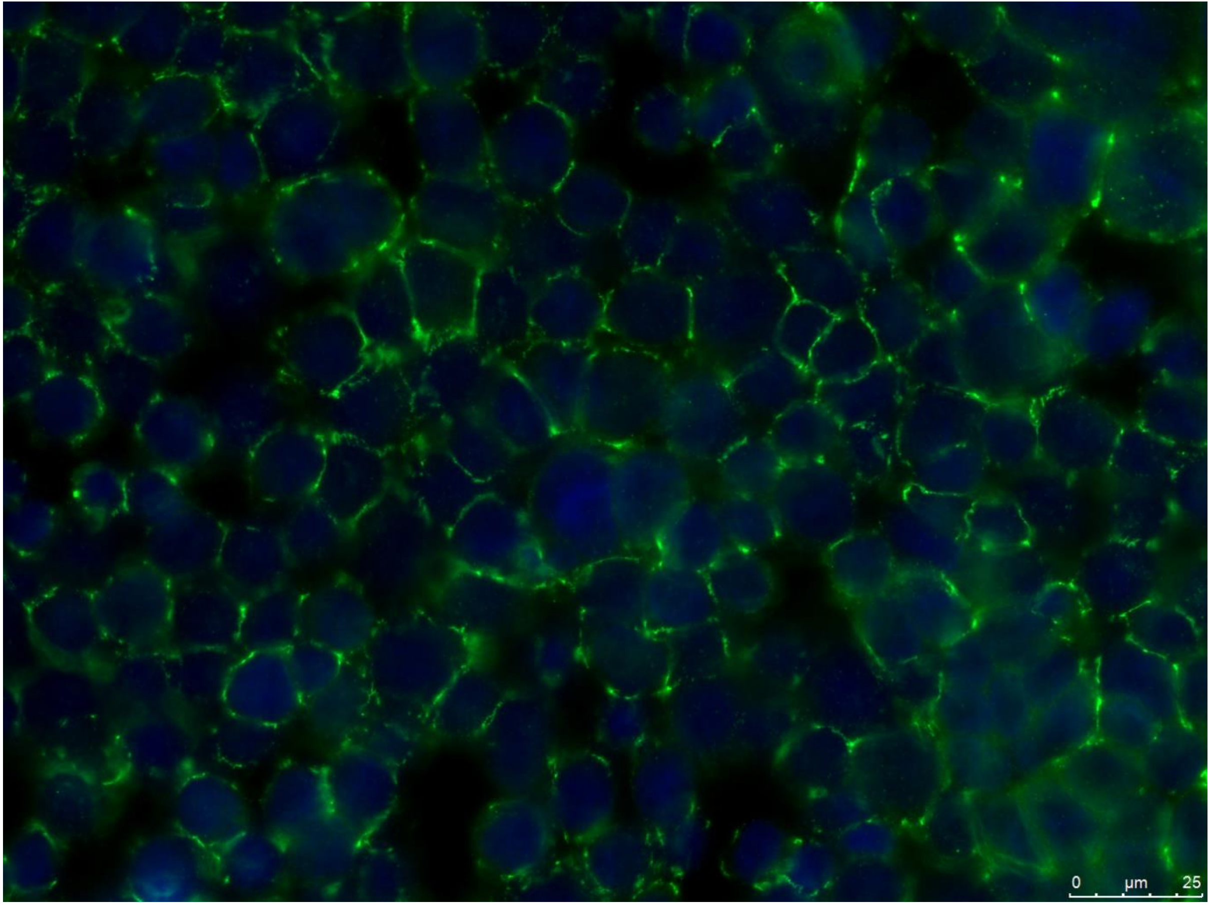
Faecalibacterium prausnitzii decreases inflammatory markers and *Akkermansia muciniphila* accumulates tight junction protein ZO-1 in cellular membrane of Caco-2 cells using HoxBan system



Name: Kim Hurkmans, S4160630
Daily supervisor: Gabriela Bravo Ruiseco
First examiner: Hermie Harmsen
Second examiner: Raphael Rosa Fagundes
Special thanks to: Klaas Nico Faber and Ali Saeed
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Abstract

Two anaerobic bacteria that are considered as next generation probiotic are *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*. *F. prausnitzii* is a butyrate-producing bacterium that has anti-inflammatory properties and *A. muciniphila* is an acetate-producing bacterium that is involved in restoring the epithelial barrier. Co-culturing those obligate anaerobic bacteria with colonocytes is a challenge, however, the Human Oxygen Bacteria Anaerobic (HoxBan) system allows this for a period of up to 36 hours. The aim of the current study was to investigate whether *A. muciniphila* individually and together with *F. prausnitzii* as a bacterial consortium could be co-cultured with Caco-2 cells using the HoxBan system. First, the medium was adjusted by adding mucin from a porcine stomach to culture *A. muciniphila*. Next, Caco-2 cells were cultured for 18 hours with *F. prausnitzii* and *A. muciniphila* individually or in a bacterial consortium. Reduction of oxygen in the media was observed and *F. prausnitzii* produced a growth rim. Furthermore, a decrease in cell viability was observed when Caco-2 cells were cultured with bacteria. Moreover, *F. prausnitzii* showed an anti-inflammatory effect in Caco-2 cells. Interestingly, *A. muciniphila* showed an accumulation of tight junction protein ZO-1 in the cell membrane of Caco-2 cells while no upregulation of mRNA levels was observed. To conclude, this study showed that *F. prausnitzii* and *A. muciniphila* can be cultured together with Caco-2 cells on a HoxBan system. Therefore, opening up opportunities for other anaerobic bacteria to be co-cultured with Caco-2 cells or other host cells in an *in vitro* co-culture system.

Introduction

The human gastrointestinal tract (GI) harbors over 100 trillion microorganisms, mostly bacteria. Gut bacteria are localized all over the GI tract with the highest density of (10^{11}-10^{12}) per gram content in the colon (Thursby & Juge, 2017). The majority of the gut bacteria belong to the phyla Firmicutes and Bacteroidetes (combined 90%) while the remaining 10% are classified as Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia (Arumugam et al., 2011). Right after birth, when gut bacteria have colonized the gut, a symbiotic relationship between host and the gut bacteria is established (Mazmanian et al., 2005). The host provides food components to the bacteria that the host cannot digest and, in return, the gut bacteria ferment these components to products such as short chain fatty acids (SCFA's) and vitamins (Yatsunenko et al., 2012; LeBlanc et al., 2013; De Medina et al., 2014). SCFA's and vitamins are essential to maintain the healthy status of the GI tract (Yatsunenko et al., 2012). Moreover, commensal gut bacteria are also involved in maturing and reshaping the immune system (Mazmanian et al., 2005), prevention of colonization by pathogenic bacteria (Kamada et al., 2013), regulating the integrity of the epithelial barrier (Derrien et al., 2008; Capaldo et al., 2017) and the brain-gut axis (Martin et al., 2018).

The symbiotic relationship of host and gut bacteria can be challenged by changes in bacterial composition, *i.e.*, dysbiosis. Dysbiosis occurs when the number of pathogenic bacteria increases or the number of beneficial bacteria and their products decrease, resulting in inflammation and disease (Lynch & Pedersen, 2016). Factors that can alter the gut bacteria composition are for example, geographic origin, age, diet, inflammation status, exercise, pre- and probiotic and antibiotic use. Moreover, the gut bacteria composition per person is unique and there is a high variability (Wang et al., 2018).

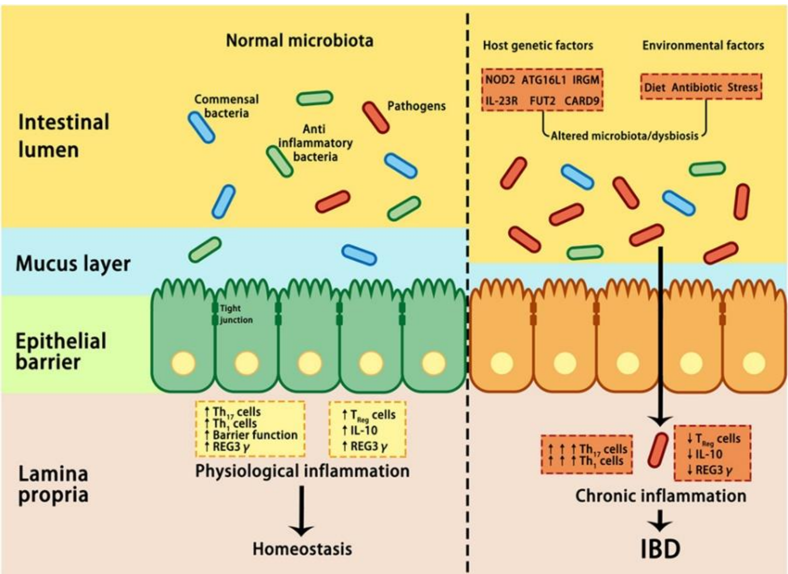

Figure 1. A schematic overview of the gut during homeostasis and inflammation (Adapted from Zhang et al., 2017)
During inflammation, the number of pathogenic bacteria is increased and the thickness of the mucus layer is decreased. As a result of the inflammation, the epithelial barrier is comprised, allowing bacteria past the epithelial barrier.

In a healthy gut, gut bacteria are located in the lumen, (outer) mucus layer or the intestinal mucosa where they compete with pathogenic bacteria, ferment food and produce SCFA's. Intestinal epithelial cells (colonocytes) feed upon these SCFA's, maintaining the mucus layer to prevent inflammation from the gut bacteria and regulating the epithelial barrier integrity via tight junctions (TJ) (Johansson et al., 2011; Schroeder, 2019) (**Figure 1, left panel**). When inflammation occurs, TJ that link the colonocytes together widen, therefore weakening the epithelial barrier (Desai et al., 2016) (**Figure 1, right panel**). The epithelial barrier is especially important since a compromised barrier integrity plays a role in gut-related diseases like irritated bowel disorder (IBD) (Capaldo et al., 2017) but also in non gut-related diseases for example eczema, food allergies and asthma (Zimmermann et al., 2019).

Alternatives to improve gut health and prevent diseases include the use of pre- and probiotics. Prebiotics alter the gut bacteria composition by administering food components, mainly insoluble fibers, that induce growth of beneficial bacteria (Lordan et al., 2020). While probiotics alter the gut bacteria composition by administering live microorganisms and when administered in adequate amounts, confer health benefits to the host (Guarner & Schaafsma, 1998; Tannock, 2002; Kerry et al., 2018). Currently the main bacteria used for probiotics are lactic acid-producing bacteria of the genus *Lactobacillus* sp and *Bifidobacterium* sp (Ljungh & Wadström, 2001; Foligné et al., 2013). Besides lactic acid producing bacteria, promising candidates for new generation probiotics are butyrate-producing bacteria (Saarela, 2019; Lordan et al., 2020).

Butyrate is a SCFA produced by certain species of gut bacteria by metabolizing indigestible carbohydrates. Butyrate is the main energy source of colonocytes, has anti-inflammatory properties and improves the integrity of the intestinal epithelial barrier by regulating the expression of genes for TJ (Hamer et al., 2008; De Medina et al., 2014; Kelly et al., 2015; Zheng et al., 2017; Venegas et al., 2019). Increasing the number of butyrate-producing bacteria could therefore decrease inflammation and increase the epithelial barrier integrity. A study showed that butyrate production and the

intestinal epithelial barrier integrity was increased after supplementing butyrate-producing bacteria *in vitro* in tissue of Crohn's disease (CD) patients (Geirnaert et al., 2017).

From the butyrate producers, a bacterium that could be a candidate for probiotic use is *Faecalibacterium prausnitzii*, a Gram-positive bacteria that comprises 5-15% of the bacteria in faeces (Flint et al., 2012). *F. prausnitzii* grows near the mucus layer, where it converts acetate into butyrate (Miquel et al., 2013). Moreover, a high abundance of *F. prausnitzii* is associated with a healthy gut and a low abundance is associated with diseases like IBD (Sadabad et al., 2015; Ni et al., 2017). Additionally, in previous studies it was shown that *F. prausnitzii* has anti-inflammatory effects (Miquel et al., 2013; Sadabad et al., 2015).

Even though it is not a butyrate producer, *Akkermansia muciniphila* is a promising candidate for probiotic use also, as *A. muciniphila* is associated with a healthy gut and epithelial gut barrier integrity. *A. muciniphila* is a Gram-negative anaerobic bacterium and comprises of 1-3% of the bacteria in faeces (Derrien et al., 2004, 2008). *A. muciniphila* produces propionate and acetate by feeding from the outer mucus layer (Derrien et al., 2004). *A. muciniphila*, then, stimulates the colonocytes to produce more mucus, keeping the inner mucus layer intact (Derrien et al., 2011). Because of its niche on the mucus layer, *A. muciniphila* also acts as a competitor to pathogenic bacteria and helps to promote epithelial barrier integrity by regulating the TJ (Chelakkot et al., 2018). TJ are a complex of multiple proteins that link epithelial cells, forming a barrier that prevents pathogens from entering the bloodstream. *A. muciniphila* has been shown to upregulate the mRNA levels of genes coding for the TJ proteins ZO-1, ZO-2, ZO-3, Occludin and Claudin-4, therefore increasing the epithelial barrier integrity (Chelakkot et al., 2018; Ashrafian et al., 2019). Furthermore *A. muciniphila* regulates inflammatory responses and its absence has been linked with metabolic diseases like diabetes and obesity (Derrien et al., 2011; Zhang et al., 2019).

A. muciniphila and *F. prausnitzii* are both associated with health and can be considered an interesting target for new generation probiotics because of their beneficial properties. Moreover, the combination of *A. muciniphila* and *F. prausnitzii* is promising, since *A. muciniphila* is an acetate producing bacterium and *F. prausnitzii* needs acetate to produce butyrate (Duncan et al., 2002). Syntrophic relationships between *F. prausnitzii* and other acetate-producing bacteria like *Bacteroides thetaiotaomicron* and *Bifidobacterium adolescentis* have been shown (Wrzosek et al., 2013; Rios-Covian et al., 2015). *A. muciniphila* was able to support the growth and promote butyrate production *of F. prausnitzii in vitro* (Belzer et al., 2017). It is promising and especially interesting how a mixture of *F. prausnitzii* and *A. muciniphila* could affect gut epithelial cells. The main challenge to succeed on culturing such strict anaerobic gut bacteria together with colonocytes, is that colonocytes require oxygen. So far only, the Transwell co-culture model, the Host-Microbiota Interaction (HMI ™) module, Human Oxygen-Bacteria anaerobic (HoxBan) system, human gut-on-a-chip and the human-microbial crosstalk modular microfluidic device (HuMiX) have been able to analyze the interaction between anaerobic gut bacteria and gut epithelial cells *in vitro* (von Martels et al., 2017).

In 2015, Sadabad and colleagues succeeded in co-culturing the anaerobic bacterium *F. prausnitzii* and Human colon carcinoma cells (Caco-2) for up to 36 hours using the HoxBan system (Sadabad et al., 2015). The HoxBan system is a two-compartment model, consisting of an anaerobic/bacterial compartment (where anaerobic bacteria are cultured anaerobically in a Yeast, Casitone, Fatty acids, Acetate, Glucose (YCFAG)-agar medium) and an oxygenated/human epithelial compartment (containing DMEM growth medium and Caco-2 cells grown on a coverslip as a monolayer) (**Figure 2A**). In the agar, an oxygen gradient occurs, which is observed as the pink colorization of the agar (**Figure 2B**). *F. prausnitzii* is able to reduce the oxygen to create an anerobic environment and a bacterial rim formation is observed (Khan et al., 2012; Sadabad et al., 2015) (**Figure 2C**). Besides the assessment of

the growth of *F. prausnitzii*, the HoxBan system also allows to study the effect of *F. prausnitzii* on, for example, the viability and gene expression of inflammatory and oxidative stress markers in the Caco-2 cells.

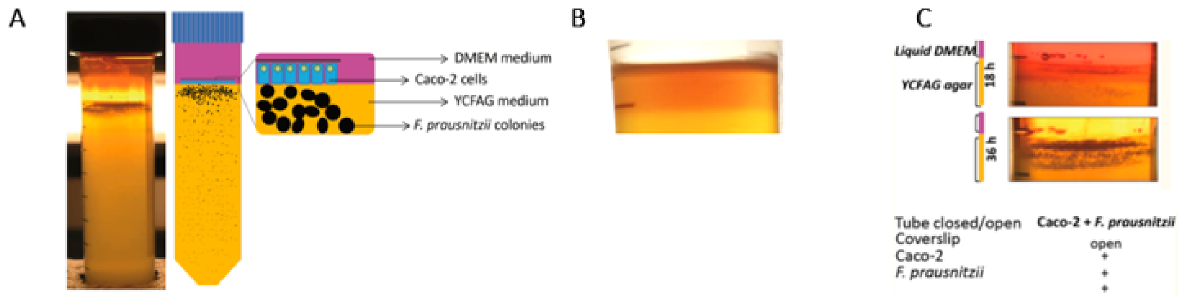

Figure 2. Culturing *F. prausnitzii* in co-culture with Caco-2 cells on the HoxBan system. A) Schematic overview of *F. prausnitzii* in co-culture with Caco-2 cells on the HoxBan system. (Adapted from Sadabad et al., 2015). B) Oxygenated (pink) area in a HoxBan without bacteria and Caco-2 cells. C) Rim formation of *F. prausnitzii* when co-cultured with Caco-2 cells after 18 and 36 hours.

So far, only a single bacterium (*F. prausnitzii),* was co-cultured together with Caco-2 cells using the HoxBan system. The aim of the current study was to investigate whether *A. muciniphila* individually and together with *F. prausnitzii* as a bacterial consortium, could be co-cultured with Caco-2 cells using the HoxBan system and how they affect the viability and gene and protein expression in Caco-2 cells. In order to culture *A. muciniphila* in a HoxBan system, the YCFAG medium was adjusted since previous studies showed that *A. muciniphila* requires a different nitrogen source then currently available at the YCFAG medium (Derrien et al., 2004). Furthermore, the pH of the medium was adjusted since pH affects the growth of *A. muciniphila* (Van Herreweghen et al., 2017). We hypothesize that A. muciniphila has a beneficial effect on epithelial barrier function, combined with an anti-inflammatory effect of F. prausnitzii, in colonocytes.

Material and methods

Bacteria and Caco-2 cell culturing

Bacteria

F. prausnitzii (A2-165, DSM 17677) and *A. muciniphila* (ATCC ® BAA-835 ™, Manassas, USA) were anaerobically grown under an atmosphere composed by ($H_2/N_2/CO_2$, 10:10:80, v/v) at 37°C in Yeast, Casitone, Fatty acids, Acetate and Glucose medium supplemented with mucin (YCFAGM) broth (as described below), unless stated otherwise, then mixed with 1:4 glycerol (80%) and stored at -80°C.

10 µL of the glycerol stock of *F. prausnitzii* and *A. muciniphila* was inoculated in 3 mL YCFAGM medium two days before the HoxBan experiment. The next day the bacteria were 'refreshed' by transferring 100 µL of the overnight culture to fresh 3 mL YCFAGM medium.

Caco-2 cells

Caco-2 cells were cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM, ThermoFischer Inc) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% PSF antibiotic cocktail (penicillin streptomycin and fungi zone; Lonza, Bazel, Switzerland) and 1% non-essential amino acids (Cibco ® NEAA). T75 flasks were used to grow the Caco-2 cells and were passaged every 3-4 days. A day prior to assembling the HoxBan, the cells were seeded with a 50-60% confluency on coverslips in a 12-well plate for 24 hours.

On the day of the HoxBan it was checked whether the coverslips had reached 80-100% confluency. Then, shortly before assembling the HoxBan, DMEM medium was replaced with antibiotic-free DMEM medium. The Caco-2 cells were always incubated at 37°C and with an atmosphere containing 5% CO_2.

Culturing *A. muciniphila* and *F. prausntzii* in YCFAG medium supplemented with mucin or gelatin

The existing Yeast, Casitone, Fatty acids, Acetate and Glucose (YCFAG) medium as used by Sadabad *et al.* (2015) needed adjustments, as previous literature showed that *A. muciniphila* required a different nitrogen source than currently available at YCFAG (Derrien et al., 2004). Therefore, the amount of casitone (10 g/L, Becton, Dickinson and Company) was cut in half (5 g/L, Becton, Dickinson and Company) and mucin from porcine stomach type lll (5 g/L, Sigma-Aldrich) or gelatin (5 g/L, Oxoid) was added as different nitrogen sources. The pH of the medium was adjusted since previous literature showed that *A. muciniphila* grew better in medium with a neutral pH (Van Herreweghen et al., 2017). The mucin from porcine stomach type lll (Sigma-Aldrich) was dissolved in water (10 g/L) and autoclaved. After autoclavation the mucin solution was kept in the fridge. When YCFAG supplemented with mucin (YCFAGM) medium was prepared, the mucin solution was added to the medium so that the mucin got autoclaved twice due to otherwise high contamination rate. When broth was used, no bacteriological agar no.1 (Oxoid) was added to the medium.

YCFAG medium supplemented with mucin or gelatin used for this experiment consisted of (all concentration per liter) casitone (5.0 g, Becton, Dickinson and Company), yeast extract (2.5 g, Oxoid), $NaHCO_3$ (4.0 g, Sigma-Aldrich), K_2HPO_4 (0.45 g, EMPROVE Merck KGaA), KH_2PO_4 (0.45 g, Merck KGaA), NaCl (0.9 g, EMSURE Merck KGaA), $MgSO_4 \cdot 7\ H_2O$ (0.09 g, Sigma-Aldrich), $CaCl_2 \cdot 2\ H_2O$ (0.12 g, Merck KGaA), CH_3COONa (2.7 g, EMSURE Merck KGaA), glucose (4.5 g, Merck KGaA), cysteine (1 g, Sigma-Aldrich), bacteriological agar no.1 (7 g, Oxoid), resazurin 0.02% (1 mg, Sigma-Aldrich), hemin 0.2% (10 mg, Sigma-Aldrich), biotin (10 µg, Sigma-Aldrich), colbalamin (10 µg, Sigma-Aldrich), *p*-aminobenzoic acid (30 µg, Sigma-Aldrich), folic acid (50 µg, AMRESCO) and pyridoxamine (150 µg, Sigma-Aldrich), propionic acid (90 mM, Sigma-Aldrich), isobutyric acid (10 mM, Sigma-Aldrich), isovaleric acid (10 mM, Sigma-Aldrich), valeric acid (10 mM, Sigma-Aldrich), 1M HCl (15 mL, Sigma-Aldrich) and mucin from porcine stomach type lll (5.0 g, Sigma-Aldrich) or gelatin (5.0 g, Oxoid). After autoclaving,

thiamine (0.05 µg/mL, Sigma-Aldrich) and riboflavin (0.05 µg/mL, Sigma-Aldrich) were added to the medium. Then the medium was adjusted to a pH of 6.5 or 7 which was checked by pH-indicator strips (MQant ®) using 37% HCL.

The inoculum was prepared by culturing *F. prausnitzii* and *A. muciniphila* anaerobically at 37°C in 3 mL of YCFAG and Brain Heart Infusion (BHI)+cysteine (37 g/L; 1 g/L, Oxoid) broth respectively. After ~24 hours, 90 µL of the bacterial suspension was added to 50 mL tubes and 45 mL of YCFAG supplemented with mucin or gelatin was added (**Supplementary Figure 1**). The control tubes did not contain bacterial suspension. The 50 mL tubes were then taken out of the anaerobic chamber to solidify with the caps screwed on tight to maintain an anaerobic environment. After solidification of the medium, the caps were unscrewed a little to allow oxygen in and the tubes were put into the 37°C incubator overnight. After ~18 hours, the tubes were taken out of the incubator and pictures of the tubes were made to assess rim formation and the reduction of oxygen.

HoxBan with Caco-2 cells

35 mL of warm (~40 °C) freshly prepared YCFAG as described in Sadabad et al. (2015) (7 g/L bacteriological agar no.1) was added to 50 mL tubes. The 50 mL tubes were stored in the fridge until further use. 35 mL of YCFAG was used to supply the bacteria with equal amounts of nutrients and to reduce the costs, since mucin is expensive.

On the day of the experiment 80 µL of bacterial suspension containing *F. prausnitzii* or *A. muciniphila* was transferred to the freshly prepared YCFAGM medium with agar. When *F. prausnitzii* and *A. muciniphila* were cultured in the same HoxBan, 30 µL of the bacterial suspension containing *A. muciniphila* was added and 50 µL of *F. prausnitzii* based on the fact that 5% of feces consist of *F. prausnitzii* and 3% of *A. muciniphila* (Arumugam et al., 2011; Derrien et al., 2008). Then in the anaerobic chamber, 10 mL warm (~40 °C) freshly prepared YCFAGM was added per tube which already contained 35 mL of solidified YCFAG.
After solidification outside the anaerobic chamber, a layer of 500 µL sterile mucin-Phosphate Buffer Solution (PBS)-agar was added in the anaerobic chamber (5 g/L mucin from porcine stomach type lll (Aldrich-Sigma), 7 g/L bacterial agar no. 1 (Oxoid) and 1x PBS). After solidification, 5 mL of prewarmed (~37 °C) DMEM (without antibiotics, with glucose) was added to the tubes containing an empty coverslip or a coverslip with Caco-2 cells on it. The coverslips were then placed on the top of the bacterial compartment, cells facing the agar (**Supplementary Figure 2**).
Lastly, the tubes were put in a 37°C, 5% CO_2 incubator with slightly unscrewed caps to allow gas exchanges into the tubes.

The controls included in this study were tubes without bacteria and tubes with an empty coverslip and DMEM on top. There was an extra tube with Caco-2 cells per condition that was used for the cell viability assay.

After 18 hours, pictures of the tubes were made and samples for further analyses were collected. Gram stains of the HoxBan were performed as described in 'Evaluation of bacterial growth'. Before taking pictures of the HoxBan, DMEM and coverslips were taken off.

Evaluation of bacterial growth

Bacterial growth was evaluated by making pictures with a phone camera. The tubes were held in front of a lamp for extra contrast. For analysis, the contrast and brightness of the pictures of the tubes were enhanced by increasing the contrast to +20% and brightness to +20% using Powerpoint.

Gram stains
By the use of a bacteriological loop, a piece of agar of the bacterial compartment of the HoxBan was placed on a glass slide in a droplet of PBS. The glass slide was then put in the microwave for several seconds until the agar was melted. Subsequently, the following protocol was used for Gram staining of the glass slides: 1 minute staining with a few droplets of Crystal violet solution (Gram's crystal violet solution, Sigma-Aldrich), rinsing with water, 1 minute of Iodine solution (Gram's Iodine solution, Sigma-Aldrich), rinsing with acetone:ethanol solution (2:1) and Fuchsin solution (Gram's Fuchsin solution, Sigma-Aldrich) for 30 seconds and rinsing with water.

The Gram stains were subsequently examined with a bright field microscope under a magnification of 100x using immersion oil. Pictures of the stained slides were taken by phone through the ocular. The contrast of the pictures were enhanced by increasing the contrast +40% and +20% brightness using Powerpoint.

<u>RNA isolation, cDNA synthesis and quantitative real-time PCR</u>
After 18 hours on the HoxBan system, the Caco-2 cells were harvested and RNA was isolated by using TRIzol (Sigma-Aldrich) according to the manufacturers protocol. In order to quantify the amount of RNA, Nanodrop 2000c Spectrophometer (Thermo-Scientific) was used.

After quantification, a mix of 10% reaction RT Buffer (500 mM Tris-HCl, 500 mM KCl, 30 mM MgCl2, 50 mM DTT), 10% dNTP mix (10 mM dATP, dGTP, dTTP, Sigma), 2% random primers (0.5 µg/µL, Sigma-Aldrich), 2% M-MLV RT (200 U/µL, Invitrogen) and 1.5% RNAse OUT (40 U/µL, Invitrogen), was added per sample containing 2.5 ng of RNA which added up to a final volume of 50 µL. Then, cDNA was made by using a thermal cycler (Bio-Rad T100) for 10 minutes at 25°C followed by 60 minutes at 37°C and 5 minutes at 95°C.

Quantitative Real-Time PCR (qPCR) was performed to assess the mRNA levels of genes in inflammatory and oxidative stress pathways like inducible nitric oxide synthase (*iNOS*, *NOS2*), heme oxygenease 1 (*HO-1*, *HMOX1*) and interleukin 1 beta (*IL-1β*, *IL1B*). Moreover, mRNA levels of *MUC2*, occludin (*OCLN*), Claudin-1 (*CLDN1*), Claudin-3 (*CLDN3*), zonula occludens-1 (*ZO-1*, *TJP1*) were used to assess the effect of bacterial exposure to Caco-2 cells on epithelial barrier markers. Lastly, glucose transporter 1 (*GLUT1*, *SCL2A1*) was used to study the HIF1-α pathway. For correction of Ct values housekeeping ribosomal *18S* was used.
The probes and primers used per gene are described in Table 1. The reaction mix used for the samples contained 4% 5 µM fluorescent probe, 1.8% 50 µM forward and reverse primers, 50% 2x qPCR reaction buffer (Eurogenic), 22.4% RNAse-free water and 20% diluted cDNA. Every sample was done in duplicate and the cDNA was diluted by using RNAse-free water. The amplification cycle that was used was 10 minutes heating to 95°C, followed by a 40-times repeated cycle, consisting of 15 seconds at 95°C and 1 min at 60°C, performed using a StepOnePlus (AB, Applied Biosystems) PCR system.

Data analysis was performed using QuantStudio Design & Analysis (v.1.5.1., Thermofisher Inc). Baseline and threshold were manually adjusted, whereas the baseline was adjusted to the cycle where the amplification started and the threshold was fixed halfway the linear area of the graph. Ct-values were calculated and normalized against *18S*. The Ct-value of the duplicates was averaged and subsequently used for statistical analysis.

Table 1. Description of probes and primers for genes measured using qPCR.

Gene	Type	Sequence
18S	Probe Sense Antisense	5'-CGC GCA AAT TAC CCA CTC CCG A-3' 5'-CGG CTA CCA CAT CCA AGG A-3' 5'-CCA ATT ACA GGG CCT CGA AA-3'
NOS2	Probe Sense Antisense	5'-TCC GAC ATC CAG CCG TGC CAC-3' 5'-GGC TCA AAT CTC GGC AGA ATC-3' 5'-GGC CAT CCT CAC AGG AGA GTT-3'
HMOX1	Probe Sense Antisense	5'-TCA GCA GCT CCT GCA ACT CCT CAA AGA G-3' 5'-GAC TGC GTT CCT GCT CAA CAT-3' 5'-GCT CTG GTC CTT GGT GTC ATG-3'
IL1B	Probe Sense Antisense	5'-CTC TGC CCT CTG GAT GGC GG-3' 5'-ACA GAT GAA GTG CTC CTT CCA-3' 5'-GTC GGA GAT TCG TAG CTG GAT-3'
SLC2A1	Probe Sense Antisense	5'- TGCTGGAGCAGCTAC-3' 5'- CGGGTTGTGCCATACTCATG-3' 5'- GCCAAAGATGGCCACGAT-3'
MUC2	Probe Sense Antisense	5'- CTCTGACGGCGTGCTCTTCAGTCCC-3' 5'- CCTGCAGAGCTATTCAGAATTCC-3' 5'- ATCTTCTGCATGTTCCCAAACTC-3'
CLDN1	Probe Sense Antisense	5'- CAGTCAATGCCAGGTACGAATTTGGTCAG-3' 5'- CCACAGCATGGTATGGCAATAG-3' 5'- CAGCCCAGCCAGTGAAGAG-3'
CLDN3	Probe Sense Antisense	5'- CCCTGCTCACCCTCGTGCCG-3' 5'- CAGGCGTGCTGTTCCTTCTC-3' 5'- GTAGAAGTCCCGGATAATGGTGTT-3'
OCLN	Probe Sense Antisense	5'- TGCAGACACATTTTTAACCCACTCCTCGA-3' 5'- GATGAGCAGCCCCCCAAT-3' 5'- GGTGAAGGCACGTCCTGTGT-3'
TJP1	Probe Sense Antisense	5'- TGGCCACAGCCCGAGGCATATTT-3' 5'- CAGTGCCTAAAGCTATTCCTGTGA-3' 5'- GCACGCCCCCATTGC-3'

Viability of Caco-2 cells

Caco-2 cells were stained for 1 minute using a 0.2% Trypan blue staining (Abcam) then after removal the cells were fixed with 4% paraformaldehyde (10 minutes at room temperature (RT)), rinsed 2 times with PBS and kept in the fridge till further use. The cells were then dehydrated by adding 1 mL of respectively 70%, 80%, 90% and 100% ethanol for 1 minute per % ethanol. The coverslips were mounted afterwards on glass slides with EUkitt (Sigma-Aldrich). After the glass slides had dried overnight, the cells were visualized by a confocal microscope with a 20x magnification.

Per glass slide, 3 fields were selected and made pictures of. In Powerpoint, with the help of a grid the blue-stained death and non-stained live cells were counted (**Supplementary Figure 3**). To enhance the visualization on the pictures of the Caco-2 cells, contrast was enhanced with +50% in Powerpoint. The % live cells was determined by using the following formula: % live cells = (N live cells / N total cells) * 100. The counted fields were used as individual datapoints, resulting in 3 data points per condition per experiment.

Immunofluorescence assay

The coverslips with Caco-2 cells were first gently washed two times with 300 µL of 1x PBS. Then the cells were permeabilized with 0.01% Triton x100 in 1x PBS for 30 minutes in a 37 °C incubator. The permeabilization buffer was then washed off again by washing the cells twice with 1x PBS and afterwards 300 µL blocking buffer (1% Bovine Serum Albumin (BSA) in 1% BSA/PBS) was added. After incubating at RT for 30 min, the blocking buffer was removed and 200 µL of ZO-1 primary antibody in PBS (1:100, Invitrogen) was added to each well. After 1 hour incubation on RT, the antibody was removed and the cells were washed twice with 300 µL of blocking buffer. Then, the cells were incubated with the secondary anti-body (anti-rabbit alexa fluor dye 1:400, Thermofisher, in 1% BSA/PBS) for 30 minutes. Lastly, after washing the cells three times with blocking buffer, the cells were mounted on a glass slide with Vectashield containing DAKO+DAPI (Vector laboratories).

The cells were visualized and pictures were made by using a Zeiss 410 inverted laser scan microscope (Leica Microsystems, Wetzlar, Germany) with a magnification of 40x using immersion oil. 3 fields per glass slide were photographed. The blue and FITC channel were captured separately and then merged by using the manufacturer's software (Leica). To enhance visualization of the Caco-2 cells on the pictures, brightness was increases +40% by using Powerpoint.

Statistical analysis

Statistical analyses were performed using Rstudio (version 4.3.4). One-way ANOVA was used for the results of the qPCR and for the cell viability assay. Both were corrected with a Bonferroni correction for multiple testing (**Appendix A-B**). The different conditions were tested against Monoculture. A p-value <0.05 was considered significant. The package ggplot2 was used to visualize the data.

Results

F. prausnitzii and *A. muciniphila* can be cultured in YCFAG medium supplemented with mucin

To assess in which medium *F. prausnitzii* and *A. muciniphila* could be cultured in the HoxBan system, adjustments to the YCFAG medium (see Material and Methods section) were made.
Figure 4 summarizes the different variables used in the experiment: different pH, cultured bacterial species (*A. muciniphila* and *F. prausnitzii)* all conditions where grown for 24 hours. The oxygenated (pink) area, is visible in all the controls, which shows that no reduction of oxygen has taken place during the culture phase. The reduction of oxygen will occur only during bacterial growth. Since *F. prausnitzii* and *A. muciniphila* are able to reduce oxygen, to create an anaerobic environment to thrive (Khan et al., 2012; Ouwerkerk et al., 2016).
In the YCFAG medium supplemented with mucin (YCFAGM) and a pH of 6.5, *F. prausnitzii* as well as *A. muciniphila* reduced oxygen present in the medium. *F. prausnitzii* even formed a growth rim (Figure 4A), which is also typically observed when using YCFAG medium (Figure 2A&2C). For the YCFAGM medium with a pH of 7, however, no reduction of oxygen took place either by *F. prausnitzii* or *A. muciniphila*, no rim was formed either (Figure 4B). Gram stains confirmed the presence of *F. prausnitzii* and *A. muciniphila* in both of the YCFAGM independent of the pH (Figure 4A-B).
For the medium supplemented with gelatin (YCFAGG) with a pH of 6.5, *F. prausnitzii* showed a reduction of the oxygen and formed a rim, however the oxygenated area of *A. muciniphila* was still clearly visible (Figure 4C).
With regard to the YCFAGG medium with a pH of 7, both *F. prausnitzii* and *A. muciniphila* reduced oxygen, however, an oxygenated area was still visible (Figure 4D). Gram stains showed that *A. muciniphila* failed to grow in the YCFAGG medium and grew in lower abundance at pH 7 compared to the YCFAGM medium. *F. prausnitzii* was abundantly present in all conditions. Based on these observation, YCFAGM and a pH of 6.5 was selected for the use in the follow-up experiments.

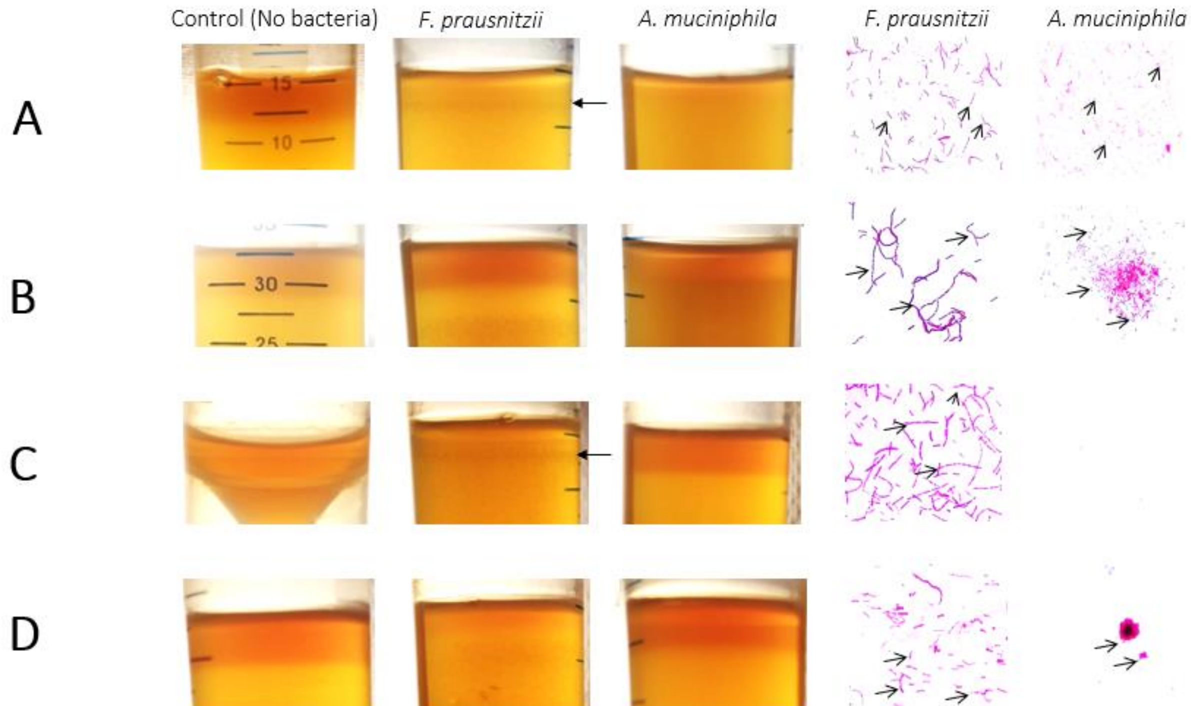

Figure 4. Comparison of the culturing of *F. prausnitzii* and *A. mucinphila* on YCFAG+Mucin and YCFAG+Gelatin media with different pH.
The magnification for the gram stains is 100x. Arrows indicate a growth rim on the HoxBan and on Gram stains indicate bacteria.
A) HoxBan and Gram stains of HoxBan using the YCFAG supplemented with mucin with a pH of 6.5 or pH 7 (B). C) HoxBan and Gram stains of HoxBan using the YCFAG supplemented with gelatin with a pH of 6.5 or pH 7 (D). Pink area on the HoxBan indicate a high oxygen concentration in the medium.

Co-culture of *F. prausnitzii* and *A. muciniphila*, individually or as bacterial consortium, with Caco-2 cells in a HoxBan system in YCFAGM medium

To assess whether *A. muciniphila* and a bacterial consortium could be co-cultured with Caco-2 cells in the HoxBan system, HoxBan experiments were performed with Caco-2 cells on top of the bacteria-containing compartment.

Figure 5A shows the oxygenated area's and rim formation between the different conditions after 18 hours of co-culture. The HoxBan without bacteria showed a clear oxygenated area. In the conditions containing bacteria, the pink area was not visible presumably due to a reduction of oxygen by *F. prausnitzii* and/or *A. muciniphila*. *F. prausnitzii* was also able to form a rim in YCFAGM, when cultured alone or together with *A. muciniphila* (**Figure 5A 2nd and 4th column**).

Gram stains of the HoxBan experiments showed the presence of *F. prausnitzii* (**Figure 5C;2E**) and *A. muciniphila* (**Figure 5D**). *A. muciniphila*'s presence in the bacterial consortium could not be confirmed, due to technical limitations. This is because of the colored background of the agar and mucin present in the medium. **Figure 5B** shows no bacteria but does show the mucin in the medium.

A

F. prausnitzii (A2-165)	-	+	-	+
A. muciniphila (BAA-835)	-	-	+	+

B C D E

Figure 5. *F. prausnitzii* forms a rim on YCFAGM media in co-culture with Caco-2 cells.
Monoculture is a HoxBan with Caco-2 cells without bacteria. Arrows indicate a growth rim on the HoxBan and on Gram stains indicate bacteria. Pink area on the HoxBan indicate a high oxygen concentration in the medium.
(**A**) Comparison of rim formation and oxygenated area's per condition (n=3). Gram stains of the HoxBan of Monoculture (**B**), *F. prausnitzii* (**C**), *A. muciniphila* (**D**) and *F. prausnitzii* + *A. muciniphila* (**E**).

Cell survival of Caco-2 cells decreased when exposed to *F. prausnitzii*, *A. muciniphila* or bacterial consortium

The total number of Caco-2 cells did not differ significantly between the different conditions (p-value>0.05) (**Supplementary Figure 4F**). The percentage of live cells, however did differ significantly between conditions: when the cells were co-cultured with *F. prausnitzii* the survival was 81±12% (p-value<0.05), for *A. muciniphila* 83±6% (p-value<0.05) and the condition containing both bacterial strains, 68±14% of cells survived (p<0.001). This is statistically significant, when compared against the Monoculture (96±3%) or 2D (97±1%) conditions. (**Figure 6**).

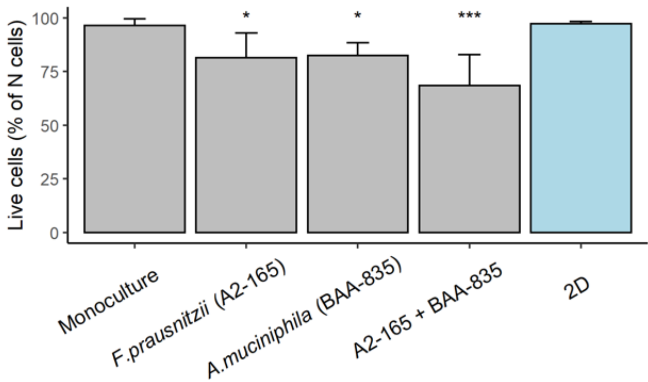

Figure 6. Live Caco-2 cells decreased when exposed to *F. prausnitzii*, *A. muciniphila* or both
2D is Caco-2 cells grown on a coverslip for 18 hours, Monoculture is a HoxBan with Caco-2 cells without bacteria. Data shows two biological replicates (n=2). Mean + SD is shown.
* p<0.05
*** p<0.001

F. prausnitzii and A. muciniphila show anti-inflammatory and anti-oxidative stress effects

To assess the effect of *F. prausnitzii* and *A. muciniphila* on oxidative stress and inflammatory markers mRNA levels of *HMOX1*, *NOS2* and *IL1B* in Caco-2 cells were investigated.
The mRNA levels for the oxidative stress marker *HMOX1* showed a reduction when Caco-2 cells were exposed to *F. prausnitzii* and the bacterial consortium when compared to the Caco-2 monoculture (**Figure 7A**). In the same way, the mRNA levels of the inflammatory markers *NOS2* and *IL1B* are downregulated in Caco-2 when cultured in the presence of *F. prausnitzii* and *A. muciniphila* whereby the bacterial consortium has the lowest mRNA levels for the inflammatory markers (**Figure 7B-C**). These results reveal trends in gene regulation, as these experiments were only performed 2 times.

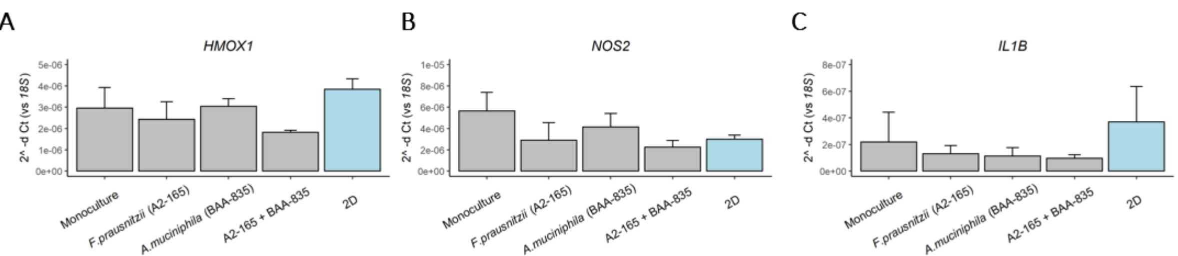

Figure 7. *F. prausnitzii and A. muciniphila* show anti-inflammatory and anti-oxidative stress effects
2D is Caco-2 cells grown on a coverslip for 18 hours, Monoculture is a HoxBan with Caco-2 cells without bacteria.
Data shows two biological replicates (n=2). Mean + SD are shown. The Y-axis shows the gene expression compared to *18s*.
(**A**) Comparison of mRNA levels of the oxidative stress marker *HMOX1* of Caco-2 cells per condition. (**B**) Comparison of mRNA levels of the inflammatory marker *NOS2* of Caco-2 cells per condition. (**C**) Comparison of mRNA levels of the inflammatory marker *IL1B* of Caco-2 cells per condition.

F. prausnitzii, A. muciniphila and bacterial consortium decrease *MUC2* mRNA levels in Caco-2 cells

To assess the effect of *F. prausnitzii* and *A. muciniphila* on epithelial barrier markers, mRNA levels of Claudin-1 (*CLDN1*), Claudin-3 (*CLDN3*), Occludin (*OCLN*), *TJP1* (encoding ZO-1) and *MUC2* were investigated.

For every gene, the bacterial consortium caused the lowest levels of mRNA in Caco-2 cells compared to Monoculture and 2D-grown Caco-2 cells. The mRNA levels for the different genes of the Caco-2 cells exposed to *F. prausnitzii* do not deviate substantially from monocultured Caco-2 cells (**Figure 8**).

Caco-2 cells exposed to *F. prausnitzii*, *A. muciniphila* or bacterial consortium compared to monocultured Caco-2 cells expressed strongly reduced levels of *MUC2*. Moreover, 2D-grown Caco-2 cells also showed lower level of *MUC2* mRNA levels compared to Monoculture (**Figure 9**). Between the conditions that contain bacteria, *A. muciniphila* caused the highest mRNA levels of *MUC2* in Caco-2 cells.

To test potential regulation of the HIF1-α pathway, mRNA levels of the glucose transporter *GLUT1* (*SLC2A1*) were also investigated. A downregulation of *SLC2A1* mRNA levels was found in Caco-2 cells exposed to *F. prausnitzii* and the bacterial consortium (**Supplementary Figure 5**).

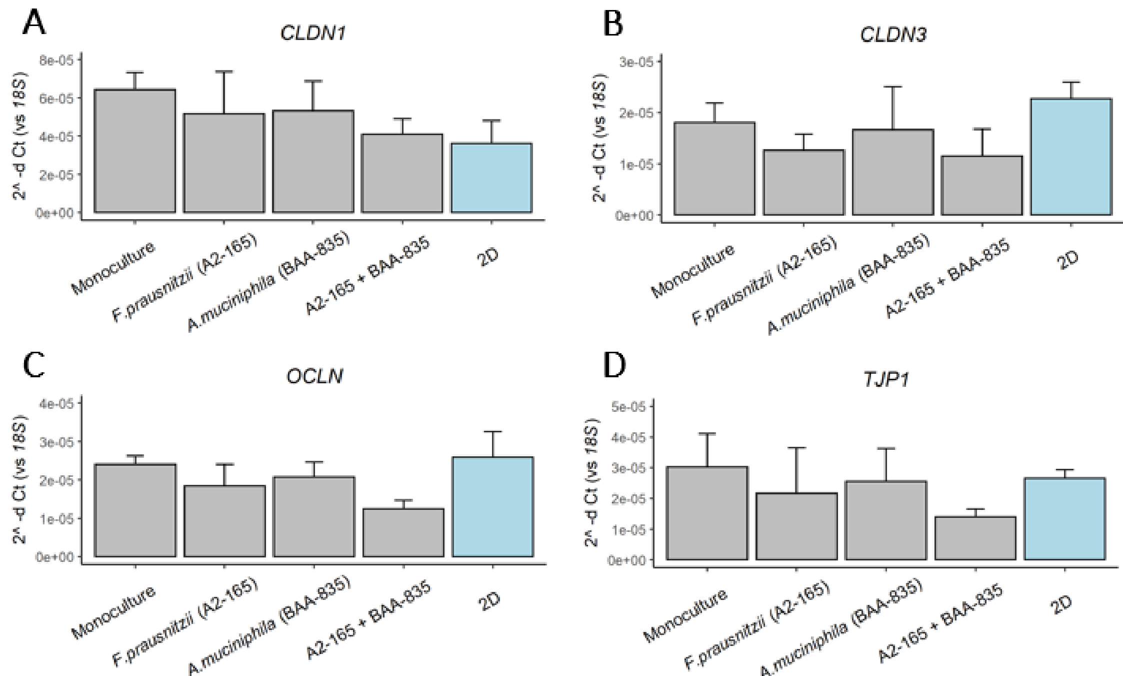

Figure 8. mRNA levels of epithelial barrier markers of Caco-2 cells per condition.
2D is Caco-2 cells grown on a coverslip for 18 hours, Monoculture is a HoxBan with Caco-2 cells without bacteria. Data shows two biological replicates (n=2). Mean + SD are shown. The Y-axis shows the gene expression compared to *18s*.
(**A**) Comparison of mRNA levels of *Claudin-1* (*CLDN1*) of Caco-2 cells per condition. (**B**) Comparison of mRNA levels of *Claudin-3* (*CLDN3*) of Caco-2 cells per condition. (**C**) Comparison of mRNA levels of *Occludin* (*OCLN*) of Caco-2 cells per condition.
(**D**) Comparison of mRNA levels of *ZO-1* (*TJP1)* of Caco-2 cells per condition.

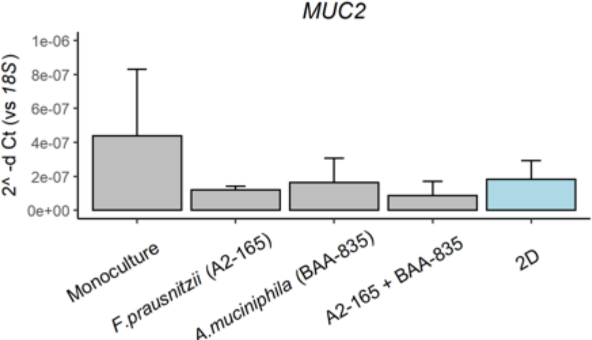

Figure 9. Caco-2 cells exposed to *F. prausnitzii, A. muciniphila* and bacterial consortium have reduced mRNA levels of *MUC2*.

2D is Caco-2 cells grown on a coverslip for 18 hours, Monoculture is a HoxBan with Caco-2 cells without bacteria. Data shows two biological replicates (n=2). Mean + SD are shown. The Y-axis shows the gene expression compared to *18s*.
Comparison of mRNA levels of *MUC2* of Caco-2 cells per condition.

A. muciniphila enhances ZO-1 protein levels that accumulate in the cellular membrane of Caco-2 cells

As intestinal barrier function is largely determined by the level and membranous location of TJ proteins, such as ZO-1, we next analyzed the cellular localization of ZO-1 by immunofluorescence microscopy (**Figure 10**). The FITC (green) signal shows ZO-1 and DAPI (blue) is used for the staining of the nuclei of the Caco-2 cells. In the 2D-grown Caco-2 cells (1st column) and the one co-cultured with *F. prausnitzii* (3rd column), the fluorescent signal appears mostly dispersed throughout the cellular cytoplasm, while in the Monoculture condition (2nd column) a predominant signal was observed in the cellular membrane. Interestingly, a very strong ZO-1 signal was observed in the cellular membrane when Caco-2 cells were co-cultured with *A. muciniphila* with focal localization as typically observed for TJs (4th column). The Caco-2 cells exposed to the bacterial consortium showed both the enhanced levels of ZO-1 in the cellular membrane (as induced by *A. muciniphila*) and a cytoplasmic fluorescent signal (as induced by *F. prausnitzii*) showed on the 5th column of Figure 10.

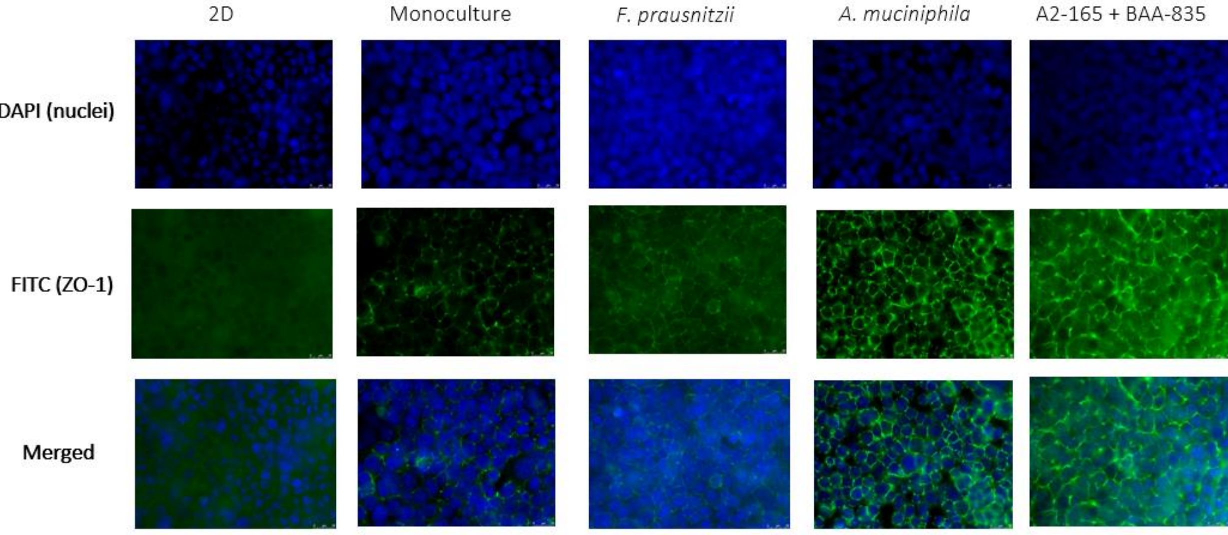

Figure 10. ZO-1 is highly expressed and located in tight junctions in Caco-2 cells exposed to *A. muciniphila* and *F. prausnitzii* + *A. muciniphila*.
2D is Caco-2 cells grown on a coverslip for 18 hours, Monoculture is a HoxBan with Caco-2 cells without bacteria.
Data shows 1 biological replicate (n=1).
DAPI is used to stain the nucleic acids and FITC-labeled antibody is used to detect zonulin-1. The magnification used is 40x.
The graph shows the comparison of ZO-1 expression of Caco-2 cells per condition.

Discussion

This study showed that *A. muciniphila* individually and together with *F. prausnitzii* as a bacterial consortium can be cultured together with Caco-2 cells in a HoxBan system. Moreover, an accumulation of ZO-1 in TJs of Caco-2 cells exposed to *A. muciniphila* was observed, while no upregulation in mRNA levels was observed. Additionally, no significant difference in mRNA levels of epithelial markers in Caco-2 cells between the different conditions was observed, except for a decrease in mRNA levels of *MUC2* when Caco-2 cells were exposed to bacteria. Caco-2 cells exposed to *F. prausnitzii* did show a decrease in inflammatory and oxidative stress markers. Lastly, a decrease in cell viability of Caco-2 cells was observed when cultured with bacteria.

Although Gram stains could not confirm the presence of *A. muciniphila* in the bacterial consortium, the cell viability, qPCR results and immunofluorescence indicated that *A. muciniphila* is present in the bacterial consortium. This is promising since the effect of specific gut bacteria on Caco-2 cells can be assessed *in vitro*. When similar results are obtained using an *in vitro* system, opposed to *in vivo*, animals can be spared. Moreover, recent effort has been directed to perform HoxBans with an inoculum of fecal samples, co-culturing numerous bacteria with Caco-2 cells (not published). Therefore, *in vitro* systems seem to be feasible in the future to replace *in vivo* experiments to assess the effect of gut bacteria on colonocytes.

As previously reported, a compromised epithelial barrier plays a role in several diseases and *A. muciniphila* is important for restoring epithelial barrier function (Liu et al., 2005; Capaldo et al., 2017; Chelakkot et al., 2018). In this study, an accumulation of the protein of ZO-1 in TJs was observed when Caco-2 cells were exposed to *A. muciniphila* while this effect was not observed at mRNA levels. Caco-2 cells that were not cultured with bacteria, showed a dispersed signal regarding ZO-1. Possibly, *A. muciniphila* recruits the ZO-1 already present to the cellular membrane, therefore maintaining the same level of mRNA although resulting in an accumulation of the protein ZO-1 in the cellular membrane of Caco-2 cells. Moreover, similar results were observed in a study with colitis-induced mice, where administration of *A. muciniphila* led to a restoration of the epithelial barrier function featuring Occludin and ZO-1 in mice with dextran sulfate sodium (DSS)-induced colitis while this effect was not observed at mRNA level (Bian et al., 2019). Interestingly, multiple experiments did find upregulation of mRNA levels of ZO-1 in the presence of *A. muciniphila*, e.g. during a high fat diet (HFD) and ethanol diet in mice (Ashrafian et al., 2019; Grander et al., 2020) or in gingival epithelial cells (Huck et al., 2020). Besides administration of *A. muciniphila*, extracellular vesicles of *A. muciniphila* (AmEVs) were able to induce an upregulation of mRNA levels of epithelial barrier markers like *ZO-1* (Chelakkot et al., 2018; Ashrafian et al., 2019). Moreover, a specific protein located in the outer membrane of *A. muciniphila* (Amuc_1100) was also shown to upregulate the mRNA levels of epithelial barrier markers and *ZO-1* after administering to mice fed a HFD. This could be an interesting outlook for pharmabiotics, which contain live, dead or components of the microbiome (Lee et al., 2018). Although, live *A. muciniphila* has already been administered in a clinical trial and no adverse effects have been observed (Plovier et al., 2017). Administration of *A. muciniphila* improved insulin sensitivity and reduced cholesterol levels, body weight and fat mass in overweight individuals in 3 months (Depommier et al., 2019).

Previous research indicates that to proliferate, colonocytes require butyrate as it is the main energy source for colonocytes (Hamer et al., 2008). Studies showed that Caco-2 cells cultured with *F. prausnitzii* had a higher viability rate than Caco-2 cells cultured without bacteria (Sadabad et al., 2015). In this study however, the Caco-2 cells cultured with *F. prausnitzii*, *A. muciniphila* or the bacterial consortium had a decreased viability. One explanation for this could be that the culturing of anaerobic bacteria is improved since the publication of Sadabad and colleagues, whereby the

anaerobic bacteria are now more able to generate an anaerobic environment at the expense of the viability of the Caco-2 cells. Furthermore, the HoxBan used in the current study had, besides a different medium for the bacteria, also an added mucin layer, thereby increasing the distance between the Caco-2 cells and (the products of) the bacteria. Moreover, a decrease in pH of the DMEM medium was observed after 18 hours of co-culturing Caco-2 cells with *F. prausnitzii*, *A. muciniphila* individually or as a bacterial consortium, however the pH was not measured. Previous literature did show that a lower pH decreases the cell viability of Caco-2 cells (Sarisaltik Yasin et al., 2019). Lastly, Caco-2 cells remain a cancer cell line, in a colon cancer cell line (HT-116) it was shown that butyrate could decrease the proliferation of the cells (Zeng et al., 2017). In this study samples for SCFA analysis were collected, although could not be processed in time.

Caco-2 cells are used as a model for colonocytes and are also used to study mucus production (Bu et al., 2011), although mucus is primarily produced by goblet cells in the intestinal epithelium (Johansson et al., 2011). In this study Caco-2 cells exposed to bacteria had decreased mRNA levels of *MUC2*. Interestingly, *A. muciniphila* is known to upregulate mRNA levels of *MUC2* (Derrien et al., 2011). When comparing different colon cancer cell lines, it was observed that Caco-2 cells expressed the lowest mRNA levels of *MUC2* and highest level of *MUC5AC* (Bu et al., 2011). Therefore, *MUC2* might not be the best candidate to study mucus production in Caco-2 cells. Therefore, it would be interesting to repeat this experiment with organoids. In concordance with previous studies a decrease in oxidative stress and inflammatory markers were observed when Caco-2 cells were exposed to *F. prausnitzii* (Miquel et al., 2013; Sadabad et al., 2015). However, the observed effect in the current study was smaller compared to other studies (Miquel et al., 2013; Sadabad et al., 2015). This could be due to lower butyrate levels, as *F. prausnitzii* is a butyrate-producer and butyrate has been shown to have anti-inflammatory effects (Hamer et al., 2008; Flint et al., 2012). However in this study, SCFA samples could not be analyzed in time.

Some things need to be taken into consideration when interpreting the results. For example, the inoculum of the bacteria needs to be standardized. As a consequence of mucin in the medium, it was difficult to use OD as a measurement for the total amount of bacteria. Especially since *A. muciniphila* consumed the mucin, resulting in a negative OD. Furthermore, the power of the statistical analysis regarding the qPCR, immunofluorescence and cell viability could be increased by repeating the experiments to gain a technical and biological triplicate.

For future studies, the presence of *A. muciniphila* needs to be confirmed in the bacterial consortium. This can be done by Fluorescent In Situ Hybridization (FISH) (Belzer et al., 2017). Moreover, to establish if there is a syntropic relationship between *F. prausnitzii* and *A. muciniphila*, acetate can be depleted from the medium, therefore *F. prausnitzii* could only use the acetate produced by *A. muciniphila*. Furthermore, future experiments could be conducted to investigate whether *A. muciniphila* could induce a translocation of TJ proteins. In this study samples for proteomics were collected, however could not be processed in time.

To conclude, this study showed that *F. prausnitzii* and *A. muciniphila* can be cultured together with Caco-2 cells on a HoxBan system with the addition of a new mucus layer. Therefore, opening up opportunities for other anaerobic bacteria to be co-cultured with Caco-2 cells or other host cells in an *in vitro* co-culture system. Furthermore, an accumulation and localization in the cell membrane of the TJ protein ZO-1 was shown when Caco-2 cells were exposed to *A. muciniphila* in an *in vitro* co-culture system, showing the importance of *A. muciniphila* in epithelial barrier integrity. *F. prausnitzii* showed anti-inflammatory and anti-oxidative stress effects in Caco-2 cells. Therefore, when *F. prausnitzii* and *A. muciniphila* could be administered together as probiotic or pharmabiotic, they could exert multiple benefits for the host.

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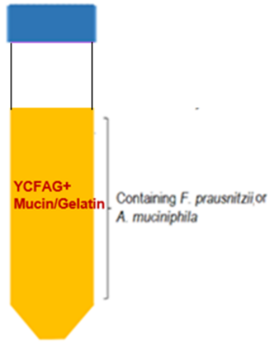
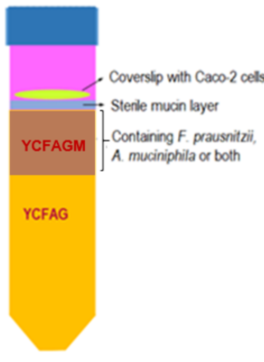
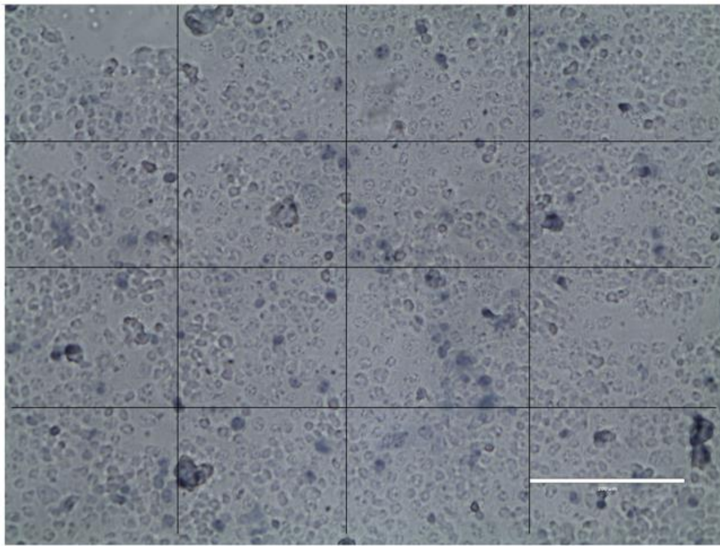
Supplementary Figures



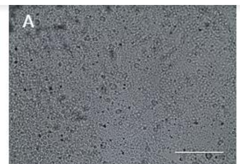
Supplementary Figure 1. Schematic overview of setup to investigate in which YCFAG media supplemented with mucin or gelatin *F. prausnitzii* and *A. muciniphila* could be cultured best.
90 µL of bacterial suspension of *A. muciniphila* or *F. prausnitzii* was added to 50 mL tubes containing 45 mL of YCFAG media supplemented with mucin or gelatin.

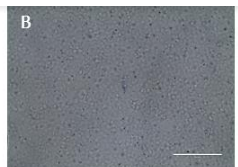


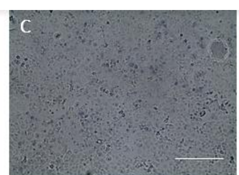
Supplementary Figure 2. Schematic overview of HoxBan setup.
50 mL tubes contained 35 mL YCFAG and on top of that 10 mL of YCFAG supplemented with mucin (YCFAGM). 80 µL of bacterial suspension of *A. muciniphila* or *F. prausnitzii* was added to the 10 mL of YCFAGM. When *A. muciniphila* and *F. prausnitzii* were cultured together, 30 µL of *A. muciniphila* and 50 µL of *F. prausnitzii* was added.
A sterile mucin layer separated bacteria from the coverslip containing Caco-2 cells. 5 mL of DMEM was added on top.

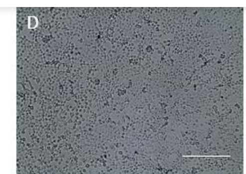


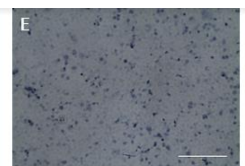
Supplementary Figure 3. Example of counting live and dead Caco-2 cells using a grid.

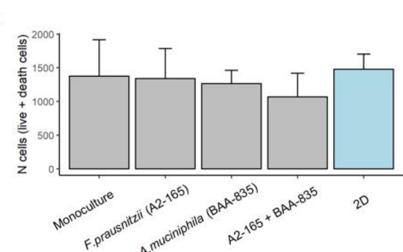




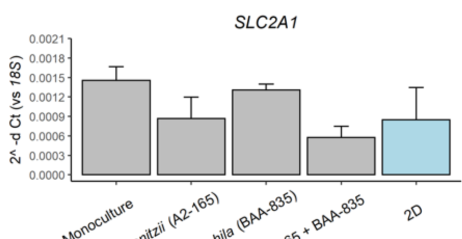








Supplementary Figure 4. Viability of Caco-2 cells per condition.
2D is Caco-2 cells grown on a coverslip for 18 hours, Monoculture is a HoxBan with Caco-2 cells without bacteria. Data shows two biological replicates (n=2).
(**A**) Trypan blue staining of Caco-2 cells without 18 hours co-culture (**A**, **B**) 2D and monoculture respectively and with 18 hours co-culture with (**C**) *F. prausnitzii,* (**D**) *A. muciniphila* and (**E**) *F. prausnitzii* + *A. muciniphila*.
(**F**) Total amount of Caco-2 cells per condition are shown as mean+SD.



Supplementary Figure 5. mRNA levels of *SLC2A1* of Caco-2 cells per condition.
2D is Caco-2 cells grown on a coverslip for 18 hours, Monoculture is a HoxBan with Caco-2 cells without bacteria. Data shows two biological replicates (n=2). Mean + SD are shown. The Y-axis shows the gene expression compared to *18s*.
Comparison of mRNA levels of glucose transporter 1 (*GLUT1*) (*SLC2A1)* of Caco-2 cells per condition.

Appendix A: Example statistical analysis of mRNA levels (*SLC2A1*)

```
#Statistical analysis of mRNA levels of SLC2A1

#####################################################################################
#Preparatory steps: installing packages
#####################################################################################
install.packages("car", dependencies = TRUE)
install.packages('FSA')

library("car") #This package is necessary for the Levene's test
library('FSA') #This package is necessary to perform a non-parametric one-way ANOVA

#####################################################################################
#Testing the normality of the data and the equality of variances
#Shapiro wilk test, to test if the data is normally distributed
#####################################################################################

shapiro.test(qPCR_GLUT1$Ct_value[qPCR_GLUT1$Condition=='F_prau'])

shapiro.test(qPCR_GLUT1$Ct_value[qPCR_GLUT1$Condition=='A_muc'])

shapiro.test(qPCR_GLUT1$Ct_value[qPCR_GLUT1$Condition=='Party'])

shapiro.test(qPCR_GLUT1$Ct_value[qPCR_GLUT1$Condition=='Nobacteria'])

shapiro.test(qPCR_GLUT1$Ct_value[qPCR_GLUT1$Condition=='2D'])

#####################################################################################
#Levene's test, to test if the variances are equal
#####################################################################################

leveneTest(Ct_value ~ Condition, data=qPCR_GLUT1)

#####################################################################################
#One-way ANOVA
#####################################################################################

res.aov_GLUT1 <- aov(Ct_value ~ Condition, data = qPCR_GLUT1)

summary(res.aov_GLUT1)

TukeyHSD(res.aov_GLUT1) #To correct for multiple testing
#####################################################################################
#One-way ANOVA non-parametric
#####################################################################################

dunnTest(Ct_value~Condition,data=qPCR_GLUT1)
```

Appendix B: Statistical analysis of % live cells & N cells per condition

```
#Statistical analysis of % of live cells

#######################################################################################
#Preparatory steps: installing packages
#######################################################################################
install.packages("car", dependencies = TRUE)
install.packages('FSA')

library("car") #This package is necessary for the Levene’s test
library('FSA') #This package is necessary to perform a non-parametric one-way ANOVA

#######################################################################################
#Testing the normality of the data and the equality of variances
#Shapiro wilk test, to test if the data is normally distributed
#######################################################################################

shapiro.test(No2D$Percentage[No2D$Condition=="2D"])

shapiro.test(No2D$Percentage[No2D$Condition=="Monoculture"])

shapiro.test(No2D$Percentage[No2D$Condition=="F_prau"])

shapiro.test(No2D$Percentage[No2D$Condition=="A_muc"])

shapiro.test(No2D$Percentage[No2D$Condition=="Party"])

#######################################################################################
#Levene's test, to test if the variances are equal
#######################################################################################

leveneTest(Percentage ~ Condition, data=No2D)

#######################################################################################
#One-way ANOVA
#######################################################################################

res.aov_Percel <- aov(Percentage ~ Condition, data = No2D)

summary(res.aov_Percel)

TukeyHSD(res.aov_Percel)
#######################################################################################
#One-way ANOVA non-parametric
#######################################################################################

dunnTest(Percentage~Condition,data=No2D)
```

```
#Statistical analysis: total N of cells per condition

##########################################################################################
#Preparatory steps: installing packages
##########################################################################################
install.packages("car", dependencies = TRUE)
install.packages('FSA')

library("car") #This package is necessary for the Levene’s test
library('FSA') #This package is necessary to perform a non-parametric one-way ANOVA

##########################################################################################
#Testing the normality of the data and the equality of variances
#Shapiro wilk test, to test if the data is normally distributed
##########################################################################################

shapiro.test(Total$Tcells[Total$Condition=="2D"])

shapiro.test(Total$Tcells[Total$Condition=="Monoculture"])

shapiro.test(Total$Tcells[Total$Condition=="F_prau"])

shapiro.test(Total$Tcells[Total$Condition=="A_muc"])

shapiro.test(Total$Tcells[Total$Condition=="Party"])

##########################################################################################
#Levene's test, to test if the variances are equal
##########################################################################################

leveneTest(Tcells ~ Condition, data=Total)

##########################################################################################
#One-way ANOVA
##########################################################################################

res.aov_Totcel <- aov(Tcells ~ Condition, data = Total)

summary(res.aov_Totcel)

TukeyHSD(res.aov_Totcel)

##########################################################################################
#One-way ANOVA non-parametric
##########################################################################################

dunnTest(Tcells~Condition,data=Total)
```