

Dept. of Food Hygiene,
Faculty of Vet. Med., Assiut University,
Head of Dept. Prof. Dr. T.A. El-Bassiony.

**SURVIVAL OF LISTERIA MONOCYTOGENES AT DIFFERENT
TEMPERATURES IN BROTH SUPPLEMENTED
WITH SODIUM CHLORIDE**
(With 3 Tables)

By
Y. HEFNAWY
(Received at 25/3/1989)

بقاء الليستيريا مونوسيتوجين في وسط سائل مضاف له كلوريد الصوديوم
على درجات حرارة مختلفة

يحيى حفناوى

تم دراسة نمو وبقاء عترتان من الليستيريا مونوسيتوجين Scott A, V7 في وسط سائل Tryptose broth مضاف له كلوريد الصوديوم بمستويات تتراوح من واحد إلى 10% وحفظ على ثلاث درجات حرارة مختلفة هي 13، 4، -18°م. وقد وجد أن قدرة الليستيريا على النمو في تركيزات عالية من كلوريد الصوديوم تزداد بازدياد درجة الحرارة. وعند درجة حرارة 13°م وجد أن نمو عترتان الليستيريا يزداد حتى في التركيزات العالية من كلوريد الصوديوم (9 : 10%). أما عند درجة 4°م فلقد لوحظ نقص في أعداد الليستيريا كلما زاد تركيز كلوريد الصوديوم وبالنسبة لدرجة -18°م فلقد تبين أن أعداد الليستيريا قد نقص بصورة ملحوظة. وقد تبين من الدراسة أن مستويات منخفضة من الملح تنبه نمو الليستيريا مونوسيتوجين والذي كان أكثر وضوحاً عند درجة حرارة 13°م. وبالتحليل الإحصائي وجدت فروق معنوية ما بين مسددة الحضنة، عترات الليستيريا، تركيزات كلوريد الصوديوم في درجات الحرارة المختلفة.

SUMMARY

Growth and survival of *Listeria monocytogenes* (strains Scott A and V7) with sodium chloride levels ranging from 0 to 10% were studied in Tryptose Broth (TB) at 13, 4 and -18°C. As the incubation temperature is increased, the organisms are able to grow in the presence of higher levels of sodium chloride. At 13°C the two serotypes were able to increase in numbers in up to 9 to 10% NaCl. At 4°C counts decreased with increasing NaCl concentrations over the 12-day period, but the organism survived in substantial numbers. At -18°C there was a large initial decline in counts with little subsequent change. Low levels of salt were found to stimulate growth of *Listeria monocytogenes* which was more pronounced at 13°C. A three way analysis of variance using the mean log CFU/ml broth showed holding time, *Listeria* strains, NaCl concentrations and their interaction to be significantly important for survival of the bacteria at different holding temperatures.

Y. HEFNAWY

INTRODUCTION

Listeria monocytogenes has been recognized as a human and animal pathogen for more than 50 years, however its prominence as a foodborne pathogen has only recently surfaced (DOYLE, 1984).

Salt is the most widely used of food preservatives. The microbiological effect of NaCl as a growth inhibitor for microbial cells in food probably depends on the osmotic withdrawal of water, and will reflect the water activity of the system. The effect of salt is often similar to that of drying (SCOOT, 1957). The lethal action of NaCl, like that of other food preservatives, is known to be reduced at low temperatures but the influence of temperature on the inhibitory (as distinct from lethal) action of salt is not well documented (INGRAM and KITCHELL, 1967).

Listeria monocytogenes is listed among the psychrotrophic food organisms (MOSSEL, 1971). Thus, the role of curing salts in the elimination of the organism has to be considered. The resistance of this organism to high salt concentrations was discussed in many published papers. *Listeria monocytogenes* was able to remain viable and fully virulent after eight weeks in 20% NaCl at 4°C but has to be compared with the culture, which without salt, could survive without loss of virulence for three to four years (MURRAY, 1955). Similar results were reported by WRAMBLY (1944), one of the early pioneers in the field who found that this organism was resistant to 20% NaCl for a period of eight weeks when incubated at 4°C. On the other hand, the viability of *L.monocytogenes* strains was shortened when exposed to 15% NaCl (BEGANOVIC, *et al.* 1971). These authors also studied growth of *L.monocytogenes* in 10% salt at 4°C and found that the organism died over 15 to 30 days, the time being dependent on inoculum size. On the contrary of other workers, KONTNICK, *et al.* (1971) illustrated that *L.monocytogenes* grew in 20% NaCl within a day. Other authors have also indicated that *L.monocytogenes* could survive for not only eight weeks in 20% NaCl but for more than eighteen weeks in 25.5% NaCl (SHAHAMAT, *et al.* 1980). The authors reported that temperature has a great influence on the action of salt and the survival time in the same concentrations can be lengthened from about 5 days at 37°C to 32 days at 22°C and more than 132 days at 4°C. Furthermore, this organism can remain viable in 16% NaCl for a year (WILSON and MILES, 1974).

Many of the studies reported in the literature have been on the lethal action of high levels of salt as used in curing brines. Little information is available on the interaction of incubation temperature and low levels of salt on the growth of *L.monocytogenes*.

As many foods contain salt at levels which are sublethal for *L.monocytogenes*, so this study was undertaken to determine the potential for *L.monocytogenes* growth in presence of salt at various temperatures.

EFFECT OF NaCl ON LISTERIA

MATERIAL and METHODS**Strains of *L.monocytogenes*:**

L.monocytogenes strain V₇ (serotype 1) and Scott A (serotype 4 b) were obtained from R.M. Twedt, Food and Drug Administration, Cincinnati, OH, USA). Cultures were maintained at 3°C on Tryptose Agar (TA) slants (Difco).

Preparation of inoculum:

Intermediate cultures were prepared by transferring isolated colonies of *L.monocytogenes* streaked TA plates into 10 ml of Tryptose Broth (TB) (Difco). Two-day-old colonies of V₇ and Scott A were used for the transfer, and the broth tubes were incubated at 35°C for 24 h. Inoculum was prepared by transferring intermediate cultures into a sufficient volume of TB and incubating it quiescently for 48 h at 25°C to provide a concentration of 10⁴-10⁵ *L.monocytogenes*/ml when added to the broth samples.

Preparation of Tryptose broth samples:

Experiments were performed to determine the effect of NaCl on the growth and survival of *L.monocytogenes* strains V₇ and Scott A at 13, 4 and -18°C in Tryptose broth. Salt levels of 0 to 10% were tested. Since formulated Tryptose broth (Difco) contains 0.5% NaCl, the medium for this experiment was prepared without this NaCl, using the following ingredients per liter: 20 g tryptose, 2 g dextrose; and appropriate amount of NaCl to produce each desired salt level.

Bottles of 100 ml and 200 ml capacity containing 50 and 100 ml of the formulated broths were inoculated with 1 ml of *L.monocytogenes* broth cultures. The contents of the second bottles were divided into test tubes, 5 ml per tube for frozen studies. *L.monocytogenes* count was made immediately to provide initial cell count. The survival time for the organism was followed at three different incubation temperatures (13, 4 and -18°C). The sampling times varied according to the incubation temperature. Replicate frozen tubes were individually thawed at room temperature (ca. 30 min.) before sampling to avoid repeat freeze-thaw microbial damage. All the experiments were duplicated.

***L.monocytogenes* counts:**

Serial dilutions of the Tryptose broths were made in 0.1% peptone water, and duplicate 0.1 ml portions of three consecutive dilutions were spread plated on McBride's *Listeria* agar (MLA) (MCBRIDE and GIRARD, 1960). Plates were incubated at 35°C for 48 h. Typical *L.monocytogenes* colonies which were smooth, bluish grey, slightly raised, translucent, watery consistency, 0.5-1.5 mm in diameter and weakly B-haemolytic were counted and counts were averaged.

RESULTS

The growth of both *L.monocytogenes* strains is greatly influenced by the incubation temperature. A three way analysis of variance was constructed using the mean

Y. HEFNAWY

log cfu/ml broth to determine the effects of holding time, *Listeria* strains, sodium chloride concentrations and their interactions on the survival of bacteria at different holding temperatures (Tables 1-3). Logarithmic transformation of cfu/ml broth obtained for each sodium chloride concentration at each holding temperature was done (SNEDECOR and COCHRAN, 1980; STEEL and TORRIE, 1980).

It is interesting that growth was obtained with both *L.monocytogenes* strains V₇ and Scott A in different levels of salt, as well as, in the controls without added salt at the different incubation temperatures.

DISCUSSION

Osmotic stress is of particular interest to food microbiologists because of the applications in preserving perishable foods. The magnitude of an osmotic stress on a population of microorganisms is conveniently expressed in terms of water activity (a_w) of the solutions. Microorganisms with a comparative bacteria in particular Gram-negative are more susceptible to plasmolysis than other microorganisms (such as Gram-positive bacteria and yeasts) containing a higher concentration of solutes. When a microbe is exposed to a very high concentration of NaCl, the effect of hypertonic solutions may be considered the main stress on it. There is some evidence that after plasmolysis has occurred a gradual return of the plasma membrane to the inside of the wall (deplasmolysis) may occur (ROSE, 1976). This may account for some of the fluctuations which appeared in the counts of cfu when *L.monocytogenes* strains V₇ and Scott A were suspended in a high concentration of NaCl and incubated at 4°C and -18°C for long periods.

The rate of growth of bacteria in media containing salt or other solutes is a function of the water activity and generally not of the concentration of a particular solute or solutes (SCOTT, 1957; WODZINSK and FRAZIER, 1960). As the solute concentration is increased, the water activity (a_w) is decreased. With a decreased a_w the log phase of the organism increases and the rate of growth decreases. A lowering of the incubation temperature from the optimum, while not materially affecting the a_w , at a given salt concentration (AYERST, 1965) does raise the lower limit of a_w for growth. Salmonellae are able to grow at 7-8% NaCl in the cultivation medium, if the growth is tested at 37°C (MATCHES and LISTON, 1972), and *Y.enterocolitica* is reported to grow in 5% NaCl at 25°C (STERN, et al. 1980).

As a rule, relatively low concentrations of salt will stimulate microorganisms, while higher concentrations inhibit them (INGRAM and KITCHELL, 1967). In this study it is shown that the range of salt concentration at which *L.monocytogenes* can grow is wide. Many investigators reported the survival of *L.monocytogenes* in higher salt concentrations at different incubation temperatures (WRAMBLY, 1944; MURRAY, 1955; WILSON and MILES, 1974; SHAHAMAT, et al. 1980).

The stimulating effect of low concentration of NaCl on the growth of bacteria may be related to Na^+ which is known to have at least two functions in the metabo-

EFFECT OF NaCl ON LISTERIA

lism of halophilic bacteria (GOW, et al. 1981). It is required specifically for the transport of a number of solutes into the cells; its function also less specifically; as an osmotic agent, in preventing the loss of intracellular solutes from the cells (MACLEOD, et al. 1978). Non halophilic bacteria possess an intracellular tonicity equivalent to that produced by about 0.85-0.9% NaCl. The other ingredients in the present Tryptose broth without added NaCl probably compensated the need of NaCl for intracellular tonicity.

It is apparent from this study that temperature has a great influence on the action of salt and the survival time. However, as the temperature was increased, the optimum sodium chloride concentration also increased. At the lower suboptimum temperature (13°C), the organisms appear to grow more rapidly when low levels of NaCl are present. This effect is evident in salt concentrations ranging from 1 up to 2% salt. In most instances the final number of cells attained is also higher at these salt concentrations than in the control samples. The growth of *E.coli* in a dilute medium was reported to be greatly stimulated by low concentration of sodium chloride (WARE, et al. 1955). The test organisms decreased in number when grown in media containing 0 to 0.2% NaCl, but the growth was stimulated at NaCl levels of 0.4% and 0.8%, with a maximum stimulation at 2.8% NaCl.

The experiments described in this report show that a cold temperature (4°C) greatly weakened the inhibitory action of NaCl and the loss of viability was very slow. On the other hand there is some evidence that the haemolytic activity of *Listeria* may be enhanced by incubation at 4°C (DURST, 1975). Also, this microorganism can increase in virulence after prolonged storage at 4°C (WOOD and WOODBINE, 1979).

From a practical point of view, application of these data to the storage of perishable food products is important. Foods with a low salt content may not support the growth of *L.monocytogenes* or other pathogens at low temperatures. If, however, storage temperatures are raised, conditions may become favourable for growth of these pathogens.

REFERENCES

- Ayerst, G. (1965): Water activity-Its measurement and significance in biology. Intern. Biodeter. Bull. 1: 13-26.
- Beganovic, H.; Milanovic, A. and Forsek, Z. (1971): The viability of *Listeria monocytogenes* in NaCl solution and pickle. Veterinaria (Sarajevo) 20: 73-80.
- Doyle, M.P. (1984): *Listeria monocytogenes*-A pathogen of renewed interest. Annual spring meeting, Food Research Institute, University of Wisconsin, Madison, USA.
- Durst, J. (1975): The role of temperature factors in the epidemiology of Listeriosis. Zbl. Bakt. Hyg., I. Abt. Orig. A 233: 72-74.
- Gow, J.A.; Macleod, R.A.; Goodbody, M.; Frank, D. and DeVoe, L. (1981): Growth characteristics at low Na⁺ concentration and the stability of the Na⁺ requirement of a marine bacterium. Canad. J. Microbiol. 27: 350-357.

- Ingram, M. and Kitchell, A.C. (1967): Salt as a preservative for foods. *J. Food Technol.* 2: 1-15.
- Kontrick, C.; Von Craevenitz, A. and Pisiciteli, V. (1971): Differential diagnosis between *Streptococcus agalactiae* and *Listeria monocytogenes* in the clinical laboratory. *Ann. Clin. Lab. Sci.* 7: 269-276.
- Macleod, R.A.; Goodbody, M. and Thomason, J. (1978): Osmotic effects on membrane permeability in a marine bacterium. *J. Bact.* 133: 1135-1143.
- Matches, J.R.; Liston, J. (1972): Effects of incubation temperature on the salt tolerance of *Salmonella*. *J. Milk Food Technol.* 35: 39-44.
- McBride, M.E. and Girard, K.F. (1960): A selective method for the isolation of *Listeria monocytogenes* from mixed bacterial populations. *J. Lab. Clin. Med.*, 55: 153-157.
- Mossel, D.A.A. (1971): Ecological essentials of antimicrobial food preservation; in: *Microbes and Biological Productivity*. Proceedings of the twenty first Symposium of the Society for General Microbiology, pp. 177-195. Cambridge University Press.
- Murray, E.G.D. (1955): A characterization of Listeriosis in man and other animals. *Canad. Med. Ass. J.* 72: 99-103.
- Rose, A.H. (1976): Osmotic stress and microbial survival, in: *The survival of Vegetative Microbes*, Proceedings of the Twenty Sixth Symposium of the Society for General Microbiology, pp. 155-182. Cambridge University Press.
- Scott, W.J. (1957): Water relations of food spoilage microorganisms. *Advances Food Res.* 7: 83-127.
- Shahamat, M.; Seaman, A. and Woodbine, M. (1980): Survival of *Listeria monocytogenes* in high salt concentrations. *Zbl. Bakt. Hyg., I. Abt. Orig. A.* 246: 506-511.
- Snedecor, G.W. and Cochran, W.G. (1980): *Statistical methods*. Iowa State University Press. Ames IA.
- Steel, R.G.D. and Torrie, J.H. (1980): *Principles and procedures of statistics. A biochemical approach*. 2nd ed. McGraw-Hill Book Co., New York.
- Stern, N.G.; Pierson, M.D. and Kotula, A.W. (1980): Effects of pH and sodium chloride on *Yersinia enterocolitica* growth at room and refrigeration temperature. *J. Food Sci.* 45: 64-67.
- Ware, G.C.; Childs, E. and Smith, H.M. (1955): The effect of salt concentration on the growth of bacteria in dilute nutrient solutions. *J. Appl. Bacteriol.* 18:446-448.
- Wilson, G. and Miles, A.A. (1974): *Topley and Wilson's Principles of Bacteriology, Virology and Immunology*, 6th ed. Vol. I, pp. 559-563; Vol. II, pp. 1716-1723. Arnold, London.
- Wodzinski, R.J. and Frazier, W.C. (1960): Moisture requirements of bacteria. I. Influence of temperature and pH on requirements of *Pseudomonas fluorescens*. *J. Bacteriol.* 79: 572-578.
- Wood, L.V. and Woodbine, M. (1979): Low temperature virulence of *Listeria monocytogenes* in the avian embryo. *Zbl. Bakt. Hyg. I. Abt. Orig. A.* 243: 74-81.
- Wrambly, G.O. (1944): Om *Listerella monocytogenes* bakteriologi och om forekomst av *Listerella* infektion nom djur. *Skand. Vet. Tidskr.* 34: 277-290. Cited by Shahamat, et al. (1980).

EFFECT OF NA CL ON LISTERIA

Table (1)

Three way analysis of variance using mean \log_{10} cfu/ml broth at 13°C

Source of variation	df	SS	MS	FS	p-level
Holding time (days)	6	2011.58	335.26	213.04	**
Listeria strains	1	69.83	69.83	44.37	**
NaCl concentrations	10	431.91	43.19	27.44	**
Listeria strains X NaCl concentrations	10	4.72	0.47	0.30	N.S.
Error	126	198.29	1.57		
Total	153	2716.34			

Table (2)

Three way analysis of variance using mean \log_{10} cfu/ml broth at 4°C

Source of variation	df	SS	MS	FS	p-level
Holding time (days)	12	129.38	10.78	33.01	**
Listeria strains	1	92.22	92.28	282.39	**
NaCl concentrations	10	141.75	14.18	43.41	**
Listeria strains X NaCl concentrations	10	23.52	2.35	7.2	**
Error	252	82.29	0.33		
Total	285	496.16			

Table (3)

Three way analysis of variance using mean \log_{10} cfu/ml broth at -18°C

Source of variation	df	SS	MS	FS	p-level
Holding time (days)	14	34.27	2.45	8.35	**
Listeria strains	1	128.72	128.27	439.01	**
NaCl concentrations	10	91.75	9.18	31.29	**
Listeria strains X NaCl concentrations	10	1.25	0.126	0.43	N.S.
Error	294	86.20	0.29		
Total	329	342.20			

** = Significant at (P/ 0.01) and (P/ 0.05).

N.S. = Not significant.