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MASTERARBEIT

„Optimising Preparation of Intestinal Mucosal Tissue and Intestinal Contents to Study Effects of *Akkermansia spp.* Supplementation during Dextran Sulfate Sodium-Induced Colitis in Mice”

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INDEX OF ABBREVIATIONS

AB	Alcian Blue
AR	Antigen-Retrieval
BSA	Bovine Serum Albumin
DSS	Dextran Sulfate Sodium
EtOH	Ethanol
FA	Formamide
FISH	Fluorescence *In-Situ* Hybridisation
HB	Hybridisation Buffer
HI	Heat Inactivated
IBD	Inflammatory Bowel Disease
MetOH	Methanol
ON	Over Night
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
RT	Room Temperature
WB	Washing Buffer
WT	Wild Type

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1 ABSTRACT

The gastrointestinal tract is filled with 10^{12} to 10^{14} bacteria, and it is assumed that the colon alone harbours over 70 % of the total number of microbes found in the human body. A disturbance of the gut microbiota has been associated with inflammatory bowel diseases, such as Crohn's disease or ulcerative colitis. The depletion of *Akkermansia muciniphila*, a known mucin degrader, is thought to be such an alteration of the gut community. Because of this, supplementation of probiotic *Akkermansia spp.* is thought to have a positive effect on colonic inflammation during dextran sulphate sodium (DSS)-induced colitis. We observed that supplementation of C57BL/6 WT mice with viable H2- and MucT-*Akkermansia* (human isolates) caused a strong weight loss in the first days of supplementation whereas mice re-gained weight after four days of administration, when H2- and MucT-*Akkermansia*-DNA was also detectable by qPCR. Heat inactivated (HI) and viable *Akkermansia* of the MG (mouse isolate) strain, as well as all other HI strains did not show any positive or negative effects on the average weight compared to the control group - neither before, nor during DSS-induced colitic inflammation. Evaluation of colonic sections with fluorescence *in situ* hybridisation (FISH) revealed the re-colonisation of *Akkermansia spp.* in all of the groups, except of the control group, but no significant correlation between inflammation scoring and the presence of *Akkermansia spp.* could be observed. Furthermore, the mucin degrading bacteria were not as expected localised exclusively next to the intestinal mucosal barrier, but distributed across the whole lumen.

Nevertheless, for better evaluation of relations between the presence of *Akkermansia spp.* in the colon and the disruption of the mucosal barrier a combined staining of both is necessary. As the main component of mucus is water (> 98 %), it is challenging to visualize it microscopically. Nine different fixatives and different staining methods were tested to find conditions for optimal tissue and mucosal preservation, as well as high signal intensities during FISH. FISH was performed on fixed cecum content samples using the general bacterial EUB338 I – III probe mix, and specific probes for the detection of *Bacteroidales* and *Clostridium* clusters XIVa and XIVb, which were determined for their average volume fraction and the probe signal intensity using DAIME. Fixation with 4 % PFA, 6/3/1 anhydrous EtOH-Carnoy, and 6/6/1 MetOH-Carnoy gave the best results concerning FISH probe signals and preservation of mucosal structures as determined during Alcian Blue staining. As Alcian Blue staining of mucin detection was not combinable with FISH, immunohistochemical staining with either the lectin Jacalin derived from Jackfruit seed, or the mucin specific anti-MUC2-antibody were performed. No clear mucin staining could be observed during lectin staining, but the antibody staining of MUC2 resulted in clear strong staining of the intestinal mucosal barrier, brush borders, and of goblet cells. Nevertheless, it

was not possible to yield good probe signal intensity when the MUC2-antibody staining was combined with FISH to determine the localisation of commensal bacteria.

Keywords: gut microbiota; *Akkermansia muciniphila*; inflammatory bowel disease; fluorescence *in situ* hybridisation; mucus; MUC2; Alcian Blue; cell shedding

2 INTRODUCTION

2.1 Gut Microbiota

Being called the biggest organ in the human body, the gastrointestinal tract is filled with 10^{12} to 10^{14} bacteria[1,2], whereas it is assumed that the colon alone harbours over 70 % of the total number of microbes found in the human body[3]. This intestinal microbiota, also called microbiome, includes anaerobic bacteria from the genera *Bacteroides*[4], *Clostridium*, *Ruminococcus*, *Bifidobacterium,* and *Akkermansia*[5], but also aerobic (facultative anaerobic) bacteria such as *Escherichia*, *Enterobacter*, *Enterococcus*, and *Lactobacillus*[4,6] can be found, whereas the ratio of anaerobes to aerobes increases from the mucosal surface to the lumen[7].

All these commensal bacteria are not only involved in several digestive processes, like for example breaking down indigestible polysaccharides to absorbable monosaccharides[3], or the synthesis of several vitamins, such as vitamin B, D, and K[8], but their composition also plays an essential physiological role in human health[9] by preventing over-colonisation of pathogens suggesting that the microbiota may be viewed as an additional multifunctional organ [10].

2.2 Mucus

Despite the high numbers of microbes, infection and irritation by commensal bacteria is rare. An important mechanism that mediates host-microbe interactions is the mucus layer. This layer is between the epithelial cells, and the intestinal content, and is rich in carbohydrates, lipids, and proteins – mainly antimicrobial peptides[11]. It therefore provides a physical protection against pathogens, and also forms the first line of defence of the innate immune system in the gut[12]. This mucus layer is composed of mucins, which are divided into two groups. The first group of mucins contains the gel-forming mucins, such as MUC2, which forms extremely large polymers (< 100 MDa) and is the main component of the mucus layers in the intestine and also form their skeleton[1]. The MUC2 mucin is a large *O*-glycosylated protein[13] (approx. 5,200 amino acids) which is characterised by abundant and variable *O*-linked glycans attached to hydroxyl amino acids clustered in proline, threonine, and serine rich PTS domains, or highly protein resistant mucin domains[12]. MUC2 is processed in the ER and the Golgi apparatus[14]. The second group of mucins is made up of the transmembrane (TM) mucins (e.g. MUC3, MUC12, MUC17), which cover the apical surface of the enterocytes and form the so-called glycocalyx which is below the mucus layer[15]. The TM mucins have a cytoplasmic tail, and are also relatively big molecules (1^6 to 1^7 Da)[1,16]. In general, mucins are produced by the goblet cells[11].

The mucus layer of the small intestine is thought to be a single layer which is permeable to bacteria, and it has also been observed, that the ileal mucus is easy to aspirate and to remove[1]. In contrast to this, the colonic mucosal layer is made up of two layers, whereas the inner mucus layer remains attached to the epithelium and cannot be removed. This inner layer is not soluble and is later on converted into the outer mucus layer by several yet unknown processes[12]. The outer "loose" mucus layer can be aspirated[17]. In general it is suggested, that in the large intestine commensal bacteria only inhabit the outer loose mucus layer. The inner firm mucus layer is devoid of bacteria what was shown e.g. by *in situ* hybridisation using a general 16S rRNA probe, but also by semi-quantitative PCR using primers specific for conserved regions of the 16s rRNA genes performed on DNA isolated from the loose and firm mucus layers[18].

Mucus in general is rather transparent and cannot be made visible microscopically as its main component is water (> 98 %)[12]. This makes the mucus hard to study and makes special treatment necessary. It was shown, that fixation with water-free Carnoy's fixatives usually gives good results concerning the preservation of the mucus layer[19]. Also, in addition to mucins, mucus layers also contain antimicrobial peptides, cytokines, and immunoglobulins[20]. As these components can be targeted in visualisation methods, such as antibody-immunostainings, but may be masked due to tissue fixation, treatments like epitope or antigen retrieval may be necessary and might provide greater diagnostic accuracy[21].

To make the mucus visible, special staining approaches are necessary. For this, several methods which stain different kinds of glycoproteins at different conditions are available. The most common histochemical techniques for mucin staining include methods that utilise a specific chemical reaction (organic, enzymatic, immunological), such as the Alcian Blue (AB) staining[22]. AB binds to the carboxyl group of sialic acid or sugars with sulfate substitution. By lowering the pH more highly acidic sulfated mucins may be visualized selectively. Usually, AB staining is performed at a pH of 2.5[23,24].

Another possibility of mucus staining offers the lectin histochemistry. Lectins bind to sugars comprising the oligosaccharide chains of glycoproteins such as mucins. Lectins are known to be rather specific, but may react only when sugars are expressed within particular structural configurations (e.g. *Sambucus nigra* lectin binds to sialic acid in alpha 2,6-linkage but not in alpha 2,3-linkage[25]). However, lectins bind only to peripherally situated sugars within oligosaccharide chains (sialic acid, fucose, *N*-acetylgalactosamine – GalNAc). As sialic acid may be attached to galactose of GalNAc, removing sialic acid is necessary[23,26].

Nevertheless, immunohistochemistry, including common antibody staining, also offers another quick and easy to apply, and reliable option for staining of intestinal mucins. Antibodies recognise specific sequences of sugars forming blood group substances or still larger molecular arrangements. This may be either carbohydrate, carbohydrate-apomucin-(*MUC* gene product)-combination, or apomucin alone[27]. In general the antibody is highly specific, but there may be a lowered sensitivity for individual components. Same as with lectin histochemistry, reactivity may be modified by the removal of sialic acid and neutral sugars. However, immunohistochemistry is prone to several technical errors. Staining patterns may be influenced by type of fixation, as well as section thickness[23].

However, there are several fixatives available, but fixatives have only been optimized either for preserving structural features of tissues, or for improving bacterial cell wall permeability for optimal probe uptake during FISH. The major goal of this study therefore was to find a fixation method that not only preserves the tissue structures and mucus very well, but also gives good results during FISH. For this, nine different fixatives were tested for their ability to preserve tissue structures as well as for their effects on probe uptake and probe signal intensity. Cecum content samples were hybridized with the general bacterial probe EUB338 I – III, and with the specific probes Bac303 (*Bacteroidales*) and Erec482 (*Clostridium* clusters XIVa and XIVb).

Furthermore, mucus staining techniques, such as Alcian Blue staining, and immunohistochemical techniques (lectin staining, antibody staining) need to be tested on paraffin sections of fixed tissues. Those stainings should then be combined with FISH to enable determination of the distribution of bacterial communities associated with the intestinal mucosal layer.

2.3 Inflammatory Bowel Diseases & *Akkermansia muciniphila*

An imbalance of the gut microbiota, which may be accompanied or caused by disturbance of the intestinal mucosal surface, has been associated with several diseases, like for example Crohn's disease or diabetes[3,28]. Such dysregulation or alteration in gut communities may be caused by antibiotic treatment[29], or also may be due to a too clean, "germ-free" childhood. Here, experiments in germ-free and specific pathogen-free mice also lead to the suggestion that early-life contact to specific microbes decreases susceptibility to diseases such as inflammatory bowel diseases (IBD)[30].

IBD is a complex chronic inflammatory disorder of the human gastrointestinal tract, mainly emerging as Crohn's disease (CD) or ulcerative colitis (UC). IBD is the result of an imbalance in the interactions of symbiotic intestinal microbiome, and epithelial barrier, and also of a

dysfunction of the immune system. It has been shown, that a down-regulation of genes encoding for the anti-inflammatory cytokine Interleukin (IL) 10 occurs during active IBD[31]. In IL-10$^{-/-}$ mice, this down-regulation of IL-10 results in a higher expression of the pro-inflammatory cytokines tumour necrosis factor α (TNFα) and IL-1β, and the transforming growth factor β (TGF-β) leading to a predisposition of knock-out mice to intestinal inflammation[32,33].

However, the causes for these misbalances still remain largely unclear, whereas an involvement of intestinal microbial community in triggering such diseases is increasingly evident[34,35]. It has been shown that the composition of the intestinal microbiome does not only differ significantly between healthy individuals and patients suffering from IBD, but also between CD and UC patients[6,36]. A dysbiosis of the microbiome in IBD patients usually is characterised by a reduced species richness and diversity of faecal microbiota, whereas compositions were more similar between healthy individuals and individuals with UC, but differed stronger between patients with CD and healthy persons[34,37,38]. It was shown, that in IBD symbionts with beneficial effects are reduced (such as *Bifidobacteria*), while the number of opportunistic pathogens increases[3,38]. Nevertheless, it cannot be said whether the increase of bacterial groups such as *Enterobacteriaceae* or *Verrucomicrobiaceae* can be called initiators or opportunists of the bacterial shifts[28]. However, due to differences in bacterial compositions between healthy individuals, and patients with either CD or UC, the presence or absence of certain bacterial genera can be used as indicators of the different phenotypes[4,28,39].

One species which abundance is known to undergo a shift in IBD is the gram-negative anaerobic *Akkermansia spp.* which belongs to the phylum of *Verrucomicrobia*. However, contradicting reports can be found when it comes to the question whether the number of *Akkermansia* is increased, reduced, or even depleted during IBD and DSS-induced colitis[40,41]. *Akkermansia muciniphila* is a known mucin degrader that can be found in the human colonic mucus layer which offers a broad range of nutrients for bacteria[42,43]. Furthermore, the mucus layer serves as a protection area as bacteria can adhere to the mucins and functions as a primary carbon and nitrogen source[44]. Furthermore, it has been suggested that mucin-degradation of bacteria provide nutrients for other resident bacteria by releasing monosaccharides or amino acids from breaking down the mucin[40].

As it has been reported that *Akkermansia* are depleted during IBD, and therefore contribute to intestinal inflammation, it has been suggested that an addition of these lacking bacteria as a probiotic might have an effect on gut microbial composition, and on DSS-induced colitis[45,46]. It is thought that an addition of lacking *Akkermansia* might support other resident

bacteria which counteract intestinal inflammation. Furthermore, the released molecules from mucus degradation (monosaccharides, amino acids, short chain fatty acids), which are produced in the mucus close to the epithelial surface, are thought to be easier available to the host[47], what might also contribute to turnover of epithelial cells and therefore repair of damaged cells during inflammation.

The major goal of this study therefore was to determine the effects of *Akkermansia*-supplementation on general characteristics that go with a colonic inflammation and a possible re-colonisation of *Akkermansia*, such as weight, and mucus preservation in general. For daily oral gavage of bacteria, three different strains of *Akkermansia* (phylogenetic intermediates), namely H2 and MucT (human), and MG (mouse), all three both viable and heat inactivated, were used. For the determination of mucosal preservation, paraffin sections of ileal, cecal, and colonic tissue fixed with 6/3/1 EtOH-Carnoy were stained with Alcian Blue to visualise mucins on the epithelial surface and in the goblet cells. Fluorescence *in situ* hybridisation was performed on 4 PFA-fixed tissue samples using the general bacterial probe EUB338 I – III, the *Akkermansia*-specific probe Akk435, and the probe EUK516 (specific for eukarya). This offered the possibility to determine whether *Akkermansia* do re-colonise in the gut before/during DSS-administration due to the oral supplementation and if so, where the bacteria was localised.

Furthermore, and more specific, the effects of the oral administration of additional *Akkermansia spp.* on DSS-induced colitis, its course of disease, and hereafter its effects on inflammation pathology scorings were determined. For this, a weight curve before and during DSS-administration was recorded. For pathology scoring, a Haematoxylin & Eosin staining was performed. The inflammation scoring was performed by a trained pathologist, results of the single groups were compared to the values of the control group, and associated to the presence of *Akkermansia spp.* determined during FISH.

3 MATERIALS AND METHODS

3.1 Mice

For all experiments, 15 weeks old male C57BL/6 WT mice were used. Mice were purchased from Charles River Laboratories (Austria) and provided from the Max F. Perutz Laboratories (MFPL), Vienna. Mice were housed in the same specific pathogen free (SPF) facility under identical conditions (insulated ventilator cages) according to recommendations of the Federation of European Laboratory Animal Science Association. Mice lived in groups of four per cage, with free access to food and water.

3.2 Gavage of *Akkermansia spp*., and DSS-Treatment

For the experiment three different strains of *Akkermansia* (phylogenetic intermediates) were used:

- MucT (human strain)
- H2 (human strain)
- MG (mouse strain).

All strains were cultured and kindly provided by the group of Prof. Dr. Willem de Vos (Laboratory of Microbiology, Wageningen University, NL) as previously described[46], snap-frozen at -80 °C and stored at 20 °C. With the bacteria, freshly prepared anaerobic PBS containing 2.5 % glycerol was provided. For oral gavage, strains were administered in viable condition, as well as heat inactivated. Heat inactivated stocks of bacteria were already in ready-to-use-dilution, living bacterial stocks needed to be diluted with anaerobic PBS/2.5 % glycerol to reach 10^9 cfu/200 µL. As control, PBS containing 2.5 % glycerol was gavaged.

Each condition was administered to a group of 4 mice, 200 µL were gavaged per mouse per day. After 6 days of *Akkermansia* supplementation, mice additionally received 2 % DSS over the provided drinking water for 7 days to provoke gastrointestinal inflammation (colitis).

3.3 Sample Preparation

3.3.1 Fixation of Mouse Intestinal Tissue

For the fixation of samples, anhydrous alcohols were used in some of the fixatives. To obtain this anhydrous (= 100 %) alcohol, methanol or ethanol were “dried” using common commercial Copper Sulphate ($CuSO_4$), which functions as desiccant, changing its colour from greyish-white to bright blue with increased volume of absorbed water. Recipes for PBS stock solution and 1x PBS can be found in Table S3 and Table S4, respectively.

Cecal content was harvested, tissue samples were taken from ileum, cecum and colon, whereas guts were prepared as “würstel” by tying them with common sewing thread to keep the content in place. All content and tissue samples were fixed by using one of the following fixatives:

- 4 % PFA
- 50/50 Ethanol/PBS
- Carnoy’s Solution:
    - 6/3/1 anhydrous Ethanol/Glacial Acetic Acid/Chloroform
    - 6/6/1 anhydrous Ethanol/Glacial Acetic Acid/Chloroform
    - 6/3/1 Methanol/Glacial Acetic Acid/Chloroform
    - 6/6/1 Methanol/Glacial Acetic Acid/Chloroform
    - 6/1/3 Methanol/Glacial Acetic Acid/Chloroform
    - 6/1/3 anhydrous Methanol/Glacial Acetic Acid/Chloroform
    - 6/6/1 anhydrous Methanol/Glacial Acetic Acid/Chloroform.

Samples fixed in Carnoy’s Solution and 50/50 EtOH/PBS were incubated for 2 hours, samples for which 4 % PFA was used as a fixative were incubated for 4 hours. All incubations were performed on ice.

Content samples were spun down (10.000 rpm, 5 min), the supernatant was discarded, and pellets were resuspended in 1x PBS for washing. Samples were spun down another time, and pellets were resuspended in 50/50 EtOH PBS for storage at -20 °C.

After incubation, one set of tissue samples (ileum, cecum, colon) were washed 2 x 20 min in 70 % EtOH/PBS, put into embedding cassettes and dehydrated in an EtOH series and xylene substitute and paraffinised. Dehydration and paraffinisation steps were performed in a tissue processor machine overnight (see Table S1). A second set of tissue samples were not washed with 70 % EtOH/PBS after incubation, but instead stored in 100 % EtOH over night (ON), before paraffinisation in the tissue processor machine. For this set of samples, dehydration steps in the tissue processor machine (steps 1 – 6; see Table S2) were skipped.

Samples were embedded in paraffin, cut transversally into 6 µm sections using a microtome (Leica; see Table S1) and mounted onto usual uncoated microscopic glass slides. Paraffin sections were dewaxed by putting it into xylene (2 x 10 min) and washed in $Ethanol_{abs}$ (10 min).

3.3.2 Akkermansia-treated mice

Every mouse was weighed every day before gavage to get an overview of/control eventual weight loss or weight gain caused by the *Akkermansia* administration and/or the DSS-induced colitis. Furthermore, to evaluate the microbial communities in every single mouse, and also an eventual shift in microbial communities, faecal pellets were harvested on days -6, -2, 6, and 8, snap-frozen in liquid nitrogen, and stored at -20 °C.

At the end of the *Akkermansia*/DSS treatment (Day 8), ileal, and cecal, and proximal colonic tissue was fixed in 4 % PFA on ice for 4 h for FISH with an *Akkermansia*-specific probe. Ileal, cecal, and distal colonic tissue was fixed in 6/3/1 EtOH-Carnoy's Solution (see 3.3.1 Fixation of Mouse Intestinal Tissue) on ice for 2 h for Alcian Blue staining to determine the mucus thickness. After incubation, tissues were stored in 100 % EtOH ON before paraffinisation in the tissue processor machine, whereas the dehydration steps (steps 1 – 6; see Table S2) were skipped. Samples were embedded in paraffin, cut transversally into 4 µm sections using a microtome (Leica; see Table S1) and mounted onto SuperFrost Plus slides (Thermo Scientific). Paraffin sections were dewaxed by putting it into xylene (2 x 10 min) and washed in $Ethanol_{abs}$ (1 x 10 min).

Leftovers of colonic tissues were also fixed with 4 % PFA, dehydrated and paraffinised in a tissue processor machine overnight, embedded in paraffin embedding cassettes, cut longitudinal into 5 µm sections using a microtome, and mounted onto SuperFrost Plus slides (Thermo Scientific). Paraffin sections were rehydrated and stained with haematoxylin and eosin (H&E) for pathological scoring of colonic inflammation using a staining machine for histology (Pathisto; see Table S1). Scorings were performed by a trained pathologist (Ao.Univ.-Prof. Dr.med.univ. Lukas Kenner, University of Vienna) in regard to inflammation severity, inflammation extent, crypt damage, and percentage of in the inflammation involved tissue.

Furthermore, luminal content from the proximal colon, ileum, and as well as colonic, cecal, and ileal tissue was snap-frozen in liquid nitrogen, and stored at -20 °C. All snap-frozen samples (including frozen faecal pellets) were sent to Wageningen University for further analysis (MITchip, qPCR, etc.).

3.4 Stainings

3.4.1 Alcian Blue Staining

To check tissue samples for preservation of mucus, 5 µm thick paraffin sections of samples fixed with 6/3/1 EtOH-Carnoy were stained with alcian blue. For this, sections were dewaxed with xylene (2 x 10 min) and $Ethanol_{abs}$ (1 x 10 min). Sections were then rehydrated in an

Ethanol series (100, 96, 80, 75, 50 %), and put in alcian blue solution (pH 2.5) for 1 hr. Afterwards, slides with sections were washed with ultrapure water two times, and counterstained with nuclear fast red for 7 min. Sections were then washed with ultrapure water, dehydrated in an Ethanol series (50, 75, 80, 96, 100 %), and mounted with Eukitt quick-hardening mounting medium. Microscopical evaluation was done using the Carl Zeiss Axio Imager M.1 microscope (see Table S1).

3.4.2 Haematoxylin & Eosin Staining

For pathology (inflammation scoring), 4 % PFA-fixed tissue samples (5 µm paraffin sections) were stained with haematoxylin and eosin. The H&E staining was performed by using a staining machine (ASS-1, Pathisto). The programme also included rehydration and dewaxing steps. The full program includes the steps which can be found in Table 1.

Table 1: Pathisto – ASS 1 – Staining Machine for Histology and Zytology; all steps are performed at RT

Step No.	Solution	Duration
1	Xylene Substitute	10 min
2	Xylene Substitute	10 min
3	100 % EtOH	5 min
4	100 % EtOH	5 min
5	90 % EtOH	2 min
6	70 % EtOH	2 min
7	Tap-H_2O wash	10 min
8	Haematoxylin	6 min
9	H_2O (tap water) wash	10 min
10	1 % HCl (in EtOH)	5 sec
11	H_2O (tap water) wash	10 min
12	70 % EtOH	30 sec
13	80 % EtOH	30 sec
14	Eosin solution	2 sec
15	90 % EtOH	1 min
16	90 % EtOH	1 min
17	100 % EtOH	1 min
18	100 % EtOH	1 min
19	Xylene Substitute	5 min
20	Xylene Substitute	5 min

After the last step, sections were left in xylene for 20 min to replace the xylene substitute, which would make mounting the sections impossible as the substitute is immiscible with most mounting media. For mounting, Eukitt quick-hardening mounting medium was used.

3.4.3 Lectin Staining

Alcian Blue is a quick and easy method for visualisation of mucus in intestinal sections. Nevertheless, it is not combinable with FISH. Therefore, to determine bacterial population and communities in and around the mucus, another method has to be used. In contrast to this, lectin staining, such as UEA-1 or Jacalin, gives a good possibility to visualise mucus and – on the same section – also the bacteria in the lumen and next to the mucus[48].

For the experiment detailed in this thesis, Jacalin (*Artocarpus integrifolia*) conjugated with Texas Red® Sulfonyl Chloride (EY Laboratories, Inc.; USA; see Table 4) was used for mucus staining. This lectin from Jackfruit seeds is specific for α-D-Galactose[26] and stains not only the freshly produced mucus in the goblet cells, but also the secreted mucus around the epithelium and the brush borders[20,49].

For fluorescence detection of the glycan epitope of the intestinal mucus using Jacalin, paraffin sections (6/3/1 EtOH-Carnoy fixed tissue) were deparaffinised and blocked with 3 % H_2O_2 in PBS for 10 min at RT. Afterwards, sections were rinsed briefly with 0.02 M $NaHCO_3$ and marked with a fatty acid pen. The Jacalin stock solution was diluted in 0.02 M $NaHCO_3$ to different final concentrations, according to manufacturer's recommendations reaching from 20 – 100 µg/mL buffer, and added onto the sections. Slides were incubated at RT for 30 min in a moist chamber in the dark and then washed three times with 0.02 M $NaHCO_3$. Nuclei of the tissue were counterstained with DAPI, sections embedded, and the staining evaluated using a CLSM at appropriate wavelengths.

For probable enhancement of the protocol, the staining was also performed with different blocking solution and buffer. Sections were blocked in 1x PBS containing 0.3 % Triton X-100 and 1 % BSA for 10 min at RT. Slides were washed once in 1x PBS, Jacalin was diluted in 1x PBS to a final concentration of 60 µg/mL PBS. Sections were incubated with the lectin in a moist chamber in the dark for 1 hr[49]. Afterwards, slides were washed five times in 1x PBS, sections counterstained with DAPI, and embedded.

For negative controls of the staining, Jacalin dilutions were pre-incubated with the Jacalin inhibitory carbohydrate D-(+)-Melibiose[20,26] at a concentration of 200 mM before applying the solution to blocked sections. Sections were incubated with inhibited Jacalin parallel to the sections incubated with the common Jacalin solutions, counterstained, and embedded. For

comparison, and for checking for unspecific binding, negative controls were evaluated at the CLSM using the same settings as for the positive stained sections.

3.4.4 MUC2 Antibody Staining

Besides lectin staining, antibody staining is a quick and easily applicable immunohistochemical staining for the visualisation of mucus. Being one of the most common mucins in the intestinal mucosal layer(s)[50,51], the gel-forming mucin MUC2 offers a broad point of application for mucosal antibody staining. Therefore, anti-MUC2 antibody[52] was used for mucus staining in paraffin embedded tissue sections.

Deparaffinised sections were rehydrated in an EtOH series (96, 80, 70, 50 % – 10 min each). For antigen retrieval (AR) slides with sections were put in a heat resistant tip box, covered with 10 mM sodium citrate buffer (see Table S5) and heated in a microwave oven (max. power) for 5 min[19,53,54]. Evaporated buffer was refilled to cover the slides and sections were boiled for another 5 min. Slides were left at RT to completely cool down before washing in 1x PBS. Sections were marked with a fatty acid pen and blocked in 1x PBS containing 1 % BSA in a humid chamber at 4 °C for 45 min in the dark. After blocking, slides were washed by dipping in 1x PBS.

Anti-MUC2 antibody (H300; rabbit polyclonal IgG, stock = 200 µg/mL – Santa Cruz Biotechnology, Inc.; USA) was diluted in blocking solution (1:100) and sections were incubated in the dark in a humid chamber at 4 °C ON. After washing in 1x PBS (3x 10 min), sections were incubated with the secondary antibody (goat anti-rabbit IgG – Jackson ImmunoResearch; USA) diluted in blocking buffer (1:500) in a humid chamber in the dark at 4 °C for 1 hr. Afterwards, slides were washed in 1x PBS (3x 10 min).

If antibody-staining of sections was combined with FISH, the hybridisation preceded the antibody-staining. As negative control, blocked sections were stained only with the secondary antibody Sections were counterstained with DAPI, embedded and evaluated using a CLSM at appropriate excitation and emission values.

3.5 Fluorescence *In-Situ* Hybridisation – FISH

All samples (cecum content samples and tissue sample) were hybridized with a general bacterial EUB338 I-III probe mix[55] (= EUBmix) to determine the presence of bacteria. If used alone, EUBmix was applied at a formamide (FA) concentration of 10 %, if applied together with other specific probes, the FA concentration of the EUBmix was adjusted to the concentration of the specific probes. Probe details can be found in Table 3, composition of hybridisation buffers and washing buffers which were used for FISH can be found in Table 2.

Table 2: Used solutions for fluorescence *in-situ* hybridization. If not noted different, volumes are in µL

Hybridisation Buffer (HB)				**Washing Buffer**			
FA %	0 %	10 %	30 %	FA %	0 %	10 %	30 %
NaCL	180	180	180	NaCL	9 mL	4.5 mL	1020
Tris-HCl	20	20	20	Tris-HCl	1 mL	1 mL	1 mL
ddH_2O	800	700	500	EDTA	-	-	500
Formamid (FA)	-	100	300	ddH_2O	ad 50 mL	ad 50 mL	ad 50 mL
SDS	1	1	1				

For determination of fixed cecum content samples, the EUBmix conjugated with the most common fluorophores – either 5,(6)-carboxfluorescein-N-hydroxysuccimidester (Fluos), indocarbocyanine (Cy3), or indodicarbocyanine (Cy5) – was applied to specify the effects of the used fixatives on the overall coverage ("biovolume fraction") and on the intensity of the EUBmix. As negative control, all samples were hybridised with a nonEUB338 probe (see Table 3) in the appropriate fluorophores in parallel.

For the hybridization, 10 µL of the 1:10 diluted fixed cecum content samples were pipetted on a 10 well glass slide and dried at 46°C in a hybridization oven. Afterwards, samples were dehydrated in an ethanol series (50 %, 80 %, 96 %) and air-dried. Then, onto the wells with the fixed and dried samples, per well 10 µL hybridization buffer (HB) + 1 µL probe were added. Slides were then placed in 50 mL Greiner tubes, which contained a piece of paper wetted with 1 mL HB (= humid chamber). Samples were incubated at 46°C in the dark for 2 hours. After incubation, samples were washed in washing buffer (see Table 2) at 48°C for 10 min. Slides were then carefully dipped into ice-cold water and air-dried.

To determine the properties of the probe in the different fixatives, cells were counterstained with DAPI. For this, samples were incubated with DAPI (2 mg/mL; 20 µL/per well) in the dark at RT for 3 min. Slides were then washed with water and air-dried.

For evaluation of bacterial colonisation on 6/3/1 Carnoy's fixed tissue samples, dewaxed sections were circled with a fatty acid pen and incubated in an humid chamber with an appropriate volume of an HB-probe solution (HB:probe = 1:10) at 46 °C in the dark. To avoid increase of auto fluorescence of the tissue, and also the detachment of tissue and mucus[56], sections were not incubated longer than 90 min. Tissue samples from WT mice for antibody-(MUC2)-staining were hybridised with the general bacterial probes EUB338 I – III, dewaxed sections from *Akkermansia*-treated mice were hybridised with the EUBmix probes, and an *Akkermansia*-specific probe (see Table 3).

Table 3: Used oligonucleotide probes

Probe Name	Long Name	Specificity	Probe Sequence (5‘ → 3‘)	FA [%]
EUB338 I*	S-D-Bact-0338-a-A-18	Most Bacteria	GCTGCCTCCCGTAGGAGT	0 - 50
EUB338 II*	S-*-BactP-0338-a-A-18	*Planctomycetales*	GCAGCCACCCGTAGGTGT	0 - 50
EUB338 III*	S-*-BactV-0338-a-A-18	*Verrucomicrobiales*	GCTGCCACCCGTAGGTGT	0 - 50
NONEUB	-	control probe complementary to EUB I – III	ACTCCTACGGGAGGCAGC	0 - 50
Bac303	S-*-Bacto-0303-a-A-17	Most *Bacteroidaceae* and *Prevotellaceae*, some *Porphyromonadaceae*	CCAATGTGGGGGACCTT	10
Erec482	S-*-Erec-0482-a-A-19	Most of the *Clostridium coccoides-Eubacterium rectale* group (*Clostridium* cluster XIVa and XIVb)	GCTTCTTAGTCARGTACCG	0
Akk435	S-G-Akk-0435	*Akkermansia spp.*	TTGCTCCCACATGACAGG	30
EUK516		Eukarya	ACCAGACTTGCCCTCC	35

*EUB338 I – III were mixed in an equimolar ratio and used at once as “EUBmix”

Table 4: Used fluorophores and most important properties

Fluorophore	Absorption maximum [nm]	Emission maximum [nm]	Molar extinction coefficient ε [1 x mol^{-1} x cm]
Fluos	494	518	7.5×10^4
Cy3	554	570	1.3×10^5
Cy5	650	667	$\geq 2 \times 10^5$
TexasRed®	586	605	8.5×10^4

For the detection, fluorophores of the labelled probes were excited with a laser. The emitted light could be detected at the appropriate emission (see Table 4) with a HD detector. Labelled organisms were visualised using a confocal laser scanning microscope (Leica CLSM TCS SP8 X; Software: LAS AF 3.2.1). To achieve comparability of the cell signal intensities for qualitative and quantitative analysis, the same instrumental settings were kept during analysis of the differently fixed samples, and also for nonEUB338-labelled cells to determine unspecific staining. Biovolumes and signal intensities were calculated in the software "daime"[57] by comparing the probe-labelled cells to the DAPI stained. Automatically selected objects were corrected manually by excluding those untypical in size and shape, and out of focus.

3.6 DAIME Quantification

For determination of the average biovolume fraction and the probe intensities, the software "daime" was used. The function "Vol%" (population probe versus general probe/stain) was used to count the targeted cells (in pixels) stained with EUBmix (= population probe) relative to the whole "bioarea" visualised by DAPI (= general stain). Probe intensities were determined by using the "measure objects" function giving the mean intensity of EUBmix-stained cells. For evaluation, all automatically selected objects were corrected manually by excluding those untypical in size and shape, and out of focus.

4 RESULTS

4.1 Fixation and Visualisation of Mucus

4.1.1 Fluorescence *In Situ* Hybridisation

The first step to determine the optimal fixative which not only preserves the mouse tissue structure at its best, but also results in good probe signals during FISH, cecum content samples of WT mice were fixed with all fixatives that could be found in the literature (see 3.3.1 Fixation of Mouse Intestinal Tissue) and hybridised with a general bacterial EUB338 I-III probe mix labelled with either Fluos, Cy3, or Cy5 (see Table 4), and the specific probes Bac303 (*Bacteriodales*), and Erec482 (*Clostridium* cluster XIVa) with appropriate formamide (FA) concentration. To find out how many cells are stained with the general bacterial probe mix, samples were counterstained with DAPI. The coverage of the EUB338 I-III general probe was then determined by performing a cell count and calculating the ratio DAPI : EUBmix using DAIME. Values of all fixatives can be found in Figure 1.A. Intensities of the probes were also determined via DAIME (see Figure 1.B and Figure 3).

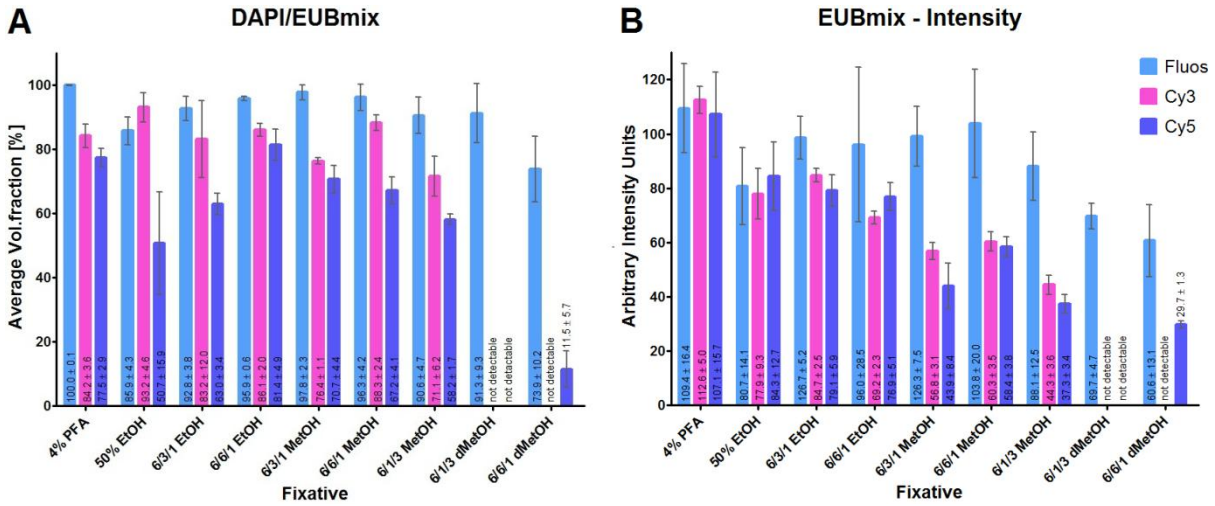


Figure 1: (A) Average volume fraction [%] of the general EUBmix probe labelled with different fluorophores (Fluos – light blue, Cy3 – pink, Cy5 – dark blue). Cells were counterstained with DAPI to determine the efficiency of the general probe. Cell numbers were calculated by using the program DAIME. (B) Probe intensity of the EUBmix probe in the different fluorophore was determined for every fixative using DAIME. For all cell quantifications and evaluations of fluorophore intensities in DAIME, the average cell number/intensity of 5 representative CLSM pictures was calculated. Error bars show the standard deviation.

As the fixation with 4 % PFA is commonly used as “standard fixation” in FISH protocols, PFA fixed samples were used as a “positive control”. This means settings for excitement of these samples at the CLSM (gating, gain, laser intensity, etc.) were used for examining all other fixed samples at appropriate wavelengths for the different fluorophores, and also DAPI.

In general it can be seen, that EUBmix labelled with Fluos yielded the highest values for average volume fraction, resulting in a visualisation of 85.9 ± 4.3 to 100.0 ± 0.1 % of all cells with EUBmix compared to DAPI stained cells (see Table 5). Compared to this, hybridisation

with EUBmix-Cy5 resulted in rather low levels of average volume fractions between approx. 50 and 81 %, therefore showing the lowest levels in comparing the three fluorophores. In Cy5, only one fixative – 6/6/1 anhydrous MetOH-Carnoy – yielded a value below 50 % (see Table 5), hybridisation of cecal content fixed with 6/1/3 anhydrous MetOH-Carnoy did not result in any detectable signals at all. These two samples were also the ones which showed lower levels of average volume fractions and intensities in Fluos, and in the set of EUBmix-Cy3-hybridised samples, no signals at all where detectable in cecal content fixed with 6/1/3 anhydrous MetOH-Carnoy, and 6/6/1 anhydrous MetOH-Carnoy (see Figure 1.A and B).

Table 5: Average volume fractions (coverage) and fluorophore intensities of EUBmix probe in the fluorophores Fluos, Cy3, Cy5. Average volume fractions and intensities were determined using DAIME. nd = not detectable

Fixative	Coverage			Intensity		
	Fluos	Cy3	Cy5	Fluos	Cy3	Cy5
4 % PFA	100.0 ± 0.1	84.2 ± 3.6	77.5 ± 2.9	109.4 ± 16.4	112.6 ± 5.0	107.1 ± 15.7
50 % EtOH	85.9 ± 4.3	93.2 ± 4.6	50.7 ± 15.9	80.7 ± 14.1	77.9 ± 9.3	84.3 ± 12.7
6/3/1 anhydr. EtOH	92.8 ± 3.8	83.2 ± 12.0	63.0 ± 3.4	126.7 ± 5.2	84.7 ± 2.5	79.1 ± 5.9
6/6/1 anhydr. EtOH	95.9 ± 0.6	86.1 ± 2.0	81.4 ± 4.9	96.0 ± 28.5	69.2 ± 2.3	76.9 ± 5.1
6/3/1 MetOH	97.8 ± 2.3	76.4 ± 1.1	70.7 ± 4.4	126.3 ± 7.5	56.8 ± 3.1	43.9 ± 8.4
6/6/1 MetOH	96.3 ± 4.2	88.3 ± 2.4	67.2 ± 4.1	103.8 ± 20.0	60.3 ± 3.5	58.4 ± 3.8
6/1/3 MetOH	90.6 ± 4.7	71.1 ± 6.2	58.2 ± 1.7	88.1 ± 12.5	44.3 ± 3.6	37.3 ± 3.4
6/1/3 anhydr. MetOH	91.3 ± 9.3	nd	nd	69.7 ± 4.7	nd	nd
6/6/1 anhydr. MetOH	73.9 ± 10.2	nd	11.5 ± 5.7	60.6 ± 13.1	nd	29.7 ± 1.3

In general, cecal content fixed with 4 % PFA did not only yield good values of average volume fraction in all three fluorophores, but also delivered the highest intensity levels of all fixatives, reaching from approx. 107 (Cy5) up to ~ 109 arbitrary intensity units (Fluos) (see Figure 1.B). Also, 4 % PFA-fixed samples showed the best cell preservation. In contrast to this, lots of lysed cells could be detected in some samples, such as the 6/1/3 anhydrous MetOH-Carnoy-, and 50 % EtOH-fixed ones (see Figure S1.A – I).

Furthermore, the fixative did not only influence the probe targeting and the intensities of the fluorophores, but also the intensity of the DAPI staining, differed between the varying fixatives, showing very strong signals in 4 % PFA fixed samples, but only weak and partly diffuse signals in samples fixed with 50 % EtOH, or one of the MetOH-Carnoy's solutions.

However, it could also be observed, that not every fixative seemed to preserve all different species in the same way. In most fixed samples only rods could be found, with DAPI staining as well as with the EUBmix probe. Only the PFA-fixed sample and that one fixed with 6/6/1 MetOH-Carnoy showed not only short and long rods, but also cocci (see Figure 2.A and C). In contrast to this, in the sample fixed with 6/3/1 EtOH-Carnoy no cocci could be found, only short and long rods could be detected with DAPI, and the general bacterial probe only stained long rods (see Figure 2.B). These differences might be caused by the fixative, but this could also be due to the fact that for each fixative the cecum content from different mice was taken and therefore the microbiota could be different.

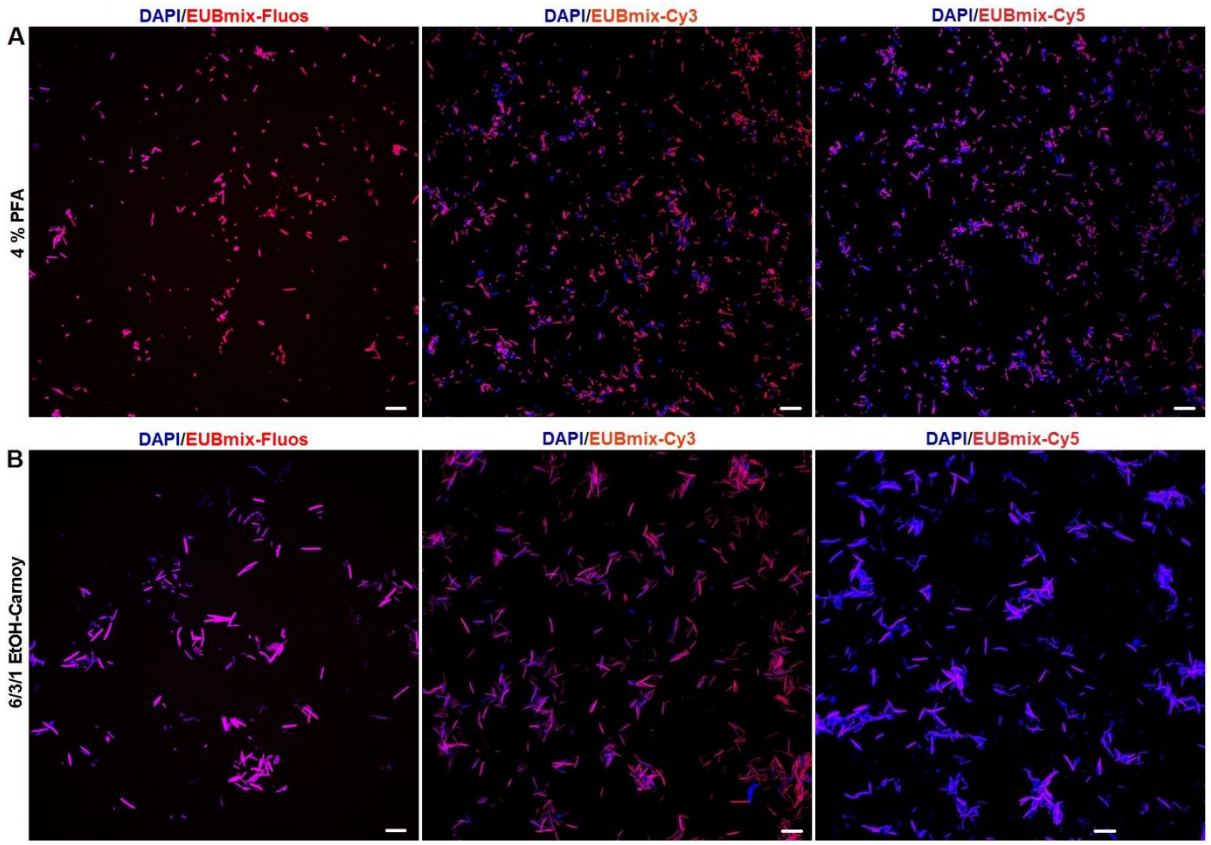
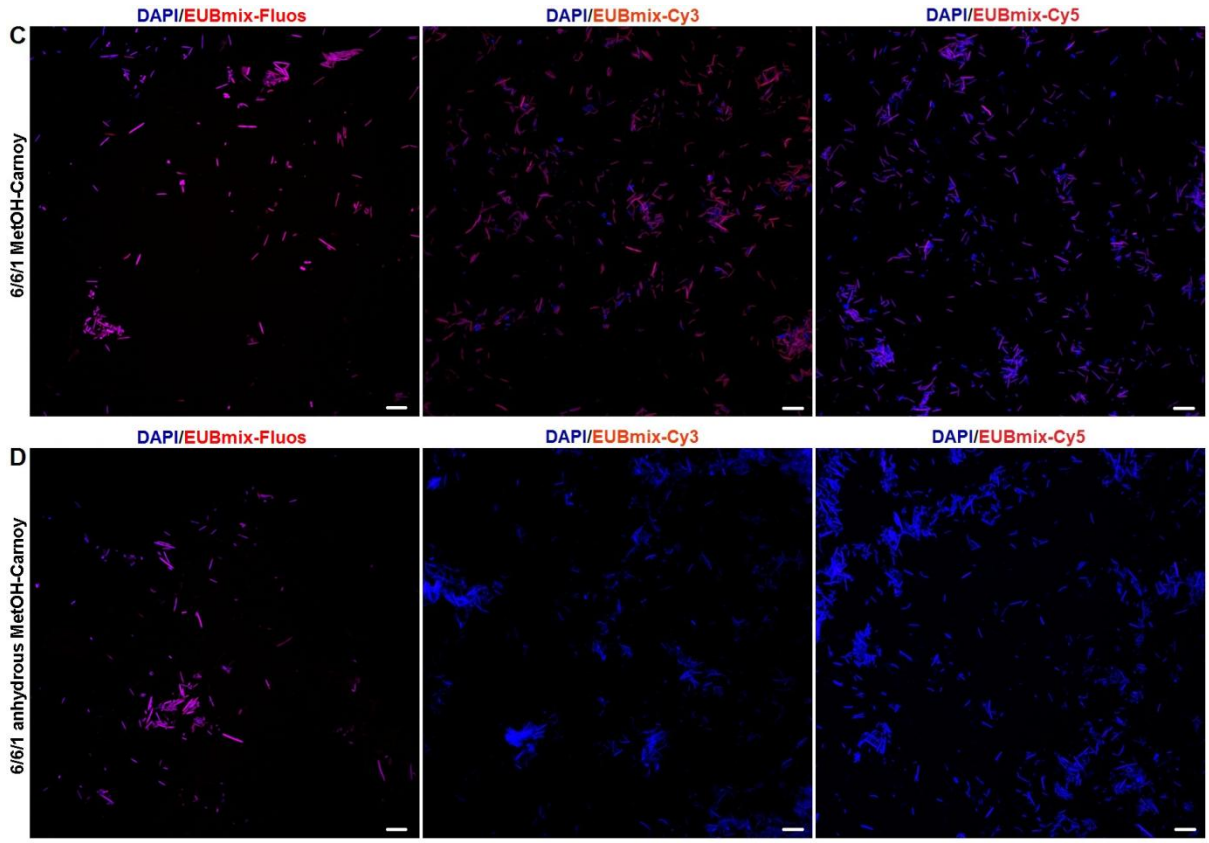


Figure 2: Testing of the general bacterial probe EUB338 I – III (EUBmix) in the fluorophores Fluos, Cy3, and Cy5; cells were counterstained with DAPI Cecum samples fixed with **(A)** 4 % PFA; cocci + rods visible; good signal intensity could be detected. **(B)** 6/3/1 EtOH-Carnoy; only short + long rods detected, no cocci visible; EUBmix probe stained mainly long rods. **(C)** 6/6/1 MetoH-Carnoy; cocci + rods visible; probe mainly stained rods. **(D)** 6/6/1 anhydrous MetOH-Carnoy; mainly long and short rods visible; good DAPI signal in all fluorophores, good visible probe intensity only with Fluos-labelled EUBmix; sample showed lots of lysed cells. Scale bars indicate a length of 10 µm.



(Continued Figure 2)

Due to different cell wall composition, Gram positive and Gram negative cells usually are preserved differently in different fixations, as a fixation with (anhydrous) alcohol might be too harsh for the thinner cell walls of Gram negative bacteria, and therefore might destroy the cell. To determine any effects of the fixation on the cells, more specific probes were used. The probe BAC303 is specific for bacteria of the *Bacteroidales*-group and therefore mainly stains Gram-negative cells with a rather sensitive cell wall. In contrast to this, the Erec482-probe stains Gram-positive bacteria which belong to the *Clostridium* clusters XIVa and XIVb.

The *Bacteroidales*-specific probe only worked for the PFA fixed cecum content samples and for two of the seven Carnoy's recipes (6/3/1 MetOH and 6/6/1 MetOH), but not for any of the fixatives that contained Ethanol (50 % Ethanol, 6/3/1 Ethanol-Carnoy, 6/6/1 Ethanol-Carnoy). However, despite working for the 6/6/1 Methanol-Carnoy, the probe could not be detected for the same Carnoy recipe when anhydrous methanol was used (see Figure 3.A).

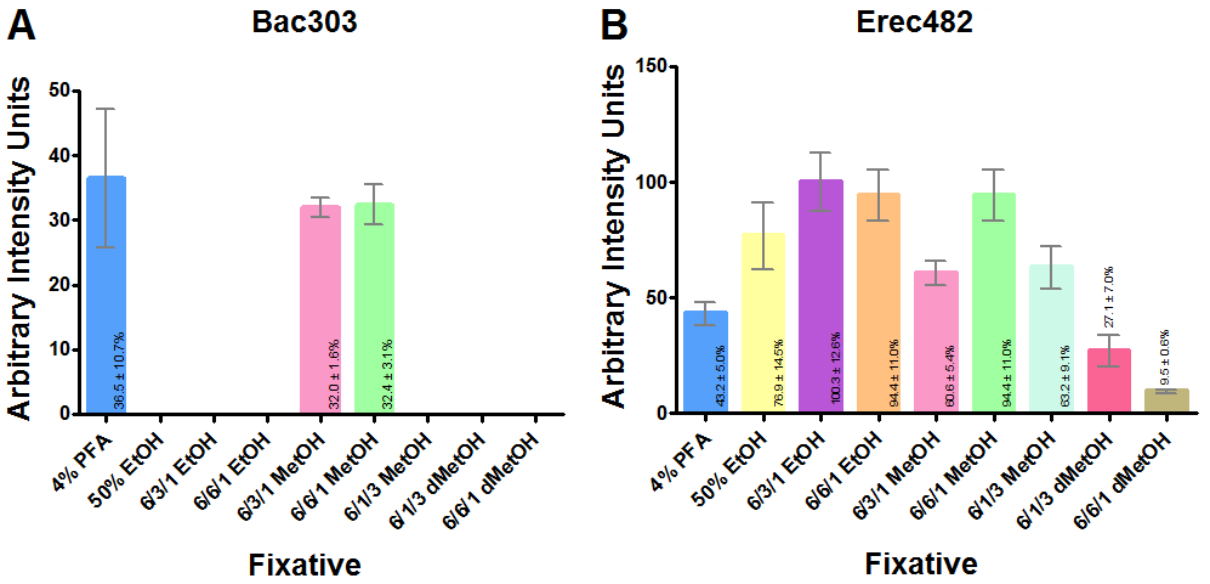


Figure 3: Probe intensity of the probes BAC303 specific for *Bacteroidales*, and Erec482 specific for *Clostridium* cluster XIVa. **(A)** Signals of the *Bacteroidales*-specific probe (BAC303) could only be detected in cecum content fixed with 4 % PFA, 6/3/1 MetOH-Carnoy, and 6/6/1 MetOH-Carnoy. **(B)** Targets of the probe Erec482 could be detected in all nine fixatives, whereas cecal content fixed with 6/3/1 EtOH-Carnoy yielded the highest intensities. Average intensities were determined from 5 representative CLSM pictures using DAIME. Error bars show the standard deviation.

The Erec482 probe, which is specific for *Clostridium* clusters XIVa and XIVb, was detected with a rather low intensity in the PFA-fixed samples (see Figure 3.B). However, higher values were yielded for most of the other fixatives. For 6/3/1 anhydrous EtOH-Carnoy, even a twofold higher intensity compared to PFA fixed samples could be measured (100.3 ± 12.6 arbitrary intensity units). Same as for the EUBmix probe, the cecum content sample fixed with 6/1/3 anhydrous MetOH-Carnoy delivered one of the weakest intensity signals (27.1 ± 7.0 arbitrary intensity units). Again, although samples fixed with 6/6/1 MetOH-Carnoy yielded rather high intensity (94.4 ± 11.0 arbitrary units), it is rather surprising that when this Carnoy composition was used with anhydrous methanol, the weakest signals for the Erec482 probe (9.5 ± 0.6 arbitrary intensity units; see Figure 3.B) were delivered.

The use of specific probes therefore showed that not every fixative is suitable for every probe due to too harsh treatment that leads to destruction of bacterial structure. Furthermore, the effects of the fixation were also noticeable during DAPI staining, which showed high levels of bacterial debris in the samples when treated with a certain fixative (e.g. 50 % EtOH; not shown). Also, it could be observed that the use of different fixatives does not only influence the efficiency and intensity of the FISH-probes, but also of the DAPI staining itself. Again, cecal content fixed with 4 % PFA showed the best DAPI staining, in contrast to this, the use of more aggressive fixatives, such as 50 % EtOH or 6/1/3 anhydrous MetOH-Carnoy, resulted not only in bad probe intensities, but also rather weak signals for DAPI could be observed (see Figure S1..A4 – I4).

4.1.2 Alcian Blue Staining

In previous experiments H&E staining of mouse intestinal tissue fixed with the nine different fixatives it was shown, that tissue fixation with 6/1/3 EtOH-Carnoy and 6/6/1 MetOH-Carnoy resulted in the best structure preservation and also showed the lowest levels of shrinkage of the luminal content. Therefore, colonic tissues fixed with either of these two fixatives, was used for alcian blue staining for visualisation of the mucosal layer. Additionally, 4 % PFA-fixed colon was stained as the FISH probes showed the highest efficiency and intensities in this fixation. Mucin staining was performed with alcian blue at a pH of 2.5, resulting in blue staining of the mucus layer, and also of the luminal content. Epithelial cells were counterstained with nuclear fast red causing reddish staining of the epithelium.

In the first set of samples, tissues were dehydrated in an EtOH-series (see Table S2) before paraffinisation (see Figure 4.A1-3), samples of the second set (see Figure 4.B1-3), were kept in 100 % EtOH for 24 h after fixation, dehydration steps in the tissue processor (see Table **S2**) were skipped, and tissues were immediately paraffinised after the EtOH-incubation to keep these samples water-free. After paraffinisation and sectioning, samples of both sets were rehydrated in an EtOH-series (100, 96, 80, 75, 50 %) before alcian blue staining. Microscopic evaluation clearly shows, that in general tissues which were processed water-free before paraffinisation show better preservation of mucus than the dehydrated ones (see Figure 4). Nevertheless, contrary to samples fixed with 6/1/3 EtOH-Carnoy which were not only kept water-free before and during the process of paraffinisation but also fixed with an anhydrous fixative (containing “dried” alcohol) and therefore kept non-aqueous throughout the whole process of sample preparation, samples fixed with 6/6/1 MetOH-Carnoy and 4 % PFA were kept in an aqueous-containing solution during fixation.

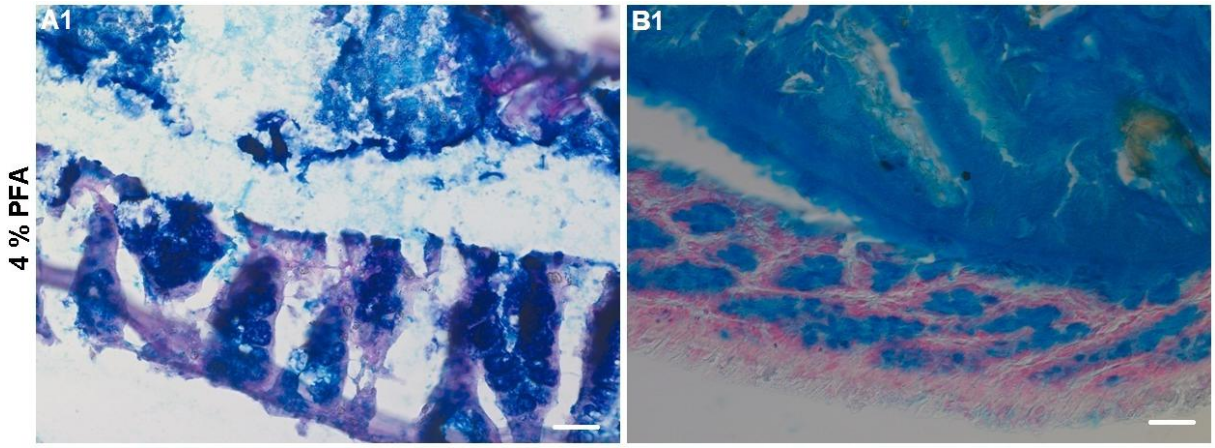
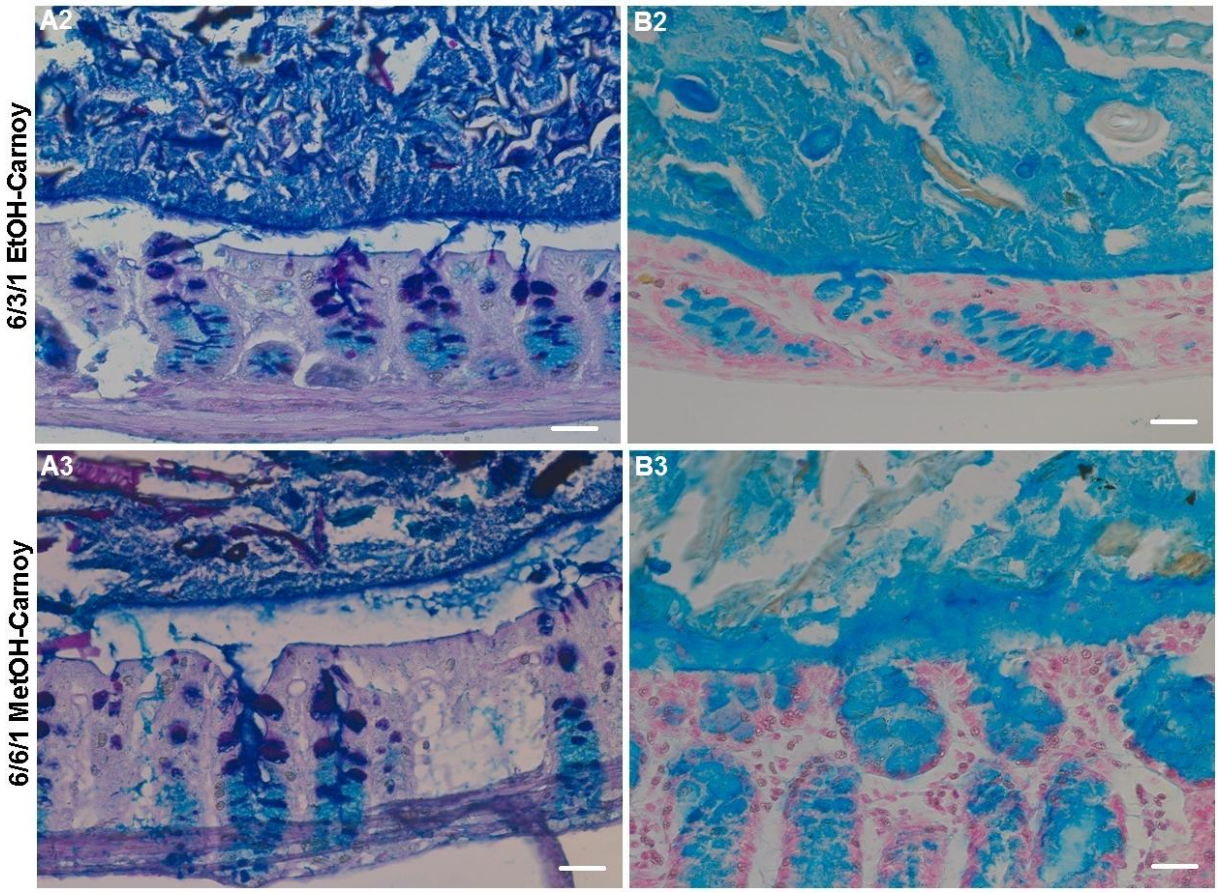


Figure 4: Alcian Blue stained paraffin sections from colon. **(A1 – 3)** Sections were dehydrated in an EtOH-series starting at 70 % after fixation and before paraffinisation. Dehydrated samples show only collapsed mucosal layers or no layer at all. Goblet cells are stained well. **(B1 – 3)** Sections were not dehydrated, but kept in 100 % EtOH after fixation and before paraffinisation. Samples which were not dehydrated, and therefore kept water-free until paraffinisation, show clear mucus layers and good staining of goblet cells. Also the reddish colour of the nuclear fast red counterstaining is better differentiable. Scale bars indicate 200 µm. Magnification: 40x



(Continued Figure 4)

Fixation with 4 % PFA resulted in a rather strong disruption of the tissue structure, whereas in dehydrated samples also the luminal content was not preserved too well (see Figure 4.A1). Also, mucus in goblet cells seems to be stained rather strong, but no mucus layer is left between the epithelium and the luminal content. In contrast to this, colonic tissue fixed with 6/3/1 EtOH-Carnoy and 6/6/1 MetOH-Carnoy show better tissue preservation, but nevertheless, also no clear mucus layer is visible, but only narrow collapsed lines of the mucus can be detected at the edges of the epithelium and the luminal content (see Figure 4.A2 – 3).

Contrary to the bad structure preservation and the lack of the mucus layers in the dehydrated samples of the first set of samples, colonic tissues which were kept water-free throughout the processing showed good structure preservation. Not only the epithelium and the luminal content can be differentiated, but also the mucus layer between these two is clearly recognisable. Also, goblet cells in all sections show good structures and can be differentiated from the surrounding epithelial tissue (see Figure 4.B1 – 3). Nevertheless, although the mucus staining seems to have worked perfectly, the mucus layer mostly is only distinguishable due to structure differences as the luminal content shows the same bluish colour as the stained mucin does.

However, whereas tissue sections fixed with 6/3/1 EtOH-Carnoy show perfect preserved structures, both in tissue and in mucus layer (see Figure 4.B2), 4 % PFA-fixed colon shows good tissue preservation and also staining of mucin, but nevertheless, the mucus is not stuck to the epithelium all over the whole section (see Figure 4.B1). In contrast to this, colonic tissue fixed with 6/6/1 MetOH-Carnoy seems to show good preservation of epithelium and mucus, but no passage from the mucus to the luminal content is clearly visible and also, the luminal content itself is not as well preserved as the rest of the section (see Figure 4.B2).

Furthermore, samples that were processed under water-free conditions did not only show better results in preservation of tissue structures and of the mucus layer, but also in mucus staining in general. These samples show a clear blue colour of the stained mucin in the mucus layer and the goblet cells, and counterstained epithelial cells can be clearly differentiated due to its pinkish colour. In contrast to this, samples which underwent dehydration before paraffinisation show a blue-violet colour over the entire section after the alcian blue staining, what makes distinguishing the different parts difficult (see Figure 4).

4.1.3 Lectin Staining

Alcian blue staining delivers good results when it comes to visualisation of mucus, but nevertheless, this staining method is not combinable with a FISH. Therefore, to determine both – bacteria and mucus – on one and the same sample, another staining technique is necessary. Therefore, 6 µm thick dewaxed paraffin section from the same colonic tissue fixed with 6/3/1 EtOH-Carnoy were stained with the lectin Jacalin. To ensure the presence of mucus, the sections from the same tissue, which was used for the alcian blue staining, was used for the lectin staining.

The first set of sample was stained according to the manufacturer's recommendations. Therefore, sections were blocked with 3 % H_2O_2 in PBS, for washing steps and diluting the Jacalin 0.02 M $NaHCO_3$ was used as buffer. Nuclei of the epithelial cells were counterstained with DAPI for a better orientation on the section.

Different dilutions of Jacalin were used to determine the concentration with the best signal intensity and the least unspecific signals. As evaluation at the CLSM did not show any significant differences, representative pictures of staining with 60 µg Jacalin/mL buffer are shown in Figure 5. This concentration was also used for further experiments with a different protocol (Figure 6).

Visualisation of the TexasRed®-labelled Jacalin on the CLSM showed that only a small collapsed mucus layer inside of goblet cells could be stained (see Figure 5.A). Furthermore, only very little signal of a narrow, collapsed layer of secreted mucus could be detected in a

gap between two crypts (see Figure 5.B). Nevertheless, despite resulting only in rather weak signals, due to the negative control, where the Jacalin was inhibited by pre-incubation with D-(+)-Melibiose, signals do not seem to be false positive ones (see Figure 5.D).

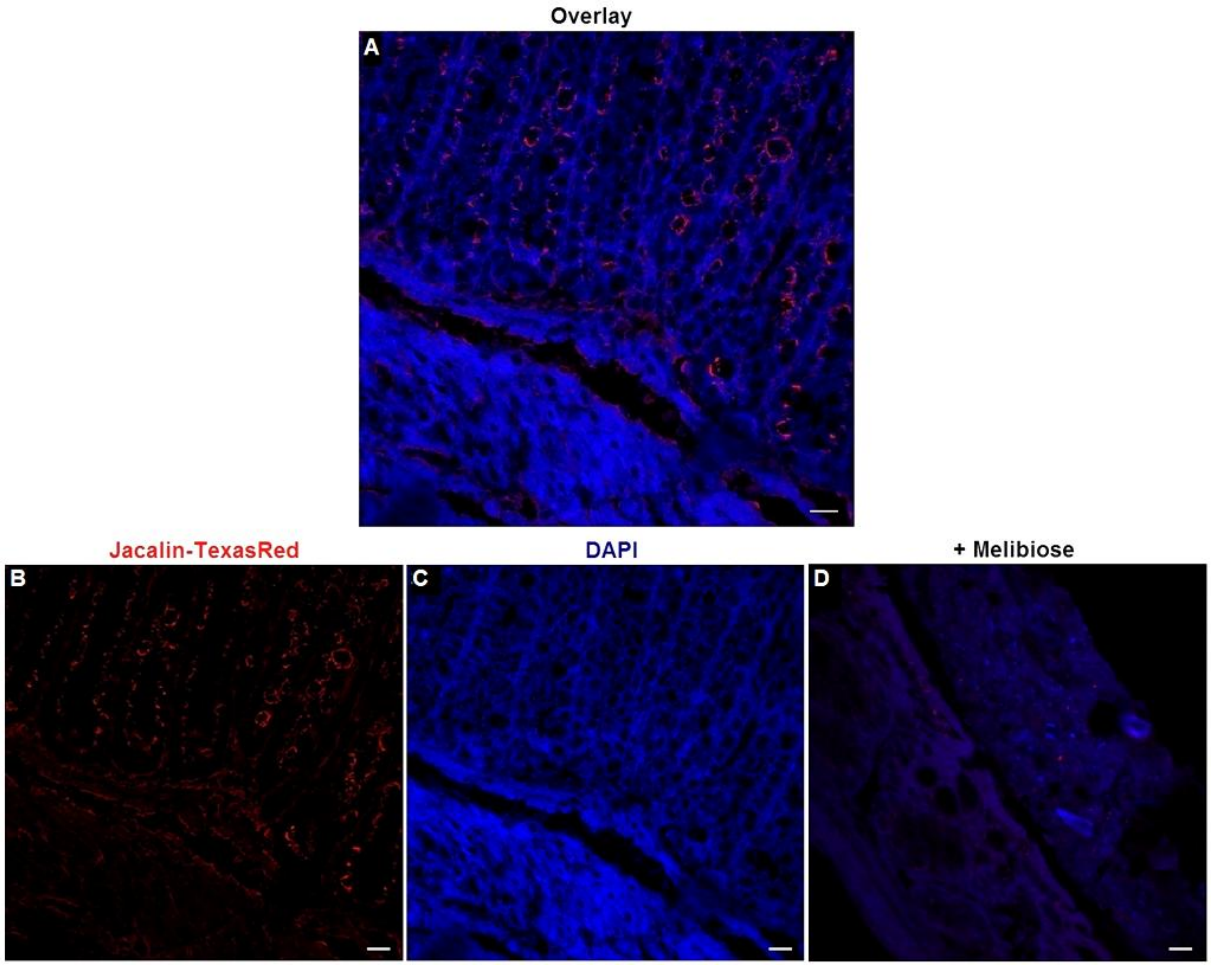


Figure 5: Jacalin staining of colonic tissue fixed with 6/3/1 EtOH-Carnoy blocked with **H_2O_2**; **(A)** Overlay; blue shows the epithelial tissue (DAPI), red shows Jacalin-stained mucus; **(B)** Mucus stained with Jacalin **(C)** DAPI-stained epithelial tissue; (A – C) show that with H_2O_2 blocking and $NaHCO_3$ as a buffer results in staining of mucus inside goblet cells, only weak signals for secreted mucus could be detected. **(D)** Negative control: Jacalin was inhibited by pre-incubation with D-(+)-Melibiose. Scale bars indicate 10 µm. Magnification: 40x

To get higher signal intensities of the Jacalin-TexasRed®, and also for better staining of secreted mucus, a different protocol[49] was applied. For this, sections were blocked with 1x PBS containing 0.3 % Triton X-100 and 1 % BSA. For washing and diluting the Jacalin, 1x PBS was used. To make the results comparable, for the evaluation of this set of samples the same settings (gating, gain, laser intensity, etc.) as for the sections of the first set were used at the CLSM.

Figure 6.A shows that the different protocol indeed did enhance signal intensity of the Jacalin, resulting in clear staining of freshly produced mucus in the goblet cells (see Figure 6.B). The negative control (see Figure 6.D) again showed that these signals seem not to be due to unspecific binding of the lectin. Nevertheless, despite getting good staining of

mucus inside of goblet cells, no staining of the mucus layer between epithelium and luminal content could be observed.

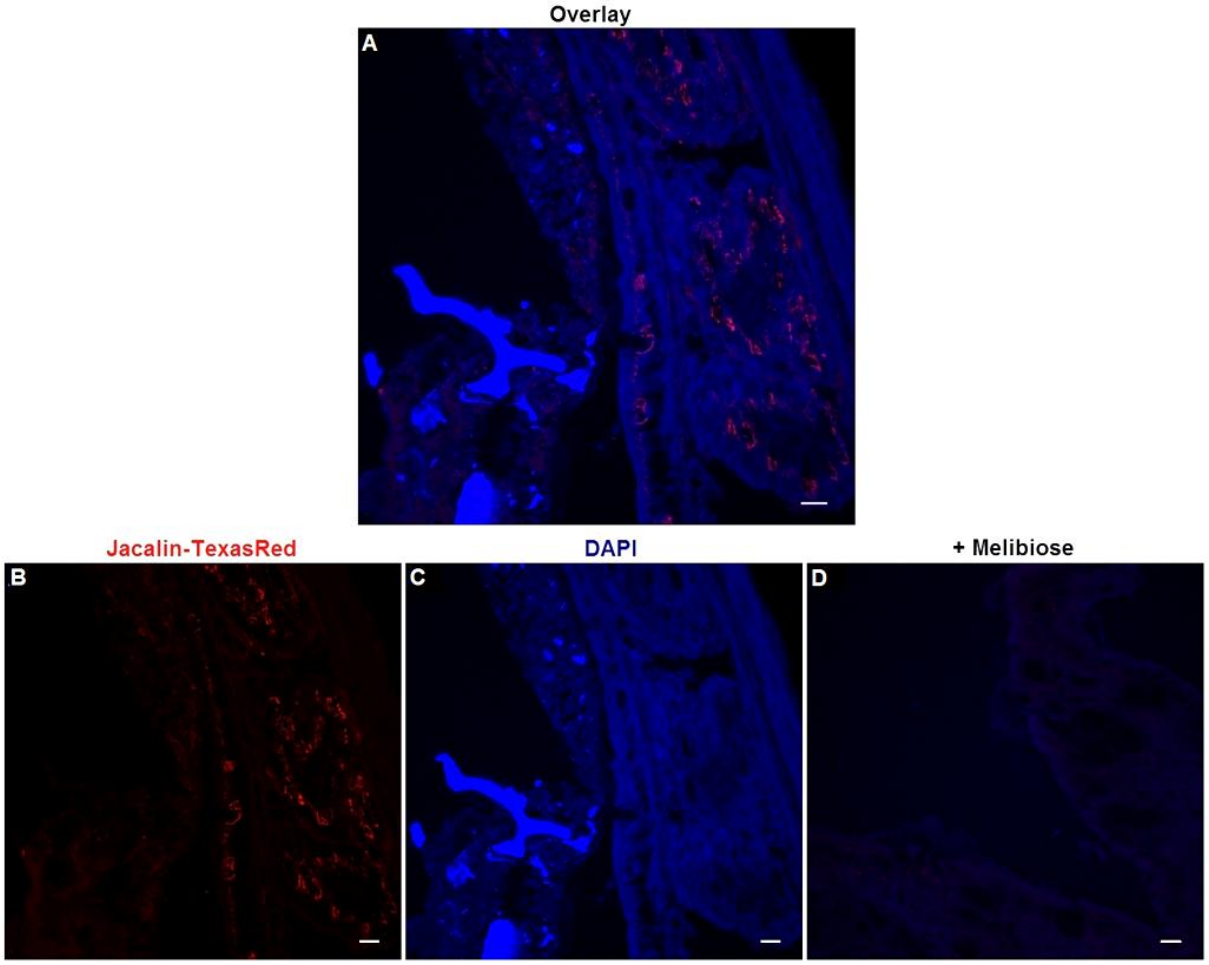


Figure 6: Jacalin staining of colonic tissue fixed with 6/3/1 EtOH-Carnoy blocked with **1x PBS containing 0.3 % Triton X-100 and 1 % BSA**; **(A)** Overlay; blue shows the epithelial tissue (DAPI), red shows Jacalin-stained mucus; **(B)** Mucus stained with Jacalin **(C)** DAPI-stained epithelial tissue; (A – C) show that with H_2O_2 blocking and $NaHCO_3$ as a buffer results in staining of mucus inside goblet cells, only weak signals for secreted mucus could be detected. **(D)** Negative control: Jacalin was inhibited by pre-incubation with D-(+)-Melibiose. Scale bars indicate 10 µm. Magnification: 40x

Nevertheless, no clear staining – neither of goblet cells, nor of secreted mucus – could be detected. Furthermore, the presented results were not reproducible, therefore other immunohistochemical methods (anti-MUC2-antibody) were tested.

4.1.4 MUC2 Antibody Staining

As only poor staining of the intestinal mucus layer was observed in Jacalin staining, the *O*-glycosylated gel-forming mucin MUC2 was visualised using an antibody. For visualisation of this, a secondary antibody labelled with Cy3 was used. 6 µm thick dewaxed and rehydrated paraffin sections fixed with 6/3/1 EtOH-Carnoy were used for the immunohistochemical staining.

When the antibody-staining was applied to sections of colonic tissue fixed with 6/3/1 EtOH-Carnoy, it was shown, that tissue structures are well preserved, and that the intestinal mucus layer (anti-MUC2-antibody, red) can be clearly distinguished from the epithelium and the luminal content (DAPI, blue) after incubation with a mucin specific antibody (see Figure 7). However, despite yielding high signal intensities for the labelled mucins, the epithelial tissue also showed a rather high autofluorescence in the Cy3-channel.

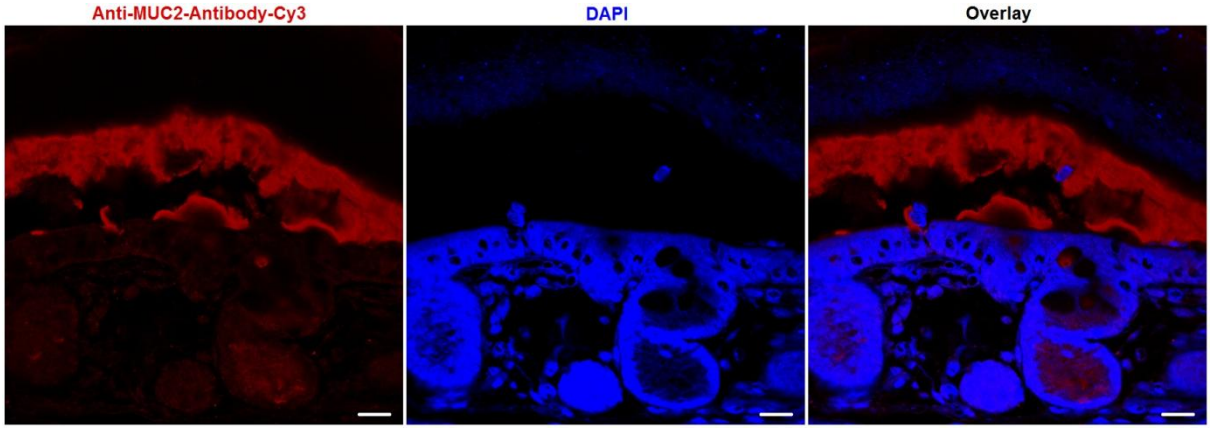


Figure 7: Staining of the intestinal mucus layer in mouse gut on 6/3/1 EtOH-Carnoy-fixed tissue using anti-MUC2-antibody in Cy3. MUC2 staining results in good visualization of the mucosal layer between the epithelium and the luminal content. Scale bars indicate 15 µm. Magnification: 63x

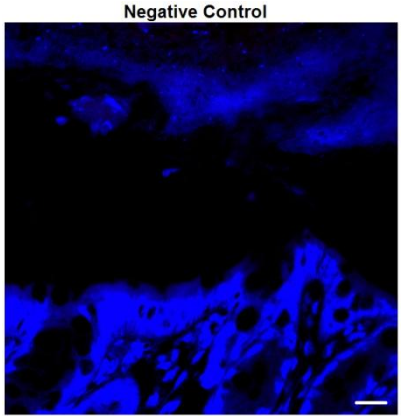


Figure 8: Negative control for the anti-MUC2-antibodystaining. For this, sections were incubated only with the secondary antibody in Cy3. Scale bar indicates 15 µm. Magnification: 63x

Furthermore, also non-secreted mucus could be detected in the goblet cells, but these mucins are not stained as strongly as the secreted mucus layer (see Figure 7).

In contrast to this, no signals could be detected on sections incubated with the Cy3-labelled secondary antibody only, showing that the staining of mucin did not result from unspecific binding of the secondary antibody (see Figure 8).

To determine the distribution of bacterial communities associated with the intestinal mucus layer, the antibody-staining of mucin was combined with fluorescence *in-situ* hybridisation using the bacterial-specific oligonucleotide probe mix EUBmix-Fluos on sections of mouse colon fixed with either 4 % PFA, or with 6/3/1 EtOH-Carnoy.

In general, combining FISH with antibody-staining of mucins on sections fixed with the different fixatives worked out for all tested samples. Nevertheless, despite resulting in detectable signals for FISH, and allowing visualisation of intestinal mucus at one time, differences in signal intensity of the bacterial labelling, and also in preservation of structures

could be observed. Microscopical evaluation of 4 % PFA-fixed sections shows that the combined staining results in good FISH signals, but although the mucus staining with antibody yields good signals, only a collapsed mucus layer at the edges of the epithelium and the luminal content could be detected (see Figure 9.A). In contrast to this, combining FISH and mucus staining on sections fixed with 6/3/1 EtOH-Carnoy resulted in good signal intensities of the antibody staining, and also reveals good preservation of the mucus layer. Nevertheless, hybridisation of the sections with the general bacterial probe EUBmix in Fluos yielded only rather weak signals of the bacterial staining (see Figure 9.B).

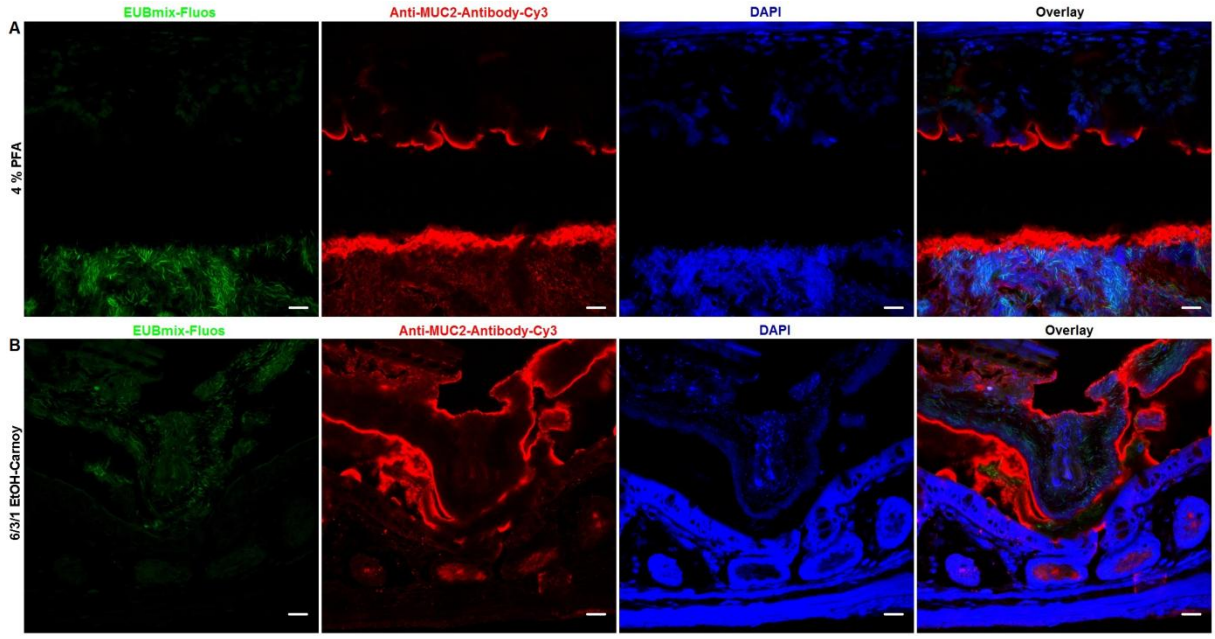


Figure 9: Combination of anti-MUC2-antibody staining and FISH with the bacterial probe mix EUBmix-Fluos on sections fixed with 4 % PFA or 6/3/1 EtOH-Carnoy. **(A)** Combined staining on sections fixed with 4 % PFA show good FISH signals, but only a collapsed mucus layer could be detected. **(B)** Combined staining on sections fixed with 6/3/1 EtOH-Carnoy shows good preservation of the mucus layer, but FISH staining shows only very low signals. Scale bars indicate 15 µm. Magnification: 63x.

To check whether the pre-treatment of the sections (antigen retrieval, blocking), or the incubation with the MUC2-specific antibody after the FISH staining had an effect on the probe signal intensity, another set of sample was tested at different condition. Pre-treatments (antigen retrieval with sodium citrate buffer, blocking with 1 % BSA in PBS) were performed equally on all sections. Furthermore, for comparison, FISH was performed on sections fixed with 4 % PFA and 6/3/1 EtOH-Carnoy which did not undergo any pre-treatment. Those sections showed good signals in the Fluos-channel for the bacterial EUBmix probe, whereas different fixations lead to preservation of different bacterial species. Sections fixed with 4 % PFA showed all morphologies from cocci to rods (see Figure 10.A). In contrast to this, in 6/3/1 EtOH-Carnoy-fixed sections, mainly long rods are stained with the general EUBmix probe (see Figure 10.B).

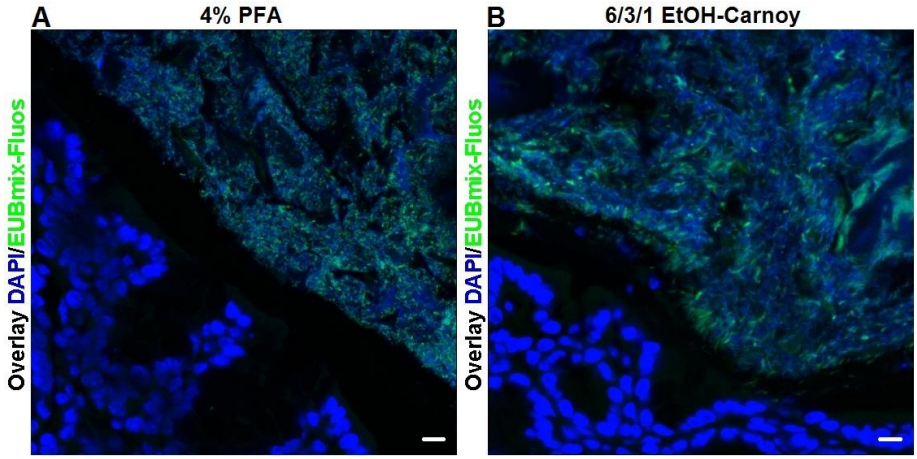


Figure 10: FISH on a dewaxed paraffin section of colonic tissue. **(A)** Tissue fixed with 4 % PFA. Sections show good and clearly distinguishable signals for the probe mix EUBmix in Fluos. Bacteria show all morphologies from cocci to rods. **(B)** Hybridisation of sections fixed with 6/3/1 EtOH-Carnoy result in higher probe signal intensities, whereas mainly long rods are preserved by this fixation. Scale bars indicate 10 µm. Magnification: 63x

In the next step, FISH was performed on dewaxed 6/3/1 EtOH-Carnoy-fixed sections of colonic tissue after antigen retrieval and blocking. The first set of samples was embedded and evaluated directly after the hybridisation (see Figure 11.A). Two other sets of samples were hybridised with the general bacterial probe, followed by the antibody-staining protocol in the absence of antibody. For this, sections were incubated only with either hybridisation buffer (HB; see Figure 11.B), or with blocking buffer (BSA; see Figure 11.C) to determine the effects of the solution used for the antibody-staining on the FISH signals.

However, all treatments resulted in weak probe intensities and furthermore, also strong background signals in the Fluos channel. Sections which were not further incubated after the fluorescence *in-situ* hybridisation showed only slightly stronger signals (see Figure 11). Nevertheless, as, signals cannot be differentiated in the different sets compared to signals of the untreated sections (see Figure 10), it can be assumed, that the FISH is affected by the pre-treatment steps of antigen retrieval and/or blocking.

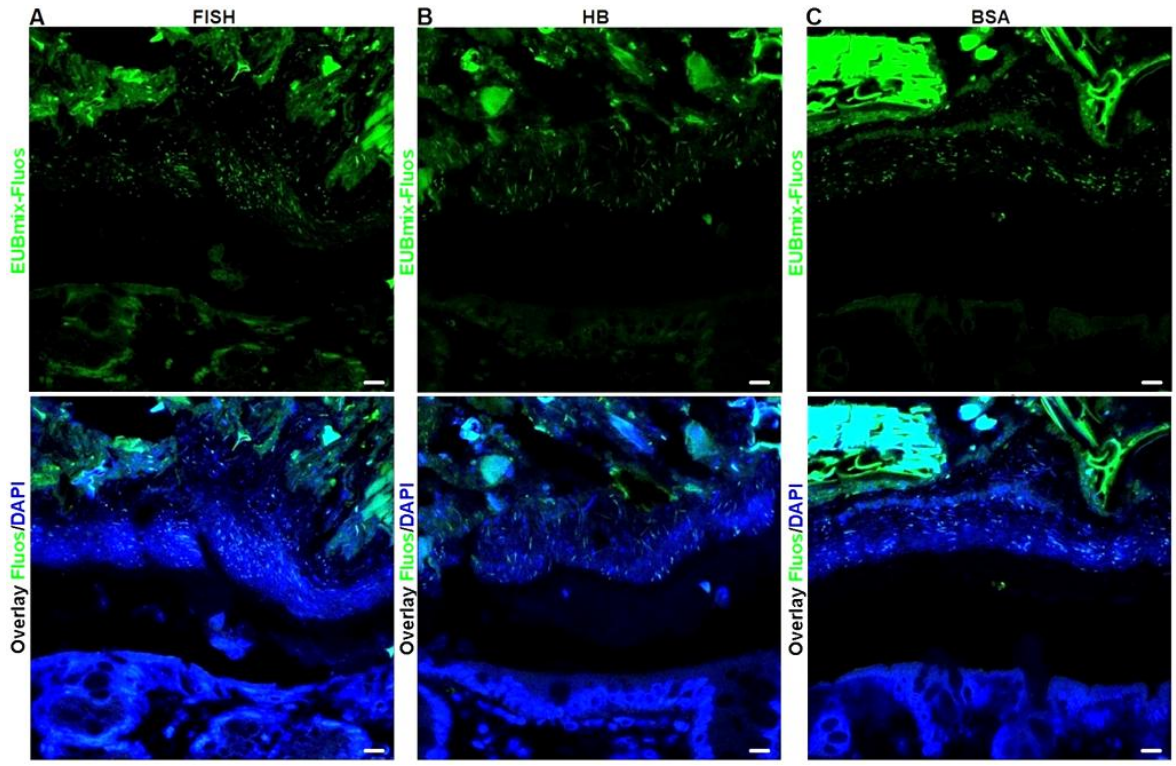


Figure 11: Different protocols for FISH staining on 6/3/1 EtOH-Carnoy-fixed sections. **(A)** Sections were only stained with the EUBmix FISH probe mix in Fluos and checked for signal intensities. **(B)** Sections were hybridised with EUBmix, followed by the protocol for MUC2 antibody staining without adding antibody. Sections were incubated with hybridisation buffer (HB) ON. **(C)** Sections were hybridised with EUBmix in fluos, followed by the standard protocol for mucin staining without adding the antibody. Sections were incubated with 1 % BSA. Scale bars indicate 10 µm. Magnification: 63x

After checking the effects of the different protocols on the fluorescence *in-situ* hybridisation, the primary and secondary antibodies were added to the incubation to see whether the different solutions also have any effects on the antibody itself, and the preservation of the mucosal structure. It was shown, that in MUC2 incubation with blocking buffer (1 % BSA in PBS) the mucus layer between epithelium and luminal content was clearly distinguishable (see Figure 12.A2), but despite labelling single cells in FISH, the intensity of the bacterial EUBmix-probe in Fluos (see Figure 12.A3) was not as strong as the ones from FISH on sections without pre-treatments (see Figure 10.A).

In contrast to this, despite showing a rather high background in the Fluos-channel, FISH staining resulted in higher signal intensity on sections on which antibody-staining was performed with hybridisation buffer (see Figure 12.B3). However, on this sections signal intensity for the Cy3-stained mucins in the intestinal mucus layer was not as strong and clear, and also resulted in a high background in the Cy3-channel (see Figure 12.B2). Furthermore, the antibody-staining of the mucin revealed a rather collapsed and patchy mucus layer when the protocol was performed with HB. In general, also the luminal debris, such as undigested fibres and food remains, show high unspecific signals in Fluos (see Table 6).

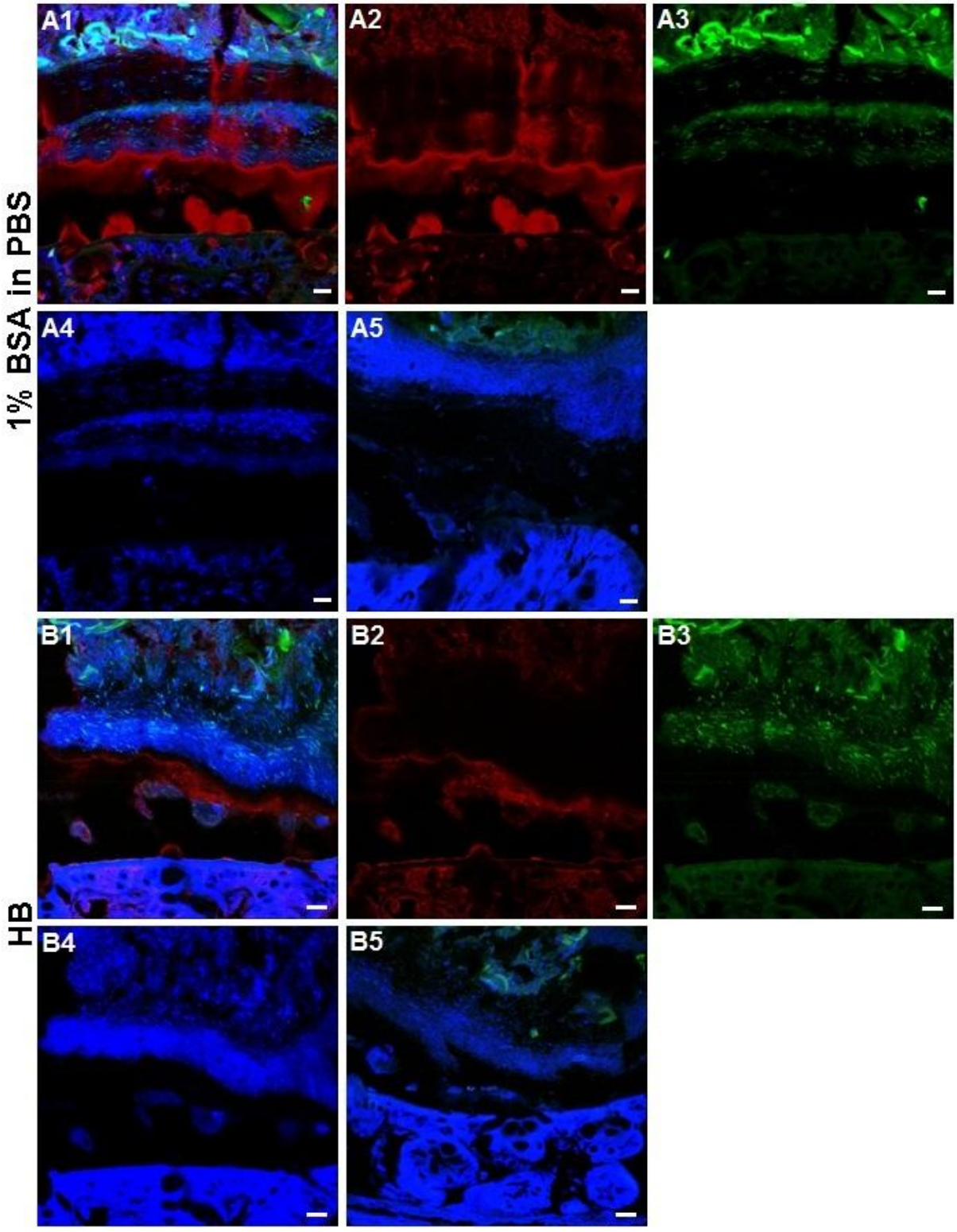


Figure 12: Combination of FISH and anti-MUC2-antibody staining on sections of colonic tissue fixed with 6/3/1 EtOH-Carnoy. **(A1-4)** FISH was performed on sections, afterwards MUC2-antibody staining was performed using blocking buffer (1 % BSA in PBS). The EUBmix-probe shows rather weak signals, but nevertheless, single cells can be detected **(A3)**. The antibody staining results in bright signals showing good preservation of the mucus layer (A2). **(B1-4)** MUC2-antibody staining was performed on dewaxed sections after FISH using hybridisation buffer (HB) for diluting the primary and secondary antibodies. FISH signals are rather strong **(B3)**, but evaluation of the antibody staining reveals weak signals, and a high background **(B2)**. **(A5, B5)** Negative control. FISH was performed using the NONEUB probe in Fluos, antibody staining was performed only with the secondary antibody in Cy3 diluted in 1 % BSA **(A5)**, or in HB **(B5)**. Scale bars indicate 15 µm. Magnification: 63x

Table 6: Evaluation of the different staining combinations of FISH and MUC2

	Quality of Bacterial Staining	Quality of Mucin Staining
MUC2 Staining Only	-	clear staining of mucosal layer, brush borders, and goblet cells; mucosal layer clearly distinguishable from luminal content and epithelium
FISH Only	clear staining of luminal bacteria; good probe signal intensities, single cells identifiable	-
FISH After Pre-Treatment	weak probe intensity; high auto-fluorescence of luminal debris and epithelial tissue	-
FISH + MUC2-HB	weak probe intensity, mainly long rods recognisable; rather high background signals	clear mucin staining, but mucosal layer rather patchy and collapsed; high background in Cy3-channel
FISH + MUC2-BSA	weak probe intensity, rods and cocci recognisable; rather high background signals of the epithelium; strong unspecific signals of luminal debris	clear staining of mucosal layer, brush borders, and goblet cells; mucins markedly destinguishable from tissue; rather weak unspecific signals of background

4.2 Effects of Bacterial Administration on DSS-Induced Colitis

4.2.1 Effects of *Akkermansia* Administration on Body Weight

Three different strains of *Akkermansia spp.* (phylogenetic intermediates) were gavaged to 15 weeks old male C57BL/6 WT mice. The human strains MucT and H2, and the mouse strain MG were gavaged at a concentration of 10^9 cfu/200 µL, both viable and heat inactivated, as well as PBS containing 2.5 % glycerol to a group of four mice as a control. After seven days of *Akkermansia* supplementation, 2 % DSS were administered to the mice over the drinking water to provoke colitic inflammation.

In the first days of supplementation an unexpected effect could be observed, as mice gavaged with MucT viable and H2 viable strains rapidly lost weight after already the first gavage (see Figure 13). On the fourth day of supplementation, these groups of mice reached the lowest average weight (see Table 7), whereas the group of H2 viable-fed mice yielded the lowest average weight of all groups at day -3 (80.5 ± 4.2 %). In contrast to this, all other groups did not show significant differences compared to the control group, which was gavaged with PBS/2.5 % glycerol solution.

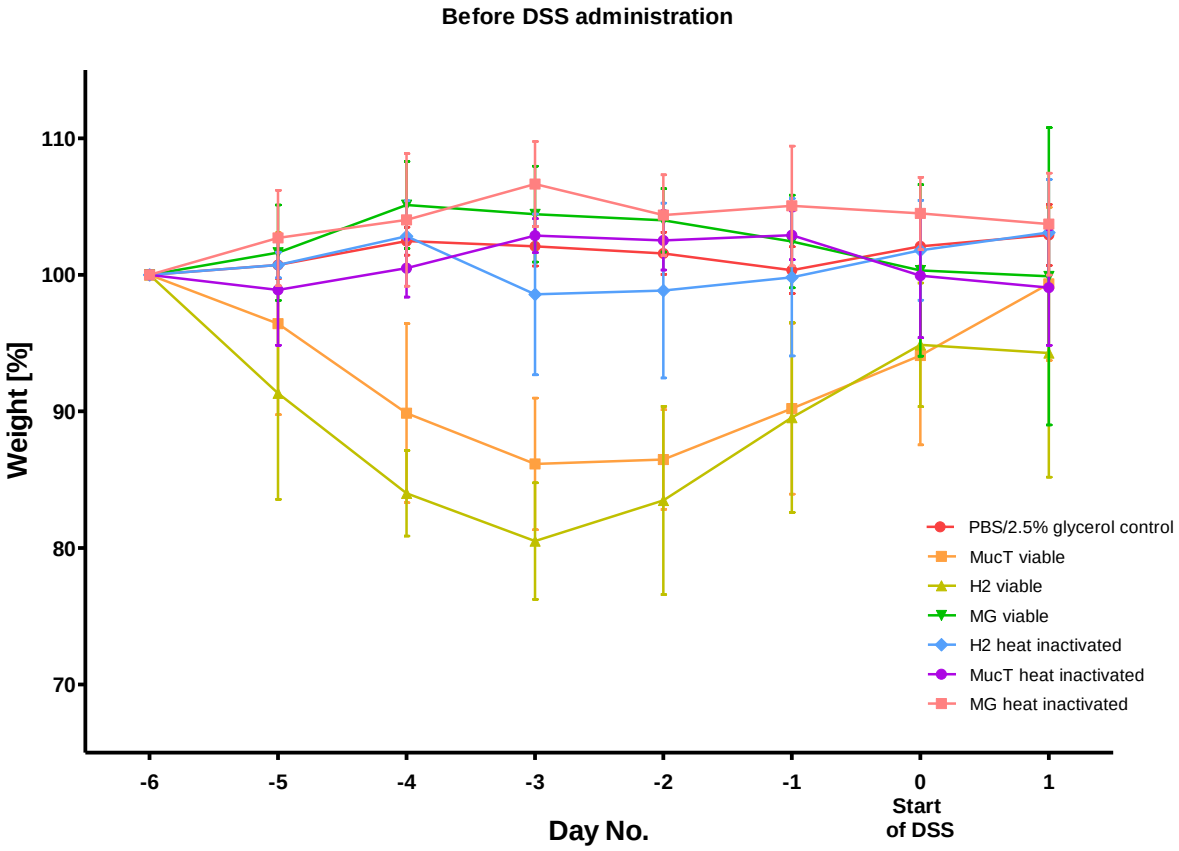


Figure 13: Weight curve of the mouse groups treated with different *Akkermansia* supplementations before start of treatment with 2 % DSS (day -6 to 0). The curves show an unexpected effect of *Akkermansia* on mice resulting in a strong and rapid weight loss in the first day of supplementation of the viable H2 and MucT strains.

Except of the group fed with the heat inactivated H2-strain (see Figure 13) all groups show increasing average weight values throughout the first days, whereas rather stable values are reached after day -4 and kept until DSS treatment started. In contrast to this, mice fed with the heat inactivated H2-strain showed a reduction of the average weight at day -3 (98.6 ± 5.9 %), whereas weight again increased over the next days until DSS treatment started (see Table 7).

Table 7: Average weight [%] of the seven different groups of treatment normalised to original body weight [g]. Average values were determined of 4 mice per group.

Day #	PBS/2.5 Glycerol Control	MucT Viable	H2 Viable	MG Viable	H2 Heat Inactivated	MucT Heat Inactivated	MG Heat Inactivated
-6	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
-5	100.7 ± 1.0	96.5 ± 6.7	91.3 ± 7.8	101.6 ± 3.5	100.7 ± 0.9	98.9 ± 4.0	102.7 ± 3.5
-4	102.5 ± 1.0	89.9 ± 6.6	84.0 ± 3.1	105.1 ± 3.2	102.8 ± 2.6	100.5 ± 2.1	104.0 ± 4.8
-3	102.1 ± 1.4	86.1 ± 4.8	80.5 ± 4.2	104.5 ± 3.5	98.6 ± 5.9	102.9 ± 1.3	106.7 ± 3.1
-2	101.6 ± 1.6	86.5 ± 3.7	83.5 ± 6.9	104.0 ± 2.3	98.9 ± 6.4	102.5 ± 2.2	104.4 ± 3.0
-1	100.3 ± 1.7	90.2 ± 6.3	89.6 ± 7.0	102.4 ± 3.4	99.8 ± 5.7	102.9 ± 1.8	105.0 ± 4.4
0	102.1 ± 2.2	94.1 ± 6.6	94.9 ± 4.5	100.3 ± 6.3	101.8 ± 3.7	100.0 ± 4.5	104.5 ± 2.7
1	102.9 ± 2.2	99.3 ± 5.6	94.3 ± 9.1	99.9 ± 10.9	103.1 ± 3.9	99.1 ± 4.2	103.7 ± 3.7
2	101.9 ± 2.7	100.3 ± 3.4	92.3 ± 12.2	96.2 ± 8.4	98.3 ± 2.2	98.3 ± 3.6	100.2 ± 7.6
3	100.6 ± 4.4	99.8 ± 3.2	99.0 ± 2.1	96.5 ± 7.7	100.7 ± 2.1	99.6 ± 1.6	98.9 ± 9.2
4	101.1 ± 4.8	98.7 ± 3.0	96.6 ± 2.6	97.2 ± 8.4	103.4 ± 2.9	101.6 ± 1.6	97.8 ± 10.0
5	100.2 ± 3.4	95.5 ± 2.2	91.8 ± 9.8	96.4 ± 7.6	103.8 ± 3.7	97.9 ± 4.0	96.6 ± 11.8
6	96.9 ± 2.7	92.4 ± 1.5	88.1 ± 11.7	92.0 ± 6.8	101.2 ± 2.7	93.2 ± 1.3	92.0 ± 11.6
7	91.8 ± 1.6	87.1 ± 2.4	87.3 ± 10.3	90.1 ± 2.2	95.7 ± 3.0	89.8 ± 2.3	89.3 ± 2.3
8	84.2 ± 3.5	77.5 ± 4.0	77.3 ± 10.7	83.5 ± 2.7	89.4 ± 4.4	80.1 ± 3.5	82.5 ± 6.7

However, after 6 days of *Akkermansia* supplementation, mice additionally received 2 % DSS over the provided drinking water to provoke gastrointestinal inflammation. Compared to the control group gavaged with 200 µL PBS/2.5 % glycerol, none of the other groups showed significant differences concerning the average weight loss (see Figure 14). However, only the group of mice supplemented with heat inactivated Akkermansia from the H2 strain showed slightly higher average weight values than the ones of the control group (see Table 7). After the minor weight loss of this group at day -3, the average weight increased slowly with each

day to reach the average level of the other groups at day 1 (103.1 ± 3.9 %). However, at day 2 there was another decrease of the average weight of the heat inactivated H2-group (98.3 ± 2.2 %), but nevertheless, the weight increased rapidly, reaching levels slightly beyond the average weight values of the control group (see Table 7) whereas this effect lasted until the end of the experiment (see Figure 14).

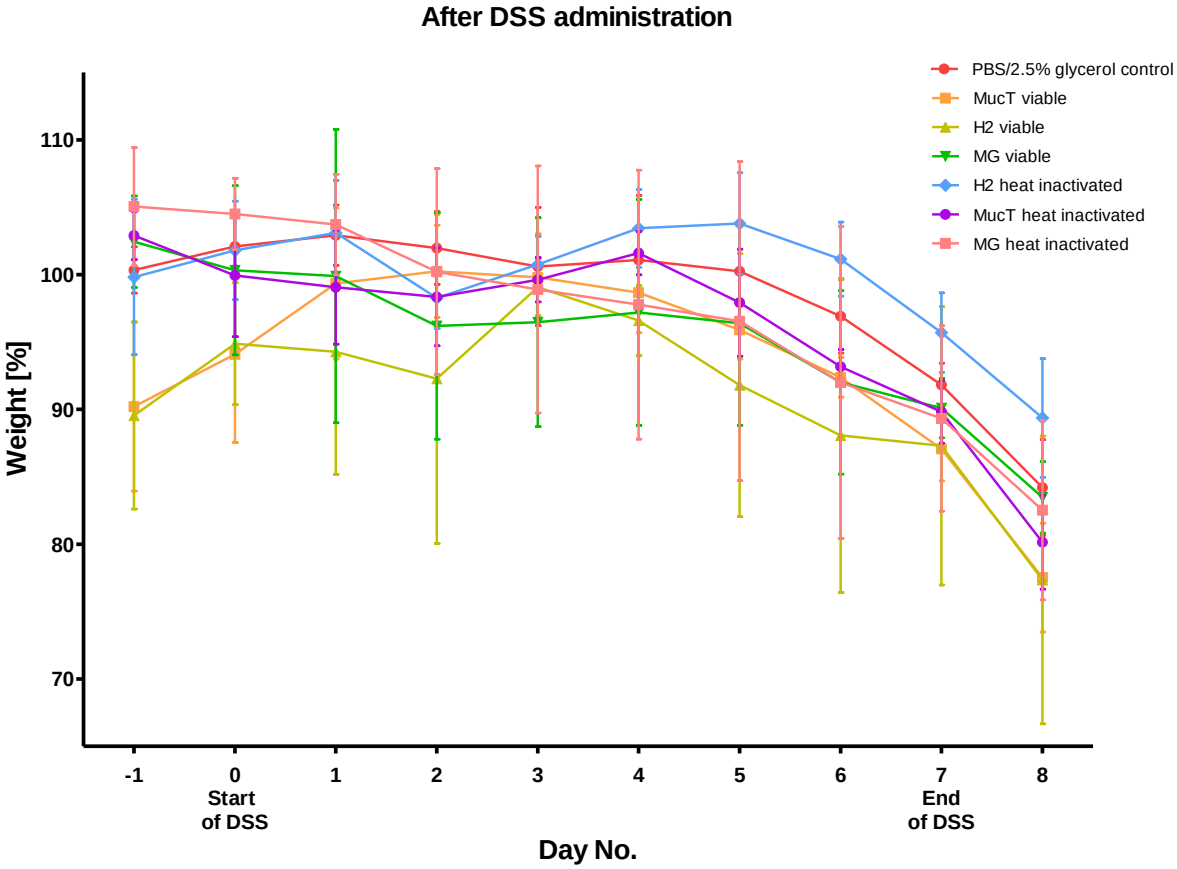


Figure 14: Weight curve of the mouse groups treated with different *Akkermansia* supplementations after start of treatment with 2 % DSS (day 0 to 8). The graph shows, that *Akkermansia* supplementation does not affect weight loss due to colitis, as mice gavaged with *Akkermansia* do not lose less weight than mice of the control group. However, also no negative effect due to the supplementation could be observed.

After the rapid weight loss in the first days of *Akkermansia* supplementation in the groups of mice gavaged with viable MucT strains, the average weight values increased reaching average levels compared to the control group at day 1 (99.3 ± 5.6 %). In contrast to this, the group of which mice were administered viable H2-*Akkermansia* showed another decrease at days 1 and 2 resulting in an average weight [%] as low as 92.3 ± 12.2 %. However, this decrease is due to only one mouse of the group rapidly losing weight after the start of DSS administration (see Table S6). The following increase of the group's average weight [%] at day 3 (99.0 ± 2.1 %) is due to the loss of this one single mouse, which had to be killed as its weight loss was more than 30 % of its original weight. All the other groups did not show any significant differences in their average weight [%] due to DSS administration or *Akkermansia* supplementation compared the control group (see Figure 14).

However, qPCR for detection of *Akkermansia*-DNA in the faeces of the different mice collected at day -6 and after 4 days of gavaging correlates with the weight curves before DSS-treatment started. Whilst no DNA *Akkermansia* could be detected in any of the mice at day -6, the amount of detected *Akkermansia*-DNA increased in faeces samples four days after the start of *Akkermansia*-supplementation (Day -2) in groups of mice gavaged with viable bacteria of the MucT- and H2-strains (see Figure 15). After the initial weight loss of the mice in these groups, day -2 when *Akkermansia*-DNA could be detected in the faeces was also the time when mice started to re-gain weight (see Figure 13).

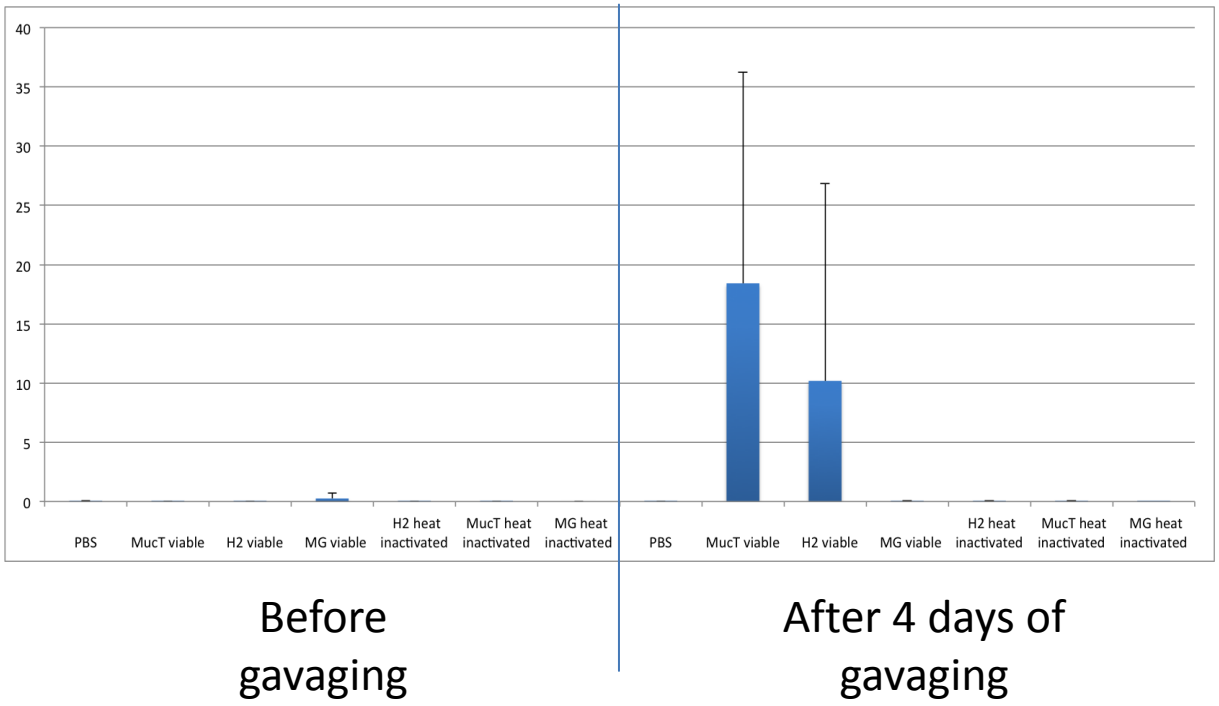


Figure 15: Detection of *Akkermansia*-DNA in faeces of mice using qPCR. Amounts show correlations of the presence of *Akkermansia spp.* in the faeces with the weight curves of the mice before DSS treatment. No DNA of *Akkermansia* could be detected at the beginning of the experiment (Day -6). In contrast to this, despite losing weight in the first days of bacterial supplementation, mice gavaged with viable *Akkermansia* from the MucT- and H2-strains re-gained weight at the same time when *Akkermansia*-DNA was detectable in the faeces. qPCR was performed at **Wageningen University** (Netherlands) by the group of Willem de Vos (Laboratory of Microbiology).

Another parameter in characterisation of murine colitis in mice is the colon length of the treated mice as this is reduced during gastrointestinal inflammation[58]. The colon length was measured at the last day of the experiment during sampling, the colon lengths [cm] were normalised to the original body weight [g] of the mice.

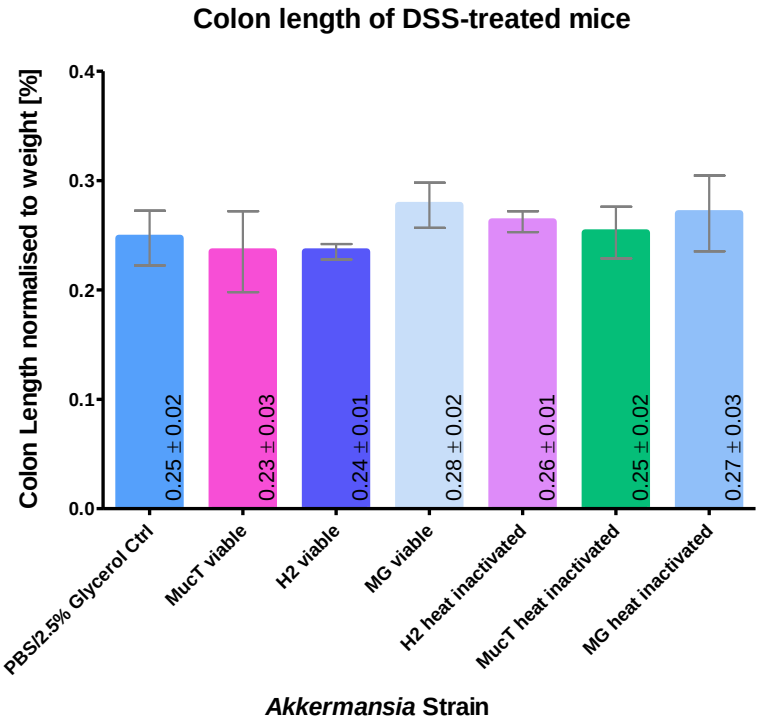


Figure 16: Average colon lengths [%] of the gavaged mice normalised to original body weight [g]; measured colon lengths of DSS-treated mice do not show any significant differences

In Figure 16 it can be seen clearly that no significant differences between the control group (PBS/2.5 % glycerol: 0.25 ± 0.02 %) and the groups of mice supplemented with the different viable and heat inactivated *Akkermansia* strains reaching from 0.23 ± 0.03 % to 0.28 ± 0.02 % could be observed.

4.2.2 Mucus Thickness

To determine mucus thickness, 6/3/1 EtOH-Carnoy-fixed tissue from ileum, cecum and colon of the *Akkermansia* supplemented mice were cut in 6 µm thick sections after paraffin-embedding. Sections were dewaxed, rehydrated in an EtOH-series and stained with alcian blue. For comparison, 6/3/1 EtOH-Carnoy-fixed tissue from a WT mouse was used as a positive control (see Figure 4.B2). The evaluation of that section also shows that the protocol for alcian blue staining does not affect the preservation or structure of the mucus and tissue.

However, alcian blue staining of the 6/3/1 EtOH-Carnoy-fixed sections of the *Akkermansia*-gavaged mice revealed that in most sections the mucus layer was too disrupted, or completely destroyed, respectively. Therefore, mucus thickness was not measureable.

In Figure 17 pictures of colon, cecum and ileum of the control group are shown, as well as pictures of mice gavaged with viable and heat inactivated *Akkermansia* of the human MucT strain which are representative, as no differences between the different gavaged strains could be observed concerning the presence of mucus. However, it can be seen, that even in sections of mice in the control group the mucus seems to be completely disrupted, in colon and cecum, as well as in ileum, whereas only a narrow collapsed layer of intestinal mucus is left at the edges of the epithelium (see Figure 17.A1-C1). Furthermore, despite showing good preservation of mucus inside the goblet cells in ileum and cecum in the control group sections (see Figure 17.B1-C1), mucus in goblet cells of the colon seems to be rather patchy and disrupted (see Figure 17.A1).

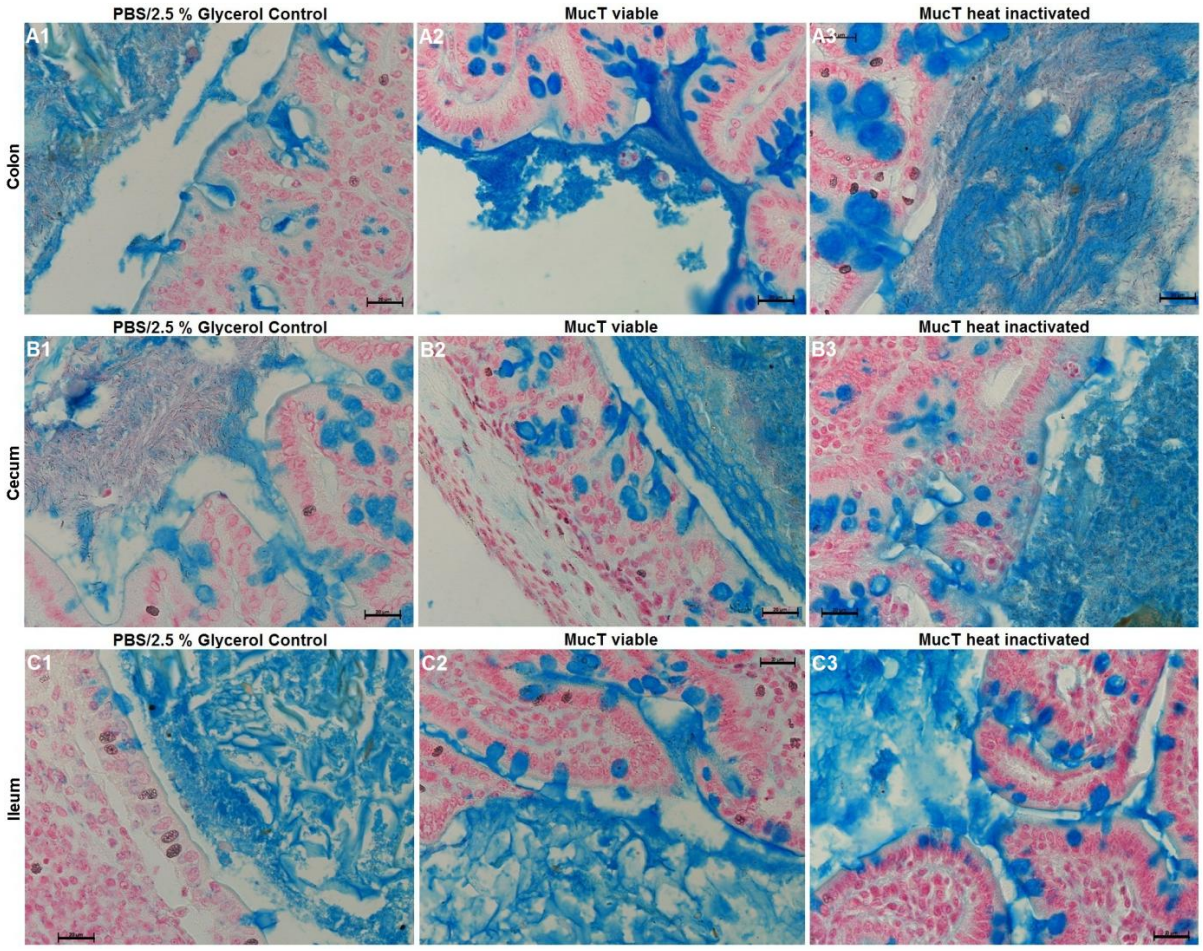


Figure 17: Alcian blue stained 6/3/1 EtOH-Carnoy sections of *Akkermansia* supplemented DSS-treated mice. **(A1-C1)** Sections of colon, cecum, and ileum of the PBS/2.5 % Glycerol Control group show only a narrow, collapsed layer of mucus next to the epithelium, and also rather patchy mucus patterns in the goblet cells in the colon. **(A2-C2)** Staining of the intestinal mucus in mice gavaged with viable *Akkermansia* of the MucT strain show mucus filled goblet cells of normal size. Only in colon (A2) the mucus layer is preserved, ileum and cecum show disrupted structures of the intestinal mucus. **(A3-C3)** Sections of mice treated with heat inactivated MucT *Akkermansia* show only a patchy preservation of the mucus layer; goblet cells show good mucin staining, whereas the ones in the colonic sections are much enlargened. Also, bacteria reach the epithelium in this section (A3). Scale bars indicate 20 µm. Magnification: 40x

However, the only good preservation of the intestinal mucus layer can be found in the colon of mice gavaged with the viable MucT strain (see Figure 17.A2), but almost no luminal content could be preserved in this section, as the colon of the presented mouse was completely empty after the DSS treatment. In contrast to this, the colon of the mouse treated with heat inactivated MucT *Akkermansia* does not have any intestinal mucus left, which enables the luminal content to reach directly to the epithelium (see Figure 17.A3).

Despite the lack of detectable mucus layers in sections of the cecum (see Figure 17.B2-B3), these mice still seemed to have a present luminal content including bacteria after the DSS treatment. Contrary to this, in the ileum almost no luminal content could be detected, but a patchy mucus layer seemed to spread out and diffuse over the whole lumen in alcian blue stained sections of mice treated with viable and heat inactivated MucT *Akkermansia*.

In general, in all sections mucus filled goblet cells of normal size could be detected. Nevertheless, some tissues, like for example the colonic sample of mice supplemented with heat inactivated *Akkermansia* of the MucT strain showed strongly enlarged goblet cells (see Figure 17.A3) compared to the cells in tissues of the control group. Furthermore, in the ileal tissue of the control group, a decreased number of goblet cells could be detected (see Figure 17.C1).

4.2.3 Detection of *Akkermansia spp.* using FISH

To determine colonisation of gavaged *Akkermansia spp.*, FISH was performed on PFA-fixed colonic paraffin sections of the treated mice using the EUBmix probe-mix in Fluos to check general bacterial colonisation together with the *Akkermansia*-specific probe Akk435 (see Table 3) in Cy3. Furthermore, the localisations of eventually occurring *Akkermansia*-colonisations were to be detected as these mucin-degrading bacteria[42,43] were expected to colonise close to the intestinal mucosal layer.

In general it was shown, that bacterial colonisation could not be detected on every section as some mice had empty colons caused by strong diarrhoea due to the DSS-induced colitis (see Table 8). Nevertheless, despite few expectations, *Akkermansia* could be found in mice of every group treated with the different strains, both active and heat inactivated (HI). Only in the control group of mice treated with a PBS/2.5 % glycerol-solution without *Akkermansia*-supplementation no *Akkermansia* could be detected in any of the single mice. Nevertheless, despite showing general bacterial population (see Table 8) labelled with EUBmix-Fluos and DAPI, only a low number of bacteria could be detected in these samples. Furthermore, bacteria were detectable mainly in the middle of the lumen rather than close to the epithelium

(see Figure 18). Also, the EUBmix probe-mix showed only rather low signal intensities and a rather high autofluorescence of the surrounding cellular structures.

Table 8: Evaluation of hybridised paraffin sections (5 µm) fixed with 4 % PFA; sections were hybridised with EUBmix-Fluos and the *Akkermansia*-specific probe Akk435-Cy3 (see Table 3). (X) probe signals detectable; (-) no probe signals detected

Group	Treatment	Mouse	EUBmix-Fluos	Akk435-Cy3
1	PBS/2.5% Glycerol Control	0	X	-
		r	X	-
		l	X	-
		b	X	-
2	MucT Viable	0	X	X
		r	-	-
		l	X	X
		b	X	X
3	H2 Viable	0	-	-
		r	-	-
		l	X	X
		b	-	-
4	MG Viable	0	X	X
		r	X	X
		l	X	X
		b	X	-
5	H2 Heat Inactivated	0	X	-
		r	X	X
		l	X	X
		b	X	X
6	MucT Heat Inactivated	0	X	X
		r	X	-
		l	X	X
		b	-	-
7	MG Heat Inactivated	0	X	-
		r	-	-
		l	X	X
		b	-	-

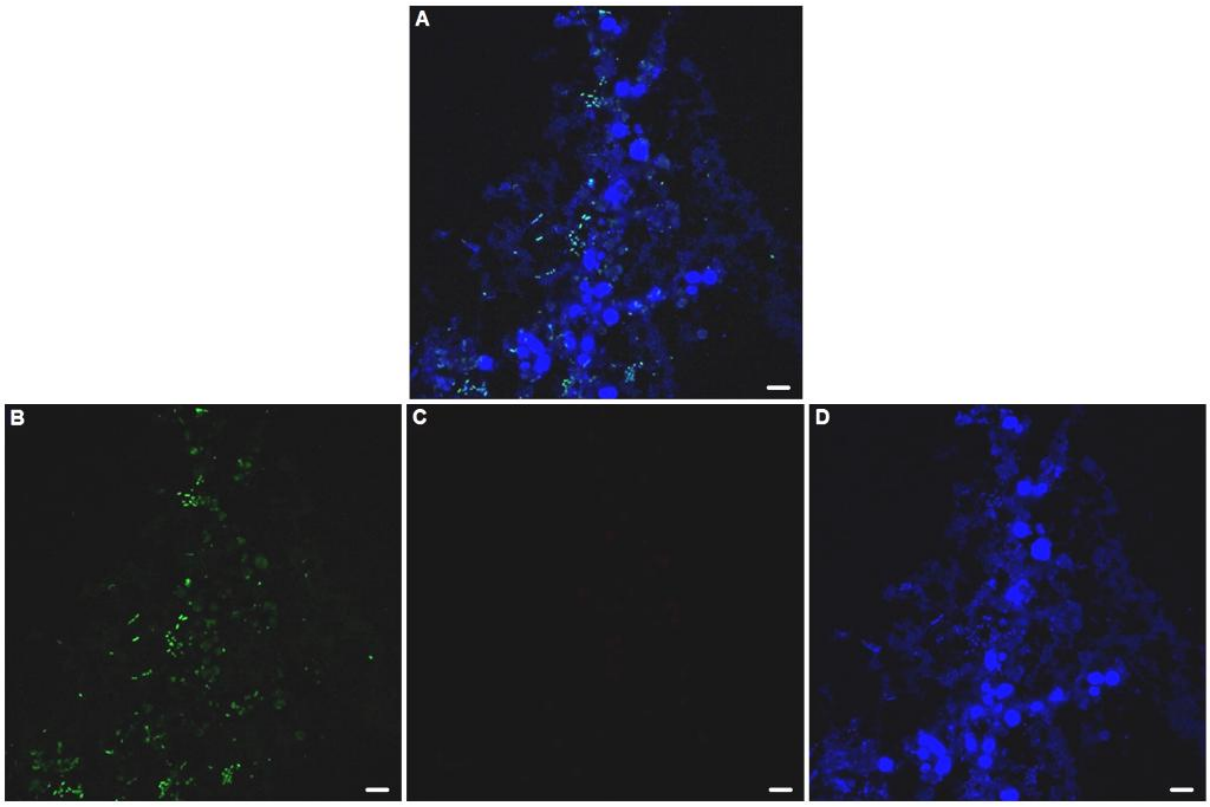


Figure 18: FISH stained PFA-fixed paraffin section of a mouse gavaged with PBS/2.5 % glycerol. **(A)** Overlay. **(B+C)** Hybridisation with EUBmix-Fluos and Akk435-Cy3 reveal bacterial population in the lumen, but no *Akkermansia* (C) could be detected. **(D)** DAPI staining showed apoptotic cells shed from the inflamed epithelium in the lumen. Scale bars indicate 10 µm. Magnification: 63x

In contrast to the control group, high numbers of *Akkermansia spp.* could be detected in mice supplemented with viable bacteria of the MucT- and H2-strains (see Table 8). The hybridisation of the sections with the Akk435-Cy3 probe resulted in strong signals, but nevertheless, only few bacteria could be detected close to the epithelium, *Akkermansia* could only be found in the middle of the lumen. Furthermore, rather strong cell shedding occurred whereas cells tended to gather around the apoptotic cells, as observed before in the control group. However, despite a strong level of cell shedding, also the tissues appeared relatively strong disrupted (pictures not shown).

Akkermansia could be found close to the intestinal mucosa only in few sections. Sections of a mouse treated with the viable MG strain did not only show high numbers of bacteria in the lumen stained with the EUBmix probe (see Figure 19.B), but also a strong colonisation of *Akkermansia spp.*, whereas the species-specific probe showed that this strain also colonised next to the epithelial surface (see Figure 19.A+C). Furthermore, in DAPI staining it could be observed, that in mice treated with viable MG *Akkermansia*, not only the *Akkermansia spp.*, but luminal bacteria in general sit directly on the epithelium making it probable that no mucosal layer was left after DSS-treatment of the mice (see Figure 19.D).

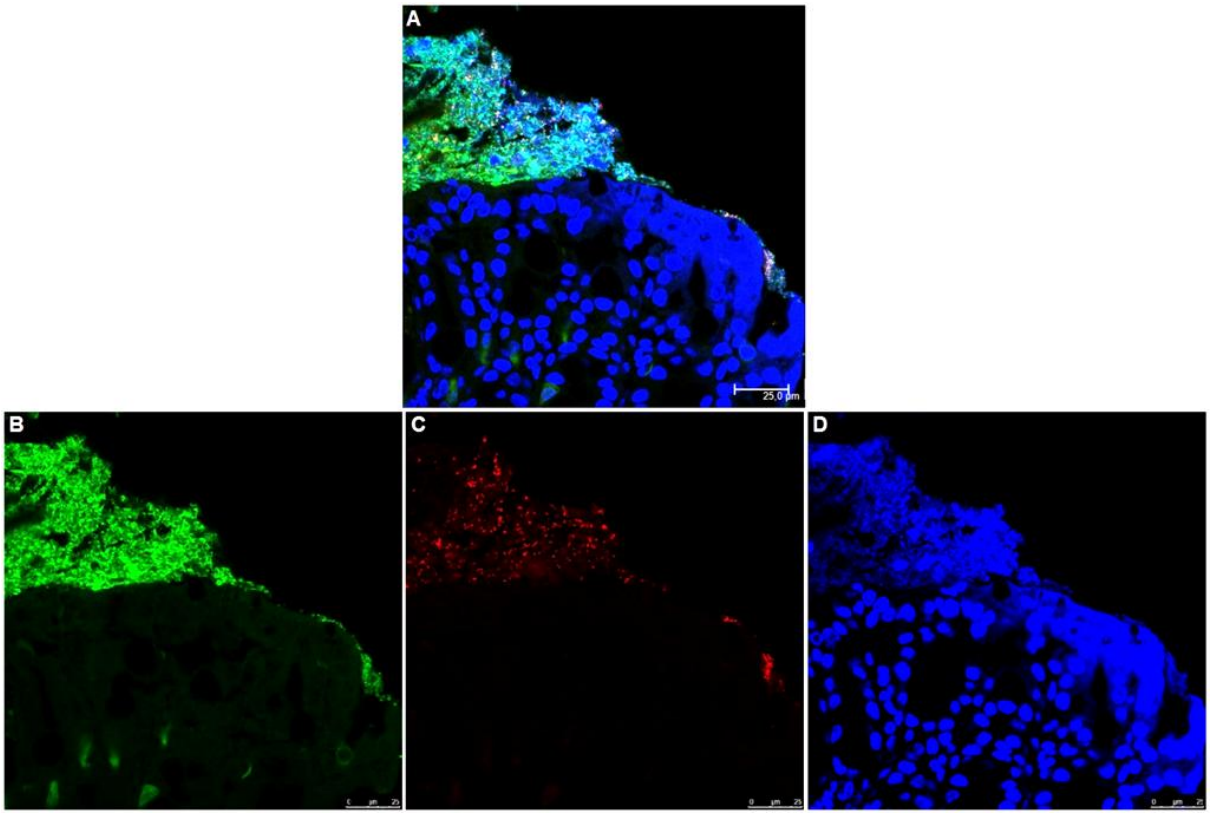


Figure 19: FISH stained PFA-fixed paraffin section of a mouse supplemented with viable *Akkermansia* of the MG strain. **(A)** Overlay. **(B+C)** Hybridisation with EUBmix-Fluos reveals bacterial colonisation next to the epithelium, no bacteria could be found in the middle of the lumen. **(C)** Strong signals could be detected all over the bacterial colonisation using the specific probe Akk435-Cy3 showing the presence of a high number of *Akkermansia* on this section. **(D)** DAPI staining reveals that bacteria sit directly on the epithelium indicating a lack of the mucosal layer. Also, cell shedding could be observed. Scale bars indicate 25 µ. Magnifiaction: 63x

However, also in samples from mice supplemented with the heat inactivated strains, *Akkermansia spp.* could be detected with the specific probe. Nevertheless, signals in both channels (Fluos and Cy3) were rather weak. Most bacteria could be found close to or directly on the epithelial surface, but in sections from half of the sampled mice extreme cell shedding could be observed. These structures of shed apoptotic epithelial cells made capturing bacterial structures hard due to their strong auto-fluorescence in Fluos and Cy3, and DAPI signals of bacteria were mostly overlaid by the ones of the cell nuclei. Especially in mice fed with heat inactivated *Akkermansia* from the H2-strain a high level of cell shedding was determined. As a result of this, an additional layer consisting of shed epithelial cells was present between the epithelium and the luminal content. On sections of one mouse, cell shedding was that strong, that almost the whole lumen was filled with apoptotic cells and only a small area of the lumen was free for passage of the luminal content. Also, basically no bacteria could be detected on this section. On most parts of this sample it was hardly possible to clearly distinguish between epithelial structures and apoptotic cells (see Figure 20.A).

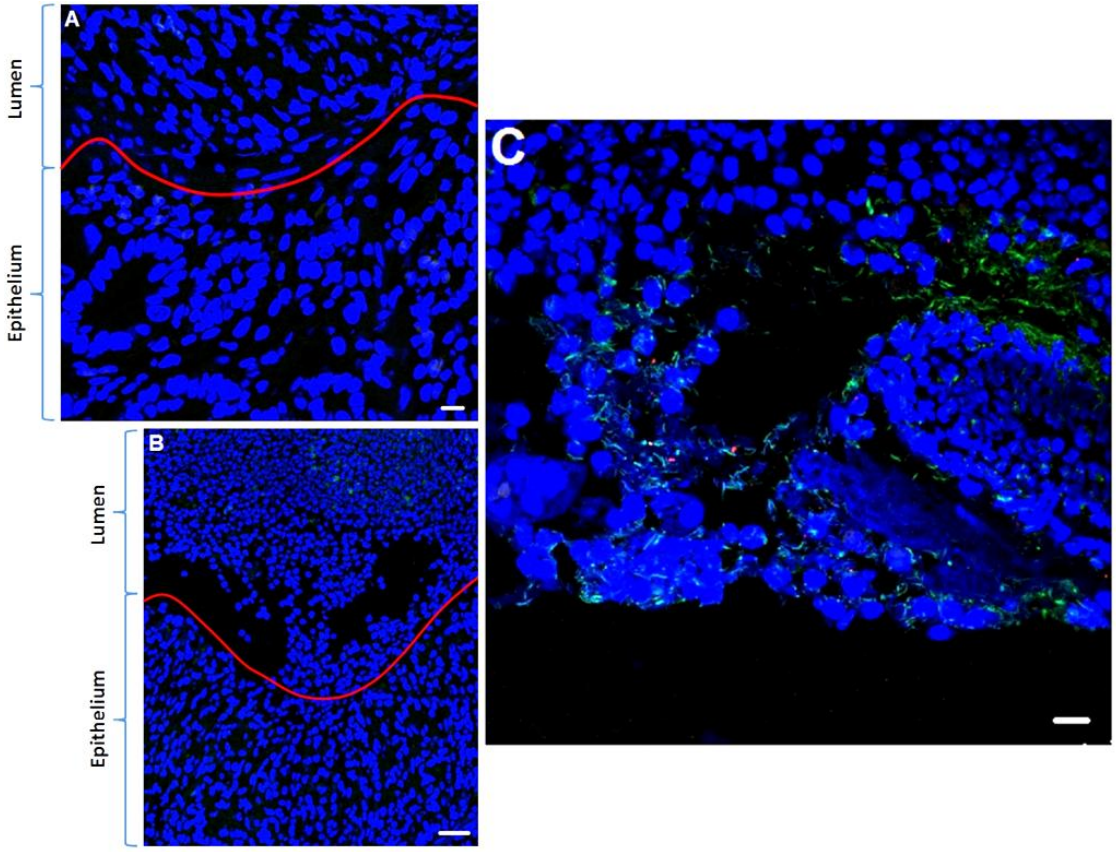


Figure 20: (A) DAPI-staining of a paraffin section of colonic tissue of a mouse supplemented with HI *Akkermansia* of the H2-strain show extreme cell shedding. The typical circular pattern of the epithelium is left only at a low level making it possible to partly distinguish epithelial cells from shed apoptotic cells that almost completely fill up the lumen. No bacteria detectable. The red line indicates the junction of epithelium to lumen. **(B)** DAPI stained colonic paraffin section of a HI MucT-supplemented mouse. Almost the whole lumen is filled up with apoptotic epithelial cells, only a small area is left free for the passage of the luminal content. As a result of the strong cell shedding the epithelium can only be differentiated due to the stronger packing of the shed cells in the lumen. The red line indicates the junction of epithelium to lumen. **(C)** FISH on colonic paraffin section of a HI MucT-treated mouse. Lower levels of cell shedding allow recognition of the epithelium. FISH reveals a low amount of bacteria (EUBmix-Fluos – green), with a proportionally high number of *Akkermansia* (Akk435-Cy3 – pink) between the shed epithelial cells, whereas bacteria tend to gather around the apoptotic cells. Scale bars indicate 100 µm. Magnification: 63x

However, the phenomenon of high level cell shedding occurred on samples of mice supplemented with heat inactivated MucT-*Akkermansia*. Almost the whole lumen was filled with shed apoptotic epithelial cells, but contrary to the colonic sections of the heat inactivated H2-supplemented mice where still some typical circular structures of the epithelium were visible in DAPI staining, this typical structure was completely disrupted in the HI MucT-samples. Furthermore, also almost no bacteria were detectable on these sections. Therefore, differentiating the lumen filled with apoptotic cells from the epithelium is only possible due to the denser packaging of the shed cells and a stronger auto-fluorescence of the epithelium in the Fluos-channel (see Figure 20.B).

However, not all sections of the HI MucT-treated mice showed such high levels of cell sheddings. These sections held only low levels of bacteria which could be detected using the EUBmix-Fluos probe-mix. But despite a low amount of bacteria, still proportionally high

numbers of *Akkermansia* could be found with the specific Akk435 probe in Cy3. Nevertheless, as still shed cells were present on these sections, bacteria tended to gather around those apoptotic cells, which showed a strong signal in DAPI and also a high auto-fluorescence in the Fluos-channel, making capturing the bacterial signals difficult despite having a strong FISH probe signal (Figure 20.C). Still, enterogastric bacteria, including *Akkermansia*, could be found next to the epithelial surface, whereas no gap was left between the bacteria and the epithelium, and also in crypts, again suggesting that there is no intestinal mucosal layer left on the samples.

Same as in HI MucT- and H2-supplemented mice, colonic sections from mice treated with heat inactivated *Akkermansia* of the MG-strain showed an additional layer of shed cells between the epithelium and the luminal content. *Akkermansia* were present, but only in rather low number due to the generally very low amount of bacteria. Furthermore, again capturing of the bacteria was difficult as most of the bacteria gathered around shed cells and FISH signals were therefore overlaid by the auto-fluorescence of the apoptotic epithelial cells.

However, although DAPI staining during FISH revealed the levels of cell shedding of different levels in the control mice (see Figure 18), and also in the *Akkermansia*-supplemented mice, the phenomenon of cell shedding was first observed in sections of MucT-supplemented mice stained with Alcian Blue (see Figure 21 – encircled).

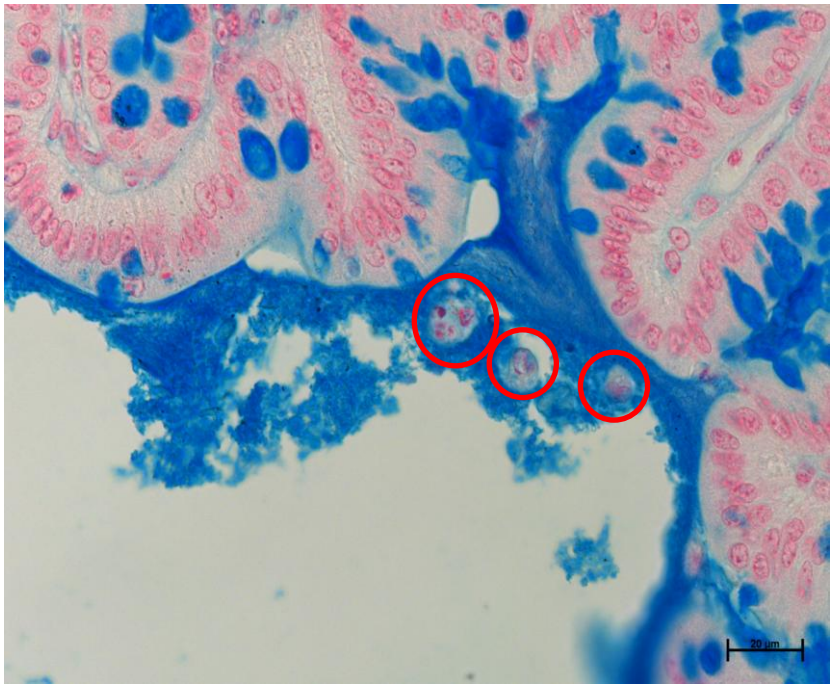


Figure 21: Alcian Blue stained colonic paraffin section of a mouse supplemented with viable *Akkermansia* of the MucT-strain. Red circles show apoptotic cells shed from the intestinal epithelium due to inflammation. Scale bar indicates 20 µm. Magnification: 40x

To better visualise the apoptotic cells which are shed from the inflamed epithelium, FISH was repeated on sections using EUBmix-Fluos and the eukaryote-specific probe EUK516-Cy3, sections were counterstained with DAPI. Microscopic evaluation revealed single apoptotic cells, as well as clumps made of several cells in the lumen of several sections (e.g. in mice supplemented with heat inactivated *Akkermansia* of the MG-strain; see Figure 22), whereas, same as in the control group (see Figure 18), bacteria were likely to cluster around the shed cells.

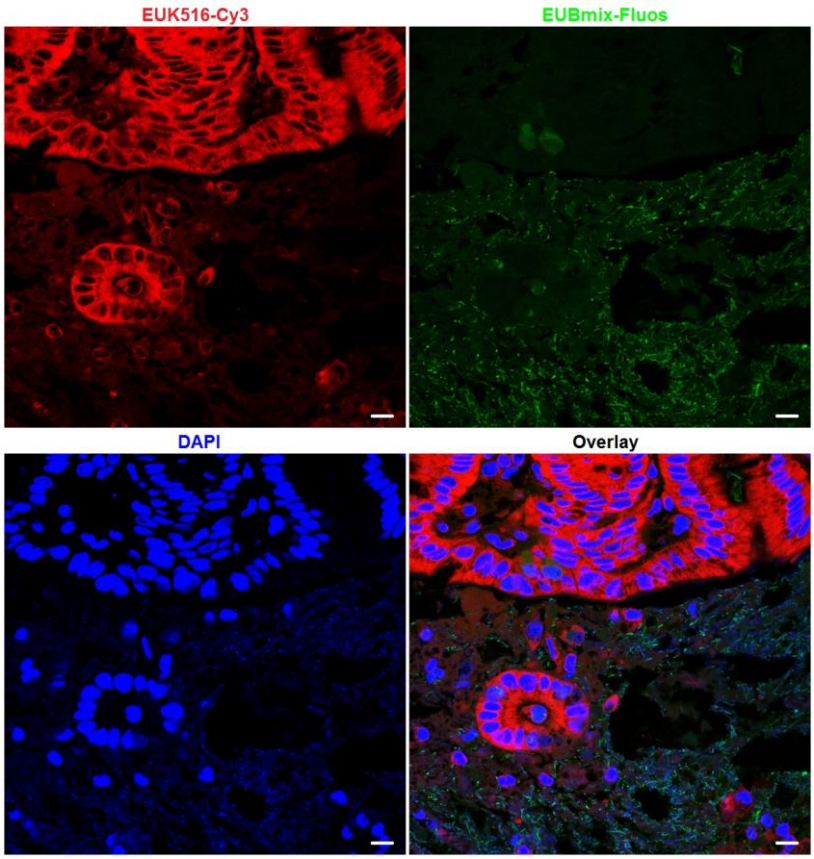


Figure 22: FISH-stained colonic paraffin section of a mouse supplemented with heat inactivated MG-*Akkermansia*. The probe EUK516-Cy3 specific for eukaryotic cells and DAPI staining reveal shed epithelial cells in the lumen surrounded by bacteria which are stained with EUBmix-Fluos. Scale bars indicate 10 µm. Magnification: 63x

Furthermore, it could be observed, that a higher level of cell shedding occurred in the intestines of mice treated with heat-inactivated bacteria. A tile scan of the section of a mouse treated with the heat inactivated MG-strain revealed that the turnover of epithelial cells in the inflamed tissue is strong enough that the original pattern of the intestinal epithelial tissue is only partially left amongst the section (see Figure 23).

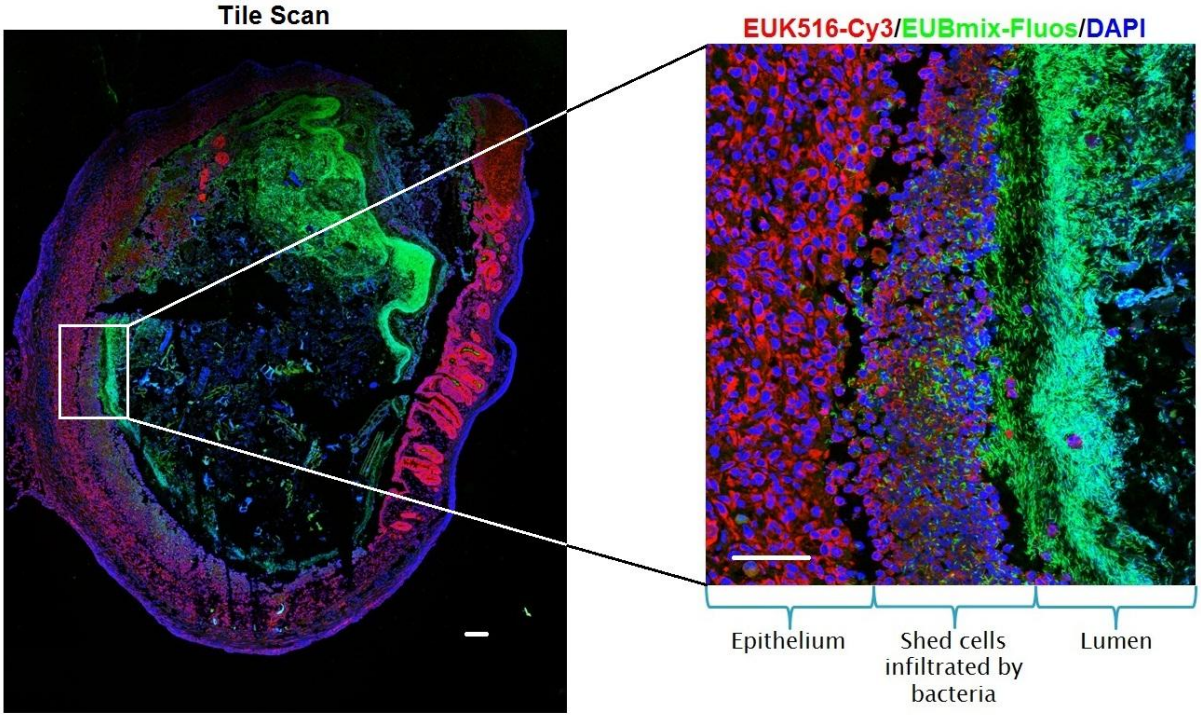


Figure 23: FISH stained colonic paraffin section of a HI MG-supplemented mouse. Colitic inflammation amplified cell turnover of the epithelium that strong, that the apoptotic cells built a protective layer between the epithelial surface and the luminal content which could be visualised by DAPI staining (blue) and the use of the eukaryote-specific probe EUK516-Cy3 (pink). The zoom clearly shows, that the epithelium and the shed cell layer can only be differentiated due to the infiltration of the shed cells by bacteria (EUBmix-Fluos – Green). Scale bars indicate 100 µm (tile scan) and 50 µm (zoom), respectively

Furthermore, a higher magnification of an area of the disrupted tissue showed that an additional layer between the epithelium and the luminal content had been formed by apoptotic shed cells what has been observed before during *Akkermansia*-FISH. Due to this additional layer, and to the lack of structural details in the intestinal epithelial, differentiation between these two is only possible because of bacterial infiltration of the shed cell-layer (see Figure 23).

One reason for cell shedding is suggested to be the intestinal inflammation due to DSS treatment. The inflammation scoring performed by a trained pathologist revealed, that sections which showed higher amounts of shed cells in FISH and DAPI staining, also had a higher inflammation scoring.

Only samples from mice treated with viable MucT *Akkermansia* seemed not to follow this pattern. H&E stained sections of these mice were evaluated with high inflammation scoring compared to the rest of the samples (see Figure 24), and although cell shedding could be observed in those samples as well, the estimated amount of apoptotic cells was not as high as in other samples which yielded lower inflammation scorings.

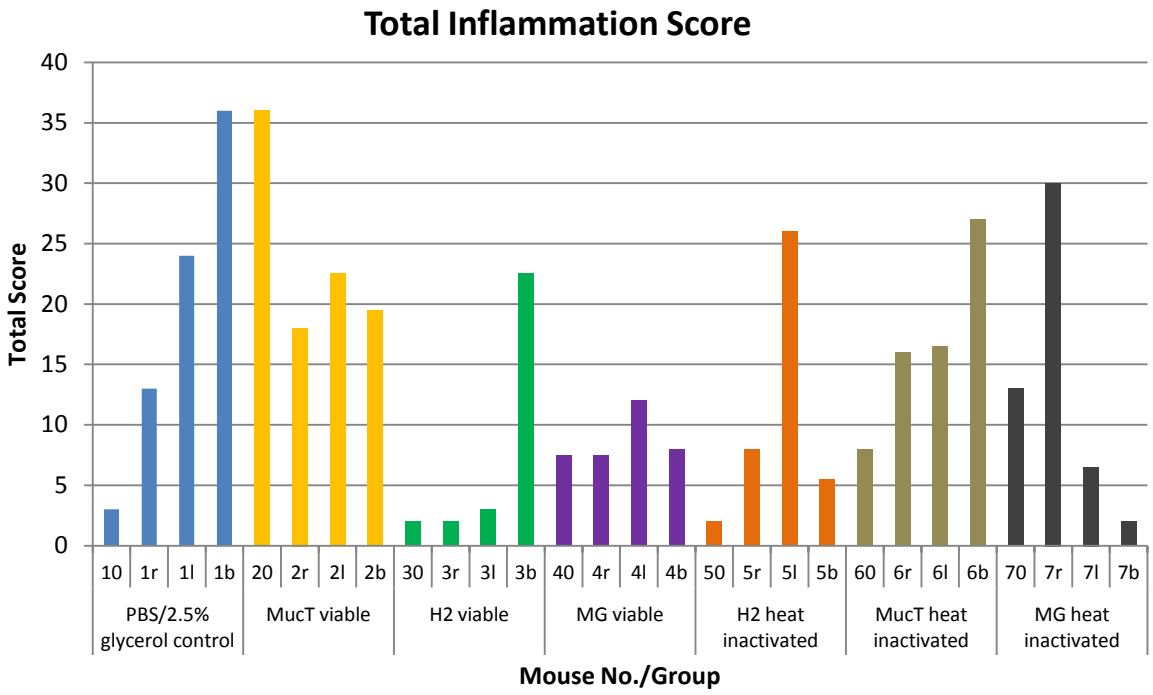


Figure 24: Total inflammation scores of gavaged mice; scorings were performed by a trained pathologist (Ao.Univ.-Prof. Dr.med.univ. Lukas Kenner, University of Vienna) in regard to inflammation severity, inflammation extent, crypt damage, and percentage of in the inflammation involved tissue. Bars show the total scores calculated from the mentioned evaluated properties.

When results of the pathology scores are compared to the presence of bacteria, or *Akkermansia* colonisation, respectively (see Table 8), there does not seem to be a trend that shows any differences between mice of the different supplementations. In general, in the groups fed with heat inactivated MucT- and MG-*Akkermansia*, the mice which had no bacteria in the lumen at all (6b, 7r; see Figure 24) are the ones with the worst scoring results in their groups. Nevertheless, despite the high inflammation levels, these mice were the ones in their groups which showed the lowest weight loss (see Table S6).

The group of the mice supplemented with viable MG-*Akkermansia*, the one mouse with no bacteria had almost the same pathology scoring like all the other mice of this group. Still, the mice in the “viable” group do have a better average scoring (see Table 9) than the group of mice treated with heat inactivated *Akkermansia* of the MG-strain. Nevertheless, the one mouse that was killed earlier before the end of the experiment due to a too strong weight loss (7b; see Table S6), and also did not have any bacteria in the lumen, was the one with the lowest level of inflammation (see Figure 24).

Table 9: Average total inflammation scores of *Akkermansia*-supplemented mice.

Group	Treatment	Average Total Score	Standard Deviation
1	PBS/2.5% Glycerol Control	19	14
2	MucT Viable	24	8
3	H2 Viable	7	10
4	MG Viable	9	2
5	H2 Heat Inactivated	10	11
6	MucT Heat Inactivated	17	8
7	MG Heat Inactivated	13	12

Nevertheless, a big difference in scoring results could be observed between the groups supplemented with either H2 viable or H2 heat inactivated *Akkermansia*. Mice treated with heat inactivated bacteria showed approx. two times higher scoring results than the ones supplemented with viable *Akkermansia*. Nevertheless, these inflammation scoring results are not thought to be due to the *Akkermansia*, as the results are better in the H2 viable gavaged mice where basically no *Akkermansia* could be detected in FISH (see Table 8). However, mice supplemented with viable H2-bacteria in general were the ones with the lowest average inflammation score (see Table 9).

In contrast to this, the scoring results of mice supplemented with heat inactivated and viable MucT-*Akkermansia* show opposite effects as the group of the viable bacteria yield worse inflammation scores (see Table 9) than the heat killed *Akkermansia*-mice (see Figure 24). Nevertheless, in the MucT-HI group, the mice in which the presence of *Akkermansia* was proven have a better scoring than the ones without *Akkermansia* (see Table 8). Therefore the MucT groups were the only ones in which the mice supplemented with heat inactivated *Akkermansia* did better than the viable ones (see Figure 24). In general, the H2-viable-gavaged mice had the best scoring results, although no *Akkermansia* could be detected after FISH (see Table 8) suggesting that *Akkermansia*-supplementation does not have any effect on DSS-induced colitis in mice.

5 DISCUSSION

5.1 Fixation of Mucus

5.1.1 Visualisation of Intestinal Microbiota

Several chemicals and solutions are available for the fixation of tissue sample, but nevertheless, fixatives have only been optimised either for preserving structural features of tissues[59], or for improving bacterial cell wall permeability for optimal probe uptake during FISH[60]. FISH offers an easy applicable and reliable method for a rapid identification of prokaryotic cells without cultivation[60] making it optimal for environmental and medical samples. Nevertheless, only few attempts have been made to combine FISH with a mucus staining technique with barely satisfying results[19]. In this study, several different fixatives were tested for the structural preservation of mouse intestinal tissue, as well for their influences on FISH probe efficiency and signal intensity. Furthermore, the fixation's impact on the intestinal mucosal layer was to be determined by Alcian Blue staining. In the next step, staining methods for mucins which are easy combinable with FISH were to be tested, firstly to determine the preservation of the mucosal layer, and secondly to visualise bacterial colonisation next to the mucus and eventual bacterial infiltration of the mucosal layer.

As first step to determine the optimal fixative for tissue and mucus preservation and for good probe signals during FISH, cecum content samples from WT mice were fixed with nine different fixatives. Samples were hybridised with the general bacterial EUB338 I-III probe mix labelled with either Fluos, Cy3, or Cy5, and counterstained with DAPI. A cell count using DAIME to determine the ratio DAPI : EUBmix revealed that Fluos seems to be the labelling of first choice as it works best for all fixation concerning the overall probe efficiency as well as the probe signal intensity (see Figure 1). In contrast to this, signal intensities of Cy3- and Cy5-labelled probes yield lower values in most of the fixatives, except of PFA and EtOH, where Cy3, or Cy5 respectively showed slightly higher values of arbitrary intensity units than Fluos (see Table 5). In contrast to this, no signals of the Cy3-labelled probe were detectable at all in Carnoy's solutions with anhydrous MetOH (6/1/3 dMetOH-Carnoy, 6/6/1 dMetOH). As the same samples showed detectable signals when hybridised with EUBmix-Fluos, it is rather clear that lacking signals are due to the different fluorescent-labels rather than to the sample. Also, hybridisations for the different fluorophores were run parallel and in triplicates whereas all repeats showed similar results indicating the dependence of the fluorophore on the fixative and its compounds. Furthermore, in contrast to this, when usual methanol was used for the 6/6/1 MetOH-Carnoy, the EUB-probemix labelled with Cy3 and Cy5 delivered rather average signal intensities compared to the rest of the fixatives tested (see Figure 1.B)

suggesting that the contained water seems to stabilise bacterial structure and prevents collapse of the bacterial cell walls.

However, cecal content samples fixed with Carnoy's solutions containing anhydrous MetOH also delivered weak signals with the DAPI staining (see Figure S1.H4, I4). In general, lots of lysed cells could be detected in the 6/1/3 anhydrous Methanol-Carnoy-fixed samples what probably is caused by the anhydrous alcohol that weakens the cellular structure of the bacteria or the hybridised rRNA. For the 6/1/3 anhydrous MetOH-Carnoy another reason may be the lower concentration of glacial acetic acid. It is known that the chloroform and the acetic acid in the Carnoy's recipe are to balance the effect of the alcohol which causes collapse of protein structures, therefore lower concentrations of these two compounds may have effects on the preservation of structures and microorganisms[59].

Ethanol fixation has been tested on normal colonic tissue before, showing that a standard FISH protocol seems to yield usable results during visualisation[61]. Experiments detailed in this thesis show that hybridisation of Ethanol-fixed cecal content in general yields a good average volume fraction compared to the PFA-fixed ones (see Figure 1.A) when hybridised with the EUB338 I-III probe mix. Furthermore, also good probe signal intensities of all three tested fluorophores were obtained (see Table 5). Nevertheless, values still are not as good as when the probe was tested with a Fluos-label.

However, due to different cell wall compositions between Gram positive and Gram negative cells not only the effects of the different fixatives on the general EUB338 I-III probe-mix and on the different fluorophores were determined, but also specific probes were tested to see the influence of the different fixation methods on the stability of the bacterial cell wall. The probe BAC303 is specific for the Gram-negative bacteria of the *Bacteroidales*-group which have a rather sensitive cell wall and therefore might react differently to the different fixation methods. Exemplary for Gram-positive bacteria, the Erec482 probe specific for the *Clostridium* clusters XIVa and XIVb was used. Experiments revealed that not every fixative is suitable for every probe (see Figure 3). Whereas the *Bacteroidales*-specific probe worked fine for PFA-fixed cecum content samples and for two of the seven Carnoy's recipes (6/3/1 MetOH, 6/6/1 MetOH), the BAC303 did not work for EtOH-fixed samples and any of the Carnoy's containing Ethanol. Furthermore, despite yielding a suitable probe signal intensity in 6/6/1 MetOH-Carnoy, no signals could be detected when anhydrous methanol was used for this Carnoy-recipe. These data show that sample preservation with fixatives containing anhydrous alcohols seem to be too harsh for gram negative bacteria leading to the destruction of bacterial structures. This can also be observed during DAPI staining

(see Figure S1) which reveals that cells mostly are destroyed and only can be visualised as bacterial debris in the corresponding samples.

In contrast to this, signals of the *Clostridium*-specific probe Erec482 were detectable in all of the fixed cecal content samples. Nevertheless, again samples fixed with 6/1/3 anhydrous MetOH-Carnoy and 6/6/1 anhydrous MetOH-Carnoy delivered the weakest intensity signals. However, the Erec482-probe was the only one in this experiment where PFA-fixation did not lead to the highest arbitrary intensity unit values in evaluating the probe signal intensity. Most of the other fixatives yielded up to twofold higher values, such as samples fixed with 6/3/1 anhydrous EtOH-Carnoy which delivered an almost twofold higher intensity.

The results obtained by testing differently fixed samples with specific probes demonstrate, that not every fixative is suitable for every probe, and also it could be observed, that not every fixative seemed to preserve all different species in the same way. Only two fixed cecal content samples (4 % PFA, 6/6/1 MetOH-Carnoy) contained not only short and long rods, but also cocci, the rest of the fixatives rather preserved only rods, whereas in DAPI staining both morphologies could be detected and the general EUBmix partly stained only long rods like in the 6/3/1 EtOH-Carnoy fixed sample (see Figure 2.B). However, these differences might be caused by the used fixative, but as the cecal content from different mice was used for each fixation, differences in the microbiota could be due to the different mice. Furthermore, the fixation method not only affects the probe efficiency and signal intensity, but also the quality of the DAPI staining resulting in weaker signals (see Figure S1).

However, it was also shown, that not only the rRNA-targeted oligonucleotide probes, but also the fluorescent dyes yield varying results in the different fixations. Probe signal quenching has been reported before[60], but in contrast to Wagner *et al.*, who reported nucleotide quenching of the probe signal intensities during FISH[60], weak or lacking signals in the tested cecal content samples are due to the different compositions of the fixatives. As Cy5 is known to be a rather sensitive fluorescent dye and to quickly bleach at too high temperatures or when exposed to light or microscopical laser light for too long, and also to degrade when exposed to certain chemicals, it is not really surprising that signals are rather weak in some of the fixatives (such as MetOH-containing Carnoy's solutions) or not detectable at all when anhydrous methanol was used. However, the cyanine dyes Cy3 and Cy5 contain two indole rings which are insoluble in and therefore not affected by the chloroform contained in the 6/1/3 anhydrous MetOH-Carnoy and 6/6/1 anhydrous MetOH-Carnoy, but nevertheless, the anhydrous methanol, same as any residual solvent, seems to influence the dye as it may excite *cis-trans*-isomerization of the linkage between the two indole rings causing a decrease or loss of the signal[62]. As the EUB338 I-III probe-mix with the fluorescein-label was the one

that did not only yield the best signal intensities throughout the experiment, but also the only one that worked for samples fixed with 6/1/3 anhydrous MetOH-Carnoy or 6/6/1 MetOH-Carnoy, respectively (see Figure 1), Fluos should be the fluorochrome of first choice for further experiments and combination with other methods.

5.1.2 Visualisation of Intestinal Mucus

In previous experiments H&E staining was performed on paraffin sections of all differently fixed tissue samples to find out which fixative causes the least structural impact and the lowest levels of shrinkage of the luminal content and therefore results in the best tissue structure preservation. Obtained results showed that colonic tissue fixed with 6/1/3 EtOH-Carnoy and 6/6/1 MetOH-Carnoy showed the best preservation in H&E staining, and also caused higher signal intensities after hybridisation with the EUB338 I-III probe-mix, and therefore was used for Alcian Blue staining for visualisation of the mucosal layer. Additionally, as FISH of cecal content fixed with 4 % PFA yielded the highest probe signal intensities for the EUBmix and also resulted in well detectable probe signals for the tested specific probes, paraffin sections of 4 % PFA-fixed colonic tissue was also stained with Alcian Blue for mucus detection. After Alcian Blue staining, mucins as well as the luminal content were stained blue, epithelial cells were counterstained with nuclear fast red and therefore visualised with a reddish colour.

For comparison two sets of samples were stained, whereas the first one was dehydrated in an EtOH-series (see Table S2) before paraffinisation, samples of the second set were kept in 100 % EtOH for 24 h after fixation before paraffinisation. In general it can be seen, that samples that were kept water-free delivered better Alcian Blue staining as first of all the blue colour of the Alcian Blue and the reddish stained cells from the counterstaining can be differentiated more clearly (see Figure 4.B1 – 3). Nuclear fast red counterstaining of the dehydrated samples resulted in a dark violet colouring of the epithelium what made a precise distinction from the dark blue luminal content and mucus almost impossible (see Figure 4.A1 – 3).

Furthermore not only the colouring of the paraffin section was better in sections that were kept water-free throughout sample preparation and paraffin embedding, but also the tissue structure was preserved at a higher level. Shrinkage of the luminal content did not occur in any of the samples, no matter whether they were kept water-free or not, but nevertheless, in samples which underwent dehydration after fixation the mucosal layer between lumen and epithelium seemed to be mostly washed away, and only a narrow, collapsed layer of mucus was left or no mucus at all, respectively. Furthermore, not only the mucosal layer was affected, but also the epithelial tissue showed rather high levels of disruption. In dehydrated

sections fixed with 4 % PFA additionally the luminal content was not preserved too well (see Figure 4.A1).

The intestinal mucosal layer is known to be highly water-soluble[12], therefore it is not surprising that Alcian Blue staining of the second set of samples, where paraffin sections were kept water-free throughout the whole sample preparation procedures, results in better tissue structure preservation. Not only the epithelium and the luminal content can be differentiated more clearly by the colours, but also the mucus layer between these two is identifiable. Therefore, in contrast to Cohen *et al.*[20] where secreted mucus could only be stained with Alcian Blue in unfixed snap-frozen tissues, staining of secreted mucus could be observed in the tested paraffin sections. Furthermore, also goblet cells were stained and can be differentiated with a clear blue colour in between the reddish epithelial cells in colon tissue fixed with 4 % PFA, 6/1/3 EtOH-Carnoy and 6/6/1 MetOH-Carnoy (see Figure 4.B1 – 3).

Nevertheless, although goblet cells can be markedly identified, the mucus layer is distinguishable rather due to structural differences to the luminal content than for the colour in some areas, as the lumen shows the same bluish colour as the stained mucin does. However, there still gaps can be found between the epithelium and the lumen in paraffin sections fixed with 4 % PFA probably indicating shrinkage of the luminal content and alterations of the colonic tissue in general during fixation (see Figure 4.B1). In contrast to this, tissue samples fixed with either 6/1/3 anhydrous EtOH-Carnoy or 6/6/1 MetOH-Carnoy were perfectly preserved not showing any gaps or tissue disruption at all.

Alcian Blue staining delivers good results in visualising the intestinal mucosal layer, but nevertheless, it is not combinable with FISH. Therefore, to determine intestinal microbiota localised next to or infiltrating the intestinal mucosal layer a different staining technique needs to be applied.

Lectin histochemistry is one possibility for mucin detection on paraffin section of colonic tissue[20]. Lectins are rather stable compounds and even survive heat treatments[48]. They bind to sugars surrounding the oligosaccharide chains of mucins[25] and can therefore be used for visualisation of these glycoproteins. For the experiment detailed in this thesis Jacalin, which is a lectin originating from Jackfruit seeds and is specific for α-D-Galactose[26], was used in a conjugation with TexasRed®. Furthermore, lectin staining makes combination with FISH and therefore visualisation of mucus-associated microbiota possible.

Firstly, Jacalin-TexasRed® was applied to paraffin sections of colonic tissue samples fixed with 6/3/1 anhydrous EtOH-Carnoy according to the manufacturer's recommendations using 3 % H_2O_2 in PBS as a blocking buffer, the lectin was diluted in 0.02 M $NaHCO_3$. Different

Jacalin concentrations were tested (20 – 100 µg Jacalin/mL buffer), whereas no significant differences could be detected. Therefore, all further experiments were performed with a concentration of 60 µg Jacalin/mL buffer.

Nevertheless, although it has been shown before that Jacalin does not only stain the mucus in goblet cells, but also the secreted mucus around the epithelium and the brush borders[20,49], only a narrow collapsed mucus layer next to the epithelium could be detected during evaluation at the CLSM (see Figure 5) when the protocol was performed according to the manufacturer's recommendations. Furthermore, also in the goblet cells only a small amount of mucus could be detected (see Figure 5.B). However, for the negative control a paraffin section from the same area of the fixed colon sample was stained with Jacalin which was inhibited by pre-incubation with D-(+)-Melibiose. As no signals could be observed on this section (see Figure 5.D) the detected signals from the Jacalin-stained sections definitely can be referred to as positive signals from stained mucins.

In general, the staining resulted in a very weak signal intensity of the TexasRed®-labelled, and also because only very small amounts of mucus could be detected at the inner walls of the goblet cells and at the epithelium, a different protocol was applied to get better signal intensities and higher staining levels. According to Freitas *et al.*[49], 1x PBS containing 0.3 % Triton X-100 and 1 % BSA was used for the blocking of the sections to prevent unspecific binding of the lectin, the Jacalin was diluted in 1 % PBS. This changed protocol did indeed enhance the signal intensity of the Jacalin-staining (see Figure 6.A), whereas clear staining of the mucus in goblet cells could be observed (see Figure 6.B). But in contrast to the first protocol where H_2O_2 was used for blocking and at least a weak signal of a narrow mucosal layer that has been preserved in between two crypts could be detected, no intestinal mucus at all could be found when 1x PBS/0.3 % Triton X-100/1 % BSA was used for blocking. Again, no signals were detectable on the negative control-section indicating, that the observed signals of the Jacalin-staining definitely are obtained from stained mucus (see Figure 6.D).

However, although lectins mostly are used on cryosections[20,63], also paraffin sections have been stained with Jacalin before[48,49] resulting in good and clear staining of mucus-filled goblet cells and the mucosal layers in the small intestine. But nevertheless, the obtained results in this study go together with other studies performed before showing that one factor influencing the quality of the staining might be the part of the intestine that was sampled. Hereby it was shown, that not only the goblet cells, but also the whole intestinal mucosal layer next to the epithelium including the brush borders, as well as mucous vesicles, goblet cells and immature vesicles were perfectly stained in fixed ileal mouse tissue[49]. In contrast to

this, when the protocol for lectin staining was performed on paraffin sections on fixed colonic tissue the Jacalin-staining all in all yielded similar results to the pictures obtained in the experiments detailed in this thesis, meaning that mainly staining of immature vesicles and goblet cells was observed, while brush borders and the intestinal mucosal layer in general was not detectable at all by using Jacalin. Nevertheless, by using Bouin's solution a different fixative was used for preserving the tissue samples what might also influence the quality of the lectin staining. FISH has been tested before on bouin-fixed samples[64] where reproducible and acceptable results were obtained. However, only isolated and cultivated cells were fixed and visualised by Fried *et al.*, but in our context the mucus staining should be combined with FISH. Therefore, the Bouin fixative needs to be tested for probe efficiency and probe signal intensity on Bouin-fixed cecal content. Furthermore, also the Jacalin-staining itself might be tested again on colon tissue samples fixed with Bouin's solution. Furthermore, another lectin, such as Wheat Germ Agglutinin (WGA) might be tested on fixed sections, as this lectin has been shown to stain mucus well in both, ileal and colonic tissues[49].

However, although the staining was repeated several times, the presented results were not reproducible with either of the two protocols. Therefore, an anti-MUC2-antibody staining was tested as another immunohistochemical method for the detection of mucins on paraffin sections.

Another option for immunohistochemical staining offers an anti-MUC2-antibody. The *O*-glycosylated gel-forming mucin MUC2 is one of the major components of the mucosal glycocalyx[1,12,14] making it an ideal target for antibody-staining. Paraffin sections of colonic tissue samples fixed with 6/3/1 EtOH-Carnoy were stained with a MUC2-specific antibody labelled with Cy3 resulting in a clear staining of the intestinal mucus layer and brush borders, and also of the mucus-producing goblet cells in the epithelium (see Figure 7). Furthermore, the stained mucus (red) can clearly be distinguished from the DAPI stained epithelium and luminal content (blue). Nevertheless, although the labelled mucins could be detected with high signal intensities, also a rather strong auto-fluorescence of the epithelium and the luminal content could be detected in the Cy3-channel.

As immunohistochemistry with antibodies is based upon antibody-epitope-binding, it may happen that the primary antibody binds to amino acids other than those within the desired epitope of the antigen causing non-specific binding[65]. To prevent any possible unspecific staining, and thus reducing background signals, blocking was performed. Another important step for pre-treatment of the sections is the antigen-retrieval (AR), which is necessary to de-mask immunoreactive antigens within the tissue[53] and therefore make it available for the specific antibodies. Several methods are available for this step, but nevertheless, AR by

heating the sections covered with 10 mM sodium citrate buffer in the microwave oven for 2 x 5 min, as this method is known to be a quick and easy to apply technique[21,54].

For the visualisation of the intestinal mucosal layer, pre-treated colonic tissue fixed with 6/3/1 EtOH-Carnoy was incubated with a primary anti-MUC2-antibody. As Fluos-labelled probes yielded the best signal intensities on fixed cecal content in previous experiments (see 5.1.1 Visualisation of Intestinal Microbiota), a Cy3-labelled secondary antibody was used. Microscopical evaluation of an incubated section showed that tissue structures are well preserved, and that the intestinal mucus layer was perfectly stained and can clearly be distinguished from the epithelium and the luminal content after application of the mucin-specific antibody (see Figure 7). Furthermore, not only the mucosal layer and brush borders could be detected using the mucin-specific antibody, but also mucins in the goblet cells were markedly stained. However, despite yielding good signal intensities for the labelled mucins, rather high auto-fluorescence of the epithelial tissue could be observed in the Cy3-channel (red, see Figure 7). Nevertheless, the negative control, where sections were incubated only with the Cy3-labelled secondary antibody, showed no unspecific binding (see Figure 8).

5.1.3 Combination of Mucus Staining and FISH

The next step was to combine the successful mucin-specific antibody staining with FISH to determine the bacterial distribution along the intestinal mucus barrier. FISH and MUC2-antibody staining have been combined before, but despite showing good mucin staining, the combination resulted in rather unclear bacterial staining[19,66]. In general it is said that FISH should always precede immunohistochemical staining[18,19,56]. As FISH on cecal content fixed with 4 % PFA yielded the best probe signal intensities in preceding experiments, the combination of FISH and antibody-staining was tested not only on 6/3/1 EtOH-fixed colonic tissue samples but also on paraffin sections of tissue samples fixed with 4 % PFA.

In general, combining FISH and antibody-staining with the specific MUC2-antibody resulted in detectable signals for both in all fixations. Nevertheless, despite allowing visualisation of the luminal bacteria, the applied EUB338 I-III probe-mix yielded only weak signal intensities, whereas on paraffin sections of PFA-fixed tissue the probe showed better signals (see Figure 9.A), as it has also been the case on PFA-fixed cecal content (see Figure 1.B). Contrary to this, FISH signals were detectable on tissue samples fixed with 6/3/1 EtOH-Carnoy, but only with a weak intensity. Furthermore, these samples also showed a rather high auto-fluorescence of the epithelial tissue in the Fluos-channel (see Figure 9.B).

Nevertheless, mucin staining with the specific antibody resulted in well detectable signals for both fixations. Still, differences can be seen in the preservation of the structures. In

6/3/1 EtOH-Carnoy-fixed paraffin sections the mucosal layer is clearly stained by the MUC2-antibody, also staining of the mucins in the goblet cells causes good signal intensity of the Cy3-labelling (see Figure 9.B). Therefore, the staining shows that the 6/3/1 EtOH-Carnoy-fixative is ideal for the preservation of structural characteristics and features of the colonic intestine. However, immunostaining of PFA-fixed samples also result in good and clear staining of the mucins in the mucosal barrier and in the goblet cells (see Figure 9.A), but in contrast to Carnoy-fixed samples only a narrow collapsed mucus layer at the epithelial brush borders were observed. This alteration of the tissue structure has been observed before in Alcian Blue-stained PFA-fixed paraffin sections (see Figure 4.B1) where the fixation also caused a gap between the luminal content and the epithelium.

However, in general the results of combining FISH and antibody-staining yield the same results that have been shown before[19,66]. In 2010, Johansson *et al.* combined these to techniques resulting in rather good signals for both, the bacterial labelling via FISH and the MUC2-antibody staining. Nevertheless, signals for the MUC2-antibody could be detected in the mucosal layer, brush border and goblet cells, but only signals in the goblet cells are clear, as all other signals are as bright as the background auto-fluorescence of the epithelium. Contrary to this, the MUC2-antibody was definitely distinguishable in the immunostaining performed for this thesis (see Figure 9). However, when Johansson *et al.* improved their results for the mucin staining with the specific antibody, the bacterial staining using oligonucleotide probes also yielded only weak signals[66] as seen in Figure 11.

To check whether the pre-treatment with blocking and antigen retrieval, or the incubation with the MUC2-specific antibody after the FISH staining were causative for the weak probe signal intensity of the general bacterial oligonucleotide probe different conditions and combinations were tested (see Table 6). Hybridisation performed on sections which did not undergo any pre-treatments showed that – same as in the cecum content samples – different fixation led to preservation of different bacterial species (see Figure 10). FISH of both, PFA- and EtOH-Carnoy-fixed paraffin sections yielded good probe signal intensities, identification of single cells was clearly possible. In contrast to this, when FISH was performed on paraffin sections of 6/3/1 EtOH-Carnoy-fixed colonic tissue samples which underwent blocking and antigen retrieval with 10 mM Sodium Citrate buffer, the EUB338 I-III probe yielded only weak signals in evaluation at the CLSM (see Figure 11.A). Furthermore, a high background auto-fluorescence of the epithelium could be observed.

Usually the blocking solution is also used to dilute the antibody to the appropriate concentration. However, to try to preserve the quality of the FISH probe signals, and to check the effects of the antibody incubation on the bacterial labelling, the antibody-staining protocol

without adding primary and secondary antibodies was performed after the FISH on two sets on samples. One set was incubated with FA-free hybridisation buffer (see Figure 11.B), to the second one 1 % BSA in PBS was added (see Figure 11.C). However, both treatments resulted in weak probe intensities and also increased background signals in the Fluos-channel. As probe signals therefore could not be enhanced by the different immunostaining protocols compared to the sections without any pre-treatment it is suggested, that the quality of the bacterial labelling is affected by the pre-treatment steps of antigen retrieval and/or blocking. It has been shown that blocking is not really necessary for successful antibody staining[65]. Therefore, as a next step, the immunostaining with the mucin-specific MUC2-antibody should be performed without pre-treatments to preserve the quality of the FISH staining which has been shown to be better on sections which did not undergo blocking and antigen retrieval.

After checking the effects of the different protocols on the FISH probe signal intensities, another incubation was run where the primary and secondary antibodies were added. With this, any effects of the modified protocol on the antibody itself and the preservation of the mucosal structure should be observed (see Table 6). When the MUC2-antibody was incubated with blocking buffer (1 % BSA in PBS) the mucus layer can be clearly differentiated from the luminal content and the epithelial cells. Furthermore, also the mucus in the goblet cells could be detected (see Figure 12.A1). However, despite obtaining good results for the Cy3-labelled antibody staining, again only weak FISH signals could be observed (see Figure 12.A3). In contrast to this, FISH staining resulted in higher signal intensity on sections incubated with antibody diluted in HB (see Figure 12.B3). But despite yielding good MUC2-antibody signals when BSA was used for incubation, the immunostaining resulted in rather weak signals of the Cy3-stained mucins when the antibodies were incubated in HB. Staining was not as strong and clear, and also resulted in a high background signal in the Cy3-channel (see Figure 12.B2). Furthermore, no mucins in goblet cells were detectable, the staining of the mucosal layer revealed a rather collapsed and patchy mucin distribution between the luminal content and the epithelium (see Figure 12.B1).

However, the intestinal mucosal layer is known to be important for protection from luminal bacteria[12]. The colonic mucosal layer has been suggested to consist of an inner layer, and an outer loose layer[17], whereas the outer layer is thought to work as a filter, as the inner layer has been shown before to be devoid of bacteria[18,67]. Pictures obtained from combination of FISH and MUC2-immunostaining also show that the mucus layer is not always separated from the luminal bacteria with a clear border, but seems to smear into the lumen and mixes

with the luminal content (see Figure 12.A). This smear might be the outer loose part of the colonic mucosal layer, but this structural feature might also be due to structure disruption during sectioning. Nevertheless, apart from this smear, no clear differentiation of inner and outer layer as it has been described before was possible.

Nevertheless, this shows that for the specific antibody incubation with 1 % BSA in PBS represents the optimal option for visualisation of intestinal mucus. However, as no consensus could be found to obtain good results for both, FISH and antibody -immunostaining, another technique such as CARD-FISH might be performed to visualise bacterial colonisation along the intestinal mucosal barrier.

5.2 Effects of Bacterial Administration on DSS-Induced Colitis

5.2.1 Effects of *Akkermansia* Administration on Body Weight

Akkermansia spp. is a group of bacteria which are known mucus degraders[40]. In healthy humans, *Akkermansia muciniphila* is an abundant resident of the gastrointestinal tract and seems to be a crucial mechanism for mucus turnover[46] and also an important modulator for gut homeostasis[45], but it is known to be depleted in human inflammatory bowel diseases[42,43]. This shift of human gut microbiota is thought to have influence on the course and progression of diseases such as Crohn's Disease or ulcerative colitis, whereas both diseases show different bacterial shifts and different ways of disease progression[34]. The most common way to study bacterial alterations in the gut microbiome is DSS-induced colitis on mouse models[58]. In this study, C57BL/6 WT were gavaged with the *Akkermansia* strains MucT and H2 (human), and MG (mouse), all three strains both viable and killed by heat inactivation to find out the effects of the change in the *A. Muciniphila* number and whether the depletion of this taxa contributes to the colitic inflammation.

The most obvious thing to observe the effects of a daily administration of *Akkermansia*, and in further consequence of the DSS-induced colitis, is the body weight of the supplemented mice. In the first days of supplementation an unexpected effect in two of the groups (H2 and MucT viable) could be observed as a dramatic weight loss soon after the beginning of the gavaging occurred (see Figure 13). This effect of weight loss has been observed before[46], when viable *A. Muciniphila* reduced diet-induced body weight gain, but also decreased body weight of mice fed with a control diet.

In this study, the phenomenon of rapid weight loss occurred even after the first gavage with the human *Akkermansia* strains (see Table 6), whereas it cannot be said what was causative for the dramatic decrease. Possibilities would be a stronger activity of the mice, which was observed in MucT and H2-gavaged groups, coupled with a refusal of food uptake. Also,

during colitic inflammation a down-regulation of IL-10 results in a higher expression of the pro-inflammatory cytokines TNFα and IL-1β[32,33] what might contributes to food refusal and therefore to weight loss. The tumor necrosis factor-alpha, same as IL-1 and IL-6, are thought to lead to anorexia, as these cytokines have been shown to cause a reduced food intake and changes in gastric emptying in rats[68].

Furthermore, also the loss of urine during weighing of the mice also contributes to the mice's weight, but as the weight loss was constant over the first four days, this possibility is rather unreasonable. However, whereas for the experiment detailed in this thesis mice were daily administered by oral gavage at the dose of 10^9 cfu/0.2 mL sterile anaerobic PBS over a period of 15 days, Everard *et al.* supplemented the mice with a lower dose (2×10^8 cfu/0.2 mL sterile anaerobic PBS) but for 4 weeks[46]. Furthermore, no detailed data of daily weighing is available for their experiment therefore it is not possible to further compare the weight curves. Therefore, it cannot be said if and how the different concentrations of *Akkermansia* supplementation affect the body weight of the treated mice.

In contrast to the weight loss observed in groups of mice treated with viable H2 and MucT *Akkermansia*, no significant differences compared to the control group supplemented with PBS/2.5 % glycerol could be observed in any of the other groups gavaged with viable and heat inactivated *Akkermansia* strains (see Figure 13). All of these groups showed increasing average weight values throughout the first days of supplementation, whereas constant values are reached at day -4 and maintained until after DSS-administration started (see Figure 13 and Figure 14). The only group that does not go with this trend is the one supplemented with heat inactivated H2-*Akkermansia*. This group shows a decrease of the average weight from 102.8 ± 2.6 % at day -4 down to 98.6 ± 5.9 % at day -3 (see Table 6), whereas this reduction of the average value can be traced down to one single mouse of this group (5r) which lost weight at day -3 (99.5 % of original body weight), when all other mice gained weight and showed values between 102.0 – 105.0 % (see Table S6). Nevertheless, average weight values again increased over the next days until DSS-treatment started (see Table 6).

However, after 6 days of *Akkermansia* supplementation, mice additionally received 2 % DSS over the provided drinking water to provoke gastrointestinal inflammation. Compared to the control group, which was supplemented with 200 µL PBS/2.5 % glycerol, no significant differences concerning the average weight loss could be observed in any of the groups (see Figure 14). Only the group supplemented with heat inactivated H2-*Akkermansia*, which showed a minor decrease of average weight before DSS-administration, reached levels

slightly beyond the average weight values of the control group (see Table 6), whereas this effect lasted until the end of the experiment (see Figure 14).

DSS-treated mice have been supplemented with *Akkermansia muciniphila*[45] before, but no preceding supplementation was done then before DSS-administration started. However, contrary to the obtained results of the experiments detailed in this thesis, a stronger weight loss compared to the control group was observed when bacteria were administered together with 2 % DSS. In contrast to this, mice did recover from DSS-induced colitis when isolated extra-cellular vesicles derived from faeces of *Akkermansia muciniphila*-supplemented mice were administered together with the DSS[45].

However, qPCR for detection of *Akkermansia*-DNA in the faeces collected from all mice revealed that *Akkermansia*-DNA was present after 4 days of bacterial supplementation only in the groups gavaged with viable H2- and MucT-*Akkermansia* (see Figure 15). This data also correlates with the average body weight curves before DSS-treatment started, as these two groups started to re-gain weight at day -2 when *Akkermansia*-DNA could be detected in the faeces (see Figure 13). Nevertheless, it is still rather surprisingly that no background for *Akkermansia*-DNA could be detected during qPCR in any of the samples.

Nevertheless, in general it can be said, according to the weight curves (see Figure 13 and Figure 14), *Akkermansia* supplementation does not contribute to DSS-induced colitic inflammation, neither causing any positive, nor any negative effects on inflammation characterics. This can also be concluded from the fact, that no significant differences could be observed in the colon lengths [cm] of treated mice (see Figure 16), which can also be used as a parameter in characterisation of murine colitis due to a reduction of this during DSS-induced gastrointestinal inflammation[45].

5.2.2 Effects of *Akkermansia* Administration on Intestinal Mucus

The colonic mucosal barrier is an important part of the immune system in the intestine[69] as it keeps a distance between commensal bacteria in the lumen and the epithelial cells[66]. In human IBD mucus thickness decreases with increased inflammation severity and the inflammation of the colon is always accompanied by disruptions of the mucus barrier[69]. In mouse models, colonic inflammation is provoked by administration of DSS in the drinking water, which is the most common way for induction of colitis in animal models. DSS penetrates the mucosal layer and decreases its thickness making it possible for bacteria to infiltrate the layer and get in contact with the epithelial surface[58,70]. This event of bacterial penetration of the mucus barrier and epithelium has been suggested to cause the

development of colitis[66]. Furthermore, it has been shown that DSS induces and amplifies the apoptosis of colonic epithelial cells[70].

During DSS-administration supplementation of mice with *Akkermansia muciniphila* has been shown to contribute to mucus formation causing a thickening of the colonic mucosal barrier in animal models[46]. However, Alcian Blue staining was done on paraffin sections of 6/3/1 EtOH-Carnoy-fixed tissue samples, revealing that mucus barrier was too disrupted, or even completely destroyed on most of the tissue samples making it impossible to measure mucus thickness (see Figure 17).

However, despite the lack of the mucosal barrier, there still was a gap between the luminal content and the epithelium so that luminal bacteria did not get in contact with the epithelial surface in most of the samples. This fact shows that bacterial infiltration might be a reason for triggering colonic inflammation but cannot be the only reason for that. Nevertheless, paraffinised tissue samples represent only a short time-lapse of the whole inflammation process what makes it not possible to say whether bacteria were in touch with the epithelium at another time-point of the experiment what would verify former results[70].

Only a few samples showed a good preservation of the mucosal layer, such as colonic paraffin sections of viable MucT-treated mice. Here, a rather thick mucus layer can be seen after Alcian Blues staining, but nevertheless, no luminal content and therefore no bacteria can be found on this section (see Figure 17.A2). In contrast to this, when mice were supplemented with heat inactivated MucT, no mucosal barrier is detectable in colonic sections, but luminal bacteria get in contact with and penetrate the epithelial surface. Furthermore, this section also shows strongly enlarged goblet cells (see Figure 17.A3).

5.2.3 Detection and Localisation of Supplemented *Akkermansia spp.*

To determine the colonisation of gavaged *Akkermansia spp.*, and to see the general localisation of luminal bacteria next to the epithelium, FISH was performed on colonic paraffin sections of the treated mice. As *Akkermansia spp.* are known mucus degraders[43,44] they are expected to be localised close to the intestinal mucosal layer, or to have even infiltrated the mucus barrier in the colon after administration of probiotic *Akkermansia spp*. The colonic mucosal layer of healthy individuals, and also of animal models, has been shown to be devoid of bacteria[18,50,66,67]. Nevertheless, during progressing inflammation in DSS-induced colitis, bacteria start penetrating the mucus and infiltrate the barrier defecting at the epithelial surface[69,71].

FISH with the *Akkermansia*-specific probe Akk435 (see Table 3) on paraffin sections of 4 % PFA-fixed colonic tissue samples revealed that supplemented *Akkermansia spp.* did

indeed seem to recolonise the gut. *Akkermansia* could be found in mice of every group treated with the different strains, both active and heat inactivated, but not in mice of the control group which was supplemented only with the diluent PBS/2.5 % glycerol. The control samples also were the ones which in general only showed a low bacterial colonisation in the colon which could be stained with the general EUBmix (see Table 8). Furthermore, here bacteria were detectable mainly in the middle of the lumen rather than close to the epithelium with only rather low probe signal intensities (see Figure 18). This lack of luminal content in general shows that the colitic inflammation triggered by the DSS-administration caused strong diarrhoea leading to a complete emptying of the colon.

In contrast to the control group, high numbers of *Akkermansia spp.* could be detected in mice supplemented with viable MucT- and H2-*Akkermansia* (see Table 8) using the *Akkermansia*-specific Akk435-Cy3 probe, whereas *Akkermansia* could only be found in the middle of the lumen but not close to the mucosal barrier. Nevertheless, the presence of the supplemented phenotypes go together with the qPCR which revealed the lack of *Akkermansia*-DNA before the beginning of the experiment but obvious presence of *Akkermansia spp.* in the gut and therefore their re-colonisation in the intestine after four days of gavage (see Figure 15).

In mice supplemented with viable bacteria of the MG-type strain no *Akkermansia*-associated DNA was detectable in faecal samples of day 4 during qPCR. Nevertheless, *Akkermansia* were present on paraffin sections at the end of the experiment (see Figure 19). This indicates, that colonisation of the administered bacteria starts at a later time point of the administration than day -2 (no QPCR data available). However, *Akkermansia* were expected to localise next to the mucosal layer in all of the paraffin sections, but nevertheless, this was the case only in few sections. Some of the samples, where *Akkermansia spp.* could be found at the expected localisation, are paraffin sections of viable MG-supplemented mice (see Figure 19). The species- specific probe showed that this strain colonised next to the epithelial surface (see Figure 19.A+C). Furthermore, excitation of the general EUBmix-probe in Fluos and also DAPI-staining revealed, that in general the interluminal bacteria sit directly on the epithelium suggesting that the intestinal mucosal barrier was completely removed during colonic inflammation due to the DSS-administration (see Figure 19.B+D). This data shows, that no protection of the mucosal layer and therefore against DSS-induced colitis is given by the colonisation and presence of *Akkermansia spp.* in the gut. Former results also suggest that not the presence of *Akkermansia muciniphila* itself has a protective effect against IBD, but its extracellular vesicles produced in the gut[45].

Nevertheless, despite being not viable anymore, re-colonisation of *Akkermansia spp.* in the gut could also be observed when FISH was performed on sections of mice supplemented

with heat inactivated bacteria (see Table 8). Hereby, growth also seems to set in at a later stage of the supplementation, as no DNA was detectable in the qPCR performed with faecal pellets samples of day -2 (four days after supplementation started).

However, the experiment of *Akkermansia*-supplementation of mice before and during DSS-induced colitis revealed that bacteria, both viable and heat inactivated do colonise in the gut at a certain point of time whereas the mucus degrading *Akkermansia spp.* are located not only next to the epithelium but all over the lumen. Nevertheless, the experiment might be repeated, whereas faecal pellets for qPCR should be sampled every day to determine the point of time at which colonisation of the supplemented *Akkermansia spp.* sets in.

Furthermore, no specific trend concerning the pathological inflammation scoring of the different samples could be observed when those data were combined to the presence of *Akkermansia spp.*, or bacterial colonisation in general, respectively. It was shown before, that mice showed worse pathology scores when supplemented with *Akkermansia muciniphila* compared to mice of the control group which were received only DSS[45]. In the experiments detailed in this thesis, in general there does not seem to be a trend that shows any differences between mice of the different supplementations compared to the control group receiving only PBS/2.5 % glycerol (see Figure 24). In general, mice with an empty lumen where no bacteria at all could be detected showed the worst coring results in there groups (see Table 8, Figure 24). Still, those mice were the ones in their groups which showed the lowest weight loss [%] (see Table S6).

However, in contrast to mice supplemented with MG- and MucT-*Akkermansia*, both viable and heat inactivated, a big difference in scoring results could be observed between the groups supplemented with either H2 viable or H2 heat inactivated *Akkermansia* (see Figure 24, Table 9). Nevertheless, although mice supplemented with viable H2-bacteria approx. two times higher scoring results, these pathology scores are not thought to be due to the *Akkermansia* strain, as basically no *Akkermansia* could be detected during FISH (see Table 8).

Nevertheless, the average total inflammation scores of the *Akkermansia*-supplemented mice ranging from approx. 7 to 17 (see Table 9) show, that in general the administration of probiotic bacteria lead to lower pathology scores compared to the control group (19 ± 14). Only the group of mice gavaged with viable *Akkermansia* of the MucT strain showed worse average total scores (24 ± 8) than the control group, whereas *Akkermansia spp.* could be detected in three out of the four mice (see Table 8). In contrast to this, in the MucT-HI group, the mice in which *Akkermansia* could be detected during FISH have a better scoring than the

ones without *Akkermansia*. Contrary, H2-viable-gavaged mice had the best scoring results above all despite having no detectable *Akkermansia* in the lumen. This shows very clearly, that *Akkermansia*-supplementation does not seem to have any effect on DSS-induced colitis in mice.

5.2.4 Further Effects of DSS-Induced Colonic Inflammation

DSS is also known to induce and amplify the apoptosis of colonic epithelial cells[70]. These strong levels of cellular turnover in the colonic tissue during DSS-induced colitis could also be observed on several tissue samples of the supplemented mice. This increased process of epithelial cells migrating from the base of a crypt to the villi where the cells then are shed and become apoptotic is also known as cell shedding[72,73]. This process of death of abnormal (inflamed) epithelial cells is important to save the organism from clonal expansion and tumour formation. Shed cells then usually are discarded together with the luminal content[74]. Nevertheless, it is not clear whether cells are shed because they are apoptotic, or whether they become apoptotic during shedding[72].

During Alcian Blue staining of 6/6/1-EtOH-Carnoy-fixed paraffin sections, the first consequences of the inflammation could be observed as it first revealed the phenomenon of cell shedding (e.g. in MucT-supplemented mice; see Figure 21). Also, during FISH the counterstaining of cells using DAPI enabled observation of the effect in several sections (see Figure 18, Figure 20). To better visualise the apoptotic cells which are shed from the inflamed epithelium, FISH was performed additionally using the eukaryote-specific probe EUK516-Cy3. On several sections, only single apoptotic cells in the lumen close to the epithelium (see Figure 22), mostly on sections treated with viable *Akkermansia spp*, which in general showed only low levels of cell shedding. In contrast to this, higher levels of cell shedding could be observed on colonic sections of mice supplemented with heat inactivated bacteria. Here, this effect partly was so strong, that the shed apoptotic epithelial cells built an additional layer separating the luminal content from the inflamed epithelium (see Figure 23) or even almost filled up the whole lumen leaving only a small area empty for the passage of luminal content (see Figure 20). This high level of cell shedding can be due to a severe inflammation of the epithelial tissue. Furthermore, it might also be an alternative to the missing mucosal layer in the colon. To verify this hypothesis a mucus staining is necessary to determine whether there is any mucosal structure left between the apoptotic structures, and also whether goblet cells are left in the epithelium or somehow disrupted so that no secretion of mucins for the formation of a protective mucosal layer is possible anymore.

Cell shedding has been shown to be triggered and amplified by the presence of the pro-inflammatory cytokine TNF-α[75–77] which expression has been shown to be increased during inflammation[32,33]. Therefore, it is thought that the level of cell shedding goes together with inflammation scores. Experiments showed, that a coherence between the level of cell shedding observed during FISH and the severity of the inflammation evaluated during pathology examinations could be observed. In general, sections with a higher level of cell shedding such as sections of mice treated with MG-HI (see Figure 23) also showed worse pathology scores indicating a more severe inflammation (see Figure 24).

Nevertheless, former publications show, that increased cell shedding of apoptotic cells already sets in after short period of DSS-administration[78]. Therefore, to determine exact point of time when this increased cell turnover sets in more sampling points necessary. Furthermore, also a better connection between inflammation severity, cell shedding, and the presence and amount of present *Akkermansia spp.* would be possible with more regular samples.

6 CONCLUSION

6.1 Fixation and Visualisation of Mucus

Several fixatives are available for tissue fixation, whereas not every fixative is also suitable for giving good results during fluorescence *in-situ* hybridization. Testing the general bacterial EUB338 I – III probe mix revealed that cecum content samples fixed with 4 % PFA delivered the best average volume fraction and highest probe intensity signals. Also, the Bac303 probe (specific for *Bacteroidales*) could be detected well, but for the Erec482 (*Clostridium* clusters XIVa and XIVb) only weak signals could be detected. During Alcian Blue staining, this fixative also showed rather bad tissue preservation on paraffin sections with shrinkage of the intestinal content, rather big gaps between epithelium and content could be seen, and the mucosal layer was disrupted on almost the whole section. In contrast to this, fixation with 6/3/1 EtOH-Carnoy gave good results, for structure preservation as well as for signals of EUBmix and Erec482 probes. Still, no signal could be detected for *Bacteroidales* due to the ethanol in the fixative. Only samples fixed with 6/6/1 Methanol-Carnoy worked for all three probes and also showed good structure preservation. Nevertheless, tissue fixed with 6/3/1 EtOH-Carnoy showed the best preservation of mucosal structures, both for the secreted mucins in the mucosal barrier, as well as for the freshly produced mucus in the goblet cells.

However, Alcian Blue staining was shown to work perfectly for the visualisation of mucus, but nevertheless, this method is not combinable with FISH. Therefore, immunohistochemical methods were tested. Firstly, mucin staining using the lectin Jacalin extracted from Jackfruit seed labelled with the fluorescent dye TexasRed® was performed on 6/3/1 EtOH-Carnoy-fixed colonic paraffin sections of a WT mouse. Two different protocols were applied, but nevertheless, no clear staining – neither of goblet cells, nor of secreted mucins in the mucosal barrier on the epithelial surface could be detected. Furthermore, the obtained results were not reproducible, therefore immunohistochemical staining of mucins using a anti-MUC2-antibody was tested. In contrast to the poor staining of the intestinal mucosal structures obtained during Jacalin staining, the glycosylated gel-forming mucin MUC2 could perfectly be visualised using an antibody. Rehydrated paraffin sections of colonic tissue fixed with 6/3/1 EtOH-Carnoy revealed clear staining of the well preserved mucosal structures, as well as of brush borders, and goblet cells in the epithelial tissues. Nevertheless, despite yielding good results in mucosal staining, the combination of antibody staining and FISH on one section resulted in only rather weak probe signal intensity with a high background. Here, also two different protocols were applied to enhance probe signal intensity of FISH which preceded the antibody staining. It was shown that for the specific antibody incubation with

1 % BSA in PBS represents the optimal option for visualisation of intestinal mucus, but nevertheless, no consensus could be found to obtain good results for both, FISH and antibody-immunostaining. Therefore, it is suggested to use another technique such as CARD-FISH to visualise bacterial localisation along the mucosal layer. Furthermore, staining should then be performed with different, more specific probes.

6.2 Effects of Bacterial Administration on DSS-Induced Colitis

Three different strains of *Akkermansia spp.* (phylogenetic intermediates were gavaged to 15 weeks old male C57BL/6 WT mice. The human strains MucT and H2, as well as the mouse strain MG were orally administered at a concentration of 10^9 cfu/200 µL, both viable and heat inactivated, as well as PBS containing 2.5 % glycerol as a control, each to groups of four mice over a period of 15 days. On the seventh day (Day 0) colonic inflammation was provoked by the administration of 2 % DSS provided over the drinking water.

All in all, neither a positive, nor a negative effect of administration of probiotic *Akkermansia spp.* could be observed. During the first days of supplementation before DSS-induction of the colitis, a considerable weight loss could be observed in the viable human MucT and H2 strains, whereas the gavaged mice recovered rather quickly from the rapid weight loss after Day -3 (see Figure 13). This recovery also goes with qPCR where *Akkermansia*-DNA was detectable in the viable MucT and H2-groups four days after gavaging started (see Figure 15). Nevertheless, all the other groups did not show any significant differences compared to the control group. After DSS-treatment started, no significant differences between the different phylogenetic intermediates compared to the control group could be observed (see Figure 14) indicating no positive or negative effect of the bacterial supplementation on the average weight [%] during colitic inflammation. Furthermore, also comparison of the colon lengths of DSS-treated mice did not show any significant differences between the single groups after normalisation of the colon lengths [cm] to the original body weight [g] of the mice.

To determine mucus thickness, paraffin sections of 6/3/1 EtOH-Carnoy-fixed tissue from ileum, cecum and colon of all mice were stained with Alcian Blue. The staining revealed rather strong disruption of the mucosal barrier in most of the sections making a measurement of mucus thickness impossible (see Figure 17). Nevertheless, a few sections did indeed show a rather goo preservation of the intestinal mucus layer, such as samples of mice gavaged with the viable MucT strain (see Figure 17.A2). Still, although mucus could be detected on those colonic sections, DSS-induced colitis caused the colon to be completely empty and therefore probably devoid of any bacteria.

Furthermore, apart from the disrupted mucosal layer, most ileal, cecal, and colonic sections showed mucus filled goblet cells of normal size. Still, some samples such as the colonic sample of mice supplemented with heat inactivated MucT-*Akkermansia* showed strongly enlarged goblet cells. However, a few sections did also show that bacteria did get in contact with and penetrate the epithelial surface due to a lack of a mucosal barrier.

FISH on paraffin sections revealed that *Akkermansia spp.* were present in all of the supplemented groups, except of the control group indicating that the supplementation of *Akkermansia* does contribute to a successful re-colonisation of lacking bacteria in the gut. Nevertheless, despite expecting the mucus degrading bacteria to infiltrate the mucosal barrier, *Akkermansia* mostly were detectable in the interluminal content rather than close to the mucus layer. Still, also a mucus association of the *Akkermansia* was observed in single samples such as sections from mice supplemented with viable MG-*Akkermansia*. Nevertheless, no significant connection could be made between the presence of *Akkermansia spp.* in the colon and the pathological inflammation scores determined on H&E stained sections. In contrast to this, coherence could be observed when it comes to cell shedding, as the level of apoptotic cells in the luminal content seemed to increase with the severity of the colitic inflammation evaluated during pathology scoring.

However, paraffinised tissue samples represent only a short time-lapse of the re-colonisation and localisation of *Akkermansia spp.,* and of the whole inflammation process. Furthermore, sampling more time points also will enable a better evaluation of the time point when cell shedding, and the re-colonisation of *Akkermansia spp.* set in. Also, it will be easier to draw a better connection between the presence of *Akkermansia spp.* and inflammation severity. Additionally, a combined staining of the intestinal mucins and of luminal bacteria would enhance the possibility to localise luminal bacteria, specifically of *Akkermansia spp.*

7 ZUSAMMENFASSUNG

Der Gastrointestinaltrakt enthält 10^{12} bis 10^{14} Bakterien. Es wird angenommen, dass allein im Dickdarm 70 % aller im humanen Körper enthaltenen Mikroben gefunden werden können. Eine Störung dieser Darmflora wurde mit chronisch entzündlichen Darmkrankheiten, wie Morbus Crohn oder Colitis ulcerosa in Zusammenhang gebracht. Als solche Veränderung der Darmflora wird die Verminderung der Anzahl an mucusabbauenden *Akkermansia muciniphila* gehandelt. Deshalb wird angenommen, dass eine Supplementierung von probiotischen *Akkermansia spp.* positive Auswirkungen auf eine Natrium-Dextransulfat (DSS)-induzierte Colitis hat. Das Projektteam hat beobachtet, dass die Supplementierung von C57BL/6 WT Mäusen mit lebensfähigen humanen H2- und MucT-*Akkermansia* zu einem starken Gewichtsverlust in den ersten Tagen führte, wobei diese Mäuse vier Tage nach Beginn der Verabreichung, als *Akkermansia*-DNA auch in einer quantitative PCR nachweisbar war, wieder an Gewicht zunahmen. Hitzeinaktivierte (HI) und lebensfähige *Akkermansia* des MG-(Maus)-Stammes, sowie alle anderen HI-Stämme hatten im Vergleich zur Kontrollgruppe keine positiven oder negativen Auswirkungen auf das durchschnittliche Gewicht, weder vor, noch während der DSS-induzierten Entzündung. Bei der Evaluierung von Dickdarmgewebedünnschnitten mittels Fluoreszenz *in situ* Hybridisierung (FISH) konnte die Rekolonisierung des Dickdarms durch *Akkermansia spp.* in allen Gruppen, außer in der Kontrollgruppe, nachgewiesen werden. Allerdings wurde kein relevanter Zusammenhang zwischen der Anwesenheit von *Akkermansia spp.* und dem Entzündungsgrad nachgewiesen. Darüber hinaus konnten die mucinabbauenden Bakterien nicht, wie erwartet, ausschließlich an der intestinalen Mucusschicht nachgewiesen werden, sondern diese verteilten sich über das gesamte Darmlumen.

Für eine bessere Bewertung des Zusammenhangs zwischen der Anwesenheit von *Akkermansia spp.* im Dickdarm und der Störung der Darmschleimhaut ist eine Kombinationsfärbung von beiden notwendig. Der Hauptbestandteil von Mucus ist Wasser (> 98 %), dadurch ist eine Visualisierung mittels Mikroskop schwierig. Neun verschiedene Fixiermittel und unterschiedliche Färbemethoden wurden getestet, um die besten Bedingungen für die optimale Gewebe- und Schleimhauterhaltung, sowie gute Signalintensität mittels FISH zu finden. FISH wurde auf fixiertem Blinddarminhalt mit der allgemeinen Sonde EUBmix I – III und den spezifischen Sonden für *Bacteroidales* und *Clostridium* clusters XIVa und XIVb durchgeführt. Mittels DAIME wurde deren Average Volume Fraction und Signalintensitäten ermittelt. Auf Signalintensität und Strukturerhaltung während einer Alzianblaufärbung bezogen, lieferten Fixierung mit 4 % PFA, 6/3/1 wasserfreiem EtOH-Carnoy und 6/6/1 MetOH-Carnoy die besten Ergebnisse. Da die Färbung der Darmschleimhaut mit Alzianblau aber nicht mit FISH kombinierbar ist, wurden

immunhistochemische Färbemethoden mit dem Lektin Jacalin aus der Jackbaumfrucht und mit dem mucinspezifischen Anti-MUC2-Antikörper getestet. Die Lektinmarkierung lieferte keine klare Färbung der intestinalen Mucusschicht, die Antikörpermarkierung von MUC2 hingegen führte zu einer eindeutigen Färbung der Mucusbarriere, des Bürstensaums und der Becherzellen. Um symbiotische Bakterien zu lokalisieren, konnten durch die Kombination von MUC2-Antikörperfärbung und FISH keine zufriedenstellenden Signalintensitäten der Sonden erzielt werden.

Schlagworte: Darmflora; *Akkermansia muciniphila*; chronisch entzündliche Darmkrankheiten; Fluoreszenz *in situ* Hybridisierung; Mucus; MUC2; Alzianblau; Cell Shedding

8 REFERENCES

1. Johansson, M. E. V *et al.* Composition and Functional Role of the Mucus Layers in the Intestine. *Cell. Mol. Life Sci.* **68,** 3635–41 (2011).

2. Young, V. & Schmidt, T. Overview of the Gastrointestinal Microbiota. *Adv. Exp. Med. Biol.* **635,** 29–40 (2008).

3. Sekirov, I., Russell, S. L., Antunes, L. C. M. & Finlay, B. B. Gut Microbiota in Health and Disease. *Physiol. Rev.* **90,** 859–904 (2010).

4. Berry, D. *et al.* Phylotype-Level 16S rRNA Analysis Reveals New Bacterial Indicators of Health State in Acute Murine Colitis. *ISME J.* **6,** 2091–106 (2012).

5. Weir, T. L. *et al.* Stool microbiome and metabolome differences between colorectal cancer patients and healthy adults. *PLoS One* **8,** e70803 (2013).

6. Qin, J. *et al.* A Human Gut Microbial Gene Catalogue Established by Metagenomic Sequencing. *Nature* **464,** 59–65 (2010).

7. O'Hara, A. M. & Shanahan, F. The Gut Flora as a Forgotten Organ. *EMBO Rep.* **7,** 688–93 (2006).

8. Cummings, J. H. & Macfarlane, G. T. Role of Intestinal Bacteria in Nutrient Metabolism. *JPEN. J. Parenter. Enteral Nutr.* **21,** 357–65 (1997).

9. De Vos, W. M. & de Vos, E. a J. Role of the Intestinal Microbiome in Health and Disease: From Correlation to Causation. *Nutr. Rev.* **70 Suppl 1,** S45–56 (2012).

10. Manson, J., Rauch, M. & Gilmore, M. The Commensal Microbiology of the Gastrointestinal Tract. *Regul. Immune Syst.* (2008). at <http://link.springer.com/chapter/10.1007/978-0-387-09550-9_2>

11. Bergstrom, K. S. B. *et al.* Muc2 Protects Against Lethal Infectious Colitis by Disassociating Pathogenic and Commensal Bacteria from the Colonic Mucosa. *PLoS Pathog.* **6,** e1000902 (2010).

12. Hansson, G. C. Role of Mucus Layers in Gut Infection and Inflammation. *Curr. Opin. Microbiol.* **15,** 57–62 (2012).

13. Gouyer, V. *et al.* The Characterization of the First Anti-Mouse Muc6 Antibody Shows an Increased Expression of the Mucin in Pancreatic Tissue of Cftr-Knockout Mice. *Histochem. Cell Biol.* **133,** 517–25 (2010).

14. Park, S., Zhen, G., Verhaeghe, C., Nakagami, Y. & Nguyenvu, L. T. The Protein Disulfide Isomerase AGR2 is Essential for Production of Intestinal Mucus. *Proc. Natl. Acad. Sci. U. S. A.* **106,** 6950–6955 (2009).

15. Atuma, C., Strugala, V., Allen, A. & Holm, L. The Adherent Gastrointestinal Mucus Gel Layer : Thickness and Physical State In Vivo. *Am. J. Physiol. Gastrointest. Liver Physiol.* **280,** G922–G929 (2013).

16. Juge, N. Microbial Adhesins to Gastrointestinal Mucus. *Trends Microbiol.* **20,** 30–9 (2012).

17. Johansson, M. E. V, Larsson, J. M. H. & Hansson, G. C. The Two Mucus Layers of Colon are Organized by the MUC2 Mucin, Whereas the Outer Layer is a Legislator of Host-Microbial Interactions. *Proc. Natl. Acad. Sci. U. S. A.* **108 Suppl,** 4659–65 (2011).

18. Johansson, M. E. V *et al.* The Inner of the Two Muc2 Mucin-Dependent Mucus Layers in Colon is Devoid of Bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **105,** 15064–9 (2008).

19. Johansson, M. E. V & Hansson, G. C. Preservation of Mucus in Histological Sections, Immunostaining of Mucins in Fixed Tissue, and Localization of Bacteria with FISH. *Methods Mol. Biol. Clift. Nj* **842,** 229–235 (2012).

20. Cohen, M., Varki, N. M., Jankowski, M. D. & Gagneux, P. Using Unfixed, Frozen Tissues to Study Natural Mucin Distribution. *J. Vis. Exp.* e3928 (2012). doi:10.3791/3928

21. Shi, S. R., Key, M. E. & Kalra, K. L. Antigen Retrieval in Formalin-Fixed, Paraffin-Embedded Tissues: An Enhancement Method for Immunohistochemical Staining Based on Microwave Oven Heating of Tissue Sections. *J. Histochem. Cytochem.* **39,** 741–748 (1991).

22. Mowry, R. W. Alcian Blue Techniques for the Histochemical Study of Acidic Carbohydrates. *J. Histochem. Cytochem.* **6,** 82 (1958).

23. Walsh, M. D. & Jass, J. R. Histologically Based Methods for Detection of Mucin. **125,** 29–44

24. Quintarelli G., Scott J. E., D. M. C. The Chemical and Histochemical Properties of Alcian Blue. I. The Mechanism of Alcian Blue Staining. *Histochemie* **4,** 73–85 (1964).

25. Sata T., Roth J., Zuber C., Stamm B., H. P. U. Expression of Alpha-2,6-Linked Sialic Acid Residues in Neoplastic but not in Normal Human Colonic Mucosa: A Lectin-Gold Cytochemical Study with Sambucus Nigra and Maackia Amurensis Lectins. *Am. J. Pathol.* **139,** 1435–1448 (1991).

26. Kabir, S. Jacalin: A Jackfruit (Artocarpus Heterophyllus) Seed-Derived Lectin of Versatile Applications in Immunobiological Research. *J. Immunol. Methods* **212,** 193–211 (1998).

27. Prenzoska J., Layton G. T., Devine P. L., McKenzie I. F., X. P. X. Second-Generation Monoclonal Antibodies to Intestinal MUC2 Peptide Reactive With Colon Cancer. *J. Natl. Cancer Inst.* **84,** 699–703 (1992).

28. Swidsinski, A., Loening-Baucke, V., Vaneechoutte, M. & Doerffel, Y. Active Crohn's Disease and Ulcerative Colitis Can Be Specifically Diagnosed and Monitored Based on the Biostructure of the Fecal Flora. *Inflamm. Bowel Dis.* **14,** 147–61 (2008).

29. Bartosch, S., Fite, A., Macfarlane, G. T. & Mcmurdo, M. E. T. Characterization of Bacterial Communities in Feces from Healthy Elderly Volunteers and Hospitalized

Elderly Patients by Using Real-Time PCR and Effects of Antibiotic Treatment on the Fecal Microbiota. *Appl. Environ. Microbiol.* **70,** 3575–3581 (2004).

30. Olszak, T. *et al.* Microbial Exposure During Early Life Has Persistent Effects on Natural Killer T Cell Function. *NIH Public Access* **336,** 489–493 (2012).

31. Schreiber, S., Heinig, T., Thiele, H. & Raedler, A. Immunoregulatory Role of Interleukin 10 in Pationts with Inflammatory Bowel Disease. *Gastroenterology* **108,** 1434–1444 (1995).

32. Sellon, R. K. *et al.* Resident Enteric Bacteria Are Necessary for Development of Spontaneous Colitis and Immune System Activation in Interleukin-10-Deficient Mice. *Infect. Immun.* **66,** 5224–31 (1998).

33. Li, M. O. & Flavell, R. A. Contextual Regulation of Inflammation: A Duet by Transforming Growth Factor-Beta and Interleukin-10. *Immunity* **28,** 468–76 (2008).

34. Gophna, U., Sommerfeld, K., Gophna, S., Doolittle, W. F. & Veldhuyzen van Zanten, S. J. O. Differences Between Tissue-Associated Intestinal Microfloras of Patients with Crohn's Disease and Ulcerative Colitis. *J. Clin. Microbiol.* **44,** 4136–41 (2006).

35. Nell, S., Suerbaum, S. & Josenhans, C. The Impact of the Microbiota on the Pathogenesis of IBD: Lessons from Mouse Infection Models. *Nat. Rev. Microbiol.* **8,** 564–77 (2010).

36. Manichanh, C. *et al.* Reduced Diversity of Faecal Microbiota in Crohn's Disease Revealed by a Metagenomic Approach. *Gut* **55,** 205–11 (2006).

37. Willing, B. P. *et al.* A Pyrosequencing Study in Twins Shows that Gastrointestinal Microbial Profiles Vary with Inflammatory Bowel Disease Phenotypes. *Gastroenterology* **139,** 1844–1854.e1 (2010).

38. Sepehri, S., Kotlowski, R., Bernstein, C. N. & Krause, D. O. Microbial Diverstiy of Inflamed and Noninflamed Gut Biopsy Tissues in Inflammatory Bowel Disease. *Inflamm. Bowel Dis.* **13,** 675–83 (2007).

39. Berry, D. & Reinisch, W. Intestinal Microbiota: A Source of Novel Biomarkers in Inflammatory Bowel Diseases? *Best Pract. Res. Clin. Gastroenterol.* **27,** 47–58 (2013).

40. Derrien, M., Vaughan, E. E., Plugge, C. M. & de Vos, W. M. Akkermansia Muciniphila Gen. Nov., Sp. Nov., a Human Intestinal Mucin-Degrading Bacterium. *Int. J. Syst. Evol. Microbiol.* **54,** 1469–76 (2004).

41. Png, C. W. *et al.* Mucolytic Bacteria With Increased Prevalence in IBD Mucosa Augment In Vitro Utilization of Mucin by Other Bacteria. *Am. J. Gastroenterol.* **105,** 2420–2428 (2010).

42. Van Passel, M. W. J. *et al.* The Genome of Akkermansia Muciniphila, A Dedicated Intestinal Mucin Degrader, and Its Use in Exploring Intestinal Metagenomes. *PLoS One* **6,** e16876 (2011).

43. Derrien, M., Collado, M. C., Ben-Amor, K., Salminen, S. & de Vos, W. M. The Mucin Degrader Akkermansia Muciniphila Is An Abundant Resident of the Human Intestinal Tract. *Appl. Environ. Microbiol.* **74,** 1646–8 (2008).

44. Derrien, M. *et al.* Mucin-Bacterial Interactions in the Human Oral Cavity and Digestive Tract. *Gut Microbes* **1,** 254–268 (2010).

45. Kang, C.-S. *et al.* Extracellular Vesicles Derived from Gut Microbiota, Especially Akkermansia Muciniphila, Protect the Progression of Dextran Sulfate Sodium-Induced Colitis. *PLoS One* **8,** e76520 (2013).

46. Everard, A. *et al.* Cross-talk Between Akkermansia Muciniphila and Intestinal Epithelium Controls Diet-Induced Obesity. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 9066–71 (2013).

47. Belzer, C. & de Vos, W. M. Microbes inside - From Diversity to Function: The Case of Akkermansia. *ISME J.* **6,** 1449–58 (2012).

48. Baintner, K., Jakab, G., Gyôri, Z. & Kiss, P. Binding of FITC-Labelled Lectins to the Gastrointestinal Epithelium of the Rat. *Pathol. Oncol. Res.* **6,** 179–83 (2000).

49. Freitas, M., Axelsson, L.-G., Cayuela, C., Midtvedt, T. & Trugnan, G. Microbial-Host Interactions Specifically Control the Glycosylation Pattern in Intestinal Mouse Mucosa. *Histochem. Cell Biol.* **118,** 149–61 (2002).

50. Corazziari, E. S. Intestinal Mucus Barrier in Normal and Inflamed Colon. *J. Pediatr. Gastroenterol. Nutr.* **48 Suppl 2,** S54–5 (2009).

51. Liquori, G. E. *et al.* In Situ Characterization of O-Linked Glycans of Muc2 in Mouse Colon. *Acta Histochem.* **114,** 723–32 (2012).

52. Van der Sluis, M. *et al.* Muc2-Deficient Mice Spontaneously Develop Colitis, Indicating that MUC2 is Critical for Colonic Protection. *Gastroenterology* **131,** 117–29 (2006).

53. Kanai, K., Nunoya, T., Shibuya, K., Nakamura, T. & Tajima, M. Variations in Effectiveness of Antigen Retrieval Pretreatments for Diagnostic Immunohistochemistry. *Res. Vet. Sci.* **64,** 57–61 (1998).

54. Shi, S. R., Chaiwun, B., Young, L., Cote, R. J. & Taylor, C. R. Antigen Retrieval Technique Utilizing Citrate Buffer or Urea Solution for Immunohistochemical Demonstration of Androgen Receptor in Formalin-Fixed Paraffin Sections. *J. Histochem. Cytochem.* **41,** 1599–1604 (1993).

55. Daims, H., Brühl, A., Amann, R., Schleifer, K. H. & Wagner, M. The Domain-Specific Probe EUB338 is Insufficient for the Detection of All Bacteria: Development and Evaluation of a More Comprehensive Probe Set. *Syst. Appl. Microbiol.* **22,** 434–44 (1999).

56. Canny, G., Swidsinski, A. & McCormick, B. Interactions of Intestinal Epithelial Cells with Bacteria and Immune Cells: Methods to Characterize Microflora and Functional Consequences. *Methods Mol. Biol.* **341,** 17–35 (2006).

57. Daims, H., Lücker, S. & Wagner, M. Daime, A Novel Image Analysis Program for Microbial Ecology and Biofilm Research. *Environ. Microbiol.* **8,** 200–213 (2006).

58. Laroui, H. *et al.* Dextran Sodium Sulfate (DSS) Induces Colitis in Mice by Forming Nano-Lipocomplexes with Medium-Chain-Length Fatty Acids in the Colon. *PLoS One* **7,** e32084 (2012).

59. Puchtler, H., Waldrop, F., Conner, H. & Terry, M. Carnoy fixation: Practical and Theoretical Considerations. *Histochemie* **16,** 361–371 (1968).

60. Wagner, M., Horn, M. & Daims, H. Fluorescence In Situ Hybridisation For The Identification and Characterisation of Prokaryotes. *Curr. Opin. Microbiol.* **6,** 302–309 (2003).

61. Baloglu, G. *et al.* The Effects of Tissue Fixation Alternatives on DNA Content: A Study on Normal Colon Tissue. *Appl. Immunohistochem. Mol. Morphol.* **16,** 485–492 (2008).

62. Kretschy, N. & Somoza, M. M. Comparison of the Sequence-Dependent Fluorescence of the Cyanine Dyes Cy3, Cy5, DyLight DY547 and DyLight DY647 on Single-Stranded DNA. *PLoS One* **9,** e85605 (2014).

63. Szentkuti, L. & Enss, M. L. Comparative Lectin-Histochemistry on the Pre-Epithelial Mucus Layer in the Distal Colon of Conventional and Germ-Free Rats. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **119,** 379–86 (1998).

64. Fried, J., Wolfgang, L., Psenner, R. & Schleifer, K. H. Improvement of Ciliate Identification and Quantification: A New Protocol for Fluorescence In Situ Hybridization (FISH) in Combination with Silver Stain Techniques. *Syst. Appl. Microbiol.* **25,** 555–571 (2002).

65. Buchwalow, I., Samoilova, V., Boecker, W. & Tiemann, M. Non-specific Binding of Antibodies in Immunohistochemistry: Fallacies and Facts. *Sci. Rep.* **1,** 28 (2011).

66. Johansson, M. E. V *et al.* Bacteria Penetrate the Inner Mucus Layer Before Inflammation in the Dextran Sulfate Colitis Model. *PLoS One* **5,** e12238 (2010).

67. Hansson, G. C. & Johansson, M. E. V. The Inner of the Two Muc2 Mucin-Dependent Mucus Layers in Colon is Devoid of Bacteria. *Gut Microbes* **1,** 51–54 (2010).

68. McCarthy, D. O. Tumor Necrosis Factor Alpha and Interleukin-6 have Differential Effects on Food Intake and Gastric Emptying in Fasted Rats. *Res. Nurs. Heal.* **23,** 222–228 (2000).

69. Swidsinski, A. *et al.* Comparative Study of the Intestinal Mucus Barrier in Normal and Inflamed Colon. *Gut* **56,** 343–50 (2007).

70. Araki, Y. *et al.* Dextran Sulfate Sodium Administered Orally is Depolymerized in the Stomach and Induces Cell Cycle Arrest Plus Apoptosis in the Colon in Early Mouse Colitis. *Oncol. Rep.* **28,** 1597–605 (2012).

71. Swidsinski, A. *et al.* Mucosal Flora in Inflammatory Bowel Disease. *Gastroenterology* **122,** 44–54 (2002).

72. Bullen, T. F. *et al.* Characterization of Epithelial Cell Shedding From Human Small Intestine. *Lab. Invest.* **86,** 1052–63 (2006).

73. Grootjans, J. *et al.* Ischaemia-Induced Mucus Barrier Loss and Bacterial Penetration are Rapidly Counteracted by Increased Goblet Cell Secretory Activity in Human and Rat Colon. *Gut* **62,** 250–8 (2013).

74. Gibson, P. R. Apoptosis or Necrosis - Colonic Epithelial Cell Survival. *Novartis Found. Symp.* **263,** 133–150, 211–218 (2004).

75. Watson, A. J. & Hughes, K. R. TNF-α-Induced Intestinal Epithelial Cell Shedding: Implications For Intestinal Barrier Function. *Ann. N. Y. Acad. Sci.* **1258,** 1–8 (2012).

76. Kiesslich, R. *et al.* Identification of Epithelial Gaps in Human Small and Large Intestine by Confocal Endomicroscopy. *Gastroenterology* **133,** 1769–78 (2007).

77. Duckworth, C. A. & Watson, A. J. Analysis of Epithelial Cell Shedding and Gaps In The Intestinal Epithelial. *Methods Mol. Biol.* **763,** 105–114 (2011).

78. Venkatraman, A., Ramakrishna, B. S., Pulimood, A. B., Patra, S. & Murthy, S. Increased Permeability in Dextran Sulphate Colitis in Rats: Time Course of Development and Effect of Butyrate. *Scand. J. Gastroenterol.* **35,** 1053–1059 (2000).

9 SUPPLEMENTARY INFORMATION

9.1 Supplementary Materials and Methods

9.1.1 Equipment and Software

Table S1: Equipment used

Name	Producer
Microtome RM2155	Leica Microsystems
Shandon Excelsior Tissue Processor	Thermo Scientific
ASS 1 – Staining Machine for Histology and Zytology	Pathisto
Confocal laser scanning microscope CLSM TCS SP8 X **Software:** LAS AF 3.2.1	Leica Microsystems
MILLI-Q Biocel water purification system	EMD Millipore
Vortex Genie 2	Carl Roth GmbH
Hybridisation oven UE-500	Memmert GmbH
Eppendorf research pipettes 1 – 1000 µL	Eppendorf AG
Incubation/Inactivation Water Bath 1004	GFL Gesellschaft für Labortechnik mbH
Pathology microscope Axio Imager M.1 **Software:** AxioVision Rel. 4.8	Carl Zeiss Microscopy

Table S2: Programme for paraffinisation of sections using the Shandon Excelsior Tissue Processor (Thermo scientific)

Step No.	Solution	Duration	Temperature
1	70 % EtOH	5 min	36 °C
2	75 % EtOH	1 h	
3	90 % EtOH	1 h	
4	96 % EtOH	1 h	
5	100 % EtOH	1 h	
6	100 % EtOH	1 h	
7	100 % EtOH	1 h	
8	Xylene Substitute	1 h	
9	Xylene Substitute	1 h	
10	Xylene Stubstitute	1 h	
11	Wax	1 h, 20 min	
12	Wax	1 h, 20 min	
13	Wax	1 h, 20 min	

9.1.2 Media, Buffers, and Solutions

Table S3: Phosphate-buffered saline (PBS) stock solution (pH 7.2 – 7.4)

Chemical	Concentration (mmol/L)
NaH_2PO_4	200
Na_2HPO_4	200
Ultrapure Water	

Table S4: 1x PBS

Chemical	Concentration (mmol/L)
PBS stock solution	10
NaCL	130
Ultrapure Water	

Table S5: 10 mM Sodium Citrate Buffer (pH 6)

Chemical	Concentration
$Na_3C_6H_5O_7$	10 mM
Tween 20	0.05 %
Ultrapure Water	

9.2 Supplementary Results

9.2.1 Supplementary Figures

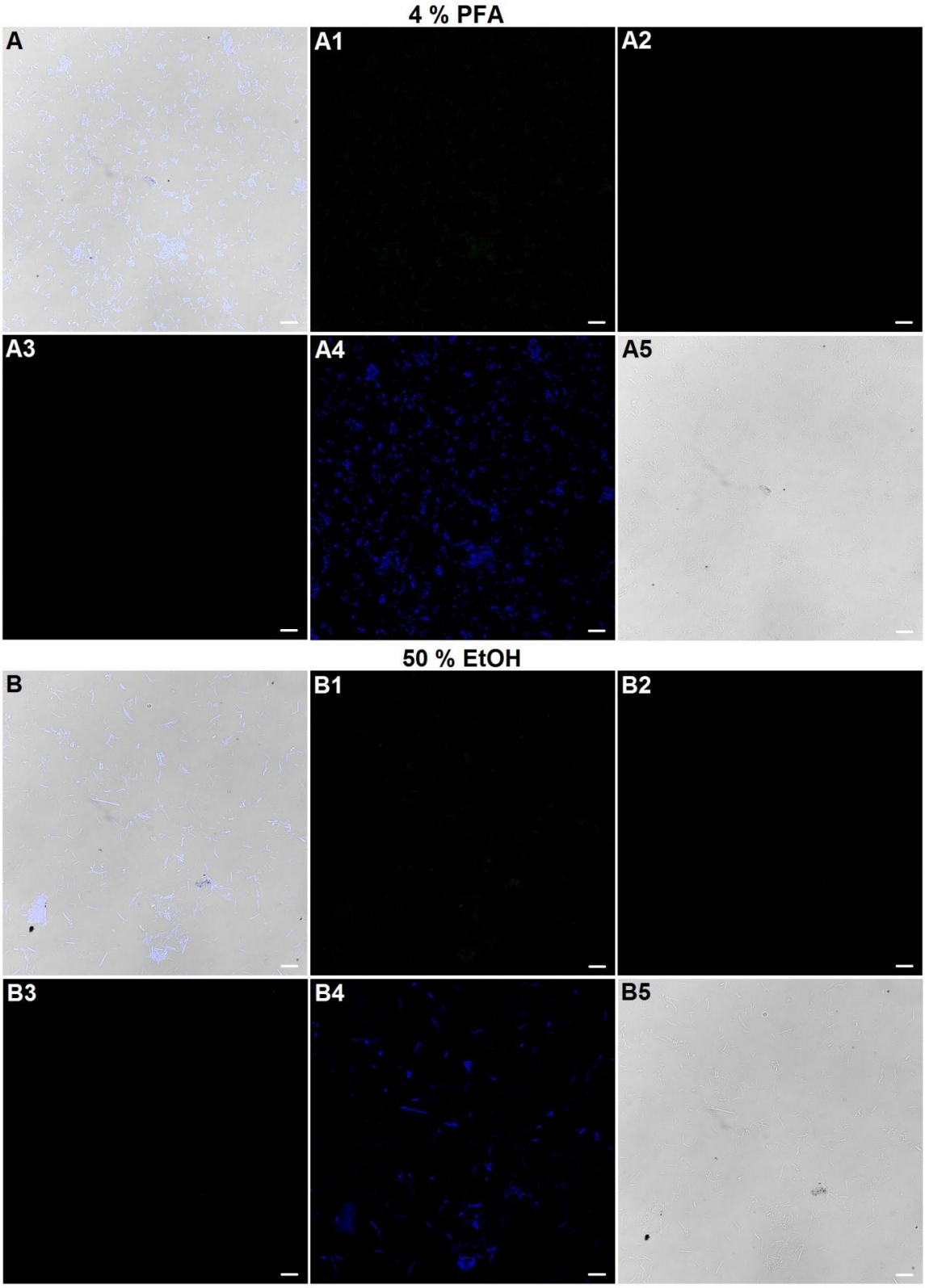
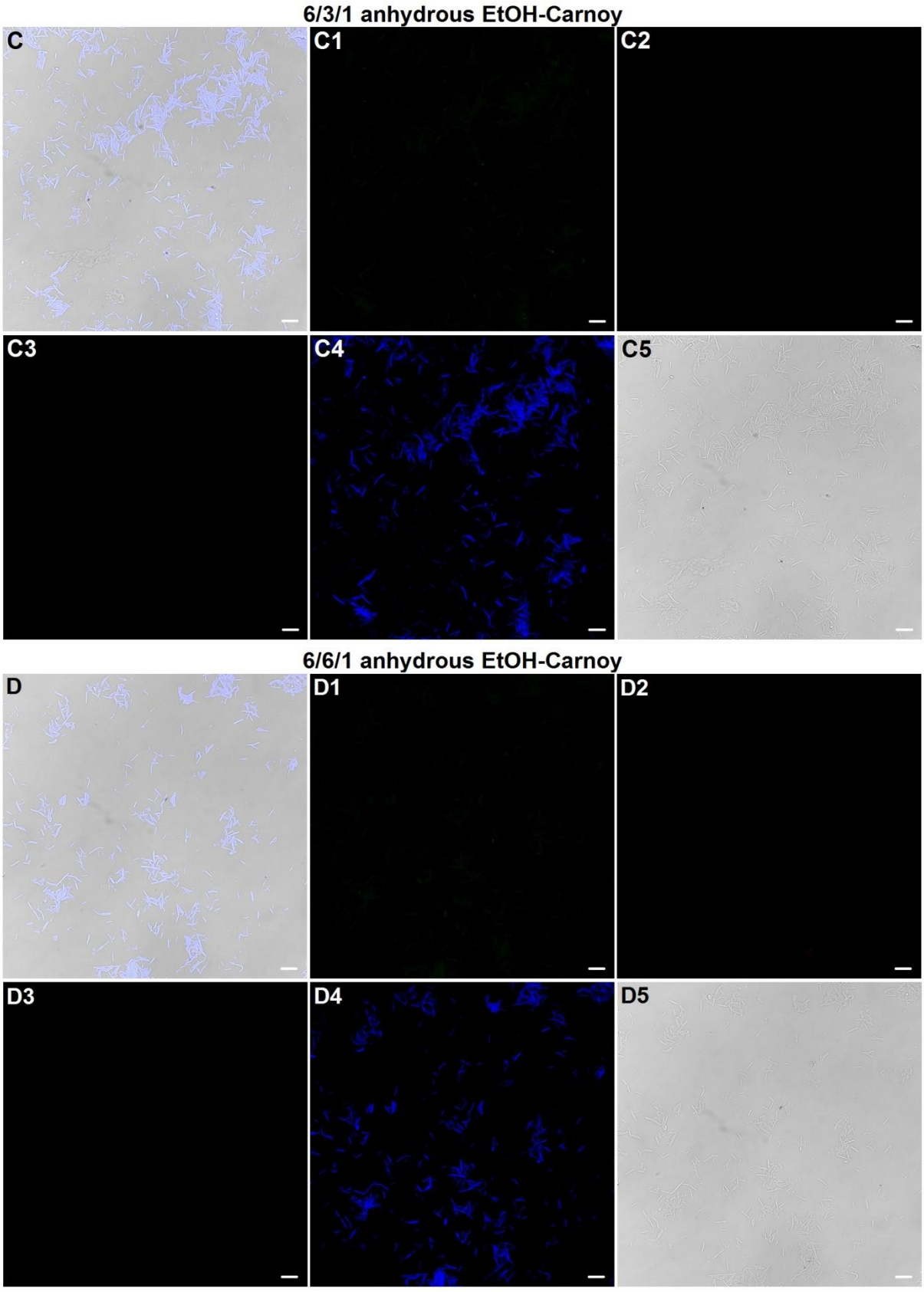
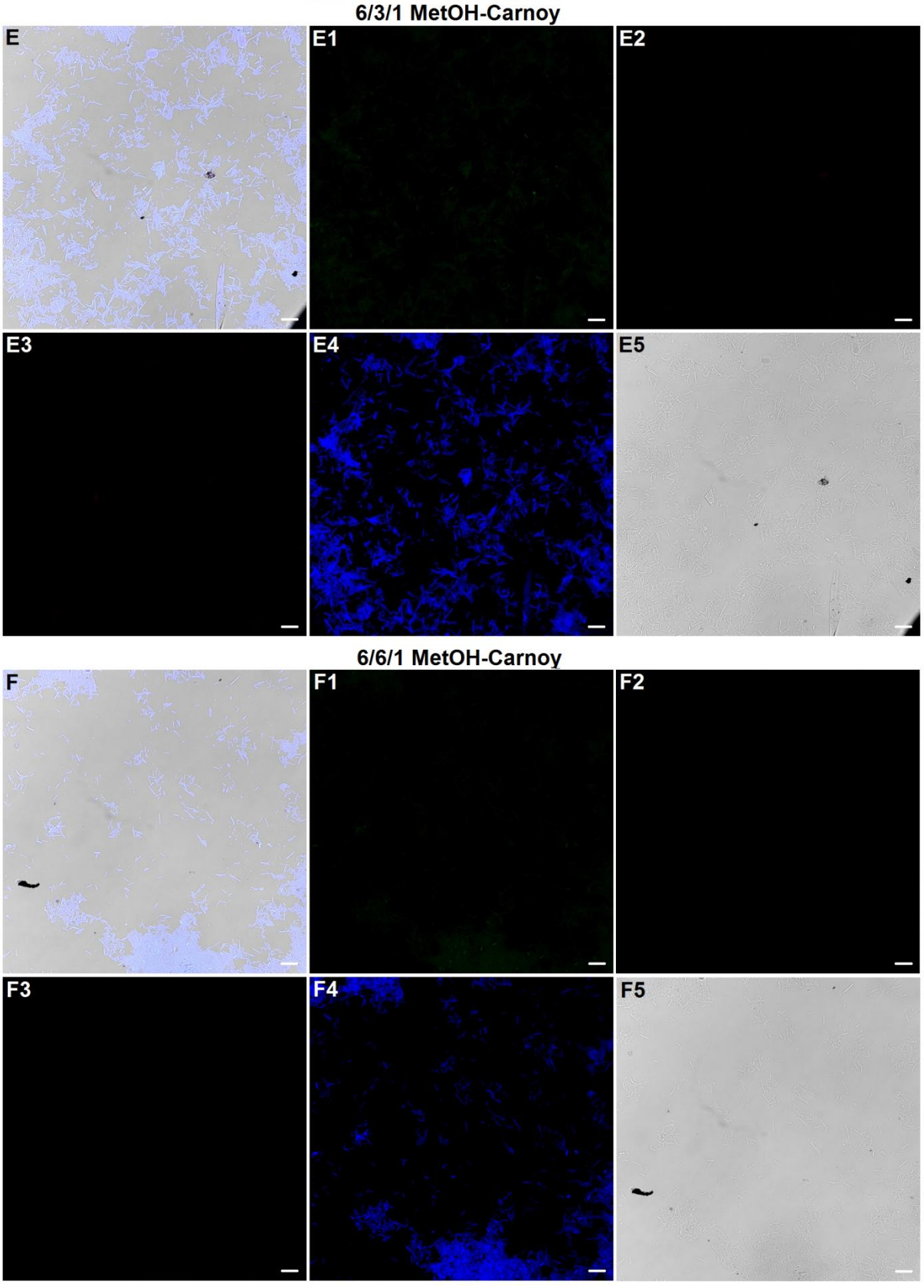


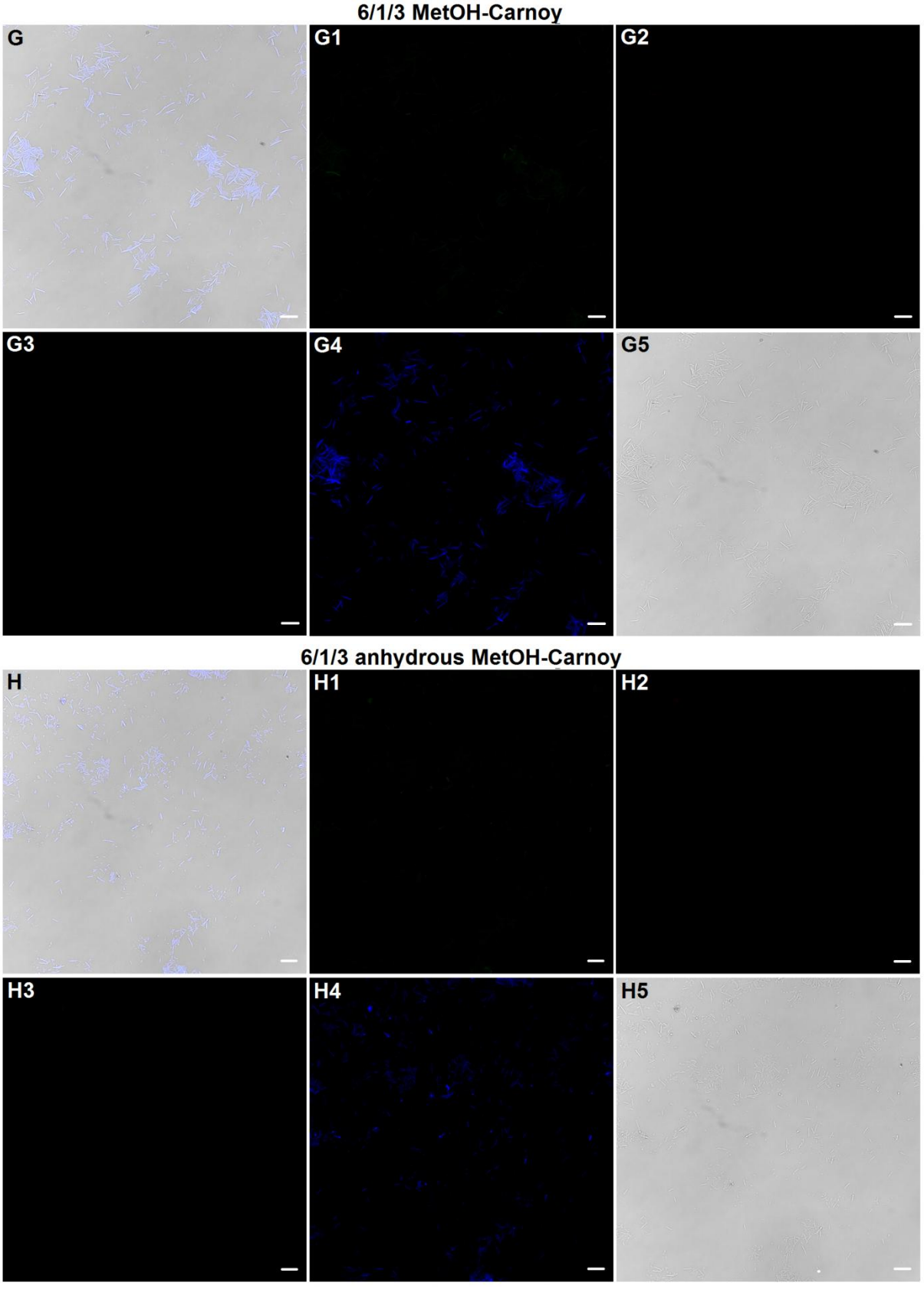
Figure S1: Images A-I show the overlay of all channels, the single channels are nonEUB-Fluos (corresponding images “1”), nonEUB-Cy3 (corresponding images “2”), nonEUB-Cy5 (corresponding images “3”), DAPI (corresponding images “4”), and brightfield images (corresponding images “5”). In general no auto-fluorescence in the channels for Fluos, Cy3, and Cy5 is detectable. DAPI images show, that signal intensities for DAPI-signals are dependent on the used fixative. Scale bars indicate 10 µm.



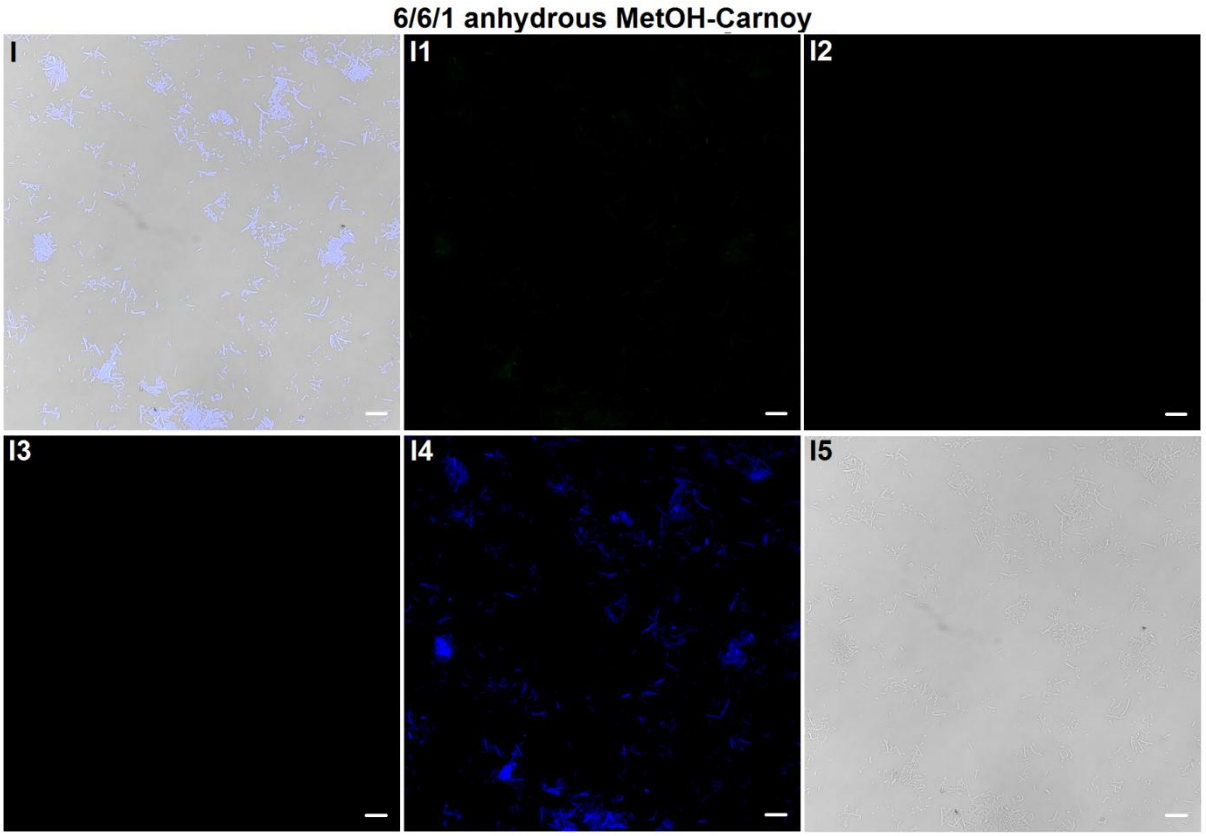
(continued Figure S1)



(continued Figure S1)



(continued Figure S1)



(continued Figure S1)

9.2.2 Supplementary Tables

Table S6: Weight [%] of the mice supplemented with different *Akkermansia spp.* strains (viable and heat inactivated, and of the control group treated with PBS/2.5 % Glycerol before and during DSS-induced colitis. Body weights have been normalised to original body weight [g].

Group	Treatment	Mouse No.	Weight [%]						
			Day -6	Day -5	Day -4	Day -3	Day -2	Day -1	Day 0
1	PBS/2.5% Glycerol Control	0	100.0	100.5	103.9	103.9	102.9	102.4	105.4
		r	100.0	101.9	102.4	100.5	100.5	99.5	101.0
		l	100.0	99.5	102.1	101.5	100.0	98.5	101.0
		b	100.0	101.0	101.5	102.5	102.9	101.0	101.0
2	MucT Viable	0	100.0	94.9	90.4	79.3	85.4	86.0	88.2
		r	100.0	99.5	96.6	75.3	89.8	95.1	100.5
		l	100.0	87.9	80.9	85.4	88.9	96.0	99.0
		b	100.0	103.4	91.6	82.0	81.8	83.7	88.7
3	H2 Viable	0	100.0	90.4	83.7	100.5	79.3	87.0	94.7
		r	100.0	85.1	82.3	108.3	81.4	90.7	95.8
		l	100.0	87.3	81.5	102.7	93.7	98.5	100.0
		b	100.0	102.5	88.5	106.3	79.5	82.0	89.0
4	MG Viable	0	100.0	96.5	101.0	100.5	103.0	100.5	91.5
		r	100.0	104.4	108.3	108.3	107.4	107.4	105.9
		l	100.0	102.7	104.4	104.4	102.2	100.0	100.5
		b	100.0	102.9	106.8	106.8	103.4	101.9	103.4
5	H2 Heat Inactivated	0	100.0	100.5	105.0	105.0	92.6	95.0	98.0
		r	100.0	99.5	99.5	99.5	98.6	98.1	100.5
		l	100.0	101.5	102.0	102.0	98.0	98.0	102.0
		b	100.0	101.4	104.8	104.8	107.2	108.2	106.7
6	MucT Heat Inactivated	0	100.0	102.0	100.0	100.0	100.5	101.0	103.4
		r	100.0	102.5	103.5	103.5	102.0	103.5	103.5
		l	100.0	94.1	98.5	98.5	102.0	102.0	99.0
		b	100.0	97.0	100.0	100.0	105.6	105.1	93.9
7	MG Heat Inactivated	0	100.0	104.0	104.0	104.0	101.5	100.5	103.0
		r	100.0	104.3	109.1	109.1	108.0	110.7	108.0
		l	100.0	105.0	105.5	105.5	105.5	106.0	105.0
		b	100.0	97.5	97.5	97.5	102.5	103.0	102.0

(continued Table S6)

Group	Treatment	Mouse No.	Weight [%]							
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
1	PBS/2.5 Glycerol Control	0	102.9	105.4	106.3	107.8	105.4	101.0	90.7	81.5
		r	102.4	101.0	100.5	100.5	98.6	95.2	93.7	89.4
		l	100.5	99.0	100.0	99.5	99.5	95.4	90.3	82.6
		b	105.9	102.5	95.6	96.6	96.6	96.1	92.6	83.3
2	MucT Viable	0	95.5	96.6	98.3	97.2	94.4	91.6	85.4	74.2
		r	106.8	104.4	104.4	102.9	93.7	90.8	85.9	77.2
		l	100.5	101.5	99.5	98.5	97.5	93.0	86.4	75.4
		b	94.6	98.5	97.0	96.1	98.0	94.1	90.6	83.3
3	H2 Viable	0	81.7	74.0	0.0	0.0	0.0	0.0	0.0	0.0
		r	101.4	98.1	97.7	95.8	94.4	90.2	80.0	69.8
		l	100.5	99.5	101.5	99.5	100.0	98.5	94.6	84.9
		b	93.5	97.5	98.0	94.5	81.0	75.5	0.0	0.0
4	MG Viable	0	84.5	84.0	85.0	85.0	88.0	83.0	88.0	84.0
		r	108.3	102.0	102.0	103.9	106.4	99.5	93.1	84.3
		l	100.0	97.3	98.9	98.9	95.1	92.3	89.0	79.7
		b	106.8	101.5	100.0	101.0	96.1	93.2	90.3	85.9
5	H2 Heat Inactivated	0	100.5	95.0	98.5	102.0	105.0	103.0	91.6	83.7
		r	99.1	99.5	100.0	100.9	103.2	98.6	98.6	94.0
		l	107.0	100.0	103.5	107.5	108.0	104.0	96.0	88.5
		b	105.8	98.6	101.0	103.4	99.0	99.0	96.6	91.3
6	MucT Heat Inactivated	0	104.4	102.0	101.5	102.0	97.1	91.7	89.3	80.5
		r	97.5	97.0	99.5	101.5	103.5	94.5	86.9	75.4
		l	100.0	100.5	100.0	103.4	94.1	92.6	90.7	80.9
		b	94.4	93.0	97.5	99.5	97.0	93.9	92.4	83.8
7	MG Heat Inactivated	0	104.0	103.0	100.0	95.0	95.0	91.0	81.4	74.9
		r	98.9	97.9	102.1	103.7	103.7	100.0	93.6	87.2
		l	108.0	109.0	107.5	107.0	107.0	101.0	93.0	85.5
		b	104.0	91.0	86.0	80.5	80.5	76.0	0.0	0.0

LIST OF FIGURES AND TABLES

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