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Characterization of the Gut Microbiota and Colitogenic Bacterial Species in Core 1 O-glycans Deficient Mice

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CHARACTERIZATION OF THE GUT MICROBIOTA AND IDENTIFICATION OF COLITOGENIC BACTERIAL SPECIES IN CORE 1 O-GLYCANS DEFICIENT MICE

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CHARACTERIZATION OF THE GUT MICROBIOTA AND IDENTIFICATION OF COLITOGENIC BACTERIAL SPECIES IN CORE 1 O-GLYCANS DEFICIENT MICE

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Mucus is one important component of the intestinal mucosal barrier, and loss of its functionality is associated with colitis in humans and mice. Mice deficient in core 1 O-glycans (TM-IEC C1*galt*1$^{-/-}$) display a thinner mucus layer upon mutation of the glycosyltransferase enzyme (C1GALT1) responsible for core 1 O-glycans attachment, thus constituting an experimental model for human Ulcerative Colitis (UC). Under conventional conditions, these mice experience spontaneous inflammation of the colon. However, the exact role of the microbiota in colitis development in these mice has not been systematically investigated.

Aimed to gain insights into the role of the microbiota in colitis when there is a breach in the mucus barrier, we characterized the gut microbiota of conventional TM-IEC C1*galt*1$^{-/-}$ mice, tested the requirement of bacteria for initiation of colitis, and identified bacterial species associated with disease in this mice model.

Analysis of fecal bacterial populations by pyrosequencing of 16S rRNA tags showed that disease in conventional TM-IEC C1*galt*1$^{-/-}$ was associated with microbial dysbiosis manifested by increases in *Lactobacillus* and *Clostridium* species, and decreases in unclassified *Ruminococcus* and unclassified *Lachnospiraceae*. Under germ-free (GF) conditions, deletion of the C1GALT1 enzyme caused decreased goblet cells, but did not result in inflammation, showing that bacteria are required for disease

development. Monoassociation of GF TM-IEC C1*galt*1$^{-/-}$ revealed that the selected bacterial species differ significantly in the ability to induce disease. *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* showed association with induction of inflammation in this mice model. *Bacteroides sartorii, Clostridium symbiosum*, *Akkermansia muciniphila*, and *Lactobacillus johnsonii* not only did not cause manifestations of disease, but they induced high levels of secretory-IgA.

Findings suggest that the relationship between the commensal microbiota and the intestinal epithelium skews towards development of inflammation in the presence of a defective mucus layer, but not all dysbiotic shifts contribute to disease. *Bacteroides* species, which were not enriched in conventional TM-IEC C1*galt*1$^{-/-}$ mice, caused inflammation, while *Lactobacillus johnsonii* (enriched during colitis) did not. This thesis represents the first reported evidence of microbial dysbiosis caused by lack of core 1 O-glycans, and forms the basis for future work aimed to indentify bacterial factors related to disease.

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Chapter 1

Microbial changes and Epithelial Barrier in Inflammatory Bowel Diseases: A Review

1.1 IBD pathogenesis

Inflammatory Bowel Diseases (IBD) are a group of intestinal inflammatory disorders, including Ulcerative Colitis (UC) and Crohn's Disease (CD), that are considered multifactorial, involving atypical interactions between colonic bacteria, mucosal immune cells, and the intestinal epithelial barrier in genetically susceptible hosts (Sartor, 2006; Sartor and Muehlbauaer, 2007; Xavier and Podolsky, 2007; Strober et al, 2007; McGukin et al, 2009). Animal models and studies in patients with IBD implicate the intestinal microbiota in the initiation and perpetuation of inflammatory processes in the disease (Sartor and Muehlbauaer, 2007; Xavier and Podolsky, 2007).

Several general hypotheses exist regarding the etiology of IBD. One states that a dysregulation of the mucosal immune system, in the form of faulty mucosal effector T cells or regulatory T cells, leads to an aberrant immune response against the normal microbiota (Sartor, 2006; Sartor and Muehlbauaer, 2007; Strober et al, 2007). A second hypothesis proposes that alterations in the constitution of the gut microbiota - either in the type of microorganisms that compose the population, or in the degree to which certain microorganisms challenge the mucosal immune barrier - trigger an immune response against the microbiota. (Chichlowski and Hale, 2008; Sartor, 2006; Sartor and Muehlbauaer, 2007; Strober et al, 2007). The third hypothesis, which has been addressed in this work, suggests that disturbances in the epithelial barrier, which includes the mucus layer, may result in increased interactions between the microbiota and the immune system, thus increasing susceptibility to the disease (Einerhand et al, 2002; McGuckin et

al, 2009; Fu et al, 2011). All these scenarios converge on a possible relationship between the gut microbiota and the development of inflammation.

Evidence of the role of bacteria in precipitating the disease is seen in the positive effects of antibiotics in amelioration of symptoms (Reviewed by Sartor, 2004); the association between pathways for recognition of microbial molecular patterns and antigen presentation in disease development (i.e. mutations in nucleotide-binding oligomeraizing domains (Nod) and Toll-like receptors (TLRs)) (Cho, 2008); and the experimental requirement for bacteria in most animal models of IBD to initiate inflammation (Sadlack et al, 1993; Elson et al, 1995). Despite this evidence, there is no convincing proof of a single bacterial species associated with the development of IBD. An imbalance between protective and harmful bacteria results from the breakdown in host-microbial mutualism interactions (Backhed et al, 2005) contributing to development of disease.

1.2 The intestinal mucosal barrier and its relation to IBD

The intestinal mucosal barrier constitutes one of the largest surface areas that is exposed to, and continually interacts with, the external environment. It has developed to maintain a sensitive balance between absorbing essential nutrients and protecting from harmful matter, and it is a vital site for innate and adaptive immune regulation. Antigens are being sampled by M cells (also known as microfold or membranous cells), dendritic cells and macrophages constantly across the epithelial layer, at the boundary between the mucosal immune system and the external luminal contents. The excessive uptake of antigens may elicit an exaggerated immune response leading to inflammation. The

capacity to manage antigen uptake is denoted as the *intestinal barrier function* (Salim and Soderholm, 2011).

The intestinal epithelial monolayer is a physical barrier between the internal and external environment. Additionally, the mucus layer, the luminal commensal microbiota, and the inner subepithelial cells and their products (i.e. dendritic cells, macrophages, and lymphocytes that produce cytokines and chemokines and form part of the innate and adaptive immune system) shape the intestinal barrier. Schematics of a normal and a disrupted intestinal mucus barrier are presented in Figures 1.1 and 1.2, respectively.

1.2.1 Intestinal Epithelial Cells

The epithelial monolayer is composed of a series of cells organized in a single thoroughly folded layer to produce crypts and villous protrusions. It is composed of four cell lineages that arise from a single epithelial stem cell (Barker et al, 2008), and that are interconnected through tight junctions (TJs) that regulate the barrier permeability. These cells include goblet cells, enterocytes, enteroendocrine cells, and Paneth cells, each one with a specific function (Salim and Soderholm, 2011; Henderson et al, 2011). Enterocytes are mainly absorptive cells that regulate nutrient and ion absorption through microvillar extensions and their receptors, essential for dietary antigen uptake. The main role of goblet cells is mucus production, while enteroendocrine cells produce hormones and neuropeptides, and Paneth cells primarily produce antimicrobial factors (Ramasundara et al, 2009). Both, Paneth cells and enterocytes have a direct influence in

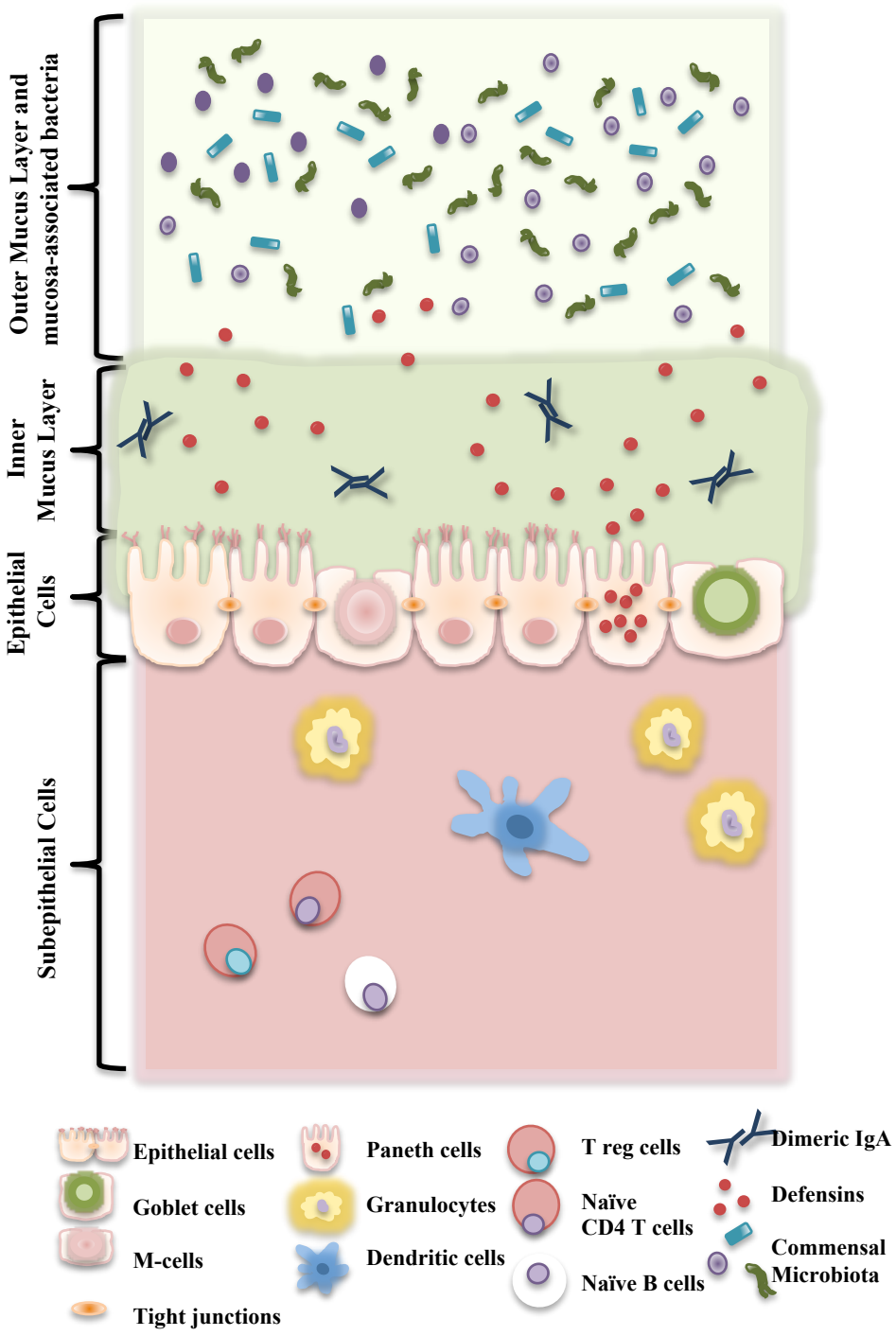

Figure 1.1 Normal O-glycosylation of the mucus layer ensures a normal intestinal barrier function by preventing aberrant interactions between commensal bacteria, epithelial cells and the mucosal immune cells.

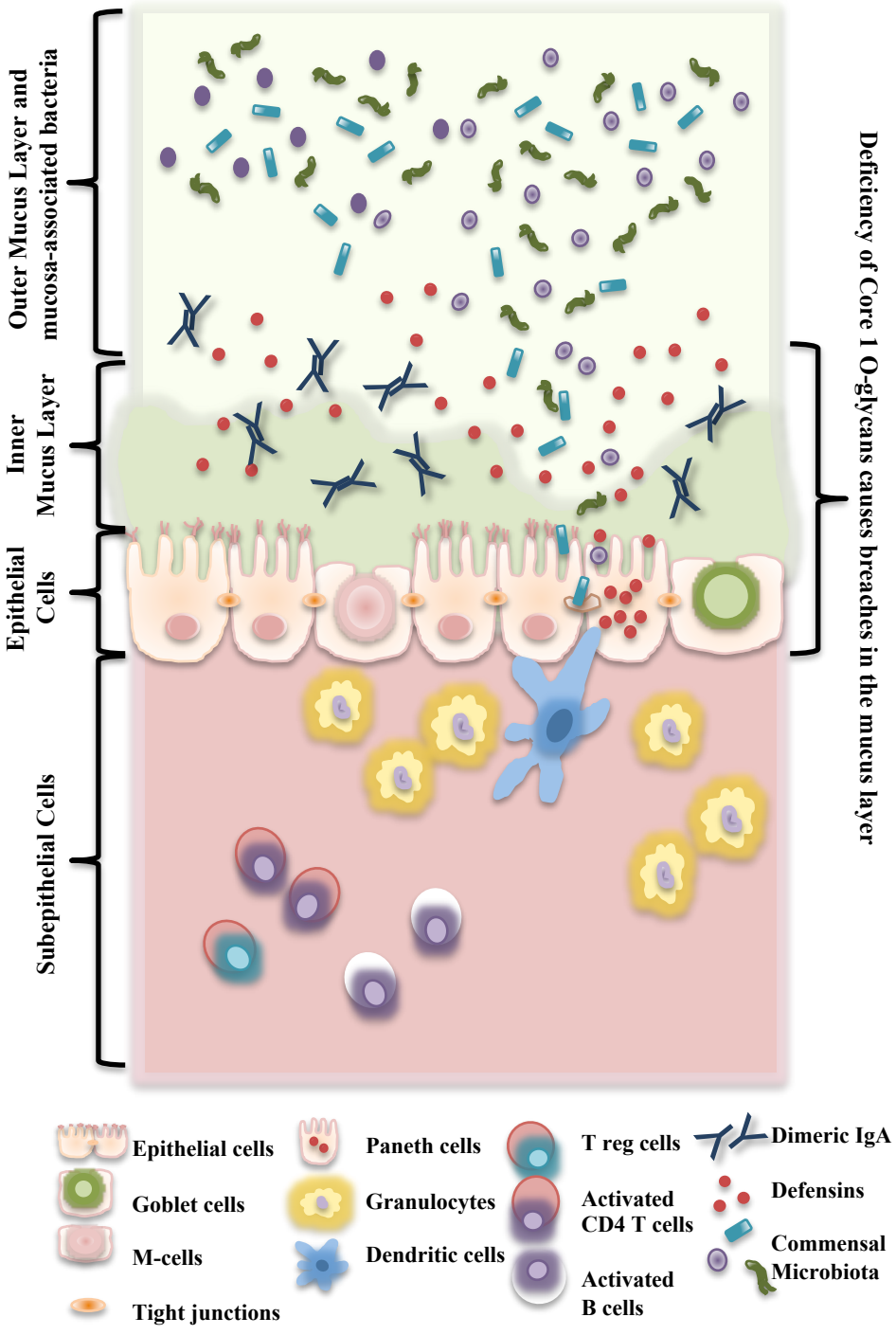

Figure 1.2 Lack or core 1 O-glycans in the mucus layer results in an impaired mucus barrier allowing closer interactions between the commensal microbiota and the epithelial layer, thus inducing an immune response towards the host's microbiota.

the normal commensal microbiota and invading pathogens. While Paneth cells produce a diversity of defensins, lectins and related antimicrobial substances, enterocytes play a role in the transcytosis of secretory immunoglobulin A (sIgA) into the intestinal lumen (Kaetzel, 2005) by putting accross a polymeric receptor that binds to the dimeric form of IgA, thus completing the formation of sIgA.

Besides forming a physical barrier, epithelial cells are equipped with pattern recognition receptors (PRRs), such as TLRs, and NOD-like receptors (NLRs) that are essential for the identification and response to microbial components (Reviewed by Kawai and Akira, 2009). Upon recognition of microbial-associated molecular patterns (MAMPs) by the PRRs, a cascade of signals is evoked either towards tolerance of resident beneficial bacteria, or for the eradication of pathogenic microbes. An abnormal signaling through PRRs could evoke inflammatory responses leading to chronic inflammation. Moreover, mutations in genes related to pathways for recognition of MAMPs and antigen presentation (i.e. *NOD2, CARD15, ATG16L1*) have been associated to predisposition to inflammatory processes and IBD (Henckaerts et al, 2007; Frank et al, 2011).

In addition, the TJs that connect the individual epithelial cells assist in the epithelium's general function as a barrier. The proteins that form the TJs aid in maintaining cellular polarity and are directly involved in epithelial cell differentiation (Schulzke et al, 2008; Hossain and Hirata, 2008). They regulate transport of water, electrolytes, and nutrients across the epithelium. Defects in permeability associated to faulty TJs have been reported in IBD patients (Secondulfo et al, 2001; Clayburgh et al, 2004).

1.2.2 Mucus Layer

Goblet cells secrete large quantities of mucin glycoproteins and other mucus components (trefoil peptides, Fcγ binding protein, and resistin-like molecule β) that come together to build a protective gel-like coat, with varying degrees of viscosity and thickness, which overlies the gastrointestinal tract (GIT) (Rubinstein and Tirosh, 1994; Atuma et al, 2001; Holm and Phillipson, 2012; Varum et al, 2012). The proportion of goblet cells was found decreased in both, UC and CD, being specifically diminished in the upper third of the crypts in UC (Gersemann et al, 2009). A low number of goblets cells directly affect the mucus gradient and its function as a barrier. The thickness gradient influences bacterial colonization and establishment of microbial niches (Jager et al, 2013), and it has been shown to be altered in active IBD (Pullan et al, 1994; Strugala et al, 2008). The vast majority of bacteria in the lumen concentrate in the outer layer of the mucus; while an inner layer, devoid of bacteria, neighbors the epithelial cells (Johansson et al, 2008). The sterility of the inner layer is due in part to the high concentration of antimicrobial peptides that localizes in the mucosal surfaces (Meyer-Hoffert et al, 2008).

The expression of mucin glycoprotein in the gut is mainly directed by the *MUC2* gene. The vital role of the mucus layer in maintaining the intestinal mucosal physical barrier was demonstrated in *MUC2* knockout mice that spontaneously developed colitis due to bacterial closeness to epithelial cells (Van der Sluis et al, 2006). Furthermore, aberrant expression of mucin genes and defective post-transcriptional processing of MUC 2 mucins have been found in human IBD (Moehle et al, 2006).

Additionally, deficiency of carbohydrate moieties attached to mucin backbones, including core 1, core 2, and core 3 O-glycans, have been linked to predisposition and development of inflammation; and, in some cases, to colorectal cancer (An et al, 2007; Stone et al, 2009; Fu et al, 2011). O-glycans are synthesized in the Golgi apparatus starting at the Tn antigen, N-Acetylgalactosamine (GalNAc) attached through an O-link to serine and threonine residues in the mucin backbone (Xia et al, 2004) (Figure 1.3). The Tn antigen is normally covered by other O-glycans that are synthesized by the action of several kinds of glycosyltransferases. The core 1 synthase, glycoprotein-N-acetylgalactosamine 3-β-galactosyltransferase 1 (C1GALT1, also known as T-synthase) specifically synthesizes core 1 O-glycans. Patients with UC were found to express the Tn antigen, and a subset of them presented mutations in the gene that encodes core 1 β1,3-galactosyltransferase-specific chaperone protein 1 (C1GALT1C1, also known as Cosmc) (Fu et al, 2011) which is essential for the action of C1GALT1 (Ju and Cummings, 2002; Aryal et al, 2010), thus limiting core 1 O-glycans synthesis.

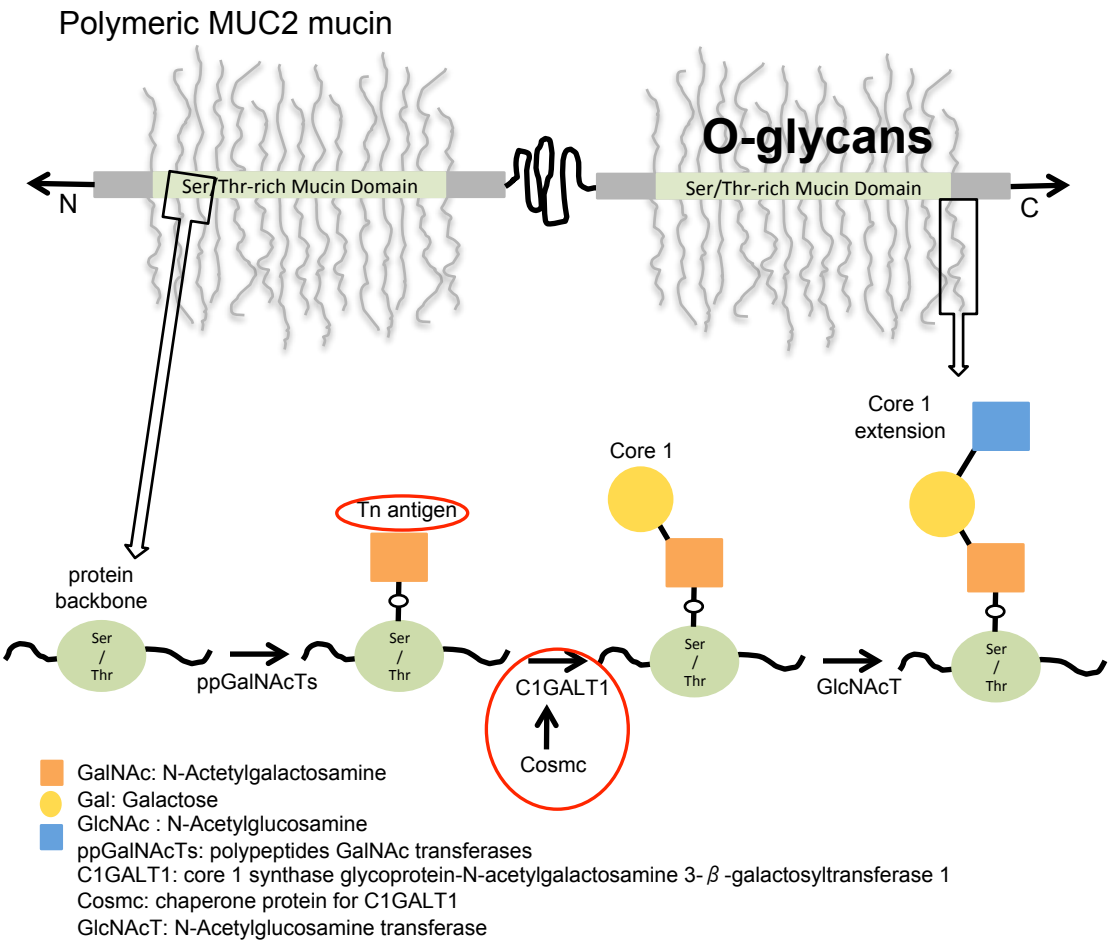

Figure 1.3 Synthesis of core 1 O-glycans starts at the Tn antigen by the action of the C1GALT1 enzyme, which in turn depends of the Cosmc chaperone protein. Other glycosyltransferases, specific for the added carbohydrates moieties, mediate core 1 O-glycans chain elongation. Mutations in Cosmc have been found in UC patients, thus having defective core 1 O-glycans synthesis. Tn antigen, C1GALT1 enzyme and Cosmc chaperone protein are circled in red.

1.2.3 Subepithelial Immune Cells

Peyer's patches are formed by aggregated lymphoid follicles with specialized epithelium called the follicular-associated epithelium (FAE). Within the FAE, specialized M cells continuously sample lumen contents by endocytosis, delivering the

sampled material to immune cells located in the subepithelial dome of the lymphoid follicle (Corr et al, 2008). Subsequently, the lumen content is collected by antigen presenting cells (APCs) and by lymphocytes (Neutra et al, 2001) that either ignore or respond to foreign antigens. The actions of the APCs could elicit a series of effects that might include T-cell-independent IgA production, and T-regulatory responses, among others (Macpherson and Uhr, 2004, Fleeton et al, 2004). After stimulation, the lymphocytes will release cytokines and chemokines capable of influencing the epithelial integrity, thus preventing or predisposing to inflammation (Suenaert et al, 2010). For example, Enss et al (2000) found that mucin expression and structure is influenced by proinflammatory cytokines in an intestinal cancer cell line.

1.2.4 Commensal Microbiota

The commensal microbiota plays several roles that are critical to the host's health. Besides contributing to nutrient acquisition (Backhed et al, 2004), the microbiota aids in limiting colonization by pathogens (Artis, 2008), and in promoting the development and maturation of the immune system (Umesaki and Setoyama, 2000; Round and Mazmanian, 2009). The general scenarios leading toward development of IBD include a role for the host microbiota (addressed in section 1.1). Changes in the microbiota identified in IBD disorders are discussed in section 1.3 and summarized in Table 1.1.

1.3 Microbial characterization in IBD

The majority of the known symbionts of the GIT establish mutualistic relationships with the host. Commensal microorganisms benefit from a steady supply of nutrients and a relatively stable environment, while the host receives nutritional and digestive benefits, protection against colonization by pathogens, and stimulation of the immune system for its development and maturation (Umesaki and Setoyama, 2000; Round and Mazmanian, 2009). Alterations in the environment and the microbiota's composition might result in the acquisition of virulence factors by some commensals, turning them into pathogens and predisposing to disease.

In general, molecular analyses of the gut microbiota in IBD have found a reduction in diversity of the microbial communities in the disease, characterized by a decrease in the population of Firmicutes (Frank et al, 2007; Willing et al, 2010; Walker et al, 2011). However, research on microbial changes for other phyla is still inconclusive with regards to which bacterial taxa are linked to inflammation. For instance, while some researchers found increased Clostridial population in the disease (Mylonaki et al, 2005), suggesting a causal role for the organism, further investigations have found that this group is depleted in the disorder (Gophna et al, 2006; Andoh et al, 2011). In fact, a decrease in *Faecalebacterium prausnitzii* in CD is the only element found in agreement between several groups or researchers (Sokol et al, 2009; Willing et al, 2010; Joossens et al, 2011; Morgan et al, 2012; Kabeerdoss et al, 2013). *F. prausnitzii* belongs to the order of the Clostridiales and has been associated with anti-inflammatory properties.

Similar issues rise for *Bacteroides* species. Swidsinski et al (2005) found *Bacteroides* species as the predominant group in inflamed colon, while other studies found no changes (Mylonaki et al, 2005), or even a decrease in population size of *Bacteroides* in active state of the disease (Frank el al, 2007; Noor et al, 2010).

Results that suggest a relationship for particular Bacteroides species with the disease, either as causative or preventive of inflammation, are uncertain and sometimes conflicting for both, animal models and IBD patients. For example, high levels of antibody against the outer membrane of *B. vulgatus* were found in UC patients suggesting a role for the bacterium in the pathogenesis of the disease (Bamba et al, 1995). In the case of *B. ovatus*, elevated IgG and IgA titers against this species were found in patients with CD and UC, suggesting a role of the organism in the immune response to the disease (Saitoh et al, 2002). Conversely, in animal models, it was found that *B. vulgatus* plays a role against the development of colitis in IL-10 KO mice (Sydora et al, 2005). On the other hand, Hudcovic et al, (2009) found that *B. ovatus* protects immunodeficient SCID mice from further inflammation in dextran sodium sulfate (DSS)-induced injury of the colon. This paradox warrants further research in order to determine a causal relationship between specific bacterial species and development of inflammation. Table 1.1 summarizes major changes in microbial communities found in human IBD.

Table 1.1 Dysbiosis in the gut microbiota in human IBD

Subjects	Methods	Main Findings	References
33 UC patients 6 CD patients 14 controls	FISH	↓ Bifidobacteria in UC ↑ E. coli in active UC and CD relative to controls ↑ Clostridia in active UC = Lactobacillus & Bacteroides	Milonaki et al, 2005
13 CD patients 13 UC patients 5 IC patients 13 controls (HS)	FISH Facs	↓ Diversity in IBD relative to HS & IC ↓ Firmicutes in IBD ↓ *Clostridium coccoides* group in UC ↓ *Clostridium leptum* group in CD ↑ Bacteroides in IC = Enterobacteria, Atopobioum & Bifidobacterium between phenotypes	Sokol et al, 2006
6 CD patients 5 UC patients 5 controls (HS)	High-throughput sequencing	↑ Proteobacteria in CD relative to UC & HS– mainly γ Proteobacteria and *E. coli* ↑ Bacteroidetes in CD – mainly *B. vulgatus* ↓ Clostridia in CD UC tissue-associated flora broadly = to HS	Gophna et al, 2006
68 CD 61 UC 61 controls (non-IBD)	Molecular-phylogenetic sequence analysis Q-PCR	↑ Actinobacteria in IBD ↑ Proteobacteria in IBD ↓ Bacteroidetes in IBD ↓Lachnospiraceae in IBD	Frank et al, 2007
32 CD patients 17 UC patients 8 IC patients 27 controls (HS)	QRT-PCR	↓ *C. leptum* & *C. coccoides* in IBD & IC ↓ *F. prausnitzii* in IBD & IC ↓ Bifidobacteria in IBD & IC	Sokol et al, 2009
15 twin pairs UC 23 twin pairs CD 2 twin pairs controls (HS)	454 FLX sequencing	UC microbiota broadly = to HS ↑ Ruminococcaceae in CD ↑ Lactobacillaceae in ICD ↑ Faecalebacterium in CD but ↓ in ICD ↓ Streptococcus in UC ↓ Prevotella in UC ↑ Enterobacteriaceae in ICD	Willing et al, 2010
13 UC patients 22 controls (HS)	PCR-DGGE	↓ Diversity in UC relative to HS ↓ Bacteroides in UC mainly *B. vulgatus*, *B. ovatus, B. uniformis* & *Parabacteroides* spp.	Noor et al, 2010
6 CD patients 6 UC patients 5 controls (HS)	High-throughput sequencing Q-PCR	↓ Diversity in CD than UC ↓ Firmicutes in IBD ↑ Bacteroidetes in IBD ↑ Enterobacteria in CD	Walker et al, 2011
31 UC patients 31 CD patients 30 controls (HS)	T-RFLP analysis	↓ Clostridium in CD & UC ↑ Bacteroides in CD	Andoh et al, 2011
68 CD patients 84 non CD relatives 55 controls	DGGE	↓ *Faecalebacterium prausnitzii* ↓ *Bacteroides adolescentis* ↓ *Dialister invisus* ↑ *Ruminoccocus gnavus*	Joossens et al, 2011
6 active UC pts 6 remission UC 6 controls (HS)	DGGE Q-PCR	↓ Lactobacillus spp. in active UC ↓ *Akkermansia muciniphila* in active UC	Vigsnaes et al, 2012
121 CD patients 75 UC patients 27 controls (HS)	454 FLX sequencing	↓ Roseburia in IBD ↓ Ruminococcaceae & *F. prausnitzii* in CD ↑ Escherichia/Shigella	Morgan et al, 2012

CD: Crohns disease, UC: Ulcerative Colitis, ICD: CD localized in the ileum, IC: Infectious colitis, HS: Healthy subjects, ↑: increase, ↓: decrease, =: similar to control

1.4 Bacterial capacity for colonization and carbohydrates utilization

Being sterile at birth, the gastrointestinal tract of the human neonate is rapidly colonized by microbes upon exposure to the environment, parental contact, and diet antigens (Palmer et al, 2007). Many factors affect the initial composition of the neonate's microbiota, including mode of delivery (vaginal or cesarean), enteric feeding (breast-fed or formula), and gestational age at birth (Penders et al, 2006; Biasucci et al, 2010), among others. The composition of the neonate microbiota varies greatly between individuals (Palmer et al, 2007). Initially governed by Bifidobacteria (Stark and Lee, 1982), the breast-fed infant gut microbiota is rapidly affected by further exposure to environmental and food antigens, being eventually transformed into an "adult" microbiota that harbors up to 100 trillion (10^{14}) microbes, dominated by Firmicutes and Bacteroidetes (Eckburg et al, 2005). What makes the microbiota such as dynamic entity?

Throughout the host life, the microbiota is persistently challenged by several physical, chemical and metabolic factors, inherent to the host, which may affect its composition (Booijink et al, 2007). Continuous epithelial cell and mucus turnover (Kim et al, 2010), immune defense mechanisms (Sansonetti and Medzhitov, 2009), peristalstic movements, pH gradients, and host's digestive enzymes (Booijink et al, 2007) are some of the elements that microbes defy for niche establishment and survival. In addition, the members of the microbiota are subjects of competitive exclusion, and they are affected by antibiotic usage during disease stages of the host.

The composition of the microbiota varies along and across the GIT (Swidinski et al, 2007; reviewed by Walter and Ley, 2011), with some species attached to the epithelium and others found in the lumen. Niche establishment within the gut requires that commensals evolve to develop strategies to avoid immune recognition by actively repressing or modulating the host response (Sansonetti and Medzhitov, 2009). Additionally, they need the capacity to utilize available nutrients within their surrounding environment. These nutrients include host's diet complex carbohydrates, as well as host's mucosa-associated glycans.

The ability of the microbiota to metabolize complex carbohydrates is granted by an abundant repertoire of degrading enzymes that are selectively expressed depending on environmental pressures (Martens et al, 2011). However, not all members of the gut microbiota have a vast enzymatic potential. Some have limited degrading capability, while others depend on metabolic products of neighboring microbes for their survival (Xu et al, 2007; Png et al, 2010).

As an example of metabolic attributes, the human symbiont *Bacteroides thetaiotaomicron* is able to express 209 paralogs of SusC and SusD (proteins related to starch and maltooligosaccharide utilization), 226 glycoside hydrolases, and 15 polysaccharide lyases, that are able to breakdown any type of diet or host glycans within the gut (Cho and Salyers, 2001; Sonnenburg et al, 2005; Xu et al, 2007). In contrast, another member of the Bacteroidetes, *Parabacteroides distasonis*, has only one candidate α-fucosidase, two classes of carbohydrate-processing enzymes, and 7 polysaccharide deacetylases, thus limiting the metabolic capacity for the utilization of carbohydrate sources (Xu et al, 2007). Still, *P. distasonis* posses a greater faculty for protein

degradation than *B. thetaiotaomicron* does (Xu et al, 2007). The increased ability of *B. thetaiotaomicron* to exploit surrounding nutrients confers the bacterium fitness advantages and categorizes it as a "generalist". *P. distasonis* is classified as a "specialist", being able to merely metabolize specific diet components (Xu et al, 2007). These metabolic characteristics have an immense influence in niche selection and establishment, since the bacterium ability to express gene clusters for nutrient utilization will decide its fate, endurance and persistence along the GIT.

1.5 Humoral Immune response in IBD

One of the major challenges that microorganisms confront in their endeavor to colonize the host is evasion of the immune system. Humoral immunity is the part of the immune system mediated by macromolecules found in extracellular fluids. It comprises secreted antibodies, specific antimicrobial peptides, and complement proteins, among other elements. Secreted antibodies perform immune exclusion as a first-line of defense, thus counteracting bacterial colonization and penetration of soluble antigens into the mucosa. This section briefly summarizes general findings in immunoglobulin (Ig) levels associated with human IBD.

Contrary to normal healthy mucosa, the mucosa of both, UC and CD patients, contains large amounts of IgG relative to healthy subjects, the levels being higher in UC than CD (Gouni-Berthold et al, 1999; Furrie et al, 2003). The IgG isotype associated with UC is IgG1 in serum and mucosal cells, while the one associated with CD is IgG2 (Kett et al, 1987; Ruthlein et al, 1992; Philipsen et al, 1995; Furrie et al, 2003),

suggesting differences in the immune mechanisms involved in these diseases. Other studies have found increases in both IgG1 and IgG3 in UC (Dorn et al, 2002). It is still uncertain whether the high IgG response in IBD patients originates in the periphery (not localized in the mucosa), or in the mucosa. However, studies do suggest that the response is originated peripherally, and IgG is transported across the enterocyte into the mucosa (Thoree et al, 2002; Hansen et al, 2006). Despite the differences in IgG levels and isotypes between the phenotypes, some researchers consider that the levels of this antibody do not constitute a reliable marker to distinguish between UC and CD since not in all patients the changes correlate positively with disease activity (Gouni-Berthold et al, 1999).

Conversely, decreased levels of mucosal IgA in IBD were found relative to controls in both, UC and CD (Philipsen et al, 1995; Brandtzaeg et al, 2006). Interestingly, mononuclear cells (MNC) from patients with CD exhibit primarily dimeric IgA and relatively small amounts of monomeric IgA, while the opposite is observed in UC (MacDermott et al, 1983). Since in normal mucosa the majority of the IgA is dimeric, the presence of monomeric IgA in the intestine could be due to homing of monomeric IgA-producing cells into the intestine, or to *in situ* proliferation due to immune alterations. However, in contrast to control MNCs, a higher percentage of the intestinal IgA subtype identified in IBD was IgA1 (MacDermott et al, 1986; Kett and Brandtzaeg, 1987), which is less resistant to proteolytic degradation (Brandtzaeg et al, 2006). Secretory IgA (sIgA) assists innate mechanisms in protecting the epithelium, thus strengthening its function as a barrier. In the absence of sIgA, gut commensals could overstimulate the innate immune system predisposing to inflammation.

Regarding IgM, the findings are conflicting. While some studies have found no changes in the levels of serum IgM in either of the two IBD phenotypes (Philipsen et al, 1995), other investigations have reported higher levels of lamina propria (LP) cells expressing IgM in CD relative to controls (Dorn et al, 2002). However, differences might arise from the nature of the samples tested (serum *versus* Ig-expressing LP cells) and variations in the methods used for immunoglobulin measurement between the studies.

Studies have found that the Igs of IBD patients are directed towards cytoplasmic bacterial components, rather than membrane proteins (Macpherson et al, 1996). Additionally, IBD patients have a higher number of Ig-coated bacteria (IgG, IgA, and IgM) in feces than controls, implying loss of tolerance to the commensal microbiota (van der Waaij et al, 2004; Harmsen et al, 2012). This supports the theory that the etiology of IBD is an aberrant immune response to the normal microbiota.

1.6 Specific Aims, Rationale and Significance

Murine models resemble certain aspects of human diseases closely enough to constitute efficient tools for the investigation and elucidation of predisposing factors, as well as alternative treatments to human illnesses. The core 1 synthase, glycoprotein-N-acetylgalactosamine 3-beta-galactosyltransferase 1 enzyme (also known as T-synthase, abbreviated C1GALT1), is the enzyme responsible for attachment of O-glycans in the mucin proteins that form the mucus layer. The core 1 O-glycans deficient (C*1galt1*$^{-/-}$) mice model resembles human UC by having an incomplete, thin mucus layer similar to that found in a subset of UC patients (Pullan et al, 1994; Strugala et al, 2008). In this report, we described the gut microbiota of conventional C1*galt*1$^{-/-}$ mice using 16s rRNA sequencing, we determined if germ-free (GF) with a Tamoxifen-induced mutation specific to intestinal cells (TM-IEC) C1*galt*1$^{-/-}$ mice develop colitis, and we colonized GF TM-IEC C1*galt*1$^{-/-}$ mice with selected single bacterial species to determine their colitogenic potential in this mice model. By characterizing the gut microbiota in the C1*galt*1$^{-/-}$ mice with a disrupted mucus layer, we elucidated: (A), the putative role of specific bacterial species in the disease; and (B), whether or not a breach in the mucus layer by itself predisposes to disease development in the absence of microbes. The identification of specific bacterial species associated with disease development or prevention could be used in the development of appropriate antimicrobial treatments against the causative microorganism, while preserving beneficial commensals. With this set of results, we can start to understand the species-specific or strain-specific character

of host-microbial interactions that promote colitis in the absence of normal mucus synthesis.

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Chapter 2

Germ-free Core 1 O-glycans Deficient Mice and Development of Inflammation

2.1 Introduction

Most gnotobiotic animal models of IBD require the presence of bacteria for the host to develop disease. The aim of the experiment addressed in this chapter was to determine if core 1 O-glycans deficient mice (C1*galt*1$^{-/-}$), which are known to spontaneously develop colitis in a conventional setting (Fu et al, 2011), would become diseased in a germ-free setting.

Gnotobiology is defined as the study of organisms that are either germ-free (GF) - containing no bacteria, viruses, fungi or any other form of commensal or pathogenic symbiont - or that are associated with known, specific bacteria (Coates, 1975). The development of gnotobiology has provided new approaches to elucidate the etiology of diseases, as well as the dynamics of microbe-host relationships and their influence in the development, establishment and maintenance of the immune system. The gnotobiological approach confers the ability to control and manipulate the environment in which an organism develops and performs, and combined with genetic technologies, is instrumental in the elucidation of the crosstalk between symbiotic and pathogenic microorganisms and both, healthy and diseased hosts (Reviewed by Falk et al, 1998).

Thompson and Trexler (1971) reviewed the differences in the gastrointestinal tract (GIT) structure and function between GF and conventionally raised animals. Murine models are the ones mostly studied by gnotobiology (as opposed to chicken, pig, dog or cattle models), and differences have been identified between GF mice and their conventional, age-matched, counterparts. Morphological modifications in the GIT of GF mice include, but are not limited to, enlargement of the cecum (5 to 10 times larger than

normal), thinner intestinal wall, thinner mucus layer, longer villi in the small intestine, and shorter crypts that contain fewer cells. In addition, GF mice have slower intestinal motility (speed of transit and gastric emptying) than conventional mice. These differences could be subsided by the introduction of bacteria in the gut, thus demonstrating a definite relationship between the microbiota and the morphology of the gut (Huseby et al, 1994).

The absence of microbes in GF mice has a direct impact in their immune system, including a reduced number and size of Peyer's patches (PP), decreased intestinal immunoglobulin (Ig) A-secreting plasma cells, reduced CD4+ T cells in the lamina propria (LP), and changes in T cell phenotype in the intraephitelial compartment (Macpherson and Harris, 2004; Macpherson et al, 2005). Regarding Igs in serum, GF mice display normal levels of IgM, reduced levels of IgA and IgG, and increased levels of natural IgE relative to conventional mice (Macpherson and Harris, 2004; McCoy et al, 2006). Although the immune system of GF mice is capable of responding to antigens, stronger responses are achieved in conventionalized (ex-germ-free) mice and, even stronger, in conventional (with a normal microbiota since birth) mice (Fubara and Freter, 1972).

The requirement of bacteria to induce inflammation has been proved in several murine models of inflammatory disorders, such as the interleukin 2 deficient (IL-$2^{-/-}$) mice, the interleukin 10 deficient (IL-$10^{-/-}$) mice, T cell receptor αβ deficient (TCR$\alpha^{-/-}$) mice, and the HLA-B27/β2m transgenic rat (which express human MHC class I and human β2 microglobuline) (Dianda el al, 1997; Reviewed by Podolsky, 1997). In these models, the host does not develop inflammation if kept germ-free.

The Core 1 synthase, C1GALT1, is the enzyme responsible for attachment of core 1 O-glycans in the mucin proteins that form the mucus layer. Conventional mice that lack the T-synthase enzyme (C1*galt*1$^{-/-}$) spontaneously develop colitis (Fu et al, 2011). However is still unknown if the C1*galt*1$^{-/-}$ mice model in a GF setting will develop inflammation. A Tamoxifen-induced mutation specific to the intestinal epithelia was generated in C1*galt*1$^{-/-}$mice (TM-IEC C1*galt*1$^{-/-}$) in a GF setting. Mutation induction was performed in GF TM-IEC C1*galt*1$^{-/-}$ mice to determine whether a breach in the mucus barrier in the absence of microbes predisposes to inflammation. GF WT mice and conventionalized (ex-germ-free) core 1 O-glycans deficient mice were used as controls.

2.2 Materials and Methods

2.2.1 TM-IEC C1*galt*1$^{-/-}$ Mice

Breeding of C1*galt*1$^{-/-}$ mice with VillinCre-ER^{T2} transgenic mice, both on a C57BL/6J congenic background, generated Tamoxifen (TM)-inducible intestinal epithelial cell-specific C1*galt*1$^{-/-}$ mice (TM-IEC C1*galt*1$^{-/-}$) who were made germ-free (GF) at Taconic Farms, Inc. Mice were bred in the Gnotobiotic Mice Facility at the University of Nebraska-Lincoln, and weaned at four weeks of age. Mice were genotyped by PCR assay on genomic DNA isolated from tail clippings. The primers used include CRE1: 5'-AGGTGTAGAGAAGGCACTTAGC-3', and CRE2: 5'-CTAATCGCCATCTTCCAGCAGG-3'. Six to ten mice, 6 to 7 weeks old were used per experiment including Cre-negative (WT), age-matched littermates as controls. Mice were colonized by orally gavaging 200μl of the total contents of cecums previously harvested from two conventional C57BL/6J mice and diluted in 4ml of sterile PBS. Tamoxifen (TM; MP Biomedicals) was injected intraperitoneally (1mg in an ethanol/sunflower oil mixture 1:9 v/v) for 5 consecutive days in a blinded fashion to both, WT (Cre^{-}) and TM IEC C1*galt*1$^{-/-}$ (Cre^{+}) mice. Therefore, a vehicle-control group - treated with ethanol/sunflower oil (without TM) - was not needed. Both, WT and TM IEC C1*galt*1$^{-/-}$ started TM treatment 48 to 72 hours post-colonization. TM treatment was completed before the mice were 8 weeks old. Mice weights were monitored and recorded every other day, and they were sacrificed 20 days after TM injections. Figure 2.1 shows a schematic representation of the experimental protocol.

2.2.2 Sample collection

Cecum contents were snap-frozen in liquid nitrogen. The distal colon was divided into three sections and preserved in liquid nitrogen, Carnoy's fixative (60% methanol, 30% chloroform, 10% glacial acetic acid), and 10% formalin, respectively.

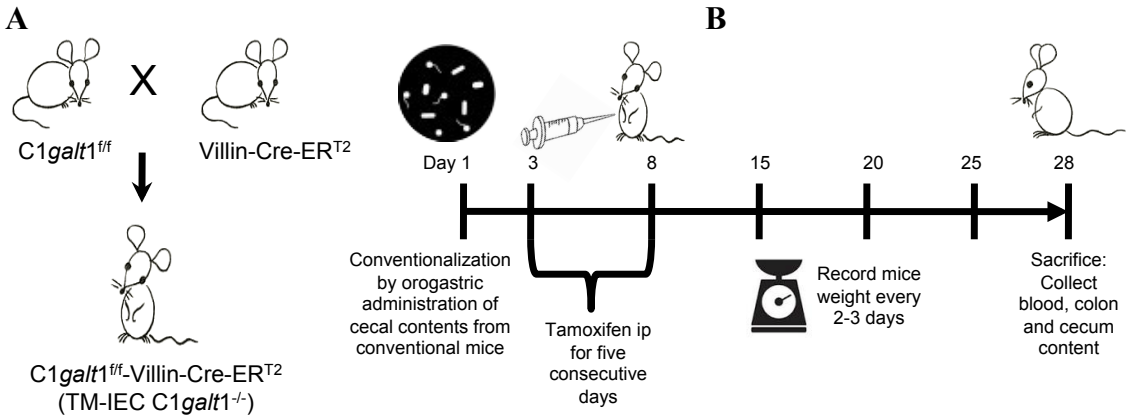

Figure 2.1 Schematic representation of the experimental protocol. [A] Generation of mice with an inducible deficiency of core 1 O-glycans specific to the intestinal epithelia (TM-IEC C1*galt*1$^{-/-}$) was achieved by breeding C1*galt*1$^{-/-}$ mice with Villin-Cre-ER^{T2} transgenic mice. They were made GF at Taconic Farms, Inc. [B] GF mice were colonized with 200µl of diluted cecal contents. Subsequently (48 to 72 hours post-colonization), 1mg of Tamoxifen (TM) diluted in an ethanol and sunflower oil mixture (1:9 v/v) was injected intraperitoneally for five consecutive days to both, WT and TM-IEC C1*galt*1$^{-/-}$ mice. Mice were sacrificed 20 days after TM treatment.

2.2.3 Immunohistochemical and Immunofluorescence staining of the distal colon tissue

2.2.3.1 Fluorescence in-situ hybridization (FISH)

FISH was performed to assess bacterial colonization and mucosal association. Samples were embedded in paraffin after fixing them in 10% formalin or Carnoy's fixative. Once paraffin embedded, they were left to dry at room temperature (RT)

overnight. Paraffin sections of 5μm thickness were deparaffinized and rehydrated as follows: 3 washes of xylene for 3 minutes; 1 wash with xylene/ethanol (50/50) for 3 minutes; 3 washes of 100% ethanol for 3 minutes each; 3 washes of 95% EtOH (3 mins/each); and one wash with dH_2O for 2 minutes. FISH probes were diluted with FISH Hybridization buffer (0.9M NaCl, 0.1M TRIS pH 7.2, 30% Formamide, 0.1%SDS) to a final concentration of 2.5ng/μl, and aliquotted onto each tissue section (100μL per slide). Slides were incubated at 37°C overnight in a humidified chamber in the dark, and then washed with pre-warmed (37°C) FISH Hybridization buffer for 15min. Excess hybridization buffer was aspirated, and tissue samples were washed with FISH Wash Buffer (0.9M NaCl, 0.1M TRIS pH 7.2) for 15 minutes. Afterwards, slides were rinsed twice for 2 minutes with distilled water, and 4′,6′-diamidino-2-phenylindole (DAPI) was applied for 3 minutes as a nuclear counter-stain. Two additional washes with distilled water were done, and slides were mounted using PermaFluor (ThermoScientific). Bacterial probes used for FISH are listed in Table 2.1.

Table 2.1 Bacterial probes used for FISH analysis

Probe	Target	Sequence	5' conjugation
EUB338	Universal bacterial probe	5'-GCT GCC TCC CGT AGG AGT-3'	Texas Red
NON338	Nonsense/non-specific probe (negative control)	5'- ACT CCT ACG GGA GGC AGC-3'	Texas Red
CFB286	Chlorobi/Fibrobacteres Bacteroidetes Superphylum	5'-TCC TCT CAG AAC CCC TAC-3'	Alexa Fluor 488

2.2.3.2 Hematoxylin and Eosin, Alcian Blue, Tn antigen and Lamina propria $CD45^+$ cells staining

Histological scoring for evaluation of colitis development was done based on epithelial hyperplasia and infiltration of $CD45^+$ lamina propria (LP) cells into the colon tissue. Hematoxylin and Eosyn (H&E) was used to address epithelial hyperplasia. Alcian Blue (AB) and Tn antigen staining were used to confirm mutation induction. Slides for H&E and AB staining were prepared following the same protocol as for FISH. H&E and AB were applied as established in the Core Imaging Facility of the Oklahoma Medical Research Foundation (OMRF) (http://imaging.omrf.org/wp-content/uploads/2011/08/hande.pdf). Briefly, starting with paraffin embedded section, the sections were washed 3 times with 100% xylene, once with 50:50 xylene and 100% ethanol, twice with 100% ethanol and twice with 95% ethanol. All washes were 3 minutes long. The sections were rinsed with distilled water for 2-3 minutes after the washes. Hematoxylin was added and incubated for 5 minutes. Sections were washed under running, tap water for approximately 5 minutes and immersed in acid alcohol (1%HCl in 70% ethanol) until sections turned pink. Next, sections were rinsed with tap water for 3 to 5 minutes and slowly immersed in ammonia water (1ml NH_4OH in 1 liter of water) 5 to 6 times until sections darken. Rinse step was repeated and Eosin was added for 1 minute. After a final rinse with tap water, 2 minutes rinses were done as follows: 3 times with 95% ethanol, 3 times with 100% ethanol, once with 50:50 xylene/100% ethanol, and 3 times with 100% xylene. Slides were kept on xylene until coverslipped.

For Lamina propria $CD45^+$ cells staining, 5µm formalin or Carnoy's-fixed, paraffin-embedded tissue sections were deparaffinized with xylene and rehydrated through an ethanol gradient to water. Sections were blocked for 15 minutes using serum-free protein block (Dako #X0909); incubated overnight at 4°C with rat-anti mouse CD45 (Abcam #ab25386, 5ug/mL) in primary antibody dilution buffer (1°ADB) (1% BSA, 0.1% Triton-x 100, 0.05% Tween-20 in PBS); and washed twice with 1°ADB, and twice with wash buffer (WB) (0.05% Tween-20 in PBS), 5 minutes/wash. Epifluorescent labeling for all stains was carried out using AlexaFluor 488-conjugated donkey anti-rat IgG (Invitrogen) in wash buffer, for 1 hr at RT in the dark, followed by rinsing in WB (3 x 5 mins). Samples were then fixed in 4% paraformaldehyde (PFA) in PBS for 5–10 mins, rinsed in WB (3 x 5 mins), stained with DAPI (for DNA counterstaining), washed 3 x 5 min in dH_2O, air-dried in the dark for 20 mins, and finally mounted with PermaFluor (ThermoScientific). Sections were viewed on an Eclipse E1000 (Nikon) microscope.

Protocol for Tn staining was done as described by Fu et al (2011). Briefly, deparaffinized sections were incubated with or without 0.5U/ml sialidase from *Arthrobacter ureafaciens* (Roche) at 37°C for 3 hours, and subsequently incubated for 30 minutes with biotinilated anti-Tn (mouse IgM) or with isotype-matched control IgM. Bound antibodies were detected with horseradish peroxidase-conjugates streptavidin (Vector Laboratories).

$CD45^+$ LP cells were stained as previously described and counted in a blinded fashion at 40X magnification, for 4 to 8 fields per sample, by counting all the nuclei in between the crypts, avoiding the nuclei associated with blood and lymphatic vessels (i.e. endothelial cells). Calculation of the number of epithelial nuclei in the crypts, by

counting 10 to 20 well-oriented crypts per section using the H&E stained slides, assessed epithelial hyperplasia. The average number of intra-epithelial cells (IECs) per crypt for each section was obtained from the raw number of IECs. The average number of LP cells and IECs for each TM IEC C1*galt*1$^{-/-}$ mice was divided by the average number obtained for each WT, and converted to percentage. Scores were assigned base on parameters established in Table 2.2.

Table 2.2 Scoring parameters for assessment of inflammation

Parameter	Score			
	0	1	2	3
Lamina Propria CD45^{+} cells per HPF (% WT)	≤105%	106-125%	126-150%	≥151%
Epithelial Hyperplasia (% WT)	≤105%	106-125%	126-150%	≥151%

2.2.4 Immunoglobulin measurement by ELISAs

ELISA protocols were followed for measurement of immunoglobulins IgA, IgM, IgG, and the IgG isotypes IgG1, IgG2a, IgG2b, and IgG3, as described in Peterson et al (2007). Briefly, plates were coated with 100µl of unlabeled goat anti-mouse (GAM) Ig heavy and light chain (H&L) (Southern Biotech #1010-01) diluted in bicarbonate buffer, and incubated overnight at 4°F. Coating buffer was decanted, and 300µl of 1% Bovine Serum Albumin in PBS (BSA-PBS) were added after 3 washes with PBS-Tween (0.05% of Tween 20 in PBS pH7.4, PBS-T). 1% BSA-PBS was used to dilute all samples and standards, and PBS-T was used for all subsequent washes. Plates were incubated for

30min at RT. Subsequently, plates were washed 3 times as previously, and standards and samples added. After a 2 hours incubation period at RT, plates were washed again, and 100µl of the appropriate HRP conjugated secondary antibody added per well, followed by a second 2hr incubation step. Finally, after 3 washes, 100µl of ABTS substrate were added per well, incubated 20min at RT, and OD read at 405nm. Specific details of isotype controls and HRP-conjugated secondary antibodies are listed in table 2.3.

Table 2.3 Antibodies used for Immunoglobulin ELISAS

Igs	Isotype Control	HRP-conjugated
IgA	Mouse IgA unlabeled Southern Biotech#0106-01	Goat Anti-mouse IgA-HRP Southern Biotech #1040-05
IgG	Mouse IgG unlabeled Southern Biotech #0107-01	Goat Anti-mouse IgG-HRP Southern Biotech #1030-05
IgG1	Mouse IgG1 unlabeled Southern Biotech #0102-01	Goat Anti-mouse IgG1-HRP Southern Biotech #1070-05
IgG2a	Mouse IgG2a unlabeled Southern Biotech #0103-01	Goat Anti-mouse IgG2a-HRP Southern Biotech #1080-05
IgG2b	Mouse IgG2b unlabeled Southern Biotech #0104-01	Goat Anti-mouse IgG2b-HRP Southern Biotech #1090-05
IgG3	Mouse IgG3 unlabeled Southern Biotech #0105-01	Goat Anti-mouse IgG3-HRP Southern Biotech #1100-05
IgM	Mouse IgM unlabeled Southern Biotech #0101-01	Goat Anti-mouse IgM-HRP Southern Biotech #1021-05

2.2.5 Data Analysis

Statistical analysis for immunoglobulin levels and histological scores between genotypes (TM-IEC C1*galt*1$^{-/-}$ *vs*. WT) was determined by unpaired t-test with Welch's correction and one-way ANOVA using GraphPad Prism 5.0 (GraphPad Software Inc., USA) statistical software.

2.3 Results

2.3.1 Assessment of colonization in the conventionalized TM-IEC C1galt1$^{-/-}$ mice

FISH staining of colon tissue confirmed absence of bacteria in the GF mice (Figure 2.2C) and showed robust bacterial colonization in the conventionalized mice, the presence of bacteria being more intense in the conventionalized TM-IEC C1*galt*1$^{-/-}$ mice than in the conventionalized WT (Figure 2.2A). The use of phylum-specific primers indicated abundance of Bacteroidetes in the conventionalized mice (Figure 2.2B).

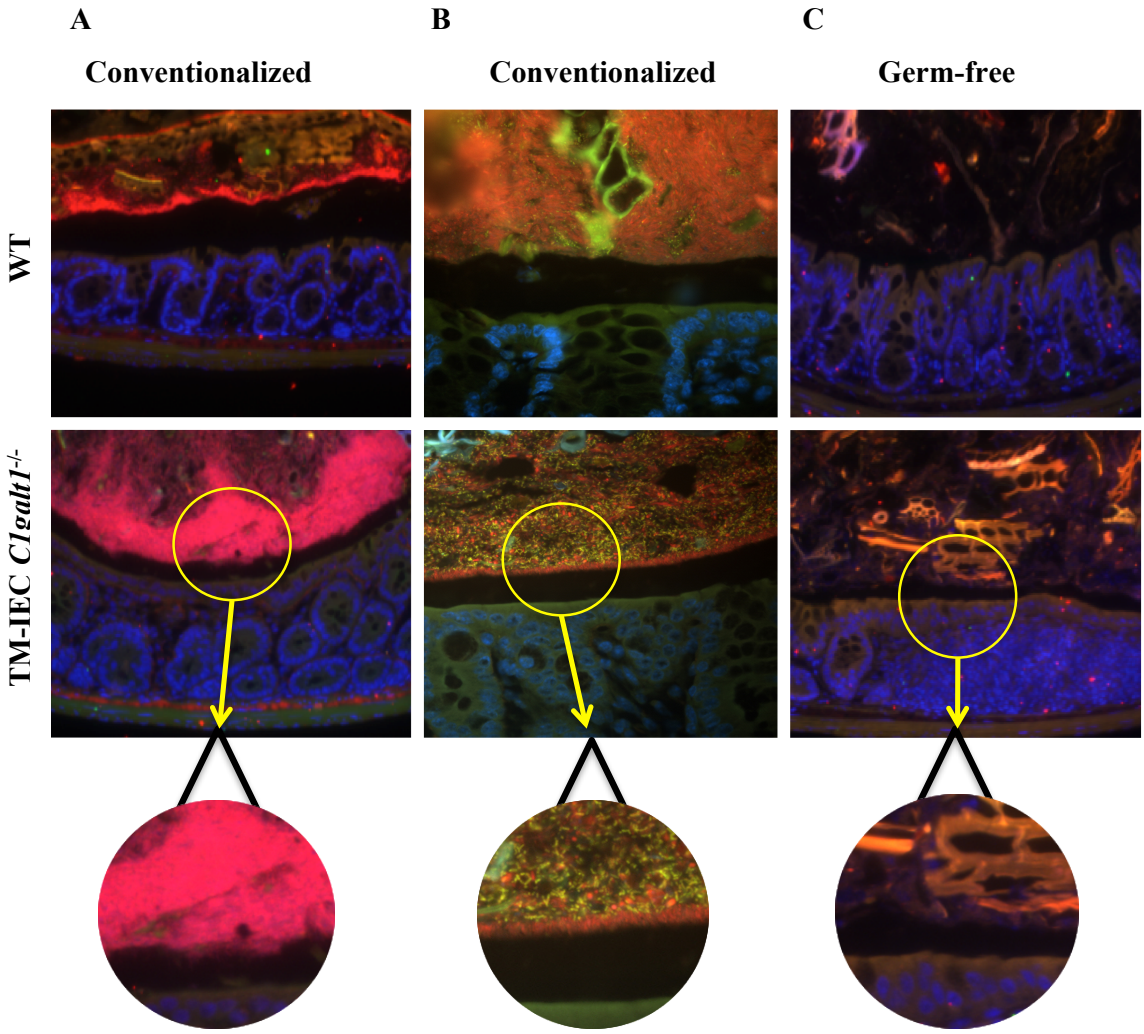

Figure 2.2 Mucosa-associated bacteria increased in the conventionalized TM-IEC C1*galt*1$^{-/-}$ mice relative to the WT, as assessed by FISH. [A] FISH staining using universal primers demonstrated robust colonization (bacteria stained in red) of the conventionalized (ex-germ-free) mice. The mucosa-associated bacteria increased in the conventionalized TM-IEC C1*galt*1$^{-/-}$ mice [B] The use of phylum specific primers showed abundance of Bacteroidetes (green) in the microbiota of the conventionalized mice. [C] FISH analysis of GF mice. [A, B, C] Note a decrease in the mucus layer (black space between bacteria-in red or green- and epithelial cells-in blue) in the mutated mice (bottom row) relative to the WT control (top row).

2.3.2 Assessment of mutation induction

Both, Alcian Blue (AB) staining and Tn antigen staining were used to assess mutation induction. Figure 2.3 shows structural features analyzed through [A] AB and [B] Tn antigen staining for the assessment of differences between genotypes (WT *vs* TM-IEC C1*galt*1$^{-/-}$) and between treatments (GF *vs* conventionalized). AB-stained colon tissue of both, conventionalized TM-IEC C1*galt*1$^{-/-}$ and GF TM-IEC C1*galt*1$^{-/-}$ mice, showed decreased number of goblet cells and a thinner mucus layer relative to their WT counterparts (Figure 2.4A). Specifically for the GF setting, counting of goblet cells in the TM-IEC C1*galt*1$^{-/-}$ mice showed a statistically significant decrease of 20% to 30% ($p=<0.05$) in the number of goblets cells (caused by the mutation) when compared to the GF WT (Figure 2.5). In addition, FISH analysis demonstrated a closer proximity of the microbiota to the epithelial layer in the conventionalized TM-IEC C1*galt*1$^{-/-}$ (Figure 2.2A-B), relative to the conventionalized WT.

The Tn antigen is a glyco-aminoacidic determinant linked to serine or threonine residues by a glycosidic bond (GalNAc-O-Ser/Thr). It is masked in normal cells by type O saccharide chain elongation; therefore, it is expressed in some abnormal or sick cells when such elongation does not occur. Upon deletion of the T-synthase enzyme (C1GALT1) responsible for core 1 O-glycosilation of the mucus layer, the Tn antigen will be detected. Both conventionalized and GF TM-IEC C1*galt*1$^{-/-}$ mice showed increased presence of Tn antigen (brown staining), compared to their WT counterparts (Figure 2.4B).

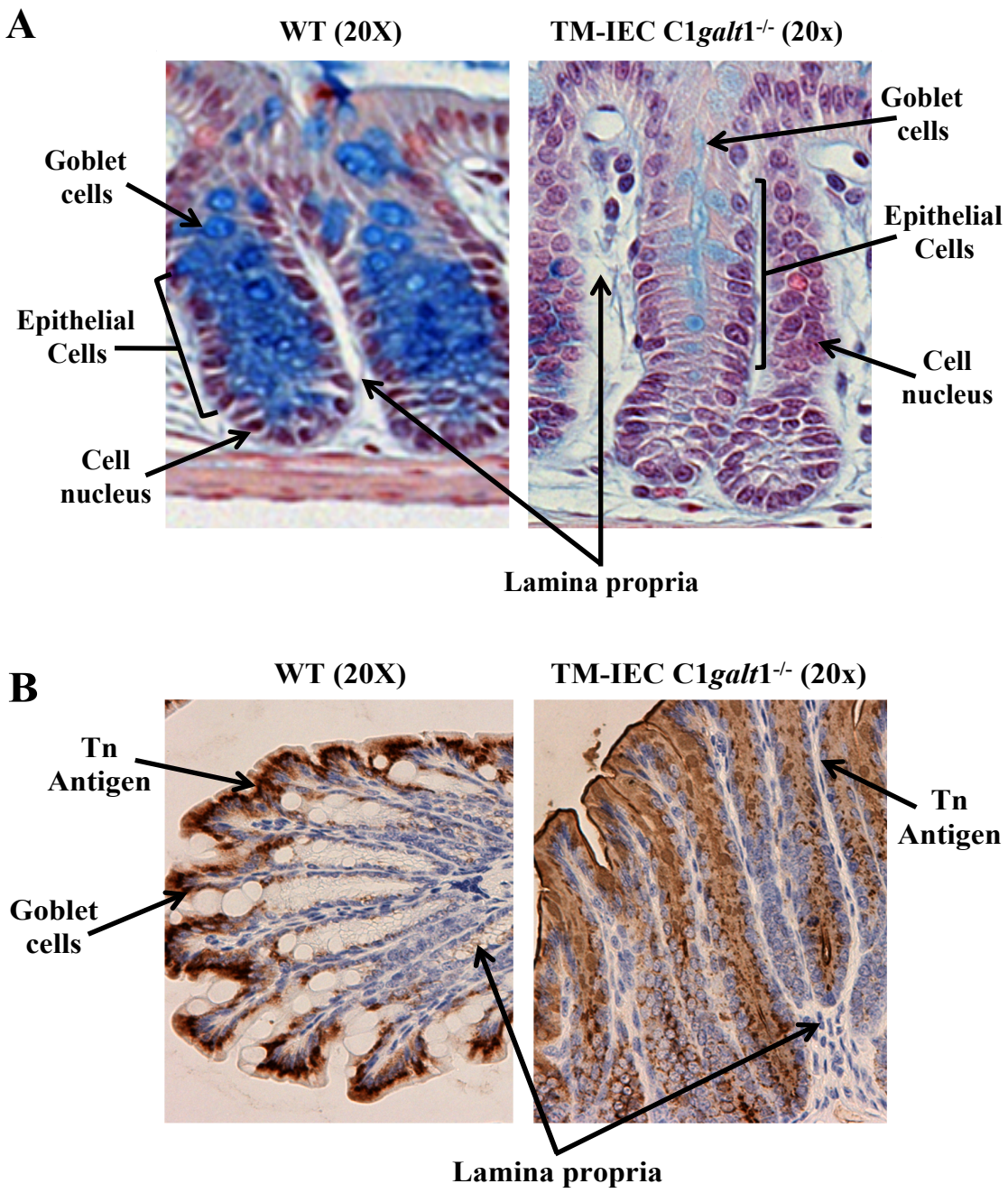

Figure 2.3 Structural features analyzed for the assessment of deficiency of core 1 O-glycans. Note differences between the genotypes in [A] decreases in mucus production and the number of goblet cells (stained blue) and [B] increases in the formation of Tn antigen (stained brown).

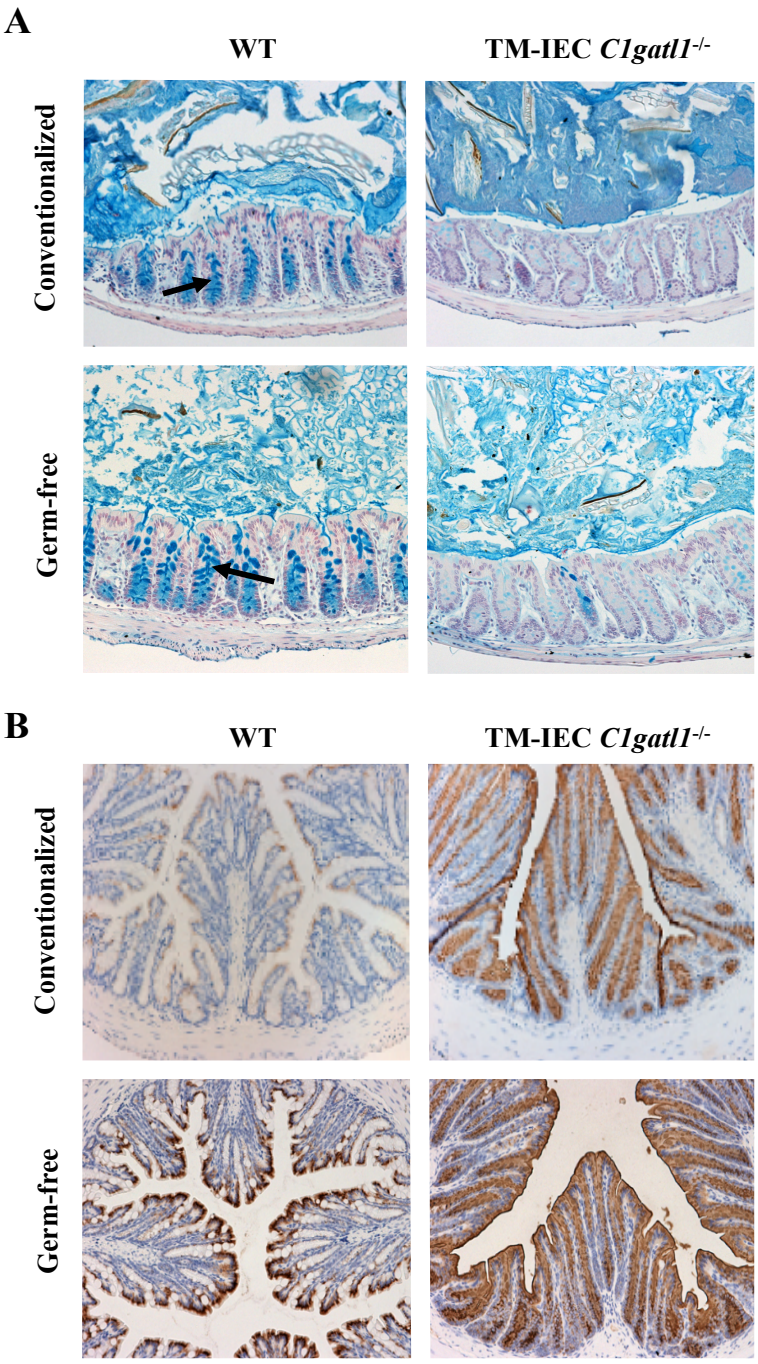

Figure 2.4 Good mutation induction was obtained after Tamoxifen treatment in the TM-IEC C1*galt*1$^{-/-}$ mice as assessed by a reduction in goblet cells and increased production of Tn antigen. [A] Alcian Blue staining showed a decrease in mucus production by goblet cells (black arrows point at goblet cells in the WT mice). [B] Tn staining (brown stain) confirmed an increased detection of Tn antigen in the TM-IEC C1*galt*1$^{-/-}$ mice in both, the conventionalized and the GF scenarios.

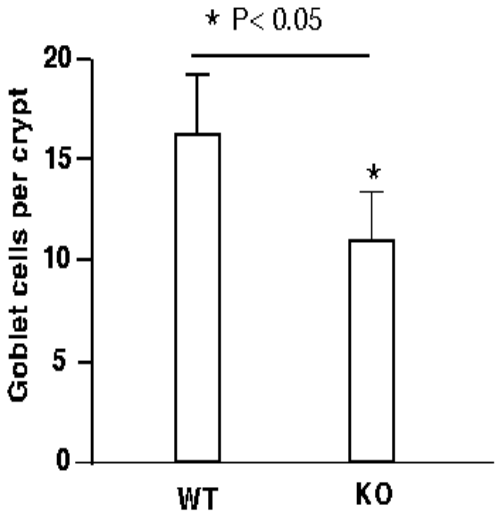

Figure 2.5 The GF TM-IEC C1*galt*1$^{-/-}$ mice displayed a reduction of 20% to 30% in the number of goblet cells relative to the GF WT mice as assessed through AB staining. GF mice inherently exhibit a decrease in the number of goblet cells relative to the conventional mice. Interestingly, mutation induction further reduced the number of goblet cells in the GF TM-IEC C1*galt*1$^{-/-}$ mice. This reduction was estimated in 20% to 30% relative to the WT GF mice.

2.3.3 Development of disease in the GF TM-IEC C1*galt*1$^{-/-}$ mice relative to Conventionalized TM-IEC C1*galt*1$^{-/-}$ mice

Development of colitis was monitored by the appearance of classic disease signs, such as development of diarrhea, weight loss and rectal prolapse. Comparison of the average daily weight gain (ADG) showed higher values in the GF mice than the conventionalized mice. Assessment of ADG between the genotypes (TM-IEC C1*galt*1$^{-/-}$ *vs*. WT) revealed opposite trends between the treatments: the ADG was greater in the GF WT mice relative to the GF TM-IEC C1*galt*1$^{-/-}$, while in conventionalized mice the TM-IEC C1*galt*1$^{-/-}$ showed greater ADG than the WT. Still, no statistically significant changes were observed regarding weight fluctuations. Average daily weight gain and standard deviations per treatment group and per genotype are listed in Table 2.4. Development of rectal prolapse was not observed in the conventionalized mice. No major alterations in stool consistency were observed.

Table 2.4 Mice average daily weight gain (ADG) and standard deviations (SD) per treatment and per genotype

Treatment	ADG WT ± SD (g)	ADG KO ± SD (g)	P-value
Germ-free	0.14±0.07	0.12±0.03	0.4769
Conventionalized	0.07±0.06	0.10±0.04	0.3511

Inflammation scores (parameters for scoring presented in table 2.2) were based on epithelial hyperplasia and on infiltration of lamina propria $CD45^+$ cells into the tissue, evaluated by H&E staining and immunolabeling of $CD45^+$ cells, respectively. Figure 2.6 schemes the determination of inflammation scores.

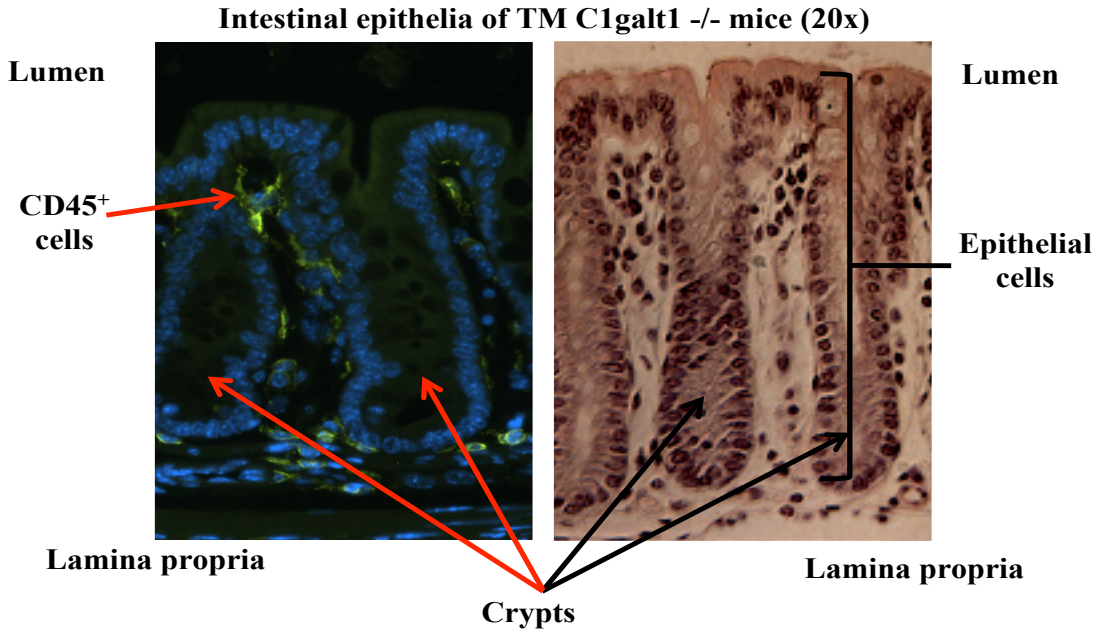

Figure 2.6 Features analyzed for the determination of inflammation scores based on lamina propria CD45+ cells infiltration in the tissue of the colon and epithelial hyperplasia. The number of CD45+ cells was estimated by counting all the nuclei in between the crypts, avoiding the nuclei associated with blood and lymphatic vessels (left). Epithelial hyperplasia was estimated by counting the number of epithelial nuclei in10 to 20 well-oriented crypts per section of colon (right).

The $CD45^+$ marker is present in all leukocytes, making it an useful indicator of inflammation. No significant differences between the germ-free genotypes (GF WT vs. GF TM-IEC C1*galt*1$^{-/-}$ mice) were found in the number of $CD45^+$ cells (Figure 2.9A). On the other hand, the number of $CD45^+$ cells in the conventionalized TM-IEC C1*galt*1$^{-/-}$ doubled the amount of cells in the conventionalized WT counterpart, going from 30 cells in the WT up to 60 cells ($p=0.0460$) on average per field in a conventionalized TM-IEC C1*galt*1$^{-/-}$ sample (Figures 2.7 and 2.9A). Appendix B includes graphs of absolute numbers of CD45+ cells per treatment and genotype.

Epithelial hyperplasia refers to an abnormal increase in the number of normal cells that increases the volume of the intestinal epithelia. Hematoxylin will stain the nuclei of cells purple-blue, while Eosyn is used as a counterstain to color the cytoplasm and extracellular proteins in shades of pink and red. Therefore, H&E staining allows the study of changes in the cellular composition of the tissue. Statistical analysis of inflammation scores showed no significant differences between the genotypes in the GF setting. However, the conventionalized TM-IEC C1*galt*1$^{-/-}$ mice developed moderate to severe inflammation, relative to the conventionalized WT counterpart, with scores values of 4 and 5 in a scale with a highest possible value of 6 ($p<0.001$) (Figures 2.8 and 2.9B).

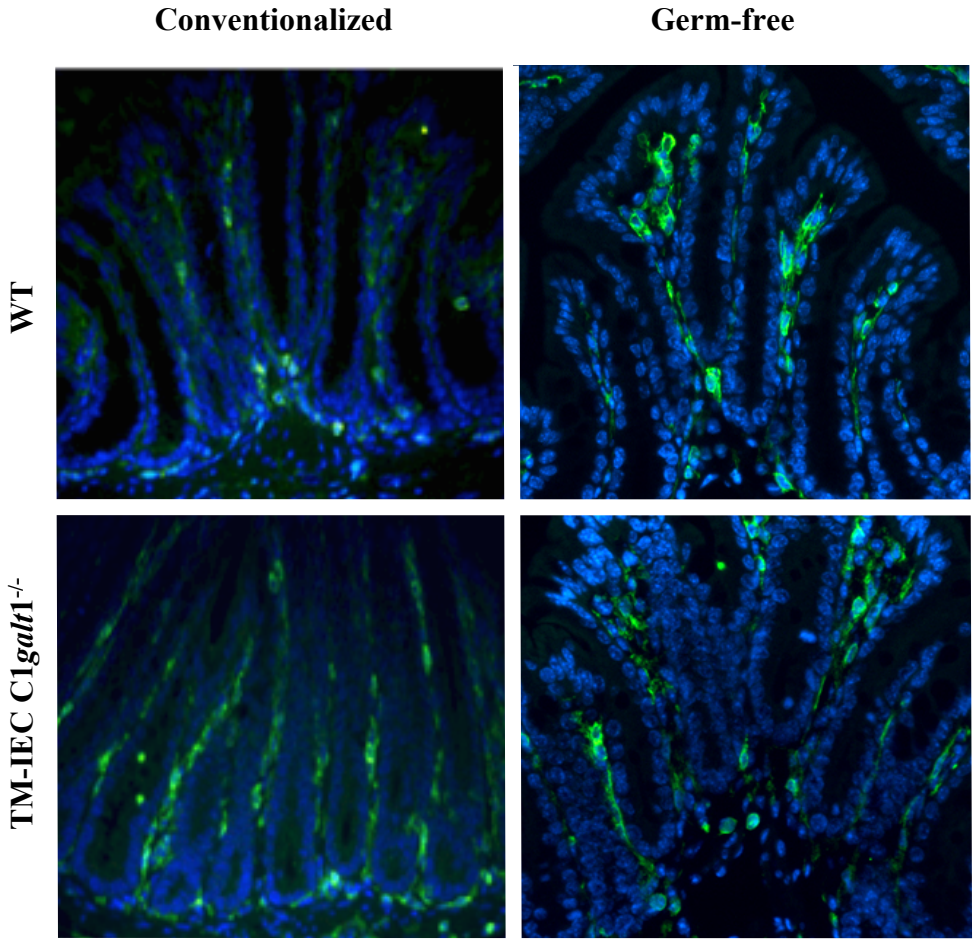

Figure 2.7 Increases in the number of infiltrated lamina propria CD45$^+$ cells were seen in the conventionalized TM-IEC C1*galt*1$^{-/-}$ relative to the conventionalized WT. The criteria for assessment of inflammation in the colon included infiltration of CD45$^+$ cells in the colon tissue. The number of CD45+ cells (stained green) in the conventionalized TM-IEC C1*galt*1$^{-/-}$ mice doubled possibly due to an increased exposure of the microbiota to the intestinal epithelia caused by a breach in the mucus layer.

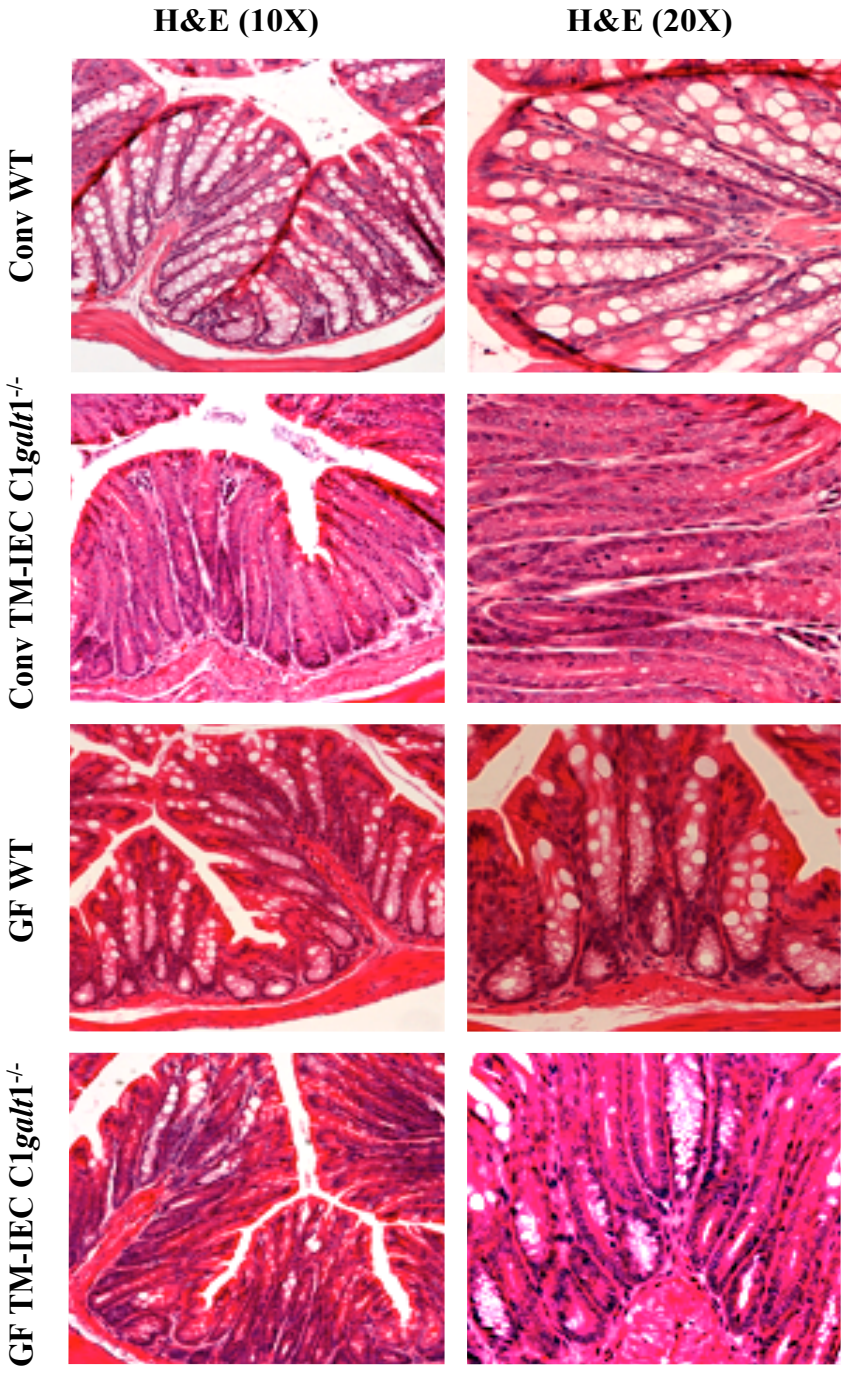

Figure 2.8 Conventionalized TM-IEC C1*galt*1$^{-/-}$ developed a high degree of epithelial hyperplasia. H&E was used to assess epithelial hyperplasia in the colon. Note differences in appearance of the tissue and length of the crypts in the conventionalized TM-IEC C1*galt*1$^{-/-}$ mice compared to the GF counterpart and the WT controls.

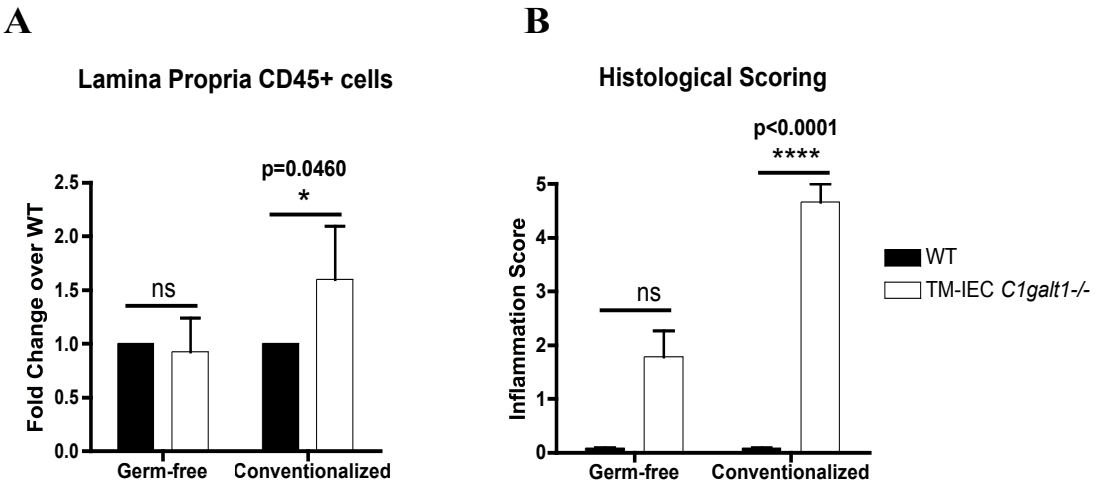

Figure 2.9 Germ-free TM IEC C1*galt*1$^{-/-}$ mice did not develop inflammation. [A] The number of CD45+ cells in the conventionalized mice doubled possibly to an increased exposure of the microbiota to the intestinal epithelia caused by a breach in the mucus layer. [B] GF IEC C1*galt*1$^{-/-}$ mice do not develop inflammation relative to conventionalized IEC C1*galt*1$^{-/-}$ mice based on the established parameters.

2.3.4 Differences in B cell responses between TM-IEC C1*galt*1$^{-/-}$ and WT mice in GF and conventionalized settings

The adaptive immunity response was assessed in the GF and conventionalized WT and TM-IEC C1*galt*1$^{-/-}$ mice by measurement of immunoglobulin levels by ELISA. Immunoglobulin levels were compared *between* genotypes (WT *vs* TM-IEC C1*galt*1$^{-/-}$), and *within* genotypes (GFWT *vs* conventionalized WT, and GF TM-IEC C1*galt*1$^{-/-}$ *vs* conventionalized TM-IEC C1*galt*1$^{-/-}$) (Figure 2.10 and Table 2.5).

A higher level of IgA in serum was found in the GF TM-IEC C1*galt*1$^{-/-}$ mice relative to the GF WT. The opposite was found in the conventionalized TM-IEC C1*galt*1$^{-/-}$ mice were serum IgA was lower than in the conventionalized WT. In regards to secretory IgA (sIgA), the levels were higher in the TM-IEC C1*galt*1$^{-/-}$ mice than in

WT, in both GF and conventionalized scenarios. However, the differences were only statistically significant between the genotypes in the GF mice (serum IgA: GF WT 30.49 ± 1.48 *vs* GF TM-IEC C1*galt*1$^{-/-}$ 50.14 ± 5.43, p= 0.004; sIgA: GF WT 97.46 ± 19.62 *vs* GF TM-IEC C1*galt*1$^{-/-}$ 429.7 ± 88.29, p=0.021). In addition, specifically for sIgA, statistically significant differences were found *within* genotypes (GF WT 97.46 ± 19.62 *vs* Conv WT 1982.0 ± 407.80, p=0.019; GF TM-IEC C1*galt*1$^{-/-}$ 429.7 ± 88.29 *vs* Conv TM-IEC C1*galt*1$^{-/-}$ 2562.0 ± 477.40, p=0.021).

No significant differences were found in total IgG or IgG subtypes between treatments or between genotypes. In spite of this, a qualitative increase in IgG1 was seen in the GF mice (both, WT and TM-IEC C1*galt*1$^{-/-}$) when compared to the conventionalized. These changes were proportional to reductions in IgG2b were the opposite occurred: higher levels of IgG2b were found in the conventionalized mice than in the GF.

Regarding IgM, levels were higher in both, the GF WT mice and the GF TM-IEC C1*galt*1$^{-/-}$ mice than in conventionalized mice. However, statistically significant differences were found exclusively for comparisons between the GF TM-IEC C1*galt*1$^{-/-}$ and the conventionalized TM-IEC C1*galt*1$^{-/-}$ (367.70 ± 41.61 *vs* 223.50 ± 36.00, respectively; p= 0.024).

Table 2.5 lists the means, standard error of the means (SEM), and p-values for comparison *between* and *within* genotypes for each immunoglobulin measured.

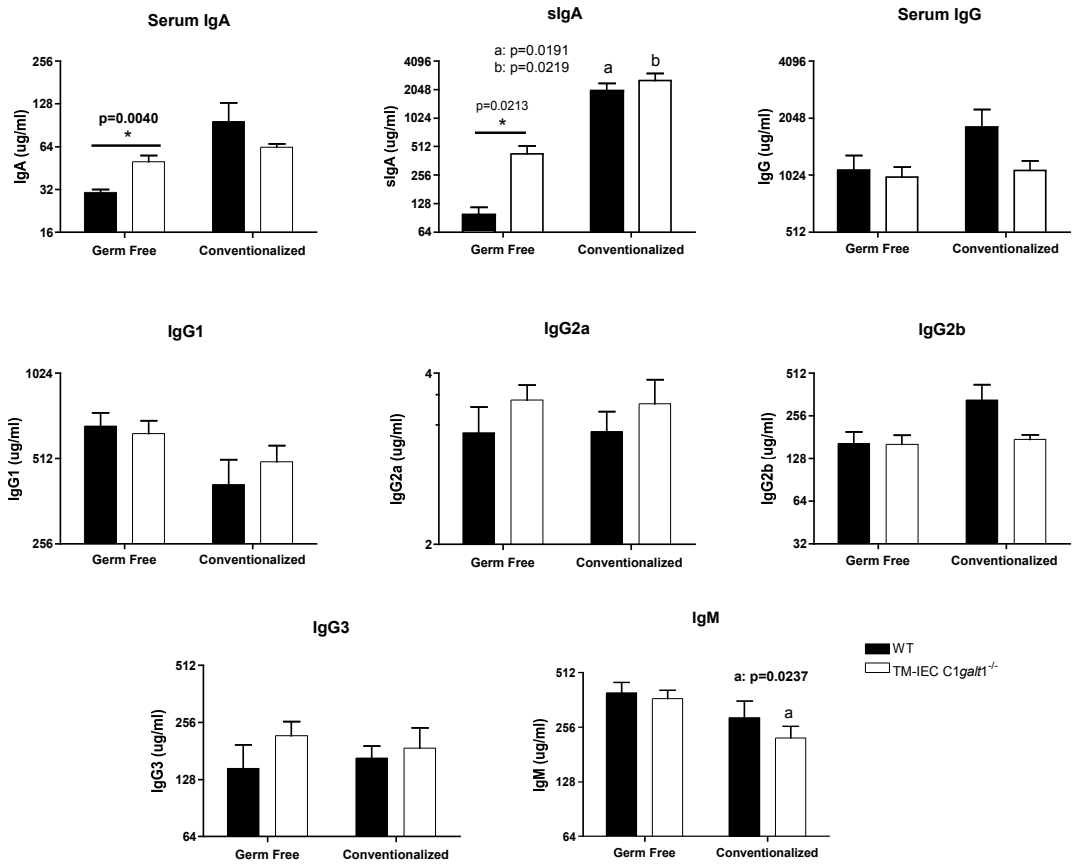

Figure 2.10 Lack of core 1-Oglycans induced production of secretory IgA in the absence of microbes. The levels of sIgA in GF mice increased when the T-synthase (C1GALT1*)* enzyme is deleted, thus creating a breach in the mucus barrier. Conventionalization of germ-free mice induced increases in the levels of sIgA in the TM-IEC C1*galt*1$^{-/-}$mice. No significant differences were found in the levels of IgG or IgG isotypes between the treatments or between genotypes. IgM levels were higher in the GF mice. Lines above bars indicate significant differences between mice of different genotypes (WT vs TM-IEC C1*galt*1$^{-/-}$), while letters above bars indicate significant differences between mice of the same genotype (GF TM-IEC C1*galt*1$^{-/-}$ *vs* conventionalized TM-IEC C1*galt*1$^{-/-}$ or GF WT *vs* conventionalized WT). Y-axes present real values in a log 2 scale. Error bars represent SD.

Table 2.5 Comparisons of Immunoglobulin levels between WT and TM-IEC C1*galt*1$^{-/-}$ in germ-free and conventionalized scenarios

Immunoglobulin (ug/ml)	Germ Free		Conventionalized		P-values per comparison			
	WT	TM-IEC C1galt1$^{-/-}$	WT	TM-IEC C1galt1$^{-/-}$	GFWT vs ConvWT	GFKO vs ConvKO	GFWT vs GFKO	ConvWT vs ConvKO
IgA	30.49 ± 1.48	50.14 ± 5.43	96.10 ± 34.33	63.38 ± 3.39	0.152	0.058	**0.004****	0.413
sIgA	97.46 ± 19.62	429.70 ± 88.29	1982.00 ± 407.80	2562.00 ± 477.40	**0.019***	**0.021***	**0.021***	0.398
IgM	395.60 ± 57.09	367.70 ± 41.61	289.10 ± 67.69	223.50 ± 36.00	0.268	**0.024***	0.698	0.440
IgG	1658.00 ± 380.30	1273.00 ± 100.00	1837.00 ± 438.60	1083.00 ± 133.80	0.767	0.298	0.348	0.199
IgG1	667.50 ± 76.03	626.60 ± 69.66	414.20 ± 92.40	498.30 ± 70.36	0.072	0.227	0.696	0.502
IgG2a	4.38 ± 0.77	4.68 ± 0.59	3.16 ± 0.26	3.53 ± 0.36	0.165	0.121	0.760	0.442
IgG2b	162.90 ± 34.43	160.70 ± 25.16	332.10 ± 93.75	174.20 ± 13.26	0.232	0.642	0.959	0.237
IgG3	146.50 ± 48.71	218.00 ± 41.20	166.10 ± 26.51	187.70 ± 51.76	0.730	0.662	0.276	0.729

Values represent means and standard errors of means (Mean ± SEM). Asterisks (*) indicate significant values

GF: Germ-free; Conv: Conventionalized; WT: Wild type mice; KO: TM- IEC C1*galt*1$^{-/-}$ mice

2.4 Discussion

2.4.1 Germ-free TM-IEC C1*galt*1$^{-/-}$ mice do not develop inflammation relative to conventionalized TM-IEC C1*galt*1$^{-/-}$

The typical symptoms and pathology associated with ulcerative colitis (development of diarrhea, rectal prolapse, weight loss, among others) were not evident in the conventionalized mice. Nevertheless, in spite of such mild phenotype, we were able to assess the development of disease through immunological and histological changes associated with inflammation. The infiltration of lamina propria CD45+ cells into epithelial tissue indicates an active immune response, and the increase in number on epithelial cells (hyperplasia) denotes the occurrence of a pathologic process.

GF mice, regardless of their genetic constitution, are known to have a distinct GIT morphology and an underdeveloped, immature immune system (Thompson and Trexler, 1971). GF WT mice normally reveal a lower number of goblet cells and thinner mucus layer in the small intestine than conventional WT mice. In the TM-IEC C1*galt*1$^{-/-}$ mice the induced breach in the mucus barrier caused a further reduction in the number of goblet cells relative to the GF WT. However, based on the established parameters for assessment of inflammation, TM-IEC C1*galt*1$^{-/-}$ mice did not develop disease as long as they were kept germ-free, and only showed sings of inflammation when conventionalized through gavage of cecal contents. Although the GF TM-IEC C1*galt*1$^{-/-}$ mice developed some degree of hyperplasia, the number of LP CD45^{+} cells found in these mice was equivalent to the one found in the GF WT counterpart. Ultimately, the scores obtained for epithelial hyperplasia evaluation, added to those obtained from LP CD45^{+} cell counts,

did not show any significant differences between the GF TM-IEC C1*galt*1$^{-/-}$ mice and the GF WT counterpart. In contrast, in conventionalized mice, deletion of the C1GALT1 enzyme resulted in increased interactions between the microbiota and epithelial surfaces (as assessed by FISH), increased infiltration of LP CD45^{+} cells and increased epithelial hyperplasia, which contributed to higher inflammation scores than in conventionalized WT mice. Therefore, results indicate that the microbiota, in conjunction with a breach in the mucus layer, has a role in disease development.

The absence of typical symptoms associated with UC in conventionalized TM-IEC C1*galt*1$^{-/-}$ could be attributed to a role of their genetic background in the pathogenesis of the disease (Mahler and Leiter, 2002; Buchler et al, 2012). Initial experiments aimed to study the relationship between lack of core 1 O-glycans and the induction of disease were performed with C1*galt*1$^{-/-}$ originated from a 129/SvlmJ mouse and bred into a C57Bl/6J genetic background (Xia et al, 2004). Even though the conventional TM-IEC C1*galt*1$^{-/-}$ mice used by Fu et al (2011) were generated in a C57Bl/6 congenic background, the mice used in our experiments were backcrossed *additional generations* into the C57Bl/6J mice. Fu's mutated conventional mice genetic background was closer to 129/SvlmJ mice than a C57Bl/6J background, and spontaneously developed severe, typical symptoms of colitis. However, in our system we found a mild disease phenotype in both, the conventionalized and monoassociated mutant. Gulati et al (2012) demonstrated that the response of the two strains towards microbial colonization differ from one another. The 129/SvImJ mice have decrease number of Paneth cells and a different profile of antimicrobial peptides relative to the C57BL/6J. These two factors affect the gut microbial composition and synergistically,

they may act to make the 129/SvImJ strain more susceptible to disease, and development of inflammation.

Additionally, the mice used in the experiments described in this dissertation were raised and bred in a GF setting, and as already established, the immune response of the GF mice is weaker than that of the conventionalized mice, and even weaker than that seen in the conventional setting. The combination of two factors, genetic background and absence of microbes, add up in disease prevention in the GF TM-IEC C1*galt*1$^{-/-}$ mice which is consistent with the hypothesis of the multifactorial etiology of IBD.

2.4.2 Breaches in the mucus layer induce systemic and localized production of IgA in the absence of microbes.

The levels of both serum and secretory IgA are, in general, higher in the conventionalized mice than the GF mice. However, the differences in systemic and localized IgA between GF TM-IEC C1*galt*1$^{-/-}$ mice and the GF WT are by far greater than those found in the conventionalized setting. In GF mice, the level of serum IgA almost doubled in the TM-IEC C1*galt*1$^{-/-}$ mice while sIgA increased four times (4x) relative to the GF WT mice. Such changes could be explained by the enhanced exposure of the intestinal epithelia- and consequently the immune system- to environmental and diet antigens granted by the absence of microbes and the presence of a defective mucus layer. In a normal, fully functional epithelial barrier, the mucus layer protects against exposure to foreign antigens and against chemical and proteolytic injury, thus controlling or preventing immune responses.

Overall, the Ig values found for the GF mice are in agreement to those found by Macpherson and Harris (2004), which were summarized in the introduction. For both, WT and TM-IEC C1*galt*1$^{-/-}$ mice we found decreases in IgA and IgG in the GF mice when compared to the conventionalized, and relatively similar values of IgM between the treatments.

Interestingly, the trends observed in the conventionalized TM-IEC C1*galt*1$^{-/-}$ are opposite to the Ig values obtained in patients with IBD. The conventionalized TM-IEC C1*galt*1$^{-/-}$ mice had increased sIgA and decreased total serum IgG, which is contrary to what has been found in humans (increased IgG and decreased IgA). These results might relate to our assessment of the microbiota associated with the TM-IEC C1*galt*1$^{-/-}$ mice, since we found the opposite of what has been found for human IBD (increases in Firmicutes in the TM-IEC C1*galt*1$^{-/-}$ mice, which contrast with the decreases in this phylum in patients with IBD).

2.4.3 Bacteroidetes exist in abundance in the microbiota of the conventionalized mice.

The analysis of the microbiota in *conventional* core 1 O-glycans deficient mice relative to *conventional* WT mice is addressed in Chapter 3 of this dissertation. The major changes found at the phylum level were increases in Firmicutes and decreases in Bacteroidetes, with the latter comprising up to 40% of the total microbiota.

The use of phylum-specific primers in our FISH analysis showed abundance of Bacteroidetes in the *conventionalized* (ex-germ-free) mice. Even though our results

indicated that Firmicutes were the most abundant phylum in the conventional WT and TM-IEC C1*galt*1$^{-/-}$mice, it is important to acknowledge the amount of Bacteroidetes that the microbiota include for two reasons: firstly, the genus *Bacteroides*, which belongs to the Bacteroidetes, is the most abundant bacterial genus in the human gut; and secondly and most importantly, *Bacteroides* species have been linked to development of inflammation in human and murine models of IBD (Gophna et al, 2006; Walker et al, 2011; Andoh et al, 2011).

Furthermore, the dissimilarities between our pyrosequencing results (addressed in chapter 3 of this thesis) for microbiota characterization and results obtained through FISH analysis might arise from differences in the environmental microbiota- and therefore, differences in the mice gut microbiota- since mice were bred and treated at different facilities. Specifically, pyrosequencing was performed in samples from conventional mice housed in the mice facility of the OMRF, Oklahoma, while mice used in the experiments reported in this dissertation were bred in a germ-free setting, and conventionalized using cecal contents from the mice facility at University of Nebraska, Nebraska.

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Chapter 3

The Gut Microbiota of

Core 1 O-glycans Deficient Mice

3.1 Introduction

The mucus layer has the main function of keeping at distance the gut microbiota from the intestinal epithelia and mucosal immune cells. Defects associated with the mucus layer disrupt the relationship between the microbiota and the host, thus potentially evoking immune responses against the microbiota predisposing to disease. The objective of the work described in this chapter was to identify changes in the microbiota caused by a disruption in the mucus layer, specifically by deficiency of core 1 O-glycans in the mucus' composition.

The intestinal epithelial barrier includes a monolayer of epithelial cells, subepithelial cells and the mucus layer. The mucus layer, in turn, consists of bioactive molecules, such as mucins, glycoproteins, and anti-microbial peptides, among other components (McGuckin et al, 2011), and it comprises an inner and an outer layer (Johanson et al, 2011). Although the thickness of each of these layers varies along the gastrointestinal tract, the outer layer is thicker and has a lower content of Muc 2 mucins than the inner layer. Both, mucin content and thickness of the layers play a role in the spatial organization of the microbiota and the intestinal mucosa, as these can limit interactions between bacteria and luminal contents with epithelial cells (Johansson et al, 2010). The outer layer is usually colonized by commensals and mucosa-associated bacteria, while the inner layer is considered sterile being devoid of bacteria (Johanson et al, 2008).

O-glycans are the main component of the intestinal mucins that form the mucus gel layer shielding the gut epithelium (Figure 3.1). They are usually attached

to serine and threonine residues in the mucin protein, and comprise up to 80% of the mucins' molecular weight (Kim and Ho, 2010). Some of their functions include shielding epithelial surfaces against chemical and physical damage, maintaining hydration of the epithelium, and serving as receptors to trap and remove bacteria. They may also affect the conformation of the protein to which they are attached, thus affecting its function (Varki and Lowe, 2009).

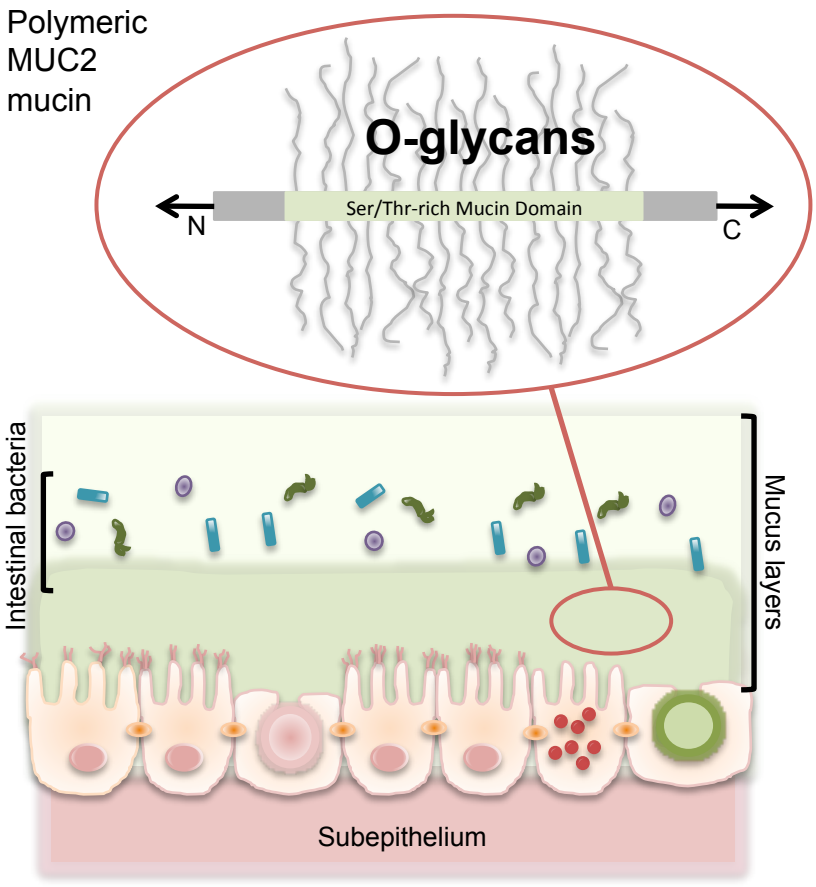

Figure 3.1 O-glycans are the main component of the intestinal mucins that form the mucus gel layer shielding the gut epithelium. O-glycans are usually attached to serine and threonine residues in the mucin glycoprotein backbone. They comprise up to 80% of the mucin's molecular weight.

Defects in the mucus layers and their O-glycans may arise from mutations in the mucin genes, lack of mucin proteins, and deletions of the glycosyltransferase enzymes needed for mucus synthesis and O-glycans attachment. A primary defect in mucus synthesis could lead to a breach in the epithelial barrier, allowing closer contact between commensals and immune cells. Consequently, altered mucosa-bacteria-immune cells interactions will ensue. Indeed, a subset of UC patients have been shown to have impaired expression of intestinal mucin-type O-glycans due to mutations in *C1galt1C1*, a chaperone protein (also known as cosmc protein) required by the core 1 β1,3-galactosyltransferase (also known as T-synthase) needed for core 1 O-glycans attachment (Podolsky et al, 1988; Holmen Larsson et al, 2011, Fu et al, 2011). However, the function of these carbohydrate moieties in the etiology of the disease remains unclear. In the absence of O-glycans, the protective functions of the mucus layer are lost and susceptibility to disease is increased, as it has been shown in conventional mice lacking core-1 O-glycans, which spontaneously develop colitis (Fu et al, 2011).

A second source of mucosal defects may arise from the ability of bacteria and pathogens to adapt and interact with mucus (Derrien et al, 2004; Cervantes-Sandoval, 2008; Gaskell et al, 2010; Martens et al, 2011; Van den Abbeele et al, 2011). Degradation of the mucus layer by bacterial enzymes, such as glycosydases, may affect mucus integrity increasing the degree of interaction between the intestinal epithelia and bacteria, bacterial products or luminal substances. Such changes may prompt inflammatory disorders. Furthermore, changes in bacterial factors during the progression of inflammation may also influence mucus synthesis and expression. In

turn, impaired mucus synthesis could have adverse effects on the immune system and the microbiota, contributing to the perpetuation of inflammation.

Differences in pathological changes in mucus expression have been found between UC and CD (Einerhand et al, 2002; Gersemann et al, 2009; McGuckin et al, 2011). While the thickness of the mucus layer and the number of goblet cells either remain unchanged or are increased in CD, both of these elements are reduced in UC (Pullan et al, 1994; Strugala el at, 2008). Both patterns of change have an impact in the number of bacteria present in the colon, as some constituents of the microbiota live in close association with the mucus layer and contribute to niche establishments for other members of the intestinal flora (Png et al, 2010). Accordingly, preserving mucus components and the mucus layer, is essential in its role as a barrier. We hypothesize that when the mucus layer is destroyed by inflammation, genetic loss, or overconsumption by gut bacteria, these changes can result in increased inflammatory signaling and, eventually, colitis.

Aiming to characterize changes in the gut microbiota associated with a breach in the mucus layer caused by lack of O-glycans, we performed molecular analysis of fecal bacterial populations by pyrosequencing of 16S rRNA tags of conventional TM-IEC C1*galt*1$^{-/-}$ mice. The TM-IEC C1*galt*1$^{-/-}$ mice have an inducible mutation of the glycosyltransferase enzyme responsible for attachment of core 1O-glycans to the mucin backbone, which results in a thinner mucus layer, thus resembling human Ulcerative Colitis (UC). The identification of specific bacterial taxa in IBD could be a stepping-stone in the development of appropriate antimicrobial therapies against pathobionts while preserving beneficial commensals.

3.2 Materials and Methods

3.2.1 Sample collection from conventional TM-IEC C1*galt*1$^{-/-}$ mice.

Samples were collected from four conventional wild type mice, and four conventional TM-IEC C1*galt*1$^{-/-}$ mice kept in a specific pathogen-free facility at the Oklahoma Medical Research Foundation (OMRF), Oklahoma City, Oklahoma. Samples included fecal pellets (FP), cecal contents (CC) and left colon contents (LCC), and they were kept at -80°F until processed for DNA isolation in the Peterson laboratory.

3.2.2 DNA Isolation

Samples (FP, CC and LCC) were weighted and diluted in phosphate buffer (pH7) in a 1:10 ratio, followed by centrifuging at 10,000rpm (Eppendorf 5424) for 5 minutes. This washing step was repeated two more times to remove inhibitors. Bacterial cell pellets were resuspended in 750µl of lysis buffer (200mM NaCl, 20mM EDTA, 100mM Tris [pH 8.0], 20 mg/ml lysozyme), transferred to a sterile microcentrifuge tube containing 300mg of 0.1mm zirconium beads, and incubated at 37°C for 30min. Afterwards, 85µl of 10% sodium dodecyl sulfate solution and 40µl of proteinase K (15mg/ml) were added, and tubes were incubated at 60°C for 30min. After incubation, 500µl of phenol-chloroform-isoamyl alcohol (25:24:1) were added, and samples were homogenized in a MiniBeadbeater-8 (BioSpec Products) at

maximum speed for 2 minutes, followed by cooling in ice. Layers were separated by centrifugation at 14,000rpm for 5min before extraction of the top layer. This step was repeated three times with phenol:chlorofom:isoamyl, and twice with chloroform-isoamyl alcohol (24:1). DNA was recovered by inducing precipitation through the addition of 100% ethanol and 3M sodium acetate. The amount of eluting reagents used depended on the final volume of sample obtained after the last extraction step (2.5 times of ethanol per volume of sample and 1/10 volume of sample of 3M sodium acetate). Samples were stored at -20°F overnight followed by centrifugation at 14,000rpm (Eppendorf 5424) for 30min. Ethanol was discarded, DNA pellets were allowed to dry at room temperature, and resuspended in 100µl of 10mM Tris-HCL buffer (pH8).

3.2.3 Pyrosequencing of 16S rRNA PCR

The variable regions 1 and 2 of the 16s rRNA genes were amplified by PCR using a composite forward primer and a reverse primer containing a unique 12-base barcode to tag PCR products to their respective samples. The primer sequences used were as follows: 454_27F 5'-*GCCTTGCCAGCCCGCTCAGTC***AGTCAGAGTTTGATCCTGGCTCAG**-3' and 454_338R 5'-*GCCTCCCTCGCGCCATCAG*NNNNNNNNNNNNCA**TGCTGCCTCCCGTAGGAGT**-3'; where the italicized sequences are the 454 Life Sciences primers B and A, and the bold sequences are the broadly conserved bacterial primer 27F and the broad-range bacterial primer 338R, respectively. The NNNNNNNNNNNN region in the reverse

primer represents the unique 12-base barcode used to tag each PCR product with a "CA" inserted as a linker between the barcode and the reverse primer. PCR conditions were: 5mins at 95°C; followed by 30 cycles of 45s at 95°C, 45s at 57°C, and 2min at 72°C; and lastly 10min at 72°C in an Eppendorf Mastercycler® thermocycler. PCR products were visualized by gel electrophoresis. Amplicons from individuals samples were mixed in equal amounts based on concentration, and purified using the QIAGEN MiniElute Gel extraction kit (QIAGEN #28604). The final, purified product, with a concentration of 29.99ng/ml was submitted to emulsion PCR and sequenced using a Roche 454 FLX pyrosequencer at the Cornell University Life Sciences Core Laboratories Center. Sequences obtained from pyrosequencing were filtered and submitted to quality control parameters.

3.2.4 Data Analysis and Statistics

Sequences generated from pyrosequencing were submitted to the open source software package *Quantitative Insights into Microbial Ecology* (QIIME; http//qiime.source-forge.net) for removal of low quality sequences. Sequences that met the following parameters were kept for analysis: (i) length between 340 and 400 nucleotides; (ii) no ambiguous bases; (iii) quality score equal or above 25; (iv) no mismatches in primers; and (v) no mismatches in barcodes. After sequences were quality controlled, chimera check was performed using the *Chimera Slayer* method in QIIME and binned by barcode. Taxonomic-based analysis was performed using the *Classifier* (Wang et al, 2007) program of the *Ribosomal Database Project* (RDP)

version 9 (Cole et al, 2009) (http://rdp.cme.msu.edu/) to assign taxonomic status to each sequence. Significance of the *Classifier* data was determined by calculating proportions and statistical analysis performed by calculating the average mean of proportions and Fisher's Z score (Appendix A) using a critical value of 3.29 (α=0.001).

Taxonomic-independent methods were used to estimate species diversity. Sequences were aligned and clustered using the *Aligner* and *Complete Linkage Clustering* algorithm available through the *Pyrosequencing pipeline* of the RDP. Clustering was done with a threshold of 97% pairwise identity for assignment into operational taxonomic units (OTUs). Initial clustering of the sequences in the dataset by *Complete Linkage Clustering* produced a total of 3,641 OTUs. After selecting representative sequences for each OTU, a single best BLAST hit was identified for all sequences in the dataset using BioEdit Blastall function by blasting them against the dataset of non-chimeric sequences. OTUs over 300 bases long, over 97% identity, and containing more than 100 hits in total were kept for analysis. This filtering step produced a total of 891 OTUs. A new set of representative sequences was selected from the group of 891 OTUs, and the blasting step was repeated. Sequences that overlapped between OTUs were determined by comparing the sequences that comprise each OTU, and were merged together, resulting in 206 OTUs, from which representative sequences were selected and blasted against the dataset. Hits were counted using Excel. The filtering step was repeated as before producing a total of 184 unique OTUs. Proportions were calculated and significance of the OTUs was determined by calculating the average mean of proportions and Fisher's Z score

(Appendix A). A critical value of 3.29 (α=0.001) was selected to determine statistical significance. Taxonomy was assigned for each OTU representative sequence by selecting the Best Blast result from a local type strain database using BioEdit Blast.

Additionally, representative sequences from each OTU were aligned using PyNast, and used to construct a reference phylogenetic tree through QIIME. This phylogenetic tree was used for Unweighted UniFrac analysis. Rarefaction analysis was performed using the *Pyrosequencing pipeline* of the RDP after alignment and complete linkage clustering of the sequences for individual samples.

3.3 Results

3.3.1. Diversity assessment and comparisons between microbial communities by non-taxonomic-based analyses: UniFrac, Principal Component Analysis, Rarefaction, Shanon and Chao indexes

To determine whether lack of core 1 O-glycans caused proportional changes in the gut microbiota of the mice, we analyzed fecal pellets (FP), cecal contents (CC), and left colon contents (LCC) of conventional TM-IEC C1*galt*1$^{-/-}$ (KO) mice (n=4) and conventional wild type (WT) mice (n=4) by pyrosequencing of the V1-V2 region of the 16S rRNA gene. A total of 406,339 reads were obtained, which, after submission to quality control parameters and chimera check, resulted in 279,912 sequences that were used in the analysis. One sample (fecal pellets from a wild type

mouse) was dropped from the analysis because of poor amplification of reads. The percentage of chimeric sequences removed was 16.4%. The average number of sequences per sample was 12,170 sequences with an average length of 370bp.

3.3.1.1 UniFrac and Principal Component Analysis

Unweighted UniFrac is a metric for distance between communities of organisms that takes into consideration absence or presence of Operational Taxonomic Units (OTU), but not their abundance (Lozupone et al, 2006). It accounts for unique OTUs and their branch length. Principal Component Analysis (PCoA) is a variable reduction procedure that finds linear combinations of a set of variables with maximum variance and removes their effect, repeating this successively to eliminate redundancy or variable correlation with one another. The purpose is to avoid measuring the same construct more than once. Unweighted UniFrac and PCoA were performed for all samples to assess differences in microbial composition between the samples. Analyses showed differences in bacterial communities between the genotypes TM-IEC C1*galt*1$^{-/-}$ *vs* WT, specifically in the LCC. Principal Component 1 (PC1) accounts for 26.83% of the variability in the data, and PC2 accounts for 18.6% in the LCC (Figure 3.1A). The UniFrac phylogenetic tree showed a distinct microbial population in the LCC of the conventional TM-IEC C1*galt*1$^{-/-}$ mice when compared to the conventional WT (Figure 3.2A). However, these differences were not seen in the fecal pellets or in cecal contents of the mice (Figure 3.2B-C).

3.3.1.2 Rarefaction and Shannon and Chao indexes

Rarefaction curves (Figure 3.3) and Shannon and Chao indexes (Figure 3.4) - methods for assessment of species diversity - showed that the most diverse microbiota is found on the cecum of the conventional WT mice, followed by cecum samples of the TM-IEC C1*galt*1$^{-/-}$ mice. Similarly, the WT LCC samples were more diverse than the LCC TM-IEC C1*galt*1$^{-/-}$ samples. There was no change in diversity between the WT and the TM-IEC C1*galt*1$^{-/-}$ fecal samples. However, no statistically significant differences ($p > 0.05$) in diversity were found between genotypes within each anatomical site sampled or between anatomical sites. It is necessary to take into consideration that rarefaction curves consider the number of species in a sample, but do not account for specific taxa present (as opposed to Unweighted UniFrac).

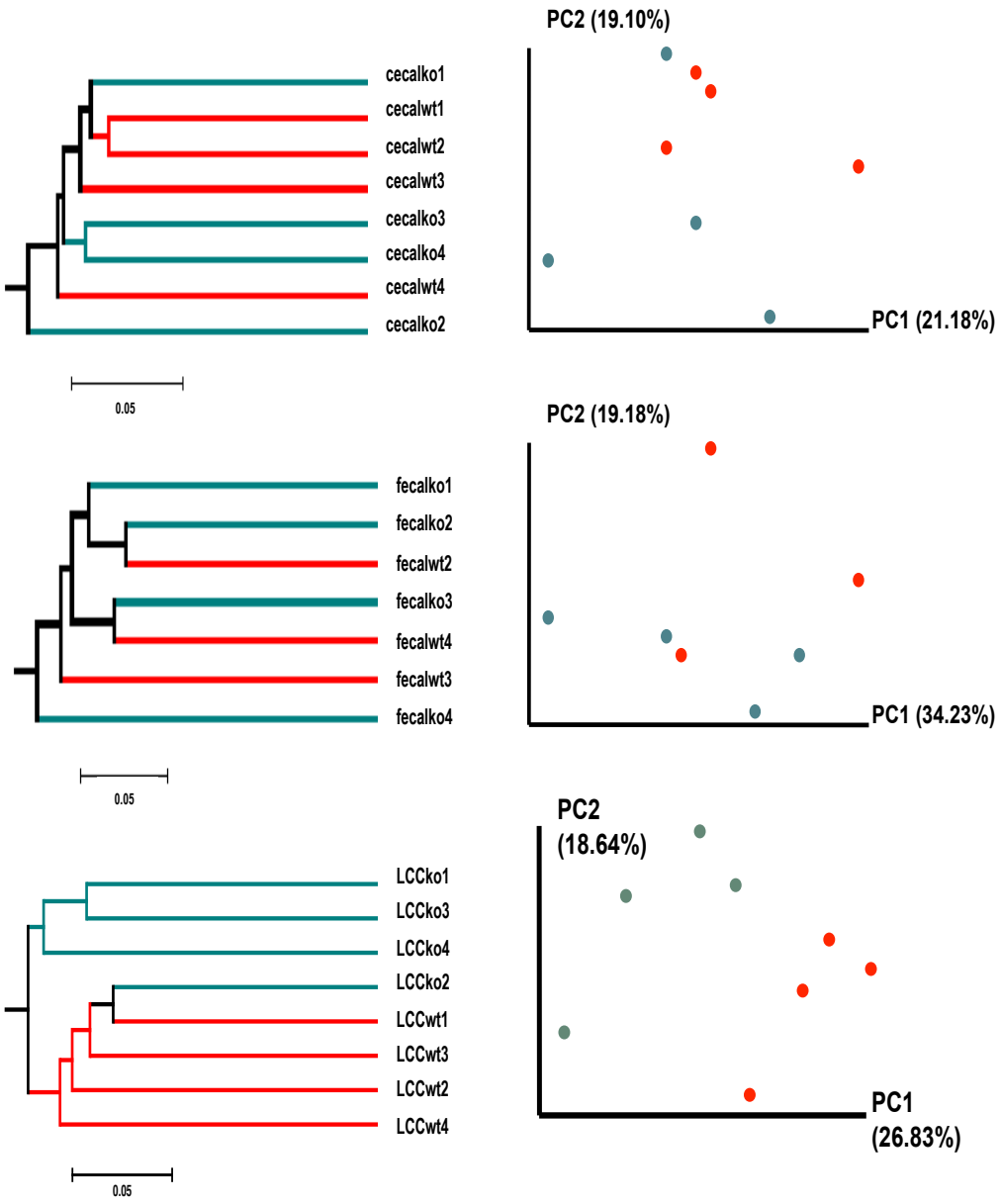

Figure 3.2 Unweighted UniFrac and Principal Component analyses reflect two distinct microbial communities in the left colon content in the TM-IEC C1*galt*$^{-/-}$ relative to the WT mice. Unweighted UniFrac (non-abundance) cluster trees and PCoA graphs are shown for [A] cecal samples, [B] fecal pellets, and [C] left colon content. These analyses were performed to assess similarities in microbial communities between the genotypes. The microbial communities from cecal contents and fecal pellets of WT and TM-IEC C1*galt*1$^{-/-}$ mice share similarities in composition. However, two distinct microbial communities were observed in LCC samples between the genotypes. KO: TM-IEC C1*galt*$^{-/-}$ mice, Red dots and lines represent the WT mice. Green dots and lines represent the TM-IEC C1*galt*$^{-/-}$ mice.

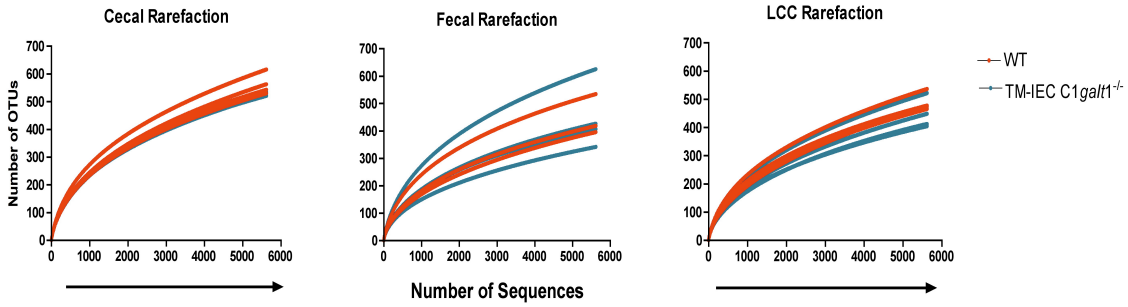

Figure 3.3 Rarefaction graphs for analysis of species diversity in the different anatomical sites showed no statistically significant differences between the genotypes. It is necessary to take into consideration that rarefaction curves consider the number of species in a sample, but do not account for specific taxa present, which is contrary to Unweighted UniFrac. Red lines represent the WT mice. Green lines represent the TM-IEC C1*galt*$^{-/-}$ mice.

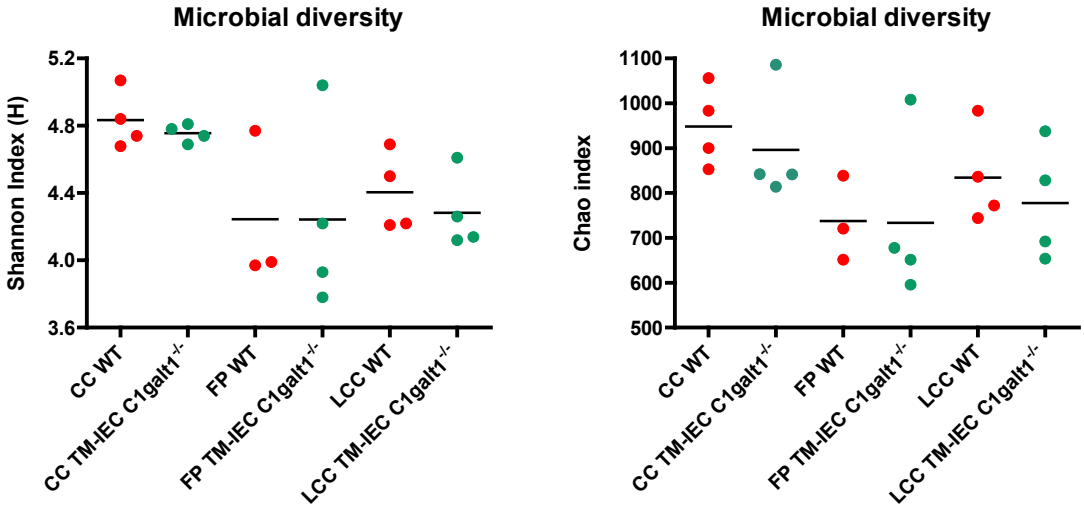

Figure 3.4 Microbial diversity decreased in the cecal contents and left colon contents of the TM-IEC C1*galt*1$^{-/-}$ mice relative to the WT. The most diverse microbiota was found in the cecal contents of the WT mice followed by the cecal contents of the TM-IEC C1*galt*1$^{-/-}$ mice. Same trend was observed in the LCC samples (higher diversity found in the WT samples than the mutant). However, no differences in diversity were found in the microbial communities of the fecal pellets between WT and TM-IEC C1*galt*1$^{-/-}$. Shannon index increases as both, richness (abundance) and evenness of the community increases. Higher Chao1 index values means higher diversity.

3.3.2 Taxonomy-based analysis of the microbial communities

The RDP Classifier is a simple probabilistic (naïve Bayesian) classifier that provides rapid taxonomic assignments, from domain to genus, to bacterial 16S rRNA sequences. After submission of the sequences to Classifier, the total of sequences per taxa per sample were summarized at different taxonomic levels, and proportions were calculated. Significance of the Classifier data was determined, as mentioned in the methods section, using a critical value of 3.29 (α=0.001) to assess significance of results.

3.3.2.1 Phylum

Taxonomy-based analysis at the phylum level showed a significant increase in the number of Firmicutes and a decrease in the number of Bacteroidetes in the TM-IEC C1*galt*1$^{-/-}$ relative to the WT (Figure 3.5). Firmicutes increased, on average, from 43.6% in the WT mice to 52.8% in the TM-IEC C1*galt*1$^{-/-}$ mice, while Bacteroidetes decreased from 41.1% to 34.4%. These changes were greater in the FP, were Firmicutes increased from 38.6% to 60.4% (Z=15.20), and Bacteroidetes decreased from 44.4% to 27.0% (Z=-12.79). A smaller change in the number of Firmicutes was found in LCC samples were they increased from 42.6% in the WT mice to 47.7% in the TM-IEC C1*galt*1$^{-/-}$ mice (Z=4.12) (Figure 3.5). These differences were not observed in CC samples of the mice.

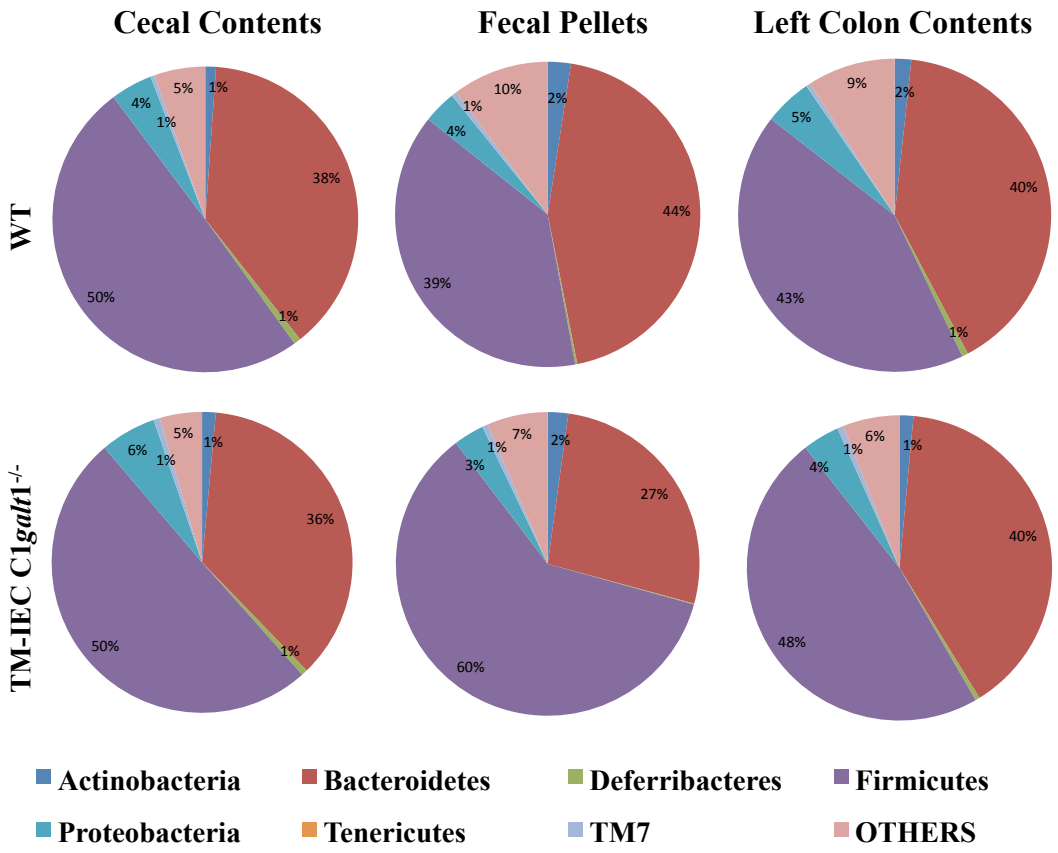

Figure 3.5 RDP Classifier analyses at the Phylum level showed that deficiency of Core 1 O-glycans causes significant increases in Firmicutes and decreases in Bacteroidetes in the fecal pellets of the mice. An increase of 22% in Firmicutes and a relative decrease of 17% in Bacteroidetes were found in the fecal population of the TM-IEC C1*galt*1$^{-/-}$ mice relative to the WT. Similar changes at a minor extent were seen in LCC samples. However, they were not observed in the contents of the cecum.

3.3.2.2 Family

At the family level, significant increases were found in the conventional TM-IEC C1*galt*1$^{-/-}$ mice, specifically in the amount of Lactobacillaceae and Clostridiaceae in all types of samples. In LCC, Lactobacillaceae increased from 14.3% to 28.7% (Z=14.2871), while Clostridiaceae increased from 0.016% to 1.022% (Z=5.9574). The number of Prevotellaceae decreased in FP and CC samples, but remained

unchanged in LCC samples. Ruminococcaceae changes in CC or FP were not significant; however, in LCC this taxon decreased to almost half its value, from 4.49% to 2.64% (Z=-3.9675). Lachnospiraceae was found decreased in both, FP and LCC, with the greater reduction in LCC, were its number changed from 7.09% to 3.55% (Z=-6.2343). Another taxonomic group found diminished were the unclassified Clostridiales (from 6.82 to 4.48%, Z=-4.03). Figures 3.6 and 3.7 show results at the Family level.

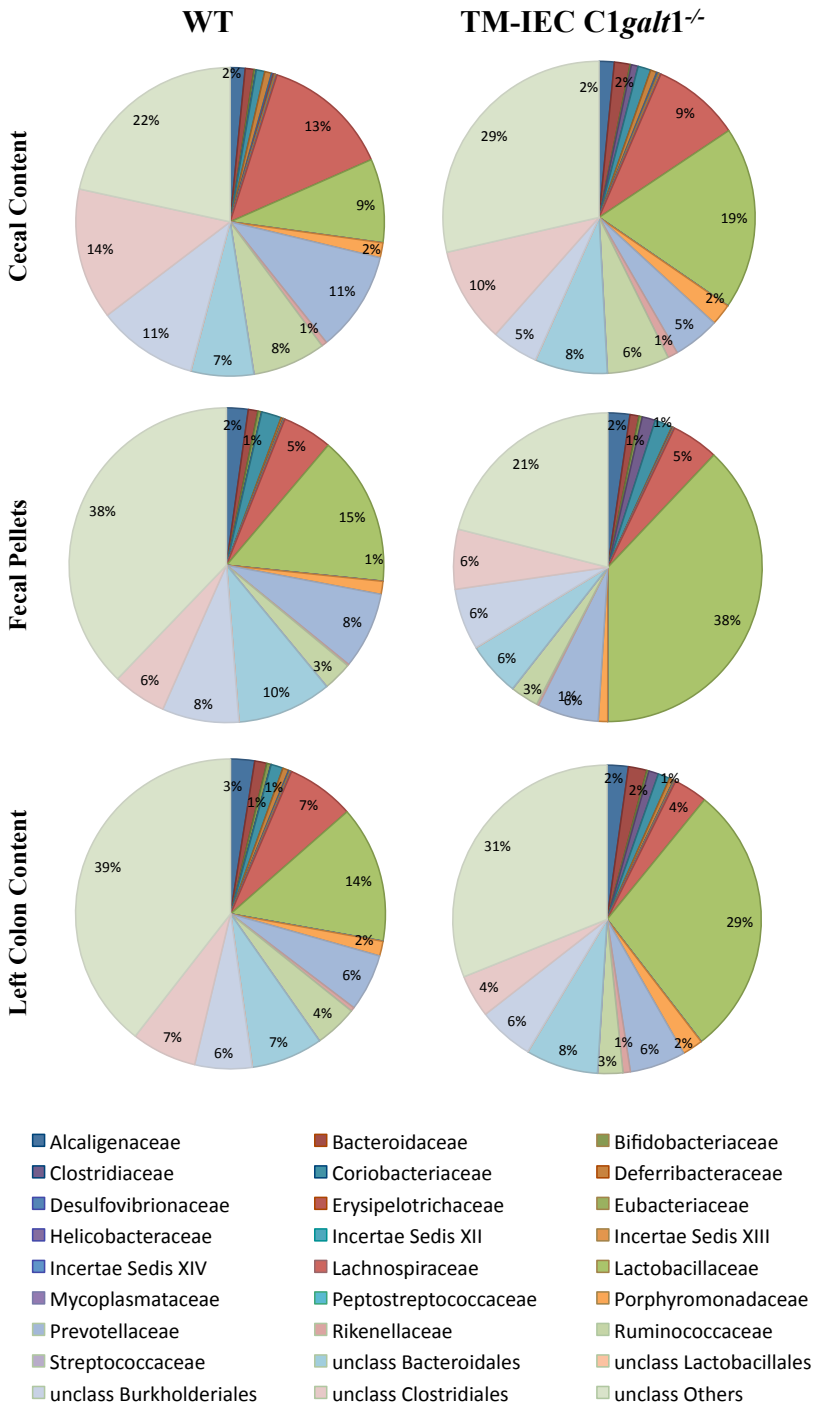

Figure 3.6 The net change in the population of Firmicutes at the Family level is the result of increases in Clostridiaceae and Lactobacillaceae, and decreases in Ruminococcaceae and Lachnospiraceae. Clostridiaceae and Lactobacillaceae consistently increased in all anatomical sites in the TM-IEC C1*galt*1$^{-/-}$ mice. Pie charts represent the abundance (in percentage) of bacterial families present in each anatomical site per genotype.

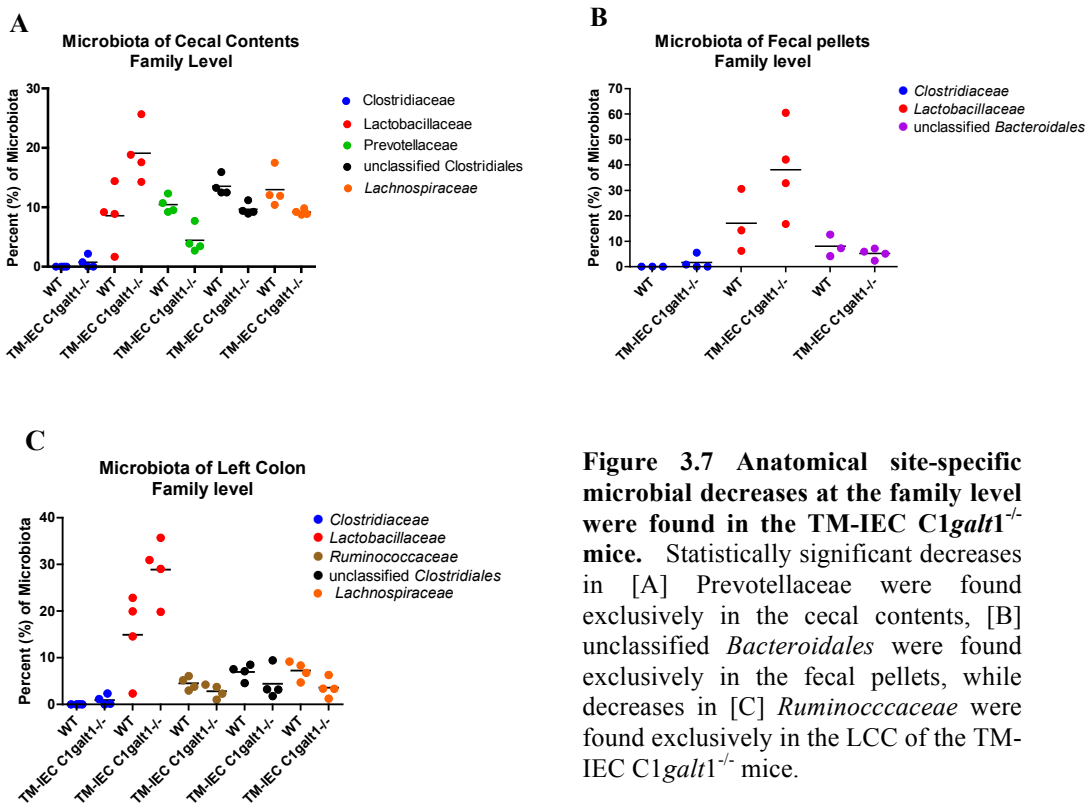

Figure 3.7 Anatomical site-specific microbial decreases at the family level were found in the TM-IEC C1*galt1*$^{-/-}$ mice. Statistically significant decreases in [A] Prevotellaceae were found exclusively in the cecal contents, [B] unclassified *Bacteroidales* were found exclusively in the fecal pellets, while decreases in [C] *Ruminocccaceae* were found exclusively in the LCC of the TM-IEC C1*galt1*$^{-/-}$ mice.

3.3.2.3 Genus

At the genus level, significant changes were found in LCC samples on populations of *Lactobacillus*, *Clostridium*, unclassified Lachnospiraceae, and unclassified *Ruminococcus* (Figures 3.8 and 3.9C). The predominant change was in the population of *Lactobacillus,* which increased from 14.26% to 28.67% (Z= 14.29). *Clostridium* also increased changing from 0.01% to 0.89% (Z= 5.56). Changes in these two groups of bacteria were found in all anatomical sites (Figure 3.9). Unclassified Lachnospiraceae diminished in LCC samples from 6.23% to 3.03% (Z=-6.00). Lastly, unclassified Ruminococcaceae decreased in all types of samples but changes were only significant in LCC (from 2.87 to 1.49; Z=-3.7391). Figure 3.8 presents the taxonomic analysis at the genus level for all anatomical sites tested.

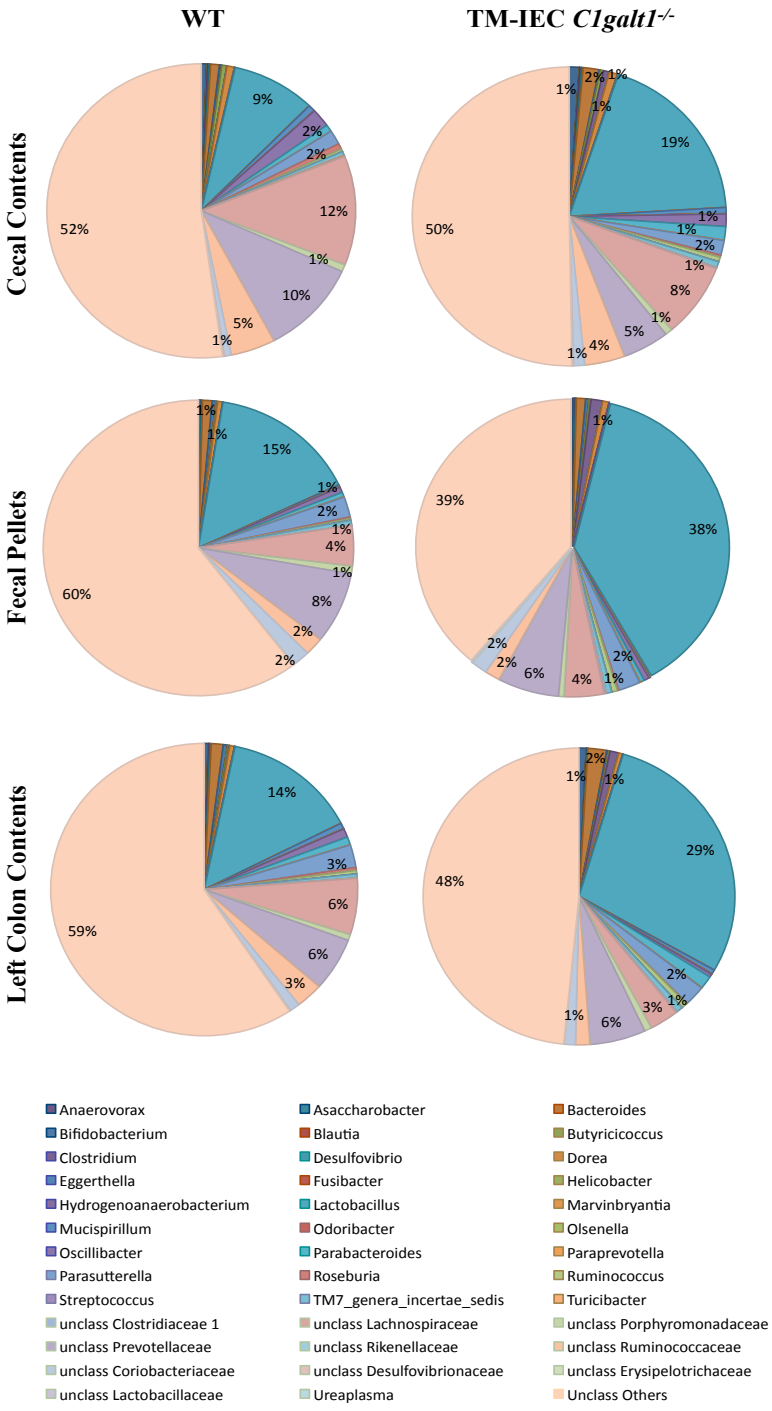

Figure 3.8 Increases in Clostridium and Lactobacillus spp. were found in all anatomical sites of the core 1 O-glycans deficient mice being the Lactobacillus population the most affected at the Genus level. Pie charts represent percentage of bacteria by anatomical site and per genotype.

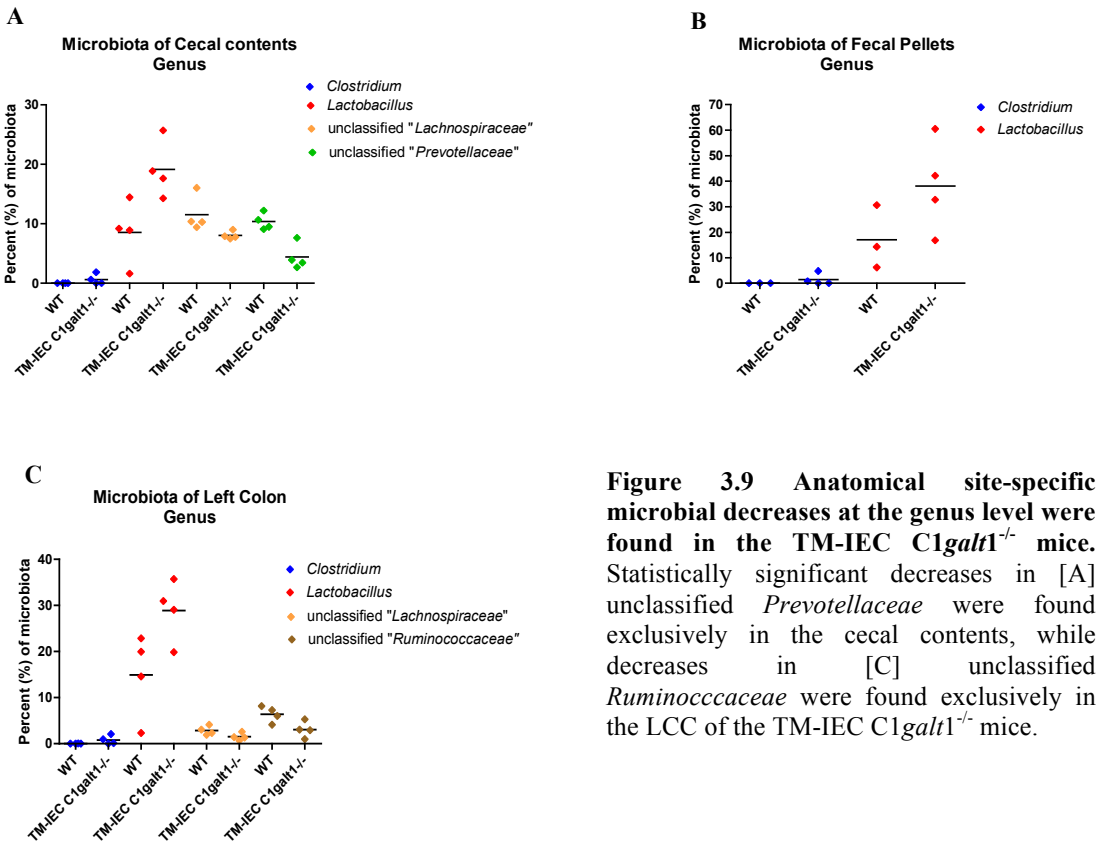

Figure 3.9 Anatomical site-specific microbial decreases at the genus level were found in the TM-IEC C1*galt*1$^{-/-}$ mice. Statistically significant decreases in [A] unclassified *Prevotellaceae* were found exclusively in the cecal contents, while decreases in [C] unclassified *Ruminocccaceae* were found exclusively in the LCC of the TM-IEC C1*galt*1$^{-/-}$ mice.

3.3.2.4 Operational Taxonomic Units

Initial clustering of sequences in the dataset by Complete Linkage Clustering of the RDP produced a total of 3,641 Operational Taxonomic Units (OTUs) using a 97% similarity threshold. After merging overlapping OTUs, and counting of Best Blast hits (as discussed in the methodology), a total of 184 unique OTUs were left for analysis, which accounted for 97.6% of the sequences. Similar to Classifier analysis, significance of the OTUs data was determined by calculating the average mean of proportions and Fisher's Z score using a critical value of 3.29 (α=0.001) to assess importance of results.

Examination of OTUs defined by 97% identity resulted in 19 OTUs significantly different between the genotypes (WT *vs* TM-IEC C1*galt*1$^{-/-}$) when considering all anatomical sites (Table 3.1). However, 8 OTUs (Figure 3.10) were specifically significant in LCC samples. Three OTUs, representing clusters associated to *Lactobacillus intestinalis* (97% identity), *Lactobacillus johnsonii* (99% identity), and *Clostridium disporicum* (97% identity), resulted statistically different in all sites, being considerably increased in the conventional TM-IEC C1*galt*1$^{-/-}$ mice. The number of *Lactobacillus johnsonii* doubled in LCC from 13.60% to 25.79% (Z=12.34), while *Lactobacillus intestinalis* increased to almost four times from 1.29% to 3.77% (Z=6.43). *Clostridium disporicum* increased from less than 0.02% in conventional WT mice samples to 1.08% (Z= 6.01) in the conventional TM-IEC C1*galt*1$^{-/-}$ mice.

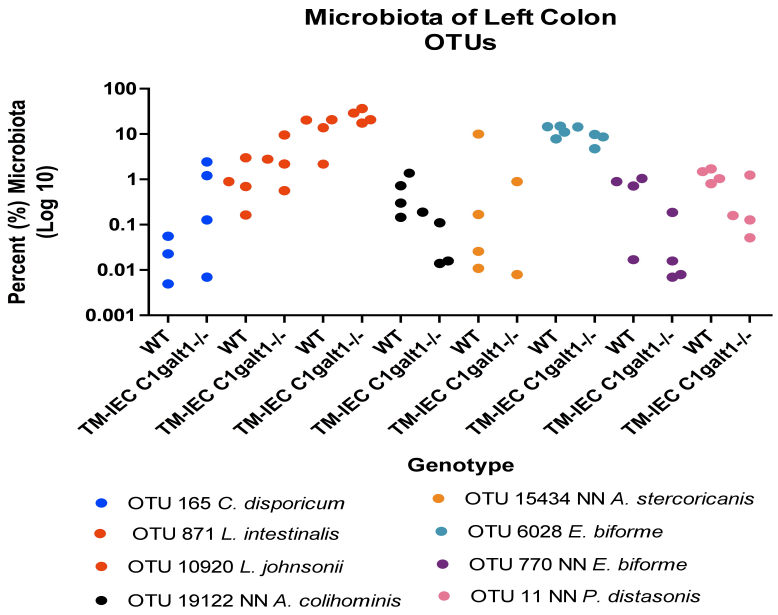

Figure 3.10 Statistical analyses of OTUs showed changes in 8 OTUs specific to the LCC of the TM-IEC C1galt1-/- mice. These OTUs are represented by *Clostridium disporicum*, *Lactobacillus intestinalis*, *Lactobacillus johnsonii*, which are increased in the LCC of the mice; and *Anaerotruncus colihominis*, *Allobaculum stercoricanis*, *Eubacterium biforme* and *Parabacteroides distasonis,* which are decreased in the mice colon.

The remaining OTUs that were found significant in the distal colon were reduced in the conventional TM-IEC C1*galt*1$^{-/-}$ mice relative to the conventional WT, some of them having over a ten-fold reduction. These were: OTU11, whose nearest neighbor (NN) shares 94% identity with *Parabacteroides distasonis*, reduced from 1.34% to 0.45% (Z=-3.6872) and OTU19122 NN (96% identity to *Anaerotruncus colihominis)*, from 0.73% to 0.09% (Z=-3.8169). Additionally, three OTUs form the family of Erysipelotrichaceae *were reduced: Eubacterium biforme* (97% identity), from 12.66 to 9.35% (Z=-4.18); OTU770 NN (94% identity to *Eubacterium biforme*), from 0.61% to 0.06% (Z=-3.6155); and OTU15434 (91% identity to *Allobaculum stercoricanis*) from 2.98% to 0.28% (Z=-8.1531). Proportions and Z-values (for statistic significance) per anatomical site for all OTUs are given in Table 3.1.

Table 3.1 Statistically Significant Operational Taxonomic Units per anatomical site

OTU number	Representative sequence	cecal TM-IEC C1*galt*1-/-	cecal WT	cecal Z value	fecal TM-IEC C1*galt*1-/-	fecal WT	fecal Z value	LCC TM-IEC C1*galt*1-/-	LCC WT	LCC Z value	Percent (%) identity	SpeciesBest Blast Results Type strain database
1744	fecalwt4_100687	0.0307	0.0161	3.3937	0.0181	0.0210	-0.7301	0.0257	0.0233	0.6134	91	Algoriphagus halophilus
15434	LCCwt2_14989	0.0032	0.0136	-4.2406	0.0008	0.0038	-2.3511	0.0028	0.0298	-8.1531	91	Allobaculum stercoricanis
19122	cecalwt4_156667	0.0036	0.0056	-1.0974	0.0013	0.0050	-2.3880	0.0009	0.0073	-3.8169	96	Anaerotruncus colihominis
79	cecalwt4_233197	0.0526	0.0463	1.0294	0.0348	0.0755	-6.3494	0.0724	0.0769	-0.6776	92	Bacteroides fragilis
17587	cecalko1_43511	0.0076	0.0080	-0.1612	0.0069	0.0172	-3.3789	0.0068	0.0062	0.3055	91	Bacteroides tectus
889	LCCko4_107859	0.0066	0.0180	-3.8147	0.0108	0.0263	-4.1128	0.0128	0.0119	0.3148	92	Barnesiella viscericola
3	cecalwt3_8802	0.0037	0.0180	-5.1414	0.0063	0.0040	1.0980	0.0025	0.0031	-0.4158	91	Blautia hydrogenotrophica
7142	cecalwt4_225040	0.0004	0.0077	-4.4140	0.0005	0.0020	-1.5281	0.0002	0.0031	-2.6719	91	Clostridium aldenense
165	cecalko4_30502	0.0077	0.0001	4.1634	0.0147	0.0000	5.5299	0.0108	0.0002	6.0149	97	Clostridium disporicum
9022	cecalwt1_107537	0.0059	0.0181	-4.1329	0.0060	0.0046	0.6411	0.0041	0.0094	-2.5225	90	Clostridium jejuense
6028	fecalko1_63023	0.0678	0.0736	-0.8158	0.1059	0.1756	-7.0355	0.0935	0.1266	-4.1777	97	Eubacterium biforme
770	LCCwt1_102773	0.0004	0.0038	-2.8031	0.0011	0.0108	-4.6699	0.0006	0.0061	-3.6155	94	Eubacterium biforme
871	fecalko3_101523	0.0332	0.0072	6.3751	0.0476	0.0168	5.8349	0.0377	0.0129	6.4325	99	Lactobacillus intestinalis
10920	LCCko1_17826	0.1689	0.0879	8.5389	0.3361	0.1399	15.6044	0.2579	0.1360	12.3351	99	Lactobacillus johnsonii
4363	cecalko3_75532	0.0250	0.0201	1.1914	0.0093	0.0251	-4.3586	0.0201	0.0296	-2.3904	94	Parabacteroides distasonis
11	cecalwt3_15009	0.0036	0.0094	-2.6655	0.0020	0.0090	-3.4410	0.0045	0.0134	-3.6872	94	Parabacteroides distasonis
8594	fecalwt2_204110	0.0144	0.0255	-2.8946	0.0052	0.0196	-4.7119	0.0168	0.0284	-3.0631	96	Parabacteroides merdae
161	LCCko3_63397	0.0058	0.0019	2.1982	0.0085	0.0011	3.4978	0.0073	0.0032	2.3174	91	Parasutterella excrementihominis
12	fecalko2_41438	0.0436	0.1098	-9.1405	0.0627	0.0709	-1.1368	0.0516	0.0536	-0.3486	86	Prevotella loescheii

Values represent Weighted Means of Proportions and Z values. Critical value = 3.29 (α=0.001)

3.4 Discussion

3.4.1 Distinct microbial communities are associated with the conventional colitic TM-IEC C1*galt*1$^{-/-}$ mice

Changes between microbial communities were found in the conventional TM-IEC C1*galt*1$^{-/-}$ mice relative to the conventional controls mice (WT). Summarizing, these changes mainly include increases in Firmicutes spp. and reductions in Bacteroidetes spp. in core 1 O-glycans deficient mice.

Sonnenburg et al (2004) propose that commensals dwell in the mucus layer by overlying the gut epithelium through the formation of a "biofilm-like community". They define biofilms as "dense cohesive communities of microbes that embed themselves within surface-associated matrices". The surface-associated matrix referenced is the one formed by the carbohydrate polymers in the mucus layer. As previously described, the TM-IEC C1*galt*1$^{-/-}$ mice have an incomplete, thin mucus layer. Consequently, they have a defective matrix for microbes to establish themselves. Accordingly, changes in the microbiota could be explained by a decrease in sites for bacterial attachment.

Furthermore, since some bacteria are capable of inducing inflammation (Waidmann et al, 2003; Balish and Warner, 2002; Kim et al, 2005), the development of inflammation in the colon, and other related immune changes (i.e. induction of proinflammatory cytokines and cell-mediated responses) could affect niche establishments for some members of the microbiota.

3.4.2 *Lactobacillus* and *Clostridium* populations are enriched in conventional colitic TM-IEC C1*galt*1$^{-/-}$ mice

Some studies in human UC have observed a decrease in Firmicutes (Sokol et al, 2006; Walker et al, 2011). In the conventional TM-IEC *C1galt1*$^{-/-}$ mice model there is an increase in this group, which more specifically are the net result of increases in *Clostridium* and *Lactobacillus*, despite decreases in unclassified Ruminococcaceae and unclassified Lachnospiraceae at the genus level. Results from our mice model show evidence that parallel results from other human studies, since both *Ruminococcus* spp. (in CD) and *Lachospiraceae* spp. (in UC and CD) were found decreased in IBD patients (Frank et al, 2007; Morgan et al, 2012).

The most significant alteration within the *Lactobacillus* was an increase in *L. johnsonii*. This is consistent with the work of Peña et al (2004), where isolates of this species were found to predominate in the IL-10 deficient C57BL/6 mice model of colitis and in which some *Lactobacillus* species isolated from these mice were unable to inhibit TNF-α while others could. In contrast, some *L. johnsonii* strains have been able to mitigate the effect of other bacteria in colitis induction, prevent disease development in murine models of cirrhosis and diabetes (Chiva et al, 2002; Valladares et al, 2010; Lee and Kim, 2011), and ameliorate inflammation in human and mice models of colitis, or DSS-induced injury of the colon (Liu et al, 2011; Oliva et al, 2012; Duary et al, 2012; Jadhav et al, 2013).

In regards to the *Clostridium* group, OTU analysis revealed that *C. disporicum* was found increased in the conventional TM-IEC C1*galt*1$^{-/-}$ mice in the OTU analysis.

This finding is in partial agreement with work from Milonaki et al (2005) who found *Clostridium* spp. increased in active UC. However, in general, studies have found a decrease in *Clostridium* spp. in human UC, especially in the *Clostridium leptum* group, to which *F. prausnitzii* belongs, and to whom a protective role in CD has been credited by some researchers (Sokol et al, 2009; Joossens et al, 2011; Morgan et al, 2012; Kabeerdoss et al, 2013). Other species of *Clostridium*, such as *C. butyricum* and *C. tyrobutyricum*, have been found to decrease colon inflammation induced by DSS (Zhang et al, 2009; Hudcovic et al, 2012).

Since our experiments were specifically aimed at the identification of taxa affected by core 1 O-glycans deficiency, these results do not allow us to ascribe a specific role for *L. johnsonii* or *C. disporicum* in our mice model in terms of whether their enrichment is a cause of lack of O-glycans, or a consequence of inflammation.

3.4.3 *Eubacterium biforme* and *Anaerotruncus colihominis,* two butyrate-producing bacteria, are decreased in conventional colitic TM-IEC C1*galt*1$^{-/-}$ mice

Short-chain fatty acids (SCFA), which include propionate, butyrate, and acetate, among others, are metabolic products of microbial fermentation with an important role in colonic health (Reviewed by Hijova and Chmelarova, 2007). The anti-inflammatory capacities of SCFA, especially butyrate, have been tested *in vitro* and *in vivo,* and are well documented (Steinhart et al, 1996; Tedelind et al, 2007). In

addition, butyrate was identified as the preferred energy source for colonic epithelial cells (Roediger, 1980).

Both, *E. biforme* (Barcenilla et al, 2000) and *A. colihominis* (Lawson et al, 2004) have been characterized as butyrate-producing bacteria, and both of these were found decreased in the conventional TM-IEC C1*galt*1$^{-/-}$ mice relative to conventional WT. They are members of the phylum Firmicutes: the first one belongs to the order Erysipelotrichales, family Erysipelotrichaceae; while the latter belongs to the order Clostridiales, family Ruminococcaceae and it is part of the *Clostridium leptum* group. Some species of *Eubacterium*, one of the most common genera in the human intestine (Schwiertz et al, 2002) have been reported as capable of attenuating experimental colitis (Kanauchi et al, 2005).

Eubacterium biforme, which represents OTU number 6028 (97% identity) in our assessment, was found an average of -1.5 fold decreased in the conventional TM-IEC C1*galt*1$^{-/-}$ considering all anatomical sites tested (Table 3.1). Fecal pellets showed the most significant change, were it decreased from 17.56% in the WT mice to 10.59% (-1.7 fold decrease) in the TM-IEC C1*galt*1$^{-/-}$ mice. The changes in *A. colihominis*, which represents OTU 19122, were not as significant considering its low abundance in the mice. Still, decreases in both of these species may affect the total amount of butyrate available for epithelial cells and therefore, potentially contribute to loss of epithelial integrity.

Besides being a major nutrient source for colonocytes, SCFA constitute a source of nutrients for various members of the microbiota, serve as modulators of colonic and intracellular pH, and ion transport, and they are regulators of proliferation,

and differentiation (reviewed by Cook and Sellin, 1998). In addition, SCFA stimulate gene expression, including expression of epithelial mucin 2 proteins (Willemsen et al, 2003). Since loss of butyrate-producing bacteria would imply destabilization of all these processes, it is to be expected that loss of the epithelial barrier function, and further dysbiotic shifts would follow, thus predisposing to disease development.

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Chapter 4

Colitogenic Bacterial Species in Core 1 O-glycans Deficient Mice

4.1 Introduction

Animal models are designed to resemble specific aspects of human diseases with the objective of elucidating the etiology of such disorders, and develop appropriate treatments. The main objective of the set of experiments described in this chapter was to identify bacterial species with the potential of inducing inflammation in the TM-IEC C1*galt*1$^{-/-}$ (core 1 O-glycans deficient) mice model. The core 1 O-glycans deficient mice model, in which the intestinal mucus layer is thinner than normal, presents a simplified version of Ulcerative Colitis (UC), as it has been shown that a subset of UC patients have this anatomical anomaly (Einerhand et al, 2002; Fu et al, 2011).

The characterization of microbial species inhabiting the GIT of IBD patients is done with the purpose of identifying causative bacteria of inflammation and to develop the appropriate antimicrobial therapies. Even though there is no consensus on the identification of specific bacterial species as causative of IBD, several species have been categorized either as "colitogenic" or as "preventive/protective" in a number of colitis murine models. Interestingly, bacterial species that have been associated with induction of inflammation in some models have been shown to have the opposite effect in others. For example, *Bacteroides vulgatus* was identified as an inducer of inflammation in the HLA-B27 transgenic rat model, but as "preventive" in the IL-2$^{-/-}$ mice model of colitis. It can be hypothesized that the pathogenicity of particular bacterial species varies depending on the type of genetic deficiency (immunological or physiological) introduced into the animal model to resemble the human disease. Examples of "inducers" and "non-inducers" of inflammation for various murine models are listed in table 4.1

Table 4.1 Inflammation inducer and non-inducer bacterial species in selected murine models of colitis and chemically-induced injury of the colon

Mice model	Inducer bacterial species	Non-inducer bacterial species	Settings	References
IL-2$^{-/-}$	*E. coli* mpk	*B. vulgatus* mpk	GF	Waidmann et al, 2003 Muller et al, 2008
IL-2$^{-/-}$	*E. coli* mpk	*E.coli* Nissle 1917 *B. vulgatus* mpk	SPF	Bohnn et al, 2006
HLA-B27	*B. vulgatus*	ND	GF	Rath et al, 1999
IL-10$^{-/-}$	*H. hepaticus*	ND	SPF	Kullberg et al, 1998
IL-10$^{-/-}$	*E. faecalis*	*Bifidobacterium* spp. Specific *Lactobacillus* spp	GF	Balish and Warner, 2002
IL-10$^{-/-}$	ND	*L. plantarum* 299V	GF & SPF	Schultz et al, 2002
IL-10$^{-/-}$	*E. faecalis*	*P. fluorescens*	GF	Kim et al, 2005
IL-10$^{-/-}$	ND	*L. plantarum* LP-Onlly	Conv.	Xia et al, 2011
DSS injury	*Y. enterocolitica*	*B. adolescentis*	Conv.	Frick et al, 2007
TNBS injury	ND	*B. longum* HY8004 *L. plantarum* AK8-4	Conv.	Lee at al, 2009
TNBS injury	*K. pneumoniae*	*L. johnsonii*	Conv.	Lee and Kim, 2011

Abbreviations- GF: Germ-free, SPF: Specific pathogen-free, Conv: Conventional, ND: not determined, DSS: Dextran sodium sulfate, TNBS: 2,4,6-trinitrobenzene sulfonic acid

We performed monoassociation experiments of GF WT and GF TM-IEC C1*galt*1$^{-/-}$ mice with selected bacterial species. The purpose of these experiments was to determine bacterial species associated with the development of inflammation in the context of a defective mucus layer. Species were chosen either based on their reported association with development of inflammation in murine models of colitis, or because

they were identified in our work as altered in the conventional TM-IEC C1*galt*1$^{-/-}$ mice, such as *Lactobacillus johnsonii,* among others (addressed in Chapter 3).

4.2 Materials and Methods

4.2.1 TM-IEC C1*galt*1$^{-/-}$ mice

Mice for monoassociation experiments were genotyped and selected for each bacterial treatment as discussed in Chapter 2. Mice were colonized by orally gavaging 200μl of oxygen-sensitive bacterial species in pure cultures, or by rubbing cultures of oxygen-tolerant bacterial species onto the mouse fur (Bry et al, 1996). Deficiency of intestinal O-glycans was induced 48 to 72 hours after colonization by intraperitoneal injections of 100 μl of 1mg of Tamoxifen (TM; MP Biomedicals) in an ethanol/sunflower oil mixture (1:9 v/v) for 5 consecutive days. For both, WT controls and TM-IEC C1*galt*1$^{-/-}$ mice, TM treatment was completed before the mice were 8 weeks old. Mice were sacrificed either 20 days or 30 days after TM injections depending on the bacterial species used for colonization. Mice weights were monitored and recorded every other day. A group of Germ-free (GF) TM-IEC C1*galt*1$^{-/-}$ and GF WT mice used as negative controls (non-colinized mice), were also treated with TM, and sacrificed at 20 or 30 days. A vehicle-control group - treated with ethanol/sunflower oil without TM) - was not used since TM injections were administered to both WT (Cre^{-}) and TM-IEC C1*galt*1$^{-/-}$ (Cre^{+}) mice. Figure 4.1 shows a schematic representation of the methodology used for the bacterial monoassociation experiments.

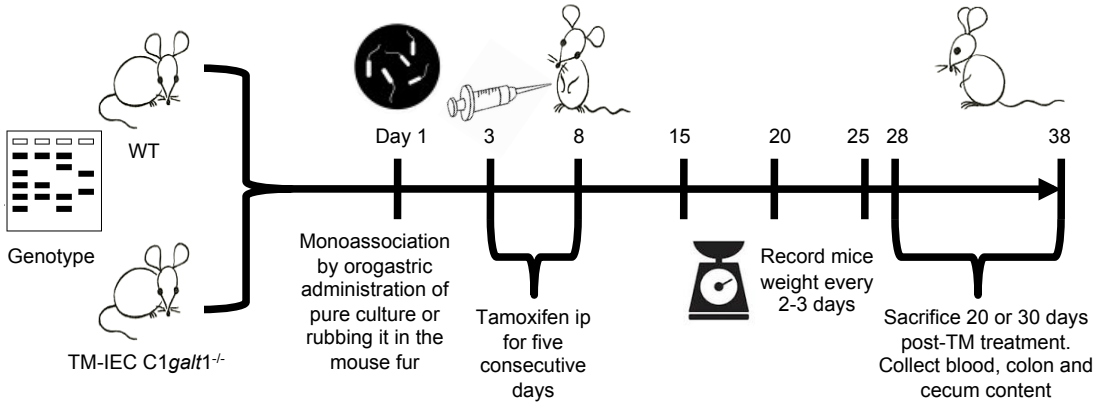

Figure 4.1 Schematic representation of the experimental protocol. Mice were genotyped for presence of the Cre gene to distinguish the TM-IEC C1*galt*1$^{-/-}$ from the WT, which were used as control. Mice were monocolonized through orogastric administration of 200µl of pure culture or by rubbing the culture onto the fur. Subsequently (48 to 72 hours post-colonization), 1mg of Tamoxifen (TM) diluted in an ethanol and sunflower oil mixture was injected intraperitoneally for five consecutive days to both, TM-IEC C1*galt*1$^{-/-}$ and WT. Mice weights were recorded every 2-3 days. Mice were sacrificed 20 or 30 days after TM treatment, depending on the bacterial treatment provided.

4.2.2 Bacterial species

The selection of bacterial species used for the monoassociation experiments was based on its identification, by pyrosequencing analysis (discussed in Chapter 3), as significantly altered in the conventional TM-IEC C1*galt*1$^{-/-}$ mice, or by being described by other authors as "colitogenic" or inflammation inducers in various murine models of UC. Additional species, not associated with development of inflammation, were selected as "control strains" (Table 4.2).

All the bacterial species used in monoassociation experiments were grown in an anaerobic chamber with environmental conditions set as follows: 37°C, 5% CO_2, 5% H

and 90% N_2. *Bacteroides* species included *Bacteriodes thetaiotaomicron* VPI 5482, *Bacteroides vulgatus* ATCC 8482, *and Bacteroides sartorii* A-C2-0. All Bacteroides were grown in TYG (per liter: 10g Tryptone, 5g Yeast Extract, and 11 mM (2g) Glucose, supplemented with 8.2mM (1g) cysteine, 1ml hematin-histidine solution (12mg hematin dissolved in 10ml of 0.2M histidine pH8), 1mg vitamin K3, 1ml of 0.8% calcium chloride solution, 2.6μM (0.4mg) ferrous sulfate, 100ml of 1M KPO_4, and 40ml of TYG salts solution (per liter: 2mM (0.5g) MgSO4 heptahydrate, 119mM (10g) NaHCO3, 34.2mM (2g) NaCl)).

Other species used included: *Clostridium disporicum* ATCC 43838, grown in Reinforced Clostridial Medium (prepared according to instructions); *Clostridium symbiosum* ATCC 14940, grown in Modified Meat Medium (per liter: 125g Cooked Meat Medium, 30.0g trypticase [BD 211921], 5.0g yeast extract, 28.7mM (5.0g) K_2HPO_4, 4.0ml 0.025% resazurin, 4mM (0.5g) cysteine, 10.0ml hemin solution [50.0mg hemin, 1.0ml 1N NaOH, 100ml distilled water], 0.2ml vitamin K1 solution [0.15ml vitamin K1, 30.0ml 95% ethanol]); *Akkermansia muciniphila* BAA-835, grown in Brain Heart Infusion Broth (prepared according to instructions); and *Lactobacillus johnsonii,* isolated from mice fecal pellets and identified by 16s rRNA sequencing using the universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1391R (5'-GACGGGCGGTGWGTRCA-3'), and grown in Modified MRS (per liter: 55g MRS, 29.2mM (10g) of maltose, and 28mM (5g) of fructose), and *Bifidobacterium longum* grown in Modified MRS with added cysteine (0.05% per volume).

Bacteroide*s*-colonized mice were euthanized at 30 days, except for *B. vulgatus*-colonized mice. Mice colonized with *B. vulgatus* and other selected bacterial species were sacrificed at 20 days post-TM treatment.

Table 4.2 Bacterial species selected for monoassociation experiments

Bacteria	Reason for selection	Reference
Akkermansia muciniphila	Mucus degrader, decreased in UC patients	Vigsnaes et al, 2012
Bacteroides sartorii	Mouse specific strain not associated with colitis	Control strain
Bacteroides thetaiotaomicron	Colitic in HLA-B27 transgenic rat model	Hansen et al, 2012
Bacteroides vulgatus	Colitic in gnotobiotic IL-10$^{-/-}$ and HLA-B27 murine models	Sydora et al, 2005 Rath et al, 1999
Bifidobacterium longum	Being study for probiotic effect	Bai et al, 2006 Ortiz-Lucas et al, 2013 Rodes et al, 2013
Clostridium disporicum	Species increased in colitic TM-IEC C1galt1$^{-/-}$ mice	Chapter 3 of this thesis
Clostridium symbiosum	Clostridium species not associated with colitis	Control strain
Lactobacillus johnsonii	Genus increased in colitic TM-IEC C1galt1$^{-/-}$ mice	Chapter 3 of this thesis

4.2.3 Sample collection

Mice were anesthetized using Isofluorane and blood collected for immunoglobulin measurement. Cecum contents were snap-frozen in liquid nitrogen. The distal colon was divided into three sections and preserved in liquid nitrogen, Carnoy's fixative (60% methanol, 30% chloroform, 10% glacial acetic acid), or 10% formalin, respectively.

4.2.4 Immunohistochemical and Immunofluorescence staining of distal colon tissue

Procedures for immunohistochemical and immunofluorescence staining, and for determination of histological scores were discussed in Chaper 2. Bacterial probes used for FISH analysis are listed in Table 4.3. Parameters used for determination of inflammation scores are specified in Table 4.4.

Table 4.3 Bacterial probes used for FISH analysis

Probe	Target	Sequence	5' conjugation
EUB338	Universal bacterial probe	5'-GCT GCC TCC CGT AGG AGT-3'	Texas Red
CFB286	Chlorobi/ Fibrobacteres Bacteroidetes Superphylum	5'-TCC TCT CAG AAC CCC TAC-3'	Alexa Fluor 488
MUC1437	*Akkermansia muciniphila*	5'-CCT TGC GGT TGG CTT CAG AT -3'	Alexa Fluor 488
NON338	Nonsense/non-specific probe (negative control)	5'- ACT CCT ACG GGA GGC AGC-3'	Texas Red
GAM42a	Gamma Proteobacter Phylum	5'- GCC TTC CCA CAT CGT TT-3'	Alexa Fluor 488

Table 4.4 Scoring parameters for assessment of inflammation

Parameter	Score			
	0	1	2	3
Lamina Propria $CD45^+$ cells per HPF (% WT)	≤105%	106-125%	126-150%	≥151%
Epithelial Hyperplasia (% WT)	≤105%	106-125%	126-150%	≥151%

4.2.5 Immunoglobulin measurement by ELISAs and Data Analysis

ELISA protocols were followed for measurement of IgA, IgM, IgG, and IgG isotypes IgG1, IgG2a, IgG2b, and IgG3 as described in Chapter 2. Statistical analyses for immunoglobulins levels *between* genotypes and *within* genotypes among bacterial treatments were determined by unpaired t-test with Welch's correction using GraphPad Prism 5.0 (GraphPad Software Inc., USA) statistical software.

4.2.6 Quantification of bacterial species by Quantitative PCR

Quantification of total bacteria was performed by quantitative PCR (qPCR) using the universal primers 8F and 1391R after DNA isolation from cecal contents of the mice. DNA was extracted as described in Chapter 3. Each PCR reaction was done in 20µl total volume, and consisted of 10µl of 2x SYBR Green QPCR master mix (Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix, Agilent Technologies), 1µl of each primer (10µM concentration), 7µl of nuclease-free water, and 1µl of genomic DNA template. Amplification was performed using a Bio-Rad CFX96 Real Time System model C1000 Thermal Cycler. The amplification program consisted of an initial denaturing step of 3 minutes at 95°C, followed by 40 cycles, each consisting of 5 seconds at 95°C (denaturing), 10 seconds at 60°C (annealing), and 10 seconds at 68°C (extension). Fluorescence was measured at each cycle during the 60°C annealing-extension step. Submitting the amplified products to dissociation analysis generated melting curves. Ten –fold serial dilutions of DNA extracted from pure cultures of each bacterium tested were

used to generate standard curves for absolute quantification. DNA for the standard curves was normalized at 50ng starting concentration per reaction. Standard curves were made by plotting the threshold cycle values obtained from DNA of the dilution series as a linear function of the base 10 logarithm of the number of bacteria. Analysis of results was done by converting the CT value obtained for each sample to ng/μl based on the standard curve. The amount of ng/μl was converted to its equivalent number of copies (based on genome size), and transformed to genome equivalents per gram of cecal contents (based on the initial amount of cecal content used for DNA extraction). The final numbers were subjected to unpaired t-test with Welch's correction using GraphPad Prism 5.0 (GraphPad Software Inc., USA) statistical software for determination of statistically significant differences between the genotypes.

4.3 Results

The main objective of these experiments was to assess the effect of individual bacterial species in development of inflammation, in the presence of a breach in the mucus layer, through the comparison of monoassociated WT *vs* monoassociated TM-IEC C1*galt*1$^{-/-}$. However, our results will be addressed in a number of ways. For both settings, the monoassociated WT (intact mucus layer) and the monoassociated TM-IEC C1*galt*1$^{-/-}$ (defective mucus layer), we used GF and conventionalized mice, as negative controls (no bacteria) and positive controls (complete microbiota), respectively. The comparison of monoassociated against GF mice (monoassociated WT *vs* GF WT, and monoassociated TM-IEC C1*galt*1$^{-/-}$ *vs* GF TM-IEC C1*galt*1$^{-/-}$) isolates the effect of the mutation in inflammation (which was addressed in chapter 3) in the absence of microbes, and it allows us to evaluate changes caused by an introduced bacterium in both, an intact and a defective mucus layer. Meanwhile, comparison of monoassociated mice with conventionalized mice (monoassociated WT *vs*. Conv WT, and monoassociated TM-IEC C1*galt*1$^{-/-}$ *vs*. Conv TM-IEC C1*galt*1$^{-/-}$) helps to evaluate how the effect of a single bacterium compares to that of a complete microbiota, which resembles with greater accuracy the scenario in human IBD. Our general results will also be discussed in comparison to those obtained by Fu et al (2011) in a conventional setting.

4.3.1 Assessment of bacterial colonization (monoassociation)

FISH and qPCR with the 16S rRNA universal primers 8F and 1391R were used to assess bacterial colonization. Evaluation of mucosal association and colonization of the

distal colon by FISH showed robust colonization by *B. thetaiotaomicron, B.vulgatus* and *B. sartorii* (Figure 4.2), moderate colonization by *C. symbiosum* and *L. johnsonii*, and weak colonization by *A. muciniphila* (Figure 4.3), for both WT and TM-IEC C1*galt*1$^{-/-}$. Similarly, statistical analysis of quantitative PCR performed on *cecal contents* of the mice showed no statistical differences in the number of bacteria between the WT and TM-IEC C1*galt*1$^{-/-}$ mice, independently of bacterial association. However, the degree of colonization of each bacterial species in the cecum differed relative to the distal colon, with *L.johnsonii* showing the strongest colonization, followed by *A. muciniphila, Bacteroides* spp. and *C. symbiosum* in decreasing order of abundance. Specifically, colonization values obtained from qPCR- measured as genome equivalents per gram of cecal contents- ranged from $2x10^8$ to $4x10^8$ for *L. johnsonii;* from $2x10^8$ to $3x10^8$ for *A. muciniphila;* from $1x10^7$ to $1.6x10^8$ for the *Bacteroides* spp.; and from $9x10^7$ to $2x10^8$ for *C. symbiosum*. Discrepancies in the degree of colonization revealed by FISH and the numbers obtained from qPCR could be related to differences in the microenvironment of the two anatomical sites (colon and cecum), as it has been shown that different microbial species segregate along the GI tract, locating themselves in that part of the tract which best suits their growth requirements (Reviewed by Walter and Ley, 2011). In addition, inflammation along the GI tract may result in differential induction of antimicrobial peptides by epithelial cells along different parts of the tract, thus creating an heterogenous environment that might include distinct regions unsuitable for colonization by certain bacterial species but fit for others (Hildebrand et al, 2013).

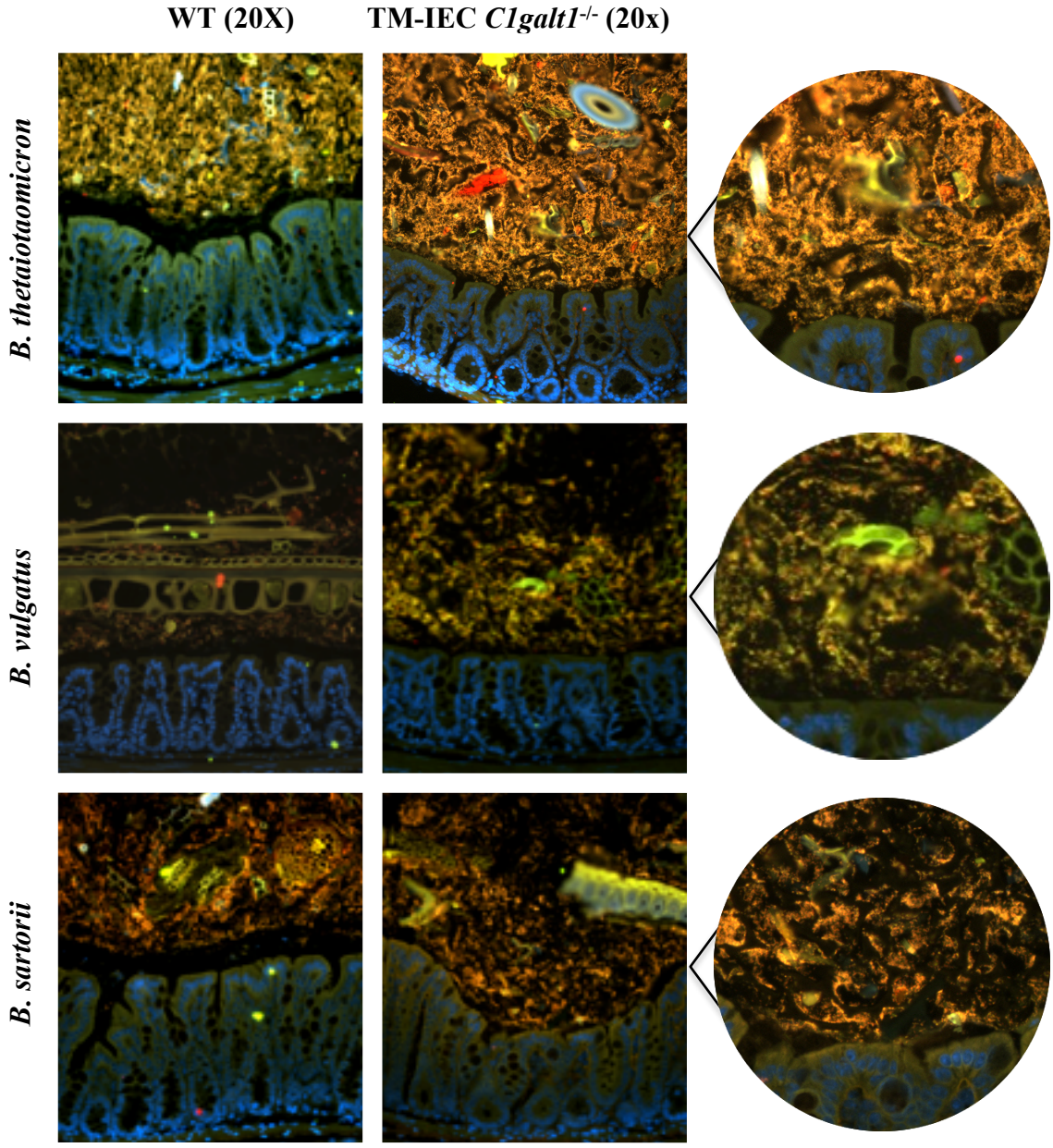

Figure 4.2 Bacteroides species colonized the colon in the absence of core 1 O-glycans. FISH analysis revealed robust colonization by *B. thetaiotaomicron, B. vulgatus* and *B. sartorii* in both WT and TM-IEC C1*galt*1$^{-/-}$ mice. Note reduced mucus layer in the TM-IEC C1*galt*1$^{-/-}$ mice - the black space between the microbiota (yellow-green) and the epithelial cells (blue). Circles to the right show an amplified view of the interface between the microbiota and the epithelia.

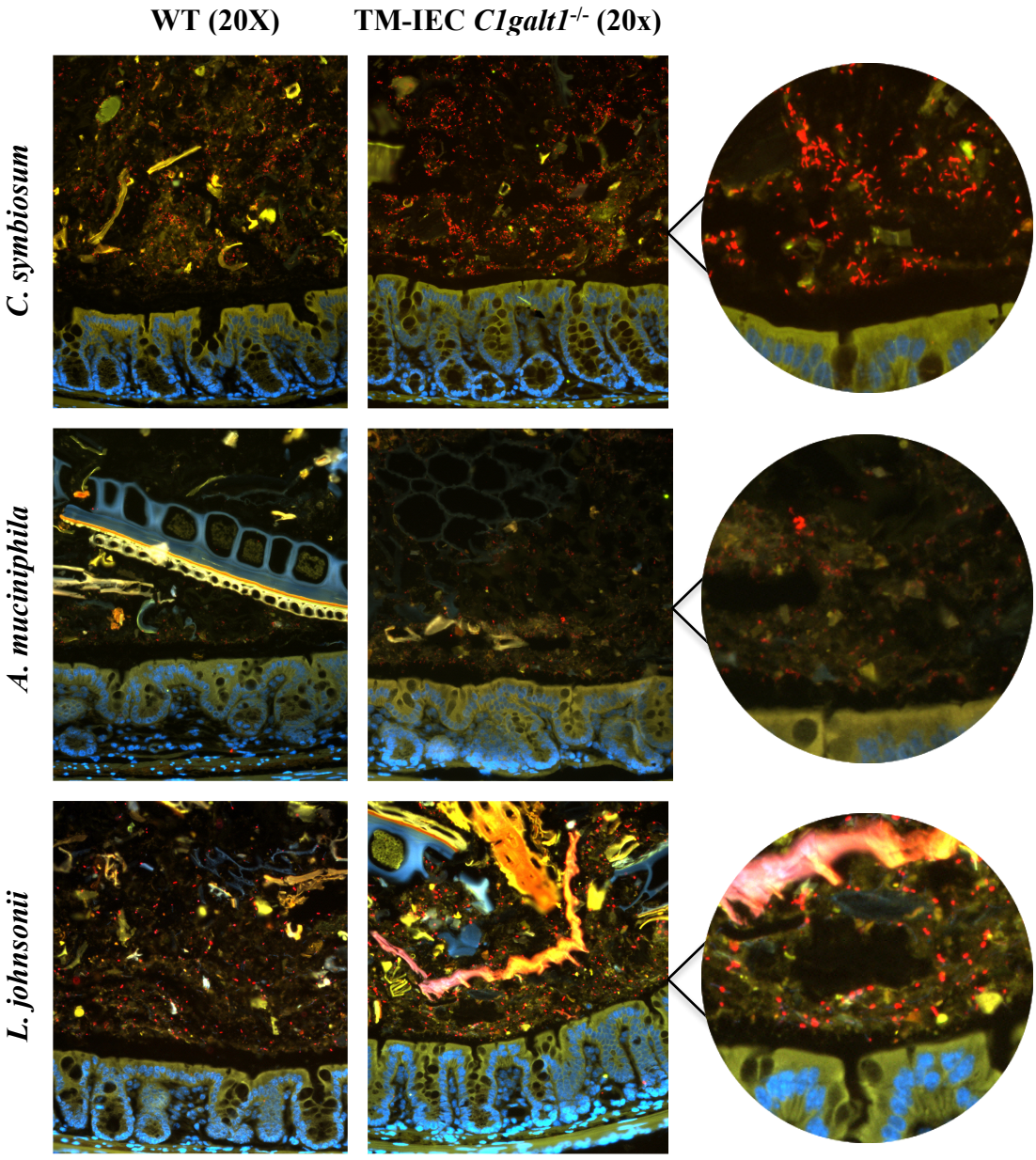

Figure 4.3 FISH analysis revealed moderate colonization by *C. symbiosum* and *L. johnsonii*, and weak colonization by *A. muciniphila* in both, WT and TM-IEC C1*galt*1$^{-/-}$ mice. The amount of bacteria seen in the distal colon of *C. symbiosum*, *L. johnsonii*, and *A. muciniphila*-colonized mice was reduced relative to that seen in *Bacteroides*-colonized mice. Interestingly, the amount of bacterium in the *C. symbiosum* - TM-IEC C1*galt*1$^{-/-}$ colonized mice seems to be greater than the WT counterpart. However, no statistically significant differences in total bacteria were found between the genotypes as measured by qPCR in cecal contents of the mice. Discrepancies between the amount of bacteria determined by qPCR and FISH analysis could be attributed to differences in anatomical sites tested. Note reduced mucus layer in the TM-IEC C1*galt*1$^{-/-}$ mice – the black space between microbiota (red) and the epithelial cells (blue). Circles to the right show an amplified view of the interface between the microbiota and the epithelia.

4.3.2 Assessment of mutation induction

Evaluation of mutation induction in the monoassociated TM-IEC C1*galt*1$^{-/-}$ mice was performed as discussed in Chapter 3 for the conventionalized and GF TM-IEC C1*galt*1$^{-/-}$ mice. Tn antigen and Alcian Blue staining were performed to evaluate an increased presence of Tn antigen in the mutated mice, and to assess reduction in the ability of goblet cells to produce mucus, respectively. Figure 4.4 shows increased amount of Tn antigen in the mutated mice relative to the WT. Figures 4.5A and 4.5B prove decreased mucus production (lack of blue staining within the crypts) in the monoassociated TM-IEC C1*galt*1$^{-/-}$ mice.

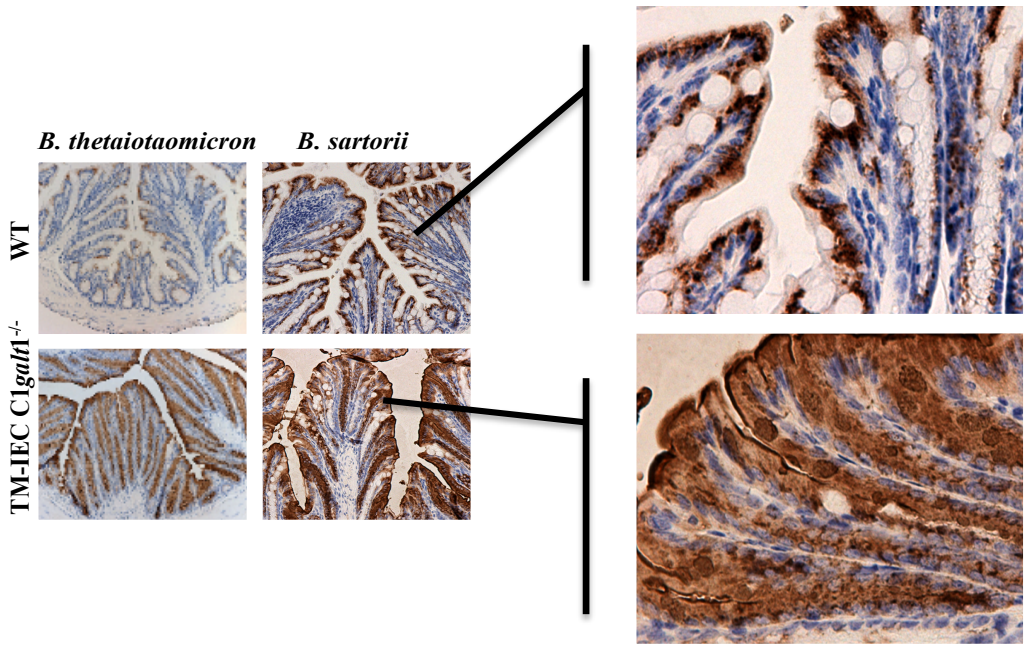

Figure 4.4 Deletion of the C1GALT1 enzyme caused increased production of Tn antigen in the TM-IEC C1*galt*1$^{-/-}$ mice. Pictures show presence of Tn antigen (stained brown) in WT and TM-IEC C1*galt*1$^{-/-}$ mice monoassociated with *B. thetaiotaomicron* (left) and *B. sartorii* (middle). The Tn antigen is a glyco-aminoacidic determinant linked to serine or threonine residues by a glycosidic bond (GalNAc-O-Ser/Thr). It is masked in normal cells by type O saccharides chain elongation; therefore, it is expressed in some abnormal or sick cells when such elongation does not occur. Upon deletion of the C1GALT1 enzyme responsible for core 1 O-glycosilation of the mucus layer, the Tn antigen was detected in higher amounts in the TM-IEC C1*galt*1$^{-/-}$ mice relative to the WT mice (right).

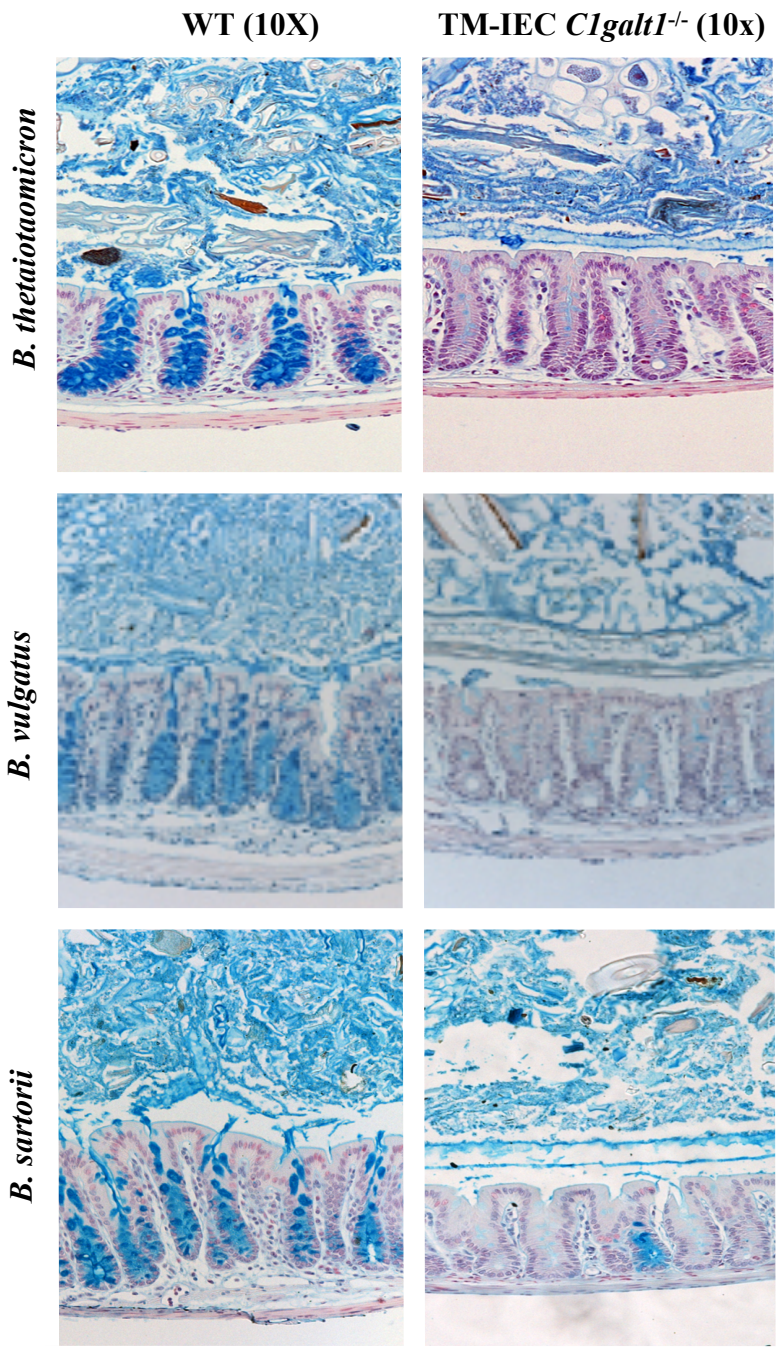

Figure 4.5A Alcian Blue staining revealed good mutation induction in the TM-IEC C1*galt*1$^{-/-}$ mice colonized with *Bacteroides* species. AB staining revealed an impaired mucus production by the goblet cells. Note "empty" crypts (lack of blue stain) in the TM-IEC C1*galt*1$^{-/-}$ mice in all bacterial treatments.

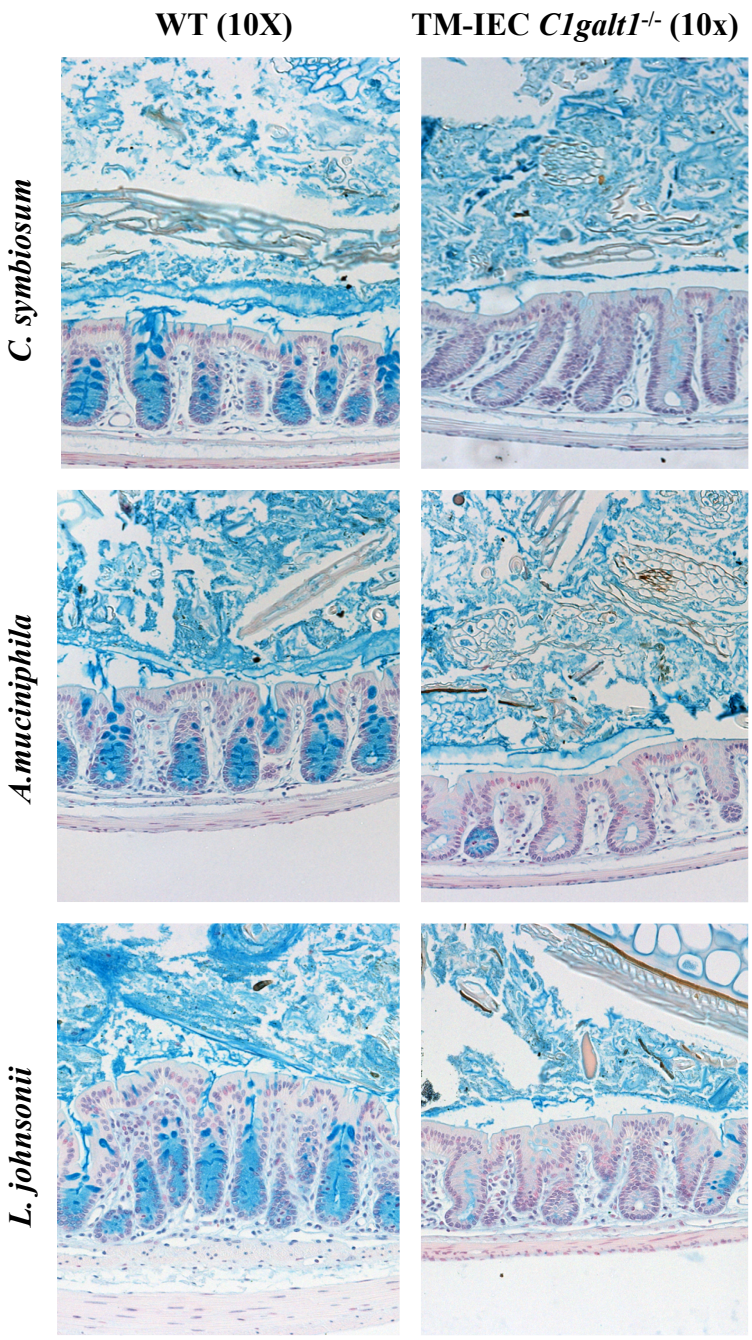

Figure 4.5B Alcian Blue staining revealed good mutation induction in the TM-IEC C1*galt*1$^{-/-}$ mice colonized with *Clostridium symbiosum*, *Akkermansia muciniphila* and *Lactobacillus johnsonii*. AB staining revealed an impaired mucus production by the goblet cells. Note "empty" crypts (lack of blue stain) in the TM-IEC C1*galt*1$^{-/-}$ mice in all bacterial treatments.

4.3.3 Development of disease in the monoassociated TM-IEC C1*galt*1$^{-/-}$ mice relative to WT mice

Development of colitis was monitored by the appearance of classic disease signs, such as weight loss, development of diarrhea and rectal prolapse. No statistically significant changes were observed regarding weight fluctuations. A general assessment of average daily weight gain (ADG) between genotypes (TM-IEC C1*galt*1$^{-/-}$ *vs* WT) in monoassociated mice revealed that the general trend was a decrease in ADG in the TM-IEC C1*galt*1$^{-/-}$ relative to the WT counterpart, except for monoassociations with *A. muciniphila* and *L. johnsonii,* were TM-IEC C1*galt*1$^{-/-}$ gained more weight (on average) than the WT.

ADG comparisons between monoassociated and GF mice- in both, WT and TM-IEC C1*galt*1$^{-/-}$ conditions- showed that mice monoassociated with *Bacteroides* species had a decreased ADG, while the ADG in mice monoassociated with *C. symbiosum, A. muciniphila* and *L. johnsonii* increased, relative to their corresponding GF TM-IEC C1*galt*1$^{-/-}$ controls. Yet, all these differences were not statistically significant. Average daily weight gain with standard deviations, and p-values per treatment group and per genotype are listed in Table 4.5. Development of rectal prolapse was not observed in any of the monoassociated TM-IEC C1*galt*1$^{-/-}$ mice. No major alterations in stool consistency were observed.

Table 4.5 Mice average daily weight gain (ADG) and standard deviations (SD) per treatment and per genotype

Treatment	ADG WT ± SD (g)	ADG TM-IEC C1*galt*1$^{-/-}$ ± SD (g)	P-value
Germ-free 20d	0.14±0.07	0.12±0.03	0.4769
Germ-free 30d	0.11±0.03	0.10±0.03	0.5993
B. thetaiotaomicron	0.10±0.04	0.07±0.04	0.2474
B. vulgatus	0.10±0.02	0.06±0.02	0.0554
B. sartorii	0.08±0.06	0.06±0.02	0.6371
C. symbiosum	0.20±0.00	0.17±0.02	N/A
A. muciniphila	0.11±0.02	0.13±0.06	0.5528
L. johnsonii	0.14±0.06	0.16±0.08	0.6850

4.3.4 Determination of colitogenic bacterial species

The parameters addressed for the determination of inflammation scores in the monoassociation experiments (and therefore, for identification of inflammation-inducing bacteria) were the same as the ones used for evaluation of inflammation in the GF TM-IEC C1*galt*1$^{-/-}$ mice (Table 4.4). Infiltration of LP CD45^{+} cells and epithelial hyperplasia, observed in TM-IEC C1*galt*1$^{-/-}$ monoassociated mice were compared to those obtained for TM-IEC C1*galt*1$^{-/-}$ mice in GF and conventionalized settings (negative and positive controls, respectively). However, determination of statistical significance was done exclusively between genotypes (monoassociated WT *vs* monoassociated TM-IEC C1*galt*1$^{-/-}$ mice). Determination of inflammation scores for monoassociations with *Bifidobacterium longum* were inconclusive, thus will be addressed in future works.

The $CD45^+$ marker, present in all leukocytes, was used to evaluate immune cell infiltration in the tissue as an indicator of inflammation (Figure 4.6). Quantification of $CD45^+$ immune cells showed that monoassociation with *B. vulgatus* caused a stronger tissue permeation with immune cells in the monoassociated TM-IEC C1*galt*1$^{-/-}$ mice than in their monoassociated WT counterpart, with up to an average of 1.9-fold increase in the number of cells (all changes in $CD45^+$ cells are expressed as fold-change over WT in Figure 4.7). Unfortunately, we only had one *B-vulgatus*-monoassociated WT control mouse, which hindered the completion of a strong statistical analysis between genotypes for this bacterial treatment. However, comparisons of the $CD45^+$ absolute numbers obtained for *B. vulgatus*-monoassociated TM-IEC C1*galt*1$^{-/-}$ mice against GF and conventionalized mice (for both, WT and TM-IEC C1*galt*1$^{-/-}$ mice), and against the other monoassociated TM-IEC C1*galt*1$^{-/-}$ mice were not significant, independently of bacterial monoassociation. The second highest fold change in $CD45^+$ cells was observed in TM-IEC C1*galt*1$^{-/-}$ mice colonized with *B. thetaiotaomicron* (an average if 1.8-fold increase, $p=0.017$), followed by the conventionalized TM-IEC C1*galt*1$^{-/-}$ mice (1.6-fold increase, $p=0.046$). In addition, monoassociation of TM-IEC C1*galt*1$^{-/-}$ mice with *L. johnsonii* showed significant infiltration of $CD45^+$ cells into the tissue (1.5-fold increase, $p=0.019$). Interestingly, the presence of *C. symbiosum* in the monoassociated TM-IEC C1*galt*1$^{-/-}$ mice caused a significant decrease in the number of $CD45^+$ cells (average of -1.4 fold decrease, $p=0.016$) relative to the monoassociated WT counterpart, which resembles the trend seen in the TM-IEC C1*galt*1$^{-/-}$ GF mice. No significant changes in $CD45^+$ cells were found in mice associated with *A. muciniphila* and *B. sartorii*. Appendix B includes absolute numbers of CD45+ cells per treatment and genotype.

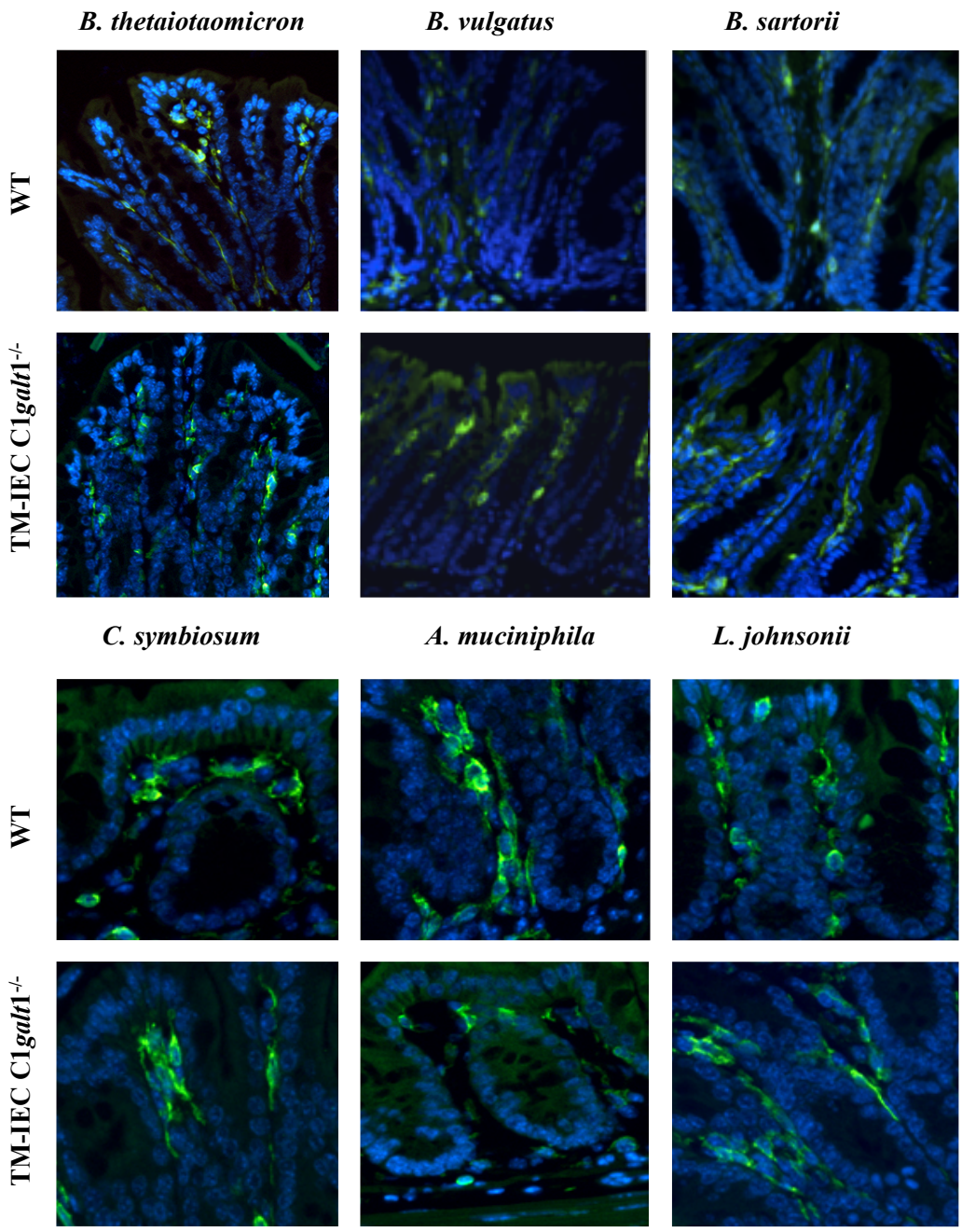

Figure 4.6 CD45^{+} cells were used as an indicator of an active immune response in the TM-IEC C1*galt1*$^{-/-}$ mice. Changes in number of CD45^{+} cells were estimated to evaluate the immune response caused by the bacteria in the case of a breach in the mucus layer, relative to bacterial association in an intact mucosal barrier.

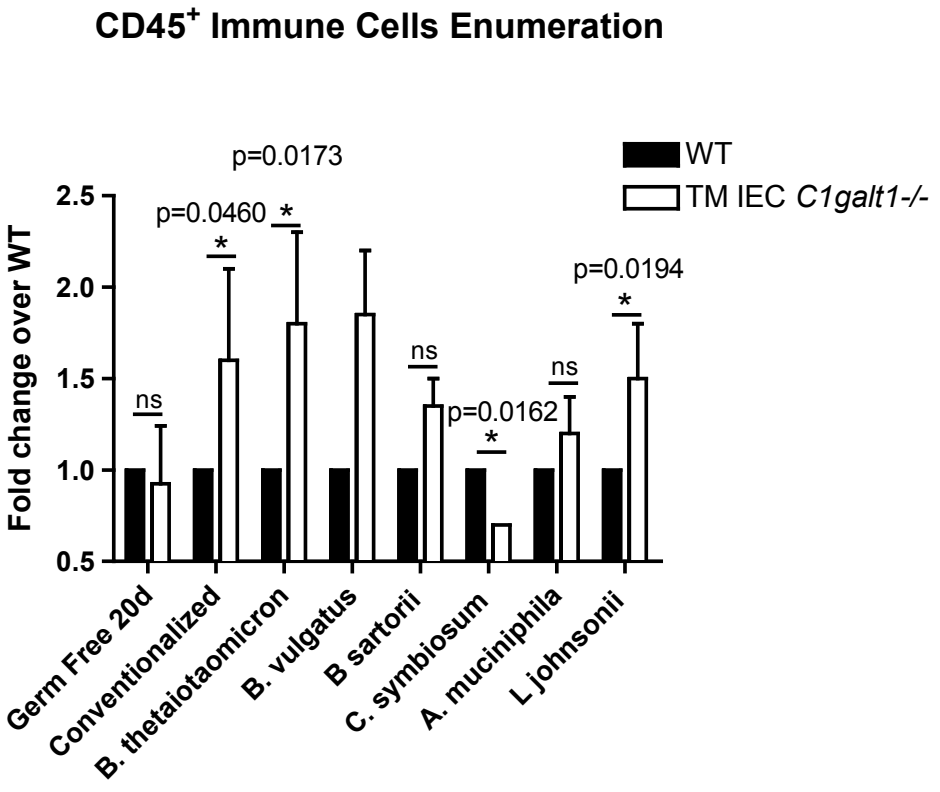

Figure 4.7 Lack of core 1 O-glycans and single association of GF mice with *Bacteroides vulgatus* and *Bacteroides thetaiotaomicron* caused stronger immune responses, as measured by changes in infiltrated lamina propria CD45$^+$ cells, than mice associated with a complete microbiota. *B. vulgatus* and *B. thetaiotaomicron* caused a 1.9 and 1.8 fold-change increase, respectively, in the number of CD45$^+$ cells in mice monoassociated with these bacteria. The observed changes were greater than those seen in the conventionalized mice that have a complete microbiota. In contrast, monoassociation with *C. symbiosum* caused a 1 fold-decrease in these cells. Bars represent fold change over WT; error bars represent standard deviations. P-values were estimated for changes between genotypes (WT *versus* TM-IEC C1*galt*1$^{-/-}$).

The second parameter used for the determination of inflammation scores was epithelial hyperplasia (Figures 4.8A and 4.8B). The conventionalized TM-IEC C1*galt*1$^{-/-}$ mice showed the highest degree of hyperplasia, with the TM-IEC C1*galt*1$^{-/-}$ mice having up to a 1.6-fold increase (average fold increase of 1.44, p=0.0323) in the number of

epithelial cells. Following, in decreasing order, monoassociation of TM-IEC C1*galt*1$^{-/-}$ mice with *B. thetaiotaomicron* and with *B. vulgatus* had an average of 1.3-fold increase in epithelial cells (p=0.0323 for *B. thetaiotaomicron* monoassociation). Contrary to B. *thetaiotaomicron* and *B. vulgatus*, *B. sartorii* and *A.muciniphila*, also gram-negative bacteria, did not cause significant changes in terms of hyperplasia. The mild degree of hyperplasia caused by monoassociation of TM-IEC C1*galt*1$^{-/-}$ mice with *L. johnsonii* was similar to that found in GF mice. Interestingly, *C. symbiosum,* which caused a statistically significant decrease in the number of CD45+ cells in TM-IEC C1*galt*1$^{-/-}$ mice, also caused significant hyperplasia (average of 1.2-fold increase, p= 0.0154). All changes in the number of epithelial cells are expressed as fold-change over WT in Figure 4.9.

Inflammation scores were estimated by the sum of scores given for changes in the numbers of CD45^{+} and epithelial cells as previously established (Table 4.4). The highest scores were obtained for the conventionalized TM-IEC C1*galt*1$^{-/-}$ mice (p<0.0001), followed by monoassociation of TM-IEC C1*galt*1$^{-/-}$ mice with *B. thetaiotaomicron* (p=0.0024) and *B. vulgatus*, relative to their WT counterparts (Figure 4.11). In addition, the differences between genotypes of *C. symbiosum*-monoassociated TM-IEC C1*galt*1$^{-/-}$ mice reached statistical significance (p=0.0310). However, the total scores are comparable to the scores obtained for the GF TM-IEC C1*galt*1$^{-/-}$ mice. Similarly, the scores obtained for mice monoassociated with *B. sartorii, A. muciniphila* and *L. johnsonii* are equivalent or lower than the ones calculated for GF TM-IEC C1*galt*1$^{-/-}$ mice (Figure 4.10).

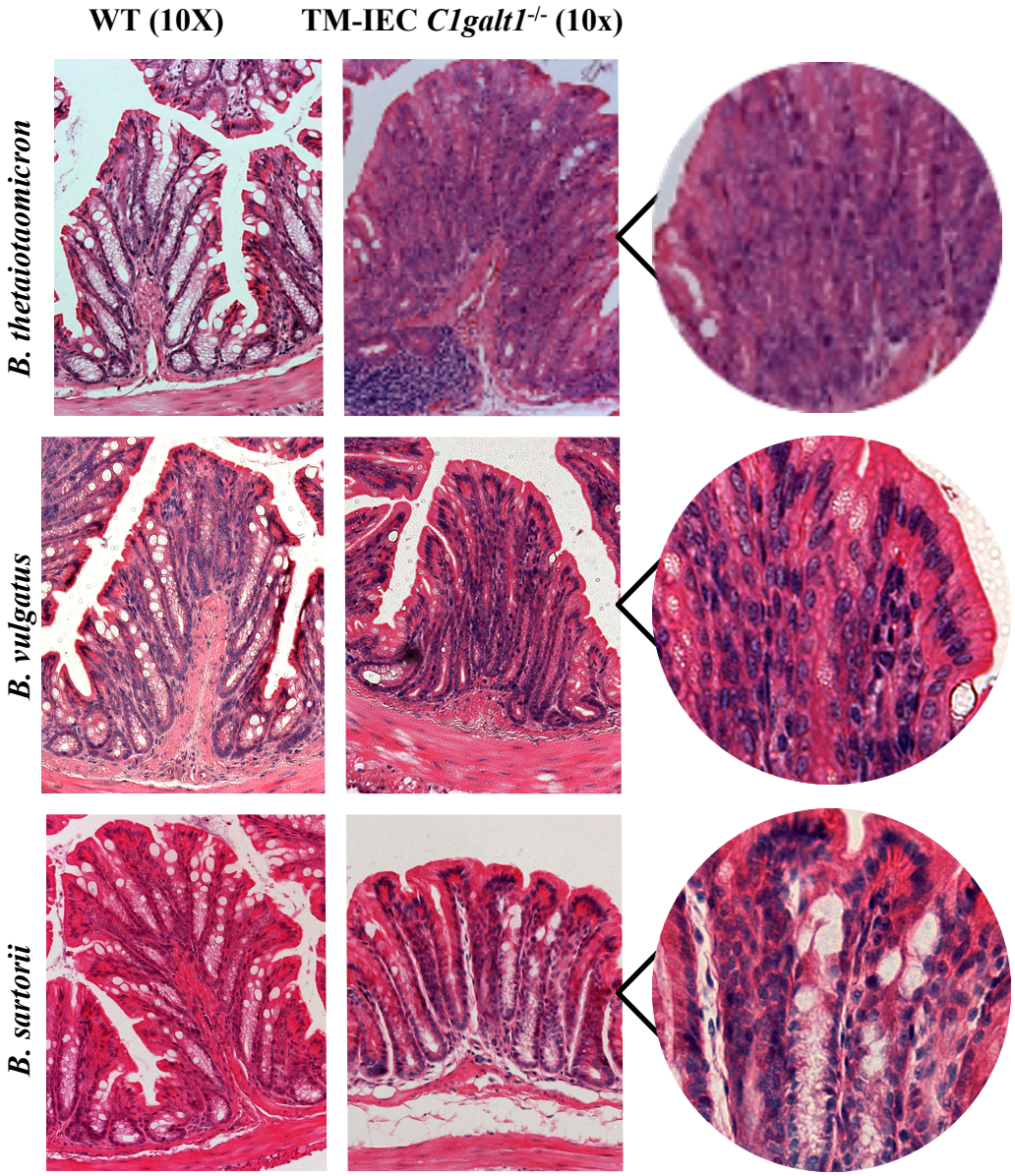

Figure 4.8A Assessment of epithelial hyperplasia in WT and TM-IEC C1*galt*1$^{-/-}$ mice monocolonized with *Bacteroides* spp. Note differences in the appearance of the tissue between the genotypes (monoassociated WT *vs* monoassociated TM-IEC C1*galt*1$^{-/-}$**)** caused by increases in volume as a consequence of increases in the number of epithelial cells. Note differences in tissue appearance between the *B. thetaiotaomicron*-monocolonized TM-IEC C1*galt*1$^{-/-}$ mice (top) and the *B. sartorii*-monocolonized mice (bottom). Circles to the right show a closer view of the tissue.

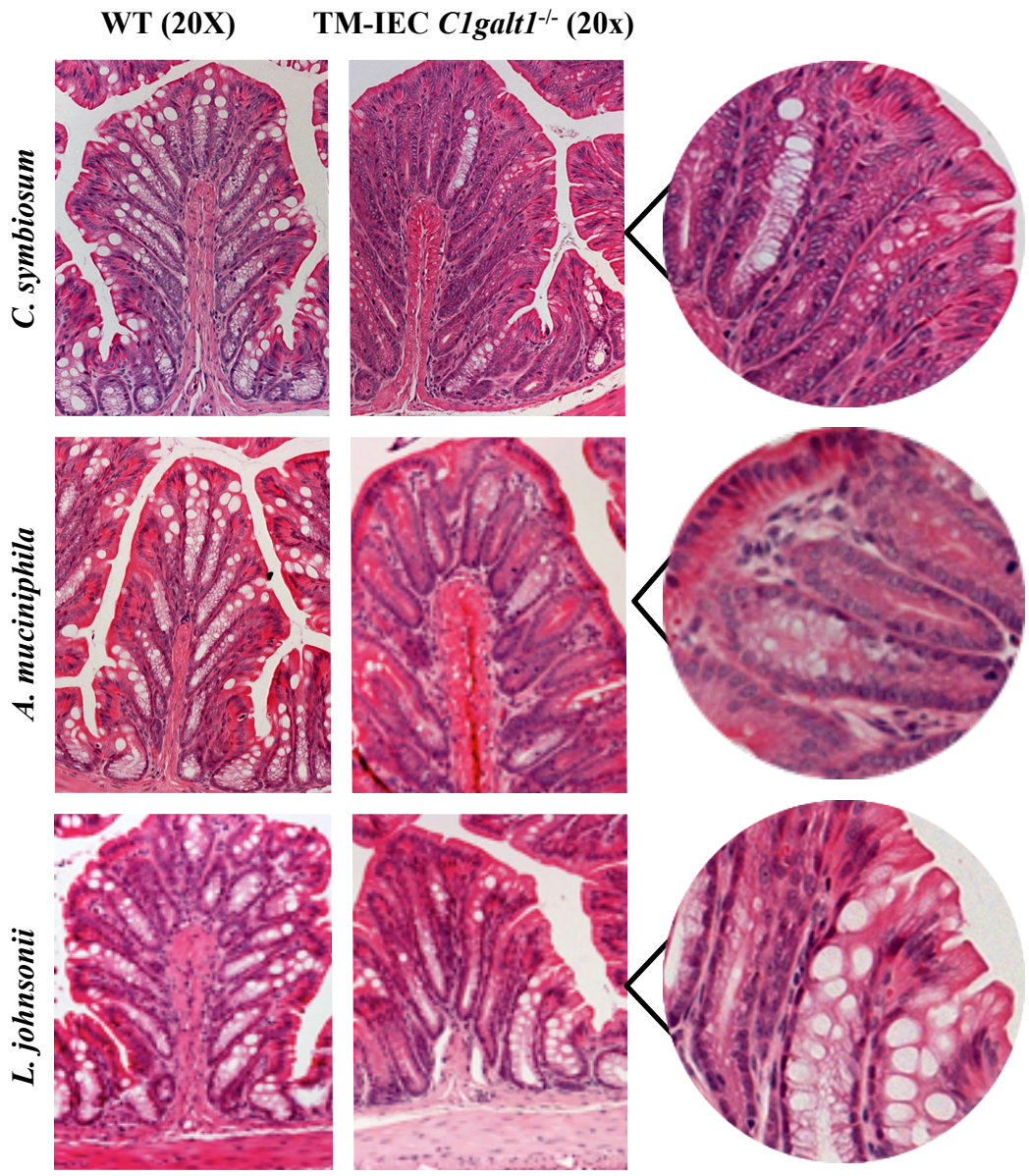

Figure 4.8B Assessment of epithelial hyperplasia in mice monocolonized with *C. symbiosum*, *A. muciniphila*, and *L. johnsonii.* Note differences in the appearance of the tissue between the genotypes (monoassociated WT *vs* monoassociated TM-IEC C1*galt*1$^{-/-}$**)** caused by increases in volume as a consequence of increases in the number of epithelial cells. Circles to the right show a closer view of the tissue.

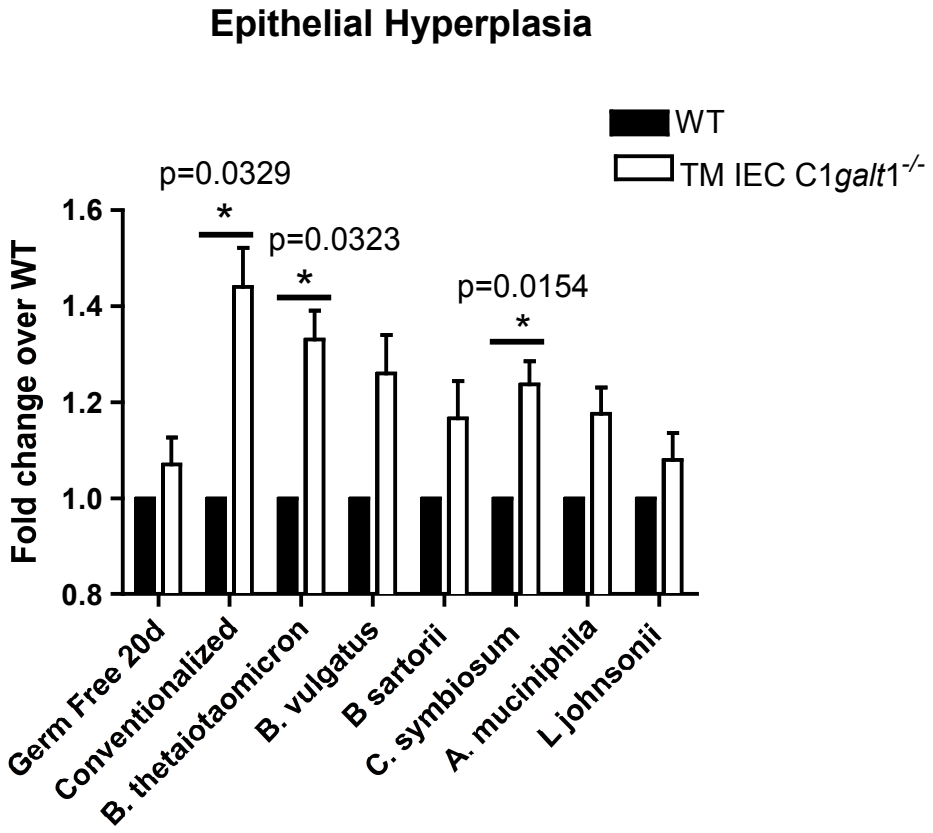

Figure 4.9 *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* caused a higher degree of hyperplasia than monoassociations with *Bacteroides sartorii* or other bacterial species tested. The TM-IEC C1*galt*1$^{-/-}$ conventionalized mice (with a complete microbiota) presented the highest degree of hyperplasia among all bacterial treatments. Monoassociation with *B. thetaiotaomicron* and *B. vulgatus* caused the highest degree of hyperplasia among the monoassociation experiments. Bars represent fold change over WT; error bars represent standard deviations. P-values were estimated for changes between genotypes (WT versus TM-IEC C1*galt*1$^{-/-}$).

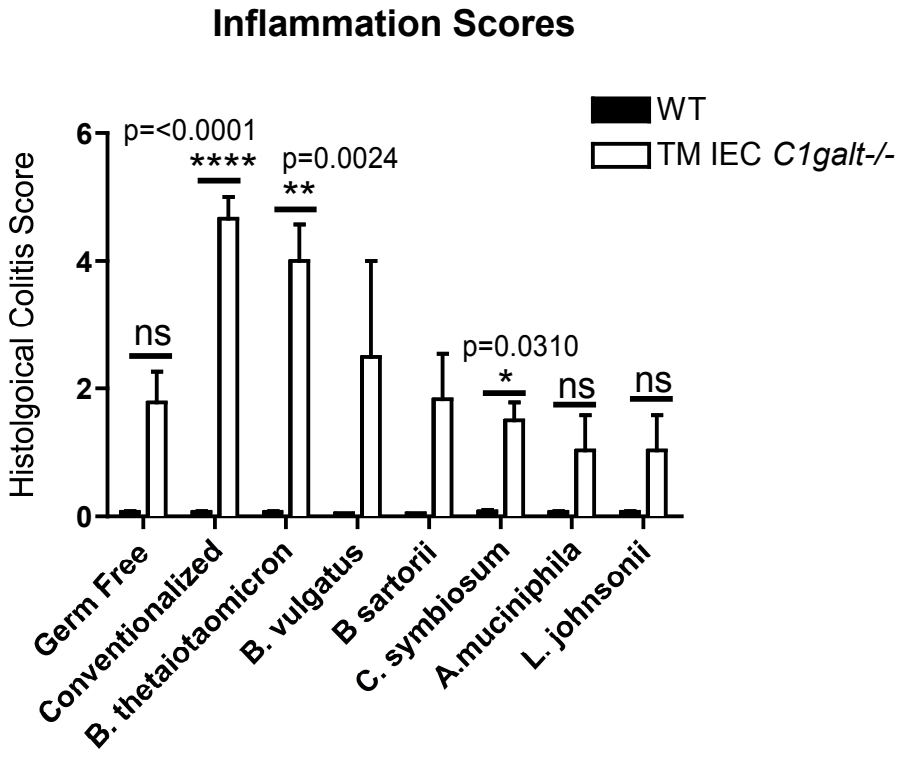

Figure 4.10 Intestinal symbionts *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* are capable of inducing colitis in the absence of core 1 O-glycans. *B. thetaiotaomicron* and *B. vulgatus* caused the highest degree of inflammation (as measured by lamina propria CD45+ cells infiltration in the colonic tissue and epithelial hyperplasia) among the monoassociation experiments. Monoassociation with *B. sartorii, A. muciniphila, C. symbiosum* and *L. johnsonii* did not induce inflammation. Error bars represent standard deviations. P-values were estimated for changes between genotypes (WT *versus* TM-IEC C1*galt*1$^{-/-}$).

4.3.5 B cell responses in monoassociated TM-IEC *C1galt1*$^{-/-}$ mice

Adaptive immunity responses were assessed by measurement of immunoglobulin levels by ELISA. Immunoglobulin levels were compared *between* genotypes (WT *vs* TM-IEC C1*galt*1$^{-/-}$), and *within* genotypes (GFWT *vs* monoassociated WT, and GF TM-IEC C1*galt*1$^{-/-}$ *vs* monoassociated TM-IEC C1*galt*1$^{-/-}$). Immunoglobulin levels for *B. thetaiotaomicron* and *B. sartorii* were compared against the GF controls 30 days after TM treatment (GF30), while for other bacteria used in these experiments comparisons were against GF controls 20 days after TM treatment (GF20). In addition, comparisons between GF20 and GF30 were performed in a similar fashion. A higher number of statistically significant differences were found *within* genotypes rather than *between* them, especially for the TM-IEC C1*galt*1$^{-/-}$ mice (Figures 4.11-4.13 and Table 4.5).

In both, GF and monoassociated settings, a breach in the mucus barrier induced increases in serum IgA production in the monoassociated TM-IEC C1*galt*1$^{-/-}$ mice relative to their monoassociated WT counterparts (Figure 4.11A) independently of the bacterial species tested. These changes were not statistically significant when comparing monoassociated WT *vs* monoassociated TM-IEC C1*galt*1$^{-/-}$ for every bacterial treatment, or when comparing monoassociated TM-IEC C1*galt*1$^{-/-}$ mice with GF TM-IEC C1*galt*1$^{-/-}$ mice. However, they were significant between the genotypes in the GF setting at both, 20 days and 30 days ($p=0.0040$ and $p=0.0222$, respectively). Additionally, changes in serum IgA reached significance when comparing *B.thetaiotaomicron*-monoassociated WT and *C. symbiosum*-monoassociated WT mice relative to the GF WT control. *B. thetatiotaomicron* induced an increase in serum IgA (GF30WT 32.15 ± 1.531 *vs*

B.thetaiotaomicron WT 43.47 ± 2.55, p=0.0067), while monoassociation with *C. symbiosum* caused a decrease in the immunoglobulin level (GF20WT 30.49 ± 1.48 *vs C. symbiosum* WT 24.46 ± 0.40, p= 0.0029).

Similar to serum IgA, an impaired mucus barrier caused increases in secretory IgA (sIgA) in the mutated mice relative to the WT in both, GF and monoassociated scenarios (Figure 4.11B). However, contrary to what was found for serum IgA, significant changes *between* genotypes were found for sIgA in TM-IEC C1*galt*1$^{-/-}$ monoassociated with *C. symbiosum* and with *L. johnsonii*, relative to their monoassociated-WT counterpart (*C. symbiosum* WT 188.90 ± 4.74 *vs C. symbiosum* TM-IEC C1*galt*1$^{-/-}$ 240.90 ± 14.29, p=0.0409; *L. johnsonii* WT 171.60 ± 7.90 *vs L. johnsonii* TM-IEC C1*galt*1$^{-/-}$ 271.00 ± 21.68, p=0.0499, respectively). The only significant changes *within* the TM-IEC C1*galt*1$^{-/-}$ mice occurred in mice monoassociated with *C. symbiosum*, and the GF30 mice relative to the GF20 control (GF TM-IEC C1*galt*1$^{-/-}$ 175.70 ± 7.42 *vs C. symbiosum* TM-IEC C1*galt*1$^{-/-}$ 240.90 ± 14.29, p= 0.0155; *vs* GF30 203.20 ± 7.704, p=0.0258).

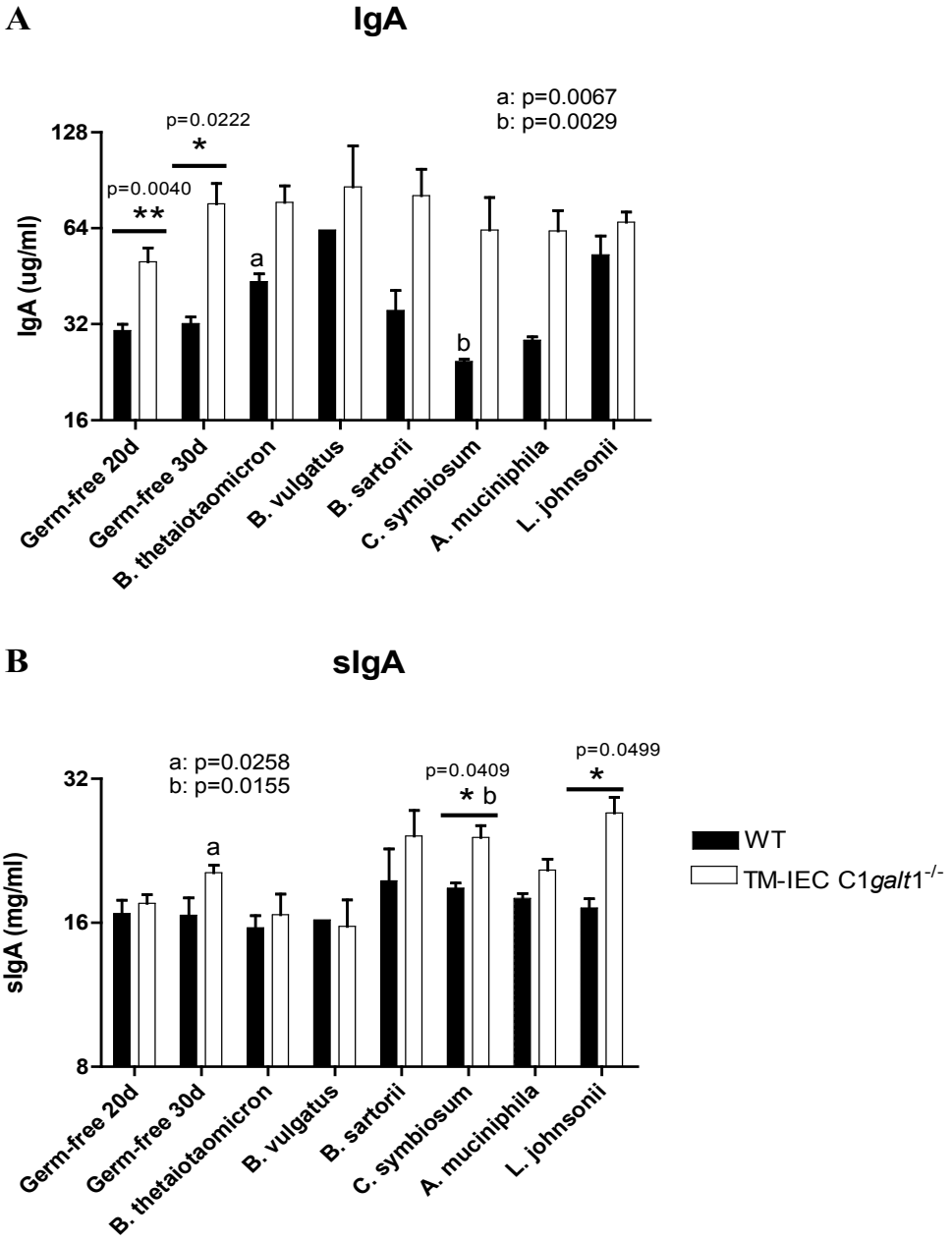

Figure 4.11 Lack of core 1 O-glycans causes increases serum and secretory IgA independently of germ-free status or bacterial species used for monoassociaton. [A] Graphic representation of serum IgA production (systemic response) induced by selected bacterial species. [B] Graphic representation of secretory IgA production (localized response) induced by selected bacterial species. [A and B] The combined effect of a breach in the mucus layer and presence of bacteria has a greater impact in systemic and localized IgA immune responses than bacterial monoassociation by itself. Lines above bars indicate significant differences between mice of different genotypes (WT *vs* TM-IEC C1*galt*1$^{-/-}$ mice), while letters above bars indicate significant differences between mice of the same genotype (GF TM-IEC C1*galt*1$^{-/-}$ mice *vs* monoassociated TM-IEC C1*galt*1$^{-/-}$ mice or GFWT *vs* monoassociated WT). Error bars represent SD.

No trends were found in IgG and IgM production. The values are dependent on bacterial monoassociation. While some bacteria caused increases in the levels of both immunoglobulins, others caused decreases. Once more, most changes were found *within* genotypes (GFWT *vs* monoassociated WT, or GF TM-IEC C1*galt*1$^{-/-}$ *vs* monoassociated TM-IEC C1*galt*1$^{-/-}$) rather than *between* genotypes (WT *vs* TM-IEC C1*galt*1$^{-/-)}$. While the levels of serum IgA and sIgA increased over time in the GF setting, the opposite occured with IgM and IgG, were the level decreased with time.

In the case of serum IgG (Figure 4.12A), significant changes occurred exclusively within the TM-IEC C1*galt*1$^{-/-}$ mice (GF TM-IEC C1*galt*1$^{-/-}$ *vs* monoassociated TM-IEC C1*galt*1$^{-/-}$). *B. thetaiotaomicron* caused an increase in IgG, while *A. muciniphila* and *L. johnsonii* induced decreases in its levels relative to the GF control (GF30 TM-IEC C1*galt*1$^{-/-}$ 978.5 ± 196.0 *vs B. thetaiotaomicron* TM-IEC C1*galt*1$^{-/-}$ 1685.00 ± 59.15, p=0.0261; GF20 TM-IEC C1*galt*1$^{-/-}$ 1273 ± 100.0 *vs A. muciniphila* TM-IEC C1*galt*1$^{-/-}$ 714.6 ± 114.7, p=0.0144; and *vs L. johnsonii* TM-IEC C1*galt*1$^{-/-}$ 908.2 ± 42.88, p=0.0052).

In the case of IgM (Figure 4.12B), no significant changes were found *between* genotypes. All bacterial monoassociations caused decreases in both WT and TM-IEC C1*galt*1$^{-/-}$ relative to their GF counterparts. The majority of statistically significant changes were found *within* the WT mice for monoassociations with *A.muciniphila*, *C. symbiosum* and *L. johnsonii* (GFWT 395.6 ± 57.09 *vs A.muciniphila* WT 176.3 ± 13.98, p=0.0033; *vs C. symbiosum* WT 183.6 ± 8.482, p=0.0043; and *vs L. johnsonii* WT 199.0 ± 15.31, p= 0.0068). Changes *within* the TM-IEC C1*galt*1$^{-/-}$ mice occurred in the GF setting and in monoassociation with *L. johnsonii* (GF TM-IEC C1*galt*1$^{-/-}$ 395.6 ± 57.09 *vs*

GF30 TM-IEC C1*galt*1$^{-/-}$ 236.4 ± 42.73, p=0.0258; and *vs L. johnsonii* TM-IEC C1*galt*1$^{-/-}$ 231.4 ± 6.62, p=0.0072).

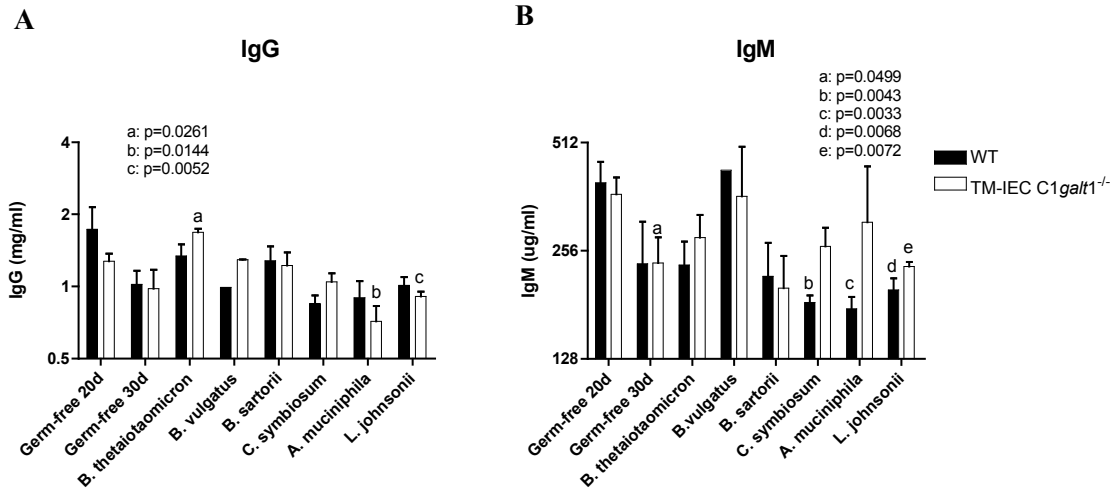

Figure 4.12 Changes in serum IgG and IgM were dependent on microbial association rather than lack of core 1 O-glycans. No specifics trends were found for IgG and IgM production between the WT and the TM-IEC C1*galt*1$^{-/-}$ mice. Bacterial association had a greater impact on the production of these immunoglobulins, as some bacteria caused the values to increase while other caused decreases, independently of presence or absence of core 1 O-glycans. Lines above bars indicate significant differences between mice of different genotypes (WT *vs* TM-IEC C1*galt*1$^{-/-}$ mice), while letters above bars indicate significant differences between mice of the same genotype (GF TM-IEC C1*galt*1$^{-/-}$ mice *vs* monoassociated TM-IEC C1*galt*1$^{-/-}$ mice or GFWT *vs* monoassociated WT). Error bars represent SD.

In regards to the IgG subtypes, no significant changes were found for IgG1 *within* or *between* genotypes (Figure 4.13A). Significant changes *between* genotypes were found in monoassociations with *B. sartorii* and *B. thetaiotaomicron* in the induction of IgG2a and IgG2b, respectively (Figures 4.15B-C) (*B. sartorii* WT 4.376 ± 0.4013 *vs B. sartorii* TM-IEC C1*galt*1$^{-/-}$ 2.508 ± 0.2316 p=0.0275; *B. thetaiotaomicron* WT 175.5 ± 17.16 *vs B. thetaiotaomicron* TM-IEC C1*galt*1$^{-/-}$ 315.7 ± 44.25, p=0.0213, respectively).

The levels of IgG2a, IgG2b and IgG3 were significant in the *L. johnsonii* monoassociated TM-IEC C1*galt*1$^{-/-}$ mice when compared to the GF TM-IEC C1*galt*1$^{-/-}$ control (Figures 4.13B-D). Even though the trend for IgG2b was an increase in the mutated mice, the majority of significant changes in IgG2b were among the WT mice: monoassociations with *B. thetaioatomicron* caused increased level of IgG2b, while *C. symbiosum* and *Akkermansia muciniphila* caused decreases in the Ig subtype relative to their corresponding WT controls (Figure 4.13C).

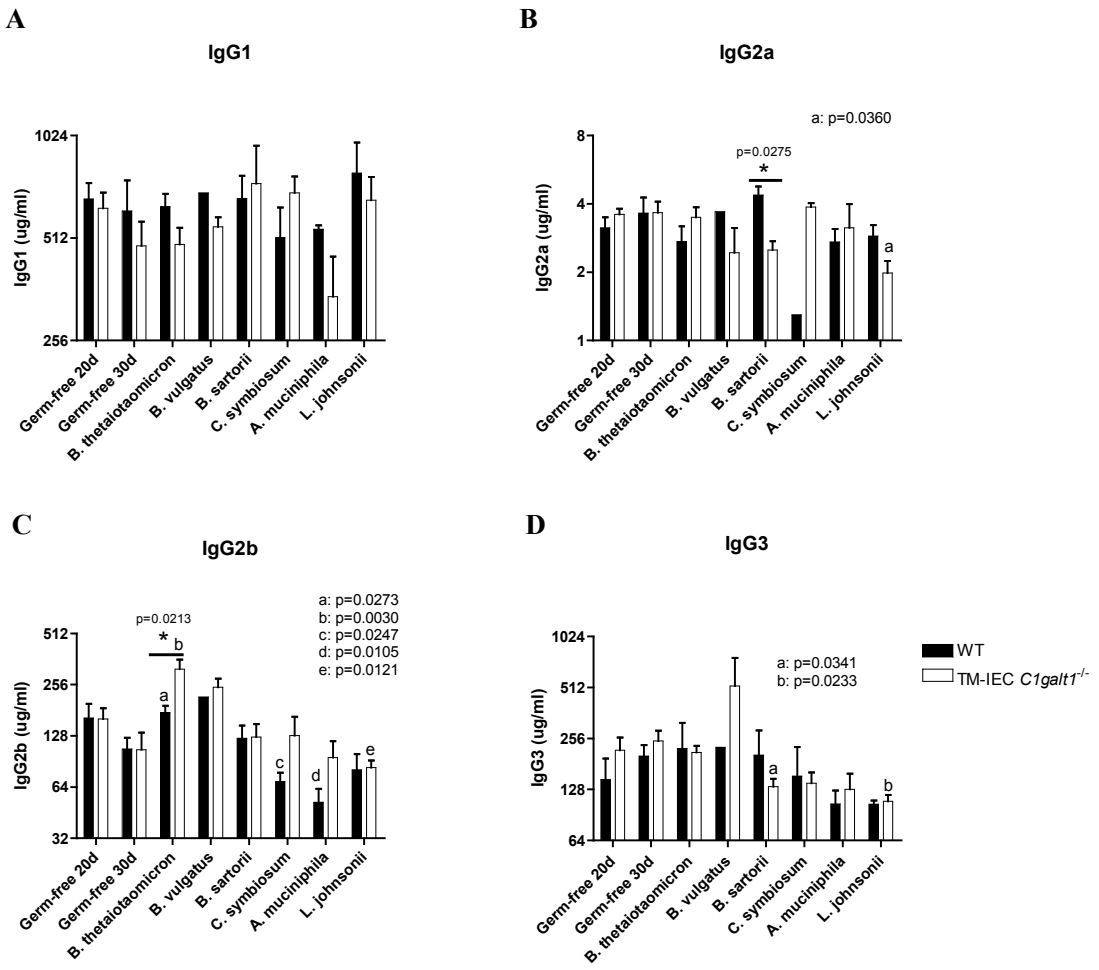

Figure 4.13 Deficiency of core 1 O-glycans caused increases in IgG2b in the TM-IEC C1*galt*1$^{-/-}$ mice independently of bacterial association. No trends were observed in the induction of IgG subtypes except for [B] IgG2b, were the mutation induced increases independently of bacterial association. Lines above bars indicate significant differences between mice of different genotypes (WT *vs* TM-IEC C1*galt*1$^{-/-}$ mice), while letters above bars indicate significant differences between mice of the same genotype (GF TM-IEC C1*galt*1$^{-/-}$ mice *vs* monoassociated TM-IEC C1*galt*1$^{-/-}$ mice or GFWT *vs* monoassociated WT). Error bars represent SD.

Table 4.6 Comparisons of immunoglobulin levels between germ-free and monoassociated WT and TM-IEC C1*galt*1$^{-/-}$ mice

Germ-free 20 days

Immunoglobulin	WT	N	TM-IEC C1*galt*1$^{-/-}$	N	GFWT vs TXWT (p values)	GFKO vs TXKO (p values)	WT vs KO (p values)
IgA (ug/ml)	30.49 ± 1.48	11	50.14 ± 5.43	13	---	---	**0.0040****
sIgA (mg/ml)	167.10 ± 11.15	11	175.70 ± 7.42	13	---	---	0.5283
IgM (ug/ml)	395.6 ± 57.09	11	367.7 ± 41.61	13	---	---	0.6975
IgG (mg/ml)	1658 ± 380.3	11	1273 ± 100.0	13	---	---	0.3483
IgG1 (ug/ml)	667.5 ± 76.03	11	626.6 ± 69.66	13	---	---	0.6960
IgG2a (ug/ml)	4.383 ± 0.77	10	4.685 ± 0.60	13	---	---	0.2075
IgG2b (ug/ml)	162.9 ± 34.43	11	160.7 ± 25.16	13	---	---	0.9591
IgG3 (ug/ml)	146.5 ± 48.71	11	218.0 ± 41.20	13	---	---	0.2759

Germ-free 30 days

Immunoglobulin	WT	N	TM-IEC C1*galt*1$^{-/-}$	N	GF20WT vs GF30WT (p values)	GF20KO vs GF30KO (p values)	WT vs KO (p values)
IgA (ug/ml)	32.15 ± 1.53	4	76.32 ± 12.08	5	0.4593	0.1050	**0.0222***
sIgA (mg/ml)	165.70 ± 14.51	4	203.20 ± 7.7	5	0.9452	**0.0258***	0.0846
IgM (ug/ml)	235.4 ± 72.60	4	236.4 ± 42.73	5	0.1264	**0.0499***	0.9914
IgG (mg/ml)	1023 ± 138.8	4	978.5 ± 196.0	5	0.1424	0.2291	0.8600
IgG1 (ug/ml)	615.0 ± 141.2	4	485.8 ± 85.94	5	0.7600	0.2349	0.4696
IgG2a (ug/ml)	3.643 ± 0.63	4	3.665 ± 0.43	5	0.4725	0.1847	0.9771
IgG2b (ug/ml)	107.1 ± 17.63	4	106.3 ± 27.57	5	0.1750	0.1761	0.9817
IgG3 (ug/ml)	201.5 ± 32.58	4	247.8 ± 36.66	5	0.3668	0.5976	0.3811

***B. thetaiotaomicron*$^{\#}$**

Immunoglobulin	WT	N	TM-IEC C1*galt*1$^{-/-}$	N	GFWT vs TXWT (p values)	GFKO vs TXKO (p values)	WT vs KO (p values)
IgA (ug/ml)	43.47 ± 2.55	6	77.15 ± 9.77	7	**0.0067****	0.7885	0.1537
sIgA (mg/ml)	163.90 ± 7.99	6	199.90 ± 20.53	8	0.9147	0.8833	0.1403
IgM (ug/ml)	233.4 ± 37.92	6	278.7 ± 43.16	8	0.9817	0.5019	0.4474
IgG (mg/ml)	1341 ± 158.3	6	1685 ± 59.15	7	0.1738	**0.0261***	0.0885
IgG1 (ug/ml)	633.4 ± 57.13	6	490.6 ± 58.77	7	0.9117	0.9646	0.1121
IgG2a (ug/ml)	2.735 ± 0.45	6	3.498 ± 0.37	8	0.2918	0.7735	0.2187
IgG2b (ug/ml)	175.5 ± 17.16	6	315.7 ± 44.25	7	**0.0273***	**0.0030****	**0.0213***
IgG3 (ug/ml)	223.4 ± 93.15	6	211.5 ± 20.87	8	0.8312	0.4220	0.9051

B. vulgatus

Immunoglobulin	WT	N	TM-IEC C1*galt*1$^{-/-}$	N	GFWT vs TXWT (p values)	GFKO vs TXKO (p values)	WT vs KO (p values)
IgA (ug/ml)	63.13	1	86.29 ± 29.82	3	n/a	0.3552	n/a
sIgA (mg/ml)	162.05	1	157.20 ± 21.21	4	n/a	n/a	n/a
IgM (ug/ml)	428.98	1	363.2 ± 135.2	3	n/a	0.9776	n/a
IgG (mg/ml)	991.64	1	1295 ± 0.90	2	n/a	0.8341	n/a
IgG1 (ug/ml)	608.95	1	527.3 ± 32.67	3	n/a	0.2195	n/a
IgG2a (ug/ml)	3.7	1	2.439 ± 0.69	3	n/a	0.1134	n/a
IgG2b (ug/ml)	216.46	1	246.9 ± 30.33	3	n/a	0.0803	n/a
IgG3 (ug/ml)	227.31	1	521.6 ± 244.7	3	n/a	0.3457	n/a

Values represent means and standard errors of means (Mean ± SEM). Asterisks (*) indicate significant p values. Pound sign (#) indicates comparison was made against GF 30 days control. GF: Germ-free; TX: Monoassociated; WT: Wild type; KO: TM-IEC C1*galt*1$^{-/-}$ mice

Table 4.6 Comparisons of immunoglobulin levels between germ-free and monoassociated WT and TM-IEC C1*galt1*$^{-/-}$ mice (continuation)

***B. sartorii*$^{\#}$**

Immunoglobulin	WT	N	TM-IEC C1*galt1*$^{-/-}$	N	GFWT vs TXWT (p values)	GFKO vs TXKO (p values)	WT vs KO (p values)
IgA (ug/ml)	35.31 ± 5.54	3	80.95 ± 17.20	3	0.6376	0.8397	0.1275
sIgA (mg/ml)	195.60 ± 32.78	3	242.80 ± 31.69	3	0.4929	0.3489	0.3765
IgM (ug/ml)	217.4 ± 51.83	3	201.4 ± 46.27	3	0.8501	0.6029	0.8327
IgG (mg/ml)	1283 ± 187.7	3	1223 ± 163.3	3	0.3459	0.3822	0.8241
IgG1 (ug/ml)	669.5 ± 110.8	3	740.9 ± 215.0	3	0.7765	0.5854	0.7957
IgG2a (ug/ml)	4.376 ± 0.40	3	2.508 ± 0.23	3	0.3795	0.0636	**0.0275***
IgG2b (ug/ml)	123.8 ± 23.37	3	126.0 ± 24.18	3	0.5996	0.6151	0.9519
IgG3 (ug/ml)	204.3 ± 81.61	3	133.0 ± 15.23	3	0.9769	**0.0341***	0.4810

C. symbiosum

Immunoglobulin	WT	N	TM-IEC C1*galt1*$^{-/-}$	N	GFWT vs TXWT (p values)	GFKO vs TXKO (p values)	WT vs KO (p values)
IgA (ug/ml)	24.46 ± 0.40	2	63.22 ± 16.69	4	**0.0029****	0.5101	0.1029
sIgA (mg/ml)	188.90 ± 4.74	2	240.90 ± 14.29	4	0.1011	**0.0155***	**0.0409***
IgM (ug/ml)	183.6 ± 8.48	2	263.0 ± 33.50	4	**0.0043****	0.0736	0.1052
IgG (mg/ml)	849.6 ± 67.50	2	1045 ± 90.77	4	0.0627	0.1217	0.1830
IgG1 (ug/ml)	514.3 ± 115.4	2	695.2 ± 81.50	4	0.3832	0.5427	0.3289
IgG2a (ug/ml)	1.3000	1	3.878 ± 0.15	3	n/a	0.4800	n/a
IgG2b (ug/ml)	69.02 ± 8.79	2	128.6 ± 37.23	4	**0.0247***	0.5026	0.2171
IgG3 (ug/ml)	153.4 ± 74.23	2	139.2 ± 22.40	4	0.9449	0.1153	0.8847

A. muniniphila

Immunoglobulin	WT	N	TM-IEC C1*galt1*$^{-/-}$	N	GFWT vs TXWT (p values)	GFKO vs TXKO (p values)	WT vs KO (p values)
IgA (ug/ml)	28.48 ± 0.74	2	62.73 ± 9.95	3	0.2551	0.3474	0.0753
sIgA (mg/ml)	179.60 ± 4.38	3	206.00 ± 11.34	3	0.3160	0.1114	0.1627
IgM (ug/ml)	176.3 ± 13.98	3	307.4 ± 131.8	3	**0.0033****	0.7050	0.4267
IgG (mg/ml)	897.3 ± 156.2	3	714.6 ± 114.7	3	0.0912	**0.0144***	0.4153
IgG1 (ug/ml)	543.1 ± 14.73	3	344.8 ± 106.8	3	0.1395	0.1141	0.2071
IgG2a (ug/ml)	2.718 ± 0.38	3	3.143 ± 0.85	3	0.0815	0.2120	0.6935
IgG2b (ug/ml)	52.11 ± 10.52	3	95.23 ± 23.94	3	**0.0105***	0.1016	0.2409
IgG3 (ug/ml)	105.1 ± 21.16	3	128.2 ± 30.68	3	0.4523	0.1112	0.5795

L. johnsonii

Immunoglobulin	WT	N	TM-IEC C1*galt1*$^{-/-}$	N	GFWT vs TXWT (p values)	GFKO vs TXKO (p values)	WT vs KO (p values)
IgA (ug/ml)	52.78 ± 7.70	3	66.72 ± 5.251	3	0.1048	0.0642	0.2317
sIgA (mg/ml)	171.60 ± 7.90	3	271.00 ± 21.68	3	0.7464	0.0532	**0.0499***
IgM (ug/ml)	199.0 ± 15.31	3	231.4 ± 6.62	3	**0.0068****	**0.0072****	0.1914
IgG (mg/ml)	1012 ± 82.30	3	908.2 ± 42.88	3	0.1278	**0.0052****	0.3443
IgG1 (ug/ml)	795.5 ± 182.1	3	662.2 ± 112.6	3	0.5830	0.8055	0.5777
IgG2a (ug/ml)	2.884 ± 0.35	3	1.986 ± 0.25	3	0.6212	**0.0360***	0.1282
IgG2b (ug/ml)	80.94 ± 19.18	3	83.48 ± 8.27	3	0.0618	**0.0121***	0.9145
IgG3 (ug/ml)	104.8 ± 5.283	6	109.0 ± 10.06	3	0.4146	**0.0233***	0.7360

Values represent means and standard errors of means (Mean ± SEM). Asterisks (*) indicate significant p values. Pound sign (#) indicates comparison was made against GF 30 days control. GF: Germ-free; TX: Monoassociated; WT: Wild type; KO: TM-IEC C1*galt1*$^{-/-}$ mice

4.4 Discussion

4.4.1 Intestinal symbionts *Bacteroides thetaiotaomicron* and *Bacteroides vulgatus* initiate colitis in the absence of O-glycans

Bacteroides thetaiotaomicron has been shown to be involved in the pathogenesis of colitis in several murine models, such as HLA-B27 transgenic rats (Rath et al, 1996; Rath et al, 1999; Hansen et al, 2012), and the double KO IL10r2- and Tgfbr2- mouse model of IBD (Bloom et al, 2011). Similarly, *Bacteroides vulgatus* was recognized as an inflammation inducer, as it caused disease in the HLA-B27 transgenic rat model of IBD (Rath et al, 1996; Rath et al, 1999). In the TM-IEC C1*galt*1$^{-/-}$ mice, monoassociation with *B. thetaiotaomicron* caused inflammation that closely resembles that seen in conventionalized mice, while inflammation caused by *B. vulgatus* could be considered moderate, compared to the same control (conventionalized mice).

Metabolic analyses of *B. thetaiotaomicron* have shown its immense capacity of adaptation to its surroundings by expressing genes for carbohydrate utilization depending on source availability (Sonnenburg et al, 2005; Bjursell et al, 2006). Specifically, it is known that this bacterium has a cluster of genes involved in glycans utilization (Martens et al, 2009). However, even *B. thetaiotaomicron* mutants lacking genes for colonization and fitness (chuR-), and for O-glycans utilization (5xECF-) were able to induce moderate inflammation (data not included). Therefore, deficiency of O-glycans does not affect *B. thetaiotaomicron* colonization capacity; on the contrary, it seems to create a favorable environment for the bacterium to initiate colitis. Lack of O-glycosylation could be key in

deciding the fate of commensals, which may either remain as symbionts, or turn into pathobionts.

4.4.2 Host specificity of *Bacteroides sartorii* and *Lactobacillus johnsonii* could have a role in prevention of inflammation in the TM-IEC C1*galt*1$^{-/-}$ mice

Contrary to *B. thetaiotaomicron*, the inflammation scores of *B. sartorii*-colonized and *L. johnsonii*- colonized TM-IEC C1*galt*1$^{-/-}$ mice are comparable to the scores seen in GF TM-IEC C1*galt*1$^{-/-}$ mice. Experimental evidence shows that gut microbes have evolved into host-adapted lineages through long-term evolutionary processes, thus developing a specialized symbiotic relationship with their host (Oh et al, 2009; Frese et al, 2011). Since both, *B. sartorii* and *L. johnsonii* strains used in these set of experiments were mice-specific, the low intensity of inflammation induced in the TM-IEC C1*galt*1$^{-/-}$ mice by these two strains suggests that strain-host evolution could play a role in reducing the pathogenic potential of different bacterial species in this mice model.

4.4.3 *Lactobacillus johnsonii* increased as a consequence of inflammation, while *Clostridium disporicum* increases caused inflammation in the *conventional* TM-IEC C1*galt*1$^{-/-}$ mice

The monoassociation experiments using the TM-IEC C1*galt*1$^{-/-}$ mice model represent a simplified scenario of the etiology of UC, as these do not account for possible

interactions between different bacterial symbionts of the GIT. In a conventional setting, development of inflammation could harm some bacterial species more than others, thus freeing resources for the surviving species, and reducing competition for nutrients and niches between the members of the microbiota. The effect of such interactions is removed in a GF setting since competition is eradicated and resources become fully available for the single bacterium introduced into the monoassociated mouse.

The *L. johnsonii* strain used in the monoassociaton experiments was isolated from *conventional* colitic TM-IEC C1*galt*1$^{-/-}$ mice, in which it was found increased relative to conventional WT mice (discussed in Chapter 2). However, the TM-IEC C1*galt*1$^{-/-}$ mice mono-colonized with this bacterium did not present inflammation (in fact, they had a lower inflammation score than the GF TM-IEC C1*galt*1$^{-/-}$ mice). On the other hand, preliminary results for monoassociation experiments with *C. disporicum,* a species that was also found increased in the conventional colitic TM-IEC C1*galt*1$^{-/-}$ mice, showed that this organism caused certain degree of inflammation (preliminary data in Appendix C) in the monoassociated TM-IEC C1*galt*1$^{-/-}$ mice. In both of these cases where bacterial species showed increases in the conventional colitic mice, the bacteria may benefit from the lack of competition and possibly also from products released as a result of inflammation. Nevertheless, their opposite behaviors in the monoassociated TM-IEC C1*galt*1$^{-/-}$ mice suggest that the increase in *L. johnsonii* is a consequence of inflammation, rather than a cause, while increases in *C. disporicum* represent a cause of inflammation, and not a consequence.

4.4.4 *Akkermansia muciniphila* and *Clostridium symbiosum* might have a role in modulating the immune response in the TM-IEC C1*galt*1$^{-/-}$ mice for prevention of inflammation

Non-colitogenic bacteria were identified for the TM-IEC C1*galt*1$^{-/-}$ mice model through these sets of experiments. The specific factors that decide why a bacterium remains a symbiont or become a pathobiont are unknown. Aside from the potential beneficial evolutionary changes implied by host-specificity, specific bacteria may have beneficial effects on the host's health, through the prevention or amelioration of inflammation.

L. johnsonii, *C. symbiosum* and *A. muciniphila* induced comparable degrees of inflammation in the monoassociated TM-IEC C1*galt*1$^{-/-}$ mice, with similar or even lower scores than the ones obtained for the GF TM-IEC C1*galt*1$^{-/-}$ mice. Even though some *Lactobacillus* species, including *L. johnsonii* in specific diseases, have been categorized as "probiotics" for their role as beneficial commensals or as species with putative roles in disease prevention or amelioration (Chiva et al, 2002; Valladares et al, 2010; Lee and Kim, 2011) this is not the case for *A. muciniphila* or *C. symbiosum*.

In humans, *A. muciniphila*, a mucolytic bacterium, is reduced in active UC and CD (Png et al, 2010; Vignaes et al, 2012). This species is unable to colonize well by itself, since the bacterium relies on of symbiotic interactions with other commensals to be able to colonize the host (Png et al, 2010). In monoassociated TM-IEC C1*galt*1$^{-/-}$ mice, *A. muciniphila* appeared to cause mild hyperplasia, a mild increase in the number of CD45+ cells, and increases in almost all the immunoglobulins tested; however, none of

these changes resulted statistically significant relative to the monoassociated WT counterpart. Still, it is possible that these mild qualitative and quantitative changes are indicative of a role of *A. muciniphila* in immune control and as an antagonist of inflammation development, as there is evidence that the bacterium induces the expression of genes involved in regulation of immune responses and maintenance of gut homeostasis (Derrien et al, 2011), and has the ability to decrease endotoxemia (Everard et al, 2013).

Given that *A. muciniphila* was found in low numbers in the colon of both, the WT and the TM-IEC C1*galt*1$^{-/-}$ mice, this raises the question of whether the low histological inflammation score obtained in monoassociation experiments with this species could be attributed to ineffective or transient colonization caused by lack of O-glycans in the mutated mice, or by lack of syntrophic relationships with other members of the microbiota. However, even though *A. muciniphila* was not able to strongly colonize the distal colon, it was found in high counts in the mice's cecum, which is the preferred niche for *A. muciniphila* in the GIT (Derrien et al, 2011). Bi- or multi-association experiments in TM-IEC C1*galt*1$^{-/-}$ mice using other "non-inflammatory" bacterial species would help to determine if this bacterium is able to strongly colonize the colon and act as a "probiotic" with several "non-inflamamtory" bacterial species.

Regarding *Clostridium*, selected *Clostridial* species have been found to prevent disease in several murine models of colitis and other human diseases (Zhang et al, 2009; Hudcovic et al, 2012). However, *C. symbiosum* specifically, which belongs to the Clostridial Cluster XIVa, has not been linked to IBD. Accordingly, we used it in our experiments as a control strain. Contrary to *C. disporicum* (preliminary data in Appendix C), the effect of *C. symbiosum* on tissue inflammation was minimal. Interestingly,

although it caused moderate hyperplasia, *C symbiosum* caused a considerable decrease in the number of infiltrated LP CD45+ cells, which is opposite to what was found for other bacterial strains used for monoassociations. In addition, the commensal significantly induced production of sIgA, which has a role in gut homeostasis and inflammation prevention (Peterson et al, 2007). Both, decreased number of CD45+ cells and increased levels of sIgA, propose an anti-inflammatory role for *C. symbiosum*. The low inflammation scores obtained for *C. symbiosum*-monoassociated mice suggest that the bacterium caused minimum insult to the GIT, if any, and the change in histological scoring observed may have been due to the mutation per se.

4.4.5 B cells responses are species-dependent

The main biological functions of immunoglobulin, produced by B cells as part of the adaptive immune response, are directed towards eradication of invading pathogens. These functions include (i) agglutination of viruses and bacteria (mostly by IgM), (ii) opsonization, (iii) antibody-dependent cellular toxicity (usually IgG), (iv) neutralization (mainly IgG and IgA), (v) protection of the mucosa (IgA), and (vi) complement activation via either the classical pathway (activated by antigen-antibody complexes, mainly IgM and IgG) or the alternative pathway (spontaneously activated on contact with pathogenic cells, mainly by IgG and IgA) (Murphy et al, 2008; Abbas et al, 2010). In regards to IgG subtypes, IgG1 and IgG3 are active in opsonization, the process by which antibodies bind antigens and activate phagocytosis by macrophages, neutrophils or polymorphonuclear leukocytes; while IgG2a, IgG2b and IgG3 act in complement

activation, one of the defense mechanisms of the humoral immune system. All these functions represent different ways by which the immune system eliminates intruding pathogens.

No trends have been found for IgM levels in humans with UC (Philipsen et al, 1995; Dorn et al, 2002). However, humans with UC have large amounts of IgG - being IgG1 the predominat subtype (Kett et al, 1987; Philipsen et al, 1995; Ruthlein et al, 1992; Furrie et al, 2003), - and decreased levels of IgA (Philipsen et al, 1995; Brandtzaeg et al, 2006).

Similar to human UC, in our monoassociation experiments, no general trend was found in the induction of IgM by monoassociated bacteria: while some bacterial species caused increases in IgM levels in the TM-IEC C1*galt*1$^{-/-}$ mice, others caused decreases, relative to the monoassociated WT counterpart. Likewise, the assessment of results obtained for IgG, including subtypes IgG1 and IgG3, revealed no specific trends regarding differences between the genotypes. Interestingly, parallel to human UC, IgG1 was the most abundant subtype in all cases. IgG2b on the other hand, showed an increase in the TM-IEC C1*galt*1$^{-/-}$ mice in all bacterial monoassociations. Though, (concerning IgG2b values) the only case that reached statistical significance was the monoassociation with *B. thetaiotaomicron* (which also obtained the highest inflammation score among the monoassociation treatments). Such an increase does make sense, since one of the functions of IgG2 is complement activation, and dysregulation of the complement cascade has been implicated in the pathogenesis of several inflammatory disorders (Harrison, 2010). Some fragments of the complement cascade (i.e. C3a, C4a and C5a) are inflammatory mediators; thus, this suggests that higher levels of Ig2 could correlate

with higher levels of inflammation. Interestingly, when *B. thetaiotaomicron* and *B sartorii* - *Bacteroides* species with the highest and lowest inflammation scores, respectively- are compared in terms of production of IgG subtypes, the trends are opposites, suggesting a causal relationship between the immune response and disease development.

4.4.6 Secretory IgA protected against development of disease by selected commensal bacteria in core 1 O-glycans deficient mice

B. thetaiotaomicron and *B. vulgatus,* previously reported as inflammation provocateurs in several mice models of colitis, also induced severe inflammation in the TM-IEC C1*galt*1$^{-/-}$ mice. On the contrary, the inflammation scores obtained for *B. sartorii*, *C. symbiosum*, *A. muciniphila* and *L. johnsonii* – monoassociated mice were comparable to those obtained for the GF counterparts; thus these species did not induce inflammation.

Secretory IgA has been characterized as a mediator of gut homeostasis due to its capacity for immune exclusion and anti-inflammatory roles through inhibition of inflammatory intermediaries (i.e. TNFα) (Peterson et al, 2007; Reviewed by Brandtzaeg, 2010). No significant differences were found in the levels of sIgA induced by *B. thetaiotaomicron* and *B. vulgatus* in monoassociated TM-IEC C1*galt*1$^{-/-}$ mice relative to their monoassociated WT couterpart, while the levels of sIgA induced by *B. sartorii*, *C. symbiosum*, *A.muciniphila* and *L johnsonni* were increased in the mutated mice relative to

the WT control. Indeed, results obtained for monoassociations with *B. thetaiotaomicron* and *B. vulgatus* resemble what has been found in human UC, were the levels of sIgA in IBD patients are lower than in controls (Philipsen et al, 1995; Brandtzaeg et al, 2006). Even though only the levels of sIgA in the *C. symbiosum* and *L johnsonii*-monoassociated mice reached statistical significance, results suggest a role of sIgA in prevention of inflammation in the TM-IEC C1*galt*1$^{-/-}$ mice.

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Chapter 5

General Conclusions, Perspectives and Future Directions

5.1 General Conclusions

5.1.1 Disease phenotype is microbe-dependent

We have identified both, potential protective and colitogenic bacteria in the TM-IEC C1*galt*1$^{-/-}$ model of UC, which complement findings from other murine models of IBD, such as the IL-2$^{-/-}$ and the IL-10$^{-/-}$ mice (Balish and Warner, 2002; Waidmann et al, 2003; Muller et al, 2008). While monoassociations with *B. thetaiotaomicron* and *B. vulgatus* induced inflammation in monoassociated TM-IEC C1*galt*1$^{-/-}$ mice relative to the corresponding monoassociated WT mice, the effects of *B. sartorii*, *C. symbiosum*, *A. muciniphila* and *L. johnsonii* were mild, and some even appeared to decrease the changes seen in GF mice. This variation is in parallel agreement with results by Frank et al (2011), who worked in human IBD patients examining the relationships between different IBD genotypes, phenotypes and gut microbial communities, and concluded that IBD phenotypes are associated with changes in the microbiota. The capacity of different bacterial species to either induce or prevent inflammation could be attributed to intrinsic characteristics of each species. The histological scoring and immunoglobulin values found for each tested bacterium suggest that the development and severity of the disease is microbe-dependent.

There is some level of disagreement between previous reports and the results of this investigation in terms of the specific effect of some bacterial species on the inflammatory process. For example, *B. vulgatus* was found to be colitogenic in the TM-IEC C1*galt*1$^{-/-}$ mice, but protective in the IL2$^{-/-}$ mice model. Discrepancies such as this could be attributed to the intrinsic characteristics of each bacterial strain, since strains of

the same species might behave differently, and intrinsic characteristics of each mice strain or animal model used, since the outcomes of experiments will be dependant on the genetic manipulation that the model has. The effects of the different treatments, in this case the bacterial species selected for monoassociations, will be conditional to how they are affected by the absence or presence of the manipulated gene. Additionally, the monoassociation approach, used for these experiments and by other authors in their studies, eliminates competition and other metabolic relationships between microbial symbionts (inherent to a specific pathogen free or conventional microbiota) whose products might interact in complex ways to predispose or to prevent development of inflammation. Therefore, it is expected that the behavior of a single bacterium will be according to the surrounding environment.

Other environmental factors might affect results. For example, the gut microbiota will be directly affected by the diet, specifically the diversity and availability of nutrient sources, since not all microbial species have the same nutritional requirements for growth and development. Additionally, microbes present throughout the housing facility could enter the gut as food "contaminants", thus creating new sources of competition, which might impact the microbiota's composition. Age is also a determinant factor in the composition of the microbial ecosystem, since it is known that the predominant taxon in the GIT of a newborn is different from that of an adult. Finally, differences in experimental treatments are expected to have different effects on the composition of the microbiome.

5.1.2 Breaches in the epithelial barrier (mucus layer) predispose to development of inflammation even in the absence of microbes

Even though comparisons between GF TM-IEC C1*galt*1$^{-/-}$ mice and conventionalized TM-IEC C1*galt*1$^{-/-}$ mice showed that GF mice did not develop inflammation, several factors suggest that inflammation could be evoked in the absence of microbes in the TM-IEC C1*galt*1$^{-/-}$ mice. Firstly, the mice for these experiments were sacrificed 20 days after TM treatment. At this time, GF TM-IEC C1*galt*1$^{-/-}$ mice developed mild hyperplasia but they had a decreased level of CD45+ cells relative to the GF WT mice, thus skewing results towards a low inflammation score. Even though we performed experiments keeping GF mice up to 30 days post TM treatment, we were unable to complete the histological analysis for this experimental group. Consequently, in the context of a known genetic mutation and the presence of an incomplete mucus layer, it is still unknown if a higher degree of hyperplasia and, accordingly, higher inflammation scores would have occurred by more prolonged exposure of the epithelia and mucosal immune cells to environmental factors.

Secondly, we found differences in B cell responses between GF TM-IEC C1*galt*1$^{-/-}$ mice sacrificed 20 days after the onset of the monoassociation experiment (GF20) and those sacrificed at 30 days (GF30). Specifically, there were higher levels of sIgA in the GF30 than in the GF20. This suggests that there is an active, strong, on-going immune response that could be attributed to a defective mucus barrier and which could, eventually, contribute to disease development.

Lastly, we hypothesize that the TM-IEC C1*galt*1$^{-/-}$ mice genetic background can contribute to development of the disease as it has been proved that genetic background greatly influences development of disease in other animal models (Mahler and Leiter, 2002; Buchler et al, 2012; Reingold et al, 2013). The C1*galt*1$^{-/-}$ mice originally produced by Xia's Laboratory (OMRF, Oklahoma) were originated from a 129/SvlmJ mouse and bred into a C57Bl/6J genetic background (Xia et al, 2004). Even though the conventional TM-IEC C1*galt*1$^{-/-}$ mice used by Fu et al (2011) were generated in a C57Bl/6 congenic background, the mice used in our experiments were backcrossed *additional generations* into the C57Bl/6J mice. While Fu's mutated conventional mice spontaneously developed colitis, in our system we found a mild disease phenotype in both, the conventionalized and monoassociated mutant. It is unknown if the *germ-free* TM-IEC C1*galt*1$^{-/-}$ mice derived in a pure 129/SvlmJ genetic background would develop inflammation giving that the disease phenotype was found severe in earlier generations of *conventional* TM-IEC C1*galt*1$^{-/-}$ that were closer to a 129/SvlmJ background rather than a C57Bl/6J genetic background (Fu et al, 2011).

5.1.3 The TM-IEC C1*galt*1$^{-/-}$ mice model of UC confirms the multifactorial etiology of IBD

The circumstances that evoke and perpetuate IBD converge along three main themes: host's genetic susceptibility, gut microbiota and specific environmental factors. The synergistic effect of these factors may induce a strong, persistent immunological

reaction whose final outcome is chronic inflammation in areas of the GIT. Our findings confirm the multifactorial nature of the disease (Figure 5.1).

Firstly, the genetic background of the mice used as an animal model for the study of the human disease affects both, the microbiota and immune responses, predisposing to disease development (Gulati et al, 2012; McKnite et al, 2012). As discussed in the previous section, inflammation was severe in the *conventional* TM-IEC C1*galt*1$^{-/-}$ mice strains that were closer to a 129/SvlmJ genetic background rather than a C57Bl/6J background, while it was mild in the *conventionalized* and *monoassociated* TM-IEC C1*galt*1$^{-/-}$ that was backcrossed multiple generations into a C57Bl/6J background. In addition, genetic manipulations impact results. While WT mice did not develop inflammation in the monoassociation experiments, monoassociated TM-IEC C1*galt*1$^{-/-}$ presented various signs of disease dependent on the introduced bacterial species.

Secondly, absence of microbes prevented development of the disease, confirming the role of the microbiota in the pathological process (Dianda el al, 1997; Reviewed by Podolsky, 1997). GF mice did not develop inflammation in several murine models of UC, including our TM-IEC C1*galt*1$^{-/-}$ system. Indeed, while association with single bacterial species was capable of skewing towards development of inflammation, a complete microbiota (conventionalization of GF mice) was capable of inducing the highest degree of inflammation among the bacterial treatments. However, we also found differences in the ability of our selected bacteria to cause disease in our mice model. Our results shares similarities with work from Stepankova et al (2007), who found that an IBD model of SCID mice reconstituted with CD45RBhigh CD4+ T cells did not develop

inflammation if kept GF, and in which single and defined combinations of bacteria could act either as colitogenic or non-colitogenic in their mice model.

A third source of evidence for the multifactorial nature of the etiology of the disease relates to environmental factors. For example, even though the TM-IEC C1*galt*1$^{-/-}$ mice used by Fu et al (2011) received exactly the same TM treatment as the TM-IEC C1*galt*1$^{-/-}$ mice used in our experiments, our mice presented a mild disease phenotype while Fu's mice spontaneously developed colitis. Aside from the differences in genetic background already addressed, these two sets of mice were also raised under different environmental conditions. Mice used by Fu's team were grown in cages (conventional setting) at the mice facility of the OMRF in Oklahoma, while the mice used for these experiments were bred and raised inside an isolator, *under germ-free conditions* at the Gnotobiotic Mice Facility in UNL. Differences in environmental conditions have an impact on immune system development. In addition, the diet that these two groups received was not the same, nor was it handled in a similar manner (food and water for the GF mice goes through a sterilization process). Indeed, for humans and other animal models the environment has been recognized as a factor predisposing to inflammation and disease (Campbell et al, 2012; Smith et al, 2012)

Finally, even though, in the monoassociation experiments, changes induced by each bacterium in the amount of adaptive immune cells (T or B cell phenotypes) were not evaluated, and measurement of inflammation markers by ELISAs (such as TNFα and IL 6) was not possible (due to low levels in serum), we were able to observe active B cell responses characteristic of each bacterial species. For example, high levels of sIgA (relative to the WT counterpart) were seen for potential protective bacteria, such as *C.*

symbiosum and *L. johnsonii*, while low levels were found for colitogenic bacteria, such as *B. thetaiotaomicron*.

Summarizing, even though dissimilarities exist in the degree of inflammation found in the conventional (Fu et al, 2011), and the conventionalized and monoassociated TM-IEC C1*galt*1$^{-/-}$ mice, results from this study confirm the multifactorial etiology of IBD. Findings suggest that, after its initiation by a breach in the epithelial barrier, the disease progresses through changes in the constitution of the microbiota, and it is perpetuated by the synergistic effect of these two factors in the immunological system. The similarities between the human disease and the results found in our mutant mice model - namely increases in *Lactobacillus* (Willing et al, 2010) and *Clostridium* (Milonaki et al, 2005) and decreases in *Lachnospiraceae* (Frank et al, 2007) - highlight the adequacy of the TM-IEC C1*galt*1$^{-/-}$ mouse model. The control afforded by this model in terms of timing of the introduction of the host's defect (a weakened intestinal mucosal layer), and in terms of the extent of microbial colonization of the GIT of germ-free mice (monoassociation vs. conventionalization) have been instrumental in the elucidation of the complex interplay of factors involved with provoking inflammation in the context of a failed mucus barrier.

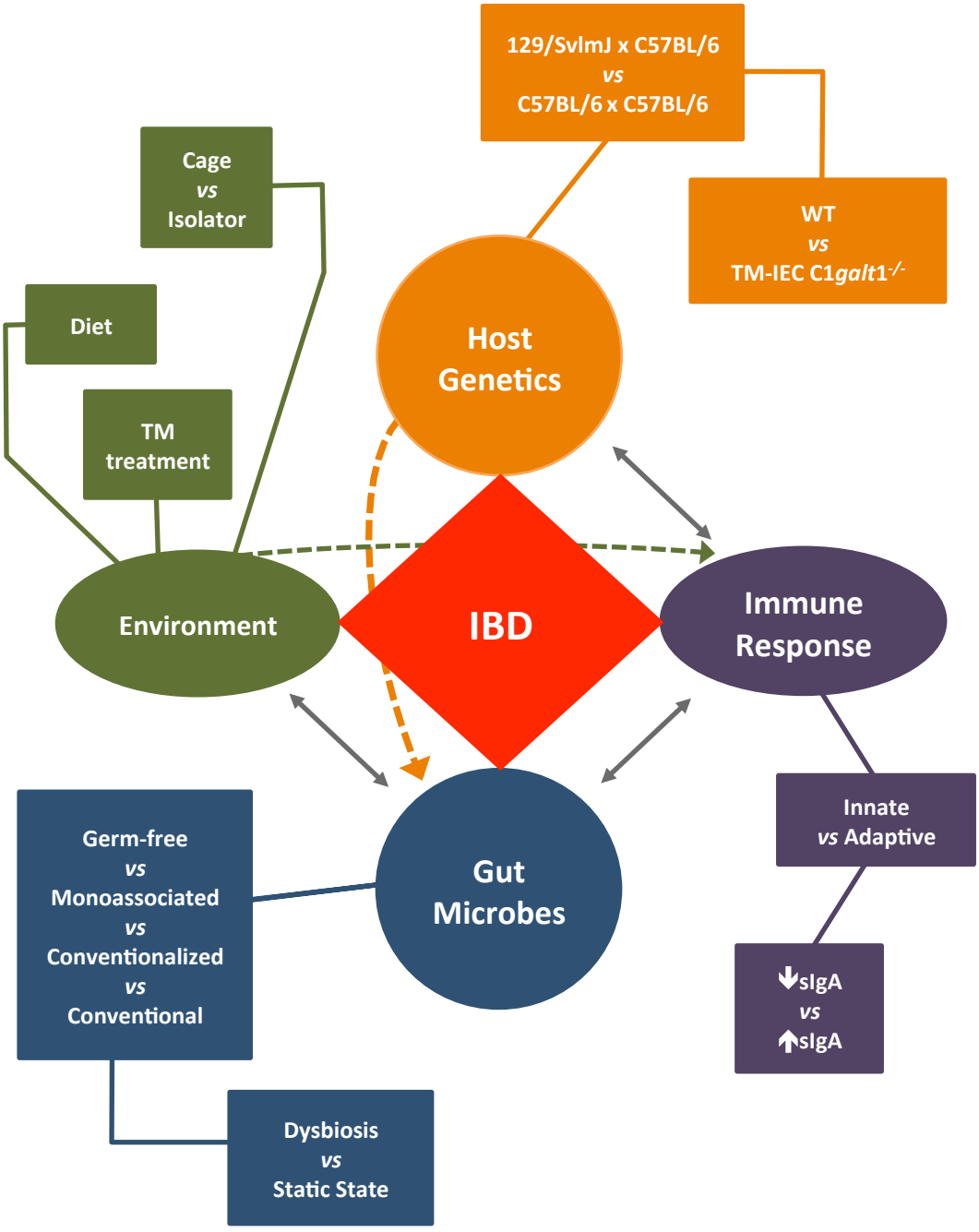

Figure 5.1 Diagram illustrating the multifactorial etiology of IBD in the TM-IEC C1*galt*1$^{-/-}$ mice. Genetic background, environment, immune response and the gut microbiota converge in the development of inflammation and IBD.

5.2 Perspectives and Future Directions

The GIT is the ecological niche of a complex microbial population constituted by over 10^{14} microorganisms, including mostly bacteria. This microbial population increases in density and complexity along the GIT, and it changes from a mostly aerobic composition in the stomach into an anaerobic environment in the ileum and distal colon (Sartor 2008). The microbiota has various physiological functions, and it benefits the host through (i) nutrient degradation, (ii) vitamin production, (iii) production of bactericidal compounds against opportunistic bacteria, (iv) contribution to epithelial cell differentiation, and (v) involvement in immune system maturation. Most importantly, in the context of the present investigation, this microbiota is also one of the components of the intestinal barrier, as it occupies niches that might otherwise be colonized by pathogens.

The commensal microbiota seems to be the most important etiologic factor in the pathogenesis of IBD. This statement is supported by experimental evidence including: (i) that antibiotics improve inflammation in IBD; (ii) that the disease is preferentially localized were the microbial density is at its maximum (terminal ileum and colon); (iii) that mutations in genes related to microbial recognition have been identified in IBD patients; (iv) that epithelial barrier dysfunction predisposes to disease in animal models; and (v), that disease can be prevented in animal models if they are kept germ-free (Reviewed by Guarner, 2005). It is apparent that certain gut bacterial species can either behave as beneficial commensals or become pathogenic, in response to either genetic or

environmental cues. But in spite of all the evidence connecting the gut microbiota to inflammatory processes, the specific factors that determine whether a bacterium remains a beneficial symbiont, or becomes a pathobiont, remain unknown.

The metabolic capacities inherent to each bacterium (for example, the ability of expressing genes depending on the type of nutrients available in the surrounding environment, and the capacity of producing bactericidal compounds to compete for niche establishment) may be considered "broad" or "limited" when compared to other members of the microbiota. These metabolic capacities are what confer a bacterium the ability to colonize and persist in the GIT. In the context of a defined genetic background, this raises the question of whether what may be seen as "ameliorating or protective effects" in a bacterium, may, in fact, represent the reduced pathogenic potential that results from a restricted ability to compete with other members of the microbiota, and with the host's immune system. This question is still unexplored and it is a prospective philosophical starting point to determine whether the metabolic capacities of individual bacterial species are key in either eliciting or preventing inflammation in the presence of an incomplete mucus layer.

Additionally, the monoassociation approach exclusively considers the effect of a single bacterium, eliminating the wide spectrum of possible interactions among competing members of the microbiota. The way in which one bacterium behaves during the process of colonization of a germ-free host might differ significantly from its behavior in a more complex scenario, such as the conventional setting. Assessment of bacterial and host's gene expression in mono-, bi- or multi-association experiments would help in elucidating with more certainty whether or

not specific bacteria elicit or prevent inflammation, and how they do so. A first order objective would be to determine if the "preventive/protective" behavior of a "non-inducer" of inflammation would remain in the presence of other bacterial species, including both, inflammation "inducers" and "non-inducers". Similarly, it would be enlightening to assess whether the effect of a purported inflammation provocateur remains in the context of an increasingly complex gut microbiome.

Furthermore, it is still unexplored how the identified "colitogenic" and "non-colitogenic" bacteria impact the adaptive immune system in regards to T cell phenotypes. Studies have associated ulcerative colitis with a skew towards Th2 cells, while in Crohn's disease there is a skew toward Th1 cells (Niessner and Volk, 1995; Bhan et al, 1999; Dong et al, 2013). It is unclear if the "protective" effect of the bacteria is due to a state of tolerance towards the bacteria, to the intervention of regulatory T cells in maintaining homeostasis, or the bacterium exclusion by specific sIgA. It is also unknown if the "colitogenicity" of the microorganism in the TM-IEC C1galt1$^{-/-}$ mice will skew towards Th2, thus resembling human UC.

5.3 Concluding remarks

The complex pathogenesis of IBD warrants more research. Clearly, specific components of the microbiota play critical roles within the gut, either in prevention or development of inflammation. Accordingly, alterations in the epithelial barrier and the mucus layer that in turn affect the microbiota could either be beneficial or detrimental to the host. Furthermore, the intrinsic and extrinsic factors of the immune system determine the progression of inflammation. Advances in our understanding of mucosal immunology and of the composition, structure and metabolic profile of the gut microbiome will help elucidate the role of each player in the development of inflammation and of appropriate therapies.

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Appendix A

Fisher's Z score

Z test for the equality of two proportions

Objective

To investigate the assumption that proportions π_1 and π_2 of elements from two populations are equal, based on two samples, one from each population.

Methods

It is assumed that the populations have proportions π_1 and π_2 with the same characteristics. Random samples of size n_1 and n_2 are taken and respective proportions p_1 and p_2 calculated. The test statistic is:

$$Z = \frac{(p_1 - p_2)}{\left\{ P(1-P)\left(\frac{1}{n_1} + \frac{1}{n_2}\right)\right\}^{1/2}}$$

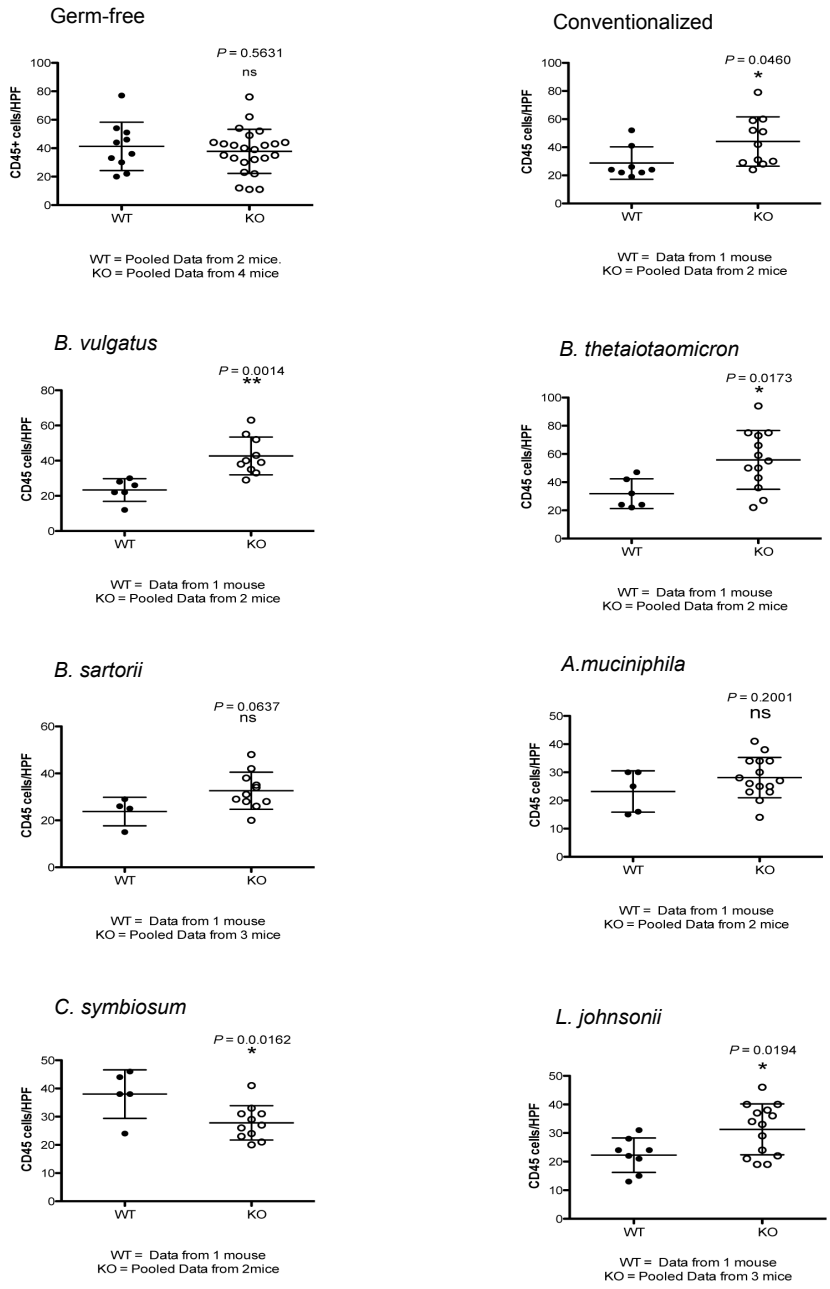
$$\text{where } P = \frac{p_1 n_1 + p_2 n_2}{n_1 + n_2}$$

Under the null hypothesis that $\pi_1 = \pi_2$, Z is approximately distributed as a standard normal deviate.

Source: Kanji, Gopal K. (2006) 100 Statistical Tests. SAGE Publications Ltd, London 3rd edition, p27

Appendix B

CD45+ cells counts per treatment

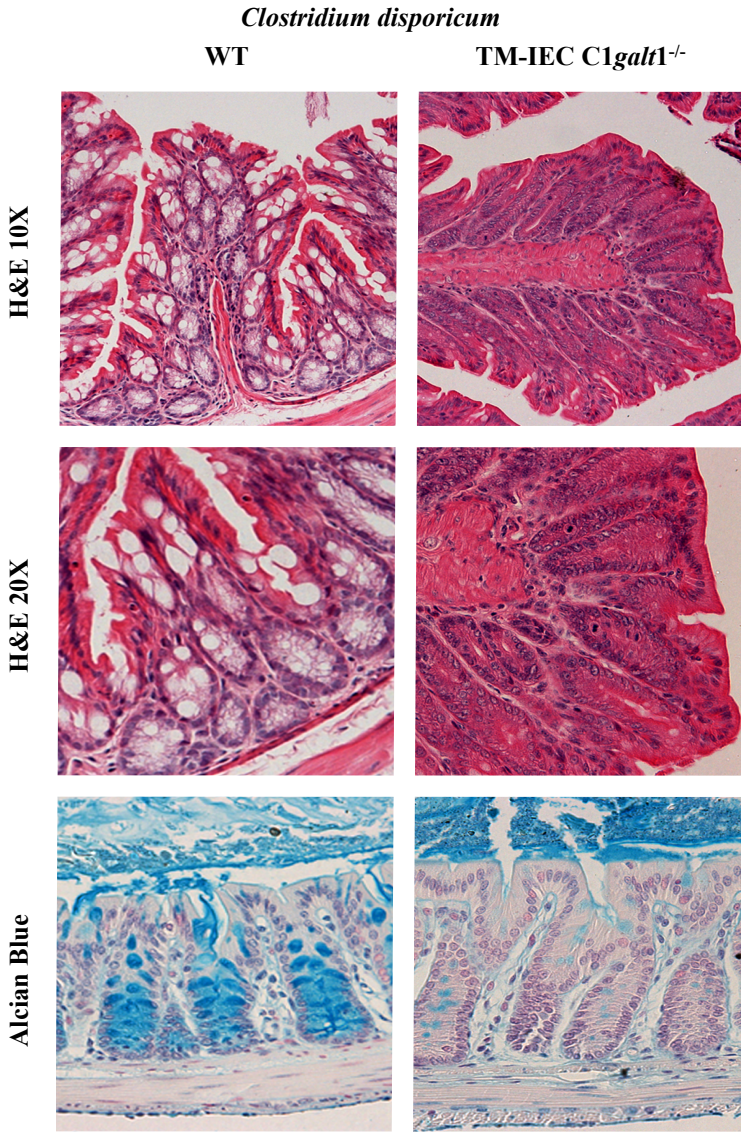


Each data point represents one high-power (40X) image
Error Bars represent Standard Deviations
Statistics represent the results of Unpaired t-tests

Appendix C

Preliminary results for monoassociation with *C. disporicum*

[A] Histology: Monoassociation with *Clostridium disporicum* caused inflammation in the TM-IEC C1*galt*1$^{-/-}$ mice. AB shows good mutation induction.



[B] Comparisons of immunoglobulin levels between germ-free and monoassociated WT and TM-IEC C1*galt*1$^{-/-}$ mice

Germ-free 20d

Immunoglobulin	WT	N	TM-IEC C1*galt*1$^{-/-}$	N	GFWT vs TXWT (p values)	GFKO vs TXKO (p values)	WT vs KO (p values)
IgA (ug/ml)	30.49 ± 1.48	11	50.14 ± 5.43	13	---	---	**0.0040****
sIgA (mg/ml)	167.10 ± 11.15	11	175.70 ± 7.42	13	---	---	0.5283
IgM (ug/ml)	395.6 ± 57.09	11	367.7 ± 41.61	13	---	---	0.6975
IgG (mg/ml)	1658 ± 380.3	11	1273 ± 100.0	13	---	---	0.3483
IgG1 (ug/ml)	667.5 ± 76.03	11	626.6 ± 69.66	13	---	---	0.6960
IgG2a (ug/ml)	4.383 ± 0.77	10	4.685 ± 0.60	13	---	---	0.2075
IgG2b (ug/ml)	162.9 ± 34.43	11	160.7 ± 25.16	13	---	---	0.9591
IgG3 (ug/ml)	146.5 ± 48.71	11	218.0 ± 41.20	13	---	---	0.2759

C. disporicum

Immunoglobulin	WT	N	TM-IEC C1*galt*1$^{-/-}$	N	GFWT vs TXWT (p values)	GFKO vs TXKO (p values)	WT vs KO (p values)
IgA (ug/ml)	32.23 ± 2.43	4	64.10 ± 4.99	4	0.5675	0.0877	**0.0046****
sIgA (mg/ml)	229.10 ± 14.98	4	243.20 ± 19.38	4	**0.0160***	**0.0474***	0.5896
IgM (ug/ml)	239.8 ± 34.82	4	252.1 ± 33.27	4	**0.038***	0.0508	0.8072
IgG (mg/ml)	1250 ± 67.03	4	1238 ± 199.2	4	0.3149	0.8812	0.9578
IgG1 (ug/ml)	720.4 ± 59.88	4	818.4 ± 85.00	4	0.5950	0.1245	0.3894
IgG2a (ug/ml)	3.483 ± 0.54	4	3.051 ± 0.14	4	0.6160	0.1726	0.4924
IgG2b (ug/ml)	79.09 ± 14.99	4	87.69 ± 11.61	4	**0.0455***	**0.0196***	0.6691
IgG3 (ug/ml)	119.2 ± 36.84	4	159.0 ± 47.23	4	0.6638	0.3744	0.5359

Values represent means and standard errors of means (Mean ± SEM). Asterisks (*) indicate significant p values. GF: Germ-free; TX: Monoassociated; WT: Wild type; KO: TM-IEC C1*galt*1$^{-/-}$ mice

[C] Mice average daily weight gain (ADG) and standard deviations (SD) per treatment and per genotype

Treatment	ADG WT ± SD (g)	ADG TM-IEC C1*galt*1$^{-/-}$ ± SD (g)	P-value
C. disporicum	0.13±0.02	0.07±0.03	0.0296*

* Sex distribution could have affected p-values (3 out of 4 mice in the WT group and 2 out of 4 in the KO group were males)