Diplomarbeit

Establishment of fluorescence in situ hybridization for the microbial analysis of the vermiform appendix

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Graz, am 2.Dezember 2020 *Valentin Trinkl eh*

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Contents

Abbreviations and Acronyms

CARD-FISH	catalyzed reporter deposition-FISH
CLASI-FISH	combinatorial labeling and spectral imaging-FISH
CLSM	confocal laser scanning microscope
Cy3	Carbocyanine 3
Cy5	Carbocyanine 5
DAPI	4′,6-diamidino-2-phenylindole
DOPE-FISH	double labeling of oligonucleotide probes-FISH
FISH	fluorescence *in situ* hybridization
GALT	gut-associated lymphoid tissue
GC	guanine-cytosine
GI	gastrointestinal
HRP	horseradish peroxidase
IBD	inflammatory bowel disease
ISH	*in situ* hybridization
MiL-FISH	multilabeled oligonucleotide-FISH
NGS	next generation sequencing
PAS	pediatric appendicitis score
PAS	periodic acid-Schiff
PE	polyethylene
PMMV	pepper mild mottle virus
RG	Rhodamine Green
RT-qPCR	real-time quantitative polymerase chain reaction
WGA	wheat germ agglutinin

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Zusammenfassung

Hintergrund: Die Pathogenese der akuten Appendizitis, einer entzündlichen Erkrankung des Wurmfortsatzes, ist nach wie vor nicht vollständig geklärt. Der aktuellen Forschungslage zur Folge, scheinen Fusobakterien eine entscheidende Rolle in der Krankheitsentstehung zu tragen. Fusobakterien sind bekannt dafür orale Infektionen zu verursachen. In endodontischen Infektionen konnten neben Fusobakterien auch vermehrt methanogene Archaeen nachgewiesen werden, was einer symbiotischen Beziehung der beiden Mikroorganismen geschuldet sein könnte. Das Ziel dieser Arbeit war es, ein FISH-Protokoll für Appendixschnitte zu etablieren, um in weiterer Folge die räumliche Verteilung und Zusammensetzung von Bakterien und Archaeen in entzündeten Appendices darzustellen. **Methodik:** Es wurden 60 Appendixproben von Kindern, welche aufgrund des Verdachts einer Appendizitis einer Appendektomie an der Universitätsklinik für Kinder- und Jugendchirurgie Graz unterzogen wurden, verwendet, um daran die Methodik zur Fixierung, Einbettung und rRNA-FISH zu testen. **Ergebnisse:** Zur Fixierung der Appendixproben wurde Carnoy'sche Lösung verwendet, welche im Vergleich mit einer Ethanolfixierung in Stuhlsuspensionen eine gleichwertige Erhaltung der Zellintegrität der Mikroorganismen zeigte. Carnoy`sche Lösung konservierte außerdem in Appendixproben Appendixzellen und die Mukusschicht. Appendixproben wurden in Technovit 8100® eingebettet, da es vor Färbungen nicht aus den Schnitten entfernt werden muss. Zur Gewährleistung der notwendigen anaeroben Umgebung wurden die Blöcke während der Aushärtung in Vakuumkammern gelagert. Ein Vergleich mehrerer Objektträger und Trockungsmethoden zeigte die beste Adhärenz der Schnitte, nach dreistündigem Trocknen bei 40°C auf SuperFrost™ Objektträgern. In Versuchen mit Zellsuspensionen war es durch die Permeabilisierung mit Lysozym und Proteinase K und anschließender rRNA-FISH von Mikroorganismen möglich, die mikrobiellen Zellen unter einem konfokalen Laser-Scanning-Mikroskop zu betrachten. Die Übertragung dieser Arbeitsprozesse auf die Appendixschnitte, war trotz mehreren Adaptionsversuchen, nicht erfolgreich und es konnten mittels rRNA-FISH keine Mikroorganismen in den Appendixproben dargestellt werden. **Schlussfolgerung:** Da die Darstellung von Mikroorganismen in den Appendixschnitten durch FISH mit Hilfe der untersuchten Fixierungs,- Einbettungs- und Hybridisierungsmethoden

nicht erfolgreich war sollten weitere Versuche zur Optimierung des Verfahrens unternommen werden.

Abstract

Background: The pathogenesis of acute appendicitis, an inflammatory disease of the appendix vermiformis, is still not fully understood. The current state of research suggests that *Fusobacteria* play a crucial role in the pathological process. *Fusobacteria* are known to cause oral infections. In endodontic infections methanogenic archaea were detected alongside *Fusobacteria*, potentially due to a symbiotic relationship between them. It was the aim of this study to establish a Fluorescence *in situ* hybridization (FISH) protocol on appendix sections and in further consequence, to visualize the spatial organization and composition of bacteria and archaea in the diseased appendix. **Methods:** 60 appendix samples were gathered from children undergoing appendectomy, due to the clinical assumption of appendicitis, at the pediatric surgery department of the LKH-Universitätsklinikum Graz, to test methods of fixation, embedding, cutting and performing of rRNA-based fluorescence *in situ* hybridization. **Results:** The appendix samples were fixed in Carnoy solution, which, compared to ethanol-based fixation on stool samples, showed an equally good preservation of the microbial cellular integrity. Furthermore Carnoy solution preserved the appindeceal mucus layer and tissue in the appendix samples. Samples were embedded in Technovit 8100®, as it does not have to be eluted from the sections prior to staining. In order to ensure the necessary anoxic polymerization conditions, blocks were put in vacuum chambers to harden. A comparison of several slides and drying regimes showed, the best adherence of sections after drying for 3 hours at 40°C on SuperFrost™ slides. In tests on cell suspensions, our permeabilization of the microorganisms with lysozyme and Proteinase K and subsequent hybridization with their respective probe enabled the examination of microbial cells with a confocal laser scanning microscope. However, despite several attempts of optimization, we were finally unable to visualize microorganisms in our appendix samples via FISH. **Conclusion:** As the visualization of microorganisms in appendix sections via FISH was not successful with the tested methods of fixation, embedding and hybridization, further experiments to optimize the process should take place.

1 Introduction

1.1 Microbiota and Microbiome

1.1.1 General

The human microbiota is defined as the entirety of living microorganisms that are present on and in our body (1). Although for quite some time it was thought, that the microbiota outnumbers our human cells by a ratio of at least 10:1, we now know that these approximately 38 trillion microbial cells more or less match the number of human cells in our body (2).

Defining the term microbiome proves to be a rather difficult task, as many different opinions about its definition exist. Sometimes the term microbiome gets mixed up with the term metagenome by wrongly defining microbiome as the collection of all genes from the members of the microbiota. A very recent concept of the microbiome, based on an over 30 year old definition by Whipps *et al*. (3), suggests that the microbiome refers to the microorganisms themselves, their microbial structural elements (nucleic acids, proteins, lipids, polysaccharides) and microbial metabolites, as well as molecules formed by the hosts. This definition also includes mobile genetic elements, such as viruses, which are not part of the microbiota (1).

Due to the development of improved biological research technologies the amount of research on the topic of the gut microbiome has shown a tremendous rise since the early 2000s (4). Scientists from various fields can now look into connections between the microbiome and illnesses such as cardiovascular diseases (5). One goal is to perhaps even find new therapeutic approaches for long known health issues by modifying our microbiota (6).

1.1.2 Function of the microbiota

Some of the proteins and enzymes produced by the organisms of our gut microbiota are beneficial if not crucial for our well-being. Not only do certain bacterial enzymes support our own mammalian enzymes by breaking down more complex carbohydrates like plant-derived, complex polysaccharides, they also synthesize vital vitamins for us (7).

Besides supplying our body with nutrients, the healthy gut microbiota also plays a role in our immune defense by inhibiting the colonization of our intestines by

pathogens. They succeed in doing so, by producing bacteriocins (8) and interrupting the adhesion of harmful microorganisms to the intestinal surface (9), to name a few mechanisms. Furthermore, the presence of the microbiota trains the human immune system (8,10) and stimulates growth and differentiation of the intestinal epithelium (8).

1.1.3 Dysbiosis

Not always do our microorganisms work in our favor. The occurrence of qualitative or quantitative changes in the configuration of the microbiota, can lead to a so called dysbiosis (11). Although the term dysbiosis is omnipresent in literature, its definition is still a controversial topic. Some describe it as a loss of diversity or bloom of potential pathogens, where others claim that dysbiosis is any compositional change of the microbiota in disease compared to healthy individuals (12).
There is growing evidence that this deviation of homeostasis could play a leading role in the pathogenesis of inflammatory bowel diseases and coeliac disease, where till now the underlying aetiology is unexplained (13). One disease that is confirmed to be caused by dysbiosis is the infective diarrhea due to an intestinal overpopulation with *Clostridium difficile.* Normally *C. difficile* is a harmless, always present bacterium whose proliferation is limited in a healthy microbiota. However, prior antibiotic treatment can induce dysbiosis that allows the overgrowth of *C. difficile* (14) leading to the most common nosocomial infection in the United States (15).

1.1.4 Composition of the microbiome

1.1.4.1 Bacteria

The domain of Bacteria represents quantitatively the vast majority of the gut microbome, with *Firmicutes* and *Bacteroides* as the most abundant bacterial phyla in our intestines. As one study showed: More than 95% of bacterial 16S rRNA sequences that scientists found in the gut microbiome were from these two phyla (16). Interestingly enough, it seems that they share an inverse association with each other, meaning that the more Firmicutes one individual has, the less Bacteroides are present and vice versa (17).

1.1.4.2 Archaea

Also existent in our microbiome are Archaea, forming an own phylogenetic domain apart from Bacteria and Eukaryotes (18). Archaea were the last microbes to gather the status of being an own domain. In the year 1990, thanks to Carl R. Woese, Archaea were put on an equal level alongside of the other two domains. Woese was not only the first scientist to discover Archaea in the year 1977 but also the first to formulate the hypothesis that Archaea should become their own domain, due to their distinctively different features from bacteria. He succeeded in doing so, by using the highly conserved 16S rRNA genes as a taxonomic marker (19).

Archaea are prokaryotes as Bacteria and have some features in common. The small subunit of their ribosomes contains the same type of rRNA and they both do not exhibit encapsulated nuclei. However, archaea not only share similarities with other prokaryotes, they do, at least on the molecular level, have characteristics in common with eukaryotes, too (20).

Although archaea share characteristics with either of the other domains, they also have features, like the unique structure of their cell wall, which clearly separates them from the other two domains of life (21).

In the beginning Archaea were considered to be exclusively extremophiles, meaning they proliferate under extreme circumstances like the halophilic archaea which have a preference for hypersaline surroundings (22). Nowadays, we know that this was a misconception and that there are indeed mesophilic archaea living with and around us (23).

Although this domain is only known for a relatively short time, 20 species of archaea were already identified in the gastrointestinal tract. The predominant phyla of archaea in our guts are methanogens, a key-stone species that is able to produce methane from bacterial fermentation products (22).

Even though the impact of archaea to the human body is little understood, it is certain that our bacteria and in further consequence we, can profit from them. Bacteria and archaea are known to coexist in a syntrophic relationship (24). As methanogenic archaea have the ability to form methane out of bacterial fermentation byproducts, like carbon dioxide, hydrogen and methyl compounds, they support bacterial digestive processes (25).

Another intriguing fact about archaea is, that to this day not a single pathogenic species was found. Considering that 0.36% of all known bacterial species are pathogenic and assuming that this relative amount is approximately the same for archaea, we should have identified more than ten different archaeal pathogens by now. One possible explanation for the lack of bona fide pathogenic archaea was proposed by Gill & Brinkman (26) and revolves around the idea that the development of pathogenesis is a multi-step procedure dependent on the acquisition of virulence factors. In Bacteria, the spread of virulence factors is mainly facilitated by mobile genetic elements that are transmitted via phages. There are known archaeal viruses, but so far none was detected carrying any genetic information comprising virulence factors (26).

Without any bona fide archaeal pathogens, their role in disease can only be based on supporting other microorganisms which are actual pathogens.

Methanogens were especially found in polymicrobial infections involving anaerobic bacteria, such as endodontic infections (27). In case of anaerobic pathogenic bacteria, methanogens can favor their proliferation by metabolizing accumulating hydrogen, thereby permitting the further growth of secondary fermenters (27,28). A similar relationship was observed in periodontal disease, were *Methanobrevibacter* were detected, along with other bacteria, in the subgingival plaque, which provides an oxygen-deprived environment (28).

1.1.4.3 Bacteriophages and viruses

Whenever in an environment bacteria or archaea are present it is likely, that viruses appear with them. Reason being, that there are many viruses which have bacterial, archaeal or eukaryotic cells as host. Viruses, which infect bacteria in order to replicate, are called bacteriophages. One study pointed out that the majority of this gut-associated phages belongs to the family of *Siphophage*. The same research group was also able to detect about 1200 different viral genotypes, making it the most divergent group of microbes in our intestines (29). Although probably not all of the identified genotypes are human-related, like the overrepresented pepper mild mottle virus (PMMV), which is actually a plant parasite. Interestingly enough, human feces containing PMMV were infectious to pepper plants, yielding the thought-provoking concept of humans being a vector for plant diseases (30).

1.1.4.4 Eukaryotes

The human gastrointestinal tract also harbors eukaryotic microorganisms, though they are of lot less quantity than the bacteria. Representatives of this domain in our gut are fungi like *Candida sp.* or facultative pathogen protozoa such as *Blastocystis sp*. (31).

1.1.5 Core microbiome

Exactly defining the human gut microbiota is an impossible task, since differences in composition among individuals are too substantial. This led to the idea of establishing a core gut microbiota, which is shared by the majority of humans. Nowadays, the consensus is, that there is no such thing as a core microbiota but rather a core microbiome. Meaning, there is a basis of microbial genes instead of microorganisms that most people have in common.
Summarized one might say we have more similarities among each other, in the function of our microbiome than composition of the microbiota (32).

1.1.6 Influences on the microbiota

But why is there such a variety in the gut microbiota of humans? Probably the most obvious influencing factor is diet. Different amounts of carbohydrates, proteins or fats in one's diet, can indeed cause alteration in the composition of the microbiota. For example, high fat diets are suspected to shift the healthy microbiota to a state of dysbiosis (33). Different culture-associated dietary habits may also be determining why we have geographically linked variations in the gut flora. The typical high-calorie Western diet is comprised of lots of animal protein, fat and simple sugars and only small portions of fiber. This diet results in a lower diversity of the gut microbiota compared to plant-based diets seen in native Africans. Although whether less variety results in a less healthy gut microbiota has yet to be subject of further investigation (34).
Besides nutrition, there are many more aspects affecting the microbes in our guts. For instance, how a baby is delivered at birth. Infants born by cesarean section never get in contact with the vaginal microbiota of the mother, therefore developing a different gut flora in their first two years of life, with less diversity, compared to

babies delivered by natural childbirth (35). Another influence early in life is infant feeding. Since mother's milk is not sterile, infants being breastfed ingest bacteria that are among the first to build up the gut flora (36).
Furthermore, gastrointestinal infections, medication, genetics and other lifestyle associated circumstances, like stress and smoking are known to play an important role in shaping the gut microbiota, too (33,34).

1.1.7 Regional differences in the gastrointestinal tract microbiota

Last but not least it is important to note, that our microbes are not evenly dispersed through our intestinal tract. It makes sense, considering the very different habitat conditions between the several sections of our gastrointestinal tract. In the mouth, small and large intestines environmental conditions are relatively mild compared to the stomach with its high selective pressure caused by the gastric acid (16).

1.1.7.1 Appendix

Regarding the microbiota, another interesting segment of our guts is the appendix and its inflammation - the appendicitis. To this day, we neither fully understand the physiologic functions of the appendix nor the pathogenesis that lies behind the appendicitis. We cannot even say for certain whether the appendix is a redundant organ, which, in case of an appendicitis, can be removed without any negative consequences (37).
On the contrary, studies have shown that appendectomy results in a belated onset of inflammatory bowel disease (IBD) (38). In case of ulcerative colitis, a prior appendectomy also led to milder manifestation of the disease (39). Finding the connection between appendectomy and IBD is a highly discussed topic. Sahami *et al.* (40) proposed three different hypotheses, why the removal of the appendix could lead to a lower risk for IBD. According to them, an appendectomy results in less aberrant IgA-producing B lymphocytes, a reduced chance of dysbiosis and fewer numbers of natural killer cells. All factors beneficial for the prevention of IBD. They came to those conclusions as for one thing the appendix holds a lot of lymphoid tissue and for another, due to the special role of this organ, its microbiota could benefit the gut (40).

Due to its small luminal diameter and secluded position, the appendix could serve as a safe haven for beneficial microorganisms. Enteric infections induce diarrhea because our body aims to remove the pathogens in our gastrointestinal tract. However, with the occurring diarrheal clearance our body loses many benign and helpful members of our microbiota, too. But since the appendix is not affected by the fecal stream and bowel movement, its microbiota is retained and recolonization of the guts can take place (41).

With the exact cause of appendicitis still unknown, the microbiota is also suspected to play a leading role in the pathogenesis. Several studies on this topic were conducted and one difference found between healthy and inflamed appendix, was an increase in *Fusobacteria* (42–45). However it is required to note that not all cases of appendicitis can be related to the phylum of *Fusobacteria* (37).

1.1.8 Microbiota and Health

Not only diseases, possibly caused by our microbiota gather medical interest. Knowing that we can have an influence on the microbes living in our guts, we could potentially try shaping the microbiome to improve our health and well-being.

The use of probiotics and prebiotics are two ways to intentionally affect our microbiota. Probiotics on the one hand are alive microorganisms, whereas prebiotics on the other hand are only substrates for already present microbes. There are many ways in which these products interact not only with our inherent microbiota but also directly with our body (46). For example the application of specific probiotics resulted in an improved gut barrier by for one increasing the expression of proteins building up tight junctions and for another boosting the production of certain intestinal mucins (47,48). But the impact of probiotics seems to go even further than the gastrointestinal tract, as the administration of certain probiotic strains lead to the reduction of blood cholesterol and blood pressure (49,50). This could open up new ways in the treatment of patients with metabolic syndrome and lower the risk for cardiovascular disease (51).

1.2 Methods to analyze the microbiota

To truly understand our microbiota and how it interacts with our body, we need methods to examine its components. Analyzing microorganisms on a genetic level

can be done with tests such as fluorescence *in situ* hybridization (FISH), real-time quantitative polymerase chain reaction (RT-qPCR) or next generation sequencing (NGS). Although these techniques all more or less deal with the study of nucleic acids, they each have their own purpose of application. *In situ* hybridization (ISH) on the one hand yields primarily qualitative results while struggling with a relatively low throughput. For the examination of nucleic acids of a larger count of genes, processes like RT-qPCR or NGS will provide more quantitative data due to their higher throughput (52).

1.2.1 FISH

Fluorescence *in situ* hybridization is a cytogenetic process to visualize specific parts of nucleic acids. As the name suggests, the great advantage of FISH is, that nucleic acid targets do not have to be isolated from their source (single cells or tissues) prior to performing FISH. This results in the ability to provide information about the localization of target nucleic acid sequences in the tissue or even the cell itself.

1.2.2 History of FISH

The first *in situ* hybridization was performed by Gall and Pardue using rRNA probes labeled with radioactive tritium in 1969 (53). A few years later Manning *et al*. (54) succeeded in the first non-radioisotopic ISH. Their concept involved an indirect labeling-method that consisted of biotin-labeled rRNA probes and an avidin-based detection system (54). In the year 1980 Bauman *et al.* (55) achieved a major breakthrough by conducting an ISH using fluorochrome-labeled RNA (55). At that time immunofluorescence-based detection methods for proteins had already been around for more than 30 years. The approach of using fluorescence instead of radioactivity to label the probes made ISH more convenient for several reasons. The long exposure times required for image-acquisition when working with radioisotopes became redundant (56) and by labeling probes with fluorochromes of different emission wavelengths, multiple nucleic acid targets can be simultaneously detected in one hybridization step. Furthermore, fluorescent probes offer better spatial resolution and are safer to handle than radioactive probes (57).

1.2.3 Direct vs. indirect labeling

The principle of *in situ* hybridization is based on sequence-specific nucleic acid probes, which hybridize with their complementary nucleic acid strands. The target of a probe can be a sequence of DNA or RNA. The different processes of probe detection can be divided in two groups:
One of them works by directly labelling the nucleotides of the probes with fluorochromes or radioisotopes; the other one is an indirect labeling method where an extra step is needed to visualize the probes via an immunological or enzymatic detection system (58).
Indirect labeling is the more elaborate method, which has the advantage of creating stronger fluorescence signals, while requiring additional steps to perform the necessary immunological or enzymatic reaction. Another disadvantage occurs when the fluorescent antibodies or avidin molecules bind unspecific to off-target structures instead of the hapten or biotin-labeled probes, therefore increasing the background signal and lowering the signal-to-noise ratio. Furthermore, the practice of multicolor FISH to visualize several nucleic acid targets at once is limited. In addition to the need of fluorochromes with varying excitation spectrums, indirect labelling also requires different binding-pairs for every single target region to precisely identify each one of them. Nevertheless, because of its benefits in signal strength, indirect labeling may be the method of choice to create significant staining when dealing with small genomic targets (59).

1.2.4 Designing of probes

Oligonucleotide probes for FISH of rRNA are typically between 15 and 25 nucleotides long. Designing probes to correctly target certain sequences is a very crucial process in order to achieve high specificity. Unfortunately, there is a lot more to consider when designing probes than just synthesizing complementary RNA strands to known 16S rRNA sequences (60).
Higher-order structure of the ribosomes, attributable to interactions among rRNA-molecules themselves and interactions between rRNA and ribosomal proteins, can decrease the accessibility of the nucleic acid targets for the oligonucleotide probes (61).

Since the guanine-cytosine content (GC-content) of a probe affects its melting behavior, it should range between 50% and 70% to avoid high amounts of unspecific binding (60). If that is not possible, parameters such as hybridization temperature need to be adjusted to guarantee necessary stringency conditions (62).
Self-complementary regions consisting of more than three nucleotides should be avoided when designing new probes as they can reduce the affinity of a probe for its target sequence. Furthermore, probes should at least have one mismatch to non-target sequences, preferentially in the middle of the oligonucleotide to minimize the stability of a possible probe to non-target binding, in order to ensure high probe specificity (63).
Nowadays, it is rarely necessary to come up with new oligonucleotide sequences for probe design, as there are so many already known sequences that are saved on online probe databases, for everyone to access. For example, the established database for microbial-targeted probes probeBase displays appropriate probes after feeding a search mask the name of the target microorganism or taxon of a higher rank (64). Other online services such as the TestProbe-Tool of the SILVA database project can be further used to test probe coverage of microbial-targeted probes *in silico* (65).
For some probes their complementary counterparts are available, which can be used as negative controls. For example, the sequence of the non-sense probe nonEUB is complementary to the actual probe EUB338 and should therefore not bind to any rRNA at all (66).

1.2.5 Different techniques of FISH

In order to overcome many of the initially described limitations, a lot of methodological progress has already happened.
Direct and indirect labeling techniques evolved and now comprise a wider spectrum of different FISH approaches.
An elaborate and fairly common used method on microorganisms is catalyzed reporter deposition-FISH (CARD-FISH), where the oligonucleotide probes are labeled with horseradish peroxidase (HRP). After adding fluorochrome-labeled tyramide, each HRP-labeled probe induces the covalently deposition of several tyramide molecules to the nucleic acid target, causing a signal stronger than that of

single-fluorochrome labeled oligonucleotide probes (67). One problem when working with CARD-FISH lies in the extensive permeabilization necessary for the large HRP-molecule to penetrate the cell. This can be a tedious process, as overdoing permeabilization can result in lysis of the cells, to a point that a total loss of signal is the consequence (68,69).

Besides CARD-FISH and other indirect labeling methods, there are modifications for direct labeling methods that increase the signal yield, too.

Double labeling of oligonucleotide probes-FISH (DOPE-FISH) is an advanced direct labeling method, which delivers stronger signals by double labeling oligonucleotide probes. By placing fluorochromes at the 5'- and 3'-end of the probes, yields are more than doubled in signal intensity (70).

Although Schimak *et al.* (71) labeled probes with four fluorochromes in their multilabeled oligonucleotide-FISH (MiL-FISH), this effect has its limits, as at some point, signals will not get stronger anymore, by attaching more and more fluorochromes to the probe. If a certain minimal distance between two molecules of the same fluorochrome is not maintained, photochemical quenching is bound to happen (71).

Regarding the problem that there is only a limited amount of fluorochromes with distinctly different excitation and emission wavelengths available for labelling, one research group came up with the new method termed combinatorial labeling and spectral imaging-FISH (CLASI-FISH). By combining different fluorochromes for the labeling of each taxa, they created many distinguishable color combinations that allowed the detection of more different microbial taxa in one single CLASI-FISH experiment (72).

1.2.6 Clinical application of FISH

In the clinical setting, FISH proved itself very useful for genetic counseling or prenatal diagnostics, where the main concern is the identification of genetic disorders before further consequences occur. Whether parents of a disabled child want to know more about the causality of their child's condition before having another child, or relatives about the potential risk of having a congenital disorder, FISH is a technique that can be used for the detection of those chromosomal aberrations.

FISH also can be applied in oncology, as some tumor cells have a higher expression of specific genes. With the right complementary probe the increased gene amplification can be quantified and used as a prognostic biomarker in certain types of cancer (58).

Before FISH was discovered, the commonly used method in the field of clinical cytogenetics was metaphase chromosome banding. This process allowed for distinct identification of each chromosome and its haploid counterpart as well as detecting structural aberrations like deletions, duplications, translocations and inversions. Although this was a great advance from solely being able to observe numerical aberrations, the relatively low resolution of the banding pattern left many subtle aberrations unnoticed. The development of FISH enabled the detection of these previously undiscovered, submicroscopic deletions or complex aberrations and filled the gap between the diseases clinical manifestation and its genotype (73). However, not only the results of FISH were a lot more appealing, also the work process in the laboratories got simplified. Being able to apply FISH on interphase chromosomes, allowed cytogeneticists to use cells at any stage of the cell cycle, making the former necessary cultivation for several days redundant (74).

1.2.7 FISH for phylogenetic staining of microorganisms

Although ISH started out on eukaryotic cells scientists adopted the technique for prokaryotes. The concept of fast and exact identification of microbes without culturing them beforehand seemed intriguing for microbiologists. Culture-depending techniques are time-consuming and yet do not display the accurate composition and spatial arrangement of bacterial communities in their natural habitat. Considering that some microbial organisms cannot even be grown in culture, diagnostic microbiology was in dire need of another method (57).

Thanks to Carl R. Woese's discovery of the highly conserved 16S rRNA of prokaryotes, those specific rRNA sequences could be utilized to create phylogenetic group-specific oligonucleotide probes.

One of the first experiments in phylogenetic staining using ISH was conducted by Giovannoni *et al.* (75) who used radiolabeled probes to identify single microbial cells (75).

In the following year DeLong *et al.* (76) were the first group to publish the use of FISH for the identification of microbes. Although their approach worked out fine, a few potential problems when working with fluorescently labeled RNA already came up (76).

Briefly, there are two main issues that can occur with FISH: False positive results, due to autofluorescence or unspecific binding, or false negative results, caused by a number of reasons such as insufficient penetration of the cell walls or low intracellular rRNA content (57).

1.3 Spatial organization of the gut microbiota

As already mentioned above, there is a great interest in the exploration of the human microbiota. Especially the gut microbiota receives a lot of attention and is highly cited in the literature with over 6500 publications over the last 5 years on PubMed as of October 6, 2020.

Many research groups that deal with the gut microbiota use sequencing to analyze its composition (77–80). Sequencing as a method with high throughput is ideal for quantifying but also identifying the microbial composition that makes up the intestinal flora. However, conventional sequencing approaches rely on homogenization of the samples to increase the accessibility and dispersion of the genetic material. Therefore, information about the original spatial distribution of the microbes in the sample is lost and does not get conveyed through this method (81). This is quite unfortunate, as the acquisition of quantitative data alone is not sufficient to fully comprehend the fundamentals of our gut microbiota.

By taking the spatial organization of the intestinal flora into consideration we can describe characteristics in its distribution patterns. Several studies already confirmed that there is a spatial heterogeneity not only in the longitudinal but also in the radial axis of the gastrointestinal (GI) tract. Some concepts suggest that the mucosal microbiota is distinctly different from the luminal microbiota (78,82,83), where others propose the concept of an incompletely mixed bioreactor without strictly defined compartments (84).

Further it is important to notice that the spatial organization discloses information about interactions between microorganisms. We already know that certain

microorganisms rely on syntrophic relationships, i.e. each other's metabolic products that are further degraded to gain energy (85).

However, the interactions between microbes are not only confined to their metabolisms. Other studies showed that in biofilms, bacteria of various species have specific other bacterial binding-partners with whom they aggregate, to adhere to the biofilm (86,87).

Just like spatial distribution in the healthy gut microbiota reveals information about its physiological functions, it could help us understand the pathophysiology behind certain inflammatory diseases of the GI tract.

The mucosa in our guts acts as a natural barrier and protects the underlying epithelial lining from direct contact with the intestinal flora and thus allows for the unproblematic coexistence of microbes in our guts (88). Our mucosa in the colon and stomach consists of two layers of different mucus. The dense inner mucus layer is attached to the intestinal wall and works as the microbial barrier in the proper sense. In contrast, the unattached and loose outer layer serves as microbial habitat that harbors commensal microorganisms (89,90).

Several studies on the topic of inflammatory bowel disease suggest, that the interaction between the mucosa and its adherent microflora is an important element in the pathogenesis of Crohn's disease or ulcerative colitis (91–94).

A loss of immunological tolerance to the own microbiota is a feature that is known to co-occur with IBD (95). Defects or dysfunction of the mucus in IBD patients could allow bacteria to penetrate it and infiltrate deeper layers of the intestinal wall, triggering an immune response and inflammation (89).

1.4 Appendicitis

1.4.1 Epidemiology

Besides inflammatory bowel disease, the inflammation of the appendix is a fairly common intestinal inflammatory disease, with an incidence of 100 (per 100,000 persons per year) in Northern America and 151 in Western Europe (96). It is one of the main causes of acute abdomen and therefore, a common indication for emergency surgery (97).

1.4.2 Aetiology

Similar to IBD, the actual cause of appendicitis is still unknown. Crohn's disease and ulcerative colitis belong to the spectrum of immune-mediated diseases (98), whereas in appendicitis evidence like clustering among cases, suggest that it is primarily an infectious disease. However, this clustering occurred usually among persons of the same household, therefore, a possible involvement of genetic predisposition cannot be ruled out (99).

Furthermore, despite knowing about numerous specific viral, bacterial, fungal and even parasitic infectious agents that potentially induce appendicitis (100), one of the longest existing and most common advocated theories considers the obstruction of the appendiceal lumen as the crucial element in the pathogenesis of appendicitis (101–103). In this pathophysiological concept, occlusion of the appendiceal lumen leads to the retention of the continuously secreted mucosal fluids, therefore, increasing the intraluminal pressure to a point where blood vessels in the appendiceal wall get compressed and the blood flow comes to a halt. Eventually the ensuing ischemia leads to necrosis, allowing bacteria to easily invade the already weakened tissue (104).

Although this theory sounds well thought out, one research group found that in the majority of patients with appendicitis, the intraluminal pressure of the appendix was not increased, hence ruling out the presence of obstruction (105,106).

In one study, faecoliths, which are supposed to be the most frequent cause of appendiceal obstruction, could only be detected in less than a fifth of the inflamed appendices (107). On top of that, patients with incidentally on CT-scans detected feacoliths, had no higher risk of contracting acute appendicitis (108).

The same applies to lymphoid hyperplasia, which appeared significantly more often in appendices without any pathology, than in inflamed ones (109).

Taking these findings into consideration, the theory of obstruction as the primary cause of appendicitis became more and more unlikely.

1.4.3 The role of the microbiota in acute appendicitis

As obstruction was ruled out as primary cause of appendicitis, the appendiceal microbiota and its part in the pathogenesis of appendicitis received more and more

attention in the scientific world. It was not until the use of culture-independent methods, that the polymicrobial processes in appendicitis were analyzed in detail (37). Several studies using sequencing of 16S rRNA gene regions evaluated the microbial composition of diseased and normal appendices and were able to point out differences in the composition of the microbiota of healthy individuals and appendicitis patients. One consistent finding was the increased amount of *Fusobacteria* in diseased samples (37,42,44,45,110). In some of the studies, this observation came along with a decrease of the genus *Bacteroides* (42,44). Whereas Salö *et al.* (37) showed that the quantity of *Bacteroides* depends on the grade of appendicitis and that in gangrenous appendicitis they can be found in high abundance (37).

1.4.4 Fusobacteria

The higher numbers of Fusobacteria in appendicitis samples are a quite interesting discovery as they are typically considered to be part of the physiological oral microbiota. *Fusobacteria* were also detected in smaller numbers in histologically normal appendix samples, but not in healthy fecal samples, emphasizing the uniqueness of this finding even more (44). *Fusobacteria are* also occurring in the oral cavity of healthy individuals, where they are well known to contribute to periodontal disease (111). Their actual infectious potential becomes obvious in the rare but very serious Lemierre syndrome, which is characterized by a *Fusobacterium i*nfection of the oropharynx (112).

Some virulence factors of these anaerobic, non-spore forming Gram-negative bacilli have already been described. For example, a strain of *Fusobacterium nucleatum* was proven to use an adhesin called FadA, which binds directly to E-Cadherin of epithelial cells, allowing the bacteria to adhere to human cells (113).

Further *Fusobacteria* are able to produce endotoxin, leukotoxin and haemolysin. In theory these virulence factors should allow the bacteria to overcome the epithelial barrier and become invasive (114).

Although numerous appearances of *Fusobacteria* in appendicitis can be proven via 16S rRNA gene sequencing, it allows only for associations and not for a definitive causal relationship between bacteria and disease to be made (45).

1.4.5 FISH and appendicitis

In order to complement the information about appendicitis, gathered through sequencing, FISH could be applied to determine a lead organism in the pathogenesis.

By applying FISH on sections of appendicitis samples, one could observe the potential underlying invasion of the mucosa by microorganisms, which causes the appendix to become inflamed.

Although microorganisms that become invasive by overcoming the mucosal barrier would be the assumed pathomechanism for appendicitis, there are dissenting voices, claiming that bacterial translocation in the appendix occurs in healthy individuals and is necessary for the development of the gut-associated lymphoid tissue (GALT) and its further upkeeping in adulthood (115).

Swidsinski *et al.* (116) were first in describing FISH on appendicitis samples. Their multicolor 16S rRNA-probes method worked great, as they were able to point out distinctive lesions of single epithelial cells in the appendiceal mucosa that were full of various bacteria. In 62% of the lesions, *Fusobacteria* could be detected by hybridizing with a probe, specific for the genus of *Fusobacteria*. For further breakdown of the genus, two probes specific for *Fusobacterium nucleatum* and *Fusobacterium necrophorum* respectively, were also applied. 79% of the detected *Fusobacteria* additionally hybridized with the probe for *Fusobacterium nucleatum* and 12% with the probe for *Fusobacterium necrophorum*. Despite the high bacterial diversity found in patients, in the appendix of 18 healthy individuals, *Fusobacteria* were only detected in the lumen, but never invading the appendiceal tissue. Furthermore *Fusobacteria* were only identified in 0.5% of cecal biopsies, 2% of faecal cylinders and in no rectal swab, suggesting that the increase of *Fusobacteria* in appendicitis is limited to the appendix itself.

By application of Koch's postulates on the aetiology of infectious diseases Swidsinski *et al*. (116) came, in consideration of their results, to the conclusion that most cases of appendicitis are indeed caused by local infection with *Fusobacteria*. Since they did not observe *Fusobacteria* in 38% of patients with histologically diagnosed appendicitis, they claimed that the absence of *Fusobacteria* does not interfere with their theory, but rather suggests that further microorganisms may also be capable of causing appendicitis (116).

Although the work of Swidsinski *et al.* had novelty value by being first in describing the epithelial lesions in appendicitis from a microbiological standpoint, there are still issues in the pathogenesis of appendicitis that remain unresolved.
What are the lead organisms in the infection of the appendix if no *Fusobacteria* are present? Why do *Fusobacteria* show higher proliferation in certain appendices and what triggers their invasion of the appendiceal tissue?

1.5 Study goal

1.5.1 Main goal

The main goal of our study was to establish a reliable protocol for the application of FISH on samples of appendix in our laboratory. In addition to the actual hybridization, this also included the development of regimes for fixation, embedding and sectioning of the tissue samples.

1.5.2 Secondary goal

After the establishment of aforementioned protocol, our second objective was to continue the work of Swidskinski *et al*.(116) in analyzing the microbial characteristics of acute appendicitis and potentially addressing some of the remaining unresolved issues in its pathogenesis.
Hence, a special emphasis of our research group lies on the exploration of archaea, as we also wanted to investigate their role in the microbiota of the inflamed appendix. Although, as mentioned before, we do not know of any *bona fide* pathogens among the domain of archaea (117), certain infectious diseases from the field of dental medicine show associations with methanogenic archaea.
Considering that *Fusobacteria* were found alongside *Methanobrevibacter oralis* in endodontic infections (118), a similar connection may apply in acute appendicitis and can be identified via FISH.

2 Materials and methods

2.1 Gathering of the samples

For our first objective we obtained 60 appendices to test fixation, embedding, cutting and performing FISH. In close collaboration with Assoz. Prof.Priv. Doz. Dr. med. univ. Georg Singer and Kevin Brunnader of the pediatric surgery department of the LKH-Universitätsklinikum Graz, 60 appendices were gathered in the stretch of 15 weeks. Prior to the appendectomy, informed consent for the further use of the removed appendix samples was obtained from a parent/legal guardian of all participants.

Out of the 60 samples, four of the appendices could not be used, because of a shortage of fixative solution over one weekend.

Every collected appendix was taken out under the clinical assumption, that an inflammation was present. Whether an appendectomy was performed or not, was decided by a pediatric surgeon.

To objectify the likelihood of an appendicitis based on clinical examination and laboratory parameters, the pediatric appendicitis score (PAS) and Alvarado score were used.

Both scores include important signs and symptoms that are indicative of appendicitis, such as: abdominal pain, tenderness in the right lower quadrant, nausea, vomiting, loss of appetite and fever. Regarding the laboratory parameters, an increased amount of white blood cells and a higher percentage of neutrophils suggest an appendicitis (119). Considering the work of Maghrebi *et al.*, which determined a threshold value for the Alvarado score, a score of 8 or above gives you a reasonable certainty for an appendicitis (120).

27 of the 60 removed appendices had an Alvarado score of 8 or higher.

Further recorded patient information were date of birth, gender and whether they received antibiotics prior to surgery or not. The average age of the included patients was 11 years, with the youngest being 3 years and the oldest 17 years old. 18 patients were female and 20 received antibiotics before the surgery.

2.2 Fixation

For the fixation of the samples Carnoy solution was used, following publications by Hasegawa *et al.* and Welch *et al.* (84,121). The solution was made fresh every seven days using the following formula.

Tab. 1 Composition of Carnoy solution

Compounds	**% (v/v)**
Ethyl alcohol	55
Chloroform	30
Glacial acetic acid	10
Isopropyl alcohol	2.5
Methyl alcohol	2.5

As individual vessels for the fixation of the appendices, we chose Eppendorf® tubes 5 ml with screw caps. The tubes were filled up with 4 ml of freshly prepared Carnoy solution and stored at the pediatric surgery department in a refrigerator at 4°C.
After the surgical removal of the appendices, an approximately 1 cm long piece of the proximal end of the appendices was cut off by the surgeon and put in the Eppendorf® tubes. For fixation, the tubes were then again stored at 4°C for at least 2 hours. When the fixation was done, every specimen was put in a series of three Eppendorf® tubes filled with precooled Ethanol (100%) for 3 minutes each. While performing this washing steps in ethanol, the tubes were kept cool in Styrofoam boxes filled with crushed ice.
Afterwards the specimens were stored in Eppendorf® tubes filled with ethanol (100%) at -20°C until further usage.

2.3 Embedment

Prior to embedding, approximately 3 mm thick sections were cut off the fixed samples using a scalpel. The remnants of the appendix samples were put back into the ethanol filled tubes and stored at -20°C again.
The fresh cut sections were then placed into Eppendorf® tubes filled with ice cold acetone for a total of 1 hour, with a switch of the acetone after about 15 minutes.

The samples were embedded using the methacrylate based system, Technovit® 8100 (Kulzer).

In order to embed the samples, they were first infiltrated in an infiltration solution. The infiltration solution was prepared according to the user instruction by combining 100 ml of Technovit® 8100 basic solution with one bag of hardener 1. Freshly prepared infiltration solution was stored for up to 4 weeks at 4°C.

At first, specimens were placed in Eppendorf® tubes with 2 ml of infiltration solution for about an hour at 4°C. After that initial infiltration step, the infiltration solution was exchanged with fresh infiltration solution and the samples were stored at 4°C to infiltrate overnight.

After infiltration, the samples were ready for polymerization. The specimens were put in 1.5 ml Eppendorf® tubes with 1 ml of fresh infiltration solution. In order to start the polymerization, 45 µl of hardener 2 were added to the infiltration solution. All solutions were precooled at 4°C. As recommended by the manufacturer, everything was mixed thoroughly by repeatedly inverting the Eppendorf® tubes for about 3 minutes until the solution turned slightly amber. Then the contents of the tubes were poured into a 1 cm x 1 cm x 1 cm ice-cube mold made out of silicone. In order to create the required anoxic polymerization conditions, a gas exchange station and a vacuum pump in our lab were used. The filled molds were placed in a precooled small vacuum chamber made out of stainless steel with a rubber plug. With a vacuum pump, air was first removed until the pressure in the chamber reached almost -1 bar. Then, pure nitrogen was introduced into the chamber until the pressure in the chamber hit 1 bar. This procedure was repeated once before putting the vacuum chamber pressurized at 1 bar in the refrigerator at 4°C to polymerize overnight. When the polymerization was completed, the hardened blocks were put into plastic bags and stored at 4°C until further use.

2.4 Sectioning

The blocks were trimmed with a bench band saw to reduce the cutting surface as much as possible and to create more surface area for the chuck of the microtome to firmly hold onto. Furthermore, the end of the sample was trimmed by 1 millimeter at the cutting side.

Sections were cut using the semi-automated rotary microtome Thermo Scientific HM™ 340E and a wedge knife made by Leica.
Before sectioning, X-tra® slides (Leica) were prepared by pipetting a large drop of distilled water on them. Sections were then cut at 5 µm thickness and placed onto the water drop using a fine paint brush. After the sections had fully stretched, the excess water on the slides was sucked up with a pipette.
Afterwards the slides were left to dry at 40°C for at least 3 hours in the slide incubator (Boekel Slide Moat™).
Slides that were not immediately used for FISH, were stored in a slide box at 4°C.

2.5 Permeabilization

Before hybridization, the sections were permeabilized using Proteinase K (VWR) and lysozyme (Roth). The permeabilization steps took 15 minutes each and were conducted at 37°C in the slide incubator.
While the slides were preheated in the incubator, the permeabilization solutions were prepared freshly. Proteinase K stock solution was diluted in a 20 mM Tris-HCL and 2 mM CaCl2 buffer [pH 8.0] to obtain a Proteinase K solution with a concentration of 10 µg/ml. Lysozyme powder was suspended in a 0.05 M EDTA and 0.1 M Tris-HCl Buffer to achieve a lysozyme solution at a concentration of 10 mg/ml. First, every section was permeabilized with 30 µl of Proteinase K solution. After each permeabilization step the slides were thoroughly rinsed with distilled water and allowed to dry for a few minutes on the slide incubator.
The second permeabilization step was done with 30 µl of lysozyme solution.

2.6 Hybridization

After the permeabilization the slides were subjected to hybridization. First, the temperature of the slide incubator was set to 46°C and distilled water was added into its wells, to create a humid environment for the hybridization to take place. Next, the hybridization buffer and the different probe solutions were prepared and preheated at 46°C in a thermoshaker. The hybridization buffer contained 0.9 M NaCl, 0.02 M Tris-Hcl (pH 8), 0.04% SDS and 20% deionized formamide (OmniPur®) and was based on the composition suggested by Swidsinski and

Loening-Baucke (122). The concentration of NaCl was adjusted to the formamide concentration of the hybridization buffer (60).

Probes targeting bacteria were EUB338, EUB338II and EUB338III. As archaeal probes, ARCH915 and MB311 were used. Also one nonsense probe NONEUB was employed as negative control. All used probes (see Table 2) were synthesized by Eurofins Genomics.

EUB338, NONEUB and ARCH915 were DOPE-probes and synthesized with a fluorophore on the 5'- and 3'-end. The probes were labeled with either Carbocyanine 3 (Cy3), Carbocyanine 5 (Cy5) or Rhodamine Green (RG).

According to probeBase the employed probes were taxon-specific at similar hybridization stringencies. Hence the formamide concentration in the hybridization buffer was chosen to be 20% for all probes.

Tab. 2 *Oligonucleotide probes*

Probe	Fluorophore	Sequence (5`->3`)	Designed target	%FA	Reference
EUB338 I	Cy3	GCT GCC TCC CGT AGG AGT	Most *Bacteria*	0-50	(64)
EUB338 II	Cy3	GCA GCC ACC CGT AGG TGT	Phylum *Planctomycetes*	0-50	(64)
EUB338 III	Cy3	GCT GCC ACC CGT AGG TGT	Phylum *Verrucomicrobia*	0-50	(64)
EUB338 I	DOPE-Cy5	GCT GCC TCC CGT AGG AGT	Most *Bacteria*	0-50	(64)
NonEUB	DOPE-Cy5	ACT CCT ACG GGA GGC AGC	Control probe complementary to EUB338	0-50	(64)
ARCH 915	RG	GTGCTCCCCCGCCAATTCCT	Domain *Archaea*	20	(123)
MB311	Cy3	ACC TTG TCT CAG GTT CCA TCT CC	Order *Methanobacteriales*	30	(64)

Stock solutions of the probes were diluted with PCR-grade water to achieve a probe concentration of 20 µM. The final working concentration in the hybridization buffer was 2 µM. On each section 20 µl of hybridization buffer were pipetted and after 25 minutes 2 µl of working probe solution were added.

To prevent cross contamination during washing, we avoided treating samples on the same slide with different probes. The sections were left to hybridize for 6 hours on the slide incubator in the dark.In the meantime wash buffer was prepared fresh. Wash buffer contained 0.225 M NaCl, 0.02 M Tris-HCl (pH 8), 0.025 M EDTA and 0.01% SDS. 40 ml of wash buffer was filled in 50 ml Falcon™ tubes and placed in

a water bath at 48°C. Every two slides, one Falcon™ tube with wash buffer was set up.

When hybridization was done, the slides were briefly rinsed with wash buffer before putting two of them with their undersides together in one Falcon™ tube and washing them for 25 minutes. Control slides with the nonsense probe were always washed separately from the positive slides.

After the 25 minutes, the slides were pulled out with tweezers and rinsed thoroughly with ice-cold distilled water to stop the reaction. The slides were covered with aluminium foil and allowed to air dry. Once the slides had dried completely, about 50 µl of VectaShield® Antifade Mounting Media with 4′,6-diamidino-2-phenylindole (DAPI) were pipetted on each slide and covered with a coverslip. As DAPI stains all present DNA in the sample it was used as a counterstain for FISH. The coverslip was gently pressed down to get rid of remaining air bubbles and mounting media before sealing the perimeter of the coverslip with nail polish. After the nail polish had hardened, slides were stored at 4°C until further usage.

2.7 Microscopy

For the analysis of the slides the confocal laser scanning microscope (CLSM) Nikon A1R at the core facility of the Center for Medical Research Graz was used. Depending on which probes and their respective fluorophores were put on the sections, various wavelengths were used. The different excitation wavelengths of 405 nm, 488 nm, 561 nm and 640 nm were applied in sequence. Emission filters used were 525/50 and 595/50.

We only used the 60x plan apochromat objective.

When taking pictures, we worked with 1024x1024 pixel scan size, an average line count of 8 and a scan speed of 0.125.

The pinhole was set to 1AU (38.3 µm). Adaption of signal strength was done by adjusting the gain and power of the lasers. The microscope settings were kept within an experiment to ensure comparability of the results.

3 Results

Since the main goal of our study revolved around establishing a FISH method for sections of appendices, we established and evaluated every step of the procedure, in order to achieve satisfying results.

3.1 Fixation

3.1.1 Fixative solution

In order to test, whether Carnoy solution is capable of fixating the appendix tissue and the microbes within, we compared it with an ethanol-based fixation method that was already established in our lab. As proxy we used stool samples that were fixated with both methods for 33 hours (time was arbitrarily chosen) before the rest of our FISH protocol was applied.
When observing the slides under the microscope, both fixation methods seemed to preserve the cellular integrity of the microbes equally well as seen in Figure 1.

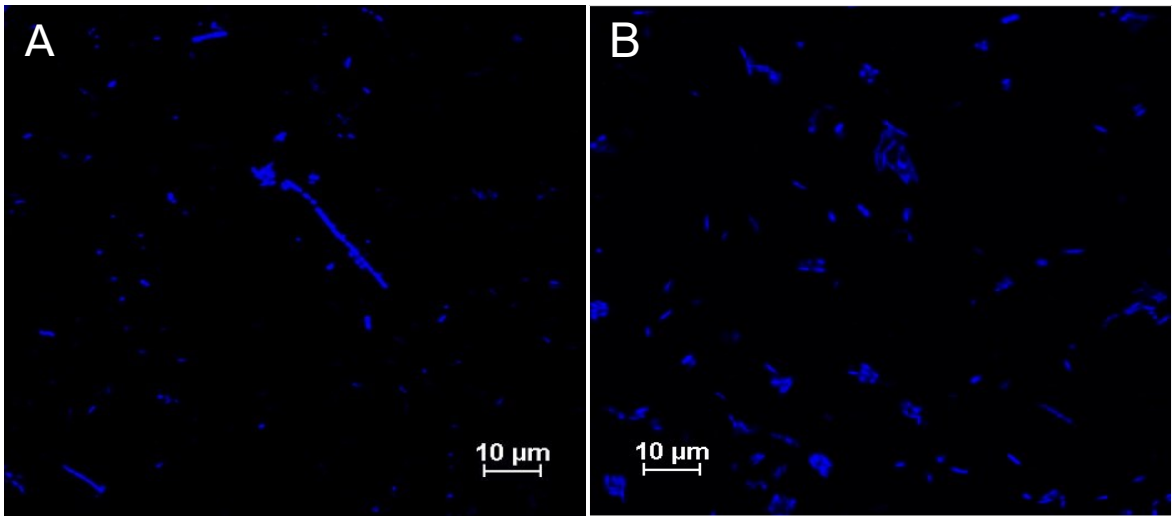

Fig. 1 DAPI-stained stool sample fixated in ethanol (A) and Carnoy solution (B). Different morphologies (rod-shaped, coccal) of the microbes are clearly recognizable in both images.

3.1.2 Fixation time

No information was available on how tissue samples need to be fixed in Carnoy solution. One reason might be, that the fixation time depends on the size or type of tissue and how long it takes for the solution to penetrate the specimen.

To find out if there are limitations to the fixation time, we tried various durations, the shortest being 2 hours and the longest being 120 hours.
Via microscopic evaluation, we found no distinguishable differences between the analyzed durations. The preservation of cells always seemed to work regardless of the fixation duration. However, as at this stage no specific probe signal was available, we were only able to assess the quality of fixation on the basis of human cells in the appendix sections.

3.2 Embedment

3.2.1 Anoxic polymerizing conditions

It is crucial to exclude oxygen during polymerization of Technovit® 8100. In our tests, oxygen occasionally interacted with the solution during polymerization, resulting in a gelatinous, very sticky and amber-colored block.
As the instructions of the manufacturer suggested, we tried to exclude oxygen by covering the filled molds with the provided polyethylene (PE) films.
However, the included PE films did not fit on our silicone molds and never sealed the top completely. Either they did not stay on the surface of the solution and sank to the bottom or the shrinking of the methacrylate during polymerization led to gaps on the surface that allowed air to get in between the films and the embedding material.
Our next try to create an anoxic polymerization environment was to use 10 ml glass vials with a rubber stopper and aluminum cap. After filling the vials with our polymerization solution, we sealed the vials and applied an anoxic atmosphere with 0.5 bar N_2. The methacrylate fully hardened and became colorless in the vials. The problem with this method was, that the hardened embedding media stuck firmly to the glass. Even after breaking the vial to retrieve the methacrylate disc, we were not able to remove every shard of glass without breaking the disc itself too.
As using glassware was inappropriate, we tried establishing an embedding method for silicone molds by using a conventional vacuum sealer to bag the filled molds. The idea was to remove most of the air and subsequently seal the bag airtight.
However, the silicone molds were not strong enough to withstand the compressing force of the vacuum bag and collapsed under the pressure. Hence a metallic frame was constructed in order to help the molds withstanding the compressing force.

Despite, the silicone molds not collapsing, due to the supporting metallic frame, and the seal being airtight, the methacrylate did not harden and it became apparent, that the vacuum sealer was not able to remove enough oxygen in the beginning for the polymerization to start.
Satisfying embedding results were only achieved using a small vacuum chamber as described above (see 2.3). With this method blocks became fully hardened, not sticky on their surfaces and only with a slight yellowish discoloration.
Several sequences of applying vacuum or gas when working with the vacuum chambers were tested. In our observations it seemed that the last step, whether the vacuum chambers were stored with low pressure or overpressure, had the greatest influence. Although blocks fully hardened under both methods, storing them with low pressure led to the formation of gas bubbles in the hardened block, resulting in an increased risk of chipping the block during cutting with the microtome. Thus, usage of 1 bar overpressure is advisable for this procedure.

3.2.2 Hardener 2 concentration

In order to further improve the polymerization of Technovit® 8100 we tried increasing the concentration of hardener 2 above the recommended 3.33% (v/v).
In our experiments raising the hardener 2 concentration to 10, 15, 20 and 25 percent did neither change the consistency of the block nor the microscopic image, even compared to the suggested concentration by the manufacturer.
On the contrary, less hardener 2 in the polymerization solution than recommended, hindered the start of any reaction and the solution remained unchanged. Due to its high viscosity, it occasionally occurred that too little hardener 2 was pipetted to the polymerization solution. Since an excess of hardener 2 did not seem to interfere with polymerization, we used a concentration of 4.5% (v/v) in further experiments, to prevent this complication.

3.2.3 Sample size

The user instruction of Technovit® 8100 states not to use tissue samples thicker than 1 mm. Our appendix samples were substantially bigger (up to 1 cm in length) which frequently resulted in unsatisfying polymerization results. Even when the

block hardened completely, the specimen itself remained rubbery and could not be cut with the microtome.
Extending the infiltration time to 12 hours did not compensate for the larger sample sizes and the biggest specimens still turned out too soft for cutting.
In order to overcome this issue, we eventually decided to cut our samples down with a scalpel to the recommended size of 1 mm thick discs. This led to great polymerization on the one hand but on the other hand, after analysis using a microscope, to tearing of the epithelium in all sections.
As a consequence, we reduced the thickness of our samples to approximately 3 mm, in order to keep the tissue integrity in the center of the sample intact.
During infiltration of the specimens overnight, the infiltration solution was replaced once. The blocks with the 3 mm thick specimens completely polymerized and after removing the ends of the embedded sample with a bench band saw, sections cut with the microtome showed no disruption of the epithelium.

3.2.4 De-embedment

Although the manufacturer claims, that the plastic of Technovit® 8100 cannot be eluted after embedding, we tested a de-embedding technique based on an article (124), which suggested that the removal of methacrylate with acetone improves signal quality of ISH.
Following the drying of the sections on the slides, sections were submerged for one hour in acetone. After one hour, the sections remained unaltered evaluated by the naked eye. Furthermore, when examining the sections under the microscope, no differences in signal quality of FISH compared to sections that were not treated with acetone, were observed. Therefore, treating methacrylate sections with acetone prior to FISH appears not to be recommendable.

3.3 Sectioning

3.3.1 Pre-cutting

In order to reduce the force needed to cut the block with the microtome, we compared two approaches of decreasing the cutting surface of the block with the bench band saw. In the first approach, the cutting surface was only trimmed down

slightly as the relatively large sections were easy to transfer with a brush or tweezers and stretched ideally. However, due to the larger size of the sections and the hybridization buffer always spreading over the whole surface area of the sections during FISH, the buffer and probes pointlessly covered areas of the section where no sample was embedded.
Another approach was to trim down the block almost to the perimeter of the specimen with only a margin of 1-2 mm remaining. Smaller sections were more difficult to handle with the brush and could not be grabbed with tweezers, as there was too little free space around the sample to take hold of. The hybridization buffer and probe solution during hybridization also spread to the borders of the section but since the sections were smaller, the majority of the liquid stayed in the region of the appendiceal section. Since it occasionally occurred, that the hybridization buffer dried out during hybridization, we chose to trim down the cutting surface of the blocks as much as possible, to restrict the spreading of the buffer, thus minimizing its evaporation.

3.3.2 Speed of sectioning

While using the microtome we noticed that it made a difference how fast the hand wheel of the microtome was operated. Slower cutting led to more ripples and wrinkles in the section which often hindered the sections to stretch adequately.
In comparison, a faster cutting motion produced almost crease-free, straight sections, which stretched properly.

3.3.3 Slides

We tested four different kinds of slides, to investigate which one guaranteed for the best adherence of the sections. Tested slides were: gelatin-coated slides, uncoated slides, Surgipath® X-tra® slides (Leica) and SuperFrost™ slides (Thermo Scientific™). In order to prepare the gelatin-coated slides, uncoated slides were dipped in Kaisers Glyceringelatine (Roth) and allowed to air dry. Sections were dried on each kind of slide at room temperature for 3 hours before performing FISH.
The sections on the gelatin-coated slides showed hardly any adherence and were rinsed off early during washing steps between both permeabilization methods (lysozyme and Proteinase K treatment).

Sections on uncoated slides almost completely detached during the washing step after hybridization, but held onto them by a corner, making it possible to still retrieve them on the slide when sections were pulled out of the wash buffer.
The Surgipath® X-tra® and SuperFrost™ slides offered the best adherence of the sections, with only slight detachments of single corners during the post-hybridization washing step.

3.3.4 Drying of the sections

In order to further increase the adherence of the sections to our slides, different drying regimes were compared. Sections were dried on SuperFrost™ slides at 37°C, 40°C and 60°C in the slide incubator for three hours without the lid on top. After drying, FISH was performed on the sections.
All temperatures tested, led to an improved adherence of the sections compared to drying at room temperature. No alterations in signal quality or tissue morphology, due to the different drying temperatures, were observed under the microscope. However, in our FISH experiments drying at 40°C for 3 hours, not a single time sections detached during the further process.

3.4 Reduction of autofluorescence

Due to the use of higher laser intensity settings in the beginning of our work, we experienced rather strong autofluorescence of the sections during microscopy (CLSM).
To reduce the autofluorescence we tested photobleaching as proposed by Neumann & Gabel (125) and bleaching with hydrogen peroxide. Both bleaching methods were applied after drying the sections on the slides. After bleaching, the sections were DAPI stained.
For the photobleaching two slides were put into a UV light box with two 15 W emitters for 8 and 24 hours, respectively. During microscopic analysis both sections delivered the same results with no change in autofluorescence but severe obliteration of the tissue, to such extent that it was not possible anymore, to identify any histological structures.

For the bleaching with hydrogen peroxide, three sections were treated with aqueous 3% H_2O_2 solution for 15, 30 and 60 minutes. The treatment with hydrogen peroxide did neither reduce autofluorescence nor alter the morphology of the tissue. None of the above mentioned methods was suitable to reduce autofluorescence and we did not apply them in further experiments anymore.

3.5 Permeabilization

Our permeabilization procedure on cell suspensions included 15 minutes of Proteinase K and 15 minutes of lysozyme permeabilization. When testing this permeabilization process on archaeal and bacterial cell suspensions, it allowed for successful hybridization of archaea and bacteria with their respective probes. Based on the morphology of the probe and DAPI signals, cells were never lysed by this procedure.

Since we did not get any signal in our sections, we tested longer permeabilization times and doubling of the concentrations of Proteinase K and lysozyme. Furthermore we tried adding 1% Triton-X-100 (Sigma-Aldrich) to the lysozyme solution, in order to increase the lysozyme activity on Gram-negative bacteria (126). Permeabilization times compared were 15, 30, 35 and 45 minutes. Increasing the permeabilization time did not result in any specific staining of microbes in the appendix sections. Neither the suspected microbial DAPI signals in the lumen nor the integrity of the appendiceal tissue were altered by the longer permeabilization time.

Raising the concentrations of the Proteinase K solution to 20 µg/ml and lysozyme solution to 20 mg/ml also brought no change in signal quality or tissue integrity, compared to the original concentration.

The same was the case for the addition of 1% Triton-X-100 to the lysozyme solution.

3.6 Hybridization

3.6.1 Hybridization time

During all our FISH experiments on appendiceal sections, we were never able to visualize microbes by hybridization with their respective probe. Before applying FISH on sections we performed it on different bacterial and archaeal cell

suspensions. Although in these experiments problems like unspecific staining occurred, in the majority of cases microorganisms were stained with their respective probe.

In order to adjust our FISH protocol to sections of the vermiform appendix, we increased the initial hybridization time of 6 hours up to 12 hours, to compensate for a potential worse accessibility of the embedded targets by the probes. Longer hybridization times did neither enhance probe-specific signal quality nor increase the amount of unspecific staining.

3.6.2 Probes

At first we diluted our working probe solutions according to weight to achieve 2 ng of probe in the mixed up hybridization solution. Due to the different molecular weights of the probes their molar concentrations in the final hybridization solution were different for each probe. However in order to compare the signal of different probes, keeping the molecular concentration the same for each probe is crucial.

Therefore, we prepared our probe solutions according to molecular concentration in further experiments. We also increased the concentration of our probes to 2 µM according to similar experiment set-ups found in literature (84,121).

Raising the probe concentration to 2 µM did not result in any specific stained microbes but slightly increased unspecific background staining in the appendix sections.

Since Cy5 is considered to fade quickly (127), we used probes that were conjugated with RG. In order to further improve the range of potential bacterial targets we applied the probe mix EUB338I, EUB338II and EUB338III at 2 µM final probe concentration in the final hybridization solution, instead of EUB338I alone.

The results were equivalent to those obtained with the DOPE-Cy5-EUB338 probe. We were neither able to observe any specific staining of bacteria nor a change in unspecific staining of non-targets.

3.7 Microscopy

In order to detect possible weak specific probe signals with the Nikon A1R we tried increasing gain, power of the lasers and pinhole size. The higher intensity laser settings did not reveal any specific probe signals but rather amplified background

signal especially in the green channel (RG) to an extent, that overall image quality was not acceptable anymore. In comparison, applying low laser intensity settings decreased the background signal tremendously, as seen in Figure 2A and 2E.
In an attempt to find out whether the background signal came from autofluorescence or unspecific probe staining, FISH-sections were compared with only DAPI-stained sections. At high intensity laser settings, DAPI-only stained sections showed the same autofluorescene intensity in the green and red channel, hence making autofluorescence the likely cause for the background signal.

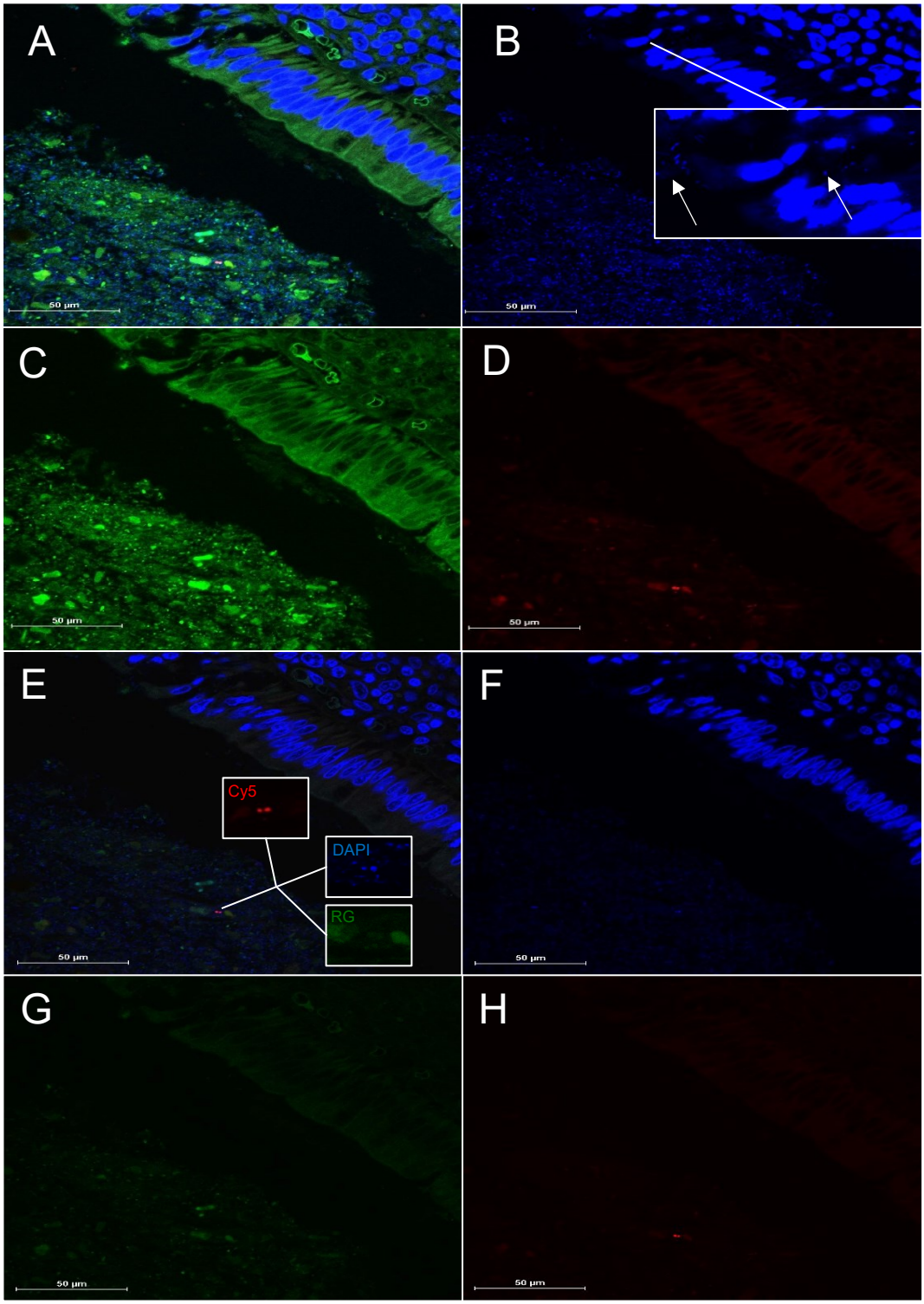

Fig. 2 Appendiceal section with cylindrical epithelium in the right upper corner and luminal content in the bottom left corner. Probes applied are DOPE-Cy5-EUB338 and RG-ARCH915. Images (A), (B), (C), (D) were taken with high laser intensity settings, whereas images (E), (F), (G), (H) display the channels in the same order but with low laser intensity settings. (A) shows the overlay image of all three channels. (B) displays only DAPI channel with bright luminal and eukaryotic DAPI signals. The white box shows in higher magnification small DAPI signals next to epithelial nuclei in and around a defect in the epithelial lining. In the single channel images (C) and (D) strong autofluorescence can be observed in the RG channel (C), whereas the autofluorescence in the Cy5 channel (D) is neglectable. Autofluorescence in the RG-channel (G) and luminal signals in the DAPI-channel (F) are only faintly visible in images with low laser intensity settings. Nevertheless, two potentially specific CY5-signals, that are congruent with DAPI but not RG-signal, as depicted in the white boxes in image (E), appear bright and are clearly visible. in the overlay image (E) and the CY5-channel (H) on low laser intensity settings.

4 Discussion

Our work revolved around the objective of establishing a FISH protocol on appendiceal sections with the subsequent goal of analyzing the microbial characteristics in inflamed appendices. During our experiments, FISH proved to be a troublesome technique whose establishment in a laboratory can be a tedious process. Although we were able to sort out some of the problems in the different steps of the FISH protocol (our final version is listed in the Appendix), we were not able to eradicate all of them, as our results showed. If potential microbial signals were seen in the lumen, they were almost always only DAPI-stained and not hybridized with a probe.

Since detection of microbial signatures requires the simultaneous hybridization with at least one group- or species- specific probe, we cannot claim, that those DAPI-signals are depicting microbes (127).

For the main part of our work, the paper of Hasegawa *et al.*(121) served us as a guideline. Although we always looked for further suggestions and ideas in literature, a lot of problem solving still happened through trial-and-error.

This discussion revolves around the approaches and ideas that still could be tried to tackle the remaining problems.

4.1 Gathering of the samples

During microscopic examination of our sections, it often occurred that both specific probe signal and also luminal content were missing at all. The comparison of sections from two appendices in Figure 3 displays clearly the difference between absence and presence of luminal content.

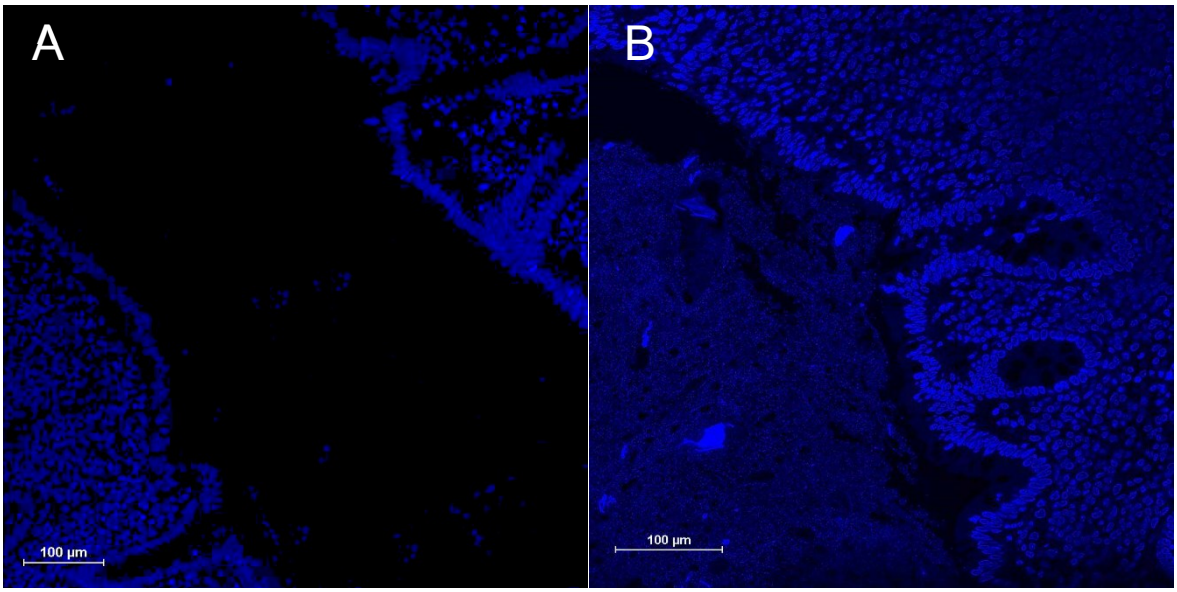

Fig. 3 DAPI-stained sections of two different appendices with (A) no luminal content and (B) luminal content adjacent to the epithelial lining

In the majority of cases, this could also be observed by evaluation of the sections with naked eye, as there was only blank embedding material to be seen in the lumen. Partial loss of luminal content as well as shrinkage of feces was noticed by Swidsinski *et al.* (128), when working with appendix sections. In another study, Swidsinski *et al.* (116) found dense luminal bacterial masses in the lumen of only 8 of their 70 investigated appendix samples. However, despite the apparent lack of luminal content in the majority of their samples, they were still able to visualize an abundance of bacteria adhering to or invading the mucosa, in all their appendix samples, via FISH (116).

Absence of luminal content was also observed in murine large and small intestinal sections by Hasegawa *et al.* (121). They came to the conclusion that the luminal contents got rinsed out by the fixative during fixation. In order to conquer this problem they coated the cut ends of the samples with low-melting point agarose (121).

Although we were already aware of this problem at the beginning, we deliberately decided against the agarose step, as we thought that due to the length of the appendix samples, only the luminal content at the ends would get washed out, leaving the luminal content in the middle of the sample intact. Furthermore implementing an agarose step in a surgery room would be quite challenging and disturbing to the work flow of the surgery.

Nevertheless, as retrospective analysis, coating at least some of the samples with agarose to prevent the loss of luminal contents should be tried, as our approach with bigger sized samples did not work well in retaining the luminal contents and later complicated the embedding of the samples.

4.2 Fixation

The decision to use Carnoy solution for the fixation of the samples was based on several studies (91,129,130), which showed, that mucus is only properly preserved when fixed with Carnoy solution. They claim, that the non-aqueous Carnoy solution does not dehydrate the mucus as much as formaldehyde-based fixations, thus conserving the mucus layer better (129,130). Furthermore, Swidsinski *et al.* (91) came to the conclusion that the mucosa adherent bacterial biofilm is completely lost upon formalin fixation (91). However, it is a controversial topic as Hasegawa *et al*. (121) conducted a direct comparison of the two fixatives and was not able to find any difference in configuration of the preserved mucus layer (121).
Still, due to certain advantages like the redundancy of dehydration steps and us proving its proper function on cell suspensions, working with Carnoy solution should be continued. Nevertheless, there are adjustments that could be made, as discussed below.

4.2.1 Composition of Carnoy solution

The composition of our Carnoy solution was based on a formula of commercially available Carnoy solution made by Ricca Chemical, due to its stated shelf life of 12 months. This led to the addition of methyl and isopropyl alcohol to the formula of 6 parts ethanol, 3 parts chloroform and 1 part glacial acetic acid. Although the ratio of 6/3/1 is quite common in Carnoy solution (121,128,129,131), there is a newer modified version available with 6 parts ethanol, 6 parts glacial acetic acid and 1 part chloroform, which is supposed to deliver better results (122).
Although our fixation seemed to work on cell suspensions as well as on appendiceal cells in the sections, the modified Carnoy solution could be tested to maybe improve the fixation of prokaryotes in our appendix samples.

4.2.2 Ratio of Carnoy solution to sample volume

According to Swidsinski & Loening-Baucke (122) the volume of Carnoy solution during fixation should ideally be at least ten times larger than the sample volume. Ignoring this ratio raises the proportion of water in the fixation solution, leading to a decrease in quality of the hybridization (122).
We fixed all samples in about 4 ml of Carnoy solution, regardless of their size. The smallest samples, barely met the required ratio of sample volume to Carnoy solution of 1:10.
Taking our larger samples into account, fixation in 20 ml of Carnoy solution per sample or cutting the samples in smaller pieces prior to fixation, should be tried, to achieve the suggested proportions.

4.3 Embedding

4.3.1 Anoxic polymerizing conditions

Since Technovit® 8100 relies on an anoxic environment to harden properly, great effort went into finding a way to keep the oxygen away during polymerization. Due to our samples being too big to use the recommended Histoforms, we initially used larger silicon ice cube molds with dimensions of 2.5 cm x 2.5 cm x 2.5 cm. These dimensions did not work with the provided PE films which led us to using a vacuum chamber for our embedding process. Retrospectively the Histoform Q should have been used as we were forced to cut the samples into smaller pieces anyway, to ensure proper hardening of the specimens.
Since we already had multiple vacuum chambers provided in our laboratory, we continued our process of embedding using the ice cube molds.
Although Histoforms are quite exensive compared to our ice cube molds, if an embedding process with Technovit® 8100 has to be established and no vacuum chambers are at the ready, one should consider using a Histoform and PE films, as the embedding is done faster by not requiring the use of a gas exchange station and vacuum pump. Our silicon forms were cheap but only tolerated 3-4 rounds of embedding before becoming brittle and breaking apart.
Further alternatives would be to try covering the smaller silicone molds we ended up using, with the provided PE films.

Instead of PE films, De Jonge *et al.* (132) covered Technovit® 8100 with a layer of paraffin oil, which also allowed the block to harden (132).
Another approach was pursued by Hasegawa *et al.*(121), who filled the Technovit® 8100 embedding solution into Eppendorf® tubes and BEEM® embedding capsules for polymerization. (121)

4.3.2 Sample size

The user instruction of Technovit® 8100 recommends keeping specimens as small as possible and not exceeding 1 mm in thickness. We ended up cutting our samples to 3 mm thickness in order to ensure successful polymerization.
However, De Jonge *et al.*(132) developed a protocol for embedding specimens with up to 2 cm^3 in Technovit® 8100. They succeeded in doing so, by drastically extending the infiltration time to 2-4 days, with multiple changes of infiltration solution, depending on the size of the sample (132).
Applying this protocol could avoid cutting down the samples prior to infiltration, thus preventing manipulation of the samples before embedding, which could lead to tissue damage or dislodgement of any luminal contents otherwise.

4.3.3 Agitation

In an official online instruction by the manufacturer of Technovit® 8100, is clearly stated, that samples have to be agitated during infiltration, in order to achieve successful polymerization. However, there is no mention of an agitation step in the user instruction that comes with the Technovit® 8100 kit. We never agitated our samples during infiltration, since storing them at 4°C in the refrigerator made continuous agitation impossible.
In order to gently agitate the samples during infiltration, the filled Eppendorf® Tubes could be placed on a tube roller in a 4°C cold room.

4.4 Hybridization

4.4.1 CARD-FISH

The rRNA content of microorganisms is heavily influenced by their current physiological state. In oligotrophic environments, which limit their growth rate, the

rRNA content decreases. In further consequence, the oligonucleotide probes have less targets to bind to, resulting in a weaker fluorescence signal and poor detectability (133). Applying CARD-FISH bypasses this problem, as the horseradish peroxidase of a single probe catalyzes the binding of multiple tyramide molecules, therefore amplifying the signal.

Although CARD-FISH could have been tried on our sections, it is possible, that it would not have solved our signal problem. Keeping the number of intestinal microbes in mind, the gastrointestinal tract cannot be considered an oligotrophic environment, hence low rRNA content as reason for our missing signal seems unlikely.

In cells with sufficient rRNA content and insufficient permeabilization, CARD-FISH may even have an inferior detection rate, compared to regular FISH, due to the relatively high molecular weight of HRP and its poor penetration of the cell walls (134).

4.4.2 Dextran sulfate

Dextran sulfate is known for its application as a molecular crowding reagent that can be used to increase the local concentration of the interacting molecules, such as probe and target DNA (135). As a consequence, probes and their respective targets are forced closer together and the hybridization rate of probe to target is increased. Recommended concentrations of dextran sulfate in the hybridization buffer are 50-100 mg/ml (136).

In FISH experiments on cell suspensions with 100 mg/ml dextran sulfate added to the hybridization buffer, we were not able to spot any difference in signal quality compared to our regular formulation. As dextran sulfate also took quite long to dissolve in the hybridization buffer, we stopped adding it and never used it on our appendix sections.

However, dextran sulfate also made the hybridization buffer more viscid, a feature that would prove itself very useful on our sections by limiting the spreading and the evaporation rate of the hybridization buffer. Since dextran sulfate never negatively affected the probe signal on our tests on cell suspensions, its effect on hybridization of appendix sections could be tested.

4.4.3 Hybridization stringency

The term hybridization stringency describes the hybridization conditions under which a probe can bind to its target regions. For example, in order for probes to bind to their target regions under high stringency conditions, probe to target complementarity has to be excellent.

The stringency is determined by the NaCl concentration in the hybridization buffer as well as the hybridization temperature and formamide concentration. Reducing the hybridization temperature and increasing the NaCl concentration leads to lower stringency (136). Adjusting the formamide concentration to optimize the hybridization stringency is a common practice in FISH (137). The higher the concentration of formamide in the hybridization buffer, the lower the stringency, as formamide facilitates the denaturation of the probe/target complex (138).

Since we had no probe signal at all, changing the NaCl concentration or hybridization temperature would only make sense towards a lower hybridization stringency that is more forgiving and allows for binding of probes to targets, which they do not match completely. However, lowering the stringency also results in higher unspecific binding of the probes.

Our formamide concentration of 20% already accounted for rather low stringency conditions. Since the other hybridization conditions were based on several studies that were able to visualize microbes in a similar set-up (84,121), too high stringency conditions seem rather unlikely to have caused the lack of probe signal in our work.

4.4.4 Washing stringency

Stringency also plays a role in the post hybridization washing. Washing with high stringency will remove all unbound or non-specifically bound probes, but may also affect target-specific bound probes. Similar to the hybridization buffer, the stringency of the washing conditions, is determined by the NaCl concentration and temperature of the wash buffer. The amount of NaCl in the wash buffer usually correlates with the formamide concentration in the hybridization buffer, as depicted in Table 3, and was in our case according to 20% formamide, 0.225 M.

Too high stringency in washing may result in a total loss of probe signal. The temperature of our wash buffer was 48°C and identical to the aforementioned

literature (84,121). Our NaCl concentration of 0.225 M in the wash buffer was 0.010 M higher compared to the 0.215 M NaCl used by Hasegawa *et al.* (121) and Welch *et al.*(84). Since the difference is small, the higher NaCl concentration was most likely not the cause of the specific probe signal to get washed off in our experiments.

4.4.5 EDTA concentration in washing buffer

In our work we mistakenly had a five times higher concentration of EDTA in our washing buffer than commonly used. As it is with the concentration of NaCl, the amount of EDTA in the wash buffer is also determined by the formamide concentration in the hybridization buffer, as seen in Table 3. For a concentration of 20% formamide, a concentration of 0.005 M EDTA is recommended for the wash buffer but we used 0.025 M.

EDTA is a chelating agent that inhibits the degradation of nucleic acids by forming chelates with divalent cations, such as Ca^{2+} or Mg^{2+}, which serve as cofactors for nucleases (139). We unfortunately did not notice the incorrect concentration of EDTA during all of our experiments. Since we used EDTA in form of disodium EDTA, we did introduce more sodium into the hybridization buffer. By using a concentration of 0.025 M disodium EDTA, we raised the sodium concentration of 0.1125 M by 0.05 M to 0.1625 M. Compared to the recommended concentration, this is an increase by almost 25%. A change in washing stringency, that could have caused specific probe signal to get washed away, cannot be ruled out. Therefore, it is important to use the correct amount of EDTA in further FISH experiments.

Tab. 3 *Composition of wash buffer*

% FA in hybridization buffer	M NaCl in wash buffer	M EDTA in wash buffer
0	0.9	0.0
5	0.636	0.0
10	0.450	0.0
15	0.318	0.0
20	0.225	0.005
25	0.159	0.005
30	0.112	0.005
35	0.080	0.005
40	0.056	0.005
45	0.040	0.005
50	0.028	0.005
55	0.020	0.005
60	0.008	0.005
70	0.0	0.0035

4.5 Mucus staining

According to Swidsinski *et al.*(128) the empty space between luminal content and epithelium in FISH-images of appendix sections, represents the mucus layer. They confirmed this theory by comparing sections with alcian blue and periodic acid-Schiff (PAS) reaction stained mucus and sections without mucus staining (128).

Judging by the space between epithelium and luminal content in Figure 2A our mucus layer seemed to be present and intact.

However, we believe that the mucus layer can only be properly assessed when it is visible, therefore, we consider mucus staining a necessity in future FISH experiments of the appendix.

Since we use a confocal laser scanning microscope, working with a staining method like alcian blue/ PAS, which cannot be assessed via fluorescence microscopy, seems impractical. Therefore, we would rather try a fluorochrome based method for staining mucus. For example, wheat germ agglutinin (WGA), a lectin that binds to sialic acid and *N*-acetylglucosaminyl residues, that can be conjugated with flurochromes, to stain mucus (121).

4.6 Microscopy

As already mentioned before, we were not satisfied with the results of our FISH. The two Cy5-signals counterstained with DAPI and no signal in the green channel that are magnified in Figure 2E were a rare sight during microscopy.

Regarding the criteria for bacterial identification by Swidsinski (127), 3 of the 4 criteria for the certain identification were fulfilled:

1. Morphological form typical for bacteria
2. Detectable in DAPI counterstain
3. No cross-hybridization with unrelated FISH probes
4. Hybridization with at least two group or species-specific FISH probe

The fourth rule, hybridization with an additional group- or species-specific probe, was not fulfilled, because we did not apply another probe with the same target.

Looking at the low laser intensity images shown in Figure 2, it also became clear that we had no problem with autofluorescence when laser intensity was decreased. Furthermore, the presumably specific Cy5-signals were still bright enough, to be clearly visible.

One problem we encountered during microscopy, were the wildly different intensities of prokaryotic and eukaryotic DAPI-signals. This led to the dilemma that prokaryotic cells in vicinity of epithelial cells are either not visible at low illumination or vanish due to overexposed nuclei at high illumination. In Figure 2B, with high laser intensity settings, small DAPI signals (marked with arrows) close to a questionable epithelial defect are barely visible next to the very bright eukaryotic nuclei.

In future experiments we would choose our lower laser intensity settings, where no information loss in the image due to overshadowing by eukaryotic DAPI-signal occurs. Prokaryotic DAPI-signal does not need to be strong, as it only serves as a counterstain to validate the specific hybridization of the probe.

Also, in the other channels, as seen in Figure 2, the higher laser intensity settings only led to bright autofluorescence, but did not reveal any specific signal, that was not visible with lower laser intensity settings.

All the images presented in this work were taken in one focal plane. However, we briefly did try out image acquisition with Z-stacks. When changing the focus, we noticed, that there was no focal plane, in which all potential microbial DAPI-signals in the lumen were seen at the same time. The first images acquired with Z-stacks

looked promising, as it allowed us to choose the focal plane with the least background signal for each channel individual. Image acquisition using Z-stacks is definitely a more sophisticated option, than working with one focal plane alone. Especially for quantitative measurements of microbial signals in a section, it could prove itself very useful, as all signals would get depicted in one image.

5 Summary

Considering the main goal of this study, establishing a reliable protocol for FISH on sections of appendix vermiformis, a lot of progress has been made, even though the intended end result has yet to be achieved. Furthermore, without the successful establishment of a FISH protocol, we were not able to address our secondary goal of investigating the microbial characteristics of appendicitis and their role in its pathogenesis. This work demonstrated that Carnoy solution is capable of fixating microbial as well as eukaryotic cells and seems to preserve the mucosal layer of the appendix. Furthermore, the methacrylate based embedding system Technovit 8100®, was shown to be great for embedment, due to the uncomplicated storage and high durability of the blocks as well as simple further processing of the sections. However, in consequence of its sensibility to oxygen during polymerization, blocks occasionally did not harden properly and had to be discarded. Sections were cut at 5µm thickness with a semi-automated rotary microtome and stretched on X-tra® slides (Leica), which offered the best adherence of the sections after drying for 3 hours at 40°C. Prior to hybridization, permeabilization steps with lysozyme and Proteinase K, which had already been established in our lab on cell suspensions, were performed on the appendix sections. While these steps did not harm the integrity of the tissue, they also did not reveal any prior undisclosed microorganisms, by making them accessible for FISH. Hybridization was performed, based on a FISH protocol applied on cell suspensions in our lab, with rRNA-targeted oligonucleotide probes for bacteria and archaea as well as one non-sense probe as negative control. DAPI was used to nonspecifically counterstain all nucleic acids in the section. In the microscopic examination, using the CLSM Nikon A1R, microorganisms could not be identified with certainty. In some samples there was a complete lack of luminal content, whereas in others luminal content with DAPI-signals, perhaps depicting microorganisms, was present. However, these DAPI-

signals did not reveal signal of the applied probes, therefore making the definitive identification of microorganisms impossible.

In order to successfully visualize microorganisms in appendix sections further modifications to our FISH protocol have to be made. Although in our discussion we proposed new ideas for each step of the process, the introduction of an agarose step to prevent dislodgement of luminal content and the adjustment in ratio of Carnoy solution to sample volume should be prioritized in their implementation.

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7 Appendix

FISH protocol

Fixation

Carnoy solution
Fresh sample is put into 5 ml Eppendorf® tubes with 4 ml of Carnoy solution to fixate for at least 2 hours at 4°C.

Carnoy solution(can be used for up to 7 days)

Compounds	**% (v/v)**
Ethyl alcohol	55
Chloroform	30
Glacial acetic acid	10
Isopropyl alcohol	2.5
Methyl alcohol	2.5

Filled Eppendorf® tubes are stored at 4°C.

Dehydration

After fixation, dehydrate samples in a series of three Eppendorf® tubes filled with precooled 100% EtOH for 3 minutes each. Store samples in fourth Eppendorf® tube filled with 100% EtOH at -20°C.

Embedding in Methacrlyate

Infiltration

Infiltration solution for Technovit® 8100-Embedment (can be used for up to 4 weeks)

- 100 ml Technovit® 8100 basic solution
- 1 bag 0.6g Technovit® 8100 hardener I

Store infiltration solution at 4°C
For infiltration fill up 5 ml Eppendorf® tubes with 4 ml of infiltration solution and put samples in it. Infiltrate overnight at 4°C. Make sure that sample length does not exceed 3 mm. Otherwise, cut appendix down with scalpel prior to infiltration.

Polymerization

When preparing for polymerization, precool vacuum chambers at 4°C in refrigerator.

Polymerization solution for Technovit® 8100 (per sample)

- 1 ml infiltration solution
- 45 µl Technovit® 8100 hardener II

Mix up polymerisation solution in 1.5 ml Eppendorf® tubes and put infiltrated sample in. Agitate tubes for 3 minutes. Pour everything in silicon mould and put the mould in vacuum chamber. Use gas-station according to the following regime: V (till barometer shows -0.9 bar) -> G (till 0 bar)-> V (till -0.9 bar)-> G (till 1 bar) Let the samples polymerize overnight.

Cutting

Retrieve samples from vacuum chamber and cut sections with microtome. Sections should be cut 5 µm thick. Pipette ~2 ml of ddH_2O on X-tra® adhesive slides and use a brush to lay cut sections on water. After sections are stretched remove the excess water with a pipette.
Dry the slides for 3 h at 40°C in incubator.

Permeabilization

Proteinase K

- 1 µl Proteinase K-stock solution
- 1 ml Tris-HCl 20mM, CaCl2 2mM

Pipette 30 µl of Proteinase K solution on section and let incubate for 35 minutes at 37°C in incubator.
Rinse slides with ddH_2O.

Lysozyme

- 10 mg Lysozyme
- 1 ml TE-Buffer

Pipette 30 µl of lysozyme solution on section and let incubate for 15 minutes at 37°C in incubator.
Rinse slides again with ddH_2O.
Let slides air-dry

FISH

Hybridization

Hybridization buffer (1 ml)

NaCl (5 M)	180 µl (≙ 0.05259g
Tris-HCl (1M, pH 6.8)	20 µl
SDS (10%)	4 µl
ddH2O	596 µL
Deionized formamide	200 µL

Prepare hybridization buffer and preheat it at 46°C in the ThermoMixer. Place slides in slide incubator at 46°C and pipette ddH_2O into its wells.
Pipette 20 µl hybridization buffer on each section. Let incubate for 25 minutes.
Meanwhile prepare the working probe solutions by diluting stock solution with PCR-grade water to achieve a probe concentration of 20 µM.
After 25 minutes add probe solutions to hybridization buffer.

Washing

Wash buffer: (500ml)

NaCl final conc.	6.5745 g 0.225 M
Tris-HCl (1M, pH 8.0)	10 ml
SDS (10%)	0.5 ml
EDTA (0.5M)	20 ml
ddH2O	464.5 ml

Prepare wash buffer and pour it into 50 ml falcons. Place falcons in water bath at 48°C.
After hybridization rinse slides with pre-warmed washing buffer before placing them in falcons for 25 minutes. Afterwards rinse slides with ice-cold ddH_2O.

Counterstaining and mounting

Counter-stain with 50 µl of VectaShield® Antifade Mounting Media with (DAPI).
Put coverslip on top of slide and fix and gently press down to remove air bubbles.
Fix coverslip with nail polish.
Store slides at 4°C until further usage.