and transmural. Symptoms include diarrhea, abdominal pain and systemic symptoms such as fatigue and weight loss, nausea, vomiting, and occasionally fevers or chills. CD diagnosis relies on a combination of symptoms, radiology, endoscopy, and histological criteria (Torres *et al.*, 2017). In addition, CD can also present extra-intestinal manifestations, among which arthropathy, dermatological lesions, such as pyoderma gangrenosum and erythema nodosum, and ocular involvement (Faggiani *et al.*, 2024).

### **1.1.3 Indeterminate colitis**

Indeterminate colitis (IC) is a term to describe a colectomy specimen that could not be classified as UC or CD because the histological features overlapped. This classification is also used when all the diagnostic tools, colonoscopies, biopsies, or imaging are inconclusive and do not show clear criteria for either disease.

### **1.1.4 IBD-risk factors**

The rapid increase in incidence of IBD is certainly related to its multifactorial pathogenesis and many factors are conditioned by the effect of globalization and lifestyle changes associated with it, but some risk factors can be modified to become prevention factors.

#### **Environmental factors**

Environmental factors that contribute to IBD risk include lifestyle choices, like increased sanitation and lack of contact with opportunistic micro-organisms, smoking, obesity, dietary habits, and antibiotic use. Furthermore, environmental factors also play a key role in early life, including mode of delivery, breastfeeding practices, and antibiotic use, as well as events occurring later in childhood, are recognized as potential factors contributing to susceptibility to IBD. Moreover, environmental

pollutants and other contaminants have been linked to IBD through their capacity to disrupt mucosal defences and activate immune pathways. Hypoxia related to high-altitude environments is also being explored as a possible trigger for IBD flare-ups. Environmental factors add significant complexity to our understanding of IBD pathogenesis; they also reinforce the notion that multifaceted diseases such as IBD require equally multifaceted therapeutic strategies that extend beyond conventional single-agent treatment. Smoking has been shown to diminish intestinal permeability, alter microcirculation and cytokine production, and increase mucin production (Jones *et al.*, 2020; Miyauchi *et al.*, 2025; Papoutsopoulou *et al.*, 2020). Alcohol abuse has been revealed to increase the risk of developing IBD, while also exacerbating disease activity by contributing to flares, inflammation, hospitalizations, and related complications; moreover, this association is dose-dependent, with heavy alcohol consumption exerting distinctly harmful effects on IBD populations (Bogale *et al.*, 2021). In addition, recent studies found a positive correlation between psychological factors (emotional, physical, sexual) and IBD risk (Anyane-Yeboah *et al.*, 2024) and a higher prevalence of trauma in IBD patients (Gnat *et al.*, 2023). A lack of physical activity can be another risk factor in the development of IBD. Recently, a systematic review and meta-analysis highlighted evidence from three large cohort studies and seven case-control studies that higher physical activity levels are inversely associated with IBD risk. However, with so many subjective factors that could influence and alter the observational study, there is no direct evidence of a correlation between lack of physical activity and the occurrence of IBD (Tiong *et al.*, 2024). The variety of environmental factors related to the risk of developing IBD adds considerable complexity to our understanding of the pathogenesis of IBD and thus the fibrosis associated with it.

### **Genetic factors**

In recent decades, genome-wide association analyses (GWAS) and transcriptome-wide association analyses (TWAS) have played a key role in the genetics of IBD. Over 200 susceptibility loci have been identified, and some loci are specifically associated with CD and UC. These genetic variants impair essential mechanisms such as microbial recognition, immune homeostasis, and epithelial barrier function, fuelling the chronic inflammation typical of IBD. Many of these genes converging on common pathways while only a minority of them is substantially validated as pivotal in IBD pathogenesis. The IBD-associated variants are localized to genes that regulate five main pathways: bacterial sensing, autophagy, endoplasmic reticulum (ER) stress, Th-17 immune pathway, and the vitamin D receptors (VDR) (Mauduit *et al.*, 2025).

- Nucleotide-binding oligomerization domain 2 (NOD2)

The NOD2 gene is a member of the evolutionarily conserved family of Nod-like receptors (NLRs), receptors that function as an intracellular sensor that primarily detects danger-associated molecular patterns (DAMPs). NOD2 plays a crucial role in detecting muramyl dipeptide (MDP), produced by the degradation of bacterial peptidoglycan. In the gut, CD-associated NOD2 polymorphisms impair MDP-downstream signalling, resulting in excessive immune and epithelial cell activation that fuels inflammation. (Claes *et al.*, 2015). In the absence of functional NOD2, TLR-2 and TLR-4 are also inadequately inhibited, leading to hyperactive NF- $\kappa$ B signalling and overproduction of inflammatory cytokines by immune cells. Similarly, NOD2 deficiency in intestinal epithelial cells (IECs) diminishes antimicrobial peptide and mucin production, while also disrupting intestinal stem cell maintenance essential for epithelial renewal (Tiong *et al.*, 2024). NOD2 polymorphisms correlate with elevated paracellular permeability in CD patients and enhanced receptor-mediated transcytosis of immune complexes, facilitating the intracellular entry of bacterial pathogens (e.g., *Salmonella*, *Yersinia*) (Rochereau *et al.*, 2021). Although the precise mechanisms by which NOD2 mutations contribute to CD development remain incompletely understood.

#### ➤ Endoplasmic reticulum stress

The ER function is to regulate protein homeostasis through ER-associated degradation, chaperone-mediated folding, and autophagy. Disruption of these processes leads to ER stress, characterized by the accumulation of misfolded/unfolded proteins, which triggers the unfolded protein response (UPR). The UPR orchestrates either adaptive restoration of ER homeostasis or maladaptive cell death through three transmembrane sensors: inositol-requiring protein 1 (IRE1 $\alpha$ ), protein kinase RNA (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6). These proteins inhibit the UPR. IRE1 detects an imbalance between misfolded proteins and chaperones, inducing unconventional splicing of X-Box Binding Protein 1 (XBP1) mRNA and promoting cytoplasmic RNase activity and the expression of various ER quality control genes. Anterior Gradient 2 (AGR2) overexpression is induced by the UPR and participates in ER homeostasis. Accumulating evidence indicates that ER stress and dysfunctional UPR in fibrosis occur via cell death, inflammation, EMT, and myofibroblast activation (X. Chen *et al.*, 2026). IBD genetic studies have identified risk alleles, such as XBP1 and AGR2, both involved in ER stress and UPR signalling (Kaser *et al.*, 2008; Mauduit *et al.*, 2025).

#### ➤ Autophagy

Autophagy plays a protective role during acute inflammation by eliminating damaged organelles, invaded pathogens, and misfolded proteins, limiting inflammatory spread. Depending on the cell type, different cells show different needs and sensitivities to autophagy. Moderate autophagy, particularly

selective autophagy, is critical in IECs to maintain barrier integrity and eliminate endogenous damage. Paneth cells, as secretory cells, rely heavily on autophagy-related proteins (e.g., ATG16L1, IRGM) to support granule secretion and antibacterial defense (R. Lin *et al.*, 2026). Of note, in chronic or persistent inflammatory states, autophagic function may be hyperactivate, be chronically suppressed, or undergo qualitative alterations, thus transforming into a pathogenic factor. Chronic exposure to LPS can suppress autophagosome formation, leading to sustained inflammasome activation. Variants in autophagy genes, such as the autophagy-related 16-like gene (ATG16L) and the immunity-related GTPase M family (IRGM), have been identified through GWAS and are considered genetic risk factors for CD (Lassen *et al.*, 2014). In CD, individuals who are homozygous for the ATG16L1 T300A variant exhibit a functional deficit in autophagy, resulting from impaired autophagosome formation. This defect compromises the cell's ability to manage and clear cytoplasmic material, including bacteria (Kuballa *et al.*, 2008; Larabi *et al.*, 2020).

### **The intestinal barrier**

The intestinal barrier plays a crucial role as an interface between the intestinal compartment and the external environment, it mediates the interaction between luminal factors and the immune system, which is crucial for preserving the mucosal immunity. In the lumen, degradation of bacteria and antigens by bile, gastric acid and pancreatic enzymes occurs, and commensal bacteria have the task of inhibiting colonization by pathogens. Also, the bacterial adhesion is prevented by the physical properties of a tiny layer of water, glycocalyx, and mucus, just above the epithelial surface, and by the action of secreted immunoglobulins in the lamina propria. To maintain the integrity of the intestinal barrier, the epithelial cells are connected by junctional complexes: tight junctions (TJ), adherent junctions and desmosomes. This integrity is, also, crucial to define permeability of molecules between adjacent epithelial cells and for the correct absorption of nutrients. Drugs and other molecules pass through the TJ via two distinct pathways based on different molecule sizes and selectivity: the *pore pathway* and the *paracellular pathway* (Dunleavy *et al.*, 2023). After the mucus and epithelium layer, a third physical barrier, more recently considered, is the gut vascular barrier: fenestrated blood vessels that prevent passage of microbial-derived large molecules and dietary compounds into the portal and systemic circulations while allowing the passage of small molecules (Spadoni *et al.*, 2015). When any disturbances disrupt the epithelial layer, its barrier function becomes compromised, resulting in increased intestinal permeability and subsequent mucosal inflammation. The loss of epithelial integrity creates an imbalance between luminal antigens and the local immune system, allowing soluble mediators, bacterial products, and even whole microorganisms to cross the intestinal wall and propagate the inflammatory cascade. This process underlies both the initiation and progression of chronic inflammatory disorders (Bergstrom *et al.*, 2022; Gunther *et al.*, 2011; Liso *et*

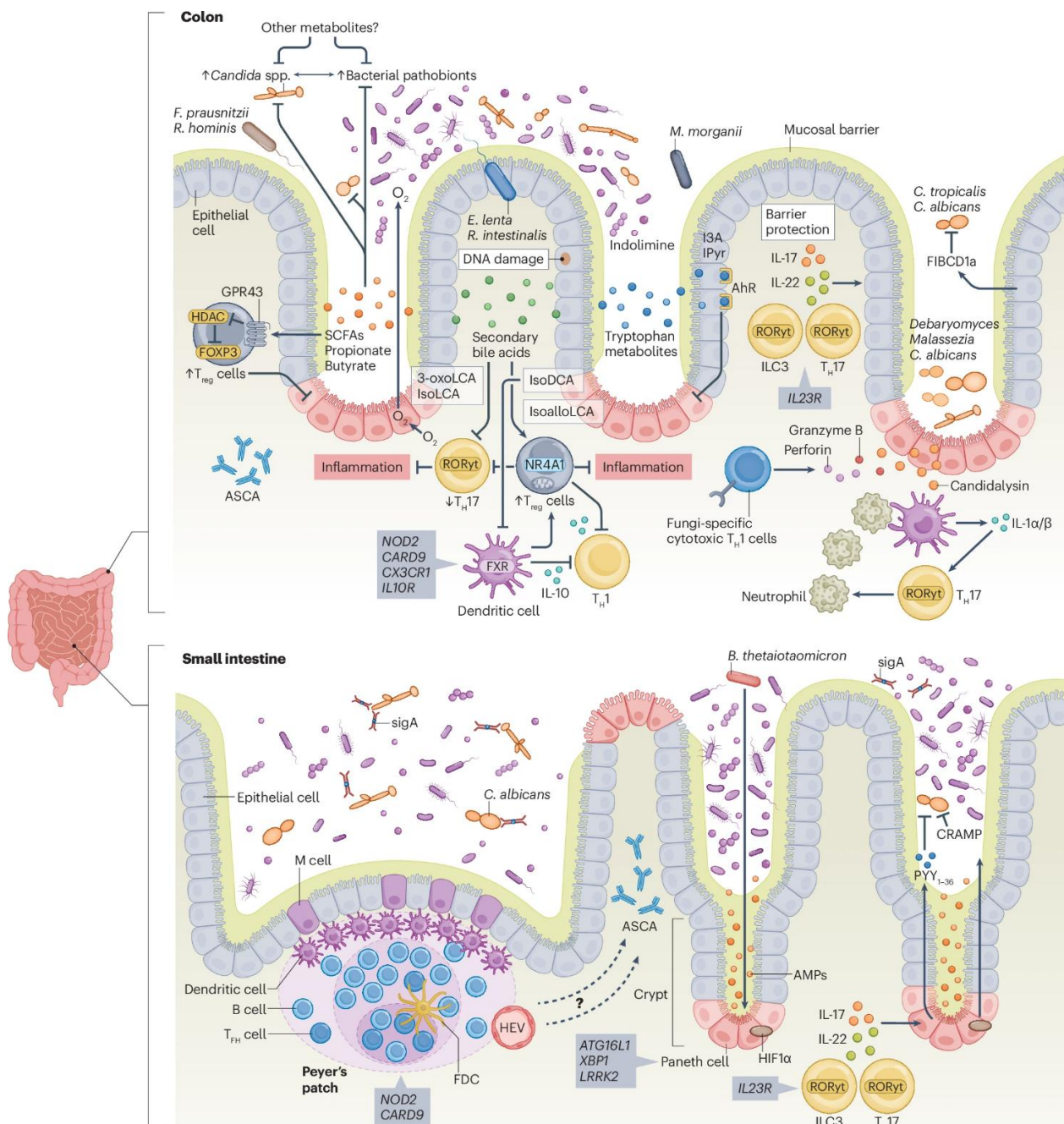
*al.*, 2020; Madsen *et al.*, 1999; Martinez-Sanchez *et al.*, 2023). Notably, the contribution of a dysfunctional intestinal barrier to intestinal inflammation has been extensively demonstrated in animal models and consistently observed not only in patients with IBD but also in individuals affected by other gastrointestinal conditions (Kotla *et al.*, 2023). Multiple studies demonstrate that dysregulated intestinal permeability can precede the clinical onset of CD by several years. Notably, asymptomatic individuals showing increased permeability on lactulose-mannitol testing have a significantly higher likelihood of developing small-intestinal CD's later on. Confocal laser endomicroscopy (CLE) has revealed characteristic features such as mucosal leakage and microerosions during active IBD, indicating compromised barrier function. Importantly, similar abnormalities are detectable in a subset of patients who appear clinically quiescent, and these findings are strong predictors of future relapse (Neurath *et al.*, 2025). As a result of this, the restoration of barrier integrity assessed by CLE emerged as a significantly better predictor of long-term outcomes in IBD than the prediction given by conventional endoscopy, which assesses the visible mucosal surface, or conventional histology, which assesses small biopsy specimens (Rath *et al.*, 2023).

### **Gut microbiome**

Over several decades, substantial evidence has established the microbiota play a key role in IBD pathophysiology. A various assortment of bacteria, viruses, and fungi residing in the intestine constitutes the intestinal microbiota and can be considered as a component of functional gut barrier. Microbes regulate immune responses through pattern recognition receptors and pathogen-associated molecular patterns (PRR-PAMP) interactions, initiating communication with macrophages and neutrophils. The term dysbiosis is used to describe the imbalances of this complex microbial community cooperating with the human host. Dysbiosis is characterized by a reduction in beneficial bacteria such as *Faecalibacterium* and *Bifidobacterium*. This imbalance, together with altered glycan profiles, disrupts the TJ, increasing paracellular permeability and promoting immune activation. Several genera, including *Adlercreutzia*, *Clostridia* UCG 014, *Clostridium sensu stricto* 1 and *Colidextribacter*, as well as their metabolic pathways involved in the synthesis of glutamate, tryptophan and threonine, have been shown to modulate the gut barrier (Leibovitzh *et al.*, 2022). Although numerous studies have attempted to identify reproducible taxonomic patterns in IBD, results have often varied due to methodological heterogeneity and the influence of geography, diet, and intraindividual variability (Guggeis *et al.*, 2025). A number of high-resolution metagenomic analyses has demonstrated that patients with active CD or UC exhibit a marked reduction of short-chain fatty acids (SCFA)-producing *Firmicutes*, including *Faecalibacterium prausnitzii*, *Roseburia hominis*, *Eubacterium rectale*, which are well-established regulators of anti-inflammatory processes and epithelial homeostasis. The loss of these taxa contributes to diminished butyrate availability,

impairing intestinal barrier integrity, modulating T regulatory cells (Treg) differentiation, and altering epithelial metabolic pathways that normally support tolerance and mucosal healing. Conversely, IBD-associated dysbiosis is frequently characterized by an overabundance of *Proteobacteria*, a phylum that includes multiple pathobionts such as *Escherichia coli*, *Fusobacterium* spp., *Ruminococcus gnavus*, and diverse *Gammaproteobacterial* species implicated in inflammatory responses. Shotgun metagenomic studies reveal consistent enrichment of these pro-inflammatory taxa during active disease, with *R. gnavus* producing inflammatory polysaccharides and mucin-degrading enzymes that compromise epithelial protection. Similarly, mucolytic species such as *Ruminococcus torques* and *Bacteroides fragilis* expand in IBD patients, reinforcing a pathological cycle in which the progressive degradation of mucus layers enhances accessibility of luminal antigens and microbial components to the mucosal immune system. Functional alterations in microbial transcriptomes and metabolomes indicate that the IBD microbiome is defined by shifts in metabolic capacity rather than solely by changes in abundance. Many commensals that generate beneficial SCFAs or metabolize bile acids are depleted, whereas bacteria involved in oxidative stress responses and inflammatory metabolite production become dominant, compromises the intestinal barrier, facilitating the translocation of microbial components such as lipopolysaccharide (LPS) into the bloodstream. This breach of mucosal defences can trigger systemic inflammation and exacerbate disease progression. Dysbiosis also drives aberrant activation of innate immune receptors (TLR2, TLR4), engages genetic susceptibility loci such as *CARD9*, *IL23R*, and *ATG16L1*, and disrupts Paneth cell function, antimicrobial peptide production, and epithelial barrier repair processes. Pathobionts, such as *Adherent-invasive E. coli* (AIEC) or *Clostridium innocuum*, induce IL-1 $\beta$ , TNF, and IL-6 production in mononuclear phagocytes and epithelial cells, amplifying inflammatory cascades and fostering T helper 17 (Th17) responses, which are central to IBD immunopathology. Conversely, the depletion of butyrate-producing bacteria undermines Treg induction, which normally restricts inflammation and preserves homeostasis (Iliev *et al.*, 2025) (**Figure 2**). Vitamin D exerts bidirectional interactions with the gut microbiota, influencing microbial composition in healthy individuals while, in turn, being modulated by microbial activity. Genetic studies show that variations in the human vitamin D receptor (VDR) gene correlate with shifts in intestinal microbial communities, and the absence of intestinal VDR in mice induces dysbiosis. Although bacteria do not express VDR themselves, vitamin D effects on the microbiota are mediated through the VDR signalling in epithelial and immune cells. VDR-deficient mice display impaired Paneth cell function, including defective autophagy, granule exocytosis, and antimicrobial peptide (AMPs) secretion changes, and thus promote inflammation and increase susceptibility to infection. Clinically, higher vitamin D levels correlate with increased circulating cathelicidin, a member of the family of AMPs, and reduced inflammation in UC patients, and vitamin

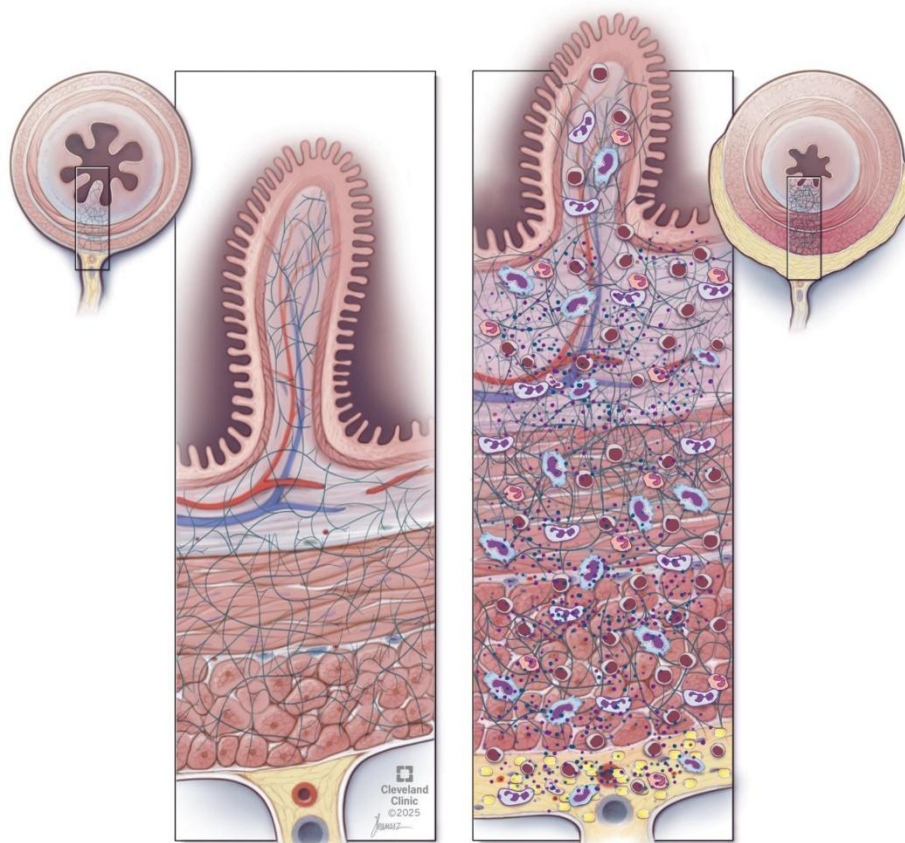
D enhances cathelicidin-mediated antimicrobial activity against *E. coli* *in vitro*, while protecting against experimental colitis *in vivo*. Current evidence suggests that commensal and pathogenic bacteria regulate VDR expression and that microbial metabolites, such as butyrate, can upregulate epithelial VDR expression. Other bacterial metabolites, including lithocholic acid, activate VDR signalling in T cells and suppress IL-2 production. Certain bacterial enzymes additionally hydroxylate steroids and contribute to vitamin D activation. The limited evidence available suggests a beneficial effect of vitamin D in both CD and UC, including increased *Enterobacteriaceae* and reduced intestinal inflammation (Vernia *et al.*, 2022).



**Figure 2. Microbiota-derived metabolites, immune and genetic mechanisms, and their effects on inflammation, intestinal biology and IBD (Iliev *et al.*, 2025)**

## 1.2 Intestinal fibrosis

Intestinal fibrosis is a common and irreversible pathological consequence of IBD. In CD, fibrotic strictures and obstructive symptoms affect over 50% of patients during their lifetime and even more requiring surgical interventions. The onset of fibrosis is less frequent in UC and can lead to motility disturbances and bowel urgency (Rieder *et al.*, 2024). Fibrogenesis initiate as a consequence of persistent inflammation that promotes excessive extracellular matrix (ECM) deposition and architectural tissue remodelling of the intestinal wall, that can lead to stricture formation and obstruction, that need surgical intervention, but the exact mechanisms of this chronic progression remain unknown (**Figure 3**). UC has long been viewed as a condition limited to the mucosal layer and not associated with fibrosis, in contrast to CD, which causes deeper, transmural inflammation and scarring. Recently, research shows that ulcerative colitis can involve more than just the surface layers and can induce structural changes included: haustral loss, colonic shortening, resulting from



progressive submucosal thickening and tissue contraction, and submucosal fibrosis (I. O. Gordon *et al.*, 2018). In CD, fibrosis can involve the full thickness of the bowel wall that includes the mucosa, submucosa, muscularis, muscularis propria and serosa layers. In addition, it can affect any part of the gastrointestinal tract, and the fibrosis can result in critical narrowing of the lumen resulting in strictures, or stenosis, that commonly lead to intestinal obstruction that requires surgery (Latella *et al.*, 2015).

**Figure 3. Bowel wall changes in stricturing CD.** Left panel normal intestinal wall: mucosa layers, muscularis mucosa, submucosa, muscularis propria and mesentery, covered by serosa. Right panel: intestinal wall in stricturing CD: a thickening of all layers in conjunction with an expansion of the mesenteric fat (S. N. Lin *et al.*, 2025).

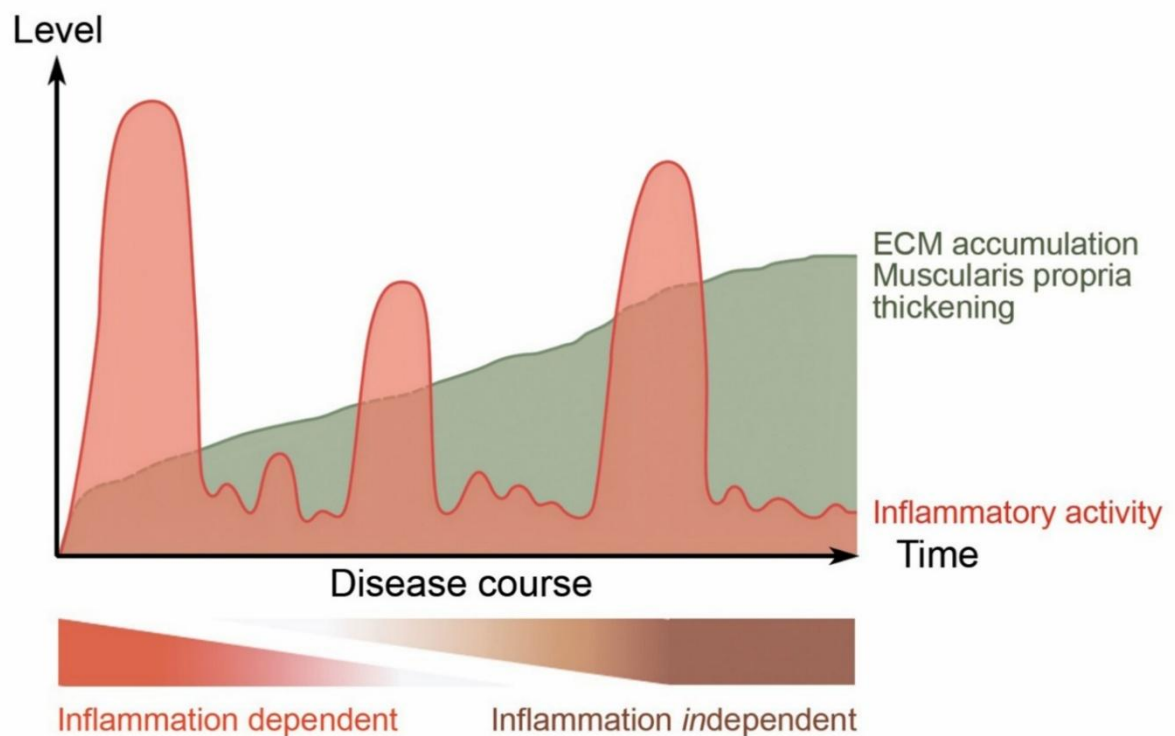
Different cell types, such as smooth muscle cells (SMCs), myofibroblasts, and fibroblasts drive the process of fibrogenesis, characterized by an abnormal accumulation of ECM primarily composed of collagen. The activation of these cells is influenced by a range of factors, including genetic and epigenetic changes, microbiota alterations, cytokines, and other soluble factors released during inflammatory responses (Mignini *et al.*, 2024). Myofibroblasts derived from mesenchymal cells are not the sole contributors to excessive ECM deposition. Activated fibroblasts from various origins can also produce ECM, including those arising from epithelial cells via epithelial-to-mesenchymal transition (EMT), endothelial cells through endothelial-to-mesenchymal transition (EndoMT), circulating fibrocytes, certain immune cells. These converging processes clearly result in excessive deposition of a disorganized ECM, ultimately contributing to intestinal fibrosis. A recent study showed that CCR2-positive monocytes could inhibit collagen degradation by upregulating tissue inhibitors of metalloproteinases (TIMP-1), a process that paradoxically triggers a feedback mechanism promoting further ECM breakdown (Kuroda *et al.*, 2019). Despite advances in IBD treatment, effective therapies targeting intestinal fibrosis remain lacking. Current anti-inflammatory treatments, including advanced therapies, do not significantly interrupt the progression of intestinal fibrosis. This can be explained by the fact that fibrosis initially triggered by inflammation but, once started, subsequently can progress independently over time (**Figure 4**). The antifibrotic effects of

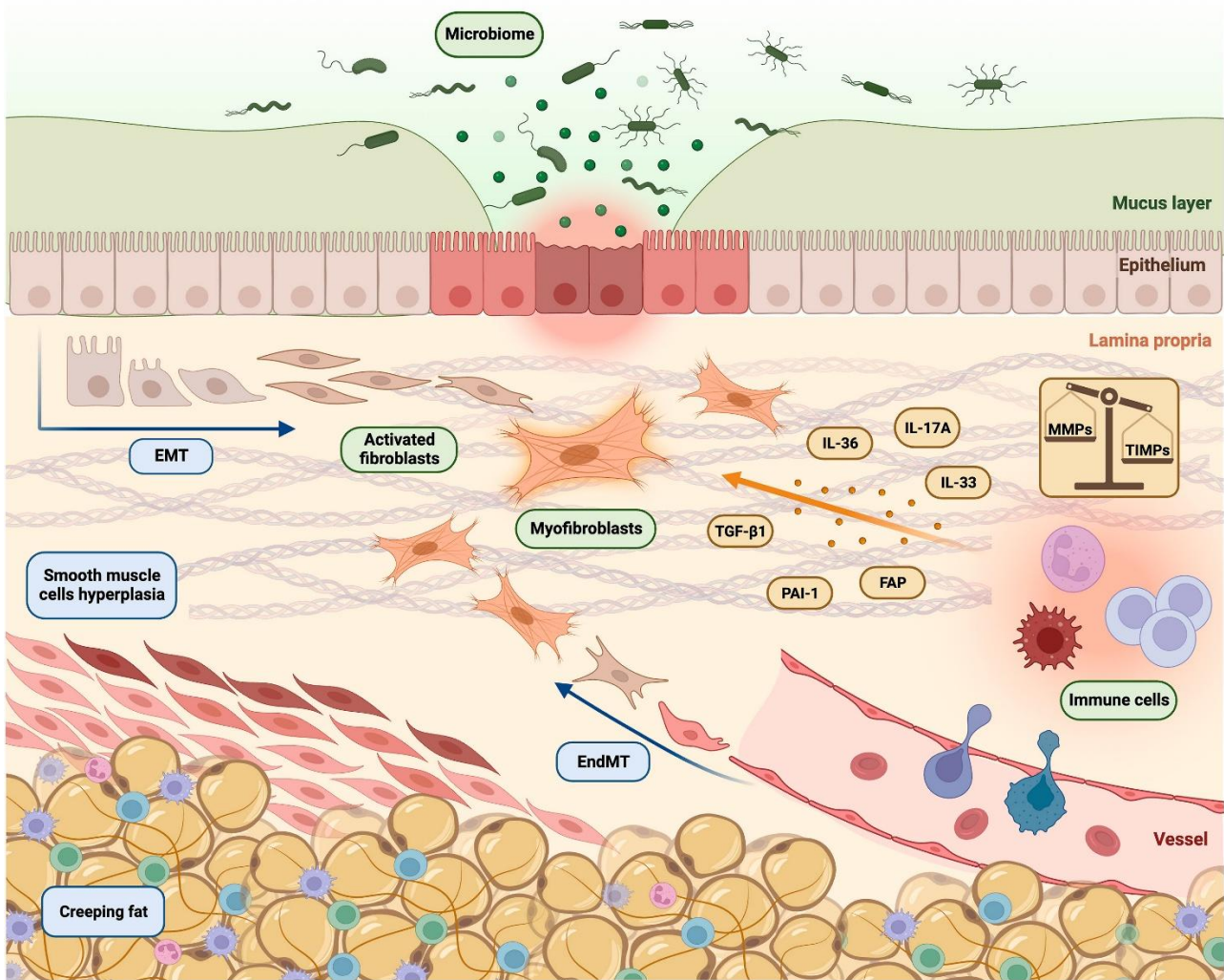
available anti-inflammatory drugs have been evaluated only in limited clinical studies (Rieder *et al.*, 2024; Su *et al.*, 2025).

**Figure 4.** Model for progressive accumulation of extracellular matrix and thickening of the muscularis propria, despite a relapsing and remitting chronic inflammatory disease course (Rieder *et al.*, 2024).

### 1.2.1 Components of intestinal fibrosis

Recurrent, or persistent, epithelial injury is crucial for initiating and sustaining intestinal inflammation and fibrogenesis (**Figure 5**). Epithelial and endothelial damage releases chemotactic factors promoting the recruitment and activation of innate and adaptive immune cells, as well as mesenchymal cell precursors of the activated myofibroblasts (**Figure 6**) (J. Wang *et al.*, 2021).





**Figure 5.** Fibrogenesis: complex and multistep process involving a multitude of cellular and molecular players (Lenti *et al.*, 2024).

## Mesenchymal cells

Mesenchymal cells are specialised stromal components of the gut wall that provide the structural framework supporting the intestinal epithelium. This heterogeneous group includes subepithelial myofibroblasts, pericytes, stromal stem cells, cells of the muscularis mucosae, and the smooth muscle cells of the villus core. Derived from mesenchymal stem cells, they are defined by their non-epithelial, non-endothelial and non-haematopoietic nature. In addition, interstitial cells of Cajal and fibrocytes, non-conventional mesenchymal cell types, also contribute to these functions.

### ➤ Fibroblast, smooth muscle cells, and myofibroblast

Fibroblasts are specialized cells in connective tissues that have a fundamental role in maintaining structural integrity by producing and remodelling ECM. They synthesize ECM components, such as collagen, elastin, and glycosaminoglycans. In a physiological state, fibroblasts contribute to normal wound healing by migrating to sites of injury, proliferating, and depositing ECM to repair damaged

tissue. In pathological conditions, particularly fibrosis, these cells become hyperactivated and under the influence of profibrotic factors, like TGF- $\beta$ , can acquire a myofibroblast phenotype. Fibroblasts also engage in extensive crosstalk with immune cells, amplifying both inflammatory and fibrotic pathways through cytokine and growth factor mediated-signalling (Antar *et al.*, 2023). SMCs, likewise, contribute to this process, as they can differentiate into myofibroblasts under specific conditions. Notably, SMCs isolated from the ileum of CD patients show marked alterations in morphology and contractility (Severi *et al.*, 2014). Interestingly, myofibroblasts retain a degree of plasticity and can revert to SMCs in response to pro-inflammatory cytokines. This bidirectional transition may promote smooth muscle hyperplasia and hypertrophy, ultimately thickening the muscularis propria, driving to stricture formation and intestinal obstruction. Myofibroblasts are characterized by increased ECM production and heightened contractility, which contribute to excessive tissue stiffness and scarring.

#### ➤ Pericytes

Tiny blood endothelial cells are surrounded by pericytes ( $\alpha$ -SMA<sup>+</sup>, desmin<sup>+</sup>) that exhibit an intermediate phenotype between fibroblasts and vascular SMCs. Pericytes can differentiate into fibroblasts and contribute to the pool of ECM-producing fibroblasts following activation by inflammation-driven mediators (Rieder *et al.*, 2024).

#### ➤ Fibrocytes

Fibrocytes are bone-marrow-derived mesenchymal progenitor cells that display features of both immune cells and fibroblasts, expressing markers such as CD45 and collagen I. They are mobilized in response to inflammation or tissue injury and subsequently migrate to affected sites, where they contribute to tissue repair and fibrogenesis by producing collagen, fibrogenic cytokines, and growth factors. Once recruited, fibrocytes can promote fibrosis through both direct mechanisms, such as ECM deposition and secretion of profibrotic mediators, and indirect mechanisms, including differentiation into myofibroblasts. This transition is driven in part by TGF- $\beta$ 1, which induces fibrocyte-to-myofibroblast conversion and further amplifies the fibrotic response (Wu *et al.*, 2023; Yang *et al.*, 2024). Evidence also links circulating fibrocytes to the fibrostenotic phenotype in CD, with elevated fibrocyte frequencies observed in affected patients compared with healthy individuals (Ueno *et al.*, 2022). Moreover, subsets such as CCR2<sup>+</sup> fibrocytes have been shown to exacerbate colonic fibrosis by inhibiting collagen degradation, thereby promoting ECM accumulation (Kuroda *et al.*, 2019).

### Non-mesenchymal cells

### ➤ Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) was first recognized through the pioneering developmental studies of Elizabeth Hay who demonstrated that epithelial cells could convert into mesenchymal cells during embryogenesis (Hay, 1995). EMT was later shown to be reactivated in adult pathological processes, like fibrosis and cancer, where it contributes to tissue remodelling, cellular plasticity, and disease progression. Particularly in the intestine, EMT has emerged as a fundamental mechanism driving fibrogenesis, in part by generating epithelial-derived fibroblasts that deposit ECM and promote scarring (Flier *et al.*, 2010; Lovisa *et al.*, 2019). Importantly, in the context of CD, EMT involvement in fistula formation and fibrosis was recognized, over the past decade, in intestinal fistulae of CD patients, where epithelial cells dislocated from the basement membrane, migrated into fistula tracts, and acquired transitional mesenchymal-like features under chronic inflammatory and impaired wound-healing conditions. Since this initial observation, a growing body of research has confirmed that EMT contributes both to CD-associated fistulas and to the pathogenesis of IBD-associated intestinal fibrosis, where epithelial cells undergoing EMT can become a direct source of fibroblasts and amplify ECM deposition (Bataille *et al.*, 2008; Jiang *et al.*, 2018). Through EMT-driven reprogramming, IECs acquire fibroblast-like properties, supplying a substantial fraction of FSP1<sup>+</sup> fibroblasts that expand the profibrotic cell pool and promote excessive extracellular matrix deposition. Key profibrogenic mediators, including TGF- $\beta$ 1 and IL-13, amplify EMT in the fibrotic intestinal mucosa, with increased TGF- $\beta$ 1 expression observed in the epithelial and submucosal layers of CD-associated fibrotic tissue. During EMT activation in intestinal fibrosis, epithelial cells progressively lose polarity and adherence, accompanied by downregulation of epithelial markers such as E-cadherin, ZO-1, claudins, cytokeratins, and type IV collagen, and a corresponding upregulation of mesenchymal markers including vimentin, FSP1,  $\alpha$ -SMA, fibronectin, N-cadherin, and collagens I/II. Experiments in TNBS-induced colitis models further demonstrate that epithelial cells in inflamed gut tissues co-express epithelial and mesenchymal markers, directly confirming EMT phenotypes during intestinal fibrogenesis (Flier *et al.*, 2010).

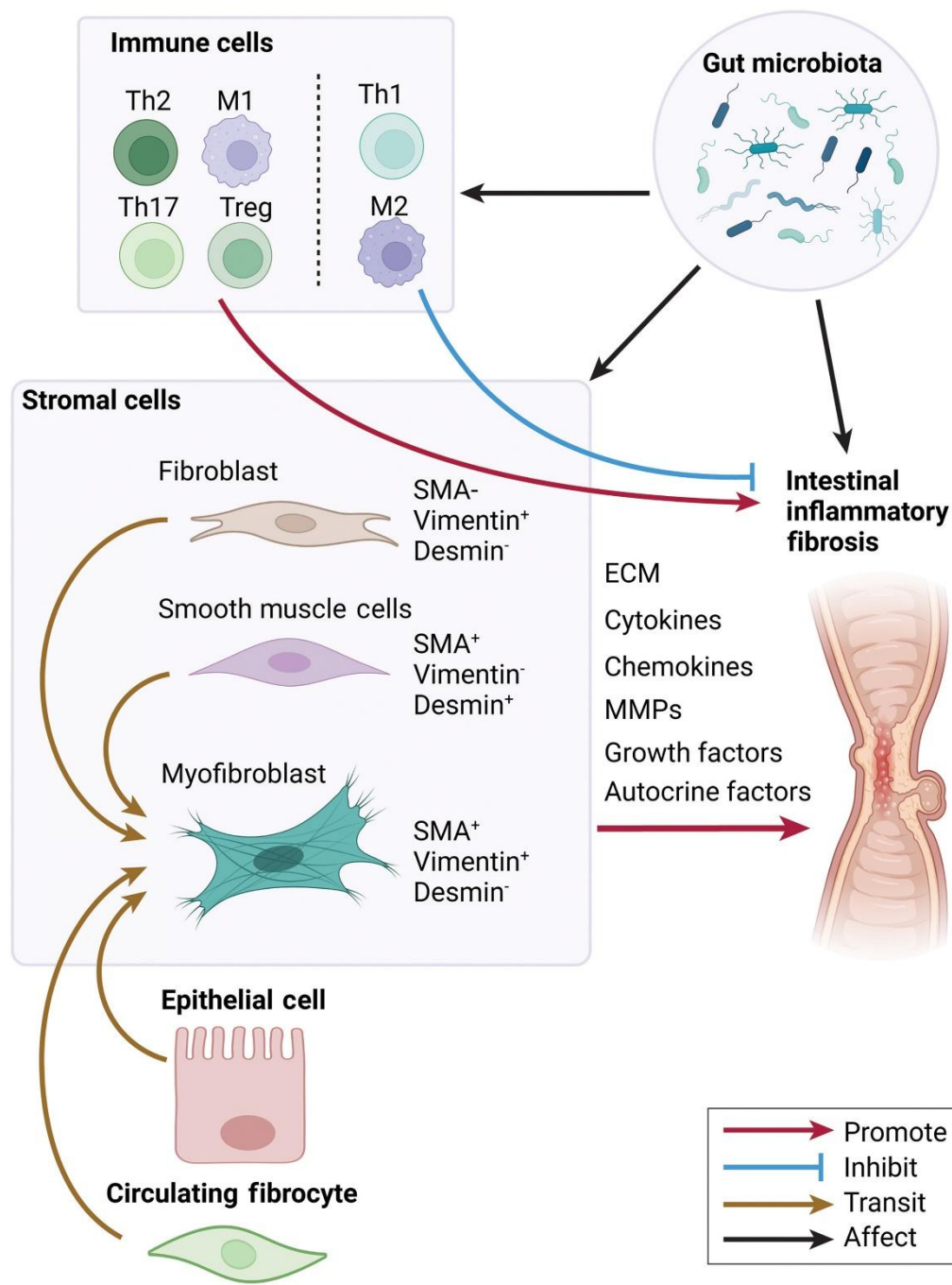
### ➤ Endothelial-mesenchymal transition

Endothelial cells can also undergo phenotypic reprogramming during chronic intestinal inflammation. Endothelial cells deprived of growth factors or stimulated by inflammatory cytokines (TGF- $\beta$ , IL-1 $\beta$ , and TNF- $\alpha$ ) may be subjected to endothelial-mesenchymal transition (EndoMT), a process in which they progressively lose endothelial markers and acquire a fibroblast-like morphology and transcriptional profile. This mechanism is increasingly recognized as a contributor to fibrotic remodelling across organ systems, including the gut. Evidence of active EndoMT has been observed *ex vivo* in microvascular vessels from patients with CD and UC, where endothelial cells co-

expressing endothelial and mesenchymal markers appear only in zones of active inflammation. Further *in vivo* validation comes from endothelial-reporter mouse models, where endothelial cells permanently tagged by fluorescent markers were found outside vascular structures and within fibrotic intestinal regions. These observations confirm that EndoMT not only occurs in intestinal tissue but also contributes directly to the mesenchymal cell pool driving fibrosis (Rieder *et al.*, 2011).

➤ Immune cells

The immune cells are essential components of fibrosis pathogenesis, with macrophages playing a noteworthy role. Specifically, the M2 phenotype of macrophages is recognized for its profibrotic role to stimulate fibroblast activation releasing key molecules such as TGF- $\beta$ 1, PDGF, FGF, and IGF (Amamou *et al.*, 2022). Also, T helper 2 and T helper 17 lymphocytes take part to the process as promotor of fibrogenesis. The Th17 response specifically triggers the production of interleukin (IL)-17 and IL-21 furthering ECM production by intestinal myofibroblasts. Additionally, the Th2 response induces the release of IL-4 and IL-13, with the latter directly promoting collagen deposition by fibroblasts. Moreover, IL-13 stimulates macrophages to promote fibrosis by secreting TGF- $\beta$ 1 and matrix metalloproteinases (MMPs) (Gieseck *et al.*, 2018; Ray *et al.*, 2014). Furthermore, recent studies have suggested a possible role for IgG4-producing plasmablasts which can promote IBD-related strictures due to the secretion of PDGF (Bushara *et al.*, 2022).



**Figure 6 Cellular components in intestinal fibrosis** (Yang *et al.*, 2024)

### Extracellular Matrix

ECM is a dynamic, non-cellular, structural and functional component of intestinal tissue, secreted by several cell types (epithelial, endothelial, fibroblasts, and myofibroblasts), and it exerts a pivotal role in cell morphogenesis, adhesion, differentiation, growth, survival, and migration. In a healthy gut, ECM is a complex mix of collagens, glycoproteins, and proteoglycans and forms two distinct compartments: the interstitial matrix (IM), a reticular scaffold for cell support; and the basement membrane (BM), a pericellular matrix between the epithelium and the lamina propria. The BM, mainly constituted by laminins and type IV collagen, provides structural support to the epithelium.

IM is rich in fibrillar collagens, especially kinds I, III, and V, and other molecules like elastin, fibronectin, and proteoglycans, which provide tensile strength and elasticity. ECM also acts as a reservoir for growth factors and cytokines, such as TGF- $\beta$ , and these actively participate in tissue signalling systems. The ECM undergoes remodelling during chronic inflammation and changes in collagen composition, such as an increased type III/I collagen ratio across intestinal layers in CD. The inability to counterbalance excessive ECM formation with appropriate degradation, accelerates the development of fibrosis. MMPs degrade the structural components of the ECM and modulate inflammatory and fibrotic processes. In a physiological state, MMPs have low activity; while in IBD, both MMP expression and activity are dysregulated. Although several MMPs, such as MMP-1, MMP-2, MMP-3, MMP-9, and MMP-1, are upregulated in fibrotic tissues, the concomitant increase in their endogenous inhibitors, TIMP-1, limits their proteolytic effects. Analyses of fibrotic ileal tissue in CD have revealed upregulated MMP-1 and MMP-3 expression in the submucosa and muscularis, along with increased MMP-2 activity. These findings indicate that MMPs may exert multifaceted functions, encompassing not only extracellular matrix degradation but also pro-fibrotic signalling mechanisms, including the activation of latent TGF- $\beta$ 1. Fibroblasts and myofibroblasts derived from stenotic tissue display a higher TIMP-1 expression and reduced ECM-degrading potential, further reinforcing ECM accumulation. This disruption of the balance between MMP-mediated breakdown and ECM overproduction is a key pathogenic mechanism underlying progressive intestinal fibrosis (Biel *et al.*, 2024; Sferra *et al.*, 2026).

### 1.2.2 Key molecules involved in intestinal fibrosis

During inflammation phase, mesenchymal cells are exposed to a highly complex microenvironment composed of luminal factors and a broad spectrum of inflammatory mediators, including cytokines and growth factors released by both immune and non-immune cells. The production and the function of ECM undergo qualitative and quantitative changes driven by numerous factors associated with local inflammation. Among these, the TGF- $\beta$  cytokine is particularly significant. Its levels likely rise as a compensatory response to inflammation due to its immunosuppressive and anti-inflammatory properties. However, in carrying out this role, TGF- $\beta$  also acts as a strong pro-fibrotic mediator, stimulating intestinal mesenchymal cells and promoting their differentiation into myofibroblasts (S. N. Lin *et al.*, 2025). This diverse milieu is thought to play a central role in initiating intestinal fibrosis and may also contribute to its persistence.

#### ➤ TGF- $\beta$ 1

In the multifunctional growth factor family, TGF- $\beta$ , plays a pivotal role in both physiological processes and pathological tissue responses, however it results upregulated in essentially all fibrotic

diseases (Budi *et al.*, 2021) and it is established that TGF- $\beta$  contributes significantly in the pathogenesis of fibrotic diseases like CD, myocarditis, diabetic nephropathy, rheumatoid arthritis, and radiation-induced fibrosis through fibroblast proliferation, extracellular matrix (ECM) accumulation, and tissue remodelling (Yun *et al.*, 2019). However, in addition to its pro-inflammatory role, TGF- $\beta$  also has significant anti-inflammatory action and promote differentiation of T regulatory cells and inhibit differentiation along the Th1 and Th2 lineage (Sanjabi *et al.*, 2017). TGF- $\beta$  is produced by numerous cell types, including epithelial cells, immune cells, and fibroblasts. Although the mechanisms regulating TGF- $\beta$  production in the human intestine are not yet fully understood, its expression can be upregulated by a variety of stimuli, such as bacteria, viruses, cytokines, apoptotic cells, and autocrine or paracrine signalling loops (Kashiwagi *et al.*, 2015; Torchinsky *et al.*, 2009). Among the three TGF- $\beta$  isoforms (TGF- $\beta$ 1, TGF- $\beta$ 2, and TGF- $\beta$ 3), each of which exhibits distinct biological activities, intestinal fibrosis is predominantly driven by TGF- $\beta$ 1. As inactive form, TGF- $\beta$ 1 is stored as a disulfide-linked homodimer bound to a latency-associated peptide (LAP). To activate the functional form, TGF- $\beta$ 1 requires LAP dissociation, a process mediated by extracellular enzymes like cathepsins, plasmin, calpain, thrombospondin, matrix metalloproteinases and integrin- $\alpha$ v $\beta$ 6, which are known to participate in latent TGF- $\beta$  activation within fibrotic intestinal tissues. The association between TGF- $\beta$ 1 levels and intestinal strictures in CD was demonstrated by Di Sabatino *et al.*, who reported a significant elevation of TGF- $\beta$ 1 expression in the intestinal mucosa overlying strictures, partially attributed to enhanced TGF- $\beta$  production by myofibroblasts near the strictured areas (Di Sabatino *et al.*, 2009). Also, Li *et al.* found that the level of active TGF- $\beta$ 1 was markedly higher in strictured intestinal muscle obtained from surgically resected ileo of CD patients compared to adjacent normal intestinal tissue (C. Li *et al.*, 2013). In addition, the serum TGF- $\beta$ 1 levels were significantly elevated in treatment-naïve patients with active UC compared to healthy controls (Sambuelli *et al.*, 2000). Lymphocytes from lamina propria of UC patients exhibited significantly higher TGF- $\beta$ 1 production compared to controls, particularly evident upon stimulation with CD2 and CD28, suggesting a heightened TGF- $\beta$ 1 response in the inflammatory environment (Del Zotto *et al.*, 2003). In summary, TGF- $\beta$  plays a pivotal role in the IBD-related fibrosis pathogenesis, and elevated levels of TGF- $\beta$ , both locally in the intestinal tissue and systemically in the serum, are closely associated with inflammation, fibrosis, and stricture formation. Activated TGF- $\beta$ 1 binds to the TGF- $\beta$  type II receptor (TGF $\beta$ RII), which is followed by formation of a complex with TGF- $\beta$  type I receptor (TGF $\beta$ RI), these receptors are expressed by many cell types, including immune cells and epithelial cells. The resultant TGF- $\beta$  receptor complex activates intracellular signalling through the canonical (Smad-dependent) and non-canonical pathways (Smad-independent).

#### ➤ IL-1 family members

IL-1 family consists of 11 cytokine members, and in addition to their well-established roles in inflammation, some members, such as IL-1, IL-33, and IL-36, are increasingly recognized for their involvement in intestinal fibrosis.

- IL-1 $\alpha$  and IL-1 $\beta$

IL-1 $\alpha$  is typically expressed in epithelial cells but can also be induced following activation in other cell types, including macrophages, monocytes, and endothelial cells. When released from dying IECs, IL-1 $\alpha$  acts as DAMP and plays a crucial role in initiating and sustaining chronic intestinal inflammation (Valatas *et al.*, 2017). In a dextran sodium sulfate (DSS)-induced colitis mouse model, IL-1 $\alpha$ -deficient mice exhibited less severe disease symptoms and showed faster recovery. Additionally, neutralizing IL-1 $\alpha$  in wild-type mice during acute colitis alleviated both clinical symptoms and histological damage, suggesting that IL-1 $\alpha$  contributes significantly to the inflammatory response in the gut (Nunberg *et al.*, 2018). On the other hand, IL-1 $\beta$ , primarily produced by mononuclear phagocytes, is a key pro-inflammatory cytokine in the early stages of intestinal inflammation (Friedrich *et al.*, 2019). Stimulating the secretion of pro-inflammatory cytokines, chemokines, and other mediators such as IL-8, monocyte chemoattractant protein (MCP)-1, and MMPs, particularly MMP-1, from intestinal myofibroblasts and fibroblasts, IL-1 $\beta$  helps to recruit more immune cells to the site of injury and drives further inflammatory responses. Increased levels of IL-1 are found in IBD, resulting in activation of IL-1 converting enzyme (ICE) and releasing of mature IL-1 $\beta$  in the colon that contributes to the activation of myofibroblasts. In contrast, some studies have shown that IL-1 $\beta$  may also inhibit collagen synthesis and promote the production of collagenases and TIMP-1 in intestinal SMCs. The precise role of IL-1 $\beta$  in intestinal fibrosis remains a subject of ongoing research, due to the both pro-fibrotic and anti-fibrotic effects depending on the stage and type of intestinal injury (J. Wang *et al.*, 2021).

- IL-36

IL-36 is a pro-inflammatory cytokine that plays a significant role in the pathogenesis of various inflammatory diseases, including intestinal fibrosis. It is part of the IL-1 family of cytokines, consisting of IL-36 $\alpha$ , IL-36 $\beta$ , IL-36 $\gamma$ , and the IL-36 receptor antagonist (IL-36Ra). In the intestinal tract, IL-36 is generated by many different cell types, including fibroblasts, myofibroblasts, epithelial cells, goblet cells, macrophages, and glial cells. IL-36 $\alpha$  and IL-36 $\gamma$  were upregulated in endoscopic biopsies of patients with active colonic CD compared to unaffected biopsies from the same patients (J. Wang *et al.*, 2021).

- IL-33

IL-33 is a potent alarmin, released from damaged or stressed cells, that can activate immune responses through its receptor, ST2. In the gut, IL-33 promotes pro-inflammatory and Th2-associated immune responses contributing to fibrosis. This cytokine is primarily expressed by fibroblasts, smooth muscle cells, endothelial cells, adipocytes, IECs, and infiltrating lamina propria mononuclear cells. Increased expression levels of IL-33 and its receptor, ST2, was found in IBD patients, particularly in UC (Kobori *et al.*, 2010). In models of AIEC-induced intestinal fibrosis, bacterial flagellin enhances ST2 expression in intestinal epithelial cells. The IL-33-ST2 axis subsequently drives intestinal fibrosis via TLR5 and NLRC4, while blockade of this pathway using an anti-ST2 antibody suppresses fibrosis-related gene expression and reduces collagen deposition in the colon (Imai *et al.*, 2019).

- IL-18

First identified in mouse serum after exposure to bacterial LPS, IL-18 was initially characterized as a factor that increases IFN- $\gamma$  production by monocytic cells. IL-18 most closely resembles IL-1 $\beta$  in structure and in its association with the inflammasome, a proteolytic complex that activates pro-IL-1 $\beta$  and pro-IL-18 through caspase-1-dependent cleavage. Clinical studies have associated elevated epithelial IL-18 secretion with higher IBD severity. Knockout mice lacking IL-1 $\beta$ , IL-18, or both cytokines were resistant to TNBS-induced colitis, with a double deficiency providing the strongest protection, likely due to blockade of convergent inflammatory pathways. Although IL-18 is predominantly considered a pro-inflammatory mediator, it exerts essential functions for intestinal homeostasis. In fact, the NLRP6 inflammasome, highly expressed in intestinal epithelial cells, contributes to the maintenance of mucosal integrity through the regulation of epithelial turnover. Loss of NLRP6 or caspase-1 results in worsening of DSS-induced colitis, likely due to the reduced availability of basal IL-18 required for proper functioning of the intestinal barrier. However, when produced in excess, IL-18 becomes a promoting factor of mucosal damage (Elinav *et al.*, 2011; Williams *et al.*, 2019).

- IL-6 family members

- IL-6

IL-6 plays a physiological role in intestinal epithelial cell differentiation and wound repair. However, its levels rise in experimental colitis and in human IBD, where it exerts pro-inflammatory effects. This activity is driven by the activation of multiple immune cell types, including antigen-presenting cells and T cells. IL-6 binds its soluble receptor (sIL-6R) and activates the STAT3 pathway, thereby preventing apoptosis and promoting the production of additional pro-inflammatory cytokines. Consistent with this, inhibition of IL-6 signalling in mouse models of colitis increases T-cell apoptosis

and significantly reduces pro-inflammatory cytokines such as IFN- $\gamma$ , TNF, and IL-1 $\beta$  in inflamed tissues (Sferra *et al.*, 2026).

- IL-11

IL-11 has been identified as a profibrotic cytokine released by myofibroblasts, injured epithelial cells, and SMCs. Tan *et al.* found that IL-11 was mainly expressed by fibroblasts obtained from severely fibrotic tissue and the immunofluorescence analysis of full-thickness intestinal sections from stenotic regions revealed the colocalization of IL11 with COL1A in CD patients (Tan *et al.*, 2025).

- Th17-related cytokines

The Th17 immune pathway plays a complex and often paradoxical role in the initiation and progression of intestinal fibrosis. Th17 cells, driven by IL-6, IL-21, IL-23, IL-1 $\beta$  and TGF- $\beta$ , produce a broad array of effector cytokines, including IL-17A, IL-17F, IL-21, IL-22 and TNF- $\alpha$ , that are implicated in chronic intestinal inflammation and tissue remodelling. Among these, IL-17A has been most extensively studied and is consistently elevated in inflamed IBD mucosa, where it exerts pronounced effects on fibroblasts and myofibroblasts. Experimental models demonstrate that IL-17A directly promotes myofibroblast proliferation, EMT, and increased collagen production, thereby contributing to the ECM deposition. In murine TNBS-induced chronic colitis, deleting IL-17 significantly down-regulates collagen III, TIMP-1, and MMP-2. Also, in clinical observations, IL-17A is markedly overexpressed in CD strictures, where it enhances production of MMP-3, MMP-12, TIMP-1, and collagen by human intestinal myofibroblasts. However, IL-17 and IL-22 contribute to maintaining epithelial integrity and promoting mucosal repair, and several studies demonstrate that blocking IL-17 can worsen epithelial injury and intensify colitis, suggesting a protective role under specific inflammatory conditions. This duality emphasizes that Th17 responses depend on context and the microenvironment (Latella *et al.*, 2020).

- Th2-related cytokines

Th2 cells can directly promote fibrosis by stimulating fibroblasts to synthesize collagen in response to IL-4 and IL-13, often by reinforcing the TGF- $\beta$ /Smad axis. IL-13 enhances pericellular accumulation of latent TGF- $\beta$ 1 complexes (LAP/TGF- $\beta$ ), increasing the amount of readily activatable TGF- $\beta$  in the stromal microenvironment and promoting downstream signalling that orchestrates fibroblast activation and ECM deposition. Recent studies show that chronic inflammatory cytokines cooperate with Th2 pathways to expand profibrotic fibroblast subsets, such as FAP<sup>+</sup> stromal cells, which are strongly enriched in stricturing CD. Furthermore, IL-4 and IL-13 drive M2 macrophage polarization, a phenotype known to produce TGF- $\beta$ 1, PDGF, and other mediators that sustain fibroblast hyperactivation, even after partial control of inflammation, thereby reinforcing fibrosis as

a self-perpetuating process. Mice deficient in the SH2 domain-containing inositolpolyphosphate 5'-phosphatase (SHIP), a negative regulator of PI3K, develop spontaneous Crohn-like ileitis. Lo Y. *et al.* hypothesized that SHIP-deficient mice develop fibrosis due to increased PI3Kp110 $\delta$  activity. IL-4 and IL-13 were shown to activate the PI3Kp110 $\delta$  pathway, and its upregulation may be sufficient to drive fibrosis. Blocking IL-13, collagen accumulation and muscle thickening decrease. Importantly, IL-13 neutralization also reduced expression of TGF- $\beta$ -related profibrotic mediators (Lo *et al.*, 2019).

➤ Interferons (INF)

IFN- $\gamma$  is the most extensively studied interferon isoform in experimental models of colitis. In DSS-induced colitis in mice, levels of this cytokine significantly increase compared to controls. In fact, mice with lack of IFN- $\gamma$  exhibit a marked reduction in DSS-induced inflammation, supporting the key role of IFN- $\gamma$  in the pathogenesis of IBD (Lee *et al.*, 2017). Additional evidence shows that IFN- $\gamma$ , released by Th1 cells, triggers the production of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$  and IL-6 (Sferra *et al.*, 2026).

➤ TNF- $\alpha$  family members

Two members of the TNF- $\alpha$  family, TNF- $\alpha$  and TNF-like ligand 1A (TL1A), are well-known for their involvement in the pathogenesis of IBD.

➤ TNF- $\alpha$

In the early stages of intestinal inflammation, TNF- $\alpha$  exerts a particularly strong pro-inflammatory influence, although its precise mechanisms remain unclear. TNF- $\alpha$ , is synthesized as a transmembrane protein and subsequently it is cleaved into its soluble form by the MMP TNF-converting enzyme (ADAM-17). As a pro-inflammatory cytokine, TNF- $\alpha$  contributes to colitis by binding to its receptors (TNFR1 and TNFR2) and activates the NF- $\kappa$ B signalling pathway. The downstream effects include enhanced angiogenesis, necroptosis of Paneth cells, elevated MMPs production, activation of macrophages and T cells, and damage to IECs. TNF- $\alpha$  can bind to key ECM components, including fibronectin and laminin, forming ECM-associated TNF- $\alpha$  complexes that potentiate its pro-inflammatory signaling stimulating the release of IL-1 and IL-6, as well as adhesion molecules, immune cells, and MMPs (Vaday *et al.*, 2000). This creates a self-reinforcing inflammatory loop that perpetuates tissue injury. Its profibrotic effects are largely mediated through TNFR2, which enhances fibroblast proliferation via ERK1/2 while inhibiting collagen degradation through STAT3-dependent induction of TIMP-1. Notably, probiotics have been shown to counteract TNF- $\alpha$ -induced epithelial damage (Xin *et al.*, 2024). In a recent study, *Lactobacillus acidophilus* inhibits the TNF- $\alpha$ -induced increase in intestinal epithelial TJ permeability via a inhibition of NF- $\kappa$ B activation (Haque *et al.*, 2024). Additionally, TNF- $\alpha$  upregulates the expression of TGF- $\beta$ 1 and

TIMP-1 in colonic epithelial cells. Furthermore, conditioned medium from epithelial cells treated with TNF- $\alpha$  can induce the production of MMP-9 in colonic subepithelial myofibroblasts.

➤ Tumor necrosis factor-like ligand 1A (TL1A)

TL1A and its receptor, DR3 (TNFRSF25), are key modulators of chronic intestinal inflammation, functioning as broad co-stimulatory signals for immune activation and as regulators of T-cell homeostasis, including Treg activity (Siakavellas *et al.*, 2015). Their contribution to intestinal fibrosis has recently become increasingly evident. Several immune pathways, such as Th2/IL-13 and Th17/IL-17A signalling, are reinforced through the TL1A/DR3 axis and their sustained activation can drive fibrogenic processes. TL1A is expressed by human intestinal myofibroblasts and is markedly upregulated in CD; likewise, both TL1A and DR3 are substantially overexpressed in the ileitis- and fibrosis-SAMP mouse models (Bamias *et al.*, 2006). Acting through DR3, TL1A increases cytokine secretion by T cells and can induce apoptosis in cells with high DR3 expression, earning its designation as a master cytokine in human intestinal inflammation (Twohig *et al.*, 2012). Experimental studies further demonstrate that TL1A stimulates colon fibroblast proliferation and collagen production, enhancing TGF- $\beta$ 1 expression and Smad3 phosphorylation, effects that are attenuated by pharmacological inhibition of the TGF- $\beta$ 1/Smad3 pathway (Song *et al.*, 2024).

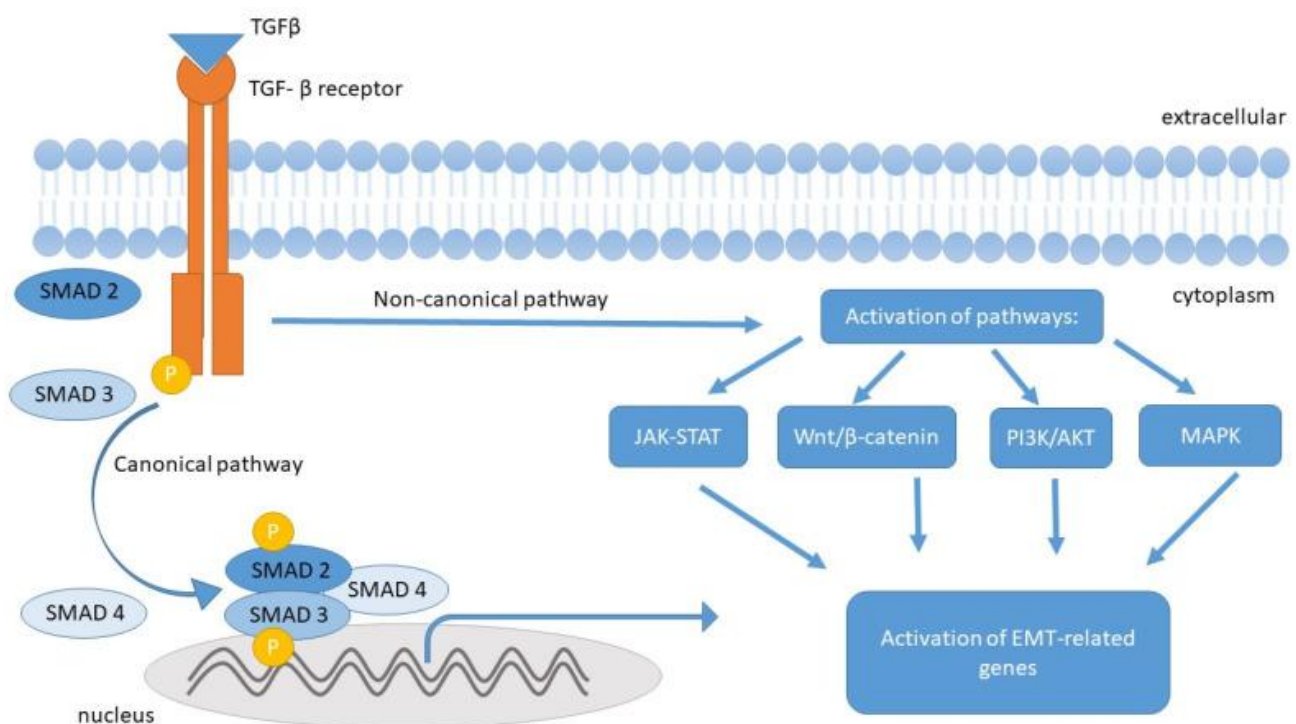
➤ Angiotensin II

Ang II, a key effector peptide of the renin-angiotensin system (RAS), is generated from angiotensin I by angiotensin-converting enzyme (ACE) and is best known for its roles in vascular tone and fluid homeostasis. In the GI tract, however, Ang II also regulates immune activity and tissue remodelling. Genetic deficiency of angiotensinogen (Atg $^{-/-}$ ) attenuates the severity of TNBS-induced colitis, highlighting a direct contribution of RAS to immune-mediated intestinal injury. Further evidence comes from human biopsy studies showing a positive correlation between mucosal fibrosis (MTC staining) and Ang II immunoreactivity, as well as *in vitro* data demonstrating that Ang II promotes myofibroblast proliferation and collagen synthesis via Angiotensin II Receptor Type 1 (AGTR1) (Garg *et al.*, 2020). Ang II and TGF- $\beta$ 1 are overexpressed in fibrostenosing CD, and Ang II enhances local TGF- $\beta$ 1 production, thereby amplifying profibrotic signalling cascades. Beyond its stromal effects, Ang II also facilitates leukocyte recruitment by inducing cytokines and chemokines such as MCP-1 in monocytes (Dai *et al.*, 2007), and promotes dendritic-cell activation (Lapteva *et al.*, 2001). Importantly, the new experimental data of Artone *et al.* demonstrate that RAS dysregulation is not merely associated with fibrosis but is functionally required for its progression. The authors show that angiotensinogen (AGT) and AGTR1 are upregulated in inflamed gut tissues from CD patients, and that Ang II directly triggers a profibrotic transcriptional program in CCD-18Co, inducing IL-13,

TGF- $\beta$ 1, COL1A1, MMP-9, insulin-like growth factor-1, and thrombospondin-2. These findings are reinforced *in vivo* using SAMP1/YitFc mice, a spontaneous model of Crohn-like ileitis and fibrosis, in which the RAS pathway becomes progressively activated as fibrosis progresses (Artone *et al.*, 2025).

### 1.2.3 TGF- $\beta$ 1 Canonical and non-canonical pathways driving intestinal fibrosis

Among fibrotic mediators, TGF- $\beta$ 1 plays a central role in driving intestinal fibrosis, it be found in inactive form as a disulfide-bonded homodimer, attached to LAP. The dissociation of the LAP and the subsequently activation of TGF- $\beta$ 1 can be brought out through several factors, including cathepsins, plasmin, calpain, thrombospondin, integrin- $\alpha$ v $\beta$ 6, and matrix metalloproteinases. Once activated, TGF- $\beta$ 1 binds its transmembrane receptors, and exerts its function through two main pathways: the canonical (Smad-dependent) pathway and the non-canonical (Smad-independent) pathway such as Wnt/ $\beta$ -catenin, PI3K-Akt, and ERK/JNK/P38 MAPK pathway (**Figure 7**). In CD, tissue TGF- $\beta$ 1 gene expression levels and phosphorylated Smad2/3 protein levels are upregulated in myofibroblasts in the strictured areas, while Smad7, a negative regulator of TGF- $\beta$ 1, is down-regulated in the same areas. TGF- $\beta$ 1 activation upregulates  $\alpha$ -SMA, collagen I, and fibronectin in human fibroblasts, SMC, and in intestinal organoid-associated mesenchymal cells (Rieder *et al.*, 2024).



**Figure 7. TGF- $\beta$ 1 main pathways** (Sputa-Grzegorzolka *et al.*, 2025)

- Canonical (Smad-dependent) pathway

The canonical TGF- $\beta$  signalling pathway is characterized by the activation of the Smad proteins, which act as transcriptional regulators. Canonical signalling is initiated when latent TGF- $\beta$ 1 is activated in the extracellular microenvironment and binds the heterotetrameric receptor complex composed of TGF- $\beta$  receptor type II (T $\beta$ RII) and type I (T $\beta$ RI/ALK5). Upon ligand binding, T $\beta$ RII recruits and phosphorylates T $\beta$ RI, enabling the propagation of intracellular signaling. Once activated, T $\beta$ RI phosphorylates the receptor-activated Smads, Smad 2 and Smad 3, which subsequently associate with the common mediator Smad4 to form a transcriptionally active complex that translocate into the nucleus. Within the nucleus, this Smad complex binds to Smad-binding elements (SBEs) on target genes and interacts with cofactors to activate transcriptional programs involved in fibrosis and primarily increased in ECM synthesis, fibroblast activation, and repression of matrix-degrading enzymes. This canonical signalling axis is tightly regulated by inhibitory Smad 7, which competes for receptor binding or recruits ubiquitin ligases to promote receptor degradation, thereby preventing excessive profibrotic signalling (Yun *et al.*, 2019); however, in intestinal fibrosis, Smad 7 levels are often inhibited (F. Chen *et al.*, 2025). Pathological conditions activate canonical TGF- $\beta$ /Smad signalling, which promotes the differentiation of resident fibroblasts into contractile myofibroblasts, which are the primary effectors of matrix deposition. TGF- $\beta$  induces the production of collagens I and III, fibronectin, and cross-linking enzymes such as Lysyl oxidase (LOX), establishing a progressively stiff and pathogenic ECM. Smad signalling also suppresses ECM degradation by TIMPs and downregulates MMPs, effectively shifting tissue homeostasis toward net accumulation of fibrotic material. Canonical TGF- $\beta$  additionally induces EMT and EndoMT in the gut, providing further sources of mesenchymal cells that amplify the fibroblast pool. EMT in IECs is driven by Smad-dependent induction of transcription factors including Snail, Slug, and ZEB1, leading to loss of epithelial markers, increased motility, decreased barrier function, and acquisition of matrix-producing properties. In addition, the TGF- $\beta$ -canonical pathway cooperates with non-canonical pathways, such as MAPK, PI3K/AKT, Rho-GTPases, and NF- $\kappa$ B, to amplify the fibroblast activation and cell survival (Giarratana *et al.*, 2024). Under normal conditions, TGF- $\beta$  maintains homeostasis and epithelial quiescence, whereas chronic inflammation shifts the TGF- $\beta$  axis toward persistent Smad2/3 activity, driving deregulated tissue remodelling. The canonical TGF- $\beta$ /Smad pathway orchestrates every stage of intestinal fibrogenesis, from initiation to progression, by activating myofibroblasts, driving EMT/EndoMT, stimulating ECM synthesis, inhibiting matrix turnover, and collaborating with secondary pathways to stabilize the fibrotic phenotype (F. Chen *et al.*, 2025).

➤ Non-canonical (Smad-independent) pathways

- Wnt/ $\beta$ -catenin pathway

In the Wnt/ $\beta$ -catenin signalling pathway, the binding of Wnt ligands to Frizzled receptors and the LRP5/6 co-receptors inhibits the  $\beta$ -catenin destruction complex, resulting in  $\beta$ -catenin stabilization, accumulation, and subsequent translocation into the nucleus. Once there,  $\beta$ -catenin associates with TCF/LEF transcription factors to regulate genes involved in fibroblast activation, EMT, and ECM production. Importantly, the Smad-dependent TGF- $\beta$  pathway and the Wnt/ $\beta$ -catenin pathway interact extensively: TGF- $\beta$ 1 can potentiate Wnt signalling by upregulating Wnt ligands or stabilizing  $\beta$ -catenin, while  $\beta$ -catenin, in turn, strengthens TGF- $\beta$ 1 signalling by enhancing Smad activity. This reciprocal reinforcement accelerates fibroblast activation, EMT, and ECM deposition, establishing a self-perpetuating fibrotic cycle within the intestinal microenvironment (Vallee *et al.*, 2017). Supporting this interplay, Ye *et al.* demonstrated that TGF- $\beta$ 1 increases  $\beta$ -catenin levels and Wnt pathway activation by inhibiting the kinase GSK3 $\beta$ , thereby promoting EMT progression (Ye *et al.*, 2013). The involvement of the Wnt/ $\beta$ -catenin axis in EMT induction is well recognized. Under homeostatic conditions, inactive  $\beta$ -catenin remains sequestered at the epithelial membrane through its association with E-cadherin, helping to maintain epithelial integrity and suppress Wnt signaling. During fibrosis, however, Wnt signalling becomes activated, leading to disruption of the  $\beta$ -catenin-E-cadherin complex. As a result,  $\beta$ -catenin becomes free to enter the nucleus, while E-cadherin undergoes degradation, thereby facilitating epithelial dedifferentiation and EMT. Notably, interventions that prevent disruption of this complex can restore epithelial characteristics, underscoring the essential role of Wnt-mediated EMT in fibrotic progression (Di Gregorio *et al.*, 2020)

- PI3K-Akt pathway

PI3K/Akt is one of the central pathways in controlling cell growth, proliferation, motility, metabolism and survival, and it has been proved to be involved in the development of lung fibrosis, cardiac fibrosis and liver fibrosis. TGF- $\beta$  was shown to activate Akt through PI3K via both Smad-dependent and Smad independent pathways. Moreover, the PI3K regulatory subunit p85 is constitutively associated with T $\beta$ RII, while its interaction with T $\beta$ RI occurs only in the presence of TGF- $\beta$ . PI3K/AKT intersects with multiple pathways, involved in autophagy, ER stress responses, and cellular senescence, shaping fibroblast fate and contributing to the chronicity of fibrotic responses. Autophagy suppression by PI3K/AKT/mTOR signaling, for example, limits ECM turnover and promotes the accumulation of dysfunctional organelles. Interestingly, targeting this pathway with inhibitors or natural products can restore autophagy and significantly interrupt its self-amplifying vicious cycle, offering therapeutic potential for alleviating fibrosis (Fang *et al.*, 2025).

Recently, RNA-seq analysis identified the PI3K/AKT pathway as a key mediator of amphiregulin (AREG)-induced fibrosis, both *in vitro* and *in vivo*. The inhibition of the PI3K/AKT pathway effectively suppressed AREG-induced activation and proliferation of isolated human intestinal fibroblasts and attenuated colitis-associated fibrosis in mice (S. Wang *et al.*, 2025).

- ERK/JNK/p38 MAPK pathway

TGF- $\beta$  triggers also the mitogen-activated protein kinase (MAPK) pathway, and once activated, regulates a wide range of biological processes, including cell survival, proliferation, differentiation, adhesion, migration, and metabolism (Z. Deng *et al.*, 2024). There are three main downstream pathways of MAPK, which include ERK, JNK, and p38/MAPK. Specifically, when TGF- $\beta$ 1 binds its receptors, the tyrosine kinase activity of T $\beta$ RI phosphorylates other molecules leading to SHCA-GRB2-SOS complex that triggers the canonical MAPK signaling cascade, which progresses through the activation of Ras/Raf proteins. Furthermore, TGF- $\beta$  can activate the signalling of p38 and JNK through a receptor kinase-independent mechanism. The TGF- $\beta$ -activated T $\beta$ R complex recruits TRAF4 and TRAF6, triggering their K63-linked polyubiquitination. Polyubiquitinated TRAF activates TAK1, a MAPKKK, which then phosphorylates MAPKKs. These MAPKKs activate p38 and JNK, with MKK3 and MKK6 specifically targeting p38, while MKK4 activates both p38 and JNK. Additionally, TGF- $\beta$ -activated Rho GTPases, such as RHOA, RAC1, and CDC42, contribute to p38 and JNK activation (Y. Zhang *et al.*, 2025). Elevated activation of JNK, along with ERK and p38, has been revealed in colonic tissue from 27 patients with moderate to severe CD or UC (Waetzig *et al.*, 2002).

- PPAR- $\gamma$

PPAR- $\gamma$ , a transcription factor within the nuclear hormone receptor superfamily, is increasingly recognized as a central molecular key in the fibrosis process. PPAR- $\gamma$  forms heterodimers with the retinoid X receptor to bind specific DNA sequences known as PPAR response elements (PPREs) in various cell types, including adipose tissue, muscles, brain, and the immune system. PPAR- $\gamma$  is also expressed in fibroblasts, where it regulates numerous target genes and influences connective tissue dynamics, mesenchymal cell activation, differentiation, and survival serving as a link between cellular metabolism and fibrogenesis. Dysregulated levels of PPAR- $\gamma$  are associated with several pathological conditions, such as obesity, cancer, diabetes, atherosclerosis, and multiple sclerosis. PPAR- $\gamma$  helps to mitigate fibrosis by counteracting TGF- $\beta$ 1 activity. Agonists of PPAR- $\gamma$  are known to reduce TGF- $\beta$ -induced collagen deposition and inhibit myofibroblast differentiation (Burgess *et al.*, 2005). The expression of PPAR- $\gamma$  is suppressed by various cytokines, chemokines, and intracellular signalling pathways, including TGF- $\beta$ 1, the canonical WNT/ $\beta$ -catenin pathway, TNF- $\alpha$ ,

and hypoxia. Conversely, transcription factors such as C/EBP, ELF proteins, and nuclear factor erythroid 2-related factor 2 (Nrf2), associated with NF-E2, are known to positively regulate PPAR- $\gamma$  expression (Abdelkarim *et al.*, 2010). PPAR- $\gamma$  is highly expressed in the colonic epithelium and plays a dual role in modulating inflammation and restraining fibrogenesis. Earlier foundational work demonstrated that activation of PPAR- $\gamma$  suppresses pro-inflammatory cytokines and interferes with pro-fibrotic mediators such as TGF- $\beta$ . A recent multi-omic analysis revealed that PPAR- $\gamma$  agonists not only downregulate EMT-related pathways but also suppress AGE/RAGE signalling and cellular senescence programs, both of which are strongly associated with ECM accumulation and fibroblast activation in IBD. Moreover, treatment with the PPAR- $\gamma$  agonist (GED 0507-34 Levo) attenuated all major pro-fibrotic pathways in DSS-induced colitis (Pompili *et al.*, 2023). Recently, the role of PPAR- $\gamma$  has been highlighted as a key regulator of macrophage polarization, epithelial barrier homeostasis, and T cell differentiation, processes intimately linked to fibrogenesis, as chronic metabolic stress and loss of barrier integrity are known amplifiers of fibroblast activation in CD. These findings are consistent with clinical observations showing impaired epithelial expression of PPAR- $\gamma$  in UC and experimental evidence that its pharmacological activation reduces mucosal injury (Bashiri *et al.*, 2026)

#### **1.2.4 Pathogenetic factors involved in intestinal fibrosis**

##### **Gut microbiota**

An expanding body of research supports the idea that the gut microbiota is a central regulator of intestinal fibrogenesis, with microbial signals acting as environmental risk factors independently of active inflammation, pointing toward a model in which dysbiosis can perpetuate ECM deposition through persistent activation of fibroblasts. Luminal contents, including bacteria, their metabolites, and cell wall components, interact directly with the intestinal stroma and immune system, accelerating stricture formation in both CD and UC. Microbiome influences fibrogenesis, drives EMT modulating cytokine networks and generating metabolites capable of reprogramming fibroblast behaviour; notably, some of these pathways are inflammation-independent, suggesting that fibrosis can persist even when mucosal inflammation is controlled pharmacologically. Complementary evidence from recent mechanistic reviews demonstrates that dysbiosis exacerbates barrier dysfunction and activates TGF- $\beta$ 1/WNT/ $\beta$ -catenin pathways, while the depletion of SCFA-producing commensals reduces anti-fibrotic metabolic signals. Multi-omics profiling of CD patients, by integrating microbiome, fecal/blood metabolomics, and MRI-based fibrosis assessment, reveals links between microbial metabolites and fibrosis-related structural alterations. Particularly, L-aspartic acid directly enhanced fibrotic activation in human intestinal myofibroblasts *in vitro* and exacerbated fibrosis *in vivo* in

TNBS-induced colitis models. These underscore the increasingly recognized influence of the gut microbiome in IBD-associated fibrosis and may actively contribute to fibrogenesis in CD. (X. Li *et al.*, 2024).

### **Exposome**

In addition to all environmental factors listed as risk factors in IBD, a subset referred to as the “exposome”, including chemical pollutants, dietary patterns, airborne toxicants, and tobacco smoke, has been increasingly linked not only to IBD but also to intestinal fibrosis. A growing body of evidence identifies the aryl hydrocarbon receptor (AhR) as a pivotal molecular node linking exposome to fibrotic outcomes. AhR is a highly conserved ligand-activated transcription factor expressed in immune, stromal, and epithelial cells, where it acts as a conserved environmental sensor governing responses to dietary metabolites, pollutants, and smoke-derived ligands. AhR activation induces effector cytokines such as IL-22 and TGF- $\beta$  and engages NF- $\kappa$ B signalling pathways, together contributing to epithelial repair, immune responses, and fibrogenesis. Dysregulated or diminished AhR activation in the inflamed gut has been observed in IBD, suggesting that impaired AhR signaling may exacerbate chronic inflammation and tilt mucosal repair toward pathological extracellular matrix deposition. Evidence demonstrates that exposure to environmental factors can activate an AhR-NF- $\kappa$ B-C/EBP $\beta$  axis in dendritic cells and T cells, driving intestinal inflammation through direct environmentally mediated transcriptional reprogramming. These findings support the hypothesis that the exposome may influence fibrogenesis by modulating immune responses, stromal activation, and epithelial barrier repair via AhR-dependent mechanisms (Coumoul *et al.*, 2026; Stockinger *et al.*, 2021).

### **Creeping fat**

The expansion of mesenteric adipose that envelops inflamed intestinal tissue segments is a hallmark of CD and it's known as “creeping fat”. Beyond its recognized structural presence, recent studies have highlighted its active immunometabolic functions and its contribution to fibrogenesis. Creeping fat is metabolically active and enriched in pro-inflammatory cytokines, including high levels of TNF. The specific free fatty acids (FFAs) released from creeping fat and their effects on neighbouring tissues have not yet been clarified. Single-cell profiling has revealed that creeping fat contains preadipocytes that are highly predisposed to adipogenic differentiation and become specifically activated during inflammation, displaying elevated cytokine signalling and strong pro-fibrotic behaviour. (Hwang *et al.*, 2023). Newer mechanistic findings further demonstrate that creeping fat harbours metabolically reprogrammed macrophages driven by microbiota-regulated metabolites, which promote adipogenesis and immune activation within the mesentery. Moreover, microbial translocation into

mesenteric fat has been shown to trigger macrophage responses, wound-healing programs, and fibroblast activation, reshaping creeping fat into a driver of inflammation and fibrosis rather than a bystander tissue (Kredel *et al.*, 2013). More recent work has identified creeping-fat-derived mechanosensitive CTHRC1<sup>+</sup> fibroblasts as direct contributors to intestinal fibrosis and stricture formation through YAP/TAZ-dependent pathways, positioning creeping fat as a central source of pro-fibrotic cells in CD strictures (Bauer-Rowe *et al.*, 2025). In addition, new research suggests that FFAs directly stimulate muscle proliferation and contribute to thickness of the muscularis propria. In a transgenic loss-of-fat model, Liu *et al.* showed that removing mesenteric adipocytes prevents inflammation-induced thickening of the muscularis propria, indicating that adipocytes are essential for this proliferative response. Lipidomic, also, revealed that creeping fat releases several FFAs, especially palmitate, suggesting active lipolysis driven by inflammatory mediators such as TNF. These findings align with recent lipidomic studies showing abnormal lipid metabolism in creeping fat from CD patients (Liu *et al.*, 2025)

### **Matrix stiffness**

The abnormal fibrotic accumulation of ECM has been traditionally considered a consequence of long-term stimulation of local mesenchymal cells by a chronic immune response, but emerging evidence suggests that the ECM itself functionally contributes to the progression of fibrosis. A change in tensile forces and tissue elasticity, such as stiffness and fiber alignment, also significantly influences ECM deposition. A rigid ECM enhances fibroblast activation and promotes further collagen deposition, creating a self-reinforcing loop of fibrosis. Mechanical signals transmitted through integrins activate FAK and ERK pathways, ultimately driving epithelial cell proliferation. Additionally, shear stress and mechanical stretching can induce cyclooxygenase-2 and pro-inflammatory mediators such as IL-6, IL-8, and monocyte chemoattractant protein-1 (Pompili *et al.*, 2021).

### **1.2.5 Current therapies for intestinal fibrosis**

In the past 20 years, the availability of biologic drugs and advanced small molecules has increased dramatically improving outcomes and quality of life of patients with CD. However, the need for surgery has decreased only modestly, indicating that current therapies are either ineffective or insufficiently effective in preventing fibrotic complications (Jeuring *et al.*, 2017). To date, no approved anti-fibrotic therapy exists. Although some anti-inflammatory agents have shown benefit in patients with CD's-related strictures, adalimumab remains the only agent with prospective evidence of significant improvement in obstructive symptoms (Bouhnik *et al.*, 2023). For example, 5-ASA has been used for more than fifty years as effective and safe for inducing and maintaining clinical and endoscopic remission in ulcerative colitis. However, evidence supporting its antifibrotic activity

remains limited, and ECCO guidelines do not recommend its use either for the treatment of active disease or for maintenance therapy in CD (H. Gordon *et al.*, 2024). Immunosuppressants remain a mainstay for chronic inflammatory diseases, including IBD, but have not been shown to reduce the risk of intestinal fibrosis or the need for surgical resections. Corticosteroids, such as prednisone, methylprednisolone, and budesonide, are effective for inducing clinical remission in moderate to severe CD and UC (Quera *et al.*, 2023), though they are not recommended for long-term maintenance. Patients who are steroid-dependent or refractory often require biological therapy. Currently used biologics target three major pathways: TNF- $\alpha$ , integrins (particularly  $\alpha 4\beta 7$ ), and IL-12/23. Anti-TNF- $\alpha$  agents have demonstrated robust efficacy, with studies showing that approximately two-thirds of stenotic CD patients can avoid abdominal surgery (Allocca *et al.*, 2017). Recent preclinical studies have identified promising anti-fibrotic targets. One approach focuses on modulating the balance between TIMPs and MMPs, regulators of ECM turnover. Thalidomide, emerging as a treatment for refractory CD, has been shown to restore this balance and reduce intestinal fibrosis in animal models (Flier *et al.*, 2010). Recombinant human BMP-7, which counteracts TGF- $\beta 1$ 's pro-fibrotic activity, has shown efficacy in animal studies by inhibiting EMT and ameliorating intestinal fibrosis (Santacrose *et al.*, 2022). One of the most promising avenues for anti-fibrotic therapy remains the targeting of TGF- $\beta$  and its signalling cascades. TGF- $\beta 1$  production is strongly stimulated by angiotensin II (ANG II), which is upregulated in the colonic mucosa of CD patients. As part of the renin-angiotensin system (RAS), ANG II makes RAS inhibitors, ACE inhibitors (ACEi) and angiotensin II receptor blockers (ARBs), attractive candidates for anti-fibrotic therapy. Preclinical and clinical studies have shown that ACEi (X. Zhang *et al.*, 2022) and ARBs can reduce fibrosis progression in liver (Gu *et al.*, 2023) and lung tissue (Amirkhosravi *et al.*, 2024). In models of intestinal fibrosis, inhibition of AGTR1, the final effector of the RAS, as well as of its upstream regulator ACE, has yielded largely positive results (Okawada *et al.*, 2011; Wengrower *et al.*, 2012). Recently, findings by Artone *et al.* have substantiated the activation of the RAS in inflamed intestinal regions of patients with CD and demonstrated that losartan exerts significant therapeutic effects in attenuating intestinal inflammation and fibrosis in both *in vitro* and *in vivo* settings, also showing a marked antifibrotic effect in an experimental model of Crohn's-like ileitis (Artone *et al.*, 2025). Despite significant progress in understanding intestinal fibrogenesis, clinical trials evaluating anti-fibrotic therapies have only recently begun (NCT 05843578) testing AGMB-129, a potent blocker of TGF $\beta$  signalling (J. Wang *et al.*, 2024).

### 1.2.6 Probiotics approach in intestinal fibrosis

Probiotics have been studied for their ability to modulate the microbiota, reduce inflammation, and suppress fibrotic signalling pathways. In 2001, an expert consultation of international scientists from

the Food and Agriculture Organization of the United Nations (FAO) and the WHO provided a definition of probiotics, revisited in 2014 by Hill *et al.*: probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (Hill *et al.*, 2014). The term probiotic derived from two words “pro” and “bios”, from the latin preposition “pro,” and the greek word “βιωτικός-biotikos”, meaning “life” microorganisms. One of the earliest studies investigating the immunomodulatory potential of probiotics in IBD demonstrated that, in patients with CD and UC, consuming yogurt supplemented with *L. rhamnosus* GR-1 and *L. reuteri* RC-14 for one month induced anti-inflammatory effects. The probiotic intake could expand the peripheral pool of CD4<sup>+</sup>CD25<sup>high</sup> Treg cells, while simultaneously reducing TNF- $\alpha$  and IL-12 production by monocytes and dendritic cells. The same results were not observed when patients consumed yogurt without probiotic supplementation (Lorea Baroja *et al.*, 2007). 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. Probiotic administration significantly reduced the levels of pro-inflammatory factors, IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , in serum and intestinal tissue, and markedly downregulated the inflammatory (TLR4/NF- $\kappa$ B) and fibrotic (TGF- $\beta$ 1/Smad) signaling pathways in intestinal tissue (X. Deng *et al.*, 2020). Han *et al.* show that the probiotic *Lactobacillus acidophilus* (LA85) in a DSS-induced mouse model of UC significantly reduced disease severity, improving weight loss, colon length, and histological damage compared with untreated colitic mice. It also modulated systemic inflammation by lowering TNF- $\alpha$  and IL-6 while partially restoring IL-10 levels. The results suggested that *L. acidophilus* restored the microbiota, decreased levels of inflammatory cytokines, and supported epithelial integrity (Han *et al.*, 2023). In any case unfortunately few are the studies that focus mainly on intestinal fibrosis among these we include the following: a study by Lombardi *et al.* demonstrated that a soluble lysate from the high-concentration multi-strain probiotic Vivomixx<sup>®</sup> formulation inhibited TGF- $\beta$ 1-induced fibrosis in CCD-18Co (Lombardi *et al.*, 2021). In a 2022 study, Vetuschi *et al.* observed anti-inflammatory and antifibrotic effects in a mouse model of chronic DSS-induced colitis maintained with a diet enriched with biologically fermented olive cream and the specific probiotic strain *Lactiplantibacillus plantarum* IMC513. The results showed a significant reduction in macro- and microscopic colitis scores and a marked decrease in the expression of inflammatory cytokines and profibrotic molecules, such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , TGF- $\beta$ 1, p-SMAD3, PI3K, p-Akt,  $\alpha$ -SMA, and collagens I-III, compared with the untreated and single-stimulus-treated groups (Vetuschi *et al.*, 2022).

## 2. AIM OF THE STUDY

Intestinal fibrosis, known as a common complication of inflammatory bowel disease (IBD), principally Crohn's disease (CD) and ulcerative colitis (UC), develops in response to chronic tissue injury caused by persistent inflammation, and is characterized by myofibroblast activation and uncontrolled and excessive deposition of extracellular matrix (ECM). Intestinal fibrosis occurs in over 50% of patients with CD, constantly leading to stenosis and fistulae, often requiring invasive interventions such as surgical resection or balloon dilation. Even though significant progress has been made in understanding the mechanisms underlying IBD-associated fibrosis, the development of effective anti-fibrotic therapies remains an open challenge, as current treatments provide only partial relief. Interestingly, the emerging role of microbiota in IBD-associated fibrosis suggests that microbiota-targeted interventions, such as probiotic use, may offer promising antifibrotic strategies. To date, few studies have directly investigated the impact of probiotic strains or their metabolites on fibrotic signalling pathways. *OxxySlab<sup>TM</sup>*, a formulation composed by eight different probiotic strains, has shown promising results in other inflammation-related diseases and in many pathways associated with the onset of fibrosis. Then, the purpose of this work was to evaluate the anti-fibrotic effects of the lysate from this probiotic formulation, focusing on the modulation of intestinal fibrosis and epithelial-to-mesenchymal transition (EMT) in *in vitro* models. To do this aim, we use a broadly recognized *in vitro* model of differentiation of intestinal fibroblasts to activated myofibroblasts, obtained by stimulating human fibroblasts (CCD-18Co) with TGF- $\beta$ 1, a key cytokine in fibrogenesis. In this model, we evaluate the probiotic lysate effect on the expression of the main markers associated with TGF- $\beta$ -induced fibrotic activation such as  $\alpha$ -SMA, collagen I, and fibronectin. Furthermore, we evaluate the probiotic modulation of key molecular mediators of TGF- $\beta$  signaling pathways, such as p-Smad2/3, p-Akt, PPAR- $\gamma$ , and  $\beta$ -catenin, and the probiotic impact on the TGF- $\beta$ 1 gene expression. Similarly, we use a well-known EMT *in vitro* model, employing differentiated epithelial intestinal cells (Caco-2-IECs) stimulated with TGF- $\beta$ 1, to evaluate the effect of the probiotic lysate on the modulation of the main EMT-associated markers, like e-cadherin, occludin and  $\alpha$ -SMA and the interference of the probiotic stimuli with the  $\beta$ -catenin activation.

### 3. MATERIALS AND METHODS

#### 3.1 Cell Lines

##### ➤ CCD-18Co

Human intestinal CCD-18Co fibroblast cell line (CRL-1459) was purchased from American Type Culture Collection (ATCC, Georgetown, DC, USA) and then cultured in Dulbecco's modified Eagle's medium (DMEM) added with 10% of Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM glutamine. Trypsin solution from bovine pancreas (Euro Clone, West York, UK), was used to detach the cells after reaching 80% confluence, and sub-cultured at 7000 cells/cm<sup>2</sup> in a sterile tissue culture flask for the subsequent experiments.

##### ➤ IEC-Caco-2

The Caco-2 cell line obtained from Sigma-Aldrich (St. Louis, MO, USA) was cultured in DMEM, supplemented with 10% of FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 1% non-essential amino acids, and medium was changed every two days. After reaching 80% confluence, cells were detached with trypsin and seeded at 60,000 cells/cm<sup>2</sup> into sterile tissue culture 12-well plates (Becton, San Jose, CA, USA) for differentiation and experimental conditions.

Both cell lines were cultured in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C.

#### 3.2. Preparation of Bacterial Lysate

The *OxySlab*<sup>™</sup> multi-strain probiotic formulation (EOS2021 Srl, Ardea, Rome, Italy) is composed by eight lyophilized bacterial strains of lactic acid bacteria and bifidobacteria as follows: *Streptococcus thermophilus* CNCM I-5570, *Lactobacillus brevis* CNCM I-5566, *Bifidobacterium animalis* subsp. *Lactis* CNCM I-5571, *Bifidobacterium animalis* subsp. *Lactis* CNCM I-5572, *Lactobacillus plantarum* CNCM I-5569, *Lactobacillus paracasei* CNCM I-5568, *Lactobacillus acidophilus* CNCM I-5567, *Lactobacillus helveticus* CNCM I-5573. The preparation of the probiotic lysate was obtained following the protocol described by Lombardi *et al.* (Lombardi *et al.*, 2021). Probiotic formulation in lyophilized form (133 × 10<sup>9</sup> CFU) was resuspended in 10 mL of cool phosphate-buffered saline (PBS, EuroClone, West York, UK), centrifuged at 8600× g, and then washed twice with cool PBS. To breakdown the bacterial cells, the resuspension was sonicated using a Vibracell sonicator (Sonic and Materials, Danbury, CT, USA) for 30 min, with 10 s of sonication followed by 10 s of pause, and keeping the sample immersed in wet ice. Before and after each sonication step, the absorbance of the resuspension was measured at 590 nm with a spectrophotometer (Eppendorf, Hamburg, Germany) to assess bacterial cell breakdown. Then, the samples were centrifuged at 17,949× g, and the supernatants were filtered through a 0.22 µm-pore filter (Corning

Incorporated, Somerville, MA, USA) to eliminate any remaining bacteria. Total protein content was quantified using a *DC* protein assay (Bio-Rad Laboratories, Hercules, CA, USA), with bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) resuspended in PBS to establish a standard curve. The probiotic lysate was then aliquoted and stored at -80°C until treatments.

### **3.3 *In vitro* models and treatments**

#### **3.3.1 Intestinal Fibrosis Experimental Design**

CCD-18Co cells were plated at 5000 cells/cm<sup>2</sup> to allow the attachment in a 25-cm<sup>2</sup> tissue culture flask, the next days the complete medium was replaced with serum-deprived medium for 24 h, then the fibroblasts were stimulated with 10 ng/mL of TGF-β1 (hTGF-β1; Cell Signaling Technology, Danvers, MA, USA) for 48 h, to induce the fibrotic phenotype, in the presence or absence of probiotic formulation lysate at different concentrations.

#### **3.3.2 Epithelial to mesenchymal transition (EMT) experimental design**

Caco-2 cells were seeded at 60,000 cells/cm<sup>2</sup> and cultured in sterile tissue culture 12-well plates to allow the cells to spontaneously differentiate, express polarized columnar epithelium and functional characteristics of the intestinal epithelial cells (IECs). After fourteen days post-confluence, Caco-2 IECs were treated for 96 h with TGF-β1 (20 ng/mL) in the serum-free medium with or without the multi-strain probiotic lysate at different concentrations. In all the experiments, untreated cells were used as “Control”.

### **3.4 Assessment of cell viability and proliferation**

#### **3.3.1. Cell Proliferation Assay of CCD-18Co**

Cell Counting Kit-8 (CCK-8) assay was used to assess the cell proliferation of CCD-18Co cells, acquired from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), following to the manufacturer's instructions. The CCD-18Co cells were added in 96-well-plated with different concentrations of the probiotic lysate: *OxySlab* (25–50–100 µg protein/mL) with or without stimulation by the TGF-β1. After treatment, 10 µL of CCK-8 assay reagent was added to each well at time points of 0, 24, and 48 h. The optical density (OD) values were measured using a spectrophotometer (BioRad, Hercules, CA, USA) at 450 nm.

#### **3.3.2. Cell Viability Assay of Caco-2 IECs**

Cell viability for the Caco-2 IECs was assessed with the 3-(4,5-diphenyl tetrazolium bromide (MTT) assay (Immunological Sciences, Rome, Italy). Caco-2 cell line was seeded into sterile tissue culture

96-well plates at 60,000 cells/cm<sup>2</sup>, to allow the differentiation of the cells as described before. Fourteen days post-confluence, the 96-well-plated Caco-2 IECs were incubated with different concentrations of probiotic lysate (10–25–50–100–200 µg protein/mL). After incubation, the MTT assay was performed at different times, 0, 24, 48, 72, and 96 h, and the absorbance signals were measured using a spectrophotometer at 570 nm and the background at 630 nm. The background absorbances have been subtracted from the signal absorbance to obtain normalized absorbance values.

### 3.5 Western blot analysis

Western blot analyses was performed at the end of the treatment, the cells were washed in PBS, harvested and lysed using a cell scraper in RIPA buffer (Merck KGaA, Darmstadt, Germany) containing a protease inhibitor mixture (Merck KGaA, Darmstadt, Germany). The quantification of protein contained in every sample was then performed by DC Protein Assay using BSA in RIPA buffer as a standard. Subsequently, the 25 µg of protein, prepared using the blue sample buffer, were boiled (5 min, 100 °C) and then subjected to electrophoresis using a 10% SDS-polyacrylamide gel. The resulted gel with the separated proteins were transferred onto a 0.45 µm nitrocellulose membrane sheet for 1 h at 4 °C at 70 V, using a Mini Trans-Blot Cell apparatus (BioRad, Hercules, CA, USA). Blocking of membranes was performed by dipping the membrane in 5% non-fat dry milk or 5% BSA for 2 h at room temperature. Overnight incubation was performed at 4 °C with several antibodies reported in **Table 1**. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and HRP-conjugated rabbit anti-mouse were used as secondary antibodies (Millipore EMD, Darmstadt, Germany). Protein immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Amersham, UK) system). Densitometric analyses of the immunoreactive bands were performed using the ALLIANCE chemiluminescence documentation system (UVITEC, Cambridge, UK) and normalized to the corresponding GAPDH or β-tubulin bands.

| Primary Antibody                       | Dilution | Company                                           |
|----------------------------------------|----------|---------------------------------------------------|
| rabbit polyclonal anti-COL1A1          | 1:1000   | Boster Biological Technology, Pleasanton, CA, USA |
| rabbit monoclonal anti-fibronectin     | 1:1000   | Cell Signaling Technology, Danvers, MA, USA       |
| rabbit monoclonal anti-p-SMAD2/SMAD3   | 1:1000   | Cell Signaling Technology, Danvers, MA, USA       |
| mouse monoclonal anti-α-SMA            | 1:1000   | OriGene, Rockville, MA, USA                       |
| rabbit monoclonal Phospho-Akt (Ser473) | 1:1000   | Cell Signaling Technology, Danvers, MA, USA       |
| rabbit polyclonal anti-Akt             | 1:1000   | Cell Signaling Technology, Danvers, MA, USA       |
| rabbit polyclonal anti-β-catenin       | 1:1000   | Cell Signaling Technology, Danvers, MA, USA       |
| rabbit monoclonal anti-PPAR-γ          | 1:1000   | Cell Signaling Technology, Danvers, MA, USA       |
| rabbit Polyclonal anti-occludin        | 1:1000   | OriGene, Rockville, MA, USA                       |
| mouse monoclonal anti-E-cadherin       | 1:1000   | Cell Signaling Technology, Danvers, MA, USA       |

|                                         |        |                                           |
|-----------------------------------------|--------|-------------------------------------------|
| mouse monoclonal anti-GAPDH             | 1:1000 | OriGene, Rockville, MA, USA               |
| mouse monoclonal anti- $\beta$ -tubulin | 1:1000 | Thermo Fisher Scientific, Boston, MA, USA |

**Table 1.** List of the primary antibodies used for Western blot.

### 3.6 Immunofluorescent staining

To assess an immunostaining after treatments of CCD-18Co and Caco-2 IEC, cells grown on coverslips in a 12-well plate (seeded at 5000 cells/cm<sup>2</sup> and 60000 cells/cm<sup>2</sup>, respectively) and stimulated as previously described. At the end of the respective treatments, the coverslips were then washed with PBS, fixed with 4% formaldehyde for 20 min, and permeabilized with 0.1% triton X-100 (Sigma-Aldrich) for 5 min, except for the evaluation of the occludin and e-cadherin that are surface proteins and therefore do not require permeabilization. Subsequently, the coverslips were blocked with 3% BSA in PBS for 20 minutes at room temperature. Then, cells were maintained overnight at 4 °C with specific primary antibodies (**Table 2**). Therefore, staining was performed using FITC conjugated goat anti-mouse secondary antibody (Bethyl Laboratories, Inc, Montgomery, TX, USA) or FITC conjugated goat anti-rabbit secondary antibody (Millipore) at 1:1000 dilution, for 1 h at room temperature. CCD-18Co were also incubated with TRITC labelled phalloidin (Sigma-Aldrich) for 45 min at room temperature. Then, the coverslips were mounted with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) and examined at 40x magnification with a fluorescent microscope (Eclipse Ts2R-FL, Nikon Corporation, Tokyo, Japan).

| Primary Antibody                     | Dilution | Company                                           |
|--------------------------------------|----------|---------------------------------------------------|
| rabbit polyclonal anti-COL1A1        | 1:250    | Boster Biological Technology, Pleasanton, CA, USA |
| rabbit monoclonal anti-fibronectin   | 1:200    | Cell Signaling Technology, Danvers, MA, USA       |
| mouse monoclonal anti- $\alpha$ -SMA | 1:250    | OriGene, Rockville, MA, USA                       |
| rabbit Polyclonal anti-occludin      | 1:50     | OriGene, Rockville, MA, USA                       |
| mouse monoclonal anti-E-cadherin     | 1:500    | Cell Signaling Technology, Danvers, MA, USA       |

**Table 2.** List of the primary antibodies used for immunofluorescent staining.

### 3.7 Total RNA extraction and quantitative Real-Time PCR (qPCR)

To evaluate the difference of TGF- $\beta$ 1 gene expression between untreated (control) and probiotic-treated CCD-18Co, Real-time RT-PCR was carried out using a ViiA7 sequence detection system (Applied Biosystems, Foster City, CA, USA). The total RNA from the cells was extracted with the RNeasy Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions and quantified by spectrophotometry: 1  $\mu$ g of total RNA was reverse transcribed in a final volume of 20

μL using a mixture of primers. 0.5 μg of each cDNA was used to perform real-time PCR. Real-time quantitative RT-PCR analysis was carried out by SYBR Green dye detection (Thermo Fisher Scientific Waltham, MA, USA). Reverse and forward primers, acquired from IDT (acquired from Integrated DNA Technologies, IDT, Coralville, IA, USA), were used at a concentration of 1 μM (hTGF-β1), and their sequences were as follows: hTGF-β1 forward 50-CAACGAAATCTATGACAAGTTCAAGCAG-30 and reverse 50-CTTCTCGGAGCTCTGATGTG-30. The mRNA level of GAPDH was used as internal control for the normalization with subsequent primers: GAPDH forward 50-TTGCCCTCAACGACCACTTT-30 and reverse 50-TGGTCCAGGGGTCTTACTCC-30. The fold-change quantification in target genes was calculated with the  $2^{-DDCt}$  method.

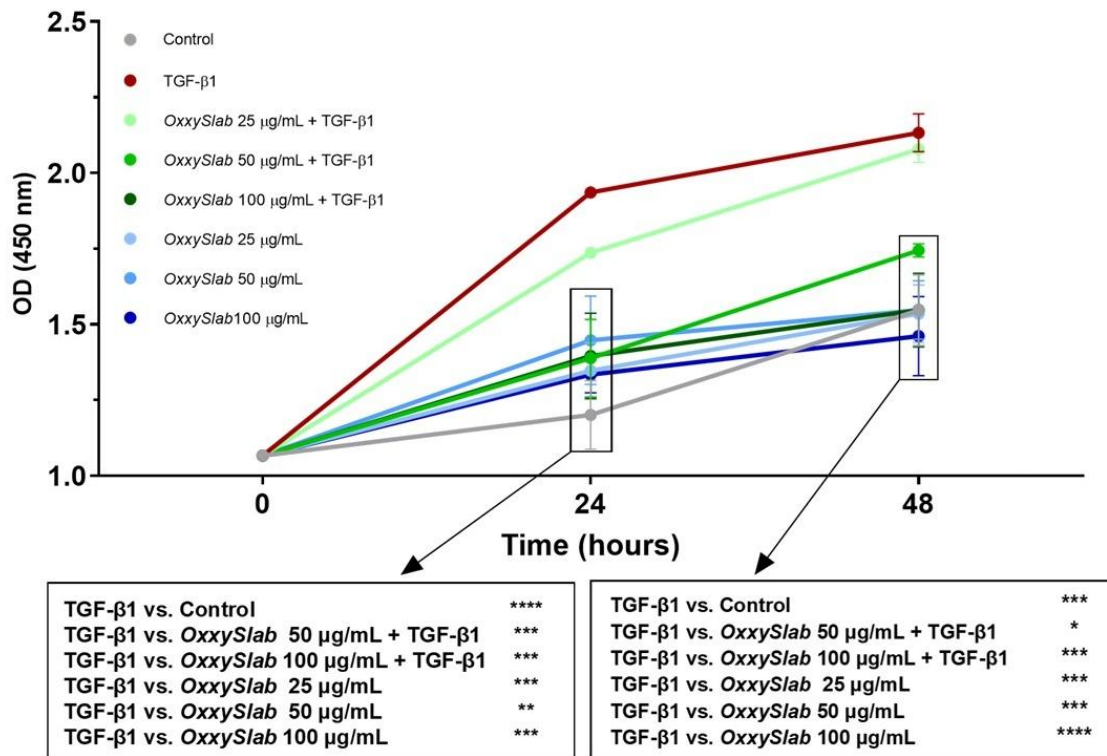
### 3.8 Statistical analysis

All data were statistically evaluated by GraphPad Prism (version 8.02, GraphPad Software, San Diego, CA, USA). One-way ANOVA or two-way ANOVA and Dunnett post hoc test, were used. Data were expressed as mean ± SD or mean ± SEM. The *p* values were considered statistically significant when lower than 0.05.

## 4. RESULTS

### 4.1 Multi-strain probiotic lysate decreased proliferation in CCD-18Co

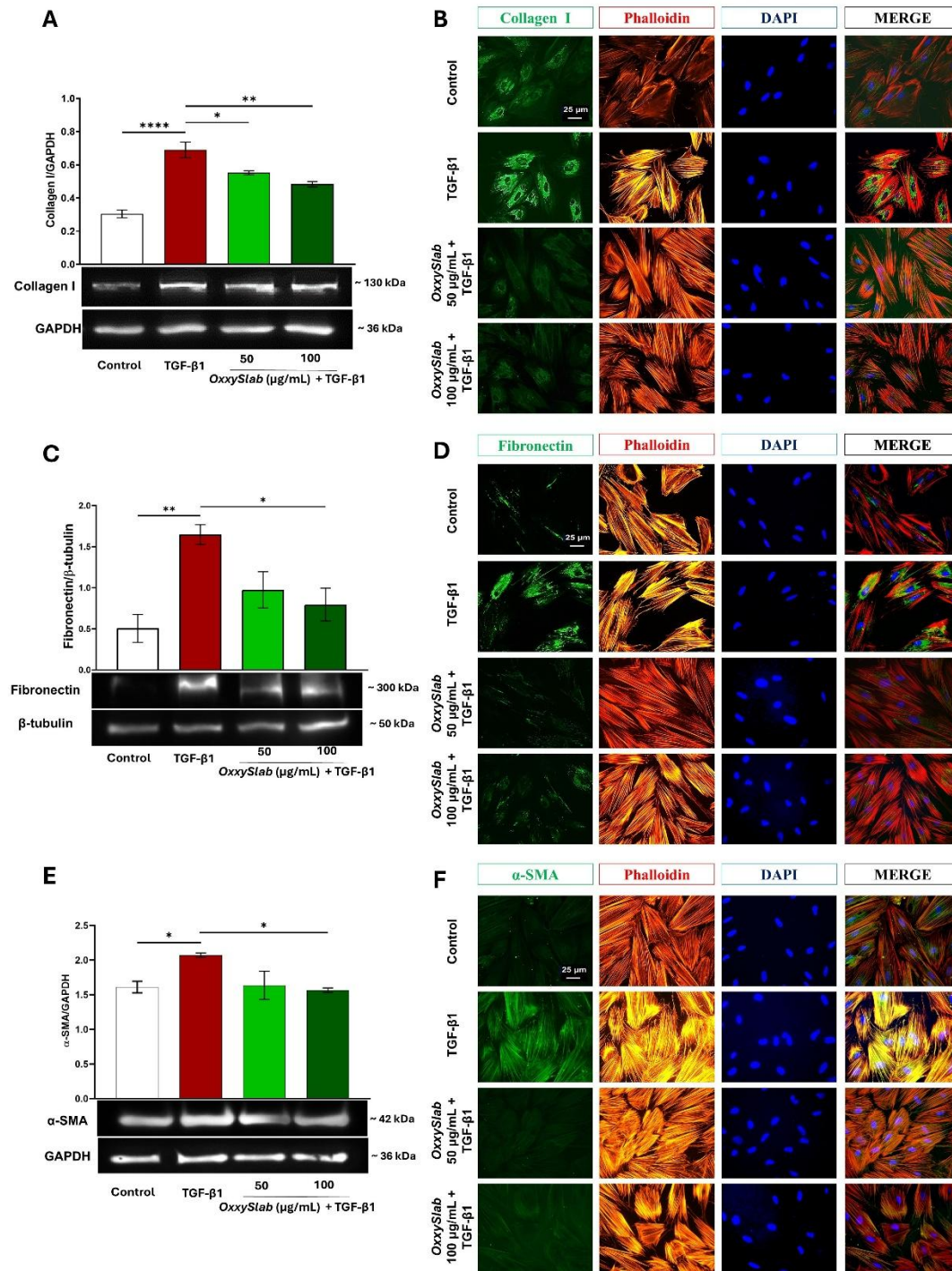
Before investigating the antifibrotic properties of the multi-strain probiotic formulation lysate, *OxxySlab*, a proliferation assay was assessed. The proliferative response of CCD-18Co fibroblasts after stimulation with TGF-β1 (10 ng/mL) in the presence or absence of increasing concentrations of probiotic lysate (25, 50, and 100 μg protein/mL) over a timeline of 48 h. Cell proliferation was quantified using the CCK-8 assay at 0, 24, and 48 h, with duplicate measures at each time point. (**Figure 8**). TGF-β1 exposure significantly enhanced fibroblast proliferation compared with untreated controls, confirming its profibrotic activity. Co-treatment with the probiotic lysate at 50 and 100 μg/mL significantly attenuated this proliferative response, restoring proliferation levels comparable to baseline conditions. Importantly, the probiotic lysate alone did not alter basal proliferative rates, indicating that its effects were specifically related to the TGF-β1-induced activation state. Given the minimal impact of the 25 μg/mL concentration on TGF-β1-induced proliferation, this concentration was not included in subsequent experiments.



**Figure 8.** *OxySlab* lysate effect on TGF-β1-induced proliferation of CCD-18Co. CCK-8 proliferation assay analysis on CCD-18Co stimulated with TGF-β1 alone or in combination with *OxySlab* lysate at the following protein concentration 25, 50, and 100 µg/mL for 48 h, after 24 h of starvation. Data are acquired by two independent experiments performed in triplicate are expressed as mean ± SEM and analyzed by two-way ANOVA. All data were compared with the TGF-β1 condition (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.0001$ , \*\*\*\*  $p < 0.0001$ ).

## 4.2 *OxySlab* Lysate impact on fibrotic marker expression on TGF-β1-stimulated CCD-18Co.

Fibroblast activation status is a key event in intestinal fibrogenesis, characterized by cytoskeletal remodelling and upregulation of fibrotic markers, including collagen type I, fibronectin, and  $\alpha$ -SMA. To evaluate the impact of probiotic lysate treatment, CCD-18Co cells are stimulated with TGF-β1 and analysed for fibrotic marker expression by Western blot. As expected, TGF-β1 stimulation induces a marked increase in collagen I and fibronectin protein levels compared to untreated controls. Co-exposure with the multi-strain probiotic lysate significantly attenuated this upregulation in a concentration-dependent manner (**Figure 9A,C**), indicating a suppressive effect on fibroblast activation. These results suggest that *OxySlab* lysate modulates key components of the fibrotic response, potentially interfering with signaling pathways that drive ECM protein overproduction.



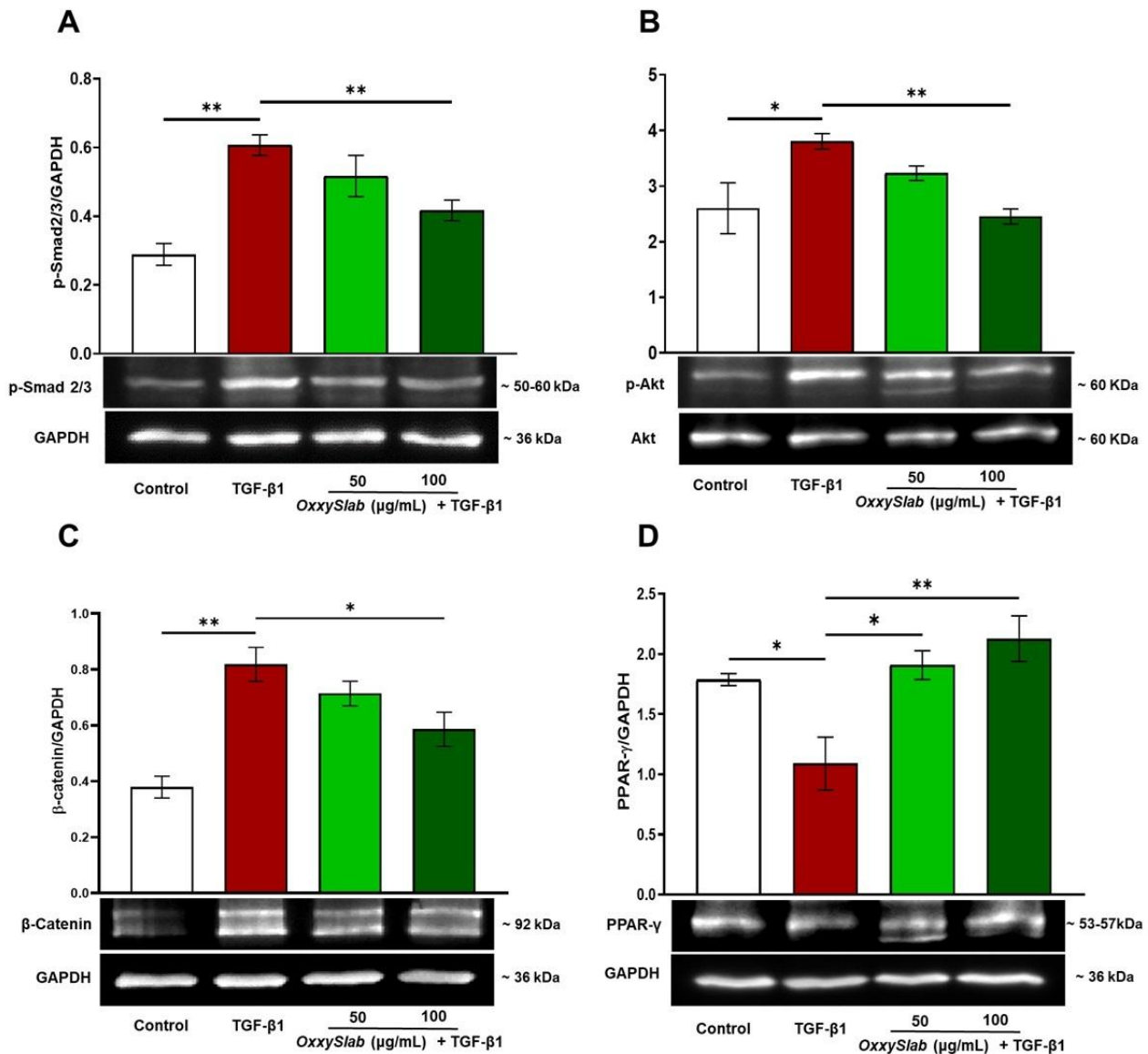
**Figure 9. Effect of *OxySlab* on fibrosis markers in *in vitro* intestinal fibrosis model.** Immunoblotting assay for (A) collagen I, (C) fibronectin, and (E)  $\alpha$ -SMA performed on CCD-18Co incubated for 48 h with TGF- $\beta$ 1 (10 ng/mL) in the presence or absence of probiotic lysate (50-100  $\mu$ g protein/mL). Densitometric analysis was normalized to GAPDH for collagen I and  $\alpha$ -SMA, and to  $\beta$ -tubulin for fibronectin. Data are from three independent experiments and are expressed as mean  $\pm$  SEM. For comparative analysis of data, a one-way ANOVA is used. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*\*  $p < 0.0001$ ). Representative immunofluorescence images (magnification 40x) of untreated and treated CCD-18Co, as above described, stained with antibodies against (B) collagen I, (D) fibronectin, and (F)  $\alpha$ -SMA (green), and TRITC-phalloidin to F-actin (red). Nuclei were counterstained with DAPI (blue).

To investigate whether the probiotic lysate modulated myofibroblast differentiation, we further assessed  $\alpha$ -SMA expression in CCD-18Co cells activated by TGF- $\beta$ 1 and treated with *OxySlab*. Western blot analysis confirms a significant upregulation of  $\alpha$ -SMA following TGF- $\beta$ 1 stimulation. Notably, co-treatment with the probiotic lysate results in a concentration-dependent reduction in  $\alpha$ -SMA levels, with the highest concentration showing a statistically significant effect (**Figure 9E**). Immunofluorescence staining corroborates these findings, showing increased signal intensity for collagen I, fibronectin, and  $\alpha$ -SMA in TGF- $\beta$ 1-treated cells compared with controls (**Figure 9B, D, F**). Probiotic co-treatment markedly reduces the staining intensity of these fibrotic markers, restoring levels to those of untreated cells. In addition, TGF- $\beta$ 1 stimulation induces pronounced cytoskeleton remodelling, as evidenced by enhanced phalloidin staining and the formation of prominent stress fibers across the cytoplasm. These features are absent in control cells. Co-treatment with probiotic lysate attenuates this effect, reducing phalloidin fluorescence and restoring cytoskeletal organization to near-baseline levels.

Overall, these results demonstrate that the multi-strain probiotic lysate exerts remarkable anti-fibrotic effects by simultaneously inhibiting fibroblast proliferation and collagen and fibronectin expression, as well as reversing cytoskeletal alterations associated with fibrogenesis.

#### **4.3 *OxySlab* interferes with the activation of TGF- $\beta$ 1 pathways**

The activation of intestinal fibroblasts, a key player of fibrosis, is triggered by chronic inflammation and is regulated by multiple cytokines, with TGF- $\beta$ 1 playing a central role. TGF- $\beta$ 1 exerts its effects through both Smad-dependent (canonical) and Smad-independent (non-canonical) signaling pathways. To investigate whether the antifibrotic effects of the probiotic formulation lysate were mediated by modulation of the canonical Smad pathway, we perform a Western blot analysis to assess the levels of phosphorylated Smad2/3 (p-Smad2/3). In **Figure 10A**, we can observe that stimulation through TGF- $\beta$ 1 markedly increases Smad2/3 phosphorylation compared to untreated cells, in parallel with the upregulation of fibrotic markers. Interestingly, co-treatment with the probiotic lysate at both tested concentrations significantly reduces p-Smad2/3 levels, suggesting attenuation of TGF- $\beta$ 1-induced transcriptional activation of profibrotic genes. This reduction is consistent with the observed downregulation of collagen I, fibronectin, and  $\alpha$ -SMA protein expression.



**Figure 10. Effects of *OxySlab* on TGF- $\beta$  signaling pathways in *in vitro* fibrosis model.** Immunoblotting analysis and representative images of (A) p-Smad2/3, (B) pAkt, (C)  $\beta$ -Catenin, (D) PPAR- $\gamma$  in CCD-18Co starved for 24 h, then incubated for 48 h with TGF- $\beta$ 1 (10 ng/mL) in the presence or absence of the selected concentrations of the probiotic lysate (50-100  $\mu$ g protein/mL). Densitometric analysis was performed by normalizing vs. GAPDH, except for p-Akt, which was normalized to the respective Akt level. Data are from three independent experiments and are expressed as mean  $\pm$  SEM. For the analysis of data, one-way analysis of variance ANOVA (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

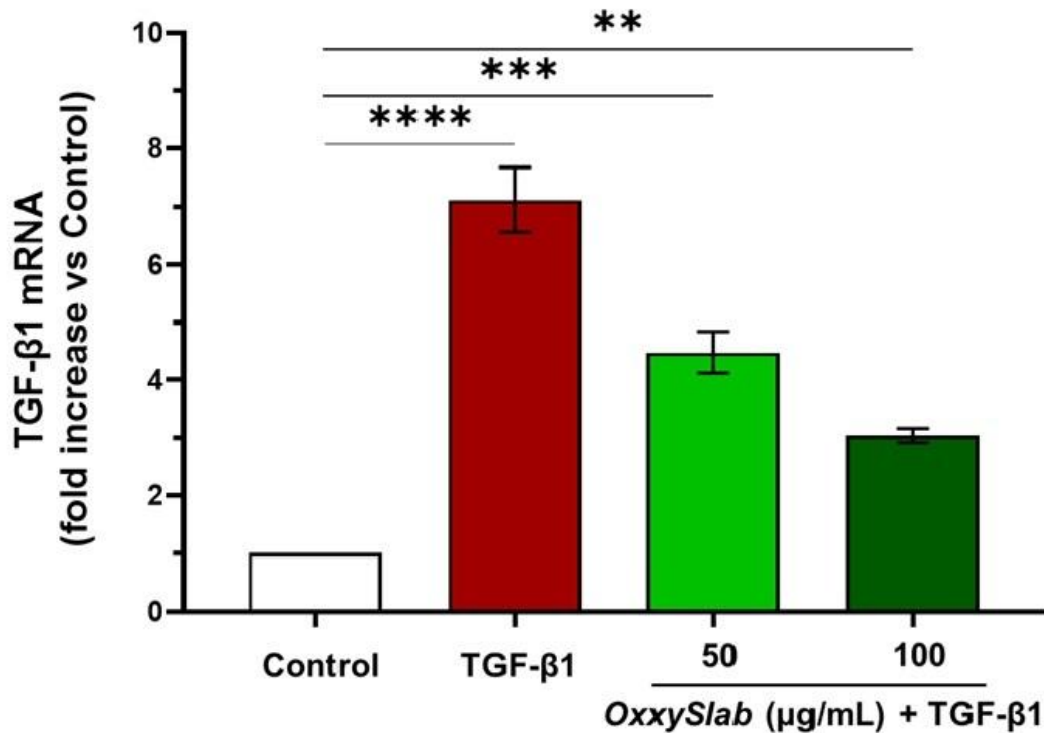
In addition to the canonical Smad pathway, TGF- $\beta$ 1 also activates the non-canonical PI3K/Akt pathway. To evaluate the involvement of this pathway, the phosphorylation status of Akt is analysed by Western blot. As shown in **Figure 9B**, TGF- $\beta$ 1 stimulation significantly increases phosphorylated-Akt (p-Akt) levels compared to control cells. Sustained Akt activation is known to promote excessive ECM production and drive fibroblast-to-myofibroblast differentiation, thereby exacerbating intestinal

fibrosis. Remarkably, co-treatment with the probiotic lysate markedly reduces p-Akt expression, indicating a modulatory effect on this signaling axis.

We have also investigated two additional pathways implicated in TGF- $\beta$ 1-mediated fibrogenesis: the WNT/ $\beta$ -catenin pathway, which promotes fibrosis, and the PPAR- $\gamma$  pathway, which exerts anti-fibrotic effects. TGF- $\beta$ 1 stimulation led to a significant increase in  $\beta$ -catenin protein levels (**Figure 10C**), consistent with its pro-fibrotic role. This upregulation is effectively suppressed by probiotic lysate treatment. Conversely, TGF- $\beta$ 1 reduced PPAR- $\gamma$  expression, while co-treatment with the probiotic lysate restores its levels (**Figure 10D**), suggesting a protective regulatory effect. Taken together, these findings demonstrate that the multi-strain probiotic formulation attenuates intestinal fibrosis by interfering with multiple intracellular signal transduction pathways activated by TGF- $\beta$ 1, including Smad-dependent signalling (p-Smad2/3) and Smad-independent pathways (PI3K/Akt, WNT/ $\beta$ -catenin, and PPAR- $\gamma$ ) (**Figure 10D**), suggesting a protective regulatory effect. Taken together, these findings demonstrate that the multi-strain probiotic formulation attenuates intestinal fibrosis by interfering with multiple intracellular signal transduction pathways activated by TGF- $\beta$ 1, including Smad-dependent signalling (p-Smad2/3) and Smad-independent pathways (PI3K/Akt, WNT/ $\beta$ -catenin, and PPAR- $\gamma$ ).

#### **4.4. Multi-Strain Probiotic Lysate modulates TGF- $\beta$ 1 gene expression**

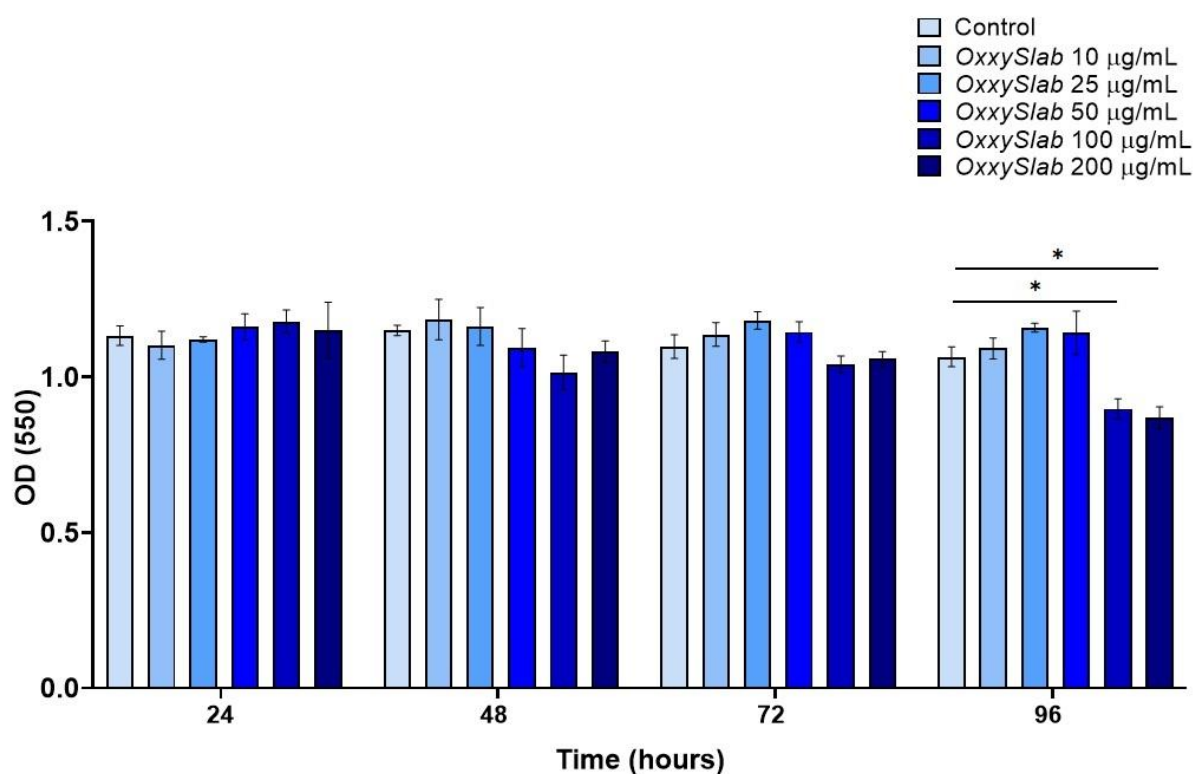
To determine whether the probiotic formulation affects transcriptional regulation, we quantified TGF- $\beta$ 1 gene expression in CCD-18Co cells using RT-qPCR. As expected and in line with previous observations, TGF- $\beta$ 1 stimulation markedly increases its own transcript levels, reflecting the presence of a self-reinforcing feedback loop that promotes fibrogenesis. Co-treatment with the *OxySlab* lysate significantly reduces TGF- $\beta$ 1 mRNA expression in a concentration-dependent manner (**Figure 11**), indicating that the probiotic lysate disrupts this autocrine amplification of TGF- $\beta$ 1 signaling. This transcriptional repression is consistent with, and better explain, the concurrent reduction in fibrotic marker expression and the attenuation of downstream signaling pathway activation.



**Figure 11. OxySlab effect on TGF-β1 gene expression in intestinal fibrosis model.** SYBR Green Real-Time PCR was performed to quantify TGF-β1 mRNA expression in CCD-18Co cells, with transcript levels normalized to GAPDH as the internal reference gene. Results represent the mean  $\pm$  SEM of three independent experiments, each conducted in triplicate. Statistical significance was assessed using one-way ANOVA followed by Dunnett's post hoc test (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  vs. Control).

#### 4.5 Cell viability of Caco-2-IECs with different concentration of *OxySlab*

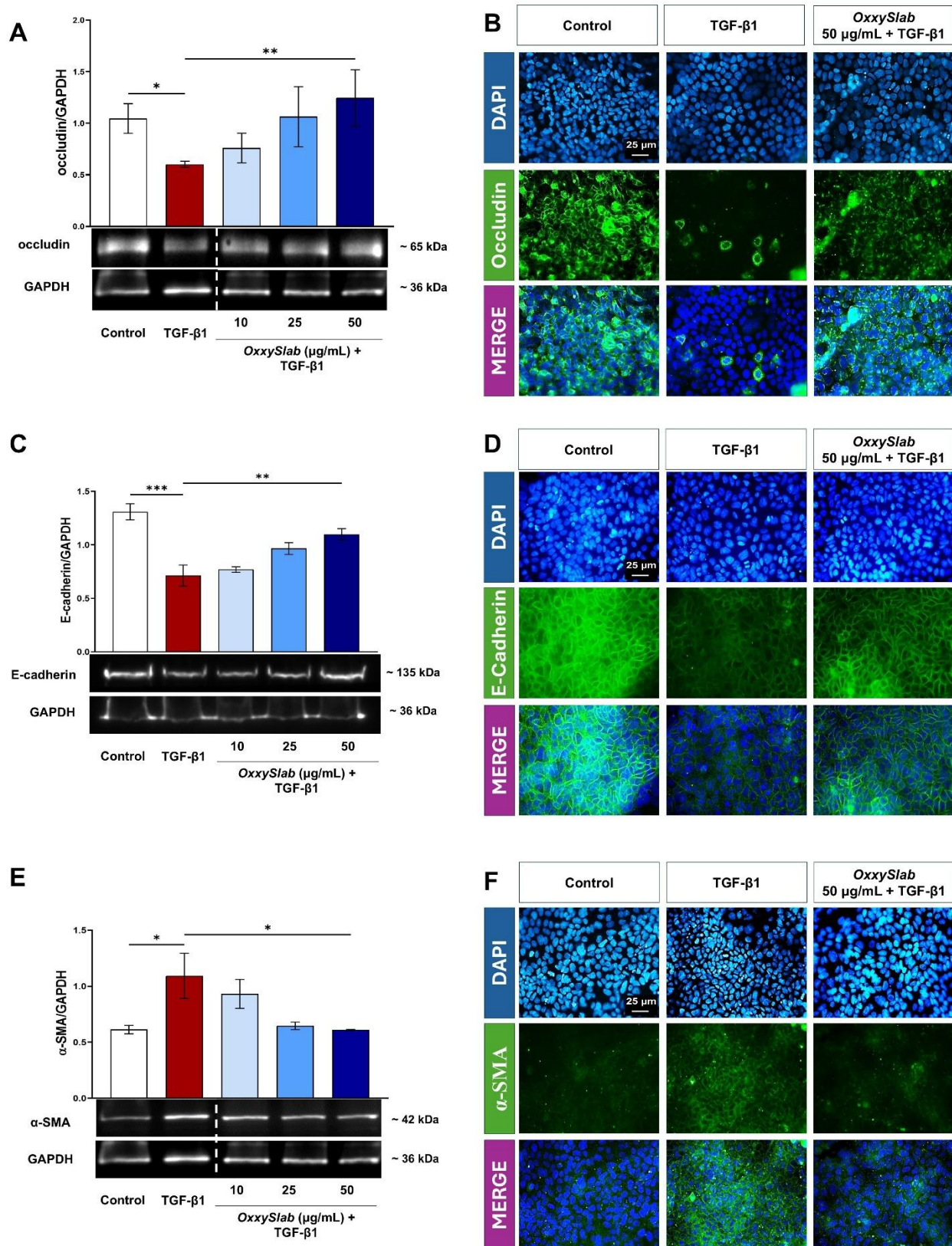
Before examining the effects of the probiotic lysate on EMT, we first assess the viability of Caco-2 IECs following exposure to increasing concentrations of *OxySlab* lysate (25, 50, 100, and 200  $\mu\text{g/mL}$ ) for up to 96 h. Cell viability was evaluated using the MTT assay. As shown in **Figure 12**, a significant decrease in absorbance, reflecting reduced cell number, is detected only at the two highest concentrations (100 and 200  $\mu\text{g/mL}$ ), and exclusively at the 96 h time point. No cytotoxic effects are observed at earlier time points or at lower concentrations. Based on these results, the 100 and 200  $\mu\text{g/mL}$  of *OxySlab* doses are excluded from subsequent EMT experiments to prevent confounding effects related to impaired cell viability.



**Figure 12 Cell viability assay of Caco-2 IECs incubated with *OxySlab*.** MTT assay analysis of Caco-2 IECs incubated with *OxySlab* lysate (10–25–50–100–200 µg protein/mL) for 96 h. Data from three independent experiments performed in triplicate are expressed as mean ± SEM and then analyzed with the two-way ANOVA (\*  $p < 0.05$  vs. Control).

#### 4.6 *OxySlab* reverted TGF-β1-induced EMT in Caco-2 IECs

EMT is one of the major mechanisms contributing to the enrichment of the intestinal myofibroblast pool, as it involves the transition of epithelial cells into mesenchymal-like cells. Among known stimuli, TGF-β1 is regarded as the most potent inducer of EMT in intestinal epithelial cells. To assess if the probiotic formulation lysate could mitigate this process, we have examined the expression levels of two key epithelial markers: occludin, a tight-junction protein essential for maintaining barrier integrity, and E-cadherin, a central component of the apical junctional complex that governs cell-cell adhesion. Caco-2 IECs are exposed to TGF-β1 for 96 h in the absence or presence of *OxySlab* lysate at selected concentrations (10, 25, and 50 µg/mL). Western blot analysis shows that TGF-β1 markedly decreases the protein levels of occludin (**Figure 13A**) and E-cadherin (**Figure 13C**), confirming EMT activation. Co-treatment with the probiotic lysate restores the expression of both epithelial markers in a concentration-dependent manner. These biochemical results are further confirmed by immunofluorescence staining, which reveals diminished occludin (**Figure 13B**) and E-cadherin (**Figure 13D**) signals in cells treated with TGF-β1 and a clear recovery following co-exposure to the probiotic and TGF-β1. Collectively, these findings indicate that *OxySlab* helps preserve epithelial identity and barrier integrity by counteracting TGF-β1-induced EMT.

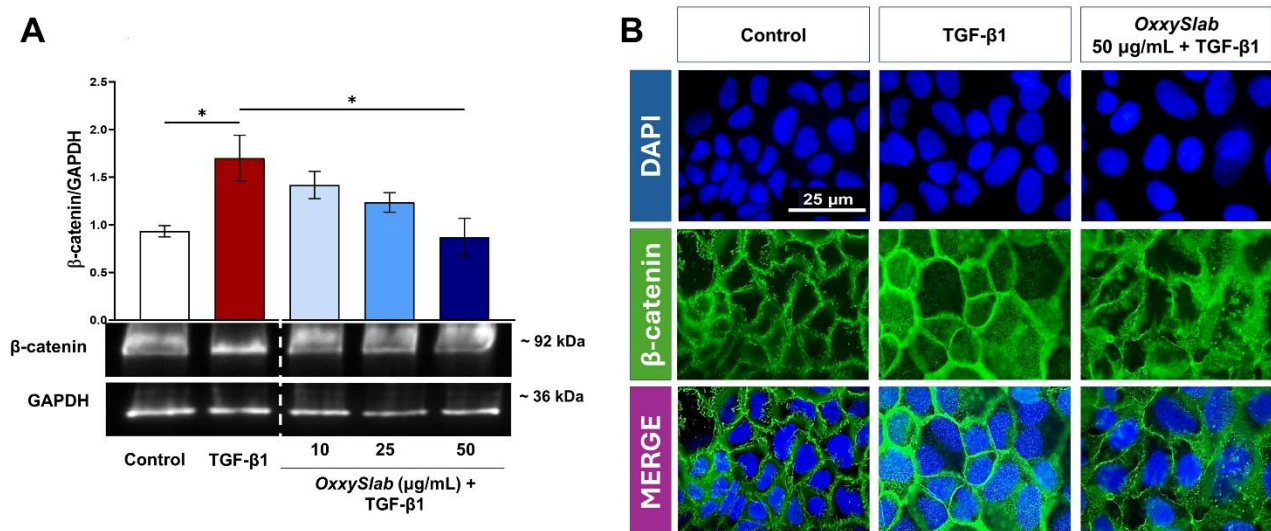


**Figure 13.** Effect of *OxySlab* on epithelial and mesenchymal markers expression in the *in vitro* EMT model. Immunoblotting assay for (A) occludin and (C) E-cadherin, and (E)  $\alpha$ -SMA was performed on Caco-2 IECs incubated for 96 h with TGF- $\beta$ 1 (20 ng/mL) alone or in combination with *OxySlab* lysate at selected concentrations (10-25-50  $\mu$ g) in the presence of TGF- $\beta$ 1.

protein/mL). Representative images of immunoblotting for occludin, E-cadherin,  $\alpha$ -SMA, and GAPDH are shown. The dotted lines indicate areas in which the images were cropped to remove lanes irrelevant for this study. Densitometric analysis was normalized to GAPDH. Data are from three independent experiments, and values are expressed as mean  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). No significant difference was detected between TGF and its combination with the lowest doses of probiotics. Representative immunofluorescence images from three independent experiments in duplicate, of untreated and treated Caco-2 IECs stained with antibodies against (B) occludin, (D) E-cadherin, (F)  $\alpha$ -SMA antibodies (green), are shown. Nuclei were counterstained with DAPI (blue) (magnification 40x).

In addition, the downregulation of epithelial markers, TGF- $\beta$ 1 stimulation in Caco-2 IECs also led to a marked increase in the mesenchymal marker  $\alpha$ -SMA, confirming the induction of the EMT program. Co-treatment with the probiotic lysate produces a dose-dependent decrease in  $\alpha$ -SMA expression, with the highest concentration exerting a significant inhibitory effect (**Figure 13E**). Immunofluorescence analyses corroborate these findings, revealing a clear reduction in  $\alpha$ -SMA signal intensity in cells exposed to the probiotic lysate (**Figure 13F**).

To further clarify the mechanisms through which the *OxxySlab* lysate suppresses EMT, we have assessed the involvement of the WNT/ $\beta$ -catenin signaling pathway. Canonical WNT signalling is known to promote EMT by increasing  $\beta$ -catenin levels and enhancing the transcription of EMT-associated genes. Consistent with this, Western blot and densitometric analyses show that TGF- $\beta$ 1 stimulation markedly increases  $\beta$ -catenin expression in Caco-2 IECs (**Figure 14A**). Representative immunofluorescence images further revealed a pronounced nuclear accumulation of  $\beta$ -catenin in TGF- $\beta$ 1-treated cells (**Figure 14B**), indicating pathway activation. Notably, co-treatment with the probiotic lysate effectively counteracts these effects, as demonstrated by reduced  $\beta$ -catenin expression and diminished nuclear localization. Together, these findings indicate that *OxxySlab* modulates the WNT/ $\beta$ -catenin pathway, thereby contributing to the inhibition of the EMT process.



**Figure 14. Effect of *OxySlab* on  $\beta$ -catenin expression in the *in vitro* EMT model.** Immunoblotting assay for (A)  $\beta$ -catenin was performed on Caco-2 IECs incubated for 96 h with TGF- $\beta$ 1 (20 ng/mL) alone or in combination with *OxySlab* lysate at selected concentrations (10-25-50  $\mu$ g protein/mL). Representative images of immunoblotting for  $\beta$ -catenin and GAPDH are shown. The dotted lines indicate areas in which the images were cropped to remove lanes irrelevant for this study. Densitometric analysis was normalized to GAPDH. Data are from three independent experiments, and values are expressed as mean  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA (\*  $p < 0.05$ ). No significant difference was detected between TGF- $\beta$ 1 and its combination with the lowest doses of probiotics. Representative immunofluorescence images of untreated and treated Caco-2 IECs stained with antibodies against (B)  $\beta$ -catenin antibodies (green), are shown. Nuclei were counterstained with DAPI (blue) (magnification 100x).

Taken together, these findings demonstrate that the multi-strain probiotic formulation attenuates intestinal fibrosis counteracting one of its key cellular drivers. By promoting the reversion of the EMT program, the probiotic lysate effectively restores the expression of epithelial markers while reducing mesenchymal marker levels, thereby preserving epithelial identity and barrier integrity.

## 5. DISCUSSION

Intestinal fibrosis represents one of the common long-term complications of IBD, including CD and UC, up 35% of patients with CD develop intestinal strictures and up to 70% of CD patients require surgery within 10 years of diagnosis (Z. Liu *et al.*, 2024). This occurs because chronic inflammation triggers fibrosis, thus cause narrowing of the lumen and structural and functional damage, leading to intestinal stenosis and obstruction. This process is driven by uncontrolled and excessive ECM protein production and accumulation in the stromal compartment, primarily collagen and fibronectin. To date, the surgical intervention remains the only effective option for managing advanced stenotic disease, and no approved, targeted, or safe antifibrotic therapies are available. The search for effective antifibrotic treatments remains an active challenge, limited primarily by an incomplete understanding of the molecular pathways underlying the onset and progression of fibrogenesis. Nevertheless, the progressive course of fibrosis, combined with the complex interactions among stromal, immune, epithelial, and microbial factors, poses a difficult challenge for identifying the precise molecular moment at which chronic inflammation transforms into a fibrotic process. When intestinal fibrosis has not yet progressed to a stage requiring surgical resection, clinical management typically relies on pharmacological therapies to control inflammation; however, these have not yet succeeded in arresting fibrosis progression. Recent studies have also highlighted that fibrosis can evolve even in the absence of active inflammation, due to the fact that inflammatory signals are not the only drivers of the fibrotic process once it has been triggered (Yang *et al.*, 2024). Available anti-inflammatory drugs have been evaluated in limited clinical trials for their anti-fibrotic potential; however, one point is certain: mere attenuation of inflammation does not resolve the progression of fibrosis. In addition, it is important to note that, until recently, no *in vitro* studies had conducted a systematic, specifically targeted evaluation of the antifibrotic potential of the pharmacological agents routinely used in the clinical management of IBD. It was only in 2024, that our group performed the first comprehensive *in vitro* investigation, testing selected IBD-drug representatives from all major drug classes currently employed in clinical practice. Their findings demonstrated that only certain agents exhibit direct antifibrotic properties in intestinal *in vitro* models: prednisone, methylprednisolone, and budesonide, and the biological adalimumab, show the ability to counteract TGF- $\beta$ 1-induced fibrosis and EMT in *in vitro* models of fibrosis and EMT (Artone *et al.*, 2024). New omics-based approaches, are allowing us to better understand the biological processes underlying fibrosis, providing more useful insights for the development of drugs that directly target these processes. Given its key profibrotic role in intestinal fibrosis, identifying compounds that block TGF- $\beta$  signalling is the most promising antifibrotic therapeutic approach. TGF- $\beta$ 1 can activate both canonical Smad-dependent signalling and others non-canonical pathways. These pathways interact with the Wnt/ $\beta$ -catenin axis and inhibit

PPAR- $\gamma$ -mediated antifibrotic signalling, supporting the persistent profibrotic phenotype. Particularly, recent studies show that WNT transcriptional profiles support ECM deposition and mesenchymal reprogramming, suggesting that WNT inhibitors may counteract the progression of fibrosis (Y. Liu *et al.*, 2024). Similarly, modulation of the nuclear receptor PPAR- $\gamma$  has emerged as a promising antifibrotic approach, due to its ability to antagonize  $\beta$ -catenin signalling and thus suppress inflammatory pathways, and recent research highlights its role as a molecular brake in the initiation of fibrosis (Cicchinelli *et al.*, 2024). The research landscape is increasingly moving towards the testing of interventions targeting the microbiota. The disruption of dynamic balance, in bacterial composition and metabolic profiles, of the microbiota, and thus altered functionality, could be responsible for the transition from chronic inflammation to irreversible structural remodelling (Watanabe *et al.*, 2022). Probiotics, postbiotics, and fecal microbiota transplantation have demonstrated the potential to modulate inflammation, immune activation, and oxidative stress, key factors upstream of fibrotic progression in IBD (Vallejos *et al.*, 2025). However, few studies have directly investigated the impact of probiotic strains or their metabolites on fibrotic signalling pathways (Bernardi *et al.*, 2024). Recent study demonstrated a beneficial effect of the Vivomixx® formulation lysate on TGF- $\beta$ 1-induced fibrosis in intestinal fibroblasts through the inhibition of the TGF- $\beta$ 1/Smad signalling pathway (Lombardi *et al.*, 2021). Building on this, the current work aims to explore the effect on fibrotic pathways of a multi-strain probiotic formulation, which has previously been reported to exert beneficial effects in various pathological disorders. (Baldassarre *et al.*, 2023; Bonfili *et al.*, 2018; Bonfili *et al.*, 2021; Castelli *et al.*, 2020; Ceccarelli *et al.*, 2021; Trinchieri *et al.*, 2022). In a recent work, *OxxySlab*, formerly *SLAB51*, has shown anti-inflammatory effects protecting Caco-2 IECs from an LPS-induced inflammatory response (Lombardi *et al.*, 2023). *OxxySlab* is formulated with eight different strains capable of modulating the gut microbiota and influencing key cellular pathways implicated in inflammation, fibrosis, and tumor progression. Recent experimental and clinical research indicate that *OxxySlab* exhibits biological activities extending beyond the canonical immunomodulatory and barrier-supporting effects typically attributed to probiotics. In this study, it has been demonstrated that *OxxySlab* effectively attenuated the profibrotic fibroblasts' proliferation, differentiation, activation, and EMT in *in vitro* models. Stimulation with TGF- $\beta$ 1 on CCD-18Co is able to induce cell proliferation and upregulate fibrotic markers, including collagen I, fibronectin, and  $\alpha$ -SMA, along with cytoskeletal reorganization, effectively recreating a widely used *in vitro* model of intestinal fibrosis. As expected, the immunofluorescence and Western blot analyses highlight a marked upregulation of collagen I and  $\alpha$ -SMA in CCD-18Co fibroblasts following TGF- $\beta$ 1 stimulation. Cytoskeletal reorganization after TGF- $\beta$ 1 exposure, characterized by the formation of thick actin stress fibers, is visualized by phalloidin staining. TGF-induced fibroblast activation,

observed in the *in vitro* model, was mediated by both canonical and non-canonical signalling pathways. In contrast, untreated cells maintained a quiescent phenotype, without the morphological hallmarks of the activated myofibroblasts. Co-treatment with probiotic lysate and TGF- $\beta$ 1 in CCD-18Co cells significantly attenuated TGF- $\beta$ 1-induced fibrotic responses in a dose-dependent manner. The bacterial lysate not only reversed the overexpression of collagen I and  $\alpha$ -SMA but also mitigated the actin cytoskeleton rearrangements, thereby inhibiting the differentiation of CCD-18Co cells into activated myofibroblasts. Furthermore, where TGF- $\beta$ 1 stimulation alone resulted in a significant increase in phosphorylated Smad2/3, co-treatment with *OxxySlab* significantly reduced Smad2/3 activation, indicating an attenuation of canonical TGF- $\beta$ 1 signalling. The PI3K/Akt signalling axis was further examined as a key non-canonical branch of the TGF- $\beta$ 1 pathway, widely implicated in the development and progression of fibrotic disorders, promoting fibroblast activation, enhancing ECM deposition, and facilitating EMT (Fang *et al.*, 2025). In the *in vitro* fibrosis model described in this work, stimulation with TGF- $\beta$ 1 increased phospho-Akt levels, while co-treatment with probiotic lysate suppressed this activation, suggesting that *OxxySlab* mitigates fibrosis also by modulating Akt signalling. TGF- $\beta$ 1-driven fibrogenesis is reinforced through extensive crosstalk with the WNT/ $\beta$ -catenin signalling cascade. Canonical WNT signalling promotes the accumulation and nuclear translocation of  $\beta$ -catenin, where it activates transcriptional programs that induce fibrosis-related genes. The WNT/ $\beta$ -catenin pathway usually remains strongly repressed in adult organs, but it becomes reactivated under chronic injury conditions, enabling  $\beta$ -catenin stabilization, nuclear translocation, and transcriptional induction of profibrotic target genes, each contributing to fibroblast activation, extracellular matrix accumulation, and EMT (Maurice *et al.*, 2025; Xue *et al.*, 2025). Notably, TGF- $\beta$ 1 and WNT/ $\beta$ -catenin form a mutually reinforcing signalling loop, where TGF- $\beta$ 1 modulates key WNT transcriptional effectors enhancing  $\beta$ -catenin-dependent gene expression; in turn,  $\beta$ -catenin activation amplifies the transcription of TGF- $\beta$ -responsive profibrotic genes (Dzialo *et al.*, 2021; Guo *et al.*, 2009; Lewis *et al.*, 2022). A crucial additional key factor in the management of fibrosis is PPAR- $\gamma$ , a nuclear receptor with established anti-fibrotic functions. Evidence demonstrates that canonical WNT/ $\beta$ -catenin signalling negatively regulates PPAR- $\gamma$ , suppressing its transcriptional activity. In radiation-induced fibrosis, overactivation of WNT/ $\beta$ -catenin pathway downregulates PPAR- $\gamma$  expression, whereas activation of PPAR- $\gamma$  has been shown to antagonize both WNT/ $\beta$ -catenin and TGF- $\beta$ 1 pathways (Vallee *et al.*, 2017). In 2016, Specca *et al.* demonstrated that the novel PPAR $\gamma$  agonist, GED-0507-34 Levo, can significantly downregulate intestinal fibrosis in the mouse model of DSS-induced chronic colitis, exerting a significant regulatory effect on key profibrotic cellular and molecular pathways (Specca *et al.*, 2016). Mechanistically, PPAR- $\gamma$  agonists promote the expression of WNT inhibitors (such as DKK1 and GSK3 $\beta$ ), which suppress both TGF-

$\beta$ 1 signalling and PI3K/Akt activation, resulting in reduced  $\beta$ -catenin stabilization and diminished transcription of profibrotic genes (D'Alessio *et al.*, 2022). The central role of TGF- $\beta$ 1, WNT/ $\beta$ -catenin, and PPAR- $\gamma$  in orchestrating the molecular events driving fibroblast activation and extracellular matrix deposition provides a robust conceptual framework to explore novel modulatory strategies targeting these pathways, including the use of probiotics. Specifically, given the antagonistic relationship between  $\beta$ -catenin activation and PPAR- $\gamma$  expression, it has been proposed that the probiotic lysate could attenuate fibrosis progression by reducing  $\beta$ -catenin levels and/or restoring PPAR- $\gamma$  activity. The findings in this study show that *OxySlab* exerts its effects as hypothesized by antagonizing multiple profibrotic signalling pathways and may offer a more effective strategy to reverse or limit fibrosis. Beyond fibroblasts, also epithelial cells contribute to fibrosis, and their plasticity significantly contributes to the profibrotic cell pool through EMT, a process characterized by the loss of epithelial markers, loss of epithelial integrity, and the gain of mesenchymal traits such as  $\alpha$ -SMA and fibronectin expression (Fousekis *et al.*, 2025; Latella *et al.*, 2017). TGF- $\beta$ 1 stimulation induce EMT in Caco-2 IECs, as evidenced by reduced epithelial marker expression and increased  $\alpha$ -SMA and  $\beta$ -catenin levels, confirming a shift towards a mesenchymal-like phenotype. Notably, probiotic treatment effectively counteracts these alterations, restoring epithelial features and limiting mesenchymal transition. This observation suggests that the probiotic lysate not only modulates fibroblast activation but may also preserve epithelial identity, thereby interrupting a key cellular mechanism that amplifies fibrogenesis. One of the crucial points in the probiotic landscape is the need of a clearer distinction between the viable and non-viable probiotic preparations as well as to differentiate bioactive compounds derived from probiotics. Traditionally, live probiotics have been praised for their ability to colonize the digestive tract, to dynamically interact with the host's microbiome, and to have many positive effects such as modulation of immune function, improving intestinal barrier and supporting gut homeostasis, due to the metabolic capacity of the probiotics and their ability to communicate with host cells in a strain-dependent manner. However, in more recent years, there has been an increasing amount of evidence to support the idea that non-viable probiotic preparations may have clinically distinct advantages. Non-viable probiotic preparations, such as bacterial lysates that retain bioactive components, are considered safer than viable or proliferating probiotics, especially in immunocompromised or elderly patients, as well as in individuals who have conditions such as IBD or other disorders in which providing live microorganisms could potentially cause harm. Non-viable probiotic preparations also are fully safe and reliable when processed or stored for a longer period than viable probiotic preparations, which would result in more consistent therapeutic results. In particular, there is increasing evidence indicating that inactivated microorganisms will continue to elicit biologically significant activity

(modulating host immune response, suppressing inflammation, and enhancing the intestinal epithelial barrier). It is also important to note that non-viable probiotic products and isolated components that are derived from probiotics can have an impact on the composition of the gut microbiota as well as on its metabolic activity. Non-viable probiotics can affect resident microbial species through direct interactions, and they can alter host-derived factors that influence the gut ecosystem. The ability of these non-viable probiotics to maintain or restore barrier function *in vivo* suggests that they could be valuable and clinically applicable substitution agents for traditional formulations of live probiotics, especially in therapeutic settings where the safety, stability, and regulated biological activity of the probiotic are essential (Calvanese *et al.*, 2025; de Simone, 2019). Stability is critical to ensuring that a probiotic product is consistently effective and practically usable. In comparison to non-viable probiotics, live probiotics must overcome significant obstacles with regard to maintaining viability and metabolic activity throughout their lifecycle. Due to their sensitivity to environmental stressors, live probiotic products are also subject to harsh acidic and enzymatic environments within the gastrointestinal tract, requiring complicated processes for manufacture, special types of containers for storage, and stringent storage conditions. The result is that, by the time live probiotics reach the target site, there are often dramatically reduced amounts of viable cells, resulting in inconsistent dosing and variable outcomes of therapeutic treatment. In contrast, bioactive compounds from bacteria have greater stability than both live probiotics and bioactive compounds from other sources. Being non-living molecules, bioactive compounds from bacteria have a much higher level of resistance to destruction by environmental conditions, extreme pH levels, and enzymatic actions. This increased resistance gives bacterial compounds a longer shelf life along the gastrointestinal tract. Furthermore, probiotic lysates are easier to store and transport, while ensuring that the active compound is administered more effectively and accurately to the host, thus ensuring reliable and reproducible clinical results (Liang *et al.*, 2023). Given this, the mechanisms shown in this work align with contemporary research identifying probiotic derivatives as promising therapeutic tools in IBD-associated fibrosis. The results highlight that the multi-strain probiotic formulation, *OxxySlab*, exerts multifaceted antifibrotic effects by modulating canonical and non-canonical TGF- $\beta$  signalling, suppressing fibroblast activation, and reversing EMT. With the use of more advanced experimental models, it is now possible to highlight the potential capabilities of probiotic lysates to act on multiple facets of intestinal disease, making them a critical element in strategies aimed at preventing or reversing fibrosis in IBD. The next steps of our research will focus on the important task of identifying and purifying the *OxxySlab*-derived factors which specifically reverse the intestinal fibrosis process. Although these results provide valuable insights into molecular mechanisms, the precise microbial compounds responsible are not yet fully understood and these multifaceted antifibrotic effects have

only been evaluated using *in vitro* models that can recreate a simple and controllable environment but are unable to mimic the complex structure of the human gut and the intricate process of intestinal fibrosis. Nevertheless, these results open the door to possible probiotic lysate therapeutic applications for intestinal fibrosis in inflammatory bowel disease. Future studies will employ more complex experimental models to better recapitulate intestinal fibrosis and clarify the roles of epithelial plasticity and immune components. The next goal is to test the antifibrotic efficacy of *OxySlab* in advanced 3D organoids and full-thickness intestinal constructs to bridge the translational gap between cell culture and clinical application.

## 6. REFERENCES

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