Biofilm formation of Listeria monocytogenes strains in food processing environments
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Food contamination by Listeria monocytogenes remains one of the major concerns of some food chains, particularly for ready-to-eat foods, including processed foods. Listeriosis in human could be a fatal infection mainly caused by consuming foods contaminated with L. monocytogenes. It is of a great importance not only due to the high case fatality rate but also due to its economic burden such as for periodic surveillance, controlling outbreaks, and recalls of suspected foods from market. A biofilm is a sessile community of bacterial cells, embedded in a self-produced extracellular polymeric substances matrix. The biofilms formed by this pathogen, both on biotic and abiotic surfaces, are a source of contamination by bacteria that have become more resistant or even persistent in food processing environments (FPE). In this study, various strains were examined to test the biofilm formation process under adverse conditions that L. monocytogenes encounters in FPE such as refrigerating temperature, nutrition deficiency, and existence of salt using various techniques.

Methods

A. Biofilm Ring Test (BRT™)

Adhesion capacity was assessed utilizing BRT™ (Biofilm Control, France). Biofilm Index (BFI) was obtained which represents an index of the blockage of the magnetic beads as the sessile cells are forming. Influence of cold stress was evaluated by exposing a sudden cold shock from 37°C to 4°C (cold-stressed cells). Cells that reached stationary phase at 4°C were used for comparison (cold-adapted cells).

B. Microtiter assay

Effect of single and synergic stress factors such as temperature down shift, existence of salt, and nutrition deficiency was evaluated on biofilm formation. After 24 h incubation under stress conditions, the final cell density was measured by measuring an optical density at 600 nm (OD600). The total biomass in biofilms was fixed by 96% ethanol and stained with 0.1% crystal violet (CV) solution. After washing and drying steps, 33% acetic acid was used to denat the CV and get homogenized solution which was quantified by obtaining an OD600 value.

C. Microscopy observations

To visualize attachment pattern and intensity of biofilm formation, phase-contrast microscope (Olympus CKX5, Olympus, Japan) was employed after CV staining of biofilms grown in a polystyrene 6-well plate. Additionally, scanning electron microscopy (SEM) was applied to visualize biofilm architecture and cell morphologies on stainless steel coupons. Briefly, coupons were washed and biofilms were fixed with a solution of 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4). Then coupons were dehydrated using a graded ethanol series (70, 90, and 100%) and a 50:50 mixture of ethanol/hexamethyldisilazane (HMDS). Samples were immersed in HMDS (Delta microscopes, France) followed by air-drying and sputter-coated with gold-palladium (JFC-1300, JEOL, Japan) and observed with a scanning electron microscope (JEOL 6800LV, JEOL, Japan) at 5 kV in high-vacuum mode.

Results

FIG. 1. Increased adherence of cold-stressed L. monocytogenes measured by BRT™. Bacterial suspension was prepared with initial inoculum at OD600 of 0.5 (A) and 0.17 (B). The results show that 19 out of the 22 strains revealed statistical difference (p < 0.05) in their adhesion level displayed by BFI between cold-stressed and cold-adapted cells, in either one of the two inoculums. Strains and serogroups are indicated in X axis. Mean ± SD, * p < 0.05.

FIG. 2. SEM (JFC-1300, JEOL, Japan) observations of L. monocytogenes biofilm formation under three different conditions on stainless steel coupons. Scale bars: 10 μm.

FIG. 3. SEM observation of cell morphology and biofilm structure. a, c, e, and j cold-stressed cells; b, f, and h cold-adapted cells; d) positive control. Scale bar in yellow with length (µm).

FIG. 4. Microtiter plate assay of 22 L. monocytogenes strains under cold-stressed and cold-adapted conditions. Adherent cells were quantified by CV staining (A) and final cell densities including planktonic and sessile cells were measured by turbidity of wells (B). Mean ± SD, * p < 0.05.

FIG. 5. Enhanced adhesion of 19 L. monocytogenes strains under nutrition deficiency measured by BRT™ at 37°C and 20°C. Serial dilution of cells were inoculated in BHI and DBHI broth and incubated for 5 h at 37°C and 10°C. Mean ± SD, * p < 0.05.

FIG. 6. Effect of salts on growth and biofilm formation of 1 L. monocytogenes strain (serogroup IIa). Cells were grown in BHI and DBHI broths containing 0, 0.85, 2, and 5 % salt. Total biomass (bar graph) and final cell density (symbol) are measured after 24 h incubation at 37°C. Even the total cell growth was inhibited, salts increased biofilm formation significantly in DBHI broth. Mean ± SD, * p < 0.05.

FIG. 7. Observation of L. monocytogenes biofilm formation under various stress conditions. Cells were grown in BHI and DBHI broths containing 0, 0.85, 2, and 5 % salt at 37°C for 24 h. Biofilms were stained with 0.1% CV solution and observed under