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**Functional Characterization of Bacterial Strains Enriched in
Colon Adenomas and Their Impact on Colonic Epithelial Cells**

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Abstract

Colorectal cancer (CRC) is one of the most common malignancies worldwide and a leading cause of cancer-related mortality. It arises from the progressive accumulation of genetic and epigenetic alterations in the epithelial cells lining the colon or rectum, often evolving from precancerous lesions such as adenomatous polyps. Lifestyle factors—including diet, obesity, and physical inactivity—as well as hereditary predispositions contribute significantly to CRC risk. In recent years, increasing attention has been directed toward the role of the gut microbiota in colorectal cancer development and progression. The intestinal microbiota, a complex and dynamic community of microorganisms, is essential for maintaining gut homeostasis, immune function, and metabolic balance. Dysbiosis—an imbalance in microbial composition—has been linked to CRC through mechanisms such as chronic inflammation, production of carcinogenic metabolites, and modulation of host signalling pathways. Certain bacterial species have been identified as potentially pro-tumorigenic, while others may exert protective effects, highlighting the microbiota as both a risk factor and a promising target for prevention, diagnosis, and therapy in colorectal cancer.

This study is based on the “Metabolic Population Hypothesis”, which proposes that distinct dysbiotic microbial communities can converge functionally to produce similar metabolic outputs, ultimately driving the same pathological outcome. In the context of colorectal cancer, this concept suggests that different configurations of the gut microbiota—though taxonomically diverse—may promote a pro-tumorigenic environment. These shared metabolic activities can promote chronic inflammation, epithelial damage, and genomic instability, contributing to drive early epithelial alterations, shifting normal mucosa toward a pre-neoplastic phenotype that can evolve into adenomas and ultimately colorectal cancer.

The aim of this study is to metabolically and functionally characterize microbial populations enriched in colorectal adenomas. By integrating analyses of microbial composition with their metabolic potential and activity, the study seeks to identify key functional traits and pathways associated with adenoma-associated communities.

In this study, we have identified adenoma associated mucosa preponderant bacterial strains and went on to evaluate their potential role in generating and promoting a pro-tumorigenic environment. We investigated these bacteria by assessing their impact on key cellular processes

relevant to colorectal carcinogenesis. In particular, we evaluated their ability to induce DNA damage and modulate epithelial cell proliferation rates, both of which are critical features of early tumour development. In addition, we examined their effects on intestinal barrier integrity, including epithelial permeability and tight junction function. Finally, we explored their interactions with the host immune system, focusing on their capacity to trigger inflammatory responses or alter immune signalling pathways. Together, these analyses provide a comprehensive view of how adenoma-associated bacteria may influence multiple aspects of host physiology linked to disease progression.

1. Introduction

1.1. Human Colorectal Cancer

1.1.1 Epidemiology and risk factors

Colorectal cancer (CRC) is currently ranked as third in the number of new cancer cases reported worldwide and is considered the fourth deadliest cancer type, ranked second in the number of malignancy-associated fatalities^{1,2}. CRC is a complex multifactorial disease, with a broad range of risk factors: genetics and environmental factors play a critical role in the onset and progression of the disease. Smoking, high consumption of red meat, high-fat and low-fibre diet, alcohol abuse and physical inactivity are among the most common lifestyle-associated risk factors. Age plays an important role, with people over 50 years old being more exposed to developing CRC. While death rates have decreased in the last decades due to better screening and better clinical practices, a significant increase in newly diagnosed cases for people under 50 years of age has been reported, leading to a potentially heavier burden for global healthcare systems^{3,4} (**Fig. 1**).

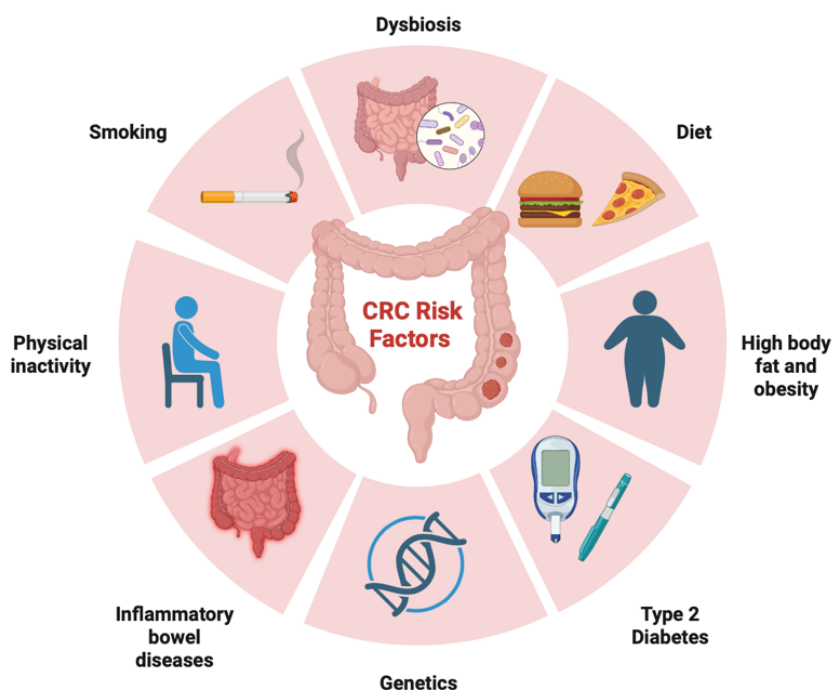


Figure 1 - Risks factors contributing to colorectal cancer (CRC) initiation and progression include genetic predispositions, environmental influences and comorbid conditions. Created with BioRender.

1.1.2 Molecular pathways of carcinogenesis and histological features of different Colorectal Cancer Subtypes

Most CRCs originate from precursor lesions called “colorectal polyps”. Polyps are generally benign macroscopically visible protrusions of the mucosa into the lumen, which can be classified based on morphology, histological features and associated molecular alterations linked to different potential pathways of carcinogenesis⁵. The polyp to CRC progression is a protracted and multistep process which involves the accumulation of driver mutations that vary among different polyp subtypes^{2,6}. CRC is currently assumed to originate from a stem cell or a stem-like cell. These cells are called “cancer stem cells” and are the result of progressively accumulated genetic and epigenetic alterations, they are located at the base of colonic crypts and are essential for tumour initiation and progression^{7,8} **(Fig. 2)**. Cancer stem cells undergo metabolic reprogramming, a process governed by significant alterations in oncogenes and tumour suppressor genes normal functioning, leading to uncontrolled cell growth^{9,10}. Currently, there are two major pathways for the polyp to CRC progression: the traditional adenoma-carcinoma pathway, and the sessile-serrated pathway^{2,4}. It is important to note that the molecular events that drive the initiation, promotion and progression of CRC are the result of interactions that happen on many levels. This process is an extremely complex equation that encompasses environmental influences and individual characteristics such as cancer susceptibility, paired with accumulated somatic mutations¹¹.

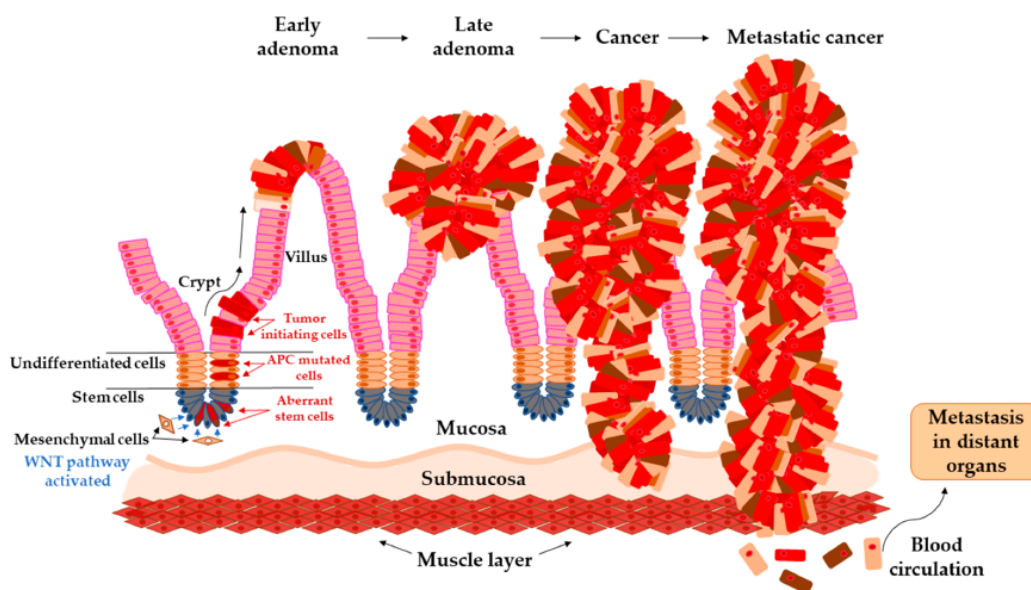


Figure 2. The adenoma-carcinoma pathway involves alterations in the WNT signalling pathway, which lead to the proliferation of aberrant colonic crypts and, over years, can ultimately result in metastatic cancer (Siskova et al., 2020).

1.1.3 Adenomatous Polyps

Adenomatous polyps account for approximately 70% of cases and encompass various subtypes: tubular, villous and tubulovillous adenomas (**Fig. 3**). Histologically, adenomatous polyps feature crowded, elongated crypts with dysplastic epithelium. These subtypes are also characterized by different levels of malignant potential: tubulovillous adenomas pose a moderate risk, while villous adenomas exhibit elongated, finger-like structures that might invade the surrounding mucosa^{2,12}. Adenomatous polyps are commonly associated with chromosomal instability (CIN), a somatic defect that causes changes in chromosomal copy number and structure. This process might lead to the physical loss of a wild-type copy of a suppressor gene, such as APC and TP53¹¹. The APC protein is part of a complex that degrades B-Catenin, a protein that binds to nuclear receptors (members of the T-cell factor-lymphocyte factor enhancer factor family) and regulates genes involved in cellular activation, such as the Wnt signaling pathway. Without a properly functioning APC, there is no control of B-catenin degradation and Wnt signaling becomes constitutively activated. This mutation is linked to adenomatous polyposis, an inherited cancer predisposition in which the risk of developing colorectal cancer by the age of 40 years is almost 100%^{11,13}. While APC changes are key factors in tumour progression, they are not sufficient. SMAD2 and SMAD4 are involved in Transforming Growth Factor B (TGF-B) signalling acting as oncosuppressors. They are inactivated through loss of heterozygosity in colorectal cancer. A similar inactivation pathway is observed for the TP53 gene which encodes for P53, a crucial protein in cell-cycle and cell-death mechanisms. The inactivation of p53 requires two steps: a missense mutation that inactivates transcription of p53 and 17p chromosomal deletion that eliminates the second allele. This alteration in p53 function is often associated with the transition from large adenoma to invasive carcinoma^{11,14,15}.

1.1.4 Serrated Polyps

Serrated Polyps differ from Adenomatous polyps both in morphology and in molecular pathways of development. They are characterized by a saw-toothed (serrated) architectural pattern of the polyp's surface and crypt epithelium on histologic examination. Serrated polyps are usually less predictable compared to adenomatous polyps, and account for about 15-30% of CRC cases¹⁶. Serrated polyps include hyperplastic polyps (HPs), sessile serrated lesions (SSLs) and traditional serrated adenomas (TSAs) – (**Fig. 3**). Hyperplastic polyps account for the majority of serrated polyps and are generally benign. While TSAs pose a higher risk of malignant transformation, they are the least encountered type of lesion, accounting for approximately 1%

of all colorectal polyps^{2,16}. SSL might be associated with the CpG island methylator phenotype (CIMP, or CIMP-high), a condition where certain promoter-associated CpG islands exhibit aberrant methylation levels, resulting in silencing of gene expression. This phenotype is associated with protein loss of function, notably MLH1, MSH2, MSH6, and PMS2 which are crucial for DNA mismatch repair (MMR), resulting in microsatellite instability (MSI) and the consequent evolution into dysplasia and high-grade dysplasia. These cells accumulate somatic mutational events and harbour high antigen burden, resulting in easier recognition by immune cells. This characteristic explains why usually MSI CRCs have a better prognosis compared to microsatellite stable (MSS) forms and respond much better to immunotherapy^{17,18}.

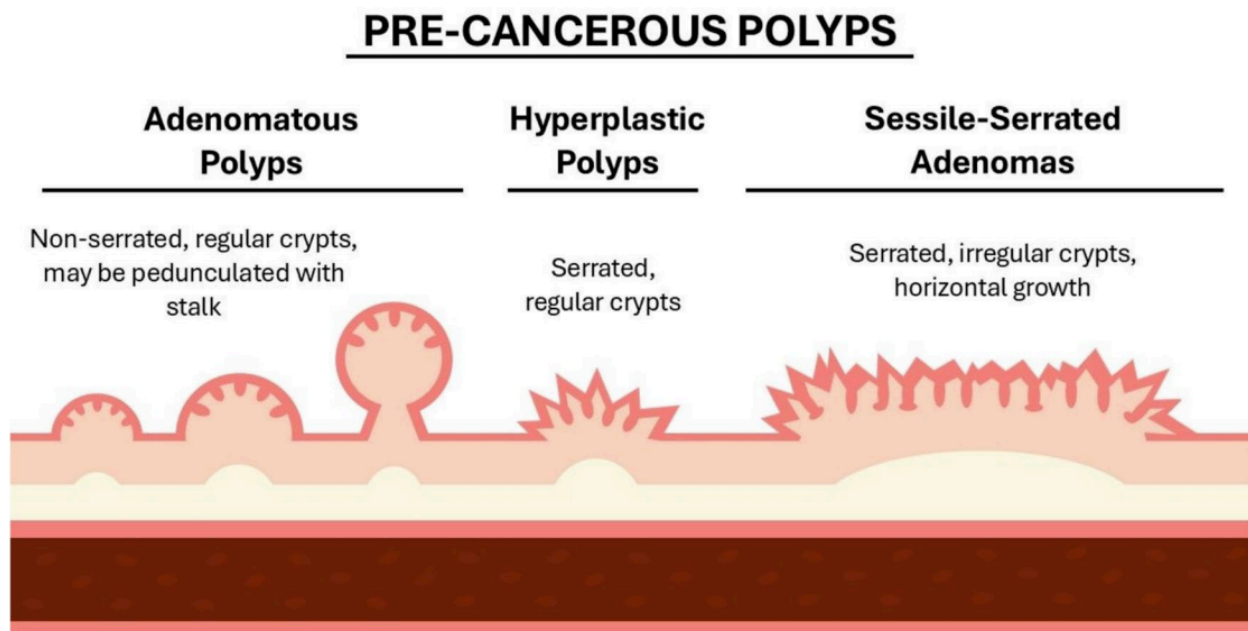


Figure 3. Three main classifications for colorectal polyps: adenomatous, hyperplastic and sessile-serrated adenomas. From (Kotula et al., 2025)

1.1.5 Adenoma-carcinoma sequence

The adenoma–carcinoma sequence describes the stepwise progression from benign precursor lesions to invasive colorectal cancer through the accumulation of genetic and epigenetic alterations. In the conventional pathway, this process typically begins with mutations in APC, leading to aberrant Wnt signalling and early adenoma formation, followed by activating mutations in KRAS, which promote proliferation and adenoma growth. The transition to carcinoma is often marked by loss of tumour suppressors such as TP53 and SMAD4, enabling genomic instability, invasion, and malignant transformation^{11,13–15}. In contrast, the serrated

pathway arises from serrated polyps and is commonly initiated by activating mutations in BRAF, accompanied by widespread CpG island methylation (CIMP) and, in some cases, microsatellite instability due to MLH1 silencing^{17,18}. While both pathways culminate in colorectal cancer, they are distinguished by their initiating events and molecular trajectories, with key late-stage alterations—such as TP53 loss or mismatch repair deficiency—marking the critical shift from benign lesions to invasive carcinoma.

1.1.6 Tumour Staging Classification, Diagnosis and Current Therapeutical Approaches

Currently, the American Joint Committee on Cancer (AJCC) staging manual is considered the most complete summary of all available information on the staging of adult cancers for all clinically important anatomic sites. It represents the standard for classifying patients with cancer, defining prognosis, and determining the best treatment approaches. The TNM classification was firstly developed by French surgeon Pierre Denoix between the 1940s and 1950s. Its main function is to standardize the description of the anatomical extent of malignant tumors^{19,20} **(Table 1)**.

Current diagnostic approaches for colorectal cancer (CRC) rely on a combination of endoscopy, imaging, stool-based and emerging molecular tests aimed at detecting cancer or its precursors at an early stage. It is important to highlight the relevance of screening practices, since with early detection of colorectal cancer polyps, clinicians can improve the likelihood of a patient's survival. Most CRC cases follow the adenoma-carcinoma pathway, which normally provides a window of 5-10 years for detection. Data show that CRC prognosis is highly correlated with diagnostic staging: TNM stage I patients have a 5 year survival rate of 90%, which drops around 12% for stage IV patients²¹. The US Preventive Services Task Force (USPSTF) recommends to initiate screening at age 45, based on modelling data for estimated life-years gained, CRC incidence, and CRC mortality if screening is started at varying ages²². Unfortunately, optimal screening practices depend on a plethora of factors, such as geographic region, insurance status (where needed) and healthcare availability²³.

Colonoscopy is currently considered the golden diagnostic and screening method for colorectal cancer, because it allows direct visualization of the colonic mucosa, enabling both sample collection and removal of precancerous polyps. While efficient, it presents several challenges in terms of patient discomfort during the procedure itself, and also patient adherence to optimal

preparation for undergoing the examination^{1,24}. FIT (Faecal Immunochemical Test) is a cheap, sensible and less invasive alternative, associated with greater patient compliance. While high-risk individuals still need more extensive screening, lower-risk people might benefit from a less invasive technique. FIT is fairly sensible for CRC detection (around 80% precision) but less effective than colonoscopy regarding precursor lesions such as polyps. Stool DNA tests are another alternative to detect mutations and are superior to FIT at detecting pre-cancerous lesions. Liquid biopsies offer minimally invasive procedure (which might enhance screening participation). CT colonography uses imaging to visually reconstruct the colon, has high sensitivity, but still requires bowel preparation. All these techniques are less invasive and fastidious than colonoscopy, but do not have the possibility of direct/live polyp remotion²⁵.

Prognosis, recurrence risk, and treatment approach for CRC vary based on the disease stage. Surgery is the standard approach for stage I, II, and III tumours. It applies to stage IV disease only if the primary mass and metastases are resectable. Following surgery and in case of non resectable tumours, adjuvant chemotherapy is the usually the first line of treatment with fluoropyrimidine (5-FU) and multiple-agent regimens, including oxaliplatin (OX), irinotecan (IRI), and capecitabine (CAP)^{26,27}. Targeted therapies offer an alternative by inhibiting specific growth pathways involved with tumour growth and spreading. These include blocking the epidermal growth factor receptor (EGFR) and suppressing the Ras–Raf–MEK–ERK pathway, which regulates cell proliferation, survival, and growth. Another crucial target is vascular endothelial growth factor-A (VEGF-A), a key promoter of tumour angiogenesis^{26,28}. Immunotherapy has provided a strong advancement in cancer treatment. Immune Checkpoint Inhibitors (ICIs) are antibodies designed to block immune checkpoint receptors such as PD-1 and CTLA-4 to restore T-cell function. Normally, immune checkpoints prevent excessive immune response by acting as a brake of the immune system, but in a cancerous contexture they might hinder tumour detection²⁹.

AJCC Stage	Stage grouping	Description of Colorectal Cancer
0	Tis N0 M0	The cancer is in its initial phase, often referred to as carcinoma in situ or intramucosal carcinoma (Tis). At this stage, it remains confined to the inner layer (mucosa) of the colon or rectum and has not extended further.
I	T1 or T2 N0 M0	The cancer has: progressed beyond the muscularis mucosa and invaded the submucosa (T1), extended into the muscularis propria (T2), infiltrated the outer layers of the colon or rectum without penetrating through them (T3) - it has not metastasized to adjacent lymph nodes (N0) or distant locations (M0).
II	T3, T4a or T4b N0 M0	The cancer has: infiltrated the outer layers of the colon or rectum without penetrating through them (T3), breached the wall of the colon or rectum, but it has not infiltrated surrounding tissues or organs (T4a), penetrated the wall of the colon or rectum and has either attached to or invaded adjacent tissues or organs (T4b) - it has not metastasized to nearby lymph nodes (N0) or distant sites (M0) at this stage.
III	Any T N1 or N2 M0	The cancer has expanded outside of the colon or rectum, up to the visceral peritoneum (T1-T4), but has not invaded any nearby organs (M0) – The cancer has also spread to at least 1 nearby lymph node or infiltrated the fatty areas surrounding the lymph node (N1-N2)
IV	Any T Any N M1	Invasion of nearby tissues and lymph nodes may or may not be present. However, the cancer has reached one or more distant organs, one or more distant lymph nodes or distant regions of the peritoneum.

Table 1. CRC staging. Modified from American Joint Committee on Cancer. Chapter 20 - Colon and Rectum in AJCC Cancer Staging Manual, 8th Edition. New York, NY, Springer; 2017

1.1.7 Immune contexture of tumour microenvironment

The colorectal cancer (CRC) microenvironment is a highly dynamic and heterogeneous ecosystem composed of malignant epithelial cells, stromal components, immune cells, vasculature, and extracellular matrix, all of which interact to influence tumour behaviour³⁰. These interactions are critical for tumour initiation, progression, and response to therapy, highlighting that CRC is not solely driven by intrinsic genetic alterations but also by its surrounding milieu. The metabolic and signalling crosstalk within the microenvironment further drives angiogenesis, epithelial–mesenchymal transition, and therapeutic resistance³¹. Immune cells, including T lymphocytes, macrophages, and dendritic cells, play dual roles by either promoting anti-tumour immunity or facilitating immune evasion depending on their phenotype and functional state^{30,32}. For instance, cytotoxic CD8⁺ T cells are generally associated with better prognosis, whereas immunosuppressive populations such as regulatory T cells and M2-like tumour-associated macrophages (TAMs) support tumour growth and progression³³. Cancer-associated fibroblasts (CAFs) represent another key component, contributing to extracellular matrix remodelling, secretion of cytokines like TGF- β and IL-6, and promotion of invasion and metastasis. Moreover, CAFs and other stromal cells undergo metabolic reprogramming, producing metabolites such as lactate that fuel cancer cell proliferation and reshape the immune landscape^{30,32}.

1.2. Gut Microbiota

The human colon hosts the most complex and densely populated microbial ecosystem of the entire gastrointestinal tract³⁴. It is estimated to contain around 100 trillion microorganisms, including Bacteria, Archaea, Eukarya and viruses. Among these, approximately 39 trillion are bacterial cells, outnumbering the estimated 30 trillion total human cells. The vast majority of these bacteria resides in the large intestine, particularly in the cecum. Under physiological conditions, these microorganisms coexist with the host in a dynamic yet stable equilibrium, establishing a mutually beneficial relationship. This state, referred as eubiosis, is characterized by high taxonomic diversity, elevated microbial gene richness, and a stable functional microbiome core³⁵. The gut microbiota exerts essential functions in host physiology. It ferments otherwise indigestible carbohydrates, synthesizing vitamins (B and K) and producing short chain fatty acids (SCFAs), which serve as key energy sources for colonocytes and as modulators

of host metabolism. Moreover, the microbiota supports the development of both innate and adaptive immunity, inhibits the colonization of pathogenic microbes, contributes to the maintenance of the intestinal barrier integrity, regulating permeability, and promotes tolerogenic response³⁶. Everyone has a specific and unique gut microbiota composition, that evolves throughout life in response to various factors such\ as diet, lifestyle, physical activity, illnesses and antibiotic use³⁷. Colonization of the intestinal tract begins at birth and is strongly influenced by maternal and perinatal factors. Infants born via vaginal delivery tend to acquire a microbiota resembling that of the maternal vaginal and intestinal flora, whereas those born via caesarean section tend to be colonized predominantly by microbes originating from the hospital environment and maternal skin. Feeding practices further shape microbial succession, with breastfed infants displaying distinct microbiota profiles compared with formula-fed infants³⁸.

Many studies have investigated gut microbiome diversity and abundance, most commonly by using 16S rRNA gene sequencing. The dominant bacterial phyla in the human include *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Verrucomicrobia*, with *Firmicutes* and *Bacteroidetes* accounting for 90% of the total (**Fig. 4**). The *Firmicutes* phylum is composed by more than 200 genera, including *Ligilactobacillus*, *Bacillus*, *Clostridium* (representing 95% of all *Firmicutes*), *Enterococcus*, and *Ruminicoccus*. Within the *Bacteroidetes* phylum *Bacteroidetes* and *Prevotella* are the most abundant genera³⁹.

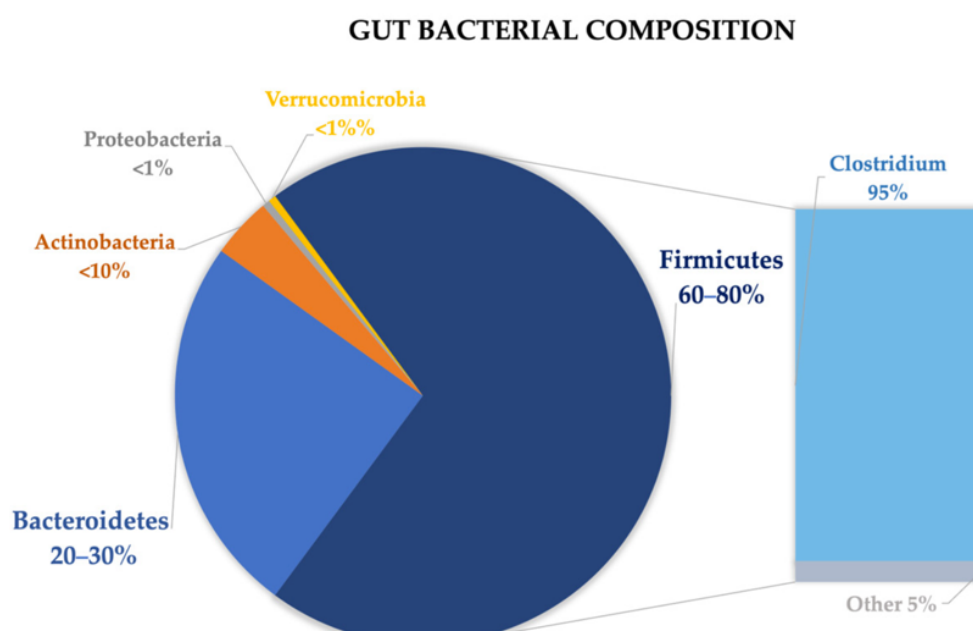


Figure 4 - Gut microbiota composition, where the main phyla are represented by Firmicutes and Bacteroides, which account together for approximately 90% of the total microbial population (Marano et al., 2025)

1.2.1 Microbial metabolites

Microbial metabolites help maintain homeostasis in physiological conditions⁴⁰. Microorganisms produce small molecules during their metabolic processes that can regulate the immune system, maintain gut epithelium integrity, and influence host metabolic pathways. It is estimated that approximately 50,000 metabolites are produced by gut microbes in the human intestine⁴¹. Gut microbiota-derived metabolites can be classified into three major groups based on their origin and function. In the first group there are metabolic end-products, metabolites produced by microbial communities fermenting luminal dietary components. This category includes SCFAs such as acetate, butyrate and propionate, produced mainly from the fermentation of dietary fibres, tryptophan, and indole derivatives. The second group is represented by *de novo* microbial products, synthesized directly by gut microbes. They include branched-chain amino acids (BCAAs), as leucine, isoleucine, valine, involved in protein synthesis, cellular metabolism and growth; polyamines (PAs), as putrescine, spermidine and spermine, essential for cell proliferation, differentiation and reduction of oxidative stress and inflammation; vitamins (K and B), necessary for DNA synthesis, energy metabolism, and red blood cell production. In the last group there are modified host-derived metabolites, compounds produced by the host and modified by gut bacteria, such as secondary bile acids (BAs)⁴². Microbial metabolites can also be distinguished in primary and secondary metabolites. Primary metabolites are essential molecules used by bacteria to grow and communicate with host cells. For this reason, dysregulation of their production could lead to cancer development. These metabolites are often derived from dietary substrates and can be secreted into the intestinal lumen, where they reach a very high concentration. An important example of primary metabolites are SCFAs, that can reach a concentration of up to 140 mM in the gut. Secondary metabolites are not essential for microbial survival but provide advantages under specific conditions. These metabolites include toxins, inhibitory compounds and signalling molecules, (example of secondary metabolites is bile acids, BAs). They influence lipid metabolism, microbiota composition and xenobiotics clearance. They don't reach the concentrations of primary metabolites⁴³. Several mechanisms have been proposed to explain how microbial metabolites may contribute to diseases, including induction of DNA damage, increased intestinal permeability, and modulation of the immune response. In case of dysbiosis, the production of microbial metabolites is altered, impacting on inflammatory processes and development of various diseases, including CRC⁴⁴.

1.2.2 Gut Dysbiosis as a Cancer Hallmark

Dysbiosis refers to the disruption of the physiological homeostasis of the gut microbial community. Multiple factors contribute to this unbalance, including age, sex, genetics, diet, inflammatory and infectious diseases, and drug exposure⁴⁵. In a dysbiotic state, there is often a reduction in commensal species diversity with a concomitant expansion of pro-inflammatory pathobionts. This shift destabilizes the intestinal barrier functions, leading to increases mucosal permeability, bacterial translocation and the creation of a pro-inflammatory environment⁴⁶. As a result, innate immune cells like dendritic cells, macrophages and natural killer (NK) cells secrete elevated levels of pro-inflammatory cytokines, including interleukin 12 (IL-12), interleukin 23 (IL-23), interferon γ (IFN γ), tumour necrosis factor α (TNF- α). These mediators drive the activation of T and B cells and a chronic inflammation⁴⁷. Chronic inflammation, along with the production of reactive oxygen species (ROS), DNA damage, and abnormal cell proliferation, contribute to the development of colorectal adenomas and CRC⁴⁶.

1.2.3 Role of microbiota in colorectal cancer initiation

Currently, gut microbiome dysbiosis is recognized as a hallmark of cancer. Approximately 20% of all cancers are associated with viral or microbial infections, in fact bacteria are now recognised as key players in tumorigenesis of different cancer types³⁴. Several studies have demonstrated that the gut bacterial composition of patients with colorectal adenomas and CRC differs from that of healthy individuals. In patients with adenomas, the gut microbiota is typically enriched in *Proteobacteria* and depleted in *Bacteroidetes*. In contrast, individuals with CRC exhibit a marked increase in *Fusobacterium* and *Prevotella*, along with a reduction in obligate anaerobes and beneficial butyrate-producing bacteria. Many studies have demonstrated that transferring the faecal microbiota from tumour-bearing mice into germ free mice leads to a twofold increase in tumour development in the recipients, compared to mice that received a healthy microbiota. These discoveries suggest that microbiota alterations may contribute to the progression of colorectal adenomas to CRC³⁶. Although our understanding of microbial mechanisms that promote cell transformation is still limited, some pathways have been identified like genotoxins, biofilm, pathogenic bacteria, inflammation, metabolites and oxidative stress⁴⁸. Overall, these mechanisms result in the disruption of the balance between

host cell proliferation and cell death, the modulation of immune system function and the alteration of host metabolism⁴⁹.

1.2.4 Genotoxic effect of bacterial metabolites

Some bacteria can produce and release toxins, classified as secondary metabolites, to compete against other microbes in intestinal niches. They can exert a genotoxic activity and could be involved in the initiation of cancer. Errors in the DNA sequence are inevitable during DNA replication, but they are generally repaired using various mechanisms. However, DNA damage may persist due to inefficient repair mechanisms, dietary components or the presence of certain bacterial species and their metabolites. Accumulation of such damage can lead to cell mutations and tumorigenic cascade⁵⁰. There are many types of DNA damage, such as mismatches, single/double-strand breaks, DNA adducts (**Fig. 5**). All of them can be induced by mechanisms as oxidation and alkylation⁵¹. DNA oxidation is often caused by DNA interaction with Reactive Oxygen Species (ROS). ROS are normally produced by mitochondrial oxidative metabolism, or directly by bacteria in their competition for intestinal niches⁵², to reduce chemical species⁵³, and to modulate host signalling pathways⁵⁴. DNA alkylation occurs when alkyl groups bind specific DNA bases, forming adducts and mutations⁴³. Around 15% of all *E. coli* harbour the polyketide synthase (pks) pathogenicity island in their genome. This region encodes for colibactin, a virulence factor capable to induce DNA damage (double strand breaks and cross-links), promoting genomic instability and significantly alter cell cycle dynamics⁵⁵. *Campylobacter jejuni* produces the Cytolethal Distending Toxin (CDT), inducing DNA double-strand breaks, cell cycle arrest and apoptosis³⁴. Moreover, a new class of molecules has been discovered, termed as “indolimines”. These are small metabolites produced by multiple strains of *Morganella morganii*, capable of inducing abnormal transcriptional signatures and cell proliferation, both in cell-based assays and mice⁵⁶.

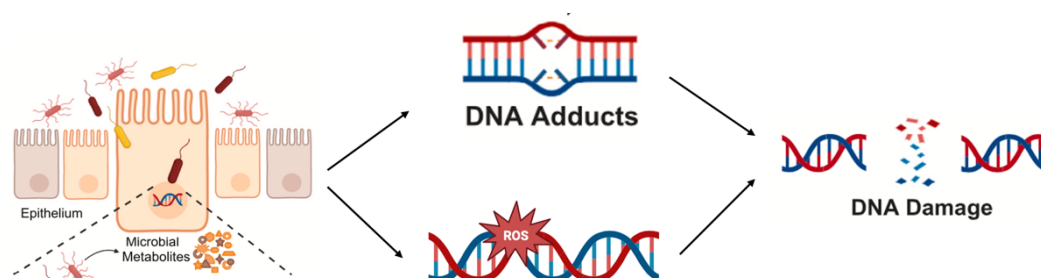


Figure 5. Microbial metabolites can induce DNA damage through different mechanisms, including the formation of DNA adducts, oxidative stress and double strand breaks. Image reworked Pérez Escrivá et al., 2025

1.2.5 Microbial metabolites effects on epithelial permeability

The gut barrier is structured in 3 interdependent layers: the luminal mucus layer, the continuous epithelial cell layer, and an internal layer that forms the mucosal immune system. Together, these layers form a physical and immunological defence system⁵⁷. Under physiological conditions the gut barrier is selectively permeable, regulating interactions with luminal content, including bacteria and their metabolites, as well as nutrient absorption and waste elimination. It prevents the entry of luminal pathogens, viruses and environmental toxins⁴³ **(Fig. 6a)**. The barrier function is supported and ensured by adhesive junctional proteins (e.g. claudins, zonula occludens proteins), that form tight junctions, gap junctions and desmosomes, all essential to control cellular permeability⁴¹. Moreover, also a balanced microbiome and microbial metabolites, produced in close proximity to the intestinal epithelium, contribute to the maintenance of the barrier integrity and functions. Notable examples include SCFAs that enhance gut barrier integrity, upregulating Claudin-1 expression and becoming an energy source for intestinal epithelial cell metabolism. Tryptophan and indole derivatives preserve gut barrier function enhancing its integrity and strengthening tight junctions. Polyamines (PAs), such as putrescine, spermidine, spermine, and microbial vitamins also support intestinal barrier integrity⁴². The loss of the integrity of the intestinal epithelium is known as “leaky gut” or intestinal wall leakage syndrome. This condition permits the translocation of bacteria, bacterial metabolites, endotoxins and inflammatory mediators into systemic circulation, leading to widespread alterations and tissue damages⁵⁸ **(Fig. 6b)**. Factors that contribute to increased intestinal permeability include mucus degradation, disruption of tight junctions, oxidative stress, dysregulation of enterocyte apoptosis. Secondary BAs such as deoxycholic acid and lithocholic acid can disrupt the epithelial barrier integrity by altering tight junction organization, promoting occludin dephosphorylation, and modulating autophosphorylation of the endothelial growth factor receptor (EGFR)⁴³. Metagenomic analysis across multiple human cohorts revealed higher occurrence of *bai* (BA inducible) operons from *Clostridium scindens* and close relatives in faeces of patients with CRC⁵⁹. This increased permeability promotes chronic inflammation and oxidative stress, both of which are key drivers of colorectal carcinogenesis. In particular, exposure of intestinal stem cells to genotoxic compounds and microbial products can enhance DNA damage and tumour initiation. Barrier dysfunction is also closely linked to microbiome dysbiosis, where pathogenic bacteria can invade the mucosa and further amplify inflammatory signalling and immune dysregulation⁶⁰. Clinically, conditions characterized by chronic barrier impairment, such as inflammatory bowel disease, are associated with a significantly increased risk of CRC, underscoring the importance of epithelial

integrity in cancer prevention. Overall, disruption of the gut barrier contributes to a pro-tumorigenic microenvironment that facilitates both the initiation and progression of colorectal cancer⁶¹.

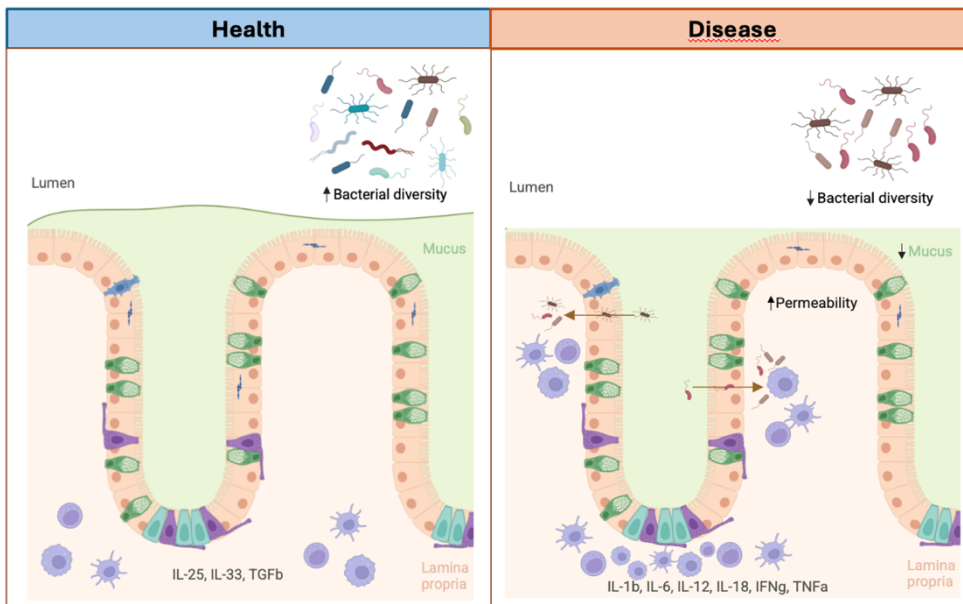


Figure 6. a) Representation of the colonic epithelial barrier under physiological conditions, characterized by high bacterial diversity, an intact mucus layer and well-structured tight junctions; b) Colon epithelial barrier under dysbiotic conditions

1.2.6 Microbial metabolites effect on immune system

Under physiological conditions, the gut microbiota regulates immune cell functions and maintains a non-inflammatory homeostasis⁶². The mucus layer, along several other mechanisms, plays a crucial role in maintaining homeostasis: key functions include minimizing contact between external pathogens and intestinal epithelial cells and the secretion of antimicrobial peptides such as lysozyme, defensins and immunoglobulin A⁶³. Furthermore, microbiota-derived metabolites, such as SCFAs, BAs and vitamins, also regulate immune responses. These compounds interact with several types of receptors including G-protein coupled receptors (GPRs), nuclear receptors and pattern recognition receptors (PRRs)⁴². When this balance is disrupted, key regulatory pathways become compromised. In fact, dysbiosis alters both the composition of gut microbiota and increases inflammatory processes, disrupting immune regulation. For instance, in a dysbiotic state, mucus secretion by goblet cells is impaired. In addition, Toll-like receptor 5 (TLR5), a PRR that induces IL-22 and IL-17 expression

in monocytes to maintain homeostasis, becomes inactivated. This dysfunction leads to altered bacterial composition, including the overgrowth of certain taxa such as *Enterobacteriaceae*, promoting spontaneous colitis and metabolic syndromes³⁵. As previously discussed, dysbiosis increases intestinal permeability, and this allows lipopolysaccharides (LPS) to enter the circulation, activating Toll-like receptor 4 (TLR4) and inducing chronic inflammation⁶⁴. Moreover, the reduced production of SCFAs and BAs associated with dysbiotic conditions leads to a pro-inflammatory response and development of autoimmune diseases⁶⁵. Some pathogenic bacteria are directly involved in promoting inflammation and carcinogenesis. For instance, colonization of the gut by *Fusobacterium nucleatum* has been shown to increase T helper 17 (Th17) cells in the colon, contributing to CRC development. Moreover, since the intestinal microbiota also plays a crucial role in the maturation of the innate immune system, its absence impairs the function of neutrophils and DCs, reducing their ability to kill pathogens and secrete Interferon-I (IFN-I)³⁵.

Rationale of the study

Evidence of dysbiosis and microbial-derived metabolites being relevant contributors in cancer initiation and progression is constantly growing. While researchers were able to identify several CRC-related bacterial strains and their metabolites, we currently lack comprehensive understanding of how bacterial communities interact in a dysbiotic environment. Research has revealed that microbiota composition was highly heterogenous in CRC patients, highlighting the absence of a specific dysbiotic signature associated with cancer development. The restricted/local metabolic framework generated by dysbiotic bacteria colonizing the intestinal adenomas, and its role in creating and sustaining a pro-tumorigenic environment remains poorly understood. Based on these data we formulated the Metabolic Population Effect Hypothesis, according to which different dysbiotic ecosystems in CRC-prone patients might generate similar tumour-promoting microenvironments enriched in specific metabolites, affecting both immune and epithelial cell biology thus initiating and promoting the adenoma-carcinoma sequence.

Aim of the study

The progressive accumulation of genetic and epigenetic alterations leads to the disruption of the intestinal epithelial barrier, a key event in colorectal cancer (CRC) development through the adenoma-carcinoma sequence.

Increasing evidence suggests that epithelial transformation is closely associated with intestinal dysbiosis. Although recent studies have highlighted a significant contribution of the gut microbiota to disease development and progression, the mechanisms by which specific bacterial communities influence this process remain incompletely understood and poorly characterized. In particular, the functional interactions between dysbiotic microbial populations, their metabolic products, and the host epithelium require further investigation to clarify how these factors may promote epithelial dysfunction and tumorigenesis.

For this reason, the aim of this project is to explore the potential role of metabolites produced by bacterial strains enriched in adenoma-associated mucosa in establishing and maintaining a pro-tumorigenic environment, either through direct effects on host cells or by disrupting epithelial barrier integrity and promoting inflammation.

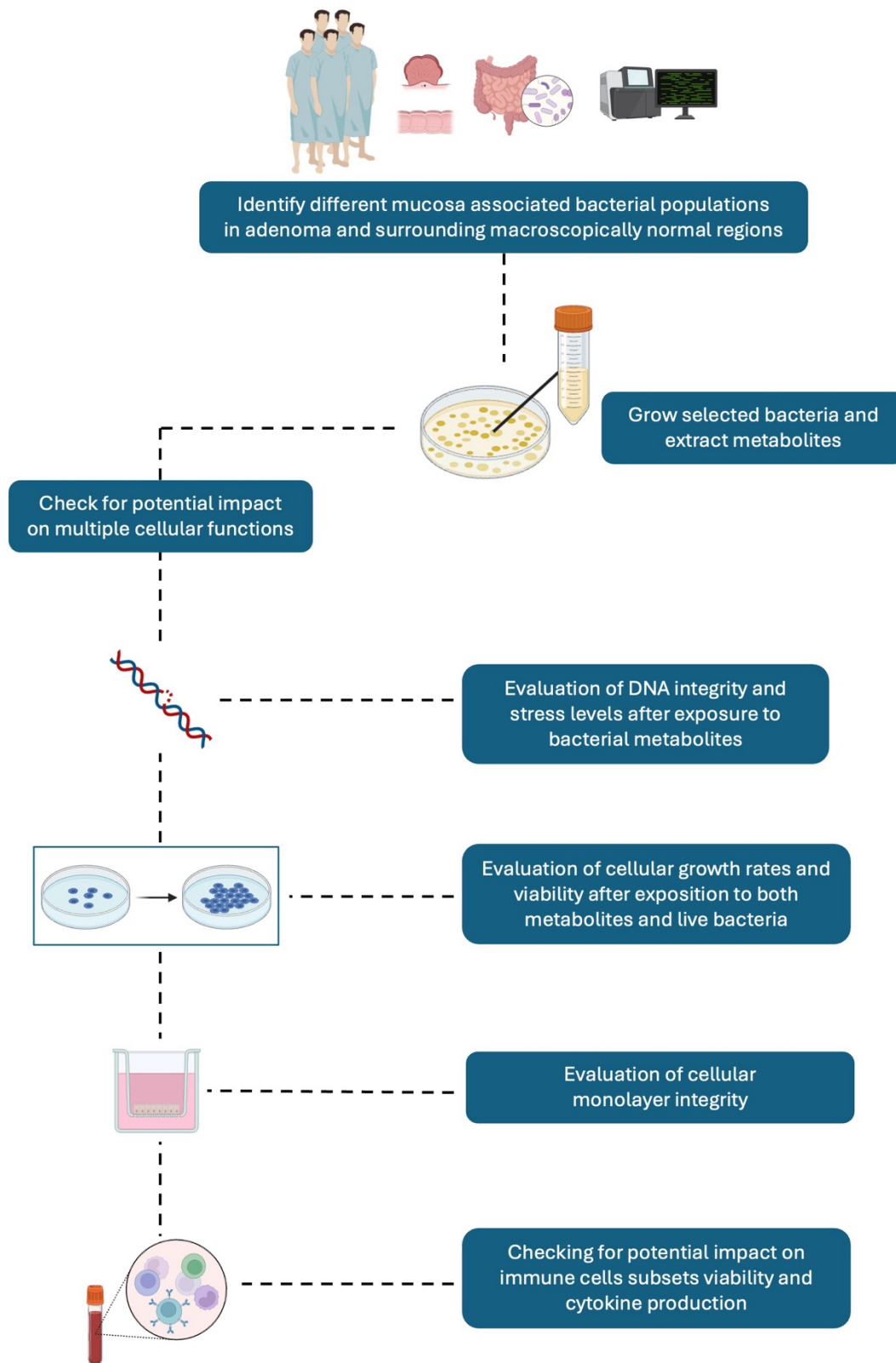


Figure 7. **Experimental design and workflow of the study.** The scheme provides a brief overview of the workflow, starting from patient-derived samples used to identify bacterial populations enriched in adenomas, followed by assessment of their effects on cellular processes potentially associated with cancer development, including DNA damage and increased proliferation rates.

3. Materials and Methods

3.1. Cell lines

For this study, we used 3 epithelial cell lines:

- CACO2, an adherent cell line isolated from colon tissue derived from a patient with colorectal adenocarcinoma. These cells express characteristics of enterocytic differentiation upon confluence.
- HT29, an adherent epithelial cell line isolated from a primary tumor obtained from a patient with colorectal adenocarcinoma. These cells express characteristics of mature intestinal cells and form a tight monolayer with an apical brush border exhibiting a similarity to enterocytes.
- NCM460D, an epithelial cell line derived from the normal colon of a 68-year-old Hispanic male and It was not infected or transfected with any exogenous genetic information.

To perform experiments on immune system response, we used PBMCs (Peripheral Blood Mononuclear Cells) which include T cells, B cells and Natural Killer Cells.

CACO2 and HT29 cell lines were maintained in Dulbecco's Modified Essential Medium (DMEM). NCM460D were cultured in Ham's F12 Nutrient Mix. PBMCs were collected from buffy coats and kept in Roswell Park Memorial Institute 1640 Medium (RPMI). All media were enriched with 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin, 1% Sodium Pyruvate (NAP), 1% Non-Essential Amino Acids (NEAA). All cells were incubated at 37° in a humidified atmosphere with 5% CO₂.

3.2. Mucus Sampling Method

Mucus samples from the adenoma surface and from the macroscopically normal tissue were collected from 5 patients undergoing elective polypectomy during dedicated endoscopy sections. The sampling method involved a newly developed endoscopic technique, which implements a cap positioned on the tip of a conventional endoscope. The cap uses a gentle aspiration to isolate the sampling area, creating a virtual chamber (**Fig 8b**). A dedicated two-ways catheter is connected to the virtual chamber through the operative channel of the scope.

The double catheter is then used to flush the mucosal aspirate directly into sterile collection tubes utilizing a pre-loaded saline solution (**Fig 8a**).

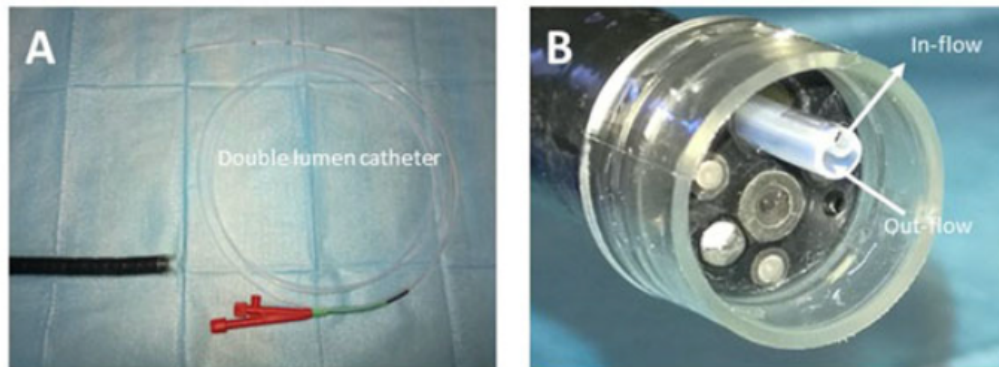


Figure 8. a) picture of the double lumen catheter used for flushing mucosal aspirates. b) detail of the newly developed cap that allows to isolate mucosal portions of interest

3.3. Identification of Bacteria Enriched in the Adenoma Associated Mucosa

DNA from mucus samples was extracted using with G NOME DNA isolation kit (MP Biomedicals) following the manufacturer's instructions. The 16S rRNA gene was initially amplified by interest-specific primers targeting V3 (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3'), V4 (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3') regions coupled with overhang adapters. The reaction was carried out in 25 μ L volume containing 6 ng/ μ L microbial DNA, 1 μ M of each primer, and 2x KAPA HiFi HotStart ReadyMix (Roche, Italy). The following PCR program was used: initial denaturation at 95 $^{\circ}$ C for 3 min, followed by 25 cycles consisting of denaturation (95 $^{\circ}$ C for 30 s), annealing (55 $^{\circ}$ C for 30 s) and extension (72 $^{\circ}$ C for 30 s), and a final extension step at 72 $^{\circ}$ C for 5 min. PCR products were analysed by 1% agarose gel electrophoresis for quantity and quality. Expected product size after the Amplicon PCR step is about 550 bp. DNA samples resulting from the PCR step were amplified with dual-index primers using Nextera Xt Index Kit V2 Set A (Illumina, San Diego, CA). Each sample possessed specific barcode sequences at the 5'- and 3'-end of the PCR amplicon for sample identification in the pooled library. Library concentration and exact product size were measured using the Agilent 2100 Bioanalyzer System (Agilent, Santa Clara, CA, USA). A 20 nM pooled library and a PhiX control v3 (20 nM) (Illumina, San Diego, CA) were mixed with 0.2 N fresh NaOH and hybridization buffer HT1 (Illumina) to produce a final concentration at 10 pM each. The resulting library was mixed with the PhiX control v3 (5%, v/v) (Illumina) and injected on a

Miseq Reagent Nano Kit V2 500 Cycles to obtain a paired-end 2×150 bp sequencing. The 250 bp 16S reads were processed through QIIME2 (version 2018.11) as follows: (1) Following visualization of demultiplexed samples and the average quality across the reads, quality filtering, dereplicating, and chimera filtering were performed using the DADA2 plugin within QIIME2, with the following parameters: `-p-trunc-len-f 250`, `-p-trunc-len-r 242`, `-p-trim-left-f 0`, `-p-trim-left-r 0`, `-p-trunc-q 2`, and using consensus as the chimera filtering method; (2) a phylogenetic tree was generated for downstream core diversity analyses using Multiple Alignment using Fast Fourier Transform (MAFFT) for multi-sequence alignment and IQ-TREE ultrafast bootstrap for tree generation, with the following parameters: `-p-perturb-nni-strength 0.2` and `-p-stop-iter 200`; (3) alpha and beta diversity core metrics were assessed using the qiime diversity command; (4) taxonomy classification was performed using the qiime feature-classifier classify-sklearn feature, using a Naïve Bayes classifier trained on SILVA database (release 132, 99% operational Taxonomic Units (OTUs) full-length 16S rRNA sequences), available from the QIIME2 website (<https://docs.qiime2.org/2018.11/data-resources/>). Taxonomy count tables for phylum, family, and species were generated after taxonomy classification and exported for manipulation and plotting outside of QIIME2.

The QIIME2 frequency table, taxonomy table, phylogenetic tree, and metadata were imported as “physeq” R objects (R Core Team, 2013) using the phyloseq package. Principal coordinate analysis of unweighted UniFrac beta-diversity distance matrix exported from QIIME2 and alpha diversity measurements of physeq object were determined by microbiomeSeq, with alpha-diversity pairwise comparisons assessed by ANOVA. Beta-dispersion pairwise comparisons on unweighted UniFrac values was performed in R using betadisper in vegan. Pairwise PERMANOVA of unweighted UniFrac values was performed separately using pairwise Adonis in R using the default Bonferroni *p*-value correction (<https://github.com/pmartinezarbizu/pairwiseAdonis>). Comparison of abundances at different taxonomic levels was visualized with bar plots using ggplot2 using the exported taxonomy tables from QIIME2, and Kruskal-Wallis for significance testing.

3.4. Bacterial culture conditions

All bacterial strains were acquired from the Leibniz Institute (DSMZ) and maintained in BD DIFCO Columbia Broth (294420) liquid culture under anaerobic conditions at 37° and 5% CO₂.

Plating for growth curves and general colony analysis was done on Columbia Agar plates enriched with 5% sheep blood (Thermo Fischer Scientific – PB5039A).

3.5. Bacterial Supernatants and Live Bacteria Handling

Bacterial strains were grown to exponential phase to minimize background nuclease activity associated with DNases released during stationary phase. The tubes containing the liquid cultures were centrifuged at maximal speed for 20 minutes in order to collect both pelleted live bacteria and their cell-free supernatants (CFS). Supernatants were then filtered with 0,22µm filters to avoid contaminations since bacteria do not grow in sterile conditions inside the anaerobic hood. Bacterial CFSs were adjusted to a neutral pH to assess the actual effect of their metabolites, rather than the influence of acidic or basic pH.

Experiments with bacterial supernatants were conducted adding 20% of bacterial CFS to cell culture media. A 20% (v/v) concentration was used to ensure sufficient exposure to bacterial secreted factors while maintaining appropriate culture conditions for host cells. Live bacteria were resuspended in PBS and added to cells at bacteria-to-cell ratios of 100:1, 50:1, and 20:1. The final volume was adjusted to maintain a constant composition of 20% PBS and 80% cell culture medium.

3.6. Comet Assay

To assess DNA damage on epithelial cells, the Oxiselect Comet Assay Kit (#MBS168415-75) was used. The comet assay was performed according to the manufacturer's instructions. Cells were seeded in a 48 well plate and then treated with bacterial CFSs for 24h. Cells were washed with PBS 1x, treated with trypsin and collected by gentle scraping. Cells were then mixed with pre-heated agarose in a 1/10 ratio, the mixtures were transferred onto the provided comet slides and allowed to solidify at 4°C for 30 min. Cells were lysed at 4°C, and electrophoresis was performed with a cold alkaline electrophoresis buffer at low voltage for 15 min. The comet slides were rinsed in pre-chilled H₂O for 5 min three times and were fixed with 70% cold ethanol for 5 min. The air-dried slides were stained with Vista Green DNA Dye and viewed using a fluorescence microscope in FITC channel.

3.7. pUC19 Damage Assay

To observe direct DNA damage pUC19 plasmid (New England Biolabs), constituted by 2685 base pairs, was linearized using EcoRI-HF (New England Biolabs) following the manufacturer's instructions and the plasmid was treated with bacterial medium and supernatants for 24h. The treated plasmid was purified and extracted using the Monarch® DNA Gel Extraction Kit 250-Preps T1020L (New England Biolabs). Purified linearized DNA samples were eluted in 20 µl DEPC-treated water. The purified plasmid was quantified using the NanoDrop spectrophotometer (Thermo Scientific NanoDrop OneC UV-Vis Spectrophotometer). For each condition, 150 ng of DNA was mixed with Orange Loading Dye 10x (LI-CORbio) and run in native conditions. Gel electrophoresis was performed using 1% agarose TAE gels for 10 min at 70 V and then for 80 min at 120 V. Gels were stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher). The ladder used is a 1Kb ladder (Solis Biodyne).

DNA damages that can be identified:

- Alkylation destabilizes glycosidic bonds, leading to depurination and fragmentation, which appear as faster-migration bands.
- DNase-like activity produces extensive fragmentation under both native and denaturing conditions, visible as a continuous smear on the gel.
- Restriction enzyme-like activity generates discrete fragments under native conditions, and following denaturation produces smaller fragments that may appear similar to DNase activity.

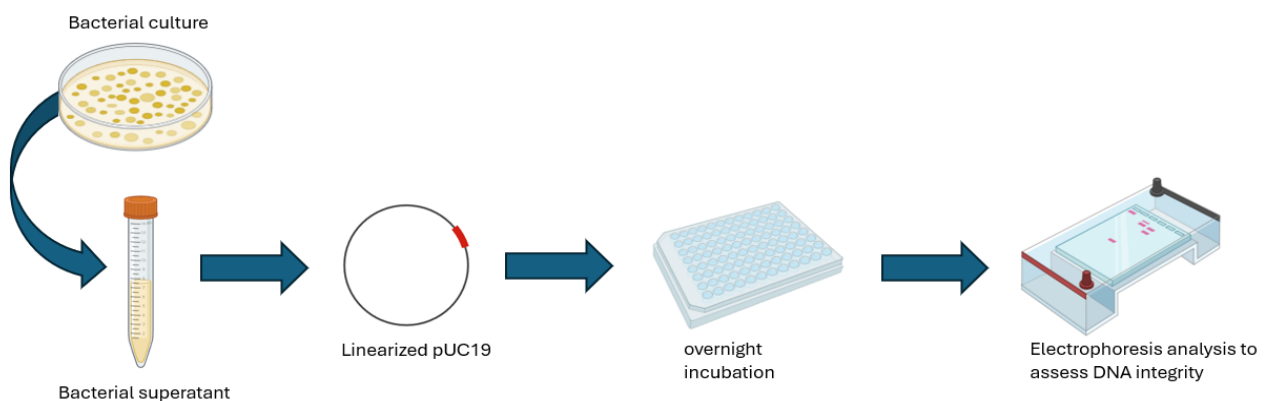


Figure 9. Schematic representation of the pUC19 Assay workflow

3.8. 8-Hydroxy-2-deoxyguanosine (8-OHdG) ELISA Assay

8-Hydroxy-2-deoxyguanosine is a ubiquitous marker of oxidative stress. Free radicals and other reactive species are normally produced during physiological cellular processes, and they are usually kept in check by various deoxidant and repair systems. When contact with Reactive Oxygen Species (ROS) is prolonged or unregulated, it becomes harmful. If DNA damage occurs, repair systems will excise single bases and release deoxynucleotides, which can then be measured to assess the level of DNA damage related to ROS.

The assay was performed according to the manufacturer's instructions. Cells were seeded in a 24 well plate and treated with 20% bacterial CFS for 24h and 72h. Cell media were collected and frozen at -20°. Standards were prepared by doing serial dilutions of the provided standard solution and assay buffer, with 9 total dilutions starting from 8000 pg/ml to 0 pg/ml. Samples were used without dilution. 50ul of samples were added to the goat anti-rabbit igG antibody-coated wells. Afterwards, 25ul of 8-Hydroxy-2'-deoxyguanosine Conjugate and 25ul of 8-Hydroxy-2'-deoxyguanosine Antibody were added to each well and left to incubate for 2 hours while shaking. Wells were washed 3 times and then incubated for 30 minutes with 100ul of TMB substrate. In the end, 50ul of Stop Solution were added to each well and the plate was read at 450nm. To generate the standard curve, a four-parameter algorithm was used.

3.9. XTT Viability Assay

The CyQUANT XTT Cell Viability Assay (Invitrogen #X12223) is a colorimetric assay used to measure the metabolic activity of cells, which can be used as an indicator of cell viability, proliferation and health. Live cells convert a tetrazolium-based salt reagent to a coloured formazan product, which can be measured with a plate reader. The absorbance correlates with cell viability and proliferation. The assay provides two solutions: the XTT reagent (2,3-bis-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) and an electron coupling reagent meant to improve sensibility and consistency of the procedure.

Cells were seeded in a 96 well plate and treated with 20% bacterial CFS for 24h (100ul total volume). According to manufacturer's instructions, 70ul of mixed reagent solution were added directed into the wells without removing cell media and incubated for 4 hours at 37°. The plate was then read at 450nm and 660nm. The second measurement is used to remove background noise.

3.10. Trans-Epithelial Electrical Resistance (TEER)

3×10^4 CACO-2 cells per well were seeded in transwell 24 well plate inserts (Costar). After one week they reached confluence and formed an epithelial monolayer. Every two days the culture medium was changed. TEER measurement was done after 24h, 36h, 48h, 60h and 72h using the Millicell® ERS 3.0 Digital Voltohmmeter (Millipore). To evaluate the monolayer permeability the resistance of the blank (only insert) was subtracted from the resistance of the sample. The resistance over the area of the insert was normalized by the following equation: $R = (R_{TOT} - R_{BLANK}) \cdot \text{area}_{WELL}$. Then delta changes (Δ) for each sample were computed at T24, T36, T48, T60, T72 (relative to T0), removing sample resistance at time T0 from sample resistance at T24, T36, T48, T60, T72.

3.11. Cell proliferation analysis

Cell proliferation monitoring was performed on the Incucyte S3 Live-cell imaging and analysis system by Sartorius. This instrument is an incubator with a built-in camera system which allows the operator to acquire images of whole plates or single wells at chosen time intervals, so that cellular growth is essentially live monitored.

The experiment was performed in a 96 well plate. CACO2 and HT29 cells were seeded with a density of 5k cells/well, while NCM460D cells were seeded with a density of 10k cells/well to compensate for slower proliferation rates. 5 mages for each well have been acquired at 6 hours intervals for a total time of 72 hours. The instrument comes with a proprietary software that allows the operator to create analysis masks that fit the specific cell type being used. These masks outline cellular borders and are used by the software to calculate cellular confluency based on the amount of well surface that is being covered by cells. The analysis was performed normalizing cell confluency to the T0 of each sample – this enhances sample specificity and rules out potential differences in initial seeding density.

3.12. BrdU Assay

Bromodeoxyuridine (BrdU) is a thymidine analog that replaces [3H] thymidine. BrdU is then incorporated into newly synthesized DNA strands of actively proliferating cells. Following partial

denaturation of double stranded DNA, BrdU is detected immunochemically allowing the assessment of cells which are actively synthesizing new DNA and therefore are proliferating.

For this experiment we used the Calbiochem BrdU Cell Proliferation Assay (Sigma Aldrich – QIA58). The assay was performed according to the manufacturer's instructions. Cells were seeded in a 96 well plate and left to attach overnight. In the morning, cells were marked with a BrdU label that will be incorporated into newly synthesized DNA and treated with both live bacteria and bacterial CFS. After 24 hours, cells were fixed, permeabilized and incubated for one hour with the anti-BrdU antibody. Cells were then washed 3 times and incubated for 30 minutes with the Peroxidase Goat Anti-Mouse IgG HRP Conjugate. The plate was washed again 3 times with the wash buffer and then filled completely with deionized water to ensure complete washing. TMB (3,3',5,5'-tetramethylbenzidine) was added and left to react in the dark for 15 minutes. Finally, the stop solution was added and the plate was read at 450nm and 540nm to remove background noise.

3.13. Sulforhodamine B Assay

The sulforhodamine B (SRB) Assay is a colorimetric used to measure cell density and proliferation. The SRB dye binds to basic amino acid residues of cellular proteins under acidic conditions, allowing a direct measure of cellular mass.

Cells were treated with both live bacteria and bacterial CFS for 72h. After, they were fixed directly adding 10% cold trichloroacetic acid (TCA) onto the wells without removing cell media. The plate must be incubated for at least one hour at 4°. Well contents are then removed by flooding the plate with tap water. The plate is then allowed to air dry for several hours (better if overnight) since residual water might dilute the SRB dye in the next step, altering the result. After the wells are completely dried, the SRB dye is added and incubated at room temperature for 30 minutes. After blotting the plate on paper towels, and any excess dye is washed with 1% acetic acid. The plate is again left to air dry over-night and Tris base is then added to solubilize the bound dye (around 30 minutes are needed, time is reduced if the plate is on a mixer). Finally, absorbance was read at 510nm.

3.14. Flow cytometry on PBMCs

PBMCs were extracted from donor buffy coats. 5×10^4 cells were seeded in a 96-well plate. For intracellular cytokine labelling, cells were incubated for 3 h at 37°C in RPMI + 10% FBS with PMA (50ng/ml, Merck), Ionomycin (1µg/ml, Merck), and Brefeldin A (10 µg/ml, Merck). After PMA/Ionomycin/Brefeldin A stimulation, cells were washed with PBS 1x. To analyse cell viability cells were stained with Zombie yellow dye (BV510). For extracellular staining cells were treated with following antibodies: CD3 (APC-Cyanine 7), CD4 (PE-Cyanine7), CD8 (Alexa Fluor 700). All of them were diluted 1:100 in PBS 1x. For intracellular staining cells were treated with the following antibodies: IFN γ (FITC) and TNF α (BV786).

3.15. Gas Chromatography-Mass Spectrometry (GC-MS)

Gas Chromatography-Mass Spectrometry is an analytical technique that allows separation, identification and quantification of chemical compounds in complex matrixes. The mixture is separated into its individual components, and molecular profiles are compared to available libraries to identify single molecules. Samples need to undergo a process called “derivatization” where chemical residues such as NH, OH and SH groups are substituted by R groups. For the hydroxyl group (OH), it is also important to avoid any water during the procedure, which might be present even in traces for example in freshly autoclaved glass ampoules. Derivatization is a key step to render the sample compatible with the GC-MS instrument.

The sample is mixed with Ethyl Acetate, which is used to extract compounds from aqueous samples and also to provide a homogenous reaction environment. 200ul of bacterial supernatant are mixed with 600ul of Ethyl Acetate and the sample is centrifuged for 5 minutes at max speed. Transparent supernatant is transferred into a 2ml dark glass bottle, and the process is repeated again with another 600ul of Ethyl Acetate. The bottle is then transferred at 50° under a constant nitrogen flow for about an hour to desiccate the sample. Finally, the sample is resuspended in 50-100ul of *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide activated I (MTSFA) - Sigma Aldrich #50992 – which is the derivatizing agent and are analysed through GC-MS.

Qualitative analysis was performed by an Agilent 7890B gas chromatograph equipped with an Agilent 7693A autosampler, fitted with an Agilent 7000D single quadrupole mass detector (Agilent, Palo Alto, CA, USA). Chromatographic separations were performed on a HP-5MS capillary column: 30 m x 0.25 mm i.d. with a film thickness of 0.25 µm (Agilent J & W GC column, Palo Alto, CA, USA).

1 μ L of sample was injected in splitless mode into the injector maintained at temperature of 250 °C. Helium at constant flow of 1 mL/min was used as carrier gas. The oven temperature ramp was as follows: from 70 °C for 1 min, to 260 °C at a rate of 10 °C/min and held isothermal for 2 min, from 260 °C to 280 °C at a rate of 30 °C/min for 2 min and from 280 °C to 330 °C at 50 °C/min maintained for 5 min as post run. MS transfer line, source and quadrupole were set at 280 °C, 280 °C and 150 °C, respectively. Electron ionization source (EI), operating at 70 eV, was applied for the experiments in full scan acquisition mode range of 50-550 m/z.

Chromatograms were acquired and analysed through the Agilent MassHunter Workstation Software (Version 10.2). The software relies on multiple analysis modules that allow to identify single metabolites and their respective intensities from a complex matrix. Peak identification and deconvolution were performed using the Unknown Analysis module. Metabolites were identified by comparing obtained mass spectra with the data available on the NIST2020 (Gaithersburg, MD, USA) commercial database. Finally, relative abundance of each metabolite was obtained through the Quantitative Analysis module.

3.16. Statistical Analysis

Results were expressed as mean value $\pm$ SD. Statistical analysis of data was performed using Unpaired t-test or One Way Anova for multiple comparisons. Differences were considered statistically significant when $P \leq 0.05$.

4. Results

4.1 The mucosa of patients with colorectal adenomas showed enrichment of *Ligilactobacillus apodemi*, *Phascolarctobacterium faecium* and *Oscillibacter ruminantium*

The main endpoint of this study was to identify and functionally characterize enriched bacterial strains in the adenoma associated mucosa to potentially find a direct link between preponderant populations and the transition from normal to dysplastic epithelium. To identify enriched populations, mucus aspirates from the adenoma surface and macroscopically normal surrounding tissue were obtained from 5 patients undergoing selective polypectomy during dedicated endoscopy sessions. Mucus-associated microbiota was taxonomically profiled through 16S rRNA sequencing and resulting data were processed using DADA2 pipeline to reconstruct amplicon sequence variants (ASVs). Analysis revealed differences in community structure between the two microenvironments: taxonomic composition showed that *Ligilactobacillus apodemi*, *Phascolarctobacterium faecium*, *Oscillibacter ruminantium* were enriched in the adenoma-associated communities, whereas *Anaerococcus vaginalis*, *Oscillibacter valericigines* and *Bacteroides vulgatus* were more prevalent in normal mucosa (**Fig. 10**).

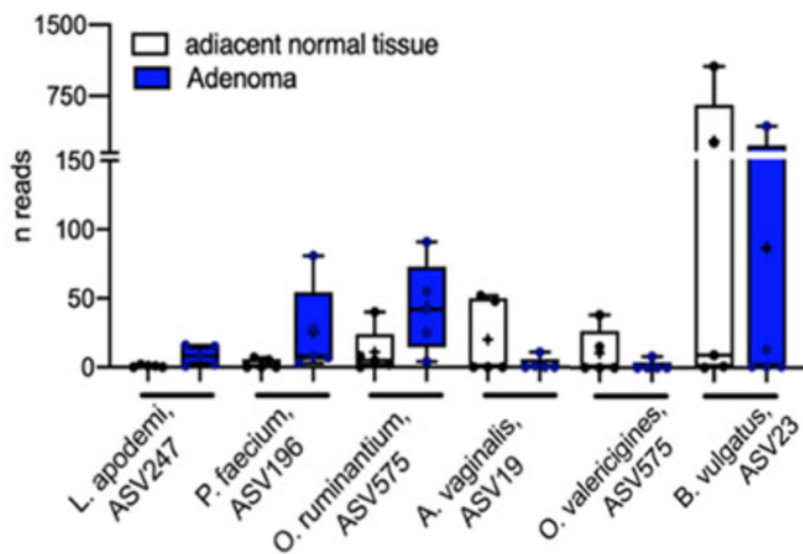


Figure 10 - Species identified through DADA2-ASV analysis of mucus-associated microbiota from adenoma tissue (blue bars) and adjacent normal tissue (white bars) in five surgical cases

4.2. Bacterial supernatants do not induce DNA damage in epithelial cells

To determine whether Cell Free Supernatants (CFSs) may play a role in cellular transformation and potentially contribute to tumorigenesis along the adenoma-adenocarcinoma sequence, we evaluated several processes implicated in tumorigenesis including DNA damage and mutation, cytotoxicity, enhanced cell proliferation, and alterations on epithelial barrier permeability.

To evaluate DNA damage in colon epithelial cells, we performed the Comet Assay. The principle of the assay is that cells are embedded in agar, and membranes are lysed so the nuclear material is exposed. Cells are then subjected to a low voltage alkaline electrophoresis so that they can migrate inside the agar creating the characteristic comet tails, from which the assay takes its name. If the DNA is not damaged, it will appear as a defined green point when observed with a fluorescent microscope, if there is damage, it will appear as a “comet”. Comet tail length (in proportion to their heads) is directly related with the amount of damage sustained. Cells were treated with 20% bacterial supernatants for 24h, and the assay was performed. No DNA damage was observed after incubation. All treatments were comparable with their respective controls for each cell line (**Fig. 11**).

After acquiring and analysing the Comet Assay images, we hypothesized that in this specific experimental setup, potential genotoxic metabolites present in the CFSs may not have been able to enter the cells. To evaluate direct genotoxic effects, we used the pUC19 plasmid from *E. coli*. In this assay the DNA is completely exposed, hence more vulnerable to toxic molecules. For this experiment, bacterial supernatants were incubated with linearized pUC19 for 24h. After incubation, the DNA was extracted and subjected to electrophoresis in native conditions. Electrophoresis allows to detect several types of damage, such as alkylation, DNase-like, and restriction enzyme-like. For alkylation and DNase like damage, the DNA band will show smearing, while for restriction enzyme-like damage, it will appear in multiple bands. In our analysis, none of the three bacterial strains exhibited DNA damage capability, as all bands were comparable with the control and showed no smearing or fragmentation (**Fig. 12**).

Both the comet assay and the pUC19 analysis were focused on DNA damage at high degrees (such as fragmentation), but it is possible that bacterial CFSs are able to induce minor forms of damage, without necessarily causing severe effects (or effects which are not detectable with the previous methods). To further assess the ability of bacterial supernatants of inducing damage, we performed an ELISA assay to detect 8-Hydroxy-2-deoxyguanosine (8-OHdG) levels, which is a byproduct of DNA repair systems. When DNA is repaired, nucleotides are released from

damaged bases and are stabilized into nucleosides. In most cases, 8-OHdG is utilized to assess reactive oxygen species (ROS) related damage. For this experiment, a secondary time point was added, in order to exclude the possibility of 24 hours being not sufficient for observing damage.

Cells were treated with bacterial CFSs for 24 and 72 hours, and the assay was performed as described in “Materials and Methods” section. In the end, we observed no significant differences between controls and treatments in both time points. At 24h, CFSs treated cells even showed lower oxidative stress levels compared to the controls. At 72h, which was used to detect potential slower induced damage, all samples showed lower levels of 8-Hydroxy-2-deoxyguanosine compared to the 24h treatment, while still exhibiting no significant difference with the control (**Fig.13**). These results allow us to exclude the possibility of severe DNA damage, since bacterial CFSs did not induce detectable damage in either cells or plasmid. We can also rule out the hypothesis of smaller DNA perturbation due to higher levels of oxidative stress, since 8-OHdG levels were comparable in all samples at both time points examined.

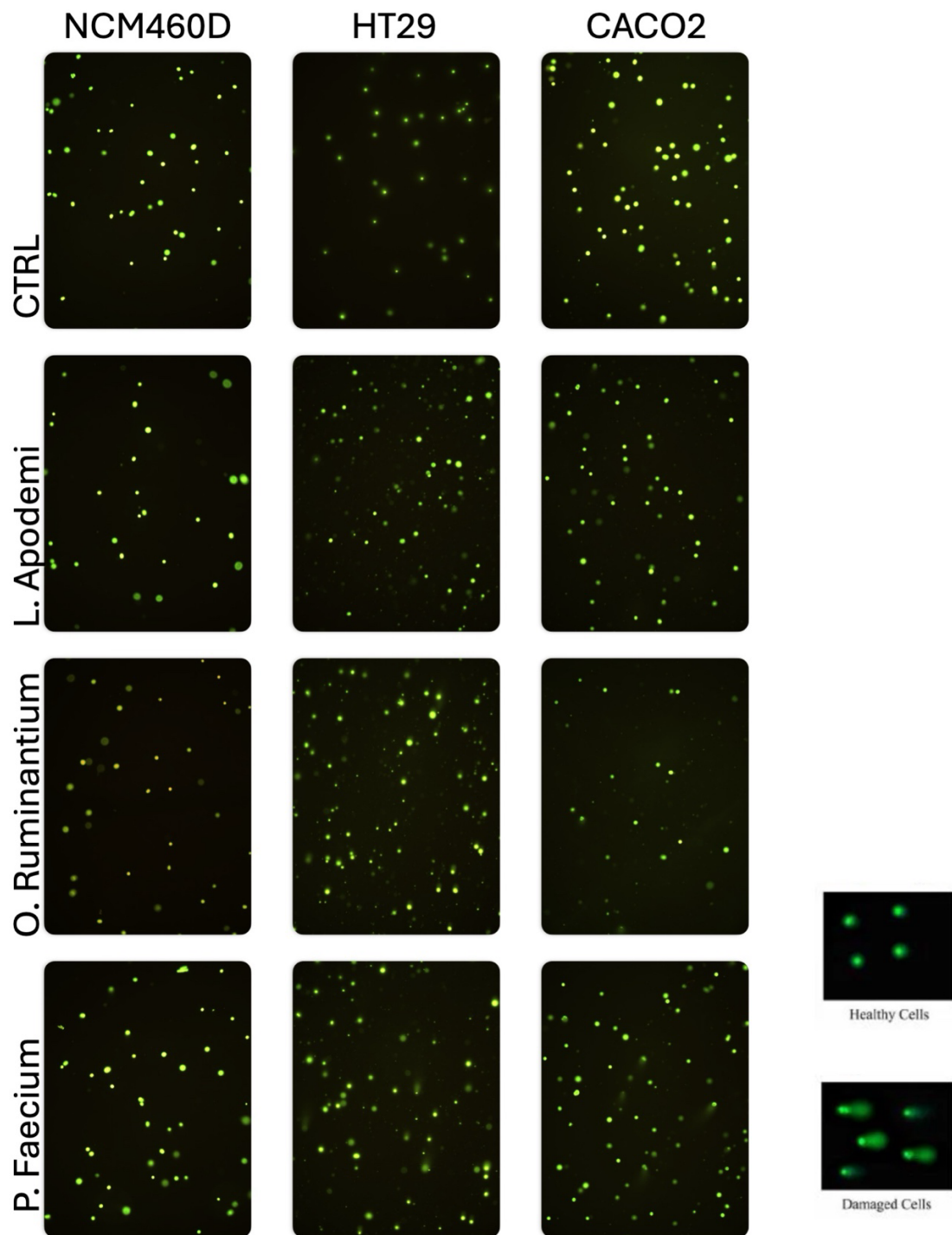


Figure 11. **Bacterial CFSs do not induce DNA damage in colon epithelial cells** - Fluorescence microscopy images of the Comet Assay performed after 24h of treatment with bacterial CFSs.

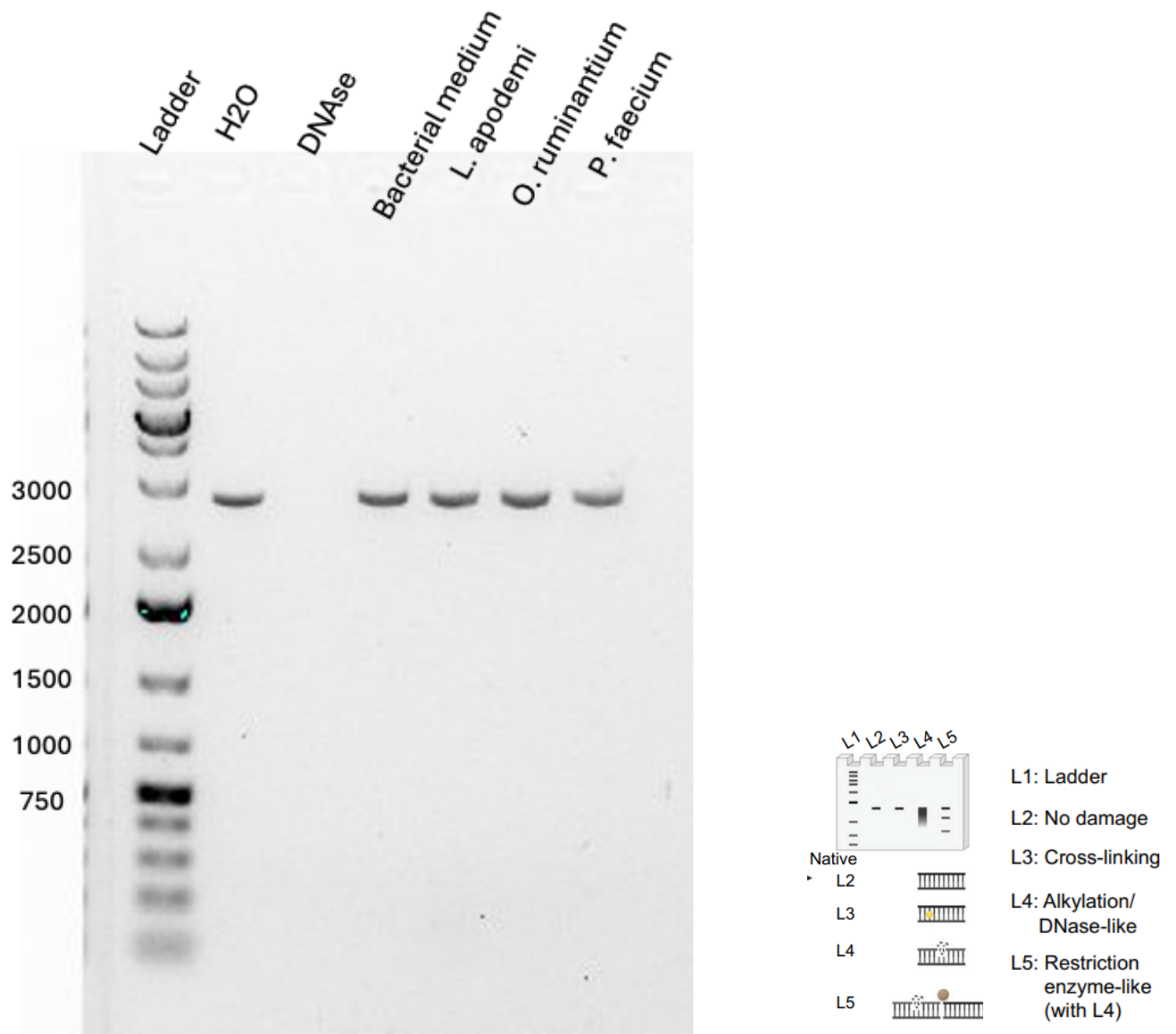


Figure 12. **Bacterial CFSs do not degrade DNA in a plasmid assay** – Native electrophoresis after 24h exposure of pUC19 to bacterial supernatant. No difference was observed in band integrity; all treatments are comparable with the control.

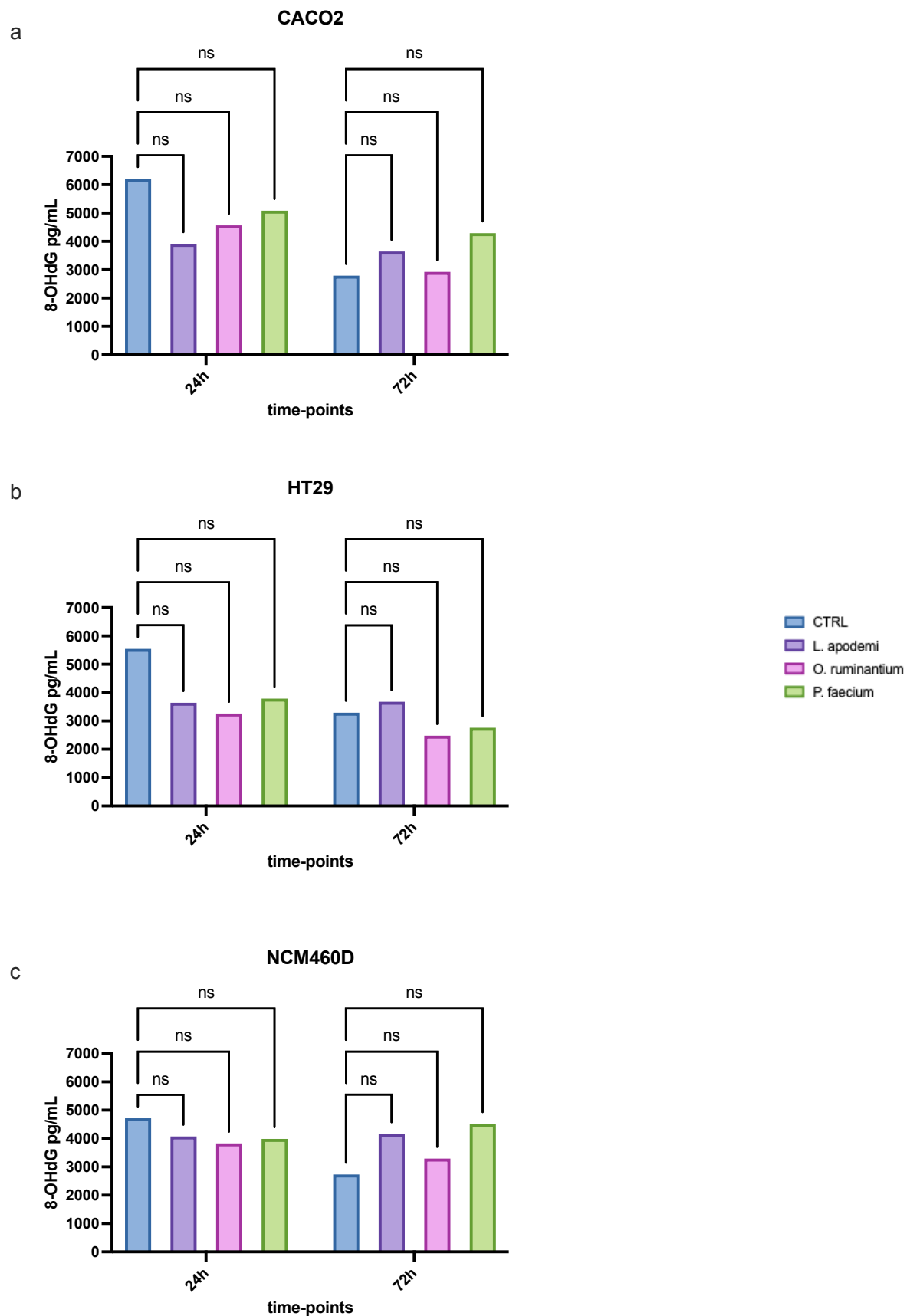


Figure 13. **Bacterial supernatants do not induce ROS related stress in colon epithelial cells** – 8-OHdG ELISA assay to detect oxygen reactive species related genomic stress in colon epithelial cells – Statistical analysis was performed using 2 Way Anova Multiple Comparisons Test (P VALUE $<0,05$). No significant difference was observed in CACO2 (a), HT29 (b) or NCM460D (c).

4.3. Cellular metabolic activity is marginally altered after bacterial CFSs exposure

DNA damage evaluation showed no direct impact of bacterial supernatants on genomic integrity, so we went on to investigate cytotoxicity as a broader measure of cellular response. Cytotoxicity was assessed using the XTT assay. In this assay, metabolically active cells convert a tetrazolium-based salt reagent to a coloured formazan product, which can be measured with a plate reader. The absorbance correlates with conversion rates, indicating higher or lower metabolic activity. Abnormal metabolic activity might indicate altered proliferation rates, with potential different outcomes: higher metabolic activity could be correlated with irregular growth rates, while lower metabolic activity might indicate cytotoxicity or cytostatic effects.

After treating the cells for 24 hours the XTT reagents were added, and cells were incubated for 4 hours. The plate was read at 450nm and 660nm, and results were analysed. Bacterial supernatants showed inconsistent effects on different cell lines: *L. apodemi* increased metabolic activity in NCM460D cells, but not in HT29 or CACO2 (**Fig. 14a**). *O. ruminantium* reduced metabolic rates in HT29 but elevated them in NCM460D (**Fig. 14b**), while *P. faecium* only affected HT29 cells by lowering their metabolic activity (**Fig. 14c**). CACO2 were the only cell type that did not respond to any of the treatments. While inconsistent among the three cell lines, XTT assay results led us to believe that higher or lower metabolic activity could be correlated with altered proliferation rates and cell numbers.

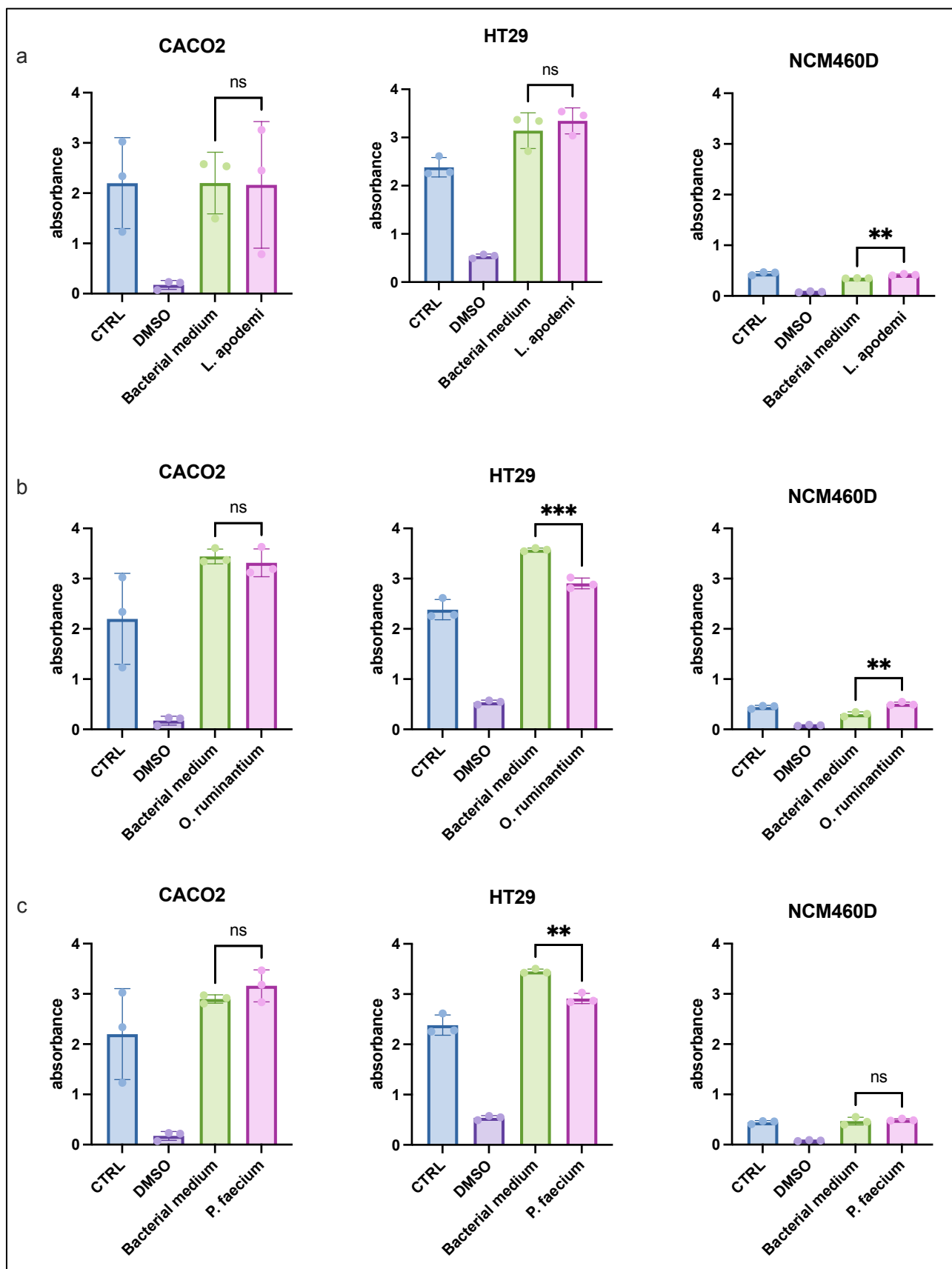


Figure 14. **Bacterial supernatants marginally influence cellular metabolic rates** – XTT analysis of colon epithelial cells after 24h of exposure to bacterial CFSs. *L. apodemi* slightly increased metabolic activity in NCM460D cells (a). *O. ruminantium* significantly reduced metabolic rates in HT29 but increased them in NCM460D cells (b). *P. faecium* has decreased metabolic activity in HT29 (c).

4.4. Cell proliferation rates were not significantly altered by bacterial CFSs or Live Bacteria

After analysing cellular metabolic activity, we went on to evaluate proliferation rates. While our hypothesis was mainly based on a metabolic context, live bacteria may potentially directly interact with and influence epithelial cells. Therefore, we monitored cell proliferation for 72h acquiring images at 6 hours intervals using the Incucyte S3 Live Imaging System.

After 72h of cell exposure to bacterial supernatants, results highlighted hindered growth for both CACO2 and HT29, but the same effect was observed also cells treated with just bacterial growth medium. NCM460D responded differently, showing higher proliferation rates when treated with both bacterial medium and bacterial CFS. This pattern was constant for *L. apodemi* (**Fig 15a**), *O. ruminantium* (**Fig. 16**), and *P. faecium* (**Fig. 17a**). Unfortunately, both observed effects seemed more related to bacterial growth medium than the supernatant itself. While untransformed cells and tumoral cells showed opposite responses to treatment, in most samples CFSs and bacterial medium are essentially overlapping, indicating that bacterial supernatants have no significant effect on cellular proliferation rates.

Live *L. apodemi* yielded similar results, proliferation analysis showed no significant difference in both tumoral and untransformed cells. Growth curves are perfectly comparable in all cell types and conditions (**Fig. 15b**). Live *P. faecium*, on the other hand, seems to enhance growth in NCM460D cells but had no effect on CACO2 and HT29 (**Fig 17b**). Live *O. ruminantium* did not allow live proliferation analysis due to its interference with the imaging system, which rendered the analysis unreliable.

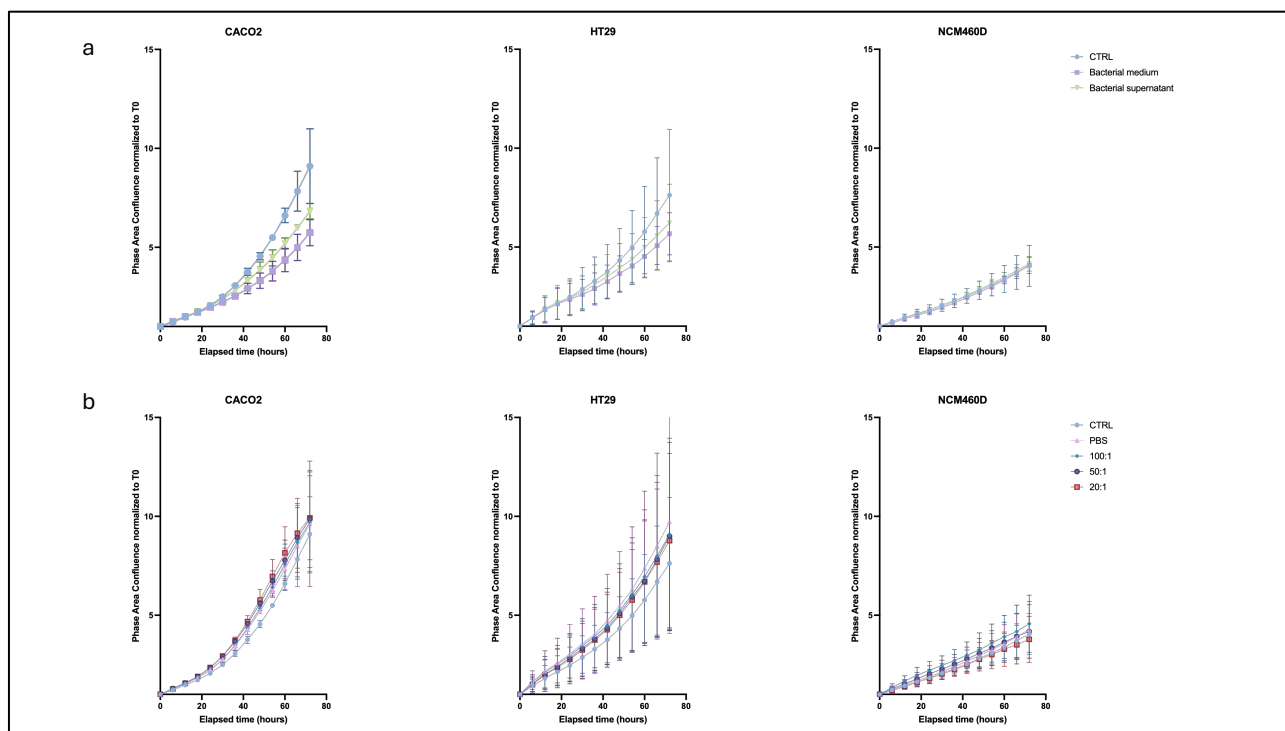


Figure 15. a) *L. apodemii* treated cells proliferation rates measured with the Incucyte S3 Live Imaging System – a) growth rates were not influenced by *L. apodemii* supernatant - b) growth rates were not influenced by live *L. apodemii*.

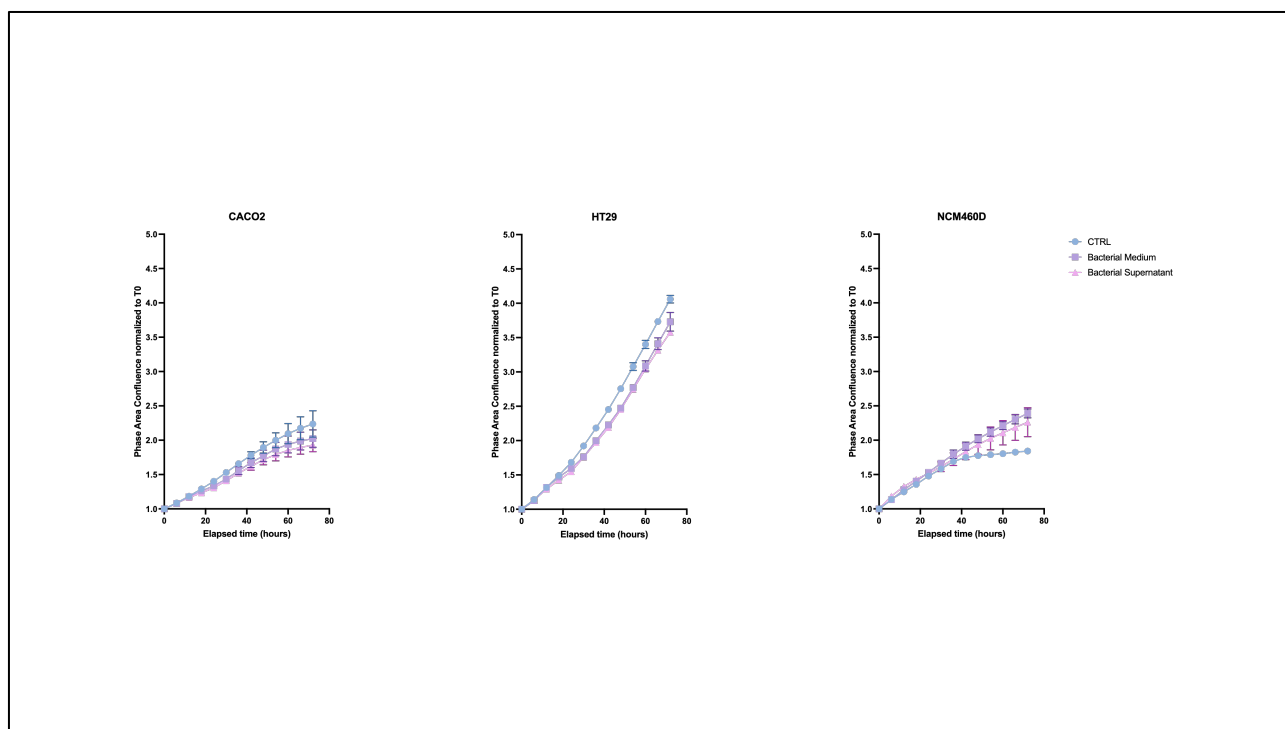


Figure 16. *O. ruminantium* CFS treated cells proliferation rates measured with the Incucyte S3 Live Imaging System – growth rates were not altered by *O. ruminantium* supernatant.

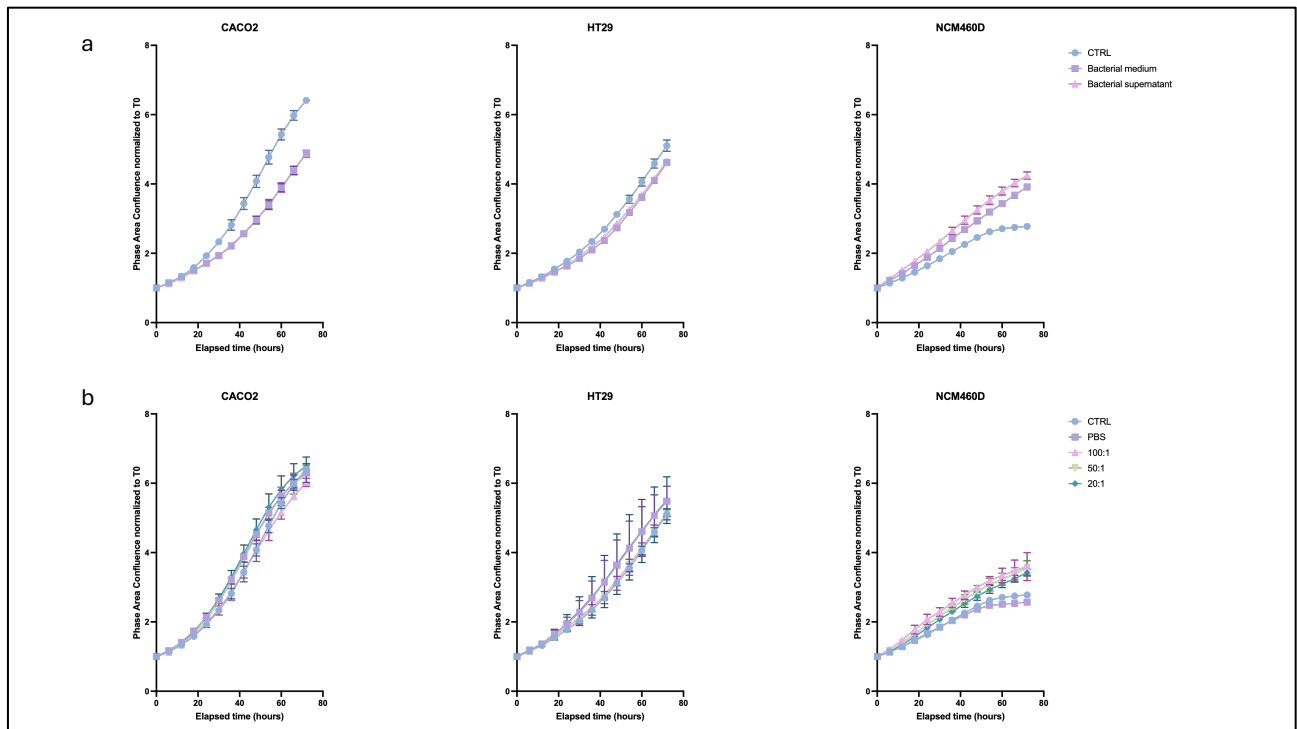


Figure 17. *P. faecium* treated cells proliferation rates monitored with the Incucyte S3 Live Imaging System – a) growth rates were not influenced by *P. faecium* supernatant – b) CACO2 and HT29 growth rates were not influenced by live *P. faecium*, while it increased proliferation rates in NCM460D.

To further explore cellular growth, we performed the BrdU (Bromodeoxyuridine) Assay and the SRB (Sulforhodamine B) Assay. BrdU is a thymidine analogue that gets incorporated into newly synthesized DNA during the S-phase of cell cycle, allowing to measure proliferation. SRB is a fluorescent dye that binds aminoacidic residues in cellular protein and is used to measure total cell mass. BrdU was measured after 24h to detect any initial boost, while SRB was measured after 72h to evaluate differences in total growth.

After treatment with bacterial supernatants, no significant difference was observed in BrdU incorporation, indicating that bacterial CFSs do not increase DNA replication in colon epithelial cells, as no discrepancy was detected in *L. apodemi* (**Fig. 18a**), *O. ruminantium* (**Fig. 19a**) or *P. faecium* supernatant treated cells (**Fig. 20a**). Live *L. apodemi* BrdU analysis showed slightly reduced DNA synthesis at a ratio of 100:1 for HT29 and a ratio of 20:1 for CACO2, while no significant effect was observed for NCM460D cells (**fig. 18b**). BrdU incorporation after live *O. ruminantium* exposure revealed decreased levels for 100:1 and 20:1 ratios in HT29 cells, while no significant difference was observed with 50:1. No statistically relevant difference was detected in CACO2 and NCM460D (**Fig. 19b**). Live *P. faecium* slightly decreased DNA synthesis in all cell lines, but statistical significance was only reached in NCM460D cells (**Fig. 20b**).

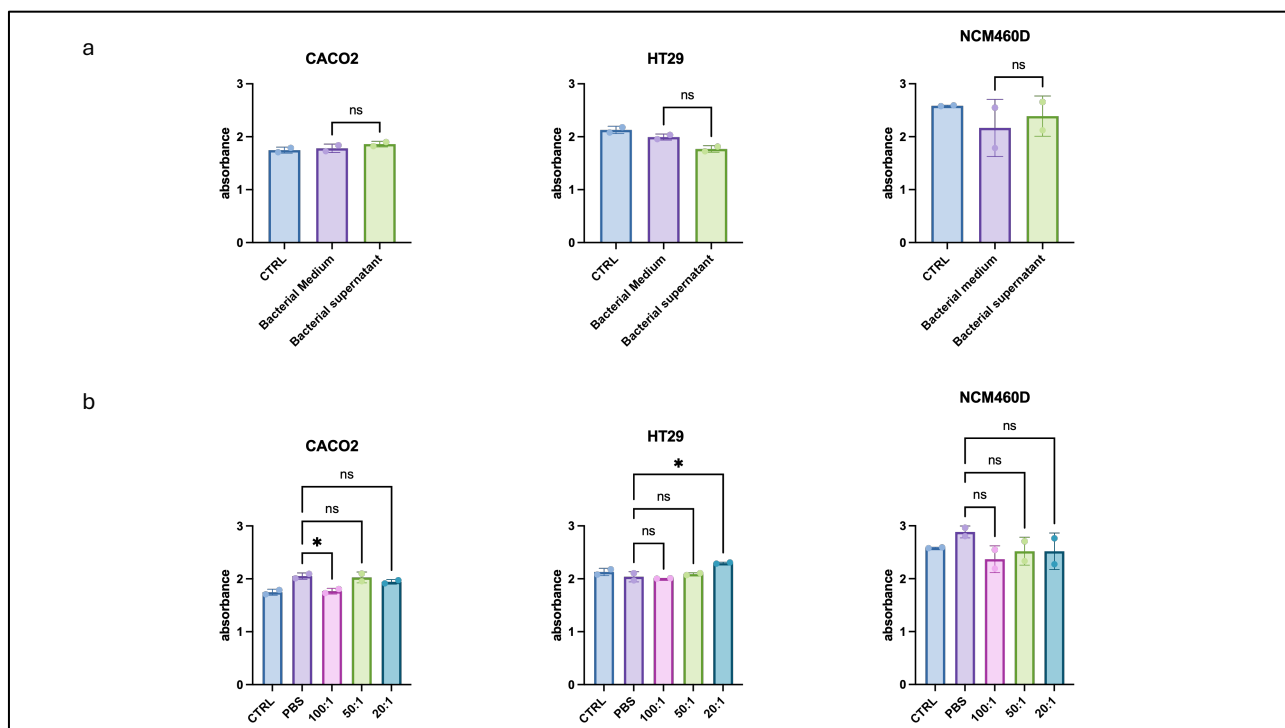


Figure 18. *L. apodemii* treated cells BrdU incorporation assay – a) *L. apodemii* supernatant did not produce any significant increase in DNA replication – b) Live *L. apodemii* slightly reduced DNA replication in CACO2 cells at 100:1 ratio while it increased it in HT29 cells at a 20:1 ratio. Statistics were performed using Unpaired t test for data set a and Ordinary One-Way Anova Multiple Comparisons Test (Dunnet's Correction) for data set b – For both data sets, statistical relevance was considered at P Value <0,05.

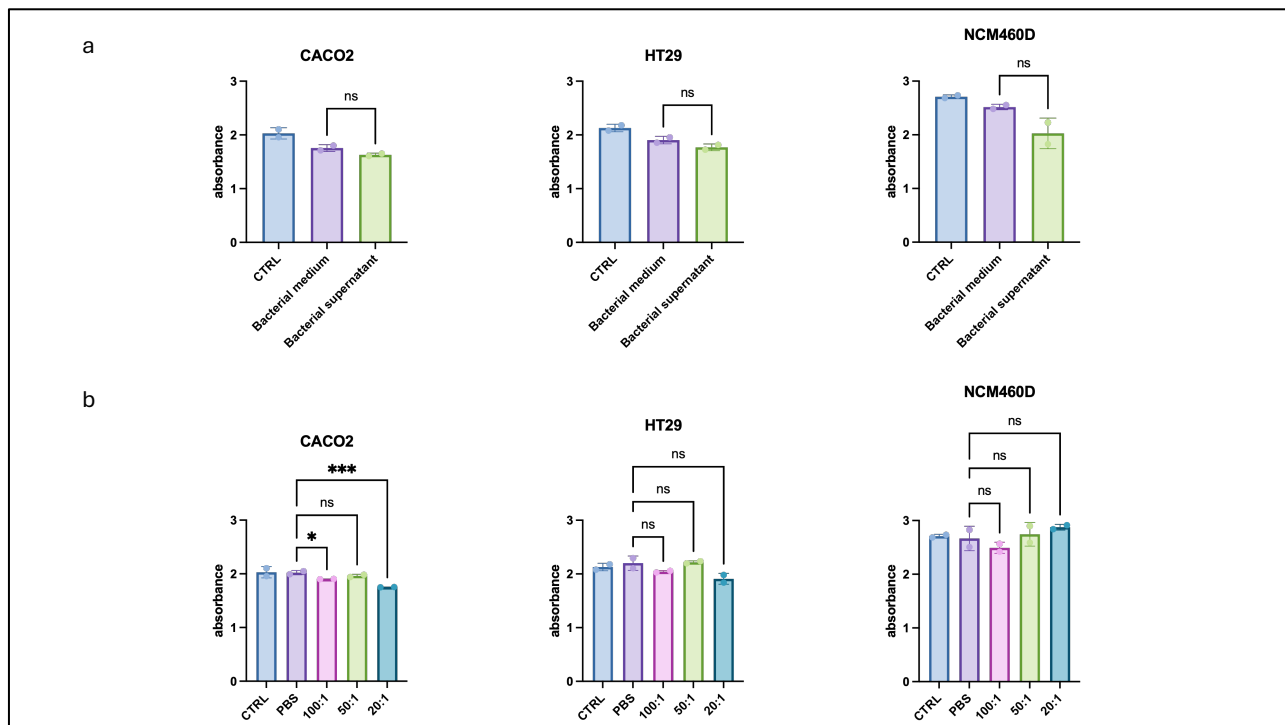


Figure 19. *O. ruminantium* treated cells BrdU Incorporation Assay – a) *O. ruminantium* supernatant did not produce any significant change in DNA replication. B) Live *O. ruminantium* decreased DNA replication in CACO2 cells at 100:1 and 20:1 but not at 50:1. Statistics were performed using Unpaired t test for data set a and Ordinary One-Way Anova Multiple Comparisons Test (Dunnet's Correction) for data set b – For both data sets, statistical relevance was considered at P Value <0,05.

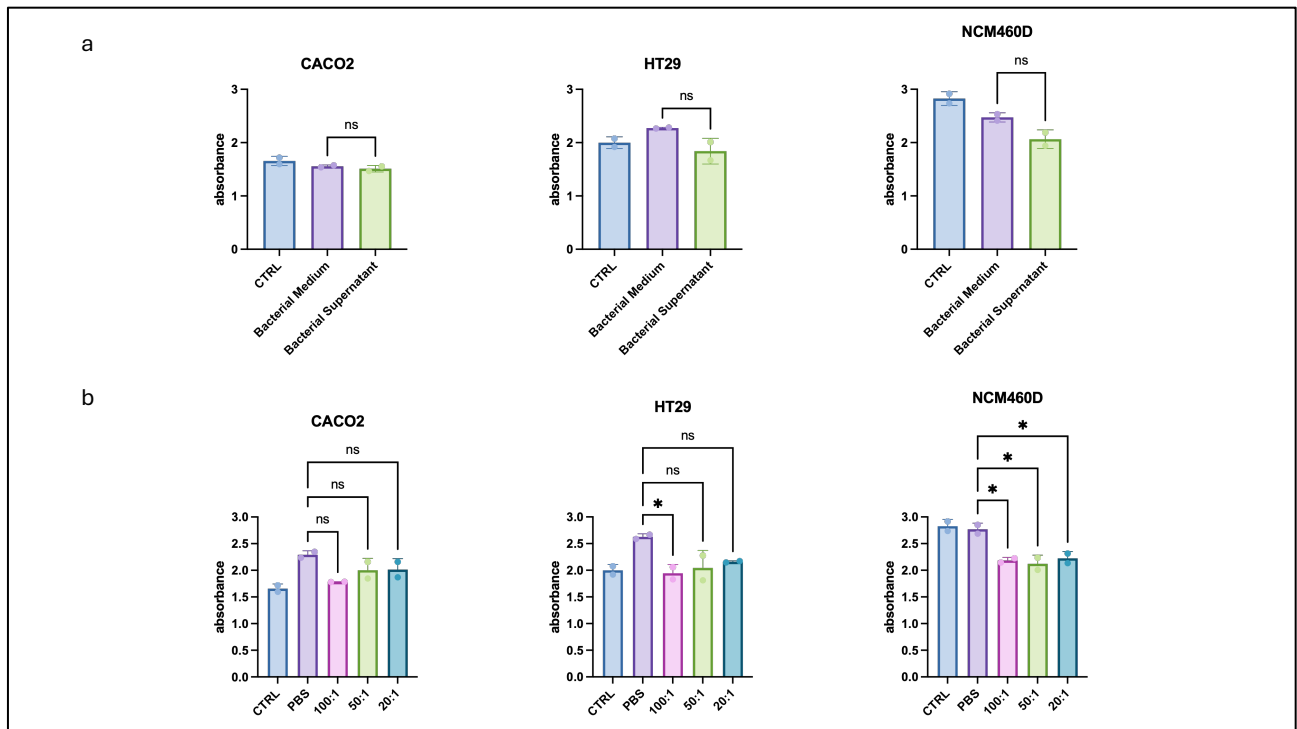


Figure 20. *P. faecium* treated cells BrdU Incorporation Assay – a) *P. faecium* supernatant did not alter DNA replication in treated cells – b) Live *P. faecium* slightly decreased DNA replication in all cell lines, statistical relevance was achieved in CACO2 cells at 100:1 ratio and in all conditions for NCM460D. Statistics were performed using Unpaired t test for data set a and Ordinary One-Way Anova Multiple Comparisons Test (Dunnet's Correction) for data set b – For both data sets, statistical relevance was considered at P Value <0,05.

SRB analysis after CFSs exposure shows no significant difference in cell mass for *O. ruminantium* (**Fig. 22a**) and *P. faecium* treated cells (**Fig. 23a**). For *L. apodemii* supernatant, CACO2 and HT29 still showed no difference, while NCM460D cells were moderately decreased after supernatant exposure (**Fig. 21a**). Live *L. apodemii* SRB analysis revealed no significant impact on CACO2 and HT29, while NCM460D showed (even if statistically relevant) a small decrease in cellular mass (**Fig. 21b**). For Live *O. ruminantium*, total cellular mass was comparable in all treatments for CACO2 and HT29, while NCM460D cells showed a significant decrease at a 100:1 ratio and a slight increase at 50:1 and 20:1 ratios (**Fig. 22b**). SRB analysis for live *P. faecium* yielded perfectly compatible results with the proliferation monitoring, as no effect was detected on CACO2 and HT29, but a significant increase was observed on NCM460D cells (**Fig. 23b**).

Overall, while some samples reached statistical significance, neither *L. apodemii* or *O. ruminantium* and their metabolites were able to induce notable changes in cell proliferation rates. On the contrary, while *P. faecium* supernatant yielded no effect, live *P. faecium* was able to increase growth rates of NCM460D cells, as confirmed by both proliferation monitoring and SRB analysis. BrdU analysis, on the other hand, suggested an initial decrease in new DNA synthesis,

but this is disputed by regular growth curves seen in proliferation monitoring. This difference in results is yet to be clarified.

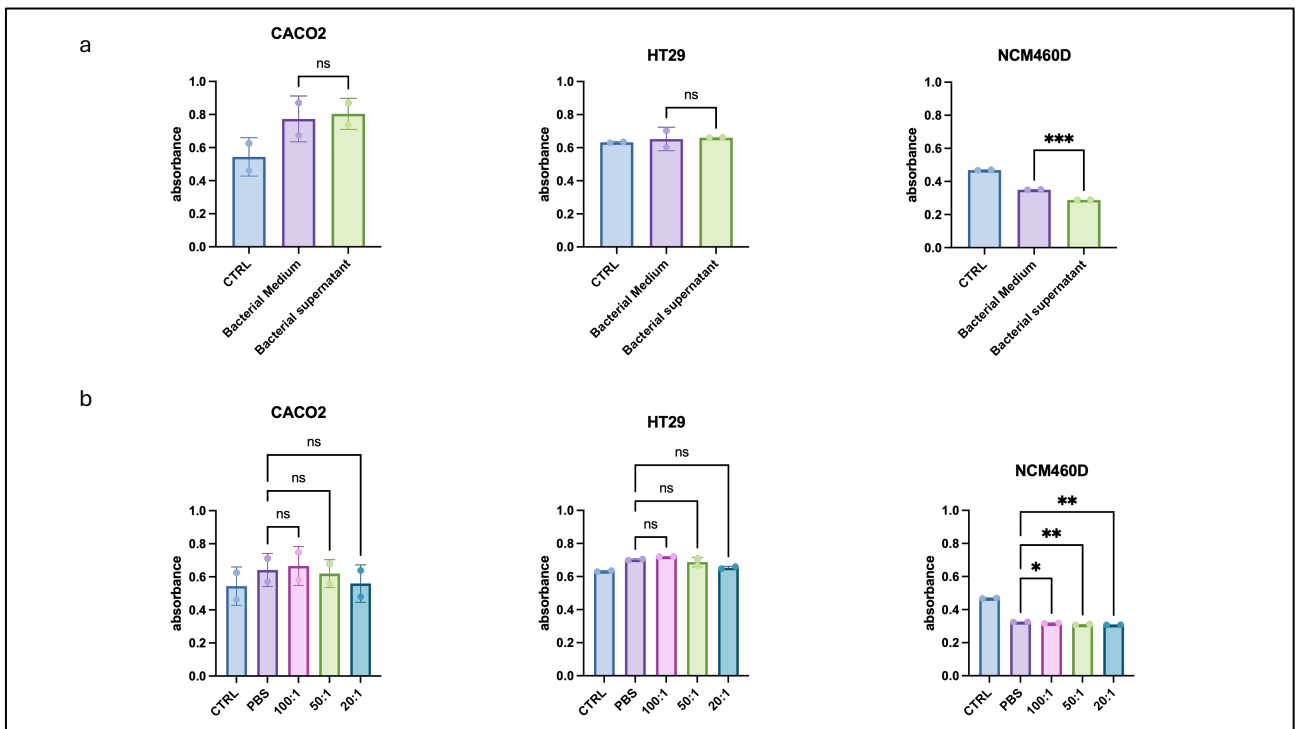


Figure 21. SRB evaluation of *L. apodemi* treated cells – a) cellular mass levels were slightly reduced by *L. apodemi* supernatant only in NCM460D cells – b) Live *L. apodemi* had no impact on CACO2 and HT29, while it produced a slight decrease in NCM460D cells at all ratios. Statistics were performed using Unpaired *t* test for data set a and Ordinary One-Way Anova Multiple Comparisons Test (Dunnet's Correction) for data set b – For both data sets, statistical relevance was considered at P Value <0,05.

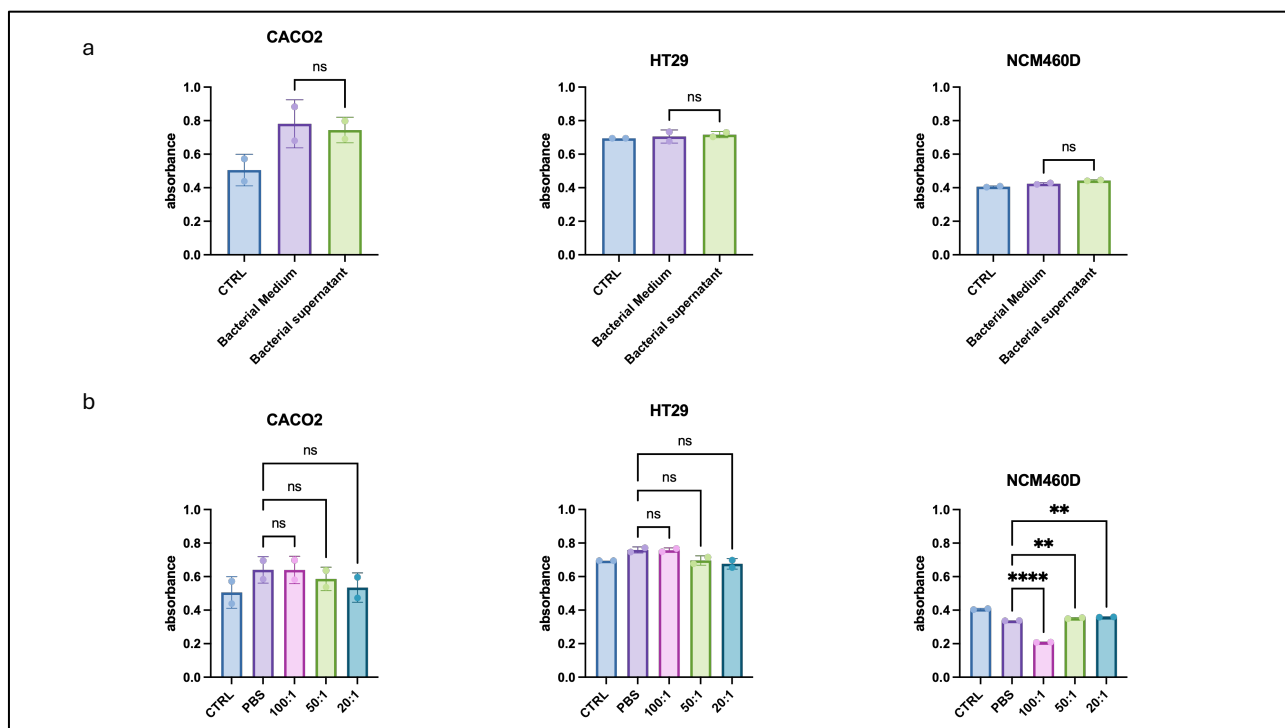


Figure 22. SRB evaluation of *O. ruminantium* treated cells – a) cellular mass levels were not affected by *O. ruminantium* supernatant – b) Live *O. ruminantium* had no impact on CACO2 and HT29, but it produced a significant decrease in NCM460D cells at 100:1 ratio, and a slight decrease at 50:1 and 20:1. Statistics were performed using Unpaired t test for data set a and Ordinary One-Way Anova Multiple Comparisons Test (Dunnet's Correction) for data set b – For both data sets, statistical relevance was considered at P Value <0,05.

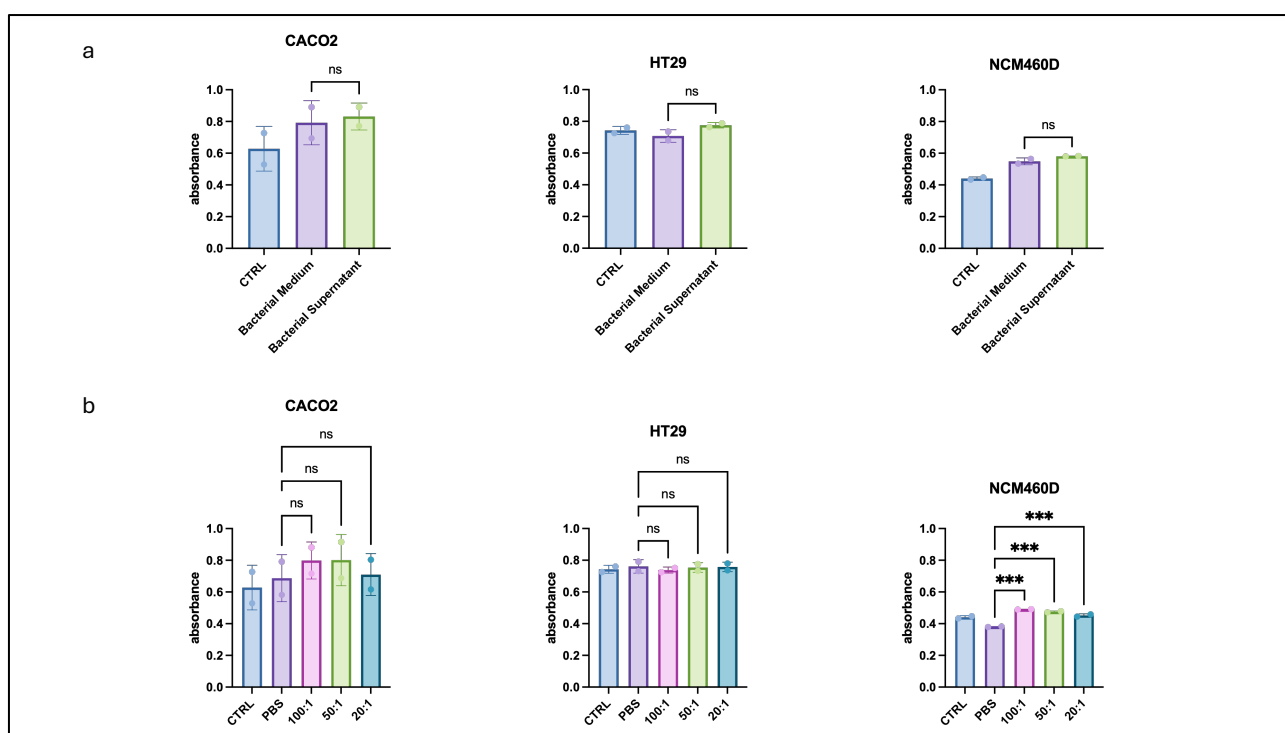


Figure 23. SRB evaluation of *P. faecium* treated cells – a) cellular mass levels were not affected by *P. faecium* supernatant – b) Live *P. faecium* had no impact on CACO2 and HT29, but it produced a significant increase in NCM460D cells at all ratios. Statistics were performed using Unpaired t test for data set a and Ordinary One-Way Anova Multiple Comparisons Test (Dunnet's Correction) for data set b – For both data sets, statistical relevance was considered at P Value <0,05

4.5. Bacterial CFSs do not alter cellular barrier permeability

During previous experiments we focused on cellular viability and proliferation. As exposure to both supernatants and live bacteria did not affect cellular growth, we subsequently evaluated other functional aspects, such as barrier integrity. As discussed before, cellular barrier integrity is a key element for maintaining homeostasis because it allows for an extremely controlled passage of molecules and metabolites. If transit is unrestricted, bacterial metabolites and endotoxins might enter inner layers of the colon, causing inflammation. Prolonged inflammation causes tissue damage and is a relevant factor in cancer development.

We evaluated permeability with CACO2, implementing Trans Epithelial Electrical Resistance (TEER) as a direct measure of barrier integrity. We used a simulated inflammatory stimulus (IL-1B + TNF α) as control, which visibly increased monolayer permeability, confirming that high level inflammation is indeed deleterious for gut barrier integrity. On the contrary, bacterial CFSs impact at the end of treatment was comparable to both the controls and bacterial growth medium only. For *O. ruminantium* (**Fig. 24b**), all conditions (except for inflammation) are essentially overlapping. For *L. apodemi* (**Fig. 24a**), there seems to be a significant increase during the first 30 hours, For *P. faecium*, a slight reduction in barrier resistance is visible throughout the time-points, indicating potential hindrance (**Fig. 24c**).

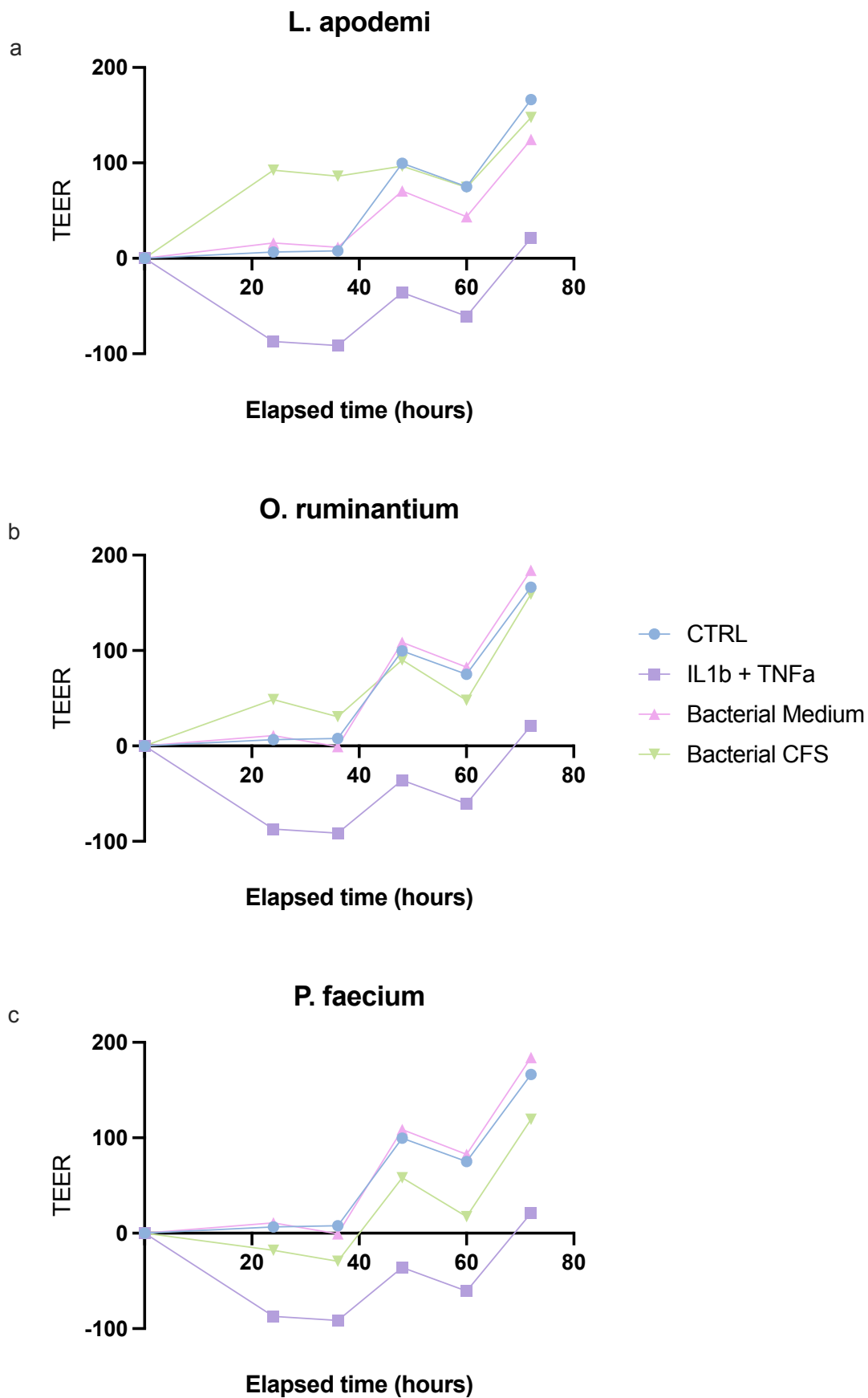


Figure 24. **Bacterial supernatants do not alter cellular barrier integrity** – Evaluation of cellular monolayer integrity through Trans Epithelial Electrical Resistance (TEER).

4.6. Bacterial supernatants do not significantly alter the immune response

Given the central role of immune cells in the tumour microenvironment and the ability of microbial metabolites to interact with them, we next investigated whether the microbial CFSs under study could modulate the immune response. To explore the effects of bacterial metabolites, we assessed both cell viability and functional responses, following stimulation with the CFSs. We extracted Peripheral Blood Mononuclear Cells (PBMCs) from Buffy Coats of healthy donors and incubated them with bacterial supernatants for 24h. Within the viable cell population, we next distinguished adaptive immune cells (CD3⁺), further classifying them into CD4⁺ and CD8⁺ subsets, and innate immune cells (CD3⁻). Similar to the overall viability results, the proportions of these subpopulations did not differ significantly after 24 hours of stimulation (**Fig. 25**). To assess the potential impact of bacterial CFSs on immune cell activity, we analysed the cytokine production profiles of CD4⁺, CD8⁺, and CD3⁻ immune cells using intracellular flow cytometry.

Given the cytotoxic and pro-inflammatory role of CD8⁺ T lymphocytes, we focused on the production of TNF α and IFN γ , two key cytokines involved in immune defence and inflammation. In CD8⁺ cells, after 4 hours of treatment only *L. apodemi* CFS induced a visible but not statistically relevant reduction in TNF α production. This effect was independent of bacterial culture medium, as the cells treated with it alone did not show similar reduction. A similar trend was observed in the proportion of TNF α /IFN γ double positive cells, which declined after treatment. In contrast, IFN γ production alone remained unchanged under all conditions (**Fig. 26**). We next investigated whether the immunosuppressive effect observed in CD8⁺ T cells extended to CD4⁺ T lymphocytes, which are key coordinators of the adaptive immune response through the production of a broad range of cytokines. Similar to CD8⁺ cells, *L. apodemi* reduced TNF α production in CD4⁺ cells after 24 hours. This was accompanied by a decrease in TNF α /IFN γ double positive cells, while IFN γ expression alone remained unaffected. Reduction in both TNF α and TNF α /IFN γ is visible but not statistically significant (**Fig. 27**). Finally, we investigated whether bacterial metabolites influenced innate immune cell CD3⁻ providing a broader view of immune modulation. CD3⁻ cells mirrored what we observed in CD4 and CD8 cells, with a reduction in TNF α and TNF α /IFN γ double positive cells (**Fig. 28**).

In conclusion, we observed visible reduction of TNF α upon *L. apodemi* supernatant exposure of CD8, CD4 and CD3⁻ cells, likely reflecting selective suppression of TNF α production, while no effect was detected for *O. ruminantium* and *P. faecium*.

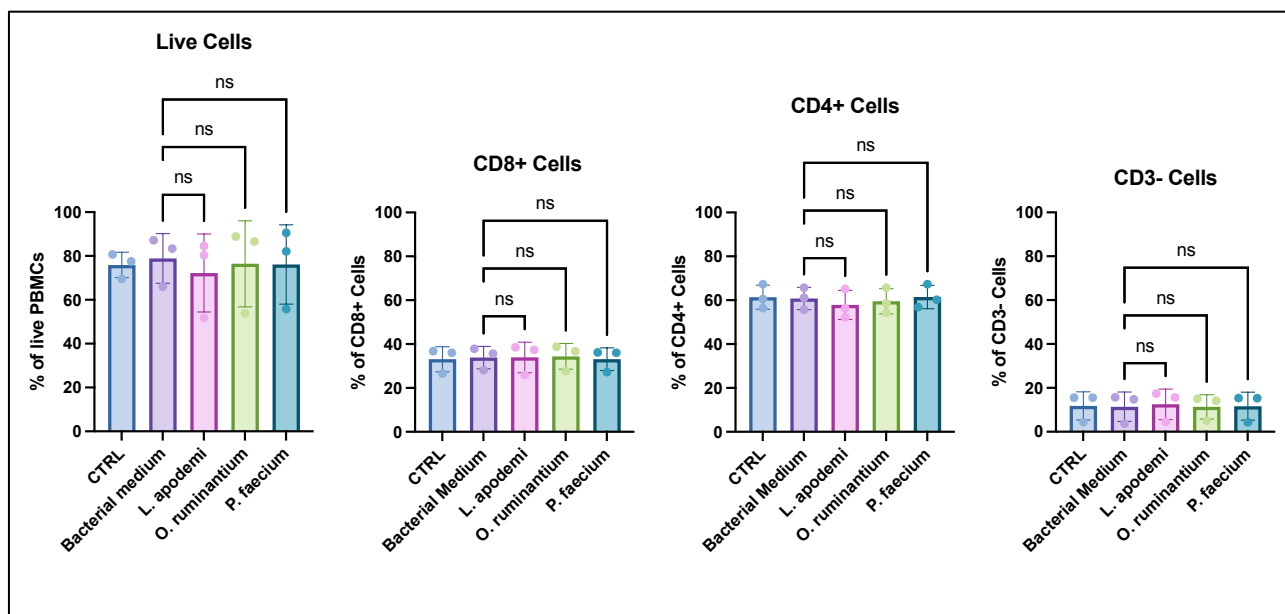


Figure 25. Overall viability and subpopulations composition of PBMCs examined through flow cytometry using the Zombie yellow dye. Bacterial supernatants did not alter overall viability or subpopulation composition. Statistical analysis performed using Ordinary One Way Anova (P VALUE $<0,05$)

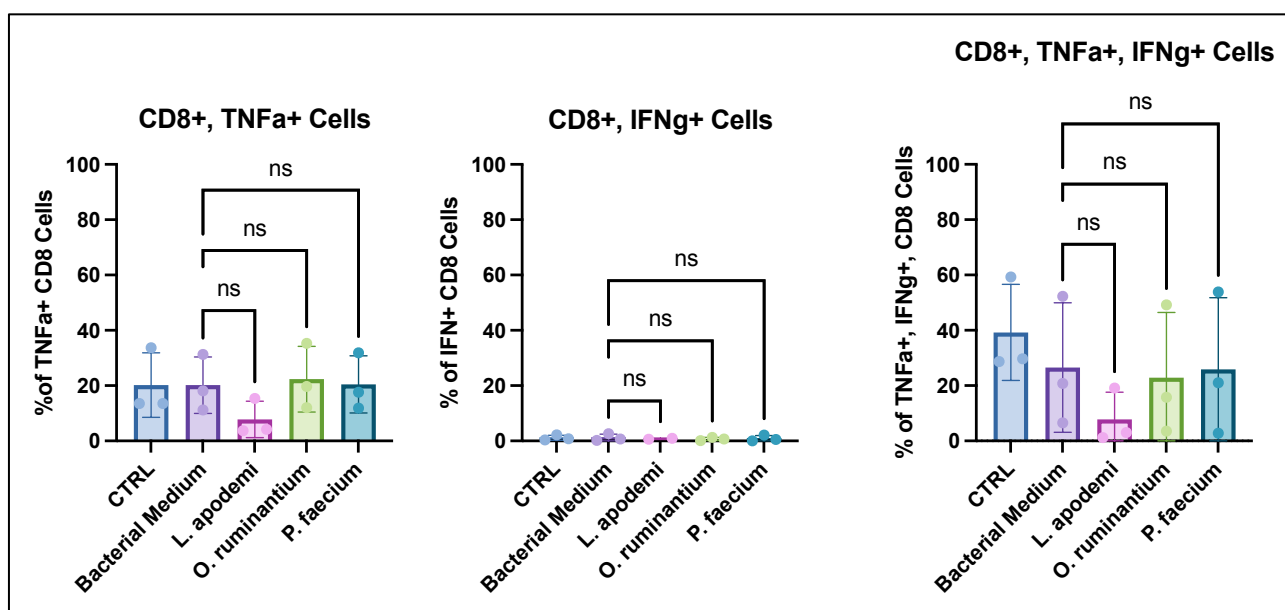


Figure 26. Flow cytometry analysis of CD8+ cells cytokine production (TNFa +, IFNg+, TNFa/IFNg double positive cells). Statistical analysis performed using Ordinary One Way Anova (P VALUE $<0,05$)

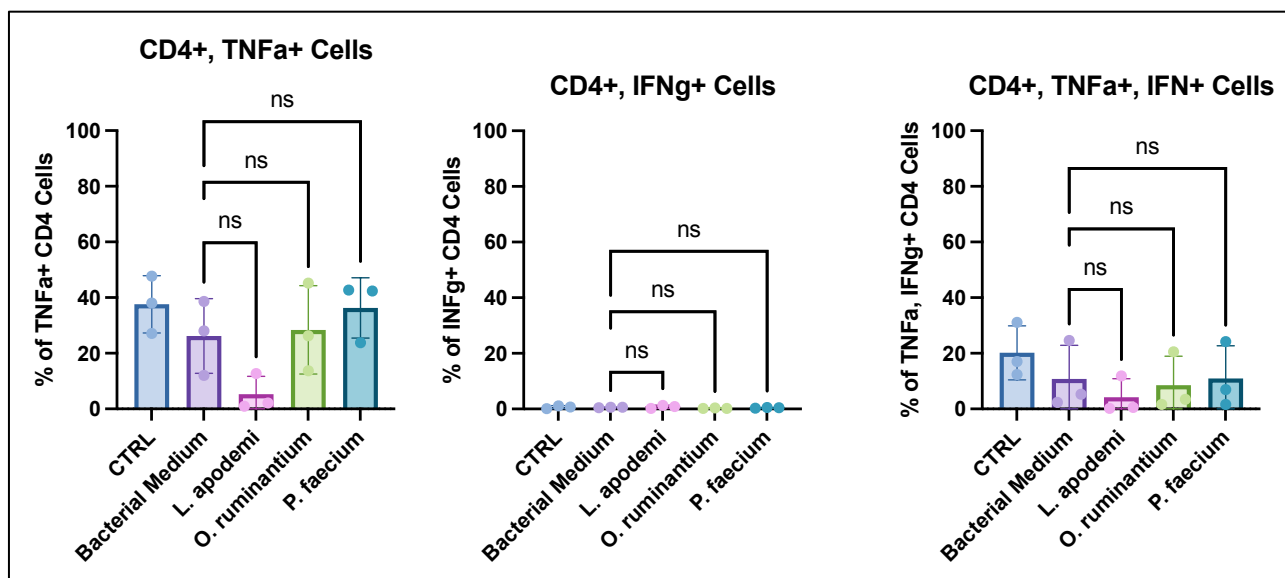


Figure 27. Flow cytometry analysis of C48+ cells cytokine production (TNFa +, IFNg+, TNFa/IFNg double positive cells). Statistical analysis performed using Ordinary One Way Anova (P VALUE <0,05)

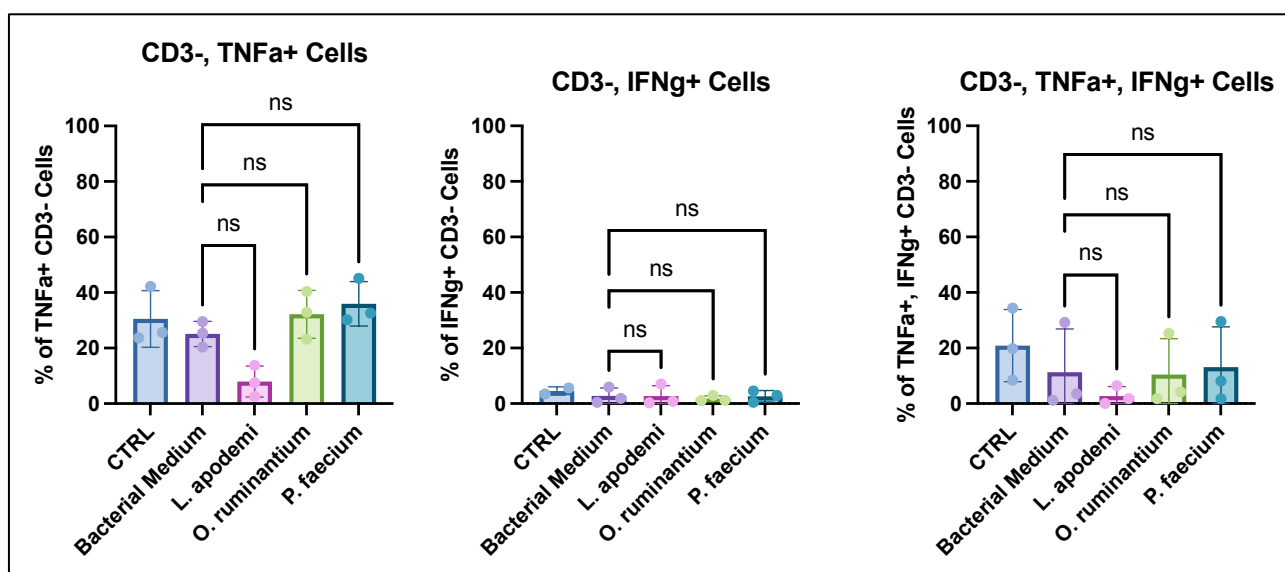


Figure 28. Flow cytometry analysis of CD3- cells cytokine production (TNFa +, IFNg+, TNFa/IFNg double positive cells). Statistical analysis performed using Ordinary One Way Anova (P VALUE <0,05)

4.7. Untargeted Metabolomics with GC-MS has not detected potentially harmful molecules

This work is based on the “Metabolic Population Effect” hypothesis, which implies that different bacterial communities, in a dysbiotic context, might contribute to promote a pro-tumorigenic environment. Our results suggested low impact on general cellular health and proliferation for the three bacteria we examined, but we still wanted to unveil potential underlying mechanisms. To further assess the metabolic microenvironment, additional analyses of the bacterial supernatants were conducted. Gas chromatography–mass spectrometry (GC–MS) was employed to characterize their metabolite composition. The resulting profiles were subsequently examined to evaluate whether the detected metabolites could offer new insights and provide a basis to formulate new hypotheses.

L. apodemi supernatant contained a range of organic acids, hydroxy acids, lipid-related compounds, and secondary metabolites (**Fig. 29**). The presence of lactic, citric, and succinic acids indicates active central carbon metabolism involving fermentative pathways and the tricarboxylic acid cycle⁶⁶. Hydroxy acids derived from branched-chain amino acids suggest ongoing amino acid catabolism. Additionally, compounds such as 3-phenyllactic acid and the detected diketopiperazine are consistent with the production of metabolites associated with microbial interactions, including antimicrobial activity or signalling⁶⁷. Glycerol and glycerol monostearate point to lipid metabolism or membrane-associated processes⁶⁸.

O. ruminantium CFS contained nucleobases and nucleosides (uracil, adenosine), vitamins (niacinamide), amino acids (L-tryptophan), sugars (D-glucopyranose), organic acids (lactic acid, 3-phenyllactic acid), lipid-related compounds (glycerol, glycerol monostearate), and a diketopiperazine derivative (3-benzylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione) (**Fig. 30**). The detection of uracil and adenosine indicates active nucleotide metabolism and turnover, while the presence of D-glucopyranose and lactic acid is consistent with ongoing carbohydrate metabolism and fermentative activity⁶⁹. L-tryptophan and niacinamide suggest amino acid metabolism. Additionally, 3-phenyllactic acid and the diketopiperazine are associated with secondary metabolism and may play roles in microbial interactions⁶⁷. Glycerol and glycerol monostearate further indicate lipid metabolism or membrane-associated processes.

P. faecium supernatant contained nucleobases and nucleosides (adenine, uracil, adenosine), a vitamin derivative (niacinamide), lipid-related compounds (glycerol, glycerol monostearate,

stearic acid), and nitrogen-containing metabolites including phenethylamine, norharmane, and a diketopiperazine derivative (3-benzylhexahydropyrrolo[1,2-a] pyrazine-1,4-dione) – (**Fig. 31**). The presence of adenine, uracil, and adenosine indicates active nucleotide metabolism and turnover, while glycerol, glycerol monostearate, and stearic acid suggest lipid metabolism and membrane-associated processes⁶⁸. Phenethylamine and norharmane are consistent with amino acid metabolism, whereas the detected diketopiperazine is associated with microbial signalling or interaction mechanisms⁶⁷.

Overall, the metabolite profile reflects a metabolically active state encompassing central carbon metabolism, nucleotide turnover, and secondary metabolite production. We did not find compounds that might be directly linked to CRC promotion or development. However, recent data have shown that *P. faecium*'s metabolism is based on succinate rather than glucose⁷⁰. Butanedioic acid (or succinic acid) is the second most abundant metabolite in *L. apodemi* and it is produced in minor quantities from *O. ruminantium*, this might suggest cross-feeding among these bacterial strains.

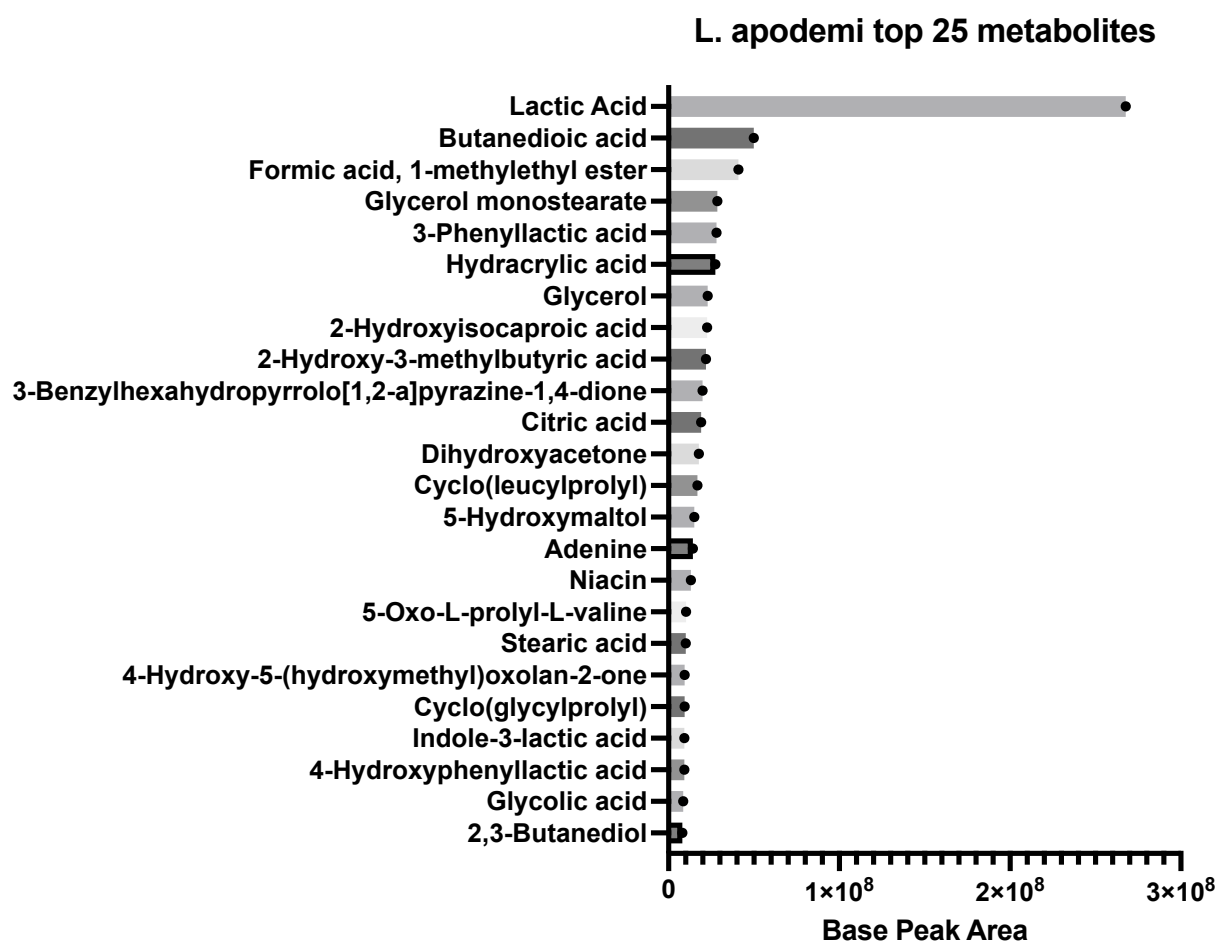


Figure 29. Untargeted metabolomics of *L. apodemi* supernatant. Data obtained through derivatization of samples and subsequent analysis using Gas Chromatography – Mass Spectrometry.

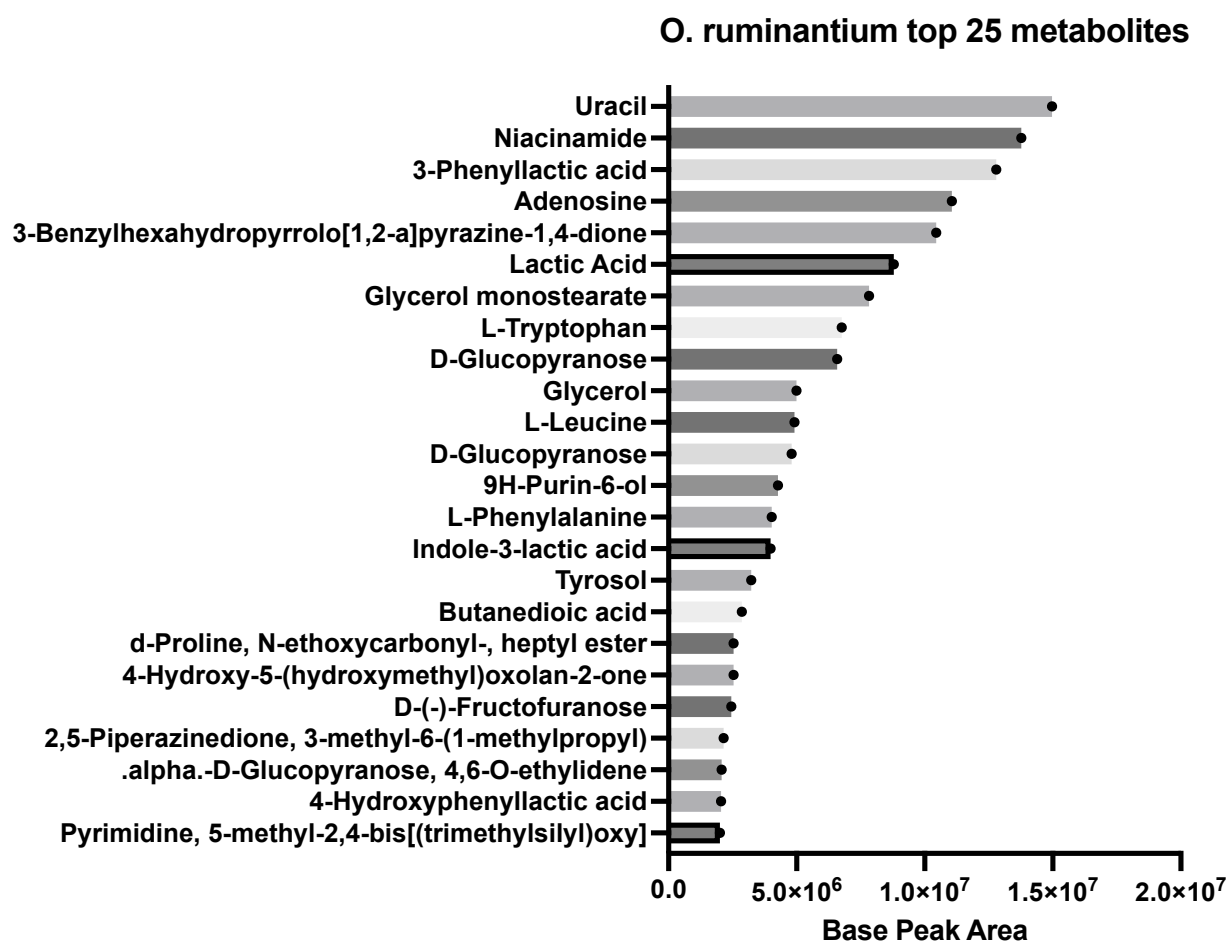


Figure 30. Untargeted metabolomics of *O. ruminantium* supernatant. Data obtained through derivatization of samples and subsequent analysis using Gas Chromatography – Mass Spectrometry.

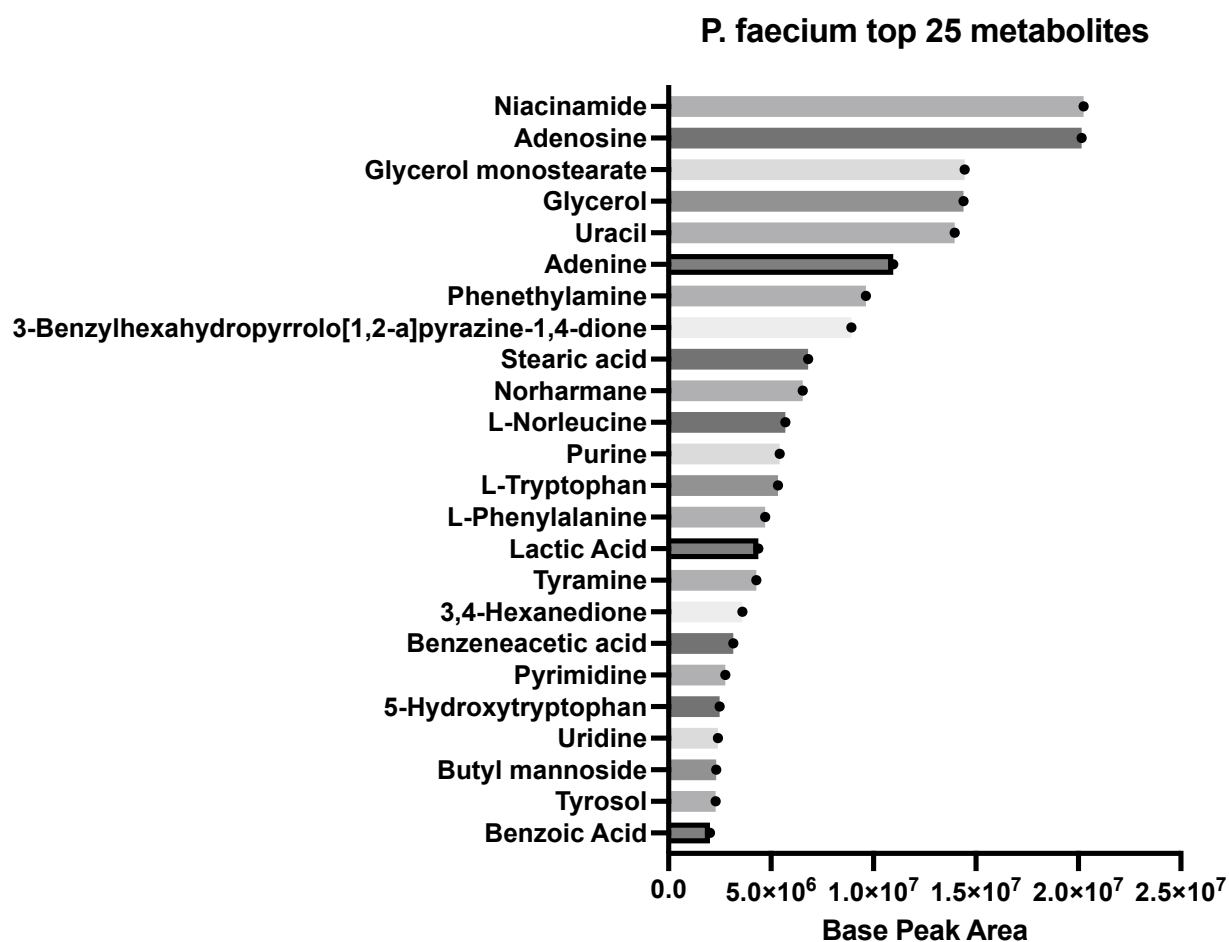


Figure 31. Untargeted metabolomics of *P. faecium* supernatant. Data obtained through derivatization of samples and subsequent analysis using Gas Chromatography – Mass Spectrometry.

Discussion

During the last years, the gut microbiota has emerged as a central factor in human health and disease, with growing evidence of its causative role in intestinal disease, such as inflammatory bowel diseases (IBD) and colorectal cancer (CRC). Research is currently focused on characterizing the gut microbiota, mainly through two sequencing approaches: whole-genome shotgun (WGS) sequencing and 16S rRNA sequencing⁷¹.

However, both the inter- and intra-individual variability driven by genetics, diets, and environment together with methodological limitations still complicate a full understanding of the gut microbiota⁷². In particular, faecal samples, mucosal biopsy and intestinal fluid, are only a proxy of the entire intestinal ecosystem. There are also several microorganisms that require very specific physiological conditions to survive, making them difficult to study outside the human body⁷³. Despite these challenges, the characterization of microbial genera and species inhabiting the intestine under both homeostatic and disease conditions may enhance diagnostic capabilities, including the detection of disease at early stages, and expand therapeutic opportunities.

Colonization of specific bacteria could be used as diagnostic markers for various intestinal diseases. It may be also possible to target dysbiosis to restore microbial balance and contribute to therapeutic strategies.

This is particularly relevant in CRC, where sporadic colorectal adenomas can represent precursors of the tumour. The progression from adenoma to carcinoma involves the transition from normal epithelial tissue to aberrant crypt foci (ACF), and then to early and advanced adenomatous polyps. These lesions can evolve into intramucosal carcinoma, followed by early colorectal cancer, and ultimately invasive adenocarcinoma. However, only a minority of early colorectal adenomas progress to invasive CRC⁷⁴.

Bacterial metabolites, in a dysbiotic context, can elicit tumorigenesis through different mechanisms: DNA damages, direct cytotoxicity, increasing of proliferation rates, altering the epithelial barrier permeability, or disrupting immune regulation. These pathways, acting in synergy, can induce mutations and cellular alterations. While most of these altered cells are normally eliminated, those that survive may acquire a selective advantage, driving tissue transformation⁷⁵. We evaluated all these mechanisms on different cell types, at different time-points and through different types of tests, to better understand how preponderant bacterial

strains found in the adenoma-associated mucosa might influence the microenvironment and promote tumour initiation and progression.

In this study, we analysed the gut microbiome of five patients with colorectal adenoma using 16S rRNA sequencing. Through this sequencing study we identified three bacterial species enriched in the adenoma mucosa of patients: *Ligilactobacillus apodemi*, *Phascolarctobacterium faecium*, and *Oscillibacter ruminantium*. These taxa are poorly characterized in literature. Given the established impact of the microbiota on the adenoma-carcinoma sequence, we wanted to investigate if these bacteria, and particularly their metabolites, could influence this pathway.

L. apodemi is an anaerobic, Gram-positive, rod-shaped, non-endospore-forming bacterium. It's able to produce L-lactate and gallic acid from tannic acid⁷⁶. Nowadays, there are no studies in which this bacterium is associated to tumours or other diseases.

O. ruminantium is a strictly anaerobic, Gram-negative, non-spore forming bacterium. It's motile by peritrichous flagella and able to produce especially butyric acid⁷⁷. It was identified in human for the first time in four patients affected of bacteremia in Denmark from 2001 to 2010. Remarkably one of these patients was affected by metastatic colon adenocarcinoma, that in fact lead to his death⁷⁸.

However, *P. faecium* is comparatively better studied. It is an obligate anaerobic, Gram-negative, non-spore-forming bacterium, able to produce propionate. This species abundantly colonizes the human gastrointestinal tract, suggesting a key role in the balance of the gut microbiota⁷⁹. *P. faecium* is not able to utilize sugars efficiently, its metabolism relies on succinate production from other bacteria, and its main product is propionate⁸⁰. Beyond its role in microbial cross-feeding and propionate production, *Phascolarctobacterium faecium* has emerged as a potentially important modulator of host immune function, highlighting a functional link between gut microbial metabolism and immune homeostasis. A recent study in mice demonstrated that *P. faecium* treatment increases the population of M2 macrophages while reducing that of M1 macrophages. It also enhances the number of T reg cells compared to untreated mice⁸¹. Another work highlighted *P. faecium* ability to reduce inflammation in a murine model of colitis by subtracting succinate from the microenvironment. Succinate acts as a signalling metabolite capable of activating SUCNR1, a receptor expressed on various immune cells that promotes pro-inflammatory pathways upon stimulation. By consuming succinate and converting it into propionate, *P. faecium* may reduce ligand availability for SUCNR1, thereby attenuating receptor-mediated inflammatory signalling⁸². While the potential role of *L. apodemi* in cancer development has not been explored yet, members of the genus *Lactobacillus* are well-

known immunomodulators. They interact with immune cells via pattern recognition receptors, influencing cytokine production and promoting regulatory T cell responses. Lactobacilli also enhance gut barrier function and produce metabolites, such as lactic acid, that support mucosal immunity⁸³. In our study, we examined TNF α and IFN γ production by different subtypes of immune cells and we have found that while not statistically significant, *L. apodemi* was able to visibly reduce cytokine production. As described earlier, *P. faecium*'s metabolism is based on succinate, which is one of the main products of *L. apodemi*. This might indicate a tight correlation between the two strains, since *P. faecium* could benefit from *L. apodemi*'s high abundance, which corresponds to an increase in succinate availability. These findings, applied to the adenoma-carcinoma sequence, suggest that higher levels of *P. faecium* and *L. apodemi* (given their ability to interact with the immune system) may polarize an immunosuppressive response, potentially supporting the proliferation of transformed cells.

While there are no available studies on *P. faecium* and *O. ruminantium*, data suggests that lactobacilli play an important role in maintaining and restoring gut barrier integrity. Mechanistically, these bacteria can enhance tight junction protein expression, reduce epithelial permeability, and modulate host immune responses⁸⁴. We evaluated barrier integrity through Trans Epithelial Electrical Resistance (TEER), but we were not able to find substantial differences among the three examined bacterial strains. Even though TEER analysis has highlighted no impact, we cannot currently affirm that neither of these bacteria has an impact on cellular barrier integrity, since more data is needed on tight-junctions protein expression.

Research has identified several bacterial strains capable of inducing DNA damage such as double strand breaks and cross-links, altering normal cell cycle dynamics and conventional transcriptional features. These are key elements in cancer initiation and progression^{34,55,56}. Given the preponderance of *L. apodemi*, *O. ruminantium* and *P. faecium* in the adenoma associated mucosa, we explored the potential impact of their metabolites on DNA through different approaches. We evaluated cellular DNA damage through the Comet Assay, ROS-related genomic stress through 8-OHdG ELISA Assay and even direct DNA damage potential with a plasmid DNA assay. Results highlighted no damage in cellular and plasmid DNA, nor increased levels of genomic stress. While we cannot rule out altered gene expression, which is yet to be evaluated, we can confidently exclude DNA damage induced by bacterial metabolites.

Bacterial metabolites are key mediators of host-microbe interactions in the gut, influencing cellular processes including proliferation. Certain metabolites, such as short-chain fatty acids, indole derivatives, and secondary bile acids, can modulate signalling pathways, epigenetic

marks, and immune responses that regulate epithelial growth^{85,86}. In this work we assessed cellular metabolic activity and proliferation rates through different techniques, comparing the effects of supernatants and live bacteria to determine if any detected outcome could be attributed to both or differed between CFSs and actively secreting bacteria. Despite small differences, cellular growth rates were not remarkably affected by either supernatants or live bacteria. Given our data, we can confidently rule out cytotoxicity since both transformed and untransformed cells maintained regular growth rates. Likewise, no enhancement in proliferation was observed, since cell growth remained comparable to the control under both live bacteria and supernatant treatments.

In conclusion, these findings highlight the limitations of simplified experimental systems in fully capturing the complexity of the adenoma microenvironment. Although the experiments did not highlight significant impact across multiple cellular parameters, the potential influence on immune-related responses suggests a more nuanced role that may only emerge in more complex settings. This underscores the need to develop and employ more representative, multi-component models that better mimic the cellular and microbial interactions present *in vivo*. Expanding toward more comprehensive ecosystem-based approaches may therefore provide a clearer understanding of how specific bacterial communities contribute to promote a pro-tumorigenic microenvironment.

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