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*Lactobacillus bulgaricus*와 *Streptococcus lactis* 발효탈지유에서의 *Listeria monocytogenes*의 생존추이

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Behavior of *Listeria monocytogenes* in skim milk during fermentation by *Lactobacillus bulgaricus* and *Streptococcus lactis*

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Abstract

Behavior of *Listeria monocytogenes* in Skim milk during fermentation by *Lactobacillus bulgaricus YI-2* and *Streptococcus lactis FYI-1* were determined. Autoclaved skim milk was inoculated with ca. 10^3 *L. monocytogenes* (Strain LM91-1 or LM 96-2) cells/㎖, and with 5.0, 1.0, 0.5 or 0.1% of a milk culture of either *L. bulgaricus YI-2* or *S. lactis FYI-1*. Skim milk containing ca. 10^3 *L. monocytogenes* was incubated at 37 or 42℃ for 15 h with *L. bulgaricus YI-2*, and at 21 or 30℃ for 15 h with *S. lactis FYI-1*. Cultured skim milks were stored at 4℃ in the refrigerater. Samples were plated on Oxford Agar with oxford antimicrobic supplement to enumerate *L. monocytogenes* and on either modified MRS agar to enumerate lactic acid bacteria. *L. monocytogenes* survived the 15-h fermentation with *S. lactis FYI-1* in all combinations of level of inoculum and temperature of incubation, but inhibition of growth ranged from 94 to 100%. When incubated with over the 1.0% of *L. bulgaricus*, *L. monocytogenes* inhibited or disappeared in fermented skim milk from 9 h after incubation. Especially, incubation at 42℃ with 5.0% *L. bulgaricus YI-2* as inoculum appeared to be the most effective inhibitory combination for strain LM 91-1, causing 100% inhibition in growth based on maximum papulation attained. In most instances of incubated with *L. bulgaricus YI-2*, growth of the pathogen appeared to be completely inhibited when the pH dropped below 4.38.

I. INTRODUCTION

Since 1983, three confirmed dairy-related listeriosis outbreaks resulted from consumption of pasteurized milk (Fleming et al. 1985)[10], Mexican-style cheese (James et al. 1985)[14], and Brie cheese contaminated with *Listeria monocytogenes* and have led to at least 150 cases of listeriosis, including 54 deaths.

Numerous dairy products have been recalled because of contamination with *L. monocytogenes* ; included are fluid milk, softripened cheeses, butter milk, Liederkranz and Romano cheese[1-6].

The most probable way for *L. monocytogenes* to enter the milk supply is via post-pasteurization contamination ; however, research indicates that the pathogen also may survive HTST pasteurization[8].

A study in Spain revealed that *L. monocytogenes* was present in 21.4% of pasteurized milk samples marketed by a Madrid processing plant[12]. Also in Korea, from 1991 to 1992, 2 of 100(2%) pasteurized market milk samples tested contained *L. monocytogenes*[17]. As is generally well known that lactic acid bacteria are antagonistic to many other bacteria. Research has shown that some starter cultures are antagonistic to various foodborne pathogens as well as food spoilage organisms in fermented dairy products[7,9,11,15,16,25,26]. These foods also have commonly been assumed to be safe and free from pathogenic microorganisms, for example, like species of *Listeria*. However, several investigators[7,13] have shown the pathogens do survive the fermentation of yogurt and then are inactivated at various rates during storage of the product.

And in fact, little work has been done to directly show the antagonistic effect that lactic cultures may have on *L. monocytogenes*.

Schaack and Marth(1988)[23,24] made lactic cultures from skim milk inoculated with *L. monocytogenes*, and studying behavior of *L. monocytogenes* during skim milk and yogurt fermentations with various lactic acid bacteria. In this work, they found that *L. monocytogenes* survived many of the fermentations, thus supporting the results on behavior of the pathogen reported by Ryser and Marth[20,21] and Ryser et al.[22]. However, in Korea, we have very few experimental informations about the relationship to survival of *L. monocytogenes* in lactic acid bacterial environment.

The purpose of this research was to determine the behavior of *L. monocytogenes* in skim milk during fermentation by *Lactobacillus bulgaricus* and/or *Streptococcus lactis*.

II. MATERIALS AND METHODS

1. Cultures and Sample preparation

Two strains of *L. monocytogenes* were used-stain LM91-1(Raw milk isolate, serotype 1) and strain LM96-2(Soil of dairy farm isolate, serotype 1). Both stock cultures were maintained on slants of Tryptic Soy Agar(TSA)(Difco laboratories, Detroit, MI, USA) contained 0.6% of yeast extract, stored at 4-5℃ and were transferred bi-monthly.

To begin an experiment, inoculum from a stock culture slant was transferred in duplicated to Bacto Listeria enrichment broth(LEB)(Difco) were contained as described previously[17] and incubated at 30℃ for 24 h under normal atmospheric conditions.

A second transfer of the cultures was made to new tubes of LEB, which were then incubated at 30℃ for 24 h.

Inocula of 0.05㎖ from the second LEB cultures were then added into 500㎖ of erlenmeyer flasks containing 200㎖ of Sterile skim milk (autoclaved 15 min, 121℃).

After incubation for 24 h at 30℃, another 0.05㎖ transfer was made into new flasks of sterile skim milk, followed by incubation at 30℃ for 48 h. These transfers were made to determine a known final population of *L. monocytogenes* achieved in milk in 48 h. The purpose of these trial was made it possible to calculate the final dilution of the 48-h-old culture needed to establish a consistent initial inoculum of ca. 1×10^3 *L. monocytogenes* cells per ml of milk in each test flask.

According to the method of Marth and Schaack[23], in each trial, five flasks were used for each incubation temperature. Three flasks were inoculated to contain ca. 10^3 *L. monocytogenes* cells/㎖ of milk. One of the three flasks served as a control and the other two flasks were further inoculated with either 5.0, 1.0, 0.5 or 0.1%(v/v) of a lactic culture preparation ; Use of *Lactobacillus bulgaricus YI-2* or *Streptococcus lactis FYI-1*.

A 4th flask was inoculated with the same percentage of lactic culture(lactic control) and a 5th flask was uninoculated, thus serving as a negative control.

For each trial, one set of flasks was incubated for 15 h at 37-42℃ and the other set for 15 h at 21-30℃ ; In case of *L. bulgaricus YI-2* did at 37-42℃ and *S. lactis FYI-1* did at 21-30℃. And all followed by refrigeration at 4℃ for further studies.

Lactic cultures were prepared in laboratory of DIEH(Dongshin Institute of Environment & Hygiene, KwangJu, Korea) as frozen concentrates(Stains *L. bulgaricus YI-2 and S. lactis FYI-1* for these experiments). Aluminum vials of frozen concentrate were thawed and a series of 0.1㎖ transfers of the culture were made to freezer cuvetts that contained 1 ㎖ of sterile skim milk. Contents of these cuvetts were frozen at -70℃ to maintain culture activity. Using cultures were prepared by thawing one cuvett and transferring 0.1㎖ of the contents to a flask containing 200㎖ of sterile skim milk. This was incubated at 37℃ 9 h. The 9-h-old culture served as the inoculum for the test flasks. Each trial was done in duplicate.

2. Sampling and Enumeration of *L. monocytogenes*

In fermented milk trials, 1㎖ samples were taken every 3 h and diluted in 0.5% peptone buffer solution, if necessary.

Samples for enumeration of *L. monocytogenes* were surface-plated in duplicate on Oxford Agar(OXA)(Difco) with Oxford antimicrobic supplement(Difco). When in the presence of a lactic cultures, and the control samples were plated in duplicate on TSA contained 0.6% of yeast extract.

To enumerate the lactic culture, samples were pour-plated in modified lactobacilli MRS agar(m-MRS broth+1.5% bacto agar (Difco)). Plates for enumeration of *L. monocytogenes* were incubated at 35℃ for 48 h under normal atmospheric condition, and plates to enumerate lactic acid bacteria were incubated at 42℃ or 30℃ for 48 h under normal atmospheric conditions(*L. bulgaricus* at 42℃ and at 30℃ for *S. lactis*). After the incubation, colonies were counted using a darkfield colony counter.

3. Measurement of pH and Calculation of percent Inhibition.

The pH of the milks was measured by aseptically removing a 5㎖ sample from a test flask and measuring the pH with a pH meter (HANNA Instruments HI 8314, Singapore).

The formula used to calculate percent inhibition is

$$\frac{A-B}{A} \times 100$$

Where,

A = Ultimate population of *L. monocytogenes* in control

B = Ultimate population of *L. monocytogenes* in the culture with the lactic acid bacterium

Ⅲ. RESULTS

1. Behavior of *L. monocytogenes* in the presence of *L. bulgaricus YI-2*

The inoculum levels of *L. bulgaricus* were 5.0, 1.0, 0.5 or 0.1% with *L. monocytogenes* strain LM91-1, but only 1.0% was used with strain LM96-2. The inoculum levels choose in accordance with the inoculum levels of Schaack and Marth[24] for comparison. Experiment with wild type strain LM96-2 was done to determine if wild type strain of the pathogen behaved as did strain LM91-1 in the

presence of the lactic acid bacteria. Table 1 shows the percent inhibition in growth of *L. monocytogenes* on various test combination.

Results obtained when using 5.0% *L. bulgaricus* and incubation at 37℃ or 42℃ are shown in Fig.1 and 2, respectively.

With *L. bulgaricus* and incubation at 37℃ or 42℃, *L. monocytogenes* began to grow in all instances but then was inhibited and the population began to decrease between 9 and 13 h of incubation without

Table 1. Percent inhibition in growth of *L. monocytogenes*[a] in skim milk during fermentation 15 h by *Lactobacillus bulgaricus* YI-2.

Inoculum of lactic culture(%)	Incubation temperature (℃)	Strain of *Listeria*	Inhibition (%)	Final pH
5.0	37	LM91-1	100	3.73
	42	LM91-1	100	3.68
1.0	37	LM91-1	100	3.80
	42	LM91-1	100	3.72
	37	LM96-2	100	3.82
	42	LM96-2	100	3.70
0.5	37	LM91-1	99.8	4.18
	42	LM91-1	100	4.00
0.1	37	LM91-1	99.4	4.23
	42	LM91-1	100	3.88

[a]Initial population of *L. monocytogenes* was ca. 1000/ml.

exception in all instances. However, when the inoculum was 0.1% lactic culture at 37℃, the population of *L. monocytogenes* decreased but viable cells were still detected at 15 h. Based on this result, inhibition effects do not correspond with the report of Schaack and Marth.[24] Data in Fig.1 shows the behavior of *L. monocytogenes* LM91-1 with 5.0% *L. bulgaricus* inoculum and incubation at 37℃. The pathogen was completely inactivated between 12 and

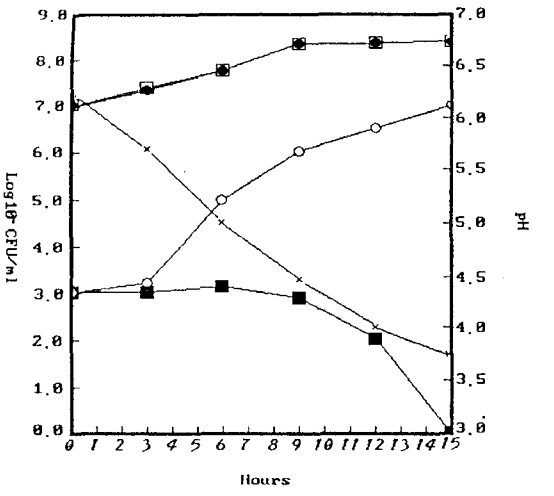

Fig. 1 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 5.0% L. bulgaricus YI-2 at 37℃*

Lm+Lb(■): Represents the population of L. monocytogenes when incubated with the L. bulgaricus.

Lb+Lm(□): Represents the population of L. bulgaricus when incubated with the L. monocytogenes.

Lb(●): Represents the population of L. bulgaricus alone.

Lm(○): Represents the population of L. monocytogenes alone.

pH(×): pH represents the pH of the milk at the time of sampling.

※ *Note : Lb+Lm and Lb are represented by the same growth curve in this figure since the population of both were essentially identical.*

15 h of incubation ; the population of *L. monocytogenes* had dropped to a non-detectable level. The final pH was 3.73.

With incubation at 42℃, the population of *L. monocytogenes* dropped to a non-detectable level at an even faster rate between 9 and 12 h of incubation(Fig.2). With 1.0% *L. bulgaricus* inoculum, yet it was still very inhibitory to survival of *L. monocytogenes* as shown in Fig.3.

However, then the population of *L. monocytogenes* leveled off and began of decrease between 12 and 15 h of incubation, remarkably when the pH dropped below 4.0 (In case of incubation at 42℃ was more

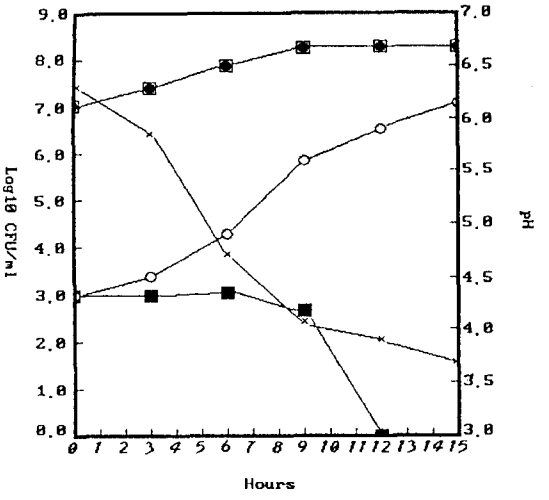

Fig. 2 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 5.0% L. bulgaricus YI-2 at 42 ℃. Key to abbreviations is in the title of Fig. 1*

※ *Note: Lb+Lm and Lb are same curve in this figure like in Fig. 1.*

detrimental to survival than that of *L. monocytogenes* which was incubated at 37℃, after 12 h).

In all experiment conditions, the population of

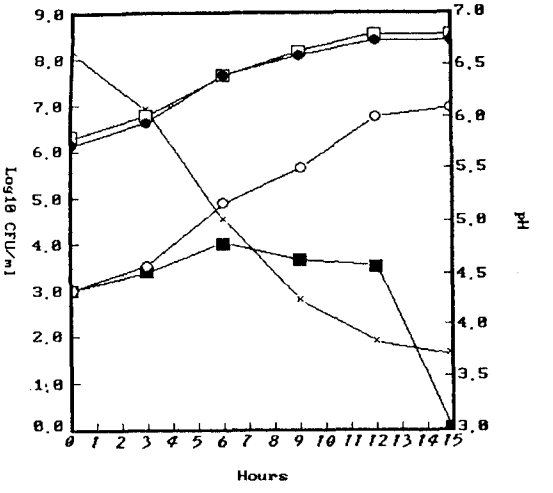

Fig. 4 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 1.0% L. bulgaricus YI-2 at 42 ℃. Key to abbreviations is in the title of Fig. 1*

L. monocytogenes LM91-1 began to increase up to 6 h of incubation. And then slowly decreased, with a rapid drop from 12-15 h of incubation, again when the pH decreased below 4.0 as shown in Fig.4. These

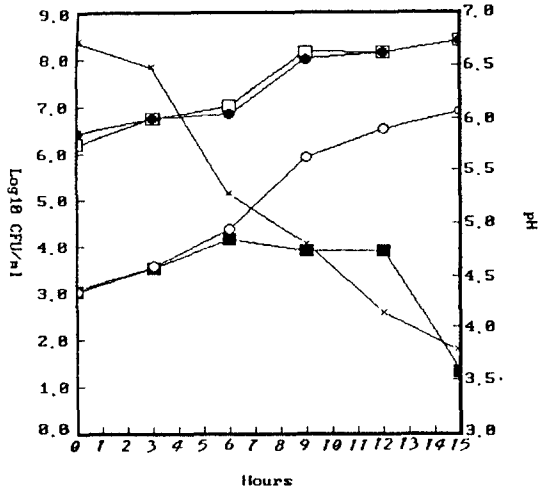

Fig. 3 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 1.0% L. bulgaricus YI-2 at 37 ℃. Key to abbreviations is in the title of Fig. 1*

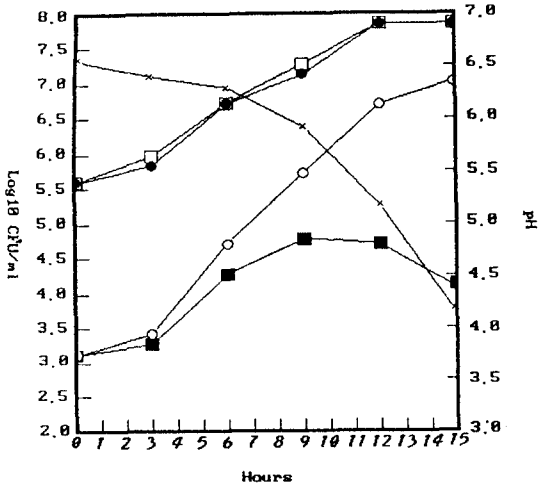

Fig. 5 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 0.5% L. bulgaricus YI-2 at 37 ℃. Key to abbreviations is in the title of Fig. 1*

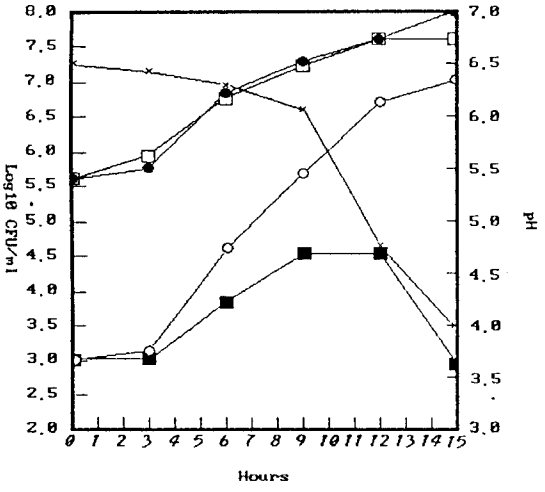

Fig. 6 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 0.5% L. bulgaricus YI-2 at 42 ℃. Key to abbreviations is in the title of Fig. 1*

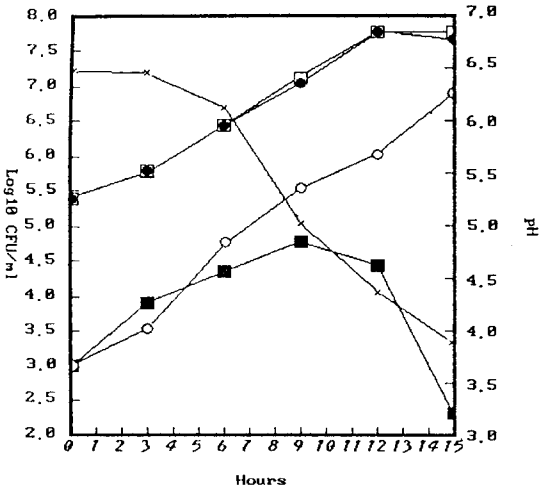

Fig. 8 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 0.1% L. bulgaricus YI-2 at 42 ℃. Key to abbreviations is in the title of Fig. 1*

results were coincide with the report of Schaack and Marth[24], distinctly.

Use of a *L. monocytogenes* strain LM96-2 was similar to those obtained with strain LM96-1(Table 1, data not shown). At 37℃, the population of strain LM96-2 began to decrease after 12 h of incubation, but was still detected in a few numbers at 15 h too. At 42℃, the population dropped to a non-detectable level between 12 and 15 h of incubation, which was similarly with strain LM91-1.

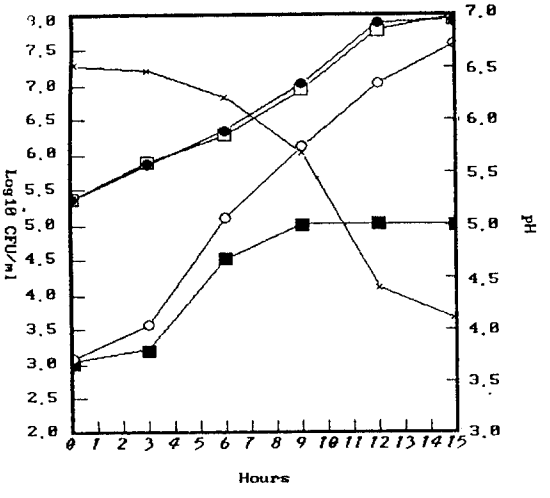

Fig. 7 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 0.1% L. bulgaricus YI-2 at 37 ℃. Key to abbreviations is in the title of Fig. 1*

When the inoculum was 0.5% *L. bulgaricus* and incubation was at 37℃, *L. monocytogenes* grew slowly but markedly and increased by over 1.0 order of magnitude during the incubation(Fig.5), but compared to the control, inhibition of growth was over 90%. Figure 6 depicts results when incubation was at 42℃. It is evident that *L. monocytogenes* again grew together with the *L. bulgaricus* for up to 9 h of incubation. After 9 h, the pH level dropped below 5.0 and the pathogen ceased growth but remained at a constant level (Fig.6). When the inoculum was 0.1% *L. bulgaricus* and incubation was at 37℃, *L. monocytogenes* grew actually and increased by over 2 orders of magnitude (Fig.7), and allowed for considerable growth of the pathogen rather than 42℃. In contrast, the population of *L. monocytogeses* at 42 ℃ was decreased rapidly between 12and 15 h of incubation, again, when the pH of the fermented milk dropped below 4.0(Fig.8).

2. Behavior of *L. monocytogenes* in the presence of *S. lactis FYI-1*

Streptococcus lactis was choosen that it is representative mesophilic lactic starter microorganism. Inoculum levels of *S. lactis* were 5.0, 1.0, 0.5 and 0.1% in trials with *L. monocytogenes* strain LM 91-1 and 1.0% with contained strain LM96-2.

S. lactis seemed less inhibitory toward strain LM91-1 than was *L. bulgaricas*. Table 2 gives the percent inhibition in growth of *L. monocytogenes* for each test combination. Results obtained when using 5.0% *S. lactis* and incubation at 21 or 30℃ are shown in Fig. 9 and 10, respectively. At 21℃ and with 5.0% inoculum, growth of *L. monocytogenes* was inhibited 99%. Although inhibition of growth were 100% at 30℃ in nearly all experiments (Fig. 9~16) except on only 0.1% inoculum of lactic culture.

Table 2. Percent inhibition in growth of *L. monocytogenes*[a] in skim milk during fermentation 15 h by *Streptococcus lactis* FYI-1.

Inoculum of lactic culture(%)	Incubation temperature (℃)	Strain of *Listeria*	Inhibition (%)	Final pH
5.0	21	LM91-1	99	4.42
	30	LM91-1	100	4.20
1.0	21	LM91-1	98	4.60
	30	LM91-1	100	4.28
	21	LM96-2	99	4.58
	30	LM96-2	100	4.32
0.5	21	LM91-1	97	4.63
	30	LM91-1	100	4.38
0.1	21	LM91-1	94	5.62
	30	LM91-1	99	4.43

[a]Initial population of *L. monocytogenes* was ca. 1000/ml.

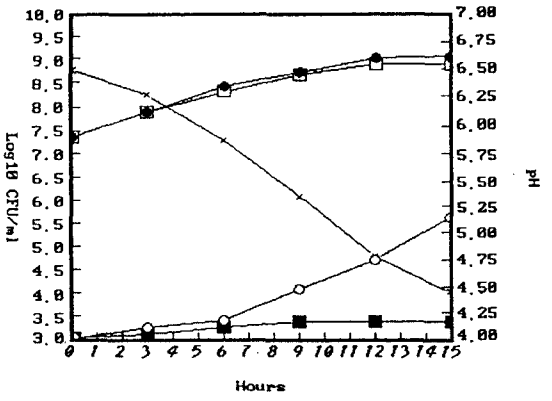

Fig. 9 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 5.0% S. lactis FYI-1 at 21℃.*

Lm+Lb(■): Represents the population of L. monocytogenes when incubated with the S. lactis FYI-1.

Lb+Lm(□): Represents the population of S. lactis FYI-1 when incubated with the L. monocytogenes.

Lb(●) : Represents the population of S. lactis FYI-1 alone.

Lm(○) : Represents the population of L. monocytogenes alone.

pH(×) : pH represents the pH of the milk at the time of sampling.

The temperature of incubation was quite important in determining the rapidity of growth of *L. monocytogenes* independent of the lactic culture, e.g.

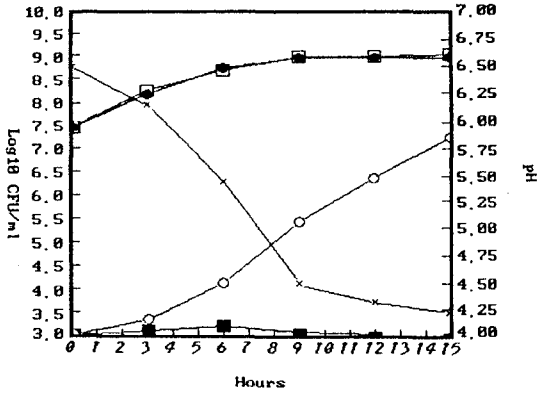

Fig. 10 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 5.0% S. lactis FYI-1 at 30℃. Key to abbreviations is in the title of Fig. 9*

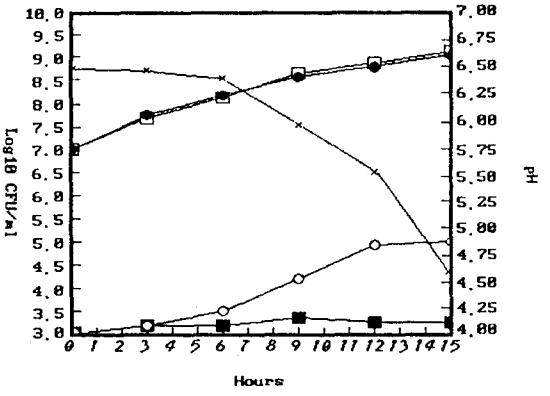

Fig. 11 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 1.0% S. lactis FY1-1 at 21 ℃. Key to abbreviations is in the title of Fig. 9*

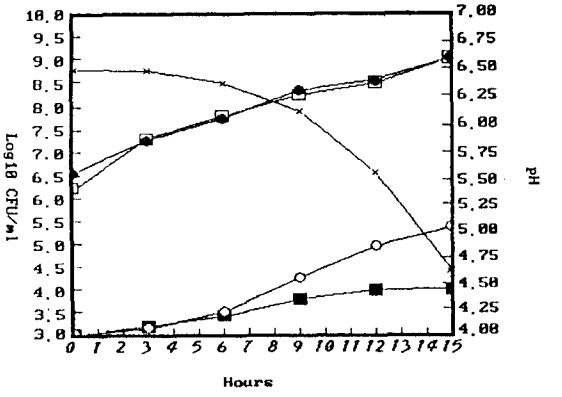

Fig. 13 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 0.5% S. lactis FY1-1 at 21 ℃. Key to abbreviations is in the title of Fig. 9*

in the control(Fig.9,10). At 30℃ and with each % inoculum, inhibition of *L. monocytogenes* was greater than at 21℃ and the population began to decrease slightly between 12 and 15 h of incubation, when a final pH of below the 4.43 was achieved, respectively. However, *L. monocytogenes* still survived at the conclusion of the fermentation process of all experiment. Distinctively, this part was different from mostly case of *L. bulgaricus*.

Figure 11 and 12 show the behavior of *L. monocytogenes* LM91-1 in milk inoculated with 1.0% *S. lactis* and incubated at 21 and 30℃, respectively. Incubation at 21℃ again was somewhat less detrimental to *L. monocytogenes* than was incubation at 30℃, with inhibition on growth of *L. monocytogenes* being 98% at 21℃ when a final pH of 4.60 was achived vs 100% at 30℃ when the final pH was 4.28.

Using strain LM96-2, inhibition of growth at 21℃ was 99%, which was more than for strain LM91-1. Maybe this was caused from the final pH of the fermented milk was lower in the culture with strain LM96-2 (data not shown).

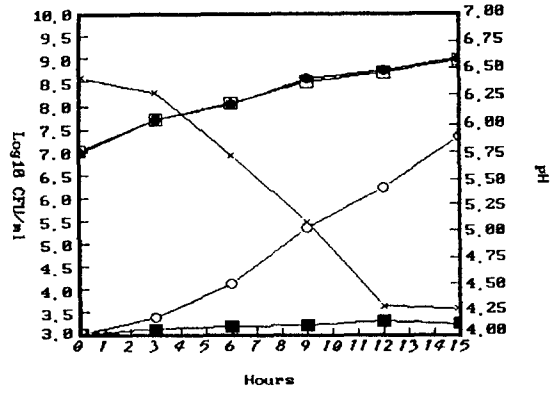

Fig. 12 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 1.0% S. lactis FY1-1 at 30 ℃. Key to abbreviations is in the title of Fig. 9*

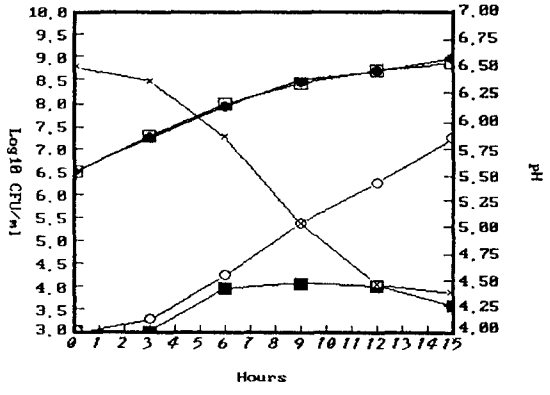

Fig. 14 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 0.5% S. lactis FY1-1 at 30 ℃. Key to abbreviations is in the title of Fig. 9*

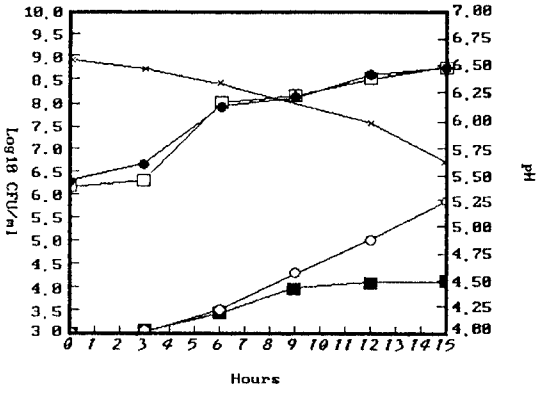

Fig. 15 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 0.1% S. lactis FY1-1 at 21 ℃. Key to abbreviations is in the title of Fig. 9*

At 30℃, the inhibition on growth of strain LM96-2 appeared to be greater than of LM91-1 even though the final pH values in the fermented milk were higher (data not shown).

When the inoculum of lactic acid bacteria was lowered to 0.5% and incubation was at 21℃, *L. monocytogenes* grew and actually increased by over 0.80 order of magnitude during the incubation (Fig.13), but, compared to the control, inhibition of growth was 97%. This tendency to over about 1.0 order of magnitude was appeared markedly in Fig.15, and Fig.16.

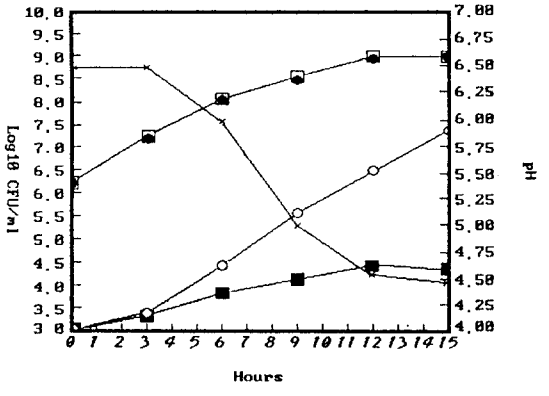

Fig. 16 *Behavior of L. monocytogenes LM91-1 in skim milk fermented with 0.1% S. lactis FY1-1 at 30 ℃. Key to abbreviations is in the title of Fig. 9*

Decreasing the inoculum to 0.1% and incubating at 21℃, the final pH of the fermented milk was only 5.62, but there was still inhibition of 94% compared to growth in the control.

However, incubation at 21℃ (Fig. 15)allowed for considerable growth of the pathogen. In the presence of 0.1% lactic starter and incubation at 30℃, *L. monocytogenes* increased in population by about over 1.2 orders of magnitude and then leveled of (Fig.16). Again, the population of *L. monocytogenes* did not decrease during the fermentation even when the final pH was 4.43. However, the inhibition percentage of growth was 99% in this instance. These data indicate what might happen when acid production by the lactic culture is delayed and inadequate.

Ⅳ. DISCUSSION

Results indicate that growth of *L. monocytogenes* was strongly inhibited by *L. bulgaricus* and to a lesser extent by *S. lactis*. The degree of inhibition was directly related to the amount of inoculum and strain of lactic culture used as well as temperature of incubation. In results of all experiments of *L. bulgaricus*, incubation at 42℃ was more inhibitory to *L. monocytogenes* than was incubation at 37℃, regardless of the lactic culture being evaluated.

Similary, in case of *S. lactis*, incubation at 30℃ was more inhibitory to *L. monocytogenes* than was incubation at 21℃. Other researchers also have found that incubation temperature affects an important role in determining growth and survival of pathogenic bacteria during fermentation. According to the data of Schaack and Marth[23], they demonstrate that incubation temperature was also an important factor on the inhibition of *L. monocytogenes* by lactic acid bacteria. Their data shown that *L. monocytogenes* actually grew better at 30℃ than at 21℃ with the lower levels of lactic inoculum. However, since growth in the control samples was much greater at 30℃ than at 21℃, the percent inhibition was greater at the warmer temperature. With higher levels of

lactic culture, *L. monocytogenes* also grew faster initially at 30℃ than it did at 21℃, but the lactic culture produced acid at a much faster rate at 30 than at 21℃ ; thus with a higher lactic population and warmer incubation temperature, growth of L. monocytogenes was repressed more than at 21℃.

Pulusani et al[18] reported antimicrobial activity of a methanol-acetone extract of lyophilized milk that had been fermented by *S. thermophilus*. Reddy and Shahani[19] isolated an antibiotic substance from *L. bulgaricus* termed bulgarican, which was active against both gram-positive and gram-negative bacteria. Schaack and Marth[23] reported that the growth of the *L. monocytogenes* completely inhibited when the pH decrease to lower than 4.75, and especially, the decrement of pH lower than 4.0 was accompanied by rapid death of the pathogen[24].

From the data presented in this paper, it is apparent that growth of *L. monocytogenes* was markedly inhibited during fermentation by *L. bulgaricus* than *S. lactis*, yet the pathogen managed to survive for at least 9 h during all milk fermentations, and survived in all lactic fermentations. From a practical viewpoint, *L. monocytogenes* survived a thermophilic fermentation(use of *L. bulgaricus*) up to 9 h, even when a high concentration of lactic culture was used together with a high (e.g. 42℃) incubation temperature ; On the other hand, *L. monocytogenes* survived a mesophilic fermentation(use of *S. lactis*) in all period of experiment(up to 15 h), actually. However, when the pH of fermented milk dropped below 4.0, L. monocytogenes could no longer survive. This emphasizes the importance of a processing conditions of lactic fermentation to achieve microbiologically safe fermented dairy products. The likelihood of finding *L. monocytogenes* in a fermented dairy product is not unrealistic,

as evidenced by recent products recalls including buttermilk and soft ripened cheese in America[1-4].

Although this data indicate that the ability of *L. monocytogenes* survived in skim milk during and after two types of lactic acid fermentations, there should be no reason for consumers to believe that the market dairy products are not safe. However, we have very few information[17] about contamination of *L. monocytogenes* in Korean dairy fermented milk, also have poor base of studying about *L. monocytogenes*.

It is possible, anytime, anywhere that *L. monocytogenes* could enter the pasteurized milk supply. If such milk is fermented, the pathogen likely would contaminate a fermented dairy products. Accordingly we must be prepare for unexpected contamination to dairy fermented products and full-scale open market of dairy products in 1997 by UR agreement, especially.

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