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The Role of Mucosal Biofilms in Ulcerative Colitis and Irritable Bowel Syndrome

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Hunter Alexander Holley

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ABSTRACT

The prevalence of bowel diseases, such as ulcerative colitis (UC) and Crohn's disease (CD), in addition to bowel disorders such as irritable bowel syndrome (IBS), has shown an increasing trend in the last decades. As the role of the intestinal microbiota in regard to intestinal dysbiosis and disease comes to the forefront of healthcare science, it is imperative to establish and understand the relationship between microbial communities as well as their microbial constituents in terms of presence, composition, and function within the human individual. The main purpose of this master's thesis is to microscopically confirm the presence of endoscopically (macroscopically) visible biofilms within individuals with IBS, UC, and healthy controls. This was accomplished by establishing a relationship between endoscopic ratings of suspected biofilm layers and the bacterial surface area coverage obtained by fluorescence *in situ* hybridization (FISH) experiments and image analyses. The results revealed that bacterial surface area coverage of biofilm-biopsies is significantly larger when compared to normal mucosa biopsies of both biofilm-positive (p-value: .0332) and biofilm-negative (p-value: 0.0039) patients.

ZUSAMMENFASSUNG

Chronisch-entzündliche Darmerkrankungen wie Colitis ulcerosa (CU) und Morbus Crohn (MC), sowie funktionelle Darmerkrankungen wie das Reizdarmsyndrom (RDS), haben einen erheblichen Zuwachs innerhalb der letzten Jahrzehnte erfahren. Da die Rolle der Darmflora in Hinsicht auf intestinale Dysbiose und Darmerkrankungen in den Fokus der Gesundheitsmedizin tritt und an Bedeutung gewinnt, ist es von besonderer Wichtigkeit den Zusammenhang zwischen mikrobiellen Gemeinschaften und ihren mikrobiellen Bestandteilen in Bezug auf das Vorhandensein, die Zusammensetzung und deren Funktion innerhalb des menschlichen Individuums zu entschlüsseln. Der Untersuchungsgegenstand dieser Masterarbeit liegt in der mikroskopischen Bestätigung des Vorhandenseins von endoskopisch (makroskopisch) sichtbaren Biofilmen. Die hierfür untersuchten Darmbiopsieproben stammen von Patienten mit RDS, CU sowie von gesunden Probanden. Innerhalb dieser Studie konnte ein Zusammenhang zwischen endoskopischen Bewertungen der Biofilme und der Oberflächenbedeckung des bakteriellen Biofilms hergestellt wurde. Die hierfür verwendeten Daten bezüglich der Oberflächenbedeckung des bakteriellen Biofilms wurden durch Fluoreszenz *in situ* Hybridisierungsexperimente (FISH) und Bildanalysen gewonnen. Die Ergebnisse zeigen, dass die Fläche, die mit Bakterien bedeckt ist bei Biofilm-Biopsien im Vergleich zu normalen Mukosabiopsien von Biofilm-positiven (p-value: 0.0332) als auch Biofilm-negativen (p-value: 0.0039) Patienten größer ist.

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INTRODUCTION

The role of microbial communities on and within the human body has been of increasing interest to researchers in the last decades due to recent developments in medical technology, which have made such research possible. Newfound discoveries, due to the diminishing costs and increased efficiency of DNA sequencing, have enabled scientists to thoroughly investigate bacterial 16S ribosomal RNA gene sequences. These investigations have allowed scientists to propose that microbial communities as well as their microbial constituents play a causal role in the development of the human immune system, as well as acting as a line of defense in commensal environments¹. As is comparable in all ecosystems, niches are frequently filled by organisms, which have adapted and evolved to serve their respective ecological requirements. As the human body is capable of housing up to 10^{14} bacterial cells², it is important to note that the human colon is one of the most densely populated areas of the body.

Origins of Commensal Microbiota

As genes are inherited by offspring from their parents, it is hypothesized that an individual's microbial community is also inherited (maternally from parent to progeny).³ This hypothesis is strengthened by the congruent phylogenies observed amongst whole microbial communities and their animal counterparts.⁴ The coevolution of humans and their respective microbial communities is a result of the

¹ Turnbaugh et al., "The Human Microbiome Project."

² Cho and Blaser, "The Human Microbiome."

³ Cho and Blaser.

⁴ Cho and Blaser.

mutualistic relationships the two have co-adapted with one another in order to persist. During the course of evolution, the human body became an advantageous environment for microbes to colonize because it provided a protective source of nutrients, water, as well as energy. Microbial communities provided their human counterparts vital substances needed for the development of a robust immune-system defense, reproduction, and metabolism⁵. Inheritance of a microbial community is further supported when investigating how the human gut is first colonized with bacteria. As the newborn leaves the sterile womb, the offspring is initially exposed to microbiota as it makes its way through the vaginal canal. These first microbial encounters are predominantly lactobacilli species found in the mother's vaginal canal. Though dependent on the mode of delivery (vaginal or cesarean), the vertical transmission of bacteria from the mother to the newborn plays an important role in the development of a commensal bacterial community within the growing human. According to Cho and Blaser, the exposure to lactobacillus in the mother's vaginal canal, as well transition through breast milk, is thought to explain the initial lactobacilli bloom in the baby's gut⁶. The colonization of an early microbial community via mother-child vertical transmission is vital in establishing an initial intestinal flora that will protect the human not only during the first months of gastrointestinal development, but also well into maturity as new species begin to colonize the gut⁷.

⁵ Cho and Blaser.

⁶ Cho and Blaser.

⁷ Cho and Blaser.

After the initial microbial colonization of the human colon, there are several factors, which come to shape the microbial composition as time progresses. The development of teeth, for example, drastically alters the species composition and abundance of microbes that come to reside in the oral cavity⁸. The succession of the microbial community of the oral cavity becomes important as Cho and Blaser denote the congruencies between bacterial species found in the oropharynx of individuals, such as from genera *Streptococcus*, *Actinomyces*, *Prevotella*, and *Gemella*, with those which are also found residing in their distal gastrointestinal tract⁹, pointing to progression of microbial species from proximal to distal locations.

The Gastrointestinal Mucosal Surface

The human gastrointestinal tract is characterized by its immense size of epithelial surface area, which is exposed to the content ingested by the body, including both nutrient sources and microbes. Acting as a line of defense, the gut is coated with two layers of protective mucus (except for in the small intestine where only one is found) that allow for absorption of micromolecules and nutrients, whilst defending the body against direct contact with pathogens. The mucus layers of the intestine, though both protective, differ in morphology. The inner mucus layer is firmly attached to the epithelial surface and is approximately between 50 - 200 μm thick, while the outermost mucus layer, though also of varying thickness (several hundred μm), can easily be sloughed off and therefore does not have a defined

⁸ Cho and Blaser.

⁹ Cho and Blaser.

boarder¹⁰. Goblet cells, which produce gel-like mucins, span the entire length of the intestinal tract and provide these layers of protection. The compositions of these mucins differ depending on site of origin, with the most abundantly produced intestinal mucin, MUC2, having been more thoroughly investigated. Integral in forming a line of protection in the distal gut, as well as supplying a vital energy source for commensal bacteria, MUC2 mucins encode for a protein whose central domains are mainly composed of the amino acids proline, threonine, and serine, thus coining the PTS-domains of intestinal mucins¹¹. These hydroxy amino acids, which are found in high abundances, allow for the numerous adhesions of O-glycans, giving rise to the structural integrity of the mucin domains¹². In addition to maintaining the mucosal framework, the large amounts of O-glycans confer advantages to the mucin domains such as protease resistance, as well as the mucins' ability to substantially bind water¹³. Furthermore, MUC2 mucins are able to create stratified sheets of mucus due to disulfide bonds, which allow the domains to form dimeric and trimeric complexes. The interaction between these complexes is what gives rise to the mucosal stratification, which is later strengthened and maintained via a series of CysD domains, which can also be found within MUC2 mucins¹⁴. The complex interactions of components comprising the mucin macromolecule, function in unison to simultaneously protect the vast surface area of otherwise exposed epithelium, whilst providing nourishment to commensal bacteria.

¹⁰ Hansson, "Role of Mucus Layers in Gut Infection and Inflammation."

¹¹ Hansson.

¹² Hansson.

¹³ Hansson.

¹⁴ Hansson.

Commensal Bacteria and the Colonic Mucosal Surface

On average, 10 - 100 trillion microbes reside in the human colon alone¹⁵. The mainly anaerobic environment, paired with an influx of nutrients, yields perfect conditions for microbes to colonize and multiply. The compact integrity and stratification of the inner-mucosal layer found in the large intestine is hypothesized to confer its ability to devoid the area of any microbes, which may be privy to invading the human body¹⁶. The tight adherence of the mucus produced by the goblet cells of the epithelial layer, is thought to function by inhibiting the passage of large (bacteria-sized) molecules. As this layer is continuously produced, previously excreted layers are pushed inward (luminally) where its exposure to “endogenous serine protease activities” allows for its expansion of “4-5 fold in volume”, creating the outer mucus layer¹⁷. The loosening of the once tight mucosal layers makes possible the colonization of the outer mucus layer by microbial communities, where readily available monosaccharides can be harvested for microbial replication and growth. Interestingly, scientist have discovered that the commensal bacteria in the gut have a large portion of their genome dedicated to the production of enzymes needed to break down glycosidic linkages, thus enabling them to take up the cleaved saccharides^{18,19}. Furthermore, studies conducted by the Png group, explain that these enzymes, normally exoglycosidases, function by cleaving one monosaccharide

¹⁵ Turnbaugh et al., “The Human Microbiome Project.”

¹⁶ Hansson, “Role of Mucus Layers in Gut Infection and Inflammation.”

¹⁷ Hansson.

¹⁸ MetaHIT Consortium et al., “A Human Gut Microbial Gene Catalogue Established by Metagenomic Sequencing.”

¹⁹ van Passel et al., “The Genome of Akkermansia Muciniphila, a Dedicated Intestinal Mucin Degradar, and Its Use in Exploring Intestinal Metagenomes.”

from the MUC2 molecule at a time²⁰. Since the MUC2 molecule is comprised of a high number of monosaccharides in form of over 100 different oligosaccharide species²¹, it is proposed that the relatively slow harvesting of mucins by commensal bacteria via secretion of oligosaccharide - specific cleaving enzymes, is in fact beneficial as it grants time for the renewal of subsequent mucus layers. An additional advantage that commensal bacteria confer to their host is the conversion of host-ingested carbohydrates into monosaccharides and subsequently short-chain fatty acids by bacterial metabolism²². Hansson et al. hypothesize that availability of these short-chain fatty acids, which are able to diffuse through the inner mucus layer, supply the epithelium with the energy source it needs in order to maintain the high turnover of mucins and mucus²³.

Shaping the Colonic Microbial Community

In investigating the theorized causal roles a microbial community enacts within its host, one must first grasp the host's ecology and respective ecosystem, which allows for the microbial community to assemble. Focusing on the microenvironment, in this case of the large intestine, theories and postulates of macro-ecology can be implemented in order to identify, as well as explain trends of microbial community assembly that have been scientifically recorded regarding the human colon. The Turnbaugh group drew aspects from the island biogeography theory of ecology to elucidate the trends of microbial community assembly.

²⁰ Png et al., "Mucolytic Bacteria With Increased Prevalence in IBD Mucosa Augment In Vitro Utilization of Mucin by Other Bacteria."

²¹ Hansson, "Role of Mucus Layers in Gut Infection and Inflammation."

²² Hansson.

²³ Hansson.

Specifically, the group calls on the *multiple stable states phenomenon* of the theory, which is used to specifically characterize the colonization and growth of a newfound community influenced strongly on the order in which species initially entered²⁴. This further supports the claims of Cho and Blaser, mentioned previously, that initial exposure of microbiota species (i.e. *Lactobacilli*) via vertical transmission is fundamental in colonizing an initial commensal community within the gut that will consequently shape the colonic habitat and influence subsequent microbial assembly.

When characterizing the community composition of microbiota within the human colon, the ecological theory of neutral community assembly best explains the trends that current data have revealed. Though the majority (> 90%) of colonic microbial constituents found in humans belong to either the Bacteroidetes or Firmucutes phyla, there proves to be a higher degree of interspecific microbial community assemblage when comparing assemblages against those of other humans²⁵. The neutral theory of community assembly suggests, “that most species will share the same general niche, or the biggest niche, and thus are likely to be functionally redundant”²⁶. This was exactly the case when Turnbaugh investigated 18, 348 fecal 16S rRNA gene sequence data from 14 unrelated adults over the course of one year, and illustrated that community assemblages between individuals

²⁴ Turnbaugh et al., “The Human Microbiome Project.”

²⁵ Turnbaugh et al.

²⁶ Hubbell, “Neutral Theory and the Evolution of Ecological Equivalence.”

was indeed functionally redundant, while the community assemblage of a specific individual was revealed to be “highly variable”²⁷.

Further exploration into community assemblages of colonic microbiota were recently conducted, that revealed the presence of specific “enterotypes” within humans. This was achieved by analyzing fecal matter, in tandem with DNA sequence homologies, which was referenced to sequences made available by the Human Microbiome Project²⁸ and the European MetaHIT consortium²⁹. The results provided evidence to the phylogenetic organization of microbial “enterotypes” amongst humans³⁰. These enterotypes are coined based on the “variation observed in the levels of one of three genera: *Bacteroides*, *Prevotella*, and *Ruminococcus*”, or by their respective enterotypes 1, 2, and 3³¹. Furthermore, Arumugam and colleagues discovered through correlation analyses that the abundances of the different enterotypes (or the three distinct genera) respectively coincide with those of the other genera³². This gives reason to believe that the “enterotypes are in fact driven by groups of species that together contribute to the preferred community composition”³³. Research conducted during the Human Microbiome Project provides insight into how these genomic variances, or enterotypes, may occur and how they are sustained within human populations, making up the core human microbiome of the gut.

²⁷ Turnbaugh et al., “The Human Microbiome Project.”

²⁸ The Human Microbiome Jumpstart Reference Strains Consortium et al., “A Catalog of Reference Genomes from the Human Microbiome.”

²⁹ The Human Microbiome Jumpstart Reference Strains Consortium et al.

³⁰ Arumugam et al., “Enterotypes of the Human Gut Microbiome.”

³¹ Arumugam et al.

³² Arumugam et al.

³³ Arumugam et al.

Understanding the core microbiome of the human gut, or the set of shared genes amongst human guts, requires the consideration of several factors as no single variable solely influences its composition. As depicted by Turnbaugh, the genotype and physiology of the host plays an important role in whether certain genotypes within the gut microbiome will be able to colonize and persist³⁴. This includes the host's disease status, age, as well as their innate and adaptive immune system. Other factors, which shape the core microbiome, include the host's lifestyle and diet. As commensal gut microbes degrade and feed upon ingested material, the contents that are ingested play a role as to available resources for microbial community development. This is also the case in regards to the environment in which the host is surrounded, including residence or place of employment. For example, a study conducted with infants who were surrounded by at least one furry pet for 3-4 months following birth, had an altered gut microbiome community (increased diversity) in comparison to those who were not in proximity of furry pets following birth³⁵. It is also important to note that a portion of the human gut microbiome is not always present or in other words is transient. Due to fluctuations in all of the above-mentioned factors during a human's life, or "micro - evolution"³⁶, certain microbial genes may be acquired while others are removed from the human core microbiome of the gut.

³⁴ Turnbaugh et al., "The Human Microbiome Project."

³⁵ the CHILD Study Investigators et al., "Exposure to Household Furry Pets Influences the Gut Microbiota of Infants at 3–4 Months Following Various Birth Scenarios."

³⁶ Turnbaugh et al., "The Human Microbiome Project."

Inflammatory Bowel Diseases & the Microbial Community

Though the relationship between the colonic microbial community and its respective human host has been described in large as commensal, it is crucial to understand that perturbations in these microbial communities can lead to intestinal environments, which can be damaging to the human's health. The onset of inflammatory bowel diseases (IBDs) is sought to originate from the host's immune response to an altered intestinal microbial community, leading to inflammation of the intestinal tissue³⁷. The degree and severity of intestinal tissue inflammation comes to characterize the two main classifications of IBD by which humans are afflicted: Ulcerative colitis (UC) and Crohn's disease (CD). UC is understood to inflame mainly the mucosa of the large intestine and rectum, while the deep - penetrating inflammatory lesions that form in CD individuals have been observed to appear anywhere in the digestive tract³⁸. Both diseases however, have been defined by "major shifts" in their microbial community compositions^{39,40,41,42,43,44} as well as

³⁷ Franzosa et al., "Gut Microbiome Structure and Metabolic Activity in Inflammatory Bowel Disease."

³⁸ Swidsinski et al., "Spatial Organization and Composition of the Mucosal Flora in Patients with Inflammatory Bowel Disease."

³⁹ Imhann et al., "Interplay of Host Genetics and Gut Microbiota Underlying the Onset and Clinical Presentation of Inflammatory Bowel Disease."

⁴⁰ Morgan et al., "Dysfunction of the Intestinal Microbiome in Inflammatory Bowel Disease and Treatment."

⁴¹ Gevers et al., "The Treatment-Naive Microbiome in New-Onset Crohn's Disease."

⁴² Haberman et al., "Pediatric Crohn Disease Patients Exhibit Specific Ileal Transcriptome and Microbiome Signature."

⁴³ Lane, Zisman, and Suskind, "The Microbiota in Inflammatory Bowel Disease."

⁴⁴ Blander et al., "Regulation of Inflammation by Microbiota Interactions with the Host."

having unique respective microbial signatures⁴⁵. Additional analyses discussed by Franzosa et al., have implemented the casual role of microbial community constituents (i.e. metabolites) in shaping the overall community assemblage within the gut via signaling pathways, roles in antibiotic activities, as well as modulation of the immune system⁴⁶. In a study conducted by Franzosa and colleagues, the role of metabolites in the prevalence of IBD was further underlined, as they linked broad metabolic shifts found amongst IBD individuals with the absence of overlapping metabolic profiles found in non-IBD controls⁴⁷. The variations observed by the group were hypothesized to originate from differences in levels of active inflammation, as the metabolic profiles of individuals suffering from UC and CD were more similar than either one to non-IBD controls⁴⁸. These discoveries, in tandem with prior analyses of the intestinal community, support the hypotheses that unique compositional organizations within the human gut may give rise to particular IBDs and gut disorders. Alternatively, gut inflammation may shape the bacterial community.

Biofilm Formation

Equally noteworthy as the composition of the microbial community is the spatial organization of said community within the gut. As described previously, microbes are able to colonize the outer-most mucus layer, in the colon specifically, which leads to the mutualistic relationship between humans and microbial gut

⁴⁵ Huttenhower, Kostic, and Xavier, "Inflammatory Bowel Disease as a Model for Translating the Microbiome."

⁴⁶ Franzosa et al., "Gut Microbiome Structure and Metabolic Activity in Inflammatory Bowel Disease."

⁴⁷ Franzosa et al.

⁴⁸ Franzosa et al.

communities. However, the abundant colonization and growth of structured communities, which are encased within a self-produced extracellular polymeric substance (EPS) matrix, can give rise to so called “biofilms” that adhere to both inert and living surfaces^{49 50}. The formation and appearance of biofilms has been hypothesized to stem primarily from the advantages, which the biofilm confers to the producing bacterial community⁵¹. In contrast to a free-moving bacterium in a liquid or mucus environment, the conglomerate of bacteria that form a biofilm layer are able to create their own protective environment, which can adhere directly to a surface. Analyses have elucidated a probable five-step biofilm formation theory, with the initial two steps pertaining to bacterial surface adherence, followed by an additional two steps devoted to cell aggregation and formation of micro-colonies, and completed with the final step of biofilm dispersal⁵². During the macroscopically visible third and fourth steps, the biofilm is able to ward against harmful substances (i.e. antimicrobial agents such as antibiotics, antimicrobial peptides, and immunoglobulin), due to the properties of the mucinous matrix. The EPS matrix not only allows the bacterial community to adhere to a surface (i.e. epithelial surface) and conglomerate, but also protects the community by binding and or neutralizing antimicrobial agents to a “sub-lethal” level before reaching all individual bacteria within the biofilm⁵³. Interestingly, the biofilm confers two more layers of antimicrobial resistance by the creation of dormant zones within the biofilm in

⁴⁹ Swidsinski et al., “Spatial Organization and Composition of the Mucosal Flora in Patients with Inflammatory Bowel Disease.”

⁵⁰ Hall-Stoodley, Costerton, and Stoodley, “Bacterial Biofilms.”

⁵¹ Hall-Stoodley, Costerton, and Stoodley.

⁵² Hall-Stoodley, Costerton, and Stoodley.

⁵³ Hall-Stoodley, Costerton, and Stoodley.

addition to housing resistant phenotypes within its composition. Scientists explain that the “stationary phase dormant zones” are beneficial to the persistence of the biofilm, in respect to antimicrobials, as these compounds target a specific aspect of microbial growth; therefore conferring a survival advantage to non-growing bacteria⁵⁴. Additionally, biofilms have been observed to contain at least a small portion of resistant phenotypes, which if the biofilm were to be disturbed, would enable the biofilm to recover and persist. Due to the nature of extensive microbial community assemblages within the mucosal layers, which reach and adhere to the intestinal epithelium, presence of said structures are described to be intestinal biofilms. Recent investigations focusing not only on the spatial organization, but also microbial density of intestinal biofilms, have further elucidated microbial ecology in IBDs, in addition to those who are afflicted by irritable bowel syndrome (IBS). This intestinal disorder is characterized by the symptoms of chronic bowel pain, which is accompanied by a feeling of discomfort and/or bloating that is relieved upon defecation, leading to altered bowel habits⁵⁵.

Implications of Biofilms in Altered Intestinal Health

Studies investigating the mucosal bacterial community assemblages in individuals with IBDs as well as IBS have illustrated differences between afflicted individuals and normal controls. Specifically, examinations of mucosal organization and composition carried out by the Swidsinski group, revealed that individuals with IBD have a mean mucosal biofilm density, which is “2 powers higher” than that of

⁵⁴ Hall-Stoodley, Costerton, and Stoodley.

⁵⁵ Durbán et al., “Structural Alterations of Faecal and Mucosa-Associated Bacterial Communities in Irritable Bowel Syndrome.”

IBS or controls⁵⁶. The high density observed comprising the biofilm layers was portrayed in fluorescent microscopy images, where the biofilm was seen to be resting directly upon the colonic epithelium, having invaded and colonized the gut's protective inner mucus layer. Direct epithelial contact with bacteria, as previously described, plays an integral role in the activation of the host's immune systems as well as subsequent inflammation within the intestine. Similar conditions were observed regarding mucosa-associated microbiota by the investigators of Zhong et al., where it was discovered that microbial invasion of the inner mucus layer, especially in those with UC, was clearly evident, in addition to certain bacterial species (i.e. *E. coli*) that invaded the intestinal crypts in addition to the lamina propria⁵⁷.

In attempts to strengthen the argument against the negative association between intestinal biofilms and human health, an intriguing experiment was conducted, which investigated the effects of human biofilms in various mouse models. Human biofilm homogenates from biofilm-positive colorectal cancer and healthy patients, in addition to biofilm-positive normal mucosa homogenates from normal flanking intestinal tissues of colorectal cancer patients and biofilm-negative healthy subjects were used in the investigations. Tomkovich et al. were able to prove that biofilm-positive homogenates induced colon tumor formation in

⁵⁶ Swidsinski et al., "Spatial Organization and Composition of the Mucosal Flora in Patients with Inflammatory Bowel Disease."

⁵⁷ Wang, Lu, and Zhong, "Tu1895 - Distinct Microbial Populations Exist in the Mucosa-Associated Microbiota of Diarrhea Predominant Irritable Bowel Syndrome and Ulcerative Colitis."

susceptible mouse models, whereas biofilm-negative homogenates did not⁵⁸. This finding supports the previously mentioned hypothesis that the composition of the biofilm layer in fact contains a unique microbial community that can lead to an altered intestinal state. Strikingly, the scientist discovered that biofilm homogenates from healthy controls induced colon inflammation and tumors similarly to their biofilm-positive tumor homogenate counterparts; providing that biofilms, regardless of human disease status, are capable of harboring a harmful microbial community⁵⁹. Supporting previously mentioned claims that invasive species associate with biofilm microbial communities, the scientist were able to provide evidence demonstrating consistent mucus and epithelium invasion by bacteria associated with biofilm-positive tumor homogenates in comparison to normal mucosa biopsies of healthy patients⁶⁰. These results elucidate the possible effect and relationship between individuals with intestinal disease and their harmful biofilm communities.

Additionally, Tomkovich et al. were able to not only identify different functional microbial communities between biofilm-positive and biofilm-negative homogenates, but also between mouse models when investigating their functional communities 12 weeks after inoculation⁶¹. This information provides a basis for confirming the compositional differences between microbial communities within biofilm layers and those residing in the normal mucosa of the intestine.

⁵⁸ Tomkovich et al., "Human Colon Mucosal Biofilms from Healthy or Colon Cancer Hosts Are Carcinogenic."

⁵⁹ Tomkovich et al.

⁶⁰ Tomkovich et al.

⁶¹ Tomkovich et al.

Furthermore, analyses conducted by Dejea et al. investigating the organizational role of microbiota in proximal colorectal cancers, revealed that the composition of the microbial community of paired normal mucosa samples of colorectal patients clustered with those of tumor microbiome communities⁶². Their findings provide for a possible connection between microbial community structure and disease progression⁶³. The scientists were also able to demonstrate that the presence of dense colonic biofilm, found on colorectal tumors and adenomas, does not only correlate with bacterial tissue invasion, but also increases in cellular proliferation; a signal for oncogenic transformation, further suggesting the biofilms ability to surpass the intestinal inner-mucosal barrier and elicit a harmful response ⁶⁴.

Interestingly, findings made by the Durbán group, provide additional evidence supporting distinct differences between microbial members that reside in the colon of IBS individuals in comparison to the microbial members that comprise their fecal samples⁶⁵. The group not only identified a few significantly over-represented taxa in the fecal samples of IBS individuals paired against controls, but also revealed that mucosal bacterial taxa of IBS individuals to be statistically underrepresented when compared against controls ⁶⁶. Additional analyses conducted by the group identifying the composition of the microbial community within IBS individuals, provided supporting evidence that the intestinal community

⁶² Dejea et al., "Microbiota Organization Is a Distinct Feature of Proximal Colorectal Cancers."

⁶³ Dejea et al.

⁶⁴ Dejea et al.

⁶⁵ Durbán et al., "Structural Alterations of Faecal and Mucosa-Associated Bacterial Communities in Irritable Bowel Syndrome."

⁶⁶ Durbán et al.

assemblage of those with IBS only subtly differs to that of normal controls⁶⁷. The subtle differences in gut microbiota composition of IBS individuals in conjunction with the data supporting distinct differences between IBS fecal and mucosal microbial communities, will be paramount when conducting future analyses aimed at classifying the IBS -associated gut microbiome.

Aims & Objectives

As scientific findings have underlined the significance of the human gut microbiota in disease, it will be imperative to continue investigations into the mechanisms of microbial communities and their constituents within the body. The study portrayed in this thesis aims to reveal the relationship between endoscopic vs. microscopic images of biofilm-positive and biofilm-negative colonic tissue. This is achieved by investigating the bacterial surface area coverage amongst biofilm samples of the different cohorts: IBS, UC, in addition to healthy controls. This is made possible by employing fluorescence *in situ* hybridization, or FISH, on intestinal mucosal biopsies. FISH is an experimental procedure that allows for the “identification, visualization, and quantification of microorganisms in environmental and [medical] samples”⁶⁸. The procedure’s effectiveness lies in targeting the ribosomal RNA (rRNA) of the microbial organisms in question through the use of (in standard cases) mono-labeled oligonucleotide probes⁶⁹. The probes, once having paired with their corresponding target rRNA sequence, will enable later analyses

⁶⁷ Durbán et al.

⁶⁸ Lukumbuzya et al., “A Multicolor Fluorescence in Situ Hybridization Approach Using an Extended Set of Fluorophores to Visualize Microorganisms.”

⁶⁹ Lukumbuzya et al.

employing fluorescence microscopy, where the excitation of the fluorophore will make image capturing possible.

An additional aim is to set a visual identification and determination standard for medical practitioners when classifying biofilm characteristics endoscopically. These characteristics include “continuity” (describing the uninterrupted coverage of the biofilm layer), and “adherence” (describing the degree to which the biofilm adheres to the colonic epithelium). Furthermore, given the additional data of the sampled cohort’s presence to intestinal adenomas, investigations were conducted to elucidate their possible correlation in altering the amount of colonic mucosal bacteria. In meeting above-mentioned aims, the investigations within this thesis will not only set a standard for endoscopically positively diagnosing biofilm layers within individuals, but also contribute to biofilm characteristics within afflicted individuals (IBS, UC, and healthy controls).

Material & Methods

All samples obtained for experimental use and analyses within the project “Mukosale Biofilme bei Colitis ulcerosa / Mucosal biofilms in ulcerative colitis” were in accordance with the Ethics Committee of the Medical University of Vienna (ethics number 1617/2014).

Tissue Section Preparation

Methacarn (60 % CH₃OH, 30 % CHCl₃, 10 % CH₃COOH)-fixed biofilm and normal-mucosa biopsy samples were prepared for FISH experiments by initially cutting each sample with a microtome, 240 µm in depth, yielding a total of 60, 4 µm sections. The 4 µm sections were placed 4 sections per super frost ultra plus slides, yielding a total of 15 slides per biopsy. Slides 1, 5, 10, and 15 were selected for haematoxylin and eosin (H&E) staining, in order to visualize which region of the biopsy would provide the best representation of biofilm coverage (interpreted by the presence of mucus) on which to conduct subsequent FISH analyses. Slides were stored at 4°C if not processed immediately.

H&E Staining:

Preparation

Slides that were selected for H&E staining were deparaffinized in two washes of xylene for 3 minutes each. Thereafter the slides were rehydrated in 6 successive washes of ethanol, in decreasing amounts respectively (100 %, 100 %, 95 %, 80 %, 70 %, 50 %). This was followed with a 3-minute wash in aqua distillata. The slides were then exposed to a 5-minute bath of pre-made Hematoxylin Mayer's Solution to allow for nuclei staining. The slides were then differentiated in HCl-alcohol (70 % EtOH, 37 % HCl) for 3-4 seconds before being rinsed with warm tap water, until a blue hue was reached. This step was followed by a 3-minute bath in an Eosin Y (0.1 %) solution for cytoplasmic staining. Slides were rinsed again with water and afterwards dehydrated with 6 increasing washes of ethanol respectively (50 %, 70 %, 80 %, 95 %, 100 %, 100 %). Finally the slides underwent one wash with N-

butylacetate before they were covered with histofluid medium and allowed to dry for 24 hours before image analyses.

Sample Selection

H&E prepared slides were analyzed employing an Olympus BX51 microscope at 40x, 100x, and 200x magnification. Upon microscopy, the sections in question were screened for the presence/absence of a mucus layer that would be a promising target for subsequent FISH analyses. Regions that were positively identified for having a mucus layer in contact with the epithelium were recorded and imaged under 40x, 100x, and 200x magnification and stored for later comparison and analyses. For biopsy samples, in which the initial 240 μm depth yielded a negative result for the presence of a mucosal (biofilm) layer, a subsequent preparation of slides was prepared according to the procedure mentioned above. Having identified the region of the biopsy (1,5,10,15) that would yield the most conclusive result, a neighboring prepared slide ((2,3,4)(6,7,8,9)(11,12,13,14)) was selected for FISH analyses.

Fluorescence in situ Hybridization (FISH):

Preparation

The selected slides were taken and baked in a 56°C oven for 1 hour in order to prime them for the deparaffinization process, which was completed by subjecting the slides to 2 washes of xylene for 10 minutes each. The slides were then rehydrated in successive ethanol washes at 95 % and 90 % respectively for 10 minutes each. Lastly, the slides were rehydrated for 10 minutes in Milli Q water, before they were subsequently air-dried with an air stream. The slides were then

taken and all individual biopsy sections encircled with a Liquid Blocker Pap Pen, in order to ensure the placement of the proceeding 19 μ l of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 7.2, 0.1 % SDS, 10 % formamide). When preparing the hybridization buffer, the probe's respective optimal formamide range (FA %) was taken into consideration, describing the percentage of formamide in hybridization buffer at which the probe is still effective. Once all sections were treated with hybridization buffer, 1 μ l of a EUB338 universal bacterial-oligonucleotide probe mix (5 μ M) (**Table 1**), was added to each section, resulting in an end concentration of 0.25 μ M for each probe. The prepared slides were subsequently placed into a hybridization chamber, which consisted of a 50 ml tube that had been prepared by adding 900 μ l of hybridization buffer to a small piece of paper within the chamber in order to prevent dehydration. The sealed chambers were placed into a 46°C hybridization oven for 16 hours, and thereafter rinsed at 48°C in a wash buffer (0.45 M NaCl, 20 mM Tris pH 7.2) for 10 minutes. This procedure was followed by a quick dip into ice-cold Milli Q water and subsequently air-stream dried. To allow for the staining of DNA, each individual section was then exposed to 30 μ l of DAPI solution (0.1 μ g/ml PBS) in the dark for 10 minutes. This was followed by a subsequent dip in ice-cold Milli Q water and thereafter air-stream dried. If the samples were not immediately analyzed, they were stored in the dark at -20°C.

Table 1. Characteristics of Employed Oligonucleotide Probes used for FISH

Probe name	Specificity	Probe sequence (5'->3')	Fluorophore	FA (%)	Reference
EUB338-I	Universal bacteria	GCTGCCTCCCGTAGGAGT	Cy3/Fluos	0-50	⁷⁰
EUB338-II	<i>Planctomycetales</i>	GCAGCCACCCGTAGGTGT	Cy3/Fluos	0-50	⁷¹
EUB338-III	<i>Verrucomicrobiales</i>	GCTGCCACCCGTAGGTGT	Cy3/Fluos	0-50	⁷²

Use of Pretreatments

In attempts to enhance the binding efficacy of the EUB338 universal bacterial oligonucleotide probe mix, and thereby yield higher bacterial signal intensity during confocal laser microscopy, the use of four different pretreatments were employed: Triton X-100, 0.1 M HCl, lysozyme, and proteinase K. The above-mentioned protocol for FISH preparation, was followed for processing the slides, however the pretreatment steps were completed after encircling the sections with the Liquid Blocker Pap Pen but before applying the hybridization buffer. Triton X-100 (0.3 % in PBS) was employed by placing 30 µl of solution to sections for 5 minutes at room temperature before rinsing with Milli Q water and continuing with hybridization. The same procedure was followed for the use of 0.1 M HCl, however the sections were rinsed with 1xPBS instead of Milli Q water. The use of lysozyme (0.01 g + 100 µl 0.5 M EDTA + 100 µl 1 M Tris/HCl + 800 µl Milli Q water) required the sections to be exposed to 30 µl of working solution within a chamber at 37°C for 60 minutes before a Milli Q water rinse and continuation of hybridization. Employing proteinase

⁷⁰ Amann et al., "Combination of 16S RRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations."

⁷¹ Daims et al., "The Domain-Specific Probe EUB338 Is Insufficient for the Detection of All Bacteria."

⁷² Daims et al.

K (dilution of 15 µl stock (10 mg/1 ml PBS) in 1 ml 1xPBS) called for the application of 30 µl of working solution for 10 minutes at 37°C before rinsing with Milli Q water and proceeding with hybridization.

Analyses

FISH analyses were conducted on an inverted Leica TCS SP8X Confocal Laser Scanning Microscope (CLSM, Leica). This microscope is “equipped with a 405 nm UV diode, a Leica supercontinuum white light laser, two photomultiplier (PMT) detectors, and three hybrid (HyD) detectors...” as well as “... a fixed wavelength diode laser (WLL)...” which was set to 70 % intensity and used to excite the fluorophores at their “respective excitation wavelengths”⁷³. Before mounting the slides with a coverslip for microscopy, they were embedded with 20 µl of Citifluor medium (glycerol in PBS), which created a uniform refractory index for imaging. A subsequent addition of glycerol to the slide surface allowed for visualization with the microscope’s HC PLAPO CS2 objective lens. Leica Application Suite X (LAS X) software was employed to capture and process FISH images under 63x magnification with a 1.30 numerical aperture, specifically at regions where mucus or mucosal biofilms were present. Before proceeding with image capturing, a coverslip correction was performed by visualizing and manipulating the “Y” axis. Leica Lightning Software was thereafter employed under 3432x3432 format at a speed of 700 Hz. The software also enabled the capturing of three-dimensional (3D) image stacks, referred to as “z-stacks”, which make it possible to record the 3D

⁷³ Lukumbuzya et al., “A Multicolor Fluorescence in Situ Hybridization Approach Using an Extended Set of Fluorophores to Visualize Microorganisms.”

fluorescence signal at a given position⁷⁴. Z-stacks were employed in these analyses, with stacks ranging between 3 - 12 image layers, with an axial distance of 0.33 μm between images. All images were captured under a smart gain of 100 % in order to enable clear structural differentiation and reduce image noise. Images of DNA stainings (DAPI) were captured at a wavelength region of 415 nm – 549 nm at 3 % laser intensity, while universal bacterial probe mix (EUB338 I, II, III) stainings were captured at a wavelength region of 564 nm-737 nm at 8 % laser intensity. The LAS X software was used to false-color fluorophores of images captured during analyses (DAPI (pink) EUB338 I, II, III mix (light blue)). All images used in further computational analyses were saved as maximum intensity projections (MIPs), or a composite image of the z-stack image layers, in order to wholly represent the area under observation⁷⁵.

Image Data Collection

The images obtained using the CLSM were subsequently investigated employing the computer software program Image J, version 2.0.0-rc-69/1.52p. The images were uploaded to the Image J software system, where respective pixel and distance measurements were set to scale (144.72 μm x 144.72 μm or 98.02 μm x 98.04 μm). Afterwards, the areas of each image that contained bacteria were hand-selected (traced) and the computed surface area values (μm^2) were recorded. The selected bacterial areas were then saved separately to an internal clipboard, where

⁷⁴ Lukumbuzya et al.

⁷⁵ Lukumbuzya et al.

further analyses will take place to identify the quantity of bacteria within each respective area.

Data Processing and Investigation

Data gleaned from image processing was uploaded and analyzed using the software program RStudio, version. 1.1.383. The program allowed for the organization and calculation of collected image data, in order to produce graphs and corresponding figures. Tests for data normality were conducted using the Shapiro-Wilk's function within RStudio. The Kruskal-Wallis test was later employed to calculate significant differences between biopsy sample types, while the pairwise Wilcoxon rank sum test was used to calculate pairwise comparisons between type classifications. The same above-mentioned functions were used to investigate levels of normality and significance amongst biofilm-positive cohorts in respect to disease status, degree of biofilm adherence, and degree of biofilm continuity.

Results

To investigate macroscopic and microscopic biofilm presence as well as possible roles that mucosal biofilms may have in UC and IBS, a similar number of samples were obtained from each patient cohort. **Table 2** provides a detailed characterization of the intestinal biopsies sampled, including disease cohort (UC, IBS, or control) as well as whether the sample was from a biofilm, the normal mucosa of a biofilm-positive patient, or the normal mucosa of a biofilm-negative patient (sample type).

Table 2. Characteristics of Analyzed Samples

Characteristic	Controls	IBS patients	UC patients
No. of biofilm samples analyzed	6	10	13
No. of normal mucosa samples analyzed from biofilm-positive patients	7	9	12
No. of normal mucosa samples analyzed from biofilm-negative patients	10	14	3

Numeric data characterizing the bacterial surface area coverage in μm^2 of the biopsies by sample type (normal mucosa from a biofilm-negative individual (NM⁻), normal mucosa from a biofilm-positive individual (NM⁺), and biofilm biopsy (B⁺)) can be found in **Table 3**. The non-normal distribution of data from each biopsy type can be seen in **Figure 7 a, b, and c** respectively. A Kruskal-Wallis test determined

that there is a significant difference between biopsy sample types (p-value = 0.0018). Wilcoxon rank sum tests provided that there is a significant difference between biofilm biopsies in comparison to normal mucosa biopsies of biofilm-positive individuals (p-value = 0.0332) and biofilm-negative individuals (p-value = 0.0039). Graphical representation of biofilm surface area coverage in relation to biopsy sample type can be seen in **Figure 1**.

Table 3. Surface Area Statistics (μm^2) of Analyzed Samples by Biopsy Sample Type

Characteristic	NM-	NM+	B+
Min.	0	0	0
1 st Quartile	453	695	4294
Median	875	2646	7966
3 rd Quartile	4368	7232	18486
Max.	41154	22291	57260
Normal Y/N (p-value)	N (6.4e ⁻⁰⁹)	N (0.0001)	N (2.27e ⁻⁰⁵)

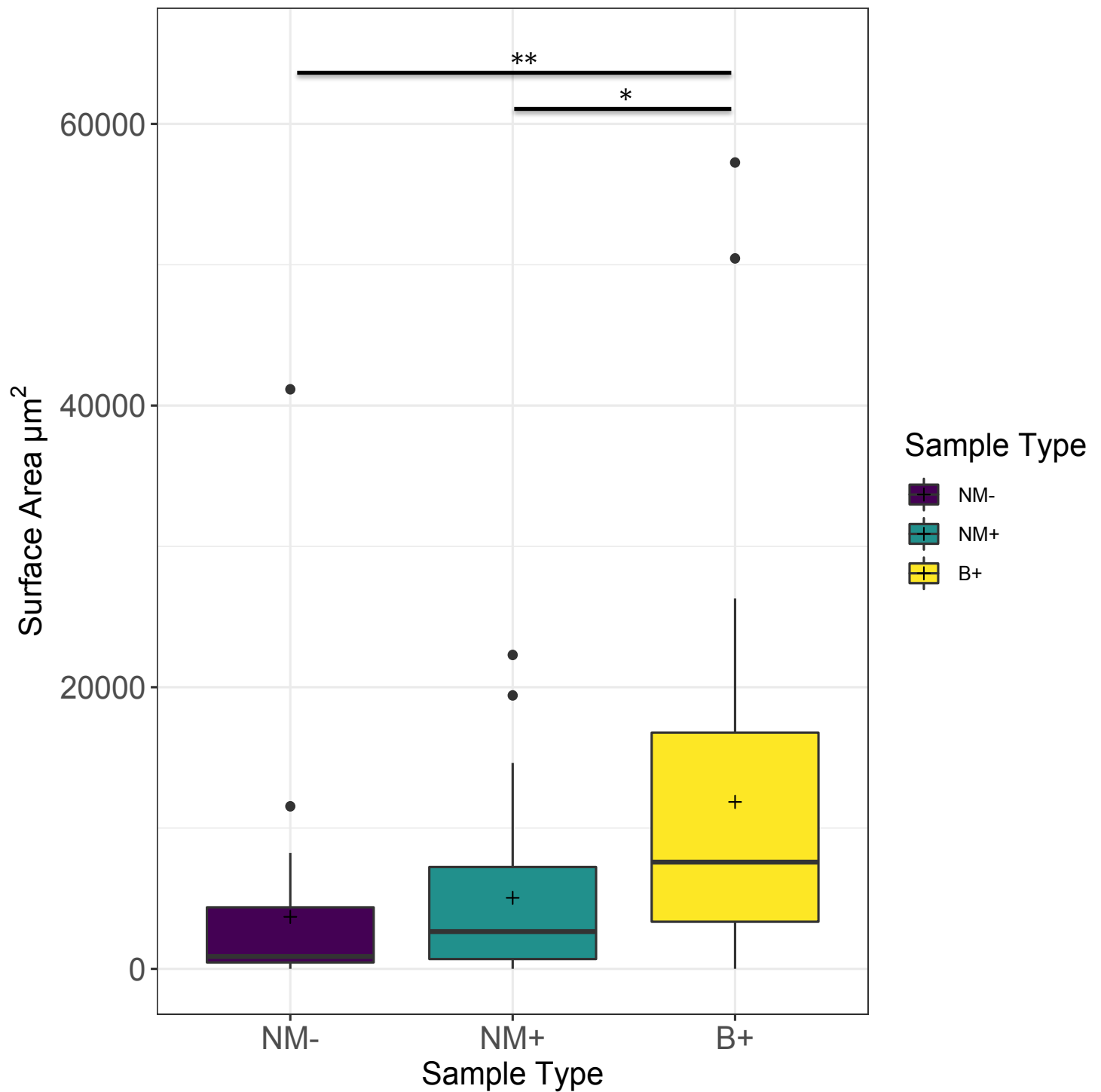


Figure 1. Biofilm Surface Area Coverage (μm^2) by Sample Type. The above boxplot depicts surface area, which is covered by bacteria (μm^2) of each biopsy sample type: normal mucosa from biofilm-negative patients (NM⁻), normal mucosa from biofilm-positive patients (NM⁺), and biofilms (B⁺). A significant difference between biopsy types B⁺ and NM⁻ (p-value: 0.0039) as well as between B⁺ and NM⁺ (p-value: 0.0332) was found. The crosshairs represent the means of the data and the solid bars the medians.

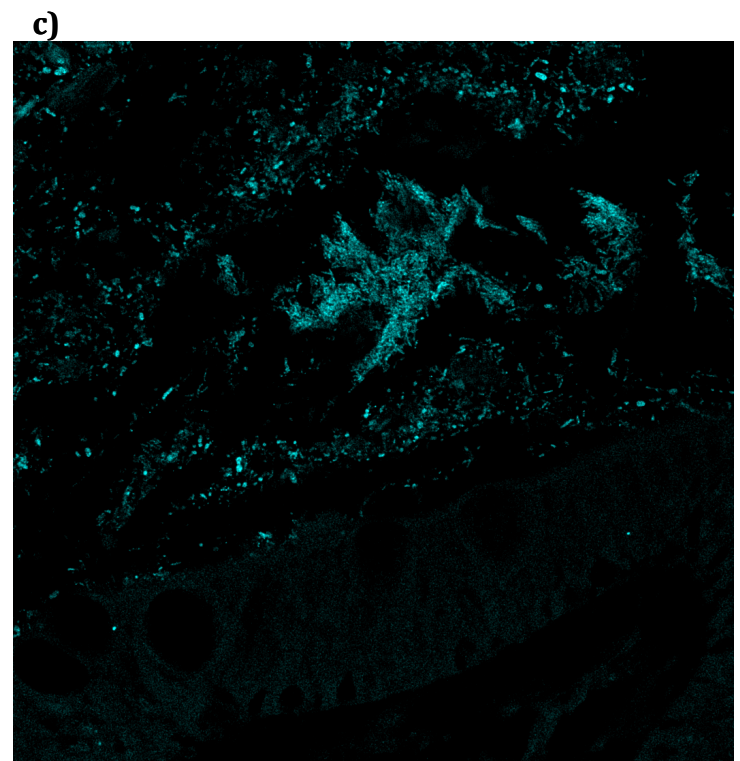
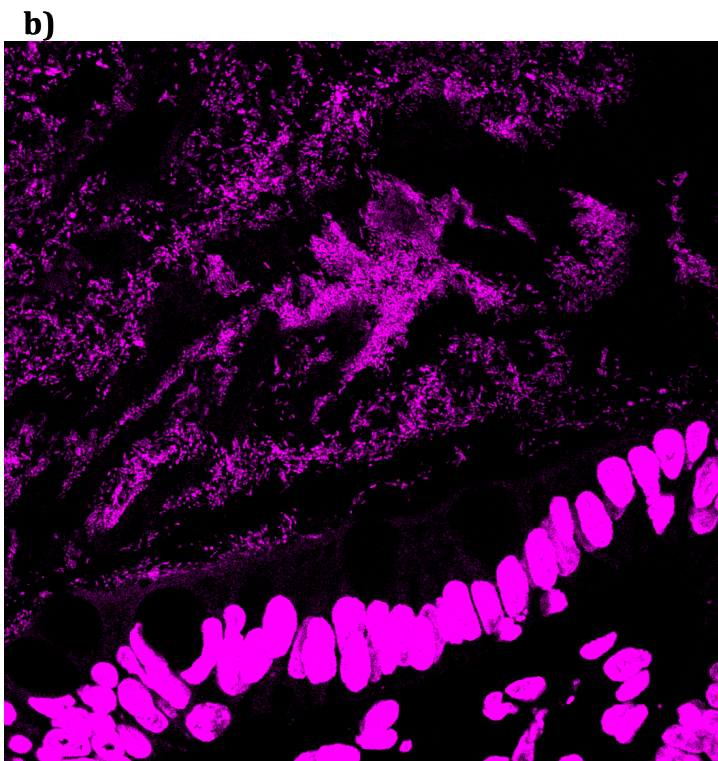
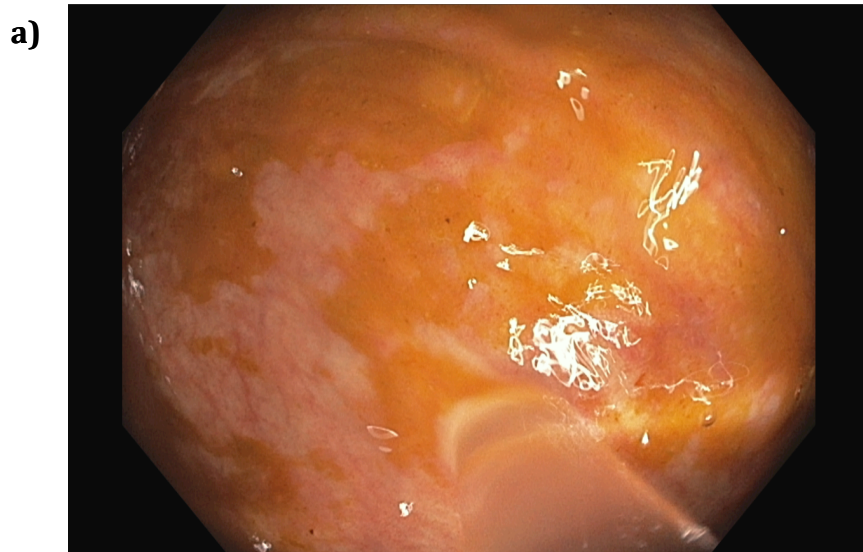


Figure 2. Biofilm Images from a B⁺ Patient. **a)** Endoscopic image of a B⁺ patient intestinal biofilm from which a biopsy specimen was obtained. The light pink color indicates the epithelium and the yellow-brown layer, which covers most of the epithelial surface, is considered to be the biofilm layer (degree of continuity 2). **b) & c)** Microscopic images of the B⁺ patient's colonic biopsy specimen. **b)** is an image stained with DAPI (pink) revealing a continuous epithelial border and a thick biofilm layer residing on the epithelial surface. **c)** is stained with EUB338 universal probe mix (light blue) confirming bacterial presence.

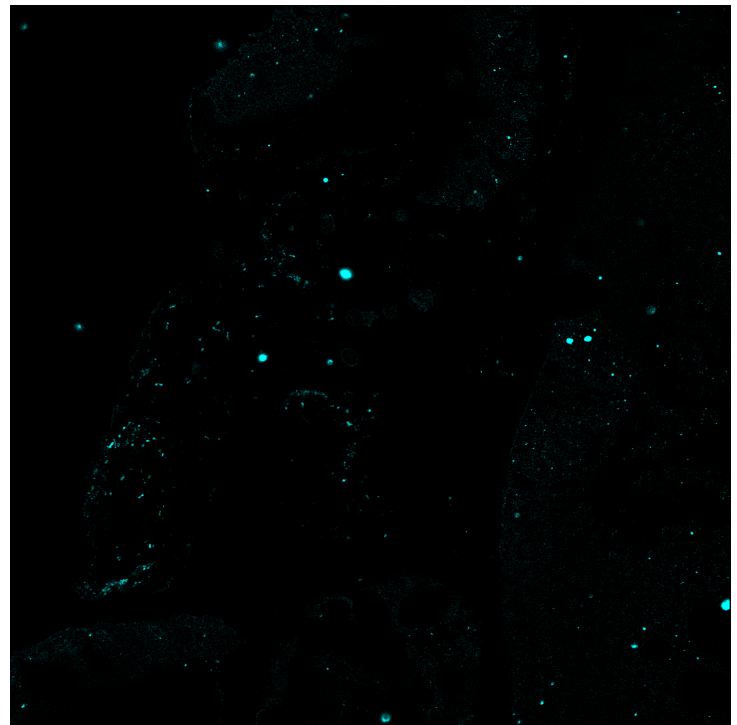
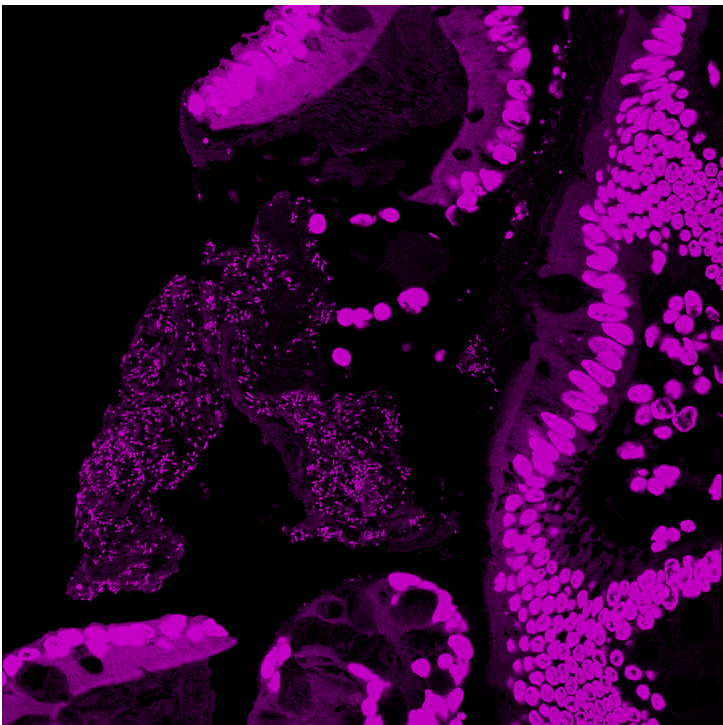
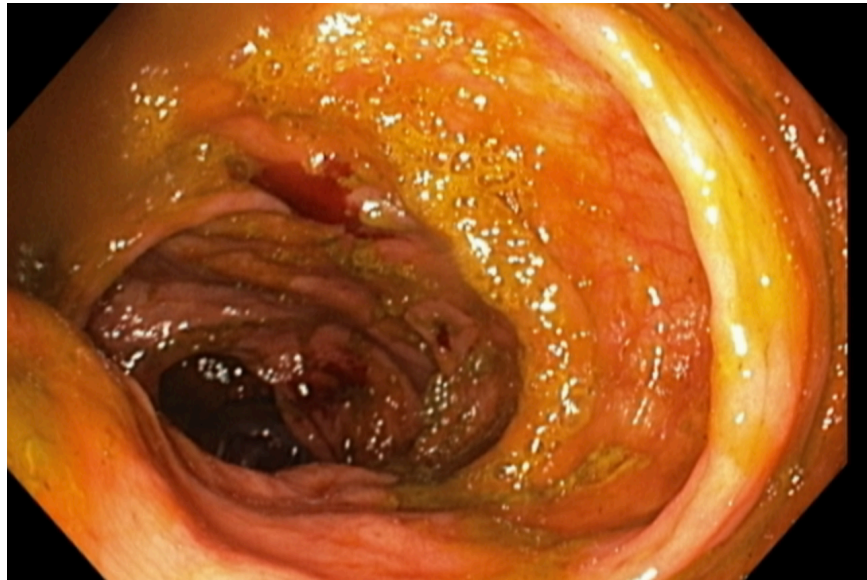


Figure 3. Biofilm Images from a B⁺ Patient. **a)** Endoscopic image of a B⁺ patient intestinal biofilm from which a biopsy specimen was obtained. The light pink color indicates the epithelium and the thick yellow-brown layer, which covers most of the epithelial surface, is considered to be the biofilm layer (degree of continuity 3). **b) & c)** Microscopic images of the B⁺ patient's colonic biopsy specimen. **b)** is an image stained with DAPI (pink) revealing a continuous epithelial border and a thick biofilm layer residing above the epithelial surface. **c)** is stained with EUB338 universal probe mix (light blue) confirming bacterial presence (weak signal intensity).

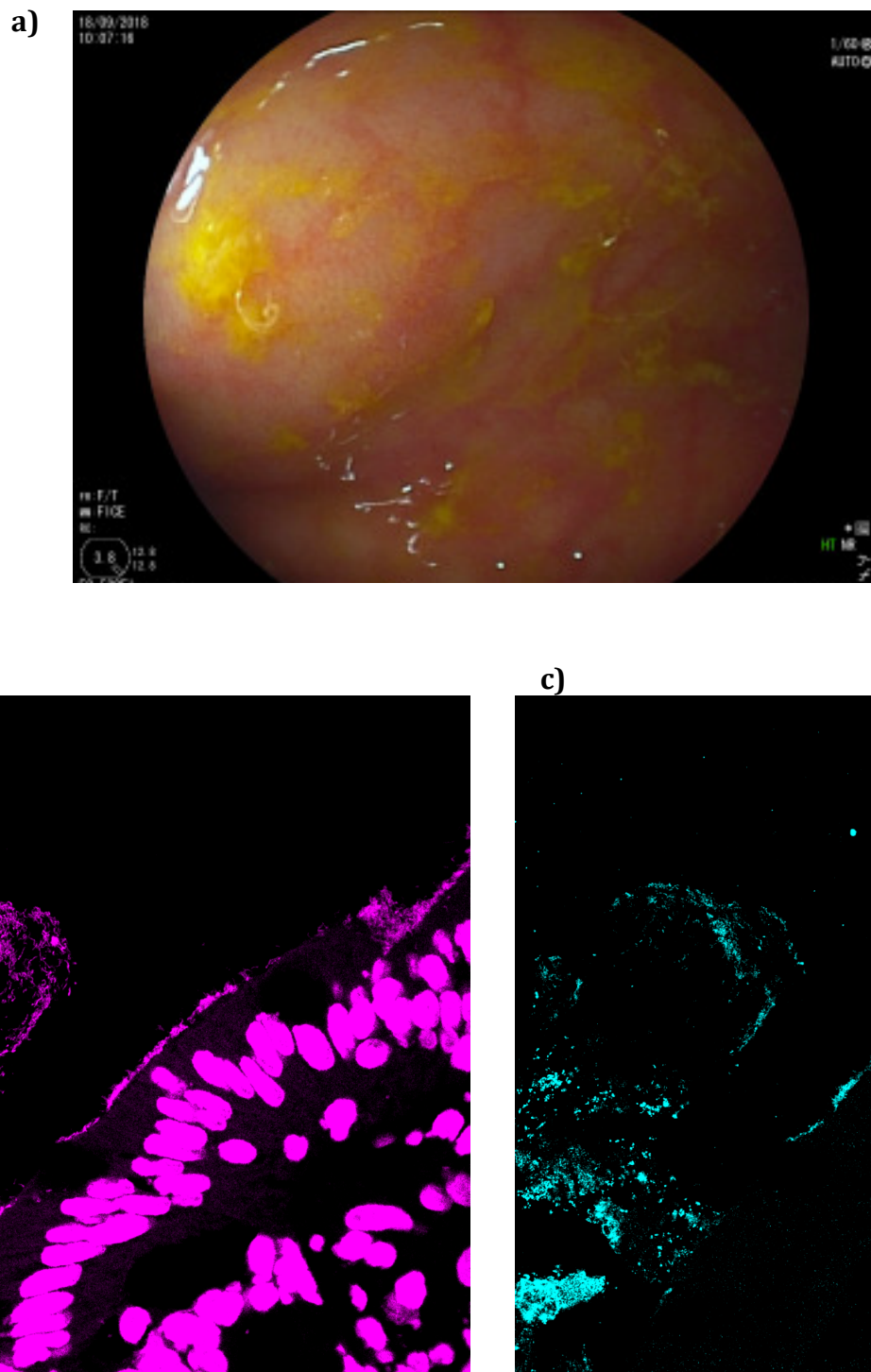
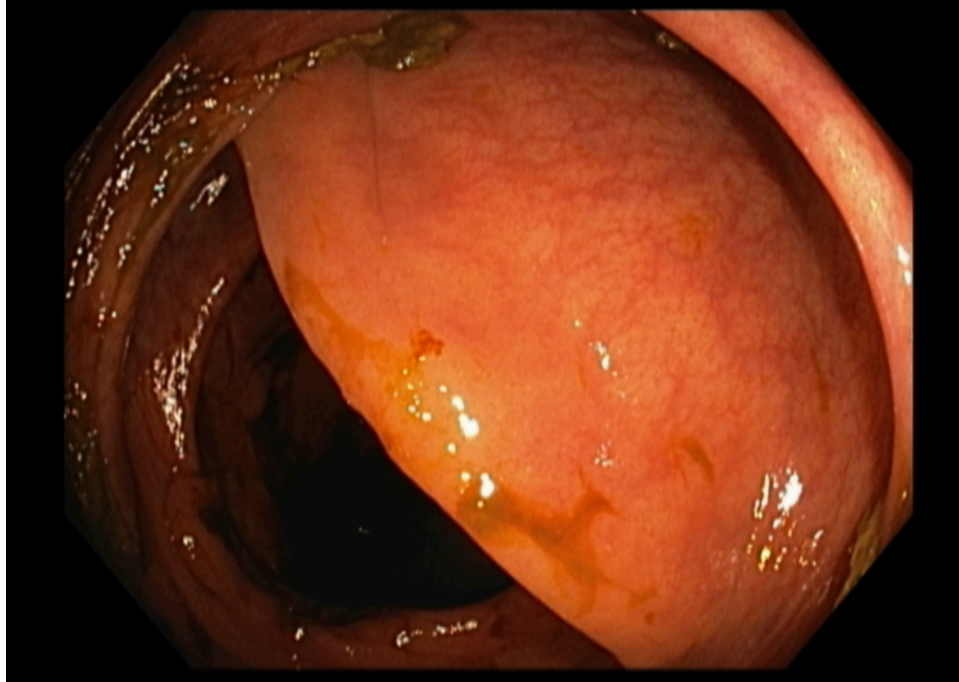
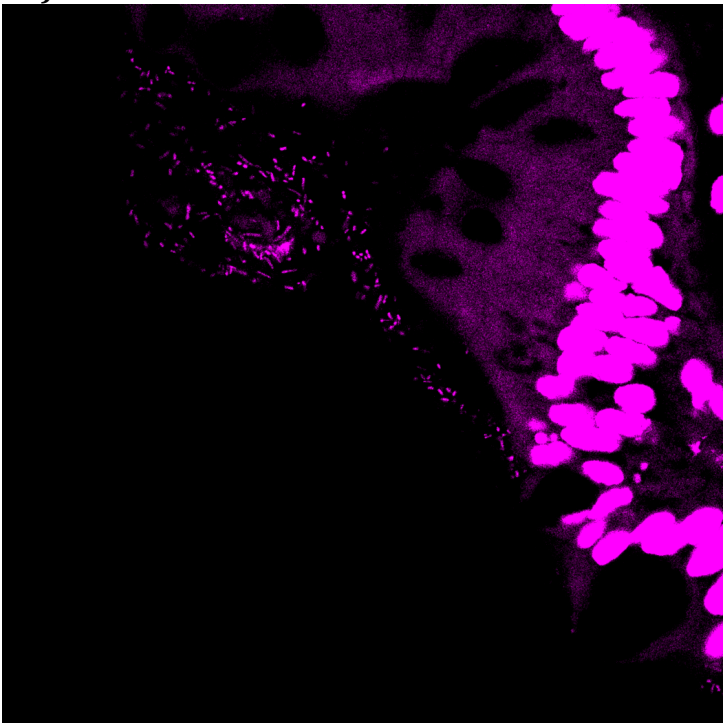


Figure 4. Biofilm Images from a B⁺ Patient. **a)** Endoscopic image of a B⁺ patient intestinal biofilm from which a biopsy specimen was obtained. The light pink/red color indicates the epithelium and the yellow layer is considered to be a patchy biofilm layer (degree of continuity 1). **b)** & **c)** Microscopic images of the B⁺ patient's colonic biopsy specimen. **b)** is an image stained with DAPI (pink) revealing a continuous epithelial border and a biofilm layer residing on the epithelial surface. **c)** is stained with EUB338 universal probe mix (light blue) confirming bacterial presence.

a)



b)



c)

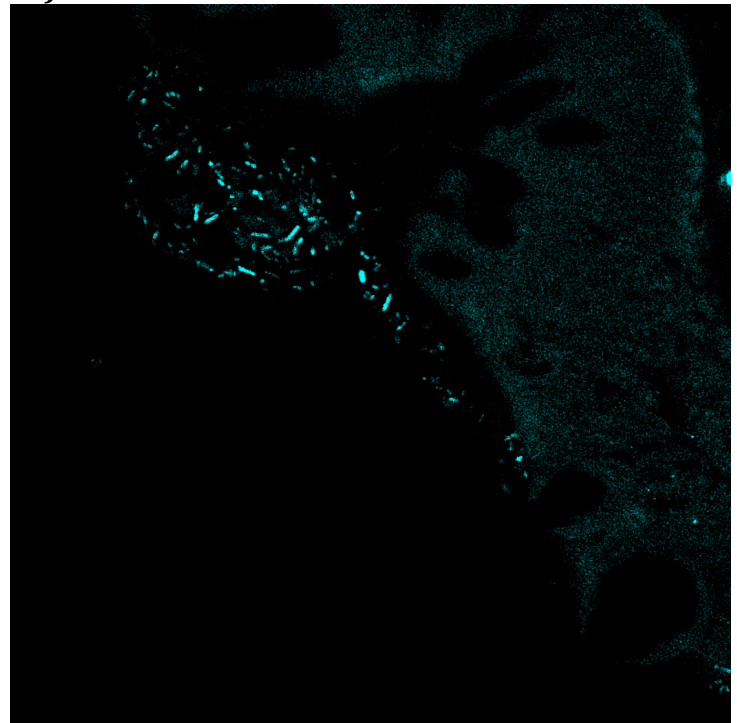


Figure 5. Normal Mucosa Images from a Biofilm-Positive Patient (NM⁺). **a)** Endoscopic image of a NM⁺ patient's normal mucosa from which a biopsy specimen was obtained. The light pink color indicates the epithelium with a translucent mucosa layer and a small patch of brown biofilm. **b) & c)** Microscopic images of the NM⁺ patient's colonic biopsy specimen. **b)** is an image stained with DAPI (pink) revealing a continuous epithelial border and a biofilm layer residing near the epithelial surface. **c)** is stained with EUB338 universal probe mix (light blue) confirming bacterial presence.

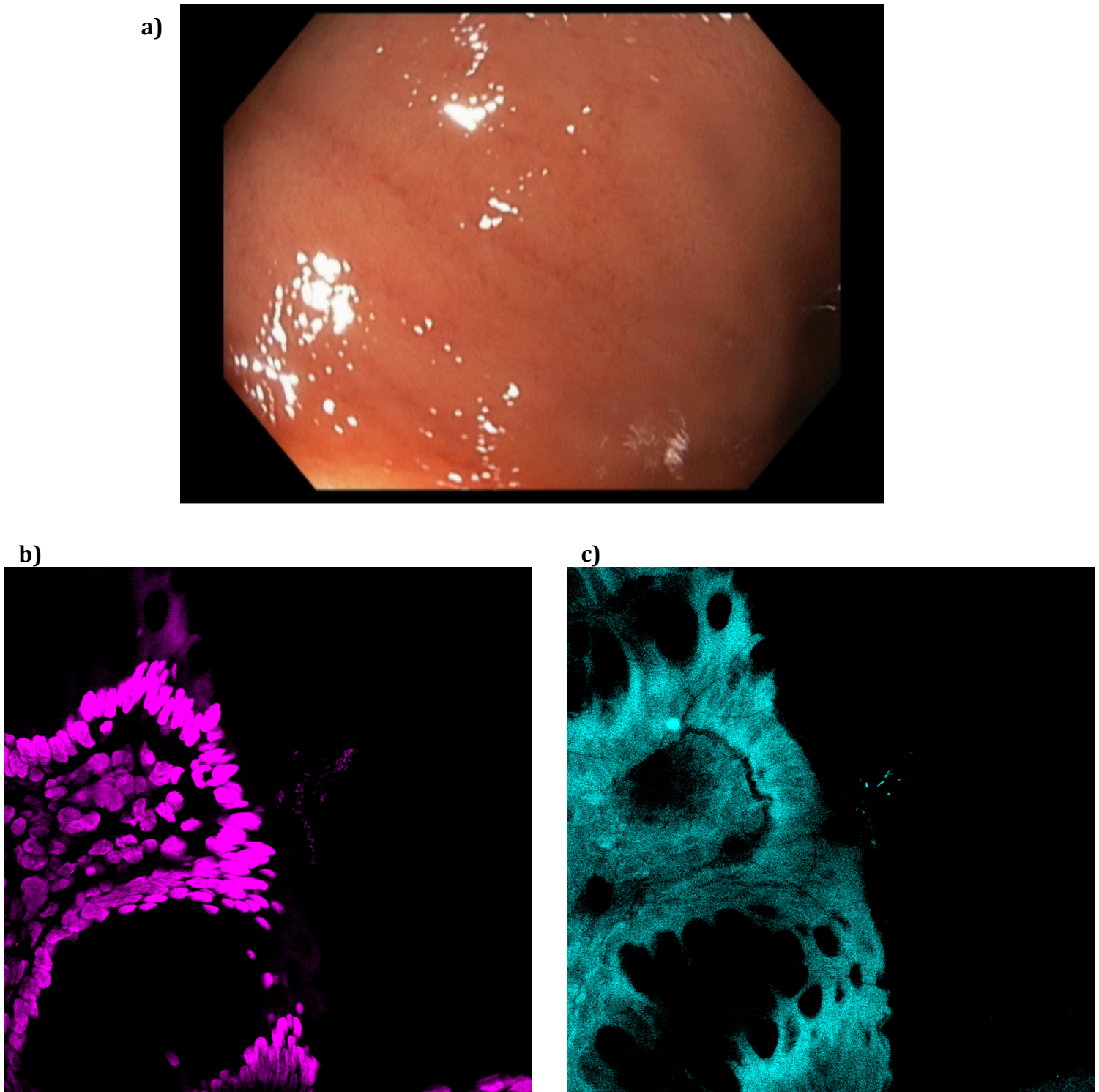


Figure 6. Normal Mucosa Images from a Biofilm-Negative Patient (NM-). **a)** Endoscopic image of a NM- patient's normal mucosa from which a biopsy specimen was obtained. The light pink color indicates the epithelium with a translucent normal mucosa layer. **b) & c)** Microscopic images of the NM- patient's colonic biopsy specimen. **b)** is an image stained with DAPI (pink) revealing a continuous epithelial border with a small amount of bacteria residing near the epithelial surface. **c)** is stained with EUB338 universal probe mix (light blue) confirming bacterial presence of a small number of bacteria.

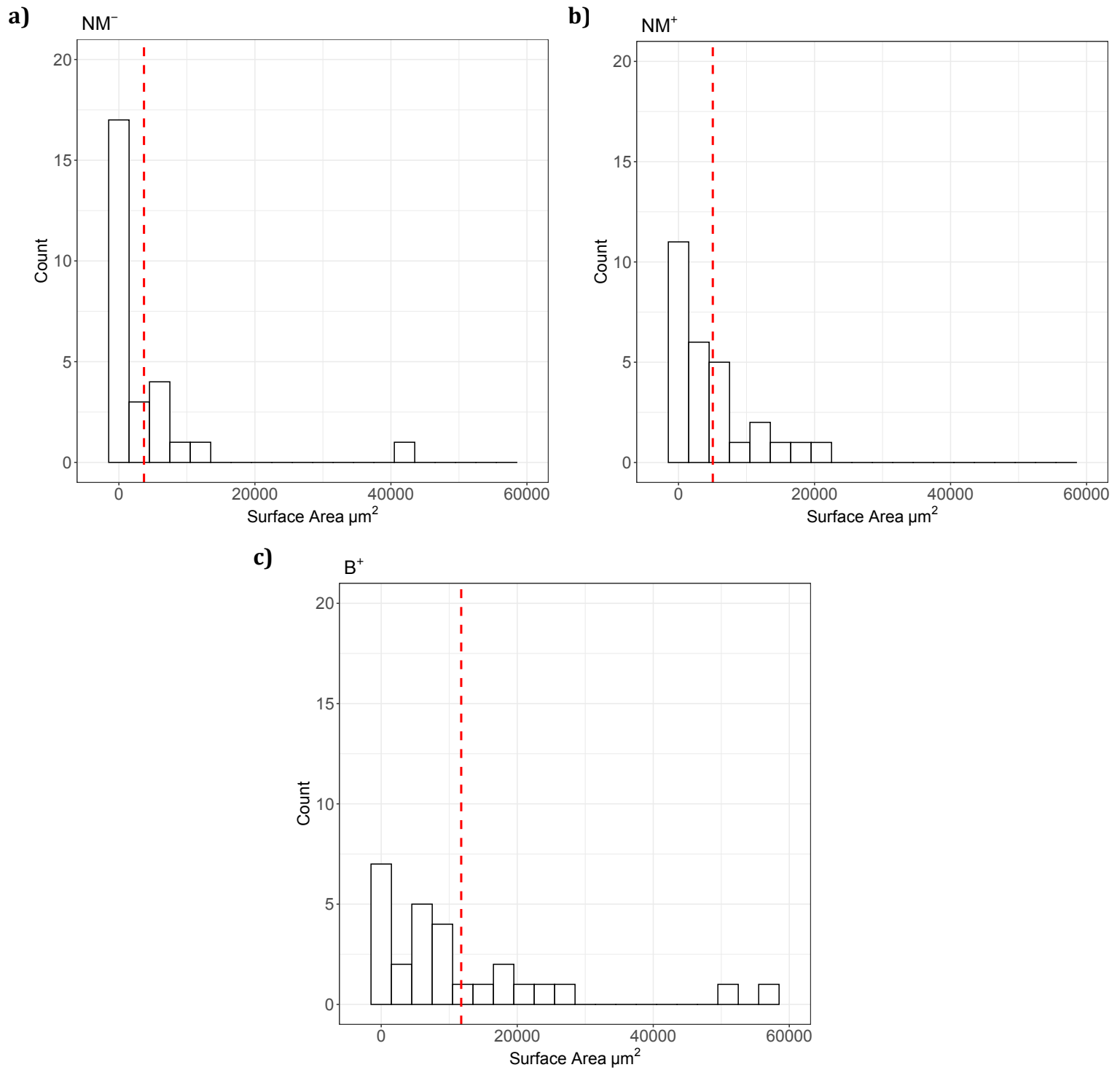
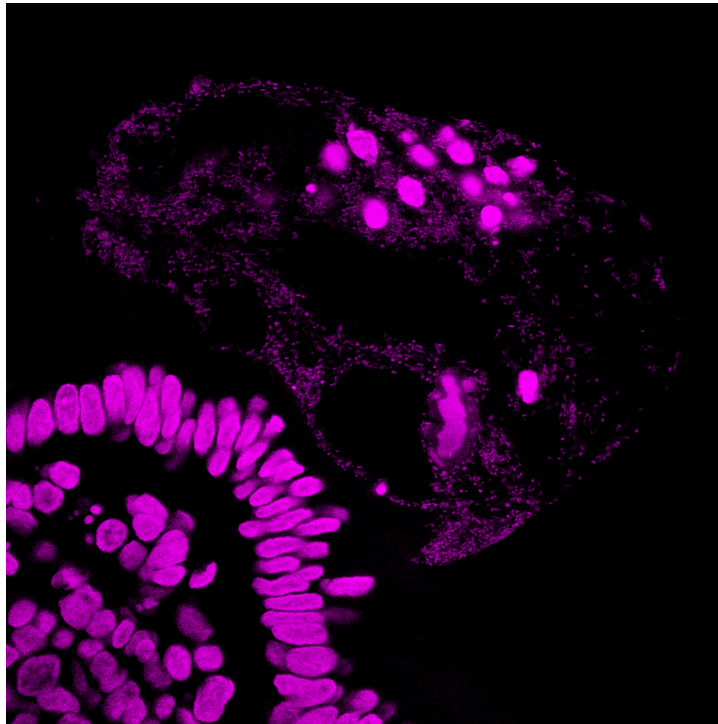
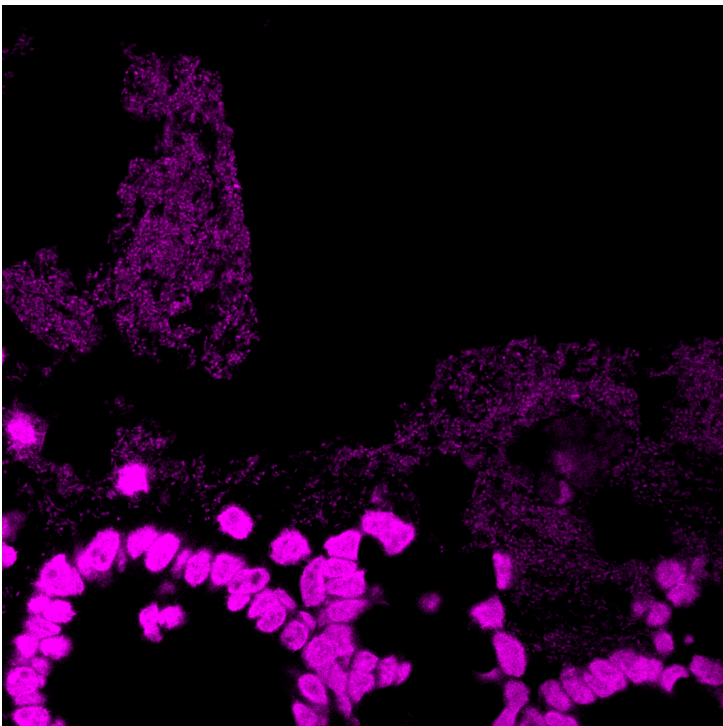


Figure 7. B⁺ Data Distribution Graphs in respect to Sample Type. The above graphs depict the distribution of surface area (μm^2) from each of the biopsy types independent of disease type: **a)** normal mucosa from biofilm-negative patients (NM⁻), **b)** normal mucosa from biofilm-positive patients (NM⁺), and **c)** biofilms (B⁺). The y-axis informs about patient counts while the x-axis informs surface area (μm^2) with a binwidth of 3000 (μm^2). The red-dashed line reveals the mean of the data in each graph.

a)



b)



c)

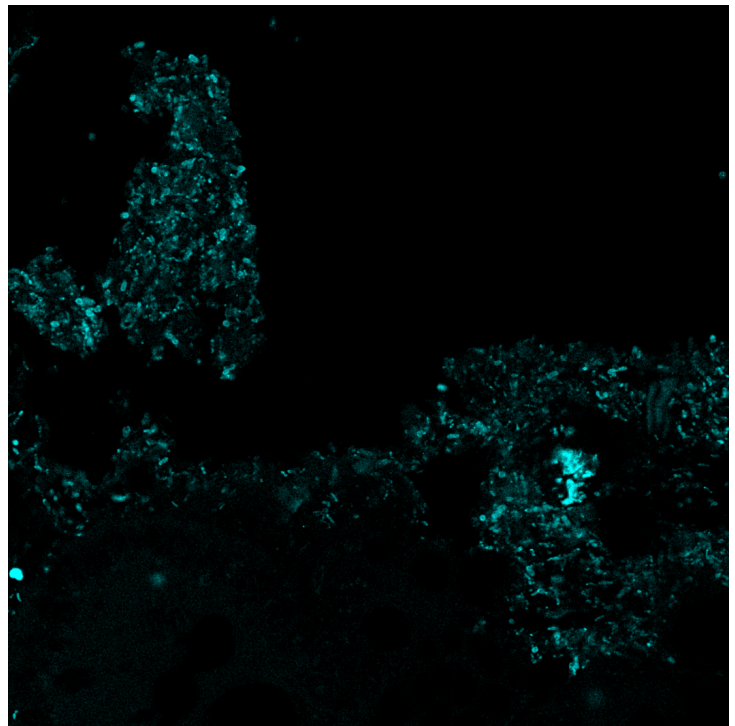


Figure 8. Additional Microscopic Biofilm Images. Microscopic images of B⁺ patients' colonic biopsy specimens. **a) & b)** are images stained with DAPI (pink) revealing a continuous epithelial border and a thick biofilm layer residing on the epithelial surface. **c)** is stained with EUB338 universal probe mix (light blue) confirming bacterial presence seen in **b)**.



Figure 9. Additional Microscopic Biofilm Images. Composite of microscopic image fields (tile image) of a B⁺ patient's colonic biopsy specimen. This tile image is stained with DAPI (pink) revealing epithelial border and thick biofilm layer residing above the epithelial surface.

Numeric data characterizing the biofilm surface area coverage of biofilm biopsies in regard to disease status (ulcerative colitis (UC), irritable bowel syndrome (IBS), and healthy control (cntrl)) can be found in **Table 4** below. The normal distribution of data from UC and IBS cohorts can be seen in **Figure 11 (a & b)**. **Figure 11 c)** depicts the non-normal distribution of the control cohort. Performing a Kruskal-Wallis test of the data determined that there is no significant difference between the disease cohorts (p-value = 0.7088) for biofilm size. Graphical representation of biofilm surface area coverage in respect to disease status can be seen in **Figure 10**.

Table 4. Surface Area Statistics (μm^2) of Biofilm Samples in regard to Disease Status

Characteristic	UC	IBS	Control
Min.	186	0	324
1 st Quartile	4013	4698	842
Median	7709	9807	3508
3 rd Quartile	10612	23943	11782
Max.	18711	50449	57259
Normal Distribution Y/N (p-value)	Y (0.4345)	Y (0.1362)	N (0.0031)

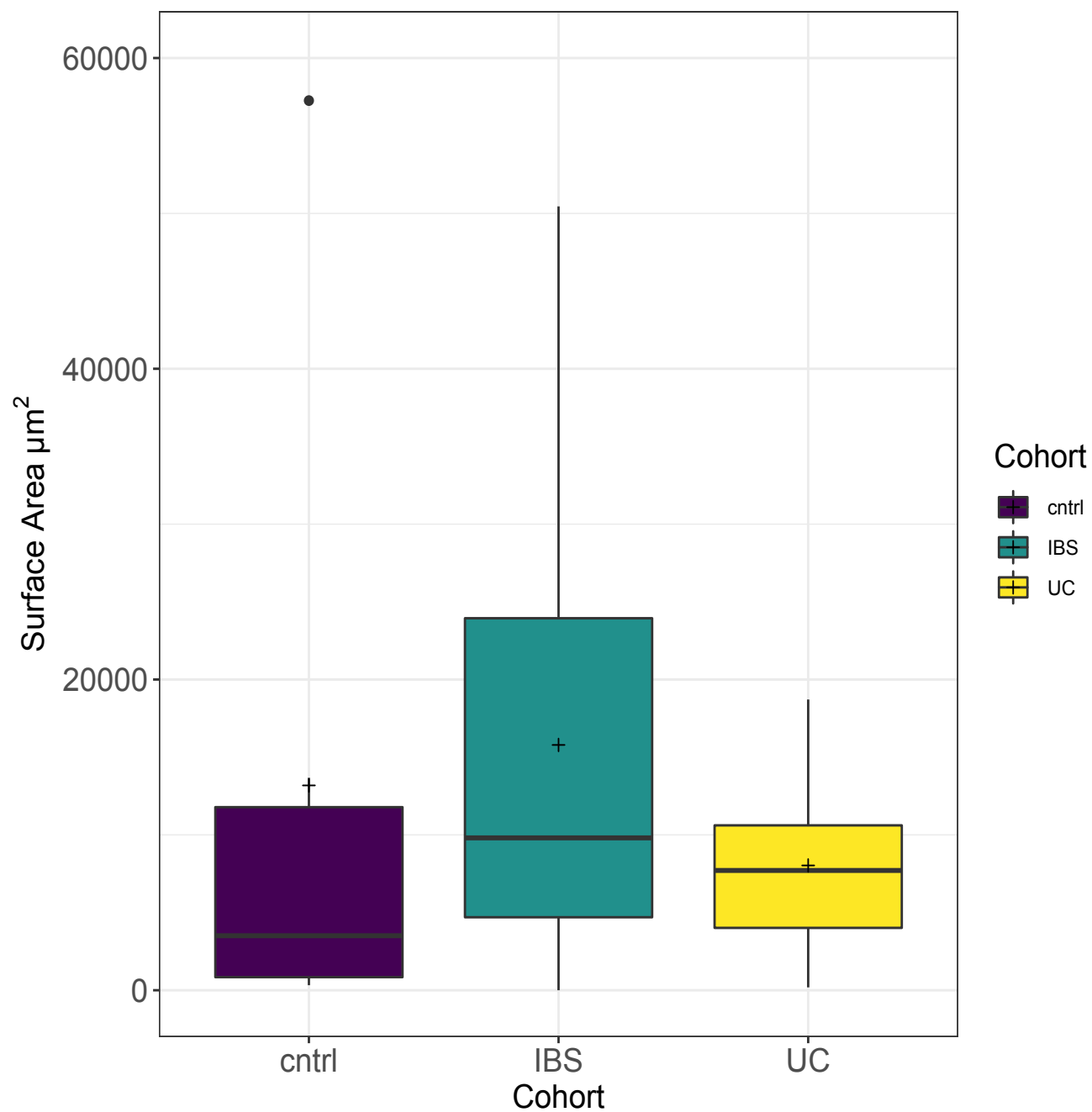


Figure 10. Biofilm Surface Area Coverage (μm^2) of Disease Cohorts. The above boxplot depicts biofilm surface area (in μm^2) in respect to each cohort: Control (cntrl), irritable bowel syndrome (IBS), and ulcerative colitis (UC). The crosshairs represent the means of the data and the solid bars the medians.

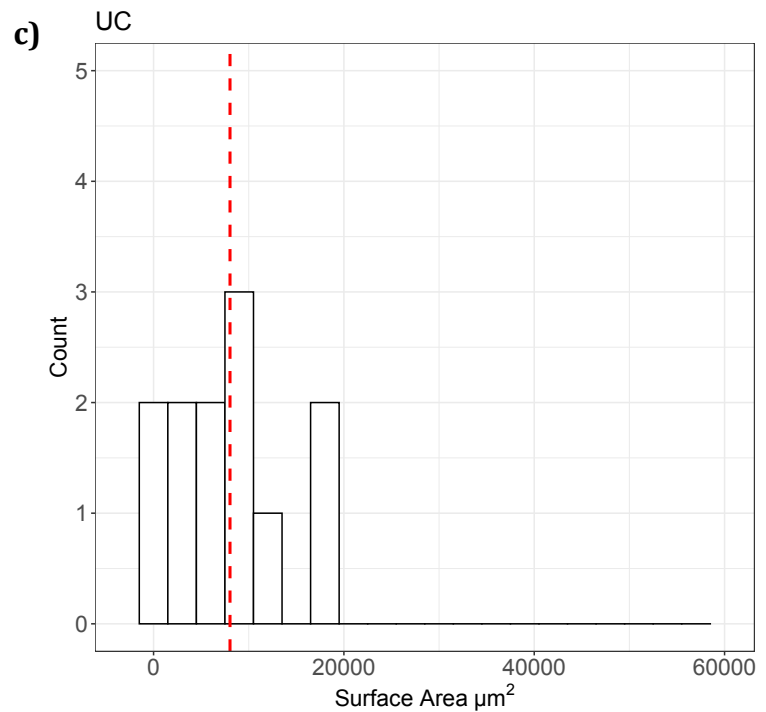
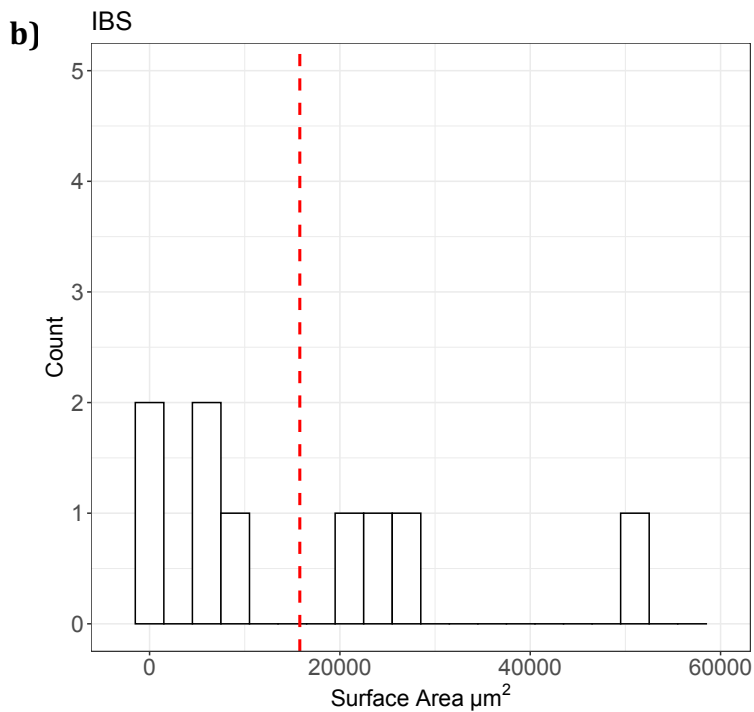
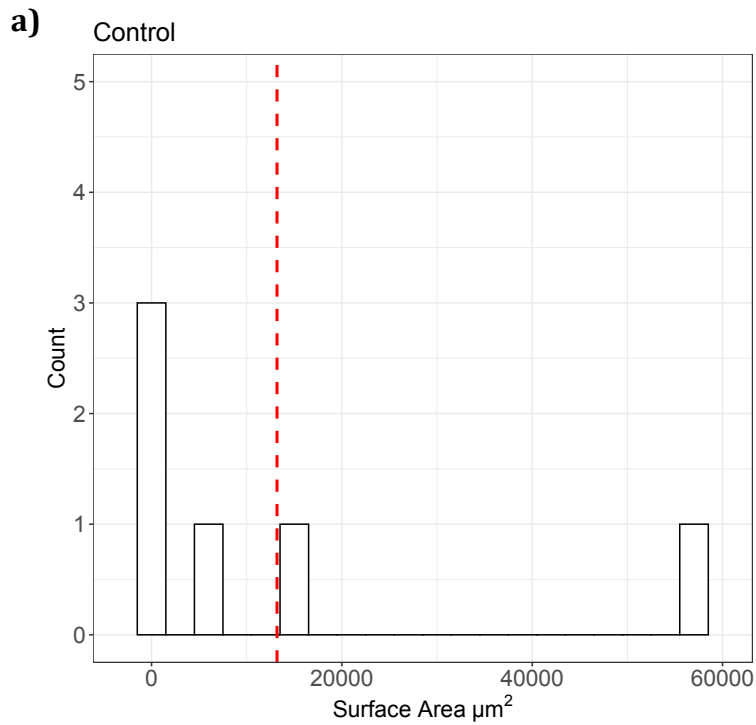


Figure 11. B+ Data Distribution Graphs in respect to Patient Cohort. The above graphs depict the biofilm surface area distribution (in μm^2) of each cohort: **a)** control (Control), **b)** irritable bowel syndrome (IBS) **c)** ulcerative colitis (UC). The y-axis informs about patient counts while the x-axis informs surface area (μm^2) with a binwidth of 3000 (μm^2). The red - dashed line reveals the mean of the data in each graph.

Numeric data characterizing the biofilm surface area coverage of biofilm biopsies in respect to degree of adherence (1, 2, or 3) can be found in **Table 5** below. The degrees of adherence can be understood as the biofilm's characteristic to stick, or adhere to the colonic epithelium. In theory, the higher the degree of adherence, the stickier the biofilm was to the epithelial surface, and thus more difficult to remove. The normal distribution of data from adherence degrees 1 and 3 can be seen in **Figure 13 a) & c)** respectively. **Figure 13 b)** depicts the non-normal distribution of the degree of adherence 2. A Kruskal-Wallis test determined that there is no significant difference between degrees of adherence (p-value = 0.7806) of the biofilm samples. Graphical representation of biofilm surface area coverage in respect to degree of adherence can be seen in **Figure 12**.

Table 5. Surface Area Statistics (μm^2) of Biofilm Samples by Degree of “Adherence”

Characteristic	1	2	3
Min.	764	0	267
1 st Quartile	2731	1766	6990
Median	4698	7710	12084
3 rd Quartile	30978	16098	21376
Max.	57259	50449	26298
Normal Distribution Y/N (p-value)	Y (0.1192)	N (0.0017)	Y (0.7163)

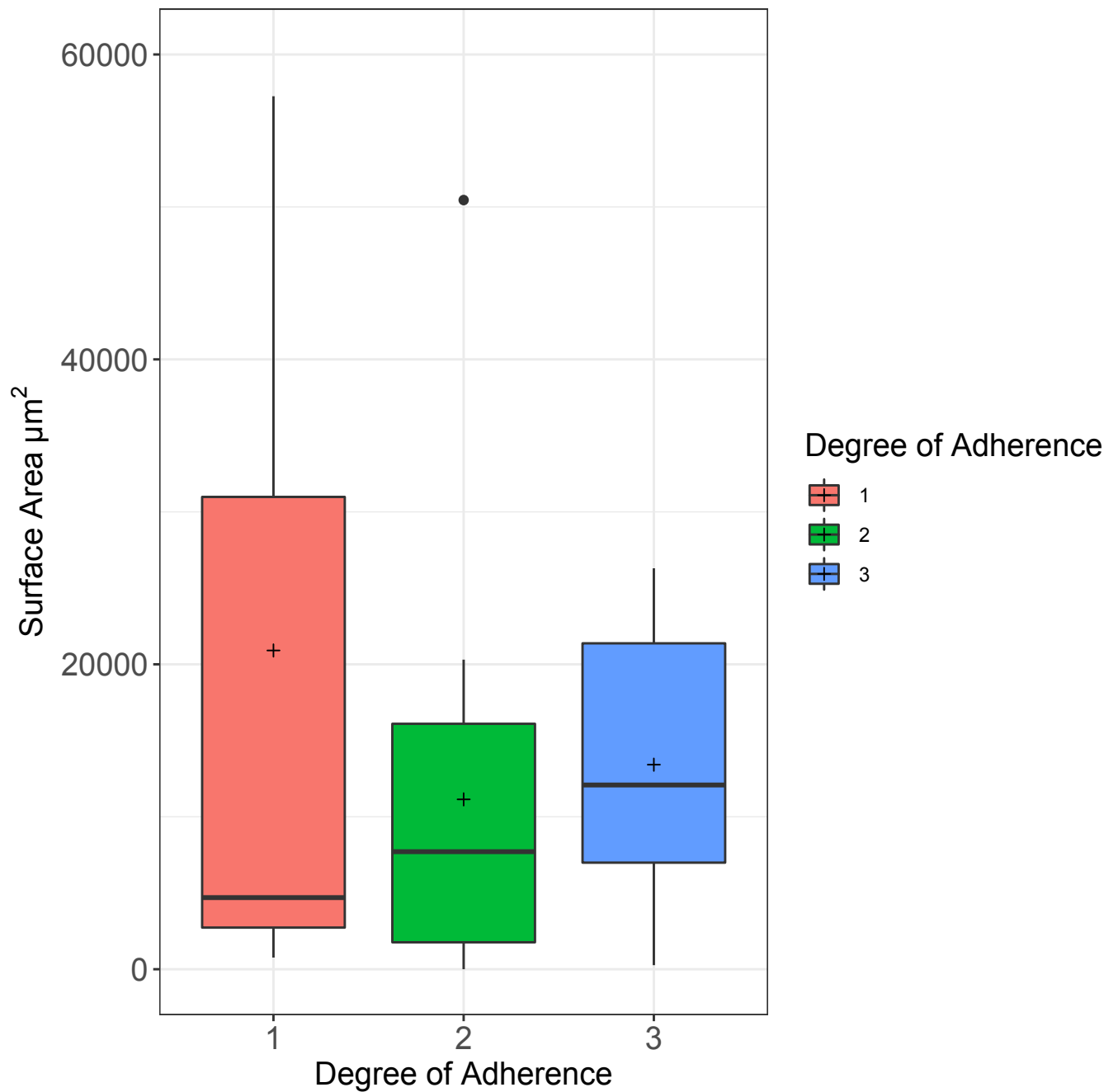


Figure 12. Biofilm Surface Area Coverage (μm^2) by Degree of Adherence. The above boxplot depicts biofilm surface area (in μm^2) in respect to degree of biofilm adherence independent of disease type: 1, 2, or 3 with 3 being the most adhesive to the epithelium, making the biofilm more difficult to remove. The crosshairs represent the means of the data and the solid bars the medians.

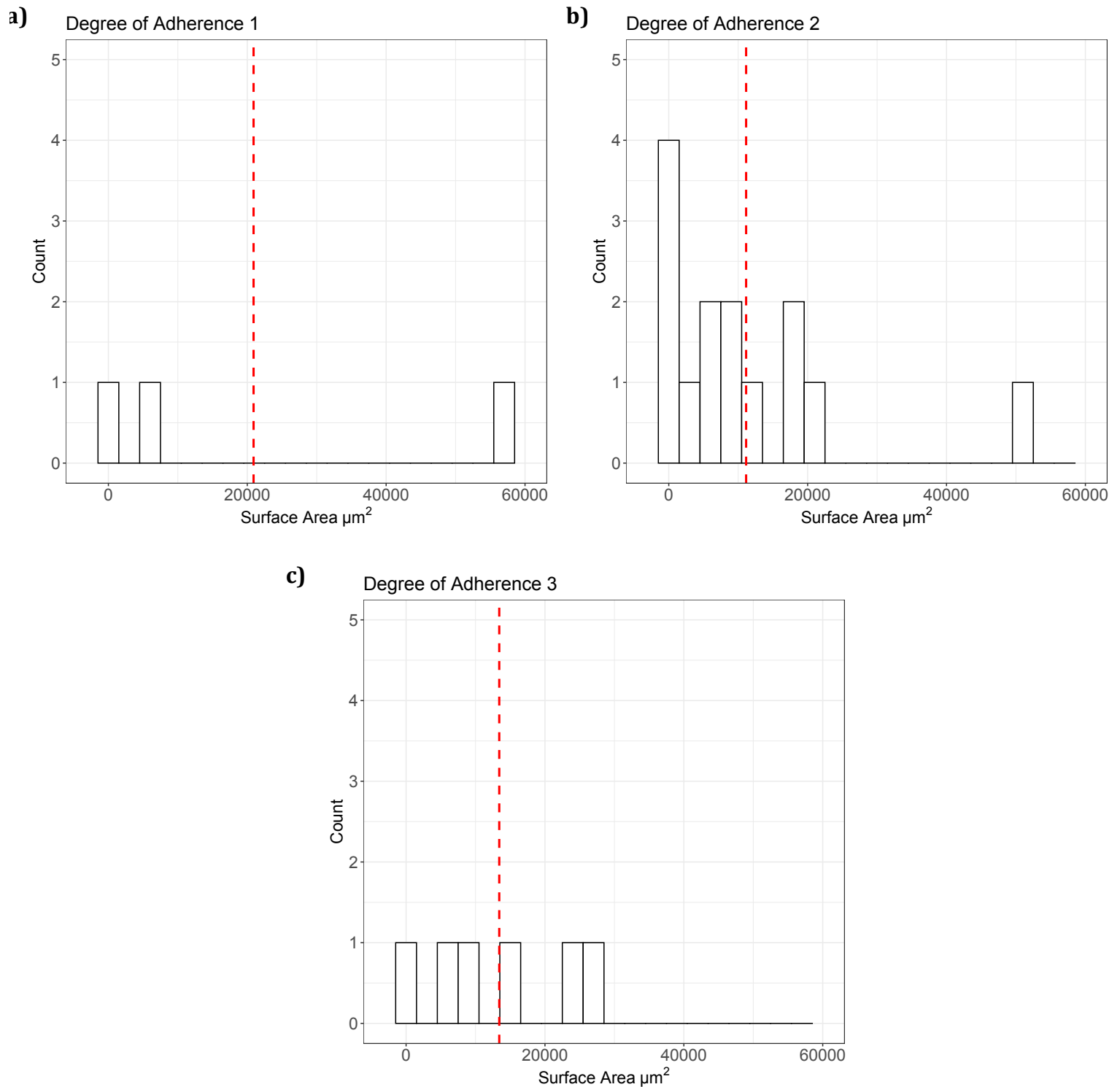


Figure 13. B+ Data Distribution Graphs in respect to Degrees of Adherence. The above graphs depict the biofilm surface area distribution (in μm^2) in regard to degree of biofilm adherence independent of disease type: **a)** 1, **b)** 2, and **c)** 3 with 3 being the most adhesive to the epithelium, making it more difficult to remove. The y-axis informs about patient counts while the x-axis informs surface area (μm^2) with a binwidth of 3000 (μm^2). The red - dashed line reveals the mean of the data in each graph.

Numeric data characterizing the biofilm surface area coverage of biofilm biopsies in respect to degree of continuity (1, 2, or 3) can be found in **Table 6** below. The degrees of continuity can be understood as the biofilm's characteristic to evenly cover, or coat the colonic epithelium without interruption. In theory, the higher the degree of continuity, the less patchy the biofilm appears to be. In other words, a degree of continuity 1 would be characterized as patchy, while a solid biofilm layer without normal mucosa visible in between would be classified as a degree of continuity 3. **Figure 15 b)** depicts the non-normal distribution of the degree of continuity 1, while the normal distribution of data from continuity degrees 2 and 3 can be seen in **Figure 15 a) & c)** respectively. A Kruskal-Wallis test determined that there is no significant difference between degrees of continuity (p-value = 0.2885) of the biofilm samples. Graphical representation of biofilm surface area coverage in respect to degree of continuity can be seen in **Figure 14**.

Table 6. Surface Area Statistics (μm^2) of Biofilm Samples by Degree of "Continuity"

Characteristic	1	2	3
Min.	267	0	820
1 st Quartile	4340	764	5822
Median	13674	6108	7320
3 rd Quartile	20305	9806	26297
Max.	57260	17800	50449
Normal Distribution Y/N (p-value)	N (0.0452)	Y (0.4494)	Y (0.2165)

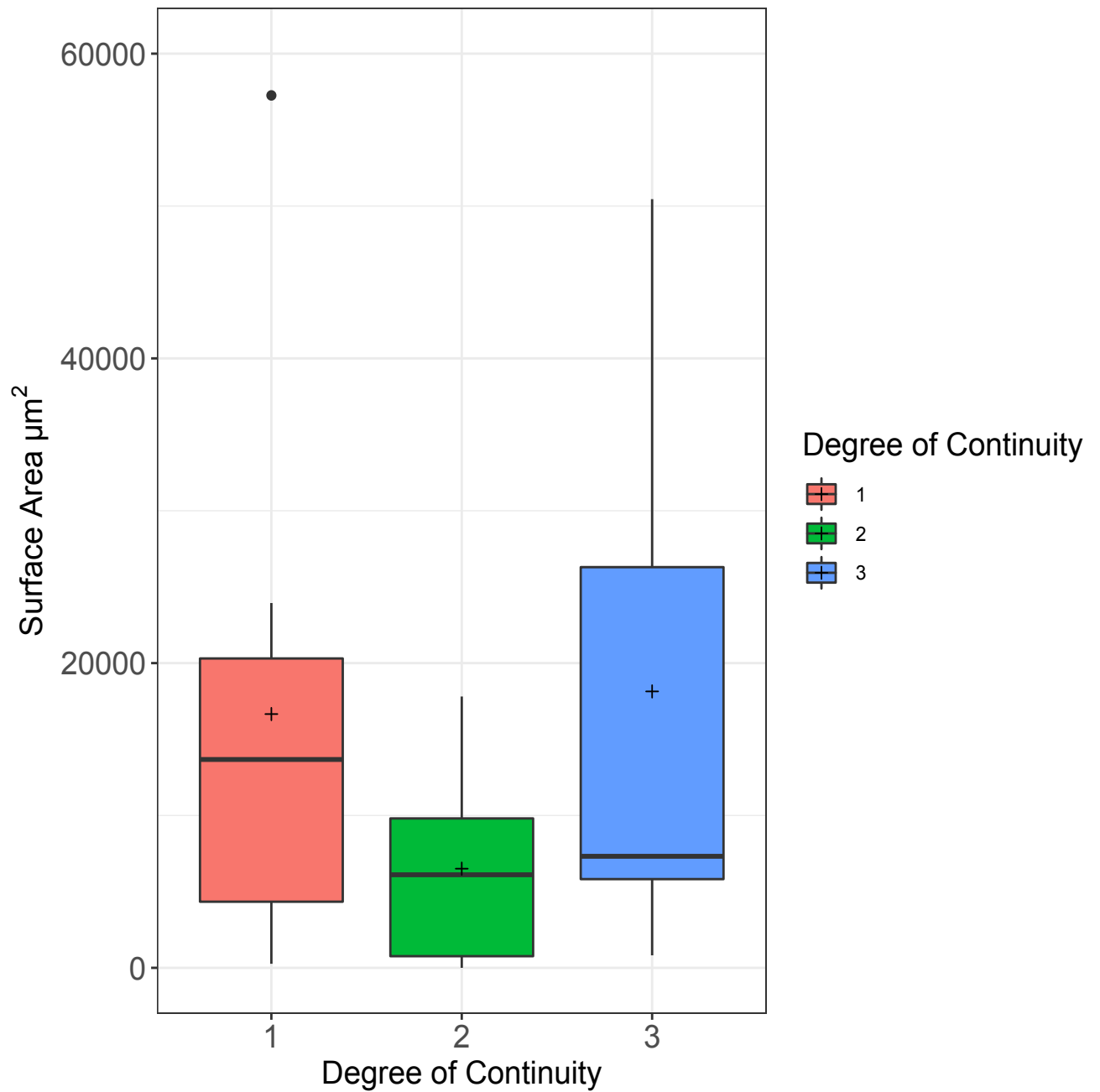


Figure 14. Biofilm Surface Area Coverage (μm^2) by Degree of Continuity. The above boxplot depicts biofilm surface area (in μm^2) in regard to degree biofilm continuity independent of disease status: 1, 2, or 3 with 3 being the most continuous or least patchy in appearance. The crosshairs represent the means of the data and the solid bars the medians.

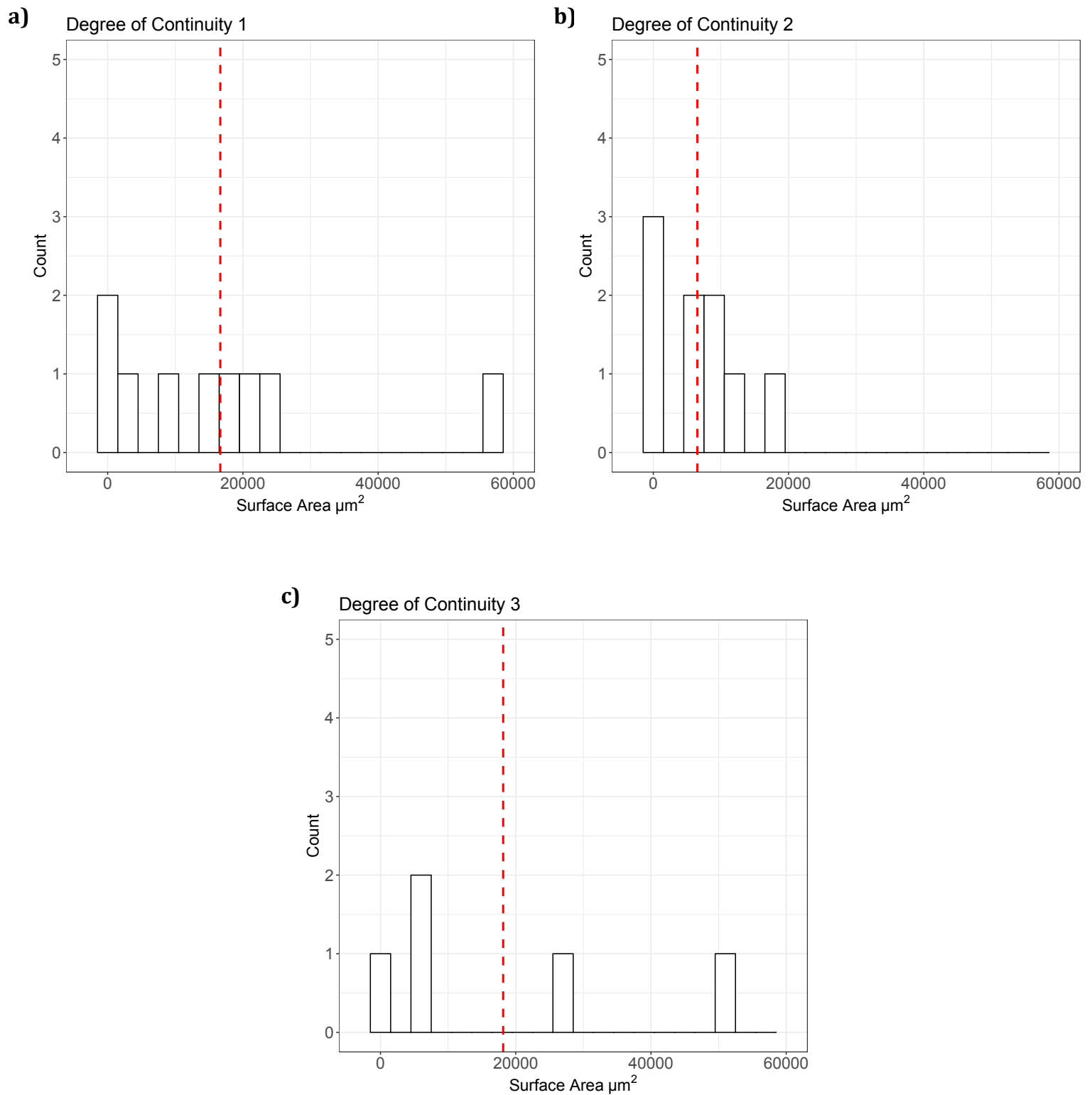


Figure 15. B+ Data Distribution Graphs in respect to Degrees of Continuity. The above graphs depict the biofilm surface area distribution (μm^2) in regard to degree of biofilm continuity independent of disease status: **a) 1**, **b) 2**, and **c) 3** with 3 being the most continuous or least patchy in appearance. The y-axis informs about patient counts while the x-axis informs surface area (μm^2) with a binwidth of 3000 (μm^2). The red - dashed line reveals the mean of the data in each graph.

Numeric data characterizing the surface area coverage of all obtained samples, independent of both sample and disease type, in respect to the absence (N) or presence (Y) of a sessile serrated adenoma (SSA) can be found in **Table 7** below. **Figure 17 a) & b)** depict the normal distribution of both adenoma absence and presence surface area data respectively. A Kruskal-Wallis test was used to determine that there is no significant difference between samples when comparing for the presence or absence of an SSA (p-value = 0.34). A trend for increased bacterial surface area for adenoma positive patient samples was found. Graphical representation of biofilm surface area coverage in respect to the presence or absence of a SSA can be seen in **Figure 16**.

Table 7. Surface Area Statistics (μm^2) of Samples by Adenoma Absence (N)/Presence (Y)

Characteristic	N	Y
Min.	0	408
1 st Quartile	564	733
Median	2646	5403
3 rd Quartile	8032	15828
Max.	50449	57259
Normal Distribution Y/N (p-value)	N (2.98e ⁻¹¹)	N (0.0042)

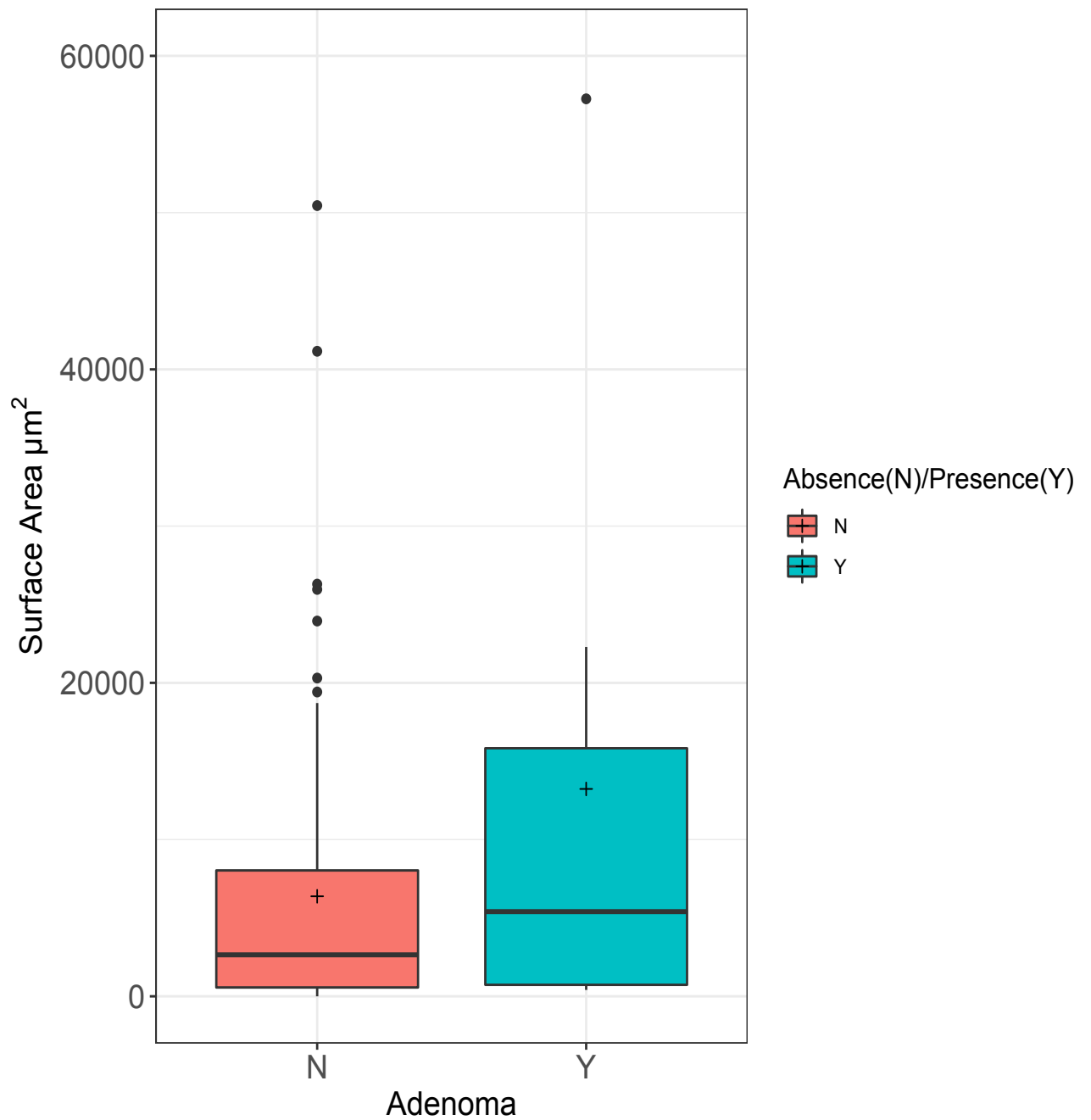


Figure 16. Bacterial Surface Area Coverage (μm^2) by Presence/Absence of SSA. The above boxplot depicts surface area data (μm^2) of all biopsy samples (B^+ , NM^+ , NM^-) in regard to the absence (N) or presence (Y) of sessile serrated adenomas (SSA). The crosshairs represent the means of the data and the solid bar the medians.

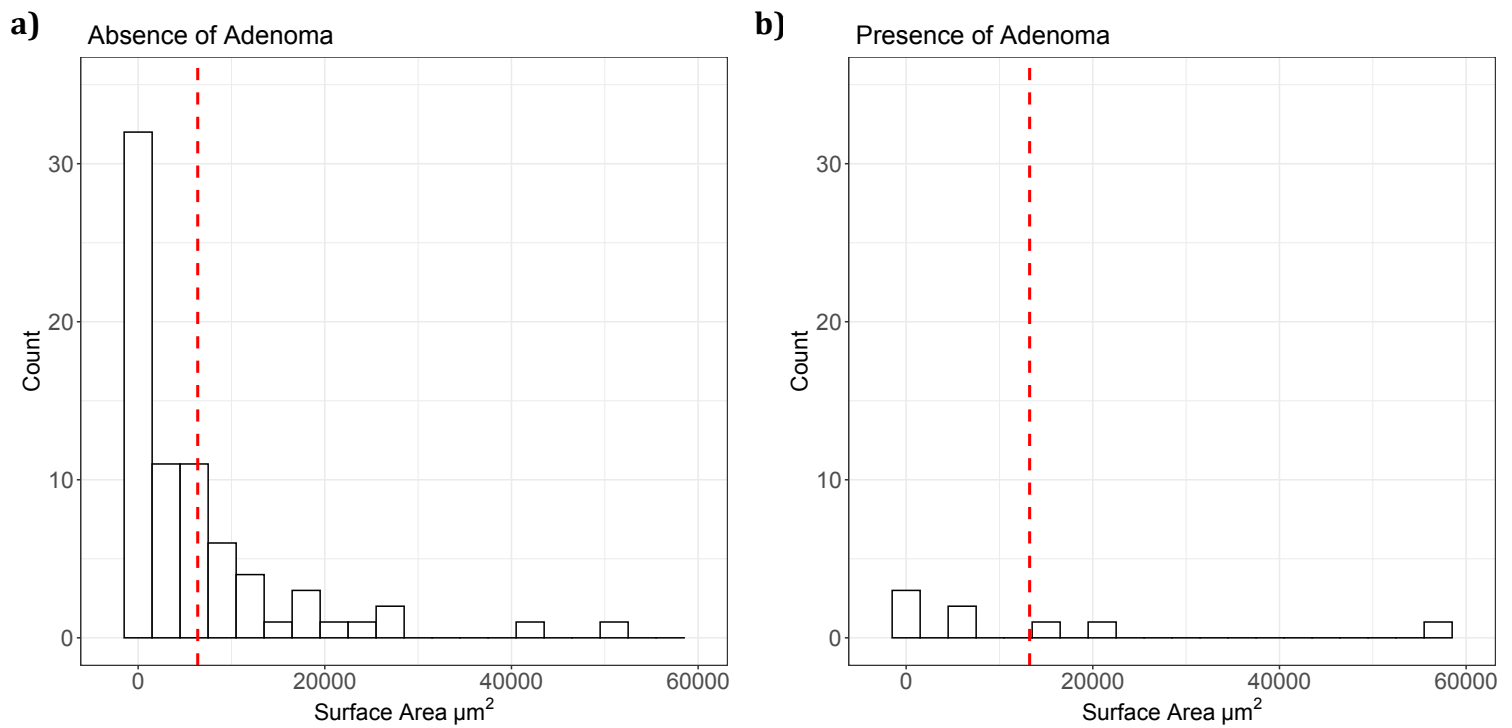


Figure 17. Data Distribution Graphs in respect to the Absence/Presence of SSAs. The above graphs depict the distribution of bacterial surface area data (μm^2) of all biopsy samples (B⁺, NM⁺, NM⁻) in regard to absence **a)** or presence **b)** of sessile serrated adenomas (SSA). The y-axis informs count of patients while the x-axis informs surface area (μm^2) with a binwidth of 3000 (μm^2). The red - dashed line reveals the mean of the data in each graph.

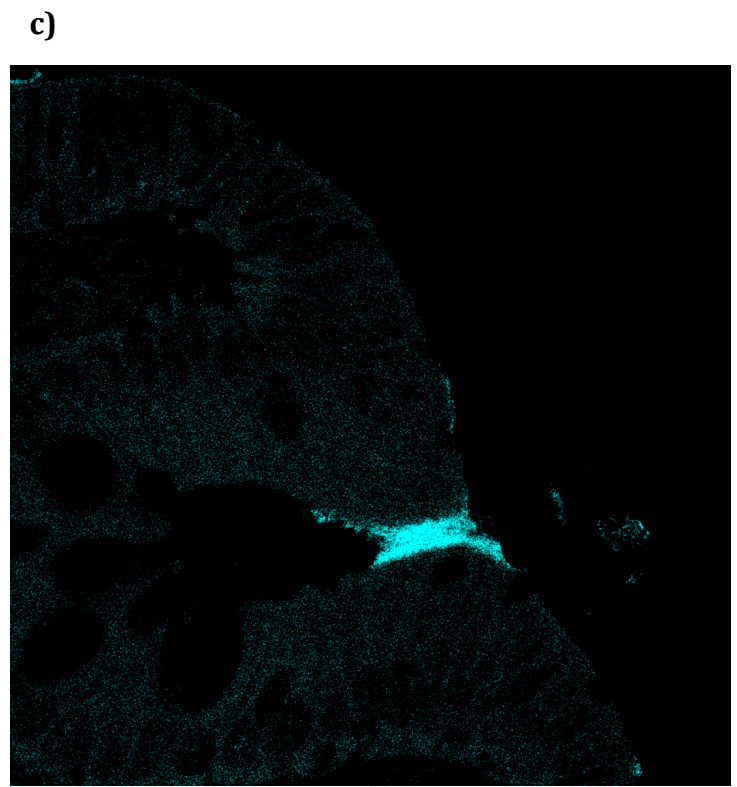
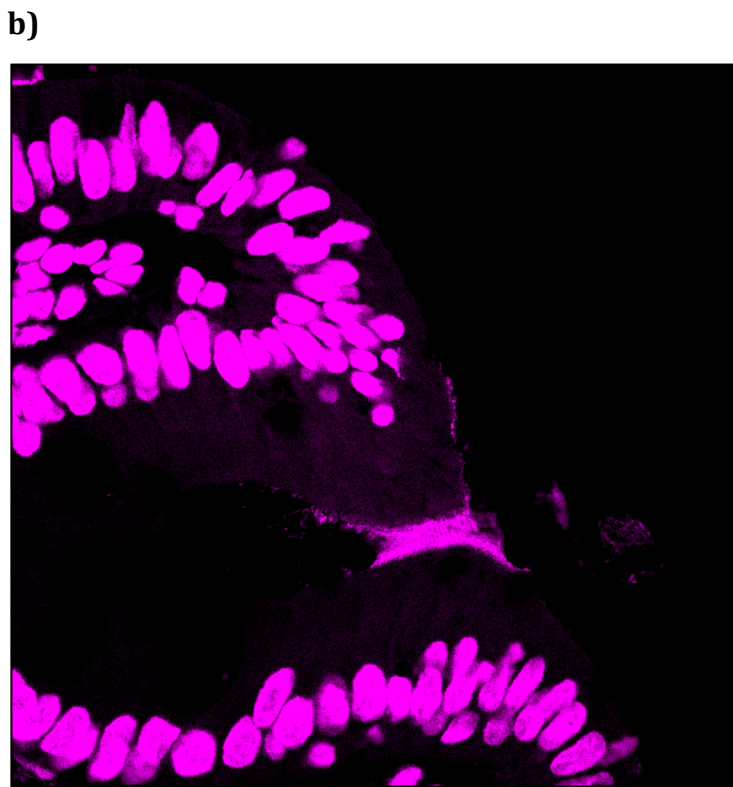
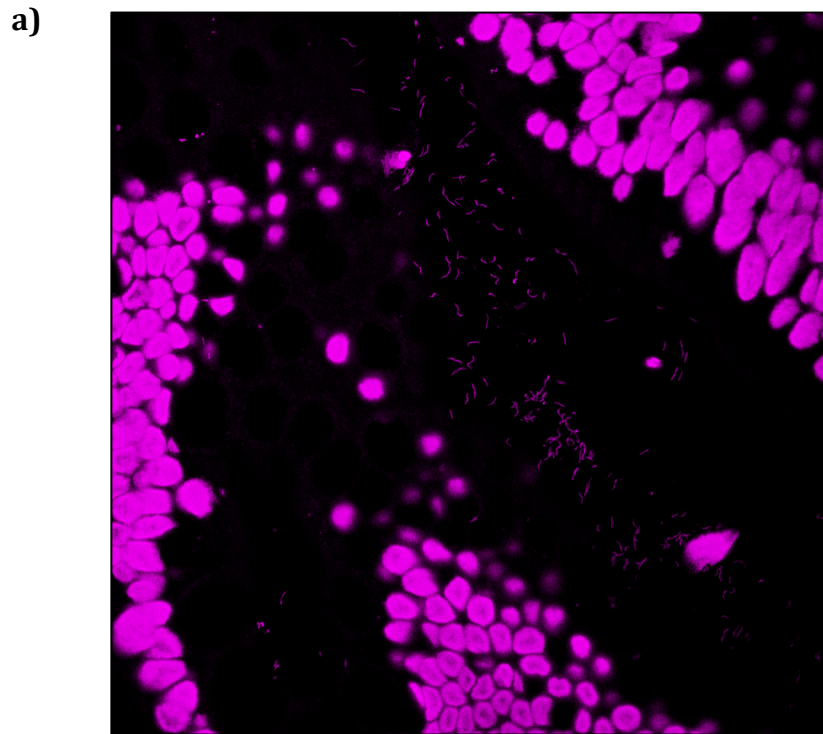


Figure 18. Additional Microscopic Images Showing Intestinal Crypt Invasion.

Microscopic images of a B⁺ patient's (a) and NM⁺ (b & c) colonic biopsy specimens a) is an image stained with DAPI (pink) revealing invasion of a colonic crypt b) is an image stained with DAPI (pink) revealing a cross section of a colonic crypt with heavy bacterial invasion at crypt entrance c) is stained with EUB338 universal probe mix (light blue) confirming bacterial presence in b).

Discussion

IBDs and IBS represent not only a growing financial burden to healthcare providers, but also, more importantly negatively affect the afflicted human in terms of quality of life (both physically and socially), in addition to their work productivity⁷⁶. Strides in the fields of microbiology as well as microbial ecology have enabled scientist to better understand not only the compositions, but also the spatial organization of microbial communities within the gut. Advancements in gene sequencing (i.e. strides in 16S rRNA sequencing, metagenomics, metatranscriptomics) in tandem with state of the art microscopy now enable scientists to visualize microbial behavior and characteristics⁷⁷.

By employing these new technological advancements, we were able to determine that there is a significant difference between the macroscopically visible biofilm layers and the normal mucosa in regard to the bacterial surface area coverage (μm^2); within individuals with UC, IBS, and healthy controls. Performing a Kruskal-Wallis test proved that there is a significant difference in bacterial biofilm surface area coverage (μm^2) between biopsy sample types. An additional Wilcoxon-pairwise analysis provided that the endoscopically assigned determinations of intestinal biofilm layers were in fact significantly different when compared against the normal mucosa samples of both biofilm-positive (p-value = 0.0332) and biofilm negative (p-value = 0.0039) patients. All surface area data used in these calculations

⁷⁶ Almquist, Törnblom, and Simrén, "Practical Management of Irritable Bowel Syndrome."

⁷⁷ Turnbaugh et al., "The Human Microbiome Project."

was gleaned from DAPI images due to the efficacy of the stain. This enabled a more accurate representation of the bacteria present.

Visual representations of the differences seen macroscopically during endoscopy versus their microscopically analyzed counterparts are provided in **Figures 2, 3, 4, 5 and 6**. In interpreting the microscopic images, important to note is that pretreatment procedures (outlined in the Results section) were not used, nor obtained images employing the pretreatment procedures used for analyses. Theoretically, the pretreatment options would have achieved the desired results by degrading the bacterial cell membrane to an extent, which would allow for an increased likelihood for the bacterial probe to cross the membrane and bind to the target ribosomal RNA sequence. However, due to all pretreatment options increased image background noise and autofluorescence in addition to reducing the sharpness of bacterial biofilms, they were not continued.

DAPI-stained images revealed to have the highest degree of efficacy, in that the staining functioned efficiently and consistently during the duration of the project for both human and bacterial (unspecific) DNA. The EUB338 probe mix (**Table1**), targeting bacterial 16S rRNA sequences however did not have the same pattern of consistency. This can be observed specifically in comparing images **b)** & **c)** of **Figure 2**. Initial experiments aimed to test whether the use of specific fluorophores, the fluorescent molecule of the probe allowing for visualization during microscopy, played a role in optimizing bacterial visualization (**Table 1**). Our tests concluded that the use of a Cy3 fluorophore probe mix elicited a higher visual representation when investigating bacterial presence in comparison to employing a

6 - Carboxyfluorescein (Fluos) fluorophore probe mix. These observations led us to employ the Cy3 EUB338 probe mix for the remainder of the study. There are an additional number of factors that could affect the efficacy of bacterial probes during FISH experiments as its functionality depends on the metabolic activity of the microbes. Understanding that “the ribosomal content of metabolically inactive bacteria is reduced”, one can explain how the reduction of available targets for the RNA-based FISH probes would lead to a decrease in signal fluorescence⁷⁸. The fact that metabolically silent bacteria can still be observed with general DNA stains, like the DAPI stain used in these experiments, suggests that the bacteria analyzed in these experiments were in part metabolically silent or “unamenable” to the FISH probe⁷⁹. As amenability of the RNA-based FISH probes can be a measure of bacterial metabolic activity, it can “therefore [be] an indirect sign of bacterial vitality”⁸⁰. Additional factors affecting the amenability of FISH probes include exposure to solutions (fixatives) in which biopsy samples are preserved. In this study, the biopsy samples were fixed in methacarn solution, allowing for the optimal preservation of nucleic acids⁸¹, thus increasing the probability of binding between the FISH probe and its intact target rRNA sequence. Another denaturing fixative includes Carnoy’s solution, which uses ethanol instead of methanol as its alcohol in solution. However though both Carnoy’s solution and methacarn function to preserve nucleic acids, the

⁷⁸ Swidsinski et al., “Azathioprine and Mesalazine-Induced Effects on the Mucosal Flora in Patients with IBD Colitis.”

⁷⁹ Swidsinski et al.

⁸⁰ Amann et al., “Combination of 16S rRNA-Targeted Oligonucleotide Probes with Flow Cytometry for Analyzing Mixed Microbial Populations.”

⁸¹ Howat and Wilson, “Tissue Fixation and the Effect of Molecular Fixatives on Downstream Staining Procedures.”

denaturing effect of Carnoy's solution (by means of dehydration)⁸² is found to be excessively drying, leading to shrinkage and hardening of the tissue sample. Denaturing of the sample could lead to damage of mucosal layer integrity, thus methacarn remained the optimal choice for fixation. The former two solutions, or "molecular fixatives", are unique in that they employ the use of alcohols instead of formalin as a fixative agent⁸³. Formalin, a solution comprised of 4 % formaldehyde in water, was widely used as a fixative agent for tissue preservation. However, as formaldehyde was observed to adversely affect the "quality of RNA and DNA available for extraction", the use of fixatives for molecular analyses has come to replace formalin⁸⁴. Although rarely used when the integrity of RNA and DNA is in question, formalin is still employed for standard treatment procedures in pathology (i.e. H & E Staining).

Apart from sample preservation procedures affecting the amenability of FISH probes, is the patient's exposure to any medications prior to the time of biopsy. As discovered by Swidsinski et al., patients who were receiving IBD medication (i.e. azathioprine and mesalazine) exhibited altered mucosal bacteria activity as well as bacterial amenability when analyzed⁸⁵. For example, the scientists discovered a significant reduction of bacterial "amenability, adherence, and [mucosal] concentrations" when investigating samples taken from patients who took

⁸² Howat and Wilson.

⁸³ Howat and Wilson.

⁸⁴ Howat and Wilson.

⁸⁵ Swidsinski et al., "Azathioprine and Mesalazine-Induced Effects on the Mucosal Flora in Patients with IBD Colitis."

mesalazine⁸⁶. After additional experiments proved that mesalazine did not have an effect on bacterial growth, the group conjectured that the anti-inflammatory medication may function by “[restoring] the [intestinal] mucus barrier function without altering the innate immunity”, leading to an indirect suppression of mucosal bacteria⁸⁷. Understanding that the two above-mentioned medications are prescribed to ameliorate IBD symptoms, is vital to know the patient’s full medication history for interpreting results accurately. Further analyses need to be conducted with the data gleaned from these experiments in order to test if bacterial vitality differs between biopsy sample types, within disease cohorts and/or upon drug treatment. The uncertainty of microbial metabolic activity, and thereby visual differences when comparing DNA stained images to EUB338 probe mix images, supports the use of DAPI images to confer a more accurate representation of bacterial surface area coverage.

Noteworthy are the additional possible sources of bacterial surface area coverage discrepancies, which may stem from biopsy collection during colonoscopy as well as tissue sample preparation while cutting sections. When the biopsies were collected, the adhesive nature of intestinal mucins and the EPS matrix of biofilms could have enabled these layers to either remain within the intestine, or remain attached to the forceps once the biopsy was released. Additionally, once the methacarn-fixed, paraffin embedded biopsies were being cut for sample analyses, the angle at which the sample was cut could also play a role in how much of the mucosal/biofilm layer would have been present for subsequent processing. For this

⁸⁶ Swidsinski et al.

⁸⁷ Swidsinski et al.

reason, we employed the technique discussed in the methods section of cutting several hundred micrometers deep, in order to ensure that we chose the most representative section area in terms of mucus/biofilm presence.

Unfortunately, the additional aims of these experiments did not yield any statistically significant data. When analyzing the degrees of “adherence” and “continuity” of the biofilm layers, their endoscopically designated degrees were inconclusive based on microscopic analyses of respective surface area data (μm^2). These results are visually confirmed in **Figures 12 & 14** respectively. **Figures 2, 3, & 4 a)** provide endoscopic representation of the varying degrees of continuity. The same non-significant result was determined when analyzing if the relationship between the presence or absence of an intestinal adenoma and bacterial surface area coverage (observed in **Figure 16**). A possible limitation of this study could be the small sample size. Increasing the number of patients sampled in regard to sample type, in addition to disease cohort, could yield more robust statistically conclusive results on which to make further conclusions.

Microscopic analyses also revealed intestinal crypt bacterial invasion in six different patients, with one comparative example portrayed in **Figure 18**. Both of these images came from the same individual who suffers from IBS, however **Figure 18 a)** was from directly from a biofilm, while **18 b)** was taken from the normal mucosa. Zhong et al. recorded similar results of crypt invasion in patients with IBS⁸⁸, providing evidence for distinct mucosa-invasive microbial communities within IBS

⁸⁸ Wang, Lu, and Zhong, “Tu1895 - Distinct Microbial Populations Exist in the Mucosa-Associated Microbiota of Diarrhea Predominant Irritable Bowel Syndrome and Ulcerative Colitis.”

individuals. However as only a general bacterial probe was utilized, additional testing would need to be conducted in order to see if microbial species would be in line with previously made discoveries.

In summary, the experiments and analyses conducted throughout this master's thesis have enabled the statistically sound differentiation between an endoscopically identified intestinal bacterial layer in comparison to the normal mucosa; confirming the presence of an intestinal biofilm. This evidence will provide the necessary means, by which acting physicians can designate the presence or absence of intestinal biofilms during colonoscopy. Further analyses utilizing the data available from the FISH images, will shed light onto the microbial density of the biofilm layer as well as the normal mucosa. This data paired with 16S RNA gene sequence analyses will aid in further characterizing the composition of microbial community as well as its ecology.

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