FUNCTIONAL STRUCTURE OF INTESTINAL MICROBIOTA IN HEALTH AND DISEASE

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INTRODUCTION

The intestine is an interface between macroorganisms and the naturally polymicrobial environment. While ingesting food, the organism encounters diverse bacteria. Intestinal microbiota are therefore basically polymicrobial. Different from monocultures, the polymicrobial community contains members that are not easily cultivated in the laboratory in isolation yet thrive in association with other microbes. The properties of isolated microorganisms do not explain how the polymicrobial community functions or why it can grow under conditions which may be deadly for each of the constituents. To understand polymicrobial communities, the composite structures as they grow and respond to challenges need to be monitored. One of the methods to visualize single bacterial species within complex communities is to use ribosomal RNA fluorescence in situ hybridization (FISH). Each bacterium possesses 10^{3-5} ribosomes. Each ribosome includes an RNA copy. Some of the regions of the ribosomal RNA are strain-specific, others are universal for groups, domains or even kingdoms. Synthetically produced oligonucleotides which are complimentary to sequences of interest can be labeled with fluorescent dye and added to samples containing bacteria. These oligonucleotides, which are called FISH probes, hybridize with RNA of bacterial ribosomes. Bacteria can be visualized with the microscope directly without additional enhancement, because of the high number of ribosomes within each bacterium [1].

The following presentation is largely based on data obtained from intestinal microbiota using FISH. The names of the FISH probes are listed according to abbreviations of probeBase online resource for rRNA targeted oligonucleotid probes (http://www.microbial-ecology.net/probebase/credits.asp) [2].

INTESTINAL MICROBIOTA

The intestinal pulp is rich in nutrients and provides a desirable environment for many bacteria. The organism develops therefore multiple mechanisms to control bacteria either through suppression (for example with lysozyme of the saliva, gastric acid of the stomach, secretion of defensins) or through separation of bacteria from the intestinal wall with a mucus barrier. Most often both suppression and separation are implicated but differently balanced. Polymicrobial communities as compared to monocultures are extremely recalcitrant. They respond in coordination to environmental challenges, resist antibiotic treatment and immune responses and are able to persist under extreme conditions. The control of bacterial growth by the host is therefore never absolute and the intestine is never sterile. The occurrence, composition and organization of intestinal microbiota in each gut segment depend on whether suppression or separation dominates. In gut regions with active suppression of microbiota, the bacteria are occasional, of variable composition and of low concentration. A complete separation of bacteria from the mucosa and low levels of suppression lead to the development of an intestinal reservoir in which bacteria can grow and reach high concentrations. Bacteria are

indigenous here. The balance of suppression and separation mechanisms depends on the evolutionary impact of bacteria on health benefits and hazards. For example, none of the eukaryotic organisms can digest cellulose. Plant feeding animals use microorganisms for this task. Bacteria, which digest cellulose are therefore indigenous in the rumen of these animals. Absorption of nutrients takes place in the small intestine. Bacteria are clear competitors here and are suppressed in all animals including ruminal mammals. But for clear pathogens, the presence of single bacterial groups is transient and concentrations of bacteria are low in the small intestine. The function of the large intestine is resorption of water and electrolytes and reduction of the fecal mass. The intestinal content which leaves the small intestine contains many non-digestible substances. These non-digestible substances can be still degraded by bacteria in the large intestine. Our presentation will be mostly restricted to microbiota of the human intestinal tract. Description of processes in the intestinal tract of rodents will be presented only if their description is necessary for understanding processes in the human intestine.

Bacteria in the upper gastrointestinal tract

Mouth

The mouth is the host region which has first contact with bacteria of the outer world. The environmental diversity is high and the bacterial diversity is high as long as samples of saliva are evaluated. In contrast, FISH-based investigations of samples taken from the oral cavity demonstrate that the stratified epithelium of the mouth in healthy persons is free of bacteria, despite high concentrations of bacteria in saliva (Figure1).

No bacteria can be found also in saliva taken directly from the salivary duct. In contrast, bacteria can be found in high concentrations on food remnants (Figure 2), the dental surface and within or attached to desquamated epithelial cells suspended in oral secretions (Figure 3).

The stratified epithelium of the oral cavity and the epithelium of the salivary glands possess efficient mechanisms to suppress bacterial adhesion and growth but are unable to control the bacterial growth on the surfaces of teeth, food remnants or on desquamated epithelial cells.

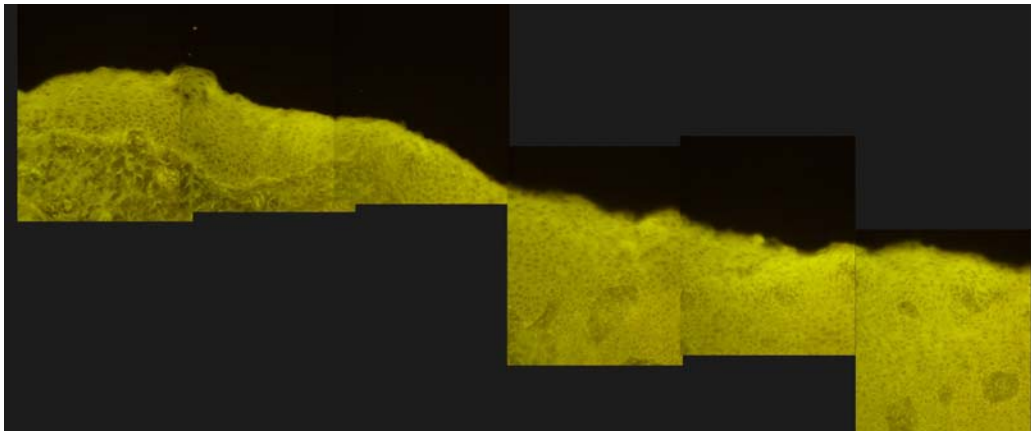


Figure 1. Healthy human mouth epithelium hybridized with the universal Eub338 Cy3 bacterial probe. The figure is composed of 6 consecutively taken microphotographs at magnification of x400. No bacterial signals can be seen.

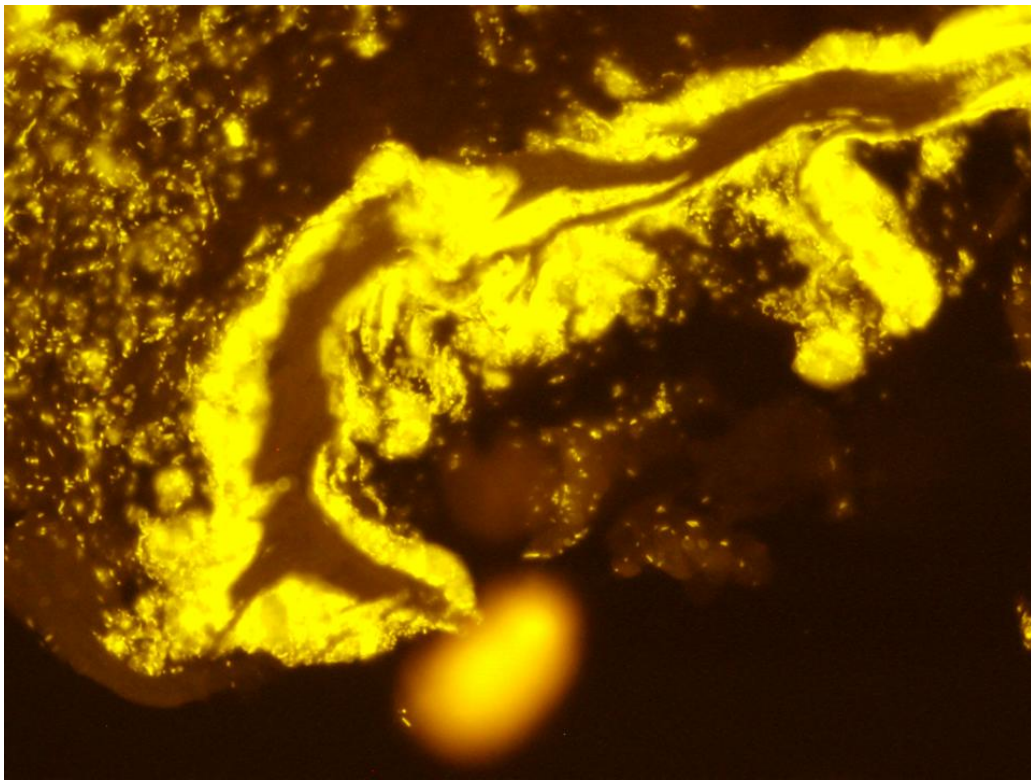


Figure 2. Massive bacterial biofilm attached to food remnants in the mouth (universal Eub338 Cy3 probe, yellow fluorescence x400).

Different from the healthy mucosal surface, food remnants can be covered with prolific bacterial biofilm.

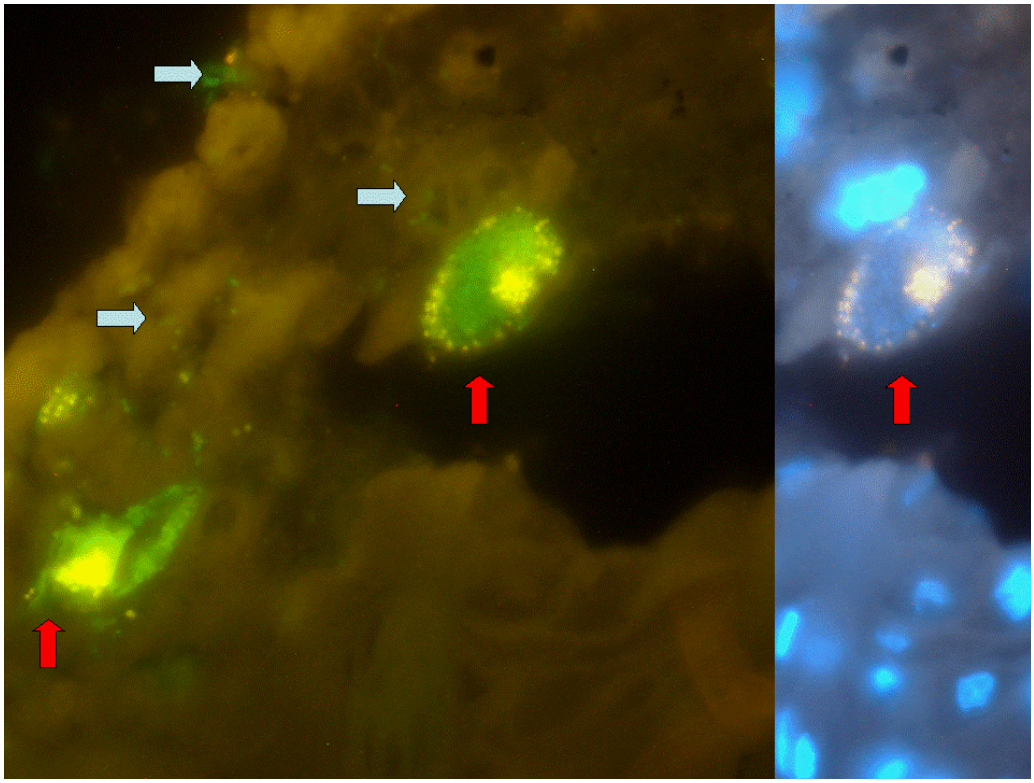
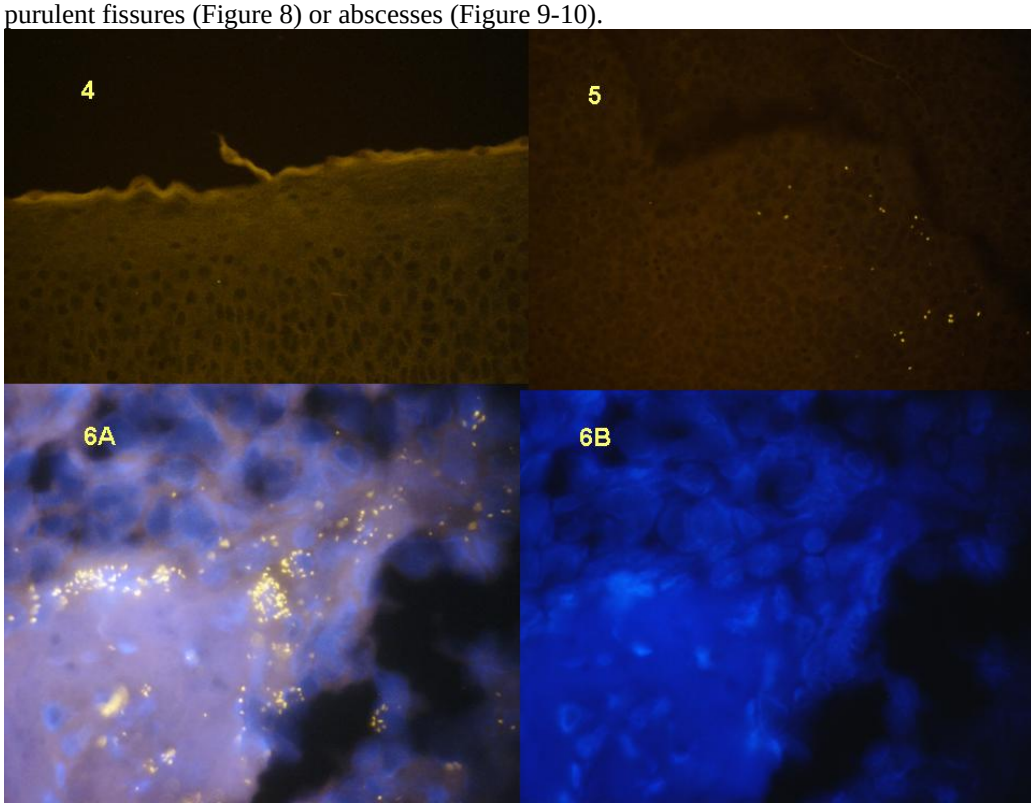


Figure 3. Left: isolated island of bacteria attached to desquamated epithelial cells in saliva (universal bacterial probe Eub338 FITC, green fluorescence) and *Burkholderia* (Burkho Cy3 probe, orange fluorescence). On the right: DAPI stain (blue fluorescence of all DNA rich structures) is overlayed with *Burkholderia* fluorescence x1000. The large blue fluorescence spots on the right are nuclei of desquamated epithelial cells within saliva. The attached bacteria are either irregularly scattered (green fluorescence, blue arrows) or organized to oval structures with exact arrangement of single bacterial groups (red arrows).

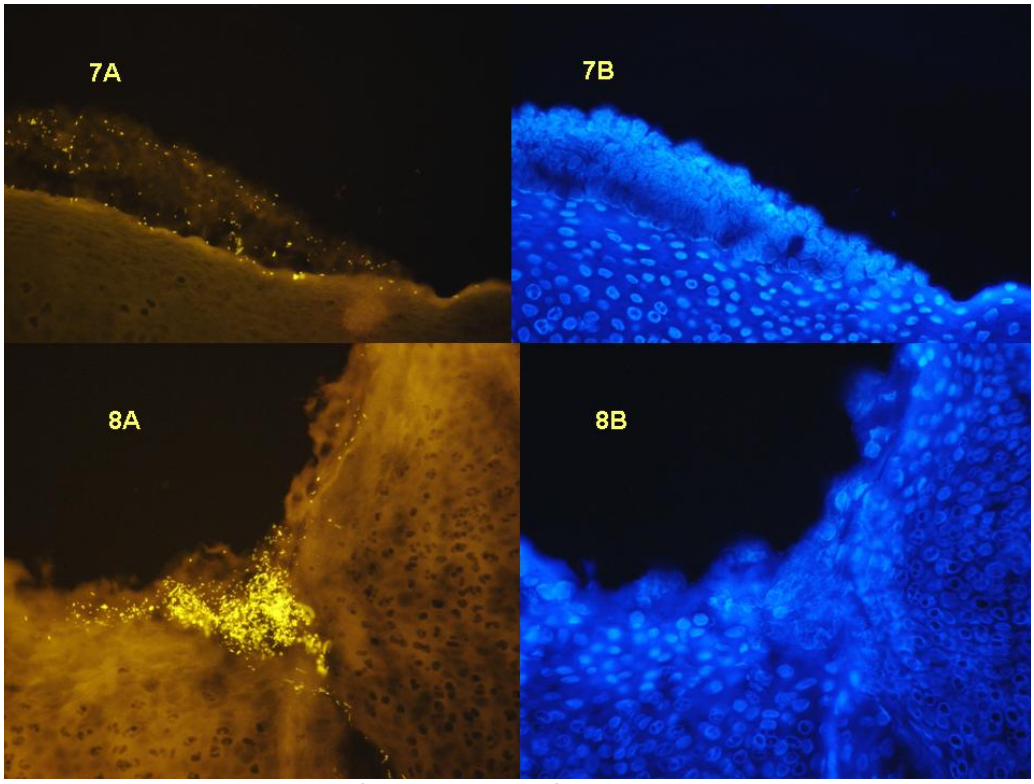
Tonsils

Similar to the situation in the mouth, no constant pattern of colonization can be found on the surface of the tonsilar epithelium [3]. Most of the epithelial surface of tonsils is free of bacteria even in tonsillectomy material after chronic tonsillitis (Figure 4). When bacteria were found, they were localized either to circumscribed regions of diffuse infiltration (Figure 5), within

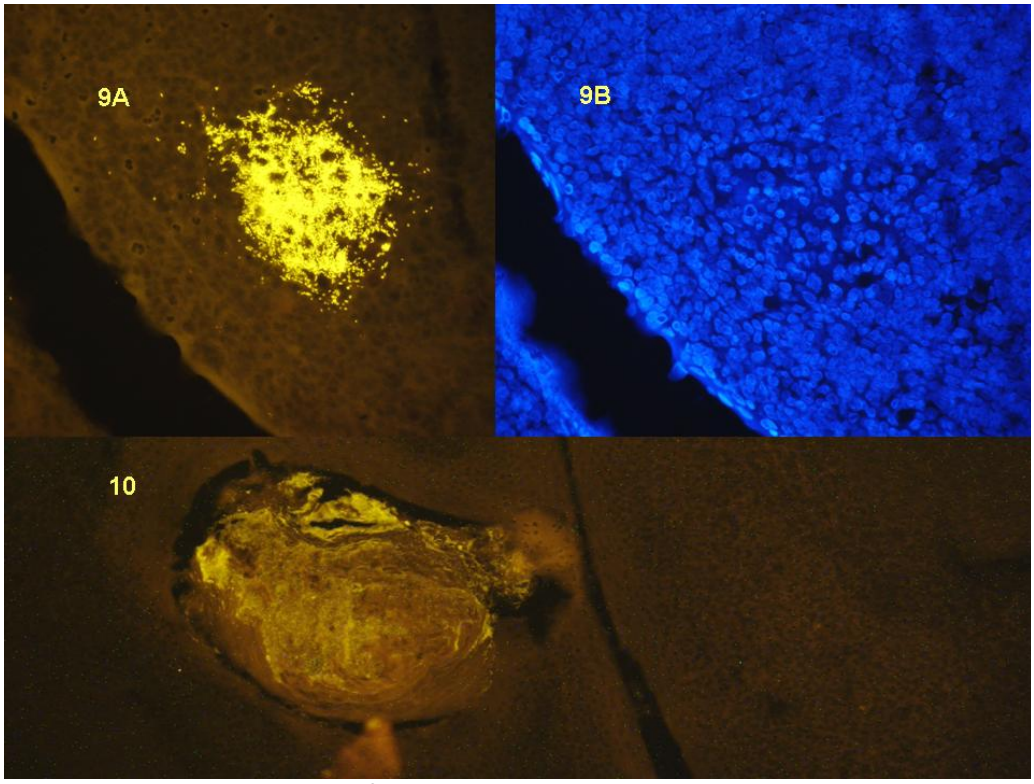
macrophages (Figures 6 A and B), superficial infiltrates (Figure 7), singular purulent fissures (Figure 8) or abscesses (Figure 9-10).



Figures 4 – 6. 4: Healthy tonsillar epithelium (universal Eub338 Cy3 probe, x400) free of bacteria. 5: Diffuse local infiltration of a tonsil with *Haemophilus influenzae* (Haeinf Cy3 probe, orange fluorescence x400). Despite diffuse distribution, bacteria are localized only in parts of the tonsil. The remainder of the tonsillar tissue is free of bacteria. 6A: Diffuse infiltration with *Streptococcus pyogenes* (Strpyo Cy3 probe, orange fluorescence) and DAPI stain (all DNA structures) overlaid. Isolated DAPI stain of the same microscopic field is presented on the right side to better outline the silhouettes of the eukaryotic cells (Figure 6B). One can see, that bacteria are ordered around the nuclei of eukaryotic cells and probably phagocytized by macrophages.



Figures 7 – 8. 7A: superficial adherence of bacteria to the tonsillar surface (universal Eub338 Cy3 probe, orange florescence). Bacteria are attached to the surface and mixed with an inflammatory infiltrate covering them. 7B: DAPI stain of the same microscopic field reveals the DNA structures. Bacteria are enveloped by a lymphatic infiltrate (large blue nuclei of leukocytes). 8A: fissure filled with bacteria. 8B: The DAPI stain of the same microscopic fields demonstrates that bacteria are surrounded by inflammatory cells indicating an active immunologic response to bacterial adhesion and invasion (Eub338 Cy3 probe, orange fluorescence).



Figures 9-10. Examples of microabscess (9A, x400, Eub338 Cy3, 9B - DAPI stain of the same microscopic field) and macroabscess (10, Eub338 Cy3, x 100) within tonsillar tissue.

The composition of bacteria within infectious tonsilar foci (Table 1) is individual and often differs even between different regions of the same tonsil, indicating that bacteria are not indigenous here but represent remnants of incompletely cured purulent processes.

Table 1. Occurrence of different bacterial groups within local tonsillar lesions such as fissures and diffuse infiltrates

Superficial Infiltration and Fissures*	%	Diffuse Infiltration*	%
Fusobacteria spp. (Fuso)	36	*Firmicutes* (LGC)	74
Pseudomonas (Ps, Pseaer A, Pseaer B)	34	*Streptococcus* (Strc493)	74
Beta-Proteobacteria inclusive. *Neisseria* (Bet42a)	33	*Haemophilus influenzae* (Haeinf)	66
Burkholderia (Burcep, Burkho)	30	*Actinobacteria* (HGC)	50
Lactobacillus and *Enterococcus* (Lab)	24	*Bacteroides/Prevotella* (Bac303)	39
Veillonella group inclusive *Veillonella parvula* (Veil,Vepa)	23	*Cytophaga-Flavobacteria* (CF319)	34
Clostridium coccoides – E. rectale (Erec)	20	*Streptococcus pyogenes* (Strpyo)	11
Staphylococcus aureus (Staaur)	11	*Atopobium* and others (Ato291)	6
Prevotella intermedia (Prin)	10		
Ruminococcus bromii, R. flavefaciens (Rbro, Rfla)	7		
	6		

Coriobacterium group (Cor653) *Listeria,Brochothrix* (Lis637,1255)	6 4		

*Bacteria which were observed within regions with diffuse infiltration were also found in fissures but not vice versa

Stomach and duodenum

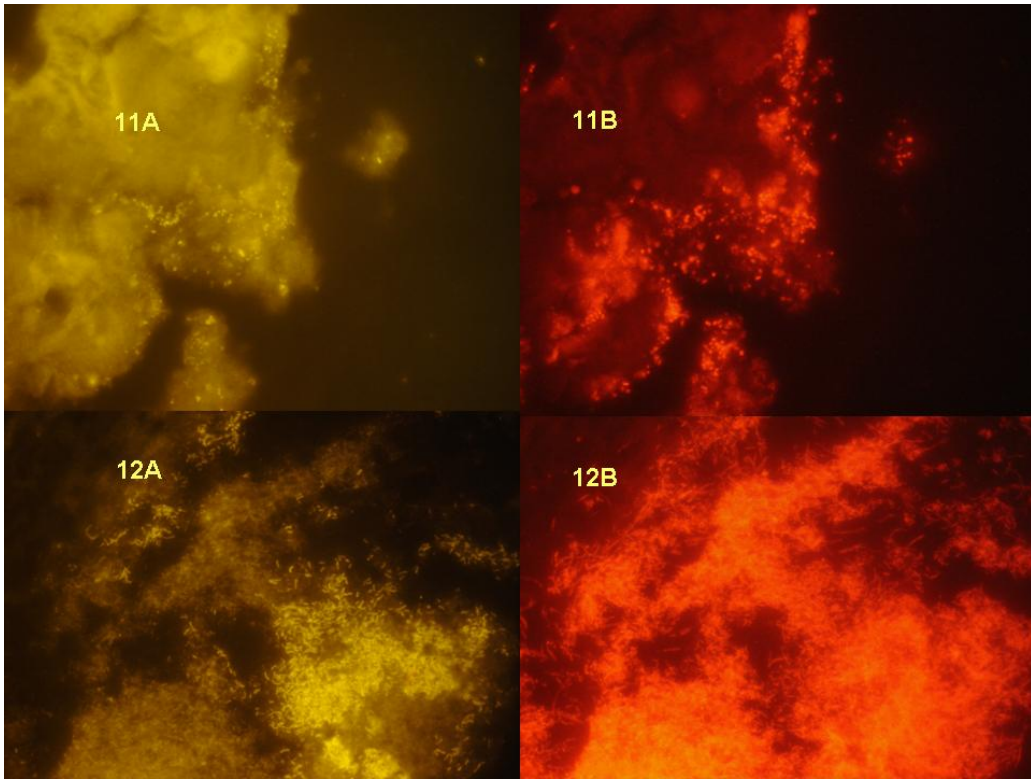
Depending on the digested food, the culture of the gastric or duodenal juices contains bacterial concentrations of $10^3 – 10^4$/ml. For comparison, a 10 µl suspension of bacteria with a concentration of 10^7 cells per ml applied to a glass surface in a circle of 1 cm results in 40 cells per average microscopic field at magnification of x1,000 [3]. At such concentrations no biofilms can be formed.

Fluorescence in situ hybridization demonstrates that bacteria in the stomach and duodenum are localized strictly within the lumen and separated from the mucosa by a mucus layer. Their composition is heterogeneous, reflecting the heterogeneous composition of the ingested flora. In patients with *Helicobacter pylori* infection, the mucosal surface of the stomach is covered with a bacterial biofilm in which *Helicobacter pylori* is predominant. The data on *H. pylori* biofilms are abundant, extensively studied and reviewed in the literature and will not be specifically referred here. It is however important to mention, that besides *H. pylori* biofilms other bacterial groups including *E. coli* can adhere in a confluent manner to the mucosa, for example in patients with polyposis of the stomach.

Pancreatic tract

Healthy pancreas tissue is not available for investigation. Biopsies taken during endoscopic retrograde cholangiopancreatography (ERCP) are restricted mainly to patients with benign or malignant obstruction. The FISH analysis of microbiota within these samples demonstrates islands of bacterial adhesion in about 70% of the biopsies (Figure 11) [4].

The anatomically normal pancreatic duct epithelium is free of bacteria. Bacterial islands are located in regions of disturbed duct anatomy. Bacteria found in the pancreatic duct are diverse, mainly represented by environmental groups, indicating an exogenous origin and its composition is highly individual. We were unable to find any constant pattern of colonization. The stability of these findings can not be verified, because repeated biopsies from these regions were not obtained.



Figures 11-12. Biofilm in a calcified pancreatic duct simultaneously hybridized with group specific (A) and universal (B, Eub338 Cy5, red fluorescence) probes x400. Figure 11 demonstrates *Enterobacteriaceae* (Ebac Cy3, orange fluorescence). Fig 12 shows *Cytophaga-Flavobacterium* group (CF319a Cy3, orange fluorescence). Bacteria are differently composed and have no characteristic form of spatial organization.

Biliary tract

Biopsies from the bile ducts are rare. Material of gallbladder resections, electively performed without preoperative antibiotics, is free of bacteria, indicating that the bile duct epithelium is normally not colonized. The situation is different in the presence of foreign bodies such as biliary stents. The colonization of the biliary stents by polymicrobial biofilm can be documented already one week after implantation. The microbial colonization starts with the distal end of the biliary stent and advances proximally. Bacteria are located mainly on the inner surface of the plastic stent. The surface of biliary stents facing normal epithelium is usually not colonized indicating that the healthy epithelial layer efficiently resists microbial colonization even upon contact with the foreign body [4]. The quick development of biofilms at the inner surface of the biliary stents indicates that the biliary and pancreatic secretions

alone are not able to prevent the development of bacterial biofilms on foreign bodies. Both aerobic and anaerobic bacteria, which are commonly found in the intestine, can be identified. Interestingly, the bacterial biofilm disappears as soon as the stent is occluded by sludge (Figure 13).

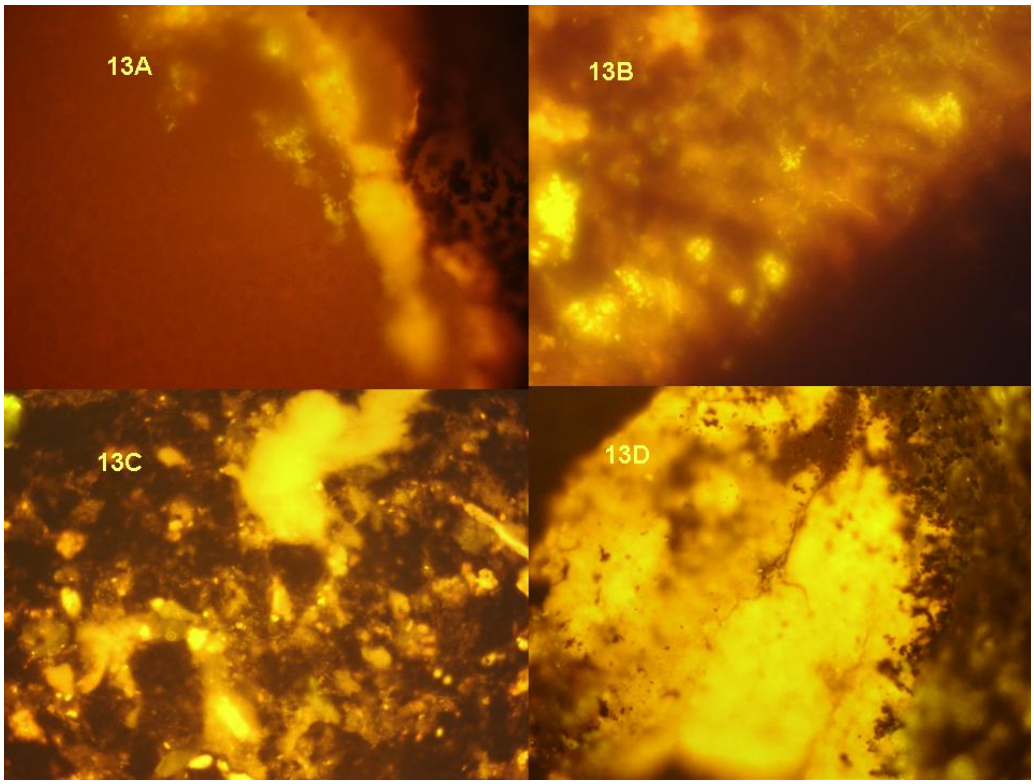


Figure 13. Sections of whole biliary stents hybridized with the universal Eub338 Cy3 probe. Bacteria (orange signal can be clearly identified as punctuate signals within the yellowish sludge covering the inner surface of stents (A,B) in partially occluded stents. Bacteria disappear from the stent lumen with increasing occlusion of the bile duct stent (C stent center, D stent wall), only large yellowish masses of cholesterol and other sludge can be seen.

Gallstones

A similar trend of the bacterial biofilm vanishing after local deposition of organic substances can be observed in gallstones. As foreign bodies, gallstones should be permanently colonized. Bacteria can be indeed found in loose brown pigment stones and sludge, which are an initial stage in the formation of gallstones. The natural history of the gallstone is a procession from brown to composite, and then to cholesterol gallstone. The cholesterol stones may reach considerable sizes and persist in the human body over many decades.

However, despite such long history and multiple episode of acute bacterial cholecystitis, the cholesterol gallstones obtained after cholecystectomy are mostly sterile. Obviously, the sedimentation of cholesterol and sludge within the bacterial biofilm is an integral part of some kind of protective mechanism against otherwise extremely recalcitrant infections [4]. This mechanism appears extremely efficient in suppressing bacterial biofilms on foreign bodies when compared to the complete ineffectiveness of presently available antibiotics.

Small intestine

The epithelial surface of the small intestine in healthy humans is not colonized (Figure 14). The bacteria are represented by occasional groups and can be found in low concentrations of 10^5 or less within the lumen. Bacteria do not form conglomerates and spatial structures and the luminal contents are separated from the mucosa by a mucus layer. The situation is similar in mice but not in rats. In most groups of wild-type rats, segmented filamentous bacteria (SFB) can be observed tightly attached to the epithelial surface and located between villi throughout the small intestine (Figure 15). The SFB adherence in rats is not accompanied by an increase in concentrations of other bacterial groups or leukocytes. It is difficult to say whether segmented filamentous bacteria are pathogens such as *Helicobacter* species, saprophytic or symbiotic bacteria with an unknown role, because of the very high prevalence of adherent SFB in the small intestines of wild-type rats. In human, the SFB adherence could not be observed either in health or disease. Pathologic conditions with altered microbiota in the small intestine are acute and chronic infections, bacterial overgrowth, and inflammatory bowel disease (IBD). Typical for all of them are a comprised mucus barrier, loss of bacterial separation between mucosa and lumen, bacterial adherence, invasion and translocation (see break of the mucus barrier in IBD below) [5].

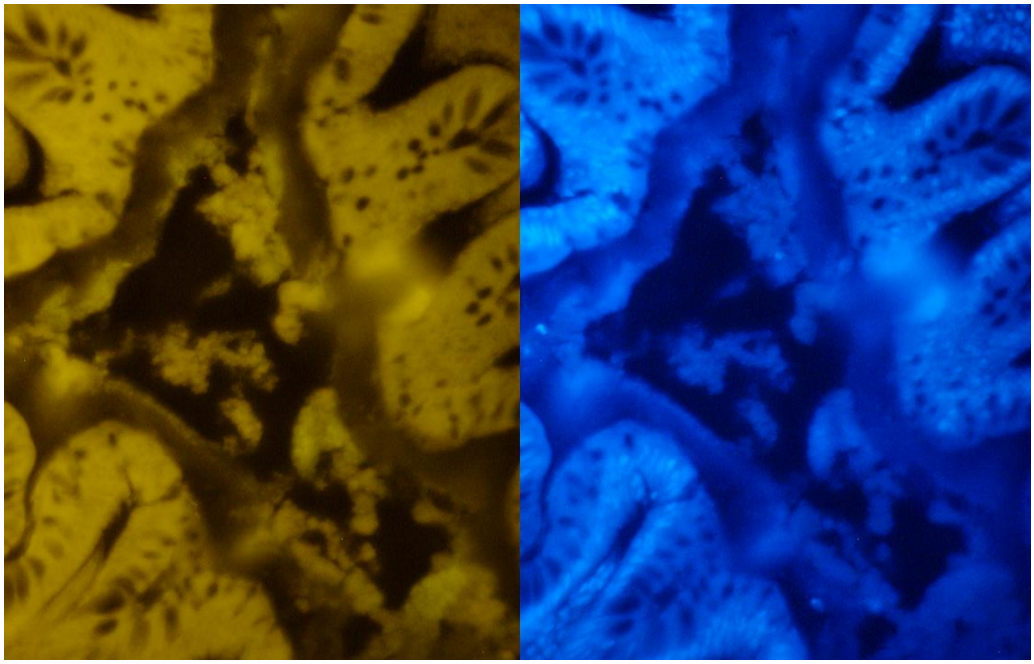


Figure 14. Ileum of a healthy person. Left: hybridization with the universal Eub338 Cy3 probe x400. Right: the same microscopic field demonstrating DAPI fluorescence of all DNA structures x400. No bacteria can be seen between crypts. The lumen is completely separated from the mucosa by a mucus layer, which is free of bacteria.

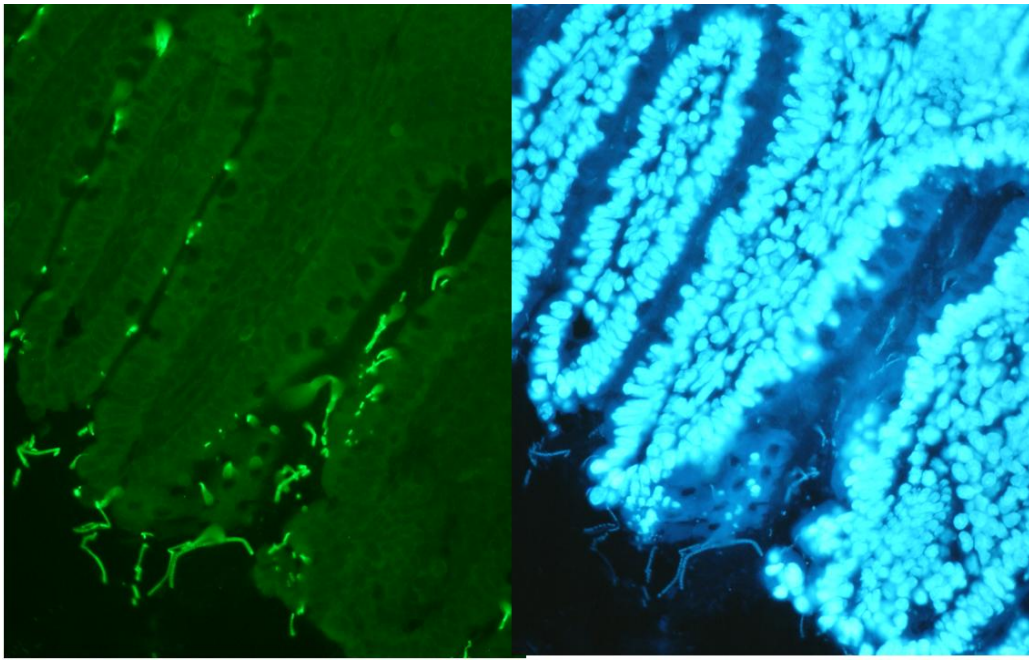


Figure 15. Rat small intestine. Left: hybridization with the SFB FITC probe (segmented filamentous bacteria). Right: the same microscopic field in DAPI fluorescence. Long curly bacteria adhere to the villi and enter deep into the crypts. No other bacterial groups are seen.

Bacteria in the large intestine

The role of microbiota in colonic function

The large intestine is a biofermenter, in which the host employs bacteria to degrade undigested leftovers. Bacteria produce valuable substances such as vitamins and short fatty acids by degrading waste products. To recover these nutrients in the small intestine, many species adopt fecophagy. The considerable differences in the size and anatomy of the large intestine observed between mammals indicate that the processes of utilization may differ and that other than fecophagic mechanisms may be available in some species, which allow them to directly absorb and utilize beneficial products of bacterial metabolism.

In homo sapiens, the absorption in the large intestine is restricted mostly to water and electrolytes and fecophagy is uncommon. Bacteria in the human colon are mainly responsible for the reduction of the fecal mass. It is not known which of the bacterial groups are important for this purpose. We can, however, assume that numerically predominant and obligatory present bacteria are indispensable for the biochemical processes which occur in the colon. *Eubacterium* rectale (*Roseburia* spp), *Faecalibacterium prausnitzii* and *Bacteroides* groups comprise each 10% to 30% and cumulative 70% of the total microbiota in humans [6,7]. Obviously these groups are important for the

function of the colonic biofermenter. All other bacterial groups are present only in subgroups of patients or parts of the colon.
Although the fecal flora is one of the most well characterized microbiota with culture and molecular-genetic methods, many of the bacterial species that inhabit the large intestine are still unknown. Strict anaerobic species are predominant. The diversity of bacteria is high and consists of about 3,000 to 5,000 species [8].
In the colon, bacteria reach concentrations of up to the $10^{11\text{-}12}$/ml and compose up to 90% of the fecal mass. Thus high bacterial concentrations can be achieved only under active facilitation of bacterial growth. Peristalsis extensively mixes bacteria with fibers and maintains optimal viscosity and temperature is one element of this process. The lack of any perceptible suppression by the host is however the most astonishing feature. It has been previously assumed that the enormous masses of intestinal bacteria directly contact the intestinal wall. The non-pathogenic bacteria are tolerated, while the pathogenic bacteria are responded to. The immune response determines which bacteria should be normally present in the colon and which not. In reality, the residents of the large bowel can not be thus clearly divided into good and evil. Many of indigenous bacteria are pathogenic: *Escherichia coli* cause sepsis, *Bacteroides* cause abscesses, *Enterococci* cause endocarditis, *Clostridium perfringens* cause gas gangrene. We call these bacterial groups normal inhabitants of the human colon since they can be found in every healthy person. “Healthy” are these bacteria in no way. The host is able to distinguish nonpathogenic from saprophytic bacteria and the pathways of such recognition are well explored. Mechanisms which could eliminate single bacterial groups from the highly concentrated mass of fecal microbiota without affecting “beneficial” bacteria have yet to be demonstrated. The FISH analysis of the mucosal flora clearly demonstrates that the host does not tolerate the indigenous microbiota, it separates them from contact with the mucosa.

Mucus barrier

Biopsies from healthy persons show that the walls are covered with mucus, which is free of bacteria throughout the colon (Figure 16-18) and ileum (Figure 14). The separation of fecal bacteria from the mucosa by mucus can be seen in sections of normal appendices, resected for suspected acute appendicitis but found to be normal (Figure 19).
A similar separation of colonic bacteria from the mucosa can be observed in the distal colon of rodent animals (Figure 20), where the intestine is filled with

a fecal mass that can be investigated in total.

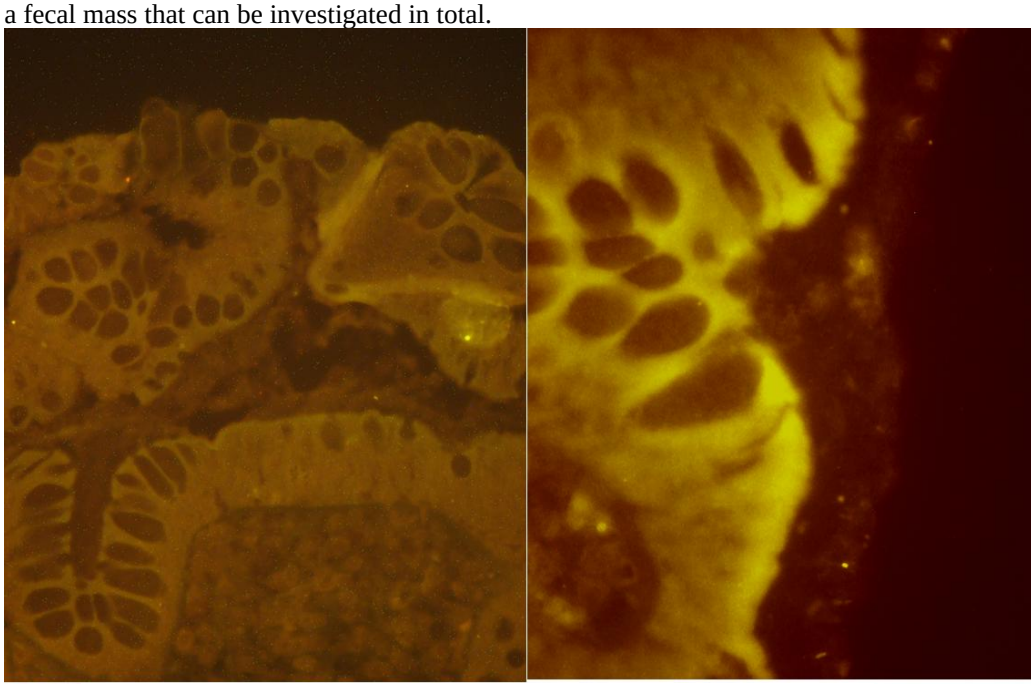


Figure 16-17. Biopsies from ascending and descending colon of a healthy person hybridized with the universal Eub338 Cy3 probe, left x 400; right x 1000. The surface of the mucosa is covered with mucus which completely omits bacteria.

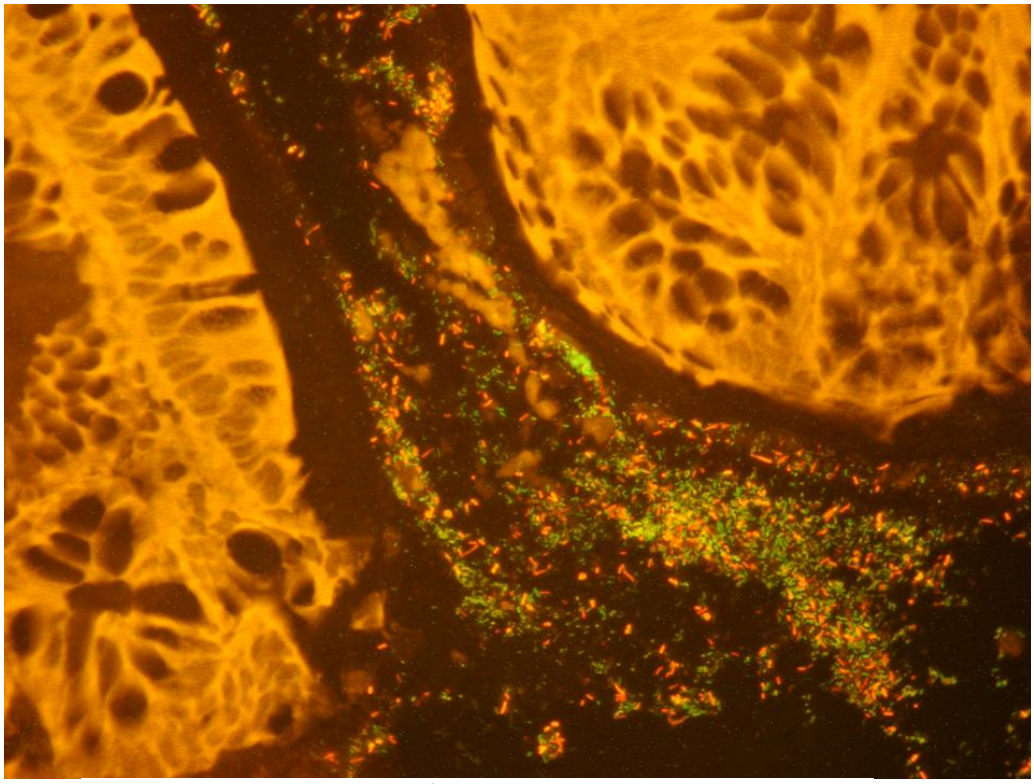


Figure 18. Cecal biopsy taken from a patient, who was inadequately purged. Remnants of feces remained in the colon. No bacteria adhere or contact the mucosa despite considerable amounts of intraluminal bacteria (multi-color FISH). *Bacteroides* is orange, *Eubacterium rectale* is green and *Faecalibacterium prausnitzii* is red x 400.

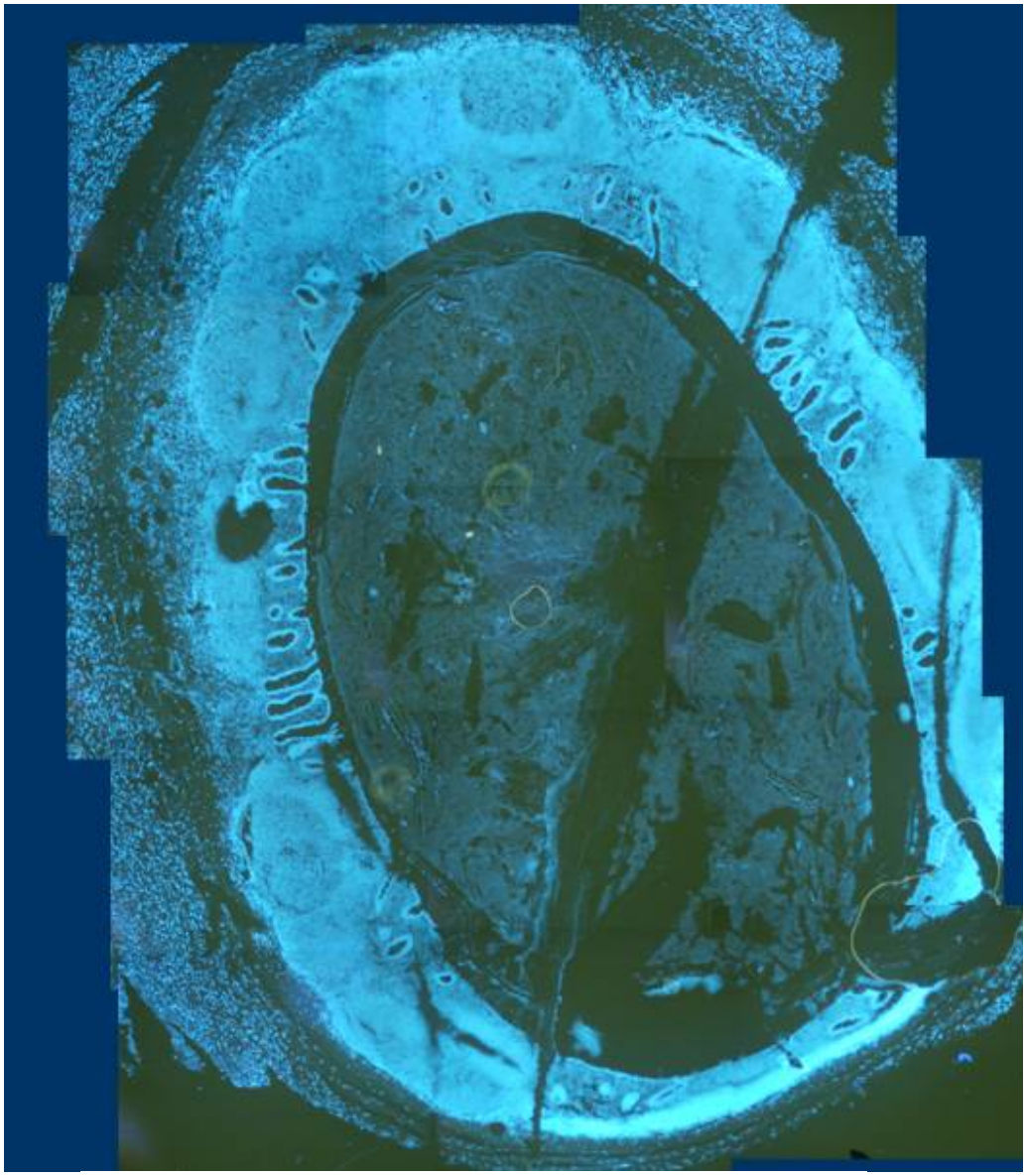


Figure 19. DAPI stain of a transverse appendix section taken at a magnification of x100. No leukocytes can be seen within the appendix confirming that the appendix is not inflamed. The bacteria within the lumen are homogeneously distributed over the lumen of appendix and clearly demarcated from the mucosa by a mucus layer. The figure is composed of many photographs taken at small magnification.

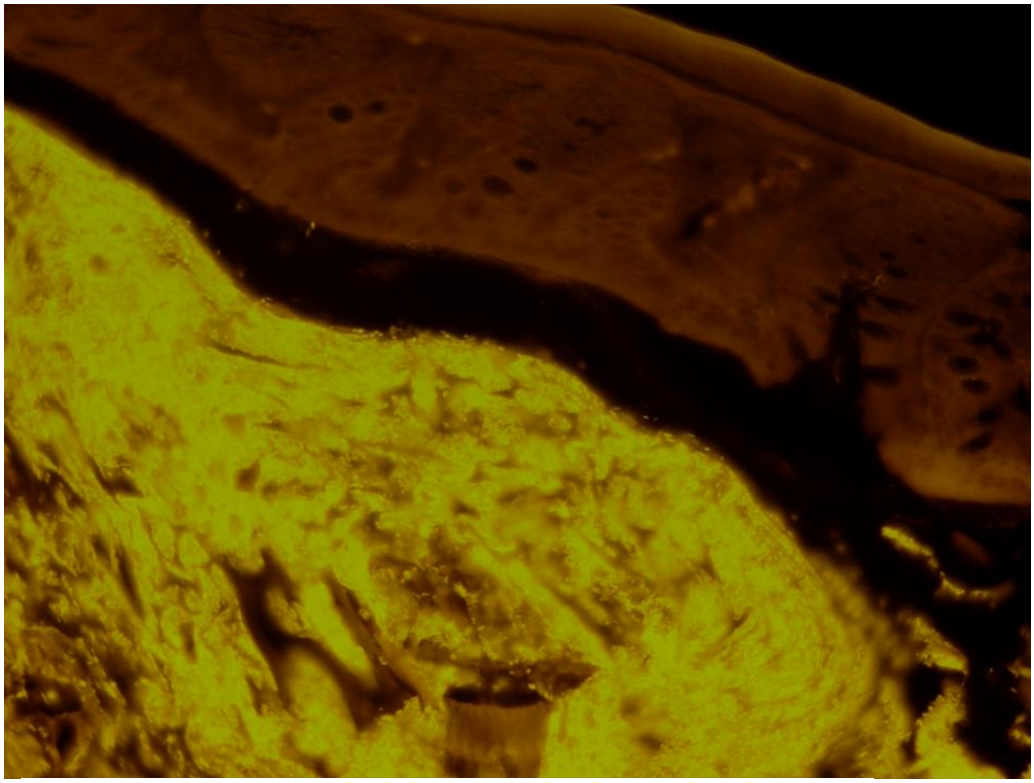


Figure 20. Distal colon of a healthy wild-type mouse, universal Eub338 Cy3 probe, x400. Bacteria (orange fluorescence) are clearly separated from the colonic wall by a mucus layer. Bacterial concentrations within the colon are high and homogeneously distributed between central regions of the intestinal lumen and mucosa adjacent regions. The water from the fecal stream softens the mucus layer. Pieces of the maturated mucus are carried away by the fecal stream. The mucus layer however builds an impenetrable wall between mucosa and feces.

Viscosity of the mucus barrier

Different from humans, in the proximal colon of mice and rats, bacteria contact the colonic wall (Figure 21) and enter crypts in high concentrations [9]. However, the contact of bacteria with the mucosa in the proximal colon of mice is selective. Long curly rods of *Eubacterium rectale* contact the mucosa and enter crypts in large numbers, while short coccoid rods of *Bacteroides* are separated from the colonic wall. The differences in arrangement of bacterial groups are especially obvious in multi-color FISH that shows simultaneously different bacterial species (Figures 22-23).

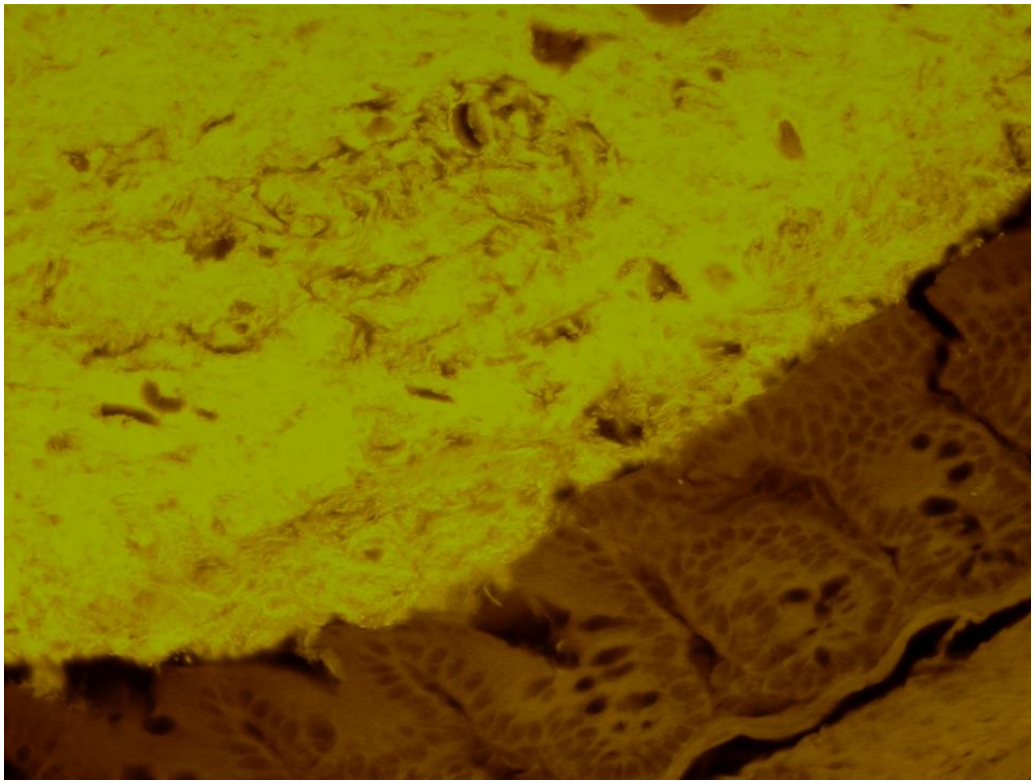
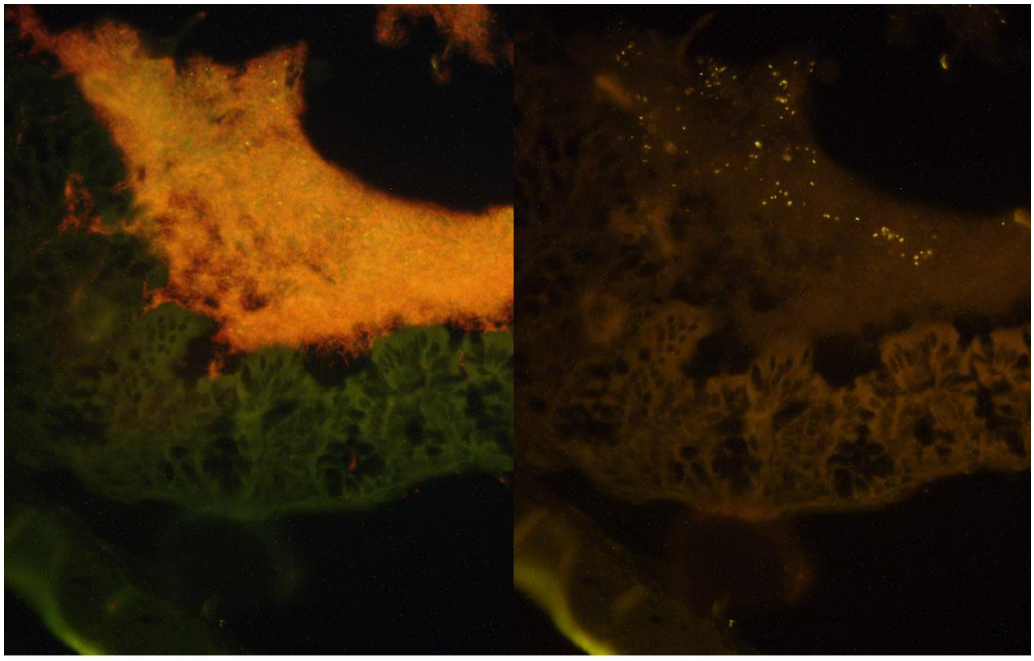
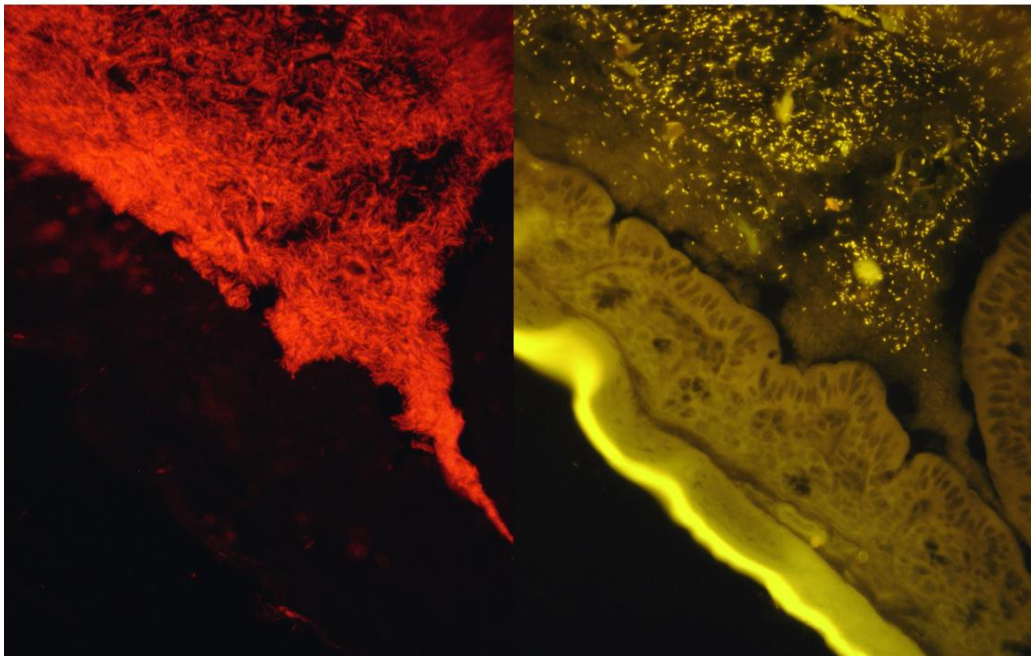


Figure 21. Bacteria are highly concentrated and evenly distributed throughout the proximal colon in a healthy mouse (Eub338 Cy3, all bacteria, orange). Bacteria contact the colonic wall and enter crypts in the proximal colon of mice, this is different in the distal colon. There is no reduction in fluorescence of the bacteria contacting the colonic wall indicating an absence of any suppressive substances produced by the colonic wall.



Figures 22. Proximal mouse colon. Left: all groups of bacteria are overlaid (*Eubacterium rectale* is red, *Bacteroides* is orange, all other bacteria are green). Right: shows only *Bacteroides* within the same microscopic field.



Figures 23. Proximal mouse colon. Left: *Eubacterium rectale*, Right: *Bacteroides* within the same microscopic field. While long rod shaped bacteria including the *Eubacterium rectale* group contact the colonic wall and enter crypts, the short coccoid rods of *Bacteroides* are completely separated from the mucosa in the same location and do not enter crypts.

Analysis of bacterial groups in the murine proximal colon reveals, that only long rods with a curly form contact the mucosa (*Eubacterium rectale*, *Bifidobacteriaceae Lactobacillius*). Short rods and coccoid bacteria such as *Bacteroides*, *Enterobacteriaceae*, *Clostridium difficile*, *Veillonella* and other groups are separated from the mucosa. The difference in the spatial distribution of differently shaped bacteria in the proximal colon of mice indicates that the mucus layer in the proximal colon of rodents is also present, however because of a lower viscosity it is penetrable for long corkscrew formed bacteria but not for short coccoid rods.

The bacterial shape is important for bacterial movement. Short rods are equipped with multiple pili. Pili enable movements in a watery environment but not in slime. Short rods may have flagella, which through propeller-like action move them through slime. Long curly rods use complex body movements to screw through gels of high viscosity, but are immobile in water. The investigations on velocity of differently shaped bacteria in simulated mucus with variable viscosity indicate that the coccoid *Bacteroides* bacteria have the highest velocity at viscosity corresponding to 0.2% agarose and are immobilized at 0.4% agarose, while the long curly rods of the *Eubacterium rectale* group have the highest velocity at viscosity of 0.5% agarose. All bacterial movements stop at viscosity of 0.7% agarose [10].

The distribution of long curly bacteria or short coccoid rods within mucus can be used to mark the areas of changing viscosity. In healthy humans, the separation of bacteria from the mucosa is equally perfect in the proximal and distal colon and bacteria are never found in crypts. In healthy rodents the viscosity of the mucus in the proximal colon is markedly lower than in the distal colon allowing bacteria with a long curly rod shape to reach and contact the mucosa. The reason for differences in mucus layer viscosity of the proximal colon in human and rodents is unclear and the adherence of selected bacterial groups here may have some evolutionary advantages. It can also be that the region between crypts in the proximal colon of rodents builds the germinal zone for the colonic biofermentor (see biostructure of fecal microbiota in health, inflammatory bowel disease and disease control groups below). The lumen of the human large intestine is much larger, and the germinal zone is located intraluminally.

The presence of the mucus barrier in the proximal colon of mice can be clearly demonstrated in germ-free mice mono-associated with *Enterobacter cloacae* – a bacterium with a short coccoid form. The distinct mucus layer and separation of bacteria from the colonic wall can be observed in both the distal and

proximal colon of these mice. Bacteria are perfectly separated in the distal colon (Figure 24), however in the proximal colon some bacteria can be found inside of isolated vacuoles of the goblet cells, especially at the bottom of crypts (Figure 25).

The undifferentiated epithelial cells at the base of the crypts are primarily mucus-secreting cells, whereas differentiated cells of the columnar epithelium are mainly absorptive cells, removing water and electrolytes from the mucus [11]. The epithelial stem cells at the crypt base proliferate and replace surface cells within 4–8 days. The dissemination of *E. cloacae* in crypt bases and goblet cells outlines zones of lower viscosity and confirms independently that during the journey from the crypt base toward the surface epithelium, crypt cells become increasingly differentiated and absorptive.

The adsorptive cells of the crypt neck and of the epithelial cells of the columnar epithelium dehydrate the mucus layer. Dehydration makes the mucus layer solid and impenetrable for bacteria and protects sites of mucus production and the mucosa from encounters with potential pathogens. The lower viscosity of the mucus at the crypt base promotes emptying of crypts and prevents obstruction, but as a drawback it may make these types of cells more vulnerable to invasion by potential pathogens. Indeed, invasion of epithelial cells by *E. cloacae* was observed exclusively at the crypt bottom, whereas no *E. cloacae*-containing cells were observed within the cytoplasm of the columnar epithelial cells in mono-associated mice. Interestingly, crypt abscesses, which are typical histomorphologic findings in human self-limiting colitis and IBD, are also more abundant in crypt bases.

The changes in viscosity of the mucus layer are controlled by two opposite processes: The resorption of water by the columnar epithelial cells solidifies the mucus at the mucosal site. The diffusion of water from the fecal stream thins (dilutes) the mucus from the luminal side. The fecal stream carries away the maturated mucus and incorporates it into the fecal mass (Figure 20). The mucus layer is continuously renewed. The viscosity of the mucus secreted by the goblet cells is significantly lower than the viscosity of the dehydrated mucus film, which is attached to the columnar epithelium. The secreted mucus can not merge with the dehydrated mucus because of differences in consistency. Instead it heaves up the semisolid dehydrated mucus and spreads below it. This mode of replacement protects freshly secreted mucus from bacterial penetration until it is in turn solidified by water resorption and can serve as impenetrable cover. The waves of secretions, displacement, and solidification lead to an onion-like stratification of the mucus. This stratification can be seen in alcian stain, or even better with FISH in patients with irritable bowel syndrome where the zones of massive secretion and imperfect solidification are visualized by bacteria which are entrapped within consecutively displaced and alternating mucus films of moderate and high viscosity (Figure 26).

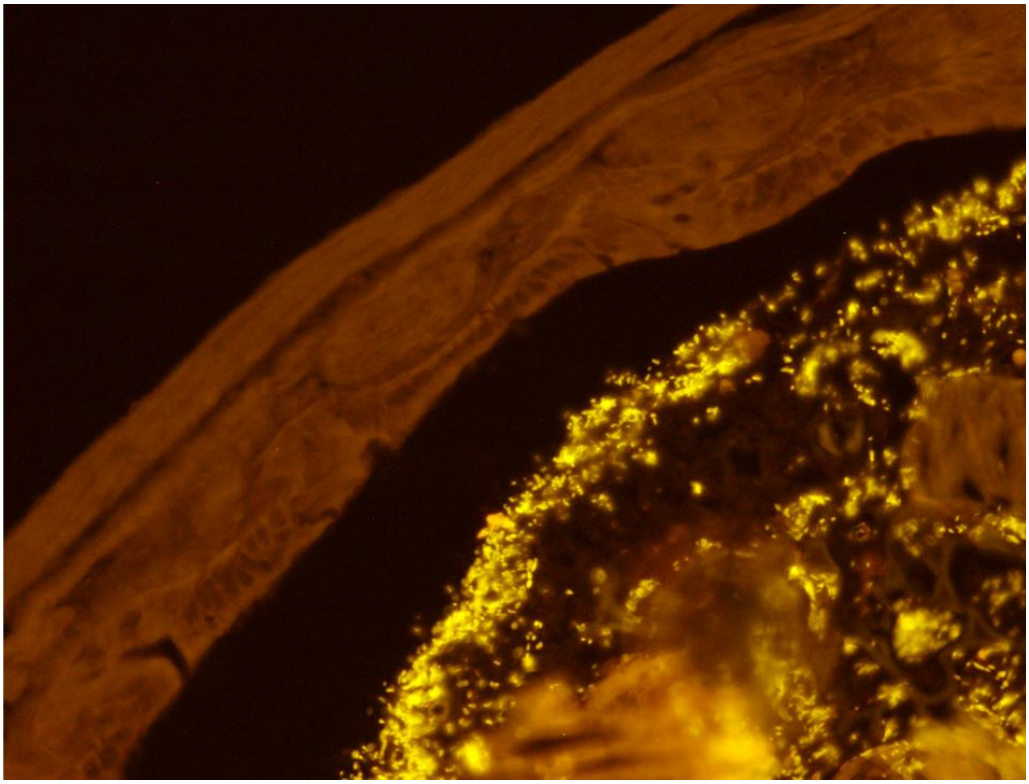


Figure 24. Distal colon of a mouse mono-associated with *Enterobacter cloacae*, a short coccoid rod. Bacteria are clearly separated from the colonic wall by a mucus layer.

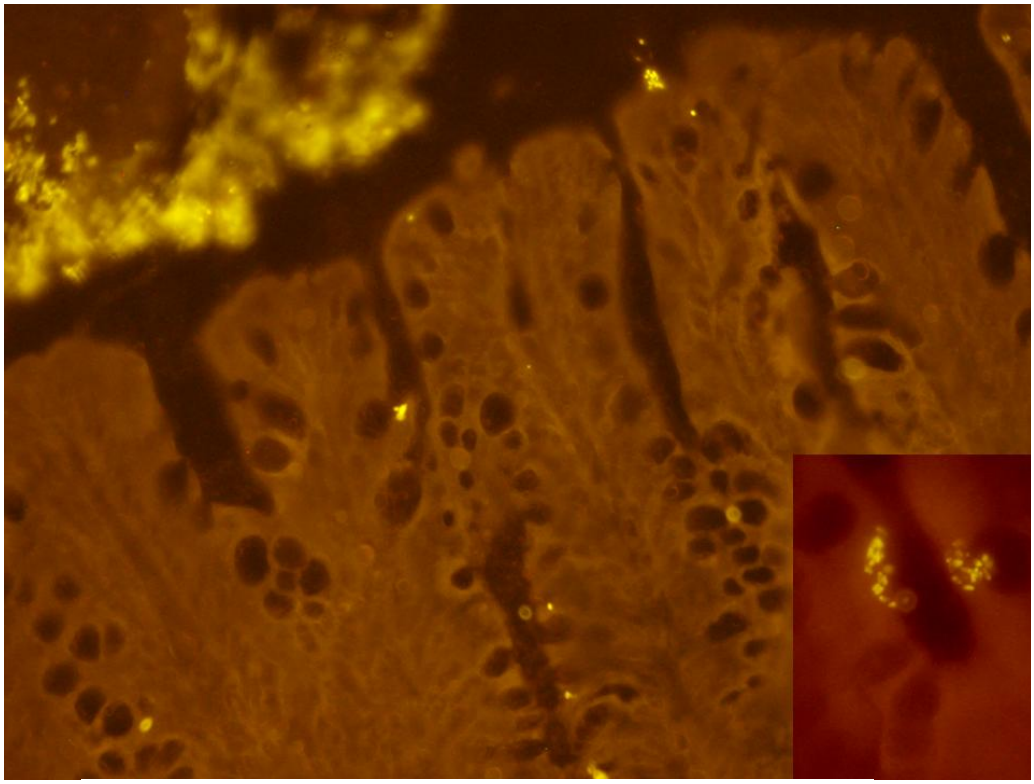


Figure 25. Proximal colon of a mouse mono-associated with *Enterobacter cloacae* (orange fluorescence x1000). The mucus layer is clearly perceivable. Bacteria can be found in regions of lower mucus viscosity at the bottom of crypts and within vacuoles of goblet cells. The insert shows bacteria in a crypt.

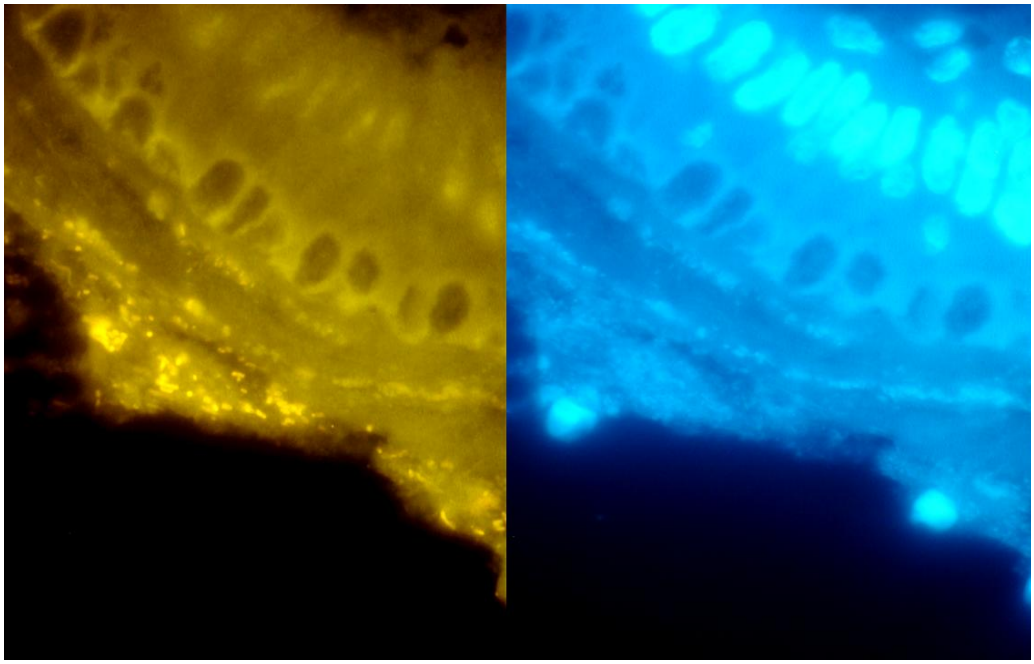


Figure 26. Cecal biopsy from a patient with irritable bowel syndrome. Bacteria are hybridized with the universal probe (Eub338 Cy3, orange, left) and counterstained with DAPI. The waves of mucus with incorporated and immobilized bacteria are moving from the mucosa to the luminal side. Goblet cells are discharging their contents below these coats of solidified mucus leading to an onion-like structure of mucus.

Fecal microbiota

Biostructure of fecal microbiota

The native structure of fecal microbiota within intact intestine cannot be directly investigated in humans, which is different from what is achievable with animal experiments. Intestine filled with feces cannot be obtained from healthy persons, except on rare occasions, from appendectomy and elective surgical resections ideally performed without prior use of antibiotics. An alternative approach is to study stool samples, since the outer regions of feces represent the luminal surface of the mucus layer and since the structural organization of defecated feces does not differ from feces located within the intestine. In analogy to core boring used for investigation of geologic formations, the spatial structure of fecal microbiota can be investigated on sections of punched out fecal cylinders, which are then fixated and embedded in paraffin [12,13].

In healthy humans, the surface of formed stools is covered with a mucus layer which is similar to the mucus covering the mucosal surface of biopsies (Figure 27). Fecal microbiota in healthy humans can be divided into habitual bacterial groups present in all subjects (Figure 28) and occasional bacteria, which are

present only in subgroups of subjects, either diffusely or locally distributed (Figure 29).

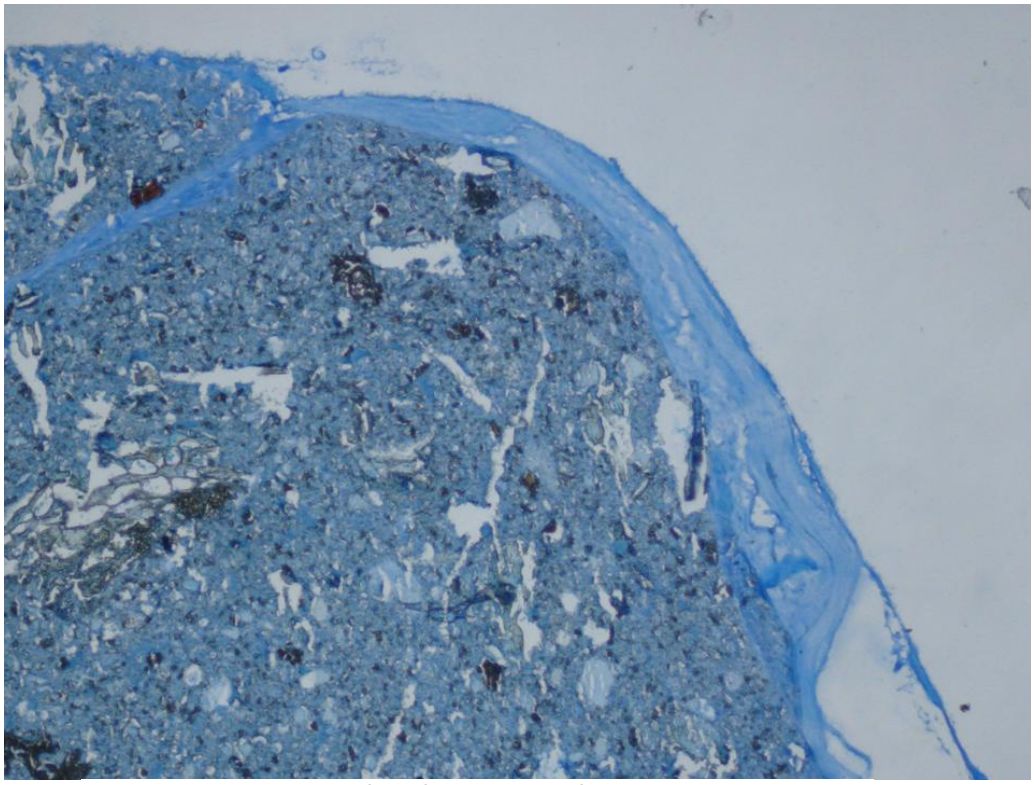


Figure 27. Alcian stain of the fecal cylinder from a healthy person. The mucus layer covers the spontaneously defecated formed stool. Note that no bacteria are in the mucus

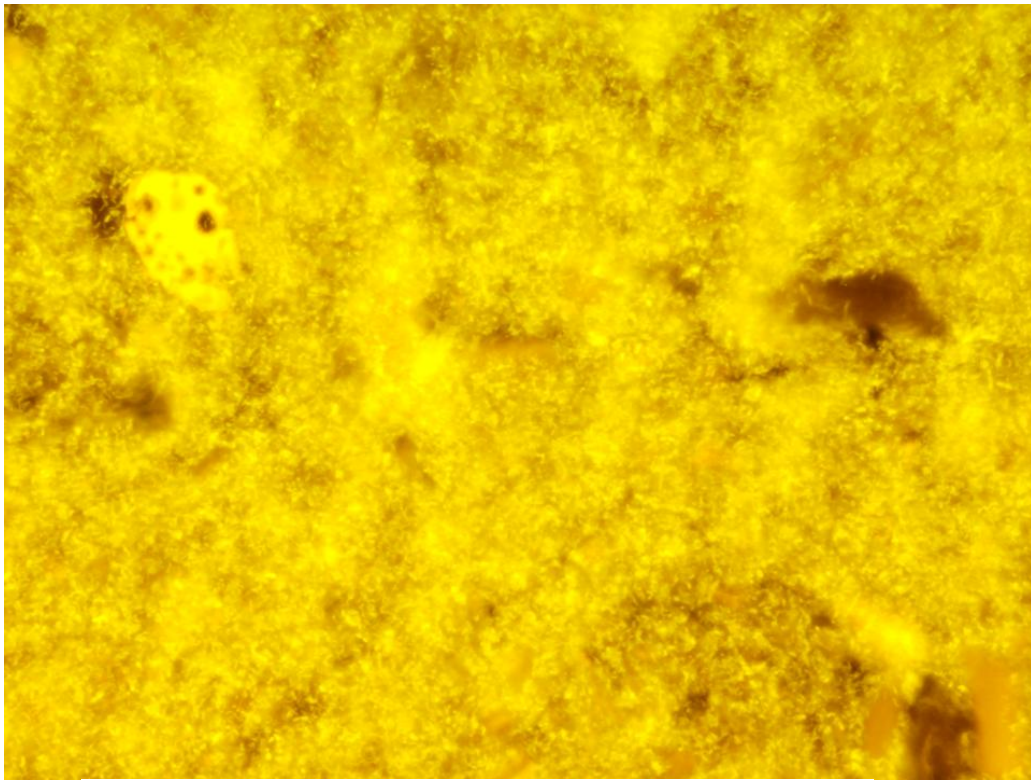


Figure 28. Habitual bacterial groups are obligatorily present in each healthy person, homogenously distributed all over the fecal cylinder and attribute each 20 to 50% to the bacterial biomass. Habitual bacterial groups are represented in human by *Eubacterium rectale* (Erec Cy3, orange fluorescence, x400), *Bacteroides* and *Faecalibacterium prausnitzii*)

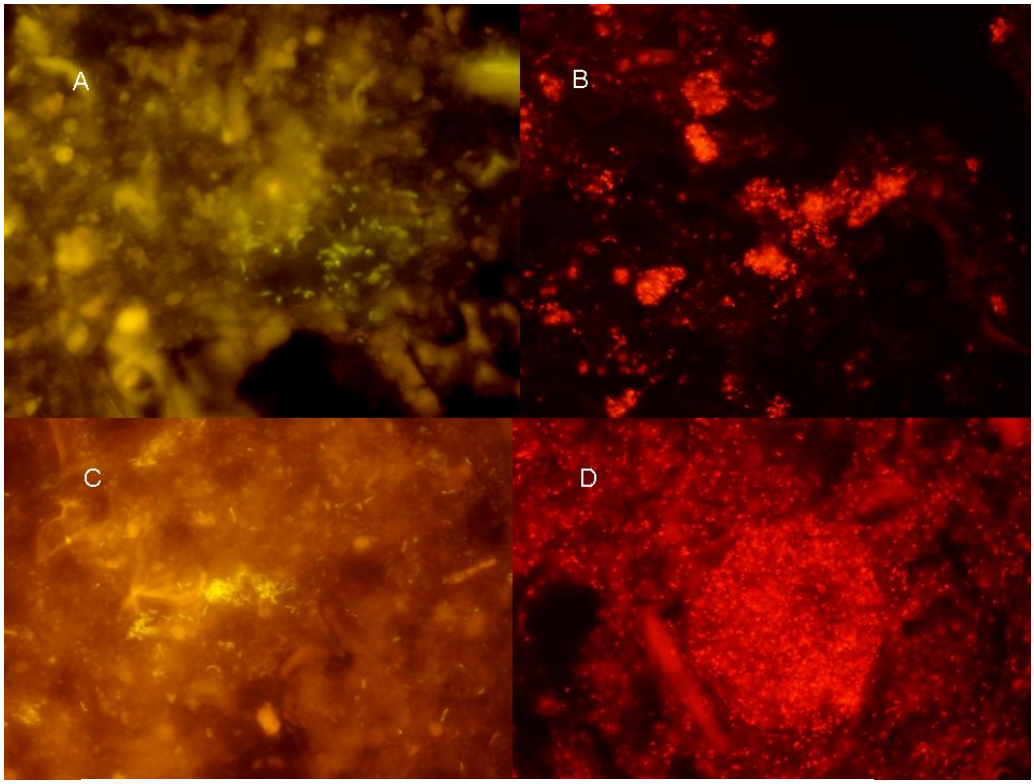


Figure 29. Examples of distribution of occasional bacteria. Occasional bacterial groups can be detected by FISH only in some of persons. They can be diffusely or locally distributed. A – *Clostridium histolyticum*, orange x1000.
B – *Bifidobacteriacae*, red fluorescence x400. C – *Eubacterium hallii*, orange x400.
D – *Enterobacteriaceae, red x400*.

Investigation of 86 different bacterial groups demonstrated that *Eubacterium rectale* (*Roseburia* spp.), *Bacteroides*, and *Faecalibacterium prausnitzii* group are habitual and compose each 20% to 50% of the fecal flora and together at least 70% of all bacteria present in feces. All other bacterial groups occur only in a subset of patients. The exact incidence and concentrations have been previously reported [11,12]. With regard to the mucus layer, bacteria can be divided in fecomucous, mucophob and mucotrop (Figure 30-31). All habitual bacteria are fecomucous. Their highest concentrations are within feces, however they also enter mucus. Their concentrations diminish with increasing distance to the fecal surface. The mucophobe bacteria, a typical representative is the *Bifidobacteriaceae* group, avoid mucus. Mucotrope bacteria such as *Enterobacteriaceae* and *Verucomicriaceae* are located on the border between feces and mucus.

The biostructure and composition of bacterial groups is individual in each case. Daily investigation of stools demonstrated a relative stability of the fecal microbiota. However, weekly investigations continued over more than 6 months have shown that the individual composition of the microbiota change significantly with time.

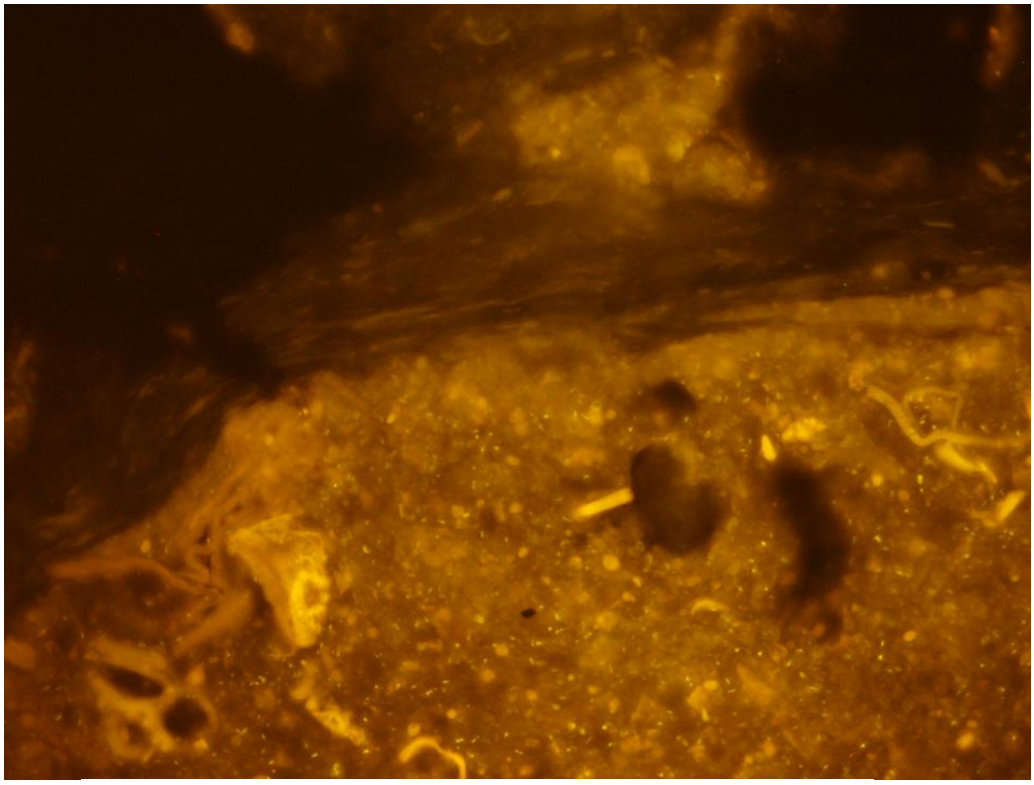


Figure 30. Example of mucophob bacteria. *Bifidobacteriaceae* avoid mucus, orange fluorescence x 400.

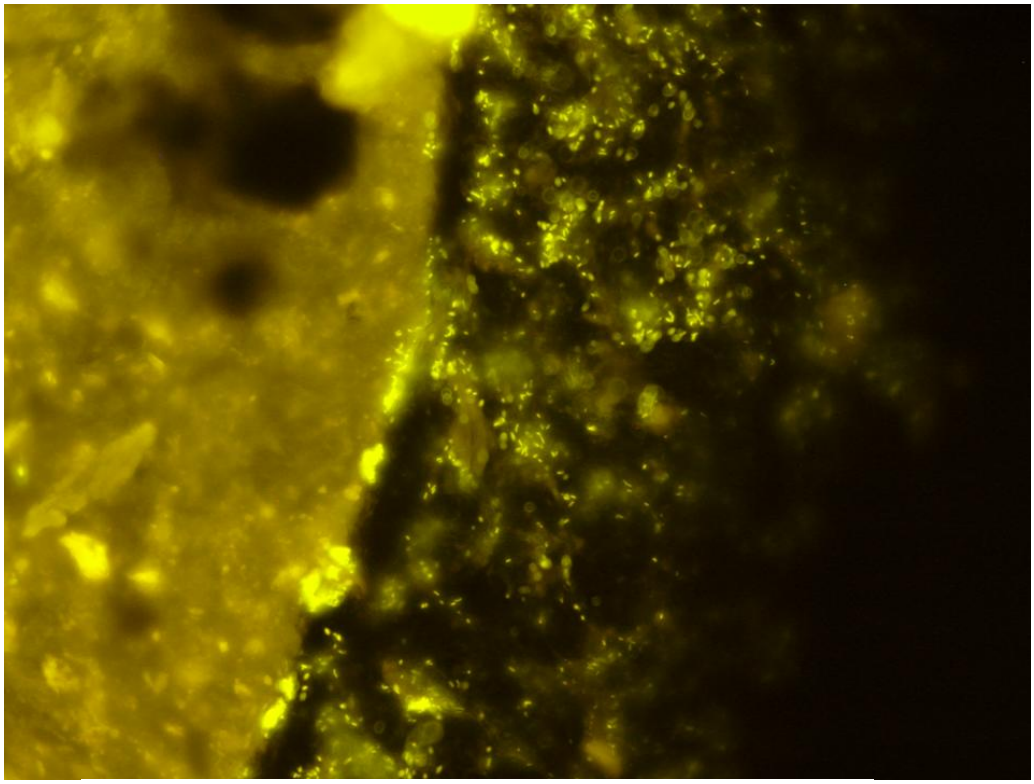


Figure 31. Example of mucotrope bacterial groups. *Enterobacteriaceae*, orange fluorescence, x400. Mucotrope bacterial groups including *Verucobacteriaceae* are preferably located in the region between feces and mucus. All mucotrope bacteria are occasional. *Verucobacteriaceae* appear to be associated with diarrhea, but can be also found in asymptomatic healthy persons.

CHANGES OF THE COLONIC MICROBIOTA IN DISEASE

Break of the mucus barrier in inflammatory bowel disease

The most prominent feature of intestinal inflammation is a break of the mucus barrier (Figures 32-33) with subsequent migration of intestinal bacteria towards the mucosa, adhesion and cytopathologic effects (Figure 34). Bacteria adhere to epithelial cells and build dense adherent layers. Despite massive adhesion, the epithelial barrier holds up against the bacterial invasion in most cases. Finding bacteria in epithelial cells or within submucosal regions is an exception. Even in severe inflammation, multiple sections of the same biopsy have to be investigated before single intraepithelial bacterial inclusions can be detected (Figure 35). They are located mainly at the bottom of the crypts, which remain often empty of bacteria. They are not present in the columnar epithelium, which has direct contact with the dense masses of bacteria (see

viscosity of the mucus barrier). Bacteria can often be seen in regions of the biopsy, which are mechanically damaged [5]. Reports of finding submucosal bacteria should be cautiously interpreted as long as they do not exactly define how far away from the biopsy edge the observations were made.
Although adherent bacterial layers are present in nearly all (94%) patients with IBD who had not been treated with antibiotics, the highest concentrations of mucosal bacteria are found, not in the highly inflamed regions of the intestine, but in less or macroscopically non-inflamed regions. This indicates that the break of the mucus barrier is primary to the inflammation. In inflamed regions, the bacterial concentrations are reduced (Figures 34,33), while leukocytes appear in the mucus in large numbers often arraying the outer regions of the mucus (Figure 36). Leukocytes within mucus are absent in biopsies from healthy persons. Bacteria reach the intestinal wall and lead to development of ulcers, fissures, and crypt abscesses, despite high concentrations of leukocytes and reduced numbers of bacteria in the mucus of inflamed gut segments.

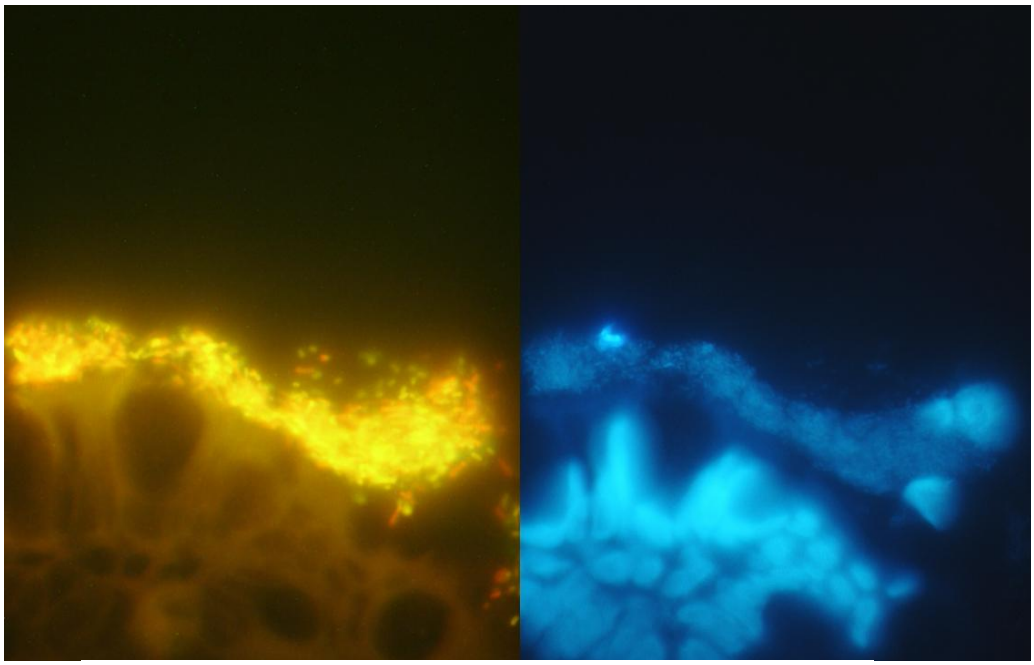


Figure 32. Prolific bacterial biofilm covers the colonic mucosa in a patient with Crohn's disease. Left: multicolor FISH. Right: DAPI stain of DNA structures. *Bacteroides* is orange, *Eubacterium rectale* and *Faecalibacterium prausnitzii* are both red, all other groups are green. Despite close attachment to the mucosa no bacteria can be found intracellular in the columnar epithelium or submucosa. In DAPI stain only a few leukocytes are seen in the biofilm. The patient was treated with azathioprine. Prolific biofilms without leukocyte response are typical for patients with IBD treated with azathioprine.

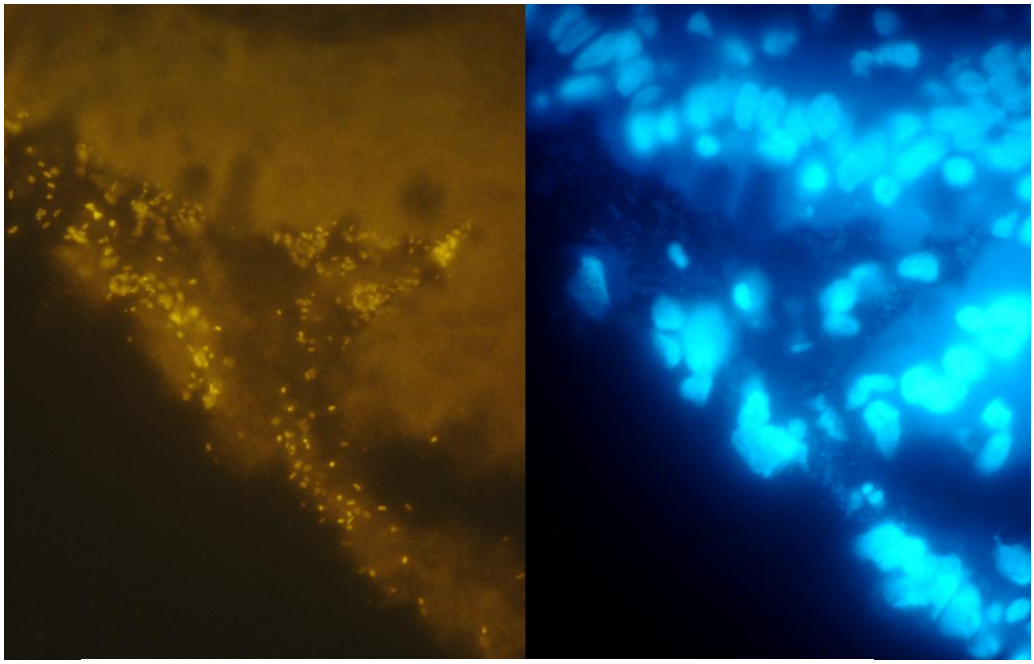


Figure 33. Ascending colon of a patient with Crohn's disease. Adhesion of *Bacteroides* (orange) to the epithelial surface and the entrance of crypts. Right: DAPI stain demonstrates the response of the leukocytes (large blue nuclei) which migrate into the mucus of crypts and the surface.

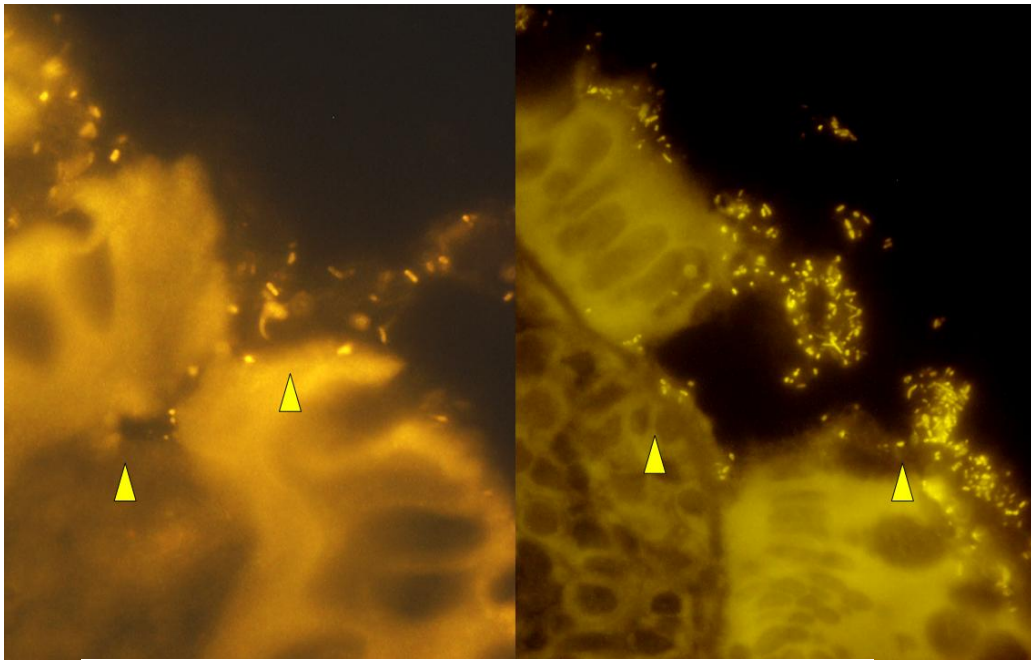


Figure 34. Ulcerative colitis, cytopathogenic effects of bacterial adhesion, with defects (ulcerations) of the epithelial layer and migration of bacteria into the submucosa. Bacteria are hybridized with the universal probe (Eub 338 Cy3, orange fluorescence x 1000). Despite the severe inflammation, the number of bacteria is reduced compared to regions without marked inflammation as shown in figure 32.

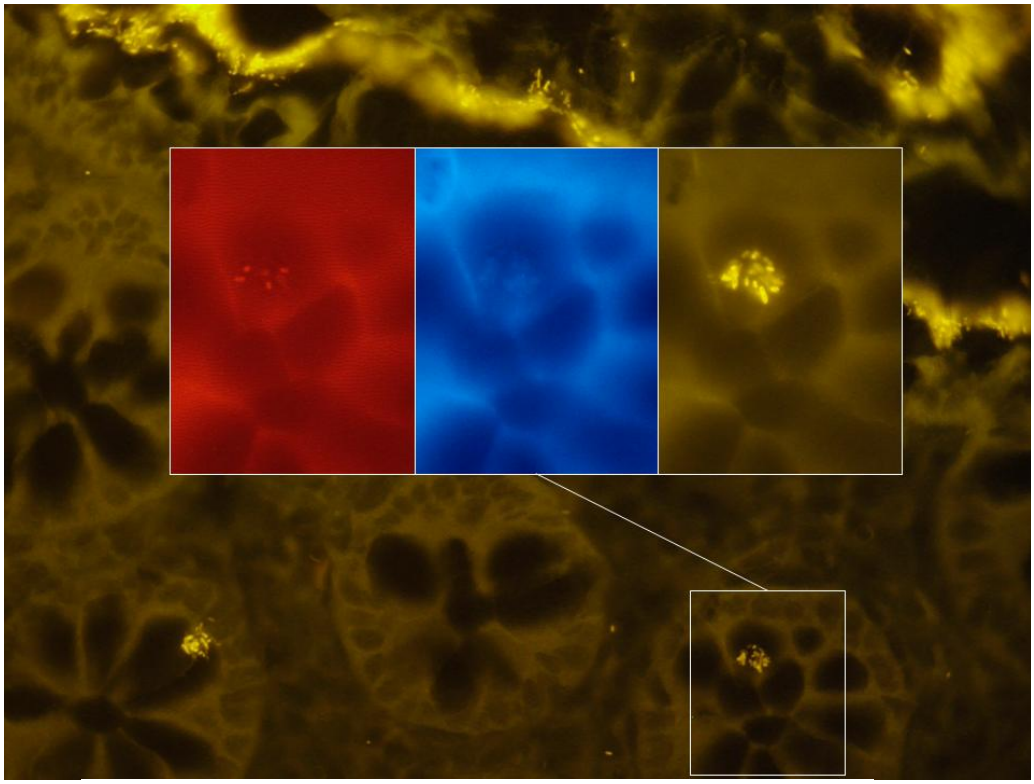


Figure 35. Bacterial inclusions in the vacuoles of goblet cells in a patient with Crohn's disease x 400 (insertions x 1000). All bacteria are orange, *Eubacterium rectale* is red. The inserted figures (DAPI) demonstrate that the individual fluorescence signals with specific bacterial probes have also signals corresponding to DNA fluorescence. The intracellular inclusions are located in crypts, which are free of bacteria.

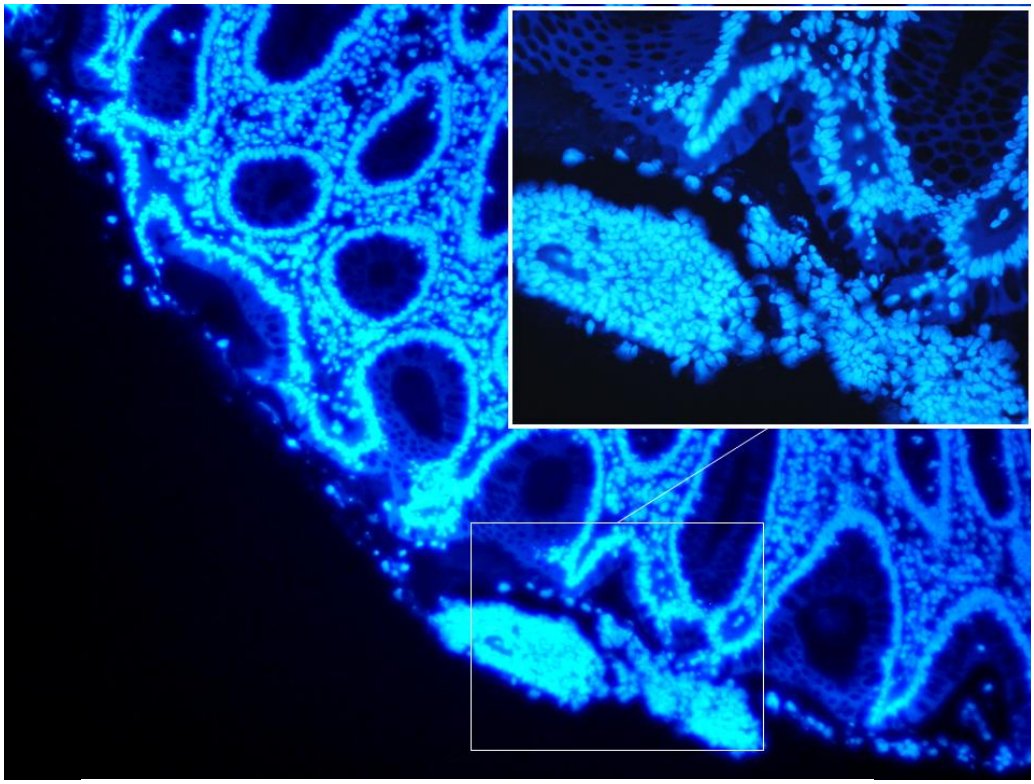


Figure 36. Sigmoid colon of a patient with ulcerative colitis (DAPI x100 and insertion x400. Leukocytes (large blue nuclei) array the outer regions of the mucus.

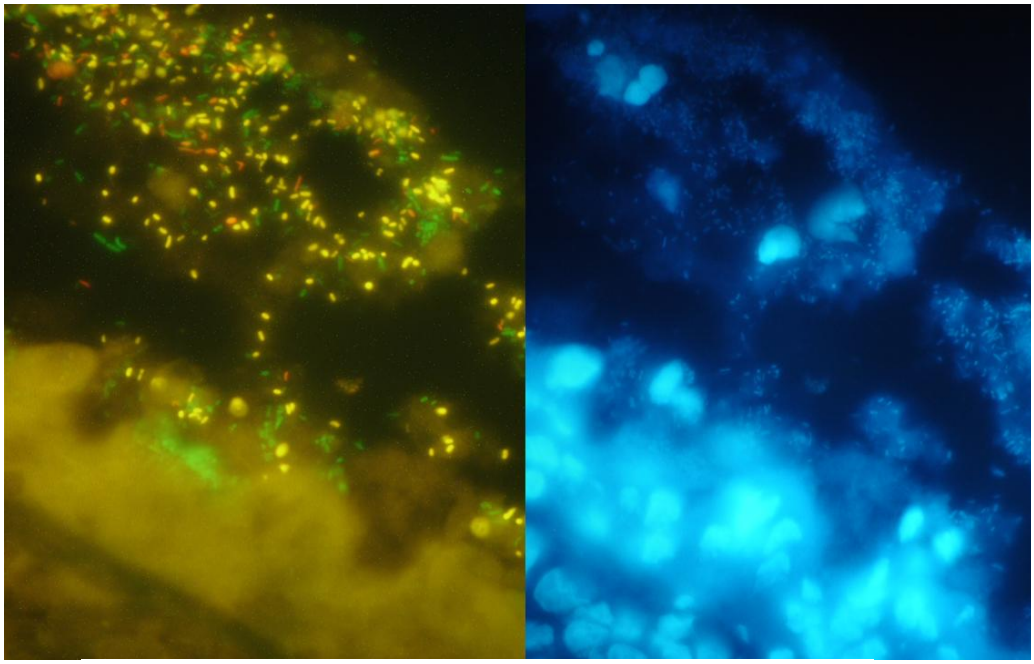


Figure 37. Self-limiting colitis, ascending colon, *Bacteroides* is yellow, *Eubacterium rectale* and *Faecalibacterium prausnitzii* are both red, all other bacteria are green. DAPI stain of the same microscopic field demonstrates leukocyte migration into the mucosa. The proportion of bacteria other than *Bacteroides+Eubacterium rectale+Faecalibacterium prausnitzii* is higher than 10% in patients with self-limiting colitis or specific infection. This is different in patients with IBD. In IBD these three bacterial groups compose together 90% of the mucosal bacteria.

The break in the mucus barrier and bacterial adherence to the mucosa is not IBD specific. Bacterial concentrations of 10^9 bacteria/ml or higher can be found within mucus in nearly all patients with IBD but also in patients with celiac disease, 60% of patients with acute diarrhea, 52% of patients with diverticulosis, 45% of patients with carcinoma or polyps, and in 38% of patients with irritable bowel syndrome (IBS). Ninety percent of bacteria found in mucus of patients with these diseases are represented by only three groups: *Bacteroides, Eubacterium rectale* and *Faecalibacterium prausnitzii* (Figure 32). The mean density of the mucosal bacteria is significantly lower in non-IBD disease and the composition of the biofilm differs. Bacteria of the *Bacteroides fragilis* group are responsible for >50% of the biofilm mass in IBD. In contrast, bacteria that positively hybridize with the Erec (*Eubacterium rectale*) and Fprau (*Faecalibacterium prausnitzii*) probes account for >50% of the biofilm in IBS patients, but only for <30% of the biofilm in IBD. The range of individual findings is however high and the differentiation between

Crohn's disease, ulcerative colitis, IBS, diverticulosis or colonic cancer based solely on the FISH analysis of the mucosal flora is at present impossible. Even *Faecalibacterium prausnitzii*, which is completely depleted in feces of Crohn's disease patients (see biostructure of fecal microbiota in IBD), cannot be used as a diagnostic criterion. It can be detected in mucus of colonoscopic biopsies from more than 50% of patients with Crohn's disease but is, for example, absent in most of the biopsies from healthy persons.
The situation is different in cases of self-limiting colitis or specific infections such as *Serpulina*, *Fusobacterium necrophorum* (*nucleatum*) or Wipple's disease. Typical for a specific break of the mucus barrier is an increase of bacterial groups other than Fprau+Erec+Bac to 10% to 70% (Figure 37).

Factors potentially comprising the mucus barrier
Since the beginning of the 20th century, there has been a steady increase in reported cases of both Crohn's disease and ulcerative colitis and the peak has obviously not been reached. This increase in IBD affects mainly the developed world, especially populations with high living standard and urban areas. Statistically, the frequency of the disease correlates with introduction of tap water, soap and improvement of the living conditions. The hygiene hypothesis argues that improved hygiene and a lack of exposure to microorganisms of various types have sensitized our immune systems, leading to excessive reactions to harmless bacteria in our environment. Out of these speculations have come recommendations to allow young children a reasonable amount of contact with dirt, pets, and other potential sources of infection. A positive effect of such lifestyle changes has not been demonstrated..

The role of facultative pathogens
The statement that exposure to microbes in city dwellers is low is basically wrong and reflects only enteral infections with marked clinical symptoms. The number and diversity of bacteria in the large intestine of the urban population is definitively as high as of those living in rural areas, if not higher. The vegetables and fruits imported from Greece, Portugal, New Zealand, South Africa, and Australia bring a vast variety of microorganisms to people on every continent, providing opportunities for new exposures. The mobility of the modern society has led to a profound and rapid exchange of bacteria worldwide which was never encountered in geographically constrained rural populations of the world. The exposure to facultative pathogens definitively increased in the last 100 years; however their spectrum shifted from *Cholera*, *Salmonella*, *Shigella* and *Yersinia* to less spectacular, clinically "noiseless" pathogens. It is only decades after the first description that *Helicobacter pylori* was accepted as pathogen and moved into the center of research interest. *Helicobacter* is however not the only pathogen capable of forming biofilms. *Serpulina*, adhesive *Enterobacteriaceae*, or *Gardnerella* which were also shown to form adherent biofilms on the epithelial surface have remained even now largely unnoticed by the medical community. These biofilms are often

completely asymptomatic, such as found with *Helicobacter pylori* infections. Their discovery was in most cases accidental, the clinical relevance remains unknown and implications controversial. Common for all of these “silent infections” is however, their ability to compromise the mucus barrier and provide niches for growth of other bacteria which normally have no access to the mucosa (Figures 38,39). The exact data on occurrence and incidence of such “silent infections” in the human population need still to be evaluated.

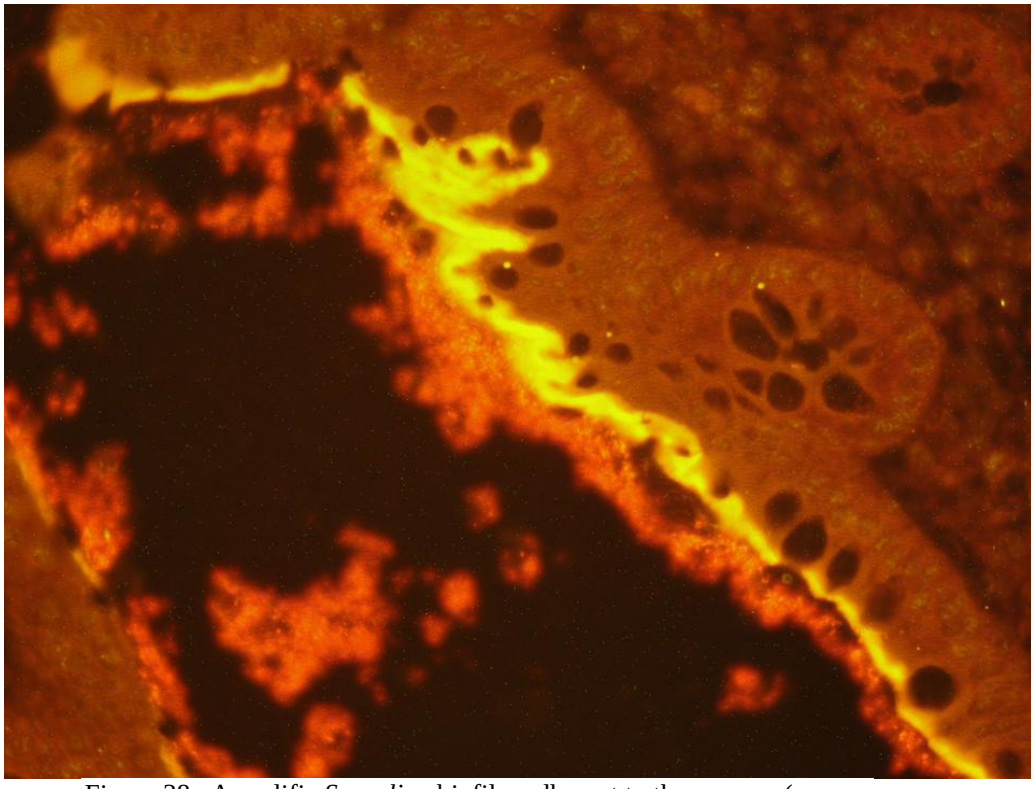


Figure 38. A prolific *Serpulina* biofilm adherent to the mucosa (orange serpentine covering the mucosa) opens access for other colonic bacteria (red fluorescence, Eub338 Cy5) x400.

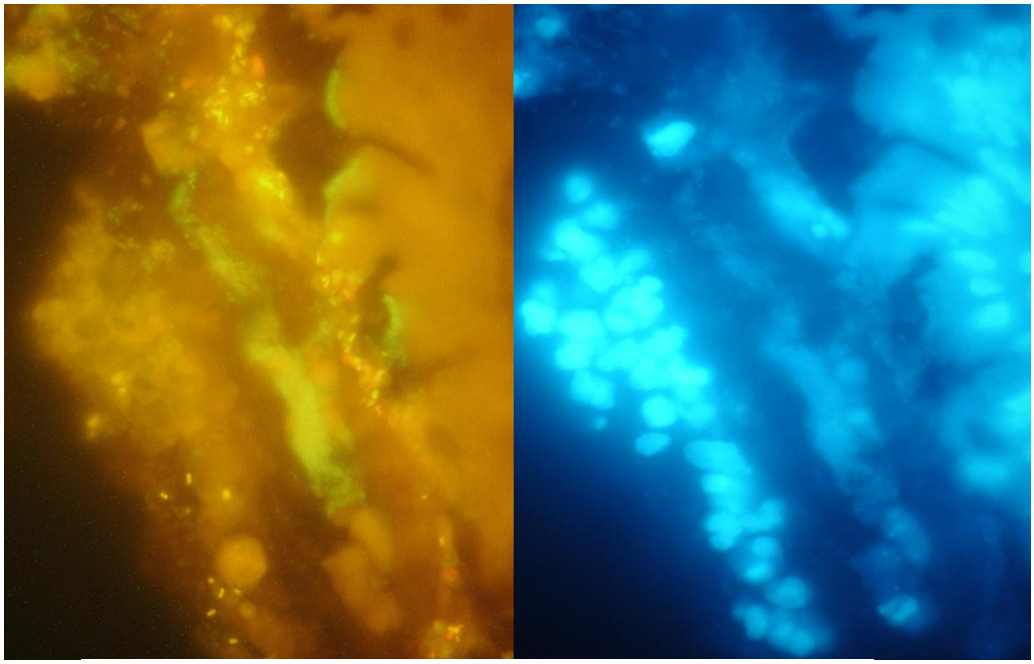


Figure 39. Sigmoid colon of a patient with Crohn's disease. Prolific biofilm by AIEC *Escherichia coli* (*E.coli* is green, *Bacteroides* is yellow, *Eubacterium rectale* is red). The adherence of *E.coli* opens access for colonic microbiota to the mucosa. Despite an extensive leukocyte response, the number of bacteria present is very high and bacteria are located below the leukocytes that array the outer mucus regions.

Substances reducing the viscosity of the mucus barrier

The stated statistical correlation of the hygiene hypothesis between increased incidence of IBD and increased cleanliness of the modern society may have a completely different and more disturbing explanation. Detergents clean objects, they do not sterilize them. We know astonishing little about their effects on the intestinal mucosa and the mucus barrier, despite the longtime use of detergents in households. In-vitro and in-vivo evidence however suggest that substances which reduce the mucus viscosity may contribute to bacterial proliferation on the intestinal mucosa.

Detergents

Addition of detergents such as dextran sodium sulfate (DSS) to an in-vitro model of stimulated mucus enables migration of bacteria through gels with a viscosity corresponding to agarose concentrations of 0.9%. *Bacteroides* migration could be seen up to concentrations of 0.6% and migration of the *Eubacterium rectale* group up to 0.9%. Without detergents *Bacteroides* is

immobilized at viscosity corresponding to agarose concentration of 0.4% and the migration of all bacterial groups stops at viscosity corresponding to agarose concentrations of 0.7% [10].
Although the effects of detergents on the mucosal barrier in human are unknown, in the mouse model, the addition of DSS to food induces acute colitis (Figure 40), which becomes chronic after repeated exposure to DSS. The DSS induced inflammation in mice is restricted to the large intestine, where bacterial concentrations are high, bypasses the small intestine, where bacterial concentrations are low. Antibiotics relieve the DSS induced inflammation. Both peculiarities stress the key role of bacteria in the pathogenesis of DSS colitis.

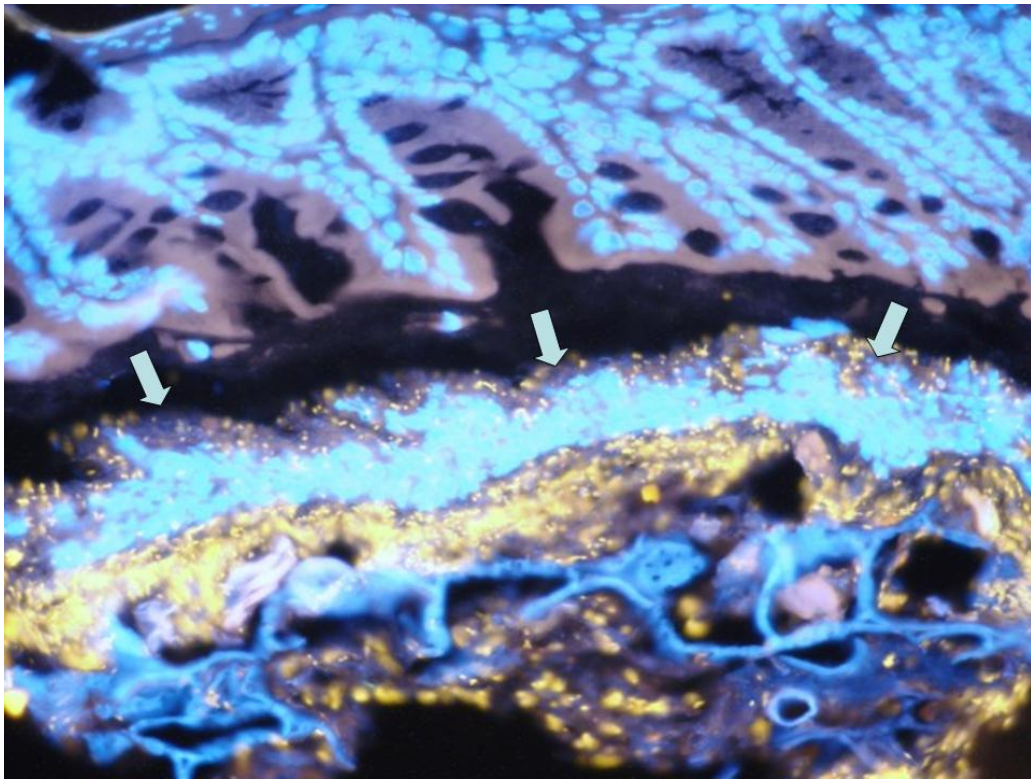


Figure 40. Inflammatory response in DSS mouse colitis. *Bacteroides* (Bac303 Cy3, orange) and DAPI stain are overlayed. Leukocytes array the outer regions of the mucus layer (arrows).

Emulsifiers
Emulsifiers are another group of substances which could potentially influence the mucus barrier and are increasingly used by the food industry since the beginning of the 20th century.

Recent data on Il-10 gene-deficient mice support the potentially detrimental role of emulsifiers, such as 2% carboxymethyl cellulose (CMC) [14]. High bacterial concentrations were found within crypts of Lieberkuhn in the ileum of all CMC treated IL-10 knock-out mice (Figure 41). The finding resembled visually observations in the ileum of Crohn's disease patients (Figure 42).

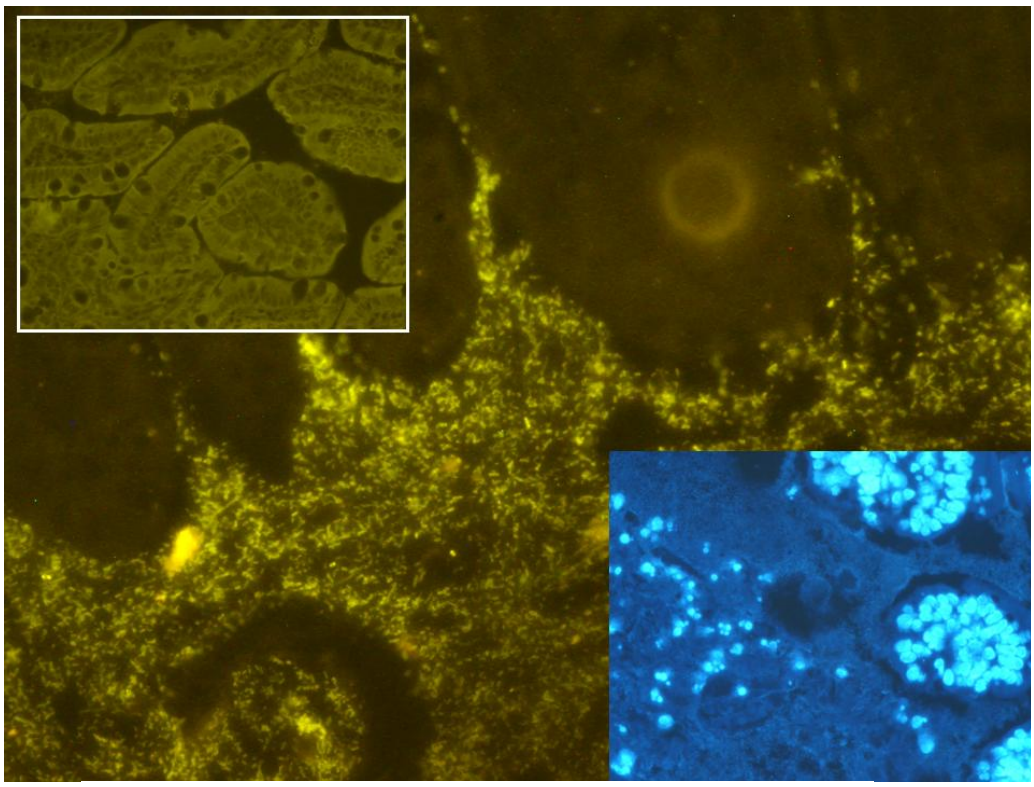


Figure 41. Comparison between ileum of the Il 10 gene-deficient mouse treated with 2% CMC and control IL-10 gene-deficient mouse receiving only water (framed insertion). The CMC ingestion is accompanied by a significant increase in intraluminal bacteria in the small intestine. Bacteria enter deep into the crypts of Lieberkuhn and leukocytes migrate into the lumen of the intestine x400 (blue insertion demonstrates the DAPI fluorescence of the same region).

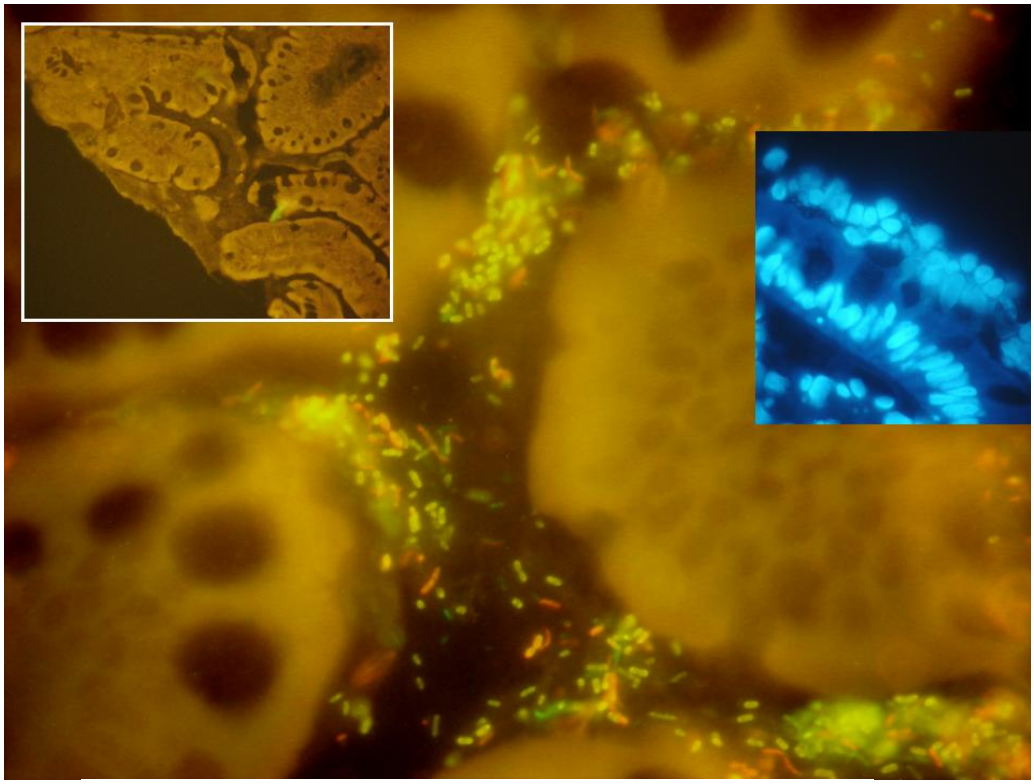


Figure 42. Prolific adherent biofilm located between villi in the ileum of a patient with Crohn's disease. *Bacteroides* is orange, *Eubacterium rectale* is red, all other bacteria are green x 1000. The blue insertion demonstrates an intense leukocyte response in the same patient in a neighboring region. The white framed insertion shows the ileum of a healthy person.

Bile acids

Many other factors can influence the mucus barrier. Bile acids, for example, are natural emulsifiers. Normally they are completely reabsorbed in the ileum and do not reach the colon. When the ileum is resected, then the reabsorption is disturbed, bile acids reach the colon and induce diarrhea.

Glutens

Celiac disease is regarded as an allergic response although the exact structure within the gluten molecule which induces an allergic reaction could not be defined. We do know that symptomatic celiac disease is always ongoing with bacterial overgrowth in the small bowel. The link between bacteria and glutens is poorly understood. Glutens are however naturally occurring emulsifiers. It could be that first bacteria make glutens harmful and that progressive

destruction of the mucosa in the small intestine leads to decreased suppression and bacterial overgrowth.

Smoking
Smoking stimulates mucus secretion. The epidemiologic studies indicate that smoke is beneficial in patients with ulcerative colitis but detrimental for patients with Crohn's disease. A thicker mucus barrier could indeed explain why smoking could be protective in UC patients but has no effect in Crohn's disease, where bacterial suppression is more important, than bacterial separation.

Stress
Stress interferes both with mucus production and regulation of the viscosity of the mucus. It is a known fact that in patients with IBD, stress leads to acute exacerbations of the disease.

Multiple other factors including defensins, probiotics, enteral pathogens, the inflammation itself, genetic background etc. interfere with the mucus barrier function. As long as the mucus barrier is compromised, a conflict between the organism and the pathogens which inhabit our colon in large numbers and diversity is inevitable.

Possible ways to remodel the mucus barrier

- Eradication of occasional pathogens, which compromise the mucus barrier (*Entero-adhesive E. coli, Fusobacterium nucleatum, Serpulina*)
- Selective control of mucus secretion and dehydration (analogs of cortisol)
- Induction of a higher differentiation of epithelial cells, which leads to switch from mainly secretary to adsorptive function (analogs of anti TNF suppressing apoptosis, (methotrexate or MTX)
- Suppression of adherent bacterial biofilms (a possible effect of 5-ASA)
- Reduction of the burden of detergents and emulsifiers in our food
- Stimulation of innate immunity (substances like GM-CSF, probiotics as living vaccines)

Antibiotics can effectively reduce the number of pathogens contacting the mucosa. They have however no direct influence on the mucus barrier and they can not sterilize the polymicrobial colonic microbiota. As soon as antibiotics are withdrawn, the situation becomes reversed. In the long-term, antibiotics are generally ineffective in IBD, because of increasing microbial resistance. The mucus barrier, however, can be compromised not only by environmental or genetic factors but also by specific pathogens such as *Serpulina, Fusobacteria, Enterobacteriaceae,* or *Gardnerella* which build biofims on the epithelial surface. The identification of adherent biofilms and their eradication could be advantageous.

Prednisolone is a very potent drug. As a glucocorticoid, it stimulates mucus secretion. Its mineralocorticoid activity increases water resorption, thereby increasing the viscosity gradient within the intestinal mucus layer. Development of substances which can selectively control the mucus barrier without the typical side effects of prednisone could be of extreme advantage for IBD treatment.

We have previously mentioned that the columnar epithelial cells are differentiated and mainly resorptive, while crypt cells are immature stem cells and mainly secretors. A balance between both is under TNF control. The cell turnover is increased in inflammation. Anti TNF agents reduce the apoptosis of differentiated epithelial cells. This may explain why, of many known cytokines, only anti-TNF antibodies have a clinically proven role in the treatment of IBD. The development of drugs with an effect on apoptosis regulation of the epithelial cell turnover should be considered in the future.

MTX is a potent agent for the in vitro transformation of immature epithelial cell lines to mucine producing goblet cells and columnar epithelial cells. Higher concentrations MTX increase the proportion of columnar to goblet cells.

The role of so called immune suppressive substances in the treatment of IBD remains to be exactly defined.

Mesalazine suppresses bacterial biofilms in vivo by mechanisms which are at present not clear. Different from antibiotic therapy, the suppression with mesalazine does not seem to induce bacterial resistance. It is possible that the suppressive effects of mesalazine could be further expanded when the mode of action is clarified.

The importance of reducing the detergent and emulsifier burden in our food has been mentioned. We do not know at present which of the substances may reach the colon and accumulate in the human body. This issue needs to be further investigated before any recommendations can be made.

Stimulation of the immune response is an intriguing approach for the treatment of IBD. Previous trials with interferon and GM-CSF were half-hearted and inconclusive. PEG interferon was for example not tested at all. The therapeutic potential could be enormous. After all, probiotics may act as living vaccines using attenuated strains to stimulate mucosal immunity. Actually we do not know how probiotics work. However, since the influence of antibiotics on polymicrobial microbiota is limited, the use of biologicals for the control of indigenous microbiota is intriguing. We must however admit that all presently available probiotics use bacterial strains which are present only in small numbers in the human large intestine (less than 0.01%). They were selected mainly for ease of culture, storage, transport and stability within food products. The probiotic potential of anaerobes, which constitute the mass of the indigenous flora of the large intestine, has not been studied.

BIOSTRUCTURE OF FECAL MICROBIOTA IN HEALTH, INFLAMMATORY BOWEL DISEASE AND OTHER GASTROINTESTINAL DISEASES

The colon is a highly efficient biofermenter. We take the colonic bacterial concentrations of $10^{11\text{-}12}$ bacteria per gram of stool and diversity of more than 3,000 species, for granted. They are however unprecedented for environment and non reproducible for man in vitro. Any dysfunction of the mucus barrier, irritation of the gut or inflammatory response could lead to malfunction of the colonic biofermenter and general or specific alterations of microbiota. These alterations could be used for diagnostic purposes. Unfortunately, all previous investigations of the fecal microbiota used homogenized samples of feces and did not reveal any diagnostically valuable features. This may be due to ignorance of the spatial organization of the colonic microbiota.

The situation changed with studies on the microbial biostructure of punched fecal cylinders which made the investigation of fecal microbiota a reliable, highly reproducible, and easy to perform diagnostic assay [see also biostructure of fecal microbiota 12,13].

Functionally, the colonic biofermenter can be divided into three zones:

- a transparent outer **mucus layer,** which is constantly dehydrated by the columnar epithelium, therefore highly viscous, impenetrable for bacteria and separates the colonic biofermenter from the mucosa;
- a **central fermenting zone** in which bacteria and fibers are stirred and fermented;
- a transitional **resting zone** between mucus layer and central zone in which mucus becomes increasingly diluted by luminal fluids and penetrable for bacteria. The softened mucus is however still less versatile for peristalsis and stays for prolonged time attached to the colonic wall. Bacteria enter these soft portions of the mucus in concentrations inverse to the growing viscosity gradient and become increasingly immobilized. Trapped within the resting zone, bacteria are protected against purging events and can be used for renewed settings of the biofermenter after occasional cleanouts, periods of fasting or even antibiotic treatment. Bacteria within the resting zone are **germinal stocks of the colonic biofermenter**.

The firm mucus can be easily perceived with alcian stain. In healthy subjects, both the resting **(germinal) zone** and the **luminal (fermenting) area** can not be distinguished from each other (Figure 43). When the colonic biofermenter properly functions the composition and density of bacteria found here are similar. In disease, however, the microbial changes in these compartments are different and disease specific (Figures 44-48).

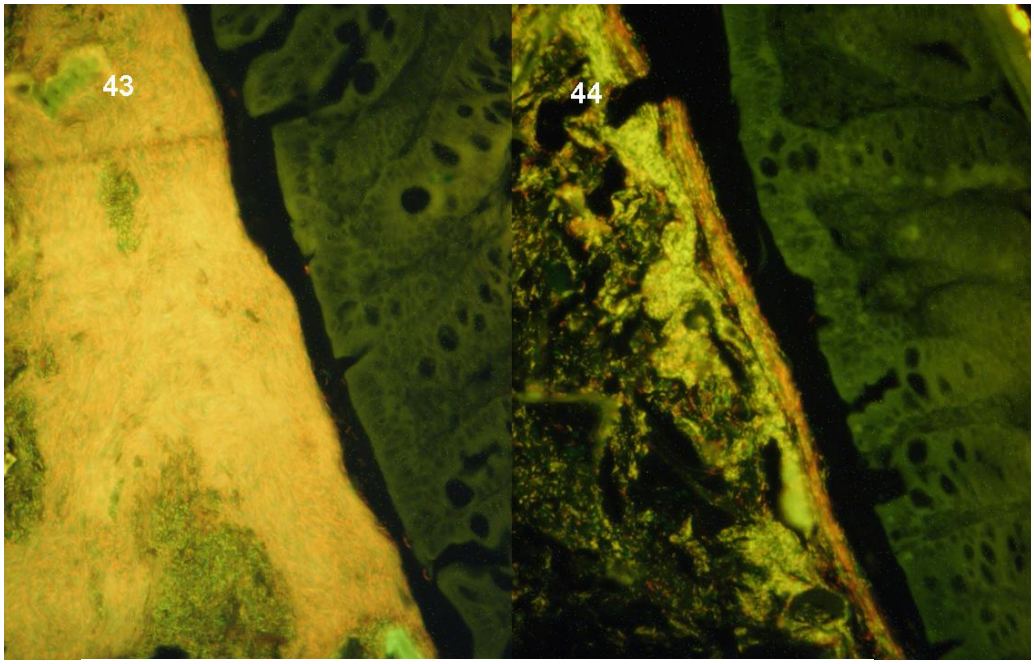
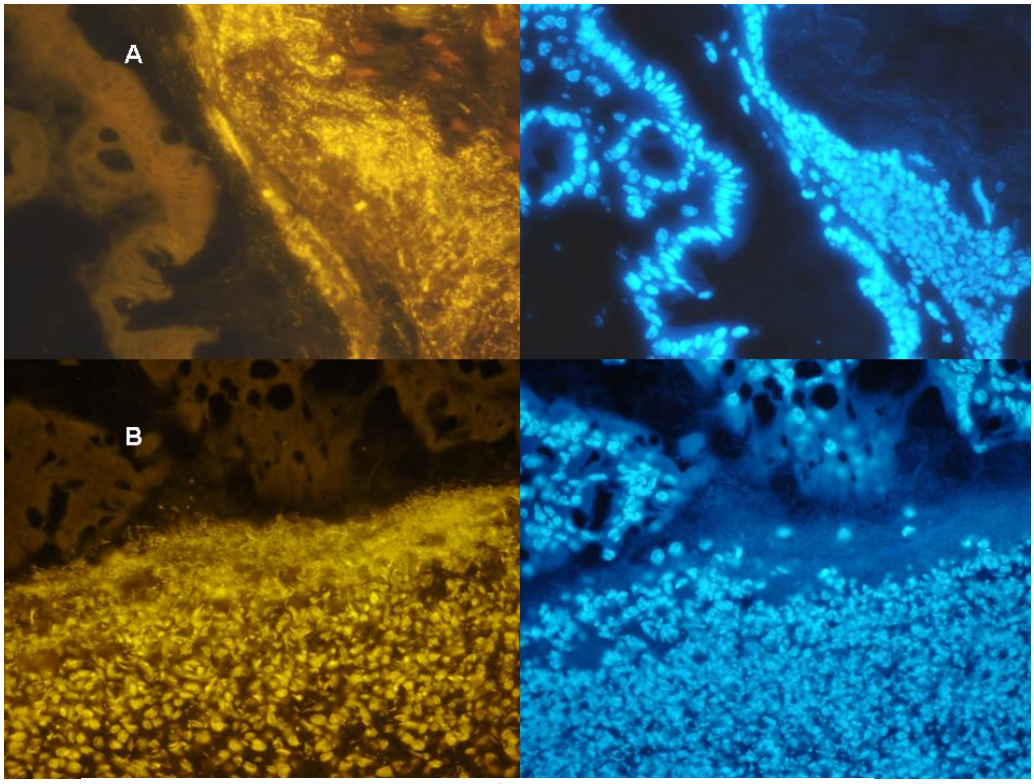


Figure 43. Colonic bacteria in the healthy wild-type mouse are diffusely distributed and have similar high concentrations at the center of feces and in the “germinal” zone.

Figure 44. Bacteria are suppressed in a 28 week-old mouse with IL-10 deficiency, especially at the center of feces. *Bacteroides* is orange, *Eubacterium rectale* is red, all other bacteria are green, x400. The suppression of bacteria precedes the actual inflammation. The animals at the time of investigation had no symptoms.



Figures 45. A: 32 week-old IL-10 deficient mouse with colitis. B: 20 week-old wild type mouse with severe dextran sodium sulfate (DSS) induced colitis. The left panels demonstrate the distribution of bacteria in feces, the right panel demonstrates the DAPI stain of the same microscopy field. Despite massive leukocyte response in both cases presented on the right (the intestinal lumen is completely filled with leukocytes in the DSS mouse) and bacterial reduction at the center of feces (left panels), bacterial concentration in the transition zone between mucus and feces are completely intact and the fluorescence of bacteria is not inhibited.

What happens in the laboratory when the biofermenter stops to work properly? The laboratory staff discharges the biofermenter, decontaminates the contents, and restocks the system. Exactly the same happens in the malfunctioning colonic biofermenter: discharge, decontamination, restocking. Problems with the germinal stock are in the foreground in IBD.

Site dependant changes of the colonic microbial biostructure

The mucus layer

Diarrhea discharges the colonic biofermenter, reduces the total number of luminal bacteria and is accompanied by massive increase in mucus production: increased thickness of the mucus layer and growing incorporation of the unstructured mucus within the fecal mass (Figure 46).
Increased mucus production is general for most intestinal disorders. The only exception is ulcerative colitis, where the mucus layer is depleted, probably as a result of exhaustion.
In acute diarrhea, the local concentrations of single bacterial groups and their fluorescence intensity is unchanged, but the homogeneous structure of healthy feces (Figure 28) is interrupted by broad large septa (unformed stools) or multiple thin striae (watery stools, Figure 46). In chronic conditions, the bacterial concentrations are in addition markedly reduced, indicating that active decontamination of the biofermenter contents takes place.

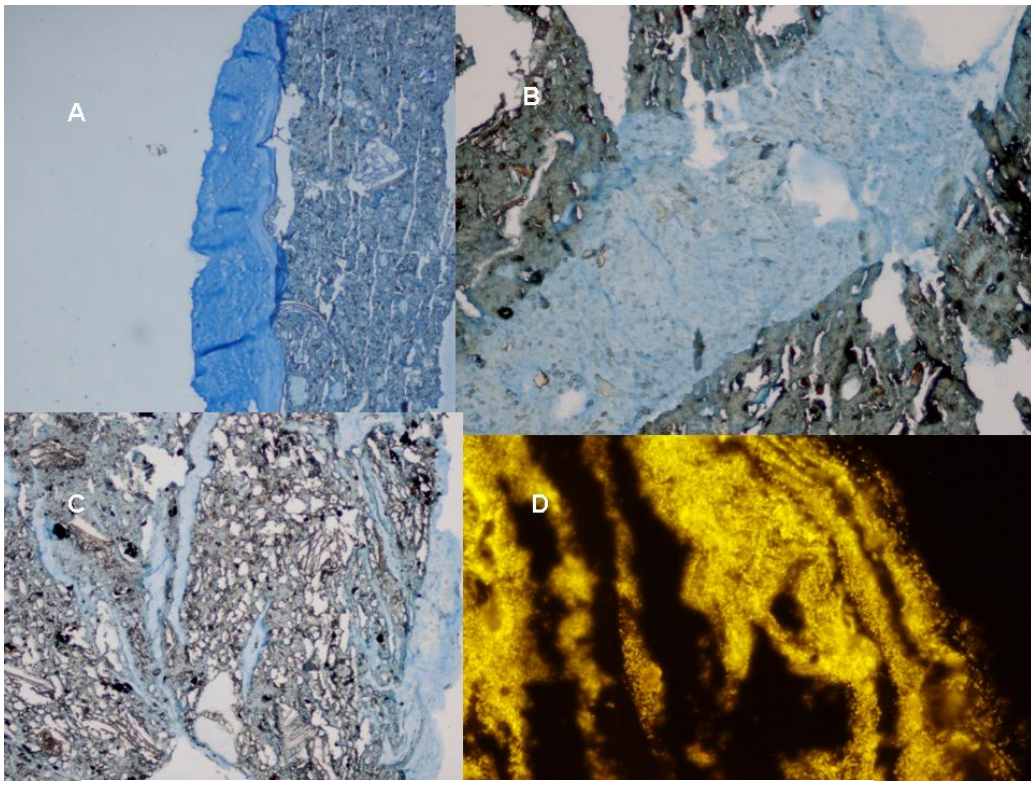


Figure 46. Increased mucus production as a sign of irritation in patients with irritable bowel syndrome (A) and diarrhea (B-D). In Figures A-C mucus can be directly seen with alcian stain. D: Disruption of the normally homogenous biostructure of the fecal cylinder and development of multiple striae of slime demonstrate the increased mucus production. A, B, C x100, D x400.

The working (luminal) area of the colonic biofermenter

The most common general feature of intestinal disturbances is a suppression of bacterial growth and metabolism at the center of the biofermenter. The suppression is general, and is most apparent in regards to the habitual bacteria. Their concentrations in healthy person are especially high and the distribution throughout the fecal cylinder is normally homogenous. In case of colonic malfunction (IBS, IBD, diarrhea, etc.) initially, only the fluorescence signals fade We call these fading a hybridization silence (Figures 47-48), because the number of bacteria stays constant from suppressed to unsuppressed regions and only hybridization signals of bacteria change (relative hybridization silence). With disease progression however, the hybridization signals of single bacterial groups may disappear completely (absolute hybridization silence). It is then impossible to discriminate between suppression and physical elimination of bacteria.

The epicenter of suppression is located in the center of feces and with an exception in Crohn's disease, does not involve the superficial "germinal" zone of the fecal cylinder, where the fluorescence of bacteria and bacterial numbers remain high (Figures 47-48). It appears that the production of the suppressive substance takes place in the small intestine or at least upstream from the colon. The extent to which single habitual bacterial groups are involved is individual and reproducible in repeated investigations of the same patient. It is probable that the involved mechanism of suppression may be different and directed more or less specifically to different bacterial groups. These changes can be exactly quantified on slides of fecal cylinders. Disease progression can be monitored in fecal cylinders obtained at different times.

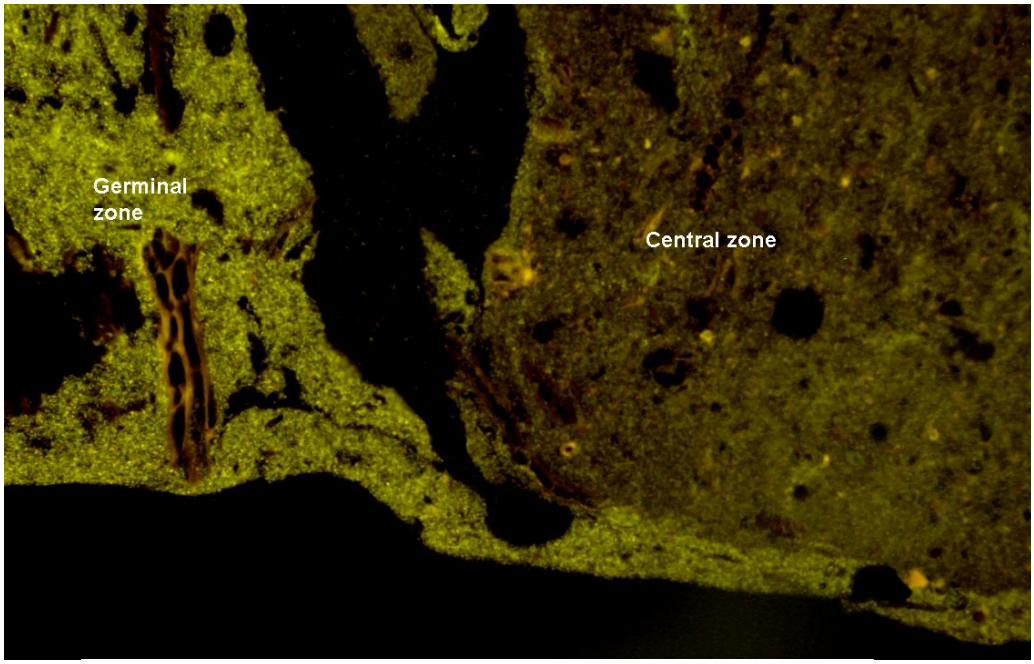


Figure 47. Hybridization silence of *Faecalibacterium prausnitzii* in a patient with idiopathic diarrhea, orange fluorescence x 200. Bacteria are suppressed at the center of the feces, suggesting reduced metabolism. The concentration and fluorescence of bacteria at the surface of feces is well preserved. Bacterial concentration and fluorescence are excellent in the periphery of feces.

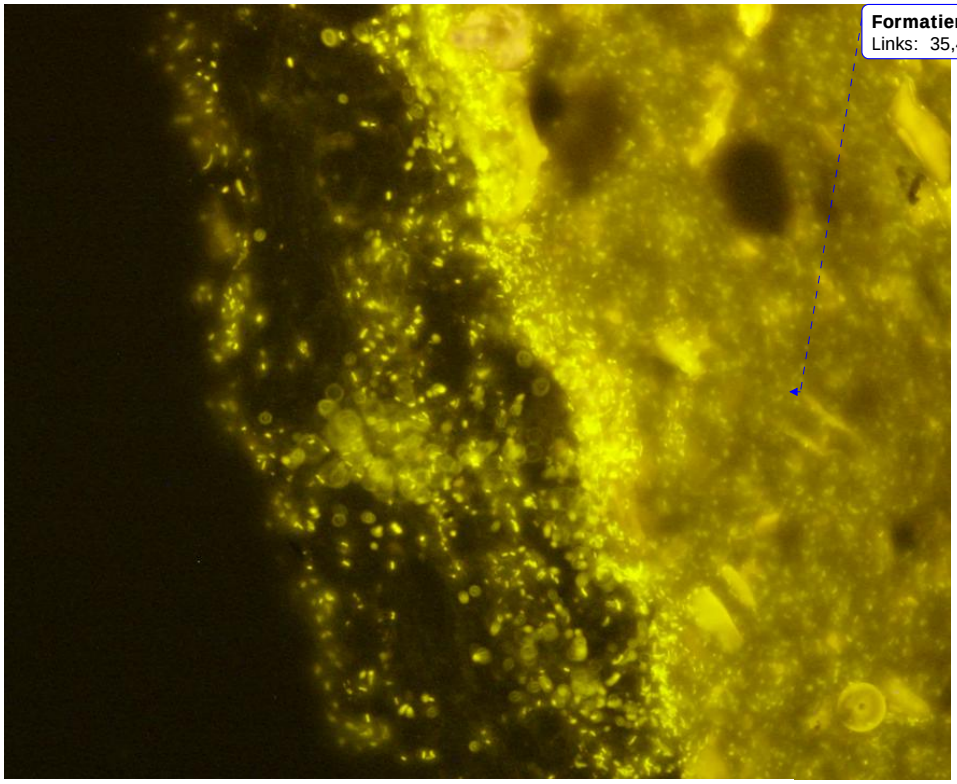


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Figure 48. Hybridization silence of *Faecalibacterium prausnitzii* in a patient with acute gastro-enteritis caused by *Campylobacter jejuni*, yellow fluorescence x 400. Bacteria are suppressed throughout the feces, reduced fluorescence of bacteria (relative hybridization silence). Bacteria are highly concentrated and have excellent fluorescence in the "germinal" zone, at the transition from feces to mucus, and in the superficial portions of the mucus.

The germinal zone of the colonic biofermenter

Changes involving the germinal bacterial zone are characteristic for active inflammatory bowel disease and absent in most other gastrointestinal diseases, with the exceptions of carcinoid of the small bowel and subgroups of patients with celiac disease. The most prominent feature of ulcerative colitis is a replacement of the mucus layer by leukocytes. The leukocytes are located in the germinal zone, which they progressively destroy (Figure 49-50). Active Crohn's disease is characterized by complete depletion of *Faecalibacterium prausnitzii* from the central and germinal zones of feces.

The reproducible detection of these two features in three consecutive fecal cylinders taken in two-weekly intervals allow the diagnosis of active CD and UC with a 79/80% sensitivity and 98/100% specificity [13].

Both leukocytes within the germinal zone and *Faecalibacterium prausnitzii* depletion are not causes but symptoms of the disease. High dose prednisolone or anti TNF therapy is able to reverse these findings within days. The reversal is not permanent. Both *Faecalibacterium prausnitzii* deletion and leukocyte infiltration of the germinal zone return quickly after the reduction of prednisolone or within weeks after Remicade infusion (anti-TNF antibody).

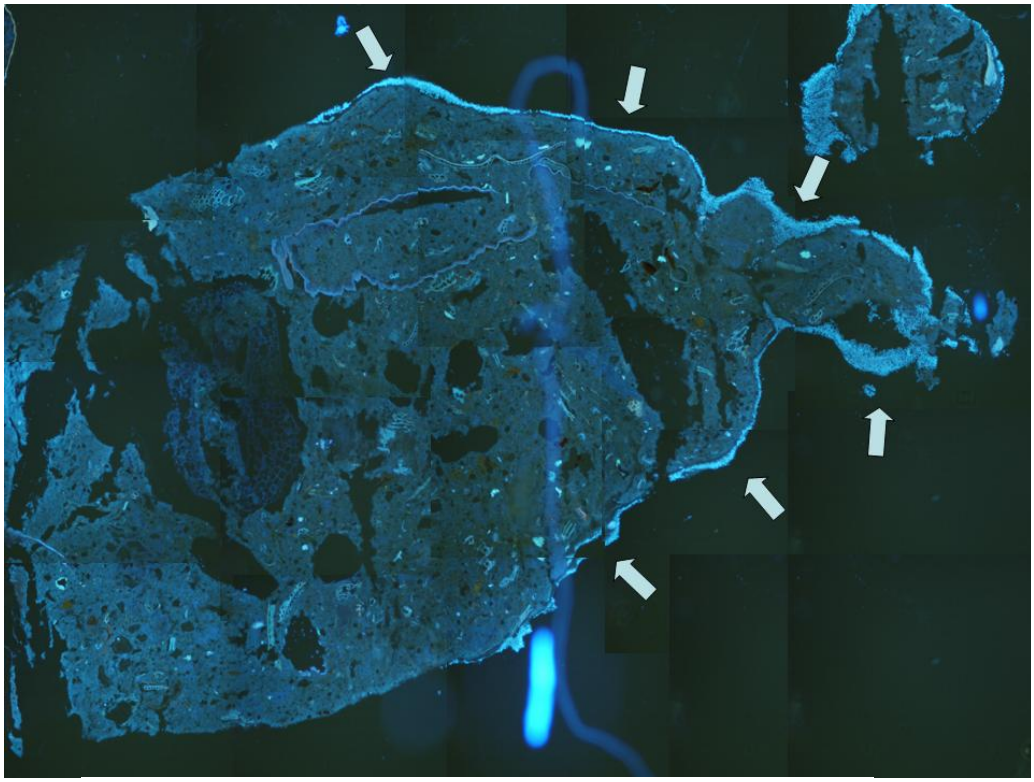

Figure 49. Fecal cylinder with DAPI stain. Multiple single microscopic fields taken at magnification of x 100 are composed to an overview. The center of the cylinder contains no leukocytes and the bacteria are homogeneously distributed. The cylinder has no mucus cover, instead the “germinal” zone is infiltrated by many leukocytes (arrows). The findings are typical for ulcerative colitis.

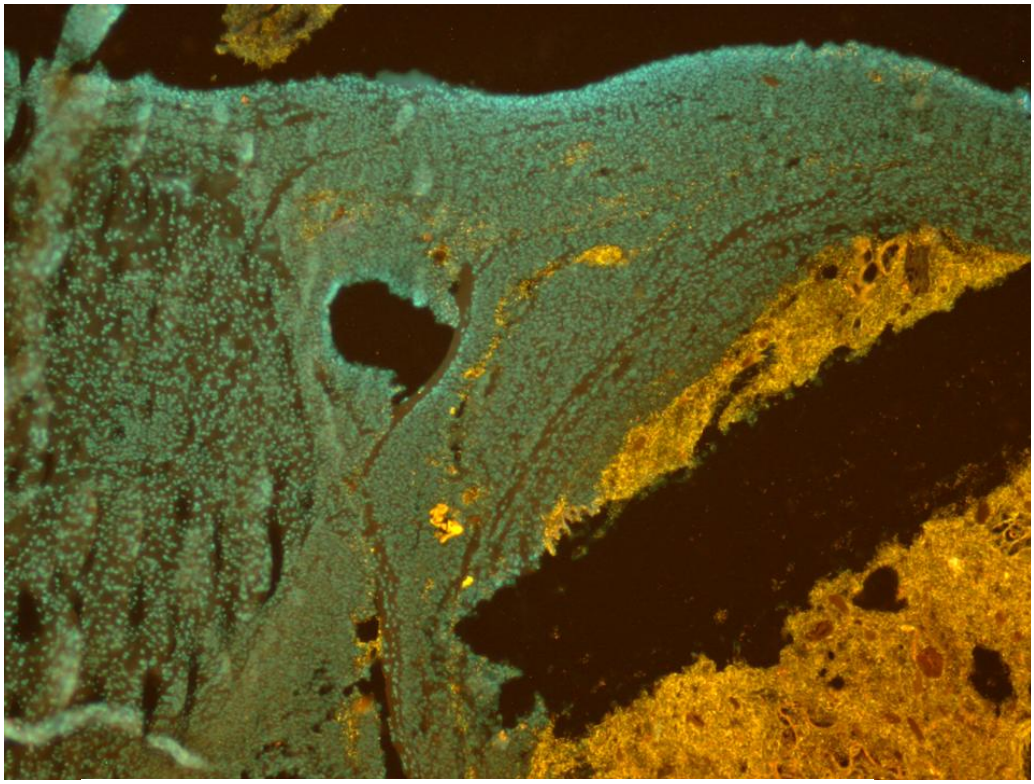

Figure 50. Fecal cylinder of a patient with ulcerative colitis. Massive infiltration of the "germinal" zone by leukocytes (DAPI stain). The fluorescence of *Faecalibacterium prausnitzii* and DAPI are overlayed. The concentrations of *Faecalibacterium prausnitzii* are not markedly reduced in ulcerative colitis.

The analysis of occasional bacterial groups within the fecal cylinders of patients with IBD demonstrated astonishing differences in occurrence and concentrations of *Enterobacteriaceae*, *Bifidobacteria*, *Atopobium*, *Eubacterium cylindroides*, *E. hallii* bacterial groups between Crohn's disease and ulcerative colitis [13]. This indicates that these diseases are distinctly different entities and not just different expressions of the same inflammatory process. However, because of inconsistent occurrence of occasional bacterial groups in single patients, the IBD specific changes of these bacterial groups can not be used for diagnosis in each case.

CONCLUSIONS

The surface of the intestinal tract in healthy humans is free of bacteria in all bowel segments. Adherence of bacteria to epithelial cells is therefore a sign of infection. In contrast to the mucosa, the intestinal lumen is never sterile. The reason for that is the polymicrobial nature of intestinal microbiota. In gut

segments like stomach or small intestine where bacteria are actively suppressed, the microbiota are accidental in occurrence, composition, and concentrations. The situation is different in the colon. The colon is a biofermenter that reduces nondigested remnants with bacterial help. Here, the bacterial growth is facilitated and the suppression is suspended. Bacterial concentrations and diversity in the colon reach astronomic numbers. Some of these bacteria are indispensable for the function of the colonic biofermenter, others occur occasionally. Many of the indigenous bacteria are potential pathogens: *Bacteroides, Enterobacteriaceae, Enterococci,* and *Clostridium histolyticum.* The control of pathogens within the colonic biofermenter is achieved by an impenetrable mucus layer. Although the mucus layer is typical for all mucosal surfaces, the intense resorption of water through the colonic columnar epithelium thickens the colonic mucus to an especially high viscosity, which immobilizes all bacterial movement. As long as the separation is complete, the number and diversity of the colonic bacteria is unimportant. Before bacteria can adhere and invade the mucosa, they must first traverse the mucus. When pathogens penetrate the mucus and adhere to the epithelial cells, inflammation clears the mucosa from the bacterial contact and the mucus from the bacteria, thus re-establishing the status quo.

The situation is different in the case of prolonged compromise of the mucus barrier. The inflammation cannot clear the mucosa from pathogens that inhabit the colonic lumen and continuously migrate towards the mucosa. The inflammation becomes chronic. Inflammatory bowel disease is a polymicrobial infection that is characterized by a sustained broken mucus barrier, subsequent bacterial migration towards the mucosa and proliferation of a complex bacterial biofilm on the epithelial surface with resulting invasive and cytopathologic effects. The reasons for malfunction of the mucus barrier could be increased urbanization that leads to increased incidence of silent infections with adherent bacteria that compromise the mucus barrier, excessive use of detergents and emulsifiers that change the viscosity of the mucus and many other reasons. As long as the mucus barrier function is impaired, the inflammatory process cannot successfully clear the bacteria from the mucosal surface and the inflammation is itself detrimental. Then immunosuppressive therapy remains the main therapeutic option. Other therapeutic principals including regulation of the mucus secretion and viscosity, suppression of bacterial biofilms, eradication of occasional pathogens, probiotics and immunostimulation are possible therapeutic interventions and should be increasingly considered and evaluated in the future.

As a consequence of the inflammatory response, the composition and structure of fecal microbiota is changed. Based on the biostructure of fecal cylinders, active Crohn’s disease and ulcerative colitis can be distinguished from each other and other gastrointestinal diseases. The specific and noninvasive monitoring of disease activity will enable us to intensify the search for alternative therapeutic strategies aimed at cure of the disease rather than symptom control.

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