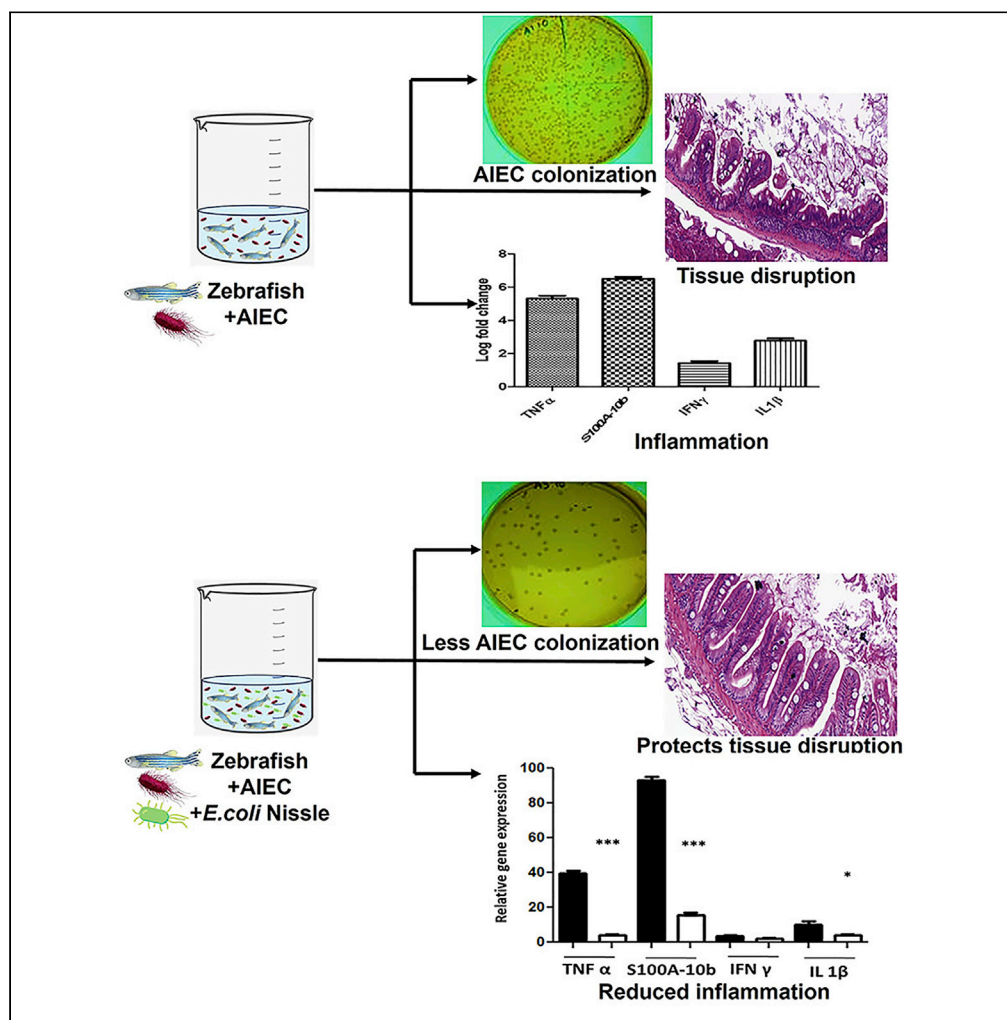


Article

# An adult zebrafish model for adherent-invasive *Escherichia coli* indicates protection from AIEC infection by probiotic *E. coli* Nissle



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**Highlights**

AIEC can colonize, invade, and induce inflammation in the zebrafish gut

Probiotic *E. coli* Nissle can protect zebrafish from AIEC infection

EcN is effective both prophylactically and therapeutically against AIEC-induced IBD



## Article

# An adult zebrafish model for adherent-invasive *Escherichia coli* indicates protection from AIEC infection by probiotic *E. coli* Nissle

Dhrubajyoti Nag,<sup>1</sup> Dustin Farr,<sup>1</sup> Saumya Raychaudhuri,<sup>2</sup> and Jeffrey H. Withey<sup>1,3,\*</sup>**SUMMARY**

**Adherent-invasive *Escherichia coli* (AIEC) is an opportunistic pathogen associated with major inflammatory bowel disease, Crohn disease, and ulcerative colitis. Unfavorable conditions push commensal AIEC to induce gut inflammation, sometimes progressing to inflammation-induced colon cancer. Recently, zebrafish have emerged as a useful model to study human intestinal pathogens. Here, a zebrafish model to study AIEC infection was developed. Bath inoculation with AIEC resulted in colonization and tissue disruption in the zebrafish intestine. Gene expression of pro-inflammatory markers including interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor alpha (TNF $\alpha$ ), interferon- $\gamma$  (IFN $\gamma$ ), and S100A-10b (akin to human calprotectin) in the zebrafish intestine was significantly induced by AIEC infection. The probiotic *E. coli* Nissle 1917 (EcN) was tested as a therapeutic and prophylactic against AIEC infection and reduced AIEC colonization, tissue damage, and pro-inflammatory responses in zebrafish. Furthermore, EcN diminished the propionic-acid-augmented hyperinfection of AIEC in zebrafish. Thus, this study shows the efficacy of EcN against AIEC in an AIEC-zebrafish model.**

**INTRODUCTION**

In the United States, about 1.5 million people suffer from inflammatory bowel disease (IBD), causing considerable distress, mortality, and economic loss every year (Ng et al., 2017; Kappelman et al., 2007). Before World War II, IBD was a rare disease in North America and most European countries (Molodecky et al., 2012). Crohn's disease (CD) and ulcerative colitis (UC) are both forms of IBD but differ in pathophysiology, affect different parts of the gastrointestinal (GI) tract, and have differing symptoms, complications, disease course, and management. The cause of CD is still unclear, but genetic, immunological, and environmental factors contribute to risk of disease onset and progression (Torres et al., 2017). Industrialized countries in Northern Europe and North America experienced a steady rise in the incidence of CD during the second half of the 20<sup>th</sup> century (Molodecky et al., 2012; Torres et al., 2017; Cosnes et al., 2011). Variation in the incidence and prevalence of CD can be found based on geographic region, environment, immigrant population, and ethnic groups. Although UC was more prevalent in the past, CD has shown increasing incidence in the past few decades, and both UC and CD may be equally prevalent in North America (Malik, 2015). Up to 15% of CD is consistent with a family history of CD, whereas a small proportion of patients report a family history of UC (Ananthkrishnan, 2015; Halme et al., 2006). Many genes are involved in CD pathogenesis, but the most commonly associated genes are NOD2, IL-23R, and ATG16L1 (Vermeire, 2004; Duerr et al., 2006; Brand, 2009). Quality of diet is directly linked with the risk of CD. Long-term dietary fruit fiber intake was associated with lower risk of CD (Ananthkrishnan et al., 2013; Galvez et al., 2005). A high saturated fat diet correlated with increased inflammatory responses in animal models, mediated by Th1 cells in interleukin-10 (IL-10) knockout mice (Devkota et al., 2012), but there was no such association in human studies (Chapman-Kiddell et al., 2010). High levels of n-6 polyunsaturated fatty acids (omega-6 PUFA) or n-3 PUFA (high n-6: n-3 ratio) in human food have been related to increased risk of CD (Martini and Brandes, 1976; Thornton et al., 1979). IBD patients have a decreased variety in gut microbiota compared with healthy individuals, and this phenomenon is more distinct in CD than UC (Gevers et al., 2014; Kostic et al., 2014). Different environmental factors, including family size, exposure to pets and farm animals, breastfeeding, hygiene, stress, and diet can affect the onset of IBD, illustrating its complexity to study (Timm et al., 2014; Castiglione et al., 2012; Ng et al., 2015).

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Adherent-invasive *Escherichia coli* (AIEC) has been described as a human pathobiont and has been implicated in development of CD. Darfeuille-Michaud et al. identified a significantly higher prevalence of AIEC in CD patients as compared with healthy individuals. AIEC can reside and replicate in macrophages after invading the intestinal epithelium (Darfeuille-Michaud et al., 2004). Microbiomes high in abundance of Bacteroides were prevalent in people having a western diet rich in animal protein and saturated fat, whereas Entero-type 2 (Prevotella) can be linked with a diet rich in carbohydrates and fiber (Wu et al., 2011). Therefore, regional differences in diet can influence development of IBD by modifying the gut microbiota. Studies showed that *Faecalibacterium prausnitzii*, a butyrate-producing bacterium from the Firmicutes phylum, confers protection against IBD (Morgan et al., 2012), which raises the possibility of treatment with probiotics.

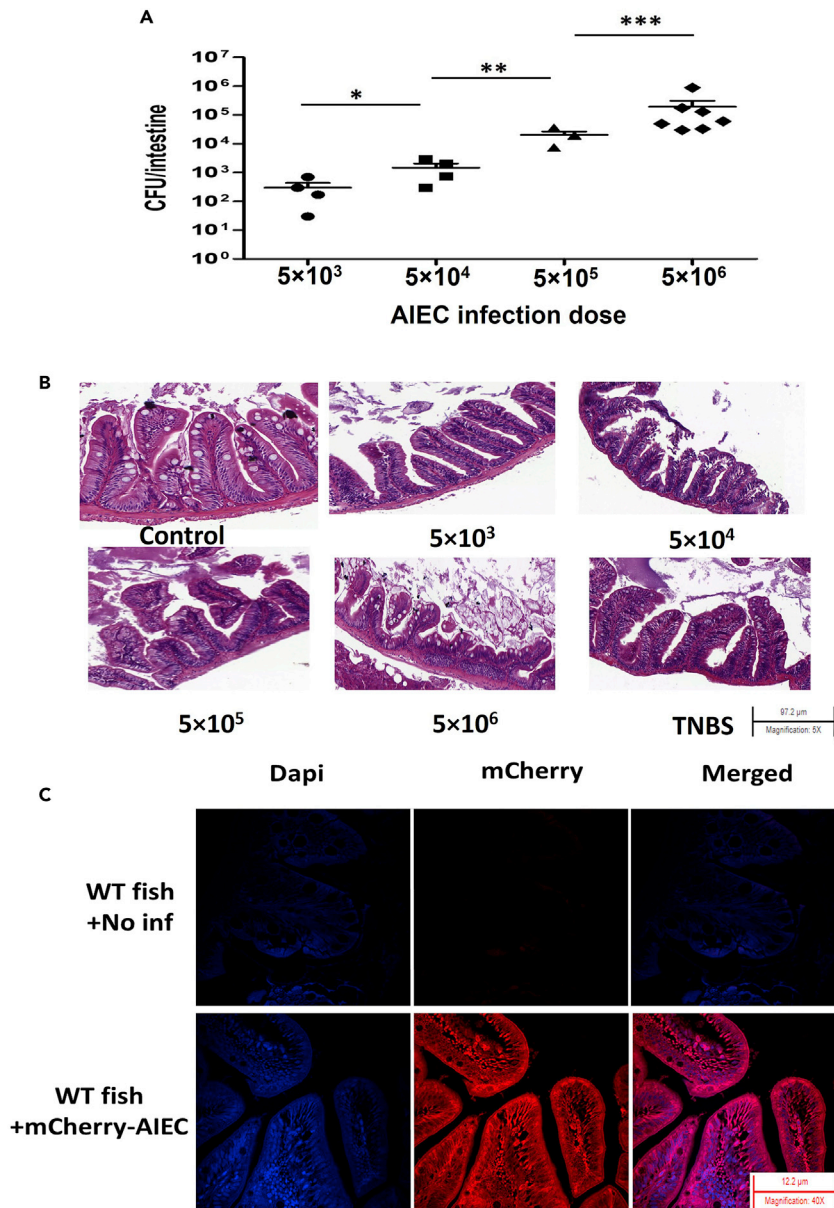
AIEC strains recovered from CD wounds can adhere to and invade cultured intestinal epithelial cells and survive and multiply within macrophages (Darfeuille-Michaud et al., 1998; Glasser et al., 2001). The pathophysiology of AIEC and its role during CD is an emerging topic of research (Elhenawy et al., 2018) but the mechanisms are yet to be revealed. The diversity of virulence factors in different AIEC strains indicates that *E. coli* strains have evolved different strategies to colonize their hosts (Tawfik et al., 2014). There is some evidence that AIEC can reduce epithelial barrier function by displacing and redistributing ZO-1, a protein required for the formation of apical tight junctions (Sasaki et al., 2007; Wine et al., 2009). This decrease in epithelial barrier integrity would result in increased translocation of AIEC across the epithelial barrier and may therefore exacerbate AIEC pathogenesis. Disintegrity of the epithelial barrier could increase the pathogenicity of other invasive enteric pathogens or opportunistic pathogens (Smith et al., 2013). AIEC can cause persisting disease by replicating and residing within macrophages. Previous work performed with murine macrophage cell lines revealed that the prototype AIEC strain, LF82, multiplies in a vacuole presenting the characteristics of a mature phagolysosome (Bringer et al., 2006; Lapaquette et al., 2012). AIEC strains have been shown to express FimH protein variants with recently acquired amino acid substitutions; these mutations confer a significantly higher ability for AIEC to adhere to carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6)-expressing intestinal epithelial cells (Dreux et al., 2013). Moreover, adherence of AIEC to human intestinal epithelial cells is mediated through the type 1 fimbriae interaction with mannosylated CEACAM6. The type 1 fimbriae of AIEC were shown to bind to CEACAM6, which is expressed at higher levels in inflamed intestinal epithelial cells of IBD patients (Mirsepasi-Lauridsen et al., 2016). All the UC-associated *E. coli* strains that caused loss of tight junctions in epithelial cell monolayers were hemolytic. AIEC isolated from UC patients can adhere to epithelial cells and disrupt epithelial tight junctions via an HlyA-dependent mechanism, providing strong evidence that this is an important novel pathogenic mechanism in UC. In addition, wild-type and colitis-susceptible IL-10<sup>-/-</sup> mice colonized with HlyA-expressing AIEC had elevated inflammation and increased epithelial permeability compared with mice colonized with the HlyA-deficient mutant (Mirsepasi-Lauridsen et al., 2016).

Despite the importance of AIEC to human IBD, animal models to study its pathogenesis are very limited (Nag et al., 2020). Instead, models for AIEC have been primarily cell-culture based, such as in murine epithelial cells and macrophages. In recent years, zebrafish have been developed as a very useful model for studying a variety of different enteric diseases (Howlader et al., 2016; Szabady et al., 2009; Kordon et al., 2018; Nag et al., 2018a, 2018b). In this study we establish a new zebrafish model for AIEC. Colonization, inflammation, and release of S100A-10b protein, the closest zebrafish relative to human calprotectin (an IBD marker), are induced by AIEC pathogenesis in the zebrafish model. The probiotic *E. coli* Nissle (EcN) was also tested as a potential therapeutic/prophylactic for IBD and results suggest that EcN reduces AIEC colonization and inflammation in zebrafish.

## RESULTS

### AIEC colonizes and invades the zebrafish gut

Previous studies established zebrafish as a model for *Vibrio cholerae* colonization, using bath exposure (Runft et al., 2014; Nag et al., 2018a, 2018b). Here, similar methods were used to test whether AIEC would colonize the zebrafish intestine. Individual fish were exposed to 5 × 10<sup>6</sup> CFU/mL of AIEC via bath inoculation, followed by enumeration of AIEC in the intestinal tract 24 h postinfection (24 hpi). AIEC LF82 was specifically selected by plating intestinal homogenates on LB agar medium containing 100 μg/mL ampicillin, and the colonies were confirmed by streaking on MacConkey agar plates, followed by colony PCR of *fimH* (Conte et al., 2014). When fish were inoculated by bath for 6 h, followed by 18 h incubation in sterile fresh water, a mean colonization of ~5 × 10<sup>5</sup> CFU per zebrafish intestine was observed 24 hpi (Figure 1A). The



**Figure 1. Colonization of AIEC in zebrafish intestines**

(A) Fish were added to 400 mL water containing  $5 \times 10^3$ ,  $5 \times 10^4$ ,  $5 \times 10^5$ , or  $5 \times 10^6$  CFU/mL of AIEC cells for 6 h and then incubated in fresh, sterile water for 18 h. Data are represented as mean  $\pm$  SEM from multiple experiments. Each dot represents the data from one fish. Total colonization per intestine was calculated after plating serial dilutions of intestinal homogenates 24 h postinfection. Statistical significance indicated above the data was determined by Student's t test. \* $p = 0.0442$ , \*\* $p = 0.0034$ , and \*\*\* $p = 0.0002$ .

(B) Representative H&E staining (5X) of zebrafish intestinal sections. Villus structures in zebrafish having different AIEC doses were compared with uninfected control zebrafish. The H&E figures are representative of three independent experiments.

(C) Fluorescent microscopy of mCherry-AIEC infection of WT zebrafish intestinal epithelium. Fish were exposed to  $5 \times 10^6$  CFU/mL of mCherry-AIEC for 6 h, moved to fresh water for 18 h, then sacrificed, fixed, and prepared for sectioning. Bacteria were visualized (Red) by expressing mCherry on the pPrps plasmid. Blue fluorescence (DAPI) represents intestinal epithelial cell nuclei. Magnification 40X. The fluorescent figures are representative of three independent experiments. An uninfected control fish is shown in the upper panel to verify there was no significant autofluorescence in zebrafish gut (no DAPI staining).

next goal was to study the dose-dependent colonization of AIEC in zebrafish. Fish were inoculated with  $5 \times 10^3$ ,  $5 \times 10^4$ , and  $5 \times 10^5$  CFU/mL of AIEC for 6 h, followed by 18 h incubation in sterile fresh water. Fish intestines showed colonization levels of  $\sim 5 \times 10^2$ ,  $\sim 2 \times 10^3$ , and  $\sim 2 \times 10^4$ , respectively (Figure 1A). As the difference of colonization 24 hpi between inoculation doses of  $\sim 5 \times 10^5$  and  $\sim 5 \times 10^6$  was most significant, all the following colonization experiments were done using  $\sim 5 \times 10^6$  CFU/mL of AIEC.

The AIEC LF82 strain is invasive to human epithelial cells and macrophages, and the main pathogenic feature is invasion of the superficial cells of the intestine (Conte et al., 2014). Therefore, fish intestines underwent histopathology to examine villi denudation 24 hpi with AIEC. Significant villi denudation and epithelial defects/tattering were seen with three different infectious doses of AIEC (Figure 1B), suggesting AIEC can effectively invade the intestinal epithelial layer and disrupt the tissue structure. A standardized scoring system was used by a trained pathologist (Reveal Biosciences) to assess the degree of tissue damage induced by AIEC in the H&E staining of tissue sections. The AIEC-infected fish had significant epithelial defects/tattering and leukocyte infiltrates/inflammation as compared with the uninfected control fish. The chart for the pathological scoring and the representative figure for epithelial defects/tattering and leukocyte infiltrates/inflammation are shown in Figures S1A and S1B. Tissue disruption from TNBS treatment was not significant compared with the control but the leukocyte infiltrates/inflammation had similar scoring to AIEC infection.

Next, the colonization of an mCherry-tagged AIEC strain (AIEC LF82 pPrpsM-mCherry; Spec<sup>f</sup> 100  $\mu$ g/mL) was examined using fluorescence microscopy. Fish were inoculated with  $5 \times 10^6$  CFU/mL of AIEC-mCherry by 6 h bath inoculation, followed by 18 h incubation in sterile fresh water. At 24 hpi,  $\sim 5 \times 10^5$  CFU per zebrafish intestine was observed by plate counts, and intestines were harvested and processed for fluorescent staining to visualize the colonization of the AIEC-mCherry strain (Figure 1C, lower panel). These images show red fluorescent (mCherry) bacteria colonizing along villi projections of the intestinal epithelial cells (nuclei stained blue, DAPI) of the zebrafish. The control fish gut with no infection did not show any red or blue fluorescence (indicating no autofluorescence; Figure 1C, upper panel).

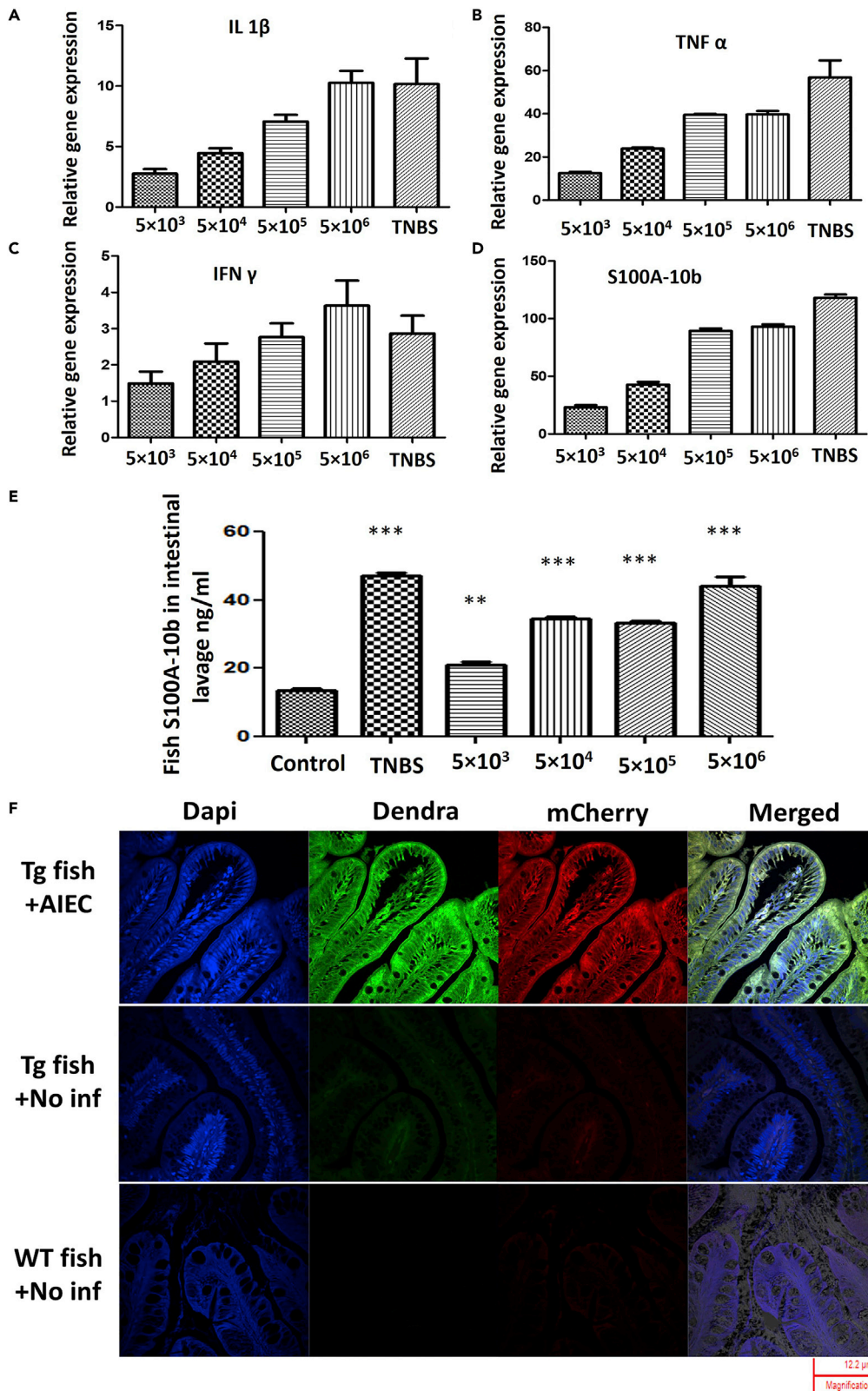
To further verify that the AIEC is invasive, a gentamicin protection assay was done. Zebrafish were infected with  $5 \times 10^6$  CFU/mL of AIEC following the aforementioned protocol. After 24 h, fish intestines were harvested and chopped in small segments. The small intestinal segments were incubated with 100  $\mu$ g/mL of gentamicin at room temperature for 25 min. Then, intestinal segments were washed three times in 1X PBS and subjected to homogenization with glass beads. The homogenate was serially diluted and plated for CFU count. The intestines treated with gentamicin followed by the homogenization showed  $2.5 \times 10^5$  CFU/intestine of zebrafish, which is not significantly different from the colonization assay result (Figure S1C). The supernatant collected before homogenization did not show any bacterial count on the LB plates. These results suggest that after 24 h of infection with AIEC, half of the AIEC invaded the epithelial cells and half were still colonizing the epithelial surface, strongly suggesting that AIEC is invasive in the zebrafish intestine.

### AIEC-induced inflammation in the zebrafish gut

Intestinal inflammation is the main immunogenic response of the host in IBD. AIEC, being an invasive bacterium, can induce an inflammatory response in patients (Strober, 2011). Previous studies showed that AIEC can also induce inflammation in a mouse model (Bretin et al., 2018). Here, the inflammatory responses of zebrafish during AIEC infection were quantified. IL-1, the “gatekeeper” of inflammation, is the apical cytokine in a signaling cascade that drives the early response to injury or infection. Tumor necrosis factor alpha (TNF $\alpha$ ) and interferon- $\gamma$  (IFN $\gamma$ ) are two of the effector cytokines during inflammation (Ogryzko et al., 2014; Xie et al., 2021; Brugman, 2016). Gene expression levels of IL-1 $\beta$ , TNF $\alpha$ , and IFN $\gamma$  from the zebrafish intestinal cells were measured to determine whether AIEC induced intestinal inflammation. TNBS (20  $\mu$ M) was used as a positive control for induction of inflammation. Gene expression of all three inflammatory markers increased with increasing AIEC infectious dose. The fold changes of IL-1 $\beta$  expression were  $\sim 2.5$ , 4.5, 7, and 10 with the infectious doses of  $5 \times 10^3$ ,  $5 \times 10^4$ ,  $5 \times 10^5$ , and  $5 \times 10^6$  CFU/mL AIEC, respectively (Figure 2A). The fold changes of TNF $\alpha$  expression were  $\sim 12.5$ , 24, 39.5, and 40 and of IFN $\gamma$  were  $\sim 1.5$ , 2, 3, and 3.5 with the increasing infectious doses (Figures 2B and 2C). Treatment with TNBS showed increases in IL-1 $\beta$ , TNF $\alpha$ , and IFN $\gamma$  expression by  $\sim 10$ , 57, and 3, respectively (Figures 2A, 2B, and 2C).

Calprotectin, released by neutrophils, is used as a marker for IBD in humans (Bjarnason et al., 2017). Among other versatile activities, calprotectin is an antimicrobial peptide (Ellis et al., 2015) and chelator of metal ions (Farr et al., 2022). All mammalian s100a (calprotectin-related) genes are clustered in the genome,





### Figure 2. Inflammation induced by AIEC in zebrafish

(A–D) WT zebrafish were infected with AIEC at  $5 \times 10^3$ ,  $5 \times 10^4$ ,  $5 \times 10^5$ , or  $5 \times 10^6$  CFU/mL for 6 h, moved to fresh water for 18 h, and then sacrificed. mRNA was isolated from intestinal tissue. Relative gene expression levels of TNF $\alpha$  (A), s100a-10b (B), IFN $\gamma$  (C), and IL-1 $\beta$  (D) were determined by qRT-PCR. Gene expression was normalized against  $\beta$ -actin and expressed as fold change. TNBS, a positive stimulator of inflammatory markers, was used as a positive control. Error bars indicate standard deviation. Data are represented as mean  $\pm$  SEM from three experiments.

(E) S100A-10b (calprotectin) protein levels were determined in intestinal homogenate via ELISA. Error bars indicate standard deviation. TNBS, a positive stimulator of inflammatory markers, was used as a positive control. Data are represented as mean  $\pm$  SEM from three experiments. \*\* $p < 0.005$  and \*\*\* $p < 0.0005$  as compared with control. (F) Fluorescent microscopy of neutrophil response to AIEC infection of transgenic (*mpx:dendra*) zebrafish intestinal epithelium. Fish were exposed to  $5 \times 10^6$  CFU/mL of AIEC for 6 h, moved to fresh water for 18 h, then sacrificed, fixed, and prepared for sectioning. Bacteria were visualized (Red) by expressing mCherry from the pPrps plasmid; blue fluorescence (DAPI) represents intestinal epithelial cell nuclei (bottom row: no DAPI staining and the merged figure contains white background), and green fluorescence (Dendra) represents neutrophils. Magnification 40X. The fluorescent figures are representative of three independent experiments.

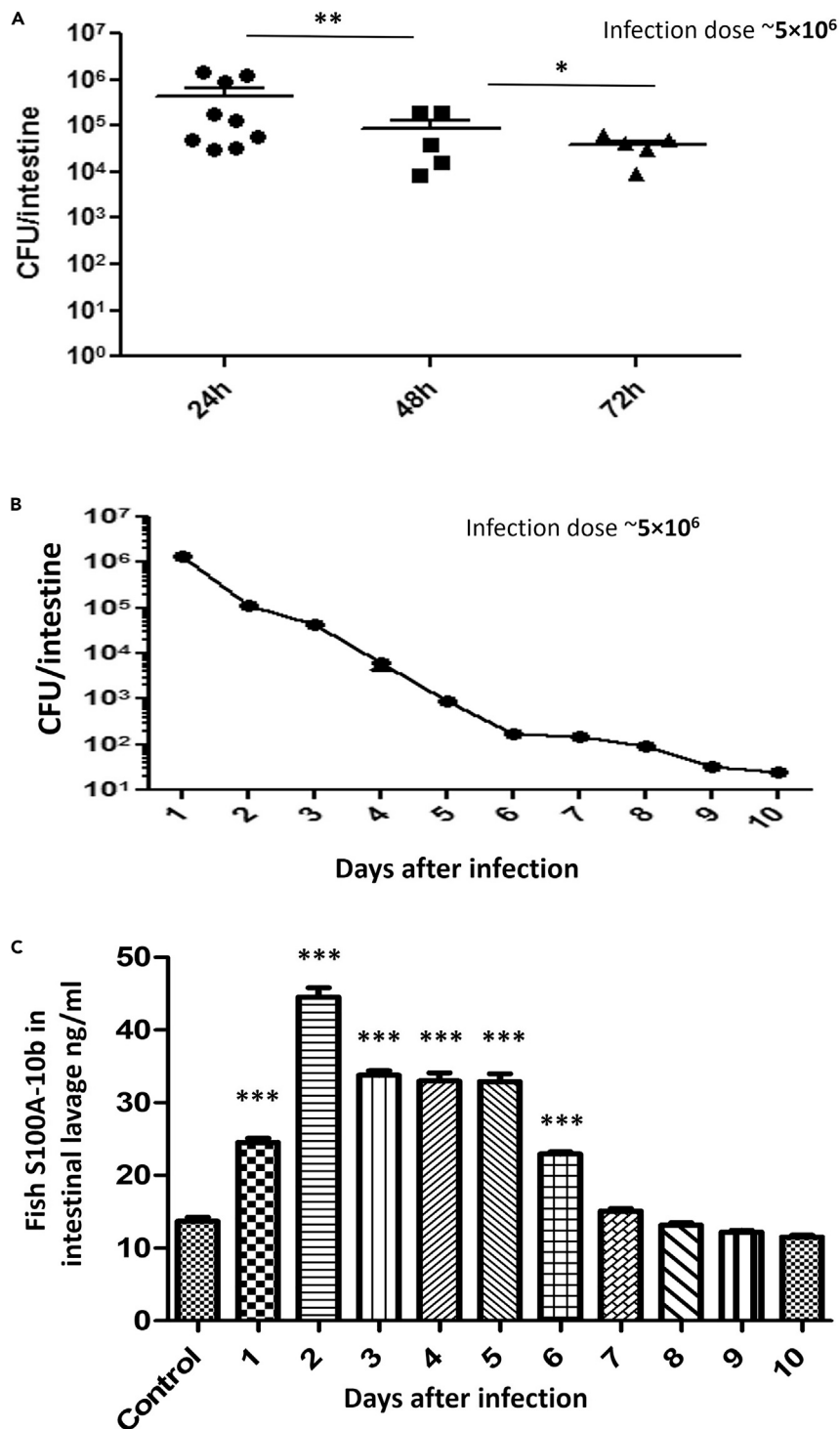
whereas S100B, G, P, and Z are located as singletons on different chromosomes (Kraemer et al., 2008). In zebrafish, the S100 genes are located in two smaller clusters of 3.5 and 1.0 MB each, on chromosomes 16 and 19, respectively, and composed of seven (S100A1, S100A-10b, S100I.1, S100I.2, S100T, S100V1, and S100W) and four (S100A10a, S100A11, S100S, and S100U) genes, respectively (Kraemer et al., 2008). Zebrafish S100A-10b protein, a calcium binding protein, is highly similar (43% amino acid identity, and 63% similarity) to the S100A8 portion of human calprotectin [S100A8/A9]) and is very likely to play the same role. We used qPCR primers for the zebrafish s100a-10b gene to assess transcriptional activity and a human calprotectin ELISA kit to assess protein levels. The ELISA kit was recommended by the manufacturer to quantify zebrafish calprotectin and cross-reacted with zebrafish intestinal homogenates in preliminary studies.

As calprotectin is a well-proven marker for intestinal inflammation, s100a-10b expression levels were measured in zebrafish intestinal tissue by qRT-PCR, and the S100A-10B protein level was measured in intestinal lavage by ELISA. s100a-10b gene expression showed a fold change of  $\sim 23.5$ , 42.5, 89.5, and 93 with the infectious doses of  $5 \times 10^3$ ,  $5 \times 10^4$ ,  $5 \times 10^5$ , and  $5 \times 10^6$  CFU/mL AIEC, respectively (Figure 2D). An infectious dose of  $5 \times 10^6$  CFU/mL AIEC induced a response most similar to the positive control for inducing inflammation, TNBS. ELISA from the intestinal lavage of zebrafish at 24 hpi by AIEC confirmed increasing calprotectin (S100A-10B) protein with increasing infectious doses. S100A-10B in intestinal lavage was measured as  $\sim 21$ , 33, 37, 42, and 44 ng/mL when infected with  $5 \times 10^3$ ,  $5 \times 10^4$ ,  $5 \times 10^5$ , and  $5 \times 10^6$  CFU/mL of AIEC or TNBS, respectively (Figure 2E).

As a vigorous increase in neutrophil-specific S100A-10b at both the gene and protein levels was observed after AIEC infection, next the recruitment of neutrophils in the intestine during the colonization of AIEC was examined in transgenic (*mpx:dendra*) zebrafish, which have fluorescent neutrophils. An infectious dose of  $5 \times 10^6$  CFU/mL of the AIEC mCherry strain was used. At 24 hpi the zebrafish intestines were harvested and subjected to fluorescent microscopy. A large recruitment of *mpx:dendra* (green)-tagged neutrophils was observed in the intestinal surface colonized by AIEC mCherry (red) (Figure 2F, top panel). Very few to no neutrophils were visualized in the control uninfected transgenic zebrafish (Figure 2F, middle panel). No or negligible red and green autofluorescence was seen in wild-type (WT) uninfected control zebrafish (Figure 2F, bottom panel).

### Persistent colonization of zebrafish by AIEC

Chronic inflammation in IBD patients is a hallmark of the disease, and persistent AIEC colonization may contribute to inducing inflammation (Small et al., 2013). Therefore, the fish model was used to examine whether persistent AIEC colonization occurred or if the infection was rapidly cleared in fish. Fish were inoculated with  $5 \times 10^6$  CFU/mL of AIEC LF82 by bath inoculation for 6 h, followed by incubation in fresh, sterile water for the desired amount of time. Fish intestines were collected for the colonization assay 24 hpi to 10 dpi (days post infection), each day at the same time. The fish showed colonization levels of  $\sim 5 \times 10^5$ ,  $\sim 9 \times 10^4$ , and  $\sim 5 \times 10^4$  CFU/intestine at 24, 48, and 72 hpi, respectively (Figure 3A). Colonization levels were significantly reduced from day 1 to day 3, but the number of colonizing bacteria remained high up to 3 dpi. To examine a longer time course of infection, the bacterial load in zebrafish intestines was assessed for 10 days. AIEC colonization decreased as the days progressed, but a detectable bacterial load (Above  $10^2$  CFU/intestine) was observed until day 8 (Figure 3B). Despite persistent AIEC colonization in the zebrafish intestine, survival was 100% at 10 dpi. To assess IBD markers over this extended time period, calprotectin (S100A-10b) protein concentration in intestinal lavage of zebrafish was quantified by ELISA up



**Figure 3. Long-term AIEC infection in zebrafish**

(A) Fish were added to 400 mL water containing  $5 \times 10^6$  CFU/mL of AIEC for 6 h and then moved to fresh water for 18 h (24 hpi), 42 h (48 hpi), or 66 h (72 hpi). Data are represented as mean  $\pm$  SEM from multiple experiments. Each dot represents the data from one fish. Total colonization per intestine was calculated by plating serial dilutions of intestinal homogenates 24 h postinfection. Statistical significance indicated above the data was determined by Student's t test. \* $p = 0.0105$  and \*\* $p = 0.0036$ .



**Figure 3. Continued**

(B) Fish were added to 400 mL water containing  $5 \times 10^6$  CFU/mL of AIEC for 6 h and then moved to fresh water for up to 10 days with daily water changes. Three fish were sacrificed per day. The graph is the presentation of the decreasing colonization of AIEC in zebrafish intestine with subsequent days. Error bars indicate standard deviation. Data shown are from three experiments.

(C) S100A-10b protein levels were determined in intestinal homogenates by calprotectin ELISA. Days after infection were plotted in the x axis. Error bars indicate standard deviation. Data are represented as mean  $\pm$  SEM from three experiments.  $***p \leq 0.0001$  compared with control.

to 10 dpi. A significant increase in intestinal S100A-10b was found until day 6, which supported the chronic inflammatory effect of AIEC on the zebrafish gut (Figure 3C).

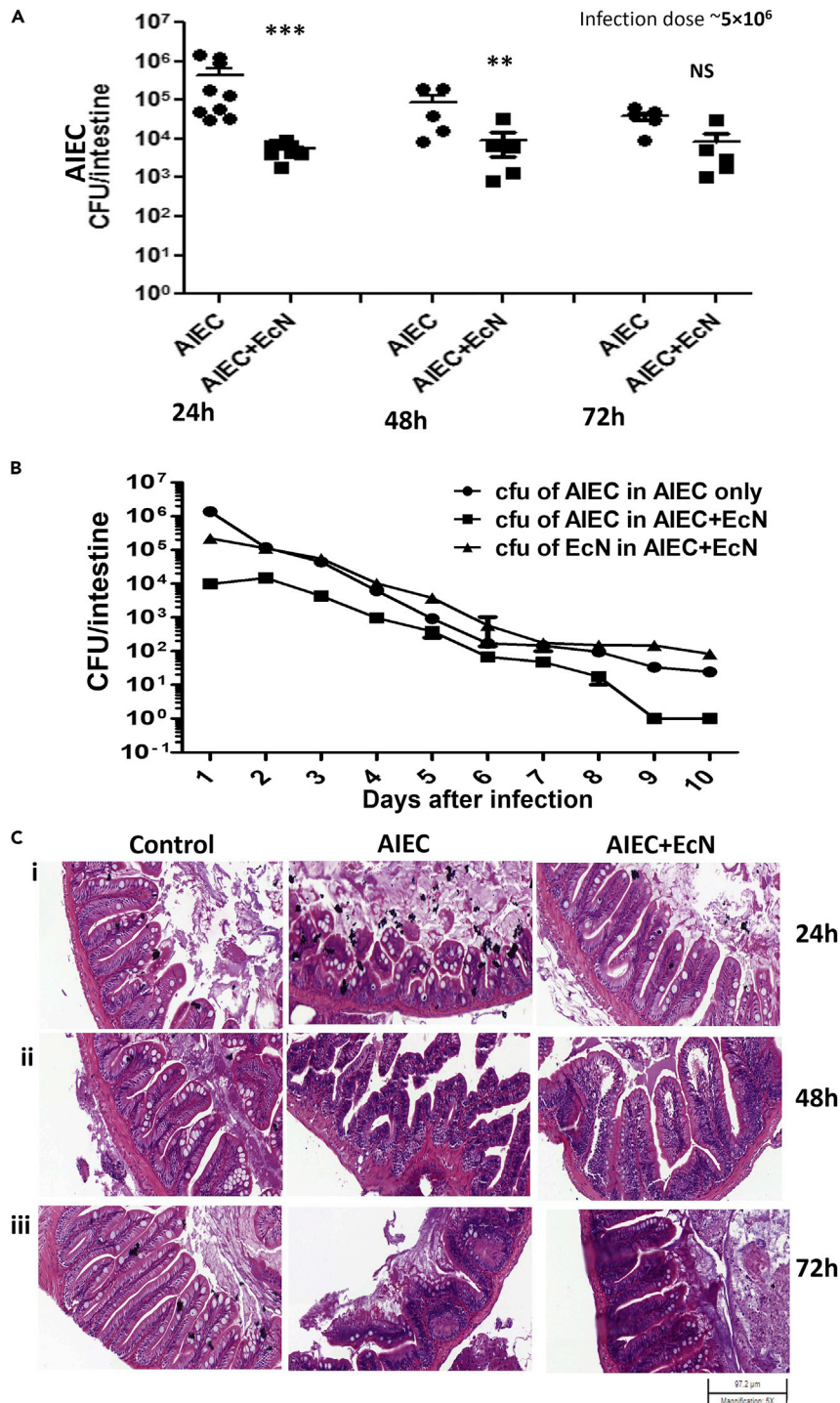
**Effect of probiotic strain, *E. coli* Nissle 1917, on AIEC infection in zebrafish**

*Escherichia coli* strain Nissle 1917 (EcN) has been used as a probiotic and therapeutic agent for over a century (Pradhan and Weiss, 2020). Reports suggest that EcN can protect mice from infection by various pathogenic *E. coli* (Pradhan and Weiss, 2020; Gronbach et al., 2010; Bury et al., 2018). In a previous study, EcN was found to reduce *V. cholerae* colonization in a zebrafish model (Nag et al., 2018a). Here, the question was whether EcN could have similar or even more dramatic effects against AIEC. First, EcN colonization in zebrafish was verified. Fish were added to 400 mL water containing  $5 \times 10^6$  CFU/mL of EcN for 6 h and then incubated in sterile, fresh water for 18 h (24 hpi), 42 h (48 hpi), or 66 h (72 hpi). EcN colonization of  $\sim 2 \times 10^5$  CFU/intestine,  $\sim 9 \times 10^4$  CFU/intestine, and  $\sim 8.5 \times 10^4$  CFU/intestine were observed at 24 h, 48 h, and 72 h postinfection, respectively (Figure S2B), suggesting stable colonization levels over this time span. Next, the effect of EcN on AIEC colonization was examined. Fish were inoculated with  $5 \times 10^6$  CFU/mL of AIEC alone or a 1:1 combination of AIEC and EcN cells for 6 h, then washed and incubated in sterile, fresh water for 18 h (24 hpi), 42 h (48 hpi), or 66 h (72 hpi). At 24 hpi, EcN co-infection reduced AIEC colonization by more than 80-fold, from  $\sim 5 \times 10^5$  CFU/intestine to  $\sim 6 \times 10^3$  CFU/intestine. At 48 hpi EcN co-infection reduced AIEC colonization by more than 11-fold. By 72 hpi a difference in colonization was still observed but was not statistically significant (Figure 4A). The fish-excreted AIEC levels were determined by dilution plating of water containing fish infected with AIEC only or by both AIEC and EcN. CFU counts in water paralleled the intestinal colonization trend (Figure S2C), as we have previously observed in a *V. cholerae* zebrafish model (42). In the long-term infection experiment, EcN colonization in zebrafish intestine was above the detection level for 10 days when EcN was co-inoculated with AIEC (Figure 4B, 3<sup>rd</sup> panel). EcN also offered protection by significantly reducing the AIEC colonization in zebrafish intestine for 10 days (Figure 4B).

To determine whether EcN can protect against villi denudation and overall rupture caused by AIEC, histopathology of fish intestine was examined at 24, 48, and 72 hpi. Fish were inoculated with AIEC only or 1:1 AIEC and EcN as described earlier; intestines were harvested and processed for H&E staining to assess villi denudation of fish intestine. EcN reduced the degree of villi denudation caused by AIEC at all three time points (Figure 4C). The chart for the pathological scoring for epithelial defects/tattering and leukocyte infiltrates/inflammation is shown in Figure S2D. The results of scoring by an independent pathologist suggested that EcN can successfully reduce the tissue rupture and inflammation caused by AIEC infection at 24 hpi and 48 hpi. At 72 hpi EcN reduced the colonization as well as the invasion of AIEC in the zebrafish gut, but the reduction of leukocyte recruitment was not significant.

**Effect of EcN on AIEC-induced inflammation in zebrafish**

EcN has anti-inflammatory effects in different animal models (Güttsches et al., 2012; Fábrega et al., 2017). EcN is also a well-known anti-inflammatory probiotic for human health use (Bury et al., 2018). Effects of EcN on inflammation in the zebrafish model were examined next. First, expression of previously mentioned inflammatory markers by qRT-PCR at 24 h, 48 h, and 72 h postinfection with AIEC was compared with the expression in uninfected control zebrafish. At 24 hpi the fold change of the inflammatory genes, i.e., TNF $\alpha$ , s100a-10b, IFN $\gamma$ , and IL-1 $\beta$ , was increased  $\sim 40$ -fold,  $\sim 95$ -fold,  $\sim 4$ -fold, and  $\sim 10$ -fold, respectively in infected fish (Figure 5A). The fold changes of these genes at 48 h after AIEC infection was enumerated as  $\sim 6$ -fold,  $\sim 45$ -fold,  $\sim 3$ -fold, and  $\sim 2.5$ -fold (Figure 5B), whereas at 72 h postinfection the changes were  $\sim 12$ -fold,  $\sim 52$ -fold,  $\sim 3$ -fold, and 2-fold, respectively (Figure 5C). When EcN was co inoculated with AIEC, the expression of inflammatory markers was significantly reduced, as compared with AIEC infection alone at 24 hpi (Figure 5A). At 48 hpi, EcN co-infection reduced TNF $\alpha$ , calprotectin (s100a-10b), and IL-1 $\beta$  gene expression by  $\sim 3$ -fold and  $\sim 2.5$ -fold, respectively (Figure 5B). At 72 hpi, TNF $\alpha$ , s100a-10b, and IFN $\gamma$



**Figure 4. Effects of EcN on AIEC colonization**

(A) Fish were added to 400 mL water containing  $5 \times 10^6$  CFU/mL of only AIEC or 1:1 combination of AIEC and EcN for 6 h and then moved to fresh water for 18 h (24 hpi), 42 h (48 hpi), or 66 h (72 hpi). AIEC colonization in zebrafish intestine is shown on the y axis and is compiled from multiple experiments. Data are represented as mean  $\pm$  SEM from multiple experiments. Each dot represents the data from one fish. Total colonization per intestine was calculated after plating serial dilutions of intestinal homogenates 24 h postinfection. Statistical significance indicated above the data was determined by Student's t test. \*\*\*p < 0.0001, \*\*p = 0.0019, and NS = nonsignificant.

**Figure 4. Continued**

(B) Fish were added to 400 mL water containing  $5 \times 10^6$  CFU/mL of only AIEC or 1:1 combination of AIEC and EcN for 6 h and then moved to fresh water for up to 10 days with daily water changes. Three fish were sacrificed per day. The graph illustrates the significant decrease in AIEC colonization in AIEC and EcN (1:1) co-infected zebrafish compared with AIEC-infected zebrafish. The graph also illustrates successful EcN colonization in the zebrafish intestine over time. Error bars indicate standard deviation. Data shown are from three experiments.

(C) Representative H&E staining (5X) of zebrafish intestinal sections. Villus structure in AIEC-only and AIEC and EcN (1:1)-infected zebrafish were compared with the control zebrafish 24 hpi (i), 48 hpi (ii), and 72 hpi (iii). The H&E figures are representative of three independent experiments.

gene expression were reduced by  $\sim 2.5$ -fold,  $\sim 2.8$ -fold, and  $\sim 2.5$ -fold, respectively (Figure 5C). These results revealed that EcN can indeed reduce the intestinal inflammation by reducing AIEC colonization and invasion in the zebrafish gut. As the zebrafish *s100a-10b* gene was highly expressed in fish intestines at 24, 48, and 72 hpi, we next evaluated using ELISA of intestinal lavage fluids whether the intestinal calprotectin level would be diminished by EcN during AIEC infection. EcN significantly reduced the S100A-10b secretion in fish intestine at 24, 48, and 72 hpi (Figure 5D). TNBS was used as a positive control for inducing S100A-10b secretion, and uninfected fish were used as negative controls.

**Prophylactic and therapeutic use of EcN against AIEC infection**

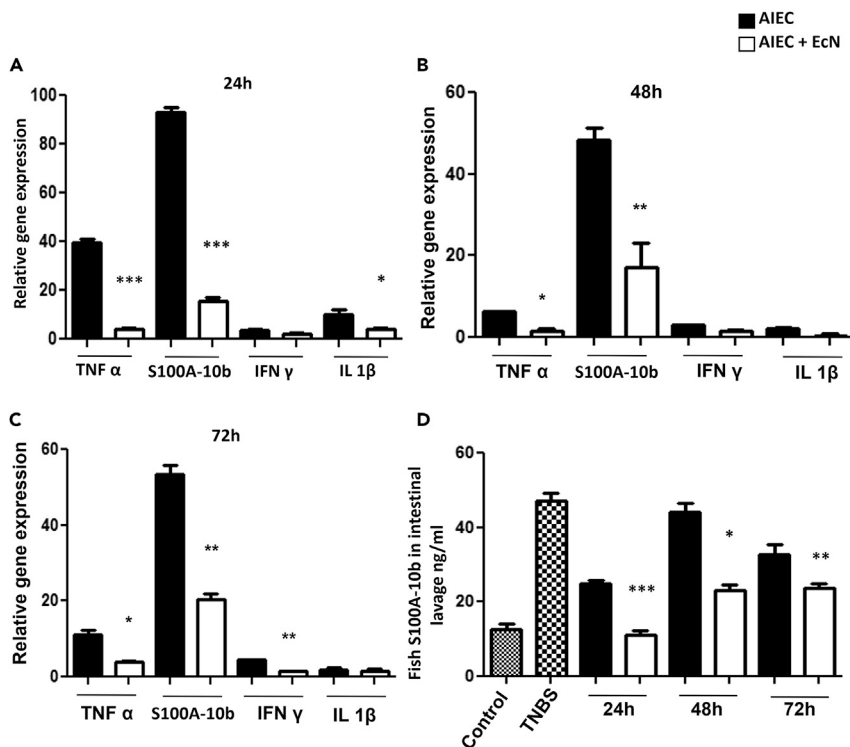
EcN reduced colonization levels and damage caused by AIEC when AIEC and EcN were co-inoculated simultaneously in zebrafish. The next question was whether EcN could be used as a prophylactic or therapeutic agent against AIEC. To test the use of EcN as a prophylactic, fish were inoculated with EcN prior to AIEC infection, and to test EcN as a therapeutic, fish were infected with AIEC prior to EcN inoculation. EcN produced significant protection under both conditions, lowering the colonization of AIEC by  $\sim 30$ -fold when added prior to AIEC and by  $\sim 32$ -fold when added after AIEC (Figure 6A). AIEC colonization was reduced to a somewhat greater degree when co-inoculated with EcN but the prophylactic or therapeutic use of EcN separately from AIEC caused a reduction in AIEC that was not significantly different from co-inoculation of EcN and AIEC.

Either prophylactic or therapeutic use of EcN reduced the gene expression of the inflammatory markers, i.e.  $\text{TNF}\alpha$ , *s100a-10b* (zebrafish equivalent of calprotectin),  $\text{IFN}\gamma$ , and  $\text{IL-1}\beta$ , that became elevated due to AIEC infection (Figure 6B). Intestinal calprotectin levels also decreased significantly due to prophylactic and therapeutic treatment of zebrafish with EcN during AIEC infection (Figure 6C). Colonization of EcN was slightly higher during the therapeutic use of EcN against AIEC at 24 hpi (Figure S3A).

**EcN offered protection in hyperinfective conditions for AIEC in zebrafish**

Propionic acid (PA), a short-chain fatty acid present in the human gut, can augment the growth and colonization of AIEC in the presence of ethanolamine (Ormsby et al., 2019). Ethanolamine is a very common metabolite in the vertebrate gut, and it is utilized by a variety of gut bacteria (Garsin, 2010; Thiennimitr et al., 2011). During IBD, AIEC is well known to utilize ethanolamine to increase its colonization and invasion into epithelium (Ormsby et al., 2019). The next question was whether growth in the presence of PA would render AIEC hyperinfective in zebrafish. AIEC was cultured overnight in LB with 20 mM PA. Then, zebrafish were infected with PA-primed AIEC following the above-described standard fish infection protocol. PA-primed AIEC showed  $\sim 10$ -fold higher colonization in the zebrafish model compared with the normal LB grown AIEC (Figure 7A), confirming that PA increases infectivity. The following question was whether EcN could reduce hyperinfectivity induced by PA. EcN was inoculated (1:1) with PA-primed AIEC, and colonization was assayed at 24 hpi. EcN reduced PA-primed AIEC colonization levels  $\sim 140$ -fold in zebrafish, confirming that EcN can effectively suppress AIEC hyperinfectivity (Figure 7A).

To evaluate whether EcN could lower the pro-inflammatory response of PA-primed AIEC in the zebrafish gut, the S100A-10b protein content in the zebrafish gut was quantified by ELISA at 24 hpi with AIEC, PA-primed AIEC, or PA-primed AIEC plus EcN (1:1). PA-primed AIEC augmented the pro-inflammatory S100A-10b secretion and induced  $\sim 42$  ng/mL of protein, whereas AIEC alone induced 32 ng/mL in zebrafish intestinal lavage. Both AIEC and PA-primed AIEC showed significantly increased S100A-10b secretion in zebrafish intestine compared with uninfected control. When EcN was applied against PA-primed AIEC, the S100A-10b secretion was reduced to 25 ng/mL, suggesting that EcN treatment can successfully reduce severe inflammation induced by hyperinfective AIEC (Figure 7B). S100A-10b excreted by zebrafish into water was also enumerated for both AIEC- and PA-primed-AIEC-infected fish. In both conditions a significant



**Figure 5. Effect of EcN on AIEC-induced inflammation**

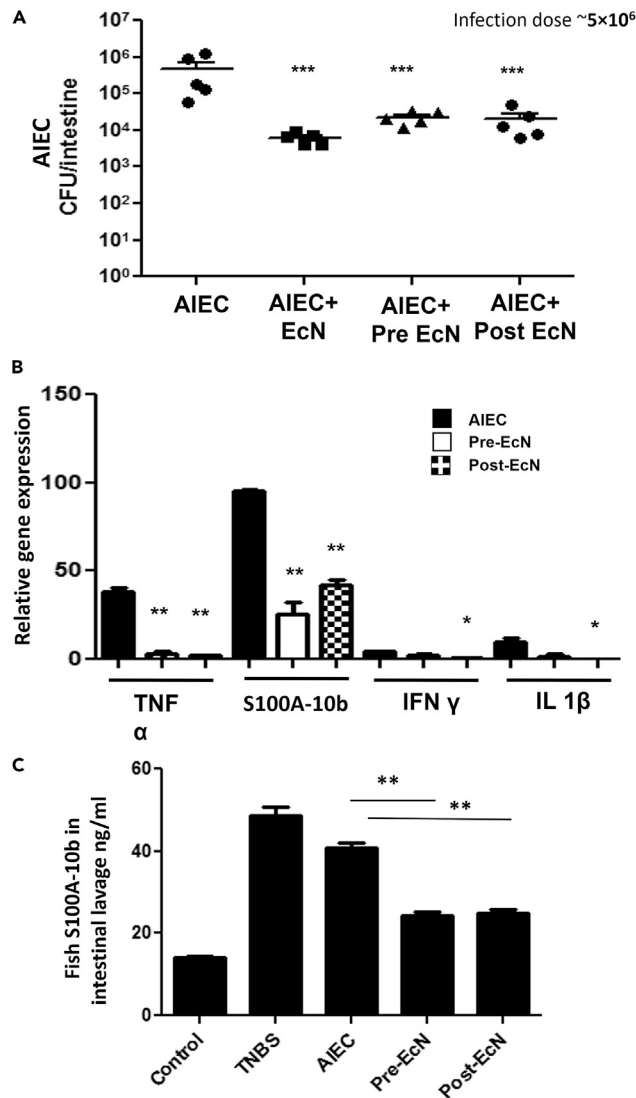
(A–D) WT zebrafish were infected with  $5 \times 10^6$  CFU/mL AIEC and simultaneously inoculated with EcN for 6 h and then moved to fresh water for 18 h (24 hpi) (A), 42 h (48 hpi), (B) or 66 h (72 hpi) (C) and then sacrificed. mRNA was isolated from intestinal tissue. Relative gene expression levels of TNF $\alpha$  (A), s100a-10b (B), IFN $\gamma$  (C), and IL-1 $\beta$  (D) were determined by qRT-PCR. Gene expression was normalized against  $\beta$ -actin and expressed as fold change. Error bars indicate standard deviation. Data are represented as mean  $\pm$  SEM from three experiments. \*\*\* $p$  < 0.0005, \*\* $p$  < 0.005, and \* $p$  < 0.05 as compared between AIEC and AIEC and EcN. (D) S100A-10b protein levels were determined in intestinal homogenates by calprotectin ELISA. Error bars indicate standard deviation. TNBS, a positive stimulator of inflammatory markers, was used as a positive control. Data are represented as mean  $\pm$  SEM from three experiments. \*\*\* $p$  = 0.0006, \*\* $p$  = 0.0025, and \* $p$  = 0.0412 compared between AIEC and AIEC and EcN.

increase in S100A-10b excreted by infected fish into the water was detected as compared with uninfected control fish (Figure S4). S100A-10b excreted into water by PA-primed-AIEC-infected zebrafish was higher than any AIEC-infected fish, but the difference was not significant.

Finally, the effect of EcN on hyper colonization of AIEC was tested by infecting fish with a 10:1 ratio of AIEC to EcN. EcN significantly reduced AIEC colonization by  $\sim$ 15-fold even at this ratio (Figure 7C).

## DISCUSSION

The evidence for microbial etiology in IBD and the interplay of host genetics and bacterial factors are an escalating research area in human disease (Sartor, 2008). Dysbiosis during IBD has a huge impact on many human biological pathways (DeGruttola et al., 2016). Large studies have suggested that the etiology of IBD involves environmental and genetic factors that lead to dysfunction of the intestinal epithelial barrier and consequent dysregulation of the mucosal immune system and responses to gut microbiota. AIEC strains, which abnormally colonize the ileal mucosa of IBD patients, have emerged as “pathobionts” implied in the pathogenesis of IBD (Carrière et al., 2014). An IBD episode can allow many enteric pathogens to colonize and invade the gut epithelium, disrupting the primary immune barrier. During dysbiosis in IBD, implanting beneficial gut bacteria (including probiotics) could be an important factor to recover healthy gut function. Here, a new AIEC-zebrafish disease model was developed, and the beneficial effects of a probiotic strain, *E. coli* Nissle 1917, were demonstrated against AIEC colonization.



**Figure 6. Prophylactic and therapeutic effects of EcN on AIEC infection**

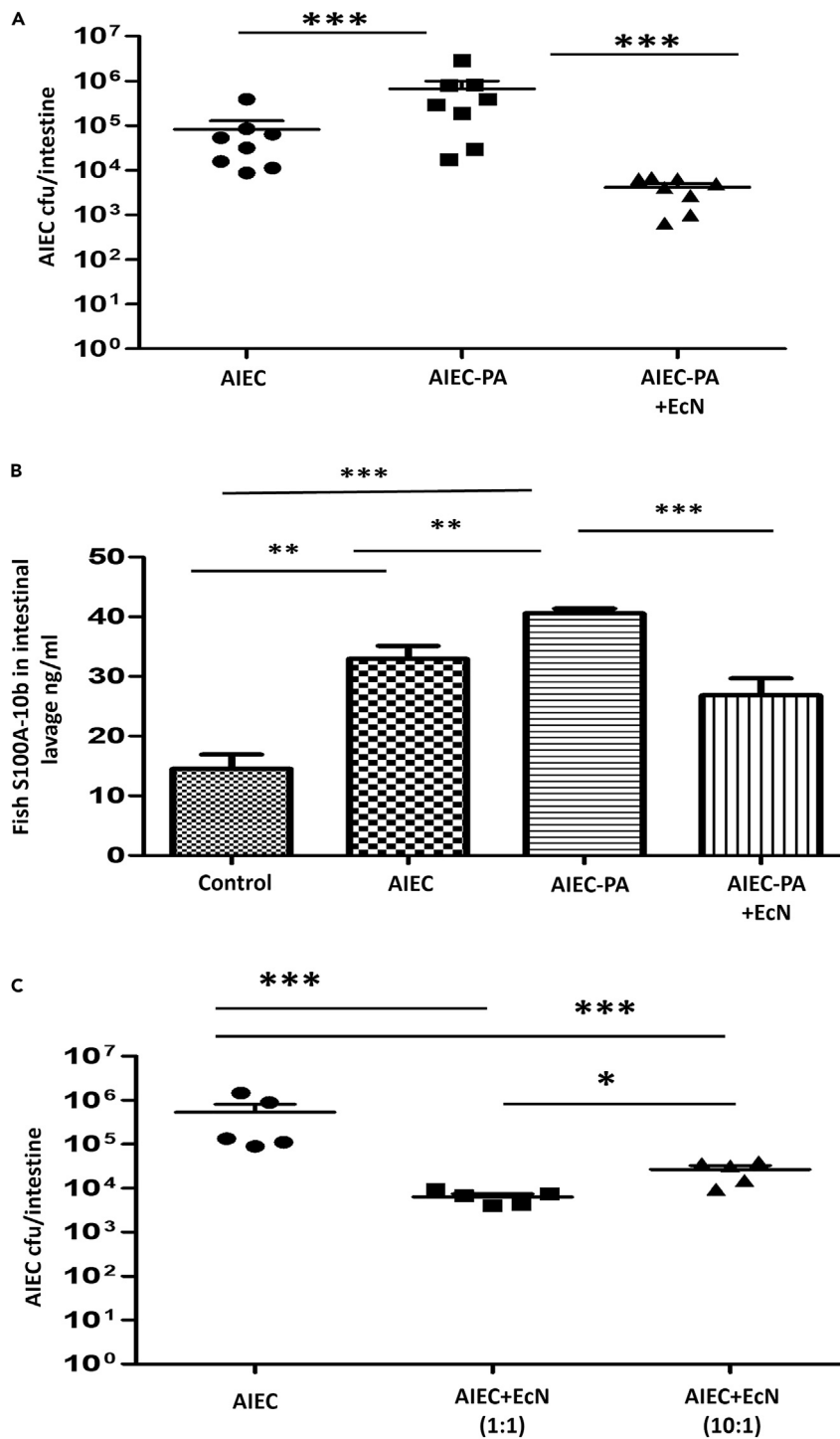
(A) Fish were added to 400 mL water containing  $5 \times 10^6$  CFU/mL of only AIEC or a 1:1 combination of AIEC and EcN cells for 6 h and then moved to fresh water for 18 h, or fish were added to 400 mL water containing  $5 \times 10^6$  CFU/mL of EcN for 6 h followed by  $5 \times 10^6$  CFU/mL of AIEC (pre-EcN, prophylactic) for 6 h and vice versa, i.e., AIEC first, then EcN (post-EcN, therapeutic), and then incubated in fresh water for 12 h. AIEC colonization in zebrafish intestine is shown on the y axis and is compiled from multiple experiments. Data are represented as mean  $\pm$  SEM from multiple experiments. Each dot represents the data from one fish. Total colonization per intestine was calculated after plating serial dilutions of intestinal homogenates 24 h postinfection. Statistical significance indicated above the data was determined by Student's t test.  $***p < 0.0005$  compared with only AIEC infection.

(B) Zebrafish were infected with  $5 \times 10^6$  CFU/mL of only AIEC, pre-EcN then AIEC (prophylactic), or AIEC then post-EcN (therapeutic) as mentioned and were sacrificed 24 hpi. mRNA was isolated from intestinal tissue. Relative gene expression levels of TNF $\alpha$ , s100a-10b, IFN $\gamma$ , and IL-1 $\beta$  were determined through qRT-PCR. Gene expression was normalized against  $\beta$ -actin and expressed as fold change. Error bars indicate standard deviation. Data are represented as mean  $\pm$  SEM from three experiments.  $**p < 0.005$  and  $*p < 0.05$  as compared with only AIEC infection.

(C) S100A-10b protein levels were determined in intestinal homogenates by calprotectin ELISA. Error bars indicate standard deviation. TNBS, a positive stimulator of inflammatory markers, was used as a positive control. Data are represented as mean  $\pm$  SEM from three experiments.  $**p < 0.005$  compared with the only AIEC infection.

Zebrafish models for several human pathogens have been described (Nag et al., 2018a, 2018b, 2020; Howlander et al., 2016; Farr et al., 2022; Bailone et al., 2020; Li et al., 2020) and indicated the potential for a zebrafish AIEC infection model. AIEC can easily and rapidly colonize the zebrafish intestine by bath inoculation,





**Figure 7. EcN protects against hyperinfective AIEC**

(A) Fish were added to 400 mL water containing  $5 \times 10^6$  CFU/mL of only AIEC, PA-primed AIEC (AIEC grown overnight in LB with 20 nM propionic acid) or a 1:1 combination of PA-primed AIEC and EcN cells for 6 h and then moved to sterile fresh water for 18 h. AIEC colonization in zebrafish intestine is shown on the y axis and is compiled from multiple experiments. Data are represented as mean  $\pm$  SEM from multiple experiments. Each dot represents the data from one fish. Total colonization per intestine was calculated after plating serial dilutions of intestinal homogenates 24 h postinfection. Statistical significance indicated above the data was determined by Student's t test. \*\*\* $p < 0.0001$ .

**Figure 7. Continued**

(B) S100A-10b protein levels were determined in intestinal homogenate via calprotectin ELISA. Error bars indicate standard deviation. Data are represented as mean  $\pm$  SEM from three experiments.  $**p < 0.005$  and  $***p < 0.0001$ . (C) Fish were added to 400 mL water containing  $5 \times 10^6$  CFU/mL of only AIEC, 1:1 and 10:1 combination of AIEC and EcN cells for 6 h, and then incubated in fresh water for 18 h. AIEC colonization in zebrafish intestine is shown on the y axis and compiled from multiple experiments. Data are represented as mean  $\pm$  SEM from multiple experiments. Each dot represents the data from one fish. Total colonization per intestine was calculated after plating serial dilutions of intestinal homogenates 24 h postinfection. Statistical significance indicated above the data was determined by Student's t test.  $***p < 0.0001$  and  $*p = 0.0109$ .

disrupt the epithelial barrier, and produce vigorous inflammation in the zebrafish gut. An infectious dose of  $5 \times 10^6$  CFU/mL produced high colonization levels in zebrafish, confirmed by immunofluorescence assays. Degree of tissue rupture, which is another very important indicator of AIEC infection in zebrafish, increased with the AIEC dose. An AIEC infectious dose of  $5 \times 10^6$  CFU/mL colonized abundantly at early time points, caused gut epithelial tissue disruption until 72 hpi and continued to colonize at low levels up to at least ten days.

AIEC can worsen the condition of IBD patients by inducing massive inflammatory responses (Bretin et al., 2018). In the zebrafish model, AIEC also induced a significant increase in inflammatory markers such as  $TNF\alpha$ ,  $IFN\gamma$ , and  $IL-1\beta$  during infection. Calprotectin, mainly secreted by neutrophils, has several different biological activities in humans (Jukic et al., 2021) and is also a very sensitive marker for IBD (Bjarnason, 2017; Hovstadius et al., 2021). AIEC infection recruited neutrophils in transgenic zebrafish with *mpx:Dendra* as seen by fluorescence microscopy. Zebrafish S100A-10b had previously been identified as having an equivalent role to human calprotectin and is highly similar in protein sequence (43% identity, 67% similarity) to human S100A8 (Farr et al., 2022). Zebrafish intestinal S100A-10b during AIEC infection was significantly elevated at both the gene expression and protein abundance levels. We attempted to measure the fecal S100A-10b as an inflammation marker (Bjarnason, 2017), but it was not detected (except once; Figure S4), as the water volume was too high compared with the fish excretions. Detection may be possible if the fecal pellets can be collected from the bottom of the beaker.

In a previous study, the efficacy of well-known probiotic strain EcN against the colonization of *V. cholerae* was demonstrated in a zebrafish model (Nag et al., 2018a). EcN is a nonpathogenic Gram-negative strain used as a therapeutic for many gastrointestinal disorders, including diarrhea (Henker et al., 2008), uncomplicated diverticular disease (Fric and Zavoral, 2003), and IBD, particularly UC (Schultz, 2008). Another study found that EcN protected against AIEC pathogenicity in a Caco-2 cell culture model (Huebner et al., 2011). Here, we have successfully tested EcN for the first time against AIEC in an animal model. AIEC and EcN did not show antagonistic effects in LB or in fish infection water (Figure S3B). EcN can colonize the zebrafish gut for at least 10 days (41; Figure 4B). When EcN was co-inoculated with AIEC in zebrafish, EcN significantly protected against AIEC by lowering the colonization level, tissue damage, and inflammation in zebrafish gut caused by AIEC. EcN protected the gut against the colonization and invasion of AIEC and reduced the inflammation caused by AIEC infection. Lower AIEC infectious dose also correlated with reduced inflammatory responses.

In clinical practice a probiotic cannot be administered to patients at the same time as the infection of any pathogen. Therefore, for practical purposes, the prophylactic and therapeutic effects of EcN on AIEC infection in zebrafish were tested. In both approaches, EcN successfully reduced AIEC colonization, tissue rupturing, and inflammation in the zebrafish gut.

The incidence of IBD can be affected by the food habits or diet of the population, and the colonization of AIEC is altered in the presence of some food derivatives. Studies showed that invasion and colonization of AIEC were increased in the presence of propionic acid and ethanolamine (59). When zebrafish were infected with PA-primed AIEC (AIEC grown overnight in LB with 20 nM PA), elevated colonization levels and inflammation in the fish gut were observed. EcN was effective in mitigating this hyperinfective condition and reduced the colonization and inflammation caused by PA-primed AIEC. EcN was also effective even when it was used as a sub-dose to the AIEC infection at a 1:10 ratio; this strongly suggests that EcN could be a powerful therapeutic and prophylactic tool against the development of AIEC-induced IBD.

In conclusion, the studies described here introduce the zebrafish as a very useful animal model to study AIEC infection and disease progression. The prophylactic and therapeutic potential of a commercially available probiotic, EcN, against AIEC were demonstrated in the zebrafish infection model. The data clearly suggest that zebrafish could be a useful model to study AIEC infection mechanisms, and EcN could be used in IBD patients to mitigate AIEC infection, which may help to ease the difficulties associated with IBD.

### Limitations of the study

Although zebrafish show potential to study AIEC induced pathogenicity and inflammation, the main limitation of the study was unavailability of a wide variety of primary antibodies and/or ELISA kits to specifically study fish inflammation. Instead, qRT-PCR was used to evaluate the inflammatory markers in this study. Another limitation of this study was difficulty in acquiring zebrafish stool samples to evaluate excreted inflammatory proteins from the stool samples. However, this could be resolved by using a more sophisticated set-up (such as using fine mesh above the container base to collect stool particles) in the zebrafish infection chamber. Excreted protein studies were done using water samples, which sometimes diluted the excreted protein to below the level of detection. Finally, fish and human intestinal tracts do have some anatomical differences, which could limit the scope of observations possible using this model.

### STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Histology & imaging of infected zebrafish intestines
- QUANTIFICATION AND STATISTICAL ANALYSIS
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### SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2022.104572>.

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### AUTHOR CONTRIBUTIONS

D.N., S.R., and J.H.W. designed the experiments; D.N. and D.F. carried out the experiments; D.N. and J.H.W. wrote the manuscript, and all the authors gave editorial input.

### DECLARATION OF INTERESTS

We declare no conflict of interest.

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## STAR★METHODS

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Bacterial and virus strains</b>		
Adherent invasive <i>E. coli</i> strain LF 82	Kind gift from Prof. Darfeuille-Michaud A	N/A
Adherent invasive <i>E. coli</i> strain LF 82 (Spec <sup>r</sup> 100 µg/mL)	Kind gift from Dr. Olivier Espeli	N/A
<i>E. coli</i> strains Nissle 1917	Lab Strain	N/A
<i>E. coli</i> strain Nissle (Rif <sup>r</sup> 75 µg/mL)	Lab Strain	N/A
<b>Chemicals, peptides, and recombinant proteins</b>		
Tryptone	BD	Ref: 211705
Yeast Extract	BD	Ref: 212750
Agar	BD	Ref: 214010
Instant Ocean salts	Aquarium Systems	Product No. SS15-10
Tricaine-S	Syndel	Lot No: 13065
Trizol	Thermo Fisher	Ref: 15596026
SuperScript <sup>TM</sup> First-Strand Synthesis System	Invitrogen	
SYBR green	Applied Biosystem	Catalog no: A25742
Calprotectin ELISA Kit	MyBioSource	MBS7606803
<b>Experimental models: Organisms/strains</b>		
Zebrafish strain AB	Lab bred	N/A
<b>Oligonucleotides</b>		
IL1β	Integrated DNA Technologies	5′CATTTCAGGCCGTCACA3′; 5′GGACATGCTGAAGCGCACTT3′
TNFα	Integrated DNA Technologies	5′CCATGCAGTGATGCGCTT3′; 5′TTGAGCGGATTGCACTGAAA3′
IFNγ	Integrated DNA Technologies	5′CTTCCAGGCAAGAGTGAGA3′; 5′TCAGCTCAAACAAGCCTTTCG3′
S100A-10b	Integrated DNA Technologies	5′GCAGAGGGGAACATCAAC3′; 5′CCCACCACAAGAGACAAAA3′
<b>Software and algorithms</b>		
GraphPad Prism 9.0	GraphPad Prism Software, Inc	<a href="https://www.graphpad.com/">https://www.graphpad.com/</a>
ZEISS ZEN lite	Zeiss	<a href="https://www.zeiss.com/microscopy/us/products/microscope-software/zen-lite.html">https://www.zeiss.com/microscopy/us/products/microscope-software/zen-lite.html</a>

## RESOURCE AVAILABILITY

## Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jeffrey Withey ([jwithey@med.wayne.edu](mailto:jwithey@med.wayne.edu)).

## Materials availability

This study did not generate new unique reagents.

## Data and code availability

All data reported in this paper will be shared by the [Lead contact](#) upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the [Lead contact](#) upon request.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

### Strains and culture conditions

Adherent invasive *E. coli* strain LF 82 (AIEC LF82 Amp<sup>r</sup>100 µg/mL), AIEC LF82 pPrpsM-mCherry (Spec<sup>r</sup> 100 µg/mL, Kind gift from Dr. Olivier Espeli), probiotic *E. coli* strains Nissle 1917 (EcN) and *E. coli* strain Nissle (Rif<sup>r</sup> 75 µg/mL, introduced by adaptive mutagenesis) were used in this study. No phenotypic difference in a motility assay (0.3% LB agar) was observed between the rifampicin resistant EcN and WT EcN strain (Figure S2A). All strains were frozen in 15% glycerol in Luria-Bertani (LB) broth (Difco, NJ, USA) at –80°C. Prior to experimentation, each strain was grown in LB broth (Difco, NJ, USA) at 37 °C under shaking conditions (100 rpm) or on plates in LB agar (Difco, NJ, USA) with the appropriate antibiotic(s). LB agar with the desired antibiotic concentrations was prepared for the selection of strains during colonization study.

### Animal model: zebrafish

Adult, wild-type AB zebrafish and transgenic zebrafish (*mpx:Dendra2<sup>uw</sup>m4/AB*, expressing green fluorescent protein in neutrophils, gifted by the Thummel laboratory, WSU), 6 to 12 months of age, were used in all experiments. The fish were housed in an automated recirculating tank system (Aquaneering, CA, USA) using water filtered by reverse osmosis and maintained at pH 7.0 to 7.5. The tank water was conditioned with Instant Ocean salts (Aquarium Systems, OH, USA) to a conductivity of 600–700 µS. The fish were fasted for at least 12 h prior to each experiment. Zebrafish were euthanized in 100 mL of 32 µg/mL Tricaine-S (tricaine methane sulfonate; MS-222 [Syndel, WA, USA]) for a minimum of 30 min after cessation of opercular movement. All animal protocols were approved by the Wayne State University IACUC.

## METHOD DETAILS

### Infection procedure in zebrafish

Four to five zebrafish per experimental group were placed into a 400 mL beaker with a perforated lid containing 200 mL of sterile infection water (autoclaved tank water). Bacterial cultures were grown with aeration in LB broth at 37° C for 16 to 18 h. Cells were centrifuged at 10,000 g for 10 min. The resulting pellet was washed twice with 1X PBS and resuspended in 1X PBS to an estimated concentration of 10<sup>9</sup> CFU/mL by measuring the OD = 1 at 600 nm. 2 mL of bacterial inoculum was added to the beaker containing the fish. The final bacterial cell density used was ~5 × 10<sup>6</sup> CFU/mL (infection dose) for this study and was verified by plating serial dilutions of the inoculated infection water. The CFU of EcN used was equal to or 1/10 times the number of AIEC CFU in the inoculum. For general infection fish were exposed to bacteria for 6 h, and then the fish were washed twice for removal of surface bacteria and kept in fresh sterile water for 18 h. EcN was inoculated either with (coinfection, 1:1) or before (prophylactic) or after (therapeutic) AIEC infection. For prophylactic experiments, fish were inoculated by bath with EcN for 6 h, infected with AIEC for 6 h, washed twice and kept in fresh infection water for 12 h, and then the fish were euthanized. For therapeutic experiments, fish were infected with AIEC for 6 h, then inoculated with EcN for 6 h, washed twice and kept in fresh infection water for 12 h, and then euthanized. To get the colonization data from more than 24 h infection, fish were infected for 6 h and then washed twice and kept in fresh infection water. Fish were sacrificed after 2 days–10 days to get the long-term colonization data. Water was changed each day and fish were fed once each day, starting from day 2 after infection. To assess hyperinfection of AIEC in zebrafish, AIEC LF82 strain was grown overnight in the presence of 20 mM propionic acid (Sodium propionate; Millipore Sigma, Burlington, MA, USA) and then fish were infected following the same protocol. Each beaker was placed into a glass-front incubator set at 28° C for the duration of the experiment.

### Colonization assay

After the specified time points, fish were euthanized, the intestine of each fish was aseptically dissected, placed into homogenization tubes (2.0-mL screw-cap tubes; Sarstedt, Numbrecht, Germany) with 1.5 g of 1.0-mm glass beads (BioSpec Products, Inc., Bartlesville, OK) and 1 mL of 1X PBS, and held on ice. Homogenization tubes were loaded into a Mini-Beadbeater-24 (BioSpec Products, Inc.) and shaken at maximum speed for two 1-min cycles, with the samples being incubated for 1 min on ice after both the first and last cycles. Intestinal homogenates from each fish were diluted and plated for enumeration on LB agar plates with appropriate antibiotics and incubated overnight at 37° C. AIEC and EcN were each selected on appropriate antibiotic media. In each colonization assay, whole intestine from a single zebrafish was homogenized and the colonization data were calculated as cfu per zebrafish intestine. Any colonization above

or equal to  $10^2$  cfu per intestine was taken as detectable colonization. Uninfected zebrafish gut homogenate (control fish) was also diluted and spread over all the antibiotic containing LB plates used in this work as a negative control. No colonies were observed after overnight incubation at 37° C

### Processing of infection water

With a 60-mL syringe (BD 309653; BD, NJ, USA), 50 mL of fish infection water was extracted before the colonization assay, in duplicate, and put into two 50 mL conical tubes. For all assays, 50 mL conical tubes were centrifuged at 10,000 rpm for 15 min at 4° C and supernatant was decanted, with care not to disturb the pellet. Each pellet was resuspended in 2 mL of 1X PBS. Then the suspension was serially diluted and plated on LB agar plates with appropriate antibiotics to enumerate the bacterial load in excreted water.

### Real Time PCR analysis of zebrafish inflammatory target genes

RNA was isolated from zebrafish intestine using Trizol (Thermo Fisher Scientific, Waltham, USA) according to company protocol (Hummon et al., 2007). Total RNA was resuspended in RNase-free water and quantified using a NanoDrop. After normalization, isolated RNA was subjected to cDNA synthesis using the SuperScript™ First-Strand Synthesis System (Invitrogen, Grand Island, NY, USA). Real time quantitative PCR was performed in an Applied biosystems 7500 real-time PCR instrument, using SYBR green (Applied Biosystems, Foster City, CA) according to the standard protocol. Primers used for real time PCR were Il1 $\beta$ : F5'CATTTGCAGGCCGTACA3'; R5'GGACATGCTGAAGCGCACTT3', TNF $\alpha$ : F5'CCATGCAGTGATGC GCTTT3'; R5'TTGAGCGGATTGCACTGAAA3', IFN $\gamma$ : F5' CTTCCAGGCAAGAGTGCAGA3'; R5'TCAGC TCAAACAAAGCCTTTCG3' and zebrafish S100A-10b protein (zebrafish calprotectin): F5'GCAGA GGGAACTCATCAAC3'; R5'CCCACCACAAGAGACACAAA3'. All the qRT-PCR and Real time PCR experiments were repeated three times. The internal control gene  $\beta$ -actin was amplified simultaneously in a separate reaction. Threshold cycle number (CT) of triplicate reactions was determined using the ABI-SDS software and the mean CT of triplicate reactions was determined. The levels of expression of the genes of interest were normalized to  $\beta$ -actin using the formula  $2^{-\Delta\Delta CT}$ , where  $-\Delta\Delta CT = \Delta CT$  (sample)  $-\Delta CT$  (calibrator) and  $\Delta CT$  is the CT of the target gene subtracted from the CT of the housekeeping gene ( $\beta$ -actin). Fish were incubated for 12 h with 20  $\mu$ M TNBS (2,4,6-Trinitrobenzenesulfonic acid solution; Sigma, St. Louis, USA) in infection water to induce non-lethal inflammation used as a positive control.

### Intestinal homogenate ELISA

Intestinal tissue was removed at specified time points and placed into 1.5 mL centrifuge tubes with 100  $\mu$ L 1X PBS and homogenized using pellet pestles. Next, 50  $\mu$ L of RIPA buffer was added and a brief centrifugation was done. Samples were then diluted 1:25 with 1X PBS and the calprotectin ELISA kit was run according to manufacturer's instructions (MyBioSource, San Diego, CA).

### Histology & imaging of infected zebrafish intestines

After euthanizing, intestines were removed and then placed in 10% zinc formalin for 24 h. Next, intestines were placed in 70% ethanol and shipped to Reveal Biosciences (Reveal Biosciences, Inc., San Diego, CA) for H&E and fluorescence staining and imaging following their routine lab protocol. A board-certified veterinary pathologist (hired by Reveal Biosciences) with experience in laboratory animals and toxicologic pathology evaluated the H&E slide images for any findings with special assessment of intestinal structural disintegration and tissue inflammation. Pathological scoring of the H&E slides was done to enumerate the degree of epithelial defects/tattering (0 = within normal limits; 1 = mild surface epithelial "tattering"; 2 = focally extensive surface epithelial tattering; 3 = erosions; 4 = ulceration) and leukocyte infiltrates/inflammation (0 = none/within normal limits; 1 = minimal or least extent discernible; 2 = mild; 3 = moderate; 4 = marked; 5 = severe or greatest extent possible). The fluorescence microscopy images of infected and uninfected tissues were generated with the same laser intensity and the images were processed identically for the generation of the figures (Reveal Biosciences, Zeiss LSM 510 software).

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Statistical analysis

Each experiment was performed a minimum of three times on separate occasions, unless otherwise specified in the figure legends. Analyzed data are presented as the mean  $\pm$  SD. Significant frequencies were compared using the  $\chi^2$  test, and continuous variables were compared using Student's t test. A two-tailed t test was performed to test against a control as \* $p \leq 0.05$ , \*\* $p \leq 0.005$ , \*\*\* $p \leq 0.0005$  unless mentioned differently in figure legends. Analyses were performed using GraphPad Prism 7.0.