Diplomarbeit

Influence of Herbal Substances on the Intestinal Epithelium and its Microbiota in an Air Liquid Interface Culture

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Graz, June 13, 2022 Senay David Zegay eh

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Zusammenfassung

Das Mikrobiom metabolisiert Substanzen, je nach individueller Zusammensetzung der Bakterien, von Mensch zu Mensch unterschiedlich. Dies kann einen bedeutenden Einfluss auf den Effekt einer Substanz haben. *Run Chang Wan* (RCW) ist eine traditionelle chinesische Kräutermischung, die häufig bei Obstipationen Anwendung findet. Die genauen Wirkmechanismen sind jedoch weitgehend unklar. Herkömmliche Zellkulturen stellen die in vivo Bedingungen nur unzureichend dar, wodurch mögliche Interaktionen zwischen einem Agens, einem Epithelgewebe und den sich darauf befindenden Mikroben nicht beobachtet werden können. Mit einer Air Liquid Interface (ALI) Kultur und beigefügten Darmbakterien, versuchten wir diese Lücke zu schließen und möglichst realistische Versuchsbedingungen zu schaffen.

Die ALI-Kultur nutzt eine Einlage mit einer Polycarbonat Membran, auf der Zellen kultiviert werden. Auf der apikalen Seite haben die Zellen Luftkontakt und auf der basalen Seite stehen sie mit einem Nährmedium in Kontakt. Diese Bedingungen führen zu einer Differenzierung der Zellen. Zur Herstellung eines Colonepithels wurden zwei Adenokarzinomlinien des Colons, CaCo2 und HT29, im Verhältnis 3 zu 1 gemischt. In der ALI Kultur bildeten die Zellen ein strukturiertes Epithel, das Schleim enthielt. Den Epithelien wurde unter anaeroben Bedingungen eine 10 %-ige Stuhlsuspension beigefügt und anschließend mit unterschiedlichen RCW-Konzentrationen inkubiert.

Dies führte zu starken Zellschädigungen, welche sowohl in der RNA-Messung als auch histologisch nachweisbar waren, wobei lediglich in Abwesenheit von RCW genug Zellen überlebten, um erfolgreich fixiert und gefärbt zu werden.

Weitere Studien werden nötig sein, um eine Methode zu finden, die eine erfolgreiche Kombination von Colon ALI Kulturen mit einem Microbiom möglich macht.

Abstract

Microbiota have been found to metabolize substances in different ways and can sometimes be the key factor of the therapeutic effectiveness of a substance. *Run Chang Wan* (RCW) is a traditional Chinese herbal medicine often used to relieve constipation, but the certain mechanisms of action are still unclear. Conventional cell cultures do not resemble the *in vivo* conditions, making it impossible to observe if there are relevant interactions between an agent, differentiated epithelial cells and microbiota. We tried to overcome this problem by combining microbiota within an Air Liquid Interface culture of colonic cells to resemble an environment as near as possible to *in vivo* conditions.

The Air Liquid Interface is a cell culturing method to support the differentiation of epithelial cells into a pseudostratified ciliated epithelium with mucus producing goblet cells. Cells are cultured in inserts on a polycarbonate membrane with pores of 0.4 µm so the cells are exposed to air on the apical side and in contact with medium from the basal side. Two colon adenocarcinoma cell lines were cultured, CaCo2 and HT29, to form a colonic epithelium. The microbiota were applied as a 10% suspension in anaerobe medium and incubated with different concentrations of RCW.

These conditions lead to cell damage as RNA measurements showed. Also, most staining procedures were not successful, probably due to a lack of vivid cells to adhere. Only the cells without RCW but with microbiota survived with visible signs of cell damage.

Further studies need to be conducted to find successful methods to combine ALI cultured colon cells with microbiota.

1 Introduction

1.1 The Colon Epithelium

The colon epithelium is responsible for the resorption of electrolytes and water. It is also responsible for the secretion of mucins. The simple columnar colon epithelium consists mainly of colonocytes, goblet cells, enteroendocrine cells (mainly EC-Cells) and a small number of Paneth-Cells. Colonocytes can not only absorb but also secret electrolytes and water (1). A characteristic feature of the colon epithelium are crypts in which cell division occurs. Colonic stem cells are located in the base of the crypt and differentiate as they migrate to the surface (2).

The goblet cells morphology is shaped by swelled theca containing mainly mucin granules (3). Studies have shown that mice deficient in MUC2, the major goblet cell mucin, have no morphologically identifiable goblet cells(3).

Goblet cells synthesize and secrete mucins to form a highly viscous mucus layer covering the epithelium. The mucus is responsible for the clearance of the epithelium and separation from bacteria to prevent inflammation and infection. The proportion of goblet cells among other epithelial cell types increases similarly as the number of microbial organisms increases from the duodenum to the distal colon (from 4 to 16%)(3). Goblet cells are smaller and fewer in germ-free mice compared to conventionally raised mice, suggesting the modulation of goblet cells by microbes (3).

The most abundant gel-forming mucin in the intestine is MUC2, a large glycoprotein with hygroscopic oligosaccharide sidechains. It consists of central tandem repeat domains (so-called mucin domains) that are rich in proline, serin and threonine (also called PTS domains). These amino acids act as attachment sides for oligosaccharide sidechains to bind O-glycosidically (3, 4) turning the mucin domains into long, stiff bottle brush-like rods. The O-glycans make up more than 80% of the mucin protein’s mass and give them their high water-binding capacity. They also make the mucin domains highly protease resistant and constitute an important energy source for commensal bacteria (4).

The mucin domains are also bordered on each side by cysteine-rich domain and domains of von Willebrand factor, which are involved in dimerization and oligomerization, giving the mucin network its highly viscous characteristics.

The mucins constitute two layers of mucus and therefore a protection barrier for the epithelium against microbes, their microbial products or ingested food which may cause physical and chemical injuries. The 20 so far discovered mucin genes and glycoproteins can be broadly classified in secretory and membrane associated mucins. Both, the inner and the outer mucus gel layers in the colon consist mainly of secretory MUC2 mucin (3) and with a lower content of MUC5AC and MUC6 (2).

The inner layer, approximately 200 μm thick, adheres to the surface of the epithelium and gets renewed within hours. The mucin net here is very densely knotted and too tight for bacteria to invade. This layer is devoid of bacteria and rich in IgA-antibodies, suggesting the protective function of the inner layer. In the outer layer the mucin net is looser and more accessible for bacteria. This is the space to live for the commensal bacteria (1).

Mice studies have shown that Muc2 deficiency leads to inflammation of the colon and add to the onset of experimental colitis. Further, the activity of mucosal inflammation correlates with a decrease in MUC synthesis and secretion in patient with ulcerative colitis. These findings suggest that mucosal barrier plays a key role in the pathogenesis of the disease. Also, Muc2 deficient mice have shown to develop adenomas in early age which progressed to invasive adenocarcinomas in the small intestine and rectum, implying that Muc2 also plays a tumor-suppressing role, whose mechanisms remain unclear (5). Various models have shown that Muc2 deficient mice did not develop severe colitis and intestinal cancer, when they were held in germ-free condition or treated with antibiotics. This supports the role of the microbiota (6).

1.2 Microbiome

The human body is home to the same number of bacterial cells as human cells (7). They reside on our skin, mouth, nose, intestinal tract and genitals and many microbes are substantial for our survival (8).The number of bacteria in the colon exceeds all other organs by two orders of magnitude and is considered to be the major contributor to the total bacterial population (7).

The human gut microbiome influences the immune system, development, and physiology of its host, as well as metabolism. Age, diet, and health status of the host strongly influence the composition of the microbial community. Recent research focuses on microbial metabolites to explain the pharmacological activity of medicinal plants. The gut microbiota can degrade big molecules with low bioavailability, like polyphenols, and make their beneficial compounds accessible to the host. Further, the microbiota metabolize substances in different ways and can sometimes be the key factor of the therapeutic effectiveness of a substance (8).

1.3 The Air Liquid Interface (ALI) Culture

Cell cultures are an important material of study and continue to be invaluable for basic research and direct application. Cell cultures using primary cells or cell lines allow research at manageable cost and can reduce ethically controversial animal testing (9). Yet, conventional cell cultures do not fully recapitulate *in vivo* tissue organization and phenotypes (10). In conventional cell cultures cells are fully covered with medium and need to adapt to oxygen supply exclusively from the apical side. This does not always resemble the natural conditions of epithelial cells (11).

Therefore, new culturing techniques have been developed. Since the year 2000 there has been a rapid increase in 3D cell culture studies, as they induce cells to behave closer to *in vivo* conditions, than conventional two-dimensional cell cultures do (9).

The ALI technique reduces the apical medium volume to a thin film and minimizes the diffusion barrier. This results in enhanced oxygen supply that likely meets the requirements of the cultured cells. To resemble the *in vivo* conditions for epithelial cells as closely as possible the ALI technique is applied to membrane cultures (11). Cells are cultivated in inserts with a porous membrane (comparable to the epithelial basement membrane) at the bottom which fit into 12-well or 24-well plates. In this way cells can be cultured with medium on both sides, apical and basal. As soon as the cells show confluency, the medium is only maintained on the basal side. This leads to cell differentiation after 1-3 weeks.

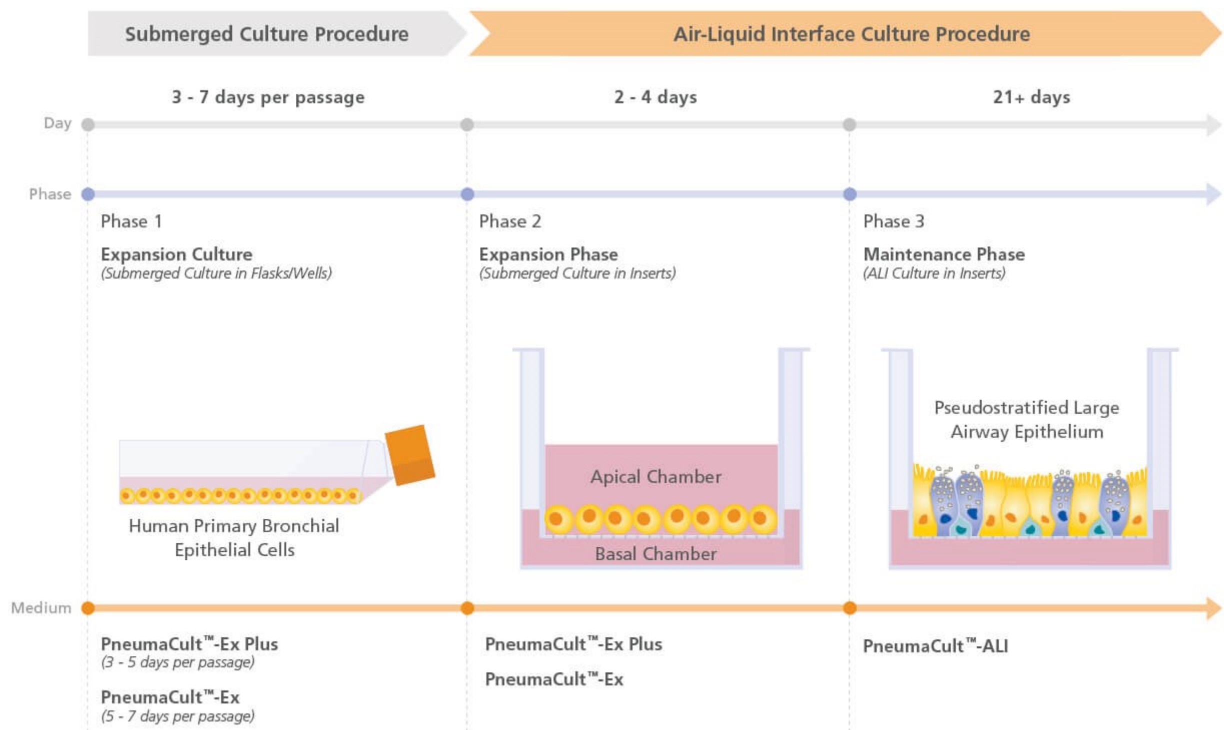

Figure 1 – Schematic overview of an ALI culture with respiratory epithelial cells: Cells are expanded in conventional cell culture. Then the cells are seeded in the inserts and cultivated with medium on both sides. When the cells reach confluency, after 3-5 days, the apical medium is removed, and the cells are maintained by the medium on the basal side. Medium change is performed every two days and after 1-3 weeks the cells differentiate. Figure by STEMCELL Technology.

1.4 ALI Culture to mimic the colon epithelium

The gastrointestinal tract is responsible for 20-25% of the human body oxygen turnover and can be even 33% when measured 60 minutes postprandial. This is due to the energy-consuming transport processes along the epithelial barrier. The reduction of hypoxia therefore may have a big impact on intestinal cells. Comparative studies with porcine intestinal cells have identified regions of hypoxia in conventionally cultured cells but none in ALI cultured cells.

Cultured in ALI cultures, intestinal cells have a sufficient supply of oxygens and should form polarized and differentiated epithelial structures under these conditions (12). Studies with two intestinal porcine epithelial cell types have shown a more specific differentiation and functional morphology of the cells when cultures in ALI compared to conventional culturing techniques (11).

In our experiment we used two cell lines. CaCo2 cells and HT29 cells, which are both cell lines derived from colorectal adenocarcinomas. CaCo2 cells form a continuous epithelium and do not produce mucus. HT29 cells produce mucus and grow in small groups. They do not form a continuous epithelium.

1.5 Traditional Chinese Medicine

Traditional Chinese Medicine (TCM) is a more than 2000 years old holistic concept of diagnosis, pathophysiology, and therapy of diseases. TCM includes herbal medication, acupuncture and other physical therapies (13). In TCM the healthy human body is seen as an entity in equilibrium. The five elements wood, fire, earth, metal, and water explain functions of the body and how it changes during disease.

Qi is described as a vital energy that flows via meridians through the body to maintain function and health of organs. It is not related to the concept of energy used in science.

Yin and *Yang* are also ancient abstract concepts and represent two complementary aspects that everything can be divided into, TCM considers a balance of *Yin* and *Yang* to contribute to a healthy body. The goal of treatments is to restore the energy (*Qi*) and balance (*Yin* and *Yang*) of these systems (14).

A particularly interesting aspect of TCM is the herbal medicine which is the most comparable to modern pharmaceuticals (15). As many modern drugs are derivatives of natural products, also Chinese herbals may deliver some new promising treatments. The discovery of the substance Artemisinin, a potent anti-malaria drug derived from *Artemisia annua*, has been a clear example of the potential held by herbal medicine (13).

Herbal formulations can be produced in various ways, one of them is called decoction. Decoction is an extraction method for herbs and plants to dissolve the chemicals of the plants. The materials are mashed to allow maximum dissolution of the ingredient and then boiled in water, usually twice to intensify the extraction (16).

This study investigates the effect of the formula RCW.

It contains five components which are listed in Table 1.1 with they role in TCM. RCW is considered to moisten the intestines and to clear heat. It’s created by decoction.

Table 1.1 - Components of RCW: Overview of the different components of RCW and their role in TCM (17).

Component	Function in TCM
Semen Cannabis Sativae (Huo Ma Ren)	moistens the intestines, strengthens the *Yin*, clears heat
Semen Pruni Persicae (Tao Ren)	activates blood circulation, breaks blood stasis, moistens intestines
Radix Angelicae Sinensis (Dang Gui)	tonifies blood, moistening the intestines
Radix Rehmanniae Glutinosae (Sheng Di Huang)	clears heat, increases fluids, nourishes the *Yin*
Fructus Citri Aurantii (Zhi Ke)	breaks stagnant *Qi*, relieves distension and accumulation

1.6 Aim of the Study

This study investigated the development of the ALI culture method for cells of the colon epithelium. Two different colon cancer cell lines were used in a mixed culture. We investigated the influence of the RCW formula on the cells in the ALI cultures of the colon epithelium and the influence on the composition of microbes.

2 Materials

Table 2.1 Materials for Histological Techniques:

Ethanol, 100%	MERCK, Vienna, Austria
Acetic acid, 100%	MERCK, Vienna, Austria
Excelsior Tissue Processor	ThermoFisher Scientific; Massachusetts, USA
Rotation Microtome HM355S	ThermoFisher Scientific; Massachusetts, USA
Superfrost Plus Microscope Slides	ThermoFisher Scientific; Massachusetts, USA
Cover glasses, 24x24mm	ThermoFisher Scientific; Massachusetts, USA
Tissue Clear	Histolab
Hematoxylin, Mayer's hemalum solution	MERCK, Vienna, Austria
Ammoniac, 25%	MERCK, Vienna, Austria
Eosin, 1%	MERCK, Vienna, Austria
Alcian Blue	Sigma-Aldrich; Vienna, Austria
Nuclear fast red	MERCK, Vienna, Austria

Table 2.2 List of Materials for Cell Culture:

CaCo2 cells	Department of Medicine III, Division of Gastroenterology and Hepatology, Graz, Austria
HT29 cells	Amin El-Heliebi, Department of Cell Biology, Histology and Embryology, Graz, Austria
PBS, pH 7.4	Gibco, ThermoFisher Scientific; Massachusetts, USA
Bovine Fibronectine, diluted in PBS, final concentration 2 µg/ml	Sigma; Missouri, USA
MEM, Minimum Essential Medium	Gibco, ThermoFisher Scientific; Massachusetts, USA
HyClone™ Fetal Bovine Serum (FBS)	ThermoFisher Scientific; Massachusetts, USA
Penicillin/Streptomycin	Gibco, ThermoFisher Scientific; Massachusetts, USA
MEM NEAA (100X), Minimum Essential Medium Non-Essential Amino Acids	Gibco, ThermoFisher Scientific; Massachusetts, USA
Sodium Pyruvate, 100 mM	Gibco, ThermoFisher Scientific; Massachusetts, USA
Trypsin-EDTA 10X	Biowest
Trypsin-EDTA 1x, diluted in PBS	Biowest
CASY Model TT	Schärfe System, Reutlingen, Germany
Run Chang Wan	Apotheke Boznerplatz, Innsbruck, Austria
12 well plate, 24 well plate, 96 well plate	Costar, Corning Incorpo-rated

Table 2.3 Other Materials:

EZ4U Nonradioacitve cell proliferation and cytotoxicity assay, BI-5000	Biomedica; Vienna, Austria
peqGOLD Total RNA Kit	Peqlab, Erlangen, Germany
GOLD cDNA Kit	Peclab, Erlangen, Germany
NanoDrop™ 1000 Spectrophotometer	ThermoFisher Scientific; Massachusetts, USA
cDNA Cycler, DNA Engine Dyad, Peltier Thermal Cycler	Bio-rad, Vienna, Austria (Hercules, USA)
iTaq Universal SYBR Green Supermix	Bio-rad, Vienna, Austria (Hercules, USA)
Primer: MUC2, MUC5AC, MUC5B	Microsynth; Vienna, Austria
qPCR cycler, CFX96 Real-Time System, C1000 Thermal Cycler	Bio-rad, Vienna, Austria (Hercules, USA)

3 Methods

3.1 Cells and Cell Culture

CaCo2 and HT29 were cultivated in growth medium composed of MEM with 10% FBS, 1% P/S, 1% NEEA and 1% sodium pyruvate. The cells were stored in an incubator with 37°C, 5% CO_2. The media were changed twice a week and cells were split as they reached 90% confluence.

10X Trypsin was used for splitting the CaCo2 cells, for the HT29 cells 1X Trypsin was used. To split the cells the medium was aspirated from the cell culture flask. Then the cells were washed with 10ml PBS which was aspirated again. Trypsin was added to the washed cells, and they were incubated for 5 minutes at 37°C. Then the cells were harvested with 8ml medium and centrifuged for 5 minutes at 1200U/min. After the medium was aspirated, the cells were resuspended in fresh growth medium and then redistributed in 75cm² flasks for expansion. The flasks were filled up to 12ml with growth medium. The splitting rate was 1:2 for both cell lines.

Former studies of the Department of Cell Biology, Histology and Embryology, Medical University Graz have shown that CaCo2 cells and HT29 cells cultured in mixed cultures had the best results when seeded in a ratio 3:1 with 3 parts of CaCo2 and 1 part HT29 cells (18).

To transfer the cells to the Transwell ALI culture medium was aspirated and the culture flask washed with 10ml of PBS. Then 2ml of trypsin were applied (10X Trypsin for CaCo2 cells, 1X Trypsin for the HT29 cells). The whole content of the flasks was transferred into tubes and 50µl of the CaCo2 and HT29 tubes were used to measure the cell concentrations with a CASY cell counting device and to calculate the necessary volume of both cell suspensions to get a 3:1 ratio.

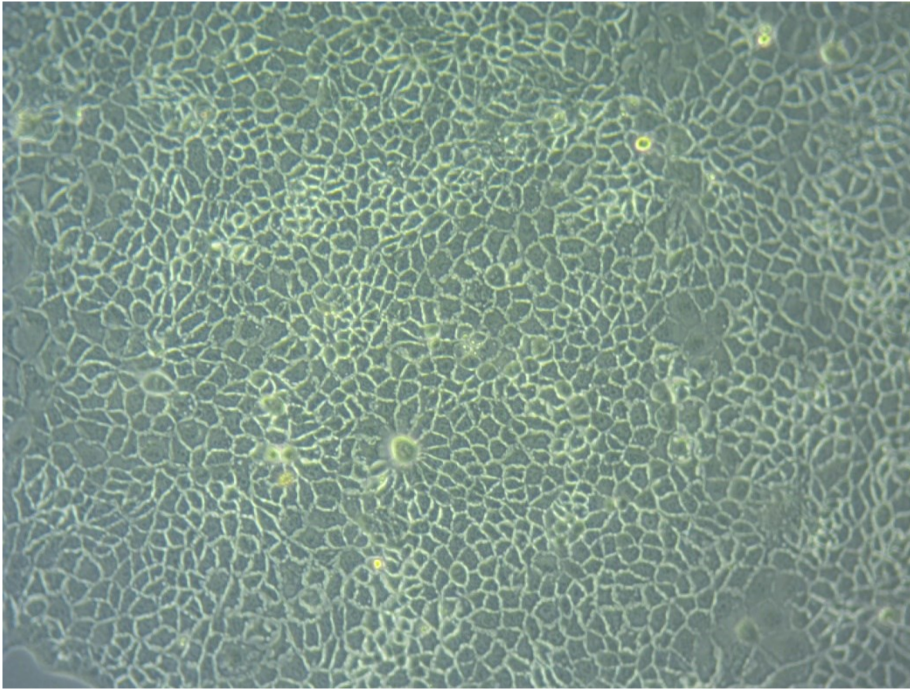

Figure 3.1 - CaCo2 in wet culture cultivated in growth medium composed of MEM with 10% FBS, 1% P/S, 1% NEEA and 1% sodium pyruvate. The cells were stored in an incubator with 37°C, 5% CO_2. The media were changed twice a week and cells were split as they reached 90% confluence.

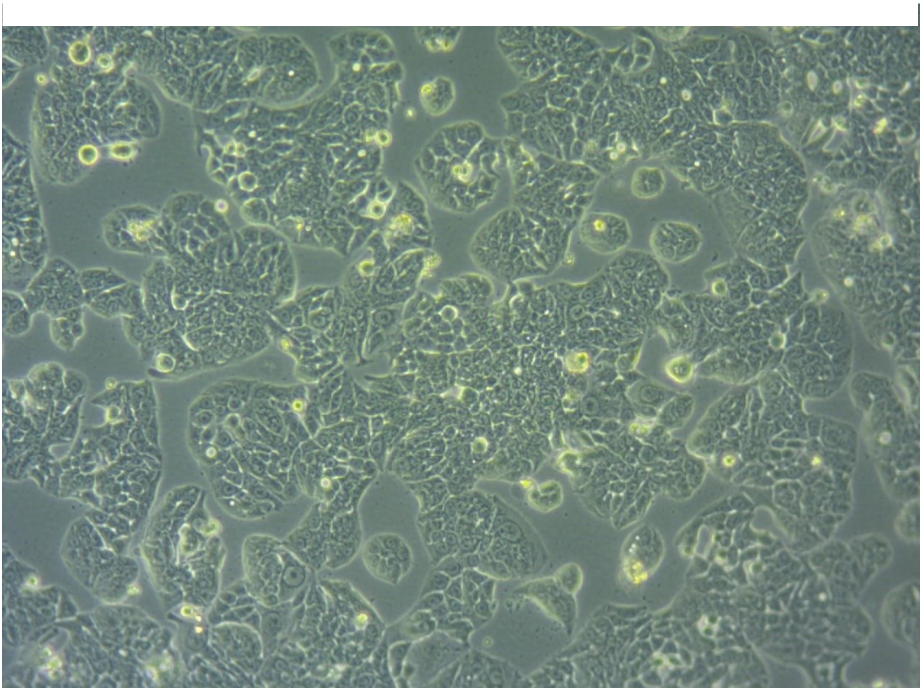

Figure 3.2 - HT29 in wet culture cultivated in growth medium composed of MEM with 10% FBS, 1% P/S, 1% NEEA and 1% sodium pyruvate. The cells were stored in an incubator with 37°C, 5% CO_2. The media were changed twice a week and cells were split as they reached 90% confluence.

3.2 ALI Culture

For the ALI cultures plates and Transwell inserts were used.

The inserts had polycarbonate membranes with a pore size of 0.4 µm and insert diameter of 12mm. These inserts were brought in 12-well plates. The inserts were coated with Fibronectin and incubated for 1 hour.

The cells were seeded into the apical chamber of the inserts as a suspension with 500ul medium for each insert containing $3x10^5$ cells. The basal chamber was filled with 1ml medium, and the cells were incubated for 3 days. After four days the medium was aspirated from the apical chambers of the inserts and the inserts were rinsed with PBS and exposed to air. The basal chamber medium was replaced with new medium. Medium change in the basal chamber was performed every second day.

3.3 Microbiome Preparation

A freshly passed feces sample was kindly provided by the Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine. The feces were donated by a healthy volunteer (female, age 41, non-smoker, normal mixed diet, no antibiotics for more than 2 years; intake of spicy food was avoided the last days before donation). After donation the sample was immediately transferred in a bottle gassed with N2 for 30 minutes to obtain anaerobe conditions. A 10% stool suspension was made with stool and anaerobe medium. Before that the medium had been gassed for 20 minutes with N2.

3.4 Treatment of Colon Epithelial Cells in ALI culture with RCW and Microbiome

Following experiments were all performed inside of an anaerobic workstation.

A) Interaction of the Microbiome and TCM

To assess interactions between the microbiome (MB) and RCW 10 ml of stool suspension were incubated with 30µl (0.3%) and 60µl (0.6%) RCW and as a control sample without RCW. MB and RCW were incubated for 24h and 72h in 15ml falcon tubes. All experiments were performed three times.

Samples of 1ml were taken after incubation time and control. All samples were frozen as soon as possible at -80°C for later DNA analysis.

Table 3.1 – Scheme of performed incubation

Incubation time	0 h	24 h	72 h	24 h	72 h	24 h	72 h
#1	MB	MB	MB	0.3% RCW+MB	0.3% RCW+MB	0.6% RCW+MB	0.6% RCW+MB
#2	MB	MB	MB	0.3% RCW+MB	0.3% RCW+MB	0.6% RCW+MB	0.6% RCW+MB
#3	MB	MB	MB	0.3% RCW+MB	0.3% RCW+MB	0.6% RCW+MB	0.6% RCW+MB

B) Interaction of MB/TCM and ALI culture of CaCo2 and HT29

To assess the interactions between CaCo2 and HT29 cells with MB and RCW, the 21 ALI culture membranes were cut out of the Transwell inserts and mixed with 5ml of 10% stool suspension in 15ml falcon tubes. Cells and MB were incubated with 15µl (0.3%), 30µl (0.6%) RCW and without RCW as a control for 24h and 72h.

After incubation the membranes were taken out of the falcon tubes, washed in PBS, and cut in two parts.

One half of each membrane was fixated in Carnoy's solution for 20 minutes at room temperature. Then the membranes were put in cassettes and embedded in paraffin for histological analysis.

The other half of the membrane was put in lysis buffer for later RNA analysis. From each falcon tube 1ml of fluid was taken for DNA analysis of the microbiome. The RNA samples and DNA samples were stored at -80°C.

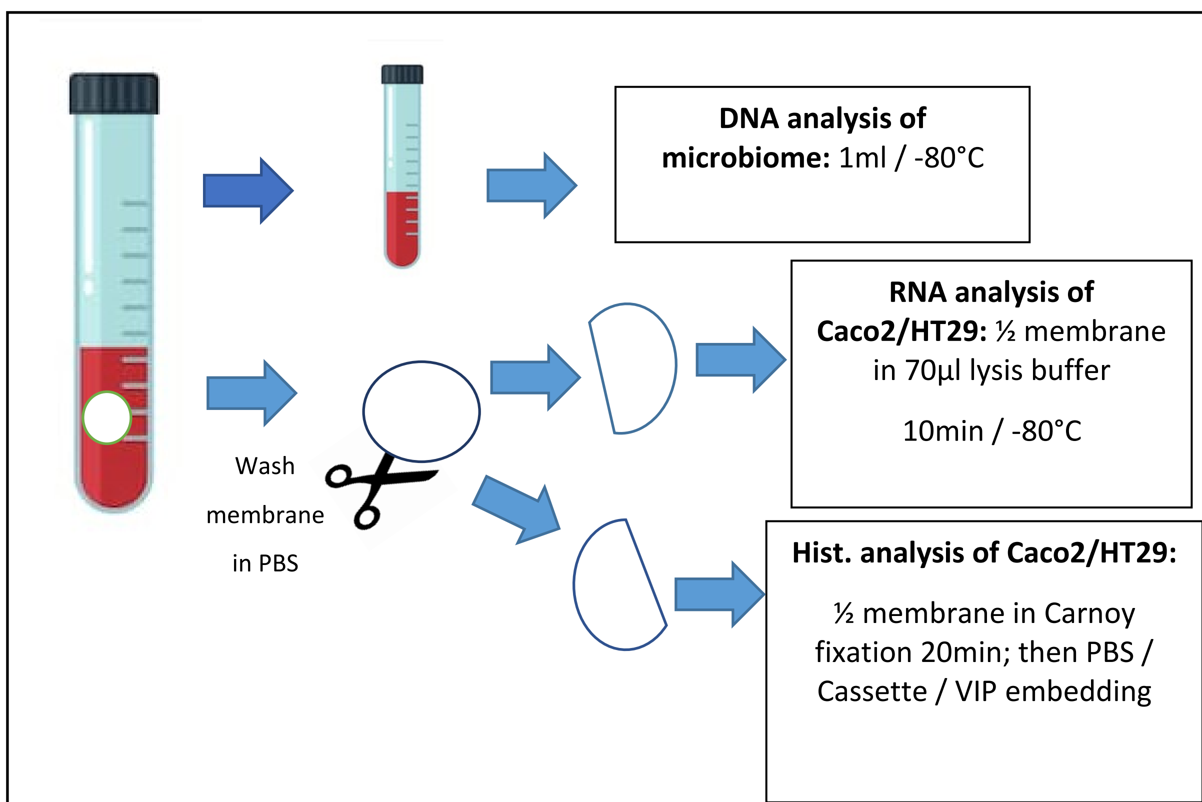

Figure 3.3: Scheme of Sampling: *After incubation, each membrane is washed in PBS and then cut in two parts. One part is put into lysis buffer for 10 minutes and then cooled down to -80°C for later RNA analysis. The other part of the membrane is fixated in Carnoy's solution for 20 minutes at room temperature for paraffin embedding and histological analysis. 1 ml of fluid is taken out of each tube for DNA analysis of the microbiome.*

3.5 Histological Techniques

3.5.1 Fixation and Paraffin Embedding

The Transwell membranes were fixed in Carnoy's solution for 20 minutes. Afterwards the membranes were put in cassettes and washed with PBS. The cassettes were put in a tissue processor (Excelsior™), dehydrated, and embedded in melted paraffin wax overnight. Then the paraffinated membranes were placed vertically in a mould form and hot paraffin was poured in to form a paraffin block and put on a cooling plate to harden.

The paraffin blocks were cut in tissue specimens of 5 µm with a rotation microtome. The specimens were fixed on object slides and dried at 53°C.

Then the tissue specimens were deparaffinated by dipping the slides in four consecutive tissue clear solutions (Histolab Clear) for 5 minutes per tub. Next, the slides were placed shortly (maximum 1 minute) in 4 descending ethanol gradient tubs (100%, 96%, 70%, 50%) and Aqua dest for rehydration. Table 3.2 shows an overview of the procedure of deparaffination and dehydration.

Table 3.2 Deparaffination and Dehydration

Tissue Clear 1a, 1b	5 minutes each
Tissue Clear 2a, 2b	5 minutes each
Tissue Clear /100% Alkohol Mischung 1:1	rinse
100% Ethanol.	rinse
96% Ethanol	rinse
70% Ethanol	rinse
50% Ethanol	rinse
A. dest.	Change multiple times

3.5.2 Hematoxylin and eosin staining (HE)

The deparaffinated and dehydrated slides were stained with HE. The procedure is described in Table 3.3. Then the slides were dried and mounted with mounting media.

Table 3.3 HE staining procedure

Hematoxylin	10 min
Aqua dest.	rinse and change aqua 2-3 times
NH_3-water (2.5ml ammonia/ 1L aqua dest.)	a few seconds until blue
1% Eosin	5-60 seconds
96% ethanol	rinsed in 2 charges
100% ethanol	shortly
tissue clear/100% ethanol (1:1)	shortly
tissue clear	10 minutes
Cover permanently	dry horizontally

3.5.3 Alcian blue staining

Alcian blue is used to stain acidic glycosaminoglycans. The staining procedure is described in the table below.

After staining the slides, they were dehydrated in ascending ethanol gradient and placed in tissue clear (HistoLab) for 10 minutes. Then the slides were dried and mounted in mounting media.

Table 3.4 Alcian blue staining procedure

3% acetic acid	3 min
1% Alcian blue in 3% acetic acid	30 min
3% acetic acid	rinsed
Aqua dest.	rinsed
0.1% nuclear fast red in 5% aqueous aluminium sulfate	5 min
Aqua dest.	rinsed

3.5.4 EZ4U-Test

To determine the influence of RCW on the colon epithelial cells a EZ4U Nonradioactive cell proliferation and cytotoxicity assay was performed.

The CaCo2/HT29 cells were seeded into a 96-well plate with $5x10^4$ cells in 100 µl medium. After 24 hours medium was exchanged with growth medium spiked with RCW in different concentrations: 0%, 0.3%, 0.6%, 1.25%, 2.5% and 5%.

The spiked medium was exchanged with 200 µl fresh medium after different incubation times: 1 day, 3 days, 5 days, and 7 days.

The substrate solution was prepared according to the instructions of the kit and 20 µl were added to each well. Then cells were incubated for 2 h at 37°C. After incubation the plate was analyzed in the spark plate reader according to the manual.

3.5.5 RNA-Isolation and cDNA synthesis

The peqGOLD Total RNA Kit by peqlab was used for RNA isolation. The cells had been lysed in lysis buffer before they were washed and centrifuged multiple times according to the protocol of the used Kit. After isolation the RNA was eluted in 30 µl or 50 µl sigma water and were kept on ice. Then Nanodrop was used to measure the RNA concentrations. RNA was then rewritten to cDNA with reverse Transcriptase as cDNA is more stable than RNA. For cDNA synthesis the peqlab GOLD cDNA kit was used. The calculated volume for 1 µg RNA diluted in 10 µl sigma water. Then per sample 1 µl of oligo and 1 µl of random primers were added to the mix. Samples were by briefly centrifuged the primers were annealed to the RNA at 65°C for 5 minutes in a thermocycler. Then 4 µl buffer solution, 1 µl RNAse-Inhibitor, 2 µl dNTP mix and 1 µl reverse transcriptase were added to the samples to a total volume of 20 µl per sample.

All samples were loaded in the thermocycler and incubated for 70 minutes at 65°C for cDNA synthesis. Then the cDNA samples were stored at -20°C.

3.5.6 qPCR

The qPCR was performed with iTaq Universal SYBR Green Supermix (BIORAD) on a qPCR 96 well (CFX96 BIORAD).

For each well 5µl SYBrGreen, 2 µl cDNA template (1 µg cDNA), 2 µl Primer (800nM) and 1 µl sigma water were mixed to 10µl. First the SYBR Green Supermix was mixed with the primers to a master mix and distributed in all wells equally, then the cDNA samples were added. The 96 well plate was sealed and centrifuged for 1 minute at 300g.

The plate was then loaded into the qPCR cycler. Primers for MUC2, MUC5AC and MUC5B and beta-actin as a control were used. Two different beta actin primers were used with melting points at 58°C and another primer with a melting point at 60°C together with MUC 5AC, MUC 2 primers.

Each protocol ran for 40 cycles. MUC2, MUC5AC and MUC5B primers had different melting points. qPCRs were performed as shown in Figure 3.2 and 3.3.

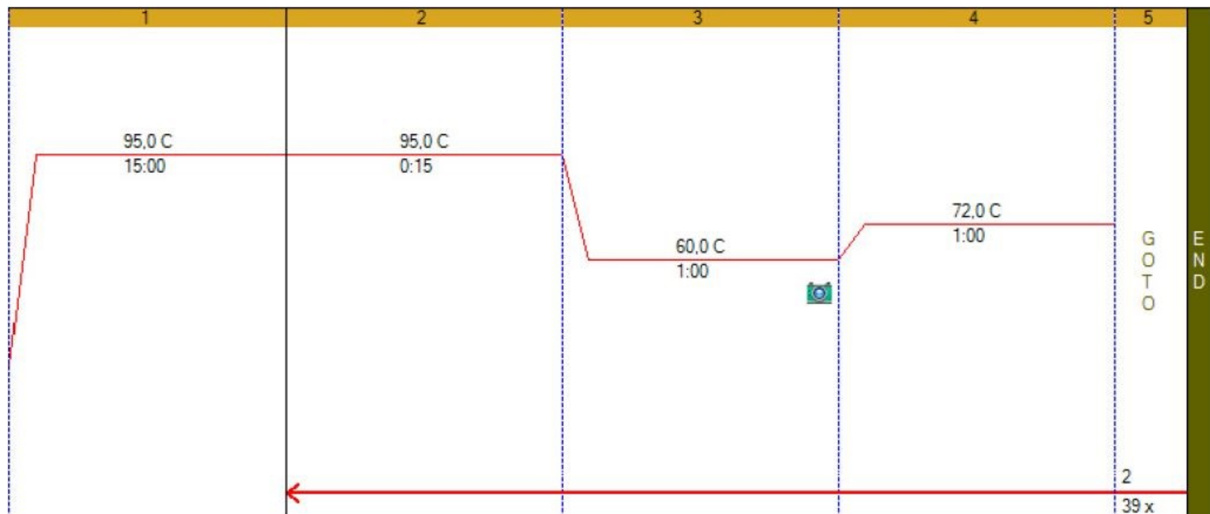

Figure 3.4 – qPCR protocol for MUC2 and MUC5AC primers: *The protocol consists of denaturation for 15 minutes at 95°C and 40 amplification cycles of 15 seconds at 95°C, 1 minute at 60°C, and 1 minute at 72°C.*

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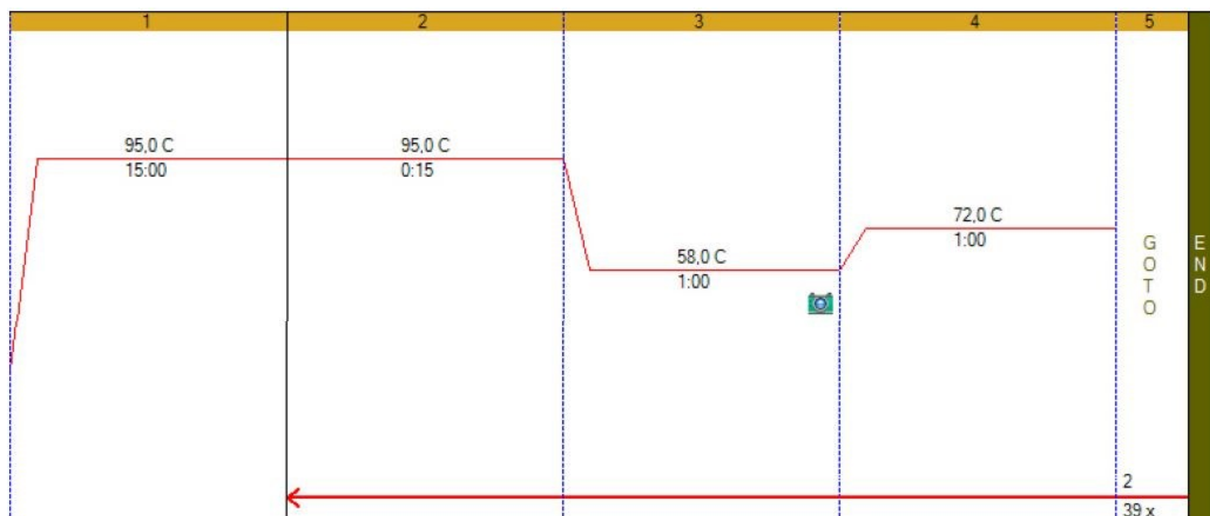


***Figure 3.5 – qPCR protocol for MUC5B* primers**: *The protocol consists of denaturation for 15 minutes at 95°C and 40 amplification cycles of 15 seconds at 95°C, 1 minute at 58°C, and 1 minute at 72°C.*

3.6. DNA Extraction and Sequencing

DNA from all sample types (untreated, 0.3% RCW, 0.6% RCW) was extracted using the EZNA stool DNA kit (Omega bio-tek), following the extraction protocol as given by the manufacturer. DNA concentration was determined via Qubit and a standardized amount was subjected to PCR. The 16S rRNA gene amplicons for the universal approach were amplified using Illumina-tagged primers F515 (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-

GTGYCAGCMGCCGCGGTAA-3′)
and R806 (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-GGACTACNVGGGTWTCTAAT-3′).

The cycling conditions were initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturing at 94°C for 45 s, annealing at 60°C for 60 s and elongation at 72°C for 90 s and a final elongation step at 72°C for 10 min. Library preparation and the sequencing were carried out at the Core Facility Molecular Biology at the Center for Medical Research at the Medical University of Graz, Austria. DNA concentrations were normalized using a SequalPrep™ normalization plate (Invitrogen), and each sample was indexed with a unique barcode sequence (8 cycles index PCR). After pooling of the indexed samples, a gel cut was carried out to purify the products of the index PCR. Sequencing was done using the Illumina MiSeq device and MS-102-3003 MiSeq® Reagent Kit v3-600cycles (2 × 251 cycles).

3.7 Data Analysis

To analyze the microbiome composition, the obtained raw reads were processed using QIMEE 2 (2021.2) with the friendly help of Univ.-Prof. Dr. Christine Moissl-Eichinger.

Data analysis was performed with Microbiome Explorer an R package for the analysis and visualization of microbial communities. During the filtering process, samples with less than 10.000 reads were removed. All taxa were included, and the samples were normalized to proportion on genus level. Shannon indices, a way to measure the diversity of species in a sample, were calculated to evaluate the alpha diversity.

Beta diversity was measured using Bray-Curtis dissimilarity distance and visualized using principal coordinates analysis for dimension reduction.

Differential abundance analysis was performed to detect changes in abundance between two incubation timepoints using the DESeq2 model.

Longitudinal analysis was performed for the genus of Alistipes to visualize abundance changes across multiple incubation timepoints.

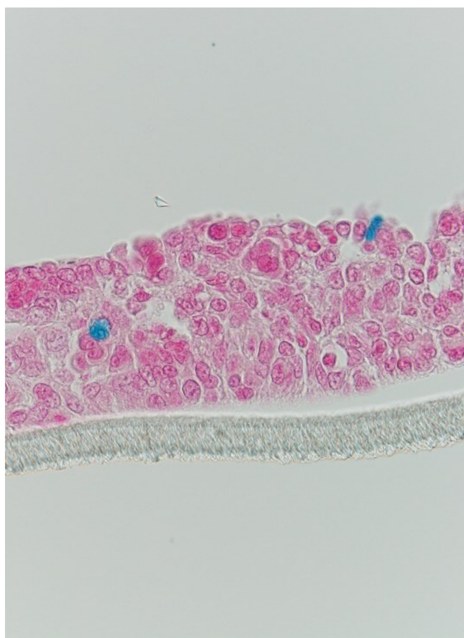
To validate the significance of the beta-diversity plots made with microbiome explorer, “Calypso” was used, a web-server application for visualizing microbiome-environment interactions.

4 Results

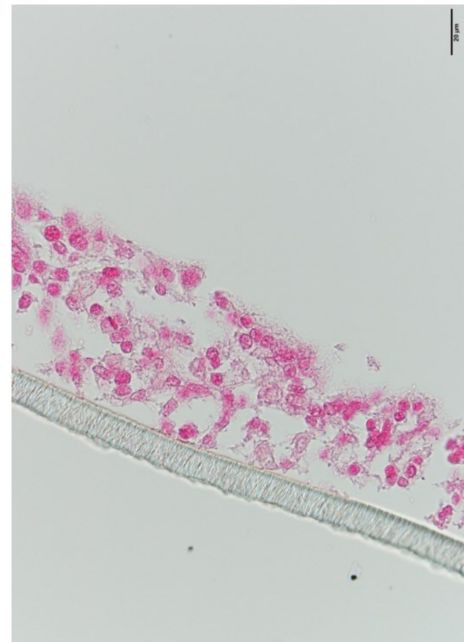
4.1 Influence of RCW and microbiome on 3D ALI cultures of the colon epithelium

Unfortunately, only 2 of the 21 samples were successfully stained. All other samples showed no cell layer after the staining procedures.

One sample with only CaCo2/HT29 cells, no stool and no RCW was stained. And one sample with cells and stool that had been incubated for 24 hours was stained.

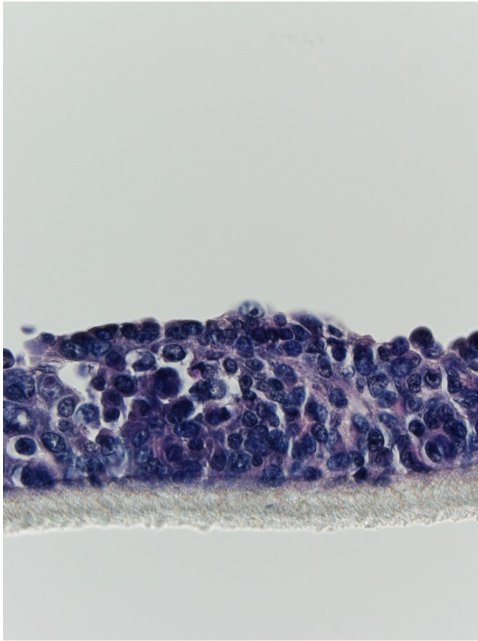


CaCo2 and HT29

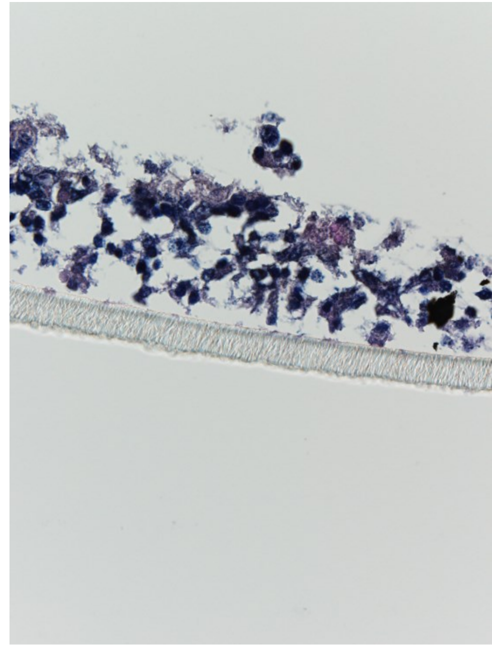


CaCo2 and HT29 + *Microbiome*

Figure 4.1 – CaCo2 and HT29 cells (ratio 3 to1) ALI culture stained in Alcian blue. *Cells were seeded on membrane inserts in growth medium. Medium was removed from the apical side after 4 days. Medium was changed every 2-3 days. After 3 weeks the membranes were cut out and incubated for 24 hours with and without microbiome. Membranes were fixed and embedded. The cells were stained in Alcian blue stain. The original magnification was 40x.*



CaCo2 and HT29



CaCo2 and HT29 +Microbiome

Figure 4.2 CaCo2 and HT29 cells (ratio 3 to1) ALI culture stained in HE. *Cells were seeded on membrane inserts in growth medium. Medium was removed from the apical side after 4 days. Medium was changed every 2-3 days. After 3 weeks the membranes were cut out and incubated for 24 hours with and without microbiome. Membranes were fixed and embedded. The cells were stained in HE. The original magnification was 40x.*

4.2 The Concentration-dependent effect of RCW on the viability of CaCo2 and HT29

To test cell proliferation and viability of CaCo2 and HT29 cells treated with RCW in different concentrations, a EZ4U viability assay was done. After 24h we only observed a slight decrease in the viability with the increasing gradient of RCW.

All assays were performed three times with different passages and per assay two determinations were done. Furthermore, viability assays had been performed for 3 days, 5 days and 7 days with concentrations of RCW of 0.15%, 0.3% and 0.6 %.

The viability assay after 3 days of incubation with RCW did not show a significant decrease in viability as shown in Figure 4.4.

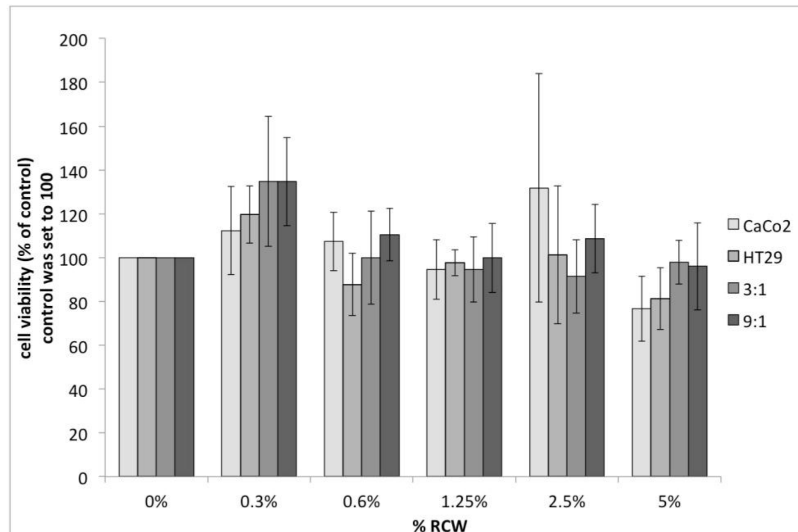


Figure 4.3 Cell viability of colon epithelial cells treated with different concentrations of RCW for 24 hours: *The cells were seeded in a 96-well plate in single and mixed cultures, incubated for 24 hours with growth medium and then incubated in growth medium spiked with different concentrations of RCW. After 24 h incubation with RCW spiked medium the EZ4U viability assay was performed according to the manufacturer's instructions. Values are expressed as the mean ± SEM.*

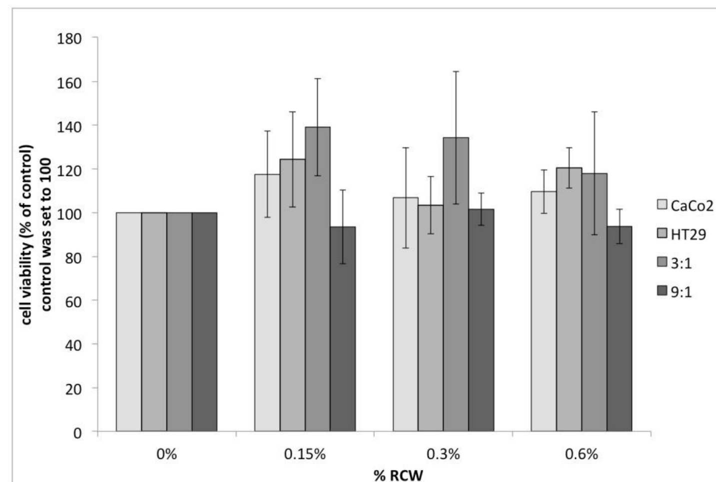


Figure 4.4 Cell viability of colon epithelial cells treated with different concentrations of RCW for 3 days: *The cells were seeded in a 96-well plate in single and mixed cultures, incubated for 3 days with growth medium and then incubated in growth medium spiked with different concentrations of RCW. After 3 days incubation with RCW spiked medium the EZ4U viability assay was performed according to the manufacturer's instructions. Values are expressed as the mean ± SEM.*

Assays performed with the cells that had been incubated with RCW for 5 and 7 days showed an overall viability of about 80% and no significant decrease of cell viability with increasing treatment concentrations. Therefore, we concluded that RCW had no effect on the viability of the cells.

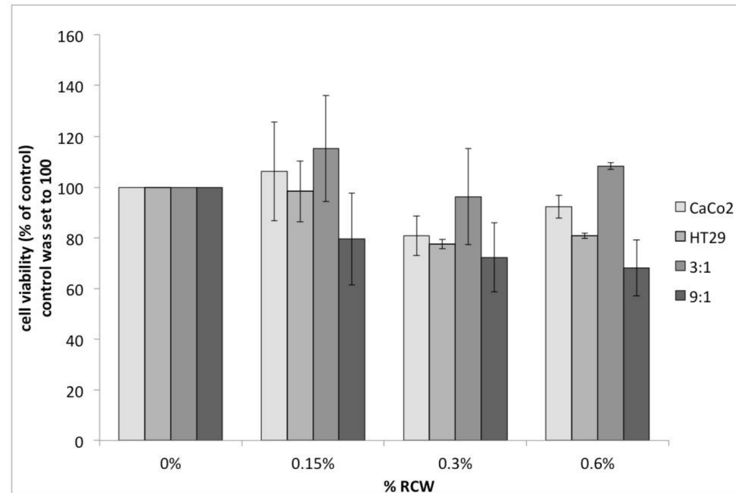


Figure 4.5 Cell viability of colon epithelial cells treated with different concentrations of RCW for 5 days: *The cells were seeded in a 96-well plate in single and mixed cultures, incubated for 5 days with growth medium and then incubated in growth medium spiked with different concentrations of RCW. After 5 days incubation with RCW spiked medium the EZ4U viability assay was performed according to the manufacturer's instructions. Values are expressed as the mean ± SEM.*

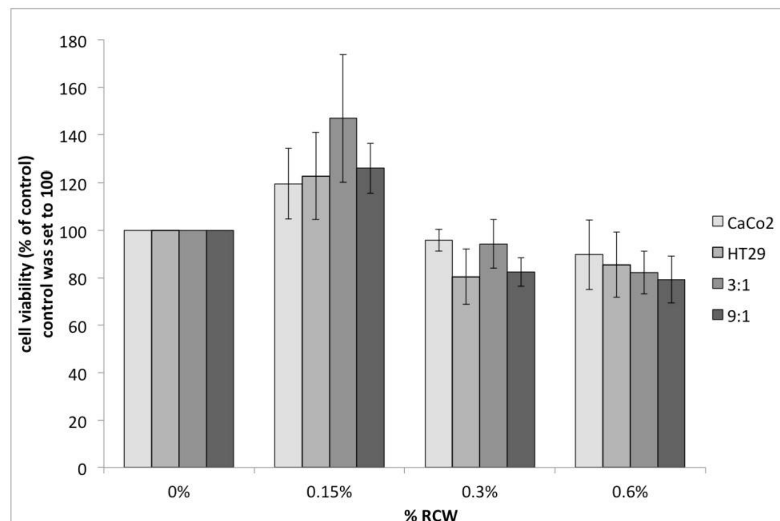


Figure 4.6 Cell viability of colon epithelial cells treated with different concentrations of RCW for 7 days: *The cells were seeded in a 96-well plate in single and mixed cultures, incubated for 7 days with growth medium and then incubated in growth medium spiked with different concentrations of RCW. After 7 days incubation with RCW spiked medium the EZ4U viability assay was performed according to the manufacturer's instructions. Values are expressed as the mean ± SEM.*

4.3 RNA Analysis

After incubation of the cells with microbes and RCW for 24 hours or 3 days, membranes were cut in half and one part of each membrane put in lysis buffer for RNA analysis.

The mean values of the NanoDrop measurements are shown in Table 4.1 and 4.2

Table 4.1: Nano Drop RNA mean values after 24h incubation:

CaCo2/HT29	315.1 ng/ µl
CaCo2/HT29+Microbiome	169.6 ng/ µl
CaCo2/HT29+Microbiome + 0.3% RCW	33.1 ng/ µl
CaCo2/HT29+Microbiome + 0.6% RCW	11.2 ng/ µl

Table 4.2: Nano Drop RNA mean values after 72h incubation:

CaCo2/HT29+Microbiome	5.4 ng/ µl
CaCo2/HT29+Microbiome + 0.3% RCW	7.4 ng/ µl
CaCo2/HT29+Microbiome + 0.6% RCW	7.5 ng/ µl

4.4 Influence of RCW on MUC mRNA expression

After cDNA synthesis, qPCRs were run for mucin primers MUC2, MUC5AC and MUC5B. The qPCR results were normalized to the housekeeping gene beta-actin and the ratio compared to the untreated cells without microbes. The results are shown in Figures 4.7, 4.8 and 4.9.

(a) MUC2

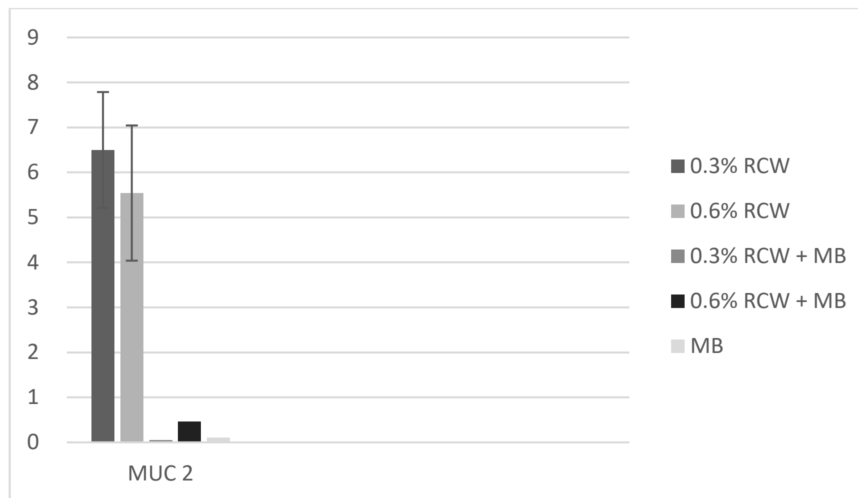


Figure 4.7 – Data of the qPCR: *The qPCR was performed with 3 different primers: MUC2, MUC5AC and MUC5B. The results were normalized to beta-actin and the ratio compared to an untreated control was calculated. Values are expressed as the mean ± SEM.*

(b) MUC 5B

Figure 4.8 – Data of the qPCR: *The qPCR was performed with 3 different primers: MUC2, MUC5AC and MUC5B. The results were normalized to beta-actin and the ratio compared to an untreated control was calculated. Values are expressed as the mean ± SEM*

(a) MUC 5AC

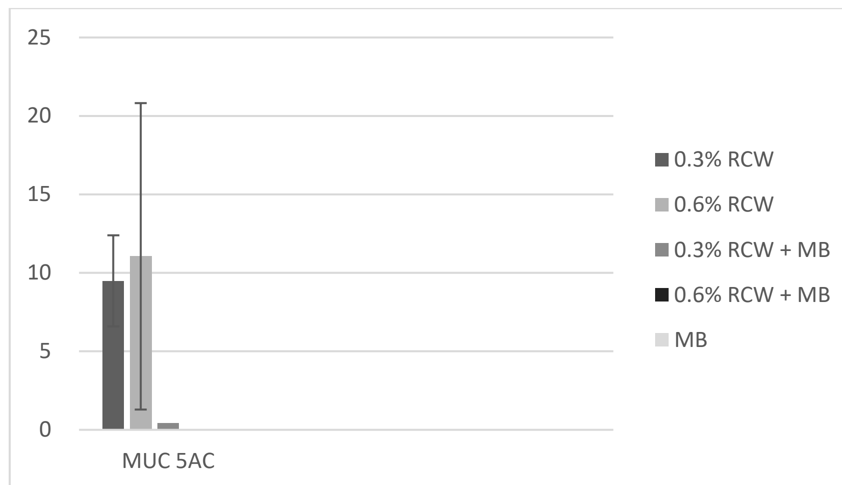


Figure 4.9 – Data of the qPCR*: The qPCR was performed with 3 different primers: MUC2, MUC5AC and MUC5B. The results were normalized to beta-actin and the ratio compared to an untreated control was calculated. Values are expressed as the mean ± SEM*

4.5 Influence of RCW on microbial composition

Using the microbiome explorer package for "R Studio" alpha diversity values of each sample were calculated and visualized. In Figure 4.10. a decrease of alpha diversity over incubation time is shown without any correlation with RCW concentration levels. The number of genera observed in the samples increased after 24h and decreased after 72h to the level of timepoint 0h (Figure 4.11).

In Figure 4.12 beta diversity, the amount of diversity between the samples is presented and show a high correlation between incubation time and similarity of the samples. An apparent shift of the microbial composition on the genus level was seen over the course of time with a rise of Bacteroides, CAG-873 and Alistipes and a decrease in abundance of Christensenellaceae_R-7-Group (Figure 4.13).

Figure 4.9.5. shows the abundance change of Alistipes bacteria. Differential analysis had shown, from the ten most abundant genus, Alistipes was the only genus to increase over time on a significant level (adjusted $p<0.05$). Figure 4.14 shows abundance of Alistipes over time.

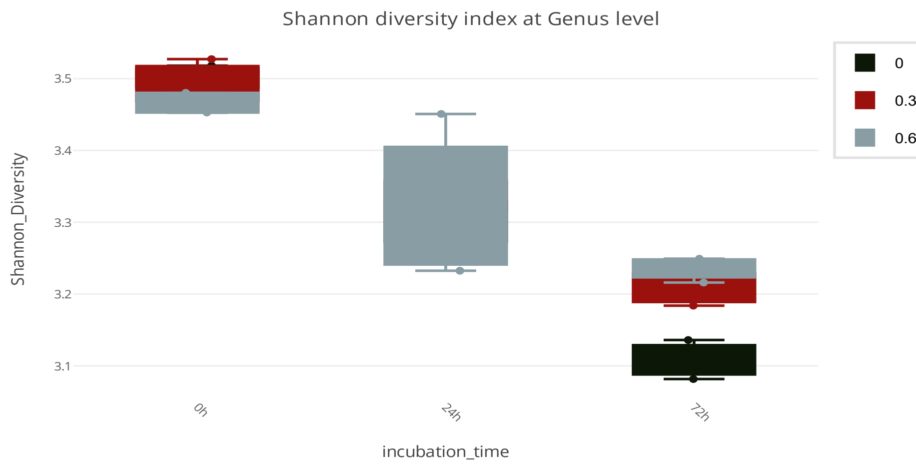


***Figure 4.10 – Alpha Diversity of all samples**. Shannon indices were calculated to evaluate the alpha diversity. Boxplots were colored in black for no treatment, red for 0.3% RCW treatment and grey for 0.6% treatment.*

Figure 4.11 – Richness diversity Index of all samples.

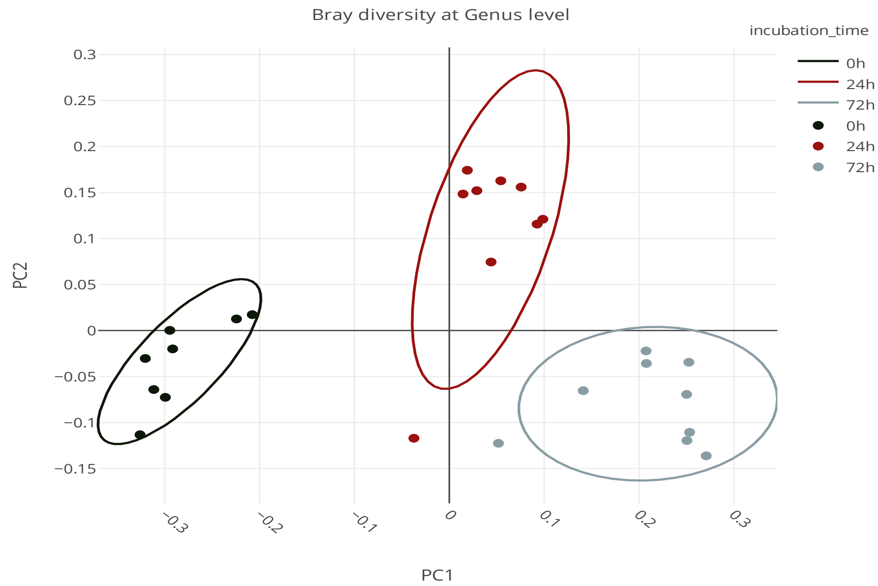


Figure 4.12 – Beta Diversity measured using Bray-Curtis dissimilarity distance. *Timepoint 0h, 24h and 72h were colored. Beta-Diversity was visualized using principal coordinates analysis for dimension reduction.*

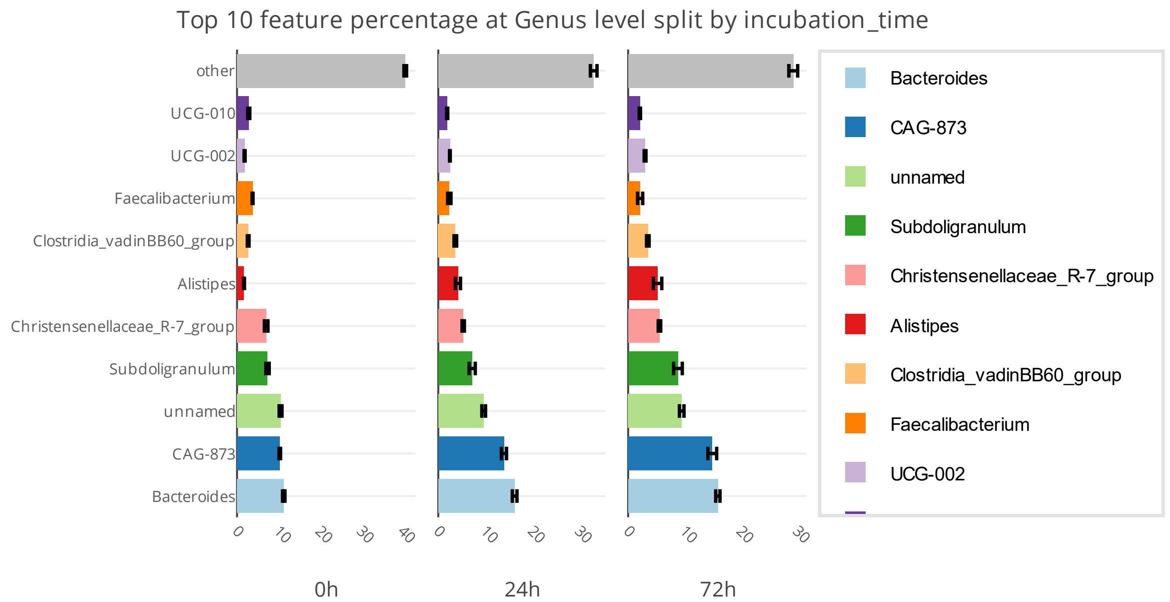


Figure 4.13 – Average relative abundance of top 10 genera across timepoints 0h, 24h, 72h*.*

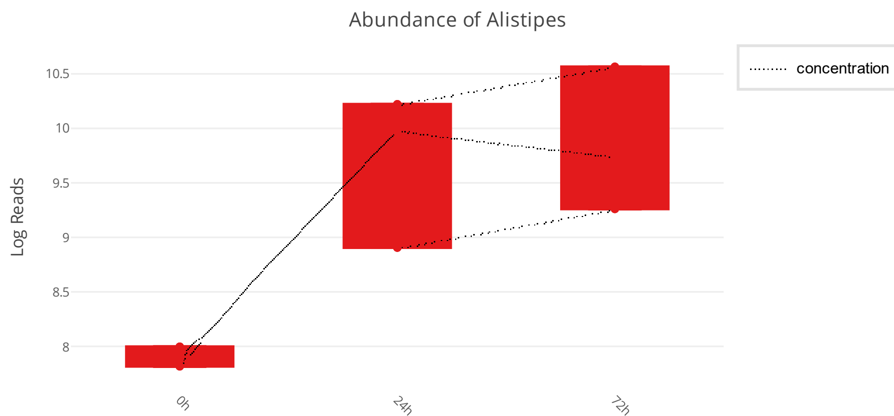


***Figure 4.14 – Abundance of Alistipes species of across timepoints 0h, 24h, 72h**. Samples with the same RCW concentration are connected with a line showing highest abundance for 0.3% followed by 0.6% and 0% at timepoint 72h.*

5 Discussion

As conventional 2D cell cultures have their limitations in resembling in vivo conditions, this study tried to bring 3D-culturing techniques one step further by adding microbiota to the experiment. Not only to have more realistic conditions for the agent and the cells, but also to detect shifts of the microbial composition during the experiment.

Therefore, 3:1 mixed CaCo2 cells and HT29 cells were cultivated for 3 weeks and then treated with different concentrations of RCW and microbiome.

Unfortunately, only the controls and the samples without RCW but Microbiome left enough adherent cells for successful staining with HE and alcian blue. An explanation why the cells have died may be that too much harm was done to the

cells by the microbiota. RCW may have contributed to cell death as samples in combination with microbiota did not even leave traces on the slides. This complies with the EZ4U assays for RCW that have shown to decrease the viability of the cells to 80% after 7 days.

RNA isolation and measurements also show a decrease of RNA amounts with increasing RCW concentration and incubation time. The conclusion of the low amounts of RNA is that most cells did not survive the experiment.

The alcian blue stained control specimens show a continuous multilayered epithelium with prismatic cells and small amounts of mucus (blue). In contrast the specimens with cells incubated with microbiota show signs of cell damage as no recognizably shaped cells, loss of cytoplasm and no mucus were visible.

The HE stained controls show a continuous multilayered epithelium with prismatic cells. Samples with cells and microbiota show a loss of cells' shapes and huge loss of cytoplasm. Only the nuclei of the damaged cells were recognizable.

The qPCR data show a decrease in the mRNA expression for the mucins MUC2 and MUC5B to almost zero expression when microbiome was added and suggests cell damage.

MUC5AC expression mean values show a slight increase from 0.3 to 0.6 RCW concentrations. Due to the relatively high standard deviation of 0.6 RCW, it is not possible to make a conclusion of this results.

Data analysis of the microbiome shows that the microbial composition shifts drastically over time which could be due to unphysiological incubation conditions.

This effect may cover up any other effects of the treatment.

These results are consistent with the loss of alpha-diversity over time (Fig. 4.10).

Alistipes was the most prominent genus, regarding its abundance at timepoint 0h, to show growth under the given incubation conditions. This could be due to a general robustness of the genus or only that Alistipes thrive under these certain conditions.

6 Conclusion and Future Prospects

In earlier studies we developed a promising ALI culture for the colon epithelium with CaCo2 and HT29 cells in a 3 to 1 ratio mixed culture.

In this study we added microbiota to the cells to come close to *in vivo* conditions and to evaluate not only changes in mucin production but how the composition of microbiota changes under RCW treatment. Because no cells survived the incubation with microbiota and RCW, it was not possible to make any conclusion about the interaction of colonic cells, microbiota, and RCW. To successfully recreate the interaction of colon epithelium, microbiota and RCW further research is needed.

Specific microbiota may enhance or reduce the effects of a treatment by metabolizing agents. With cell cultures that contain microbiota, more realistic disease models could be recreated to get a better understanding of these interactions.

References

1. Lüllmann-Rauch R. Taschenlehrbuch Histologie. 6 ed: Stuttgart ; New York, : Georg Thieme Verlag, [2019]; 2019.
2. Sellers RS. The colon: from banal to brilliant. Toxicol Pathol. 2014;42(1):67-81.
3. Kim YS. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep. 2010;12(5):319-30.
4. Hansson GC. Role of mucus layers in gut infection and inflammation. Curr Opin Microbiol. 2012;15(1):57-62.
5. Van der Sluis M. Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology. 2006;131(1):117-29.
6. van der Post S. Structural weakening of the colonic mucus barrier is an early event in ulcerative colitis pathogenesis. Gut. 2019;68(12):2142-51.
7. Sender R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. PLoS Biol. 2016;14(8):e1002533.
8. Pferschy-Wenzig E. A Combined LC-MS Metabolomics- and 16S rRNA Sequencing Platform to Assess Interactions between Herbal Medicinal Products and Human Gut Bacteria in Vitro: a Pilot Study on Willow Bark Extract. Frontiers in Pharmacology. 2017;8(893).
9. Ravi M. 3D cell culture systems: advantages and applications. J Cell Physiol. 2015;230(1):16-26.
10. Usui T. Preparation of Human Primary Colon Tissue-Derived Organoid Using Air Liquid Interface Culture. Curr Protoc Toxicol. 2018;75:22.6.1-.6.7.
11. Nossol C. Air-liquid interface cultures enhance the oxygen supply and trigger the structural and functional differentiation of intestinal porcine epithelial cells (IPEC). Histochem Cell Biol. 2011;136(1):103-15.
12. Klasvogt S. Air-liquid interface enhances oxidative phosphorylation in intestinal epithelial cell line IPEC-J2. Cell Death Discov. 2017;3:17001.
13. Wang J. What has traditional Chinese medicine delivered for modern medicine? Expert Rev Mol Med. 2018;20:e4.
14. Traditional chinese medicine.
15. Cheung F. TCM: Made in China. Nature. 2011;480(7378):S82-3.
16. Nagalingam A. Chapter 15 - Drug Delivery Aspects of Herbal Medicines. In: Arumugam S, Watanabe K, editors. Japanese Kampo Medicines for the Treatment of Common Diseases: Focus on Inflammation: Academic Press; 2017. p. 143-64.
17. https://www.sacredlotus.com/go/chinese-formulas/medicine/run-chang-wan.
18. Giuliani S. Air Liquid Interface Culture to Mimic the Epithelium of the Respiratory Tract and the Colon. 2018:63.