In Vivo Bioluminescence Imaging for Targeting Acute Hypoxic/Ischemic Small Intestine with Engineered Salmonella typhimurium

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This study aimed at investigating the feasibility of bioluminescence imaging (BLI) with engineered Salmonella typhimurium (ΔppGpp S. typhimurium) for visualizing acute hypoxic/ischemic bowels. At the start of 12- or 24-h reperfusion, ΔppGpp S. typhimurium was injected into the lateral tail veins of rats in which three segments of the small intestine were respectively subjected to 2, 3, and 4 h of ischemia. BLI and magnetic resonance imaging were performed at each reperfusion time point. Bioluminescence was exclusively detected in the hypoxic/ischemic segment of the intestine, showing the ability of ΔppGpp S. typhimurium to specifically target and proliferate in a hypoxic/ischemic area. Serial monitoring of these rat models revealed a progressive increase in bacterial bioluminescence in the ischemic intestines in conjunction with viable bacterial counts. The viable bacterial counts were positively correlated with lactate dehydrogenase levels after 24 h of reperfusion following 3 or 4 h of ischemia as well as interleukin-6 levels after 24 h of reperfusion following 4 h of ischemia. Our findings demonstrated that BLI was able to detect the acute hypoxic/ischemic bowel via monitoring of the distribution, internalization, and activity of administered ΔppGpp S. typhimurium. These findings may be useful for the early diagnosis of ischemic bowel disease.

More importantly, evaluating intestinal viability is essential to surgical decision-making in patients with AMI, but it can be challenging because the appearance of the re-perfused ischemic intestine can be deceptive. Although the standard intraoperative assessment of intestinal viability involves evaluating color and motility as well as bleeding of the cut ends, this method might lead to a non-specific misclassification and observational biases and requires extensive clinical experience. Another method for determining intestinal viability is second-look laparotomy within 48 h to reinspect areas of questionable viability. However, up to 57% of patients have been shown to require further bowel resection. Therefore, it might be clinically important to identify new diagnostic methods to effectively manage patients with intestinal I/R injury at an earlier stage.

Various imaging modalities have emerged for preclinical studies of small animal models to understand human biology and disease, such as magnetic resonance imaging (MRI), positron emission tomography, and single photon-emission computed tomography. In particular, bioluminescence imaging (BLI), an optical molecular imaging modality that allows highly sensitive and quantitative detection of target tissue in animal models, has become a popular strategy for the real-time analysis of dynamic biological processes.

In AMI, damage to the bowel wall occurs because of hypoxia resulting from the interruption of blood flow in the mesenteric vessels. These...
hypoxic and anoxic microenvironments may be targeted by obliga-
tory or facultative anaerobic bacteria such as Salmonella typhimu-
rium that are defective in the synthesis of ppGpp (ΔppGpp S. typhi-
murium), as reported in a previous study7 that demonstrated the selective
accumulation of the bacteria in the infarcted myocardium. Indeed,
such bacteria could accumulate and actively proliferate within
infarcted tissue, resulting in a 1,000-fold or even greater increase in
bacterial numbers relative to those in normal organs such as the liver
and spleen. Based on these prior findings, we assumed that insight
into the specific localizations and real-time monitoring of ΔppGpp
S. typhimurium migration and proliferation could be obtained using
BLI in an animal model with I/R-induced bowel injury.

This preclinical study aimed to investigate the feasibility of BLI with
engineered ΔppGpp S. typhimurium for visualizing the acute hypox-
ic/ischemic bowel.

RESULTS

Serum Biochemistry

The study timeline is presented in Figure 1. In I/R models, the lactate
dehydrogenase (LDH) levels after the 12 and 24 h of reperfusion were
significantly higher than baseline levels (p < 0.05) (Figure 2). The aspar-
tate aminotransferase (AST) level after 12 h reperfusion was signifi-
cantly higher than the baseline level (p < 0.05). Although the interleukin
(IL)-6 level was significantly elevated after 24 h of reperfusion compared
to the value after 12 h of reperfusion, it was undetectable at baseline.
In sham controls, no significantly different serum enzymatic activity levels
were detected between baseline and after 12 and 24 h of reperfusion for
AST (108.40 ± 19.78 versus 106.50 ± 21.92 versus 118.33 ± 38.89), ALT
(51.80 ± 15.82 versus 51.50 ± 14.85 versus 59.67 ± 26.58), albumin
(1.91 ± 0.59 versus 1.63 ± 0.54 versus 1.66 ± 0.27), and LDH
(467.40 ± 122.69 versus 493.50 ± 38.89 versus 486.33 ± 50.30). The
IL-6 level in sham controls was not detected at any time point.

Histological and Immunofluorescence Analyses

Regarding the degree of I/R injury, the 33 separate bowel segments of
11 rats were categorized as grade 1 (n = 4) in I/R bowels that under-
grew 2 h of ischemia followed by 12 h of reper-
fusion; grade 1 (n = 3) or grade 2 (n = 4) in I/R
bowels with 2 h of ischemia followed by 24 h of reper-
fusion; grade 1 (n = 1), grade 2 (n = 2), or grade 3 (n = 1) in I/R
bowels with 3 h of ischemia followed by 12 h of reperfusion; grade 3
(n = 2), grade 4 (n = 3), or grade 5 (n = 2) in I/R bowels with 3 h of
ischemia followed by 24 h of reperfusion; grade 2 (n = 1), grade 3 (n =
2), or grade 4 (n = 1) in I/R bowels with 4 h of ischemia followed by
12 h of reperfusion; and grade 2 (n = 1), grade 3 (n = 2), or grade 4 (n =
2), or grade 6 (n = 4) in I/R bowels with 4 h of ischemia followed by 24 h of reper-
fusion (Table 1).

Inflammatory cells including neutrophils were observed in the I/R
injured bowel wall. Furthermore, the degree of inflammatory cell
infiltration was proportional to the severity of the mural injury. Ac-
cording to the pathological findings, the degree of mural damage of
the intestines was positively correlated with I/R duration. However,
the sham group did not show obvious macroscopic or microscopic
injury in the bowel wall. Moreover, the heart, liver, and spleen of
the I/R group showed no pathological findings on hematoxylin and
eosin (H&E) staining.

Immunofluorescence staining showed that ΔppGpp S. typhimurium
was visible in the hypoxic/ischemic bowel wall (Figure 3). Further-
more, the intensity of signals in the bowel wall, where the bacteria
colonized, was positively correlated with I/R duration.

Localization of S. typhimurium in the I/R Intestine

The engineered ΔppGpp S. typhimurium was injected intravenously
into I/R rats. Thereafter, whole-body BLI was performed after 12
and 24 h of reperfusion using an in vivo imaging system. Biolumines-
cence was detected in the hypoxic/ischemic intestine (Figure 4). How-
ever, no significant signals were detected in the remaining bowel loop
and other organs. Serial monitoring of the rats with I/R injury re-
vealed a progressive increase in bioluminescence in the I/R bowel, re-
flecting an increase in the number of bacteria.

The bacterial colony-forming units (CFU)/g of segmented bowel tis-
sues were significantly increased in rats subjected to the mesenteric I/
R compared to those in the sham group (Figure 5). To correlate the
localized bioluminescent signals with bacterial load in the I/R intestine, the numbers of bacteria were counted in various organs. Each I/R-injured bowel segment had a relatively small bacterial burden after 12 h of reperfusion. The number of CFU in I/R tissue increased dramatically, reaching a maximum of $\sim 10^7$ CFU/g after 24 h of reperfusion. Bacterial colonies were also found in other organs such as the heart, liver, and spleen, reflecting the reticuloendothelial nature of these organs as reported previously. However, the bacterial burden in the liver and spleen did not increase during the same period of reperfusion. The intensity of the bacterial bioluminescence from the I/R intestine correlated closely with the bacterial count in rats that underwent 2, 3, and 4 h of ischemia accompanied by 24 h of reperfusion ($R^2 = 0.73$, $p < 0.001$).

**Assessment of Intestinal I/R Injury Using MRI**

T1-weighted gadolinium-enhanced MRI was performed after 12 and 24 h of reperfusion. However, these images did not show the I/R bowel segment specifically (Figures 4I and 4J).

**DISCUSSION**

Our study demonstrated that genetically engineered bacteria, $\Delta$ppGpp S. typhimurium, exhibited significant bacterial colonization at the acute hypoxic/ischemic bowel wall. In patients with AMI, confirming the resection margin is among the most important factors contributing to postoperative mortality and morbidity. However, in cases of intestinal I/R injuries, there appears to be no clear difference between reversibly and irreversibly damaged bowel tissue in conventional imaging studies. Given this limitation, our study is noteworthy, as BLI using $\Delta$ppGpp S. typhimurium that was systemically injected helped detect and monitor the I/R-injured small intestine. Indeed, $\Delta$ppGpp S. typhimurium could be a potential biomarker for

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**Figure 2. Results of Serum Enzymatic Activity at Baseline and after 12 and 24 h of Reperfusion in the Intestinal Ischemia/Reperfusion Group**

The boxplots represent interquartile range (including median, upper and lower quartile) and maximum and minimum values. Serum enzymatic activity levels are also expressed as mean ± standard deviation above or below each boxplot. ND, not detected. *p < 0.05, by Kruskal-Wallis test.

**Correlation of Bacterial Counts with LDH and IL-6 Activities**

Bacterial counts were positively correlated with LDH levels after 3 h ($R^2 = 0.73$, $p = 0.013$) and 4 h ($R^2 = 0.85$, $p = 0.004$) of mesenteric ischemia followed by 24 h of reperfusion (Figure 6). In addition, bacterial counts were also positively correlated with IL-6 levels after 24 h of reperfusion following 4 h of ischemia ($R^2 = 0.86$, $p = 0.014$). There was no significant correlation between bacterial counts and LDH or IL-6 activity in the other bowel segments.

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**Assessment of Intestinal I/R Injury Using MRI**

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monitoring intestinal viability and evaluating the degree of intestinal I/R injury.

AMI is an abdominal emergent condition that results from the occlusion of the mesenteric artery or vein. Interruption of the blood flow to the bowel wall can lead to macro- and micro-circulatory failure with abrupt onset and induce bowel necrosis. Furthermore, intestinal I/R tends to cause inflammatory reactions, as evidenced by the increased LDH and IL-6 activity at 24 h of reperfusion in our study. A histological change in the I/R-injured bowel wall is characterized by necrosis and a loss of villous epithelium, villus length shortening, and inflammatory cell invasion, which gradually extends outward from the mucosa to the outer muscular layer. In our rat model, we observed damage to the villous mucosa, including cellular detachment, particularly in the villi’s apical regions, giving rise to degraded mature epithelial cells within the lumen.

In the present study, significant morphological changes were observed in rats in which 2, 3, or 4 h of ischemia was followed by 12 or 24 h of reperfusion. In the 2-h ischemia group, the degree of mucosal injury was minimal to moderate; this was observed as a morphological spectrum from subepithelial edema at the villous tips to extensive edema and congestion. In the group with 3 h of ischemia followed by 24 h of reperfusion, some areas with denuded tips, intact muscularis mucosae, and submucosal necrosis were detected. The most severe injury, transmural infarction including complete necrosis of the mucosa, submucosa, and proper muscle layer, was observed in the 4-h ischemia group. Moreover, the neutrophil infiltration intensity was correlated with the degree of mural damage. This observation suggests that a localized inflammatory change played a role in the exclusive distribution of *S. typhimurium* in the hypoxic/ischemic bowel wall. In particular, the degree of ischemic injury in the bowel dramatically increased with irreversible damage occurring in a group in which 4 h of ischemia was followed by 24 h of reperfusion. Contrary to the above findings, on contrast-enhanced MRI, the bowel segments, which were considered irreversibly injured histologically, still appeared viable after 3 or 4 h of ischemia, reflecting the limitation of MRI for evaluating the viability of the I/R-injured bowel.

The mechanism underlying bacterial targeting of hypoxic/ischemic tissue is an intriguing question. A molecular imaging study demonstrated that ΔppGpp *S. typhimurium* showed a selective propensity for infarcted myocardium as well as for sites of inflammation induced by the localized injection of complete Freund’s adjuvant. Thus, it is plausible that the I/R-injured bowel wall with inflammatory cells might attract *S. typhimurium*. Moreover, the augmented, infection-induced O₂ demands and impaired local tissue perfusion might contribute to the low O₂ microenvironment in the infected tissues. Under hypoxic conditions, the replication rate of *S. typhimurium* is boosted, reflecting that low O₂ conditions impair the elimination of t3ss2-non-expressing *S. typhimurium*. As described in a previous study, hypoxia regulates the immune response. We observed high IL-6 levels after 24 h of *S. typhimurium* infection under hypoxic conditions. Based on the positive correlation between viable bacterial counts and IL-6 levels, hypoxia presumably had a strong impact on the bacterial activity of *S. typhimurium* in conjunction with increased expression of pro-inflammatory cytokines. Additionally, LDH levels were higher in the I/R groups after both 12 and 24 h of reperfusion than those at baseline, which indicated that the levels increased in proportion to the increase in reperfusion time. A clinical study of acute intestinal obstruction demonstrated that LDH was abundant in the intestinal mucosa and that the serum LDH level increased under tissue hypoxia/ischemia. Furthermore, in our study, there was a positive correlation between LDH levels and viable bacterial count. Taken together, our findings suggest that inflammatory cells that accumulated in the ischemic bowel tissue, the low O₂ level, and cyto-kines secreted in the I/R-injured intestine together facilitated the creation of a metabolic environment favorable for bacterial deposition and proliferation. Elucidating this mechanism was beyond the scope of the present work, and further studies will be necessary to completely understand the mechanism by which *S. typhimurium* targets the I/R-injured intestine.

It is possible that the bacteria could systemically spread through the bloodstream after intravenous injection of bacteria. Indeed, in our study, bacteria were also found in several organs such as the liver, spleen, and heart other than the I/R-injured bowel, most likely captured by phagocytic macrophages. Although the inadvertent transfection of non-target organs might be inevitable, this issue might not be serious because previous studies demonstrated that the bacterial burden in the liver and spleen after administration of ΔppGpp *S. typhimurium* began to decline rapidly after 24 h, reaching undetectable levels with no systemic inflammatory reactions in non-target regions, whereas the number of CFU in hypoxic regions of the infarcted tissue increased dramatically during the same period of time. Also, there was no sign of serious local or systemic inflammatory reactions following administration of ΔppGpp *S. typhimurium*, and

### Table 1. Histopathological Grading of Intestinal Injury for 2, 3, and 4 h of Ischemia followed by 12 or 24 h of Reperfusion

| Grade | Features | 12-h Reperfusion | 24-h Reperfusion |
|-------|----------|-----------------|-----------------|
|       |          | Ischemic Period | Ischemic Period |
|       |          | 2 h 3 h 4 h     | 2 h 3 h 4 h     |
| 0     | no pathological change |               |                 |
| 1     | focal loss of surface epithelium | n = 4 | n = 1 | n = 3 |
| 2     | mucosal infarction | n = 2 | n = 1 | n = 4 |
| 3     | submucosal infarction | n = 1 | n = 2 | n = 2 |
| 4     | mural infarction | n = 1 | n = 3 | n = 1 |
| 5     | mural infarction | n = 2 | n = 2 | n = 2 |
| 6     | transmural infarction | n = 4 |               |                 |

*For histopathological grading guidelines, see Strand-Amundsen et al.*

*a* There is loss of muscularis mucosae with complete necrosis of mucosa and submucosa.

*b* Involvement of the inner layer of muscularis propria with the complete necrosis of the mucosa and submucosa.
bioluminescence began to decline rapidly in target regions since 7 days after bacteria injection. Consequently, ΔppGpp S. typhimurium used in our experimental protocol appears to specifically target and proliferate in the hypoxic/ischemic tissue without causing significant systemic infection.

BLI has emerged as a useful tool in experimental studies using small animal models. However, this technique might be limited by the depth of the penetration and size of the imaging system for large animal studies, especially in human applications. Indeed, there are still many hurdles that BLI technology needs to surpass in order to become effectively applicable to human studies. Nevertheless, during the next 5–10 years, the field of BLI is expected to expand with the development of more stable, higher yielding luciferases and substrates capable of producing a palette of signal wavelengths enabling multisource imaging, resulting in more accurate data collection and active use in large animal models. After BLI methods are established in large animal models for testing clinical hypotheses and upon confirming biocompatibility and bioactivity, translation to clinical trials using well-established nuclear imaging in humans may be possible.

There are several limitations to the present study. First, in our study, the bacterial burden in the I/R-injured bowel was evaluated at two time points after the intravenous injection of bacteria. A follow-up study that includes more frequent evaluation of the bacterial burden in the target tissue may be needed to understand temporal changes in the localization of injected bacteria more concretely with the help of BLI. Second, technical considerations are needed to apply this BLI approach to larger animal models. Because the depth of the targeted organs is a critical factor in the use of BLI, the organ of interest would need to be exposed by incising the abdominal wall to facilitate the entry of the BLI detector. Third, even though we measured IL-6 levels, other inflammatory cytokines such as IL-1β were not included in our study.

In conclusion, our study demonstrated that in vivo BLI is a feasible method for detecting the acute hypoxic/ischemic bowel by monitoring the distribution, internalization, and activity of injected ΔppGpp S. typhimurium. These findings might be useful for the early diagnosis of ischemic bowel disease.

**MATERIALS AND METHODS**

**Animal Models**

All animal experiments were approved by the Institutional Animal Care and Use Committee of Chonnam National University. Fourteen 7-week-old pathogen-free male Sprague-Dawley rats (weighing 331–340 g) were obtained from Orient Co. (Iksan, Korea). After 2 weeks of adaptation, the rats were housed in individual cages in a temperature-controlled room with alternating 12-h light/12-h dark cycles. After 12-h fasting, the rats were randomly assigned to the following two groups: (1) the sham group (n = 5), designed to assess the risks of the surgery and of anesthetic agent use, in which the same surgical procedure as in the intestinal I/R group was performed, but without superior mesenteric artery (SMA) clamping; and (2) the intestinal I/R group (n = 11), subjected to occlusion of the SMA branches that supply three separate intestinal segments for 2, 3, and 4 h accompanied by 12 (n = 4) or 24 h (n = 7) of reperfusion (Figure 1).

The mean body weights of the rats were not significantly different between the groups (sham versus I/R group after 12-h reperfusion and sham versus I/R group after 24-h reperfusion: 298.00 ± 2.65 g versus 293.00 ± 4.24 g and 300.67 ± 1.53 versus 294.63 ± 6.70 g, respectively).

**Surgical Procedures**

After being placed in the supine position, each rat was anesthetized with 2.5%–3% isoflurane mixed with oxygen (1 L/min), after which a 2.5-cm midline abdominal laparotomy was performed. The surgical area was covered with a sterile non-adherent pad moistened with
saline. The jejunal and ileal small bowel loop and the corresponding mesentery were isolated and exteriorized using cotton swabs moistened in saline. The branches of the SMA were then occluded using non-crushing microvascular clips to create three separate ischemic bowel segments with a length of 7 cm each with more than 2-cm intervals between the ischemic segments. Each SMA branch supplying the corresponding bowel segments was occluded for 2, 3, and 4 h and marked with different colored silk threads, and heparin solution (6 USP U/mL) was administered every 30 min through the intravenous catheter in the tail vein to prevent blood clotting. The breathing rate was monitored with a pressure transducer (model 1025; SA Instruments, Stony Brook, NY, USA) and maintained at 60–70 breaths per minute. Core body temperature was maintained at approximately 36°C by a warming lamp and a heating pad that was supplied by a circulating warm water pump. A wipe moistened with saline was applied to the surgical area during the entire procedure. Reperfusion was initiated by releasing the microvascular clips and was verified by observing the return of color and pulsation in the previously non-perfused bowel segments. The intestine was then pushed back into the abdominal cavity using saline-moistened cotton tips. The anterior abdominal wall was then closed by sterile sutures. Resuscitation was performed by intraperitoneal administration of 0.5 mL/100 g of normal saline. The rats were maintained in heated clean cages for 12 or 24 h.

Serum Biochemical Analysis
Blood samples (0.3 mL/rat) were taken from all rats 10 h before ischemia (baseline), after 12 h of reperfusion, and at the end of 24 h of reperfusion. However, the blood could not be collected at each hour during ischemia since blood sampling under ischemic conditions could lead to risks of complications and adverse effects such as hemolysis. Serum samples were collected by centrifugation at 3,000 rpm for 15 min and stored at –80°C until analysis. The level of IL-6, an inflammation marker, was quantified by enzyme-linked immunosorbent assay kits (BD Pharmingen, San Diego, CA, USA) according to the manufacturer’s instructions. Based on a previous study, which showed that harmful effects caused by intestinal ischemia were not limited to the affected intestine (e.g., hepatic hypoperfusion and acute hepatic damage after I/R), serum biochemistry

Figure 4. BLI and MRI in Representative Rat Models
(A) Normal intestine. (B) Three segments for 2, 3, and 4 h of ischemia accompanied by 24 h of reperfusion in the small intestine. (C) A fresh culture of ΔppGpp S. typhimurium (2 × 10^6 CFU). (D–G) Representative in vivo bioluminescence images before (D and F) and after (E and G) laparotomy at 12 h of reperfusion (D and E) and 24 h of reperfusion (F and G) following an injection of ΔppGpp S. typhimurium through the lateral tail vein in a rat model with intestinal ischemia/reperfusion injury. (H) Bioluminescence images of the indicated isolated organs from a representative animal. (I and J) T1-weighted MR images before (I) and after (J) gadolinium enhancement taken after 24 h of reperfusion. (K and L) Bioluminescence images before (K) and after (L) laparotomy after 24 h of reperfusion following an injection of ΔppGpp S. typhimurium through the lateral tail vein in a sham control. The colored arrowheads indicate the corresponding bowel segments that were subjected to 2, 3, and 4 h of ischemia.
analysis also included AST, alanine aminotransferase (ALT), and albumin as part of a liver function test, as well as LDH as a general indicator of the presence and severity of acute or chronic tissue damage in this study. These serum levels were measured by an autoanalyzer using a commercially available test kit (VET TEST 8008; IDEXX Laboratories, Westbrook, ME, USA).

**Bacterial Strain Injections into Rats**

We used the attenuated S. typhimurium strain with depletion of relA and spoT genes (ΔppGpp), which encode ribosome-bound and cytosolic ppGpp synthetase, respectively. Salmonellae were grown in Luria-Bertani broth medium (Becton Dickinson, Franklin Lakes, NJ, USA) with vigorous aeration at 37°C. For monitoring bioluminescence, the bacterial luciferase gene (lux) from S. typhimurium-Xen26 (Caliper Life Sciences, Hopkinson, MA, USA) was transduced into the chromosome of ΔppGpp strain by P22HT int transduction. At the start of the reperfusion through the SMA, a fresh culture of ΔppGpp S. typhimurium (2 × 10^7 CFU) resuspended in 100 μL of phosphate-buffered saline (PBS) was injected through the lateral tail vein using a 1-mL insulin syringe (BD Ultra-Fine II, Becton Dickinson, Franklin Lakes, NJ, USA). Sham control rats were also injected with the bacteria at the same dose.

**Optical BLI**

Following each 12 or 24 h of reperfusion since bacteria were injected, BLI was performed using an in vivo imaging system (Berthold Technologies, Germany) before and immediately after the laparotomy. Imaging signals were quantified as maximum photons per second per centimeter squared per steradian (p/s/cm^2/sr) within the regions of interest.

**MRI**

Abdominal MRI was performed after 12 and 24 h of reperfusion before and after the injection of 0.025 M gadoterate meglumine (Dotarem; Guerbet, Paris, France) using a clinical 3.0 T magnetic resonance scanner (MR750; GE Healthcare, Milwaukee, WI, USA) with a rat coil. A two-dimensional T1-weighted fast spin-echo pulse
sequence was used (repetition time/echo time, 565/9.6 ms; echo chain, 1/1; bandwidth, 31.2 kHz). Seventeen slices of 1.2-mm thickness each and a field of view of 100 × 100 mm² were used to display the rat abdomen.

Quantification of S. typhimurium in Different Organs of Rats

After sacrificing the rats after 12 h (n = 4) or 24 h (n = 7) of reperfusion, the hearts, livers, spleens, and half of the three bowel segments for all rats were extracted, weighed, and then stored individually in sterile tubes containing PBS at 4°C. Samples were transferred to sterile homogenization tubes, homogenized, and returned to the original tubes for preparation of serial dilutions with PBS. Luria-Bertani agar plates containing kanamycin (50 μg/mL) were inoculated with the homogenate and the plates were incubated overnight at 37°C. Colonies were counted and bacterial loads were expressed as CFU per gram of tissue.

Histological and Immunofluorescence Staining

For histological and immunofluorescence analyses, the bowel tissue samples were divided into two portions. First, tissue samples were fixed in 10% formaldehyde. After 24 h of fixation, the fragments were dehydrated, cleared, and embedded in paraffin. The paraffin blocks were cut into 3-μm-thick sections and stained with H&E for assessment of the degree of I/R injury. The histopathological images were acquired using an IEEE 1394 digital camera (FO124SC; FOculus, Finning, Germany). One pathologist, who was blinded to the information regarding the occlusion time of SMA branches and the duration of reperfusion, evaluated the degree of intestinal tissue injury according to the Swerdlow scoring system using the 7-point grading scale as presented in Table 1.

Second, the remaining tissue samples were processed simultaneously for immunofluorescence. Preparation of the samples for heat-induced epitope retrieval was performed for 5 min in a 125°C pressure cooker with 10 mmol/L citrate buffer (pH 6.0) for mouse anti-desmin (1:100; Dako, Copenhagen, Denmark), rabbit anti-Salmonella (1:200; AbD Serotec, Oxford, UK), and mouse anti-RLuc (1:50; Millipore, Billerica, MA, USA) antibodies. The slides were then incubated with each primary antibody overnight at 4°C. To identify the damaged intestine using anti-desmin antibody, a streptavidin-horseradish peroxidase detection system was used, and visualization was done based on chromogen reactions of the tissue sections that were initially treated with 0.02% diaminobenzidine.8 To detect S. typhimurium located in the damaged intestinal wall, anti-Salmonella antibody and anti-desmin antibody stained with Alexa Fluor 488-conjugated chicken anti-rabbit antibody (Molecular Probes, Eugene, OR, USA) and Cy3-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories, Westgrove, PA, USA), which emit green and red fluorescence, respectively, were used. After being treated with a fluorescently labeled secondary antibody, the slides were mounted with 4′,6-diamidino-2-phenylindole/antifade solution (Millipore). Antibody diluent (Dako) was applied as a negative control. The sections were analyzed using a Zeiss LSM 510 META confocal laser-scanning microscope (Carl Zeiss, Göttingen, Germany).8

Statistical Analysis

All statistical analyses were performed using MedCalc software version 19.0.4 (MedCalc Software, Ostend, Belgium). Data were analyzed using the Kruskal-Wallis one-way analysis of variance with a post hoc test by Conover. In addition, the correlation of bacterial viable counts with serum enzymatic activity was analyzed by Spearman’s correlation test with a 95% confidence interval. A p value less than 0.05 was considered significant.

AUTHOR CONTRIBUTIONS

C.-M.M., S.-S.S., and S.-H.H. designed the study; C.-M.M. and J.H.Z. performed the majority of experiments; C.-M.M., S.-S.S., S.-H.H., J.-
J.M., and Y.Y.J contributed to the analysis and interpretation of results; C.-M.M. wrote the first draft of the manuscript; S.-S. and S.-H.H. have approved the final manuscript and completed manuscript; also, all authors agreed with the contents of the manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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