Nigerian Journal of Microbiology, Vol. 24(2): 2235 – 2242 2010
www.nsmjournal.org

EFFECT OF pH AND TEMPERATURE ON CELL VIABILITY AND HAEMOLYSIN PRODUCTION BY *Listeria* spp. ISOLATED FROM UNCOOKED MEAT AND VEGETABLE SAMPLES IN NSUKKA, NIGERIA.

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Abstract

Haemolysin (Listeriolysin O) is a major virulence factor in pathogenic strains of *Listeria*. Presently, the conditions for its production are not fully elucidated. This study examined the influence of culture pH and temperature on cell viability, multiplication and haemolysin production by fresh isolates of *L. monocytogenes, L. ivanovii* and *L. seeligieri.* At 5°C and pH 3.8 to 4.8, there was a significant ($p < 0.05$) decline in cell viability and multiplication. However, both parameters remained in a steady state from pH 5.0 to 5.6 at the same temperature. At 30°C, there was also decline in cell viability and multiplication from pH 3.8 to 4.8 but significant cell multiplication from pH 5.0 to 5.6. Haemolysin production was concomitant with conditions allowing growth of the listerial organisms and the highest increases in haemolysin production were recorded during the log phase of growth. For all isolates, haemolysin was produced from pH 5.0 to 9.0 at the above temperatures and productivity was highest with *L. ivanovii*.

Keywords: Listeria monocytogenes*;* L. ivanovii*; haemolysin; pH; temperature*
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Introduction

The genus *Listeria* consists of Gram-positive, catalase-positive, facultatively anaerobic, rod-shaped bacteria. Seven species of *Listeria* have been identified but *L. monocytogenes* is the principal pathogen in humans and animals (Gellin and Broome, 1989; Bille and Doyle, 1991; Low and Donachie, 1997; Beverly, 2004; Peiris, 2005).

L. monocytogenes has become established as a food-borne pathogen. The organism is of particular concern to the food industry and public health regulatory agencies because of its ability to grow and multiply under unfavourable conditions such as direct sunlight, repeated freezing and thawing, drying and heating, and extended pH ranges (FSIS, 2001; Peiris, 2005; Ramaswamy *et al*., 2007). Refrigeration does not prevent growth of the pathogen (NFPA, 1999; Beverly, 2004; Peiris, 2005; Ramaswamy *et al*., 2007). Studies have also shown that it exhibits some measure of heat tolerance although it will not survive pasteurization except when present in high numbers (NFPA, 1999; Stehulak, 1992; Casadei *et al*., 1998; FSIS, 2001).

L. monocytogenes secretes a haemolytic protein, listeriolysin O (LLO) belonging to the group of sulfhydryl-activated cytolysins and homologous to Streptolysin O and pneumolysin (Mengaud *et al*., 1987). This haemolysin is believed to be the major virulence factor of *L. monocytogenes* (Mengaud *et al*., 1987; Buncic *et al*., 1996; NFPA, 1999; Portnoy *et al.,* 2002; Haas *et al*., 2007).

Although the pathogenicity of *L. monocytogenes* has been widely reported, there is little information on conditions influencing the production of the main pathogenicity factor, the LLO. In this study, the dynamics of haemolysin production by *Listeria* spp. isolated from uncooked meat and vegetable samples were investigated.

Materials and Methods

Organisms and culture conditions

All listerial isolates used in this study were isolated from meat and vegetable samples obtained from a local market at Nsukka, Enugu State, Nigeria. The listerial organisms were isolated on PALCAM agar using the USDA-enrichment method (Hitchins, 2001). Typical

listerial organisms were identified to species level using standard confirmatory tests as described by Bille and Doyle (1991). Two isolates of *L. monocytogenes* (T and N), two isolates of *L. ivanovii* (O and R) and one isolate of *L. seeligeri* were used in this study. All cells were maintained in trypticase soy broth plus yeast extract (TSBYE) at pH 7.3.

Haemolytic activity on blood agar

All isolates were initially tested for haemolytic activity on *Listeria monocytogenes* blood agar (LMBA), using sterile sheep blood. Test isolates were streaked onto the agar plates and incubated at 37^{o}C for 24 to 48 h. Haemolytic *Listeria* species grew as small light-coloured colonies surrounded by narrow zones of beta haemolysis.

Quantitation of haemolysin production

Haemolysin production was determined semi-quantitatively by the microplate technique using washed human erythrocytes (Dominguez-Rodriguez *et al*., 1986) and by the procedure of Buncic *et al*., (1996). Briefly, the isolates were cultured in TSBYE, at 37^{o}C for 24 h. The bacterial cells were harvested by centrifugation for ten minutes at 4600 x g and were re-suspended in 15 ml of 0.1% peptone water. Washed erythrocytes were prepared by centrifugation of 20 ml human blood (containing anticoagulant) at 2600 x g for five minutes. After discarding the plasma, the packed erythrocytes were re-suspended in 20 ml sterile 0.9% saline, re-centrifuged, and the saline wash aspirated. The wash procedure was repeated until no pink colouration was detected in the saline wash. Washed packed erythrocytes (3 ml) were added to 100 ml of sterile 0.9% saline solution.

Titration was carried out in microtitre plates with U-form wells in which 50 µl of saline solution (0.9%) had been placed in each well (11 wells were used for each isolate). Two-fold serial dilutions were carried out with the test isolates in the wells. To each bacterial dilution, 100 µl of the 3% suspension of washed human erythrocytes was added. The microtitre plates were then incubated at 37^{o}C for 8 h, after which wells were examined for complete or partial haemolysis. The haemolytic activity titres were expressed as complete haemolysis units (CHU; the reciprocal of the highest dilution at which 100% haemolysis took place) and minimal haemolysis units (MHU; the reciprocal of the highest dilution at which haemolysis was detected). The titres were converted to logarithms before their mean values were calculated.

Time course of haemolysin production

The temporal synthesis of haemolysin was studied with *L. monocytogenes* (isolates T and N), *L. ivanovii* (isolates O and R), and *L. seeligeri* (isolate F). The cells (9 x 10^{6} cfu.ml^{-1}) were cultured in 250 ml TSBYE (pH 7.3) and incubated at 35^{o}C. At six hourly intervals, 100 µl portions of the cultures were taken and analyzed for haemolytic activity as previously described. Samples were taken for up to 108 h.

Effect of pH and temperature on the growth of L. monocytogenes

Two isolates of *L. monocytogenes* (T and N) were used in this study. TSBYE broth in 250 ml Erlenmeyer flasks was adjusted to pH 3.8 to 5.6 using hydrochloric acid (HCl) or sodium hydroxide (NaOH) to achieve final pH values of 3.8, 4.2, 4.4, 4.8, 5.0 and 5.6. The broths were inoculated with 9 x 10^{6} cells per ml of the listerial isolates and incubated at 5^{o}C and 30^{o}C for up to 63 days. At various time points, viable population (CFU/ml) was determined by serially diluting samples in phosphate buffer (pH 7.2) and plating out on trypticase soy agar (TSA) plates.

Effects of pH and temperature on haemolysin production

Five isolates of *Listeria* (two of *L. monocytogenes,* two of *L. ivanovii* and one of *L. seeligeri*) were used. TSBYE broth measures (5 ml) in test tubes were adjusted to pH 5.0, 5.5, 7.0 or 9.0 using HCl or NaOH. The tubes were inoculated with 9.0 x10^{6} bacteria per ml before incubating at 5^{o}C and 30^{o}C. Haemolysin titres from the various tubes were determined on the seventh day of incubation as previously described.

Results

Quantitation of haemolytic activity

The two isolates of *L. monocytogenes* showed similar haemolytic patterns of complete haemolysis up to 1:4 dilution (CHU = 4). Isolate N showed partial haemolysis up to 1:16 dilution (MHU = 16) while isolate T showed partial haemolysis up to1:128 (MHU = 128). The *L. ivanovii* isolate examined displayed more intense haemolysis than the *L. monocytogenes* isolates, showing complete haemolysis up to 1:16 (CHU = 16) and partial haemolysis up to 1: 1024 (MHU = 1024).

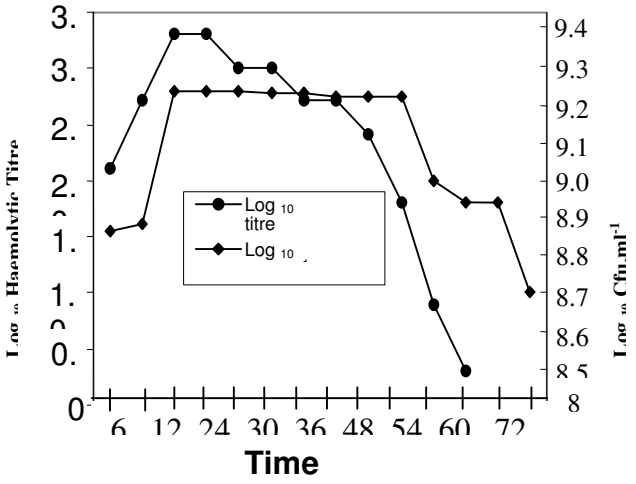

Fig. 1. Temporal synthesis of Haemolysin by *L. monocytogenes* T. Note peak production of haemolysin (log_{10} haemolytic titre 3.31) at 24h, which corresponds with the end of the log phase of growth.

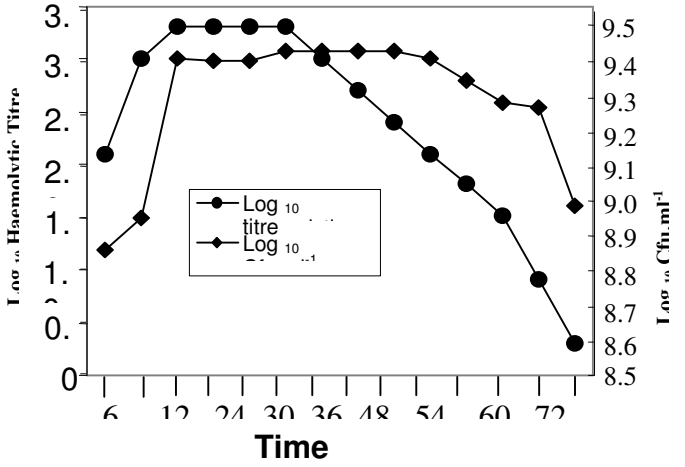

Fig. 2. Temporal synthesis of Haemolysin by *L. ivanovii* O. Peak haemolysin production was observed for this isolate at 24 h as was observed for *L. monocytogenes*. However, the peak was maintained for a longer period for this isolate.

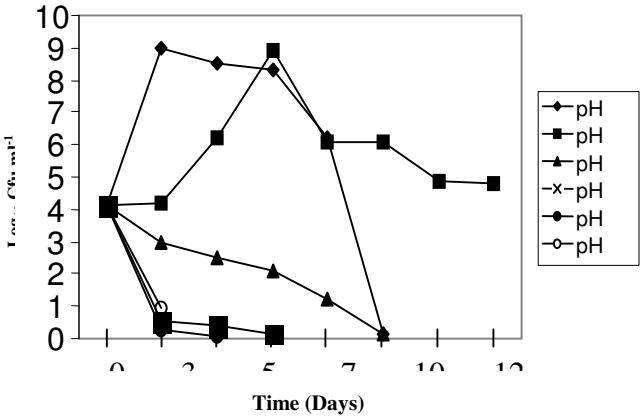

Fig. 3. Effect of pH on the growth of *L. monocytogenes* T at 30°C. The cells maintained greater viability at the higher pH values (pH 5.0 – 5.6) than at pH values 3.8 – 4.8. At pH values 3.8 to 4.8, no growth was recorded.

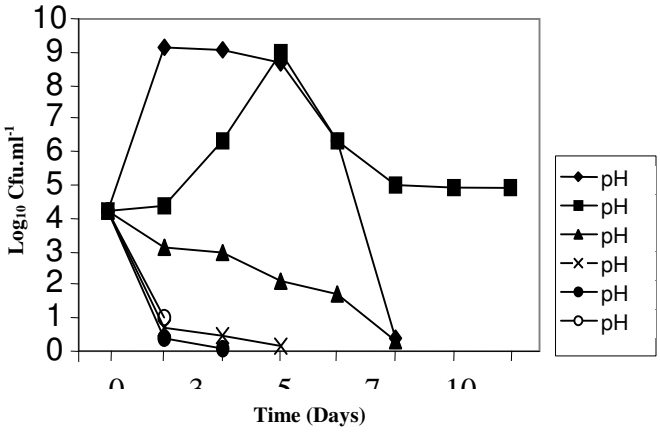

Fig. 4. Effect of pH on the growth of *L. monocytogenes* N at 30°C. The cells maintained greater viability at the higher pH values (pH 5.0 – 5.6) than at pH values 3.8 – 4.8. At pH values 3.8 to 4.8, no growth was recorded.

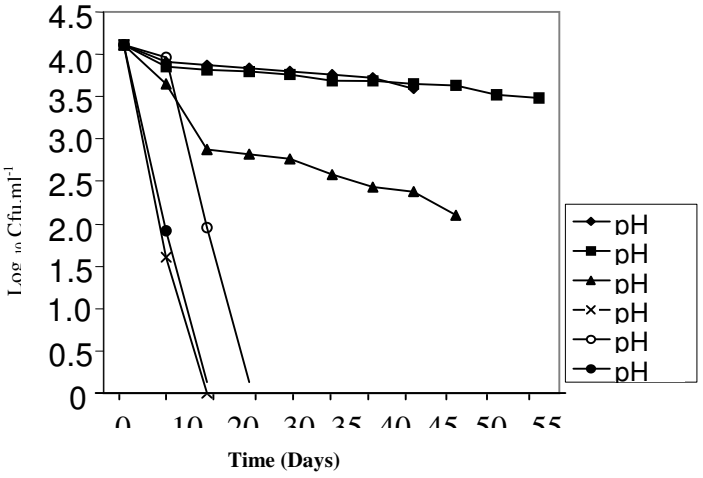

Fig. 5. Effect of pH on growth/ viability of *L. monocytogenes* T at 5°C. The cells maintained greater viability at pH 5.0 and pH 5.6 but rapidly lost viability at pH values 3.8 to 4.4.

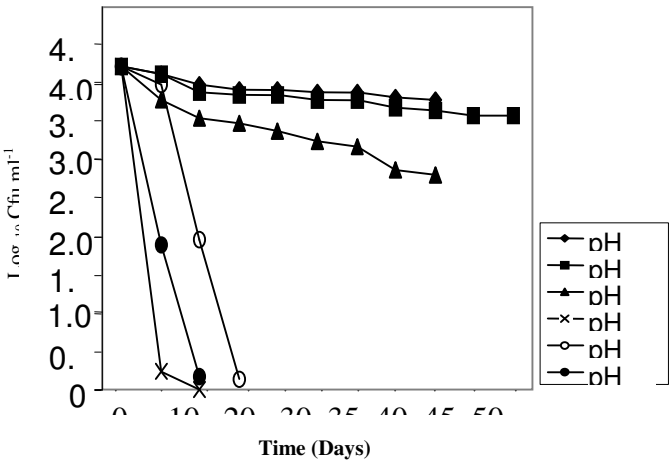

Fig. 6. Effect of pH on growth/ viability of *L. monocytogenes* N at 5°C. The cells maintained greater viability at pH 5.0 and pH 5.6 but rapidly lost viability at pH values 3.8 to 4.4.

Table 1. Effects of pH and temperature on haemolysin production

ISOLATES	PH							
	CHU at 5°C				CHU at 30°C			
	5	5.5	7	9	5	5.5	7	9
L. monocytogenes T	4	4	16	8	16	16	64	32
L. monocytogenes N	4	4	16	8	8	8	64	8
L. ivanovii O	4	4	16	8	16	16	128	64
L. ivanovii R	4	4	16	8	8	8	64	32
L. ivanovii F	2	2	8	4	16	16	64	32

CHU = Complete haemolytic unit. Values represent mean of three replicates.

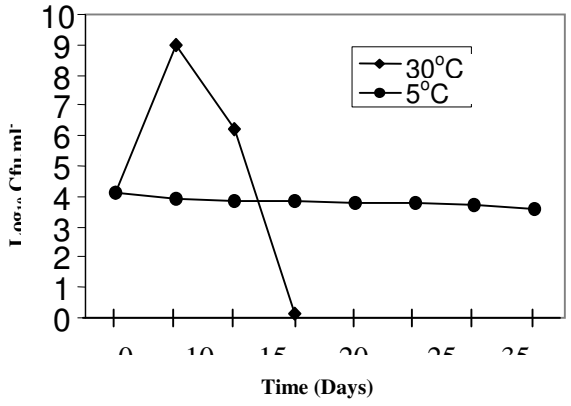

Fig. 7. Effect of temperature on the growth of *L. monocytogenes* T at pH 5.6. Growth was recorded only at 30°C with rapid loss of viability. Conversely, at 5°C, there was no growth but cells retained viability for up to 60 days.

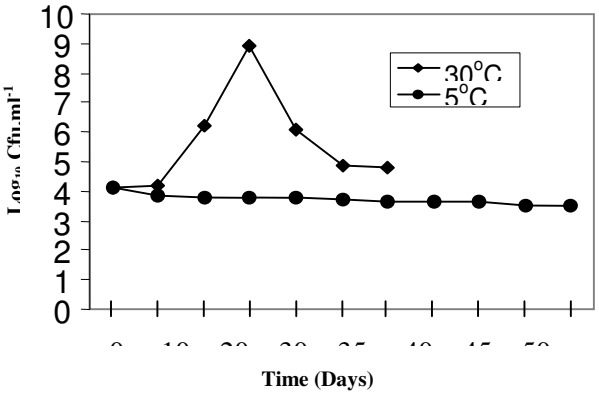

Fig. 8. Effect of temperature on the growth of *L. monocytogenes* T at pH 5.0. Growth was recorded only at 30°C. At 5°C, there was no growth but cells retained viability for up to 60 days.

Time course of haemolysin production

The temporal synthesis of haemolysin was studied with two isolates of *L. monocytogenes*, two isolates of *L. ivanovii* and one isolate of *L. seeligeri*. All isolates showed similar patterns of haemolysin synthesis, attaining peak production (log_{10} haemolytic titres 2.71 – 3.31) at 24h, which corresponded with the end of the log phase of growth. The titres remained near peak level until mid stationary phase after which there was a rapid decline in haemolytic activity. The data for *L. monoctogenes* T and *L. ivanovii* O are shown in Figs. 1 and 2. For *L. monocytogenes*, a peak titre of 3.31 was recorded at 24 h. The titre remained at that level till 30 h after which haemolytic activity began to decline dropping to near zero by 96 h. Similarly, a peak of 3.31 was recorded for *L. ivanovii*. However, for this isolate the titre remained near peak till 48 h before declining to near zero by 108 h.

Effect of pH on growth/viability of L. monocytogenes

The viability and growth of *L. monocytogenes* T and *L. monocytogenes* N were monitored over a range of pH values (3.8 to 5.6). The results are shown in Figures 3 – 6. The results showed that, generally, the cells maintained greater viability at the higher pH values (pH 4.8 – 5.6) than at pH values 3.8 – 4.4 at both 30°C and 5°C. At pH 5.6, both isolates attained their peaks in Log_{10} cfu.ml^{-1} (9.00 and 9.15 respectively) on the third day of incubation at 30°C. The numbers dropped after ten days and then declined rapidly to near extinction after 12 days (Figs. 3 and 4). At pH

5.0, the isolates attained their peaks (8.91 and 8.98 respectively) on the seventh day. The numbers remained near peak level until the 10th day after which the numbers began to decline. At pH values 3.8 to 4.8, for both isolates, no growth was recorded. Instead there was a steady decline in viable population to extinction within five to twelve days of incubation.

At 5°C, again the cells maintained greater viability at pH 5.0 and pH 5.6 but rapidly lost viability at pH values 3.8 to 4.4 (Fig. 5 and 6).

Effect of incubation temperature on growth/survival of L. monocytogenes

At the permissive pH values (5.0 and 5.6), growth was observed only at 30°C. At 5°C, however, no growth (actual increase in Log_{10} viable population) was recorded but the cells retained viability for over 60 days (Figs. 7 and 8).

Effects of pH and temperature on haemolysin production

For the five haemolytic isolates studied, the highest yields of haemolysin were produced in a medium of pH 7. This was true for cultures incubated at 5°C and for those incubated at 30°C. The highest activity of CHU 128 was observed with *L. ivanovii*, isolate O at pH 7 and 30°C (Table 1). Appreciable amounts of haemolysin were produced at the alkaline pH of 9 and at the acidic pH of 5. Furthermore there was about a four-fold higher production of haemolysin at 30°C than at 5°C.

Discussion

All isolates of *L. monocytogenes* and *L. ivanovii* used in this study were haemolytic with *L. ivanovii* displaying more intense haemolysis than *L. monocytogenes*. It has been reported that pathogenicity of listerial organisms, particularly *L. monocytogenes,* appears to be associated with the production of haemolysin (Brackett and Beuchat, 1990; Buncic *et al*., 1996; NFPA, 1999; Portnoy *et al.,* 2002; Haas *et al*., 2007). However, the conditions that determine production of the listerial haemolysin or listeriolysin and hence pathogenicity remain to be clarified.

Some investigators have attempted to define the temperature and pH conditions required for growth as well as haemolysin production of *L. monocytogenes* but no clear consensus has been reached. It has been reported that *L. monocytogenes* is capable of growth at temperature ranges of –0.4 to 50°C and pH values between 4.4 and 9.6 (NFPA, 1999; Beverly, 2004; Peiris, 2005). In a comprehensive study, Buncic *et al*. (1996) evaluated the dynamics of haemolysin production and pathogenicity of *L. monocytogenes* strains stored at refrigeration temperature. They concluded that haemolysin production and hence pathogenicity was dependent on whether cells were growing or non-growing at the time of evaluation. Growing cells were said to produce haemolysin and to have increased pathogenicity while non-growing cells that had ceased haemolysin production had reduced pathogenicity. This view is supported by the findings in this study, which show that for all isolates studied, haemolysin production was highest during the log phase of growth after which there was a rapid decline in haemolytic titres.

The effect of pH on viability and growth of the listerial organisms was investigated, using two isolates of *L. monocytogenes*. The results showed that pH values between 5.0 and 5.6 were more favourable for the viability and growth of the cells than the lower pH values from 3.8 to 4.8, at which the cells rapidly lost viability. The effect was, however, dependent on temperature. At 30°C, growth (increase in Log_{10} cfu.ml^{-1}) was recorded at the permissive pH values while at 5°C no growth was recorded although the cells maintained a reasonable level of viability (decrease in viable population of only 0.51 to 0.62) for over 60 days.

The effect of pH on haemolysin production was also evaluated at 5°C and 30°C respectively. The results showed that for all isolates, the highest haemolysin titres were recorded at neutral pH at both temperatures. These results are in agreement with those of some other researchers who showed that *L. monocytogenes* cells gave maximum haemolysin production at neutral pH and substantially reduced yields at pH 5.5 or below

(Mckellar, 1992; Khan *et al*., 1993; Datta and Kothary, 1993; Dimming *et al*., 1994). Although the influence of pH on haemolysin titres was shown, in this study, to be similar at both 5°C and 30°C, there was about a four-fold higher amount of haemolysin production at 30°C than at 5°C. This agrees with the reports of some workers (Czuprynski *et al*., 1989; Myers and Martin, 1994), but is in contrast with those of others (Durst, 1975; Shahamat *et al*., 1980; Stephens *et al*., 1991).

From published reports, the issue of effect of temperature on LLO production and pathogenicity seems to be a controversial one. Some researchers have speculated that refrigerated foods might constitute a greater hazard for listeriosis than non-refrigerated foods based on reports that production of haemolysin is greater at 4°C than at 37°C (Gray and Killinger, 1966; Durst, 1975; Shahamat,*et al*., 1980; Stephens *et al*., 1991). Some others, however, have rejected this view reporting that production of listeriolysin is significantly reduced at 4°C (Czuprynski *et al*., 1989; Myers and Martin, 1994; Buncic *et al*., 1996) and still others have reported not recording any significant effects of temperature (Brackett and Beuchat, 1990). Buncic *et al.,* (1996), in their report expressed the view that the effect of refrigeration on pathogenicity was not merely a question of temperature but more a question of the biological and metabolic status of the cells. They opined that if cells were growing and metabolically active at refrigeration temperature, they would still produce the haemolysin whereas if the cells were non-growing, haemolysin production would cease and therefore pathogenicity would be reduced.

As stated earlier, this study showed that at 5°C, no growth was recorded but viability was maintained over 60 days, particularly at pH between 4.8 and 5.6. At 30°C, however, growth occurred within the first seven days after which the cell numbers rapidly declined. When considered from the point of view of Buncic *et al*., (1996), then, one should expect haemolysin production to be higher at 30°C than at 5°C as was observed in this study. The implication of these results is that although *L. monocytogenes* maintains better viability at refrigeration temperature, haemolysin production is only enhanced when temperatures are raised to room temperature and above. It can be extrapolated from this then, that in terms of food safety, the risk of infection from the organism is greater when foods are stored at wrong temperatures or when foods are exposed to fluctuations in temperature. In other words, foods contaminated with *L. monocytogenes* may not necessarily pose a serious threat if cooked promptly and adequately after removal from the refrigerator. If, however, such food is allowed to attain temperatures between 28 to 30°C and left at that temperature for prolonged periods, the organisms multiply. If subsequently refrigerated, the cells survive refrigeration temperature only to multiply again when taken out. Thus, such practices may lead to increase in number of organisms found in the affected food to levels that may then pose a more serious threat to susceptible individuals.

It can be concluded therefore, that while preservation at refrigeration temperature (less than 5°C) and acidification to pH 4.4 and below may confer some protection against listeriosis, through the inhibition of cell proliferation and haemolysin production, the most suitable option for food safety remains the complete elimination of these organisms from food substances and the prevention of recontamination of cooked food.

References

Beverly, R. L. (2004).The control, survival and growth of *Listeria monocytogenes* on food products. *Ph.D. Dissertation.* Department of Food Science, Louisiana State University.

Bille, J. and Doyle, M. P. (1991). *Listeria* and *Erysipelothrix.* In: Balows, A., Hausler, W. J., Herrmann, L. K., Isenberg, H. D. and Shadomy, H. J. (eds.) *Manual of Clinical Microbiology* 5th ed. American Society for Microbiology, Washington, DC. Pp. 287-292.

Brackett, R. E. and Beuchat, L. R. (1990). Pathogenicity of *Listeria monocytogenes* grown on crabmeat. *Applied and Environmental Microbiology* 56: 1216-1220.

Buncic, S., Avery, S. M. and Rogers, A. R. (1996). Listeriolysin O production and pathogenicity of non-growing *Listeria monocytogenes* stored at refrigeration temperature. *International Journal of Food Microbiology* 31: 133-147.

Casadei, M. A., Esteves de Matos, R., Harrison, S. T. and Gaze, J. E. (1998). Heat resistance of *Listeria monocytogenes* in dairy products as affected by the growth medium. *Journal of Applied Microbiology* 84: 234-239.

Czuprynski, C. J., Brown, J. F. and Roll, J. T. (1989). Growth at reduced temperatures increases the virulence of *Listeria monocytogenes* for intravenously but not intragastrically inoculated mice. *Microbiological Pathogen* 7: 213-223.

Datta, A. R and Kothary, M. H. (1993). Effects of glucose, growth temperature and pH on listeriolysin O production in *Listreria monocytogenes*. *Applied and Environmental Microbiology* 59: 3495-3497.

Dimming, L. K., Myers, E. R. and Martin, S. E. (1994). Catalase, superoxide dismutase and listeriolysin O production by *Listeria monocytogenes* in broth containing acetic and hydrochloric acids. *Journal of Food Protection* 57: 626-628.

Dominguez-Rodriguez, L., Vazquez Boland, J. A., Fernandez Garayzabal, J. F., Echalecu Tranchant, P., Gomez-Lucia, E., Rodriguez Ferri., E. F. and Suarez Fernandez, G. (1986). Microplate technique to determine haemolytic activity for routine typing of *Listeria* strains. *Journal of Clinical Microbiology* 24: 99-103.

Durst, J. (1975). The role of temperature factors in the epidemiology of listeriosis. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A* 233: 72-74.

Food Safety and Inspection Services (FSIS) and United States Department of Agriculture (1999). *Listeriosis and Food Safety Tips.* USDA Consumer Publication List.

Gellin, B. G. and Broome, C. V. (1989). Listeriosis. *Journal of the American Medical Association* 261: 1313-1320.

Gray, M. L. and Killinger, A. H. (1996). *Listeria monocytogenes* and listeric infections. *Bacteriological Reviews* 30: 309-382.

Haas, J., Kusinski, K., Pore, S., Shadman, S. and Vahedi, M. (2007). A ride with *Listeria monocytogenes*: A Trojan horse. *Eukaryon* 3: 47-54.

Hitchins, A. D. (2001). *Listeria monocytogenes. Bacteriological Analytical Manual,* 8th ed. United States Food and Drug Administration and Center for Food Safety and Nutrition.

Khan, S. A., Khalid, S. M. and Siddiqui, R. (1993). The effect of pH and temperature on haemolysin production by *Listeria* species. *Letters in Applied Microbiology* 17: 14-16.

Low, J. C. and Donachie, W. (1997). A review of *Listeria monocytogenes* and listeriosis. *The Veterinary Journal* 153: 9-29.

McKellar, R. C. (1992). Effect of reduced pH on secretion, stability and activity of *Listeria monocytogenes* listeriolysin O. *Journal of Food Safety* 12: 283-293.

Mengaud, J., Chenevert, J., Geoffroy, C., Gaillard, J. and Cossart, P. (1987). Identification of the structural gene encoding the SH-activated haemolysin of *Listeria monocytogenes*: listeriolysin O is homologous to streptolysin O and pneumolysin. *Infection and Immunity* 55: 3225-3227.

Myers, E. R. and Martin, S. E. (1994). Virulence of *Listeria monocytogenes* propagated in NaCl containing media at 4°C, 25°C and 37°C. *Journal of Food Protection* 59: 2082-2086.

National Food Processors Association (1999). NFPA Fact Sheet on *Listeria monocytogenes.* NFPA.

Peiris, W. I. P. (2005). *Listeria monocytogenes*, a food borne pathogen. *M.Sc. Thesis Report.* Section of Food-associated Pathogens, Faculty of Veterinary Medicine and Animal Science. Swedish

University of Agricultural Sciences, Uppsala.

Portnoy, D. A., Auerbuch, V. and Glomski, I. J. (2002). The cell biology of *Listeria monocytogenes* infection: the intersection of bacterial pathogenesis and cell-mediated immunity. *The Journal of Cell Biology* 158: 409-414.

Ramaswamy, V., Cresence, V. M., Rejitha, J. S., Lekshmi, M. U., Dharsana, K. S., Prasad, S. P. and Vijila, H. M. (2007). *Listeria* – review of epidemiology and pathogenesis. *Journal of Microbiology, Immunology and Infections* 40: 4-13.

Shahamat, M., Seaman, A. and Woodbine, M. (1980). Survival of *L. monocytogenes* in high salt concentrations. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A* 246: 506-511.

Stehulak, N. (1992). Listeriosis. One tough bug. Ohio State university Extension Fact Sheet and United States Department of Agriculture.

Stephens, J. C., Roberts, I. S., Jones, D. and Andrew, P. W. (1991). Effect of growth temperature on virulence of strains of *Listeria monocytogenes* in the mouse: evidence for dose dependence. *Journal of Applied Bacteriology* 70: 239-244.