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Effects on morphology and motility of Listeria monocytogenes cells subjected to sublethal stress of temperatures

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Abstract- Survival and growth of *Listeria monocytogenes* L56 (IMR isolate) was studied in trypticase soy broth (TSB) grown at 37ºC before being subjected to three selected sublethal stress temperatures (55ºC, 28ºC and 4ºC). The morphological changes and motility as affected by temperature stresses were determined using Scanning Electron Microscopy (SEM) and motility media, respectively. For this purpose, 10 ml broth containing *L. monocytogenes* previously grown in 300 ml of TSB at 37ºC at log phase (12 hour) and stationary phase (19 hour) to 55ºC, 28ºC and 4ºC were taken every 2 h intervals for measurement of morphological changes and motility. A total of 180 cells were measured lengthwise at 9,500x Scanning Electron Microscopy (SEM) magnification using SEMafore for each temperature treatments. The study demonstrated cells of both log and stationary phase of stressed temperature of *L. monocytogenes* showed a significant variation in morphology. Cells of log phase become elongated at 55ºC but not at 28ºC and 4ºC, whereas cells of stationary phase were shorter and more coccoidal rather than elongated as in log phase cells at the three temperatures studied. Loss of motility was observed in cells stressed at stationary phase cells but not at log phase; suggesting that motility plays a role in survival of the organism under temperature stress.

Index Terms- *Listeria monocytogenes*, temperature stress, morphology, motility, survival

I. INTRODUCTION

Microorganism is able to devise mechanisms that assist them in overcoming stresses that are designed to limit their growth. Therefore, the exposure of cells to some form of stress may induce and allow the survival of microorganisms with usually high durability to a given inactivation process. Temperature is a major factor in microbial destruction of spoilage and pathogenic microorganisms in foods. If the heat treatment is insufficient, it imposes a stress on the organism, which result in damage or injury of bacterial cells. Nevertheless, the bacteria have physiological mechanisms that trigger adaptive strategies which enables them to survive in environments that preclude their growth [1].

Listeria monocytogenes has become a major concern to the food industry as indicated by several reports of listeriosis outbreaks associated with contaminated dairy and food products in America and Europe. The Centers for Disease Control and Prevention (CDC) reported that twenty-four confirmed listeriosis outbreaks during 1998–2008, resulting in 359 illnesses, 215 hospitalizations, and 38 deaths from 1998-2008 [2]. Listeriosis includes a spectrum of clinical illnesses ranging from febrile gastroenteritis to potentially fatal bacteremia and meningitis in groups at higher risk for invasive diseases, including older adults and persons with certain medical conditions [3, 4].

Many processes in food preservation such as freezing, heating and drying caused sublethal injury to the pathogen, which resulted in surviving cells being unable to grow on selective media containing NaCl, bile salts or other detergents [5]. *Listeria* can be sublethally injured by a variety of processing treatments. Injured *Listeria* may be present in foods, yet escape detection because current techniques are inadequate for the recovery of injured *Listeria*. Injured *Listeria*, which escapes detection, possesses the potential to repair in food, thereby poses a serious public health threat.

L. monocytogenes can grow at a wide range of temperatures (-1°C to ~ 45°C) [6]. Foods are subjected to different temperature during handling and storage that may influence the growth and survival of pathogens. Growth of 4°C was selected because most foods are kept in refrigeration temperature. Growth of ambient temperature (28°C) was selected because many foods are handled at this temperature and *L. monocytogenes* may often be present on surfaces and utensils and become a major source of contamination. A temperature of 55°C was chosen because food may be subjected to temperature abuse during food handling or storage and this temperature can be stressful to the microorganism [7]; thus this study was conducted to determine the survival of growth characteristic of *L. monocytogenes* in these stressful temperature environments.

Scanning Electron Microscopy (SEM) was used to observe the morphological changes of *L. monocytogenes* subjected to various temperature stresses. Motility of *L. monocytogenes* during adaptation of temperature stress was also studied.

Therefore, the objective of this study was to examine the morphology and growth as indicated by motility of temperature stressed *L. monocytogenes*.

II. EXPERIMENTAL METHOD

A. Source of Microorganism

Listeria monocytogenes (L56) used in this study was obtained from Institute for Medical Research (IMR) of Malaysia. The

source of this culture was from Royal College of Pathologists of Australasia (RCPA) Quality Assurance Programme M8/92 Daving Change (UHN 009967), which was a clinical isolate from infected human blood (serotype 4b). Cultures were maintained at –20°C in trypticase soy broth (Merck, Germany) containing 10% glycerol.

B. Confirmation of L. monocytogenes Culture

Biochemical and morphological tests were performed to confirm the purity of the isolate. Isolate was streaked on trypticase soy agar (Merck, Germany) with 0.6% yeast extract (TSAYE) prior to subculturing into PALCAM agar (Merck, Germany) and incubated at 37°C for 24 to 48 h. Presumptive positive *Listeria* colonies that were grey green with a black sunken center which exhibited black halo in PALCAM agar (Merck, Germany) after incubation 24-48 h at 37°C were selected and further purified by streaking on Trypticase Soy Agar (Merck, Germany). Isolates that were Gram (+), catalase (+), motile at 21°C (+), methyl red (+), β hemolysis (+), nitrate reduction (-), rhamnose (+) and xylose (-) were considered to be *L. monocytogenes* [8].

C. Survival of L. monocytogenes under Temperature Stress

For preparation of inoculum for survival studies, a single isolated colony of the bacteria on Trypticase Soy Agar (Merck, Germany) after incubation of 24 h at 37°C was transferred to 100 ml Trypticase Soy Broth (Merck, Germany) and incubated at 37°C for 30 min. One ml from this broth was transferred into 300 ml of TSB with an initial 10^3 CFU/ml. The bacterium was grown at 37°C until the bacterium reached log and stationary phase after 12 and 19 h, respectively before subjected to selected sublethal temperature stresses (55°C, 28°C and 4°C). Prior to sudden shifting temperature, the inoculum size for log and stationary phase were 10^8 and 10^9 CFU/ml, respectively.

During the stressing of *L. monocytogenes* from 37°C to 55°C, 28°C and 4°C, 10 ml broth containing stressed cells of *L. monocytogenes* were taken from 300 ml of TSB and transferred into the sterile centrifuge tubes. The sampling was taken at every 2 h interval during the growth of *L. monocytogenes* at three temperature stress (55°C, 28°C and 4°C) at log and stationary phases. Then, the broth of stressed cells of *L. monocytogenes* was harvested by centrifugation (Kubota 5100) at 6,000 rpm for 15 minutes at 4°C. The supernatant was replaced with 4% glutaraldehyde solution for fixation purpose and kept at refrigeration until further used for SEM's sample preparation.

D. SEM's Sample Preparation

The cells were then washed in 0.1M sodium cacodylate buffer (repeated three times), post-fixed in 1% buffered osmium tetroxide for 24 h at 4°C, washed again in 0.1 M sodium cacodylate buffer (repeated three times), subjected to 0.1M graded acetone; 35%, 50%, 75%, 95% and 100% (repeated three times), and critical point dried in liquid CO_2. Each sample was then individually mounted on stubs, splutter coated with a gold-palladium complex and examined using a JSM 6400 Model Scanning Electron Microscopy (JOEL) at 15 kV.

E. Measurement of Cells Length Using SEMafore

Each SEM image consisted of approximately 60-100 stressed cells of *L. monocytogenes*. In order to get at least 60% of overall population cells for each observation, a total of 180 measurements from triplicate SEM images for each sampling time were recorded. The sampling time was done at 2 h interval for 6 h at 55°C, 28°C, and 4°C for cells at log and stationary phases. The SEM magnification at 9,500x was used throughout this study.

F. Effect of Temperature Stress on Motility of L. monocytogenes

Aseptically, at one hourly interval the motility media (Merck, Germany) was stabbed inoculated with temperature stressed *L. monocytogenes* followed by incubation at 25°C for 3 d. Results were recorded as positive or negative based on umbrella-shaped growth pattern in motility media.

G. Statistical Analysis

The length of cells at log and stationary phase exposed to different stressed temperatures were statistically analysed using SAS (SAS Institute, Cary, NC, USA). One-way ANOVA was used to determine significant different ($P \leq 0.05$) between samples. Duncan's test was used as post hoc comparison between means of variables of samples.

III. RESULTS AND DISCUSSIONS

A. Mean Cells Length of L. monocytogenes As Affected by Sublethal Stress Temperatures

Upon exposure to sublethal stress of temperatures, *L. monocytogenes* undergoes various morphogenesis as observed under SEM. There phenotypic variations observed include cell elongation, coccoid and intact cells. Cell width was neglected due to the insignificant changes observed in this study.

Table 1: Comparison of mean cells length (μm) of log and stationary phase *L. monocytogenes* on exposure to sublethal temperature stresses

Bacterial phase	Temperature and hour of stress	Mean (μm) ± Standard deviation
Log	55°C (2 h)	$0.94 \pm 0.46^{a,a}$
Stationary	55°C (2 h)	0.87 ± 0.27^{a}
Log	55°C (4 h)	2.07 ± 0.97^{a}
Stationary	55°C (4 h)	0.74 ± 0.27^{b}
Log	55°C (6 h)	1.33 ± 0.77^{a}
Stationary	55°C (6 h)	0.84 ± 0.30^{b}
Log	28°C (2 h)	0.83 ± 0.24^{a}
Stationary	28°C (2 h)	0.89 ± 0.25^{b}
Log	28°C (4 h)	0.88 ± 0.24^{a}
Stationary	28°C (4 h)	1.01 ± 0.31^{b}
Log	28°C (6 h)	0.87 ± 0.27^{a}
Stationary	28°C (6 h)	0.94 ± 0.28^{b}
Log	4°C (2 h)	1.02 ± 0.35^{a}
Stationary	4°C (2 h)	0.72 ± 0.20^{b}
Log	4°C (4 h)	0.92 ± 0.32^{a}

Stationary	4°C (4 h)	1.05 ± 0.25^{b}
Log	4°C (6 h)	0.89 ± 0.29^{a}
Stationary	4°C (6 h)	1.01 ± 0.30^{b}

Table 1 demonstrates the variation of cells length (μm) of log phase cells after exposure to various temperature stresses, which ranging between 0.83 μm to 2.07 μm. Log phase cells exposed to 55°C resulted in filament formation. The mean cells length increased from 0.94 μm to 2.07 μm at 2 and 4 h, but decreased to 1.33 μm after 6 h exposure. In contrast, no elongation of cell length occurred when *L. monocytogenes* cells were stressed at 28°C and 4°C. Cells of log phase at 28°C were relatively shorter in length as compared to cells at 4°C. However, cells at 4°C showed a continuous decrease of cells length throughout the duration of stress. There was no difference in cell length stressed at 28°C within the six hour of stress.

The length of log phase cells length increased as the growth environment became more challenging. The mean of cells length increased at 28°C, followed by 4°C, and 55°C. Cell elongation was evident at 55°C than 28°C (Fig. 1 and 2). This study showed that the log phase cells *L. monocytogenes* undergo changes in phenotypic morphologically in cell length when exposed to high temperature stress. Previous reports demonstrated long filamentous structures occurred when *Listeria* were propagated on agar containing 8 to 9% NaCl [9, 10]. In other study, filament formation of *L. monocytogenes* occurred at NaCl concentration above 1,000 mM with an increase in filament length as NaCl concentration increased [11]. The formation of filaments of *L. monocytogenes* was also reported when this organism was exposed to salt, acid and alkali stress at 3°C [12], and 5% NaCl and 7% KCl [13]. They concluded that the cells length increased as the growth environment became more challenging. The formation of filaments is required for the repair of DNA lesions which give rise to mode one killing, which involved DNA damage.

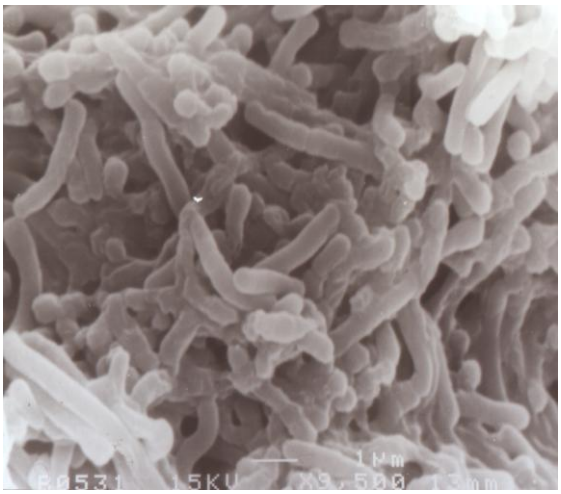

Fig. 1. SEM of log phase cells *L. monocytogenes* became elongated at 55°C

Bacterial cells exposed to mild heating temperature (55°C) had an accelerated growth rate [14] and became filamentous rather than at other stress conditions. It was concluded that the faster bacterial cells grow, the longer they are, and the size seems to be an exponential function of growth rate at a given temperature [15]. Another hypothesis is that mild heating of bacterial cells would adapt 'thermal expansion'. According to Serway [16], as growth temperature increases, its volume increases. The overall thermal expansion of a bacterial cell is a consequence of the changes in the average separation between its constituent liquid atoms and molecules.

However, a different phenomenon was observed during stationary phase of *L. monocytogenes* (Table 1). Most of *L. monocytogenes* cells became shorter and coccoid with mean ranged between 0.72 μm to 1.05 μm. Exposure to 55°C resulted in shortening of cells. The mean of cells length was decreased from 0.87 μm (2 h) to 0.74 μm (4 h), however the cells length increased to 0.84 μm (6 h). Cells of stationary phase at 28°C were 0.89 μm to 1.01 μm whilst, the cells length of stationary phase at 4°C were 0.72 μm to 1.05 μm. The cells length increased from 0.72 μm (2 h) to 1.05 μm (4 h), and the filament decreased to 1.01 μm (6 h). It was observed that cells of stationary phase at 55°C (4 h) and 4°C (2 h) demonstrated shorter cells length than other stress condition with the mean cells length of 0.74 μm and 0.72 μm, respectively.

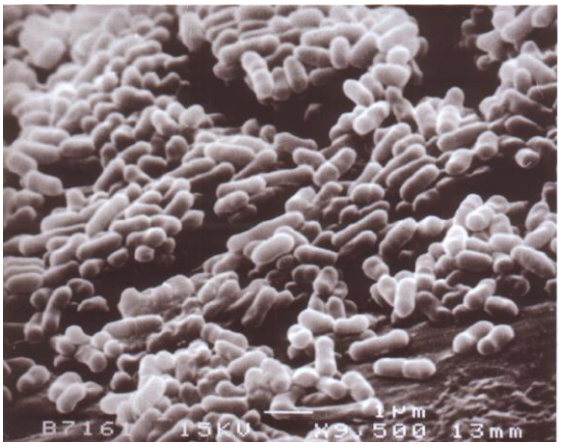

Fig. 2. SEM of log phase cells *L. monocytogenes* at 28°C

As shown in Fig. 2, no apparent differences in average size (below than 1.01μm) was observed at 28°C. Although both log and stationary phase cells were shorter, they were statistically different at $P \leq 0.05$. Cells growths at 28°C were more intact and this suggests that at this temperature which is near to optimal growth (30-37°C) were not disrupted by temperature stress. It was reported that young cultures of *L. monocytogenes* consist of short rod organism measuring from 0.5 by 1 to 2 μm; the ends that may be slightly pointed, with short chains and diploforms often seen as V or Y-shaped [17].

At 4°C, the decreased in cells length was observed constantly from 2 to 6 h during log phase of stressed cells of *L. monocytogenes*. In contrast, during stationary phase, it was an increase of stressed cells length, which observed after 2 hour. Stressed cells of stationary phase of *L. monocytogenes* at 4°C were slightly longer (0.72 to 1.05 μm) than cells at 28°C (0.83 μm to 1.01 μm). The possibility of cells were structural damaged

due to temperature stress is described as "fussy phenomenon', which was reported earlier when *L. monocytogenes* was subjected to long-term chilling storage [18]. In addition, SEM showed the bacteria tend to clump together when cells at stationary phase was stressed at 4°C as shown in Fig. 3. The organism being presented in clumps is associated with the occurrence of shoulder present in survival curves [19].

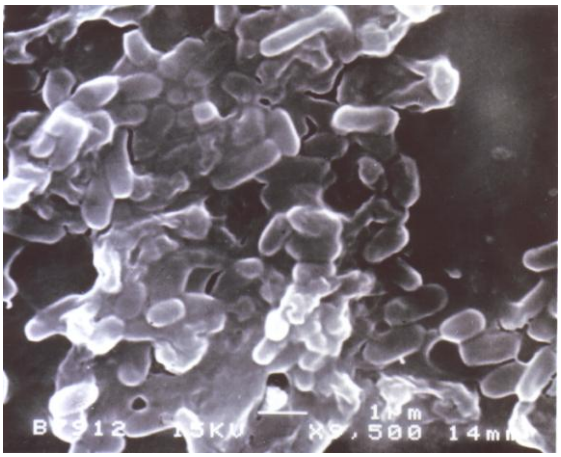

Fig. 3. SEM of stationary phase cells *L. monocytogenes* showing cell clumping at 4°C

Based on the statistical analysis, the present study demonstrated that the changes in morphology of *L. monocytogenes* were dependent on the bacterial growth phase environment. It is suggested that alteration and changes of morphology of *L. monocytogenes* is a part of microbial cells adaptation for their continued survival and successful growth [20].

B. Motility of L. monocytogenes As Affected by Sublethal Stress of Temperatures

Studies using motility agar was used to determine whether cells from temperature stresses could influence listerial motility when subjected to sublethal stress of temperatures. Motility character of *L. monocytogenes* was affected by growth phase of the pathogen (Table 2). It was observed that loss of motility was observed for cells at stationary phase compared to cells at log phase at the three stressed temperatures studied.

Table 2: Motility results of *L. monocytogenes* subjected to sublethal stress of temperatures at log and stationary phases

Bacterial phase	Temperature and hour of stress	Motility
Log	55°C (2 h)	Motile
Stationary	55°C (2 h)	Non-Motile
Log	55°C (4 h)	Motile
Stationary	55°C (4 h)	Non-Motile
Log	55°C (6 h)	Motile
Stationary	55°C (6 h)	Non-Motile
Log	28°C (2 h)	Motile
Stationary	28°C (2 h)	Non-Motile
Log	28°C (4 h)	Motile
Stationary	28°C (4 h)	Non-Motile
Log	28°C (6 h)	Motile
Stationary	28°C (6 h)	Non-Motile
Log	4°C (2 h)	Motile
Stationary	4°C (2 h)	Non-Motile
Log	4°C (4 h)	Motile
Stationary	4°C (4 h)	Non-Motile
Log	4°C (6 h)	Motile
Stationary	4°C (6 h)	Non-Motile

Little is known concerning listerial motility character as affected by environmental stress. The present study demonstrated that cells during stationary phase lost their motility character, whereas listerial motility during log phase was not affected. Very little is known about the influence of temperature on motility of *L. monocytogenes*, however, a previous study reported that 30 (68.2%) of 44 strains tested showed swimming at 22°C and 4 (9.1%) of those were also motile at 12°C. Their finding showed that swimming motility was observed only at 22°C, so confirming the temperature-dependent flagellum production in *Listeria* spp [21].

The result is of this study clearly suggested that stress may affect some of phenotypic characteristic of organism as adaptation for survival. The finding of this study is very useful in preventing misidentification of *L. monocytogenes* in food systems, as most of food systems present in stress environment. This study provides a better understanding about filament and coccoid formation of *L. monocytogenes* in stress environment and its potential implication to food safety.

IV. CONCLUSIONS

L. monocytogenes cells are able to grow and survive throughout the sublethal temperature stress evaluated and undergo changes in morphology and motility in order to adapt to the new environment.

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