Effects of *Shigella flexneri* Exposure on Fluid Secretion and Histopathological Changes in Mouse Ileum and Colon

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ABSTRACT
Diarrheal disease remains one of the leading causes of mortality worldwide, and in Indonesia *Shigella flexneri* constitutes a major etiological agent of endemic diarrhea. The clinical manifestations range from watery diarrhea to bacillary dysentery, or a combination of both, reflecting the bacterium's biphasic pathogenic mechanisms. However, the specific processes by which *S. flexneri* induces watery diarrhea have not been fully elucidated. This study aimed to compare intestinal fluid secretion and histopathological alterations in the ileum and colon of mice, thereby contributing to a deeper understanding of *Shigella* pathogenesis. Four experimental groups were examined: negative control ileum, ileum exposed to *S. flexneri*, negative control colon, and colon exposed to *S. flexneri*. Using the ex vivo Mice Ligated Ileal Loop (MLIL) method, intestinal segments were incubated for 30 minutes, and intestinal weight was recorded at 5-minute intervals. Data were analyzed using an unpaired t-test, while histological findings served as supplementary qualitative evidence. The results demonstrated a greater increase in intestinal weight in the *S. flexneri*–exposed groups compared with controls, although the differences did not reach statistical significance. Histological evaluation was limited due to tissue autolysis, which hindered optimal interpretation. In conclusion, exposure to *S. flexneri* tended to enhance intestinal fluid secretion, particularly in the colon at the 30-minute mark; however, this effect was not statistically significant under short-term ex vivo conditions. Longer incubation periods and improved tissue preservation techniques are required to more accurately capture the full pathogenic effects of *S. flexneri*.
Keywords: Mice Ligated Ileal Loop (MLIL); *Shigella flexneri*; intestinal fluid secretion; ex vivo

INTRODUCTION
Diarrhea is broadly defined as an alteration in normal bowel habits characterized by increased stool liquidity, volume, or frequency, typically exceeding three episodes within a 24-hour period [1]. As a clinical syndrome, diarrhea encompasses a wide spectrum of etiologies, with infectious diarrhea representing one of the most prevalent forms worldwide. Such infections are commonly caused by bacteria, viruses, or parasites transmitted through contaminated food, unsafe water, inadequate sanitation, or poor personal hygiene practices. Despite substantial global progress in water safety and public health interventions, diarrheal disease continues to impose a significant burden, with an estimated 1.7 billion cases occurring annually and approximately 760,000 deaths among children under five years of age. These figures highlight not only the persistent vulnerability of young children but also the broader challenges faced by low- and middle-income countries in controlling enteric infections. In Indonesia, diarrhea remains endemic, with recurrent outbreaks reported across multiple provinces, reflecting ongoing disparities in environmental sanitation, access to clean water, and the effectiveness of disease surveillance systems [2].

Within the spectrum of bacterial pathogens responsible for diarrheal illness, *Shigella* species occupy a prominent position. They are recognized as one of the most frequent causes of diarrhea requiring hospitalization in Indonesia, accounting for 27.3% of confirmed cases. Shigellosis, also known as bacillary dysentery, typically presents with fever, abdominal cramps, and diarrhea containing mucus or blood, manifestations that reflect the organism's invasive nature and its ability to elicit intense inflammatory responses in the colonic mucosa [3]. Four species are formally recognized within the genus; *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei* each differing in geographic distribution, virulence, and epidemiological patterns. Among these, *S. flexneri* and *S. dysenteriae* are considered the most virulent, particularly in developing countries where sanitation infrastructure remains limited and person-to-person transmission is facilitated by crowded living conditions [4].

The pathogenesis of shigellosis is complex and multifactorial, involving epithelial invasion, intracellular replication, lateral cell-to-cell spread, and the production of cytotoxins and enterotoxins. These mechanisms collectively contribute to the characteristic clinical manifestations. Watery diarrhea is generally associated with early involvement of the small intestine, where enterotoxins disrupt absorptive and secretory processes, whereas dysentery is linked to colonic invasion, epithelial destruction, and ulceration [5–7]. Experimental studies have demonstrated that watery diarrhea may develop rapidly, occurring within 90 minutes to 2 days following exposure, underscoring the efficiency with which *Shigella* perturbs intestinal homeostasis [8]. Despite these insights, important gaps remain in understanding the differential effects of *S. flexneri* on various intestinal segments, particularly regarding the magnitude of fluid secretion and the nature of histopathological changes in the ileum versus the colon.

Given these scientific and public health considerations, the present study was designed to provide a detailed comparison of the effects of *S. flexneri* exposure on ileal and colonic segments in mice, with specific attention to fluid secretion dynamics and histopathological alterations. The objective of this study is to determine how *S. flexneri* differentially influences secretory responses and tissue pathology in the ileum and colon, thereby contributing to a more comprehensive understanding of its pathogenic mechanisms.

METHODS
An experimental laboratory study was conducted using a repeated-measures design. An ex vivo intestinal loop incubation model, adapted from the classical Mice Ligated Ileal Loop (MLIL), was employed to assess fluid secretion and histopathological changes in mouse ileum and colon following exposure to *Shigella flexneri*. Unlike the conventional in vivo MLIL performed in anesthetized animals with intact circulation, this study utilized excised intestinal segments incubated ex vivo after euthanasia, with repeated measurements of intestinal weight recorded at 5-minute intervals over a 30-minute observation period.

Twenty male BALB/c mice (8–12 weeks old) were used. Animals were euthanized under ketamine anesthesia, and 10 cm segments of ileum and colon were excised. Each segment was ligated at both ends with sterile thread to prevent leakage and randomly assigned to one of four groups: 1) ileum + Brain Heart Infusion (BHI, negative control) 2) ileum + *S. Flexneri*; 3) colon + BHI (negative control); and 4) colon + *S. Flexneri.* The required sample size was calculated using the formula p (n–1) ≥ 15, where p represents the number of treatment groups and n the number of replicates. With four treatment groups in this study (ileum control, ileum + *S. flexneri*, colon control, colon + *S. flexneri*), the minimum number

of replicates required was five per group ($4 \times [n-1] \geq 15 \rightarrow n \geq 5$). Accordingly, a total of 20 intestinal segments were used, consisting of 10 ileal and 10 colonic segments, each randomly assigned to one of the four groups with five replicates per group.

Shigella flexneri isolates were obtained from the Microbiology Laboratory, Faculty of Medicine, Universitas Brawijaya. Cultures were grown on MacConkey agar at 37°C for 24 hours, harvested in phosphate-buffered saline (PBS, pH 7.4), and transferred into BHI broth. Standardization was performed using the McFarland 0.5 turbidity standard (≈1–1.5 × 10^8 CFU/mL). To obtain the desired working concentration, 1 mL of the standardized suspension was diluted in 9 mL of BHI broth, yielding ~1–1.5 × 10^7 CFU/mL. A further ten-fold dilution was performed to achieve a final inoculum of ~1–1.5 × 10^6 CFU/mL, which was used for ex vivo intestinal loop incubation. Prior to application, bacterial suspensions were incubated for 4 hours at 37 °C to allow adaptation and toxin release.

Each intestinal segment received 100 µL of inoculum (BHI or *S. flexneri* suspension, OD=1) and was immersed in Roswell Park Memorial Institute (RPMI) medium maintained at 37°C with gentle agitation. Baseline weights were recorded prior to inoculation. Subsequent weights were measured every 5 minutes for 30 minutes using an analytical balance (Ohaus Adventurer™). Changes in weight were calculated as the difference between baseline and subsequent measurements, representing net fluid secretion.

At the end of the 30-minute incubation, intestinal segments were fixed in 10% neutral-buffered formalin overnight. Tissues were processed using an automatic tissue processor, embedded in paraffin, and sectioned at 3–5 µm thickness. Sections were stained with hematoxylin and eosin (HE) following Bancroft's protocol [9]. Slides were examined under light microscopy for evidence of epithelial damage, necrosis, villous architecture disruption, and inflammatory cell infiltration.

Weight measurements were recorded at each time point. Normality of data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variance with Levene's test. Differences between groups were analyzed using unpaired t-tests. A p-value <0.05 was considered statistically significant. Histological findings were qualitatively described to support quantitative data.

RESULTS

Intestinal weight changes

Figure 1 illustrates the mean intestinal weight changes (±SD) across all groups at each time point. All groups demonstrated an initial decrease in weight at 5 minutes, followed by progressive increases up to 30 minutes. Ileal segments exposed to *S. flexneri* showed greater increases compared to controls beginning at 15 minutes, while colonic segments exposed to *S. flexneri* exhibited a sharp rise at 30 minutes, surpassing both their control and the ileum treatment group. To account for baseline differences, changes in intestinal weight were calculated at each interval, and cumulative mean weight gain was assessed after 30 minutes (Table 1). The initial decrease in intestinal weight at 5 minutes likely reflects osmotic fluid shifts into the surrounding RPMI medium. Subsequently, weight increased progressively across groups. Ileal segments exposed to *S. flexneri* showed greater increases than controls between 15 and 20 minutes, although fluctuations were observed at 25 minutes. By 30 minutes, the colon exposed to *S. flexneri* exhibited a sharp rise in weight, surpassing both its control and the ileum treatment group. Cumulative analysis revealed that both ileum and colon segments exposed to *S. flexneri* had higher total weight gain compared to controls (ileum: 0.094 g vs. 0.046 g; colon: 0.086 g vs. 0.030 g).

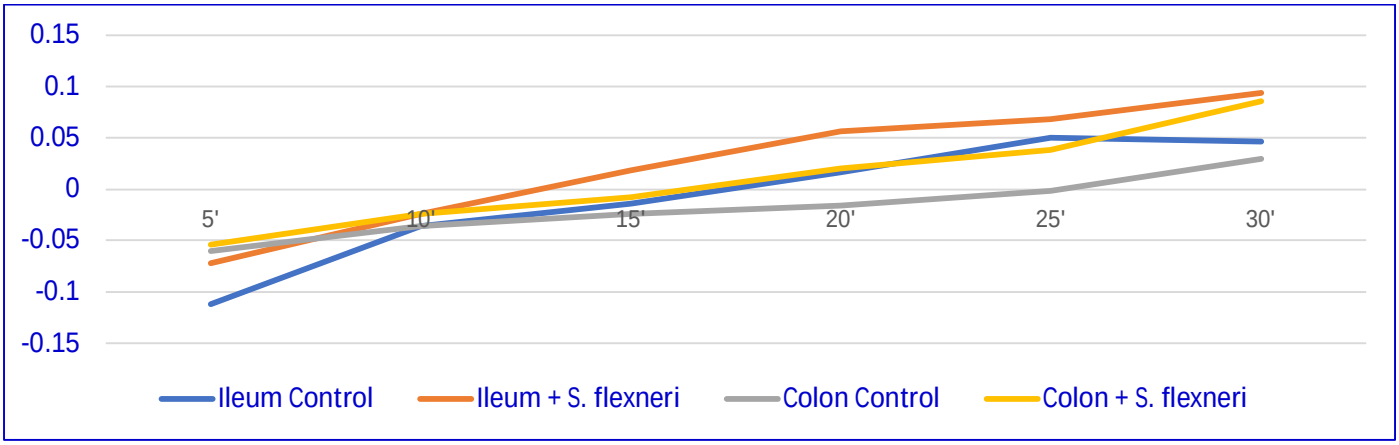

Figure 1. Sequential changes in intestinal weight (mean ± SD) in ileum and colon segments exposed to *Shigella flexneri* or negative control over a 30-minute incubation period.

Table 1. Delta intestinal weight gain (mean ± SD, g) and p-values from unpaired t-tests

Time (minute)	Δ ileum control (g)	Δ ileum + *S. flexneri* (g)	p-value*	Δ colon control (g)	Δ colon + *S. flexneri* (g)	p-value*	ileum + *S. flexneri* vs colon + *S. flexneri* (p-value)
5	-0.112 ± 0.043	-0.072 ± 0.059	0.256	-0.060 ± 0.070	-0.054 ± 0.054	0.883	0.628
10	0.076 ± 0.025	0.048 ± 0.022	0.096	0.024 ± 0.070	0.030 ± 0.019	0.858	0.197
15	0.022 ± 0.001	0.042 ± 0.041	0.321	0.012 ± 0.031	0.016 ± 0.022	0.820	0.250
20	0.030 ± 0.020	0.038 ± 0.018	0.524	0.008 ± 0.023	0.028 ± 0.023	0.203	0.463
25	0.034 ± 0.024	0.012 ± 0.036	0.292	0.014 ± 0.011	0.018 ± 0.013	0.620	0.737
30	-0.004 ± 0.061	0.026 ± 0.045	0.403	0.032 ± 0.019	0.048 ± 0.023	0.265	0.359
Total Δ (0–30 min)	0.046	0.094		0.030	0.086		

*Pairwise comparisons using unpaired t tests.

Mauchly's test indicated that the assumption of sphericity had been violated ($p = 0.001$); therefore, degrees of freedom were corrected using Greenhouse-Geisser estimates. For the ileum dataset, repeated measures ANOVA revealed a significant main effect of time ($F(2.423, 19.381) = 22.543$, $p < 0.001$), indicating that intestinal weight in the ileum changed significantly across measurement intervals. The main effect of group was not

significant ($F(1, 8) = 0.774$, $p = 0.405$), and the interaction between time and group was also not significant ($F(2.423, 19.381) = 0.553$, $p = 0.617$). Similarly, for the colon dataset, repeated measures ANOVA revealed a significant main effect of time ($F(2.361, 23.615) = 14.004$, $p < 0.001$), indicating that intestinal weight in the colon also changed significantly across measurement intervals. The main effect of group was not significant ($F(1, 10) = 0.481$, $p = 0.504$), and the interaction between time and group was likewise not significant ($F(2.361, 23.615) = 0.605$, $p = 0.580$).

Pairwise comparisons using unpaired t-tests did not reveal significant differences between treatment and control groups at any individual time point ($p > 0.05$). These findings suggest that while exposure to *S. flexneri* tended to increase fluid secretion in both ileum and colon, the effect was modest and did not reach statistical significance within the 30-minute observation period.

Histopathological findings

Histological examination revealed evidence of structural damage in both control and treatment groups, which limited the validity of interpretation. In the ileum, loss of mucosal integrity, detachment of the muscularis propria, disruption of villous architecture, and epithelial shedding into the lumen were observed. In the colon, glandular coagulation, mucosal discontinuity, and epithelial detachment were noted. More generally, necrosis, widened intercellular spaces, and erosions were present across all groups. Lymphocytes were identified in some areas, but polymorphonuclear infiltration was absent. Assessment of tight junction integrity was not possible due to widespread autolysis and tissue degradation.

Representative histological images are shown in Figure 2, illustrating mucosal discontinuity and epithelial detachment in both ileum and colon segments. These findings indicate that tissue damage occurred in both control and treatment groups, most likely as a consequence of ex vivo incubation and autolysis, thereby rendering the histological data invalid as supplementary evidence for this study.

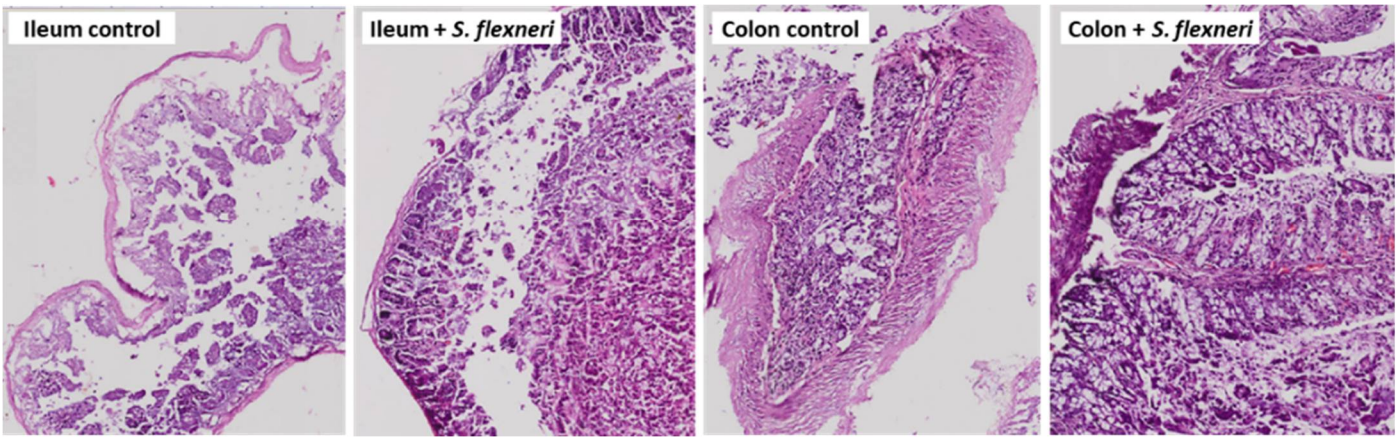

Figure 2. Representative histological sections of ileum and colon segments exposed to *Shigella flexneri* or negative control, showing mucosal discontinuity and epithelial detachment (H&E stain, 40×)

DISCUSSION

This study investigated the early secretory and histopathological responses of ileal and colonic segments following *Shigella flexneri* exposure using an ex vivo ligated loop model. Although statistically significant differences were not observed, a consistent trend toward increased fluid accumulation in exposed segments, particularly in the colon at the 30-minute interval was detected. Rather than indicating an absence of pathogenic activity, these findings likely reflect the temporal dynamics of *Shigella* infection and the inherent biological constraints of the ex vivo system. Early events in enteric infection often unfold before measurable statistical separation emerges, especially when virulence factor expression and epithelial interaction require time to reach maximal effect.

Clinical manifestations of shigellosis range from early watery diarrhea to inflammatory dysentery, reflecting temporally evolving pathogenic processes during infection [10,11]. Experimental models demonstrate that disruption of epithelial barrier integrity and induction of proinflammatory cytokines can occur shortly after *Shigella* exposure, preceding overt inflammatory tissue destruction [6]. Early secretory responses have been attributed primarily to enterotoxins such as ShET1, which stimulate electrolyte and water secretion prior to extensive epithelial invasion and have been shown to induce significant fluid accumulation in rabbit ileal loop models [12]. However, those in vivo loop systems involve intact vascular perfusion, immune cell recruitment, and longer incubation periods conditions that may potentiate toxin-mediated secretion. In contrast, the short-term ex vivo conditions employed in the present study likely limited maximal toxin expression and secretogenic activity, as secretory responses depend on active virulence factor production and sufficient epithelial interaction [5]. Accordingly, the absence of statistical significance within 30 minutes most plausibly reflects submaximal toxin-mediated effects rather than a true lack of biological response.

In contrast to toxin-mediated secretion, the invasive phase of *Shigella* infection depends on the type III secretion system (T3SS), which mediates epithelial invasion, actin cytoskeletal remodeling, and intercellular dissemination [13,14]. This process promotes acute inflammatory responses with neutrophil recruitment, ultimately resulting in mucosal ulceration and dysentery [15]. Importantly, in vivo models demonstrate that polymorphonuclear infiltration and epithelial injury require several hours to become histologically evident [16]. The delayed increase in colonic weight observed at 30 minutes in our study may therefore represent the earliest epithelial interaction preceding overt invasion and inflammatory amplification. The temporal distinction between ileal and colonic responses observed here is biologically plausible, as the colon is the principal site of invasive pathology in human shigellosis [11].

The modest magnitude of secretion observed in this model may also be attributable to species-specific virulence differences. While *S. flexneri* produces ShET1 and related plasmid-encoded factors [12], it lacks the Shiga toxin produced by *S. dysenteriae*, a potent cytotoxin associated with more severe mucosal injury [11]. Comparative epidemiological and experimental data indicate that disease severity varies among *Shigella* species [17], and the absence of robust secretion in short-term experiments may reflect intrinsic differences in toxin potency, expression kinetics, and tissue tropism.

Methodological considerations are critical for interpreting these findings. Unlike conventional in vivo ligated loop models [16], the ex vivo approach eliminates vascular perfusion, immune cell trafficking, and neurohumoral signaling. These systemic factors are known to amplify inflammatory and epithelial responses [18]. The significant main effect of time observed in both ileum and colon suggests that fluid shifts occurred

independently of bacterial exposure, likely reflecting osmotic equilibration within the incubation environment. Consequently, pathogen-specific effects may have been partially masked by physiological fluid redistribution inherent to the ex vivo system.

Histopathological evaluation further underscores the importance of temporal and methodological context. Extensive autolysis observed in both control and treatment groups precluded reliable assessment of inflammatory infiltration or tight junction integrity. Previous investigations have demonstrated that *Shigella* disrupts tight junction–associated proteins and compromises epithelial barrier integrity during infection [19]. In addition, *Shigella* dynamically targets colonic crypts and interacts with epithelial architecture in a spatially regulated manner [20]. Such alterations may precede gross morphological damage and are not always detectable using conventional H&E staining, particularly in partially degraded tissue. Advanced imaging and molecular approaches indicate that early barrier dysfunction involves modulation of junctional complexes rather than immediate necrosis [19]. Therefore, the absence of definitive histological differences in this study should not be interpreted as absence of epithelial interaction, but rather as a limitation of tissue preservation and analytical sensitivity.

Importantly, the observed trend toward sequential ileal and colonic fluid responses is consistent with clinical and experimental evidence indicating that early watery diarrhea may precede invasive colonic disease in shigellosis [11]. Enterotoxin-mediated secretion, including effects attributed to ShET1, is primarily demonstrated in small intestinal loop models [12], whereas invasive and inflammatory processes driven by the T3SS culminate in colonic pathology [11,14]. By capturing the earliest measurable fluid dynamics, this study contributes to understanding the temporal transition between epithelial secretory responses and subsequent inflammatory injury. Although the short incubation period limited detection of maximal pathogenic effects, the data suggest that secretory alterations may begin before overt inflammatory damage becomes histologically apparent.

Several limitations merit acknowledgment. The 30-minute observation period was likely insufficient to capture peak toxin expression or T3SS-mediated invasion. Quantification of enterotoxins and inflammatory mediators was not performed, preventing direct mechanistic correlation between virulence factor expression and fluid accumulation. Moreover, the absence of immune and vascular components restricts extrapolation to clinical disease. Nonetheless, isolating epithelial responses in an ex vivo setting may offer a controlled framework for dissecting early secretory mechanisms without confounding systemic variables.

Future investigations should extend incubation times, incorporate molecular quantification of ShET1 and T3SS activity, and employ organoid or in vivo models to replicate the full complexity of host–pathogen interactions. Integrating functional secretion assays with barrier integrity markers would clarify the temporal relationship between toxin-mediated secretion and invasive inflammatory damage. Such approaches are essential for elucidating the mechanistic continuum from watery diarrhea to dysentery in *S. flexneri* infection.

CONCLUSION

This study demonstrated that exposure of mouse ileal and colonic segments to Shigella flexneri tended to increase intestinal fluid secretion compared to controls, with the most pronounced rise observed in the colon at 30 minutes. Although differences were not statistically significant under short-term ex vivo conditions, the observed trends are consistent with the recognized progression of shigellosis in which early watery diarrhea precedes colonic involvement. These findings highlight the potential of the ex vivo ligated intestinal segment model to capture early fluid secretion responses, while also underscoring the need for extended incubation and complementary approaches to fully characterize the pathogenic mechanisms of S. flexneri. Future investigations incorporating longer observation periods, toxin assays, and improved histological preservation will be essential to clarify the role of S. flexneri in the development of watery diarrhea and dysentery.

Ethical consideration, competing interest and source of funding

-This study was conducted in 2014 as part of an undergraduate thesis project at the Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia. Ethical approval for animal experimentation was obtained from the Institutional Research Ethics Committee of Universitas Brawijaya prior to study initiation. As the research was conducted more than a decade ago, the original approval number is no longer retrievable due to archival limitations. All animal procedures were performed in accordance with institutional guidelines and standard laboratory practices in the Microbiology and Pathology laboratories. No human subjects were involved in this study.

-The authors have no competing interests.

-Source of funding is authors.

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