Human Primary Organoid-Derived Epithelial Monolayers as a Novel Strategy for the Study of Adherent Invasive *Escherichia coli* pathogenicity and the effects of Postbiotics on Intestinal Epithelial Function

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Doctoral Thesis

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La flor más bonita de mi jardín
tiene fragancia de jazmín.

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ABBREVIATIONS

(d)-ODMs	(differentiated)-Organoid-Derived Monolayers	**EIEC**	Enteroinvasive *E. coli*
2D	Bidimensional	**EII**	Enfermedad Inflamatoria Intestinal
3D	Tridimensional	**ELISA**	Enzyme-Linked ImmunoSorbent Assay
AIEC	Adherent Invasive *Escherichia coli*	**EMEM**	Minimum Essential Medium – Eagle with Earle's BSS
AMPs	Antimicrobial Peptides	**EPEC**	Enteropathogenic *E. coli*
ATF4	Activation Transcription Factor 4	**EpOC**	Epithelial Organoid Culture
AU	Arbitrary Units	**ETEC**	Enterotoxigenic *E. coli*
BHI	Brain Heart Infusion	**ExPEC**	Extraintestinal Pathogenic *E. coli*
BSA	Bovin Serum Albumin	**FACS**	Fluorescent Activated Cell Sorter
CD	Crohn's Disease	**FBS**	Fetal Bovine Serum
CDEIS	Crohn's Disease Endoscopic Index of Severity	**FC**	Fold Change
cfu	Colony Forming Units	**FDR**	False Discovery Rate
cpm	cycles per minute	**FLA**	Flagellin
CRC	Colorectal Cancer	**GALT**	Gut Associated Lymphoid Tissue
DAEC	Diffusely Adherent *E. coli*	**GI**	Gastrointestinal
DAPI	4',6-diamidino-2-phenylindole	**GM-CSF**	Granulocyte Macrophage Colony-Stimulating Factor
DCs	Dendritic Cells	**GWAS**	Genome Wide Association Studies
DEC	Diarrhoeagenic *E. coli*	**HBSS**	Hank's Balanced Salt Solution
DEG(s)	Differentially Expressed Gene(s)	**HILIC**	Hydrophilic Interaction Liquid Chromatography
DMEM	Dulbecco's Modification of Eagle's Medium	**h**	Hour(s)
DPBS	Dulbecco's Phosphate-Buffered Saline	**HPLC**	High-Performance Liquid Chromatography
DSS	Dextran Sulfate Sodium	**I407**	Intestine 407
DTT	Dithiothreitol	**IBD**	Inflammatory Bowel Disease
EAEC	Enterohemorrhagic *E. coli*	**IECs**	Intestinal Epithelial Cells
EDTA	Ethylene Diamine Tetra-acetic Acid	**IELs**	Intraepithelial Lymphocytes
EHEC	Enteroaggregative *E. coli*	**IFN**	Interferon

IL	Interleukin	**PCR**	Polymerase Chain Reaction
ILC	Innate Lymphoid Cells	**PFA**	Paraformaldehyde
INV-I	Invasion Index	**PRRs**	Pattern Recognition Receptors
IPA	Ingenuity Pathway Analysis	**PSM**	Peptide Spectrum Match
JAK	Janus kinase	**qToF**	quadrupole Time-of-Flight
L/D	Live/Dead	**RNAseq**	RNA sequencing
LB broth	Luria-Bertani broth	**rpm**	revolutions per minute
LC-MS/-MSMS	Liquid Chromatography Mass Spectrometry	**RPMI**	Roswell Park Memorial Institute
LPS	Lipopolysaccharide	**RT**	Room Temperature
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry	**RT-qPCR**	Quantitative Multiplex Real-time PCR
MAMPs	Microbe-Associated Molecular Patterns	**SCFAs**	Short-Chain Fatty Acids
MII	Malaltia Inflamatòria Intestinal	**SEM**	Standard Error of the Mean
Min	Minute(s)	**SN**	Supernatant
MM	Minimal Media	**SNPs**	Single Nucleotide Polymorphisms
MNEC	Meningitis-Associated *E. coli*	**STAT**	Signal Transducer and Activator of Transcription
moDCs	monocyte derived Dendritic Cells	**STEC**	Shiga toxin-producing Escherichia coli
MOI	Multiplicity of Infection	**TEER**	Transepithelial Electrical Resistance
MTT	Thiazolyl blue tetrazolium bromide	**TGF**	Transforming Growth Factor
NAD	Nicotinamide Adenine Dinucleotide	**Th**	T helper
NF-kB	Nuclear Factor kappa B	**TJ**	Tight Junctions
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs	**TJAP**	TJ-Associated Proteins
NTEC	Necrotoxic *E. coli*	**TLR**	Toll-Like Receptors
OD	Optical Density	**TNF**	Tumor Necrosis Factor
OMVs	Outer Membrane Vesicles	**UC**	Ulcerative Colitis
ON	Overnight	**UPEC**	Uropathogenic *E. coli*
PAMPs	Pathogen Associated MolecularPatterns	**UPLC**	Ultra-High Performance Liquid Chromatography
PBMCs	Peripheral Blood Mononuclear Cells	**WM**	Washing Medium
PCA	Principal Component Analysis	**ZO**	Zonula Occludens

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ABSTRACT

Inflammatory bowel disorders – such as Inflammatory Bowel Disease (IBD) – are rising worldwide. A well-known feature of IBD is dysbiosis of the gut microbiota, characterized by a significant reduction of beneficial strains and a sharp increase in facultative anaerobes, as is the case of Adherent Invasive *E. coli* (AIEC). Even though the implication of the microbiota in persistent inflammation has been studied for years, a direct causal relationship between dysbiosis and IBD has not been established. To date, several strategies, such as the use of probiotics, have been proposed to counteract the microbial imbalance. Nevertheless, probiotics are thought to impair the return of the indigenous microbiome, and to aggravate inflammation in immune compromised patients. Recently, postbiotics – bacterial-free metabolites secreted by probiotic strains – have been proposed as a better and safer strategy to counterbalance the effects of intestinal inflammation.

The intestinal epithelium is the first layer the luminal bacteria interact with. It is composed by a thin monolayer of cells that form a protective barrier against potential detrimental antigens along the whole gut. Thus, the study of epithelial responses to bacteria or their derived metabolites is of great importance to understand intestinal health and disease. Recent advances in the use of primary epithelial cell culture using freshly isolated human intestinal crypts as starting material offers a more faithful representation of the human gut compared to immortalized cell lines.

In the first study of this thesis, we describe for the first time the use of a primary epithelial bidimensional (2D) model derived from 3D organoid cultures as a successful tool to study AIEC infection and its effects on the host epithelium at early and extended infection periods. We proved that this *ex vivo* culture adopts an appropriate cell polarization and orientation, thus becoming a promising resource to study the interactions of the luminal content with host epithelial cells. Importantly, we showed a strain and time-specific response of primary human intestinal epithelial cells when infected with AIEC and non-AIEC strains thus supporting the use of our system to study the functional consequences of AIEC infection on the intestinal epithelium.

This 2D primary cell culture system derived from intestinal organoids was also employed in the second part of this thesis to explore the putative beneficial properties of postbiotics in the epithelial response to inflammatory stimuli. Transcriptomic analysis of 2D cultures pre-treated with postbiotics points towards an effect of the metabolite-cocktail in reverting the inflammatory status of the intestinal epithelium.

Moreover, postbiotics induced the differential expression of several genes on intact primary epithelial cells, demonstrating their potential in contributing to maintenance of homeostasis.

Overall, the human primary organoid-derived monolayer provides a promising tool for elucidating the potentially detrimental or beneficial mechanisms underlying the crosstalk of bacteria and its metabolites with the intestinal epithelium. Moreover, the capacity of this culture to respond and mimic the pro-inflammatory environment *in vitro*, may expand its use in modeling bacteria-host interactions in the context of intestinal inflammatory disorders, such as IBD.

RESUMEN

Los trastornos inflamatorios intestinales, como la enfermedad inflamatoria intestinal (EII), están aumentando en todo el mundo. Un rasgo muy conocido de la EII es la disbiosis de la microbiota intestinal, caracterizada por una reducción significativa de las cepas beneficiosas y un fuerte aumento de los anaerobios facultativos, como es el caso de la *E. coli* Adherente Invasiva (AIEC). Aunque la implicación de la microbiota en la inflamación persistente se ha estudiado durante años, no se ha establecido una relación causal directa entre la disbiosis y la EII. Hasta la fecha, se han propuesto varias estrategias, como el uso de probióticos, para contrarrestar el desbalance microbiano. Sin embargo, se cree que los probióticos perjudican el retorno del microbioma autóctono y pueden agravar la inflamación en los pacientes inmunocomprometidos. Recientemente, los postbióticos – metabolitos libres de bacterias secretados por cepas probióticas – se han propuesto como una mejor y más segura estrategia para mitigar los efectos de la inflamación intestinal.

El epitelio intestinal es la primera capa con la que interactúan las bacterias del lumen intestinal. Éste está compuesto por una fina monocapa de células que forman una barrera protectora, a lo largo de todo el intestino, contra posibles antígenos perjudiciales. Por ello, el estudio de las respuestas del epitelio a las bacterias o a sus metabolitos es de gran importancia para comprender la función intestinal en un estado tanto saludable como de inflamación. Los recientes avances en el uso de cultivos primarios de células epiteliales utilizando células derivadas de criptas intestinales humanas como material de partida, ofrecen una representación más fiel del intestino humano en comparación con las líneas celulares inmortalizadas.

En el primer estudio de esta tesis, describimos por primera vez el uso de un modelo de epitelio intestinal en conformación bidimensional (2D) derivado de organoides intestinales como herramienta para estudiar la infección por AIEC así como sus efectos en el epitelio del huésped en períodos de infección tempranos y prolongados. Demostramos que este cultivo *ex vivo* adopta una polarización y orientación celular adecuadas, convirtiéndose así en un recurso prometedor para estudiar las interacciones del contenido del lumen intestinal con las células epiteliales del huésped. Es importante destacar que mostramos una respuesta de las células epiteliales primarias del intestino humano que es específica de la cepa empleada (AIEC o no-AIEC) y del tiempo de infección. Estos resultados dan soporte al uso de nuestro sistema para estudiar las consecuencias funcionales de la infección por AIEC en el epitelio intestinal.

Este sistema de cultivo celular primario 2D derivado de organoides intestinales también se empleó en la segunda parte de esta tesis para explorar las supuestas propiedades beneficiosas de los postbióticos en la respuesta del epitelio a varios estímulos inflamatorios. El análisis transcriptómico de los cultivos 2D pre-tratados con postbióticos apunta a un efecto del cóctel de metabolitos en la reversión del estado inflamatorio del epitelio intestinal.
Además, los postbióticos indujeron la expresión diferencial de varios genes en las células epiteliales primarias intactas, demostrando su potencial para contribuir al mantenimiento de la homeostasis intestinal.

En general, el cultivo primario en monocapa derivado de organoides humanos constituye una herramienta prometedora para dilucidar los mecanismos potencialmente perjudiciales o beneficiosos que subyacen a la interacción de las bacterias y sus metabolitos con el epitelio intestinal. Además, la capacidad de este cultivo para responder y simular el entorno pro-inflamatorio *in vitro*, puede ampliar su uso en la modelización de las interacciones bacteria-huésped en el contexto de los trastornos inflamatorios intestinales, como la EII.

RESUM

Els trastorns inflamatoris intestinals, com la malaltia inflamatòria intestinal (MII), estan augmentant a tot el món. Un tret molt conegut de la MII és la disbiosis de la microbiota intestinal, caracteritzada per una reducció significativa de les soques beneficioses i un fort augment dels anaerobis facultatius, com és el cas de l'*E. coli* Adherent Invasiva (AIEC). Encara que la implicació de la microbiota en la inflamació persistent s'ha estudiat durant anys, no s'ha establert una relació causal directa entre la disbiosis i la MII. Fins avui, diverses estratègies tals com l'ús de probiòtics, han estat proposades per intentar contrarestar el desequilibri microbià. No obstant això, es creu que els probiòtics perjudiquen el retorn del microbioma autòcton i agreugen la inflamació en els pacients immuno-compromesos. Recentment, els postbiòtics – metabòlits lliures de bacteris secretats per soques probiòtiques – s'han proposat com una millor i més segura estratègia per a mitigar els efectes de la inflamació intestinal.

L'epiteli intestinal és la primera capa amb la qual interactuen els bacteris de la llum intestinal. Aquest està composat per una fina monocapa de cèl·lules que formen una barrera protectora al llarg de tot l'intestí contra possibles antígens perjudicials. Per això, l'estudi de les respostes de l'epiteli als bacteris o als seus metabòlits és de gran importància per a comprendre la funció intestinal en un estat tant saludable com d'inflamació. Els recents avanços en el cultiu primari de cèl·lules epitelials utilitzant criptes intestinals humanes com a material de partida, ofereixen una representació més fidel de l'intestí humà en comparació amb les línies cel·lulars immortalitzades.

En el primer estudi d'aquesta tesi, descrivim per primera vegada l'ús d'un model d'epiteli intestinal en conformació bidimensional (2D) derivat d'organoides intestinals com a eina per a estudiar la infecció per AIEC així com els seus efectes en l'epiteli de l'hoste en períodes d'infecció curts i prolongats. Demostrem que aquest cultiu *ex vivo* adopta una polarització i orientació cel·lular adequades, convertint-se així en un recurs prometedor per a l'estudi de les interaccions del contingut de la llum intestinal amb les cèl·lules epitelials de l'hoste. És important destacar que vam demostrar una resposta de les cèl·lules epitelials primàries de l'intestí humà específica de la soca emprada (AIEC o no-AIEC) i del temps d'infecció. Aquests resultats donen suport a l'ús del nostre sistema per estudiar les conseqüències funcionals de la infecció per AIEC en l'epiteli intestinal.

Aquest sistema de cultiu cel·lular primari 2D derivat d'organoides intestinals també es va emprar en la segona part d'aquesta tesi per tal d'explorar les suposades propietats beneficioses dels postbiòtics en la resposta de l'epiteli a diversos estímuls inflamatoris. L'anàlisi transcriptòmic dels cultius 2D pre-tractats amb postbiòtics apunta a un efecte del còctel de metabòlits en la reversió de l'estat inflamatori de l'epiteli intestinal.
A més, els postbiòtics van induir l'expressió diferencial de diversos gens en les cèl·lules epitelials primàries intactes, demostrant el seu potencial per a contribuir al manteniment de l'homeòstasi.

En general, el cultiu primari en monocapa derivat d'organoides humans constitueix una eina prometedora per a dilucidar els mecanismes potencialment perjudicials o beneficiosos subjacents a la interacció dels bacteris i els seus metabòlits amb l'epiteli intestinal. A més, la capacitat d'aquest cultiu per a respondre i simular l'entorn pro-inflamatori *in vitro*, pot ampliar el seu ús en la modelització de les interaccions bacteri-hoste en el context dels trastorns inflamatoris intestinals, com la MII.

INTRODUCTION

SECTION 1: Inflammatory Bowel Disease

Crohn's Disease (CD) and Ulcerative Colitis (UC) are chronic inflammatory bowel diseases (IBD) that are thought to arise as a result of a dysregulated immune response towards gut commensal microbiota in genetically predisposed individuals. CD and UC are characterized by alternating periods of remission (without clinical and endoscopic manifestations) and relapse (with active inflammation). Even though both diseases share some features, phenotype and disease location are significantly different: while CD can affect the entire gastrointestinal (GI) tract causing transmural inflammation, UC only involves the colonic surface mucosa or, occasionally, the submucosa (1).

1. Epidemiology

Incidence (the probability of occurrence of a given medical condition in a population within a specified period of time) and prevalence (the proportion of a particular population found to be affected by a medical condition at a specific time) rates for both CD and UC are found to be higher in the North of Europe and North America. In fact, IBD is more commonly found in industrialized and developed regions, suggesting that environmental factors might greatly influence IBD occurrence. Nevertheless, the incidence of IBD is particularly increasing in countries or areas, such as Asia or Eastern Europe, where the number of cases was relatively low hitherto (2).

Within Europe, the incidence of IBD is characterized by a north–south gradient, where the incidence, between 2010-2019, of CD was 6.3 per 100,000 in Northern Europe in comparison to only 3.6 per 100,000 in Southern Europe, and the incidence of UC in Northern and Southern Europe was 11.4 and 8.0 per 100,000, respectively (3–5).

Regarding age, IBD patients usually present the first episode of the disease between the ages of 20-30 years. Nevertheless, 5-15% of the patients are for the first time diagnosed between the age of 50-60. In relation to gender distribution, the incidence of IBD appears to be similar for both females and males (even though in pediatric disease, boys are more affected than girls) (6,7).

2. Pathogenesis

Even though the etiology of IBD is still unknown, several studies have suggested that IBD are multifactorial diseases where diverse factors (genetic susceptibility, immune response, intestinal microbiota, or environmental factors (8,9)) contribute to the development of the

disease. Thus, this assumption supports the main hypothesis, which points out that susceptible patients suffer from a dysregulation of the immune system towards their commensal gut microbiota (10).

2.1 Genetic Susceptibility

Thanks to next generation sequencing technologies, in particular Genome Wide Association Studies (GWAS) which can identify single nucleotide polymorphisms (SNPs), and the worldwide-publicly available databases, there has been great advance in the study of this field (11). The first identified gene which appeared to be mutated in CD was *NOD2* (also known as *CARD15* or *IBD1*) (12). Later, another study identified 163 SNPs associated to IBD (110 shared in both CD and UC, 30 specifics for CD and 23, for UC) thanks to the integration of 15 different GWAS. From the 163 identified SNPs, some genes – mainly related to autophagy (*ATG16L1* or *IRGM*) and the immune system (interleukin (*IL*)*-23R*, *JAK2* or *STAT3*) – have been identified to be altered in IBD patients (13). Moreover, some of these genes have also been found to be modified in patients suffering from other autoimmune diseases, suggesting that IBD patients might present common pathways to other diseases (9).

Despite the great variety of SNPs that have been associated to IBD, altogether they only account for the 20-25% of the disease hereditable factor. This phenomenon does not only happen in IBD but in many other polygenic diseases. Hence, future studies should be focused on studying gene-gene or gene-environment interactions rather than detecting new SNPs (13).

2.2 Immune Response

Immune responses in the intestinal mucosa are mainly ensured by the gut associated lymphoid tissue (GALT), constituted by Peyer's patches, mesenteric lymph nodes and lymphoid follicles within the lamina propria (14). Deregulation of the mucosal immune system (both innate and adaptive responses) has been associated with the pathogenesis of IBD (15,16).

Genetically predisposed patients when exposed to certain environmental factors can activate immune responses against microbials or self-antigens which in turn, may

impair the mucosal barrier of the intestinal mucosa, the first physical barrier on the mucosal surface. Therefore, the loss of integrity on this barrier enables the intestinal luminal bacteria to access and cross the intestinal epithelium and to interact with the immune system underneath it (17).

The intestinal epithelium is considered the second line of defense against bacterial invasion. Even though its composition will be detailed in later sections, intestinal epithelial cells (IECs) play a key role in maintaining the integrity of the intestinal mucosa, as they prevent the invasion of antigens, pathogens, and commensal microorganisms (17) not only physically but also secreting antimicrobial peptides (AMPs) and defensins (**Figure 1**). This function is known to be altered in IBD patients (18–20).

The immune response can be triggered when a tissue is damaged or right after the recognition of any foreign particle (21). IECs, as well as macrophages or dendritic cells (DCs), express pattern recognition receptors (PRRs) namely the toll-like receptors (TLRs) on their surface, and the NOD-like receptors, in the cell cytoplasm. The PRRs recognize pathogen associated molecular patterns (PAMPs) and start an intracellular cascade producing chemokines and cytokines which ends up activating the transcription and production of pro-inflammatory mediators to ensure an effective innate response towards any pathogen. PRRs also enhance antigen presentation and consequently, the activation of T cells, thus playing an important role in the interaction between the innate and adaptive immune system (22). In this regard, mutations in *CARD15* gene encoding the NOD2 protein were associated with the occurrence of IBD, especially CD, as we already mentioned. NOD2 is an intracellular microbial sensor that acts as a potent activator and regulator of inflammation. Therefore, deficiency in this protein promotes important changes on the immune response in the lamina propria, leading to a chronic tissue inflammation (15).

Macrophages and DCs are antigen presenting cells and they also secrete several cytokines (such as IL-6, IL-10, IL-12, IL-23, transforming growth factor (TGF)β, or tumor necrosis factor (TNF)α) to activate other immune cells (**Figure 1**) (23). DCs are crucial to cross-link innate and adaptive immune responses by antigen

presentation in the mesenteric lymph nodes, where naïve T lymphocytes differentiation takes place.

As mentioned, PRRs are expressed in various cell types including IECs, monocytes, macrophages, or DCs (24,25). In healthy individuals, TLR are less expressed compared to CD patients, thus supporting its role in triggering an exacerbate response (24,26).

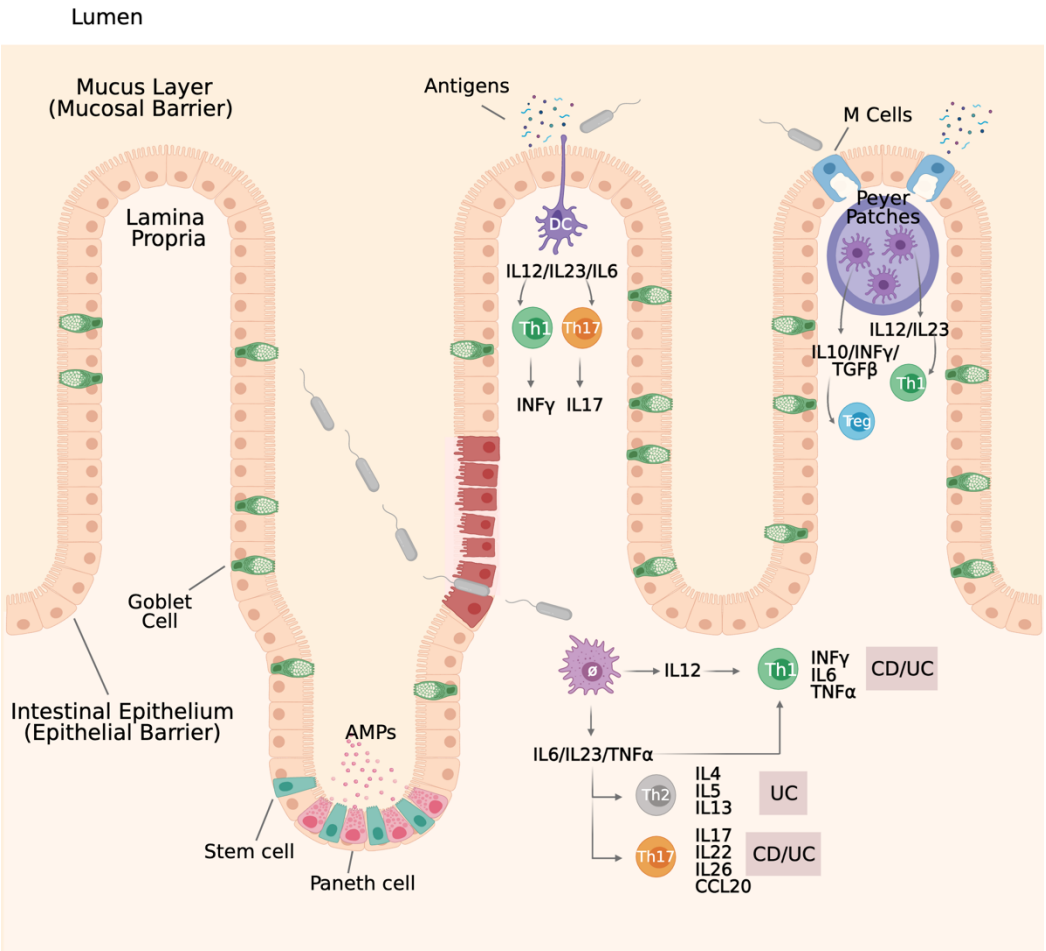


Figure 1. Cytokines and the immune system in IBD. Cytokines produced by different immune cell types under intestinal inflammation. Dendritic cells can stimulate the differentiation of effector Th1, Th2, Th17 or Treg cells as well as promote the differentiation of T and B cells in Peyer's patches (in the small intestine, as is the case of this figure) or in the mesenteric lymph nodes. This figure has been adapted from Silva et al. and Neurath et al. (16, 21) and created with BioRender.

PRRs also potentiate antigen presentation and co-stimulation, consequently driving potent T cell activation (22). $CD4^+$ T cells proliferate in secondary lymphoid tissues (i.e., lymph nodes, Peyer's patches, etc.) and differentiate into T helper (Th) cells in the presence of antigens and cytokines. The main subtypes of effector $CD4^+$ Th cells

are Th1, Th2, Treg, and Th17. Th1 differentiate after exposure to IL-12 secreted by DCs. When activated later by antigen presentation at effector sites (i.e., the intestinal mucosa), Th1 cells secrete effector cytokines such as interferon (INF)γ, IL-6 or TNFα which have been implicated in the pathophysiology of both UC and CD. DCs can also promote differentiation of the Th17 subpopulation (**Figure 1**). The cytokines promoting Th17 differentiation are less well characterized in human than in mice where TGFβ together with a pro-inflammatory cytokine (IL-6 or IL-1β) have been implicated. Upon differentiation, Th17, as well as other innate lymphoid IL-17 or IL-22 producing cells (i.e., ILC3), acquire the expression of the IL-23R. IL-23, which can be secreted also by intestinal DCs and macrophages, promotes survival and proliferation of Th17 cells and other IL-17-producing cells which play a crucial role in intestinal inflammatory manifestations (15). IL-17 and IL-22 produced by Th17 cells appear to be related to the induction of colitis, since these cytokines initiate and amplify the local inflammatory signs and promote the activation of counterregulatory mechanisms targeting IECs (27). IL-23 also activates signal transducer and activator of transcription (STAT)4 in memory T lymphocytes, stimulating the production of IFNγ. In turn, IFNγ is responsible for triggering the production of inflammatory cytokines in cells of the innate immune system, contributing to the increase of the inflammation present in IBD (28).

Th2 cells maturation is mainly due to cytokine secretion by macrophages. This cell population has been linked to UC pathogenesis. In fact, the imbalance between Th1 and Th2 cytokines released by the intestinal mucosa determines the intensity and duration of the inflammatory response in experimental colitis (29). Finally, Treg cells, which are involved in tissue repair and tolerance to self-antigens are mainly differentiated after exposure to cytokines such as IL-10, IFNγ and TGFβ.

Overall, the secretion of certain cytokines as well as the response to self-antigens by immune cells (30–32) seem to be related to the onset and establishment of IBD. Thus, it makes it evident that T cells play a crucial role in the regulation of the immune response in IBD.

2.3 Microbiota

The human intestinal microbiota is a large reservoir of microorganisms (bacteria, fungi, viruses, and unicellular eukaryotes) that coexist within the gastrointestinal tract, reaching densities between 10^{13}-10^{14} microbial cells. These numbers are derived from the total bacterial cells in the colon (3.8×10^{13} bacteria), the organ that harbors the highest density of microbes (33). This microbial community exerts different functions in the human body, including nutrient metabolism, immune system maturation, and suppression of harmful microorganisms' growth. Nonetheless, many factors can alter the composition of the microbiota, from genetics, diet, age, drugs, or tobacco among others.

It is widely known that the gut microbiota has a key role in IBD pathogenesis. In fact, IBD has been characterized by a remarkable dysbiosis, a breakdown in the balance between beneficial and harmful bacteria that are present in the human gut (34,35), compared to healthy individuals. This leads to a drastic decrease in the overall microbial biodiversity in IBD patients, who show a reduction in the total number of Bacteroidetes and Firmicutes – particularly *Faecalibacterium prausnitzii* (*F. prausnitzii*) – (36–38), while members of the Proteobacteria phylum – mainly *Escherichia coli* (*E. coli*) – are increased (39–41) (**Figure 2**). In fact, these findings suggest that the abundance of these two bacterial species could be a reliable indicator of dysbiosis in IBD patients (42–44).

However, a cause-effect relationship has been challenging to prove. Therefore, studies trying to determine whether dysbiosis is truly causative or merely a consequence of inflammation have suffered from a number of limitations, making it difficult to achieve robust conclusions.

It is also well established by studies performed both in fecal or mucosa-associated communities, that CD patients' microbiota differs from that found in patients with UC and to that in healthy controls (45). In fact, Sankarasubramanian et al. (46) have recently demonstrated that several unique microbial species can distinguish healthy controls from UC and CD patients, a feature that was also studied by Lopez-Siles et al. (42).

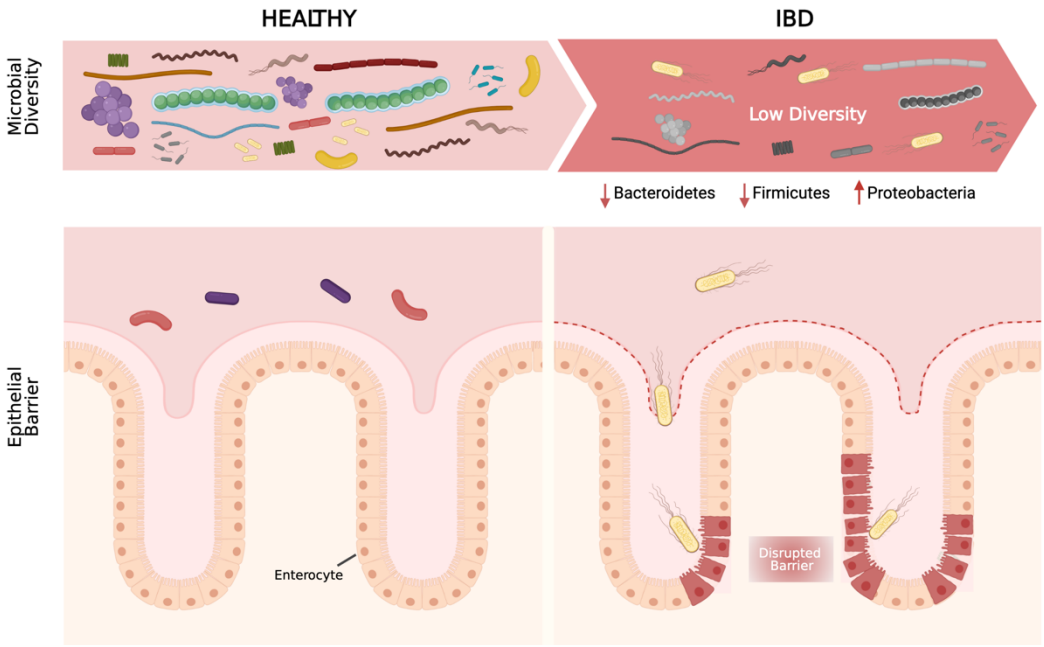


Figure 2. Microbial composition and epithelial barrier status in healthy versus IBD gut. Under healthy conditions, the intestinal microbiota is highly diverse and enriched in Bacteroides and Firmicutes. However, these phyla are known to be reduced in patients with IBD leading to a less diverse microbial composition with an expanded growth of Proteobacteria such as *Escherichia coli*. Therefore, the mucosal function is also altered promoting a decrease in the mucus thickness, a reduction in the mucosal integrity and an impaired barrier function. This increases bacterial translocation and stimulates activation of the inflammatory immune response. This Figure has been adapted from Sommer et al. (41) and created with BioRender.

These differences were also evident when studying the Adherent Invasive *Escherichia coli* (AIEC), an *E. coli* pathotype – which has been associated with IBD – that will be discussed more in detail in further sections. AIEC strains are mainly found in ileal and colonic samples of CD patients while its association with UC is less clear. Nevertheless, a recent meta-analysis study suggests that this pathotype could actually be involved in UC disease progression more than earlier thought (47).

Another determinant fact that supports the implication of intestinal bacteria into the inflammatory status is the response of some IBD patients with mild disease to any treatment that modifies their gut microbiota (48,49). For instance, fecal microbiota transplantation has been considered as an encouraging option for patients suffering from IBD (50,51). Moreover, probiotics have also been shown to provide some efficacy in remission in UC patients (52). Nonetheless, Tsilingiri et al. demonstrated that postbiotics (a group of microbial metabolites that we will discuss later) were

better than probiotics in reducing pro-inflammatory cytokine secretion in IBD tissues (53).

Overall, all the available studies regarding the role of the microbiota in IBD supports the fundamental idea that studying the gut microbiota with an eye on therapeutics may be crucial to guide future personalized therapies.

2.4 Environmental Factors

Chronic inflammatory disorders and neoplasms have become the main cause of morbidity and mortality during the last century in the Western world (54). The increase in chronic autoimmune and inflammatory diseases (such as IBD) has been linked to the social and economic progress, as well as the increase in life expectancy that first took place in northern Europe and America, but which can actually be seen in other parts of the world (rest of Europe, Japan and South America) (55). The "hygiene theory", or the dramatic decrease in human exposure to microbes, has been proposed as a possible contributor to this mentioned shift. This lack of contact with microbial antigens early in life, affects the proper maturation of the immune system so that it would not be equipped to effectively act, prompting to a much more ineffective immune response (56).

Other environmental factors related to IBD include tobacco, diet, certain drugs, and stress, among others. Tobacco is the most influential environmental factor in IBD with opposite effects on UC and CD. In CD, tobacco is a risk factor that increases the risk of relapse and/or surgical resection. In UC, smoking cessation worsens the disease, suggesting a protective effect of tobacco (57). Another environmental factor that has been linked to IBD are pharmacological treatments. For example, oral contraceptives have been related to a higher risk of developing IBD, especially CD (58). On the other hand, nonsteroidal anti-inflammatory drugs (NSAIDs) are shown to be important in causing relapse (59). Stress also plays an important role in the disease; anxiety and depression may be crucial in relapse and deterioration of the disease (60).

Many other environmental factors have been linked to IBD; however, there is no sufficiently strong evidence to support a causative effect of these factors in the

development of the disease. Thus, it has been accepted that all these factors might only have an accumulative effect on genetically predisposed patients (8).

3. Diagnosis

3.1 Symptomatology

IBD symptomatology can include hemorrhagic diarrhea, abdominal pain, tenesmus, urgency to evacuate, weight loss and anorexia (61–63). Nevertheless, these clinical characteristics vary depending on whether patients suffer from CD or UC. UC patients usually experience pain in the lower left abdomen as well as diarrhea (64). As a result, they may undergo weight loss and residual blood is found during rectal examination. On the contrary, patients suffering from CD feel pain in the lower right side of the abdomen and rectal bleeding is less usual. The most common complication in CD is intestinal obstruction, which results from intestinal wall thickening due to inflammation. In addition, CD patients suffer from malnutrition complications as a consequence of a decreased nutrient absorption (65,66).

Moreover, among 50-60% of IBD patients also suffer from extraintestinal manifestations (64) such as arthritis, hypertrophic osteoarthropathy, vitiligo or psoriasis (67).

3.2 Disease Extension

As we already mentioned, disease extension significatively differ between both diseases: while UC only affects the colon (**Figure 3A-C**), CD can affect the entire GI tract (**Figure 3D-H**) (1).

Around 1/3 of UC patients suffer from proctitis (**Figure 3A**), a type of intestinal inflammation that only affects the rectum. When the inflammation is extended along the colon, we can distinguish between distal colitis (**Figure 3B**) and pancolitis (**Figure 3C**). The extent of the disease and its severity correlate with clinical prognosis in UC. Thus, patients with proctitis usually have a better prognosis than those with a more extensive disease (68). Parallelly, extensive colitis is commonly associated with a higher risk of colectomy (69) and colorectal cancer (70).

Regarding CD patients, according to the Montreal classification (71,72), intestinal inflammation might be present on the terminal ileum – L1 – (**Figure 3D**), the colon

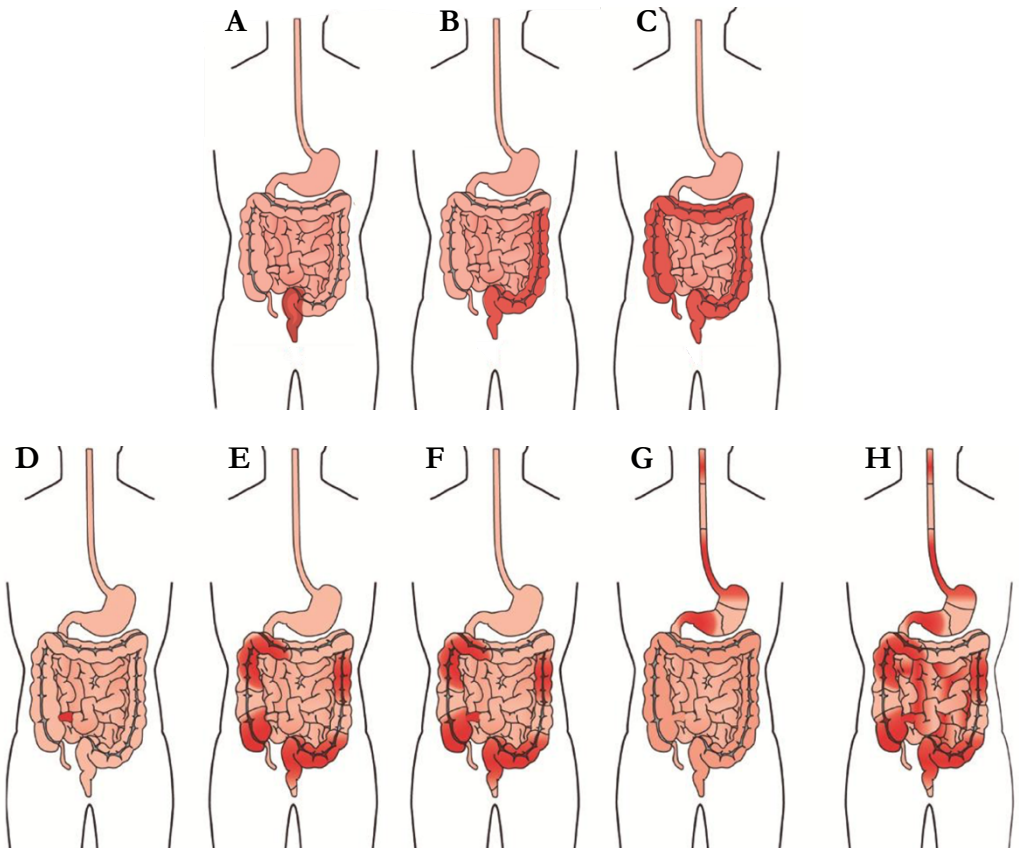


Figure 3. Disease Extension in IBD according to the Montreal classification. (A) Proctitis; **(B)** Distal Colitis; **(C)** Pancolitis; **(D)** Terminal Ileum Inflammation -L1-; **(E)** Colonic Affectation -L2-; **(F)** Ileocolonic Disease -L3-; **(G)** Upper GI tract Affectation -L4-; **(H)** Distal and Upper GI tract Inflammation -L3+L4-. This figure has been adapted from Baumgart et al. (66)

– L2 – (**Figure 3E**), both the ileum and colon – ileocolonic disease, L3 – (**Figure 3F**) or on the upper GI tract – L4 – (**Figure 3G**). Moreover, some patients might suffer from both L3 and L4 manifestations thus affecting the lower and upper GI tract (**Figure 3H**) (66).

The Montreal classification also describes CD according to its behavior, defining the disease as B1 when it is non-stenotic and non-penetrating; B2, for the stenotic disease and B3, when it is penetrating (73).

3.3 Treatment

The aim of the currently available treatments for IBD is to induce and maintain remission. Rather than reversing pathogenic mechanisms, they intend to decrease the side effects associated with the disease. Corticosteroids, immunosuppressants and biologic treatments are the most routinely used, although some other drugs such as antibiotics or metronidazole might also be useful in some cases (74). Nevertheless,

when the mentioned treatments are not effective, surgical resection may be required. Some of the most common drugs used in IBD are mentioned below:

- Corticosteroids are usually used to induce remission in moderate or severe IBD since they act as potent anti-inflammatory drugs. They inhibit cyclooxygenases and regulate immune cells by reducing their pro-inflammatory capacity. Corticosteroids can be given orally (prednisolone, prednisone, budesonide) or intravenously (hydrocortisone or methylprednisolone). Liquid suppositories or enemas may also be given. Different strategies have been developed to maximize their topical effects while limiting the systemic side effects of steroids. In that sense, budesonide is a poorly absorbed corticosteroid with limited biodistribution that has therapeutic benefits with reduced systemic toxicity in CD (75) or UC (76). Nevertheless, these agents cannot be used as maintenance therapy due to the large number of associated side effects (77,78).

- Immunosuppressants (including azathioprine, mercaptopurine or methotrexate) inactivate key processes, such as proliferation and survival of T lymphocytes that can promote inflammation. Except for methotrexate, this group of drugs might also have toxic effects due to their capacity to intercalate within nucleic acids (79). Immunosuppressants are usually prescribed when patients do not respond to corticosteroids, or they are corticosteroid dependent. Specifically, in CD patients immunosuppressants are useful for both inducing and maintaining remission (80) while in UC they are mostly indicated for maintenance (81).

- Biologics used in IBD are monoclonal antibodies directed against different targets involved in its pathogenesis such as cytokines (TNFα, IL-12, IL-23) or α4β7 integrins, involved in lymphocyte homing to the gut. Anti-TNFα therapy is the most used biologic among IBD patients. Infliximab, adalimumab and certolizumab are the three anti-TNFα drugs that have been proved to reduce IBD-related hospitalization and the risk of surgery since they can induce mucosal healing and improve patients' life quality (82). However, 30-50% of patients receiving this treatment fail to respond. Thus,

novel strategies that block alternative targets have emerged. New drugs include ustekinumab (which blocks the p40 subunit, common for both IL-12 and IL-23 cytokines) or vedolizumab (which blocks the α4β7 integrin) (83,84).

- Small molecules are synthetic compounds known to ameliorate IBD. Among them, tofacitinib is the only drug currently approved for the treatment of moderate to severe UC. Tofacitinib is a pan Janus kinase (JAK) inhibitor shown to induce and maintain remission in a percentage of patients with UC (85–87).

- Despite the use of newly available drugs, approximately 30-40% of the patients are refractory to any treatment (88) and they are forced to undergo surgical resection either because of disease complications or due to uncontrolled inflammation. Surgery usually consists in removing the inflamed area. There are different surgical procedures but colectomy (total removal of the colon) with ileostomy is the most commonly used (89).

Other strategies used to treat IBD are hematopoietic stem cell transplant (90,91) for CD patients with highly refractory and severe disease, or the mesenchymal stem-cell therapy to treat fistulizing perianal disease in CD patients (92,93). Overall, all the efforts are focused on the achievement of long-term maintenance of remission in IBD patients, either through using conventional or emergent therapies.

SECTION 2: The Intestinal Epithelium in physiology and IBD

The human intestine is divided in two different anatomic regions: the small (duodenum, jejunum, and ileum) and the large intestine or colon. From the outside, serosa, muscularis externa, submucosa and mucosa are the four principal layers that form the intestine. The intestinal mucosa is a dynamic barrier that separates the intestinal lumen from the internal environment of the human body. The intestinal mucosa is formed by the **mucus layer**, the **intestinal epithelium**, and the underlying lamina propria that contains numerous immune cells near the epithelial barrier (94).

1. Physiology of the Intestinal Epithelium

The **intestinal epithelium** serves as a defense against food antigens and microorganisms. It is formed by a single layer of different cell types (**Table 1**) that are organized into villi (which are only found in the small intestine and enlarge the surface area of the epithelium in this region) and crypts (**Figure 4**) (95). This epithelial layer has the ability to renew every 4-5 days. Renewal relies on intestinal pluripotent stem cells that are located at the base of the crypt (**Figure 4**) (96,97). Stem cells divide, differentiate by migrating along the crypt and are finally released into the lumen. Eventually, cells undergo spontaneous apoptosis and are also drive out into the intestinal lumen. Stem cell proliferation and apoptosis are tightly controlled by paracrine signals, so a balance is achieved (97). In this way, the intestinal epithelium

Table 1. IECs: Types, function, and localization.

Cell Type	Function	Localization (Ileum/Colon)
Enterocyte	Nutrient absorption	Ileum and Colon
Goblet Cell	Mucus secretion	Ileum and Colon
Tuft Cell	Chemosensory cells – Immune response activation	Ileum and Colon
Paneth Cell	AMPs' production	Ileum
M Cell	Sample and transport of antigens/pathogens from the luminal surface to the sub-epithelium	Ileum and Colon
Enteroendocrine Cell	Hormone secreting / Synapse with nerves	Ileum and Colon

is constantly renewed while the number of cells forming the epithelial layer remains stable. There are different types of differentiated cells in the epithelium, including enterocytes, Goblet cells, Tuft cells, Paneth cells, M cells, and enteroendocrine cells (97) (**Table 1**). The organization of these cells along the small and large intestines differs, being Paneth cells exclusively located at the base of the small intestinal crypts (**Figure 4**) (95).

In addition to IECs, we also find intraepithelial lymphocytes (IELs), which are located within epithelial cells. IELs form a heterogeneous population with cytotoxic or regulatory functions. In that way, IELs can interact with each other and with other immune cells located underneath the intestinal epithelium (98). IELs provide defense against pathogens, possibly through the removal of infected epithelial cells and the secretion of AMPs (99).

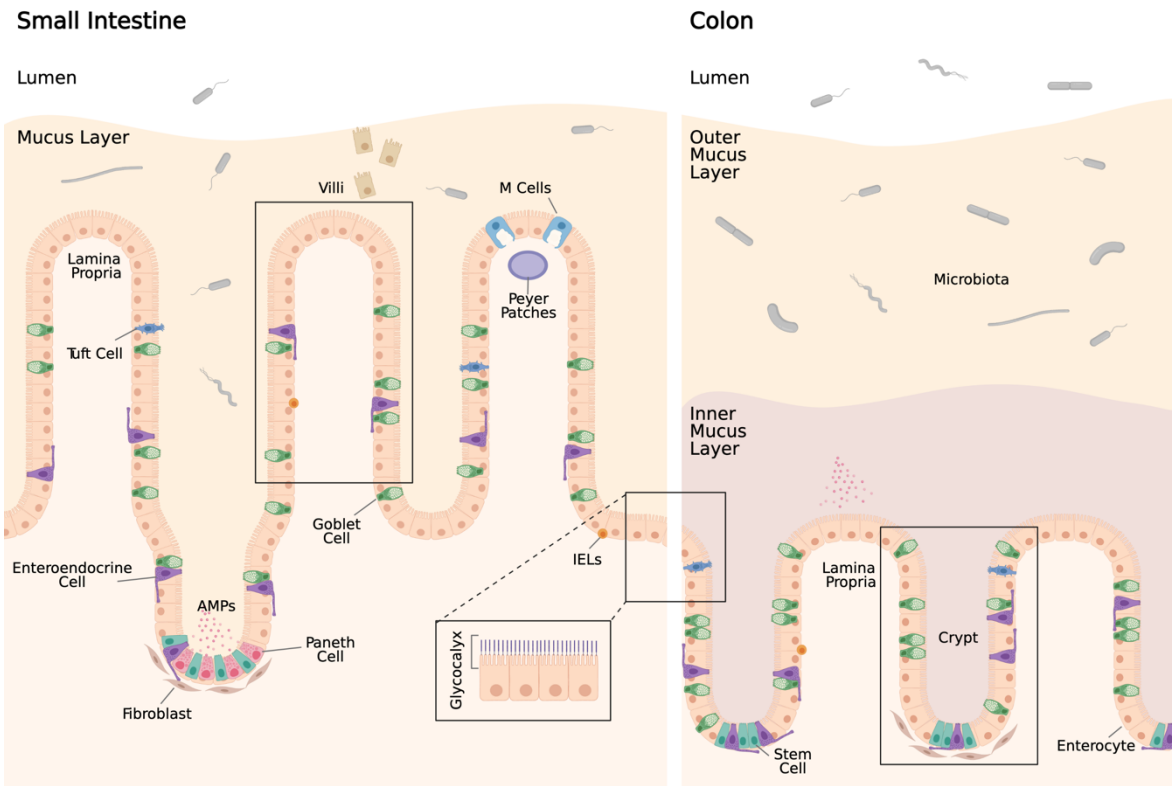


Figure 4. Small intestine and colonic healthy mucosa. On the left side, the ileal intestinal mucosa, containing crypts and villus is represented. The mucus layer covers the entire intestinal epithelium which is formed by different cell types including Paneth cells that are exclusively located at the base of the ileal crypts. On the right side, we show the colonic mucosa, whose epithelial barrier is covered by two mucus layers: a thinner and outer and a more robust and inner one.
This Figure has been adapted from Burgueño et al. (26) and created with BioRender

On the other hand, the **mucus layer** is formed due to the secretion of mucus and AMPs by IECs, IELs and the underlying macrophages. The main role of this layer is to protect the

mucosa from commensal microbes and invasive microorganisms thanks to two different types of barriers: the physical and the chemical.

- The physical barrier consists of the mucus, the glycocalyx and the cell junctions. This barrier physically inhibits the microbial invasion of the mucosa.

  Goblet cells are in charge of mucus production, thus forming a viscous layer enriched in mucins: glycoproteins that form large polymers (100). These secretory cells are more abundant in the colon compared to the small intestine. The small intestine harbors a single, tightly attached mucus layer, whereas in the colon, mucus is organized into two distinct layers: an outer loose layer (highly colonized by different microbial components) and an inner denser layer that is firmly attached to the epithelium (a feature that, together with the presence of high AMPs concentrations, prevents bacteria from reaching it) (**Figure 4**) (101). Since mucus is continuously secreted, the outer layers are sloughed off and carried into the fecal stream (102).

  On the other hand, the glycocalyx (**Figure 4**) is a mesh of carbohydrates, glycoproteins, glycolipids, and transmembrane mucins that block bacterial invasion (103).

  Finally, cell junctions are structures specialized in cell-to-cell attachment. Altogether provide structural support (104) and only allow the entry of certain solutes and fluids, creating a selective permeable barrier. They include tight junctions (TJ), adherent junctions and desmosomes.

  - TJ are complexes mainly formed by:
    - Claudins. A family of proteins that regulate the paracellular transport by forming channels of a determined size and charge that allow the flowthrough of solutes, water, and macromolecules (105).
    - Occludins (together with tricellulin and marvelD3 (105)) are TJ-associated proteins (TJAP) shown to regulate the formation, maintenance, and function of tight junctions.
    - Zonula Occludens (ZO). Membrane proteins that connect transcellular proteins to the intracellular cytoskeleton, thus playing a role in the

assembly and maintenance of binding proteins and paracellular permeability (106).

- Adherent junctions are multiprotein complexes – located in the basolateral side of the bound cells – that play an important role in cell-cell adhesion and signaling (104).
- Desmosomes, which are transmembrane adhesion proteins of the cadherin family, provide mechanical cohesion to the epithelium thus maintaining the function of the epithelial barrier (104).

- The chemical barrier is formed by AMPs, defensins (among other anti-microbial molecules) and oxygen. Both AMPs and oxygen are secreted by the epithelium and immune cells adjacent or inserted within the IECs (107). Its accumulation at high concentrations within the mucosa, especially in the small intestine where Paneth cells are responsible for their production, greatly restrict the presence of potential microbial inhabitants. AMPs and defensins bind to the microbial cell membrane and induce its disruption by forming pore-shaped structures (108). Defensins can be classified into α-, β- and θ-defensins, being the first ones the most expressed by Paneth cells. In addition, Paneth cells localization in the crypt niche (**Figure 4**) suggests an essential role in stem cells protection (109). In the colon, defensins are produced by IECs, IELs and macrophages from the lamina propria (110).

Finally, if bacteria subvert the mucus and the epithelial barrier, the autophagy process (through the action of the phagocytes found in the lamina propria) will be in charge of preventing bacterial replication and persistence.

2. Intestinal Epithelium alterations in IBD

Defects in the intestinal epithelium are a characteristic feature of IBD and can manifest in different forms, from defective autophagy mechanisms and mucus production, to altered secretion of AMPs thus predisposing the epithelium to bacterial invasion (**Figure 5**). These alterations in the host's defense mechanisms promote bacterial-IECs interaction which can result in the perpetuation of the intestinal inflammation (111). In addition, the risk of developing IBD is associated with genetic variants related to epithelial functions, mainly those affecting bacterial clearance and autophagy. As mentioned before, one of the strongest risk factors for IBD is mutations in NOD2, an important intracellular sensor for bacterial

muramyl peptide (112). Moreover, a great number of cellular processes, such as autophagy, have been found to be altered in IBD patients (113). For example, patients with active UC showed a decrease in the expression of activating transcription factor 4 (ATF4), an important autophagy-related protein of the intestinal mucosa (114). In CD, mutations of ATG16L1 and IRGM, two important autophagy-related proteins, have been highly related to the pathogenesis of the disease (115,116).

Overall, autophagy would appear to be involved in the pathogenesis and progression of IBD. Thus, some autophagy regulators have been suggested as a possible target for IBD treatment, although most of them have not yet undergone clinical development.

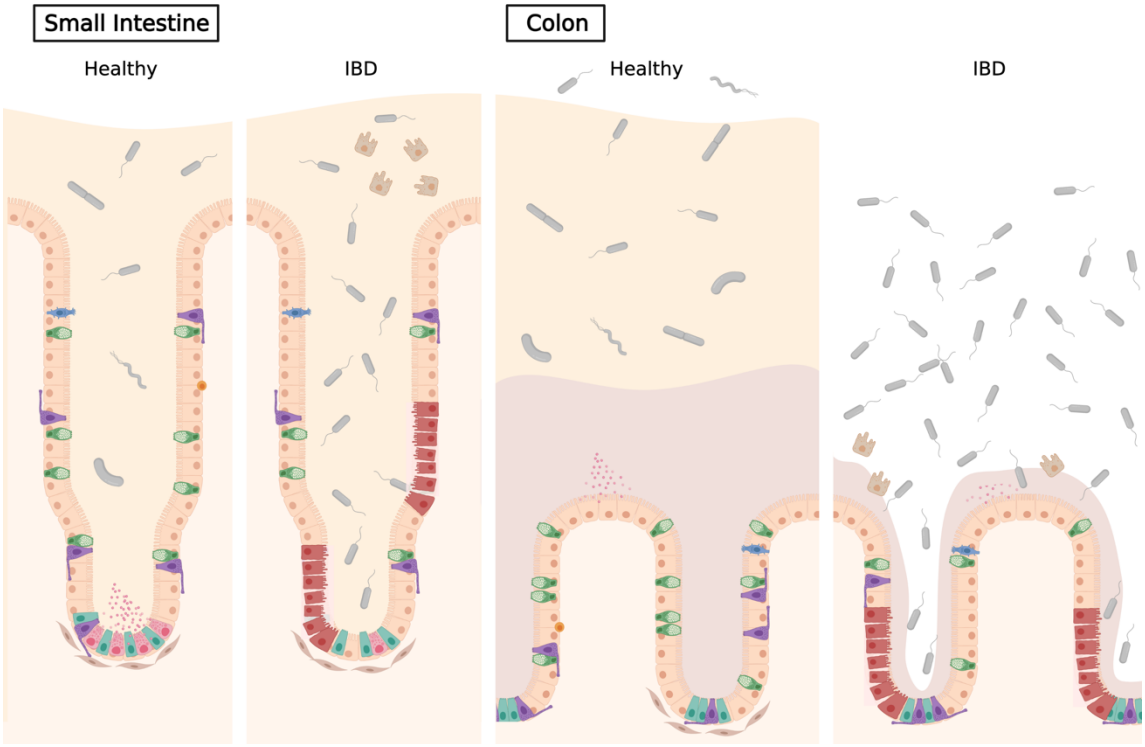


Figure 5. Intestinal Epithelium Alterations in IBD. On the left, schematic representation of the healthy and the IBD small intestine. Patients with ileal affectation present a reduction in the number of Paneth cells, a decrease in the production of defensins, an increased epithelial permeability as well as a higher cell death. On the right panel, the healthy and IBD colon are represented. Patients with colonic inflammation show a reduction in the number of Goblet cells as well as a thinner mucus layer. This leads to higher epithelial permeability and cell death.
This figure has been adapted from Peterson et al. (95) and created with BioRender.

As already mentioned, mucus integrity is essential to prevent mucosal inflammation. In UC, however, the mucus layer is usually weaker due to an alteration in mucins glycosylation and a reduction in the number of goblet cells (117–119) (**Figure 5**). In fact, epithelial permeability increases in both CD and UC (120–122). In CD, there is an increase in Claudin-2 while a reduction of Occludins and Claudins 5 and 8 have been observed (121). Something similar

happens in UC patients, in which apoptotic mechanisms have also been found to be altered (111,120,122), thus affecting the intestinal epithelial permeability.

Finally, CD has been highly associated with alterations in Paneth cells population. Paneth cells differentiation factor is reduced in ileal CD, thus decreasing the availability of these cells in the ileum (123). In turn, this phenomenon led to a decrease in the production of α-defensins and AMPs (124). On the contrary, β-defensins have been found increased in some UC patients, thus boosting their antimicrobial response (125).

SECTION 3: Adherent Invasive *Escherichia coli* in IBD

As we have described in previous sections, the most affected GI areas in IBD patients are the distal ileum and the entire colon which are the highest bacterial-colonized segments in the GI tract. Therefore, this supports again the fundamental role of the microbiota in IBD (126). In this section, we will first describe the microbial composition of the human gut, emphasizing on its impact on health and disease. Then, we will focus on *E. coli* and, more specifically, on Adherent Invasive *E. coli* and its role on IBD pathogenesis.

1. Intestinal Microbial Composition in Human Gut

In humans, the lower GI tract is colonized by a complex microbial population that exhibit variations in density and composition due to chemical, nutrient, and immunological gradients along the tract (**Figure 6A-B**).

The small intestine has a more acidic environment, with higher levels of oxygen and antimicrobial agents than the colon (**Figure 6B**); therefore, its microbiome it's mainly composed by facultative anaerobes (127). On the other hand, the most diverse microbial community of the entire human body is contained within the colon and cecum. In these regions, microorganisms are responsible of degrading polysaccharides that have not been degraded in the small intestine. Moreover, the AMPs concentration is decreased, the GI transit is slower, and simple carbon sources are scarce. Altogether, these conditions facilitate the growth of fermenting anaerobes such as Bacteroidaceae (family) and Clostridia (class) (127).

Bacterial diversity not only differs longitudinally but within the lumen and the inter-fold regions of the colon (transversally). (**Figure 6A**) (127).

Among the different factors that can affect the microbial composition, diet is one of the most influential. In fact, diet-derived polysaccharides can control the microbial composition of the colon, suggesting the strong repercussion of diet on quickly changing the microbial composition of any individual (128).

1.1 Microbial Impact on Health and Disease

The crosstalk between the commensal microbiota and the immune system is crucial for a proper immune activity (129,130). Commensal bacteria colonize the host shortly

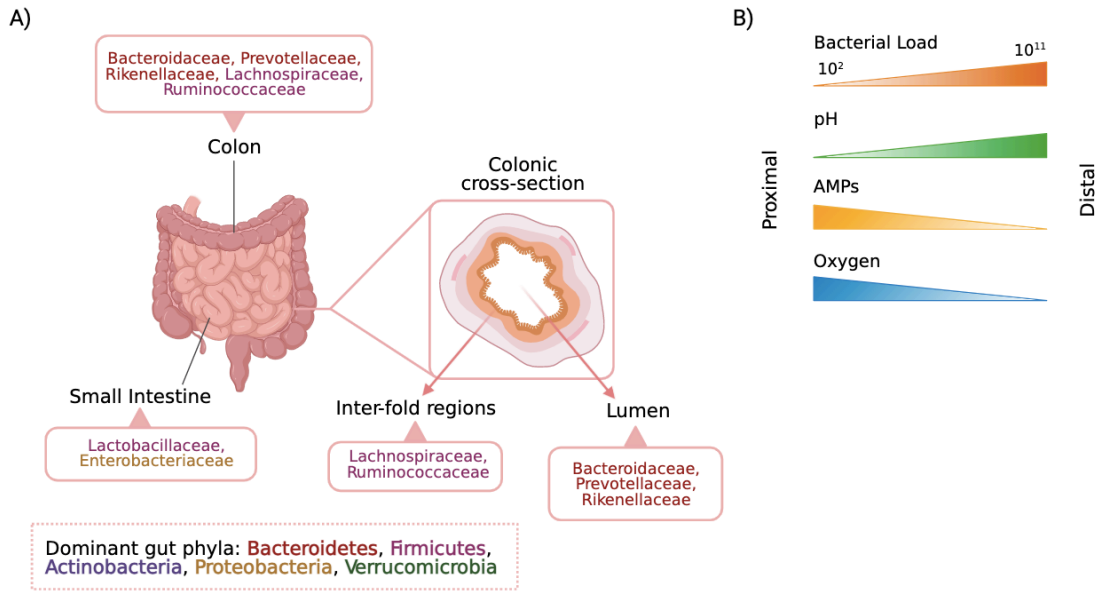


Figure 6. Microbial habitats in the human lower gastrointestinal tract. (A) The dominant bacterial phyla in the gut are Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia. The dominant bacterial families of the small intestine and colon reflect physiological differences along the length of the gut. In the small intestine, the families Lactobacillaceae and Enterobacteriaceae dominate, whereas the colon is characterized by the presence of species from the families Bacteroidaceae, Prevotellaceae, Rikenellaceae, Lachnospiraceae and Ruminococcaceae (colors correspond with the relevant phyla). A cross-section of the colon shows the lumen – which is dominated by Bacteroidaceae, Prevotellaceae and Rikenellaceae – and the inter-fold regions of the lumen – dominated by Lachnospiraceae and Ruminococcaceae –. **(B)** The changes in the bacterial diversity along the intestine are due to differences in the oxygen concentration, the antimicrobial peptides (AMPs) and the pH, which limits the bacterial density in the small intestinal community, while promoting the high bacterial loads in the colon. This Figure has been adapted from Donaldson et al. (127) and created with BioRender.

after birth. This community continuously develops into a highly diverse ecosystem during host growth (131). Over time, host-bacterial associations have developed into beneficial relationships. Gut microbes exert a wide range of functions that are crucial for maintaining the integrity of the mucosal barrier, providing certain nutrients such as vitamins, metabolizing indigestible compounds, or protecting against pathogens, among many others (132). Therefore, the intestinal microbiota plays a key role in maintaining energy homeostasis in the human being. For example, dietary fibers such as xyloglucans, which are commonly found in vegetables, cannot be digested on the stomach nor the small intestine. Thus, specific species of *Bacteroides* (133) residing in the colon are the responsible of their metabolization. Other non-digestible fibers, such as oligosaccharides, can be used by beneficial microbes, such as *Lactobacillus* and *Bifidobacterium* (134). The healthy gut microbiome produces 50–100 mmol/L per day of short-chain fatty acids (SCFAs), such as acetic, propionic, and butyric acid (135). SCFAs can be quickly absorbed in the colon and play many different roles in

regulating gut motility, inflammation, glucose homeostasis, or energy accumulation (136,137). In addition, the intestinal microbiome stimulates the normal development of the humoral and cellular mucosal immune systems (138). Microbial signals and secreted metabolites can be sensed by the hematopoietic and non-hematopoietic cells of the innate immune system and are then translated into tolerogenic responses (139). Therefore, an imbalance in the microbial composition may lead to the development or exacerbation of GI diseases (gastric, colorectal, and esophageal cancer, Inflammatory Bowel Syndrome or IBD) among many others (140).

As we already mentioned, whether microbial changes occur as a result of local inflammation or contribute to IBD pathogenesis remains unclear. What it has been established is that IBD patients (as we introduced in Section 1) present a decrease in gut microbial richness, a depletion of anaerobic species and SCFA producers while an increase of facultative anaerobic bacteria, such as *E. coli* (141,142). Even though a lot of effort has been made to unravel the composition and role of the intestinal microbiota in IBD, there are still a lot of unknowns about host-to-microbe, microbe-to-host and inter-microbe crosstalk.

2. *Escherichia coli* in human gut

One of the up to 10^{13}-10^{14} microorganisms (143) found in the human gut is *E. coli*, a gram-negative bacterium belonging to the Enterobacteriaceae family that normally interacts mutualistically with the host. *E. coli* is a lifelong colonizer (144,145) of the human gut: being settled right after birth and maintaining its presence through life. Normally, it persists as a harmless commensal in the mucous layer of the cecum and colon (146). Gut commensal *E. coli* strains are highly diverse in terms of phylogenetic origin (146–148) and they frequently express adhesins, capsular antigens, toxins (such as the α-hemolysin), as well as the siderophore system aerobactin to sustain their persistence in the gut (146).

2.1 *E. coli* pathogenic groups

E. coli species comprise both harmless commensal and pathogenic strains. The latter may have acquired different sets of virulence genes via horizontal transfer of DNA (through plasmids, transposons, bacteriophages, or pathogenicity islands) allowing them to adapt to pathogenic conditions and cause a wide variety of diseases. Pathogenic *E. coli* strains are grouped in pathotypes according to its clinical spectrum and virulence factors (145,149–151) (**Table 2**). The extraintestinal pathogenic *E. coli*

(ExPEC) group comprises strains causing urinary tract infections, sepsis, or meningitis. These groups are the uropathogenic *E. coli* (UPEC) and the meningitis-associated *E. coli* (MNEC). Interestingly, ExPEC are believed to originate in the gut, belonging to the commensal microbiota of many healthy individuals. Therefore, it is not simple to differentiate ExPEC from commensal *E. coli* strains (146,147) except for their plasmid content (152).

Table 2. *E. coli* classification in pathogroups and pathotypes

Pathogroups and pathotypes	Description
ExPEC	
UPEC	Responsible of urinary infections
MNEC	Causative of gram-negative neonatal meningitis and sepsis
DEC	
EAEC	Causes persistent diarrhea
EPEC	Responsible for fatal infant diarrhea
ETEC	Infant and traveler's diarrhea due to their secreted endotoxins
EHEC	Can cause bloody and non-bloody diarrhea as well as hemolytic uremic syndrome
EIEC	Responsible for inflammatory colitis and dysentery
DAEC	Urinary tract infections and diarrhea in children
NTEC	Neonatal enteritis as well as urinary tract infections

Another group comprises the diarrhoeagenic *E. coli* (DEC) strains, which encloses those causing intestinal infections. These strains carry specific surface adhesins necessary to colonize the GI tract even though they rarely translocate into the intestinal epithelium (145,153). Seven well-defined pathotypes are found in this group: enterohemorrhagic *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC), and necrotoxic *E. coli* (NTEC) (**Table 2**). Two decades ago, a new pathotype named AIEC has been proposed to be associated with IBD (154).

2.1.1 Adherent Invasive *E. coli*

The most studied strain from the AIEC pathotype is the LF82 which was isolated from a CD patient with ileal affectation (154). It was proposed as a new pathotype since its pathogenic traits were different from the previously described DEC pathotypes (155) and its phenotype differed from that of ExPEC strains even though several virulence attributes were common (156–159).

For an *E. coli* strain to be considered AIEC has to: adhere to intestinal IECs (≥1 bacteria/cell) (154), invade IECs through the involvement of host cell actin polymerization and microtubule recruitment (≥0.1% of the original inoculum) (155), survive and replicate within macrophages without inducing cell death (≥100% of intracellular bacteria 24h post-infection) (160), and lack any of the already-known virulence or invasive determinants (161).

3. AIEC in IBD

3.1 AIEC in Crohn's Disease

As we described in previous sections, patients suffering from CD present a compromised epithelial barrier function thus promoting a dysregulated mucosal immune response and facilitating invasion of pathogens (such as AIEC) which can invade IECs and, in turn, promote chronic inflammation.

According to a recently published meta-data analysis, AIEC prevalence among CD patients and healthy controls is 21-62% and 0-19%, respectively (47,162).

Mainly by the use of *in vitro* cultures, several studies have demonstrated that AIEC strains are able to compromise the epithelial-barrier structure due to the presence of several virulence mechanisms thus potentially contributing to its disruption and playing a role in the pathogenesis of CD. These mechanisms are listed below:

I. AIEC is able to adhere to and invade the intestinal mucosa and translocate across the human intestinal barrier, thus promoting mucosal colonization and tissue damage (163,164).
II. In CD patients there is an increase in the levels of particular receptors (i.e., CEACAM6, Gp96 or CHI3L1) which may facilitate AIEC adhesion and

invasion to IECs (163,165–167). Moreover, the already mentioned genetic deficiencies such as defects in the immune system have also been reported to contribute to excessive AIEC intracellular replication and persistent infection inside IECs (168–170).

III. AIEC is able to survive within macrophages while preventing cell apoptosis (171,172). Recognition and uptake by macrophages drive secretion of inflammatory cytokines thus promoting a constant inflammatory response that can also induce the formation of granulomas, common histopathological features of CD (173).

IV. AIEC can modulate host autophagy via the inhibition of *ATG5* and *ATG16L1* expression, which have already been reported to be diminished in ileal samples of CD patients (174).

In addition, dysregulation of apical junctional complex has been observed in IBD patients. This might be explained, in part, by AIEC infection as it may also stimulate higher epithelial permeability and a decrease in the transepithelial electrical resistance (TEER) by inducing a re-distribution of tight junctional proteins (175).

Altogether, AIEC leads to a disruption of the barrier function which, together with the inflammatory status of IBD patients, can prompt the loss of microbiome diversity and promote AIEC expansion (176–178), reinforcing the link between AIEC pathogenicity mechanisms and CD clinical manifestations.

Although a lot of effort has been put into the research of AIEC virulence molecular mechanisms and its relationship with CD pathogenesis, AIEC contribution to the disease progression remains unclear (the cause-effect relationship that we already mentioned before). Nonetheless, the assumption to consider AIEC as a pathobiont (bacteria that can be part of the normal microbiota but turns pathogenic under specific conditions) has gained plausibility.

In vivo studies have demonstrated that AIEC does not colonize mice spontaneously but under a particular context (177–180) such as gut inflammation caused by the presence of another pathogen (180), suggesting that AIEC alone could not lead to any inflammatory disease.

Moreover, it is known that AIEC is present in healthy subjects without inducing inflammation. Actually, AIEC capacity to damage a particular host depends on both

the microbial composition of the GI tract and the exposure of the host to other risk factors. Altogether, this evidence supports the most remarkable hypothesis regarding AIEC implication in CD, which associates these bacteria with the disease in a context of susceptibility thus considering this microorganism a pathobiont rather than a truly pathogen (181–185).

3.2 AIEC in Ulcerative Colitis

AIEC implication in UC is less clear than in CD. Nevertheless, recent studies suggest a similar prevalence of AIEC strains in both CD and UC, suggesting a stronger association of this bacteria with IBD than earlier thought (47,186).

Nadalian et al. conducted a data synthesis and meta-analysis study in which they estimate the overall prevalence of AIEC among CD and UC patients compared with non-IBD controls. To do so, they analyzed published data from 12 previous studies in 8 different countries (between 2004-2019). Eight out of the twelve analyzed studies presented data on the occurrence of AIEC in UC. According to this analysis, AIEC prevalence among UC patients and healthy controls was 12% and 5%, respectively. Thus, their results pave the way to a deeper study of the implication of this pathobiont in UC, trying to understand if its pathogenicity mechanisms in this disease are similar to those already described in CD.

4. AIEC pathogenicity

As mentioned, AIEC virulence mechanisms, while not unique to this pathobiont, allow its adhesion and invasion of IECs as well as its survival within macrophages. Therefore, AIEC is able to evade host defense mechanisms and to disrupt the intestinal epithelial barrier. Nevertheless, the vast majority of the available studies concerning AIEC virulence mechanisms only test a single AIEC strain (generally the LF82) and they mainly use human immortalized cell lines (Caco-2, Hep-2, and Intestine 407 (I407)) or murine derived cell lines (J774) (187). Even though these methods have so far allowed the identification of the virulence mechanisms of this pathobiont, new strategies might be essential to better comprehend the interaction of AIECs with the human gut, but also to unravel the pathophysiology that underlies inflammatory intestinal disorders.

4.1 AIEC and the Intestinal Epithelial Barrier

Even though the most well-known and defined AIEC characteristic is its ability to adhere to and invade IECs, before getting in contact with these cells, AIEC needs to cross the mucus layer. Virulence factors involved in early stages of mucosal invasion while evading host AMPs have been described in AIEC strains. Secretion of proteases enhances mucins degradation and therefore facilitates bacterial spread through the mucus layer (188–190). Moreover, proteins such as FliC (involved in flagella polymerization) have been reported to be involved in AIEC motility throughout the mucus layer (191,192). Although FliC is present in most of the enteric bacteria, its expression seems to be induced by the presence of mucus in AIEC strains but not in commensal bacteria (192). AIEC can also impact on the host ability to secrete AMPs through the presence of arlA and arlC genes (encoded in plasmids of some AIEC strains) (**Figure 7.1**) (193).

Once AIEC crosses the mucus layer, adhesion to and invasion of IECs starts. This process has been reported to occur through a micropinocytosis-like process in Hep-2 cells, where villi elongate and engulf the bacterium (155,194). A different mechanism has been recently reported for AIEC internalization using a colonic cell line – Caco2 – (195). In this case, uptake of AIEC via lipid rafts was proven.
Protein-receptor interactions may also induce AIEC internalization. Adhesion through type 1 pili is one of the most studied virulence mechanisms of the AIEC pathotype. It specifically binds to oligomannosidic glycans that are present on host cells surface. Remarkably, it has been hypothesized that AIEC strains preferably bind those glycans exposed on early apoptotic IECs to promote its invasion (196). Meprins, a group of proteases secreted by human IECs that degrade type-1 pili (among many other proteins and peptides), have been demonstrated to be decreased in CD patients, thus enhancing AIEC colonization (197). On the other hand, FimH (an adhesin whose expression has been described to be higher in AIEC compared to non-AIEC strains (192)) interacts with the CEACAM6 receptor (**Figure 7.2**) (165,166,191,198), which is found to be overexpressed on CD patients; an expression that can be further exacerbated after AIEC infection by the induction of pro-inflammatory cytokines secretion (199).

Flagella are also crucial in mediating AIEC-induced cellular responses through their binding to IECs-TLR5 (**Figure 7.2**) (200). Moreover, CHI3L1 and Gp96 receptors, which have been described to be highly expressed in ileal and colonic IECs of active CD patients respectively, bind to AIEC ChiA (167) and OmpA (201) proteins and promote adhesion and invasion of IECs (**Figure 7.2**). Of note, increased cytokine secretion induces CHI3L1 expression and, as a result, AIEC invasion of IECs is enhanced under inflammatory conditions. On the other hand, OmpA is the main component of the outer membrane vesicles (OMVs) (**Figure 7.2**).

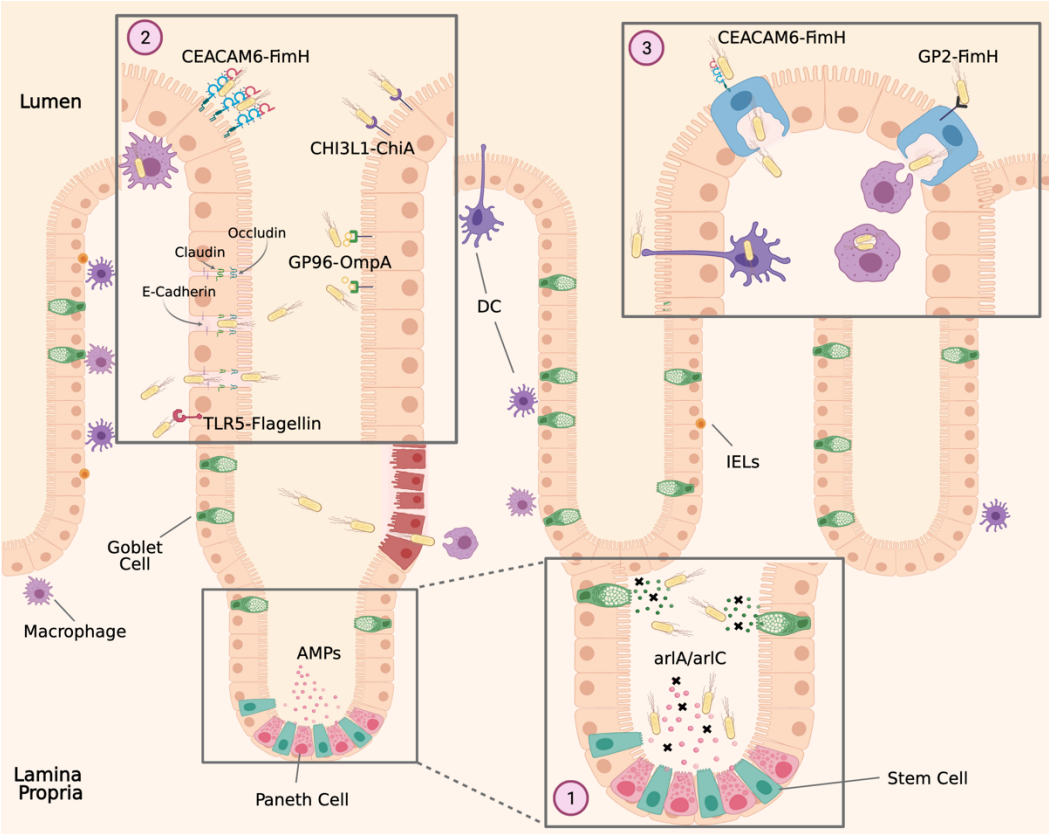


Figure 7. Interaction of adherent-invasive *Escherichia coli* (AIEC) with the intestinal mucosa in the context of IBD. (1) AIEC can cross the mucous layer and impact on the host ability to secrete AMPs through the presence of *arlA* and *arlC*; **(2)** AIEC adheres to intestinal epithelial cells (IECs) through interaction with some cell receptors leading to their invasion; **(3)** AIEC can interact with immune cells by penetrating the lamina propria and the Peyer's patches via M cells.
This Figure has been adapted from Palmela et al. (182) and created with BioRender.

OMVs can deliver virulence factors into host cells that contribute to the invasion process when in contact with IECs through the interaction of OmpA-Gp96 (202). In fact, Rolhion et al. stated that internalization of AIEC in the ileal mucosa of CD patients takes place in part by means of the interaction of OmpA and Gp96 rather than the quantity of OMVs released (201).

Other mechanisms, independent from the mentioned above, help AIEC translocation through cell-junction modifications or M-cells internalization. As we mentioned, once cell-to-cell junctions are altered, cell structures are modified, and epithelial permeability is increased. Dysregulation of tight junctions has been observed in IBD patients (203–206) and AIEC has been described to disrupt these complexes while reducing the TEER (207). Some *in vitro* studies support the view that AIEC LF82 strain, as well as other invasive *E. coli* strains, use E-cadherin displacement as a potential mechanism to decrease epithelial barrier integrity (175,207) (**Figure 7.2**).

Moreover, AIEC-induced cytokine-release by macrophages can impact the expression of several cell-junction components on epithelial cells. In fact, ZO-1 (175,207) and occludin (204) are down-regulated resulting in increased gaps between cells (**Figure 7.2**).

In addition, AIEC have evolved to use M-cells as a gateway to invade the epithelium without the loss of the barrier integrity (208). AIEC translocation through M-cells occurs via type-1 pili interaction with GP2 (**Figure 7.3**) and via the binding of LpfA to a receptor that has not yet been identified (163,209).

During AIEC invasion several inflammatory mechanisms are activated. AIEC interaction with IECs induces secretion of several cytokines (i.e., IL-8, TNFα and IFNγ) which, in turn, enhance transmigration of immune cells and reduction of the epithelial barrier resistance (199,207). Moreover, AIEC blocks STAT1 activation after IFNγ stimulation in IECs (210). By intercepting this pathway, inflammatory responses to microbial infections are impaired: lower numbers of immune cells move to the infection site and the transcription of IFNγ-dependent genes is abrogated (210).

Cell-to-cell communication via exosomes – small membrane vesicles that can be released from different cell types such as IECs and macrophages (211) – might be considered also as an alternative pathway for AIEC infection. Exosomes are generally involved in several immune regulatory processes (212). After AIEC infection, high amounts of exosomes are released, nuclear factor kappa B (NF-kB) pathway is activated, and IL-8 production occurs without damaging the integrity of the epithelial barrier (213).

Altogether, AIEC proteins and mechanisms responsible for mediation of host-bacteria interaction enhance AIEC fitness and gives a selective advantage to other strains in the gut.

4.2 AIEC and Immune Cells

The immune cells located in the lamina propria are in charge of killing pathogens. Nonetheless, AIEC have been shown to be able to survive and replicate within macrophages while preventing cell apoptosis (160,168,171,172). The ability to survive and replicate inside host macrophages implies supporting environments with low pH, low nutrient content, and high oxidative and stress conditions (160,214–216).

Currently, the mechanism by which AIEC evade cell-killing processes and adapt to the phagolysosome environment is still poorly understood. It seems that after internalization in macrophages, AIEC is able to form large vacuoles rather than escape from them (214,215). Vacuoles have been reported to probably induce AIEC persistence by disabling this pathobiont to replicate intracellularly (215).

At this point, AIEC induces the secretion of large amounts of pro-inflammatory cytokines, particularly TNFα, without inducing host cell death (160,214). In fact, the amount of TNFα secreted positively correlates with the quantity of intracellular bacteria (172). This cytokine production induces damages to the intestinal epithelia thus favoring AIEC colonization. Nevertheless, once TNFα levels are lower, intramacrophage-bacterial loads are reduced (215).

Moreover, the LF82 strain has been proved to be able to invade and replicate within human neutrophils, but contrary to its behavior inside macrophages and IEC, AIEC induces the autophagic death of the infected neutrophils (217). Additionally, LF82 can replicate within monocytes isolated from patients with CD for the first 20 hours (h) after infection before being cleared (218).

4.3 AIEC and Cytokine Production

As we introduced, some AIEC strains induce increased expression of TNFα, IFNγ and IL-8 in IECs and macrophages (182). Transcripts of these cytokines have been detected to be increased in colonic biopsies of patients with CD after AIEC infection. This process has been demonstrated to affect the cell cycle distribution in Caco-2 cell lines (199).

In addition, flagella can trigger IL-8 and CCL20 secretion in polarized IECs, which in turn leads to the recruitment of macrophages and dendritic cells to the site of infection (219,220). IFN-γ and TNF-α secretion by macrophages and lymphocytes also leads to increased CEACAM6 expression, which further enhances AIEC colonization.

Finally, AIEC also modulate the turnover of the ubiquitin proteasome system in infected IECs by means of NF-kB activation thus preventing host protection from an over-reactive inflammatory response (221).

4.4 AIEC and Autophagy

In general, bacteria invading macrophages or IECs are rapidly targeted to follow a lysosomal degradative pathway specialized in the identification and elimination of intracellular pathogens, known as autophagy. As mentioned in Section 1, CD patients have mutations in some genes (*ATGL16L1, IRGM* and *NOD2*) that might impair autophagic responses (168). Hence, given that autophagy restricts AIEC intracellular replication, gene defects in this pathway could contribute to the overgrowth of this bacteria. However, AIEC have developed mechanisms to abolish autophagy. As we already mentioned, it has been reported that intracellular LF82 activates NF-kB (174,222), which leads to a decrease in expression of the autophagy mediators ATG5 and ATG16L1. Hence, AIEC is able to inhibit autophagy and thus enhance the inflammatory response.

5. AIEC Characterization Methods

Even though a lot is known about AIEC mechanisms of pathogenicity, deciphering molecular markers for the rapid identification of this pathotype is of great importance. This would help determine AIEC colonized patients, prevalence, abundance, host range or transmission paths which, in turn, might pave the way to prevent AIEC infection or provide personalized treatment for AIEC carriers. One of the main reasons that hamper progress in this area is the absence of an AIEC-dependent molecular signature. Therefore, a lot of effort has been put into the research of putative genetic and phenotypic markers for rapid AIEC identification.

First studies, based on polymerase chain reaction (PCR)-based gene prevalence (159,223), demonstrated that AIEC strains did not harbor a genetic signature that could distinguish

them from commensal *E. coli* nor present identical virulence genes already described in other *E. coli* pathotypes. Even though PCR-based studies focusing on gene content reported some genes to be more prevalent in AIEC versus non-AIEC strains, differences were not significant except for one gene (187).

In 2010, the first AIEC genomes were sequenced. This paved the way to comparative genomic studies that attempted to elucidate the AIEC-genome characteristics in order to identify a genetic biomarker (224). Nevertheless, still no gene or genetic sequence have yet been identified as exclusive for AIEC pathotype. As a consequence, whole genome analysis of SNPs attracted attention. The first study, which dates from 2015, used this methodology and identified 29 SNPs that could differentiate 4 AIEC strains from commensal and ExPEC strains, but unfortunately these SNPs were no exclusive for the AIEC pathotype (225).

Hence, AIEC identification currently relies on phenotypic traits requiring cell-line-culture infection assays. In order to go one step further, the use of primary human-derived bidimensional (2D) culture instead of immortalized cell lines might overcome all the limitations that these could present thus serving as a useful model to study the host-pathogen interaction to better understand the pathogenicity mechanisms of this pathotype.

SECTION 4: Postbiotics and Intestinal Epithelial Cells

1. Postbiotics definition

The relationship between the intestinal mucosa and the microbiota is considered a synergistic one. Gut microorganisms rely on host's metabolites to grow, while at the same time they produce small molecules that regulate their self-growth or enhance other species' development and protection. Moreover, some of these metabolites (secreted by live bacteria or after bacterial lysis) released into the host environment are known to have beneficial effects for the host. In addition, they have been proposed as potential surrogate markers of disease exacerbation, as has already been proven in cardiovascular disease (226–228).

As reviewed elsewhere, (226,229–233) a limited but diversified group of microbially-derived metabolites (SCFAs, indole derivatives, polyamines, secondary bile acids, bacterial polysaccharides, vitamins, or adenosine triphosphate) has been extensively studied over the past two decades.

Nevertheless, bacteria live in complex communities and IECs are constantly exposed to the sum of all gut bacteria-derived metabolites, rather than a specific strain or single metabolite. Both independently and collectively, microbial metabolites shape the immune system promoting, in the case of probiotic-derived metabolites, mainly beneficial effects (227,234). The metabolic cocktail composed of soluble factors secreted by life probiotic bacteria – living microorganisms which, when administered in adequate amounts, confer health benefits on the host (53,235–238)– or any bacterial-released molecule capable of providing health benefits through a direct or indirect mechanism, has been collectively known as postbiotics since 2012 (53). In other reports, they have also been referred to as metabiotics, biogenics, or cell-free supernatants (239,240).

Different approaches could be employed to define "types" of postbiotics; for example, by their physiological function or effect – either local or systemic – (immunomodulatory, anti-inflammatory, anti-microbial, hypocholesterolemic, anti-obesogenic, anti-hypertensive, anti-proliferative, anti-oxidant) (**Figure 8**); by their components (if they are mainly composed of proteins, lipids, carbohydrates, organic acids, etc.); or by their bacterial sources (*Lactobacillus* spp., *Streptococcus* spp., *Bifidobacterium* spp., *Escherichia* spp., or *F. prausnitzii*, among many other probiotic strains) (241,242). Given the fact that a postbiotic is composed of a complex mixture of metabolites derived from one or more bacterial strains and is thus characterized

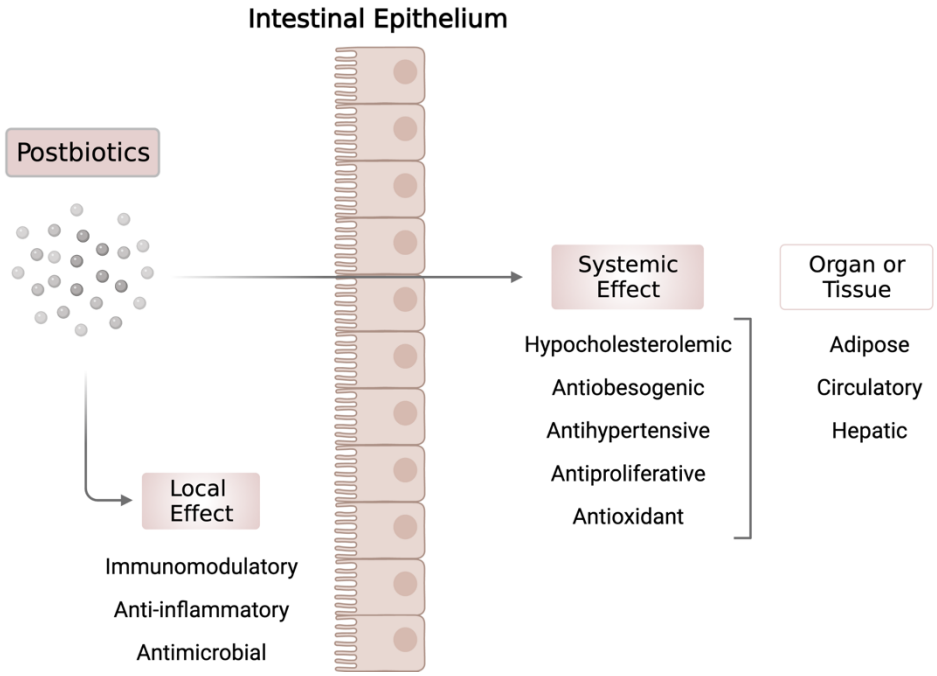


Figure 8. Postbiotics and their potential local and systemic beneficial effects in the host. One of the available postbiotics classification has been determined according to their physiological function or effects (local or systemic). Therefore, postbiotics can exert immunomodulatory, anti-inflammatory, anti-microbial, hypocholesterolemic, anti-obesogenic, anti-hypertensive, anti-proliferative or anti-oxidant effects.
This Figure has been adapted from J.E. Aguilar-Toalá et al. (227) and created with BioRender.

by a wide variety of different effects, sources, and compositions, the classification of postbiotics as a whole remains a challenging task.

2. Postbiotics production and characterization

Postbiotics' production encompasses diverse techniques such as enzymatic treatment, sonication, heat application, centrifugation and filtration, dialysis, freeze-drying, or column purification. Once collected, identification of its components is not a trivial task and can require several analytical approaches. Some of the more commonly employed techniques include matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), high performance liquid chromatography (HPLC), high-field proton nuclear magnetic resonance spectroscopy, ultra-performance liquid chromatography (UPLC), or liquid chromatography mass spectrometry (LC-MS and LC-MSMS) (226,227,243). Those are only some of the preferred options to elucidate postbiotics'

composition. Choosing any of these procedures is a decision that will always depend on the need for quantitative or qualitative characterization, as well as the nature of the molecules to be identified.

In fact, most of the published studies about postbiotics have not provided a description of the molecular composition underlying this "metabolite-cocktail" (244–248). Optimizing all the available methods to determine each postbiotic component will be essential in order to dissect their molecular mechanisms of action, which are currently unknown. Nonetheless, the effects of postbiotics on the intestinal epithelium are being intensively studied as a whole. In fact, and as we already mentioned, what researchers conceive of as postbiotics are "the set of microbial molecules" rather than single metabolites and therefore, the putative beneficial effect than postbiotics might exert is always due to the sum of every single metabolite by which it is constituted (249).

3. The impact of postbiotics in health and disease

Postbiotics have recently been proposed as food supplements to promote intestinal homeostasis in lieu of probiotics when the use of live bacteria may pose some risks to the patient due to the presence of microbe-associated molecular patterns (MAMPs) that potentially activate innate immunity and could further promote inflammation, such as in cases involving inflammatory GI diseases. In that sense, postbiotics are thought to mimic probiotics' beneficial effects while avoiding the risks of administering live bacteria (53).

During the past years, the effect of microbial-derived molecules has been mainly studied individually by focusing on understanding the response of the human body to a single microbial molecule. The actions of individual metabolites need to be considered before the sophisticated interplay of hundreds of them can be deciphered. For that reason, butyrate, polyamines, vitamins, bile acids or indole derivatives – among many others – are being proposed as food supplements due to their immunomodulatory or anti-inflammatory properties (250).

Nonetheless, some microbial molecules interact among themselves to finally exert their various effects. Thus, postbiotics might be the best models for a better understanding of the convoluted interactions that persistently occur within our body.

To date, several studies suggest that these benefits (anti-inflammatory, antioxidant, antimicrobial, etc.) are better than those conferred by probiotics, concluding that postbiotics exert their effects with improved safety profiles (53,245,248,251). Moreover, several disadvantages position probiotics backward of postbiotics: 1) probiotic strains might have

antibiotic resistant genes that can be acquired by pathogenic bacteria through horizontal gene transfer (252); 2) using probiotics in any product requires the viability of bacterial cells to be maintained in order to ensure accurate administration of the desired amount of microorganisms. This can be easily altered by different variables such as temperature, pH or interaction with other microbes (253); 3) when probiotics colonize the gut, they may inhibit the return of the indigenous microbiome (254,255).

Table 3. Current available Clinical Trials using postbiotics to treat or prevent gastrointestinal disorders

Name of the study	Phase	Status	Pathology/ Condition	Drug/ Intervention	Location
Effect of POSTbiotics Supplementation on Microbiome in OBese Children: the POST-OB Study	Phase 4	Recruiting	Childhood obesity	Vitamin D3 and Immunofos	Ospedale San Paolo, Milan - Italy
Randomised, Controlled Study to Assess Safety and Tolerance of Infant Formula With Prebiotics and Postbiotics in Healthy Infants	N.A.	Not yet recruiting	Healthy Term Infants	Milk Based Infant Formula	Nutricia Research
To Assess the Safety and Tolerance of Infant Formula With Locust Bean Gum in Infants With Regurgitation	N.A.	Recruiting	Regurgitation	Milk Based Anti-Regurgitation Infant Formula	Poliklinika Ginekologicz no-Położnicza Sp. z o.o. Sp. k. Białystok - Poland
Gut Health, Inflammation, Hormones	N.A.	Active, not recruiting	Aging Well	VMK223 and cellulose	University of Roehampton, London - United Kingdom
MS-20 on Patients With Ulcerative Colitis (UC)	N.A.	Not yet recruiting	Ulcerative Colitis	MS-20 oral solution	National Taiwan University Hospital
Gastrointestinal Tolerance and Safety of an Infant Formula Containing Prebiotics, Probiotics and Postbiotics.	N.A.	Recruiting	Gastrointestinal Tolerance	Infant formula containing prebiotics, probiotics and postbiotics	Poliklinika Ginekologicz no-Położnicza Sp. z o.o. Sp. k. Białystok - Poland

N.A.: Not Applicable. This Table has been created with the available information at www.clinicaltrials.gov site by searching for "postbiotics".

Despite the challenge of translating all this scientific knowledge to a clinical setting, there are already six ongoing clinical trials using postbiotics in infant formula, to treat obese patients or to improve UC severity (**Table 3**). Results are not yet available since most of them are still in the recruiting phase.

In addition, several cell-free products are already commercially available. These include Colibiogen (Laves-Arzneimittel GmbH, Schötz, Switzerland), a product derived from *E. coli* Laves 1931 cultures that has been shown to be effective in reducing skin lesions from patients with polymorphous light eruptions (256–258). Hylak Forte (Ratiopharm/Merckle GmbH, Germany) is another bacterial-free liquid containing metabolic products from different bacterial strains (*E. coli, Streptococcus faecalis, Lactobacillus acidophilus*, and *Lactobacillus helveticus*), which has been proven to control salmonellosis in infants and intestinal dysbiosis in patients with chronic gastritis. Moreover, it also reduces diarrhea induced by radiation in oncologic patients (259–261). CytoFlora (BioRay Inc., Laguna Hills, CA, USA) is composed of several microorganism-cell-wall lysates and it also helps in correcting intestinal dysbiosis (262). Another commercialized set of products is the MATRIX line (Smartfarma, Milan, Italy), which includes three baby-care vitaminic and mineral supplements (Smart D3 Matrix, Polivit Matrix, and IdraMatrix). All three contain ImmunoFOS (Postbiotica, Milan, Italy), a patented postbiotic produced using an innovative fermentative process (PBTECH) that helps strengthen the immune system and restore the intestinal microbiota (263,264). This same postbiotic is used in a pet-care product: Renal N (Candioli Pharma, Beinasco, Piemonte, Italy), a group of antioxidant products that favor pets' immune defense and restores normal intestinal functionality. In fact, postbiotics have also been studied for animal care, as reported by Izuddin et al. (265,266).

Therefore, the available data suggests that postbiotics might be a safer alternative for treating intestinal inflammatory diseases due to their lack of immune activating molecules (i.e., PAMPs, MAMPs). Even though some of them are already commercialized, further investigation is needed to completely characterize their exact composition and determine their mechanisms of action.

4. Use of Postbiotics to attenuate Intestinal Inflammation

Even though this is a very recent field, a lot of effort is being put into the elucidation of the putative beneficial effects of metabolites derived from probiotics. To do so, postbiotics are generally tested in the presence of a strong inflammatory agent in order to elucidate their potency and mechanisms of action.

With this purpose, several strategies are being applied, from human immortalized cell lines to *in vivo* assays, going through *ex vivo* cultures such as intestinal organoids or tissue explants, among others. As we will briefly introduce in this section, most of the available reports using these techniques have demonstrated the feasibility of studying postbiotics using the intestinal epithelium (250).

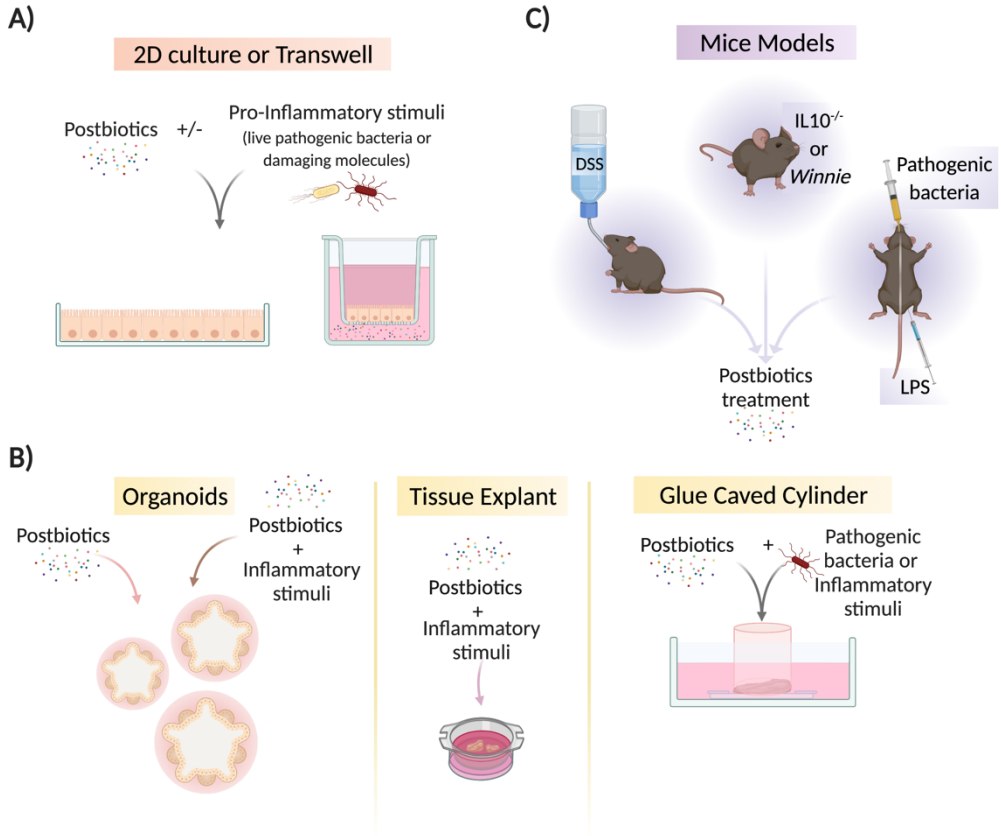


Figure 9. Schematic representation of the available models for the study of postbiotics effects in the intestine. A) *In vitro* models mainly consist of cell lines seeded as monolayers in culture plates (left) or Transwells (right). Transwells have been used to collect the apical and basolateral supernatant after postbiotics treatment of the selected cell-culture. On the other hand, cells seeded in plates can be used to test postbiotics' properties, for example, in the absence or presence of a pro-inflammatory stimuli. Both *in vitro* approaches help in the initial screening of probiotic-derived molecules to determine their beneficial effects. **B)** To test postbiotics using *ex vivo* cultures, the currently available studies have used epithelial organoids, intestinal tissue explants, and the glue caved cylinder. By exposing the cultures to either a pro-inflammatory stimulus, pathogenic bacteria, and/or postbiotics, the effects of the latter on mice or human samples can be studied. **C)** *In vivo* experimental models of intestinal inflammation (e.g., the acute model of DSS-induced colitis, the spontaneous model developed by IL10$^{-/-}$ (knock out) or Winnie mice, or mice inoculated with pathogenic bacteria or LPS) have aided investigations of the potential therapeutic effects of postbiotics. Postbiotics have been administered intra-gastrically, -peritoneally or -rectally. Improvement in colitis recovery as well as enhancement of the immune response are some of the benefits that have been attributed to postbiotics thanks to these studies.
This Figure has been adapted from Mayorgas et al. (250) and created with BioRender.

In vitro strategies represent the first approach to characterize the biological effects of postbiotics as they offer many advantages (**Figure 9A**). In fact, by using cell lines seeded on a Transwell chamber, researchers have demonstrated the anti-inflammatory properties of lactic acid bacteria-secreted metabolites after their interaction with the intestinal epithelium (267). Cytotoxic and antiproliferative effects on intestinal epithelial cells have also been proved by the use of cell lines (239,244,268–272).

These cell cultures were also used to study the capacity of postbiotics to block the binding of pathogenic bacteria. In fact, Mack et al. demonstrated that a determined probiotic strain and its derived metabolites equally reduced the adherence of pathogenic bacteria to intestinal cells. Therefore, they were able to demonstrate that some of the beneficial effects of the probiotic strains were derived from their secreted metabolites (251,273).

Overall, the available studies demonstrate that human intestinal cell lines are an effective and useful tool for initially screening the possible beneficial effects of postbiotics (either anti-inflammatory, antiproliferative, anti-adherent, or anti-invasive) on the intestinal epithelium.

Nevertheless, and as we will detail in later sections, despite their many advantages, immortalized cell lines cannot reproduce the conditions in tissues and end up posing some limitations. Therefore, researchers frequently opt for *in vivo* experimental models (250). These models allow for a complete study of the effects that the tested product is exerting. *In vivo* models are not only one of the most advantageous tools in research, but also a necessary one before a product or drug can reach clinical development. Furthermore, *in vivo* experiments are not only desirable to test efficacy of a candidate drug, but necessary to determine its potential toxicity. Definitely, *in vivo* models have been used to fully understand the role of postbiotics – administered by oral gavage, intraperitoneally, or intrarectally – in improving gut health and life nutrition. These studies can be performed with a broad range of animal models: from spontaneous chronic colitis in IL-10 knock out mice to dextran sulfate sodium (DSS)-induced acute colitis, in addition to models of inflammation induced by lipopolysaccharide (LPS) (243) or life pathogenic bacteria (**Figure 9C**) (274). *In vivo* studies are essential to understand how postbiotics act in a multi-organ life system, their capacity to enhance the immune system by boosting cell differentiation, their ability to strengthen cell-to-cell attachments to improve the intestinal epithelial barrier, and their capability to increase mucin production for enhanced protection of the whole intestinal tissue. Altogether, several studies have demonstrated that postbiotics do provide protection in models of intestinal inflammation by reducing weight loss, lowering the impact of inflammation on the epithelial

structure and, on the whole, by stimulating the immune system to help protecting the body against intestinal immune diseases (243,245–247,274–278).

Nonetheless, when an *in vivo* strategy is not accessible, *ex vivo* cultures become a useful tool since they offer the possibility to model the pathophysiology of the gut. Intestinal epithelial organoids (**Figure 9B**) have been used to study the impact of postbiotics on host peripheral lipid metabolism and histone acetylation (279) as well as to explore the anti-inflammatory effects of certain postbiotics. In the latter case, colonic organoids from healthy individuals, as well as from CD and UC patients were generated in order to determine the anti-inflammatory effects of postbiotics derived from different bacterial strains (246). Other strategies such as tissue explants or the glued caved cylinder have been proposed as more physiological culture types (**Figure 9B**). In fact, the latter is able to maintain apical to basolateral polarity during stimulation via the use of a glued cylinder. This culture system was used to confirm the immunomodulatory properties of *Lactobacillus*-secreted metabolites against bacterial infection (53).

Nevertheless, new strategies might be applied to better understand postbiotics' effects. More specifically, due to their polarized distribution, organoid-derived 2D cultures generated from human samples, in lieu of tridimensional (3D) organoids, could provide a better approach to study the anti-adhesive, anti-invasive, or anti-inflammatory properties of postbiotics (280).

SECTION 5: Use of Human Primary Organoid-Derived Monolayers to study Bacterial-Epithelium Interactions

As introduced in previous section, different culture systems have been used to study the pathophysiology of several GI diseases, such as IBD, at the epithelial level. Immortalized cell lines (*in vitro* 2D cultures), are easy to obtain, handle and share among research groups. They can also be expanded and used over time with reproducible results. Nevertheless, cell lines lack important physiological features such as tissue cytoarchitecture, inter-individual variability and gut location-specific attributes; in fact, they do not represent all of the epithelial cell types that are known to populate the intestinal epithelium. These limitations can be overcome by using human primary cultures. Epithelial cells isolated from their *in vivo* environment and cultured *ex vivo*, provide an experimental set up that is closer to physiology. This is possible due to a well-conserved cytoarchitecture, as well as to the maintenance of most of the intercellular connections and interactions (281).

As an example, epithelial organoid cultures generated from intestinal samples mimic the epithelial phenotype of origin and thus represent a promising tool for studying the physiopathology of the intestinal epithelium (282–284). Gut-derived organoids have been extensively used for the study of particular microbial metabolites and even whole bacteria-epithelium interactions (285–288). Nonetheless, they pose some limitations, such as difficult accessibility on the apical side. This has also prompted the investigation, development and use of other relevant strategies easier to handle such as organoid-derived monolayers (ODMs). This *ex vivo* cell culture exhibits an appropriate cell polarization and orientation for a more physiological bacteria- and/or microbial metabolites-host cell interplay, thus representing a powerful tool for the study of IBD pathophysiology. Different methods have been reported for generating short-term cultures of polarized cell monolayers derived from mechanically and enzymatically dissociated organoid cultures (129,280,289–294). These studies also demonstrated that ODMs can give rise to most of the differentiated cell lineages that are present in the intestinal crypts. This is a crucial point that must be considered when studying IBD, where an alteration on the intestinal epithelial composition has been proved (280,295).

During the last years, a lot of effort have been put into demonstrating the usefulness of this culture type to study host-bacterial interactions. Upon apical infection of ODMs with pathogenic *Listeria monocytogenes*, *Shigella flexneri, Salmonella enterica Typhi*, or shiga toxin-producing *Escherichia coli* (STEC), TEER and fluorescein isothiocyanate–dextran assays showed increased permeability in organoid monolayers (296). Infected cultures also showed

an increased secretion of IL-8, along with other pro-inflammatory responses unique to each pathogen (297–300). Similarly, another study added flagellin to a colonoid-derived monolayer and observed that TLR5 activation promoted IL-8 secretion (301). In contrast, Ruan et al. demonstrated that the addition of other pathogenic bacterial products (including lipoteichoic acid or LPS) to enteroid-derived monolayers did not trigger IEC innate immune responses (302).

Moreover, Sayed et al. have recently published a study in which AIEC infection of organoid-derived 2D cultures is applied to explore host engulfment in IBD (293). Thus, their research clearly supports the suitability of ODMs as a tool to study AIEC pathogenicity.

Therefore, these and other recent studies pave the way to the use of ODMs to study microbial-epithelium interactions (292,297,303–305) endorsing the advantages of epithelial monolayers over 3D organoids (289,290,306,307). Overall, ODMs may represent a very useful tool in the study of the intestinal epithelium, both in healthy and diseased status, as well as its response to any external agent involved in the pathophysiology of GI diseases. Hence, in this thesis we have employed this *ex vivo* culture for the study of IECs-infection by AIEC as well as the elucidation of the putative beneficial properties of postbiotics in the intestinal epithelium.

OBJECTIVES

STUDY 1: Determination of AIEC capability to invade Organoid-Derived Epithelial Monolayers and its derivative effects on the Intestinal Epithelium

Since two decades ago, AIEC has been reported to be associated with the pathogenesis of IBD (181,182,308,309). To date, the only available approach to identify AIEC strains is to evaluate their ability to adhere to and invade IECs and to survive within macrophages by using *in vitro* cultures (187). *In vivo* strategies have also helped characterize the invasive strategy of this pathobiont. Altogether, both *in vitro* and *in vivo* methods have supported the study of AIEC's mechanisms of pathogenicity including its capacity to cross the intestinal mucosal layer, its interaction with IECs-receptors used to facilitate IECs' invasion, its effects on the epithelial barrier function or their evasion of the immune response (162,310).
Nevertheless, the use of *ex vivo* cultures such as human primary isolated IECs (i.e., organoid-derived monolayers) might provide a better experimental setting more closely reflecting the tissue of origin, phenotype, and structure. Infecting human organoid-derived monolayers could serve as a useful model to study the host-pathogen interaction to better understand the pathogenicity mechanisms of this pathotype. Overall, this approach could lead to the discovery of new disease biomarkers and therapeutic targets. Therefore, **we hypothesize that human primary organoid-derived monolayers (ODMs) could serve as a tool to understand AIEC pathogenicity in human intestine.**

For that purpose, the specific objectives of this study were:

- To generate and characterize ODMs and differentiated ODMs (d-ODMs) from healthy human colonic samples.
- To establish an infection method using colonic d-ODMs and examine the ability of AIECs to adhere to and invade primary human epithelial cells.
- To evaluate the gene and protein expression changes induced by AIEC-infection on d-ODMs.

STUDY 2: Production of Postbiotics and their effects on Human Blood Immune Cells and Organoid-Derived Epithelial Monolayers

Most of the available studies exploring the effects of bacterial metabolites on the gut epithelium have used isolated single molecules (311–314). Nevertheless, the intestinal epithelium is constantly exposed to complex bacterial communities and consequently their secreted metabolites, thus gathering importance the study of the complex metabolic cocktails to unveil their potential effects.

Few years ago, Tsilingiri et al. demonstrated that the whole set of bioactive compounds produced by probiotics (beneficial bacteria) can provide health benefits through direct or indirect mechanisms. In fact, these group of metabolites, now called postbiotics, were shown to exert stronger immunomodulatory properties in both human blood immune cells and IECs compared to probiotics (53).

To date, several bacterial strains – mainly lactic acid bacteria – have been used to produce postbiotics and different culture methods have been applied to elucidate their properties (227,232,250). Moreover, some probiotic strains – such as *Streptococcus salivarius* subs. *thermophilus* and *Escherichia coli* Nissle (315–325) – have been already proved to ameliorate IBD. Nevertheless, the potential beneficial effects of their secreted metabolites have not yet been deeply studied.

Therefore, **here we hypothesize that postbiotics from *Streptococcus salivarius* subs. *thermophilus* and *Escherichia coli* Nissle could exert an immunomodulatory effect on human blood immune cells and the intestinal epithelium.**

To test our hypothesis, we proposed the following specific objectives:

- To produce and characterize postbiotics derived from *Streptococcus salivarius subs. thermophilus* and *Escherichia coli* Nissle.
- To test the immunomodulatory properties of the produced postbiotics on peripheral blood mononuclear cells (PBMCs) and monocyte-derived dendritic cells (moDCs).
- To analyze the effects of the selected postbiotics on the transcriptional profile of both uninflamed and artificially-inflamed d-ODMs.

MATERIALS&METHODS

I. Human Primary Organoid-Derived Epithelial Monolayer (ODM) Generation

1. Patients population and sample collection

Healthy intestinal mucosal samples were obtained from patients undergoing surgery for left-sided colorectal cancer (CRC) or routine endoscopy for CRC screening. For surgical pieces, a segment of healthy mucosa was collected at least 10 cm from the margin of the affected area. Biopsy samples showed no evidence of neoplastic lesions. However, samples were not specifically assessed for signs of microscopic inflammation.

All patients were recruited at the Department of Gastroenterology, Hospital Clinic de Barcelona, after obtaining written informed consent. The study protocol was approved by the Ethics Committee of the Hospital Clinic of Barcelona (registration number HCB/2016/0546).

Blood samples from healthy donors were directly processed for peripheral blood mononuclear cells (PBMCs) isolation. Samples were obtained at the Department of Gastroenterology – Hospital Clinic de Barcelona –, the Ospedale Pavia (Pavia, Italy) and at the Banc de Sang i Teixits (Barcelona, Spain). **Table 4** and **5** show the clinical and demographic characteristics of the subjects enrolled to develop each of the studies described below.

Table 4. Clinical and demographic characteristics of the subjects enrolled in the first study.

N	Age (years)	Gender (M/F)	Sample Type (Surgical/Biopsy)
Group 1 (ODMs/d-ODM characterization: qPCR)			
5	53-74 (55.6)	3/2	4/1
Group 2 (d-ODMs culture: AIEC infection and qPCR)			
13	52-69 (59.5)	7/6	5/8
Group 3 (ODMs/d-ODMs culture: Immunofluorescence)			
8	52-72 (55.05)	6/2	3/5

N: number of subjects included in the analysis.
Age: Range (mean).
qPCR: quantitative real-time PCR

Table 5. Clinical and demographic characteristics of the subjects enrolled in the second study.

N	Age (years)	Gender (M/F)	Sample Type (Whole blood/Buffy coat) (Surgical/Biopsy)
Group 1 (blood samples)			
13	24-32 (27,8)	3/3	6/**7***
Group 2 (d-ODMs for RNAseq)			
4	54-94 (69.5)	4/0	3/1

N: number of subjects included in the analysis.
Age: Range (mean).
***** Demographic and clinical data from buffy coat healthy donors was not available due to privacy issues. We can however ensure that the included donors were adults between the ages of 18-90.

2. Crypts Isolation

The fresh colonic tissue samples (1-2 cm^2 surgical sample or 6-8 biopsies) were collected on a conical tube containing Hank's Balanced Salt Solution (HBSS; Lonza) at room temperature (RT). Samples were immediately processed or kept at 4°C before proceeding with the protocol (326–329).

Only for surgical samples, the submucosa and large contaminating blood vessels were removed using scissors and a scalpel after washing them with cold Dulbecco's phosphate-buffered saline without Ca^{2+} and Mg^{2+} (DPBS; Gibco). Then, pieces of 0.5-1 cm^2 were cut and transferred to a new conical tube.

After one or two washes with cold DPBS by gentle manual flipping for a few seconds, samples were incubated with antimicrobial cocktail (**see Annex I Table 1**) for 20 minutes (min) at RT under gentle agitation on a platform rocker. After 1-2 additional washes with DPBS at RT to remove the antimicrobial cocktail, the samples processing differed according to their origin: surgical samples were incubated with 10 mM dithiothreitol (DTT; Roche) in DPBS for 8 min at RT under gentle agitation on a platform rocker to remove the mucus, washed with DPBS and submerged in crypt isolation buffer (**see Annex I Table 2**). For endoscopic biopsies, samples were incubated in crypt isolation buffer containing 500 µM DTT. All samples were then incubated for 45 min at 4ºC under gentle agitation.

Samples were again resuspended in cold DPBS and vigorous hand shaking was then performed for 15-30 seconds for crypts release. Crypts were collected and inactivated Fetal

Bovine Serum (FBS; Applied Biosystems) was added to a 5% final concentration to maintain cell viability. This step was repeated until the desired number of crypts was obtained.
The crypt fractions were centrifuged at 150g for 3 min at 4°C. Each crypt pellet was then gently resuspended in cold Washing Medium (WM; **see Annex I Table 3**). All fractions were merged in one single conical tube for an optimal crypt count [1].

3. Crypt Culture

After spinning the samples at 150-200g for 3 min at 4°C, the desired number of crypts was carefully resuspended in ice-cold Matrigel (Corning) considering that each Matrigel dome should contain 80-100 crypts. Crypt-Matrigel drops (25 µl/each per well) were seeded in pre-warmed 48-well plates and incubated with 250 µl of STEM medium (**see Annex I Table 4**) at 37ºC 5% CO_2. The crypt culture was usually ready to be expanded after 2 days.

4. Crypts and Organoids Culture Passage

As mentioned, 2 days after crypt seeding, an expansion step was required. The same procedure was performed to passage a previously expanded epithelial organoid culture (EpOC), 5-6 days after seeding. As a rule, the dilution rate was 1:2-1:3 for the crypt culture, and 1:4-1:5 for previously expanded EpOCs.
First, the STEM medium was removed, and Matrigel-embedded domes were washed once with 300 µl cold DPBS per well. The same volume of Cell-Recovery solution (Corning) was added to each Matrigel drop. Drops containing either colonic crypts or EpOCs were collected in ice-cold 15 ml conical tubes.
After 30-40 min incubation on ice, inverting the tubes every 5-10 min to facilitate Matrigel depolymerization, each tube was filled with additional 5-7 ml cold WM and spined at 400g for 3 min at 4°C. The pellet was resuspended with 5 ml/tube of pre-warmed dissociation solution (**see Annex I Table 5**). Cells were then incubated at 37ºC for 15-20 min.
Subsequently, mechanic dissociation was accomplished with a G20-G21 needle mounted on a 5 ml syringe. Dissociated cells were centrifuged at 800g for 4 min at 4°C and washed 3 times with 5 ml cold WM at the same speed (at this point, mechanically dissociated EpOCs could be alternatively used for generating a 2D culture as we will detail in the next section).

[1] Since the number of crypts isolated from biopsy samples is limited, we usually skipped this step and directly embedded the crypts in Matrigel (see next section, *3.Crypt Culture*). Based on our experience, we calculate that on average, 12-16 Matrigel drops of 25 µl each are obtained from 8 biopsies.

The last washing step was performed on a 1.5 ml tube. After that, the cell pellet was resuspended in Matrigel supplemented with 10 μM Y-27632 (Merck).
Matrigel drops (22 μl/each) were seeded in pre-warmed 48-well plates (1 drop/well) and incubated with 250 μl of STEM medium supplemented with 10 μM Y-27632.
The medium was changed with fresh STEM medium (without Y-27632) every 48h. EpOCs grown for 5-6 days were usually ready to be used for downstream applications or for further expansion repeating this protocol.

5. Monolayer culture generation

Monolayer cultures were generated from EpOCs after 5 days of expansion. Prior to EpOCs dissociation, 48-well plates were pre-coated with a thin layer of diluted (1:20) Matrigel in DPBS to promote cell adhesion.

To generate ODMs from EpOCs, the followed procedure was identical to the described on the previous section (*see 4.Crypts and Organoids Culture Passage*). Once dissociated, cells were centrifuged and washed twice with cold WM before being resuspended in 1-2 ml of WM for manual cell counting with Trypan blue Solution (1:1; Gibco). The pellet was then resuspended in the required volume of STEM medium – supplemented with 10 μM Y-27632 – to achieve $2x10^5$ cells/well/250 μl. Cells were seeded on Matrigel pre-coated 48-well plates and incubated for 24h at 37ºC 5% CO_2.
Based on our experience, every EpOCs drop contains around 40,000-100,000 cells. Thus, depending on the final number of ODMs needed, a determined number of EpOCs drops will be used at the starting point.

5.1 Monolayer culture Differentiation

Twenty-four hours after seeding, ODMs were induced to differentiate. To this end, STEM medium supplemented with Y-27632 was discarded and ODMs were washed twice: first with DPBS and then, with Advanced Dulbecco's Modification of Eagle's Medium (DMEM)/F12 medium (Invitrogen) (300 μl/well) at RT to remove dead cells. DIFF medium (250 μl/well) (**see Annex I Table 6**) was then added and ODMs were incubated at 37ºC 5% CO_2 for 48 additional hours.
Under these conditions, the differentiated monolayer (d-ODMs) reached 90-100% confluence 1-2 days after differentiation.

To characterize the monolayer gene expression pattern in ODM versus d-ODM Quantitative Multiplex Real-time PCR (RT-qPCR) and Immunofluorescence staining were then conducted.

5.2 RNA extraction and Quantitative Multiplex Real-time Polymerase Chain Reaction

Both ODMs and d-ODMs were harvested in Trizol (Life Technologies) for RNA extraction (**Table 4 Group 1**) and isolation using the RNeasy Kit (Qiagen). RNA was transcribed to cDNA at a final concentration of 250 ng/50 µl using the reverse transcriptase High-Capacity cDNA RT kit (Applied Biosystems) with RNase inhibitor (Applied Biosystems). RT-qPCR was then conducted as follows: 96-well microplates contained a final volume of 10 µl/well (1 µl cDNA+0.5 µl each TaqMan Assay diluted in TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and H_2O). Target genes were amplified and quantified using *ACTB* (Applied Biosystems) as the endogenous control. PCR reaction was run in the ABI PRISM 7500 Fast RT-PCR System.

Target gene expression values relative to ACTB were expressed as arbitrary units (AU) following this formula:

$$AU = 2^{-(Ct\ target\ gene\ -\ Ct\ ACTB)} \times 1000$$

5.3 Immunofluorescence staining

Monolayer cultures (**Table 4 Group 3**) – seeded in µ-Slide 8 Well ibiTreat chambers (IBIDI) for an optimal image acquisition – were processed for immunofluorescent staining as follows:
After two DPBS washes, the cell monolayer was fixed with 2% paraformaldehyde (PFA; Electron Microscopy Sciences) (1:1 4% PFA + DPBS) for 5 min at RT and then with 4% PFA for 10 min at RT. Cells were then washed three times with DPBS at RT. Next, 250 µl of 20mM Glycine (Sigma-Aldrich) were added for 10 min at RT to reduce background staining. Cells were washed again with DPBS three times, and 250 µl of 0.25% Triton X100 (Sigma-Aldrich) were added for 20 min at RT to permeabilize the cell membranes. To block non-specific binding, 250 µl of 1% bovine serum albumin (BSA; Sigma-Aldrich) were added to the culture and incubated at RT for 30-45 min.

Primary antibodies (150-200 µl/well) – mouse anti-EpCAM (1:100; Dako), rabbit anti-E-Cadherin (1:100; Cell Signalling Technology), rabbit anti-MUC2 (1:250; Santa Cruz Biotechnology), mouse anti-KI67 (1:100; Leica) or mouse anti-Villin (1:100; Dako) – were diluted in 1% BSA and incubated overnight (ON) at 4ºC. After 3 more DPBS washes, cells were incubated with 150-200 µl/well of the secondary antibodies – Anti-mouse Cy3 (1:400; Jackson ImmunoResearch) and Anti-rabbit 488 (1:400; Jackson ImmunoResearch) – at the specified dilutions in 1% BSA for 1h at RT. From that point, cells were kept in the dark. For DNA counterstaining, 150-200 µl/well of 4',6-diamidino-2-phenylindole (DAPI; diluted 1:10000 in DPBS; Invitrogen) were added and incubated at RT for 10 min. Finally, cells were covered in 200 µl/well of mounting medium (80% Glycerol (Sigma-Aldrich) in DPBS). Samples were stored at 4ºC for subsequent fluorescent microscope observation[2].

6. Statistical Analysis

Quantitative data are expressed as the standard error of the mean (SEM). A paired t-test was performed to examine statistically different expression patterns between 2 groups. A p-value of <0.05 was considered statistically significant. Data were analyzed and graphed using Graphpad Prism 8 (version 8.2.1).

II. AIEC infection of d-ODM

1. Bacterial Strains

The AIEC reference strain LF82, which was isolated from a chronic ileal lesion of a patient with CD (154), and the non-pathogenic strain *E. coli* K12 C600 (a prototypical derived laboratory strain which has been extensively used for molecular microbiology and bacterial physiology studies since its isolation in 1954 (330)), were used in this study. Both strains were provided in 2006 by Prof. Arlette Darfeuille-Michaud (Université d'Auvergne, Clermont-Ferrand, France) to Dra. Margarita Martínez-Medina (Universitat de Girona), with whom we conducted this study in collaboration.

Prior to infection, LF82 and K12 strains were cultured in 1.5 ml of Luria-Bertani (LB) Broth (**see Annex I Table 7**) and incubated for 12-18h at 37ºC without shaking.

[2] For short-term storage, stained cells were kept at 4ºC or at -20ºC for up to 6 months.

2. Reference Model of Infection: Intestine 407 cell line

The I407 cell line (ATCC CCL-6, RRID: CVCL_1907), originally employed for AIEC-pathotype identification (161), was used as the reference method of the gentamicin protection assay in order to ensure that the bacterial ON cultures show the expected phenotype. Cells were passaged every 2-3 days via 5-min incubation with 1 ml of Trypsin-EDTA (Lonza) after a washing step with DPBS. After collection, cells were centrifuged at 500g for 5 min at 20ºC. Pelleted cells were resuspended in Minimum Essential Medium – Eagle with Earle's BSS (EMEM) complete medium (**see Annex I Table 8**) and seeded in T75 flasks. Twenty-four hours before infection, $4x10^5$cells/well were plated on 24-well plates.

The assay was performed at Multiplicity of Infection (MOI) 10, as described previously (refs 46,47 paper A). Infection lasted 3h followed by 1h of gentamycin treatment. During the entire procedure, EMEM-MM (**see Annex I Table 9**) was employed. Invasive ability was quantified as the percentage of the intracellular bacteria from the initial inoculum ($4x10^6$ colony forming units (cfu)/ml):

$$\text{INV-I (\%)} = (\text{intracellular bacteria}/4x10^6 \text{ bacteria inoculated}) \times 100$$

3. d-ODM-based gentamicin protection assay

3.1 d-ODM cell counting

To infect cells with a determined MOI, it is crucial to know the exact number of cells seeded as a monolayer at the time of infection. In our particular case, we seeded $2x10^5$ EpOCs-derived single cells/well in 48-well plates based on previous experience (data not showed). To monitor the number of cells present in the plate at 100% confluency, experiments were performed seeding the above number of cells/well and counting cells present in d-ODM prior to infection. This step proved decisive in order to adjust the needed inoculum of bacteria and achieve the desired MOI.

Briefly, d-ODMs were washed with DPBS to remove non-attached cells. Trypsin-EDTA (150 µl) was added to the culture for 10-15 min at 37ºC 5% CO_2. Detached cells were collected and resuspended in Advanced DMEM/F12 + 10% FBS. These last two steps were repeated until complete cell-detachment was achieved. Cells were centrifuged at 800g for 4 min and at 4ºC and resuspended in 200 µl of Advanced DMEM/F12 + 10% FBS for manual cell counting as explained in a previous section

(see *5. Monolayer culture generation* (p.70)). We found it critical to not exceed the 10-15 min incubation with Trypsin-EDTA in order to prevent cell death.

On average, we recovered approximately $1.8x10^5$ cells/well prior to infection, which is close to the number of cells initially seeded (**see Annex I Supplementary Figure 1**). For the infection assay, two different MOI – 20 and 100 – were assessed on d-ODM-based assays. Thus, d-ODM counted-cells ($1.8x10^5$ cells/well) were multiplied 20- or 100-times to determine the bacterial cfu/ml required for reaching each MOI value. In that case, $3.6x10^6$ or $18x10^6$ *E. coli* cfu/ml were needed.

3.2 Bacterial Colony Forming Unit adjustment

The study of the *E. coli* growth curve in LB allowed us to estimate the cfu/ml at every measured Optical Density (OD) (**Figure 10**). Prior to infection, ON bacterial cultures (both from LF82 and K12 strains) were adjusted to OD = 0.1, corresponding to $1.6x10^8$ cfu/ml. This OD was chosen since it represents an adequate inoculum volume for the infection assay for both of the assessed MOIs. The bacterial suspension was prepared following these steps:
ON bacterial cell suspensions were diluted 1:1 with LB medium and 1 ml was transferred to a cuvette. The OD was measured with a spectrophotometer at a wavelength (λ) of 600 nm. OD adjustment was achieved in accordance with the following formula:

$$iV = (fOD\ (0.1) \times fV) / (mOD \times 2)$$

iV; Initial Volume (required volume of the ON culture)
fOD; Final OD (0.1 in this case)
fV; Final Volume (1 ml)
mOD; Measured OD

The calculated iV and the required volume of DIFF-MM (**see Annex I Table 10**) up to 1 ml total volume were added to a 1.5 ml tube.

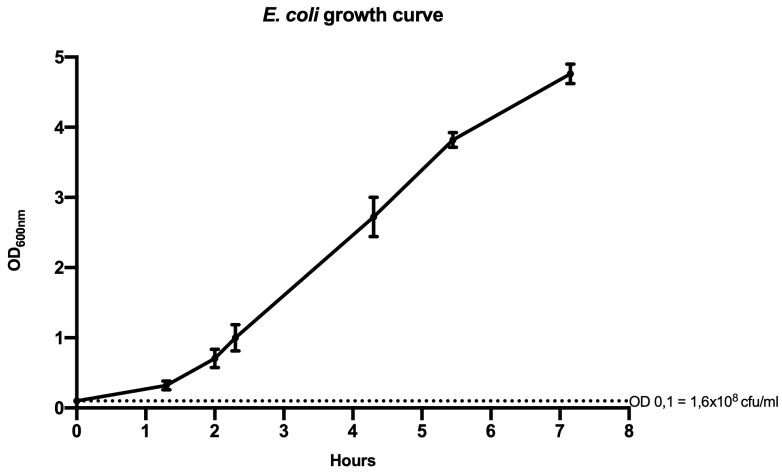


Figure 10. Mean value of the OD measurement and cfu/ml quantification of the *E. coli* growth curve. *E. coli* growth curve in LB was monitored for 7h by measuring the OD and by seeding the culture for cfu/ml quantification (n = 3 experimental replicates).

3.3 d-ODM Infection Kinetics and Gentamicin Protection Assay

As already mentioned, LF82 and K12 strains were used as positive (invasive) and negative (non-invasive) control, respectively. Infection-kinetics was performed using d-ODMs generated from 7 different subjects (**Table 4 Group 2**) as the starting material. Every experiment was conducted in duplicate. Therefore, the period between cells seeding and infection was 72h (cells were incubated for 24h after seeding and before differentiation, and 48h after differentiation and before infection).

Table 6. Adjustment of the added bacterial-culture volume to the d-ODM culture depending on the tested MOI.

MOI 20	MOI 100
Number of d-ODM-cells: 180,000	Number of d-ODM-cells: 180,000
Final cfu/ml (fC): 3,600,000	Final cfu/ml (fC): 18,000,000
Final volume/well (fV): 500 µl	Final volume/well (fV): 500 µl
Initial cfu/ml (iC): $1.6x10^8$	Initial cfu/ml (iC): $1.6x10^8$
Added volume ($_{add}V$): **11.25 µl**	Added volume ($_{add}V$): **56.25 µl**

DIFF medium was then discarded from 100% confluent d-ODMs; cells were washed twice with DPBS at RT (500 µl/well) and fresh DIFF-MM was added (500 µl/well). Next, the corresponding volume of OD 0.1 bacterial suspension (**Table 6**) was inoculated to reach each assessed MOI by gently releasing the drop.

Infected d-ODMs were incubated for 4, 5, 6 or 7h at 37ºC 5% CO_2 for the complete infection-kinetics study. At the end of each time point, cells were washed 3 times with DPBS at RT and DIFF-MM containing 100 µg/ml of gentamicin was added for 1 additional hour in order to remove the extracellular bacterial cells. Three more DPBS washes at RT were required after gentamicin treatment. Cells were then treated with 1% Triton X-100 (250 µl/well) to release the internalized bacteria. Vigorous pipetting to generate bubbles was required to efficiently detach and break the eukaryotic cell membranes.

It was critical that the Triton X-100 step did not take longer than 30 min in order to avoid bacterial cell death.

3.3.1 d-ODM Infection and Low-Gentamicin Treatment

In parallel, d-ODMs were either infected (with *E. coli* LF82 and K12) or non-infected and incubated for 5 and 6h (n=3, **Table 4 Group 2**) without gentamycin treatment to analyze and compare both gene and protein expression levels.

On the other hand, infected and non-infected d-ODMs were also incubated for 5h + 1h of gentamycin treatment as performed for the infection-kinetics (see previous section *3.3 d-ODM Infection Kinetics and Gentamicin Protection Assay*). Cells were then washed twice with DPBS at RT before starting a third incubation period in DIFF medium (without antibiotics) supplemented with 15 µg/ml of gentamicin for further 6 or 18h (n=2 or 3 respectively, **Table 4 Group 2**).

Both assays were performed in duplicate and at MOI 100.

3.4 Invasion Index

To be able to count cfu/ml, the bacterial suspension resulting from the Triton X-100 treatment was serially diluted in Ringer Solution (Scharlau). Dilutions of 10^{-1} and 10^{-2}, as well as the non-diluted samples, were plated (25 µl) in LB agar plates

(**see Annex I Table 11**) and incubated ON at 37ºC. For a homogeneous mixture of bacterial dilutions, vortexing solutions is highly recommended.

Grown colonies in each dilution were only taken into consideration when the counting was between 15 - 150. Intracellular bacterial counts were obtained as follows:

$$\text{Intracellular bacteria} = \frac{\Sigma \text{ colonies}}{(0.025 \text{ x } (n_1 + 0.1 \text{ x } n_2) \text{ x DF})} \text{ x well volume (0.25)} = \text{cfu/well}$$

n_1 = number of plates at the more concentrated dilution
n_2 = number of plates at the less concentrated dilution
DF = dilution factor of the more concentrated dilution

Once the number of cfu/well was obtained, the invasion index (%) was calculated considering the amount of bacteria initially inoculated to d-ODMs:

Invasion Index (INV-I) = (Intracellular bacteria/Inoculated Bacteria†) x 100 = %

†: in this context, 3.6×10^6 for MOI 20 or 18×10^6 for MOI 100.
As previously described by Darfeuille-Michaud et al., who studied AIEC infection by using immortalized cell lines (161), we considered a strain to be invasive when the INV-I% was > 0.1%

4. Fluorescent Cyto-staining

To visualize bacterial internalization, LF82- and K12-infected monolayer cultures (at 5h of infection followed by 1h of gentamicin treatment (5+1) and MOI 100) seeded in µ-Slide 8 Well ibiTreat chambers, were processed for fluorescent cyto-staining. This procedure was identical to that used for ODM/d-ODM characterization (see section *5.3 Immunofluorescence staining* (p.71)) except for the used antibodies. After incubation with the blocking solution, 150-200 µl/well of Phalloidin diluted 1:40 in 1% BSA was added for staining of the actin filaments. After 1-h incubation at RT in the dark, cells were washed 3x with DPBS at RT. DAPI (250 µl/well) was then added and the protocol continued as described in the above-mentioned section. The assay was performed with cells obtained from 5 different subjects (**Table 4 Group 3**).

5. CellTox Green Cytotoxicity Assay

To assess cell viability and cytotoxicity after AIEC infection, we used the CellTox Green Cytotoxicity Assay following the protocol recommended by the manufacturer. Briefly, after the infection assay, infected and non-infected d-ODMs were incubated with the CellTox reagent (1:1, 150 µl DIFF-MM + 150 µl CellTox) previously diluted according to the manufacturer's instructions (1:500 in Assay Buffer). After ≥15 min of incubation at 37ºC in the dark, cultures were observed using a fluorescence microscope. A positive control of cell death was included by adding 100 µg/ml of digitonin (Sigma-Aldrich) in the uninfected d-ODM for 1h. The assay was performed with samples from 3 different donors (**Table 4 Group 3**).

6. Soluble Proteins Measurement (ELISA)

Supernatants from non-infected and LF82- and K12-infected d-ODMs were collected for the detection of soluble proteins (CCL20 and IL-8) by using commercial Enzyme-Linked ImmunoSorbent Assay (ELISA, both from R&D Systems) kits. The followed procedure was the recommended by the manufacturer. Absorbance (450 nm) was measured using a microplate reader (Molecular Devices).

7. RNA extraction and RT-qPCR

RNA extraction and RT-qPCR of the *E. coli* infected d-ODMs as well as de non-infected controls was the same as detailed in section *5.2 RNA extraction and Quantitative Multiplex Real-time Polymerase Chain Reaction* (p.71).

8. Statistical Analysis

Quantitative data are expressed as the SEM. A 2-way RM ANOVA test was performed to examine statistical significance in paired multiple group data sets with two independent variables. A One-way RM ANOVA was conducted to examine statistical significance in paired multiple group data sets with only one independent variable. Both tests were followed by a Tukey test correction for multiple testing. A paired t-test was performed to examine statistically different expression patterns between 2 groups. A p-value of <0.05 was considered statistically significant. Data were analyzed and graphed using Graphpad Prism 8 (version 8.2.1).

III. Postbiotics production, characterization, and application

1. Bacterial Strains

Streptococcus salivarius subs. *thermophilus* (Orla-Jensen 1919) Farrow and Collins 1984 – ATCC 19258 – (*S. thermophilus*) (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH), *E. coli* Nissle 1917 (isolated from Mutaflor ® - Ardeypharm) and *E. coli* K12 C600 (provided by Dra. Margarita Martínez-Medina, Universitat de Girona) were grown to further collect their metabolites. *S. thermophilus* was grown in Brain Heart Infusion – BHI – (37 g/L; BD Biosciences) while both *E. coli* strains were cultured in LB medium. For the preparation of agar plates, 15 g/L of Bacto Agar (BD Difco) was added to the culture media mixture.

Only *S. thermophilus* and *E. coli* Nissle are considered as probiotic strains thereby we only consider as postbiotics the microbial metabolites derived from these two strains. *E. coli* K12 was used as a "non-probiotic" control.

1.1 Bacterial strains isolation

Lyophilized *S. thermophilus* was reconstituted as recommended by the manufacturer. Briefly, to open the glass ampoule containing the vial with the dried pellet, the tip of the ampule was heated in a flame (which, in turn, also provided the required sterile environment) and 4-5 water drops were placed onto the hot tip to crack the glass. The inner vial was taken out with forceps and, under sterile conditions, was uncovered to add 0.5 ml of BHI. Thirty minutes later, the pellet was completely rehydrated. After gently mixing the content, half of the whole amount was transferred to a test tube with 5 ml of liquid BHI. The other half was plated onto a BHI agar plate. Both cultures were incubated ON at 37ºC (at 180 revolutions per minute – rpm – for the liquid culture).

On the other hand, one capsule of Mutaflor® containing 2.5-25x10^9 cfu of *E. coli* Nissle was reconstituted in 3 ml of DPBS at RT. The powder was completely dissolved in 30 min. After gently mixing, the solution was serially diluted (10^{-1} to 10^{-9}) and 100 µl of each dilution were plated on LB agar plates. Cultures were then incubated ON at 37ºC.

E. coli K12 was directly cultured on liquid or LB agar plates since no reconstitution was required (incubation was ON at 37ºC and 180 rpm for liquid cultures).

All strains were stored at -80ºC in 18% glycerol (Sigma-Aldrich) diluted in DPBS.

1.2 Bacterial strains identification

Bacterial strains' identity was confirmed by MALDI-TOF MS by the Microbiology Department at the Hospital Clínic de Barcelona.

Briefly, after growing each strain in blood agar plates (Becton Dickinson) ON at 37ºC, a small fraction of a single colony of each one was spotted onto a MALDI target plate (MSP 96 target ground steel; Bruker Daltonics), subsequently overlaid with 1 µl of matrix solution (α-cyano-4-hydroxy-cinnamic acid; Bruker Daltonics) and air-dried at RT.

MALDI-TOF MS was conducted in a Microflex LT (Bruker Daltonics) benchtop instrument as described previously (331,332).

Spectra were analyzed with the MALDI BioTyper software (version 3.1; Bruker Daltonics) using the pre-processing and BioTyper main spectrum identification standard methods (mass range 2,000-20,000 m/z) against the default Bruker database (v.8.0.0.0). Accuracy of the identification was determined by a logarithmic score value resulting from the alignment of peaks to the best matching reference spectrum (332).

1.3 Determination of the Bacterial growth curves

In order to establish the desired OD in which postbiotics would be produced, the study of all the strains growth curves was performed. Since *E. coli* growth curve was already followed-up (as detailed in section *3.2 Bacterial Colony Forming Unit adjustment* (p.74)), only *S. thermophilus* was analyzed in this occasion. To do so, and due to the low growth rate of *S. thermophilus*, measurements were performed every 15 min for 24h with and Epoch2 Reader (BioTek, Serial Num. 1505291) using 96-well plates. The reader was pre-heated at 37ºC and a double orbital shaking (180 cycles per minute – cpm –) was maintained during the entire incubation. As we already mentioned, the OD was measured at a λ of 600 nm. BHI medium was used as a blank.

2. Postbiotics Production

All bacterial strains were grown ON at 37ºC in agar plates of their respective culture media. For *S. thermophilus*, a single colony was plated again into a BHI agar plate (passage 1) and

incubated ON at 37ºC. An individual colony (passage 0 for *E. coli*, passage 1 for *S. thermophilus*) was then cultured ON at 37ºC and 180 rpm in liquid culture medium (10:1 flask-culture volume ratio). The ON liquid culture was then re-started to OD = 0.1 for *E. coli* strains or 0.02 for *S. thermophilus* and the growth curves were followed until they reached mid-exponential phase. Bacterial cultures were then centrifuged (4000g for 10 min at 4ºC) and supernatants were filtered through a sterile Syringe Filter (0.20 μm PES; Corning) and stored at -80ºC (non-purified postbiotics).

Purified postbiotics were produced in collaboration with Postbiotica S.r.l by removing the culture media of each strain through an innovative fermentative process (PBTECH) – undisclosed method –, thereby unveiling the true effect of the microbial bioactive components (263,264). Purified postbiotics, which are in solution in an inert liquid (here called vehicle – undisclosed composition) were stored at 4ºC.

3. Postbiotics characterization

Once the postbiotics were obtained, we proceeded with their proteomic (for both purified and non-purified metabolite-cocktails) and metabolomic (only for purified postbiotics) characterization. The former was conducted at the Proteomics Platform of Barcelona Scientific Park (Barcelona, Spain), a member of ProteoRed. The metabolomic analysis was carried out in collaboration with Dr. Xavier Domingo-Almenara at the Centre for Omics Sciences (COS) – EURECAT (Reus, Spain).

3.1 Proteomic characterization

Liquid samples, that were analyzed by LC-MSMS, were first cleaned-up from salts and other mass spectrometry interfering substances by loading them into molecular weight cutoff filters (Amicon Ultra 3 KDa, 0.5 ml, Millipore). Proteins were recovered and quantified with the Micro BCA™ Protein Assay Kit (Thermo Scientific). Proteins were then in-solution digested. The resulting peptide mixtures were acidified with formic acid and desalted in a C18 tip (P200 Toptip, PolyLC) – as per the manufacturer's indications – and dried in a SpeedVac system.

The peptide mixtures were analyzed in a nanoAcquity liquid chromatographer (Waters) coupled to an LTQ-Orbitrap Velos (Thermo Scientific) mass spectrometer. Eluted peptides were subjected to electrospray ionization in an emitter needle (PicoTipTM, New Objective) with an applied voltage of 2,000V. Peptide masses (m/z 300-1,600) were analyzed in data dependent mode where a full Scan MS was

acquired in the Orbitrap with a resolution of 60,000 FWHM at 400m/z. Up to the 15th most abundant peptides (minimum intensity of 500 counts) were selected from each MS scan and then fragmented in the linear ion trap using CID (38% normalized collision energy) with helium as the collision gas. The scan time settings were: Full MS: 250 ms (1 microscan) and MSn: 120 ms. The generated raw data file was collected with Thermo Xcalibur (v.2.2).

Raw data files were used to search against specific databases for every microorganism. Database search was performed with Sequest HT search engine using Thermo Proteome Discover (v.1.4.1.14).

Both a target and a decoy database were searched in order to obtain a false discovery rate (FDR), and thus estimate the number of incorrect peptide-spectrum matches that exceed a given threshold. To improve the sensitivity of the database search, Percolator (semi-supervised learning machine) was used in order to discriminate correct from incorrect peptide spectrum matches. Percolator assigns a q-value to each spectrum, which is defined as the minimal FDR at which the identification is deemed correct. These q-values are estimated using the distribution of scores from the decoy database search.

3.2 Metabolomic characterization

Metabolites from all postbiotics (100 µl) were extracted with 400 µl of MeOH, dried down and resuspended in 50 µl of ACN/H_2O (9:1). Metabolites were then analyzed by ultra-high performance liquid chromatography (UPLC) (Agilent Technologies, Santa Clara, CA) using a hydrophilic interaction liquid chromatography (HILIC) column, coupled to an Agilent quadrupole time-of-flight (qToF) mass spectrometer. The injection volume was 10 µl and data was acquired in positive mode. For identification purposes, retention time and in-source fragments were compared with reference data from an in-house database, as described elsewhere (333).

4. Postbiotics' effects in human blood immune cells

Once characterized, postbiotics were tested for their putative immunomodulatory properties by using both stimulated and non-stimulated human blood immune cells. These included PBMCs (comprising both innate and adaptive cells) and monocyte derived dendritic cells (moDCs) cultures.

4.1 PBMCs

To test the immunomodulatory capacity of postbiotics, PBMCs were isolated and treated with all postbiotics and their vehicle. To activate innate responses, PBMCs were stimulated with LPS and postbiotics were then added.

4.1.1 PBMCs Isolation

Blood samples were diluted (5X) with DPBS and centrifuged at 300g for 10 min at RT (speed: 1/1) for purification of the mononuclear cells. After discarding the supernatant, the pellet was resuspended with DPBS up to 30 ml. The diluted cell suspension was carefully layered over 15 ml of Ficoll (Limphoprep) in a 50 ml conical tube. The samples were then centrifuged at 300g for 30 min at RT (speed 1/0).

The ring containing PBMCs was aspirated with a Pasteur pipette and carefully passed through a humidified 70 μm filter in a 50 ml tube. DPBS was added up to 40 ml and the samples were again centrifuged (300g; 10 min; RT; speed: 9/9). After carefully removing the supernatant, the cell pellet was resuspended in 50 ml of DPBS and centrifuged at 160g for 10 min at RT (speed: 9/9) for platelet removal.

The cell pellet was finally resuspended in complete RPMI medium (**see Annex I Table 12**) and manually counted.

4.1.2 PBMCs Stimulation

PBMCs were seeded in 96-well plates (flat bottom) at a final concentration of 200,000 cells/well. Cells were incubated in complete RPMI medium with the postbiotic (purified (n=6, **Table 5 Group 1**) or non-purified (n=4, **Table 5 Group 1**)) or its control (vehicle/culture medium), at different concentrations (25%; 12.5%; 6.25%; 3.125%; 1.5625%; 0.78125%; 0.390625%. Final volume 200 μl/well). Then, cells were stimulated with or without LPS (100 ng/ml; Sigma-Aldrich) for 24h at 37ºC 5%CO_2. Cells incubated with complete RMPI ± LPS were used as controls.

4.2 Monocyte Derived Dendritic Cells

Given that DCs are crucial to cross-link the innate and adaptive immune responses, we tested postbiotics on moDCs to elucidate their potential immunomodulatory effects.

4.2.1 moDCs Differentiation

After PBMCs isolation, cells were induced to differentiation into DCs (n = 3, **Table 5 Group 1**). To do so, $CD14^+$ cells were selected by magnetic labeling and separation. A total of $15x10^7$ cells were labeled with CD14 Microbeads (Miltenyi Biotec) and magnetically separated using MACS LS Columns as recommended by the manufacturer.

Isolated $CD14^+$ cells were resuspended in RPMI complete medium and plated into 6-well plates at a final concentration of $3x10^6$ cells/well. Human IL-4 (20 ng/ml; Peprotech) and Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF; 50 ng/ml; Biolegend) were added to induce differentiation into DCs.

Cells were incubated at 37ºC 5% CO_2 for 7 days, adding fresh RMPI medium containing both cytokines on alternate days.

4.2.2 moDCs Characterization

To ensure that $CD14^+$ cells were accurately differentiated into DCs, cell surface markers were stained for Flow Cytometry (FACS) analysis (n =3, **Table 5 Group 1**). **Table 13 in Annex I** lists the used antibodies, clones and conjugated fluorochromes.

Cells collected at day 7 after culture with IL-4 and GM-CSF (200,000 cells/tube) were washed in 2 ml of FACS buffer (**see Annex I Table 14**) and centrifuged at 400g for 5 min at 4ºC. The cell pellet was resuspended in 50 µl of DPBS containing the Live/Dead – L/D – (1:1000; Zombie Aqua™ Fixable Viability Kit, Biolegend) dye to exclude dead cells and the Fc Receptor Blocking Solution (Human TruStain FcX™; 1:50; Biolegend). After 10 min at 4ºC, antibodies were added at the quantity recommended by the manufacturer and incubated for 20 min at 4ºC in the dark. Cells were then washed with FACS buffer (2 ml/tube; 400g for 5 min at 4ºC) and the cell pellet was resuspended and fixed with 250 µl/tube of 1X Stabilizing Fixative

(BD Biosciences). Samples were acquired using a BD FACSCanto II flow cytometer (BD) and analyzed with FlowJO software (BD).

4.2.3 moDCs Stimulation

Similar to PBMCs, moDCs (n=2, **Table 5 Group 1**) were seeded in 96-well plates (flat bottom) at a final concentration of 20,000 cells/well. Cells were incubated in complete RPMI medium with each postbiotic or its vehicle, at different concentrations (see section *4.1.2 PBMCs Stimulation* (p.83). Final volume 200 µl/well). Cells were then stimulated with or without LPS (100 ng/ml) for 24h at 37ºC 5% CO_2. Cells incubated with complete RMPI ± LPS in the absence of postbiotics were used as controls.

4.3 MTT assay

After simulation of either PBMCs or moDCs, plates were centrifuged (300g, 5 min, RT) and half of the supernatant (~100 µl/well) was collected and frozen at -20ºC for subsequent soluble protein analysis.

To check cell viability, sterile MTT solution (**see Annex I Table 15**) was added to every well to a final concentration of 0.45 mg/ml. Plates were then incubated for 4h at 37ºC 5% CO_2. Next, 100 µl/well of Solubilization Solution (**see Annex I Table 16**) was added to dissolve formazan crystals. Before reading the absorbance at 570 nm, plates were mixed to ensure complete solubilization. Data was acquired with a microplate reader (Molecular Devices).

4.4 Soluble Protein Measurement (ELISA)

Collected supernatants from stimulated and non-stimulated PBMCs and moDCs cultures were analyzed for the detection of soluble proteins (IL-10, IL-12/IL-23 p40 and IL-12p70) by using commercial ELISA kits (all from R&D Systems) and following the manufacturer's instructions. Absorbance (450 nm) was measured with a microplate reader (Molecular Devices).

5. Postbiotics effects in d-ODM

After testing their properties in human blood immune cells, postbiotics effects were assessed in ODMs. This *ex vivo* intestinal culture was not only used intact but also artificially-inflamed to simulate the inflamed human intestine.

5.1 Artificial inflammation of d-ODM

As mentioned, to *ex vivo* mimic the intestinal inflammatory status of IBD patients, d-ODMs (n = 4, **Table 5 Group 2**) were exposed to 4 different stimuli and a combination of two of them (here called, inflammatory cocktail) (334–338)

Two days after induction of differentiation (see section *5.1 Monolayer culture Differentiation* (p.70)), d-ODMs were incubated with TNFα (25 ng/ml; R&D Systems), IL-1β (25 ng/ml; Peprotech), Flagellin – FLA – (100 ng/ml; InvivoGen), IFNγ (10 ng/ml; Peprotech), and the inflammatory cocktail (IFNγ +TNFα) for 24h. Subsequently, RNA was collected and stored for further analysis[3].

5.2 Postbiotics treatment of d-ODM

The non-inflamed and artificially-inflamed d-ODMs were incubated in the presence of postbiotics in order to assess their effects on this cell culture.

Thus, postbiotics or their vehicle (10% v/v) were added to the d-ODMs 17-19h before the artificial inflammation was induced (ON pre-conditioning).

Cells only treated with postbiotics, vehicle or pro-inflammatory cytokines were used as control (n = 4, **Table 5 Group 2**).

RNA collection was performed 24h after adding the pro-inflammatory cytokines[3].

5.3 RNA sequencing analysis

Barcoded RNAseq libraries from treated and untreated artificially-inflamed d-ODMs (n = 4, **Table 5 Group 2**, 120 samples in total) were prepared from 500 ng total RNA using Illumina's TruSeq stranded Total RNA with Ribo-Zero Gold kit according to the manufacturer's instructions. Libraries were subjected to paired-end sequencing (150 bp) on a NovaSeq6000 platform (Illumina). Later, the cutadapt

[3] The followed procedure for RNA extraction and RT-qPCR of the treated and control d-ODMs was the same as detailed in section *5.2 RNA extraction and Quantitative Multiplex Real-time Polymerase Chain Reaction* (p. 71).

software (version 1.7.1) was used for quality filtering and the libraries were mapped against the human reference genome using the STAR aligner (2.5.2a) with Ensembl annotation (release GRCh38.10). Read counts per gene were obtained with RSEM (version 1.2.31) as previously described (91). Analysis was performed using R (version 3.6.1) and Bioconductor (Version 3.10) on Ubuntu 18.04. The human transcriptome was visually inspected for batch effects in a principal component analysis (PCA). Outliers and the top 10% genes using the coefficient of variation and non-coding protein genes were removed (46,036 - with remaining 14,624 genes). Data was normalized using the trimmed mean of M-values and log transformed into counts per millions using edgeR (version 3.28).

5.4 Comparison between artificially-inflamed d-ODMs and IBD patients expression signature

To link the transcriptional signatures induced on artificially-inflamed d-ODMs with that from IBD patients with inflamed mucosa, an in-house dataset (339) including 53 samples of the colon from non-IBD subjects, as well as CD and UC patients at the time of inclusion in the study – week 0 – (see **Annex I Table 18**) was used. After data normalization using edgeR (version 3.26.8 (340)) and voom (limma version 3.40.6 (341)), the obtained number of protein coding genes was 16,053.

6. Statistical Analysis

Quantitative data are expressed as the standard error of the mean (SEM). A 2-way RM ANOVA or a standard 2-way ANOVA test were performed to examine statistical significance in paired and unpaired, respectively, multiple group data sets. A One-way RM ANOVA was conducted to examine statistical significance in paired multiple group data sets with only one independent variable. All analyses were followed by a Tukey test correction for multiple testing. A p-value of <0.05 was considered statistically significant. Data were analyzed and graphed using Graphpad Prism 8 (version 8.2.1).

For the RNAseq data, differential expression analysis was performed with edger v.3.28 package, adjusting for inter-individual differences. To correct for multiple testing, the FDR was estimated using the method of Benjamini and Hochberg. A gene was considered differentially expressed when it was significant at 5% p-value and showed a fold-change (FC) different than $|0|$.

RESULTS

STUDY 1: Determination of AIEC capability to invade Organoid-Derived Epithelial Monolayers and its derivative effects on the Intestinal Epithelium

1. Establishment of Human Primary Organoid-Derived Epithelial Monolayers as an *ex vivo* model to study host-bacterial interactions

The intestinal crypt is organized so that the stem-cell compartment resides at the bottom, thereby protected from the luminal content, while the differentiated and surface epithelium is more directly in contact with the microbiota and its metabolites. In order to develop a model that would more faithfully reproduce the upper crypt epithelium, which is closer to the lumen and thus, to bacteria and their metabolites, and based on previous results from our lab (282,327), we established a monolayer of differentiated epithelial cells derived from epithelial organoid cultures (d-ODMs).

First, we aimed to determine the optimal culture conditions for the ODMs to acquire a differentiated phenotype while reaching an appropriate confluence (~100%) for the AIEC invasion assay. Based on previous experiments in our lab, seeding $2x10^5$ single cells/well was required to get 100% confluence at the time of the experiment.

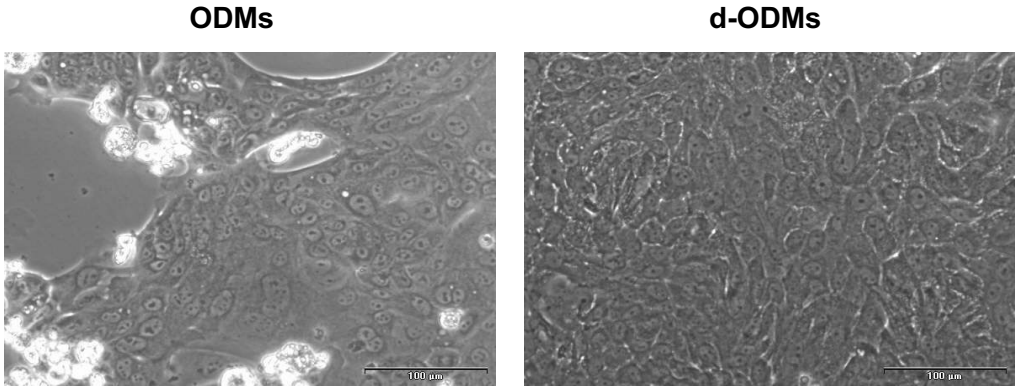


Figure 11. Organoid-Derived Monolayer (ODMs) culture. ODMs (left panel) 24h after seeding showed a confluence of around 70-80% while d-ODMs (right panel), 48h after differentiation, showed 100% confluence. Scale bars: 100 µm.

On day 1, indeed, cells created clusters that alternated with empty areas, while on day 3 (two days after induction of differentiation), the monolayers reached ~100% confluence (**Figure 11**). At that point, cells were collected and counted, obtaining an average of approximately $1.8x10^5$ cells/well (**see Annex I Supplementary Figure 1**). Once the number of cells at ~100% confluency was determined, we characterized the phenotype of the d-ODMs by measuring key genes and proteins whose expression changes dramatically upon epithelial stem cell differentiation (290,329).

As shown in **Figure 12A**, mRNA levels of the stemness marker *AXIN2* and the proliferation markers *MYC* and *MKI67*, were significantly higher in ODMs compared to d-ODMs. On the other hand, transcriptional levels of the differentiation markers *TFF3* and *MUC2* showed a trend towards up-regulation in d-ODMs compared to ODMs. Similarly, *TJP3*, a representative marker of epithelial cell junctions, was significantly up-regulated in d-ODM. Other markers used to characterize the phenotype of the d-ODM culture (282) are described in **Supplementary Figure 2** (**see Annex I**).

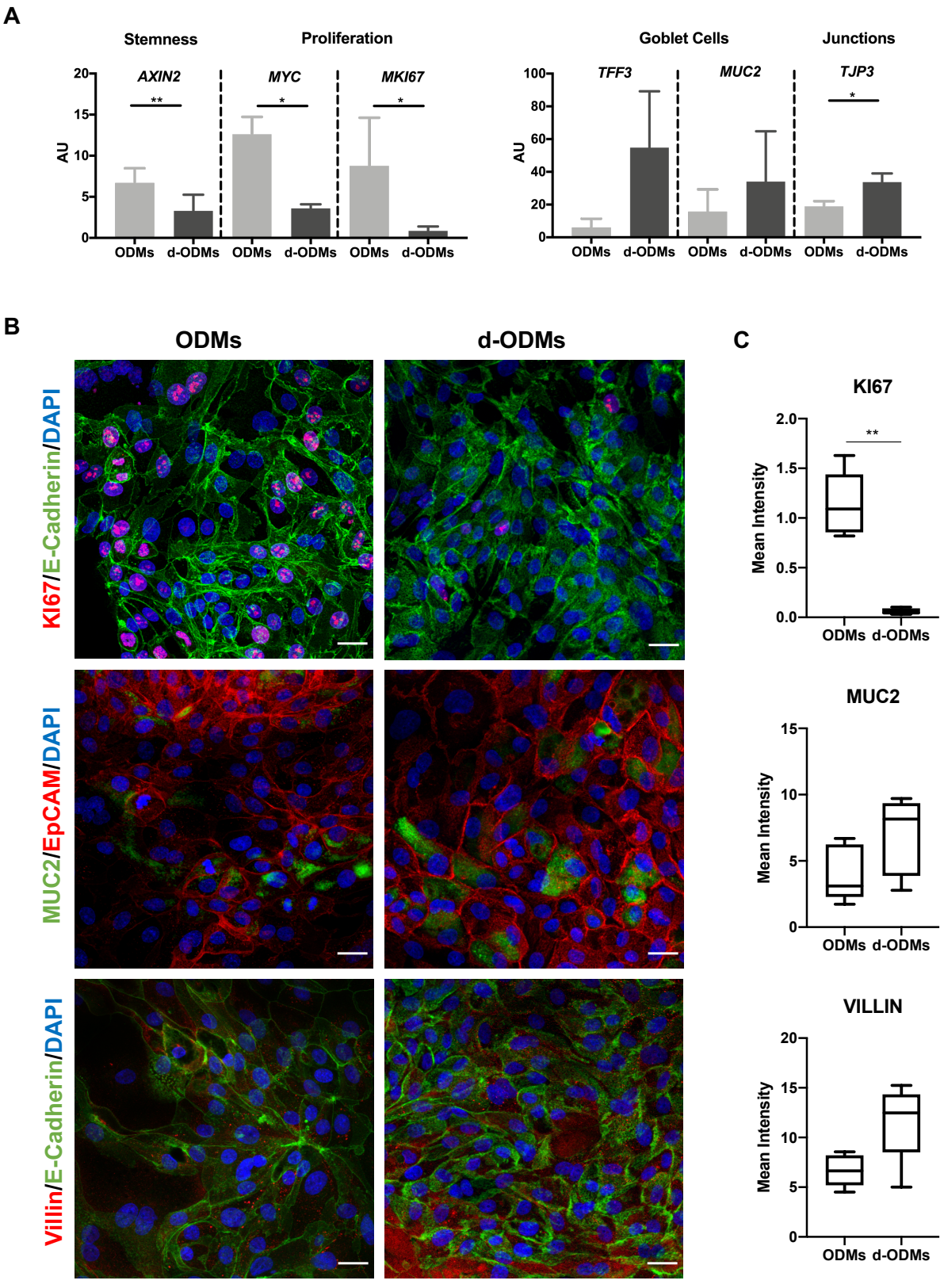
Using transcriptional analysis is a convenient and accessible readout to monitor the differentiation status – or other phenotypic features – of cell cultures. Nevertheless, protein staining was also included in the analysis to evaluate not just protein expression but also localization within the cell monolayer.

As an example, we determined the protein expression of KI67, MUC2 and Villin proteins by immunofluorescence (**Figure 12B**)**.** In agreement with the differentiated phenotype achieved in d-ODMs, KI67 was markedly decreased while MUC2 and Villin were increased compared to ODMs. These results were confirmed by fluorescence quantification analysis (**Figure 12C**). In addition, localization of MUC2 and Villin at the apical side (assessed in an orthogonal view of the d-ODM (**Figure 13**)), confirmed the appropriate cell polarization of the 2D culture.

Altogether, our results demonstrate that primary cells derived from human EpOCs can establish a stable monolayer that preserves the intestinal identity thus mimicking the tissue of origin. Moreover, we achieved a differentiated and polarized phenotype in the d-ODMs at optimal confluence to support the study of AIEC-infection.

(figure on next page)

Figure 12. Organoid-Derived Monolayers (ODMs) characterization. (A) Gene expression analysis of ODMs and d-ODMs (n = 5 for each culture type). *AXIN2, MYC, MKI67, TFF3, MUC2* and *TJP3* genes were analyzed by RT-qPCR to determine their expression levels in ODM vs. d-ODMs. A paired t-test was performed to examine statistically different expression patterns between the two groups (ODMs/d-ODMs). A P value of <0.05 was considered statistically significant. *AXIN2*: ** indicates P = 0.0012. *MYC*: * indicates P = 0.0135. *MKI67*: * indicates P = 0.0335. *TJP3*: * indicates P = 0.0365. **(B)** Protein expression analysis by immunofluorescence. KI67, MUC2 and Villin were analyzed to confirm the proliferation and differentiation status of ODMs and d-ODMs. E-Cadherin and EpCAM were used as epithelial cell-wall markers. DAPI, in blue, was used to counterstain the cell nuclei. Scale bars: 25 µm. Images are representative of n = 3 independent experiments performed with samples from two different donors. **(C)** Box-plot distribution of the fluorescent signal of KI67, MUC2 and Villin proteins in ODMs and d-ODMs, expressed as Mean Intensity. Fluorescence was quantified in 5 different fields per sample. A paired t-test was performed to examine statistically different expression patterns between the two groups (ODMs/d-ODMs). A P value of <0.05 was considered statistically significant. KI67: ** indicates P = 0.0013.


A
Stemness
Proliferation
Goblet Cells
Junctions
AXIN2
MYC
MKI67
TFF3
MUC2
TJP3
AU
ODMs
d-ODMs
B
ODMs
d-ODMs
KI67/E-Cadherin/DAPI
MUC2/EpCAM/DAPI
Villin/E-Cadherin/DAPI
C
KI67
MUC2
VILLIN
Mean Intensity

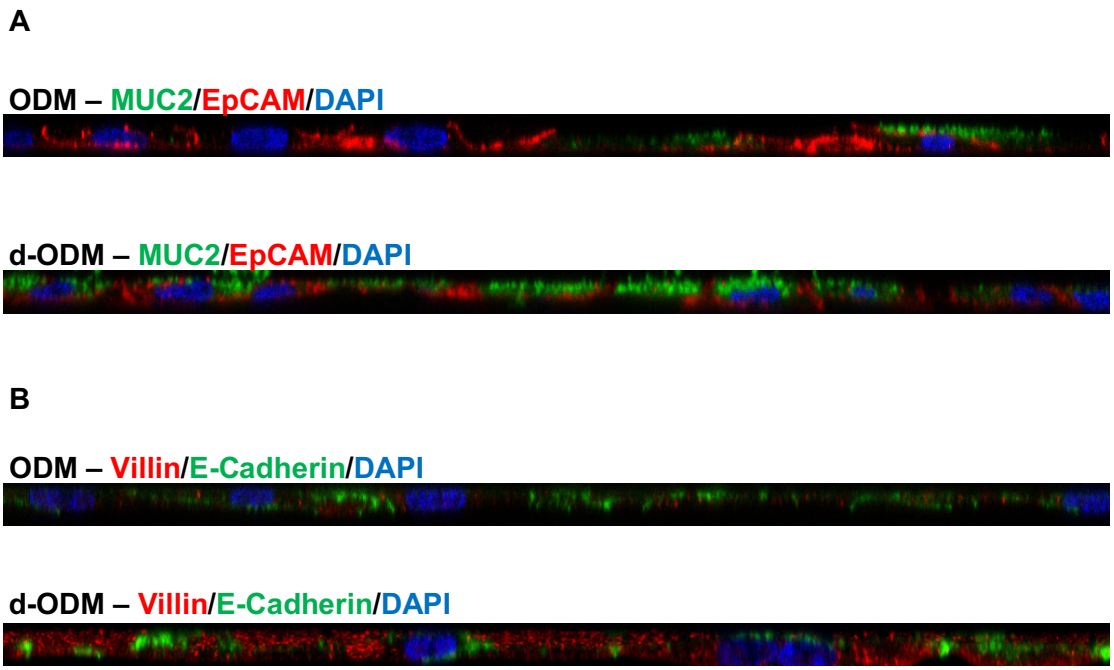


Figure 13. Orthogonal views of the protein expression in ODMs and d-ODMs analyzed by immunofluorescence. MUC2 **(A)** and Villin **(B)**, both markers of differentiated IECs, were mainly expressed in the apical surface of d-ODMs compared to ODMs. EpCAM and E-Cadherin were used as epithelial cell-wall markers. DAPI counterstained the cell nuclei. Images are representative of n = 3 independent experiments performed using samples from two different donors.

2. Adherent-Invasive *Escherichia coli* can invade Human Primary Organoid-Derived Epithelial Monolayers

To date, the characteristics and pathogenicity of AIECs have been studied by employing immortalized cell lines (187). Here, we studied the capability of AIECs to interact and invade a primary intestinal monolayer culture. First, we designed a kinetics infection assay to determine the time course of bacterial entry and/or intracellular survival in our culture system. To verify the strains' invasiveness capacity, the I407 cell line was used as the reference model of the gentamicin protection assay. Both invasion assays (d-ODM and I407) were carried out in parallel; thus, the *E. coli* ON cultures used to infect IECs were the same for each experiment performed. Results represented in **Figure 14** show an INV-I% in I407 cells of 0.99±0.225 and 0.0025±0.00094 for the LF82 and K12 strains, respectively. These results were in agreement with previously published data (155,161).

We next examined AIEC-d-ODMs invasion by determining the percentage of internalized bacteria every hour for 7h of infection followed by 1h of gentamicin treatment as detailed in the Materials & Methods section (p.75).

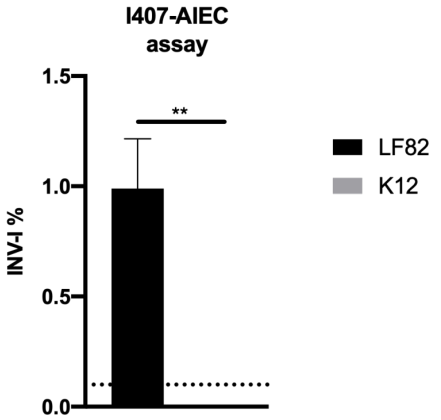
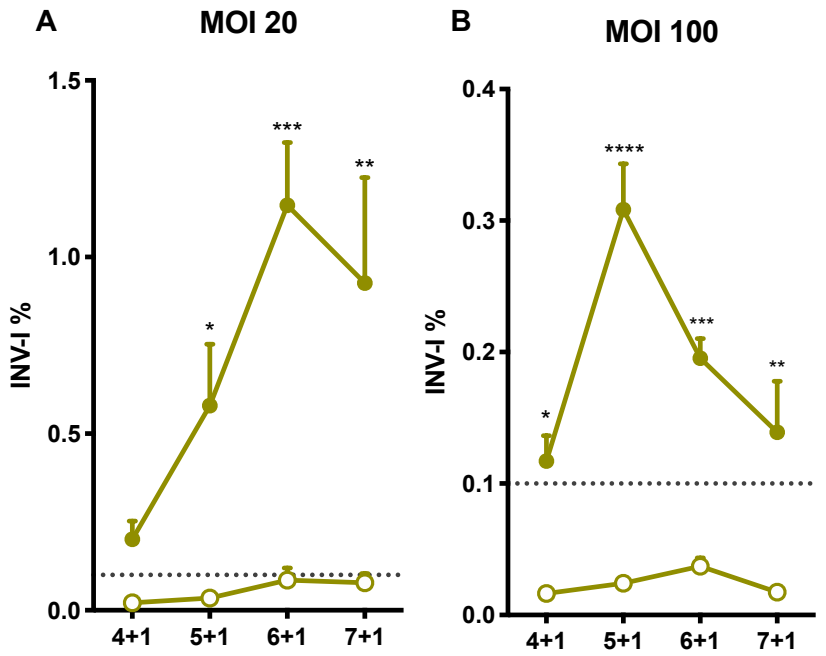


Figure 14. *E. coli* LF82 and K12 Invasion Indexes (INV-I %) in the I407 cell line. The dashed line represents the established threshold (0.1) that determines the invasive capacity of the *E. coli* strains tested in I407 cells (n = 5 experimental replicates). A paired t-test was performed to examine the statistical significance between LF82 and K12 INV-I %. A P value of <0.05 was considered statistically significant. ** indicates P = 0.0024.

The assessed MOIs were 20 and 100. As shown in **Figure 15A-B**, the AIEC LF82 strain was able to invade d-ODMs, while the non-invasive *E. coli* strain (K12) showed an INV-I% below the established threshold (0.1%) at both MOIs. Moreover, LF82 showed a time-dependent increment of the INV-I%, and thus of its invasion capacity and/or intracellular multiplication. This capability was significantly higher compared to the K12 strain at 5, 6 and 7h after infection for MOI 20 (**Figure 15A**) and at all time points for MOI 100 (**Figure 15B**). In fact, 5h of infection followed by 1h of gentamicin treatment (5+1) at MOI 100 showed the greatest difference; the LF82 INV-I% measured almost 13 times greater than the K12 INV-I%. This occurred despite the fact that all INV-I% were lower when the MOI was higher (MOI 100) and vice versa. Furthermore, working with a greater number of bacteria/cell (higher MOI) ensured a remarkable reproducibility over time with highly consistent numbers of internalized bacteria in every experiment performed (**Figure 15C**). Cell viability was monitored throughout time using the CellTox Green assay and no changes were observed in any of the conditions studied (data not shown).

Moreover, by staining the eukaryotic actin filaments as well as the DNA (both bacterial and eukaryotic (**Figure 16**)), we confirmed the presence of high amounts of intracellular LF82 bacteria in most of those cells that formed the d-ODMs compared to the K12 strain.



C

		K12		LF82		
		Mean	**SEM (±)**	**Mean**	**SEM (±)**	**Adjusted p-value**
MOI 20	**4+1**	0.021	0.002	0.201	0.051	0.8981
	5+1	0.034	0.011	0.579	0.174	**0.0370**
	6+1	0.085	0.034	1.146	0.177	**0.0002**
	7+1	0.078	0.026	0.926	0.298	**0.0013**
MOI 100	**4+1**	0.016	0.004	0.117	0.019	**0.0256**
	5+1	0.024	0.001	0.308	0.034	**<0.0001**
	6+1	0.037	0.006	0.195	0.014	**0.0007**
	7+1	0.017	0.004	0.139	0.038	**0.0066**

Figure 15. Graphic representation of *E. coli* LF82 and K12 invasion indexes on d-ODMs. INV-I% of both *E. coli* strains (n = 5 for each represented point in the graph) at MOI 20 **(A)** and 100 **(B)** relative to the increasing infection time points. The dashed line represents the established threshold (0.1) over which *E. coli* strains were considered to be invasive. The error bars correspond to the SEM. **(C)** Mean, SEM and adjusted p-values obtained by a 2-way RM ANOVA test to examine statistical significance between LF82 and K12 INV-I% for each infection timepoint. This analysis was followed by a Tukey test correction for multiple testing. A P value of <0.05 was considered statistically significant, and it is highlighted in bold.

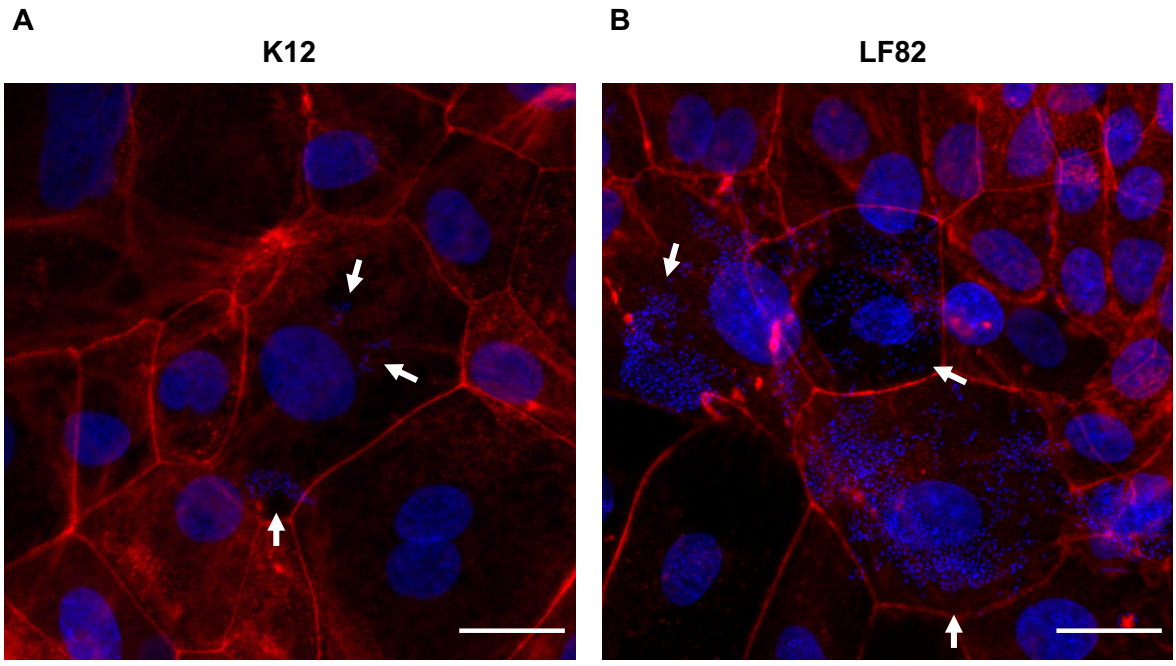


Figure 16. *E. coli* K12 and LF82 invasion of d-ODMs as determined by the gentamicin protection assay. Fluorescent staining was performed to visualize the LF82 infection of d-ODMs compared to the non-invasive control, K12 strain, after 5h of infection and 1h of gentamicin treatment at MOI 100. Phalloidin marked the eukaryotic actin filaments while DAPI bound to the DNA of both epithelial and bacterial cells. White arrows show bacterial localization inside the IECs. Scale bars: 25 μm. Images are representative of n = 3 independent experiments performed with samples from two different donors.

3. Human Primary Organoid-Derived Epithelial Monolayers respond to Adherent-Invasive *Escherichia coli* infection

3.1 LF82 and K12 strains induce similar effects on d-ODMs 5- or 6-hours post-infection

Interaction of AIEC bacteria with epithelial cells is not limited to cell invasion since it can also lead to the secretion of pro-inflammatory cytokines, destabilization of cell-to-cell junctions, and changes in the expression of diverse cell molecules and proteins (e.g., mucins, cell receptors, autophagy, etc.) by the host's cells (182). Therefore, to better characterize the interaction of this pathobiont with the epithelium, we studied the response that AIEC infection elicits on the d-ODM model.

As demonstrated in our previous experiments (**Figure 15**), 5+1h at MOI 100 was the most advantageous condition to study the invasive capacity of AIEC in d-ODMs. Hence, RNA and supernatants from non-infected d-ODMs (control) as well as *E. coli* infected d-ODMs at MOI 100 for 5 and 6h were collected for further analysis. No gentamicin treatment was conducted in this occasion based on previous published data (219). For this analysis, alterations in the transcriptional levels of genes codifying for AIEC cell receptors, junction proteins, cytokines, and other molecules

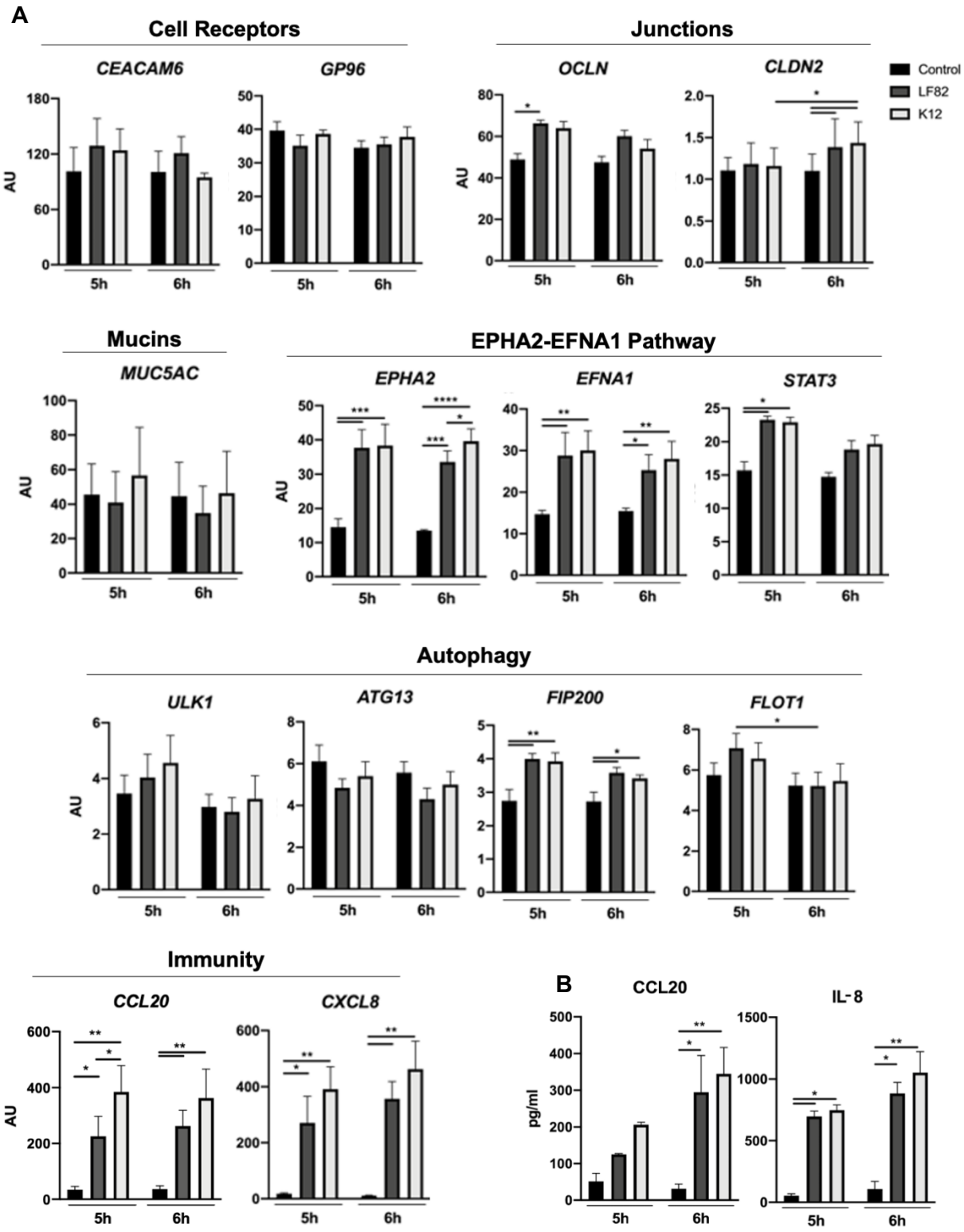


Figure 17. Characterization of d-ODMs response to AIEC infection after 5 and 6 hours. (A) Gene expression analysis of d-ODMs after exposure to *E. coli* LF82 and K12 strains. Non-infected d-ODMs were used as a control (n = 3 for each condition). The more relevant genes/pathways related to AIEC interaction with IECs were analyzed by RT-qPCR to determine their expression levels in infected vs. non-infected d-ODMs. **(B)** CCL20 and IL-8 secreted proteins of non-infected vs. *E. coli* infected d-ODMs were measured by ELISA (n = 3 for each condition). The error bars correspond to the SEM. A 2-way RM ANOVA test was applied to examine statistical significance between the three conditions for each infection timepoint. This analysis was followed by a Tukey test correction for multiple testing. * indicates $P < 0.05$; ** indicate ≤ 0.01; *** indicate ≤ 0.001; **** indicate < 0.0001.

involved in AIEC invasion of IECs were explored (**Figure 17A** and **Supplementary Figure 3** (see Annex I)).

AIEC infection did not significantly regulate the transcriptional expression of *CEACAM6, CHI3L1* and *GP96* which codify for proteins used by LF82 strain as receptors for adhesion to host IECs. In contrast, *OCLN* and *CLDN2* levels, both codifying for proteins involved in the formation of cell-to-cell junctions, were significantly increased after 5 and 6h of infection, respectively. In fact, alterations in *CEACAM6* and *CLDN2* have been reported to be associated with an increase in the intestinal barrier permeability (175,194,198,342). Other genes encoding for junction proteins (*TJP3*, *CEACAM7* and *JAM2*) did neither significantly change upon *E. coli* infection (**Supplementary Figure 3**).

AIEC have also been reported to reduce the expression of several mucins, nonetheless the differences we observed in *MUC5AC* or *MUC2* did not reach statistical significance.

On the other hand, even though an up-regulation of various Eph/ephrin proteins in response to inflammation has already been proved (343) and they have been recently been implicated in the infection of IEC by multiple viruses (344), there are no available reports analyzing its association in AIEC infection. Therefore, we evaluated the role of EPHA2-EFNA1 pathway (including several downstream genes such as *STAT3*, *JAK2* or *FYN*) in AIEC infection of IECs. We detected a significant increase in the expression of some of them (*EPHA2*, *EFNA1* and *STAT3*) after d-ODMs exposure to both *E. coli* strains and at 5- and 6-h post-infection. In contrast *JAK2* and *FYN* were not significantly changed upon bacterial infection.

Next, we measured the expression of several genes involved in mediating autophagy. Only expression of *FIP200* (a ULK1-ATG13 interacting protein required for autophagosome formation in mammalian cells (345,346)), but not *ULK1*, *ATG13*, *FLOT1* or *ATG5*, was significantly increased both at 5 and 6h of infection regardless of the *E. coli* strain. Finally, to monitor the inflammatory response induced by AIEC we measured changes in gene and protein expression of CCL20, *CXCL8*/IL-8, *CXCL10* and *IL-6* (**Figure 17A and B**). Both LF82 and K12 strains induced a significant increase in the mRNA levels as well as in the secretion of both CCL20 and IL-8.

The results from these experiments, thus far, demonstrate that exposure for 5 or 6h to *E. coli* induced a response in d-ODMs, affecting expression of genes involved in

cell-to-cell junctions, EPHA2-EFNA1 pathway, autophagy, and immune response. Nevertheless, we found no significant differences between the responses elicited by the two analyzed *E. coli* strains (as shown by others (342)). Similarly, the time of infection (5 or 6h) did not appear to be crucial since no significant differences were detected among them.

3.2 Extended d-ODMs Infection Periods Induce a Strain Specific Response in the Intestinal Epithelium

Given that mRNA and protein expression was measured right at the end of the infection period, the changes we observed so far could be a consequence of both the invasive as well as the adhered *E. coli* which, moreover, appear to induce similar responses regardless of the invasive capacity of the *E. coli* strain. Thus, we decided to perform additional experiments in which transcriptional and protein changes were measured after exposing them to *E. coli* for 12 or 24h under gentamicin treatment. In brief, d-ODMs were infected for 5h at MOI 100 followed by 1h of gentamicin treatment (100 μg/ml). The monolayer was then incubated with DIFF medium (without antibiotics) supplemented with 15 μg/ml of gentamicin (low-gentamicin treatment) for an additional 6 or 18h (being the total incubation period of 12 or 24h, respectively). At 12h, the INV-I% was 0.5735±0.1205 for the LF82 and 0.021±0.008 for the K12 strain, thus being 27.3 times higher in the LF82 compared to the K12 strain (**Figure 18**). On the other hand, at 24h the LF82 INV-I% was 11.15 times higher than that from the non-invasive strain (0.474±0.207 and 0.0425±0.0215 for LF82 and K12 strains, respectively). No bacterial cells were detected in the non-infected control. DAPI staining confirmed these results (**see Annex I Supplementary Figure 4**), showing the presence of high amounts of intracellular LF82 bacteria in most d-ODMs cells compared to the K12 strain and the non-infected control (292).

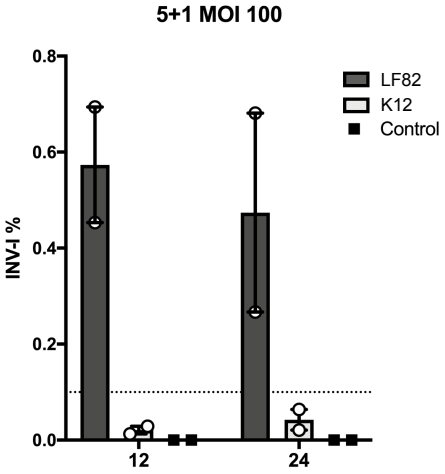


Figure 18. *E. coli* LF82 and K12 Invasion Indexes (INV-I %) in d-ODMs after low-gentamicin treatment. The dashed line represents the established threshold (0.1) that determines the invasive capacity of the *E. coli* strains after 12 and 24h. The assay was repeated twice (n = 2) and in duplicate. No statistic test could be performed due to insufficient data.

As in the previous analysis, expression of genes codifying for AIEC cell receptors, junction proteins, cytokines, and other molecules involved in the invasion of IECs by AIEC were measured (**Figure 19A** and **Supplementary Figure 5** - see Annex I). Overall, gene expression modulation was more consistent at 24 rather than 12h after AIEC infection. AIEC, but not *E. coli* K12, induced a significant increase of *OCLN* and a significant decrease of *CLDN2* – both markers of cell junctions – 24h after AIEC infection. Comparable to what we observed at earlier time points, mucin genes (*MUC5AC* and *MUC2*) were not significantly changed in response to AIEC or the non-invasive *E. coli*.

In addition to *OCLN* and *CLDN2*, *CHI3L1*, *EPHA2*, *EFNA1* and the autophagy gene *ATG13* were significantly regulated 24h post-AIEC infection. At 12h post-infection *ULK1* presented the greatest differences between strains, showing increased levels only in AIEC infected d-ODMs. Unfortunately, changes at that timepoint could not be statistically evaluated due to insufficient data (n = 2).

Similar to *EPHA2*, *EFNA1* and *ATG13*, significantly different effects were observed 24h after infection in the immune response genes *CCL20* and *CXCL8*. Nonetheless this selective effect was not confirmed by secretion of CCL20 and IL-8 proteins which were highly expressed by both *E. coli* stains after 24h (**Figure 19B**).

Altogether, and despite the low number of experimental replicates, we show that while at early time points (≤6h) post-infection and before gentamicin treatment, invasive and non-invasive *E. coli* strains trigger comparable responses in d-ODMs, AIEC promotes a significantly different response in d-ODMs compared to the non-invasive *E. coli* K12 strain 24h post-infection.

While we here provide only an initial characterization of this novel model of infection, our results clearly support the use of primary epithelial monolayers as a tool to further explore the effects of AIEC infection in human epithelium.[4]

[4] Part of these results are published in Frontiers in Immunology (see Annex II)

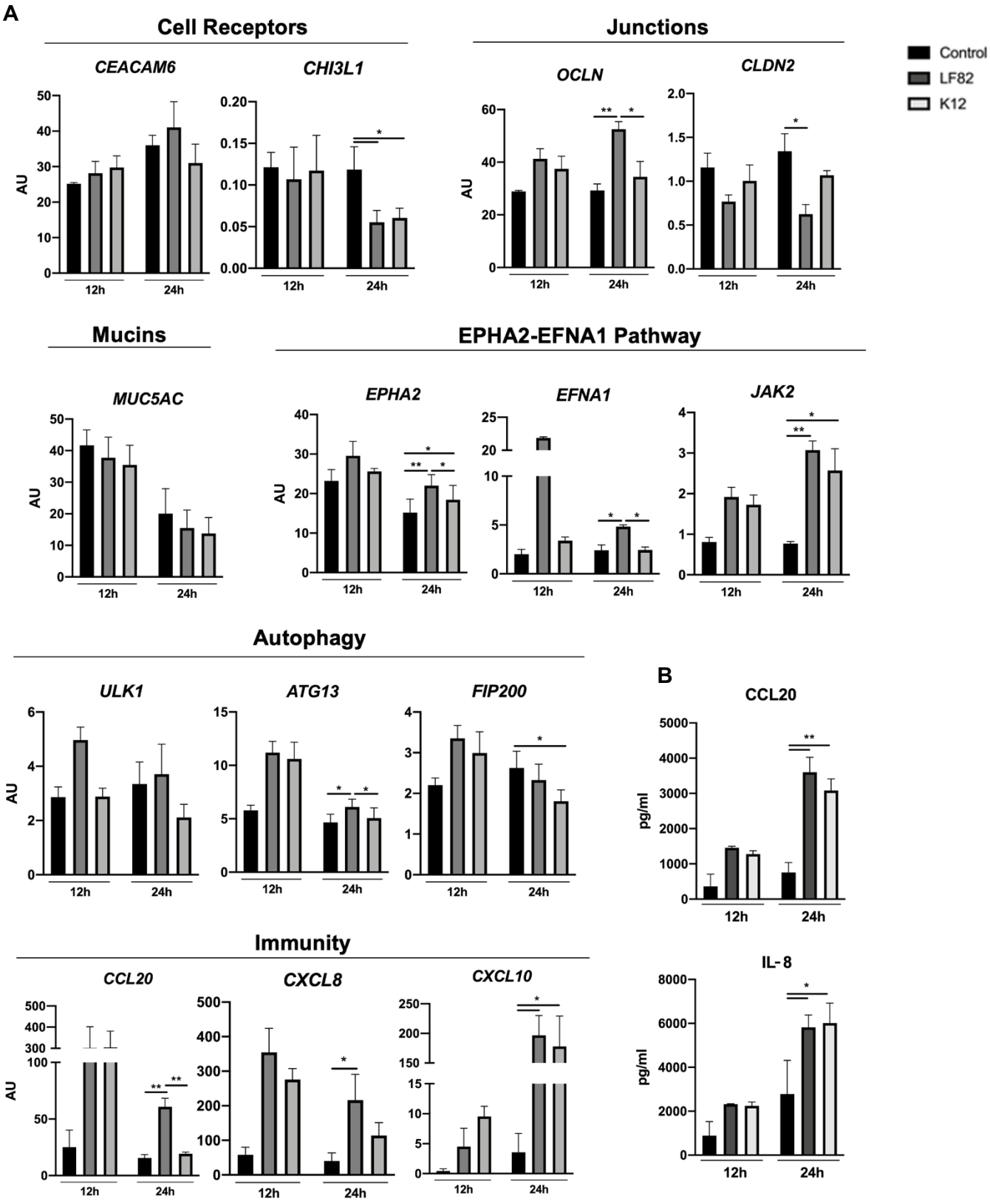


Figure 19. Characterization of d-ODMs response to AIEC infection after 12 and 24 hours. (A) Gene expression analysis of d-ODMs after exposure to *E. coli* LF82 and K12 strains. Non-infected d-ODMs were used as a control (n = 2 for 12h of infection and n = 3 for 24h). The more relevant genes/pathways related to AIEC interaction with IECs were analyzed by RT-qPCR to determine their expression levels in infected vs. non-infected d-ODMs. **(B)** CCL20 and IL-8 secreted proteins of non-infected vs. *E. coli* infected d-ODMs were measured by ELISA (n = 2 for 12h of infection and n = 3 for 24h).
The error bars correspond to the SEM. A One-way RM ANOVA test was applied to examine statistical significance between the three conditions at 24 hours. This analysis was followed by a Tukey test correction for multiple testing. * indicates $P < 0.05$; ** indicate ≤ 0.01; *** indicate ≤ 0.001; **** indicate < 0.0001.

STUDY 2: Production of Postbiotics and their effects on Human Blood Immune Cells and Organoid-Derived Epithelial Monolayers

1. Analysis and Characterization of Postbiotics derived from *S. thermophilus* and *E. coli*

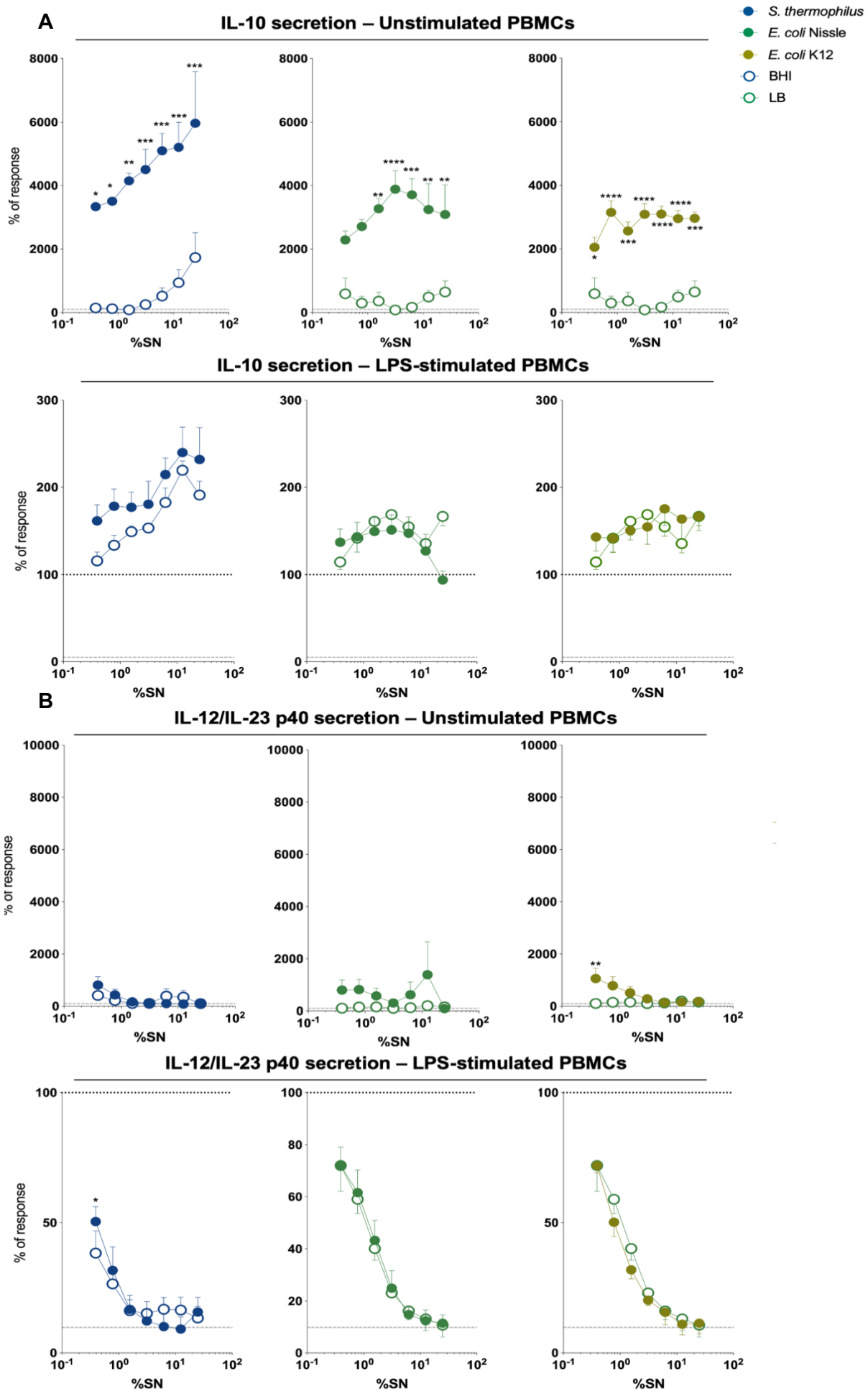
1.1 Bacterial culture media mask the putative effects of the secreted bacterial metabolites

As introduced in earlier sections, *S. thermophilus* and *E. coli* Nissle (both probiotic strains) have been shown to ameliorate IBD (315–325). Nevertheless, the use of probiotics to treat inflammatory disorders has also been demonstrated to pose some risks to patients due to the presence of MAMPs that could further promote inflammation (53). Therefore, we used their secreted metabolites (or postbiotics), as well as metabolites derived from *E. coli* K12 (non-probiotic control) – obtained as detailed in the Materials and Methods section (p.80) – to characterize its composition and explore their exerted properties on the epithelium.

Serial dilutions of postbiotics grown in their respective culture medium were tested on PBMCs stimulated with or without LPS for 24h. Moreover, to explore the contribution of the bacterial growth medium to the postbiotics effects, PBMCs were also cultured with serial dilutions of the *S. thermophilus* and *E. coli* culture media (BHI and LB, respectively). Cell viability under all culture conditions, determined using the MTT assay, did not change, thus supporting a non-toxic effect of the tested products (data not shown). At 24h of culture, supernatants were collected to analyze the

(figure on next page)

Figure 20. Percentage of response of the unstimulated and LPS-stimulated PBMCs to postbiotics and culture media. Postbiotics from *S. thermophilus* (blue) and *E. coli* Nissle (green), as well as the secreted metabolites from *E. coli* K12 (yellow), were assessed for their effects in human isolated PBMCs. The culture media (BHI and LB) were included as the "vehicle". Postbiotics or the culture media were serially diluted (0.391-25%) and added to the cell cultures. **(A)** IL-10 levels relative to their respective control (cells incubated ± LPS). The dashed line in the upper panel (unstimulated cells) represents the untreated cells – control – (100%). The dashed lines in the lower panel (LPS-stimulated cells) represent the untreated and the LPS-treated cells (4.93% and 100%, respectively). **(B)** IL-12/IL-23 p40 levels relative to their respective control (cells incubated ± LPS). The dashed line in the upper panel (unstimulated cells) represents the untreated cells – control – (100%). The dashed lines in the lower panel (LPS-stimulated cells) represents the untreated and the LPS-treated cells (9.82% and 100%, respectively).
The assay was performed with cells from 4 different donors and in duplicate. A 2-way RM ANOVA was performed to examine the statistical significance between postbiotics and the culture media. This analysis was followed by a Tukey test correction for multiple testing. * indicate $P < 0.05$; ** indicate ≤ 0.01; *** indicate ≤ 0.001; **** indicate < 0.0001. SN: supernatant


A
IL-10 secretion – Unstimulated PBMCs
IL-10 secretion – LPS-stimulated PBMCs
B
IL-12/IL-23 p40 secretion – Unstimulated PBMCs
IL-12/IL-23 p40 secretion – LPS-stimulated PBMCs
S. thermophilus
E. coli Nissle
E. coli K12
BHI
LB
% of response
%SN

concentration of IL-10 (an anti-inflammatory cytokine (347)) and IL-12/IL-23 (both heterodimeric cytokines sharing the p40 subunit and secreted in response to infection (348)). **Figure 20** shows the percentage of response (relative to their respective controls) for each evaluated protein.

PBMCs treated with postbiotics or K12 secreted-metabolites triggered a significant increase of IL-10 compared to the culture media alone while they did not induce a change in IL-12/IL-23 p40 secretion at any of the concentrations studied (**Figure 20A and Figure 20B** - upper panels), indicating a *per se* anti-inflammatory effect of the tested postbiotics. LPS-treated PBMCs also showed an increase in IL-10 secretion, although non-significant (**Figure 20A** - lower panel).

IL-12/IL-23 p40 was also secreted by PBMCs in response to LPS (**Figure 20B** lower panel). In addition, both the bacterial metabolites from all three species and their corresponding culture media alone, decreased – in a dose dependent manner – the secretion of IL-12/IL-23 by LPS-stimulated PBMCs.

Overall, these results suggest that bacterial secreted-metabolites may have an anti-inflammatory effect (decrease in IL-12/IL-23 and increase in IL-10 secretion) in LPS-stimulated cells. Nevertheless, since BHI and LB media also regulated to a comparable degree the amount of IL-10 and IL-12/IL-23 p40 produced by LPS-stimulated PBMCs, we hypothesized that the media present in the metabolite cocktail might affect the immune cell response to LPS and hinder the interpretation of our results.

To confirm our hypothesis, some of the tested metabolite-cocktails and culture media were analyzed in parallel by LC-MSMS at the Proteomics Platform of the Barcelona Scientific Park (Barcelona, Spain). The descriptive results represented in **Supplementary Figure 6** (see Annex I) show that the top 10 isolated proteins in the postbiotics' solution are the same as for their culture medium, confirming a remarkable contribution of the latter to the effects of the metabolite-cocktails on cell responses, thus interfering on their characterization.

Altogether, these results strongly suggested that the bacteria culture media had a critical impact on the postbiotics' composition masking their possible immunomodulatory effects.

1.2 Purified Postbiotics Characterization

Based on the previous results, we decided to purify the postbiotics (and *E. coli* K12 secreted metabolites) before testing their effects *in vitro*. Hence, in collaboration with Postbiotica S.r.l, bacterial supernatants were purified thus removing the effect of the media (undisclosed method). Then, postbiotics were analyzed again by LC-MSMS and UPLC-HILIC-qTOF for proteomic and metabolomic characterization, respectively.

From the entire list of identified proteins (97 for *S. thermophilus*, 922 for *E. coli* Nissle and 843 for *E. coli* K12), **Figure 21A** shows the top 10 secreted proteins of each bacterial strain with the highest peptide spectrum match (PSM), namely those proteins whose detected peptides were more frequently identified. Even though these results are merely descriptive, characterizing the composition of a metabolite-cocktail is a valuable step before conducting any further analysis.

On the other hand, **Figure 21B** shows the relative quantification of the listed metabolites among every analyzed metabolite-cocktail. In this case, due to technical limitations (i.e., lack of bacterial-metabolites databases), we could only identify 7 metabolites for each bacterial strain. As an example, levels of hypoxanthine in *S. thermophilus* postbiotics were lower than those found in both *E. coli* strains secreted-metabolites. The same behavior was observed with almost all the detected metabolites except for tryptophan, whose levels were similar among the bacterial strains. These results suggest that *S. thermophilus'* metabolomic profile differs from that of *E. coli* Nissle and *E. coli* K12. These results are consistent with the fact that *S. thermophilus* and *E. coli* are bacterial strains phylogenetically greatly diverse.

2. Postbiotics show an Immunomodulatory Effect on Human Blood Immune Cells

Based on the results showed in the previous section (**Figure 20**), we explored the potential immunomodulatory effects of the purified postbiotics on human blood immune cells.

2.1 Effects on PBMCs

First, different concentrations (see Section *4.1.2 PBMCs Stimulation* (p.83)) of the purified postbiotics or their vehicle (undisclosed composition) were tested for their

immunomodulatory effects on PBMCs stimulated with or without LPS for 24h. None of the metabolite-cocktails decreased cell viability based on the MTT assay (data not shown). The supernatants were collected for IL-10 and IL-12/IL-23 p40 protein secretion analysis.

None of the purified metabolite-cocktails induced a significant change in secretion of IL-10 or IL-12/IL-23 p40 cytokines in unstimulated PBMCs (data not shown).

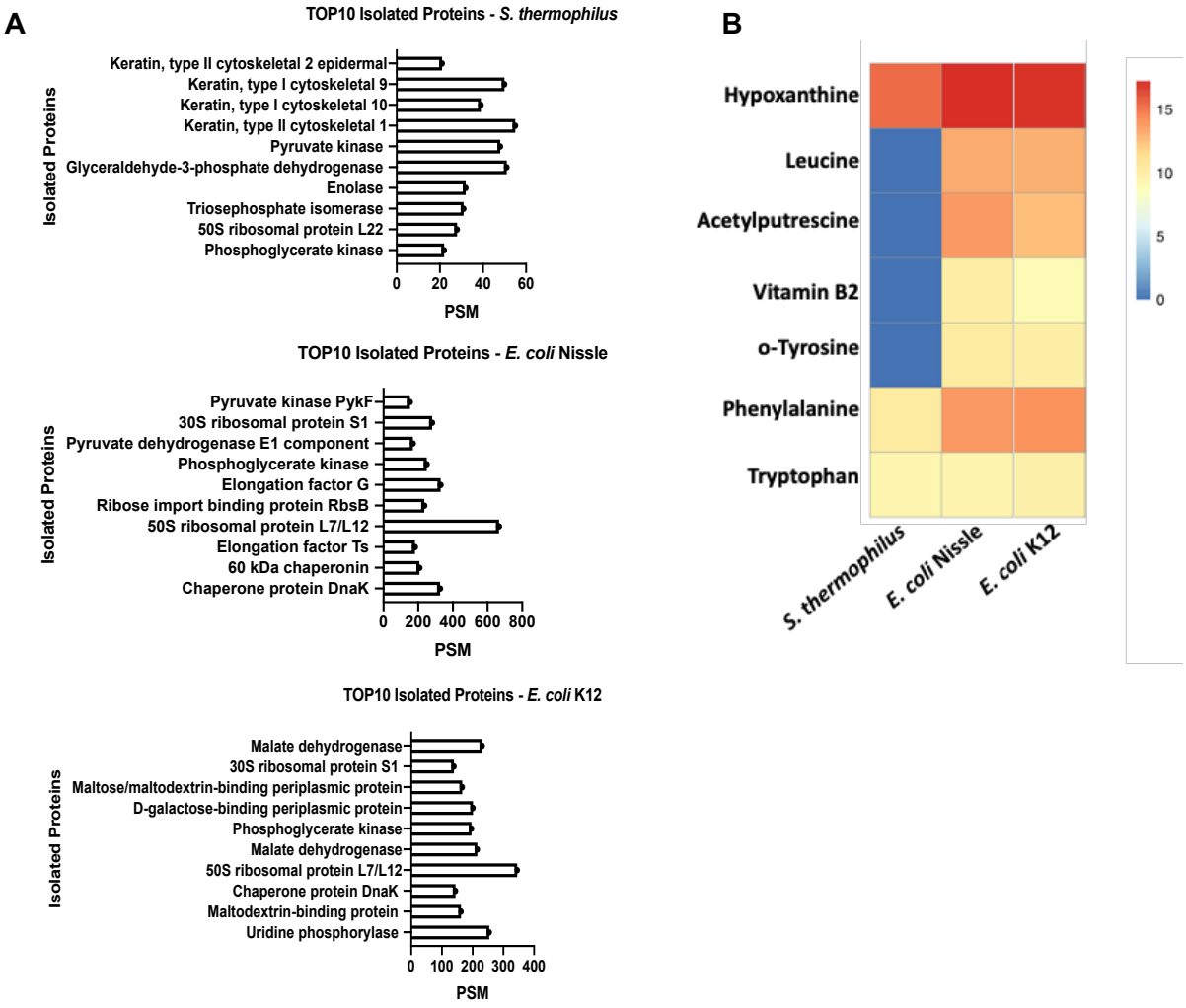


Figure 21. Protein and metabolite identification of the purified metabolite-cocktails. *S. thermophilus* and *E. coli* Nissle postbiotics as well as *E. coli* K12 secreted metabolites were analyzed to characterize their protein and metabolite content by LC-MSMS or UPLC-HILIC-qTOF, respectively. **A)** The represented descriptive data shows the top 10 identified proteins with a higher peptide spectrum match (PSM): those proteins whose detected peptides were more frequently identified. **B)** Relative quantification (counts) of each detected metabolite among every analyzed metabolite-cocktail. This data demonstrates that *S. thermophilus* levels of each detected metabolite are lower than those from both *E. coli* strains.

Similarly, LPS-stimulated cells did not show an increase in the secretion of IL-10 (**Figure 22A**) after treatment. On the contrary, *E. coli* Nissle significantly reduced IL-10 production when applied at the highest concentration (25%) to LPS-stimulated

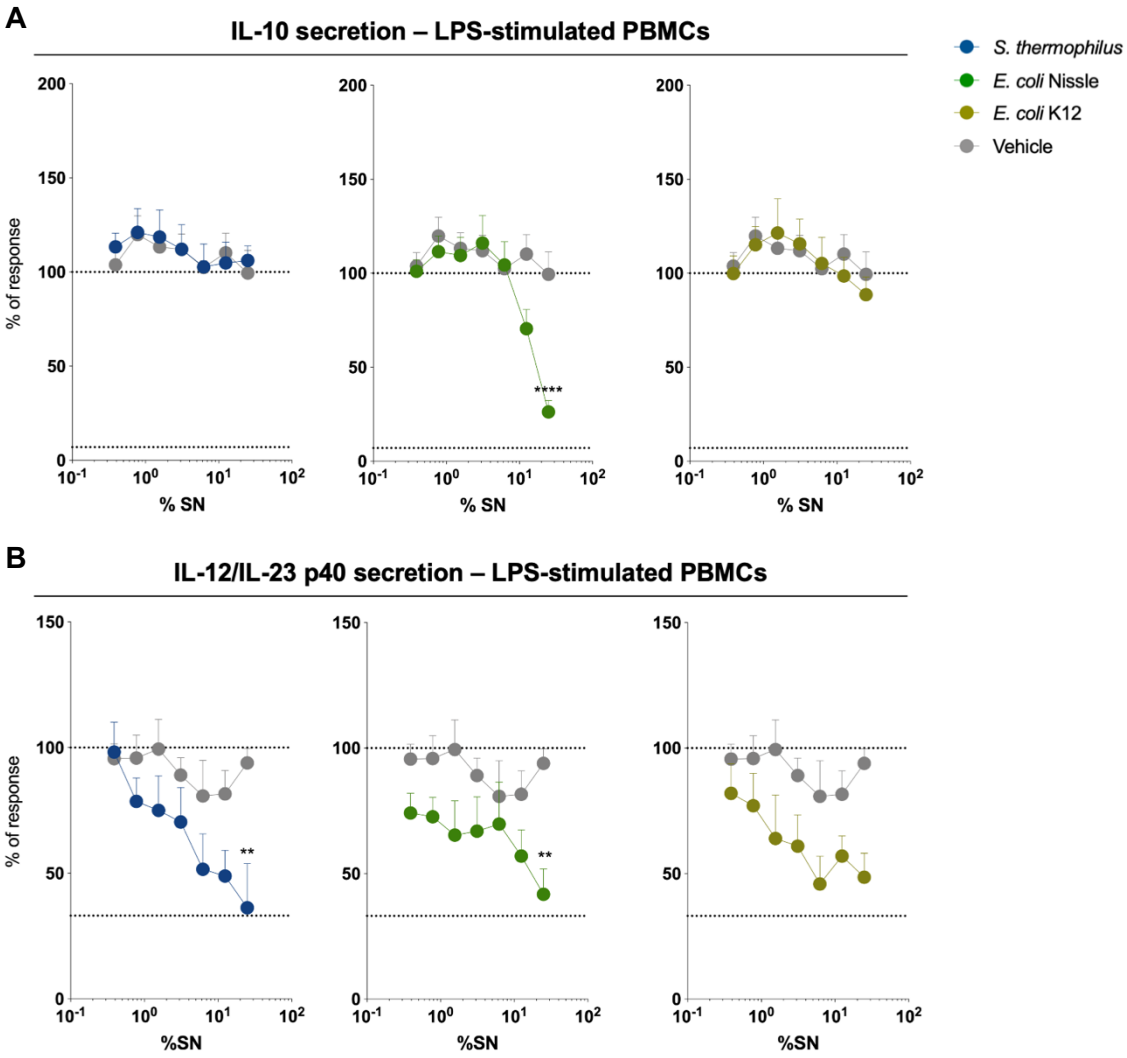


Figure 22. Percentage of response of LPS-stimulated PBMCs upon treatment with purified postbiotics. Purified postbiotics from *S. thermophilus* (blue) and *E. coli* Nissle (green), as well as secreted metabolites from *E. coli* K12 (yellow), were assessed for their anti-inflammatory effects in human isolated PBMCs. Postbiotics or the vehicle were serially diluted (0.391-25%) and added to the cell cultures. **(A)** IL-10 levels relative to the control (cells incubated only with LPS). The dashed lines represent the untreated and the LPS-treated cells (7% and 100%, respectively). **(B)** IL-12/IL-23 p40 levels relative to the control (cells only incubated with LPS). The dashed lines represent the untreated and the LPS-treated cells (33.13% and 100%, respectively).
The assay was performed with cells from 6 different donors and in duplicate. A 2-way ANOVA was performed to examine the statistical significance between Postbiotics and the vehicle. This analysis was followed by a Tukey test correction for multiple testing. * indicates $P < 0.05$; ** indicate ≤ 0.01; *** indicate ≤ 0.001; **** indicate < 0.0001. SN: supernatant

cells. Moreover, IL-12/IL-23 p40 secretion was significantly reduced in LPS-stimulated PBMCs when cells were treated with the highest concentration (25%) of *S. thermophilus* and *E. coli* Nissle postbiotics (**Figure 22B**).

Finally, we obtained an anti-inflammatory index calculated as the ratio of IL-10 to IL12/IL23 p40 responses in LPS-stimulated PBMCs upon postbiotics treatment (**Figure 23**). This analysis revealed a significantly higher anti-inflammatory index of *S. thermophilus* postbiotics at the highest tested concentration (25%). These effects were not observed when using *E. coli* Nissle postbiotics, or the metabolite mixture derived from the non-probiotic *E. coli* strain K12, thus suggesting an advantage of *S. thermophilus* postbiotics in promoting a protective immunomodulatory response upon LPS stimulation.

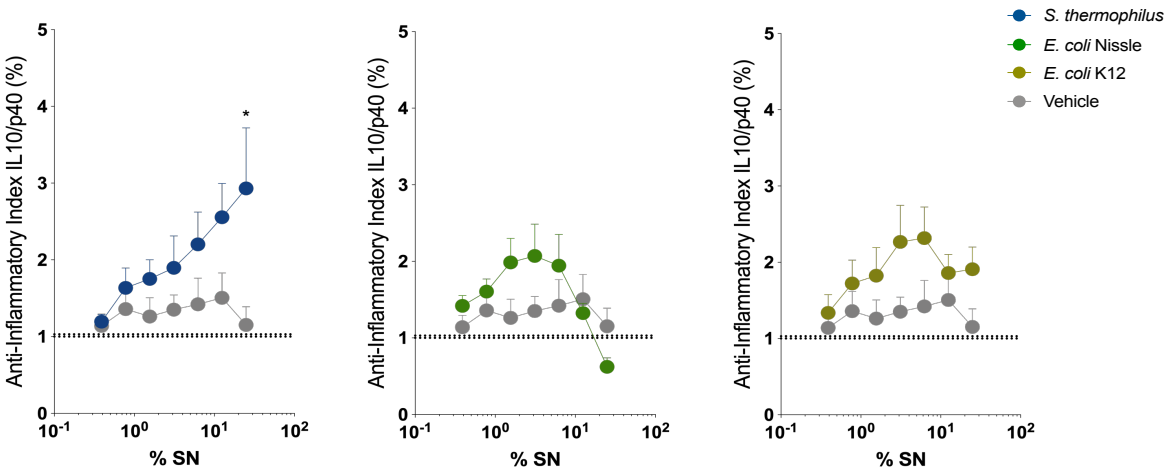


Figure 23. Postbiotics' Anti-Inflammatory Index. From the three analyzed metabolite-cocktails, only postbiotics derived from *S. thermophilus* showed a significant anti-inflammatory profile when added at the highest concentration (25%) to LPS-stimulated PBMCs.
The dashed lines represent the untreated and the LPS-treated cells (1% and 1,03%, respectively). The assay was performed with PBMCs from 6 different donors and in duplicate. A 2-way ANOVA was performed to examine the statistical significance between Postbiotics and the vehicle. This analysis was followed by a Tukey test correction for multiple testing. * indicates $P < 0.05$; ** indicate ≤ 0.01; *** indicate ≤ 0.001; **** indicate < 0.0001. SN: supernatant

2.2 Effects on moDCs

DCs are crucial to cross-link the innate and adaptive immune responses. We thus tested for the postbiotics' potential immunomodulatory effects in this relevant immune cell subset.

To generate DCs, monocytes were positively selected from PBMCs and induced to differentiate in the presence of GM-CSF and IL-4. Immature moDCs were then

characterized by Flow Cytometry analysis. **Table 14 in Annex I** lists the antibodies used (clones and conjugated fluorochromes) to stain the selected cell surface markers. As shown in **Figure 24**, we obtained a highly homogeneous population of DCs positive for the DC markers CD11c and HLA-DR and negative for CD14 (marker of monocytes), after differentiation.

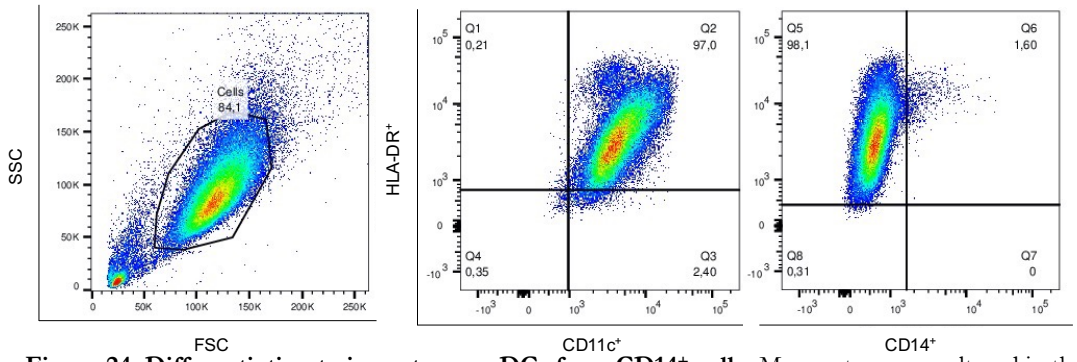


Figure 24. Differentiation to immature moDCs from CD14⁺ cells. Monocytes were cultured in the presence of GM-CSF and IL-4 to induce their differentiation to immature DCs. Expression of cell surface markers of DCs (HLA-DR and CD11c) and monocytes (CD14) was analyzed at day 7 by flow cytometry showing a complete differentiation to the desired cell type. The graphs are representative from 3 independent experiments. SSC: side-scattered; FSC: forward-scattered

Purified postbiotics were then tested for their putative immunomodulatory effects on moDCs stimulated with or without LPS for 24h. None of the metabolite-cocktails affected cell viability measured using the MTT assay (data not shown). Supernatants from cultured moDCs were collected after 24h to measure IL-10 and IL-12 p70 protein secretion

As shown in **Figure 25A** (upper panel), all the tested metabolite-cocktails induced the secretion of IL-10 on immature (unstimulated) moDCs. LPS-stimulated moDCs showed an increase in the secretion of IL-10 (**Figure 25A**, lower panel) that was higher in cells that were exposed to *S. thermophilus* postbiotics and *E. coli* K12 secreted metabolites. These results, together with the analyzed IL-12 p70 secretion (data not shown), allowed the determination of the anti-inflammatory index (IL-10/p70) in LPS-treated moDCs (**Figure 25B**). Although we did not perform a statistical analysis due to the low number of replicates, these results suggest an anti-inflammatory potential of *S. thermophilus* postbiotics (**Figure 25B** – in blue) and *E. coli* K12 metabolites (**Figure 25B** – in yellow) on moDCs.

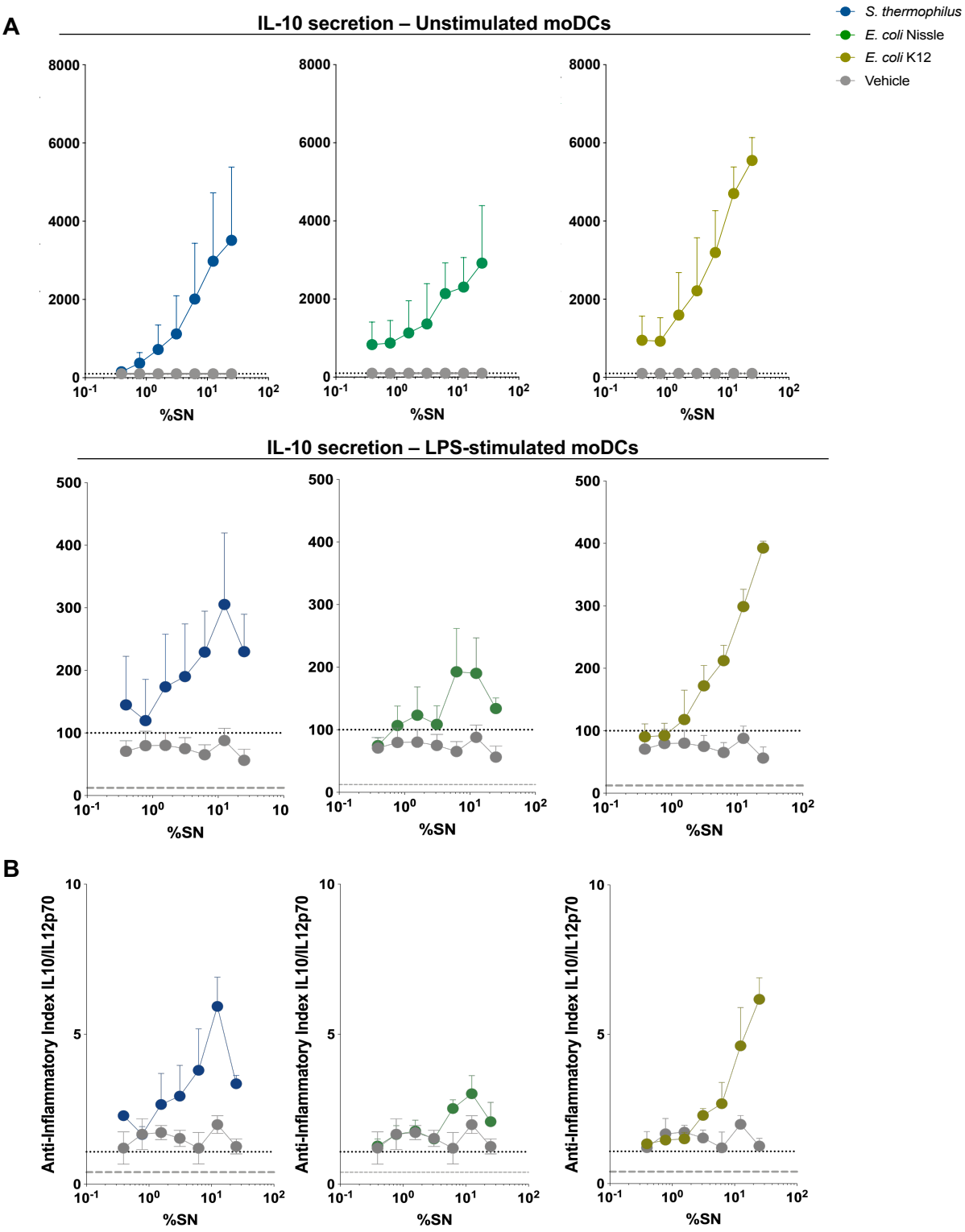


Figure 25. Percentage of response of LPS-stimulated moDCs upon treatment with purified postbiotics. Purified postbiotics from *S. thermophilus* (blue) and *E. coli* Nissle (green), as well as secreted metabolites from *E. coli* K12 (yellow), were assessed for their anti-inflammatory effects in moDCs. Postbiotics or the vehicle were serially diluted (0.391-25%) and added to the cell cultures. **(A)** IL-10 protein levels relative to their respective control (cells incubated ± LPS). The dashed line in the upper panel (unstimulated cells) represents the untreated cells (100%). The dashed lines in the lower panel (LPS-stimulated cells) represent the untreated and the LPS-treated cells (12.26% and 100%, respectively). **(B)** Postbiotics' Anti-Inflammatory Index. Data derived from the analysis of IL-10 and IL-12 p70 secretion (data not shown) in LPS-stimulated cells after postbiotics treatment was used to obtain the anti-inflammatory index (IL-10/IL-12 p70). The dashed lines represent the untreated and the LPS-treated cells (0.4% and 1.08%, respectively). The assay was performed with cells from 2 different donors and in duplicate. SN: supernatant

3. Postbiotics regulate the expression signature of Human Primary Organoid-Derived Epithelial Monolayers

Apart from the already demonstrated effects of *S. thermophilus* and *E. coli* Nissle probiotics on the improvement of IBD severity, their postbiotics have been shown to block pathogen adhesion to IECs (349), to limit DNA damage (350) and to enhance immune responses (247). Nevertheless, most of the available *in vitro* studies have been performed using human or mouse immortalized cell lines.

For this reason, and as we already did in the previous study, ODMs were employed to analyze the effects of the selected postbiotics on the intestinal epithelium. For that purpose, d-ODMs were incubated in the presence of *S. thermophilus* or *E. coli* Nissle postbiotics, as well as *E. coli* K12 secreted metabolites. Total RNA from d-ODMs was isolated after 24h. Transcriptomic analysis performed by RNA sequencing (RNAseq) showed that *S. thermophilus*, *E. coli* Nissle and *E. coli* K12 secreted metabolites induced the differential expression of 189, 143 and 158 genes, respectively, compared to the vehicle. Only 6 of these genes were similarly affected by the metabolite-cocktails of the 3 bacterial strains (**Figure 26A**). Apart from two that remain uncharacterized, the expression of the other 4 genes is showed in **Figure 26B** (RNAseq data). Of note, the 3 microbial metabolite-cocktails induced a significant decrease in the expression of *ORC1* gene, and an increase in *RIMBP3* expression levels compared to the unstimulated culture. *ORC1* is known to be essential for DNA replication and its expression is induced by cell growth stimulation while *RIMBP3* encodes for a protein crucial for spermiogenesis; nonetheless, their role in epithelial cell function remains unknown (351,352).

On the other hand, the 3 tested metabolite-cocktails induced the expression of *FABP6,* a cell receptor of long-chain fatty acids and bile acids, mainly expressed in the small intestine (353), whose expression has been reported to be decreased in IBD patients (354).

Similarly, *C2,* which encodes for a protein of the complement system that is part of the innate immune defense, was significantly increased in d-ODMs by the 3 microbial metabolite-cocktails.

Beyond these 4 commonly regulated genes, we also looked at some other involved in pathways that are relevant to intestinal immunity and that were differentially expressed in d-ODMs upon treatment with the postbiotics individually.

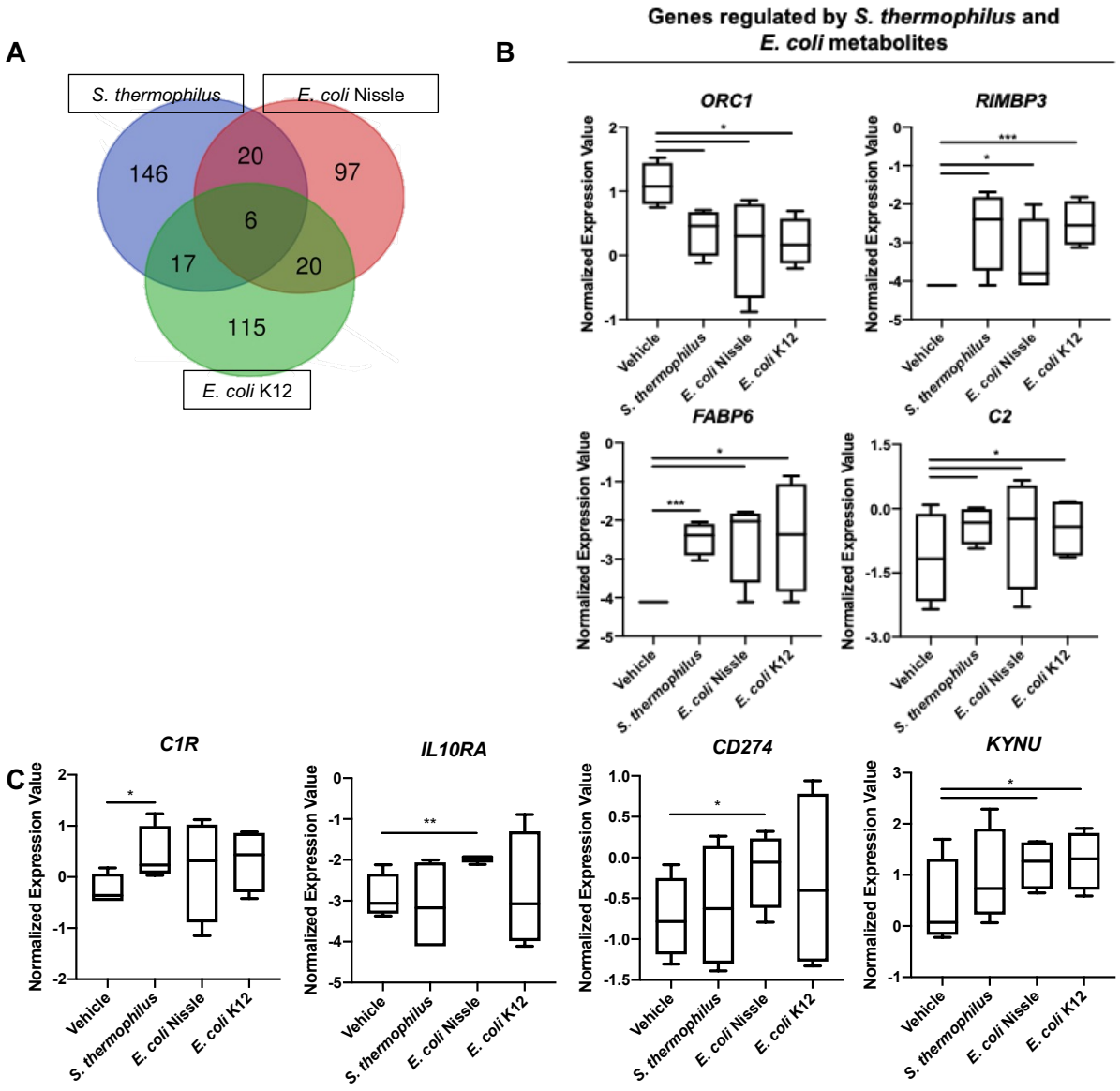


Figure 26. Genes regulated by *S. thermophilus* and *E. coli* secreted metabolites in d-ODMs. A) Venn diagram showing the number of genes that are differentially expressed in ODMs treated with *S. thermophilus*, *E. coli* Nissle and *E. coli* K12 secreted metabolites compared to the vehicle.
Normalized expression values of **B)** genes commonly deregulated by the three metabolite-cocktails and **C)** genes specifically deregulated by *S. thermophilus* postbiotics (*C1R*), *E. coli* Nissle postbiotics (*IL10A* and *CD274*) or *E. coli* secreted metabolites (*KYNU*). A gene was considered differentially expressed when it was significant at 5% p-value and showed a fold-change different than |0|. * indicates $P < 0.05$; ** indicate ≤ 0.01; *** indicate ≤ 0.001

For example, *S. thermophilus* postbiotics significantly induced the expression of *C1R* (**Figure 26C**), a proteolytic subunit in the complement system that cleaves C2 (whose expression in d-ODMs is also increased after *S.thermophilus* postbiotic treatment, as we just mentioned). Therefore, our data suggests that postbiotics derived from *S. thermophilus* may have a role in innate immunity regulation of IECs (355). When focusing on postbiotics from *E. coli* Nissle, a significant increase on the expression of *IL10RA* and *CD274*, both related to the immune

response, was shown. Indeed, IL10RA mediates the immunosuppressive signal of IL-10 and inhibits the synthesis of pro-inflammatory cytokines, while CD274 blocks exacerbated T cell activation and is also important in IECs-T cell interactions (356,357).

Finally, both *E. coli* Nissle and K12 strains increased *KYNU* levels in d-ODMs. *KYNU* encodes for an enzyme involved in the biosynthesis of nicotinamide adenine dinucleotide (NAD) cofactors from tryptophan (358). This data matched with the results displayed in **Figure 21B**, where we identified similar levels of tryptophan in the metabolite-cocktails of the three bacterial strains. Thus, the presence of tryptophan in the media might induce its metabolization by IECs through the activation of *KYNU*.

Altogether, these results would support the capacity of postbiotics to modulate the transcriptional signature of non-inflamed d-ODMs. To further confirm these results, transcriptional, proteomic as well as functional validation in an additional cohort of postbiotic-treated d-ODMs would need to be conducted.

4. Effect of Postbiotics on Inflamed Human Primary Organoid-Derived Epithelial Monolayers

Next, we investigated the potential ability of postbiotics to modulate the response of intestinal epithelial cells (d-ODMs) to relevant inflammatory signals.

4.1 Artificial Inflammation of d-ODMs mimics the transcriptional profile of active IBD patients

First, we aimed to determine the response of the intestinal epithelium to pro-inflammatory stimuli. Artificial inflammation was achieved by exposing d-ODMs to 4 different stimuli (TNFα, IL-1β, FLA and IFNγ) and to a combination of two of them (IFNγ +TNFα, here called inflammatory cocktail or cocktail). Twenty-four hours after stimulation, RNA was collected and RNAseq analysis was conducted (as detailed in Materials and Methods section (p.86)) to identify the differentially expressed genes under all the stimulating conditions.

A total of 588 genes were differentially expressed by IFNγ, 180 by TNFα, 356 by the inflammatory cocktail, 329 by IL-1β and 304 by FLA, compared to the vehicle. **Table 17** (see Annex I) shows the top 50 genes regulated by each stimulus highlighting in yellow those that are common to, at least, 2 stimuli. The inflammatory cocktail, for instance, shared 194 genes with the transcriptional signature induced by IFNγ and 20 genes with TNFα-signature (**Figure 27A**). This data suggests a major implication of

IFNγ rather than TNFα on the effects induced by the inflammatory cocktail. In fact, when analyzing – using Ingenuity Pathway Analysis (IPA) – the top upstream regulators identified for both IFNγ and the inflammatory cocktail (**Figure 27B**, upper panel), most of them were common. Similarly, although to a lesser extent, IL-1β and FLA (**Figure 27B**, lower panel) shared part of their induced signature (94 differentially expressed genes - **Figure 27A**).

Next, to elucidate if the *ex vivo* artificially-inflamed epithelial culture could mimic the transcriptional profile of the active IBD mucosa, we compared the signatures induced by the pro-inflammatory stimuli on artificially-inflamed d-ODMs to an in-house RNAseq database from inflamed biopsies of active CD and UC patients (**Table 18** – see Annex I).

As we show in **Table 19** (see Annex I), from the total number of genes differentially expressed in d-ODMs after exposure to IFNγ, TNFα, the inflammatory cocktail, IL-1β and FLA, 45% and 36% of them were common in CD and UC patients, respectively. Moreover, 73-76% of the common differentially expressed genes followed the same trend (i.e., genes up-regulated in artificially-inflamed d-ODMs, were also found to be up-regulated in the IBD cohort). This result indicates that the epithelial response of d-ODMs to inflammatory signals mimics the transcriptional landscape in IBD.

4.2 Postbiotics Effects on Inflamed Human Primary Organoid-Derived Epithelial Monolayers

Finally, to reveal the capacity of postbiotics to prevent the induced inflammatory status of the d-ODMs, postbiotics or the vehicle (10% v/v) were added to the cell culture 17-19h before the artificial inflammation was induced (ON pre-conditioning). Twenty-four hours after d-ODMs inflammation, RNA was collected and RNAseq was conducted as detailed in Materials and Methods section (p.86).

4.2.1 *S. thermophilus* Postbiotics tune the Effect of Inflammation induced by IL-1β and the pro-inflammatory cocktail in d-ODMs

A PCA was performed to visualize the distribution of the transcriptional profiles of artificially-inflamed d-ODMs with or without postbiotics pre-

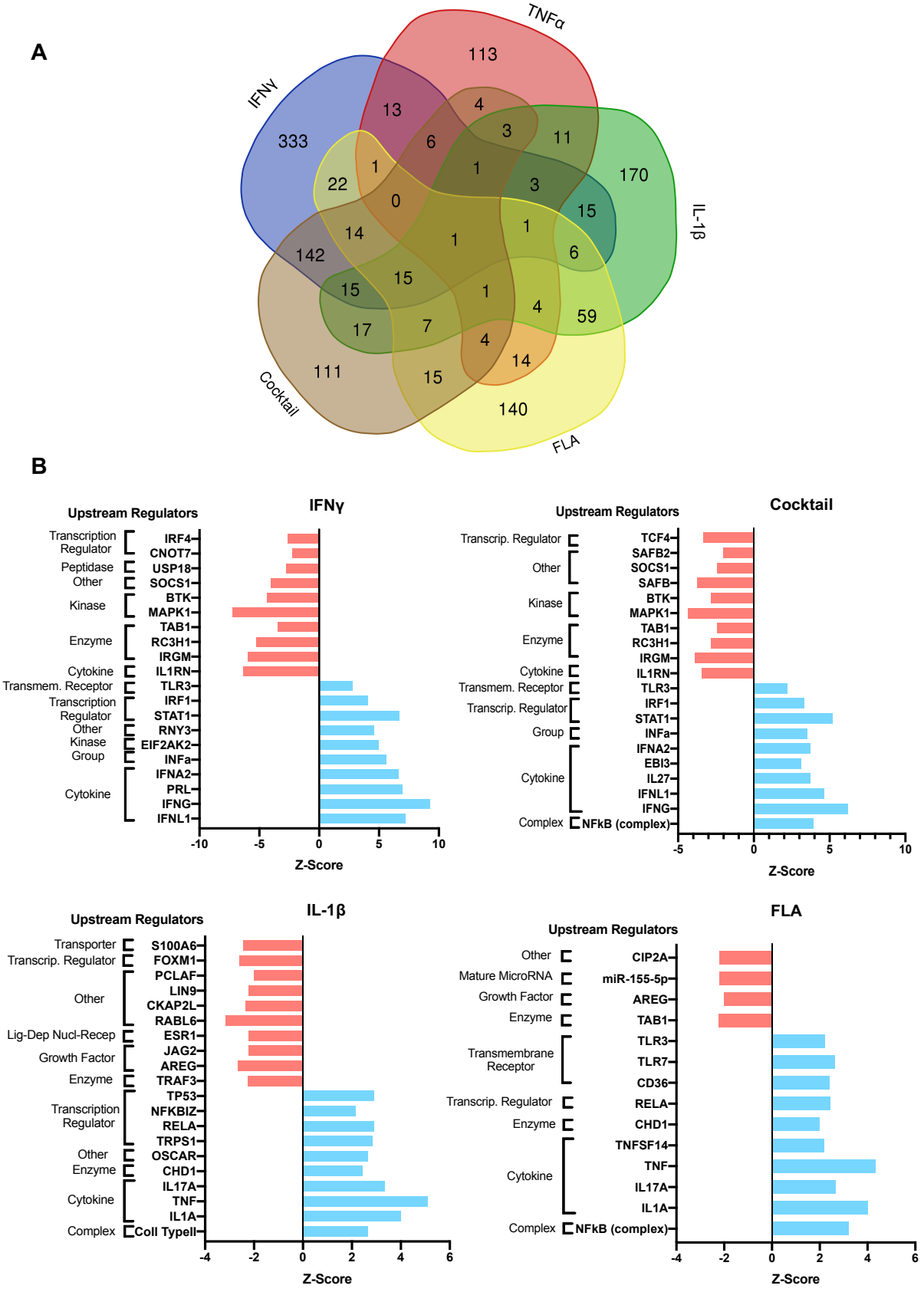


Figure 27. Transcriptional signature of artificially-inflamed d-ODMs. A) Venn diagram showing the number of differentially expressed genes in artificially-inflamed d-ODMs upon stimulation with the different cytokines compared to the vehicle. **B)** Bar plot showing the activation z-score of the top upstream regulators of d-ODMs inflamed with IFNγ, the inflammatory cocktail, IL-1β and FLA compared to the vehicle (data obtained by IPA).

treatment. As shown in **Figure 28**, this analysis suggested that only exposure to *S. thermophilus* postbiotics modulated the response of d-ODMs to inflammation, and that this modulation was present exclusively when d-ODMs were stimulated with IL-1β and the inflammatory cocktail. Thus, we specifically analyzed the genes whose expression was reversed by *S. thermophilus* postbiotics under these conditions (**Table 20** in Annex I shows the complete list of genes). As a result, 43 and 39 genes were identified when d-ODMs (pre-treated with *S. thermophilus* postbiotics) were stimulated with IL-1β and the inflammatory cocktail, respectively. Three of them (highlighted in bold in **Table 20** - Annex I) attracted our attention due to their well-known implication in IBD pathogenesis: *CHI3L1, NOD2* and *CARD9*. *CHI3L1* encodes for a glycoprotein that has been reported as a novel biomarker of IBD activity in CD patients (359,360). In colitis, CHI3L1 exacerbates intestinal inflammation by binding to bacterial chitin-binding protein, (167,361) thus enhancing bacterial adhesion and invasion as is the case of AIEC strains (362). Accordingly, our dataset revealed an increase in the expression of *CHI3L1* in IL-1β-inflamed d-ODMs (**Table 20** in Annex I). This effect was counteracted by *S. thermophilus* postbiotics to levels similar to the control (**Figure 29**). Similarly, transcriptional levels of *NOD2* – a gene that has been strongly associated to the risk to develop CD and that is known to play a key role in the recognition and handling of bacterial signals – were also regulated by *S. thermophilus* postbiotics in d-ODMs inflamed with IL-1β. On the other hand, the expression of *CARD9*, whose deficiency has been reported to impair the immune responses in IBD patients and increase their predisposition to undergo microbial infections (363–365), was significantly decreased by the inflammatory cocktail in d-ODMs.

Again, this effect was counteracted by *S. thermophilus* postbiotics.

Overall, we showed that *S. thermophilus* postbiotics hold potential beneficial properties on the epithelium under an inflammatory status. Thus, postbiotics might presumably reduce the effects of inflammation in IECs, not only by regulating the immune response but also by affecting bacterial recognition and clearance by IECs.

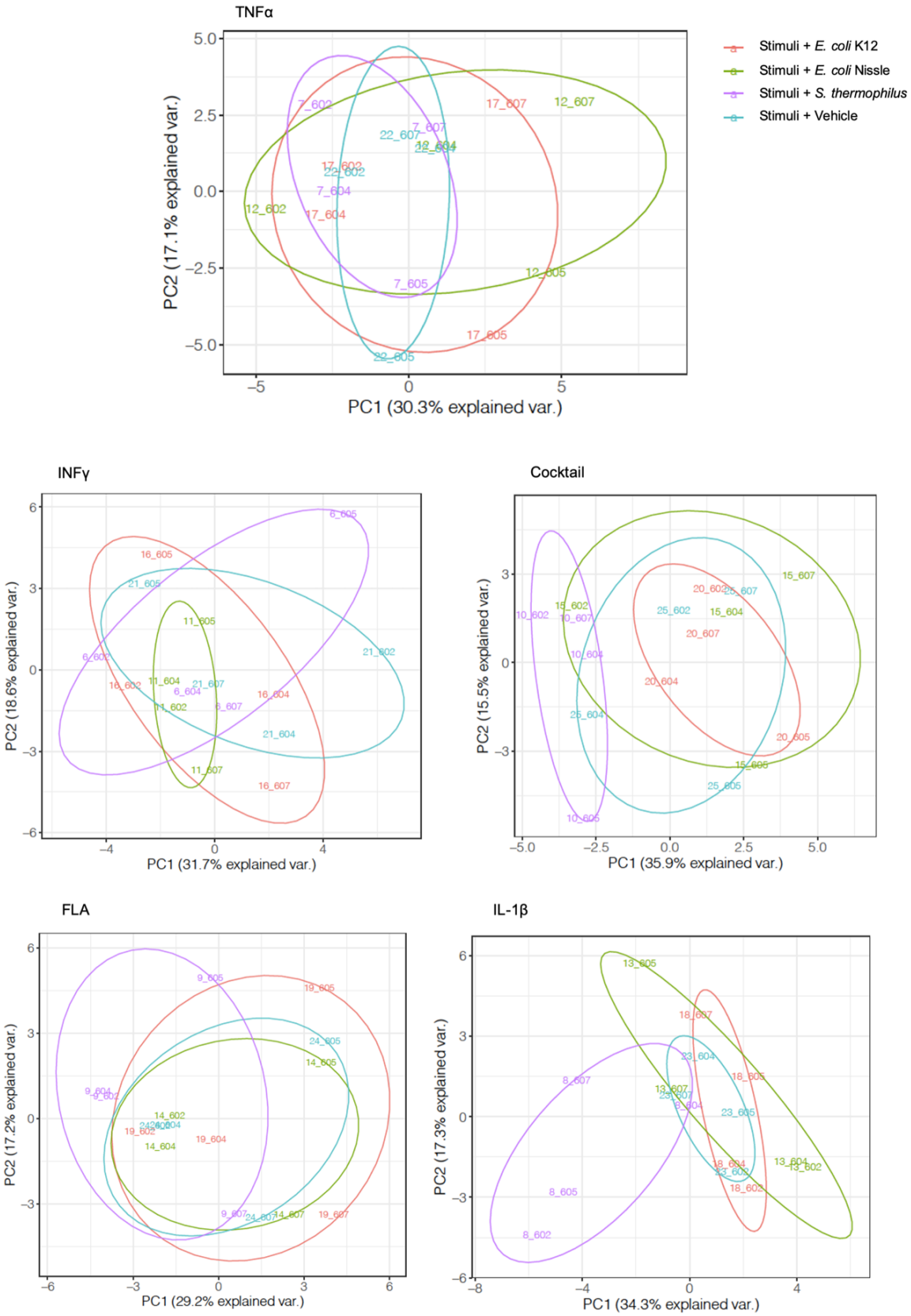


Figure 28. Principal Component Analysis of artificially-inflamed d-ODMs transcriptional profiles after postbiotics treatment. In the graphs, PCA separated samples according to pre-treatment with the different metabolite-cocktails followed by artificial inflammation with the selected panel of stimuli.

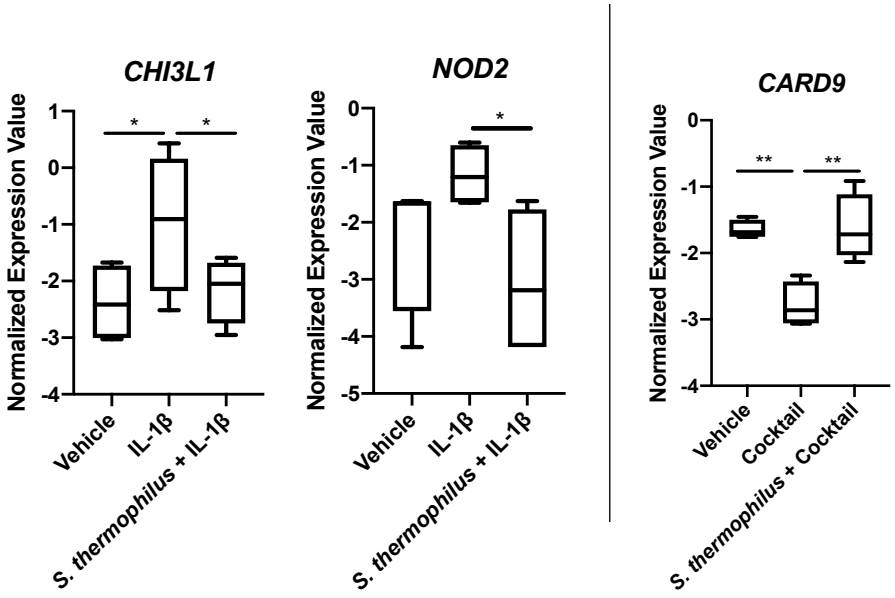


Figure 29. *S. thermophilus* postbiotics reverts the expression of *CHI3L1, NOD2* and *CARD9*. Postbiotics from *S. thermophilus* down-regulate the expression of *CHI3L1* and *NOD2* induced by IL-1β and increase the expression of *CARD9* suppressed by the inflammatory cocktail reaching similar levels than those of control (vehicle) d-ODMs. A One-way RM ANOVA was performed to examine statistically different expression patterns between the two groups. A P value of <0.05 was considered statistically significant.

DISCUSSION

STUDY 1: Determination of AIEC capability to invade Organoid-Derived Epithelial Monolayers and its derivative effects on the Intestinal Epithelium

In this study we describe the steps required to develop a novel and reproducible human intestinal epithelial model for the study of microbial-epithelial interactions such as enteric bacterial infections – particularly AIEC-related infections. Similar strategies have been applied to study the interaction between AIEC (or other enteric pathogens and *E. coli* pathotypes) and human isolated IECs (165,290,297,366,367). Indeed, there is a recent and relevant publication in which organoid-derived 2D cultures are infected with AIEC (293). Nonetheless, this report does not include a detailed description of the steps taken to optimize infection efficacy. In contrast, our principal focus was to describe the steps required to obtain optimal 2D culture from EpOCs, that can be used as a reproducible model of primary epithelial cell infection with different *E. coli* strains. We could achieve this through the accurate optimization of the presented protocol, from cell counting prior to infection to ODMs differentiation, and from infection kinetics to MOI testing. To the best of our knowledge, this is the first publicly accessible protocol that demonstrates the capacity of AIEC, compared to a non-invasive strain, to infect human primary IECs in a 2D configuration.

The model we presented takes into consideration the variability of human biological responses to any pathogens, something that other models based on the use of immortalized cell lines cannot fully address (297,368). Indeed, one of the main advantages of working with *ex vivo* primary cultures is that these might offer a more physiological and personalized view of the host's response to bacterial infections. However, unpredictable biological variability could hinder the obtainment of the necessary cell concentration at the starting point. In that context, establishing an accurate and standardized protocol is crucial to reach experimental reproducibility and ensure robust results comparison. In our case, reproducibility was assessed first by testing the gene and protein expression levels of KI67 and MUC2 (among many others) in the 2D cultures derived from different donors. In this context, our results suggest that ODMs and d-ODMs preserve the characteristics of the intestinal epithelium *in vivo*, resembling cells at the base and top of colonic crypts. Moreover, AIEC infections were carried out in duplicate, exposing d-ODMs from seven different individuals to *E. coli*. This validation approach is of great importance in host-pathogen interaction studies, considering the real differences in infection susceptibility among individuals and the divergence in host responses to a pathogen (367).

Then, we performed a more extensive characterization of the d-ODM response to AIEC infection at gene and protein level to add robustness to our culture system. By exposing cells to *E. coli* (both the LF82 and K12 strains) under different conditions (distinct infection timepoints and gentamicin treatments) we could better explore the response of d-ODMs.

Determining the number of cells that form the monolayer at the time of infection is a crucial step that we optimized to 1) achieve the optimal differentiated phenotype of the monolayer cultures, and 2) properly adjust the number of bacterial cells added to the d-ODMs (MOI), which can greatly affect the results.

In addition, AIEC infection of d-ODMs was performed at different time points to analyze and select the best condition for achieving high reproducibility and maximum specificity (lowest infection by non-invasive *E. coli*) of infection. Over time, increasing amounts of invasive bacteria were detected, with higher values detected when lower amounts of bacteria (MOI 20) were added to the culture at the starting point. Based on this finding, we concluded that adding more bacteria did not translate into increased invasiveness. Similar results were obtained by Boudeau et al. in 1999 with Hep-2 cells (155). A 5-fold increase in the inoculum only represented an increase of 2.06±0.7–fold (mean value of the fold-change increase for each timepoint) in intracellular bacteria. As d-ODMs cells were verified as viable with the CellTox Green assay, differences in the invasion indices were related to the initial inoculum and not to epithelial cell viability. We believe that the d-ODMs can harbor a limited number of intracellular bacteria and, therefore, upon a given quantity of initial inoculum the invasion index will start decreasing. Even so, working with higher bacterial loads ensures a remarkable reproducibility of the results.

Another observation concerns the dramatic decrease in the invasion index at the most extended time of infection on LF82 INV-I% for both MOI 20 and 100. Other authors similarly reported a decrease in the intracellular bacteria 4h after infection in mouse embryonic fibroblasts and HeLa, Hep-2 and I407 cell lines (169). Initially, we hypothesized that this event might be a consequence of eukaryotic cell death due to the bacterial infection process. Based to this assumption, at higher bacterial loads (MOI 100), eukaryotic cells would have begun to die at earlier time points. Nonetheless, and as we just mentioned, using the CellTox Green assay we observed that cell viability was maintained over time after infection (data not shown). Although AIECs are capable of evading IECs and macrophage-defense mechanisms aimed to eliminate intracellular pathogens (155,161,210), decreases in the intracellular bacteria could reflect the capacity of IECs to restrict AIEC replication after a certain infection period (169). Testing the intracellular-bacteria viability at each time point

would help confirm our hypothesis. It would also be attractive to determine, using this model, the presence of intracellular AIEC cells with a persistent phenotype, i.e., viable bacteria in a non-replicating state. This non-replicative population of LF82 has been proved to tolerate antibiotics when they are phagocytosed by macrophages (369). In fact, persisters are suspected to be the cause of relapsing in infectious chronic diseases (370), and so verification of its presence in human IEC would be of great interest.

Moreover, in our study we also evaluated the impact of AIEC infection on epithelial cells including expression of bacterial sensing molecules, tight junction proteins, or immune response secreted proteins (219,371). Thus, to explore this response at early timepoints after AIEC infection, cells were first exposed to *E. coli* during 5 or 6h without gentamicin treatment in agreement with previous reported studies (219). The results from these experiments demonstrated a response of *E. coli* stimulated d-ODMs compared to the uninfected control, however no significant differences were shown between the two analyzed *E. coli* strains, for any of the genes analyzed (342). Similarly, the time of infection (5 or 6h) did not appear to be crucial since, in general, no significant differences were detected among them. These results might probably be a consequence of extracellular interactions between bacteria and IECs. Hence, cells were then exposed to *E. coli* for 5h and then treated for 1 additional hour with 100 µg/ml to kill extracellular bacteria. Next, host cells were incubated with low gentamicin concentration (15 µg/ml) for further 6 or 18h in complete medium without antibiotics as reported by others (196). In that case, at 24h post-infection, AIEC promoted a significantly different response on d-ODMs compared to the non-invasive *E. coli* K12 strain.

An increase in the expression of AIEC receptors (*CEACAM6*, *GP96* and *CHI3L1*) has been reported in inflammation thus potentially favoring AIEC adhesion and invasion of IECs (162,362,372–374). In our study, although there was some variability in the response, we did not observe any significant change in expression of *CEACAM6* or *GP96* 24h after infection. In contrast, at the same timepoint, *CHI3L1* was significantly down-regulated with either one of the *E. coli* strain. Interestingly, in the second study of this thesis we detected no differences in the expression of *CEACAM6* or *GP96* under artificial inflammation of the human primary 2D culture. Conversely, *CHI3L1* expression was significantly increased in artificially-inflamed d-ODMs. Our results suggest an increased possibility of this pathobiont to adhere to and invade d-ODMs under inflammatory conditions.

On the other hand, disruption of the apical junctional complex (comprising several proteins such as occludin, claudin-2 or E-cadherin) have been demonstrated to occur in response to

exposure to AIEC or other *E. coli* strains (206,304,375,376) leading to a decrease of IECs' resistance (175,377). Specifically, an increase of *CLDN2* after AIEC infection has been also reported in IBD patients (194). Interestingly, in our system *CLDN2* mRNA levels were significantly decreased 24h after AIEC infection. Similarly, a down-regulation of *OCLN* has been associated to AIEC and other pathogenic *E. coli* infections (304,378), however our results support a significant up-regulation of this gene after AIEC infection at this timepoint. While our results may contradict previous observations, with this 2D culture system we can clearly demonstrate that AIEC – but not non-invasive *E. coli* – induce a marked change in the transcription of key genes involved in the formation and stability of tight junctions. Whether these signatures lead to changes in protein expression and cell junction reorganization, as well as in epithelial permeability, remains to be addressed.

Otherwise, transcriptional levels of genes or molecules involved in the immune response are also known to be dysregulated after AIEC infection (371,379). Indeed, overproduction of pro-inflammatory cytokines occurs after the interaction of AIEC with IECs (219,380). Accordingly, in our study we show a significant increase in mRNA levels of both CCL20 and CXCL8 24h after AIEC infection compared to uninfected cells, and cells exposed to non-invasive *E. coli*. This selective effect was not confirmed by protein production which was highly expressed by both *E. coli* stains. This finding could be due to the fact that changes in protein production may only be detected at later time points (>24h). Similarly, mRNA levels of the autophagy gene *ATG13* were significantly up-regulated 24h post-AIEC infection but not by the non-invasive K12 stain. These results, together with those observed for *CLDN2*, are in agreement with the data published by Nighot et al., who demonstrated that autophagy regulates intestinal epithelial tight junctions by targeting claudin-2 protein degradation, thus controlling paracellular permeability (379,381). Hence, AIEC infection might promote the activation of the IEC autophagic process thus affecting the intestinal epithelial barrier function.

Finally, Eph receptors and their Ephrin ligands have been recently reported to be involved in inflammation and viral infections (343,344). More specifically, EphA2 (encoded by *EPHA2* gene) has been reported to serve as an entry receptor of several viruses to infect epithelial cells. Even though no available reports demonstrate the role of this receptor and its ligand EphrinA1 (encoded by *EFNA1*) in AIEC pathogenesis, our results showed an up-regulation of *EHPA2* and *EFNA1* genes 24h after AIEC infection, thus suggesting its possible implication in AIEC cell invasion.

Overall, our results provide an initial characterization of the human primary 2D culture as a novel model of AIEC infection. Nevertheless, a more detailed description of this method by employing other techniques (i.e., whole transcriptome or proteomic analysis) is required to better comprehend and elucidate AIEC pathogenic mechanisms. Moreover, other approaches such as the use of d-ODMs derived from the inflamed mucosa of IBD patients or exposed to inflammatory signals *ex vivo*, as well as d-ODMs exposed to other *E. coli* strains (both AIEC and commensal) could be used. This might lead not only to the development of a more comprehensive approach for studying the interaction of AIECs with the human gut, but also to a better understanding of the pathophysiology underlying inflammatory intestinal disorders thus offering the possibility of testing personalized treatment approaches against AIEC infections.

STUDY 2: Production of Postbiotics and their effects on Human Blood Immune Cells and Organoid-Derived Epithelial Monolayers

In this study, we have been able to produce postbiotics and to explore their effects on primary human cells. Production and characterization of postbiotics can be conducted using different techniques and analytical approaches. Either way, here we have demonstrated that the bacterial culture media can mask the intrinsic effects of postbiotics on *in vitro* cultures, thus stressing the importance of obtaining purified bacterial metabolite-cocktails (in our particular case, achieved in collaboration with Postbiotica S.r.l) before their use and characterization.

We also attempted to determine the composition of the generated postbiotics. In our case, the descriptive proteomic characterization by LC-MSMS could identify an extended list of bacterial secreted proteins. This analysis revealed that the two *E. coli* strains used (the probiotic Nissle and the non-probiotic K12) shared 35-45% of their proteomic profiles (compared by accession number) while *S. thermophilus* only shared 3% of its identified proteins with the other two strains. Similar results were obtained through the metabolomic analysis by UPLC-HILIC-qTOF. We found these results very consistent with the fact that, apart from belonging to different bacterial groups, *E. coli* and *S. thermophilus*' growth curves were markedly different. While *E. coli* reached an OD of 0.6 at mid-exponential phase, that from *S. thermophilus* was 0.1. Differences in their growth rates may probably correlate with strong dissimilarities in secreted amounts of metabolites. Among the seven identified metabolites, tryptophan was the only one whose relative quantification appeared to be similar between the three metabolite-cocktails. Remarkably, Kepert et al. demonstrated the influence of probiotic-derived tryptophan on gut microbiome and allergic airway disease. Indeed, when screening supernatants derived from several probiotic strains (including *E. coli* Nissle and *S. thermophilus*) for their ability to down-regulate CCL17 in a T cell line, only a *Lactobacillus* and a *Lactococcus* strain showed a significant effect in that study. After fractionation of these probiotics supernatants, they found D-tryptophan to be the bioactive compound that modulated the profile of cytokines and chemokines produced in the employed cell lines (382). These results suggest an impact of this metabolite in modulating the immune response, thus paving the way to its analysis in other culture systems such as d-ODMs.

Although the analysis of the composition of postbiotics is a challenging task, it might help understand the implication of each metabolite within the postbiotics mix in any of the analyzed biological scenarios. Nonetheless, when working with undescribed metabolite-cocktails, such as in our case, one should be aware that the posed limitations, including the

lack of publicly available data bases, might prevent to the precise characterization and quantification of the cocktail's composition. Therefore, despite the different techniques that could be used to detect, identify, and later quantify the components of these metabolite-cocktails, the development of improved isolation protocols and analytical tools is necessary to allow the accurate characterization of novel postbiotics.

Besides, understanding how postbiotics work in culture models that more physiologically resemble the human intestinal mucosa would allow the selection of the strains to further produce postbiotics that are more suitable for clinical development or commercial use (249). Of all the cells that form the mucosal surface, epithelial cells as well as intraepithelial lymphocytes are the ones in closer contact to the microbiota, and thus may be the ones primarily perceiving and responding to the adjacent microorganisms. Nevertheless, beyond the epithelial barrier, cells in the underlaying lamina propria can sense bacteria and are fully equipped to recognize and respond to them as well. Indeed, below the epithelial layer, the intestinal mucosa is populated by the largest repertoire of lymphocytes in the entire organism (383). In addition, the lamina propria is densely populated by phagocytic and antigen presenting cells, including macrophages and DCs that upon bacterial encounter can mount tolerogenic or inflammatory responses depending on the environment (22,23). Moreover, in IBD patients, neutrophils and monocytes, normally absent from the mucosa, are rapidly recruited to the lamina propria and are key effector drivers of disease (384). Since the interaction of bacteria and their derived metabolites with the host mucosa (particularly in the gut) is complex, the development of a model system that simulates the human intestine would therefore be of great value for testing the action of postbiotics on both healthy and diseased tissues (53,234). In the absence of working models that contain all intestinal cell types and that reliably reproduce the human mucosa, we tested the effects of postbiotics in several isolated cellular systems. The use of PBMCs (249,267) showed to be a valuable *in vitro* tool to assess the immunomodulatory effects of postbiotics in primary human immune cells. PBMCs contain a mixture of immune cells, including lymphocytes (T, B, and innate lymphocytes) and myeloid cells (monocytes, neutrophils, eosinophils, and very low numbers of circulating DCs) (385). On the other hand, moDCs form a highly homogeneous population of dendritic cells (expressing MHC Class II and CD11c) that have been widely used as a reliable source of antigen presenting cells for *in vitro* studies.

To mimic the status of immune cells in the context of inflammation, we stimulated PBMCs and moDCs with LPS, a relevant immune activating stimulus. Using these cell systems, we showed a potential anti-inflammatory and immunoregulatory effect of *S. thermophilus*

postbiotics in both LPS-activated PBMCs and moDCs cultures, as shown by the modulation of IL-10, IL-12 and IL-23 cytokines. In moDC, *E. coli* K12 also exerted immunomodulatory effects, suggesting that bacterial metabolites secreted by non-probiotic strains may also hold putative beneficial capacities. Although the number of replicates used in our study was too low to perform the appropriate statistical analysis, this is an observation that warrants further investigation. Indeed, to our knowledge, there are no published studies assessing the effects of *E. coli* K12 metabolites on epithelial cell cultures.

Besides using PBMCs and moDCs, we took advantage of the polarized human primary 2D cultures (d-ODMs), which preserve their physiologic intestinal identity as we already showed in the first study. Doing so, we could explore the effects of the purified postbiotics on the intestinal epithelium.

Whole genome RNA sequencing analysis revealed that the three metabolite cocktails induced the differential expression of a comparable number of genes (189 by *S. thermophilus* postbiotics, 143 by *E. coli* Nissle postbiotics and 158 by *E. coli* K12 secreted metabolites) in d-ODMs. Nonetheless, our analysis revealed that the signatures were widely different, with only 4 genes (*ORC1, RIMBP3, C2* and *FABP6*) commonly regulated by the three microbial metabolite-cocktails. Of note, *C2* expression appeared to be significantly increased by all tested microbial metabolite-cocktails. *C2* encodes for the complement component 2 of the complement system, a potent innate immune defense mechanism against microbes. Deficiency of *C2* has been associated to autoimmune diseases(386), showing the importance of the complement system in the adequate activation of immune responses. Remarkably, *S. thermophilus* postbiotics also showed to significantly increase the expression of another component of the complement system, *C1R*, involved in the cleavage of C2. Therefore, *S. thermophilus* postbiotics might have a role in innate immunity regulation on IECs, or even in promoting bacteria cell wall breakage upon infection since this is one of the main roles of the complement system.

We also found interesting the increase of *KYNU* transcripts by both *E. coli* strains. KYNU is an enzyme involved in the biosynthesis of NAD cofactor from tryptophan. Given the fact that we identified similar levels of tryptophan in the three metabolite-cocktails, we hypothesize that the presence of bacterial-secreted tryptophan in the media might induce its metabolization by IECs through the activation of *KYNU*. Considering that the lack of tryptophan has been reported to lead to impaired intestinal immunity and promote dysbiosis (387,388), our results would suggest that an increase in tryptophan metabolism by epithelial cells may be another so far unrecognized effect of bacterial-derived metabolites.

Moreover, another important hypothesis of this study was that postbiotics may not only promote protective immune responses under homeostatic conditions, but also alter the response to inflammatory mediators. In contrast to peripheral immune cells that are sensitive to the TLR4 agonist LPS, epithelial cells respond to the TLR5 ligand, flagellin, as well to inflammatory cytokines (i.e., IFNγ, TNFα or IL-1β) relevant to IBD.

Initial characterization of the response of d-ODMs to these pro-inflammatory stimuli further helped us characterize this culture system and validate its relevance as a model to study intestinal inflammation. As expected from their implication in NF-kB signaling pathway, FLA and IL-1β shared about 30% of the identified differentially regulated genes. Nonetheless, of all the stimuli tested, IFNγ was by far the most potent activator of the epithelium driving a characteristic response such as the up-regulation of *JAK1* and *JAK2, STAT1, IRF1*, *ICAM1* and *IDO1* or the induction of *CXCL10* (389). In contrast, TNFα showed the weakest effect on the epithelial culture either alone or in combination with IFNγ, a condition that resulted mostly in the stimulation of the IFNγ signature. Other available studies also applied TNFα in combination with other stimuli (such as IFNγ or IL-1β), possibly to potentiate its effects (336–338). Nevertheless, we cannot exclude that changing the concentration of the stimuli or incubation times could produce different results.

While each of the different tested activators drove a significant transcriptional signature, all of them were represented in the transcriptional profile of biopsies derived from inflamed mucosa of IBD patients. Indeed, when comparing artificially-inflamed d-ODMs and active IBD samples at the transcriptional level, we found that stimulation of d-ODMs induced the regulation of a large number of genes that also appear to be altered in IBD patients. Hence, our "inflamed" culture system partially mimics the transcriptional profile of active patients as similarly showed by others (337).

Finally, we pre-treated d-ODMs with the purified postbiotics prior to activation with the different inflammatory signals to assess their potential as modulators of intestinal inflammation. RNAseq analysis unveiled a modest but detectable effect of *S. thermophilus* postbiotics over inflamed d-ODMs. Particularly, *CHI3L1* expression was reverted by this postbiotic. As we already mentioned, *CHI3L1* levels – whose basal expression is very low in intact IECs – are reported to be increased upon inflammation, predominately on IECs and macrophages in the lamina propria (167). Indeed, other authors have reported the implication of IL-1β (and other cytokines) in promoting its up-regulation in several cell lines (362,390,391). CHI3L1 has been implied not only in IBD (rather in UC than in CD (362,392)) but many other diseases (391). One of its main roles is to serve as a receptor for

gut pathogens such as AIEC or *Salmonella enterica serovar typhimurium* (*S. typhimurium*). A report from 2006, demonstrated a significantly decreased capacity of AIEC and *S. typhimurium* to invade colonocytes in cells lacking CHI3L1 (362). Thus, given the fact that 1) IL-1β increases the expression of *CHI3L1* while 2) *CHI3L1* is significatively reduced after postbiotics pre-treatment of IL-1β inflamed d-ODMs, it would be of great interest to check whether d-ODMs pre-treated with *S. thermophilus* postbiotics could be protective towards AIEC internalization promoted by IL-1β stimulation.

Similarly, *S. thermophilus* postbiotics did also revert the expression of *NOD2* in IL-1β stimulated d-ODMs. As already mentioned, *NOD2* mutations confer an increased risk to CD development (12). Dysregulation of Nod2 signaling contributes to increased predisposition to infection in animal models and humans (393) and, consequently, a dysregulation of bacterial diversity (394). Thus, *S. thermophilus* postbiotics might play a role in reestablishing *NOD2* levels and thus regulate gut bacterial composition.

S. thermophilus postbiotics also reverted the down-regulation of *CARD9* gene induced by the pro-inflammatory cocktail. *CARD9* is a signaling adaptor known to play a major role in the sensing of pathogenic microorganisms in the gut. It has been reported to be either a risk factor or protective against IBD, and its deficiency is known to impair intestinal immune responses and microbial gut homeostasis (365). Remarkably, *CARD9* deficiency has also been associated with the inability to metabolize tryptophan, a defect that could in turn, aggravate intestinal inflammation (395). Thus, by restoring *CARD9* levels in artificially-inflamed d-ODMs, *S. thermophilus* postbiotics might have a role in the reestablishment of tryptophan metabolization as well as the gut immune response thus promoting intestinal homeostasis.

Hence, in this study we demonstrate the regulatory effects of bacterial postbiotics, more specifically those secreted by *S. thermophilus* on human immune cells and intestinal epithelium. However, depending on the biological system used, postbiotics might promote different responses. Thus, in-depth characterization of the postbiotics generated here will require further testing in other systems including pluricellular cultures, tissue explants and finally, experimental animal models. Moreover, it would be also attractive to assess whether postbiotics treatment can impact the epithelial interaction and response to AIEC. According to our results, we hypothesize that bacterial metabolite-cocktails (particularly *S. thermophilus* postbiotics) could modulate the inflammatory response of infected d-ODMs or even affect the interaction of the pathobiont with the host by regulation of its cell receptors. Therefore, assessing the impact of postbiotic-treatment on cytokine release, epithelial permeability, or

expression of tight-junction proteins in artificially-inflamed or infected d-ODMs, would provide a valuable characterization of these metabolite mixtures in biologically relevant conditions and thus delve into their beneficial effects.

CONCLUSIONS

STUDY 1: Determination of AIEC capability to invade Organoid-Derived Epithelial Monolayers and its derivative effects on the Intestinal Epithelium

1. Human 3D organoid cultures derived from human intestinal crypts can give rise to a polarized monolayer of differentiated cells (i.e., differentiated organoid-derived monolayers, d-ODMs) that preserves their intestinal identity thus mimicking the tissue of origin. Therefore, this is a suitable model to study the function of the human epithelium and explore its interactions with luminal and mucosal signals.

2. Using the d-ODMs infection model, we determined the optimal conditions to achieve high experimental reproducibility by maximizing invasiveness of AIEC relative to non-AIEC strains.

3. At early infection times, the response of the d-ODMs to AIEC and non-AIEC strains is comparable while it becomes strain specific at later timepoints. These observations strongly support the applicability of the d-ODMs cultures to study the functional consequences of AIEC infection on human intestinal epithelium.

Overall, we provided a characterization of the human primary epithelial 2D culture as a novel model of AIEC infection. Our results clearly support the use of this system as a robust tool to study intestinal host-pathogen interactions, as is the case of AIEC infection. Therefore, d-ODMs can serve as a reliable model to study the pathogenicity mechanisms of this pathotype and thus, the pathophysiology underlying inflammatory intestinal disorders.

STUDY 2: Production of Postbiotics and their effects on Human Blood Immune Cells and Organoid-Derived Epithelial Monolayers

1. Production of postbiotics for downstream applications requires a purification step that eliminates the bacterial culture media from the final product.

2. *S. thermophilus*-derived postbiotics and *E. coli* K12-derived metabolites (although to a less extent) show promising immunomodulatory effects on human blood immune cells, suggesting that metabolites derived from both probiotic as well as non-probiotic bacteria may be potentially used to modulate immune responses.

3. The response of d-ODMs to inflammatory signals represents an important component of the total transcriptional landscape in the intestinal mucosa of IBD. Based on this, we speculate that regulating the response of the epithelium to these cues could hold significant promise in dampening inflammation and promoting mucosal healing in IBD patients.

4. Postbiotics regulate the expression profile in uninflamed d-ODM. Moreover, *S. thermophilus* postbiotics show the potential to tune the response of the epithelium under an inflammatory environment.

Hence, we demonstrated the beneficial effects of postbiotics in different culture systems. Considering the effects of *S. thermophilus* postbiotics proven here and being aware of the different responses that these metabolite-cocktails might promote depending on the biological scenario, we believe that *S. thermophilus* postbiotics are a promising candidate to modulate the response of the intestinal mucosa during inflammation.

FUTURE PERSPECTIVES: Analyzing the Effects of *S. thermophilus* Postbiotics in AIEC-infected Organoid-Derived Epithelial Monolayers

In our studies, we have first set up a robust and reproducible approach for studying the mechanisms of epithelial cell response upon AIEC infection. Moreover, we have showed the immunomodulatory capacity of *S. thermophilus* postbiotics as well as their ability to regulate the expression of certain genes that are known to be involved in AIEC internalization into IECs (such as *CHI3L1*) under inflammatory conditions. In addition, we showed that postbiotics activate innate immunity by upregulating the expression of genes involved in the complement cascade, thus potentially increasing the ability of epithelial cells to recognize and target pathogens. Therefore, we believe that our results pave the way to the analysis of *S. thermophilus* postbiotics effects in the AIEC infection model we developed using d-ODMs. We think it could be relevant to first analyze the invasive capacity of AIEC strains in artificially-inflamed d-ODMs or d-ODMs derived from the inflamed mucosa of IBD patients. Moreover, treating the infected cultures with *S. thermophilus* postbiotics' might help elucidate the potential capacity of this postbiotic to 1) modulate the inflammatory response in infected d-ODMs and 2) regulate the interaction of this pathobiont with the receptors expressed on the host cells. Results derived from all these suggested *in vitro* studies would support or discourage the use of postbiotics in *in vivo* models.

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Bea, gracias por ayudarme a entender, a entenderme. Por hacerme crecer y quererme más. Por ayudarme a cuidarme, a ser consciente. Eres clave en esto, y en lo que venga. Eres parte de ese "click". Infinitas gracias, de verdad.

A toda mi familia, a mis tíos y mis primos, siempre pendientes de a donde va la pequeña de la familia, de lo que hago, pero sobretodo de que sea feliz. Soy consciente de la familia tan maravillosa que tengo. Os quiero mucho a todos. Gracias por confiar en mi y quererme tanto.

Y mi motor principal en la vida. Mama, Miguel, Albert. Os amo infinito. Sois las personas que quiero ser. Sois mi inspiración, mis ganas de seguir adelante y mi orgullo más grande. Habéis estado sosteniéndome en cada paso que he decidido dar, por loco que fuera. Siempre contentos y orgullosos de mi. Y siempre os digo, orgullosa yo de tener a la mejor madre del mundo, la que me enseñó a levantarme y a seguir siempre. El papi más bonito que alguien puede tener, he aprendido siempre tanto de ti… tanto, que nos parecemos hasta en los andares. Y mi pequeño, que orgullosa estoy de ti, niño. Gracias por ayudarme a crecer, por confiar, por admirar, por dejarme ser. Eres mi pilar. Os quiero. Esta tesis es absolutamente vuestra, porque sin vosotros nada de esto hubiese sido real. Gracias por darme siempre un poquito más de lo que habéis podido. No me imagino nada de esto sin vosotros ¡Sois la mejor familia del mundo!

Alberto, te adoro. Esta historia empezó a la vez que esta tesis. ¡Y míranos! Has sido mi apoyo más incondicional siempre. Cada palabra, cada abrazo, cada consejo. Contigo he crecido. Y gracias a ti, he sacado fuerzas cuando no las tenía. Porque, ¿sabes?, todos estos años he tenido a mi lado alguien que es "hogar", alguien que no para de hacerme reír y de hacerme sentir. Alguien de quien aprendo todos los días, sobretodo a quererme más y mejor. Te quiero mucho, mivi. Gracias por recorrer este camino, a veces duro, a mi lado. Eres un regalo de la vida, te lo aseguro. ¡Ahora nos espera lo mejor! Te amo.

Y a ti, que sé que has estado a mi lado todos los días y que estás leyendo esto ahora. Te quiero, yaya. Esto, y todo lo que venga, es para ti. Eres mi persona favorita del mundo mundial.

P.S. Who run the world? ☺

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ANNEX I

TABLES

For Tables 1-16: Final concentrations (%, Molarity, dilutions (X)) are expressed relative to purchase concentration.

Table 1. Antimicrobial cocktail

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
DPBS	Gibco	100 %
Fungizone	ThermoFisher	2.5 µg/ml
Normocin	Invivogen	500 µg/ml
Gentamicin	Lonza	500 µg/ml

Table 2. Crypt Isolation Buffer

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
DPBS	Gibco	100 %
Ethylene Diamine Tetra-acetic Acid (EDTA)	Promega	8 mM

Table 3. Washing medium composition

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
Advanced DMEM/F12 medium	Gibco	100%
HEPES	Gibco	10mM
GlutaMAX	Gibco	1X
FBS	Gibco	5%

Table 4. STEM medium composition

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
Advanced DMEM/F12	Gibco	50% v/v
Wnt3a-conditioned medium	Produced using an L-Wnt3a cell line, ATCC CRL-2647	50% v/v
HEPES	Gibco	10 mM
GlutaMAX	Gibco	1X
N-2	Gibco	1X
B-27 without retinoic acid	Gibco	1X
Nicotinamide	Sigma-Aldrich	10 mM
N-Acetyl-L cysteine	Sigma-Aldrich	1 mM
Normocin	InvivoGen	100 μg/ml
Gastrin I	Tocris Bioscience	1 μg/ml
Human Noggin	Peprotech	100 ng/ml
Human Epidermal Growth Factor (EGF-1)	Gibco	50 ng/ml
SB202190	Sigma-Aldrich	10 μM
LY2157299	Axon MedChem	500 nM
R-Spondin-1 (RSPO1)	Sino Biologicals	500 ng/ml
Prostaglandin E2 (PGE2)	Sigma Aldrich	100 nM

Table 5. Dissociation medium composition

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
Advanced DMEM/F12 medium	Invitrogen	100%
HEPES	Gibco	10 mM
GlutaMAX	Gibco	1X
N-2	Gibco	1X
B-27 without retinoic acid	Gibco	1X
Nicotinamide	Sigma-Aldrich	10 mM
N-Acetyl-L cysteine	Sigma-Aldrich	1 mM
Prostaglandin E2 (PGE2)	Sigma-Aldrich	2.5 µM
Y-27632	Merck	10 µM
Dispase	Gibco	400 µg/ml

Table 6. DIFF medium composition

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
Advanced DMEM/F12 medium	Invitrogen	100 %
HEPES	Gibco	10 mM
GlutaMAX	Gibco	1X
N-2	Gibco	1X

B-27 without retinoic acid	Gibco	1X
N-Acetyl-L cysteine	Sigma-Aldrich	1 mM
Normocin	InvivoGen	100 µg/ml
Gastrin I	Tocris Bioscience	1 µg/ml
Human Noggin	Peprotech	100 ng/ml
Human Epidermal Growth Factor (EGF-1)	Invitrogen	50 ng/ml
LY2157299	Axon MedChem	500 nM
R-Spondin-1 (RSPO1)	Sino Biologicals	250 ng/ml

Table 7. LB Broth

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
LB Broth	Lennox	16 g/L
H_2O	-	100%

Table 8. Complete EMEM medium composition

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
Minimum Essential Medium – Eagle with Earle's BSS (EMEM)	Lonza	100%
FBS	Gibco	10%
Antibiotic Antimycotic Solution	Sigma-Aldrich	1%
MEM Vitamin Solution	Gibco	1%
MEM Non-Essential Amino Acids Solution	Gibco	1%
L-glutamine	Gibco	1%

Table 9. EMEM-MM

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
EMEM	Lonza	100%
FBS	Gibco	10%

Table 10. DIFF-MM

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
Advanced DMEM/F12 medium	Invitrogen	100 %
HEPES	Gibco	10 mM
GlutaMAX	Gibco	1X
N-2	Gibco	1X
B-27 without retinoic acid	Gibco	1X

Table 11. LB agar plates for AIEC invasion assay

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
LB Broth	Lennox	16 g/L
Bacto Agar	BD Biosciences	20 g/L

Table 12. Complete RPMI medium composition

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
Roswell Park Memorial Institute (RPMI)-1640, without L-glutamine	Hyclone (Cultek)	100%
FBS	Gibco	10%
Antibiotic Antimycotic Solution	Sigma-Aldrich	1%
Sodium Pyruvate (NaP)	Gibco	1%

MEM Non-Essential Amino Acids Solution	Gibco	1%
L-glutamine	Gibco	1%

Table 13. Antibodies for FACS staining

ANTIBODY	FLUOROCHROME	CLONE	MANUFACTURER
CD3	FITC	UCHT1	BD Biosciences
CD19	FITC	HIB19	BD Biosciences
CD20	FITC	2H7	Biolegend
CD56	FITC	NCAM16.2	BD Biosciences
CD83	PE	HB15e	Miltenyi Biotech
HLA-DR	PerCp	L243	BD Biosciences
CD14	APC	M5E2	Biolegend
CD11c	BV	3.9	Biolegend

Table 14. FACS buffer composition

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
DPBS	Gibco	100%
FBS	Gibco	2%
Sodium Azide (NaN3)	Sigma-Aldrich	1%

Table 15. MTT Solution

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
DPBS	Gibco	100%
Thiazolyl blue tetrazolium bromide (MTT)	Sigma-Aldrich	5 mg/ml

Table 16. Solubilization Solution

COMPONENT	MANUFACTURER	FINAL CONCENTRATION
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	50%
Isopropanol	Sigma-Aldrich	50%

Table 17. Top 50 genes (sorted by increasing values of p-value) regulated by every stimulus compared to the vehicle.
FC: Fold Change. FDR: Fold Discovery Rate

INFγ vs Vehicle					TNFα vs Vehicle					Cocktail vs Vehicle					IL-1β vs Vehicle					FLA vs Vehicle				
Gene	Expression (UP/DW)	FC	FDR	p-nominal	Gene	Expression (UP/DW)	FC	FDR	p-value	Gene	Expression (UP/DW)	FC	FDR	p-value	Gene	Expression (UP/DW)	FC	FDR	p-value	Gene	Expression (UP/DW)	FC	FDR	p-value
GBP5	UUP	4855.78	1.85E-05	1.27E-09	CORO7-PAM16	DW	-32.81	0.5115	6.88E-05	CHRD	UP	51.28	0.3905	5.34E-05	BEST4	UP	6.72	0.3039	2.08E-05	CPXM1	UP	62.17	0.2519	3.32E-05
CXCL9	UUP	7048.28	0.0001	1.87E-08	MAST1	DW	-10.68	0.5115	8.23E-05	MUC19	DW	-16.93	0.5281	0.0001	SNPH	UP	3.46	0.4208	8.82E-05	ACTG2	DW	-5.74	0.2519	3.44E-05
GBP4	UUP	135.56	0.0002	3.62E-08	BOC	DW	-4.89	0.5115	0.0001	CORO7-PAM16	DW	-25.75	0.5281	0.0001	CD274	UP	2.72	0.4208	0.0001	CCDC87	UP	2.44	0.8270	0.0002
CD274	UUP	55.85	0.0004	9.65E-08	SPDYE6	DW	-15.78	0.5115	0.0001	SSTR2	UP	14.62	1.0000	0.0005	GOLGA8Q	UP	8.20	0.4208	0.0001	NKD1	UP	6.80	0.9999	0.0004
CXCL11	UUP	241.34	0.0005	1.80E-07	RIMBP3	UP	20.09	0.5115	0.0002	FAM151A	DW	-8.07	1.0000	0.0006	CCL20	UP	6.65	0.9504	0.0004	JSRP1	UP	2.28	0.9999	0.0004
KLHDC7B	UUP	41.01	0.0006	2.42E-07	GNRHR	DW	-3.20	1.0000	0.0005	DAO	UP	7.76	1.0000	0.0010	CRTAC1	UP	4.80	0.9504	0.0004	MYLPF	UP	2.62	0.9999	0.0005
GBP7	UUP	40.69	0.0012	5.92E-07	VTN	UP	2.17	1.0000	0.0010	DPYD	UP	3.11	1.0000	0.0013	CYP2C8	DW	-4.27	0.9504	0.0006	TNFRSF18	UP	17.51	0.9999	0.0005
HLA-DMB	UUP	111.59	0.0012	6.32E-07	BEST4	UP	6.37	1.0000	0.0013	VWCE	DW	-12.51	1.0000	0.0015	PKIG	UP	1.98	0.9504	0.0007	SERPINB7	UP	12.56	0.9999	0.0006
CIITA	UUP	29.05	0.0015	8.95E-07	CCDC87	UP	2.21	1.0000	0.0014	CD274	UP	26.47	1.0000	0.0021	S100A9	UP	7.10	0.9504	0.0007	ANKRD61	DW	-4.20	0.9999	0.0007
CXCL10	UUP	1863.51	0.0017	1.27E-06	CELA3B	UP	2.69	1.0000	0.0015	FBXL22	DW	-9.35	1.0000	0.0023	KIRREL2	UP	23.79	0.9504	0.0008	DUOXA1	UP	2.28	0.9999	0.0008
SERPING1	UUP	168.58	0.0017	1.32E-06	ASCL2	UP	3.91	1.0000	0.0018	FAM72D	DW	-8.94	1.0000	0.0025	STMN3	UP	2.43	0.9504	0.0008	NCCRP1	UP	10.55	0.9999	0.0010
IDO1	UUP	14440.07	0.0017	1.47E-06	NKX6-2	UP	6.65	1.0000	0.0025	PTHLH	UP	6.15	1.0000	0.0025	CCDC28B	DW	-2.04	0.9504	0.0009	C2CD6	DW	-6.81	0.9999	0.0012
CCL8	UUP	155.11	0.0017	1.50E-06	TRPC5	DW	-8.92	1.0000	0.0034	CDCA7	DW	-2.02	1.0000	0.0028	CCDC87	UP	2.39	0.9504	0.0009	CCL5	UP	24.28	0.9999	0.0015
C2	UUP	26.78	0.0056	5.33E-06	WDR97	DW	-2.87	1.0000	0.0035	TMEM132C	DW	-5.83	1.0000	0.0029	C8orf44-SGK3	UP	15.56	0.9504	0.0009	CLEC2B	UP	13.48	0.9999	0.0016
IRF1	UUP	4.96	0.0058	5.99E-06	CENPA	UP	2.38	1.0000	0.0038	SERPINA1	UP	1.78	1.0000	0.0031	BIRC3	UP	2.41	0.9504	0.0010	EBI3	UP	28.11	0.9999	0.0018
WARS1	UUP	17.34	0.0075	8.22E-06	SYN2	UP	4.63	1.0000	0.0038	HLA-DMB	UP	45.84	1.0000	0.0032	CPXM1	UP	8.70	0.9999	0.0011	SCUBE2	UP	9.48	0.9999	0.0019
IL15RA	UUP	4.26	0.0121	1.47E-05	ZIK1	UP	11.88	1.0000	0.0040	C4B	UP	4.71	1.0000	0.0034	WNT4	UP	2.09	0.9999	0.0012	C1QTNF2	UP	2.24	0.9999	0.0020
HLA-DMA	UUP	8.39	0.0121	1.52E-05	GLYATL1	UP	4.96	1.0000	0.0041	DTX3	DW	-9.06	1.0000	0.0035	NOS2	UP	44.71	0.9999	0.0022	FAM72D	DW	-8.18	0.9999	0.0021
IFI30	UUP	7.12	0.0121	1.63E-05	IL10RA	DW	-4.34	1.0000	0.0041	CLEC2B	UP	11.79	1.0000	0.0036	FBXO27	UP	3.93	0.9999	0.0022	UPK1B	DW	-7.44	0.9999	0.0023
C1R	UUP	4.95	0.0121	1.68E-05	TBC1D3D	DW	-14.87	1.0000	0.0044	HLA-DMA	UP	4.47	1.0000	0.0043	FOXM1	DW	-2.08	0.9999	0.0025	TMEM106A	UP	2.45	0.9999	0.0023
PSMB9	UUP	5.89	0.0121	1.74E-05	SERPINI1	UP	1.53	1.0000	0.0049	LYPD6B	UP	1.96	1.0000	0.0044	ZC3H12C	UP	1.81	0.9999	0.0027	ZNF519	DW	-2.45	0.9999	0.0023
GBP1	UUP	8.57	0.0137	2.06E-05	ZNF772	UP	1.43	1.0000	0.0052	LTB4R2	DW	-3.84	1.0000	0.0045	HMGCLL1	UP	4.53	0.9999	0.0029	CCL20	UP	3.72	0.9999	0.0023
CHRD	UUP	42.98	0.0144	2.27E-05	AP001781.2	DW	-7.68	1.0000	0.0055	C1R	UP	3.38	1.0000	0.0047	H3C12	DW	-1.75	0.9999	0.0033	S100A9	UP	18.21	0.9999	0.0024
MMP25	UUP	24.05	0.0169	2.84E-05	MTSS1	UP	1.57	1.0000	0.0056	CDH13	DW	-4.72	1.0000	0.0047	SLC9A4	DW	-4.47	0.9999	0.0037	APOBEC3G	UP	4.56	0.9999	0.0029
APOL1	UUP	9.55	0.0169	2.89E-05	RPS10-NUDT3	UP	1.55	1.0000	0.0056	TAS2R43	DW	-6.04	1.0000	0.0048	SEMA3C	UP	1.81	0.9999	0.0037	TIGD3	UP	2.06	0.9999	0.0032

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INFγ vs Vehicle					TNFα vs Vehicle					Cocktail vs Vehicle					IL-1β vs Vehicle					FLA vs Vehicle				
Gene	Expression (UP/DW)	FC	FDR	p-value	Gene	Expression (UP/DW)	FC	FDR	p-value	Gene	Expression (UP/DW)	FC	FDR	p-value	Gene	Expression (UP/DW)	FC	FDR	p-value	Gene	Expression (UP/DW)	FC	FDR	p-value
SAMD9L	UUP	5.77	0.0171	3.04E-05	DCHS1	DW	-6.19	1.0000	0.0066	HLA-DRA	UP	82.93	1.0000	0.0049	APOBEC3G	UP	2.53	0.9999	0.0038	UNC5A	UP	9.71	0.9999	0.0032
CLC	UUP	15.16	0.0183	3.46E-05	KIRREL2	UP	4.26	1.0000	0.0068	APOL4	UP	32.49	1.0000	0.0051	SPNS3	UP	1.72	0.9999	0.0042	DEPDC1	DW	-2.07	0.9999	0.0034
HLA-DRA	UUP	224.79	0.0183	3.50E-05	NCALD	DW	-1.93	1.0000	0.0071	KLHDC7B	UP	10.62	1.0000	0.0052	TRERF1	UP	1.77	0.9999	0.0043	PI3	UP	3.96	0.9999	0.0034
MCUB	UUP	5.74	0.0183	3.63E-05	CHI3L1	UP	3.08	1.0000	0.0073	G0S2	UP	2.51	1.0000	0.0052	DUOXA2	UP	5.32	0.9999	0.0045	RHCG	UP	4.95	0.9999	0.0036
HLA-DPA1	UUP	604.84	0.0198	4.05E-05	CHGA	DW	-5.88	1.0000	0.0074	C2	UP	14.75	1.0000	0.0052	KIF4A	DW	-2.37	0.9999	0.0047	SERPINA1	UP	2.06	0.9999	0.0036
ETV7	UUP	6.45	0.0233	5.06E-05	SLC9A5	DW	-5.20	1.0000	0.0077	UNC5A	UP	11.71	1.0000	0.0053	UNC5A	UP	6.82	0.9999	0.0048	RPS27AP5	UP	2.32	0.9999	0.0036
NOS2	UUP	1551.26	0.0233	5.09E-05	PRICKLE3	DW	-1.48	1.0000	0.0078	CFAP73	DW	-4.67	1.0000	0.0058	RBAK-RBAKDN	DW	-6.15	0.9999	0.0048	POU5F1	UP	1.77	0.9999	0.0038
CCL5	UUP	48.11	0.0242	5.46E-05	VWF	DW	-5.03	1.0000	0.0079	C8orf44-SGK3	UP	10.27	1.0000	0.0059	CCNA2	DW	-2.00	0.9999	0.0051	DUOX1	UP	1.77	0.9999	0.0038
TRIM69	UUP	4.03	0.0266	6.19E-05	ZNF771	DW	-1.73	1.0000	0.0097	VAMP5	UP	3.24	1.0000	0.0061	CHI3L1	UP	4.00	0.9999	0.0052	KIRREL2	UP	10.21	0.9999	0.0038
CCL22	UUP	35.83	0.0280	6.70E-05	SERPIND1	DW	-4.52	1.0000	0.0100	FBXO24	UP	3.12	1.0000	0.0063	IL1A	UP	5.47	0.9999	0.0054	SIRT4	UP	2.18	0.9999	0.0040
HLA-DPB1	UUP	159.81	0.0297	7.51E-05	SLC9B1	DW	-6.45	1.0000	0.0105	PRKCG	DW	-6.22	1.0000	0.0064	MAP3K8	UP	1.97	0.9999	0.0056	MATN4	UP	4.42	0.9999	0.0041
LAP3	UUP	3.96	0.0297	7.63E-05	SLX1B	UP	4.53	1.0000	0.0106	CXCL9	UP	1133.59	1.0000	0.0066	QPCT	UP	1.43	0.9999	0.0057	NMUR2	UP	2.16	0.9999	0.0049
IFIT3	UUP	8.30	0.0297	7.71E-05	ANGPTL2	DW	-2.15	1.0000	0.0114	TNFSF13B	UP	5.73	1.0000	0.0066	ANGPTL2	DW	-2.19	0.9999	0.0059	SERPINI1	UP	1.93	0.9999	0.0051
XAF1	UUP	8.35	0.0297	7.92E-05	TCEA2	UP	2.44	1.0000	0.0114	ENKUR	UP	1.91	1.0000	0.0068	CFAP251	UP	1.67	0.9999	0.0059	EID2B	DW	-8.31	0.9999	0.0054
TCAF2	UUP	6.16	0.0304	8.31E-05	CGREF1	UP	2.06	1.0000	0.0119	OBSCN	DW	-1.88	1.0000	0.0069	EXOSC5	DW	-1.47	0.9999	0.0060	RRM2	DW	-1.54	0.9999	0.0054
NLRC5	UUP	5.21	0.0314	8.79E-05	CARD14	UP	2.91	1.0000	0.0119	ZP3	DW	-1.84	1.0000	0.0073	PPBP	UP	2.63	0.9999	0.0061	TAS2R46	DW	-1.81	0.9999	0.0054
TNFSF13B	UUP	9.66	0.0348	0.0001	SAP25	DW	-5.34	1.0000	0.0123	FTCDNL1	DW	-3.11	1.0000	0.0073	NAV3	UP	1.73	0.9999	0.0062	COL9A2	UP	3.05	0.9999	0.0056
APOBEC3G	UUP	13.04	0.0348	0.0001	C11orf53	UP	9.03	1.0000	0.0125	SCUBE2	UP	4.24	1.0000	0.0074	DUOX2	UP	3.43	0.9999	0.0063	CDC20	DW	-1.73	0.9999	0.0058
ZBP1	UUP	15.31	0.0357	0.0001	CA14	UP	9.00	1.0000	0.0127	MMP25	UP	8.95	1.0000	0.0077	ZNF653	UP	1.74	0.9999	0.0065	C6orf226	DW	-2.06	0.9999	0.0062
STAT1	UUP	4.22	0.0357	0.0001	HIRIP3	DW	-2.03	1.0000	0.0127	GINS2	DW	-1.93	1.0000	0.0077	PRRX2	UP	4.67	0.9999	0.0067	C19orf18	UP	2.94	0.9999	0.0062
TAP1	UUP	3.82	0.0357	0.0001	GRIN3A	DW	-1.36	1.0000	0.0129	GBP7	UP	16.64	1.0000	0.0080	RSPH1	UP	1.79	0.9999	0.0067	CFB	UP	3.45	0.9999	0.0065
MSRB1	UUP	2.85	0.0359	0.0001	LGALS7B	UP	4.72	1.0000	0.0132	TMEM150A	DW	-1.63	1.0000	0.0081	CEP112	UP	2.71	0.9999	0.0067	CD14	DW	-2.33	0.9999	0.0066
OAS3	UUP	5.93	0.0380	0.0001	FGF11	DW	-3.11	1.0000	0.0132	CCL5	UP	19.96	1.0000	0.0083	NCAPG	DW	-2.00	0.9999	0.0068	LYN	UP	1.49	0.9999	0.0068
BATF2	UUP	6.37	0.0422	0.0001	LIPJ	DW	-1.93	1.0000	0.0135	IL15	UP	1.98	1.0000	0.0084	C4BPA	UP	2.27	0.9999	0.0068	ATF7-NPFF	DW	-8.61	0.9999	0.0069
TAP2	UUP	4.14	0.0437	0.0001	TMEM143	UP	1.59	1.0000	0.0138	NOS2	UP	288.49	1.0000	0.0085	PI3	UP	3.33	0.9999	0.0069	KLHL6	DW	-4.48	0.9999	0.0070

Table 18. Clinical and demographic characteristics of the subjects whose mucosal samples were used for transcriptomic analysis.

	Controls	UC	CD
Patients/Samples	8	25	20
Gender (F/M)	5/3	10/15	10/10
Age at diagnostic (≤16/17-40/>40 years)	-	1/21/3	0/5/15
***CDEIS of the segment (median±SD)**	-	-	20±9.20
***Endoscopic MAYO (median±SD)**	-	3±0.5	-
Ulcers (yes/no)	0/8	20/5	17/3

*Endoscopic UC and CD activity at the time of the colonoscopy was categorized according to the Mayo endoscopic subscore (396) and the Crohn's Disease Endoscopic Index of Severity (CDEIS), respectively (397). Active UC was defined when the Mayo endoscopic subscore was ≥1. Endoscopic CD activity was defined as a CDEIS ≥4 for the segment from which the biopsies were obtained. SD: standard deviation.

Table 19. Percentages (%) of differentially expressed genes (DEGs) common in artificially-inflamed d-ODMs and IBD active patients.

Stimuli	DEGs	DEGs in active UC	DEGs in active CD	Genes involved in UC (%)	Genes involved in CD (%)	Genes involved in UC with same trend (%)	Genes involved in CD with same trend (%)
IFNγ	588	309	269	52.55	45.74	87.05	89.59
TNFα	180	55	38	30.55	21.11	54.54	50
Cocktail	356	179	146	50.28	41.01	75.97	80.82
IL-1β	329	160	134	48.63	40.72	75	80.59
FLA	304	137	117	45.06	38.48	76.64	81.19
Mean				**44.46**	**35.95**	**72.52**	**73.47**

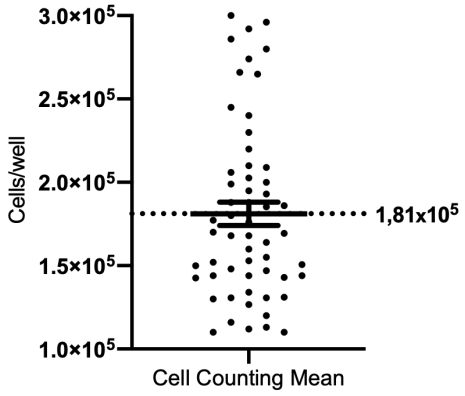
DEGs: differentially expressed genes
UC: Ulcerative Colitis
CD: Crohn's disease

Table 20. Genes regulated by *S. thermophilus* postbiotics in d-ODMs artificially-inflamed with IL-1β and the inflammatory cocktail

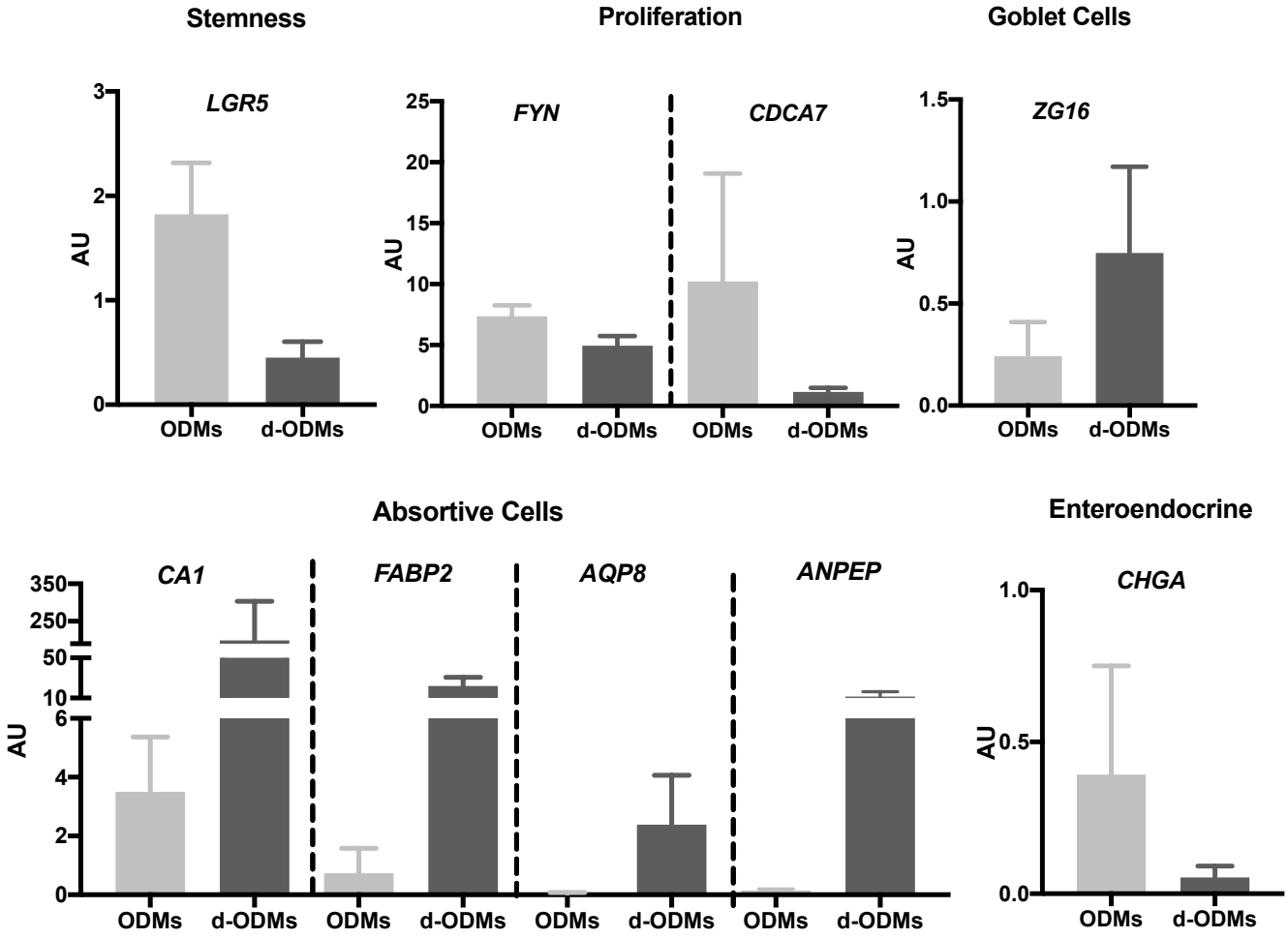
Gene	IL-1β vs Vehicle	*S. thermophilus* vs IL-1β	Gene	Cocktail vs Vehicle	*S. thermophilus* vs Cocktail
USH2A	DW	UP	MRPL4	DW	UP
BORCS8-MEF2B	DW	UP	PASK	DW	UP
ASPM	DW	UP	PRKCG	DW	UP
SCML2	DW	UP	COQ3	DW	UP
SETD6	DW	UP	GDF7	DW	UP
FANCE	DW	UP	HES6	DW	UP
RCL1	DW	UP	PRPS1	DW	UP
DLGAP5	DW	UP	CCDC141	DW	UP

TOP2A	DW	UP	PROSER3	DW	UP
ZFHX2	DW	UP	VWCE	DW	UP
POLR1E	DW	UP	TMEM150A	DW	UP
CCNA2	DW	UP	KLHL6	DW	UP
CLIC2	DW	UP	ZNF491	DW	UP
CCDC28B	DW	UP	DTX3	DW	UP
UBE2C	DW	UP	TMEM132C	DW	UP
ATAD5	DW	UP	CFAP73	DW	UP
NPIPB4	DW	UP	**CARD9**	**DW**	**UP**
CYB5RL	DW	UP	MAPK12	DW	UP
ASB14	DW	UP	ZP3	DW	UP
DTNBP1	UP	DW	ZNF257	DW	UP
SCT	UP	DW	HAUS7	DW	UP
MCOLN1	UP	DW	LTB4R2	DW	UP
LYL1	UP	DW	FAM72D	DW	UP
PGF	UP	DW	SAA2-SAA4	DW	UP
PRKCG	UP	DW	TAS2R43	DW	UP
CHI3L1	**UP**	**DW**	PKP1	UP	DW
ZMYND15	UP	DW	AKAP3	UP	DW
ACKR2	UP	DW	PACRG	UP	DW
FBXO27	UP	DW	PRPH2	UP	DW
NOD2	**UP**	**DW**	UNC5A	UP	DW
PKIG	UP	DW	G0S2	UP	DW
GPR37	UP	DW	ABI3BP	UP	DW
ADGRF3	UP	DW	SCUBE2	UP	DW
NUDT17	UP	DW	YOD1	UP	DW
NAP1L2	UP	DW	CTNNA3	UP	DW
SPATA21	UP	DW	FAM167B	UP	DW
C19orf67	UP	DW	CHRM5	UP	DW
CFAP54	UP	DW	CNTF	UP	DW
STMN3	UP	DW	UGT1A4	UP	DW
SELENOM	UP	DW			
CAPN14	UP	DW			
SAPCD1	UP	DW			
HLA-DPA1	UP	DW			

FIGURES



Supplementary Figure 1. Mean number of the d-ODMs-cells/well before AIEC infection. d-ODMs obtained from 7 different donors were plated in several replicates (n = 57) to determine the cell number at the time of infection.



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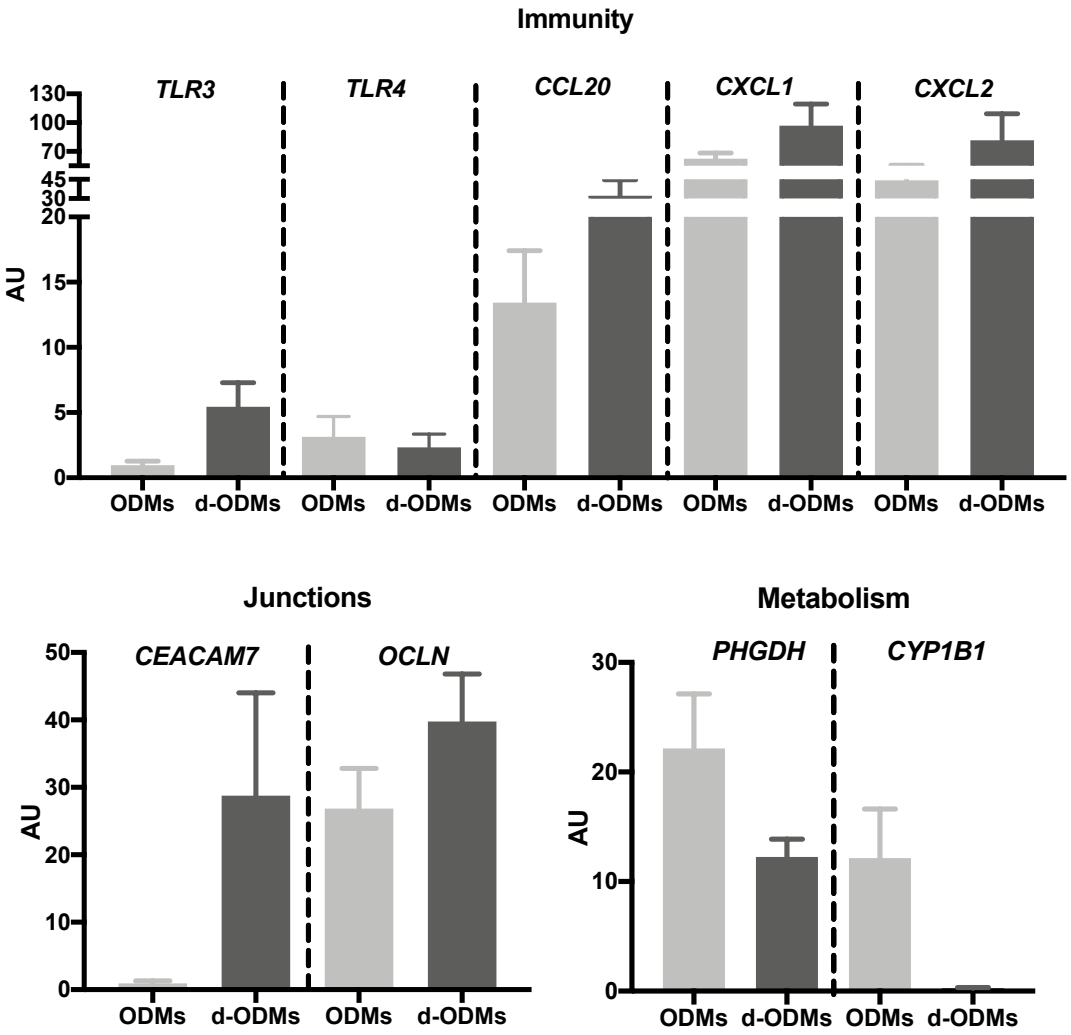
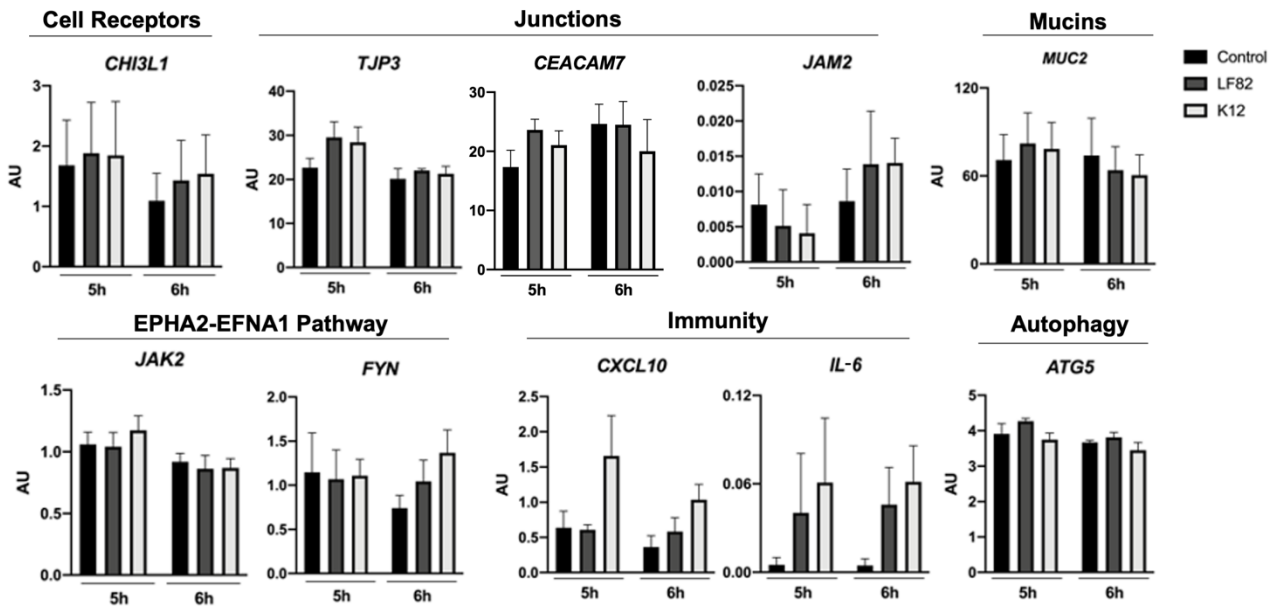
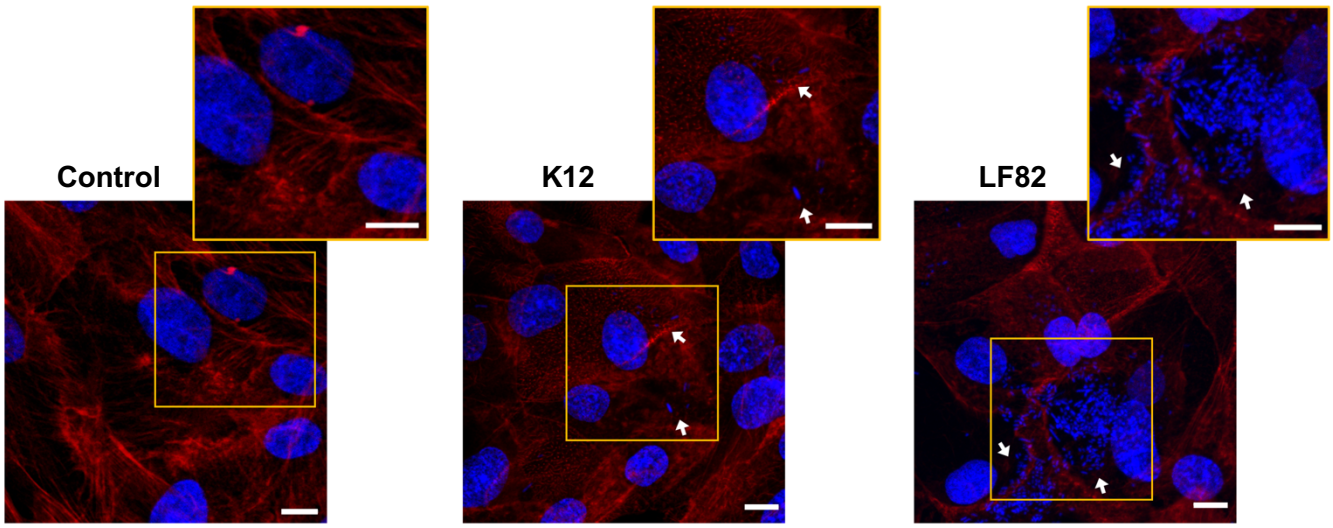


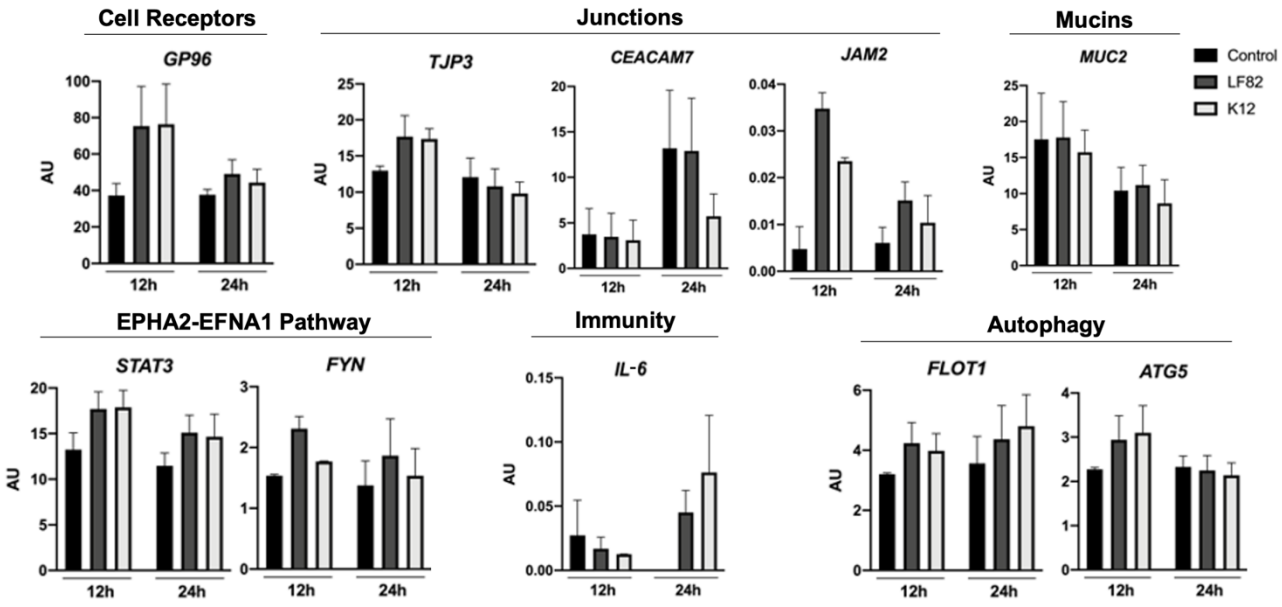
Supplementary Figure 2. Gene expression analysis in ODMs and d-ODMs. Genes, which have been grouped according to functional classification, were analyzed by RT-qPCR to determine their expression levels in ODM vs. d-ODMs. A paired t-test was performed to examine statistically different expression patterns between the two groups (ODMs/d-ODMs; n = 5 for each culture type). A P value of <0.05 was considered statistically significant.



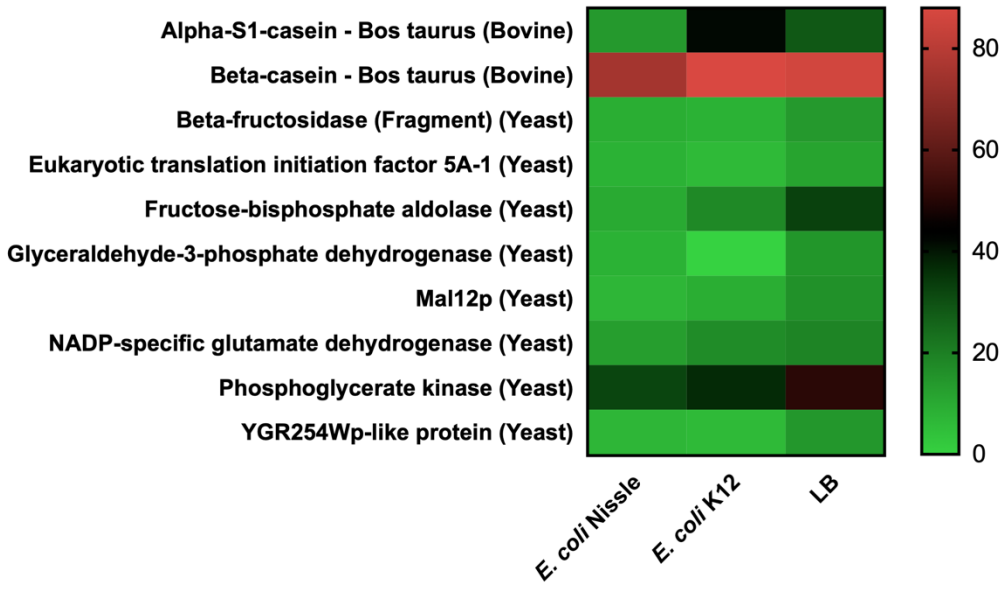
Supplementary Figure 3. Gene expression analysis of d-ODMs 5- and 6-h post-infection with *E. coli*. Genes, which have been grouped according to functional classification, were analyzed by RT-qPCR to determine their expression levels in d-ODMs after exposure to *E. coli* LF82 and K12 strains. Non-infected d-ODMs were used as a control (n = 3 for each condition). The error bars correspond to the SEM. A 2-way RM ANOVA test was applied to examine statistical significance between the three conditions for each infection timepoint. This analysis was followed by a Tukey test correction for multiple testing.



Supplementary Figure 4. *E. coli* LF82 and K12 invasion of d-ODMs 24h after infection. Fluorescent staining was performed to visualize the LF82 and K12 strains invasion of d-ODMs 24h after infection at MOI 100. Phalloidin marked the eukaryotic actin filaments while DAPI bound to the DNA of both epithelial and bacterial cells. White arrows show bacterial localization inside the IECs. Scale bars: 10 µm. Images are representative of n = 2 independent experiments.



Supplementary Figure 5. Gene expression analysis of d-ODMs 12- and 24-h post-infection with *E. coli*. Genes, which have been grouped according to functional classification, were analyzed by RT-qPCR to determine their expression levels in d-ODMs after exposure to *E. coli* LF82 and K12 strains. Non-infected d-ODMs were used as a control (n = 2 for 12h of infection and n = 3 for 24h). The error bars correspond to the SEM. A One-way RM ANOVA test was applied to examine statistical significance between the three conditions at 24h. This analysis was followed by a Tukey test correction for multiple testing.



Supplementary Figure 6. Top 10 proteins identified by LC-MSMS. *E. coli* Nissle postbiotics, *E. coli* K12 secreted metabolites and LB medium were analyzed to characterize their protein content. The represented data shows the peptide spectrum match (PSM) of each described protein for every analyzed liquid cultures. These results demonstrate the marked impact of the medium on the metabolite-cocktails composition.

ANNEX II

frontiers in Immunology

METHODS
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A Novel Strategy to Study the Invasive Capability of Adherent-Invasive *Escherichia coli* by Using Human Primary Organoid-Derived Epithelial Monolayers

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Over the last decades, Adherent-Invasive *Escherichia coli* (AIEC) has been linked to the pathogenesis of Crohn's Disease. AIEC's characteristics, as well as its interaction with the gut immune system and its role in intestinal epithelial barrier dysfunction, have been extensively studied. Nevertheless, the currently available techniques to investigate the cross-talk between this pathogen and intestinal epithelial cells (IECs) are based on the infection of immortalized cell lines. Despite their many advantages, cell lines cannot reproduce the conditions in tissues, nor do they reflect interindividual variability or gut location-specific traits. In that sense, the use of human primary cultures, either healthy or diseased, offers a system that can overcome all of these limitations. Here, we developed a new infection model by using freshly isolated human IECs. For the first time, we generated and infected monolayer cultures derived from human colonic organoids to study the mechanisms and effects of AIEC adherence and invasion on primary human epithelial cells. To establish the optimal conditions for AIEC invasion studies in human primary organoid-derived epithelial monolayers, we designed an infection-kinetics study to assess the infection dynamics at different time points, as well as with two multiplicities of infection (MOI). Overall, this method provides a model for the study of host response to AIEC infections, as well as for the understanding of the molecular mechanisms involved in adhesion, invasion and intracellular replication. Therefore, it represents a promising tool for elucidating the cross-talk between AIEC and the intestinal epithelium in healthy and diseased tissues.

Keywords: organoid-derived epithelial monolayers (ODM), adherent-invasive *E. coli* (AIEC), bacterial infection, intestinal epithelial cells (IECs), inflammatory bowel disease (IBD)

INTRODUCTION

Escherichia coli (*E. coli*) strains are widely known inhabitants of the healthy human gut microbiota, being one of the first colonizers as well as among the most prevalent microorganisms in the intestines (1, 2). *E. coli* promotes health benefits to its hosts by preventing the colonization of pathogens and thus, positively contributes to intestinal homeostasis (3, 4). However, several *E. coli* strains, including the Adherent-Invasive *E. coli* (AIEC) pathotype, have acquired a virulent nature. Despite the lack of typical enteropathogenic *E. coli* virulent factors in AIEC isolates, these are able not only to adhere to and invade intestinal epithelial cells (IECs), but also to replicate within macrophages without inducing cell death, thus evading protective host immune responses (5–7).

AIEC was first identified in the ileal mucosa of patients with Crohn's Disease (CD) and may constitute more than the 50% of the total number of bacteria both in early and chronic ileal lesions (8, 9). AIEC prevalence in Inflammatory Bowel Disease (IBD) – which comprises CD and Ulcerative Colitis (UC) – patients is significantly higher than in non-IBD subjects and, in general, AIEC strains are found in ileal and colonic samples of CD patients (6, 10–17). In UC, although the prevalence of this pathobiont is less clear, a recent meta-analysis suggests that this pathotype could be involved in its pathogenesis (18). Both *in vitro* and *in vivo* assays helped explain the molecular basis of AIEC pathogenicity in CD (9, 19). AIEC mechanisms to cross the mucus layer include the secretion of bacterial proteases (20, 21) as well as the alteration of host antimicrobial peptides (22). Adhesion and invasion to IECs occurs through the interaction between, among others, AIEC type 1 pili and the eukaryotic glycoprotein CEACAM6 (23, 24). On the other hand, flagella are crucial in mediating AIEC-induced cellular responses through their binding to IECs-toll like receptor (TLR)-5 (25). All these events end up triggering a cytokine release which, in turn, promotes intestinal epithelial permeability (26) and intestinal inflammation in compromised patients (27). AIEC are also able to invade M cells and translocate through Peyer's patches reaching the lamina propria and rapidly spreading through the mesenteric lymph nodes (28–30), and to translocate across the intestinal barrier due to tight junctions expression alteration (31). Overall, it has been demonstrated that AIEC infections affect a wide variety of host cell processes such as protein synthesis, signal transduction, cell division, and cytoskeletal function among many others (32).

AIEC identification is currently challenging, as it relies on phenotypic assays based on infected cell cultures, which are highly time-consuming. Therefore, the identification of AIEC molecular markers is of great importance since it would support detection of AIEC carriers, which is necessary to carry out epidemiological studies and to eventually establish prevention protocols (33–35). Different immortalized cell lines have been applied to assess the AIEC phenotype. The most common ones for the study of AIEC adhesion and invasion capacity are Caco2, Intestine-407 (I407), T84 and Hep2 as reviewed by Camprubí-Font et al. (36). Even though cell lines are easy to obtain, handle and expand over time, they lack important physiological features such as tissue cytoarchitecture, inter-individual variability and gut location-specific attributes. All of these limitations can be overcome by using human primary cultures. Organs or tissues isolated from their *in vivo* environment offer the advantage of providing a more physiological experimental setting due to their mimesis of the tissue of origin, phenotype and structure. Hence, infecting human colonocytes derived from patient biopsies might represent a promising strategy for studying the intestinal epithelium response to AIEC, as well as new pathogenicity mechanisms associated with this pathobiont. Such an approach could lead to the discovery of new disease biomarkers and new therapeutic targets. To our knowledge, there are few publicly available reports that analyze the interaction between enteric pathogens and human isolated IECs (23, 37–41). More recently, Sayed et al. published a study in which AIEC infection of organoid-derived 2D cultures is applied to explore host engulfment in IBD. Their research supports the suitability of human organoid-derived epithelial monolayers (ODMs) as a tool to study AIEC pathogenicity (42). Here, we deeply describe our recently developed infection method that uses colonic ODMs to examine the ability of AIECs to adhere to and invade primary human epithelial cells. This *ex vivo* cell culture exhibits an appropriate cell polarization for a more physiological-like bacteria-host cell interplay and thus represents a powerful tool for AIEC-infection studies. Throughout the next sections we will detail the entire procedure by which ODMs are obtained and lately infected with AIEC. To that end, we will also specify the performed infection-kinetics assay to determine the ideal time of infection and the bacteria/IEC ratio for this pathobiont to efficiently invade ODMs.

MATERIALS AND EQUIPMENT

Reagents

Biological Reagents

- Human Epithelial Organoid 3D Cultures (EpOCs): intestinal samples of healthy sigmoid colon with no evidence of macroscopic inflammatory lesions were obtained from subjects undergoing surgery for left-sided colorectal cancer (CRC) or routine endoscopy for CRC screening. For surgical pieces, a segment of healthy mucosa was collected at least 10 cm from the margin of the affected area. Biopsy samples showed no evidence of neoplastic lesions. However, biopsies were not specifically assessed for signs of microscopic inflammation.

Surgical or biopsy samples were immediately used for generating EpOCs. **Supplementary Table 1** shows the clinical and demographic characteristics of the subjects enrolled to develop this protocol and from which 3D cultures were obtained. EpOCs samples were used on day 5 of expansion and were distributed among different subgroups based on the experimental approaches used. Patients were recruited at the Department of Gastroenterology, Hospital Clinic Barcelona. The study protocol was approved by the Ethics Committee of the Hospital Clinic of Barcelona (registration number HCB/2016/0546).

- Cell lines: Intestine-407 – I407 – (ATCC CCL-6, RRID: CVCL_1907) cell line.
- Bacterial Strains: The AIEC strain LF82, which was isolated from a chronic ileal lesion of a patient with CD, and the non-pathogenic strain *E. coli* K12 C600 [a prototypical derived laboratory strain which has been extensively used for molecular microbiology and bacterial physiology studies since its isolation in 1954 (43)], were provided in 2006 by Prof. Arlette Darfeuille-Michaud (Université d'Auvergne, Clermont-Ferrand, France).

Primary Cell Culture Reagents

All concentrations shown here correspond to the used working concentration (WC).

- Heat inactivated – at 56°C for 30 minutes – fetal bovine serum – FBS – South American (Applied Biosystems, Foster City, CA, USA. Ref. 10270106).
- Washing medium (WM) (**Supplementary Table 2**).
- Matrigel Growth Factor Reduced Basement Membrane (Corning, NY, USA. Ref. 356231): -80°C stored bottles were thawed overnight (ON) on ice. 500 µl aliquots were prepared and frozen at -20°C for later use. Once thawed, aliquots were stored at 4°C for no longer than one week.
- Cell Recovery solution (Corning, NY, USA. Ref. 354253).
- Dissociation medium (**Supplementary Table 3**).
- Wnt3a-conditioned medium + Y (STEM+Y medium) (**Supplementary Table 4**).
- Trypan blue Solution (Gibco, Grand Island, NY, USA. Ref. 15250061).
- Differentiation medium (DIFF medium) (**Supplementary Table 5**).

Cell Line Reagents

- Trypsin-EDTA (Lonza, Basel, Switzerland. Ref. H3BE17-161E). WC: 170,000 U/L trypsin and 200mg/L EDTA.
- EMEM Complete Medium (**Supplementary Table 6**).

Bacterial Culture Reagents

- Liquid Luria-Bertani (LB) Broth (Sigma-Aldrich, Saint Louis, MO, USA. Ref. L3022).

Gentamicin Protection Assay Reagents

- Minimal media (EMEM-MM/DIFF-MM; **Supplementary Tables 7** and **8**, respectively).
- Minimal media containing 100 µg/ml of gentamicin (Lonza, Basel, Switzerland. Ref. 17-519Z).
- Ringer Solution (Scharlau, Barcelona, Spain. Ref. 06-073-500).
- LB Agar (**Supplementary Table 9**).

RNA Isolation and Quantitative Multiplex Real-Time Polymerase Chain Reaction Reagents

- TRIzol reagent (Life Technologies, Carlsbad, CA, USA. Ref. 15596018).
- Chloroform (Sigma-Aldrich, Saint Louis, MO, USA. Ref. C2432-500).
- RNeasy Kit (Qiagen, Hilden, Germany. Ref. 74106).
- High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA. Ref. 4368813).
- RNAse Inhibitor (Applied Biosystems, Carlsbad, CA, USA. Ref. N8080119).
- TaqMan™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG (Applied Biosystems, Carlsbad, CA, USA. Ref. 4366073).
- Nuclease Free Water (Promega, Madison, WI, USA. Ref. P1193).
- Pre-designed TaqMan Assays (Applied Biosystems, Carlsbad, CA, USA.): *MYC* (Mm00487804_m1), *MKI67* (Mm01278617_m1), *AXIN2* (Hs00610344_m1), *TJP3 (*Hs00274276_m1*), TFF3* (Hs00902278_m1), *MUC2* (Hs03005094_m1), *LGR5* (Hs00173664_m1), *FYN* (Hs00176628_m1), *CDCA7* (Hs00230589_m1), *ZG16* (Hs00380609_m1), *TLR3* (Hs01551078_m1), *TLR4* (Hs00152939_m1), *CCL20* (Hs01011368_m1), *CXCL1* (Hs00605382_gH), *CXCL2* (Hs00601975_m1), *ANPEP* (Hs00952642_m1), *FABP2* (Hs01573164_g1), *AQP8* (Hs00154124_m1), *CA1* (Hs01100176_m1), *CHGA* (Hs00154441_m1), *CEACAM7* (Hs03988977_m1), *OCLN* (Hs00170162_m1), *PHGDH* (Hs01106330_m1), *CYP1B1* (Hs00164383_m1), (all of them conjugated with FAM dye) and *ACTB* (endogenous control; Ref. 4310881E) with VIC dye.

Immunostaining Assay Reagents

- Paraformaldehyde aqueous solution – PFA – (Electron Microscopy Sciences, Hatfield, PA, USA. Ref. 15710. WC: 4%.
- Glycine (Sigma-Aldrich, Saint Louis, MO, USA Ref. G7126). WC: 20 mM.
- Bovine serum albumin – BSA – (Sigma-Aldrich, Saint Louis, MO, USA Ref. T8787). WC: 1%.
- Primary antibodies: mouse anti-EpCAM (1:100; Dako, Denmark. Ref. M0804), rabbit anti-E-Cadherin (1:100, Cell Signaling Technology, Danvers, MA, USA. Ref. 3195S), mouse anti-KI67 (1:100, Leica, Wetzlar, Germany. Ref. NCL_L-KI67_MM1), rabbit anti-MUC2 (1:250, Santa Cruz Biotechnology, Dallas, TX, USA. Ref. sc-15334), mouse anti-VILLIN (1:100; Dako, Denmark. Ref. M3637) all diluted in 1% BSA.
- Secondary antibodies: anti-mouse Cy3 (1:400, Jackson ImmunoResearch, Cambridge, UK. Ref. 115-165-205. RRID: AB_2338694) and anti-rabbit Alexa 488 (1:400, Jackson ImmunoResearch, Cambridge, UK. Ref. 111-545-144. RRID: AB_2338052) all diluted in 1% BSA.
- 4′,6-diamidino-2-phenylindole (DAPI) (diluted 1:10000 in DPBS, Invitrogen, Carlsbad, CA, USA. Ref. D1306).

- Alexa Fluor™ 555 Phalloidin (diluted 1:40 in 1%BSA; Invitrogen, Carlsbad, CA, USA. Ref. A34055).
- Mounting medium: glycerol (Sigma. Ref. G5516-500). WC: 80%.

Other Reagents

- Dulbecco phosphate-buffered saline – DPBS – (Gibco, Grand Island, NY, USA. Ref. 14190-169).
- Triton X-100 (Sigma-Aldrich, Saint Louis, MO, USA. Ref. T8787).
- Distilled H_2O.
- CellTox™ Green Cytotoxicity Assay (Promega, Madison, WI, USA. Ref. G8741).
- Digitonin (Sigma-Aldrich, Saint Louis, MO, USA., Ref. D141). WC: 100 µg/ml.

Equipment

Consumables

- 1.5 ml tubes (Eppendorf, Hamburg, Germany. Ref. 211-2130).
- 1.5 ml tubes RNAse free (Invitrogen, Carlsbad, CA, USA Ref. AM12400).
- Falcon 15ml Sterile Disposable Conical Centrifuge Tubes (BD Biosciences, San Jose, CA, USA. Ref. 352096).
- Falcon 50ml Sterile Disposable Conical Centrifuge Tubes (BD Biosciences, San Jose, CA, USA. Ref. 352070).
- Filtered pipette tips – 10 µl, 20 µl, 200 µl, 1000 µl – (VWR International Eurolab, Barcelona, Spain. Refs. 732-1148/732-1150/732-1153/732-1154).
- Serological pipettes: 5, 10, 25 ml and 50 ml (VWR International Eurolab, Barcelona, Spain. Refs. 357543/357551/357535/734-1740).
- Scalpels (VWR, International Eurolab, Barcelona, Spain. Ref. SWAN6608).
- Microscope slides (DDBiolab, Barelona, Spain. Ref. 37519).
- KOVA® Glasstic Slide 10 With Counting Grids (Kova, Garden Grove, CA, USA. Ref. 87144E).
- BD Emerald 5 ml syringes (BD Biosciences, San Jose, CA, USA. Ref. 1026307731).
- BD Microlance® 3 21Gx1" 0.8mmx25mm (BD Biosciences, San Jose, CA, USA. Ref. 301156).
- MicroAmp™ Optical Adhesive Film (Applied Biosystems, Foster City, CA, USA. Ref. 4311971).

Plates and Flasks

- 48-well plates (Corning, NY, USA. Ref. 3548).
- 24-well plates (Jet Biofil, Guangzhou, China. Ref. TCP-011-024).
- µ-Slide 8 Well ibiTreat: #1.5 polymer coverslip, tissue culture treated, sterilized (IBIDI, Gräfelfing, Germany. Ref. 80826).
- T25 and T75 tissue culture flasks (BioLab, Barcelona, Spain. Refs. 55400/55402).
- 120x120mm Petri dishes (Corning, NY, USA. Ref. GOSSBP124-05).
- Microplate 96 well qPCR FAST THERMAL CYCLING (Applied Biosystems, Foster City, CA, USA. Ref. 4346907).

Lab Equipment

- Vortex mixer.
- Thermo Scientific™ NanoDrop™ One^C Microvolume UV-Vis Spectrophotometer Precision Scale.
- Veriti 96-well Thermal Cycler (Applied Biosystems).
- Benchtop shaker (BOECO Mini-Rocker Shaker MR-1).
- Benchtop refrigerated centrifuge (for 1.5 ml, 15 ml and 50 ml conical tubes).
- Inverted microscope (Olympus X51 Inverted Microscope).
- Fluorescence Inverted Microscope Nikon S Ti.
- Cell incubator (37°C, 5% CO_2).
- Biosafety hood.
- Autoclave.
- Spectrophotometer.
- ABI PRISM 7500 Fast RT-PCR System (Applied Biosystems).
- Leica TCS_SP5 scanning spectral confocal microscope (Leica Microsystems, Germany) equipped with an DMI 6000 inverted fluorescence microscope, blue diode (405nm), Argon (488nm), diode pumped solid state (561nm) lasers and a Apochromat 63X oil immersion objective (NA 1.4).
- Zeiss LSM880 laser scanning spectral confocal microscope (Carl Zeiss, Jena, Germany) equipped with an Axio Observer 7 inverted microscope, blue diode (405nm), Argon (488nm), diode pumped solid state (561nm) and HeNe (633nm) lasers and a Plan Apochromat 63X oil (NA 1.4) immersion objective lenses.

Other Equipment

- Micropipettes and Pipettor.
- Tube racks.
- Refrigerated racks.
- Aluminum foil.
- Cell-counter.
- Forceps.
- Scissors.
- Spectrophotometer Cuvettes.

Software Equipment

- Image processing software (Image J Fiji, https://imagej.net/Fiji).
- Data software analysis Graphpad Prism 5 (GraphPad Software, http://www.graphpad.com/).

METHODS

Our prime aim was to develop a new model of infection using primary human intestinal epithelium. For that purpose, ODMs were generated from EpOCs and differentiated (d-ODMs) before being infected by *E. coli*.

In this section we will accurately describe the optimized protocol for ODM generation from EpOCs, ODM differentiation and AIEC infection of d-ODMs to evaluate AIEC's invasive capacity in differentiated primary epithelial cells.

Organoid-Derived Monolayer (Timing ⏲ 4d)

Generation of Organoid-Derived Monolayers

EpOCs were generated as previously described (44, 45). Briefly, crypts were isolated from intestinal samples after an incubation of 45' with 8mM EDTA at 4°C. Crypts were then embedded in 25 µl of Matrigel and covered with 250 µl of STEM medium (**Supplementary Table 4** – modified without Y). After 2-3 days, the crypt culture was mechanically dissociated to single cells using a dispase-based solution (**Supplementary Table 3**) and expanded at a 1:3 dilution. EpOCs were used after 5 days of expansion to generate ODMs as detailed below. Prior to EpOCs dissociation, 48-well plates were pre-coated with a thin layer of diluted (1:20) Matrigel in DPBS to promote cell adhesion. A volume of 150 µl/well was added and plates were incubated at room temperature (RT) for 1h. Excess Matrigel was discarded and the diluted-Matrigel layer was covered with Advanced DMEM/F12 medium and kept at RT until immediate use. Alternatively, coated plates were stored at 4°C covered in DPBS for up to 7 days.

Δ CRITICAL. Based on our experience, every EpOCs drop contains around 40,000-100,000 cells. Thus, depending on the final number of single cells needed for the invasion assay, a determined number of EpOCs drops will be used at the starting point.

To generate ODMs from EpOCs, the protocol was as follows:

(1) Matrigel drops containing EpOCs were washed with cold DPBS and collected in Cell Recovery solution (300 µl/well) at 4°C for 40 minutes. Every 5-10 minutes, cell suspensions were gently inverted upside-down.

(2) 4-5 ml of washing medium (WM) (**Supplementary Table 2**) were added, and the cell suspensions were centrifuged at 400*g* for 4 minutes at 4°C.

(3) Supernatant was discarded and the pellet was resuspended in Dissociation Medium (**Supplementary Table 3**) followed by 15-20 minutes of incubation at 37°C. On average, 5 ml Dissociation Medium were used for every 20-25 Matrigel drops.

(4) After organoid release, cells were mechanically disaggregated using a 5 ml syringe with a 21G needle until the cells were totally dissociated (20-50 strokes were conducted depending on the sample (**Figure 1A**)). To evaluate the extent to which EpOCs were dissociated to single cells, microscope observation was performed. If required, additional rounds of 10-20 strokes followed by microscope observation were performed until complete cell dissociation was reached.

(5) Cells were centrifuged at 800*g* at 4°C for 4 minutes and washed with 5 ml of WM after supernatant removal. This step was repeated twice.

(6) The remaining pellet was resuspended in 1-2 ml of WM for manual cell counting:

a. Cells (10 µl) were diluted 1:1 with Trypan blue Solution.

b. 10 µl of the cell suspension was loaded into a Glasstic Slide 10 With Counting Grids and the cell number was estimated according to the manufacturer's recommendations. The mortality rate (% of dead cells over the total number of cells) was usually below 10%.

(7) Single cells were again centrifuged, and the pellet was resuspended in the required volume of STEM+Y medium (**Supplementary Table 4**) to achieve $2x10^5$ cells/well/250 µl.

(8) Cells were seeded on Matrigel pre-coated 48-well plates and incubated for 24h at 37°C 5% CO_2 (**Figure 1B**).

Differentiation of Organoid-Derived Monolayers

After incubation, ODMs were induced to differentiation. To this end, STEM + Y medium was discarded and ODMs were washed with DPBS and Advanced DMEM/F12 medium (300 µl/well) at RT to remove dead cells. DIFF medium (250 µl/well) (**Supplementary Table 5**) was then added and ODMs were incubated at 37°C 5% CO_2 for an additional 48h.

Under these conditions, the differentiated monolayer (d-ODMs) reached 100% confluence 1-2 days after differentiation (**Figure 2A**). Therefore, the period between cells seeding and infection was 72h (cells were incubated for 24h after seeding and before differentiation, and 48h after differentiation and before infection).

Quantitative Multiplex Real-Time Polymerase Chain Reaction and Immunofluorescence

Δ FOR SYSTEM SET UP ONLY. The methodology described in this section was only utilized during optimization and until the protocol we established was entrenched (**Figure 1C**).

RT-qPCR

Both ODMs and d-ODMs were harvested in Trizol for RNA extraction (**Supplementary Table 1 Group 1**) and isolation using the RNeasy Kit. RNA was transcribed to cDNA at a final concentration of 250 ng/50 µl using the reverse transcriptase High-Capacity cDNA RT kit with RNase inhibitor. Reverse transcription was performed using a Programmable Thermal Cycler for 10 minutes at 25°C followed by 2 hours at 37°C. Quantitative Multiplex Real-time PCR (qPCR) was then conducted to characterize the monolayer gene expression pattern in ODMs versus d-ODMs. qPCR 96-well microplates contained a .volume of 10 µl/well (1 µl cDNA+0.5 µl each TaqMan Assay diluted in TaqMan Fast Universal PCR Master Mix and H_2O). Target genes were amplified and quantified using *ACTB* as the endogenous control. PCR reaction was run in the ABI PRISM 7500 Fast RT-PCR System using the following program: a holding stage for 20 seconds at 95°C and a cycling stage for 3 minutes at 95°C and 30 seconds at 60°C during 40 cycles. Target gene expression values relative to *ACTB* were expressed as arbitrary units (AU) following this formula:

$$AU = 2^{-(\text{Ct target gene} - Ct\ ACTB)} \times 1000$$

Immunofluorescence Staining

Monolayer cultures (both ODMs and d-ODMs, **Supplementary Table 1 Group 3**) seeded in µ-Slide 8 Well ibiTreat chambers

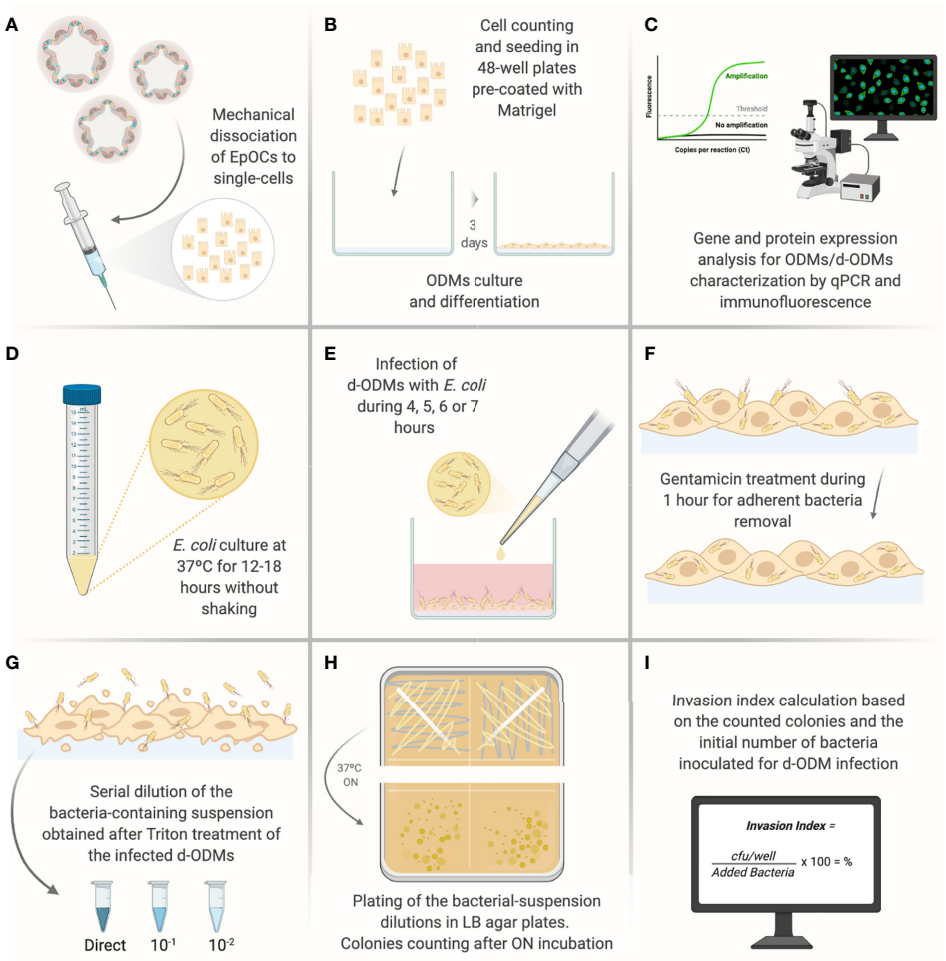


FIGURE 1 | Illustrated experimental workflow of the most critical steps of the d-ODMs-*E. coli* infection protocol. **(A)** Mechanical dissociation of EpOCs with the help of a 5 ml syringe with a 21G needle until achievement of single-cells. **(B)** Single-cell counting and seeding ($2x10^5$ cells/well on pre-coated 48-well plates with diluted Matrigel (1:20). Cells were incubated until ODM formation for further differentiation. **(C)** Characterization of ODMs and d-ODMs by qPCR and immunostaining (only during the protocol set-up). **(D)** ON growth of *E. coli* LF82 and K12 strains were grown in liquid LB. **(E)** Infection of d-ODMs with *E. coli* strains performed by gently releasing the drop. Infection times were from 4-7 hours. **(F)** Gentamicin (100 µg/ml) addition for 1 hour to eliminate adherent bacteria. **(G)** Cell treatment with 1% Triton X-100 to facilitate intracellular bacteria release. The bacterial suspension was then serially diluted and seeded. **(H)** ON incubation of bacterial dilutions in LB agar plates. **(I)** After colony counting, the Invasion Index for each strain was determined. This figure was created using BioRender.com.

(for optimal image acquisition) were processed for immunofluorescent staining as follows:

(1) After two DPBS washes, the cell monolayer was fixed with 2% PFA (1:1 4% PFA + DPBS) for 5 minutes at RT and then with 4% PFA for 10 minutes at RT.

(2) Cells were washed three times with DPBS: 1st fast; 2nd and 3rd 5 minutes at RT.

Δ CRITICAL. STOP POINT – Cells were stored at 4°C covered in DPBS (300 µl) or were immediately used for staining.

(3) 250 µl of 20mM Glycine was added for 10 minutes at RT to reduce background staining.

(4) DPBS washes were conducted as described in step (2).

(5) For permeabilization, 250 µl of 0.25% Triton X100 were added for 20 minutes at RT.

(6) Cells were then washed 3 additional times – 5 minutes each – with DPBS.

(7) To block non-specific binding, 250 µl of 1% BSA was applied and incubated at RT for 30-45 minutes.

(8) Primary antibodies (150-200 µl/well) – EpCAM, E-Cadherin, MUC2, Villin or KI67 – were added at the specified dilutions (in 1% BSA) and incubated ON at 4°C.

(9) After 3 DPBS washes (as in step 6), cells were incubated with 150-200 µl/well of the secondary antibodies – Anti-mouse Cy3 and Anti-rabbit 488 – at the specified dilutions (see *Materials* section) in 1% BSA for 1h of incubation at RT. Cells were washed 3x with DPBS at RT as described in step 6.

(10) For DNA counterstaining, DAPI (250 µl/well) was added and incubated at RT for 10 minutes. Washes were repeated as in step 6.

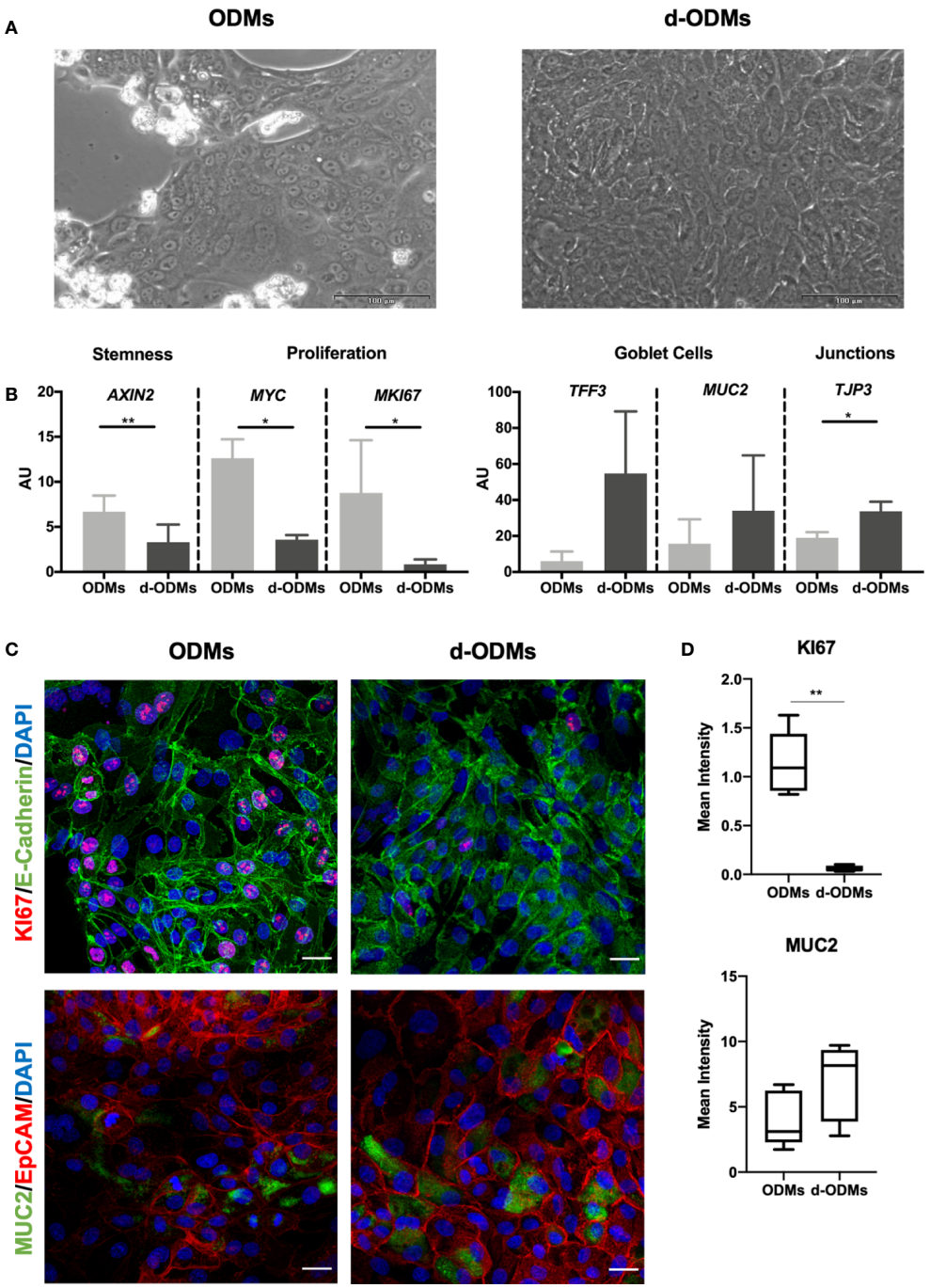


FIGURE 2 | Organoid-Derived Monolayers (ODMs) characterization. **(A)** ODMs (left panel) 24 hours after seeding showed a confluence of around 70-80% and d-ODMs (right panel) 48 hours after differentiation, showed 100% confluence. **(B)** Gene expression analysis of ODMs and d-ODMs (n = 5 for each culture type). *AXIN2, MYC, MKI67, TFF3*, *MUC2* and *TJP3* genes were analyzed by qPCR to determine their expression levels in ODM vs. d-ODMs. A paired t-test was performed to examine statistically different expression patterns between the two groups (ODMs/d-ODMs). A P value of <0.05 was considered statistically significant. *AXIN2*: **indicates P = 0.0012. *MYC*: *indicates P = 0.0135. *MKI67*: *indicates P = 0.0335. *TJP3*: *indicates P = 0.0365. **(C)** Protein expression analysis by immunofluorescence. KI67 and MUC2 were analyzed to confirm the proliferation and differentiation status of both ODMs and d-ODMs. E-Cadherin and EpCAM were used as epithelial cell-wall markers. DAPI was used to counterstain the cell nuclei. Scale bars: 25 µm. Images are representative of n = 3 independent experiments performed with samples from two different donors. **(D)** Box-plot distribution of the fluorescent signal of KI67 and MUC2 proteins in ODMs and d-ODMs, expressed as Mean Intensity. Fluorescence was quantified in 5 different fields per sample. A paired t-test was performed to examine statistically different expression patterns between the two groups (ODMs/d-ODMs). A P value of <0.05 was considered statistically significant. KI67: **indicates P = 0.0013.

(11) Finally, 200 µl/well of mounting medium (80% Glycerol in DPBS) were added. Samples were stored at 4°C for subsequent fluorescent microscope observation.

Δ CRITICAL. After adding the secondary antibodies, cells were kept in the dark.

Δ CRITICAL. For short-term storage, stained cells were kept at 4°C or at -20°C for up to 6 months.

AIEC Infection of Differentiated Organoid-Derived Monolayer (Timing ◴ 3d)

Bacterial Strains

Prior to infection, LF82 and *E. coli* K12 strains were cultured in 1.5 ml of LB Broth and incubated for 12-18 hours at 37°C without shaking (**Figure 1D**).

Reference Model of Infection

The I407 cell line, originally employed for AIEC-pathotype identification (6), was used as the reference method of the gentamicin protection assay in order to ensure that the bacterial strains ON cultures show the expected phenotype. Cells were passaged every 2-3 days *via* 5-minute incubation with 1 ml of Trypsin-EDTA after a washing step with DPBS. After collection, cells were centrifuged at 500*g* for 5 minutes and 20°C. Pelleted cells were resuspended in EMEM complete medium (**Supplementary Table 6**) and seeded in T75 flasks. Twenty-four hours before infection, $4x10^5$cells/well were plated on 24-well plates.

The assay was performed at Multiplicity of Infection (MOI) 10, as described previously (46, 47). Infection lasted 3 hours followed by 1 hour of gentamycin treatment. During the entire procedure, EMEM-MM (**Supplementary Table 7**) was employed. Invasive ability was quantified as the percentage of the intracellular bacteria from the initial inoculum (4×10^6 cfu/ml):

$$\text{I-INV}\,(\%) = \left(\text{intracell. bacteria}/4\times10^6\right)\times 100$$

Δ FOR SYSTEM SET UP ONLY. This model of infection was only performed until establishment of the d-ODM-based gentamicin protection assay.

d-ODM-Based Gentamicin Protection Assay

d-ODM Cell Counting

To infect cells with a determined MOI, it is crucial to know the exact number of cells seeded as a monolayer at the time of infection. In our particular case, we seeded $2x10^5$ EpOCs-derived single cells/well in 48-well plates based on previous experience (data not published), although this may need to be adjusted by each lab as culture conditions can vary slightly. To monitor the number of cells present in the plate at 100% confluency, experiments were performed seeding the above number of cells/well and counting cells present in d-ODM prior to infection. This step proved decisive in order to adjust the needed inoculum of bacteria and achieve the desired MOI. Briefly, d-ODMs were washed with DPBS to remove non-attached cells. Trypsin-EDTA (150 µl) was added to the culture for 10-15 minutes at 37°C 5% CO_2. Detached cells were collected and resuspended in Advanced DMEM/F12 + 10% FBS. These last two steps were repeated until complete cell-detachment was achieved. Cells were centrifuged at 800*g* for 4min and at 4°C and resuspended in 200 µl of Advanced DMEM/F12 + 10% FBS for cell counting as explained in a previous section (see the *ODM generation* section).

Δ CRITICAL. It is important to not exceed the 10-15 minutes incubation with Trypsin-EDTA in order to prevent cell death.

On average, we recovered approximately $1.8x10^5$ cells/well prior to infection (**Supplementary Figure 1**), which is close to the number of cells initially seeded. Notice that these numbers may have to be adjusted by each lab, as mentioned above.

For the infection assay, two different MOI – 20 and 100 – were assessed on d-ODM-based assays.

Thus, d-ODM counted-cells ($1.8x10^5$ cells/well) were multiplied 20- or 100-times to determine the bacterial colony forming units (cfu)/ml required for reaching each MOI value. In our case, $3{,}6x10^6$ or 18 $x10^6$ *E. coli* cfu/ml were needed.

Δ CRITICAL. Working at a confluence as close as possible to 100%, is essential to ensure the optimal ratio of bacterial cells/eukaryotic cells in order to reach the desired MOI.

Bacterial Optical Density and Colony Forming Unit Adjustment

The study of the *E. coli* growth curve in LB allowed us to estimate the cfu/ml at every measured Optical Density (OD) (**Supplementary Figure 2**). Prior to infection, ON bacterial cultures (both from LF82 and K12 strains) were adjusted to OD = 0.1, corresponding to $1.6x10^8$ cfu/ml. This OD was chosen since it represents an adequate inoculum volume for the infection assay for both of the assessed MOIs. The bacterial suspension was prepared following these steps:

(1) ON bacterial cell suspensions (500 µl) were diluted 1:1 with LB medium and 1 ml was transferred to a cuvette.

(2) The OD was measured with a spectrophotometer at a wavelength (λ) of 600 nm.

(3) OD adjustment was achieved in accordance with the following formula:

$$iV = fOD\ \ (0.1)\times fV/(mOD)\times\ \ 2$$

iV; Initial Volume (required volume for the ON culture)
fOD; Final OD (0.1 in this case)
fV; Final Volume (1 ml)
mOD; Measured OD

(4) The calculated iV and DIFF-MM (**Supplementary Table 8**) up to 1 ml total volume were added to a 1.5 ml tube.

ODM Infection and Gentamicin Protection Assay

As already mentioned, LF82 and K12 strains were used as positive (invasive) and negative (non-invasive) control, respectively. Infection was performed using d-ODMs generated from 7 different subjects (**Supplementary Table 1 Group 2**) as the starting material. Every experiment was conducted in duplicate.

DIFF medium was discarded from 100% confluent d-ODMs; cells were washed twice with DPBS at RT (500 µl/well) and fresh DIFF-MM was added (500 µl/well). Then, the corresponding

volume of OD 0.1 bacterial suspension (**Table 1**) was inoculated to reach each assessed MOI by gently releasing the drop (**Figure 1E**). Infected d-ODMs were incubated for 4, 5, 6 or 7 hours at 37°C 5% CO_2 for the complete infection-kinetics study. At the end of each time point, cells were washed 3 times with DPBS at RT – as explained above – and DIFF-MM containing 100 µg/ml of gentamicin was added for 1 additional hour (**Figure 1F**) in order to remove the extracellular bacterial cells. Three more DPBS washes at RT were required after gentamicin treatment. 1% Triton X-100 (250 µl/well) was added to d-ODMs to release the internalized bacteria. Vigorous pipetting to generate bubbles was required to efficiently detach and break the eukaryotic cell membranes (**Figure 1G**).

Δ CRITICAL. The Triton X-100 step should not take longer than 30 minutes in order to avoid bacterial cell death.

Invasion Index

To be able to count cfu/ml, the bacterial suspension resulting from the Triton X-100 treatment was serially diluted in Ringer Solution (**Figure 1G**). Dilutions of 10^{-1} and 10^{-2}, as well as the non-diluted samples, were plated (25 µl) in LB agar plates (**Supplementary Table 9**) and incubated ON at 37°C.

♦ **TIP**. 120x120mm square plates were used to plate up to 4 different dilutions. Plating was performed with the pipette-tip itself immediately after inoculation. The inoculum was streaked homogeneously through the plate-section (**Figure 1H**).

Δ CRITICAL. For a homogeneous mixture of bacterial dilutions, vortexing solutions is highly recommended.

Grown colonies in each dilution were only taken into consideration when the counting was between 15 - 150 (**Figure 1I**).

Intracellular bacteria

$$= \frac{\Sigma\ colonies}{(0.025\ \times\ (n_1 + 0.1\ \ x\ \ n_2)\ \times\ DF)}\ \times\ well\ \ volume\ \ (0.25)$$

$$= cfu/well$$

n_1 = number of plates at the more concentrated dilution
n_2 = number of plates at the less concentrated dilution
DF = dilution factor of the more concentrated dilution

Once the number of cfu/well was obtained, the invasion index (%) was calculated considering the amount of bacteria initially inoculated to d-ODMs:

$$Invasion\ \ Index = \frac{Intracellular\ \ bacteria}{Inoculated\ \ Bacteria\ \dagger}\ \times\ 100 = \%$$

†: in this context, $3.6x10^6$ for MOI 20 or $18\ x10^6$ for MOI 100.

TABLE 1 | Adjustment of the added bacterial-culture volume to the d-ODM culture depending on the tested MOI.

MOI 20	MOI 100
Number of d-ODM-cells: 180,000	Number of d-ODM-cells: 180,000
Final cfu/ml (fC): 3,600,000	Final cfu/ml (fC): 18,000,000
Final volume/well (fV): 500 µl	Final volume/well (fV): 500 µl
Initial cfu/ml (iC): $1.6x10^8$	Initial cfu/ml (iC): $1.6x10^8$
Added volume ($_{add}$V): **11.25 µl**	Added volume ($_{add}$V): **56.25 µl**

As previously described by Darfeuille-Michaud et al., who studied AIEC infection by using immortalized cell lines (6), we considered a strain to be invasive when the Invasion Index was > 0.1%

Fluorescent Cyto-staining and CellTox Green Cytotoxicity Assay

Notice that even though the techniques detailed herein are not mandatory, they were performed to obtain a deeper understanding of the results obtained from the AIEC infection of d-ODM (see *Anticipated Results* section).

Fluorescent Cyto-Staining

To visualize the bacterial internalization, LF82- and K12-infected monolayer cultures (at 5 hours of infection followed by 1 hour of gentamicin treatment (5 + 1) and MOI 100) seeded in µ-Slide 8 Well ibiTreat chambers, were processed for fluorescent cyto-staining. This procedure was identical to that used for ODM/d-ODM characterization until step (7) of the Immunofluorescence Staining section. After incubation with the blocking solution, 150-200 µl/well of Phalloidin diluted 1:40 in 1% BSA was added for staining of the actin filaments. After 1-hour incubation at RT, cells were washed 3x with DPBS at RT as in step 6 (see *Immunofluorescence Staining* section). DAPI (250 µl/well) was then added and the protocol continued as described in steps 10 and 11. The assay was performed with cells obtained from 3 different subjects (**Supplementary Table 1 Group 3**).

CellTox Green Cytotoxicity Assay

The protocol for d-ODMs cytotoxicity assessment corresponded to that recommended by the manufacturer. Briefly, after the infection assay, infected and non-infected d-ODMs were incubated with the CellTox reagent (1:1, 150 µl DIFF-MM + 150 µl CellTox) previously diluted according to the manufacturer's instructions (1:500 in Assay Buffer). After ≥15 minutes of incubation at 37°C in the dark, cultures were observed using a fluorescence microscope. A positive control of cell death was included by adding 100 µg/ml of digitonin in the uninfected d-ODM for 1 hour.

Data and Statistical Analysis

Quantitative data are expressed as the standard error of the mean (SEM). A paired t-test was performed to examine statistically different expression patterns between 2 groups, and a 2-way ANOVA test to examine statistical significance in multiple group data sets, followed by a Tukey test correction for multiple testing. A p-value of <0.05 was considered statistically significant. Data were analyzed using Graphpad Prism 8 (version 8.2.1).

ANTICIPATED RESULTS

Establishment of Differentiated Human Intestinal Epithelial Monolayer Cultures

The intestinal crypt is organized so that the stem-cell compartment resides at the bottom, thereby protected from the luminal content, while the differentiated and surface epithelium is more directly in

contact with the microbiota and its metabolites. In order to develop a model that would more closely resemble the type of upper crypt epithelium that is more susceptible to bacterial interactions and based on previous results from our lab (48, 49), we used a monolayer of differentiated epithelial cells derived from epithelial organoid cultures (d-ODMs).

First, we aimed to determine the optimal culture conditions for the ODMs to acquire a differentiated phenotype while reaching an appropriate confluence (100%) for the AIEC invasion assay. Based on previous experiments by our lab, we seeded $2x10^5$ single cells/well. On day 1, cells created clusters that alternated with empty areas, while on day 3, the monolayers reached 100% confluence, the requirement for AIEC infection (**Figure 2A**). Under these conditions, cells were collected and counted, obtaining an average of approximately $1.8x10^5$ cells/well (**Supplementary Figure 1**). Once the d-ODM number of cells at ~100% confluency was determined, we confirmed the differentiated phenotype of the monolayer by measuring key genes and proteins whose expression changes dramatically upon epithelial stem cell differentiation (38, 50).

As shown in **Figure 2B**, mRNA levels of *AXIN2*, *MYC* and *MKI67*, (the first, marker of stemness and the two last, markers of proliferation), were significantly higher in ODMs compared to d-ODMs. On the other hand, transcriptional levels of the differentiation markers *TFF3* and *MUC2*, showed an up-regulation, despite not statistically significant, in d-ODMs compared to ODMs. Similarly, *TJP3*, representative marker of epithelial cell junctions, was significantly up-regulated in d-ODM. Other markers included in the analysis (**Supplementary Figure 3**) confirmed the differentiated phenotype of the d-ODM culture (48).

Although using transcriptional analysis to easily screen cultures for their differentiation status – or other phenotypic features – is valuable, protein staining of the intact 2D cultures would help evaluate not just protein expression but also localization within the cell monolayer.

As an example, here we determined the protein expression of KI67, MUC2 and Villin by immunofluorescence. **Figure 2C** and **Supplementary Figure 4A** show representative images from 3 independent experiments. In agreement with the differentiated phenotype achieved in d-ODMs, KI67 was markedly decreased while MUC2 and Villin were increased compared to ODMs. These results were confirmed by fluorescence quantification analysis (**Figure 2D** and **Supplementary Figure 4B**).

Finally, to prove that the 2D culture exhibited an appropriate cell polarization, orthogonal views of MUC2 and Villin were analyzed (**Supplementary Figure 5**), showing a marked up-regulation of these two differentiation markers at the apical side of the d-ODM.

Altogether, both approaches demonstrated that primary cells derived from human EpOCs can establish a stable monolayer that preserves the intestinal identity thus mimicking the tissue of origin. Moreover, we achieved a differentiated and polarized phenotype in the d-ODMs at optimal confluence for the AIEC-infection study.

AIECs Can Invade d-ODMs

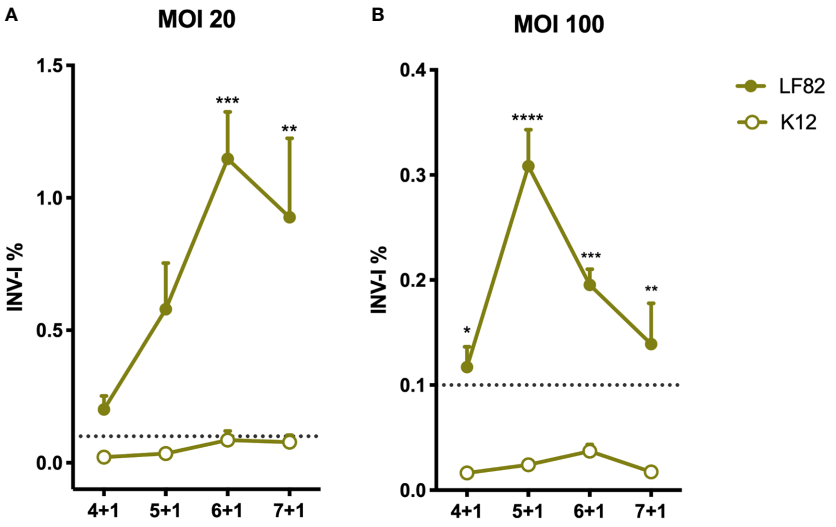
To the date, the characteristics and pathogenicity of AIECs have been studied so far by employing immortalized cell lines (36). Here, we studied the capability of AIECs to interact and invade a primary intestinal monolayer culture. First, we designed a kinetics infection assay to determine the time course of bacterial entry and/or intracellular survival in our culture system. To verify the strains' invasiveness capacity, I407 cell line was used as the reference method of the gentamicin protection assay. Both invasion assays (d-ODM and I407 infection) were carried out in parallel; thus, the *E. coli* ON cultures used for their infection were the same for each experiment performed. Results represented in **Supplementary Figure 6** show an INV-I% in I407 cells of 0.99 ± 0.225 and 0.0025 ± 0.00094 for the LF82 and K12 strains, respectively. These results were in agreement with previously published data (6, 46). Therefore, we conducted an infection-kinetics study to examine AIEC-d-ODMs infection by determining the percentage of internalized bacteria every hour for 7 hours of infection followed by 1 hour of gentamicin treatment as detailed in the previous sections. The assessed MOIs were 20 and 100. As shown in **Figures 3A, B**, we could quantitatively prove that the AIEC LF82 strain was able to invade d-ODMs, while the non-invasive *E. coli* strain (K12) showed an invasion index (INV-I%) below the established background (<0.1%). Moreover, LF82 showed a time-dependent increment of the INV-I%, and thus of the invasion capacity and/or intracellular multiplication in the AIEC-reference strain. Nevertheless, this capability was significantly higher compared to the K12 strain, both at 6 and 7 hours after infection for MOI 20 (**Figure 3A**) and at all time points for MOI 100 (**Figure 3B**). In fact, 5 hours of infection followed by 1 hour of gentamicin treatment at MOI 100 showed the greatest difference; the LF82 INV-I% measured almost 13 times greater than the K12 INV-I%. This occurred despite the fact that all INV-I % were lower at MOI 100 than at MOI 20. Furthermore, working with a greater number of bacteria/cell (higher MOI) ensured a remarkable reproducibility over time with highly consistent numbers of internalized bacteria in every experiment performed (**Figure 3C**). Nevertheless, this does not ensure higher INV-I%; in fact, this proved to be higher when the MOI was lower, as shown in **Figures 3A, B**. Maintenance of the d-ODMs cells' viability throughout all of the timepoints was observed *via* the CellTox Green assay (data not shown).

By staining the eukaryotic actin filaments (**Figure 4**), we confirmed the presence of high amounts of intracellular LF82 bacteria in the majority of those cells that formed the d-ODMs compared to the K12 strain.

In summary, we demonstrated the capacity of AIECs to invade the epithelial cells of d-ODMs. Thus, we present here a method that can be applied in multiple AIEC-IEC cross-talk studies, not only to discover new AIEC pathogenic mechanisms and host implicated molecules, but also, and more relevantly, to establish a possible starting point for further clinically oriented applications.

ADVANTAGES AND DISADVANTAGES

In the following section we will highlight which, in our opinion, are the most noteworthy advantages and disadvantages that this protocol presents. By doing so, we can focus on its practicality and try to overcome its limitations.



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		K12		LF82		
		Mean	SEM (±)	Mean	SEM (±)	Adjusted p-value
MOI 20	4+1	0.021	0.0028	0.2012	0.0510	0.9824
	5+1	0.0346	0.0114	0.5794	0.1742	0.1401
	6+1	0.0856	0.0345	1.1468	0.1773	**0.0002**
	7+1	0.0782	0.026	0.9266	0.2980	**0.0033**
MOI 100	4+1	0.0164	0.0048	0.1172	0.0193	**0.0300**
	5+1	0.0242	0.0017	0.3084	0.0348	**<0.0001**
	6+1	0.0372	0.0063	0.1954	0.0148	**0.0001**
	7+1	0.0174	0.0049	0.139	0.0388	**0.0048**

FIGURE 3 | Graphic representation of *E. coli* LF82 and K12 invasion indexes on d-ODMs. INV-I% of both *E. coli* strains (n = 5 for each represented point in the graph) at MOI 20 **(A)** and 100 **(B)** relative to the increasing infection time points. The dashed line represents the established threshold (0,1) over which *E. coli* strains were considered to be invasive. The error bars correspond to the SEM. **(C)** Mean, SEM and adjusted p-values obtained by a 2-way ANOVA test to examine statistical significance between LF82 and K12 INV-I% for each infection timepoint. This analysis was followed by a Tukey test correction for multiple testing. A P value of <0.05 was considered statistically significant, and it is highlighted in bold.

Advantages

- Working with samples isolated from their natural surroundings (the human intestine in this case), preserves the cytoarchitecture and most of the intercellular connections and interactions. Moreover, it also provides the option to consider the interindividual variability that exists between different subjects.
- Working with ODMs and d-ODMs offers accessibility to the IECs-apical side, contrary to 3D-organoid structures which may be required for infectious models.
- We also demonstrate its great reproducibility, a highly relevant feature when one considers the differences between individuals and their responses to microbes.
- Given the fact that ODMs and d-ODMs can be generated from potentially any individual, including patients suffering from IBD, this method offers the possibility of testing personalized treatment approaches.

Disadvantages

- Time-consuming. EpOCs and ODM cultures are time intensive. Nonetheless, once the system is set up, organoid-derived single cells can be more rapidly obtained, shortening the time required for the entire procedure.
- Costly. EpOCs and ODM cultures could remain unaffordable for some research groups due to the high costliness of most of the reagents that are required.
- Access to patient specimens is required.
- Sample-to-sample variability might lead to differences in the number of cells obtained from every EpOCs drop. This might

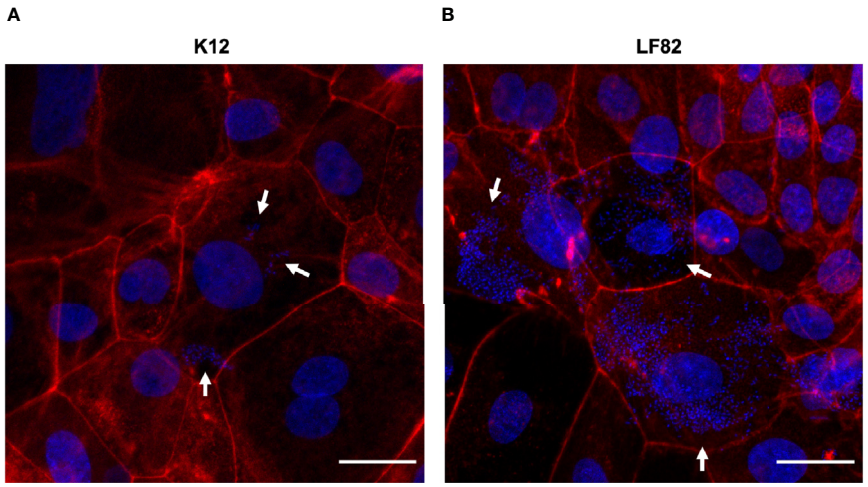

FIGURE 4 | *E. coli* LF82 and K12 invasion of d-ODMs as determined by the gentamicin protection assay. Phalloidin staining was performed to visualize the non-invasive control strain K12 **(A)** and the invasive LF82 **(B)** in d-ODMs after 5 hours of infection and 1 hour of gentamicin treatment at MOI 100. Phalloidin marked the eukaryotic actin filaments while DAPI bound to the DNA of both epithelial and bacterial cells. White arrows show bacterial localization inside the IECs. Scale bars: 25 µm. Images are representative of n = 3 independent experiments performed with samples from two different donors.

be an important limiting aspect that should be considered when applying the method described here.

DISCUSSION

In this manuscript we describe the steps required to develop a novel and reproducible human intestinal epithelial model for the study of enteric bacterial infections, particularly AIEC-related infections. Our model takes into consideration the variability of human biological responses to any pathogens, something that other models based on the use of cell lines cannot fully address (41, 51). Indeed, one of the main advantages of working with *ex vivo* primary cultures (as we mentioned in the previous section) is that these might offer a more physiological view of the host's response to AIEC infections. However, unpredictable biological variability could hinder the obtainment of the necessary cell concentration at the starting point. In that context, establishing an accurate and standardized protocol is crucial to facilitating reproducibility and enabling results comparisons. In our case, reproducibility was assessed first, by testing the gene and protein expression levels of the 2D cultures derived from the different donors. Moreover, AIEC infections were carried out in duplicate, exposing those EpOCs-derived d-ODMs from seven different individuals to *E. coli*. This validation approach is of great importance in host-pathogen interaction studies, considering the real differences in infection susceptibility among individuals and the divergence in host responses to a pathogen (39).

While a more extensive characterization of the d-ODM at protein level would add robustness to our culture system, our results suggest that ODMs and d-ODMs preserve the characteristics of the intestinal epithelium *in vivo*, resembling cells at the base and top of colonic crypts, respectively. Determining the number of cells that form the monolayer at the time of infection is a crucial step to better adjusting the working conditions in order to (1) achieve the optimal differentiated phenotype of the monolayer cultures, and (2) to properly adjust the number of exposed bacterial cells to the d-ODMs (MOI), which can greatly affect the results.

AIEC infection of d-ODMs was performed at different time points to analyze and select the best condition for achieving high reproducibility of infection and maximum specificity (lowest infection by non-invasive *E. coli*). Over time, increasing amounts of invasive bacteria were detected, with higher values evident when smaller amounts of bacteria (MOI 20) were added to the culture at the starting point. Based on this finding, we concluded that adding more bacteria does not directly correlate with higher invasion values. Similar results were obtained by Boudeau et al. in 1999 with Hep-2 cells (46). A 5-fold increase in the inoculum only represented an increase of 2.06 ± 0.7–fold (mean value of the fold-change increase for each timepoint) in intracellular bacteria. As d-ODMs cells were verified as viable with the CellTox Green assay, differences in the invasion indices were related to the initial inoculum. We believe that the d-ODMs can harbor a limited number of intracellular bacteria and, therefore, upon a given quantity of initial inoculum the invasion index will be lower. Even so, working with higher bacterial loads ensures a remarkable reproducibility of the results. This observation is not only valid for the invasive LF82 strain but also for the non-invasive control, K12.

Another observation concerns the dramatic decrease in the invasion index at the longest time of infection on LF82 INV-I% for both MOI 20 and 100. Other authors have similarly reported a decrease in the intracellular bacteria 4 hours after infection in mouse embryonic fibroblasts and HeLa, Hep-2 and I407 cell lines (52). Initially, we hypothesized that this event might be a consequence of eukaryotic cell death due to the bacterial infection process. Based to this assumption, when the initial

bacterial load was higher (MOI 100), eukaryotic cells would have begun dying at earlier time points. Nonetheless, using the CellTox Green assay we observed that infected cells viability was maintained over time (data not shown). Although AIECs are capable of evading IECs and macrophage-related stress responses in order to eliminate intracellular pathogens (6, 7, 46), decreases in the intracellular bacteria could reflect the capacity of IECs to restrict AIEC replication after a certain infection period (52). Testing the intracellular-bacteria viability at each time point would help confirm our hypothesis. It would also be interesting to determine, using this model, the presence of intracellular AIEC cells with a persistent phenotype; i.e. viable bacteria in a non-replicating state (53).

Similar strategies have been applied to study the interaction between AIEC, or other enteric pathogens and *E. coli* pathotypes, and human isolated IECs (23, 37–39, 41) and there is a recent and relevant publication in which organoid-derived 2D cultures are infected with AIEC (42). Nonetheless, this report does not include a detailed description of the steps taken to optimize infection efficacy. In contrast, our focus was to describe the steps required to obtain optimal ODM from EpOCs, that can be used as a model of primary epithelial cell infection with different *E. coli* strains. In particular, we go over the optimized steps from cell counting prior to infection to ODMs differentiation, and from infection kinetics to MOI testing. To the best of our knowledge, this is the first publicly accessible protocol that demonstrates the capacity of AIEC, compared to a non-invasive strain, to infect human primary IECs in a 2D configuration. Nonetheless, in our study we did not evaluate the impact of AIEC infection on epithelial cells including expression of bacterial sensing molecules, tight junctions, or immune response secreted proteins (54, 55). Such studies deserve further attention and will help elucidate how the epithelium differentially responds to invasive compared to non-invasive *E. coli*.

In conclusion, we can report the successful development of a human primary organoid-derived epithelial monolayer model of infection. Further application of this model, such as growing the d-ODMs in transwell-chambers in order to co-culture monolayers with AIECs and other human intestinal cell types (56) or the generation of d-ODMs derived from IBD patients, might lead not only to the development of a more comprehensive approach for studying the interaction of AIECs with the human gut, but also to a better understanding of the pathophysiology underlying inflammatory intestinal disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethic Committee of the Hospital Clinic of Barcelona with the registration number HCB/2016/0546. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AM, ID, AS, and MM-M designed the study. AM designed and conducted the experiments, acquired and analyzed the data, performed the biostatistics analysis, and wrote the manuscript. ID, designed and conducted experiments. MM-P and ME collected samples and provided technical support. QB-R provided technical support. ER recruited patients and collected samples. ID, AS, and MM-M supervised the experiments, analyzed data, and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.646906/full#supplementary-material

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Microbial Metabolites, Postbiotics, and Intestinal Epithelial Function

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Chronic inflammatory disorders are rising worldwide. The implication of the microbiota in persistent inflammation has been studied for years, but a direct causal relationship has not yet been stablished. Intestinal epithelial cells (IECs) form a protective barrier against detrimental luminal components. Indeed, a decrease in epithelial integrity may trigger a severe inflammatory reaction due to the infiltration of potentially harmful molecules and microorganisms. Bacterial imbalance, more commonly known as dysbiosis, occurs during inflammation and several strategies have been proposed to counteract this condition. Probiotics have been widely used to positively alter the inherited microbial composition and recover a eubiotic status. Nevertheless, probiotics are thought to impair the return of the indigenous microbiome, and to aggravate inflammation in compromised patients. In contrast, postbiotics—bacterial-free metabolites secreted by probiotic strains—have been proposed as a better and safer strategy. Recent scientific studies that have demonstrated the immunomodulatory properties and epithelial protection of postbiotics are summarized in this review, with an emphasis on the available methods that are currently in use to better understand the role of postbiotics in health and nutrition.

1. Introduction

The gastrointestinal (GI) tract is a complex ecosystem, populated by large amounts of microorganisms including bacteria, fungi, archaea, protozoa, and viruses that constitute the intestinal microbiota. As a result of its close evolution with the intestinal environment, the intestinal microbiota has adopted a remarkable spectrum of vital functions for the host including nutrient absorption and digestion, fermentation of dietary fibers, generation of energy, synthesis of vitamins, and pathogen defense. Some of the roles exerted by the intestinal microbiota result from the effects of their metabolite production.[1–3] Perturbations such as dietary changes can alter the balance of live bacteria within the healthy intestine, as well as the type of metabolites that they produce.[4] This phenomenon is commonly referred to as dysbiosis and has been associated with many diseases including inflammatory bowel disease (IBD; comprising Crohn's disease (CD) and ulcerative colitis (UC)),[5] obesity,[6] diabetes,[7] autism,[8] depression,[9] and colorectal cancer (CRC).[10–12]

Given the fact that the GI tract is constantly exposed to foreign antigens, intestinal epithelial cells (IECs), which constitute the outmost layer of the intestinal mucosa, are considered to be the first line of defense, forming a protective wall against all the potentially harmful luminal components. Indeed, disruption of the epithelial barrier and increased epithelial permeability may lead to the development of the diseases mentioned above due to antigen infiltration. In addition to the physical barrier function, IECs also produce antimicrobial molecules and other defense mechanisms to keep at bay the microbial communities, to preserve their integrity and to prevent infiltration of the previously mentioned luminal antigens. Thus, IECs play a bidirectional role by both responding to microbial products, such as microbial metabolites, as well as by modulating microbial functions.[3]

Microbial metabolites are any molecule that is modified or synthesized by the microbiota. Microbial metabolites, or more specifically bacterial metabolites, are commonly classified into three different groups: A) metabolites produced by bacteria from dietary components, B) those produced by bacterial biochemical modification of host bioproducts, and C) those synthetized de novo by bacteria (**Table 1**).[1,2,4] In addition, microbial metabolites have also been differentiated by their elemental composition (proteins, polysaccharides, organic acids, lipids, lipoteichoic acids, peptidoglycan, etc.) or their bioactive effect (immunomodulatory, anti-inflammatory, antimicrobial, antioxidant, antiproliferative, etc.).[13]

The term postbiotics refers to any soluble factor resulting from the metabolic activity of a probiotic bacteria—living microorganisms which, when administered in adequate amounts, confer health benefits on the host[14–18]—or any bacterial-released molecule capable of providing health benefits through a direct or indirect mechanism. Most of the microbial metabolites included in the available classifications could be regarded as postbiotics. Nevertheless, what researchers conceive of as postbiotics are "the set of microbial molecules" rather than single metabolites. In fact, most of the published studies about postbiotics have not

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Table 1. Microbial metabolites produced by intestinal commensal bacteria.

Metabolite	Bacterial-producers	Source	Mechanisms of action	Effects on IECs	Refs.
Metabolites produced by gut bacteria from dietary components					
SCFA (acetate, propionate, and butyrate)	Acetate: Bacteroidetes (*Bacteroides* spp., *Prevotella* spp.) Propionate: Bacteroidetes and Firmicutes (*Veillonella* spp., *Dialister* spp., *Ruminococcus* spp.) Butyrate: Firmicutes (*Roseburia* spp., *Faecalibacterium prausnitzii*)	Fermentation of polysaccharides contained in dietary starches and fibers	Once in the lumen, binding to G protein-coupled receptors and induction of IL-1β and IL-18 production in cells. Intracellularly, SFCA can reduce cell proliferation by regulating transcription through inhibition of histone deacetylases and activation of histone acetyltransferases.	Anti-inflammatory properties. Reduction of pathogens growth while promoting expansion of beneficial microorganisms.	Morrison et al.[28] Louis et al.[30] Koh et al.[31] Bloemen et al.[32] Donohoe et al.[33,38] Ferrer-Picón et al.[47]
Indole derivatives	*Lactobacillus* spp., *Clostridium* spp., *Bacteroides* spp.	Dietary tryptophan catabolism	Binding to the AHR ligand promotes IL-22 production by innate lymphoid cells.	Reduction of the inflammation, IECs survival, proliferation and induction of antimicrobial peptides production which leads to intestinal homeostasis.	Wikoff et al.[56] Zelante et al.[59] Shi et al.[60] Qiu et al.[61] Melo-González et al.[62]
Polyamines (putrescine, spermidine and spermine)	*Bacteroides* spp., *Fusobacterium* spp., *Escherichia coli*	Arginine metabolism	Unclear. Block LPS-induced expression of pro-inflammatory cytokines in monocytes and macrophages.	Enhancement of IECs proliferation, cellular signaling, stress resistance. Boost tight junctions formation.	Kitada et al.[72] Rooks et al.[64] Zhu et al.[76] Zhang et al.[77] Haskó et al.[78]
Metabolites produced by the host and modified by gut bacteria					
Secondary bile acids	*L.monocytogenes*, *Bacteroides* spp., *Escherichia* spp., *Clostridium* spp., *Eubacterium* spp., *Lactobacillus* spp.	Transformation of host Primary Bile Acids	Binding to bile acid receptor and G protein-coupled bile acid receptor 1 triggers the reduction of pro-inflammatory cytokines.	Increase of IL-18 by free taurine helps IECs barrier repair and stability. Reduction of pro-inflammatory cytokines.	Martinez-Augustin et al.[81] Ridlon et al.[82,86] Wahlström et al.[84] Guo et al.[89] Cipriani et al.[91] Nijmeijer et al.[93]
Metabolites synthesized de novo by gut bacteria					
Bacterial polysaccharides	*B. fragilis* and *Clostridium* spp.	Mainly produced by *B. fragilis*	$CD4^+$ T cells activation through presentation of bacterial polysaccharides.	Induction of the anti-inflammatory cytokine IL-10 inhibits IL-17-producing cells. Protection of IECs from inflammation.	Mazmanian et al.[95] Dasgupta et al.[96] Cobb et al.[97] Atarashi et al.[98]
Vitamins (B and K)	Vitamin B: lactic acid bacteria and *Bifidobacterium* spp. Vitamin K: *E. coli*, *B. subtilis* and *M. philei*	De novo synthesized by lactic acid bacteria and other gut commensals	Act as cofactors of several enzymatic reactions to end up reducing cell stress.	Regulates energy generation as well as gene expression. Modulates intestinal immunity.	Kunisawa et al. LeBlanc et al.[105] Walther et al.[106] Yoshii et al.[103]
ATP	Not clear which species are the main producers of microbially-derived ATP.	Secreted by gut bacteria	Activation of ATP-receptors.	Serves as energy source and act on immune cells activity. Promotes intestinal homeostasis. Drives $CD4^+$ cell polarization toward T_H17 cells.	Iwase et al.[107] Mempin et al.[108] Faas et al.[109] Atarashi et al.[110] Ivanov et al.[111] Perruzza et al.[114]
Postbiotics	Any probiotic strain (*F. prausnitzii*, *S. thermophilus*, *E. coli* Nissle, *Bifidobacterium* spp., *Lactobacillus* spp. among many others)	Released by probiotic bacteria after fermentation.	Unknown.	Immunomodulatory, anti-inflammatory, hypocholesterolemic, anti-obesogenic, antihypertensive, antiproliferative, antimicrobial, antioxidant, anti-adherent, anti-invasive.	Tsilingiri et al.[15] Cicenia et al.[119] Shin et al.[120] Nakamura et al.[121] Gao et al.[122] Mileti et al.[123]

SCFA: Short chain fatty acids, AHR: Aryl hydrocarbon receptor, IECs: Intestinal epithelial cells, LPS: Lipopolysaccharide, ATP: adenosine triphosphate.

provided a description of the molecular composition underlying this "metabolite-cocktail".[19–23]

Postbiotics have recently been proposed as food supplements to promote intestinal homeostasis in lieu of probiotics, such as in cases involving inflammatory GI diseases, when the use of live bacteria may pose some risks to the patient due to the presence of microbe-associated molecular patterns (MAMPs) that potentially activate innate immunity and could further promote inflammation. In that sense, postbiotics are thought to mimic probiotics' beneficial-effects while avoiding the risks of administering live bacteria.[15]

In the following sections we will describe the different types of bacterial-derived metabolites known to date and how they can be obtained. Moreover, their function and effects on human gastrointestinal epithelial cells will also be discussed with a special focus on postbiotics' effects on in vitro, ex vivo, and in vivo models and their potential beneficial applications both in health and disease.

2. Types of Microbial-Derived Molecules, Sources, and Bioactivities

The relationship between the gut and the microbiota is considered a synergistic one. Gut microorganisms rely on host's metabolites to grow, while at the same time they produce small molecules that regulate their self-growth or enhance other species' development and protection. Moreover, some of these metabolites (secreted by live bacteria or after bacterial lysis) released into the host environment are known to have beneficial effects for the host. In addition, they have been proposed as potential surrogate markers of disease exacerbation, as has already been proven in cardiovascular disease.[2,13,24]

As reviewed elsewhere,[1,2,4,25] a limited but diversified group of microbially-derived metabolites has been extensively studied over the past two decades. In the following sections we discuss the better characterized bacterial metabolites, emphasizing their source and bioactivity (Table 1).

2.1. Short Chain Fatty Acids

Of all microbial metabolites, short chain fatty acids (SCFA) have been the most exhaustively investigated. SCFA are saturated aliphatic organic compounds with a backbone of 1–6 carbons that are produced by bacterial fermentation of dietary products such as starch and fiber, plant-derived polysaccharides that cannot be digested by host enzymes.[26,27]

Acetate (C2, two carbons), propionate (C3), and butyrate (C4) are by far the most abundant SCFA in the human intestine.[28] Acetate is mainly produced by members of the Bacteroidetes phylum such as *Bacteroides* spp. or *Prevotella* spp., among others, via the Wood-Ljungdahl and acetyl-CoA pathways. Acetate can either be absorbed by IECs and used as substrate for butyrate production, or travel intact through the intestinal epithelium to the liver where it is released into the systemic circulation.[28–30]

Propionate is produced not only by Bacteroidetes, but also by Firmicutes members such as *Veillonella* spp., *Dialister* spp. or *Ruminococcus* spp., via the succinate, acrylate, and propanediol pathways. Propionate is mainly metabolized in the liver after absorption from the gut mucosa.[29,31,32]

Butyrate is, by far, the best studied SCFA. Members of the Firmicutes phylum are responsible for its synthesis generally via the butyryl-CoA:acetate CoA-transferase routes. Butyrate serves as the primary energy source for colonocytes and is basically metabolized within the intestinal mucosa, while what remains is finally degraded in the liver.[31,33]

SCFA in the GI tract act both extra- and intra-cellularly. From the outside, SCFA bind to the transmembrane G protein-coupled receptors, most of which have been reviewed by Postler et al. and Neumann et al. G protein-coupled receptors-SCFA complexes induce inflammasome activation and consequently interleukin (IL)-1β and IL-18 gene transcription. Both of them are important cytokines involved in maintaining gut homeostasis, specifically acting on epithelial barrier function and integrity as well as promoting healthy microbiome composition.[1,4]

Once in the intracellular compartment, propionate and butyrate regulate transcription by inhibiting nuclear class I histone deacetylases and activating histone acetyltransferases. As a result, propionate, and to a greater extent butyrate, can induce cell death and reduce proliferation of abnormal cells such as cancer cells.[34–36] On the other hand, healthy cells are able to metabolize butyrate and instead use it as a primary energy source, thereby preventing its accumulation and inhibition of histone deacetylases.[37,38]

Most of the studies related to SCFA, particularly those concerning butyrate, have demonstrated its anti-inflammatory properties on murine models of intestinal inflammation.[39–41] Alterations in stool SCFA content have been reported in IBD[42–44] and CRC patients[45,46] compared to healthy subjects. Nonetheless, a recent study from our group showed that despite having decreased butyrate-producing bacteria in feces, IBD patients presented no changes in the concentration of butyrate or any other SCFA in their stools.[47] We suggested that this was related to an inflammation-induced alteration in epithelial butyrate transport and metabolism. Nevertheless, it has also been demonstrated that measurements of SCFA fecal content do not precisely reflect the concentration of these metabolites in the gut, since only around 5% of it is estimated to be found in feces after consumption by colonic epithelium.

Besides their anti-inflammatory effects, SCFA can also reduce Enterobacteriaceae (*Escherichia* spp., *Salmonella* spp.) growth while promoting Firmicutes expansion.[25] Hence, due to their capacity to regulate gut microbial composition and balance colonic pH, SCFA—mainly butyrate—have been proposed as dietary supplements as well as a preventive or therapeutic option in intestinal diseases such as IBD or CRC.[45,48,49] Nonetheless, tumor necrosis factor (TNF)α was shown to impair butyrate transport and metabolism,[47] suggesting that supplementation of active patients with butyrate or SCFA extracts may not provide any beneficial effects as long as inflammation persists.[50] This effect appears to be reversed when treating patients with anti-inflammatory biological drugs (i.e., anti-TNFα).[47] Furthermore, another recent study using an epithelial primary cell monolayer showed that butyrate synergized with TNFα and interferon (INF) ɣ to induce increased IL-8 transcription and production of inflammatory proteins by the epithelium.[51]

2.2. Indole Derivatives

Beyond SCFA, gut bacteria produce many other immunologically active metabolites from ingested food.[2] Dietary tryptophan is an essential amino acid whose levels have been reported to decrease in IBD patients' serum and plasma compared to healthy controls, while levels in stool have been shown to increase in these patients.[52–55] Tryptophan is degraded by bacterial enzymes to obtain indole derivatives which can subsequentially act as ligands for the aryl hydrocarbon receptor (AHR) in host cells.[56] Indole derivatives are not the only ligands for AHR; other diet components such as flavones, isoflavones, flavanones, carotenoids, or indole-3-carbinol and indole-3-acetronile are also AHR agonists.[57,58] AHR plays an essential role in the gut mucosa, where *Lactobacillus* spp., *Clostridium* spp., or *Bacteroides* spp. catabolize tryptophan and its derivatives to obtain AHR ligands that will impact on the microbial composition by inducing the expansion of protective bacteria (*Lactobacillus reuteri*) while suppressing the growth of pathogenic microorganisms. In fact, lack of endogenous AHR ligands in mice provides a growth advantage and niche colonization by tryptophan metabolizing-bacteria; the resulting indole derivatives can then compensate for the absence of inner-produced AHR ligands.[59–61]

In addition, production of IL-22 by innate lymphoid cells is induced after activation of AHR signaling. This cytokine, which has been involved in certain autoimmune diseases, also plays a protective role by driving epithelial cell survival and proliferation, as well as synthesis of antimicrobial peptides.[62] As a result, microbial-produced AHR ligands promote intestinal homeostasis and limit inflammation by protecting mucosal barrier functions.

Indeed, AHR deficiency results in a higher susceptibility to *Candida albicans*, *Citrobacter rodentium*, or *Listeria monocytogenes* infection.[60,63]

2.3. Polyamines

Arginine is also an amino acid metabolized by gut bacteria to generate immunomodulatory metabolites: polyamines, small polycationic molecules derived from food or synthesized by the intestinal microbiota (via amino acid decarboxylase enzymes) or mammalian cells (with ornithine decarboxylase being the limiting enzyme).[64] Putrescine (N2, diamine), spermidine (N3), and spermine (N4) are the major polyamines produced within the human GI tract. In fact, polyamine levels in the urine and serum of CRC patients are higher than those of healthy individuals, suggesting its possible application as a biomarker for early diagnosis.[65,66] Similarly, spermidine and spermine levels in the colonic mucosa of CRC individuals are threefold higher compared to normal colonic mucosa.[67–69] *Bacteroides* spp., *Fusobacterium* spp., and *Escherichia coli* are the main bacteria responsible for arginine metabolization to polyamines.[70–72] The latter are crucial for an optimal function of the intestinal epithelium including cell proliferation, cellular signaling, stress resistance, or RNA and protein synthesis.[73] Moreover, polyamines boost bacterial longevity.[74,75]

Together with other polyamines, spermine exerts anti-inflammatory effects by regulating cells of the innate immune system.[76] Spermine blocks lipopolysaccharide (LPS)-induced expression of pro-inflammatory cytokines in macrophages and monocytes.[77] IL-10 production is increased while INFγ expression is reduced, as demonstrated in several in vivo studies.[78] On the other hand, it has been shown that spermine reduces IL-18 secretion, a cytokine involved in epithelial repair and barrier stability. This is counterbalanced by taurine, another microbial metabolite described below.[79]

In addition, polyamines can contribute to the formation of tight junctions by promoting E-cadherin transcription, a cell-to-cell adhesion molecule. Spermine, spermidine, and putrescine have been reported to be increased during intestinal mucosal repair in vitro, while its absence is related to reduction in IECs proliferation and migration to the site of injury.[80]

Unfortunately, little is understood about the molecular mechanisms by which polyamines function.

2.4. Secondary Bile Acids

The gut microbiota not only synthesize metabolites from dietary compounds, but also use metabolic products secreted by host cells into the intestinal lumen as substrate. This is true of bile acids, which are released into the duodenum by the gall bladder to help internalize lipidic molecules thanks to the hydrophilic and hydrophobic hemispheres that they contain, which associate with dietary fats forming micelles.[81] Primary bile acids (PBA) (cholic acid and chenodeoxycholic acid) are synthesized in the liver from cholesterol and conjugated with taurine or glycine before being secreted via bile into the intestine.[82] Cholesterol-derived bile acids can be returned to the liver or directed to the small and large intestines where they are either absorbed or converted into secondary bile acids (SBA) by bacteria.[83] Various reactions are involved in this process: deconjugation of glycine or taurine, oxidation and epimerization, dehydroxylation or esterification, among others. These result in the formation of 16–20 different SBA.[84,85] Several different bacterial species are known to be responsible for these reactions. *Bacteroides fragilis*, *Bacteroides vulgatus*, and *Listeria monocytogenes* are some of the most common species facilitating deconjugation; oxidation and epimerization are driven by intestinal Firmicutes, *Bacteroides* spp., and *Escherichia* spp.; dihydroxylation occurs after deconjugation and is catalyzed by Firmicutes phylum members such as *Clostridium* spp. and *Eubacterium* spp.; and esterification is performed by *Bacteroides* spp., *Eubacterium* spp., and *Lactobacillus* spp.[86,87]

As mentioned above, free taurine (obtained after PBA deconjugation) is able to increase IL-18 production via inflammasome activation, thus helping epithelial barrier function, similar to SCFA.[79]

Other immunomodulatory effects of both PBA and SBA have also been described. Both are capable of reducing the expression of pro-inflammatory cytokines secreted by several immune cells through, at least, two receptors: the bile acid receptor and the G protein-coupled bile acid receptor 1; in fact, a significant reduction in bile acid transporters and consequently, a decrease in bile-acids levels in stool samples has been described in CD disease patients.[88–90] Nevertheless, some reports have suggested that bile acids have some possibly harmful effects in the context of inflammatory disorders in the gut such as irritable bowel

syndrome (IBS) where bile acids malabsorption (detected as a variation of its concentration in serum) can result in diarrhea.[91,92] Imbalances in the amount of PBA and SBA can result from the well-known dysbiosis in IBD patients. Hence, impaired bile acid metabolism may enhance the inflammatory response, thus worsening IBD. Some authors have shown the potential damage that bile acids can exert on bacterial membranes and DNA, potentially leading to an imbalanced microbial composition in IBD.[93,94] Either way, the physiological consequences of bile acids for bacteria, and vice versa, as well as their effects in the context of inflammatory diseases, require further study.

2.5. Bacterial Polysaccharides, Vitamins, and Adenosine Triphosphate

Gut microbes are also able to synthesize and secrete de novo molecules. The most studied ones are bacterial polysaccharides, vitamins, and adenosine triphosphate (ATP).

2.5.1. Bacterial Polysaccharides

Polysaccharides derived from commensal bacteria positively contribute to intestinal health. Polysaccharide A (PSA), produced by *Bacteroides fragilis*, is the most commonly studied bacterial polysaccharide due to its important immunomodulatory properties. Deeply discussed by Postler et al., the main immune effect of PSA is the induction of IL-10 secretion by immune cells.[4] IL-10 is an anti-inflammatory cytokine that inhibits the activity of IL-17- and INFγ-producing cells (T_H17 and T_H1, respectively), protecting IECs from exacerbated inflammation (as occurs in colitis).[95,96] Moreover, bacterial polysaccharides are essential for efficient gut colonization by intestinal commensal bacteria.[97,98]

Jiang et al. have recently demonstrated that not only immune cells, but also enterocytes, are influenced by PSA. They published a study on human fetal enterocytes in which they elucidated the role of PSA in inhibiting IL-1β-induced inflammation (while IL-1β, as mentioned above, exerts a key protective role in the epithelium, when secreted at higher doses acts as a potent pro-inflammatory cytokine[99,100]) by lowering pro-inflammatory IL-8 levels. This could prove of great value in diminishing colitis in premature infants.[101]

2.5.2. Vitamins

Since humans are not able to produce vitamins, they fully depend on diet or bacteria to access them. Gut bacteria are able to de novo synthesize vitamin K and produce several B-group vitamins (mainly B12, but also B2, B6, and B9), which are either absorbed or converted to their derivatives in the intestinal epithelium to later be released into the blood. Even though IECs mainly act as transporters of the bacterial-produced vitamins, these metabolites can also have beneficial effects on intestinal cells such as boosting epithelial barrier function or gut immunosurveillance. Indeed, vitamin B affects the metabolism of the lipid mediator sphingosine 1-phosphate, which controls lymphocyte trafficking into the intestine. In particular intraepithelial lymphocytes (located between gut epithelial cells) help protect against pathogens. By this manner, vitamins demonstrate their contribution in maintaining the intestinal homeostasis.[102,103] Vitamin B has been shown to act as a cancer-preventing metabolite, a property that has been attributed to this group of vitamins due to their role in energy generation and gene regulation, as well as in intestinal immunity modulation. In particular, low vitamin B intake has been associated with an increased IL-10/IL-12 ratio and an exacerbation of clinical symptoms in patients suffering from IBS.[104] The most studied vitamin-producing bacteria are lactic acid bacteria and other gut commensals such as *Bifidobacterium* spp. Some researchers have studied the genome and transcriptome of these groups of bacteria to elucidate the complex mechanisms and pathways followed to finally obtain B vitamins.[105,106] The same happens with vitamin K2 (also known as menaquinone), which is part of lactic acid bacterial membranes and protects them from oxidation. Nevertheless, its synthesis has mostly been ascribed to *E. coli*, *Bacillus subtilis*, and *Mycobacterium philei*, and its absorption mainly occurs at the terminal ileum and distal colon. Menaquinone is being studied for its potential beneficial effects on bone and cardiovascular health. Its structure, sources, and the bacterial metabolic pathways for its synthesis are well described by Walther et al.[106]

2.5.3. Adenosine Triphosphate

As is well known, ATP is synthesized by bacteria and eukaryotic cells. Although its main role is to serve as an energy source, it has also been shown to affect immune cell activity when secreted into the extracellular space.[107,108] Contrary to all the microbial-derived metabolites discussed thus far, ATP displays pro-inflammatory properties. Necrotic or stressed cells can release ATP during inflammatory processes. Among its many other effects on the immune system, bacterial-released ATP not only drives $CD4^+$ T cells polarization toward T_H17 cells, but also limits the production of protective immunoglobulin A (IgA) by plasma cells. This phenomenon has been linked to a higher susceptibility to develop colitis or to exacerbate it in colitis-mouse models.[109,110] It is not yet clear which bacterial species are the main producers of microbial-derived ATP. Although segmented filamentous bacteria induce T_H17 cells in the ileal lamina propria in mice, no evidence of ATP production by these bacteria has been reported. The capacity of other T_H17-inducing intestinal bacteria to produce ATP remains to be elucidated, but they all share the ability to adhere to the intestinal epithelium.[111–113] On the other hand, some authors have demonstrated that microbially derived ATP promotes a healthy microbial-immune crosstalk and intestinal homeostasis.[114–116]

Finally, adenosine obtained after ATP-hydrolyzation has been studied for its immunosuppressive properties in the tumor environment.[117] Thus, numerous questions surrounding ATP need answering. This is also true of bacterial produced ATP and its immunomodulatory properties.

2.6. Postbiotics

Thus far we have described the effects that individual metabolites produced by different bacteria can have on the intestinal

epithelium. Nevertheless, bacteria live in complex communities and IECs are constantly exposed to the sum of all gut bacteria-derived metabolites, rather than a specific strain or single metabolite. Both independently and collectively, microbial metabolites shape the immune system promoting, in the case of probiotic-derived metabolites, mainly beneficial effects.[13,118] The metabolic cocktail composed of soluble factors secreted by life probiotic bacteria has been collectively known as postbiotics since 2012.[15] Nevertheless, they have also been referred to as metabiotics, biogenics, or cell-free supernatants.[10,119]

Different approaches could be employed to define "types" of postbiotics; for example, by their physiological function or effect (immunomodulatory, anti-inflammatory, hypocholesterolemic, antiobesogenic, antihypertensive, antiproliferative, antibiotic, antioxidant); by their components (if they are mainly composed of proteins, lipids, carbohydrates, organic acids, etc.); or by their bacterial sources (*Lactobacillus* spp., *Streptococcus* spp., *Bifidobacterium* spp., *Escherichia* spp., or *Faecalibacterium prausnitzii,* among many other probiotic strains).[120,121] Given the fact that a postbiotic is composed of a complex mixture of metabolites derived from one or more bacterial strains, and is thus characterized by a wide variety of different effects, sources, and compositions, the classification of postbiotics as a whole remains a challenging task.

Postbiotics' production encompasses diverse techniques such as enzymatic treatment, sonication, heat application, centrifugation and filtration, dialysis, freeze-drying, or column purification. Once collected, identification of its components is not a trivial task and can require several analytical approaches. Some of the commonly employed techniques include matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, high performance liquid chromatography, high-field proton nuclear magnetic resonance spectroscopy, ultra-performance liquid chromatography, or liquid chromatography mass spectrometry.[2,13,122] Those are only some of the preferred options to elucidate postbiotics' composition. Choosing any of these procedures is a decision that will always depend on the need for quantitative or qualitative characterization, as well as the nature of the molecules to be identified.

Optimizing all the available methods to determine each postbiotic component will be essential in order to dissect their molecular mechanisms of action, which are currently unknown. Nonetheless, the effects of postbiotics on the intestinal epithelium are being intensively studied. Most commonly, postbiotics are tested in the presence of a strong inflammatory agent in order to elucidate their potency and mechanisms of action.[123]

In the next sections we will review the available scientific literature, examining the in vitro, ex vivo, or in vivo assays that have been used to explore these effects.

3. Study of Postbiotics' Effects in In Vitro Models of Intestinal Epithelial Cells

The most common way to test the effects of a metabolite or set of metabolites on IECs is by using cell monolayers (**Figure 1**A) that mimic the physiological conditions of study. The main human immortalized cell lines used for the study of postbiotics and its derived properties are discussed below.

3.1. HT-29

More than fifteen years ago Ménard et al. studied the anti-inflammatory properties of lactic acid bacteria-secreted metabolites after their interaction with the intestinal epithelium. They collected bacterial postbiotics (or, as they refer to it, bacterial conditioned medium) and applied them to a monolayer of the human colonic cancer cell line HT29-19A seeded on a transwell, a microporous filter separating the apical and the basolateral compartments. To confirm their assumption, they collected the basolateral medium after adding postbiotics from two different lactic acid bacteria (*Bifidobacterium breve* and *Streptococcus thermophilus*) at the apical side for 24 h. Peripheral blood mononuclear cells (PBMCs) were stimulated with LPS, a component of the Gram-negative bacterial wall that induces pro-inflammatory cytokine release in blood cells through interaction with the toll-like receptor (TLR)-4 on the cell membrane. After stimulation, PBMCs were treated with the previously collected basolateral medium hypothetically containing postbiotics. A reduction in TNFα release by LPS-stimulated PBMCs was detected, confirming that the inhibitory capacity of bacterial postbiotics during inflammation was retained after crossing the intestinal epithelial monolayer of the HT29-19A cell line.[124]

Postbiotics have also been studied for their cytotoxic and antiproliferative effects on epithelial cells.[19] Chuah et al. used a HT-29 monolayer to demonstrate the antiproliferative capacity of postbiotics (or postbiotic metabolites as they refer to them) derived from six different bacteriocin-producing *Lactobacillus plantarum* strains. The total number of HT-29 cells was reduced in a time-dose dependent manner when the six postbiotics were individually applied to the cell culture. To confirm this effect, they conducted a growth arrest study by trypan blue cell counting. On this occasion they only tested one of the postbiotics derived from *L. plantarum* and treated cells at 15% (v/v) and 30% (v/v) dilution. Again, the postbiotics exhibited time-and dose-dependent antiproliferative effects on HT-29 cells. In addition, other studies have reported the antiproliferative effects of lactic acid bacteria on CRC using cell lines,[125–129] as also reviewed by Konstantinov et al.[10]

Human immortalized cell lines were also used to study the capacity of postbiotics to block the binding of pathogenic bacteria. Mack et al. tested the capacity of the *L. plantarum* probiotic strain to inhibit pathogenic *E. coli* binding to HT-29 cells. An enterohaemorrhagic and an enteropathogenic *E. coli* (EHEC and EPEC, respectively) were chosen for that purpose. They demonstrated the capacity of the probiotic strain to reduce the adherence of both EHEC and EPEC to the tested intestinal cells. Interestingly, they repeated the experiment (only with EPEC) but using *L. plantarum* supernatant instead; EPEC was unable to bind to HT-29 cells when the microbial postbiotics were applied undiluted or at a 1:10 dilution. Furthermore, they were able to demonstrate that some of the beneficial effects of the probiotic strains were derived from their secreted metabolites.[130,131]

3.2. Caco-2

E. coli does not always act as an intestinal pathogenic strain. *E. coli* Nissle 1917 (EcN) is a well-studied probiotic that has been

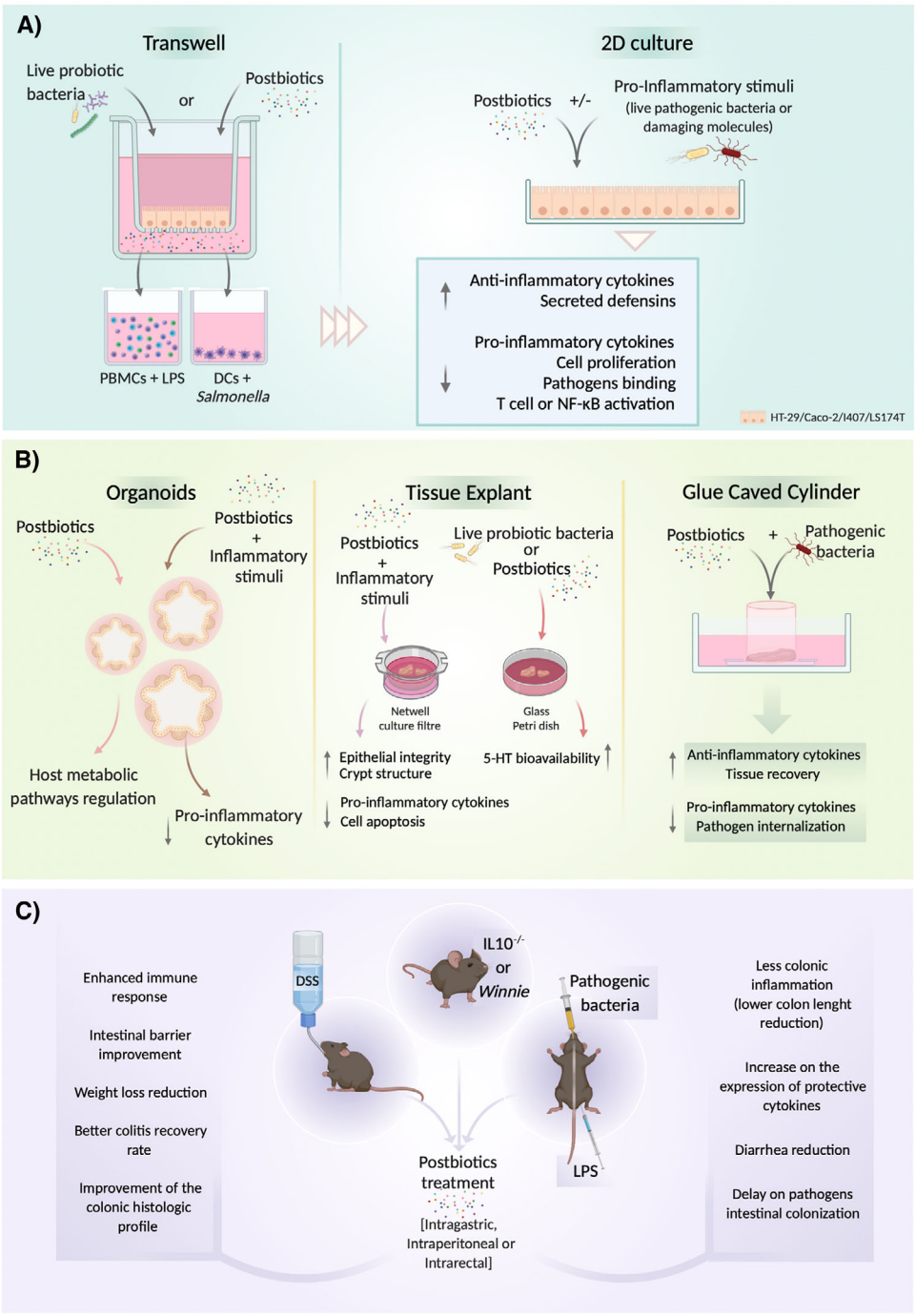

Figure 1. Schematic representation of the available models for the study of postbiotics effects in the intestine. A) In vitro models mainly consist of cell lines seeded as monlayers in Transwells (left) or culture plates (right). Transwells have been used to collect the basolateral supernatant after live probiotic bacteria or postbiotics are added to the top chamber. Collected supernatants can be then tested on stimulated immune cells: peripheral blood mononuclear cells (PBMCs) treated with lipopolysaccharide (LPS), or dendritic cells (DCs) cultured in the presence of a pathogen such as *Salmonella*. On the other hand, cells seeded in plates can be used to test postbiotics' properties in the absence or presence of a pro-inflammatory stimuli. Both in vitro approaches help in the initial screening of probiotic-derived molecules to determine their beneficial effects. B) To test postbiotics using ex vivo cultures, studies have used epithelial organoids, intestinal tissue explants, and the glue caved cylinder. By exposing the cultures to either pro-inflammatory stimuli, live probiotics, pathogenic bacteria, and/or postbiotics, the effects of the latter on mice or human samples that closer resemble the whole organ can be studied. C) In vivo experimental models of intestinal inflammation (e.g., the acute model of DSS-induced colitis, the spontaneous model developed by IL10$^{-/-}$ (knock out) or *Winnie* mice, or mice inoculated with pathogenic bacteria or LPS) have aided investigations of the potential therapeutic effects of postbiotics. Postbiotics have been administered intragastrically, -peritoneally or -rectally. Improvement in colitis recovery as well as enhancement of the immune response are some of the benefits that have been attributed to postbiotics thanks to these studies. 5-HT: Serotonin. This figure has been created with BioRender.[202]

used to maintain remission in UC patients.[132–138] In fact, some of the in vitro studies that we will discuss here, demonstrate that EcN blocks the adherence to IECs of several pathogenic strains by using different strategies.[139–141] Schlee et al. suggested a role for EcN soluble factors on the inhibition of pathogens adherence to IECs. Furthermore, they propose that this effect could be related to the capacity of EcN postbiotics to induce human β-defensin 2 (hBD-2) expression, a widely studied molecule with a strong antimicrobial activity. Therefore, hBD-2 production might prevent pathogen adhesion to epithelial cells. hBD-2 levels in Caco-2 enterocyte-like cell line were analyzed after adding EcN resuspended bacterial pellet (as also did Möndel et al.) or supernatant.[142] hBD-2 expression was significantly higher with the supernatant than when the bacterial pellet was cocultured with Caco-2, meaning that bacterial postbiotics are able to induce antimicrobial peptides secretion in IECs. Moreover, they demonstrated that EcN flagellin accounted for this effect since supernatants from flagellin mutants lost their capacity to induce hBD-2.

A different group of pathogenic *E. coli* are the adherent invasive *E. coli* (AIEC). As its name suggests, AIEC are able to adhere and invade IECs.[143–146] These pathogens have been extensively studied for their role in the pathogenesis of IBD.[147–151] LF82 is the AIEC reference strain, isolated from a chronic ileal lesion of a patient with CD.[152] Huebner et al. investigated the impact of EcN on LF82 infection of Caco-2 cells. EcN showed an inhibitory effect on invasion of the epithelial cell line by LF82, which was also similarly demonstrated by Boudeau et al. and He et al.[153,154] EcN also modified the cytokine production of Caco-2 cells after bacterial challenge with LF82. Indeed, mRNA levels of pro-inflammatory cytokines were decreased when cells were cocultured with EcN and LF82 compared with infection with LF82 alone. Unfortunately, no assays were performed with the bacterial supernatant of EcN, so no evidence shows the implication of its metabolites on AIEC adherence and invasion inhibition.

Similar to one of the mentioned studies, Mileti et al. incubated dendritic cells (DCs)—derived from human PBMCs—with supernatants derived from *Lactobacillus paracasei* treated Caco-2 cells. In that case, the objective was not assessing bacterial or postbiotics translocation but the study of the obtained metabolites after probiotic-stimulation of IECs. Thus, supernatants from the bottom chamber of the transwell—where Caco-2 cells were seeded—were collected for DCs stimulation. After incubation, DCs showed to be affected in their ability to release pro-inflammatory cytokines in response to *Samonella* infection. Moreover, they also noticed a drastic reduction of DCs ability to activate T cells and drive their polarization to Th1 T cells. This indicated that the incubation of IECs with *L. paracasei* has a strong effect on the ability of DCs to activate an inflammatory response to *Salmonella*.[123]

3.3. Others

Cuív et al. demonstrated the immunomodulatory effects of metabolites secreted by *Enterococcus faecalis*—isolated from infant feces by metaparental mating[155]—by using LS174T goblet cell-like (in addition to Caco-2) cell lines. Postbiotics (or cell-free supernatants as they refer to microbial supernatants) were obtained at different time-points of the *E. faecalis* growth phase. Both cell lines were stimulated for 6 h with the pro-inflammatory cytokines TNFα and IL-1β. A decrease in the activation of the nuclear factor kappa B (NF-κB) could be observed due to the conjugation of the luciferase reporter gene to both cell lines. The decrease was shown in the presence of the microbial supernatant, suggesting a decrease in cell activation. This effect was confirmed by measuring nuclear translocation of NF-κB using an immunofluorescence assay.

A recent study from the same group showed similar results using 5 different human gut bacterial strains from the *Clostridia* genus, also isolated by metaparental mating.[21] Postbiotics derived from the isolated bacteria were able to suppress NF-κB activation in a strain-specific manner: they compared the suppressive capacity of bacterial isolates assigned to the same operational taxonomic units (≥97% 16S rRNA sequence identity) and found differences between species in their ability to repress NF-κB activation—as also showed by others.[131] Going one step further, they also determined that the suppressive capacity of almost all the examined strains corresponded to the <3 kDa fraction of the postbiotics.

Oxidative stress, a phenomenon that takes place in the inflamed gut as seen in IBD patients, can damage biological molecules such as DNA or proteins.[156–160] A report used the ileocyte cell line I407 to examine the antioxidative properties of two lactic acid bacterial intracellular contents. In their study, however, Ou et al. did not use postbiotics or bacterial supernatants but their intracellular extracts (obtained after sonication of the bacterial cell suspension).[161] *S. thermophilus* and *Lactobacillus delbrueckii* spp. *bulgaricus* intracellular extracts were added to I407 cells cultured in the presence of H_2O_2. They observed a significant decrease on the DNA damage, especially when *S. thermophilus* intracellular extracts were applied. In addition, inhibition of H_2O_2 cytotoxicity to I407 cells was also higher with *S. thermophilus* than with *L. bulgaricus* intracellular extracts. These findings suggest the ability of probiotic's intracellular extracts, in particular of *S. thermophilus*, to limit cellular DNA damage by reactive oxygen species. Consequently, they suggest a potential role of probiotics intracellular extracts in preventing intestinal inflammation.

Overall, all the above-mentioned studies demonstrate that human intestinal cell lines are an effective and useful tool for initially screening the possible beneficial effects of postbiotics (either anti-inflammatory, antiproliferative, anti-adherent, anti-invasive, or antioxidant) on the intestinal epithelium. Nevertheless, despite their many advantages cell lines cannot reproduce the conditions in tissues, nor do they reflect interindividual variability. In that sense, ex vivo and in vivo assays represent a necessary step forward.

4. Study of Postbiotics' Effects in Ex Vivo Models of Intestinal Epithelial Cells

Working with an organ or tissue isolated from its natural in vivo environment provides an experimental set up that is closer to physiology. This is possible due to a well-conserved cytoarchitecture, as well as to the maintenance of most of the intercellular connections and interactions.[162] As such, ex vivo models—both

from human and mice—have also become an important tool for examining the impact of postbiotics (Figure 1B), as we describe in this next section.

4.1. Intestinal Epithelial Organoids

Epithelial organoid cultures generated from small-size intestinal samples mimic the tissue of origin and thus represent a promising ex vivo tool for studying the physiopathology of the intestinal epithelium.[163–165]

Gut-derived organoids have been extensively used for the study of particular microbial metabolites and even whole bacteria-epithelium interactions.[166–169] Nevertheless, little is known about their application to the field of postbiotics. Here we discuss the few available, to the best of our knowledge, postbiotics studies involving organoids.[47,170–172]

The first study used mouse ileal organoids to study the impact of postbiotics on host peripheral lipid metabolism and histone acetylation.[173] Organoids were exposed to supernatants from *Akkermansia muciniphila* and *F. prausnitzii,* as well as to individual specific SCFA. Genes involved in cell cycle-control, adipocyte function, and peripheral lipid metabolism were later analyzed. Their results reveal a strain-specific effect on intestinal organoids with *A. muchiniphila* and *F. prausnitzii* postbiotics showing distinct transcriptomic responses. In fact, when comparing bacterial postbiotics with their respective culture media, only *A. muchiniphila* exhibited a distinct pattern of gene expression, meaning that these postbiotics had a remarkable effect on the epithelial transcriptional program. Indeed, *A. muciniphila* postbiotics regulated the expression of those transcription factors and genes involved in host metabolic pathways, with lipid metabolism being among of the top 10 altered associated networks. Thus, this study demonstrated the strong effect of microbial postbiotics on the intestinal epithelial function, which differs according to the bacterial species.

In a different work, Giri et al. used human organoids to explore the anti-inflammatory effects of postbiotics. In particular, they generated organoids from healthy individuals, as well as from CD and UC patients, in order to study the beneficial effects of postbiotics derived from different bacterial strains. Organoids were stimulated with IL-1β. IL-8 secretion was measured after treatment with postbiotics. All the analyzed postbiotics exhibited significant suppressing activity, measured as a reduction in the production of IL-8 in response to IL-1β, to an equivalent or even better degree than the positive control (*F. prausnitzii* postbiotics). Similar results were observed in *Winnie* mice organoids. Hence, Giri et al. demonstrated the usefulness of organoid culture for studying the anti-inflammatory effects derived from bacterial postbiotics.[21]

Overall, the little data available to date indicate that organoids could be an appropriate gut model system to study epithelial-microbial interactions. Nonetheless, they pose some limitations, such as cellular polarization or difficult accessibility on the apical side. This has also prompted the investigation and use of other more physiologically relevant strategies, that is, tissue explants.

4.2. Tissue Explants

Intestinal tissue explants have been used since the 1960's for such areas of study as the pathogenesis of IBD or the effects of diverse stimuli on intestinal cell proliferation and differentiation. The importance of this culture type derives from the fact that they contain all cellular components of the intestinal mucosal and not only IECs.

This approach has been demonstrated to be useful when results from cell culture and in vivo models are inconclusive.[174,175]

Ileal and colonic human explants have been used to study the potential benefits of *Lactobacillus* postbiotics in post-infectious irritable bowel syndrome (PI-IBS). Explants were placed on netwell culture filters with the mucosa exposed uppermost and incubated with the inflammatory stimuli (LPS), and subsequently with whole *Lactobacillus* or its postbiotic.[23] A reduction in mRNA levels of pro-inflammatory cytokines and an increase in IL-10 was observed after treating explants with both *Lactobacillus* and its postbiotics. TLR-4 protein was also significantly decreased only when explants were treated with the postbiotics, but not the whole bacteria. Remarkably, this effect was more pronounced in ileal tissues compared to colonic explants. Thus, this study was able to measure the anti-inflammatory effects of postbiotics using an ex vivo organ culture of PI-IBS disease, lending credence to the potentially favorable biological effects of *Lactobacillus* postbiotics.

Yan et al. obtained *Lactobacillus rhamnosus* GG postbiotics and, through an ion exchange procedure, purified two of the contained proteins.[131] These postbiotics-purified proteins were tested on a murine colon organ culture with or without TNF as inflammatory stimuli. Histologic sections showed that TNF induced massive damage by disrupting the epithelium and necrotizing the mucosa. Postbiotics-purified proteins restored epithelial integrity, as well as the colonic crypt structures in cultures previously stimulated with TNF. Similarly, the pro-apoptotic effect of TNF was reverted by the postbiotics-purified proteins. To confirm this result, immunostaining was used to detect expression levels of caspase 3, a regulator of the apoptotic program that is induced by TNF.[176] As expected, postbiotics-purified proteins reduced TNF-induced caspase-3 expression compared to TNF-only treated conditions.

In their study, Nzakizwanayo et al.[177] used murine explants to determine the role of EcN postbiotics in enhancing serotonin (5-hydroxytryptamine, 5-HT) bioavailability through its interaction with secreted host-derived factors. They performed several experiments to investigate the effects of an array of cell-free supernatants on 5-HT overflow; EcN postbiotics, untreated mice ileal tissue supernatant (I-SNT) or EcN postbiotics obtained after bacterial growth in I-SNT (I-EcN-SNT) were used. All the supernatants were tested on fresh tissue sections placed on glass petri dishes. Treatment with I-SNT, as well as EcN postbiotics, resulted in no significant alterations to extra-cellular 5-HT (contrary to treatment with EcN cell suspension). Nonetheless, I-EcN-SNT treatment significantly raised 5-HT levels, thus demonstrating EcN's interaction with ileum-secreted factors to regulate 5-HT availability in IECs. These results prove that tissue explants are a good strategy to study bacterial interactions with host-cells secreted metabolites.

Thus, tissue explants represent a feasible and useful strategy for studying the gut mucosa's interaction with microbial metabolites. Nevertheless, beyond the conventional explant culture, new approaches have emerged to better control the conditions of the entire process. These approaches provide several advantages including the use of smaller tissue sections and optimal tissue orientation (apical side up) throughout the experiment.[15]

4.3. Glued Caved Cylinder

Tsilingiri et al. developed a novel organ culture system of intestinal tissue that maintains apical to basolateral polarity during stimulation via the use of a glued caved cylinder. Briefly, the tissue of interest is fixed with surgical glue to the glass cylinder. The preparation is then placed on sterile metal grids positioned on a center-well culture dish. Culture medium is added both in the center-well plate and inside the cylinder, where the appropriate stimuli is also included. The incubation takes place in a 5% carbon dioxide incubator for 2 h. After removing the medium from the inside of the cylinder, the tissue is transferred to a pressurized oxygen chamber. Finally, the cylinder is discarded, the tissue histologically analyzed and the media collected for cytokine production study.[15]

This new culture system was used to study the immunomodulatory properties of *Lactobacillus*-secreted metabolites. *L.paracasei* B21060 was grown for postbiotics collection. The anti-inflammatory properties of B21060 postbiotics were confirmed in an infection model of *Salmonella typhimurium*, one of the most threatening pathogens of the GI tract. When the postbiotics were added together with *Salmonella* in the cylinder, TNF levels diminished while IL-10 secretion increased, thus counteracting *Salmonella* inflammatory effects by the use of postbiotics. This not only illustrated a dramatic effect of the postbiotics during *Salmonella* infection, but also a stimulation of the anti-inflammatory response driven by the tissue.

Moreover, histological analysis confirmed the usefulness of this ex vivo culture to mimic a classic *Salmonella* infection and to evaluate the NF-κB-p65 translocation, showing that the vast majority of the tissue surface treated with postbiotics after infection resembled that of the non-infected tissue.

By using the cylinder, they could also reach the following additional conclusions: 1) in the presence of postbiotics, *Salmonella* was unable to penetrate the lamina propria, as the total number of internalized bacteria remained 30% less than in the absence of postbiotics; 2) inhibition of *Salmonella* invasion was due to a direct effect of the postbiotics on the tissue and not on the bacteria; 3) B21060 postbiotics were able to reduce pro-inflammatory cytokine secretion in IBD tissues, in contrast to the *Salmonella*-infected tissues, where it failed to induce IL-10 release; 4) NF-κB-p65 translocation was significantly reduced on ileal CD tissues after postbiotics treatment.

Given the fact that the glued caved cylinder is a reliable ex vivo tool, Zagato et al. also used it for the study of fermented infant formula on murine colonic samples.[20] They queried whether fermented infant formula without living bacteria could recapitulate any of the beneficial effects of breast-feeding, and how this could affect the immunological development of infants. Specifically, the hypothesis of this group was that *L. paracasei* CBA L74-fermented milk (milk powder containing *L. paracasei* CBA L74 postbiotics) could, similarly to breast milk, offer protection against enteric pathogens. Mice colonic tissue was stimulated with *S. typhimurium* in the presence or absence of CBA L74 postbiotics. Indeed, they reported a drastic reduction in the amount of pro-inflammatory cytokines produced in response to *S. typhimurium* and lower tissue destruction in the presence of fermented milk. Their results show the positive effects of postbiotics present in fermented milk preparation and support the potential use of postbiotics in protecting the intestine from inflammation and providing immune support to newborns and infants.

Thus, ex vivo culture systems have steadily improved over time, offering the possibility to model what might happen in the gut in any given biological situation, from health to disease, when an in vivo strategy is not possible.

5. Study of Postbiotics' Effects on Intestinal Epithelial Cells in In Vivo Models

The last step for a biological product or drug to be tested before its clinical development is the in vivo assay. In vitro and ex vivo assays can help reveal the more relevant characteristics and effects that the trial product might possess. Indeed, in vivo studies are necessary to understand how the body, as a whole, responds to a particular substance.[179]

Hence, several studies employed the in vivo assay to fully understand the role of postbiotics—administered by oral gavage, intraperitoneally, or intrarectally (Figure 1C)—in improving gut health and life nutrition.

One of the earliest reports regarding mice treated with postbiotics dates from 2005.[22] Ménard et al. treated IL-10 deficient C57BL/6 mice with postbiotics derived from *B. breve* and *S. thermophilus*. Two other groups of mice were either treated with living bacteria or received no treatment (control) for 10 weeks. The authors analyzed the colon and the mesenteric lymph nodes. Cells from the latter were subsequently stimulated with *E. coli* supernatant to test their immunogenicity. When compared to the other groups, mesenteric lymph node cells from mice treated with postbiotics showed the highest secretion levels of pro-inflammatory cytokines (TNFα, IL-12, and INFγ), which was also confirmed by qPCR. This may indicate a Th1 polarization of the immune response. In agreement with these results, they observed an increase in the number of INFγ-secreting $CD4^+$ and $CD8^+$ subsets in mesenteric lymph nodes cells. The colonic mucosa was also examined for epithelial barrier integrity, exhibiting a higher electrical resistance with a concomitant lower paracellular diffusion of small molecules in mice treated with postbiotics, compared to littermates receiving living bacteria or control. Moreover, mice treated with live bacteria showed a lower capacity than those treated with postbiotics to recover from the induced colitis (less weight gain). Overall, these results demonstrated the value of bacterial-secreted metabolites (but not living bacteria) in enhancing of the immune response and improving the intestinal barrier in vivo.

In a different report, dextran sulfate sodium (DSS)-induced colitis in C57BL/6 mice was treated with *Lactobacillus* postbiotics.[20] Mice appeared to be protected against colitis due

to a significant reduction in weight loss, as well as a better recovery rate in the treated group. This was confirmed by histological improvement of the colonic tissue. DSS mice were also used in the same study to better understand the colitis-protecting effect of fermented infant formula. Mice receiving fermented milk—milk with *Lactobacillus* postbiotics—also lost less weight and recovered faster. This was supported by less severe colon length reduction (a sign that correlates with the severity of colitis). Interestingly, an increase in the expression of protective cytokines such as IL-33 was also observed in the colonic mucosa. Similar results were obtained by Yoda et al. and Segawa et al.[180,181]

Giri et al. also studied the anti-diarrheal properties of two postbiotics derived from *Clostridium bolteae* in *Winnie* mice.[21] One of the tested postbiotics not only reduced diarrhea scores, but it also significantly diminished colonic inflammation. Moreover, goblet cells (a population of mucus secreting epithelial cells) were recovered and their mucin production increased. These results not only indicated the strong beneficial effects of postbiotics in reducing the severity of colitis, but also the value of in vivo assays for identifying the most promising postbiotics.[182]

Another study showed that postbiotics can help ameliorate *E. coli* K1 neonatal meningitis[183] in a neonatal rat model.[154] The authors demonstrated that pre-treatment with *L. rhamnosus* postbiotics delayed intestinal colonization of *E. coli* K1, as well as intestinal barrier injury induced by this microorganism. *E. coli* K1 counts in blood, liver, and spleen were significantly decreased in rat pups pre-treated with postbiotics. Moreover, they also demonstrated that *L. rhamnosus* postbiotics have considerable potential to promote the maturation of neonatal intestinal defense, including the upregulation of Ki67 (a marker or proliferative cells), MUC2 (a goblet cell produced mucin), and IgA in the intestine of treated pups. Comparable results were also published by the same group but characterizing the bioactive compounds of *L. rhamnosus* postbiotics.[122]

Thus, in vivo studies of postbiotics can be performed with a broad range of animal models: from spontaneous chronic colitis in IL-10 knock out mice to DSS-induced acute colitis, in addition to models of inflammation induced by LPS[122] or life pathogenic bacteria.[154] Overall, in vivo studies are essential to understanding how postbiotics act in a multi-organ life system, their capacity to enhance the immune system by boosting cell differentiation, their ability to strengthen cell-to-cell attachments to improve the intestinal epithelial barrier, and their capability to increase mucin production for enhanced protection of the whole intestinal tissue. Altogether, in vivo studies show that postbiotics do provide protection in models of intestinal inflammation by reducing weight loss, lowering the impact of inflammation on the epithelial structure and, on the whole, by stimulating the immune system to help protecting the body against intestinal immune diseases.

6. Potential Applications and Concluding Remarks

Microbial-human crosstalk in the GI tract has been extensively studied for years due to the well-established importance of microbes in health and disease. Whether the observed microbial effects on the intestine were due to the entire bacteria or their secreted products was addressed later. Microbial metabolites are essential contributors to the interaction between microorganisms and host cells. Scientific studies have mainly focused on understanding the response of the human body to a single microbial-derived molecule. The actions of individual metabolites need to be considered before the sophisticated interplay of hundreds of them can be deciphered. Nonetheless, as we have shown in this review, some microbial molecules interact among themselves to finally exert their various effects. Here, we contend that postbiotics, defined as the mixture of products released by probiotic bacteria, are the best models for better understanding of the convoluted interactions that persistently occur within our body. Nonetheless, and supporting what Wegh et al. discussed in their review,[184] a consensus-based nomenclature is required (as formulated, for example, for prebiotics or probiotics by the WHO, FAO, or ISAPP) if postbiotics are to be universally accepted as food supplements with potential health benefits. To the date, several studies suggest that these benefits (anti-inflammatory, antioxidant, antimicrobial, etc.) are very real, in comparison to those conferred by probiotics, concluding that postbiotics exert their effects with better safety profiles due to the absence of MAMPs.[15,20,23,130] Moreover, several other advantages position postbiotics ahead of probiotics: 1) probiotic strains might have antibiotic resistant genes that can be acquired by pathogenic bacteria through horizontal gene transfer;[185] 2) using probiotics in any product requires the viability of bacterial cells to be maintained in order to ensure accurate administration of the desired amount of microorganisms. This can be easily altered by different variables such as temperature, pH or interaction with other microbes;[186] 3) when probiotics colonize the gut, they may inhibit the return of the indigenous microbiome.[187,188]

Most of the studies discussed in this review demonstrated the feasibility of studying postbiotics using the intestinal epithelium. The current available IECs systems used to study postbiotics are highly diverse. In vitro strategies represent the first approach to characterize the biological effects of postbiotics as they offer several advantages. Cell lines are easy to obtain, handle, and share among research groups. In addition, they can be expanded and used over time with reproducible results. Nevertheless, cell lines lack important physiological aspects, such as interactions between cell populations, tissue cytoarchitecture or interindividual variability. All of them appear to be fulfilled by ex vivo cultures. Nonetheless, access to human tissues is not always feasible or ethically justified; therefore, researchers frequently opt for experimental models. Working with in vivo models allows for a complete study of the effects that the tested product is exerting. In vivo models are not only one of the most advantageous tools in research, but also a necessary one before a product or drug can reach clinical development. Furthermore, in vivo experiments are not only desirable to test efficacy of a candidate drug, but necessary to determine its potential toxicity.

By using all the culture systems discussed herein, researchers have been able to determine several of postbiotics' characteristics: 1) postbiotics are able to block pathogen adhesion to IECs; this is not due to their antimicrobial capacities but because of a protective effect toward IECs—possibly triggering IECs defensin-secretion, a well-known antimicrobial peptide; 2) postbiotics are able to translocate through the epithelial barrier while maintaining their immunomodulatory properties—this might help elucidate the downstream mechanisms of action; 3) postbiotics are capable of reducing the tissue damage caused

Table 2. Current available clinical trials using postbiotics to treat or prevent gastrointestinal disorders.

Name of the study	Phase	Status	Pathology/Condition	Drug/Intervention	Location
Effect of postbiotics supplementation on microbiome in obese children: the POST-OB study	Phase 4	Recruiting	Childhood obesity	Vitamin D3 and immunofos	Ospedale San Paolo, Milan—Italy
Randomised, controlled study to assess safety and tolerance of infant formula with prebiotics and postbiotics in healthy infants	N.A.	Not yet recruiting	Healthy term infants	Milk based infant formula	Nutricia research
To assess the safety and tolerance of infant formula with locust bean gum in infants with regurgitation	N.A.	Recruiting	Regurgitation	Milk based anti-regurgitation infant formula	Poliklinika Ginekologiczno-Położnicza Sp. z o.o. Sp. k. Białystok - Poland
Gut health, inflammation, hormones	N.A.	Enrolling by invitation	Aging Well	VMK223 and cellulose	University of Roehampton, London—United Kingdom

N.A.: Not applicable. This table has been created with the available information at www.clinicaltrials.gov site by searching for "postbiotics".

by reactive oxygen species; 4) they can decrease the proliferative capacity of cancer cells; 5) postbiotics can interact with IECs and affect the expression of transcription factors involved in cellular metabolism, thereby improving its function; 6) improve mucosal inflammation by restoring epithelial integrity, crypt structures and villus size; and 7) enhance the immune system both in health and disease.[20,180] Nevertheless, new strategies might be applied to better understand postbiotics' effects. For example, 2D cultures derived from human samples, in lieu of 3D organoids, could provide a better approach to study the anti-adhesive, anti-invasive, or antimicrobial properties of specific postbiotics against human intestinal pathogens.[189] Going one step further, the microfluidic human gut-on-chip could replicate the characteristics of an in vivo model, mimicking the mechanical, structural, absorptive, and pathophysiological properties of the human gut, combined with microbial postbiotics.[190]

Despite the challenge of translating all this scientific knowledge to a clinical setting, there are already four ongoing clinical trials using postbiotics in infant formula or to treat obese patients (**Table 2**). Results are not yet available since most of them are still in the recruiting phase. In addition, several cell-free products are already commercially available. These include Colibiogen (Laves-Arzneimittel GmbH, Schötz, Switzerland), a product derived from *E. coli* Laves 1931 cultures that has been shown to be effective in reducing skin lesions from patients with polymorphous light eruptions.[191–193] Hylak Forte (Ratiopharm/Merckle GmbH, Germany) is another bacterial-free liquid containing metabolic products from different bacterial strains (*E. coli, Streptococcus faecalis, Lactobacillus acidophilus,* and *Lactobacillus helveticus*), which has been proven to control salmonellosis in infants and intestinal dysbiosis in patients with chronic gastritis. Moreover, it also reduces diarrhea induced by radiation in oncologic patients.[194–196] CytoFlora (BioRay Inc., Laguna Hills, CA, USA) is composed of several microorganism cell-wall lysates and it also helps in correcting intestinal dysbiosis.[197] Another commercialized set of products is the MATRIX line (Smartfarma, Milan, Italy), which includes three baby-care vitaminic and mineral supplements (Smart D3 Matrix, Polivit Matrix, and IdraMatrix). All three contain ImmunoFOS (Postbiotica, Milan, Italy), a patented postbiotic produced using an innovative fermentative process (PBTECH) that helps strengthen the immune system and restore the intestinal microbiota.[198,199] This same postbiotic is used in a pet-care product: Renal N (Candioli Pharma, Beinasco, Piemonte, Italy), a group of antioxidant products that favor pets' immune defense and restores normal intestinal functionality. In fact, postbiotics have also been studied for animal care, as reported by Izuddin et al.[200,201]

Therefore, the available data suggests that postbiotics might be a safer alternative for treating intestinal inflammatory diseases due to their lack of immune activating molecules (i.e., PAMPs, MAMPs). Even though some of them are already commercialized, further investigation is needed to completely characterize their exact composition and determine their mechanisms of action.

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Conflict of Interest

The authors declare no conflict of interest.

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anti-inflammatory effect, inflammatory bowel disease, intestinal epithelial cell, microbial metabolites, postbiotics

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