HHS Public Access

Author manuscript

Cell Microbiol. Author manuscript; available in PMC 2021 March 01.

Published in final edited form as:

Cell Microbiol. 2020 March ; 22(3): e13152. doi:10.1111/cmi.13152.

The Zebrafish as a Model for Gastrointestinal Tract - Microbe Interactions

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Summary

The zebrafish (*Danio rerio*) has become a widely used vertebrate model for bacterial, fungal, viral, and protozoan infections. Due to its genetic tractability, large clutch sizes, ease of manipulation, and optical transparency during early life stages, it is a particularly useful model to address questions about the cellular microbiology of host-microbe interactions. Although its use as a model for systemic infections, as well as infections localized to the hindbrain and swimbladder have been thoroughly reviewed, studies focusing on host-microbe interactions in the zebrafish gastrointestinal tract have been neglected. Here, we summarize recent findings regarding the developmental and immune biology of the gastrointestinal tract, drawing parallels to mammalian systems. We discuss the use of adult and larval zebrafish as models for gastrointestinal infections, and more generally, for studies of host-microbe interactions in the gut.

Keywords

Zebrafish; *Danio rerio*; microbiome; microbiota; host-pathogen interactions; infection model; gastrointestinal tract

1. Introduction

Due to its fecundity, genetic tractability, small size, rapid development, and optical transparency during early development, the zebrafish *(Danio rerio)* has emerged as one of the most well-used vertebrate model organisms in cellular microbiology. Several reviews have focused on zebrafish as a model for infectious disease (Tobin *et al.,* 2012; Sullivan *et al.,* 2017; Duggan and Mostowy, 2018), but all were focused on systemic disease models or localized infections of the swim bladder or hindbrain; none discussed infections of the

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gastrointestinal (GI) tract. Over the last few years however, literature in this area has grown considerably, as has literature focusing on the microbiota, and characterization of the transcriptional profiles and cell types of the zebrafish GI tract, allowing us to draw comparisons to the mammalian GI tract. These studies have made it clear that the development and physiology of the zebrafish GI tract bear many similarities to humans. For example, ~70% of human genes have a corresponding orthologue in zebrafish (Howe *et al.,* 2013), and the transcriptional regulatory networks controlling intestinal development and physiology are highly conserved between fish and mammals (Lickwar *et al.,* 2017). These findings highlight the utility of the zebrafish model to study *in vivo* host-microbe and host-pathogen interactions in the GI tract. With this review, we summarize recent literature highlighting structural and molecular similarities between zebrafish and mammalian GI tract architecture and immune surveillance. We further discuss examples of studies looking at host-microbe and host-pathogen interactions in the zebrafish GI tract, highlighting experimental features and summarizing the most important findings. We hope this review will highlight the utility of the zebrafish model for GI tract- microbe studies, bridging the gap from whole animal studies to single cell and molecular, mechanistic experiments.

2. Organization and development of the zebrafish intestine

2.1. Intestinal segmentation and architecture in zebrafish

The primary functions of the intestine include digestion and absorption of nutrients, and the elimination of waste products. The zebrafish intestine is highly homologous with the mammalian intestine in its development, organization, and function (Pack *et al.,* 1996; Wallace *et al.,* 2005; Ng *et al.,* 2005; Carten and Farber, 2009). Gene expression and transcriptional regulation in intestinal epithelial cells are highly conserved along the segmental regions of the mammalian and zebrafish intestines (Lickwar *et al.,* 2017), and many metabolic functions are also conserved (Schlegel and Gut, 2015; Quinlivan and Farber, 2017). Both zebrafish and mammalian GI tracts are covered by a protective mucus layer that predominantly consists of the gel-forming mucin Muc2 as its structural component (Johansson *et al.,* 2011; Jevtov *et al.,* 2014). Mucin is primarily secreted by goblet cells, which in adult zebrafish are distributed throughout the gut (albeit more abundant in the middle and posterior gut) (Wallace *et al.,* 2005; Ng *et al.,* 2005).

There are, however, some key functional and architectural differences between zebrafish and mammalian GI systems to consider (Figure 1). The mammalian gastrointestinal tract is generally composed of five distinct parts: (1) the stomach, which partially digests food by mixing it with acid and digestive enzymes, (2) the duodenum, which aids chemical digestion, (3) the jejunum, which absorbs nutrients, (4) the ileum, which absorbs bile salts, and (5) the colon, which absorbs water and salts. The zebrafish intestine is divided into three histologically defined segments, including (1) the anterior intestinal bulb, (2) the middle intestine, and (3) the posterior intestine. This nomenclature for the zebrafish gut is typically maintained from larval to adult stages. In contrast to mammals, zebrafish lack the typical signatures of a stomach organ and the intestinal bulb undergoes no acidification. The passage of microbes through the acidic environment of the human stomach, which can reach pH values as low as 1.4 (Dressman *et al.,* 1990), serves as a regulatory cue for some GI

pathogens (Ramos-Morales, 2012), and its absence may impact the outcome of infection. Zebrafish do, however, express several digestive enzymes that are functionally equivalent to mammalian gastric markers, including rennin, nothepsin, several cathepsins, and the lipase Lipf (Wallace *et al.,* 2005; Wang *et al.,* 2010). The different regions of the adult zebrafish gut have distinct functions analogous to the mammalian small intestine and colon. Functionally, the anterior intestinal bulb likely plays a role in bile salt recovery (Lickwar *et al.,* 2017), while the anterior and middle regions may aid in lipid and protein absorption (Ng *et al.,* 2005; Brugman, 2016). Like the mammalian colon, the posterior region of the zebrafish gut is responsible for ion and water absorption (Wallace *et al.,* 2005). Established markers of the mammalian small and large intestine (e.g., *villin* and *fabp2)* are differentially expressed along the length of the zebrafish intestine (Wang *et al.,* 2010). Transcriptional domains identified in larval zebrafish are maintained in adults, and a model delineating five transcriptional and functional domains has been proposed, and includes *ada* for the anterior, duodenum-like region, *fabp2* and *rbp2a* for the anterior, jejunum-like region, *fabp6* and *slc10a2* for the middle, ileum-like region, and *lamp2* for the middle to posterior, colon-like region (Lickwar *et al.,* 2017).

The intestinal architecture of zebrafish is also less complex than that of mammals as it consists only of the mucosa, muscularis externa, and serosa layers. Underneath the mucosa and epithelium is the lamina propria containing blood capillaries, muscle fibers, and lymphatic vessels. Surrounding this layer is the muscularis externa composed of circular and longitudinal smooth muscle fibers and enteric neurons (Wallace and Pack, 2003). The lining of the mucosa is folded into large, randomly-shaped intestinal folds instead of the mammalian villi and intestinal crypts (crypts of Lieberkühn) (Pack *et al.,* 1996; Wallace *et al.,* 2005; Wang *et al.,* 2010). Since zebrafish lack crypts of Lieberkühn, which in mammals are the source of intestinal stem cells, cell division instead occurs at the base of the folds and cells migrate to the tip of the folds where they become apoptotic (Wallace *et al.,* 2005; Wang *et al.,* 2010). The time course for cell migration in the anterior intestine is 5–7 days, while migration takes 7–10 days in the middle intestine (Wallace *et al.,* 2005). Goblet cells are still present in zebrafish, but distributed throughout the mucosa rather than localized to crypts (Pack *et al.,* 1996; Wang *et al.,* 2010).

The enteric nervous system (ENS) is a functionally important component of the GI system both in zebrafish and mammals, and consists of enteric neurons and glial cells. These cells modulate several key functions including gut peristalsis, hormone secretion, water balance, and absorption. In contrast to the more architecturally complex mammalian ENS which is composed of two plexuses (i.e., myenteric and submucosal), each with their own interconnected ganglia, the zebrafish ENS develops into a single myenteric layer of neurons, glia, and other cell types (e.g., interstitial cells of Cajal) without any clearly defined ganglia (Wallace *et al.,* 2005).

2.2. Development of the zebrafish gastrointestinal tract

Intestinal development in zebrafish larvae can be categorized into 3 major stages (Ng *et al.,* 2005). Stage 1 is defined by formation of the lumen; a thin rod of endodermal cells that undergo anterior-to-posterior differentiation and proliferation (Wallace and Pack, 2003); by

60 hours post-fertilization (hpf) the lumen has expanded rostrocaudal (Kimmel *et al.,* 1995; Alvers *et al.,* 2014). During stage 2, the intestinal epithelium becomes polarized via cell type differentiation (Pack *et al.,* 1996; Wallace *et al.,* 2005), and by 76 hpf, enteroendocrine cells are present throughout the anterior to posterior regions of the intestine. Enteroendrocrine cells produce and release active compounds (e.g., hormones) into the surrounding tissues as well as modulate enzyme secretions and muscle contractions (Wallace *et al.,* 2005; Takashima *et al.,* 2013). At 76 hpf, the beginning of stage 3, intestinal folds have developed in the anterior and middle intestinal regions, and peristaltic contractions have begun (Pack *et al.,* 1996; Wallace *et al.,* 2005). Growth and differentiation of the digestive tract continues, and by 5 days post-fertilization (dpf), the majority of the system is functional (Wallace and Pack, 2003; Ng *et al.,* 2005); at this point the larval zebrafish digestive tract is comprised of the mouth, pharynx, esophagus, intestinal bulb, intestine, and anal opening. Extensive folding can be found within the anterior intestine, but at 5 dpf the posterior intestinal regions have no folds (Ng *et al.,* 2005), and by 6 to 8 dpf, cell proliferation begins to decrease (Cheesman *et al.,* 2011). As the larval zebrafish ages, folding continues and the folds themselves become shorter in the caudal direction (Menke *et al.,* 2011; Li *et al.,* 2019), and the lumen begins to widen at the anterior end and becomes progressively smaller towards the posterior region (Wallace *et al.,* 2005).

Enteric precursors originating from the neural crest migrate into the intestinal tract at 32 hpf and reach the posterior end by 66 hpf (Olden *et al.,* 2008; Heanue *et al.,* 2016). By 4 dpf the zebrafish gut exhibits spontaneous, coordinated contractions and the digestive system is fully functional by 7 dpf (Holmberg *et al.,* 2007). We refer readers to excellent recent reviews of ENS function and its interactions with the various other cells types of the zebrafish intestine (Ganz *et al.,* 2016; Ganz, 2018).

3. Immune surveillance of the gastrointestinal tract

Zebrafish have made valuable contributions to our understanding of vertebrate immunity (Martins *et al.,* 2019). Multiple comprehensive reviews discuss the teleost immune system (Traver *et al.,* 2003; Trede *et al.,* 2004; Sullivan and Kim, 2008a; Meeker and Trede, 2008; Uribe *et al.,* 2011; Renshaw and Trede, 2012; Rauta *et al.,* 2012), and zebrafish immunity in the context of infectious disease (Sullivan and Kim, 2008b; Meijer and Spaink, 2011; Masud *et al.,* 2017). The zebrafish and human immune systems share many similarities, including both innate and adaptive components (Traver *et al.,* 2003; Renshaw and Trede, 2012).

3.1. Innate immunity and the GI tract

Larval zebrafish solely depend on their innate immune system to fend off invading microbes since the adaptive immune system is not functional until 4–6 weeks post-fertilization (Willett *et al.,* 1999; Lam *et al.,* 2004). During this time, colonization of the larval gut by commensals can prime innate immune cells against infections through a Toll-like receptor (TLR) and myeloid differentiation primary response 88 (MyD88) dependent pathway (Galindo-Villegas *et al.,* 2012). One of the benefits of zebrafish is their optical transparency, allowing for the use of transgenic lines with fluorescently-marked innate immune cells like macrophages (Ward, 2003; Redd *et al.,* 2006; Hall *et al.,* 2007; Ellett *et al.,* 2011; Walton *et*

al., 2015; Sanderson *et al.,* 2015; Nguyen-Chi *et al.,* 2017), neutrophils (Mathias *et al.,* 2006; Renshaw *et al.,* 2006; Buchan *et al.,* 2019), and eosinophils (Balla *et al.,* 2010). These resources have given valuable insights into the role of innate immune cells in intestinal homeostasis and pathogenesis.

Intestinal macrophages play a role in microbiome homeostasis and complement regulation. Loss of intestinal macrophages in adult zebrafish *irf8* mutants causes dysbiosis of the resident gut microbiota, and a reduction in the complement component C1q (Earley *et al.,* 2018). Neutrophils are recruited to the intestine in response to inflammatory stimuli such as the pathogen-associated molecular pattern (PAMP) lipopolysaccharide (LPS). Immersion of larvae in LPS activates MyD88 and tumor necrosis factor-alpha (TNF-α), and leads to intestinal neutrophil influx (Bates *et al.,* 2007). Colonization of larvae by a conventional microbiota promotes production of alkaline phosphatase by the gut epithelium, which detoxifies microbiota-derived LPS and prevents excessive inflammation to maintain intestinal homeostasis (Bates *et al.,* 2007). Zebrafish TLRs that recognize PAMPs share key structural similarities with mammalian TLRs, but some TLRs may differ in functional aspects (Palti, 2011). For example, in mammals TLR4 responds to LPS, while zebrafish TLR4 paralogs (Tlr4a and Tlr4b) fail to recognize and respond to LPS (Sullivan *et al.,* 2009). Since zebrafish are, however, able to mount a MyD88-dependent inflammatory response upon LPS stimulation (Bates *et al.,* 2007; Yang *et al.,* 2017), it is likely that a hitherto unidentified TLR could be functionally equivalent to mammalian TLR4. At least 17 TLRs have been identified in zebrafish (Jault *et al.,* 2004; Meijer *et al.,* 2004), suggesting redundant and potentially overlapping functions; we refer readers to another review of TLRs and their known functions in teleost species (Palti, 2011).

Zebrafish neutrophils typically migrate to sites of infection or injury at a faster rate than macrophages (Ellett *et al.,* 2011), thus forming the first line of defense against pathogen insult. The presence of a commensal intestinal microbiota increases the number of circulating neutrophils, enhances neutrophil migratory velocity, and recruitment to extra-intestinal sites of injury (Kanther *et al.,* 2014). Zebrafish neutrophils are functionally largely equivalent to mammalian neutrophils: they are capable of phagocytosis and degranulation, produce cytokines and reactive oxygen species (ROS), and can form neutrophil extracellular traps (NETs) (Lieschke *et al.,* 2001; Harvie and Huttenlocher, 2015; Palie *et al.,* 2007).

The precise role of eosinophils has been difficult to delineate due to a lack of specific markers, but they may play a role in responding to intestinal helminth infections (Balla *et al.,* 2010). Adult zebrafish eosinophils share morphology and differential gene expression with mammalian eosinophils (Lieschke *et al.,* 2001). When challenged with helminth antigens or - infection, eosinophil numbers within the zebrafish intestine increase, indicating conservation of eosinophil-mediated immune responses between zebrafish and mammals (Balla *et al.,* 2010).

Secretion of antimicrobial molecules into the intestinal mucosal layer, both constitutive and in response to TLR activation by PAMPs, is also conserved from mammals to zebrafish. Zebrafish produce antimicrobial peptides (AMPs), of which β-defensins, cathelicidins, hepcidins, and histone-derived peptides are also found in mammals, and piscidins are fish

specific (Zou *et al.,* 2007; Katzenback, 2015). Secreted peptidoglycan recognition proteins (PGRPs) (Chang *et al.,* 2007) and antibacterial lectins functionally similar to mammalian pore forming C-type lectins (Brinchmann *et al.,* 2018) also contribute to zebrafish intestinal mucosal immunity. Secreted antimicrobial factors are expressed throughout the zebrafish intestine, both in larvae and adults (Oehlers *et al.,* 2011a).

3.2. Adaptive immunity and the GI tract

The zebrafish adaptive immune system reaches morphological and functional maturity by 4–6 weeks post-fertilization (Willett *et al.,* 1999; Lam *et al.,* 2004) and consists of B- and T-cells capable of antigen receptor rearrangement in response to pathogens via recombination activating genes 1 and 2 *(ragl* and *rag2)* (Trede and Zon, 1998; Langenau and Zon, 2005). Other initiators of the adaptive immune response, like major histocompatibility complex class I and II molecules, are also present in zebrafish (Sültmann *et al.,* 1993; Sültmann *et al.,* 1994; Takeuchi *et al.,* 1995; Rauta *et al.,* 2012).

The zebrafish lymphatic system shares many morphological, molecular, and functional features with those of mammals, but lacks some lymphoid tissues, like lymph nodes (Jung *et al.,* 2017) and Peyer's patches (Boehm *et al.,* 2003; Brugman, 2016). However, there is evidence that the enterocytes of the larval and adult zebrafish mid-intestine are highly endocytic (Oehlers *et al.,* 2011a) and perform a specialized function in luminal antigen sampling. Consistent with that, a labeled antigen derived from a *Yersinia ruckeri* immersion-vaccine is initially detected in the mid-intestine enterocytes of zebrafish larvae, followed by the spleen (Korbut *et al.,* 2016). In adult zebrafish orally infected with *Mycobacterium marinum,* bacteria are taken up into vacuoles by antigen-sampling cells, which make up the majority of the epithelium of the posterior mid-intestine. Bacteria are trafficked to the basal side of the intestine, where they subsequently co-localize with leukocytes, before eventually travelling to the liver and spleen (Løvmo *et al.,* 2017).

Teleost B-cells exhibit additional phagocytic and microbicidal functions not typically seen in higher vertebrates (Li *et al.,* 2006; Øverland *et al.,* 2010), and can also function as initiating antigen-presenting cells (APCs), linking the innate and adaptive immune systems in zebrafish (Zhu *et al.,* 2014; Lewis *et al.,* 2014). In teleosts, intestinal mucosal immunity is largely dependent on B-cells acting as the primary responders to perturbation, but our understanding of their origins and full spectrum of functions in maintaining gut homeostasis is still limited (see Parra *et al.,* 2016 for review).

4. Zebrafish infection models of gastrointestinal pathogens

4.1. Common routes of infection

Several approaches are currently in use to establish infections with GI pathogens in the zebrafish host, each with distinct advantages and disadvantages to consider. One of the most commonly used routes of infection is by immersion of larvae or adults in a suspension containing a defined pathogen concentration (for detailed protocols see e.g. Varas *et al.,* 2019 for larvae and Mitchell *et al.,* 2017 for adults). The former also offers a side-by-side comparison of infection resulting from immersion vs. caudal vein injection. Immersion

infection can be used for larvae beginning when the mouth first opens at around 3 dpf (Ng *et al.,* 2005). It is advantageous due to its extremely high throughput and does not require much specialized equipment. Its disadvantage is that the exact dose of pathogen taken up by the fish, although it can be measured, can only be indirectly controlled by varying the concentration of pathogen in the suspension. Immersion cannot be applied to strictly anaerobic microbes. For some GI pathogens, in particular those for which zebrafish are not a natural host, immersion does not lead to robust intestinal colonization (e.g., Tysnes *et al.,* 2012; Stones *et al.,* 2017 etc.). More recently, the protozoan *Paramecium caudatum,* a natural prey for larval zebrafish, has been adapted as a vehicle for food-borne infection models (Stones *et al.,* 2017; Flores *et al.,* 2019; Fan *et al.,* 2019). The protozoan internalizes bacteria into storage vacuoles and following uptake of *Paramecia* by the zebrafish and degradation of the protozoan in the foregut, the bacterial load is released into the middle intestine. This approach, although more laborious than simple immersion, still allows for high throughput and mimics human exposure to GI pathogens by consumption of contaminated food. Another advantage is that *P. caudatum* passages the pathogen through acidifying storage vacuoles prior to its release into the zebrafish gut, and the low pH may prime the pathogen similarly to the low pH environment of the human stomach, as discussed in section 2. A third, more labor intensive route of infection is oral gavage of adults or microgavage of larval zebrafish (see Cocchiaro and Rawls, 2013 for a detailed protocol). A defined amount of pathogen suspension is directly delivered into the foregut via a fine capillary that is inserted into the mouth and esophagus of the animal. This technique comes with several costs: it is time consuming, requires specialized equipment, and a skilled experimenter is necessary to prevent injury or death of the infected animal. Sufficient sample sizes are necessary to compensate for attrition or failure of the inoculum to reach the intestine due to regurgitation (Runft *et al.,* 2014). Lastly, some studies inject GI pathogens into the caudal vein or peritoneum of zebrafish, which introduces the pathogen into the blood stream. Despite the difference in administration, these studies have contributed valuable insights into microbial factors modulating inflammation and tissue damage, which often drive mortality as a result of infection (Dong *et al.,* 2013; Okuda *et al.,* 2014).

Below, we discuss GI pathogens studied in zebrafish to date, along with relevant outcomes from those studies. The developmental stage and route of infection used in each case are listed in Table 1.

4.2. Edwardsiella *and* Aeromonas *infections in zebrafish*

Major aquaculture and opportunistic human pathogens include members of the *Edwardsiella* and *Aeromonas* genera (Lee and Wendy, 2017). The consumption of food contaminated with these bacteria can cause gastroenteritis in healthy persons, and more severe diarrheal disease in elderly and immunocompromised individuals (Clarridge *et al.,* 1980; Gracey *et al.,* 1982). *Edwardsiella* species known to infect fish include *E. ictaluri, E. hoshinae, E. piscicida,* and *E. tarda,* but only the latter is known to affect humans (Jordan and Hadley, 1969; Hawke *et al.,* 1981; Nomura and Aoki, 1985). Like other fish pathogens, the *Edwardsiella* and *Aeromonas* species enter their host through the skin and/or gills (Ventura and Grizzle, 1987; Menanteau-Ledouble *et al.,* 2011). Infection of zebrafish with *E. tarda* (Pressley *et al.,* 2005) or *E. ictaluri* (Hawke *et al.,* 2013) induces the pro-inflammatory cytokines IL-β and TNF-α,

leads to the development of hemorrhagic septicemia and increases mortality. Furthermore, studies using zebrafish infection models have elucidated *E. tarda* virulence factors such as (1) the type III secretion system essential for invasion and intracellular replication in phagocytic cells (Okuda *et al.,* 2014), (2) an invasin protein important for haemolytic activity and biofilm formation (Dong *et al.,* 2013), and (3) flagellar components involved in motility, biofilm formation, and adhesion (Xu *et al.,* 2014). As with *E. tarda,* a type III secretion system dependent increase in mortality is seen with *E. piscicida,* although a type VI secretion system was identified as an additional virulence factor in the latter (Hu *et al.,* 2019). The same model was used to show that the NOD-like receptor mediated immune response is required for upregulation of antibacterial genes, including beta-defensins and major histocompatibility complex (MHC) related genes, in response to *E. piscicida* infection (Wu *et al.,* 2019). Failure to induce the pathway leads to increased bacterial burden and mortality in response to infection. Several studies have used zebrafish to investigate the efficacy and mechanism of action of potential novel therapeutics to combat *Edwardsiella* infections, including vaccines (Guo *et al.,* 2015) and immunomodulatory nanoparticles that fortify resistance against pathogens (Udayangani *et al.,* 2017).

Members of the genus *Aeromonas* that affect humans include *A. hydrophila, A. caviae, A. veronii,* and *A. dhakensis,* while *A. salmonicida* is fish-specific (Clarridge *et al.,* 1980; Sacho *et al.,* 1990; Joseph and Carnahan, 1994). Immersing larval or injured adult zebrafish in *A. hydrophila* upregulates the production of IL-ip and TNF-a in response to colonization, and increases neutrophil recruitment to the wound site (Saraceni *et al.,* 2016). *Aeromonas* sp. isolated from the zebrafish gut exhibit intrinsic antibiotic resistance and harbor extracellular enzymes such as lipase, hemolysin, proteases, and DNase with the potential to degrade host cells, indicative of their potential to cause disease in the zebrafish host (Hossain *et al.,* 2018). Gut colonization with *A. hydrophila* alters the adult zebrafish intestinal microbiota composition, concomitantly increasing pathogen abundance and decreasing beneficial intestinal bacteria (Yang *et al.,* 2017). Although both *A. hydrophila* and *A. veronii* are able to colonize the intestine and cause mortality (Saraceni *et al.,* 2016; Ran *et al.,* 2018), *A. veronii* expresses more aerolysin toxin, which causes intestinal lesions and invasion of the intestinal barrier, and is associated with increased virulence compared to *A. hydrophila* (Ran *et al.,* 2018).

4.3. Vibrio *infections in zebrafish*

Members of the *Vibrio* genus are natural inhabitants of warm coastal waters. The consumption of raw or contaminated seafood often leads to vibriosis in humans, a disease characterized by diarrhea, nausea, and/or abdominal cramps (Johnston *et al.,* 1986). Though gastroenteritis is most closely associated with *V. cholerae, V. parahaemolyticus* and *V. vulnificus* can also cause intestinal disease, serious wound and soft tissue infections, as well as bacteremia (Johnston *et al.,* 1986; Lee *et al.,* 2003; Tsai *et al.,* 2009). Various groups have attempted to model *Vibrio*-induced diseases in both mammalian and non-mammalian systems, however the animals used were not natural hosts and required tedious manipulation (Rowe *et al.,* 2014). *Vibrios* naturally colonize zebrafish, and some strains are natural fish pathogens, so using zebrafish as a host to model disease may offer valuable insights into *Vibrio* pathogenesis and aid the identification of therapies to combat infection (Runft *et al.,*

2014). The *V. cholerae* serogroup O1 biotype El Tor responsible for cholera efficiently colonizes the zebrafish intestine and forms microcolonies in close contact with the intestinal epithelial surface similar to those seen in human specimen (Runft *et al.,* 2014). The pathogen is shed into the water and can be transmitted to naïve fish (Runft *et al.,* 2014). Adults immersed in *V. cholerae* develop cholera toxin-independent diarrhea characterized by increased mucin production and excretion (Mitchell *et al.,* 2017). Although cholera toxin is dispensable for colonization and pathogenesis in zebrafish, accessory toxins including RTX and HlyA are induced during intestinal colonization, and the metabolic regulator CRP is required for their activation inside the zebrafish host (Manneh-Roussel *et al.,* 2018). *V. cholerae* uses a host-directed type VI secretion system to enhance intestinal contractions in the zebrafish gut and to expel resident intestinal microbiota to allow for pathogen colonization (Logan *et al.,* 2018). Treating zebrafish infected with *V. vulnificus* with the AMP epinecidin-1 increases host survival, possibly through modulation of immunomodulatory genes (Pan *et al.,* 2011). Inoculating zebrafish with *V. parahaemolyticus* via intraperitoneal injection causes abdominal hemorrhaging, swelling, and secretion of two major markers of host sepsis, TNF-α and IL-1β (Zhang *et al.,* 2016).

4.4. Salmonella *infections in zebrafish*

More recently, the use of zebrafish has been expanded to model infection of non-fish pathogens in the GI tract. *Salmonella enterica* serovar Typhimurium is a major causative agent of human foodborne gastroenteritis (Hoelzer *et al.,* 2011). Immersing zebrafish larvae in S. Typhimurium leads to gut colonization and inflammation of the intestine and cloaca (Howlader *et al.,* 2016; Varas *et al.,* 2017). While the precise molecular mechanisms responsible for inflammation are not fully understood, expression of *Salmonella* virulence plasmid (*spv*) genes in adult zebrafish suppresses protective host responses such as expression of type II IFN-γ, IL-12, and TNF-α, and promotes expression of cytokines known to facilitate intracellular pathogen survival such as IL-4, IL-10, and IL-13 (Wu *et al.,* 2017). Furthermore, *in vivo* clearance of S. Typhimurium is mediated by inflammasome activation in neutrophils (Tyrkalska *et al.,* 2016). Mechanistically, infected zebrafish release CXC chemokine 18 and leukotriene B4 that trigger both the recruitment of neutrophils to the infected site and phagocytosis of *S.* Typhimurium. Once engulfed, *S.* Typhimurium activates the Gbp4 inflammasome that modulates the activity of cytosolic phospholipase A2 and production of prostaglandins, ultimately leading to bacterial clearance (Tyrkalska *et al.,* 2016). Strains of S. Typhimurium with non-optimal translational fidelity recruit fewer neutrophils following colonization via the food-borne route, and are outcompeted by a wild-type strain *in vivo* (Fan *et al.,* 2019).

4.5. E. coli *infections in zebrafish*

Another human enteric pathogen successfully modeled in zebrafish is enterohemorrhagic *Escherichia coli* (EHEC), which colonizes the intestine and causes bloody diarrhea in the human host. Following foodborne delivery via *P. caudatum,* EHEC colonizes the middle intestine of zebrafish larvae despite the presence of endogenous microbiota, and by 4 days post-infection survival rates decrease by ~40% (Stones *et al.,* 2017). In humans and cattle, intestinal colonization by EHEC and the formation of attaching and effacing lesions on enterocytes is mediated by a set of virulence genes encoded by the locus of enterocyte

effacement (LEE), (Phillips *et al.,* 2000; Elliott *et al.,* 2000). Following foodborne infection of larval zebrafish, EHEC induce the LEE during early colonization of the GI tract, and LEE induction is required for optimal colonization and pathogenesis (Stones *et al.,* 2017). The ability to colonize the zebrafish intestine, however, is not limited to pathogenic *E. coli* strains; Commensal *E. coli* isolates from the gut of healthy human volunteers also colonize zebrafish, and can reduce colonization by *V. cholerae* by decreasing the intestinal pH as a result of glucose metabolism (Nag *et al.,* 2018).

4.6. Non-bacterial GI infections in zebrafish

Gastrointestinal microbes studied in zebrafish are not limited to bacteria; Eukaryotic microorganisms, such as the protozoan *Giardia duodenalis* can cause intestinal damage in humans and a zebrafish model of inoculation with cysts has been established (Tysnes *et al.,* 2012). Excystation and infection of the zebrafish intestine by trophozoites, however, remains to be shown. Altan *et al.* recently described a naturally occurring picornavirus in zebrafish (Altan *et al.,* 2019). Picornavirus-1 ZfPV-1 selectively infects a subset of enterocytes and cells in the lamina propria, providing a natural model to study virus-GI tract interactions in a vertebrate host. Human norovirus persists in the larval zebrafish intestine and hematopoietic cells following yolk injection, replicates, and can be transmitted to naïve hosts (Van Dycke *et al.,* 2019).

Zebrafish were initially used to study natural fish pathogens with the intention to limit the impact of infection on aquaculture, but the studies discussed above highlight a rapidly expanding movement to use zebrafish to model zoonotic and human bacterial infections, as well as protozoan and viral infections.

5. Zebrafish as a model to study the intestinal microbiome

The gut microbiome is the collective population of microorganisms that reside in the host GI tract. Many members of the microbiome have beneficial roles, such as regulating digestion, nutrient absorption, and immune system maturation (Umesaki *et al.,* 1999; Semova *et al.,* 2012; Thaiss *et al.,* 2016). Perturbation of the gut microbiome has been linked to a wide range of disease states including neurodevelopmental disorders, obesity, and inflammatory bowel diseases (IBD) (Turnbaugh *et al.,* 2008; Frank *et al.,* 2011; Luna *et al.,* 2017; Felice and O'Mahony, 2017). The application of metagenomics has enabled the differentiation of microbes preferentially residing in healthy versus diseased hosts, but it is presently unclear if there is a conserved 'core' microbiome, or how host genetics, the microbiome, and environmental factors interact to determine physiological and pathophysiological states of the host.

Zebrafish are a powerful model to address these unknowns and begin to unravel complex host-microbiota interactions in a more controlled, simple model system compared to other vertebrates used in microbiome studies. For example, derivation of germ-free (GF) larvae is simpler, more expedient, and more cost-effective than using murine GF models (Pham *et al.,* 2008). Studies using conventionally raised (i.e., born and raised in the presence of their normal microbiota), GF, and conventionalized (i.e., derived GF and then colonized with microbiota) larvae are beginning to elucidate the mechanisms governing host-microbiota

homeostasis as well as the effects of the microbiota on host organ development and differentiation. Comparative studies of conventional and GF zebrafish larvae demonstrate that the microbiota influences host gene expression, particularly genes related to intestinal epithelial proliferation, nutrient metabolism, and the innate immune response (Rawls *et al.,* 2004; Reikvam *et al.,* 2011). Other studies support the hypothesis that host factors have a dominant effect on determining the intestinal microbiome composition (Rawls *et al.,* 2006; Roeselers *et al.,* 2011). For example, transplantation of intestinal microbiota from donor mice into GF zebrafish larvae reveals that, while the resulting GI microbiota in zebrafish resembles that of the donor mice, the number of microbial lineages present in the recipient community changes drastically. This suggests host-mediated selective pressures restrict the microbiome composition (Rawls *et al.,* 2006). This observation is further supported by microbiome profiling of recently caught and domesticated zebrafish reared in multiple research facilities; there exists a 'core' microbiome that is shared among zebrafish lineages regardless of environmental exposure (Roeselers *et al.,* 2011). *In vivo* analyses indicate the intestinal microbiome is required for intestinal epithelium development and differentiation in zebrafish (Bates *et al.,* 2006). Several developmental deficiencies have been observed in GF larvae, including the absence of brush border intestinal alkaline phosphatase activity, immature glycan expression, a decrease in secretory cell numbers, and altered gut motility. These effects can be reversed with the re-introduction of microbiota from conventional donors (Bates *et al.,* 2006). For an in-depth review of the gut microbiome and its analysis in zebrafish and other teleosts, see Tarnecki *et al.,* 2017.

The mammalian and zebrafish gut microbiome share six bacterial divisions, but the human GI tract is primarily dominated by Firmicutes, Bacteroidetes, and Actinobacteria while Proteobacteria are the predominant phylum in zebrafish (Rawls *et al.,* 2004; Eckburg *et al.,* 2005; Bates *et al.,* 2006). Several studies demonstrate the GI tract of zebrafish can be colonized with aerobic and anaerobic species derived from human fecal communities (Toh *et al.,* 2013; Arias- Jayo *et al.,* 2018; Valenzuela *et al.,* 2018). The successful colonization of the larval gut with anaerobic species, including *Lactobacillus paracasei* and strict anaerobes such as *Eubacterium limosum* and *Akkermansia muciniphila,* in particular, was an important stepping stone in establishing zebrafish as a surrogate host for studies on the human gut microbiome (Toh *et al.,* 2013; Arias-Jayo *et al.,* 2018).

The emerging consumer interest in probiotics as a supplement to promote health and well-being has further propelled microbiome studies in zebrafish. The lactic acid bacteria *Lactobacillus plantarum* is of particular interest and is currently commercialized as a beneficial probiotic that alleviates stress and anxiety in humans (Chong *et al.,* 2019). In zebrafish, *L. plantarum* protects against stress-induced dysbiosis of the gut microbiota and ameliorates anxiety-related behavior. These changes in microbiome composition and behavior correlate with an increase in expression of gamma-aminobutyric acid receptors, and of serotonin transporters in zebrafish brains following administration of *L. plantarum* (Davis *et al.,* 2016). Colonization of adult zebrafish with the probiotic *Lactobacillus rhamnosus* decreases the expression of host genes associated with dietary lipid metabolism (Falcinelli *et al.,* 2017). Consequently, shifts in microbiome community structure resulting from high dietary fat intake and associated weight gain are ameliorated by supplementation

with *L. rhamnosus,* highlighting its potential to attenuate diet-related metabolic disorder (Falcinelli *et al.,* 2017).

6. Zebrafish models of inflammatory bowel diseases

While the etiologies of multifactorial IBDs like ulcerative colitis and Crohn's disease are unknown, two key features of the pathology are intestinal epithelial damage and intestinal dysbiosis. The intestinal epithelium provides a physical barrier between the intestinal lumen and surrounding tissues, and works in concert with immune cells to sense and respond to both normal microbiota and opportunistic infection. Therefore, factors such as host genetics and environmental conditions that impair intestinal epithelial homeostasis and disrupt the interplay with resident microbiota can significantly impact IBDs (Bellaguarda and Chang, 2015). Novel murine models of intestinal epithelial damage and inflammation have advanced our understanding of IBDs, but several limitations associated with murine models such as cost, imaging capacity, and genetic manipulation still constrict elucidation of how the aforementioned disease modifiers converge and affect pathogenesis *in vivo* (Kiesler *et al.,* 2015).

The zebrafish model circumvents murine-associated limitations and provides the opportunity to rapidly explore factors associated with increased inflammation and dysbiosis in response to intestinal epithelial damage and is thus relevant to IBDs in humans. Zebrafish models of chemically-induced IBD mimic some key aspects of the pathological condition in humans, including enterocolitis, intestinal epithelial damage, disruption of intestinal architecture, and shifts in microbiome composition (He *et al.,* 2013). For example, intra-rectal administration of oxazolone in adult zebrafish causes disruption of the intestinal fold architecture, depletes goblet cells, increases immune cell infiltration of the gut, and upregulates pro-inflammatory cytokines (Brugman *et al.,* 2009). Likewise, immersion of larval and adult zebrafish in 2,4,6-trinitrobenzenesulfonic acid (TNBS) induces intestinal inflammation, inhibits peristalsis, and disrupts epithelial integrity by impairing the tight junctions between cells. This suggests TNBS disrupts intestinal barrier function and promotes features observed in IBD patients (Fleming *et al.,* 2010; Oehlers *et al.,* 2011b). It has been proposed that increased intestinal epithelial cell shedding after damage compromises barrier integrity, which in turn fuels more inflammation (Blander, 2016). In support of this, leukocytes mobilize from the caudal hematopoietic tissue to the periphery and accumulate around the damaged intestine following TNBS insult in zebrafish (Oehlers *et al.,* 2011b). Disruption of the intestinal barrier by TNBS promotes microbial infiltration of the lamina propria, initiating a cascading pro-inflammatory response characterized by secretion of TNF-α, via activation of TLR3, MyD88, TIR-domain-containing adapter-inducing interferon-β (TRIF), and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) (He *et al.,* 2013). Together, these data support the idea that zebrafish provide a high-throughput model to interrogate genetic pathways and drug candidates that can restore epithelial barrier function and modulate immune cell function to protect against microbial infiltration during IBD.

7. Perspective

Zebrafish have been a well-used model for infection biology for many years. More recent research highlighting similarities between the zebrafish gut and the GI tract of mammals has expanded to include studies on GI tract-pathogen interactions. The zebrafish as a model organism particularly lends itself to questions that cannot be addressed easily in other model systems thanks in part to the ease of intravital imaging and raw statistical power. Its use in this context may help unravel new details of the cellular microbiology underpinning infection, lead to the identification of novel virulence factors and aspects of their regulation within the GI niche, and aid the discovery of effective therapies by enabling high-throughput drug screening in the live host. Future studies that use zebrafish to study host-microbiome interactions will continue to identify host-intrinsic and - extrinsic factors and selective pressures critical for establishing and shaping the host microbiome. Such discoveries may aid in the development of novel therapies for combating microbiome- associated diseases.

Acknowledgements

We thank members of the Krachler and Eisenhoffer labs for useful comments on earlier versions of the manuscript. We thank Jordan Pietz (UT MD Anderson Cancer Center) for designing Figure 1. We would like to apologize to everyone whose work we could not include it the current version of the review due to space limitations.

Funding and Conflicts of Interest

This work was supported by the NIH, National Institute of Allergy and Infectious Diseases (Grant R01 AI132354-01A1 to AMK and a Diversity Supplement supporting EF (3R01AI132354-02S1), a John S. Dunn Foundation Grant supporting MAO, a MBID Fellowship supporting ATN, and the Cancer Prevention Institute of Texas, RR14007, National Institutes of General Medical Sciences, R01GM124043, and the Linda and Mark Quick Award for Basic Science to GTE.

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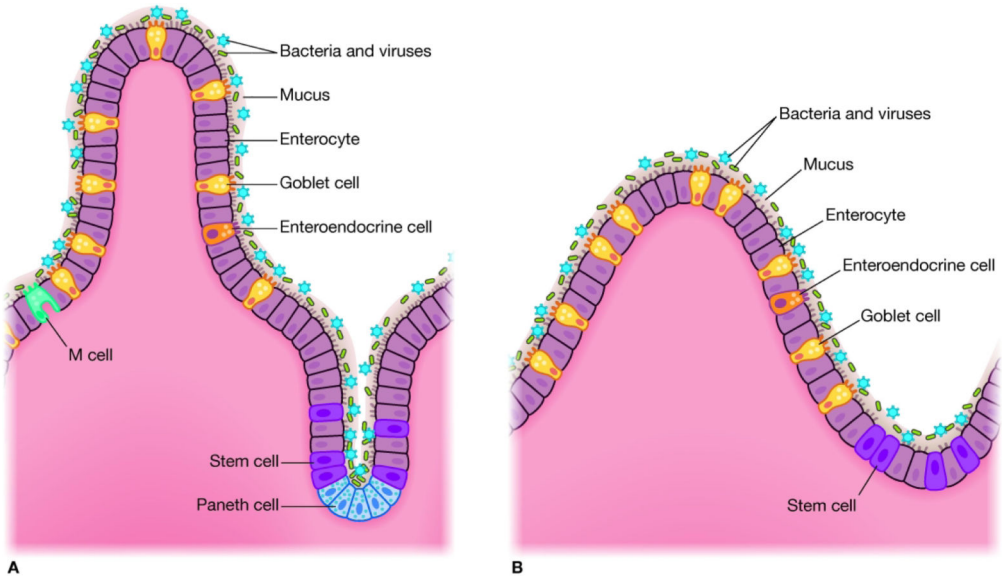

Figure 1.

Comparison of cell types and structures between the mammalian (A) and zebrafish (B) gastrointestinal tract. The zebrafish intestine is organized into irregular folds as opposed to villi, and lacks crypts. Mammalian and zebrafish intestines share stem cells, enterocytes, enteroendocrine cells, and goblet cells. Paneth cells and M-cells are absent in zebrafish.

Table 1.

Summary - Examples of gastrointestinal pathogens and their disease profiles in the zebrafish host.

The table is organized first by pathogen (in alphabetical order), followed by developmental stage, followed by route of infection. Some of the studies summarized below discuss additional infection models which were not included in the table due to space constraints.

Pathogen	Route of infection	Zebrafish developmental stage	Disease phenotype/ finding	Reference
Aeromonas hydrophila	Immersion	Larvae, 4 dpf	Colonization and increased mortality	Saraceni *et al.*, 2016
Aeromonas hydrophila	Immersion	Adults, 4 months	Change in microbiota composition; induction of innate immune response	Yang *et al.*, 2017
Aeromonas hydrophila, Aeromonas veronii	Immersion	Germ-free larvae, 4–5 dpf; Adults	Intestinal colonization; increased mortality; intestinal lesions in adults; Aerolysin-dependent virulence	Ran *et al.*, 2018
Edwardsiella ictaluri	Co-housing	Adults	Skin hemorrhage; abdominal hemorrhage; nephritis; septicaemia; mortality	Hawke *et al.*, 2013
Edwardsilla piscicida	Immersion	Larvae, 5 dpf	Type III secretion system- and type VI secretion system-dependent increase in mortality	Hu *et al.*, 2019
Edwardsilla piscicida	Immersion	Larvae, 4 dpf	Histone H2A and RIP2 are required for induction of antibacterial genes and decrease bacterial burden and mortality in response to infection	Wu *et al.*, 2019
Edwardsiella tarda	Immersion	Larvae, 1 dpf	Increased mortality; systemic infection	Pressley *et al.*, 2005
Edwardsiella tarda	Immersion	Larvae, 1 dpf	Flagella-dependent increase in mortality	Xu *et al.*, 2014
Edwardsiella tarda	Immersion	Healthy adults; Wounded adults (skin abrasion)	(Healthy) Colonization; no mortality (Wounded) Increased mortality; septicaemia; colonization of intestinal smooth muscle tissue	Pressley *et al.*, 2005
Edwardsiella tarda	Injection, i.p.	Adults	Type III secretion system-dependent increase in mortality	Okuda *et al.*, 2014
Edwardsiella tarda	Injection, i.p.	Adults	Invasin-dependent increase in mortality (LD_{50})	Dong *et al.*, 2013
Escherichia coli (EHEC)	Food-borne	Larvae, 4 dpf	Intestinal colonization; neutrophil recruitment; type III secretion system induction; increased mortality; transmission to naïve fish	Stones *et al.*, 2017
Escherichia coli (Nissle, strain 40)	Immersion	Adults	Intestinal colonization; metabolize glucose and decrease intestinal pH; decrease burden of *V. cholerae* during co-infection	Nag *et al.*, 2018
Giardia duodenalis	Oral gavage	Adults	Intestinal deposition and retention of cysts, excretion of cysts; no detection of trophozoites; no intestinal damage	Tysnes *et al.*, 2012
Human norovirus (HuNoV GI and GII)	Injection, yolk sac	Larvae, 3 dpf	Viral replication; viral persistence in the intestine and hematopoietic cells; transmission to naïve fish; antiviral treatment	Van Dycke *et al.*, 2019
Picornavirus-1	Naturally occuring	Adults	Intestinal colonization; viral shedding	Altan *et al.*, 2019
Salmonella enterica serovar Typhimurium	Injection, otic vesicle; Injection, yolk	Larvae, 2 dpf	Increased SPI-1- and SPI-2-dependent mortality; caspase-1 activation (WT); Gbp4 inflammasome mediated response to infection	Tyrkalska *et al.*, 2016
Salmonella enterica serovar Typhimurium	Food-borne	Larvae, 5 dpf	Colonization of intestine; neutrophil recruitment; translational fidelity-dependent bacterial fitness *in vivo*	Fan *et al.*, 2019

Pathogen	Route of infection	Zebrafish developmental stage	Disease phenotype/ finding	Reference
Salmonella enterica serovar Typhimurium	Immersion	Larvae, 6 dpf	Colonization of intestine and cloaca; inflammation and swelling of the cloaca; neutrophil recruitment to site of infection	Varas *et al.*, 2017
Salmonella enterica serovars Typhimurium, Enteritidis, Weltevreden	Immersion	Adults	Colonization and replication; shedding and transmission to naïve fish; intestinal inflammation and destruction of intestinal epithelium; diarrhea; increased mortality; protective immunity via bath vaccination with heat-killed bacteria	Howlader *et al.*, 2016
Salmonella enterica serovar Typhimurium	Oral gavage	Adults, 8 months	Colonization of intestine; intestinal inflammation and ulceration; macrophage and lymphocyte infiltration; tissue damage; Increased mortality; *spv*-dependent pathology	Wu *et al.*, 2017
Vibrio cholerae	Immersion	Larvae, 4 dpf	CRP- and TcpA-dependent intestinal colonization and mortality; *rtx, hlyA* induction during colonization	Manneh-Roussel *et al.*, 2018
Vibrio cholerae	Immersion	Larvae, 5 dpf	Colonization of the intestine	Runft *et al.*, 2014
Vibrio cholerae	Immersion	Germ-free larvae, 5 dpf	Intestinal colonization; decrease in commensal *Aeromonas*; type VI secretion system dependent increase in gut peristalsis	Logan *et al.*, 2018
Vibrio cholerae	Oral gavage; Immersion	Adults, 6–9 months	Colonization of intestine; microcolony formation; transmission to naïve fish	Runft *et al.*, 2014
Vibrio cholerae	Immersion	Adults	Intestinal colonization; diarrhea; increased mucin production and excretion	Mitchell *et al.*, 2017
Vibrio parahaemolyticus	Injection, i.p.	Adults, 7–8 months	Increased mortality; necrosis of kidneys, liver and intestinal villi; intestinal bleeding	Zhang *et al.*, 2016
Vibrio vulnificus	Injection, i.p.	Adults	Increased mortality; inflammatory response; protection by AMP epinecidin-1	Pan *et al.*, 2011