UNIVERSITA' POLITECNICA DELLE MARCHE

DIPARTIMENTO DI SCIENZE DELLA VITA E DELL'AMBIENTE

Corso di Laurea Magistrale

BIOLOGIA MOLECOLARE E APPLICATA

curriculum Tecnologie Biologiche

Organoidi Intestinali Umani per la Valutazione della Tossicità del BPA: utilizzo di Probiotici come Strategia Innovativa di Mitigazione

Human Intestinal Organoids for BPA Toxicity Assessment and Probiotics as Innovative Mitigation Strategy

Tesi di Laurea Magistrale di:

Cristina D'Amelio

Relatore:

Chiar.ma Prof.ssa

Oliana Carnevali

Correlatori:

Dott. Christian Giommi

Dott. Jordi Guiu Sagarra

Anno Accademico 2023/2024

Sessione di Laurea Luglio 2024

A chi mi ha da sempre sostenuto.

Ai miei genitori, pilastri della mia vita,

per il loro Amore incondizionato e l'instancabile sostegno.

Table of Contents

ABSTRACT

Il Bisfenolo A (BPA) è un plastificante ampiamente diffuso e uno degli interferenti endocrini più comuni nell'ambiente. Gli esseri umani sono frequentemente esposti al BPA attraverso l'ingestione di alimenti e bevande contaminati, permettendo al BPA di raggiungere l'intestino. Studi *in vivo*, sia su modelli mammiferi che non mammiferi, hanno dimostrato gli effetti tossici del BPA sul tratto gastrointestinale, associandolo a infiammazione, alterazioni della struttura e della permeabilità della barriera intestinale, cambiamenti istopatologici nel tessuto intestinale e alterazioni nella composizione del microbiota intestinale, con conseguente disbiosi o comparsa di disturbi metabolici. Contemporaneamente, numerosi studi *in vivo* hanno evidenziato i molteplici effetti benefici dei probiotici sulla salute umana e animale. Attualmente, vi è un grande interesse verso gli effetti preventivi o terapeutici dei probiotici contro gli interferenti endocrini, incluso il BPA. Basandoci sulle evidenze riguardanti la tossicità intestinale del BPA e i benefici dei probiotici, il nostro studio ha ipotizzato che il BPA possa esercitare una tossicità diretta sulle cellule intestinali, mentre il probiotico potrebbe contrastare la tossicità indotta dal BPA a livello intestinale. Per verificare questa ipotesi, abbiamo

utilizzato organoidi intestinali umani, ottenuti da biopsie intestinali di tre pazienti volontari, come modello 3D *in vitro* per l'esposizione a diverse concentrazioni di BPA e per il successivo co-trattamento con il probiotico SLAB51. Sugli organoidi esposti al BPA sono state condotte analisi morfometriche e molecolari per valutare l'espressione ed i livelli di vari marcatori. Le analisi morfometriche sono state utilizzate per valutare la crescita e la proliferazione degli organoidi esposti al BPA e co-trattati con il probiotico SLAB51. Le analisi di RT-qPCR sono state eseguite sugli organoidi esposti al BPA per valutare l'espressione genica di marker coinvolti nei processi di proliferazione cellulare, differenziamento, funzionalità cellulare, stress ossidativo e infiammazione, mentre l'immunoistochimica è stata utilizzata per valutare i livelli proteici di Ki-67, un marcatore di proliferazione, ALPi, un enzima secreto dagli enterociti che indica la funzionalità delle cellule intestinali, e CASP3, una proteina esecutrice dell'apoptosi. Le analisi morfometriche hanno mostrato una significativa riduzione del diametro e dell'area degli organoidi esposti a diverse concentrazioni di BPA, identificando 0.56 pM, 56 pM e 56 μM come le concentrazioni più tossiche di questo contaminante. La co-somministrazione del probiotico SLAB51 ha dimostrato un effetto mitigante sulla tossicità del BPA. In particolare, questo effetto era ben evidente analizzando le morfometrie degli organoidi esposti a 56 pM di BPA, dove il

probiotico ha contribuito a ripristinare valori di area e diametro comparabili al gruppo di controllo non esposto al BPA. L'immunoistochimica ha rivelato una significativa diminuzione dei livelli proteici di Ki-67 nel gruppo trattato con la dose più alta di BPA, 56 μM, suggerendo un effetto antiproliferativo sugli organoidi. Inoltre, l'aumento dell'espressione di ALPi e i cambiamenti nell'attività enzimatica indicano potenziali disfunzioni intestinali dovute all'esposizione al BPA. Un aumento significativo dei livelli di CASP3 è stato osservato a basse concentrazioni di BPA, in particolare 0.56 pM, indicando un effetto pro-apoptotico non evidente a concentrazioni più elevate, confermando il comportamento tipico del BPA con risposte non monotoniche ed effetti variabili a seconda della dose. In conclusione, il nostro studio dimostra che il BPA può avere effetti tossici sugli organoidi intestinali a seconda della concentrazione, sottolineando la complessità della risposta tossica. Pertanto, questo studio contribuisce a una comprensione più approfondita dei rischi associati all'esposizione al BPA e allo sviluppo di strategie per proteggere la salute intestinale di fronte a sfide ambientali. Le nostre analisi molecolari in corso mirano a chiarire ulteriormente l'impatto del BPA sugli organoidi intestinali e a valutare le proprietà benefiche del probiotico contro la tossicità del BPA a livello molecolare e funzionale.

INTRODUCTION

1.1 Endocrine Disruptive Chemicals (EDCs)

The first definition of endocrine disruptive chemicals (EDCs) was provided in 1996 at the European seminar on endocrine disruptors (EDs) held in Weybridge, United Kingdom. “An endocrine disruptor is an exogenous substance that induces adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function”. An additional definition regarding potential endocrine disruptors was also agreed upon: “A potential endocrine disruptor is a substance that possesses properties that could lead to endocrine disruption in an intact organism” (Schug et al., 2016). Endocrine disruptors, by interfering with the endocrine (or hormonal) system, are closely implicated in the global decline of metabolic health, leading to metabolic disorders such as insulin resistance, diabetes and alcoholic fatty liver disease (Maradonna & Carnevali, 2018). This large and multifaceted family includes plasticizers such as phthalates and bisphenols (Warner & Flaws, 2018), industrial chemicals including alkylphenols and flame retardants (Chokwe et al., 2015), atmospheric pollutants such as polycyclic aromatic hydrocarbons (Patel et al., 2020), along with pesticides, heavy metals, and dioxins (Alengebawy et al., 2021).

EDCs can act through various mechanisms and exhibit different properties (Vandenberg et al., 2012):

(i) they can be toxic at very low concentrations, showing a mechanism of action similar to that of hormones;

(ii) display of non-monotonic U-shaped responses, where low contaminant concentrations can cause phenotypic alterations not necessarily observed with higher doses or observed only at very high concentrations;

(iii) agonistic or antagonistic properties following interaction with hormonal receptors resulting in complex and unpredictable biological responses mimicking stimulation or inhibition of hormonal action;

(iv) alteration of the metabolism of natural hormones, leading to variations in the amount of hormone available for receptor binding and as consequence: the hormone can be produced in greater or lesser quantities than usual or can be degraded at an abnormal rate. Additionally, some EDCs affect hormone transport, further modifying the amount of hormone available to bind to receptors;

(v) some EDCs are able to accumulate in the adipose tissue due to their lipophilic nature.

Initially, it was thought that endocrine disruptors exerted their effects exclusively through binding to nuclear hormone receptors (NRs). For example, Bisphenol A (BPA) binding to estrogen receptors (Huang et al., 2020); some pesticides, phthalates, and polyhalogenated compounds binding to androgen receptors; dioxins, perchlorates, and furans binding to thyroid receptors, with retinoid receptors being affected as well (Schug et al., 2011; Diamanti-Kandarakis et al., 2009). Other less-known mechanisms of action of EDCs include modification of gene expression and alteration of epigenetic processes (Lopez-Rodriguez et al., 2021). Epigenetic alterations can also be transmitted through the germline to unexposed generations, causing intergenerational and transgenerational effects (Skinner, 2016).

Among the disorders associated with exposure to EDCs there are metabolic disorders. EDCs can be ingested and reach the intestine, where they affect various types of intestinal cells, compromising the function of the mucosal-intestinal barrier (Coruzzi, 2010). Additionally, the disruption of the hormonal environment induced by EDCs can lead to changes in the gut microbiota leading to dysbiosis (Gálvez-Ontiveros et al., 2020). EDCs can have a direct impact on the gut microbiota, especially during the perinatal period when the microbiota begins to colonize the intestine (Hampl & Starka, 2020). Several studies have in fact shown that postnatal exposure of rodents to various EDCs, such as BPA

(Lai et al., 2016), diethyl phthalate, methylparaben, triclosan, and a mixture of these chemicals (Hu et al., 2016) or polychlorinated biphenyls (Choi et al., 2013), can alter the growth of specific intestinal microbial populations, evidencing that exposure to EDCs compromises the normal intestinal microbiota and has a potential negative impact on metabolic health.

Gut microbiota plays an essential role in regulating fat accumulation (Bäckhed et al., 2004), controlling intestine ability to catabolize and assimilate nutrients and modulating the intestinal production of hormones that regulate appetite or nutrient absorption, such as insulin. In fact, disorders in the gut microbiota have been associated with obesity (Teixeira et al., 2012) and diabetes (Larsen et al., 2010). The interactions between gastrointestinal microbiota and EDCs are multifaceted and interdependent. In fact, while on one hand environmental contaminants alter the composition of gastrointestinal bacteria and/or the metabolic activity that shapes the composition and the characteristics of the host's microbiome, on the other hand, the microbiota can extensively metabolize environmental chemicals, thus modulating their toxicity in the host (Claus et al., 2016). In recent years research has highlighted that, alterations in the gut microbiota and intestinal health and functionality, including those induced by EDCs, can have significant repercussions on overall health and on the functioning of other organs, such as the brain. For instance, changes in the

composition of the gut microbiota or in the permeability of the gastrointestinal barrier can trigger alterations in the gut-brain axis, eliciting inflammatory responses that may contribute to neurological conditions (Balaguer-Trias et al., 2022). Ni et al., (2021) demonstrated that cognitive impairments in male mice exposed to BPA were associated with alterations in gut microbiota diversity, decreased production of short-chain fatty acids (SCFAs), damage to intestinal barrier function and blood-brain barrier, increased neuroinflammation, and colon inflammation (Ni et al., 2021). Thus, EDCs can cross the intestinal barrier and directly enter the intestinal environment. These compounds can also disrupt the enteric nervous system, which controls gastrointestinal functions and microbiota composition and activity through neuropeptides and neurotransmitters, such as serotonin and catecholamines. By altering the synthesis, release and degradation of these neurotransmitters, EDCs can influence the composition and functionality of the intestinal microbiota, potentially leading to dysbiosis (Cresci & Bawden, 2015; Heintz-Buschart & Wilmes, 2018; Salvucci, 2019). A dysbiotic microbiota can compromise the intestinal barrier, causing the release of molecules from the diet and from the microbiota itself that can accumulate in organs and tissues and that can have a negative impact on the host's metabolism and health (Hrncir, 2022).

Therefore, studies examining the interactions between EDCs, intestinal cells and the gut microbiota will help determine the effects of EDCs at the intestinal level and beyond. By understanding how EDCs influence the microbiota and *vice versa*, researchers can gain insight into the intricate mechanisms through which EDCs impact overall health, including intestinal and metabolic health, immune function, and neurological function. This comprehensive understanding is crucial for developing strategies to mitigate the adverse effects of EDCs exposure and protect human health.

1.1.1 The Plasticizer Bisphenol A (BPA)

BPA (4,4'-isopropylidenediphenol, 2,2'-bis(4-hydroxyphenyl) propane) is a chemical substance used in combination with other chemicals to produce certain plastics and resins and is classified as an endocrine disruptor chemical. BPA is used, for example, in polycarbonate plastic, a type of transparent and rigid plastic used to make water dispensers, food containers, and reusable beverage bottles. This chemical is also used to produce epoxy resins found in protective coatings and linings for cans and vats for food and beverages (European Food Safety Authority, 2023). The binding of BPA to plastic polymers is not covalent, leading to the release of this compound into packaged foods and liquids, as well as into the environment (Cousins et al., 2002). The transfer of BPA from the

coating to food depends on the food composition, whether there is direct contact with the food, the duration of contact, the thickness of the coating material, the contact temperature, and pH values (Repossi et al., 2016). In humans, the main routes of exposure to BPA are direct skin contact, oral exposure through the ingestion of contaminated foods and beverages, or inhalation (Chianese et al., 2018) (**Figure 1**).

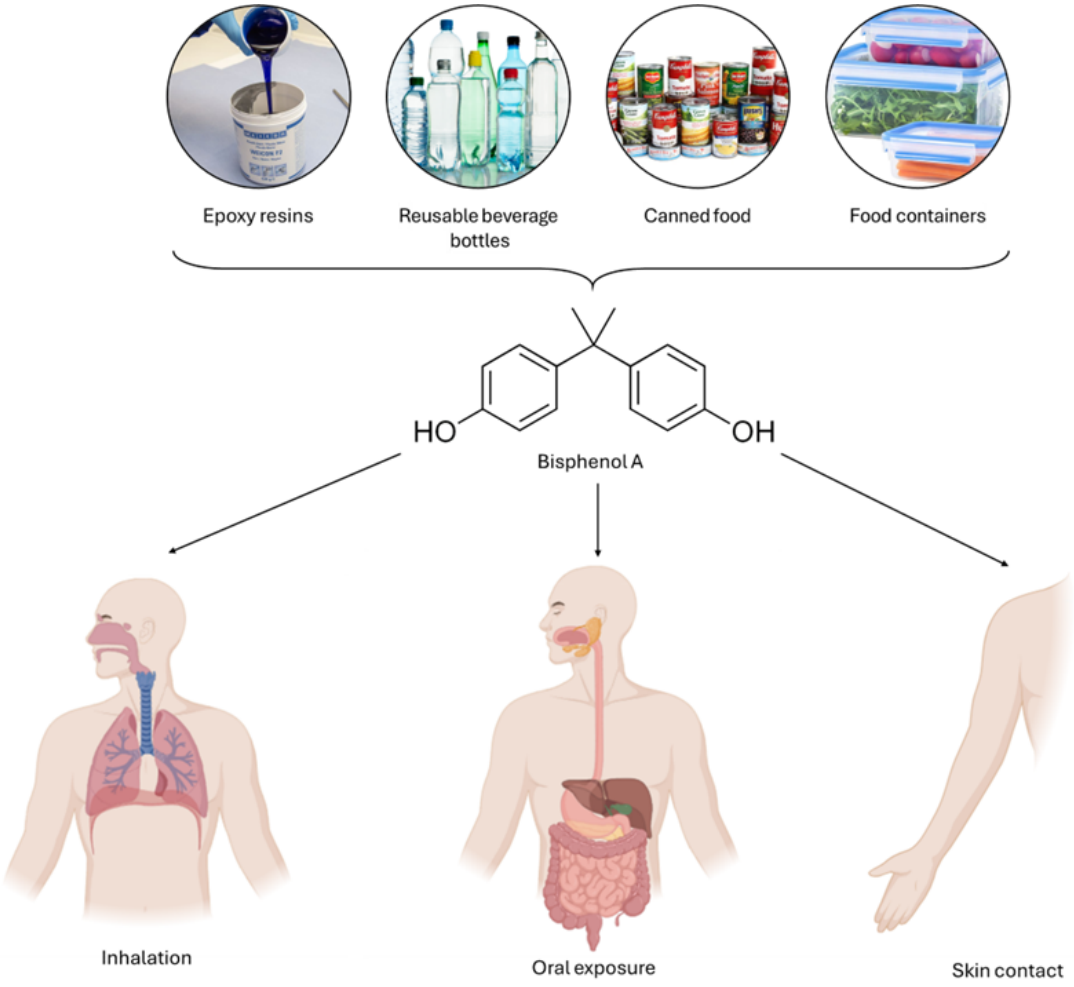

Figure 1. Common sources of BPA and main routes of BPA exposure in humans (Figure created with BioRender.com).

There is substantial evidence indicating that BPA exposure contributes to the risk of cancer development, developmental issues, diabetes, obesity, metabolic syndrome, as well as infertility and subfertility. The mechanisms underlying these multiple effects are numerous and involve BPA binding to both estrogen nuclear receptors as well as estrogen membrane receptors, interference with other nuclear and non-nuclear receptors, alterations in hormone synthesis or metabolism, and epigenetic dysregulation (Vandenberg et al., 2009; Santangeli et al., 2016-2019).

Since one of the main routes of BPA exposure in humans is through ingestion, BPA can affect the gastrointestinal tract (GIT), where digestion and absorption processes occur. Numerous studies on the intestinal effects of BPA have traditionally focused on its impact on the composition of the gut microbiota, which can lead to dysbiosis and metabolic disorders. However, in recent years, there has been a growing interest in evaluating the direct effects of BPA on intestinal cells and intestinal function. Regarding the effect of BPA on gut microbiota, Reddivari et al., (2017) demonstrated that BPA exposure can reduce the richness and diversity of the gut microbiota and thus the levels of beneficial short-chain fatty acids. These alterations in composition of the gut microbiota are linked to disruption of intestinal permeability and increase of systemic lipopolysaccharide (LPS), resulting in chronic subclinical inflammation and

altered lipid and glucose homeostasis (Reddivari et al., 2017). BPA is capable of significantly altering species diversity in the gut microbiota and increasing the abundance of *Proteobacteria*, a microbial marker of dysbiosis (Lai et al., 2016). In fact, it has been shown that BPA can induce intestinal dysbiosis in obese individuals by interfering with estrogen receptors, as estrogens are involved in stimulating mucus formation in the intestine, which allows beneficial bacteria to populate the intestinal environment (Kaliannan et al., 2018). Recent *in vivo* and *in vitro* studies on various mammalian models have highlighted the ability of BPA and other plasticizers to induce inflammation and intestinal damage (Feng et al., 2019; Liu et al., 2022; Wang et al., 2021). BPA specifically affects the small intestine, having significant health implications. The integrity of the intestinal barrier can be compromised by BPA, leading to an increased permeability which allows harmful substances to enter the bloodstream, triggering systemic inflammation (Nair et al., 2023). The alteration of the intestinal barrier was demonstrated by Wang et al., (2021) in the intestine of mice exposed to BPA. BPA significantly reduced the gene expression levels of the tight junction proteins ZO-1, occludin, claudin-1, and claudin-4, leading to disruption of the permeability and functionality of the intestinal barrier (Wang et al., 2021). BPA can interact with estrogen receptors, including estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), in

the epithelial cells of the intestine, disrupting signaling pathways and inducing oxidative stress, contributing to its impact on the small intestine (Nair et al., 2023). In addition, it was demonstrated that in mice intestine, BPA can induce the overproduction of reactive oxygen species (ROS) and inhibition of antioxidant enzyme capacity, resulting in an imbalance between oxidant and antioxidant actions and a tendency towards an oxidative state in intestinal tissues (Wang et al., 2021). In addition to oxidative stress, inflammation is hypothesized as one of the underlying causes of the harmful effects of BPA. BPA can activate inflammatory signaling pathways and increase the production of proinflammatory cytokines, contributing to inflammation in the intestine (Nair et al., 2023). Exposure to BPA induced inflammatory responses in mice intestine by increasing the gene expression of key factors of the innate immune system and enhancing the transcriptional activity of NF-kB, which promoted the secretion of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, and TNF-α (Wang et al., 2021).

Moreover, in the epithelial cells of both small and large intestine exposed to BPA a correlation with alteration of cell growth, proliferation and apoptosis was found (Nair et al., 2023). Concomitantly, in the intestine of BPA-treated mice, the gene expression and enzymatic activity of the pro-apoptotic factor Bax were significantly increased, while those of the key anti-apoptotic factor

Bcl-2 were significantly decreased. BPA treatment also significantly increased the gene expression and enzymatic activity of the apoptotic executor protein Caspase-3, suggesting a strong pro-apoptotic effect (Wang et al., 2021). The ability of BPA to disrupt cellular pathways involved in proliferation and apoptosis is associated with the development of cancers. Epidemiological studies have also linked BPA exposure to a higher risk of colon cancer, particularly colorectal cancer (Nair et al., 2023). Chen et al., (2015) demonstrated that exposure to BPA in SW480 colorectal cancer cells altered more than 56 proteins involved in cell structure, motility, proliferation, ATP production, oxidative stress, and protein metabolism. Additionally, it has been detected the overexpression of *CDH2* and *SNAI1* genes, which stimulate cancer progression, and the activation of the anti-apoptotic factor BCL-XL, which inhibits apoptosis while promoting migration and invasion of colon cancer cells (Chen et al., 2015).

Due to increasing concerns about the safety of BPA and the evidence of its impact on human health, BPA was banned in the production of baby bottles in the European Union (EU) in 2011. The United States Food and Drug Administration (FDA) banned the use of BPA in baby bottles, spill-proof cups, and infant formula packaging materials in 2012-2013. In April 2023, the European Food Safety Authority (EFSA) published a new safety assessment of

BPA, drastically reducing the tolerable daily intake (TDI) of BPA from 4 µg/kg body weight per day in 2015 to 0.2 ng/kg (approximately 20.000 times lower) (EFSA, 2023). Nevertheless, due to the environmental presence of BPA and its presence in certain household items, it continues to pose a threat to the health and safety of humans, animals, and the environment.

1.2 The Human Intestine

The adult human intestine is a complex organ comprised of roughly 7 meters of small intestine and 2 meters of large intestine. This system finalizes the digestive process that starts in the oral cavity and stomach. In the small intestine, water and small-molecule nutrients, such as sugars, monovalent ions, and amino acids, are absorbed. The large intestine then accumulates larger molecules, like fiber, and serves as an anaerobic fermentation chamber. This chamber facilitates the breakdown and absorption of by-products and the synthesis, often operated by gut microbiota, and absorption of additional nutrients such as vitamins (Boland, 2016). The small intestine is phenotypically heterogeneous, consisting of three morphologically distinct regions: the duodenum, jejunum, and ileum (Cronin et al., 2010). The large intestine, also known as colon, can be divided into four main regions: the ascending colon, transverse colon, descending colon, and sigmoid colon. Each of these anatomical regions hosts a wide variety of

phenotypically and morphologically distinct cell types. Within the intestine, there is a diverse population of epithelial, stromal, and immune cells, each consisting of multiple subtypes (Kabat et al., 2014). In the surface, the human intestine is composed of a mucosa called “gastrointestinal mucosa” made by multiple layers, each of them with a specific role (Kabat et al., 2014). The outer layer comprises the mucus layer, the commensal gut microbiota and defense proteins such as antimicrobial proteins (AMPs) and secretory immunoglobulin A (sIgA). The middle layer is composed of intestinal epithelial cells (IECs), while the inner layer is composed of immune cells of innate and adaptive immunity (Vancamelbeke & Vermeire, 2017).

The mucosal surface of the gastrointestinal tract is indeed covered by a protective layer of mucus, which serves several important functions such as protecting intestinal cells from external agents and facilitating nutrient absorption (Paone & Cani, 2020). Intestinal mucus is composed mainly of water (normally >98%) and the protein mucin-2 (MUC2) and is produced by goblet cells (Pelaseyed et al., 2014). In the stomach and colon there are two layers of mucus, whereas in the small intestine there is only one layer (Johansson et al., 2008). Underneath the mucus layer, there is the epithelium of the gastrointestinal barrier made by different types of cells, such as absorptive enterocytes, goblet cells, enteroendocrine cells, Paneth cells and microfold cells

(M cells). Those cells are renewed by a pool of stem cells residing at the bottom of the intestinal crypts (Barker et al., 2007; Vancamelbeke & Vermeire, 2017). Focusing on enterocytes and their counterparts in the colon, the colonocytes, they are columnar cells characteristic of the small intestine and are the primary absorptive cells in the intestinal tract. They have apical microvilli that significantly increase the absorptive surface area. In fact, these cells possess hydrolytic and absorptive functions and are responsible for the catabolism of nutrients (De Santa Barbara et al., 2003). Enterocytes are joined together by tight junctions that are essential for establishing and maintaining enterocytes polarity, on which the integrity of the epithelial barrier depends (Snoeck et al., 2005). Some markers are expressed in both enterocytes and colonocytes. In particular, *KLF4*, *FABP2* and *KRT20*, which is also present in goblet cells and enteroendocrine cells, while *CA1* is characteristic of colonocytes (Bustamante-Madrid et al., 2024). These markers play crucial roles in intestinal function. *KLF4* encodes for the Krüppel-like factor 4 (KLF4) transcription factor, also called gut-enriched Krüppel-like factor (GKLF), and is involved in regulating cell proliferation and differentiation, and intestinal epithelial integrity. It is essential for maintaining the intestinal protective barrier, preventing the loss of fluids and harmful substances from the body (Ghaleb & Yang, 2017). Cytokeratin 20 (KRT20) is a distinctive marker of mature

epithelial cells in the intestine, especially colon and small intestine enterocytes, and plays a role in cell structural stabilization by forming the cytoskeleton of epithelial cells (Moll et al., 1993). *CA1* encodes for an enzyme, carbonic anhydrase 1 (CA1), involved in regulating the acid-base balance in the intestine. Its activity is important for bicarbonate secretion and maintaining optimal pH in the intestinal lumen, thus contributing to digestion and nutrient absorption (Kivelä et al., 2005). Finally, fatty acid binding protein 2 (FABP2) is involved in transporting fatty acids within enterocyte cells. This protein aids in the absorption and utilization of dietary fatty acids, playing a key role in lipid metabolism and energy homeostasis in the body (Gajda & Storch, 2015).

Goblet cells, named for their goblet or cup-like appearance, are characterized by the presence of mucin granules. The primary function of goblet cells is to synthesize and secrete mucin glycoproteins, such as MUC2, which are the major macromolecular components of mucus. Goblet cells play a crucial role in maintaining intestinal homeostasis (Birchenough et al., 2015). These cells can act as antigen importers by capturing antigens from the intestinal lumen and delivering them to immune cells in the underlying lamina propria. The antigens are internalized and transported across the goblet cells to interact with dendritic cells and macrophages, which then present these antigens to T cells. This function is crucial for initiating and regulating immune responses, helping

maintain immune surveillance and tolerance in the gut, and contributing to intestinal homeostasis (Birchenough et al., 2015). Moreover, goblet cells help maintain intestinal homeostasis by providing bicarbonate for proper mucin unfolding in the small intestine and form a line of defense at the intestinal mucosa through their secretory role (Birchenough et al., 2015). Goblet cells are identified by the expression of specific lineage markers, such as *TFF2*, *TFF3* and *AGR2* (Bustamante-Madrid et al., 2024). Trefoil factor family peptides, including TFF2 and TFF3, play crucial roles in maintaining the integrity and function of the mucosal lining in the gastrointestinal tract. TFF2, primarily found in the stomach, enhances mucosal protection and repair by promoting epithelial cell migration and inhibiting apoptosis. This peptide forms a complex with mucin 6 (MUC6), which stabilizes the gastric mucus layer, providing a defense against gastric acids and pathogens (Hoffmann, 2020). Similarly, TFF3, mainly expressed in the intestinal tract, supports mucosal integrity and repair. It promotes cell migration and prevents cell death, contributing to the rapid restitution of damaged epithelial surfaces. TFF3 also forms heterodimers with proteins such as FCGBP, enhancing the innate immune defense by preventing microbial infiltration (Hoffmann, 2020). AGR2, a protein disulfide isomerase, is essential for the proper folding and secretion of mucins, critical components of the mucus barrier. Highly expressed in secretory cells, AGR2 ensures the

integrity of the epithelial barrier in the gastrointestinal tract and other mucosal surfaces. It also plays significant roles in various physiological and pathological processes, including cancer, by influencing the proper folding and function of mucin proteins (Jach et al., 2021).

Enteroendocrine cells (EECs) are scattered as individual cells throughout the gastrointestinal mucosa, residing in both the crypts and villi of the intestine (Rehfeld, 2004; Sternini et al., 2008). Traditionally, EECs can be classified into over 10 different cell types based on the primary hormones they secrete. These hormones include members of the chromogranin/secretogranin family, serotonin (5-HT), somatostatin, neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), substance P (SP), cholecystokinin (CCK), glucagon-like peptides (GLP-1 and GLP-2), and ghrelin (Fothergill & Furness, 2018; Gunawardene et al., 2011). These hormones produced by EECs may play essential roles in the regulation of nutrient absorption, intestinal immune response, epithelial barrier defense, colonic motility and visceral hyperalgesia, an increased sensitivity to pain within the intestine often associated with conditions such as irritable bowel syndrome or inflammatory bowel disease (IBD) (Eissa et al., 2018).

Intestinal immunity is maintained at the mucosal surface by intestinal epithelial cells, intraepithelial lymphocytes, and Paneth cells in the small intestine. The gut-associated lymphoid tissue (GALT) is present in the small intestine as

large aggregates of lymphoid tissue known as Peyer's patches (PP) and as isolated lymphoid follicles (ILF) located in both the upper intestine and the colon (Mörbe et al., 2021; Mowat & Agace, 2014). PP and ILF are made of specialized epithelial cells called M cells. M cells are responsible for monitoring the intestinal lumen and for capturing and transcytosing microparticles, and actively transporting small particles such as antigens, bacteria, or viruses across the intestinal epithelium. This process involves endocytosis of antigens/pathogens from one side of the M cells followed by secretion from the other side to the underlying immune cells (Kraehenbuhl & Neutra, 2000; Neutra et al., 1996). The transcytosis function of M cells requires a range of specialized cellular adaptations to capture the luminal particles/pathogens, to transport this cargo across the mucosal barrier, and to its delivery to submucosal tissues. In fact, the surveillance in the mucosal lumen is specifically targeted at viruses and bacteria, which represent the primary threats to the host and require a proper immune response. Other mechanisms exist for the uptake of soluble antigens, such as food antigens or microbial toxins (Dillon & Lo, 2019). Intestinal M cells also participate in mucosal immunological surveillance by inducing IgA-secretion by plasma cells. Secreted IgA localized in the mucus layer can modulate microbiota–host interactions (Di Tommaso et al., 2021). The gut immune action is reinforced by Paneth cells, another important

component of the immune system of gastrointestinal mucosa. These columnar cells reside at the base of the crypts in the small intestine and serve as the first line of defense against pathogens by secreting cytokines and antimicrobial products, such as defensins (Chassaing et al., 2014). Paneth cells also release lysozyme and other immunoregulatory proteins and have a microbiota-modulating role (Elphick & Mahida, 2005). Moreover, Paneth cells are a crucial element of the stem cell niche. They not only protect stem cells by producing bactericidal compounds but also provide essential niche signals, such as EGF/TGFα, Notch, and Wnt, for Lgr5-expressing stem cells in the small intestine (Sato et al., 2011).

Intestinal epithelial cells interact with the gut microbiota, a huge community of microorganisms, including bacteria, viruses and fungi (Vemuri et al., 2020). The gut microbiota and the intestinal barrier are engaged in a complex network of interactions that, under physiological conditions, maintain a balance essential for the human body homeostasis and health (Di Tommaso et al., 2021). Environmental factors, xenobiotics, dietary habits and alterations in the gut microbiota composition can compromise the integrity of the intestinal epithelial and vascular barriers. This disruption facilitates bacterial translocation and release of endotoxins, such as lipopolysaccharide, triggering a systemic inflammatory response that exacerbates metabolic disorders, including type 2

diabetes (Massier et al., 2020), obesity (Cani et al., 2008) and alcoholic or non-alcoholic fatty liver disease (Harte et al., 2010). In this context, the gut microbiota is an active participant and plays a crucial role in regulating intestinal permeability, both positively and negatively, through various metabolic and immune pathways (Di Tommaso et al., 2021).

1.2.1 Gut Microbiota

The human GIT is inhabited by the largest microbial community within the human body consisting of approximately 100 trillion of micro-organisms (most of them are bacteria, but also viruses, fungi and protozoa) called "gut microbiota" (Bull & Plummer, 2014). Microbial colonization of the human gut begins at birth. Although the infant's intestine is believed to be sterile or nearly sterile at birth, the gastrointestinal tract is rapidly colonized during and after delivery. As a neonate passes through the birth canal, he is exposed to the mother's vaginal microbiota, which significantly influences the development of the infant's intestinal microbiota, leading to initial similarities with the mother's vaginal microbial population after delivery (Huurre et al., 2008; Jiménez et al., 2008). Despite the relative similarities of the gut microbiota in mothers and their offspring, microbial colonization in the GIT is also influenced by numerous external and internal host-related factors. External factors include the microbial

load of the immediate environment, type of food eaten, and feeding habits. Additionally, dietary and temperature-related stresses can impact the establishment and composition of microbes. Internal factors include intestinal pH, microbial interactions, environmental temperature, physiological factors such as peristalsis and bile acids, host secretions, immune responses, drug therapy, and receptors located on the mucosal surface of the intestine that interact with bacteria, also known as bacterial mucosal receptors (Mackie et al., 1999). Despite the several factors that influence the composition of the gut microbiota, the microbial community in the human intestine remains relatively stable at the phylum level (Sekirov et al., 2010). *Bacteroidetes* and *Firmicutes* are preserved in nearly all individuals, although the relative proportions of these phyla may vary (Lozupone et al., 2012). However, at the bacterial species level, the variation in the composition of microbial communities among human individuals is significantly greater than that observed at the phylum level (Eckburg et al., 2005). Bacteria are unevenly distributed along the length of the gastrointestinal tract. The number of bacteria present can vary, starting from 10^2 to 10^3 bacteria per gram of stomach and duodenal contents, increasing to 10^4-10^7 bacteria per gram in the small intestine, and further increasing to 10^{11}-10^{12} bacteria per gram in the large intestine (Sekirov et al., 2010). In addition to the heterogeneity in the number of bacteria along the intestinal tract, there is also a

large variability in bacterial composition depending on the intestinal district. In fact, the intestine consists of different microenvironments where the microbiota differs significantly. In the intestinal lumen, typical genera include *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Clostridium*, *Lactobacillus*, and *Ruminococcus*, while the genera *Clostridium*, *Lactobacillus*, and *Enterococcus* are present only in the intestinal mucus and in intestinal crypts (Swidsinski et al., 2005). The composition of the gut microbiota can be modified by diet based on the amount of protein, saturated and unsaturated fats, carbohydrates, and dietary fiber provided (Valdes et al., 2018).

The microbiota performs several essential functions for the organism. One of the main functions of the gut microbiota is to secure nutrients to intestinal cells and metabolize undigested products of diet such as protein and dietary fiber (Adak & Khan, 2019). Through anaerobic fermentation of undigested complex carbohydrates, the gut microbiota can produce short-chain fatty acids. SCFAs include butyric, propionic, and acetic acids, which serve as energy substrates for intestinal epithelial cells and are also involved in regulatory functions (Macfarlane & Macfarlane, 2003). SCFAs can also increase mucus layer production by modulating the transcription of mucin genes in goblet cells (Rooks & Garrett, 2016). Intestinal bacteria not only produce SCFAs, but they are also capable of synthesizing a variety of vitamins and all essential and non-

essential amino acids (Vyas & Ranganathan, 2012). The gut microbiota also plays a significant immune role for the organism. The intestinal epithelium is the primary interface between the immune system and the external environment. The development of the host's immune system is influenced by continuous and dynamic interactions with the intestinal microbiota and its metabolites (Guarner & Malagelada, 2003), its microorganism-associated molecular patterns (MAMPs), including pathogen-associated molecular patterns (PAMPs), and its antigens (Ruff et al., 2020). Many intestinal bacteria produce antimicrobial compounds, such as bacteriocins, and compete for nutrients and attachment sites in the gut lining, thereby preventing colonization by pathogens. This action is known as the "barrier or competitive exclusion effect" (Guarner & Malagelada, 2003). The intestinal microbiota influences the functioning of other organs beyond the intestine, including the brain. In addition, it communicates with the brain through neural, immunological, and hormonal signaling pathways (Collins et al., 2012). Overall, the gut microbiota has multiple effects on human health and so acting to enhance the role of the gut microbiota can provide benefits that extend beyond digestive health. Studying the gut microbiota can pave the way for the development of clinical interventions to improve human health and to handle with health issues related to modern lifestyle (Mohajeri et al., 2018).

1.3 Human Intestinal Organoids 3D in vitro Model

During the years, much of the research on human cells has focused on the use of two-dimensional (2D) cell lines. These classic cell lines models are widely employed due to their cost-effectiveness, ease of handling, and adaptability to a wide range of experimental techniques. However, they exhibit significant limitations. For instance, their initial establishment is inefficient, often requiring extensive genetic and phenotypic adaptation to culture conditions. Additionally, many of these cell lines originate from cancers or have acquired oncogenic potential *in vitro*, thus failing to adequately replicate physiological conditions. Furthermore, there are limited 2D cell lines available representing differentiated cell types from various organs and tissues, and since these cell lines typically consist of a single cell type, they lack the complexity required for accurate comparisons with the organ of interest. To overcome these limitations, organoid cultures have been developed in recent years (Sato et al., 2009; Schutgens & Clevers, 2020). Organoids are three-dimensional (3D) self-organized structures made of both multipotent tissue-specific stem cells and their more differentiated progeny (Zachos et al., 2016). When referring to intestinal organoids, the source of the collected tissue further defines the structure and characteristics of the resulting organoids. Intestinal organoids can be derived from the differentiation of human pluripotent stem cells (PSCs), known as Human Intestinal Organoids

or hIOs. Alternatively, they can be derived from multipotent stem cells or progenitor cells isolated from intestinal crypts biopsies, in this case they are referred to as Enteroids (Stelzner et al., 2012). When sufficiently differentiated, intestinal organoids recapitulate the crypts and villi that harbor proliferative intestinal stem cells (ISCs) and progenitor cells, as well as differentiated enterocytes, goblet cells, and Paneth cells (**Figure 2**) (Onozato et al., 2021; Taelman et al., 2022).

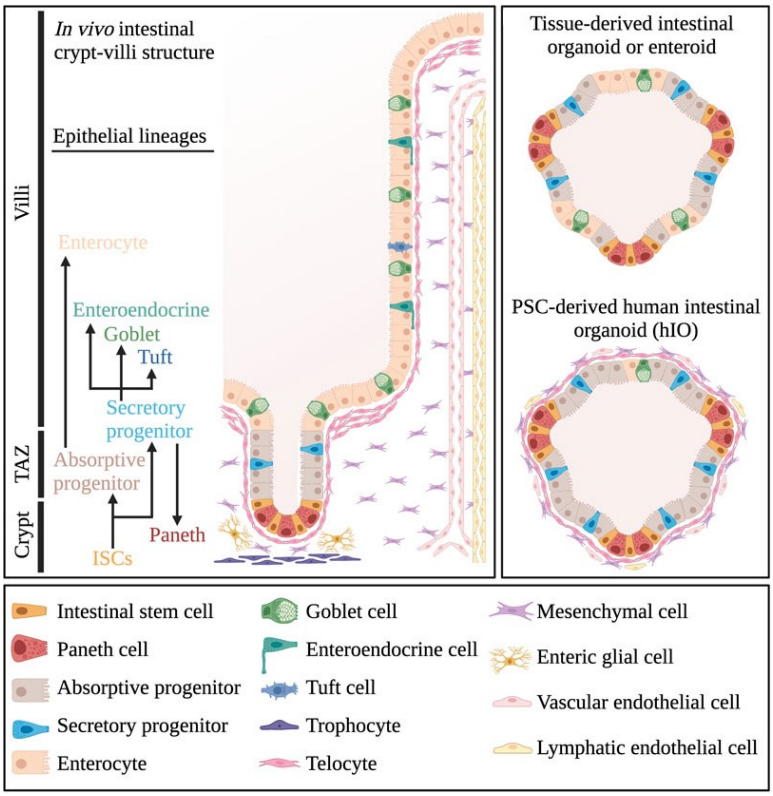

Figure 2. Cellular composition of human intestinal tissue and human intestinal organoids (Taelman et al., 2022).

Intestinal organoids can be derived from proliferative intestinal stem cells (ISCs). These cells reside at the bottom of the crypts and are characterized by the expression of the G protein-coupled receptor Lgr5 (Barker et al., 2007).

Additionally, they can replenish the entire intestinal epithelium within a short turnover time of approximately 5 days. The Lgr5+ cells that reside at the base of the crypts (Lgr5+ CBC) actively divide, generating transit-amplifying (TA) cells. These cells undergo proliferation and differentiation, giving rise to the different types of functional epithelial cells that perform essential intestinal functions (e.g., enterocytes, Paneth cells, enteroendocrine cells, goblet cells, tuft cells, Peyer's patches, and M cells). The population dynamics of these cells are tightly influenced by the action of four signaling pathways namely Wnt, epidermal growth factor (EGF), Notch, and bone morphogenetic protein (BMP) (Clevers, 2013). Wnt represents the key pathway for maintaining stem cell fate and driving the proliferation of stem and TA cells (Korinek et al., 1998). Wnt also guides the terminal differentiation of Paneth cells, which are always in direct contact with stem cells (Farin et al., 2012). Wnt, through its binding to Frizzled and Lrp5/6 receptors, leads to the stabilization of β-catenin, which binds and activates the transcription factor 4 (Tcf4), responsible for inducing a genetic program that supports stemness. Notch is also essential for maintaining the undifferentiated state. When Notch signaling is blocked in proliferative stem and TA cells, these cells differentiate into secretory lineage cells (Van Es et al., 2005). EGF signals exert strong mitogenic effects on stem and TA cells following the engagement of their EGFR receptors (Wong et al., 2012).

Finally, BMP signals are active in the villus compartment. When BMP signaling in the villi is inhibited by transgenic Noggin, crypt-like structures appear along the sides of the villi (Haramis et al., 2004). Lgr5+ CBC cells are in close contact with Paneth cells, which secrete EGF and Wnt3 and express Notch ligands. The depletion of Paneth cells *in vivo* results in the concomitant loss of Lgr5+ CBC cells in different animal models (Geiser et al., 2012; Sato et al., 2011). Signaling induced by extracellular matrix (ECM) proteins, such as laminin and collagen, is also vital for the proliferation, differentiation, and proper functioning of stem cells. Therefore, an ECM must support epithelial stem cells in propagating and maintaining the organoid form. Currently, the most used extracellular matrix to produce most organoids is Matrigel, a complex protein matrix of the basal membrane isolated from Engelbreth-Holm-Swarm mouse sarcoma cells (Aisenbrey & Murphy, 2020; Jee et al., 2019). Like stem cell lines, organoids can potentially be propagated indefinitely (Sato et al., 2009) and can be cryopreserved and expanded relatively quickly (Clinton & McWilliams-Koeppen, 2019). Organoids allow the *in vitro* recapitulation of the tissue architecture present *in vivo*, the heterogeneity among different cell types, and their interactions. Therefore, they are a potential model for tissue functionality and physiology being more accurate than 2D models (Kim et al., 2020; Lancaster & Knoblich, 2014) and so they have the advantage of reducing

the animal experimentation (satisfying the 3R reduction). Organoids are highly versatile as they are compatible with routine imaging techniques (Dekkers et al., 2019; Mahe et al., 2013), live-cell imaging (Zietek et al., 2015), methods for assessing metabolic activity (Berger et al., 2016), whole-genome gene expression analysis and protein analysis (Kip et al., 2021; Lindeboom et al., 2018; Norkin et al., 2021), and genetic editing techniques (Driehuis & Clevers, 2017; Koo et al., 2011; Schwank et al., 2013; Schwank & Clevers, 2016). For all these advantages, organoid technology may fill a need in medical research and holds promise for a wide range of translational applications. For instance organoids, like intestinal organoids, can be used for studying genetic diseases such as Cystic Fibrosis; testing the efficacy and safety of drugs; testing personalized therapies and genome editing techniques to treat genetic diseases; studying infection mechanisms and developing new treatments against infectious diseases, such as understanding the role of gut microbiota in infection; finally organoids can be used in toxicology and can be exposed to contaminants and endocrine disruptors to assess their toxicological effects on intestinal cells, providing insights into environmental health risks (Tang et al., 2022).

1.4 Probiotics Effects on Gut Microbiota and Health

"Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host" (Hill et al., 2014). Probiotics not only include bacterial cultures, mainly *Lactobacillus*, *Bacillus*, and *Bifidobacterium*, but also some strains of yeast from the genera *Saccharomyces* (Dahiya & Nigam, 2022). Probiotics can exert a positive effect on the human body through these main mechanisms (Plaza-Diaz et al., 2019): competitive exclusion of pathogens for nutrients and receptor binding sites, making their survival in the intestine difficult; improvement of intestinal barrier functions; immunomodulation of the host organism; and production of neurotransmitters. Probiotics also act as antimicrobial agents by producing SCFAs, organic acids, hydrogen peroxide (Ahire et al., 2021), and bacteriocins (Fantinato et al., 2019), thereby reducing pathogenic bacteria in the gut. Additionally, probiotics enhance intestinal barrier function by stimulating mucin protein production (Chang et al., 2021), regulating the expression of tight junction proteins, including occludin and claudin, and modulating the immune response in the intestine (Bu et al., 2022; Ma et al., 2022). The health benefits induced by probiotics are associated with the prevention and reduction of many diseases, such as allergic diseases, cancer, hypercholesterolemia, lactose intolerance, inflammatory bowel diseases, diarrhea, and irritable bowel syndrome (Grom et

al., 2020). The frequently used strains belong to the divergent group of *Bifidobacterium* and *Lactobacillus*, which significantly influence health through various actions.

An *in vitro* study on human intestinal models (M-SHIME system) evaluated the effects of an aqueous suspension of probiotics (Symprove®TM, containing *Lactobacillus acidophilus* NCIMB 30175, *Lactobacillus plantarum* NCIMB 30173, *Lactobacillus rhamnosus* NCIMB 30174, and *Enterococcus faecium* NCIMB 30176), which led to an increase in lactate production in the proximal and distal colon. Lactate, in turn, resulted in increased production of SCFAs, particularly butyrate, by bacteria metabolizing lactic acid. Additionally, an immunomodulatory effect of probiotics was observed: the production of anti-inflammatory cytokines (IL-10 and IL-6) was increased, and the production of inflammatory chemokines (IL-8, CXCL10, and MCP-1) was reduced (Moens et al., 2019). The probiotic VSL#3 has been tested in the prevention and treatment of obesity and diabetes in various murine models. Yadav and colleagues, (2013) revealed that lactic acid-producing bacteria, including *Bifidobacterium* and *Lactobacillus*, support the regeneration of intestinal epithelial cells. Lactate binds the G protein-coupled receptor 81 on Paneth cells, which promotes intestinal regeneration in a Wnt3/β-catenin dependent manner (Yadav et al., 2013).

Moya-Perez et al., (2015) demonstrated that in mice subjected to a high-fat diet, the probiotic formula VSL#3 has suppressed body weight gain and insulin resistance through modulation of the gut microbiota composition. Additionally, VSL#3 promoted the release of the hormone GLP-1, resulting in reduced food intake and improved glucose tolerance. These changes induced by VSL#3 were associated with an increase in butyrate levels (Moya-Pérez et al., 2015).

Many studies have demonstrated the potential of probiotics to counteract the harmful effects of environmental contaminants. In rats, *Bifidobacterium breve* and *Lactobacillus casei* reduced the intestinal absorption of orally administered BPA (Oishi et al., 2008), while in zebrafish, *L. rhamnosus* antagonized perfluorobutanesulfonate (PFBS)-induced metabolic alterations (Chen et al., 2020; Hu et al., 2021; Liu et al., 2021) and neurotoxicity (Liu et al., 2020). Additionally, *L. plantarum ST-III* reduced the pro-inflammatory actions of triclosan in zebrafish (Zang et al., 2019). Shi et al., (2021) demonstrated that the administration of lactic acid bacteria to mice exposed to PFOA reduced hepatotoxicity, restored the composition of the gut microbiota, and regulated the production of SCFAs (Shi et al., 2021). Recently, Giommi et al., (2024) demonstrated that the probiotic mixture SLAB51 mitigated BPA-induced toxicity in zebrafish and restored the homeostasis of the gut-liver-brain-microbiota axis. Co-administration of SLAB51 in male zebrafish led to an

increase in the number of goblet cells, responsible for producing acidic mucins, thus improving epithelial protection. Moreover, the administration of the probiotic mitigated BPA-induced alterations of the gut architecture, restoring values to control levels in both sexes (Giommi et al., 2024). These studies suggest that probiotic administration can represent a valid strategy to counteract the toxic effects of EDCs and improve human and animal health.

1.4.1 The Probiotic Mixture SLAB51

SLAB51 (SivoMixx®) is a probiotic mixture composed of *Streptococcus thermophilus* (DSM 32245), *B. lactis* (DSM 32246), *B. lactis* (DSM 32247), *L. acidophilus* (DSM 32241), *L. helveticus* (DSM 32242), *L. paracasei* (DSM 32243), *L. plantarum* (DSM 32244), and *L. brevis* (DSM 27961), species primarily hosted in the most proximal parts of the intestine such as the duodenum, jejunum and ileum, where there is normally a limited bacterial load. Several studies have demonstrated that administration of the probiotic mixture SLAB51 improves the health conditions of treated animals. Administration of SLAB51 in healthy dogs led to a decrease in the abundance of some potentially pathogenic species, such as *C. perfringens*, and promoted immune parameters systemically (plasma IgG titers) and in mucosal tissues (fecal IgA titers).

An increase in intestinal IgA can help prevent the entry or colonization of enteropathogenic strains in the intestine (Rossi et al., 2020).

In a murine model of Alzheimer's disease (AD), treatment with SLAB51 led to a significant reduction in oxidative stress through SIRT1-dependent mechanisms. Behavioral tests also revealed a positive effect of oral SLAB51 treatment on behavioral performance in AD mice, suggesting the restoration of hippocampal functions and supporting the idea that cognitive functions are influenced by bacteria acting through the gut-brain axis (Bonfili et al., 2018). In zebrafish, SLAB51 has demonstrated the ability to antagonize/mitigate BPA-induced reproductive toxicity, particularly in males, where the probiotic showed a positive interaction with spermatogenesis (Giommi et al., 2021). Zheng et al., (2023) demonstrated that chronic sleep restriction (CSR) in mice induced oxidative stress in the brain, promoted both cerebral and systemic inflammation with the release of the pro-inflammatory cytokines TNF-α and IL-1β, and disrupted the gut-brain axis, highlighting the importance of sleep for maintaining brain and systemic functions. The administration of the probiotic SLAB51 enhanced the brain's antioxidant capacity, thereby limiting the oxidative damage caused by sleep loss. Additionally, it restored the levels of gut-brain axis hormones, such as ghrelin, leptin and GLP-1, and mitigated the

development of CSR-induced peripheral and cerebral inflammation (Zheng et al., 2023).

Recently, Lombardi et al., (2023) tested the probiotic SLAB51 on *in vitro* cultures of Caco-2 cells exposed to lipopolysaccharide to evaluate its effect on the hypoxia-inducible factor (HIF) pathway, which is a central and ubiquitous cellular mechanism promoting and coordinating the transcriptional response to low-oxygen (O_2) environment (Semenza, 2011). HIFs are heterodimers composed of a hypoxia-inducible α-subunit (such as HIF-1α, HIF-2α, and HIF-3α) and a β-subunit (aryl hydrocarbon receptor nuclear translocator (ARNT)/HIF-1β and ARNT2) (Singhal & Shah, 2020). In the intestine, several genes targeted by HIF play crucial roles in maintaining the mucosal barrier. These genes are involved in microbial defense mechanisms, clearance of xenobiotics, production of mucins, and regulation of cellular energetics (Kumar et al., 2020). The data obtained from this study have shown that exposure of Caco-2 cells to SLAB51 under normoxic conditions positively influenced the HIF pathway. Specifically, a dose-dependent upregulation of HIF-1α protein levels was associated with an increase in L-lactate levels and the related glycolysis rate in treated cells. The ability of SLAB51 to stabilize HIF-1α was associated with the inhibition of prolyl hydroxylase domain 2 (PHD2) activity and expression. PHD2 is one of the hypoxia-inducible factor prolyl 4-

hydroxylases (PHDs), primarily responsible for hydroxylating prolyl residues in the O_2-dependent degradation domain of HIF-α subunits, contributing to its ubiquitination (Strowitzki et al., 2019; Wong et al., 2013). Additionally, was verified the involvement of the PI3K/AKT pathway, a pathway playing a central role in regulating energy metabolism. Activated PI3K/AKT upregulates HIF-1α transcription and translation, stabilizing and trans-activating HIF-1α regardless of O_2 levels (Dong et al., 2022; Manning & Cantley, 2007), underlying the ability of SLAB51 to induce an increase in HIF-1α levels. Furthermore, it was demonstrated that SLAB51 was able to counteract the activation of inflammatory factors, such as NF-κB and IL-1β, and the expression and activity of NOS2 induced by LPS treatment (Lombardi et al., 2023).

1.5 Hypothesis

BPA is one of the most prevalent EDCs in the environment, to which humans are frequently exposed through the ingestion of contaminated food and beverages, allowing BPA to reach the intestine (Chianese et al., 2018; Kantiani et al., 2010). Over the years, multiple *in vivo* studies on both mammalian and non-mammalian experimental models have demonstrated the toxic effects of BPA on the GIT. BPA have been correlated to inflammation (Reddivari et al., 2017), disruption of the intestinal barrier (Feng et al., 2019), histopathological

alterations in intestinal tissue (Giommi et al., 2024), and alterations of the gut microbiota composition, leading to dysbiosis (Velmurugan et al., 2017). However, there is limited information regarding the effects of BPA on *in vitro* cultured intestinal cells. At the same time, several *in vivo* studies have highlighted the multiple beneficial effects of probiotics on human and animal health. Currently, there is a growing body of research investigating the preventive or therapeutic effects of probiotics against EDCs, including BPA (Sevim & Kara, 2021). Based on the evidence regarding BPA intestinal toxicity and the beneficial effects of probiotics, the leading hypothesis of this study is that BPA can exert direct toxicity on intestinal cells, while the probiotic co-administration can mitigate the BPA-induced toxicity at the intestinal level.

1.6 Aim of the Study

In the last years, intestinal organoids have been developed as an *in vitro* model for studying physiological functions and intestinal diseases thanks to their ability to mimic the structure and function of the intestinal epithelium (Okkelman et al., 2017). Thus, the use of intestinal organoids can represent a novel and appropriate model for studying the toxicological effects of environmental pollutants on the intestine. In our study we used human intestinal organoids to assess the direct action of BPA on intestinal cells. Specifically,

the aim of our study was to investigate pathways involved in intestinal cell proliferation, intestinal cell functionality, and cell damage, including oxidative stress, inflammation, and apoptosis, to understand the influence of BPA on these processes. To address these aspects, we firstly performed a morphometric analysis to detect variations in the area and diameter of the organoids, evaluating the effect of BPA on the proliferation and growth of exposed intestinal organoids. Additionally, analyses were conducted on different markers by using Immunohistochemistry and RT-qPCR. After establishing the BPA toxicity on intestinal organoid cells, we focused on evaluating the potential of the probiotic mixture SLAB51 to mitigate BPA toxic effects. Therefore, an initial assessment was conducted on the organoids co-treated with BPA and probiotic through morphometric analysis.

MATERIALS AND METHODS

2.1 Human Intestinal Organoid Culture

Human intestinal biopsies were obtained from Biobank HUB-ICO-IDIBELL founded by Instituto de Salud Carlos III (PT20/00171) and by Xarxa de Bancs de Tumors de Catalunya sponsored by Pla Director d'Oncologia de Catalunya (XBTC) with the ethical committee approval number PR368/20. The preparation and isolation of intestinal crypts from these biopsies involved several steps. Firstly, the intestinal villi were eliminated by pressing the biopsy samples with a Neubauer chamber cover glass. The samples were then cut into small pieces using a razor blade. These tissue fragments were incubated in 40 mL of cold 10 mM EDTA in phosphate-buffered saline (PBS) for 30 minutes at 4 °C with a horizontal rotator. After incubation, the tissue was allowed to settle at the bottom of the tube, and the supernatant was removed. The tissue was then washed twice with 50 mL of cold PBS and subsequently resuspended in 15 mL of cold PBS and vigorously shaked. The tissue fragments were allowed to settle by gravity, and the supernatant was passed through a 100 µm cell strainer into 5 mL of 1% BSA-PBS. The filtered suspension was centrifuged for 3 minutes at 500xg, and the resulting pellet was resuspended in 3 mL of 0.1% BSA-PBS.

The crypts were then counted in a petri dish, and approximately 250 to 500 crypts were suspended in Matrigel growth factor reduced (Corning, New York, USA), centrifuged for 3 minutes at 500xg at 4 °C, and resuspended in Matrigel. 25 µL of Matrigel containing crypts were seeded into each well of a pre-warmed 48-well flat-bottom plate. The plate was incubated for 20 minutes at 37 °C in 5% CO_2. Subsequently, 250 µL of IntestiCult OGM Human medium (Stem Cell Technologies, Vancouver, Canada) supplemented with 10% penicillin/streptomycin was added to each well.

For organoid passage, medium was aspirated from each well and the organoids were resuspended in 700 µL of cold 1% BSA-PBS and recovered in a tube. Organoids were centrifuged for 3 min at 500xg at 4 °C and resuspended in cold 1% BSA-PBS and partially disaggregated by pipetting. The organoids were then centrifuged for 3 min at 500xg at 4 °C. The supernatant was then removed, and the organoid pellet was washed with cold 1% BSA-PBS. After an additional centrifugation with the same conditions, the supernatant was removed and the pellet containing organoids was resuspended in Matrigel based on the number of well needed and on the initial number of wells recovered. Organoids were then seeded in a 48-well flat-bottom plate pre-warmed at 37 °C with 25 µL of Matrigel containing crypts per well. After an incubation of 20 min at 37 °C in 5% of CO_2, 250 µL of medium IntestiCult OGM Human

(Stem Cell Technologies, Vancouver, Canada) supplemented with 10% penicillin/streptomycin was added to each well.

2.2 Bisphenol A Exposure

In order to select the most toxic concentrations of the endocrine disruptor Bisphenol A, a pilot study was conducted on 3 human intestinal derived organoid lines. After seeding, organoids were left forming for one day at 37 °C in 5% of CO_2 and then exposed to different concentrations of BPA (98% analytical purity, Sigma-Aldrich, Milan, Italy), using DMSO as vehicle, for 3 days (72 hours) as follow: untreated control group (CTRL); untreated control group + vehicle (DMSO 0.02%) (CTRL+DMSO); 0.56 pM of BPA (EFSA tolerable daily intake concentration) (Lambré et al., 2023); 56 pM of BPA (estimated to be the daily dietary exposure concentration to BPA in human) (Manzoor et al., 2022); 0.56 nM of BPA; 56 nM of BPA; 0.56 μM of BPA; 56 μM of BPA (**Figure 3**).

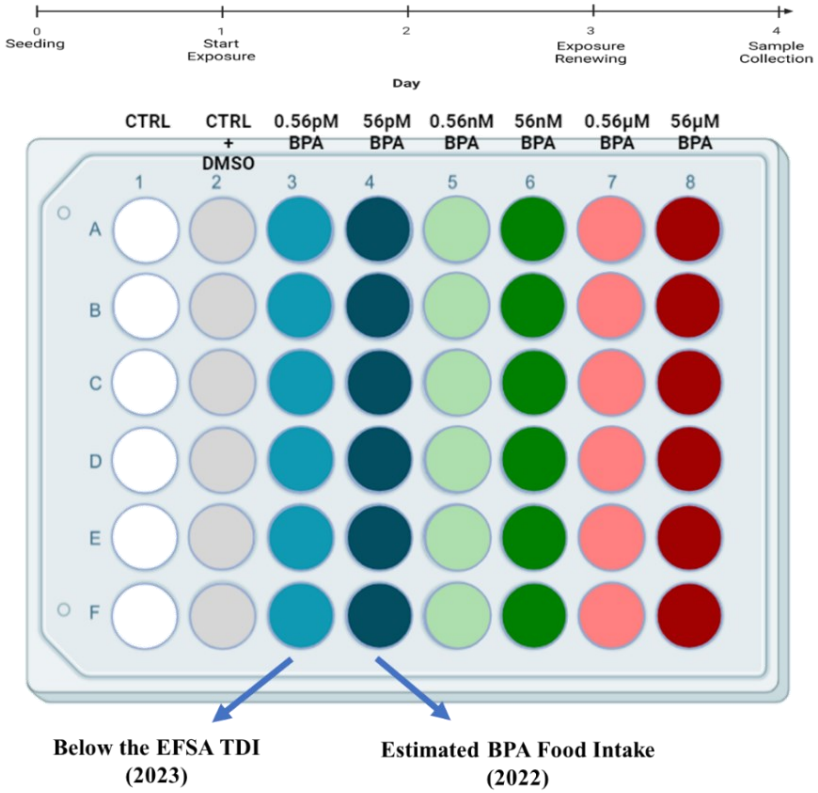

Figure 3. BPA exposure pilot study (Figure created with BioRender.com).

2.3 SLAB51 Administration

3 human intestinal derived organoid lines were also subjected to a pilot study in order to select the proper and the most effective dose of the probiotic mixture SLAB51 (Sivomixx®, Ormendes, Jouxtens-Mézery, Swiss) on the organoids. During organoid seeding, SLAB51 was administered through Matrigel by mixing organoids with lyophilized SLAB51 resuspended in 3 µL ultra-pure water or by adding the same volume of SLAB51 resuspended in the medium. The treatment lasted 4 days (96 hours) and was set up as follow: untreated

control group + 3 µL ultra-pure water during the seeding or in the medium (CTRL); 10^3 CFU of SLAB51; 10^4 CFU of SLAB51; 10^5 CFU of SLAB51; 10^6 CFU of SLAB51; 10^7 CFU of SLAB51 (**Figure 4**).

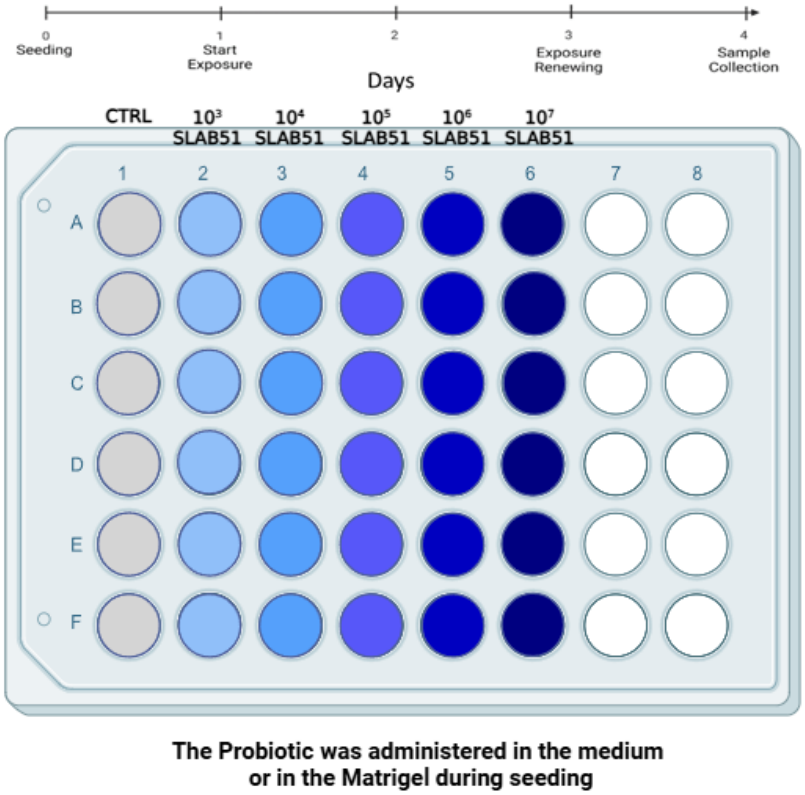

Figure 4. SLAB51 administration pilot study (Figure created with BioRender.com).

Another preliminary study was conducted for the SLAB51 concentration selection by administering the probiotic through both Matrigel and medium. The treatment lasted 4 days (96 hours) and was set up as follow: untreated control group + 3 µL ultra-pure water during seeding in Matrigel and in the medium (CTRL); 10^6 CFU of SLAB51 during seeding both in Matrigel and in the medium. The experiment was conducted only on the organoids from one

patient. In order to let bacteria growth, the penicillin and streptomycin medium addition was avoided in all the experiment from this pilot study.

2.4 Bisphenol A and SLAB51 Co-exposure

For the co-treatment of the 3 human intestinal derived organoid lines with BPA and SLAB51, the probiotic SLAB51 was administered during organoid seeding through the Matrigel by mixing organoids with lyophilized SLAB51 resuspended in 3 µL ultra-pure water and by adding the same volume of SLAB51 resuspended probiotic in the medium. The treatment lasted 4 days (96 hours) in total with probiotic treatments starting at seeding and BPA exposure starting one day after seeding (72 hours). The experimental design was set up as follow: untreated control group + vehicle and 3 µL ultra-pure water during the seeding in Matrigel and in medium (CTRL); 10^6 CFU of SLAB51 (P); 0.56 pM BPA (0.56 pM); 0.56 pM BPA + 10^6 CFU of SLAB51 (0.56 pM+P); 56 pM BPA (56 pM); 56 pM BPA + 10^6 CFU of SLAB51 (56 pM+P); 56 µM BPA (56 µM); 56 µM BPA + 10^6 CFU of SLAB51 (56 µM+ P) (**Figure 5**). In order to let bacteria growth, the penicillin and streptomycin medium addition was avoided in all the experiment from this study.

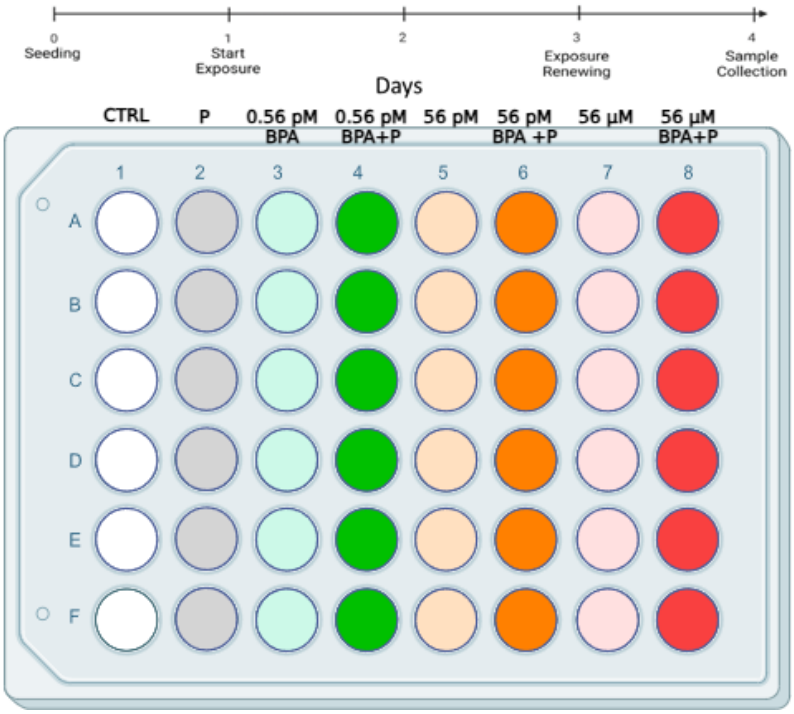

Figure 5. Co-treatment with BPA and SLAB51 (Figure created with BioRender.com).

2.5 PrestoBlue Assay for Viability Determination

To determine the viability of human intestinal organoid cells at the end of the exposure to the different concentrations of BPA, the PrestoBlue™ assay (ThermoFisher Scientific, USA) was performed. This assay detects the reducing environment in live cells. The PrestoBlue reagent contains resazurin, a blue, non-fluorescent, non-toxic, cell-permeable dye, and PBS. The reagent was added to the culture medium containing the organoids and incubated for 10 minutes at 37 °C. Once added to the culture medium, the reagent is rapidly absorbed by cells. Within viable cells, the reducing environment converts resazurin to resorufin, a dye with intense red fluorescence. After an incubation

of 3 hours, the conversion of resazurin to resorufin in live cells was detected by measuring absorbance (A < 570 nm) in a spectrophotometer. The amount of absorbance is proportional to the number of live cells and corresponds to the metabolic activity of cells.

2.6 Morphometric Analysis: Quantification of area and diameter

Human intestinal organoid lines exposed to the different concentrations of BPA, treated with the different concentrations of SLAB51, and co-treated with BPA and SLAB51, were maintained in a 48-well flat bottom-plate inside the Matrigel and were microphotographed at day 4 (96 hours) after seeding using a ZEISS Axio Observer microscope at 5 X magnification, mounted in an incubator chamber at 37 °C and 5% of CO_2, for the measurement of organoid diameter and area. The obtained microphotographs were analyzed using the ImageJ software. The "Straight" function was used to assess the diameter of the organoids, while the "Freehand selections" function was used to assess the area of the organoids (**Figure 6**).

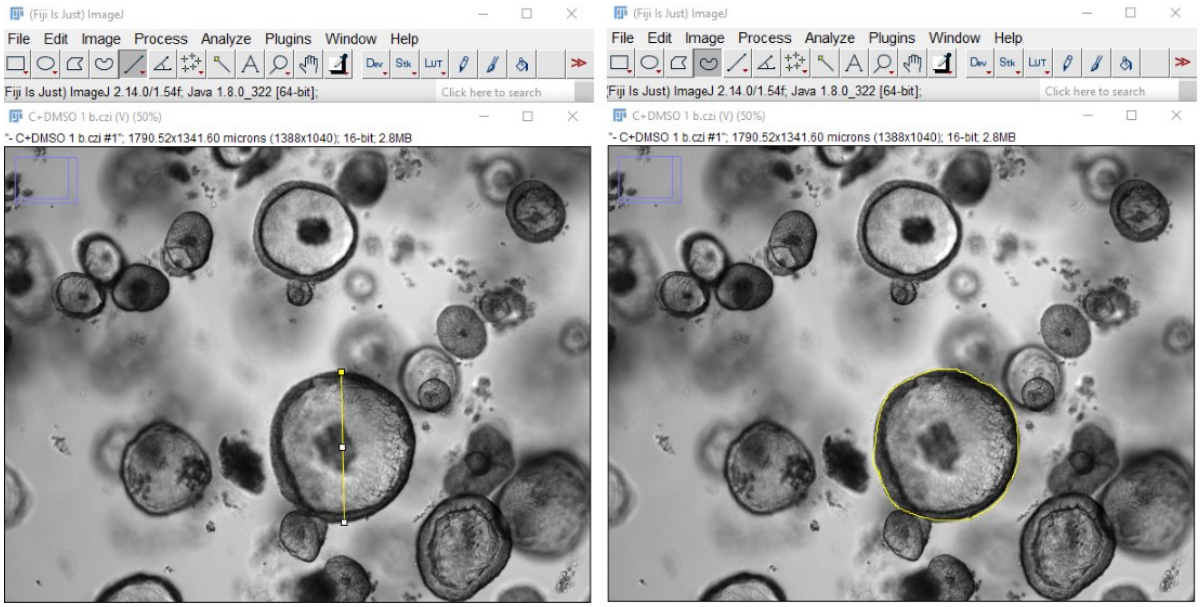

Figure 6. Morphometric analysis. Quantification of organoid diameter and area using ImageJ software.

2.7 RNA Extraction and cDNA Synthesis

For each organoid line and for each experimental group, total RNA was extracted from human intestinal organoids using the RNeasy Mini Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. The RNA final concentrations and 260/230 and 260/280 ratios were determined with the NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA), and RNA integrity was confirmed by Gel Red staining of the 28S and 18S ribosomal RNA bands on a 1% agarose gel. The extracted RNA was then stored at -80 °C. Before use, the total RNA was treated with DNase to remove genomic DNA (1 µl DNAse I and 1 µl of Reaction Buffer 10X at 37 °C for 10 minutes, Sigma-Aldrich, St. Louis, USA).

Subsequently, 1 μg of total RNA was used for cDNA synthesis with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Swiss). The synthesized cDNA was stored at −20 °C until further use.

2.8 Real Time-PCR

Quantitative real-time PCRs (qRT-PCRs) were performed using SYBR Green on a CFX thermal cycler (Bio-Rad, Milano, Italy). All samples were analyzed in duplicate. Reactions contained 1 μl cDNA diluted 1:10, 5 μl SYBR Green PCR Master Mix 2X (Bio-Rad), containing SYBR Green as a fluorescent intercalating agent, 0.1 μM of forward and reverse primers and 3.8 μl of deionized and autoclaved water. Fluorescence was monitored at the end of each cycle. The mRNAs of the housekeeping genes Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and TATA-box binding protein (*TBP*) or Ribosomal protein 18S (*RPS18*) and 60S acidic ribosomal protein P0 (*RPLP0*) were used as internal standards in each sample. For gene names and primers forward and reverse sequences, see **Table 1**.

GENE	FORWARD (5'-3')	REVERSE (3'-5')
GAPDH	GCACCGTCAAGGCTGAGAAC	AGGGATCTCGCTCCTGGAA
TBP	ATCCCTCCCCCATGACTCCCATG	ATGATTACCGCAGCAAACCGC
RPS18	TGCGAGTACTCAACACCAACA	GCATATCTTCGGCCCACA
RPLP0	CTGGAAAACAACCCAGCTCT	GAGGTCCTCCTTGGTGAACA
SOD1	GGTCCTCACTTTAATCCTCTATCCAG	CCAACATGCCTCTCTTCATCC
CAT	TCCGGGATCTTTTTAACGCCATTG	TCGAGCACGGTAGGGACAGTTCAC
GPX1	GCGGGGCAAGGTACTACTTA	CTCTTCGTTCTTGGCGTTCT
IL1β	GCTGAGGAAGATGCTGGTTC	TCCATATCCTGTCCCTGGGAG
TNFα	CCCCAGGGACCTCTCTCTAA	TGAGGTACAGGCCCTCTGAT
NOTCH1	GCACTGCGAGGTCAACAC	AGGCACTTGGCACCATTC
NOTCH2	TCAACTGCCAAGCGGATGT	CTTGGCTGCTTCATAGCTCC
LYZ	TTGGGAATGGATGGCTACAGG	GTAACCACTCTCCCATTTGGC
ALPi	TACACGTCCATCCTGTACGG	CTCGCTCTCATTCACGTCTGG
LGR5	CCACTTCCTGCATGTCTCAATC	TTTCTCAGGCTCACCAGATCCT
KRT20	AGTGGTACGAAACCAACGCC	GGACACACCGAGCATTTTGC
TFF3	TGCAGGAAGCAGAATGCACC	AAGCTGAGATGAACAGTGCCT

Table 1. Gene names and primers sequences for qRT-PCR.

Furthermore, the calculation of mRNA levels of target genes analyzed was performed using the geometric mean of the two reference genes after demonstrating that they were stably expressed by the geNorm algorithm, both applications implemented in the Bio-Rad CFX Manager 3.1. software.

2.9 Immunohistochemistry and Image Analysis

For immunohistochemistry analysis, the intestinal organoids were manually fixed using 4% paraformaldehyde (PFA) and then embedded in paraffin. The samples underwent dehydration through a series of alcohol baths with increasing concentrations: 70% ethanol for 20 minutes; 96% ethanol for 20 minutes; 100% ethanol for 30 minutes twice. Subsequently, the samples were immersed in xylene three times for 30 minutes each to remove the alcohol and facilitate paraffin infiltration. The samples were then incubated in liquid paraffin at 58-60 °C, first for 1 hour, then overnight, and finally for another hour. For the preparation of paraffin blocks, the samples were oriented to be positioned at the center of the blocks. Once the paraffin blocks with the embedded organoids were obtained, sectioning was performed using a microtome to obtain slices with a thickness of 5 µm. Before immunodetection, the paraffin sections were maintained at 60 °C for 15-30 minutes and subsequently dewaxed and rehydrated. Antigen retrieval was performed in a Decloaking Chamber at pH6. After a 20-minute incubation, immunodetection was carried out.

For the immunodetection of Ki-67, a protocol using Diaminobenzidine (DAB) was employed. Paraffin sections were rinsed with wash buffer 1X and then the peroxidase activity was blocked using a solution of 70% methanol, 30%

distilled water and 2% of hydrogen peroxide for 5 minutes at room temperature (RT), followed by another rinse with wash buffer. Next, a blocking buffer (1X TBS + 0.5% Triton + 8% donkey serum) was applied for 30 minutes to 1 hour at RT. The primary antibody for Ki-67 (mouse IgG; sc-23900 Santa Cruz Biotechnology, Dallas, Texas), diluted 1:100 in Green Diluent, was then incubated for 72 hours at 4 °C. After incubation with the primary antibody, the paraffin sections were rinsed with 1X wash buffer for 3 times, 5 minutes each. Then, EnVision Dual Link (Anti-mouse IgG conjugated with HRP) was applied for 30 minutes at room temperature. Following another rinse with 1X wash buffer for 3 times, 5 minutes each, DAB was added. Afterward, the paraffin sections were rinsed with 1X wash buffer three times for 5 minutes each. Nuclear staining was performed with Lillie's Hematoxylin for 30 seconds, followed by another rinse with distilled water three times for 5 minutes each. Finally, mounting was done using Faramount aqueous medium (Dako, ref: S3025).

For the immunodetection of cleaved CASP3, the same protocol used for Ki-67 was employed and using the primary antibody for cleaved CASP3 (rabbit IgG; Asp 175, Cell Signaling Technology, Danvers, United States), diluted 1:400 in Green Diluent, incubated for 72 hours at 4 °C.

For the detection of ALPi, an AP staining protocol was employed. The protocol utilized the Kit Alkaline Phosphatase Blue Membrane Substrate Solution (Sigma, ref: AB0300). Solution A and Solution B from the kit were mixed in a 1:1 ratio and incubated in the dark for 20 minutes at room temperature. Then, the sections were rinsed with 1X PBS three times for 5 minutes each. Nuclear fast red counterstaining was performed for 10 minutes at room temperature, followed by a 5-minute rinse with tap water. Finally, quick DAM step was performed.

For the Image Analysis and quantification of Ki-67, ALPi, and CASP3 signals, the ImageJ software was utilized. Images were converted to 8-bit and an initial threshold was applied to calculate the total area of the organoids. Subsequently, a stricter threshold was applied to calculate the area of the signal corresponding to the marker of interest (**Figure 7**). The results were reported as % of DAB-positive area on total area of organoids.

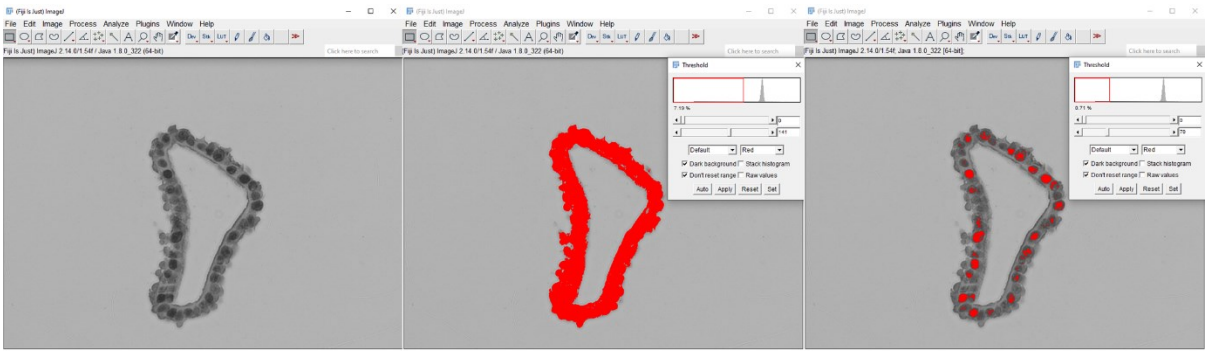

Figure 7. Image analysis and quantification of DAB signal using ImageJ software.

2.10 Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 8.0.2. For the quantification of organoid area and diameter, the data were analyzed based on the results of Normality and Lognormality test, employing one of the following methods: Kruskal-Wallis test, Mann-Whitney test, or One-way ANOVA test. For gene expression analysis via RT-qPCR, data were subjected to Normality and Lognormality test, and subsequently analyzed using the Unpaired t-test and One-way ANOVA test. For immunohistochemistry results, Normality and Lognormality were also assessed and the results were analyzed using either the One-way ANOVA test or the Kruskal-Wallis test. Statistical significance was defined as $p < 0.05$. All data were reported as mean ± standard deviation (SD).

RESULTS

3.1 Organoid Viability

Human intestinal organoids exposed to different BPA concentrations were analyzed using the PrestoBlue assay, which measures cellular metabolic activity as an indicator of cell viability. The results showed no significant changes in cell viability comparing the different experimental groups vs CTRL, despite a non-significant trend in reduction was evident only for the two highest concentrations 0.56 µM and 56 µM of BPA (**Figure 8**).

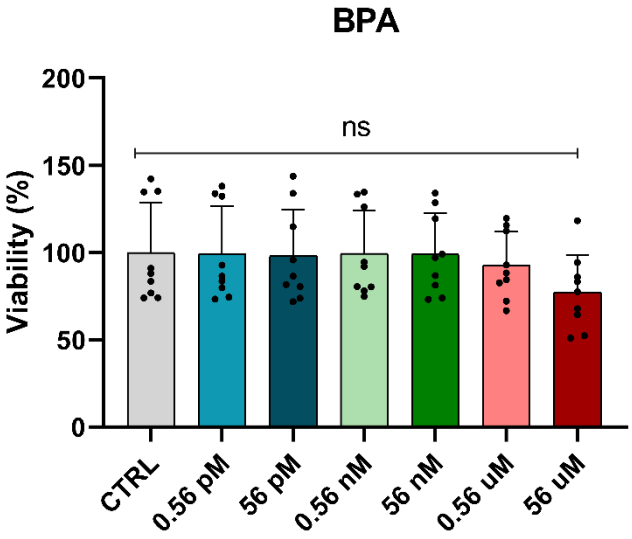

Figure 8. Organoid viability assay. Analysis of human intestinal organoid viability after 72 hrs exposure to different BPA concentrations vs CTRL. For statistical analysis, the Kruskal-Wallis statistical test was performed. Data are reported as mean ± standard deviation (SD) with n = 9. ns indicates no statistically significant differences between the experimental groups and CTRL.

3.2 Morphometric Analysis

Morphometric analysis was performed on human intestinal organoids exposed to different concentrations of BPA in order to detect variations in diameter and area among the treatment groups and to identify the most toxic concentrations of BPA. The results demonstrated a significant reduction in the diameter of the organoids exposed to 0.56 pM, 56 pM and 56 μM of BPA compared to CTRL (**Figure 9a**). Similarly, a significant reduction in the area was present in the organoids exposed to 0.56 pM, 56 pM and 56 μM of BPA compared to CTRL group (**Figure 9b**).

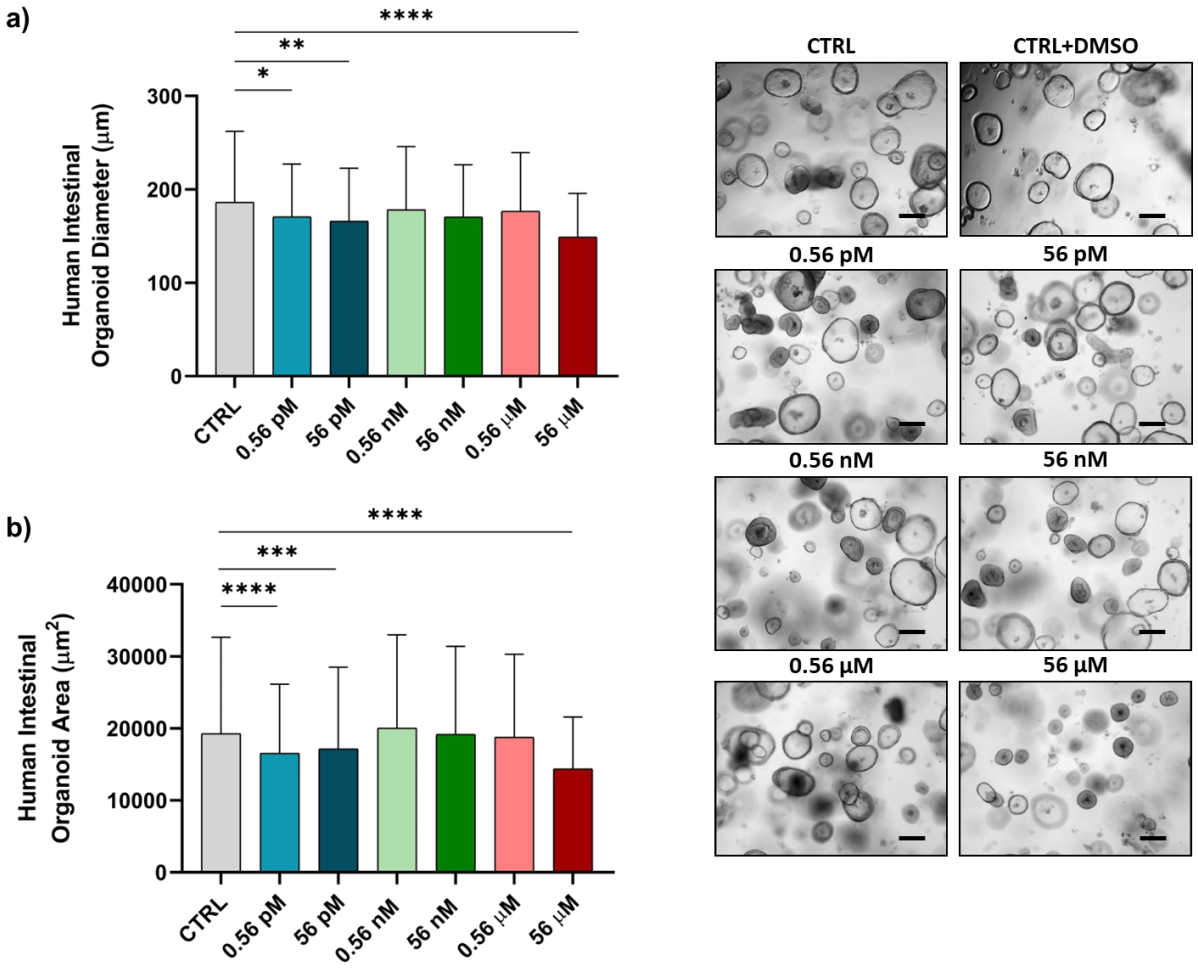

Figure 9. Morphometric analysis. a) Organoid Diameter and b) Organoid Area in organoids exposed to different BPA concentrations. For statistical analysis, the Kruskal-Wallis test followed by the Mann-Whitney test were performed. Data are reported as mean ± standard deviation (SD) with $n = 216$. Asterisks represent significance levels as follow: one asterisk (*) for $p<0.05$, two asterisks (**) for $p<0.01$, three asterisks (***) for $p<0.001$, four asterisks (****) for $p<0.0001$. Microphotographs show representative human intestinal organoids from the different experimental groups. Scale bar = 200 µm.

This pilot experiment provided the information for the next exposure where only the concentrations of BPA providing size alterations were used.

Morphometric analysis was also performed on the organoids treated with different concentrations of the probiotic mixture SLAB51, administered in Matrigel or in the culture medium, in order to identify the optimal concentration of the probiotic bacteria that could be the most effective on the organoids. In human intestinal organoids treated with different concentrations of SLAB51 probiotic bacteria in Matrigel (**Figure 10c**), the results obtained showed only a significant reduction in the diameter of the organoids treated with 10^7 CFU of SLAB51 compared to CTRL (**Figure 10a**), while the other experimental groups did not differ from the CTRL. No significant differences in the area of the organoids were found comparing the treated groups with the CTRL (**Figure 10b**).

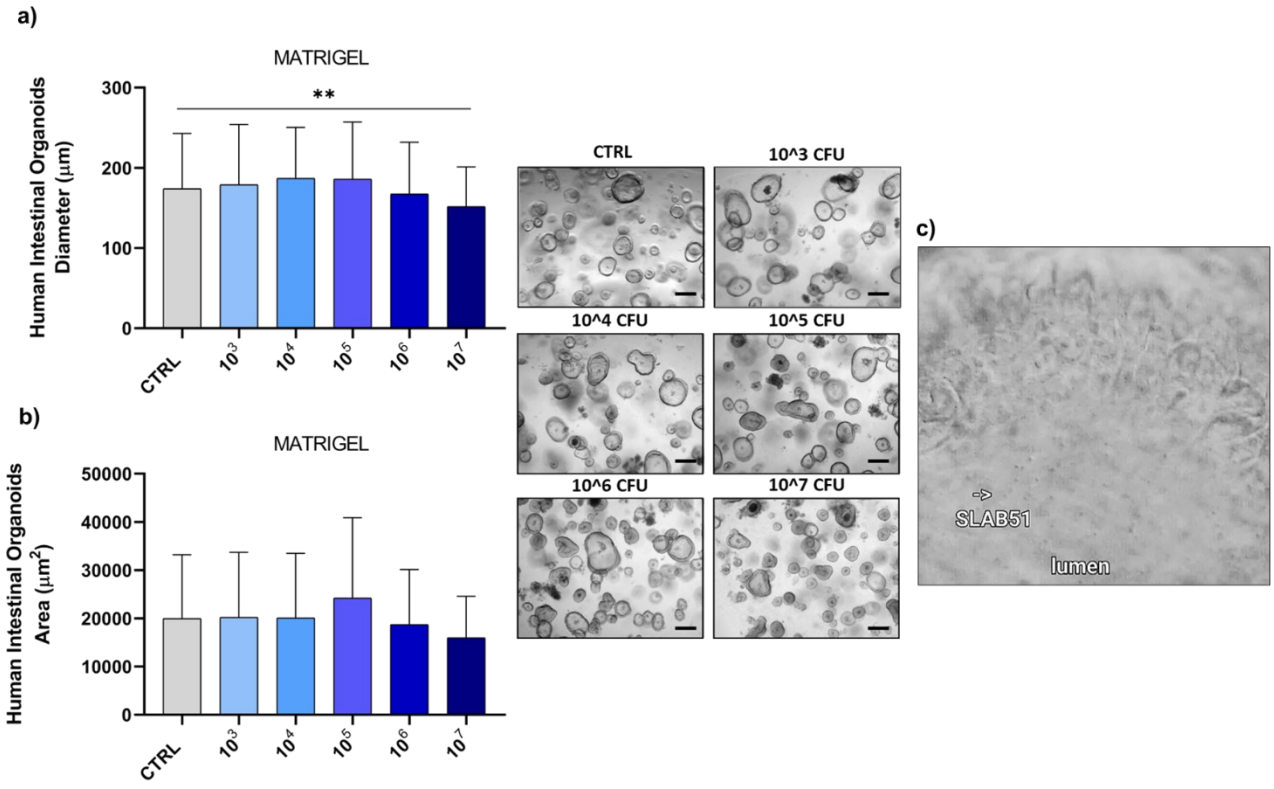

Figure 10. Morphometric analysis. a) Organoid Diameter and b) Organoid Area between the experimental groups exposed to SLAB51 by in Matrigel. For statistical analysis, the Kruskal-Wallis test followed by the Mann-Whitney test were performed. Data are reported as mean ± standard deviation (SD) with $n = 216$. Asterisks represent significance levels as follow: two asterisks (**) for $p<0.01$. Microphotographs show representative human intestinal organoids from the different experimental groups. Scale bar = 200 µm. c) Higher magnification showing the presence of SLAB51 probiotic bacteria in the organoids' lumen.

The same analysis was conducted on human intestinal organoids treated with different concentrations of SLAB51 probiotic bacteria added to the culture medium. The results showed a significant reduction in the diameter of the organoids of the group treated with 10^7 CFU when compared to CTRL (**Figure**

11a), as well as a significant reduction in the area in the same treated group when compared to CTRL (**Figure 11b**).

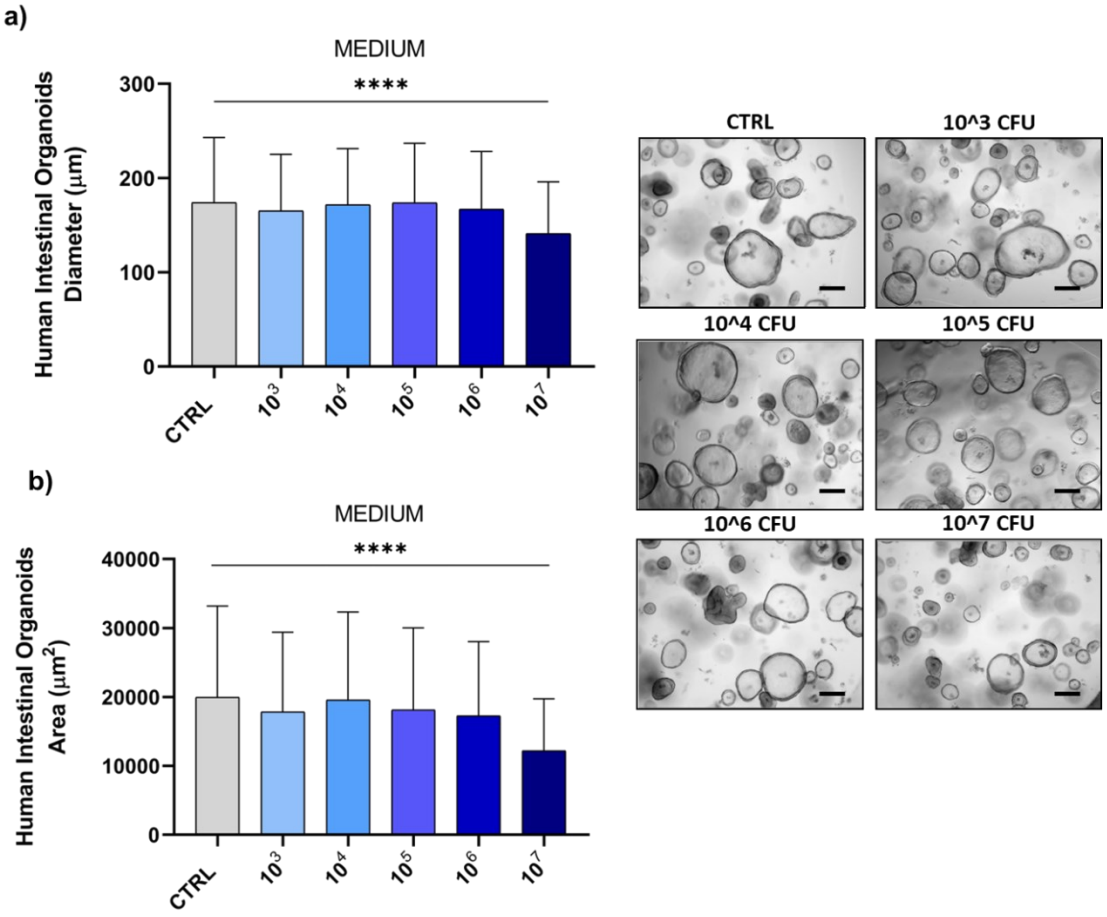

Figure 11. Morphometric analysis. a) Organoid Diameter and b) Organoid Area between the experimental groups exposed to SLAB51 through the medium. For statistical analysis, the Kruskal-Wallis test followed by the Mann-Whitney test were performed. Data are reported as mean ± standard deviation (SD) with $n = 216$. Asterisks represent significance levels as follow: four asterisks (****) for $p<0.0001$. Microphotographs show representative human intestinal organoids from the different experimental groups. Scale bar = 200 µm.

These results demonstrate that, both in Matrigel and in medium, SLAB51 probiotic mix administration did not provide differences in terms of viability in

all groups except for the 10^7 CFU. Based on these results, a concentration of SLAB51 of 10^6 CFU probiotic bacteria was chosen for further organoid exposure. The highest non-toxic concentration of SLAB51 probiotic bacteria (10^6 CFU) was chosen to maximize potential benefits and ensure observed effects are due to the probiotic. This approach helps establish a baseline for lower doses and ensures maximum efficacy while maintaining organoid viability. This strategy lays a strong foundation for understanding probiotic impact and guiding future research. To verify the efficacy of SLAB51 on human intestinal organoids, one organoid line (MF10) was exposed to a bacterial concentration of 10^6 CFU, administered simultaneously in Matrigel and in the culture medium. The results obtained demonstrated that the bacterial concentration of 10^6 CFU of SLAB51, added both in Matrigel and in medium, didn't led to significant changes in the diameter (**Figure 12a**) and in the area (**Figure 12b**) of the organoids compared to CTRL. Therefore, the concentration of 10^6 CFU of the probiotic SLAB51 was selected for the co-treatment experiment with BPA and probiotic administered both in Matrigel and medium.

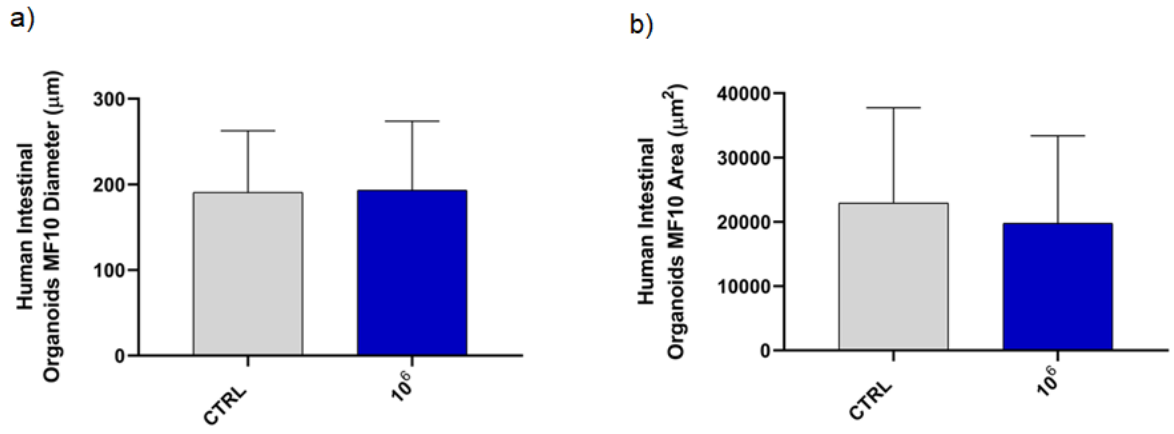

Figure 12. Morphometric analysis. a) Organoid Diameter and b) Organoid Area in MF10 line among the CTRL group and the treated group with 10^6 CFU of SLAB51 administered both in Matrigel and medium. For statistical analysis, the Mann-Whitney test was performed. Data are reported as mean ± standard deviation (SD) with $n = 72$.

To test the ability of SLAB51 to mitigate the toxic effects of BPA, the organoids were exposed to the three major toxic doses of BPA (0.56 pM, 56 pM and 56 µM of BPA) concomitantly with 10^6 CFU of SLAB51 in both Matrigel and medium. The morphometric analysis of human intestinal organoids subjected to co-treatment with BPA and the probiotic SLAB51, showed a significant reduction in the diameter of the organoids of the group treated with 56 pM of BPA compared to CTRL and Probiotic-treated organoids (P). A significant reduction in the diameter of the organoids was also observed in the groups exposed to 0.56 pM and 56 µM of BPA compared to CTRL. The reduction of the diameter observed in these groups was partially recovered by the use of probiotics when compared to CTRL. Mitigation with the probiotic was in fact

evident in the 0.56 pM+P and 56 pM+P treatment groups, where the addition of the probiotic resulted in an increase in the diameter of the organoids compared to the groups treated only with the same dose of BPA, showing that SLAB51 had the ability to restore organoid diameter to levels comparable to the CTRL group (**Figure 13a**).

Regarding the area, the results showed a significant reduction in the area of all the organoids exposed to the three BPA concentrations 0.56 pM, 56 pM and 56 μM when compared to CTRL. A clear mitigation of the toxic effects of BPA on the intestinal organoid area was only observed in the 56 pM+P treated group compared to the group only exposed to the same dose of BPA (56 pM), confirming for this concentration the results obtained for the diameter in the same treatment group, restoring organoid area to levels comparable to the control group (**Figure 13b**). Collectively these results indicate that BPA is able to impair intestinal organoid growth and that SLAB51 probiotic mixture has the ability to counteract the toxic effect of BPA on intestinal cells.

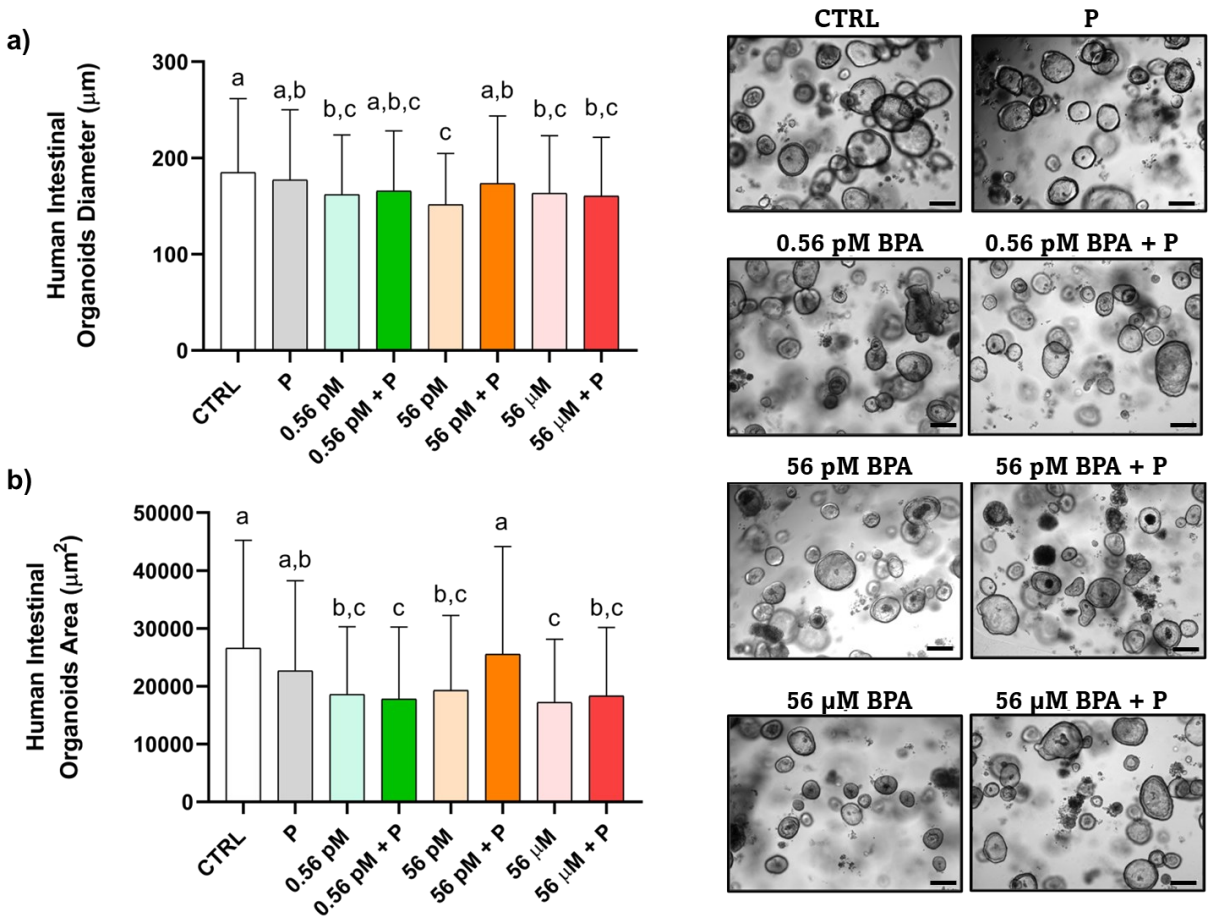

Figure 13. Morphometric analysis. a) Organoid diameter and b) Organoid area among the experimental groups. For statistical analysis, the Kruskal-Wallis test was performed. Data are reported as mean ± standard deviation (SD) with n = 276. Groups with the same letters do not present statistically significant differences among them, whereas groups with different letters do present statistically significant differences among them (p<0.05). Microphotographs show human intestinal organoids from the control group and treatment groups. Scale bar = 200 µm.

3.3 Organoid Proliferation

The results obtained from the morphometric analysis of the organoids provide an overview of the effect of BPA on organoid growth and proliferation. To further investigate the impact of BPA on organoid proliferation, RT-qPCR

analysis was conducted on *LGR5*, *NOTCH1* and *NOTCH2* genes, involved in cell proliferation at intestinal level. The results obtained showed that BPA treatment did not alter the expression of *LGR5* (**Figure 14a**), *NOTCH1* (**Figure 14b**) and *NOTCH2* (**Figure 14c**) in human intestinal organoid cells and no significant changes were detected between the treated groups.

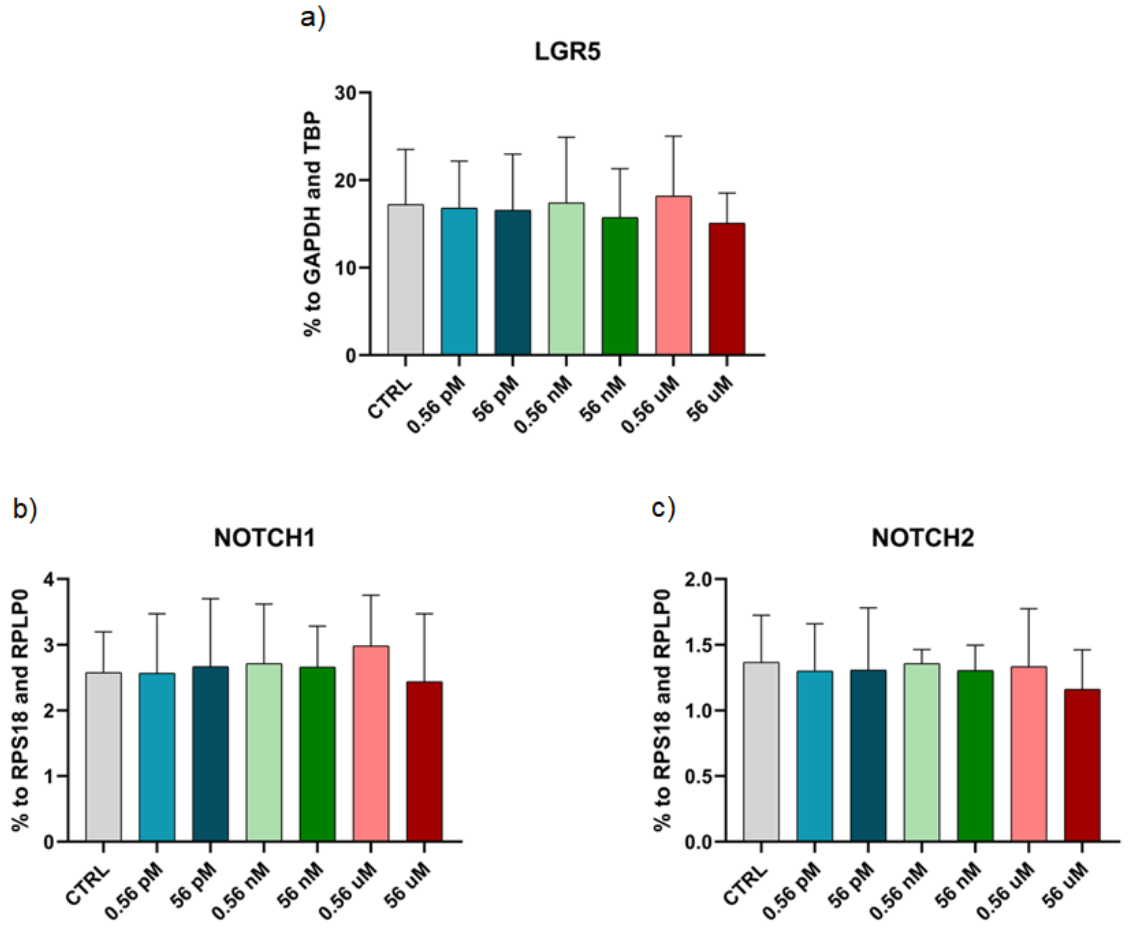

Figure 14. RT-qPCR transcript analysis. mRNA expression levels of a) *LGR5* gene, b) *NOTCH1* gene and c) *NOTCH2* gene in human intestinal organoid cells among the experimental groups. For statistical analysis, One-way ANOVA test was performed followed by Dunett's multiple comparison test. Data are reported as mean ± standard deviation (SD).

The proliferation of intestinal organoid cells was further investigated via Immunohistochemistry in order to assess the protein levels of Ki-67, a nuclear marker of cell proliferation. Consistent with the observed effects of BPA on organoid size in the morphometric analysis, the results revealed a significant decrease in Ki-67 protein levels in the organoids exposed to 56 µM of BPA compared to CTRL (**Figure 15**).

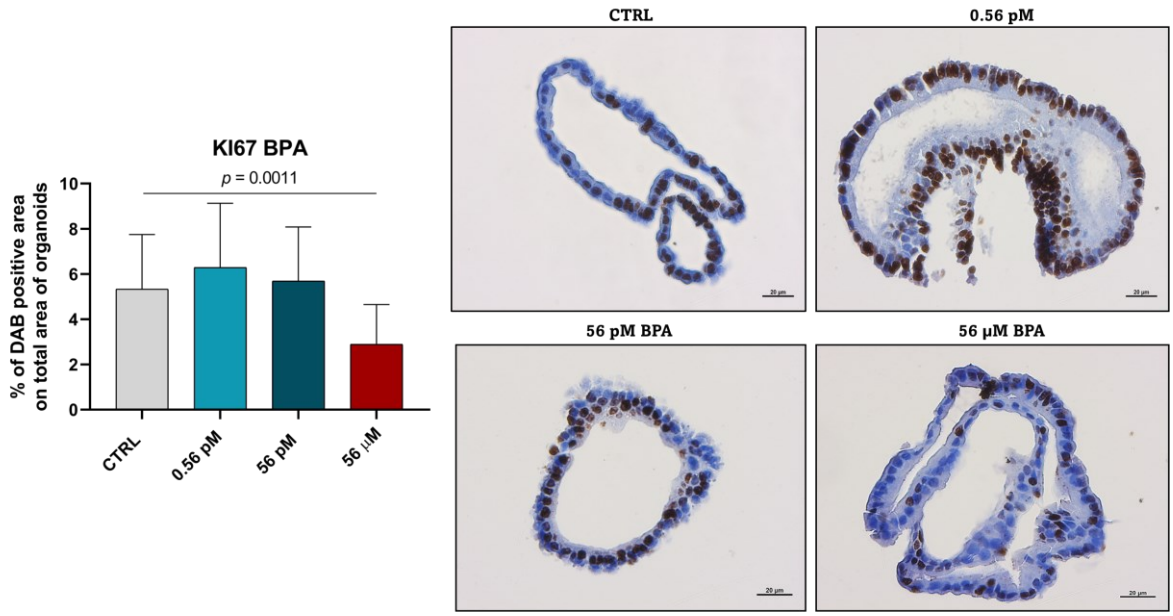

Figure 15. Immunohistochemistry. Quantification of Ki-67 protein levels among the experimental groups. For statistical analysis, One-way ANOVA test was performed followed by Kruskal-Wallis test. Data are reported as mean ± standard deviation (SD) with n = 49 for CTRL; n = 54 for 0.56 pM; n = 45 for 56 pM; n = 16 for 56 µM. Microphotographs show representative DAB staining for Ki-67 in the different experimental groups. Scale bar = 20 µm.

3.4 Organoid Differentiation

The expression of marker genes codifying for factors involved in intestinal epithelial cells differentiation, like *TFF3*, a marker of goblet cells, and *KRT20*, a marker of enterocytes, was investigated. The results obtained showed no significant variation in the expression of *TFF3* (**Figure 16a**), and *KRT20* (**Figure 16b**) among the treatment groups.

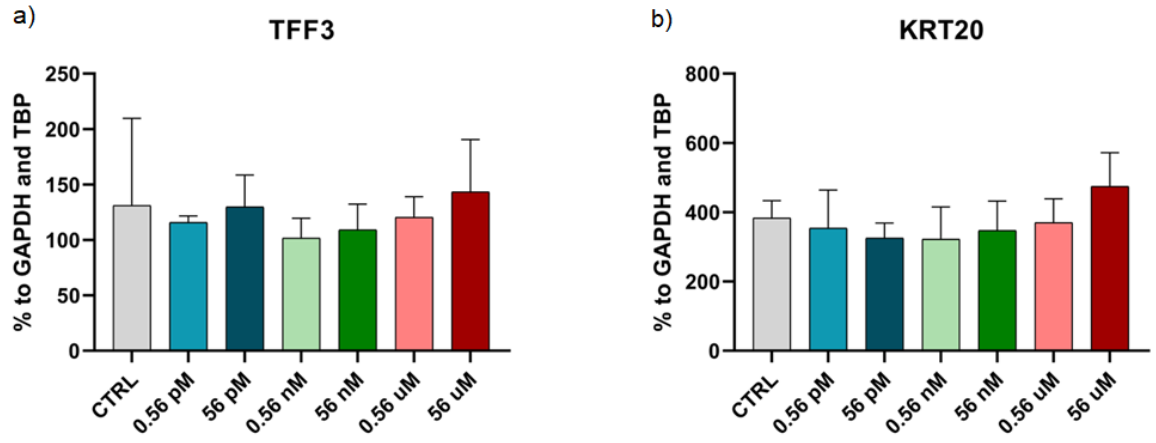

Figure 16. RT-qPCR transcript analysis. mRNA expression levels of a) *TFF3* gene and b) *KRT20* gene in human intestinal organoid cells among the experimental groups. For statistical analysis, One-way ANOVA test was performed followed by Dunett's multiple comparison test. Data are reported as mean ± standard deviation (SD).

3.5 Organoid Functionality

To assess the functionality of intestinal organoid cells treated with BPA, the expression of *LYZ*, encoding for lysozyme secreted by Paneth cells, and *ALPi*, encoding for intestinal alkaline phosphatase secreted by enterocytes, was evaluated. These genes also serve as markers of cell differentiation within the

organoids. In our study, the treatment with different concentrations of BPA did not alter the expression of *LYZ* in human intestinal organoid cells, but a non-statistically significant increase trend in *LYZ* expression was evident in the group treated with 56 µM of BPA (**Figure 17a**). Regarding *ALPi*, a significant overexpression was detected in the intestinal organoids exposed to 56 µM of BPA compared to CTRL (**Figure 17b**).

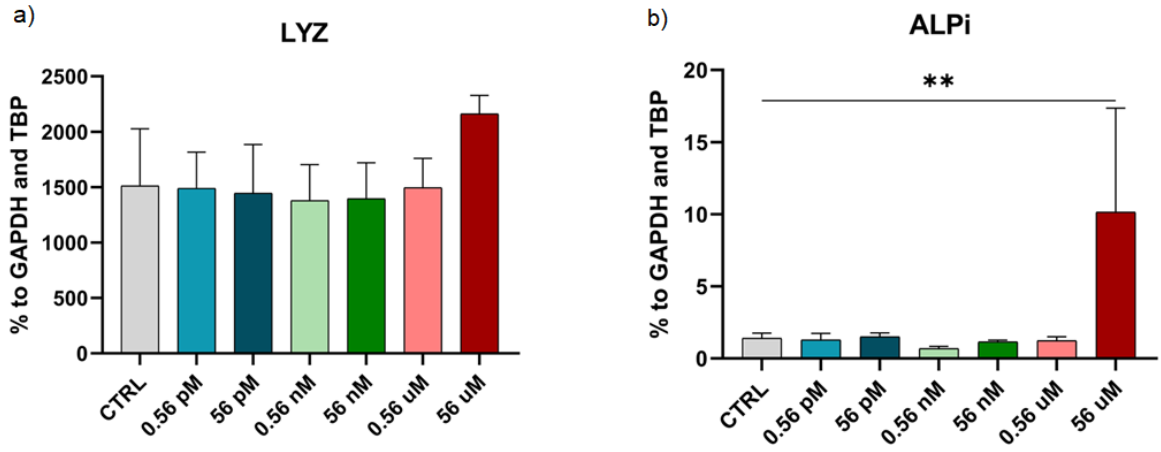

Figure 17. RT-qPCR transcript analysis. mRNA expression levels of a) *LYZ* gene and b) *ALPi* gene in human intestinal organoid cells among the experimental groups. For statistical analysis, One-way ANOVA test was performed followed by Dunett's multiple comparison test. Data are reported as mean ± standard deviation (SD). Asterisks represent significance levels as follow: two asterisks (**) for $p<0.01$.

Histological staining was performed to assess ALPi enzymatic activity in human intestinal organoid cells. The results obtained demonstrated a non-statistically significant decrease in ALPi activity in the organoids exposed to 56 µM of BPA if compared to CTRL and a significant decrease in ALPi activity in the organoids exposed to 56 pM of BPA compared to CTRL (**Figure 18**).

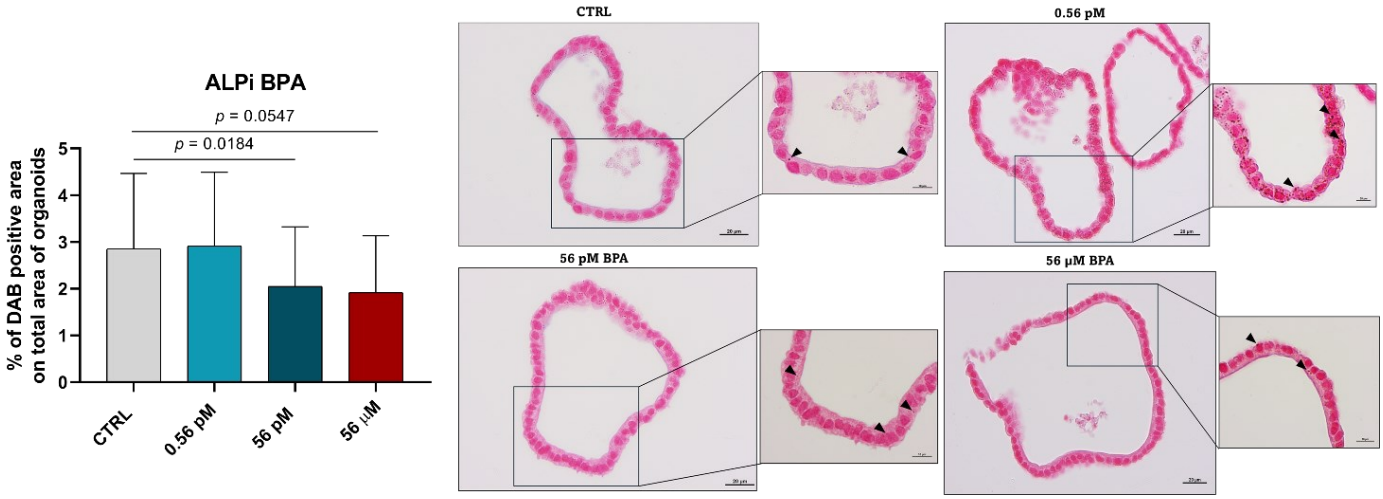

Figure 18. Histological staining. Quantification of ALPi enzymatic activity among the experimental groups. For statistical analysis, One-way ANOVA test was performed followed by Kruskal-Wallis test. Data are reported as mean ± standard deviation (SD) with n = 49 for CTRL; n = 54 for 0.56 pM; n = 45 for 56 pM; n = 16 for 56 µM. Microphotographs show representative staining for ALPi (arrow heads) in the different experimental groups. Scale bar = 20 µm; scale bar = 10 µm for 100 X magnification.

3.6 Organoid Damage and Apoptosis

To assess the toxicity of BPA on human intestinal organoid cells, the expression of genes involved in oxidative stress and inflammatory response was evaluated. For oxidative stress, *SOD1*, *CAT* and *GPX1,* representing the first line of

defense against reactive oxygen species, transcript levels were analyzed. The results obtained showed no significant changes in the expression of *SOD1* (**Figure 19a**), *CAT* (**Figure 19b**) and *GPX1* (**Figure 19c**) genes among the treatment groups.

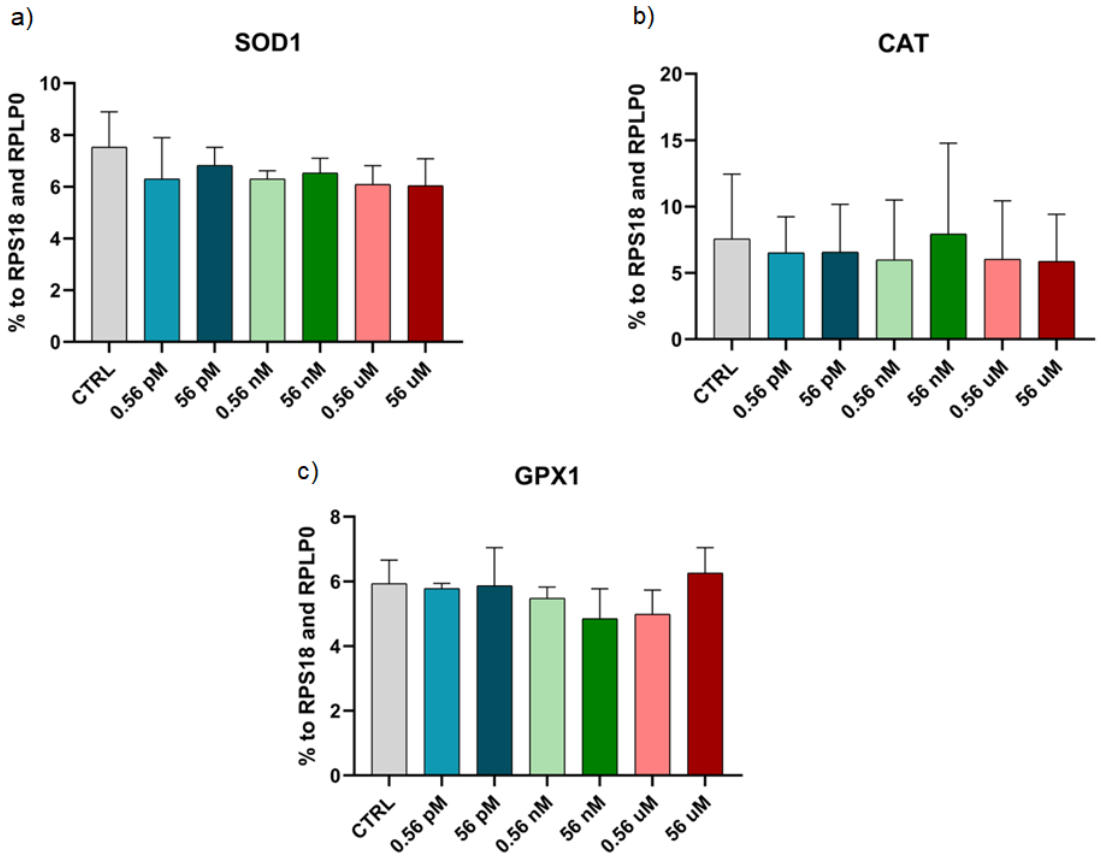

Figure 19. RT-qPCR transcript analysis. mRNA expression levels of a) *SOD1* gene, b) *CAT* gene and c) *GPX1* gene in human intestinal organoid cells among the experimental groups. For statistical analysis, One-way ANOVA test was performed followed by Dunett's multiple comparison test. Data are reported as mean ± standard deviation (SD).

Concerning the evaluation of inflammatory response, genes selected and analyzed were *IL-1β* and *TNF-α*, which are involved in inflammation induced

by injury, infection or stress. The results showed that the treatment with different concentrations of BPA did not alter the expression of *IL-1β* (**Figure 20a**) and *TNF-α* (**Figure 20b**) in human intestinal organoid cells.

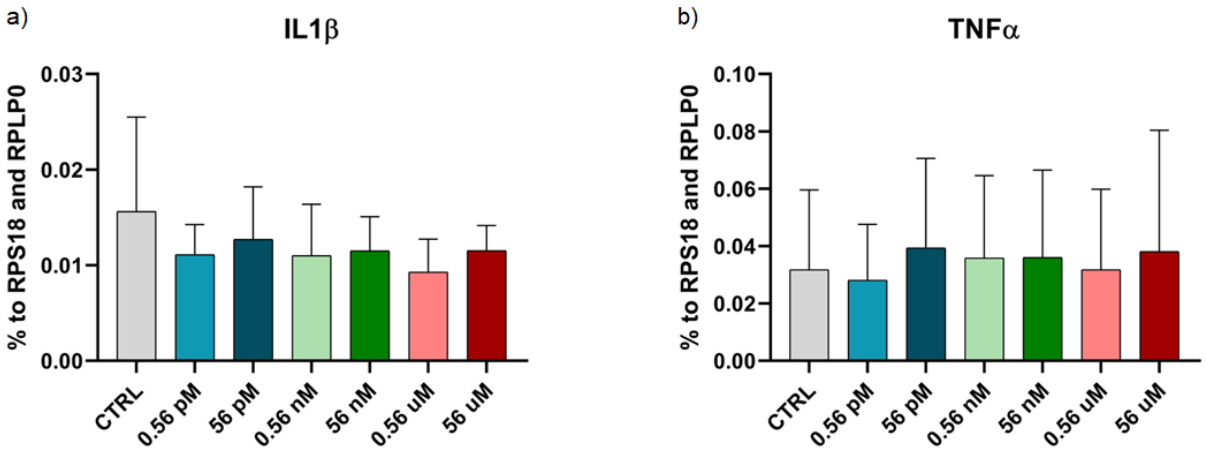

Figure 20. RT-qPCR transcript analysis. mRNA expression levels of a) *IL-1β* gene and b) *TNF-α* gene in human intestinal organoid cells among the experimental groups. For statistical analysis, One-way ANOVA test was performed followed by Dunett's multiple comparison test. Data are reported as mean ± standard deviation (SD).

Apoptosis is a programmed cell death process that can be activated in response to stress stimuli or cellular damage. To assess the ability of BPA to activate apoptosis in human intestinal organoid cells, the protein levels of cleaved Caspase-3 (CASP3) were evaluated via Immunohistochemistry. The findings of our study demonstrated a significant increase in the protein levels of CASP3 in the organoids treated with 0.56 pM of BPA compared to CTRL (**Figure 21**).

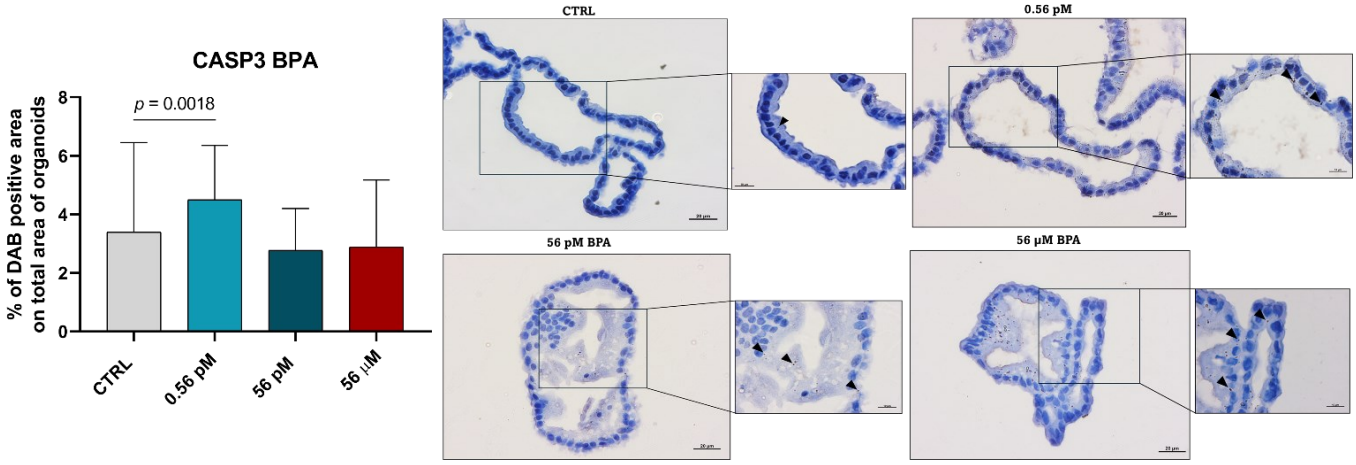

Figure 21. Immunohistochemistry. Quantification of CASP3 protein levels among the experimental groups. For statistical analysis, the Kruskal-Wallis test was performed. Data are reported as mean ± standard deviation (SD) with n = 49 for CTRL; n = 58 for 0.56 pM; n = 43 for 56 pM; n = 21 for 56 µM. Microphotographs show representative DAB staining for CASP3 (arrow heads) in the different experimental groups. Scale bar = 20 µm; scale bar = 10 µm for 100 X magnification.

DISCUSSION

Several studies conducted on experimental *in vivo* and *in vitro* models have described the intestinal toxicity resulting from the exposure to EDCs, such as BPA. Due to ethical constraints, it is challenging to study the effects of BPA *in vivo* in humans; thus, research has largely relied on animal models and *in vitro* systems. To address this limitation, we selected human intestinal organoids as a 3D *in vitro* model, which provides a more realistic representation of what might occur in the human intestine. Based on existing evidence, our research examined the direct effects of BPA at various concentrations on human intestinal organoids, focusing on pathways involved in cell proliferation and differentiation, cell functionality, cell damage, and apoptosis. Recently, the mitigation of the toxic effects of EDCs exposure, including BPA, by probiotics has garnered attention. Considering this, we evaluated if the probiotic mixture SLAB51 was able to mitigate the BPA-induced toxicity on proliferation, morphology, and growth of the exposed organoids. Additionally, using probiotics as a strategy to counteract BPA effects represents an innovative approach that has yet to be extensively assessed in research. One of the adverse effects of BPA is its potential to penetrate the intestinal barrier, leading to

significant damage in the intestinal tract. This damage manifests particularly as oxidative stress and inflammation (Luo et al., 2024). Multiple studies have demonstrated that exposure to BPA can significantly elevate the production of reactive oxygen species, leading to heightened oxidative stress and cellular damage. The stimulation of ROS production by BPA has been demonstrated *in vitro* in human colon cancer cells, where an increase in both intracellular and mitochondrial ROS was observed following BPA exposure (Qu et al., 2018). These findings were confirmed *in vivo* in mice exposed to BPA, where it induced an overproduction of ROS and inhibited the activity of antioxidant enzymes, including SOD, GPx, CAT, and T-AOC. This led to an imbalance between oxidant and antioxidant actions in intestinal tissues, promoting an oxidative state (Wang et al., 2021). Based on this evidence, our study investigated the expression of genes involved in oxidative stress, including *SOD1*, *CAT*, and *GPX1*, that represent the first line of defense against ROS. Specifically, SOD1 catalyzes the conversion of superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) (Thorpe et al., 2013), which is converted into water and oxygen by the action of CAT (Deisseroth & Dounce, 1970), while GPX1 reduces intracellular hydrogen peroxide and lipid peroxides (Huang et al., 2018). Despite previous indications that BPA induces oxidative stress, in our study the selected doses of BPA did not alter the expression of *SOD1*, *CAT*,

and *GPX1*. Therefore, to better elucidate a possible impact of BPA on oxidative stress response, it would be interesting to investigate more deeply the mechanism of action of BPA in intestinal organoids by evaluating not only the expression of genes involved in oxidative stress, but also the protein levels, the enzymatic activity of these antioxidant enzymes and the levels of intracellular and/or mitochondrial ROS.

Another effect of BPA exposure at the intestinal level is inflammation. As demonstrated both *in vitro* and *in vivo* models, exposure to BPA can activate the gut innate immune system leading to elevated levels of pro-inflammatory cytokines (Della Rocca et al., 2023). Wang et al., (2021) proved that BPA treatment significantly increased the concentrations of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and IL-8 in mice gut and in mice colonic epithelial cells (CT-26). Together, these results suggest that BPA-induced impairment of the gut barrier function is closely related to the activation of innate immune responses (Wang et al, 2021). Intestinal inflammation induced by pro-inflammatory cytokines has been linked with disruption of the intestinal TJ barrier, which allows intestinal penetration of luminal antigens (Rawat et al., 2020). Surprisingly, our study showed that BPA did not affect the expression of *IL-1β* and *TNF*-α genes, which encode pro-inflammatory cytokines involved in inflammatory responses to different

stimuli, such as infection, injury, and stress (Baud & Karin, 2001; Dinarello, 1996). Despite the lack of impact of BPA on genes involved in inflammation at transcriptional level, a possible modulation of the protein levels of cytokines and their secretion into the medium could be present. The evaluation of these markers should be of help in elucidating the possible implication of these pathways in BPA direct toxicity at intestinal level. The innate immune response in the intestine is regulated by the activity of two enzymes secreted by intestinal epithelial cells: intestinal alkaline phosphatase (ALPi or IAP) and lysozyme (LYZ). ALPi is an innate defense protein, located in the brush border of enterocytes (Calhau et al., 2000; Nakano et al., 2009), involved in maintaining intestinal homeostasis and barrier function by inhibiting pro-inflammatory cytokines, including TNF-α and IL-1β, which disrupt intestinal barrier function (Singh & Lin, 2021). ALPi also detoxifies lipopolysaccharide, which is responsible for intestinal permeability and inflammation (Beumer et al., 2003), and indirectly regulates bacterial populations by upregulating antimicrobial proteins such as lysozyme, an enzyme secreted by Paneth cells that shapes the intestinal microbial composition and eliminates pathogens in the intestine (Ostaff et al., 2013). Thus, the activity of these two enzymes protects the intestine, contributing to its optimal functionality and preventing inflammatory conditions and intestinal dysfunctions. Noteworthy, it has been observed that

exposure to BPA can alter the expression of ALPi and lysozyme, compromising their activity and protective functions. The effect of BPA on the expression of *ALPi* in human and mammalian intestine has been poorly investigated, but in the vertebrate model *Xenopus laevis*, exposure to BPA at concentrations in the nanomolar range resulted in a linear increase in the expression of *ALPi*, suggesting a stimulation of enterocytes differentiation and an estrogenic effect promoting intestinal development in vertebrates (Zhu et al., 2020). Consistent with the findings from this study, we observed an increase in *ALPi* gene expression in the intestinal organoid cells exposed to a concentration of 56 μM of BPA. However, the evaluation of ALPi enzymatic activity in the organoids demonstrated instead a reduction in the enzyme activity both in the group exposed to 56 μM of BPA and in the group exposed to 56 pM of BPA. The reduction in ALPi activity in the exposed organoids could result from either direct or indirect effects of BPA on the enzyme itself, cellular metabolism, cell differentiation, or other cellular components and processes. To better understand the specific cause, further investigations may be needed, including analyses of cellular function, metabolism, and altered signaling pathways.

Regarding the effect of BPA on lysozyme, a study conducted on mice subjected to perinatal exposure of BPA demonstrated a possible correlation between BPA and inflammatory bowel disease, which can be linked to alterations in lysozyme

production (Yu et al., 2020). The exposure to BPA led to a reduction in lysozyme expression in Paneth cells of the ileum, without altering the number of cells present, and contributed to decreased fecal lysozyme activity against bacterial peptidoglycan, resulting in reduced antimicrobial activity against commensal bacteria, such as *E.coli*. These findings indicate a defect in the protection of gut against infection and injury and a dysregulation in the development of gut immune homeostasis, which may account for the predisposition to inflammation in offspring during adulthood (Malaisé et al., 2018). Despite evidence suggesting that BPA can interfere with the expression and activity of lysozyme, our study did not detect any variations in *LYZ* gene expression in the cells of intestinal organoids exposed to BPA. However, a non-significant trend towards increased *LYZ* expression was observed in the organoids exposed to 56 µM of BPA, indicating a possible induction of the innate immune response following BPA exposure. This observation, however, does not correlate with the reduction of ALPi enzymatic activity and the expression of pro-inflammatory cytokines as expected.

The cellular damage induced by BPA in intestinal cells has been associated in many studies with the ability of BPA to induce apoptosis. Apoptosis is a programmed death of a self-protective nature after the host cell senses external risk factors (Elmore, 2007). Intestinal epithelial cells have the capability for

self-repair but when exposure to exogenous harmful substances exert toxic effects that exceed the threshold for cellular repair, intestinal damage occurs (Lei et al., 2018; Liu et al., 2019). In mice exposed to BPA the gene expression of the pro-apoptotic factor Bax were significantly increased, whereas those of the anti-apoptotic key factor Bcl-2 were significantly decreased in the intestine. Moreover, the gene abundance and enzyme activity of the apoptotic executive protein Caspase-3 were also significantly increased after BPA treatment, suggesting a strong pro-apoptotic effect of BPA. These findings demonstrate that BPA may cause the functional impairment of the gut barrier by inducing cellular damage and disrupting cellular homeostasis (Wang et al, 2021). Regarding the induction of apoptosis by BPA, in our research an increase in the levels of the cleaved form of the executive CASP3 was demonstrated in the intestinal organoid cells exposed to the lowest dose of BPA. This further illustrates that BPA can exhibit non-linear effects depending on the dose, with harmful effects observed also at lower doses and not present at higher ones. Alterations in the apoptosis rate in the intestinal epithelium can cause corresponding changes in the proliferation rate of stem cells in the crypts and their subsequent differentiation to replace lost or damaged cells (Bao et al., 2020). To maintain tissue homeostasis, it is essential to balance cell proliferation, differentiation, and death. Recent studies have shown that

bisphenols, including BPA, can disrupt intestinal homeostasis. Zhu et al., (2022) found that in mice the exposure to BPA injured intestinal homeostasis and mucosal barrier. Specifically, the pollutant decreased cell proliferation in crypts, down-regulated expression of stem cell markers as well as secretory cell markers, reduced goblet cell number and mucin protein, and declined expression levels of tight junction markers. These alterations have been associated with the ability of BPA to interfere with Lgr5, Wnt and Notch signaling in both vertebrates and mammalian intestine. Exposure to BPA led to the transcriptional upregulation of *LGR5* in *X. laevis* intestine *in vivo* and in mammalian IEC-6 cells *in vitro* (Zhu et al., 2020). However, in mice intestine BPA induced a downregulation of *LGR5*. Moreover, Notch and Wnt signaling were disrupted leading to mucosal barrier dysregulation and intestinal inflammation in adult mice (Zhu et al., 2022). Despite the findings obtained by Zhu et al. (2020-2022), our results indicate that BPA did not affect the transcript levels of *LGR5* and *NOTCH1/2* in human intestinal organoid cells. This discrepancy could be due to differences in experimental conditions, such as variations in BPA concentrations, time of exposure, or the specific characteristics of the experimental model used. To address these inconsistencies, future research should focus on considering the cellular heterogeneity within intestinal organoids. Single-cell RNA sequencing studies

could help identify if specific cell populations respond differently to BPA. Additionally, since BPA might influence protein levels through post-transcriptional mechanisms, it would be worthwhile to examine protein expression and potential post-translational modifications, such as phosphorylation.

Disruption of Notch or Wnt signaling impairs stem cell homeostasis and prevents the maintenance of the balanced composition of cell types (Ratanasirintrawoot & Israsena, 2016; Sailaja et al., 2016). Different studies have shown that BPA can disrupt the expression of key markers in differentiated intestinal epithelial cells. In a study conducted on pig exposed to BPA, it was demonstrated that BPA led to the reduction of the number of goblet cells in the colon and cecum and reduced the expression of TFF3 peptide, a mucin-associated peptide that is secreted by goblet cells in response to injury and inflammation and that plays a crucial role in maintaining and restoring gastrointestinal mucosal homeostasis (Aihara et al., 2017). The alteration of TFF3 peptide expression by BPA suggests a possible compromise of the intestinal barrier function and a potential onset of inflammation (Liu et al., 2024). BPA exposure can potentially alter the expression of *KRT20*, a gene involved in insulin secretion (Liu et al., 2023). *KRT20* is primarily expressed in enterocytes, and its expression can be

influenced by metabolic changes, such as diabetic ketoacidosis. Specifically, the ketone body β-hydroxybutyrate can induce the differentiation of intestinal cells, as evidenced by the increased expression of some differentiation markers in the intestine, including *KRT20* (Wang et al., 2016). Several epidemiological studies have shown that BPA, acting as an endocrine disruptor, can cause metabolic disorders, such as obesity and type 2 diabetes in which the pancreas, being an important endocrine organ, may be involved. This highlights that BPA, by disrupting metabolism, can have a negative impact on various interconnected organs, such as pancreas, liver, and intestine. Despite the potential influence of BPA on the expression of *TFF3* and *KRT20*, in our study we did not detect any alteration in the expression of these genes in the intestinal organoid cells exposed to BPA. This lack of effect may be attributed to the fact that the organoids were cultured in expansion media, where the number of differentiated cells is very limited. To investigate more accurately the effects of BPA on specific cell lineages, it would be appropriate to first differentiate the organoids and then expose them to BPA. This approach could be considered a potential direction for future research, aiming to provide a more comprehensive understanding of BPA's impact on intestinal cells.

As previously discussed, the process of intestinal cell differentiation is closely linked to their proliferation. In the intestine, cells actively divide to constantly

renew the epithelial lining (Bao et al., 2020). Multiple studies have evaluated the effect of BPA on the proliferation of distinct types of intestinal cells, both *in vivo* and *in vitro* models. Wang et al., (2021) demonstrated that treating the mouse colonic epithelial cell line (CT-26) with BPA significantly impaired the cells proliferative capacity. Their study showed that BPA exposure led to a marked reduction in both the gene expression and enzymatic activity of key proliferation markers (Wang et al., 2021). In another study conducted by Qu et al., (2018) the potential toxicity of BPA was assessed using human colon cancer cells (HCT116). Cells were exposed to different concentrations of BPA, ranging from 0 to 400 μM, for 24 hours. The findings revealed a significant decrease in cell viability following the treatment with BPA concentrations exceeding 100 μM, with the median lethal dose (LD50) being determined at 250 μM. Furthermore, cytotoxic effects were evident after a 24-hour incubation period with 250 μM BPA. The decline in cell proliferation was associated with the activation of apoptosis induced by BPA. Moreover, BPA induced damage in mitochondria, the main cellular organelles responding to oxidative stress triggered by BPA, demonstrating that the accumulation of intracellular and mitochondrial ROS may play a pivotal role in BPA-induced toxicity of colonic epithelial cells (Qu et al., 2018). Contrary to the findings presented in these studies, Jun et al., (2021) demonstrated that BPA was able to decrease

proliferation at low doses and increase proliferation at higher doses in HT-29, a human colorectal adenocarcinoma cell line. Cells were treated for five days with different doses of BPA, ranging from 0 to 100 μM. They demonstrated that five days after BPA treatment, cell proliferation increased in a dose-dependent manner for BPA concentrations ranging from 0.05 to 50 μM but decreased in cells treated with 100 μM of BPA (Jun et al., 2021). It is widely known that the different impact of BPA on cell proliferation can be influenced by multiple factors. Firstly, cell type specificity, such as metabolic activity, receptors expression and functional specialization, can influence the response to BPA. Moreover, the concentration of BPA is pivotal, and the duration of exposure further influences outcomes. Additionally, environmental and experimental conditions, including culture environment and assay techniques, can introduce variability in the observed effects. In biological research, Ki-67 is used to study and investigate cell proliferation in various contexts. Ki-67 presence is closely correlated with cellular proliferative activity, making it a valuable indicator for assessing cell growth and division. It is highly expressed in cycling cells but strongly downregulated in resting G0 cells, therefore it has been considered a marker of cell proliferation (Gerdes et al., 1983). In addition, Ki-67 has been recently recognized as a marker for various types of cancers, including gastrointestinal and colorectal cancers. Due to its role in cell proliferation,

assessing Ki-67 expression can be useful for evaluating cancer's prognosis (Sun & Kaufman, 2018). It is interesting to note that some studies reported a correlation between BPA exposure and the development of colorectal cancer. Particularly, Jun et al., (2021) highlighted that both in mice with colorectal cancer and in HT-29 colon cancer cells, BPA exposure contributed to the proliferation, migration, and growth of cancer cells. These findings underscore a possible link between BPA and colorectal cancer (Jun et al., 2021). Despite the information regarding the correlation between Ki-67 and gastrointestinal cancers, including colorectal cancer, and the correlation between the latter and BPA exposure, few studies have evaluated Ki-67 expression in intestinal cells after BPA exposure. In our study, assessing the protein levels of Ki-67 in intestinal organoids provided a valuable approach to establish the role of BPA in intestinal cell proliferation. Our finding revealed a significant decrease in Ki-67 protein levels in the organoid cells treated with the highest dose of BPA. These results suggest that BPA may have an inhibitory effect on cell proliferation at higher concentration, with potential negative implications for human health. Notably, this effect was not observed at the lowest and middle doses of BPA. These differences observed are consistent with what is already known about the action of BPA and the non-monotonic responses it can induce depending on its concentration (Vandenberg et al., 2012). This finding warrants

further investigation to fully understand the mechanisms of BPA action and its biological consequences.

Another approach to assess the effects of BPA on cell proliferation could involve the morphological analysis of the target cells or tissues. To evaluate the impact of BPA on the proliferation and growth of the exposed intestinal organoids, we assessed potential alterations in the area and diameter of the organoids. The results obtained revealed intriguing insights into toxic effects of BPA on the organoids. Specifically, BPA exposure resulted in a non-monotonic reduction in the area and diameter of the intestinal organoids. Since intestinal organoids are *in vitro* models that partially recreate the environment and physiological characteristics of the human intestine (Kim et al., 2020; Lancaster & Knoblich, 2014), the results obtained suggest that exposure to BPA could induce a potential inhibition of proliferation or impairment of growth mechanisms in intestinal cells and can pose a risk to intestinal health. The morphological structure of the intestinal epithelium is an important indicator of intestinal health. In mice, the exposure to BPA led to a severe disruption of the morphological structure of the colonic epithelium and resulted in an increased histopathological score (Feng et al., 2019). Another study, conducted on rats exposed to BPA, showed that the endocrine disruptor induced impairment of the small intestinal architecture and disruption of the

intestinal villi structure (Ambreen et al., 2019). A study conducted on pregnant pigs examined the impact of maternal BPA exposure during pregnancy on intestinal digestion and absorption function in offspring. Morphological analysis of the intestine revealed that the ratio of jejunum villus height to crypt depth in newborn and weaning pigs was significantly reduced due to maternal BPA exposure. This reduction probably indicates a reduction in proliferation and differentiation of intestinal epithelial cells (Liu et al., 2017). Many hormonal receptors, including estrogen receptors (ERs) and thyroid hormone receptors (TRs), are implicated in the development and regulation of the intestinal barrier (Chen et al., 2022; Sirakov & Plateroti, 2011). Several studies have indicated that Bisphenols (BPs), including BPA, possess estrogenic and thyroid-disrupting properties; therefore, it is not surprising that BPs could potentially interfere with intestinal development and homeostasis (Zhu et al., 2024). Considering these interactions, it would be interesting to further investigate these receptors to gain a more comprehensive understanding of BPA's potential toxicity and grant a clearer picture of BPA's impact on intestinal organoid cells. This analysis could provide crucial insights into the mechanisms through which BPA disrupts intestinal barrier functions and homeostasis.

Multiple studies have highlighted the potential of probiotics to influence growth and proliferation of intestinal cells, offering promising therapeutic avenues for various gastrointestinal disorders. In our study, we selected the probiotic mixture SLAB51 to evaluate its potential to counteract BPA-induced toxicity in human intestinal organoids. Giommi et al., (2024) recently demonstrated that SLAB51 co-administrated with BPA recovered the gut architecture and restored intestinal homeostasis in zebrafish exposed to BPA (Giommi et al., 2024). Consistently with these previous observations, in the present study an improvement in the morphological parameters of the organoids co-treated with BPA and the probiotic was observed, specifically, in the middle dose tested. The strong antagonistic effect of the probiotic on BPA toxicity, restoring the morphology of the organoids and resulting in area and diameter values similar to those of the control group, was here evidenced. Similarly, other studies have highlighted the beneficial properties of probiotics on intestinal cells. Seth et al., (2007) demonstrated the properties of two soluble proteins produced by *Lactobacillus rhamnosus* in maintaining intestinal epithelial cell (IEC) homeostasis. These proteins, called P40 and P75, have been shown to prevent cytokine-induced apoptosis in the intestinal epithelium of mice and humans by modulating key signaling pathways. Specifically, P40 and P75 transactivated the epidermal growth factor receptor (EGFR), leading to

the expression of a proliferation-inducing ligand (APRIL) in the epithelium. Additionally, P40 and P75 promoted the production of heat shock proteins Hsp72 and Hsp25 in IECs. These heat shock proteins are essential for protecting the integrity of strong binding proteins, thereby supporting the proliferation and viability of intestinal epithelial cells (Seth et al., 2007). In a study conducted on pig intestinal organoids, the probiotic bacterium *B. amyloliquefaciens* was able to stimulate the growth and proliferation of organoid cells. The continuous administration of 10^8 CFU/mL led to a significant increase in the area of the organoids. Additionally, the probiotic induced an increase in the protein levels of Ki-67, a marker for cell proliferation, as well as an increase in the number of Lgr5+ stem cells and goblet cells, suggesting that *B. amyloliquefaciens* not only enhanced ISC proliferation and viability but also supported their differentiation and function (Wang et al., 2024). In line with these studies, our research highlights the potential of the SLAB51 probiotic formulation in mitigating the toxicity of BPA at the intestinal level. However, further molecular analyses are required to investigate more deeply the pathways involved in these protective effects.

CONCLUSION

In conclusion, our study sheds light on the multifaceted toxicity of BPA on intestinal organoids. We observed significant alterations in the organoid morphology upon BPA exposure, particularly at concentrations of 0.56 pM, 56 pM, and 56 μM, indicating the pollutant toxicity. Interestingly, co-exposure to BPA and the probiotic SLAB51 showed promising results in mitigating BPA-induced toxicity, particularly at the concentration of 56 pM+P at 10^6 CFU, where the probiotic effectively restored organoid morphology. While our investigation did not reveal significant changes in the expression of key genes associated with oxidative stress, inflammation, proliferation, and intestinal cell differentiation, we did observe an anti-proliferative effect of BPA and an impact on organoid function and homeostasis after BPA exposure at high concentration and also non-monotonic effects of BPA which resulted in an increase in CASP3 levels at lowest BPA concentration, indicating a pro-apoptotic effect at low concentrations that was not seen at highest BPA concentrations. Additional molecular analyses are in progress to further clarify the impact of BPA on the intestinal organoid cells and to evaluate the beneficial properties of the probiotic against BPA toxicity not only on

organoid morphology and growth but also at the molecular and cellular functionality levels. Furthermore, our study adds to the expanding body of evidence indicating that BPA exposure can lead to non-monotonic effects, with responses differing based on the concentration. This emphasizes the importance of conducting thorough assessments of BPA toxicity, encompassing a spectrum of doses and endpoints, which have significant implications in risk assessment. In summary, our study provides valuable insights into the complex interactions between BPA, intestinal organoids, and probiotics. By elucidating these mechanisms, we can further better understand the risks associated with BPA exposure and develop strategies to protect intestinal health in the face of environmental challenges.

BIBLIOGRAPHY

- Adak, A., & Khan, M. R. (2019). An insight into gut microbiota and its functionalities. *Cellular and Molecular Life Sciences : CMLS*, *76*(3), 473–493. https://doi.org/10.1007/S00018-018-2943-4

- Ahire, J. J., Jakkamsetty, C., Kashikar, M. S., Lakshmi, S. G., & Madempudi, R. S. (2021). *In Vitro* Evaluation of Probiotic Properties of Lactobacillus plantarum UBLP40 Isolated from Traditional Indigenous Fermented Food. *Probiotics and Antimicrobial Proteins*, *13*(5), 1413–1424. https://doi.org/10.1007/S12602-021-09775-7

- Aihara, E., Engevik, K. A., & Montrose, M. H. (2017). Trefoil Factor Peptides and Gastrointestinal Function. *Annual Review of Physiology*, *79*, 357. https://doi.org/10.1146/ANNUREV-PHYSIOL-021115-105447

- Aisenbrey, E. A., & Murphy, W. L. (2020). Synthetic alternatives to Matrigel. *Nature Reviews. Materials*, *5*(7), 539–551. https://doi.org/10.1038/S41578-020-0199-8

- Alengebawy, A., Abdelkhalek, S. T., Qureshi, S. R., & Wang, M. Q. (2021). Heavy Metals and Pesticides Toxicity in Agricultural Soil and Plants: Ecological Risks and Human Health Implications. *Toxics*, *9*(3), 1–34. https://doi.org/10.3390/TOXICS9030042

- Ambreen, S., Akhtar, T., Hameed, N., Ashfaq, I., & Sheikh, N. (2019). *In Vivo* Evaluation of Histopathological Alterations and Trace Metals Estimation of the Small Intestine in Bisphenol A-Intoxicated Rats. *Canadian Journal of Gastroenterology and Hepatology*, *2019*. https://doi.org/10.1155/2019/9292316

- Bäckhed, F., Ding, H., Wang, T., Hooper, L. V., Gou, Y. K., Nagy, A., Semenkovich, C. F., & Gordon, J. I. (2004). The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(44), 15718–15723. https://doi.org/10.1073/PNAS.0407076101

- Balaguer-Trias, J., Deepika, D., Schuhmacher, M., & Kumar, V. (2022). Impact of Contaminants on Microbiota: Linking the Gut-Brain Axis with Neurotoxicity. *International Journal of Environmental Research and Public Health*, *19*(3). https://doi.org/10.3390/IJERPH19031368

- Bao, L., Shi, B., & Shi, Y. B. (2020). Intestinal homeostasis: A communication between life and death. *Cell and Bioscience*, *10*(1), 1–3. https://doi.org/10.1186/S13578-020-00429-9/FIGURES/1

- Barker N, van Es JH, Kuipers J, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature*. 2007;449(7165):1003-1007. doi:10.1038/nature06196

- Baud, V., & Karin, M. (2001). Signal transduction by tumor necrosis factor and its relatives. *Trends in Cell Biology*, *11*(9), 372–377. https://doi.org/10.1016/S0962-8924(01)02064-5

- Berger, E., Rath, E., Yuan, D., Waldschmitt, N., Khaloian, S., Allgäuer, M., Staszewski, O., Lobner, E. M., Schöttl, T., Giesbertz, P., Coleman, O. I., Prinz, M., Weber, A., Gerhard, M., Klingenspor, M., Janssen, K. P., Heikenwalder, M., & Haller, D. (2016). Mitochondrial function controls intestinal epithelial stemness and proliferation. *Nature Communications*, *7*. https://doi.org/10.1038/NCOMMS13171

- Beumer, C., Wulferink, M., Raaben, W., Fiechter, D., Brands, R., & Seinen, W. (2003). Calf intestinal alkaline phosphatase, a novel therapeutic drug for lipopolysaccharide (LPS)-mediated diseases, attenuates LPS toxicity in

mice and piglets. *The Journal of Pharmacology and Experimental Therapeutics*, *307*(2), 737–744. https://doi.org/10.1124/JPET.103.056606

- Birchenough, G. M. H., Johansson, M. E. V., Gustafsson, J. K., Bergström, J. H., & Hansson, G. C. (2015). New developments in goblet cell mucus secretion and function. *Mucosal Immunology*, *8*(4), 712. https://doi.org/10.1038/MI.2015.32

- Boland, M. (2016). Human digestion--a processing perspective. *Journal of the Science of Food and Agriculture*, *96*(7), 2275–2283. https://doi.org/10.1002/JSFA.7601

- Bonfili, L., Cecarini, V., Cuccioloni, M., Angeletti, M., Berardi, S., Scarpona, S., Rossi, G., & Eleuteri, A. M. (2018). SLAB51 Probiotic Formulation Activates SIRT1 Pathway Promoting Antioxidant and Neuroprotective Effects in an AD Mouse Model. *Molecular Neurobiology*, *55*(10), 7987–8000. https://doi.org/10.1007/S12035-018-0973-4

- Bu, Y., Liu, Y., Liu, Y., Wang, S., Liu, Q., Hao, H., & Yi, H. (2022). Screening and Probiotic Potential Evaluation of Bacteriocin-Producing Lactiplantibacillus plantarum *In Vitro*. *Foods*, *11*(11). https://doi.org/10.3390/FOODS11111575

- Bull, M. J., & Plummer, N. T. (2014). Part 1: The Human Gut Microbiome in Health and Disease. *Integrative Medicine: A Clinician's Journal*, *13*(6), 17. /pmc/articles/PMC4566439/

- Bustamante-Madrid, P., Barbáchano, A., Albandea-Rodríguez, D., Rodríguez-Cobos, J., Rodríguez-Salas, N., Prieto, I., Burgos, A., Martínez de Villarreal, J., Real, F. X., González-Sancho, J. M., Larriba, M. J., Lafarga, M., Muñoz, A., & Fernández-Barral, A. (2024). Vitamin D opposes multilineage cell differentiation induced by Notch inhibition and BMP4 pathway activation in human colon organoids. *Cell Death & Disease 2024 15:4*, *15*(4), 1–16. https://doi.org/10.1038/s41419-024-06680-z

- Calhau, C., Martel, F., Hipólito-Reis, C., & Azevedo, I. (2000). Differences between duodenal and jejunal rat alkaline phosphatase. *Clinical Biochemistry*, *33*(7), 571–577. https://doi.org/10.1016/S0009-9120(00)00171-5

- Cani, P. D., Bibiloni, R., Knauf, C., Waget, A., Neyrinck, A. M., Delzenne, N. M., & Burcelin, R. (2008). Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes*, *57*(6), 1470–1481. https://doi.org/10.2337/DB07-1403

- Chang, Y. H., Jeong, C. H., Cheng, W. N., Choi, Y., Shin, D. M., Lee, S., & Han, S. G. (2021). Quality characteristics of yogurts fermented with short-chain fatty acid-producing probiotics and their effects on mucin production and probiotic adhesion onto human colon epithelial cells. *Journal of Dairy Science*, *104*(7), 7415–7425. https://doi.org/10.3168/JDS.2020-19820

- Chassaing, B., Kumar, M., Baker, M. T., Singh, V., & Vijay-Kumar, M. (2014). Mammalian Gut Immunity. *Biomedical Journal*, *37*(5), 246. https://doi.org/10.4103/2319-4170.130922

- Chen, L., Lam, J. C. W., Tang, L., Tang, L., Hu, C., Liu, M., Liu, M., Lam, P. K. S., & Zhou, B. (2020). Probiotic Modulation of Lipid Metabolism Disorders Caused by Perfluorobutanesulfonate Pollution in Zebrafish. *Environmental Science and Technology*, *54*(12), 7494–7503. https://doi.org/10.1021/ACS.EST.0C02345/SUPPL_FILE/ES0C02345_SI_001.PDF

- Chen, P., Li, B., & Ou-Yang, L. (2022). Role of estrogen receptors in health and disease. *Frontiers in Endocrinology*, *13*. https://doi.org/10.3389/FENDO.2022.839005

- Chen, Z. J., Yang, X. L., Liu, H., Wei, W., Zhang, K. S., Huang, H. Bin, Giesy, J. P., Liu, H. L., Du, J., & Wang, H. S. (2015). Bisphenol A modulates

colorectal cancer protein profile and promotes the metastasis via induction of epithelial to mesenchymal transitions. *Archives of Toxicology*, *89*(8), 1371–1381. https://doi.org/10.1007/S00204-014-1301-Z

- Chianese, R., Troisi, J., Richards, S., Scafuro, M., Fasano, S., Guida, M., Pierantoni, R., & Meccariello, R. (2018). Bisphenol A in Reproduction: Epigenetic Effects. *Current Medicinal Chemistry*, *25*(6), 748–770. https://doi.org/10.2174/0929867324666171009121001

- Choi, J. J., Eum, S. Y., Rampersaud, E., Daunert, S., Abreu, M. T., & Toborek, M. (2013). Exercise attenuates PCB-induced changes in the mouse gut microbiome. *Environmental Health Perspectives*, *121*(6), 725–730. https://doi.org/10.1289/EHP.1306534

- Chokwe, T. B., Okonkwo, J. O., Sibali, L. L., & Ncube, E. J. (2015). Alkylphenol ethoxylates and brominated flame retardants in water, fish (carp) and sediment samples from the Vaal River, South Africa. *Environmental Science and Pollution Research*, *22*(15), 11922–11929. https://doi.org/10.1007/S11356-015-4430-X/FIGURES/4

- Claus, S. P., Guillou, H., & Ellero-Simatos, S. (2016). The gut microbiota: a major player in the toxicity of environmental pollutants? *NPJ Biofilms and Microbiomes*, *2*. https://doi.org/10.1038/NPJBIOFILMS.2016.3

- Clevers, H. (2013). The intestinal crypt, a prototype stem cell compartment. *Cell*, *154*(2), 274. https://doi.org/10.1016/J.CELL.2013.07.004

- Clinton, J., & McWilliams-Koeppen, P. (2019). Initiation, Expansion, and Cryopreservation of Human Primary Tissue-Derived Normal and Diseased Organoids in Embedded Three-Dimensional Culture. *Current Protocols in Cell Biology*, *82*(1). https://doi.org/10.1002/CPCB.66

- Collins, S. M., Surette, M., & Bercik, P. (2012). The interplay between the intestinal microbiota and the brain. *Nature Reviews. Microbiology*, *10*(11), 735–742. https://doi.org/10.1038/NRMICRO2876

- Coruzzi, G. (2010). Overview of gastrointestinal toxicology. *Current Protocols in Toxicology*, *Chapter 21*(SUPPL. 43). https://doi.org/10.1002/0471140856.TX2101S43

- Cousins, I. T., Staples, C. A., Klečka, G. M., & Mackay, D. (2002). A multimedia assessment of the environmental fate of bisphenol A. *Human and Ecological Risk Assessment*, *8*(5), 1107–1135. https://doi.org/10.1080/1080-700291905846

- Cresci, G. A., & Bawden, E. (2015). The Gut Microbiome: What we do and don't know. *Nutrition in Clinical Practice : Official Publication of the*

American Society for Parenteral and Enteral Nutrition, *30*(6), 734. https://doi.org/10.1177/0884533615609899

- Cronin, C. G., Delappe, E., Lohan, D. G., Roche, C., & Murphy, J. M. (2010). Normal small bowel wall characteristics on MR enterography. *European Journal of Radiology*, *75*(2), 207–211. https://doi.org/10.1016/J.EJRAD.2009.04.066

- Dahiya, D., & Nigam, P. S. (2022). The Gut Microbiota Influenced by the Intake of Probiotics and Functional Foods with Prebiotics Can Sustain Wellness and Alleviate Certain Ailments like Gut-Inflammation and Colon-Cancer. *Microorganisms*, *10*(3). https://doi.org/10.3390/MICROORGANISMS10030665

- De Santa Barbara, P., Van Den Brink, G. R., & Roberts, D. J. (2003). Development and differentiation of the intestinal epithelium. *Cellular and Molecular Life Sciences* , *60*(7), 1322. https://doi.org/10.1007/S00018-003-2289-3

- Deisseroth, A., & Dounce, A. L. (1970). Catalase: Physical and chemical properties, mechanism of catalysis, and physiological role. *Physiological Reviews*, *50*(3), 319–375. https://doi.org/10.1152/PHYSREV.1970.50.3.319

- Dekkers, J. F., Alieva, M., Wellens, L. M., Ariese, H. C. R., Jamieson, P. R., Vonk, A. M., Amatngalim, G. D., Hu, H., Oost, K. C., Snippert, H. J. G., Beekman, J. M., Wehrens, E. J., Visvader, J. E., Clevers, H., & Rios, A. C. (2019). High-resolution 3D imaging of fixed and cleared organoids. *Nature Protocols*, *14*(6), 1756–1771. https://doi.org/10.1038/S41596-019-0160-8

- Della Rocca, Y., Traini, E. M., Diomede, F., Fonticoli, L., Trubiani, O., Paganelli, A., Pizzicannella, J., & Marconi, G. D. (2023). Current Evidence on Bisphenol A Exposure and the Molecular Mechanism Involved in Related Pathological Conditions. *Pharmaceutics*, *15*(3). https://doi.org/10.3390/PHARMACEUTICS15030908

- Di Tommaso, N., Gasbarrini, A., & Ponziani, F. R. (2021). Intestinal Barrier in Human Health and Disease. *International Journal of Environmental Research and Public Health*, *18*(23), 12836. https://doi.org/10.3390/IJERPH182312836

- Diamanti-Kandarakis, E., Bourguignon, J. P., Giudice, L. C., Hauser, R., Prins, G. S., Soto, A. M., Zoeller, R. T., & Gore, A. C. (2009). Endocrine-disrupting chemicals: an Endocrine Society scientific statement. *Endocrine Reviews*, *30*(4), 293–342. https://doi.org/10.1210/ER.2009-0002

- Dillon, A., & Lo, D. D. (2019). M Cells: Intelligent Engineering of Mucosal Immune Surveillance. *Frontiers in Immunology*, *10*(JUL), 1499. https://doi.org/10.3389/FIMMU.2019.01499

- Dinarello, C. A. (1996). Biologic Basis for Interleukin-1 in Disease. *Blood*, *87*(6),2095–2147. https://doi.org/10.1182/BLOOD.V87.6.2095.BLOODJOURNAL8762095

- Dong, S., Liang, S., Cheng, Z., Zhang, X., Luo, L., Li, L., Zhang, W., Li, S., Xu, Q., Zhong, M., Zhu, J., Zhang, G., & Hu, S. (2022). ROS/PI3K/Akt and Wnt/β-catenin signalings activate HIF-1α-induced metabolic reprogramming to impart 5-fluorouracil resistance in colorectal cancer. *Journal of Experimental & Clinical Cancer Research : CR*, *41*(1). https://doi.org/10.1186/S13046-021-02229-6

- Driehuis, E., & Clevers, H. (2017). CRISPR/Cas 9 genome editing and its applications in organoids. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *312*(3), G257–G265. https://doi.org/10.1152/AJPGI.00410.2016

- Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., & Relman, D. A. (2005). Diversity

of the Human Intestinal Microbial Flora. *Science (New York, N.Y.)*, *308*(5728), 1635. https://doi.org/10.1126/SCIENCE.1110591

- EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP), Lambré C, Barat Baviera JM, et al. (2023) Re-evaluation of the risks to public health related to the presence of bisphenol A (BPA) in foodstuffs. *EFSA J*. 2023;21(4):e06857. doi:10.2903/j.efsa.2023.6857

- Eissa, N., Hussein, H., Hendy, G. N., Bernstein, C. N., & Ghia, J. E. (2018). Chromogranin-A and its derived peptides and their pharmacological effects during intestinal inflammation. *Biochemical Pharmacology*, *152*, 315–326. https://doi.org/10.1016/J.BCP.2018.04.009

- Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology*, *35*(4), 495–516. https://doi.org/10.1080/01926230701320337

- Elphick, D. A., & Mahida, Y. R. (2005). Paneth cells: their role in innate immunity and inflammatory disease. *Gut*, *54*(12), 1802–1809. https://doi.org/10.1136/GUT.2005.068601

- European Food Safety Autority (2023): Bisphenol A | EFSA (europa.eu)

- Fantinato, V., Ramalho Camargo B., H., Lúcia, A., & Pilleggi De Sousa, O. (2019). Probiotics study with Streptococcus salivarius and its ability to produce bacteriocins and adherence to KB cells. *Revista de Odontologia Da UNESP*, *48*, 20190029. https://doi.org/10.1590/1807-2577.02919

- Farin, H. F., Van Es, J. H., & Clevers, H. (2012). Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells. *Gastroenterology*, *143*(6). https://doi.org/10.1053/J.GASTRO.2012.08.031

- Feng, L., Chen, S., Zhang, L., Qu, W., & Chen, Z. (2019). Bisphenol A increases intestinal permeability through disrupting intestinal barrier function in mice. *Environmental Pollution*, *254*, 112960. https://doi.org/10.1016/J.ENVPOL.2019.112960

- Fothergill, L. J., & Furness, J. B. (2018). Diversity of enteroendocrine cells investigated at cellular and subcellular levels: the need for a new classification scheme. *Histochemistry and Cell Biology*, *150*(6), 693–702. https://doi.org/10.1007/S00418-018-1746-X

- Gajda, A. M., & Storch, J. (2015). Enterocyte Fatty Acid Binding Proteins (FABPs): Different Functions of Liver- and Intestinal- FABPs in the Intestine. *Prostaglandins, Leukotrienes, and Essential Fatty Acids*, *93*, 9. https://doi.org/10.1016/J.PLEFA.2014.10.001

- Gálvez-Ontiveros, Y., Páez, S., Monteagudo, C., & Rivas, A. (2020). Endocrine Disruptors in Food: Impact on Gut Microbiota and Metabolic Diseases. *Nutrients*, *12*(4). https://doi.org/10.3390/NU12041158

- Geiser, J., Venken, K. J. T., de Lisle, R. C., & Andrews, G. K. (2012). A mouse model of acrodermatitis enteropathica: loss of intestine zinc transporter ZIP4 (Slc39a4) disrupts the stem cell niche and intestine integrity. *PLoS Genetics*, *8*(6). https://doi.org/10.1371/JOURNAL.PGEN.1002766

- Gerdes, J., Schwab, U., Lemke, H., & Stein, H. (1983). Production of a mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *International Journal of Cancer*, *31*(1), 13–20. https://doi.org/10.1002/IJC.2910310104

- Ghaleb, A. M., & Yang, V. W. (2017). Krüppel-like factor 4 (KLF4): What we currently know. *Gene*, *611*, 27. https://doi.org/10.1016/J.GENE.2017.02.025

- Giommi, C., Habibi, H. R., Candelma, M., Carnevali, O., & Maradonna, F. (2021). Probiotic Administration Mitigates Bisphenol A Reproductive Toxicity in Zebrafish. *International Journal of Molecular Sciences 2021, Vol. 22, Page 9314*, *22*(17), 9314. https://doi.org/10.3390/IJMS22179314

- Giommi, C., Lombó, M., Habibi, H. R., Rossi, G., Basili, D., Mangiaterra, S., Ladisa, C., Chemello, G., Carnevali, O., & Maradonna, F. (2024). The probiotic SLAB51 as agent to counteract BPA toxicity on zebrafish gut microbiota -liver-brain axis. *The Science of the Total Environment*, *912*. https://doi.org/10.1016/J.SCITOTENV.2023.169303

- Grom, L. C., Coutinho, N. M., Guimarães, J. T., Balthazar, C. F., Silva, R., Rocha, R. S., Freitas, M. Q., Duarte, M. C. K. H., Pimentel, T. C., Esmerino, E. A., Silva, M. C., & Cruz, A. G. (2020). Probiotic dairy foods and postprandial glycemia: A mini-review. *Trends in Food Science & Technology*, *101*, 165–171. https://doi.org/10.1016/J.TIFS.2020.05.012

- Guarner, F., & Malagelada, J. R. (2003). Gut flora in health and disease. *Lancet (London, England)*, *361*(9356), 512–519. https://doi.org/10.1016/S0140-6736(03)12489-0

- Gunawardene, A. R., Corfe, B. M., & Staton, C. A. (2011). Classification and functions of enteroendocrine cells of the lower gastrointestinal tract. *International Journal of Experimental Pathology*, *92*(4), 219–231. https://doi.org/10.1111/J.1365-2613.2011.00767.X

- Hampl, R., & Starka, L. (2020). Endocrine disruptors and gut microbiome interactions. *Physiological Research*, *69*(Suppl 2), S211–S223. https://doi.org/10.33549/PHYSIOLRES.934513

- Haramis, A. P. G., Begthel, H., Van Den Born, M., Van Es, J., Jonkheer, S., Offerhaus, G. J. A., & Clevers, H. (2004). De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science (New York, N.Y.)*, *303*(5664), 1684–1686. https://doi.org/10.1126/SCIENCE.1093587

- Harte, A. L., Da Silva, N. F., Creely, S. J., McGee, K. C., Billyard, T., Youssef-Elabd, E. M., Tripathi, G., Ashour, E., Abdalla, M. S., Sharada, H. M., Amin, A. I., Burt, A. D., Kumar, S., Day, C. P., & McTernan, P. G. (2010). Elevated endotoxin levels in non-alcoholic fatty liver disease. *Journal of Inflammation (London, England)*, *7*. https://doi.org/10.1186/1476-9255-7-15

- Heintz-Buschart, A., & Wilmes, P. (2018). Human Gut Microbiome: Function Matters. *Trends in Microbiology*, *26*(7), 563–574. https://doi.org/10.1016/J.TIM.2017.11.002

- Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., &

Sanders, M. E. (2014). Expert consensus document. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews. Gastroenterology & Hepatology*, *11*(8), 506–514. https://doi.org/10.1038/NRGASTRO.2014.66

- Hoffmann, W. (2020). Trefoil Factor Family (TFF) Peptides and Their Diverse Molecular Functions in Mucus Barrier Protection and More: Changing the Paradigm. *International Journal of Molecular Sciences*, *21*(12), 1–20. https://doi.org/10.3390/IJMS21124535

- Hrncir, T. (2022). Gut Microbiota Dysbiosis: Triggers, Consequences, Diagnostic and Therapeutic Options. *Microorganisms 2022, Vol. 10, Page 578*, *10*(3), 578. https://doi.org/10.3390/MICROORGANISMS10030578

- Hu, C., Liu, M., Tang, L., Liu, H., Sun, B., & Chen, L. (2021). Probiotic intervention mitigates the metabolic disturbances of perfluorobutanesulfonate along the gut-liver axis of zebrafish. *Chemosphere*, *284*, 131374. https://doi.org/10.1016/J.CHEMOSPHERE.2021.131374

- Hu, J., Raikhel, V., Gopalakrishnan, K., Fernandez-Hernandez, H., Lambertini, L., Manservisi, F., Falcioni, L., Bua, L., Belpoggi, F.,

Teitelbaum, S. L., & Chen, J. (2016). Effect of postnatal low-dose exposure to environmental chemicals on the gut microbiome in a rodent model. *Microbiome*, *4*(1). https://doi.org/10.1186/S40168-016-0173-2

- Huang, J. Q., Zhou, J. C., Wu, Y. Y., Ren, F. Z., & Lei, X. G. (2018). Role of glutathione peroxidase 1 in glucose and lipid metabolism-related diseases. *Free Radical Biology & Medicine*, *127*, 108. https://doi.org/10.1016/J.FREERADBIOMED.2018.05.077

- Huang, W., Ai, W., Lin, W., Fang, F., Wang, X., Huang, H., Dahlgren, R. A., & Wang, H. (2020). Identification of receptors for eight endocrine disrupting chemicals and their underlying mechanisms using zebrafish as a model organism. *Ecotoxicology and Environmental Safety*, *204*. https://doi.org/10.1016/J.ECOENV.2020.111068

- Huurre, A., Kalliomäki, M., Rautava, S., Rinne, M., Salminen, S., & Isolauri, E. (2008). Mode of delivery - effects on gut microbiota and humoral immunity. *Neonatology*, *93*(4), 236–240. https://doi.org/10.1159/000111102

- Jach, D., Cheng, Y., Prica, F., Dumartin, L., & Crnogorac-Jurcevic, T. (2021). From development to cancer - an ever-increasing role of AGR2.

American Journal of Cancer Research, *11*(11), 5249. /pmc/articles/PMC8640830/

- Jee, J. H., Lee, D. H., Ko, J., Hahn, S., Jeong, S. Y., Kim, H. K., Park, E., Choi, S. Y., Jeong, S., Lee, J. W., Cho, H. J., Kwon, M. S., & Yoo, J. (2019). Development of Collagen-Based 3D Matrix for Gastrointestinal Tract-Derived Organoid Culture. *Stem Cells International*, *2019*. https://doi.org/10.1155/2019/8472712

- Jiménez, E., Marín, M. L., Martín, R., Odriozola, J. M., Olivares, M., Xaus, J., Fernández, L., & Rodríguez, J. M. (2008). Is meconium from healthy newborns actually sterile? *Research in Microbiology*, *159*(3), 187–193. https://doi.org/10.1016/J.RESMIC.2007.12.007

- Johansson, M. E. V., Phillipson, M., Petersson, J., Velcich, A., Holm, L., & Hansson, G. C. (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(39), 15064–15069. https://doi.org/10.1073/PNAS.0803124105

- Jun, J. H., Oh, J. E., Shim, J. K., Kwak, Y. L., & Cho, J. S. (2021). Effects of bisphenol A on the proliferation, migration, and tumor growth of colon cancer cells: *In vitro* and *in vivo* evaluation with mechanistic insights

related to ERK and 5-HT3. *Food and Chemical Toxicology*, *158*, 112662. https://doi.org/10.1016/J.FCT.2021.112662

- Kabat, A. M., Srinivasan, N., & Maloy, K. J. (2014). Modulation of immune development and function by intestinal microbiota. *Trends in Immunology*, *35*(11), 507–517. https://doi.org/10.1016/J.IT.2014.07.010

- Kaliannan, K., Robertson, R. C., Murphy, K., Stanton, C., Kang, C., Wang, B., Hao, L., Bhan, A. K., & Kang, J. X. (2018). Estrogen-mediated gut microbiome alterations influence sexual dimorphism in metabolic syndrome in mice. *Microbiome*, *6*(1). https://doi.org/10.1186/S40168-018-0587-0

- Kantiani, L., Llorca, M., Sanchís, J., Farré, M., & Barceló, D. (2010). Emerging food contaminants: a review. *Analytical and Bioanalytical Chemistry*, *398*(6), 2413–2427. https://doi.org/10.1007/S00216-010-3944-9

- Kim, J., Koo, B. K., & Knoblich, J. A. (2020). Human organoids: model systems for human biology and medicine. *Nature Reviews. Molecular Cell Biology*, *21*(10), 571–584. https://doi.org/10.1038/S41580-020-0259-3

- Kip, A. M., Soons, Z., Mohren, R., Duivenvoorden, A. A. M., Röth, A. A. J., Cillero-Pastor, B., Neumann, U. P., Dejong, C. H. C., Heeren, R. M. A.,

Olde Damink, S. W. M., & Lenaerts, K. (2021). Proteomics analysis of human intestinal organoids during hypoxia and reoxygenation as a model to study ischemia-reperfusion injury. *Cell Death & Disease*, *12*(1). https://doi.org/10.1038/S41419-020-03379-9

- Kivelä, A. J., Kivelä, J., Saarnio, J., & Parkkila, S. (2005). Carbonic anhydrases in normal gastrointestinal tract and gastrointestinal tumours. *World Journal of Gastroenterology : WJG*, *11*(2), 155. https://doi.org/10.3748/WJG.V11.I2.155

- Koo, B. K., Stange, D. E., Sato, T., Karthaus, W., Farin, H. F., Huch, M., Van Es, J. H., & Clevers, H. (2011). Controlled gene expression in primary Lgr5 organoid cultures. *Nature Methods*, *9*(1), 81–83. https://doi.org/10.1038/NMETH.1802

- Korinek, V., Barker, N., Moerer, P., Van Donselaar, E., Huls, G., Peters, P. J., & Clevers, H. (1998). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nature Genetics*, *19*(4), 379–383. https://doi.org/10.1038/1270

- Kraehenbuhl, J. P., & Neutra, M. R. (2000). Epithelial M cells: differentiation and function. *Annual Review of Cell and Developmental*

Biology, *16*, 301–332. https://doi.org/10.1146/ANNUREV.CELLBIO.16.1.301

- Kumar, T., Pandey, R., & Chauhan, N. S. (2020). Hypoxia Inducible Factor-1α: The Curator of Gut Homeostasis. *Frontiers in Cellular and Infection Microbiology*, *10*, 227. https://doi.org/10.3389/FCIMB.2020.00227

- Lai, K. P., Chung, Y. T., Li, R., Wan, H. T., & Wong, C. K. C. (2016). Bisphenol A alters gut microbiome: Comparative metagenomics analysis. *Environmental Pollution (Barking, Essex : 1987)*, *218*, 923–930. https://doi.org/10.1016/J.ENVPOL.2016.08.039

- Lancaster, M. A., & Knoblich, J. A. (2014). Organogenesis in a dish: modeling development and disease using organoid technologies. *Science (New York, N.Y.)*, *345*(6194). https://doi.org/10.1126/SCIENCE.1247125

- Larsen, N., Vogensen, F. K., Van Den Berg, F. W. J., Nielsen, D. S., Andreasen, A. S., Pedersen, B. K., Al-Soud, W. A., Sørensen, S. J., Hansen, L. H., & Jakobsen, M. (2010). Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PloS One*, *5*(2). https://doi.org/10.1371/JOURNAL.PONE.0009085

- Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K. M., & He, D. (2018). Microplastic particles cause intestinal damage and

other adverse effects in zebrafish Danio rerio and nematode Caenorhabditis elegans. *Science of The Total Environment*, *619–620*, 1–8. https://doi.org/10.1016/J.SCITOTENV.2017.11.103

- Lindeboom, R. G., van Voorthuijsen, L., Oost, K. C., Rodríguez-Colman, M. J., Luna-Velez, M. V, Furlan, C., Baraille, F., Jansen, P. W., Ribeiro, A., Burgering, B. M., Snippert, H. J., & Vermeulen, M. (2018). Integrative multi-omics analysis of intestinal organoid differentiation. *Molecular Systems Biology*, *14*(6). https://doi.org/10.15252/MSB.20188227

- Liu, H., Wang, J., Mou, D., Che, L., Fang, Z., Feng, B., Lin, Y., Xu, S., Li, J., & Wu, D. (2017). Maternal Methyl Donor Supplementation during Gestation Counteracts the Bisphenol A-Induced Impairment of Intestinal Morphology, Disaccharidase Activity, and Nutrient Transporters Gene Expression in Newborn and Weaning Pigs. *Nutrients 2017, Vol. 9, Page 423*, *9*(5), 423. https://doi.org/10.3390/NU9050423

- Liu, H., Guo, D., Kong, Y., Rui, Q., & Wang, D. (2019). Damage on functional state of intestinal barrier by microgravity stress in nematode Caenorhabditis elegans. *Ecotoxicology and Environmental Safety*, *183*, 109554. https://doi.org/10.1016/J.ECOENV.2019.109554

- Liu, H., Zhou, Y., Li, Y., & Gong, Z. (2023). Important roles of Hif1a in maternal or adult BPA exposure induced pancreatic injuries. *Scientific Reports*, *13*(1). https://doi.org/10.1038/S41598-023-38614-8

- Liu, M., Song, S., Hu, C., Tang, L., Lam, J. C. W., Lam, P. K. S., & Chen, L. (2020). Dietary administration of probiotic Lactobacillus rhamnosus modulates the neurological toxicities of perfluorobutanesulfonate in zebrafish. *Environmental Pollution*, *265*, 114832. https://doi.org/10.1016/J.ENVPOL.2020.114832

- Liu, M., Tang, L., Hu, C., Huang, Z., Sun, B., Lam, J. C. W., Lam, P. K. S., & Chen, L. (2021). Antagonistic interaction between perfluorobutanesulfonate and probiotic on lipid and glucose metabolisms in the liver of zebrafish. *Aquatic Toxicology*, *237*, 105897. https://doi.org/10.1016/J.AQUATOX.2021.105897

- Liu, R., Cai, D., Li, X., Liu, B., Chen, J., Jiang, X., Li, H., Li, Z., Teerds, K., Sun, J., Bai, W., & Jin, Y. (2022). Effects of Bisphenol A on reproductive toxicity and gut microbiota dysbiosis in male rats. *Ecotoxicology and Environmental Safety*, *239*, 113623. https://doi.org/10.1016/J.ECOENV.2022.113623

- Liu, Z., Liu, M., Wang, H., Qin, P., Di, Y., Jiang, S., Li, Y., Huang, L., Jiao, N., & Yang, W. (2024). Glutamine attenuates bisphenol A-induced intestinal inflammation by regulating gut microbiota and TLR4-p38/MAPK-NF-κB pathway in piglets. *Ecotoxicology and Environmental Safety*, *270*, 115836. https://doi.org/10.1016/J.ECOENV.2023.115836

- Lombardi, F., Augello, F. R., Palumbo, P., Bonfili, L., Artone, S., Altamura, S., Sheldon, J. M., Latella, G., Cifone, M. G., Eleuteri, A. M., & Cinque, B. (2023). Bacterial Lysate from the Multi-Strain Probiotic SLAB51 Triggers Adaptative Responses to Hypoxia in Human Caco-2 Intestinal Epithelial Cells under Normoxic Conditions and Attenuates LPS-Induced Inflammatory Response. *International Journal of Molecular Sciences*, *24*(9). https://doi.org/10.3390/IJMS24098134/S1

- Lopez-Rodriguez, D., Franssen, D., Bakker, J., Lomniczi, A., & Parent, A. S. (2021). Cellular and molecular features of EDC exposure: consequences for the GnRH network. *Nature Reviews. Endocrinology*, *17*(2), 83–96. https://doi.org/10.1038/S41574-020-00436-3

- Lozupone, C. A., Stombaugh, J. I., Gordon, J. I., Jansson, J. K., & Knight, R. (2012). Diversity, stability and resilience of the human gut microbiota. *Nature*, *489*(7415), 220–230. https://doi.org/10.1038/NATURE11550

- Luo, D., Tang, X., Wang, Y., Ying, S., He, Y., Lin, H., Khoso, P. A., & Li, S. (2024). Selenium deficiency exacerbated Bisphenol A-induced intestinal toxicity in chickens: Apoptosis and cell cycle arrest mediated by ROS/P53. *Science of The Total Environment*, *913*, 169730. https://doi.org/10.1016/J.SCITOTENV.2023.169730

- Ma, X. Y., Son, Y. H., Yoo, J. W., Joo, M. K., & Kim, D. H. (2022). Citation: Tight Junction Protein Expression-Inducing Probiotics Alleviate TNBS-Induced Cognitive Impairment with Colitis in Mice. *Nutrients*, *14*(14). https://doi.org/10.3390/NU14142975/S1

- Macfarlane, S., & Macfarlane, G. T. (2003). Regulation of short-chain fatty acid production. *The Proceedings of the Nutrition Society*, *62*(1), 67–72. https://doi.org/10.1079/PNS2002207

- Mackie, R. I., Sghir, A., & Gaskins, H. R. (1999). Developmental microbial ecology of the neonatal gastrointestinal tract. *The American Journal of Clinical Nutrition*, *69*(5). https://doi.org/10.1093/AJCN/69.5.1035S

- Mahe, M. M., Aihara, E., Schumacher, M. A., Zavros, Y., Montrose, M. H., Helmrath, M. A., Sato, T., & Shroyer, N. F. (2013). Establishment of Gastrointestinal Epithelial Organoids. *Current Protocols in Mouse Biology*, *3*(4), 217–240. https://doi.org/10.1002/9780470942390.MO130179

- Malaisé, Y., Ménard, S., Cartier, C., Lencina, C., Sommer, C., Gaultier, E., Houdeau, E., & Guzylack-Piriou, L. (2018). Consequences of bisphenol a perinatal exposure on immune responses and gut barrier function in mice. *Archives of Toxicology*, *92*(1), 347–358. https://doi.org/10.1007/S00204-017-2038-2/FIGURES/6

- Manning, B. D., & Cantley, L. C. (2007). AKT/PKB signaling navigating downstream. *Cell*, *129*(7), 1261–1274. https://doi.org/10.1016/J.CELL.2007.06.009

- Manzoor MF, Tariq T, Fatima B, et al. An insight into bisphenol A, food exposure and its adverse effects on health: A review. *Front Nutr*. 2022;9:1047827. Published 2022 Nov 3. doi:10.3389/fnut.2022.1047827

- Maradonna, F., & Carnevali, O. (2018). Lipid Metabolism Alteration by Endocrine Disruptors in Animal Models: An Overview. *Frontiers in Endocrinology*, *9*. https://doi.org/10.3389/FENDO.2018.00654

- Massier, L., Chakaroun, R., Tabei, S., Crane, A., Didt, K. D., Fallmann, J., Von Bergen, M., Haange, S. B., Heyne, H., Stumvoll, M., Gericke, M., Dietrich, A., Blüher, M., Musat, N., & Kovacs, P. (2020). Adipose tissue derived bacteria are associated with inflammation in obesity and type 2

diabetes. *Gut*, *69*(10), 1796–1806. https://doi.org/10.1136/GUTJNL-2019-320118

- Moens, F., Van den Abbeele, P., Basit, A. W., Dodoo, C., Chatterjee, R., Smith, B., & Gaisford, S. (2019). A four-strain probiotic exerts positive immunomodulatory effects by enhancing colonic butyrate production *in vitro*. *International Journal of Pharmaceutics*, *555*, 1–10. https://doi.org/10.1016/J.IJPHARM.2018.11.020

- Mohajeri, M. H., Brummer, R. J. M., Rastall, R. A., Weersma, R. K., Harmsen, H. J. M., Faas, M., & Eggersdorfer, M. (2018). The role of the microbiome for human health: from basic science to clinical applications. *European Journal of Nutrition*, *57*(Suppl 1), 1. https://doi.org/10.1007/S00394-018-1703-4

- Moll, R., Zimbelmann, R., Goldschmidt, M. D., Keith, M., Laufer, J., Kasper, M., Koch, P. J., & Franke, W. W. (1993). The human gene encoding cytokeratin 20 and its expression during fetal development and in gastrointestinal carcinomas. *Differentiation; Research in Biological Diversity*, *53*(2), 75–93. https://doi.org/10.1111/J.1432-0436.1993.TB00648.X

- Mörbe, U. M., Jørgensen, P. B., Fenton, T. M., von Burg, N., Riis, L. B., Spencer, J., & Agace, W. W. (2021). Human gut-associated lymphoid tissues (GALT); diversity, structure, and function. *Mucosal Immunology*, *14*(4), 793–802. https://doi.org/10.1038/S41385-021-00389-4

- Mowat, A. M., & Agace, W. W. (2014). Regional specialization within the intestinal immune system. *Nature Reviews. Immunology*, *14*(10), 667–685. https://doi.org/10.1038/NRI3738

- Moya-Pérez, A., Neef, A., & Sanz, Y. (2015). Bifidobacterium pseudocatenulatum CECT 7765 Reduces Obesity-Associated Inflammation by Restoring the Lymphocyte-Macrophage Balance and Gut Microbiota Structure in High-Fat Diet-Fed Mice. *PLoS ONE*, *10*(7). https://doi.org/10.1371/JOURNAL.PONE.0126976

- Nakano, T., Inoue, I., Alpers, D. H., Akiba, Y., Katayama, S., Shinozaki, R., Kaunitz, J. D., Ohshima, S., Akita, M., Takahashi, S., Koyama, I., Matsushita, M., & Komoda, T. (2009). Role of lysophosphatidylcholine in brush-border intestinal alkaline phosphatase release and restoration. *American Journal of Physiology - Gastrointestinal and Liver Physiology*, *297*(1), G207. https://doi.org/10.1152/AJPGI.90590.2008

- Neutra, M. R., Pringault, E., & Kraehenbuhl, J. P. (1996). Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annual Review of Immunology*, *14*, 275–300. https://doi.org/10.1146/ANNUREV.IMMUNOL.14.1.275

- Ni, Y., Hu, L., Yang, S., Ni, L., Ma, L., Zhao, Y., Zheng, A., Jin, Y., & Fu, Z. (2021). Bisphenol A impairs cognitive function and 5-HT metabolism in adult male mice by modulating the microbiota-gut-brain axis. *Chemosphere*, *282*, 130952. https://doi.org/10.1016/J.CHEMOSPHERE.2021.130952

- Norkin, M., Ordóñez-Morán, P., & Huelsken, J. (2021). High-content, targeted RNA-seq screening in organoids for drug discovery in colorectal cancer. *Cell Reports*, *35*(3), 109026. https://doi.org/10.1016/J.CELREP.2021.109026

- Oishi, K., Sato, T., Yokoi, W., Yoshida, Y., Ito, M., & Sawada, H. (2008). Effect of probiotics, Bifidobacterium breve and Lactobacillus casei, on bisphenol A exposure in rats. *Bioscience, Biotechnology, and Biochemistry*, *72*(6), 1409–1415. https://doi.org/10.1271/BBB.70672

- Okkelman, I. A., Foley, T., Papkovsky, D. B., & Dmitriev, R. I. (2017). Live cell imaging of mouse intestinal organoids reveals heterogeneity in their

oxygenation. *Biomaterials*, *146*, 86–96. https://doi.org/10.1016/J.BIOMATERIALS.2017.08.043

- Onozato, D., Ogawa, I., Kida, Y., Mizuno, S., Hashita, T., Iwao, T., & Matsunaga, T. (2021). Generation of Budding-Like Intestinal Organoids from Human Induced Pluripotent Stem Cells. *Journal of Pharmaceutical Sciences*, *110*(7), 2637–2650. https://doi.org/10.1016/J.XPHS.2021.03.014

- Ostaff, M. J., Stange, E. F., & Wehkamp, J. (2013). Antimicrobial peptides and gut microbiota in homeostasis and pathology. *EMBO Molecular Medicine*, *5*(10), 1465–1483. https://doi.org/10.1002/EMMM.201201773

- Paone, P., & Cani, P. D. (2020). Mucus barrier, mucins and gut microbiota: the expected slimy partners? *Gut*, *69*(12), 2232–2243. https://doi.org/10.1136/GUTJNL-2020-322260

- Patel, A. B., Shaikh, S., Jain, K. R., Desai, C., & Madamwar, D. (2020). Polycyclic Aromatic Hydrocarbons: Sources, Toxicity, and Remediation Approaches. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/FMICB.2020.562813

- Pelaseyed, T., Bergström, J. H., Gustafsson, J. K., Ermund, A., Birchenough, G. M. H., Schütte, A., van der Post, S., Svensson, F., Rodríguez-Piñeiro, A. M., Nyström, E. E. L., Wising, C., Johansson, M. E.

V., & Hansson, G. C. (2014). The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunological Reviews*, *260*(1), 8–20. https://doi.org/10.1111/IMR.12182

- Plaza-Diaz, J., Ruiz-Ojeda, F. J., Gil-Campos, M., & Gil, A. (2019). Mechanisms of Action of Probiotics. *Advances in Nutrition (Bethesda, Md.)*, *10*(suppl_1), S49–S66. https://doi.org/10.1093/ADVANCES/NMY063

- Qu, W., Zhao, Z., Chen, S., Zhang, L., Wu, D., & Chen, Z. (2018). Bisphenol A suppresses proliferation and induces apoptosis in colonic epithelial cells through mitochondrial and MAPK/AKT pathways. *Life Sciences*, *208*, 167–174. https://doi.org/10.1016/J.LFS.2018.07.040

- Ratanasirintrawoot S, Israsena N. Stem Cells in the Intestine: Possible Roles in Pathogenesis of Irritable Bowel Syndrome. *J Neurogastroenterol Motil*. 2016;22(3):367-382. doi:10.5056/jnm16023

- Rawat, M., Nighot, M., Al-Sadi, R., Gupta, Y., Viszwapriya, D., Yochum, G., Koltun, W., & Ma, T. Y. (2020). IL1B Increases Intestinal Tight Junction Permeability by Up-regulation of MIR200C-3p, Which Degrades Occludin

mRNA. *Gastroenterology*, *159*(4), 1375–1389. https://doi.org/10.1053/J.GASTRO.2020.06.038

- Reddivari, L., Veeramachaneni, D. N. R., Walters, W. A., Lozupone, C., Palmer, J., Hewage, M. K. K., Bhatnagar, R., Amir, A., Kennett, M. J., Knight, R., & Vanamala, J. K. P. (2017). Perinatal Bisphenol A Exposure Induces Chronic Inflammation in Rabbit Offspring via Modulation of Gut Bacteria and Their Metabolites. *MSystems*, *2*(5), 93–110. https://doi.org/10.1128/MSYSTEMS.00093-17

- Rehfeld, J. F. (2004). A centenary of gastrointestinal endocrinology. *Hormone and Metabolic Research = Hormon- Und Stoffwechselforschung = Hormones et Metabolisme*, *36*(11–12), 735–741. https://doi.org/10.1055/S-2004-826154

- Repossi, A., Farabegoli, F., Gazzotti, T., Zironi, E., & Pagliuca, G. (2016). Bisphenol A in Edible Part of Seafood. *Italian Journal of Food Safety*, *5*(2), 98–105. https://doi.org/10.4081/IJFS.2016.5666

- Rooks, M. G., & Garrett, W. S. (2016). Gut microbiota, metabolites and host immunity. *Nature Reviews. Immunology*, *16*(6), 341–352. https://doi.org/10.1038/NRI.2016.42

- Rossi, G., Pengo, G., Galosi, L., Berardi, S., Tambella, A. M., Attili, A. R., Gavazza, A., Cerquetella, M., Jergens, A. E., Guard, B. C., Lidbury, J. A., Stainer, J. M., Crovace, A. M., & Suchodolski, J. S. (2020). Effects of the Probiotic Mixture Slab51® (SivoMixx®) as Food Supplement in Healthy Dogs: Evaluation of Fecal Microbiota, Clinical Parameters and Immune Function. *Frontiers in Veterinary Science*, *7*. https://doi.org/10.3389/FVETS.2020.00613

- Ruff, W. E., Greiling, T. M., & Kriegel, M. A. (2020). Host-microbiota interactions in immune-mediated diseases. *Nature Reviews. Microbiology*, *18*(9), 521–538. https://doi.org/10.1038/S41579-020-0367-2

- Sailaja BS, He XC, Li L. The regulatory niche of intestinal stem cells. *J Physiol*. 2016;594(17):4827-4836. doi:10.1113/JP271931

- Salvucci, E. (2019). The human-microbiome superorganism and its modulation to restore health. *International Journal of Food Sciences and Nutrition*, *70*(7), 781–795. https://doi.org/10.1080/09637486.2019.1580682

- Santangeli, S., Maradonna, F., Gioacchini, G. *et al.* BPA-Induced Deregulation Of Epigenetic Patterns: Effects On Female Zebrafish Reproduction. *Sci Rep* 6, 21982 (2016). https://doi.org/10.1038/srep21982

- Santangeli, S., Consales, C., Pacchierotti, F., Habibi, H.R., Carnevali, O. (2019) Transgenerational effects of BPA on female reproduction. The Science of the total environment. 685:1294-1305. https://doi.org/10.1016/j.scitotenv.2019.06.029

- Sato, T., Vries, R. G., Snippert, H. J., Van De Wetering, M., Barker, N., Stange, D. E., Van Es, J. H., Abo, A., Kujala, P., Peters, P. J., & Clevers, H. (2009). Single Lgr5 stem cells build crypt-villus structures *in vitro* without a mesenchymal niche. *Nature*, *459*(7244), 262–265. https://doi.org/10.1038/NATURE07935

- Sato, T., Van Es, J. H., Snippert, H. J., Stange, D. E., Vries, R. G., Van Den Born, M., Barker, N., Shroyer, N. F., Van De Wetering, M., & Clevers, H. (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature*, *469*(7330), 415–418. https://doi.org/10.1038/NATURE09637

- Schug, T. T., Janesick, A., Blumberg, B., & Heindel, J. J. (2011). Endocrine disrupting chemicals and disease susceptibility. *The Journal of Steroid Biochemistry and Molecular Biology*, *127*(3–5), 204–215. https://doi.org/10.1016/J.JSBMB.2011.08.007

- Schug, T. T., Johnson, A. F., Birnbaum, L. S., Colborn, T., Guillette, L. J., Crews, D. P., Collins, T., Soto, A. M., Vom Saal, F. S., McLachlan, J. A., Sonnenschein, C., & Heindel, J. J. (2016). Minireview: Endocrine Disruptors: Past Lessons and Future Directions. *Molecular Endocrinology*, *30*(8), 833. https://doi.org/10.1210/ME.2016-1096

- Schutgens, F., & Clevers, H. (2020). Human Organoids: Tools for Understanding Biology and Treating Diseases. *Annual Review of Pathology: Mechanisms of Disease*, *15*(Volume 15, 2020), 211–234. https://doi.org/10.1146/ANNUREV-PATHMECHDIS-012419-032611/CITE/REFWORKS

- Schwank, G., Koo, B. K., Sasselli, V., Dekkers, J. F., Heo, I., Demircan, T., Sasaki, N., Boymans, S., Cuppen, E., Van Der Ent, C. K., Nieuwenhuis, E. E. S., Beekman, J. M., & Clevers, H. (2013). Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell*, *13*(6), 653–658. https://doi.org/10.1016/J.STEM.2013.11.002

- Schwank, G., & Clevers, H. (2016). CRISPR/Cas9-Mediated Genome Editing of Mouse Small Intestinal Organoids. *Methods in Molecular*

Biology (Clifton, N.J.), *1422*, 3–11. https://doi.org/10.1007/978-1-4939-3603-8_1

- Sekirov, I., Russell, S. L., Caetano M Antunes, L., & Finlay, B. B. (2010). Gut microbiota in health and disease. *Physiological Reviews*, *90*(3), 859–904. https://doi.org/10.1152/PHYSREV.00045.2009

- Semenza, G. L. (2011). Oxygen sensing, homeostasis, and disease. *The New England Journal of Medicine*, *365*(6), 537–547. https://doi.org/10.1056/NEJMRA1011165

- Seth A, Yan F, Polk DB, Rao RK. Probiotics ameliorate the hydrogen peroxide-induced epithelial barrier disruption by a PKC- and MAP kinase-dependent mechanism. *Am J Physiol Gastrointest Liver Physiol*. 2008;294(4):G1060-G1069. doi:10.1152/ajpgi.00202.2007

- Sevim, Ç., & Kara, M. (2021). Can probiotics win the battle against environmental endocrine disruptors? *Arhiv Za Farmaciju*, *71*(6), 565–580. https://doi.org/10.5937/ARHFARM71-34237

- Shi, L., Pan, R., Lin, G., Liang, X., Zhao, J., Zhang, H., Chen, W., & Wang, G. (2021). Lactic acid bacteria alleviate liver damage caused by perfluorooctanoic acid exposure via antioxidant capacity, biosorption

capacity and gut microbiota regulation. *Ecotoxicology and Environmental Safety*, *222*. https://doi.org/10.1016/J.ECOENV.2021.112515

- Singh, S. B., & Lin, H. C. (2021). Role of Intestinal Alkaline Phosphatase in Innate Immunity. *Biomolecules*, *11*(12). https://doi.org/10.3390/BIOM11121784

- Singhal, R., & Shah, Y. M. (2020). Oxygen battle in the gut: Hypoxia and hypoxia-inducible factors in metabolic and inflammatory responses in the intestine. *The Journal of Biological Chemistry*, *295*(30), 10493–10505. https://doi.org/10.1074/JBC.REV120.011188

- Sirakov, M., & Plateroti, M. (2011). The thyroid hormones and their nuclear receptors in the gut: From developmental biology to cancer. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1812*(8), 938–946. https://doi.org/10.1016/J.BBADIS.2010.12.020

- Skinner, M. K. (2016). Endocrine disruptors in 2015: Epigenetic transgenerational inheritance. *Nature Reviews. Endocrinology*, *12*(2), 68. https://doi.org/10.1038/NRENDO.2015.206

- Snoeck, V., Goddeeris, B., & Cox, E. (2005). The role of enterocytes in the intestinal barrier function and antigen uptake. *Microbes and Infection*, *7*(7–8), 997–1004. https://doi.org/10.1016/J.MICINF.2005.04.003

- Stelzner, M., Helmrath, M., Dunn, J. C. Y., Henning, S. J., Houchen, C. W., Kuo, C., Lynch, J., Li, L., Magness, S. T., Martin, M. G., Wong, M. H., & Yu, J. (2012). A nomenclature for intestinal *in vitro* cultures. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *302*(12). https://doi.org/10.1152/AJPGI.00493.2011

- Sternini, C., Anselmi, L., & Rozengurt, E. (2008). Enteroendocrine cells: a site of "taste" in gastrointestinal chemosensing. *Current Opinion in Endocrinology, Diabetes, and Obesity*, *15*(1), 73–78. https://doi.org/10.1097/MED.0B013E3282F43A73

- Strowitzki, M. J., Cummins, E. P., & Taylor, C. T. (2019). Protein Hydroxylation by Hypoxia-Inducible Factor (HIF) Hydroxylases: Unique or Ubiquitous? *Cells*, *8*(5). https://doi.org/10.3390/CELLS8050384

- Sun, X., & Kaufman, P. D. (2018). Ki-67: more than a proliferation marker. *Chromosoma*, *127*(2), 175. https://doi.org/10.1007/S00412-018-0659-8

- Swidsinski, A., Loening-Baucke, V., Lochs, H., & Hale, L. P. (2005). Spatial organization of bacterial flora in normal and inflamed intestine: A fluorescence in situ hybridization study in mice. *World Journal of Gastroenterology : WJG*, *11*(8), 1131. https://doi.org/10.3748/WJG.V11.I8.1131

- Taelman, J., Diaz, M., & Guiu, J. (2022). Human Intestinal Organoids: Promise and Challenge. *Frontiers in Cell and Developmental Biology*, *10*. https://doi.org/10.3389/FCELL.2022.854740

- Tang, X. Y., Wu, S., Wang, D., Chu, C., Hong, Y., Tao, M., Hu, H., Xu, M., Guo, X., & Liu, Y. (2022). Human organoids in basic research and clinical applications. *Signal Transduction and Targeted Therapy*, *7*(1). https://doi.org/10.1038/S41392-022-01024-9

- Teixeira, T. F. S., Souza, N. C. S., Chiarello, P. G., Franceschini, S. C. C., Bressan, J., Ferreira, C. L. L. F., & Peluzio, M. do C. G. (2012). Intestinal permeability parameters in obese patients are correlated with metabolic syndrome risk factors. *Clinical Nutrition*, *31*(5), 735–740. https://doi.org/10.1016/J.CLNU.2012.02.009

- Thorpe, G. W., Reodica, M., Davies, M. J., Heeren, G., Jarolim, S., Pillay, B., Breitenbach, M., Higgins, V. J., & Dawes, I. W. (2013). Superoxide radicals have a protective role during H2O2 stress. *Molecular Biology of the Cell*, *24*(18), 2876–2884. https://doi.org/10.1091/MBC.E13-01-0052

- Valdes, A. M., Walter, J., Segal, E., & Spector, T. D. (2018). Science and Politics of Nutrition: Role of the gut microbiota in nutrition and health. *The BMJ*, *361*, 36–44. https://doi.org/10.1136/BMJ.K2179

- Van Es, J. H., Van Gijn, M. E., Riccio, O., Van Den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D. J., Radtke, F., & Clevers, H. (2005). Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature*, *435*(7044), 959–963. https://doi.org/10.1038/NATURE03659

- Vancamelbeke, M., & Vermeire, S. (2017). The intestinal barrier: a fundamental role in health and disease. *Expert Review of Gastroenterology & Hepatology*, *11*(9), 821–834. https://doi.org/10.1080/17474124.2017.1343143

- Vandenberg, L. N., Maffini, M. V., Sonnenschein, C., Rubin, B. S., & Soto, A. M. (2009). Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. *Endocrine Reviews*, *30*(1), 75–95. https://doi.org/10.1210/ER.2008-0021

- Vandenberg, L. N., Colborn, T., Hayes, T. B., Heindel, J. J., Jacobs, D. R., Lee, D. H., Shioda, T., Soto, A. M., vom Saal, F. S., Welshons, W. V., Zoeller, R. T., & Myers, J. P. (2012). Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses. *Endocrine Reviews*, *33*(3), 378–455. https://doi.org/10.1210/ER.2011-1050

- Velmurugan, G., Ramprasath, T., Gilles, M., Swaminathan, K., & Ramasamy, S. (2017). Gut Microbiota, Endocrine-Disrupting Chemicals, and the Diabetes Epidemic. *Trends in Endocrinology and Metabolism: TEM*, *28*(8), 612–625. https://doi.org/10.1016/J.TEM.2017.05.001

- Vemuri, R., Shankar, E. M., Chieppa, M., Eri, R., & Kavanagh, K. (2020). Beyond Just Bacteria: Functional Biomes in the Gut Ecosystem Including Virome, Mycobiome, Archaeome and Helminths. *Microorganisms*, *8*(4). https://doi.org/10.3390/MICROORGANISMS8040483

- Vidhya A Nair, Shrinithivihahshini N.D., and Sivaramakrishnan S. Effects of BPA on The Gastro Intestinal Track (GIT). Bull. Env. Pharmacol. Life Sci., Vol 12 [8] July 2023: 324-332

- Vyas, U., & Ranganathan, N. (2012). Probiotics, Prebiotics, and Synbiotics: Gut and Beyond. *Gastroenterology Research and Practice*, *2012*, 16. https://doi.org/10.1155/2012/872716

- Wang, K., Qiu, L., Zhu, J., Sun, Q., Qu, W., Yu, Y., Zhao, Z., & Shao, G. (2021). Environmental contaminant BPA causes intestinal damage by disrupting cellular repair and injury homeostasis *in vivo* and *in vitro*. *Biomedicine & Pharmacotherapy*, *137*, 111270. https://doi.org/10.1016/J.BIOPHA.2021.111270

- Wang, Q., Zhou, Y., Rychahou, P., Fan, T. W. M., Lane, A. N., Weiss, H. L., & Evers, B. M. (2016). Ketogenesis contributes to intestinal cell differentiation. *Cell Death & Differentiation 2017 24:3*, *24*(3), 458–468. https://doi.org/10.1038/cdd.2016.142

- Wang, Q., Wang, F., Tang, L., Wang, Y., Zhou, Y., Li, X., Jin, M., Fu, A., & Li, W. (2024). Bacillus amyloliquefaciens SC06 alleviated intestinal damage induced by inflammatory via modulating intestinal microbiota and intestinal stem cell proliferation and differentiation. *International Immunopharmacology*, *130*, 111675. https://doi.org/10.1016/J.INTIMP.2024.111675

- Warner, G. R., & Flaws, J. A. (2018). Bisphenol A and Phthalates: How Environmental Chemicals Are Reshaping Toxicology. *Toxicological Sciences*, *166*(2), 246. https://doi.org/10.1093/TOXSCI/KFY232

- Wong, B. W., Kuchnio, A., Bruning, U., & Carmeliet, P. (2013). Emerging novel functions of the oxygen-sensing prolyl hydroxylase domain enzymes. *Trends in Biochemical Sciences*, *38*(1), 3–11. https://doi.org/10.1016/J.TIBS.2012.10.004

- Wong, V. W. Y., Stange, D. E., Page, M. E., Buczacki, S., Wabik, A., Itami, S., Van De Wetering, M., Poulsom, R., Wright, N. A., Trotter, M. W. B.,

Watt, F. M., Winton, D. J., Clevers, H., & Jensen, K. B. (2012). Lrig1 controls intestinal stem-cell homeostasis by negative regulation of ErbB signalling. *Nature Cell Biology*, *14*(4), 401–408. https://doi.org/10.1038/NCB2464

- Yadav, H., Lee, J. H., Lloyd, J., Walter, P., & Rane, S. G. (2013). Beneficial metabolic effects of a probiotic via butyrate-induced GLP-1 hormone secretion. *The Journal of Biological Chemistry*, *288*(35), 25088–25097. https://doi.org/10.1074/JBC.M113.452516

- Yu, S., Balasubramanian, I., Laubitz, D., Tong, K., Bandyopadhyay, S., Lin, X., Flores, J., Singh, R., Liu, Y., Macazana, C., Zhao, Y., Béguet-Crespel, F., Patil, K., Midura-Kiela, M. T., Wang, D., Yap, G. S., Ferraris, R. P., Wei, Z., Bonder, E. M., … Gao, N. (2020). Paneth cell-derived lysozyme defines the composition of mucolytic microbiota and the inflammatory tone of the intestine. *Immunity*, *53*(2), 398. https://doi.org/10.1016/J.IMMUNI.2020.07.010

- Zachos, N. C., Kovbasnjuk, O., Foulke-Abel, J., In, J., Blutt, S. E., De Jonge, H. R., Estes, M. K., & Donowitz, M. (2016). Human Enteroids/Colonoids and Intestinal Organoids Functionally Recapitulate Normal Intestinal Physiology and Pathophysiology. *The Journal of*

Biological Chemistry, *291*(8), 3759–3766. https://doi.org/10.1074/JBC.R114.635995

- Zang, L., Ma, Y., Huang, W., Ling, Y., Sun, L., Wang, X., Zeng, A., Dahlgren, R. A., Wang, C., & Wang, H. (2019). Dietary Lactobacillus plantarum ST-III alleviates the toxic effects of triclosan on zebrafish (Danio rerio) via gut microbiota modulation. *Fish & Shellfish Immunology*, *84*, 1157–1169. https://doi.org/10.1016/J.FSI.2018.11.007

- Zheng, Y., Zhang, L., Bonfili, L., de Vivo, L., Eleuteri, A. M., & Bellesi, M. (2023). Probiotics Supplementation Attenuates Inflammation and Oxidative Stress Induced by Chronic Sleep Restriction. *Nutrients*, *15*(6). https://doi.org/10.3390/NU15061518/S1

- Zhu, M., Li, Y., Niu, Y., Li, J., & Qin, Z. (2020). Effects of bisphenol A and its alternative bisphenol F on Notch signaling and intestinal development: A novel signaling by which bisphenols disrupt vertebrate development. *Environmental Pollution*, *263*, 114443. https://doi.org/10.1016/J.ENVPOL.2020.114443

- Zhu, M., Wei, R., Li, Y., Li, J., Dong, M., Chen, X., Lv, L., & Qin, Z. (2022). Bisphenol chemicals disturb intestinal homeostasis via Notch/Wnt signaling and induce mucosal barrier dysregulation and inflammation.

Science of The Total Environment, *828*, 154444. https://doi.org/10.1016/J.SCITOTENV.2022.154444

- Zhu, M., Zeng, R., Wu, D., Li, Y., Chen, T., & Wang, A. (2024). Research progress of the effects of bisphenol analogues on the intestine and its underlying mechanisms: A review. *Environmental Research*, *243*, 117891. https://doi.org/10.1016/J.ENVRES.2023.117891

- Zietek, T., Rath, E., Haller, D., & Daniel, H. (2015). Intestinal organoids for assessing nutrient transport, sensing and incretin secretion. *Scientific Reports 2015 5:1*, *5*(1), 1–10. https://doi.org/10.1038/srep16831

SPECIAL THANKS

Con immensa gratitudine, desidero ringraziare tutte le persone che hanno contribuito alla realizzazione di questa tesi e che mi sono state vicine durante questo percorso di crescita personale e professionale.

Un sentito ringraziamento al mio Relatore, la Prof.ssa Oliana Carnevali, per la sua attenta supervisione, la sua guida esperta, la sua disponibilità costante e i preziosi consigli durante la ricerca e la scrittura di questa tesi.

Desidero ringraziare il Dott. Christian Giommi, Co-relatore di questa tesi, per la sua premura e disponibilità nell'avermi affiancato in questo percorso, per il suo incoraggiamento costante, per il suo aiuto e per i suoi preziosi suggerimenti.

Ringrazio il Dott. Jordi Guiu Sagarra per il contributo fornito alla stesura di questa tesi e per la collaborazione nella realizzazione di questo progetto, per il materiale e il supporto fornito nella sua esecuzione.

Il ringraziamento più grande va alla mia Famiglia, ai miei genitori e a mio fratello Antonio. A voi devo tutto, la persona che sono e i traguardi che ho raggiunto. Grazie per i valori che mi avete trasmesso, per aver da sempre creduto in me e per avermi supportato con costanza nel mio percorso.

A papà Michele, che sin da bambina mi ha trasmesso la sua passione per la Scienza e per la Natura, che è stata per me fonte di ispirazione. Grazie per il tuo amore incondizionato. Grazie ai i tuoi insegnamenti, ai tuoi sacrifici e al tuo inesauribile sostegno senza i quali non avrei potuto raggiungere questo traguardo.

A mamma Antonella, ai suoi consigli, alla sua guida. Grazie per essere stata da sempre per me un'amica e confidente su cui poter contare. Grazie per avermi sostenuto e supportato costantemente, soprattutto nei momenti difficili. Grazie per non aver mai dubitato delle mie capacità e per avermi spronato a migliorare e a dare sempre il massimo.

A mio fratello Antonio, la mia opposta metà. Grazie per l'orgoglio e l'affetto nei miei confronti che dimostri con timidezza. Grazie per avermi accompagnato in questo percorso e per avermi regalato piccoli momenti di svago. Grazie per avermi fatto sentire più piccola la distanza da casa. Spero che il tuo impegno e la tua determinazione ti portino a realizzare i tuoi obiettivi.

Un ringraziamento speciale va a Bruno, che mi ha da sempre supportata e sopportata in questo percorso per me così intenso ed impegnativo. Grazie per essermi sempre stato a fianco nelle difficoltà, nei momenti belli e in quelli brutti. Grazie per aver creduto sempre in me e nelle mie potenzialità, grazie

per aver sempre gioito di ogni mio successo, anche piccolo, trasmettendomi costantemente la stima e la fiducia che provi nei miei confronti.

Grazie alle mie amiche, Carmela e Costanza, lontane ma vicine. Mi avete dimostrato che l'amicizia è un valore raro e mi avete insegnato che l'amicizia vera è fatta di sincerità e piccoli gesti, e che non vi è invidia, ma solo sostegno reciproco e il gioire dei successi dell'altra persona come fossero i propri.

Un ringraziamento alle mie compagne di corso, che hanno condiviso con me questo percorso intenso, seppur breve. Grazie per il vostro supporto e per i momenti di confronto e di condivisione che abbiamo vissuto insieme.

Grazie alla Scienza e alla Biologia, che sono per me non solo una passione, ma anche una fonte inesauribile di curiosità e scoperta. Lo studio di queste discipline mi ha trasmesso valori come la curiosità, il rispetto per la vita in ogni sua forma e l'importanza dell'osservazione, della sperimentazione e del continuo apprendimento, principi che porto con me in ogni ambito della mia vita.

Infine, un grazie vorrei dedicarlo a me stessa, per non aver mai permesso ai giudizi altrui di condizionarmi, per aver sempre creduto nelle mie potenzialità e per aver dato il massimo in ogni occasione. Sono grata per il coraggio che ho avuto nel seguire la mia passione per la Biologia, per la mia costanza e forza di

volontà, per la mia voglia di conoscere, di imparare e di mettermi in gioco. La conclusione di questo percorso segna per me un nuovo inizio, che affronterò con la stessa tenacia e determinazione. Sono pronta a intraprendere questa nuova avventura con entusiasmo, fiduciosa che, con il supporto delle persone care, riuscirò a raggiungere nuovi traguardi, a migliorarmi e a crescere, sia professionalmente che personalmente.

Grazie.

Per Aspera ad Astra.