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Growth and Cell Morphology of *Listeria monocytogenes* as Affected by Various Concentrations of NaCl and KCl

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ABSTRACT

In this study, the effects of various concentrations of NaCl and KCl on the growth characteristics and cell morphology of *L. monocytogenes* were evaluated. It was found that *L. monocytogenes* can grow in the presence of 1-9 % NaCl and 1-11 % KCl. The higher the concentration of NaCl used, the longer the lag phase induced. In addition, it was observed that *L. monocytogenes* tolerate KCl better than NaCl when using the same percents in broth. Microscopic analyses of *L. monocytogenes* following incubation revealed cell elongation under high salt conditions. The beginning of filament formation was apparent at 5 % NaCl and 7% KCl with an increase in filament length with increasing salt concentration.

Key Words: *Listeria monocytogenes*, KCl, NaCl, salt, morphology.

INTRODUCTION

Listeria monocytogenes is a foodborne pathogen that can cause serious invasive disease in humans. This microorganism is widespread in the environment and is able to survive and growth, under environmental conditions that are lethal or inhibitory to many other non-sporeforming foodborne pahogens. This pathogen is remarkably salt tolerant and can survive under high salt concentrations (McClure et al. 1989). *Listeria monocytogenes* has been isolated from a range of salt containing food including cheese, salted fish, cooked ham and other cured meat and seafood products (Sheridan et al. 1994; Rocourt 1994; Greenwood et al. 1991; Akhondzadeh Basti et al. 2006). NaCl is one of the most commonly employed agents for food conservation, allowing considerable increase in storage time by reducing water activity. Historically, NaCl was among the very few effective preserving methods known. With the advent of refrigeration, better processing, packaging, transport and storage, there is less need for high salt levels to maintain product quality and safety. Furthermore, in recent years there is a tendency for reducing sodium in foods due to its relationship with hypertension, but where salt has been added as a preservation hurdle, removal or reduction of the salt will reduce shelf-life and could affect safety in more microbiologically fragile products. Potassium chloride (KCl) is the most obvious replacement for salt (NaCl) in food products (Bidlas and Lambert 2008). Previous report on the survival of microorganisms at low a_w values revealed that the response to a_w was solute dependent and for the growth of *Clostridium perfringens*, solute identity had a bearing on the amount of growth for a given a_w, with KCl having a demonstrably greater effect than NaCl (Strong et al. 1970). In addition, NaCl was found to be more inhibitory than glycerol for *Salmonella* cells at the same a_w (Marshall et al. 1971). Beuchat (1974), however, reported that at equivalent a_w, NaCl and KCl had equivalent effects against *Vibrio parahaemolyticus*. Furthermore, it was observed that in fermented meat products, the replacement of NaCl with KCl did not affect the degree of inhibition and or inactivation, but did alter the taste of the foodstuffs (Gimeno et al. 1999, 2001).

Therefore, in the present study, the effect of various concentrations of NaCl and KCl on the growth characteristics of *L. monocytogenes* in broth was evaluated. In addition, the effect of these conditions on morphological characteristics of the cells was also evaluated.

MATERIALS AND METHODS

Microorganism and growth medium

The stock culture of *Listeria monocytogenes* (a food isolate) was stored at -20 °C in Tryptic Soy Broth (Merck) supplemented with 25 % (vol/vol) sterile glycerol (Merck). To prepare the inoculum, 0.1 ml of stock culture was added to 10 ml of TSB and incubated without shaking for 18 to 24 h at 35°C. NaCl and KCl were added to TSB prior to autoclaving and pH was adjusted to 6.0 with HCl after autoclaving.

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Growth studies

The behaviour of *L. monocytogenes* in TSB (pH, 6.0) was determined at 30°C in the presence of 1, 3, 5, 7, 9, and 11 % NaCl or KCl. To achieve this purpose, according to the correlation between optical density (O.D.) and viable cell count, portions (10 ml each) of sterile TSB containing 1, 3, 5, 7, 9 and 11 % NaCl or KCl were inoculated with 100 µl of diluted *L. monocytogenes* culture to produce an initial level of ca 10^5 CFU/ml. It was confirmed by plating 100 µl on TSA. For the inoculated TSB medium, 300 µl were dispensed in six wells and the same volume of non-inoculated medium was dispensed in four wells of micro-titre plates in order to determine the O.D. of the growth medium and to detect possible contamination. A synergy HT microplate reader (BioTek Instruments) was used to follow the growth of *L. monocytogenes* in the micro-titre plates. Optical density was read every one hour for the first 24 h. and then every two hour until 70 h. at a wavelength of 600 nm.

Morphological observations

Changes in cell morphology were assessed by Gram staining; microscopic observations were made using the oil immersion lens of a light microscope (Olympus Instruments).

RESULTS

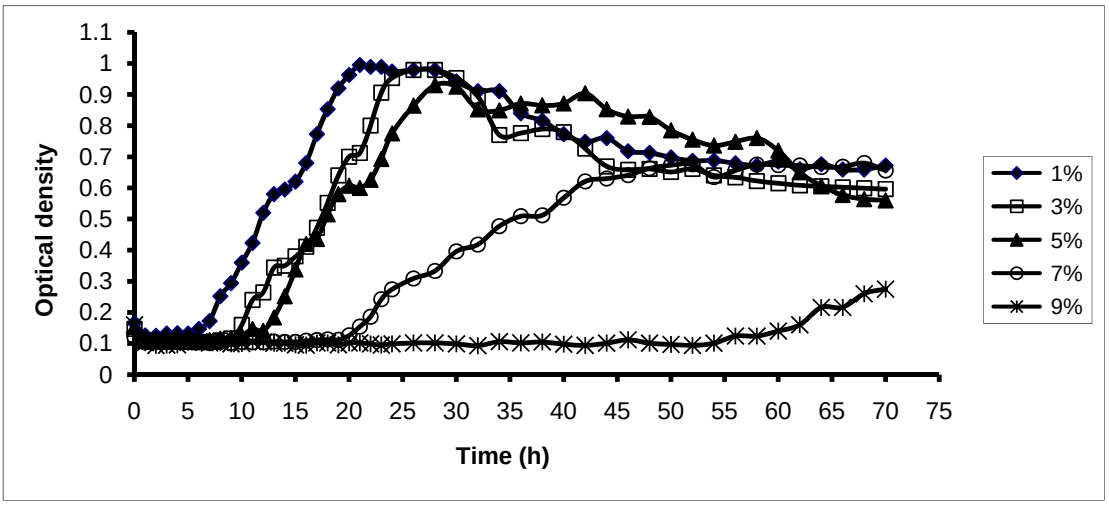
Growth studies

The growth curves of *L. monocytogenes* in the presence of various concentrations of NaCl and KCl can be seen in Fig 1a and 1b. As shown, *L. monocytogenes* can grow in the presence of 1, 3, 5, 7, and 9 % NaCl. The higher the concentration of NaCl used, the longer the lag phase induced. For example, the growth was occurred in the presence of 7 and 9 % NaCl after a lag phase of approximately 20 and 52 h, respectively. According to our results, the growth curve of *L. monocytogenes* was more affected by the presence of NaCl than by the presence of the same concentrations of KCl. Apart from the presence of 1, 3 and 5 % NaCl, where no significant differences were observed as compared to treatments having the same percents of KCl, addition of higher concentrations of salts resulted in a more inhibitory environment in NaCl supplemented broths. For example, the growth in the presence of 7 and 9 % NaCl induced longer lag times compared to the presence of the same concentrations of KCl. Furthermore *L. monocytogenes* was not able to grow in TSB containing 11 % NaCl until 120 h, but in case of 11 % KCl, the growth was occurred after about 50 h lag phase.

Morphological studies

Young culture of *L. monocytogenes* consist of coccoid organisms measuring 0.5 by 1.0 to 2.0 µm; the ends that may be slightly pointed, which short chains and diploforms often seen as V- or Y- shaped. In this study, microscopic analyses of *L. monocytogenes* following incubation revealed morphological changes under high salt conditions. Filaments were observed in KCl or NaCl supplemented broths as well as in all the combination treatments (Fig. 2a and 2b). The beginning of filament formation was apparent at 5 % NaCl and 7% KCl with an increase in filament length with increasing salt concentration.

a)



b)

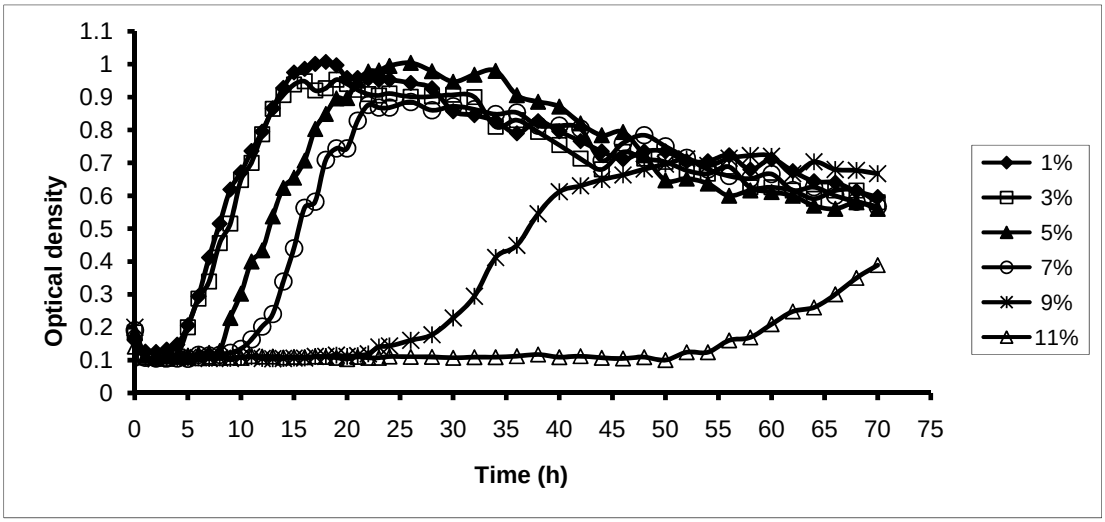


Figure 1. Growth curves of *L. monocytogenes* in TSB containing various concentrations of NaCl (a) and KCl (b) at 30°C.

a)

b)

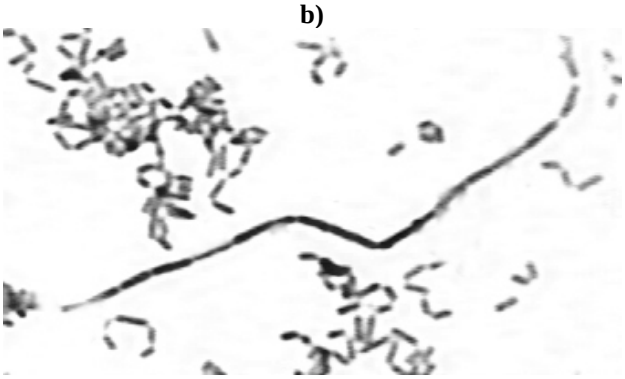


Figure 2. Filament formation in *Listeria monocytogenes* in TSB containing (a) 9 % NaCl and (b) 11 % KCl at pH 6.0.

DISCUSSION

Reducing the available water in food is a long-established method for controlling bacterial growth. Desiccation or increasing the humectant content of a food results in a reduced water activity (a_w) (Brown 1976). Optimal growth of *L. monocytogenes* occurs at an a_w of 0.99, but this bacterium tolerate many stressful conditions and can survive in low-a_w foods for long periods (Larson et al. 1999). Various solutes are incorporated into food in order to reduce a_w and maintain a reasonable safety margin before growth of microorganisms can occur. In addition to impart salty flavor, NaCl is commonly used in various steps of food preparation to inhibit the growth of spoilage and pathogenic bacteria. For example, salting of cheese by immersion in brine is a common industry practice which improves taste and also as a preserving agent. Several cheese varieties are brine salted, including pasta filata types (mozzarella, provolone, salami, and giganti), brick, Hispanic, and feta. The salt content of brines is maintained at 18 to 24% for most varieties and at 5 to 10% for feta. However, consumers want products with reduced level of sodium and the consumer pressure has resulted in increases in the a_w in intermediate-moisture-level foods (a_w, 0.6-0.9) (Li and Torres 1993). This affects primarily the spoilage stability of the products at the lower end of this a_w range, but pathogens, including *L. monocytogenes*, have caused illness following survival in foods at the higher end.

It was reported previously that potassium chloride has an equivalent antimicrobial effect on some microorganisms when calculated on a molar basis (Boziaris et al. 2007; Bidlas and Lambert 2008). As the amount of added salt is generally calculated on a percent basis in the food industry, in this study we used different percents of the salts. According to the results of the present study, although *L. monocytogenes* can grow in the presence of up to 9 % NaCl and up to 11 % KCl, the lag times increased as the concentration of the salts

increased. According to McClure et al. (1989), *L. monocytogenes* is able to grow in nutrient broth supplemented with up to 10% (w/v) NaCl at pH 5.0 to pH 8.0 at 25°C. Furthermore, our results indicate that, *L. monocytogenes* tolerate KCl better than NaCl when using the same percents in broth. On the other hand, KCl has not an equivalent antimicrobial effect on *L. monocytogenes* when calculated on a percent basis. It seems that due to a larger a_w effect of NaCl, *L. monocytogenes* was more affected by the osmotic conditions made by NaCl when using the same percents as KCl.

According to Hazeleger et al. (2006), exposure to salt stress resulted in high amounts of elongated cells in *Listeria* at concentrations of more than 7 % which is higher than that observed in our study. In our study filament formation was started at 5 % NaCl and 7 % KCl and the filament length and the extent of filamentation were increased as the concentration of the salts increased. Morphological changes in *L. monocytogenes* grown in the presence of high levels of NaCl at 10°C and 37°C were studied by Brzin (1973, 1975), who found that growth on agar media containing 8 to 9 % NaCl (pH, 7.0) was accompanied by cell elongation. The degree and extent of elongation increased as the growth temperature increased. Incubation at 37°C and 30°C resulted in the longest cells, while the effects on morphological characteristics were much less pronounced after incubation at 10°C. According to Bereksi et al. (2002), cell elongation in *L. monocytogenes* was only observed when NaCl addition and acidification were applied concomitantly. They reported remarkable morphological changes that mainly consisted of the presence of filamentous structures. Some filaments were barely apparent when *L. monocytogenes* Scott A was grown at pH 5.0 with 5% NaCl, but became predominant when the concentration of NaCl was increased to 10 %. However, Isom et al. (1995) observed morphological changes in *L. monocytogenes* grown at 37°C at pH 7.0 in TSB containing various NaCl concentrations (up to 8.8 %). Filament formation was apparent at NaCl concentration of 5.8 % and filament length increased as the NaCl concentration increased. They also reported the presence of filamentous structures when cells were grown at 37°C at pH 5.0 in the absence of additional NaCl. We observed filamentation at pH 6.0 in the presence of 5 % NaCl or 7 % KCl, and filament length and the number of filamentous cells were increased as the concentration of the salts increased. The filamentous cells observed in our study presumably formed as a result of a continued increase in biomass in the absence of cell septation during the low-a_w stress. According to Hazeleger et al. (2006), these elongated cells are actually on the verge of division and, when transferred to more favorable conditions, will split up rapidly in single cells and start growing. If this happens in practice, it could have a significant impact on food safety, for instance when elongated pathogens are present. If that food is subsequently kept at conditions where growth is possible, the filaments will split up rapidly into many cells, resulting in a highly contaminated food product.

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