

**TITLE PAGE**  
**- Food Science of Animal Resources -**  
**Upload this completed form to website with submission**

ARTICLE INFORMATION	Fill in information in each box below
<b>Article Type</b>	Research article
<b>Article Title</b>	Inhibition of <i>Salmonella</i> Typhimurium biofilm formation, adhesion, and invasion by whey beverage supplemented with <i>Triticum dicoccum</i> (farro) enzyme
<b>Running Title (within 10 words)</b>	Antagonistic effects of farro enzyme-enriched whey beverage against <i>S. Typhimurium</i>
<b>Author</b>	Jihyeon Baek <sup>1</sup> , Jinho Lee <sup>1</sup> , Yong Jin Jeong <sup>2,3</sup> , Shin Young Oh <sup>2</sup> , Seok-Seong Kang <sup>1,*</sup>
<b>Affiliation</b>	<sup>1</sup> Department of Food Science and Biotechnology, Dongguk University, Goyang 10326, Republic of Korea <sup>2</sup> KMF Co., Ltd., Daegu, 41605, Republic of Korea <sup>3</sup> Department of Food Science and Technology, Keimyung University, Daegu 42601, Republic of Korea
<b>Special remarks – if authors have additional information to inform the editorial office</b>	None
<b>ORCID (All authors must have ORCID) <a href="https://orcid.org">https://orcid.org</a></b>	Jihyeon Baek ( <a href="https://orcid.org/0009-0007-6642-8797">https://orcid.org/0009-0007-6642-8797</a> ) Jinho Lee ( <a href="https://orcid.org/0000-0002-1793-8017">https://orcid.org/0000-0002-1793-8017</a> ) Yong Jin Jeong ( <a href="http://orcid.org/0000-0002-5712-2856">http://orcid.org/0000-0002-5712-2856</a> ) Shin Young Oh ( <a href="http://orcid.org/0009-0008-4988-6320">http://orcid.org/0009-0008-4988-6320</a> ) Seok-Seong Kang ( <a href="https://orcid.org/0000-0001-7029-9122">https://orcid.org/0000-0001-7029-9122</a> )
<b>Conflicts of interest</b> List any present or potential conflicts of interest for all authors. (This field may be published.)	The authors declare no potential conflict of interest.
<b>Acknowledgements</b> State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available. (This field may be published.)	This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (NRF-2020R1A2C1010010).
<b>Author contributions</b> (This field may be published.)	Conceptualization: Jeong YJ, Kang SS. Data curation: Baek J, Lee J. Formal analysis: Baek J. Methodology: Baek J, Lee J, Oh SY. Validation: Baek J, Lee J, Oh SY. Investigation: Baek J. Writing - original draft: Baek J, Jeong YJ, Kang SS. Writing - review & editing: Baek J, Lee J, Jeong YJ, Oh SY, Kang SS.
<b>Ethics approval (IRB/IACUC)</b> (This field may be published.)	This article does not require IRB/IACUC approval because there are no human and animal participants.

**CORRESPONDING AUTHOR CONTACT INFORMATION**

For the <u>corresponding</u> author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Seok-Seong Kang
Email address – this is where your proofs will be sent	sskang@dgu.ac.kr
Secondary Email address	kangelo72@gmail.com
Postal address	Department of Food Science and Biotechnology, College of Life Science and Biotechnology, Dongguk University, 32 Dongguk-ro, Ilsandong-gu, Goyang-si 10326, Republic of Korea.
Cell phone number	+82-10-3669-6409

Office phone number	+82-31-961-5150
Fax number	+82-31-961-5108

ACCEPTED

## Abstracts

*Triticum dicoccum* (Farro)—an ancient wheat species—has recently gained attention for its exceptional health benefits. However, research on its antibacterial and anti-biofilm properties remains limited. Additionally, a growing trend has been observed in releasing enriched or fortified whey beverages to enhance their functionality. Therefore, this study aims to investigate the inhibitory effects of whey beverages supplemented with enzyme-rich fermented farro (WF) on *Salmonella* Typhimurium biofilm formation and explore the underlying mechanisms. Treatment with WF significantly reduced biofilm formation and viability of *S. Typhimurium*. Moreover, WF decreased the bacterial adhesion to and invasion of human intestinal epithelial cells. WF also inhibited gene expression associated with motility and initial adhesion in *S. Typhimurium*, as well as genes involved in quorum sensing (QS), in a dose-dependent manner. Furthermore, WF suppressed the production of the QS signaling molecule autoinducer-2 in a similar dose-dependent manner. Consequently, our findings indicate that the addition of enzyme-rich fermented farro to whey beverage enhances anti-biofilm activity, which is probably attributed to its antimicrobial effects, inhibition of initial adhesion, and QS reduction. These findings offer a promising basis for developing fortified dairy beverages that can enhance food safety and promote human health.

**Key words:** farro; whey beverage; *Salmonella* Typhimurium, anti-biofilm, antibacterial effect

## Introduction

Whey—a by-product of cheese production in the dairy industry, often regarded as waste—has an estimated global production of approximately 153 million tons annually (Kadyan et al., 2021).

However, whey is now recognized as a rich source of bioactive components, including  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, glycomacropeptide, and immunoglobulins (Song et al., 2022).

Recently, in the food industry, innovative products with higher functional value compared to that of traditional options are continually developed, including those fortified with whey (Purkiewicz and Pietrzak-Fiećko, 2021). Ahmadi et al. (2020) demonstrated that a whey beverage fortified with magnesium and vitamin C reduces inflammatory cytokine levels while improving skeletal muscle mass and strength indices. Moreover, homemade whey beverages enriched with up to 50% fruit and vegetable components exhibit significantly higher antioxidant activity and increased levels of phenolic compounds, flavonoids, and lipophilic pigments (Purkiewicz and Pietrzak-Fiećko, 2021). Nonetheless, investigation on the antibacterial and anti-biofilm effects in fortified whey beverages remains insufficient.

*Triticum dicoccum*, commonly referred to as "farro," is among the most ancient wheat species in the world (Arzani and Ashraf, 2017; Padulosi, 1996). While being largely replaced by higher-yielding wheat varieties, such as *Triticum aestivum* (common wheat), by the early 20th century, interest in *T. dicoccum* wheat has recently grown. This resurgence is attributed to its superior nutritional profile, including lower starch digestibility, higher antioxidant content, and abundant dietary fiber, all of which are associated with significant health benefits (Dhanavath and Prasada Rao, 2017). *T. dicoccum* is particularly known for its high concentrations of proteins, dietary fiber, and antioxidant compounds (Lachman et al., 2012). Additionally, its lower starch digestibility makes it particularly suitable for patients with diabetes. Studies show that it can reduce glycemic response in type 2 diabetes models and significantly lower total cholesterol,

triglycerides, and LDL-cholesterol levels compared to that of modern wheat diets (Yenagi et al., 2001). However, research on its antibacterial and anti-biofilm activities against foodborne pathogens remains largely limited.

The fermentation processes of grains have recently emerged as a promising technological approach (Gabriele and Pucci, 2022). The fermentation process alters the chemical composition of grains, enhances the levels of functional compounds, and further boosts the health benefits of the resulting products (Verni et al., 2019). The fermentation of *Oryza sativa* (indica rice) extract with lactic acid bacteria enhances its antibacterial and anti-biofilm effects against foodborne pathogens, including *Salmonella* Typhimurium, *Yersinia enterocolitica*, and *Staphylococcus aureus* (Kwon et al., 2022). Additionally, a study shows that sourdough fermentation enhances the nutraceutical and functional properties of spelt (Gabriele et al., 2023), supporting its potential therapeutic applications in managing hypertension, oxidative stress and inflammatory intestinal diseases.

*Salmonella* is a Gram-negative, rod-shaped bacterium recognized as a major causative agent of acute gastroenteritis (Seo and Kang, 2020). Globally, nontyphoidal *Salmonella* is estimated to be responsible for 93,757,000 cases of gastroenteritis annually, leading to approximately 155,000 deaths annually (Majowicz et al., 2010). *Salmonella* can also be found in various dairy products and dairy herds on farms (Adhikari et al., 2009; Sánchez-Vargas et al., 2011). In food processing environments, biofilms formed by *Salmonella* represent a contamination critical point in the food chain (Soni et al., 2013). Biofilm formation plays an essential role in the survival of bacteria within feed factory environments (Vestby et al., 2009). Therefore, the persistence of *Salmonella* significantly increases the risk of infection, particularly in environments that support bacterial survival. Addressing this issue necessitates a strategic focus on inhibiting biofilm formation, which serves as a protective mechanism for bacteria and an essential factor in its resistance to environmental stressors and sanitation measures.

Consequently, this study aims to evaluate the effects of whey beverages supplemented with enzyme-rich fermented farro (referred to as “farro enzyme”) in inhibiting *S. Typhimurium* biofilm formation. Additionally, the study investigates the inhibitory effect of whey beverage supplemented with farro enzyme on the adhesion and invasion of *S. Typhimurium* in human intestinal epithelial cells.

## **Materials and methods**

### **Preparation of whey beverage supplemented with farro enzyme**

The whey beverage was prepared following the method previously described (Song et al., 2022), with a few modifications. Whey powder was obtained from Samik Dairy and Food Co. Ltd. (Seoul, Korea) and suspended with distilled water in a 1:10 ratio. The whey suspension was sterilized by stirring in a water bath at 80°C for 3 min. The sterilized whey suspension was then mixed with 1% and 10% farro enzyme (v/v), which was provided from GrainOn Co. Ltd. (Seoul, Korea), and subjected to a secondary sterilization process at 60°C for 5 h. The mixture was then centrifuged at  $15,000 \times g$  for 20 min, and the resulting supernatants were adjusted to a pH 7.2–7.4 using NaOH and HCl. The whey beverage was then filtered through a 0.2  $\mu\text{m}$  syringe filter.

### **Bacteria culture and biofilm formation inhibition**

*S. Typhimurium* KCTC 1925 was obtained from the Korean Collection for Type Cultures (Jeongup, Korea) and incubated in Luria–Bertani (LB) medium (LPS solution, Daejeon, Korea) at 37°C. *S. Typhimurium* was incubated for 15 h at 37°C and adjusted to  $2 \times 10^7$  CFU/mL using LB broth. Subsequently, 100  $\mu\text{L}$  of bacterial suspension was added to each well of a 96-well plate, along with 100  $\mu\text{L}$  of whey beverage supplemented with 1% farro enzyme (WF1) or 10% farro enzyme (WF10). The plate was incubated at 37°C for 24 h. Additionally, the bacterial suspension was also incubated with whey beverage without farro enzyme (WF0) under the same

condition as a control. In a separate experiment, a 96-well plate was pretreated with 100  $\mu\text{L}$  of WF0, WF1, or WF10 for 24 h. Following this, 100  $\mu\text{L}$  of *S. Typhimurium* ( $2 \times 10^7$  CFU/mL) was added and incubated at 37°C for an additional 24 h. Conversely, 100  $\mu\text{L}$  of *S. Typhimurium* ( $2 \times 10^7$  CFU/mL) was preincubated at 37°C for 24 h in a 96-well plate and then treated with 100  $\mu\text{L}$  of WF0, WF1, or WF10 for 24 h. Following this, the culture supernatants were discarded, and the culture plates were gently rinsed with phosphate-buffered saline (PBS). *S. Typhimurium* biofilm was stained with 0.1% crystal violet solution for 30 min at 25°C, then washed with PBS to remove excess dye. The bound stain was resolubilized in a solution of 0.1% acetic acid and 95% ethanol, and absorbance was measured at 595 nm using a microplate reader (AMR-100, Allsheng, Hangzhou, China).

### **Confocal laser scanning microscopy**

After confocal dishes were pretreated with 100  $\mu\text{L}$  of WF0, WF1, or WF10 at 37°C for 24 h, 100  $\mu\text{L}$  of *S. Typhimurium* culture ( $2 \times 10^7$  CFU/mL) was added and incubated at 37°C for an additional 24 h. After discarding the culture supernatants, the confocal dishes were gently washed with PBS. The *S. Typhimurium* biofilm was stained using a LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions and observed using a confocal laser scanning microscope (Eclipse Ti-E, Nikon, Tokyo, Japan) at  $\times 600$  magnification.

### **Viability of planktonic *S. Typhimurium***

To assess the viability of planktonic *S. Typhimurium*, a 96-well plate was pretreated with 100  $\mu\text{L}$  of WF0, WF1, or WF10 at 37°C for 24 h. *S. Typhimurium* culture was then added and incubated at 37°C for an additional 24 h. Following incubation, the supernatants were harvested to obtain

planktonic *S. Typhimurium*, which was then serially diluted in PBS. The viability of planktonic *S. Typhimurium* was determined by plating on LB agar.

### **Inhibition of adhesion to and invasion of HT-29 cells**

The adhesion and invasion assay were conducted following the method as previously described (Lee et al., 2022), with some modifications. The human intestinal epithelial cell line HT-29 was maintained in Dulbecco's Modified Eagle's Medium (DMEM; Welgene, Gyeongsan, Korea) containing 10% fetal bovine serum (FBS; Gibco, Burlington, ON, Canada), 100 U/mL penicillin, and 100 µg/mL streptomycin (HyClone, Logan, UT, USA) at 37°C in a 5% CO<sub>2</sub> atmosphere in a humidified incubator. To investigate the inhibitory effects of WF0, WF1, or WF10 on the adhesion of *S. Typhimurium* to HT-29 cells, HT-29 cells were plated in a 12-well plate at  $5 \times 10^5$  cells/mL and grown to full confluence. *S. Typhimurium* cultures were washed with PBS and resuspended in antibiotic-free DMEM containing 10% FBS. The monolayer of HT-29 cells was carefully washed with PBS and subsequently cotreated with the bacterial culture in antibiotic-free DMEM and WF0, WF1, or WF10. Following a 1 h incubation with gentle shaking, the cells were carefully washed with PBS to remove non-adherent bacteria and treated with 0.2% Triton X-100 for 20 min to lyse the adherent bacteria. The adherent bacteria counts were determined by plate count method using LB agar. For the invasion assay, fully confluent HT-29 cells were treated with WF0, WF1, or WF10 and *S. Typhimurium*, as previously described above. After washing with PBS, the cells were treated with gentamycin (100 µg/mL) and suspended in antibiotic-free DMEM for 1 h to eliminate extracellular bacteria. The cells were then lysed with 0.2% Triton X-100, and the invasion of *S. Typhimurium* was quantified by plating on LB agar.

### **Quantitative reverse transcription polymerase chain reaction**



The inhibitory effect of WF0, WF1, or WF10 on *S. Typhimurium* biofilm formation was evaluated by analyzing the expression of biofilm-associated genes using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Following the incubation of WF0, WF1, or WF10 in a 6-well plate for 24 h, *S. Typhimurium* culture was added in each well and incubated at 37°C for an additional 24 h to allow biofilm formation. The biofilm was detached by pipetting, transferred to a 1.7 mL microtube, and centrifuged at  $15,000 \times g$  for 5 min. The resulting bacterial pellets were resuspended in 1.5 mg/mL lysozyme (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for 30 min. Total RNA was extracted from *S. Typhimurium* using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription was conducted using random hexamers and M-MLV reverse transcriptase (Promega, Madison, WI, USA) to synthesize complementary DNA (cDNA). The resulting cDNA was then amplified using the SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan) on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR conditions included an initial denaturation at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s and annealing/extension at 60°C for 31 s. Relative gene expression was quantified using the  $2^{-\Delta\Delta Ct}$  method, with normalization to 16S rRNA. The sequences of primers used in this study are presented in Table 1.

### **S. Typhimurium autoinducer-2 production assay**

To determine autoinducer-2 (AI-2) signaling molecule production of *S. Typhimurium*, a bioluminescence assay was conducted with some modifications to the methodology as previously described (Taga and Xavier, 2011; Wattanavanitchakorn et al., 2014). *S.*

*Typhimurium* cultures were treated with or without WF0, WF1, or WF10 and incubated at 37°C for 24 h. Following incubation, cell-free supernatants were collected by centrifugation at  $12,000 \times g$  for 10 min and subsequently filtered through a 0.2  $\mu\text{m}$  membrane. The AI-2 reporter strain,

*Vibrio campbellii* BB-170, was cultured overnight in an autoinducer bioassay (AB) medium at 30°C. This culture was subsequently diluted 1:5000 in fresh AB medium. The diluted *V. campbellii* BB-170 culture was combined with the cell-free supernatants at a 1:9 ratio and incubated at 30°C for 7 h. Bioluminescence was then measured using a Victor X4 Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). The relative AI-2 production was calculated as a percentage compared to that of the untreated control.

### **Statistical analysis**

All data in this study are presented as the mean  $\pm$  standard deviation from triplicate experiments. Statistical analysis was conducted to compare the results of each assay to that of the respective control group. Statistical significance ( $p < 0.05$ ) was determined using an unpaired two-tailed *t*-test (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA, USA) or one-way analysis of variance (ANOVA) (IBM SPSS Statistics 23, IBM, Armonk, NY, USA).

### **Results**

#### **Inhibitory effect of whey beverage supplemented with farro enzyme on *S.***

#### ***Typhimurium* biofilm formation**

The inhibitory effect of whey beverages on *S. Typhimurium* biofilm formation was evaluated using crystal violet staining. The whey beverage was observed to effectively inhibit biofilm formation in a farro enzyme dose-dependent manner when applied as a pretreatment and cotreatment. Figure 1A shows that biofilm formation was decreased by approximately 19% and 31% when pretreated with WF1 and WF10, respectively, compared to that of the untreated control. Additionally, when cotreated with WF1 and WF10, biofilm formation was significantly reduced by approximately 22% and 60%, respectively, compared to that of pretreatment ( $p < 0.05$ ) (Figure 1B). However, WF0 was unable to inhibit *S. Typhimurium* biofilm formation in

pretreatment and cotreatment conditions ( $p > 0.05$ ). Posttreatment with WF0, WF1, and WF10 did not consistently reduce biofilm formation. Confocal laser scanning microscopy further confirmed the dose-dependent inhibitory effect of farro enzyme supplementation during cotreatment with WF1 and WF10 on *S. Typhimurium* biofilm formation. Untreated *S. Typhimurium* samples demonstrated a dense, thick, and fully matured biofilm, whereas samples treated with WF10 exhibited significantly less dense biofilms, characterized by dispersed individual colonies. These findings indicate the significant inhibitory effect of whey beverage supplemented with farro enzyme in disrupting *S. Typhimurium* biofilm formation.

### **Whey beverage supplemented with farro enzyme suppresses the viability of *S. Typhimurium***

To evaluate whether whey beverage supplemented with farro enzyme inhibits *S. Typhimurium* biofilm formation by reducing bacterial viability in the planktonic state, the viability of planktonic *S. Typhimurium* cultured with WF0, WF1, or WF10 was quantified. Figure 2 shows a dose-dependent decrease in the viability of planktonic *S. Typhimurium* with increasing concentrations of farro enzyme. Incubation with WF10 for 24 h significantly reduced the viability of planktonic *S. Typhimurium* by approximately 88%.

### **Inhibitory activity of whey beverage supplemented with farro enzyme on adhesion and invasion of *S. Typhimurium* in HT-29 cells**

Figure 4A shows that the adhesion of *S. Typhimurium* to HT-29 cells was significantly inhibited by WF1 and WF10 compared to that of WF0 (74% inhibition), with reductions of 44% and 32%, respectively. Similarly, Figure 4B shows that the invasion of *S. Typhimurium* into HT-29 cells was not significantly reduced by WF0. However, WF1 and WF10 (47% and 17%, respectively)

significantly suppressed the invasion of *S. Typhimurium* into HT-29 cells in a dose-dependent manner with respect to farro enzyme supplementation.

### **Whey beverage supplemented with farro enzyme downregulates genes responsible for *S. Typhimurium* biofilm formation**

To investigate the gene regulation involved in *S. Typhimurium* biofilm formation in the presence or absence of WF0, WF1, and WF10, the transcriptional profiles of *filC*, *lsrD*, *adrA*, *sdiA*, *luxS*, and *csgD* were analyzed. The expression levels of genes associated with adhesion and biofilm formation, such as *filC* and *csgD*, were significantly reduced by > 80% in *S. Typhimurium* treated with WF0, WF1, and WF10, compared to that of the untreated control (Fig. 5).

Additionally, gene expression levels associated with QS and regulation, such as *lsrD* and *luxS*, as well as those associated with virulence and motility, including *adrA* and *sdiA*, were significantly decreased. The reduction in gene expression by at least 80% is considered to correlate with the reduction in the viability of planktonic *S. Typhimurium* following treatment with WF0, WF1, and WF10. A significant reduction of *filC*, *lsrD*, *adrA*, *sdiA*, and *luxS* in a supplemented farro enzyme dose-dependent manner was observed ( $p < 0.05$ ). Although not statistically significant, a dose-dependent decrease in *csgD* gene expression was observed with farro enzyme supplementation ( $p > 0.05$ ).

### **AI-2 production reduction following treatment with whey beverage supplemented with farro enzyme**

To assess whether QS is involved in the reduction of *S. Typhimurium* biofilm formation, AI-2 production by *V. campbellii* BB-170 was measured after treatment WF0, WF1, and WF10.

Figure 6 shows that WF0, WF1, and WF10 treatment demonstrated a dose-dependent inhibitory effect of AI-2 in a supplemented farro enzyme, reducing it by > 90%. The reduction in AI-2

production by > 90% is probably associated with the lowered viability of planktonic *S.*

*Typhimurium* following WF0, WF1, and WF10 treatment. This finding suggests that whey beverage supplemented with farro enzyme inhibits QS by decreasing the AI-2 signaling molecule.

## Discussion

*S. Typhimurium* is a major zoonotic pathogen responsible for foodborne outbreaks, with its AI-2/*luxS* QS system influencing virulence, biofilm formation, and food spoilage, ultimately leading to food poisoning and disease transmission (Nahar et al., 2021; Zhao et al., 2017). Previous studies show the antimicrobial activity of whey and its peptide against various foodborne pathogenic bacteria (Abdel-Hamid et al., 2016; Osman et al., 2016). Whey, a by-product of the dairy industry, contains several bioactive components, including branched-chain amino acids, essential amino acids, and functional peptides, and offers health benefits against various metabolic diseases (Boscaini et al., 2023; Patel, 2015). However, to date, the potential biofilm inhibitory effect of unmodified whey beverages remains uninvestigated. Our results demonstrated the potential supplementation of whey with farro enzyme in effectively inhibiting biofilm formation by reducing the adhesion and QS of *S. Typhimurium* along with decreasing its viability. This finding suggests that the functionality of whey can be enhanced by adding farro enzyme, offering the potential to expand the application of whey-based products and generate added value.

To our knowledge, this study is the first to report the anti-biofilm properties of whey beverages supplemented with *T. dicoccum* (farro enzyme). *T. dicoccum*, an ancient wheat species, has recently garnered attention for its numerous health benefits and suitability for organic farming practices (Dhanavath and Prasada Rao, 2017). Fermentation processes have been widely employed to enhance the nutritional and nutraceutical properties of plant-based foods (Garrido-

Galand et al., 2021). A study shows that fermentation improves the functional properties of farro, including its anti-inflammatory and antioxidant activities (Gabriele et al., 2023). Another study shows that whole flours sourced from different species of *Triticum* genus exhibit antimicrobial activity against enteric pathogenic bacteria, including *Escherichia coli* and *S. Typhimurium* (Grande et al., 2023). Another related study shows the antimicrobial potential of a novel peptide WBP-1 from wheat bran (Zou et al., 2025). Furthermore, whey stimulates the growth of probiotic bacteria, demonstrating its potent prebiotic effect (Sánchez-Moya et al., 2017). Whey and butter milk-based formula also modulate gut microbiota by preventing or restoring antibiotic-induced alterations (Bellés et al., 2023). Thus, it is plausible that whey beverages supplemented with farro enzymes could also influence the intestinal microbiome. However, the antibacterial and antibiofilm activity of *T. dicoccum* remains extensively uninvestigated. Therefore, this study is the first to demonstrate that whey beverages supplemented with farro enzyme effectively inhibit *S. Typhimurium* biofilm formation, which also explains the underlying mechanisms involved. The biofilm has the potential to serve as a virulence factor, playing an essential role in the pathogenic mechanisms of *S. Typhimurium* (Eran et al., 2020; Seo and Kang, 2020). The intestinal mucosa is a primary target for *Salmonella*, where, following ingestion, it traverses the gastrointestinal tract to adhere to and invade host tissues (Gagnon et al., 2013; Wagner and Hensel, 2011). These processes are essential steps in pathogenesis; therefore, inhibiting the adhesion and invasion of intestinal epithelial cells is crucial for preventing infection. The initial adhesion and early biofilm formation of *Salmonella* are primarily driven by flagella, with *fliC* encoding the major constituent of the flagellin structure, essential for forming the helical filament (Vilas Boas et al., 2024; Wang et al., 2020). Subsequently, *csgD* coregulates the synthesis of curli fimbriae, whereas the *adrA* gene controls the production of extracellular matrix components, such as cellulose, which contribute to biofilm formation and maintaining its structural integrity (González et al., 2024; Li et al., 2024). Our findings indicate that treatment

with WF1 and WF10 inhibits adhesion and invasion of intestinal epithelial cells by reducing the expression of the *flaA*, *csgD*, and *adrA* genes. These findings correspond with that of Salehi et al. (2017), who demonstrated that flagellar  $\Delta$ *fliC* $\Delta$ *fliB* mutants exhibit reduced adhesion and invasion to intestinal epithelial cells.

Furthermore, the findings of this study indicate that treatment with WF1 and WF10 significantly suppressed the expression of additional QS-related genes in *S. Typhimurium*, including *lsrD*, *sdia*, and *luxS*. QS is a bacterial cell-to-cell communication mechanism in which the production, release, and detection of signaling molecules, known as Ais, occur in response to fluctuations in population density. This process plays an essential role in the behavior of bacteria that cause foodborne diseases (Choi et al., 2012; Escobar-Muciño et al., 2022). QS is commonly targeted as a strategic mechanism for inhibiting biofilm formation (Koutsoudis et al., 2006). The inhibition of *sdia* and *luxS* may be attributed to the suppression of signal molecule biosynthesis or interference with the reception of acyl-homoserine lactone. Alternatively, WF1 and WF10 might promote enzyme inactivation or degrade molecules involved in the QS system (Eran et al., 2020; Mi et al., 2023). 4,5-Dihydroxy-2,3-pentanedione, commonly referred to as “AI-2,” is a key compound in the QS pathway that exists in multiple forms. In *S. Typhimurium*, AI-2 plays a significant role in controlling cellular processes critical to pathogenesis, including the production of virulence factors and biofilm formation (Almasoud et al., 2016; Vijayababu et al., 2018). Our findings indicate that AI-2 production of *S. Typhimurium* treated with whey beverage supplemented with farro enzyme is reduced in a dose-dependent manner. Our study revealed that AI-2 production in *S. Typhimurium* treated with WF1 and WF10 was reduced in a dose-dependent manner with the supplementation of the farro enzyme. Consequently, the reduction in AI-2 production triggered by WF leads to a disruption in biofilm stability and a decrease in the virulence of *S. Typhimurium* by impairing its adhesion and motility.

## **Conclusions**

This study highlights the potential of whey beverages supplemented with farro enzyme as a functional component for enhancing the antibacterial and anti-biofilm properties of whey beverage. The addition of whey beverage supplemented with farro enzyme significantly inhibited *S. Typhimurium* biofilm formation and reduced bacterial adhesion as well as invasion of intestinal epithelial cells. The dose-dependent suppression of biofilm formation was associated with the downregulation of motility, adhesion, and QS-related genes, as well as inhibiting AI-2 production. These findings suggest that whey beverage supplemented with farro enzyme exerts anti-biofilm activity via multiple mechanisms, including antimicrobial action, interference with initial bacterial adhesion, and disrupting QS signaling. This research offers a promising foundation for developing fortified dairy beverages aimed at improving food safety and human health. However, challenges remain for practical application, maintaining sensory quality as drink, and confirming efficacy against a broader range of pathogens. Further research is needed to optimize formulation and evaluate the long-term health benefits of these functional beverages.

## **Conflicts of interest**

The authors declare that they have no conflicts of interest.

## **Acknowledgments**

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MSIT) (NRF-2020R1A2C1010010).

## **Author contributions**

Conceptualization: Jeong YJ, Kang SS. Data curation: Baek J, Lee J. Formal analysis: Baek J. Methodology: Baek J, Lee J, Oh SY. Validation: Baek J, Lee J, Oh SY. Investigation: Baek J.



Writing - original draft: Baek J, Jeong YJ, Kang SS. Writing - review & editing: Baek J, Lee J, Jeong YJ, Oh SY, Kang SS.

## Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

## References

- Abdel-Hamid M, Goda HA, De Gobba C, Jenssen H, Osman A. 2016. Antibacterial activity of papain hydrolysed camel whey and its fractions. *Int Dairy J* 61:91-98.
- Adhikari B, Besser T, Gay J, Fox L, Davis M, Cobbold R, Berge AC, Mcclanahan R, Hancock D. 2009. Introduction of new multidrug-resistant *Salmonella enterica* strains into commercial dairy herds. *J Dairy Sci* 92:4218-4228.
- Ahmadi A, Eftekhari MH, Mazloom Z, Masoompour M, Fararoei M, Eskandari MH, Mehrabi S, Bedeltavana A, Famouri M, Zare M. 2020. Fortified whey beverage for improving muscle mass in chronic obstructive pulmonary disease: A single-blind, randomized clinical trial. *Respir Res* 21:1-11.
- Almasoud A, Hettiarachchy N, Rayaprolu S, Babu D, Kwon YM, Mauromoustakos A. 2016. Inhibitory effects of lactic and malic organic acids on autoinducer type 2 (ai-2) quorum sensing of *Escherichia coli* o157: H7 and *Salmonella* Typhimurium. *LWT-Food Sci Technol* 66:560-564.
- Arzani A, Ashraf M. 2017. Cultivated ancient wheats (*Triticum* spp.): A potential source of health-beneficial food products. *Compr Rev Food Sci Food Saf* 16:477-488.
- Bellés A, Abad I, Sánchez L, Grasa L. 2023. Whey and buttermilk-based formulas modulate gut microbiota in mice with antibiotic-induced dysbiosis. *Mol Nutr Food Res* 67:2300248.

- Boscaini S, Skuse P, Nilaweera KN, Cryan JF, Cotter PD. 2023. The ‘whey’ to good health: Whey protein and its beneficial effect on metabolism, gut microbiota and mental health. *Trends Food Sci Technol* 133:1-14.
- Choi J, Shin D, Kim M, Park J, Lim S, Ryu S. 2012. Lsr-mediated quorum sensing controls invasiveness of *Salmonella typhimurium* by regulating *spi-1* and *flagella* genes. *PloS one* 7:e37059.
- Dhanavath S, Prasada Rao U. 2017. Nutritional and nutraceutical properties of *Triticum dicoccum* wheat and its health benefits: An overview. *J Food Sci* 82:2243-2250.
- Eran Z, Akçelik M, Yazıcı BC, Özcengiz G, Akçelik N. 2020. Regulation of biofilm formation by *Salmonella Typhimurium*. *Mol Biol Rep* 47:5041-5050.
- Escobar-Muciño E, Arenas-Hernández MM, Luna-Guevara ML. 2022. Mechanisms of inhibition of quorum sensing as an alternative for the control of *E. coli* and *Salmonella*. *Microorganisms* 10:884.
- Gabriele M, Arouna N, Ārvay J, Longo V, Pucci L. 2023. Sourdough fermentation improves the antioxidant, antihypertensive, and anti-inflammatory properties of *Triticum dicoccum*. *Int J Mol Sci* 24:6283.
- Gabriele M, Pucci L. 2022. Fermentation and germination as a way to improve cereals antioxidant and antiinflammatory properties. In *Current advances for development of functional foods modulating inflammation and oxidative stress*. B. Hernández-Ledesma and C. Martínez-Villaluenga (ed). Academic Press, Cambridge, MA, USA. pp 477-497.
- Gagnon M, Berner AZ, Chervet N, Chassard C, Lacroix C. 2013. Comparison of the Caco-2, HT-29 and the mucus-secreting HT29-mtx intestinal cell models to investigate *Salmonella* adhesion and invasion. *J Microbiol Methods* 94:274-279.
- Garrido-Galand S, Asensio-Grau A, Calvo-Lerma J, Heredia A, Andrés A. 2021. The potential of fermentation on nutritional and technological improvement of cereal and legume flours: A

- review. *Food Res Int* 145:110398.
- González JF, Laipply B, Sadowski VA, Price M, Gunn JS. 2024. Functional role of the biofilm regulator *csgD* in *Salmonella enterica* sv. Typhi. *Front Cell Infect Microbiol* 14:1478488.
- Grande T, Souid A, Ciardi M, Della Croce CM, Frassinetti S, Bramanti E, Longo V, Pozzo L. 2023. Evaluation of antioxidant and antimicrobial activities of whole flours obtained from different species of triticum genus. *Eur Food Res Technol* 249:1575-1587.
- Kadyan S, Rashmi H, Pradhan D, Kumari A, Chaudhari A, Deshwal GK. 2021. Effect of lactic acid bacteria and yeast fermentation on antimicrobial, antioxidative and metabolomic profile of naturally carbonated probiotic whey drink. *LWT-Food Sci Technol* 142:111059.
- Koutsoudis MD, Tsaltas D, Minogue TD, Von Bodman SB. 2006. Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii*. *Proc Natl Acad Sci USA* 103:5983-5988.
- Kwon H-J, Ahn H, Kim BS, Kang S-S, Lee K-G. 2022. Anti-bacterial and anti-inflammatory activities of lactic acid bacteria-bioconverted indica rice (*Oryza sativa* L.) extract. *Chem Biol Technol Agric* 9:44.
- Lachman J, Orsák M, Pivec V, Jirů K. 2012. Antioxidant activity of grain of einkorn (*Triticum mono-coccum* L.), emmer (*Triticum dicoccum* schuebl [schrack]) and spring wheat (*Triticum aestivum* L.) varieties. *Plant Soil Environ* 58:15-21.
- Lee HB, Kim KH, Kang GA, Lee K-G, Kang S-S. 2022. Antibiofilm, antiadhesive and anti-invasive activities of bacterial lysates extracted from *Pediococcus acidilactici* against *Listeria monocytogenes*. *Foods* 11:2948.
- Li Z, Zhang M, Lei G, Lu X, Yang X, Kan B. 2024. A single base change in the *csgD* promoter resulted in enhanced biofilm in swine-derived *Salmonella* Typhimurium. *Microorganisms* 12:1258.
- Majowicz SE, Musto J, Scallan E, Angulo FJ, Kirk M, O'brien SJ, Jones TF, Fazil A, Hoekstra RM,

- Studies ICOEDBOI. 2010. The global burden of nontyphoidal *Salmonella* Gastroenteritis. Clin Infect Dis 50:882-889.
- Mi J, Yu Z, Yu H, Zhou W. 2023. Quorum sensing systems in foodborne *Salmonella* spp. and corresponding control strategies using quorum sensing inhibitors for food storage. Trends Food Sci Technol 144:104320.
- Nahar S, Jeong HL, Kim Y, Ha AJ-W, Roy PK, Park SH, Ashrafudoulla M, Mizan MFR, Ha S-D. 2021. Inhibitory effects of flavourzyme on biofilm formation, quorum sensing, and virulence genes of foodborne pathogens *Salmonella* Typhimurium and *Escherichia coli*. Food Res Int 147:110461.
- Osman A, Goda HA, Abdel-Hamid M, Badran SM, Otte J. 2016. Antibacterial peptides generated by alcalase hydrolysis of goat whey. LWT-Food Sci Technol 65:480-486.
- Padulosi S. 1996. Hulled wheats: Proceedings of the first international workshop on hulled wheats. Vol 4. S. Padulosi, K. Hammer, and J. Heller (ed). Bioersity International, Castelvechchio Pascoli, Tuscany, Italy. pp 4
- Patel S. 2015. Emerging trends in nutraceutical applications of whey protein and its derivatives. J Food Sci Technol 52:6847-6858.
- Purkiewicz A, Pietrzak-Fiećko R. 2021. Antioxidant properties of fruit and vegetable whey beverages and fruit and vegetable mousses. Molecules 26:3126.
- Salehi S, Howe K, Lawrence ML, Brooks JP, Bailey RH, Karsi A. 2017. *Salmonella enterica* serovar kentucky flagella are required for broiler skin adhesion and Caco-2 cell invasion. Appl Environ Microbiol 83:e02115-02116.
- Sánchez-Moya T, López-Nicolás R, Planes D, González-Bermúdez C, Ros-Berruezo G, Frontela-Saseta C. 2017. In vitro modulation of gut microbiota by whey protein to preserve intestinal health. Food Funct 8:3053-3063.
- Sánchez-Vargas FM, Abu-El-Haija MA, Gómez-Duarte OG. 2011. *Salmonella* infections: An

- update on epidemiology, management, and prevention. *Trop Med Infect Dis* 9:263-277.
- Seo H-J, Kang S-S. 2020. Inhibitory effect of bacteriocin produced by *Pediococcus acidilactici* on the biofilm formation of *Salmonella* Typhimurium. *Food Control* 117:107361.
- Song D, Lee HB, Kim G-B, Kang S-S. 2022. Whey fermented by *Enterococcus faecalis* M157 exhibits antiinflammatory and antibiofilm activities against oral pathogenic bacteria. *J Dairy Sci* 105:1900-1912.
- Soni KA, Oladunjoye A, Nannapaneni R, Schilling MW, Silva JL, Mikel B, Bailey RH. 2013. Inhibition and inactivation of *Salmonella* Typhimurium biofilms from polystyrene and stainless steel surfaces by essential oils and phenolic constituent carvacrol. *J Food Prot* 76:205-212.
- Taga ME, Xavier KB. 2011. Methods for analysis of bacterial autoinducer-2 production. *Curr Protoc Microbiol* 23:1C. 1.1-1C. 1.15.
- Verni M, Rizzello CG, Coda R. 2019. Fermentation biotechnology applied to cereal industry by-products: Nutritional and functional insights. *Front Nutr* 6:42.
- Vestby LK, Møretro T, Langsrud S, Heir E, Nesse LL. 2009. Biofilm forming abilities of *Salmonella* are correlated with persistence in fish meal-and feed factories. *BMC Vet Res* 5:1-6.
- Vijayababu P, Samykanu G, Antonyraj CB, Thomas J, Narayanan S, Ahamed SIB, Piramanayagam S. 2018. Patulin interference with atp binding cassette transferring auto inducer- 2 in *Salmonella* typhi and biofilm inhibition via quorum sensing. *Inform Med Unlocked* 11:9-14.
- Vilas Boas D, Castro J, Araújo D, Nóbrega FL, Keevil CW, Azevedo NF, Vieira MJ, Almeida C. 2024. The role of flagellum and flagellum-based motility on *Salmonella* Enteritidis and *Escherichia coli* biofilm formation. *Microorganisms* 12:232.
- Wagner C, Hensel M. 2011. Adhesive mechanisms of *Salmonella enterica*. *Bacterial Adhesion:*

- Chemistry, Biology and Physics. D. Linke and A. Goldman (ed). Springer, Amsterdam, The Netherlands. pp 17-34.
- Wang F, Deng L, Huang F, Wang Z, Lu Q, Xu C. 2020. Flagellar motility is critical for *Salmonella enterica* serovar Typhimurium biofilm development. *Front Microbiol* 11:1695.
- Wattanavanitchakorn S, Prakitchaiwattana C, Thamyongkit P. 2014. Rapid and simple colorimetric method for the quantification of ai-2 produced from *Salmonella* Typhimurium. *J Microbiol Methods* 99:15-21.
- Yenagi N, Hanchinal R, Patil C, Koppikar V, Halagi M. 2001. Glycaemic and lipidemic response to dicoccum wheat (*Triticum dicoccum*) in the diet of diabetic patients. *Int J Diab Dev Countries* 21:153-155.
- Zhao X, Zhao F, Wang J, Zhong N. 2017. Biofilm formation and control strategies of foodborne pathogens: Food safety perspectives. *RSC Adv* 7:36670-36683.
- Zou Z, Purnawan MA, Wang Y, Ismail BB, Zhang X, Yang Z, Guo M. 2025. A novel antimicrobial peptide wbp-1 from wheat bran: Purification, characterization and antibacterial potential against *Listeria monocytogenes*. *Food Chem* 463:141261.

## Figure legends

**Fig. 1.** Inhibition of *S. Typhimurium* biofilm formation by whey beverage supplemented with farro enzyme. (A) *S. Typhimurium* ( $1 \times 10^7$  CFU/mL) was added to WF0, WF1, and WF10 preincubated at 37°C for 24 h, followed by a further 24-h incubation. (B) *S. Typhimurium* ( $1 \times 10^7$  CFU/mL) was co-incubated with WF0, WF1, and WF10 at 37°C for 24 h. (C) *S. Typhimurium* ( $1 \times 10^7$  CFU/mL) was preincubated at 37°C for 24 h, followed by adding WF0, WF1, and WF10, followed by a further 24-h incubation. After incubation, *S. Typhimurium* biofilm formation was measured using crystal violet staining. Data are expressed as means  $\pm$  standard deviations from at least three independent experiments. Statistically significant differences ( $p < 0.05$ ) among groups are indicated by different superscript letters (a–c).

**Fig. 2.** Changes in *S. Typhimurium* biofilm formation in the presence of WF0, WF1, and WF10. *S. Typhimurium* ( $1 \times 10^7$  CFU/mL) was co-incubated with WF0, WF1, and WF10 on confocal dish. *S. Typhimurium* biofilm was stained with green fluorescent SYTO 9, and formation was analyzed using confocal microscopy. All images shown are representative of at least three independent and consistent observations. The size bars represent 50  $\mu$ m.

**Fig. 3.** Viability of planktonic *S. Typhimurium* in the presence of WF0, WF1, and WF10. *S. Typhimurium* ( $1 \times 10^7$  CFU/mL) was co-incubated with WF0, WF1, and WF10 in a 96-well plate at 37°C for 24 h. The viability of planktonic *S. Typhimurium* was determined using the agar plating method. Data are expressed as means  $\pm$  standard deviations from at least three independent experiments. Statistically significant differences ( $p < 0.05$ ) among groups are indicated by different superscript letters (a–c).

**Fig. 4.** *S. Typhimurium* adhesion and invasion to HT-29 cells in the presence or absence of WF0, WF1, and WF10. HT-29 cells ( $5 \times 10^5$  CFU/mL) were plated and grown to full confluence. The cells were co-incubated with *S. Typhimurium* ( $1 \times 10^7$  CFU/mL) and WF0, WF1, and WF10 for 1 h. (A) For adhesion assays, non-adherent bacteria were removed, and adherent bacteria were lysed with 0.2% Triton X-100 and quantified by plating on LB agar. (B) For invasion assays, extracellular bacteria were eliminated using gentamycin (100  $\mu$ g/mL), and intracellular bacteria were quantified after lysis. Data are expressed as means  $\pm$  standard deviations from at least three independent experiments. Statistically significant differences ( $p < 0.05$ ) among groups are indicated by different superscript letters (a–c).

**Fig. 5.** Downregulation of gene transcription associated with *S. Typhimurium* biofilm formation in the presence of WF0, WF1, and WF10. *S. Typhimurium* ( $1 \times 10^7$  CFU/mL) was co-incubated with WF0, WF1, and WF10 at 37°C for 24 h. The mRNA expression levels of gene transcription associated with *S. Typhimurium* biofilm formation were measured using qRT-PCR. Data are expressed as means  $\pm$  standard deviations from at least three independent experiments. Statistically significant differences ( $p < 0.05$ ) among groups are indicated by different superscript letters (a–c).

**Fig. 6.** Inhibition of AI-2 production by *S. Typhimurium* in the presence of WF0, WF1, and WF10. *S. Typhimurium* was cultured with WF0, WF1, and WF10 at 37°C for 24 h. Cell-free supernatants were mixed with a diluted suspension of *V. campbellii* BB170 culture and incubated at 30°C for 7 h. AI-2 production was measured using bioluminescence, and the relative AI-2 production is presented as a percentage of the control. Data are expressed as means  $\pm$  standard



deviations from at least three independent experiments. Statistically significant differences ( $p < 0.05$ ) among groups are indicated by different letters (a–c).

ACCEPTED

**Table 1.** Primer sequences for qRT-PCR analysis.

Target gene	Sequence (5'-3')
<i>filC</i>	F: GGCGATTGCTAACCGTTTTA R: GCAATGGAGATACCGTCGTT
<i>lsrD</i>	F: ATCGGGCTATGCGCGATTAC R: TCCGGCATTATCAGCCCAC
<i>adrA</i>	F: GGGCGGCGAAAGCCCTTGA R: GCCCATCAGCGCGATCCACA
<i>sdiA</i>	F: CGATCCGGTATTAAAGCCAGAGA R: GGTTCCGGCAACATCACACAC
<i>luxS</i>	F: ACGGTAACGGCGTTGAGATT R: AGCTCCGGGATCTGGTTTTG
<i>csgD</i>	F: CGGCCGGTTGCATTGTTTTA R: TGTTCCCTGGTCTTCAGTGGC
16s rRNA (internal standard)	F: AAAGACTGACGCTCAGGTGC R: AGGGCACAACCTCCAAGTAG

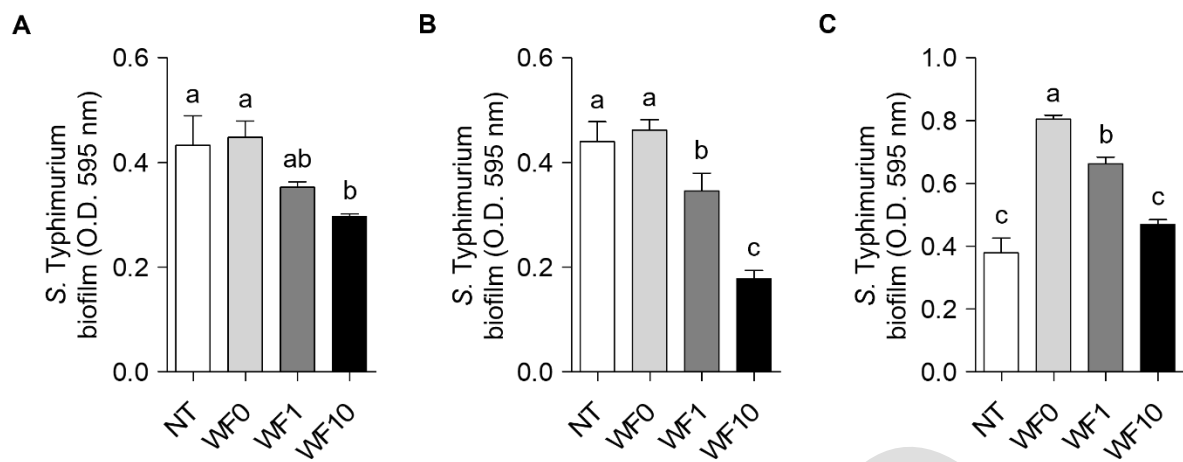


Figure 1

ACCEPTED

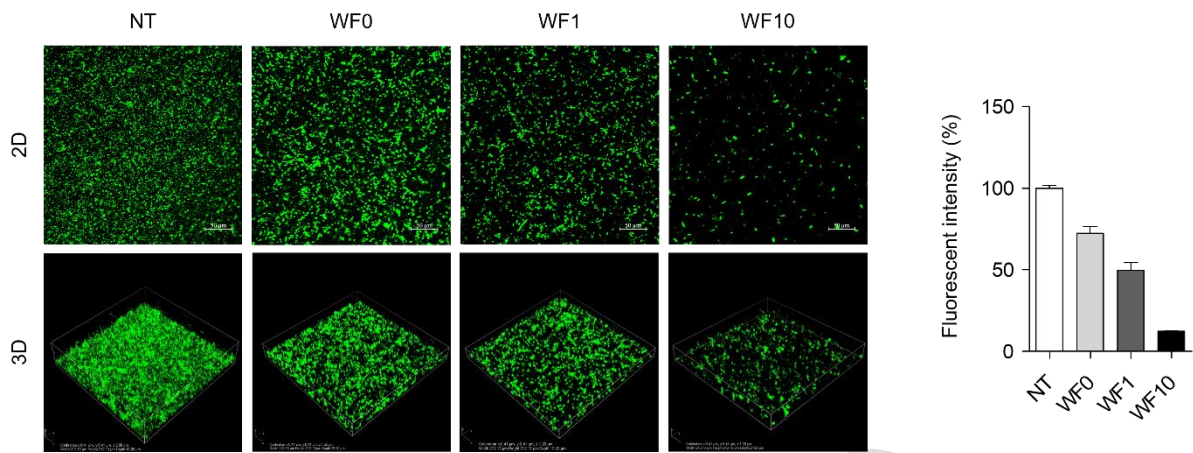


Figure 2

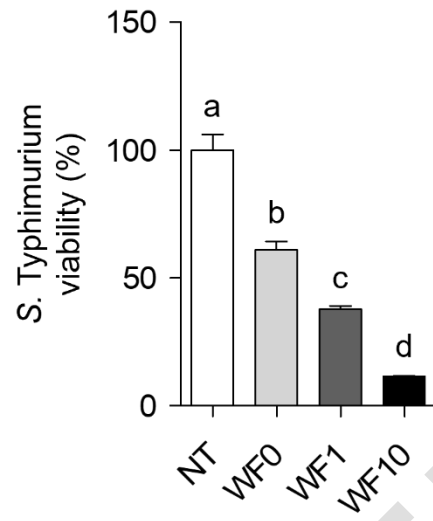


Figure 3

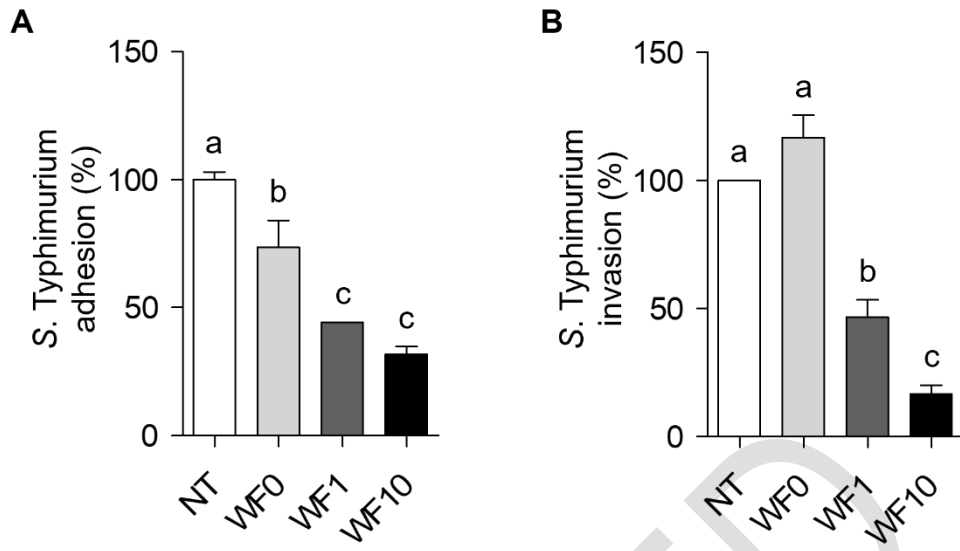


Figure 4

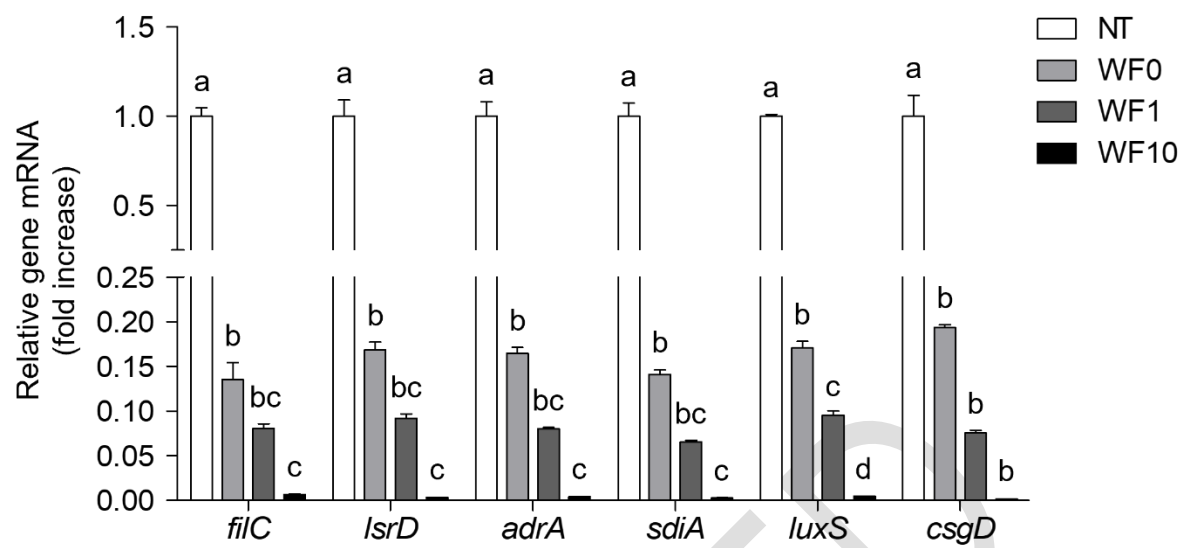


Figure 5

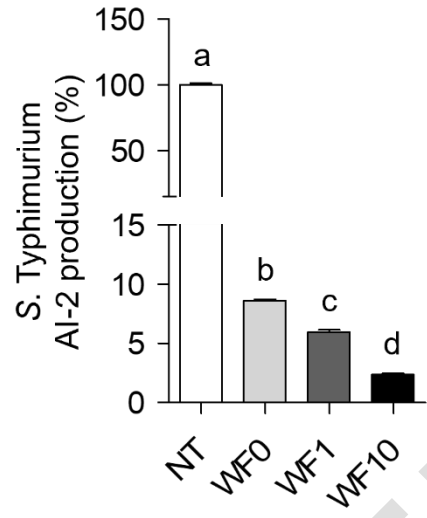


Figure 6

ACCEPTED