**One day before experiment---preparation**

1. Media and plates preparation

* TSA-0.7%YE, PBS, Listeria selective media, MacConkey
* tweezers, glasswares, glass petridish, tips.

1. Wash & sterilization

* Stainless steel (304), size: 1.5 × 0.5”, 20 pieces for each experiment and one pathogen
* PE
* SS (314)

SS (314) coupons with the dimensions 2×1×0.2 cm were degreased by sonication in a detergent solution for 30 min and then in 70% (v/v) ethanol for 15 min. After a rinse in distilled water, the coupons were air-dried and autoclaved at 121 °C for 15 min. (Xinyi Pang, Hyun-Gyun Yuk. Effects of the colonization sequence of Listeria monocytogenes and Pseudomonas fluorescens on survival of biofilm cells under food-related stresses and transfer to salmon)

1. Bacteria preparation

* One loop of listeria, E. Coli and P. fluorescens was transferred into 50mL TSB-0.7%YE.
* Cultivate listeria and E.Coli at 37C for 18h. Cultivate P. fluorescens at 28C overnight (~24h)
* Centrifuge and wash twice with PBS
* After wash at the last time, the pellet was resuspended in **25mL TSB-0.7%YE**. (~108-9log/mL)

**0.99mL+0.01mL =990uL +10uL double dilution**

**0.9mL+0.1mL =900uL+100uL one dilution**

**Biofilm formation:**

**For single-species biofilm formation:**

**Method 1---Bacteria attachment using media**

1. 1mL bacteria (*L.m*, *E.Coli* or *P.f.)* mix with 100mL TSB-0.7%YE
2. Divide into 6 wells in 6-well plate. Each well contains 10mL inoculated TSB-0.7%YE
3. 6 pieces of coupons were placed in each well
4. Cultivate at 37C for 4h
5. Each coupon was gently washed with 5mL PBS to remove non-attached bacteria
6. The coupons were transferred in new wells with 10mL fresh 10-fold diluted TSB-0.7%YE (?) or 2000ppm lettuce juice extract solution
7. Store at 4C for 7days
8. CV staining and plate counting

**Method 2---Bacteria attachment using inoculation directly**

1. Coupons were placed in sterilized petri dish
2. Each coupon were inoculated with 0.1 mL bacteria (*L.m*, *E.Coli* or *P.f.)*
3. Incubate at 37C for 4h with lid
4. Each coupon was gently washed with 5mL PBS to remove non-attached bacteria
5. The coupons were transferred in new wells with 10mL fresh 10-fold diluted TSB-0.7%YE (?) or 2000ppm lettuce juice extract solution
6. Store at 4C for 7days
7. CV staining and plate counting

**Reference:**

[1]Leriche & Carpentier. Limitation of adhesion and growth of Listeria monocytogenes on stainless steel surfaces by Staphylococcus sciuri biofilms

[2] Stepanovic et al.. Biofilm formation by Salmonella spp. And Listeria monocytogenes on plastic surface.

**For dual-species biofilm formation:**

**According to Pang &Yuk,**