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Measuring the Effects of Bacteria and Chemicals on the Intestinal Permeability of Caenorhabditis elegans

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TITLE:

Measuring the Effects of Bacteria and Chemicals on the Intestinal Permeability of *Caenorhabditis elegans*

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KEYWORDS:

3,3'-diindolylmethane, *Caenorhabditis elegans*, FITC-dextran, gut health, high-throughput image analysis, intestinal permeability, *Pseudomonas aeruginosa*.

SUMMARY:

This protocol describes how to measure intestinal permeability of *Caenorhabditis elegans*. This method is helpful for basic biological research on intestinal health related to the interaction between intestinal bacteria and their host and for screening to identify probiotic and chemical agents to cure leaky gut syndrome and inflammatory bowel diseases.

ABSTRACT:

In living organisms, intestinal hyperpermeability is a serious symptom that leads to many inflammatory bowel diseases (IBDs). *Caenorhabditis elegans* is a nonmammalian animal model that is widely used as an assay system due to its short lifespan, transparency, cost-effectiveness, and lack of animal ethics issues. In this study, a method was developed to investigate the effects of different bacteria and 3,3'-diindolylmethane (DIM) on the intestinal permeability of *C. elegans* with a high-throughput image analysis system. The worms were infected with different gut bacteria or cotreated with DIM for 48 h and fed with fluorescein isothiocyanate (FITC)-dextran overnight. Then, the intestinal permeability was examined by comparing the fluorescence images and the fluorescence intensity inside the worm bodies. This method may also have the potential to identify probiotic and pathogenic intestinal bacteria that affect intestinal permeability in the animal model and is effective for examining the effects of harmful or health-promoting chemicals on intestinal permeability and intestinal health. However, this protocol also has some considerable limitations at the genetic level, especially for determining which genes are altered to control illness, because this method is mostly used for

phenotypic determination. In addition, this method is limited to determining exactly which pathogenic substrates cause inflammation or increase the permeability of the worms' intestines during infection. Therefore, further in-depth studies, including investigation of the molecular genetic mechanism using mutant bacteria and nematodes as well as chemical component analysis of bacteria, are required to fully evaluate the function of bacteria and chemicals in determining intestinal permeability.

INTRODUCTION:

Intestinal permeability is considered as one of the main barriers related to the intestinal microbiota and mucosal immunity and is likely to be affected by several factors, such as gut microbiota modifications, epithelial impairment, or mucus layer alterations[1]. Recent papers have reported effective protocols to measure the intestinal permeability of cultured human intestinal cells by analyzing the fluorescence flux rates across the intestinal cell layer[2], but fewer research papers present a suitable procedure for measuring the gut permeability in nematodes, particularly in *C. elegans*, by using FITC-dextran staining.

There are two representative protocols for measuring the gut permeability in *C. elegans* using Nile red[3] and erioglaucine disodium (or the Smurf assay)[4,5]. In this protocol, we used FITC-dextran (average molecular weight 10,000), which has a much higher molecular weight than Nile red (MW = 318.37) and erioglaucine disodium (MW = 792.85). FITC-dextran is more similar than Nile red or erioglaucine disodium dyes to actual macromolecular nutrients such as carbohydrates, which are absorbed through the intestinal layer. The intestinal permeability of *C. elegans* fed with erioglaucine disodium (blue Smurf dye) can be easily evaluated without fluorescence microscopy. However, in the Smurf assay, quantitative analysis of intestinal permeability is difficult due to the lack of standardization and should be evaluated manually[4,5]. In the case of the Nile red assay, Nile red also stains lipid droplets in cells, which may interfere with the exact determination of gut permeability in *C. elegans*[6]. The present protocols enable rapid and precise quantitative analysis of intestinal permeability in *C. elegans* treated with various intestinal bacteria and chemicals while avoiding unspecific lipid staining.

C. elegans is a typical model in biological fields due to its affordable price, easy manipulation, limited animal ethics issues, and short lifespan, which is beneficial for rapid experimentation[7]. In particular, after the entire *C. elegans* genome was published, nearly 40% of genes in the *C. elegans* genome were found to be orthologous to genes that cause human diseases[8]. Moreover, the transparent body allows observation inside the organism, which is advantageous for researching cellular events and for fluorescence applications in cell biology, for example, stem cell staining with DAPI or immunohistochemistry[9]. *C. elegans* is often used as an experimental animal to study the interaction between the gut microbiota and the host; in addition, *C. elega*ns is used to screen health-promoting probiotic bacteria[10-12] as well as dietary chemicals promoting intestinal health[13,14].

Pseudomonas aeruginosa and *Enterococcus faecalis* are well-known gut bacteria that negatively affect the gastrointestinal system, especially the colonic epithelial cells of the intestinal tract[15,16]. Therefore, measuring the gut permeability triggered by these bacteria is necessary for

the screening and development of new drugs that can recover and reduce the damage caused by bacterial inflammation and infection. In this protocol, we tested the effects of these intestinal bacteria on the intestinal permeability of *C. elegans*.

We also report an optimized protocol for testing chemicals on the intestinal permeability of *C. elegans*. For this purpose, we used 3,3'-diindolylmethane (DIM) as a model chemical because DIM is a bioactive metabolite compound derived from indole-3-carbinol, which is present in *Brassica* food plants, and has been reported to have therapeutic effects on IBD in mice[17,18]. In addition, we recently discovered that DIM improves intestinal permeability dysfunction in both cultured human intestinal cells as well as the model nematode *C. elegans*[19].

In this study, we used three different experimental conditions. First, we measured the effects of the different bacteria, *P. aeruginosa* and *E. faecalis,* on intestinal permeability (**Figure 1**). Second, we measured the effects of live and heat-inactivated *P. aeruginosa* on intestinal permeability (**Figure 2**). Third, we measured the effects of DIM (a model chemical) on the intestinal permeability of *C. elegans* fed with *P. aeruginosa* (**Figure 3**).

The objective of this study was to develop optimized protocols that measure the intestinal permeability of *C. elegans*, which is changed by treatment with various intestinal bacteria as well as with chemicals.

PROTOCOL:

1. Preparation of *P. aeruginosa* PAO1 and *Escherichia coli* OP50 culture

1.1. Prepare 500 mL of sterilized Luria-Bertani (LB) medium (**Table 1**) and inoculate a single colony of *P. aeruginosa* into the medium. Incubate the culture for 14 to 15 h at 37 °C with a shaking speed of 150 rpm.

1.2. Equally distribute the bacterial culture into two 500-mL centrifuge tubes and centrifuge the tubes at 3,220 *x g* at 4 °C for 30 min.

1.3. Remove the supernatant until the volume is 50 mL (one-tenth of the initial volume) and resuspend the bacterial pellet.

1.4. Store the concentrated bacterial culture at 4 °C until use. The storage period of *P. aeruginosa culture* can be up to 1 month, but fresh culture is better for inducing intestinal damage.

NOTE: The protocol can be paused here. In addition to the whole live bacteria, the supernatant of the bacterial culture can be used to test the effects of the intestinal bacteria[19].

1.5. To prepare *E. coli* OP50, culture *E. coli* OP50 in DYT medium (**Table 1**) and then apply similar steps to those described above but decrease the volume to one-sixth of the original

volume. For example, if the initial volume is 500 mL, after removing supernatant, the remaining volume is approximately 83 mL.

NOTE: The protocol can be paused here.

2. Preparation of *Enterococcus faecalis* KCTC 3206 culture

2.1. Prepare 500 mL of sterilized brain heart infusion (BHI) broth (**Table 1**).

2.2. Inoculate one colony into 500 mL of BHI broth and incubate the culture for 14 – 15 h in a shaking incubator at 37 °C and 150 rpm.

2.3. Equally distribute the bacterial culture into two 500-mL centrifuge tubes and centrifuge the tubes at 3,220 *x g* at 4 °C for 30 min.

2.4. Remove the supernatant until the volume is 50 mL (one-tenth of the initial volume) and resuspend the pellet.

2.5. Store the concentrated bacterial culture at 4 °C until use. The fresh culture is better for testing the effects on the intestinal permeability.

NOTE: The protocol can be paused here.

3. Preparation of heat-inactivated *E. coli* OP50 and heat-inactivated *P. aeruginosa* PAO1 cultures

3.1. Culture and harvest the bacteria as described above (steps 1.1-1.3).

3.2. Heat-inactivate the *E. coli* OP50 or *P. aeruginos*a PAO1 culture as described previously[20-22]. For heat inactivation, incubate the resuspended bacteria at 65 °C (water bath) for 30 min.

3.3. Cool the concentrated bacterial culture to room temperature and store it at 4 °C until use. The storage period can be up to 1 month.

NOTE: The protocol can be paused here.

4. Preparation of nematode growth medium (NGM) plates for testing the effects of different bacteria on the intestinal permeability of *C. elegans*

4.1. Place 1.25 g of peptone, 1.5 g of NaCl, 8.5 g of agar, a magnetic stirrer and 487.5 mL of distilled water in a 500-mL glass bottle (**Table 1**).

4.2. Mix the mixture well, autoclave the mixture for 15 min at 121 °C and cool the mixture to 55 °C in a water bath for 30 min.

4.3. Remove the medium from the water bath, add the components (0.5 mL of 1 M $CaCl_2$, 0.5 mL of cholesterol, 0.5 mL of $MgSO_4$, 12.5 mL of KPO_4) (**Table 1**) to the NGM, and mix well.

NOTE: All the individual components must be autoclaved except for cholesterol (dissolved in absolute ethanol), and every experimental step must be carried out on a clean bench.

4.4. Distribute 20 mL of NGM to each 90 x 15 mm Petri dish and allow the agar to solidify on a clean bench at room temperature (approximately 20 °C). The NGM plates can be stored for up to one month at 4 °C.

NOTE: The protocol can be paused here. The NGM plates used for FITC-dextran staining were prepared by the same procedure (from steps 4.1-4.3). 10 mL of NGM was distributed to each 60 x 15 mm Petri dish and allowed to solidify on a clean bench at room temperature (approximately 20 °C).

4.5. Remove the bacterial culture from the 4 °C refrigerator and vortex the culture thoroughly before spreading the culture onto the NGM plates.

4.6. Add a total of 800 µL of the bacterial culture to each fresh NGM plate, and allow the plates to dry in a 20 °C incubator overnight.

NOTE: For the first experiment (**Figure 1**), two NGM plates with *E. coli* OP50, one NGM plate with *P. aeruginosa* PAO1, and one NGM plate with *E. faecalis* KCTC3206 were prepared. For the second experiment (**Figure 2**), two NGM plates with live *E. coli* OP50, one NGM plate with live *P. aeruginosa* PAO1, and one NGM plate with heat-inactivated *P. aeruginosa* PAO1 were prepared.

5. Preparation of NGM plates for testing the effects of a chemical (DIM) on the intestinal permeability of *C. elegans* fed with *P. aeruginosa*

5.1. Add 0.5 g of peptone, 0.6 g of NaCl, 195 mL of distilled water, a magnetic stirrer, and 6.8 g of agar to a 500-mL glass bottle (NGM agar).

5.2. Add 0.5 g of peptone, 0.6 g of NaCl, 195 mL of distilled water, and a magnetic stirrer without agar to another 500-mL glass bottle (NGM broth).

5.3. Autoclave the two bottles (from steps 5.1-5.2), one empty 100-mL glass bottle, and one empty 500-mL glass bottle for 15 min at 121 °C. Then, allow the medium containing bottles to cool to 55 °C in the water bath for 30 min. Keep the agar medium in the water bath and remove the bottle containing broth (from step 5.2) for the next step.

5.4. Add the additive chemicals to the NGM broth: 0.4 mL of 1 M $CaCl_2$, 0.4 mL of cholesterol in ethanol (mL), 0.4 mL of 1 M $MgSO_4$ and 10 mL of 1 M KPO_4 (all of these components must be

sterilized apart from the cholesterol in ethanol). Then, stir the mixture thoroughly with a magnetic stirrer at 55 °C.

5.5. Label the autoclaved empty 500-mL glass bottle with dimethyl sulfoxide (DMSO) and the autoclaved empty 100-mL glass bottle with DIM.

5.6. Transfer 50 mL of NGM broth into the DIM-labeled 100-mL bottle. Add 500 µL of 20 mM DIM stock into the bottle and mix well.

NOTE: DIM is dissolved in DMSO (20 mM DIM stock).

5.7. Quickly remove the NGM agar medium from the water bath, add 50 mL of the NGM agar medium into the DIM-labeled bottle and mix thoroughly.

5.8. Place a 20-mL aliquot of DIM-containing NGM medium into each 90 x 15 mm Petri dish. Approximately five DIM-containing NGM plates can be made from this step.

NOTE: The final concentration of DIM in each NGM plate will be 100 µM.

5.9. To prepare the DMSO-containing NGM plate, transfer 150 mL of NGM broth into the DMSO-labeled 500-mL bottle. Add 1.5 mL of DMSO to the bottle and mix well.

5.10. Add 150 mL of the NGM agar medium to the DMSO-labeled bottle and mix thoroughly.

5.11. Place a 20-mL aliquot of DMSO-containing NGM medium in each 90 x 15 mm Petri dish. Approximately fifteen DMSO-containing NGM plates can be made from this step.

NOTE: The final concentration of DMSO in each NGM plate will be 0.5%.

5.12. Solidify the plates at room temperature for at least 3 h and store them at 4 °C until use.

NOTE: The protocol can be paused here.

5.13. Remove the *E. coli* OP50 or *P. aeruginosa* PAO1 culture from the 4 °C refrigerator (from step 1.4) and vortex the culture properly before spreading onto the NGM plates.

5.14. Put 800 µL of the *E. coli* OP50 or *P. aeruginosa* PAO1 bacterial culture onto each fresh NGM agar plate and allow the plates to dry in a 20 °C incubator overnight.

NOTE: The protocol can be paused here. For the third experiment (**Figure 3**), two DMSO-containing NGM plates coated with live *E. coli* OP50, one DMSO-containing NGM plate coated with live *P. aeruginosa* PAO1, and one DIM-containing NGM plate coated with live *P. aeruginosa* PAO1 were prepared.

6. Preparation of age-synchronized *C. elegans*

6.1. Grow worms on a solid NGM plate and feed them with *E. coli* OP50 until the desired population is reached.

6.2. Synchronize the eggs by using either the timed egg laying method or the bleaching solution treatment as described previously[20,23,24].

7. Treatment of bacteria or DIM and FITC-dextran feeding

7.1. Incubate the age-synchronized eggs at 20 °C for 64 h on NGM plates supplemented with live *E. coli* OP50 as food.

7.2. Wash the age-synchronized L4 larva with S-buffer and transfer them (more than approximately 500 worms) to the treatment NGM plates containing different bacteria and chemicals (described in NOTE following step 4.6 and 5.14,). Incubate them at 20 °C for 48 h.

NOTE: The treatment time can range from 24 to 72 h, according to the bacteria and chemicals used. The therapeutic or preventive effect of DIM can also be evaluated by pretreating the worms with pathogens and then treating the worms with DIM (the therapeutic effect) or by pretreating the worms with DIM and then treating with pathogens (the preventive effect)[19].

7.3. To prepare the FITC-dextran-supplemented plates, mix 2 mL of heat-inactivated *E. coli* OP50 with 4 mg of FITC-dextran. Then, divide 100 µL of the FITC-dextran and *E. coli* OP50 mixture into twenty fresh NGM agar plates (60 x 15 mm) and allow the plates to dry for 1 h on a clean bench.

NOTE: The final concentration of FITC-dextran in each NGM plate will be 20 µg/mL.

7.4. Prepare five *E. coli* OP50-containing NGM plates without FITC-dextran for the vehicle control treatment. For this purpose, divide 100 µL heat-inactivated *E. coli* OP50 to each fresh NGM agar plates (60 x 15 mm) and allow the plates to dry for 1 h on a clean bench.

NOTE: For each independent experiment, fifteen FITC-dextran-supplemented NGM plates and five NGM plates without FITC-dextran are required.

7.5. After 48 h of treatment (from step 7.2), wash the worms with S-buffer, transfer the worms to the FITC-dextran supplemented plates and the NGM plates without FITC-dextran, and incubate the plates overnight (14-15 h).

NOTE: For each treatment group, 5 replicates of FITC-dextran staining (or vehicle control feeding) are required.

7.6. Wash the worms with S-buffer and allow them to crawl in the fresh NGM agar plate for

1 h.

NOTE: For each independent experiment, a total of 20 fresh NGM plates are needed. In this step, you can use the NGM plate supplemented without or with *E. coli* OP50.

7.7. Add 50 µL of 4% formaldehyde solution to each well of a black 96-well flat-bottom plate. Transfer approximately 50 worms from each NGM plate into each well for fluorescence measurements. After 1 to 2 min, thoroughly remove all the formaldehyde from each well and add 100 µL of mounting medium to coat the wells.

NOTE: Formaldehyde solution (4%) is used to immobilize and fix the worms. For each treatment group, 5 wells (5 replicates) are used for image analysis.

8. Imaging *C. elegans* with the Operetta imaging system and determination of intestinal permeability by measuring the FITC-dextran fluorescence uptake

NOTE: Fluorescent stereomicroscopy can be used for image analysis instead of the Operetta system.

8.1. Capture fluorescence images and measure the fluorescence intensity using the Operetta High-Content Imaging System and analyze the images with Harmony software.

8.2. In the Harmony software, press the icon **Open lid** to open the lid and to put the plate into the machine.

8.3. Set up the parameters.

8.4. Click **Set up**, select the plate type (96-well corning flat-bottom) and add the channels (bright-field and EGFP channels).

8.5. Adjust the layout. Go to the **Layout selection**, and then select **Track** and adjust the parameters (first picture at 1 µm, number of planes are 10, distance is 1 µm).

8.6. Select one of the treatment wells and one capture field and press **Test** to check whether the pictures are satisfactory in the **Run experiment** section.

8.7. If the pictures are satisfactory, return to the **Set up** section and press the **Reset** icon at the end of the screen. Then, select all the target wells and a suitable number of capture fields.

8.8. Go to **Run experiment** again and enter the plate name, then press **Start** to begin processing.

8.9. To measure the intensity of the fluorescence, go to the image analysis section and input the image. Find the cell by choosing the EGFP channel and method B. Adjust the common

threshold to 0.5, area to >200 µm^2, split factor to 3.0, individual threshold to 0.18 and contrast to more than 0.18. Then, calculate the intensity properties and choose the mean as the output. Press the **Apply** icon to save the setup.

8.10. Go to the **Evaluation** section to measure the intensity by obtaining a heatmap and data table. Set the **Readout parameter** to **Cells – Intensity Cell EGFP Mean – Mean per Well** and start the evaluation.

NOTE: The protocol can be paused here.

8.11. To extract the data from Operetta, click the **Setting** button and choose **Data management**.

8.12. Choose **Write archive**, and then open the browse and select the file.

8.13. To select the file, click the small **+** signal at the left corner, and then click **Measurement** and choose **Plate name**.

8.14. Select the file and click **OK**.

8.15. Select the path to save the file by clicking the **Browse** signal at the active path.

8.16. Click **Start** to save the data file.

NOTE: The protocol can be paused here.

9. Statistical analysis of the FITC-dextran fluorescence of *C. elegans*

9.1. Import the data and calculate the mean and standard deviation (SD) using a statistics software.

9.2. Analyze the significant difference with one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test.

REPRESENTATIVE RESULTS:

After incubation with *P. aeruginosa* PAO1, *C. elegans* showed a significant increase in FITC-dextran fluorescence in the worm body compared to the fluorescence shown after incubation with the other two bacterial strains (**Figure 1**). The fluorescence intensities of worms fed with *E. coli* OP50, *P. aeruginosa* PAO1, and *E. faecalis* KCTC3206 were 100.0 ± 6.6, 369.7 ± 38.9, and 105.6 ± 10.6%, respectively. The data emphasize that *P. aeruginosa* caused more vital damage to the epithelial gut barrier, and therefore, the worms exhibited a dramatic increase in their intestinal permeability. Based on this result, FITC-dextran can easily penetrate through the intestinal layer, so *P. aeruginosa* was selected as a potential candidate pathogen for screening the effects of DIM. Although *E. faecalis* is a gut pathogen that can produce extracellular

superoxide and hydrogen peroxide, which damage colonic epithelial cell DNA[15], in some cases, *E. faecalis* is also known as a potential probiotic bacteria due to its ability to produce bacteriocins against some pathogens[25,26]. The functions of a probiotic include adherence to epithelial surfaces, persistence in the human gastrointestinal tract, immune stimulation and antagonistic activity against intestinal pathogens[26]. Therefore, the gut permeability of worms incubated with *E. faecalis* remained unchanged compared with that of the vehicle control worms. This result shows that the amount of the infection can be studied by the increase in the fluorescence intensity, and *P. aeruginosa* causes more intestinal permeability than other strains.

Figure 2 shows the difference between live and heat-inactivated *P. aeruginosa* PAO1 based on the intensity of FITC-dextran fluorescence in the worm body. Both the fluorescence images and statistical data indicate that the pathogen could not trigger any toxicity to nematodes after heat inactivation. *P. aeruginosa* can produce exotoxin A – a potent extracellular cytotoxin that is lethal for many animals[27]. Exotoxin A can be rapidly abolished by heating at 45 °C to 60 °C[28]. Therefore, heat-inactivated *P. aeruginosa* was unable to damage the permeability of the worms' intestinal epithelia. The supernatant of *P. aeruginosa* PAO1 culture significantly damaged intestinal permeability, and therefore, the culture supernatant instead of whole *P. aeruginosa* cells can be used to induce intestinal permeability dysfunction[19]. The supernatant contains endotoxins, exotoxin A, and lipopolysaccharides[29], and these components are known to induce cell toxicity[30,31]. Therefore, these components of the supernatant may affect the intestinal permeability, although we did not check the direct effect of exotoxins and lipopolysaccharides on the intestinal permeability in *C. elegans*.

DIM cotreatment for 48 h significantly decreased the FITC-dextran fluorescence intensity inside the guts of worms compared with the *P. aeruginosa* single treatment (**Figure 3C, D**). Statistical analysis by one-way ANOVA and Tukey's multiple comparison test showed that after treatment with DIM, the mean fluorescence intensity was significantly decreased in comparison with the fluorescence intensity of the *P. aeruginosa*-only treatment. The fluorescence intensities of the worms treated with *P. aeruginosa*-only and *P. aeruginosa* plus DIM were 486.3 ± 41.7 and 414.2 ± 25.0%, respectively (**Figure 3E**). Based on this result, DIM can be considered a good natural product to cure intestinal permeability dysfunction caused by bacterial infections. This result indicated that DIM can attenuate cell inflammation in gut cells, which reduces the permeability of the intestine[19]. This result is similar to the results obtained in a mouse model, in which DIM showed a significant reduction in inflammation in the colon[32].

FIGURE LEGENDS:

Figure 1: The effects of different bacteria on the intestinal permeability of *C. elegans.* Microscopy images of the worms including bright-field, FITC fluorescence (green channel), and merged images. Microscopy images of worms from (**A**) *E. coli* OP50 without FITC-dextran feeding, (**B**) *E. coli* with FITC-dextran feeding, (**C**) *P. aeruginosa* PAO1 with FITC-dextran feeding, and (**D**) *E. faecalis* KCTC3206 with FITC-dextran feeding (white scale bar = 1 mm and black scale bar = 200 μm). Age-synchronized L4 larvae were incubated for 48 h in NGM plates seeded with *E. coli* (**A**, **B**), *P. aeruginosa* (**C**), and *E. faecalis* (**D**). Then, the worms were transferred to plates

containing FITC-dextran (**B**-**D**), except for the vehicle control (**A**). (**E**) The FITC fluorescence intensity of the different bacterial treatments. A higher percentage of FITC fluorescence indicated a higher gut permeability. Columns and error bars indicate the mean ± SD. ***P < 0.001 for significant difference from the vehicle control. ###P < 0.001 for significant difference from the FITC-dextran-treated worms fed with *P. aeruginosa* PAO1 (ANOVA, n = 5). This graph is representative of two independent experiments.

Figure 2: The effects of live and heat-inactivated *P. aeruginosa* PAO1 on the intestinal permeability of *C. elegans*. Microscopy images of worms from (**A**) live *E. coli* OP50 without FITC-dextran feeding, (**B**) live *E. coli* with FITC-dextran feeding, (**C**) live *P. aeruginosa* PAO1 with FITC-dextran feeding, and (**D**) heat-inactivated *P. aeruginosa* with FITC-dextran feeding (white scale bar = 1 mm and black scale bar = 200 µm). Age-synchronized L4 larvae were incubated for 48 h in NGM plates seeded with live *E. coli* (**A**, **B**), live *P. aeruginosa* (**C**), and heat-inactivated *P. aeruginosa* (**D**). Then, the worms were transferred to plates containing FITC-dextran (**B**-**D**), except for the vehicle control (**A**). (**E**) FITC fluorescence intensity comparing live and heat-inactivated *P. aeruginosa* PAO1. Columns and error bars indicate the mean ± SD. ***P < 0.001 and **P < 0.01 for significant difference from the vehicle control. ###P < 0.001 for significant difference from the FITC-dextran-treated worms fed with live *P. aeruginosa* PAO1 (ANOVA, n = 5). This graph is representative of two independent experiments.

Figure 3: The effect of DIM on the intestinal permeability of *C. elegans* fed *P. aeruginosa*. Microscopy images of worms from (**A**) *E. coli* OP50 without FITC-dextran feeding, (**B**) *E. coli* with FITC-dextran feeding, (**C**) *P. aeruginosa* PAO1 with FITC-dextran feeding, and (**D**) *P. aeruginosa* and DIM (100 µM) cotreatment with FITC-dextran feeding (white scale bar = 1 mm and black scale bar = 200 µm). Age-synchronized L4 larvae were incubated for 48 h in NGM plates seeded with live *E. coli* (**A**, **B**), live *P. aeruginosa* (**C**), live *P. aeruginosa* and DIM (**D**). Then, the worms were transferred to plates containing FITC-dextran (**B**-**D**), except for the vehicle control (**A**). (**E**) The FITC fluorescence intensity indicates that the gut permeability of *C. elegans* was affected by DIM. Columns and error bars indicate the mean ± SD. ***P < 0.001 for significant difference from the vehicle control. ###P < 0.001 and ##P < 0.01 for significant difference from the FITC-dextran-treated worms fed with *P. aeruginosa* PAO1 (ANOVA, n = 5). This graph is representative of two independent experiments.

DISCUSSION:

By utilizing this new method for determining gut permeability in *C. elegans,* which combines automated fluorescence microscopy and quantitative image analysis, the differences caused by intestinal microorganisms or chemicals can be determined in vivo, specifically in the *C. elegans* intestine. This protocol is useful for gut permeability investigations and applicable to many tasks, such as reactive oxygen species (ROS) determination under stress conditions and morphological examinations, due to its convenience and easy manipulation. Moreover, this method can be used to determine the effects of therapeutic and preventive methods against multiple pathogens in *C. elegans.* In particular, it may be an efficient protocol to investigate the mechanisms of different bacterial strains, including both harmful and useful bacteria. Some pathogens and probiotic bacteria with similar structures exert different effects on the host[33,34].

This procedure can assist in evaluating which mechanism the bacteria are utilizing and where the targeted substrates can be secreted extracellularly or intracellularly. In addition, this method can also help to strengthen the hypothesis that heat treatment plays a vital role in pathogen inactivation.

However, there are also some difficulties during this procedure. First, the number of worms in each plate is important for statistically meaningful results and robust worm detection, so 70 – 90% confluency (at least 50 worms per well) is recommended. Accordingly, trial experiments should be performed to obtain suitable worms. Second, the plate properties are one of the important factors that affect the quality of images and intensity determination, particularly for the bottom material. In some advanced experiments, a glass bottom is the best option for the measurement of fluorescence due to its excellent optical quality. However, because of the high price, a polystyrene bottom is often used, although the quality is not as high as that of the glass bottom. Finally, the plate coating methods for *C. elegans* require optimization. In this experiment, fluorescent mounting medium was applied because it can preserve and enhance the labeling of tissue sections, but it is unable to fix the worms on the plate bottom properly.

This protocol has limitations; as *C. elegans* is a nematode, further experiments, such as evaluation in human intestinal cell monolayers and in mammals, should be performed. In this method, the phenotypic changes in *C. elegans* fed pathogenic bacteria and chemicals were determined. Therefore, the molecular and genetic mechanisms underlying the pathogenic or probiotic effects of intestinal bacteria as well as the therapeutic effects of chemicals should be further elucidated. Specific signaling pathways exerted by pathogenic bacterial infection and therapeutic effects of chemicals can be evaluated using both various mutant bacteria and mutant worms. In addition, further in-depth studies identifying the chemical components responsible for the pathogenic and probiotic effects of intestinal bacteria would be beneficial.

Here, we report an experimental protocol for measuring intestinal permeability in *C. elegans* treated with different bacteria and chemicals by using FITC-dextran feeding. We believe that these protocols will be helpful for basic biological research, such as studying the interaction between the intestinal microbiota and gut health, as well as for the development of probiotics and nutraceuticals for the prevention and treatment of intestinal health problems.

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DISCLOSURES:

The authors have nothing to disclose.

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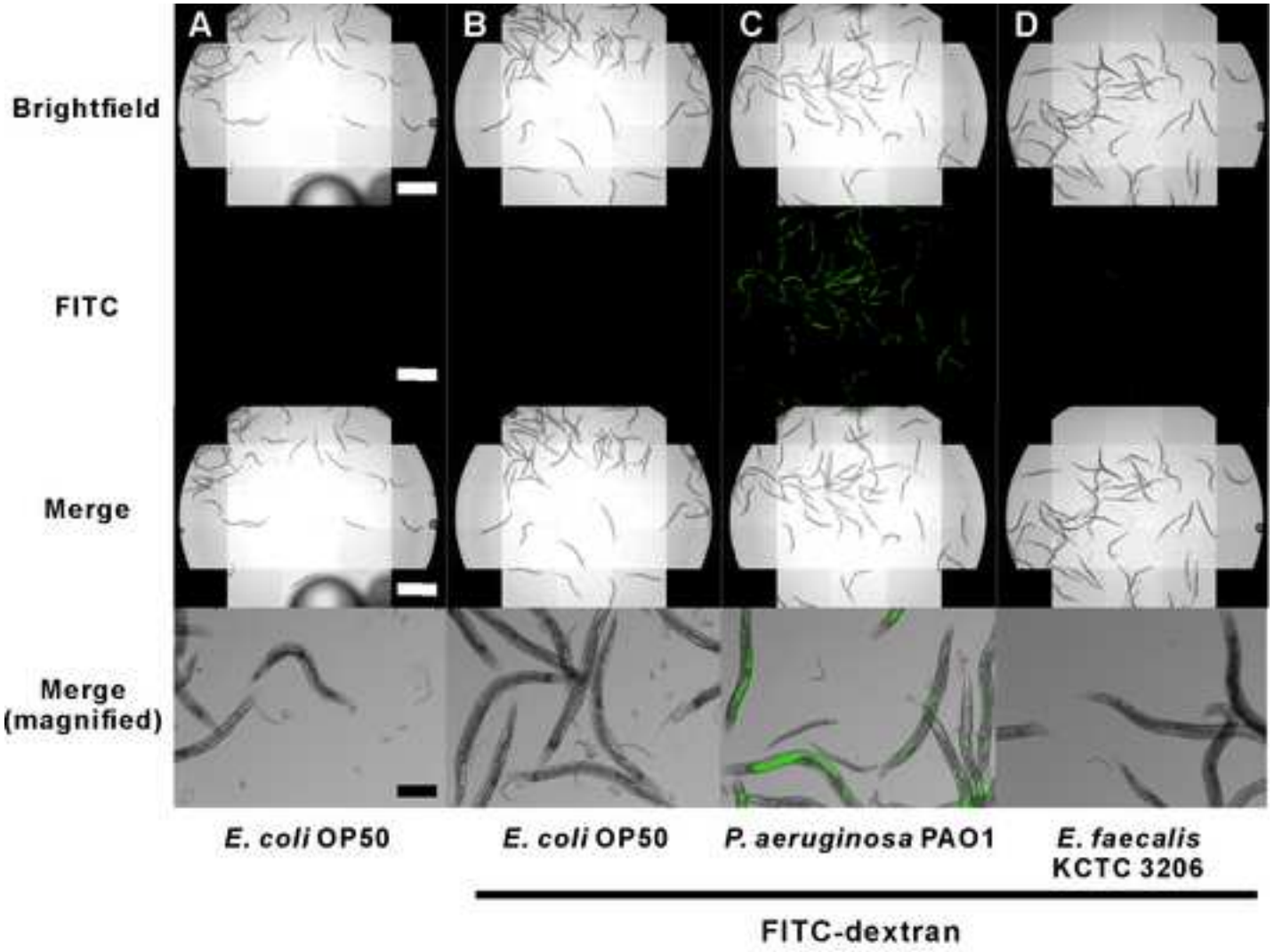
Figure 1



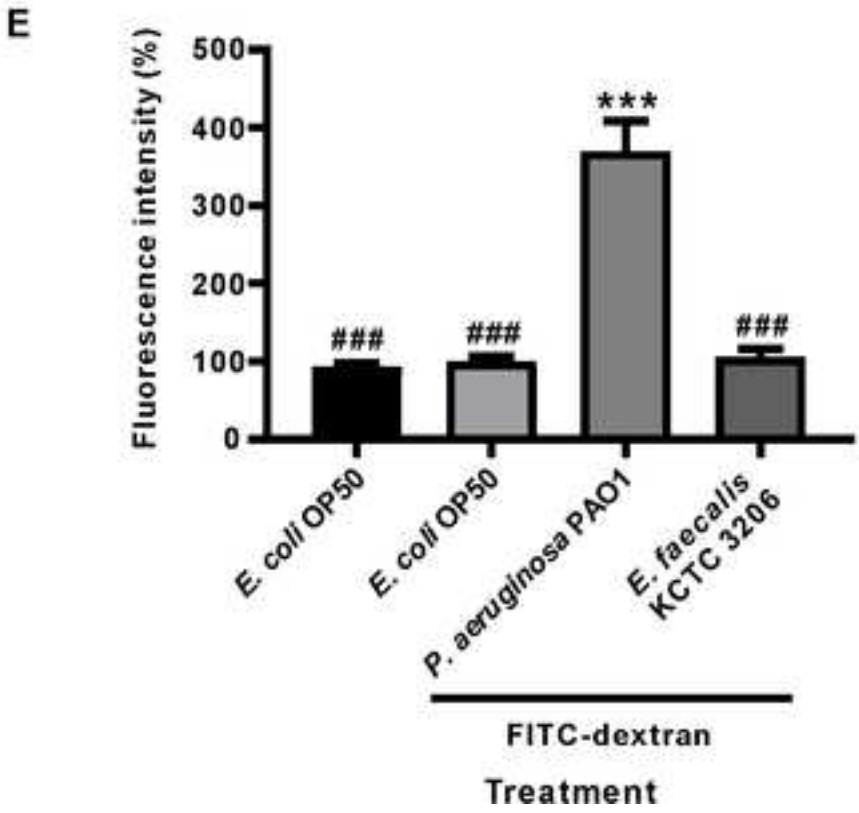
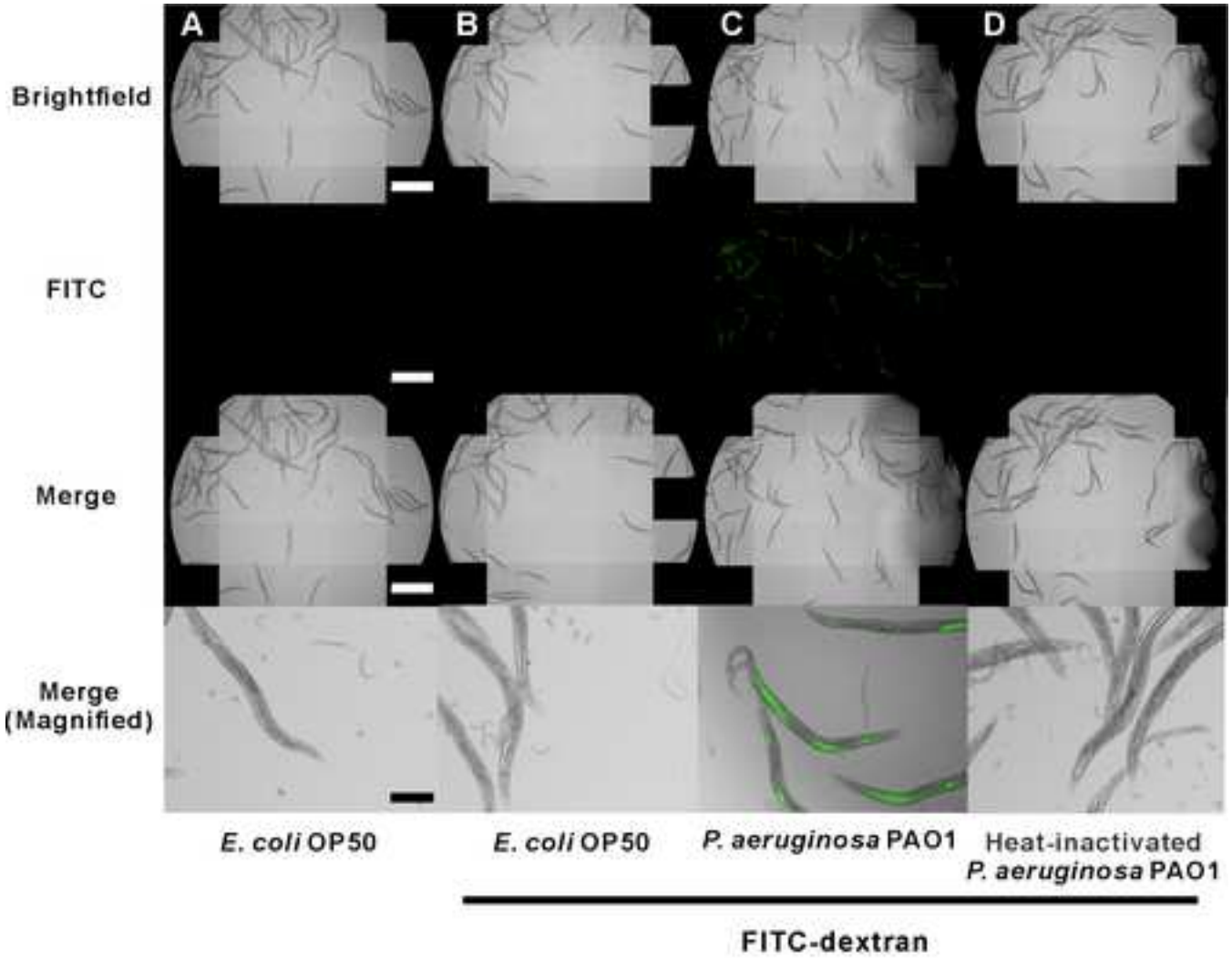


Figure 2



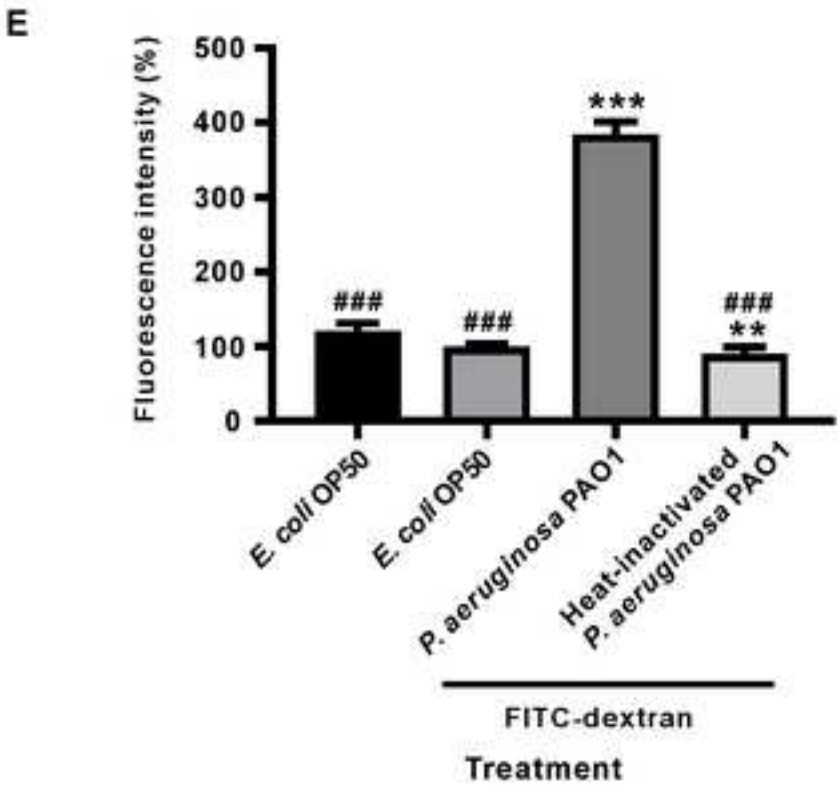
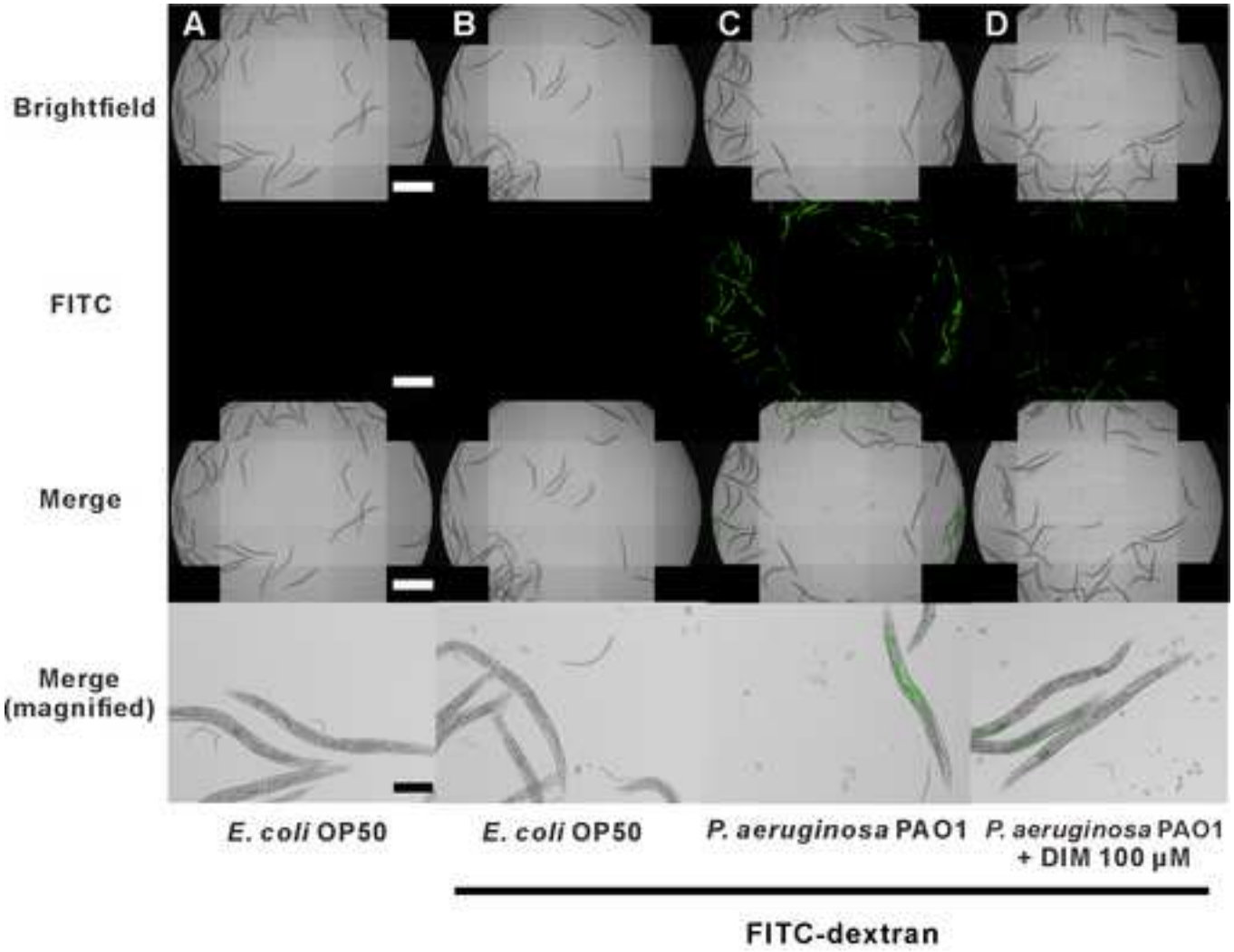


Figure 3



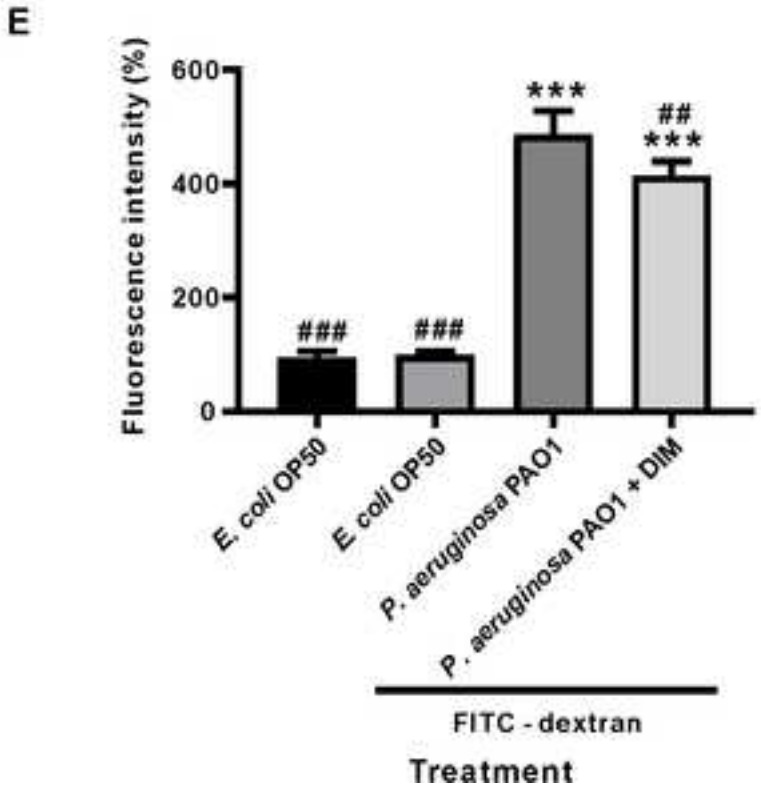


Table 1: Recipes of culture growth media and buffers.

Normal NGM agar media – 500 ml for maintenance and testing the effect of different bacteria
1. Add 1.25 g of peptone, 1.5 g of NaCl, 8.5 g of agar, and 487.5 mL of distilled water into a glass bottle.
2. Autoclave for 15 min at 121 °C.
3. Cool down in a water bath for 30 min at 55 °C, and then add 0.5 mL of 1 M $CaCl_2$, 0.5 mL of 5 mg/mL cholesterol (dissolved in ethanol), 0.5 mL of 1 M $MgSO_4$, 12.5 mL of KPO_4.
4. Mix with magnetic stirring, and then aliquot into Petri dishes (90 × 15 mm dishes for the maintenance and the bacterial treatment; 60 × 15 mm dishes for the FITC-dextran feeding).
5. Store the NGM plates at 4 °C until use.

DYT media for the cultivation of *E. coli* OP50 – 500 ml
1. Add 3 g of NaCl, 6 g of yeast extract, 9.6 g of peptone, and 580 mL of distilled water into an Erlenmeyer flask.
2. Autoclave for 15 min at 121 °C.
3. Cool down and store at room temperature until use.

LB media for the cultivation of *P. aeruginosa* PAO1 – 500 mL
1. Add 12.5 g LB medium powder (or 5 g tryptone, 2.5 g yeast extract and 5 g sodium chloride) and 487.5 mL distilled water into an Erlenmeyer flask.
2. Autoclave for 15 min at 121 °C.
3. Cool down and store at room temperature until use.

BHI media for the cultivation of *E. facalis* KCTC3206 – 500 mL
1. Add 26 g of BHI medium power (or 3.85 g of calf brains, 4.9 g of beef heart, 5 g of proteose peptone, 1 g of dextrose, 2.5 g of sodium chloride, and 1.25 g of disodium phosphate) and 474 mL of distilled water into an Erlenmeyer flask.
2. Autoclave for 15 min at 121 °C.
3. Cool down and store at room temperature until use.

S-buffer – 1000 ml
1. Add 5.85 g of NaCl, 6 g of KH_2PO_4, 1 g of K_2HPO_4, and 987 mL of distilled water into a glass bottle.
2. Adjust the pH of the solution to 6.0.
3. Autoclave for 15 min at 121 °C.
4. Cool down and store at room temperature until use.

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We have revised the manuscript to address the concerns of the editors, and we resubmit in the hope that it will now be acceptable for publication. A detailed response to each comment is appended. All the changes are shown in red in the revised manuscript.

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