UNIVERSITÀ DEGLI STUDI DELL'AQUILA
DIPARTIMENTO DI MEDICINA CLINICA, SANITÀ PUBBLICA, SCIENZA DELLA VITA E DELL'AMBIENTE
Dottorato in Scienze della Salute e dell'Ambiente
Curriculum: Medicina Traslazionale
Ciclo: XXXVI

Titolo della tesi

STUDIO DEI MECCANISMI BIOMOLECOLARI DELLA FIBROSI CUTANEA UTILIZZANDO UN MODELLO SPERIMENTALE IN VITRO E VALUTAZIONE DELL'EFFICACIA DI UN CEPPO SELEZIONATO DI PROBIOTICO NEL CONTRASTARE IL FENOTIPO FIBROTICO.

SSD MED/04

Dottoranda

Dott.ssa **Blerina Bahiti**

Coordinatrice del Corso

Prof.ssa Maria Grazia Cifone

Relatrice

Prof.ssa Benedetta Cinque

Co-Relatrice

Prof.ssa Paola Palumbo

A.A. 2022/2023

ACKNOWLEDGEMENTS

"Non esiste VENTO se non si conosce PORTO"- "There is no WIND if you don't know PORT"

I read this quote in the wonderful building of the Rectory of the University of L'Aquila, but as I approach the end of my PhD, its deep meaning has taken on a new importance in my life.

Success is no accident. It is hard work, perseverance, learning, studying, sacrifice and most of all, love of what you are doing or learning to do. Completing a PhD is a monumental achievement, and I still find it hard to believe that I have reached this milestone. I want to raise a toast to everyone who has been a part of this incredible journey with me.

First and foremost, I would like to thank my PhD supervisors, **Prof.ssa Maria Grazia Cifone**, for providing an environment in which I could thrive as an independent researcher. You have fostered a community in your group that attracts kind, generous, and hardworking people, and I am so glad to have been a part of that. I appreciate your support of my own professional endeavors. Your exceptional academic expertise, coupled with your commitment to support early career researchers truly make you a role model in the often-competitive realm of academia. Your guidance and encouragement have been invaluable.

I would like to express my deep and sincere gratitude to my research tutor and co/tutor, **Prof.ssa Benedetta Cinque and Prof.ssa Paola Palumbo**, which with their constant support, guidance and encouragement have been invaluable throughout the process. From the initial stages of refining my research work to the final submission of my thesis, their steadfast presence and wealth of wisdom have been instrumental in shaping my growth. A special appreciation goes to Prof.ssa Benedetta Cinque which have deeply inspired me with her dynamism, vision and motivation. She has taught me the methodology to carry out the research and to present the research works as clearly as possible. It was a great privilege and honor to work and study under her guidance.

Special mention goes to the Rector of the University of Elbasan, **Professor Skender Topi**, without whom all this would not have been possible. His vision and support made this dream of mine come true.

I would like to say thanks to my friends and research colleagues for their constant encouragement.

Last but not the least, I want to thank my parent, for their love, caring and sacrifices for educating and preparing me for my future.

Undoubtedly, pursuing a doctorate presented its fair share of challenges. However, what initially appeared as obstacles eventually turned out to be blessings in disguise. These challenges allowed me to refocus my efforts. Apologies to everyone who patiently put up with my enthusiastic ramblings about my research.

As I bring these years of PhD journey to a close, I can genuinely say that I am immensely proud of my accomplishments. This transformative journey has not only shaped me as a researcher but also as an individual, imparting the invaluable lesson that perseverance yields fruitful rewards in the long run. The unwavering commitment and determination displayed by those around me have profoundly influenced my character.

In conclusion, I dedicate this thesis to my sons Ajni and Laert, who are my strength and the greatest success of my life. I apologize that this sacrifice was also theirs, but I know that they are proud of this victory of mine. My guardian angel, my husband, gave me the greatest strength to move forward and give my best.

Thank you, God, for letting me through all the difficulties!

List of Abbreviations

Abbreviation	Definition
S. thermophilus	Strephtococuss thermophilus
NHDF	Normal human dermal fibroblasts
ECM	Extracellular matrix
TGF-β	Transforming growth factor-β
TGF-βR	Transforming growth factor-β receptor
PDGF	Platelet-derived growth factor
IL	Interleukin
IFN-γ	Interferon-γ
VEGF	Vascular endothelial growth factor
FGF	Fibroblast growth factor
α-SMA	Alpha smooth muscle actin
MMPs	Matrix-metalloproteases
MFB	Myofibroblast
HA	Hyaluronic Acid
FN	Fibronectin
EDA-FN	Extra domain A fibronectin
Co-Smad	Co-mediator Smad
R-Smad	Receptor-regulated Smad
I-Smad	Inhibitory Smad
Smurf1	Smad ubiquitin-related factor-1
Dkk-1	Dickkopf-related protein-1
PPARγ	Peroxisome proliferator activated receptor γ
MAPK	Mitogen-activated protein kinase

Summary

Abstract

The protracted inflammatory phase in skin wound healing may lead to dermal fibrosis, and existing therapeutic methods have shown limited efficacy. Exploring alternative approaches, such as those involving specific probiotic strains, could offer potential therapeutic avenues. This study aims to assess the potential of *Strephtococuss thermophilus* lysate in counteracting the fibrogenic impact of TGF-β1 in normal human dermal fibroblasts (NHDF).

NHDF underwent exposure to TGF-β1 to induce a fibrotic phenotype. The assessment included measuring proliferation rates and cell numbers through the IncuCyte® Live Cell Imager system and the trypan blue dye exclusion test. Western blot and immunofluorescence techniques were employed to analyse phenoconversion markers (α-SMA and fibronectin) and collagen I levels. RT-PCR was used to evaluate TGF-β1 mRNA levels. Western blot was also utilized to assess Smad2/3 phosphorylation, β-catenin, and PPARγ expression. Collagen gel retraction and scratch wound healing assays were conducted to study cell contractility function and migration of NHDF. The impact of *S. thermophilus* lysate, either alone or in combination with TGF-β, was examined across all the aforementioned parameters and markers linked to the TGF-β-induced fibrotic phenotype.

The application of *S. thermophilus* lysate demonstrated a substantial reduction in key mediators and events associated with the abnormal activation of myofibroblasts induced by TGF-β1 in the fibrotic profile. The treatment with *S. thermophilus* significantly lowered cell proliferation, migration, and myo-differentiation. Furthermore, the probiotic lysate treatment resulted in decreased expression levels of α-SMA, fibronectin, and collagen-I, impacting the collagen contraction ability of activated dermal fibroblasts. Additionally, the probiotic intervention targeted TGF-β1 signaling by reducing Smad2/3 activation, TGF-β1 mRNA levels, and β-catenin expression through the upregulation of PPARγ.

This is the first report showing that *S. thermophilus* lysate had a remarkable anti-fibrotic effect in TGF-β1-activated NHDF by inhibiting Smad signaling. Notably, the probiotic was able to reduce β-catenin and increase PPARγ levels. The findings support our point that *S. thermophilus* may help prevent or treat hypertrophic scarring and keloids.

1 INTRODUCTION

1.1 Skin Fibrosis

Fibrosis is a gradual pathological process characterized by the slow development of tissue degeneration, eventually culminating in severe consequences for various organ systems such as the heart, lungs, liver, kidneys, and skin. The insidious progression of fibrosis poses significant challenges, underscoring the critical need for comprehensive understanding and effective therapeutic interventions to mitigate its devastating impact on diverse physiological systems (Antar et al, 2023). Consequently, the harmful impact of organ fibrosis is estimated to play a significant role in approximately 45% of fatalities within industrialized countries (Lurje et al, 2023) (Figure 1).

Skin fibrosis constitutes a significant public health concern globally, impacting over 100 million individuals annually in developed countries, with an even higher prevalence worldwide. This condition disrupts the normal physiological function of soft tissue, giving rise to both functional challenges and potential aesthetic issues, contributing to psychological distress among affected individuals (Wang et al, 2023).

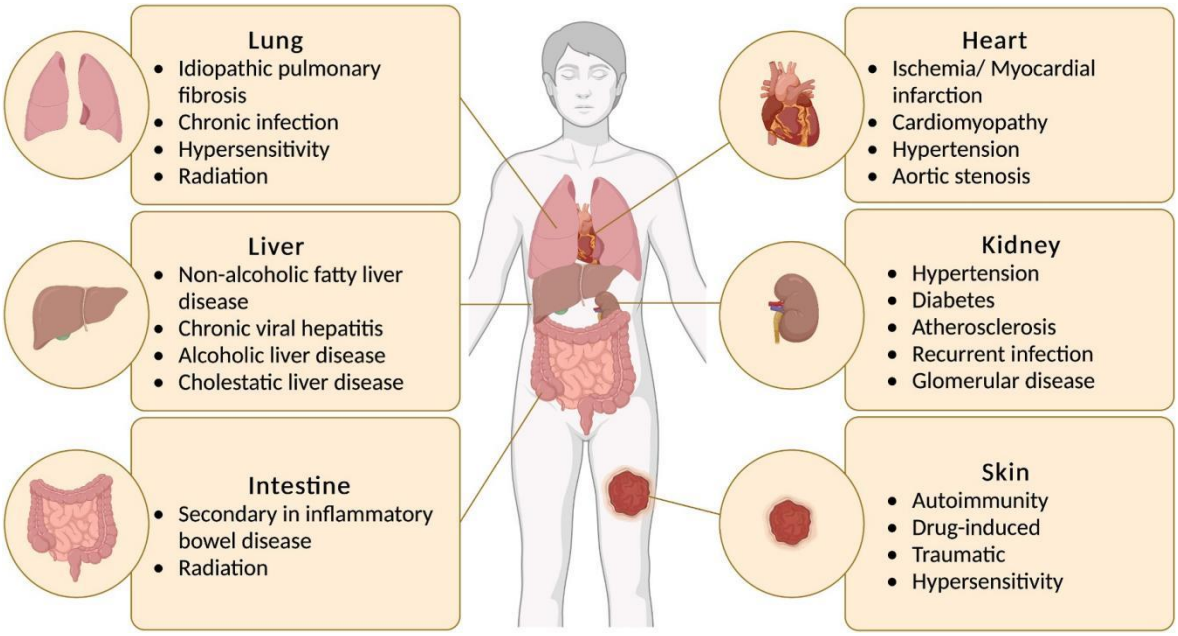

Figure 1. Common manifestations and etiologies of fibrosis (Lurje et al, 2023)

Skin fibrosis is a pathological condition characterized by the abnormal accumulation of extracellular matrix (ECM) components, primarily collagen, in the dermal layers, leading to tissue scarring and functional impairment. In cases of severe injuries characterized by significant loss of dermal and epidermal tissue, including instances of extensive surgery, traumatic events, and notably severe burns, a pathological wound healing process may result. This aberrant pattern is often marked by prolonged inflammation, ultimately culminating in dermal fibrosis and the formation of excessive scars. Notably, postburn scarring, exemplified by hypertrophic scars (HSC) and keloids, stands as a prevalent complication, with the occurrence of HSC observed in as much as 70% of burn patients. Simultaneously, keloids also manifest as a common pathology in individuals who have experienced burn injuries. The classification of burns hinges on two primary factors: the extent of the burn and its depth. The severity of scarring following a burn injury is primarily influenced by the depth of the burn itself (Faour et al, 2023).

The ECM constitutes a meticulously arranged, collagen-based dense meshwork comprising intricate macromolecules, including proteins and polysaccharides. This elaborate structure is secreted by resident cells, predominantly fibroblasts. Beyond its fundamental role in offering structural support to cells and tissues, the ECM has demonstrated substantial influence on the proliferation, differentiation, and metabolism of parenchymal cells (Wang et al, 2023). Fibrosis is often a consequence of damage to the epidermis or skin due to chronic exposure to chemicals or trauma. It is characterized by the proliferation of fibroblasts and abnormal deposition of collagen fibers in the dermis or around hair follicles, usually oriented parallel to the epidermis. In more severe cases, fibrosis can extend deeper into the dermis and subcutaneous tissue. In early fibrosis, fibroblasts usually accompanied by inflammatory cells, tend to be larger and more active, and collagen fibers are less compact and more disorganized.

Substantial evidence unequivocally supports the integral involvement of mechanical alterations in the ECM in the initiation and progression of diverse skin fibrotic diseases. Elevated ECM stiffness, identified as a crucial indicator, stands prominently as a significant hallmark in the landscape of fibrotic diseases, underscoring its pivotal role in the intricate pathophysiology of these conditions. The nuanced understanding of these ECM mechanical changes emerges as a key focal point for unraveling the complexities inherent in various forms of skin fibrosis.

While some degree of fibrosis is a normal part of the wound-healing process, excessive or abnormal fibrosis can lead to various skin conditions.

Numerous diseases manifest with the clinical presentation of skin fibrosis, exhibiting a diverse range of aetiologies that encompass physical factors such as radiation or mechanical stimulation, chemical exposures, biological influences, and immune-related factors (Table 1). The multifaceted origins of these diseases underscore the complexity of skin fibrosis, necessitating a comprehensive understanding of the interplay between various causative factors for effective diagnosis and management.

Skin fibrosis exhibits substantial overlap in molecular signaling pathways with fibrosis in other organs, establishing a shared molecular framework. Notably, the convergence of signaling cascades involves pivotal pathways, including but not limited to the transforming growth factor-β (TGF-β) signaling pathway and the Hippo signaling pathway. This intricate interconnection in molecular mechanisms underscores the systemic nature of fibrotic processes, necessitating a comprehensive understanding of these shared pathways for the development of targeted therapeutic interventions across various organ systems. Nonetheless, given that the skin constitutes the outermost layer of the human body, it is inherently exposed to various mechanical stresses arising from both internal physiological processes and external factors. Consequently, the exploration of mechanical stress-mediated regulatory mechanisms within the skin emerges as a particularly intriguing avenue of investigation. The unique accessibility and visibility of the skin, in comparison to other internal tissues, presents an advantageous opportunity for deliberate and controlled artificial modifications to the mechanical microenvironment. This distinctive attribute holds significant promise for innovative interventions aimed at curtailing skin fibrosis, thus offering a novel perspective in the realm of therapeutic developments dedicated to addressing skin fibrotic disorders.

Within the intricate progression of skin fibrosis, a cascade of major biological events unfolds, encompassing inflammatory cell infiltration, intricate cytokine secretion, and the consequential proliferation and differentiation of fibroblasts. This orchestrated sequence of biological phenomena represents a multifaceted interplay that underscores the dynamic and complex nature of the skin fibrotic process, requiring in-depth exploration to unravel the intricate mechanisms governing each facet of these critical events (Wang et al, 2023).

TABLE 1 Diseases with skin fibrosis symptoms (Wang et al, 2023)

Name of disease	Etiology	Clinical manifestation of skin	Biomarkers
Dermatofibroma	Mild trauma such as an insect bite, related to genomic aberrations in 17q and 22q	Hard solitary slow-growing papules (rounded bumps)	CD34-
		Appear in a variety of colors, usuallybrownish to tan	Stromelysin-3+
		Most often found on the legs and arms	Factor XIIIa+
			CD64
Scleroderma	Autoimmune disease, caused by gene mutations (e.g., *DNASE1L3*, *STAT4*, HLA class II genes) or exposure to certain chemical compounds (e.g., silica, organic solvents)	Symmetrical skin thickening	Anti-scl70
		Skin stiffness increase	Anticentromere antibodies
		Raynaud's phenomenon	Anti-U3
		Nail-fold capillary changes	Anti-RNA polymerase
			Nucleolar antigens
			CCL18
Keloids	Related to skin tension, autoimmunity, genetic and epigenetic factors (such as HLAgenes, TGF-β signalling pathway related genes) but not fully understood	Preferably in front of the sternum, earlobes,back, shoulders	STC2, SDC4, NOX4, DAAM1
		Firm, rubbery lesions or shiny, fibrous nodules	TNC
		Sometimes producing a lump many timeslarger than that of the original scar	CD138
		Vary from red to dark brown in colour	
		Sometimes accompanied by severe itchiness, pain, and changes in texture	
Hypertrophic scars	Mechanical tension	Not extend beyond the boundary of theoriginal wound	MiRNA-365a/b-3p
	Inherited tendency (such as *ASAH1 g*ene, but not fully understood)	Not to the degree observed with keloids	Gal-1
		May be itchy or painful	
		Often contain nerves and blood vessels	
		Develop after thermal or traumatic injury	
Dystrophic epidermolysis bullosa	Genetic defects within the human *COL7A1* gene	Highly susceptible to severe blistering	*COL7A1*
		Chronic scarring	Tumor serine proteases C1r and C1s
			HMGB1
Porphyria cutanea tarda	Inherited mutations	Individuals with PCT present with increasingly fragile skin on the back of the hands and the forearms. Other sun-exposed sites such as the face, scalp, neck, and arms may also be affected	Granular and homogeneous deposits of C5b-9 in vessels are characteristic immunofluorescence findings in patients with PCT
	Environmental impact		
Radiation dermatitis	Ionizing radiation, radiotherapy	Pain, sclerosis, hair loss and ulcers	StefinA3 and S100 calcium binding proteinA8 (S100A8)
	Some genes involved in the inflammatory pathway have been implicated in cutaneous radiation injury, such as *IL12RB2 rs3790568*	Advanced skin damage: dryness, scaly skin hyperpigmentation and loss of skin appendages	
Nephrogenic Fibrosing Dermopathy	Renal failure	Patients present with hard, indurated,sometimes peau d'orange plaques	CD34, CD68 and factor XIIIa
Nephrogenic systemic fibrosis	Unknown, possibly related to exposure to certain conventional gadolinium-containingcontrast agents in magnetic resonance imaging	Swelling, tightness, red or dark patches, thickening and hardening of the skin of thetrunk, burning, itching or severe tingling inthe affected area	Fibroblast growth factor (FGF)23, osteoblasttranscription factors Runt-related transcription factor 2, and osterix

1.2 Molecular and cellular players in the fibrosis process

Fibrosis, characterized by a gradual onset, evolves into a condition culminating in tissue degeneration, leading to skin disorders. The pathological process ensues when an abnormal accumulation of fibrous connective tissue manifests in the ECM following tissue injury. Excessive fibrosis results in persistent healing challenges, ultimately leading to functional impairment of organs or tissues. The intricate network of pro-fibrotic cells, mediators such as growth factors and cytokines, and other contributing factors, including ECM dynamics, vascular injury, mechanical tension, and oxidative stress, collectively contribute to the multifaceted landscape of fibrosis in diverse tissues (Antar et al, 2023) (Figure 2). Inflammatory cytokines and growth factors play a crucial role in the initiation and progression of skin fibrosis.

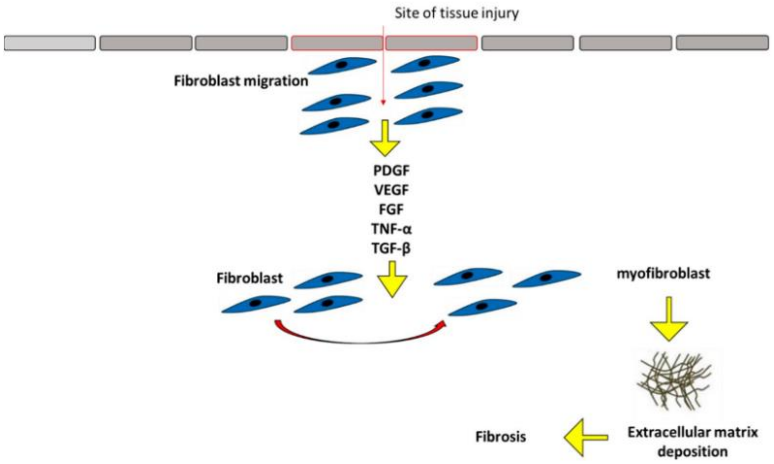

Figure 2. The major mechanisms of fibrosis. PDGF: Platelet-derived growth factor; VEGF: Vascular endothelial growth factor; FGF: Fibroblast growth factor; TNF-α Tumor necrosis factor-alpha; TGF-β: Transforming growth factor beta (Antar et al, 2023)

Efficient wound healing can prevent abnormal scar formation, yet prolonged inflammation during this process may worsen skin fibrosis. The wound healing process encompasses hemostasis, inflammation, proliferation, and remodeling phases, each requiring precise coordination and timely execution for successful tissue repair.

During the hemostasis phase, a cascade of events unfolds, releasing pro-inflammatory cytokines and growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor 2 (FGF 2 or bFGF), interleukin 8 (IL-8), and transforming growth factor-beta TGF-β. These factors emanate from the newly formed clot and surrounding damaged

tissues. Subsequently, the inflammatory phase ensues, marked by the infiltration of inflammatory cells from the immune system in response to chemokine signaling. This phase is characterized by the sequential influx of neutrophils, macrophages, and lymphocytes into the wound area. The copious presence of neutrophils serves a dual role in the wound healing process. Not only do they serve as a critical defense mechanism against invading microbes, but they also play a pivotal role in clearing both pathogens and tissue debris within the wound area. This clearance is achieved through phagocytosis, accompanied by the release of nitrogen species and reactive oxygen species (ROS). Neutrophils play a pivotal role in the orchestration of the wound healing process by facilitating the recruitment of other inflammatory cells, including monocyte-derived macrophages. These macrophages undergo differentiation into mature wound macrophages under the influence of local cytokines like interferon-γ (IFN-γ) and bacterial products such as lipopolysaccharide. Classified as the M1 type, mature macrophages continue the process of clearance through phagocytosis of tissue debris, microbial organisms, and apoptotic cells. Following this, a subset of M1 macrophages undergo apoptosis, and the remaining ones undergo transition into the M2 type of macrophage due to changes in cytokine expression, such as IL-13 and IL-4. M2 macrophages assume a distinct role in the wound healing process, particularly in steering the transition to the proliferative phase. They release essential growth factors, including vascular endothelial growth factor (VEGF), PDGF, and TGF-β, contributing significantly to the initiation of the proliferative phase. In the proliferative phase of wound healing, a cascade of growth factors, including vascular VEGF, PDGF, and TGF-β, orchestrates crucial events such as angiogenesis, re-epithelialization, and collagen production. Fibroblasts play a central role during this phase, being recruited to the wound site, where they commence the synthesis of key components of the ECM like collagen, glycosaminoglycans, proteoglycans, fibronectin, and elastin. This synthesis is accompanied by the proliferation, migration, and differentiation of fibroblasts, induced by the presence of growth factors such as fibroblast growth factor (FGF), PDGF, and TGF-β. Fibroblasts, in collaboration with endothelial cells, are pivotal for capillary growth and the formation of granulation tissue within the reparative dermis. An essential process in wound healing, wound contraction, is driven by myofibroblasts, specialized contractile cells expressing α-smooth muscle actin (α-SMA). Following the proliferative phase and the synthesis of the ECM, wound healing advances into the protracted and intricate remodeling phase. This phase, spanning weeks to months and occasionally extending to years, involves a delicate equilibrium between ECM production, breakdown, and

remodeling processes. In this stage, marked by diminished cell proliferation, extensive apoptosis of endothelial cells, macrophages, and myofibroblasts takes place. The transformation involves the substitution of glycosaminoglycans with proteoglycans and the replacement of collagen III by collagen I, orchestrated by collagenases and matrix-metalloproteases (MMPs). The pivotal outcome of this ECM reorganization is the remodeling that culminates in an architectural structure closely mirroring that of normal tissues (Zhang et al, 2020)

TGF-β, a cytokine with ubiquitous expression, plays a crucial role in the intricate orchestration of the wound healing process. TGF-β1 serves as a potent inducer of fibroblast activation, leads to the differentiation into myofibroblasts, marked by increased contractility and synthetic activity.

It is synthesized by various entities such as damaged tissues, fibroblasts, and M2 macrophages. Belonging to an extensive superfamily, TGF-β shares structural similarities with other polypeptide growth factors like nodal, activins, bone morphogenetic proteins (BMPs), inhibins, and growth and differentiation factors (GDFs). The normal functioning of TGF-β involves the modulation of essential cellular processes, including but not limited to cell growth, migration, apoptosis, differentiation, and the regulation of collagen production (Zhang et al, 2020). TGF-β exists in three isoforms (TGF-β1, TGF-β2, and TGF-β3) and they are central regulators of cell differentiation, migration, proliferation, and gene expression, with TGF-β1 being the most extensively studied in the context of fibrosis. TGF-β3 is predominantly located within mesenchymal cells, while TGF-β1 is present in endothelial, hematopoietic, and connective tissue cells. On the other hand, TGF-β2 is expressed in epithelial, neuronal, and connective tissue cells. Intriguingly, TGF-β2 has the capacity to downregulate cell junctions, promoting cell proliferation and enhancing survival, particularly in the context of inflammatory diseases (Antar et al, 2023). Every isoform encodes a precursor protein that includes an amino-terminal signal peptide essential for secretion, an extensive pro-segment, and a mature TGF-β polypeptide consisting of 112 amino acids, situated at the carboxy-terminal region (Budi et al, 2021). The trio of TGF-β isoforms originates from distinct genetic entities, each governed by its unique gene TGF-β1: gene for TGF-β1, TGF-β2 gene for TGF-β2, and TGF-β3 gene for TGF-β3. The regulatory landscape of each gene is intricately composed, featuring a multifaceted promoter equipped with an array of negative and positive regulatory elements. This promoter exhibits receptivity to a diverse spectrum of regulatory proteins, encompassing repressors and activators with varied functional roles. There are 3 TGF-β receptors (TGF-βR1, TGF-βR2, and TGF-βR3), and all three isoforms of TGF-β signal via these

three receptors. Due to its central role in fibrosis, TGF-β1 has emerged as a therapeutic target (Frangogiannis et al, 2020).

The dysregulation of TGF-β1 plays a central role in driving fibrotic phenotypes in various tissues, including the skin. It is a potent inducer of fibroblast activation, driving these cells toward a myofibroblast phenotype. Myofibroblasts exhibit increased the synthesis of ECM, particularly collagen.

The increase of TGF-β1 expression marked consistently fibrosis, although an increased TGF-β2 and TGF-β3 mRNA expression has also been noted. Activated fibroblasts and myofibroblasts represent a major cell population to respond to TGF-β1 consistent with the notion of a self-perpetuating response to injury and chronic inflammation. Macrophages and epithelial cells are also important in the expression of TGF-β1. In certain fibrotic models, reduced macrophage recruitment results in diminished levels of TGF-β1 within the fibrotic lesion, while in other instances, epithelial cells are responsible for regulating TGF-β1 activity. The roles played by various cell types in expressing TGF-β1 within the fibrotic lesion are less clearly understood, although platelets, epithelial cells, T-cells, and mast cells have also been identified as expressing TGF-β1, whereas epithelial cells upregulate TGF-β1 activity in others. The contributions of these different cell populations to increased fibrosis TGF-β1 expression varies between different fibrosis types and disease states. The induction of TGF-β1 expression is triggered by growth factors, pro-inflammatory cytokines activating MAPK pathways, and various other stimuli (Budi et al, 2021).

Fibroblasts: Primary cellular component in Skin Fibrosis

In the skin, fibroblasts, primary cellular components of connective tissue, respond to injury by forming granulation tissue during the proliferation phase and transitioning into a myofibroblast phenotype during the remodeling phase, primarily influenced by TGF-β/Smad signaling pathways. (El Ayadi et al, 2020). Fibroblasts appear as central players in the intricate process of skin fibrosis and play a significant role in maintaining tissue integrity by proliferating, differentiating, and collaborating with other cells both in homeostasis and disease states. Fibroblast activation takes place in response to injury, inflammation, or profibrotic stimuli and exhibit distinct characteristics, including increased contractility and synthetic activity. Beyond the metamorphosis of resident mesoderm-derived fibroblasts, the acquisition of myofibroblast (MFB) properties extends to diverse cell types, showcasing organ-specific variations in their origin. In the context of the skin,

MFBs can trace their lineage back to subcutaneous adipocytes, whereas in epithelial organs like the lung, epithelial cells emerge as contributors to the MFB pool. The liver MFB pool, on the other hand, witnesses the transformation of hepatic stellate cells (HSCs), also known as pericytes. Intriguingly, even mesothelial cells can undergo mesothelial-mesenchymal transition, precipitating the generation of liver MFBs during episodes of fibrotic liver injury. This intricate network underscores the dynamic and heterogeneous nature of MFB origin across different tissue contexts (Lurje et al, 2023).

From a physiological standpoint, fibroblasts undergo apoptosis, as an integral component of the latter stages of the wound healing cascade, signifying the conclusion of their role once an ample degree of mechanical coherence has been established via their production of the ECM. However, the persistence of the growth factors, such as TGF-β, coupled with organ-specific anti-apoptotic signals, creates an abundance of stimuli that effectively counteract the apoptotic process, thereby inhibiting the natural programmed cell death of fibroblasts (Lurje et al, 2023).

The myofibroblast phenotype is marked by the α-SMA, a contractile protein associated with the enhanced contractility of myofibroblasts. Myofibroblasts play a key role in the excessive deposition of ECM components leading to fibrosis: type I and type III fibrillar collagens, hyaluronan (HA), fibronectin (FN), and extra domain A fibronectin (EDA-FN). Activated fibroblasts, or myofibroblasts, exhibit distinct characteristics, including increased contractility and synthetic activity and they are a major contributor to collagen synthesis, with collagen type I being a predominant fibrotic matrix component. Dysregulated collagen production and deposition by fibroblasts contribute to tissue stiffness and architectural distortion.

The transition from fibroblasts to myofibroblasts is a critical step in fibrosis. This phenotypic switch is characterized by distinct cellular and molecular changes that contribute significantly to the fibrotic process.

The process of fibroblast-myofibroblast differentiation requires: the activation of the TGF-β1/Smad signaling pathway; cell-ECM mechanotransduction signaling; and the synthesis of modulators that promote and maintain the myofibroblast phenotype (EDA-FN, HA) (Figure 3) (Tai et al, 2021). During the activation of fibroblasts there is an intermediate stage known as the proto-myoblast. The transformation to proto-myoblasts derivates to mechanical changes, such as the stiffness of the tissue which is usually influenced by external stretching forces and the contractile forces exerted on the ECM by the myofibroblasts. This initiates a positive feedback

loop, sustaining a balance between proto-myoblasts and myoblasts, thereby maintaining stable states. The chemical changes can also activate the secretion of TGF-β, a crucial factor during fibrosis.

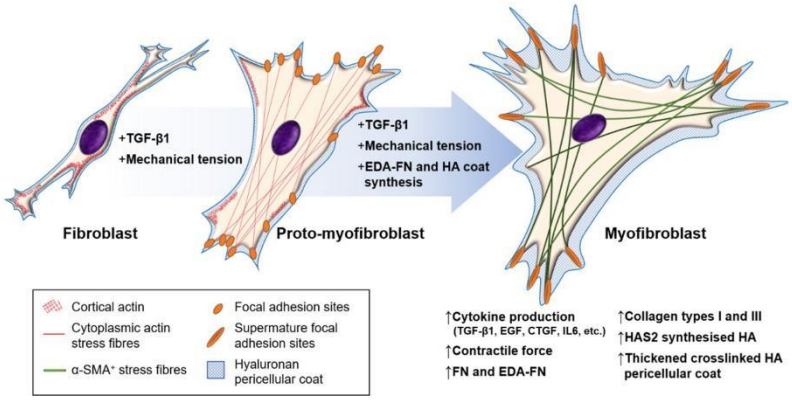

Figure 3. Fibroblast–myofibroblast differentiation (Tai et al, 2021)

1.3 TGF-β1 signaling pathway

The process of scar formation serves as a proficient reparative mechanism crucial for the genetic evolution of human beings. However, when a newly formed scar extensively occupies the normal tissue, it has the potential to result in diverse levels of disfigurement and functional impairment, thereby impacting the overall structural and physiological integrity of the affected area. The activation of myofibroblasts and subsequent production of ECM are integral components in the pathogenesis of all fibrotic diseases. Myofibroblasts play a pivotal role in orchestrating the remodeling of fibrotic tissue and the Smad2/3 signaling is the pathway mainly involved. Anomalies in the TGF-β/Smad signaling pathway within myofibroblasts have been specifically linked to the development of pathological scar formations, including the occurrence of hypertrophic scars. The dysregulation of this signaling cascade contributes significantly to the aberrant tissue remodeling observed in fibrotic conditions.

The activation of TGF-β signaling cascades remains a consistent hallmark in fibrotic tissues, irrespective of the initial injury's etiology. The initiation of TGF-β signaling necessitates not only the *de novo* synthesis and secretion of TGF-β isoforms but also entails the spatially restricted generation of active TGF-βs from latent stores. Within fibrotic tissues, diverse pathways contribute to the induction of TGF-β isoform expression. Neurohumoral mediators, exemplified by angiotensin II and norepinephrine, are liberally released in numerous fibrotic conditions. These

mediators play a pivotal role in stimulating TGF-β transcription and subsequent secretion of latent TGF-βs, exhibiting multifaceted effects across various cell types implicated in the fibrotic process (Frangogiannis et al, 2020).

The TGF-β1-activated pathway plays a central role in coordinating cellular responses, especially in the realm of fibrosis. Serving as a pivotal regulator, TGF-β1 initiates a series of interconnected events that impact a wide range of cellular functions. Whether it's triggering fibroblast activation or orchestrating changes in the extracellular matrix, grasping the subtleties of the TGF-β1-activated pathway provides insights into the complex molecular mechanisms underpinning pathological fibrotic processes. TGF-β1 initiates signaling by binding to its receptors (TGFβR), notably the TGF-β type I and type II receptors, on the surface of fibroblasts, upregulating α-SMA expression and promoting collagen secretion and cell proliferation.

The initiation of TGF-β signals involves the formation of a serine/threonine kinase complex, consisting of a type I receptor (activin-like kinase (ALK) 1–7) and a type II receptor present on the cell surface. Upon binding of the ligand to the receptor, the type II receptor becomes activated and subsequently phosphorylates the type I receptor.

Furthermore, an expanding repertoire of type III receptors has been identified, serving as co-receptors that interact with TGF-β and modulate the intricacies of TGF-β signaling.

The canonical TGF-β1 pathway involves the Smad proteins, particularly Smad2 and Smad3. The Smad protein family comprises eight distinct members classified into three functional categories: Co-mediator Smad (Co-Smad), receptor-regulated Smad (R-Smad), and inhibitory Smad (I-Smad). Each category plays a specific role in the intricate network of cellular responses orchestrated by the TGF-β signaling pathway (Zhang et al, 2020). TGFβRI phosphorylates Smad2 and Smad3, which form complexes with Smad4 and translocate into the nucleus, regulating the transcription of fibrosis-associated genes. Following phosphorylation, the type I receptor exhibits specificity in recognizing and phosphorylating the R-Smads, specifically Smad2 and Smad3. This phosphorylation event disrupts the interaction with the Smad-anchor for receptor activation (SARA) and enhances the binding affinity for Smad4, the Co-Smad. Upon liberation from SARA, Smad2 and Smad3 can associate with Smad4 to form a transcriptional complex. This complex gains the freedom to translocate into the nucleus, where it can engage with transcriptional co-activators or co-repressors, ultimately modulating the transcriptional activity of various genes. Moreover, Smad2 and Smad3 interact with closely related activin and nodal receptors, while other

receptor-regulated Smads, such as Smad1, Smad5, and Smad8, are primarily engaged by BMP. Once the TGF-β-induced Smad pathway is activated, multiple feedback mechanisms come into play to regulate the signaling duration. I-Smads, namely Smad6 and Smad7, are upregulated by both TGF-β and BMP signaling. These I-Smads operate by directly interfering with the association between receptor-regulated Smads and their respective type I receptors, resulting in reduced phosphorylation of the receptor-regulated Smads. The mechanism to terminate TGF-β signaling involves ubiquitination of Smads in both the nucleus and cytoplasm, followed by proteasome-mediated degradation of the Smad proteins. Notably, three main types of E3 ubiquitin ligases orchestrate the ubiquitination process of Smads (Zhang et al, 2020).

Within the cytoplasm, the Smad ubiquitin-related factor-1 (Smurf1) selectively engages with Smads 1, 5, and 8, orchestrating their ubiquitination process. Simultaneously, Smurf2 specifically targets activated Smad2 within the nucleus, facilitating its degradation. This intricate regulatory mechanism ensures the controlled turnover of Smad proteins in response to TGF-β signaling. It is noteworthy that Smurf1 and Smurf2 can be enlisted by Smad7, culminating in the establishment of a stable complex. This complex actively participates in the ubiquitination of the TGF-β type I receptor, a process during which Smad7 itself undergoes ubiquitination and subsequent degradation. This intricate interplay underscores the dynamic regulatory network within the TGF-β signaling pathway (Figure 4).

TGF-β1 is capable of initiating Smad-independent pathways and co-receptor signaling cascades, including but not limited to mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK1/2), Rho/Rho-associated protein kinase (ROCK), phosphatidylinositol-3-kinase (PI3K)/AKT, protein phosphatase 2A (PP2A), p38/c-Jun N-terminal kinase (JNK), protein kinase C (PKC), and tumor necrosis factor receptor-associated factor (TRAF)-4/6.

From initiating fibroblast activation to influencing ECM dynamics, this pathway stands at the forefront of therapeutic exploration, offering insights into novel strategies for managing and treating fibrotic disorders.

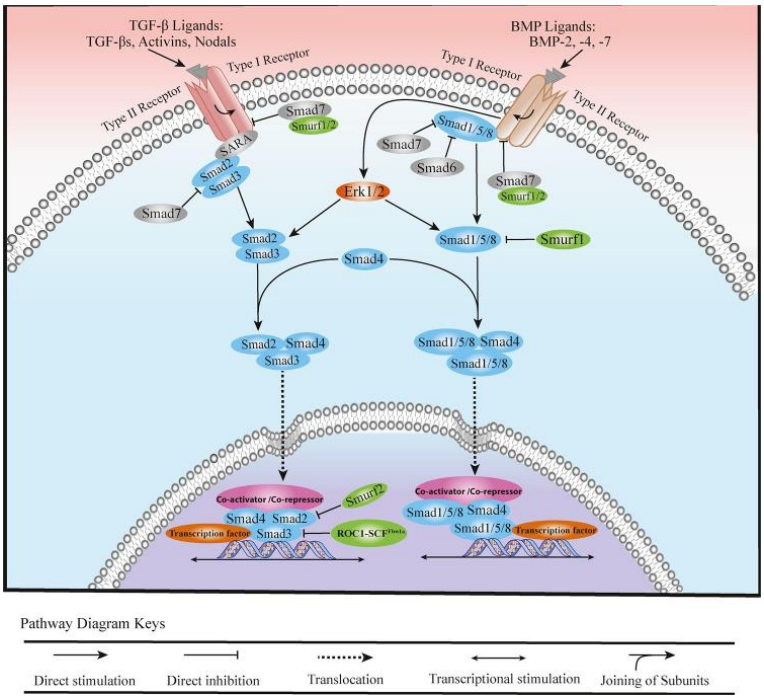

Figure 4. TGF-β-induced Smad-dependent pathway (Zhang et al, 2020)

A multitude of scientific investigations has consistently provided evidence supporting the assertion that the altered of the TGF-β1/Smad signaling pathway stands as a pivotal pathogenic element in the context of tissue fibrosis. It is well-established that a central mechanism driving progressive fibrosis within TGF-β signaling is orchestrated through the intricate network of Smad signaling pathways. This intricate interplay plays a key role in shaping the trajectory of fibrotic processes observed in diverse tissues and organs (Antar et al, 2023). As above described, Smads constitute a family of proteins that serve as the main intracellular signaling transporters for TGFBR (Transforming Growth Factor Beta Receptor). There are three types of Smad proteins based on their function: R-Smads that include Smad1, Smad2, Smad3, Smad5, and Smad8; Co-Smad, Smad4 is the only Co-Smad; and I-Smads that include Smad6 and Smad7 (Figure 5).

Primarily activated by TGF-β, this pathway plays a central role in transducing extracellular signals to the nucleus, influencing gene expression, and contributing significantly to various physiological and pathological processes. Initiation of the pathway occurs when TGF-β ligands bind to their respective cell surface receptors, activating a series of events.

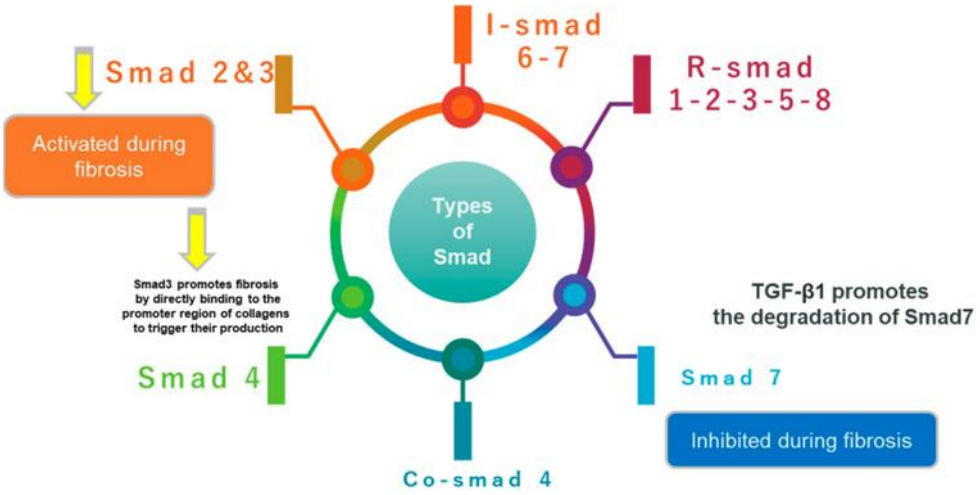

Figure 5. The schematic diagram illustrates the different types of Smad: receptor regulated Smad(R-Smad); common partner-Smad (co-Smad); and Inhibitory Smad (I-Smad) (Antar et al, 2023)

The receptor regulated Smad (R-Smad), namely Smads 1, 2, 3, 5, and 8, predominantly reside in the cytoplasm and become activated through phosphorylation by the TGFBR. Once activated, these R-Smads have the capacity to associate with specific DNA sequences or regions rich in G/C content. Smad4 resides as a single Co-Smad, forms associations with activated R-Smads and facilitates their translocation across the nuclear membrane. In response to TGF-β stimulation, Smad6 and Smad7 predominantly reside in the nucleus but translocate to the plasma membrane. Furthermore, Smad7 serves as a modulatory protein, facilitating the interaction between TGFBR1 and E3 ligases. This interaction, in turn, orchestrates the degradation of TGFBR1 through the ubiquitin pathway. TGF-β not only stimulates Smad7 transcription, but also motivates Smad7 breakdown by turning on Smad3. Smad3 emerges as a critical element in the signal transduction pathway implicated in the progression of fibrosis. More significantly, it was demonstrated that an imbalance between Smad3 and Smad7 serves as a key pathway in mediating the fibrotic response. In this context, the effectiveness of treating fibrosis seems promising through the dual approach of downregulating Smad3 while concurrently upregulating Smad7, thereby restoring the disrupted Smad3/Smad7 ratio. Moreover, alterations in the equilibrium between Smad3 and Smad7 contribute to the accumulation and activation of myofibroblasts, and excessive production, or diminished degradation of ECM.

The cDNA microarray promoter transactivation approach has allowed to identify Smad3/4 gene targets in cultured dermal fibroblasts, as: COL1A1, COL3A1, COL5A2, COL6A1, COL6A3 and

TIMP-1. Hence, the activation of skin fibrillar collagen genes is dependent on the crucial role played by the TGF-β/Smad signaling pathway (Wang et al, 2023).

1.4 Wnt/β-catenin Signaling Pathway

The Wnt/β-catenin pathway is a cellular signaling pathway critical for controlling key biological functions, including embryonic development, tissue homeostasis, and disease processes. It involves a series of molecular events triggered by Wnt ligands, ultimately leading to the activation of β-catenin and its involvement in the regulation of target genes. (Moretti et al, 2022). The two types of Wnt signaling are catenin-dependent ("canonical" Wnt signaling) and catenin-independent ("non-canonical" WNT signaling). Canonical Wnt signaling activation may play a significant role in fibrogenesis and is adequate and necessary for fibrotic tissue remodeling. In the canonical pathway, Wnt proteins, as secreted ligands, interact with Frizzled receptors and co-receptors (LRP5/6) to traverse the plasma membrane and initiate signaling. Upon binding to their receptors, Wnt proteins initiate a cascade of intracellular signaling events involving Dishevelled, Axin, Adenomatosis Polyposis Coli, and Glycogen Synthase Kinase 3, culminating in the stabilization of β-catenin. Subsequently, β-catenin translocates into the nucleus, where it acts as a transcription factor. When the ligand-binding event is absent, glycogen synthase kinase-3β phosphorylates the N terminus of β-catenin, marking it for ubiquitination and subsequent degradation. Additionally, TGF-β signaling mediators, such as the Wnt antagonist Dkk-1(Dickkopf-related protein-1), are influenced by this pathway, since their expression is downregulated by TGF-β, as depicted in Fig. 5 (Moretti et al, 2022). DKK1 plays a pivotal role in regulating the fate of β-catenin, a key modulator of Wnt signaling. However, when DKK1 is present, it triggers a cascade of events that lead to the degradation of β-catenin instead of its accumulation. By promoting the degradation of β-catenin, DKK1 prevents its translocation into the nucleus. As a result, β-catenin is unable to bind to transcription factors and activate the expression of Wnt-associated genes involved in fibrosis and other cellular processes (Tai et al, 2021).

Furthermore, the intricate web of the TGF-β signaling cascade encounters modulation by mediators within its domain, notably influenced by TGF-β itself, as evidenced by the downregulation of the Wnt antagonist, Dkk-1, as depicted in Figure 6. Investigations have suggested a compelling association between excessive Wnt signaling and phenomena such as

endothelial to mesenchymal transition in keloids and dermal scars. Additionally, the identification of accumulated beta-catenin in fibroblastic foci of idiopathic pulmonary fibrosis (IPF) further underscores the relevance of the Wnt pathway in fibrotic processes. These compelling findings designate Wnt as an additional pivotal target for therapeutic interventions aiming to alter the pathway dynamics and attain scarless healing. The introduction of CXXC5 through in vitro transfection, serving as a zinc finger catenin inhibitor, demonstrated a notable reduction in αSMA and collagen I expression within fibroblasts. Concurrently, in murine models, the topical administration of XAV-939 (a small molecule compound that acts as a tankyrase inhibitor) and pyrvinium (an anthelmintic medication who has the ability to inhibit Wnt signaling) exhibited promising outcomes, characterized by the emergence of structures akin to intact skin and a significant decrease in fibrosis (Moretti et al, 2022).

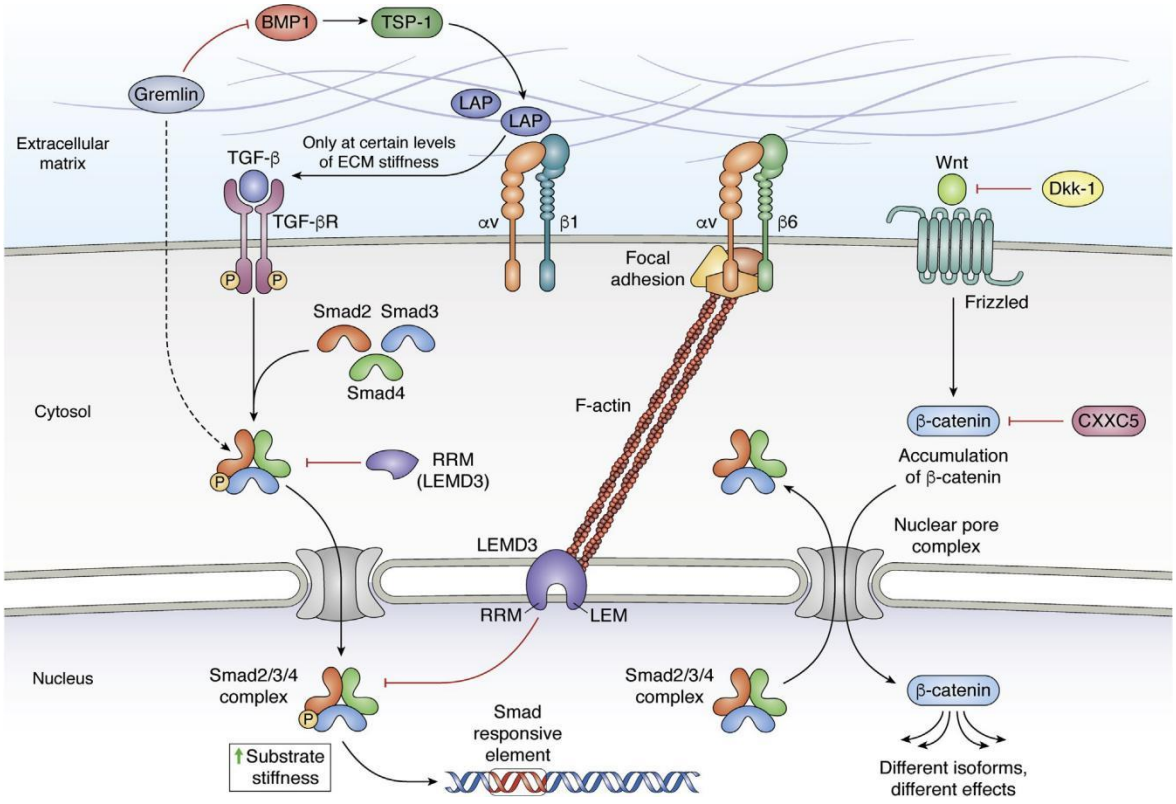

Figure 6. Summary of TGF-β signaling and interplay with Wnt (Moretti et al, 2022)

The activation of Wnt signaling, triggered by the TGF-β1-induced suppression of DKK1, was demonstrated to prompt myofibroblast differentiation, enhance the secretion of ECM components, particularly collagens, and induce fibrotic processes.

Dysregulation of this pathway is often linked to pathological conditions and provides a target for therapeutic interventions. Thorough exploration of Wnt signaling is imperative, as, akin to TGF-β, distinct isoforms of Wnt exert nearly opposite downstream effects. Administration of Wnt3a exhibited heightened proliferation and collagen I production in postnatal murine fibroblasts, contrasting with fetal fibroblasts. Intriguingly, this Wnt isoform escalated TGF-β1 levels while diminishing the secretion of TGF-β3 (known for its anti-fibrotic properties) in both fetal and postnatal fibroblasts. Conversely, Wnt6 expression demonstrated a reduction in epithelial-to-mesenchymal transition following TGF-β exposure, suggesting a broader protective impact. Wnt signaling unveils diverse therapeutic targets with potential to suppress fibrosis (Moretti et al, 2022).

1.5 Role of PPARγ in Skin Fibrosis

The outermost layer of the skin, known as the epidermis, is avascular and predominantly composed of keratinocytes. These keratinocytes, arranged in multiple layers, actively secrete growth factors and keratin. This layer serves as the frontline defense, directly encountering environmental challenges such as temperature variations, pathogens, and other external factors.

The vascularized dermal layer plays a crucial role in supplying nutrients to the entire skin, encompassing the avascular epidermis. Within the dermis, fibroblasts emerge as the predominant cell type, capable of transitioning into myofibroblasts in response to signals associated with wound healing or repair. These myofibroblasts become the primary contributors to the production of extracellular matrix proteins, particularly collagen, in the dermal region, playing a pivotal role in skin wound healing and maintaining the skin's texture and aesthetics. However, when myofibroblast activity becomes dysregulated, leading to the excessive synthesis of collagen in the dermis, it becomes a major factor in the development of skin fibrosis. Subsequent to the dermal layer, the layer of white adipose tissue, characterized by the presence of fat and numerous adipocytes producing adipokines, assumes a pivotal role in various physiological processes. This includes serving as a significant reservoir for energy storage, actively participating in thermoregulation, influencing the hair follicle cycle, and contributing to the regulation of obesity. Notably, this adipose tissue layer secretes a spectrum of crucial adipokines such as leptin, interleukin-6, and adiponectin, each playing essential roles in diverse metabolic and regulatory pathways (Ghosh et al, 2021).

PPARγ is a type of nuclear receptor and transcription factor, part of the PPAR family, which includes three subtypes: PPARα, PPARδ (also known as PPARβ), and PPARγ. PPARs play crucial roles in regulating various physiological processes, including metabolism, inflammation, and cell differentiation.

By suppressing the expression of pro-inflammatory genes and signaling pathways, PPARγ demonstrates anti-inflammatory characteristics. In conditions like skin fibrosis, where inflammation commonly contributes, the activation of PPARγ holds the potential to alleviate inflammatory responses. PPARγ is known to modulate the activity of fibroblasts, the key cells involved in the synthesis and deposition of extracellular matrix (ECM) components. The excessive production of collagen and other ECM proteins by fibroblasts contributes to skin fibrosis, and this process could potentially be regulated by PPARγ.

TGF-β activates Type I collagen synthesis, the principal matrix protein contributing to skin fibrosis, by initiating serine phosphorylation through TGF-β receptor 1 kinase. This activation leads to downstream Smad2/3 signaling and its interaction with the epigenetic regulator acetyltransferase p300 on the collagen gene promoter in human skin fibroblasts. Exploring the function of PPARγ in skin fibrosis offers valuable insights that may inform therapeutic approaches for the effective management of fibrotic skin disorders (Figure 7). Current research endeavors focus on deciphering the molecular mechanisms through which PPARγ influences fibrosis, with the ultimate goal of devising precise interventions to enhance clinical outcomes.

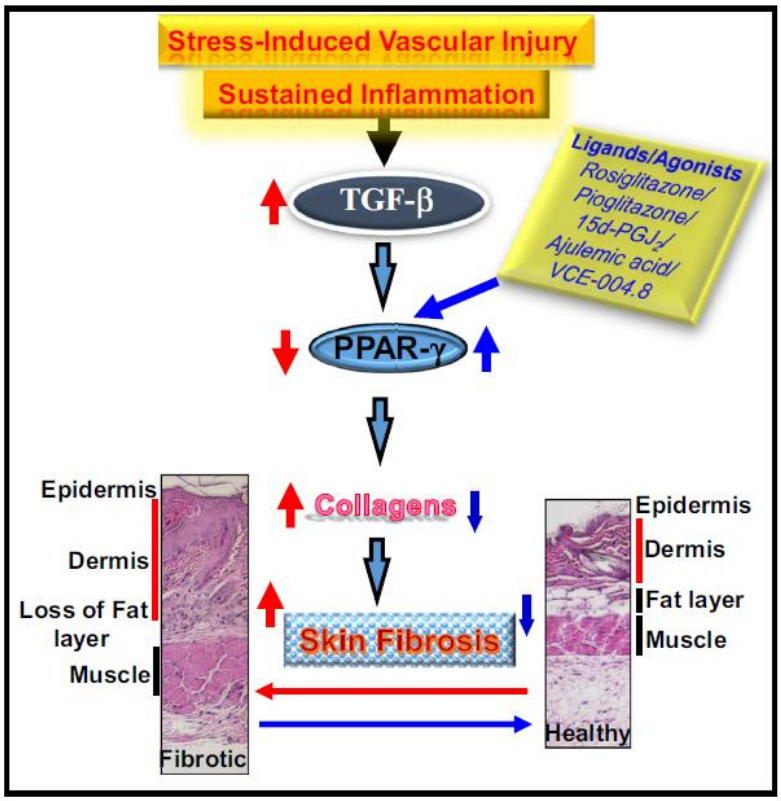

Figure 7. Involvement of PPARγ as an antifibrotic factor and a druggable target for skin fibrosis therapy (Ghosh et al, 2021)

1.6 Crosstalk between Wnt/β-catenin, TGF-β1/Smad Pathway, and PPARγ

The intricate interplay between the Wnt/β-catenin signaling pathway, the TGF-β1/Smad pathway, and peroxisome proliferator-activated receptor gamma (PPARγ) forms a dynamic network of crosstalk with significant implications in cellular regulation. This communication between pathways influences diverse cellular processes and holds relevance in various physiological and pathological contexts. The Wnt/β-catenin and TGF-β1/Smad pathways exhibit reciprocal regulation, displaying instances of cross-inhibition or synergistic effects contingent upon the cellular context. The modulation of Smad activity, influenced by Wnt signaling, contributes to variations in TGF-β1 responses. The interaction between Wnt/β-catenin and PPARγ is intricate, encompassing both positive and negative regulatory aspects. The expression of PPARγ, essential in adipogenesis and inflammation, may be influenced by Wnt signaling.

The interplay between the TGF-β1/Smad pathway and PPARγ entails the modulation of Smad activity by PPARγ, indicating a complex regulatory network. Understanding the crosstalk between these pathways provides insights into the complexity of cellular regulation and offers potential

targets for therapeutic interventions in diseases where these pathways are dysregulated, such as fibrosis (Ghosh et al, 2021).

1.7 Potential Therapeutic Targets and Anti-Fibrotic Approaches

The evolving understanding of fibrosis as a dynamic and reversible phenomenon, coupled with advancements in non-invasive assessment techniques, has sparked increased interest in the development of effective antifibrotic medications. Developing therapeutic anti-fibrotic approaches is a critical area of research and intervention. When addressing the outcomes of wound healing, it is crucial to assess the potential for hypertrophic scarring or keloid formation by considering factors such as the patient's medical history, genetic predisposition, the type of wound, and its anatomical location. Following the proliferative phase, the process of scar formation enters a subsequent stage of remodeling, characterized by the gradual reduction of scar tissue through the activity of matrix metalloproteinases (MMP). This remodeling phase can extend for up to a year post-injury. While this can lead to the development of a more mature scar, it often falls short of achieving the normal architecture of collagen fiber deposition. Interventions during this phase focus on minimizing scar tissue and restoring normal collagen architecture, with the ultimate goal of preventing scar recurrence. Treatment strategies in this stage often involve the transdermal administration of drugs, such as triamcinolone (TAC), through injections (Coentro et al, 2019). Multiple avenues exist for the development of antifibrotic drugs, encompassing diverse strategies: (I) address the root causes and mediators of the underlying injury; (II) diminish immunological activity and inflammation; (III) target specific signaling pathways, including intracellular signaling and receptor-ligand interactions; (IV) inhibit matrix production and reduce fibrogenesis; (V) mitigate fibrosis by expediting the breakdown of scar matrix, inducing stellate cell death, or employing cell transplantation; (VI) explore miscellaneous approaches. As of now, there is a notable absence of specific antifibrotic medications tailored to counteract fibroblast activation and curb enhanced ECM production. Emerging antifibrotic methodologies, such as employing specific inhibitors of intracellular tyrosine kinases, are under active investigation, exemplifying the continual exploration of innovative strategies in the pursuit of effective antifibrotic interventions (Antar et al, 2023). Various materials can serve as drug carriers to effectively traverse the basement membrane. Additionally, occlusive dressings, such as silicone dressings, stand out as key approaches in the management of elevated scars, representing a gold standard in treatment (Figure

8). Once the indentation process concludes, a noticeable mark remains, marked by an excessive accumulation of collagen in the affected region. This excess collagen compromises the skin's functionality and mechanical properties. Post-scar formation, treatment approaches often involve surgical interventions for partial correction or, in numerous instances, direct injection of steroids into the scar tissues. Historically, intralesional corticosteroid injections have been a conventional therapeutic approach for scarring conditions. However, the contemporary promotion of creams containing corticosteroids has not demonstrated a comparable level of success in addressing these issues.

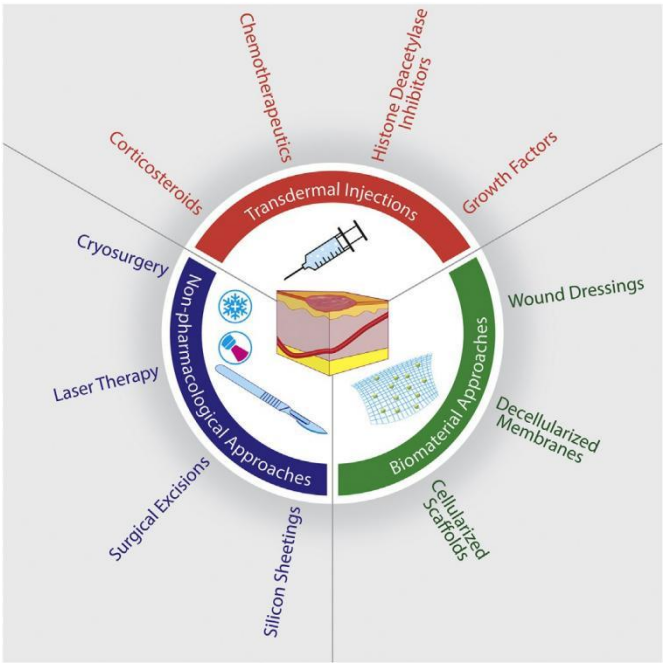

Figure 8. Post-scarring therapies in skin fibrosis to emulate normal remodelling (Coentro et al, 2019)

Corticosteroids, including hydrocortisone acetate, dexamethasone, and methylprednisolone, have been the most widely used. Hepatocyte growth factor (HGF), known for its mitogenic, morphogenic, and anti-apoptotic properties, is being explored as a promising antifibrotic treatment. This consideration arises from its involvement in collagen fibril metabolism and its regulatory impact on TGF-β1. Investigations have extended to studying the effects of HGF through subcutaneous injections, both for the treatment of pre-existing skin scars and the delivery of vectors carrying the HGF gene, particularly in cases of scleroderma.

Skin transplantation via surgical methods is also a way of treatment. This method is far from flawless and frequently results in notable skin abnormalities and undesired cosmetic distinctions for the patient, not to mention the elevated recurrence rates post-surgery. Wound dressings with functionalized properties have demonstrated efficacy in expediting wound healing processes, promoting angiogenesis, regulating the formation of new collagen structures, and enhancing the structure of pre-existing collagen to minimize its bulk around the injury site. When coupled with methods like electrospinning, leveraging electrical forces to craft fibrous scaffolds at a nano to micro scale with precisely controlled porosity, an advanced healing bandage can be manufactured. As understanding of TGF-β's significance in fibrosis grows, there is a heightened interest in exploring TGF-β inhibition as a broad therapeutic strategy aimed at mitigating, arresting, or even reversing fibrotic processes across various organ systems. The majority of therapeutic strategies targeting TGF-β can be categorized into four main groups: (1) small-molecule inhibitors designed to block the activity of TGF-β receptor kinases, (2) monoclonal antibodies intended to hinder the binding of TGF-β to its receptors, (3) ligand traps composed of dimerized ectodomains of type II TGF-β receptors (TβRII, TGFBR2) aimed at sequestering TGF-β and preventing its interaction with receptors, and (4) small molecules or antibodies that specifically disrupt the activation of TGF-β1. While the latter group focuses on achieving target selectivity, the first three classes are anticipated to exert systemic inhibition of TGF-β unless coupled with an antibody that provides target specificity (Budi et al, 2021).

1.8 Probiotics

The term "probiotic," derived from Greek, translates to "for life." This concept was pioneered around 1900 by Nobel laureate Elie Metchnikoff, who observed that the consumption of live bacteria, specifically *Lactobacillus bulgaricus* found in yogurt or fermented milk, led to improvements in various biological aspects of the gastrointestinal tract. The term "probiotic" is believed to have been coined by Ferdinand Vergin in 1954, in his article titled "Anti-und Probiotika," where he examined various microorganisms to compile a list of beneficial bacteria while also investigating the adverse effects of antibacterial agents and antibiotics on the intestinal microbiota. Subsequently, Lilly and Stillwell further defined probiotics as advantageous microorganisms capable of promoting the growth of other beneficial microorganisms (Azad et al, 2018).

Probiotics, defined as living microorganisms offering health benefits when consumed in adequate amounts, primarily consist of Lactic acid bacteria species (LAB) and Bifidobacterium. This definition has been established by the World Trade Organization (WTO) and the Food and Agriculture Organization (FAO) since 2001 and has since gained widespread recognition. LAB encompasses a diverse group of microorganisms, including Lactobacillus, Lactococcus, Enterococcus, and Streptococcus. Lactobacillus (including *L. acidophilus, L. fermentum, L. plantarum, L. casei, L. paracasei, L. reuteri, L. rhamnosus, L. satsumensis, and L. johnsonii*) are the predominant group in the gastrointestinal and digestive systems of both animals and humans. They have demonstrated efficacy in maintaining and restoring health. From a metabolic standpoint, LAB can be categorized into two groups: homofermentative and heterofermentative. Homofermentative LAB exclusively produce lactic acid using the Embden-Meyerhof-Parnas pathway. In contrast, heterofermentative LAB generate not only lactic acid but also other metabolites such as ethanol, acetic acid, and carbon dioxide through the pentose monophosphate pathway. Additionally, LAB have the capability to synthesize secondary metabolites like bacteriocins, exopolysaccharides, and enzymes. These compounds play crucial roles in enhancing the quality and extending the shelf life of fermented foods (de Melo Pereira et al, 2018). These microorganisms naturally inhabit the human intestines and are commonly introduced through the consumption of fermented foods like yogurt, cheese, and other fermented products. Probiotics are known to bolster various defense mechanisms, regulate both innate and adaptive immunity, lower intestinal pH levels, produce antibacterial agents, and aid in the elimination of toxins, carcinogens, and pathogens, either directly or by influencing the host flora. Numerous trials have demonstrated the beneficial effects of probiotics on human health, indicating their potential for disease treatment and prevention (Zheng et al, 2023).

Additional research of probiotics has led to the development of prebiotics. The term prebiotics is defined as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon for improving the host health” (Gibson et al, 1995). Subsequently, an expert panel of the International Scientific Association for Probiotics and Prebiotics (ISAPP) updated the definition of prebiotics in wider perspective as "a substrate that is selectively utilized by host microorganisms conferring a health benefit” (Gibson et al, 2017). These are generally short-chain carbohydrates which escape digestion but are used as substrates for the growth of probiotics in the upper gastrointestinal tract.

High potential is attributed to the simultaneous use of probiotics and prebiotics. In 1995, Gibson and Roberfroid introduced the term "synbiotic" to describe a combination of synergistically acting probiotics and prebiotics. Additionally, there is a growing interest in the application of postbiotics as a safer alternative to probiotics (Zolkiewicz et al, 2020). The term refers to all products obtained from non-viable probiotic microorganisms, including non-viable microbial cells, cell walls, lysates, fractions, secretions, components, and metabolites that, when received in adequate quantities (postbiotic supplements) confer benefit health on the host. Furthermore, the postbiotics used in a delivery system strengthen the endogenous probiotics of each host (Homayouni et al, 2020).

Mechanisms of action of probiotics

The Probiotics can exert numerous positive effects on the human body through complex, heterogeneous, and specific to probiotic strains mechanisms of action: competitive exclusion of pathogens the ability to colonize the intestine (Wang et al, 2018), intestinal barrier function improvement (Gaspar et al, 2018), and immune system modulation, and production of neurotransmitters (Latif et al, 2023).

Competitive exclusion

Competitive pathogen exclusion is a condition in which one bacterial species has a greater potential to attach the epithelia, through a receptor, than other species. One of the key mechanisms for this probiotic function is the decrease of luminal pH, competition for nutritional stores, and production of bacteriocin or bacteriocin-like compounds (Tegegne et al, 2022).

Production of antimicrobial substances

The production of antimicrobial compound is another mode of action through which the probiotics can modulate the microbiota composition in a positive way. The antimicrobial chemical substances are secreted by several species of Lactobacilli and Bifidobacteria and are characterized as 'bacterially generated chemicals with a physiologically active protein moiety and bactericidal activity'. The main bacteriocins are Hydrogen peroxide, diacetyl, and short-chain fatty acids (Prajapati et al, 2023). The general mechanisms of bacteriocin-mediated pathogen killing include the induction of cytoplasmic membrane permeabilization of sensitive bacteria, inhibition of DNA and RNA synthesis, and cell wall protein synthesis.

Bacteriocins possess immunomodulatory properties with pronounced anti-inflammatory effects during pathogenic infections. Several studies showed that certain kinds of probiotics inhibit many types of pathogenic bacteria by the action of their bacteriocins (Mandal et al, 2016). Bacteriocins from *Lactobacillus salivarius* inhibit bacteria, such as *Listeria monocytogenes*, many genera of Staphylococcus, Neisseria gonorrhoeae, Bacillus, and Enterococcus. *Lactobacillus plantarum* also exerts antimicrobial activities by producing many types of bacteriocins with antimicrobial effects against food spoilage bacteria, such as *Alicyclobacillus acidoterrestris* (Borrero et al. 2017), *Listeria monocytogenes, Staphylococcus aureus*, and *Escherichia coli* (Oleskin et al, 2019). Apart from bacteria, some bacteriocins from *L. plantarum* are also effective against yeast and molds, such as Fusarium, Candida, and Aspergillus. Bacteriocins of other probiotic species markedly induce apoptosis and inhibit tumor formation, cancer cell proliferation, and membrane depolarization of cancer cells during treatment (Baindara et al, 2018).

Modulation of immune system

Probiotic bacteria can exert regulatory effects on host innate and adaptive immune responses increasing immunoglobulin IgA production (Raghuwanshi et al, 2018), the numbers of natural killer cells, or improving the phagocytic activity of macrophages (Aziz and Bonavida, 2016).

Moreover, the probiotics can affect the function of the intestinal barrier modulating signal transduction pathways and gene expression in epithelial and immunological cells.

By stimulating of the host immune responses, probiotics can displace pathogens in the gastrointestinal tract and prevent intestinal diseases. Probiotics are able to trigger an anti-inflammatory response by signaling DCs to secrete anti-inflammatory cytokines such as interleukin 10 (IL-10). Moreover, they can also downregulate the secretion of pro-inflammatory cytokines during inflammation (van Zyl et al, 2020). NF-κB and MAPK pathways are activated by enteric pathogens to stimulate the secretion of pro-inflammatory cytokines, that lead to the recruitment of inflammatory immune cells to the infection sites resulting in severe inflammation, tissue damage, and disease (Llewellyn et al, 2017). The ability of probiotics to inhibit the inflammatory signaling, above reported, determines the downregulation of pro-inflammatory cytokine secretion from immune cells. Several studies have identified probiotic strains with the ability to suppress the production of pro-inflammatory cytokines to avoid pathogen-induced inflammation at infection sites (van Zyl et al, 2020).

Intestinal barrier function improvement

The intestinal epithelial barrier acts as a physical and biochemical barrier and is important to prevent the systemic entry of toxins, bacteria, and other foreign unwanted compounds. It has been reported in many studies that LAB can improve intestinal epithelial barrier damage induced by pathogenic infection (Heeney et al, 2019). Strains of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* upregulate tight junction proteins (occludin, claudin-1) resulting in enhanced barrier stability (Wang et al, 2014). *Lactobacillus plantarum* WCFS1 significantly increases occludin and zonulin-1 (ZO-1) expression by Toll-like receptor 2 (TLR2) dependent pathway and protects against tight junction disruption by toxins, pathogens, and cytokines (Karczewski et al, 2010). Qin *et al*. also showed that *L. plantarum* has protective effects on the intestinal barrier by rearranging tight junction proteins (occludin, claudin-1, and ZO-1) damaged by *Escherichia coli* and ameliorates barrier function (Qin et al, 2009). E. coli Nissle 1917 ameliorates E. coli induced intestinal epithelial barrier dysfunction by regulating the expression of occludin and claudin (Alvarez et al, 2019). *Lactobacillus rhamnosus* and *Lactobacilluus fermentum* significantly improve the *E. coli* disturbed tight junction proteins (occludin, ZO-1, cingulin-1, claudin-1) in Caco-2 cells (Bhat et al, 2020). Several studies on *Lactobacilli* have also shown that they ameliorate the intestinal barrier damage and pro-inflammatory cytokines production induced by *Salmonella* (Wang et al, 2019).

1.9 Applications of probiotics in the skin disorders

In the last ten years, the therapeutic applications of probiotics have expanded strongly not only supporting the health of the gut. Indeed, considerable research is aimed at assessing the effectiveness of selected strains in a wide range of health concerns. Beyond the gut, studies have highlighted the ability of probiotics in countering allergic responses, skin-related conditions, and even mental health challenges.

In this context, probiotics have been extensively explored in intervention studies aimed at preventing and/or treating different skin conditions like atopic dermatitis (AD), allergic rhinitis, and wound healing, which represent significant areas of focus (Di Marzio et al, 1999; Di Marzio et al, 2003; Cinque et al, 2006; Di Marzio et al, 2008; Guéniche et al, 2008; Kang et al, 2009; Peral et al, 2009; Guéniche et al, 2010; Peral et al, 2010; Muizzuddin et al, 2012; Zoccali et al, 2016;

Blanchet-Réthoré et al, 2017; Nakatsuji et al, 2017; Twetman et al, 2018, Lolou et al, 2019) (Figure 9).

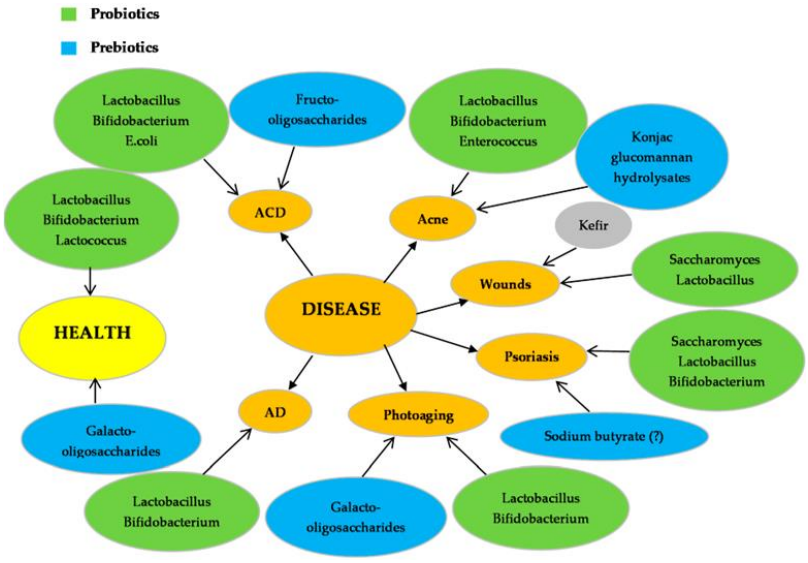

Figure 9. The role of probiotics and prebiotics on skin health and disease (Lolou et al, 2019)

Probiotic bacteria with anti-inflammatory properties have beneficial effects on people suffering from neurogenic skin inflammation or autoimmune skin diseases. Also, probiotics can be helpful for non-healing wounds thanks to the ability to protect the keratinocytes from oxidative stress or to induce skin re-epithelization. Moreover, it has been demonstrated that specific probiotic strains counteract skin aging by reversing photodamage. This finding underlines the potential of probiotics as supporting agents in skin health. However, additional clinical studies are needed to support their routine utilization in medical practice.

It is essential to keep in mind that on cutaneous or mucosal surfaces, the microorganisms present form the autochthonous microbiota, which serves a significant function in both maintaining health and contributing to the development of disease pathological conditions (Moreira et al, 2021). The diverse array of commensal bacteria residing on the skin significantly impacts the immune response within wounds and plays a vital role in maintaining the integrity of the epithelial barrier function. Additionally, these microorganisms may offer protection against bacterial wound infections or limit their severity. The influence of the skin microbiota on wound healing is mediated through various mechanisms, which can vary depending on the specific bacterial species present. These mechanisms encompass a wide range of processes within the skin, including

regulation of keratinocyte proliferation, modulation of epithelial differentiation, promotion of epidermal blood vessel growth, and modulation of cell signaling pathways (Zielińska et al, 2023). The skin ecosystems consist of a myriad of microorganisms that engage in intricate interactions with the human body, including epithelial and immune cells, as well as other microbes inhabiting the same environment. In order to establish the microbiota, the skin furnishes crucial nutrients derived from various sources, such as amino acids resulting from protein hydrolysis, fatty acids obtained from the stratum corneum, sweat, lipid breakdown, or sebum, and lactic acids originating from sweat glands. Microbiota colonization initiates shortly after birth, with its makeup being shaped by a multitude of both intrinsic and extrinsic factors. Intrinsic influences include characteristics such as skin site, age, ethnicity, gender, and interpersonal variability, while extrinsic factors encompass lifestyle choices, cosmetic usage, antibiotic consumption, hygiene practices, climate conditions, seasonal variations, and geographical location. The composition of the skin microbiota varies not only from person to person but also across different regions of the body (De Almeida et al, 2023). Of interest, probiotics, well known for their important ability to interact with the gut microbiota through various mechanisms, are also studied for their role in maintaining microbial balance in other organs like the skin when topically or orally applied (Bustamante et al, 2020). Research findings have recognised a link between the impaired gut microbiota and inflammatory skin diseases, thereby reinforcing the potential of oral probiotic administration for skin disorders (Szántó et al, 2019). Moreover, probiotic bacteria through topical applications are able to affect the skin's natural defense mechanisms and promote the production of antimicrobial peptides, contributing to its health. Even if the scientific evidence and clinical studies aimed at the study of effectiveness of topically applied probiotic products are still limited (Abdi et al, 2024), the demonstration of the beneficial effects of probiotics on skin health continues to grow in several papers and patents. The topical probiotic application is evaluated for the management of several skin disorders such as acne, atopic dermatitis, psoriasis, ageing, seborrheic dermatitis, altered wound healing. However, the precise mechanisms by which probiotics augment skin health have not yet been fully clarified.

Even though a few studies have evaluated the effects of probiotics on scar formation, probiotics might also carry out a positive effect directly in fibrotic tissue associated with impaired wound healing, such as hypertrophic scar and keloids. The ability of *Lactobacillus plantarum*, topically applied, to alleviate the infection and reduce the scarring of burn wounds, both conditions often

associated with the development of a hypertrophic scar, has been described (Baker et al. 2007). Moreover, a recent study proved that the oral administration of *Lactobacillus rhamnosus* is associated with accelerated wound closure, as well as with the alleviation of scar formation in a murine model of excisional skin wounds (Moreira et al, 2021).

Recently, Lombardi et al. reported the first experimental evidence relative to the ability of the Vivomixx® high-concentration, multi-strain probiotic formulation to counteract the TGF-β1-induced fibrotic phenotype in colonic intestinal fibroblast cells (CCD-18Co cell line). TGF-β1-activated CCD-18Co treated with this probiotic product showed a significant reduction of collagen-I and α-SMA expression due to interfering with Smad2/3 signaling and counteracting TGF-β1 neosynthesis (Lombardi et al, 2021). Of interest, previous results of research group of Professor Cifone, obtained from *in vivo* studies, highlighted the beneficial anti-inflammatory and anti-aging efficacy of the *S. thermophilus* DSM 24731, one of the components of Vivomixx® formulation. Moreover, *S. thermophilus* was able to significantly increase the skin ceramide levels in healthy subjects when topically applied with a cream containing its lysate (Di Marzio et al, 1999). The increase of stratum corneum ceramide levels, underlying the improvement in barrier function and preservation of stratum corneum elasticity, could be justified by the presence of sphingomyelinase activity in this probiotic strain. Also, the same group demonstrated that topical application of *S. thermophilus* in patients suffering from atopic dermatitis promoted a significant improvement in erythema, scaling, and pruritus (Di Marzio et al, 2003). Subsequently, it was also demonstrated the ability of the topical treatment of an *S. thermophilus*-containing cream to increase the ceramide level on the stratum corneum of healthy elderly women as well as to decrease the transepidermal water loss and capacitance of the treated skin (Di Marzio et al, 2008). In another study, the beneficial effects of topical application of *S. thermophilus* in slowing the process of photoaging, decreasing oxidative stress, and ameliorating the skin barrier function were also reported (Zoccali et al, 2016).

2 AIM OF THE STUDY

The fibrosis process can occur in almost all organs, such as the lungs, liver, heart, gut and skin. There is broad agreement between the pathological mechanisms of different fibrotic diseases, even if there are some differences. In the skin, the fibrosis belongs to a class of conditions triggered by impaired tissue regeneration and faulty repair and its main feature is the excessive deposition of ECM in the skin. The skin fibrosis is the clinical manifestation of many diseases with different etiology involving physical (radiation or mechanical stimulation), chemical, biological, and immune factors.

Several signaling pathways such as transforming growth factor-β (TGF-β) and Hippo signaling pathway are involved in skin fibrosis and shared with the other organs.

Today, several treatments derived by basic and clinical research are accessible to patients with skin fibrosis, such as surgical, drug and combination treatments. However, data on the efficiency of these approaches are still preliminary and show limited efficacy; it is, therefore, necessary to develop more satisfactory therapies as the targeted therapies able to reduce side effects and improve the efficacy of treatment. The main anti-fibrotic therapies are developed to target TGF-β1 signaling and modulate profibrotic mediators. Although potentially potent, such therapeutic approaches are usually expensive and not free from adverse events. Numerous studies have demonstrated the potential beneficial effects of selected probiotic strains on pathophysiological alterations of the skin and on skin health improvement through different mechanisms, including modulating the skin microbiota, maintaining immune responses, inducing an anti-inflammatory effect, suppressing oxidative stress, restoring skin barrier function, and releasing bioactive molecules able to inhibit the growth of pathogens.

In this context, previous *in vitro* and *in vivo* studies have demonstrated the ability of *S. thermophilus* lysate to increase the ceramide levels in human keratinocytes and in the skin of healthy subjects or elderly women, thus leading to an improvement in the lipid barrier and flexibility of stratum corneum. Therefore, the main objective of this thesis project has been to evaluate the anti-fibrotic potential of *S. thermophilus* lysate on normal human dermal fibroblasts (NHDFs) activated by TGF-β1. In particular, NHDFs cultured in the presence of TGF-β1 to induce the development of a fibrotic phenotype, were exposed to different concentrations of probiotic

lysate, alone or combined with TGF-β1, and the main parameters and markers associated with TGF-β1-induced fibrotic phenotype were evaluated.

3 MATERIALS AND METHODS

3.1 Preparation of Bacterial Samples for Cell Treatments

Streptococcus thermophilus DSM 24731® in a pure lyophilized form was generously provided by Prof. Claudio De Simone, MD. To prepare the bacterial fraction, stocks of $600x10^9$ CFU/g of lyophilized probiotic resuspended in Phosphate Buffered Saline (PBS, Euro Clone, West York, UK) underwent centrifugation at 8600 ×g, followed by two washes and resuspension in 10 ml of PBS. Subsequently, sonication was performed for 30 minutes, with alternating 10-second bursts of sonication and 10-second pauses, using a Vibracell sonicator (Sonic and Materials, Danbury, CT, USA). The sample's absorbance at 590 nm was measured before and after each sonication step using an Eppendorf spectrophotometer (Hamburg, Germany) to assess bacterial cell disruption. Following sonication, the samples were centrifuged at 17,949 × g, and the resulting supernatants were filtered through a 0.22-µm-pore filter (Corning Incorporated, Corning, NY, USA) to remove any remaining whole bacteria and obtain the bacterial lysate. The total protein content was determined using the DC Protein Assay (BioRad, Hercules, CA, USA) with bovine serum albumin as the standard.

For cell treatments, the bacterial lysate was prepared to achieve final concentrations of 25, 50, or 100 µg protein/ml, corresponding to $1.87x10^8$ CFU/ml, $3.75x10^8$ CFU/ml, and $7.5\ x10^8$ CFU/ml, respectively.

3.2 Cell Line and Culture Conditions

Normal adult primary human dermal fibroblast (NHDF, Adult CC-2511) cell line, sourced from Lonza/Cell Applications (Basel, Switzerland), was cultured at early passages (N=6-9) in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. Culturing was performed in a controlled environment with 95% air and 5% CO_2 at 37°C. All necessary reagents and consumables for cell biology were procured from EuroClone (EuroClone, West York, UK), unless stated otherwise. Upon reaching 80% confluence, the cells underwent sub-culturing, and experiments commenced at the 15th passage.

3.3 Cell treatment and Analysis of Cell Growth and Viability

The assessment of cell growth was conducted using the IncuCyte® Live Cell Imager system (Essen BioSciences, Inc., Ann Arbor, MI, USA) for continuous monitoring of cell confluence in real-time. Initially, cells were seeded in a 96-multiwell culture plate at a density of $2.5x10^3$ cells/cm^2

and allowed to adhere overnight. Following this, cells were subjected to a 24-hour starvation period in DMEM containing 0.5% FBS. Subsequently, the cells were exposed to various treatments, including TGF-β1 (10 ng/ml), lysate from *S. thermophilus* (at concentrations of 25, 50, or 100 µg protein/ml), or combinations thereof, in DMEM supplemented with 0.5% FBS. The culture plates were then positioned within the IncuCyte® Live Cell imager, capturing images at 4-hour intervals over a 72-hour period using the phase-contrast channel. Multiple image sets were acquired from different locations within each well using a 10X objective lens, with all treatment conditions performed in triplicate. Upon completion of the experiment, the percentage of cell confluence was quantified and analyzed using the IncuCyte ZOOM™ software (Essen BioSciences, Inc.), serving as an indicator of proliferation rate.

For all subsequent experiments, NHDF were seeded at a density of 8000 cells/cm2, allowed to grow overnight, and then subjected to a 24-hour starvation period in DMEM containing 0.5% FBS. Following this, cells were stimulated for 48 hours with human transforming growth factor (hTGF-β1; obtained from Cell Signaling Technology, MA, USA) at a concentration of 10 ng/ml to induce the fibrotic phenotype. Stimulation was carried out in the presence or absence of bacterial fraction from S. thermophilus at varying final concentrations (25, 50, or 100 µg protein/ml) to observe their effects. Post-treatment, cells were harvested, centrifuged for 10 minutes at 400 ×g, and incubated with 0.04% trypan blue (procured from Euro Clone, West York, UK) for 5 minutes to assess cell number and viability. Untreated cells were utilized as controls, and cell counting was performed using a Bürker chamber under microscopy (Eclipse 50i, Nikon Corporation, Tokyo, Japan).

3.4 Scratch Wound Healing Assays

To assess the impact of *S. thermophilus* lysate at varying concentrations on TGF-β1-induced migration in NHDF, a scratch assay was carried out following established procedures [40]. Initially, cells were seeded at a density of $20x10^4/cm^2$ in multiwell plates and allowed to grow until reaching confluence. Once confluence was achieved, the DMEM was aspirated, and the cell monolayers were carefully scratched using a 200 µl pipet tip to create a standardized wound. Following scratching, the cells were gently washed with PBS to remove any debris, and then treated with TGF-β1 (10 ng/ml) either alone or in combination with bacterial lysate (at concentrations of 25, 50, or 100 µg protein/ml).

Subsequently, images capturing cell migration were acquired using an inverted light microscope (Eclipse TS 100, Nikon) at 10X magnification at various time points post-injury (0-48 h). The

experiments were performed in duplicate, with six fields analyzed for each condition. Quantitative analysis of the images was conducted using the standalone TScratch software, which employs a mathematical model to automatically determine the percentage of wound closure based on the area occupied by migrating cells.

3.5 Western Blot Analysis

To conduct protein analysis via western blotting, the collected cell pellets underwent a series of steps. Initially, they were washed in phosphate-buffered saline (PBS) and then lysed using RIPA buffer, which contained a mixture of protease inhibitors to prevent protein degradation. The protein concentration within the samples was determined using the DC Protein Assay kit, with bovine serum albumin (BSA) serving as the standard for calibration. Subsequently, 25 μg of proteins were combined with sample buffer and subjected to boiling to denature the proteins and ensure uniformity in their mobility during gel electrophoresis. The proteins were then separated based on their molecular weight by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE).

Following electrophoresis, the separated proteins were transferred onto a nitrocellulose membrane via a process known as electroblotting. This transfer, conducted at a constant voltage, enabled the proteins to immobilize onto the membrane surface, preserving their spatial arrangement from the gel. To prevent non-specific binding of antibodies and reduce background noise, the membrane was blocked with 5% non-fat dry milk solution for an hour at room temperature. Once adequately blocked, the membrane was then incubated overnight at 4°C with primary antibodies specific to the proteins of interest. These antibodies included a rabbit monoclonal antibody against human phospho-Smad2/3, a mouse monoclonal antibody against α-actin smooth muscle (ACTA2), a rabbit monoclonal antibody against Fibronectin (FN1), a rabbit polyclonal antibody against COL1A1, a rabbit polyclonal antibody against β-catenin, a rabbit monoclonal antibody against PPARγ, and a mouse monoclonal antibody against GAPDH. Following primary antibody incubation, the membrane underwent a series of washes to remove excess unbound antibodies. Next, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies, specific to the host species of the primary antibodies. This secondary antibody binding enabled the detection of primary antibody-bound proteins through chemiluminescent reactions, facilitated by the application of enhanced chemiluminescence (ECL) reagents. Finally, the immuno-reactive protein bands were visualized using a chemiluminescence documentation

system, and band intensities were quantified using imaging software. To ensure accurate quantification, band densities were normalized relative to the intensity of GAPDH bands, a commonly used loading control. The resulting values were expressed in relative units, providing insights into the protein expression levels within the samples.

3.6 Immunofluorescence Staining for Fibrotic Markers

NHDF were cultured on coverslips in a 12-well plate at a seeding density of 8x103 cells/cm2 and subjected to the specified treatments for the indicated durations. Following treatment, the coverslips were rinsed with PBS, fixed with 4% formaldehyde for 20 minutes, permeabilized with 0.1% Triton X-100 for 5 minutes, and blocked with 3% BSA for 20 minutes at room temperature. Subsequently, the coverslips were incubated overnight at 4°C with primary antibodies: mouse monoclonal antibody anti-α-actin smooth muscle (ACTA2, α-SMA) (OriGene) at a dilution of 1:250, rabbit monoclonal anti-fibronectin/FN1 (Cell Signaling Technology) at a dilution of 1:200, or rabbit polyclonal antibody anti-COL1A1 (Boster Biological Technology) at a dilution of 1:250. Following primary antibody incubation, the coverslips were stained using FITC-conjugated goat anti-rabbit polyclonal IgG secondary antibody (Millipore EMD) at a dilution of 1:1000 or FITC-conjugated goat anti-mouse polyclonal IgG secondary antibody (Bethyl Laboratories, Inc.) for 1 hour at room temperature. Additionally, all samples were treated with TRITC-labeled phalloidin (Sigma-Aldrich) for 45 minutes at room temperature to visualize actin filaments. Finally, the coverslips were mounted using VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Inc.) and observed under fluorescence microscopy at 100× magnification (Eclipse 50i, Nikon).

3.7 Collagen gel retraction assay

To assess the contraction capacity of NHDF, the cells were seeded into three-dimensional collagen lattices based on a previously described technique (Ngo et al, 2006). Briefly, the acid-extracted type I collagen (5 mg/ml) mixture was prepared on ice with rat tail collagen I (Enzo Life Sciences, Lausen, Switzerland) in acetic acid 0.2% and then diluted at 3 mg/mL in filter-sterilized water. NHDF were resuspended in complete media at $8x10^5$ cells/ml.

Resuspended cells ($8x10^4$/100 µl) were added to 300 µl complete media, 200 µl collagen mixture (3 mg/ml) and brought to neutral pH with NaOH on ice. 500 µl of the collagen/cell mixture were added to each well of a pre-warmed 12-well non-tissue culture treated plate (Corning Incorporated, Corning, NY, USA). Gels polymerized at 37°C for 1 h and covered with media containing or not

10 ng/ml TGF-β1, *S. thermophilus* lysate (25, 50, or 100 µg protein/ml) and their combination. After the addition of treatments, the gels were carefully detached from the well by gently running the tip along the gel edges. Lattices were imaged with a digital camera at a fixed distance above gels, to obtain an image before release and at multiple times after release. Lattice area was measured with ImageJ and normalized to pre-release area (T0).

3.8 Total RNA Extraction and Quantitative Real-time PCR (qPCR)

Gene expression analysis of TGF-β1 was conducted in untreated (CNTR) and treated NHDF, following the same protocol as for protein expression experiments. Total RNA was extracted from cells using the RNeasy Mini Kit (QIAGEN) as per the manufacturer's guidelines and quantified using spectrophotometry. Subsequently, 1 µg of total RNA was reverse transcribed into cDNA in a final volume of 20 µL using a mixture of random primers. For real-time PCR, 0.5 µg of each cDNA was utilized. The analysis was performed using SYBR Green dye detection (Thermo Fisher Scientific) according to the manufacturer's instructions. Reverse and forward primers, procured from IDT, were employed at a concentration of 1 µM for hTGF-β1. The primer sequences for hTGF-β1 were as follows: forward 50-CAACGAAATCTATGACAAGTTCAAGCAG-30 and reverse 50-CTTCTCGGAGCTCTGATGTG-30. Additionally, in NHDF, GAPDH mRNA levels were measured for gene expression normalization using the following primers: GAPDH forward 50-TTGCCCTCAACGACCACTTT-30 and reverse 50-TGGTCCAGGGGTCTTACTCC-30.

The fold-change quantification of target genes was determined using the $2^{-\Delta\Delta Ct}$ method. Each sample was run in triplicate, and the experiment was replicated twice.

3.9 Statistical Analysis

The data underwent thorough statistical analysis using GraphPad Prism version 6.01 (GraphPad Software, San Diego, CA, USA). To compare mean values across groups, either a one-way ANOVA or a two-way ANOVA was employed, followed by the Tukey post hoc test for further comparisons.

Results are presented as mean ± SD or mean ± SEM, reflecting the variability and precision of measurements from three independent experiments performed in triplicate. Statistical significance was attributed to P values ≤ 0.05, ensuring robust conclusions.

4 RESULTS

4.1 Effect of *S. thermophilus* lysate on TGF-β1-induced cell proliferation and migration of NHDF

Cell proliferation was dynamically monitored every 6 h over a 72-h time period in NHDF monolayers, previously incubated with 0.5 % FBS supplemented medium for 24 h and then stimulated with TGF-β1 at 10 ng/ml, alone or in presence of *S. thermophilus* lysate at 25, 50, or 100 µg protein/ml. As showed in Figure 10A, the TGF-β1-induced activation of NHDF was confirmed. The treatment with TGF-β1 at 10 ng/ml also enhanced the proliferation rate expressed as cell confluence percentage by day 1, producing an effect that increased over the 72 h (~50% more than the untreated control cells). When the cells were exposed to *S. thermophilus* lysate plus TGF-β1, the percentage of cell confluence significantly decreased in time- and concentration-dependent manners with respect the TGF-β1 alone assuming values comparable to those determined for basal levels. Such findings provide evidence that bacterial lysate was able to inhibit TGF-β1-induced proliferation (Figure 10A). Differently, the bacterial lysate alone did not significantly affect the cell proliferation rate, which remained similar to the basal level in control cells.

Evaluation of the impact of *S. thermophilus* lysate on cell number and viability gave similar results. After the treatment with different concentrations of bacterial lysate, no change has been observed in the cell number of the control group, while the addition of *S. thermophilus* significantly inhibited the increase in the number of NHDF induced by TGF-β1 at 48 h (Figure 10B). Notably, the observed effect was more evident by exposing cells to 50 and 100 µg/ml of bacterial lysate. The microscopic images of the cell density under the different treatment conditions confirmed the reduction in cell number contemporary highlighting that both the parallel oriented spindle-like shape, as well as the evident increase of cell density in the presence of TGF-β1, were clearly affected by co-treatment with bacterial lysate (Figure 10B). The quantitative *in vitro* analysis of wound-closure rate showed that the closure percentage at 24 and 48 h increased in TGF-β1-activated NHDF compared with the control, while it decreased in a dose-dependent manner after the co-treatment with *S. thermophilus* lysate (Figure 10C). Representative microscopy images taken at 48 h are also shown (Figure 10C).

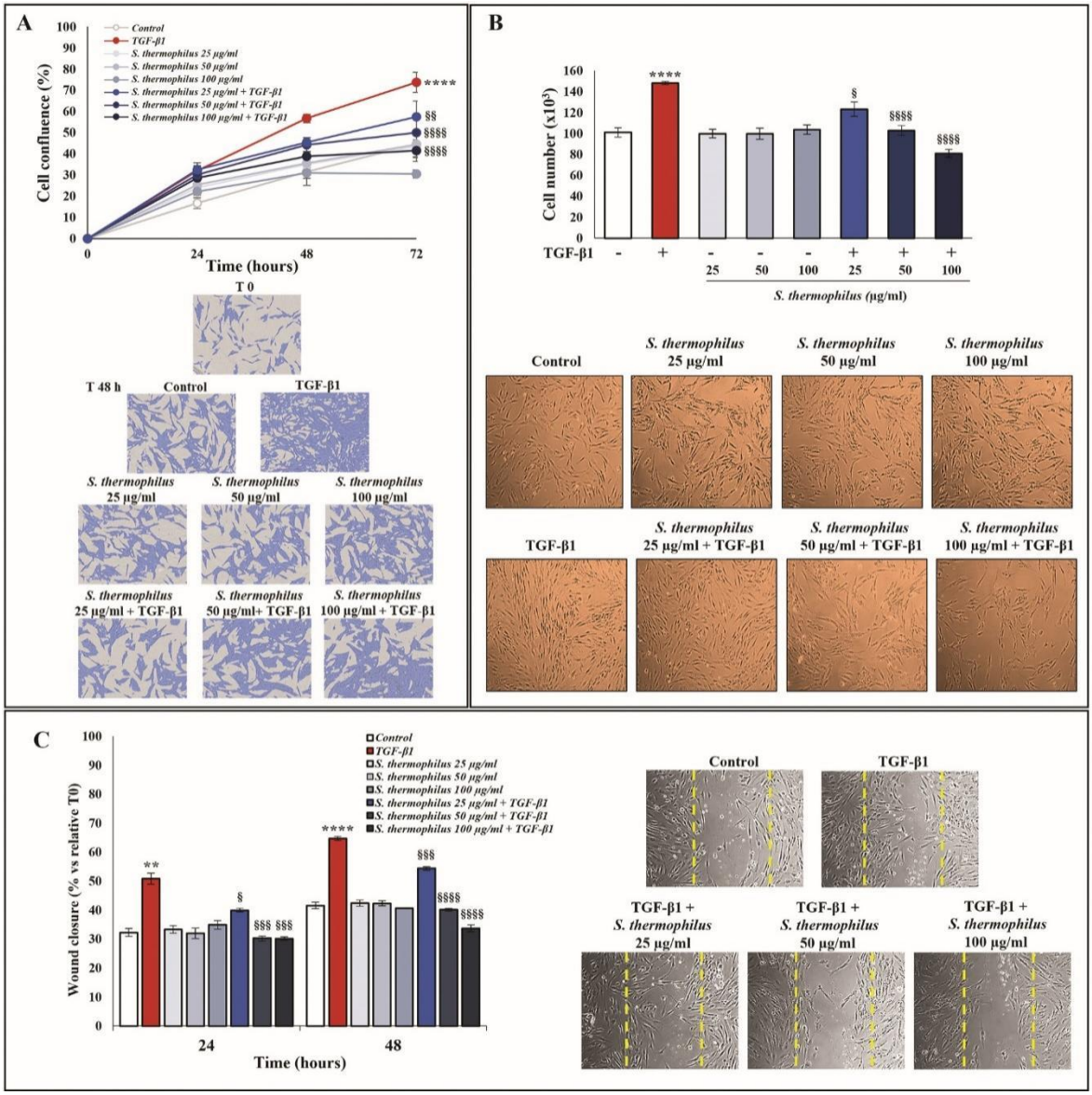

Figure 10. Effect of *S. thermophilus* lysate on TGF-β1-induced proliferation and migration of NHDF. (A) Growth curves of NHDF were analyzed as cell confluence percentage through IncuCyte® Live Cell Imager and monitored dynamically from 0 h to 72 h. NHDF were starved for 24 h and then incubated until 3 days with TGF-β1 (10 ng/ml) in the presence or absence of *S. thermophilus* lysate (25, 50 or 100 µg/ml), in 0.5% FBS medium. Results relating to one representative out of three experiments performed in triplicate, are expressed as mean ± SD. **(B)** NHDF were treated for 48 h as reported in A and cell number was counted by trypan blue staining. The results, derived from three experiments performed in duplicate, are expressed as mean ± SEM. **(C)** Quantitative analysis of wound healing assay in NHDF treated as above in A. The wound closure was captured at 0, 24, and 48 h after scratch generation and expressed as the wound closure rate (% vs. relative T0) of scratched monolayers. Values are expressed as mean ± SEM of two independent experiments in duplicate. In each case, the comparative analysis of groups of data has been performed by the two-way analysis of variance (ANOVA) followed by post hoc Tukey's test (** $P < 0.01$, **** $P < 0.0001$ vs. CNTR; § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$, §§§§ $P < 0.0001$ vs TGF-β1). Representative images of cell confluence, cell number and NHDF monolayer re-epithelialization (the yellow dashed lines indicate wound edges at T0) are inserted in A, B and C respectively.

4.2 Effects of *S. thermophilus* lysate on TGF-β1-induced myofibroblast phenoconversion

The degree of skin fibrosis can be determined by the expression of typical markers of phenoconversion of fibroblasts into myofibroblast such as α-SMA and fibronectin and by cytoskeleton re-organization (Frangogiannis et al, 2020). Thus, the effect of *S. thermophilus* lysate was evaluated on the α-SMA expression in TGF-β1-activated NHDF. Western blot analysis indicated that α-SMA protein levels were significantly up-regulated compared with the control cells (Figure 11A). The addition of the bacterial lysate is associated to significant marked dose-dependent decrease of α-SMA expression in NHDF exposed to TGF-β1 for 48 h. The immunofluorescence analysis confirmed that in response to TGF-β1 activation, the network of actin-myosin bundles stained by phalloidin (red fluorescence) was highly decorated with α-SMA, as observed by intense and overlapped yellow/orange fluorescence. On the other hand, the addition of the bacterial lysate caused a weaker fluorescent signal relative to α-SMA, restraining the myofibroblast phenoconversion by TGF-β1 (Figure 11B).

Immunoblotting and immunofluorescence analysis results showed that fibronectin protein expression followed a pattern similar to that of α-SMA characterized by a significant increase in TGF-β1-treated NHDF compared to control. The exposure to *S. thermophilus* lysate was associated to a dose-dependent significant decrease in the stimulatory effect of TGF-β1 also on fibronectin expression concerning either protein quantity or determined positive cells (Figure 11C and D). No effects on α-SMA and fibronectin expression were associated with the exposure to the *S. thermophilus* lysate alone.

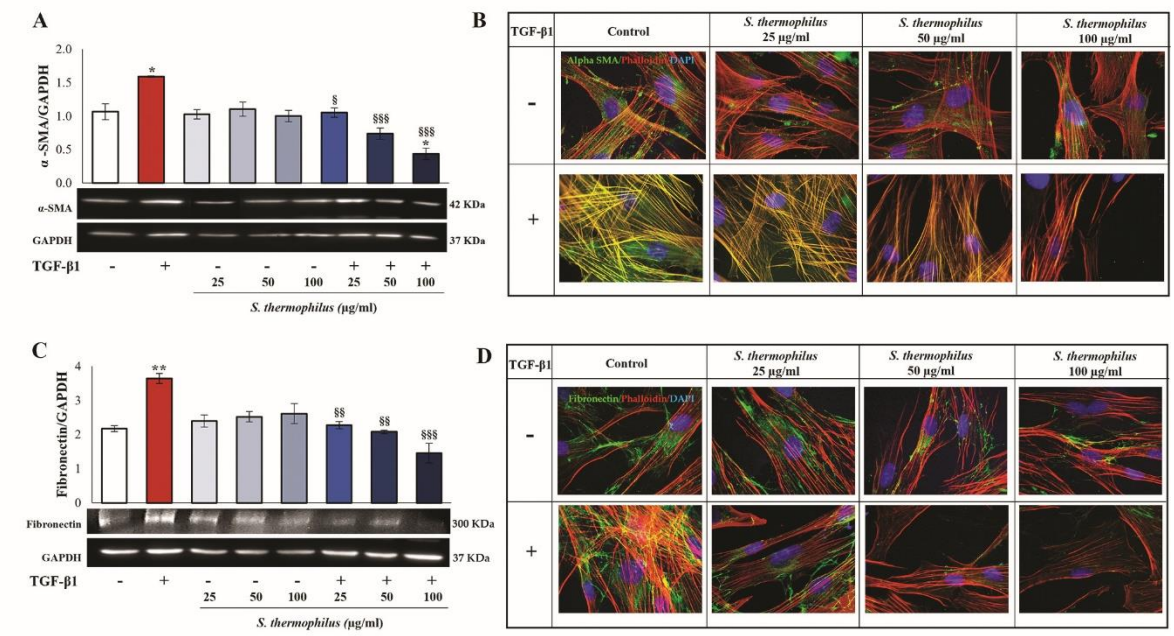

Figure 11. Effects of *S. thermophilus* lysate on TGF-β1-induced myofibroblast phenoconversion. Immunoblotting assays for **(A)** α-SMA and **(C)** fibronectin were performed on NHDF treated as previously reported. Densitometric analysis was performed by normalizing vs. GAPDH. Values are expressed as mean ± SEM of three independent experiments. For comparative analysis of data, a one-way analysis of variance (ANOVA) with post hoc Tukey's test was used. (* $P < 0.05$, ** $P < 0.01$ vs. CNTR; § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$ vs TGF-β1). Representative images of each immunoblotting are shown. Representative immunofluorescence images of NHDF stained with anti-α-SMA antibody **(B)** (green) or anti-fibronectin antibody **(D)** (green) and with TRITC-phalloidin (red) to reveal F-actin. Nuclei were counterstained with DAPI (blue) (magnification 100 x). The images are representative of three independent experiments in duplicate.

4.3 Ability of *S. thermophilus* lysate to affect collagen I production in the TGF-β1-actived NHDF and ECM remodeling

We next studied if the *S. thermophilus* lysate affected the ability of TGF-β1-activated NHDF to produce collagen I, as a mark of extracellular matrix deposition. As shown in Figure 12A, western blot analysis revealed that NHFD exposed to TGF-β1 (10 ng/ml) for 48 h and showing a change in their phenotype towards activated myofibroblasts, expressed higher levels of Coll I when compared to the control. Conversely, the exposure to *S. thermophilus* lysate (50 and 100 μg/ml) for 48 h led to a marked decrease of the Coll I level in TGF-β1-activated NHDF. Immunofluorescence analysis confirmed these results (Figure 12B). TGF-β1-treated cells appeared intense with Coll I stain, while the *S. thermophilus* lysate at the two highest concentrations induced a substantial reduction of staining for Coll I, to the extent where the relative fluorescent signals appeared to be weak and diffuse similarly to the untreated cells. The lower 25 μg/ml concentration of bacterial lysate slightly reduced the TGF-β1-induced effect at 48 h.

To investigate the functional consequences of *S. thermophilus* lysate exposure on the contractile machinery of NHDF, a 3D collagen gel contraction assay consisting of cell-collagen I matrices was used to mimic the exertion of traction forces representing a critical feature of activated myofibroblasts. Obtained results showed that TGF-β1 stimulation for 48 h led to a marked decrease in the size of the collagen lattices, as a sign of collagen gel shrinkage, compared with the control group, indicating the increased contractility of fibroblasts embedded in collagen lattices (Figure 12C and D). Of note, in presence of *S. thermophilus* treatment, the decrease in the collagen lattice size induced by TGF-β1 was hindered in a concentration-dependent manner, thus supporting the effect of the bacterial lysate on the collagen contraction ability of activated dermal fibroblasts. No statistically significant differences were observed between the retraction profiles determined for controls and NHDF treated with *S. thermophilus* alone. Similarly, their diameters did not decrease respect to the T0 considering a similar area (Figure 12D).

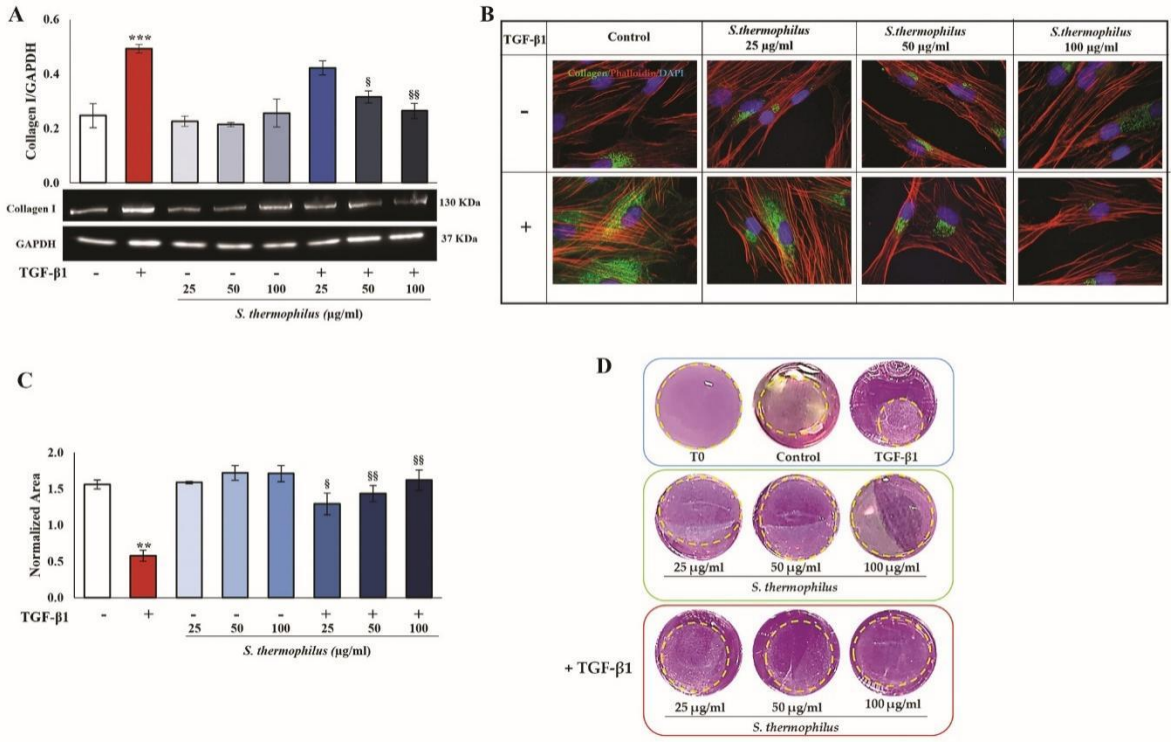

Figure 12. Effects of *S. thermophilus* lysate on collagen I production in TGF-β1-activated NHDF and ECM remodeling. (A) Immunoblotting assay for collagen I was performed on NHDF treated as previously described. Following densitometric analysis, obtained values were normalized vs. GAPDH. Values are expressed as mean ± SEM of three independent experiments performed in duplicate. Images from one representative out of three independent experiments are presented. **(B)** Representative immunofluorescence images of NHDF stained with anti-collagen I antibody (green) and with TRITC-phalloidin (red) to reveal F-actin. Nuclei were counterstained with DAPI (blue) (magnification 100 x). The images are representative of three independent experiments in duplicate. **(C)** Collagen gel

retraction assay of NHDF-populated collagen lattices. The gel contraction was digitally photo-documented, and the gel area was measured with ImageJ and normalized to pre-release area (T0). Values of normalized area are expressed as mean ± SEM of two independent experiments in duplicate. **(D)** Representative images of collagen gel pre-release (T0) and taken 48 h after treatments are shown. In all cases, the comparative analysis of data has been carried out by using one-way analysis of variance (ANOVA) followed by post hoc Tukey's test (** $P < 0.01$ vs. CNTR; *** $P < 0.001$ vs. CNTR; § $P < 0.05$, §§ $P < 0.01$ vs TGF-β1).

4.4 Effects of *S. thermophilus* lysate on TGF-β1-induced Smad activation

In the canonical pathway, TGF-β1 acts through Smad signaling responsible for controlling collagen deposition and fibrogenesis (Zhang et al, 2020). As expected, the treatment of NHDF with TGF-β1 induced phosphorylation of Smad2/3 at 48 h (Figure 13A). The simultaneous addition of probiotic prevented the TGF-β1-induced activation, thus keeping Smad2/3 phosphorylation levels in line with those of the untreated control.

Moreover, TGF-β1/Smad signaling pathway contributed to fibrotic phenotype promoting the induction of TGF-β1 gene transcription, as shown by qRT-PCR analysis, confirming previous studies (Dabiri et al, 2006; Liu et al, 2019; Juhl et al, 2020). Of note, the significant increase of TGF-β1 mRNA expression seen in activated-NHDF was strongly reduced by co-treatment with bacterial lysate for 48 h at all used concentrations (Figure 13B). No effect on TGF-β1 gene expression was observed when the cells were treated with *S. thermophilus* lysate alone.

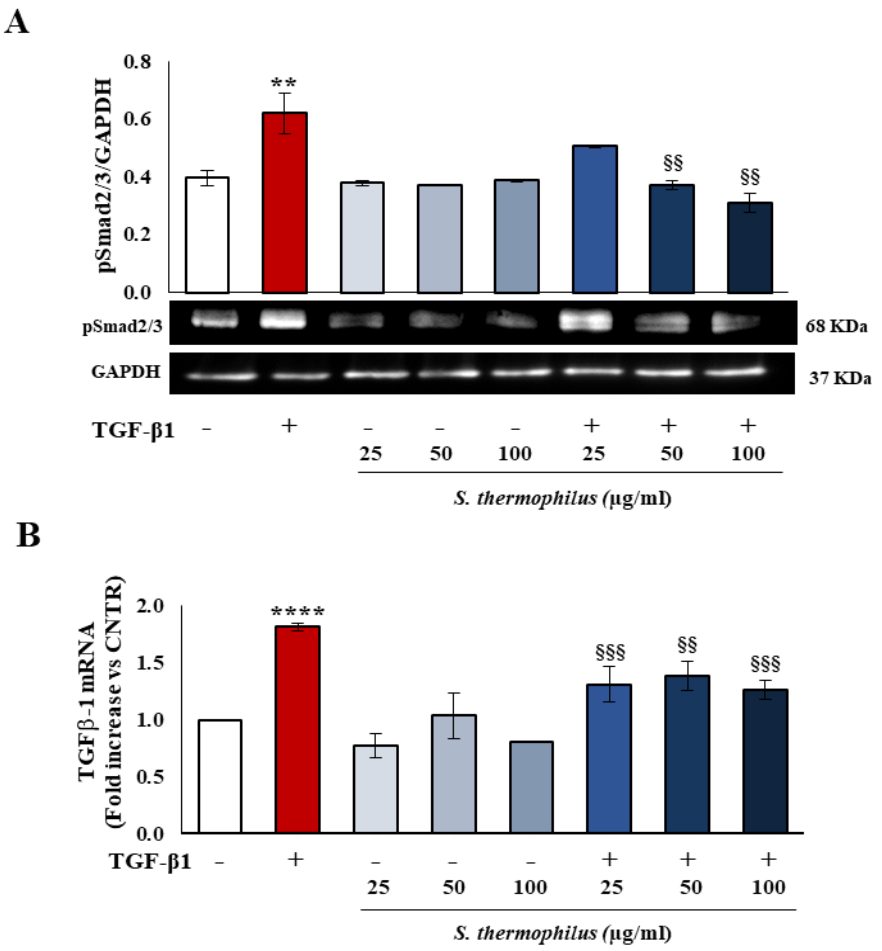

Figure 13. Effects of *S. thermophilus* lysate on TGF-β1-induced Smad signaling. (A) Immunoblotting assay for pSmad2/3 protein expression was performed on NHDF treated as described above. Following densitometric analysis, obtained values were normalized vs. GAPDH. Values are expressed as mean ± SEM of two independent experiments performed in duplicate. Representative images of immunoblotting for pSmad2/3 and GAPDH are shown. **(B)** The SYBRGreen Real-Time PCR analysis of the TGF-β1 gene was performed on NHDF. mRNA levels were relative to the amount of GAPDH mRNA. Data from one of two independent experiments in duplicate are shown as mean ± SD. In all cases, the comparative analysis of data has been carried out by using one-way analysis of variance (ANOVA) followed by post hoc Tukey's test (** $P < 0.01$ vs. CNTR; **** $P<0.0001$ vs. CNTR; §§ $P < 0.01$ vs TGF-β1, §§§ $P<0.001$ vs. TGF-β1).

4.5 Effect of *S. thermophilus* lysate on β-catenin and PPARγ expression in TGF-β1-activated NHDF

To further assess potential targets of *S. thermophilus* lysate within TGF-β1 signaling pathways, we investigated the effects of bacterial lysate on β-catenin, a key component involved in Wnt/β-catenin signaling whose aberrant activation occurs in the development of skin fibrosis (Griffin et al, 2022). A significant up-regulation of β-catenin levels was found in TGF-β1-activated NHDF, while no significant difference was registered between the control and *S. thermophilus* lysate-

treated cells (Figure 14A). Notably, *S. thermophilus* lysate treatment abolished the TGF-β1-induced increase of β-catenin levels at all tested concentrations, thus suggesting that the anti-fibrotic activity of the probiotic also involved the inhibition of β-catenin signaling.

Finally, by using western blot assays, we explore whether the β-catenin decrease observed in TGF-β1-treated NHDF after exposure to the probiotic lysate was associated with an increased PPARγ level. Interestingly, an increase of PPARγ was observed following the addition of bacterial lysate to TGF-β1-activated NHDF for 48 h at all concentrations. Although not significantly, PPARγ levels showed a trend to decreased in activated NHDF with respect to the control (Figure 14B).

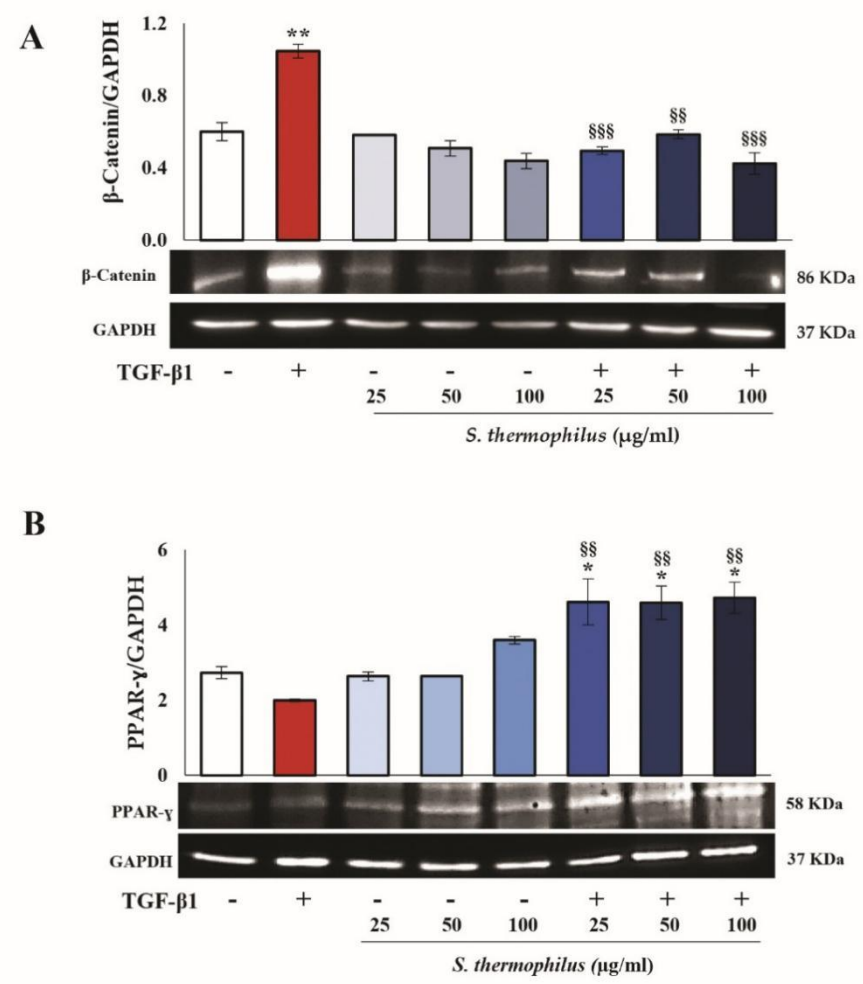

Figure 14. Effect of *S. thermophilus* lysate on β-catenin and PPARγ expression in TGF-β1-activated NHDF. (A) β-catenin and **(B)** PPARγ protein expressions were evaluated on NHDF incubated as above described, by western blot analysis. Following densitometric analysis, obtained values were normalized vs. GAPDH. Values are expressed as mean ± SEM of two independent experiments in duplicate. For comparative analysis of data, a one-way analysis of variance (ANOVA) with post hoc Tukey test was used (*P<0.05, **P<0.01 vs. CNTR; §§ P < 0.01, §§§ P < 0.001 vs TGF-β1). Representative images of immunoblotting for β-catenin, PPARγ, and GAPDH are shown.

5 DISCUSSION

Probiotics are well-known to exert beneficial activities against a plethora of pathological conditions including, but not limited to, infectious diseases, diabetes, obesity, inflammatory diseases, cancer, and allergy (Cristofori et al, 2021). In the context of skin disorders, the beneficial effects associated with oral consumption of probiotics have been proven to be effective in promoting healthy skin microbioma composition and in achieving clinically satisfactory results against different clinical conditions such as atopic dermatitis, acne, rosacea, and psoriasis (Lolou et al, 2019; Pistone et al, 2021). Moreover, the use of the topical application of probiotics as an effective therapeutic approach for treating patients with dermatological diseases remains undervalued (Knackstedt et al, 2020). Even though a few studies have investigated the effects of probiotics on scar formation, probiotics might also play a beneficial role directly in fibrotic tissue associated with dysregulated wound healing, such as hypertrophic scar and keloids. The topical application of *Lactobacillus plantarum* has been reported to be able in reducing the scarring of burn wounds and in mitigating infection, often associated with the development of a hypertrophic scar (Baker et al, 2007). Furthermore, a recent study proved that the oral administration of *Lactobacillus rhamnosus* is associated with accelerated wound closure, as well as with the alleviation of scar formation in a murine model of excisional skin wounds (Moreira et al, 2021).

Here, we provide evidence that the exposure to lysate from *S. thermophilus* DSM 24731 is able to counteract the fibrotic process induced in vitro by TGF-β1 by inhibiting the TGF-β1/Smad signaling. Fibroblast migration and proliferation are known to play a crucial role in the pathological healing process and are regulated by numerous factors. Such cellular processes were strongly affected by the presence of the probiotic lysate. Also, the phenoconversion of myofibroblasts was prevented by the exposure to *S. thermophilus* lysate in a dose-dependent manner, as showed by the reduction of α-SMA and fibronectin levels up-regulated by TGF-β1. Furthermore, the bacterial lysate was able to inhibit the capacity of TGF-β1 activated fibroblasts to synthesize Coll-I and contract collagen gel. Of note, *S. thermophilus* also abrogated the transcription of the TGF-β1 gene, thus blocking the TGF- β1 induced self-maintaining machinery. The peculiar fibrogenic actions of TGF-β1 require the co-activation of multiple molecular pathways often interconnected, including the classical (Smad-dependent) or non-classical signaling, among which, the Wnt/β-catenin pathway plays a central role in regulating the dermal fibrosis (Griffin et al, 2022). As expected, β-catenin levels were significantly increased in TGF-

β1-activated NHDF while treatment with *S. thermophilus* was able to reduce them. The activation of Wnt signaling is closely associated with the downregulation of PPARγ, being these two systems able to act in an opposite manner. PPARγ acts in decreasing the fibrotic process by opposing TGF-β1 activity. Numerous agonists of PPARγ minimize fibrotic events such as fibro-proliferation and collage deposition, encouraging the correct repair of skin tissue. The increase of β-catenin by TGF-β1 treatment in NHDF was associated with reduced PPARγ levels. Notably, the probiotic was able to counteract the effect of TGF-β1, reducing β-catenin and increasing PPARγ levels, acting as an anti-fibrotic agent. A limitation of our study is the lack of the identification of the active component/s which individuation will help to deepen further the mechanisms of action behind the observed anti-fibrotic effects of the probiotic lysate. Therefore, the next steps of our research should focus on the important task of identifying and purifying the *S. thermophilus*-derived factor(s), which specifically reverses the skin fibrosis process and affects the involvement of multiple fibrotic pathways. In this context, of particular interest will be to investigate the potential involvement of Toll-like Receptors (TLRs) expressed by fibroblasts able to recognize one or more components from microbes-associated molecular patterns (MAMPs) in the probiotic lysate and the downstream effects in terms of synthesis of cytokines known to influence TGF-β1-induced fibrotic process. Of note, the ability of *S. thermophilus* to activate the heterodimer TLR2/TLR6, which could elicit IL-10 secretion by phorbol myristate acetate-differentiated THP1 macrophages, has been reported (Ren et al, 2016). Thus, lactic acid bacteria strains, including *S. thermophilus*, stimulating TLR2/TLR6 pathways, might modulate excessive inflammatory reactions. It could be crucial to demonstrate that one or more components of MAMPs from *S. thermophilus* lysate can interact with the TLR2/TLR6 heterodimer by inducing the release of IL-10 by fibroblasts. This could explain, at least in part, the anti-fibrotic effect exerted by the used probiotic strain. In fact, IL-10 is considered a potent anti-inflammatory cytokine which plays a pivotal role in wound healing and scar formation, exerting inhibitory effects on the excessive deposition of extracellular matrix components and fibroblast-to-myofibroblast transition induced by TGF-β1 (Shi et al, 2013; Shi et al, 2014).

6 CONCLUSIONS

To the best of our knowledge, the results of the present study provide the first evidence of the ability of *S. thermophilus* to counteract the pro-fibrotic effects induced by TGF-β1 in a fibrosis skin model. In the current scenario, the available therapeutic strategies may have variable success rates or weak responses with recurrences after treatment. Moreover, the considerable side effects that follow the treatment cause discontinuation of the therapy. Therefore, the medical need is directed toward well- tolerated therapies able to prevent or reverse fibrosis without side effects as far as possible. In light of the encouraging results obtained in vitro, our next goal is to plan a prospective study in collaboration with clinicians to investigate the effect topical probiotic lysate on surgical wounds in patients with skin healing disorders. At the same time, we will be also interested to study the effects of topical application of *S. thermophilus* lysate on hypertrophic scars and keloids by selecting appropriate probiotic lysate delivering system.

7 REFERENCES

Abdi A, Oroojzadeh P, Valivand N, Sambrani R, Lotfi H. Immunological aspects of probiotics for improving skin diseases: Influence on the Gut-Brain-Skin Axis. Biochem Biophys Res Commun. 2024; 702:149632. doi: 10.1016/j.bbrc.2024.149632.

Alvarez CS, Giménez R, Cañas MA, Vera R, Díaz-Garrido N, Badia J, Baldomà L. Extracellular vesicles and soluble factors secreted by Escherichia coli Nissle 1917 and ECOR63 protect against enteropathogenic E. coli-induced intestinal epithelial barrier dysfunction. BMC Microbiol. 2019; 19(1):166. doi: 10.1186/s12866-019-1534-3.

Antar SA, Ashour NA, Marawan ME, Al-Karmalawy AA. Fibrosis: Types, Effects, Markers, Mechanisms for Disease Progression, and Its Relation with Oxidative Stress, Immunity, and Inflammation. Int J Mol Sci. 2023 16;24(4):4004. doi: 10.3390/ijms24044004.

Azad MAK, Sarker M, Wan D. Immunomodulatory Effects of Probiotics on Cytokine Profiles. Biomed Res Int. 2018:8063647 DOI: 10.1155/2018/8063647

Aziz N, Bonavida B. Activation of Natural Killer Cells by Probiotics. For Immunopathol Dis Therap. 2016;7(1-2):41-55. doi: 10.1615/ForumImmunDisTher.2016017095.

Baindara P, Korpole S, Grover V. Bacteriocins: perspective for the development of novel anticancer drugs. Appl Microbiol Biotechnol. 2018; 102(24):10393-10408. doi: 10.1007/s00253-018-9420-8.

Baker RH, Townley WA, McKeon S, Linge C, Vijh V. Retrospective study of the association between hypertrophic burn scarring and bacterial colonization. J Burn Care Res. 2007;28(1):152-6. doi: 10.1097/BCR.0B013E31802CB860.

Bhat MI, Kapila S, and Kapila R. 2020. 'Lactobacillus fermentum (MTCC-5898) supplementation renders prophylactic action against Escherichia coli impaired intestinal barrier function through tight junction modulation', Lwt-Food Science and Technology, 123.

Blanchet-Réthoré S, Bourdès V, Mercenier A, Haddar CH Verhoeven PO, Andres P. Effect of a lotion containing the heat-treated probiotic strain Lactobacillus johnsonii NCC 533 on Staphylococcus aureus colonization in atopic dermatitis. Clin. Cosmet. Investig. Dermatol. 2017;10:249–257.

Borrero J, Kelly E, O'Connor PM, Kelleher P, Scully C, Cotter PD, Mahony J, van Sinderen D. Plantaricyclin A, a Novel Circular Bacteriocin Produced by Lactobacillus plantarum NI326: Purification, Characterization, and Heterologous Production. Appl Environ Microbiol. 2017; 84(1):e01801-17. doi: 10.1128/AEM.01801-17.

Budi EH, Schaub JR, Decaris M, Turner S, Derynck R. TGF-β as a driver of fibrosis: physiological roles and therapeutic opportunities. J Pathol. 2021; 254(4):358-373. doi: 10.1002/path.5680.

Bustamante M, Oomah BD, Oliveira WP, Burgos-Díaz C, Rubilar M, Shene C. Probiotics and prebiotics potential for the care of skin, female urogenital tract, and respiratory tract. Folia Microbiol (Praha). 2020;65(2):245-264. doi: 10.1007/s12223-019-00759-3.

Cinque B, Di Marzio L, Della Riccia DN, Bizzini F, Giuliani M, Fanini D, De Simone C, Cifone MG. Effect of Bifidobacterium infantis on Interferon- gamma- induced keratinocyte apoptosis: a potential therapeutic

approach to skin immune abnormalities. Int J Immunopathol Pharmacol. 2006;19(4):775-86. doi: 10.1177/039463200601900407.

Coentro JQ, Pugliese E, Hanley G, Raghunath M, Zeugolis DI. Current and upcoming therapies to modulate skin scarring and fibrosis. Adv Drug Deliv Rev. 2019; 146:37-59. doi: 10.1016/j.addr.2018.08.009.

Cristofori F, Dargenio VN, Dargenio C, Miniello VL, Barone M, Francavilla R. Anti-infammatory and Immunomodulatory Efects of Probiotics in Gut infammation: a door to the body. Front Immunol. 2021;12:578386.

Dabiri G, Campaner A, Morgan JR, Van De Water L. A TGF-beta1-dependent autocrine loop regulates the structure of focal adhesions in hypertrophic scar fibroblasts. J Invest Dermatol. 2006; 126:963–70.

De Almeida CV, Antiga E, Lulli M. Oral and Topical Probiotics and Postbiotics in Skincare and Dermatological Therapy: A Concise Review. Microorganisms. 2023; 11(6):1420. doi: 10.3390/microorganisms11061420.

de Melo Pereira GV, de Oliveira Coelho B, Magalhães Júnior AI, Thomaz-Soccol V, Soccol CR. How to select a probiotic? A review and update of methods and criteria. Biotechnol Adv. 2018; 36(8):2060-2076. doi: 10.1016/j.biotechadv.2018.09.003.

Di Marzio L, Cinque B, De Simone C, Cifone MG. Effect of the lactic acid bacterium Streptococcus thermophilus on ceramide levels in human keratinocytes in vitro and stratum corneum in vivo. J Invest Dermatol. 1999; 113:98–106. doi: 10.1046/j.1523-1747.1999.00633.

Di Marzio L, Centi C, Cinque B, Masci S, Giuliani M, Arcieri A, Zicari L, De Simone C, Cifone MG. Effect of the lactic acid bacterium Streptococcus thermophilus on stratum corneum ceramide levels and signs and symptoms of atopic dermatitis patients. Exp Dermatol. 2003; 12:615–20. doi: 10.1034/j.1600-0625.2003.00051

Di Marzio L, Cinque B, Cupelli F, De Simone C, Cifone MG, Giuliani M. Increase of skin-ceramide levels in aged subjects following a short-term topical application of bacterial sphingomyelinase from Streptococcus thermophilus. Int J Immunopathol Pharmacol. 2008; 21(1):137-43. doi: 10.1177/039463200802100115.

El Ayadi A, Jay JW, Prasai A. Current Approaches Targeting the Wound Healing Phases to Attenuate Fibrosis and Scarring. Int J Mol Sci. 2020; 21(3):1105. doi: 10.3390/ijms21031105.

Faour S, Farahat M, Aijaz A, Jeschke MG. Fibrosis in burns: an overview of mechanisms and therapies. Am J Physiol Cell Physiol. 2023; 325(6):C1545-C1557. doi: 10.1152/ajpcell.00254.2023.

Frangogiannis N. Transforming growth factor-β in tissue fibrosis. J Exp Med. 2020; 217(3):e20190103. doi: 10.1084/jem.20190103.

Gaspar C, Donders GG, Palmeira-de-Oliveira R, Queiroz JA, Tomaz C, Martinez-de-Oliveira J, Palmeira-de-Oliveira A. Bacteriocin production of the probiotic Lactobacillus acidophilus KS400. AMB Express. 2018; 8(1):153. doi: 10.1186/s13568-018-0679-z.

Ghosh AK. Pharmacological activation of PPAR-γ: a potential therapy for skin fibrosis. Int J Dermatol. 2021; 60(3):376-383. doi: 10.1111/ijd.15388.

Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr. 1995 Jun;125(6):1401-12. doi: 10.1093/jn/125.6.1401.

Gibson GR, Hutkins R, Sanders ME, Prescott SL, Reimer RA, Salminen SJ, Scott K, Stanton C, Swanson KS, Cani PD, Verbeke K, Reid G. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nat Rev Gastroenterol Hepatol. 2017; 14(8):491-502. doi: 10.1038/nrgastro.2017.75.

Griffin MF, Huber J, Evan FJ, Quarto N, Longaker MT. The role of wnt signaling in skin fibrosis. Med Res Rev. 2022; 42:615–28.

Guéniche A, Cathelineau AC, Bastien P, Esdaile J, Martin R, Queille Roussel C, Breton L. Vitreoscilla filiformis biomass improves seborrheic dermatitis. J. Eur. Acad. Dermatol. Venereol. 2008; 22(8):1014-1015. DOI: 10.1111/j.1468-3083.2007.02508.x

Guéniche A, Bastien P, Ovigne JM, Kermici M, Courchay G, Chevalier V, Breton L, Castiel-Higounenc I. Bifidobacterium longum lysate, a new ingredient for reactive skin. Exp Dermatol. 2010;19(8):e1-8. DOI: 10.1111/j.1600-0625.2009.00932.x

Heeney DD, Zhai Z, Bendiks Z, Barouei J, Martinic A, Slupsky C, Marco ML. Lactobacillus plantarum bacteriocin is associated with intestinal and systemic improvements in diet-induced obese mice and maintains epithelial barrier integrity in vitro. Gut Microbes. 2019;10(3):382-397. doi: 10.1080/19490976.2018.1534513.

Homayouni Rad A, Aghebati Maleki L, Samadi Kafil H, Fathi Zavoshti H, Abbasi A. Postbiotics as novel health-promoting ingredients in functional foods. Health Promot Perspect. 2020; 10(1):3-4. doi: 10.15171/hpp.2020.02.

Juhl P, Bondesen S, Hawkins CL, Karsdal MA, Bay-Jensen AC, Davies MJ, et al. Dermal fibroblasts have different extracellular matrix profiles induced by TGF-beta, PDGF and IL-6 in a model for skin fibrosis. Sci Rep. 2020; 10:17300. doi: 10.1038/s41598-020-74179-6.

Kang BS, Seo JG, Lee GS, Kim JH, Kim SY, Han YW, Kang H, Kim HO, Rhee JH, Chung MJ, Park YM. Antimicrobial activity of enterocins from Enterococcus faecalis SL-5 against Propionibacterium acnes, the causative agent in acne vulgaris, and its therapeutic effect. J Microbiol. 2009;47(1):101-109.

Karczewski J, Troost FJ, Konings I, Dekker J, Kleerebezem M, Brummer RJ, Wells JM. Regulation of human epithelial tight junction proteins by Lactobacillus plantarum in vivo and protective effects on the epithelial barrier. Am J Physiol Gastrointest Liver Physiol. 2010; 298(6):G851-9. doi: 10.1152/ajpgi.00327.2009.

Knackstedt R, Knackstedt T, Gatherwright J. The role of topical probiotics on skin conditions: a systematic review of animal and human studies and implications for future therapies. Exp Dermatol. 2020; 29:15–21. doi: 10.1111/exd.14032.

Latif A, Shehzad A, Niazi S, Zahid A, Ashraf W, Iqbal MW, Rehman A, Riaz T, Aadil RM, Khan IM, Özogul F, Rocha JM, Esatbeyoglu T, Korma SA. Probiotics: mechanism of action, health benefits and their application in food industries. Front Microbiol. 2023 17;14:1216674. doi: 10.3389/fmicb.2023.1216674.

Liu J, Deng T, Wang Y, Zhang M, Zhu G, Fang H, Wang J. Calycosin inhibits intestinal fbrosis on CCD-18Co cells via modulating transforming growth Factor-beta/Smad signaling pathway. Pharmacology. 2019; 104:81–9. doi: 10.1159/000500186.

Llewellyn A, Foey A. Probiotic Modulation of Innate Cell Pathogen Sensing and Signaling Events. Nutrients. 2017;9(10):1156. doi: 10.3390/nu9101156.

Lolou, V.; Panayiotidis, M.I. Functional Role of Probiotics and Prebiotics on Skin Health and Disease. Fermentation 2019, *5*, 41. https://doi.org/10.3390/fermentation5020041.

Lombardi F, Augello FR, Palumbo P, Mollsi E, Giuliani M, Cimini AM, Cifone MG, Cinque B. Soluble Fraction from Lysate of a High Concentration Multi-Strain Probiotic Formulation Inhibits TGF-β1-Induced Intestinal Fibrosis on CCD-18Co Cells. Nutrients. 2021; 13(3):882. doi: 10.3390/nu13030882.

Lurje I, Gaisa NT, Weiskirchen R, Tacke F. Mechanisms of organ fibrosis: Emerging concepts and implications for novel treatment strategies. Mol Aspects Med. 2023; 92:101191. doi: 10.1016/j.mam.2023.101191.

Mandal SM, Pati BR, Chakraborty R, Franco OL. New insights into the bioactivity of peptides from probiotics. Front Biosci (Elite Ed). 2016; 8(3):450-9. doi: 10.2741/E779.

Moreira CF, Cassini-Vieira P, Canesso MCC, Felipetto M, Ranfley H, Teixeira MM, Nicoli JR, Martins FS, Barcelos LS. Lactobacillus rhamnosus CGMCC 1.3724 (LPR) Improves Skin Wound Healing and Reduces Scar Formation in Mice. Probiotics Antimicrob Proteins. 2021; 13(3):709-719. doi: 10.1007/s12602-020-09713-z.

Moretti L, Stalfort J, Barker TH, Abebayehu D. The interplay of fibroblasts, the extracellular matrix, and inflammation in scar formation. J Biol Chem. 2022; 298(2):101530. doi: 10.1016/j.jbc.2021.101530.

Muizzuddin N, Maher W, Sullivan M, Schnittger S, Mammone T. Physiological effect of a probiotic on skin. J Cosmet Sci. 2012;63(6):385-95.

Nakatsuji T, Chen TH, Narala S, Chun KA, Two AM, Yun T, Shafiq F, Kotol PF, Bouslimani A, Melnik AV, Latif H, Kim JN, Lockhart A, Artis K, David G, Taylor P, Streib J, Dorrestein PC, Grier A, Gill SR, Zengler K, Hata TR, Leung DY, Gallo RL. Antimicrobials from human skin commensal bacteria protect against Staphylococcus aureus and are deficient in atopic dermatitis. Sci Transl Med. 2017;9(378):eaah4680. doi: 10.1126/scitranslmed.aah4680.

Ngo P, Ramalingam P, Phillips JA, Furuta GT. Collagen gel contraction assay. Methods Mol Biol. 2006; 341:103–9. doi: 10.1385/1-59745-113-4:103.

Oleskin AV, Shenderov BA. Probiotics and Psychobiotics: the Role of Microbial Neurochemicals. Probiotics Antimicrob Proteins. 2019; 11(4):1071-1085. doi: 10.1007/s12602-019-09583-0.

Peral MC, Martinez MA, Valdez JC. Bacteriotherapy with Lactobacillus plantarum in burns. Int. Wound J. 2009;6(1):73-81. DOI: 10.1111/j.1742-481X.2008.00577.x

Peral MC, Rachid MM, Gobbato NM, Huaman Martinez MA, Valdez JC. Interleukin-8 production by polymorphonuclear leukocytes from patients with chronic infected leg ulcers treated with Lactobacillus plantarum. Clin Microbiol Infect. 2010;16(3):281–6. DOI: 10.1111/j.1469-0691.2009.02793.x

Pistone D, Meroni G, Panelli S, D'Auria E, Acunzo M, Pasala AR, et al. A journey on the skin microbiome: Pitfalls and opportunities. Int J Mol Sci. 2021; 22:9846. DOI: 10.3390/ijms22189846.

Prajapati, K., Bisani, K., Prajapati, H. *et al.* Advances in probiotics research: mechanisms of action, health benefits, and limitations in applications. Syst Microbiol and Biomanuf (2023). doi.org/10.1007/s43393-023-00208-w.

Qin H, Zhang Z, Hang X, Jiang Y. L. plantarum prevents enteroinvasive Escherichia coli-induced tight junction proteins changes in intestinal epithelial cells. BMC Microbiol. 2009; 9:63. doi: 10.1186/1471-2180-9-63.

Raghuwanshi S, Misra S, Sharma R, Bisen PS. Probiotics: nutritional therapeutic tool. J Probiotics Heal. 2018; 6:1–52.

Ren C, Zhang Q, de Haan BJ, Zhang H, Faas MM, de Vos P. Identification of TLR2/TLR6 signalling lactic acid bacteria for supporting immune regulation. Sci Rep. 2016; 6:34561. doi: 10.1038/srep34561.

Shi JH, Guan H, Shi S, Cai WX, Bai XZ, Hu XL, Fang XB, Liu JQ, Tao K, Zhu XX, et al. Protection against TGF-beta 1-induced fibrosis effects of IL-10 on dermal fibroblasts and its potential therapeutics for the reduction of skin scarring. Arch Dermatol Res. 2013; 305:341–52. doi: 10.1007/s00403-013-1314-0.

Shi JH, Li J, Guan H, Cai WX, Bai XZ, Fang XB, et al. Anti-fibrotic actions of Interleukin-10 against hypertrophic scarring by activation of PI3K/AKT and STAT3 signalling pathways in scar-forming fibroblasts. Plos One. 2014; 9:e98228. doi: 10.1371/journal.pone.0098228.

Szántó M, Dózsa A, Antal D, Szabó K, Kemény L, Bai P. Targeting the gut-skin axis-Probiotics as new tools for skin disorder management? Exp Dermatol. 2019; 28(11):1210-1218. doi: 10.1111/exd.14016.

Tai Y, Woods EL, Dally J, Kong D, Steadman R, Moseley R, Midgley AC. Myofibroblasts: Function, Formation, and Scope of Molecular Therapies for Skin Fibrosis. Biomolecules. 2021; 11(8):1095. doi: 10.3390/biom11081095.

Tegegne BA, Kebede B. Probiotics, their prophylactic and therapeutic applications in human health development: A review of the literature. Heliyon. 2022; 8(6):e09725. doi: 10.1016/j.heliyon.2022.e09725.

Twetman S, Pedersen AML, Yucel-Lindberg T. Probiotic supplements containing Lactobacillus reuteri does not affect the levels of matrix metalloproteinases and interferons in oral wound healing. BMC Res Notes. 2018;11(1):759. doi: 10.1186/s13104-018-3873-9.

van Zyl WF, Deane SM, Dicks LMT. Molecular insights into probiotic mechanisms of action employed against intestinal pathogenic bacteria. Gut Microbes. 2020; 12(1):1831339. doi: 10.1080/19490976.2020.1831339.

Wang J, Zeng Y, Wang S, Liu H, Zhang D, Zhang W, Wang Y, Ji H. Swine-Derived Probiotic Lactobacillus plantarum Inhibits Growth and Adhesion of Enterotoxigenic Escherichia coli and Mediates Host Defense. Front Microbiol. 2018; 9:1364. doi: 10.3389/fmicb.2018.01364.

Wang Y, Kasper LH. The role of microbiome in central nervous system disorders. Brain Behav Immun. 2014 May;38:1-12. doi: 10.1016/j.bbi.2013.12.015.

Wang Y, Yan X, Han D, Liu Y, Song W, Tong T, Ma Y. Lactobacillus casei DBN023 protects against jejunal mucosal injury in chicks infected with Salmonella pullorum CMCC-533. Res Vet Sci. 2019; 127:33-41. doi: 10.1016/j.rvsc.2019.09.010.

Wang K, Wen D, Xu X, Zhao R, Jiang F, Yuan S, Zhang Y, Gao Y, Li Q. Extracellular matrix stiffness-The central cue for skin fibrosis. Front Mol Biosci. 2023 Mar 8;10:1132353. doi: 10.3389/fmolb.2023.1132353.

Zhang T, Wang XF, Wang ZC, Lou D, Fang QQ, Hu YY, Zhao WY, Zhang LY, Wu LH, Tan WQ. Current potential therapeutic strategies targeting the TGF-beta/Smad signaling pathway to attenuate keloid and hypertrophic scar formation. Biomed Pharmacother. 2020; 129:110287. DOI: 10.1016/j.biopha.2020.110287.

Zheng BD, Gan L, Tian LY, Chen GH. Protein/polysaccharide-based hydrogels loaded probiotic-mediated therapeutic systems: A review. Int J Biol Macromol. 2023; 253(Pt 2):126841. doi: 10.1016/j.ijbiomac.2023.126841.

Zielińska M, Pawłowska A, Orzeł A, Sulej L, Muzyka-Placzyńska K, Baran A, Filipecka-Tyczka D, Pawłowska P, Nowińska A, Bogusławska J, Scholz A. Wound Microbiota and Its Impact on Wound Healing. Int J Mol Sci. 2023; 24(24):17318. doi: 10.3390/ijms242417318.

Zoccali G, Cinque B, La Torre C, Lombardi F, Palumbo P, Romano L, Mattei A, Orsini G, Cifone MG, Giuliani M. Improving the outcome of fractional CO2 laser resurfacing using a probiotic skin cream: Preliminary clinical evaluation. Lasers Med Sci. 2016; 31(8):1607-1611. doi: 10.1007/s10103-016-2024-6.

Żółkiewicz J, Marzec A, Ruszczyński M, Feleszko W. Postbiotics-A Step Beyond Pre- and Probiotics. Nutrients. 2020; 12(8):2189. doi: 10.3390/nu12082189.