Biofilm formation of Listeria monocytogenes strains in food processing environments

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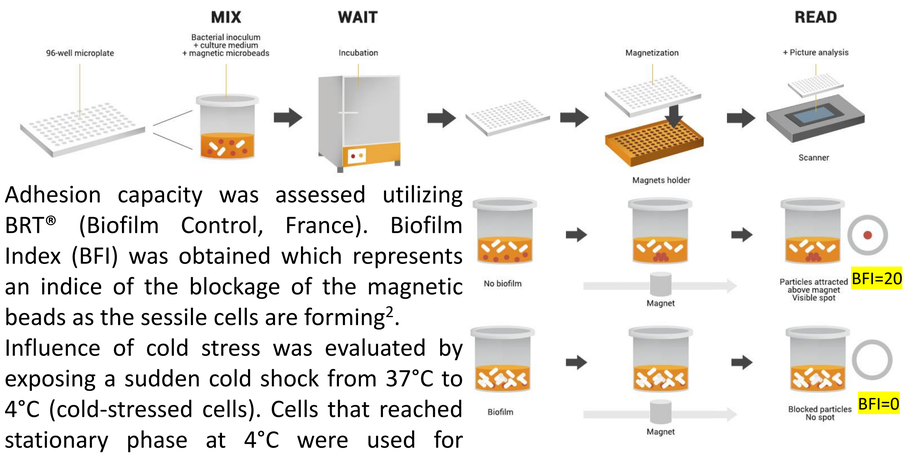
Introduction

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 (EPS) 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)[1]. 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 indice of the blockage of the magnetic beads as the sessile cells are forming[2].

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).

To examine single and synergic effects of sudden stress of cold and nutrition deficiency, cells grown under optimal condition (37°C on BHI agar) were exposed to the 10 fold diluted brain heart infusion (dBHI) broth at 10°C .

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 24h incubation under stress conditions, the final cell density was measured by measuring an optical density at 600 nm (OD_{600}). 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 destain CV and get homogenized solution which was quantified by obtaining an OD_{600} value.

C. Microscopy observations

To visualize attachment pattern and intensity of biofilm formation, phase-contrast microscope (Olympus CKX53, Olympus, Japan) was employed after CV staining of biofilms grown in a polystyrene 6-well plate. Additionally, scanning electron microscopy (SEM) was applied to visualized 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 microscopies, France) followed by air-drying and sputter-coated with gold-palladium (JFC-1300, JEOL, Japan) and observed with a scanning electron microscope (JEOL 6060-LV, JEOL, Japan) at 5 kV in high-vacuum mode.

Results

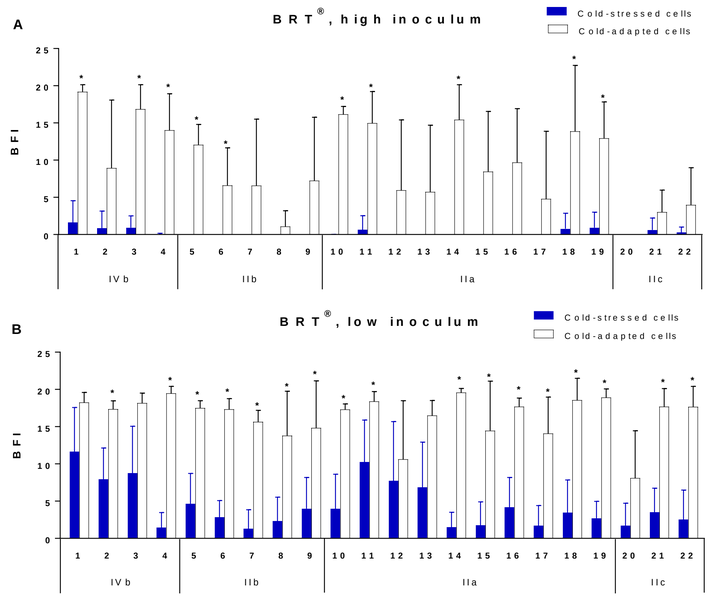


FIG. 1. Increased adherence of cold-stressed *L. monocytogenes* measured by BRT®. Bacterial suspension was prepared with initial inoculum at OD_{600} of 0.5 (A) and 0.17 (B). The results show that 19 out of the 22 strains revealed statistical difference ($p < 0.05$) in their adhesion level displayed 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, *$p < 0.05$..

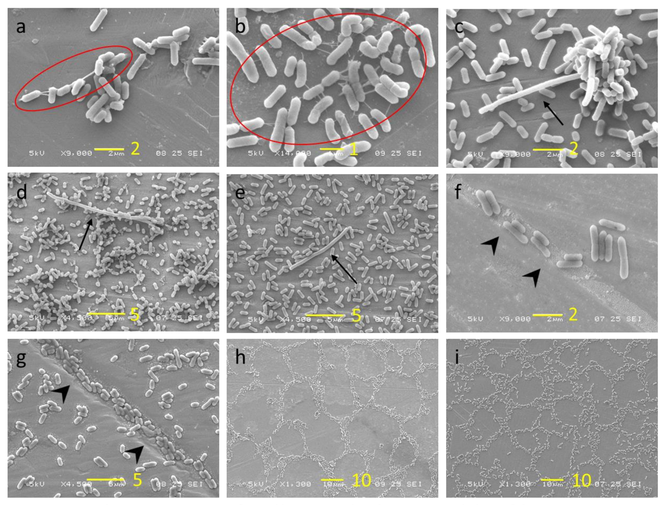


FIG. 3. SEM observation of cell morphology and biofilm structure. a, c, e, g, and i) cold-stressed cells; b, f, and h) cold-adapted cells; d) positive control. Scale bar in yellow with length (µm).

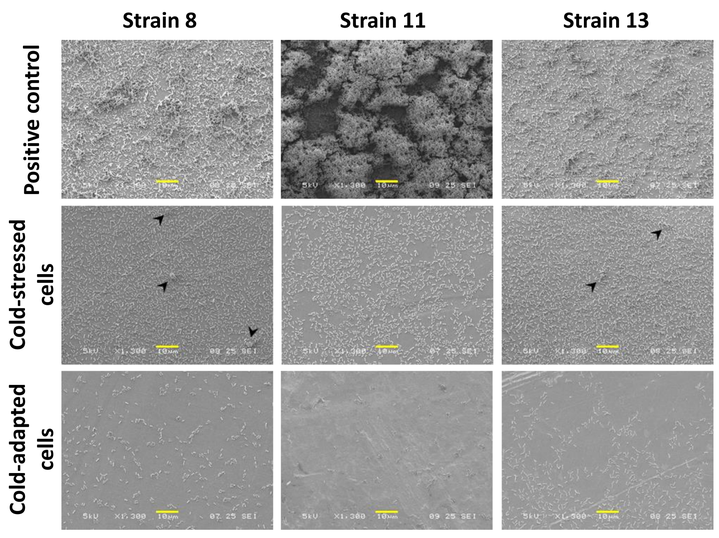


FIG. 2. SEM (JFC-1300, JEOL, Japan) observations of *L. monocytogenes* biofilm formation under three different conditions on stainless steel coupons. Scale bars: 10 µm.

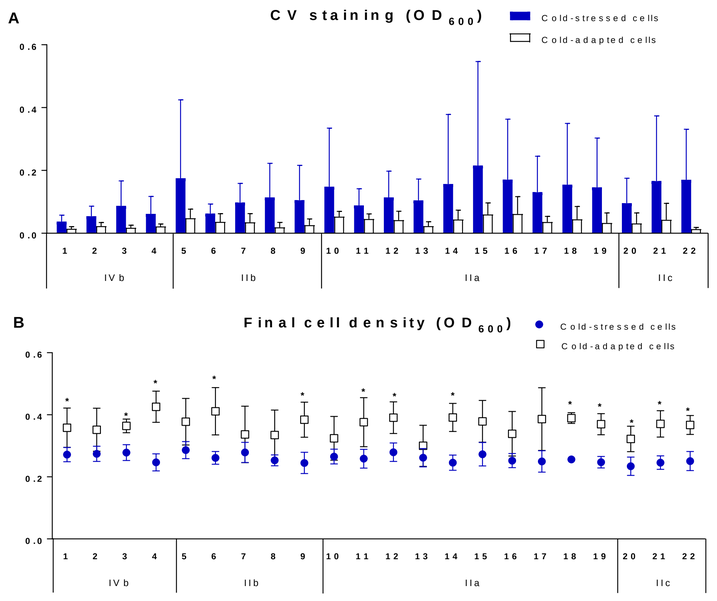


FIG. 4. Microtiter plate assay of 22 *L. monocytogenes* strains under cold-stressed and cold-adapted conditions. Adherent cells were quantified by CV staining (A) and final cell densities including planktonic and sessile cells were measured by turbidity of wells (B). Mean ± SD, *$p < 0.05$.

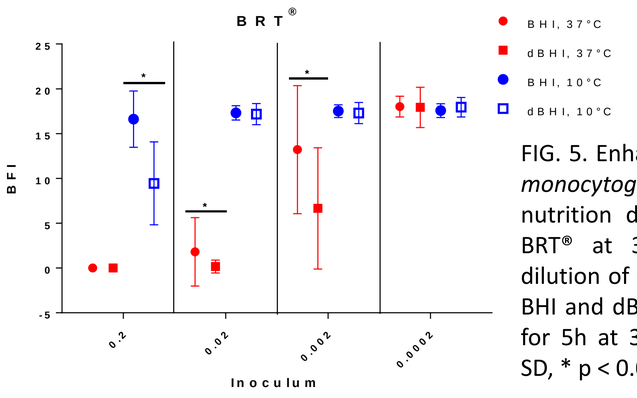


FIG. 5. Enhanced adhesion of 19 *L. monocytogenes* strains under nutrition deficiency measured by BRT® at 37°C and 10°C. Serial dilution of cells were inoculated in BHI and dBHI broth and incubated for 5h at 37°C and 10°C. Mean ± SD, * $p < 0.05$.

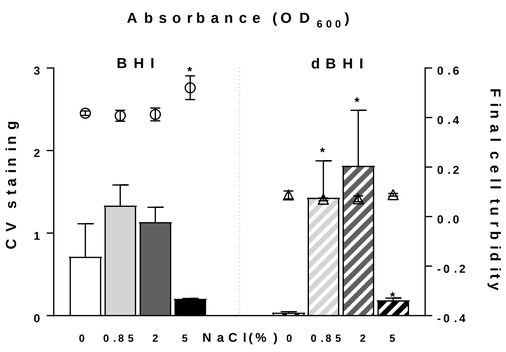


FIG. 6. Effect of salts on growth and biofilm formation of 1 *L. monocytogenes* strain (serogroup IIa). Cells were grown in BHI and dBHI broths containing 0, 0.85, 2, and 5 % salt. Total biomass (bar graph) and final cell density (symbol) are measured after 24h incubation at 37°C. Even the total cell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

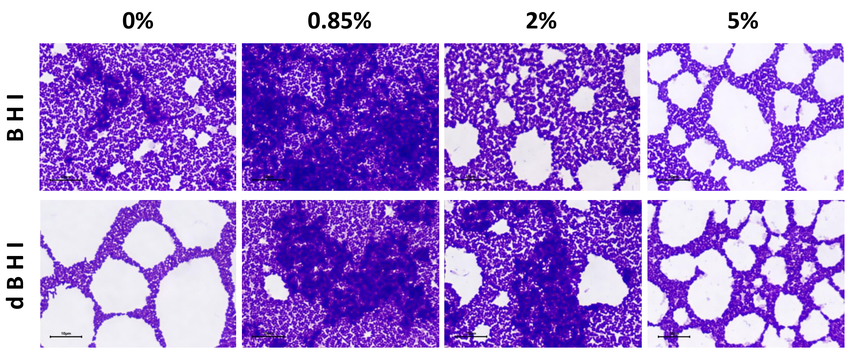


FIG. 7. Observation of *L. monocytogenes* biofilm formation under various stress conditions. Cells were grown in BHI and dBHI broths containing 0, 0.85, 2, and 5 % salt at 37°C for 24h. Biofilms were stained with 0.1% CV solution and observed under

Conclusion & Discussion

- *L. monocytogenes* is well known to survive and produce biofilms under adverse conditions and a study revealed that a long term storage of *L. monocytogenes* strains at -20°C increased biofilm formation[1,3]. In the current study, we observed that the sudden cold stress (4°C) increased adhesion of the bacteria to abiotic surfaces for the first time using BRT®. It shows that the surpassing total cell densities could not result in adhesion and biofilm formation implying that enhanced adhesion is distinct feature of cold-stressed cells.
- SEM observation supported BRT® results showing that the higher bacterial adhesion of cold-stressed cells along with cellular aggregates formation while cold-adapted cells were only able to form a single sparse layer of adherent cells. This demonstrates that the application of the device would be beneficial for testing the first step of the biofilm formation.
- Sudden stress of nutrition deficiency increased adhesion of *L. monocytogenes* under both optimal (37°C) and cold (10°C) temperatures regardless of total cell growth. Existence of salt up to certain concentration in growth medium increased biofilm formation of *L. monocytogenes* significantly and the phenomenon was more evident in nutrition deficient condition. This demonstrates that the multiple adverse conditions can stimulate more the biofilm formation.
- In sum, the findings may contribute in better understanding of the biofilm formation of *L. monocytogenes* and its contamination cycle in FPE. Further studies would be required to elucidate molecular background of the phenomenon as an adaptation strategy of *L. monocytogenes* under stress conditions.

Acknowledgements & References

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1. Ferreira, V., Wiedmann, M., Teixeira, P., and Stasiewicz, M.J. (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J. Food Prot. 77, 150–170.
2. Chavant, P., Gaillard-Martinie, B., Talon, R., Hébraud, M., and Bernardi, T. (2007). A new device for rapid evaluation of biofilm formation potential by bacteria. J. Microbiol. Methods 68, 605–612.
3. Slama, R.B., Bekir, K., Miladi, H., Noumi, A., and Bakhrouf, A. (2012). Adhesive ability and biofilm metabolic activity of *Listeria monocytogenes* strains before and after cold stress. Afr. J. Biotechnol. 11, 12475–12482.

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