# INFORMATION TO USERS

Studies on the effect of heat shock, culture conditions, and
packaging conditions on the heat resistance, recovery, and
virulence of *Listeria monocytogenes* in ground pork

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Studies on the effect of heat shock, culture conditions, and packaging

conditions on the heat resistance, recovery, and virulence of

*Listeria monocytogenes* in ground pork

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# GENERAL INTRODUCTION

In the last decade, pathogenic bacteria not previously associated with foodborne illness have emerged and their incidence in outbreaks has been increasing. The reasons for the appearance of such previously unknown organisms have been summarized by Cox (1989). These include: 1) changes in eating habits increasing the acceptability of fresh or wholesome foods, which do not contain as many barriers for control of microorganisms, 2) advances in epidemiology and more rapid and complete notification of outbreaks, 3) demographic changes in the number of ill and older people, 4) larger-scale food production and distribution, 5) changes in food processing such as vacuum packaging or chill storage, which promote survival of certain pathogens, 6) changes in handling and preparation practices, and 7) changes in the behavior and virulence of microorganisms by genetic exchange between pathogenic and nonpathogenic strains. *Listeria monocytogenes* is considered one of these newly-emerging pathogens due to its ability to grow in refrigeration conditions and to its resistance to various environmental conditions. In fact, cases of foodborne listeriosis have increased since the 1980's.

Because of its wide distribution in nature, the ecological niche of *Listeria* is difficult to define. The organism is found in soil, water, and the intestinal tract of animals and humans. Consumption of foods contaminated with this organism cause septicemia, meningitis, endocarditis, spontaneous abortions, and stillbirths.

*Listeria monocytogenes* is resistant to processing conditions used in food production such as salting, freezing, or sanitization. In particular, it is somewhat resistant to heat, which is a subject of concern to food processors. Heating is used for pasteuriza-

tion and cooking, and some organisms are found to become more heat-resistant after exposure to mild heating that occurs during precooking. This phenomenon is called 'heat shock', and these cells produce heat shock proteins that can protect the cells from subsequent heat treatments. Therefore, the heating rate of food products in thermal processing can affect the survival of microorganisms by inducing a heat shock response. Coupled to this phenomenon, storage conditions such as vacuum packaging after cooking could affect the ability of heat-injured cells to recover at low temperatures, thus increasing the hazard to the consumer.

*Listeria monocytogenes* has been detected routinely in dairy products and occasionally in meat products. Ground pork is one of the most common products used in production of processed meats. It has a higher potential for contamination with pathogens due to having a larger surface area, high nutrient content, neutral pH, and high water activity.

Thus, the possibility of outbreaks of listeriosis due to consumption of contaminated ground pork is a source of concern. However, information on the effect of heat shock on survival of *Listeria* in ground pork and on its effect on virulence of this organism is not available. For this reason, heat processing and storage methods must be evaluated and the virulence of heat shocked *Listeria* in ground pork should be assessed. This information can help processors to design appropriate conditions for the destruction of *Listeria monocytogenes* in meat processing.

The objectives of this study were:

1) To determine the effect of heat shock on survival of *Listeria monocytogenes* and on production of listeriolysin in ground pork.

2) To determine the effect of varying the rate of heating on survival of cells to subsequent processing in ground pork.

3) To determine the effect of meat age, storage temperature, storage atmosphere, and presence of antioxidants on recovery of *Listeria* after heating in ground pork.

## An Explanation of the Dissertation Organization

This thesis includes three manuscripts to be submitted to the Journal of Food Protection. A literature review precedes PAPER I and a general discussion of the research follows PAPER III. A separate reference section has been used for each of the manuscripts. References cited in the general introduction, listerature review, and summary and conclusions follow the summary and conclusions.

## LITERATURE REVIEW

### 1. History

Descriptions of the involvement of gram-positive rods in illnesses that may have been listeriosis were reported as early as 1891 (Gray and Kilinger, 1966). *Listeria monocytogenes* was first isolated from rabbit liver by Hülphers in Sweden in 1911, at which time this organism was named *Bacillus hepatis*. In 1915 and 1919, the organism was isolated from a patient with meningitis. Murray *et al.* (1926) reported that a gram-positive rod-like organism was isolated from blood of infected rabbits and pigs in 1924, and the organism was named *Bacterium monocytogenes* since it produced monocytosis as one of the symptoms. In 1927, Pirie (1940) also isolated and described a bacterium from an African mouse, and named it *Listerella hapatolytica*. Both *Listerella* and *Bacterium* were determinated to be the same organism, so the name of both was changed to *Listerella monocytogenes*. Subsequently, it was changed to *Listeria monocytogenes* in 1940, because the name *Listerella* had already been given to a Mycetozoan by Jahn in 1927. In the United States, research about *Listeria* was accomplished principally by M. L. Gray. He used a cold enrichment method for isolation of *Listeria* (Gray *et al.*, 1948) and developed the use of oblique lighting for detecting *Listeria* on isolating agar (Gray *et al.*, 1957). He also isolated this organism from the brain of an infected sheep from a flock fed contaminated silage. The cultures were all found to be serotype 4b (Gray, 1960), and the silage was shown to serve as a vehicle of infection.

Before 1960, most research on listeriosis was based on animals, but sporadic

cases of listeriosis were reported often in workers who were in contact with diseased

animals (Cain and McCann, 1986). As a result of outbreaks of foodborne listeriosis in

the 1980's, interest in *Listeria* has grown rapidly among food industry, government, and

health officials. Likewise, the amount of published information on the physiology,

pathogenesis, and survival of this organism has increased significantly.


## 2. General characteristics of *Listeria monocytogenes*


*Listeria monocytogenes* is a small, short, gram-positive rod, 0.4-0.5 $\mu$m in diameter

and 0.5-2 $\mu$m in length, with rounded ends. *Listeria* cells are flagellated, exhibiting a

characteristic tumbling or slightly rotating motility. The degree to which flagella are

produced is temperature related: higher temperatures suppress expression of motility,

although most cultures grown between 30°C and 37°C will still demonstrate some

motility. Motility is best demonstrated at 20°C in 0.2-0.4% semi-solid agar in U-shaped

tubes and confirmed by stabbing a tube of dextrose gelatine medium. This organism is

catalase-positive (which is indicated by the evolution of gas from $H_2O_2$), oxidase-

negative (which denotes the absence of cytochrome oxidase), methyl red-positive

(which exhibits the production of acids by fermentation of sugars), Voges-Proskauer(-

VP)-positive (which denotes the presence of acetylmethylcarbinol), and indole-negative

(which denotes the absence of tryptophanase). In addition, this organism utilizes

glucose and esculin with the production of acid but not gas. It can not utilize mannitol

or xylose. *Listeria* spp. grow well on simple laboratory media in the pH range of 5.0-

9.0. On solid media such as nutrient agar, *Listeria* colonies are translucent, dew-drop-like and bluish when viewed by 45° incident transmitted light. After 48 hr at 37°C, colonies are 0.2-0.4 mm in diameter. *Listeria* are resistant to some antibiotics such as polymyxin, nalidixic acid, cefolaxime, or acriflavine, which have been used as supplements in selective media.

*L. monocytogenes* serotypes produce listeriolysin (LLO) and β-hemolysin, which are pore forming cytolysins that are essential for its pathogenicity. These are discussed in more detail in section 4. of this review.

*L. monocytogenes* can grow in a wide pH range. Conner *et al.* reported the growth range in cabbage juice to be pH 5.0-9.0. Environments with pH values less than 4.5 and more than 9.5 are hostile to this organism. However, the pH value for growth is dependent on temperature. Ahamad and Marth (1989) reported that the minimum pH required for initiation of growth ranged from 5.0 to 5.7 at 4°C and from 4.3 to 5.2 at 30°C.

*Listeria* is a psychrotroph. Junttila *et al.* (1988) reported that growth of 78 *L. monocytogenes* strains on tryptose soy agar occurred at a mean minimum temperature of 1.1°C. Although growth of this organism in laboratory media at 1°C is very slow, when incubated at higher temperatures (3-6°C), the growth rate increases so the organism attains a final population of approximately $10^8$ CFU/ml of media after several weeks of incubation (El-Shenawy, 1988).

Humans, animals, and other environments serve as reservoirs of *Listeria*. This organism has been isolated from a wide variety of animals, including sheep, dog, rat, cat, rabbit, and mouse. While it was once thought that the principal source of human

listeriosis was animal contact, it is now believed that most human listeriosis results from ingestion of foods contaminated with this organism.

## 3. Isolation and Identification methods

### Serology

The basis for the present serotyping scheme for *Listeria* was established by Paterson (1940). His scheme was based on both somatic and flagellar antigens, resulting in four serotypes (serotype 1, 2, 3, and 4). Seelinger (1961) expanded the number of serotypes to five (serotype 1, 2, 3, 4a, and 4b) and Donker-Voet (1972) added more to the number of serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7). *L. monocytogenes* serotynes isolated from pathological sources are most likely to belong to serotypes 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, and 4b. Seeliger and Horhn (1979) reported that 98% of several thousand isolates associated with illness belong to those seven serotypes plus *L. ivanovii*. The serotypes that were identified differed according to region where found. For example, serotype 4b predominates in most of Europe, but there appears to be an even distribution of serotypes 1/2a, 1/2b, and 4b in Canada and the United States. Some serotypes are cross-reactive with some enterococci, certain *Escherichia coli* strains, *Staphylococcus aureus*, and some motile corynebacteria.

### Strain typing

Phage typing has been proposed as a means of providing another epidemiological

tool that expands and improves the capacity to identify relationships between *Listeria* isolates. Rocourt *et al.* (1989) increased the phage set to 31 by expanding the search for phages to include all recently recognized *Listeria* spp. Although phage typing is reproducible and discriminatory (McLauchlin *et al.*, 1986), it has a limitation of 90% of typable strains of serotype 1/2. There is a lack of phages for other serotypes such as 3 and 7 which are resistant to the phages but are not pathogenic. Recently, Audurler and Martin (1989) reported that *L. monocytogenes* was typed by using 34 phages in studies of listeriosis outbreaks and that the proportion of phage-typable *L. monocytogenes* serotype 1 and 4 was 61.1%. There is an international phage typing system for *L. monocytogenes* which is useful in epidemiological studies. The Pasteur Institute Laboratory is recognized as the International Center for *L. monocytogenes* phage typing. Loessner and Busse (1990) developed a bacteriophage typing scheme for differentiating *Listeria* isolates from dairy products and various other foodstuffs.

Isoenzyme typing is another method for *Listeria* typing (Selender *et al.*, 1991). Piffaretti *et al.* (1989) isolated 175 *L. monocytogenes* from various sources and found 45 allele profiles (or electrophoretic types (ET)) ('allele' is defined as alternative forms of a gene that can occupy a particular chromosomal site). Bibb *et al.* (1989) found 56 ETs from 310 strains. They discovered that all strains that cause listeriosis have the same or similar multilocus genotypes of enzymes. The typing is done by comparing the genetic relationships or genetic distance among the ETs. The method appears to be useful in either confirming or eliminating a common source as the cause of an outbreak of food-borne listeriosis. Later, Bibb *et al.* (1990) found 11 different ETs by using 390 isolates from patients with listeriosis in the United States during 1986 and 1987, ruling

out a single common source as a cause of that outbreak in the United States.

DNA fingerprinting is a method that uses restriction enzyme analysis (REA). It has been used to characterize strains causing outbreaks of listeriosis associated with cheese in Los Angeles, as well as the Nova Scotia outbreaks. By using this method, Wesley and Ashton (1991) reported that *L. monocytogenes* serotypes isolated from the Nova Scotia, Los Angeles, and Massachusetts outbreaks each have a unique restriction enzyme pattern. As a modified method, rRNA typing involving both radioactive and nonradioactive methods has been evaluated recently and, along with DNA fingerprinting, was found to be more discriminatory than either serotyping or phage typing, and equivalent to isoenzyme typing (Olander *et al.*, 1990).

## Media for Isolation and enumeration of Listeria monocytogenes

The isolation of *L. monocytogenes* from foods and environmental sources requires enrichment culture. In general, cold enrichment (CE) has been used at 4°C for several weeks (Heyes *et al.*, 1986), because this organism is psychotropic, which enables it to outgrow mesophiles present in the sample. But, because CE requires a long incubation time, selective agents have been used by many researchers for warm enrichment broths using many kinds of antibiotics which do not affect the growth of *L. monocytogenes*. Major enrichment media for enumeration of *L. monocytogenes* are shown in TABLE 1.

As a next step, many kinds of selective media have been suggested by many researchers for effective isolation of *L. monocytogenes* and for exact differentiation from other organisms after enrichment. As a physical method of differentiation of *Listeria*,

TABLE 1. Major enrichment media for *L. monocytogenes*[1]

| Media | Applied samples | Remarks |
| --- | --- | --- |
| FDA Enrichment Broth | milk, dairy products, poultry | warm enrichment broth |
| University of Vermont Medium (UVM) | meat, poultry, milk seafood | warm enrichment broth used as first enrichment broth in USDA procedure for meat and poultry |
| USDA Listeria Enrichment broth I (LEB I) | meat, poultry, seafood | UVM + nalidixic acid |
| USDA Listeria Enrichment broth II (LEB II) | meat, poultry, seafood | USDA enrichment broth II + acriflavine |
| Fraser broth | meat, poultry, factory environment | modified USDA II. Used as secondary enrichment broth in USDA procedure for meat and poultry samples |
| Tryptose Soy Broth (TSB) | milk, cheese, meat, tissue, environmental samples | cold enrichment broth |
| Oxoid Nutrient Broth no. 2 | milk, cheese, cabbage, sludge, water | warm enrichment broth |
| TSB + potassium tellurate | feces, water | cold or warm enrichment |

[1] cited from Elliot and Marth, 1991

obliquely reflected light has been used to observe the characteristic blue to blue-gray color when the plates are illuminated. Many kinds of antibiotics have been added into selective media. In particular, nalidixic acid has been used as a common ingredient in a large percentage of the formulations. It suppresses the growth of gram-negative bacteria. But, *Pseudomonas* spp, *Proteus* spp., and *Streptococcus* spp. can grow in media with this reagent. Watkins and Sleath (1981) reported that a combination of nalidixic acid and potassium thiocyanate was effective in selecting for *Listeria* while

suppressing the growth of other bacteria. Rodriguez *et al.* (1984) evaluated nalidixic acid and trypan blue including esculin and ferric ammonium citrate which allowed detection of esculin hydrolysis by *Listeria* to produce a black precipitate. However, even in such a formulation, enterococci were found to grow. Lovett *et al.* (1987) utilized acriflavine which suppresses the growth of gram-positive bacteria such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, and nalidixic acid in combination with cycloheximide which suppresses fungal growth, as the selective basis for the FDA Enrichment Broth. Donelly and Baigent (1986) developed the UVM Listeria Enrichment Broth by a modification of the Rodriguez Enrichment Broth substituting acriflavine for trypan blue and eliminating the glucose and ferric ammonium citrate. Recently, McBride's Listeria agar was developed, which has served as the basis for a number of other formulations. The media was incorporated with phenylethanol which suppress the growth of gram-negative bacteria and *Proteus* spp., lithium chloride (LiCl) which suppresses the growth of gram-negative bacteria except *Pseudomonas* spp., and glycine and selective reagents including blood. Lovett *et al.* (1987) developed Modified McBride Agar by eliminating blood and adding cycloheximide to suppress eucaryotic microorganisms. Lee and McClain (1986) further modified Modified McBride Listeria Agar (MLA) by increasing the LiCl concentration to 5 g/L and adding 20 mg of moxalactam/L, and this is called Lithium chloride-phenylethanol-moxalactam (LPM) agar. It inhibits the growth of many bacteria that interfere with the recovery of *L. monocytogenes* such as enterococci and *Pseudomonas* spp. Hayes *et al.* (1991) compared the cold enrichment (CE) and USDA methods by examining 402 food samples. The USDA method is a modified method of Lee and McClain for isolating *L.*

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TABLE 2. Major important selection media for *Listeria monocytogenes*[1]

| Media | Samples | Remarks |
|---|---|---|
| Macbride Listeria Agar (MLA) | dairy products, vegetables | phenylethanol, glycine, LiCl, and Sheep blood used. Streptococci and staphylococci can grow. |
| Modified MLA (MMLA, or MLA 2) | nonfat dry milk, cheese, meat, poultry | more selective than MLA |
| FDA-Modified McBride Listeria Agar (FDA-MMLA) | dairy, vegetables, seafood | MLA2 + cycloheximide |
| Trypticase Soy Agar (AC) | soft cheese, cheese factory environment | superior to FDA-MMLA in these products |
| LiCl-Phenylethanol-Moxalactam (LPM) Agar | dairy, poultry, raw meat, seafood | improved MLA2 by increasing LiCl and moxalactam |
| LPM with Xylose (LPMX) agar | meat factory environment | modified LPM, Xylose + and - *Listeria* are distinguished |
| Agricultural Research Service-modified MLA (ARS-MMLA) | milk, dairy, meat | more selective than MVJA |
| Modified Vogel-Johnson Agar (MVJA) | dairy, seafood, vegetable, coleslaw | more readily discernible (Black colony in red media) and better recovery yield than ARS-MMLA |
| Oxford Agar (OXA) | cheese, milk | used in FDA procedure for isolation |
| Modified Oxford Agar (MOX) | meat, poultry | used in USDA procedure for isolation |

TABLE 2. (continued)

| Media | Samples | Remark |
|---|---|---|
| Merck Listeria Agar | dairy product, meat, food processing environment | commercially available in Europe |
| Gum Basic Nalidixic Acid Medium (GBNA) | soil, raw milk, meat, poultry | modified MLA 2 |
| Trypaflavin Nalidixic Acid Serum Agar (TNSA) | throat swab, feces, organs,and other clinical specimens | inhibition of all non-*Listeria* |
| Acriflavine- Phenylethanol- Aesculin- Mannitol-Egg Yolk emulsion (RAPAMY) agar (by Ralivich) | meat, milk | modified TNSA. *S. faecalis* and *S. faecium* are not inhibited but can be differentiated easily |
| Acriflavine-LiCl-Phenyletha- nol-Aesculin-Mannitol-Egg Yolk (ALPAMY) agar | raw milk, soft cheese, vegetables, chicken | modified RAPANY (added LiCl and omitted nalidixic acid). *S. aureus* and *Micrococcus* spp. can grow. |

⁺cited from Elliot and Marth, 1991

*monocytogenes*, in which they omitted the KOH treatment of enrichment broths before plating on LPM agar. They found that the USDA method was significantly better than the cold enrichment method in enumeration of *L. monocytogenes* isolated from 51 food samples. The isolation efficiencies of the USDA and the CE methods were 96 and 59%, respectively.

Although MLA has long been used to isolate *L. monocytogenes* directly from highly contaminated materials as well as after cold enrichments, LPM is also used for direct plating in conjunction with popular isolation procedures. Al-Zoreky and Sandine (1990)

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also developed media by using acriflavine, ceftazidime, and moxalactam as selective

agents. This medium was found to inhibit micrococcus and enterococcus. TABLE 2

shows major selective media for isolation of *L. monocytogenes* from various samples.

To enumerate *Listeria* more effectively in foods, many kinds of media have been

developed but no one medium appears to be suitable in all situations. To evaluate the

ability of various methods of recovery and enumeration of *L. monocytogenes*, it is

important to consider the food that is to be analyzed, since the efficacy of the method

may vary depending on its composition. Particulates, high lipid content, or pigmentation

in foods can influence the performance of a medium by affecting distribution of the

inoculum on the medium or by making the colonies more difficult to detect. Cassiday *et*

*al.* (1989) evaluated three developed direct plating media for the enumeration of *Listeria*

from whole milk, ice cream mix, cheese, and cabbage. In this study, LPM agar

(LPMA), ARS agar, and Modified Vogel Johnson agar (MVJA) were used and compared

with previously tested plating media which were gum base-nalidixic acid-tryptose-soy

medium (GBNTSM) and modified Despierres agar (MDA). From their result, LPMA was

the most suitable for analyzing cheese and cabbage, but GBNTSM was most suitable

for analyzing milk and ice cream mix. Another important factor in selecting a medium is

the type and population of indigenous microorganisms present. In addition, the state of

health of *Listeria* cells can affect the efficacy of specific direct plating media. In

particular, during food processing, one of the most obvious and important consid-

erations is the efficacy of a given medium to recover the injured *L. monocytogenes* from

heating or freezing. Plating methods without a recovery step or with ineffective

recovery steps for injured *Listeria* in foods can result in false colony-counting in the

evaluation of processing conditions. Although MLA, GBNTSM, MDA, MVJA, and modified MLA are among the best for recovery, they often vary greatly in their ability to allow recognition and counting of L. monocytogenes colonies in different foods. Therefore, comparison studies with various media must be conducted in order to select the most effective media for enumeration of Listeria in a particular food product. For example, non-fermented dairy products have the fewest problems when direct plating is used because few contaminants are usually detected on the test media. However, fermented dairy products such as cheese and yogurt are among the most challenging foods from which to isolate and enumerate L. monocytogenes. The most suitable media for these products are LPMA, MLA, or MVJA. In meat products, which contain high populations of contaminating microorganisms (primarily gram-positive cocci), MVJA has been used as the most effective medium. With this medium, contaminants do not present a major problem when enumerating L. monocytogenes because the colony types are sufficiently different to be easily distinguished from L. monocytogenes.

Figure 1 and Figure 2 show procedures for isolating L. monocytogenes from foods used by the FDA and USDA, respectively. The FDA procedure was designed for milk and dairy processed food products, and the USDA procedure was designed for poultry and meat products.

As a confirmation step of Listeria colonies on the media, several tests are required: hemolytic activity by using sheep blood cells, methyl-red test, and oxidase test.

**Rapid methods for detection of L. monocytogenes in foods**

Recently, many methods have been reported for more rapid isolation and identifica

25 (g or ml) of sample + 225 ml of FDA Enrichment Broth

↓

blending or stomaching

↓

incubation for 1 to 2 day at 30°C


Streak on Oxford Agar          Streak on LPM Agar


incubation for 2 days at 30°C

↓

Check for typical bluish-green colonies under oblique-transmitted light
and
Confirmation ($\beta$-hemolysis, Methyl-red test, Gram staining, Catalase test)
and
Enumeration of colonies
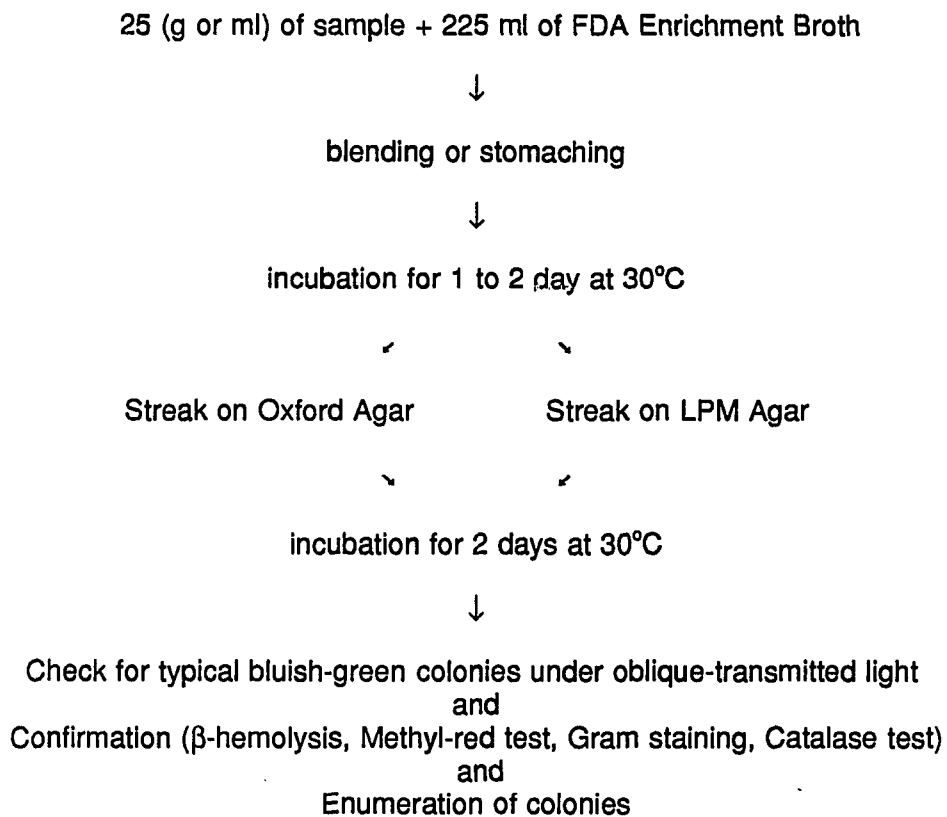

Figure 1. FDA procedure for isolating *L. monocytogenes* from food
(Elliot and Marth, 1991).

25 (g or ml) of sample + 225 ml of LEB I
↓

blending or stomaching for 2 min
↓

incubation for 1 day at 30°C
↓

adding 0.1-10 ml Fraser Broth
↓

incubation for 1 to 2 days at 35°C
↓

check Fraser Broth for blackening
↓

If positive, streak on Modified Oxford Agar (MOX)
↓

incubation for 1 to 2 days at 35°C
↓

streak typical *Listeria* colonies exhibiting black halos
on Horse Blood Overlay Agar
↓

incubation for 1 day at 35°C
↓

confirmation (β-hemolysis, oxidase, Methyl-red test, Gram staining, Catalase test)
and
Enumeration of colonies
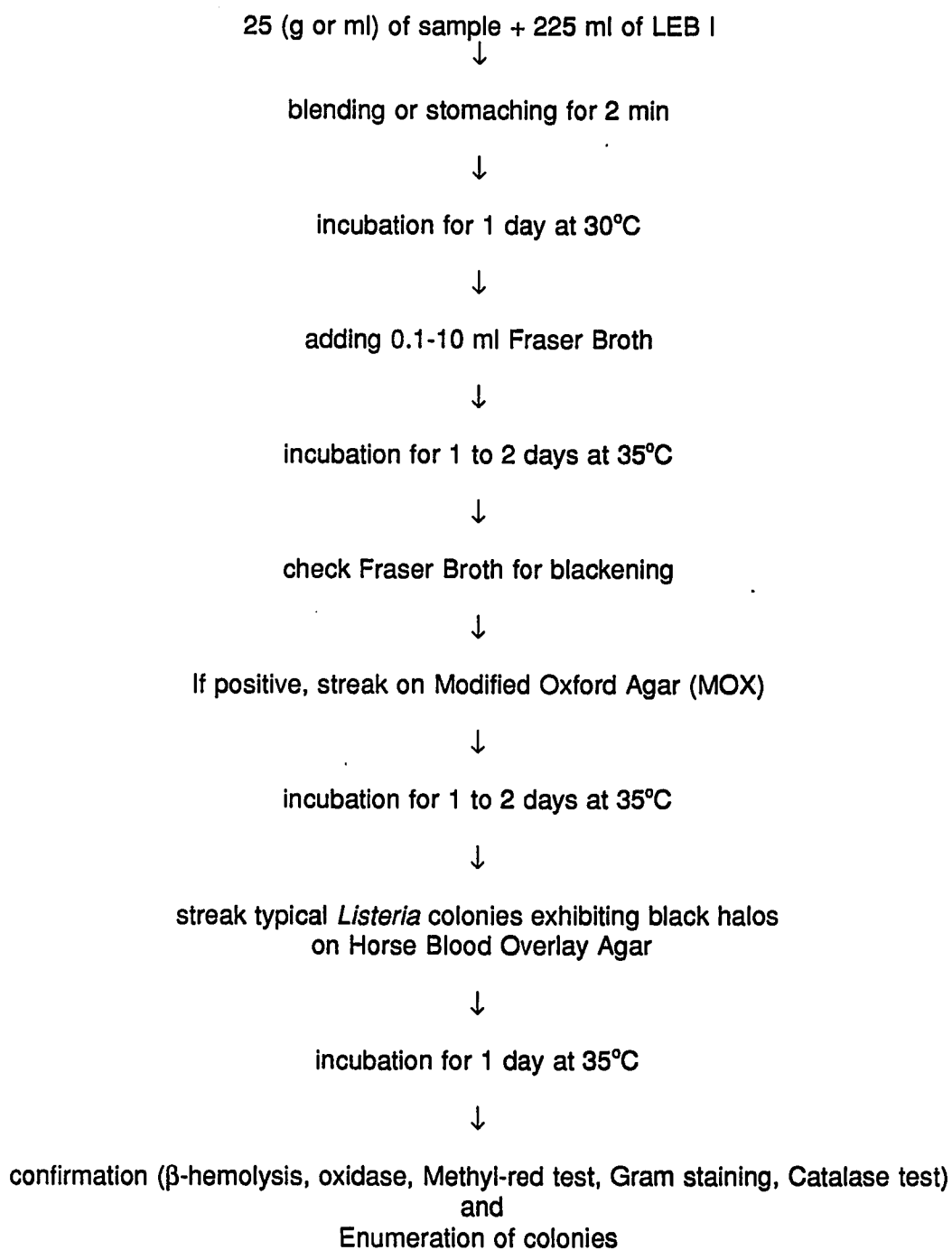
Figure 2. USDA procedure for isolating *L. monocytogenes* from food
(Elliot and Marth, 1991)

tion of *L. monocytogenes.*

Flow cytometry is a method of measuring cells in a liquid suspension. In essence, suspended cells (one by one) are brought to a detector by means of a flow channel. Fluidic devices under laminar flow define the trajectories and velocities that cells traverse the detector. Donelly and Baigent (1986) have adapted flow cytometry for the detection of *L. monocytogenes* in milk. However, the method is not practical for most laboratories due to the cost of the equipment involved.

Farber and Speirs (1987) have described an enzyme-linked immunosorbent assay (ELISA) method for detecting *L. monocytogenes* in raw milk that uses monoclonal antibodies directed against flagellar antigens (A, B, or C). The monoclones tested did not cross-react with any other 30 non-*Listeria* cultures including *Staphylococcus aureus* and *Staphylococcus faecalis.* However, although this method has good potential for foods that have sufficiently high *Listeria* counts to enable detection on direct plating, it is not suitable for foods with low levels of contamination, unless the culture is enriched prior to testing. Datta *et al.* (1987) described a ^{32}P-labeled DNA probe, a fragment of about 500 base pairs of the β-hemolysin gene from *L. monocytogenes.* Klinger *et al.* (1988), of the GENE-TRAK systems company, have developed a nucleic acid hybridization assay for *Listeria* spp. in dairy foods and environmental samples based on detection of *Listeria* 16s rRNA sequences using a ^{32}P-labeled synthetic DNA probe. They reported that the GENE-TRAK *Listeria* assay can be done in 2 days, compared to 9-14 days required for traditional cultural methods, and has a lower false negative rate (6.7%) compared to conventional culture methods (22%) in milk, gouda cheese, ice cream, and sandwiches.

Monoclonal antibodies to extracts of β-hemolytic, CAMP-positive strains of *L.*

*monocytogenes* have been used to develop an enzyme-linked immunosorbent assay

(ELISA) for *Listeria* spp. that does not cross-react with non-*Listeria* organisms (Mattingly

*et al.*, 1988). This method has been developed to enumerate *Listeria* in dairy and meat

products as well as environmental samples (FDA-Modified McBride Listeria Agar or

Fraser Broth). After enriching food samples 40-52 hr in a selective media, a double

monoclonal antibody sandwich ELISA procedure (which is known as the Listeria-Tek

Assay) is performed on the enrichment culture. From the results, presumptive results

about presence of *Listeria* in samples are available an average of 48 hr after initiation

of enrichment. In addition to providing fast results that are easily interpreted, the

Listeria-Tek Assay also is safe to do since only heat-killed organisms are used.

However, the procedure is complicated and the cost for the test is expensive (about

$15/test).

## 4. Virulence of *Listeria monocytogenes*

In general, two of eight *Listeria* species, *L. monocytogenes* and *L. ivanovii*, can

cause human and/or animal infection. In human infections, *L. ivanovii* has rarely been

isolated with only three documented cases which accounted for about 10% of total

animal listeriosis cases in Bulgaria (Rocourt and Seeliger, 1985). Therefore, this

organism is considered to be far less virulent than *L. monocytogenes*. The mechanism

of virulence of *Listeria* is highly complex and remains poorly understood. The proce-

dure involves penetration of the host cells (or phagocytosis), survival and reproduction

within the host, and invasion of target tissue. Phagocytosis is defined as a process in which particulate matter is ingested by a cell, involving the engulfment of that matter by the cell membrane. Macrophage engulfment is the animal's protection against many bacterial invaders. Macrophages have mechanisms for producing various oxygen-containing compounds such as singlet oxygen, peroxide, and superoxide anion that can inactivate enzymes and result in microbial death (1987). However, *L. monocytogenes* contains highly active catalase to which can inactivate peroxide, and superoxide dismutase (SOD) which converts superoxide anion to an inactive oxygen derivative. Shortly after *Listeria* is phagocytosed by a macrophage, it can dissolve the phagosomal membrane and enter the cytoplasm of the phagocyte, thus eluding the action of toxic oxygen radicals of the host. Bacterial actin filaments then coat the bacterial cell and become rearranged to form a tail with which the cell moves to the macrophage surface as a prelude to exiting and spreading (Tilney *et al.* 1990). Racz *et al.* (1972) reported that epithelial cells lining the intestine serve both as a site of entry and as a site for bacterial reproduction before phagocytosis of the organism in the intestine. A correlation between virulence and lipase content has been observed in *L. monocytogenes*, where only virulent strains are considered to be lipolytic. Holder and Sword (1969) examined a lipid fraction of *Listeria* species that is responsible for monocytosis classically associated with listeriosis. They determined that the lipid fraction (monocytosis-producing agent, MPA) contributed to pathogenesis by disturbing steroid metabolism and altering the gluconeogenic process in the host. Once *L. monocytogenes*, through hydrophobic interactions or other mechanisms, has attached to the cells, it then becomes intracellular.

In 1934, Burn (1934) reported the presence of a soluble, filterable hemolysin produced by *L. monocytogenes*, believed to be present only in this hemolysin was termed 'listeriolysin'. Njoku-Obi *et al.* (1963) characterized the water soluble hemolysin as a heat-labile protein inactivated by filtration but reactivated by addition of sodium thiosulfate. Hemolytic activity of listeriolysin was found to be optimal at pH 5.5 but greatly reduced at pH 7 (1964). Later work (Kingdon and Sword, 1970) showed that this hemolysin reacted with antibodies to streptolysin O and, like streptolysin O, also possessed cardiotoxic activity, which prompted its being called listeriolysin O (LLO). LLO, a membrane-damaging cytotoxin, enables the cells to invade and grow within macrophages through lysis of the membrane-bound phagocytic vacuole (Mounier, *et al.*, 1990). Currently, LLO is generally recognized as a sulfhydryl-activated, cholesterol-inhibited cytolysin and is composed of a single polypeptide chain of molecular weight of approximately 60 kDa. Geoffroy *et al.* (1989) showed there was a the difference in production of LLO between 26 *Listeria* subsp. and 5 main *Listeria* spp. including *L. monocytogenes, L. ivanovii,* and *L. seeligeri,* and reported that no LLO was produced by either *L. innocua* or *L. welshimeri.*

Parrisius *et al.* (1986) isolated and characterized another hemolysin in *L. monocytogenes* by using SDS polyacrylamide gel electrophoresis and immunoblotting. They suggested that the LLO related to streptolysin O should be called α-listeriolysin, and the remaining hemolysins that do not cross-react with streptolysin O be called β-listeriolysin. This latter hemolysin is immunologically, genetically, and molecularly different from LLO. The CAMP test is definitive for identifying β-hemolysis activity because metabolites of β-hemolytic *Staphylococcus aureus* and *Rhodococcus equi* are

used to enhance *Listeria* spp. in sheep blood agar (Skalka and Smola, 1983). The hemolytic action of *L. monocytogenes* is enhanced by proximity to *S. aureus* culture but not by the *R. equi* streak. Present findings indicate that $\beta$-listeriolysin may be responsible for the positive CAMP reaction typical of *L. monocytogenes* (Groves and Welshimer, 1977). By using the CAMP test, Skalka *et al.* (1982) set up a routine test for the *in vitro* determination of the pathogenicity of *Listeria* strains, in which the exosubstance of *R. equi* in a prepurified form strongly enhanced the hemolytic effect of *Listeria*, and the *Listeria* strains that produced positive synergistic hemolysis with this exosubstance were also pathogenic for guinea pigs and white mice.

## 5. Characteristics of listeriosis

Most cases of human listeriosis are sporadic and the incubation period is from one to several weeks in adults. The process may be accompanied by transitory flu-like symptoms that may include malaise, diarrhea, and mild fever, which is frequently so mild as to be unnoticed.

### Risk factors

The majority of human cases of listeriosis occur in individuals who have an underlying condition which leads to suppression of their T-cell-mediated immunity. For example, listeriosis is associated with immunocompromised patients, pregnant women, patients at the extremes of age, as well as people suffering from diabetes mellitus, alcoholism, cardiovascular and renal collagen disease, and hemodialysis failure.

The highest incidence of listerial infection is usually seen in neonates, followed by people over 60 years old, because of the immaturity of the immune system in the neonate and the waning of immunity with advanced age (Cieslelski, 1988). From a survey conducted on listeriosis in 1989 from 16 countries (Farber and Peterkin, 1991), 31 and 22 % of the total cases occurred in patients older than 60 years and younger than 1 month, respectively. Pregnancy also is a condition that predisposes the patient to infection with *L. monocytogenes* because it is associated with a decrease in T cell mediated immunity. During pregnancy, infection with *Listeria* commonly results in a variety of 'flu-like' symptoms which include fever, chills, headache, diarrhea, and backache. Abortion, delivery of premature infants, and inflammation of the pelvis and of the kidney also can occur (Schwartz *et al.*, 1989). Besides listeriosis in the pregnant woman, perinatal listeriosis can subsequently occur, in which two distinct clinical syndromes have been seen: an early onset illness (Anonymous, 1980, Filice *et al.*, 1978) and a late onset infection (Winslow *et al.*, 1981). The early onset illness appears shortly after birth and/or generally in a prematurely delivered child with low body weight. The major symptom is septicemia. These infants also have respiratory distress, pneumonia and microabscesses (also called 'granulomatosis infantiseptica'). *L. monocytogenes* can be isolated from the external ear, nose, blood, throat, and amniotic fluid of these patients. In addition, this organism can be found in the neonatal lung and gut. The manifestation appears as petechial eruptions or papules on the skin, or lesions in other tissues such as liver, esophagus, and lung. The mortality rate is 15-50%. In the late onset neonatal infection, infants are colonized at birth and experience a delayed onset of infection, in which infants are born apparently healthy but, after 2nd

to 4th week of birth, meningitis appears as the most common symptom. The mortality is lower (10-20%) than in the early onset infection. The conditions are fever, diarrhea, irritability and poor feeding.

AIDS patients are more likely than the normal public to contact listeriosis, estimated by the FDA to be 670 times more susceptible than the healthy adults (Anonymous. 1988) However, the incidence of listeriosis among AIDS patients is known to be less than that of normal humans. The reason is unknown, but Jacob and Murray (1986) suggested that the use of rigorous antibiotics such as trimethoprim and sulfamethoxazole for their therapy, and the presence of partially active genetic determinants, make AIDS patients resistant to listeric infection.


**Symptoms of listeriosis**

Listeriosis causes many symptoms to humans as well as animals. Bacteremia is diagnosed in adults. In this case, fever is a common symptom, but other complaints vary nonspecifically according to the individual, such as fatigue and malaise, and include nausea, vomiting, pain, and diarrhea. The mortality rate is about 30% when the patient is immunocompromised, old, or newborn (Nieman and Lorber, 1980).

A serious form of listeriosis occurs by infection of the central nervous system. The symptoms as meningitis, encephalitis (also called 'cerebritis'), or brain abscesses. The most common manifestation of listeriosis is meningitis, which develops predominantly in newborns and aged persons. In meningitis, the gastrointestinal tract can serve as an entry for bacteremic spread to the meninges (Schlech, 1984). In general, meningitis develops with high fever, headache and nuchal rigidity, which is fulminant. Mortality in

untreated patients, or those treated late, is approximately 70% (Seeliger and Finger,

1983). In encephalitis, *L. monocytogenes* affects the brain parenchyma in as many as

20% of all cases, and this appears to be more common in renal transplant recipients

than in other patients (Stamm *et al.*, 1982). In brain abscesses, it includes fever,

headache, and focal neurologic signs. Mortality is approximately 57% (Dee and Lorber,

1986).

From a survey on listeriosis of 782 cases in 1989 from 16 countries (Farber and

Peterkin, 1991), 43% were maternal (pregnant women) and neonatal infections, 29%

were bacteremia, and 24% were central nervous system infections. Other localized

forms were endocarditis and skin infections. Endocarditis follows central nervous

system infection and bacteremia as the third most commonly recognized form of listerial

infection. It is associated with malignancy and renal transplantation, and preexisting

cardiac disease (Carvajal and Frederiksen, 1988). The mortality is as high as 48%.

Direct inoculation of the skin and eye with *L. monocytogenes* results in skin infection

(Amstrong, 1985). These infections are a hazard to veterinarians, slaughterhouse

workers and laboratory workers. Bacteremic spread in the body can produce metastatic

local infection in virtually any organ such as the spleen, gallbladder, and lymph nodes.

## 6. Food-borne outbreaks by *Listeria monocytogenes*

### Foods as reservoirs

Listeria *monocytogenes* has been detected in a wide range of foods. In meat

products, most of the observed contamination is on the surface. However, Johnson *et*

*al.* (1990) recently found *L. monocytogenes* in the interior muscle cores of 5 of 110 total

samples of beef (50), pork (50), and lamb roasts (10). Serotype 1 is the prominent

serotype found in meats worldwide. Chicken also seems to be heavily contaminated

with *L. monocytogenes* as surveys show contamination rates ranging from 12 to 60%.

Bailey *et al.* (1990) have recently examined the factors influencing colonization of

broiler chickens with *Listeria monocytogenes.* Although *L. monocytogenes* did not

colonize chickens as easily as do salmonellae or *Campylobacter jejuni,* younger birds

were more susceptible to colonization than older birds. Glass and Doyle (1989) found

that growth of *L. monocytogenes* on meat was highly dependent on product type and

pH. The organism tended to grow well on meat products with a pH value near or

above 6.0, whereas it grew poorly or not at all on meats near or below pH 5.0. For

roast beef, summer sausage, and hot dogs, the inhibitory factors appeared to be pH,

combined pH and water activity, and liquid smoke. Some investigators studying the

fate of *L. monocytogenes* in fermented sausages have found at least a 100-fold

reduction in the level of the organism during the manufacture of fermented sausages

(Berry *et al.,* 1990).

The number of foodborne outbreaks of listeriosis caused by highly virulent strains

of *L. monocytogenes* have produced unprecedented surveillance of dairy foods by

health officials. Although *L. monocytogenes* has been identified in various dairy prod-

ucts, cheese has been the most intensively examined because of its known association

with food-borne listeriosis. In particular, soft cheese was found to be especially

vulnerable to contamination by *Listeria* spp. Actually, the main source is raw milk and

this organism can usually be concentrated in the curd. *Listeria* can survive the

manufacture and ripening of many kinds of cheeses. Ryser *et al.* (1987) reported that contamination with *Listeria* on the surface of cheese results from an increase in pH during ripening. In yoghurt, *Listeria* also can survive for up to 30 days with pH values as low as 4.0 in storage (Griffiths and Deibel, 1989). Because *L. monocytogenes* appears to be hardier than coliforms in buttermilk, yoghurt, and cheese (Choi *et al.* 1988), this organism can be present even in coliform-free dairy products. In addition, *Listeria* is affected by the presence of other microorganisms in dairy products. In skim milk fermentation, the presence of *Lactobacillus bulgaricus* was found to be more detrimental to the growth and survival of *Listeria* than *Streptococcus cremoris* or *Staphylococcus lactis* (Schaack and Marth, 1988).

Vegetables can also be a source of listeriae, as indicated by an outbreak of listeriosis linked to consumption of *L. monocytogenes*-contaminated coleslaw (Schlech *et al.*, 1983). Conner *et al.* (1986) reported that cabbage juice is a good growth medium for *L. monocytogenes*. Ho *et al.* (1986) investigated an outbreak of *L. monocytogenes* serotype 4b infection involving patients from eight Boston hospitals. They concluded that celery, tomatoes, and lettuce may have been the vehicles of infection. But, *L. monocytogenes* does not appear to be able to grow well on uncooked carrots (Beuchat and Brackett, 1990). Sources of contamination of vegetables include soil, water, animal manure, decaying vegetation, and effluent from sewage treatment plants. The association of *Listeria* spp. with food crops would be expected considering the distribution of the organism in nature. *L. monocytogenes* can survive for long periods in soils (Welshimier, 1960). Weis and Seeliger (1975) examined soils and vegetation from a variety sites. About 20% of soils and plant materials contained *L.*

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*monocytogenes*, with the highest prevalence found in uncultivated fields. The predominant serotypes were serotype 1/2b and 4b. Surface waters are also often contaminated with *Listeria* spp. Watkins and Sleath (1981) isolated *L. monocytogenes* from every sample of sewage, river water, and trade effluent examined. The numbers of listeriae exceeded those of *Salmonella* spp. found in the same sources. When fields to which sewage sludge had been applied were assayed for *L. monocytogenes*, there was no decrease in the number of *Listeria* for more than 8 weeks.

In the case of seafood, Weagant *et al.* (1988) examined 57 samples of frozen seafood products, and found 15 samples, including shrimp, crab-meat, lobster tail, fin fish, and surimi-based seafood, to be positive for *Listeria monocytogenes*. Fish products have been epidemiologically implicated in two listeriosis outbreak and have been thought to be the cause of one case of sporadic listeriosis.

**Outbreaks of Listeriosis**

Between 1917 and 1943, about 36 outbreaks of listeriosis have been recorded (Kaplan, 1945) and during 1949 through 1987, approximately 1300 cases of listeriosis have been documented (Ryser and Marth, 1990).

In the Maritime Province of Nova Scotia in Canada, 34 cases of perinatal listeriosis and 7 cases of adult disease occurred between March 1 and September 1 in 1981 (Schlech *et al.*, 1983). In this outbreak, perinatal cases were characterized by acute febrile illness in pregnant women followed by spontaneous abortion (5 cases), stillbirth (4 cases), live birth of a seriously ill premature or term infant (23 cases), or live birth of a well infant (2 cases). The case fatality rate for infants born alive was 27%. The

outbreak strain was L. monocytogenes 4b, and the investigation incriminated coleslaw as the vehicle. Because of this outbreak, it is now recognized that foods other than dairy products can be contaminated with L. monocytogenes.

In between June 30 and August 30, 1983, there was an outbreak in which consumption of pasteurized milk was related to 49 cases of listeriosis in Massachusetts (Fleming et al., 1985). Seven cases were fetus- or infant-related and 42 cases occurred in patients. The serotype detected was serotype 4b. The mortality was 29%. A specific brand of pasteurized whole or 2% milk was epidemiologically implicated as the vehicle.

The ability of this organism to cause foodborne illness was re-estimated between January 1 and August 15, 1985, when consumption of Jalisco brand Mexican-style cheese was related to at least 142 cases of listeriosis in Los Angeles county, California (Linnan et al., 1988). Ninety three cases (65.5%) occurred in pregnant women or their offspring. There were 48 deaths (20 fetuses, 10 neonates, and 18 nonpregnant adults). Of the L. monocytogenes isolates, 82% (86 of 105) were serotype 4b.

In Philadelphia between 1986 to 1987, 36 cases of listeriosis occurred and 32 of 36 (16 deaths) were nonpregnant adults and 4 were newborns (Schwartz et al., 1989). Twenty-four of 32 nonpregnant adults were immunosuppressed and 4 of the remaining 8 were 80 years old or older. the serotypes identified were 4b, 1/2b, 1/2a and 3b. Ice cream, salami, or vegetables were suspected.

In Texas in 1988, a woman with cancer was hospitalized with sepsis caused by L. monocytogenes (Terplan and Steinmeyer, 1989). She had eaten 1 turkey frank which had been heated in a microwave oven. Serotype 1/2a was isolated from the patient, as

well as from opened and unopened packages of the franks. The same serotype was found in the processing plant 4 months later. Turkey franks were declared to have been the most likely source of listeriosis.

Because of these and other listeriosis outbreaks in Europe where dairy products were not implicated, worldwide concern over the presence of *Listeria* in foods such as meat products, seafoods, and vegetables has been generated.

## 7. Effect of food processing

Although *L. monocytogenes* has a temperature growth range of 2.5-44°C (Seeliger, 1961), growth at 0°C has been reported (Khan *et al.*, 1973). This organism can remain viable as long or longer than most other *Listeria* spp. during extended storage at less than 0°C. Palumbo and Williams (1989) reported that *L. monocytogenes* populations decreased only 1-3 orders of magnitude in inoculated samples of canned milk, 10% Karo corn syrup, ground beef, ground turkey, frankfurters, ice cream mix during 8 weeks of frozen storage at -18°C. Two reports (Brasher *et al.*, 1984, Wood and Woodbine, 1979) have noted that virulence of *L. monocytogenes* is related to growth temperature. Both reports indicated that virulence, as assayed in the chick embryo, is enhanced at lower temperatures. Future investigations need to more clearly define how the viability and pathogenicity of this organism are affected in food during extended frozen storage.

*L. monocytogenes* is resistant to drying. Some investigators (Doyle *et al.*, 1985) determined that *L. monocytogenes* could survive the manufacture and storage of nonfat

dry milk when the initial concentration was $10^5$-$10^6$ listeriae/ml. The heat of the spray

dryer was 165°C at the inlet and 67°C at the outlet. *L. monocytogenes* survived storage

at 25°C in all packages up to 4 weeks and in some of the packages up to the 12th

week.

Several studies (Fain *et al.*, 1991, Boyle *et al.*, 1990) have focused on the ability of

*L. monocytogenes* to survive a heat treatment. The heat effect on *Listeria* appeared to

vary according to the serotype and the type of food (Lemaire *et al.*, 1989). Bradshaw *et

al.* (1985) heated *Listeria* at 52.2 to 74.4°C in milk. Temperatures of 52.2-68.9 °C were

achieved in sealed glass tubes. The D-value for *L. monocytogenes* was estimated to

be 0.9 sec at 71.7°C, and the z-value was 6.3°C. In this study, they concluded that

current pasteurization process guidelines of the Food and Drug Administration were

adequate to destroy *L. monocytogenes* in raw whole milk. But, investigators of the

1983 Massachusetts outbreak of listeriosis (Fleming *et al.*, 1985) suggested that

pasteurized milk was the vehicle of infection and that the intracellular position of *L.

monocytogenes* in naturally infected cows' milk protected the organism from heat inacti-

vation by an unknown mechanism. To examine this hypothesis, Bunning *et al.* (1986)

did parallel experiments in raw whole milk using freely suspended bacteria and bacteria

internalized by murine peritoneal phagocytes. The two suspensions in raw whole milk

were heated in sealed glass tubes. The thermal inactivation kinetics of the two

bacterial cell treatments were analyzed statistically and the differences were found not

to be significant. In another study, Doyle *et al.* (1987) heated milk containing intracel-

lular *L. monocytogenes* produced in experimentally infected cows. Cows were inoculat-

ed by the feed, and milk from these cows, had *L. monocytogenes* within leukocytes in

concentrations of $10^2$-$10^4$ cells/ml of milk. When processed at 71.7°C for 16.4 sec in a small, commercial-type plate pasteurizer, *Listeria* survivors could be detected occasionally in the pasteurized products, but only by extensive testing of the milk using several different enrichment procedures. These results indicated that, if *L. monocytogenes* within leukocytes were present in milk in unusually large numbers, the organism may survive the minimum treatment of high-temperature, short-time pasteurization. But, in practice, the usual levels of *L. monocytogenes* in farm milk is about 1 cell or less per ml and most dairy plants pasteurize milk at temperatures and holding times greater than the minimum requirements.

*L. monocytogenes* can survive at 37°C for 15 days in 10.5% NaCl, 10 days in 13% NaCl, and 5 days in concentrations of 20-30% NaCl. When the temperature is lowered to 22°C, survival times more than double. At 4°C, *L. monocytogenes* can survive more than 100 days in 10.5-30.5% NaCl (Schamat, *et al.*, 1980). Some reports (Seeliger, . 1961) indicate that *L. monocytogenes* is capable of growing in up to 10% NaCl and surviving for 1 year at 16 % NaCl when the pH is 6.0. However, in cabbage juice, *L. monocytogenes* cells appear to grow in 2% NaCl but not in 5% NaCl.

*L. monocytogenes* with a D value of 0.28-0.61 kGy (Cirigliano and Hartman, 1989) is more resistant to gamma irradiation in tryptose soy broth than are other commonly encountered non-spore forming foodborne pathogens such as *Salmonella typhimurium* (D value 0.28 kGy, Urbain, 1986), *Staphylococcus aureus* (D value 0.24 kGy, Urbain, 1986) and *Yersinia enterocolitica* (D value 0.11 kGy, El-Zawahry and Rowley, 1979). But irradiation sensitivity of *L. monocytogenes* is also affected by age of the culture, irradiation menstruum, and the type of medium used for enumeration of cells after

irradiation. Huhtanen *et al.* (1989) reported that cells incubated for 1.5 and 2.5 hr are more irradiation resistant than those incubated for 5 and 18 hr.

Addition of acids can affect the growth of *Listeria*. Ahamad and Marth (1989) studied the effect of acetic, citric, and lactic acid in preventing growth of *L. monocytogenes* in tryptose soy broth. According to their results, the acids were more effective at 7 °C than at 13, 21, and 35°C and increasing the concentration of acids to 0.2% completely suppressed growth of the organism with death of the cells occurring in the presence of more than 0.3%. Acetic acids had the highest antilisterial activity followed by lactic and citric acid.

## 8. Heat shock response

When cells or whole organisms are exposed to elevated sublethal temperatures for a short time, they respond by becoming more tolerant to a subsequent heat treatment. This increase in thermotolerance has been ascribed to production of a small number of highly conserved proteins, termed 'heat shock proteins' (hsps) by the cells during heat shock.

The function of hsps has been extensively studied, but their role in protection of the cells during heating is unknown. Bensaude *et al.* (1990) proposed that unfolding of cellular proteins during heat shock would expose hydrophobic internal domains which would bind several constitutive hsp molecules. This binding, which is reversed by ATP hydrolysis, would prevent aggregation of the unfolded proteins polypeptides to an inactive form while protecting them from the effects of a subsequent heat treatment.

Studies on the heat shock response began in 1962 (Ritossa, 1962), describing a

new set of alternated puffs on the salivary gland of a fruit fly induced by heat, dinitro-

phenol, or sodium salicylate. Some investigators discovered that many other factors

such as alcohol or arsenate as well as heat could induce the synthesis of similar pro-

teins in yeast (Miller et al., 1979) and in cultured avian cells (Kelly and Schlesinger,

1982). In eucaryotes, the molecular weight and intracellular locations of hsps are

different according to species. For example, hsp 95 (molecular weight, 95 kDa ) is

located in the golgi apparatus of vertebrate or plant cells, and is modified by phosphory-

lation into an active form, while hsp 84 (83-90 kDa) is located in the cytosol of yeast,

plants, or Drosophila cells and is modified by methylation. Hsps can also be produced

by procaryotes. In E. coli, initial observations on the heat shock phenomenon were

made on proteins visualized by two-dimensional gels (Neidhardt et al., 1981). Allan et

al. (1988) discovered two hsps (the m.w. 76 kDa and 61 kDa) from Pseudomonas

aeruginosa. Whitaker and Batt (1991) observed the enhanced synthesis of approxi-

mately 13 proteins in heat shocked Lactococcus lactis, and Streips and Polio (1985)

detected a 66 kDa hsp in heat shocked Bacillus subtilis.

In food processing, the heat resistance of L. monocytogenes can be increased

during heat treatment such as in preheating, or if a slow rate of heating is used. Fedio

and Jackson (1989) reported that cultures of Listeria preheated at 48°C for 2 hr in broth

and UHT milk before heating at 60 °C increased the heat resistance of the cells com-

pared with controls. Linton et al. (1992) reported that the $D_{55}$-value of cells heat-

shocked at 48°C for 15 min was 2.1-fold higher than nonheat shocked cells. However,

Bunning et al. (1990) compared the effect of heat shock of L. monocytogenes with that

of *Salmonella typhimurium* and found that the induction of increased thermotolerance

by heat shock at 45°C for 30 min was not significant in *Listeria* in comparison with that

of *S. typhimurium*. They suggested that induced thermotolerance was not long lived

unless the shock temperature was maintained. Sokolovic *et al.* (1990) suggested that

the increased heat resistance of heat-shocked *Listeria* was because of the production

of heat shock proteins that protected the cell from the heat-treatment. Sokolovic and

Goebel (1990) showed that listeriolysin was still very efficiently synthesized intracellul-

ary and induced under heat shock conditions.

### 9. Heat injury and recovery of *Listeria monocytogenes*

When microorganisms are subjected to environmental stresses such as heat,

freezing, radiation, and sanitizer, cells can become injured. In general, injured cells can

not grow in selective media which uninjured cells can tolerate. Therefore, whether a

culture has suffered metabolic injury can be determined by plating aliquots separately

on a nonselective and a selective medium and enumerating the colonies that develop

after suitable incubation. The mechanisms of cell injury can vary according to the type

of environmental stress to which the cells are exposed.

In this review, discussion will be limited to heat injury. Metabolic injury as a result

of heating is accompanied by damage to the cell wall, cell membrane, ribosomes, DNA,

and proteins (Hurst, 1977). In particular, the cell membrane appears to be the most

commonly affected because the lipid components of the membrane are susceptible to

the effect of heat. Loss of sodium, potassium, and magnesium from injured cells as a

result of heating has been reported (Hurst, 1974). Damage to ribosomes has also been documented, and is considered an important event due to the destruction of 16S RNA. Strange and Shon (1964) reported that the primary event in the damage to ribosomes was the loss of magnesium which was required for the integrity of ribosomes and, moreover, it served to inhibit a ribonuclease. Heat or freezing treatment can cause single strand breaks in bacterial DNA. However, the single strand breaks in DNA are thought not to be the direct result of heating but rather the consequence of nuclease activity. Heat can also degrade proteins such as enzymes. Dehydrogenases are particularly heat sensitive, but most of their activity returns during the recovery period. However, this is not true of all enzymes. For example, heated *Staphylococcus aureus* cells had 75% of the fructose diphosphate aldolase and 51% of the lactate dehydrogenase activity of normal cells after recovery (Tomlins *et al.*, 1971, Bluhm and Ordal, 1969).

The effect of various nutrients on heat injury has been studied. Smith (1990) reported that the addition of sugars, polyols, or salts to the heating menstruum protected the *L. monocytogenes* cells against heat injury, and fructose and $NH_4Cl$ were unusual in not being protective and actually potentiating the killing of cells by the normally sublethal heat treatment. The recovery or repair process has been defined as a restoration of the capabilities lost after damage from environmental stress. It also has been termed resuscitation, implying that cells are revived from apparent death. In order for injured cells to recover certain conditions are required. These include the presence of specific nutrients, pH, temperature, gaseous atmosphere, culture age, redox potential, osmolality, water activity, ionic strength, salt content, surface tension, and storage

conditions. Studies on the effect of pyruvate or catalase on recovery of injured cells have been conculuded by many researchers (McDonald *et al.*, 1983, Martin *et al.*, 1976). During injury, the accumulation of hydrogen peroxide is a universal response in cells and injured cells have an increased sensitivity to its toxic effects. Pyruvate and catalase have been shown to degrade hydrogen peroxide during recovery periods, thus allowing the cells to repair themselves.

Many researchers have also studied the recovery and growth of *Listeria* after a heat treatment. Dallmier and Martin (1988) reported that catalase activity of heat stressed cell extracts decreased sharply between 55 and 60°C and SOD was more heat labile than catalase, both of which are important factors for cell recovery. Knabel *et al.* (1990) reported that anaerobic conditions resulted in a increase in recovery of the cell in media because of absence of oxygen. Petran and Zottola (1989) studied the effects of pH, carbohydrates, and temperature on recovery and growth of *Listeria*. They showed that this organism can grow in solutions of up to 39.4% sucrose. Busch *et al.* (1992) developed media for recovery of heat-injured *Listeria* in trypticase soy broth supplemented with divalent cations, yeast extract, carbohydrates, pyruvate, and catalase. Temperature is also an important factor for recovery of injured cells. Lovett (1988) found that 25°C was the optimum temperature for repair of heat injured *L.* *monocytogenes* in pasteurized milk. Smith (1990) reported that complete or nearly complete recovery of heat injured cells was observed between 6-9 h incubation at temperatures ranging 20 to 40°C on tryptose phosphate broth agar.

As we discussed previously, *L. monocytogenes* is a pathogenic bacterium which cause many fatal syndromes particularly to immunocompromised patients. However, the ubiquity of *L. monocytogenes* in the environment, its widespread occurrence in food, and the drastic changes connected with modern food production have created an unprecedented reservoir for this organism. To be effective, preventative steps should start at the place of food and feed production, making use of heat to destroy *Listeria* when practical. In particular, during processing the main factor to be considered is the potential for the organism to survive heating if exposed to a heat shock. Because the organism is able to recover and grow during storage, the packaging conditions are also important. However, information on heat resistance of heat shocked *L. monocytogenes* in ground pork during pasteurization conditions and the effect of packaging style during storage have not been elucidated. The objectives of this study were to determine the effect of heat shock on survival of *L. monocytogenes* after heating and on production of LLO in ground pork, to determine the effect of varying the rate of heating on survival of cells to subsequent processing, and to determine the effect of storage conditions on recovery of heat-injured *L. monocytogenes*.

PAPER I. EFFECT OF HEATING AND STORAGE CONDITIONS ON SURVIVAL AND RECOVERY OF *Listeria monocytogenes* SEROTYPES IN GROUND PORK

Effect of Heating and Storage Conditions on Survival and Recovery of

*Listeria monocytogenes* Serotypes in Ground Pork[*]

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## ABSTRACT

*Listeria monocytogenes* serotype 1 and Scott A were examined to determine their survival to heating in ground pork according to: 1) heating rate during come-up time, 2) meat age, and 3) aerobic *vs.* anaerobic packaging of ground pork. The D-value was calculated by plotting the log number of survivors after heating for 0 min, 10 min, 20 min, or 30 min at 62°C. In addition, *L. monocytogenes* serotype 1 was examined to determine the recovery of heat-injured cells during storage at 4, 20, and 30°C in aerobically packaged or vacuum-packaged ground pork at various temperatures. The effect of 100, 200, and 300 ppm of butylated hydroxyanisole (BHA) and 300, 500, and 700 ppm of butylated hydroxytoluene (BHT) in this recovery was examined in aerobically packaged ground pork stored at 7°C and 30°C. The results show that *L. monocytogenes* serotype 1 was more heat-resistant than Scott A in ground pork. With serotype 1, a heating rate of 1.3 °C/min resulted in the highest D value (9.2 min), when compared with a rate of 8.0 °C/min, which resulted in a D value of 5.5 min after heating at 62°C. More survivors of both subspecies were detected after heating in fresh ground pork compared with those in long-stored (3 months) ground pork. During storage, higher number of survivors after heating were counted when the meat was packaged aerobically before heating than when packaged anaerobically for both serotypes. After heat treatment at 62°C for 10 min, *Listeria* grew more rapidly in vacuum-packaging at 4°C than in aerobic packaging. Antioxidants did not significantly affect the cell growth after stationary phase at 7°C or at 30°C.

## INTRODUCTION

*Listeria monocytogenes* is a non-spore-forming, gram-positive, and pathogenic bacterium that causes septicemia and meningitis in patients who are immunocompromised, as well as induces stilibirths in pregnant women. It is widely distributed in the environment because it can survive or reproduce over a wide range of environmental conditions. Moreover, this organism is not only resistant to heat (5, 12, 20), salt (28), freezing (9), drying (8), and sanitizers (26) during food processing, but it can even grow in vacuum packaged foods (7, 15) after processing. Although it has been recognized as a human and animal pathogen for more than 50 years, this organism has only recently emerged as a serious food borne pathogen. The survival of *L. monocytogenes* in different food products can threaten the food safety. It has been isolated from a wide range of foods such as dairy products, meat products, vegetables, and seafood, and in the 1980's, it was the causative agent of outbreaks of foodborne illness all over the world (13).

The heat-resistance of *L. monocytogenes* has been studied by many researchers as one of the factors that must be understood in order to determine if current food processing methods are adequate to destroy it. Some researchers reported that exposing *Listeria* cells to a mild heat treatment, or heat shock, resulted in production of heat shock proteins, which are thought to protect the organism from the harmful effects of further heating (29, 30). Studies on the heat shock response of *L. monocytogenes* have been done in meat products (12), milk (14) as well as broth media (6, 20).

Some investigators have questioned whether heat shocking of cells actually occurs

in nature, or whether it is a phenomenon only to be found in the laboratory. Mackey and Derrick (21) showed that varying the rate at which *Salmonella* were heated resulted in significant changes in the ability of the cells to survive a heat treatment at the target temperature. It is possible that cells are exposed to conditions similar to a heat-shock during a slow heating and thus become better able to tolerate the final heat treatment compared with cells exposed to fast heating.

*L. monocytogenes* has been studied for its ability to recover from heat treatments in various products and under various conditions. Knabel *et al.* (18) reported that enumeration under anaerobic conditions resulted in a higher number of survivors after a heat treatment in milk when compared with aerobic plating. These investigators suggested that the lack of oxygen radicals in anaerobic conditions offers the cells an opportunity to recover from injury over cells incubated in the presence of oxygen. However, Hart *et al.* (17) reported higher numbers of survivors in chicken packaged in air *vs.* modified atmosphere. In both of these studies, the cells were heated in the presence of air and then packaged aerobically or anaerobically. It would be of significance to determine whether exposure to air *vs.* vacuum during heating (prior to storage) affects the ability of *Listeria* to survive a heat treatment.

The D-values of *Listeria* have been established in different foodstuffs such as cheese (27), ground beef (2, 10), and milk (3, 5), as well as in Trypticase Soy Broth with 0.6 % yeast extract (TSBYE) (20). Even though ground pork is one of the major ingredients used in the manufacture of processed meats, the D-value of *L. monocytogenes* in this product has not been determined. Investigators have suggested that D-values of this organism may vary in many food products according to the *Listeria*

subspecies present (19).

Because this organism can grow well in refrigerated food products such as ham, sausage, cheese, and milk, concerns about the possible role of these food products as vehicles of listeriosis have emerged (4). Furthermore, this organism can survive freezing even at -18°C for 1 month in tryptose broth and phosphate buffer (9). *L. monocytogenes* is relatively more heat-resistant than other non-spore-forming microorganisms (20). Inadequate processing during pasteurization could lead to recovery of heat-injured cells (23), and to growth of this organism during refrigerated storage. Ryser *et al.* (27) reported that, in cottage cheese, heat-injured *L. monocytogenes* Scott A and serotype 1 were recovered during storage at 3°C for up to 28 d. Packaging atmosphere also is a very important factor for survival and recovery of *L. monocytogenes* in food products (15, 31). Razavilar and Genigeorgis (15) reported that 100% $CO_2$ was significantly more inhibitory to growth initiation than any other atmospheric condition in blood agar. Knabel *et al.* (18) reported higher numbers of heat-injured cells in strictly anaerobic conditions *vs.* aerobic condition.

Antioxidants are substances that can delay the onset, or slow the oxidation rate of, autooxidizable materials such as lipids. This occurs by inhibition of formation and propagation of free radicals (16). In meat products, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylated butyhydroquinone (TBHQ) and similar compounds have been used for this purpose. Prabhu *et al.* (24) and Yousef *et al.* (32) demonstrated that BHA and BHT inhibited *L. monocytogenes* by prolonging the logarithmic phase of growth while decreasing the overall growth of the organism. Payne *et al.* (22) reported that TBHQ was more effective than BHA and BHT in media. However, it

is not known whether *Listeria* will be affected in these same way in ground pork containing the antioxidants during storage.

The objectives of this study were to determine the effect of heating rate, packaging atmosphere, and meat age on heat resistance of *L. monocytogenes* serotype 1 and Scott A to heating at 62°C in ground pork, to determine the effect of storage temperature on recovery of heat-injured *L. monocytogenes* in aerobic- or vacuum-packaged ground pork, and to investigate the effect of antioxidants on this organism during storage.

## MATERIALS AND METHODS

### Bacterial culture conditions

*Listeria monocytogenes* serotype 1 (ATCC 19111) was obtained from the American

Type Culture Collection in Rockville, Maryland, and *L. monocytogenes* Scott A was

obtained from the National Animal Disease Center Laboratory in Ames, IA. The

microorganisms were incubated in Trypticase Soy Broth (BBL Beckton Dickinson,

Cockeysville, MD) with 0.6% yeast extract (TSBYE) at 37°C for 12 h. The plating

media used were Trypticase Soy Agar (TSA, BBL Beckton Dickinson, Cockeysville, MD)

with 0.6% yeast extract as a nonselective medium, and Oxoid Agar (OXA, Oxford,

Unipath LTD, Hampshire, England) as a selective medium.

### Sublethal heat treatment for determination of heat resistance and injury

After 10 h of incubation in TSBYE media, the culture of each serotype was heat-

treated at 48°C for 2 h in a water bath, immediately cooled to room temperature in an

ice bath and diluted in 80 ml of 0.1% peptone (Difco, Laboratories, Detroit, MI) solution.

The samples were blended in a Stomacher (Lab-Blender 400, Tekmar Company,

Cincinnati, Ohio) for 3 min, serially diluted, and plated on TSAYE (nonselective) or OXA

agar (selective). The plates were incubated at 37°C for 48 h, and the colonies were

counted. In TSAYE media, only *Listeria* colonies were counted. These were identified

by colony morphology and gram strain. The number of injured cells was determined by

subtracting the CFU/g of ground pork in selective from CFU/g of ground pork in

nonselective media.

**Heat treatment for determination of D-value in ground pork**

One-hundred microliters of the cultured broth were added into 5 ml of TSBYE and incubated at 37°C for 6 h to achieve the mid-logarithmic phase of growth based on standard growth curve at 625 nm.

Twenty-grams of ground pork obtained from a local retailer were placed into screw capped tubes and heated to 62°C in a water bath. When the temperature of the samples reached 62°C, 0.2 ml of *Listeria* cells (serotype 1 or Scott A) was injected into the geometric center of each sample and incubated at 62°C for 0, 10, 20, or 30 min. The numbers of total and healthy cells were detected as previously described.

**Effect of heating rate**

Ten grams of ground pork in a test tube were inoculated into the geometric center with *L. monocytogenes* serotype 1 and placed in a water bath at 35°C. The temperature was increased in the water bath to achieve 62°C at a rate of either 1.3 °C/min, 2.2 °C/min, or 8.0 °C/min. The heating rate was controlled by adjusting the controller of the water bath (Model 730, Fisher Scientific, Pittsburgh, PA). The temperature was detected with a J type thermocouple (Omega Engineering Inc., Stamford, CT) and datalogger (Model LI-1000, LI-COR, Lincoln, NE). In this experiment, the heating rate (or time required for the temperature to increase from 35°C to 62°C) was 20.5 min at a rate of 1.3 °C/min, 12.3 min at 2.2 °C/min, and 3.3 min at 8.0 °C/min. After 0 min, 10 min, 20 min, and 30 min of heating at 62°C, each sample was cooled rapidly in an ice bath. One-hundred milliliter of 1% peptone solution was added to each sample, blended, diluted properly and plated on the media to determine total, healthy, and

injured cells as described before.

## Effect of meat age

Fresh pork was defined as pork obtained and used within 3 days of slaughter, and long-stored ground pork was defined as pork used after storage for 3 months at -10°C. Twenty-grams of each sample was placed into screw capped tubes and 0.1 ml of *Listeria* cells (serotype 1 or Scott A) were injected into samples as described before. The heat treatment was 62°C for 0, 10, 20, or 30 min and the samples were cooled immediately in an ice bath to room temperature. The number of survivors was determined by plating onto TSBYE and OXA as previously described.

## Effect of vacuum environment during heating

Twenty-grams of ground pork were placed into each of eight 863 Saran pouches (6" x 4") (Curwood Inc., Oshkosh, WI) and heated to a target temperature of 62°C in a water bath (Model 730, Fisher Scientific, Pittsburgh, PA). After the target temperature was reached, 0.2 ml of *Listeria* cells (serotype 1 or Scott A) were injected into each bag and mixed. Half of the bags were sealed in air and half were vacuum-sealed (Fresh Vac. Model A300, CVP Systems Inc., Downers Grove, Il) at 380 torr. All samples were heat-treated for 0, 10, 20, and 30 min at 62°C by submerging the bags in a water bath set at 62°C. After heating, the samples were cooled rapidly to room temperature in an ice bath. Eighty-milliliters of a 0.1% peptone solution were added into each sample, which were blended in a Stomacher, serially diluted, plated onto appropriate media, and incubated at 37°C. The number of survivors was determined by colony counting after

48 h.

**Effect of storage conditions on recovery of heat-injured *L. monocytogenes***

In order to determine the effect of packaging conditions on recovery of heat treated *Listeria*, the cells and the meat samples were heat-treated separately, and then the cells were inoculated into the meat, which was then packaged and stored. In this procedure, 100 µl of the cultured broth were added into 30 ml of TSBYE and incubated at 37°C for 12 h to achieve stationary phase of growth. The culture was heat-treated at 62°C for 10 min. The come-up time was 3 min as determined by a J-type thermocouple (Omega Engineering Inc., Stamford, CT) and datalogger (Model LI-1000, LI-COR, Lincoln, NE). The heat-treated culture was cooled rapidly to room temperature in an ice bath.

Twenty-grams of ground pork were placed into pouches (Curwood Inc., Oshkosh, WI) and vacuum-sealed (Fresh Vac. Model A300, CVP Systems Inc., Downers Grove, II) at 380 torr, and the pork was heat-treated for 1 h at 62°C by submerging the bags in a water bath (Model 730, Fisher Scientific, Pittsburgh, PA). Point-three milliliter of heat-treated *Listeria* cells was injected into each bag aseptically. After that, the samples were vacuum-sealed and stored at 7, 20, 30°C until tested. To count the organisms in each sample, 80 ml of 0.1% peptone solution was added into the samples, blended in a Stomacher (Lab-Blender 400, Tekmar Company, Cincinnati, Ohio) for 3 min, serially diluted, and plated on TSAYE or OXA. After incubation at 37°C for 48 h, the number of survivors was determined by colony counting.

**Effect of antioxidants on enumeration of *Listeria* in ground pork during storage**

Butylated hydroxyanisole (BHA, Sigma Chemical Co., St. Louis, MO) and butylated hydroxytoluene (BHT, Sigma Chemical Co., St. Louis, MO) were used as antioxidants in this study. One-milliliter of 1%, 2%, and 3% BHA solution in 50% (v/v) ethanol, or 3%, 5%, and 7% BHT in 95% (v/v) ethanol was added to 100 g of ground pork and blended for 5 min per sample. In preliminary experiments, it was found that addition of ethanol to achieve a 1% solution in sample did not affect survival of *Listeria*. Ten grams of each sample blended with antioxidant were then placed in a 863 Saran pouch (6" x 4") and heated to 62°C in a water bath (Model 70 Isotemp Immersion Circulator, 28 L) of 62°C. The come-up time which was calculated as the time required for the temperature to increase from 35°C to 62°C in ground pork, was set-up to be 3 min in this experiment. After heating at 62°C for 1 h, each sample was cooled rapidly in an ice bath. Point-three milliliters of the heat treated *Listeria* was then injected into the geometric center of the ground pork, aerobically packaged, and stored at 7, 20, or 30 °C until tested. Enumeration was done as described previously.

## RESULTS AND DISCUSSION

### Heat resistance of *Listeria monocytogenes* serotypes

In this study, *L. monocytogenes* serotype 1 and *L. monocytogenes* Scott A were

tested for their ability to survive a heat treatment in ground pork. From Figure 1, 98%

of the cells of serotype 1 survived the sublethal heat treatment at 48°C, and the number

of healthy cells decreased to 43% of the healthy cells after heating. In contrast, only

32% of Scott A cells survived and the number of healthy cells decreased to 12% of

total healthy cells. Lemaire *et al.* (19) reported that strains belonging to serotype 1

were more heat-resistant than those belonging to serotype 4 in milk. Bradshaw *et al.*

(3) reported that Scott A was more heat-resistant than serotype 1 in milk. From the

data presented here (Figure 2), the opposite results were obtained, namely that

serotype 1 was found to be slightly more heat resistant than Scott A in ground pork.

Perhaps serotype 1 is better adapted to the conditions found in meat than Scott A.

Because serotype 1 has been detected more in heat-processed pork products than

Scott A, a higher heat treatment than usual should be required in processing of these

products to ensure complete destruction of *Listeria monocytogenes* serotype 1.

### Effect of heating rate on survival of *Listeria monocytogenes* in ground pork

In this study, the heating rate during heat treatment was tested in ground pork by

using *L. monocytogenes* serotype 1 because this organism has been more commonly

detected in meat products than Scott A. After heating from 35°C to 62°C at a rate of

1.3 °C/min, 2.2 °C/min, or 8.0 °C/min, the concentration of cells dropped from $1.2 \times 10^8$
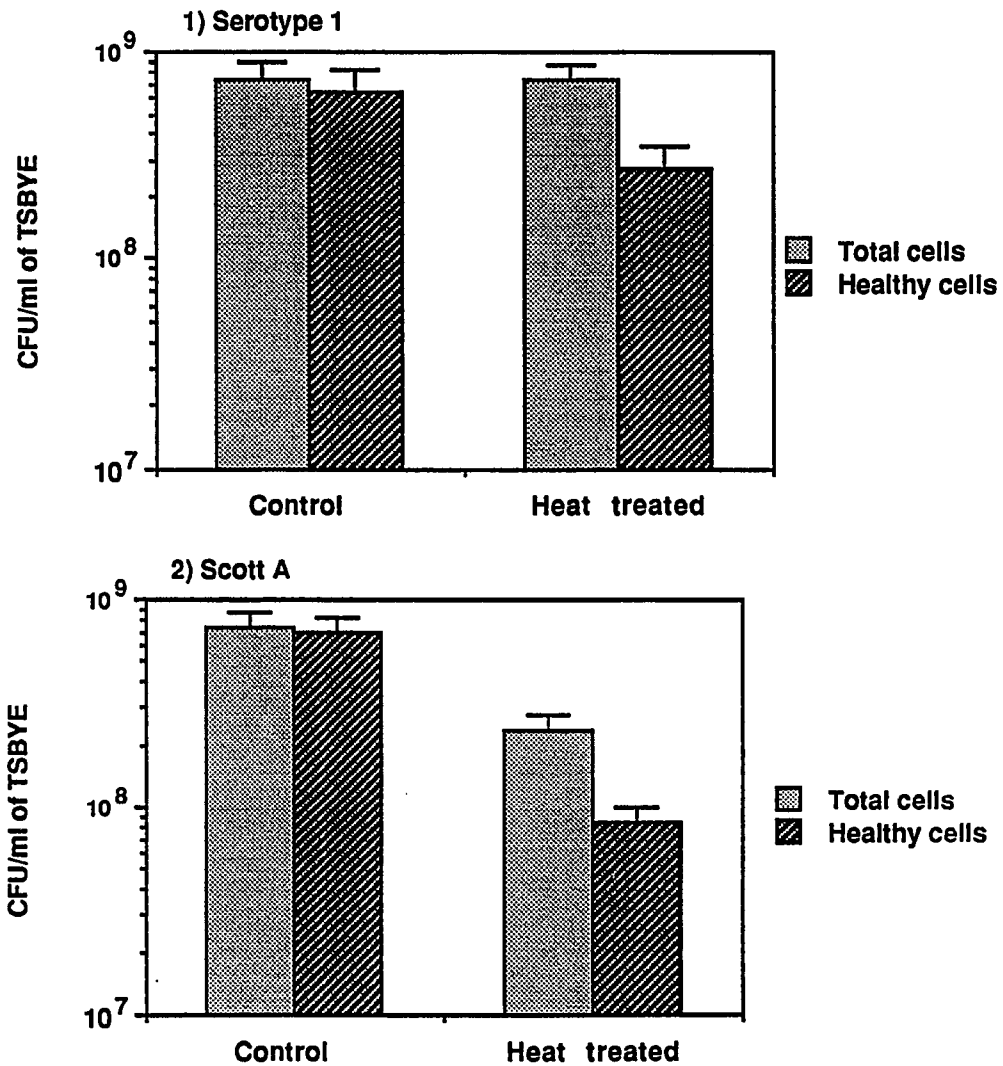
Figure 1. Comparisons of heat resistances and injury of *Listeria monocytogenes* serotype 1 and Scott A after heating at 48°C for 2 hrs. (TSAYE medium was used for total cell counting and OXA medium was used for healthy cell counting.)
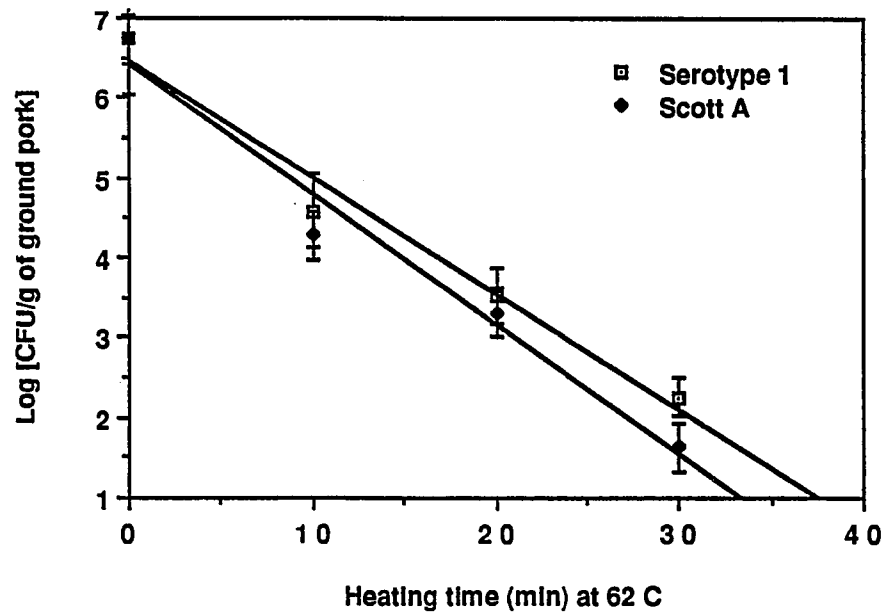
Figure 2. Heat resistance of *Listeria monocytogenes* serotype 1 and Scott A during heat treatment at 62°C in ground pork

(initial inoculum ) to 1.3 x $10^7$, 6.7 x $10^7$, and 4.77 x $10^7$, respectively. Thus, no significant difference in the effect of heating rate on *Listeria* was noted before heating to the target temperature. After heating at 62°C, higher numbers of survivors were found when the cells were initially heated at the lowest heating rate (Figure 3). The D-values were calculated to be 9.2 min, 6.2 min, and 5.5 min at the rates of 1.3 °C/min, 2.2 °C/min, and 8.0 °C/min, respectively. Mackey and Derrick (21) reported that the heat resistance of *Salmonella typhimurium* was dependent on the heating rate and that the slower the temperature rise, the greater the increase in resistance. The results of these studies on *L. monocytogenes* serotype 1 concur with those on *Salmonella* by Mackey and Derrick (21), and point to a concern that the rate at which products are heated can have a significant impact on their microbiological safety.

**Effect of meat age on survival of *Listeria monocytogenes***

*Listeria* inoculated into three-months-old ground pork was more sensitive to heating than *Listeria* inoculated into fresh ground pork (TABLE 1). The reasons for this are not very clear. It is possible that, although the meat was stored frozen, chemical reactions such as oxidation (which still occur during storage (1)) and chemical by-products produced during storage could have damaged the cells upon inoculation. Moreover, during heat treatment, lipids in pork could contain peroxides, radicals, or other organic materials (16) which could have made the injured cells more fragile. For example, it is known that the reaction of hydroxy radicals with deoxyribose results in fragmentation of DNA with loss of the bases and strand breaks (11). Therefore, it is possible that freezing may have affected these cells as suggested above. These results show that
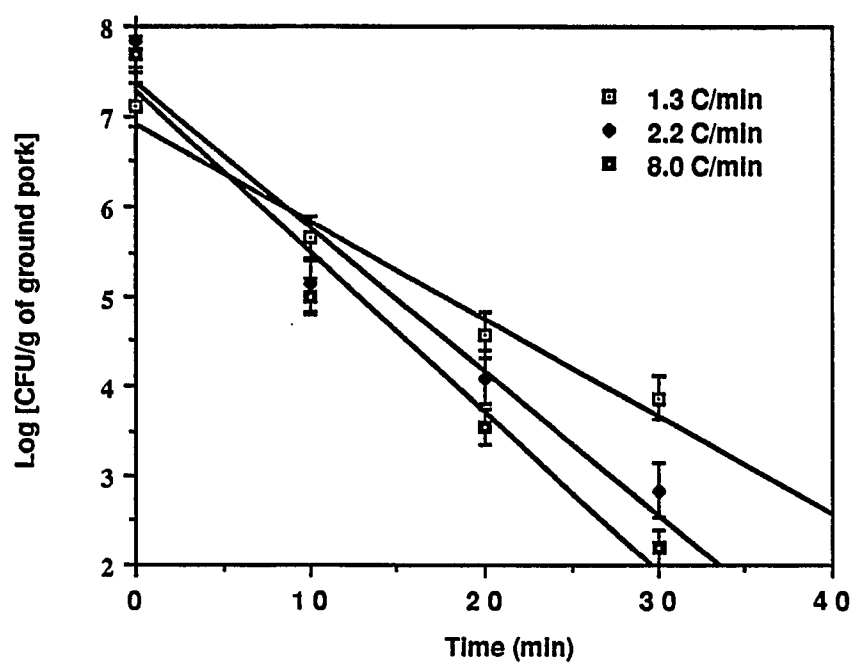
Figure 3. Effect of initial heating rate on heat resistance of *Listeria monocytogenes* serotype 1 to heating at 62°C in ground pork

TABLE 1. Effect of meat age on heat resistance of *Listeria monocytogenes* serotype 1 and Scott A.

| | D-values at 62°C | |
| --- | --- | --- |
| | Serotype 1 | Scott A |
| Fresh ground pork[1] | $7.7 \pm 0.5^2$ | $6.5 \pm 0.6$ |
| Old ground pork | $5.2 \pm 0.4$ | $4.3 \pm 0.5$ |

[1] The fresh ground pork was obtained within 3 days after slaughter and the old ground pork was obtained after 3 months of storage at -10°C.

[2] Average values of three replications ± standard deviation.

the conditions at which meats are stored may play an important role in the survival of *Listeria* to a subsequent heat treatment.

**Effect of vacuum packaging during heating on survival of *Listeria monocytogenes* in ground pork**

TABLE 2 shows that higher numbers of survivors were detected after heating when the meat was heated aerobically than anaerobically. Knabel *et al.* (18) reported that higher numbers of *Listeria* were recovered if the cells were plated under anaerobic conditions compared with aerobic conditions. They suggested that the reason for this is that, after heating, catalase and superoxide dismutase are inactivated, which prevents the cells from growing in the presence of toxic oxygen radicals found in aerobic storage. Linton *et al.* (20) also reported that enumeration of heat-shocked cells in

TABLE 2. Effect of packaging atmosphere on thermal destruction of *Listeria*
*monocytogenes* during heating in ground pork

|  | D-values at 62°C |
| --- | --- |
| Atmospheric package | 7.8 ± 0.3[1] |
| Vacuum package[2] | 6.2 ± 0.4 |

[1] Average values of three replications ± standard deviation.
[2] Vacuum-packaged at 380 torr prior to heating.

anaerobic media resulted in higher numbers than those in aerobic media. The results
presented here show that anaerobic conditions, although beneficial to the cells during
recovery after a heat treatment, are detrimental to their survival during heating. The
rapid change in atmosphere from the aerobic conditions of the original culture to the
anaerobic conditions of vacuum packaging may have made the cells more susceptible
to destruction during the heat treatment.

In addition, after heating, the cells which had been packaged anaerobically were
enumerated aerobically. It is possible that the subsequent change of atmosphere
placed an additional stress on the organism, which resulted in lower counts than cells
which were kept under aerobic conditions before, during and after heating.

**Effect of package environment on recovery of heat-treated *Listeria***

To determine the effect of packaging on recovery of heat-injured *L. monocytogenes*
serotype 1 in ground pork, *Listeria* was heat-treated at 62°C for 10 min, packaged

aerobically or anaerobically in ground pork heated at 62°C for 1 h, and stored at 4°C

(Figure 4), 20°C (Figure 5), and 30°C (Figure 6). No difference in recovery between

sample packaged aerobically *vs.* vacuum was detected at 20 and 30°C due to rapid

growth of cells at these temperatures. However, a difference in the growth rate of the

organism was detected between aerobic *vs.* vacuum packaging at 4°C of storage. The

recovery of heat-injured *Listeria* was also determined at 4°C in ground pork. After heat

treatment at 62°C for 1 h, the number of cells was enumerated by using TSAYE and

OXA media. The numbers of total and healthy cells were $1.09 \times 10^4$ CFU/g of ground

pork and $4.62 \times 10^3$ CFU/g of ground pork, respectively, in which the percentage of

healthy cells was 42.4%. Figure 7 shows the trends of recovery of heat-injured *Listeria*

in aerobically-packaged and vacuum-packaged ground pork during storage at 4°C for 0,

9, 18, and 26 days. From Figure 7, the growth rate of the cells was greater in vacuum-

packaged samples than in aerobically-packaged samples during storage at 4°C.

Knabel *et al.* (18) indicated that the use of strictly anaerobic culture conditions signifi-

cantly increased recovery in TSAYE with aerobically incubated controls. They ex-

plained that the heat treatment could result in inactivation of catalase which serves as

protection against oxygen radicals present in aerobic environments.


**Effects of storage temperature and packaging on growth of *Listeria***

Listeria monocytogenes in meat inadequately pasteurized may grow during long

time storage even at low temperatures. In these studies, the effect of storage condi-

tions on recovery of heat-injured *L. monocytogenes* serotype 1 was determined during

storage at 4, 20, or 30°C. The maximum cell number in ground pork was detected after

**1) Nonheat-treated Listeria**
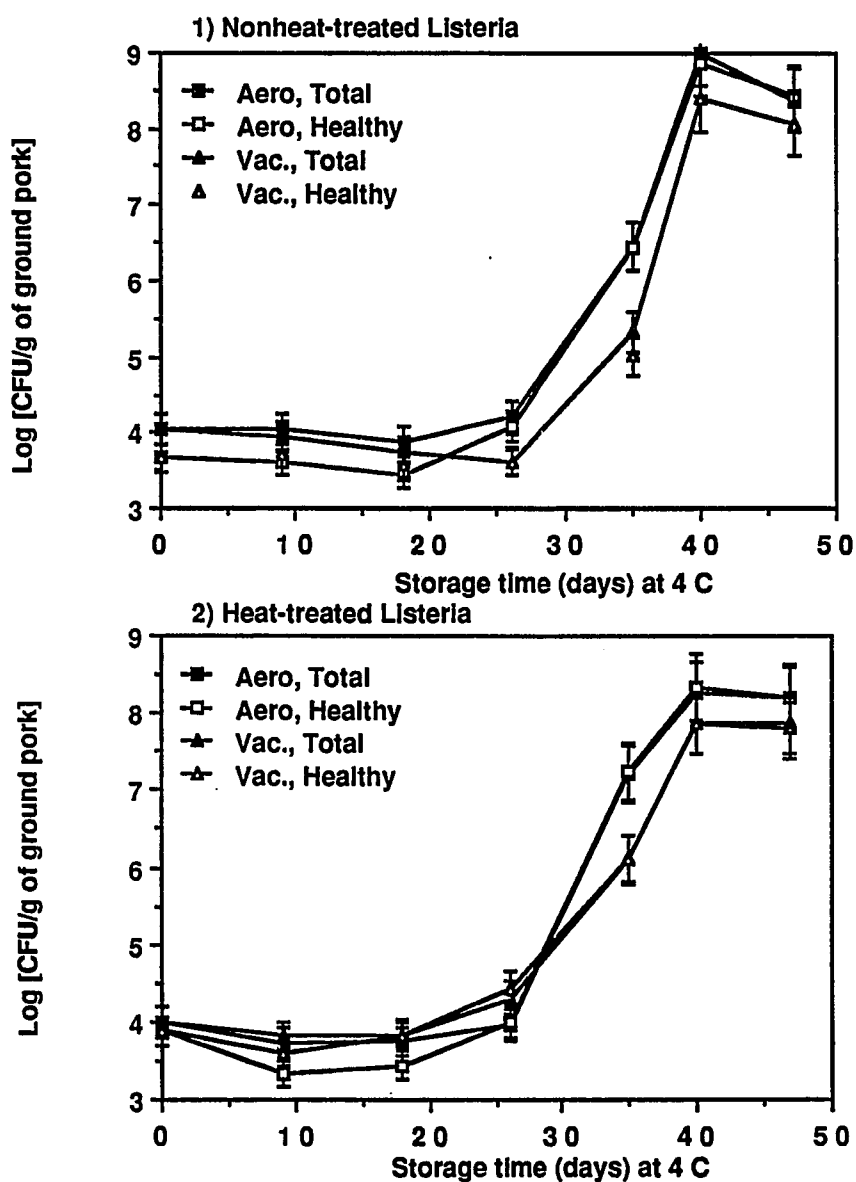


**2) Heat-treated Listeria**



Figure 4. Growth curves of heated *Listeria monocytogenes* serotype 1 during storage at 4°C in ground pork. Cells were heated at 62°C for 10 min. (Aero; aerobically packaged, Vac.; vacuum-packaged, Total; number of cells on TSAYE, Healthy; number of cells on OXA)

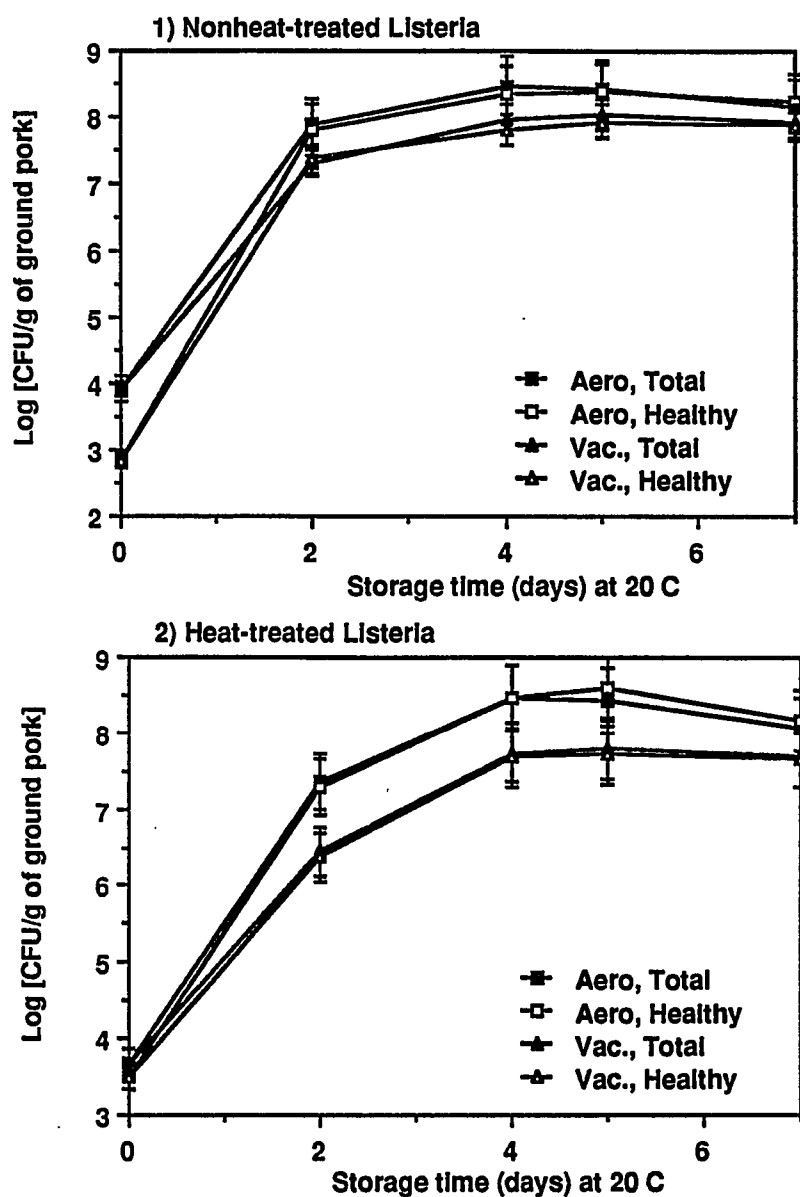## 1) Nonheat-treated Listeria



## 2) Heat-treated Listeria



Figure 5. Growth curves of heated *Listeria monocytogenes* serotype 1 during storage at 20°C in ground pork (Aero; aerobically packaged, Vac.; vacuum-packaged, Total; number of cells on TSAYE, Healthy; number of cells on OXA)

**1) Nonheat-treated Listeria**



- Aero, Total
- Aero, Healthy
- Vac., Total
- Vac., Healthy

*Storage time (days) at 30 C*

Log [CFU/g of ground pork]

**2) Heat-treated Listeria**



- Aero, Total
- Aero, Healthy
- Vac., Total
- Vac., Total

*Storage time (days) at 30 C*
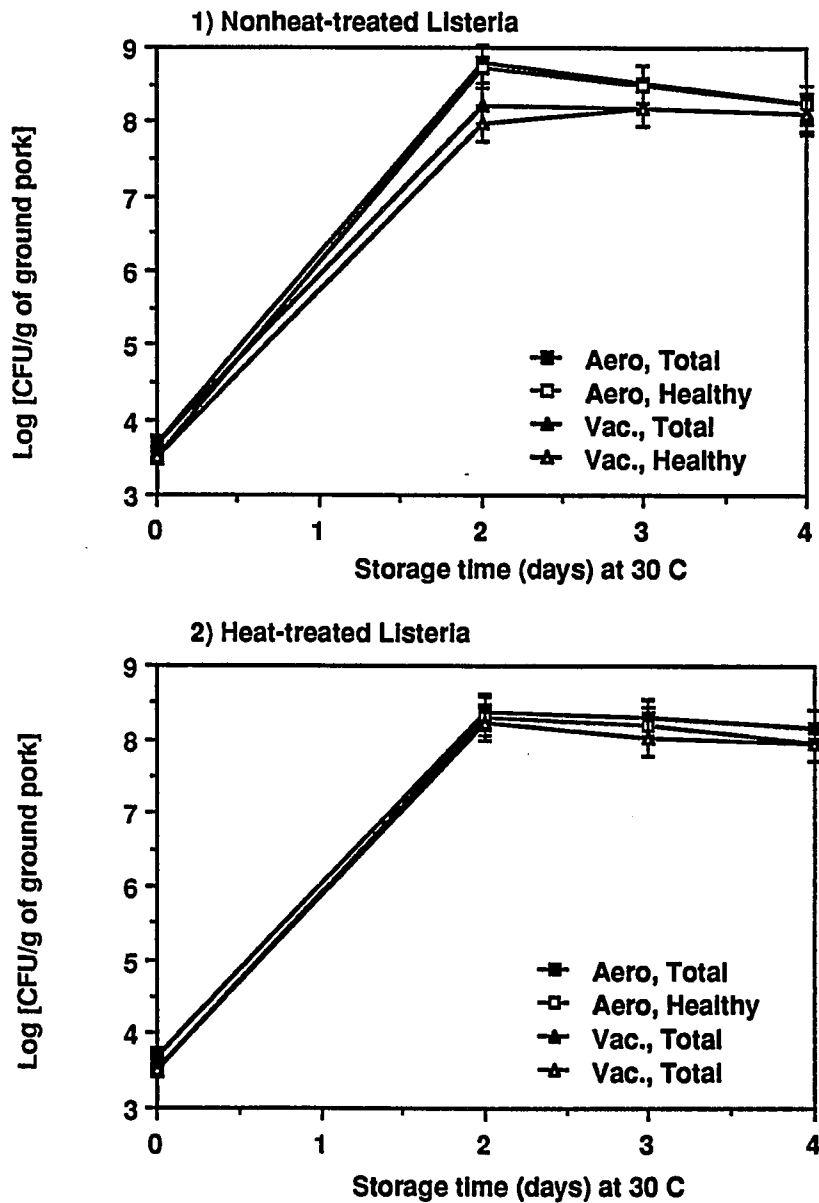
Log [CFU/g of ground pork]

Figure 6. Growth curves of *Listeria monocytogenes* serotype 1 during storage at 30°C (Aero; aerobically packaged, Vac.; vacuum-packaged, Total; number of cells on TSAYE, Healthy; number of cells on OXA)
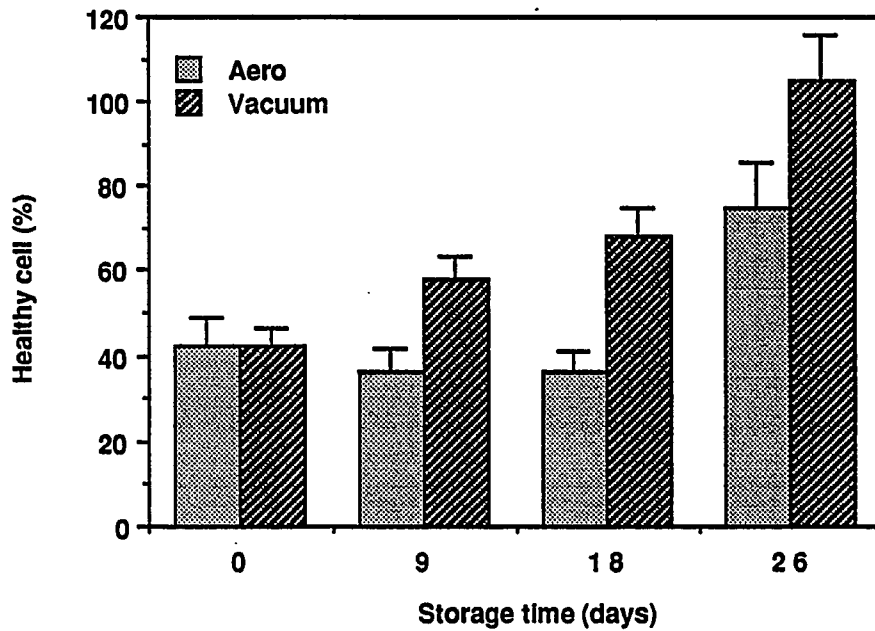
Figure 7. The recovery rates of heat-injured *Listeria monocytogenes* serotype 1 during storage at 4°C. Percent of healthy cells was calculated by percentage of CFU on TSAYE media divided by CFU on OXA media. Listeria were heat-treated at 62°C for 10 min. (Aero; aerobically packaged, Vacuum; vacuum-packaged)

40 days at 4°C, 4 days at 20°C, and 2 days at 30°C. The number of cells during storage was also affected by packaging atmosphere. Lower numbers were detected in vacuum-packaged ground pork than in ground pork packaged aerobically. Carpenter and Harrison (7) reported that *L. monocytogenes* grew more slowly on vacuum-packaged chicken breast than on film-overwrapped samples.

**Effects of antioxidants on survival of *Listeria* in ground pork**

Yousef *et al.* (32) reported that *Listeria* exhibited increasingly longer lag phase periods and generation times as well as lower maximum populations in the presence of BHA at 200 ppm in Tryptose broth. In practice, these antioxidants have two opposite roles in food products: the quenching of oxidative components which are toxic to organisms, and the inhibition of growth of organisms. However, the role which antioxidants play under various storage conditions has not been determined. To determine the effect of antioxidants in ground pork during storage, BHA and BHT were tested at concentration of 100, 200, and 300 ppm BHA, or 300, 500, and 700 ppm BHT at 7°C (Figure 8) and 30°C (Figure 9). Neither antioxidant significantly affected the growth of cells in ground pork regardless each concentrations of antioxidants. These results show that the practical concentrations of BHA and BHT for meat processing do not affect the cell growth in ground pork during storage regardless temperatures.

**1) BHA**



**2) BHT**


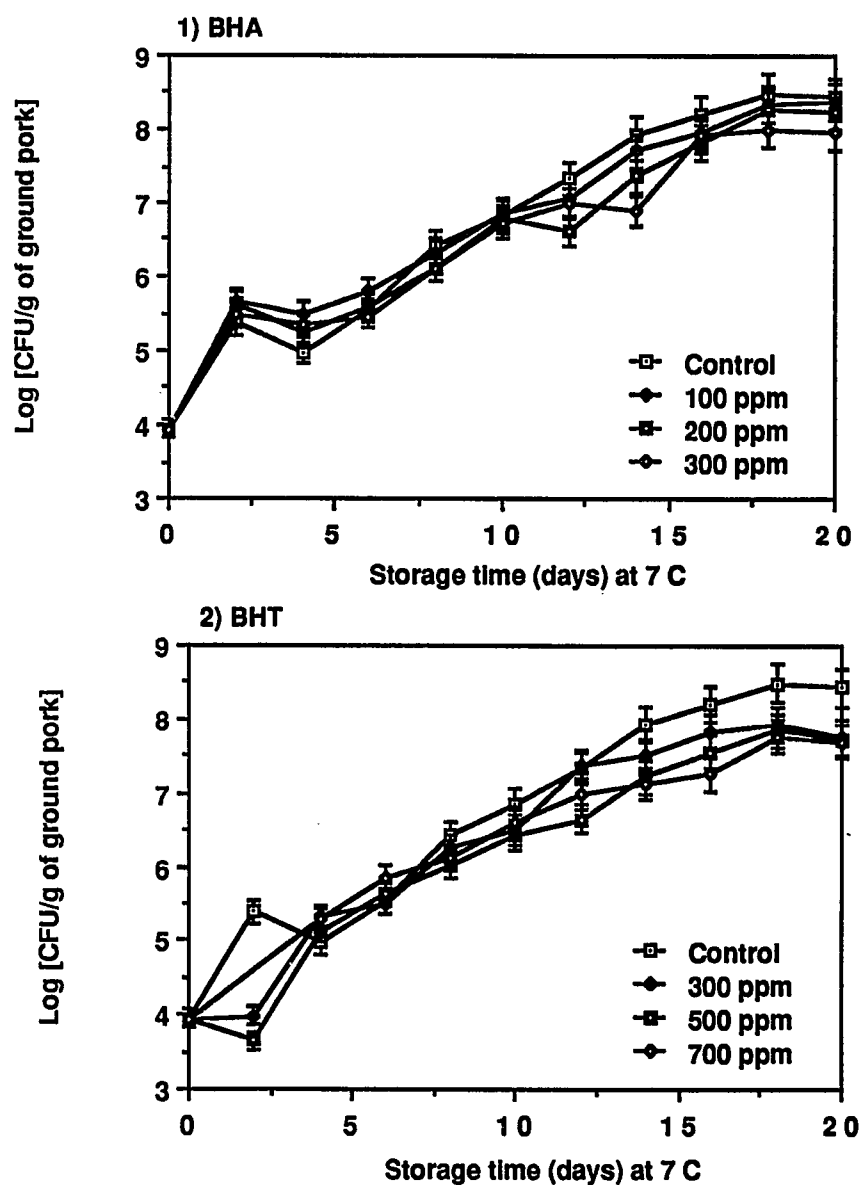
Figure 8. Effects of BHA and BHT on the growth of *Listeria monocytogenes* serotype 1 during storage at 7°C in ground pork.
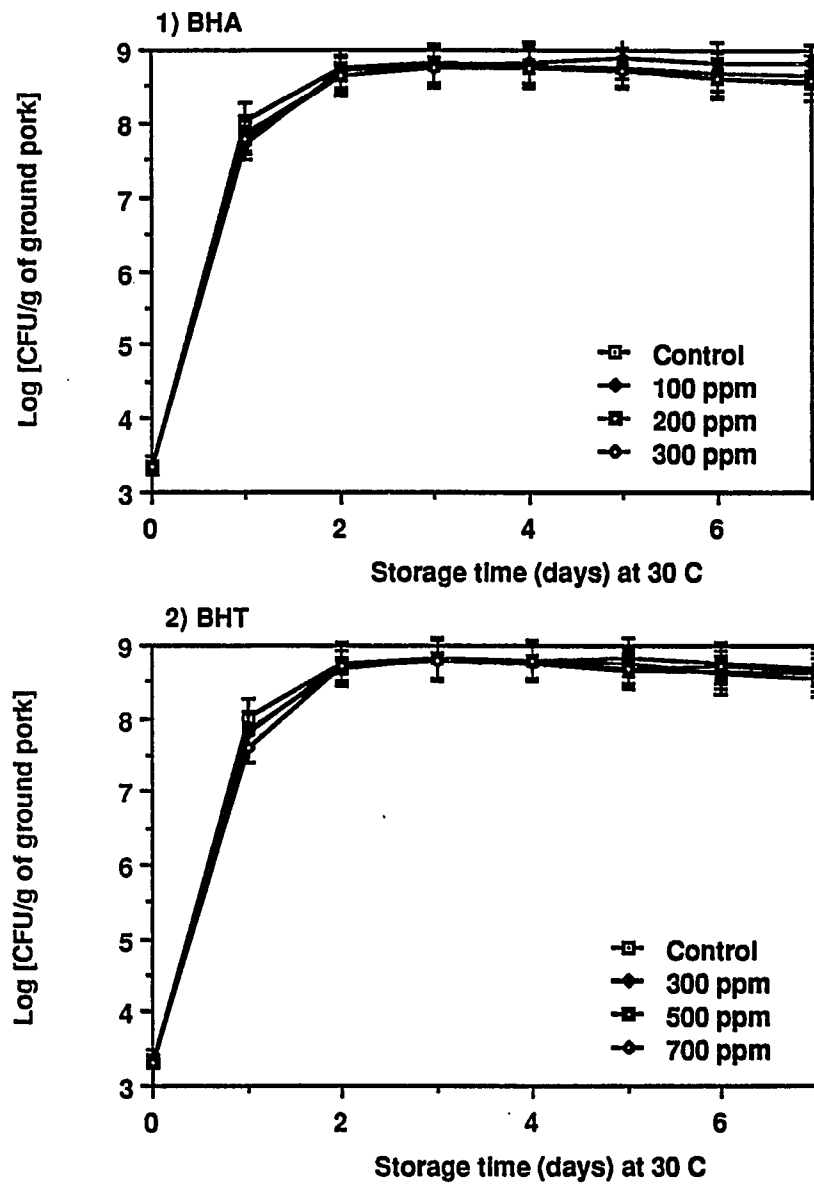
**1) BHA**



**2) BHT**



Figure 9. Effects of BHA and BHT on the growth of *Listeria monocytogenes* serotype 1 during storage at 30°C in ground pork

## Summary

The heat resistance of *L. monocytogenes* can differ according to subspecies of the organism, heating rate during a heat treatment, packaging atmosphere during heating, and meat age. In the case of ground pork, serotype 1 was more resistant than Scott A after heat treatment. The heating rate was a very important factor in the heat resistance of this organism. According to these studies, a shorter initial heating time such as pre-cooking and pasteurization would be needed to minimize the heat resistance of pathogens. The heat-treated *Listeria* packaged anaerobically was less heat resistant than that packaged aerobically.

Given the results presented here, further studies on the combined effects of heating rate, meat age, and packaging atmosphere on the heat resistance of *L. monocytogenes* serotype 1 should be conducted. Such information should aid processors to design processing procedures that would prevent or minimize the chances for survival of pathogens like *L. monocytogenes*.

During storage of ground pork, heat-injured *L. monocytogenes* were recovered more rapidly in vacuum packaging although the total number of cells was lower than controls in aerobically packaged ground pork. In addition, the number of cells after maximum growth in vacuum-packaged ground pork decreased slower than cells packaged in air. While primarily used to prevent oxidation of fats, some of these antioxidants also posses antimicrobial activity. Payne *et al.* (22) indicated that the antimicrobial activity differed according to the kinds and concentration of antioxidants used. However, antioxidants at added concentrations approved by FDA did not affect the cell growth of *Listeria* in ground pork at 7 and 30°C. In the food industry, antioxidants should be

evaluated in order to achieve both shelf-life extension by preventing lipid oxidation, as well as increased food safety by inhibiting the growth of pathogens such as *Listeria* before practically using in food products.

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PAPER II. PRODUCTION OF LISTERIOLYSIN O BY *Listeria monocytogenes* UNDER VARIOUS CULTURE CONDITIONS

# Production of Listeriolysisn O by *Listeria monocytogenes* under Various Culture Conditions[*]

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# ABSTRACT

*Listeria monocytogenes* serotype 1 and Scott A strains were examined to deter-

mine the effect of culture strain on production of listeriolysin O (LLO).  The effects of pH

(between 5.0 and 7.0), incubation time, and culture methods also were examined after

the addition of 0.5%, 1.0%, or 1.5% glucose.  As another culture method, a repeat fed-

batch method was tested.  The hemolytic activity of the culture was quantitated by

hemolysis of red blood cells.  LLO was produced in greater concentrations by serotype

1 than by Scott A.  The fed-batch method resulted In higher levels of LLO than one-time

addition of glucose.  In the fed-batch method, maximum production was detected at pH

5.5 through 6.5 after 2 h of stationary phase.  The addition of more than 1.0% glucose

for cell growth significantly reduced the production of LLO by the cells.  In addition, the

heat stability of the listerlolysin was also determined.  Heat shocking of *Listeria* cells at

48°C for 2 h resulted in almost total reduction of listeriolysin O.  Within 4 h of incubation

at 37°C, heat-shocked cells resumed production of listeriolysin, achieving on activity

level 40-times higher than that immediately after heat shock.  However, LLO activity of

control cells was higher than that of heat-skocked cells throughout the incubation

period.  Thus, heat shock did not result in an increase in the total concentration of LLO

compared with nonheat-shocked controls.  Inactivatlon of listerlolysin increased with

temperature, with none detected immediately after exposure to 62°C.  Even though the

heat shocking induce a production of LLO more rapidly, the heat-treated meat may be

less vilulent than nonheat-treated meat due to lower concentration of LLO in heat-

treated meat.

## INTRODUCTION

*Listeria monocytogenes* is a facultative anaerobic, gram-positive, and intracellular

pathogenic bacterium that can cause septicemia, meningitis, and stillbirths in humans.

In general, serious listeriosis can occur in patients who have underlying conditions

which lead to suppression of their immunity such as AIDS, cancer, or alcoholism (8). In

particular, most cases of listeriosis occur in individuals at the extremes of age (newborn

and elderly). This organism is widely distributed in the environment and is transmitted

to humans through contamination of foodstuffs such as dairy products, meat products,

and vegetables (5, 19, 20). The infection has relatively low morbidity but a high case-

fatality. Pregnant women and immunocompromised patients are very sensitive to this

microorganism (19). In particular, the mortality rate for infected infants is 36% (8)

despite aggressive supportive care and appropriate antibiotic therapy. *Listeria* is a

psychrotroph, capable of growing at refrigerator temperatures. As consumption of

refrigerated ready-to-eat foods has increased, so has isolation of *Listeria* in these

products. In addition, this organism is somewhat resistant to conventional processing

treatments such as heating (7, 13). The degree of heat resistance of this organism has

been reported to vary depending on bacterial strain (3, 6) and packaging conditions (9).

Listeriolysin O (LLO, molecular weight; 60,000) is a water-soluble, heat-labile

protein produced by *L. monocytogenes* that exhibits cardiotoxic activity. This protein is

activated by sulfhydryl groups such as sodium thiosulfate and is antigenically similar to

streptolysin O (1, 17). Listeriolysin aids the cells in invading host macrophages and

triggers lysis of the membrane-bound phagocytic vacuole in these cells by forming

pores in the membrane of phagocytes, which enables *Listeria* to escape and be free to invade other phagocytes (12, 16, 18, 21, 22, 23). Many recent reports indicate that the gene coding for the hemolysin produced by *L. monocytogenes* is at least partly responsible for the organism's virulence (11, 21, 22). Until now, LLO has been considered one of the main virulence factors of *Listeria* spp. Sokolovic *et al.* (22) reported that growth at 48 °C (rather than heat shock) resulted in resumption of listeriolysin production by a nonhemolytic mutant strain of *L. monocytogenes*. However, these investigators did not provide information on whether heat shock actually enhances the ability of cells to produce listeriolysin, or whether it simply induces it. With the help of LLO, the organism can invade macrophages, epithelial cells, and fibroblasts in the host. When the bacteria escape from a host cell vacuole, this event is mediated partly by the action of LLO which can form pores that enable the organism to leave (16, 23). For this reason, LLO has been used as a good marker for evaluating the virulence of heat-stressed *L. monocytogenes* (11, 15). However, the production of LLO may differ depending on environmental conditions such as pH, availability of nutrients, and growth phase of various subspecies.

The objectives of this study were to determine the effect of subspecies type, and of various culture conditions on production of LLO by *L. monocytogenes*, to determine the maximum amount of LLO that can be produced by this organism by using optimized conditions, to quantitate production of listeriolysin by heat-shocked *vs.* nonheat-shocked cells, and to determine the heat stability of listeriolysin at various temperatures.

# MATERIALS AND METHODS

## Bacterial culture conditions for productivity of LLO

**Species**    As test organisms, *L. monocytogenes* serotype 1 (ATCC 19111) was

obtained from the American Type Culture Collection in Rockville, Maryland, and  *L.*

*monocytogenes* Scott A was obtained from the National Animal Disease Center in

Ames, IA.  The plating media used were trypticase soy agar (TSA, BBL Beckton

Dickinson, Cockeysville, MD) with 0.6 % yeast extract as a nonselective medium and

Oxford Agar (OXA, Oxoid, Unipath LTD, Hampshire, England) as a selective medium.

**pH**    At the stationary phase, the pH of the culture was detected with pH meter

(Accumet 910, Fisher Scientific,  Pittsburgh, PA) and adjusted to 5.5, 6.0, 6.5 or 7.0 by

adding 1 N NaOH.   After incubation at 37°C for 4 h, each culture was tested for

hemolytic activity.

**Single-time glucose addition**    To determine the effect of glucose on production of

LLO, 0.1 ml of an 12 h broth culture was inoculated into 100 ml of brain heart infusion

broth (BHI, Difco Laboratories, Detroit, MI) supplemented with 0.5%, 1.0%, or 1.5%

glucose and incubated at 37°C.  Hemolysis activity of LLO was determined for each

culture every hour.  The pH of the culture was monitored and readjusted to 7.0 whenev-

er it dropped to 5.5 because low pH has been shown to destroy LLO activity (1).

**Repeat fed-batch procedure**    Glucose was added to the culture to achieve 0.5% in 100 ml BHI broth. Hemolysis activity was measured every hour, and the pH readjusted to 7.0 whenever it dropped to 5.5. Cells were at 37°C, with the stationary phase being reached after 12 h. This time was referred to as 'stage I'. After 'stage I', 0.5% glucose was added until the stationary phase was reached for a second time (referred to as 'stage II'). Glucose (0.5 g) was added to the culture broth an additional time until stage III was reached (see Figure 1). Therefore, a total of 1.5 g of glucose was added by the end of stage III (0.5 g x 3 times = 1.5 g).

To determine the effect of pH on production of LLO after 'stage I', 0.5 g of glucose was added to the culture after 'stage I' and the pH was adjusted in three ways. In method 1, the pH was readjusted to 7.0 whenever it dropped to 5.5 until the stationary phase in 'stage II' and again until 'stage III' was reached. In method 2, the pH was adjusted to 6.3 whenever it dropped to 5.5. This also was done two times (in 'stage II' and 'stage III'). In method 3, the pH of the BHI culture broth was adjusted to 6.0 but glucose was not added. The pH in all methods was detected and adjusted as described above.

For the studies involving the effect of incubation time on production of LLO by the fed-batch procedure, the pH of the culture in BHI with 0.5% glucose at the stationary phase was readjusted to 7.0 whenever it dropped to 5.5. During incubation, the culture was assayed for hemolysis at various time intervals of incubation at 37 °C.

**Preparation of crude LLO**

Crude LLO was prepared by modifying the method of Bhakdi *et al.* (2). Briefly,

bacteria were sedimented in a Beckman J2-21 centrifuge (Beckman Spinco division, Palo Alto, CA)(rotor JA-10, 11,000 x $g$) and the supernatant was filtered through a 0.45 μm cellulose acetate membrane filter (COSTAR, Cambridge, MA). For inhibition of protease, phenylmethylsulfonyl fluoride (SIGMA Chemical co., St. Louis, MO) was added as a 1 mM solution. Fifty-three grams of ammonium sulfate (Fisher Scientific, Fair Lawn, NJ) was then added per 100 ml of solution to precipitate the proteins, and the sample was stirred in an ice bath for 60 min. The precipitate was collected by centrifuging at 13,000 x $g$, resuspended in distilled water (final volume, 70 to 80 ml), and dialyzed with Spectra/Por membrane tubing (Spectrum Medical Industries Inc., Houston, TX, MWCO : 25,000) overnight against 5 L of 50 mM NaCl-4 mM EDTA at 4°C. Twenty-five grams of polyethylene glycol (PEG) 4000 (Fisher Scientific, Fair Lawn, NJ) was added to 100 ml of sample solution and stirred at 4°C for 30 min. After centrifugation at 30,000 x $g$ for 60 min, the supernatant was discarded and the precipitate was resuspended in 50 ml distilled water containing 3 mM $NaN_3$ and was refrigerated until used.


**Preparation of red blood cells (RBC) for hemolysis assay**

Defibrinated sheep blood (Adam Scientific, Warwick, RI) was centrifuged at 600 x $g$ for 5 min with a Beckman Model TJ-6 centrifuge. The red blood cells were resuspended in phosphate-buffered saline solution (PBS, pH 7.4) and washed, stirring gently. The cells then were centrifuged at 600 x $g$, and the washing steps were repeated two times. The final cell concentration was adjusted to 2.0 x $10^8$ cells per ml of red blood cell (RBC) solution in PBS containing 3 mM $NaN_3$, and the resulting solution was stored

at 4°C.

**Hemolysis assay**

The assay was performed as a modified titration method of Bhakdi *et al.* (2).

Briefly, samples were diluted with distilled water and 0.5-ml aliquots of each diluted

sample were added into 0.5 ml of 20 mM dithiothreitol in PBS solution and incubated

for 10 min in a 37°C water bath to activate the listeriolysin O. One ml of sheep red

blood cell suspension was added and the mixture was incubated for 60 min at 37°C.

After hemolysis, the mixture was diluted to 1:1 with PBS solution and resuspended with

a vortex mixer for even turbidity of the solution. The turbidity was detected spectropho-

tometrically at 650 nm by using a Spectro 20 spectrophotometer (Bausch & Lomb

Analytical System Div., Rochester, NY), and the hemolytic activities were calculated as:

$$\text{Hemolysis \%} = (1 - \frac{OD_s}{OD_t}) \times 100$$

$OD_s$ : difference of optical density at 650 nm between sample and

100% hemolyzed RBC solution

$OD_t$ : difference of optical density at 650 nm between nonhemolyzed and

100% hemolyzed RBC solution

When the RBC solution was not hemolyzed at all, the OD value was $0.62 \pm 0.02$.

Therefore, the dilution ratio of sample solution required for 50% hemolysis of RBC was

that which resulted in an OD value of $0.31 \pm 0.02$.

**Heat-shock treatment of *Listeria***

At the stationary phase, $3.5 \times 10^9$ *Listeria* cells per ml of culture broth were heat-shocked by exposing the cells to 48°C for 2 h and then cooling them to room temperature in an ice bath (7). BHI broth was then added into the heat-shocked culture (1:1) and the cells were incubated at 37°C for 4, 8, or 10.5 h. Each sample was assayed for hemolysis, and plate counts were done on appropriate agar as described previously.

**Heat treatment of listeriolysin O**

One ml of crude listeriolysin O solution was heat treated at 48, 55, or 62°C for various periods of time in a water bath. After heat treatment, each sample was immediately cooled in an ice bath, and the hemolysin activity was assayed as described previously.

# RESULTS AND DISCUSSION

## Effect of pH on production of LLO

*Listeria monocytogenes* serotype 1 and Scott A may have different physiological

characteristics because they have been isolated from different sources. Serotype 1 has

been isolated predominantly from meat products and Scott A has been isolated mainly

from dairy products (8). In this research, we examined whether the serotype responded

differently to the pH needed for optimum production of LLO in BHI media. We found

that the hemolytic activity of serotype 1 was greater than that of Scott A (TABLE 1)

since a dilution about 20 times greater than that of the Scott A sample was required to

achieve the same amount of activity. However, the range of pH required for optimum

LLO production by serotype 1 and Scott A was about pH 6.0-6.2 and pH 5.8-6.0,

respectively (Figure 1). However, at pH 7.0, which is an optimum condition for *Listeria*

growth, the production of LLO decreased In both strains.

## Effect of culture method on production of LLO

Some researchers have used 0.5 % glucose as a supplement in BHI for *Listeria*

growth (17, 18). In these studies, the effect of glucose on growth of this organism was

tested to determine the relationship between cell growth and LLO production. Glucose

was added into BHI media at 0.5, 1.0, or 1.5% in a single addition procedure. Addition-

ally, a repeat fed-batch method was used for more effective recovery of LLO, in which

glucose was added after the stationary phase was reached and the pH was adjusted to

various specific levels. In this procedure, 0.5% glucose was added three times
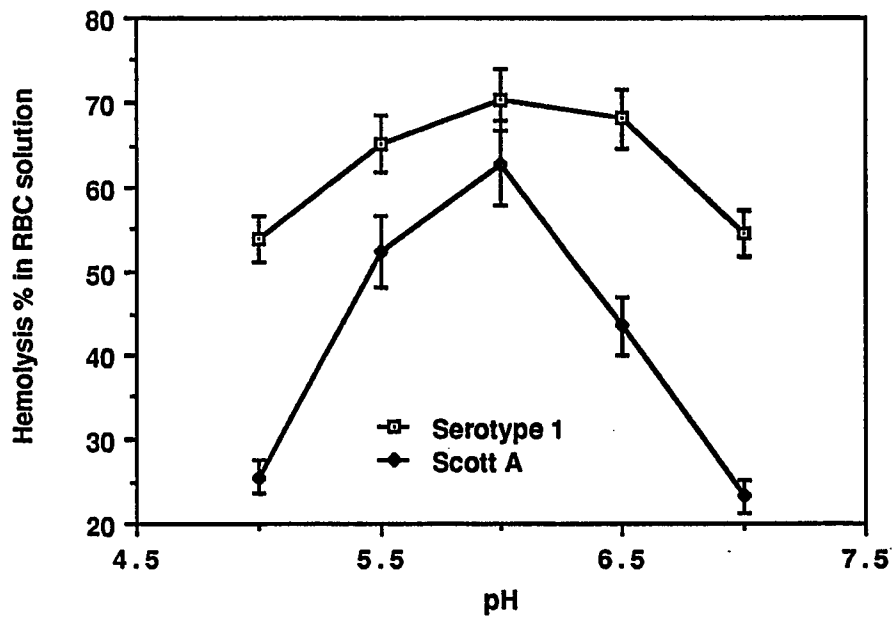
Figure 1. Effect of pH on LLO hemolytic activity of *Listeria monocytogenes* serotype 1 and Scott A. (Hemolytic activity of LLO samples produced culture after incubation for 4 h at 37°C at the stationary phase. D.I.; serotype 1; 360, Scott A;16)

TABLE 1. Comparison of LLO hemolytic activity between *Listeria monocytogenes* serotype 1 and Scott A strains

| Hemolysis % in RBC solution[1] | | | |
|---|---|---|---|
| D.I.[2] | Serotype 1 | D.I. | Scott A |
| 160 | $98.7 \pm 0.5$[3] | 8 | 100.0 |
| 320 | $70.3 \pm 3.1$ | 16 | $62.8 \pm 4.6$ |
| 640 | $26.8 \pm 3.9$ | 32 | $26.2 \pm 5.1$ |

[1] Hemolytic activity of LLO samples produced in BHI culture after 4 h incubation at pH 6.0.

[2] Dilution index : reciprocal of dilution ratio.

[3] Average hemolysis % of RBC of three trials ± standard deviation.incubation period by

throughout the incubation period by each of 3 methods. As expected, an increase in

glucose concentration resulted in an increase in cell growth (Figure 2); it also resulted

in a decrease in LLO activity by these cells (TABLE 2). The reduction may have

occurred because of a negative feedback mechanism, in which higher levels of glucose

caused inhibition of LLO synthesis by the cells. In the repeat fed-batch procedure,

about a two-fold increase in the concentration of LLO was seen through the three

stages, compared with the single addition procedure. However, the LLO was produced

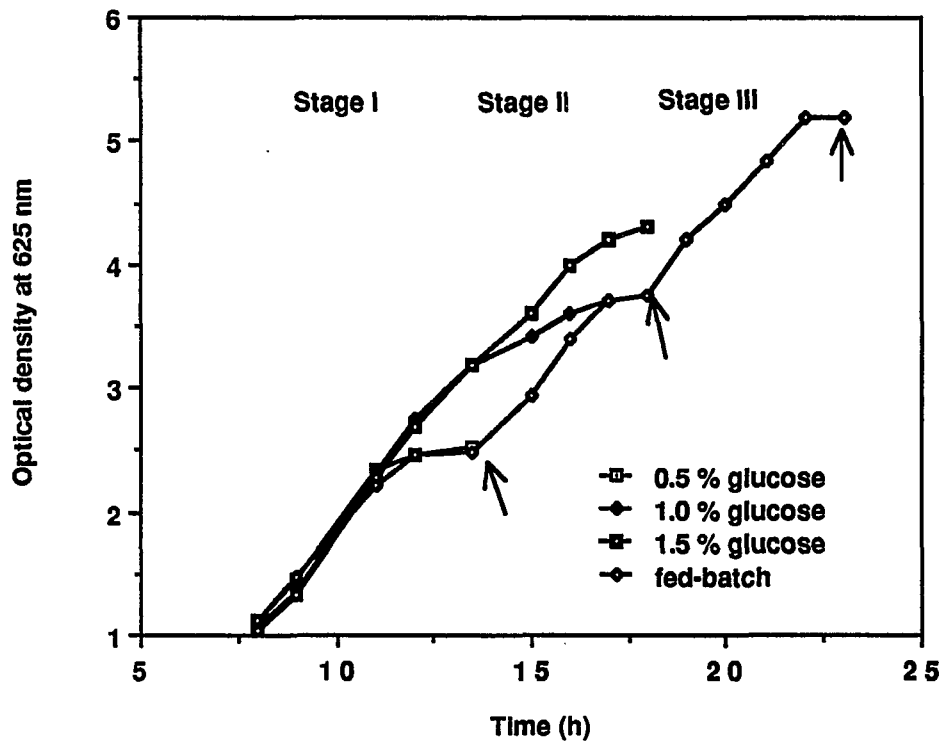more effectively at stage II than at any other stages of glucose feeding (TABLE 3).

Figure 2. Growth curves of *Listeria monocytogenes* Scott A in BHI broth grown with 0.5%, 1.0%, or 1.5% added glucose, or by fed-batch method; In the fed-batch procedure, the arrows indicate point of addition of 0.5% glucose and the pH was adjusted to 7.0 whenever it dropped to 5.5

TABLE 2. Effect of culture method on production of LLO by *Listeria monocytogenes* Scott A

| D.I.[2] | Hemolysis % in RBC solution[1] | | | |
|---|---|---|---|---|
| | 0.5% glucose | 1.0% glucose | 1.5% glucose | fed-batch[3] |
| 5 | 97.9 ± 2.0[4] | 93.6 ± 2.1 | 93.5 ± 0.8 | 98.4 ± 1.6 |
| 10 | 50.0 ± 4.0 | 38.4 ± 5.9 | 27.4 ± 1.9 | 98.9 ± 1.2 |
| 20 | 19.9 ± 4.1 | 15.1 ± 2.5 | 8.1 ± 3.2 | 59.7 ± 4.8 |

[1] Hemolytic activity of LLO samples produced in culture after incubation for 4 h at 37°C at the stationary phase.
[2] Dilution index : reciprocal of dilution ratio.
[3] Repeat fed-batch : pH of the culture was adjusted to 7.0 whenever it dropped to 5.5.
[4] Average hemolysis % of RBC of three trials ± standard deviation.

**Effect of pH control on LLO production in fed-batch procedure**

To more effectively recover LLO by neutralization of acidic conditions during growth, three methods were used. Method 1 was set up for both cell growth and LLO production; method 2 was set up only for more production of LLO but less cell growth than method 1, and method 3 was set up just for production of LLO without growth of cells. The growth rate of *Listeria* in method 2 was slower than that of method 1 (Figure 3). Hemolytic activity of LLO was detected with greater numbers of *Listeria* regardless of the methods used (TABLE 3). Production of LLO was affected more by the number of cells in culture than by the pH of the culture. The cell numbers in the early stage of method 1 were as high as the numbers at the last stage of method 2 (9.8 Log[CFU/ml]).
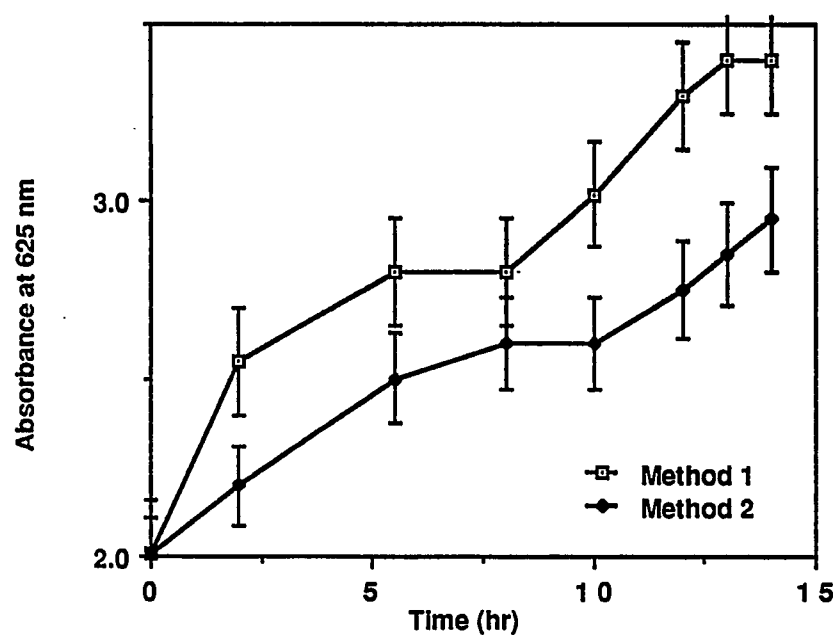
Figure 3. Growth curves of method 1 and method 2 in stage II (pH of culture; method 1; pH 5.5 ~ pH 7.0, with 0.5% glucose, method 2; pH 5.5 ~ pH 6.3, with 0.5% glucose)

TABLE 3. Effect of pH on production of LLO by *Listeria monocytogenes* Scott A in fed-batch culture

| Stage[1] | D.I.[2] | Hemolysis % in RBC solution | | | |
|---|---|---|---|---|---|
| | | Control[3] | Method 1[4] | Method 2[5] | Method 3[6] |
| I | 16 | 80.8 ± 2.0[7] | 80.8 ± 2.0 | 80.8 ± 2.0 | 80.8 ± 2.0 |
| | 32 | 12.3 ± 1.5 | 12.3 ± 1.5 | 12.3 ± 1.5 | 12.3 ± 1.5 |
| II | 16 | 69.0 ± 2.7 | 94.5 ± 0.6 | 96.7 ± 1.6 | 95.1 ± 1.6 |
| | 32 | -0.5 ± 1.8 | 57.2 ± 1.6 | 45.4 ± 2.0 | 31.0 ± 3.1 |
| III | 16 | ND | 96.0 ± 0.5 | 96.4 ± 0.3 | 90.0 ± 0.8 |
| | 32 | ND | 54.6 ± 3.5 | 56.7 ± 1.6 | 18.7 ± 2.5 |

[1] Point during growth curve at which the culture had reached stationary phase and at which glucose was added.
[2] Dilution index : reciprocal of dilution ratio.
[3] Sample of the stationary phase of Stage I, pH 7.0.
[4] pH of culture : pH 5.5 ~ pH 7.0, 0.5% glucose.
[5] pH of culture : pH 5.5 ~ pH 6.3, 0.5% glucose.
[6] pH of culture : pH 6.0, no addition of glucose.
[7] Average hemolysis % of RBC of three trials ± standard deviation.

Therefore, method 1 was more effective than method 2 in production of LLO because, even though the hemolysis activity between the two methods was not significantly different, a shorter incubation time was required for production of the same amount of LLO by method 1 than by method 2 (TABLE 3). In both the control in cells exposed to

method 3, the cells did not grow. However, in method 3, a small amount of LLO was produced.

### Effect of culture time on LLO production in repeat fed-batch procedure

Njoku-Obi *et al.* (17) reported that productivity of LLO varies depending on culture time and *Listeria* species. In general, productivity of LLO is greater at the stationary phase, but in some *Listeria* species, these investigators showed that the hemolytic activity decreases during the stationary phase because of instability of this protein, caused by oxidation during incubation of the culture. In our studies, serotype 1 and Scott A were used to detect the pattern of LLO production in BHI broth with 0.5% glucose. Figure 4 shows that, although the growth curves were different between the two strains, the maximum hemolytic activity occurred after 2 h at stage II regardless of serotype. When glucose was not added to the culture, cell growth did not occur but maximum hemolytic activity occurred after 6 h at stationary phase and, after that, the activity decreased (TABLE 4). Jenkins *et al.* reported that LLO, which contains -S-S-linkages, is deactivated by oxidizing agents (10). It is possible that LLO was affected by metabolites such as acids present during cell growth or by oxygen. The pH of the culture dropped below 4.8 during incubation, which may also have affected LLO production.

### Effect of heat shock on LLO production

After heat shock in trypticase soy broth with 0.6% yeast extract (TSBYE) at the stationary phase, cell growth and LLO production were determined. Figure 5 shows
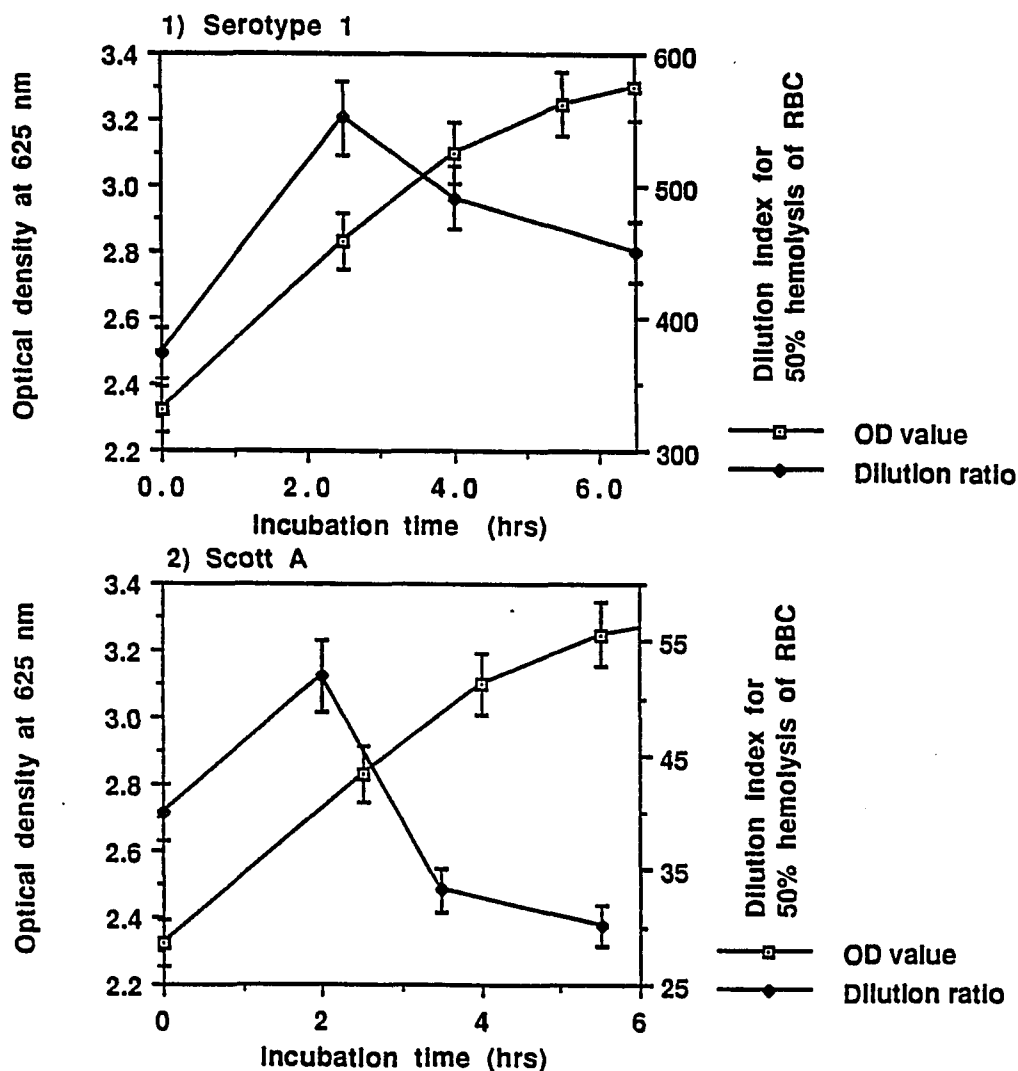
Figure 4. Effect of incubation time on production of LLO during growth of *Listeria monocytogenes* at stage II of fed-batch procedure. The pH was adjusted to 7.0 whenever it dropped to 5.5. Dilution index indicates the reciprocal of dilution ratio needed for LLO to hemolyze 50% of RBC solution (see 'Materials and Methods'.)
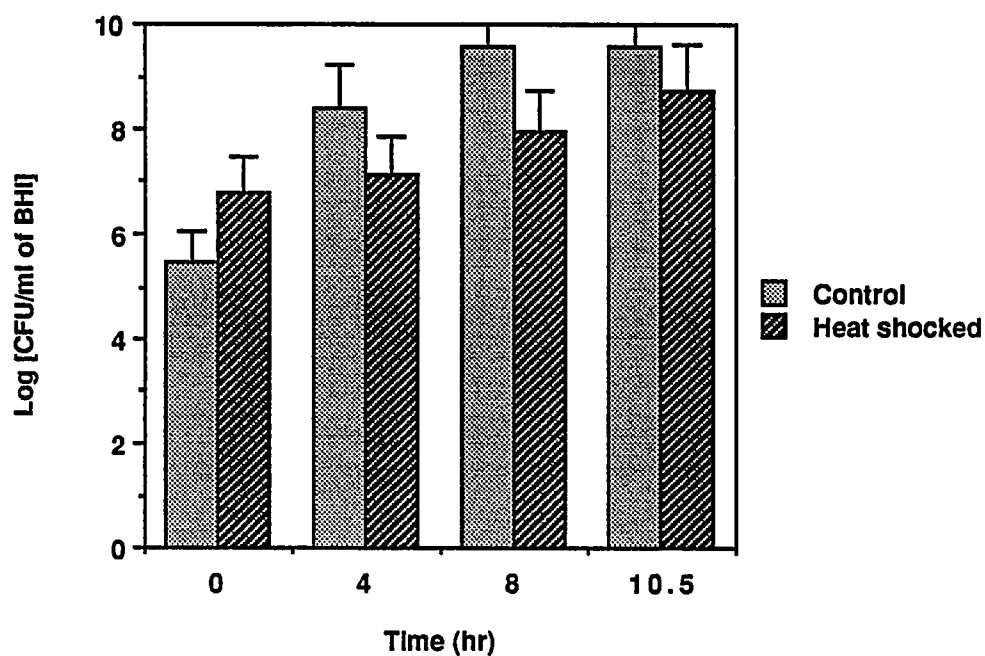
Figure 5. Growth of heat-shocked *Listeria monocytogenes* serotype 1 after dilution (1:1) with BHI broth. Both heat shocked and nonheat-shocked *Listeria* (control) cells were heat-treated at 62°C for 10 min. Total numbers of survivors and healthy cells were enumerated by plating onto nonselective media (TSA + 0.6% yeast extract)

TABLE 4. Hemolytic activity of listeriolysin O during the incubation time at stage II
without addition of glucose

| Incubation time(hr)[1] | Hemolytic activity(%)[2] |
|---|---|
| 0 | 65.5 ± 5.2 |
| 2 | 74.4 ± 8.1 |
| 4 | 88.2 ± 4.4 |
| 6 | 95.1 ± 4.2 |
| 8 | 83.8 ± 3.6 |

[1] Incubation time during stage II.
[2] Dilution index : 160

that the number of survivors after a 62°C heat treatment for 10 min was significantly

higher in heat-shocked *Listeria* immediately after heating (time 0). However, incubation

at 37°C resulted in an increase in the number of healthy cells, with control cells

recovering faster than heat-shocked cells. Immediately after heat shock, the hemolytic

activity of LLO was not detected even in undiluted culture sample, regardless of

whether the cells were heated at 62°C or not (TABLE 5). This may be because of a

decrease in the production of LLO as a result of heat shock, or because of inactivation

of LLO by the heat shock treatment. McCarthy (15) showed that heat-stressed cells

were less pathogenic at $10^4$ cells per immunocompromised mouse than resuscitated

cells. Within 4 h of incubation after heat shock, the production rate of LLO by

TABLE 5. Effect of heat-shock treatment on production of LLO by *Listeria monocytogenes* serotype 1

| Time[2] (h) | Control | | | | Heat shocked[1] | | | |
|---|---|---|---|---|---|---|---|---|
| | Not heated | | heated[3] | | Not heated | | heated | |
| | D.I.[4] | Hemolysis%[5] | D.I. | Hemolysis% | D.I. | Hemolysis% | D.I. | Hemolysis% |
| 0 | 200 | $36.5 \pm 9.7$ | 1 | ND[6] | 1 | ND | 1 | ND |
| 4 | 400 | $46.8 \pm 4.4$ | 10 | $17.4 \pm 7.3$ | 40 | $62.7 \pm 3.2$ | 1 | ND |
| 8 | - | | 20 | $59.1 \pm 4.4$ | 80 | $64.2 \pm 3.1$ | 1 | $28.1 \pm 1.8$ |
| 10.5 | - | | - | | 80 | $59.1 \pm 4.4$ | 8 | $27.5 \pm 2.7$ |

[1] Heat shocked at 48°C for 2 h in BHI broth.
[2] Cells incubated at 37°C after a heat treatment.
[3] Cells heated at 62°C for 10 min.
[4] Dilution index: reciprocal of dilution ratio.
[5] Hemolytic activity of LLO. See 'Materials and Methods'.
[6] Not detected.

heat-shocked cells without heat treatment increased more than 40 fold over the initial hemolytic activity, in comparison with a two-fold rate increase for the nonheat-shocked controls (TABLE 5). These results agreewith those of Sokolovic (22) who reported that heat shocking induced synthesis of LLO in mutants. However, the hemolytic activity of control cells was higher than that of heat-shocked cells before and after heating, and throughout the time of incubation at 37°C. This delay in the synthesis of LLO by heat-shocked cells compared with controls was probably due to the fact that heat shock itself results in injury of cells even before heating at 62°C.

**Heat stability of LLO**

LLO is a heat-labile protein (17). To determine its heat stability, a concentrated LLO solution, which is able to hemolyze 50 % of RBC at 1:1,600 dilution, was used. According to Farber *et al.* (7), the heat resistance of *Listeria* has been known to be maximized after a heat shock at 48°C for 2 h, and Sokolovic *et al.* showed that listeriolysin is still synthesized by *Listeria*, even intracellularly, under heat-shock conditions. According to Figure 6, LLO was deactivated rapidly after heating. When the sample was incubated at 48°C for 2 h, the hemolytic activity of the culture was not detected even without dilution.

**Summary**

*Listeria monocytogenes* serotype 1 produced more LLO than Scott A. The pH range of the culture for maximum production of LLO by *L. monocytogenes* serotype 1 and Scott A was determined to be pH 5.8-6.0 and pH 6.0-6.2. The highest amount of LLO was obtained by using the repeat fed-batch procedure where 0.5% glucose was added than by using the single addition of any concentration of glucose. In the repeat fed-batch procedure, the optimum conditions for production of LLO were determined when the pH of the culture was readjusted to between 5.5 and 7.0 at stage II using method 1. The final, concentrated, crude LLO solution was able to hemolyze 50% of RBC solution at a 1:1,600 dilution ratio.

In food products, the productivity of LLO could vary under different conditions. From the results, although the environmental conditions may be optimum for growth of the cells, they may not be ideal for production of LLO. Future studies need to be
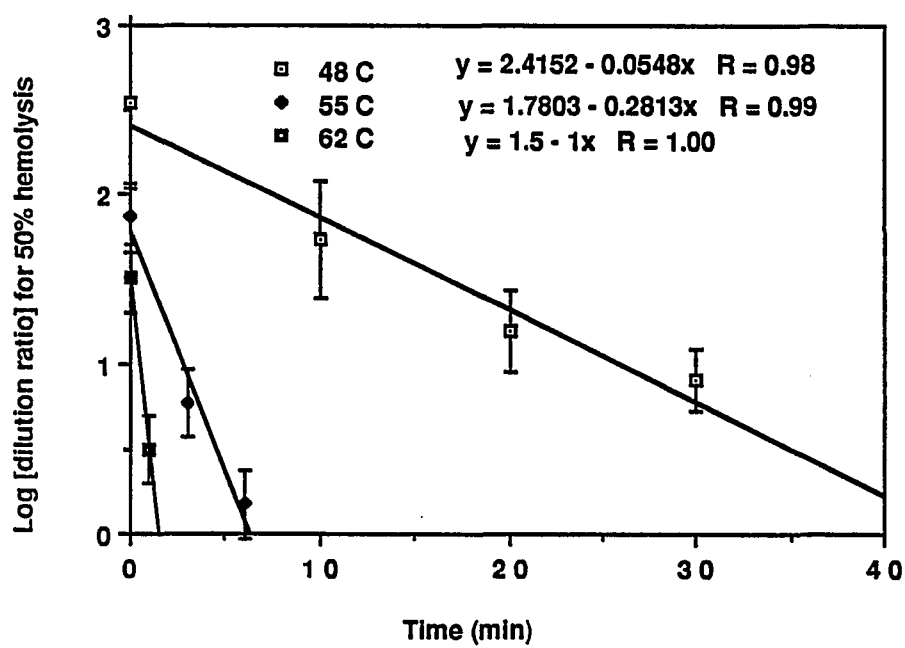
Figure 6. Effect of heat on stability of listeriolysin O at 48, 55, and 62°C

conducted on the effect of other growth parameters on the production of LLO by this organism, especially, to determine whether pH changes above 7.0 affect synthesis of this protein.

Exposure of *Listeria* to a stress such as heat shock during processing can result in an increase in the number of survivors to a subsequent heat treatment (14). This has serious implications for the industry and methods of processing must be designed in such a way as to minimize the possibility of this occurring. Heat shocking also seems to induce synthesis of LLO. However, this induction does not appear to increase the virulence of the organism, since the levels of LLO never exceeded those of nonheat-shocked controls. Christman *et al.* (4) found that synthesis of heat-shock proteins by *Salmonella typhimurium* is necessary for virulence factors to develop. However, this does not necessarily mean that virulence of such a pathogen can be enhanced by induction of the heat shock response. More research is needed on the effect of incubation temperature and atmosphere on the ability of heat-shocked cells to recover and synthesize LLO after a heat treatment. In addition, we must determine whether any change in virulence factors determined in vitro can occur in a host system, and whether these changes are transitory or even reversible.

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PART III. DEVELOPMENT OF AN ENZYME-LINKED IMMUNOSORBENT
ASSAY (ELISA) FOR ANALYSIS OF LISTERIOLYSIN O
PRODUCED BY *Listeria monocytogenes*

Development of an Enzyme-Linked Immunosorbent Assay (ELISA) for

Analysis of Listeriolysin O produced by

*Listeria monocytogenes*

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# ABSTRACT

Listeriolysin O (LLO) is a heat-labile hemolysin produced by *Listeria monocytogenes*. Its hemolytic activity has been evaluated qualitatively by sodium dodecyl sulfate (SDS) electrophoresis and immunoblotting. In this experiment, an enzyme-linked immunosorbent assay (ELISA) was developed for quantitative analysis of LLO by using Streptolysin O (SLO) and antistreptolysin O (ASO) as the reagents. The selected coating and blocking buffers were 0.05 M Tris buffer (pH 8.5) and 0.25% casein solution with phosphate-buffered saline solution (PBS) + 0.05% Tween 20, respectively. A relationship between antigen and antibody was achieved with 5 mg/ml ASO and a 1:1,000 dilution of conjugate. The heat stability of LLO at 48, 62, 72, and 80°C was examined by using this method. Although the LLO is inactivated easily at those temperatures, the protein structure was not affected at temperatures lower than 80°C for 3 min.

## INTRODUCTION

Listeriolysin O [LLO, molecular weight, 60,000 (3)] is a water-soluble, heat-labile

protein produced by *Listeria monocytogenes* that possesses cardiotoxic activity. This

protein is activated by sulfhydryl groups such as sodium thiosulfate, and the primary

sequence of the protein is almost identical to that of streptolysin O (SLO) (9), which

means that LLO can be bound by antibodies specific for SLO. Many researchers have

reported that the gene coding for the hemolysin produced by *L. monocytogenes* is at

least partly responsible for the organism's virulence (8, 14). When the bacteria escape

from a host vacuole, this event is mediated partly by the action of LLO, which forms

pores that enable the organism to escape (10). According to Sokolovic *et al.* (14), this

protein seems to be produced even under heat shock conditions and is the only major

extracellular protein produced at this point. To evaluate LLO activity qualitatively,

sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting

using antiLLO antibodies conjugated with an enzyme such as horseradish peroxidase

have been used (12, 14). However, with these methods, it has been difficult to deter-

mine whether LLO is inactivated or actually denatured during a heat treatment such as

heat shock. Enzyme-linked immunosorbent assay (ELISA) is a method that can be

used for the quantitative analysis of protein because it is specific as well as accurate (5,

15). However, it is a procedure that can be expensive inasmuch as it often requires

preparation of antibodies specific for the antigen being studies. SLO reagent and

antiSLO are standardized commercial products that have been used in clinical hemolyt-

ic titrations. As such, they offer an inexpensive and convenient alternative to the

purification of antibody and antigen reagents for the measurement of LLO.

The objectives of this study were to develop an ELISA method for the quantitative measure of LLO by using commercial ASO and SLO reagents and to determine the heat stability of LLO using this method.

## MATERIALS AND METHODS

### Bacterial culture conditions for production of listeriolysin O

*Listeria monocytogenes* serotype 1 (ATCC 19111) was obtained from the American Type Culture Collection in Rockville, MD. One milliliter of the strain was incubated in 500 ml Brain Heart Infusion broth (BHI, Difco Laboratories, Detroit, MI) supplemented with 0.5% glucose at 37°C for 12 h. At the stationary phase of growth, 0.5% glucose was added, and the cells were incubated for another 2 h. During incubation, the pH of the BHI culture with 0.5% glucose was adjusted to 7.0 and readjusted whenever the pH of the culture dropped to 5.5.

### Preparation of crude listeriolysin O

Crude listeriolysin O was prepared by a modified method of Bhakdi *et al.* Bacteria were sedimented in a Beckman J2-21 centrifuge (Beckman Spinco division, Palo Alto, CA) (rotor JA-10, 11,000 x *g*), and the supernatant was filtered through a 0.45-μm cellulose acetate membrane filter (COSTAR, Cambridge, MA). For inhibition of protease, phenylmethylsulfonyl fluoride (SIGMA Chemical Co., St. Louis, MO) was added as a 1 mM solution. Fifty-three grams of ammonium sulfate (Fisher Scientific, Fair Lawn, NJ) were then added per 100 ml of solution to precipitate the proteins, and the sample was stirred in an ice bath for 60 min. The precipitate was collected by centrifuging at 13,000 x *g*, resuspended in distilled water (final volume, 70 to 80 ml), and dialyzed with Spectra/Por membrane tubing (Spectrum Medical Industries Inc., Houston, TX, MWCO : 25,000) overnight against 5 L of 50 mM NaCl-4 mM EDTA in

the cold room. Twenty-five grams of polyethylene glycol (PEG) 4000 (Fisher Scientific, Fair Lawn, NJ) was added to 100 ml of sample solution and stirred at 4°C for 30 min. After centrifugation at 30,000 x $g$ for 60 min, the supernatant was discarded, and the precipitate was resuspended in 50 ml distilled water containing 3 mM $NaN_3$ and stored in the refrigerator until used. The final solution was able to hemolyze 50% of red blood cells (RBC) at 1:1,600 dilution.

## Determination of heat-treated listerlolysin O

One milliliter of crude and concentrated listerlolysin O solution was heat-treated at 48, 62, 72, and 80°C in a water bath for 3 min. The come-up time for each temperature was about 30 sec. The temperature was detected with J type thermocouple (Omega Engineering Inc., Stamford, CT) and datalogger (Model LI-1000, LI-COR, Lincoln, NE). After heat treatment, each sample was immediately cooled in an ice bath and diluted 20 times with 0.05 M Tris buffer (pH 8.5). An equal volume of various concentrations of SLO (10, 5, 2.5, or 0 mg/ml) was added. The overall dilution ratio was 1:40. One-hundred microliter of the mixture was tested for activity by ELISA.

## Indirect ELISA

**Antibody and antigen**     Streptolysin O (SLO, Difco Laboratories, Detroit, MI) and antistreptolysin O (ASO, Difco Laboratories, Detroit, MI) were used as antigen and antibody, respectively. These reagents are shipped in lyophilized desiccated powder form.

**Selection of coating buffer**     Phosphate-buffered saline (pH 7.6) (PBS, Sigma,

SIGMA Chemical Co., St. Louis, MO), 0.05 M Tris buffer (pH 8.5)(Sigma, SIGMA

Chemical Co., St. Louis, MO), and 0.05 M bicarbonate buffer (pH 9.5) (Sigma, SIGMA

Chemical Co., St. Louis, MO) were tested for their effect on binding of LLO onto wells

of an activated microtiter plate (96 wells, Corning Laboratory Sciences Company,

Corning, NY). One-hundred microliter of 5 mg/ml ASO solution was used, and the

dilution ratio of antibody conjugated with enzyme used for this experiment was 1:1,000.

**Selection of blocking buffer**    Point-five percent casein (Sigma Chemical Co., St.

Louis, MO) and bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO)

solution in PBS buffer containing 0.05% Tween 20 (Sigma, SIGMA Chemical Co., St.

Louis, MO) were examined to determine which provided more effective blocking of

unbound antibodies.

**Optimization of concentration of enzyme-conjugated antibody**    Monoclonal

antihuman immunoglobulin ($\gamma$-chain specific) conjugated with GG-5-alkaline phospha-

tase (Sigma Chemical Co., St. Louis, MO) was diluted with PBS buffer containing

0.05% Tween 20 to 1:500, 1:1,000, 1:2,000, 1:3,000, and 1:4,000. One milligram per

milliliter of p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) with 1 M

diethanolamine buffer + 0.5 mM $MgCl_2$ reaction mixture (pH 9.8) was used as the

substrate solution.

**Method**    A modified ELISA was used according to the method of Hornbeck (5). One

hundred microliter of sample or standard solution was added into the microtiter plate,

incubated at 37°C for 30 min, transferred to 4°C and incubated for 16 h. The Antigen-

bound microwell was rinsed three times with deionized distilled water, and the wells

were filled with blocking buffer and incubated for 30 min at room temperature. After the

blocking step, the plate was rinsed three times with deionized distilled water. ASO solution containing PBS and 0.05% Tween 20 was added into each well and the plate was incubated at room temperature for 2 h. After binding of antibody, the plate was rinsed three times in deionized distilled water, the wells filled with blocking buffer, and incubated for 10 min at room temperature. After blocking, the plate was rinsed three times with deionized distilled water. After the final rinse, residual liquid was removed by wrapping the plate in tissue paper and gently tapping it face down onto several paper towels. As the next step, 100 µl of alkaline phosphatase-linked antibody solution (anti-human immunoantibody, SIGMA Chemical Co., St. Louis, MO) was added into each well, and the plate was incubated at room temperature for 2 h. After incubation, the plate was rinsed three times with deionized distilled water, and 100 µl of p-nitrophenyl phosphate as a substrate was added as the substrate. The enzymatic reaction was carried out at 30°C for 30 min and stopped by addition of 50 µl of 3 N NaOH. The optical density was detected at 405 nm by using a Kinetic Microplate Reader spectro-photometer (Molecular Devices, Palo Alto, CA).

## RESULTS AND DISCUSSION

### Selection of coating buffer

In general, aqueous diluents at neutral or alkaline pH of various buffer solution

have been used successfully for ELISA of most proteins, but the coating effect of each

buffer is different according to the protein. For example, Barlough et al. (1) showed that

carbonate buffer (which is the most popular coating buffer for ELISA) used to bind

coronavirus antigens resulted in diffuse and nonspecific staining but that sodium

phosphate buffer, sodium chloride, or distilled water gave excellent results with that

antigen. In our study, PBS buffer (pH 7.5), 50 mM Tris buffer (pH 8.5), and 50 mM

carbonate buffer (pH 9.5) were tested as coating buffers. Figure 1 indicates that Tris

buffer was the most effective because the slope of the line obtained with this buffer was

greater (indicating greater SLO binding) and that the optical density without SLO was

less than with of any other buffers (indicating minimal background). Therefore, we

selected the 50 mM Tris buffer (pH 8.5) as the coating buffer.

### Selection of blocking buffer

A 0.25% BSA solution and a 0.25% casein solution were tested for their ability to

block effectively any residual binding capacity and to prevent nonspecific adsorption

while enhancing specific interaction of antigen and antibody. In this ELISA method, the

blocking step was used twice: the first was after binding of SLO and, the second, after

binding of ASO. TABLE 1 shows that the casein solution was more effective in

blocking than the BSA solution inasmuch as addition of ASO resulted in lower
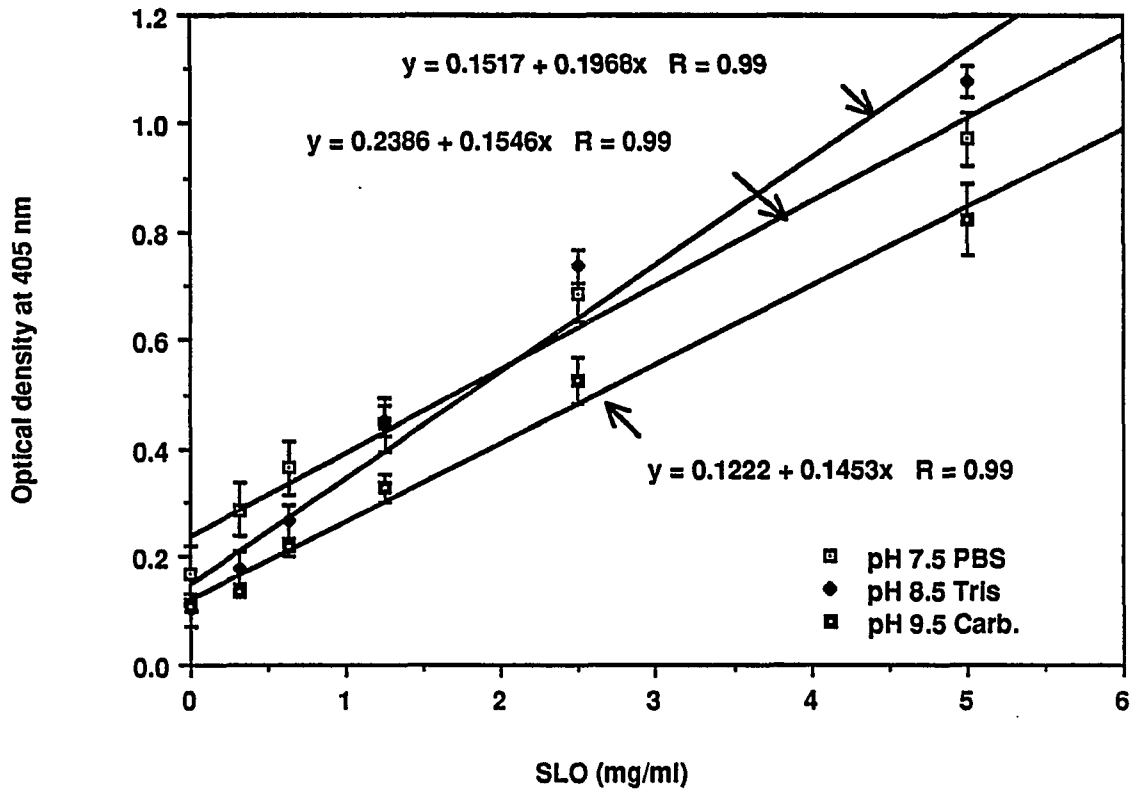
Figure 1. Effect of type of coating buffer on SLO binding (for conditions used, see 'MATERIALS AND METHODS')

TABLE 1. Comparison of BSA and casein solutions for their blocking effect in ELISA[1]

| ASO | Optical density at 405 nm | | | |
| | 0.25 % BSA | | 0.25 % Casein | |
| (mg/ml) | 1st+2nd[2] | 2nd[3] | 1st+2nd | 2nd |
|---|---|---|---|---|
| 5.00 | 0.698 | >3.000 | 0.192 | 2.720 |
| 2.50 | 0.164 | 1.224 | 0.040 | 0.641 |
| 1.25 | 0.031 | 0.120 | 0.008 | 0.022 |
| 0.61 | 0.008 | 0.030 | 0.002 | 0.012 |

[1] The binding step of SLO was omitted in this experiment.
[2] The blocking steps were done twice; before adding of each concentration of ASO (1st step) and after binding of ASO for 2 h (2nd step).
[3] The blocking step was done just after binding of ASO for 2 h (2nd step).

absorbance readings when casein was used as the blocking buffer when compared with BSA.

## Determination of anti-ASO antibody conjugated with alkaline phosphatase by a criss-cross matrix analysis

Serial dilution titration analyses were performed to determine the optimal concentration of enzyme-conjugated antibody that would result in detection of SLO. According to TABLE 2, a ASO dilution of 1:1,000 resulted in the highest optical density reading at 250 μg/ml ASO, and a dilution of 1:500 resulted in highest optical density at 62.5 μg/ml ASO. Because the concentration of ASO used in our experiments was greater than 125 μg/ml, an enzyme dilution rate of 1:1,000 was established. At this dilution, the

TABLE 2. Optimization of anti-ASO antibody concentration (conjugated with alkaline phosphatase) in ELISA

| ASO μg/ml | Optical density at 405 nm | | | | |
|---|---|---|---|---|---|
| | 1:500[1] | 1:1,000 | 1:2,000 | 1:3,000 | 1:4,000 |
| 250 | 2.528 | 1.248 | 0.458 | 0.367 | 0.225 |
| 62.5 | 1.078 | 0.514 | 0.180 | 0.144 | 0.089 |
| 15.6 | 0.055 | 0.021 | 0.004 | 0.012 | 0.005 |
| 3.9 | 0.007 | 0.005 | 0.003 | 0.003 | 0.000 |

[1] Dilution ratio of anti-ASO antibody conjugated with alkaline phosphatase.

enzymatic activity was 630 units/ml (one unit refers to the amount of enzyme necessary to hydrolyze 1.0 μmole of p-nitrophenylphosphate to form p-nitrophenol and inorganic phosphate per min).

**Determination of ASO and SLO concentration for the standard curve**

The standard curves of ASO concentration vs. SLO concentration were plotted by using the optimal enzyme conjugate concentration and appropriate blocking and coating buffer. At 0.63 mg/ml of ASO, the linear relationship was detected at less than 0.63 mg/ml of SLO, and, at 1.25 mg/ml of ASO, it was detected at less than 1.25 mg/ml of SLO. The predicted equations were:

$y = 0.134 + 0.172X$        (R = 1.00) at 5.00 mg/ml of ASO

$y = 0.034 + 0.093X - 0.007X^2$ (R = 1.00) at 2.50 mg/ml of ASO

$y = 0.068 + 0.144X - 0.011X^2$ (R = 1.00) at 1.25 mg/ml of ASO

$y = 0.015 + 0.043X - 0.002X^2$ (R = 1.00) at 0.63 mg/ml of ASO

y: Optical density at 405 nm

X: The concentration of SLO (mg/ml)

Therefore, the same concentration of SLO and ASO (1:1) resulted in a linear

relationship. However, the OD value obtained when less than 1.25 mg/ml of ASO was

used was too low to detect. Figure 2 shows that less than 5.0 mg/ml SLO at 5.0 mg/ml

ASO resulted in a linear relationship. Therefore, a concentration of ASO of 5.0 mg/ml

was selected to be used in determining concentrations of SLO of up to 5.0 mg/ml of

SLO.

**Heat stability of LLO**

After heating of samples, the absorbance at 405 nm was determined by ELISA.

The corresponding concentration of LLO was obtained from the standard curve of

absorbance vs. SLO concentration (Figure 2). Concentrations of LLO in samples

heated at 48 through 72°C showed similar values, whereas samples detected at 80°C

had significantly lower LLO concentration (Table 3).

In addition, the concentrations of LLO of the heat-treated samples were less than

that of the standard curve of 5 mg/ml of ASO, which indicates that there was an
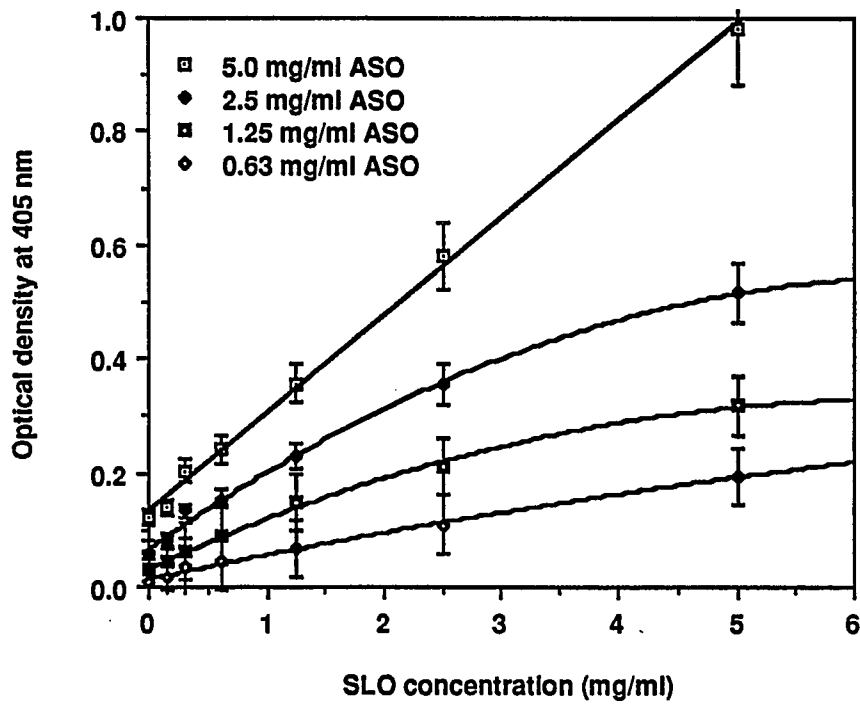
Figure 2. Standard curves of SLO on ASO concentration (The dilution ratio of antiASO antibody conjugated with enzyme was 1:1,000)

TABLE 3. Effect of heat treatment on stability of LLO

| | 25°C | 48°C | 62°C | 72°C | 80°C |
|---|---|---|---|---|---|
| LLO (mg/ml)[1] | $0.18 \pm 0.02$[2] | $0.17 \pm 0.03$ | $0.17 \pm 0.05$ | $0.15 \pm 0.03$ | $0.05 \pm 0.02$ |

[1] Corresponing concentration of LLO was calculated from the standard curve by using SLO (Figure 2).
[2] Average values of three replications $\pm$ standard deviation.

interference effect in the sample solutions against detection of LLO. This interference could be attributable to possible binding of some components onto the wells, essentially competing with antibodies. Also, protein-protein interactions in the absorption process could occur (known as 'protein-stacking'). Because these are not stable, detachment of the protein from the surface could result (7). In this experiment, the interference was defined as:

The interference (%) = 100 - (slope of sample/slope of SLO standard curve)

The interference percentages of the samples heated at 48, 62, and 72°C were calculated to be in the range of 19.8 - 24.8%, whereas the sample heated at 80°C resulted in the least interference % (14.5%). From these data, the inhibitors can be denatured or inactivated by heating to decrease the interference effect.

**Summary**

The optimal conditions for quantitative determination of LLO by an ELISA method were established. The selected coating buffer and blocking buffer were 0.05 M Tris buffer (pH 8.5) and 0.25% casein solution in PBS buffer with 0.05% Tween 20. The optimal dilution ratio of anti ASO antibody conjugated with alkaline phosphatase was 1:1,000, and the optimal concentration of ASO was 5 mg/ml. The amount of LLO in heat-treated samples was analyzed by ELISA using these conditions. According to the results, the structure of LLO was stable at temperatures less than 80°C, at which it is inactivated (11).

In general, evaluation of the hemolytic activity of listeriolysin of *Listeria monocytogenes* is usually determined *in vitro* by the CAMP test in BHI agar by using sheep blood cells for β-hemolysis (4, 13) and by microtitration assays (2). These methods do not offer an accurate measure of LLO produced because conditions such as pH and presence of oxidant often affect them (3, 6). Assays such as ELISA provide a quantitative measure of the production of LLO while not being affected by these factors. In this ELISA, the effective concentration range of SLO was 5 mg/ml through 0.156 mg/ml. The protein composition in SLO was analyzed to be 48% of the total. Therefore, the minimum concentration of SLO that can be detected by this test can be estimated to be less than 0.075 µg/µl. Although ELISA does not measure a hemolytic activity by itself, ELISA coupled to hemolytic assays can provide an indication of activity where the sensitivity or accuracy of hemolytic assays may be questionable.

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## SUMMARY AND CONCLUSIONS

Recently, concerns about the prevalence of *Listeria monocytogenes* in foods have been increasing. Detection of this pathogen in many food products has prompted recalls and has led to staggering financial losses for the industry. According to a survey by Todd (1989), even though the estimated cases of listeriosis were lower in comparison with other pathogens, he estimated that the number of deaths per year due to this organism was higher than by any other pathogen, and that the average cost per case for listeriosis was estimated to be $12,520 in the United States.

In the food industry, the process that is most commonly used to increase food quality and shelf-life is heating. In the heat treatment of foods, such as in precooking of ham or sausage, or in pasteurization of milk, *Listeria* may be exposed to a heat shock during processing and thus may become more heat resistant to a subsequent heat treatment. In addition, the cells may become more virulent, which has serious implications for the industry. It is imperative that processing methods be designed in such a way as to minimize the possibility of this occurring.

Murray and Young (1992) suggested that pathogens can adapt to hostile conditions such as heating through changes in gene expression. In heat processing, a slow rate of heating during come-up time or pre-heating for a short time can cause an increased heat resistance of *L. monocytogenes* because of production of heat shock proteins, and virulence can also be increased because of increased production of listeriolysin. More research is needed on the effect of environmental stresses like heat shock on the survival and virulence of foodborne pathogens. In particular, we must determine whether the increase in virulence factors translates to a host system, and whether this

increase in virulence is transitory or even reversible.

In these studies, the conclusions are:

1) The heat resistance of L. monocytogenes can vary according to subspecies of the organism, growth state, packaging atmosphere, storage time of sample, and heating rate during the heat treatment. In the case of ground pork, serotype 1 was more resistant than Scott A, and heat-shocking of both subspecies increased the ability of the cells to survive a heat treatment at 62°C in ground pork compared with nonheat-shocked cells. Heating of Listeria in anaerobic conditions resulted in lower number of survivors compared to cells heated in aerobic packaging in ground pork. The rate of heating is also a very important factor in heat resistance of this organism. According to results presented here, a heat rate of 1.3 °C/min resulted in a significant increase in survival of cells exposed to a heat treatment compared with cells heated at a rate of 8.0 °C/min. Therefore, a shorter preheating time during heat treatments such as pasteurization should be required to minimize the heat resistance of pathogens as well as to increase food quality.

During storage of the ground pork, the heat-injured L. monocytogenes cells were recovered more rapidly in vacuum packaging although the total number of cells was lower than control in aerobically packaged ground pork. In addition, the number of cells after the maximum growth in vacuum-packaged ground pork decreased slower than pork packaged aerobically. Some antioxidants possess antimicrobial activity, while primarily used to prevent oxidation of fats. Payne et al. (1989) indicated that the antimicrobial effect also varied according to type of antioxidant and concentration. However, from these studies, antioxidant at the allowed concentration by FDA did not

affect significantly the growth of *Listeria* during storage at 7 and 30°C.

2) In designing a heating process for food products information about the heat resistance of pathogens such as *Listeria* should be considered. Various growth conditions and methods were studied to determine the optimal conditions for maximum production of LLO by *L. monocytogenes*. The maximum amount of LLO was obtained by *L. monocytogenes* serotype 1 by using the fed-batch method with BHI+0.5% glucose. The pH range of the culture was pH 5.5 through 6.5. The final, concentrated, crude LLO solution was able to hemolyze 50% of RBC solution at 1:1,600 dilution ratio. In food products, the productivity of LLO could be vary under different conditions. From these results, environmental conditions that are optimum for cell growth and reproduction may not be optimal for LLO production.

Even though the concentrated, crude LLO solution was able to hemolyze 50% of RBC solution at a 1:1,600 dilution ratio, LLO was not stable at temperatures greater than 62°C, and LLO was easily deactivated at 48°C. However, it was produced by *Listeria* cells exposed to 48°C for 2 hrs at a faster rate than by nonheat-shocked cells, even after a heat treatment. However, the total activity of LLO was higher in nonheat-shocked cells than heat-shocked cells.

3) To evaluate the virulence of heat-shocked cells, the hemolytic activity of *L. monocytogenes* is usually determined *in vitro* by the CAMP test in BHI agar by using sheep blood cells for β-hemolysis (Groves and Welshiemer, 1977, and Skalka *et al.*, 1982) or by microtitration assays (Bhakdi *et al.* 1984). But, both methods do not offer an accurate measure of hemolysin produced because conditions such as pH and

presence of oxidant often affect them (Geoffioy *et al.*, 1987 and Jenkins *et al.*, 1964). Therefore, quantitative analysis methods as well as qualitative methods should be performed. Assays such as ELISA provide a quantitative measure of the production of LLO while not being affected by these factors. In this study, the optimal conditions for quantitative determination of LLO by an ELISA method were established. The selected coating buffer and blocking buffer were 0.05 M Tris buffer (pH 8.5) and 0.25% casein solution in PBS buffer with 0.05% Tween 20. The optimal dilution ratio of anti ASO antibody conjugated with alkaline phosphatase was 1:1,000, and the optimal concentration of ASO was 5 mg/ml. In this ELISA, the effective concentration range of SLO was 5 mg/ml through 0.156 mg/ml. The protein composition in SLO was analyzed to be 48% of the total. Therefore, the minimum concentration of SLO that can be detected by this test can be estimated to be less than 0.075 $\mu g/\mu l$. By using this ELISA, the structure of LLO appeared to be stable at temperatures less than 80°C, at which it is inactivated. Although ELISA does not measure hemolytic activity by itself, ELISA coupled to hemolytic assays can provide an indication of activity where the sensitivity or accuracy of hemolytic assays may be questionable.

Listeriosis can not be prevented solely by improved hygienic practices because of the ubiquitousness of the organism. However, it is possible to stop the reproduction of *L. monocytogenes* during and after food processing if we apply an appropriate heating process. In heat processing, a number of cells undergo injury when they are subjected to the stress of heating and the effect can vary according to heating conditions. More intense heating conditions should be used in fresh pork than in old pork due to the ability of *Listeria* to survive in the former. Faster heating rates In come-up time and

vacuum packaging should required for more effective pasteurization. In particular, slow heating and pre-heating can cause the increase of thermotolerance of *Listeria* cells and thus increase the number of survivors during subsequent heat treatment. Moreover, heat shocked *L. monocytogenes* can be more virulent because it can produce listeriolysin at a faster rate than nonheat-shocked cells. In addition, the importance of microbial injury to the safety of food products should not be underestimated. During storage of processed products containing ground pork, injured *Listeria* cells can recover more rapidly in vacuum packaging conditions, but storage at low temperature and the use of appropriate concentration of antioxidants can inhibit recovery and growth of cells.

For further research, we need to study the combined effect of meat age, vacuum packaging, and heating rate during processing, which may be the most effective way to destroy *L. monocytogenes* in meat products.

In addition, basically, little is known concerning the underlying mechanisms involved in the injury and recovery processes nor is there much information concerning the nutritional and physical conditions necessary for recovery of injured cells and more information is needed concerning the minimum temperature for recovery because this organism is found in refrigerated foods. Considering the importance of *L. monocytogenes* today, the heat resistance and recovery during meat processing is a problem that warrants further study.

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