

**Host glycoconjugates in *Salmonella* infection: a
bittersweet symphony**

**A thesis submitted for the degree of
Doctor of Natural Sciences (Dr. rer. nat.)
in the subject of *Microbiology*
by
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April 2020**

**Hannover Medical School
International PhD program “Infection Biology”
in Hannover Biomedical Research School (HBRS)**

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Date of public defense	26.06.2020

PhD project funded by:

- Deutsche Forschungsgemeinschaft (DFG) Collaborative Research Centre (SFB) 900 TP8 (Projektnummer 158989968);
- DFG priority program SPP1656/2;
- The graduate program “Infection Biology” of the Hannover Biomedical Research School (HBRS) and Center for Infection Biology (ZIB).

Table of contents

Abbreviations	6
Abstract	7
1. Introduction	9
1.1 Gut microbiota and the host	9
1.2 Structure and function of the intestinal mucosal barrier	10
1.3 Glycans and protein glycosylation	13
1.3.1 Mucins.....	15
1.3.2 Fucosylation in the gut. The secret(or) of blood sugars.....	18
1.3.3 Proteoglycans and glycosaminoglycans.....	21
1.4 Trick or treat: glycans as mediators of host-pathogen-microbiota interactions	24
1.4.1 Glycan foraging – there's always room for dessert	25
1.4.2 Sticking around: glycan-mediated bacterial adhesion.....	28
1.5 “Sweet but Psycho”. <i>Salmonella</i> infection and host glycans	30
2. Aims of the thesis.....	33
3. Summary of results	34
Study I: Std fimbriae-fucose interaction increases <i>Salmonella</i> -induced intestinal inflammation and prolongs colonization.	34
Study II: Proteoglycan-dependent endo-lysosomal fusion affects intracellular survival of <i>Salmonella</i> Typhimurium in epithelial cells.	37
4. Discussion	41
Part I.	41
Part II.....	45
5. Conclusions and outlook	50
6. List of references.....	51
7. Appendices.....	74
List of publications.....	74

Publication: Std fimbriae-fucose interaction increases <i>Salmonella</i> -induced intestinal inflammation and prolongs colonization.	75
Publication: Proteoglycan-dependent endo-lysosomal fusion affects intracellular survival of <i>Salmonella</i> Typhimurium in epithelial cells.	108
Acknowledgments.....	134
Curriculum vitae.....	135
Declaration	136

Abbreviations

AMPs	antimicrobial peptides
DC	dendritic cell
D-GlcA	D-glucuronic acid
ECs	enterochromaffin cells
GAGs	glycosaminoglycans
GalNAc	N-acetylgalactosamine
GALT	gut associated lymphoid tissue
GIT	gastrointestinal tract
GlcNAc	N-acetylglucosamine
HMOs	human milk oligosaccharides
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IECs	intestinal epithelial cells
IELs	intestinal intraepithelial lymphocytes
L-IdoA	L-iduronic acid
Neu5Ac	N-Acetylneuraminic acid, sialic acid
PGs	proteoglycans
PGM	pig gastric mucins
PIKfyve	FYVE finger-containing phosphoinositide kinase
PRRs	Pattern Recognition Receptors
PtdIns(3,5)P ₂	phosphatidylinositol 3,5-bisphosphate
PtdIns(4,5)P ₂	phosphatidylinositol 4,5-bisphosphate
PULs	Polysaccharide-Utilization Loci
SCFAs	short chain fatty acids
SFBs	segmented filamentous bacteria
SIgA	secretory immunoglobulin A
SNPs	single nucleotide polymorphisms

Abstract

Glycosylation is an enzyme-directed, site-specific, post-translational modification of proteins and lipids. Numerous studies highlighted the roles of glycosylation in various metabolic diseases, cancers, inflammatory diseases, and in susceptibility to infectious diseases. Fucosylation is a type of glycosylation involving an enzymatic addition of fucose (Fuc). It is estimated that approximately 20% of Caucasians are “non-secretors” i.e., have an inactive fucosyltransferase FUT2 – an enzyme facilitating terminal $\alpha(1,2)$ -fucosylation in mucosae. “Non-secretor” individuals do not express ABO and most of the Lewis histo-blood group antigens on epithelial cells of the GIT and on secreted glycoproteins in mucus. Previous studies highlighted an association of *FUT2* nonsense SNPs with an increased or decreased susceptibility to various viral and bacterial pathogens.

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is a pathogenic gram-negative bacterium possessing multiple virulence factors, including type 3 secretion systems, flagella, and fimbriae. Previously, it was shown that the purified core π -class Std fimbriae, encoded in *S. Typhimurium* by the *std* fimbrial operon, can specifically bind terminal Fuc α 1,2 in vitro. Nevertheless, the role of host fucosylation in *Salmonella* pathogenesis and the extent of Std fimbriae production in vivo were not known.

To determine the role of $\alpha(1,2)$ -fucosylated glycans in *Salmonella* infection, in **study I**, we orally infected streptomycin-pretreated *Fut2*^{+/+} and *Fut2*^{-/-} mice with *S. Typhimurium* and analyzed bacterial colonization and intestinal inflammation. We discovered that *S. Typhimurium* colonization and *Salmonella*-induced pathology and inflammation of the murine large intestine is *Fut2*-dependent. In **study I**, we demonstrated that *Salmonella* employs Std fimbriae to adhere to $\alpha(1,2)$ -fucosylated host glycoproteins or glycolipids in the large intestine of *Fut2*^{+/+} mice, as well as in the fucosylated primary epithelial organoid monolayers. We also showed that Std fimbriae are important for *S. Typhimurium* persistence in the gut of secretor *Fut2*^{+/+} mice. We concluded that in the fucosylated environment, *S. Typhimurium* benefits from Std-mediated binding to host Fuc α 1,2 residues which promotes persistence of the pathogen in the murine gut.

Proteoglycans (PGs) are heavily glycosylated proteins found on cytoplasmic membranes of virtually every cell and in extracellular matrices. All PGs consist of the core protein linked to extensively branched glycosaminoglycans (GAGs), such as chondroitin sulfate, heparan sulfate, and heparin. The *XylT2* gene encodes the β -D-xylosyltransferase

enzyme which initiates GAGs assembly in mammals. While PGs are crucial for cell-matrix and cell-cell interactions, previous studies demonstrated important roles of cell surface PGs in bacterial adhesion, invasion, and in immune responses. However, little is known about a role of PGs in *Salmonella* infection.

To investigate the role of host PGs in *S. Typhimurium* infection, in **study II**, we utilized Chinese hamster ovary (CHO) cells, wild-type and xylosyltransferase-deficient ($\Delta XylT2$) mutant lacking PGs. We employed a widely used gentamicin protection assay to assess bacterial adhesion, invasion, and replication in CHO cells. While *S. Typhimurium* adhered to and invaded into CHO WT and CHO $\Delta XylT2$ cells at comparable levels, we observed that 24 hours after infection, proteoglycan-deficient CHO cells were significantly less colonized by *S. Typhimurium*. Notably, we could “rescue” this proteoglycan-dependent phenotype by addition of GAGs (heparin) to the cell culture medium, as well as by complementation of the CHO $\Delta XylT2$ cells with human *XYLT2* gene.

By using chloroquine resistance assay and immunostaining in **study II**, we discovered that, in proteoglycan-deficient cells, bacteria within *Salmonella*-containing vacuoles (SCVs) are more susceptible to endocytosed gentamicin. Moreover, we were able to significantly increase (“rescue”) the *S. Typhimurium* burdens in CHO $\Delta XylT2$ cells by inhibiting the activity of phosphatidylinositol 3-phosphate 5-kinase PIKfyve which orchestrates endo-lysosomal fusion. We also discovered that CHO cells lacking PGs display elevated intra-lysosomal pH levels; this observation confirms an important role of PGs for PIKfyve-dependent vesicle fusion. To conclude, our results demonstrate, for the first time, that cell surface PGs affect survival of intracellular *Salmonella* in epithelial cells via modulation of PIKfyve-dependent endo-lysosomal fusion.

Overall, the findings of our studies presented in this cumulative thesis highlight an importance of host glycosylation and glycoconjugates in *Salmonella* infection and provide a new insight into the mechanisms of glycan-mediated host-pathogen interactions.

1. Introduction

1.1 Gut microbiota and the host

The human gastrointestinal tract (GIT) is heavily colonized by commensal microbes: the colon alone contains 10^{12} bacterial cell per gram representing over 70% of all the microbes in the human body (1). The intestinal microbiota consists of hundreds of species; its composition varies across the length of the GIT, as well as within its width (so-called spatial distribution). For example, *Clostridium*, *Lactobacillus*, and *Enterococcus* spp. were detected only in the mucus layer and epithelial crypts of the murine small intestine, while the proximal and distal segments of the colon had a distinct microbial composition (2). The differences in composition between the mucosa-associated and the fecal microbiota has been identified in several disease conditions as well as in healthy volunteers (3,4).

Commensal bacteria of the human gut provide essential nutrients, metabolize indigestible compounds, and hamper colonization by opportunistic pathogens (5). Furthermore, the bacterial symbionts modulate the host immune system via certain metabolites or pattern recognition receptors (PRRs) (6–8), regulate the multiple metabolic pathways (**Figure 1**), and even affect the behavior of the host (the microbiota-gut-brain axis) (9,10). Dramatic changes in composition and/or in functional configuration of the microbial community (for example, upon antibiotic treatments or due to an infection) can disrupt the homeostasis between the host and the gut microbiota (11). Such state is referred to as dysbiosis – a concept coined by Ilya Metchnikov more than a hundred years ago. Dysbiotic gut microbiota had since been associated with a number of serious diseases, including inflammatory bowel disease (IBD) (12), colon cancer (13), irritable bowel syndrome (IBS) (14), obesity (15,16), and asthma (17).

It should be noted that it is often difficult to establish or prove a causal link between a given condition/phenotype and the composition or function of the gut microbiota. Indeed, the interactions of the gut microbiota and a host are highly complex and reciprocal due to simultaneous contributions of the host genetics (18,19), diet (20,21), lifestyle (22), mode of birth (21,23), and the environment (24,25). Moreover, the diverse species of the gut microbiota interact with each other, cross-feeding or competing for nutrients (26). While a host can be viewed as an ecosystem providing a niche and able to manipulate commensals to its benefit, the “meta-organism” or “holobiont” paradigm considers the host organism and all

its associated microbes, including bacteria, viruses, archaea, and fungi, as one biomolecular network/entity (27,28). Such holistic approach may be also applied in a context of infection, to study host-pathogen-microbiota interactions.

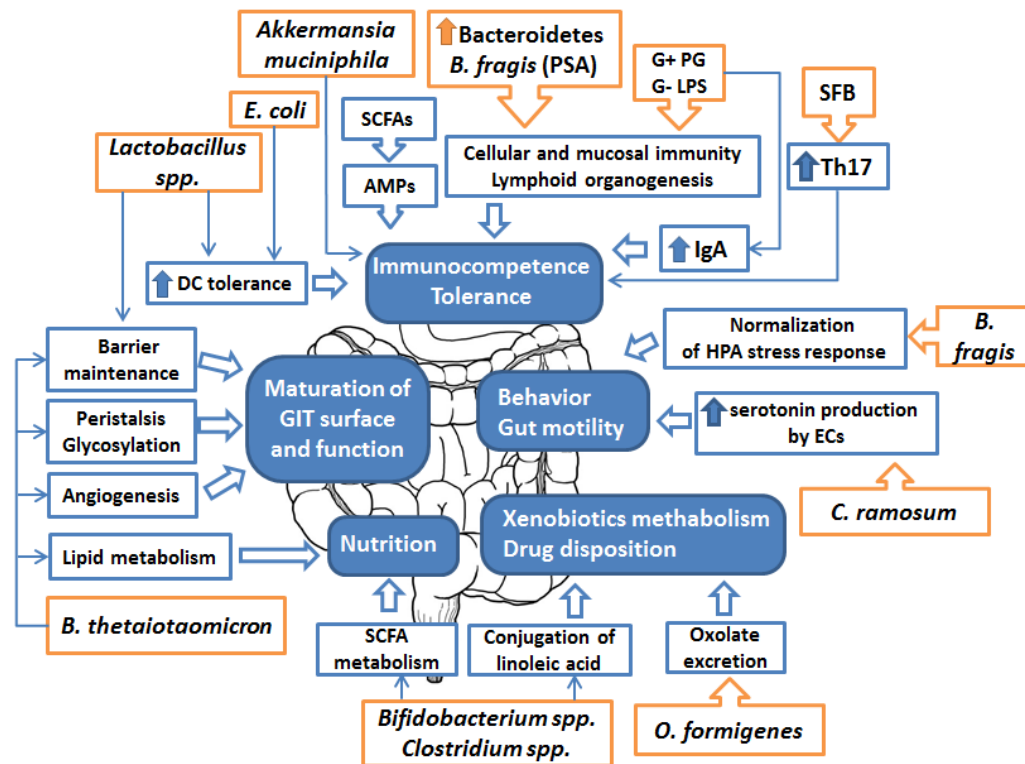


Figure 1. Gut microbiota alter host physiology. The affected host phenotypes are presented in the blue ellipses. AMPs, antimicrobial peptides; DC, dendritic cells; G+/G-, gram-positive/-negative bacteria; HPA, hypothalamus-pituitary-adrenal; PG, peptidoglycan; PSA, polysaccharide A; SCFAs, short chain fatty acids; SFB, segmented filamentous bacteria; ECs, enterochromaffin cells. Own work, adapted from (1,7,29,30).

1.2 Structure and function of the intestinal mucosal barrier

The epithelial surface of the human GIT comprises different cell types, namely, absorptive cells (enterocytes and colonocytes), stem cells, mucus-producing goblet cells, Paneth cells which produce antimicrobials, intestinal intra-epithelial lymphocytes (IELs), and enteroendocrine cells (**Figure 2**). Intestinal epithelial cells (IECs), interconnected with tight junctions and overlaid with mucus, constitute the mucosal barrier limiting bacterial invasion and, ultimately, prevent an access to the systemic circulation (31). Compromised gut barrier may lead to bacterial translocation with serious pathological consequences, such as autoimmunity (32), infection and inflammation (reviewed in 33). Increased paracellular permeability (a condition referred to as “leaky gut”) due to a combination of genetic predisposition and environmental factors also causes inflammation and loss of antigen tolerance which may trigger the onset of IBD (34).

The mucosal barrier contains a plethora of glycoconjugates including high molecular weight mucins, forming the mucus gel, and membrane-associated glycoproteins and glycolipids, forming the glycocalyx (described in details in the **Chapter 1.3**). The hydrated mucus gel covers glycocalyx, overlays the feces, and acts as a selective molecular filter, which entraps particles and bacteria (35). The thickness of the mucus gel varies throughout the GIT, being thickest in the colon (e.g, approximately 0.7 mm in rats) (**Figure 2**) (36). Accordingly, the thickness, composition, and glycosylation of the glycocalyx depend on the anatomical location and on a cell type (37,38). It was estimated that the inner mucus layer in the distal colon of mice is being continuously replaced at the rate of 50 μm / hour by secretion from goblet cells (39). The conversion from the inner colonic mucus layer to the loosely attached outer mucus layer is facilitated by endogenous proteases (39). The constant renovation of the glycocalyx and mucus is crucial for maintaining barrier properties of the gut mucosa.

The inner mucus layer of the large intestine is firmly attached to the epithelium and impenetrable to most bacteria, while the expanded outer mucus layer provides a niche for the microbiota (40,41). In contrast, the mucus layer in the small intestine is penetrable, but bacteria do not normally colonize the epithelium due a high concentration of AMPs (e.g.,

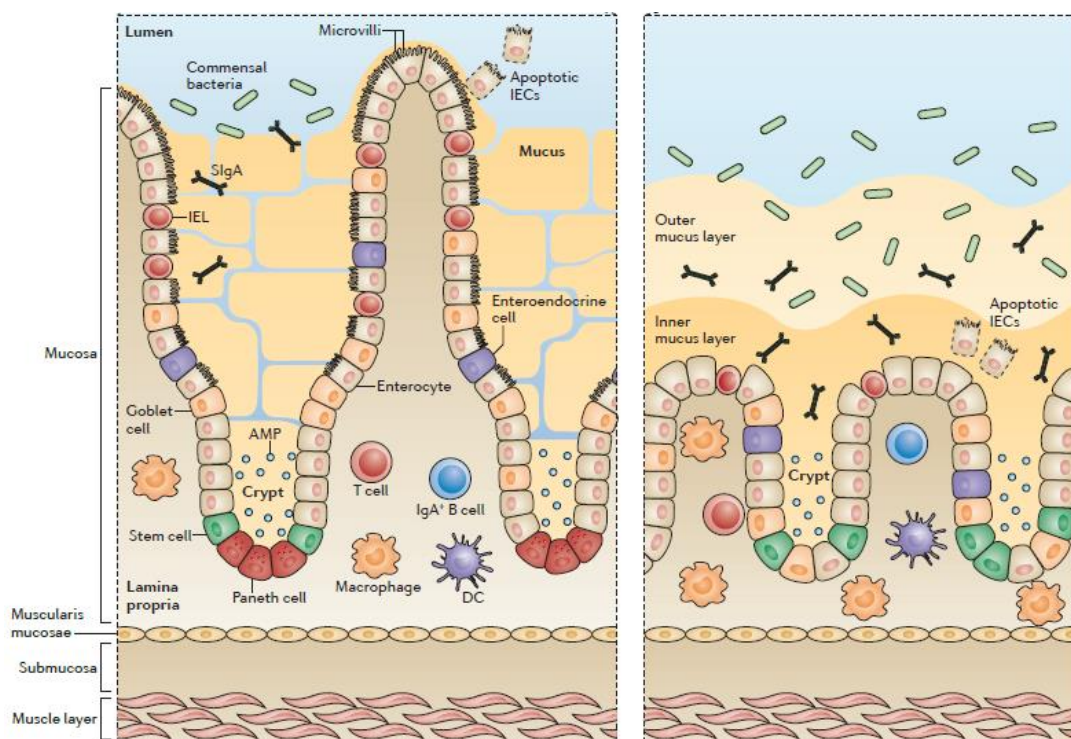


Figure 2. The intestinal mucosae of jejunum (on the left) and colon (right panel) have a distinct structure. IECs, intestinal epithelial cells; AMPs, antimicrobial peptides; IELs, intestinal intraepithelial lymphocytes; DC, dendritic cell; SIgA, secretory immunoglobulin A. Reproduced from (42) with permission from the publisher.

lysozyme and secretory phospholipase A2) (43). The notable exception is segmented filamentous bacteria (SFB) or “*Candidatus Arthromitus*” – clostridia-related, spore-forming anaerobes commonly detected in the gut of vertebrates, including humans, mice, and chickens (44). SFB tightly adhere to absorptive epithelial cells and to Peyer’s patches in the ileum. The SFB abundance in the gut has been associated with reduced colonization and growth of pathogenic bacteria, including *Salmonella* (45), enteropathogenic *E. coli* (46), and *Citrobacter rodentium* (7), in rats, rabbits, and mice respectively. The recent study (47) demonstrated that SFB-colonized mice had accelerated epithelial cell turnover and were protected against rotavirus-induced diarrhea and infection.

Indeed, the gut microbiota can modulate post-natal development and function of the intestinal mucosal barrier. For example, the important role of Paneth cells and indigenous microbiota in the development of the intestinal villus microvasculature was demonstrated by Stappenbeck et al. (48). Mice colonized with a probiotic strain *Escherichia coli* Nissle 1917 were less susceptible to dextran sodium sulfate-induced colitis and displayed reduced colonic epithelial permeability (49). Notably, IECs isolated from these mice exhibited a more pronounced expression of Zonula occludens-1 (ZO-1) protein (49) – the scaffold protein which cross-links and anchors the tight junctions to the actin cytoskeleton (50). More recent data indicated that the indigenous gut microbiota affects barrier properties of colon mucus in C57BL/6 mice (51).

The second line of defense against bacteria (commensal or pathogenic) that breached the mucosal barrier includes the liver (52) and the mesenteric lymph nodes (mLNs) (53). An enhanced microbial translocation was observed in burn victims (54), patients with HIV (55), and in rats subjected to hemorrhagic shock (56), indicating an effect of the systemic stress and immunity on the intestinal barrier function. A contributions of the different host factors to the gut barrier integrity was demonstrated in the different models of murine colitis: mice deficient in either Muc2 mucins, core 1 O-glycans, Toll-like receptors 5 (Tlr5), or interleukin 10 (IL-10), production had bacteria in a close proximity to the colonic epithelium (57).

Gut associated lymphoid tissue (GALT) and the lamina propria are crucial for the immune response in the gut. In the lamina propria, the myeloid-derived dendritic cells (DCs) act as antigen-presenting cells (APCs): DCs collect luminal antigens via long dendrites projected between the epithelial cells (58). In the small intestine, antigens are also taken up and transported to the APCs by the microfold cells (M cells) overlying the lymphoid follicles of the Peyer's patches and isolated lymphoid follicles (59). Furthermore, the epithelial and

leukocyte cells of the mucosal barrier express pattern recognition receptors (PRRs) and secrete various protective and antibacterial proteins, such as mucins, antibodies, lysozyme, defensins, and galectins (42). Many of these proteins are glycosylated and the role of such glycosylation in mucosal defense is not fully understood.

Secretory IgA (SIgA) is one of the key elements of the mucosal immunity able to modulate the gut microbiota and to keep pathogens at bay (60). Upon activation (e.g., by DCs) and class switching, newly generated IgA-producing B-cells leave the GALT and home to the intestinal lamina propria via the blood (61). About 3-5 g of dimeric IgA in humans, or 40-60 mg/kg in mice, is being secreted per day under steady-state conditions (62). Notably, SIgA was detected in the mucus, co-localized with the gut bacteria (63). Indeed, preferential coating of the fecal microbiota with SIgA (64), as well as its tolerance to the commensal bacteria (65), had been shown previously. Interactions of SIgA with the microbiota are complex: monoclonal SIgA displayed cross-species reactivity (66) and can bind antigens in a canonical Fab-dependent and non-canonical glycan-dependent manner (67). Furthermore, SIgA can block bacterial toxins (e.g., cholera toxin (68) and toxin A of *Clostridium difficile* (69)) and directly interfere with an adherence of the pathogen (murine reovirus) to the intestinal epithelium (70). Most importantly, SIgA is able to limit access of pathogens to the epithelium in a stepwise process called “immune exclusion” (71).

1.3 Glycans and protein glycosylation

Glycoconjugates, or glycans, are complex biopolymers comprised of different sugar residues, such as N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), fucose (Fuc), sialic acid (NeuAc), mannose, glucose, and xylose (72). Glycans are essential for protein maturation and turnover, for cell differentiation, adhesion and trafficking, and for receptor binding and activation of signal transduction pathways (73). Because protein glycosylation is involved in most physiological processes and diseases, its dysfunction has been linked with a development of various infectious and chronic diseases, including cancer and severe inflammation (reviewed in 74,75). The inherited defects of nucleotide-sugar biosynthesis, lack of glycosyltransferases or dysfunction of vesicular transport lead to the congenital disorders of glycosylation (CDG); most types of CDG are associated with neurological impairments (76).

Glycosylation, the attachment of sugar moieties, is an intricate and tightly regulated (77) post-translational modification involving the integrated action of multiple proteins, such as glycosylation enzymes, sugar nucleotide transporters, and trafficking proteins. It was

estimated that more than half of all proteins and many lipids in a human body are glycosylated (78). Glycosylation takes place in the endoplasmic reticulum (ER) and the Golgi complex: specific receptors in the trans-Golgi network bind the sugar moieties of soluble proteins and facilitate their transport to different cell organelles or to the cell membrane (Figure 3).

Glycosylation drastically increases the diversity of the proteome due to highly variable, non-templated activity of specific enzymes which determines a glycosidic linkage, glycan composition, structure, and length. Glycosylation activity varies between the different cell type and intracellular compartments (79). Glycosyltransferases are enzymes that independently transfer certain mono- or oligosaccharides from a donor (a sugar nucleotide or dolichol) to growing oligosaccharide chains or proteins (80). Glycan processing in the ER and Golgi also requires glycosidases, enzymes that catalyze the hydrolysis of glycosidic bonds resulting in removal of sugars moieties. Each glycosidase cleaves a particular sugar (e.g., galactosidase). Importantly, glycosylation enzymes act in a step-wise manner, i.e., the completion of one enzyme reaction triggers the next one (81).

To date, five different types of protein glycosylation were described. In the N-linked glycoproteins, the oligosaccharide is attached to the nitrogen atom of the lateral chain of asparagine in the ER, while the O-linked glycosylation implies an attachment to the hydroxyl group of the lateral chain of serine or threonine in the ER, Golgi, cytosol and nucleus. Glypiation is a special case of glycosylation of surface glycoproteins in eukaryotes and some

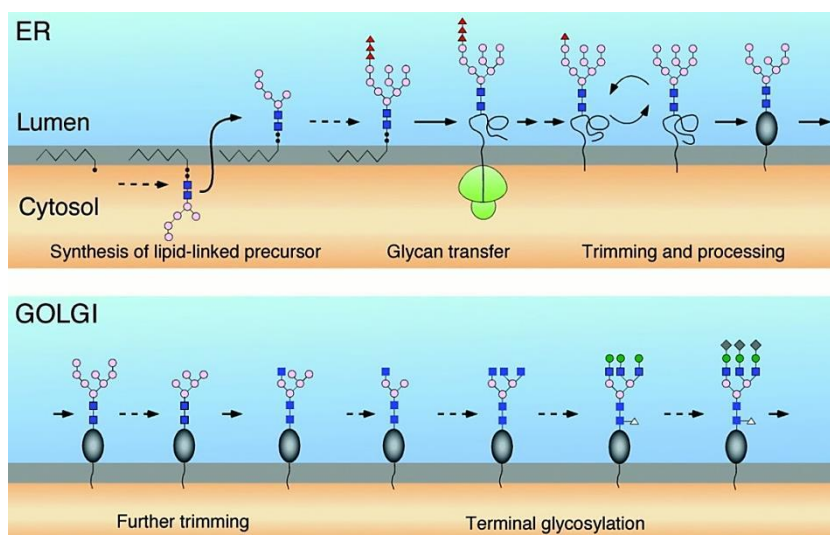


Figure 3. Biosynthesis of the N-linked oligosaccharide in a nutshell. A lipid precursor with attached sugars is translocated across the cytosol into the lumen of the endoplasmic reticulum (ER). Then, the core oligosaccharide attaches to the nascent polypeptide, which is then folded (depicted as grey oval). Further modifications of the glycoprotein occur in the Golgi complex, including an addition of GlcNAc, GalNAc, galactose, fucose, and/or sialic acid to the oligosaccharide branches. Reproduced from (82) with permission from AAAS.

archaea that involves the covalent attachment of a glycosylphosphatidylinositol (GPI) anchor (83). Other unusual examples of glycosylation include C-mannosylation which produces carbon-carbon bonds by attaching mannose to the indole ring of tryptophan (84) and phosphoglycosylation (linking of glycans to serine or threonine via phosphodiester bonds) which is limited to parasites and slime molds (85).

Glycosylation of proteins (N-linked or O-linked) generates extensively branched structures of glycans, which overcomes the limitations of the linear polypeptide chains (82). While up to 90% of proteins are N-glycosylated, O-glycosylation is crucial for the biosynthesis of mucins, proteoglycan core proteins, and some antibodies. “Mucin-type” and other mammalian O-linked glycans will be reviewed in the following subchapter. Glycoproteins may undergo a number of other modifications, such as methylation, phosphorylation, sulfation, O-acetylation, and addition of GlcNAc, mannose, or fucose (74). A role of fucosylated proteins and lipids in the intestine will be described in the **Chapter 1.3.2**. Proteoglycans – heavily glycosylated proteins – are discussed in the **Chapter 1.3.3**.

1.3.1 Mucins

Mucins are a family of GalNAc-based glycoproteins located at the mucosal surface of the GIT. The molecular structure of mucins determines their functions: the secreted O-glycosylated mucins of high molecular weight and size, such as MUC2, MUC5AC, MUC6, and MUC19, have an ability to form viscoelastic gels. Membrane-associated mucins (i.e., MUC1, MUC3, MUC4, MUC12, MUC13, and MUC17) do not form gels, but form the glycocalyx attached to the membrane of epithelial cells (86).

Glycocalyx covers the apical cell membrane of enterocytes, the major cell type in the intestinal epithelium. Interestingly, a tissue-specific expression of the transmembrane mucins was reported: MUC3 was detected throughout the whole intestine, while MUC12 was mainly found in the colon (87), and MUC17 – in the small intestine (88). The membrane-associated mucins are very long monomers containing more than 4000 amino acids and extending for up to 1 μm into the gut lumen – further than any other membrane proteins (35). A recent study of Sun et al. (89) demonstrated the micrometer-long mucin filaments emerging from the tips of microvilli and forming an intricate three-dimensional columnar network (**Figure 4**). Thus, the glycocalyx protects the cell membrane of enterocytes by establishing a glycan-rich barrier.

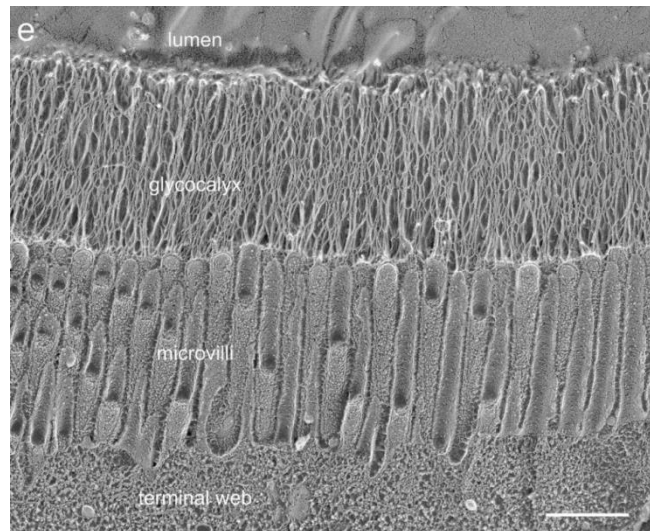


Figure 4. An electron micrograph shows the apical region of an enterocyte of the murine small intestine. Tuft-like glycocalyx separates the brush border microvilli from the intestinal lumen. Scale bar, 500 nm. Adopted from (89) under a Creative Commons Attribution 4.0 International License (CC BY 4.0).

Both membrane-bound and secreted mucins have a protein core (200–500 kDa) arranged into distinct regions (**Figure 5A**). Most MUC genes contain unique variable number tandem repeat (VNTR) sequences, encoding a central glycosylated region, rich in serine, threonine, and proline (STP repeats). The less O-glycosylated regions with a high proportion of cysteine are located at the N and C termini, or sometimes they are interspersed between the STP repeats (90). C-terminal Cystine Knot and von Willebrand factor (vWF)-type domains are found in cysteine-rich regions and involved in dimerization of secreted mucins and subsequent polymerization of the dimers via disulfide bond formation (**Figure 5B-C**).

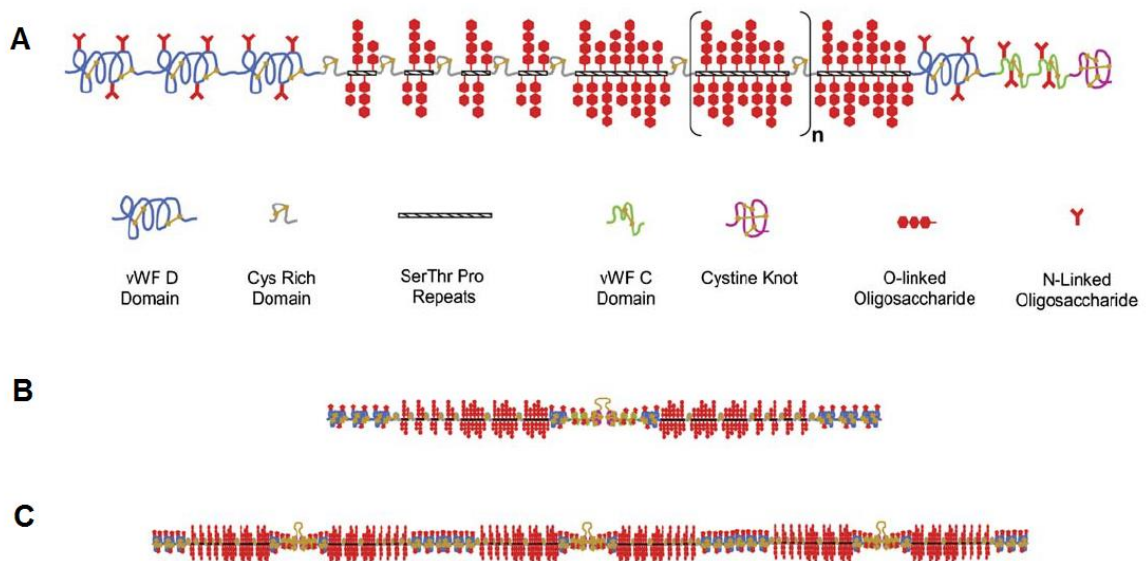


Figure 5. Schematic representation of the pig gastric mucin's structure. Mucin monomer (**A**) contains heavily O-glycosylated regions. Dimers (**B**) and multimers (**C**) are formed via disulfide bonds in the non-glycosylated regions giving rise to the high molecular weight of secretory mucins. Reproduced from (91) with permission from the publisher.

The oligosaccharide content and structures of mucins vary according to cell type, tissue location and developmental stage. The carbohydrate chain of mucins typically contains a GalNAc covalently attached to the Ser or Thr residue, eight different core structures, the backbone region produced by elongation units, and various terminal residues (**Table 1**). For example, in humans, core 4 structures were discovered in the highly fucosylated mucins of the small intestine, core 2 glycans have been found in the distal colon, while core 3 structures were widely distributed in the gut (92). Notably, many mucins, e.g., membrane-associated MUC1, MUC4 and MUC16, include regions rich in N-glycosylation sites (93). Bell *et al.* (94) demonstrated that two N-glycans located near the first and second cysteines of the knot, are crucial for dimer formation of the secreted MUC2 mucin.

Terminal structures of glycan chains present in mucins (secreted and membrane-associated) include Fuc, Gal, GalNAc or Neu5Ac residues forming histo-blood group antigens, such as ABH and Lewis antigens (**Table 1, Figure 6**). The ABH antigens are expressed in most epithelial tissues, on membrane glycoproteins and glycolipids, as well as soluble, secreted forms of these glycans (reviewed in 95). Histo-blood group antigens act as

Table 1. Common O-linked oligosaccharide structures found in mucins. Adapted from (96).

Nomenclature	Structure
Core type	
Core 1	-Gal β 1-3GalNAc α 1-Ser/Thr
Core 2	-Gal β 1-3(-GlcNAc β 1-6)GalNAc α 1-Ser/Thr
Core 3	-GlcNAc β 1-3GalNAc α 1-Ser/Thr
Core 4	-GlcNAc β 1-3(-GlcNAc β 1-6)GalNAc α 1-Ser/Thr
LacNAc elongation units	
Type 1 chain	-Gal β 1-3GlcNAc β 1-
Type 2 chain	-Gal β 1-4GlcNAc β 1-
Branching	
i-antigen	-Gal β 1-3GlcNAc β 1-3Gal β 1- (unbranched)
I-antigen	-Gal β 1-3GlcNAc β 1-3(-Gal β 1-3GlcNAc β 1-6)Gal β 1- (branched)
Terminal structures	
Blood group H	Fuc α 1-2Gal β 1-
Blood group A	Fuc α 1-2(-GalNAc α 1-3)Gal β 1-
Blood group B	Fuc α 1-2(-Gal α 1-3)Gal β 1-
Type 1 chain-based	
Lewis ^a	Gal β 1-3(-Fuc α 1-4)GlcNAc β 1-
Lewis ^b	Fuc α 1-2Gal β 1-3(-Fuc α 1-4)GlcNAc β 1-
Sialyl-Lewis ^a	Neu5Ac α 2-3Gal β 1-3(-Fuc α 1-4)GlcNAc β 1-
Type 2 chain-based	
Lewis ^x	Gal β 1-4(-Fuc α 1-3)GlcNAc β 1-
Lewis ^y	Fuc α 1-2Gal β 1-4(-Fuc α 1-3)GlcNAc β 1-
Sialyl-Lewis ^x	Neu5Ac α 2-3Gal β 1-4(-Fuc α 1-3)GlcNAc β 1-
Sulfation	
3-Sulfation	HSO ₃ -3Gal β 1-
6-Sulfation	HSO ₃ -6GlcNAc β 1-
Combined epitopes	
H-1 antigen	Fuc α 1-2Gal β 1-3GlcNAc β 1-
Sialylated type-2 chain	Neu5Ac α 2-3-Gal β 1-4GlcNAc β 1-

ligands for the cell adhesion molecules (selectin, galectin families) and for bacterial adhesins (discussed in the **Chapters 1.3.2 and 1.4**).

HMOs (human milk oligosaccharides) are a family of diverse unconjugated oligosaccharides structurally related to mucin glycans which are present in the abundance in human milk (5-15 g/L), as well as in the gut of breastfed babies (97). Recent studies suggested that HMOs can modulate epithelial and immune cell responses (e.g., mucosal leukocyte recruitment) and contribute to brain development by providing nutrients (reviewed in 98). HMOs are composed of the five basic monosaccharides: glucose, galactose, GlcNAc, fucose and sialic acid (Neu5Ac); notably, 50-80% of HMOs are fucosylated (98).

1.3.2 Fucosylation in the gut. The secret(or) of blood sugars.

Fucosylation is a type of glycosylation involving an enzymatic addition of fucose residues to a molecule. Fucosyltransferases transfer fucose from GDP-Fuc to Gal in an $\alpha(1,2)$ -linkage and to GlcNAc in $\alpha(1,3)$ -, $\alpha(1,4)$ -, or $\alpha(1,6)$ -linkages (99). In humans, the *FUT* gene family comprises 13 different genes encoding fucosyltransferases displaying a complex tissue- and cell type-specific pattern of an expression (100). Alpha-2-fucosyltransferases (encoded in mammals by *Fut1*, *Fut2*, and *Sec1* genes) are enzymes required for the biosynthesis of the terminal glycan motif Fuc α 2-Gal β -R (see **Table 1, Figure 8B-C**). Human *FUT1* (H) and *FUT2* (Secretor) genes are responsible for synthesis of ABH and Lewis histo-blood groups (**Figure 6**).

Upon glycosylation, $\alpha(1,2)$ -fucosylated proteins and lipids (101) can be directly secreted into the gut lumen or can be anchored to the apical side of the mucosal epithelium (102). Indeed, polyfucosylated (mostly Fuc α 1,2) N-linked tri- and tetra-antennary glycopeptides with blood group determinants were found in abundance in human small intestinal epithelial cells (103). In individuals with the functional α -2-fucosyltransferase FUT2 (so-called “secretor” phenotype, Se), ABH blood group antigens (analogous to those found on a surface of erythrocytes) are expressed on epithelial cells of higher respiratory tract, lower genito-urinary tract, on gastrointestinal epithelia and on mucins (**Table 1**) (104). For example, “secretors” produce Lewis^b and H type 1 antigens (mirroring blood group O); H antigen structure can be further decorated by another glycosyltransferases with GalNAc- or Gal-residues thus creating A or B antigens (**Figure 6A-B**).

In contrast, “non-secretors” (Se⁰) subjects are homozygous for the FUT2-inactivating nonsense mutation and, thus, express only the Lewis^a antigen (with *FUT3*-dependent

Fuc α 1,4) instead of Lewis^b and ABH antigens (105) (**Figure 6A-B**). Moreover, the “weak-secretor” phenotype (Se^w) which is characterized by an expression of both Lewis^a and Lewis^b antigens (**Figure 6C-D**) has been identified in Taiwanese, Polynesians, Japanese, and Australian aborigines (106). Se^w phenotype is the result of a weak (mutated) form of the α -2-fucosyltransferase (107). To conclude, a secretor status is determined by a concurrent action of two different enzymes, Secretor (FUT2) and Lewis (FUT3). Of note, Serpa et al. reported that only nine out of 47 Lewis-negative (i.e., FUT3-negative) individuals lacked the expression of Lewis antigens in gastric mucosa (Le^a-, Le^b-) indicating a possible contribution of another α -1,3/4 fucosyltransferase to Lewis antigens biosynthesis in the mucosa (108).

Naturally occurring polymorphisms of *FUT1* and *FUT2* genes (due to fully or partially inactivating mutations) have significant epidemiological and functional consequences, highlighting the importance of α (1,2)-fucosylated glycans. Lack of both functional *FUT1* and *FUT2* alleles results in the very rare Bombay blood group (109) characterized by an absence of ABH antigens on erythrocytes (regardless of the blood group genotype) and in secretions (110). Individuals with the Para-Bombay phenotype are also FUT1-negative, but do express the *FUT2*-encoded α (1,2)-fucosyltransferase, and thus “secretors” (111).

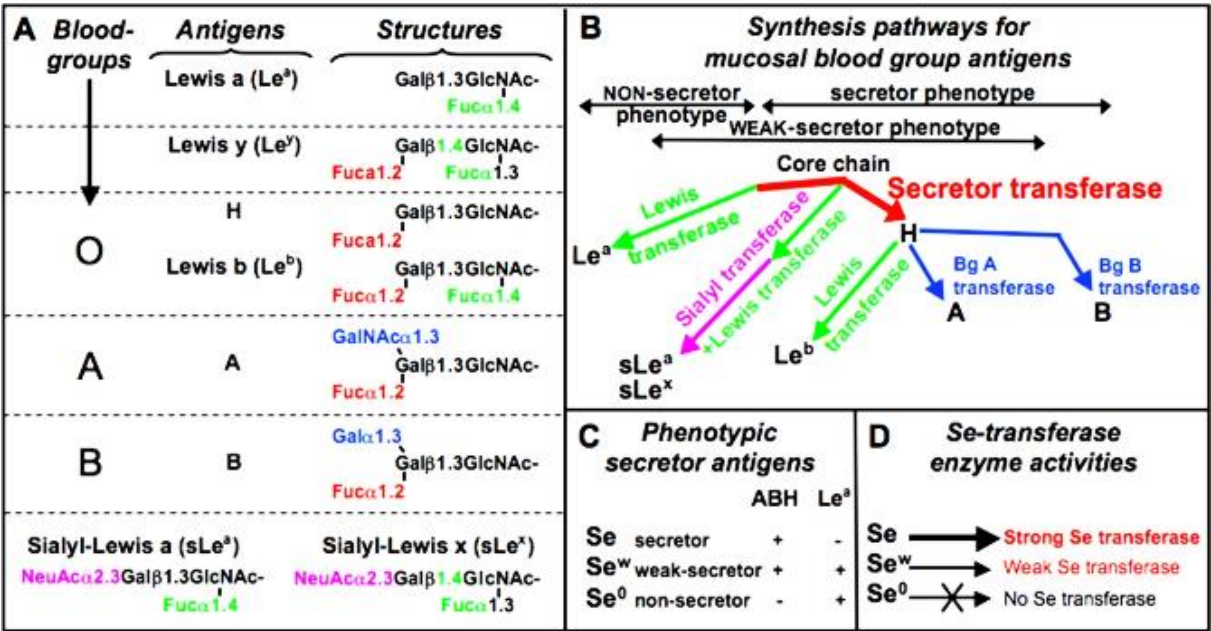


Figure 6. Fucosylated blood group antigens and associated secretor phenotypes. **(A)** The α (1,2)-fucosylated (in red) H type 1 and Lewis^b antigens correspond to blood group O. Group A and B antigens have additional GalNAc or Gal residues (blue), respectively. Sialylated Lewis antigens (depicted in pink) are up-regulated during inflammation and infection. **(B)** Biosynthesis pathways for blood group antigens with corresponding Se phenotypes. **(C)** Humans of Se, Se⁰, or Se^w phenotype are characterized by the presence/absence of ABH and Lewis^a antigens in salivary, milk, and GI tract secretions. **(D)** An activity of α 1.2fucosyltransferase defines the secretor status. Reproduced from (112) under CC0 1.0 Universal Public Domain Dedication (no copyright).

FUT2 gene polymorphism is more common: it is estimated that approximately one-fifth of the global population are “non-secretors” unable to express $\alpha(1,2)$ -fucosylated glycans on mucosa (102). *FUT2* gene, located on Chr19q13.33, has length of 9,980 bp, and it is composed of two exons separated by a 6,865-bp intron. Two functional alleles of *FUT2* are commonly found in many populations: one is a so-called reference allele (Se), and the other is the Se³⁵⁷ with a 357 C→T synonymous SNP (113). Nevertheless, more than 20 different single nucleotide polymorphisms (SNPs) in *FUT2* conferring the “non-secretor” phenotype were described (114).

Similarly to the population-specific Se^w phenotype, the frequency of Se⁰ varies in different ethnic groups. For example, in populations of Caucasian, African, and central Asian descent, the most common nonsense SNP is a G→A substitution at base pair 428 (rs601338) (105). In contrast, Se³⁸⁵, an A→T substitution at base pair 385 (rs1047781), is the most frequent cause of the “non-secretor” phenotype in east Asians (107). Notably, within their respective populations, both aforementioned SNPs are distributed at similar frequencies ($\approx 20\%$) (113). It seems that, at least in Eurasian and African populations, many *FUT2* variants had a long history of (probably pathogen-driven) balancing selection ensuring both Se and Se⁰ had an advantage (115). It is estimated that the Se⁴²⁸ mutation is at least 1.87 million years old (116). Fast spreading of the most recent, H8 haplotype (Se³⁸⁵), which appeared in East Asia about 256,000 years ago, might reflect a positive selection (113). To conclude, the world-wide distribution of Se⁰ phenotypes may be a result of on-going selective pressure providing an advantage for “non-secretor” in the certain cases.

Possible associations between the secretor status and susceptibility to infections and diseases have been extensively studied. For instance, a recent study claimed that *FUT2* SNPs rs601338 (Se⁴²⁸) and rs602662 were associated with a higher risk of diarrhea and respiratory illnesses in infants, while a longer period of breastfeeding reduced risk of diarrhea, independent of *FUT2* genotype (117). Furthermore, “secretors” were shown to be more susceptible to norovirus, rotavirus, HIV, *Helicobacter pylori*, and graft-versus-host disease, while “non-secretors” are more prone to *Candida albicans*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenza* infections, as well as to gastric cancer, primary sclerosing cholangitis, and Crohn’s disease (reviewed in 102).

Some of the pathogens mentioned above can directly bind and adhere to $\alpha(1,2)$ -fucose residues (discussed in the **Chapter 1.4.2**), which explains the observed differences in colonization. Interestingly, while *Fut2* gene expression affected the gut community assembly

in mice (118), no strong correlation between a secretor status and the composition of the human gut microbiota was detected in a twin cohort study (119). Finally, it should be noted, that “non-secretors” had increased levels of sialylated glycans (incl. Sialyl-Lewis^x) expressed on MUC5B isolated from saliva, probably, due to reduced competition between glycosyltransferases (120). Enhanced sialylation may mask or aggravate Se⁰-dependent phenotypes.

1.3.3 Proteoglycans and glycosaminoglycans

Proteoglycans (PGs) are heavily glycosylated proteins involved in the cell-matrix and cell-cell interactions. Importance of PGs for bacterial adhesion/invasion and for immune responses has been previously demonstrated in a number of studies (reviewed in 121). All PGs consist of a core protein which is covalently linked to glycosaminoglycans (GAGs) chains. GAGs are long linear polysaccharides comprised of repeating disaccharide units of the amino sugar (D-GlcNAc or D-GalNAc) and an uronic acid, either D-glucuronic (D-GlcA) or L-iduronic acid (L-IdoA), or a galactose. Based on the structure of the disaccharide unit, GAGs are divided into four distinct families: heparan sulfate/heparin, chondroitin/dermatan sulfate, keratan sulfate, and hyaluronan (**Table 2**).

Biosynthesis of the first two classes of GAGs is initiated by assembly of the common tetrasaccharide linker (D-GlcA- β 1-3-Gal- β 1-3-Gal- β 1-4-Xyl- β -Ser) on a core protein. In humans, two β -D-xylosyltransferase enzymes, encoded by the *XYLT1* and *XYLT2* genes, facilitate the synthesis of a linker by transferring UDP-xylose residues to the serine moieties of a protein (122). A type of a linkage within the repeating units defines GAG structure: the β -glycosidic bond at D-GalNAc results in a synthesis of chondroitin sulfate, while the α -glycosidic bond characterizes a heparan sulfate.

Table 2. Types of GAGs and respective PGs. Adapted from (123,124).

Glycosaminoglycan	Structure of the repeating unit	Relevant PGs
Chondroitin-sulfate (CS)	-4)- β -D-GlcA-(1 $\rightarrow$ 3)- β -D-GalNAc4[6](OSO ₃)-(1-	Phosphacan, versican, biglycan.
Dermatan sulfate (DS)	-4)- α -L-IdoA-(1 $\rightarrow$ 3)- β -D-GalNAc4(OSO ₃)-(1-	Decorin, epiphykan.
Heparan sulfate (HS)	-4)- β -D-GlcA-(1 $\rightarrow$ 4)- α -D-GlcNAc-(1-	Syndecan 1-4, glypican 1-6, testican 1-3, agrin.
Heparin	-4)- α -L-IdoA2(OSO ₃)-(1 $\rightarrow$ 4)- α -D-GlcNSO ₃ -6(OSO ₃)-	Serglycin (intracellular PG!)
Keratan sulfate (KS)	-3)- β -D-Gal-(1 $\rightarrow$ 4)- β -D-GlcNAc6(OSO ₃)-(1-	Lumican, keratocan, fibromodulin.
Hyaluronan (HA)	-4)- β -D-GlcA-(1 $\rightarrow$ 3)- β -D-GlcNAc-(1-	* not linked to a protein core.

Heparan sulfate proteoglycans (HSPGs) are predominantly found on the cell surface: either in the pericellular matrix, or intimately associated with the plasma membranes via a transmembrane domain (syndecans 1–4, CD44v3, and betaglycan) or via a GPI anchor (glypicans 1–6) (**Figure 7**). Few HSPGs, namely, collagen type XVIII, agrin, and perlecan, are secreted into the extracellular matrix (125). Notably, serglycin (mostly CS- and heparin-based PG) is the only truly intracellular PG which was originally discovered in the secretory granules of endothelial, endocrine, and hematopoietic cells (126).

A main function of HSPGs localized at the basement membranes is to anchor cells (e.g., epithelial cells to lamina propria) by interacting with each other and with other constituents of the membrane, such as laminins, integrins, collagen type IV, and nidogen (127). Cell surface and pericellular HSPGs also act as receptors for proteases and protease inhibitors, bind cytokines and chemokines (128). Moreover, HSPGs participate in a formation of growth factor gradients, and, thus, are crucial in processes of embryogenesis, regeneration, and neural differentiation (reviewed in 129). Recently, a role of HSPGs in the intracellular events was acknowledged. It was shown that syndecan-2 enters the endosomal compartment and interacts with phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) – a phospholipid component of the intracellular membranes(130).

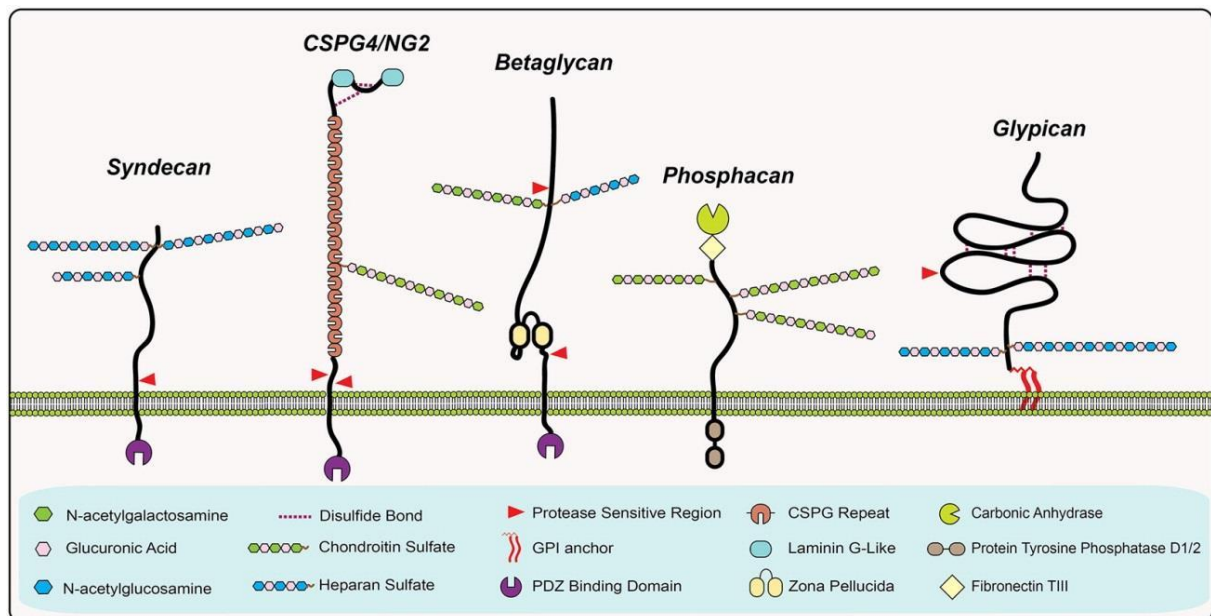


Figure 7. Schematic representation of the cell surface PGs, which include transmembrane type I (the N-terminus is outside of the plasma membrane) PGs (four syndecans, CSPG4/NG2, betaglycan and phosphacan) and six GPI-anchored proteoglycans, glypicans 1–6. The type of GAG chain and the major protease-sensitive sites are indicated. Reproduced from (121) under the Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND) license.

Chondroitin- and dermatan sulfate-containing proteoglycans (CSPGs and DSPGs, respectively) are typically found in cartilages, intervertebral discs, tendons, corneas and in the brain. For example, phosphacan (**Figure 7**) is a CSPG which was originally isolated from rat brain; it was demonstrated that phosphacan can directly interact with neurons and neural cell-adhesion molecules (131). Overall, CSPGs and DSPGs contribute to viscoelastic, osmotic, and structural properties of the extracellular matrix (ECM). The small leucine-rich proteoglycans (SLRPs) belong to the largest family of PGs structurally characterized by a protein core containing multiple leucine-rich repeats and covalently linked to CS or DS GAG chains. SLRPs (i.e., biglycan, epiphykan, podocan, decorin, lumican, osteoadherin) act both as structural constituent of ECM and as signaling molecules (reviewed in 132).

Hyaluronan- and lectin-binding proteoglycans (hyalectans) represent another distinct family including four different PGs, namely aggrecan, versican, neurocan, and brevican (121). Hyalactans share the unique tri-domain structure: a hyaluronan-binding N-terminal domain, a central domain containing the GAG side chains, and a C-terminal domain that binds lectins (133). Similarly to phosphacan, versican together with brevican were shown to be a major component of the ECM in mature brain (134); versican is also produced by stromal cells and leukocytes. An eponym “versican” highlights versatile functionality of this PG which acts as a ligand for many receptors on the surface of immune cells (e.g., CD44, P-selectins, and TLRs), notably, versican expression is increased during inflammation (135).

Keratan sulfate-based proteoglycans (KSPGs) are abundant in the cornea, cartilages, intervertebral discs, and in the neural tissues (136). Due to high water retention ability, KSPGs in joints act as a cushion absorbing mechanical shock. KS GAGs were originally discovered in the cornea and identified as a linear polymer composed of GlcNAc, galactose, and sulfate (137). The repeating disaccharide units of KS (**Table 2**) can be sulfated at C6 position of either or both the monosaccharides, thus creating the regions of high sulfation, mono-sulfation, and no-sulfation on KS chains (138). Unlike HSPGs and CSPGs, KSPGs are formed by an attachment of KS GAGs to a protein core via N-linked asparagine, or O-linked serine/threonine or mannose residues. Many SLRPs, such as fibromodulin, lumican, and keratocan, as well as hyalactans (aggrecan and versican) bear small N-linked KS-I chains (136). Importantly, the KS chains can be heavily fucosylated and/or sialylated (139).

Finally, hyaluronan (HA) is a unique type of GAG which is not covalently attached to any PG and has no chain modifications (123). The HA polymerase enzymes, HA synthases, are inserted into the plasma membrane and release HA chains in the extracellular space (140).

HA synthases have two enzymatic components adding the monosaccharides (GlcA and GlcNAc) at the reducing end of the HA chain. In mammals, three phylogenetically conserved HA synthases have been found; interestingly, a number of bacterial spp. also possess HA synthases and can produce HA (141). Within ECM, HA is usually associated with proteins and/or PGs (with aggrecan, for instance) forming the extracellular HA-rich regions. A number of studies highlighted a role of such HA-rich matrices in skin inflammation and barrier function (reviewed in 142), in morphogenesis and fertility (143), and in the tumor microenvironment (144).

1.4 Trick or treat: glycans as mediators of host-pathogen-microbiota interactions

As discussed in the previous chapters, the GIT epithelium expresses a repertoire of glycoconjugates of different “flavors”. Mucosal glycoconjugates represent sensitive markers of intestinal and gastric epithelial cell differentiation (145). The overall GIT glycosylation pattern and structures of glycoconjugates are genetically pre-determined for each given individual by genes encoding specific, carbohydrate-active enzymes (e.g., glycosidases, glycosyltransferases, glycoside hydrolases). The degree and nature of mucosal glycosylation affects GIT colonization by the gut microbiota (146), which, in turn, influences mucosal protection, digestion, and gut homeostasis. Moreover, by comparing the glycosylation patterns of germ-free (GF) and conventional mice, it has been demonstrated that the gut microbiota affect the cellular and the subcellular distribution of glycans (147).

Indeed, certain members of the gut microbiota can initiate a production of host glycoconjugates *in vivo* (148) or alter the state of glycosylation of the epithelial cells in the intestine (149). While alterations in mucin glycosylation have been associated with a number of human diseases, such as colonic cancer, ulcerative colitis and Crohn’s disease (86,150), a role of particular commensals/pathobionts in a development of the aforementioned conditions remains elusive. Finally, the diet which can shape gut microbiota and change the mucosal architecture and the mucus composition (151) is adding an additional layer of complexity to glycan-host-microbiota crosstalk.

The carbohydrate moieties of mucosal glycans can serve as microbial attachment sites and/or as nutrients for the commensal microbes (152). The following two subchapters will explore these two functions of host sugars.

1.4.1 Glycan foraging – there's always room for dessert

Previous studies demonstrated that several members of the human gut microbiota can degrade complex carbohydrates provided by the host and thus occupy a niche (153). So-called “mucin-degrading specialists” spp. include *Akkermansia muciniphila*, *Bifidobacterium bifidum*, *Bacteroides thetaiotaomicron*, *Ruminococcus gnavus*, and *Ruminococcus torques*, among others (154) (**Table 3**). Notably, a degree of glycan-degrading specialization can be substantial. For instance, Crociani et al. screened 290 strains of 29 *Bifidobacterium* spp. isolated from humans and animals for the ability to metabolize various glycan substrates: it was found that porcine gastric mucin was utilized exclusively by *B. bifidum*, *B. infantis* was the only species to degrade D-glucuronic acid; strains of *B. longum* fermented arabinogalactan and gum arabic; while α -L-fucose was a substrate for strains of *B. breve*, *B. infantis* and *B. pseudocatenulatum* (155).

Comparative genomic analysis of the human gut microbiome revealed that 86% of the analyzed genomes had genes responsible for cleavage of mucin glycans; 89% genomes – for the catabolism of derived monosaccharides; 81% of the genomes had the predicted ability to degrade at least one type of mucins (156). Interestingly, it was also suggested that 82% of the analyzed commensals could potentially form a mutualistic relationship and degrade glycans which are not degradable by any of the paired members alone (156). Commensal bacteria may also indirectly assist in colonization by other species by stimulating a production of the specific host glycoconjugates. For instance, it was demonstrated that SFB induce an expression of fucosyl asialo GM1 glycolipids on epithelium in the small intestine of BALB/c mice (157).

To thrive on mucosal glycoconjugates, commensals employ multiple enzymes cleaving either sialic acids (Neu5Ac and Neu5Gc), Fuc, Gal, GlcNAc, or GalNAc (158). Structurally-related, catalytic and carbohydrate-binding modules of enzymes that degrade, modify, or create glycosidic bonds are divided into families and described in the curated CAZy database (159). According to the CAZy classification, glycan-degrading enzymes of commensals represent different glycosyl hydrolase (GH) families. In the bacterial genomes, the corresponding genes are organized into polysaccharide-utilizing loci (PULs, see **Table 3**). To date, 1146 PUL-containing genomes belonging almost exclusively to the Bacteroidetes phylum were identified (<http://www.cazy.org/PULDB/>) (160).

Table 3. Mucus degradation and utilization by the members of the gut microbiota and by pathogens. PULs, Polysaccharide-Utilization Loci; PGM, pig gastric mucin; HMOs, human milk oligosaccharides. Adapted and modified from (161,162).

Microbe	Substrates tested	Characterized enzymes/PULs	Enzymatic activity	Reference
Human fecal microbiota	PGM		Sialidases, β -N-acetylglucosaminidase, β -galactosidase.	(163)
<i>Akkermansia muciniphila</i>	PGM, human MUC2. PGM type III		α/β -galactosidase, α/β -fucosidase, α/β -N-acetylglucosaminidase, β -glucosidase, α -mannosidase, β -N-acetylglucosaminidase. Sulfatase and glycosidase activity.	(164–166)
<i>Bacillus indicus</i> and <i>Bacillus firmus</i>	PGM		Several candidate deacetylases, N-acetyl β -glucosaminidase.	(167)
<i>Bacteroides fragilis</i>	PGM, pig colon mucin	4 PULs are described in literature, 56 predicted PULs; <i>sgu</i> gene cluster.	α/β -galactosidase, glucose-6-sulfatase, α/β -N-acetylglucosaminidase, β -D-galactose-3/6-sulfatase, N-acetylneuraminidase, α -fucosidase. Sialidase.	(168,169) (170)
<i>Bacteroides thetaiotaomicron</i>	HMOs, PGM	96 PULs described in literature	α -fucosidase, β -galactosidase, α/β -N-acetylglucosaminidase, glycosulphatase, neuraminidase, xylosidase/arabinosidase.	(171–173)
<i>Bifidobacterium bifidum</i>	PGM, human MUC2, HMOs	AfcA AfcB AgaBb AgnB	1,2- α -L-fucosidase; 1,3-1,4- α -L-fucosidase; α 1,3-galactosidase; α -N-acetylglucosaminidase (specific to GlcNAc α 1-4Gal structure on O-glycans); Putative α -N-acetylglucosaminidase.	(174) (175) (176)
<i>Bifidobacterium longum</i> subsp. <i>infantis</i> <i>Prevotella</i> spp.	Human intestinal mucin, HMOs PGM	Lacto-N-biose phosphorylase (LNBP) enzyme 20 predicted PULs, MdsA (mucin-sulfatase) Sulfoglycosidase SGL	β -galactosidases specific for lacto-N-tetraose, lactose (Lac) and type-2 HMOs. α/β -galactosidase, glucose-6-sulfatase, α/β -N-acetylglucosaminidase, β -D-galactose-3/6-sulfatase, α -fucosidase, N-acetylneuraminidase. Cleaves terminal 2-acetamido-2-deoxy- β -D-glucopyranoside 6-sulfate.	(177) (178,179) (180)
<i>Ruminococcus</i> AB strain VJ-268	Blood group B salivary glycoproteins		Blood group B-degrading α -galactosidase activity. Degradation is not complete due to an absence of β -N-acetylhexosaminidases.	(181) (182)
<i>Ruminococcus torques</i> strains IX-70 and VIII-239	PGM		Blood group A- and H-degrading α -glycosidase, β -glycosidase and sialidase activities.	(182)
<i>Clostridium perfringens</i>	PGM, blood group B glycoprotein	<i>eabC</i> (Endo- β -galactosidase)	EabC liberated the A trisaccharide, GalNAc α 1-3(Fuc α 1-2)Gal, and B trisaccharide, Gal α 1-3(Fuc α 1-2)Gal, from the blood group A and B glycotopes.	(183)
Enterococcal <i>Escherichia coli</i> (EAEC), <i>Shigella flexneri</i>	Bovine submaxillary mucin; mouse cecal mucus	Mucin serine protease	Promoted growth in cecal mucus and induced its secretion.	(184,185)
Enterohemorrhagic <i>Escherichia coli</i> (EHEC)	MUC 7; glycoprotein 340	StcE metalloprotease	StcE cleaves MUC7 and gp340, assists an adhesion.	(186,187)
<i>Vibrio cholerae</i>	PGM, salivary mucin and goblet cell surface mucin.	HapA and TagA mucin proteases	HapA is assisting in mucus gel penetration. TagA cleaves glycoproteins of mucin.	(188,189)

The degradation of mucosal O-glycans usually starts from the terminal non-reducing end where bacterial neuraminidases/sialidases (eg., of GH family 33) cleave the α 3- and α 6-linked sialic acids, allowing further degradation (190) (**Figure 8B**). The genes encoding sialidases and genes needed for sialic acid metabolism are often arranged in one cluster (*nan* clusters); such clusters were found in 46 bacterial species including 33 pathogens and 9 gut commensals (191). It is generally accepted that commensals are mostly involved in the cleavage of host glycans, while GIT-infecting pathogens utilize monosaccharides released by commensals (reviewed in 190,192). Moreover, considering that SIgA is heavily N- and O-glycosylated (193) and it is very abundant in the gut, some members of microbiota may also degrade SIgA-linked glycans (67). The commensal and pathogenic bacteria which are able to degrade mucin sugars, as well as their relevant enzymes, are presented in the **Table 3**.

Bacteroides thetaiotaomicron and *Bifidobacterium bifidum* are well-studied human gut symbionts known to thrive on dietary (e.g., HMOs, **Figure 8C**) and endogenous glycans (mucins, **Figure 8B**) by employing highly specific genes (**Table 3**, **Figure 8D**). It was estimated that approximately 60% of the GH-encoding enzymes identified in *B. bifidum* PRL2010 are involved in the degradation of mucin-derived oligosaccharides (194). Notably, it was shown that *B. thetaiotaomicron* is able to induce fucosylation of the intestinal glycocalyx

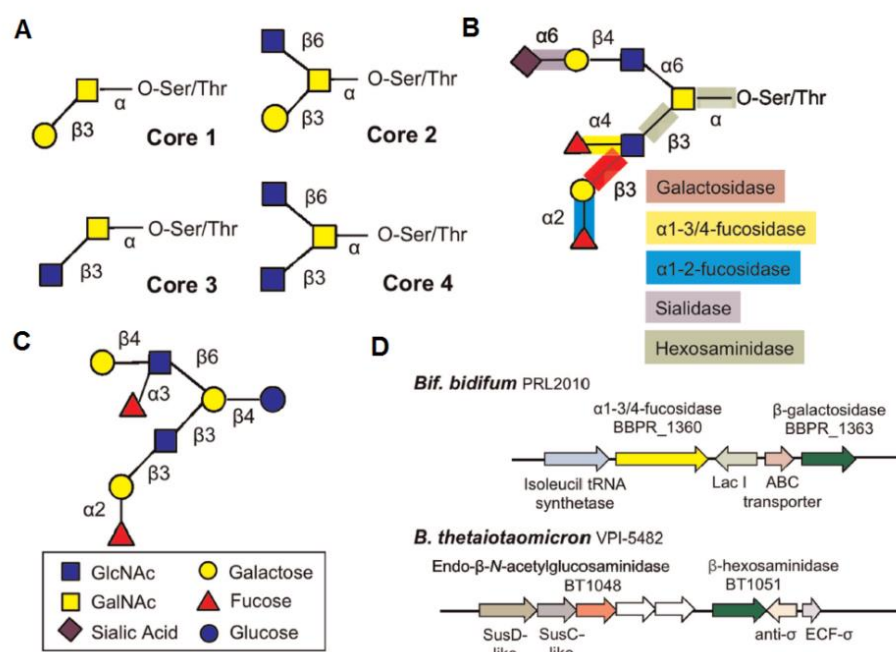


Figure 8. Intestinal mucin glycans and HMOs feed the commensals. **(A)** The predominant core-1/2/3/4 structures of gut mucin glycans (see also **Table 1**). **(B)** An example of a mucin O-glycan with the extended core-4 structure isolated from the ileum (92) and the set of GHs required to cleave monosaccharides off the mucin to make them available for fermentation. **(C)** An example of branched HMO built upon a lactose core; note the similarity to the mucin glycan structure. **(D)** Putative operons of *Bifidobacterium bifidum* and *Bacteroides thetaiotaomicron* that encode multiple GHs. Reproduced from (195) with permission from the publisher.

(148), moreover, this bacterium utilizes FucR, a molecular sensor of L-fucose availability, which regulates expression of *fucRIAK* operon encoding enzymes of fucose metabolism (196). These findings indicate that “mucin-degrading specialists” can also create a suitable physiological niche by modulating the structure of gut glycans.

In the pioneering studies of Hoskins and colleagues, it was proposed that the bacteria able to degrade complex glycans are adapted to a certain energy source (i.e., ABH blood group sugars) provided by a specific host (163,197). Interestingly, in the in vitro study employing a continuous-flow culture of anaerobic isolates from the gut microbiota, an addition of mucins to bacterial cultures significantly increased production of cell-bound and extracellular glycosidases, implying a regulatory effect of host glycoproteins on bacterial growth and physiology (198).

1.4.2 Sticking around: glycan-mediated bacterial adhesion.

Significantly increased susceptibility to gram-negative enteric pathogens among individuals with blood groups B and AB was reported 50 years ago (199). Since then, various pathogenic and commensal bacteria were shown to directly bind glycosylated receptors expressed in the gut glycocalyx and on mucins using highly-specialized adhesins (**Table 4**). These adhesins are often fimbriae or pili – thin proteinaceous appendages expressed on a surface of many gram-positive and gram-negative bacteria (**Figure 9**). Apart from adhesion to biotic and abiotic surfaces, pili/fimbriae are also involved in biofilm formation, bacterial conjugation, phage transduction, and motility (reviewed in 200).

Fimbriae/pili are important virulence factors of pathogens because they assist in initial attachment of bacteria to host tissues (201). In gram-negative bacteria, the best characterized are type I, type IV fimbriae and curli pili (**Figure 9**). Type 1 fimbriae are assembled via chaperone-usher pathway including non-covalent polymerization of fimbrial subunits into nascent rod structure (202). Many Proteobacteria, including human pathogens belonging to the *Salmonella*, *Yersinia*, *Pseudomonas*, *Klebsiella*, and *Haemophilus* genera use type 1 fimbriae to mediate attachment (reviewed in 203). The canonical example of such interaction is FimH-mediated adherence of *Escherichia coli* to mannosylated receptors expressed on epithelial cells (204,205). Recent studies highlighted roles of pili in the pathogenesis of all three main streptococcal pathogens: group A and group B streptococci (201) and *Streptococcus pneumoniae* (**Table 4**).

Table 4. Glycan-specific adhesion of the selected commensals and pathogens. Adapted from (206).

Bacterium	Specific carbohydrate receptor of a host	Bacterial adhesin	Reference
<i>Bacteroides fragilis</i>	Neu5Ac	NanU protein	(207)
<i>Bacteroides thetaiotaomicron</i>	N-acetyllactosamine	BT1043, an outer membrane lipoprotein	(208)
<i>Lactobacillus reuteri</i>	Unknown components of PGM and hen intestinal mucus.	mucus-binding protein MUB	(209)
<i>Lactobacillus rhamnosus</i> GG	Terminal β -galactosyl residues of intestinal glycoconjugates	SpaC sub-unit of SpaCBA pilus	(210)
<i>Bifidobacterium bifidum</i>	N- and O-linked glycoproteins	sortase-dependent pili	(211)
<i>Escherichia coli</i>	D-Mannose	FimH, type 1 fimbriae	(204)
<i>Escherichia coli</i> O157:H7 (EHEC)	Fibronectin, laminin, collagen IV.	Long polar fimbriae (Lpf)	(212)
Enterotoxigenic <i>E. coli</i> (ETEC)	Gal, Neu5Ac	FY and F41 pili	(213)
	GalNAca1-3(Fuca1,2)Gal β , blood group A Lewis ^a antigen.	EtpA adhesin	(214)
		CFA/I fimbriae	(215)
<i>Clostridium difficile</i>	Gal α (1,3) Gal β (1,4)GlcNAc	Toxin A	(216)
<i>Fusobacterium nucleatum</i>	GalNAc, Gal	Lectin-like adhesin	(217)
<i>Helicobacter pylori</i>	Neu5Aca(2,3)Gal β (1,4), part of Sialyl-Lewis ^x	SabA adhesin	(218,219)
	Fuca1,2 of the Lewis ^b and H-1 blood group antigens.	BabA adhesin	(220)
<i>Pseudomonas aeruginosa</i>	D-Gal	Lectin PA-IL	(221)
	Lewis ^a antigen	Lectin PA-III	(222)
<i>Streptococcus pneumoniae</i>	Maltose/cellobiose, Gal α / β and blood group A and H antigens.	Pilus-1 proteins: RrgA and RrgC	(223)
	Mannose	RrgB	
<i>Listeria monocytogenes</i>	Heparan sulfate PGs (syndecan-1)	Surface protein ActA	(224)
<i>Salmonella enterica</i> serovar Typhimurium	HSPGs and heparin (presumably)	PagN adhesin/invasin	(225)
<i>Neisseria meningitidis</i>	HSPGs, heparin	Opc adhesin	(226)
Noroviruses (8 strains)	H types 1 and 2, Lewis ^b , and Le ^y antigens.	Capsid proteins	(227)

It was shown that the gut commensals, e.g., probiotic spp. of *Lactobacillus* and *Bifidobacterium* genera, utilize pili to adhere to mucus (**Table 4**). For example, *Lactobacillus acidophilus* strain lacking aggregation-promoting factor ApF displayed reduced adherence (of 63.5% of the parental strain) to mucin in vitro (228).

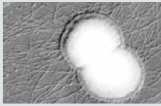
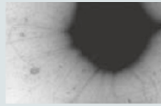
	Gram-negative bacteria			Gram-positive bacteria	
	Type I pili	Type IV pili	Curli pili	Fibrils	Pili
Examples of species	<i>Escherichia coli</i> (EPEC)	<i>Escherichia coli</i> , <i>Neisseria</i> spp. and <i>Pseudomonas</i> spp.	<i>Escherichia coli</i> (some strains)	<i>Streptococcus salivarius</i> K ⁺	<i>Streptococcus</i> spp. and <i>Corynebacterium</i> spp.
Electron microscopy					
Morphology	Rigid rod with flexible tip adhesin	Flexible rod; some form bundles (BFP/TFP)	Coiled, aggregative filament	Short, thin rod	Flexible rod
Length and diameter	1–2 μ m; 7-nm shaft and 2–3-nm tip	1–2 μ m; 5–6 nm	1–2 μ m; 1–2 nm	0.07–0.5 μ m; 1–2 nm	0.3–3 μ m; 3–10 nm
Pilin proteins	4–5	More than 2	2	2	2–3
Assembly components	3	Up to 30	4	ND	1–4
Pilus biogenesis	Chaperone/usher pathway	Type II secretion system	Extracellular	ND	Sortase mediated (some species)

Figure 9. Different types of pili found in gram-positive and gram-negative bacteria. Reproduced from (201) with permission from the publisher.

It is crucial to identify receptor-adhesin interactions, because it is feasible to apply endogenous glycans as soluble decoy receptors to reduce or prevent an adhesion of bacterial pathogens. For example, it was demonstrated that HMOs can hamper binding of *Campylobacter jejuni* to infant mucosal surfaces and thus decrease the number of *Campylobacter*-induced diarrhea incidents (229,230). Similarly, HMOs inhibited invasion of *Salmonella enterica* serovar Typhimurium of human intestinal epithelial cells (231). Furthermore, many receptors of immune cells, including the B cell antigen receptor (BCR), are highly glycosylated and may be potential targets for bacterial adhesins. For instance, it was shown that two fucose-binding lectins, BambL from *Burkholderia ambifaria* and LecB from *Pseudomonas aeruginosa*, activated B cells by binding, independently of BCR signaling (232).

1.5 “Sweet but Psycho”. *Salmonella* infection and host glycans

Salmonella is a genus of facultative anaerobic, non-spore-forming, gram-negative bacteria of the Enterobacteriaceae family. *Salmonella* spp. are motile rods of 2-5 µm length possessing 6-8 peritrichous flagella (233). The *Salmonella* genus comprises two species: *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is divided into six subspecies which are ubiquitously found in the environment and in warm-blooded animals worldwide (234). *S. bongori* is generally restricted to cold-blooded animals, particularly reptiles, however, it was reported that *S. bongori* 48:z35:-strains endemic to Southern Italy cause acute enteritis in children (235). Aforementioned *Salmonella* taxa are further subdivided into more than 2,600 serotypes (serovars) based on antigenic variation of flagella (“H-antigen”) and lipopolysaccharide (“O-antigen”) assessed by Kauffmann-White-Le Minor typing scheme (236). *Salmonella* was originally discovered in 1880, in spleens and in the Peyer's patches of typhoid patients, by Karl Joseph Eberth (237). In 1884, Georg Theodor Gaffky (Robert Koch's disciple, born in Hannover) was first to obtain a pure culture of the “Gaffky-Eberth bacillus”, *S. enterica* serovar Typhi (238).

Human-restricted typhoidal *S. enterica* serovars (Typhi, Sendai, and Paratyphi A, B, or C) are highly adapted pathogens causing systemic disease (typhoid or paratyphoid fever) which can only be transferred from human-to-human via the fecal-oral route (239). In contrast, many non-typhoidal *Salmonella* (NTS) serovars, e.g., Typhimurium and Enteritidis, are zoonotic pathogens with broad host specificity which usually invade only the GIT and cause self-limiting gastroenteritis and diarrhea in humans (reviewed in 240). However, in sub-Saharan Africa, probably due to HIV-, malaria-, or malnutrition-related immunodeficiency,

some NTS serovars can invade systemic tissues and cause bloodstream infection, with an associated case fatality of 20–25% (241). In Europe, *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is a common food-borne pathogen and a major cause of gastroenteritis. According to the European Food Safety Authority, 91,857 confirmed cases of salmonellosis in humans were reported in the EU in 2018, including 1,580 cases of food-borne outbreaks (242).

S. Typhimurium is an intracellular pathogen employing a plethora of virulence factors, such as flagella, fimbriae, type 3 secretion systems (T3SS) and effectors encoded in *Salmonella* Pathogenicity Islands (SPI)-1 and SPI-2, to infect host cells (reviewed in 243). Adhesion is a first, crucial step of *Salmonella* pathogenicity, as it is required for bacterial invasion. Not surprisingly, a large number of adhesins of different structure and specificity was found in *Salmonella* spp., including multiple fimbriae (**Figure 10**), SPI-2-inducible PagN adhesin/invasin, autotransporter adhesins (e.g., ShdA, MisL, and SadA), and the type I secreted large adhesins SiiE and BapA (reviewed in 244). While both ShdA and MisL adhesins of *S. Typhimurium* were shown to bind fibronectin (a high-molecular weight glycoprotein of the ECM) (245,246), the giant adhesin SiiE targets glycans with terminal GlcNAc and/or $\alpha(2,3)$ -linked sialic acid expressed on polarized epithelial cells (247).

Salmonella fimbriae can be produced via the chaperone/usher (CU) pathway, precipitation/nucleation, and type IV pathway. Thirty-eight unique fimbrial gene clusters (FGCs) were described in 34 different *Salmonella* serovars: each strain contains curli (*csg*) and on average 12 different CU fimbriae displaying some functional redundancy, while only a few serovars possess the type IV fimbriae (248). The “fimbriome” of *S. Typhimurium* consists of 13 fimbriae: seven core fimbriae, *csg* and CU (*fim*, *bcf*, *sth*, *stb*, *stc-peg* and *std*), four highly conserved FGCs (*saf*, *stf*, *sti* and *lpf*) and accessory *pef* and *stj* fimbriae (248). While a majority of fimbriae in *S. Typhimurium* are very poorly expressed in vitro (except *fim* and *csg*), at least 11 different fimbriae were shown to be produced in vivo as indicated by seroconversion of infected mice (249); moreover, six fimbrial operons (*lpf*, *bcf*, *stb*, *stc*, *std*, and *sth*) were required for long-term intestinal persistence (250).

However, function and specificity of only few fimbriae of *S. Typhimurium* were characterized to date (**Figure 10**). For example, purified Std fimbriae was shown to bind terminal α -1,2 fucose residues in the tissue sections of murine cecum (251). A role of the fimbrial subunit FimH in binding mannosylated receptors and in bacterial aggregation on enterocyte surfaces was demonstrated in vivo (252). Specific binding of purified plasmid-

encoded fimbriae (*pef*) of *S. Typhimurium* to the trisaccharide Gal β 1–4(Fuc α 1–3)GlcNAc (Lewis^x antigen) was detected by glycan arrays and ELISA (253). Long polar fimbriae *lpf* promoted an adhesion of *S. Typhimurium* to murine Peyer's patches (254), presumably, via attachment to ECM proteins (255). Moreover, different fimbriae and adhesins of *Salmonella* may be expressed in vivo concurrently, enhancing binding of host glycans via different moieties.

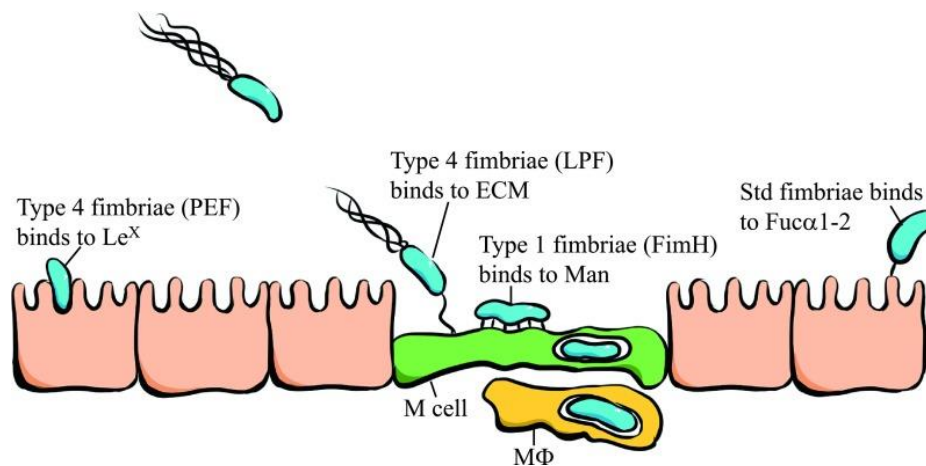


Figure 10. *S. Typhimurium* employs different fimbriae to adhere to specific glycoconjugates expressed on intestinal epithelial cells. Relevant fimbriae and glycan receptors are indicated. PEF – plasmid encoded fimbriae, LPF – long polar fimbriae (in black), ECM – extracellular matrix. Adapted from (255) under a Creative Commons Attribution 4.0 International License (CC BY 4.0).

Salmonella utilizes intestinal glycoconjugates not only for attachment, but also as energy source. According to the CAZY database, 22 different glycoside hydrolases (GH) families that encompass 48 glycosidases are present in *Salmonella enterica* (159). So far, only two GHs of *S. Typhimurium*, sialidase nanH and amylase malS, were linked to degradation of the glycocalyx of a colonic epithelial cell line (256). It is suggested that *Salmonella* spp. lack enzymes needed for a degradation of complex carbohydrates; however, they can thrive on simple sugars liberated by the gut microbiota. Indeed, it was shown that *S. Typhimurium* catabolizes microbiota-liberated free fucose and sialic acid (257).

Overall, more than 60 carbon sources can be utilized by *S. Typhimurium*, including fructose, mannose, ribose, galactose, glucose, glycerol, galactitol, meso-inositol, and mannitol (258,259). Under anaerobic conditions, *Salmonella* can also degrade 1,2-propanediol – a terminal sugar of mucins and a product of the of L-fucose fermentation (260). Notably, Stecher et al. (261) demonstrated that the inflamed intestine expresses high levels of α (1-2)-fucosylated and galactosylated mucins and that *S. Typhimurium* can sense gradients of galactose. To conclude, mucosal glycoconjugates act as receptors for adhesion and as nutrients for which *Salmonella* has to compete with resident microbiota.

2. Aims of the thesis

We hypothesized that the differences in host glycosylation will affect the establishment of infection with *S. Typhimurium* and development of disease. Therefore, the main aim of this study was to investigate the roles of host glycoconjugates (PGs and *Fut2*-dependent glycoproteins/glycolipids) in *S. Typhimurium* infection.

The specific objectives were:

1. To determine the role of host fucosylation for the interaction with *S. Typhimurium* and the outcome of *S. Typhimurium* infection.
2. To characterize the role of proteoglycans in *S. Typhimurium* infection of epithelial cells.

3. Summary of results

Study I: Std fimbriae-fucose interaction increases *Salmonella*-induced intestinal inflammation and prolongs colonization.

by Suwandi A, Galeev A, Riedel R, Sharma S, Seeger K, Sterzenbach T, Pastor LG, Boyle EC, Gal-Mor O, Hensel M, Casadesús J, Baines JF, Grassl GA.

Published in: PLoS Pathogens. 2019 Jul 22;15(7):e1007915.

<https://doi.org/10.1371/journal.ppat.1007915>

Author contributions: Alibek Galeev (co-first author) planned and performed mouse infection experiments (Figures 5, 6, 7, and S9) and assessed bacterial burden and histopathology (Figure 1B, 2AC, 6B), carried out in vitro infection experiments using epithelial cell lines (Figures 3AB and Figure S4) and organoids (Figure 4), measured bacterial gene expression (Figure S5), evaluated fimbrial expression in vivo by immunostaining (Figures 5, S6, S7), performed western blotting (Figure 3D) and motility assay (Figure S8), generated *E. coli stdABCD*, *S. Typhimurium ΔaroA stdAstop::gfp*, and *S. Typhimurium ΔaroAΔstdA* strains; analyzed the data, wrote and edited the manuscript.

Summary.

In **study I**, we investigated the role of *Fut2*-dependent mucosal glycans in *Salmonella* infection. We proposed that *S. Typhimurium* may benefit from Std-mediated binding to α(1,2)- fucose residues which are present in abundance on the intestinal epithelium and in the mucus layer. To test our hypothesis, we utilized B6.129X1-*Fut2*^{tm1Sdo/J} (*Fut2*^{-/-}) mice – widely used *Fut2*-knockout mice to study non-secretor phenotype (262). *Fut2*^{+/+} and *Fut2*^{-/-} mice, pre-treated with streptomycin (day -1), were infected with either *S. Typhimurium* WT or attenuated *S. Typhimurium ΔaroA* strain, and sacrificed at day 1, 3 7, or day 14 post infection (p.i.). We found that compared to WT mice, mice lacking *Fut2* were more susceptible to *S. Typhimurium* infection at an early time point (d1 pi.). In contrast, at the later stages of infection (d7 and d14 p.i.), *Fut2*^{-/-} mice had significantly lower bacterial colonization and decreased histopathological changes in the colon and cecum. Moreover, 7 days p.i., in colons and ceca of *Fut2*^{+/+} mice, we detected significantly higher levels of the inflammation-associated marker lipocalin-2. Accordingly, while compared to *Fut2*^{-/-} mice, *Fut2*^{+/+} mice had significantly higher numbers of recruited neutrophils and macrophages in the colon tissue, as well as a significantly stronger infiltration of CD4⁺ T lymphocytes in the colonic lamina propria, as assessed by immunocytochemistry and flow cytometry.

To test, whether gut fucosylation directly affects *Salmonella* colonization via interaction with Std fimbriae, we deleted *stdAB* genes (encoding the major fimbrial subunit and the membrane usher protein) in *S. Typhimurium* Δ *aroA* parental strain. In contrast to the infection with *S. Typhimurium* Δ *aroA*, upon infection with a *S. Typhimurium* Δ *aroA* Δ *stdAB* strain, *Fut2*^{+/+} and *Fut2*^{-/-} mice had comparable levels of bacterial colonization and gut inflammation (d7 p.i.). The role of Std fimbriae in vivo has been further confirmed by competitive index assays: the *S. Typhimurium* Δ *aroA* Δ *stdAB* strain was significantly outcompeted by the Std-expressing *S. Typhimurium* Δ *aroA* strain in the large intestine and in feces of *Fut2*^{+/+} mice, but not in *Fut2*^{-/-} mice. These results demonstrate that Std fimbriae are important in *Fut2*^{+/+} mice which implies a direct Std-fucose interaction.

In addition, we assessed Std fimbriae production in vivo using RT-qPCR analysis, immunostaining with anti-Std serum, and reporter *S. Typhimurium* strains containing *stdA*stop::gfp fusion. These reporter strains produce sfGFP when *stdA* is expressed. Similar levels of *stdA* gene expression were detected in the colons of both infected *Fut2*^{+/+} and *Fut2*^{-/-} mice. Notably, we observed that Std fimbriae are produced in the lumen of the large intestine, but not after invasion into the mucosa of both *Fut2*^{+/+} and *Fut2*^{-/-} mice on day 1 and day 7 p.i.

To assess the role of fucose-Std fimbriae interaction during bacterial infection in vitro, we utilized human intestinal epithelial cell lines, Caco-2 and mucus-producing HT29-MTX-E12. Notably, differentiated, goblet cell-like HT29-MTX-E12 cells displayed extensive α (1,2)-fucosylation of the cell surface, detected by *Ulex europaeus* agglutinin I (UEA-I) lectin staining. Because only very small fraction of *S. Typhimurium* produces Std fimbriae in vitro, we expressed *stdABCD* in *Escherichia coli* (*E. coli*) under an anhydrotetracycline-inducible promoter and confirmed the Std production by flow cytometry and Western blotting. We found that *stdABCD*-expressing *E. coli* adhered significantly better to fucosylated cell lines compared to Std-negative control. Accordingly, in primary epithelial organoids isolated from *Fut2*^{+/+} mice, co-localization of Std fimbriae-expressing bacteria was observed with α (1,2)-fucose residues, while Std-deficient bacteria were randomly distributed across all cells.

Previously, differences in microbiota composition between *Fut2*^{+/+} and *Fut2*^{-/-} mice have been reported (118). To evaluate a possible effect of commensals on *S. Typhimurium* colonization and infection, we collected the fecal samples of each mouse before and after infection and sequenced the 16S rRNA gene. In our mice, we did not detect significant differences in α -diversity (within sample) in species richness (Chao1), nor in evenness of distribution (Shannon's H) (**Figure 11A**), before infection or on day 7 after infection.

Reduction of species richness at day 7 p.i. was probably caused by the presence of inflammation. We also assessed β -diversity (between samples) by applying UniFrac distance metrics and a constrained Principal Coordinate Analysis. Host *Fut2* status, as well as *stdA* expression by *Salmonella*, did not influence gut community variability between the studied mice (**Figure 11B**). Thus, because we did not observe differences in microbiota composition between *Fut2*^{+/+} and *Fut2*^{-/-} mice, we concluded that the phenotype observed in our study is due to a direct interaction between Std fimbriae and Fuc α 1,2.

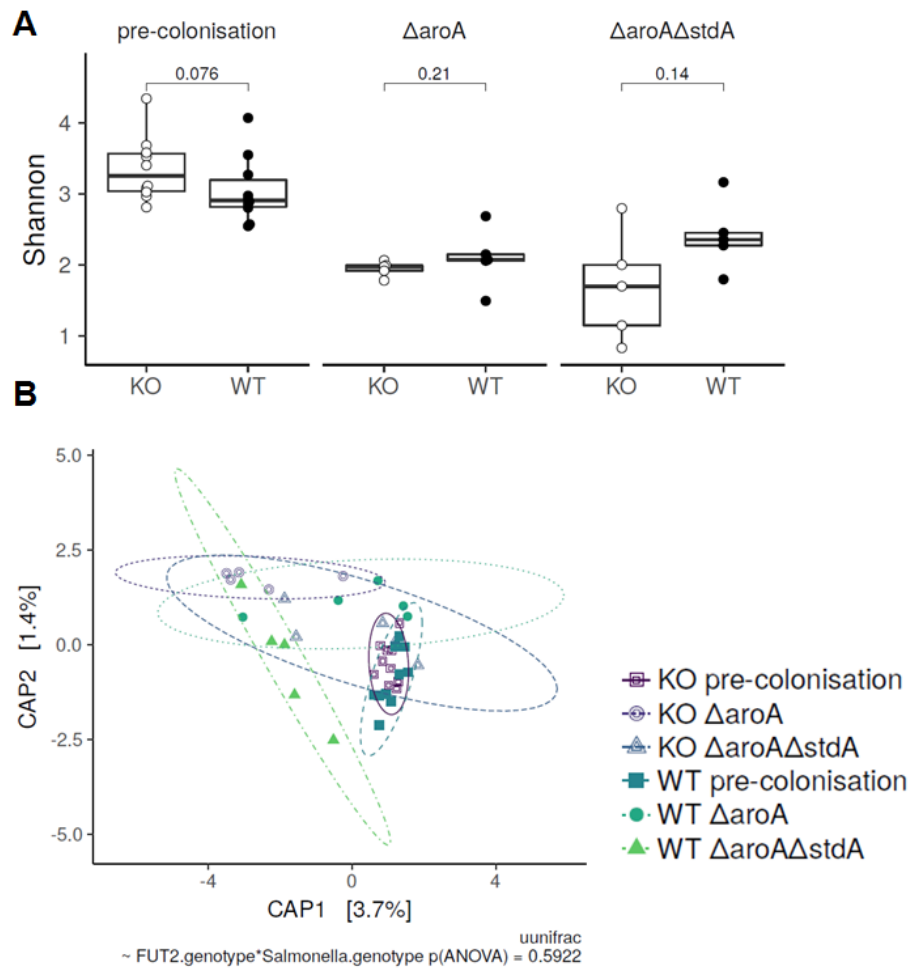


Figure 11. Unpublished data of the study I. (A) Analysis of microbial alpha diversity of *Fut2*^{+/+} (WT) and *Fut2*^{-/-} (KO) mice, uninfected, or infected with either *S. Typhimurium* Δ aro or with *S. Typhimurium* Δ aroA Δ stdA. (B) Principle Coordinate Analysis (unweighted UniFrac based on sequence distances) of untreated and *S. Typhimurium*-infected mice. Microbial diversity was estimated from 97% species level OTUs. Shannon diversity index was calculated in R using the phyloseq package. Constrained analysis of principal coordinates (“capscale”) was done within phyloseq; the “anova.cca” function of the vegan R library was applied to evaluate significance. P values are indicated. Figures and data provided by Dr. Rene Riedel.

In conclusion, our results demonstrate that *Fut2*-mediated fucosylation in the intestine enhanced *Salmonella*-triggered inflammation and colonization of the large intestine, due to a direct binding of bacterial Std fimbriae to host α (1,2)-fucosylated glycans.

Study II: Proteoglycan-dependent endo-lysosomal fusion affects intracellular survival of *Salmonella* Typhimurium in epithelial cells.

by Galeev A, Suwandi A, Bekker H, Oktiviary A, Routier F, Krone L, Hensel M, Grassl GA.

Published in: Frontiers in Immunology. 2020 Apr 29;11:731.

<https://doi.org/10.3389/fimmu.2020.00731>

Author contributions: Alibek Galeev (first author) planned and carried out the following experiments: cell cultures infections and evaluation of bacterial burdens (Figures 1A-C, 2, 3C-E, 7, S3B, S7), cytotoxicity measurements (Figure S1B), gene expression assessment (Figures S2A, S9), infections with the *S. Typhimurium* reporter strains (Figure 3AB), cell labelling experiments (Figures 4A, 6A, S4, S5, S8), antibody uptake assay (Figure 5), endo-lysosomal fusion assay (Figure 8), heparin internalization assay (Figure S10); performed related immunocytochemistry, microscopy, and data analysis; wrote and edited the manuscript.

Summary.

In **study II**, we utilized Chinese hamster ovary (CHO) epithelial cell lines, WT and proteoglycan-deficient CHO pgsA745 (referred hereafter as CHO $\Delta XylT$), to elucidate the role of proteoglycans (PGs) in *S. Typhimurium* infection. Previously, proteoglycan-dependent adhesion of several pathogens, including *Listeria monocytogenes* and *Neisseria meningitides*, was reported. Lambert et al. (225) demonstrated that PagN adhesin of *S. Typhimurium* promoted bacterial invasion of CHO WT cells, but not of CHO $\Delta XylT$ cells. However, PagN is only expressed under intracellular (SPI-2)-inducing conditions. Furthermore, a role of PGs in intracellular survival or replication of *S. Typhimurium* was not known. While, our initial hypothesis was that bacterial adhesion to and/or invasion of host epithelial cells will be affected by PGs absence, we discovered that lack PGs affected intracellular *Salmonella* via altered endo-lysosomal trafficking.

To assess a role of PGs in *Salmonella* infection, we infected CHO WT and CHO $\Delta XylT$ cells with *S. Typhimurium*. Surprisingly, we found that adhesion (30 min p.i), invasion (1.5 h p.i), and early replication (4 h p.i) of *S. Typhimurium* WT were comparable between CHO WT and CHO $\Delta XylT$ cells. Unexpectedly, 24 hours after infection, proteoglycan-deficient CHO cells were significantly less colonized by *S. Typhimurium* compared to CHO WT cells. Notably, the reduction of bacterial burden within CHO $\Delta XylT$ cells was dependent on the dose of gentamicin applied to the cell culture medium in order to eliminate extracellular bacteria.

In general, epithelial cells do not efficiently take up gentamicin. However, several transporters, including the transient receptor potential channels (Trpv), Trpv1 and Trpv4, as well as the multidrug resistance protein 2 (Mrp2), have been shown to uptake and transport gentamicin in epithelial cells (263–265). In our study, non-infected and infected CHO WT and CHO $\Delta XylT$ cells had similar levels of expression of *Trpv1*, *Trpv4*, and *Mrp2* genes. Moreover, no differences in the levels of total intracellular gentamicin concentration between CHO WT and CHO $\Delta XylT$ cells were detected by ELISA. Thus, our results indicate that the absence of PGs in CHO cells has no effect on active or passive gentamicin uptake.

Intriguingly, gentamicin-dependent reduction of *S. Typhimurium* burden in CHO $\Delta XylT$ cells could be rescued by addition (upon bacterial invasion, for 24 hours) of the GAG heparin (a structural analog of heparan sulfate) to the CHO cell culture medium a dose-dependent manner, but not by other GAGs (e.g., chondroitin sulfate). Notably, heparin did not have an effect on *S. Typhimurium* numbers in CHO WT cells, neither on growth and gentamicin-mediated killing of *S. Typhimurium* in LB broth. In addition, the infected CHO $\Delta XylT$ cells, which were genetically complemented with the human *XYLT2* gene (CHO cXylT cells), harbored significantly more bacteria at 24 h p.i. than the parental cells, but lower *S. Typhimurium* loads while compared to CHO WT cells. Accordingly, flow cytometry and immunostaining using anti-HS/heparin antibody revealed only partial complementation of PGs in CHO cXylT cells. Taken together, these data indicated that the phenotype observed in CHO $\Delta XylT$ cells is indeed proteoglycan-dependent.

Upon invasion into epithelial cells, *S. Typhimurium* establishes a specific niche where bacteria can reside and multiple: *Salmonella*-containing vacuoles (SCVs) and *Salmonella*-induced filaments (SIFs). Next, we questioned if PGs are important for intracellular localization of *Salmonella*. To address this, we utilized a *S. Typhimurium* reporter strain with constitutive DsRed protein expression and inducible sfGFP expression under control of the *uhpT* (hexose-6-phosphate transporter) promoter. Glucose-6-phosphate, abundant in eukaryotic cytoplasm, is a carbon source for *Salmonella* (266); the dual fluorescence reporter strain was used before to monitor release of *S. Typhimurium* into host cell cytosol (267). Microscopy revealed that in the CHO $\Delta XylT$ cells, significantly less bacteria were associated with SCVs compared to WT CHO cells at 24 hours p.i. We could corroborate this finding using chloroquine resistance assay. Chloroquine is an antimalarial drug, a weak base that increases intralysosomal pH (268). Treatment of cells with chloroquine, in combination with gentamicin protection assay, results in selective killing of bacteria within SCVs, thus, it is

possible to quantify cytosolic and intra-SCV *Salmonella* separately (269). Using this method, we found that infected CHO $\Delta XylT$ cells had more cytosolic bacteria comparing to CHO WT cells. We also utilized a *S. Typhimurium* $\Delta sifA$ mutant, unable to maintain SCVs and replicating only in the cytoplasm. Notably, even in presence of high concentrations of gentamicin in medium, cytosolic *S. Typhimurium* $\Delta sifA$ bacteria had similar replication rates in CHO WT and CHO $\Delta XylT$ cells. To conclude, by using three different approaches (microscopy, chloroquine resistance assay, *Salmonella* mutants) we demonstrate that reduced *S. Typhimurium* colonization of CHO $\Delta XylT$ cells, 24 h p.i., was due to a killing of bacteria in SCVs, while cytosolic *S. Typhimurium* were mostly unaffected by gentamicin.

Then, we decided to look closely on gentamicin distribution within CHO cells. We conjugated gentamicin to Cy3-labelled NHS ester and used it in gentamicin protection assay. In agreement with our previous findings, we discovered that intracellular, Cy3-labelled gentamicin is associated with SCVs and bacterial debris in CHO $\Delta XylT$ cells, but not in CHO WT cells. We hypothesized that an intracellular routing of endocytosed cargo (other than gentamicin) may be altered in the absence of PGs. To investigate a role of PGs in vesicle trafficking, we used antibody uptake assay: CHO cells were infected with *S. Typhimurium* expressing sfGFP, and, after invasion of bacteria, an anti-*Salmonella* antibody was added to cell culture medium. We detected significantly higher numbers of double-positive, intracellular bacteria in CHO $\Delta XylT$ cells compared to CHO WT cells. Importantly, heparin-treatment of CHO cells abrogated such difference. These data indicated an important role of PGs in intracellular vesicle trafficking.

To test whether lack of PGs affects endo-lysosomal trafficking and formation of acidic late endosomes and lysosomes, we stained acidic vacuoles with Lysotracker. Lysotracker probes consisting of a fluorophore linked to a weak base are highly selective for acidic organelles. Notably, CHO $\Delta XylT$ cells (both non-infected and infected) displayed a significantly reduced staining with Lysotracker comparing to CHO WT cells, indicating abnormal endo-lysosomal and SCVs acidification. To further explore this phenomenon, we generated and utilized *S. Typhimurium* acid shock reporter strain expressing sfGFP under control of the acid shock response-activated promoter P_{asr} . In agreement with Lysotracker staining data, we detected (using flow cytometry) reduced SCV acidification within CHO $\Delta XylT$ cells infected with the acid shock reporter *S. Typhimurium*.

Next, we asked, at which stage trafficking of endocytosed cargo is affected by the lack of PGs. Infected CHO WT and CHO $\Delta XylT$ cells were treated (upon invasion of bacteria)

with either inhibitor of clathrin-mediated endocytosis (dynasore), of phosphoinositide 3-kinase PI3K (wortmannin), or of FYVE finger-containing phosphoinositide kinase PIKfyve (YM201636) activity. While treatment of CHO cells with wortmannin did not affect intracellular *S. Typhimurium* numbers, inhibition of clathrin-mediated endocytosis resulted in significantly higher recovery of bacteria from CHO $\Delta XylT$ cells compared to the non-treated controls (as expected, see **Discussion, Part II**). Strikingly, PIKfyve inhibition increased *Salmonella* burdens in CHO $\Delta XylT$ cells, but decreased in CHO WT cells. YM201636-mediated PIKfyve inhibition could be abrogated by heparin treatment demonstrating a direct effect of GAGs on PIKfyve-mediated endo-lysosomal fusion.

To further investigate endo-lysosomal fusion, we utilized a modified dextran pulse-chase assay: CHO WT and CHO $\Delta XylT$ cells were first incubated with dextran-Alexa568 for 4 h, followed by 18 h chase in dextran-free CHO medium, to label lysosomes/late endosomes. Afterwards, cells were pulsed with dextran-Alexa488 for 10 min and then chased for 30 min, to label early endosomes. In order to evaluate endo-lysosomal fusion events, we calculated Pearson's correlation coefficient and Manders split coefficients (M1 and M2). Notably, compared to CHO WT cells, CHO $\Delta XylT$ cells displayed significantly lower degree of co-localization between the two labelled dextrans. Because these dextrans were initially present either in endosomes or lysosomes, low co-localization reflects a diminished occurrence of endo-lysosomal fusion events. Thus, we report here that endo-lysosomal fusion is delayed or reduced in proteoglycan-deficient CHO cells.

To conclude, we could show that proteoglycans influence survival of SCV-associated intracellular *Salmonella* by affecting PIKfyve-dependent endo-lysosomal fusion which regulates vacuolar pH and re-distribution of endocytosed cargo.

4. Discussion

Part I.

Fucosylation is the final step in the biosynthesis of oligosaccharides. In humans, a fucosyltransferase encoded by *FUT2* gene governs terminal $\alpha(1,2)$ -fucosylation of mucosal epithelial surfaces and of the secretions, such as saliva and mucus. Genome-wide association studies link the *FUT2* gene variants with an increased risk for Crohn's disease (150,270) and primary sclerosing cholangitis (271), as well as with increased or decreased susceptibility to various bacterial and viral infections (102). In **study I**, we investigated the role of *Fut2* expression for *S. Typhimurium* infection in mice and in human primary epithelial cells and immortalized epithelial cell lines. We demonstrate that Std fimbriae-fucose interaction was important for *Salmonella*-induced inflammation and intestinal colonization.

***Fut2* expression affects susceptibility to *Salmonella*-induced colitis.**

We utilized *Fut2*-knockout mice generated by Domino et al. (262). *Fut2*^{-/-} mice lack L-fucosylated epithelial glycans throughout antrum, cecum, and colon (272); analysis of oligosaccharides released from colonic mucins (mostly from Muc2) revealed complete absence of terminally fucosylated O-linked glycans (273). In the small intestine, fucosyltransferases Fut1 and Fut2 can both catalyze the addition of Fuc α 1,2: it was shown that fucosylation of M-cells in Peyer's patches is *Fut1*-dependent, while *Fut2* governs fucosylation of the intestinal epithelial cells (274). As expected, we detected strong α 1,2-fucosylation throughout GIT mucosa (especially, in the goblet cells of cecum and colon) in *Fut2*^{+/+} mice, while our *Fut2*^{-/-} mice displayed residual Fuc α 1,2 in the small intestine only (jejunum and ileum).

We confirmed the finding of Goto et al. (275) showing that mice lacking *Fut2* are more susceptible to *S. Typhimurium* infection at an early time point (d1) post infection. However, we discovered that, at later time points of infection (d7, d14 p.i.), *Fut2*^{-/-} mice are characterized by decreased intestinal colonization, by reduced pathology and inflammatory responses in the large intestine. We also observed a diminished recruitment of neutrophils, macrophages, and CD4⁺ T cells in colons of infected *Fut2*^{-/-} mice, which correlated with a reduction in bacterial burdens in these mice. Of note, we detected the similar frequencies of B cells, DCs, and IELs in colonic lamina propria of both infected *Fut2*^{+/+} and *Fut2*^{-/-} mice. Because a role of gut fucosylation in mucosal immunity is still not clear, more studies focusing on an immune response of *Fut2*^{+/+} and *Fut2*^{-/-} mice (also at steady state) are needed.

It was shown that *Fut2* expression in the ileum of BALB/c mice is induced by IL-22 produced by type 3 innate lymphoid cells (275), and is downregulated by IL-10-producing CD4⁺ T cells (276). Pickard et al. showed that the large intestine is constitutively fucosylated (277). In accordance with Pickard et al. data (277), uninfected and infected *Fut2*^{+/+} mice have comparable *Fut2* gene expression in colons (unpublished data of **study I**). Thus, it is tempting to speculate that IL-22-mediated “rapid fucosylation” is important for an early response to bacterial infections in the small intestine. In contrast, our data show that constitutive fucosylation of the large intestine enhances susceptibility to *S. Typhimurium* infection.

Std fimbriae-fucose interaction is critical for *Salmonella*-induced inflammation and colonization.

It is well known that host glycans can modulate bacterial adhesion to host tissues (96). Many eukaryotic, bacterial, and viral pathogens produce adhesins which can selectively bind specific glycan moieties present in the GIT. Among the pathogens which bind $\alpha(1,2)$ -fucosylated glycans, *H. pylori* utilizing adhesin BabA to adhere to the fucosylated gastric mucosa and norovirus GII.4, were studied the most (220,227). Previously, it was shown that purified Std fimbriae of *S. Typhimurium* can bind terminal $\alpha(1,2)$ -fucose residues in vitro (251). Moreover, Weening et al. demonstrated that Std fimbriae are required for the long-term persistence of *S. Typhimurium* in the large intestine of resistant CBA mice (250). Based on the previously discussed data, we proposed that *S. Typhimurium* is able to benefit from Std-mediated adhesion to $\alpha(1,2)$ -fucosylated glycans present in the large intestine of *Fut2*^{+/+} mice.

Chaperone-usher Std fimbriae of *S. Typhimurium* are encoded by the *std* operon containing six genes (*stdABCDEF*) which are co-transcribed from a promoter located upstream of *stdA*. While StdA constitutes the major fimbrial subunit, StdB is a putative outer membrane usher protein, and StdC is a putative periplasmic chaperone; StdE and StdF are involved in Dam-dependent regulation of *Salmonella* SPI-1 expression (278). We generated and utilized *S. Typhimurium* Δ *aroA* Δ *stdAB* mutant strain lacking Std fimbriae, but characterized by the same growth rate and motility as parental *S. Typhimurium* Δ *aroA* strain.

We demonstrated a direct interaction between bacterial Std fimbriae and host Fuca1,2 in vivo by using *S. Typhimurium* Δ *aroA* Δ *stdAB* strain and competitive index (CI) infection approach. Interestingly, in our study, Std fimbriae did not provide an advantage in the ileums of *Fut2*^{+/+} mice. Whether Std fimbriae are differentially produced in vivo, was not previously known. We observed, for the first time, a spatial expression of Std fimbriae in the murine intestine: Std-positive subpopulations of *S. Typhimurium* were detected in the lumen of the

ceca and colons, but not among invaded bacteria. This observation is in line with the fact that constitutive expression of StdE and/or StdF represses SPI-1 (278). Considering the fact that *S. Typhimurium* possesses multiple fimbriae and adhesins which are produced in vivo and may be important for persistence in the gut (e.g. *lpf*, *sth* fimbriae) (250), the differential expression of Std fimbriae may represent a mechanism of time/space-dependent bacterial colonization of the large intestine.

Microbiota of *Fut2*^{+/+} and *Fut2*^{-/-} mice and microbiota-liberated glycans did not influence *S. Typhimurium* infection.

Mucosal glycans moderate a composition of the gut microbiota and thus may indirectly influence the susceptibility to various infections (102). For example, our group previously found that an expression of the histo-blood group-related glycosyltransferase *B4galnt2* in the murine gut altered the microbial composition, which in turn affected *Salmonella* colonization (279). Previous studies highlighted a role of IL-22-mediated gut fucosylation in promoting of colonization resistance to murine pathogen *Citrobacter rodentium* (277) and to opportunistic *Enterococcus faecalis* (280). It should be noted that, in humans, it is still not clear whether a secretor status affects the gut microbiota composition (119,281). In the study of Rausch and colleagues (118), *Fut2*^{+/+} mice (housed and bred at the University of Kiel) were significantly associated with genus *Odoribacter*, while bacteria belonging to genera *Prevotella*, *Lachnobacterium* and *Ruminococcus* were enriched in the fecal microbiota of *Fut2*^{-/-} mice.

In contrast, *Fut2*^{+/+} and *Fut2*^{-/-} mice (of the same genetic background, but housed and bred at the animal facility of the Hannover Medical School) used in our **study I** were similar in terms of the fecal microbiota (indicator spp. and α/β -diversity). Such discrepancies may be caused by differences in certain aspects of animal husbandry between the facilities in Kiel and in Hannover. Indeed, a considerable variation in the proportions of major phyla, in α - and β -diversity of fecal microbiota of isogenic C57BL/6J mice were found in a study comparing 21 different animal facilities across Germany (including Kiel and Hannover) (282). Further studies are needed to clarify an impact of *Fut2* expression on the murine gut microbiota composition.

Fucosylated complex glycans of mucins are degraded by the gut commensals; released free fucose can be then consumed by enteropathogens. Ng et al. showed that in mice mono-associated with *B. thetaiotaomicron*, but not in germ-free mice, *S. Typhimurium* upregulate genes of the *fucOAPIKUR* operon responsible for fucose utilization (257). Notably, fucose utilization operon and 1,2-propanediol (a product of Fuc metabolism) utilization (*pdu*) operon

were found in all *S. enterica* serovars sequenced so far (260). The onset of intestinal inflammation is a hallmark of *S. Typhimurium* infection (283). Previous studies indicated that in the inflamed murine large intestine, *S. Typhimurium* produces at least three fucose utilization proteins, FucI, FucU, and FucA, (284) and gains growth advantage from 1,2-propanediol utilization while using both aerobic and anaerobic respiration (285).

However, no differences in the expression levels of *fucI* (encoding L-fucose isomerase) and *pduBC* (propanediol dehydratase) genes in feces and in colons of infected *Fut2^{+/+}* and *Fut2^{-/-}* mice were detected in our study. Pacheco et al. (286) described the FusKR, two-component signal transduction system of EHEC, which senses fucose and represses an expression of certain virulence and metabolism genes in the mucus layer. Whether *S. Typhimurium* can sense fucose is not known. Furthermore, considering that “non-secretors” may have an increased abundance of sialylated glycans (120), additional studies on host glycan utilization by *S. Typhimurium* are required.

Std fimbriae mediate adhesion of *S. Typhimurium* to intestinal epithelial cells in a fucose-dependent manner.

We also investigated an interaction between Std fimbriae of *S. Typhimurium* and host Fuc α 1,2 in vitro. Due to a complex and tight regulation, *fimH*, *lpf*, and *csg* are the only fimbriae readily detected in *S. Typhimurium* grown under laboratory conditions (287). Expression of the *std* operon is bistable and produces a minor (0.3%) subpopulation of Std-positive bacteria in vitro (288). Thus, we utilized an inducible heterologous expression of *stdABCD* genes in an afimbriated *E. coli* strain. We detected significantly increased adherence of Std-expressing *E. coli* to fucosylated human epithelial cell lines, which could be abrogated by the pretreatment of the cells with α (1,2)-fucose-binding lectin UEA-I.

Accordingly, using confocal microscopy, we observed a preferential binding of Std-producing *E. coli* to α (1,2)-fucosylated moieties in primary epithelial monolayers derived from *Fut2^{+/+}* mice. These observations are in line with the findings of Chessa et al. (251), who described Std-mediated adherence of *S. Typhimurium* to Caco-2 cells which could be blocked by co-incubation with H type 2 oligosaccharide (Fuc α 1-2Gal β 1-4GlcNAc) or by pretreatment of cells with α 1-2 fucosidase. To conclude, expression of Std fimbriae resulted in increased bacterial adhesion to fucosylated human cell lines and murine intestinal crypt organoids.

Part II.

Proteoglycans (PGs) are heavily glycosylated (50-60% carbohydrates) glycoconjugates containing a GAG chain attached to a protein core. PGs are abundant in connective tissues; nevertheless, they are also found in plasma membranes of virtually every cell type (121). PGs are crucial for ECM formation, moreover, a role of PGs in bacterial adhesion and invasion is acknowledged (123). In **study II**, we investigated how PGs (and absence thereof) influence *S. Typhimurium* infection of CHO epithelial cell line. We discovered that PGs represent a novel host factor crucial for PIKfyve-dependent endo-lysosomal fusion and for *Salmonella* survival within epithelial cells in a context of gentamicin protection assay.

Heparan sulfate PGs are crucial for *Salmonella* survival within CHO cells

In wild-type CHO cells, biosynthesis of PGs is initiated by the xylosyltransferase-II (Xylt2) enzyme adding xylose to a tetrasaccharide linker. We utilized PG-deficient CHO pgsA745 ($\Delta XylT2$, referred to as $\Delta XylT$) cell line generated by Esko et al. (122). A number of studies used this cell line to assess the contribution of PGs to the entry of bacteria and viruses into host cells (123). It should be noted, that hyaluronan GAG and keratan sulfate GAGs/KSPGs might still be present in CHO $\Delta XylT$ cells due to a different biosynthesis pathways (138,140). Moreover, it was recently reported that CHO $\Delta XylT$ cells carry an unspecific mutation in the *Lama2* gene resulting in a deletion of the long isoform of the laminin subunit α -2. It was proposed that this mutation in CHO $\Delta XylT$ cells caused significantly reduced invasion of group B *Streptococcus* compared to CHO WT cells (289).

Indeed, our CHO $\Delta XylT$ cells also lacked the expression of long laminin-2 isoform. Nevertheless, we detected similar levels of *S. Typhimurium* adhesion to and invasion into the CHO WT and CHO $\Delta XylT$ cell lines. Previously, Lambert et al. (225) observed reduced invasion of *S. Typhimurium* grown under *pagN*-inducing conditions into CHO $\Delta XylT$ cells. PagN is a SPI-2-induced adhesin and invasin which supposedly can bind HSPGs and heparin. While we did not evaluate *pagN* expression in our *S. Typhimurium* strains, we did not use *pagN*-inducing growth medium in the infection experiments. Importantly, previous studies in BALB/c mice identified that *pagN* gene is expressed during infection (290) and that *S. Typhimurium pagN* mutant had decreased competitiveness compared to the parental strain (291). Thus, further studies are needed to investigate a possible effect of PagN-mediated adhesion/invasion in vivo.

Notably, using a gentamicin protection assay, 24 h p.i., we detected a significant reduction of *S. Typhimurium* burden in PG-deficient CHO cells. This reduction could be abrogated by the addition of heparin, but not chondroitin sulfate, to cell culture medium. We also restored HSPGs production and “rescued” CHO $\Delta XylT$ cells by complementation with the human *XYLT2* gene. Heparin is a degradation product of heparan sulfate (HS) isolated from porcine intestine or equine lungs; heparin is widely utilized as an anticoagulant drug and as cheap structural analog of HS. Compared to HS (20-100kDa), heparin is smaller (7-20 kDs) and contains a higher percentage of sulfated residues (125). Taken together, our data indicated an important role of HS GAG (but not of HSPGs core proteins) in a survival of *S. Typhimurium* within CHO cells in a context of gentamicin protection assay.

SCV-associated *Salmonella* are killed by gentamicin in proteoglycan-deficient CHO cells.

Upon invasion of host cell, in order to establish a replication niche, *S. Typhimurium* recruits multiple host factors, including the small GTPases (RAB5, RAB7, ARL8), early endosome antigen 1 (EEA1), and Lysosome-associated membrane proteins (LAMPs) (292). We observed a recruitment of host ARL8B and Lamp1 to the *Salmonella*-containing vacuoles (SCVs) and to *Salmonella*-induced filaments (SIFs) in both CHO WT and CHO $\Delta XylT$ cells. Thus, we concluded that *S. Typhimurium* hijack endo-lysosomal trafficking and establish the SCVs/SIFs in both cell lines. However, 24 h p.i., CHO $\Delta XylT$ cells had significantly less SCV-associated *S. Typhimurium*, and more cytosolic bacteria in comparison to WT CHO cells, as was assessed by three different methods.

Because lack of PGs affected the intracellular “lifestyle” of *Salmonella*, we hypothesized that the intracellular distribution of gentamicin might be altered in infected CHO $\Delta XylT$ cells, which may result in an increased exposure of certain bacterial populations to the antibiotic. Of note, a widely accepted notion of plasma membranes being impermeable to gentamicin is not entirely correct. Epithelial cells can take up gentamicin and other aminoglycosides via endocytosis-dependent and -independent pathways (293). Indeed, an accumulation of endocytosed gentamicin in lysosomes of kidney epithelial cells was previously observed (294). While we did not detect differences in the levels of total intracellular gentamicin between CHO WT and CHO $\Delta XylT$ cells (non- and infected), we discovered an increased co-localization of Cy3-labelled gentamicin and SCVs/bacterial debris in proteoglycan-deficient CHO cells. Brumell et al. (295) demonstrated that SPI-2 T3SS effector SifA is required for maintenance of the SCVs: *Salmonella* lacking SifA were

characterized by extensive cytosolic replication in epithelial cells. In our study, enhanced cytosolic growth of *S. Typhimurium* Δ *sifA* mutant was largely unaffected by increasing gentamicin concentrations, in both CHO cell lines.

Thus, we postulate that a reduction of total *S. Typhimurium* counts in CHO Δ *XylT* cells, as well as an enhanced killing of bacteria in SCVs, is due to the altered intracellular redistribution of gentamicin within CHO Δ *XylT* cells.

Proteoglycans are important for PIKfyve-dependent endo-lysosomal fusion.

We detected an accumulation of anti-*Salmonella* IgG1 antibody (added to cell culture medium), 24 h p.i., within SCVs/SIFs in proteoglycan-deficient CHO cells. Notably, it could be abrogated by addition of heparin to cell culture medium. The neonatal Fc receptor, FcRn, a MHC class I-related protein, mediates specific IgG uptake and transcytosis in many tissues, including primary human intestinal epithelial cells, the fetal yolk sac of rats, human placenta and fetal enterocytes (296). Epithelial CHO cells have *Fcgrt* gene which has 74% sequence similarity to a human *FCGRT* ortholog encoding FcRn (BLAST (297) data). It should be noted that we did not assess FcRn expression in our CHO cells. Because anti-*Salmonella* antibody reached (as was evident from immunostaining) intracellular bacteria in SCVs/SIFs, we reasoned that antibody was non-specifically endocytosed.

Recently, Liss et al. (298) demonstrated that *S. Typhimurium* SIFs can merge with endocytosed vesicles and expose bacteria to extracellular nutrients and/or antibiotics. To investigate a possible contribution of the SIFs to delivery of gentamicin to SCVs, we infected CHO cells with a *S. Typhimurium* Δ *ssaR* strain – SPI-2 mutant lacking SIFs. In agreement with Liss et al. findings (298), intracellular *S. Typhimurium* lacking SIFs were less susceptible to a high gentamicin doses; but this was only seen in CHO WT cells. In contrast, in CHO Δ *XylT* cells, *S. Typhimurium* Δ *ssaR* were as sensitive to gentamicin treatment as WT bacteria. Based on these findings we hypothesized that PGs may directly affect endo-lysosomal trafficking of endocytosed cargo.

Previously, no defects in endocytosis/phagocytosis were reported in CHO Δ *XylT* cells (289), which is also supported by our data regarding uptake of gentamicin in CHO cells. Accordingly, by inhibiting clathrin-mediated and caveolin-mediated endocytosis with dynasore, we could abrogate gentamicin-mediated killing of *Salmonella* in our CHO Δ *XylT* cells. We also utilized inhibitors targeting phospholipids of late endosomes and lysosomes, including wortmannin – an inhibitor of phosphoinositide 3-kinases, and more specific

inhibitor of phosphatidylinositol 3-phosphate 5-kinase (PIKfyve), YM201636. PIKfyve converts phosphatidylinositol 3-phosphate (PtdIns3P) into phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P₂). A role of PIKfyve kinase in the fusion of *S. Typhimurium* macropinosomes with the late endosomes/lysosomes has been indicated by Kerr et al. (299). In our study, in agreement with Kerr et al. data (299), a specific inhibition of PIKfyve kinase activity in CHO WT cells resulted in a reduction of bacterial loads compared to untreated cells (*Salmonella* was unable to establish a niche). In contrast, inhibition of PIKfyve in PG-deficient CHO Δ XylT cells resulted in increased *S. Typhimurium* counts.

Moreover, using a dextran pulse-chase assay, we detected an inhibited/delayed endo-lysosomal fusion in CHO Δ XylT cells. Thus, we propose that PGs are required for PIKfyve-dependent endo-lysosomal fusion and associated trafficking/recycling of endocytosed cargo. We postulate that in the absence of PGs, more endocytosed gentamicin (or other cargo) retains within endosomes which are then merged with bacterial macropinosomes. Further studies are required to confirm this hypothesis.

To our knowledge, **study II** is the first report on specific interactions between host phosphoinositides and cell surface GAGs/PGs in a context of infection. Our findings are in line with a study of Krishna et al. who showed a role of PIKfyve kinase in the re-distribution of endocytosed cargo from/to lysosomes (300). It was also shown that PtdIns(3,5)P₂ (ergo, PIKfyve) is required for syntenin-mediated syndecan recycling pathway (130) and for a delivery of endocytic cargo to multivesicular endosomes or bodies (MVBs) (301). MVBs can fuse with lysosomes or can be exported as exosomes. Notably, previous studies also highlighted an involvement of HSPGs (syndecans) in the formation of MVBs and exosomes (302,303). On the other hand, *S. Typhimurium* interferes with endo-lysosome trafficking, which disturbs assembly of the endosomal sorting complex required for transport (ESCRT) system (304) and exocytosis (305,306). Overall, the mechanisms of PGs/GAGs – endo-lysosomal system – SCV/SIF interactions need further clarification.

Proteoglycan-dependent endo-lysosomal fusion alters vacuolar pH.

We also discovered that CHO Δ XylT cells had reduced LysoTracker staining, which indicated elevated pH levels within late endosomes/lysosomes. By using an acid shock response reporter *Salmonella* strain, we found that CHO WT and CHO Δ XylT cells had comparable early acidification of the SCVs, however, at a later time point, acidification of SCVs in PG-deficient CHO cells was indeed impaired. Previous studies demonstrated that phagolysosomal alkalization (e.g., caused by chloroquine or ammonium chloride) can lead

to increased killing of intra-vacuolar *Staphylococcus aureus* (307) and *Coxiella burnetii* (308), probably, by improving efficacy of antibiotics. Indeed, it was shown that the acidic pH of lysosomes depresses the antibacterial activity of gentamicin and of other aminoglycosides and prevents their accumulation in vacuoles (309). Previously, an important role of intraphagosomal pH for susceptibility of *S. Typhimurium* to gentamicin was shown in macrophages (310).

We propose that alkalinization of lysosomes in PG-deficient CHO $\Delta XylT$ cells may be a result of abnormal PIKfyve-dependent endo-lysosomal fusion and may enhance gentamicin-mediated killing of *Salmonella*. Thus, the observed differences in pH could represent another mechanism (apart from a re-distribution of gentamicin discussed earlier) of the PG-dependent phenotype of intracellular *Salmonella* survival. Additional studies are required to actually measure pH values within organelles of CHO $\Delta XylT$ cells and to further investigate the functional consequences.

5. Conclusions and outlook

In conclusion, our results show a substantial role of host glycoconjugates in the susceptibility to *S. Typhimurium* infection. In **study I**, we demonstrated that Std fimbriae binding of terminal α -1,2 fucose residues mediate bacterial adherence to host glycoproteins or glycolipids. Our results conclusively demonstrate that during the course of infection, *S. Typhimurium* benefits from host fucosylation in the large intestine, in a mechanism that requires an expression of Std fimbriae by *Salmonella* in the gut lumen.

In **study II**, we discovered that in the absence of cell surface proteoglycans (PGs), epithelial cells display reduced or delayed endo-lysosomal fusion resulting in abnormal intracellular vesicle trafficking and in reduced vesicle acidification. We showed that PIKfyve-dependent fusion and intra-vacuolar pH are crucial for intracellular bacteria to survive in a replicative niche, *Salmonella*-containing vacuole. To conclude, we describe a novel role of host PGs in *S. Typhimurium* infection.

Finally, our findings raised the following questions which can be addressed in the future studies:

- Does secretor status affect a composition of mucus-associated and fecal microbiota in mice?
- Will *Salmonella* mutants unable to utilize fucose have a reduced colonization in secretor mice? Can sialic acid utilization compensate this defect?
- Is it possible to modulate *S. Typhimurium* colonization of the large intestine by using fucosylated HMOs?
- Shang et al. (311) reported that keratan sulfate isolated from shark cartilage altered the gut microbiota taxonomy and increased the prevalence of *Lactobacillus* spp. Do HSPGs influence a composition or function of the gut microbiota? If so, how exactly?
- Can gut commensals degrade/utilize glycans present of GAG chains of PGs?
- Do PGs mediate bacterial adhesion or intracellular survival of commensals and of pathogens in the gut?
- Can *S. Typhimurium* bind cell surface PGs by utilizing PagN adhesin in vivo?
- What is a role of PG-dependent endo-lysosomal fusion in macrophages?
- What are the other functional consequences of the elevated intra-lysosomal pH in PG-deficient CHO cells? Is there an effect on lysosomal ROS production?

6. List of references

1. Sekirov I, Russell SL, Antunes LCM, Finlay BB. Gut microbiota in health and disease. *Physiol Rev*. 2010 Jul;90(3):859–904.
2. Swidsinski A. Spatial organization of bacterial flora in normal and inflamed intestine: a fluorescence *in situ* hybridization study in mice. *World J Gastroenterol*. 2005;11(8):1131.
3. Lepage P, Seksik P, Sutren M, de la Cochetière M-F, Jian R, Marteau P, et al. Biodiversity of the mucosa-associated microbiota is stable along the distal digestive tract in healthy individuals and patients with IBD. *Inflamm Bowel Dis*. 2005 May 1;11(5):473–80.
4. Bajaj JS, Hylemon PB, Ridlon JM, Heuman DM, Daita K, White MB, et al. Colonic mucosal microbiome differs from stool microbiome in cirrhosis and hepatic encephalopathy and is linked to cognition and inflammation. *Am J Physiol-Gastrointest Liver Physiol*. 2012 Jul 19;303(6):G675–85.
5. Xu J, Gordon JI. Honor thy symbionts. *Proc Natl Acad Sci*. 2003 Sep 2;100(18):10452–9.
6. Round JL, Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol*. 2009 May;9(5):313–23.
7. Ivanov II, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*. 2009 Oct 30;139(3):485–98.
8. Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y, et al. Microbiota-dependent crosstalk between macrophages and ILC3 promotes intestinal homeostasis. *Science* [Internet]. 2014 Mar 28 [cited 2020 Jan 9];343(6178). Available from: <https://science.sciencemag.org/content/343/6178/1249288>
9. Yano JM, Yu K, Donaldson GP, Shastri GG, Ann P, Ma L, et al. Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell*. 2015 Apr 9;161(2):264–76.
10. Lyte M, Chapel A, Lyte JM, Ai Y, Proctor A, Jane J-L, et al. Resistant starch alters the microbiota-gut brain axis: implications for dietary modulation of behavior. *PLOS ONE*. 2016 Jan 8;11(1):e0146406.
11. Gordon JI. Honor thy gut symbionts redux. *Science*. 2012 Jun 8;336(6086):1251–3.
12. Schaubeck M, Clavel T, Calasan J, Lagkouravdos I, Haange SB, Jehmlich N, et al. Dysbiotic gut microbiota causes transmissible Crohn’s disease-like ileitis independent of failure in antimicrobial defence. *Gut*. 2016 Feb 1;65(2):225–37.
13. Feng Q, Liang S, Jia H, Stadlmayr A, Tang L, Lan Z, et al. Gut microbiome development along the colorectal adenoma–carcinoma sequence. *Nat Commun*. 2015 Mar 11;6(1):1–13.
14. Crouzet L, Gaultier E, Del’Homme C, Cartier C, Delmas E, Dapoigny M, et al. The hypersensitivity to colonic distension of IBS patients can be transferred to rats through their fecal microbiota. *Neurogastroenterol Motil*. 2013;25(4):e272–82.

15. Turnbaugh PJ, Ley RE, Mahowald MA, Magrini V, Mardis ER, Gordon JI. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature*. 2006 Dec;444(7122):1027–31.
16. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, et al. Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* [Internet]. 2013 Sep 6 [cited 2020 Jan 8];341(6150). Available from: <https://science.sciencemag.org/content/341/6150/1241214>
17. Dzidic M, Abrahamsson TR, Artacho A, Björkstén B, Collado MC, Mira A, et al. Aberrant IgA responses to the gut microbiota during infancy precede asthma and allergy development. *J Allergy Clin Immunol*. 2017 Mar 1;139(3):1017-1025.e14.
18. Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS, et al. Evolution of mammals and their gut microbes. *Science*. 2008 Jun 20;320(5883):1647–51.
19. Bonder MJ, Kurilshikov A, Tigchelaar EF, Mujagic Z, Imhann F, Vila AV, et al. The effect of host genetics on the gut microbiome. *Nat Genet*. 2016 Nov;48(11):1407–12.
20. David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, et al. Diet rapidly and reproducibly alters the human gut microbiome. *Nature*. 2014 Jan;505(7484):559–63.
21. Bokulich NA, Chung J, Battaglia T, Henderson N, Jay M, Li H, et al. Antibiotics, birth mode, and diet shape microbiome maturation during early life. *Sci Transl Med*. 2016 Jun 15;8(343):343ra82-343ra82.
22. Clayton JB, Vangay P, Huang H, Ward T, Hillmann BM, Al-Ghalith GA, et al. Captivity humanizes the primate microbiome. *Proc Natl Acad Sci*. 2016 Sep 13;113(37):10376–81.
23. Jakobsson HE, Abrahamsson TR, Jenmalm MC, Harris K, Quince C, Jernberg C, et al. Decreased gut microbiota diversity, delayed Bacteroidetes colonisation and reduced Th1 responses in infants delivered by Caesarean section. *Gut*. 2014 Apr 1;63(4):559–66.
24. Kembel SW, Jones E, Kline J, Northcutt D, Stenson J, Womack AM, et al. Architectural design influences the diversity and structure of the built environment microbiome. *ISME J*. 2012 Aug;6(8):1469–79.
25. Rothschild D, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, et al. Environment dominates over host genetics in shaping human gut microbiota. *Nature*. 2018 08;555(7695):210–5.
26. Coyte KZ, Schluter J, Foster KR. The ecology of the microbiome: Networks, competition, and stability. *Science*. 2015 Nov 6;350(6261):663–6.
27. Bosch TCG, McFall-Ngai MJ. Metaorganisms as the new frontier. *Zoology*. 2011 Sep 1;114(4):185–90.
28. Deines P, Bosch TCG. Transitioning from microbiome composition to microbial community interactions: the potential of the metaorganism Hydra as an experimental model. *Front Microbiol* [Internet]. 2016 [cited 2020 Jan 9];7. Available from: <https://www.frontiersin.org/articles/10.3389/fmicb.2016.01610/full#h5>

29. Mandić AD, Woting A, Jaenicke T, Sander A, Sabrowski W, Rolle-Kampczyk U, et al. *Clostridium ramosum* regulates enterochromaffin cell development and serotonin release. *Sci Rep*. 2019 Feb 4;9(1):1–15.
30. Derrien M, van Baarlen P, Hooiveld G, Norin E, Muller M, de Vos W. Modulation of mucosal immune response, tolerance, and proliferation in mice colonized by the mucin-degrader *Akkermansia muciniphila*. *Front Microbiol* [Internet]. 2011 [cited 2020 Jan 13];2. Available from: <https://www.frontiersin.org/articles/10.3389/fmicb.2011.00166/full#h4>
31. Mueller C, Macpherson AJ. Layers of mutualism with commensal bacteria protect us from intestinal inflammation. *Gut*. 2006 Feb 1;55(2):276–84.
32. Vieira SM, Hiltensperger M, Kumar V, Zegarra-Ruiz D, Dehner C, Khan N, et al. Translocation of a gut pathobiont drives autoimmunity in mice and humans. *Science*. 2018 Mar 9;359(6380):1156–61.
33. Buckley A, Turner JR. Cell biology of tight junction barrier regulation and mucosal disease. *Cold Spring Harb Perspect Biol*. 2018 Jan 1;10(1):a029314.
34. Teshima CW, Dieleman LA, Meddings JB. Abnormal intestinal permeability in Crohn's disease pathogenesis. *Ann N Y Acad Sci*. 2012;1258(1):159–65.
35. Johansson MEV, Hansson GC. Immunological aspects of intestinal mucus and mucins. *Nat Rev Immunol*. 2016 Oct;16(10):639–49.
36. Atuma C, Strugala V, Allen A, Holm L. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. *Am J Physiol-Gastrointest Liver Physiol*. 2001 May 1;280(5):G922–9.
37. Egberts HJA, Koninkx JFJG, Dijk JE van, Mouwen JMVM. Biological and pathobiological aspects of the glycocalyx of the small intestinal epithelium. A review. *Vet Q*. 1984 Sep 1;6(4):186–99.
38. Gebert A, Posselt W. Glycoconjugate expression defines the origin and differentiation pathway of intestinal M-cells. *J Histochem Cytochem*. 1997 Oct 1;45(10):1341–50.
39. Johansson MEV. Fast renewal of the distal colonic mucus layers by the surface goblet cells as measured by in vivo labeling of mucin glycoproteins. *PLOS ONE*. 2012 Jul 17;7(7):e41009.
40. van der Waaij LA, Harmsen HJM, Madjipour M, Kroese FGM, Zwieters M, van Dullemen HM, et al. Bacterial population analysis of human colon and terminal ileum biopsies with 16s rRNA-based fluorescent probes: commensal bacteria live in suspension and have no direct contact with epithelial cells. *Inflamm Bowel Dis*. 2005 Oct 1;11(10):865–71.
41. Johansson MEV, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC. The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci*. 2008 Sep 30;105(39):15064–9.
42. Mowat AM, Agace WW. Regional specialization within the intestinal immune system. *Nat Rev Immunol*. 2014 Oct;14(10):667–85.

43. Meyer-Hoffert U, Hornef MW, Henriques-Normark B, Axelsson L-G, Midtvedt T, Pütsep K, et al. Secreted enteric antimicrobial activity localises to the mucus surface layer. *Gut*. 2008 Jun 1;57(6):764–71.
44. Yin Y, Wang Y, Zhu L, Liu W, Liao N, Jiang M, et al. Comparative analysis of the distribution of segmented filamentous bacteria in humans, mice and chickens. *ISME J*. 2013 Mar;7(3):615–21.
45. Garland CD, Lee A, Dickson MR. Segmented filamentous bacteria in the rodent small intestine: their colonization of growing animals and possible role in host resistance to *Salmonella*. *Microb Ecol*. 1982 Oct 1;8(2):181–90.
46. Heczko U, Abe A, Brett Finlay B. Segmented filamentous bacteria prevent colonization of enteropathogenic *Escherichia coli* O103 in rabbits. *J Infect Dis*. 2000 Mar 1;181(3):1027–33.
47. Shi Z, Zou J, Zhang Z, Zhao X, Noriega J, Zhang B, et al. Segmented filamentous bacteria prevent and cure rotavirus infection. *Cell*. 2019 Oct 17;179(3):644–658.e13.
48. Stappenbeck TS, Hooper LV, Gordon JI. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proc Natl Acad Sci*. 2002 Nov 26;99(24):15451–5.
49. Ukena SN, Singh A, Dringenberg U, Engelhardt R, Seidler U, Hansen W, et al. Probiotic *Escherichia coli* Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. *PLOS ONE*. 2007 Dec 12;2(12):e1308.
50. Wittchen ES, Haskins J, Stevenson BR. Protein Interactions at the tight junction actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. *J Biol Chem*. 1999 Dec 3;274(49):35179–85.
51. Jakobsson HE, Rodríguez-Piñero AM, Schütte A, Ermund A, Boysen P, Bemark M, et al. The composition of the gut microbiota shapes the colon mucus barrier. *EMBO Rep*. 2015 Feb 1;16(2):164–77.
52. Balmer ML, Slack E, Gottardi A de, Lawson MAE, Hapfelmeier S, Miele L, et al. The liver may act as a firewall mediating mutualism between the host and its gut commensal microbiota. *Sci Transl Med*. 2014 May 21;6(237):237ra66–237ra66.
53. Berg RD. Bacterial translocation from the gastrointestinal tract. *Trends Microbiol*. 1995 Apr 1;3(4):149–54.
54. Deitch EA, Berg R. Bacterial translocation from the gut. A mechanism of infection. *J Burn Care Rehabil*. 1987 Nov 1;8(6):475–82.
55. Dinh DM, Volpe GE, Duffalo C, Bhalchandra S, Tai AK, Kane AV, et al. Intestinal microbiota, microbial translocation, and systemic inflammation in chronic HIV infection. *J Infect Dis*. 2015 Jan 1;211(1):19–27.
56. Baker JW, Deitch EA, Li MA, Berg RD, Specian RD. Hemorrhagic shock induces bacterial translocation from the gut. *J Trauma Acute Care Surg*. 1988 Jul;28(7):896.

57. Johansson MEV, Gustafsson JK, Holmén-Larsson J, Jabbar KS, Xia L, Xu H, et al. Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut*. 2014 Feb 1;63(2):281–91.
58. Chieppa M, Rescigno M, Huang AYC, Germain RN. Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement. *J Exp Med*. 2006 Dec 25;203(13):2841–52.
59. Mabbott NA, Donaldson DS, Ohno H, Williams IR, Mahajan A. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. *Mucosal Immunol*. 2013 Jul;6(4):666–77.
60. Mantis NJ, Rol N, Corthésy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol*. 2011 Nov;4(6):603–11.
61. Mora JR, Iwata M, Eksteen B, Song S-Y, Junt T, Senman B, et al. Generation of gut-homing IgA-Secreting B Cells by intestinal dendritic cells. *Science*. 2006 Nov 17;314(5802):1157–60.
62. Tezuka H, Ohteki T. Regulation of IgA production by intestinal dendritic cells and related cells. *Front Immunol* [Internet]. 2019 [cited 2020 Jan 14];10. Available from: <https://www.frontiersin.org/articles/10.3389/fimmu.2019.01891/full#B3>
63. Rogier EW, Frantz AL, Bruno MEC, Kaetzel CS. Secretory IgA is concentrated in the outer layer of colonic mucus along with gut bacteria. *Pathogens*. 2014 Jun;3(2):390–403.
64. Waaij LA van der, Limburg PC, Mesander G, Waaij D van der. In vivo IgA coating of anaerobic bacteria in human faeces. *Gut*. 1996 Mar 1;38(3):348–54.
65. Duchmann R, Kaiser I, Hermann E, Mayet W, Ewe K, Büschenfelde K-HMZ. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin Exp Immunol*. 1995;102(3):448–55.
66. Bunker JJ, Erickson SA, Flynn TM, Henry C, Koval JC, Meisel M, et al. Natural polyreactive IgA antibodies coat the intestinal microbiota. *Science* [Internet]. 2017 Oct 20 [cited 2020 Jan 14];358(6361). Available from: <https://science.sciencemag.org/content/358/6361/eaan6619>
67. Pabst O, Slack E. IgA and the intestinal microbiota: the importance of being specific. *Mucosal Immunol*. 2020 Jan;13(1):12–21.
68. Apter FM, Lencer WI, Finkelstein RA, Mekalanos JJ, Neutra MR. Monoclonal immunoglobulin A antibodies directed against cholera toxin prevent the toxin-induced chloride secretory response and block toxin binding to intestinal epithelial cells in vitro. *Infect Immun*. 1993 Dec 1;61(12):5271–8.
69. Stubbe H, Berdoz J, Kraehenbuhl J-P, Corthésy B. Polymeric IgA is superior to monomeric IgA and IgG carrying the same variable domain in preventing *Clostridium difficile* toxin A damaging of T84 monolayers. *J Immunol*. 2000 Feb 15;164(4):1952–60.

70. Helander A, Miller CL, Myers KS, Neutra MR, Nibert ML. Protective immunoglobulin A and G antibodies bind to overlapping inter-subunit epitopes in the head domain of type 1 reovirus adhesin $\sigma 1$. *J Virol*. 2004 Oct 1;78(19):10695–705.
71. Stokes CR, Soothill JF, Turner MW. Immune exclusion is a function of IgA. *Nature*. 1975 Jun;255(5511):745–6.
72. Wormald MR, Petrescu AJ, Pao Y-L, Glithero A, Elliott T, Dwek RA. Conformational studies of oligosaccharides and glycopeptides: complementarity of NMR, X-ray crystallography, and molecular modelling. *Chem Rev*. 2002 Feb 1;102(2):371–86.
73. Varki A. Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology*. 1993 Apr 1;3(2):97–130.
74. Varki A, Lowe JB. Biological Roles of Glycans. In: Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, et al., editors. *Essentials of Glycobiology* [Internet]. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009 [cited 2020 Jan 8]. Available from: <http://www.ncbi.nlm.nih.gov/books/NBK1897/>
75. Pinho SS, Reis CA. Glycosylation in cancer: mechanisms and clinical implications. *Nat Rev Cancer*. 2015 Sep;15(9):540–55.
76. Hennet T. Diseases of glycosylation beyond classical congenital disorders of glycosylation. *Biochim Biophys Acta BBA - Gen Subj*. 2012 Sep 1;1820(9):1306–17.
77. Kasper BT, Koppolu S, Mahal LK. Insights into miRNA regulation of the human glycome. *Biochem Biophys Res Commun*. 2014 Mar 21;445(4):774–9.
78. Steen PV den, Rudd PM, Dwek RA, Opdenakker G. Concepts and principles of O-Linked glycosylation. *Crit Rev Biochem Mol Biol*. 1998 Jan 1;33(3):151–208.
79. Dove A. The bittersweet promise of glycobiology. *Nat Biotechnol*. 2001 Oct;19(10):913–7.
80. Burda P, Aeby M. The dolichol pathway of N-linked glycosylation. *Biochim Biophys Acta BBA - Gen Subj*. 1999 Jan 6;1426(2):239–57.
81. Sears P, Wong C-H. Enzyme action in glycoprotein synthesis. *Cell Mol Life Sci CMLS*. 1998 Mar 1;54(3):223–52.
82. Helenius A, Aeby M. Intracellular functions of N-Linked glycans. *Science*. 2001 Mar 23;291(5512):2364–9.
83. Ikezawa H. Glycosylphosphatidylinositol (GPI)-anchored proteins. *Biol Pharm Bull*. 2002;25(4):409–17.
84. Hofsteenge J, Müller DR, de Beer T, Löffler A, Richter WJ, Vliegthart JF. New type of linkage between a carbohydrate and a protein: C-glycosylation of a specific tryptophan residue in human RNase U. *Biochemistry*. 1994 Nov 22;33(46):13524–30.
85. Haynes PA. Phosphoglycosylation: A new structural class of glycosylation? *Glycobiology*. 1998 Jan 1;8(1):1–5.

86. Corfield AP, Carroll D, Myerscough N, Probert CS. Mucins in the gastrointestinal tract in health and disease. *Front Biosci J Virtual Libr.* 2001 Oct 1;6:D1321-1357.
87. Williams SJ, McGuckin MA, Gotley DC, Eyre HJ, Sutherland GR, Antalis TM. Two novel mucin genes down-regulated in colorectal cancer identified by differential display. *Cancer Res.* 1999 Aug 15;59(16):4083–9.
88. Gum JR, Crawley SC, Hicks JW, Szymkowski DE, Kim YS. MUC17, a novel membrane-tethered mucin. *Biochem Biophys Res Commun.* 2002 Mar 1;291(3):466–75.
89. Sun WW, Krystofiak ES, Leo-Macias A, Cui R, Sesso A, Weigert R, et al. Nanoarchitecture and dynamics of the mouse enteric glycocalyx examined by freeze-etching electron tomography and intravital microscopy. *Commun Biol.* 2020 Jan 7;3(1):1–10.
90. Dekker J, Rossen JWA, Büller HA, Einerhand AWC. The MUC family: an obituary. *Trends Biochem Sci.* 2002 Mar 1;27(3):126–31.
91. Bansil R, Turner BS. Mucin structure, aggregation, physiological functions and biomedical applications. *Curr Opin Colloid Interface Sci.* 2006 Jun 1;11(2):164–70.
92. Robbe C, Capon C, Coddeville B, Michalski J-C. Structural diversity and specific distribution of O-glycans in normal human mucins along the intestinal tract. *Biochem J.* 2004 Dec 1;384(2):307–16.
93. Hattrop CL, Gendler SJ. Structure and function of the cell surface (tethered) mucins. *Annu Rev Physiol.* 2008;70(1):431–57.
94. Bell SL, Xu G, Khatri IA, Wang R, Rahman S, Forstner JF. N-linked oligosaccharides play a role in disulphide-dependent dimerization of intestinal mucin Muc2. *Biochem J.* 2003 Aug 1;373(3):893–900.
95. Ravn V, Dabelsteen E. Tissue distribution of histo-blood group antigens. *APMIS.* 2000;108(1):1–28.
96. Moran AP, Gupta A, Joshi L. Sweet-talk: role of host glycosylation in bacterial pathogenesis of the gastrointestinal tract. *Gut.* 2011 Oct 1;60(10):1412–25.
97. Gopal PK, Gill HS. Oligosaccharides and glycoconjugates in bovine milk and colostrum. *Br J Nutr.* 2000 Nov;84 Suppl 1:S69-74.
98. Bode L. Human milk oligosaccharides: Every baby needs a sugar mama. *Glycobiology.* 2012 Sep 1;22(9):1147–62.
99. Breton C, Oriol R, Imberty A. Conserved structural features in eukaryotic and prokaryotic fucosyltransferases. *Glycobiology.* 1998 Jan 1;8(1):87–94.
100. Miyoshi E, Uozumi N, Sobajima T, Takamatsu S, Kamada Y. Roles of fucosyltransferases in cancer phenotypes. In: Furukawa K, Fukuda M, editors. *Glycosignals in Cancer: Mechanisms of Malignant Phenotypes* [Internet]. Tokyo: Springer Japan; 2016 [cited 2020 Mar 27]. p. 3–16. Available from: https://doi.org/10.1007/978-4-431-55939-9_1

101. Björk S, Breimer ME, Hansson GC, Karlsson KA, Leffler H. Structures of blood group glycosphingolipids of human small intestine. A relation between the expression of fucolipids of epithelial cells and the ABO, Le and Se phenotype of the donor. *J Biol Chem*. 1987 May 15;262(14):6758–65.
102. Taylor SL, McGuckin MA, Wesselingh S, Rogers GB. Infection's sweet tooth: how glycans mediate infection and disease susceptibility. *Trends Microbiol*. 2018 Feb 1;26(2):92–101.
103. Finne J, Breimer ME, Hansson GC, Karlsson KA, Leffler H, Vliegthart JF, et al. Novel polyfucosylated N-linked glycopeptides with blood group A, H, X, and Y determinants from human small intestinal epithelial cells. *J Biol Chem*. 1989 Apr 5;264(10):5720–35.
104. Marionneau S, Cailleau-Thomas A, Rocher J, Le Moullac-Vaidye B, Ruvoën N, Clément M, et al. ABH and Lewis histo-blood group antigens, a model for the meaning of oligosaccharide diversity in the face of a changing world. *Biochimie*. 2001 Jul 1;83(7):565–73.
105. Kelly RJ, Rouquier S, Giorgi D, Lennon GG, Lowe JB. Sequence and expression of a candidate for the human Secretor blood group alpha(1,2)fucosyltransferase gene (*FUT2*). Homozygosity for an enzyme-inactivating nonsense mutation commonly correlates with the non-secretor phenotype. *J Biol Chem*. 1995 Mar 3;270(9):4640–9.
106. Henry SM, Benny AG, Woodfield DG. Investigation of Lewis phenotypes in polynesians: evidence of a weak secretor phenotype. *Vox Sang*. 1990;58(1):61–6.
107. Yu LC, Yang YH, Broadberry RE, Chen YH, Chan YS, Lin M. Correlation of a missense mutation in the human Secretor α 1,2-fucosyltransferase gene with the Lewis(a+b+) phenotype: a potential molecular basis for the weak Secretor allele (*Se^w*). *Biochem J*. 1995 Dec 1;312(2):329–32.
108. Serpa J, Almeida R, Oliveira C, Silva FS, Silva E, Reis C, et al. Lewis enzyme (α 1–3/4 fucosyltransferase) polymorphisms do not explain the Lewis phenotype in the gastric mucosa of a Portuguese population. *J Hum Genet*. 2003 Apr;48(4):183–9.
109. Bhende YM, Deshpande CK, Bhatia HM, Sanger R, Race RR, Morgan WTJ, et al. A “new” blood group character related to the ABO system. *Lancet Lond Engl*. 1952 May 3;1(6714):903–4.
110. Koda Y, Soejima M, Johnson PH, Smart E, Kimura H. Missense mutation of *FUT1* and deletion of *FUT2* are responsible for Indian Bombay phenotype of ABO blood group system. *Biochem Biophys Res Commun*. 1997 Sep 8;238(1):21–5.
111. Kelly RJ, Ernst LK, Larsen RD, Bryant JG, Robinson JS, Lowe JB. Molecular basis for H blood group deficiency in Bombay (Oh) and para-Bombay individuals. *Proc Natl Acad Sci*. 1994 Jun 21;91(13):5843–7.
112. Lindén S, Mahdavi J, Semino-Mora C, Olsen C, Carlstedt I, Borén T, et al. Role of ABO Secretor status in mucosal innate immunity and *H. pylori* infection. *PLoS Pathog* [Internet]. 2008 Jan [cited 2020 Mar 27];4(1). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2174967/>

113. Ferrer-Admetlla A, Sikora M, Laayouni H, Esteve A, Roubinet F, Blancher A, et al. A natural history of *FUT2* polymorphism in humans. *Mol Biol Evol.* 2009 Sep 1;26(9):1993–2003.
114. Soejima M, Fujimoto R, Agusa T, Iwata H, Fujihara J, Takeshita H, et al. Genetic variation of *FUT2* in a Vietnamese population: identification of two novel Se enzyme–inactivating mutations. *Transfusion (Paris).* 2012;52(6):1268–75.
115. Fumagalli M, Cagliani R, Pozzoli U, Riva S, Comi GP, Menozzi G, et al. Widespread balancing selection and pathogen-driven selection at blood group antigen genes. *Genome Res.* 2009 Feb 1;19(2):199–212.
116. Silva LM, Carvalho AS, Guillon P, Seixas S, Azevedo M, Almeida R, et al. Infection-associated *FUT2* (Fucosyltransferase 2) genetic variation and impact on functionality assessed by in vivo studies. *Glycoconj J.* 2010 Jan 1;27(1):61–8.
117. Barton SJ, Murray R, Lillycrop KA, Inskip HM, Harvey NC, Cooper C, et al. *FUT2* genetic variants and reported respiratory and gastrointestinal illnesses during infancy. *J Infect Dis.* 2019 Feb 15;219(5):836–43.
118. Rausch P, Künzel S, Suwandi A, Grassl GA, Rosenstiel P, Baines JF. Multigenerational influences of the *Fut2* gene on the dynamics of the gut microbiota in mice. *Front Microbiol* [Internet]. 2017 [cited 2019 Feb 6];8. Available from: <https://www.frontiersin.org/articles/10.3389/fmicb.2017.00991/full>
119. Davenport ER, Goodrich JK, Bell JT, Spector TD, Ley RE, Clark AG. ABO antigen and secretor statuses are not associated with gut microbiota composition in 1,500 twins. *BMC Genomics.* 2016 Nov 21;17(1):941.
120. Thomsson KA, Schulz BL, Packer NH, Karlsson NG. MUC5B glycosylation in human saliva reflects blood group and secretor status. *Glycobiology.* 2005 Aug 1;15(8):791–804.
121. Iozzo RV, Schaefer L. Proteoglycan form and function: a comprehensive nomenclature of proteoglycans. *Matrix Biol.* 2015 Mar;42:11–55.
122. Esko JD, Stewart TE, Taylor WH. Animal cell mutants defective in glycosaminoglycan biosynthesis. *Proc Natl Acad Sci.* 1985 May 1;82(10):3197–201.
123. Taylor KR, Gallo RL. Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. *FASEB J.* 2006 Jan;20(1):9–22.
124. Mulloy B, Forster MJ. Conformation and dynamics of heparin and heparan sulfate. *Glycobiology.* 2000 Nov 1;10(11):1147–56.
125. Xu D, Esko JD. Demystifying heparan sulfate–protein interactions. *Annu Rev Biochem.* 2014;83(1):129–57.
126. Kolset SO, Tveit H. Serglycin – structure and biology. *Cell Mol Life Sci.* 2008 Apr 1;65(7):1073–85.

127. Noonan DM, Fulle A, Valente P, Cai S, Horigan E, Sasaki M, et al. The complete sequence of perlecan, a basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein-receptor, and the neural cell adhesion molecule. *J Biol Chem*. 1991 Dec 5;266(34):22939–47.
128. Sarrazin S, Lamanna WC, Esko JD. Heparan sulfate proteoglycans. *Cold Spring Harb Perspect Biol*. 2011 Jul 1;3(7):a004952.
129. Bülow HE, Hobert O. The molecular diversity of glycosaminoglycans shapes animal development. *Annu Rev Cell Dev Biol*. 2006;22(1):375–407.
130. Zimmermann P, Zhang Z, Degeest G, Mortier E, Leenaerts I, Coomans C, et al. Syndecan recycling is controlled by syntenin-PIP2 interaction and Arf6. *Dev Cell*. 2005 Sep 1;9(3):377–88.
131. Maurel P, Rauch U, Flad M, Margolis RK, Margolis RU. Phosphacan, a chondroitin sulfate proteoglycan of brain that interacts with neurons and neural cell-adhesion molecules, is an extracellular variant of a receptor-type protein tyrosine phosphatase. *Proc Natl Acad Sci*. 1994 Mar 29;91(7):2512–6.
132. Merline R, Schaefer RM, Schaefer L. The matricellular functions of small leucine-rich proteoglycans (SLRPs). *J Cell Commun Signal*. 2009 Dec;3(3–4):323–35.
133. Aumailley M, Gayraud B. Structure and biological activity of the extracellular matrix. *J Mol Med*. 1998 Feb 1;76(3):253–65.
134. Schmalfeldt M, Dours-Zimmermann MT, Winterhalter KH, Zimmermann DR. Versican V2 is a major extracellular matrix component of the mature bovine brain. *J Biol Chem*. 1998 Jun 19;273(25):15758–64.
135. Wight TN, Kang I, Merrilees MJ. Versican and the control of inflammation. *Matrix Biol*. 2014 Apr 1;35:152–61.
136. Melrose J. Keratan sulfate (KS)-proteoglycans and neuronal regulation in health and disease: the importance of KS-glycodynamics and interactive capability with neuroregulatory ligands. *J Neurochem*. 2019;149(2):170–94.
137. Meyer K, Linker A, Davidson EA, Weissmann B. The mucopolysaccharides of bovine cornea. *J Biol Chem*. 1953 Dec;205(2):611–6.
138. Caterson B, Melrose J. Keratan sulfate, a complex glycosaminoglycan with unique functional capability. *Glycobiology*. 2018 Apr 1;28(4):182–206.
139. Foyez T, Takeda-Uchimura Y, Ishigaki S, Narentuya, Zhang Z, Sobue G, et al. Microglial keratan sulfate epitope elicits in central nervous tissues of transgenic model mice and patients with amyotrophic lateral sclerosis. *Am J Pathol*. 2015 Nov 1;185(11):3053–65.
140. Itano N, Kimata K. Mammalian hyaluronan synthases. *IUBMB Life*. 2002;54(4):195–9.
141. Weigel PH. Functional characteristics and catalytic mechanisms of the bacterial hyaluronan synthases. *IUBMB Life*. 2002;54(4):201–11.

142. Muto J, Sayama K, Gallo RL, Kimata K. Emerging evidence for the essential role of hyaluronan in cutaneous biology. *J Dermatol Sci*. 2019 Apr 1;94(1):190–5.
143. Zhuo L, Yoneda M, Zhao M, Yingsung W, Yoshida N, Kitagawa Y, et al. Defect in SHAP-hyaluronan complex causes severe female infertility. A study by inactivation of the bikunin gene in mice. *J Biol Chem*. 2001 Mar 16;276(11):7693–6.
144. Passi A, Vigetti D, Buraschi S, Iozzo RV. Dissecting the role of hyaluronan synthases in the tumor microenvironment. *FEBS J*. 2019;286(15):2937–49.
145. Falk P, Roth KA, Gordon JI. Lectins are sensitive tools for defining the differentiation programs of mouse gut epithelial cell lineages. *Am J Physiol-Gastrointest Liver Physiol*. 1994 Jun 1;266(6):G987–1003.
146. Patsos G, Corfield A. Management of the human mucosal defensive barrier: evidence for glycan legislation. *Biol Chem*. 2009;390(7):581–590.
147. Freitas M, Axelsson L-G, Cayuela C, Midtvedt T, Trugnan G. Microbial–host interactions specifically control the glycosylation pattern in intestinal mouse mucosa. *Histochem Cell Biol*. 2002 Aug 1;118(2):149–61.
148. Bry L, Falk PG, Midtvedt T, Gordon JI. A Model of host-microbial interactions in an open mammalian ecosystem. *Science*. 1996 Sep 6;273(5280):1380–3.
149. Pusztai A, Ewen SWB, Grant G, Peumans WJ, van Damme EJM, Coates ME, et al. Lectins and also bacteria modify the glycosylation of gut surface receptors in the rat. *Glycoconj J*. 1995 Feb 1;12(1):22–35.
150. Rausch P, Rehman A, Künzel S, Häslér R, Ott SJ, Schreiber S, et al. Colonic mucosa-associated microbiota is influenced by an interaction of Crohn disease and *FUT2* (Secretor) genotype. *Proc Natl Acad Sci*. 2011 Nov 22;108(47):19030–5.
151. Sharma R, Schumacher U, Ronaasen V, Coates M. Rat intestinal mucosal responses to a microbial flora and different diets. *Gut*. 1995 Feb 1;36(2):209–14.
152. Dai D, Nanthkumar NN, Newburg DS, Walker WA. Role of oligosaccharides and glycoconjugates in intestinal host defense. *J Pediatr Gastroenterol Nutr*. 2000;30:S23.
153. Falk PG, Hooper LV, Midtvedt T, Gordon JI. Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol Mol Biol Rev*. 1998 Dec 1;62(4):1157–70.
154. Ouwerkerk JP, de Vos WM, Belzer C. Glycobiome: Bacteria and mucus at the epithelial interface. *Best Pract Res Clin Gastroenterol*. 2013 Feb 1;27(1):25–38.
155. Crociani F, Alessandrini A, Mucci MM, Biavati B. Degradation of complex carbohydrates by *Bifidobacterium* spp. *Int J Food Microbiol*. 1994 Dec 1;24(1):199–210.
156. Ravcheev DA, Thiele I. Comparative genomic analysis of the human gut microbiome reveals a broad distribution of metabolic pathways for the degradation of host-synthesized mucin glycans and utilization of mucin-derived monosaccharides. *Front Genet* [Internet].

- 2017 [cited 2020 Mar 8];8. Available from: <https://www.frontiersin.org/articles/10.3389/fgene.2017.00111/full#h4>
157. Umesaki Y, Okada Y, Matsumoto S, Imaoka A, Setoyama H. Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. *Microbiol Immunol*. 1995;39(8):555–62.
 158. Corfield AP, Wagner SA, Clamp JR, Kriaris MS, Hoskins LC. Mucin degradation in the human colon: production of sialidase, sialate O-acetyltransferase, N-acetylneuraminidase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. *Infect Immun*. 1992 Oct 1;60(10):3971–8.
 159. Lombard V, Golaconda Ramulu H, Drula E, Coutinho PM, Henrissat B. The carbohydrate-active enzymes database (CAZy) in 2013. *Nucleic Acids Res*. 2014 Jan 1;42(D1):D490–5.
 160. Terrapon N, Lombard V, Drula É, Lapébie P, Al-Masaudi S, Gilbert HJ, et al. PULDB: the expanded database of Polysaccharide Utilization Loci. *Nucleic Acids Res*. 2018 04;46(D1):D677–83.
 161. Stecher B, Berry D, Loy A. Colonization resistance and microbial ecophysiology: using gnotobiotic mouse models and single-cell technology to explore the intestinal jungle. *FEMS Microbiol Rev*. 2013;37(5):793–829.
 162. Tailford LE, Crost EH, Kavanaugh D, Juge N. Mucin glycan foraging in the human gut microbiome. *Front Genet* [Internet]. 2015 [cited 2020 Mar 14];6. Available from: <https://www.frontiersin.org/articles/10.3389/fgene.2015.00081/full>
 163. Hoskins LC, Boulding ET. Mucin degradation in human colon ecosystems: evidence for the existence and role of bacterial subpopulations producing glycosidases as extracellular enzymes. *J Clin Invest*. 1981 Jan 1;67(1):163–72.
 164. Derrien M, Vaughan EE, Plugge CM, de Vos WM. *Akkermansia muciniphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *Int J Syst Evol Microbiol*. 2004;54(5):1469–76.
 165. Derrien M, Vos WM de, Zoetendal EG. Mucin utilisation and host interactions of the novel intestinal microbe *Akkermansia muciniphila* [Internet]. 2007 [cited 2020 Mar 14]. Available from: <http://edepot.wur.nl/2279>
 166. Png CW, Lindén SK, Gilshenan KS, Zoetendal EG, McSweeney CS, Sly LI, et al. Mucolytic bacteria with increased prevalence in IBD mucosa augment in vitro utilization of mucin by other bacteria. *Am J Gastroenterol*. 2010 Nov;105(11):2420–2428.
 167. Manzo N, D’Apuzzo E, Coutinho PM, Cutting SM, Henrissat B, Ricca E. Carbohydrate-active enzymes from pigmented *Bacilli*: a genomic approach to assess carbohydrate utilization and degradation. *BMC Microbiol*. 2011 Sep 5;11(1):198.
 168. Robertson AM, Stanley RA. In vitro utilization of mucin by *Bacteroides fragilis*. *Appl Environ Microbiol*. 1982 Feb 1;43(2):325–30.

169. Cao Y, Rocha ER, Smith CJ. Efficient utilization of complex N-linked glycans is a selective advantage for *Bacteroides fragilis* in extraintestinal infections. *Proc Natl Acad Sci U S A*. 2014 Sep 2;111(35):12901–6.
170. Nakayama-Imaohji H, Ichimura M, Iwasa T, Okada N, Ohnishi Y, Kuwahara T. Characterization of a gene cluster for sialoglycoconjugate utilization in *Bacteroides fragilis*. *J Med Invest*. 2012;59(1,2):79–94.
171. Marcobal A, Barboza M, Sonnenburg ED, Pudlo N, Martens EC, Desai P, et al. *Bacteroides* in the infant gut consume milk oligosaccharides via mucus-utilization pathways. *Cell Host Microbe*. 2011 Nov 17;10(5):507–14.
172. Tsai HH, Hart CA, Rhodes JM. Production of mucin degrading sulphatase and glycosidases by *Bacteroides thetaiotaomicron*. *Lett Appl Microbiol*. 1991;13(2):97–101.
173. Wu M, McNulty NP, Rodionov DA, Khoroshkin MS, Griffin NW, Cheng J, et al. Genetic determinants of in vivo fitness and diet responsiveness in multiple human gut *Bacteroides*. *Science*. 2015 Oct 2;350(6256):aac5992.
174. Ashida H, Miyake A, Kiyohara M, Wada J, Yoshida E, Kumagai H, et al. Two distinct α -l-fucosidases from *Bifidobacterium bifidum* are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates. *Glycobiology*. 2009 Sep 1;19(9):1010–7.
175. Wakinaka T, Kiyohara M, Kurihara S, Hirata A, Chaiwangsri T, Ohnuma T, et al. Bifidobacterial α -galactosidase with unique carbohydrate-binding module specifically acts on blood group B antigen. *Glycobiology*. 2013 Feb 1;23(2):232–40.
176. Shimada Y, Watanabe Y, Wakinaka T, Funeno Y, Kubota M, Chaiwangsri T, et al. α -N-acetylglucosaminidase from *Bifidobacterium bifidum* specifically hydrolyzes α -linked N-acetylglucosamine at nonreducing terminus of O-glycan on gastric mucin. *Appl Microbiol Biotechnol*. 2015 May 1;99(9):3941–8.
177. Turrone F, Bottacini F, Foroni E, Mulder I, Kim J-H, Zomer A, et al. Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. *Proc Natl Acad Sci*. 2010 Nov 9;107(45):19514–9.
178. Yoshida E, Sakurama H, Kiyohara M, Nakajima M, Kitaoka M, Ashida H, et al. *Bifidobacterium longum* subsp. *infantis* uses two different β -galactosidases for selectively degrading type-1 and type-2 human milk oligosaccharides. *Glycobiology*. 2012 Mar 1;22(3):361–8.
179. Ward RE, Niñonuevo M, Mills DA, Lebrilla CB, German JB. In vitro fermentation of breast milk oligosaccharides by *Bifidobacterium infantis* and *Lactobacillus gasseri*. *Appl Environ Microbiol*. 2006 Jun 1;72(6):4497–9.
180. Wright DP, Rosendale DI, Robertson AM. *Prevotella* enzymes involved in mucin oligosaccharide degradation and evidence for a small operon of genes expressed during growth on mucin. *FEMS Microbiol Lett*. 2000 Sep 1;190(1):73–9.
181. Rho J, Wright DP, Christie DL, Clinch K, Furneaux RH, Robertson AM. A Novel mechanism for desulfation of mucin: identification and cloning of a mucin-desulfating

- glycosidase (sulfoglycosidase) from *Prevotella* strain RS2. J Bacteriol. 2005 Mar 1;187(5):1543–51.
182. Hoskins LC, Agustines M, McKee WB, Boulding ET, Kriaris M, Niedermeyer G. Mucin degradation in human colon ecosystems. Isolation and properties of fecal strains that degrade ABH blood group antigens and oligosaccharides from mucin glycoproteins. J Clin Invest. 1985 Mar 1;75(3):944–53.
183. Anderson KM, Ashida H, Maskos K, Dell A, Li S-C, Li Y-T. A Clostridial endo- β -galactosidase that cleaves both blood group a and b glycotopes the first member of a new glycoside hydrolase family, GH98. J Biol Chem. 2005 Mar 4;280(9):7720–8.
184. Harrington SM, Sheikh J, Henderson IR, Ruiz-Perez F, Cohen PS, Nataro JP. The Pic protease of enteroaggregative *Escherichia coli* promotes intestinal colonization and growth in the presence of mucin. Infect Immun. 2009 Jun 1;77(6):2465–73.
185. Navarro-Garcia F, Gutierrez-Jimenez J, Garcia-Tovar C, Castro LA, Salazar-Gonzalez H, Cordova V. Pic, an autotransporter protein secreted by different pathogens in the Enterobacteriaceae family, is a potent mucus secretagogue. Infect Immun. 2010 Oct 1;78(10):4101–9.
186. Grys TE, Siegel MB, Lathem WW, Welch RA. The StcE protease contributes to intimate adherence of enterohemorrhagic *Escherichia coli* O157:H7 to host cells. Infect Immun. 2005 Mar 1;73(3):1295–303.
187. Grys TE, Walters LL, Welch RA. Characterization of the StcE protease activity of *Escherichia coli* O157:H7. J Bacteriol. 2006 Jul;188(13):4646–53.
188. Silva AJ, Pham K, Benitez JA. Haemagglutinin/protease expression and mucin gel penetration in El Tor biotype *Vibrio cholerae*. Microbiology. 2003;149(7):1883–91.
189. Szabady RL, Yanta JH, Halladin DK, Schofield MJ, Welch RA. TagA is a secreted protease of *Vibrio cholerae* that specifically cleaves mucin glycoproteins. Microbiology. 2011 Feb;157(Pt 2):516–25.
190. Josenhans C, Müthing J, Elling L, Bartfeld S, Schmidt H. How bacterial pathogens of the gastrointestinal tract use the mucosal glyco-code to harness mucus and microbiota: New ways to study an ancient bag of tricks. Int J Med Microbiol. 2020 Feb 1;310(2):151392.
191. Almagro-Moreno S, Boyd EF. Insights into the evolution of sialic acid catabolism among bacteria. BMC Evol Biol. 2009 May 26;9(1):118.
192. Cameron EA, Sperandio V. Frenemies: signaling and nutritional integration in pathogen-microbiota-host interactions. Cell Host Microbe. 2015 Sep 9;18(3):275–84.
193. Royle L, Roos A, Harvey DJ, Wormald MR, Gijlswijk-Janssen DV, Redwan E-RM, et al. Secretory IgA N- and O-Glycans provide a link between the innate and adaptive immune systems. J Biol Chem. 2003 May 30;278(22):20140–53.
194. Turroni F, Duranti S, Bottacini F, Guglielmetti S, Van Sinderen D, Ventura M. *Bifidobacterium bifidum* as an example of a specialized human gut commensal. Front

- Microbiol [Internet]. 2014 Aug 21 [cited 2020 Apr 1];5. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4140077/>
195. Marcobal A, Southwick AM, Earle KA, Sonnenburg JL. A refined palate: bacterial consumption of host glycans in the gut. *Glycobiology*. 2013 Sep 1;23(9):1038–46.
 196. Hooper LV, Xu J, Falk PG, Midtvedt T, Gordon JI. A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. *Proc Natl Acad Sci*. 1999 Aug 17;96(17):9833–8.
 197. Hoskins LC, Boulding ET. Degradation of blood group antigens in human colon ecosystems. I. In vitro production of ABH blood group-degrading enzymes by enteric bacteria. *J Clin Invest*. 1976 Jan 1;57(1):63–73.
 198. Macfarlane GT, Hay S, Gibson GR. Influence of mucin on glycosidase, protease and arylamidase activities of human gut bacteria grown in a 3-stage continuous culture system. *J Appl Bacteriol*. 1989;66(5):407–17.
 199. Robinson MG, Tolchin D, Halpern C. Enteric bacterial agents and the ABO blood groups. *Am J Hum Genet*. 1971 Mar;23(2):135–45.
 200. Proft T, Baker EN. Pili in Gram-negative and Gram-positive bacteria — structure, assembly and their role in disease. *Cell Mol Life Sci*. 2008 Oct 27;66(4):613.
 201. Telford JL, Barocchi MA, Margarit I, Rappuoli R, Grandi G. Pili in Gram-positive pathogens. *Nat Rev Microbiol*. 2006 Jul;4(7):509–19.
 202. Lillington J, Geibel S, Waksman G. Biogenesis and adhesion of type 1 and P pili. *Biochim Biophys Acta BBA - Gen Subj*. 2014 Sep 1;1840(9):2783–93.
 203. Nuccio S-P, Bäumler AJ. Evolution of the chaperone/usher assembly pathway: fimbrial classification goes greek. *Microbiol Mol Biol Rev*. 2007 Dec 1;71(4):551–75.
 204. Krogfelt KA, Bergmans H, Klemm P. Direct evidence that the FimH protein is the mannose-specific adhesin of *Escherichia coli* type 1 fimbriae. *Infect Immun*. 1990 Jun 1;58(6):1995–8.
 205. Ofek I, Mirelman D, Sharon N. Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. *Nature*. 1977 Feb;265(5595):623–5.
 206. Miguel Freitas CC. Microbial modulation of host intestinal glycosylation patterns. *Microb Ecol Health Dis*. 2000 Jan 1;12(2):165–78.
 207. Phansopa C, Roy S, Rafferty JB, Douglas CWI, Pandhal J, Wright PC, et al. Structural and functional characterization of NanU, a novel high-affinity sialic acid-inducible binding protein of oral and gut-dwelling Bacteroidetes species. *Biochem J*. 2014 Mar 15;458(3):499–511.
 208. Koropatkin N, Martens EC, Gordon JI, Smith TJ. Structure of a SusD homologue, BT1043, involved in mucin O-glycan utilization in a prominent human gut symbiont,. *Biochemistry*. 2009 Feb 24;48(7):1532–42.

209. Roos S, Jonsson H. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiology*,. 2002;148(2):433–42.
210. Nishiyama K, Ueno S, Sugiyama M, Yamamoto Y, Mukai T. *Lactobacillus rhamnosus* GG SpaC pilin subunit binds to the carbohydrate moieties of intestinal glycoconjugates. *Anim Sci J*. 2016;87(6):809–15.
211. Turrone F, Serafini F, Foroni E, Duranti S, Motherway MO, Taverniti V, et al. Role of sortase-dependent pili of *Bifidobacterium bifidum* PRL2010 in modulating bacterium–host interactions. *Proc Natl Acad Sci*. 2013 Jul 2;110(27):11151–6.
212. Farfan MJ, Cantero L, Vidal R, Botkin DJ, Torres AG. Long polar fimbriae of enterohemorrhagic *Escherichia coli* O157:H7 bind to extracellular matrix proteins. *Infect Immun*. 2011 Sep;79(9):3744–50.
213. Mouricout MA, Julien RA. Pilus-mediated binding of bovine enterotoxigenic *Escherichia coli* to calf small intestinal mucins. *Infect Immun*. 1987 May 1;55(5):1216–23.
214. Kumar P, Kuhlmann FM, Chakraborty S, Bourgeois AL, Foulke-Abel J, Tumala B, et al. Enterotoxigenic *Escherichia coli*–blood group A interactions intensify diarrheal severity. *J Clin Invest*. 2018 Aug 1;128(8):3298–311.
215. Mottram L, Liu J, Chavan S, Tobias J, Svennerholm A-M, Holgersson J. Glyco-engineered cell line and computational docking studies reveals enterotoxigenic *Escherichia coli* CFA/I fimbriae bind to Lewis a glycans. *Sci Rep*. 2018 Jul 26;8(1):1–12.
216. Krivan HC, Clark GF, Smith DF, Wilkins TD. Cell surface binding site for *Clostridium difficile* enterotoxin: evidence for a glycoconjugate containing the sequence Gal alpha 1-3Gal beta 1-4GlcNAc. *Infect Immun*. 1986 Sep 1;53(3):573–81.
217. Weiss EI, Shanitzki B, Dotan M, Ganeshkumar N, Kolenbrander PE, Metzger Z. Attachment of *Fusobacterium nucleatum* PK1594 to mammalian cells and its coaggregation with periodontopathogenic bacteria are mediated by the same galactose-binding adhesin. *Oral Microbiol Immunol*. 2000;15(6):371–7.
218. Syder AJ, Guruge JL, Li Q, Hu Y, Oleksiewicz CM, Lorenz RG, et al. *Helicobacter pylori* attaches to NeuAca2,3Galβ1,4 glycoconjugates produced in the stomach of transgenic mice lacking parietal cells. *Mol Cell*. 1999 Mar 1;3(3):263–74.
219. Mahdavi J, Sondén B, Hurtig M, Olfat FO, Forsberg L, Roche N, et al. *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. *Science*. 2002 Jul 26;297(5581):573–8.
220. Ilver D, Arnqvist A, Ögren J, Frick I-M, Kersulyte D, Incecik ET, et al. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. *Science*. 1998 Jan 16;279(5349):373–7.
221. Garber N, Guempel U, Belz A, Gilboa-Garber N, Doyle RJ. On the specificity of the d-galactose-binding lectin (PA-I) of *Pseudomonas aeruginosa* and its strong binding to hydrophobic derivatives of d-galactose and thiogalactose. *Biochim Biophys Acta BBA - Gen Subj*. 1992 Jun 12;1116(3):331–3.

222. Mitchell E, Houles C, Sudakevitz D, Wimmerova M, Gautier C, Pérez S, et al. Structural basis for oligosaccharide-mediated adhesion of *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients. *Nat Struct Biol.* 2002 Dec;9(12):918–21.
223. Day CJ, Paton AW, Harvey RM, Hartley-Tassell LE, Seib KL, Tiralongo J, et al. Lectin activity of the pneumococcal pilin proteins. *Sci Rep.* 2017 Dec 19;7(1):1–6.
224. Alvarez-Domínguez C, Vázquez-Boland JA, Carrasco-Marín E, López-Mato P, Leyva-Cobán F. Host cell heparan sulfate proteoglycans mediate attachment and entry of *Listeria monocytogenes*, and the listerial surface protein ActA is involved in heparan sulfate receptor recognition. *Infect Immun.* 1997 Jan 1;65(1):78–88.
225. Lambert MA, Smith SGJ. The PagN protein mediates invasion via interaction with proteoglycan. *FEMS Microbiol Lett.* 2009 Aug;297(2):209–16.
226. Vries FPD, Cole R, Dankert J, Frosch M, Putten JPMV. *Neisseria meningitidis* producing the Opc adhesin binds epithelial cell proteoglycan receptors. *Mol Microbiol.* 1998;27(6):1203–12.
227. Huang P, Farkas T, Marionneau S, Zhong W, Ruvoën-Clouet N, Morrow AL, et al. Noroviruses bind to human ABO, Lewis, and Secretor histo-blood group antigens: identification of 4 distinct strain-specific patterns. *J Infect Dis.* 2003 Jul 1;188(1):19–31.
228. Goh YJ, Klaenhammer TR. Functional roles of aggregation-promoting-like factor in stress tolerance and adherence of *Lactobacillus acidophilus* NCFM. *Appl Environ Microbiol.* 2010 Aug 1;76(15):5005–12.
229. Morrow AL, Ruiz-Palacios GM, Altaye M, Jiang X, Guerrero ML, Meinzen-Derr JK, et al. Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. *J Pediatr.* 2004 Sep;145(3):297–303.
230. Ruiz-Palacios GM, Cervantes LE, Ramos P, Chavez-Munguia B, Newburg DS. *Campylobacter jejuni* binds intestinal H(O) antigen (Fuc alpha 1, 2Gal beta 1, 4GlcNAc), and fucosyloligosaccharides of human milk inhibit its binding and infection. *J Biol Chem.* 2003 Apr 18;278(16):14112–20.
231. Liu B, Yu Z, Chen C, Kling DE, Newburg DS. Human milk Mucin 1 and Mucin 4 inhibit *Salmonella enterica* serovar Typhimurium invasion of human intestinal epithelial cells in vitro. *J Nutr.* 2012 Aug 1;142(8):1504–9.
232. Wilhelm I, Levit-Zerdoun E, Jakob J, Villringer S, Frensch M, Übelhart R, et al. Carbohydrate-dependent B cell activation by fucose-binding bacterial lectins. *Sci Signal* [Internet]. 2019 Mar 5 [cited 2020 Apr 1];12(571). Available from: <https://stke.sciencemag.org/content/12/571/eaao7194>
233. Leifson E. Staining, shape, and arrangement of bacterial flagella. *J Bacteriol.* 1951 Oct;62(4):377–89.
234. Criscuolo A, Issenhuth-Jeanjean S, Didelot X, Thorell K, Hale J, Parkhill J, et al. The speciation and hybridization history of the genus *Salmonella*. *Microb Genomics* [Internet]. 2019 Jul 26 [cited 2020 Apr 3];5(8). Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6755497/>

235. Giammanco GM, Pignato S, Mammina C, Grimont F, Grimont PAD, Nastasi A, et al. Persistent endemicity of *Salmonella bongori* 48:z35:– in Southern Italy: molecular characterization of human, animal, and environmental isolates. J Clin Microbiol. 2002 Sep 1;40(9):3502–5.
236. Grimont, P. A., Weill, F. X. Antigenic formulae of the *Salmonella* serovars. In WHO collaborating centre for reference and research on *Salmonella*; 2007. p. 1–166.
237. Eberth CJ. Die Organismen in den Organen bei Typhus abdominalis. Arch Für Pathol Anat Physiol Für Klin Med. 1880 Jul 1;81(1):58–74.
238. Gesundheitsamt GK. Mittheilungen aus dem Kaiserlichen Gesundheitsamte: Bd. Die Aetiologie der Tuberkulose. Norddeutsche Buchdruckerei und Verlaganstalt ; zu beziehen durch Louis Gerschel Verlagsbuchhandlung (G. Gossmann); 1884. 548 p.
239. Baker S, Holt KE, Clements ACA, Karkey A, Arjyal A, Boni MF, et al. Combined high-resolution genotyping and geospatial analysis reveals modes of endemic urban typhoid fever transmission. Open Biol. 2011;1(2):110008.
240. Gal-Mor O, Boyle EC, Grassl GA. Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. Front Microbiol [Internet]. 2014 [cited 2020 Apr 3];5. Available from: <https://www.frontiersin.org/articles/10.3389/fmicb.2014.00391/full>
241. Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. Invasive non-typhoidal *Salmonella* disease: an emerging and neglected tropical disease in Africa. Lancet. 2012 Jun 30;379(9835):2489–99.
242. The European Union One Health 2018 Zoonoses Report. EFSA J. 2019;17(12):e05926.
243. Ibarra JA, Steele-Mortimer O. *Salmonella* – the ultimate insider. *Salmonella* virulence factors that modulate intracellular survival. Cell Microbiol. 2009;11(11):1579–86.
244. Wagner C, Hensel M. Adhesive Mechanisms of *Salmonella enterica*. In: Linke D, Goldman A, editors. Bacterial Adhesion: Chemistry, Biology and Physics [Internet]. Dordrecht: Springer Netherlands; 2011 [cited 2020 Apr 3]. p. 17–34. (Advances in Experimental Medicine and Biology). Available from: https://doi.org/10.1007/978-94-007-0940-9_2
245. Kingsley RA, Santos RL, Kestra AM, Adams LG, Bäumler AJ. *Salmonella enterica* serotype Typhimurium ShdA is an outer membrane fibronectin-binding protein that is expressed in the intestine. Mol Microbiol. 2002;43(4):895–905.
246. Dorsey CW, Laarakker MC, Humphries AD, Weening EH, Bäumler AJ. *Salmonella enterica* serotype Typhimurium MisL is an intestinal colonization factor that binds fibronectin. Mol Microbiol. 2005;57(1):196–211.
247. Wagner C, Barlag B, Gerlach RG, Deiwick J, Hensel M. The *Salmonella enterica* giant adhesin SiiE binds to polarized epithelial cells in a lectin-like manner. Cell Microbiol. 2014;16(6):962–75.

248. Dufresne K, Daigle F. *Salmonella* fimbriae: what is the clue to their hairdo? Curr Top *Salmonella* Salmonellosis [Internet]. 2017 Apr 5 [cited 2020 Apr 4]; Available from: <https://www.intechopen.com/books/current-topics-in-salmonella-and-salmonellosis/salmonella-fimbriae-what-is-the-clue-to-their-hairdo->
249. Humphries A, DeRidder S, Bäumler AJ. *Salmonella enterica* serotype Typhimurium fimbrial proteins serve as antigens during infection of mice. Infect Immun. 2005 Sep 1;73(9):5329–38.
250. Weening EH, Barker JD, Laarakker MC, Humphries AD, Tsolis RM, Baumler AJ. The *Salmonella enterica* serotype Typhimurium *lpf*, *bcf*, *stb*, *stc*, *std*, and *sth* fimbrial operons are required for intestinal persistence in mice. Infect Immun. 2005 Jun 1;73(6):3358–66.
251. Chessa D, Winter MG, Jakomin M, Bäumler AJ. *Salmonella enterica* serotype Typhimurium Std fimbriae bind terminal $\alpha(1,2)$ fucose residues in the cecal mucosa. Mol Microbiol. 2009;71(4):864–75.
252. Thankavel K, Shah AH, Cohen MS, Ikeda T, Lorenz RG, Curtiss R, et al. Molecular basis for the enterocyte tropism exhibited by *Salmonella* typhimurium type 1 fimbriae. J Biol Chem. 1999 Feb 26;274(9):5797–809.
253. Chessa D, Dorsey CW, Winter M, Bäumler AJ. Binding specificity of *Salmonella* plasmid-encoded fimbriae assessed by glycomics. J Biol Chem. 2008 Mar 28;283(13):8118–24.
254. Bäumler AJ, Tsolis RM, Heffron F. The *lpf* fimbrial operon mediates adhesion of *Salmonella* Typhimurium to murine Peyer's patches. Proc Natl Acad Sci. 1996 Jan 9;93(1):279–83.
255. Kato K, Ishiwa A. The role of carbohydrates in infection strategies of enteric pathogens. Trop Med Health. 2015 Mar;43(1):41–52.
256. Arabyan N, Park D, Foutouhi S, Weis AM, Huang BC, Williams CC, et al. *Salmonella* degrades the host glycocalyx leading to altered infection and glycan remodeling. Sci Rep. 2016 Jul 8;6(1):1–11.
257. Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, Gopinath S, et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. Nature. 2013 Oct;502(7469):96–9.
258. Gutnick D, Calvo JM, Klotkowski T, Ames BN. Compounds which serve as the sole source of carbon or nitrogen for *Salmonella* Typhimurium LT-2. J Bacteriol. 1969 Oct;100(1):215–9.
259. Nolle N, Felsl A, Heermann R, Fuchs TM. Genetic characterization of the galactitol utilization pathway of *Salmonella enterica* serovar Typhimurium. J Bacteriol [Internet]. 2017 Feb 15 [cited 2020 Apr 5];199(4). Available from: <https://jb.asm.org/content/199/4/e00595-16>
260. Staib L, Fuchs TM. Regulation of fucose and 1,2-propanediol utilization by *Salmonella enterica* serovar Typhimurium. Front Microbiol [Internet]. 2015 Oct 12 [cited 2019 May

261. Stecher B, Barthel M, Schlumberger MC, Haberli L, Rabsch W, Kremer M, et al. Motility allows *S. Typhimurium* to benefit from the mucosal defence. *Cell Microbiol.* 2008;10(5):1166–80.
262. Domino SE, Zhang L, Gillespie PJ, Saunders TL, Lowe JB. Deficiency of reproductive tract alpha (1,2)fucosylated glycans and normal fertility in mice with targeted deletions of the FUT1 or FUT2 alpha (1,2)fucosyltransferase locus. *Mol Cell Biol.* 2001 Dec 15;21(24):8336–45.
263. Myrdal SE, Steyger PS. TRPV1 regulators mediate gentamicin penetration of cultured kidney cells. *Hear Res.* 2005 Jun;204(1–2):170–82.
264. Karasawa T, Wang Q, Fu Y, Cohen DM, Steyger PS. TRPV4 enhances the cellular uptake of aminoglycoside antibiotics. *J Cell Sci.* 2008 Sep 1;121(17):2871–9.
265. Notenboom S. Increased apical insertion of the Multidrug Resistance Protein 2 (MRP2/ABCC2) in renal proximal tubules following gentamicin exposure. *J Pharmacol Exp Ther.* 2006 May 23;318(3):1194–202.
266. Götz A, Goebel W. Glucose and glucose 6-phosphate as carbon sources in extra- and intracellular growth of enteroinvasive *Escherichia coli* and *Salmonella enterica*. *Microbiology*,. 2010;156(4):1176–87.
267. Röder J, Hensel M. Presence of SopE and mode of infection result in increased *Salmonella*-containing vacuole damage and cytosolic release during host cell infection by *Salmonella enterica*. *Cell Microbiol.* 2020;22(5):e13155.
268. Ohkuma S, Poole B. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc Natl Acad Sci.* 1978 Jul 1;75(7):3327–31.
269. Knodler LA, Nair V, Steele-Mortimer O. Quantitative assessment of cytosolic *Salmonella* in epithelial cells. *PLOS ONE.* 2014 Jan 6;9(1):e84681.
270. Forni D, Cleynen I, Ferrante M, Cassinotti A, Cagliani R, Ardizzone S, et al. ABO histo-blood group might modulate predisposition to Crohn's disease and affect disease behavior. *J Crohns Colitis.* 2014 Jun 1;8(6):489–94.
271. Folseraas T, Melum E, Rausch P, Juran BD, Ellinghaus E, Shiryaev A, et al. Extended analysis of a genome-wide association study in primary sclerosing cholangitis detects multiple novel risk loci. *J Hepatol.* 2012 Aug 1;57(2):366–75.
272. Iwamori M, Domino SE. Tissue-specific loss of fucosylated glycolipids in mice with targeted deletion of alpha(1,2)fucosyltransferase genes. *Biochem J.* 2004 May 15;380(1):75–81.
273. Hurd EA, Holmén JM, Hansson GC, Domino SE. Gastrointestinal mucins of *Fut2*-null mice lack terminal fucosylation without affecting colonization by *Candida albicans*. *Glycobiology.* 2005 Oct 1;15(10):1002–7.

274. Terahara K, Nochi T, Yoshida M, Takahashi Y, Goto Y, Hatai H, et al. Distinct fucosylation of M cells and epithelial cells by Fut1 and Fut2, respectively, in response to intestinal environmental stress. *Biochem Biophys Res Commun*. 2011 Jan;404(3):822–8.
275. Goto Y, Obata T, Kunisawa J, Sato S, Ivanov II, Lamichhane A, et al. Innate lymphoid cells regulate intestinal epithelial cell glycosylation. *Science* [Internet]. 2014 Sep 12 [cited 2020 Apr 15];345(6202). Available from: <https://science.sciencemag.org/content/345/6202/1254009>
276. Goto Y, Lamichhane A, Kamioka M, Sato S, Honda K, Kunisawa J, et al. IL-10-producing CD4 + T cells negatively regulate fucosylation of epithelial cells in the gut. *Sci Rep*. 2015 Nov 2;5(1):1–11.
277. Pickard JM, Maurice CF, Kinnebrew MA, Abt MC, Schenten D, Golovkina TV, et al. Rapid fucosylation of intestinal epithelium sustains host–commensal symbiosis in sickness. *Nature*. 2014 Oct;514(7524):638–41.
278. López-Garrido J, Casadesús J. Crosstalk between virulence loci: regulation of *Salmonella enterica* pathogenicity island 1 (SPI-1) by products of the *std* fimbrial operon. *PLOS ONE*. 2012 Jan 23;7(1):e30499.
279. Rausch P, Steck N, Suwandi A, Seidel JA, Künzel S, Bhullar K, et al. Expression of the blood-group-related gene *B4galnt2* alters susceptibility to *Salmonella* infection. *PLOS Pathog*. 2015 Jul 2;11(7):e1005008.
280. Pham TAN, Clare S, Goulding D, Arasteh JM, Stares MD, Browne HP, et al. Epithelial IL-22RA1-mediated fucosylation promotes intestinal colonization resistance to an opportunistic pathogen. *Cell Host Microbe*. 2014 Oct 8;16(4):504–16.
281. Wacklin P, Tuimala J, Nikkila J, Tims S, Makivuokko H, Alakulppi N, et al. Faecal microbiota composition in adults is associated with the *FUT2* gene determining the secretor status. *PLoS ONE* [Internet]. 2014 Apr 1 [cited 2020 Apr 15];9(4). Available from: <https://go.gale.com/ps/i.do?p=HRCA&sw=w&issn=19326203&v=2.1&it=r&id=GALE%7CA375584025&sid=googleScholar&linkaccess=abs>
282. Rausch P, Basic M, Batra A, Bischoff SC, Blaut M, Clavel T, et al. Analysis of factors contributing to variation in the C57BL/6J fecal microbiota across German animal facilities. *Int J Med Microbiol*. 2016 Aug 1;306(5):343–55.
283. Stecher B, Robbiani R, Walker AW, Westendorf AM, Barthel M, Kremer M, et al. *Salmonella enterica* serovar Typhimurium exploits inflammation to compete with the intestinal microbiota. *PLoS Biol*. 2007 Oct;5(10):2177–89.
284. Kaiser BLD, Li J, Sanford JA, Kim Y-M, Kronewitter SR, Jones MB, et al. A multi-omic view of host-pathogen-commensal interplay in *Salmonella*-mediated intestinal infection. *PLOS ONE*. 2013 Jun 26;8(6):e67155.
285. Faber F, Thiennimitr P, Spiga L, Byndloss MX, Litvak Y, Lawhon S, et al. Respiration of microbiota-derived 1,2-propanediol drives *Salmonella* expansion during colitis. Sperandio V, editor. *PLOS Pathog*. 2017 Jan 5;13(1):e1006129.

286. Pacheco AR, Curtis MM, Ritchie JM, Munera D, Waldor MK, Moreira CG, et al. Fucose sensing regulates bacterial intestinal colonization. *Nature*. 2012 Dec;492(7427):113–7.
287. Bäumlér AJ, Tsolis RM, Heffron F. Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by *Salmonella* Typhimurium. *Infect Immun*. 1996 May 1;64(5):1862–5.
288. García-Pastor L, Sánchez-Romero MA, Gutiérrez G, Puerta-Fernández E, Casadesús J. Formation of phenotypic lineages in *Salmonella enterica* by a pleiotropic fimbrial switch. Herman C, editor. *PLOS Genet*. 2018 Sep 25;14(9):e1007677.
289. van Wijk XM, Döhrmann S, Hallström BM, Li S, Voldborg BG, Meng BX, et al. Whole-genome sequencing of invasion-resistant cells identifies Laminin $\alpha 2$ as a host factor for bacterial invasion. Park P, Pier GB, editors. *mBio* [Internet]. 2017 Mar 8 [cited 2019 Jan 18];8(1). Available from: <http://mbio.asm.org/lookup/doi/10.1128/mBio.02128-16>
290. Heithoff DM, Conner CP, Hanna PC, Julio SM, Hentschel U, Mahan MJ. Bacterial infection as assessed by in vivo gene expression. *Proc Natl Acad Sci*. 1997 Feb 4;94(3):934–9.
291. Conner CP, Heithoff DM, Julio SM, Sinsheimer RL, Mahan MJ. Differential patterns of acquired virulence genes distinguish *Salmonella* strains. *Proc Natl Acad Sci*. 1998 Apr 14;95(8):4641–5.
292. Kehl A, Göser V, Reuter T, Liss V, Franke M, John C, et al. A trafficome-wide RNAi screen reveals deployment of early and late secretory host proteins and the entire late endo-/lysosomal vesicle fusion machinery by intracellular *Salmonella*. *bioRxiv*. 2019 Nov 19;848549.
293. Nagai J, Takano M. Entry of aminoglycosides into renal tubular epithelial cells via endocytosis-dependent and endocytosis-independent pathways. *Biochem Pharmacol*. 2014 Aug;90(4):331–7.
294. Denamur S, Tyteca D, Marchand-Brynaert J, Van Bambeke F, Tulkens PM, Courtoy PJ, et al. Role of oxidative stress in lysosomal membrane permeabilization and apoptosis induced by gentamicin, an aminoglycoside antibiotic. *Free Radic Biol Med*. 2011 Nov;51(9):1656–65.
295. Brumell JH, Tang P, Zaharik ML, Finlay BB. Disruption of the *Salmonella*-containing vacuole leads to increased replication of *Salmonella enterica* serovar Typhimurium in the cytosol of epithelial cells. *Infect Immun*. 2002 Jun 1;70(6):3264–70.
296. Rojas R, Apodaca G. Immunoglobulin transport across polarized epithelial cells. *Nat Rev Mol Cell Biol*. 2002 Dec;3(12):944–56.
297. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol*. 1990 Oct 5;215(3):403–10.
298. Liss V, Swart AL, Kehl A, Hermanns N, Zhang Y, Chikkaballi D, et al. *Salmonella enterica* remodels the host cell endosomal system for efficient intravacuolar nutrition. *Cell Host Microbe*. 2017 Mar;21(3):390–402.

299. Kerr MC, Wang JTH, Castro NA, Hamilton NA, Town L, Brown DL, et al. Inhibition of the PtdIns(5) kinase PIKfyve disrupts intracellular replication of *Salmonella*. *EMBO J*. 2010 Apr 21;29(8):1331–47.
300. Krishna S, Palm W, Lee Y, Yang W, Bandyopadhyay U, Xu H, et al. PIKfyve regulates vacuole maturation and nutrient recovery following engulfment. *Dev Cell*. 2016 Sep;38(5):536–47.
301. Shaw JD, Hama H, Sohrabi F, DeWald DB, Wendland B. PtdIns(3,5)P₂ is required for delivery of endocytic cargo into the multivesicular body. *Traffic*. 2003;4(7):479–90.
302. Friand V, David G, Zimmermann P. Syntenin and syndecan in the biogenesis of exosomes: Syndecan-syntenin pathway in exosome biogenesis. *Biol Cell*. 2015 Oct;107(10):331–41.
303. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci*. 2018 Jan;75(2):193–208.
304. Dukes JD, Lee H, Hagen R, Reaves BJ, Layton AN, Galyov EE, et al. The secreted *Salmonella dublin* phosphoinositide phosphatase, SopB, localizes to PtdIns(3)P-containing endosomes and perturbs normal endosome to lysosome trafficking. *Biochem J*. 2006 Apr 15;395(2):239–47.
305. Kuhle V, Abrahams GL, Hensel M. Intracellular *Salmonella enterica* redirect exocytic transport processes in a *Salmonella* Pathogenicity Island 2-dependent manner. *Traffic*. 2006 Jun;7(6):716–30.
306. Perrett CA, Zhou D. *Salmonella* type III effector SopB modulates host cell exocytosis. *Emerg Microbes Infect*. 2013 Jan;2(1):1–6.
307. Maurin M, Raoult D. Phagolysosomal alkalization and intracellular killing of *Staphylococcus aureus* by amikacin. *J Infect Dis*. 1994 Feb 1;169(2):330–6.
308. Maurin M, Benoliel AM, Bongrand P, Raoult D. Phagolysosomal alkalization and the bactericidal effect of antibiotics: the *Coxiella burnetii* paradigm. *J Infect Dis*. 1992 Nov 1;166(5):1097–102.
309. Tulkens P, Trouet A. The uptake and intracellular accumulation of aminoglycoside antibiotics in lysosomes of cultured rat fibroblasts. *Biochem Pharmacol*. 1978 Feb 15;27(4):415–24.
310. Menashe O, Kaganskaya E, Baasov T, Yaron S. Aminoglycosides affect intracellular *Salmonella enterica* serovars Typhimurium and Virchow. *Antimicrob Agents Chemother*. 2008 Mar 1;52(3):920–6.
311. Shang Q, Li Q, Zhang M, Song G, Shi J, Jiang H, et al. Dietary keratan sulfate from shark cartilage modulates gut microbiota and increases the abundance of *Lactobacillus* spp. *Mar Drugs*. 2016 Dec;14(12):224.

7. Appendices

List of publications

Schramm G, Suwandi A, **Galeev A**, Sharma S, Braun J, Claes AK, Braubach P, Grassl GA. *Schistosoma* eggs impair protective Th1/Th17 immune responses against *Salmonella* infection. *Frontiers in Immunology*. 2018 Nov 14;9:2614.

Aviv G, Cornelius A, Davidovich M, Cohen H, Suwandi A, **Galeev A**, Steck N, Azriel S, Rokney A, Valinsky L, Rahav G. Differences in the expression of SPI-1 genes pathogenicity and epidemiology between the emerging *Salmonella enterica* serovar Infantis and the model *Salmonella enterica* serovar Typhimurium. *The Journal of Infectious Diseases*. 2019 Aug 9;220(6):1071-81.

Suwandi A, **Galeev A**, Riedel R, Sharma S, Seeger K, Sterzenbach T, Pastor LG, Boyle EC, Gal-Mor O, Hensel M, Casadesús J. Std fimbriae-fucose interaction increases *Salmonella*-induced intestinal inflammation and prolongs colonization. *PLoS Pathogens*. 2019 Jul 22;15(7):e1007915.

Galeev A, Suwandi A, Bakker H, Oktiviyari A, Routier FH, Krone L, Hensel M, Grassl GA. Proteoglycan-dependent endo-lysosomal fusion affects intracellular survival of *Salmonella* Typhimurium in epithelial cells. *Frontiers in Immunology*. 2020 Apr 29;11:731.

RESEARCH ARTICLE

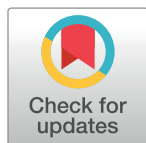
Std fimbriae-fucose interaction increases *Salmonella*-induced intestinal inflammation and prolongs colonization

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OPEN ACCESS

Citation: Suwandi A, Galeev A, Riedel R, Sharma S, Seeger K, Sterzenbach T, et al. (2019) Std fimbriae-fucose interaction increases *Salmonella*-induced intestinal inflammation and prolongs colonization. PLoS Pathog 15(7): e1007915. <https://doi.org/10.1371/journal.ppat.1007915>

Editor: Andreas J. Baumler, University of California Davis School of Medicine, UNITED STATES

Received: February 21, 2019

Accepted: June 13, 2019

Published: July 22, 2019

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Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This work was funded by the German Research Foundation (DFG) priority program SPP1656/1 and SPP1656/2 to GAG and JFB (<http://www.dfg.de>). Work in the GAG lab is also supported by and German Federal Ministry of Education and Research (BMBF) Infect-ERA consortium grant 031L0093B (<https://www.bmbf.de>) and DFG collaborative research center SFB 900

Abstract

Expression of ABO and Lewis histo-blood group antigens by the gastrointestinal epithelium is governed by an α -1,2-fucosyltransferase enzyme encoded by the *Fut2* gene. Alterations in mucin glycosylation have been associated with susceptibility to various bacterial and viral infections. *Salmonella enterica* serovar Typhimurium is a food-borne pathogen and a major cause of gastroenteritis. In order to determine the role of *Fut2*-dependent glycans in *Salmonella*-triggered intestinal inflammation, *Fut2*^{+/+} and *Fut2*^{-/-} mice were orally infected with *S. Typhimurium* and bacterial colonization and intestinal inflammation were analyzed. Bacterial load in the intestine of *Fut2*^{-/-} mice was significantly lower compared to *Fut2*^{+/+} mice. Analysis of histopathological changes revealed significantly lower levels of intestinal inflammation in *Fut2*^{-/-} mice compared to *Fut2*^{+/+} mice and measurement of lipocalin-2 level in feces corroborated histopathological findings. *Salmonella* express fimbriae that assist in adherence of bacteria to host cells thereby facilitating their invasion. The *std* fimbrial operon of *S. Typhimurium* encodes the π -class Std fimbriae which bind terminal α (1,2)-fucose residues. An isogenic mutant of *S. Typhimurium* lacking Std fimbriae colonized *Fut2*^{+/+} and *Fut2*^{-/-} mice to similar levels and resulted in similar intestinal inflammation. *In vitro* adhesion assays revealed that bacteria possessing Std fimbriae adhered significantly more to fucosylated cell lines or primary epithelial cells in comparison to cells lacking α (1,2)-fucose. Overall, these results indicate that *Salmonella*-triggered intestinal inflammation and colonization are dependent on Std-fucose interaction.

TP8 (Projektnummer 158989968). Alibek Galeev and Samriti Sharma were supported by the Center for Infection Biology (ZIB) at the Hannover Biomedical Research School (HBRS). JFB was supported by DFG under Germany's Excellence Strategy – EXC 22167-390884018. MH was supported by DFG SFB 944, project P4 and BMBF Infect-ERA consortium grant 031L0093A. TS was funded by the DFG through grant TS 1921/3-1. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Author summary

The intestinal epithelium is a crucial biological interface, interacting with both commensal and pathogenic microorganisms. It's lined with heavily glycosylated proteins and glycolipids which can act as both attachment sites and energy sources for intestinal bacteria. Fut2, the enzyme governing epithelial α 1,2-fucosylation, has been implicated in the interaction between microbes and intestinal epithelial cells. *Salmonella* is one of the most important bacterial gastrointestinal pathogens affecting millions of people worldwide. *Salmonella* possesses fimbrial and non-fimbrial adhesins which can be used to adhere to host cells. Here we show that *Salmonella* expresses Std fimbriae in the gastrointestinal tract *in vivo* and exploit Std fimbriae to bind fucosylated structures in the mucus and on the intestinal epithelium. Furthermore, we demonstrate that the Std fimbriae-fucose interaction is necessary for bacterial colonization of the intestine and for triggering intestinal inflammation. These data lend new insights into bacterial adhesion-epithelial interactions which are essential for bacterial pathogenesis and key factors in determining tissue tropism and host susceptibility to infectious disease.

Introduction

Glycosylation is an important type of post-translational modification of proteins and lipids and is involved in the regulation of a wide range of processes at the cellular and molecular level. The gastrointestinal tract is home to a vast array of glycan structures and glycoconjugates [1], where the mucosal surface is the site of complex interactions between the intestinal microbiota, intestinal barrier, and immune system. The mucosal surface is characterized by a heavily glycosylated mucus layer produced by goblet cells as well as membrane-bound glycosylated proteins and lipids that form them [2]. These glycoconjugates can be utilized by intestinal commensal bacteria and pathogens as molecular attachment sites or as nutrients [3]. Importantly, host-derived glycans can help foster beneficial relationships with symbiotic microbes, such as *Bacteroides thetaiotaomicron*, by providing an energy source in the absence of dietary polysaccharides [4].

The *FUT2* gene encodes the α -1,2-fucosyltransferase, a glycosyltransferase well known for its role in the expression of ABH and Lewis histo-blood group antigens on the gastrointestinal epithelium and in bodily secretions. Individuals expressing a functional allele are commonly described as 'secretors' whereas those homozygous for loss-of-function mutations display a 'non-secretor' phenotype. Variation in host glycosylation may directly influence susceptibility to enteric pathogens such as enterotoxigenic *Escherichia coli* [5], *Helicobacter pylori* [6], and norovirus [7]. Recent studies have shown the importance of host glycans in supporting a beneficial relationship with the endogenous microbiota by nourishing the microbiota during the stress of systemic infection [8] or by controlling opportunistic pathogens within the microbiota in the context of infection (e.g. *Enterococcus faecalis*) [9]. The non-secretor phenotype is also associated with an increased risk to develop chronic inflammatory bowel diseases [10]. This is possibly due to the altered composition of the intestinal microbiota, which may in turn influence the capacity of pathogenic bacteria to bind to host mucosal surface structures [11].

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is one of the most successful mucosal pathogens, colonizing the human gastrointestinal tract and causing severe inflammatory diarrhea [12]. *S. Typhimurium* carries several virulence genes including fimbrial adhesins, which are hair-like appendages on the outer membrane and are involved in adherence to host epithelial cells. Adhesion to host tissues is critical for invasion and pathogenicity of *S.*

Typhimurium [13]. Type 1 fimbriae are one of the best characterized fimbrial adhesins and are encoded by the *fim* operon. FimH, a lectin-like protein, directly binds to high mannose oligosaccharides conjugated to surface glycoproteins of eukaryotic cells [14,15]. Another fimbrial operon, *std*, encodes the π -class Std fimbriae, which have been described to bind terminal α -1,2 fucose residues [16].

The expression of bacterial adhesins possibly involved in binding fucosylated host proteins suggests that these fimbriae may facilitate *Salmonella* to establish or maintain infection in the highly fucosylated large intestine. Here, we investigated the role of host fucosylation in disease development during *Salmonella* infection using mice with and without expression of the *Fut2* gene (*Fut2*^{+/+} and *Fut2*^{-/-}). Taken together, our results demonstrate that Std-fucose interaction contributes to *S. Typhimurium* persistence and inflammation.

Results

Fut2 expression affects susceptibility to *Salmonella*-induced colitis

To test the hypothesis that expression of *Fut2* influences host susceptibility to enteric pathogens, a model of *S. Typhimurium*-induced colitis was utilized. *Fut2*^{+/+} and *Fut2*^{-/-} littermates were treated with streptomycin, and 24 hours later, infected with wild-type *S. Typhimurium*. One day post infection (p.i.), the cecal tissue of *Fut2*^{-/-} mice contained more *S. Typhimurium* than *Fut2*^{+/+} mice (S1A Fig) in agreement with the observations of Goto and colleagues [17]. However, in contrast to their results, we found the total cecum weight and histopathology scores (S1B–S1D Fig) were comparable between *Fut2*^{+/+} and *Fut2*^{-/-} mice.

Under most conditions wild-type *Salmonella* kill C57BL/6 mice within approximately one week. Therefore, in order to follow the infection to later time points mice were infected with the *S. Typhimurium* Δ *aroA* mutant strain which is attenuated for systemic disease but causes extensive intestinal inflammation [18]. There was no significant difference in bacterial colonization or resulting inflammation of *Fut2*^{+/+} and *Fut2*^{-/-} mice on day 1 p.i. (S1 Fig) or on day 3 p.i. (S2A–S2C Fig). However, on day 7 and day 14 p.i., a significantly reduced *Salmonella* burden in the intestine of *Fut2*^{-/-} compared to *Fut2*^{+/+} mice was detected (Fig 1A, S2A–S2C Fig). Furthermore, 7 days p.i., the histopathological changes in the colon were significantly less severe in *Fut2*^{-/-} mice compared to *Fut2*^{+/+} animals (Fig 1B and 1C). Notably, the colons of infected *Fut2*^{+/+} mice were characterized by a higher number of detached epithelial cells within the colon lumen, increased inflammatory cell infiltration within the mucosa, and stronger submucosal edema. Additionally, the levels of the inflammation-associated marker lipocalin-2 were quantified in the large intestine after *S. Typhimurium* infection. The concentration of lipocalin-2 in the colon and cecum 7 days p.i. were significantly higher in *Fut2*^{+/+} mice in comparison to *Fut2*^{-/-} (Fig 1D, S2D Fig).

Next, colon tissue sections were analyzed by immunohistochemical staining and subsequent quantification of CD68- and MPO-positive cells, which represent macrophages and neutrophils, respectively. Consistent with elevated histopathological scores, significantly higher numbers of recruited neutrophils and macrophages were detected in the colon tissue of *Fut2*^{+/+} mice compared to *Fut2*^{-/-} mice (Fig 2A–2C). In addition, a significantly stronger infiltration of CD4⁺ T lymphocytes in the colonic lamina propria of *Fut2*^{+/+} mice compared to *Fut2*^{-/-} mice was detected by immunofluorescence staining and by flow cytometry (Fig 2A and 2D). No statistically significant differences were found in the numbers or infiltrate composition with respect to cytotoxic T lymphocytes, B lymphocytes, or dendritic cells by flow cytometric quantification of CD8⁺, CD19⁺, and CD11c^{hi} cells, respectively (S3 Fig).

To summarize, *Fut2*-expressing mice exhibited higher bacterial load in the intestine at later time points, which was also associated with an increase in inflammation assessed by

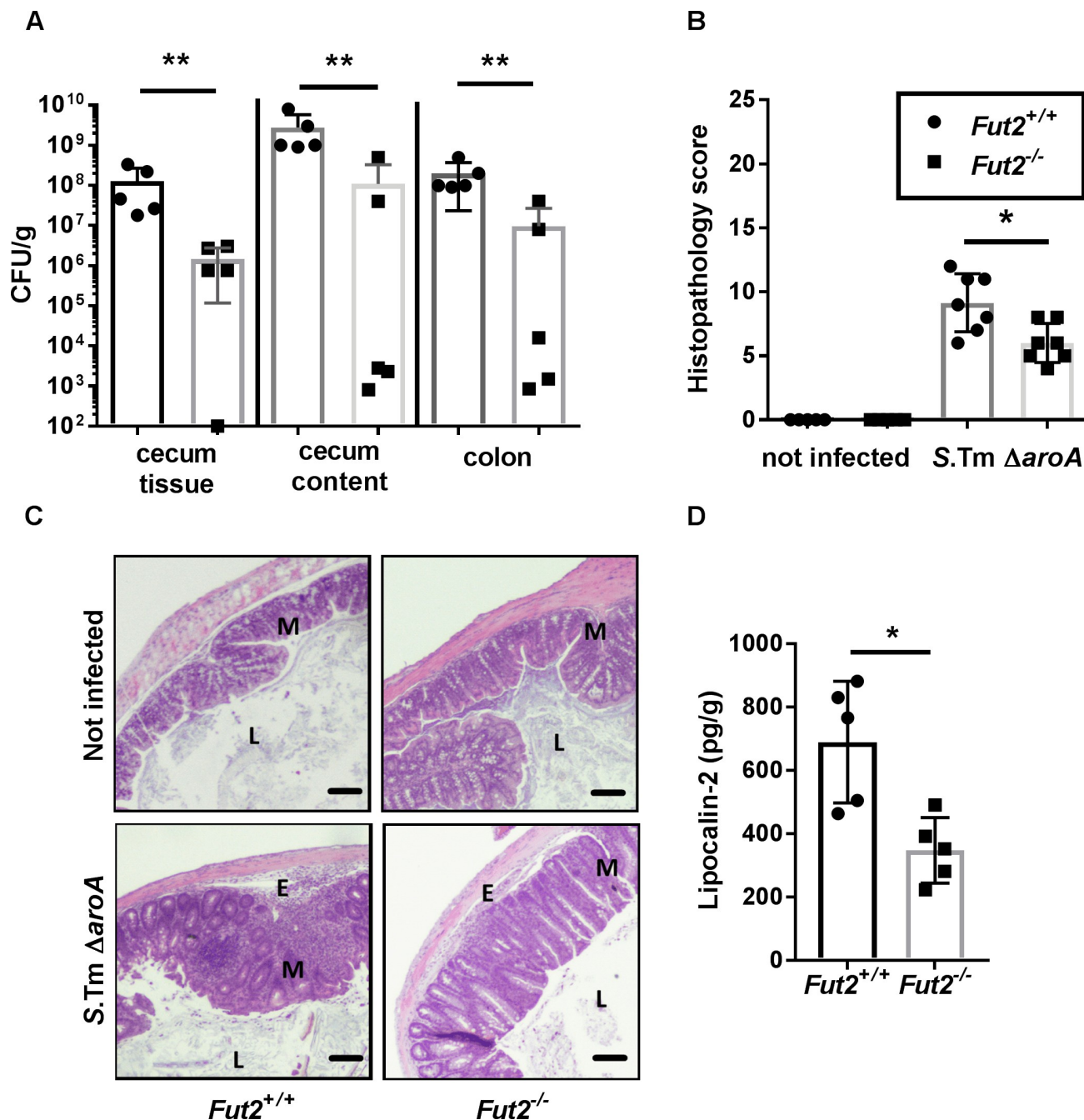


Fig 1. *Fut2* expression affects susceptibility to *Salmonella* induced colitis. Streptomycin-treated mice were infected with *S. Typhimurium* Δ aroA for 7 days. (A) *S. Typhimurium* loads were determined in cecum tissue, cecum content, and colon tissue by plating homogenates on LB agar supplemented with streptomycin (n = 5 mice per group). (B) Histology scoring revealed higher inflammation in *Fut2*^{+/+}-infected colons in comparison to *Fut2*^{-/-}-infected colons at day 7 post infection (p.i.). Uninfected colons of *Fut2*^{+/+} and *Fut2*^{-/-} mice had low histology scores. (C) H&E staining of colon tissue sections at 7 days p.i. Scale bars, 50 μ m. *Fut2*^{+/+} and *Fut2*^{-/-} uninfected mice had a normal tissue and no signs of pathology. Higher numbers of cells in the lumen (L), an increased number of inflammatory cells in mucosa (M), elevated epithelial cell desquamation, and the formation of submucosal edema (E) upon *S. Typhimurium* infection were observed in *Fut2*^{+/+} mice comparing to *Fut2*^{-/-} mice. (D) Lipocalin-2 levels measured by ELISA in supernatants of colon tissues homogenates (n = 5 per group) were higher in *Fut2*^{+/+} mice compared to *Fut2*^{-/-} mice. Graphs are representative of three independent experiments. *p<0.05; **p<0.01, Mann-Whitney test.

<https://doi.org/10.1371/journal.ppat.1007915.g001>

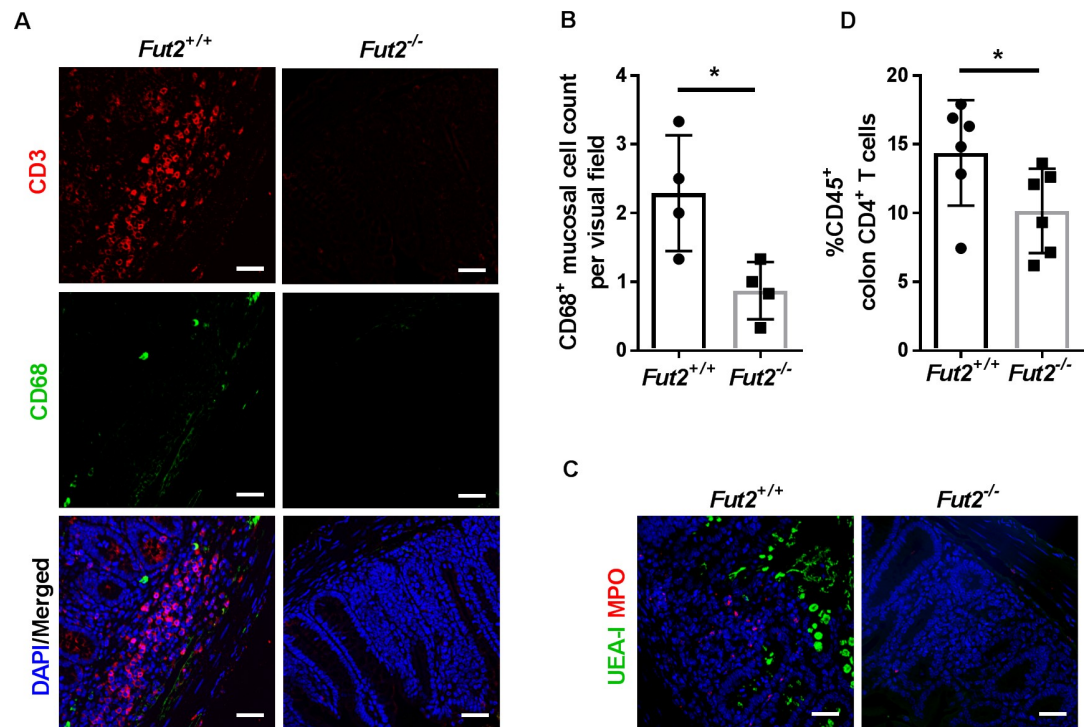


Fig 2. Increased infiltration of immune cells in *Fut2*-expressing mice after *Salmonella* infection. (A) Immunofluorescence staining showed that *Fut2*^{+/+} mice have higher numbers of CD3⁺ (red) and CD68⁺ (green) positive cells in the colon mucosa at day 7 p.i. Nuclei were stained with DAPI (blue). (B) Enumeration of stained cells indicated that *Fut2*^{+/+} mice have higher numbers of CD68⁺ cells in colon mucosa at day 7 p.i. (C) MPO signal (red) showed increased recruitment of neutrophils in *Fut2*^{+/+} mice. UEA-I (green) positive staining was detected only in *Fut2*^{+/+} mice. Scale bars 50 μ m. (D) Flow cytometry revealed a higher frequency of CD4⁺ (CD3⁺CD4⁺) cells present in colonic lamina propria of *Fut2*-expressing mice compared to *Fut2*-deficient mice (n = 4–6). Scale bars, 50 μ m. *p < 0.05, Mann-Whitney test.

<https://doi.org/10.1371/journal.ppat.1007915.g002>

histopathology and lipocalin-2 levels, demonstrating that *Fut2*-mediated fucosylation in the intestine plays an important role in *Salmonella*-triggered inflammation and colonization of the intestine.

Std fimbriae mediate adhesion to intestinal epithelial cells in a fucose-dependent manner

The *Fut2* protein facilitates intestinal epithelial fucosylation by catalyzing the addition of L-fucose residues via an $\alpha(1,2)$ linkage to the terminal β -D-galactose residue of glycans. The *std* operon of *S. Typhimurium* encodes a fimbrial adhesin known to be important for the attachment to fucosylated structures on intestinal epithelial cells [16]. To investigate the role of fucose-Std fimbriae interaction during *Salmonella* adherence, human intestinal epithelial cell lines HT29-MTX-E12 and Caco-2 were utilized. HT29-MTX-E12 are colon epithelial cells that differentiate into goblet-like cells and produce mucus after three weeks of *in vitro* culture [19]. *Ulex europaeus* agglutinin I (UEA-I) lectin staining revealed extensive fucosylation of cell surface and mucus in the differentiated HT29-MTX-E12 cells in contrast to undifferentiated HT29-MTX-E12 cells. Wheat germ agglutinin (WGA) lectin staining for the ubiquitously expressed N-acetylglucosamine was positive in both differentiated and undifferentiated HT29-MTX-E12 cells (Fig 3A).

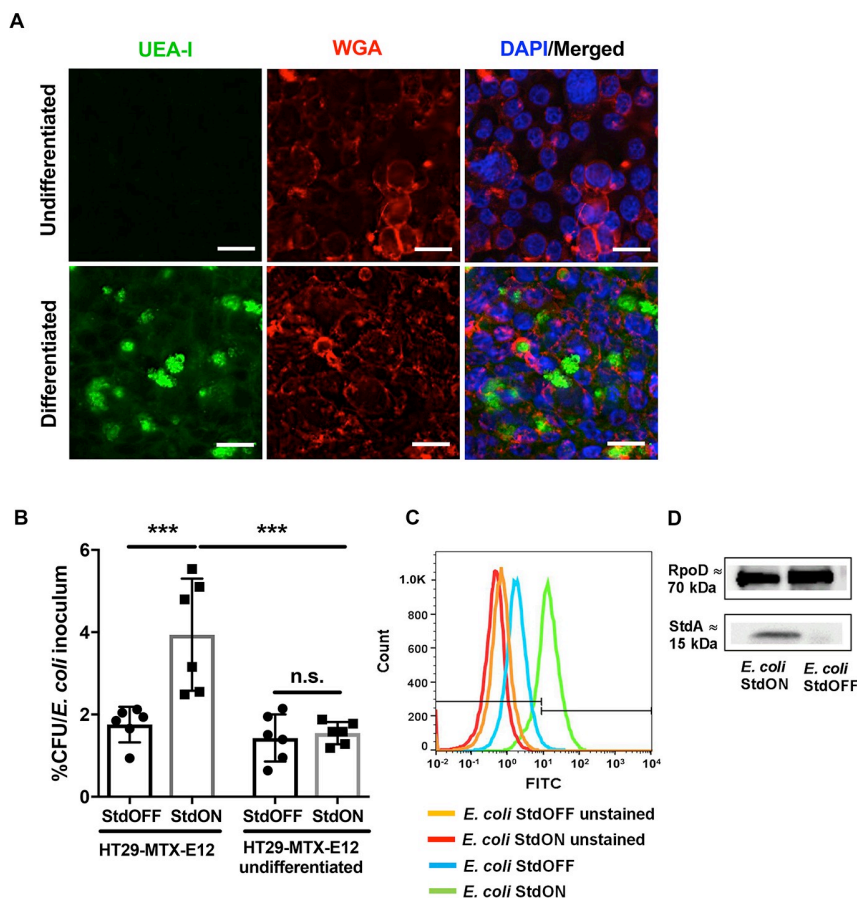


Fig 3. Std fimbriae mediate adhesion to intestinal epithelial cell culture in a fucose-dependent manner. (A) UEA-I (green) and WGA (red) staining in formalin-fixed HT29-MTX-E12 cells, at day 1 after seeding (undifferentiated) and at day 21 after seeding (differentiated). Positive UEA-I staining was present only in differentiated cells. Scale bars, 20 μ m. (B) *E. coli* overexpressing *stdABCD* operon (StdON) displayed higher adherence to differentiated HT29-MTX-E12 cells; this effect was absent in differentiated HT29-MTX-E12 cells infected with *E. coli* StdOFF and in undifferentiated HT29-MTX-E12 cells infected with either strain. *** $p < 0.001$; n.s. = not significant, ANOVA with Tukey's multiple comparison test. (C) Flow cytometry analysis of *std* expression in *E. coli* strains. (D) Specificity of the serum and Std expression were additionally confirmed by Western blotting. *E. coli* RpoD (70kDa) was used as a control.

<https://doi.org/10.1371/journal.ppat.1007915.g003>

Previous studies have shown that bacterial expression of Std fimbriae is a subject of complex and tight regulation, both *in vivo* and *in vitro* [20,21]. Only a very small proportion of the *Salmonella* population express *std* fimbriae *in vitro* [22] and the *std* operon is completely absent in *E. coli*. Therefore, to analyze the role of Std fimbriae *in vitro*, an inducible expression plasmid containing the *Salmonella stdABCD* operon encoding the structural genes of Std fimbriae was transformed into a common laboratory *E. coli* K-12 and afimbriated *E. coli* ORN172. Upon induction with anhydrotetracycline, Std fimbriae were expressed by *E. coli* (referred as *E. coli* StdON) as confirmed by flow cytometry and Western blotting (Fig 3C and 3D). HT29-MTX-E12 cells were infected with either *std*-expressing (*E. coli* StdON) or non-expressing bacteria (*E. coli* StdOFF). In contrast to *E. coli* StdOFF, only *E. coli* StdON strain showed increased adherence to differentiated HT29-MTX-E12 cells (Fig 3B). Importantly, expression of Std fimbriae had no effect on adhesion of *E. coli* StdON to undifferentiated HT29-MTX-E12 cells which do not contain fucosylated glycoproteins (Fig 3B). Similarly, *E. coli* ORN172

StdON bacteria adhered significantly better to Caco-2 cells compared to *E. coli* lacking Std expression. Addition of fucose-binding UEA-1 lectin to the cells prior to infection abrogated the adhesion of the *std*-expressing strain. In contrast, addition of dolichus biflorus agglutinin (DBA), which binds to N-acetylgalactosamine, did not affect binding of *E. coli* ORN172 StdON to Caco-2 cells (S4A and S4B Fig). Atomic force microscopy showed Std piliation of *E. coli* ORN172 StdON and the absence of pili in the empty vector control bacteria (S4C Fig). In conclusion, Std fimbriae are important for binding $\alpha(1,2)$ -fucosylated residues on cell lines corroborating the results by Chessa and colleagues [16].

Next, Std-dependent bacterial adherence to primary epithelial cells was investigated. To this end, primary intestinal epithelial cells were isolated from *Fut2*^{+/+} mice and cultivated as three-dimensional organoids in matrigel. These enteroids were expanded and seeded onto transwell filters resulting in the formation of a 2D monolayer consisting of various primary epithelial cell types. Monolayer barrier integrity and the degree of differentiation were evaluated by measuring transepithelial electrical resistance. Polarized monolayers were infected with *E. coli* StdON and StdOFF bacteria and adherence was analyzed by immunofluorescence. We counted the number of *E. coli* bacteria attached to UEA-1-positive and -negative cells. *E. coli* StdON bacteria were primarily associated with fucosylated cells, while *E. coli* StdOFF adhered equally to fucosylated and non-fucosylated cells (Fig 4A). Furthermore, a significantly higher number of the $\alpha(1,2)$ -fucose-associated *E. coli* StdON cells compared to the $\alpha(1,2)$ -fucose-associated *E. coli* StdOFF bacteria was detected (Fig 4B). Overall, this data demonstrate that *std*-expressing bacteria preferentially bind to fucosylated cells.

Std fimbriae-fucose interaction is critical for Salmonella-induced inflammation and colonization

Using cut sections of the cecum of CBA/J mice, it was previously demonstrated that purified Std fimbriae of *S. Typhimurium* are able to bind terminal $\alpha(1,2)$ -fucose residues in the mucosa [16]. However, the functional consequences of this interaction for disease development, as well as the extent of Std fimbriae expression *in vivo* are not known.

To assess Std fimbriae production *in vivo*, *stdA* gene expression was first examined using RT-qPCR. Similar levels of *stdA* gene expression were detected in the colon of both *Fut2*^{+/+} and *Fut2*^{-/-} mice infected with *S. Typhimurium* Δ aroA strain (S5A Fig). In order to determine whether the absence of *std* or the presence of intestinal fucosylated glycans affects expression of fucose or 1,2-propanediol utilization pathways we quantified levels of *fucI* and *pduBC* by RT-qPCR. We saw comparable levels of these genes expressed in either mouse strain in *std*-containing and *std*-deficient bacteria (S5B and S5C Fig). In order to look more closely at the spatial regulation of Std expression, we used reporter strains of *S. Typhimurium* containing a *stdAstop::gfp* fusion [22] and staining of tissue sections with anti-Std serum. Std was observed to be specifically expressed in the lumen of the large intestine of both *Fut2*^{+/+} and *Fut2*^{-/-} mice on day 1 (S6 Fig) and day 7 p.i. (Fig 5 and S7 Fig). In contrast, Std-expressing *Salmonella* were not observed after invasion of the mucosa (Fig 5, S6 and S7 Figs). These data demonstrate that there is a tight spatial regulation of Std-expression whereby Std fimbriae are expressed prior to invasion of the large intestine.

Fut2^{-/-} mice lack terminal fucose on intestinal epithelium [23]. To test whether epithelial fucosylation directly affects *Salmonella* colonization via interaction with Std fimbriae, the *stdA* mutation was transferred into the *S. Typhimurium* Δ aroA background strain. This mutant strain lacked functional Std fimbriae (*S. Typhimurium* Δ aroA Δ stdA) yet had the same growth rate and motility as the parental *S. Typhimurium* Δ aroA strain (S8 Fig). *Fut2*^{+/+} and *Fut2*^{-/-} mice were then infected with *S. Typhimurium* Δ aroA Δ stdA via oral gavage for 7 days. In

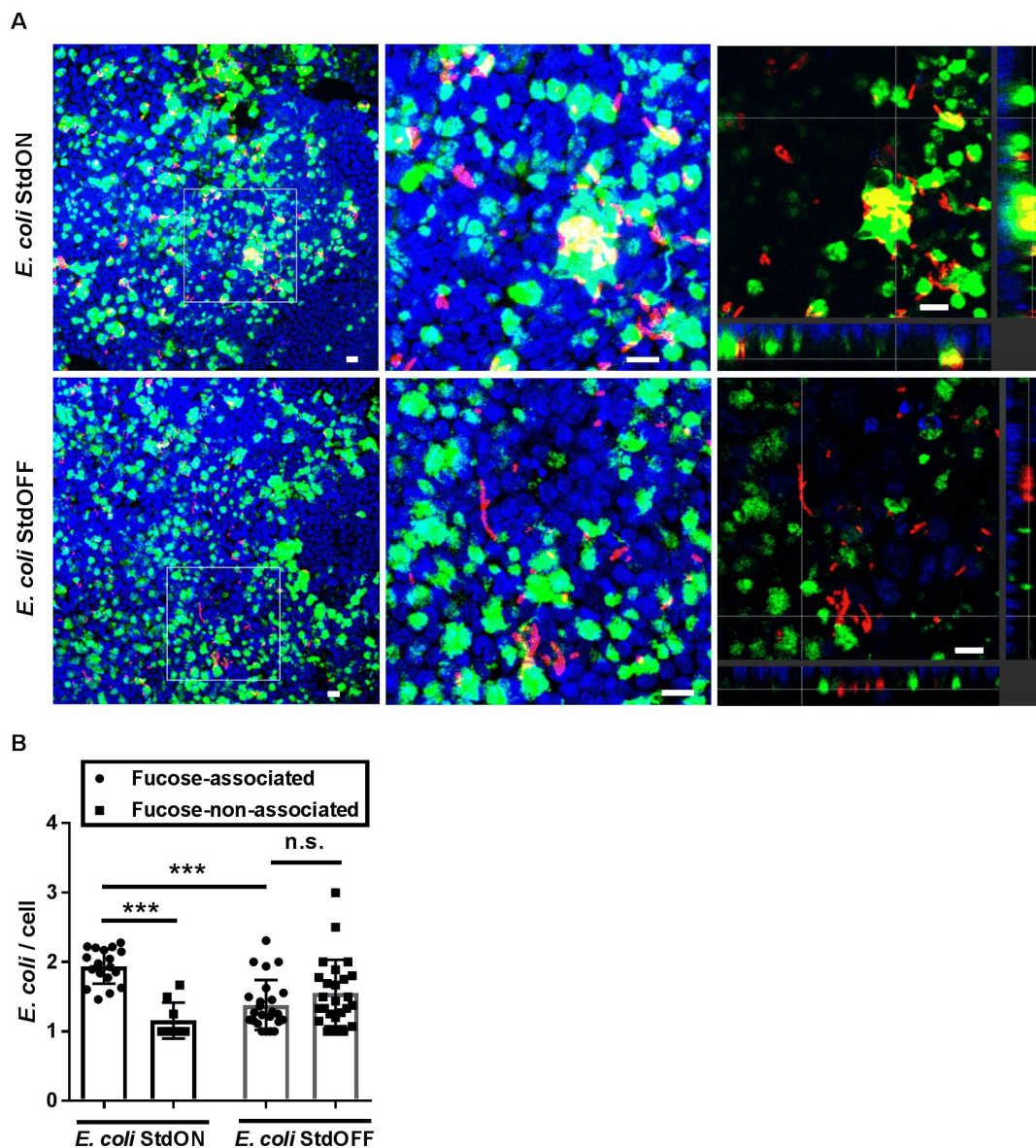


Fig 4. *E. coli* overexpressing Std fimbriae bind to fucosylated cells in primary intestinal epithelial monolayers. (A) 2D monolayers of *Fut2*^{+/+} murine colon organoids were infected with either *E. coli* StdON or *E. coli* StdOFF and immunofluorescently stained using anti-*E. coli* and UEA-1 lectin. Image analysis of the infected monolayers revealed co-localization of Std-expressing *E. coli* bacteria (red) and the $\alpha(1,2)$ -fucosylated cells (green) (A, enlarged region and its orthogonal section). Nuclei were stained with DAPI (blue). Scale bar, 20 μ m. (B) Fucose-associated and non-associated *E. coli* were quantified microscopically using at least 20 fields of view (FOV) per sample. A significantly higher numbers of the fucose-associated bacteria were detected when Std fimbriae were expressed. Data represents four independent biological repetitions. *** $p < 0.001$; n.s. = not significant, ANOVA with Tukey's multiple comparison test.

<https://doi.org/10.1371/journal.ppat.1007915.g004>

contrast to *S. Typhimurium* Δ *aroA* (Fig 1), the *S. Typhimurium* Δ *aroA* Δ *stdA* strain colonized the colon of *Fut2*^{+/+} and *Fut2*^{-/-} mice to similar levels (Fig 6A). In addition, histopathology scores showed similar intestinal inflammation of *Fut2*^{+/+} and *Fut2*^{-/-} mice. H&E staining of colonic tissue showed moderate numbers of necrotic epithelial cells, mild inflammatory cell infiltration within intestinal mucosa and mild submucosal edema (Fig 6B and 6C), which was

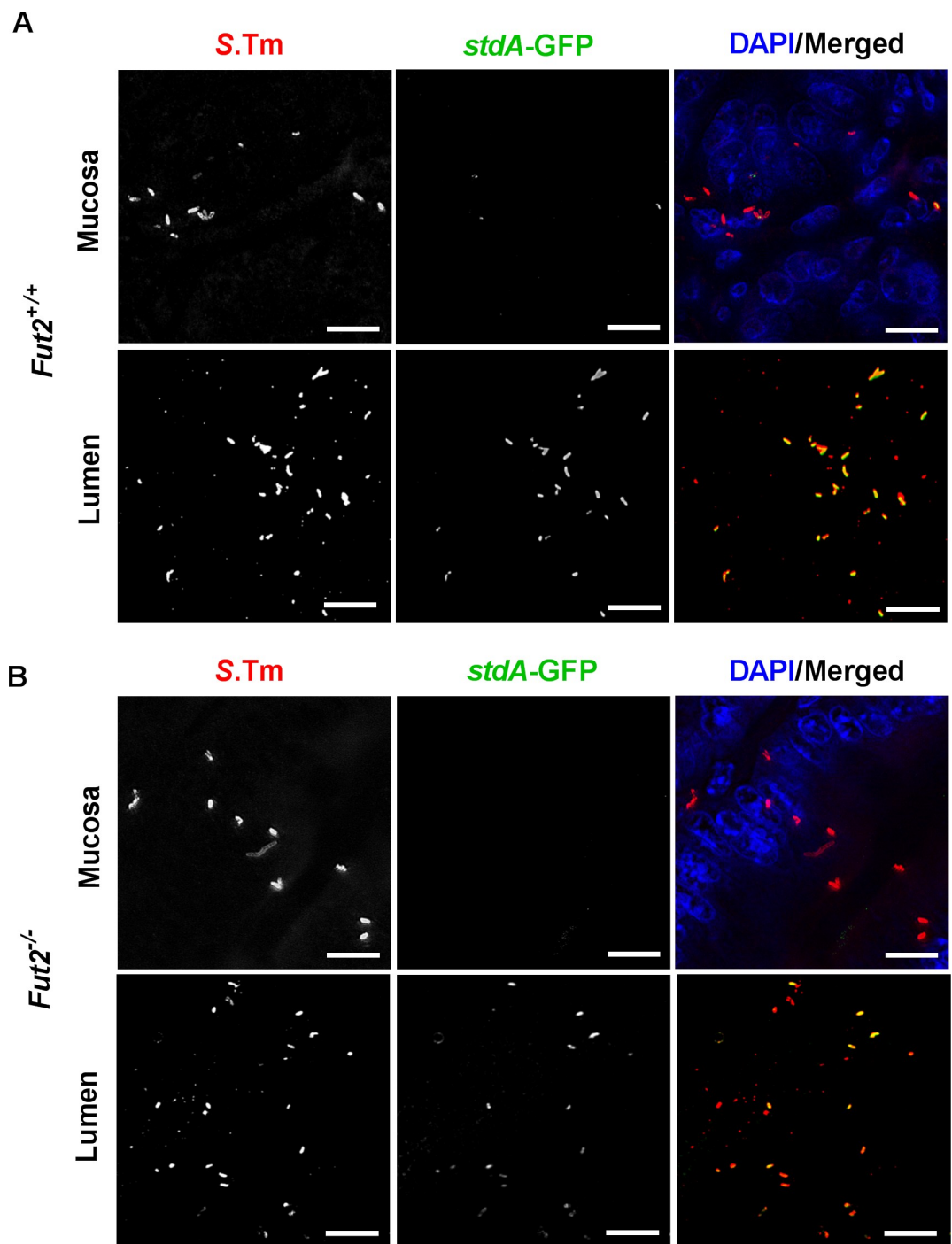


Fig 5. Std fimbriae are differentially expressed *in vivo*. *Fut2*^{+/+} (A) and *Fut2*^{-/-} (B) mice (n = 4) were infected with the reporter *S. Typhimurium* Δ aroA *stdA*stop::gfp strain. Mice were sacrificed at day 7 p.i. and colon sections were subjected to immunohistochemistry. GFP-positive bacteria were detected with an anti-GFP antibody. Std(GFP)-expressing *S. Typhimurium* were localized in the lumen of the colon, but not within the mucosa. Scale bars, 10 μm.

<https://doi.org/10.1371/journal.ppat.1007915.g005>

similar in both mouse genotypes. In addition, lipocalin-2 concentrations were similar in colons of both *Fut2*^{+/+} and *Fut2*^{-/-} mice after infection with *S. Typhimurium* Δ aroA Δ stdA (Fig 6D).

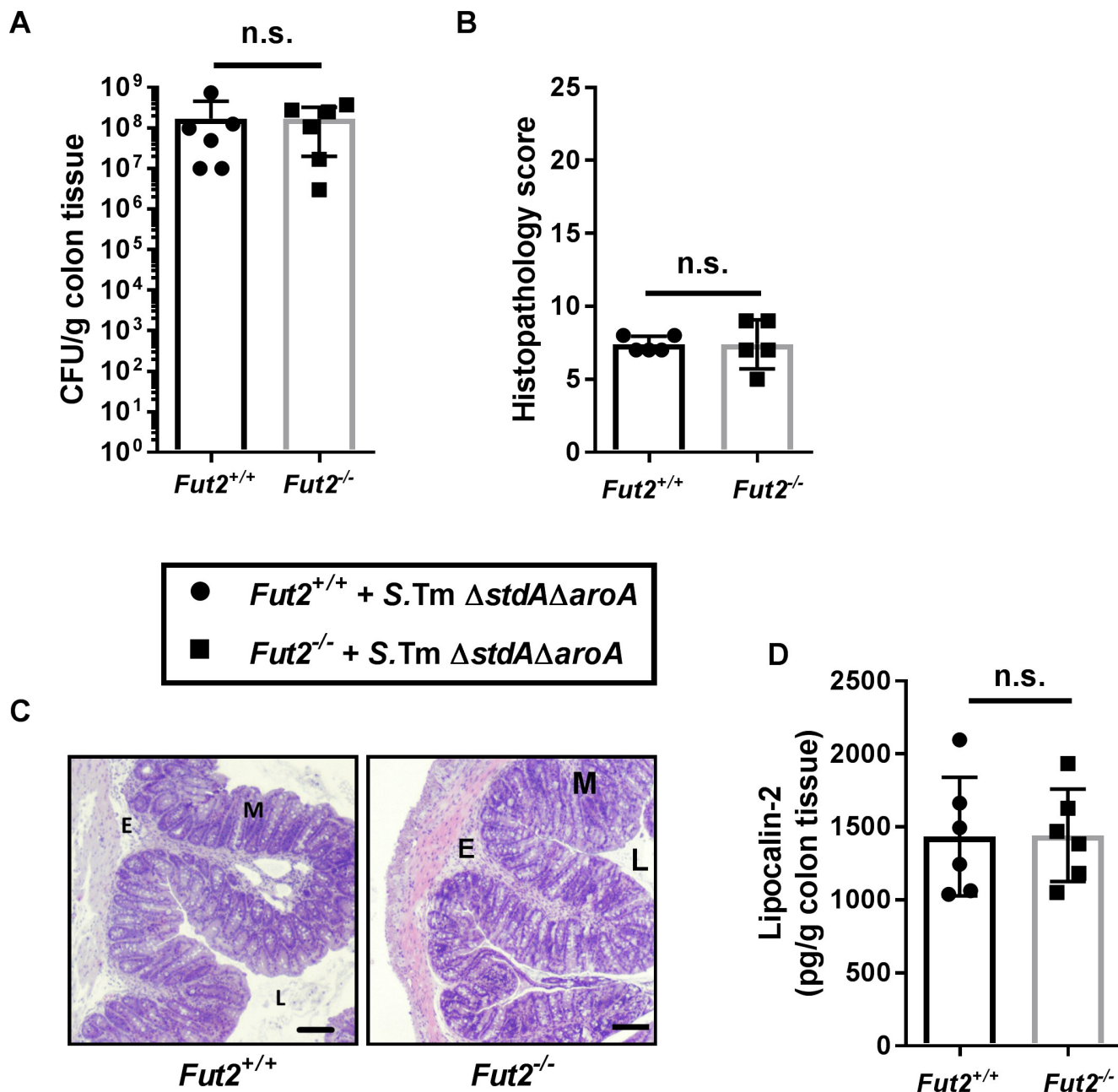


Fig 6. Std fimbriae-fucose interaction impacts *Salmonella*-induced inflammation and colonization of the colon. (A) *S. Typhimurium* $\Delta aroA \Delta stdA$ loads were determined in colon by plating homogenates on LB agar with streptomycin. Both $Fut2^{+/+}$ and $Fut2^{-/-}$ mice showed similar bacterial loads. (B) Histology scoring revealed comparable inflammation in $Fut2^{+/+}$ and $Fut2^{-/-}$ infected colons at day 7 p.i. (C) H&E staining of colon tissue sections at 7 days p.i. Scale bars, 50 μ m. Moderate numbers of necrotic epithelial cells, mild inflammatory cell infiltration within intestinal mucosa, and mild submucosal edema were observed in both $Fut2^{+/+}$ and $Fut2^{-/-}$ mice upon *S. Typhimurium* $\Delta aroA \Delta stdA$ infection. (D) Lipocalin-2 levels were measured by ELISA in supernatants of colon tissue homogenates. Graph shows representative data of three independent experiments ($n = 5-6$ per group). n.s. = not significant, Mann-Whitney test.

<https://doi.org/10.1371/journal.ppat.1007915.g006>

In order to further explore whether Std fimbriae play a role in the colonization of *S. Typhimurium* in the presence or absence of host intestinal fucosylation, competitive index (CI) experiments were performed by orogastrically infecting both $Fut2^{+/+}$ and $Fut2^{-/-}$ mice with equal numbers of *S. Typhimurium* $\Delta aroA$ and *S. Typhimurium* $\Delta aroA \Delta stdA$. Fecal pellets

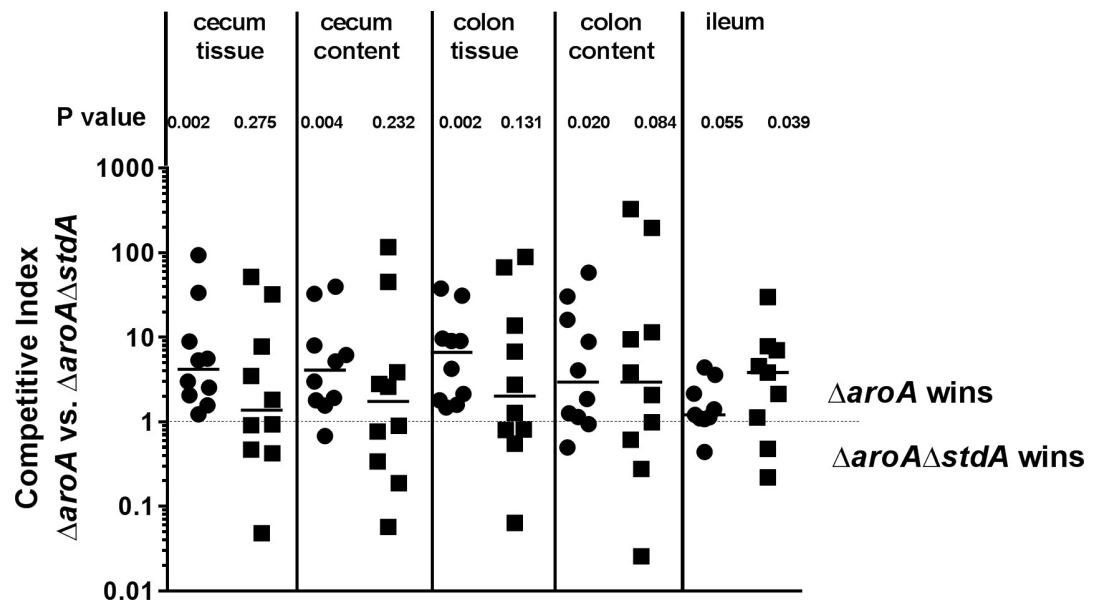


Fig 7. Std fimbriae provides a competitive advantage in a fucosylated environment. Competitive index (CI) was determined by infecting *Fut2*^{+/+} and *Fut2*^{-/-} mice (n = 10 per group) with an equal amount of *S. Typhimurium* Δ aroA and *S. Typhimurium* Δ aroA Δ stdA. Organ and feces homogenates were plated on LB agar containing streptomycin (total *Salmonella*) and on LB plates with streptomycin+kanamycin (*S. Typhimurium* Δ aroA Δ stdA only). (A) In cecum and colon, *S. Typhimurium* Δ aroA outcompeted the isogenic Δ stdA mutant in *Fut2*^{+/+} mice, but not in *Fut2*^{-/-} mice. Graph shows data for individual mice and median values, p values are indicated for each group as determined by Wilcoxon signed-rank test.

<https://doi.org/10.1371/journal.ppat.1007915.g007>

were collected at 1, 3, and 5 days p.i. and bacterial counts of both strains were determined. After 7 days, the bacterial load in intestinal tissues and luminal content was determined and the CI ratio of the two strains was calculated. Interestingly, in cecum and colon, *S. Typhimurium* Δ aroA significantly outcompeted the isogenic Std-deficient strain in *Fut2*^{+/+} mice, but not in *Fut2*^{-/-} mice (Fig 7). Accordingly, CI results from feces at day 1, 3, and 5 showed that *S. Typhimurium* Δ aroA outcompeted the isogenic Δ stdA mutant in *Fut2*^{+/+} mice (S9 Fig). Taken together, our *in vivo* data demonstrate that Std fimbriae are important for *Salmonella* colonization, persistence, and induction of inflammation in a fucosylated host environment.

Discussion

Variation in human glycosylation influences various metabolic diseases, cancers, inflammatory diseases, and susceptibility to infectious pathogens. Genome-wide association studies show that *FUT2* nonsense polymorphisms are associated with increased risk for Crohn's disease [10] and primary sclerosing cholangitis [24]. Genetic variation in *FUT2* is also linked to susceptibility to infections with bacterial and viral pathogens including *Helicobacter pylori* [25], norovirus [26, 27], Enterotoxigenic *E. coli* [5], and progression of HIV [28]. In this study, we investigated the role of *Fut2* expression for *S. Typhimurium* infection and found that Std fimbriae-fucose interaction was important for *Salmonella*-induced inflammation and colonization.

Host mucosal glycans can influence the susceptibility to infection indirectly or directly. Indirectly, glycan-dependent differences in microbiota composition may contribute to the susceptibility to infection with a particular pathogen. For example, we previously reported an influence of the histo-blood group related glycosyltransferase gene *B4galnt2* on host susceptibility to *S. Typhimurium* infection. The expression of *B4galnt2* in the gut results in differences

in microbial composition which in turn affect the extent of *Salmonella* colonization, and hence, disease pathology [29]. Many complex carbohydrates degraded by the intestinal microbiota produce metabolites that can be utilized by *Salmonella* Typhimurium and *Clostridium difficile* thereby facilitating their expansion within the gut [4]. Host glycans can also directly influence a host's susceptibility to infection by modulating bacterial attachment to host tissues. Many bacteria produce specific adhesins which bind to host glycans. For example, the *H. pylori* adhesin BabA mediates adherence to the gastric mucosa of individuals with fucosylated ABO (H)/Lewis b blood group antigens [30, 31]. Norovirus (strain GII.4) and rotavirus (strains with spike protein VP8) encode adhesins which bind $\alpha(1,2)$ -fucosylated glycans resulting in increased susceptibility in individuals with a secretor phenotype [32,33].

While *Fut2* expression in the small intestine is inducible by ILC3-derived IL-22 [17], the large intestine is constitutively fucosylated [8]. Mice lacking *Fut2* are more susceptible to *S. Typhimurium* infection at an early time point post infection, as demonstrated by Goto and colleagues [17] and confirmed in the present study. In contrast, we demonstrate that at later time points, a lack of *Fut2* expression is associated with decreased intestinal colonization, pathology, and inflammatory responses. It has been hypothesized that at one day post *S. Typhimurium* infection, $\alpha(1,2)$ -fucose-containing glycans secreted from goblet cells may interfere with the attachment of *Salmonella* to intestinal epithelial cells, although this has not been proven so far [34]. Our study suggests that at later time points post infection, *Salmonella* exploits $\alpha(1,2)$ -fucose-containing glycans present in the intestine of *Fut2*^{+/+} mice to their advantage. Fucose and its metabolic products such as 1,2-propanediol can be utilized by *S. Typhimurium* as carbon and energy sources [35]. *In vivo*, during intestinal inflammation, 1,2-propanediol is generated and serves as a nutrient source for *Salmonella* [36]. Our data do not show any differences in the expression of *fucI* and *pduBC* in *Fut2*^{+/+} and *Fut2*^{-/-} mice, even in the absence of *stdABCD* genes, suggesting that these pathways are not regulated by Std fimbriae.

It was previously shown that purified Std fimbriae of *S. Typhimurium* can bind terminal $\alpha(1,2)$ -fucose residues [16]. *Salmonella enterica* encodes up to 13 fimbrial and at least 7 non-fimbrial adhesins depending on the serovar [13,37]. The production of adhesins involves complex and tight regulation since inappropriate expression could be detrimental for bacterial colonization and pathogenesis. While the majority of adhesins are expressed in temporal and spatially highly controlled manner during animal infections, they are often not produced under laboratory conditions [20, 38]. *In vitro* expression of the *S. Typhimurium* *std* operon is bistable resulting in the emergence of a minor subpopulation of Std-positive cells [22]. Therefore, to investigate the role of Std fimbriae *in vitro* we took advantage of an Std-expressing *E. coli* strain. Our *in vitro* infection experiments revealed enhanced attachment of Std-expressing *E. coli* to fucosylated cell lines, which could be specifically blocked by the addition of the $\alpha(1,2)$ -fucose-binding lectin UEA-I. These observations confirm the findings of Chessa and colleagues [16] and were further corroborated by preferential binding of Std-expressing *E. coli* to fucosylated cells in primary epithelial monolayers. Altogether, bacteria expressing Std fimbriae exhibited increased adhesion to human cell lines and murine intestinal crypt organoids when terminal $\alpha(1,2)$ -fucose was present.

The extent and localization of Std fimbriae expression *in vivo* was not previously known. Notably, we observed the spatial expression pattern of Std fimbriae *in vivo*. In this work, we present, for the first time, evidence that Std fimbriae are differentially produced during intestinal infection. Std-positive *Salmonella* were found predominantly in the intestinal lumen, which corroborates the existence of a regulatory fimbrial switch.

Previous work has shown that Std fimbriae are required for the long-term *S. Typhimurium* colonization of CBA mice [39]. In agreement with these data, we show that Std fimbriae are

important for persistence in C57Bl/6 mice and demonstrate that this is strictly dependent on the ability of Std fimbriae to adhere to fucosylated host glycoproteins or glycolipids in the large intestine. While the expression of $\alpha(1,2)$ -fucose on enterocytes and goblet cells is facilitated by Fut2, M cells in the follicle associated epithelium overlying Peyer's patches [40] and Paneth cells [41] are $\alpha(1,2)$ -fucosylated via Fut1. Thus, based on our CI data, we can conclude that *Fut2*-dependent fucosylation is important for *Salmonella* persistence only in the large intestine. Furthermore, we present evidence that Std fimbriae are also important for the induction of host intestinal pathology and inflammation in a *Fut2*-dependent manner.

In conclusion, our results demonstrate a substantial role for glycosylation of the intestinal mucosa in the susceptibility to *S. Typhimurium* infection. Std fimbriae binding of terminal α -1,2 fucose residues mediate bacterial adherence to host glycoproteins or glycolipids. Taken together, our results conclusively demonstrate that host fucosylation in the intestine is exploited by *S. Typhimurium* during the course of infection in a mechanism that requires *Salmonella* expression of Std fimbriae.

Material and methods

Mice

B6.129X1-*Fut2*^{tm1Sdo}/J (*Fut2*^{-/-}) [42] mice were purchased from the Jackson Laboratory and intercrossed with wild-type C57BL/6J (*Fut2*^{+/+}) mice. Mice were backcrossed for 14 generations. Heterozygous breeding pairs produced litters of mixed genotypes. *Slc11a1* (Nramp1) is an important resistance gene for *S. Typhimurium*. 129X1 mice harbor *Slc11a1* resistant alleles while in C57BL/6J mice a point mutation results in *Slc11a1* sensitive alleles. The *Slc11a1* genotype of mice was verified as described [[43]] using a common reverse primer and forward primers (S2 Table) specific for the sensitive and resistant allele, respectively. All mice were homozygous for the *Slc11a1* sensitive allele. Mice were housed together under specific pathogen-free conditions in individually ventilated cages (IVC). Standard chow and water were provided *ad libitum*. Experiments were conducted in the animal facilities of the University of Kiel and Hannover Medical School in Germany.

Ethics statement

Animal experiments were conducted in direct accordance with the German Animal Protection Law consistent with the ethical requirements and approval of the Animal Care Committee of the Ministry of Energy, Agriculture, the Environment and Rural Areas of Schleswig-Holstein, Germany (protocol # V244-7224.121.3 (99-10/10)) and by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (protocol # 33.12-42502-04-16/2071).

Bacteria

S. Typhimurium SL1344 (*S. Tm*) [44], *S. Tm* Δ aroA [18] were grown at 37°C with shaking in lysogeny broth (LB) supplemented with 100 μ g/ml streptomycin. *S. Tm* Δ aroA Δ stdAB (referred to as *S. Tm* Δ aroA Δ stdA) double mutant was generated by P22 phage transduction of the Δ stdAB deletion from the *S. Tm* NCTC 12023 Δ stdAB [38] to the *S. Tm* SL1344 Δ aroA background. *S. Tm* Δ aroA *stdAstop::gfp* strain was created by P22 transduction of the Δ aroA deletion into the parental *S. Tm* *stdAstop::gfp* strain [22]. For *in vivo* infection, *S. Tm* Δ aroA Δ stdA and *S. Tm* Δ aroA *stdAstop::GFP* strains were grown overnight in LB broth supplemented with kanamycin 50 μ g/ml at 37°C. The *E. coli* Std strain containing the anhydrotetracyclin (AHT)-inducible *Salmonella* *stdABCD* operon was generated by electroporation of the plasmid p4394 [45] into *E. coli* Turbo (New England Biolabs) K-12 strain (referred as WT).

For *in vitro* infection experiments, *E. coli stdABCD* was grown overnight in LB broth supplemented with carbenicillin 50 µg/ml at 37°C and then grown to logarithmic phase in the presence (StdON) or absence (StdOFF) of AHT (IBA, 100 ng/ml). The non-fimbriated *E. coli* ORN172 strain [38] harboring the empty vector or a plasmid for expression of the *Salmonella stdABCD* operon under control of the *tetR P_{tetA}* expression cassette were grown in LB broth. Expression was induced by addition of 100 ng/ml anhydrotetracycline (AHT). Imaging of bacteria by atomic force microscopy (AFM) was performed as previously described [45].

S. Typhimurium infection of mice

Fut2^{-/-} and wild-type (*Fut2*^{+/+}) littermates were pretreated by oral gavage with 20 mg of streptomycin (Sigma-Aldrich) 24 hours before infection. Mice were orally gavaged with either 3x10⁶ *S. Tm*, *S. Tm ΔaroA*, *S. Tm ΔaroAΔstdA*, *S. Tm stdAstop::gfp*, or *S. Tm ΔaroA stdAstop::gfp* in 100 µl HEPES buffer (100 mM, pH 8.0; PAA Laboratories). Control mice (mock infection) were given 100 µl HEPES buffer. To enumerate luminal and tissue-invaded bacteria, colon and cecum tissues were harvested and the intestinal contents were separated from the tissues. Tissues were then treated with PBS containing 100 µg/ml gentamicin at 4°C for 30 minutes to kill bacteria on the tissue surface. Intestinal tissues and intestinal contents were homogenized, serially diluted, and plated on LB agar containing streptomycin (100 µg/ml).

Competitive index (CI)

Fut2^{+/+} and *Fut2*^{-/-} mice were pre-treated with 20 mg of streptomycin and infected with a mixture of *S. Tm ΔaroA* and *S. Tm ΔaroAΔstdA* strains (1:1, 3x10⁶ total bacteria per mice). During the infection, fecal pellets were collected at days 1, 3, and 5 p.i. At 7 days p.i., mice were sacrificed and bacterial loads in cecum tissue, cecum content, colon tissue, colon content, and ileum were enumerated. Feces and intestinal tissues were homogenized and plated on selective LB agar plates supplemented with either streptomycin (100 µg/ml) alone (to determine total *Salmonella* load) or with streptomycin (100 µg/ml) and kanamycin (50 µg/ml) (to enumerate *S. Tm ΔaroAΔstdA*). The number of *S. Tm ΔaroA* was calculated by subtracting the CFU counts of *S. Tm ΔaroAΔstdA* from the total *Salmonella* counts. Competitive index (CI) was calculated as the ratio of ($\Delta\text{aroA} / \Delta\text{aroA}\Delta\text{stdA}$) at the time of sampling divided by ($\Delta\text{aroA} / \Delta\text{aroA}\Delta\text{stdA}$) of the inoculum.

Motility assay

Motility was assessed by inoculating motility agar plates (10 g/l tryptone, 5 g/l NaCl, and 0.3% Bacto-agar) with saturated bacterial cultures grown overnight in LB broth. Motility halos were compared after incubation at 37°C for 6 hours.

Pathology and histology

Organs were fixed in 10% formalin, dehydrated with ethanol, and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin (H&E) according to standard laboratory procedures. Histological scores of ceca and colons were determined as previously described [46]. Briefly, pathological changes were assessed by evaluating the presence of necrotic epithelial cells and neutrophils in lumen; desquamation and ulceration in surface epithelial cells; crypt abscesses; infiltrating inflammatory and immune cells in mucosa and submucosa layer; and the formation of edema in submucosa layer.

***In vitro* infection**

Human colon epithelial clonal cell line, HT29-MTX-E12 [47,48] (a kind gift from Marguerite Clyne, University College Dublin), and the colorectal carcinoma cell line, Caco-2BBE1 (ATCC CRL-2102), were grown in DMEM supplemented with 10% fetal bovine serum (Biocrom) and 1% MEM non-essential amino acids solution (Gibco, Life Technologies). Cells were seeded in 24 well plates and incubated at 37°C in a humidified 5% CO₂ atmosphere. Cells were grown for 7 days (Caco-2-BBE1) or 21 days (HT29-MTX-E12) to achieve differentiation of the monolayers. Cells were then infected with *E. coli* StdOFF or *E. coli* StdON, *E. coli* ORN172 StdOFF or *E. coli* ORN172 StdON for 30 min at an MOI of 50. For quantification of adherence, cells were washed four times with PBS and lysed in PBS containing 1% (v/v) Triton X-100. The number of adherent bacteria was determined by serial dilutions plating. Where indicated, 30 min prior to infection, cells were incubated with medium in presence of 0.3 mM UEA-I or DBA lectins (CosmoBio) at 37°C in a humidified 5% CO₂ atmosphere.

Intestinal epithelial organoids

Primary colonic and ileal crypts were isolated from *Fut2*^{+/+} mice as described [49] with modifications. Briefly, mice were sacrificed by cervical dislocation. Intestines were opened longitudinally, cut into small pieces and washed three times with 5 ml of ice-cold DPBS. Tissues were then incubated in 10 ml of ice-cold crypt chelating buffer (10 mM EDTA in DPBS) for 90 min on an orbital shaker. The supernatant was discarded and the settled tissue fragments were resuspended twice in 5 ml ice-cold DPBS. Crypts were centrifuged for 5 min at 800 rpm at 4°C and pellets were resuspended in 1 ml ice-cold DPBS. About 100 crypts were resuspended in 25 µl organoid medium (Advanced DMEM/F12 medium (Thermo Fischer Scientific) supplemented with 2 mM GlutaMax, 50% L-WRN-Supernatant (ATCC CRL3276), 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, B27 supplement, 50 ng/ml recombinant mouse epidermal growth factor (rm EGF), 500 nM A83-01 (Tocris), 10 µM SB202190 (Tocris), 10 nM Gastrin I (Tocris), 1 mM N-Acetyl-L-cysteine (Sigma), and 10 µM Y27623 (Tocris)). 25 µl Matrigel (Corning) was added into a well of a pre-warmed 24-well plate. The plate was incubated for 0.5 h in a 37°C incubator with 5% CO₂ to allow complete polymerization of the Matrigel. Crypts were covered with 500 µl of the organoid medium. To form 2D monolayers, 3D organoids were resuspended in ice-cold PBS and centrifuged at 1500 rpm for 10 min at 4°C. Pellets were resuspended in 1 ml warm 0.05% trypsin/EDTA and incubated for 5 min at 37°C in a water bath. Organoids were dissociated by pipetting and washed with ice-cold DMEM/10% FCS and resuspended in monolayer medium (Advanced DMEM/F-12, 50% L-WRN-Supernatant, 20% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 µM Y-27632, 50 ng/ml rm-EGF). Cell suspensions were seeded onto Transwell permeable supports (polyester; 6.5 mm diameter; 0.4 µm pore size; Corning) that had been coated for 2 h at 37°C with Matrigel (diluted 1:40 in PBS). Monolayer medium was replaced every two days and monolayer barrier integrity was evaluated by measuring transepithelial electrical resistance (TEER) using a volt-ohmmeter (Millipore). On day 5 after seeding, medium was changed to differentiation medium (Advanced DMEM/F-12, 5% L-WRN-Supernatant, 20% fetal bovine serum, 2 mM L-glutamine, 50 ng/ml rm-EGF, 5 µM DAPT). For the next two days, differentiation medium was changed every day and TEER was measured. 2D monolayers were infected with either *E. coli* StdON or *E. coli* StdOFF (7 x 10⁷ bacteria per Transwell), incubated for 30 min at 37°C, washed four times with PBS, and fixed with 4% paraformaldehyde (PFA). Adherent fucose-associated and non-associated bacteria were counted microscopically, using at least 20 fields of view (FOV) per sample.

Immunofluorescence

Formalin-fixed paraffin-embedded tissue sections (5 μ m) were deparaffinized and rehydrated. Heat-induced epitope retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) and blocking was achieved using 2% normal goat serum (NGS). The following antibodies were used for immunohistochemistry (see S1 Table for a full description): anti-StdA serum [38], *Salmonella* O Antiserum Group B (BD Difco), anti-GFP (DSHB), CD3 (Abcam), CD68 (Abcam), myeloperoxidase (MPO) (Thermo Fisher Scientific), and fluorescently-labeled secondary antibodies (Invitrogen). Counterstaining of nuclei was done with 4,6-Diamidin-2-phenylindol (DAPI) (Invitrogen). HT29-MTX-E12 and Caco-2 Bbe1 cells were seeded on coverslips in 24 well plates and fixed with 4% PFA before and after differentiation. Blocking of non-specific binding was done using 2% NGS. Fluorescently-labeled lectins UEA-1 (*Ulex Europaeus* agglutinin-1) (CosmoBio) and WGA (wheat germ agglutinin) (Vector laboratories) were used to visualize α (1,2)-fucosylation and the presence of sialic acid / N-acetylglucosaminyl residues, respectively. Fixed primary epithelial cell monolayers were stained with the anti-*E.coli* antibody (Abcam), UEA-1 lectin (CosmoBio), and DAPI. Images were obtained on a Zeiss Apotome.2 microscope using AxioVision 4.9.1 software (Zeiss) and on a Leica DMI8 confocal laser scanning microscope using LAS X 3.3.0.16799 software (Leica). Brightness and contrast were adjusted using ImageJ 1.52e software.

Flow cytometry

Isolation of colonic lamina propria cells was achieved using the Lamina Propria Dissociation Kit (Miltenyi Biotec) according to the manufacturer's protocol. Leukocyte isolation was performed with 40% / 80% discontinuous Percoll gradient (GE Healthcare). Cells were incubated with Fc γ R blocking reagent (rat anti-mouse CD16/CD32, BD Biosciences) for 30 minutes on ice prior to incubation with the other antibodies. Antibodies used for flow cytometry analysis are listed in S1 Table. To detect expression of Std fimbriae, flow cytometry was performed as previously described [16] with modifications. In brief, approximately 5×10^8 bacteria were fixed with 10% formalin and incubated at room temperature for 20 minutes. After washing with PBS, cells were resuspended in 2% NGS diluted in PBS and incubated at room temperature for 30 minutes. Polyclonal rabbit anti-StdA serum [38] was added to the cell suspensions following incubation at room temperature for 30 min. After washing with PBS, fluorescently labeled secondary antibodies (Invitrogen) were added. Flow cytometry was performed using a MACS-Quant Analyzer 10 (Miltenyi Biotec). The data were analyzed using FlowJo v.10 software (TreeStar).

ELISA

Supernatants from the organ homogenates were collected and stored at -20°C . Lipocalin-2 levels were detected using mouse lipocalin-2/NGAL DuoSet ELISA (R&D Systems) according to the manufacturer's protocol. Absorbance was measured using a Synergy HTX microplate reader and acquired using Gen5 software (Biotek).

Western blot

E. coli StdOFF and *E. coli* StdON were grown in LB broth supplemented with carbenicillin (50 $\mu\text{g/ml}$) and to induce *std* fimbrial expression anhydrotetracycline (100 ng/ml) at 37°C until an OD₆₀₀ of 0.6 was reached. 10^8 bacteria were pelleted, resuspended in PBS, mixed with an equal volume of Laemmli buffer supplemented with 10% DTT, and boiled for 10 min. These whole-cell lysates were spun down and supernatants were loaded immediately onto a

SDS-PAGE gel (15%). Proteins were transferred to Hybond-P 0.45 PVDF (Amersham) membranes using a Trans-Blot semi-dry transfer cell (Bio-Rad). After blocking with Roti-block (Carl Roth), membranes were incubated first with anti-StdA [38] serum (Humphries et al., 2003) diluted 1:500 in blocking buffer, and then with a goat anti-rabbit-HRP conjugate, and finally with Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). Images were obtained using the ImageQuant LAS 4000 system (GE Healthcare).

***Salmonella* gene expression in vivo**

Feces of the infected *Fut2*^{+/+} and *Fut2*^{-/-} mice (day 7 p.i.) was immediately stored in RNeasy lysis buffer (Qiagen). Total RNA was extracted using the High Pure RNA Tissue Kit (Roche) and reverse transcription was performed with the cDNA Synthesis Kit (Roche) in accordance with the manufacturer's instructions. Quantitative real-time PCR (qPCR) was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad) using the Power SYBR Green PCR Master Mix (Applied Biosystems). Gene-specific primers are listed in S2 Table. Data were normalized to the house-keeping gene *rpoD* and analyzed by $\Delta\Delta C_t$ method [50] with median values of *Fut2*^{+/+} mice infected with *S. Tm* Δ *aroA* as calibrators.

Statistical analyses

All data were analyzed using GraphPad Prism V7.0d software. Statistical analyses were performed using one-way analysis of variance followed by Tukey's multiple comparison test or Wilcoxon-Mann-Whitney test as indicated. Graphs display the mean values $\pm$ SD, unless stated otherwise. Competitive index data were analyzed by the Wilcoxon signed-rank test by comparing medians with a hypothetical value of 1.

Supporting information

S1 Table. Antibodies and lectins used in this study.

(DOCX)

S2 Table. Primers used in this study.

(DOCX)

S1 Fig. *Fut2*-deficient mice are more susceptible to *Salmonella* infection on day 1 p.i. Streptomycin-treated mice were infected with WT *S. Typhimurium* SL1344 or *S. Typhimurium* Δ *aroA* and sacrificed at day 1 p.i. (A) *S. Typhimurium* loads were determined in the cecum by plating homogenates on LB agar with streptomycin (n = 4–5 mice per group). Higher bacterial loads were observed in *Fut2*^{-/-} mice infected with wildtype *Salmonella*. (B) Histology scoring revealed similar levels of inflammation in both *Fut2*^{+/+} and *Fut2*^{-/-} ceca at day 1 p.i. (C) H&E staining of cecum tissue sections at 1 day p.i. Scale bars, 50 μ m. Ceca of *Fut2*^{+/+} and *Fut2*^{-/-} mice were characterized by high numbers of cells in the lumen (L), increased numbers of inflammatory cells in mucosa (M), massive epithelial cell desquamation, and the formation of submucosal edema (E) upon *S. Typhimurium* wildtype and Δ *aroA* infection. (D) Similar cecum weights in both *Fut2*^{+/+} and *Fut2*^{-/-} mice were observed (n = 4–5 mice per group). *p<0.05; n.s = not significant, Mann-Whitney test. (TIF)

S2 Fig. *Fut2*^{+/+} mice carry higher bacterial burden at later infection time points. Streptomycin-treated *Fut2*^{+/+} and *Fut2*^{-/-} mice were infected with *S. Typhimurium* Δ *aroA* for 3 and 14 days. (A–C) *S. Typhimurium* loads were determined in cecum tissue, cecum content and colon by plating homogenates on LB agar supplemented with streptomycin (n = 4–8 mice per

group). (D) Lipocalin-2 levels measured by ELISA in supernatants of cecum tissues homogenates ($n = 5$) were higher in *Fut2*^{+/+} mice comparing to *Fut2*^{-/-} mice (day 7 p.i.). * $P < 0.05$; n.s. = not significant, Mann-Whitney test.

(TIF)

S3 Fig. Immune cells in murine colon tissue after *Salmonella* infection at day 7 p.i. Similar frequencies of (A) CD19⁺, (B) CD11c⁺ and (C) CD3⁺CD8⁺ cells were detected in colonic lamina propria of both *Fut2*^{+/+} and *Fut2*^{-/-} using flow cytometry ($n = 6$ mice per group). n.s. = not significant, Mann-Whitney test.

(TIF)

S4 Fig. Std fimbriae mediate adhesion to intestinal epithelial cell culture in a fucose-dependent manner. (A) Caco-2 cells were infected with *E. coli* ORN172 expressing Std fimbriae (StdON) or not (empty vector). UEA-I (green), F-actin (purple) and StdA (red) staining of formalin-fixed Caco-2 cells displaying StdA-expression by StdON strain (top) and different degrees of fucosylation by Caco-2 cells. Scale bars, 20 μ m. (B) *E. coli* ORN172 overexpressing Std (StdON) exhibited higher adherence to differentiated Caco-2 cells compared to *E. coli* ORN172 (empty). Adherence of *E. coli* StdON was abrogated upon addition of UEA-I lectin but not by addition of DBA lectin prior to infection. ** $p < 0.002$; n.s. = not significant, ANOVA with Tukey's multiple comparison test. (C) Imaging of Std fimbriae on *E. coli* ORN172 StdON by atomic force microscopy (AFM). The height profile is indicated by heatmaps. Insert show 2.5-fold enlarged details of the cell envelope.

(TIF)

S5 Fig. Bacterial gene expression *in vivo*. *Salmonella* gene expression in feces of *Fut2*^{+/+} and *Fut2*^{-/-} mice infected with either *S. Typhimurium* Δ aroA or *S. Typhimurium* Δ aroA Δ stdA was measured. Gene expression was normalized to *rpoD*. Comparable levels of *stdA* expression was observed in both *Fut2*^{+/+} and *Fut2*^{-/-} mice infected with *S. Typhimurium* Δ aroA (A). Neither *fucI* (B) nor *pduBC* (C) transcription was affected by *Fut2* genotype or the presence or absence of *stdAB* genes. No significant differences were detected using one-way ANOVA with Tukey's post test.

(TIF)

S6 Fig. Std fimbriae are differentially expressed *in vivo* on day 1 p.i. *Fut2*^{+/+} and *Fut2*^{-/-} mice ($n = 5$ of each genotype) were infected with the reporter strain *S. Typhimurium* *stdA*-stop::gfp. Mice were sacrificed on day 1 p.i. and colon sections were stained with anti-GFP antibody to detect GFP-positive bacteria. Std(GFP)-expressing *S. Typhimurium* were found mostly in lumen of colon, and only few bacteria expressed Std within mucosa. Scale bars, 10 μ m.

(TIF)

S7 Fig. Std producing *Salmonella* *in vivo* on day 7 p.i. stained with anti-Std antiserum. Colon sections of *Fut2*^{+/+} and *Fut2*^{-/-} mice were stained with an anti-*Salmonella* antibody (green). A subset of *Salmonella* stained positive with anti-Std antiserum (red). Fucosylation was visualized with UEA-1 lectin staining (grey) and nuclei were stained with DAPI (blue). Scale bars, 10 μ m.

(TIF)

S8 Fig. Deletion of *stdAB* genes does not affect motility and growth of *S. Typhimurium*. (A) *S. Typhimurium* SL1344 WT and *S. Typhimurium* SL1344 Δ stdA are similar in terms of motility. Deletion of *stdAB* genes has no effect on bacterial growth rate in WT (B) and Δ aroA

(C) backgrounds.
(TIF)

S9 Fig. Competitive index in feces. Competitive index (CI) was determined by infecting *Fut2*^{+/+} and *Fut2*^{-/-} mice (n = 10 per group) with an equal amount of *S. Typhimurium* Δ *aroA* and *S. Typhimurium* Δ *aroA* Δ *stdA*. Fecal homogenates from day 1, 3, 5 p.i. of both *Fut2*^{+/+} and *Fut2*^{-/-} mice were plated on LB agar containing streptomycin (total *Salmonella*) and on LB plates with streptomycin+kanamycin (*S. Typhimurium* Δ *aroA* Δ *stdA* only). Wilcoxon signed-rank test, p values are indicated.
(TIF)

Acknowledgments

We would like to thank Nicole Hansmeier for her support in atomic force microscopy imaging.

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References

1. Taylor SL, McGuckin MA, Wesselingh S, Rogers GB. Infection's Sweet Tooth: How Glycans Mediate Infection and Disease Susceptibility. *Trends Microbiol.* 2018; 26: 92–101. <https://doi.org/10.1016/j.tim.2017.09.011> PMID: 29079498
2. Moran AP, Gupta A, Joshi L. Sweet-talk: role of host glycosylation in bacterial pathogenesis of the gastrointestinal tract. *Gut.* 2011; 60: 1412–1425. <https://doi.org/10.1136/gut.2010.212704> PMID: 21228430
3. Dai D, Nanthkumar NN, Newburg DS, Walker WA. Role of oligosaccharides and glycoconjugates in intestinal host defense. *J Pediatr Gastroenterol Nutr.* 2000;30 Suppl 2: S23–33.
4. Ng KM, Ferreyra JA, Higginbottom SK, Lynch JB, Kashyap PC, Gopinath S, et al. Microbiota-liberated host sugars facilitate post-antibiotic expansion of enteric pathogens. *Nature.* 2013; 502: 96–99. <https://doi.org/10.1038/nature12503> PMID: 23995682
5. Mottram L, Wiklund G, Larson G, Qadri F, Svennerholm A-M. FUT2 non-secretor status is associated with altered susceptibility to symptomatic enterotoxigenic *Escherichia coli* infection in Bangladeshis. *Sci Rep.* 2017; 7. <https://doi.org/10.1038/s41598-017-10854-5> PMID: 28878367
6. Magalhaes A, Gomes J, Ismail MN, Haslam SM, Mendes N, Osorio H, et al. Fut2-null mice display an altered glycosylation profile and impaired BabA-mediated *Helicobacter pylori* adhesion to gastric mucosa. *Glycobiology.* 2009; 19: 1525–36. <https://doi.org/10.1093/glycob/cwp131> PMID: 19706747
7. Currier RL, Payne DC, Staat MA, Selvarangan R, Shirley SH, Halasa N, et al. Innate Susceptibility to Norovirus Infections Influenced by FUT2 Genotype in a United States Pediatric Population. *Clin Infect Dis Off Publ Infect Dis Soc Am.* 2015; 60: 1631–1638. <https://doi.org/10.1093/cid/civ165> PMID: 25744498
8. Pickard JM, Maurice CF, Kinnebrew MA, Abt MC, Schenten D, Golovkina TV, et al. Rapid fucosylation of intestinal epithelium sustains host-commensal symbiosis in sickness. *Nature.* 2014; 514: 638–641. <https://doi.org/10.1038/nature13823> PMID: 25274297
9. Pham TAN, Clare S, Goulding D, Arasteh JM, Stares MD, Browne HP, et al. Epithelial IL-22RA1-mediated fucosylation promotes intestinal colonization resistance to an opportunistic pathogen. *Cell Host Microbe.* 2014; 16: 504–516. <https://doi.org/10.1016/j.chom.2014.08.017> PMID: 25263220
10. McGovern DP, Jones MR, Taylor KD, Marcianti K, Yan X, Dubinsky M, et al. Fucosyltransferase 2 (FUT2) non-secretor status is associated with Crohn's disease. *Hum Mol Genet.* 2010; 19: 3468–76. <https://doi.org/10.1093/hmg/ddq248> PMID: 20570966
11. Pacheco AR, Curtis MM, Ritchie JM, Munera D, Waldor MK, Moreira CG, et al. Fucose sensing regulates bacterial intestinal colonization. *Nature.* 2012; 492: 113–117. <https://doi.org/10.1038/nature11623> PMID: 23160491
12. Hohmann EL. Nontyphoidal salmonellosis. *Clin Infect Dis Off Publ Infect Dis Soc Am.* 2001; 32: 263–269. <https://doi.org/10.1086/318457> PMID: 11170916
13. Wagner C, Hensel M. Adhesive mechanisms of *Salmonella enterica*. *Adv Exp Med Biol.* 2011; 715: 17–34. https://doi.org/10.1007/978-94-007-0940-9_2 PMID: 21557055
14. Krogfelt KA, Bergmans H, Klemm P. Direct evidence that the FimH protein is the mannose-specific adhesin of *Escherichia coli* type 1 fimbriae. *Infect Immun.* 1990; 58: 1995–1998. PMID: 1971261
15. Jones CH, Pinkner JS, Roth R, Heuser J, Nicholes AV, Abraham SN, et al. FimH adhesin of type 1 pili is assembled into a fibrillar tip structure in the Enterobacteriaceae. *Proc Natl Acad Sci U S A.* 1995; 92: 2081–2085. <https://doi.org/10.1073/pnas.92.6.2081> PMID: 7892228
16. Chessa D, Winter MG, Jakomin M, Baumler AJ. *Salmonella enterica* serotype Typhimurium Std fimbriae bind terminal alpha(1,2)fucose residues in the cecal mucosa. *Mol Microbiol.* 2009; 71: 864–75. <https://doi.org/10.1111/j.1365-2958.2008.06566.x> PMID: 19183274
17. Goto Y, Obata T, Kunisawa J, Sato S, Ivanov II, Lamichhane A, et al. Innate lymphoid cells regulate intestinal epithelial cell glycosylation. *Science.* 2014; 345: 1254009. <https://doi.org/10.1126/science.1254009> PMID: 25214634
18. Grassl GA, Valdez Y, Bergstrom KS, Vallance BA, Finlay BB. Chronic enteric salmonella infection in mice leads to severe and persistent intestinal fibrosis. *Gastroenterology.* 2008; 134: 768–80. <https://doi.org/10.1053/j.gastro.2007.12.043> PMID: 18325390

19. Pontier C, Pachot J, Botham R, Lenfant B, Arnaud P. HT29-MTX and Caco-2/TC7 monolayers as predictive models for human intestinal absorption: Role of the mucus layer. *J Pharm Sci.* 2001; 90: 1608–1619. <https://doi.org/10.1002/jps.1111> PMID: 11745719
20. Humphries A, DeRidder S, Bäuml AJ. *Salmonella enterica* Serotype Typhimurium Fimbrial Proteins Serve as Antigens during Infection of Mice. *Infect Immun.* 2005; 73: 5329–5338. <https://doi.org/10.1128/IAI.73.9.5329-5338.2005> PMID: 16113248
21. Jakomin M, Chessa D, Bäuml AJ, Casadesus J. Regulation of the *Salmonella enterica* std Fimbrial Operon by DNA Adenine Methylation, SeqA, and HdfR. *J Bacteriol.* 2008; 190: 7406–7413. <https://doi.org/10.1128/JB.01136-08> PMID: 18805972
22. García-Pastor L, Sánchez-Romero MA, Gutiérrez G, Puerta-Fernández E, Casadesus J. Formation of phenotypic lineages in *Salmonella enterica* by a pleiotropic fimbrial switch. Herman C, editor. *PLOS Genet.* 2018; 14: e1007677. <https://doi.org/10.1371/journal.pgen.1007677> PMID: 30252837
23. Iwamori M, Domino SE. Tissue-specific loss of fucosylated glycolipids in mice with targeted deletion of alpha(1,2)fucosyltransferase genes. *Biochem J.* 2004; 380: 75–81. <https://doi.org/10.1042/BJ20031668> PMID: 14967068
24. Folseraas T, Melum E, Rausch P, Juran BD, Ellinghaus E, Shiryayev A, et al. Extended analysis of a genome-wide association study in primary sclerosing cholangitis detects multiple novel risk loci. *J Hepatol.* 2012; 57: 366–75. <https://doi.org/10.1016/j.jhep.2012.03.031> PMID: 22521342
25. Ikehara Y, Nishihara S, Yasutomi H, Kitamura T, Matsuo K, Shimizu N, et al. Polymorphisms of two fucosyltransferase genes (Lewis and Secretor genes) involving type I Lewis antigens are associated with the presence of anti-*Helicobacter pylori* IgG antibody. *Cancer Epidemiol Biomark Prev.* 2001; 10: 971–7. Available: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11535550
26. Hutson AM, Atmar RL, Graham DY, Estes MK. Norwalk virus infection and disease is associated with ABO histo-blood group type. *J Infect Dis.* 2002; 185: 1335–7. <https://doi.org/10.1086/339883> PMID: 12001052
27. Marionneau S, Airaud F, Bovin NV, Pendu JL, Ruvoën-Clouet N. Influence of the Combined ABO, FUT2 and FUT3 Polymorphism on Susceptibility to Norwalk Virus Attachment. *J Infect Dis.* 2005; 192: 1071–1077. <https://doi.org/10.1086/432546> PMID: 16107962
28. Kindberg E, Hejdeman B, Bratt G, Wahren B, Lindblom B, Hinkula J, et al. A nonsense mutation (428G →A) in the fucosyltransferase FUT2 gene affects the progression of HIV-1 infection. *AIDS Lond Engl.* 2006; 20: 685–689. <https://doi.org/10.1097/01.aids.0000216368.23325.bc> PMID: 16514298
29. Rausch P, Steck N, Suwandi A, Seidel JA, Künzel S, Bhullar K, et al. Expression of the Blood-Group-Related Gene B4galnt2 Alters Susceptibility to *Salmonella* Infection. *PLOS Pathog.* 2015; 11: e1005008. <https://doi.org/10.1371/journal.ppat.1005008> PMID: 26133982
30. Nell S, Kennemann L, Schwarz S, Josenhans C, Suerbaum S. Dynamics of Lewis b Binding and Sequence Variation of the babA Adhesin Gene during Chronic *Helicobacter pylori* Infection in Humans. Blaser MJ, editor. *mBio.* 2014; 5: <https://doi.org/10.1128/mBio.02281-14> PMID: 25516619
31. Ilver D, Arnqvist A, Ögren J, Frick I-M, Kersulyte D, Inceci ET, et al. *Helicobacter pylori* Adhesin Binding Fucosylated Histo-Blood Group Antigens Revealed by Retagging. *Science.* 1998; 279: 373–377. <https://doi.org/10.1126/science.279.5349.373> PMID: 9430586
32. Hu L, Crawford SE, Czako R, Cortes-Penfield NW, Smith DF, Le Pendu J, et al. Cell attachment protein VP8* of a human rotavirus specifically interacts with A-type histo-blood group antigen. *Nature.* 2012; 485: 256–259. <https://doi.org/10.1038/nature10996> PMID: 22504179
33. de Rougemont A, Ruvoën-Clouet N, Simon B, Estienne M, Elie-Caille C, Aho S, et al. Qualitative and quantitative analysis of the binding of GII.4 norovirus variants onto human blood group antigens. *J Virol.* 2011; 85: 4057–4070. <https://doi.org/10.1128/JVI.02077-10> PMID: 21345963
34. Goto Y, Uematsu S, Kiyono H. Epithelial glycosylation in gut homeostasis and inflammation. *Nat Immunol.* 2016; 17: 1244–1251. <https://doi.org/10.1038/ni.3587> PMID: 27760104
35. Staib L, Fuchs TM. Regulation of fucose and 1,2-propanediol utilization by *Salmonella enterica* serovar Typhimurium. *Front Microbiol.* 2015; 6: <https://doi.org/10.3389/fmicb.2015.01116> PMID: 26528264
36. Faber F, Thiennimitr P, Spiga L, Byndloss MX, Litvak Y, Lawhon S, et al. Respiration of Microbiota-Derived 1,2-propanediol Drives *Salmonella* Expansion during Colitis. Sperandio V, editor. *PLOS Pathog.* 2017; 13: e1006129. <https://doi.org/10.1371/journal.ppat.1006129> PMID: 28056091
37. Yue M, Rankin SC, Blanchet RT, Nulton JD, Edwards RA, Schifferli DM. Diversification of the *Salmonella* Fimbriae: A Model of Macro- and Microevolution. *PLOS ONE.* 2012; 7: e38596. <https://doi.org/10.1371/journal.pone.0038596> PMID: 22701679

38. Humphries AD, Raffatellu M, Winter S, Weening EH, Kingsley RA, Droleskey R, et al. The use of flow cytometry to detect expression of subunits encoded by 11 *Salmonella enterica* serotype Typhimurium fimbrial operons. *Mol Microbiol*. 2003; 48: 1357–1376. PMID: [12787362](#)
39. Weening EH, Barker JD, Laarakker MC, Humphries AD, Tsois RM, Baumler AJ. The *Salmonella enterica* Serotype Typhimurium *lpf*, *bcf*, *stb*, *stc*, *std*, and *sth* Fimbrial Operons Are Required for Intestinal Persistence in Mice. *Infect Immun*. 2005; 73: 3358–3366. <https://doi.org/10.1128/IAI.73.6.3358-3366.2005> PMID: [15908362](#)
40. Terahara K, Nochi T, Yoshida M, Takahashi Y, Goto Y, Hatai H, et al. Distinct fucosylation of M cells and epithelial cells by Fut1 and Fut2, respectively, in response to intestinal environmental stress. *Biochem Biophys Res Commun*. 2011; 404: 822–828. <https://doi.org/10.1016/j.bbrc.2010.12.067> PMID: [21172308](#)
41. Rausch P, Künzel S, Suwandi A, Grassl GA, Rosenstiel P, Baines JF. Multigenerational Influences of the Fut2 Gene on the Dynamics of the Gut Microbiota in Mice. *Front Microbiol*. 2017;8. <https://doi.org/10.3389/fmicb.2017.00008>
42. Domino SE, Zhang L, Gillespie PJ, Saunders TL, Lowe JB. Deficiency of Reproductive Tract alpha (1,2) Fucosylated Glycans and Normal Fertility in Mice with Targeted Deletions of the FUT1 or FUT2 alpha (1,2)Fucosyltransferase Locus. *Mol Cell Biol*. 2001; 21: 8336–8345. <https://doi.org/10.1128/MCB.21.24.8336-8345.2001> PMID: [11713270](#)
43. Weintraub BC, Eckmann L, Okamoto S, Hense M, Hedrick SM, Fierer J. Role of alphabeta and gamma-delta T cells in the host response to *Salmonella* infection as demonstrated in T-cell-receptor-deficient mice of defined Ity genotypes. *Infect Immun*. 1997; 65: 2306–2312. PMID: [9169768](#)
44. Hoiseth SK, Stocker BA. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature*. 1981; 291: 238–9. Available: <http://www.ncbi.nlm.nih.gov/pubmed/7015147> <https://doi.org/10.1038/291238a0> PMID: [7015147](#)
45. Hansmeier N, Miskiewicz K, Elpers L, Liss V, Hensel M, Sterzenbach T. Functional expression of the entire adhesiome of *Salmonella enterica* serotype Typhimurium. *Sci Rep*. 2017; 7: 10326. <https://doi.org/10.1038/s41598-017-10598-2> PMID: [28871183](#)
46. Coburn B, Grassl GA, Finlay BB. *Salmonella*, the host and disease: a brief review. *Immunol Cell Biol*. 2007; 85: 112–118. <https://doi.org/10.1038/sj.icb.7100007> PMID: [17146467](#)
47. Lesuffleur T, Porchet N, Aubert JP, Swallow D, Gum JR, Kim YS, et al. Differential expression of the human mucin genes MUC1 to MUC5 in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations. *J Cell Sci*. 1993; 106 (Pt 3): 771–783.
48. Dolan B, Naughton J, Tegtmeyer N, May FEB, Clyne M. The Interaction of *Helicobacter pylori* with the Adherent Mucus Gel Layer Secreted by Polarized HT29-MTX-E12 Cells. Ahmed N, editor. *PLoS ONE*. 2012; 7: e47300. <https://doi.org/10.1371/journal.pone.0047300> PMID: [23056622](#)
49. Miyoshi H, Stappenbeck TS. In vitro expansion and genetic modification of gastrointestinal stem cells as organoids. *Nat Protoc*. 2013; 8: 2471–2482. <https://doi.org/10.1038/nprot.2013.153> PMID: [24232249](#)
50. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2⁻ $\Delta\Delta$ CT Method. *Methods*. 2001; 25: 402–408. <https://doi.org/10.1006/meth.2001.1262> PMID: [11846609](#)

Suwandi A, **Galeev A**, Riedel R, Sharma S, Seeger K, Sterzenbach T, Pastor LG, Boyle EC, Gal-Mor O, Hensel M, Casadesús J. Std fimbriae-fucose interaction increases *Salmonella*-induced intestinal inflammation and prolongs colonization. PLoS Pathogens. 2019 Jul 22;15(7):e1007915.

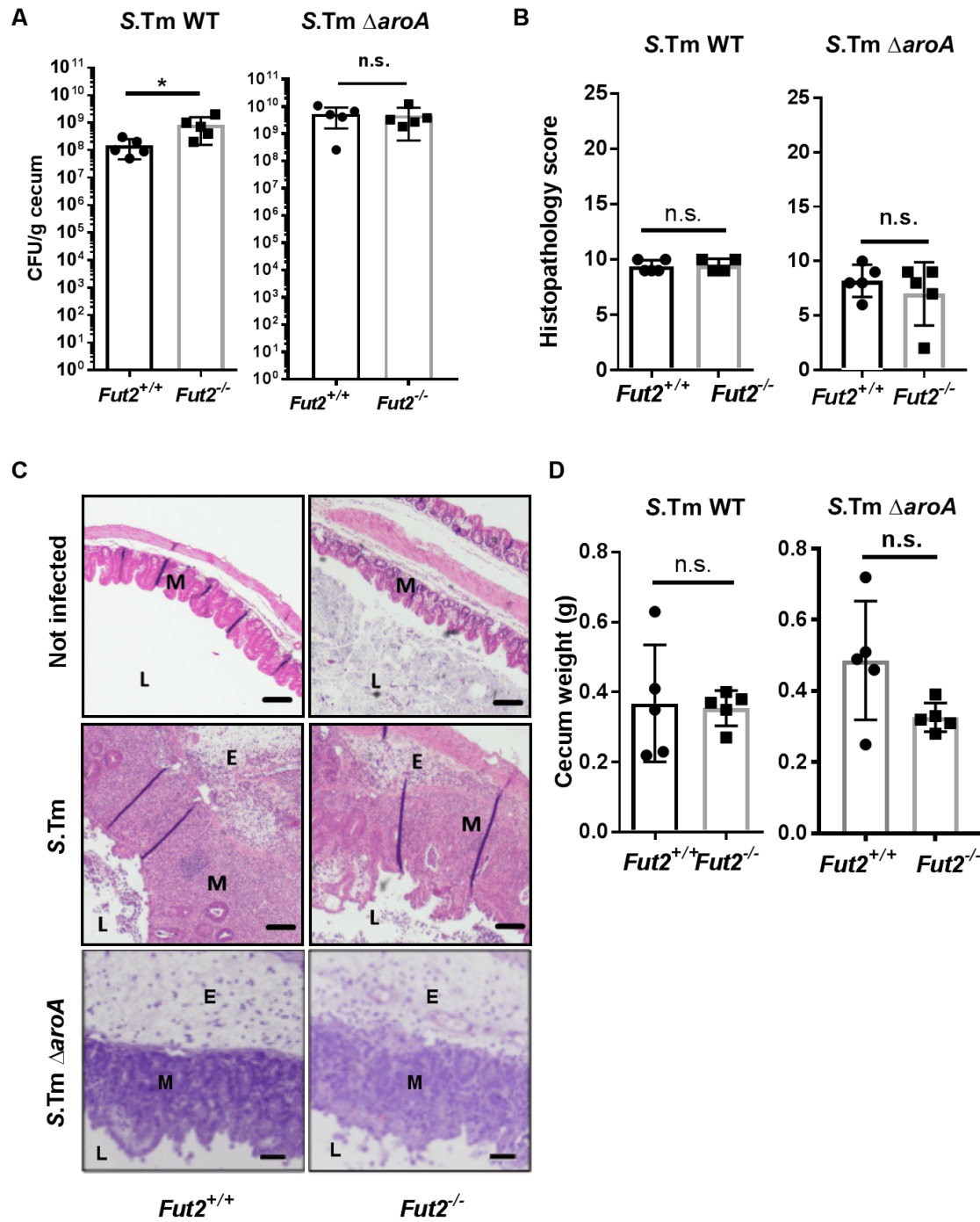
Supplementary data:

S1 Table. Antibodies and lectins used in this study.

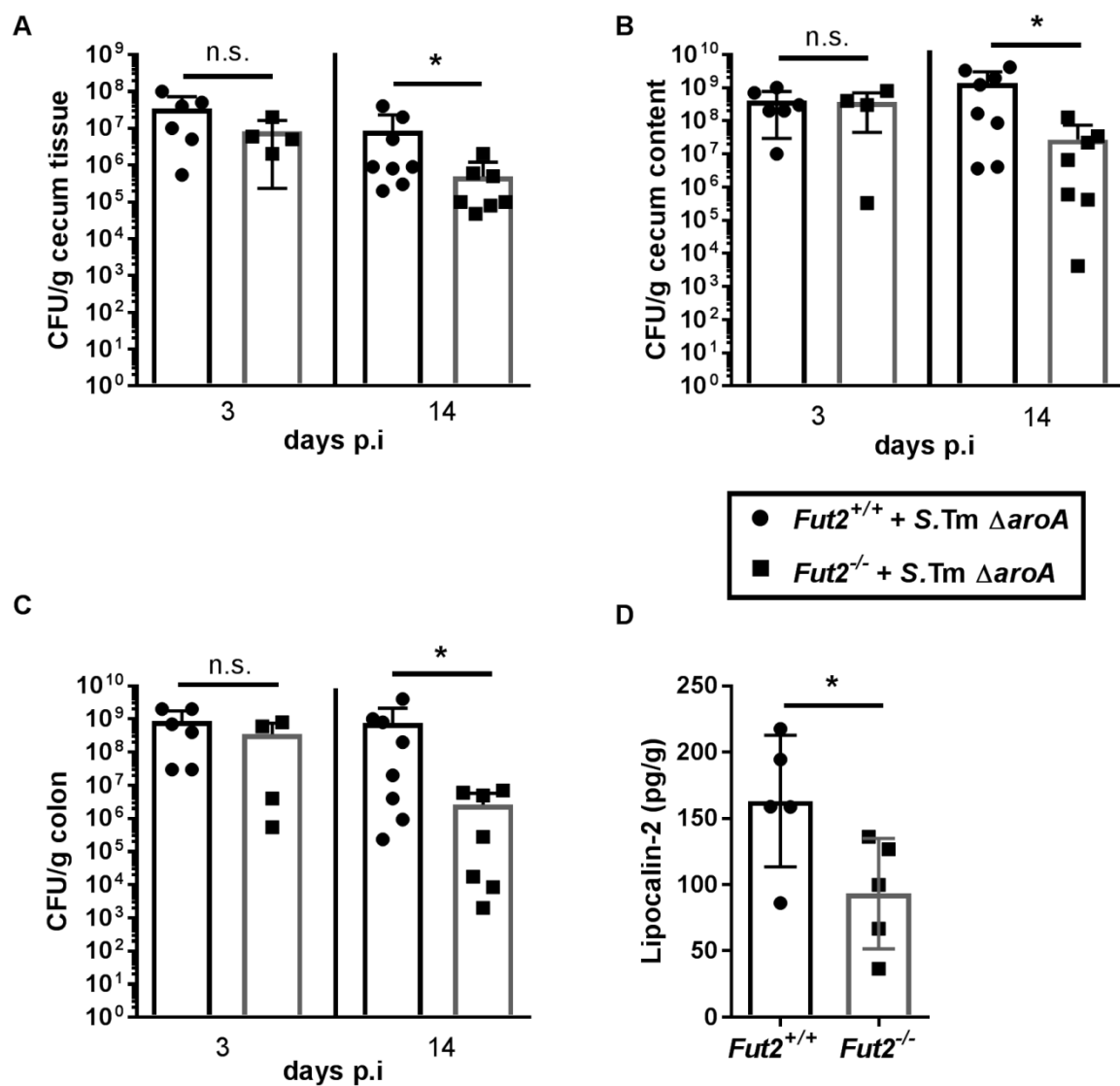
Antibody	Reference/Manufacturer, RRID and catalog number
Polyclonal rabbit anti-StdA serum	Humphries et al., 2003
F(ab)2-Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP	Thermo Fisher Scientific Cat# A10547, RRID:AB_2534046
CD3 antibody	Abcam Cat# ab5690, RRID:AB_305055
CD68 antibody [KP1], prediluted	Abcam Cat# ab845, RRID:AB_306567
Anti-Myeloperoxidase (Mature / Immature Myeloid Cell Marker) Ab-1 Polyclonal Antibody, Unconjugated	Lab Vision Cat# RB-373-A, RRID:AB_59597
F(ab)2-Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 633	Thermo Fisher Scientific Cat# A-21053, RRID:AB_2535720
F(ab)2-Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	Thermo Fisher Scientific Cat# A-11071, RRID:AB_2534115
CD16/CD32 antibody	BD Biosciences Cat# 553142, RRID:AB_394657
APC/Cyanine7 anti-mouse CD45 antibody	BioLegend Cat# 103116, RRID:AB_312981
APC anti-mouse CD4 antibody	BioLegend Cat# 100411, RRID:AB_312696
PE/Cy7 anti-mouse Ly-6G antibody	BioLegend Cat# 127618, RRID:AB_1877261
CD3 Monoclonal Antibody (17A2), FITC, eBioscience(TM)	Thermo Fisher Scientific Cat# 11-0032-80, RRID:AB_2572430
CD11c Monoclonal Antibody (N418), FITC, eBioscience(TM)	Thermo Fisher Scientific Cat# 11-0114-82, RRID:AB_464940
CD8a Monoclonal Antibody (53-6.7), PE, eBioscience(TM)	Thermo Fisher Scientific Cat# 12-0081-81, RRID:AB_465529
F4/80 Monoclonal Antibody (BM8), PerCP-Cyanine5.5, eBioscience(TM)	Thermo Fisher Scientific Cat# 45-4801-82, RRID:AB_914345
CD11b Monoclonal Antibody (M1/70), PE, eBioscience™	Thermo Fisher Scientific Cat# 12-0112-82, RRID:AB_2734869
Rat Anti-Mouse Ly-6C Monoclonal Antibody, APC Conjugated, Clone AL-21	BD Biosciences Cat# 560595, RRID:AB_1727554
PerCP-Cy™5.5 Rat Anti-Mouse CD19 Clone 1D3	BD Biosciences Cat# 561113, RRID:AB_10563071
Purified anti- <i>E. coli</i> RNA Sigma 70 antibody	BioLegend Cat# 663202, RRID:AB_2564410
Polyclonal rabbit anti- <i>E. coli</i> antibody	Abcam Cat# ab137967
Anti-CD324 (E-Cadherin) Alexa Fluor® 647 100 ug antibody	Thermo Fisher Scientific Cat# 51-3249-82, RRID:AB_1210532
Green fluorescent protein (GFP) antibody - DSHB; University of Iowa	DSHB Cat# DSHB-GFP-12A6, RRID:AB_2617417
<i>Salmonella</i> O Antiserum Group B Factors 1, 4, 12, 27	BD Difco Cat# 229731
F(ab)2-Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific Cat# A-11017, RRID:AB_2534084
Lectin	Manufacturer, RRID and catalog number
Ulex europaeus [UEA-I] (FITC) lectin	CosmoBIO Cat# CAC-JOM-J519, accession #P22972
Wheat germ agglutinin [WGA] (Rhodamine) lectin	Vector Laboratories Cat# RL-1022, RRID:AB_2336871
Dolichos biflorus agglutinin [DBA] (FITC) lectin	Vector Laboratories Cat# FL-1031, RRID:AB_2336394

Table S2. Primers used in this study.

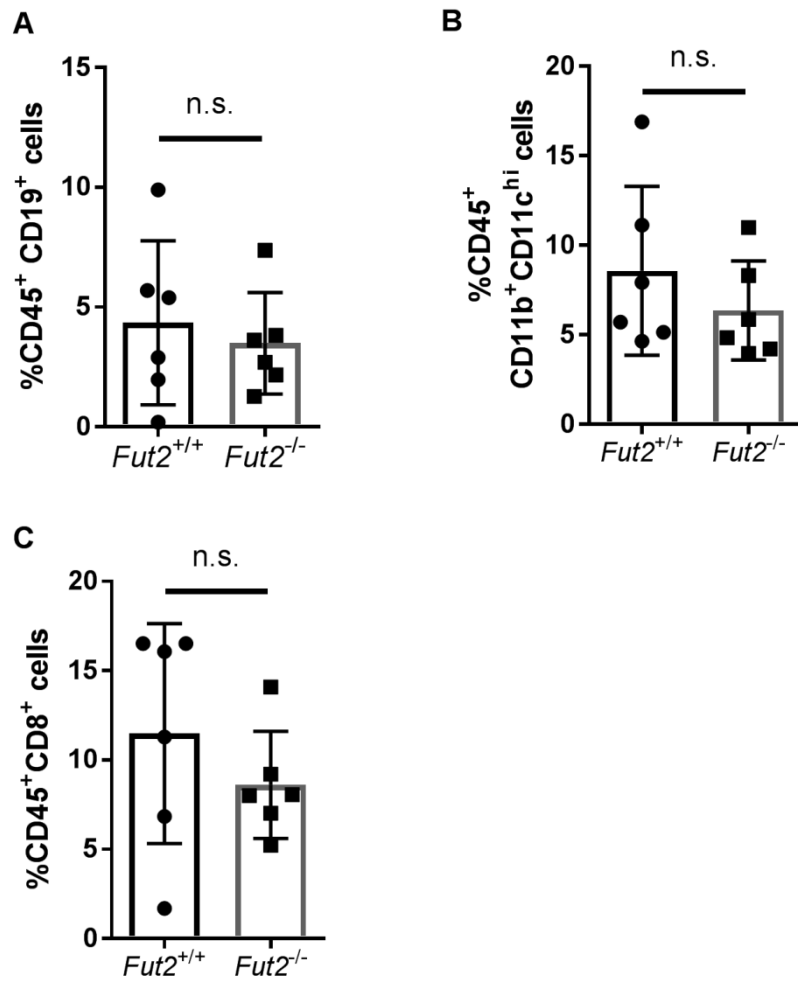
Primer	Sequence (5' to 3')	Description
gNRAMP1 fw s	CAGCATCCCGCTGTGGGA	<i>NRAMP1</i> genotyping
gNRAMP1 fw r	CAGCATCCCGCTGTGGGG	
gNRAMP1 re	ACAGCCCCGGACAGGTGGG	
rpoD-fw	GGTCTGACCATCGAACAGGTG	<i>rpoD</i> housekeeping gene
rpoD-re	ATCAGACCGATGTTGCCTTC	
stdA-fw	CGG CTG CCG GTA TGA TGT	<i>stdA</i> gene expression
stdA-rw	GGG CCT GCT GTG GGT GTA	
STm fucI-fw	CGCCGCCTGTGAAGAAAAAT	<i>fucI</i> gene expression
STm fucI-re	GTAAACCGCCCCAGGACGCT	
pduBC-F	CACCAGCTTTAGTAACGAAGC	<i>pduBC</i> genes expression; Fuchs and Staib, 2015
pduBC-R	CAAAGCCGTCCTGATTCAC	



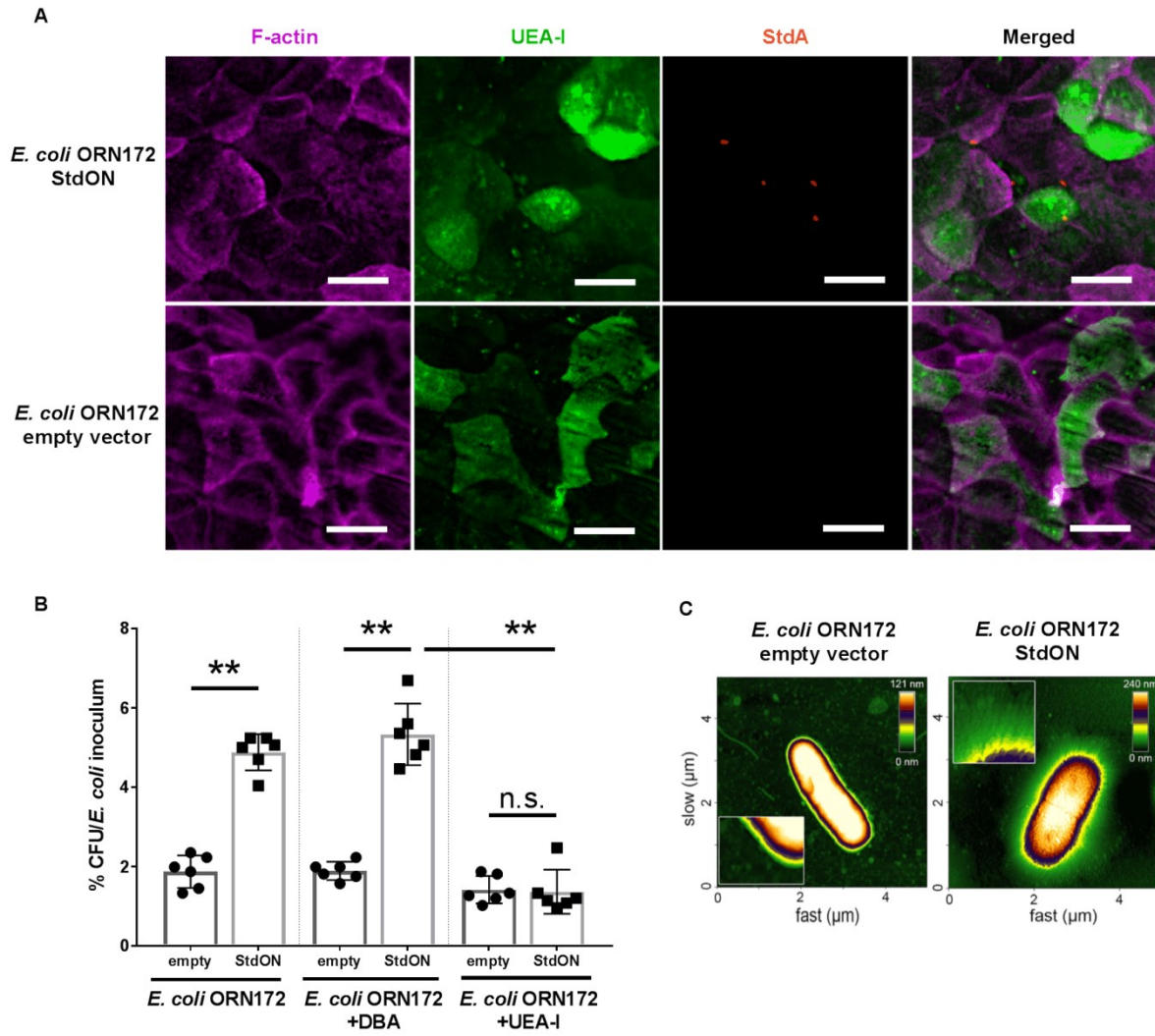
S1 Fig. *Fut2*-deficient mice are more susceptible to *Salmonella* infection on day 1 p.i.



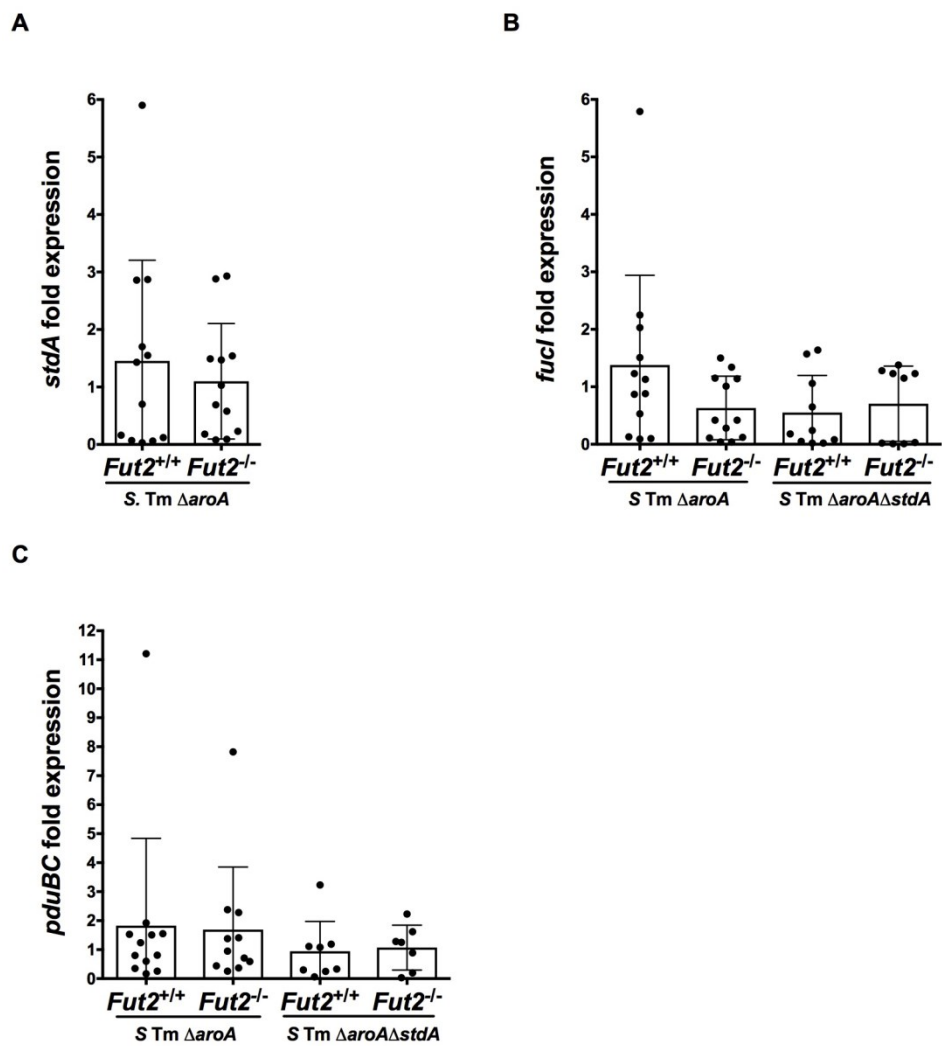
S2 Fig. *Fut2*^{+/+} mice carry higher bacterial burden at later infection time points.



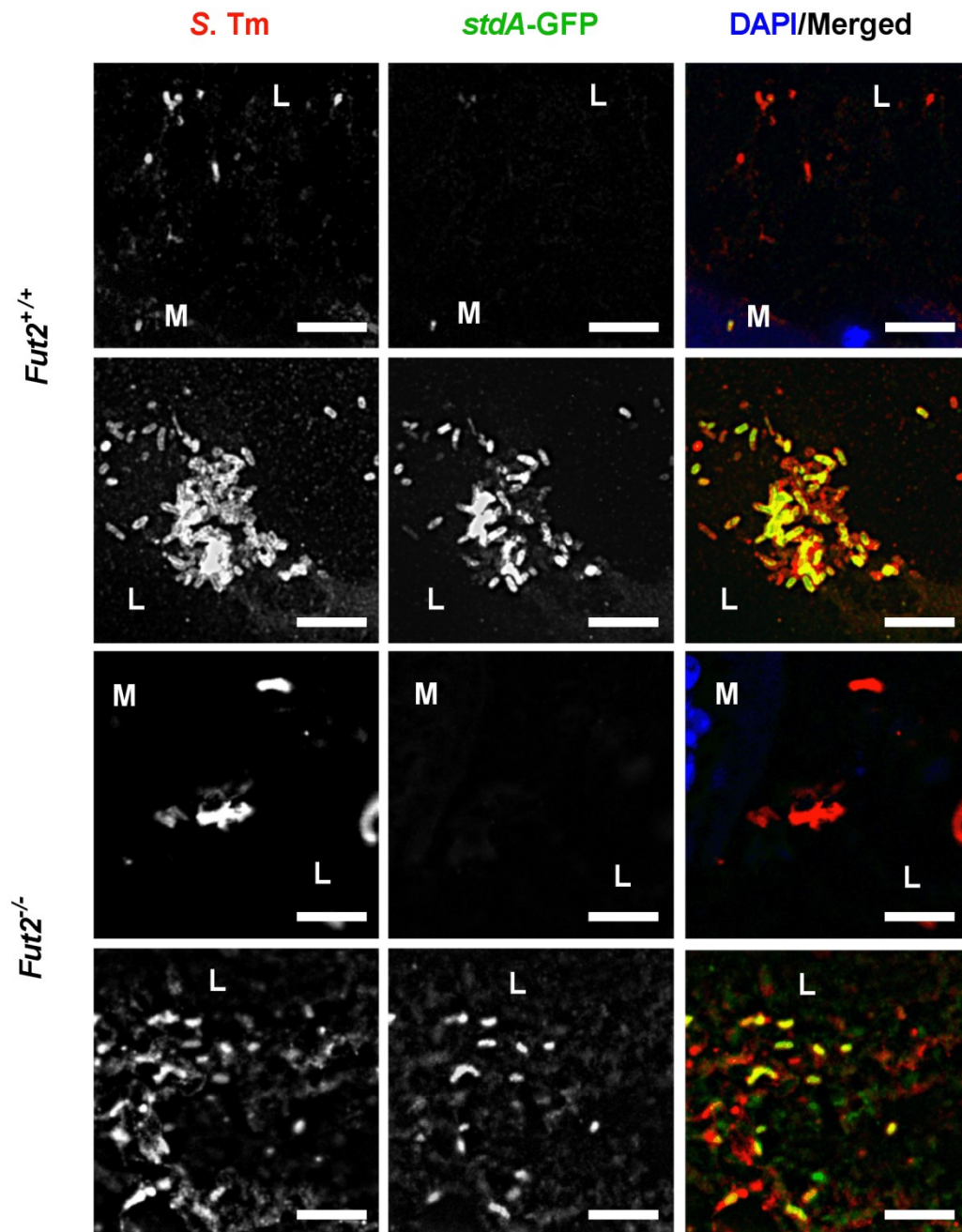
S3 Fig. Immune cells in murine colon tissue after *Salmonella* infection at day 7 p.i.



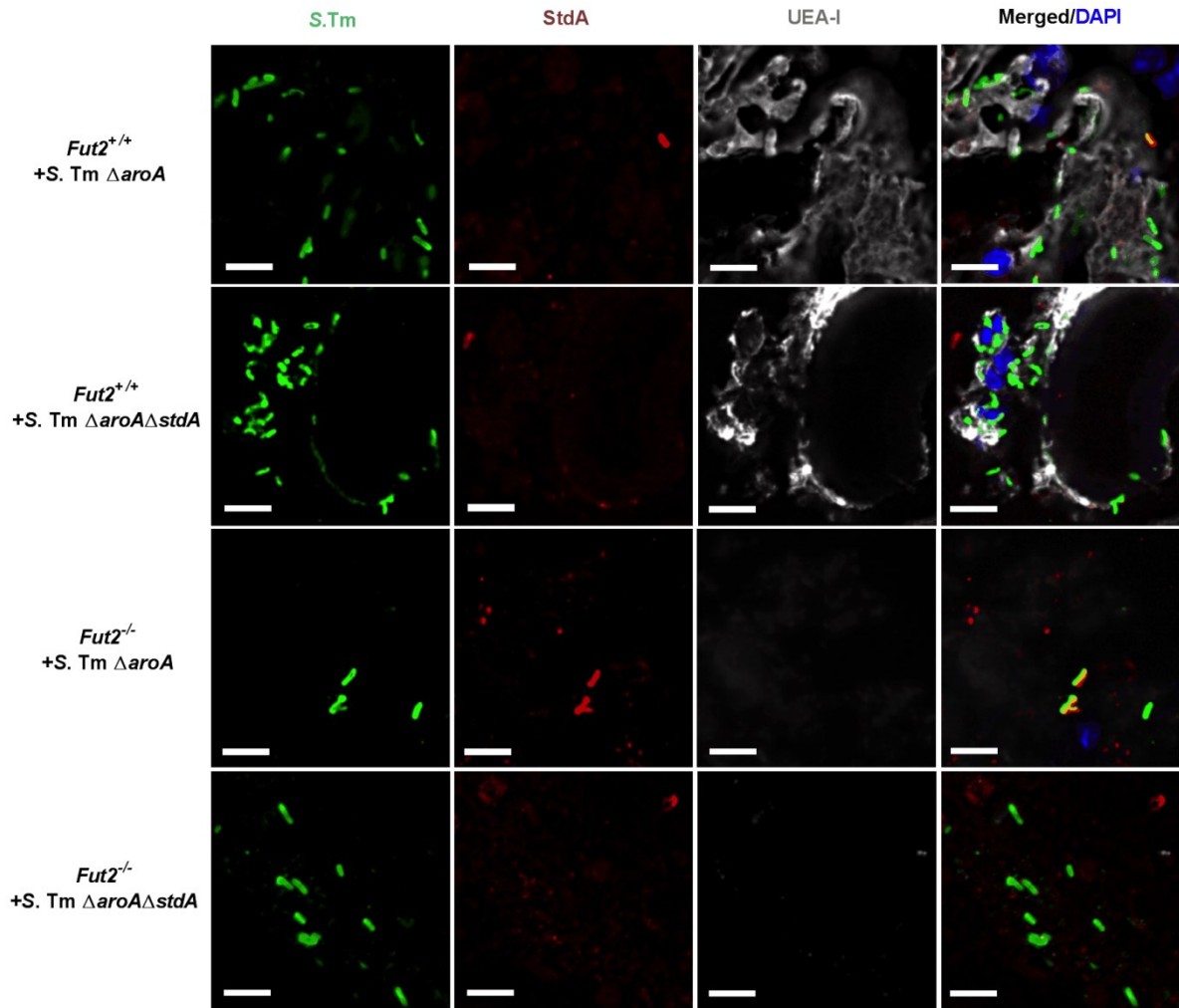
S4 Fig. Std fimbriae mediate adhesion to intestinal epithelial cell culture in a fucose-dependent manner.



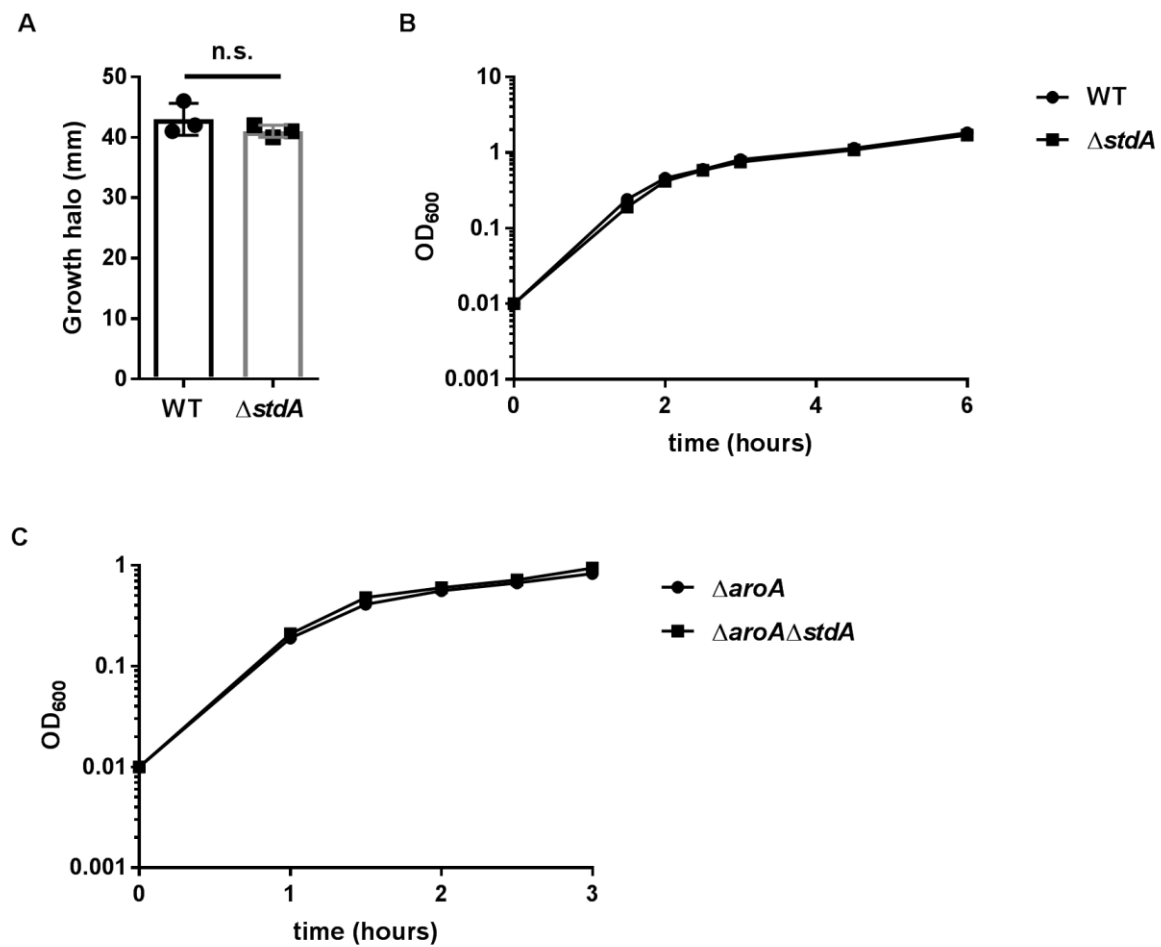
S5 Fig. Bacterial gene expression in vivo.



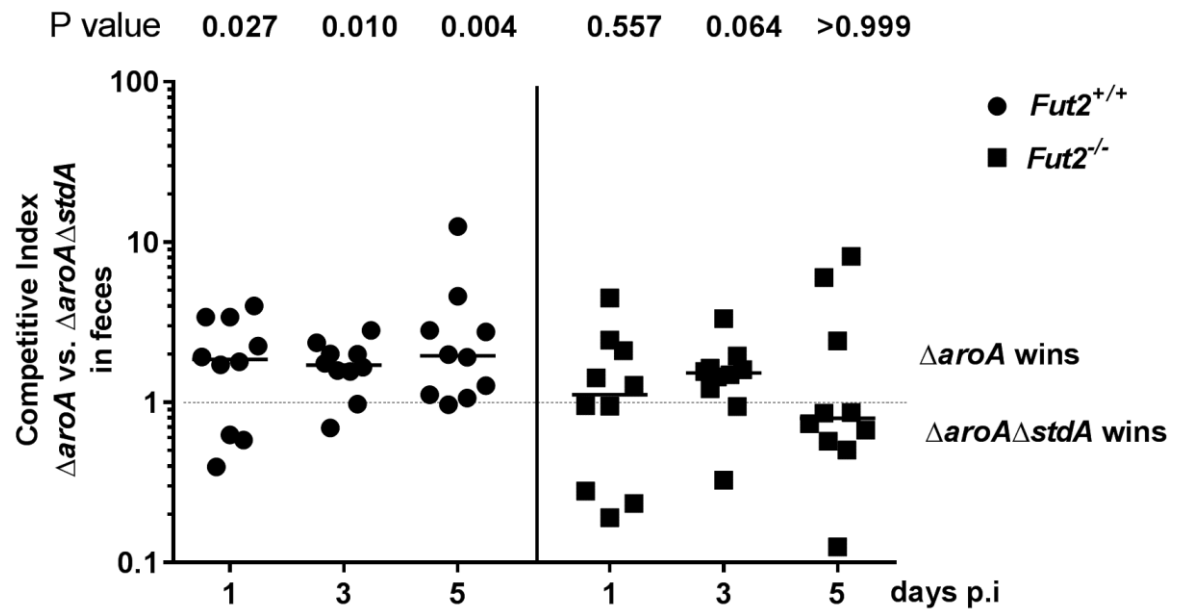
S6 Fig. Std fimbriae are differentially expressed in vivo on day 1 p.i.



S7 Fig. Std producing *Salmonella* in vivo on day 7 p.i. stained with anti-Std antiserum.



S8 Fig. Deletion of *stdAB* genes does not affect motility and growth of *S. Typhimurium*.



S9 Fig. Competitive index in feces.



Proteoglycan-Dependent Endo-Lysosomal Fusion Affects Intracellular Survival of *Salmonella* Typhimurium in Epithelial Cells

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OPEN ACCESS

Edited by:

Megan S. Lord,
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Oslo University Hospital, Norway

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Specialty section:

This article was submitted to
Molecular Innate Immunity,
a section of the journal
Frontiers in Immunology

Received: 13 December 2019

Accepted: 31 March 2020

Published: 29 April 2020

Citation:

Galeev A, Suwandi A, Bakker H,
Oktiviyari A, Routier FH, Krone L,
Hensel M and Grassl GA (2020)
Proteoglycan-Dependent
Endo-Lysosomal Fusion Affects
Intracellular Survival of *Salmonella*
Typhimurium in Epithelial Cells.
Front. Immunol. 11:731.
doi: 10.3389/fimmu.2020.00731

Proteoglycans (PGs) are glycoconjugates which are predominately expressed on cell surfaces and consist of glycosaminoglycans (GAGs) linked to a core protein. An initial step of GAGs assembly is governed by the β -D-xylosyltransferase enzymes encoded in mammals by the *XylT1/XylT2* genes. PGs are essential for the interaction of a cell with other cells as well as with the extracellular matrix. A number of studies highlighted a role of PGs in bacterial adhesion, invasion, and immune response. In this work, we investigated a role of PGs in *Salmonella enterica* serovar Typhimurium (S. Typhimurium) infection of epithelial cells. Gentamicin protection and chloroquine resistance assays were applied to assess invasion and replication of S. Typhimurium in wild-type and xylosyltransferase-deficient (Δ XylT2) Chinese hamster ovary (CHO) cells lacking PGs. We found that S. Typhimurium adheres to and invades CHO WT and CHO Δ XylT2 cells at comparable levels. However, 24 h after infection, proteoglycan-deficient CHO Δ XylT2 cells are significantly less colonized by S. Typhimurium compared to CHO WT cells. This proteoglycan-dependent phenotype could be rescued by addition of PGs to the cell culture medium, as well as by complementation of the *XylT2* gene. Chloroquine resistance assay and immunostaining revealed that in the absence of PGs, significantly less bacteria are associated with *Salmonella*-containing vacuoles (SCVs) due to a re-distribution of endocytosed gentamicin. Inhibition of endo-lysosomal fusion by a specific inhibitor of phosphatidylinositol phosphate kinase PIKfyve significantly increased S. Typhimurium burden in CHO Δ XylT2 cells demonstrating an important role of PGs for PIKfyve dependent vesicle fusion which is modulated by *Salmonella* to establish infection. Overall, our results demonstrate that PGs influence survival of intracellular *Salmonella* in epithelial cells via modulation of PIKfyve-dependent endo-lysosomal fusion.

Keywords: *Salmonella*, proteoglycans, glycosaminoglycans, xylosyltransferase, PIKfyve, gentamicin

INTRODUCTION

Proteoglycans (PGs) are heavily glycosylated proteins facilitating cell-matrix and cell-cell interactions and are also playing an important role in bacterial adhesion, invasion, and immune response (1). All PGs consist of a core protein substituted with glycosaminoglycans (GAGs) – long linear polysaccharides comprised of repeating disaccharide units. Based on the structure of the disaccharide unit, GAGs are divided into four distinct families: heparan sulfate (HS)/heparin, chondroitin/dermatan sulfate, keratan sulfate, and hyaluronan (2). The biosynthesis of the first two GAGs is initiated by the assembly of the tetrasaccharide linker D-GlcA- β 1-3-Gal- β 1-3-Gal- β 1-4-Xyl- β -Ser. The initial, rate-limiting step is the transfer of Xyl from UDP-D- α -xylose to serine moieties of the core protein and is catalyzed by the isoenzymes β -D-xylosyltransferase-I and -II (EC 2.4.2.26) (3) encoded in humans by the *XYLT1* and *XYLT2* genes, respectively.

In the past years, a number of studies highlighted an importance of PGs in bacterial pathogenesis. Proteoglycan-mediated adhesion and invasion has been previously reported for various gram-positive and gram-negative bacteria, including *Listeria monocytogenes* (4), *Neisseria gonorrhoeae* (5), *Borrelia burgdorferi* (6), and *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) (7). *S. Typhimurium* is a successful food-borne pathogen able to colonize the human gastrointestinal tract and to cause severe diarrhea. *S. Typhimurium* manipulates actin and membrane trafficking pathways of epithelial cells initiating entry via actin-mediated macropinocytosis (8). A hallmark of *Salmonella* infection is an extensive alteration of the endo-lysosomal system and phosphoinositide metabolism of the host (9). After invasion, *S. Typhimurium* translocates effectors through a type 3 secretion system (T3SS) encoded by genes in *Salmonella* pathogenicity island 2 (SPI2) into the host cytoplasm in order to establish a replicative niche called the *Salmonella*-containing vacuole (SCV). At later stages of infection, mature SCVs acquire late endosomal markers, such as LAMP1, and fuse with the late endosomes and other organelles (ER, Golgi apparatus) forming an extensive network of tubules called *Salmonella*-induced filaments (SIFs) (10, 11). Previous studies identified that SIF formation by *S. Typhimurium* is dependent on various host factors, including the lysosomal glycoproteins LAMPs (12), vacuolar vATPase (13), the late endosomal small GTPase Rab7 (14), and secretory carrier membrane proteins (SCAMPs) (15). Moreover, there is a growing body of evidence suggesting that *Salmonella* interferes with the exocytic transport machinery and with secretory pathways (16, 17).

Phosphatidylinositol (PI) belongs to the class of the phosphatidylglycerides – glycerol-based phospholipids which are a major component of biological cell membranes. Phosphorylated forms of PI (called phosphoinositides) play important roles in cell signaling, cell growth and death, and membrane trafficking (18). For example, the monophosphorylated phosphatidylinositol-3-phosphate (PtdIns3P) orchestrates recruitment and membrane association of early endosomal proteins EEA1 and Rab5 (19). Furthermore, it was demonstrated that phosphatidylinositol-3,5-bisphosphate (PtdIns(3,5)P₂) regulates endosomal fission

and fusion, as well as multivesicular body (MVB) formation and detachment (20). PIKfyve is a lipid kinase that converts PtdIns3P into PtdIns(3,5)P₂ in the endocytic microdomains of mammalian cells (21). While it is known that *Salmonella* can modulate phosphoinositide pathways in host cells (22, 23), limited knowledge exists on possible interactions of phosphoinositides with PGs.

To investigate the contribution of surface and intracellular PGs to *Salmonella* infection we utilized a proteoglycan-deficient Chinese hamster ovary (CHO) cell line (3). We demonstrate that absence of PGs in epithelial CHO cells results in an altered PIKfyve-dependent endo-lysosomal trafficking affecting intracellular *Salmonella* survival.

MATERIALS AND METHODS

Cell Lines

Chinese hamster ovary (CHO) CHO-K1 WT and CHO-K1 pgsA745 (aka Δ XylT2, referred to as Δ XylT) (3) cells were routinely cultured in DMEM/F-12 GlutaMAX growth medium (Life Technologies) supplemented with 10% (v/v) fetal bovine serum (Biochrom).

CHO Δ XylT Cell Line Complementation

CHO Δ XylT mutant was complemented with a plasmid expressing human XYLT2 as described (24). Complementation of a G418-selected clone was confirmed by flow cytometry using the heparan sulfate-specific phage display antibody AO4B08 (25). The overlaid histograms with the peak heights were normalized to mode (% of Max). The data were analyzed using FlowJo v.10 software (TreeStar).

Bacteria and Growth Conditions

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) 14028s (26), *S. Typhimurium* SL1344 WT (27), *S. Typhimurium* SL1344 eGFP (pFPV25.1) (28), *S. Typhimurium* SL1344 Δ ssaR (29), and *S. Typhimurium* SL1344 Δ sifA (30) were grown overnight at 37°C with shaking in lysogeny broth (LB) supplemented with 100 μ g/mL streptomycin, 100 μ g/mL ampicillin, or 50 μ g/mL kanamycin, when appropriate. The reporter strain *S. Typhimurium* SL1344 p4889 (31) was grown in presence of carbenicillin 50 μ g/mL. *Listeria monocytogenes* EGD strain (32) was grown at 37°C in Brain Heart Infusion (BHI) broth. For infection, overnight cultures of bacteria were sub-cultured and grown for 3 h at 37°C to mid-log phase.

Generation of Acid Shock Reporter Plasmid

The acid shock-responsive promoter of *asr* was used to control expression of sfGFP. A dual fluorescence reporter was generated based on p4889, and P_{uhpT} in p4889 was replaced by P_{asr} by Gibson assembly (GA) cloning. Primers Vf-p4889 and Vr-p4889 were used to PCR amplify the vector backbone of p4889, and 1f p4889-Pasr and 1r Pasr-sfGFP were used to amplify the P_{asr} region from genomic DNA of *S. Typhimurium*. GA resulted

in plasmid p5386 that was confirmed by DNA sequencing, and functional analyses of response of sfGFP expression upon acid shock exposure in synthetic media buffered to various pH.

Gentamicin Protection Assay

CHO WT, CHO Δ XylT, or complemented CHO Δ XylT cells (CHO cX) were seeded in 24-well plates (10^5 cells/well) and incubated overnight in 5% CO₂ at 37°C. The next day, cells were infected with either *Listeria monocytogenes* EGD, or with different *Salmonella* strains at MOI 10, 50, or 100 (as indicated). For quantification of adherent bacteria, 30 min (or 60 min for *Listeria*) post infection (p.i.), cells were washed 3 times with PBS and lysed in PBS containing 1% (v/v) Triton X-100 and 0.1% (v/v) sodium dodecyl sulfate (SDS). The cell lysates were then serially diluted in PBS and plated on LB agar or on BHI agar for colony-forming unit (CFU) count. For later time points, upon washing, culture medium was replaced with a medium supplemented with 100 µg/mL gentamicin (Sigma) to kill extracellular bacteria. The number of invaded bacteria was determined by plating the cells lysates 1.5 h p.i. (2.5 h p.i. for *Listeria*). Medium was replaced by medium containing the indicated concentrations of gentamicin and bacterial intracellular survival or replication was assessed 4 and 24 h p.i.

Gene Expression Analysis

Total RNA was extracted from CHO cells using the High Pure RNA Isolation Kit (Roche) following the manufacturer's guidelines. Reverse transcription of 1 µg RNA of each sample was done with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was assessed by qPCR using the Power SYBR® Green PCR Master Mix (Applied Biosystems) using gene specific primers (Supplementary Table S1). Relative gene expression was calculated by the $\Delta\Delta$ Ct method (33) and normalized to *Gapdh* and *Rps9* housekeeping genes.

Gentamicin ELISA

To determine levels of intracellular gentamicin, CHO WT and CHO Δ XylT cells were washed four times with PBS and lysed. The concentration of gentamicin in cell lysates was measured using the GEN ELISA Kit (Cusabio) according to the manufacturer's protocol.

Gentamicin Cy3 Conjugation and Cell Labeling Experiments

Gentamicin sulfate salt (Sigma-Aldrich) was mixed with the Sulfo-Cyanine3 NHS ester (Lumiprobe) in 50:1 molar ratio and incubated for 1 h at room temperature. The conjugate (Gen-Cy3) was isolated by reversed-phase chromatography (column C18), aliquoted, dried, and stored in the dark at -20°C. Prior to usage, the conjugate was resuspended in sterile water, absorbance at 548 nm was measured, and a concentration was calculated using the molar attenuation coefficient of the Sulfo-Cyanine3 NHS ester. In the cell labeling experiments, gentamicin sulfate used in a protection assay was replaced with Cy3-conjugated gentamicin (GEN-Cy3) at the indicated concentrations. CHO WT and CHO

Δ XylT cells incubated with GEN-Cy3 were fixed at different time points post infection.

CHO WT and CHO Δ XylT cells were seeded on coverslips and then infected with *S. Typhimurium* eGFP at an MOI of 50. Upon bacterial invasion, CHO cells were incubated for 2, 7, or 24 h in presence of 50 nM LysoTracker Red DND-99 (Sigma-Aldrich). Then, CHO cells were extensively washed with PBS, fixed with 4% paraformaldehyde (PFA) and stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) (1:1000) to visualize nuclei. Images were recorded on a Zeiss Apotome.2 microscope using AxioVision 4.9.1 software (Zeiss).

Immunocytochemistry

CHO WT and CHO Δ XylT cells were seeded on coverslips in 24 well plates, fixed with 4% PFA, washed 3 times with PBS, and permeabilized with Triton-X100 (0.1%). Unspecific binding was blocked using 2% normal goat serum (NGS), cells were then incubated with the AO4B08 antibody (25) (1:100) recognizing both heparin and HS. Infected cells were additionally stained with rabbit anti-*Salmonella* antibody (1:100). Upon washing, bound AO4B08 antibodies were detected by incubation with mouse anti-VSV tag IgG antibody P5D5 (1:400), followed by Alexa 488-conjugated goat anti-mouse IgG (1:1000) and Alexa 568-conjugated goat anti-rabbit IgG (1:1000) (Thermo Fisher Scientific). Phalloidin-iFluor 647 (1:1000) (Abcam) and DAPI (Invitrogen) were applied to visualize F-actin and nuclei, respectively. For a list of antibodies used see Supplementary Table S2.

Chloroquine Resistance Assay

To determine the number of cytosolic *S. Typhimurium* within the CHO cells, chloroquine (CHQ) resistance assay was performed as described (34). Briefly, CHO WT and CHO Δ XylT cells were infected as described above. 24 h p.i., the cells were incubated for 1 h in the presence of CHQ (400 µM) and gentamicin (cytosolic bacteria) or with gentamicin only (total intracellular bacteria). CHO cells were then lysed, and serial dilutions plated on LB agar plates.

Infection With *S. Typhimurium* Reporter Strains

CHO WT and CHO Δ XylT cells seeded on coverslips were infected with *S. Typhimurium* p4889 reporter strain at MOI 100. Upon bacterial invasion, CHO medium was supplemented with 100 µg/mL gentamicin. 24 h p.i., the infected cells and uninfected controls were fixed with 4% PFA, and then incubated with DAPI (1:1000). Images were recorded with a Zeiss Apotome.2 microscope using AxioVision 4.9.1 software (Zeiss). Bacteria in cytoplasm and in *Salmonella*-containing vacuole were enumerated in 20 random fields of view (FOV) using the Fiji software (35).

To test exposure of intracellular bacteria to acidic endosomal environments, CHO WT and CHO Δ XylT were infected with *S. Typhimurium* harboring p5386 at MOI of 10. Cells were incubated with or without gentamicin as indicated. CHO cells were detached 2 h after infection, chloramphenicol was added

in a final concentration of 200 $\mu\text{g/mL}$ to stop further bacterial protein biosynthesis, and incubation was continued at 4°C in order to allow full maturation of all synthesized sfGFP molecules. To control the effect of host cell endosomal acidification on intracellular *S. Typhimurium*, acidification was abrogated by vATPase inhibitor bafilomycin added after infection in a final concentration of 100 nM. For quantification, at least 50,000 CHO cells were analyzed by flow cytometry on an Attune NxT (Thermo Fisher) instrument. Cells were gated for DsRed-positive, i.e., *S. Typhimurium*-infected population, and sfGFP fluorescence of this population was determined as proxy for the level of acidification.

Transfection

10^6 CHO WT and CHO ΔXylT cells were resuspended in 100 μl Nucleofector Solution and mixed with 5 μg of p4605 plasmid encoding human ARL8B (ADP Ribosylation Factor Like GTPase 8B) (36) and transfected using the Nucleofector Program U-027 (Lonza). Immediately after transfection, cells were resuspended in CHO medium, seeded onto cover slips in 24 well plates, and incubated for at least 18 h prior to infection.

Antibody Uptake Assay

CHO WT and CHO ΔXylT cells seeded on coverslips were infected with *S. Typhimurium* WT or with *S. Typhimurium* WT eGFP at an MOI of 50. 1.5 h p.i., medium was replaced with medium containing 10 $\mu\text{g/mL}$ gentamicin and rabbit anti-*Salmonella* antibody (Difco). 24 h p.i., cells were fixed with 4% PFA, and bacteria were stained with the Alexa 546-conjugated donkey anti-rabbit IgG. In case of WT bacteria, samples were additionally stained with mouse anti-*Salmonella* antibody (Meridian Life Science) and Alexa 488-conjugated donkey anti-mouse IgG secondary antibody (Thermo Fisher Scientific). Bacteria were enumerated in 20 random FOVs.

Endo-Lysosomal Fusion Assay

CHO WT and CHO ΔXylT cells were seeded on coverslips for pulse-chase experiments. In brief, CHO cells were first incubated with dextran-Alexa568 (10,000 MW, 0.4 mg/mL) (Invitrogen) for 4 h, washed and incubated in dextran-free CHO medium for 18 h. Cells were then pulsed with dextran-Alexa488 (10,000 MW, 0.4 mg/mL) for 10 min, washed and incubated in CHO medium for 30 min. CHO WT and CHO ΔXylT cells were fixed with 4% PFA, and stained with phalloidin-iFluor 647 Reagent (1:1000) (Abcam) and DAPI. Images of 20 random FOVs were acquired on Zeiss Apotome.2 microscope with 63 $\times$ oil immersion objective using AxioVision 4.9.1 software (Zeiss). Spatial resolution of images was 9.7674 pixels per micron, pixel size: 0.1024×0.1024 micron². Endo-lysosomal fusion was scored by quantifying colocalization between the two labeled dextrans using ImageJ version 1.52e and JACoP plugin for pixel intensity spatial correlation analysis (37). Pearson's correlation coefficient and Manders split coefficients (M1 and M2, thresholds set manually for both channels) were calculated.

Cytotoxicity Assay

CHO cells were infected with *S. Typhimurium* WT strain as described previously. 24 h p.i., supernatants were collected and an activity of the lactate dehydrogenase (LDH) was measured using the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

Statistical Analysis

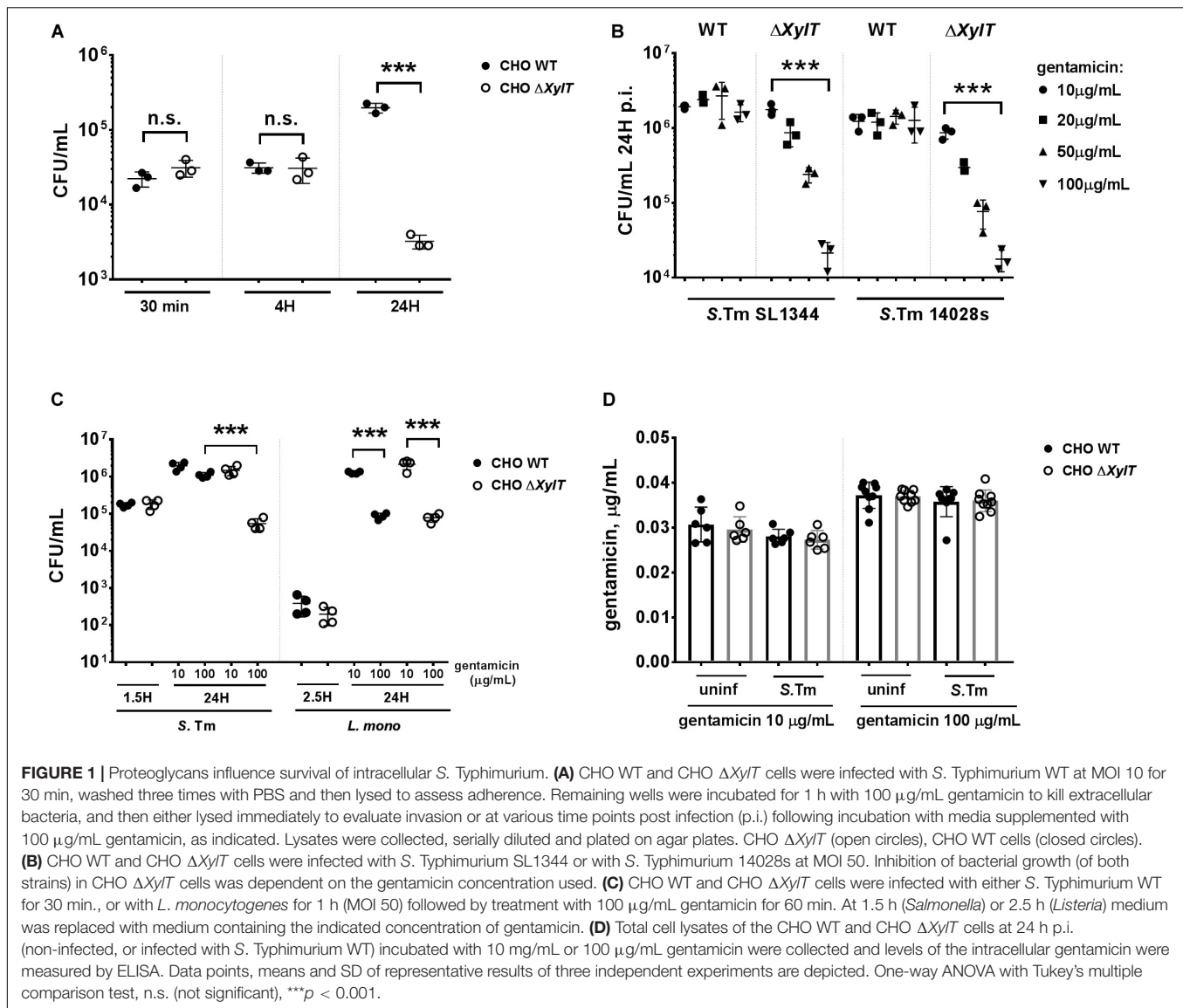
Data were analyzed using Prism V7.0d software (GraphPad). Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, or Dunnett's multiple comparison test, the Kruskal-Wallis test followed by Dunn's multiple comparison test, unpaired two-tailed t-test, or Wilcoxon-Mann-Whitney test as indicated. The results were considered statistically significant when *p*-values were smaller than 0.033. Graphs display the mean values $\pm$ SD and represent three independent biological repetitions unless stated otherwise.

RESULTS

Proteoglycans Are Crucial for *Salmonella* Survival Within CHO Cells

Proteoglycans were shown to contribute to *Salmonella* invasion via interaction with the bacterial adhesin PagN (7). However, PagN is only expressed under intracellular (SPI2)-inducing conditions and when PagN is not expressed, invasion of host cells is PG-independent. Furthermore, it is not known if PGs are important for intracellular survival or replication. To test this, CHO WT and proteoglycan-deficient CHO ΔXylT cells were infected with *S. Typhimurium* WT strains. Bacterial adhesion (30 min p.i), invasion (1.5 h p.i), and early replication (4 h p.i) were comparable between CHO WT and CHO ΔXylT cells. However, 24 h p.i., we detected a significant reduction of intracellular bacteria in CHO ΔXylT compared to CHO WT cells incubated in presence of 100 $\mu\text{g/mL}$ gentamicin (Figure 1A). Gentamicin-mediated killing of *S. Typhimurium* 14028S and SL1344 strains CHO ΔXylT cells was dose-dependent (Figure 1B). In contrast, when ampicillin was applied instead of gentamicin, a dose-dependent reduction of bacterial intracellular numbers was detected in the both CHO WT and CHO ΔXylT cells infected with *S. Typhimurium* 14028S at 24 p.i. (Supplementary Figure S1). To determine whether the effect on intracellular survival is *Salmonella*-specific, we infected CHO WT and CHO ΔXylT cells with another intracellular pathogen, *Listeria monocytogenes*. In contrast to *Salmonella*, reduction of intracellular *Listeria* was dependent on the gentamicin dose but not dependent on the presence of proteoglycans (Figure 1C).

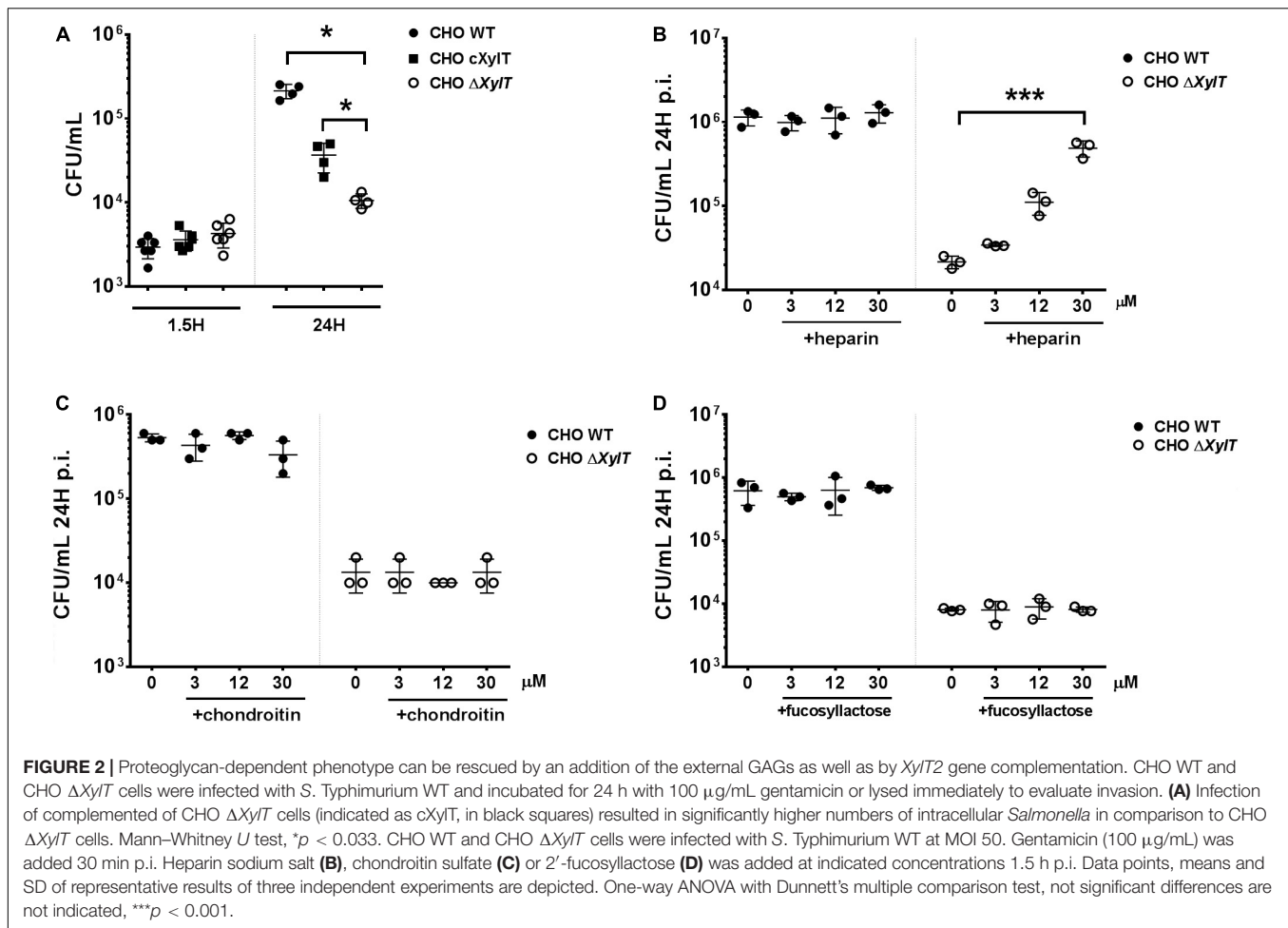
Next, we asked whether proteoglycan can affect the uptake of gentamicin into CHO cells as increased uptake of gentamicin by CHO ΔXylT cells could contribute to increased killing of intracellular *Salmonella*. CHO cells were infected with *S. Typhimurium* WT as described above and intracellular gentamicin concentrations were measured by ELISA. No differences in intracellular gentamicin levels were detected



between CHO WT and CHO $\Delta Xyl/T$ cells, or between uninfected and infected cells (**Figure 1D**). Previous studies emphasized a role of the transient receptor potential channels (Trpv), Trpv1 (38) and Trpv4 (39), and the multidrug resistance protein 2 (Mrp2 or Abcc2) (40) in the cellular uptake and transport of gentamicin, respectively. Expression of *Trpv1*, *Trpv4*, and *Mrp2* genes was comparable in non-infected and infected CHO WT and CHO $\Delta Xyl/T$ cells (**Supplementary Figures S2A–C**). Collectively, these findings indicate that a lack of proteoglycans in CHO cells does not affect active or passive gentamicin uptake.

To verify that the observed phenotype is indeed due to the proteoglycan deficiency, we complemented the CHO $\Delta Xyl/T$ cells with the human *XYLT2* gene. When compared to the proteoglycan-deficient CHO $\Delta Xyl/T$ cells, complemented CHO $cXyl/T$ cells harbored similar levels of *Salmonella* after invasion (at 1.5 h p.i.), but significantly more bacteria at 24 h p.i. (**Figure 2A**). However, while compared to CHO WT cells, complemented

CHO $cXyl/T$ cells still had lower *S. Typhimurium* loads 24 h p.i., which correlated with lower amounts of proteoglycans present on CHO $cXyl/T$ cells, as assessed by flow cytometry (**Supplementary Figure S3A**), indicating only partial complementation of PGs. Next, we tested if addition of proteoglycans to the medium could also complement *Salmonella* survival in CHO $\Delta Xyl/T$ cells. Addition of heparin (a structural analog of heparan sulfate) to the medium increased bacterial survival in CHO $\Delta Xyl/T$ cells in a dose-dependent manner, but did not affect *Salmonella* survival in CHO WT cells (**Figure 2B**). In contrast, addition of equimolar amounts of chondroitin sulfate A (**Figure 2C**) or 2-fucosyllactose (**Figure 2D**) did not affect intracellular bacterial numbers in either CHO cell line. Notably, heparin did not support or inhibit growth of *S. Typhimurium* in LB medium and did not affect killing of *Salmonella* in LB broth supplemented with 100 $\mu\text{g/mL}$ gentamicin (**Supplementary Figure S3B**). To summarize, these results indicate that host proteoglycans are important for the



survival of *Salmonella* in epithelial cells when gentamicin is added to the tissue culture medium.

CHO Cells Lacking Proteoglycans Display a Lower Abundance of SCV-Associated *Salmonella*

To investigate if a lack of proteoglycans might affect subcellular localization of intracellular bacteria, CHO WT and CHO $\Delta XylT$ cells were infected with a *S. Typhimurium* reporter strain expressing DsRed protein constitutively and sfGFP only when bacteria are located in the cytosol (31). Microscopy revealed that CHO $\Delta XylT$ cells incubated with 100 $\mu\text{g/mL}$ gentamicin had significantly lower numbers of total bacteria, but significantly higher numbers of GFP-expressing, cytosolic *Salmonella* compared to CHO WT at 24 h p.i., as shown by an elevated ratio of cytosolic/total bacteria in infected cells (Figures 3A,B). Addition of chloroquine selectively kills *Salmonella* within SCVs (34). Therefore, we used a combination of chloroquine resistance assay and gentamicin protection assay to determine cytosolic and intra-SCV bacteria corroborating our results obtained with the reporter strains (Figure 3C). To further investigate the subcellular localization of *Salmonella*, *S. Typhimurium* $\Delta sifA$ strain was utilized. This mutant is not

able to maintain SCV integrity upon infection, which results in an extensive cytosolic replication of bacteria (30). Intracellular replication was analyzed by gentamicin protection assay, and expressed as a ratio of replicated (CFU at 24 h p.i.)/invaded (CFU at 1.5 h p.i.) bacteria. Intracellular proliferation of *S. Typhimurium* $\Delta sifA$ in CHO WT cells in the presence of 100 $\mu\text{g/mL}$ gentamicin was about two times higher when compared to *S. Typhimurium* WT. In contrast, in CHO $\Delta XylT$ cells, *S. Typhimurium* $\Delta sifA$ intracellular replication was about 50 times higher compared to *S. Typhimurium* WT strain (Figure 3D). The differences in late replication were even more pronounced in CHO and CHO $\Delta XylT$ cells incubated with 200 $\mu\text{g/mL}$ gentamicin (Figure 3D). Overall, these data indicate that the reduction of bacterial burden in CHO $\Delta XylT$ cells was due to a diminished number of bacteria in SCV, while cytosolic bacteria were largely unaffected by increasing concentrations of gentamicin.

Recently, it has been shown that *Salmonella*-induced filaments (SIFs) can increase the exposure of bacteria to internalized antibiotics in the SCV (41). To evaluate a contribution of the SIF network to the observed phenotype, CHO WT and CHO $\Delta XylT$ cells were infected with either *S. Typhimurium* WT or *S. Typhimurium* $\Delta ssaR$ (a SPI-2 mutant lacking SIFs) (29). In

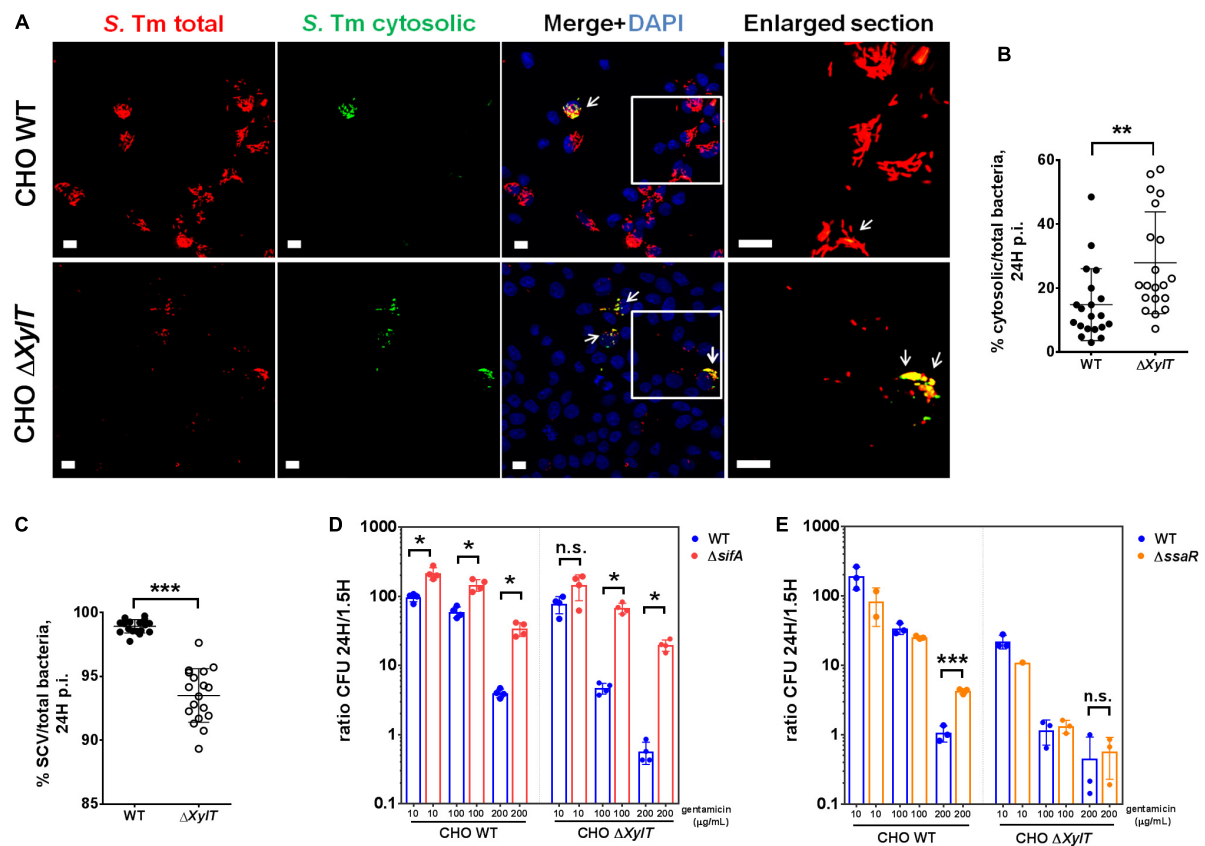


FIGURE 3 | *Salmonella* in SCV, but not cytosolic bacteria, are affected by gentamicin in proteoglycan-deficient cells. **(A,B)** CHO WT and CHO $\Delta XylT$ cells were infected at MOI 100 with *S. Typhimurium* [p4889] and then incubated in presence of 100 $\mu\text{g/mL}$ gentamicin. 24 h p.i., cell monolayers were washed, fixed with 4% PFA and stained with DAPI. Microscopy revealed a significantly higher number of GFP-expressing, cytosolic *Salmonella* (indicated by arrows) in CHO $\Delta XylT$ cells compared to CHO WT. Scale bars, 10 μm . Mann-Whitney *U* test, $^{**}p < 0.002$. **(C)** CHO WT and CHO $\Delta XylT$ cells were infected with *S. Typhimurium* WT at MOI 50, and gentamicin (100 $\mu\text{g/mL}$) was added 30 min after infection. 24 h p.i., cells were treated with 400 μM chloroquine for 1 h, then washed with PBS and lysed. Mann-Whitney *U* test, $^{***}p < 0.001$. **(D-E)** CHO WT and CHO $\Delta XylT$ cells were infected with either *S. Typhimurium* WT, *S. Typhimurium* $\Delta sifA$, or *S. Typhimurium* $\Delta ssaR$ at MOI 50. Gentamicin (100 $\mu\text{g/mL}$) was added 30 min after infection. 1.5 h p.i. medium was replaced with medium containing gentamicin at 10, 100, or 200 $\mu\text{g/mL}$ as indicated. Intracellular CFU counts were determined 1.5 and 24 h p.i. and depicted is the intracellular replication (CFU ratio of 24 to 1.5 h). **(D)** Cytosolic bacteria ($\Delta sifA$) showed higher intracellular replication than WT *Salmonella* in both CHO cell types. Data points, means and SD of representative results of two independent experiments are depicted. Mann-Whitney *U* test, n.s. (not significant), $^{*}p < 0.033$. **(E)** Intracellular replication of *S. Typhimurium* $\Delta ssaR$ mutant compared to WT *S. Typhimurium* was increased in CHO WT but not in CHO $\Delta XylT$ cells. Data points, means and SD of representative results of three independent experiments are depicted. Unpaired *t*-test, n.s. (not significant), $^{***}p < 0.001$.

agreement with the findings by Liss et al. (41), incubation of the infected CHO WT cells with 200 $\mu\text{g/mL}$ gentamicin for 24 h resulted in a significantly higher intracellular proliferation of *S. Typhimurium* $\Delta ssaR$ compared to the WT strain. In contrast, numbers of both intracellular *Salmonella* WT and $\Delta ssaR$ were strongly decreased in CHO $\Delta XylT$ cells (Figure 3E) indicating that the gentamicin-mediated inhibition of bacterial growth in CHO $\Delta XylT$ cells is independent of SIFs.

Proteoglycans Are Important for PIKfyve-Dependent Endo-Lysosomal Fusion

Our observation that CHO WT and CHO $\Delta XylT$ cells had similar levels of intracellular gentamicin was based on ELISA measurements of whole cell lysates. Next, we tested whether

intracellular localization of gentamicin is altered in the absence of proteoglycans. Indeed, in infected CHO $\Delta XylT$ cells Cy3-labeled gentamicin was found close to *Salmonella*, or bacterial debris, while in CHO WT cells gentamicin-Cy3 was distributed more randomly (Figure 4A). Of note, uninfected CHO WT and $\Delta XylT$ cells were similar in terms of a distribution of labeled gentamicin (Supplementary Figure S4). Such re-distribution of an antibiotic may enhance *Salmonella* killing within modified compartments of CHO $\Delta XylT$ cells. Association of bacteria with vacuolar markers such as LAMP-1 or ARL8B was similar in CHO WT and CHO $\Delta XylT$ cells (Figure 4B).

We hypothesized that a lack of PGs may also alter intracellular routing of cargo other than antibiotics. To test this, we employed an antibody uptake assay. Cells were infected with GFP-expressing *S. Typhimurium*, and an anti-*Salmonella* antibody was added to cell culture medium 1.5 h p.i. (after invasion of

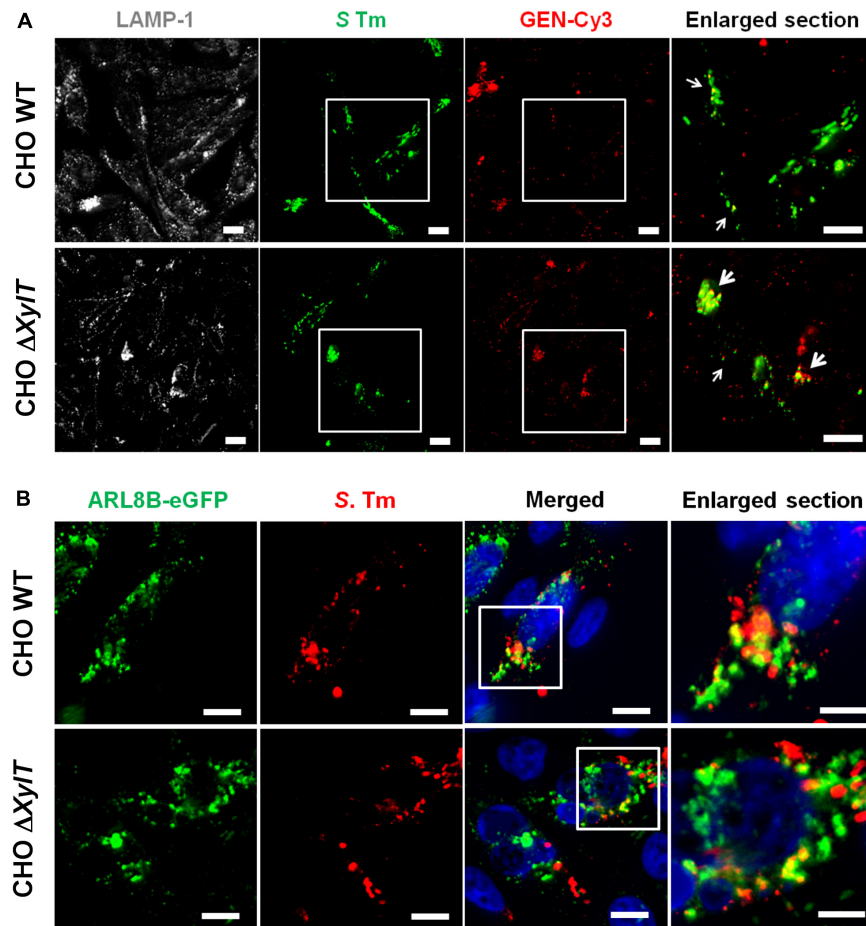


FIGURE 4 | Intracellular gentamicin is associated with *Salmonella* in CHO $\Delta XylT$ cells. **(A)** CHO cells were infected with *S. Typhimurium* WT, incubated with Cy3-labeled gentamicin 30 min p.i., and 7 h p.i. fixed with 4% PFA, stained with anti-*Salmonella* antibody, Phalloidin-iFluor647 and DAPI. Microscopy revealed an enhanced co-localization of Cy3-labeled gentamicin and *Salmonella* in CHO $\Delta XylT$ cells (bottom row) resulting in a degradation of bacteria (enlarged section, indicated by arrows). Scale bars, 10 μ m. A representative image of three biological repetitions is shown. **(B)** CHO WT and CHO $\Delta XylT$ cells were transfected with a plasmid encoding the human ARL8B gene fused to eGFP. Cells were infected with *S. Typhimurium* WT at MOI of 50. Eight hours p.i. cells were fixed and stained for *S. Typhimurium* (in red). Representative images of two biological repetitions, scale bars, 10 μ m or 5 μ m (in enlarged sections).

bacteria). 24 h p.i., we observed that significantly higher numbers of intracellular *S. Typhimurium* were stained with the anti-*Salmonella* antibody in infected CHO $\Delta XylT$ cells compared to CHO WT cells (Figures 5A,B). Addition of heparin to the cell culture medium 1.5 h p.i. reduced the number of double-positive bacteria in the infected CHO $\Delta XylT$ cells, while the total number of *S. Typhimurium* increased. These data indicates an important role of PGs in proper vesicle trafficking (Figure 5C).

Vacuole acidification is sensed and manipulated by *Salmonella*. To test whether endo-lysosomal trafficking and acidic vacuole formation is affected by proteoglycans we stained acidic organelles by incubation with Lysotracker. Strikingly, CHO $\Delta XylT$ cells (both non-infected and infected) were less stained than CHO WT cells when incubated with Lysotracker (Figure 6A). Interestingly, complemented CHO $\Delta XylT$ cells displayed an intermediate degree of Lysotracker staining (Supplementary Figure S5). To test if a lack of PG affects SCV acidification, we used a *Salmonella* strain harboring

dual fluorescence reporter p5386 to monitor exposure of intracellular *Salmonella* to acidic pH (Supplementary Figures S6A,B). The reporter features constitutive expression of DsRed, allowing the localization of intracellular *Salmonella*, and sfGFP under control of the acid shock response-activated promoter P_{asr} (42, 43). *In vitro* analyses demonstrated the P_{asr} is activated if *Salmonella* is exposed to media of pH 5.0 or lower. Exposure to media with higher pH did not lead to synthesis of sfGFP under control of P_{asr} (Supplementary Figure S6C). Inhibition of acidification of the SCV by addition of vATPase inhibitor bafilomycin fully ablated expression of P_{asr} :sfGFP (Supplementary Figure S6D). Expression of P_{asr} :sfGFP at 2 h p.i. was not affected by presence or absence of gentamicin in the cell culture medium (Figure 6B). However, at 8 h p.i. we observed lower expression of P_{asr} :sfGFP in CHO $\Delta XylT$ cells compared to CHO WT cells (Figure 6C). Taken together, the acidification of endosomal compartments is impaired in PG-deficient cells as indicated by the lower signal intensity

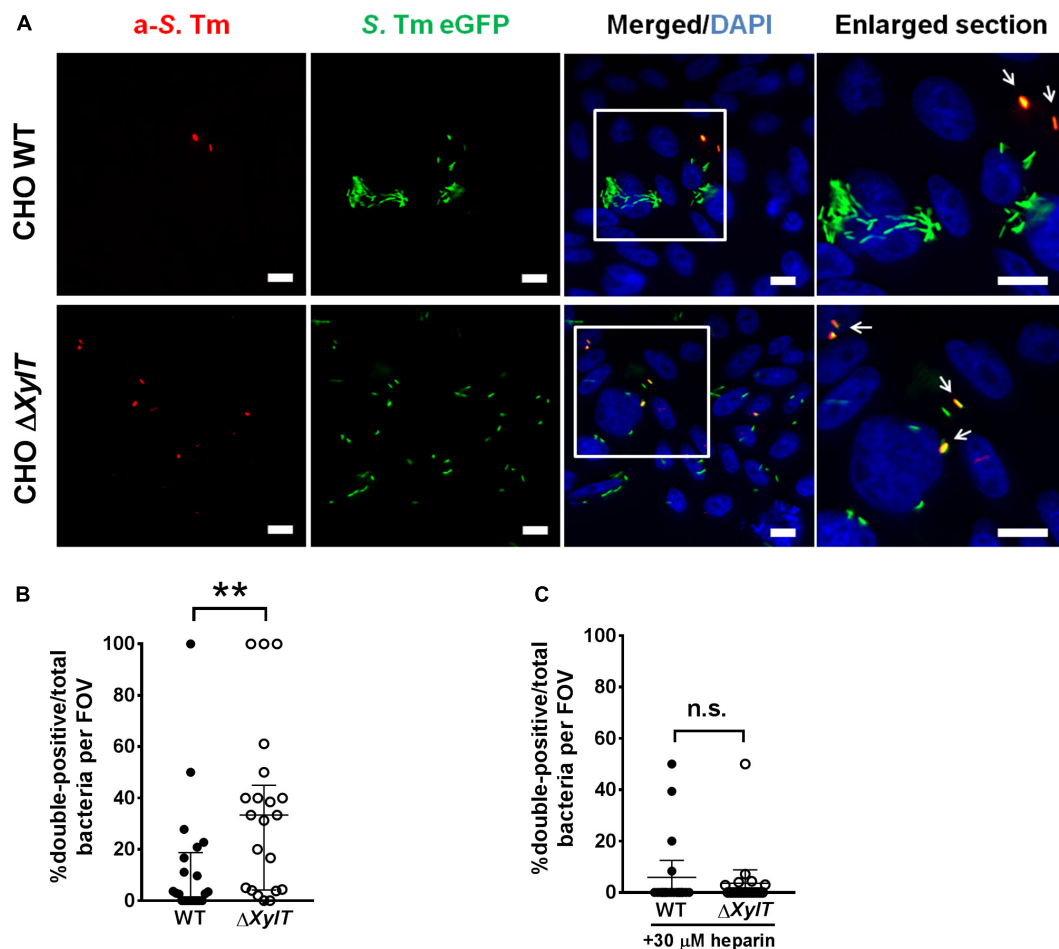


FIGURE 5 | Endocytosed cargo in infected proteoglycan-deficient CHO $\Delta XylT$ cells co-localizes with *Salmonella*. CHO cells were infected with *S. Typhimurium* eGFP. Gentamicin (100 $\mu\text{g}/\text{mL}$) was added 30 min after infection and 1.5 h p.i. anti-*Salmonella* antibody was added to the medium. 24 h p.i. cells were fixed with 4% PFA, and stained with DAPI. **(A)** Double-positive bacteria are indicated with arrows in enlarged section. Scale bars, 10 μm . **(B)** Number of double-positive bacteria was counted in 20 FOVs. Mann-Whitney U test, $**p < 0.002$. **(C)** 1.5 h p.i. heparin (30 μM) was added to growth medium containing anti-*Salmonella* antibody. 20 random FOVs were counted, mean values with 95% CI are shown. Mann-Whitney U test, n.s., not significant.

of LysoTracker labeling and lower expression of $P_{asr}::\text{sfGFP}$ in $\Delta XylT$ cells.

To identify, at which stage trafficking of endocytosed cargo is affected by the lack of PGs, we utilized inhibitors of clathrin-mediated endocytosis (dynasore), phosphoinositide 3-kinase PI3K (wortmannin), as well as an inhibitor of FYVE finger-containing phosphoinositide kinase (PIKfyve) activity (YM201636). Application of dynasore 1.5 h p.i. (added after invasion of bacteria) resulted in a significantly higher recovery of *S. Typhimurium* from CHO $\Delta XylT$ cells compared to the non-treated controls. In contrast, dynasore treatment had no significant effect on intracellular bacterial numbers in CHO WT cells (Figure 7A). In addition, treatment of either CHO WT or CHO $\Delta XylT$ cells with wortmannin had no significant effect on intracellular *S. Typhimurium* numbers (Supplementary Figure S7). When PIKfyve activity in CHO WT cells was inhibited with YM201636, *S. Typhimurium* numbers were reduced in a dose-dependent manner in agreement with the

results by Kerr et al. (44). However, YM201636 treatment of CHO $\Delta XylT$ cells resulted in significantly more intracellular bacteria compared to non-treated cells (Figure 7B). Heparin treatment abrogated the dose-dependent effect of YM201636 on *Salmonella* survival (Figure 7C) implying a direct effect of PGs on PIKfyve activity. Soluble heparin was detected within endosomal compartments and SCVs in the heparin-treated CHO $\Delta XylT$ cells as revealed by immunostaining (Supplementary Figure S10). Application of YM201636 also resulted in diminished numbers of double-positive bacteria in both CHO cell lines in an antibody uptake assay (Figure 7D). Furthermore, incubation of CHO WT and CHO $\Delta XylT$ cells with PIKfyve-inhibitor dramatically enhanced the size of acidic lysosomes (Supplementary Figure S8). These data demonstrate a critical role of proteoglycans in PIKfyve-mediated fusion events.

To assess if endo-lysosomal fusion is abrogated in CHO $\Delta XylT$ cells, we employed a modified pulse-chase assay using Alexa568- and Alexa488-labeled dextrans to label lysosomes/late

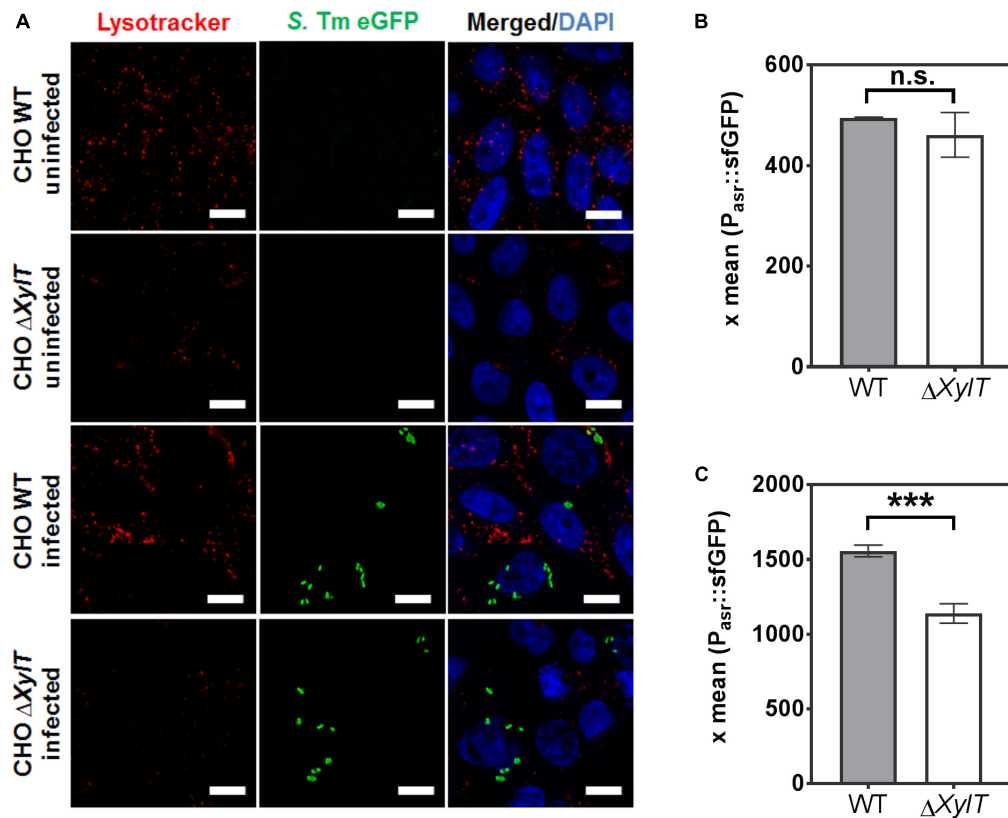


FIGURE 6 | Acidic organelles in CHO Δ XylT cells display reduced labeling by Lysotracker. **(A)** Uninfected CHO WT and CHO Δ XylT cells, or cells infected with *S. Typhimurium* EGFP at MOI of 50, were incubated for 2 h with 50 nM Lysotracker Red. Cells were fixed with 4% PFA. Representative images of three biological repetitions, scale bars, 10 μ m. **(B,C)** Acidification of *Salmonella* is dependent on the presence of PGs. CHO WT or CHO Δ XylT cells were infected with *Salmonella* WT harboring an acid shock sensor. Infection and analyses by flow cytometry were performed as described in **Supplementary Figure S6**. After infection for 30 min, cells were treated with 100 μ g/mL gentamicin for 1 h followed by 10 μ g/mL gentamicin for 1 h **(B)** or 7 h **(C)**. The mean sfGFP fluorescence intensity is displayed for CHO cells harboring DsRed-positive *Salmonella*. X-means and standard deviations are shown for triplicate samples, and the data shown are representative for three biological replicates with similar outcome. Unpaired *t*-test, n.s., not significant, ****p* < 0.001.

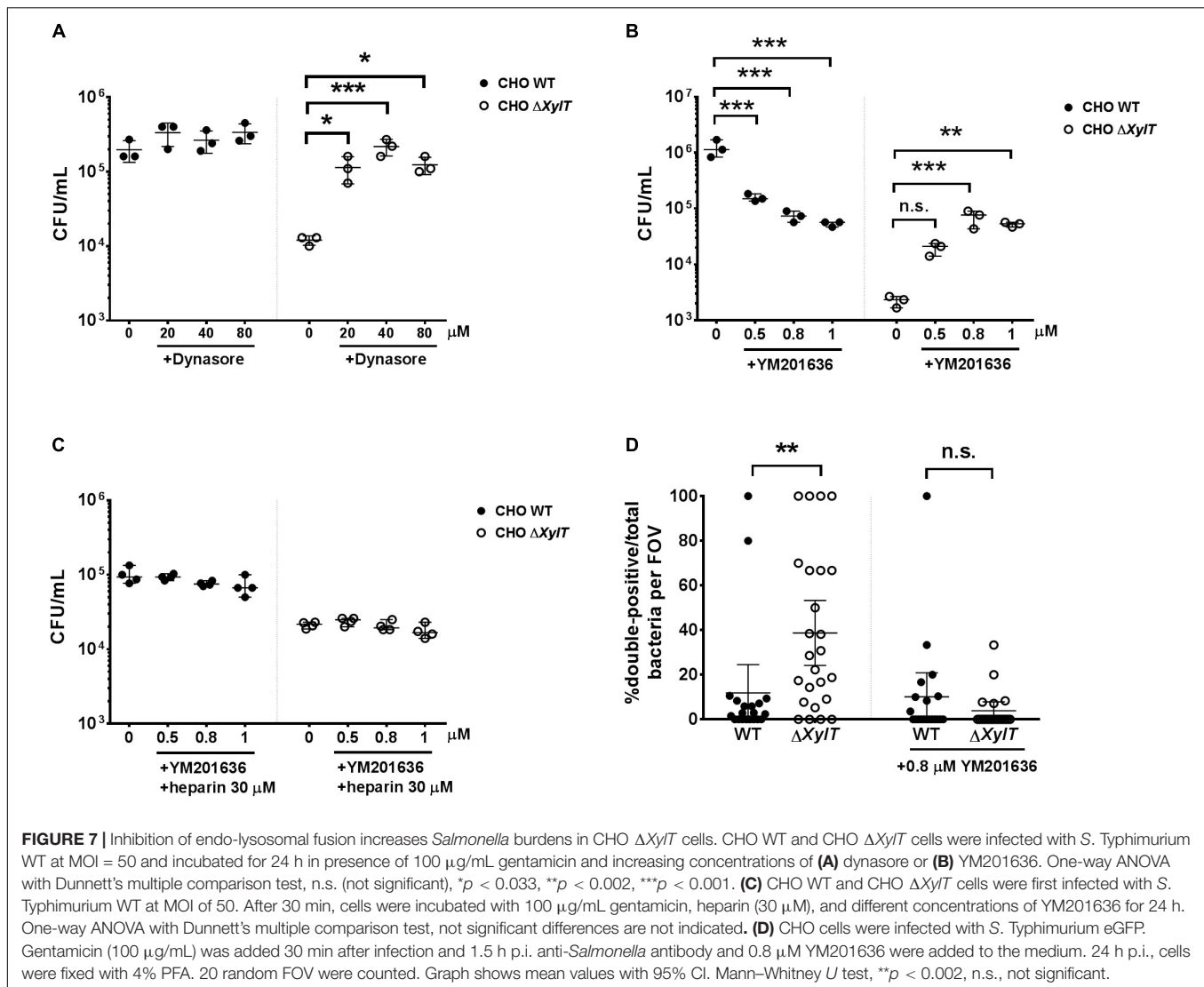
endosomes and early endosomes, respectively, as described by Kerr et al. (44). CHO WT and CHO Δ XylT cells were incubated with dextran-Alexa568 for 4 h, followed by incubation in dextran-free CHO medium. 18 h later, cells were incubated with dextran-Alexa488 for 10 min, washed and incubated in CHO medium for another 30 min before fixation with 4% PFA. To assess vesicle fusion events, Pearson's correlation coefficient and Manders split coefficients (M1 and M2, thresholds set manually for both channels) were calculated. CHO Δ XylT cells had significantly lower degree of co-localization between the two dextrans compared to CHO WT cells indicating a delayed or reduced endo-lysosomal fusion in proteoglycan-deficient cells (**Figure 8**).

DISCUSSION

In the present study, we report a novel role of PGs for endo-lysosomal fusion. PG deficiency abrogates endo-lysosomal fusion which affects *Salmonella* survival within epithelial cells in a

context of gentamicin protection assay. Wild-type CHO cells exclusively utilize *Xylt2* for PGs biosynthesis and lack detectable *Xylt1* gene expression (45). CHO pgsA745 (Δ XylT2) cells lack the xylosyltransferase-II enzyme and thus, are PG-deficient (3). This cell line has been extensively used to investigate the contribution of PGs to the entry of bacterial and viral pathogens into host cells (46). Recently, it has been identified that CHO Δ XylT cells also carry a mutation in the *Lama2* gene. The resulting deletion of the long isoform of the laminin subunit α -2 significantly reduced invasion of group B *Streptococcus* in CHO Δ XylT compared to CHO WT cells (47). Indeed, while both the short and the long isoforms of laminin-2 were expressed in our CHO WT cells, CHO Δ XylT cells lacked the long isoform expression (**Supplementary Figure S9**). In addition, it was shown that when *Salmonella* is grown under *pagN*-inducing conditions there was a reduced uptake into CHO Δ XylT cells (7). However, in our study, no differences in terms of *S. Typhimurium* adhesion to and invasion into the CHO WT and CHO Δ XylT cell lines were detected.

Upon invasion, *Salmonella* hijack endo-lysosomal trafficking and acquire host factors including LAMP1 and ARL8B in



order to establish a *Salmonella*-containing vacuole (SCV) and later on, *Salmonella*-induced filaments (SIFs). Microscopy and chloroquine resistance assays revealed that in the absence of PGs, significantly less bacteria were associated with SCVs when compared to WT CHO cells, while similar numbers of cytosolic bacteria were found in CHO WT and CHO $\Delta Xyl/T$ cells. It should be noted that no differences in the levels of a total intracellular gentamicin between CHO WT and CHO $\Delta Xyl/T$ cells were detected. Thus, we reasoned that an intracellular localization of gentamicin might be altered in the infected CHO $\Delta Xyl/T$ cells, which leads to an increased exposure of specific bacterial populations to the antibiotic. Although cell membranes are generally regarded to be impermeable to gentamicin, aminoglycosides can be transported into epithelial cells via endocytosis-dependent and -independent pathways (48). It was previously reported that endocytosis of gentamicin resulted in its accumulation within lysosomes and in increased lysosomal ROS production in kidney epithelial cells (49). CHO $\Delta Xyl/T$

cells are not defective in terms of endocytosis/phagocytosis (47), which is supported by our data regarding uptake of gentamicin (Cy3-labeled and by ELISA). However, during infection, we observed a co-localization of gentamicin and SCV-associated bacteria in proteoglycan-deficient CHO cells. Indeed, the *S. Typhimurium* $\Delta sifa$ mutant, which cannot establish a functional SCV and therefore localizes to the cytoplasm (50), was significantly less affected by increasing gentamicin concentrations than WT *Salmonella*, in both CHO cell lines. To conclude, the intracellular re-distribution of gentamicin in proteoglycan-deficient CHO cells was associated with a drastic reduction of *Salmonella* counts.

In addition, we detected increased accumulation of an anti-*Salmonella* antibody in SCV/SIF compartments in CHO $\Delta Xyl/T$ compared to CHO WT cells. This process could be blocked by addition of heparin to cell culture medium or by inhibiting of PIKfyve activity in CHO $\Delta Xyl/T$ cells. CHO WT cells treated with a specific PIKfyve inhibitor

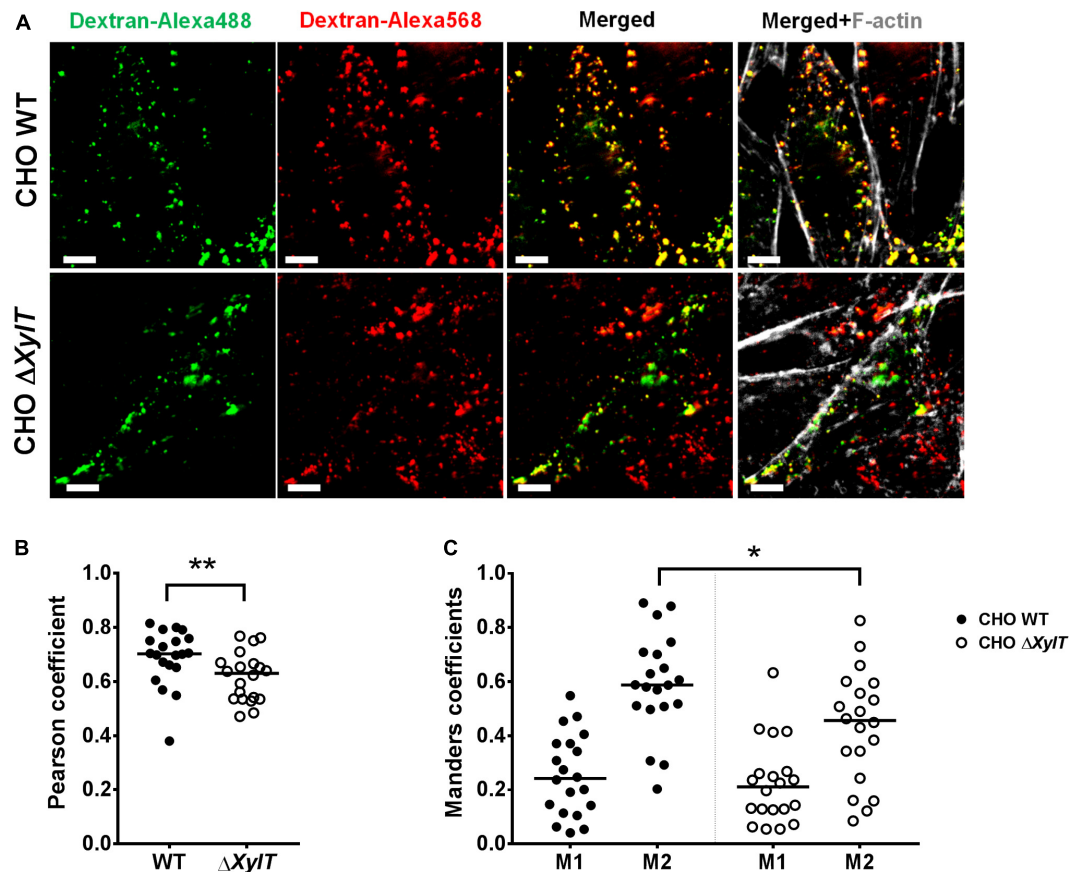


FIGURE 8 | CHO $\Delta XylT$ cells display reduced degree of endo-lysosomal fusion. CHO WT and CHO $\Delta XylT$ cells were seeded on cover slips and pulsed for 4 h with dextran-Alexa568, followed by incubation with CHO medium. 18 h later dextran-Alexa488 was added for 10 min, cells were washed and 30 min later, cells were fixed with 4% PFA. **(A)** Co-localization of Alexa-labeled vesicles can be seen in the merged section. Scale bars, 5 μ m. **(B)** Pearson correlation coefficient was calculated for 20 FOVs per cell line. **(C)** CHO $\Delta XylT$ cells have same fraction of late endosomes overlapping with early endosomes (M1), but smaller fraction of early endosomes co-localizing with late endosomes (M2). Mander's overlap coefficient calculated for each of 20 FOVs, Mann-Whitney U test, $*p < 0.033$, $**p < 0.002$. Data are representative of two biological repetitions, median values are indicated on graphs.

(YM201636) were characterized by significantly reduced bacterial loads compared to untreated controls which is in line with observations by Kerr et al. (44). In contrast, inhibition of PIKfyve in CHO $\Delta XylT$ cells increased *S. Typhimurium* counts. PIKfyve is a kinase that converts PtdIns3P into PtdIns(3,5)P₂. It has been suggested that PIKfyve orchestrates the fusion of *Salmonella* macropinosomes with organelles of the late endosomal/lysosomal system (44), and more recent data link PIKfyve activity with the recycling of tight junction proteins (51) and with a re-distribution of endocytosed cargo from/to lysosomes occurring at late stages of endocytic vacuole maturation (52). As we observed a different distribution of Cy3-labeled gentamicin and endocytosed antibody within the CHO WT and CHO $\Delta XylT$ cells, we speculated that proteoglycans are required for PIKfyve-dependent endo-lysosomal fusion and subsequent trafficking/recycling pathways. Interestingly, CHO $\Delta XylT$ cells displayed reduced labeling with LysoTracker, which was increased upon treatment with YM201636. The pH inside the SCV can affect bacterial survival in multiple ways: for example, phagosomal pH in macrophages is important for susceptibility of

S. Typhimurium to gentamicin (53). In addition, there is evidence that autophagosome-lysosome fusion in CHO cells is affected by the pH in acidic compartments (54). While, in our experiments, early acidification of the SCV in CHO WT and CHO $\Delta XylT$ cells was similar as determined by acid shock response reporter strains, at the later time points acidification of SCV in CHO $\Delta XylT$ cells was impaired.

Our results raise the question of specific interactions between phosphoinositides and PGs in the context of infection. For example, it was shown that binding of the transmembrane heparan sulfate PG syndecan-4 to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) is required for formation of focal adhesions (55). Because phosphoinositides are essential for actin assembly, it is not surprising that *Salmonella* can deplete PtdIns(4,5)P₂ by the effector SigD which results in membrane fission during bacterial invasion (56). However, the role of PGs in SCV/SIF biogenesis and endo-lysosomal trafficking is less clear. Several studies showed that syndecans, along with the endosomal sorting complex required for transport (ESCRT) proteins, are involved in the formation of multivesicular endosomes or bodies

(MVBs) (57, 58). MVBs can fuse with lysosomes or be exported as exosomes. It is known that *Salmonella* disturbs normal endosome to lysosome trafficking, affecting the ESCRT system (23) and exocytosis (16, 17). PtdIns(3,5)P₂ (hence, PIKfyve) regulates endosomal fission and fusion, and MVB formation (20).

Taken together, our data show that altered routes of endocytosed cargo in PG-deficient epithelial cells interfere with vesicle acidification and *Salmonella*-modulated PIKfyve-dependent fusion to establish a replicative niche and thereby elucidate a novel role of PGs in intracellular vesicle trafficking and SCV formation.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

AG, AS, HB, FR, MH, and GG: conceptualization. AG, AO, AS, HB, and LK: investigation. AG, AO, AS, LK, FR, HB, MH, and GG: data analysis. AG, AS, and GG: manuscript writing. AG, AO, AS, HB, FR, LK, MH, and GG: manuscript editing and approval.

FUNDING

GG was supported by the Deutsche Forschungsgemeinschaft (DFG) priority program SPP1656/2, the German Federal Ministry of Education and Research (BMBF) Infect-ERA consortium grant 031L0093B and DFG collaborative research center SFB 900 TP08 (Project number 158989968). AG was supported by the Hannover Biomedical Research School (HBRS) and by the Center for Infection Biology (ZIB). MH was supported by the DFG SFB 944 P4 and Infect-ERA consortium grant 031L0093A.

ACKNOWLEDGMENTS

We would like to thank Nicoletta Schwermann for excellent help in performing inhibitor experiments, and Janina Noster for experimental support in flow cytometry of the P_{asr} reporter.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00731/full#supplementary-material>

FIGURE S1 | Intracellular *Salmonella* are sensitive to treatment with ampicillin. (A) CHO cells were infected with *S. Typhimurium* 14028s WT at MOI 10, and ampicillin was used instead of gentamicin in a protection assay. 24 h p.i., cells had similar low CFU counts. Dotted line indicates limit of detection, ND – not detected. (B) Infected CHO WT and CHO Δ XylT cells had comparable levels of released lactate dehydrogenase as measured by LDH assay kit. Dotted line indicates limit of detection. Kruskal–Wallis test with Dunn's multiple comparison test, n.s. (not significant).

FIGURE S2 | CHO WT and Δ XylT cells had comparable expression levels of gentamicin transporters genes. Gene expression was measured in CHO WT and CHO Δ XylT cells, non-infected and infected with *S. Typhimurium* WT. Control CHO cells were incubated without gentamicin. Gene expression was normalized to *Gapdh* and *Rps9*, and control CHO WT data used as a calibrator. Comparable levels of *Abcc2* (A), *Trpv1* (B), and *Trpv4* (C), expression were observed in both CHO WT and Δ XylT cells, regardless infected or not. One-way ANOVA with Tukey's multiple comparison test and Mann-Whitney test, not significant differences are not indicated.

FIGURE S3 | Presence of PGs in CHO cells. (A) CHO WT, CHO Δ XylT, and complemented CHO Δ XylT cells (indicated as CHO cXylT) were stained with heparan sulfate-specific antibody and analyzed by flow cytometry. (B) *S. Typhimurium* WT was grown for 6 h in LB broth in presence/absence of 30 μ M heparin, and OD600 was recorded every 10 min. Mean values of three technical replicates of a representative experiment out of two are shown.

FIGURE S4 | Gentamicin uptake in CHO WT and CHO Δ XylT cells. CHO cells were incubated for 7 h with gentamicin-Cy3 conjugate and then fixed with 4% PFA. Microscopy revealed a similar distribution of the labeled antibiotic within the CHO WT and CHO Δ XylT cells.

FIGURE S5 | Labeling by Lysotracker correlates with PGs expression in CHO cells. CHO WT, CHO Δ XylT, and complemented CHO cXylT cells were infected with *S. Typhimurium* EGFP at MOI 50 and incubated for 24 h with 50 nM Lysotracker Red added upon invasion of bacteria. CHO cXylT cells had an intermediate staining (compare to Supplementary Figure S3A). Representative images of two biological repetitions. Scale bars, 10 μ m.

FIGURE S6 | A dual fluorescence reporter for acid shock exposure of *Salmonella*. (A) Plasmid map of p5386, encoding DsRed constitutively under control of promoter P_{EM7}, and sfGFP under control the acid shock-inducible promoter P_{asr}. (B) Flow cytometry and gating of *Salmonella* without fluorescent protein expression, or constitutive expression of DsRed or sfGFP. (C) Acid shock of cultured bacteria induces sfGFP expression. *Salmonella* WT harboring p5386 (*S. Tm* WT) was grown in PCN, pH 7.5 to mid-log phase. Bacteria were pelleted, washed twice in sterile saline, and resuspended in PCN buffered to the indicated pH. Culture was continued for 1 h, bacteria were harvested by centrifugation and resuspended in PCB containing 200 μ g/mL chloramphenicol to stop further protein biosynthesis. The bacteria were incubated for at least 2 h at 4°C to allow full maturation of sfGFP, and subjected to flow cytometry. (D) For *in vivo* analyses, *S. Tm* WT harboring p5386 was subcultured for 3 h and used to infect ca. 2×10^5 CHO WT or CHO Δ XylT cells at MOI of 10. If indicated (+ Baf), bafilomycin was added to a final concentration of 100 nM. Cells were infected for 30 min, washed three times to remove non-internalized bacteria and incubated 2 h with or without gentamicin addition as indicated in Fig 6B. A representative example of an assay with a constant concentration of 10 μ g/mL gentamicin is shown. After washing, cells were detached using biotase, chloramphenicol was added to final concentration of 200 μ g/mL and incubated for at least 4 h at 4°C for allow full maturation of sfGFP. Flow cytometry was performed by gating of CHO cells and the level of DsRed and sfGFP fluorescence was determined for at least 50,000 infected host cells.

FIGURE S7 | Inhibition of PI3K does not affect intracellular *Salmonella*. CHO WT and CHO Δ XylT cells were infected with *S. Typhimurium* WT at MOI of 50 and incubated for 24 h with 100 μ g/mL gentamicin and increasing concentrations of specific inhibitors. Addition of wortmannin had no effect on *Salmonella* survival in either CHO cell line. One-way ANOVA with Dunnett's multiple comparison test, only significant differences are indicated.

FIGURE S8 | PIKfyve kinase inhibition increased labeling by Lysotracker in both CHO WT and CHO Δ XylT cells. CHO cells infected with *S. Typhimurium* EGFP were incubated for 24 h with 50 nM Lysotracker Red added upon invasion, in presence/absence of 0.8 μ M YM201636. Microscopy revealed enlarged lysosomes/endosomes in YM201636-treated CHO cells. Representative images, scale bars, 10 μ m.

FIGURE S9 | CHO Δ XylT cells lack *Lama2* expression. cDNA of the uninfected CHO cells was used to screen for an expression of *Lama2* isoforms. ND – not detected.

FIGURE S10 | Addition of heparin to the medium results in intracellular accumulation of heparin in the endo-lysosomal system. CHO $\Delta XyIT$ cells, uninfected and infected with *S. Typhimurium* WT at MOI of 50 were incubated for 24 h with 100 $\mu\text{g/mL}$ gentamicin and with 30 μM heparin. Heparin (green) was detected inside CHO $\Delta XyIT$ cells, in the same compartment as bacteria (red). In CHO WT cells, in the absence of added heparin, HS staining shows localization of

HS at the cell surface but also in endo-lysosomal compartments. Representative images of two biological repetitions, scale bars, 10 μm or 5 μm (in enlarged sections).

TABLE S1 | Primers used in this study.

TABLE S2 | Antibodies used in this study.

REFERENCES

- Iozzo RV, Schaefer L. Proteoglycan form and function: a comprehensive nomenclature of proteoglycans. *Matrix Biol.* (2015) 42:11–55. doi: 10.1016/j.matbio.2015.02.003
- Taylor KR, Gallo RL. Glycosaminoglycans and their proteoglycans: host-associated molecular patterns for initiation and modulation of inflammation. *FASEB J.* (2006) 20:9–22. doi: 10.1096/fj.05-4682rev
- Esko JD, Stewart TE, Taylor WH. Animal cell mutants defective in glycosaminoglycan biosynthesis. *Proc Natl Acad Sci USA.* (1985) 82:3197–201. doi: 10.1073/pnas.82.10.3197
- Henry-Stanley MJ, Hess DJ, Erickson EA, Garni RM, Wells CL. Role of heparan sulfate in interactions of *Listeria monocytogenes* with enterocytes. *Med Microbiol Immunol (Berl).* (2003) 192:107–15. doi: 10.1007/s00430-002-0165-7
- van Putten JP, Paul SM. Binding of syndecan-like cell surface proteoglycan receptors is required for *Neisseria gonorrhoeae* entry into human mucosal cells. *EMBO J.* (1995) 14:2144–54. doi: 10.1002/j.1460-2075.1995.tb07208.x
- Leong JM, Wang H, Magoun L, Field JA, Morrissey PE, Robbins D, et al. Different classes of proteoglycans contribute to the attachment of *Borrelia burgdorferi* to cultured endothelial and brain cells. *Infect Immun.* (1998) 66:994–9.
- Lambert MA, Smith SGJ. The PagN protein mediates invasion via interaction with proteoglycan. *FEMS Microbiol Lett.* (2009) 297:209–16. doi: 10.1111/j.1574-6968.2009.01666.x
- Francis CL, Starnbach MN, Falkow S. Morphological and cytoskeletal changes in epithelial cells occur immediately upon interaction with *Salmonella typhimurium* grown under low-oxygen conditions. *Mol Microbiol.* (1992) 6:3077–87. doi: 10.1111/j.1365-2958.1992.tb01765.x
- Alonso A, García-del Portillo F. Hijacking of eukaryotic functions by intracellular bacterial pathogens. *Int Microbiol.* (2004) 7:181–91. doi: http://hdl.handle.net/10261/2135
- Knuff K, Finlay BB. What the SIF Is Happening—The Role of Intracellular *Salmonella*-Induced Filaments. *Front Cell Infect Microbiol.* (2017) 7:335. doi: 10.3389/fcimb.2017.00335
- Rajashekar R, Liebl D, Seitz A, Hensel M. Dynamic remodeling of the endosomal system during formation of *Salmonella* -induced filaments by intracellular *Salmonella enterica*. *Traffic.* (2008) 9:2100–16. doi: 10.1111/j.1600-0854.2008.00821.x
- García-del Portillo F, Zwick MB, Leung KY, Finlay BB. *Salmonella* induces the formation of filamentous structures containing lysosomal membrane glycoproteins in epithelial cells. *Proc Natl Acad Sci USA.* (1993) 90:10544–8. doi: 10.1073/pnas.90.22.10544
- Steele-Mortimer O, Meresse S, Gorvel J-P, Toh B-H, Finlay BB. Biogenesis of *Salmonella typhimurium*-containing vacuoles in epithelial cells involves interactions with the early endocytic pathway. *Cell Microbiol.* (1999) 1:33–49. doi: 10.1046/j.1462-5822.1999.00003.x
- Meresse S. The rab7 GTPase controls the maturation of *Salmonella typhimurium*-containing vacuoles in HeLa cells. *EMBO J.* (1999) 18:4394–403. doi: 10.1093/emboj/18.16.4394
- Mota LJ, Ramsden AE, Liu M, Castle JD, Holden DW. SCAMP3 is a component of the *Salmonella* -induced tubular network and reveals an interaction between bacterial effectors and post-Golgi trafficking. *Cell Microbiol.* (2009) 11:1236–53. doi: 10.1111/j.1462-5822.2009.01329.x
- Kuhle V, Abrahams GL, Hensel M. Intracellular *Salmonella enterica* redirect exocytic transport processes in a *Salmonella* pathogenicity Island 2-dependent manner: *Salmonella* redirect exocytosis. *Traffic.* (2006) 7:716–30. doi: 10.1111/j.1600-0854.2006.00422.x
- Perrett CA, Zhou D. *Salmonella* type III effector SopB modulates host cell exocytosis. *Emerg Microbes Infect.* (2013) 2:1–6. doi: 10.1038/emi.2013.31
- Toker A. Phosphoinositides and signal transduction. *Cell Mol Life Sci CMLS.* (2002) 59:761–79. doi: 10.1007/s00018-002-8465-z
- Gillooly DJ, Morrow IC, Lindsay M, Gould R, Bryant NJ, Gaullier J-M, et al. Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. *EMBO J.* (2000) 19:4577–88. doi: 10.1093/emboj/19.17.4577
- Shaw JD, Hama H, Sohrabi F, DeWald DB, Wendland B. PtdIns(3,5)P₂ is required for delivery of endocytic cargo into the multivesicular body. *Traffic.* (2003) 4:479–90. doi: 10.1034/j.1600-0854.2003.t01-1-00106.x
- Sbrissa D, Ikonomov OC, Shisheva A. PIKfyve, a mammalian ortholog of yeast fab1p lipid kinase, synthesizes 5-phosphoinositides effect of insulin. *J Biol Chem.* (1999) 274:21589–97. doi: 10.1074/jbc.274.31.21589
- Drecktrah D, Knodler LA, Steele-Mortimer O. Modulation and utilization of host cell phosphoinositides by *Salmonella* spp. *Infect Immun.* (2004) 72:4331–5. doi: 10.1128/IAI.72.8.4331-4335.2004
- Dukes JD, Lee H, Hagen R, Reaves BJ, Layton AN, Galyov EE, et al. The secreted *Salmonella* dublin phosphoinositide phosphatase, SopB, localizes to PtdIns(3)P-containing endosomes and perturbs normal endosome to lysosome trafficking. *Biochem J.* (2006) 395:239–47. doi: 10.1042/BJ20051451
- Bakker H, Oka T, Ashikov A, Yadav A, Berger M, Rana NA, et al. Functional UDP-xylose transport across the endoplasmic reticulum/golgi membrane in a chinese hamster ovary cell mutant defective in UDP-xylose synthase. *J Biol Chem.* (2009) 284:2576–83. doi: 10.1074/jbc.M804394200
- Dam GB, Hafmans T, Veerkamp JH, van Kuppevelt TH. Differential expression of heparan sulfate domains in rat spleen. *J Histochem Cytochem.* (2003) 51:727–39. doi: 10.1177/002215540305100604
- Matsuura M, Kawasaki K, Kawahara K, Mitsuyama M. Evasion of human innate immunity without antagonizing TLR4 by mutant *Salmonella enterica* serovar Typhimurium having penta-acylated lipid A. *Innate Immun.* (2012) 18:764–73. doi: 10.1177/1753425912440599
- Hoiseth SK, Stocker BAD. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature.* (1981) 291:238–9. doi: 10.1038/291238a0
- Cormack BP, Valdivia RH, Falkow SF. ACS-. optimized mutants of the green fluorescent protein (GFP). *Gene.* (1996) 173:33–8. doi: 10.1016/0378-1119(95)00685-0
- Brumell JH, Goosney DL, Finlay BB. SifA, a Type III Secreted Effector of *Salmonella typhimurium*, directs *Salmonella*-induced filament (Sif) formation along microtubules. *Traffic.* (2002) 3:407–15. doi: 10.1034/j.1600-0854.2002.30604.x
- Beuzon CR. *Salmonella* maintains the integrity of its intracellular vacuole through the action of SifA. *EMBO J.* (2000) 19:3235–49. doi: 10.1093/emboj/19.13.3235
- Noster J, Chao T-C, Sander N, Schulte M, Reuter T, Hansmeier N, et al. Proteomics of intracellular *Salmonella enterica* reveals roles of *Salmonella* pathogenicity island 2 in metabolism and antioxidant defense. *PLoS Pathog.* (2019) 15:e1007741. doi: 10.1371/journal.ppat.1007741
- Bécavin C, Bouchier C, Lechat P, Archambaud C, Creno S, Gouin E, et al. Comparison of widely used listeria monocytogenes strains EGD, 10403S, and EGD-e highlights genomic differences underlying variations in pathogenicity. *mBio.* (2014) 5:e969-14. doi: 10.1128/mBio.00969-14
- Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ $\Delta\Delta\text{CT}$ method. *Methods.* (2001) 25:402–8. doi: 10.1006/meth.2001.1262
- Knodler LA, Nair V, Steele-Mortimer O. Quantitative assessment of cytosolic *Salmonella* in epithelial cells. *PLoS One.* (2014) 9:e84681. doi: 10.1371/journal.pone.0084681
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* (2012) 9:676–82. doi: 10.1038/nmeth.2019

36. Reuter T, Vorwerk S, Liss V, Chao T-C, Hensel M, Hansmeier N. Proteomic analysis of *Salmonella*-modified membranes reveals adaptations to macrophage hosts. *Mol Cell Proteomics MCP*. (2020) [epub ahead of print]. doi: 10.1074/mcp.RA119.001841
37. Bolte S, Cordelières FP. A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc*. (2006) 224:213–32. doi: 10.1111/j.1365-2818.2006.01706.x
38. Myrdal SE, Steyger PS. TRPV1 regulators mediate gentamicin penetration of cultured kidney cells. *Hear Res*. (2005) 204:170–82. doi: 10.1016/j.heares.2005.02.005
39. Karasawa T, Wang Q, Fu Y, Cohen DM, Steyger PS. TRPV4 enhances the cellular uptake of aminoglycoside antibiotics. *J Cell Sci*. (2008) 121:2871–9. doi: 10.1242/jcs.023705
40. Notenboom S. Increased apical insertion of the multidrug resistance protein 2 (MRP2/ABCC2) in renal proximal tubules following gentamicin exposure. *J Pharmacol Exp Ther*. (2006) 318:1194–202. doi: 10.1124/jpet.106.104547
41. Liss V, Swart AL, Kehl A, Hermanns N, Zhang Y, Chikkaballi D, et al. *Salmonella enterica* remodels the host cell endosomal system for efficient intravacuolar nutrition. *Cell Host Microbe*. (2017) 21:390–402. doi: 10.1016/j.chom.2017.02.005
42. Allam US, Krishna MG, Sen M, Thomas R, Lahiri A, Gnanadhas DP, et al. Acidic pH induced STM1485 gene is essential for intracellular replication of *Salmonella*. *Virulence*. (2012) 3:122–35. doi: 10.4161/viru.19029
43. Seputiene V, Motiejūnas D, Suziedelis K, Tomenius H, Normark S, Melefors O, et al. Molecular characterization of the acid-inducible *asr* gene of *Escherichia coli* and its role in acid stress response. *J Bacteriol*. (2003) 185:2475–84. doi: 10.1128/jb.185.8.2475-2484.2003
44. Kerr MC, Wang JTH, Castro NA, Hamilton NA, Town L, Brown DL, et al. Inhibition of the PtdIns(5) kinase PIKfyve disrupts intracellular replication of *Salmonella*. *EMBO J*. (2010) 29:1331–47. doi: 10.1038/emboj.2010.28
45. Cuellar K, Chuong H, Hubbell SM, Hinsdale ME. Biosynthesis of chondroitin and heparan sulfate in chinese hamster ovary cells depends on xylosyltransferase II. *J Biol Chem*. (2007) 282:5195–200. doi: 10.1074/jbc.M611048200
46. Rostand KS, Esko JD. Microbial adherence to and invasion through proteoglycans. *Infect Immun*. (1997) 65:1–8.
47. van Wijk XM, Döhrmann S, Hallström BM, Li S, Voldborg BG, Meng BX, et al. Whole-genome sequencing of invasion-resistant cells identifies laminin $\alpha 2$ as a host factor for bacterial invasion. *mBio*. (2017) 8:e2128–16. doi: 10.1128/mBio.02128-16
48. Nagai J, Takano M. Entry of aminoglycosides into renal tubular epithelial cells via endocytosis-dependent and endocytosis-independent pathways. *Biochem Pharmacol*. (2014) 90:331–7. doi: 10.1016/j.bcp.2014.05.018
49. Denamur S, Tyteca D, Marchand-Brynaert J, Van Bambeke F, Tulkens PM, Courtoy PJ, et al. Role of oxidative stress in lysosomal membrane permeabilization and apoptosis induced by gentamicin, an aminoglycoside antibiotic. *Free Radic Biol Med*. (2011) 51:1656–65. doi: 10.1016/j.freeradbiomed.2011.07.015
50. Brumell JH, Tang P, Zaharik ML, Finlay BB. Disruption of the *Salmonella*-containing vacuole leads to increased replication of *Salmonella enterica* serovar typhimurium in the cytosol of epithelial cells. *Infect Immun*. (2002) 70:3264–70. doi: 10.1128/IAI.70.6.3264-3270.2002
51. Dukes JD, Whitley P, Chalmers AD. The PIKfyve inhibitor YM201636 blocks the continuous recycling of the tight junction proteins claudin-1 and claudin-2 in MDCK cells. *PLoS One*. (2012) 7:e28659. doi: 10.1371/journal.pone.0028659
52. Krishna S, Palm W, Lee Y, Yang W, Bandyopadhyay U, Xu H, et al. PIKfyve regulates vacuole maturation and nutrient recovery following engulfment. *Dev Cell*. (2016) 38:536–47. doi: 10.1016/j.devcel.2016.08.001
53. Menashe O, Kaganskaya E, Baasov T, Yaron S. Aminoglycosides affect intracellular *Salmonella enterica* serovars typhimurium and virchow. *Antimicrob Agents Chemother*. (2008) 52:920–6. doi: 10.1128/AAC.00382-07
54. Kawai A, Uchiyama H, Takano S, Nakamura N, Ohkuma S. Autophagosome-lysosome fusion depends on the pH in acidic compartments in CHO cells. *Autophagy*. (2007) 3:154–7. doi: 10.4161/auto.3634
55. Oh E-S, Woods A, Lim S-T, Theibert AW, Couchman JR. Syndecan-4 proteoglycan cytoplasmic domain and phosphatidylinositol 4,5-bisphosphate coordinately regulate protein kinase C activity. *J Biol Chem*. (1998) 273:10624–9. doi: 10.1074/jbc.273.17.10624
56. Terebiznik MR, Vieira OV, Marcus SL, Slade A, Yip CM, Trimble WS, et al. Elimination of host cell PtdIns(4,5)P₂ by bacterial SigD promotes membrane fission during invasion by *Salmonella*. *Nat Cell Biol*. (2002) 4:766–73. doi: 10.1038/ncb854
57. Friand V, David G, Zimmermann P. Syntenin and syndecan in the biogenesis of exosomes: syndecan-syntenin pathway in exosome biogenesis. *Biol Cell*. (2015) 107:331–41. doi: 10.1111/boc.201500010
58. Hessvik NP, Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci*. (2018) 75:193–208. doi: 10.1007/s00018-017-2595-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Galeev A, Suwandi A, Bakker H, Oktiviyari A, Routier FH, Krone L, Hensel M, Grassl GA. Proteoglycan-dependent endo-lysosomal fusion affects intracellular survival of *Salmonella* Typhimurium in epithelial cells. *Frontiers in Immunology*. 2020 Apr 29;11:731.

Supplementary data

Table S1. Primers used in this study.

Gene/Primer name	Sequence
<i>Gapdh</i>	Fw: CGTATTGGACGCCTGGTTAC Rv: GGCAACAACCTCCACTTTGC
<i>Rps9</i>	Fw: TTTACCCTGGCCAAGATCC Rv: TGGGTCCTTTTCGTCCAG
<i>Trpv1</i>	Fw: GGTGCTACATGCCCTGGT Rv: GTCACGAACCTTGGTGTGTCA
<i>Trpv4</i>	Fw: GAAGGTGTGTGACGAGGATG Rv: GATGGTGAGCTTGAACAGGTC
<i>Abcc2</i>	Fw: CACATGGCTCCTGGTGTTAG Rv: ACTCAGAAATGAAGCCGTGG
Vf-p4889 Vr-p4889	Fw: ATGCGCAAAGGCGAAGAAGTGTACCGGTGT Rv: GGCCGGCATCACCGGCGCCACAG
1f Pasr-sfGFP 1r Pasr-sfGFP	Fw: GCGCGCGGTGATGCCGGCCACGCCTGAAAAGAAATAATCC Rv: GTTCTTCGCCTTTGCGCATTTTGATACCCTCGATTTGGTTTTC

Table S2. Antibodies used in this study.

Antibody	Reference/Manufacturer, RRID and catalog number
Mouse Anti- <i>Salmonella</i> Typhimurium-LPS Monoclonal Antibody, Unconjugated, Clone 1E6	Meridian Life Science Cat# C86309M, RRID:AB_152809
Rabbit <i>Salmonella</i> O Antiserum Group B Factors 1, 4, 12, 27	BD Difco Cat# 229731
F(ab)2-Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	Thermo Fisher Scientific Cat# A-11071, RRID:AB_2534115
F(ab)2-Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific Cat# A-11017, RRID:AB_2534084
AO4B08, Anti-heparan sulfate Phage Display Antibody with VSV tag	Dam <i>et al.</i> , 2003
Mouse Anti-VSV-G tag Monoclonal Antibody, Unconjugated, Clone P5D4	Abcam Cat# ab50549, RRID:AB_883494

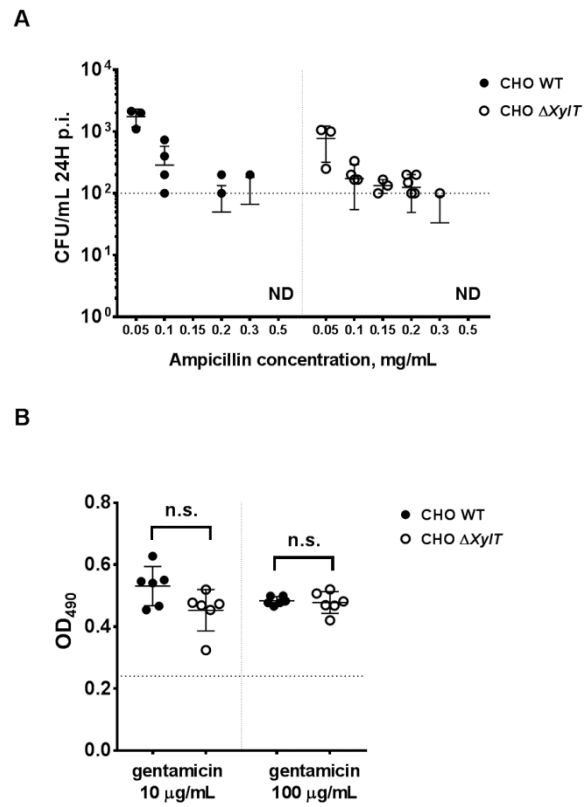


FIGURE S1. Intracellular *Salmonella* are sensitive to treatment with ampicillin.

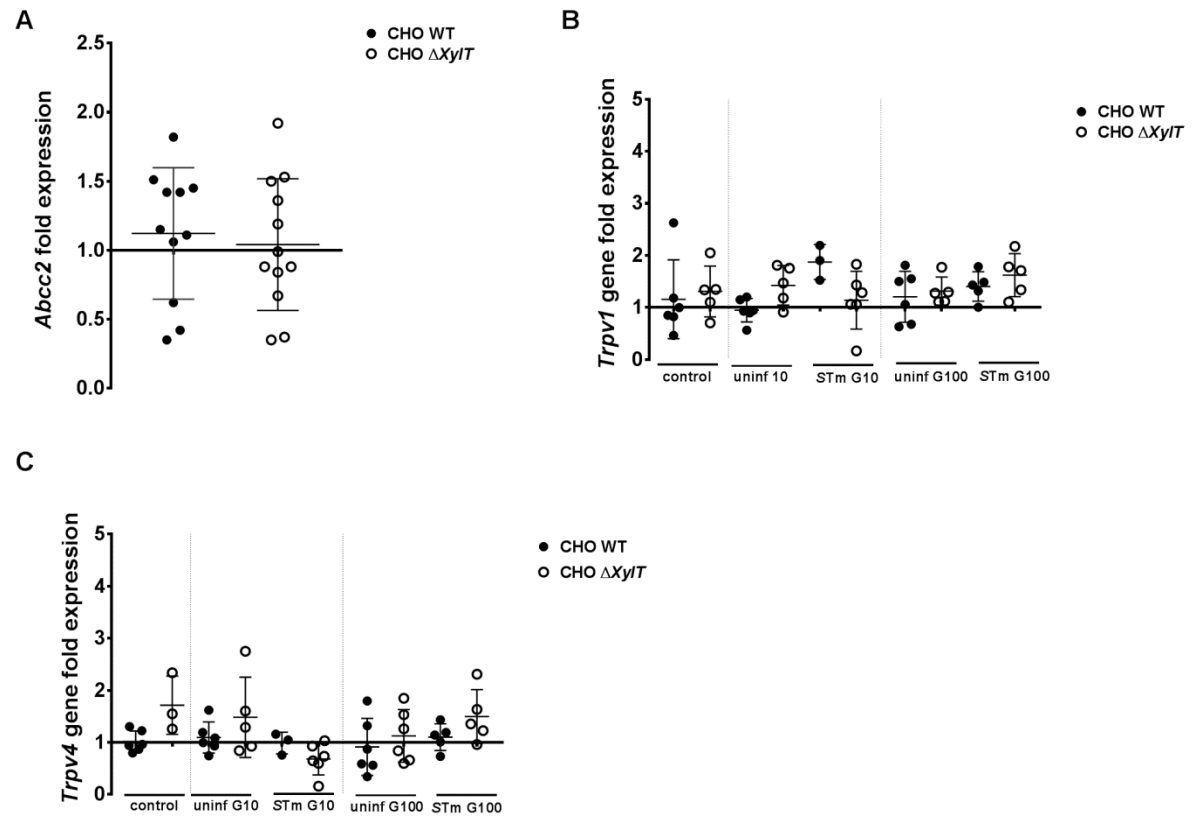


FIGURE S2. CHO WT and CHO $\Delta XylT$ cells had comparable expression levels of gentamicin transporters genes.

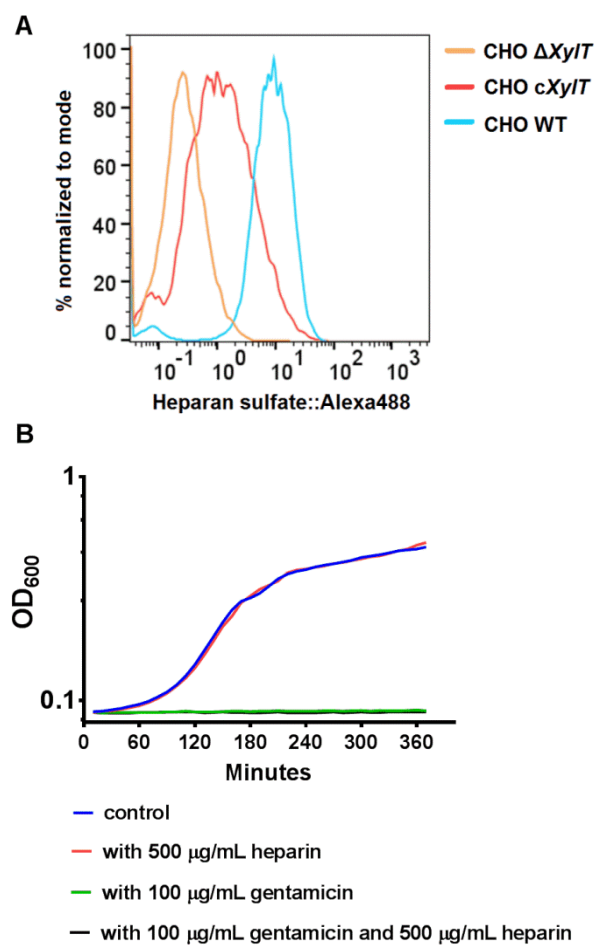


FIGURE S3. Presence of PGs in CHO cells.

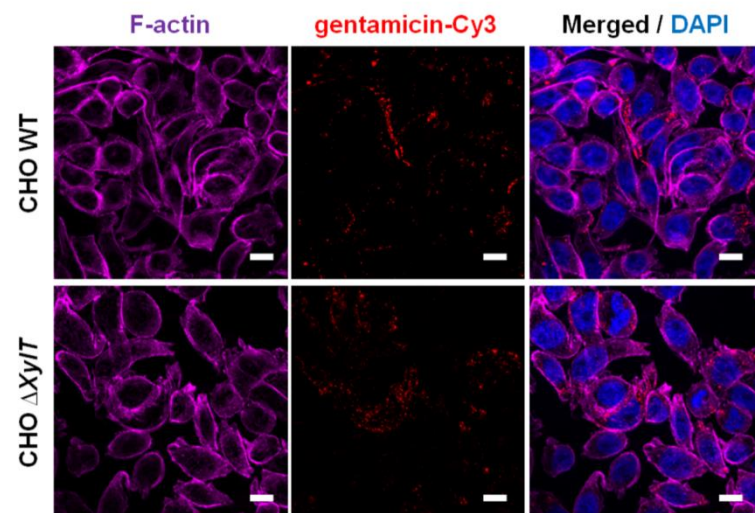


FIGURE S4. Gentamicin uptake in CHO WT and CHO Δ XyIT cells.

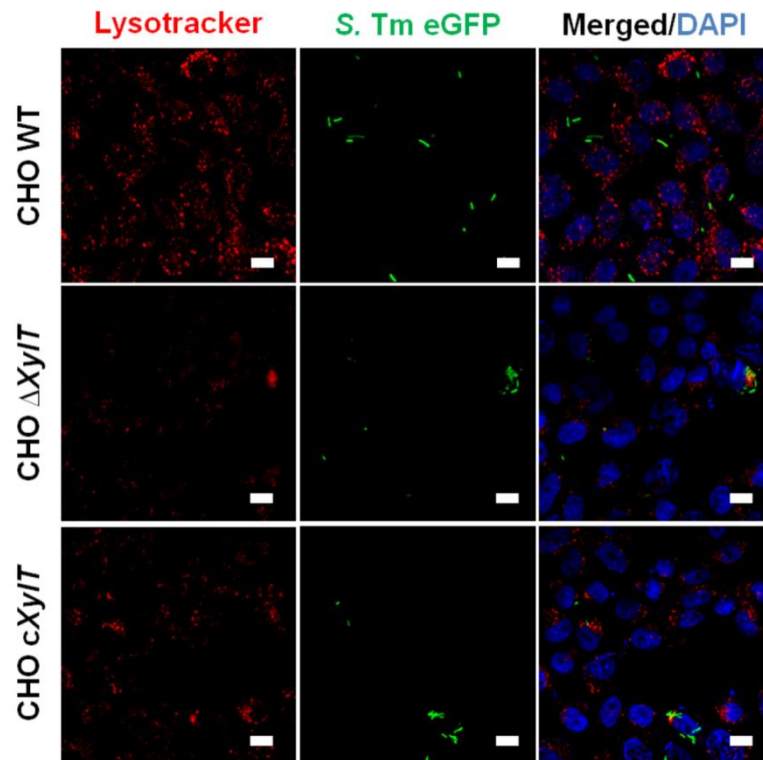


FIGURE S5. Labeling by Lysotracker correlates with PGs expression in CHO cells.

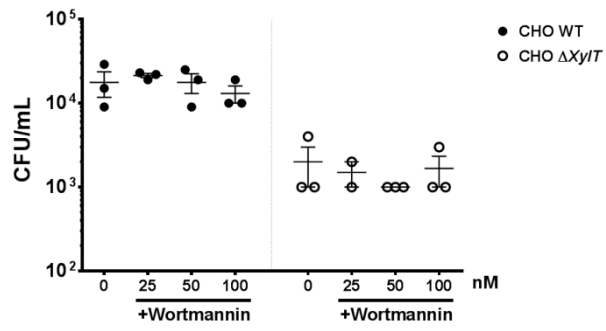


FIGURE S7. Inhibition of PI3K does not affect intracellular *Salmonella* cells.

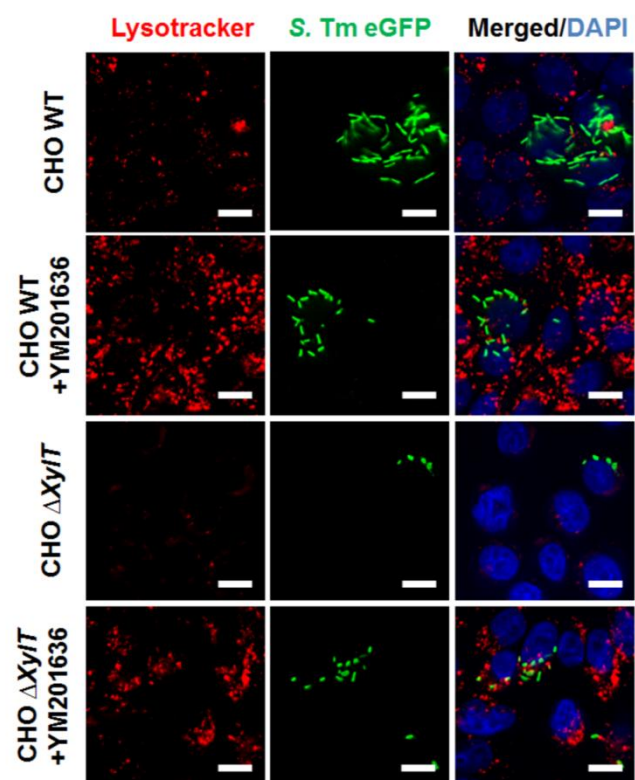


FIGURE S8. PIKfyve kinase inhibition increased labeling by Lysotracker in both CHO WT and CHO Δ XylT cells.

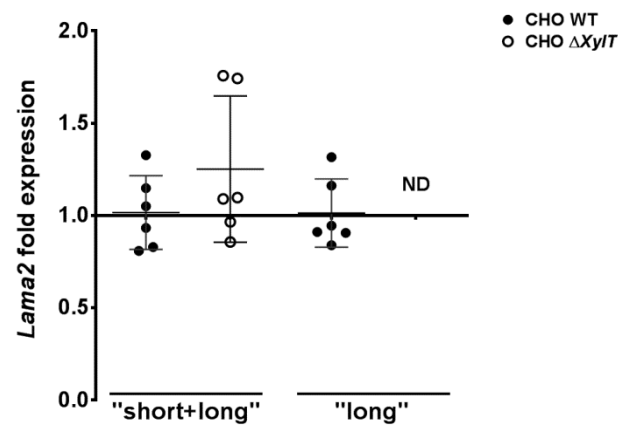


FIGURE S9. CHO $\Delta XylT$ cells lack *Lama2* expression.

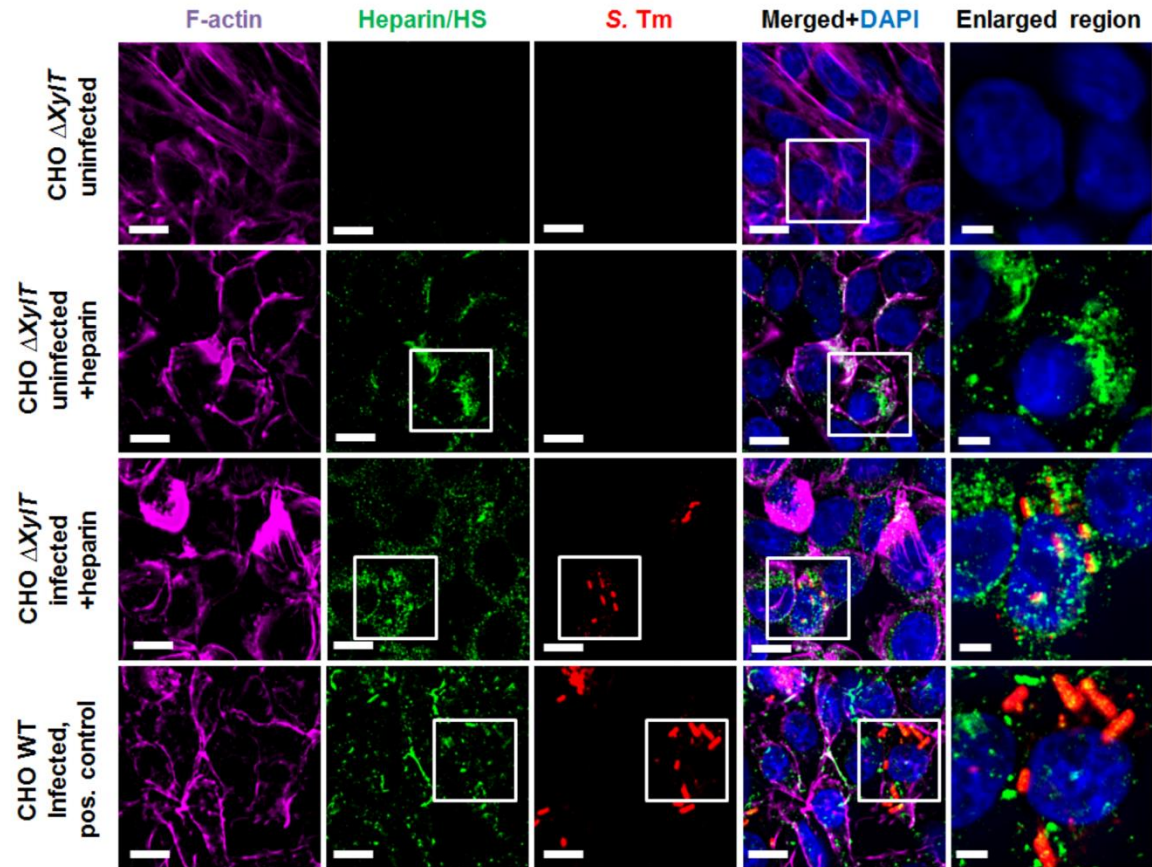


FIGURE S10. Addition of heparin to the medium results in intracellular accumulation of heparin in the endo-lysosomal system.

Acknowledgments

I dedicate this thesis to my family: because I owe it all to you. To my mother who devoted 40 years of her life to Medicine; her strength of will keeps inspiring me. To my father who always believed in me. To my younger sister who likes gummies. Thank you for your moral and emotional support during all those years!

I am also thankful to my scientific family – Arbeitsgruppe Graßl. Prof. Guntram Graßl was always supportive and kind, I was truly lucky to get the awesome PhD project and the amazing mentor! I want to express my deepest gratitude to Dr. Abdulhadi Suwandi for his continuous help with the sugars' project and for introducing me to our little furry friends. I am grateful to Dr. Katrin Erkhart, Samriti Sharma, Eva Wunschel, Kris Alvarez, Kathrin Seeger, Ana Becker, and Nicolette Schwermann, for a time we shared in and out of the lab; I will miss you guys!

I was privileged to work and to thrive at the MHH's Institute of Medical Microbiology and Hospital Epidemiology. I want to acknowledge the former and current heads of the Institute, Prof. Dr. Sebastian Suerbaum and Prof. Dr. Dirk Schlüter, respectively, for establishing a first-class research environment. Special thanks are to my friends and colleagues: Shruti Chopra, Alexander Permyakov, Martin Kohn, Vania Passos, Franjo Banovic, Charu Gupta, Dr. Erin Boyle, Dr. Marius Vital, and Dr. Winstel Volker, for the fruitful scientific and non-scientific conversations. Many thanks go to the German Research Foundation (DFG) and, ultimately, to all German (and non-German) tax-payers for the provided funding. "Give, and it will be given to you".

I sincerely thank my thesis co-supervisors, Prof. Françoise H. Routier and Prof. John Baines, as well as Dr. Hans Bakker, for their support and contributions to the project. I am also grateful to the ZIB program coordinators, Dr. Sabine Johan and Simone Zimmer, for their guidance and assistance throughout my PhD studies, especially at the final stages (in the dark times of COVID-19). I also want to thank the thesis examiners, Prof. Dr. Bärbel Stecher and Prof. Dr. Til Strowig.

Finally, I would like to thank Ivan Reitman, Rick Moranis, Dan Aykroyd, Bill Murray, and Harold Ramis (R.I.P.) for creating and portraying heroes of my childhood, the Ghostbusters. Drs. Venkman, Stantz, and Spengler embodied the best virtues of a good scientist: creativity, rigor, curiosity, wit, and a good sense of humor.

"We came, we saw, we kicked its ass!"

Curriculum vitae

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EDUCATION

10.2016-06.2020 **Hannover Medical School** (Hannover, Germany)

Institute of Medical Microbiology and Hospital Epidemiology

Dr. rer. nat. in Microbiology

Academic dissertation: " Host glycoconjugates in *Salmonella* infection: a bittersweet symphony"

Supervisor: Prof. Dr. Guntram Grassl

09.2014-06.2016 **University of Helsinki** (Helsinki, Finland)

M.Sc. in Food Safety (Microbiology)

Master's thesis: "Evaluating the effect of different growth conditions on glycoconjugate expression in *Campylobacter coli*: a methodological perspective", *eximia cum laude*.

Supervisor: Prof. Mirko Rossi

09.2005-06.2011 **South Ural State Agrarian University** (Troitsk, Russia)

Specialist degree in Food Science

SKILLS AND TECHNIQUES

- *in vivo*: *Salmonella* infection of mice, *Pseudomonas* infection of *Galleria mellonella* larvae.
- *in vitro*: 3D/2D organoids models, Lambda Red mutagenesis, IHC, IF staining, western blot, SDS-PAGE, gentamicin protection assay, anaerobic cultivation of commensals.
- software: GraphPad Prism, ImageJ, Serial Cloner, Genome Compiler, Imaris, R.
- microscopy: Zeiss Apotome2, Leica SP8 confocal, Nikon live cell imaging system.

PRESENTATIONS

- Mucosal Immunology Course & Symposium Oxford, UK
Poster presentation. 2018
- CSHL meeting "Microbial Pathogenesis & Host Response" Cold Spring Harbor, USA
Poster presentation. 2019
- 6th Joint Conference of DGHM&VAAM Leipzig, Germany
Talk: "Proteoglycan-dependent endo-lysosomal fusion affects intracellular survival of *Salmonella* Typhimurium" 2020



Declaration

Herewith, I confirm that I have written the present PhD thesis myself and independently in compliance with “the policy of Hannover Medical School on the safeguarding of good scientific practice and procedural rules for dealing with scientific misconduct” and that I have not submitted it at any other university worldwide.

Herewith, I agree that MHH can check my thesis by plagiarism detection software as well as randomly check the primary data. I am aware that in case of suspicion, ombudsman proceedings according to § 9 of MHH ‘Guidelines of Hannover Medical School to guarantee good scientific practice and dealing with scientific fraud’ will be initiated. During such proceedings, the PhD process is paused.

Kiel, (July 2020)
