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# The Inhibitory Effect of Lactic Starter Culture Against Food Borne Pathogenic Bacteria in Skim Milk

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## Abstract
The antibacterial effect of mixed culture of *Lactococcus lactis* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus* against some food borne pathogenic organisms were investigated. Much amount of acidity was produced by lactic acid bacteria and increased gradually with the fermentation time. Lating of reaching pathogens to milk lead to the over increasing of acidity produced by lactic acid bacteria. Over production of acidity aod reduction of pH level and other substances not determined lead to prevent or inhibit the growth of all pathogens. These factors clearly showed the inhibition effect of lactic acid bacteria against pathogens. The count of all pathogenic, organisms decreased sharply with fermentation time until it disappeared completely after 3 days. This shows the importance of the consumption of fermented milks. Cell-free extract of lactic acid bacteria shows clearly an inhibitory activity against all pathogenic organisms used. But, non-boiled extract was highly active than those of boiled ones. This means that, lactic acid bacteria produced thermolabile substances inhibit or prevent the growth of pathogens. Finally all these observations show the hygienic and nutritional importance of the consumption of fermented milks.

## Introduction
An essential feature of lactic acid bacteria (LAB) is their efficient carbohydrate fermentation, producing lactic acids as the major end product. These organic acids leading to inhibit the growth of food borne pathogenic and spoilage microorganisms in milk, dairy products, meat, vegetables and cereals (Reinhamier *et al*., 1990; Bevilacqua and Califano, 1992; Gonzalez de Llano, *et al*., 1996; Abd-El-Ghani and Hosny, 1998). Such antimicrobial activity was reported to be due to the lactic acid produced, either solely or in combination with other low-molecular weight organic acids, decreased pH value, hydrogen peroxide production and other substances, i.e., diacetyl and bacteriocins (Reinhamier *et al*., 1990; Daeschel, 1993; Davidson and Hoover, 1993; Santos *et al*., 1994, 1996). So, organic acids extend stability of an important foodstuff likely to undergo rapid spoilage (Piard and Desmazeand, 1991; Gonzalez de Llano *et al*., 1996).

In dairy products, organic acids resulting from the hydrolysis of fatty acids, normal bovine metabolism processes or direct addition as acidulants are essential for the coagulation process and greatly improves the hygienic quality of dairy products. Organic acids are also important for nutritional reasons as well as in flavour studies, because these acids contribute to the flavour and aroma characteristics of most cheeses. The reduction of pH was due to lactic bacteria metabolism, greatly influenced texture through water and mineral contents and have further repercussions on some chemical changes (Adda *et al*., 1982; Gonzalez de Llano *et al*., 1996).

Therefore, the present study was focused to illustrate the disappearance or inhibition of some food borne pathogenic organisms by the mixed culture of *Lactococcuus lactis* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus*.

## Materials and Methods
**Cultures as fermentation organisms:** *Lactobcoccus lactis* subsp. lactis (*Streptococcus lactis*) DRI-VAC 0012209 and *Lactobacillus delbrueckii* subsp. bulgaricus (*Lactobacillus bulgaricus*) DRI-VAC 0023307 used in this study were kindly obtained from DRIVAC Lactic Culture CHR Hansen's Laboratories, Copenhagen, Denmark.

The strains were subcultured weekly in slopes of lactose-M 17 broth and incubated at 37°C for 18-24 hrs. Stock cultures were stored at 4°C between transfers. Before use, stock culture was activated by two successive transfers at 18-24 hrs intervals. A second transfer of the cultures was made to skim milk (SM), 10% w/v, solids, which was then incubated at 37°C for 18 h. Inocula were prepared from the second culture.

**Skim milk:** Skim milk powder (SM; Difco), which have the following composition, protein 36 percent, lactose 51 percent, fat 0.7 percent, ash 8.2 percent and moisture 3 percent, was reconstituted in distilled water to a final concentration of a 10 per cent (w/v), autoclaved at 121°C for 15 min and added as required (McKellar, 1982).

**Indicator pathogenic organisms:** *Salmonella typhimurium, Escherichia coli, Staphylococcus aureus* and *Listeria monocytogenes* were obtained from Dairy Department, Faculty of Agriculture, Mansoura University, Egypt. *Bacillus cereus* 1086 was obtained from Cairo Mercin, Ain Shams Univ., Cairo, Egypt.

The pathogenic strains were reactivated twice using brain heart infusion (BHI) broth (Difco, 1984) at 37°C for 20-24 hrs before use in this study and were transferred weekly. These organisms were maintained on tryptic soy agar (Massa *et al*., 1997) and the activating inoculum was prepared after two successive 24-h cultures in tryptic soy

broth (TSB) inoculated and incubated at 37°C for 48 hrs.

**Associative growth of the mixed lactic culture with pathogens in skim milk:** The mixed lactic culture was inoculated (1% v/v) into 250 ml Erlenmeyer flasks containing 100 ml of skim milk (pH 7.2±0.1). The flasks were additionally inoculated with the pathogens. In other groups of flasks the additionally inoculation with the pathogens was carried out after 24 h from inoculation with. lactic acid bacteria and other one was inoculated with pathogens only used as control. The control and associative cultures were incubated (without shaking) for 72 h at 37°C. pH and titratable acidity Were 'carried out and the determination of cfu $ml^{-1}$, at intervals (0, 12, 24, 36, 48 and 72 hrs), was made. The cfu $ml^{-1}$ of pathogens was obtained by plating the appropriate dilutions on appropriate medium for each pathogen. All the experimepts were carried out in duplicate.

**Detection of antagonistic activity of lactic acid bacteria spent medium by the agar diffusion techniques:** The mixed lactic culture (*Lactococcus lactis* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) was grown in lactose-M 17 broth (pH 7.2 ± 0.2) for 48 hrs. at 37°C and other one was incubated for 72 hrs. at the same temperature. The supernatant culture was collected by centrifugation (13 000 g for 10 min at 4°C and divided into two portions. One portion was boiled at 100°C for 10 min. and both portions were sterilized by filtration through micro pore filter (pore size 0.22 μm). The resultant sterilized filtrate for each culture was tested for its inhibitory activity against *Listeria monocytogenes, Escherichia coli, Staphylococcus aureus, Salmonella typhimurium* and *Bacillus cereus* using the diffusion disc assay method (Hassan *et al*., 1994). In this method two petri dishes were filled with 15 ml of nutrient agar medium and inoculated with 0.1 ml of the test organisms. After the agar had solidified, two sterilized filter paper Whatman No. 3 (disks) were immersed in each filtrate exactly for three seconds then were placed on the agar surface. A third petri dish was only inoculated with the pathogenic organism as a control. The same steps were repeated with the other pathogenic organisms. Then petri dishes were kept in the refrigerator for 2h for diffusion then incubated at 37°C for 24 h before examination for zones of inhibition.

**Determination of titratable acidity:** Titratable acidity was determined according to the standard method reported by Ling (1963), and the results were expressed as percentage of lactic acid.

**The measurement of pH value:** The pH value was measured using laboratory pH-meter with a glass electrode (KnickDigital-pH meter 646) according to Ling (1963).

**Media:** Lactose-M 17 (0.5% w/v) was used for lactic acid bacteria propagation, which consisted of (g/l): peptone from soymeal 5.0, peptone from meat, 2.5; peptone from casein, 2.5; yeast extract, 2.5; meat extract, 5.0; lactose monohydrate, 5.0; ascorbic acid, 0.5; sodium β-glycerophosphate, 19.0; magnesium sulphate, 0.25; agar-agar, 12.75. For preparation, 55 g lactose M-17 agar/litter or 42.5 g M-17 broth/litter was dissolved, dispensed the broth into test tubes, and autoclaved (15 min at 121°C). The pH was adjusted to pH 7.2 ± 0.1 (Brinchmann *et al*., 1983).

***Listeria monocytogenes*:** *L. monocytogenes* was counted on Mc Brid's *Listeria* agar (Lovett *et al*., 1985).

***Escherichia coli*:** The coliform group was counted on violet red bile agar (VRBA) (APHA, 1972).

***Staphylococcus aureus*:** The cfu $ml^{-1}$ of *Staphylococcus aureus* obtained by plating on Baird-Parker medium (Oxoid). The plates were incubated at 37°C for 48 h, then counted (Otero *et al*., 1988).

***Salmonella typhimurium*:** *S. typhimurium* was counted on the high selective *Salmonella Shigella* (SS) agar (Difco Laboratories,1984) The plates were incubated at 37°C for 24 hr.

***Bacillus cereus*:** The cultures were propagated in trypticase soy broth (TSB) at 37°C (Coventry *et al*., 1996).

## Results and Discussion

It is well known that different food-borne pathogenic bacteria, spore forming and non-spore forming, bacilli and cocci, Gram-negative and Gram-positive bacteria (*Listeria, Escherichia, Staphylococcus, Salmonella* and *Bacillus*) reached to foods especially milk and dairy products, which caused different illness to human. Thus, this study was carried out to show the inhibitory effects of lactic acid bacteria against some of the pathogenic organisms. Results in Table 1 and 2 show one of the different mechanisms used by lactic acid bacteria especially in milk and dairy products to prevent the pathogenic organisms. It can be easily observed that lactic acid bacteria when used as a starter in fermented milks produced excess amount of organic acids. Thus, the acidity was increased gradually with prolongation of fermentation time, with the continuity hydrolysis and fermentation of milk lactose, protein and fat. Also, data in Table 1 show that acidity was produced with little amount when skim milk was inoculated with pathogens individually. In other words the absence of lactic acid bacteria greatly affected the production of acidity. Hence, lactic acid bacteria are essentially responsible for the production of acidity. Inoculation of milk with lactic acid bacteria incubated at 37°C for 24 h, followed by inoculation with pathogenic organisms give more time to lactic acid bacteria to produce great amount of organic acids (Table 2). The reduction of pH values (Table 4) was very pronounced than those found in Table 3. Also, the reduction of the count of all pathogenic organisms was higher (Table 6) than those found in the same inoculation (Table 5). The amount of organic acids produced by lactic acid bacteria differed with the different pathogenic organism with all fermentation time. Therefore, acidity produced lactic acid bacteria were inhibit and prevent the growth of pathogenic organisms.

Table 1: Acidit evaluation b lactic acid bacteria with some atho enic bacteria individuall in skim milk

| Incubation time (hrs.) | *L. monocytogenes* | | *Escherichia coil* | | *S. aureus* | | *S. typhimurium* | | *B. cereus* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | A | B | A | B | A | B | A | B |
| Zero | 0.7 | 0.7 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 |
| 12 | 0.7 | 2.5 | 0.7 | 2.7 | 0.9 | 1.9 | 0.6 | 2.2 | 0.7 | 1.5 |
| 24 | 0.8 | 2.3 | 0.9 | 3.1 | 1.0 | 2.7 | 0.7 | 3.0 | 0.8 | 2.2 |
| 36 | 0.9 | 4.1 | 1.3 | 3.6 | 1.3 | 3.21 | 0.7 | 4.1 | 0.9 | 2.9 |
| 48 | 1.1 | 4.6 | 1.7 | 4.2 | 1.6 | 4.0 | 0.9 | 4.6 | 1.1 | 3.8 |
| 72 | 1.6 | 4.8 | 1.9 | 5.1 | 1.0 | 4.5 | 1.4 | 5.1 | 1.8 | 4.3 |

A = control (SM incoulated with pathogens only), B = The incoculation of lactic acid bacteria and pathogenic organisms is in the same time at zero time. Acidity was calculated as % of lactic acid

Table 2: Acidity evaluation by lactic acid bacteria with some pathogenic bacteria individually in skim milk

| Incubation time (hrs.) | *L. monocytogenes* | *Escherichia coli* | *S. aureus* | *S. typhimurium* | *B. cereus* |
|---|---|---|---|---|---|
| Zero | 1.5 | 3.2 | 2.1 | 2.4 | 1.8 |
| 12 | 3.0 | 3.9 | 2.7 | 3.2 | 2.5 |
| 24 | 3.3 | 4.8 | 5.5 | 6.1 | 6.5 |
| 36 | 4.3 | 5.5 | 3.9 | 4.6 | 3.7 |
| 48 | 4.9 | 6.1 | 4.8 | 5.2 | 4.1 |
| 72 | 5.2 | 6.5 | 5.3 | 5.8 | 4.6 |

Pathogenic organisms were inoculated after 24 h from inoculation of lactic acid bacteria. Acidity was caolculated as % of lactic acid.

Table 3: The reduction of pH level in skim milk inoculated with lactic acid bacteria and some individual pathogens.

| Incubation time (hrs.) | *L. monocytogenes* | | *Escherichia coli* | | *S. aureus* | | *S. typhimurium* | | *B. cereus* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | A | B | A | B | A | B | A | B |
| Zero | 6.3 | 6.3 | 6.4 | 6.4 | 6.4 | 6.4 | 6.2 | 6.2 | 6.4 | 6.4 |
| 12 | 6.1 | 5.5 | 6.2 | 5.4 | 6.2 | 5.7 | 6.2 | 5.7 | 6.2 | 5.9 |
| 24 | 5.9 | 4.4 | 6.1 | 4.3 | 6.0 | 5.3 | 6.1 | 5.1 | 6.0 | 5.6 |
| 36 | 5.7 | 4.2 | 6.0 | 4.1 | 6.0 | 4.8 | 6.1 | 4.7 | 5.8 | 5.2 |
| 48 | 5.5 | 4.0 | 5.8 | 3.9 | 5.8 | 4.5 | 6.0 | 4.2 | 5.4 | 4.8 |
| 72 | 5.0 | 3.5 | 5.5 | 3.3 | 5.5 | 3.8 | 6.5 | 3.7 | 5.2 | 4.2 |

A = control (SM incoulated with pathogens only), B = The incoculation of lactic acid bacteria and pathogenic organisms is in the same time at zero time. Acidity was calculated as % of lactic acid

Table 4: Acidity evaluation by lactic acid bacteria with some pathogenic bacteria individual in skim milk

| Incubation time (hrs.) | *L. monocytogenes* | *Escherichia coli* | *S. aureus* | *S. typhimurium* | *B. cereus* |
|---|---|---|---|---|---|
| Zero | 5.3 | 5.4 | 5.6 | 5.2 | 5.6 |
| 12 | 4.6 | 4.8 | 5.1 | 4.8 | 5.1 |
| 24 | 4.0 | 4.1 | 4.8 | 4.2 | 4.8 |
| 36 | 3.7 | 3.9 | 4.2 | 4.0 | 4.6 |
| 48 | 3.2 | 3.7 | 3.9 | 3.8 | 4.4 |
| 72 | 3.0 | 3.1 | 3.3 | 3.5 | 3.9 |

Pathogenic organisms were inoculated after 24 h from inoculation of lactic acid bacteria. Acidity was caolculated as % of lactic acid.

Also, prevent the production of toxins from these organisms. Thus, the consumption of fermented milk is useful from hygienic and nutritional viewpoints.

As regards to Table 3 and 4 it can be observed that pH level was in the reversible line with acidity in Table 1 and 2. Thus, the inhibitory activity of lactic acid bacteria against pathogenic organisms used here was very high. Also, it can be observed that, the reduction of pH values to a level 3 to 4 prevented completely the growth of all pathogenic organisms (Table 5 and 6). Thus, at such pH level, all pathogenic organisms were not detected. As regards to the results presented in Table 3 easily observed that, the pH was reduced slightly in the absence of lactic acid bacteria. Massa *et al*. (1997) indicated that *Escherichia coli* 0157: H7 survived during the fermentation and storage of both traditional and bifido yoghurt and pH levels obtained during fermentation of the two products were within the range that has been demonstrated to allow the survival of

Table 5: The counts of pathogenic bacteria (X $10^5$ cfu/ml) in skim milk with the presence or basence of lactic acid bacteria

| Incubation time (hrs.) | *L. monocytogenes* | | *Escherichia coli* | | *S. aureus* | | *S. typhimurium* | | *B. cereus* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | A | B | A | B | A | B | A | B |
| Zero | 20 | 19 | 23 | 24 | 17 | 15 | 28 | 30 | 32 | 30 |
| 12 | 26 | 14 | 29 | 11 | 20 | 3 | 31 | 13 | 51 | 11 |
| 24 | 32 | 11 | 33 | 7 | 27 | 2 | 37 | 3 | 70 | 5 |
| 36 | 37 | 7 | 38 | 1 | 32 | 1 | 45 | 1 | 91 | 3 |
| 48 | 40 | 2 | 44 | 0 | 44 | 1 | 51 | 0 | 113 | 2 |
| 72 | 41 | 0 | 50 | 0 | 55 | 0 | 70 | 0 | 140 | 0 |

A = control (SM incoulated with pathogens only), B = The incoculation of lactic acid bacteria and pathogenic organisms is in the same time at zero time.

Table 6: The count of pathogenic bacteria (x 105 cfu) in skim milk inoculated with lactic acid bacteria (LAB).

| Incubation time (hrs.) | *L. monocytogenes* | *Escherichia coli* | *S. aureus* | *S. typhimurium* | *B. cereus* |
|---|---|---|---|---|---|
| Zero | 47 | 12 | 2 | 21 | 20 |
| 12 | 14 | 4 | 1 | 9 | 7 |
| 24 | 9 | 1 | 0.5 | 0 | 2 |
| 36 | 2 | 0 | 0 | 0 | 0 |
| 48 | 1 | 0 | 0 | 0 | 0 |
| 72 | 0 | 0 | 0 | 0 | 0 |

Skim milk as a medium was inoculated with pathogenic organisms after 24 hrs of the inoculation of. LAB, i.e., zero time.

Table 7: Inhibition activity of culture-fluids of lactic acid bacteria against some pathogenic bacteria

| Test organism | Diameter of inhibition zone in mm | | | |
|---|---|---|---|---|
| | Before boiling | | After boiling | |
| | A | B | A | B |
| *Listeria monocytogenes* | 9 | 20 | 5 | 7 |
| *Escherichia coli* | 8 | 12 | 7 | 8 |
| *Staphylococcus aureus* | 8 | 14 | 5 | 7 |
| *Salmonella typhimurium* | 10 | 15 | 7 | 12 |
| *Bacillus cereus* | 5 | 10 | 3 | 6 |

A = Cell-free extract of lactic acid bacteria incubated for 48 hrs. B = Cell-free extract of lactic acid bacteria incubated for 72 hrs. Disk diameter of 15 mm was excluded.

the pathogen. These observation and findings are similar to those reported by Spelhaug and Harlander (1989), Reinhamier *et al*. (1990), Hassan *et al*. (1994) and Abd-El-Ghani and Hosny (1998).

Data presented in Table 5 and 6 clearly show a reduction in the count of the present pathogenic organisms, which reduced sharply and start to disappear after 24-hrs incubation with the presence of lactic acid bacteria. Pathogens disappeared completely after 72-hrs incubation with the present lactic acid bacteria. Data in Table 6 show clearly the rapid disappearance of pathogens. This result back to the lating inoculation of pathogens with 24 h about the inoculation of lactic acid bacteria, this period (24 h) gave more time to lactic acid bacteria to produce more amount of acidity, which caused the rapid disappearance of pathogens. But, data in Table 5 show that the absence of lactic acid bacteria gives a chance to pathogenic organisms to grow, multiply and increased gradually. This is due to the absence of factors produced by lactic acid bacteria, which inhibit and prevent the growth of these pathogens. Therefore, the count of these pathogens was in the reversible line, when lactic acid bacteria was absent. Lactic acid bacteria produced organic acids and other substances with the beginning of fermentation time and were raising with the prolongation of storage time. Thus, the inhibitory activity was increased with time. However, counts of pathogens were different as compared with those of Table 5 and 6, this means that the inhibitory activity of lactic acid bacteria was different against the pathogenic organisms, but, with the increasing of the reduction of pH, all pathogens disappeared after 3 days incubation. Otero *et al*. (1988) reported that lactic acid bacteria had a slight inhibitory effect on *Staphylococcus aureus* population and only during the late stages of growth. These findings are similar to those obtained by Hassan *et al*. (1994) and Abd-El-Ghani and Hosny (1998).

In order to illustrate the inhibitory potential of lactic acid bacteria against food borne pathogens. Data presented in Table 7 show clearly the inhibitory potential of test organisms against pathogens. Inhibition zones were detected in the agar disc diffusion. However, diffuse zones indicating that the boiling of cell-free extract of lactic acid

bacteria was little effect than those without boiled. This means that, lactic acid bacteria were produced substances inhibits or prevents the growth of pathogens, which is not thermostable. So, the boiling decreased its effect. The effect of cell-free extract of lactic acid bacteria fermented milk for 72 hrs was highly inhibit pathogens than those of 48 hrs as fermentation time, which more producing organic acid (Table 1 and 2). Inhibitory zones differed with the different of pathogens. But, the cell-free extracts of lactic acid bacteria were greatly affected and show highest inhibitory activity against all pathogenic organisms used, both spore forming and non spore forming bacilli and cocci as well as Gram negative and Gram positive bacteria. This means that lactic acid bacteria have wide spectrum inhibition against a wide range of pathogenic organisms. This may be back to the production of organic acid, reduction of pH, production of hydrogen peroxide or the production of antibiotics, i.e., nisin, bacteriocins and brevicin. Spelhaug and Harlander (1989) reported that, numerous lactic acid bacteria produce hydrogen peroxide and in some cases, in sufficient quantity to inhibit pathogenic organisms. Coventry *et al*. (1996) and Santos *et al*. (1996) reported that lactic acid bacteria produced bacteriocins, which are used commercially as antimicrobials in food preservation and other biological applications. Abd-El-Ghani and Hosny (1998) reported similar observations. Finally, it can be easily concluded that the consumption of fermented foods especially fermented milk, which contained lactic acid bacteria are very useful, which repressed and inhibited the food pathogens as well as protect the human from its illness. Therefore, these foods are important from hygienic and nutritional viewpoints.

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