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VISUAL EXPERIMENT

# Antibacterial activity of *Weissella confusa* by disc diffusion method

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## ABSTRACT

A visual experiment was conducted to investigate the antimicrobial potential of cell-free culture supernatant of *Weissella confusa* (DD_A7) against the foodborne pathogenic bacteria *Escherichia coli* 0157: H7 by disc diffusion method. The result revealed that DD_A7 exerted effective antibacterial potential to control the growth of *E. coli* upon treatment with 100 µg/mL of the culture supernatant. This study demonstrated that DD_A7 is a potent probiotic candidate to reduce the risk factor associated with Shiga toxin-producing *E. coli*.

## INTRODUCTION

Animal harbors are usually contaminated with microbes. These microbes sometimes can also contaminate the bovine-derived food products with toxins and can cause a risk of infections to human (Kudva, et al., 1999). Shiga toxin is the key virulence factor of *Escherichia coli* 0157: H7 (ATCC 43889), which causes life-threatening complications to the patients such as severe colorectal infections (Ogura et al., 2015). Moreover, multidrug-resistivity of *E. coli* has increased the prevalence of its infections and caused a problem to global health (Miryala and Ramaiah, 2018). As per the reports, this bacterium has also become an important target organism of food animal carcass decontamination interventions and attracted the attention of investigators dealing with foodborne pathogens (Samelis et al., 2005). Currently, there are very limited antibiotics available to inhibit the growth of *E. coli* (Schroeder, et al., 2002).

Several reports suggest that probiotic treatment could be an effective strategy to control the growth and pathogenicity of these microbes (Medellin-Peña and Griffiths, 2009; Yang et al., 2014). Here, we used *W. confusa*, a lactic acid producing bacteria, previously isolated from a Korean cuisine Kimchi (Dey et al., 2019). The aim of this study was to evaluate if the cell-free culture supernatant of *W. confusa* (DD_A7) loaded on the disc could affect the survivability or growth of *E. coli*. Keeping this in mind, in this visual experiment, we described the cost-effective strategy to screen the antibacterial activity of cell-free culture supernatant by disc diffusion method *in vitro*.

## MATERIALS AND EQUIPMENT

### Reagents

De Man, Rogosa and Sharpe (MRS) was purchased from, Neogen Culture Media, USA. Other remaining chemicals such as tryptone, NaCl, yeast extract, agar, and NaOH were purchased from Sigma Aldrich, USA.

### Preparation of cell-free culture supernatant

DD_A7 was isolated from the Korean famous cuisine, Kimchi. Its probiotic potential has been studied and reported recently (Dey et al., 2019a). It was routinely cultured in MRS broth medium at 37°C to maintain its growth. In order to prepare the cell-free culture supernatant, the previously reported

protocol was followed (Dey et al., 2019b) with minor modifications. Briefly, the supernatant was collected from the culture medium by centrifugation at 12,000 × g for 15 min at 4°C, followed by neutralizing the supernatant to pH 7.0 by the adding 1 N sodium hydroxide. Further, the supernatant was filtered using a 32 mm syringe filter having pore size of 0.2 µm (Acrodisc, Pall Life Sciences, USA) and lyophilized the supernatant. The concentrated cell-free culture supernatant was stored at -20°C until used further.

### Culture of pathogenic bacteria

*E. coli* 0157: H7 (ATCC 43889) was cultivated in Luria broth medium. It is best to grow the bacteria one generation in the liquid medium before initiating the experiment. To this aim, Luria broth media was prepared, by adding 10 g of tryptone, 5 g of yeast extract and 10 g of sodium chloride in a 2-L conical flask and make up the volume up to 1000 mL with deionized/Milli-Q water. Media was sterilized before using by autoclaving it for 20 min at 15 lb/sq. in. (psi) from 121-124°C on liquid cycle. The bacteria culture (1%) was inoculated from the 50% bacterial glycerol stock in the Luria Broth medium and further cultured at 37 °C, 150 rpm on a rotary shaker for next 12 hours.

### Disposables

Sterile plastic pipettes

Tips for micro-pipettes

Sterile Eppendorf tubes (5 mL)

Luria broth media

*E. coli* 0157: H7 (ATCC 43889)

Tryptone

Sodium chloride

Conical flask (2-L)

Bacterial glycerol stock

Syringe filter (32 mm having pore size of 0.2 µm)

MRS broth medium

*Weissella confusa* (DD_A7)

Paper disc

### General equipment and glassware

Autoclave to sterilize the necessary materials

Laminar flow hood

Incubator to maintain the optimum temperature for bacterial growth

Measuring cylinder (100 mL)

Volumetric flask (100 mL)

Eppendorf tubes

Glass tubes (Size: 25.4 × 76.2 mm; Thickness: 1-1.2 mm)

Forcef

### Major equipment

Desktop centrifuge

Vortex

Rotary shaker

## VIDEO CLIPS

Duration: 4 min 36 sec

## METHOD

1. Add 250 mL Luria broth medium to a sterilized 1L flask.

2. In order to make the primary culture, inoculate 1% *E. coli* 0157: H7 bacteria from the glycerol stock into the medium with an appropriate selective antibiotic.

3. Keep the flask on a shaker at 37°C for overnight. Use fairly vigorous shaking at 150 rpm so that the culture was well oxygenated.

4. For the preparation of secondary culture, inoculate 1% *E. coli* 0157: H7 bacteria from the primary culture in a freshly prepared 250 mL Luria broth medium to a sterilized 1L flask with appropriate selective antibiotic and keep on a shaker at 37°C for overnight for the optimum growth of the bacteria.

5. Next, to evaluate the antibacterial potential of lyophilized cell-free culture supernatant of *W. confusa* DD_A7 by disc diffusion method, we prepared the Luria broth agar plates with a selective antibiotic.

6. *E. coli* (100 µL) were taken from the secondary culture and were spread over the surface of Luria broth agar plate.

7. Paper disc was placed on the surface of Luria broth agar plate and the disc was loaded with the different concentrations of the antibacterial compound.

8. Plates were placed inside the incubator with a maintained temperature of 37°C for overnight.

9. A clear zone of inhibition around the paper disc was measured in diameter.

### Effect of cell-free culture supernatant on the bacterial cell viability

To further validate the antibacterial potential of cell-free culture supernatant, broth dilution technique was used. Briefly, culture was monitored in the presence of different concentrations of cell-free culture supernatant and optical density (OD) was recorded at 600 nm for 24 hours at the interval of 3 hours under the spectrophotometer. To support the results, cultures at 24 hours before and after treatment with cell-free culture supernatant (100 µg/mL) were monitored under the fluorescent microscope in the presence of acridine orange/ethidium bromide stain.

## RESULTS

To determine the antibacterial activity of cell-free culture supernatant against the 0157: H7 serotype Shiga toxin-producing *E. coli*, we used the disc diffusion method. As we observed in Figure 1, the plate, disc number 1, 2, 3, 4 was loaded with ampicillin (100 µg/mL), cell-free culture supernatant (100, 50, 25 µg/mL). The result shows that 100 µg/mL of cell-free culture supernatant efficiently inhibited the growth of the pathogenic bacteria with 18 mm clear zone of inhibition, in a similar manner with the positive control (ampicillin) taken during the study, with 19.5 mm clear zone of inhibition.

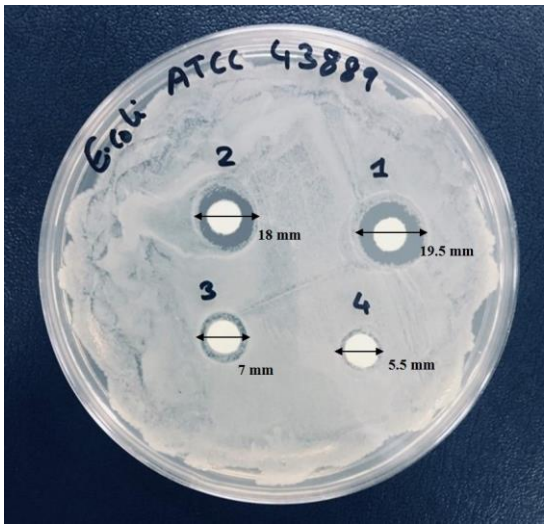


Figure 1: The antibacterial effect of cell-free culture supernatant was evaluated by disc diffusion method and compared with ampicillin on LB agar against *E. coli* 0157: H7. Ampicillin (100 µg/mL in Disc 1); cell-free culture supernatant (100 µg/mL in disc 2; 50 µg/mL in disc 3; 25 µg/mL in disc 4)

The results were further validated by exposing the exponentially grown secondary cultures of *E. coli* to the different concentrations of cell-free culture supernatant. As shown in Figure 2, at 24 hours the bacterial growth was highly reduced when treated with a higher concentration of cell-free culture supernatant (100 µg/mL) as compared with the control bacterial sample.

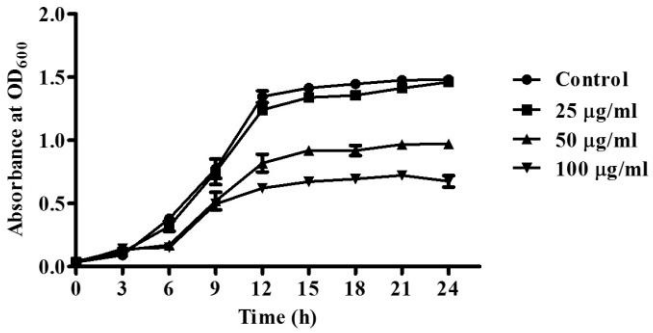


Figure 2: The growth of *E. coli* 0157: H7 in Luria broth medium in the presence or absence of cell-free culture supernatant at different concentrations were evaluated and measured the OD at 600 nm for 24 hours at 37°C

To support our findings further, we next used acridine orange/ethidium bromide staining. Acridine orange is an intercalating dye which stains both live and dead bacterial cells, however, the loss of cell membrane integrity allows ethidium bromide to stain the nucleated cells only (Hameed et al., 2016). As depicted in Figure 3, live bacterial cells appeared in green color due to acridine orange staining, whereas dead cells appeared in red color due to the loss in membrane integrity of *E. coli* 0157: H7 cells. The ratio of red fluorescent was comparatively higher after treatment with cell-free culture supernatant (100 µg/mL) than before treatment (control sample).

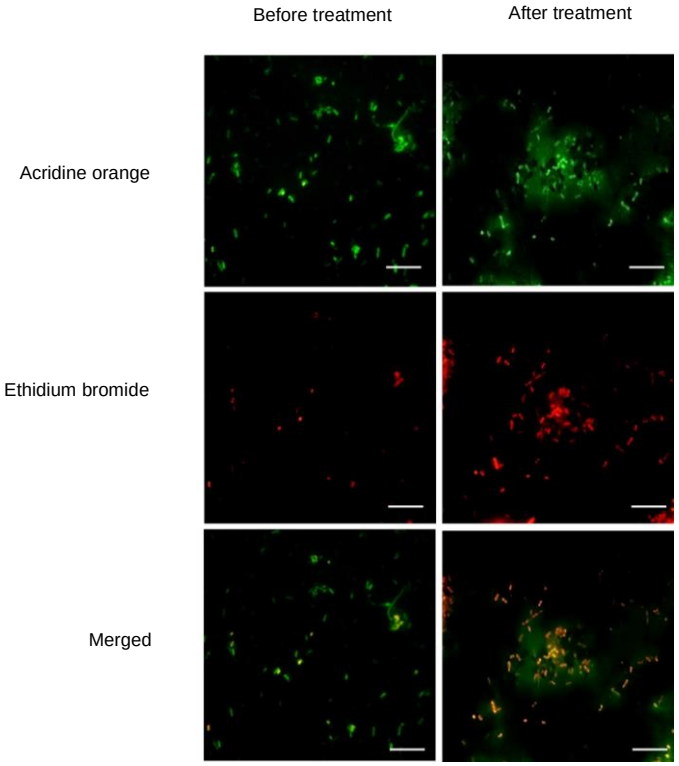


Figure 3: The viability of pathogenic *E. coli* 0157: H7 was evaluated by acridine orange/ethidium bromide staining under a fluorescent microscope after and before cell-free culture supernatant (100 µg/mL) treatment. The green fluorescence represents the live bacterial cells, whereas the dead bacterial cells are represented by red fluorescence [The scale bar is equal to 10 µm]

## DISCUSSION

There are numerous antibacterial drugs commercially available to treat infectious diseases, but despite that very drugs is inefficient to cure the diseases. Major reason lying behind this failure is the resistance

capacity of bacteria against the drug or the drug used for the treatment is not capable to control the virulence of the bacterial species (Li et al., 2018). Therefore, screening of drugs against the pathogen is necessary before suggesting the drug for the treatment of infection induced by the pathogen. Nowadays, researchers are investigating natural products as a source of new bioactive molecules (Monciardini et al., 2014; Ory et al., 2019). To this purpose, a variety of methods has been proposed which works on different principles (Valgas et al., 2007). Moreover, the results obtained from the various methods are greatly influenced by the selection of method and also depends on the selection of microorganism taken under consideration to conduct the test (Hadacek et al., 2000). Apart from the method and microorganisms, the degree of solubility, temperature, diffusion rate, molecular weight and concentration of the compounds can also influence the result of the study (Yff et al., 2002). Therefore, there is always a need for preliminary *in vitro* study to analyze the potential of the drug before being used for *in vivo* study.

Disc diffusion is a basic and important laboratory method which can be used routinely to screen the antibacterial activity of a compound *in vitro* (Balouiri et al., 2016; Canli et al., 2017). The study confirmed that cell-free culture supernatant of DD_A7 has the potential to diffuse from the disc and can effectively control the growth of the pathogenic *E. coli*. 100 µg/mL concentration of cell-free culture supernatant was enough to kill the pathogenic bacterial population, in a similar manner to ampicillin. The results of three different cost-effective experiments i.e. disc diffusion method, broth dilution, and acridine orange/ethidium bromide staining were complementing the results.

## CONCLUSION

The disc diffusion could be another alternative preliminary experiment to analyze the concentration, time required for the diffusion of active metabolites, and efficiency of the cell-free culture supernatant to inhibit the growth of targeted microbial pathogens.

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## PRECAUTION

Use sterilize water for the preparation of growth media

Open the media inside the laminar flow hood to avoid any type of contamination

Always use selective antibiotic in the growth media to avoid cross-contamination