Behavior of *Listeria monocytogenes* in the Presence of *Streptococcus lactis* in a Medium with Internal pH Control

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ABSTRACT

Growth of *Listeria monocytogenes* strains V7, Scott A, and California (initial inoculum 10²/ml) at 21 or 30°C in the presence of *Streptococcus lactis* (initial inoculum 0.25 or 1.0%) was determined using a medium with internal pH control (IPCM-1). The pH of the uninoculated medium (control) was 7.0 before and after incubation. Populations of *L. monocytogenes* in IPCM-1 without *S. lactis* after 30 h at 21°C were ca. 10⁷/ml for strains V7 and Scott A and ca. 10⁶/ml for strain California, and at 30°C they were ca. 10⁸/ml for all three strains. When data were plotted, areas of graphs between curves representing controls and treatments were calculated to quantitate the extent of inhibition of *L. monocytogenes* caused by *S. lactis*. Each such area is called the "area of inhibition" (AI). Growth of the pathogen was inhibited by *S. lactis*; the degree was dependent on temperature and concentration of lactic culture and, in some instances, strain of *Listeria*. Greatest inhibition of each strain occurred with the largest inoculum of *S. lactis* and at the highest temperature. No significant difference (p>0.05) in AI or pH among the three strains was observed at 21°C. At 30°C, strain California was inhibited significantly more (p<0.05) than V7 or Scott A by both concentrations of *S. lactis* at 24 and 30 h of incubation. No significant difference (p>0.05) in AI or pH was found at this temperature regardless of concentration of *S. lactis* or strain of *Listeria*. IPCM-1 inoculated with a lactic starter culture is ready for use at pH 5.5 after 15-18 h of incubation. Inhibition of *Listeria* was not complete at this pH under any of the experimental conditions. Substantial numbers of *L. monocytogenes* (10⁷-10⁹ CFU/ml) were present when this medium was ready for use to produce cultured dairy foods.

Conventional bulk starter preparation methods involve addition of lactic starter bacteria to milk or milk-based media which are incubated (6). Milk buffers some of the acid produced by the fermenting organisms, but as their numbers increase and they continue to produce acid, the buffering capacity is overcome. As the pH drops, acidic conditions become unfavorable for continued growth of the starter bacteria, acid production stops, and cells of the starter may be damaged. For *Streptococcus lactis*, two mechanisms may cause the damage (9). The low pH may directly inactivate several enzymes, or it may cause loss of control of the rates at which individual enzymes are synthesized. When bulk starter containing damaged cells is used to prepare fermented dairy products, the amount used may need to be increased so the quantity of lactic acid formed in a given time is comparable to that produced by healthy cells (23).

Control of pH when preparing bulk starter is not a new concept. Over the past 10 years, it has become a standard procedure in the industry for growing bulk starter (23). There are two methods to control pH of bulk starter when it is being prepared: external and internal pH control. External control methods maintain pH by automatic or manual addition of neutralizer during the fermentation process (14). The present study focused on the internal method for pH control of bulk starter cultures. This type of medium is usually a blend of whey, autoalyzed yeast, and chemical buffers that provide internal control of acidity (15). The buffer salts are insalable at pH values near 7.0, but as the pH drops, buffers are gradually and continuously solubilized to neutralize the acid.

This type of control maintains the pH of the medium between 5.1-5.2, thus allowing maximum starter growth with minimal acid damage to cells.

Bulk starter prepared with this type of medium contains approximately 10 times more cells per unit volume than does the conventional culture (23). Consequently, inoculum rates can be reduced because maintenance of optimum pH results in a very active culture. It is not uncommon for inoculum rates to be reduced by one-half compared to the amount of conventional culture that is used.

The primary function of starters is to ferment lactose of the milk into lactic acid. The decreased pH promotes syneresis of cheese curd while inhibiting growth of spoilage or pathogenic microorganisms (11). It is well known that certain starter cultures are markedly antagonistic to various foodborne pathogens and food spoilage organisms (22). Thus, many fermented dairy products have commonly been considered as free of pathogens. Some starter cultures were inhibitory to a limited number of pathogens in studies where the pH of the growth medium was controlled. *Salmonella* and *Staphylococcus aureus* grew in neutralized media, but inhibition of the pathogens occurred in the presence of lactic starter cultures (8,16). To date no research has examined the behavior of *Listeria monocytogenes* in media in which the pH is controlled.

The overall objective of this project is to develop additional information which will be useful in improving the
safety of cheese and other cultured dairy foods. The present study sought to answer the question, "how will Listeria monocytogenes behave if present when a starter culture is prepared using a bulk starter medium with internal pH control?"

MATERIALS AND METHODS

Preparation of cultures

Three strains of L. monocytogenes were used in this study: V7 (milk isolate, serotype 1), Scott A (SA) (clinical isolate, serotype 4b), and California (CA) (serotype 4b, isolated from Mexican-style cheese implicated in a 1985 outbreak of listeriosis in California). All cultures were maintained in tryptose agar (TA) (Difco Laboratories, Detroit, MI), stored at 7°C and transferred bimonthly. To begin an experiment, inoculum from the stock culture was transferred to duplicate tubes of tryptose broth (TB) (Difco) and incubated in air at 35°C for 24 h. A second transfer was made to duplicate tubes of TB which were incubated under the same conditions. Inocula of 0.05 ml each from the second set of TB tubes were added to duplicate 500-ml screw cap Erlenmeyer flasks each containing 200 ml of sterile skim milk (autoclaved at 121°C for 17 min) which were then incubated at 35°C for 24 h. Each of a second set of sterile skim milk samples was inoculated with 0.05 ml of the first milk culture and incubated at 35°C for 48 h. These transfers were made to obtain a consistent L. monocytogenes population of ca. 10^6 CFU/ml in milk which was used to inoculate test samples.

Frozen S. lactis culture was obtained from Chr. Hansen’s Laboratories (Milwaukee, WI). The culture was thawed and a series of 0.01 ml transfers was made into sterile freezer vials each containing 1.0 ml of sterile skim milk. Vials containing the culture were stored at -84°C until time of use. A flask containing 200 ml of sterile skim milk was inoculated with 0.1 ml of the thawed culture and incubated for 18 h. This served as inoculum for test samples.

Preparation of internal pH control medium (IPCM-1)

IPCM-1 (Galloway West, Fond du Lac, WI) (15.0 g) was added to a 500-ml Erlenmeyer flask containing 200 ml of cold tap water (21°C) and stirred for approximately 5 min. The pH of each sample was measured with a pH meter (Fisher Accumet model 825MP, Springfield, NJ) equipped with an Orion gel-filled combination electrode. Flasks containing the medium were steam-steriled at 121°C for 45 min and then placed in a cold water bath (15°C) for 20 min. After cooling, the medium was tempered at 21 or 30°C for approximately 18 h.

Inoculation, sampling, and pH determination

Duplicate samples of IPCM-1 were inoculated with L. monocytogenes (strains V7, SA, or CA) to yield an initial population of ca. 10^6 CFU/ml and with 0.25 or 1.0% (v/v) S. lactis controls were included which contained Listeria alone, S. lactis alone, or were uninoculated. Samples were incubated at 21 or 30°C for 30 h. Those incubated at 21°C were sampled at 0, 3, 6, 12, 18, 24, and 30 h, whereas those incubated at 30°C were sampled at 0.3, 6, 9, 12, 18, 24, and 30 h. Five ml from each test flask were removed aseptically and pH was determined at 6-h intervals.

Enumeration of L. monocytogenes and S. lactis

One-ml samples were diluted in sterile aqueous 0.5% peptone (Difco) solution, if necessary. Samples were surface-plated onto McBride’s listeria agar (MLA) (Difco) with 0.5% added lithium chloride for enumeration of L. monocytogenes in the presence of S. lactis. For controls containing Listeria alone, samples were surface-plated in duplicate onto TA. Both MLA and TA plates were incubated in air at 35°C for 48 h. To enumerate S. lactis, samples were surface-plated in duplicate in air at 35°C for 48 h. To enumerate S. lactis, samples were surface-plated in duplicate on APT agar (Difco). These plates were incubated at 25°C for 48 h and then colonies were counted using a Darkfield Quebec Colony Counter (American Optical Corp., Buffalo, NY).

Calculation of area of inhibition (AI)

The following formula was used to calculate the area of inhibition at each hour of incubation (Fig. 1):

\[ \text{Area of Inhibition} = \frac{(t_2 - t_1) / 2 \times [(d + c) - (b + a)]}{(t_2 - t_1)} \]

where:

- \( t_2 \) = hour at time 2
- \( t_1 \) = hour at time 1
- and a, b, c, and d are log populations of Listeria in IPCM-1 containing:
- a = Listeria + S. lactis at \( t_1 \)
- b = Listeria + S. lactis at \( t_2 \)
- c = Listeria alone at \( t_1 \)
- d = Listeria alone at \( t_2 \)

Log populations of Listeria in the presence of S. lactis (a, b) are means of four plate counts (duplicate plateings of two samples). Log populations of Listeria alone (c, d) are means of two plate counts (duplicate plating of one sample).

RESULTS

IPCM-1 supported growth of each of the three strains of L. monocytogenes when the medium contained the...
pathogen alone (controls) and when it contained Listeria plus the lactic culture (treatment samples). Final L. monocytogenes populations in control after 30 h at 21°C were ca. 10^7 CFU/ml for stains V7 and SA and ca. 10^6 CFU/ml for CA. At 30°C, the final population for all three strains in controls was ca. 10^8 CFU/ml. S. lactis populations in samples initially containing 0.25 or 1.0% lactic culture were ca. 3.0 x 10^5 and 1.3 x 10^6 CFU/ml, respectively. After the 30-h incubation, S. lactis populations were ca. 4.0 x 10^9 regardless of temperature or amount of initial inoculum. The initial pH of all samples was 7.0 and did not change in uninoculated or Listeria controls during the 30-h incubation.

**Incubation at 21°C**

The three strains of L. monocytogenes behaved similarly at this temperature. Thus to avoid repetition, only results obtained with strain V7 are given. Data in Fig. 2 show the behavior of strain V7 when the medium was initially inocu-

![Figure 2](http://example.com/figure2.png)

**Figure 2. Behavior of L. monocytogenes V7 in IPCM-1 in the presence of (a) 0.25% and (b) 1.0% S. lactis at 21°C. Key: V7 (+ SL) represents the population of L. monocytogenes strain V7 grown in the presence of S. lactis in IPCM-1, pH represents the pH in IPCM-1 containing V7 and S. lactis at the time of sampling, SL represents the population of S. lactis when grown with V7, V7 represents the population of L. monocytogenes strain V7 grown alone in IPCM-1.**

luted with 0.25 or 1.0% S. lactis. Inhibition of growth by the pathogen began after 18 h of incubation when the initial inoculum was 0.25% S. lactis. At the 1.0% level of added lactic culture, inhibition began after 12 h of incubation. At the end of the 30-h incubation, populations of L. monocytogenes in samples inoculated with 0.25% S. lactis were 1.5-2.0 orders of magnitude less than populations of the pathogen in controls. At the 1.0% inoculum level of S. lactis, populations of L. monocytogenes were 2.5-3.0 orders of magnitude less than in controls. After the 30-h incubation, the pH of treatment samples was 5.5-5.7 regardless of percentage of lactic culture added.

**Incubation at 30°C**

At this temperature, strains V7 and SA behaved similarly, but behavior of these two strains differed markedly from that of strain CA. Thus, results from strains V7 and CA but not SA are presented in Fig. 3 and 4. In general, growth of the pathogen was inhibited earlier at 30 than 21°C. After 30 h of incubation, the pH of all treatment samples was between 5.0-5.2, regardless of strain of the pathogen or initial percentage of added lactic culture.

![Figure 3](http://example.com/figure3.png)

**Figure 3. Behavior of L. monocytogenes V7 in IPCM-1 in the presence of (a) 0.25% and (b) 1.0% S. lactis at 30°C. Key to abbreviations is in title of Fig. 2.**
Strain CA. Of the three strains examined, CA was most sensitive to the effects of \( S. \) \( \text{lactis} \). Inhibition of growth began after 9 h of incubation when 0.25% \( S. \) \( \text{lactis} \) was added (Fig. 4a) and 6 h when 1.0% \( S. \) \( \text{lactis} \) was added to the medium (Fig. 4b). The greatest difference in populations of \( L. \) \( \text{monocytogenes} \) between treatment and control samples was noted with strain CA. When the initial lactic inoculum was 0.25%, \( L. \) \( \text{monocytogenes} \) populations in treatment samples were 4.75 orders of magnitude less than those in controls after 30 h of incubation. Populations of the pathogen in samples inoculated with 1.0% \( S. \) \( \text{lactis} \) were 6.25 orders of magnitude less than populations in controls.

**Listeria populations at pH 5.5**

According to the manufacturers, IPCM-1 is ready for use when a pH of 5.5 has been reached after 16-18 h of incubation. Table 1 gives the numbers of \( L. \) \( \text{monocytogenes} \) present in the medium at this pH value. Data for growth of \( L. \) \( \text{monocytogenes} \) at 21°C in IPCM-1 inoculated with 0.25% \( S. \) \( \text{lactis} \) are not included because pH 5.5 was not reached in most instances even after the 30 h of incubation.

**TABLE 1. Listeria monocytogenes populations present in IPCM-1 after fermentation to pH 5.5 by \( S. \) \( \text{lactis} \).**

<table>
<thead>
<tr>
<th>Condition</th>
<th>V7</th>
<th>Scott A</th>
<th>California</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00% ( S. ) ( \text{lactis} ), 30°C</td>
<td>2.0 ( \times 10^6 )</td>
<td>4.0 ( \times 10^4 )</td>
<td>1.2 ( \times 10^3 )</td>
</tr>
<tr>
<td>0.25% ( S. ) ( \text{lactis} ), 30°C</td>
<td>1.7 ( \times 10^6 )</td>
<td>3.1 ( \times 10^5 )</td>
<td>8.7 ( \times 10^4 )</td>
</tr>
<tr>
<td>1.00% ( S. ) ( \text{lactis} ), 21°C</td>
<td>2.4 ( \times 10^4 )</td>
<td>4.9 ( \times 10^4 )</td>
<td>6.9 ( \times 10^3 )</td>
</tr>
</tbody>
</table>

*Populations represent trial means; trials consisted of four plate counts each (duplicate platings of two samples for each strain, temperature, and concentration of \( S. \) \( \text{lactis} \)).

**DISCUSSION**

IPCM-1 supported growth of each of the three strains of \( L. \) \( \text{monocytogenes} \). Growth of the pathogen was inhibited when \( S. \) \( \text{lactis} \) was present; the degree of inhibition was dependent on temperature, percentage of \( S. \) \( \text{lactis} \) added to the medium, and, in some instances, strain of the pathogen.

The calculated “area of inhibition” was useful for expressing the degree to which \( L. \) \( \text{monocytogenes} \) was inhibited by the presence of \( S. \) \( \text{lactis} \). The question could be asked, “why not simply express the results as populations of \( L. \) \( \text{monocytogenes} \) found after incubation together with \( S. \) \( \text{lactis} \)?” Results expressed as populations are useful but are limited in that they do not take into account the difference between \( L. \) \( \text{monocytogenes} \) populations in treatment samples and controls, i.e., inhibition. For example, this would occur when comparing growth of two different strains of \( L. \) \( \text{monocytogenes} \) where treatment samples behaved similarly but growth of controls was different. Expressing results simply as populations of the pathogen in treatment samples may not give a complete description of the inhibition that occurred. In the present study, the AI calculation was used primarily for statistical analysis. The purpose of the analyses was to determine if any significant differences existed among trials of the same strain and among strains under the same conditions.

Inhibition as measured by AI was directly related to the percentage of \( S. \) \( \text{lactis} \) added and temperature of incubation. Thus, for any given strain of \( L. \) \( \text{monocytogenes} \), greatest inhibition occurred at 30°C when the initial inoculum was 1.0% \( S. \) \( \text{lactis} \). Our results are consistent with those of...
Schaack and Marth (20) who studied behavior of L. monocytogenes when grown in skim milk together with mesophilic lactic starter cultures. Their data indicate that L. monocytogenes grew better at 30 than at 21°C. Not only did the pathogen grow better at the higher temperature, but lactic cultures did as well. Schaack and Marth (20) suggested the greater inhibition of Listeria seen at 30 than at 21°C could be explained by the increase rate of acid production by the lactic culture at the higher temperature.

Statistical analysis using a one-way ANOVA examined significant differences in AI and pH among the three strains of L. monocytogenes for each temperature and initial concentration of S. lactis at 6, 24, and 30 h of incubation. At 21°C, no significant difference in AI or pH was found among the three strains regardless of other test conditions. Growth of the pathogen at this temperature was minimal as was the degree of inhibition. At 30°C, strains did not differ in AI or pH at 6 h of incubation. Any significant differences at 6 h would indicate inconsistency in treatment of the three strains. At this same temperature, the AI values for strains V7 and SA after 24 and 30 h of incubation were significantly different from that of CA. Strain CA was inhibited significantly more than were strains V7 and SA regardless of the initial concentration of S. lactis. This supports recent findings (17, 18) that strain CA is generally less hardy than the other two. Listeria was inhibited to a greater degree when the initial inoculum of S. lactis was 1.0% rather than 0.25%. It is noteworthy that regardless of the initial concentration of lactic culture, the pH of the growth medium in which the three strains grew did not differ significantly. This indicates that pH may have had a role in the degree of inhibition but was not the sole factor responsible for it.

Gilliand and Speck (8) found intensity of antagonistic action cannot be predicted by the rate at which acid is produced by streptococci. They studied the effectiveness of streptococci in repressing growth of salmonellae and staphylococci with and without pH control of milk. Even though pH was maintained at that of uninoculated milk, the antagonistic action of the streptococci toward Salmonella was still evident. Similar results were found with S. aureus and a Cheddar cheese starter culture. The authors concluded the antagonism exhibited by streptococci did not result entirely from low pH.

It has been recognized that lactic acid bacteria can produce inhibitory substances, other than organic acids, that are antagonistic toward other microorganisms (7, 10, 12, 13, 16). These substances are produced in much smaller amounts than is acid and include hydrogen peroxide, diacetyl, bacteriocins and secondary reaction products such as hypohydrocyanate generated by the action of lactoperoxidase on hydrogen peroxide and thiocyanate. Whatever factor or combination of factors is responsible for inhibition of L. monocytogenes, our results show it was enhanced by use of a larger rather than smaller percentage of lactic culture inoculum and by a higher rather than lower temperature of incubation.

By far the most obvious and most important result of this study is that L. monocytogenes reproduced and survived during fermentation in IPCM-1. Thus, significant numbers of the pathogen were present in the medium at pH 5.5, when it normally would be ready for use in making products. Inhibition of the pathogen was not complete and depending on percentage of added lactic culture and temperature of incubation, 10^4 to as many as 10^5 CFU of L. monocytogenes/ml were present when the medium with its lactic culture could be used. Manufacturers currently using internal pH control media to prepare bulk starter culture need to be aware of the importance of strict sanitation practices in preventing contamination of bulk starter with L. monocytogenes.

L. monocytogenes has been found in food processing environments (4, 5) and in some finished products resulting in product recalls. Recalls have involved fermented dairy products such as cheese (12) and buttermilk (3). Our research supports the findings of others (18, 19, 20, 21) that L. monocytogenes can survive the fermentation involved in the manufacture of cultured dairy products.

The World Health Organization (WHO) (24) has made recommendations to the food industry regarding control of Listeria. Stressed was the importance of gathering information about the ability of various foods to support growth of L. monocytogenes. Use of Hazard Analysis Critical Control Points (HACCP) was recommended as the best way to assure safety and quality of foods. These recommendations need to be extended to manufacturers currently using internal pH control media in the preparation of bulk starter. The WHO concluded that because Listeria spp. are probably more common and more tenacious than most pathogens, it is probable that measures for their control will have to be applied rigorously with much attention to detail.

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REFERENCES

LISTERIA MONOCYTOGENES AND STREPTOCOCCUS LACTIS