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Sviluppo di una barriera intestinale quale modulo di una piattaforma in vitro 3D per lo studio dell'interazione tra microbiota intestinale e tessuto osseo

Development of an intestinal barrier as a module of a 3D in vitro platform to study of the crosstalk between gut microbiota and bone tissue

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1. INTRODUCTION

In research, experimentation on biological models is an essential component for the development of new therapies. Traditionally, animal experiments are used for scientific purposes. Animal experiment is a very widespread phenomenon, and their use allows for basic research investigations, toxicity studies, and the development of new drugs. The practice of animal experimentation is regulated by law. The main references are European law, Directive 2010/63/EU and the Italian legislation, legislative decree No.26 of 2014.

The main animals used in experiments are rodents, like rats and mice, followed by fishes and rabbits. (Redazione, R. 2023). For several years now, the use of animals has been restricted, and researchers are trying to promote the use of alternative/complementary methods such as:

-*In vitro* cell cultures using tubs, Petri dishes and flasks, which allow the behaviour of cells to be mimicked outside a living organism.

- *In silico* method to perform via computer simulation

- Bioartificial organ for the *in vitro* reconstruction of tissues and organs like semipermeable polymer membranes.

- Pharmacogenomics, is a branch of molecular biology that investigates the effects of a specific drug based on the individual's genotype.

- Mathematical methods.

- Epidemiology and statistics.

- Autopsy and biopsy.

In parallel with the use of in vitro experiments, it is important to adopt an ethical guide to scientific research, such as the 3Rs principles, proposed by Russell and Burch.

EFSA (European Food Safety Authority) promotes approaches that minimise the use of animals in experiments and where possible they promote the use of alternative or more complementary methods. EFSA is committed to the application and respect of the 3Rs principles.

1.1 *The 3Rs principles*

The 3Rs - replacement, reduction and refinement - are important principles formulated and introduced by Russell and Burch in 1959. These principles are intended to guide developments in the research community towards a more ethical and sustainable use of animals in research. The 3Rs are not independent of each other, but when applied together they create an ethical, efficient and innovative environment. The benefits of research based on the 3Rs are not only to reduce the costs of experimentation associated with animal use, but also to encourage the search for new research methods and to conduct research in a responsible manner.

1.1.1 Replacement

Russel and Burch define Replacement as “the substitution for conscious living higher animals of insentient material” (Russell WMS, Burch RL. 1959). This insentient material can include non-animal entities such as higher plants, microorganisms, or highly degenerate animal parasites with almost completely reduced nervous and sensory systems. It is important to note that Russell and Burch did not simply define substitution as the non-use of animals. On the contrary, they emphasised the use of insentient material, animal or non-animal, in place of sentient beings capable of experiencing distress. (Tannenbaum et al. 2016).

Based on these assumptions we can define:

- *full replacement* by methods that avoid the use of animals for research and testing purposes, which are collectively known as non-animal technologies (NATs), such as in vitro experiments or computational models.

- *partial replacement,* which involves the use of some animals that, based on current scientific thinking, are not considered to experience suffering according to current scientific knowledge. These include invertebrates (e.g., Drosophila, nematodes and social amoebas) and immature forms of vertebrates. Partial replacement also includes the use of primary cells (and tissues) derived from animals that have been killed solely for this purpose (i.e., have not been used in a scientific procedure that causes suffering).

1.1.2 Reduction

Russell and Burch defined reduction as “decreasing the number of animals used to obtain specific scientific information” (Russell WMS, Burch RL. 1959). This means that the aim is to use fewer animals in experiments without compromising the quality or accuracy of the data. (Tannenbaum et al. 2016) through methods that maximise the information collected per animal in an experiment to reduce the use of additional animals.

Strategies to meet this criterion include, for example, the use of imaging modalities that allow longitudinal measurements in the same animal, the sharing of data and resources (e.g., animals, tissues and equipment) between research groups and organisations can also contribute to reduction, and the microsampling of blood, where small volumes allow repeated sampling from the same animal.

1.1.3 Refinement

The concept of refinement has also evolved. Russell and Burch describe in the "Principles" that refinement of experimental procedures means not only addressing animal welfare, but also improving the quality of life across all procedures in research. (Tannenbaum et al. 2016). Following this line, in 2005 other researchers proposed a new definition of refinement as any approach that avoids, alleviates or minimises pain and suffering throughout the lifetime of the animals involved. The key to this principle is that wellbeing is not only the absence of pain, but also the effort required to improve the state of wellbeing of the animals in the experiment. It should be emphasised that pain and distress can alter an animal's behaviour, physiology and immunology and that such changes can lead to variations in experimental results, affecting both the reliability and repeatability of studies.

1.2 2D and 3D in vitro models

In vitro experiments come from the Latin word meaning 'in glass'. In vitro experiments are a fundamental research technique for studying biological processes outside of living organisms, typically using isolated cells from organs maintained in the laboratory. In vitro assays allow the analysis of cellular mechanisms of normal and abnormal development, the testing of drug efficacy and the investigation of biochemical interactions with high precision. In vitro studies offer advantages such as controlled conditions and reproducibility, making them valuable for testing hypotheses before moving on to in vivo studies. However, in vitro studies may not truly represent the whole living organism with all its associated biological information, which could lead to uncertainties in predicting human responses. (Saeidnia et al. 2016) (National Academies Press 2001).

Cell culture models are used in pharmaceutical, medical and biological research, particularly for bioavailability and toxicity. Improved in vitro models using human cells help to reduce animal testing and could facilitate the study of molecular mechanisms in a simple and reproducible manner. Cells are classified as primary when they are directly extracted from tissues and cultured under optimised conditions. These cells closely resemble their natural state in vivo and have normal physiological properties. This similarity

makes them an excellent model for studying various biological processes, including metabolism, signalling and ageing. In addition, the use of primary cells can lead to cost savings in research. However, a major drawback is their limited lifespan; primary cells can only undergo a limited number of divisions before entering a state of senescence due to their restricted growth potential. As a result, they can be difficult to culture and maintain as continuous cell lines unless optimal conditions are maintained. Variability in the behaviour of primary cells can result from differences between donors, and the availability of specific tissues can also be a limiting factor. Primary cells can be converted into immortalised secondary cell lines by a process known as transformation, which can be chemically or virally induced. (Primary Cell Culture Basics, n.d.). Unlike primary cells, continuous cell lines are clonal strains of cells that can grow indefinitely due to random or induced mutations that prevent senescence. These immortalised lines can proliferate indefinitely; indeed, many immortalised cell lines are cells derived from tumour tissue. Cell lines can be obtained by viral infection or transfection of specific genes. Essential requirements for the maintenance of in vitro cell cultures include nutrients such as glucose, amino acids, vitamins and mineral salts, and growth factors such as fetal bovine serum (FBS), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and insulin-like growth factor (IGF).

Continuous cell lines are more robust and easier to work with than primary cells; they have faster growth rates and lower costs than primary cells. However, they may not accurately reflect the in vivo environment over time, especially after extensive passaging.

Currently, most cell culture models are performed under two-dimensional (2D) conditions, using one or more cell lines in co-culture. A major challenge with these models is the development of a three-dimensional (3D) culture system. Advances in technology have led to a significant increase in the use of 3D in vitro models, which allow a more accurate representation of how cells interact in a natural environment compared to traditional 2D cultures. This is crucial for the study of tissue development, disease modelling and drug testing. (Del Carmen Ponce De León-Rodríguez et al.2018).

The further transition from 3D cultures to in vitro platforms for tissue models represents a significant advance in biomedical research.

In vitro platforms, such as organ-on-a-chip technologies, take this a step further by mimicking the physiological conditions of human tissues. These platforms can replicate the mechanical and biochemical environment of tissues, providing researchers with valuable insights into cellular behaviour, drug responses and disease mechanisms.

Some examples of 3D cultures and in vitro platforms used to model tissues that are helping to understand biology and improve medical research include:

3D Bioprinting: This technology uses bioinks made from living cells to create complex tissue structures layer by layer. It can be used to create skin, cartilage, and even organ-like structures.

3D Spheroids: These are clusters of cells that form a spherical shape, providing a more realistic environment for studying cell behaviour, drug efficacy, and cancer research.

Organoids: These are miniaturized and simplified versions of organs grown from stem cells. For example, brain organoids can be used to study neurological diseases, while intestinal organoids can help to understand intestinal disorders.

Organ-on-a-Chip: These microfluidic devices simulate the functions of human organs. For example, a lung-on-a-chip can be used to model respiratory diseases and test drug responses, while a heart-on-a-chip can be used to study cardiac function and drug toxicity.

Tissue Engineering Scaffolds: These are 3D structures made from biomaterials that support cell growth and tissue formation. They can be used to regenerate damaged tissues, such as bone or cartilage.

1.3 In vitro intestinal barrier

1.3.1 Intestinal barrier

The intestinal barrier is a highly specialised structure that plays an essential role in protecting the organism by acting as an interface between the intestinal lumen and the circulatory system. (Schoultz & Keita, 2020). It is primarily composed of enterocytes, epithelial cells that form a continuous layer maintained by tight junction (TJ) proteins, including transmembrane proteins such as occludin and claudin, together with the cytoplasmic scaffolding protein zonula occluden 1 (ZO-1), which limit permeability. (Nathani et al, 2023). This epithelial layer is coated with a protective layer of mucus that acts as an additional barrier to pathogens and pollutants. (Schoultz & Keita, 2020). The main function of the intestinal barrier is to select nutrients for absorption while preventing the passage of pathogens and toxins, thereby helping to maintain homeostasis and overall health. The health of the intestinal barrier is essential to prevent conditions such as 'leaky gut syndrome', where permeability increases, allowing unwanted molecules to enter the bloodstream and triggering inappropriate immune responses. (Zhang et al., 2023). In this context, in vitro models of the intestinal barrier have become valuable tools for scientific research. These models allow the study of interactions between epithelial cells and the microbiota, as well as the effects of various dietary and

pharmacological compounds on barrier functionality. Using cell culture techniques, such as the Caco2 cell line expressing TJ proteins, researchers can simulate the physiological conditions of the human gut, facilitating the analysis of permeability mechanisms and cellular responses to external stimuli. This approach offers new insights into the development of targeted therapies aimed at restoring or improving the integrity of the intestinal barrier, thus contributing to the prevention of systemic diseases associated with intestinal dysfunction. (De Santis et al., 2015) (Schoultz & Keita, 2020).

1.3.2 Microbiota

The microbiota is the collection of microorganisms, including bacteria, viruses, fungi and protozoa, that colonise the human body, primarily in the intestinal tract. This microbial ecosystem consists of trillions of cells, far outnumbering human cells, and varies greatly from individual to individual. The composition of the microbiota is influenced by genetic, environmental and dietary factors and is essential for maintaining health. The main groups of bacteria that make up the gut microbiota include *Firmicutes, Bacteroidetes, Proteobacteria* and *Actinobacteria*. These microorganisms perform vital functions for human health, contributing to digestion, synthesising vitamins and modulating the immune system. The functions of the microbiota are diverse and include digestion, where microorganisms help break down

nutrients that cannot be digested by the human body, such as fibre. This process produces short-chain fatty acids, which serve as an energy source for intestinal epithelial cells and are essential for gut health. The microbiota are also involved in the synthesis of essential vitamins, such as B vitamins and vitamin K. Another fundamental function is immunity: the microbiota helps to form a protective barrier against pathogens and foreign substances by stimulating the host's immune response and preventing infection. The health of the microbiota is defined as eubiosis, while an imbalanced state is known as dysbiosis, which can lead to several diseases, including inflammatory bowel disease and metabolic disorders. Dysbiosis is often associated with poor diet, overuse of antibiotics and other environmental factors. Maintaining a balanced microbiota is therefore crucial for overall health. In the laboratory, in vitro models of the gut microbiota are used to study the interactions between microorganisms and human cells. These models allow researchers to simulate the physiological conditions of the human intestine and analyze ow different factors affect the composition and functionality of the microbiota. In conclusion, the microbiota represents an essential element for human well-being by influencing not only digestion and immunity but also psycho-emotional aspects.

The gut microbiota is involved in the absorption of nutrients essential for bone health, such as calcium and vitamin D. Some studies suggest that gut bacteria may increase the bioavailability of these nutrients, thereby facilitating their absorption at the intestinal level. The crosstalk between the gut microbiota and different tissues, including bone, is an area of increasing interest in biomedical research, as it highlights how human health can be influenced by the composition and activity of gut microorganisms.

Recent studies have shown that the gut microbiota plays a crucial role in bone metabolism, affecting both the formation and resorption of bone tissue. Dysbiosis, or an alteration in the composition of the gut microbiota, has been associated with an increase in inflammatory cytokines such as IL-1 and TNF-alpha, which activate osteoclasts, the cells responsible for bone resorption. This process can lead to reduced bone mineral density and contribute to the development of conditions such as osteoporosis. In addition, probiotics have shown potential in modulating immune responses and reducing systemic inflammation, helping to maintain a good environment for bone health. Probiotic supplementation has been shown to help prevent postmenopausal bone loss, suggesting that maintaining a balanced microbiota may be an effective strategy to combat osteoporosis.

1.3.3 In vitro gut model

The Caco-2 cell line, derived from human colorectal adenocarcinoma, has become a prominent model in gastrointestinal research for studying absorption, transport and bioavailability. Caco-2 cells exhibit morphological and functional characteristics similar to small intestinal enterocytes after 21 days, forming a polarised monolayer with tight junctions and microvilli. These cells express various digestive enzymes and transporters typical of intestinal epithelial cells, making them suitable for assessing drug absorption and metabolism. The differentiation process of Caco-2 cells significantly enhances their functionality, allowing them to closely mimic the characteristics of intestinal enterocytes. In addition, differentiated Caco-2 cells exhibit improved transepithelial electrical resistance (TEER), reflecting their ability to form a tight barrier that regulates paracellular transport. As the number of passages increases, Caco-2 cells acquire different properties and also parameters such as transepithelial electrical resistance (TEER) increase. (Verhoeckx et al., 2015) (Chapter 9). Transepithelial electrical resistance (TEER) is used to measure and monitor monolayer integrity.

Despite their advantages, Caco-2 cells have limitations, including the formation of heterogeneous monolayers that are influenced by culture conditions such as medium composition, growth factors such as glutamine,

pH and seeding density, and very high passage numbers. They are also unable to differentiate into goblet cells, resulting in a lack of mucus production. To overcome these challenges, several Caco-2 clones have been developed, such as the TC7 clone, which shows faster differentiation and more homogeneous characteristics compared to the parental line. (Del Carmen et al.,2018).

In addition, Caco-2 cells can produce inflammatory markers in response to cytokines and external stimuli such as lipopolysaccharides (LPS), although they are hyporesponsive to LPS due to limited expression of Toll-like receptors.

1.3.3.2 HT29 cell line

The HT-29 cell line is derived from human colon adenocarcinoma and is a valuable model for studying intestinal epithelial cells. The HT-29 cell line, when differentiated, displays characteristics of small intestinal enterocytes, particularly in terms of structural features, the presence of brush border-associated hydrolases and the time course of the differentiation process, which mirrors that observed in the small intestine. Despite these similarities, HT29 cells have several limitations: (1) they are malignant and have a high rate of glucose consumption coupled with impaired glucose metabolism; (2) although they replicate certain features of small intestinal enterocytes, they are derived from colonic tissue; (3) direct comparison with enterocytes from

normal colon is not possible because of their expression of brush border-associated hydrolases; (4) they cannot be equated with absorptive enterocytes because they do not specifically express all the necessary hydrolases, lack lactase and maltase-glucoamylase, and have different ion transport properties. It has been suggested that HT29 cells are similar to human fetal colon cells based on the types of hydrolases expressed and the level of intracellular glycogen accumulation. (Verhoeckx et al., 2015). In addition, villin expression levels in differentiated HT29 cells are comparable to those found in freshly isolated normal colonocytes. HT-29 cells can also differentiate into goblet-like cells capable of producing mucus under certain conditions, which is a significant distinction from Caco-2 cells that do not exhibit this capability. The mucus produced plays a critical role in forming a protective intestinal barrier. The differentiation process can be modulated by treatments such as sodium butyrate, which promotes the formation of mucus-secreting clones resembling intestinal goblet cells. In addition, villin expression levels in differentiated HT-29 cells are comparable to those found in freshly isolated normal colonocytes. Unlike the Caco-2 cell line, HT-29 cells are pluripotent and can differentiate into different enterocyte types depending on culture conditions. This differentiation is influenced by factors such as glucose availability; for example, HT-29 cells cultured in glucose-free media can

develop into enterocyte-like cells expressing specific hydrolases, whereas those cultured in glucose remain undifferentiated. HT-29 cells can also differentiate into goblet-like cells capable of producing mucus under certain conditions, a significant difference from Caco-2 cells, which do not have this ability. The mucus produced plays a critical role in the formation of a protective intestinal barrier. The differentiation process can be modulated by treatments such as sodium butyrate, which promotes the formation of mucus-secreting clones similar to intestinal goblet cells. In addition, specific subpopulations of HT-29 cells, such as HT29-MTX, have been isolated for their resistance to methotrexate and their ability to spontaneously differentiate into mucus-producing cells. These clones are often used in co-culture systems with Caco-2 cells to simulate the human intestinal barrier for absorption and permeability studies. Overall, HT-29 cells have been instrumental in the study of intestinal mechanisms and bioavailability, providing insights into epithelial differentiation and physiological functions of intestinal cells. (Del Carmen et al.,2018).

2. AIM

This work is part of a larger research project, carried out in collaboration with the Centro di Ricerca Piaggio at the University of Pisa, aimed at developing a three-dimensional (3D) in vitro platform to study the crosstalk between intestinal microbiota and bone tissue.

Here, we created a 3D in vitro model simulating the intestinal barrier by culturing Caco2 cells on electrospun gelatin scaffolds capable of mimicking the intestinal ECM. The developed model was evaluated using morpho-functional techniques. In addition, the effect on intestinal barrier integrity was tested in the presence of a supernatant derived from normal human microbiota. The results of this research, and the project in general, represent a step forward in the development of diagnostic tools based on the 3Rs.

3. MATERIALS AND METHODS

3.1. Bioengineered development of the intestinal model

3.1.1 Gelatin electrospun scaffolds

Gelatin electrospun scaffolds were prepared by the Research Center "E. Piaggio" at the University of Pisa.

Briefly, for electrospinning a solution of a 10% w/v of gelatin in glacial acetic acid (99.8%) was first prepared and then crosslinked with 3.68% v/v 3-(Glycidoxypropyl)-trimethoxysilane - GPTMS). The electrospinning process was carried out for 1 hour, with a direct current voltage of 30 kV and a feed rate of 1.2 ml/h. A distance of 10 cm was maintained between the metal needle (21 gauge) and the collector. After electrospinning, the structure was dried at room temperature for one week to ensure complete evaporation of the solvent. (Biagini et al., 2020). After the electrospinning, scaffolds were mechanically and physically characterized. Tensile tests were performed using a Zwick/Roell model Z005 with a 100 N load cell in both dry and wet conditions. Stress-strain curves were plotted for each specimen to calculate the modulus of elasticity. The permeability and the diffusion coefficients of the electrospun gelatin structure were also evaluated (Fig. 3.1 a and b). (Biagini et al., 2020). Finally, Scanning Electron Microscopy (SEM) was

used to evaluate fibre diameters and porosity using ImageJ software (Fig. 3.1.1 c). For more details on scaffold preparation, see Biagini et al, 2020.

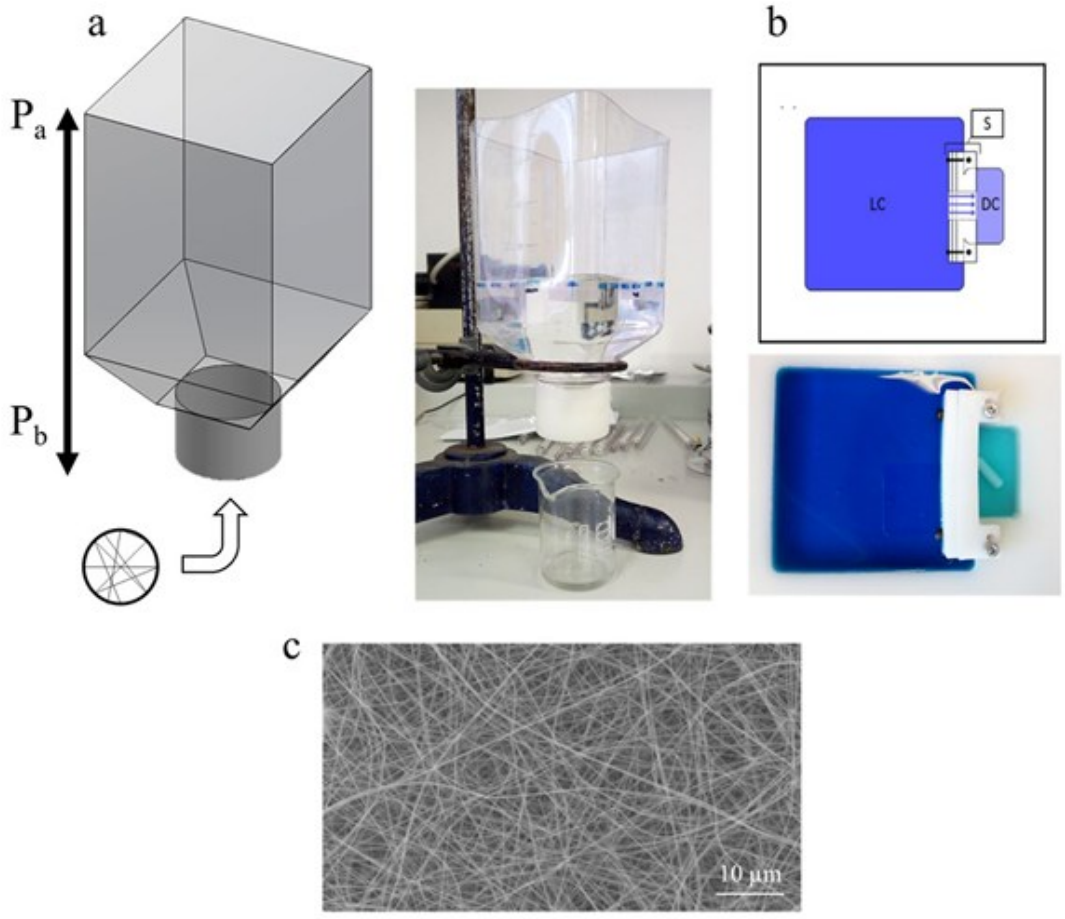

Figure 3.1. From Biagini et. al. 2020 Mechanical and physical characterization of the electrospun gelatin structure (a) Permeability test set-up. Pa represents the water pressure and Pb the atmospheric pressure. (b) Diffusion test set-up. (c) SEM image of the electrospun gelatin structure (2000x magnification).

3.1.2 Production of microbiota supernatant

The in vitro development of a normal human gut microbiota was performed at the University of Pisa. For this purpose, stool samples were screened to prevent infectious diseases, according to the European Guidelines for fecal microbiota transplantation. (Cammarota et al., 2017) and used. Prior to culturing, the samples were analyzed by multiplex PCR amplification to confirm the absence of pathogenic microorganisms and then stored at -80 °C in 10% *v/v* glycerol. (Biagini et al.,2022). For the development of normal microbiota samples were the cultured on the gelatin electrospun scaffolds for 24h, 42h and 7 days. At each time point SEM observations, Real-Time qPCR and Next Generation Sequencing (NGS) were performed. SEM showed the bacterial colonization of the scaffolds (Fig. 3.2) and the metagenomic analysis displayed the preservation of all phyla present in the faecal sample, as well as the biodiversity and richness of bacterial consortia. Moreover, at each time point the supernatant from the cultured human gut microbiota was collected and treated with a dialysis membrane (cut-off 14 kDa) to avoid side-effects of endotoxins. Supernatants derived from this in vitro model were then used in contact with the intestinal barrier

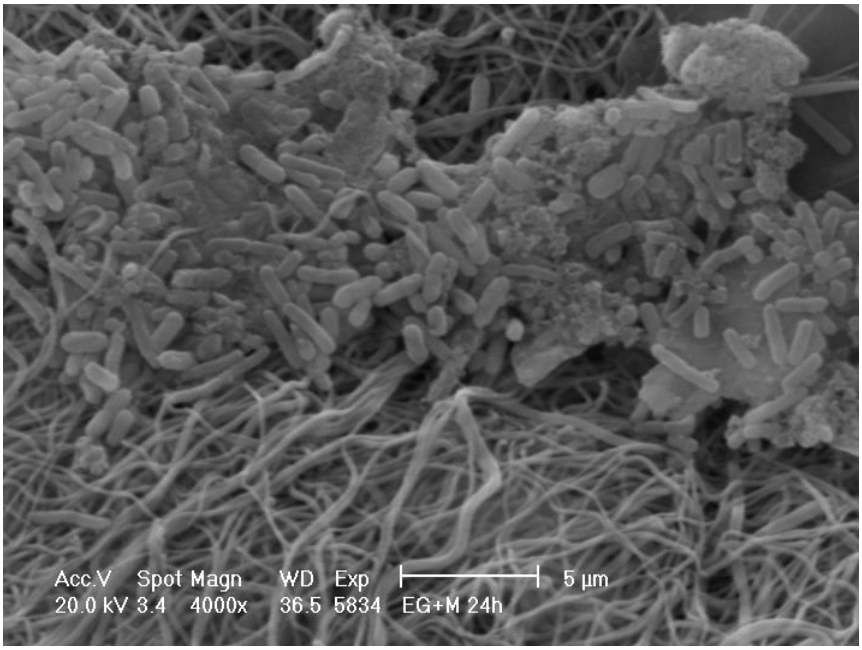

Figure 3.2. Representative SEM image of bacterial colonization on the electrospun gelatin scaffold at 24h

3.2 Intestinal Model Experimental Procedure

3.2.1 Cell culture

Caco2 cell line were used to perform experiments. Caco2 cells were plated in T25 flasks and cultured in a complete culture medium Dulbecco's Modified Eagle's Medium High Glucose (DMEM HG) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1% glutamine at 37°C and 5% CO_2.

Caco2 cells were detached from culture flasks using trypsin 1X and then seeded at a density of $3x10^5$ cells on the Transwell inserts and on the electrospun scaffolds, which were placed on the Transwell insert as a support. Before seeding gelatin electrospun, placed in 24-well plates, were incubated with ethanol 70% for 15 minutes and then were sterilized at each side under UV for 15 minutes. Scaffolds were then conditioned by rinsing in Dulbecco's Modified Eagle's Medium High Glucose (DMEM HG) overnight in a humified incubator (37 °C at 5% CO_2). After media removing, scaffolds were considered ready for cell seeding.

For seeding, cells at a density of $3x10^5$ cells/gelatin electrospun in a volume of 50 µL. After 30 minutes of incubation at 37 °C 500 µL of DMEM HG was added in each well. For Transwell alone, 700 µL of the cell suspension was added to the upper chamber of the Transwell, while 1 mL of growth medium was placed in the lower chamber. After seeding, the cells were incubated in a

humified incubator (37 °C at 5% CO_2). Once the cells reached confluence, this time point was designated as day zero. Following this initial phase, the culture continued to be monitored at regular intervals, specifically at 7, 14, 21 days post-seeding and analysed. Media were changed every two days.

All data obtained from the Caco2 seeded on the electrospun scaffolds were compared with those obtained on the insert of the Transwell alone.

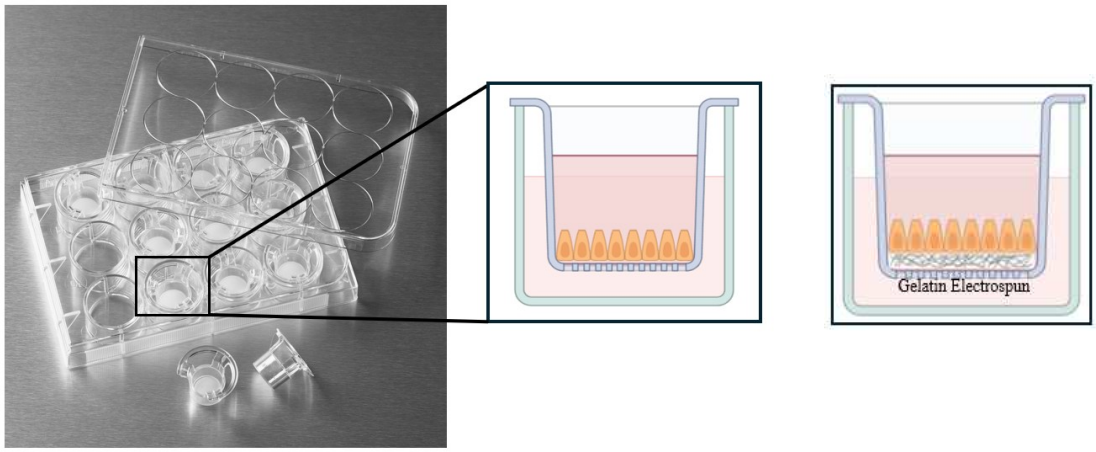

Fig. 3.3 Overview of a 24-well Transwell plate, with an inset showing a magnified view of one of the Transwell inserts and one of the gelatin electrospun, placed on top of the transwell inserts. The insert features a porous membrane that allows for the study of cell migration and permeability across the epithelial barrier, facilitating investigations into cellular interactions.

For Microbiota interaction the intestinal barrier obtained on gelatin electrospun scaffolds and on Transwell were treated for 24h with a 1:2 dilution of dialyzed supernatant.

3.2.2 Morphological assessment

3.2.2.1 Histological analysis

Cells seeded on the structures were fixed in 4% paraformaldehyde for 30 minutes and then processed for paraffin embedding. After fixation, samples were dehydrated with increasing series of alcohols and then incubated in xylene for 1 hour for clearing. After removing the xylene, the samples were placed in melted paraffin overnight and then included. Serial sections (5 μm thick) were mounted on slides, deparaffinized with xylene, rehydrated in a graded series of ethyl alcohols, and stained by Haematoxylin and Eosin (H&E) or Alcian Blue.

For H&E staining sections were placed in the haematoxylin solution, washed with running tap water and distilled water, placed in the eosin solution, washed with running tap water and distilled water again. The sections were mounted dehydrated and mounted with a Xylene based mounting medium with low viscosity (Eukitt, Sigma).

Alcian blue pH 2.5 (Sigma-Aldrich) was used to detect acidic muco-polysaccharides. The protocol involves incubation with Alcian blue solution, pH 2.5 for 3 minutes, rinsing with running tap water and distilled water, incubation with Nuclear fast red-aluminum sulfate solution 0,1% for 5

minutes and a further rinsing with water. After passing through a graded series of alcohols, treated with xylene histological and mounted with Eukitt.

Nikon Eclipse 600 light microscopy was used for the image observation.

3.2.2.2 Immunohistochemistry

For immunohistochemical analysis, 5-µm-thick tissue sections were deparaffinized and rehydrated by xylene and a graded series of ethyl alcohols (from 100% to 50%), before incubation with 3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Antigen retrieval was performed in Citrate buffer pH 6 at 70°C for 10 min. Sections were then incubated overnight at 4°C with the primary antibody E-cadherin. Antigens were visualized by Envision Dako REAL™ EnVision™ Detection System. Sections were counterstained with Mayer's hematoxylin, dehydrated, and mounted with Eukitt. Nikon Eclipse 600 light microscopy was used for observation.

3.2.2.3 Immunofluorescence

For immunofluorescence, cells were permeabilized with 0,1% Triton X100 in PBS (PBS-Triton) for 15 minutes at RT and then washed for 3 times with PBS. Blocking was performed in 3% bovine serum albumin (BSA) solution in PBS for 15 minutes and then samples were washed again with PBS. The primary antibody to ZO1 (1:100 dilution) was incubated overnight at 4°C.

After incubation, samples were washed and then incubated with a 488-labelled secondary antibody (1:1000 dilution in PBS/BSA 3%) es at RT. Cells were then incubated with phalloidin (1:5000 dilution in PBS) for 45 minutes and, after rising in PBS, incubated with 4′,6-diamidino-2-phenylindole (DAPI) (1:1000 dilution in PBS) for 15 minutes for nuclear detection. After mounted with VECTASHIELD samples were observed at Nikon E600 Fluorescence microscope (Nikon, Milan, Italy).

3.2.2.4 Scanning electron microscopy (SEM)

For SEM observation, cell-seeded scaffolds were fixed at the different time points (7, 14, 21 days) in 2% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in 1% osmium tetroxide, and dehydrated in increasing ethanol concentrations (25, 50, 70, 80, and 100%) and hexamethyldisilazane (HDMS)-dried. For the acquisition, samples were mounted on aluminum stubs, gold-sputtered and observed at FESEM ZEISS SUPRA 40 scanning electron microscopy.

3.2.3 Functional analyses

3.2.3.1 Transepithelial electrical resistance (TEER)

The Caco2 cell monolayer integrity was evaluated by Transepithelial electrical resistance (TEER). As described in section “Cell culture”, microbiota samples were added to the cells 24 hours before measurement.

TEER was recorded using an epithelial volt-ohm meter with a chopstick electrode. The electrode is sterilized with 70% ethanol and then the resistance of blank culture medium is measured. The shorter electrode is then placed in the centre and at the bottom of the gelatin electrospun or transwell inserts, while the long tip is just touching the bottom of the outerchamber (Fig.3.2.3.1); resistance is directly measured by a portable voltmeter.

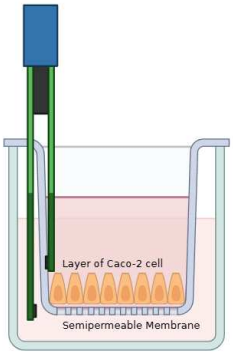

Fig.3.4 Transwell insert displaying the placement of the chopstick electrode used to measure the integrity of the intestinal barrier. This setup allows for real-time assessment of barrier function and permeability in the cultured epithelial cells.

TEER was recorded from three successive readings of each gelatin electrospun and Transwell insert. TEER values were expressed as ohms (Ω)

(resistance) × cm^2 (effective membrane area) after subtracting the blank value. The experiment was repeated two times.

3.2.3.2 Lucifer yellow

The permeability and integrity of a Caco2 cell monolayer can also be studied by measuring the passive transport of small hydrophilic molecules across the monolayer. These molecules cross the monolayer via the paracellular pathway, specifically through the tight junctions (TJ). (Verhoeckx et al., 2015). Lucifer yellow (LY) is a fluorescent marker commonly used to assess TJ integrity in cell monolayers, due to its hydrophilic nature and small molecular size (~450 Da).

Briefly, LY (100 µg/mL) was added to Hank's Balanced Salt Solution (HBSS) at 37 °C, and 500 µL of this solution was incorporated into the apical portion of the culture system. A further 2.3 mL of HBSS was then added to the basolateral part of the culture system. After incubation for three hours at 37°C in 5% CO_2, samples were read using a spectrofluorometer at excitation wavelengths of 485/535 nm (LY). Using a standard LY concentration curve, the pass rate for each sample was determined using relative fluorescence units (RFU) and quantitative permeability data was obtained as follows:

RFU (equilibrium)= $concentration\ of\ Lucifer\ Yellow\ \frac{\mu g}{mL}$

RFU (equilibrium)= $\frac{Lucifer\ yellow\ starting\ concetration \times volume\ (apical)}{volume\ (apical) + volume\ (basolateral)}$

RFU (equilibrium)= $\frac{100\frac{\mu g}{mL} \times 0.5\ mL}{0.5\ mL + 2.3\ mL} = 17.85\ \mu g/mL$

% Lucifer Yellow Passage= $\frac{RFU\ (test) - RFU\ (blanck)}{RFU\ (equilibrium) - RFU\ (blank)} \times 100$

If the passage of LY through the membrane is less than five percent, the epithelial barrier is considered intact.

4. RESULTS

4.1 Development of in vitro 3D model

4.1.1 Morphological Analysis

Caco-2 cell morphology was examined over periods of 7, 14, and 21 days in order to assess the intestinal barrier development. Both Haematoxylin and Eosin (Figs. 4.1 A-C) and Alcian blue/Nuclear fast red staining (Figs. 4.1 D-F) highlighted adhesion, progressive proliferation, and differentiation of the cells throughout the culture period. Light microscopy observation confirmed that Caco-2 cells were successfully cultured on the gelatin electrospun scaffold, demonstrating good adhesion and stable retention on the scaffold. In addition, we noted distinct morphological changes as the culture progressed, indicating improved cellular organization and maturation. In detail:

- at day 7, the cells formed a monolayer with a cuboidal morphology (Figs. 4.1 A and D).
- at day 14, whilst many cells retained the same cuboidal morphology as at day 7, other began to exhibit a columnar shape. This transition indicated a more pronounced epithelial organization that begin to resemble the structure of intestinal epithelium (Figs. 4.1 B and E).

- at day 21, the Caco-2 cells exhibited features typical of mature enterocytes, including the formation of the brush border (Fig. 4.1 G) and the glycocalyx together with goblet cells (Fig. 4.1 H), features essential for the establishment of a functional intestinal barrier with absorption and permeability properties.

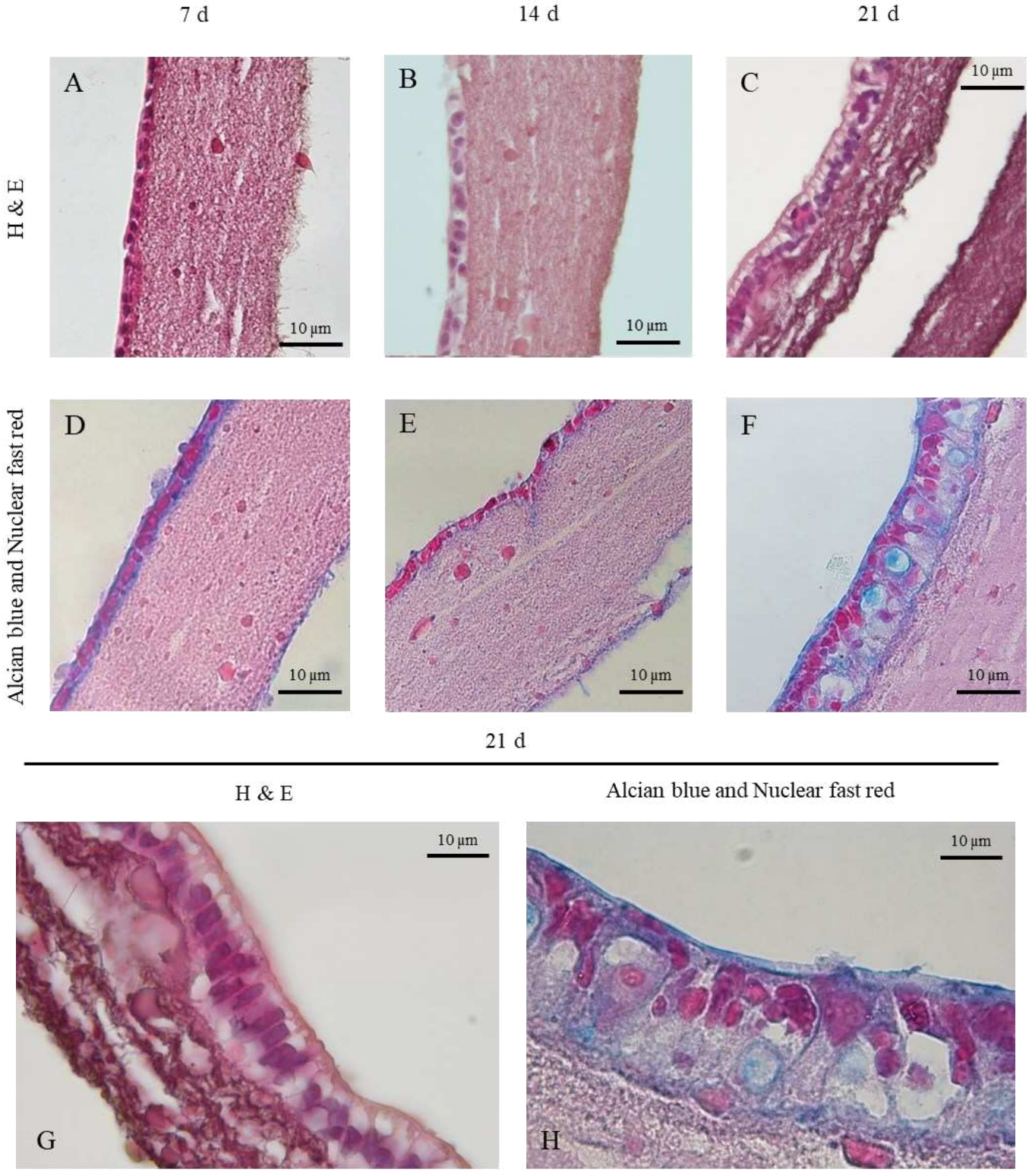

Fig. 4.1 Histological images of Caco-2 cells on electrospun structures at 7d, 14d and 21d.

A-C) Haematoxylin and eosin staining at different time points. A) Day 7: cells form a cuboidal monolayer. B) Day 14: some cells have a columnar morphology. C) Day 21: columnar epithelium with basal nuclei and a visible brush border. (40x magnification). D-F) Alcian blue/Nuclear fast red staining at different time points. D) Day 7. E) Day 14: F) Day 21 with clearly visible goblet cells and glycocalyx (in blue). (40x magnification)

G-H) High-magnification images at f day 21 sections confirming the presence of the brush border (G), and glycocalyx and goblet cells (H). (100x magnification).

The structure of the Caco-2 cell monolayers was further characterized by SEM analysis. At day 7, SEM observations showed that the Caco-2 cells were present on the surface of the gelatin scaffold but appeared dispersed and disorganized. At day 14, there was a marked increase in cell density and cells began to arrange themselves more cohesively on the scaffold surface. This progression continued until day 21, when SEM revealed the presence of well-defined and dense microvilli on the cell surfaces, indicating increased cell differentiation and maturation. These results highlight the ability of the electrospun gelatin scaffold to support Caco-2 cell growth and facilitate their organization into a more structured epithelial layer over time.

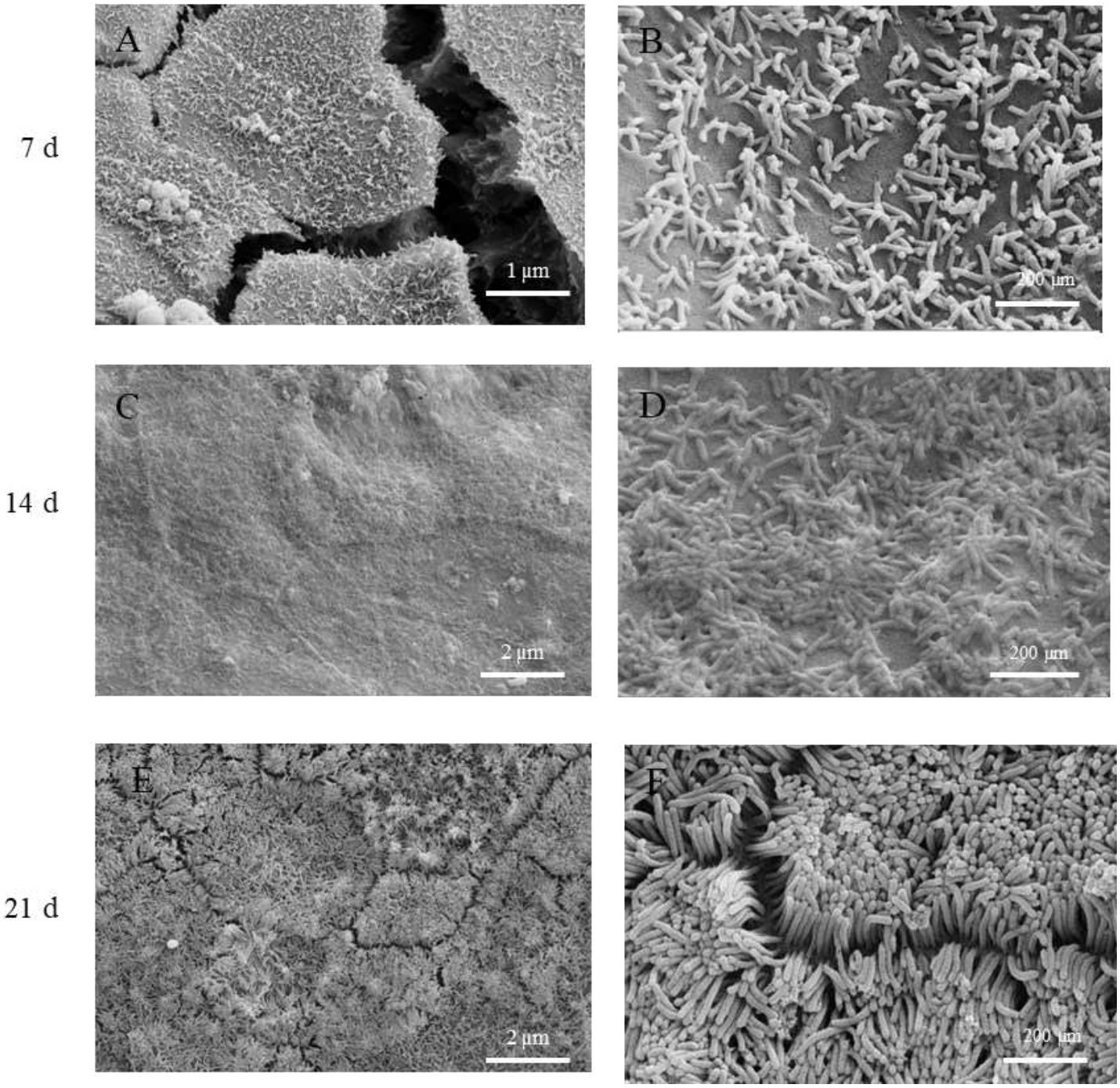

Fig. 4.2 SEM images of Caco-2 cells on electrospun structures at 7d, 14d and 21d.

A-B) Day 7: Cells forming a cuboidal monolayer, at higher magnification (B) disorganized microvilli are present
C-D) Day 14: Tightly packed cells; at higher magnification (D) an increased number of microvilli still disorganized are present
E-F) Day 21: well-formed epithelium with microvilli visible at both low (E) and high (F) magnification

4.1.2 Integrity

To assess the integrity of tight junctions (TJs), analogous to those found in the intestinal epithelium in vivo, the presence of both tight junctions and adherens junctions was assessed in Caco-2 cells cultured on gelatin electrospun scaffolds for 21 days.

Immunofluorescence staining for zonula occludens 1 (ZO1) was used to detect the tight junctions (Fig. 4.3). The results showed a prominent green fluorescent signal along the cell borders, indicating effective tight junctions assembly and the formation of a cohesive epithelial barrier. This finding is critical, as tight junctions play a vital role in regulating paracellular permeability and maintaining the integrity of the intestinal barrier. In addition, staining with phalloidin revealed red fluorescence highlighting cytoskeletal structure, further supporting the idea that Caco-2 cells have developed a well-organized cytoskeleton necessary for maintaining cell shape and function. The combination of these markers confirmed the successful maturation of the Caco-2 monolayer, reflecting its potential as an in vitro model for studying intestinal absorption and barrier function.

Adherents junctions were detected by immunohistochemistry targeting E-cadherin, which revealed the presence of distinct adherents junctions, confirming their crucial role in maintaining intercellular adhesion and

preserving the structural integrity of the epithelial layer (Fig. 4.4). These results demonstrated the development of functional epithelial junctions, indicative of a mature and physiologically relevant intestinal barrier.

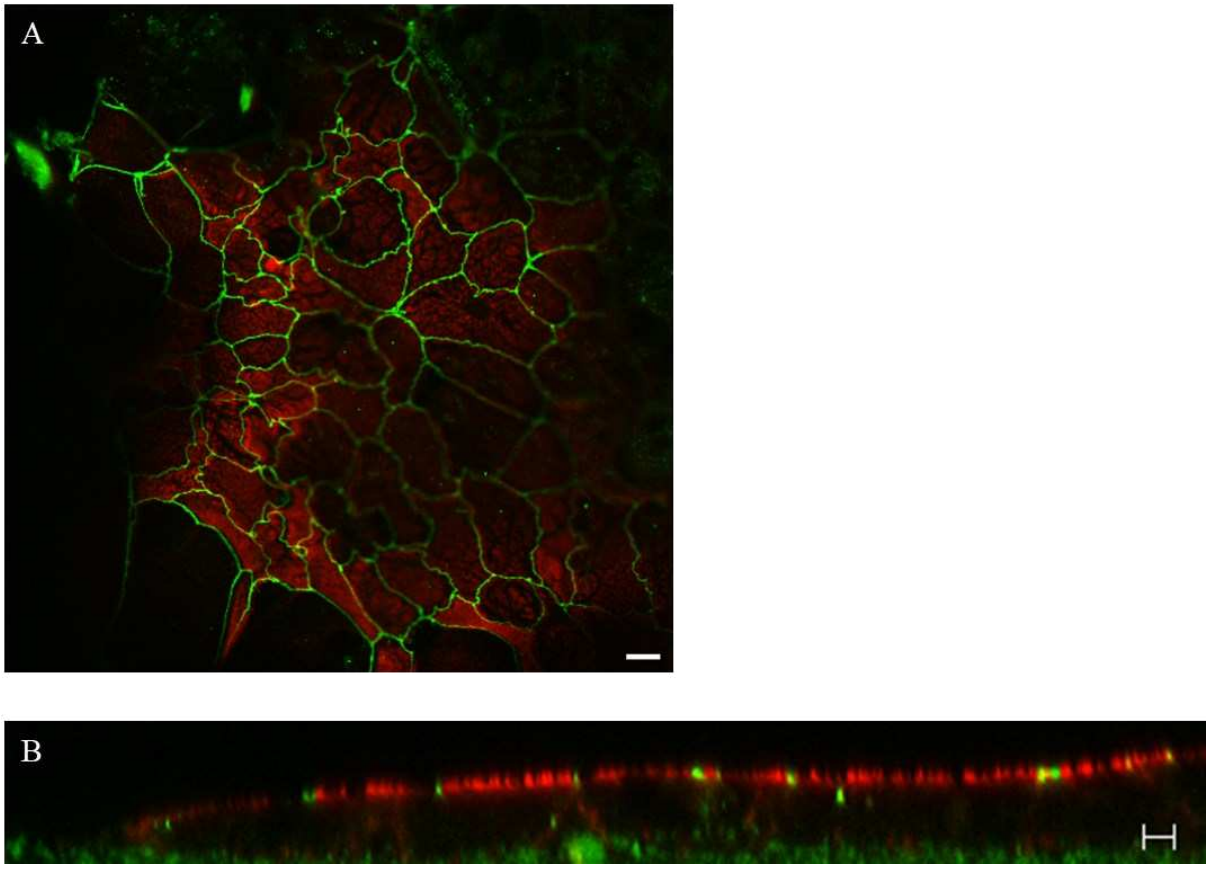

Fig. 4.3 Immunofluorescence analysis of Caco2 cells seeded on gelatin electrospun scaffold for 21 days without microbiota.

(A) Detection of zonula occludens 1 (ZO1) and actin. Scale bar:10 um

(B) The cross-sectional images by Z stack reveal zonula occludens 1 (ZO1) and actin. Scale bar: 5 um

In order to validate and improve our in vitro gut model the effect of the supernatants of dialysed human gut microbiota was tested to assess their effect on barrier integrity and functionality. Specifically, analyses were performed after 14 and 21 days in cells seeded on the electrospun scaffold

placed on a Transwell support and in Caco-2 cells seeded directly on the Transwell insert as a control.

4.2.1 Integrity

Immunofluorescence images of microbiota-treated Caco-2 cells (Figs. 4.5 A-B), showed well-defined cell borders, defined by the localization of zonula occludens 1 (ZO-1) and a good organization of the actin cytoskeletal structures. These observations confirmed the maintenance of epithelial barrier integrity even in the presence of microbiota supernatant.

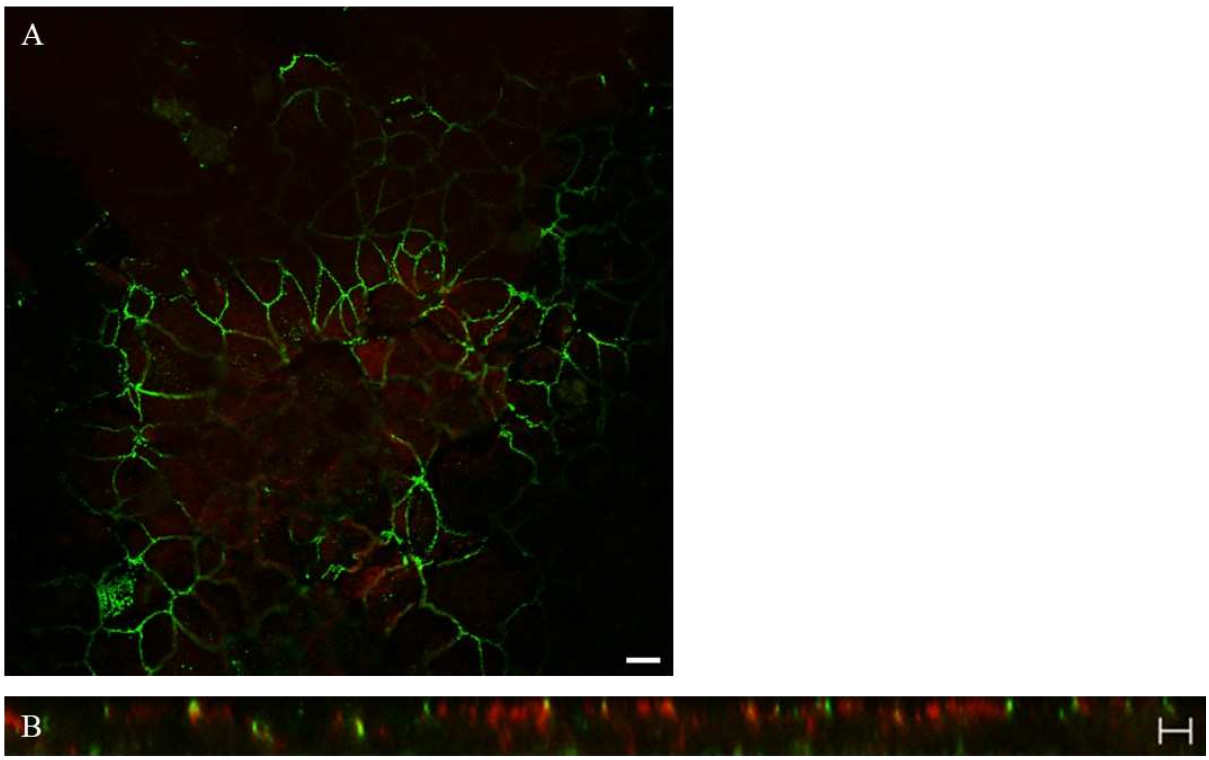

Fig. 4.5 Representative immunofluorescence image of Caco2 cells seeded on gelatin electrospun scaffold for 21 days in the presence of microbiota supernatant. (A) Detection of zonula occludens 1 (ZO1) and actin. Scale bar:10 um (B) The cross-sectional images by Z stack reveal zonula occludens 1 (ZO1) and actin. Scale bar: 5 um

4.2.2 Functionality

An intact epithelial cell monolayer is important for the construction of an in vitro intestinal model that mimics the in vivo situation. Therefore, the integrity and functionality of the Caco-2 cell monolayer grown on the electrospun scaffold was verified by TEER and LY permeability assays. Analyses were performed after 14 and 21 days in the absence or presence of dialysed microbiota supernatant. Cells seeded on the Transwell insert represented the control cultures.

4.2.2.1 Transepithelial Electrical Resistance (TEER)

The TEER values obtained at 14 and 21 days in the presence of the microbiota supernatant were normalized to respective controls (i.e. gelatin electrospun scaffold or Transwell). For cells cultured on Transwell (blue pilots), a significant decrease ($p < 0.01$) in TEER values was observed from day 14 to day 21, suggesting that the presence of microbiota modifies the transepithelial electrical resistance over time. It should be noted that the TEER values at 21 days were still higher than of 300 Ω-cm², which is considered an indication of a well-developed and functional barrier. On the contrary, in the epithelial barrier developed on the electrospun scaffolds (grey pilots), a significant increase ($p<0.001$) from 14 to 21 days was detected,

although with TEER values lower than those found in the barrier developed on Transwell inserts (Fig.4.6).

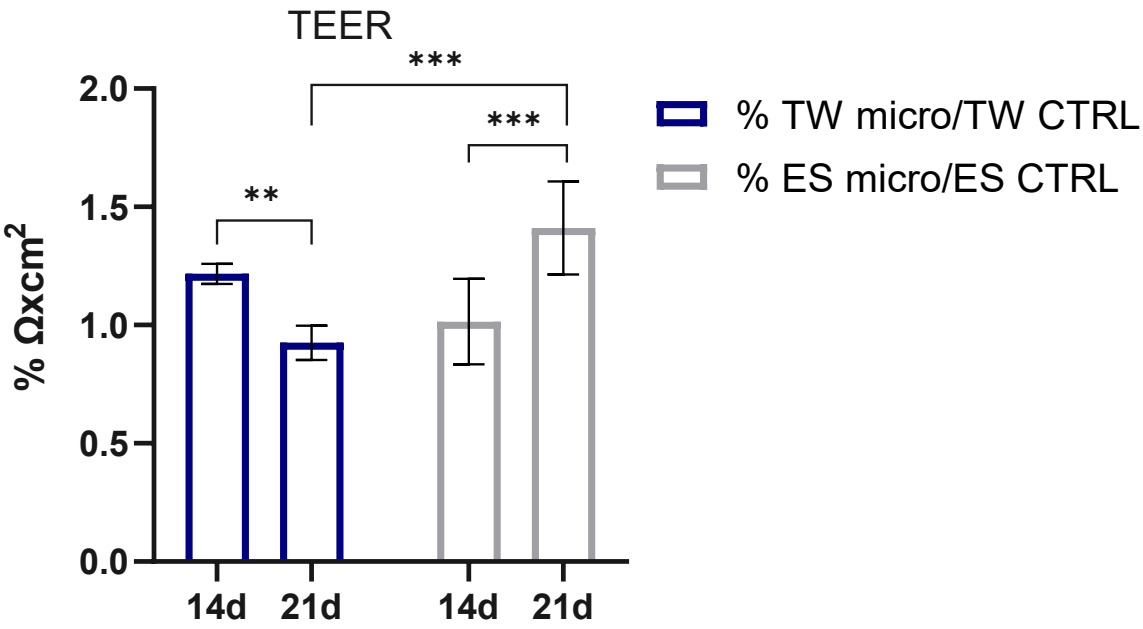

*Fig. 4.6 Histogram showing normalized TEER values of Caco-2 cells on gelatin scaffolds (ES) and Transwell (TW) systems. From day 14 to day 21, TEER decreases in Transwell with microbiota compared to the control, while it increases in gelatin scaffolds with microbiota, indicating different effects on epithelial barrier integrity based on the culture system. $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.*

4.2.2.2 Lucifer yellow (LY)

The permeability of the Caco-2 monolayers was also assessed using Lucifer Yellow on the same samples. The passage of this fluorescent marker must be less than 5% to indicate an intact membrane integrity. In both culture systems (Transwell and electrospun gelatin scaffold), the Lucifer Yellow permeability

values remained below this threshold, indicating that the epithelial barrier was generally intact. However, the values obtained from the Transwell system were higher than those from the gelatin electrospun scaffold. Notably, between day 14 and day 21, the percentage of Lucifer Yellow permeability in the Transwell system increased slightly, remaining below 5%. In contrast, the percentage of permeability decreased for the Caco-2 barrier developed on the electrospun gelatin scaffold, (Fig.4.7).

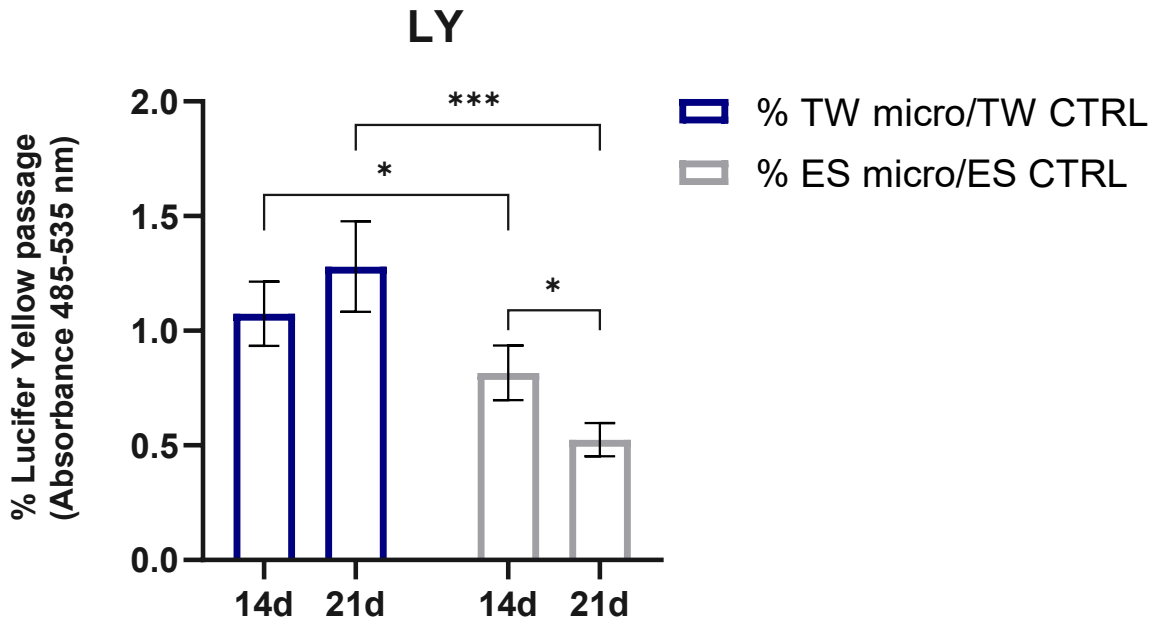

*Fig. 4.7 Histogram showing Lucifer Yellow permeability values for Caco-2 monolayers cultured on gelatin electrospun scaffolds and Transwell systems. Permeability remained below 5% for all conditions, indicating intact membrane integrity. However, from day 14 to day 21, permeability increased in the Transwell system, while it decreased further in the gelatin electrospun scaffold system. $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.*

5. DISCUSSION

The development of in vitro models that follow to the principles of the 3Rs (Replacement, Reduction, and Refinement) is essential for the advancement of biomedical research, innovation in diagnostics, and the development of new therapies. These principles, introduced by Russell and Burch in 1959, aim to improve animal welfare and ensure that scientific experiments are ethically justified and scientifically valid. The use of alternative models, such as in vitro cellular models and organoids, make it possible to reduce the use of animals in experiments, thus contributing to more sustainable and responsible research (Modelli Sperimentali Nella Ricerca Biomedica - Enciclopedia - Treccani, n.d.).

In particular, 3D models represent a significant innovation in the field of biofabrication. These techniques allow for the creation of cellular structures that more closely mimic the physiological environment in which cells grow compared to 2D in vitro cell culture systems, which are unable to accurately represent and simulate the complex processes observed in vivo (Wang et al., 2021). 3D models can provide more detailed information on cellular dynamics and interactions between different cell types and with the ECM, making them valuable tools for studying disease pathogenesis and/or screening new drugs (Modelli Sperimentali Nella Ricerca Biomedica -

Enciclopedia - Treccani, n.d.). In this context, 3D intestinalmodels, such as 3D bioprinting, spheroids, organoids, organ-on-a-chip, and tissue engineering scaffolds offer unique opportunities to study intestinal physiology and related pathologies. The systems will not only allow for the investigation of interactions between epithelial cells and microbiota but could also be used to test the efficacy of innovative therapies in an environment that better simulates in vivo conditions (Istituto Nazionale Tumori).

The aim of our research was to develop a reliable and valid in vitro model to recreate the human intestinal barrier. The experiments performed in this thesis are part of a larger research project, carried out within the framework of a collaboration between the Laboratory of Human Histology of UNIVPM and the Centro di Ricerca Piaggio of the University of Pisa, aimed at developing a three-dimensional (3D) in vitro platform to study the crosstalk between intestinal microbiota and bone tissue.

The intestinal barrier was developed starting from a biofabricated electrospun gelatin structure that served as a cell scaffold for recreating the extracellular matrix (ECM).

We demonstrated that the gelatin scaffolds were suitable for the seeding and culturing of Caco-2 cells up to 21 days. Morphological analysis using haematoxylin and eosin and Alcian blue/nuclear fast red staining showed that

the cells seeded on the scaffold started from a thin cuboidal epithelium and at 21 days reached the correct morphology of the intestinal epithelia with cylindrical cells, formation of glycocalyx and brush border, and the presence of goblet cells. Scanning electron microscopy (SEM) observation confirmed that the brush border consisted of well-formed microvilli. Overall, also considering the changes in cell morphology throughout the culture period, we concluded that the use of electrospun gelatin provides a suitable three-dimensional environment that mimic the native ECM supporting cell growth and differentiation and enhancing the physiological relevance of our in vitro model for studying intestinal barrier functions.

Previous studies have investigated different electrospun materials for the development of an intestinal barrier. A polycaprolactone (PCL) combined with cellulose acetate and ethyl cellulose (EC) has been used to form an intestinal epithelial tissue. (Gonçalves et al.,2023). Other studies have instead used Polyethylene terephthalate (PET) nanofibrous scaffolds for the in vitro intestinal barrier, which show superior tight junction formation and drug permeability compared to an in vitro 2D model. (Patient et al., 2019). A study from Hu et al. (2021) demonstrated that the use of polylactic acid (PLA) and polyacrylonitrile (PAN) nanofibrous electrospun scaffold improved the adhesion, proliferation and differentiation of Caco2 cells. The comparison of

our results with these studies allowed us to highlight the versatility and efficacy of gelatin scaffolds.

Tight junctions (TJs), including occludins, claudins, and zonula occludens proteins, as well as adherens junctions, are essential for maintaining the integrity of an intestinal barrier. In our in vitro system, we observed the expression of both ZO-1 and E-cadherin by immunofluorescence and immunohistochemistry, respectively. In particular, ZO-1 was clearly at the apical region of the cell layer corresponding to junction points between cells and its presence suggested a good cell integration and effective formation of the epithelial barrier, both crucial elements for maintaining the integrity of the intestinal barrier. Previous studies have shown that the absence or reduction of ZO-1 expression correlates with increased intestinal permeability, leading to impaired barrier function. For example, alterations in ZO-1 localization or expression have been observed in pathological conditions such as inflammatory bowel disease (IBD), where loss of epithelial barrier integrity occurs (Wei et al., 2017) (Liu et al., 2005).

Having developed an in vitro intestinal barrier that morphologically mimics intestinal characteristics, we wanted to test its functionality in the presence of gut microbiota. First, we evaluated the effect of the supernatant of a normal human gut microbiota on the barrier integrity using immunofluorescence for

ZO-1. TJs were detectable also in cells treated with microbiota with no structural differences compared to untreated barrier. On the contrary, differences between the barrier developed on the electrospun gelatin scaffold and the control system (Transwell) were quantitatively assessed by measuring intestinal barrier integrity using transepithelial electrical resistance (TEER) and lactate clearance (LY). The data showed that microbiota treatment led to a change in intestinal barrier resistance, confirming the importance of microbiota in maintaining intestinal barrier functionality; recent studies support that intestinal microbiota are involved in regulating both integrity and function within a homeostatic balance (Di Tommaso et al., 2021).

Overall, these data allow a comparison of the efficacy of gelatine scaffolds against Transwell systems. Although both models demonstrated support for an intact intestinal barrier, the gelatine scaffolds proved superior. This can be attributed to their ability to provide a more favourable three-dimensional environment for microbial proliferation and biofilm formation, which are critical factors for barrier stability and functionality (Capurso, 2016). The three-dimensionality of gelatin scaffolds allows them to better mimic physiological intestinal conditions, while promoting more complex interactions between epithelial cells and microbiota. These results confirm the role of microbiota in intestinal permeability and highlight the importance of

developing optimal in vitro models to study intestinal barriers. The use of biofabricated scaffolds could be a promising approach for future research, contributing to new therapeutic strategies for gastrointestinal diseases or potentially integrated into a more complex model to create a platform linking intestinal microbiota with bone remodelling.

In conclusion, this work contributed to the development of advanced in vitro models to study the gut-bone crosstalk, addressing the increasing need to reduce animal testing by adhering to the 3Rs principle.

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