

# *F. nucleatum* OMV characterisation and a comparative proteomic analysis of outer membrane proteins with gram-negative bacteria

Aizhan Uteubayeva

## Abstract

Gram-negative bacteria, such as *B. fragilis*, *B. thetaiotaomicron*, *C. jejuni*, *H. pylori*, *E. coli* and *F. nucleatum*, are known to be implicated in colorectal cancer (CRC) development by promoting inflammation and disrupting gut epithelium. Adhesins, the outer membrane proteins on the surface of bacteria, are the first line mechanism of interaction with epithelial cell lining via direct binding or indirect secretion as a content of outer membrane vesicles. Theoretically, adhesins may have common and complementary function of the aforementioned bacteria in carcinogenesis proposed by “driver-passenger” model (Tjalsma *et al.*, 2012). Here, the *F. nucleatum* ATCC 10953, 25586, 51191 outer membrane vesicles (OMVs) were successfully purified and characterised to an average size of 90-110 nm. The OMVs of ATCC 25586 were shown to be internalised by intestinal organoids. Further, bioinformatical analysis of bacterial proteomes was used to characterise the adhesin proteins and to propose a combined models of CRC development. and establish common pathways across bacteria, such as ToB, quorum sensing system,  $\beta$ -lactam resistance, etc. The research proved the ground for further experimental research in establishing direct effects between CRC-associated bacteria and it's sharing properties.

Acknowledgements: Nathalie Juge, Dimitra Lamprinaki, and Tanja Suligoj.

# Introduction

The normal life of a cell includes cell growth, cell death and renewal. A misregulation in these mechanisms is described by a broad term such as cancer. Carcinogenesis encompasses a group of hallmarks including but not limited to genome instability, tumour-promoting inflammation and uncontrolled proliferation (Galluzzi *et al.*, 2018).

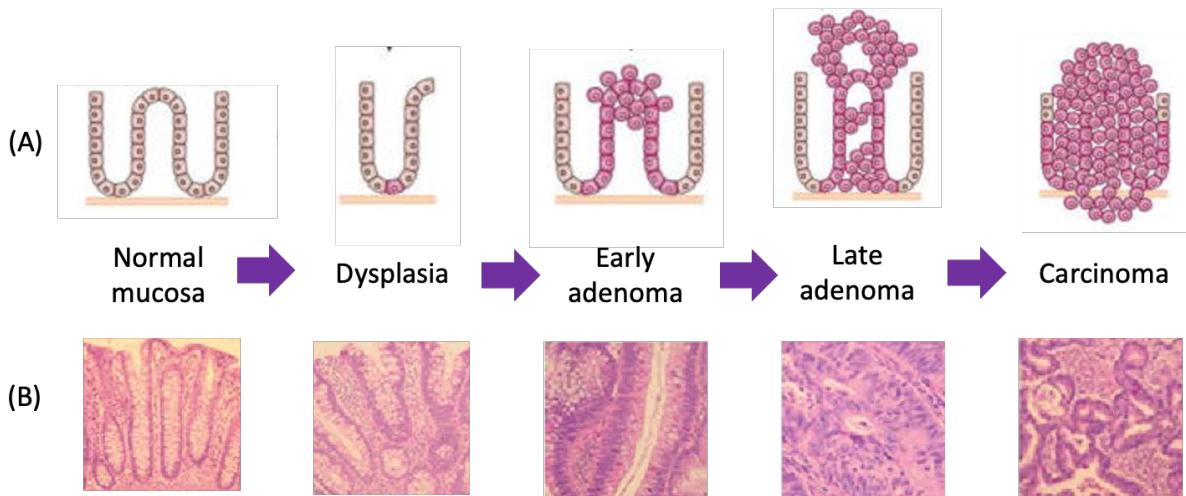
A microbiome instability, as well as various pathogens have been strongly associated with tumorigenesis. Colorectal cancer is one of the prevalent oncologies in the world and the second cause of deaths in the United Kingdom (“Deaths - Office for National Statistics,” n.d.)

## 1.1 Colorectal carcinoma is a third most frequent malignancy in the world

Colorectal cancer (CRC) is the third most common cancer type worldwide with the majority of the cases being sporadic, with no family history or hereditary genetic alterations (Keum and Giovannucci, 2019; Rawla *et al.*, 2019). The clinical outcome of CRC varies according to the timing of the diagnosis. An early detection/screening of the tumor allows an increased chance of survival by eight-fold as opposed to later diagnosis at stage IV (Zeller *et al.*, 2014). Thus the development of early detection biomarkers would significantly impact survival rates amongst CRC patients.

## 1.2 Colon epithelial cells acquire the oncogenic hallmarks behaviours due alterations in microbiota

The intestinal epithelium is a constantly renewing tissue, creating a paradigm for cancer originating from stem and stem-like cells at the basement of colonic crypts (Zeki *et al.*, 2011). The progression of the disease follows a distinct pattern starting from aberrant crypt or dysplasia, further developing into neoplastic lesions of early and late adenoma, and finally leading to carcinoma (Fig.1). The CRC is a complex disease that encompasses neoplastic formations, commonly arisen from glandular and epithelial cells due to accumulation of genetic, epigenetic and environmental factors, including the gut microbiota (Morgillo *et al.*, 2018; Ogino *et al.*, 2011). Alterations in the gut microbiota, immune system and cancerous cells are long recognised to be involved in CRC progression (Drewes *et al.*, 2016; Mehta *et al.*, 2017).



*Figure 1. Colorectal cancer progression (A) oversimplified schematic representation featuring normal colonic crypts, followed by dysplasia at the bottom of the crypt due to accumulation of epigenetic changes in stem-like cells, leading to proliferation into early and late stages of adenoma disrupting the normal structure of the epithelium, and finally forming a carcinoma with metastatic lesions (adapted from Kuipers et al., 2015) ; (B) histological level representing the forementioned steps (adapted from Bosman and Yan, 2014).*

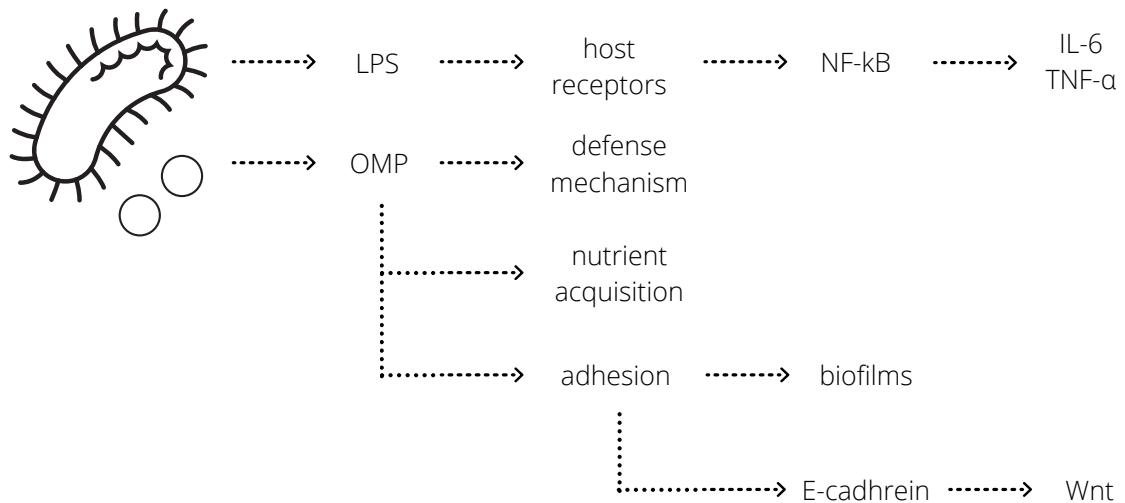
### 1.3 Gram-negative bacteria are implicated in CRC development

Intestinal microbiota is a stable-dynamic equilibrium of microbial communities, their gene and gene products are confined within the human gut (Hernandez *et al.*, 2016). *Firmicutes*, *Actinobacteria* and *Bacteroides* are the dominant phyla in healthy individuals with the stable ratio across healthy individual, meanwhile various metagenomic studies have linked CRC microbiome dysbiosis associated with gram-negative enteric pathogen such as *Fusso**bacterium***, *Escherichia*, *Campylobacter* and *Bacteroides spp.* (Allali *et al.*, 2015; Warren *et al.*, 2013; Wu *et al.*, 2013).

#### 1.3.1 *Fusso**bacterium***.

*Fusso**bacterium nucleatum*** is an anaerobic bacterial species known to be present in the healthy human oral microbiota forming oral plaque biofilms (Avila *et al.*, 2009; Kolenbrander *et al.*, 2010). The biofilm is a collection of bacteria that adhere to surfaces and are associated with antibiotic resistance (Berleman and Auer, 2013; Ellis and Kuehn, 2010). *F. nucleatum* has also shown to be implicated in other non-oral diseases, such as human deficiency virus and primary sclerosing cholangitis (Atarashi *et al.*, 2017; Olsen and Yamazaki, 2019). *F. nucleatum* is shown to be prevalent in CRC patients compared to healthy individuals via qPCR analysis and has been suggested as a potential fecal biomarker and a diagnostic tool for detection of CRC (J. Liu *et al.*, 2019; Mira-Pascual *et al.*, 2015; Mo *et al.*, 2020; Wong *et al.*, 2017).

A tumorigenic property of *F. nucleatum* associated with CRC include interaction with signalling pathways, modulation of the immune system response and mediators. In particular, the bacterium has an adhesion A (FadA) protein on the outer membrane of *F. nucleatum* that has been shown to interact with the extracellular domains of E-cadherin. E-cadherin has long been implicated in cancer progression (Christou *et al.*, 2017). There is also an increase in *FadA* gene expression in CRC leading to increased CRC cell proliferation in comparison to normal individuals (Guo *et al.*, 2020). Epithelial cell borders are sealed by three major groups of junctional proteins. E-cadherin provides a platform for the cell to establish mechanical strength. The breaching of an architecture may promote metastasis formation (Harris and Tepass, 2010). The most apical junctional proteins, tight junctions, provide regulation of molecular compounds into the cell suggesting that homeostasis can contribute to changes in proliferation, differentiation, etc (Etienne-Manneville, 2013). Indeed, this binding to E-cadherin can activate wingless-related integration site (Wnt) signalling pathway associated with cell proliferation and differentiation (Rubinstein *et al.*, 2013). An infection of mice with *F. nucleatum* has shown to promote proliferation and invasiveness in colorectal cancer tissue (Yang *et al.*, 2017).



*Figure 2. The mechanisms involved in cancer development via gram-negative bacteria. Gram-negative bacteria contain lipopolysaccharides (LPS) on the the outer membrane surface that bind to host receptors and activate immune response via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), further leading to increased levels of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). In addition, the outer membrane contains outer membrane proteins (OMP) that are known to participate in defense mechanisms against antibiotics and host immune system, essential nutrient acquisition, as well as having and adhesion property to form biofilms or activate Wnt signalling pathways by binding to E-cadherin.*

Moreover, the Wnt pathway is associated with the modulation of cancer microenvironment through immunosuppression of the adaptive host response (Kostic *et al.*, 2013). For example, a study carried out by Mima *et al.* revealed a lower density of CD3+ lymphocytes in those with higher numbers of *F. nucleatum*. This showed that those with higher densities of CD3+ lymphocytes had better patient survival outcomes in CRC (Li *et al.*, 2016; Mima *et al.*, 2015). However, *F.nucleatum* has also shown to evade the immune system, by mediating the inhibition of natural killer cells (NK), a direct relation to immune cell type for tumor eradication. Importantly, the lower rate of tumor-infiltrating immune cells and lessened anti-tumor response correlate with poorer clinical prognosis in CRC (Pagès *et al.*, 2018; Rozek *et al.*, 2016). In particular, density of CD45RO+ showed favourable results for CRC patients due to high expression of chemokines such as CXCL9 and CXCL10, which induce NK cells and T lymphocyte recruitment (Nosho *et al.*, 2010). Therefore, *F. nucleatum* has an ability impede normal functioning of immune system.

### 1.3.2 *Bacteroides*.

*Bacteroides* are rod-shaped anaerobes normally residing in the human oral cavity, intestine, respiratory and reproductive tracts. A controversial finding on whether *Bacteroides* are abundant or depleted in CRC suggests variation among different strains (Gao *et al.*, 2015; Thomas *et al.*, 2016; Wang *et al.*, 2012; Weir *et al.*, 2013). An upregulated expression of the conserved commensal colonisation factor (CCF) gene across *Bacteroides* is known to contributes to the ability of *Bacteroides fragilis* to colonise the mucus of the intestinal crypts, that usually also require immunoglobulin A (Donaldson *et al.*, 2018; Lee *et al.*, 2013). In addition, *B. fragilis* secretes metalloprotease-dependent toxin, BFT, that cleaves a tight junctional protein, E-cadherin, causing to the disruption of the intestinal barrier, promoting inflammation via interleukin 17 (IL-17) and causing DNA damage due generated reactive oxygen species (ROS)(Chung *et al.*, 2018; Goodwin *et al.*, 2011)

### 1.3.3 *Campylobacter*.

*Campylobacter* is a curved-shaped bacterium abundant in CRC patients and enriched in neighbouring non-tumorigenic samples compared to tumorigenic samples (Allali *et al.*, 2015; de Carvalho *et al.*, 2019). The *C. jejuni* adhesins, CadF and FlpA, promote intestinal colonisation via binding to extracellular matrix glycoprotein of epithelial cells called fibronectin (Konkel *et al.*, 2010; Schmidt *et al.*, 2019). Further, the cytolethal distending toxin (CDT) of *C. jejuni* promotes CRC in germ-free mice (He *et al.*, 2019). Although, the mechanism of *C. jejuni* and CRC development remain unclear, it is linked to inflammatory bowel disease progression that further increases the risk of carcinoma (Gradel *et al.*, 2009).

#### *1.3.4 Escherichia.*

*Escherichia* is a rod-shaped bacteria with significant enrichment in CRC patients and various adherence properties (Gao *et al.*, 2015; Yang *et al.*, 2019). *Escherichia coli* is able to adhere and invade epithelium, as well as up-regulate VEGF via amfibrial adhesin Afa-1 (Prorok-Hamon *et al.*, 2014). The colibactin toxin of *E. coli* is also able to cause genomic instability via DNA damage, and knock-out of *pks* gene which encodes for colibactin, was found to decrease carcinogenesis in mice (Arthur *et al.*, 2012; Cuevas-Ramos *et al.*, 2010)

#### *1.3.4 Helicobacter pylori.*

*H. pylori* is a gastric bacterium infecting the mucus of the stomach lining. There is growing evidence for its association to increased risk of CRC with *H. pylori* infection with inconclusive mechanisms. Although, colorectum is not a natural habitat for *H. pylori*, a number of studies have identified its presence in CRC tissue or polyps (Grahn *et al.*, 2005; Jones *et al.*, 2007; Soylu *et al.*, 2008). First, *H. pylori* may alter the gut microbiome favouring colonisation of carcinogenic bacteria, showing differences in microbiota between healthy and *H. pylori*-related gastric lesions (Gao *et al.*, 2018; Tongtawee *et al.*, 2018, 2016). Secondly, by promoting systemic intestinal inflammation.

### 1.4 Gram-negative bacteria produce outer membrane vesicles that are potentially implicated in carcinogenesis

All known gram-negative bacteria produce nanoscale vesicles called outer membrane vesicles (OMVs) that resemble another tumorigenic property. Before extrapolating on the role of OMVs in CRC it is important to address their function. OMVs are spherical buds of outer membrane naturally secreted by Gram-negative bacteria (Kulp and Kuehn, 2010; Schwechheimer *et al.*, 2013). The OMVs architecture is comprised of an outer and inner membranes between which is a layer of peptidoglycans (PG). The latter provide shape and stability to an OMV via formation of protein crosslinks. The outer portion of an OMVs is a nontypical layer of phospholipids and lipopolysaccharides (LPS), compared to the inner membrane (Silhavy *et al.*, 2010). Together these carry a periplasmic content such as DNA, enzymes and toxins (Ellis and Kuehn, 2010).

LPS are surface-bound endotoxins found on the cell wall of Gram-negative bacteria (Di Lorenzo *et al.*, 2019; Okahashi *et al.*, 1988). These polysaccharides are implicated in a variety of diseases. For instance, obesity is shown to alter gut permeability and immunological profile of intestinal epithelium via gut microbiome alterations (Brun *et al.*, 2007; Cani *et al.*,

2009; Luck *et al.*, 2015). Thus, allowing bacterial LPS to escape into the bloodstream. LPS is known to interact with toll-like receptors-4 (TLR4) localised on the surface of the immune cells (Molinaro *et al.*, 2015). LPS is recognised by the TLR4 *via* adaptor protein fetuin A, further activating downstream signalling resulting in chemokines secretion (Pal *et al.*, 2012; Shi *et al.*, 2006). For instance, the *in vitro* stimulation of TLR-4-expressing immune cells in dorsal root ganglia by LPS show calcium influx and production of monocyte chemoattractant protein-1 (MCP-1). However, the knockout of TLR-4 blocked these responses (Miller *et al.*, 2015).

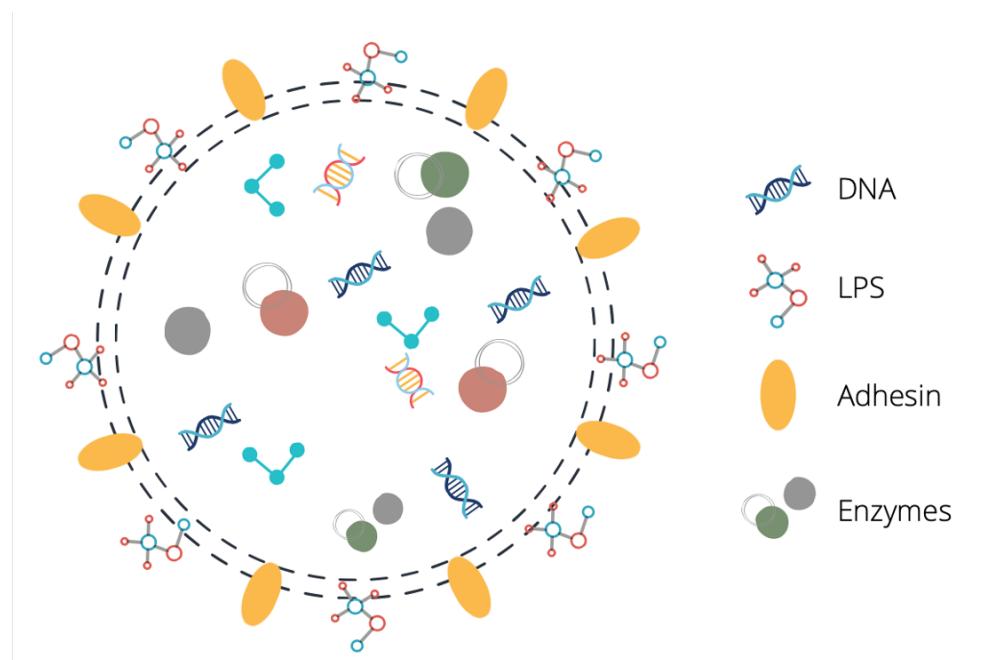


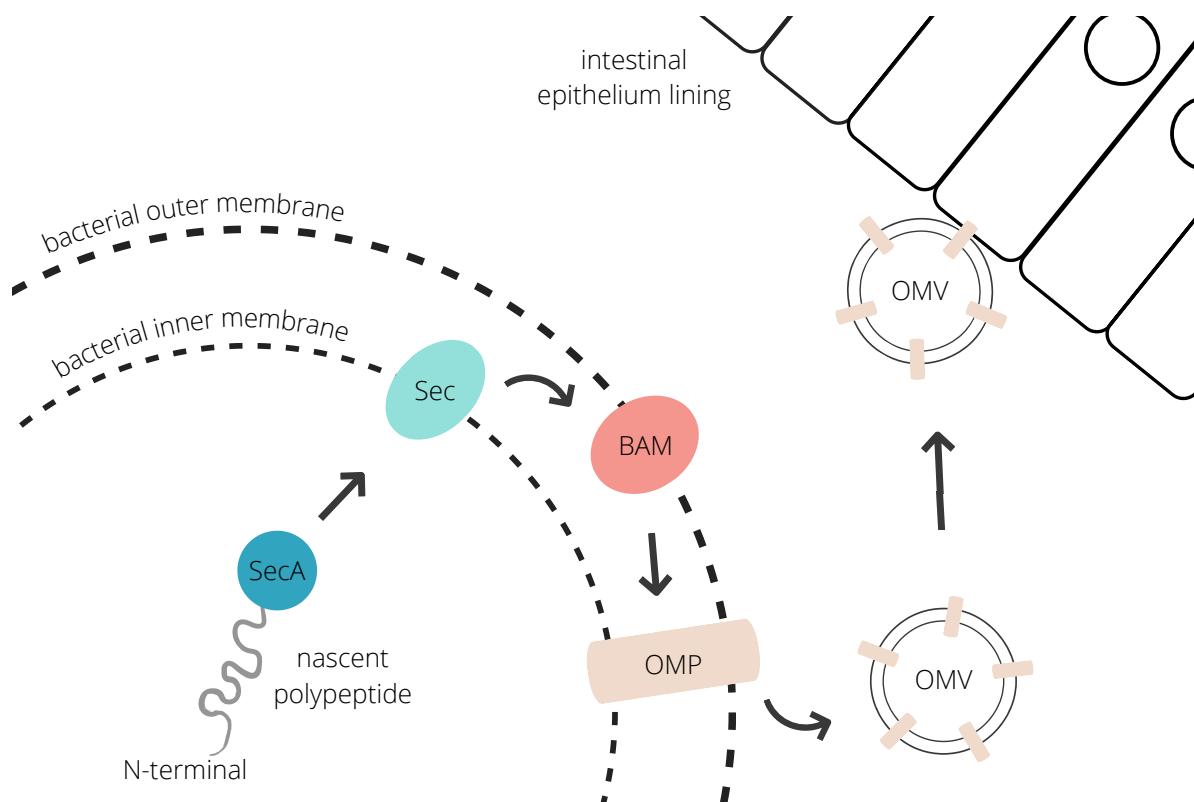
Figure 3. Composition and structure of an outer membrane vesicle featuring two-layered membrane; an internal component such as DNA and enzymes; components membrane such as adhesins and lipopolysaccharide (LPS).

## 1.5 OMVs contain outer membrane proteins contributing to adhesion and invasion of epithelial cells

Investigations on *E. coli* and *Pseudomonas aeruginosa* has shown to incorporate approximately 1% of outer membrane proteins (OMP) in vesicles (Kesty and Kuehn, 2004; Mug-Opstelten and Witholt, 1978). In some strains in *Neisseria meningitidis* the percentage of OMPs in outer membrane reaches 8-12% (Bauman and Kuehn, 2006). In *Enterobacteriaceae*, *Vibrionaceae*, and *Pseudomonaceae*, the cell envelope is stabilized by multiple inner and outer membrane components including the highly abundant  $\beta$ -barrel OMPs, such as OmpA, Peptidoglycan-associated lipoprotein (Pal), and Lpp (Samsudin *et al.*, 2016).

The OmpA proteins have important pathogenic roles including bacterial adhesion, invasion, or intracellular survival as well as evasion of host defence mechanisms or stimulation of pro-inflammatory cytokine production.

The OMPs are synthesised in the cytoplasm and destined across the inner membrane and periplasm via an N-terminal leader sequence by Sec system (Hagan *et al.*, 2011; Knowles *et al.*, 2009; Ricci and Silhavy, 2012). The Sec periplasmic chaperons, SurA and Skp, bind to OMPs for outer membrane translocation where these are further incorporated into outer membrane via the  $\beta$ -barrel assembly machinery (BAM) (Rollauer *et al.*, 2015). The OMP adopt the  $\beta$ -barrel into the membrane with loops, both inside the periplasm and outside the extracellular space. The  $\beta$ -barrel is highly stable that allows to withstand harsh living conditions and a first point of contact with the surrounding environment incorporating a variety of physiological and structural functions such as adhesion, nutrient acquisition, signal transduction, biogenesis, enzymatic activity, etc. (Fairman *et al.*, 2011; Noinaj *et al.*, 2012).



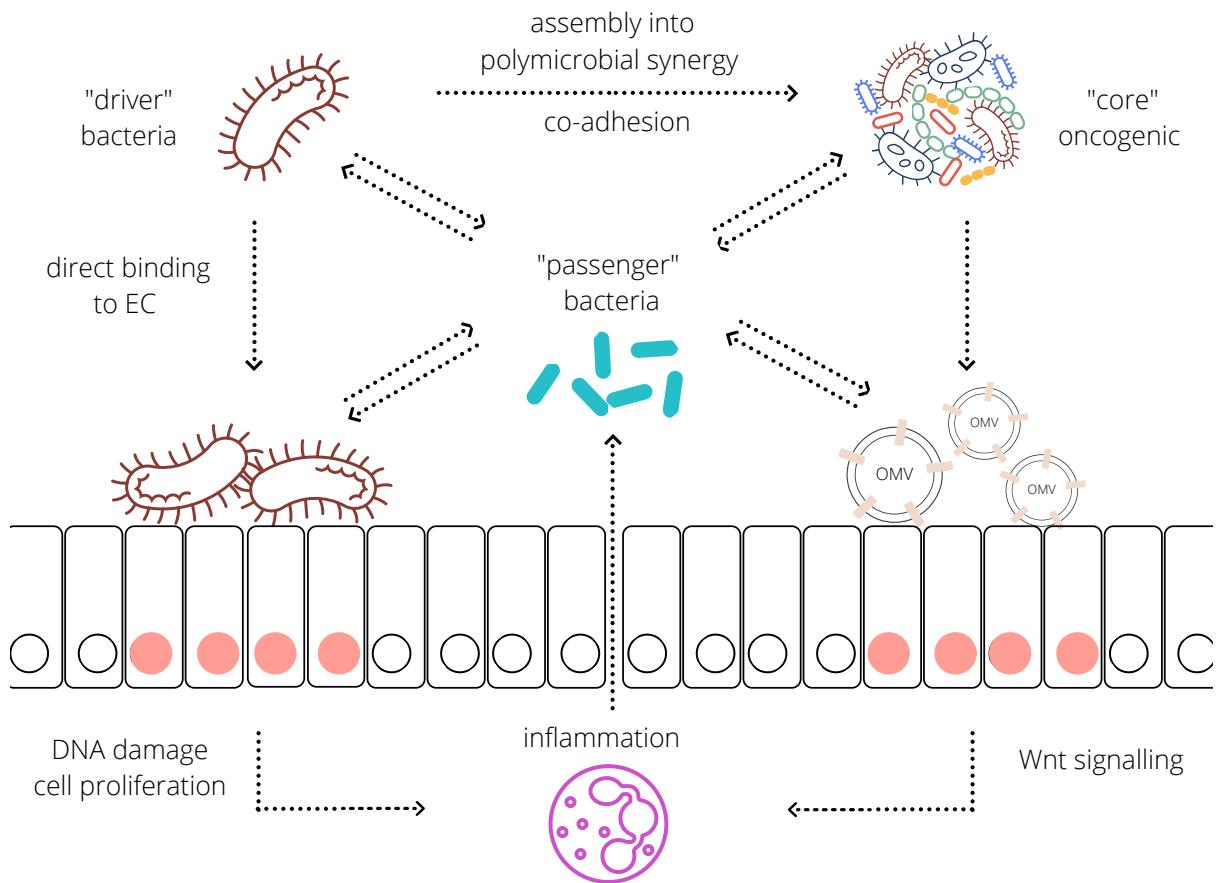
*Figure 4. The diagram representing outer membrane protein (OMP) assembly and their contribution to the formation of outer membrane vesicles (OMV) that can further bind to the intestinal epithelium lining. The OMP assembly begins with a nascent polypeptide with an N-terminal end, that is chaperoned of secretory system (SecA) across the bacterial inner membrane into the  $\beta$ -barrel assembly machinery (BAM) that folds the OMP.*

The proteomic analysis of gram-negative bacteria OMVs has only recently been of research interest. The proteomics analysis of *E. coli* OMVs has shown abundant presence of OmpA (Confer and Ayalew, 2013). The OMV analysis of *C. jejuni* has detected OMPs such as pore-forming proteins (PorA and Omp50), fibronectin-binding proteins (CadF and Cj1279c) and a surface antigen (CjaA), each known for their virulence factors (Jang *et al.*, 2014). The analysis of *H. pylori* OMV has revealed putative OMP virulent factors, such as adhesins HopZ and HorB proteins, the blood group antigen binding adhesin (BabA, BabB), sialic acid-binding adhesion (SabA, SabB), *H. pylori* adhesin A (HpaA), and adherence-associated lipoproteins (AlpA, AlpB) (Q. Liu *et al.*, 2019; Turner *et al.*, 2018, 2015). In Liu et.al proteomic characterisation of *F. nucleatum* OMV virulence components such as YadA-like domain and FadA has been identified in addition with six autotransporters constituting a majority of the vesicular molecular weight. Autotransporters were initially thought to be responsible for its own translocation into outer membrane, but recently it's associated with other particle transport and folded protein domain (Pizarro-Cerdá and Cossart, 2006).

## 1.5 Gram-negative species drive carcinogenesis as a synergy of direct and indirect “driver-passenger” effects

A recently proposed “driver-passenger” theory suggests that, firstly, there are pro-carcinogenic species driving the damage of intestinal mucosa and creating favourable environment for further CRC development by opportunistic bacteria (Tjalsma *et al.*, 2012). In this model *Bacteroides* and *Enterobacteriaceae* play role as the “driving” microbes due toxin-based direct epithelial cell damage, whereas *Fusobacterium* start to thrive in the new pro-inflammatory environment. In conjunction, the CRC-associated bacteria were suggested to overpopulate the ‘protective’ microbiome and cause tumorigenesis.

Although, the microbiome is known for its heterogenic compositions, another model of ‘polymicrobial synergy and dysbiosis’ suggests that, as a ‘core microbiome’ exists within each individual, a ‘core’ pathogenic microbiome can be acquired with a set combination of oncogenic genes (Arumugam *et al.*, 2011; Claesson *et al.*, 2011; Shaikh *et al.*, 2018). This concept was developed based on association between *Porphyromonas gingivalis* and microbial dysbiosis of periodontitis (Nath and Raveendran, 2013). Similarly, *F. nucleatum* has also been associated with periodontitis as an opportunistic agent (Hajishengallis and Lamont, 2012).



*Figure 5. The representation of a combined approach of "driver-passenger" and "polymicrobial synergy" models that include OMVs. A "driver" bacteria can potentially cause damage directly via epithelial cell adhesion (EC), indirectly via outer membrane vesicles (OMV) or contributing to the "core" oncogenic microbiome. In turn, the pathogenic mechanisms ( genotoxins or adhesions etc.) cause DNA damage, activation of Wnt signalling and increased cellular proliferation. A further induced inflammation via microbial species or EC promotes a favourable environment to "passenger" bacteria (such as *F. nucleatum*). The opportunistic species continue to contribute to carcinogenesis via "polymicrobial synergy", OMVs and direct binding.*

## 1.6 *F. nucleatum* OMVs exhibit immunogenic properties in intestinal epithelia

Recently, *F. nucleatum* OMVs, have been shown to interact with human intestinal epithelial cells, leading to activation of Toll-like receptors (TLRs) which can lead to the pro-inflammatory cytokine release and inducing the NF- $\kappa$ B immune response (Garcia-Vello *et al.*, n.d.; J. Liu *et al.*, 2019). In our laboratory group *F. nucleatum* strains and their derived OMVs were shown to interact with lectins expressed by immune cells via LPS (Di Lorenzo *et al.*, 2019; Okahashi *et al.*, 1988). *F. nucleatum* LPS structure has been described showing strain-specific differences in composition of the carbohydrates, with similarities in amino sugars and

amino acetylating groups. Garcia-Vello *et al.* investigated the O-Antigen & Lipid A structures of the LPS in the *F. nucleatum* ATCC 51191 strain. They showed that there were 3 monosaccharide repeating unit residues that composed the O-antigen such as  $\beta$ -d-GlcNAcA,  $\beta$ -d-GlcNAc3NAlaA and  $\alpha$ -d-FucpNAc4NAc (Garcia-Vello *et al.*, n.d.). In addition, interaction of *F. nucleatum* with immune system mediators is capable of stimulating the production of pro-inflammatory cytokines, such as interleukin-8 and tumour necrosis factor-alpha (TNF- $\alpha$ ) *in vivo* potentially leading to the development of proinflammatory state favouring the progression of cancer (Krisanaprakornkit *et al.*, 2000).

In the latest *F. nucleatum* OMVs related research conducted by Engevik *et al.* it has been suggested that those who have an intact microbiome may have better protective response against *F. nucleatum* as they found that mice that were not pre-treated with antibiotics showed no alteration in pro-inflammatory status after oral administration of *F. nucleatum* (Engevik *et al.*, 2021). In the study, both *in vitro* and *in vivo* models were applied. Using the colonic T84 cell line *F. nucleatum* was shown to adhere to mucin molecules adjacent to the intestinal epithelium cell layer, meanwhile in HT29 cell monolayers the size-fractioned medium of *F. nucleatum* suggested an increase in inflammatory response (IL-8, TNF, NF- $\kappa$ B) due purified OMVs and compounds larger than 50kD. However, a similar pro-inflammatory effect in human intestinal enteroids was only observed in relation to IL-8 and did not apply for other inflammatory markers. The *in vivo* model of a single gavage of *F. nucleatum* in mice with humanized microbiome revealed no changes in inflammatory status. An infiltration of immune cells, loss of mucin and cellular architecture occurred after an antibiotic cocktail was applied in a model. Interestingly, less of the *F. nucleatum* effect was observed on day 5 compared to day 3 of the experiment. Thus, suggesting that restoring microbiome and epithelium architecture play a protective role against *F. nucleatum*. With that being said, the *in vivo* model did not reflect the OMVs role in intestinal alternation nor was it clear if *F. nucleatum* was viable.

Here we will aim to characterise the *F. nucleatum* OMVs, to gain insight into internalisation at epithelium surface that can potentially lead to alterations in immune response, as well as compare the adhesins of gram-negative bacteria. To address this, we will use the Nonasclase particle machine, advanced model systems based on human-derived organoids recapitulating intestinal crypts and various computational bioinformatics tools (Almeqdadi *et al.*, 2019).

## Method

### 2.1 *F. nucleatum* OMV purification

**2.1.1 Bacterial strains and cultivation.** *F. nucleatum* ssp. ATCC 25586, ATCC 10953 and 51191 were previously frozen in the laboratory. The cells were cultured in tryptic soy broth (TSB) supplemented with 1 mg/ml menadione and 5 mg/ml hemin overnight in anaerobic cabinet (85% N<sub>2</sub>, 5% CO<sub>2</sub>, 10% H<sub>2</sub> at 37°C) until reaching an OD600nm of approximately 1.2 (for *F. nucleatum* ATCC 25586 and ATCC 10953) or 0.7 (for *F. nucleatum* ATCC 51191). The OMV purification process is shown in Fig.6(A).

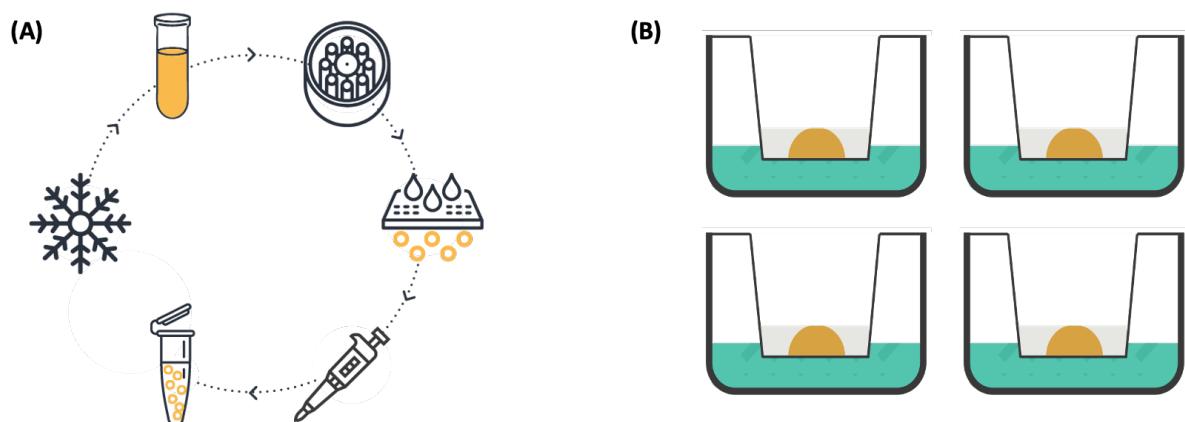


Figure 6. (A) The process of OMV purification depicting steps such as bacterial culture, ultra-centrifugation, filtration and OMV collection. (B) Graphic representation of intestinal organoids in Transwells, featuring a cell layer in a well surrounded by a basolateral medium at the bottom and apical medium on top.

**2.1.2 OMV isolation and purification.** Sample was centrifuged at 10,000 x g for 15 min at 4°C by using centrifuge 5810R or Avanti J-26XP (with rotor JLA-10.5). A formed supernatant was vacuum filtered through a 0.22 mm filter unit membrane (Sartolab-stores). For small volumes of filtered supernatant a spin-filtration unit of 100K molecular weight cut-off filter unit (MWCO) (Sartorius) at 5,500 x g and 4°C (small volumes) was applied for further concentration. For larger volumes of supernatant, an ultra-centrifuge LE-80 (Rotors: SW41 Ti, Type 45 Ti) (Beckman Coulter) at 200,500 x g (or 41,600 rpm) for 2 h at 4°C was applied before spin-filtration. OMVs were recovered from the filter using sterile phosphate buffered saline (PBS) (Lonza).

**2.1.2 OMV purification by gradient ultra-centrifugation.** In order to create 2 ml gradients of 35%, 30%, 25% and 20% densities, Optiprep medium (60% w/v, Sigma) was diluted in

Optiprep buffer (0.85% w/v NaCl and 10 mM Tricine-NaOH at pH 7.4). For the final 40% Optiprep gradient OMVs recovered in PBS were used. The 2 ml fractions were further inserted into 13.2 ml Ultra-clear tube (Beckman Coulter) starting from the bottom with highest gradient of 40 % density followed by the fractions of decreasing density. The tube was ultra-centrifuged using the SW41 Ti rotor at 135,000 x g (or 28,100 rpm) for 16 h at 4°C with “slow” acceleration and “no brake” deceleration. The 1ml fractions were collected from top to bottom for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The OMV-containing fractions were diluted 10 times (vol/vol) with sterile PBS. Small volumes were either spin-filtrated at 5,500 x g in 4°C using a 100K MWCO (Sartorius), whereas large volumes were first ultra-centrifuged by using Type 45 Ti roto at 200,500 x g (or 41,600 rpm) for 2 h at 4°C. OMVs were resuspended in sterile PBS and filtered through a 0.22 mm membrane (Sartolab-stores). Concentrated in PBS OMVs were stored at -80°C. The used equipment and surfaces were washed up by Vikron tables and Bioguard spray accordingly.

2.1.3. *SDS-page*. The collected sample fraction of 12 µl were added with 4 µl of four-times diluted buffer. These were incubated at 100C for 5 minutes and loaded in gel to run at 200 V for 35 minutes. The images were collected by using

2.1.4 *Zetaview*. The purified vesicles diameter was identified by using the Nanoparticle tracking analyzer (Analytik). The unlabelled OMVs were first used to make the calibration solutions (1:1000 into 1:5000, 1:10,000 and 1:20,000) and washed up with 5mlx6 miliQ waster. The 1ml sample was then injected, the data was analysed using the Zetaview report.

2.1.5. *FITC labelling of OMVs*. OMVs were incubated at room temperature with 500 mg/ml fluorescein isothiocyanate isomer (FITC) (Sigma) in FITC buffer (50 mM Na<sub>2</sub>CO<sub>3</sub> and 100 mM NaCl at pH 9.2). OMVs were ultra-centrifuged (LE-80 (Rotors: SW41 Ti, Type 45 Ti) (Beckman Coulter) at 52,000 x g for 30 min, washed three times with sterile PBS (Lonza) and resuspended in PBS (Lonza). For short period of time up to two weeks stored at 4°C (up to two weeks) or frozen at -80 °C.

## 2.2 Intestinal organoid culture on Transwell

The organoids on Transwell were previously generated by Quadram laboratory team (Fig.6(B)). Cells were maintained in Intesticult medium and changed every 2 days. Upon reaching the confluence in approximately 7-10 days passing was performed by incubating

the organoids in polymerized Matrigel in Cell Recovery Solution (Corning, NY, USA) for 45 min, at 4 °C, followed by mechanical dissociation, and the culture typically expanded 1:4.

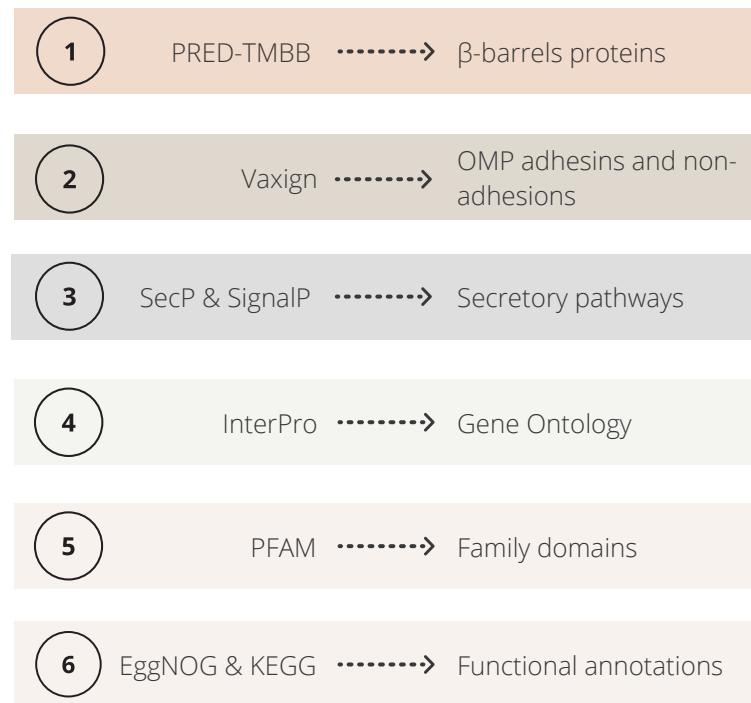
## 2.3 Bioinformatics analysis

The bacterial proteome sequences were acquired from UniProt database (<https://www.uniprot.org>) (Tab.1).

Table 1. Table summarising the UniProt Proteome IDs of gram-negative bacteria (*B. thetaiotaomicron*, *B. fragilis*, *E. coli*, *H. pylori*, *F. nucleatum*, *C. jejuni*) used for analysis and their proteome sizes.

Bacterial name	Proteome ID	Proteome size
<i>B. thetaiotaomicron</i> (ATCC 29148 / DSM 2079 / NCTC 10582 / E50 / VPI-5482	UP000001414	4,782
<i>B. fragilis</i> (ATCC 25285 / DSM 2151 / JCM 11019 / NCTC 9343)	UP000006731	4,234
<i>E. coli</i> (O157:H7)	UP000000558	5,062
<i>H. pylori</i> (ATCC 700392 / 26695)	UP000000429	1,552
<i>F. nucleatum</i> (ATCC 51191)	UP000005392	2,277
<i>C. jejuni</i> (ATCC 700819 / NCTC 11168)	UP000000799	1,623

The bacterial proteome sequences were acquired from UniProt database (<https://www.uniprot.org>) (Tab.1). The OMV proteome sequences of *F. nucleatum* subsp. *animalis* 7–1 were acquired from Liu et. al. Proteins with β-barrels were predicted by using PRED-TMBB2 (<http://www.compgen.org/tools/PRED-TMBB2>, Tsirigos et al., 2016)(cutoff is 0.6). Subcellular localization of “outer membrane” and the adhesion probability were identified using Vaxign (<http://www.violinet.org/vaxign/>, Xiang and He, 2013) (cutoff for adhesion score is 0.51). The secretory pathways were identified with SecretomeP 2.0 (<http://www.cbs.dtu.dk/services/SecretomeP/>, Bendtsen et al., 2005)) for adhesin due larger volume of data and SinglalP 5.0 (<https://services.healthtech.dtu.dk/service.php?SignalP-5.0>, Nielsen et al., 2019) for non-adhesins. Metabolic pathways and functional groups were identified via InterPro , KEGG and EggNOG 4.5.1 using OmicsBox platform (<https://www.biobam.com>). The summary of bioinformatic analysis is shown in the Figure 7.



*Figure 7. The summary of bioinformatic tools and the outcomes. (1) The bacterial proteome is analysed using PRED-TMBB to identify transmembrane domains; (2) Vaxign is used to identify the “outer membrane” localisation, adhesin and non-adhesin (cutoff is 0.51); (3) SecP and SignalP were used to determine secretory pathways needed for translocation of outer membrane proteins (OMP); (4) InterPro was used to identify gene ontologies; (5) PFAM was used to identify protein family domains; (6) EggNOG and KEGG were used to identify functional annotation for pathway analysis.*

## Results

### 3.1 Purified *F. nucleatum* OMVs displayed an average size of 90-110 nm

The presence of OMVs was identified by SDS-page (Appendix A). The Zetaview reports of Nanoparticle analyser helped to reveal OMVs average diameter in strains ATCC 10953 (100.9 nm), ATCC 25586 (93 nm), ATCC 51191 (105.2 nm) shown in Fig.7.

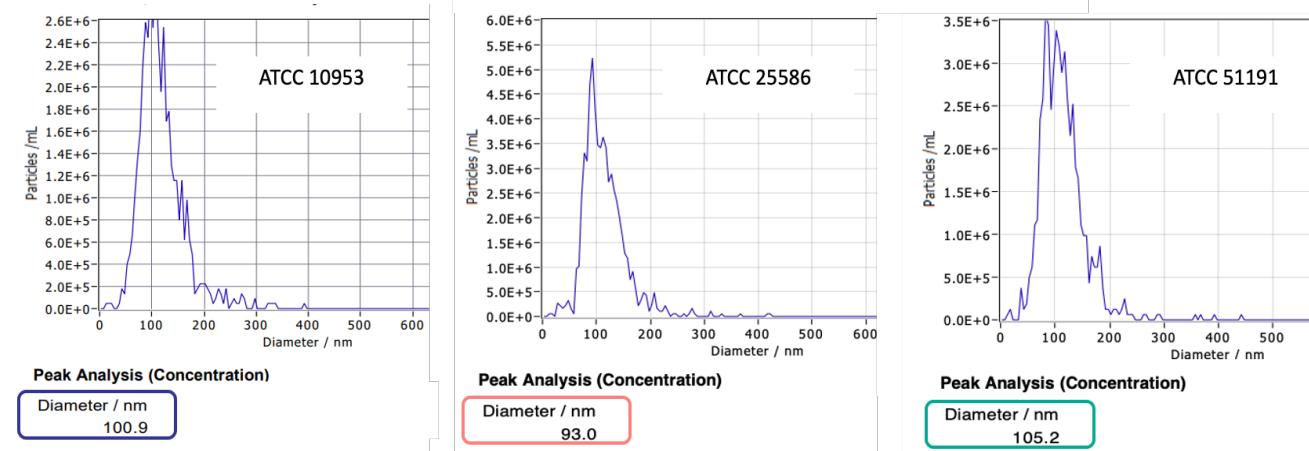


Figure 8. The Zetaview reports of Nanosize revealing the average size of OMVs in strains ATCC 10953 (100.9 nm), ATCC 25586 (93 nm), ATCC 51191 (105.2 nm).

### 3.2 Purified OMVs are internalised by intestinal organoids

The purified OMVs stained with FITC were incubated with intestinal organoids and fixed for microscopy analysis, revealing potential internalisation of the *F. nucleatum* ATCC 25586 OMVs (Fig.8).

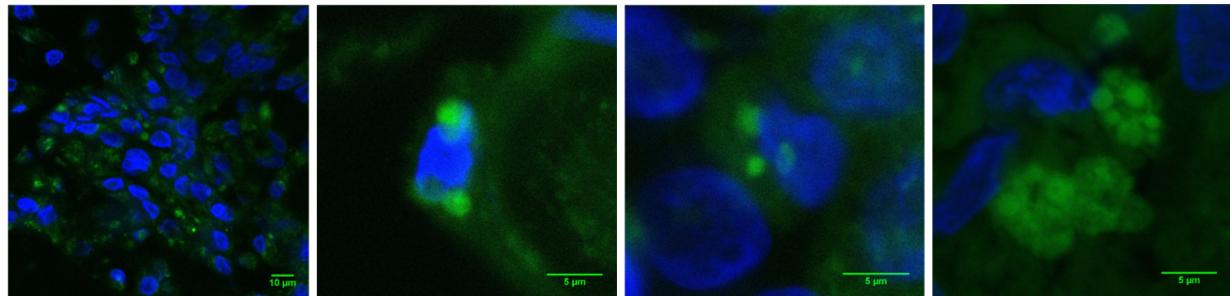


Figure 9. Confocal microscopy images of colonic organoids on trans-wells were obtained after 24 hr incubation period with *F. nucleatum* ATCC 25586 OMVs; (nucleus are labelled in blue (DAPI); OMVs are labelled in green (FITC)).

### 3.3 Gram-negative species have more adhesins than non-adhesins

The outer membrane proteins were selected using PRED-TMB ( $p \geq 0.9$  and  $\beta$ -score  $\geq 0.25$ ) and Vaxign (localisation of “Outer Membrane” and  $p \geq 0.9$ ). The total number of identified OMPs and its percentage to the whole bacterial proteome is shown in Tab.1.

Table 2. The total number of outer membrane proteins (OMPs) identified in gram-negative bacteria () and its percentage to the size of the consecutive proteomes.

	<i>B. thetaiotaomicron</i>	<i>B. fragilis</i>	<i>E. coli</i>	<i>H. pylori</i>	<i>F. nucleatum</i>	<i>C. jejuni</i>
<b>Number of OMPs</b>	183	134	77	25	27	17
<b>Percentage to the whole proteome</b>	3.83	3.16	1.52	1.61	1.19	1.05

In order to classify adhesins the probability of 0.51 used as a cut-off (Vaxign,  $F_p \geq 0.51$ ), the rest were classified as non-adhesins (Fig.9)

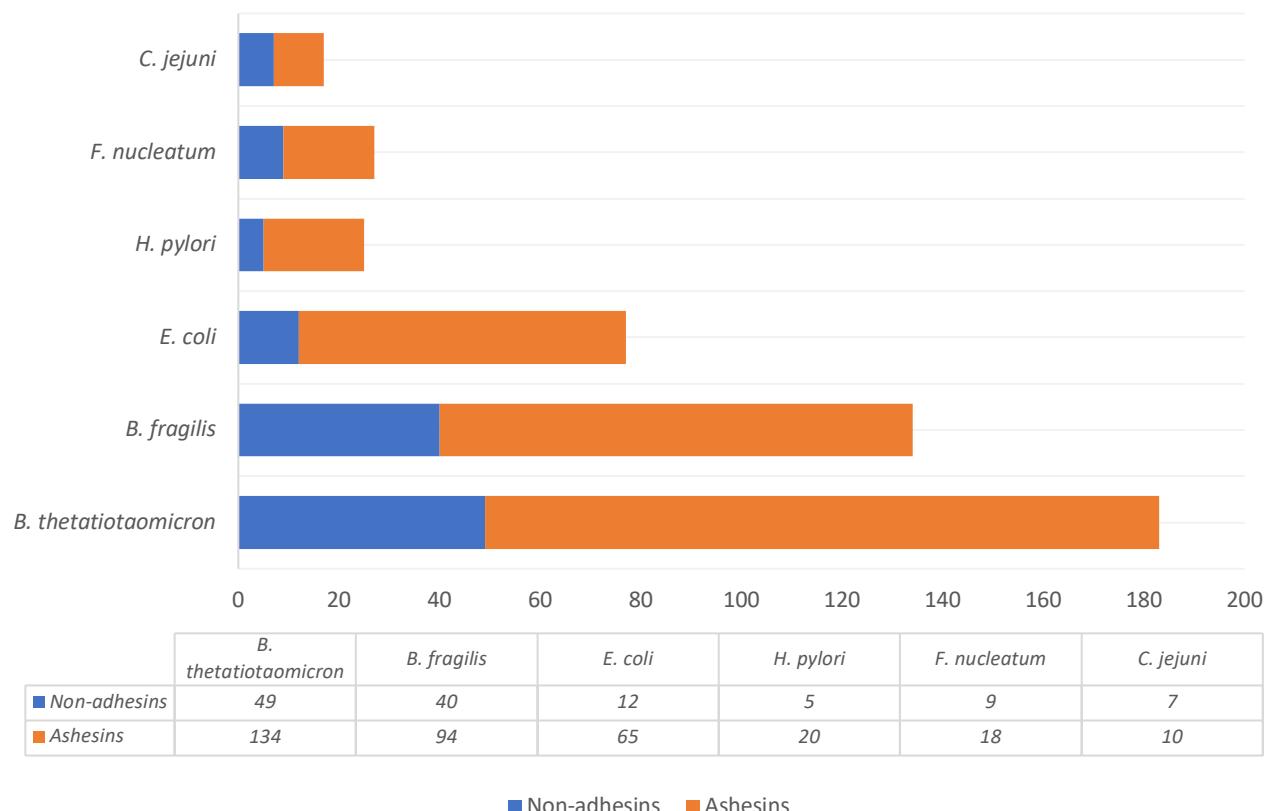


Figure 10. The number of adhesins and non-adhesins identified in gram-negative bacteria using Vaxign (cutoff for adhesins in  $p \geq 0.51$ ).

### 3.4 Gram-negative species share a variation of pathways relate to antimicrobial resistance, OMP assembly and quorum sensing system

For functional annotations, the overlapping EggNOG and KEGG pathway description were summarised for adhesins in Table 3 and non-adhesins in Table 4. Non-adhesins in gram-negative bacteria seem to share more iron-realted and OmpA-like proteins (Tab.4).

*Table 3. Overlapping EggNOG and KEGG pathways of adhesin proteins in gram-negative bacteria. (B.F: *B. fragilis*; B.T: *B. thetaiotaomicron*; C.J: *C. jejuni*; F.N: *F. nucleatum*; H.P: *H. pylori*.)*

Annotation	Descriptions	B.F	B.T	C.J	E.C	F.N	H.P
EGGNOG	Carboxypeptidase regulatory-like domain	•	•				
EGGNOG	Outer membrane efflux protein		•		•		
EGGNOG	Outer membrane protein assembly complex, YaeT protein	•	•		•		
EGGNOG	Secretin and TonB N terminus short domain	•	•				
EGGNOG	TonB dependent receptor	•	•	•	•	•	
ko02020	Two-component system	•	•	•	•		
ko03070	Bacterial secretion system	•	•	•	•	•	•
ko01501	beta-Lactam resistance	•	•	•	•	•	•
ko01503	Cationic antimicrobial peptide (CAMP) resistance	•	•	•	•	•	•
ko05133	Pertussis	•	•	•	•	•	
ko04626	Plant-pathogen interaction	•	•	•	•	•	
ko02024	Quorum sensing			•	•	•	•
ko00564	Glycerophospholipid metabolism			•	•	•	•
ko00540	Lipopolysaccharide biosynthesis			•	•	•	•
ko00592	alpha-Linolenic acid metabolism			•	•		•
ko00590	Arachidonic acid metabolism			•	•		•
ko02010	ABC transporters			•		•	•
ko02040	Flagellar assembly			•		•	•
ko00591	Linoleic acid metabolism			•	•		•
ko00270	Cysteine and methionine metabolism			•		•	•
ko00565	Ether lipid metabolism			•	•		•
ko05111	Biofilm formation			•		•	•

Table 4. Overlapping EggNOG and KEGG pathways of non-adhesin proteins in gram-negative bacteria. (B.F: *B. fragilis*; B.T: *B. thetaiotaomicron*; C.J: *C. jejuni*; F.N: *F. nucleatum*; H.P: *H. pylori*.)

Annotations	EggNOG description	B.F	B.T	C.J	E.C	F.N	H.P
<b>K07277</b>	Outer membrane protein insertion porin family		•	•		•	•
<b>EGGNOG</b>	TonB-dependent Receptor Plug Domain	•	•			•	
<b>EGGNOG</b>	TonB-dependent receptor	•	•			•	
<b>K02014</b>	Iron complex outer membrane receptor protein	•	•			•	
<b>EGGNOG</b>	TonB-linked outer membrane protein, SusC RagA family	•	•				
<b>EGGNOG K21572</b>	SusD family	•	•				
<b>K16087</b>	Hemoglobin/transferrin/lactoferrin receptor protein	•	•				
<b>K02016</b>	Iron complex transport system substrate-binding protein	•	•				
<b>K16089</b>	Outer membrane receptor for ferrienterochelin and colicins		•	•			
<b>EGGNOG K18139</b>	Outer membrane efflux protein		•	•	•		
<b>K03286</b>	OmpA-OmpF porin, OOP family	•	•	•			
<b>EGGNOG K09800</b>	Translocation and assembly module TamB			•			•
<b>EGGNOG K07289</b>	AsmA-like C-terminal region			•			•
<b>EGGNOG</b>	Outer membrane protein, OMP85 family	•				•	

### 3.4 A variation in PFAM is identified between adhesins and non-adhesins

The adhesin proteins seem to be more associated with TonB proteins and Omp protein family (Tab.5), whereas non-adhesins with bacterial surface antigen (D15) and POTRA domains (Tab.6).

Table 5. The comparison between PFAM of outer membrane adhesins in gram-negative bacteria (B.F: *B. fragilis*; B.T: *B. thetaiotaomicron*; C.J: *C. jejuni*; F.N: *F. nucleatum*; H.P: *H. pylor*)

PFAM descriptions	PFAM ID	B.F	B.T	C.J	E.C	F.N	H.P
TonB-dependent receptor, plug domain	IPR012910	•	•	•	•	•	•
TonB-dependent receptor-like, beta-barrel	IPR000531	•	•	•	•	•	•
Outer membrane protein transport protein (OMPP1/FadL/TodX)	IPR005017	•	•		•	•	
Phosphate-selective porin O/P	IPR010870	•	•				
Bacterial surface antigen (D15)	IPR000184	•	•		•		
Outer membrane efflux protein	IPR003423	•	•		•		
Alpha-2-macroglobulin	IPR001599	•				•	
Secretin/TonB, short N-terminal domain	IPR011662	•	•				
CarboxypepD_reg-like domain	PF13715	•	•				
Secretin/TonB, short N-terminal domain	IPR011662	•	•				
OmpA-like domain	IPR006665	•	•				
Phospholipase A1	IPR003187			•	•		

Table 6. The comparison between PFAM of outer membrane non-adhesins in gram-negative bacteria (B.F: *B. fragilis*; B.T: *B. thetaiotaomicron*; C.J: *C. jejuni*; F.N: *F. nucleatum*; H.P: *H. pylor*)

PFAM descriptions	PFAM ID	B.F	B.T	C.J	E.C	F.N	H.P
Bacterial surface antigen (D15)	IPR000184	•	•	•	•	•	•
Outer membrane efflux protein	IPR003423	•	•	•	•		
OmpA-like domain	IPR006665	•	•	•	•		
TonB-dependent receptor, plug domain	IPR012910	•	•	•			•
TonB-dependent receptor-like, beta-barrel	IPR000531	•	•	•			•
Patatin-like phospholipase domain	IPR002641	•	•				•
Secretin/TonB, short N-terminal domain	IPR011662	•	•				
Outer membrane protein beta-barrel domain 3	IPR041700	•	•				
CarboxypepD_reg-like domain	PF13715	•	•				
POTRA domain, BamA/TamA-like	IPR010827			•	•	•	•

Table 7. Master table representing *C. jejuni*'s outer membrane adhesion proteins master table featuring sequence name, length, number of transmembrane domains (#TM), SecP score for secretory pathways analysis (cutoff is 0.5), InterPro IDs, InterPro gene ontology (GO) IDs (P=biological process, F=molecular function, C=cellular component), gene names derived from EggNOG, EggNOG descriptions and KEGG pathway IDs or KO. The master tables for the rest of the species is provided in the supplementary material.

#	Protein Accession	Protein Length	#TM	Adhesin Probability	SecP score	InterPro IDs	InterPro GO IDs	InterPro GO Names	Gene Name	EggNOG Description	KEGG KO
1	sp_P80672_PORA_CAMJE	424	14	0.72	0.9	IPR008439	no GO terms	no GO terms	porA	major outer membrane protein	
2	tr_Q0P814_Q0P814_CAM	709	22	0.59	0.8	IPR000531 ; IPR012910	no GO terms	no GO terms	chuA	TonB dependent receptor	K02014
3	tr_Q0P8Q8_Q0P8Q8_CAM	329	12	0.66	0.5	IPR003187	P:GO:0006629; F:GO:0004620; C:GO:0016020	P:lipid metabolic process; F:phospholipase activity; C:membrane	pldA	phospholipase	K01058
4	tr_Q0P937_Q0P937_CAM	846	10	0.57	0.7	no IPS match	no IPS match	no IPS match	ytfN	Protein conserved in bacteria	K09800
5	tr_Q0P9S7_Q0P9S7_CAM	574	14	0.65	0.9	IPR005565 ; IPR013686	no GO terms	no GO terms	hxuB	Haemolysin secretion/activation protein ShlB/FhaC/HecB	
6	tr_Q0P9T3_Q0P9T3_CAM	762	10	0.74	0.8	no IPS match	no IPS match	no IPS match			
7	tr_Q0PA96_Q0PA96_CAM	426	2	0.61	1	no IPS match	no IPS match	no IPS match		Annotation was generated automatically without manual curation	
8	tr_Q0PAV5_Q0PAV5_CAM	309	14	0.61	0.7	IPR016896	no GO terms	no GO terms		Protein of unknown function (DUF2860)	
9	tr_Q0PBW1_Q0PBW1_CAM	755	20	0.7	0.9	IPR012910 ; IPR000531	no GO terms	no GO terms		TonB dependent receptor	K02014,K16087
10	tr_Q0PC44_Q0PC44_CAM	400	2	0.73	0.9	no IPS match	no IPS match	no IPS match		curli production assembly transport component CsgG	

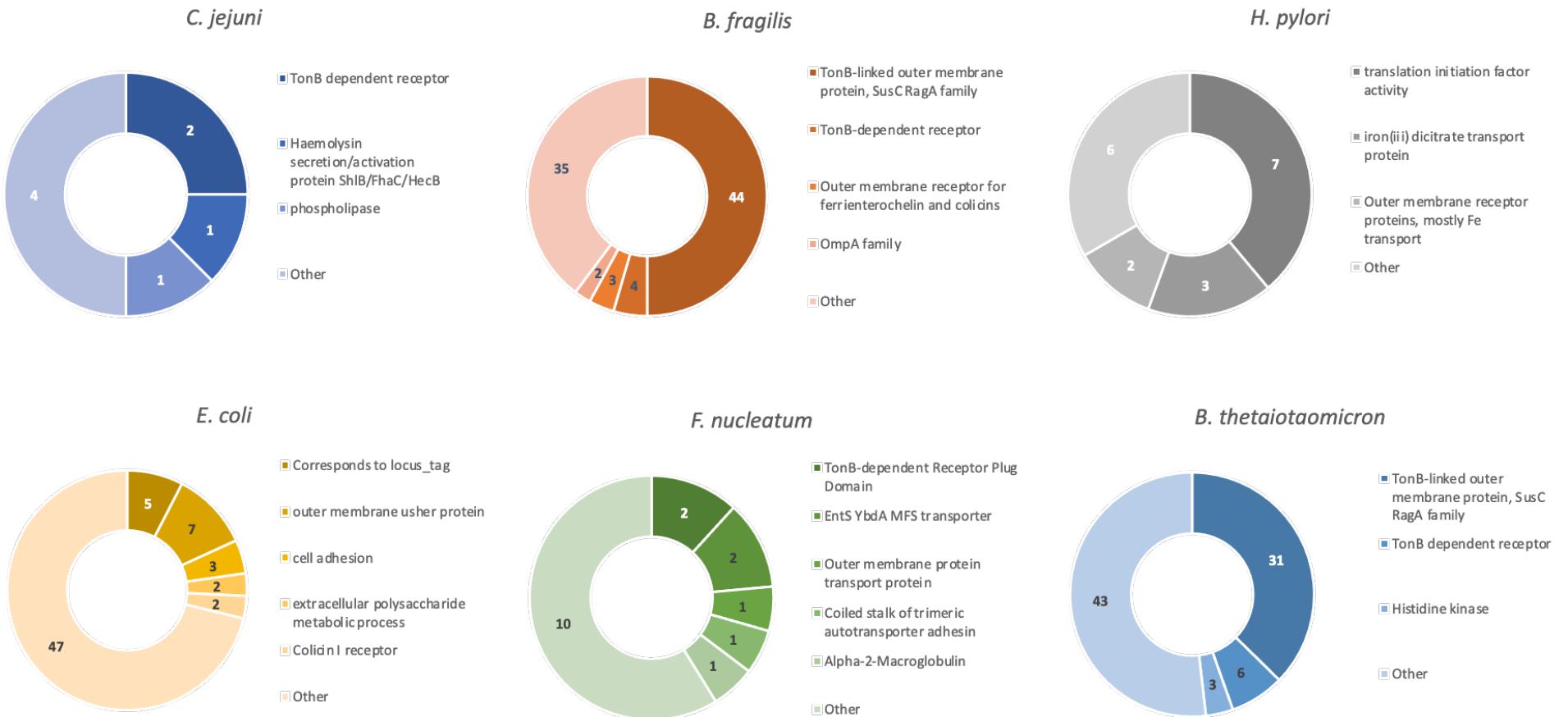


Figure 11. The pie-charts for gram-negative bacterial species (*C. jejuni*, *B. fragilis*, *H. pylori*, *E. coli*, *F. nucleatum*, *B. thetaiotaomicron*) with top EggNOG annotations. The similarity is seen in the *Bacteroides* spp. with overlapping majority of TonB-related OMPs; *E. coli* and *C. jejuni* share mostly the Usher proteins, cell adhesion proteins and polysaccharide

metabolism; *F. nucleatum* has common features with both *Bacteroides* spp. and shows higher amount of transport proteins; *H. pylori* has shown great number of protein related to initiation of translation, as well as iron-related activity.

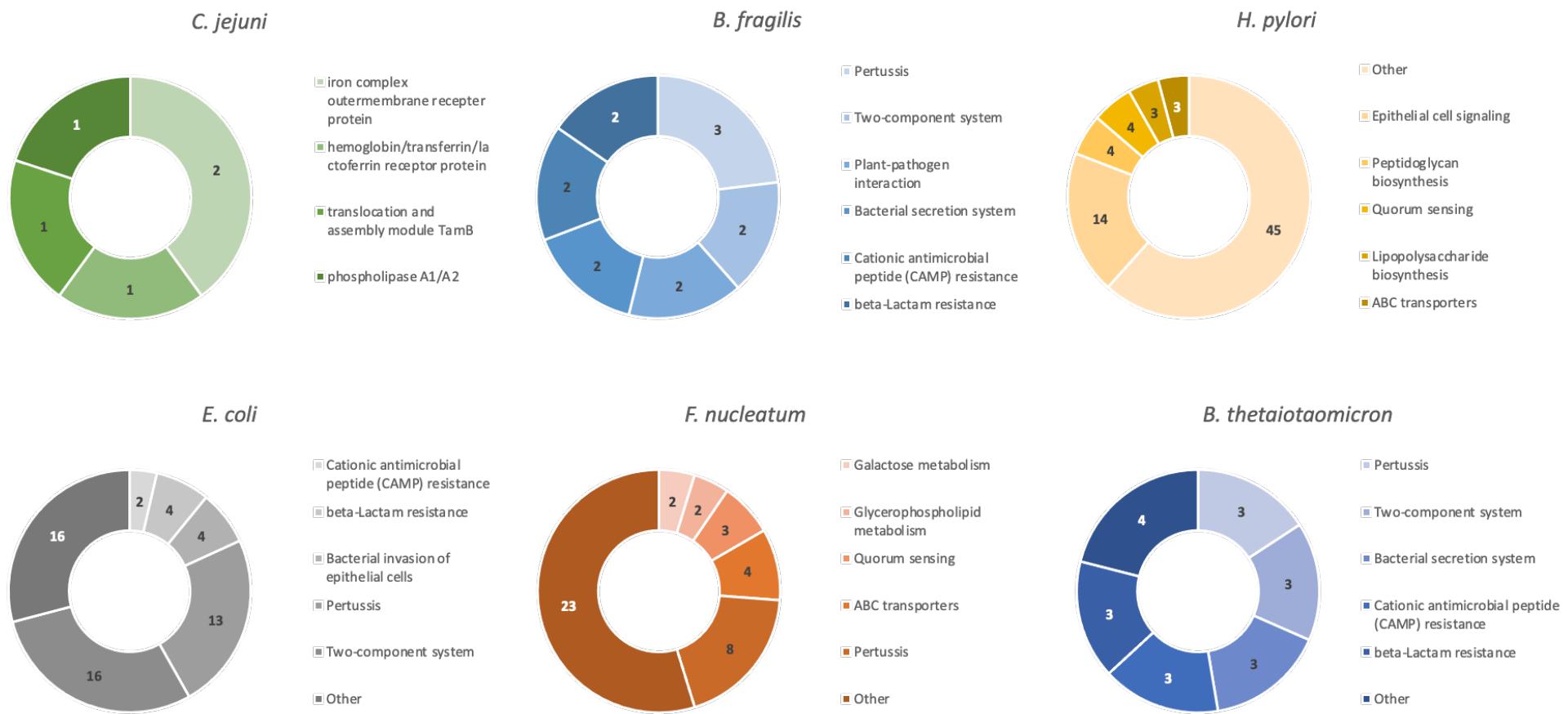


Figure 12. The pie-charts for gram-negative bacterial species (*C. jejuni*, *B. fragilis*, *H. pylori*, *E. coli*, *F. nucleatum*, *B. thetaiotaomicron*) with top KEGG annotations. The similarity is seen in the *Bacteroides* spp. with overlapping majority of pathways; *F. nucleatum* and *H. pylori* share the top functions of ABC transporters and Quorum sensing peptides; as well as *F. nucleatum* exhibits pertussis common with *E. coli*;

## Discussion

Here, the *F. nucleatum* sp. ATCC 25586, ATCC 10953 and 51191 were successfully purified and characterised as an average of 90-110 nm (Fig.7), that suggest a slight heterogeneity in size. The current method of purification is up to date and utilised the “separation by gradient” technique that allowed to further purify the OMVs and precisely measure using a Nanosize.

An optimised staining of *F. nucleatum* ATCC 25586 OMVs with FITC, incubation with intestinal organoids and microscopy analysis suggested potential vesicular internalisation (Fig.8). OMVs are typically produced in all types of environmental conditions and have an average diameter between 20-200 nm (Bonnington and Kuehn, 2014). Internalisation or endocytosis is an uptake of particles across the cellular lipid bilayer (Doherty and McMahon, 2009). The main pathways utilised for internalisation in bacteria seem to correlate with the size of the OMVs. For example, for OMVs over 200nm an actin-driven micropinocytosis is used, whereas clathrin-covered pits are used for OMVs smaller than 200nm (Vercauteren *et al.*, 2010; Weiner *et al.*, 2016). More mechanisms such as lipid-rafts and caveolin have been associated with the uptake of *H. pylori* OMVs and *C. jejuni* via epithelial cells accordingly (Elmi *et al.*, 2012; Kaparakis *et al.*, 2010). Thus, suggesting further exploration of such mechanisms in *F. nucleatum* with regards to endocytic pathways in future research.

The experiment was completed only using one strain of bacteria and need to be replicated, as the variation may occur across the species. On the strong side, a biopsy-derived adult human organoid tissue used *via* Transwell allowed to recapitulate the primary tissue up to most innovating standards (Almeqdadi *et al.*, 2019). The differentiated epithelial cell types include goblet cells, enterocytes, enteroendocrine cells and Paneth cells (E *et al.*, 2021). In future studies, new gut-on-chip technologies can be used to model cancer cell behaviour *in vitro* alongside mimicking environments that would be found in humans. These models may be useful in personalised medicine as they are being used to investigate how certain drugs and cancers may interact. These models will be useful in order to monitor the interaction between *F. nucleatum* OMVs and colon epithelial cells. The OMVs effect on gut epithelial barrier and immune system can be studied by expression of E-cadherin and identification of affected cell types using flow cytometry, as well as estimating the amount of TNF- $\alpha$  or other cytokines *via* ELISA.

An additional bioinformatic analysis of the OMPs of CRC-related gram-negative bacteria (*B. thetaiotaomicron*, *B. fragilis*, *E. coli*, *H. pylori*, *F. nucleatum*, *C. jejuni*) was applied to functionally characterise these proteins and give an insight in the potential overlapping mechanisms, important for future pharmaceutical targeting (Noinaj *et al.*, 2017; Sikora *et al.*, 2018).

The OMPs sequences were carefully selected using the PRED-TMB and Vaxign based on the presence of  $\beta$ -barrels and outer membrane localisation probability the  $p \geq 0.9$ . More pathogenic bacterial species such as *B. thetaiotaomicron* and *B. fragilis* revealed greater percentage of OMPs compared to less pathogenic *C. jejuni* (Tab.2), consistent with the proposed virulence property of OMPs. The OMPs were further classified to non-adhesins and adhesins (cutoff  $p \geq 0.51$ ) to assess the importance of adhesion in a “holistic” CRC model. The number of adhesins in gram-negative bacteria is consistently higher than non-adhesins (Fig.9) supporting the correlation between the pathogenicity and adhesin levels. The *B. thetaiotaomicron* constituting with the highest number of adhesins compared the lowest number of *C. jejuni*. Using the functional analysis of OMPs of gram-negative bacteria (*C. jejuni*, *B. fragilis*, *H. pylori*, *E. coli*, *F. nucleatum*, *B. thetaiotaomicron*) certain pathways were identified overlapping across the species. These mechanisms involve TonB-related, iron-related, pertussis, outer membrane efflux system, OMP assembly-related system, etc.

TonB transporters are outer membrane proteins important for passive nutrient acquisition and is mediated via proton-dependent energy expenditure anchored to the inner membrane (Celia *et al.*, 2016). These families IPR012910 and IPR000531 of TonB-dependent receptors and TonB-dependent receptors-like were identified in the OMPs across the selected gram-negative bacteria (Tab.4 and Tab.5). The TonB proteins were found to constitute the majority of *B. fragilis*, *B. thetaiotaomicron* and *F. nucleatum* EggNOG descriptions, suggesting a greater importance of its function. One of the first crystallised structures of TonB-dependent receptors were identified in *E. coli*, ferrichrome transporter (FhuA). The data extrapolated revealed that TonB-dependent receptors genes are present and include: *fadA* in *E. coli*, *chuA* in *C. jejuni*, *scrI* in *B. fragilis* (Table 3 and Supplementary Data). However, most of the annotated functions in EggNOG have non-identified gene due lack of existent research in the database.

Iron is one of the most abundant element by mass on the planet, and exhibit an important role in living cells, such as DNA polymerases, helicases and other co-factors (Netz *et al.*, 2011). The iron uptake in bacterial cells is normally acquired by siderophores but

requires TonB proteins for an active transport when needed for heme or vitamin B12 acquisition in a form of cobalt (Bradbeer, 1993; Neilands, 1995; Newton *et al.*, 2010; Stojiljkovic and Hantke, 1992). The haemoglobin receptor protein (K16087) and iron complex transport system substrate-binding protein (K02016) were common in *Bacteroides* spp. (Tab.7), whereas iron-related uptake gene such as *fiu* and *fhuA* were identified in *E. coli*, *tdhA* in *H. pylori*, and haemolysin *hxuB* in *C. jejuni* (*Supplementary Data*). Nevertheless, iron acquisition is a tightly regulated process due to the oxidation capability and DNA damage *via* mutagenic radicals. Tumour cells are associated with an upregulation of Fe-regulatory genes due an increased metabolic need in uncontrolled proliferation, survival and metastasis formation (Bauckman *et al.*, 2015; Kwok and Richardson, 2002; Pfeifhofer-Obermair *et al.*, 2018; Steegmann-Olmedillas, 2011). Thus, it is plausible to assume that TonB proteins can mediate an increased iron acquisition when released as components of the OMVs outer membrane, thus leading to an increase pro-oncogenic DNA damage and epigenetic changes.

In addition to influx proteins, bacterial species have developed a seemingly conserved antimicrobial resistance mechanisms of bacterial secretion system (ko03070),  $\beta$ -lactam resistance (ko01501) and cationic antimicrobial peptide (CAMP) resistance (ko01503) (Tab.4). The small molecules, such as antibiotics, penetrate the bacterial membrane *via* passive transport of OMPs (Delcour, 2009). For example, in *E. coli*  $\beta$ -lactams pass into the cells *via* the OmpF and it's mutations are associated with  $\beta$ -lactam resistance (Pagès *et al.*, 2008; Zier vogel and Roux, 2013). In contrast, OmpA was found to maintain the membrane integrity in *E. coli* and *Acinetobacter baumannii* suggesting a multifaceted role of porins (Choi and Lee, 2019). Across the adhesins *E. coli*, *B. thetaiomicrobion* and *C. jejuni* had common outer membrane efflux pathway (K18139) (Tab.4), further sharing the IPR003423 (PFAM) with *B. fragilis*. The similar pattern is followed by OmpA-OmpF porin pathway (K03286) and OmpA-like family protein (IPR006665). Moreover, an efflux system of antibiotic compound called multidrug resistance system (MDR), which is important for toxin clearance and pathogenicity. One of the major proteins of MDR is tolC protease, which has been here in *E. coli* and *B. fragilis* as well (Donner *et al.*, 2017).

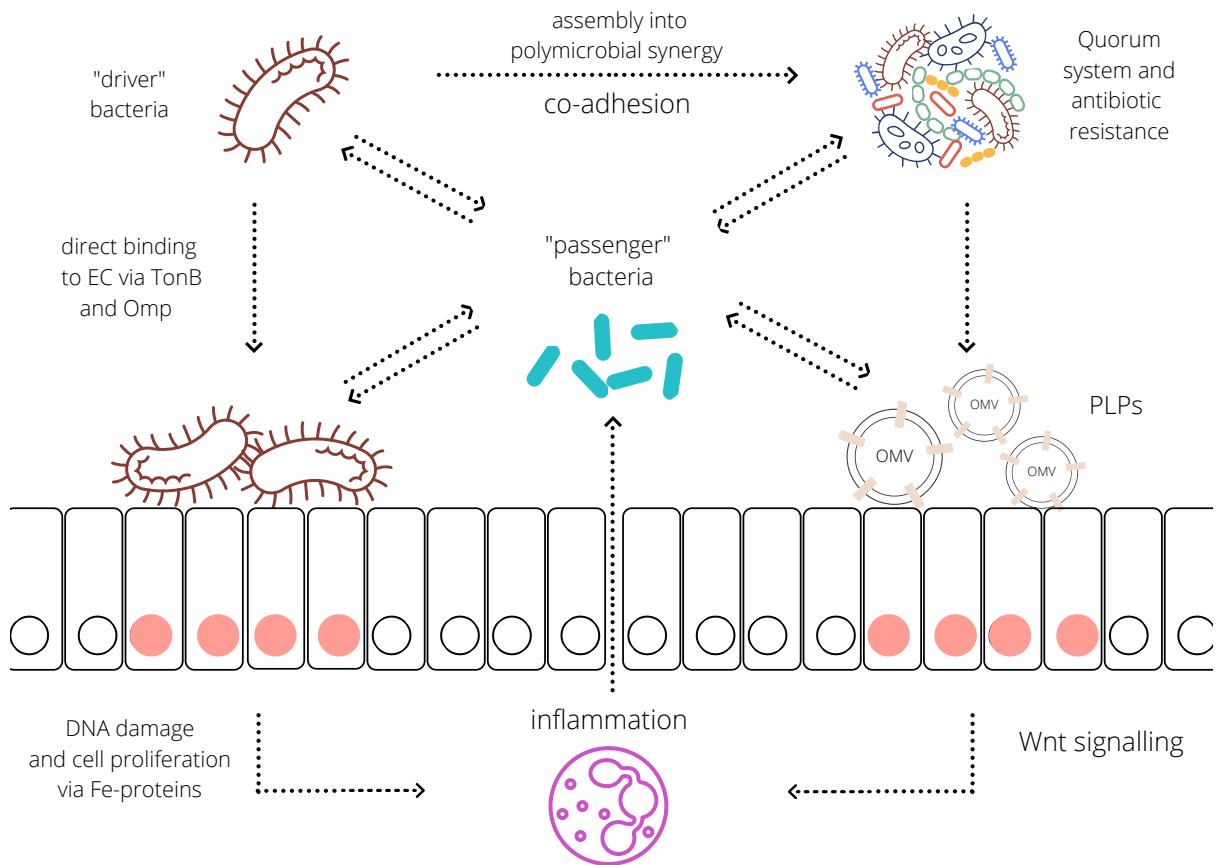
As mentioned previously in the “polymicrobial synergy” the microbes are proposed to communicate intracellularly and regulate intracellular processes. The monitoring system of own population density, biofilm formation and virulence, signalling is called quorum sensing (QS) and is maintained *via* small signal molecules (Rasmussen *et al.*, 2005). The QS pathway was identified in *F. nucleatum*, *H. pylori* and *C. jejuni* (Fig.11), meanwhile these also share a common biofilm formation (ko05111) (Tab.3). The QS mechanism involves a tight threshold

concentration of signal autoinducers leading to alterations in gene expression (Sifri, 2008). The co-culture of *P. aeruginosa* was found to induce the *E. coli* apoptosis mediated by the QS system (Kumar *et al.*, 2013.). It has also been associated with cell death and oncogenesis *via* interaction with host epithelial cells (Tornesello *et al.*, 2018).

Some other common functions recognised by the tool include patatin-like phospholipase domain, two-component system, flagellar assembly and carboxypeptidases. Phospholipases (PLPs) are enzymes that hydrolyse phospholipids on the surface of the epithelial cells releasing immunogenic products, such as fatty acids (Flores-Díaz *et al.*, 2016; Hiller *et al.*, 2018; Wilson and Knoll, 2018). The PLPs are associated with alternating host immune function, nutrient absorption and tissue virulence *via* providing the source energy for cell growth in a form of free fatty acids (Köhler *et al.*, 2006; Rameshwaram *et al.*, 2018). Here the IPR002641 family of patatin-like phospholipase domain proteins was identified common among *Bacteroides spp.* and *F. nucleatum*. In addition, the PF13715 family of carboxypeptidase regulatory-like domain in *Bacteroides spp.* that is involved in catalytic activity in acidic pH (Cerdà-Costa and Gomis-Rüth, 2014), as well as associated with E-cadherin targeting in *B. fragilis* (Shiryaev *et al.*, 2013).

In conclusion, the bacterial OMPs exhibit a variety of heterogenous functions that may contribute to the development of CRC (Fig.12). Here were established common links across the gram-negative bacteria such as *B. thetaiotaomicron*, *B. fragilis*, *E. coli*, *H. pylori*, *F. nucleatum*, *C. jejuni*. These include an association between TonB- and iron-related proteins in order to promote DNA damage, outer membrane protein efflux system in antimicrobial resistance, quorum sensing mechanism in intramicrobial signalling and catalytic OMP activities. The data provided in this study provides a base for future experimental work, for example, knocking TonB-related genes *fadA* in *E. coli*, *chuA* in *C. jejuni*, and *scrl* in *B. fragilis* to monitor iron levels, as well as fluorescently label *fiu* and *fhuA* in *E. coli* to establish whether the iron-associated proteins are embedded within the intestinal epithelium and promote iron-uptake.

Although, the functional annotations of the microbial sequences are useful in determining the overlapping mechanisms for further investigations. The whole bacterial proteomes could not show quantitative analysis of RNA expression to identify protein expression. An RNA sequence analysis of OMVs and intestinal organoids would have been beneficial for future analysis and potential pharmaceutical targets.



*Figure 13. The representation of a combined approach of "driver-passenger" and "polymicrobial synergy" models that include OMVs. A "driver" bacteria can potentially cause damage directly via epithelial cell adhesion (EC), indirectly via outer membrane vesicles (OMV) or contributing to the "core" oncogenic microbiome via mediating intramicrobial signalling via quorum sensing system. The pathogenic bacteria can adhere to EC via TonB and OmpA-like proteins. Incorporation and activation of iron-like proteins further causes oxidative stress and DNA damage, activation of Wnt signalling and increased cellular proliferation. A further induced inflammation via microbial species or EC promotes a favourable environment to "passenger" bacteria (such as *F. nucleatum*). The opportunistic species continue to contribute to carcinogenesis via "polymicrobial synergy", OMVs and direct binding.*

## References

- Allali, I., Delgado, S., Marron, P.I., Astudillo, A., Yeh, J.J., Ghazal, H., Amzazi, S., Keku, T., Azcarate-Peril, M.A., 2015. Gut microbiome compositional and functional differences between tumor and non-tumor adjacent tissues from cohorts from the US and Spain. *Gut Microbes* 6, 161–172. <https://doi.org/10.1080/19490976.2015.1039223>
- Almeqdadi, M., Mana, M.D., Roper, J., Yilmaz, Ö.H., 2019. Gut organoids: mini-tissues in culture to study intestinal physiology and disease. *Am J Physiol Cell Physiol* 317, C405–C419. <https://doi.org/10.1152/ajpcell.00300.2017>
- Arthur, J.C., Perez-Chanona, E., Mühlbauer, M., Tomkovich, S., Uronis, J.M., Fan, T.-J., Campbell, B.J., Abujamel, T., Dogan, B., Rogers, A.B., Rhodes, J.M., Stintzi, A., Simpson, K.W., Hansen, J.J., Keku, T.O., Fodor, A.A., Jobin, C., 2012. Intestinal inflammation targets cancer-inducing activity of the microbiota. *Science* 338, 120–123. <https://doi.org/10.1126/science.1224820>
- Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R., Fernandes, G.R., Tap, J., Bruls, T., Batto, J.-M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Kurokawa, K., Leclerc, M., Levenez, F., Manichanh, C., Nielsen, H.B., Nielsen, T., Pons, N., Poulain, J., Qin, J., Sicheritz-Ponten, T., Tims, S., Torrents, D., Ugarte, E., Zoetendal, E.G., Wang, J., Guarner, F., Pedersen, O., de Vos, W.M., Brunak, S., Doré, J., MetaHIT Consortium, Antolín, M., Artiguenave, F., Blottiere, H.M., Almeida, M., Brechot, C., Cara, C., Chervaux, C., Cultrone, A., Delorme, C., Denariaz, G., Dervyn, R., Foerstner, K.U., Friss, C., van de Guchte, M., Guedon, E., Haimet, F., Huber, W., van Hylckama-Vlieg, J., Jamet, A., Juste, C., Kaci, G., Knol, J., Lakhdari, O., Layec, S., Le Roux, K., Maguin, E., Mérieux, A., Melo Minardi, R., M'rini, C., Muller, J., Oozeer, R., Parkhill, J., Renault, P., Rescigno, M., Sanchez, N., Sunagawa, S., Torrejon, A., Turner, K., Vandemeulebrouck, G., Varela, E., Winogradsky, Y., Zeller, G., Weissenbach, J., Ehrlich, S.D., Bork, P., 2011. Enterotypes of the human gut microbiome. *Nature* 473, 174–180. <https://doi.org/10.1038/nature09944>
- Atarashi, K., Suda, W., Luo, C., Kawaguchi, T., Motoo, I., Narushima, S., Kiguchi, Y., Yasuma, K., Watanabe, E., Tanoue, T., Thaiss, C.A., Sato, M., Toyooka, K., Said, H.S., Yamagami, H., Rice, S.A., Gevers, D., Johnson, R.C., Segre, J.A., Chen, K., Kolls, J.K., Elinav, E., Morita, H., Xavier, R.J., Hattori, M., Honda, K., 2017. Ectopic

- colonization of oral bacteria in the intestine drives TH1 cell induction and inflammation. *Science* 358, 359–365. <https://doi.org/10.1126/science.aan4526>
- Avila, M., Ojcius, D.M., Yilmaz, O., 2009. The oral microbiota: living with a permanent guest. *DNA Cell Biol* 28, 405–411. <https://doi.org/10.1089/dna.2009.0874>
- Bauckman, K., Haller, E., Taran, N., Rockfield, S., Ruiz-Rivera, A., Nanjundan, M., 2015. Iron alters cell survival in a mitochondria-dependent pathway in ovarian cancer cells. *Biochem J* 466, 401–413. <https://doi.org/10.1042/BJ20140878>
- Bauman, S.J., Kuehn, M.J., 2006. Purification of outer membrane vesicles from *Pseudomonas aeruginosa* and their activation of an IL-8 response. *Microbes and Infection* 8, 2400–2408. <https://doi.org/10.1016/j.micinf.2006.05.001>
- Bendtsen, J.D., Kiemer, L., Fausbøll, A., Brunak, S., 2005. Non-classical protein secretion in bacteria. *BMC Microbiol* 5, 58. <https://doi.org/10.1186/1471-2180-5-58>
- Berleman, J., Auer, M., 2013. The role of bacterial outer membrane vesicles for intra- and interspecies delivery. *Environ Microbiol* 15, 347–354. <https://doi.org/10.1111/1462-2920.12048>
- Bonnington, K.E., Kuehn, M.J., 2014. Protein selection and export via outer membrane vesicles. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, Protein trafficking and secretion in bacteria* 1843, 1612–1619. <https://doi.org/10.1016/j.bbamcr.2013.12.011>
- Bosman, F., Yan, P., 2014. Molecular pathology of colorectal cancer. *Pol J Pathol* 65, 257–266. <https://doi.org/10.5114/pjp.2014.48094>
- Bradbeer, C., 1993. The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli*. *J Bacteriol* 175, 3146–3150. <https://doi.org/10.1128/jb.175.10.3146-3150.1993>
- Brun, P., Castagliuolo, I., Di Leo, V., Buda, A., Pinzani, M., Palù, G., Martines, D., 2007. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. *Am J Physiol Gastrointest Liver Physiol* 292, G518–525. <https://doi.org/10.1152/ajpgi.00024.2006>
- Cani, P.D., Possemiers, S., Van de Wiele, T., Guiot, Y., Everard, A., Rottier, O., Geurts, L., Naslain, D., Neyrinck, A., Lambert, D.M., Muccioli, G.G., Delzenne, N.M., 2009. Changes in gut microbiota control inflammation in obese mice through a mechanism involving GLP-2-driven improvement of gut permeability. *Gut* 58, 1091–1103. <https://doi.org/10.1136/gut.2008.165886>
- Celia, H., Noinaj, N., Zakharov, S.D., Bordignon, E., Botos, I., Santamaria, M., Barnard, T.J., Cramer, W.A., Lloubes, R., Buchanan, S.K., 2016. Structural insight into the role of the

- Ton complex in energy transduction. *Nature* 538, 60–65. <https://doi.org/10.1038/nature19757>
- Cerdà-Costa, N., Gomis-Rüth, F.X., 2014. Architecture and function of metallopeptidase catalytic domains. *Protein Sci* 23, 123–144. <https://doi.org/10.1002/pro.2400>
- Choi, U., Lee, C.-R., 2019. Distinct Roles of Outer Membrane Porins in Antibiotic Resistance and Membrane Integrity in *Escherichia coli*. *Frontiers in Microbiology* 10, 953. <https://doi.org/10.3389/fmicb.2019.00953>
- Christou, N., Perraud, A., Blondy, S., Jauberteau, M.-O., Battu, S., Mathonnet, M., 2017. E-cadherin: A potential biomarker of colorectal cancer prognosis. *Oncol Lett* 13, 4571–4576. <https://doi.org/10.3892/ol.2017.6063>
- Chung, L., Thiele Orberg, E., Geis, A.L., Chan, J.L., Fu, K., DeStefano Shields, C.E., Dejea, C.M., Fathi, P., Chen, J., Finard, B.B., Tam, A.J., McAllister, F., Fan, H., Wu, X., Ganguly, S., Lebid, A., Metz, P., Van Meerbeke, S.W., Huso, D.L., Wick, E.C., Pardoll, D.M., Wan, F., Wu, S., Sears, C.L., Housseau, F., 2018. *Bacteroides fragilis* Toxin Coordinates a Pro-carcinogenic Inflammatory Cascade via Targeting of Colonic Epithelial Cells. *Cell Host Microbe* 23, 203-214.e5. <https://doi.org/10.1016/j.chom.2018.01.007>
- Claesson, M.J., Cusack, S., O'Sullivan, O., Greene-Diniz, R., de Weerd, H., Flannery, E., Marchesi, J.R., Falush, D., Dinan, T., Fitzgerald, G., Stanton, C., van Sinderen, D., O'Connor, M., Harnedy, N., O'Connor, K., Henry, C., O'Mahony, D., Fitzgerald, A.P., Shanahan, F., Twomey, C., Hill, C., Ross, R.P., O'Toole, P.W., 2011. Composition, variability, and temporal stability of the intestinal microbiota of the elderly. *Proc Natl Acad Sci U S A* 108 Suppl 1, 4586–4591. <https://doi.org/10.1073/pnas.1000097107>
- Confer, A.W., Ayalew, S., 2013. The OmpA family of proteins: roles in bacterial pathogenesis and immunity. *Vet Microbiol* 163, 207–222. <https://doi.org/10.1016/j.vetmic.2012.08.019>
- Cuevas-Ramos, G., Petit, C.R., Marcq, I., Boury, M., Oswald, E., Nougayrède, J.-P., 2010. *Escherichia coli* induces DNA damage in vivo and triggers genomic instability in mammalian cells. *Proc Natl Acad Sci U S A* 107, 11537–11542. <https://doi.org/10.1073/pnas.1001261107>
- de Carvalho, A.C., de Mattos Pereira, L., Datorre, J.G., Dos Santos, W., Berardinelli, G.N., Matsushita, M. de M., Oliveira, M.A., Durães, R.O., Guimarães, D.P., Reis, R.M., 2019. Microbiota Profile and Impact of *Fusobacterium nucleatum* in Colorectal Cancer Patients of Barretos Cancer Hospital. *Front Oncol* 9, 813. <https://doi.org/10.3389/fonc.2019.00813>

- Deaths - Office for National Statistics [WWW Document], n.d. URL <https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriages/deaths> (accessed 8.18.21).
- Delcour, A.H., 2009. Outer membrane permeability and antibiotic resistance. *Biochim Biophys Acta* 1794, 808–816. <https://doi.org/10.1016/j.bbapap.2008.11.005>
- Di Lorenzo, F., De Castro, C., Silipo, A., Molinaro, A., 2019. Lipopolysaccharide structures of Gram-negative populations in the gut microbiota and effects on host interactions. *FEMS Microbiol Rev* 43, 257–272. <https://doi.org/10.1093/femsre/fuz002>
- Doherty, G.J., McMahon, H.T., 2009. Mechanisms of endocytosis. *Annu Rev Biochem* 78, 857–902. <https://doi.org/10.1146/annurev.biochem.78.081307.110540>
- Donaldson, G.P., Ladinsky, M.S., Yu, K.B., Sanders, J.G., Yoo, B.B., Chou, W.-C., Conner, M.E., Earl, A.M., Knight, R., Bjorkman, P.J., Mazmanian, S.K., 2018. Gut microbiota utilize immunoglobulin A for mucosal colonization. *Science* 360, 795–800. <https://doi.org/10.1126/science.aaq0926>
- Donner, J., Reck, M., Bunk, B., Jarek, M., App, C.B., Meier-Kolthoff, J.P., Overmann, J., Müller, R., Kirschning, A., Wagner-Döbler, I., 2017. The Biofilm Inhibitor Carolacton Enters Gram-Negative Cells: Studies Using a TolC-Deficient Strain of *Escherichia coli*. *mSphere* 2, e00375-17. <https://doi.org/10.1128/mSphereDirect.00375-17>
- Drewes, J.L., Housseau, F., Sears, C.L., 2016. Sporadic colorectal cancer: microbial contributors to disease prevention, development and therapy. *Br J Cancer* 115, 273–280. <https://doi.org/10.1038/bjc.2016.189>
- E, K., M, F., E, S., T, S., XI, Z., M, E., Rn, H., 2021. Culture and differentiation of rabbit intestinal organoids and organoid-derived cell monolayers. *Sci Rep* 11, 5401–5401. <https://doi.org/10.1038/s41598-021-84774-w>
- Ellis, T.N., Kuehn, M.J., 2010. Virulence and Immunomodulatory Roles of Bacterial Outer Membrane Vesicles. *Microbiol. Mol. Biol. Rev.* 74, 81–94. <https://doi.org/10.1128/MMBR.00031-09>
- Elmi, A., Watson, E., Sandu, P., Gundogdu, O., Mills, D.C., Inglis, N.F., Manson, E., Imrie, L., Bajaj-Elliott, M., Wren, B.W., Smith, D.G.E., Dorrell, N., 2012. *Campylobacter jejuni* outer membrane vesicles play an important role in bacterial interactions with human intestinal epithelial cells. *Infect Immun* 80, 4089–4098. <https://doi.org/10.1128/IAI.00161-12>
- Engevik, M.A., Danhof, H.A., Ruan, W., Engevik, A.C., Chang-Graham, A.L., Engevik, K.A., Shi, Z., Zhao, Y., Brand, C.K., Krystofiak, E.S., Venable, S., Liu, X., Hirschi, K.D., Hyser, J.M., Spinler, J.K., Britton, R.A., Versalovic, J., 2021. *Fusobacterium nucleatum*

Secretes Outer Membrane Vesicles and Promotes Intestinal Inflammation. *mBio* 12.  
<https://doi.org/10.1128/mBio.02706-20>

Etienne-Manneville, S., 2013. Microtubules in cell migration. *Annu. Rev. Cell Dev. Biol.* 29, 471–499. <https://doi.org/10.1146/annurev-cellbio-101011-155711>

Fairman, J.W., Noinaj, N., Buchanan, S.K., 2011. The structural biology of  $\beta$ -barrel membrane proteins: a summary of recent reports. *Curr Opin Struct Biol* 21, 523–531. <https://doi.org/10.1016/j.sbi.2011.05.005>

Flores-Díaz, M., Monturiol-Gross, L., Naylor, C., Alape-Girón, A., Flieger, A., 2016. Bacterial Sphingomyelinases and Phospholipases as Virulence Factors. *Microbiol Mol Biol Rev* 80, 597–628. <https://doi.org/10.1128/MMBR.00082-15>

Galluzzi, L., Vitale, I., Aaronson, S.A., Abrams, J.M., Adam, D., Agostinis, P., Alnemri, E.S., Altucci, L., Amelio, I., Andrews, D.W., Annicchiarico-Petruzzelli, M., Antonov, A.V., Arama, E., Baehrecke, E.H., Barlev, N.A., Bazan, N.G., Bernassola, F., Bertrand, M.J.M., Bianchi, K., Blagosklonny, M.V., Blomgren, K., Borner, C., Boya, P., Brenner, C., Campanella, M., Candi, E., Carmona-Gutierrez, D., Cecconi, F., Chan, F.K.-M., Chandel, N.S., Cheng, E.H., Chipuk, J.E., Cidlowski, J.A., Ciechanover, A., Cohen, G.M., Conrad, M., Cubillos-Ruiz, J.R., Czabotar, P.E., D'Angiolella, V., Dawson, T.M., Dawson, V.L., Laurenzi, V.D., Maria, R.D., Debatin, K.-M., DeBerardinis, R.J., Deshmukh, M., Daniele, N.D., Virgilio, F.D., Dixit, V.M., Dixon, S.J., Duckett, C.S., Dynlacht, B.D., El-Deiry, W.S., Elrod, J.W., Fimia, G.M., Fulda, S., García-Sáez, A.J., Garg, A.D., Garrido, C., Gavathiotis, E., Golstein, P., Gottlieb, E., Green, D.R., Greene, L.A., Gronemeyer, H., Gross, A., Hajnoczky, G., Hardwick, J.M., Harris, I.S., Hengartner, M.O., Hetz, C., Ichijo, H., Jäättelä, M., Joseph, B., Jost, P.J., Juin, P.P., Kaiser, W.J., Karin, M., Kaufmann, T., Kepp, O., Kimchi, A., Kitsis, R.N., Klionsky, D.J., Knight, R.A., Kumar, S., Lee, S.W., Lemasters, J.J., Levine, B., Linkermann, A., Lipton, S.A., Lockshin, R.A., López-Otín, C., Lowe, S.W., Luedde, T., Lugli, E., MacFarlane, M., Madeo, F., Malewicz, M., Malorni, W., Manic, G., Marine, J.-C., Martin, S.J., Martinou, J.-C., Medema, J.P., Mehlen, P., Meier, P., Melino, S., Miao, E.A., Molkentin, J.D., Moll, U.M., Muñoz-Pinedo, C., Nagata, S., Nuñez, G., Oberst, A., Oren, M., Overholtzer, M., Pagano, M., Panaretakis, T., Pasparakis, M., Penninger, J.M., Pereira, D.M., Pervaiz, S., Peter, M.E., Piacentini, M., Pinton, P., Prehn, J.H.M., Puthalakath, H., Rabinovich, G.A., Rehm, M., Rizzuto, R., Rodrigues, C.M.P., Rubinsztein, D.C., Rudel, T., Ryan, K.M., Sayan, E., Scorrano, L., Shao, F., Shi, Y., Silke, J., Simon, H.-U., Sistigu, A., Stockwell, B.R., Strasser, A., Szabadkai, G., Tait, S.W.G., Tang, D., Tavernarakis, N., Thorburn, A., Tsujimoto, Y., Turk, B., Berghe, T.V., Vandenebeele, P., Heiden, M.G.V., Villunger, A., Virgin, H.W., Vousden, K.H., Vucic,

- D., Wagner, E.F., Walczak, H., Wallach, D., Wang, Y., Wells, J.A., Wood, W., Yuan, J., Zakeri, Z., Zhivotovsky, B., Zitvogel, L., Melino, G., Kroemer, G., 2018. Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ* 25, 486–541. <https://doi.org/10.1038/s41418-017-0012-4>
- Gao, J.-J., Zhang, Y., Gerhard, M., Mejias-Luque, R., Zhang, L., Vieth, M., Ma, J.-L., Bajbouj, M., Suchanek, S., Liu, W.-D., Ulm, K., Quante, M., Li, Z.-X., Zhou, T., Schmid, R., Classen, M., Li, W.-Q., You, W.-C., Pan, K.-F., 2018. Association Between Gut Microbiota and Helicobacter pylori-Related Gastric Lesions in a High-Risk Population of Gastric Cancer. *Front Cell Infect Microbiol* 8, 202. <https://doi.org/10.3389/fcimb.2018.00202>
- Gao, Z., Guo, B., Gao, R., Zhu, Q., Qin, H., 2015. Microbiota dysbiosis is associated with colorectal cancer. *Front Microbiol* 6, 20. <https://doi.org/10.3389/fmicb.2015.00020>
- Garcia-Vello, P., Lorenzo, F.D., Lamprinaki, D., Notaro, A., Speciale, I., Molinaro, A., Juge, N., Castro, C.D., n.d. Structure of the O-Antigen and the Lipid A from the Lipopolysaccharide of *Fusobacterium nucleatum* ATCC 51191. *ChemBioChem* n/a. <https://doi.org/10.1002/cbic.202000751>
- Goodwin, A.C., Destefano Shields, C.E., Wu, S., Huso, D.L., Wu, X., Murray-Stewart, T.R., Hacker-Prietz, A., Rabizadeh, S., Woster, P.M., Sears, C.L., Casero, R.A., 2011. Polyamine catabolism contributes to enterotoxigenic *Bacteroides fragilis*-induced colon tumorigenesis. *Proc Natl Acad Sci U S A* 108, 15354–15359. <https://doi.org/10.1073/pnas.1010203108>
- Gradel, K.O., Nielsen, H.L., Schønheyder, H.C., Ejlertsen, T., Kristensen, B., Nielsen, H., 2009. Increased short- and long-term risk of inflammatory bowel disease after salmonella or campylobacter gastroenteritis. *Gastroenterology* 137, 495–501. <https://doi.org/10.1053/j.gastro.2009.04.001>
- Grahn, N., Hmani-Aifa, M., Fransén, K., Söderkvist, P., Monstein, H.-J., 2005. Molecular identification of *Helicobacter* DNA present in human colorectal adenocarcinomas by 16S rDNA PCR amplification and pyrosequencing analysis. *J Med Microbiol* 54, 1031–1035. <https://doi.org/10.1099/jmm.0.46122-0>
- Guo, P., Tian, Z., Kong, X., Yang, L., Shan, X., Dong, B., Ding, X., Jing, X., Jiang, C., Jiang, N., Yu, Y., 2020. FadA promotes DNA damage and progression of *Fusobacterium nucleatum*-induced colorectal cancer through up-regulation of chk2. *Journal of Experimental & Clinical Cancer Research* 39, 202. <https://doi.org/10.1186/s13046-020-01677-w>

- Hagan, C.L., Silhavy, T.J., Kahne, D., 2011.  $\beta$ -Barrel membrane protein assembly by the Bam complex. *Annu Rev Biochem* 80, 189–210. <https://doi.org/10.1146/annurev-biochem-061408-144611>
- Hajishengallis, G., Lamont, R.J., 2012. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Mol Oral Microbiol* 27, 409–419. <https://doi.org/10.1111/j.2041-1014.2012.00663.x>
- Harris, T.J.C., Tepass, U., 2010. Adherens junctions: from molecules to morphogenesis. *Nat. Rev. Mol. Cell Biol.* 11, 502–514. <https://doi.org/10.1038/nrm2927>
- He, Z., Gharaibeh, R.Z., Newsome, R.C., Pope, J.L., Dougherty, M.W., Tomkovich, S., Pons, B., Mirey, G., Vignard, J., Hendrixson, D.R., Jobin, C., 2019. *Campylobacter jejuni* promotes colorectal tumorigenesis through the action of cytolethal distending toxin. *Gut* 68, 289–300. <https://doi.org/10.1136/gutjnl-2018-317200>
- Hernandez, C.J., Guss, J.D., Luna, M., Goldring, S.R., 2016. Links Between the Microbiome and Bone. *J Bone Miner Res* 31, 1638–1646. <https://doi.org/10.1002/jbmr.2887>
- Hiller, M., Lang, C., Michel, W., Flieger, A., 2018. Secreted phospholipases of the lung pathogen *Legionella pneumophila*. *Int J Med Microbiol* 308, 168–175. <https://doi.org/10.1016/j.ijmm.2017.10.002>
- Jang, K.-S., Sweredoski, M.J., Graham, R.L.J., Hess, S., Clemons, W.M., 2014. Comprehensive proteomic profiling of outer membrane vesicles from *Campylobacter jejuni*. *J Proteomics* 98, 90–98. <https://doi.org/10.1016/j.jprot.2013.12.014>
- Jones, M., Helliwell, P., Pritchard, C., Tharakan, J., Mathew, J., 2007. *Helicobacter pylori* in colorectal neoplasms: is there an aetiological relationship? *World J Surg Oncol* 5, 51. <https://doi.org/10.1186/1477-7819-5-51>
- Kaparakis, M., Turnbull, L., Carneiro, L., Firth, S., Coleman, H.A., Parkington, H.C., Le Bourhis, L., Karrar, A., Viala, J., Mak, J., Hutton, M.L., Davies, J.K., Crack, P.J., Herzog, P.J., Philpott, D.J., Girardin, S.E., Whitchurch, C.B., Ferrero, R.L., 2010. Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. *Cell Microbiol* 12, 372–385. <https://doi.org/10.1111/j.1462-5822.2009.01404.x>
- Kesty, N.C., Kuehn, M.J., 2004. Incorporation of Heterologous Outer Membrane and Periplasmic Proteins into *Escherichia coli* Outer Membrane Vesicles\*. *Journal of Biological Chemistry* 279, 2069–2076. <https://doi.org/10.1074/jbc.M307628200>
- Keum, N., Giovannucci, E., 2019. Global burden of colorectal cancer: emerging trends, risk factors and prevention strategies. *Nat Rev Gastroenterol Hepatol* 16, 713–732. <https://doi.org/10.1038/s41575-019-0189-8>

- Knowles, T.J., Scott-Tucker, A., Overduin, M., Henderson, I.R., 2009. Membrane protein architects: the role of the BAM complex in outer membrane protein assembly. *Nat Rev Microbiol* 7, 206–214. <https://doi.org/10.1038/nrmicro2069>
- Köhler, G.A., Brenot, A., Haas-Stapleton, E., Agabian, N., Deva, R., Nigam, S., 2006. Phospholipase A2 and Phospholipase B Activities in Fungi. *Biochim Biophys Acta* 1761, 1391–1399. <https://doi.org/10.1016/j.bbalip.2006.09.011>
- Kolenbrander, P.E., Palmer, R.J., Periasamy, S., Jakubovics, N.S., 2010. Oral multispecies biofilm development and the key role of cell-cell distance. *Nat Rev Microbiol* 8, 471–480. <https://doi.org/10.1038/nrmicro2381>
- Konkel, M.E., Larson, C.L., Flanagan, R.C., 2010. *Campylobacter jejuni* FlpA binds fibronectin and is required for maximal host cell adherence. *J Bacteriol* 192, 68–76. <https://doi.org/10.1128/JB.00969-09>
- Kostic, A.D., Chun, E., Robertson, L., Glickman, J.N., Gallini, C.A., Michaud, M., Clancy, T.E., Chung, D.C., Lochhead, P., Hold, G.L., El-Omar, E.M., Brenner, D., Fuchs, C.S., Meyerson, M., Garrett, W.S., 2013. *Fusobacterium nucleatum* potentiates intestinal tumorigenesis and modulates the tumor-immune microenvironment. *Cell Host Microbe* 14, 207–215. <https://doi.org/10.1016/j.chom.2013.07.007>
- Krisanaprakornkit, S., Kimball, J.R., Weinberg, A., Darveau, R.P., Bainbridge, B.W., Dale, B.A., 2000. Inducible expression of human beta-defensin 2 by *Fusobacterium nucleatum* in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infect Immun* 68, 2907–2915. <https://doi.org/10.1128/iai.68.5.2907-2915.2000>
- Kuipers, E.J., Grady, W.M., Lieberman, D., Seufferlein, T., Sung, J.J., Boelens, P.G., van de Velde, C.J.H., Watanabe, T., 2015. Colorectal cancer. *Nat Rev Dis Primers* 1, 1–25. <https://doi.org/10.1038/nrdp.2015.65>
- Kulp, A., Kuehn, M.J., 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annu Rev Microbiol* 64, 163–184. <https://doi.org/10.1146/annurev.micro.091208.073413>
- Kumar, S., Kolodkin-Gal, I., Engelberg-Kulka, H., n.d. Novel Quorum-Sensing Peptides Mediating Interspecies Bacterial Cell Death. *mBio* 4, e00314-13. <https://doi.org/10.1128/mBio.00314-13>
- Kwok, J.C., Richardson, D.R., 2002. The iron metabolism of neoplastic cells: alterations that facilitate proliferation? *Crit Rev Oncol Hematol* 42, 65–78. [https://doi.org/10.1016/s1040-8428\(01\)00213-x](https://doi.org/10.1016/s1040-8428(01)00213-x)

- Lee, S.M., Donaldson, G.P., Mikulski, Z., Boyajian, S., Ley, K., Mazmanian, S.K., 2013. Bacterial colonization factors control specificity and stability of the gut microbiota. *Nature* 501, 426–429. <https://doi.org/10.1038/nature12447>
- Li, Y.-Y., Ge, Q.-X., Cao, J., Zhou, Y.-J., Du, Y.-L., Shen, B., Wan, Y.-J.Y., Nie, Y.-Q., 2016. Association of *Fusobacterium nucleatum* infection with colorectal cancer in Chinese patients. *World J Gastroenterol* 22, 3227–3233. <https://doi.org/10.3748/wjg.v22.i11.3227>
- Liu, J., Hsieh, C.-L., Gelincik, O., Devolder, B., Sei, S., Zhang, S., Lipkin, S.M., Chang, Y.-F., 2019. Proteomic characterization of outer membrane vesicles from gut mucosa-derived *fusobacterium nucleatum*. *Journal of Proteomics* 195, 125–137. <https://doi.org/10.1016/j.jprot.2018.12.029>
- Liu, Q., Li, X., Zhang, Y., Song, Z., Li, R., Ruan, H., Huang, X., 2019. Orally-administered outer-membrane vesicles from *Helicobacter pylori* reduce *H. pylori* infection via Th2-biased immune responses in mice. *Pathog Dis* 77, ftz050. <https://doi.org/10.1093/femspd/ftz050>
- Luck, H., Tsai, S., Chung, J., Clemente-Casares, X., Ghazarian, M., Revelo, X.S., Lei, H., Luk, C.T., Shi, S.Y., Surendra, A., Copeland, J.K., Ahn, J., Prescott, D., Rasmussen, B.A., Chng, M.H.Y., Engleman, E.G., Girardin, S.E., Lam, T.K.T., Croitoru, K., Dunn, S., Philpott, D.J., Guttman, D.S., Woo, M., Winer, S., Winer, D.A., 2015. Regulation of obesity-related insulin resistance with gut anti-inflammatory agents. *Cell Metab* 21, 527–542. <https://doi.org/10.1016/j.cmet.2015.03.001>
- Mehta, R.S., Nishihara, R., Cao, Y., Song, M., Mima, K., Qian, Z.R., Nowak, J.A., Kosumi, K., Hamada, T., Masugi, Y., Bullman, S., Drew, D.A., Kostic, A.D., Fung, T.T., Garrett, W.S., Huttenhower, C., Wu, K., Meyerhardt, J.A., Zhang, X., Willett, W.C., Giovannucci, E.L., Fuchs, C.S., Chan, A.T., Ogino, S., 2017. Association of Dietary Patterns With Risk of Colorectal Cancer Subtypes Classified by *Fusobacterium nucleatum* in Tumor Tissue. *JAMA Oncol* 3, 921–927. <https://doi.org/10.1001/jamaoncol.2016.6374>
- Miller, R.E., Belmadani, A., Ishihara, S., Tran, P.B., Ren, D., Miller, R.J., Malfait, A.-M., 2015. Damage-associated molecular patterns generated in osteoarthritis directly excite murine nociceptive neurons through Toll-like receptor 4. *Arthritis Rheumatol* 67, 2933–2943. <https://doi.org/10.1002/art.39291>
- Mima, K., Sukawa, Y., Nishihara, R., Qian, Z.R., Yamauchi, M., Inamura, K., Kim, S.A., Masuda, A., Nowak, J.A., Noshio, K., Kostic, A.D., Giannakis, M., Watanabe, H., Bullman, S., Milner, D.A., Harris, C.C., Giovannucci, E., Garraway, L.A., Freeman, G.J., Dranoff, G., Chan, A.T., Garrett, W.S., Huttenhower, C., Fuchs, C.S., Ogino, S.,

2015. *Fusobacterium nucleatum* and T Cells in Colorectal Carcinoma. *JAMA Oncol* 1, 653–661. <https://doi.org/10.1001/jamaoncol.2015.1377>
- Mira-Pascual, L., Cabrera-Rubio, R., Ocon, S., Costales, P., Parra, A., Suarez, A., Moris, F., Rodrigo, L., Mira, A., Collado, M.C., 2015. Microbial mucosal colonic shifts associated with the development of colorectal cancer reveal the presence of different bacterial and archaeal biomarkers. *J Gastroenterol* 50, 167–179. <https://doi.org/10.1007/s00535-014-0963-x>
- Mo, Z., Huang, P., Yang, C., Xiao, S., Zhang, G., Ling, F., Li, L., 2020. Meta-analysis of 16S rRNA Microbial Data Identified Distinctive and Predictive Microbiota Dysbiosis in Colorectal Carcinoma Adjacent Tissue. *mSystems* 5. <https://doi.org/10.1128/mSystems.00138-20>
- Molinaro, A., Holst, O., Di Lorenzo, F., Callaghan, M., Nurisso, A., D'Errico, G., Zamyatina, A., Peri, F., Berisio, R., Jerala, R., Jiménez-Barbero, J., Silipo, A., Martín-Santamaría, S., 2015. Chemistry of lipid A: at the heart of innate immunity. *Chemistry* 21, 500–519. <https://doi.org/10.1002/chem.201403923>
- Morgillo, F., Dallio, M., Della Corte, C.M., Gravina, A.G., Viscardi, G., Loguercio, C., Ciardiello, F., Federico, A., 2018. Carcinogenesis as a Result of Multiple Inflammatory and Oxidative Hits: a Comprehensive Review from Tumor Microenvironment to Gut Microbiota. *Neoplasia* 20, 721–733. <https://doi.org/10.1016/j.neo.2018.05.002>
- Mug-Opstelten, D., Witholt, B., 1978. Preferential release of new outer membrane fragments by exponentially growing *Escherichia coli*. *Biochimica et Biophysica Acta (BBA) - Biomembranes* 508, 287–295. [https://doi.org/10.1016/0005-2736\(78\)90331-0](https://doi.org/10.1016/0005-2736(78)90331-0)
- Nath, S.G., Raveendran, R., 2013. Microbial dysbiosis in periodontitis. *J Indian Soc Periodontol* 17, 543–545. <https://doi.org/10.4103/0972-124X.118334>
- Neilands, J.B., 1995. Siderophores: structure and function of microbial iron transport compounds. *J Biol Chem* 270, 26723–26726. <https://doi.org/10.1074/jbc.270.45.26723>
- Netz, D.J.A., Stith, C.M., Stümpfig, M., Köpf, G., Vogel, D., Genau, H.M., Stodola, J.L., Lill, R., Burgers, P.M.J., Pierik, A.J., 2011. Eukaryotic DNA polymerases require an iron-sulfur cluster for the formation of active complexes. *Nat Chem Biol* 8, 125–132. <https://doi.org/10.1038/nchembio.721>
- Newton, S.M., Trinh, V., Pi, H., Klebba, P.E., 2010. Direct measurements of the outer membrane stage of ferric enterobactin transport: postuptake binding. *J Biol Chem* 285, 17488–17497. <https://doi.org/10.1074/jbc.M109.100206>
- Nielsen, H., Tsirigos, K.D., Brunak, S., von Heijne, G., 2019. A Brief History of Protein Sorting Prediction. *Protein J* 38, 200–216. <https://doi.org/10.1007/s10930-019-09838-3>

- Noinaj, N., Easley, N.C., Oke, M., Mizuno, N., Gumbart, J., Boura, E., Steere, A.N., Zak, O., Aisen, P., Tajkhorshid, E., Evans, R.W., Gorringe, A.R., Mason, A.B., Steven, A.C., Buchanan, S.K., 2012. Structural basis for iron piracy by pathogenic Neisseria. *Nature* 483, 53–58. <https://doi.org/10.1038/nature10823>
- Noinaj, N., Gumbart, J.C., Buchanan, S.K., 2017. The β-barrel assembly machinery in motion. *Nat Rev Microbiol* 15, 197–204. <https://doi.org/10.1038/nrmicro.2016.191>
- Nosho, K., Baba, Y., Tanaka, N., Shima, K., Hayashi, M., Meyerhardt, J.A., Giovannucci, E., Dranoff, G., Fuchs, C.S., Ogino, S., 2010. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol* 222, 350–366. <https://doi.org/10.1002/path.2774>
- Ogino, S., Chan, A.T., Fuchs, C.S., Giovannucci, E., 2011. Molecular pathological epidemiology of colorectal neoplasia: an emerging transdisciplinary and interdisciplinary field. *Gut* 60, 397–411. <https://doi.org/10.1136/gut.2010.217182>
- Okahashi, N., Koga, T., Nishihara, T., Fujiwara, T., Hamada, S., 1988. Immunobiological properties of lipopolysaccharides isolated from *Fusobacterium nucleatum* and *F. necrophorum*. *J Gen Microbiol* 134, 1707–1715. <https://doi.org/10.1099/00221287-134-6-1707>
- Olsen, I., Yamazaki, K., 2019. Can oral bacteria affect the microbiome of the gut? *Journal of Oral Microbiology* 11, 1586422. <https://doi.org/10.1080/20002297.2019.1586422>
- Pagès, F., Mlecnik, B., Marliot, F., Bindea, G., Ou, F.-S., Bifulco, C., Lugli, A., Zlobec, I., Rau, T.T., Berger, M.D., Nagtegaal, I.D., Vink-Börger, E., Hartmann, A., Geppert, C., Kolwelter, J., Merkel, S., Grützmann, R., Van den Eynde, M., Jouret-Mourin, A., Kartheuser, A., Léonard, D., Remue, C., Wang, J.Y., Bavi, P., Roehrl, M.H.A., Ohashi, P.S., Nguyen, L.T., Han, S., MacGregor, H.L., Hafezi-Bakhtiari, S., Wouters, B.G., Masucci, G.V., Andersson, E.K., Zavadova, E., Vocka, M., Spacek, J., Petruzelka, L., Konopasek, B., Dundr, P., Skalova, H., Nemecova, K., Botti, G., Tatangelo, F., Delrio, P., Ciliberto, G., Maio, M., Laghi, L., Grizzi, F., Fredriksen, T., Buttard, B., Angelova, M., Vasaturo, A., Maby, P., Church, S.E., Angell, H.K., Lafontaine, L., Bruni, D., El Sissy, C., Haicheur, N., Kirilovsky, A., Berger, A., Lagorce, C., Meyers, J.P., Paustian, C., Feng, Z., Ballesteros-Merino, C., Dijkstra, J., van de Water, C., van Lent-van Vliet, S., Knijn, N., Mušină, A.-M., Scripcariu, D.-V., Popivanova, B., Xu, M., Fujita, T., Hazama, S., Suzuki, N., Nagano, H., Okuno, K., Torigoe, T., Sato, N., Furuhata, T., Takemasa, I., Itoh, K., Patel, P.S., Vora, H.H., Shah, B., Patel, J.B., Rajvik, K.N., Pandya, S.J., Shukla, S.N., Wang, Y., Zhang, G., Kawakami, Y., Marincola, F.M., Ascierto, P.A., Sargent, D.J., Fox, B.A., Galon, J., 2018. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and

- accuracy study. *Lancet* 391, 2128–2139. [https://doi.org/10.1016/S0140-6736\(18\)30789-X](https://doi.org/10.1016/S0140-6736(18)30789-X)
- Pagès, J.-M., James, C.E., Winterhalter, M., 2008. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. *Nat Rev Microbiol* 6, 893–903. <https://doi.org/10.1038/nrmicro1994>
- Pal, D., Dasgupta, S., Kundu, R., Maitra, S., Das, G., Mukhopadhyay, S., Ray, S., Majumdar, S.S., Bhattacharya, S., 2012. Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nat Med* 18, 1279–1285. <https://doi.org/10.1038/nm.2851>
- Pfeifhofer-Obermair, C., Tymoszuk, P., Petzer, V., Weiss, G., Nairz, M., 2018. Iron in the Tumor Microenvironment-Connecting the Dots. *Front Oncol* 8, 549. <https://doi.org/10.3389/fonc.2018.00549>
- Pizarro-Cerdá, J., Cossart, P., 2006. Bacterial Adhesion and Entry into Host Cells. *Cell* 124, 715–727. <https://doi.org/10.1016/j.cell.2006.02.012>
- Prorok-Hamon, M., Friswell, M.K., Alswied, A., Roberts, C.L., Song, F., Flanagan, P.K., Knight, P., Codling, C., Marchesi, J.R., Winstanley, C., Hall, N., Rhodes, J.M., Campbell, B.J., 2014. Colonic mucosa-associated diffusely adherent afaC+ Escherichia coli expressing lpfA and pks are increased in inflammatory bowel disease and colon cancer. *Gut* 63, 761–770. <https://doi.org/10.1136/gutjnl-2013-304739>
- Rameshwaram, N.R., Singh, P., Ghosh, S., Mukhopadhyay, S., 2018. Lipid metabolism and intracellular bacterial virulence: key to next-generation therapeutics. *Future Microbiol* 13, 1301–1328. <https://doi.org/10.2217/fmb-2018-0013>
- Rasmussen, T.B., Bjarnsholt, T., Skindersoe, M.E., Hentzer, M., Kristoffersen, P., Köte, M., Nielsen, J., Eberl, L., Givskov, M., 2005. Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. *J Bacteriol* 187, 1799–1814. <https://doi.org/10.1128/JB.187.5.1799-1814.2005>
- Rawla, P., Sunkara, T., Barsouk, A., 2019. Epidemiology of colorectal cancer: incidence, mortality, survival, and risk factors. *Prz Gastroenterol* 14, 89–103. <https://doi.org/10.5114/pg.2018.81072>
- Ricci, D.P., Silhavy, T.J., 2012. The Bam machine: A molecular cooper. *Biochim Biophys Acta* 1818, 1067–1084. <https://doi.org/10.1016/j.bbamem.2011.08.020>
- Rollauer, S.E., Sooreshjani, M.A., Noinaj, N., Buchanan, S.K., 2015. Outer membrane protein biogenesis in Gram-negative bacteria. *Philos Trans R Soc Lond B Biol Sci* 370, 20150023. <https://doi.org/10.1098/rstb.2015.0023>
- Rozek, L.S., Schmit, S.L., Greenson, J.K., Tomsho, L.P., Rennert, H.S., Rennert, G., Gruber, S.B., 2016. Tumor-Infiltrating Lymphocytes, Crohn's-Like Lymphoid Reaction, and

- Survival From Colorectal Cancer. *J Natl Cancer Inst* 108.  
<https://doi.org/10.1093/jnci/djw027>
- Rubinstein, M.R., Wang, X., Liu, W., Hao, Y., Cai, G., Han, Y.W., 2013. *Fusobacterium nucleatum* promotes colorectal carcinogenesis by modulating E-cadherin/β-catenin signaling via its FadA adhesin. *Cell Host Microbe* 14, 195–206.  
<https://doi.org/10.1016/j.chom.2013.07.012>
- Samsudin, F., Ortiz-Suarez, M.L., Piggot, T.J., Bond, P.J., Khalid, S., 2016. OmpA: A Flexible Clamp for Bacterial Cell Wall Attachment. *Structure* 24, 2227–2235.  
<https://doi.org/10.1016/j.str.2016.10.009>
- Schmidt, A.-M., Escher, U., Mousavi, S., Tegtmeier, N., Boehm, M., Backert, S., Bereswill, S., Heimesaat, M.M., 2019. Immunopathological properties of the *Campylobacter jejuni* flagellins and the adhesin CadF as assessed in a clinical murine infection model. *Gut Pathog* 11, 24. <https://doi.org/10.1186/s13099-019-0306-9>
- Schwechheimer, C., Sullivan, C.J., Kuehn, M.J., 2013. Envelope control of outer membrane vesicle production in Gram-negative bacteria. *Biochemistry* 52, 3031–3040.  
<https://doi.org/10.1021/bi400164t>
- Shaikh, H.F.M., Patil, S.H., Pangam, T.S., Rathod, K.V., 2018. Polymicrobial synergy and dysbiosis: An overview. *J Indian Soc Periodontol* 22, 101–106.  
[https://doi.org/10.4103/jisp.jisp\\_385\\_17](https://doi.org/10.4103/jisp.jisp_385_17)
- Shi, H., Kokoeva, M.V., Inouye, K., Tzameli, I., Yin, H., Flier, J.S., 2006. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* 116, 3015–3025.  
<https://doi.org/10.1172/JCI28898>
- Shiryayev, S.A., Remacle, A.G., Chernov, A.V., Golubkov, V.S., Motamedchaboki, K., Muranaka, N., Dambacher, C.M., Capek, P., Kukreja, M., Kozlov, I.A., Perucho, M., Cieplak, P., Strongin, A.Y., 2013. Substrate cleavage profiling suggests a distinct function of *Bacteroides fragilis* metalloproteinases (fragilysin and metalloproteinase II) at the microbiome-inflammation-cancer interface. *J Biol Chem* 288, 34956–34967.  
<https://doi.org/10.1074/jbc.M113.516153>
- Sifri, C.D., 2008. Quorum Sensing: Bacteria Talk Sense. *Clinical Infectious Diseases* 47, 1070–1076. <https://doi.org/10.1086/592072>
- Sikora, A.E., Wierzbicki, I.H., Zielke, R.A., Ryner, R.F., Korotkov, K.V., Buchanan, S.K., Noinaj, N., 2018. Structural and functional insights into the role of BamD and BamE within the β-barrel assembly machinery in *Neisseria gonorrhoeae*. *J Biol Chem* 293, 1106–1119. <https://doi.org/10.1074/jbc.RA117.000437>
- Silhavy, T.J., Kahne, D., Walker, S., 2010. The bacterial cell envelope. *Cold Spring Harb Perspect Biol* 2, a000414. <https://doi.org/10.1101/cshperspect.a000414>

- Soylu, A., Ozkara, S., Alis, H., Dolay, K., Kalayci, M., Yasar, N., Kumbasar, A.B., 2008. Immunohistochemical testing for Helicobacter Pylori existence in neoplasms of the colon. *BMC Gastroenterol* 8, 35. <https://doi.org/10.1186/1471-230X-8-35>
- Steegmann-Olmedillas, J.L., 2011. The role of iron in tumour cell proliferation. *Clin Transl Oncol* 13, 71–76. <https://doi.org/10.1007/s12094-011-0621-1>
- Stojiljkovic, I., Hantke, K., 1992. Hemin uptake system of *Yersinia enterocolitica*: similarities with other TonB-dependent systems in gram-negative bacteria. *EMBO J* 11, 4359–4367.
- Thomas, A.M., Jesus, E.C., Lopes, A., Aguiar, S., Begnami, M.D., Rocha, R.M., Carpinetti, P.A., Camargo, A.A., Hoffmann, C., Freitas, H.C., Silva, I.T., Nunes, D.N., Setubal, J.C., Dias-Neto, E., 2016. Tissue-Associated Bacterial Alterations in Rectal Carcinoma Patients Revealed by 16S rRNA Community Profiling. *Front Cell Infect Microbiol* 6, 179. <https://doi.org/10.3389/fcimb.2016.00179>
- Tjalsma, H., Boleij, A., Marchesi, J.R., Dutilh, B.E., 2012. A bacterial driver–passenger model for colorectal cancer: beyond the usual suspects. *Nat Rev Microbiol* 10, 575–582. <https://doi.org/10.1038/nrmicro2819>
- Tongtawee, T., Kaewpitoon, S., Kaewpitoon, N., Dechsukhum, C., Leeanansaksiri, W., Loyd, R.A., Matrakool, L., Panpimanmas, S., 2016. Helicobacter Pylori Associated Gastritis Increases Risk of Colorectal Polyps: a Hospital Based-Cross-Sectional Study in Nakhon Ratchasima Province, Northeastern Thailand. *Asian Pac J Cancer Prev* 17, 341–345. <https://doi.org/10.7314/apjcp.2016.17.1.341>
- Tongtawee, T., Simawaranon, T., Wattanawongdon, W., 2018. Role of screening colonoscopy for colorectal tumors in Helicobacter pylori-related chronic gastritis with MDM2 SNP309 G/G homozygous: A prospective cross-sectional study in Thailand. *Turk J Gastroenterol* 29, 555–560. <https://doi.org/10.5152/tjg.2018.17608>
- Tornesello, A.L., Buonaguro, L., Tornesello, M.L., Buonaguro, F.M., 2018. The Role of Sensing Peptides in the Cross-talk between Microbiota and Human Cancer Cells. *Mini Rev Med Chem* 18, 1567–1571. <https://doi.org/10.2174/1389557518666180713112119>
- Tsirigos, K.D., Elofsson, A., Bagos, P.G., 2016. PRED-TMBB2: improved topology prediction and detection of beta-barrel outer membrane proteins. *Bioinformatics* 32, i665–i671. <https://doi.org/10.1093/bioinformatics/btw444>
- Turner, L., Bitto, N.J., Steer, D.L., Lo, C., D'Costa, K., Ramm, G., Shambrook, M., Hill, A.F., Ferrero, R.L., Kaparakis-Liaskos, M., 2018. Helicobacter pylori Outer Membrane Vesicle Size Determines Their Mechanisms of Host Cell Entry and Protein Content. *Front Immunol* 9, 1466. <https://doi.org/10.3389/fimmu.2018.01466>

- Turner, L., Praszkier, J., Hutton, M.L., Steer, D., Ramm, G., Kaparakis-Liaskos, M., Ferrero, R.L., 2015. Increased Outer Membrane Vesicle Formation in a *Helicobacter pylori* tolB Mutant. *Helicobacter* 20, 269–283. <https://doi.org/10.1111/hel.12196>
- Vercauteren, D., Vandenbroucke, R.E., Jones, A.T., Rejman, J., Demeester, J., De Smedt, S.C., Sanders, N.N., Braeckmans, K., 2010. The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. *Mol Ther* 18, 561–569. <https://doi.org/10.1038/mt.2009.281>
- Wang, T., Cai, G., Qiu, Y., Fei, N., Zhang, M., Pang, X., Jia, W., Cai, S., Zhao, L., 2012. Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers. *ISME J* 6, 320–329. <https://doi.org/10.1038/ismej.2011.109>
- Warren, R.L., Freeman, D.J., Pleasance, S., Watson, P., Moore, R.A., Cochrane, K., Allen-Vercoe, E., Holt, R.A., 2013. Co-occurrence of anaerobic bacteria in colorectal carcinomas. *Microbiome* 1, 16. <https://doi.org/10.1186/2049-2618-1-16>
- Weiner, A., Mellouk, N., Lopez-Montero, N., Chang, Y.-Y., Souque, C., Schmitt, C., Enninga, J., 2016. Macropinosomes are Key Players in Early *Shigella* Invasion and Vacuolar Escape in Epithelial Cells. *PLoS Pathog* 12, e1005602. <https://doi.org/10.1371/journal.ppat.1005602>
- Weir, T.L., Manter, D.K., Sheflin, A.M., Barnett, B.A., Heuberger, A.L., Ryan, E.P., 2013. Stool microbiome and metabolome differences between colorectal cancer patients and healthy adults. *PLoS One* 8, e70803. <https://doi.org/10.1371/journal.pone.0070803>
- Wilson, S.K., Knoll, L.J., 2018. Patatin-like phospholipases in microbial infections with emerging roles in fatty acid metabolism and immune regulation by Apicomplexa. *Mol Microbiol* 107, 34–46. <https://doi.org/10.1111/mmi.13871>
- Wong, S.H., Kwong, T.N.Y., Chow, T.-C., Luk, A.K.C., Dai, R.Z.W., Nakatsu, G., Lam, T.Y.T., Zhang, L., Wu, J.C.Y., Chan, F.K.L., Ng, S.S.M., Wong, M.C.S., Ng, S.C., Wu, W.K.K., Yu, J., Sung, J.J.Y., 2017. Quantitation of faecal *Fusobacterium* improves faecal immunochemical test in detecting advanced colorectal neoplasia. *Gut* 66, 1441–1448. <https://doi.org/10.1136/gutjnl-2016-312766>
- Wu, N., Yang, X., Zhang, R., Li, J., Xiao, X., Hu, Y., Chen, Y., Yang, F., Lu, N., Wang, Z., Luan, C., Liu, Y., Wang, B., Xiang, C., Wang, Y., Zhao, F., Gao, G.F., Wang, S., Li, L., Zhang, H., Zhu, B., 2013. Dysbiosis signature of fecal microbiota in colorectal cancer patients. *Microb Ecol* 66, 462–470. <https://doi.org/10.1007/s00248-013-0245-9>
- Xiang, Z., He, Y., 2013. Genome-wide prediction of vaccine targets for human herpes simplex viruses using Vaxign reverse vaccinology. *BMC Bioinformatics* 14 Suppl 4, S2. <https://doi.org/10.1186/1471-2105-14-S4-S2>

- Yang, Y., Misra, B.B., Liang, L., Bi, D., Weng, W., Wu, W., Cai, S., Qin, H., Goel, A., Li, X., Ma, Y., 2019. Integrated microbiome and metabolome analysis reveals a novel interplay between commensal bacteria and metabolites in colorectal cancer. *Theranostics* 9, 4101–4114. <https://doi.org/10.7150/thno.35186>
- Yang, Y., Weng, W., Peng, J., Hong, L., Yang, L., Toiyama, Y., Gao, R., Liu, M., Yin, M., Pan, C., Li, H., Guo, B., Zhu, Q., Wei, Q., Moyer, M.-P., Wang, P., Cai, S., Goel, A., Qin, H., Ma, Y., 2017. *Fusobacterium nucleatum Increases Proliferation of Colorectal Cancer Cells and Tumor Development in Mice by Activating Toll-Like Receptor 4 Signaling to Nuclear Factor-κB, and Up-regulating Expression of MicroRNA-21*. *Gastroenterology* 152, 851-866.e24. <https://doi.org/10.1053/j.gastro.2016.11.018>
- Zeki, S.S., Graham, T.A., Wright, N.A., 2011. Stem cells and their implications for colorectal cancer. *Nat Rev Gastroenterol Hepatol* 8, 90–100. <https://doi.org/10.1038/nrgastro.2010.211>
- Zeller, G., Tap, J., Voigt, A.Y., Sunagawa, S., Kultima, J.R., Costea, P.I., Amiot, A., Böhm, J., Brunetti, F., Habermann, N., Hercog, R., Koch, M., Luciani, A., Mende, D.R., Schneider, M.A., Schrotz-King, P., Tournigand, C., Tran Van Nhieu, J., Yamada, T., Zimmermann, J., Benes, V., Kloos, M., Ulrich, C.M., von Knebel Doeberitz, M., Sobhani, I., Bork, P., 2014. Potential of fecal microbiota for early-stage detection of colorectal cancer. *Mol Syst Biol* 10, 766. <https://doi.org/10.15252/msb.20145645>
- Ziervogel, B.K., Roux, B., 2013. The binding of antibiotics in OmpF porin. *Structure* 21, 76–87. <https://doi.org/10.1016/j.str.2012.10.014>

## Appendix A

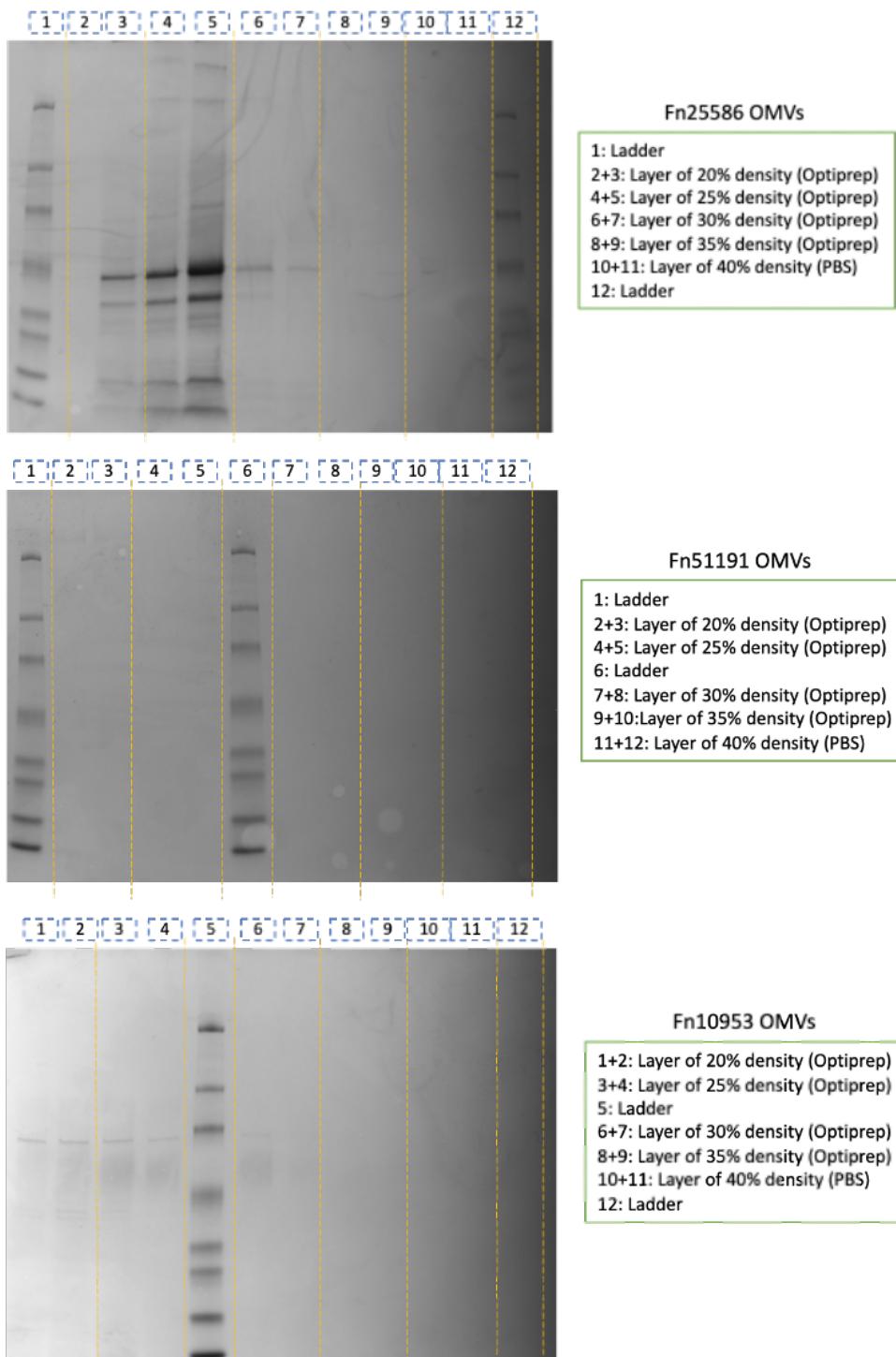


Figure 14. The SDS-page results for identification of OMV for each *F. nucleatum* strains (ATCC 25586, ATCC 10953 and 51191).

