EFFECT OF LACTIC ACID PRODUCING BACTERIA ON COLIFORMS IN RAW MILK

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ABSTRACT

The culture of lactic acid producing bacteria (LAB) was added to refrigerated raw milk in order to inhibit the development of coliform bacteria. The inhibitory activity of different inoculation levels of LAB and pH changes were observed during storage at 7°C. The survival of E. coli $O_{157}:H_7$ during storage at 7°C in sterilized milk inoculated with LAB were evaluated and inoculation levels of 5×10^7 and 1×10^8 cfu/ml of LAB were significantly reduced the growth of E. coli $O_{157}:H_7$. This study suggests that LAB could be used as an effective control of E. coli $O_{157}:H_7$.

INTRODUCTION

Many microorganisms can spoil milk and dairy products. The of shortage in the milk supply to large processing facilities at certain times of the year has led to an increased interest in ways of prolonging the storage life of raw milk (**Griffiths et al., 1987**). Dairy industry practices responsible for extended refrigerated storage before pasteurization (**Cousin, 1982**). Storage of raw milk under refrigeration selects for growth of psychrotrophic coliforms. These bacteria diminish milk quality by means of growth and production of proteolytic enzyme that degrade various milk components (**Fairbairn and Law, 1986**). More attention has been focused on the use of lactic acid bacteria to inhibit these bacterial growth in raw milk (**Juffs and Babel, 1975**). Probiotic bacteria and their health effects are a focus of intensive international research. Focus has generally been on incorporation of selected strain of Lactobacillus spp. into milk in order to exert positive health effect, the microorganisms need to be viable, active and abundant in the concentration of at least 106 cfu g^{-1} in the product throughout the specific shelf life according to **Vinderola et al. (2000)**. Due to the severity of the food borne illness caused by Escherichia coli $O_{157}:H_7$. It has become one of the food industry's primary concerns in the safety of food products. Consuming food contaminated with E. coli $O_{157}:H_7$ can lead to a severe intestinal disorder such as hemorrhagic colitis, which if not treated, can result in hemolytic uremic

syndrome (HUS) which may cause renal failure and possible death. Food of animal origin have been the primary source of E. coli $O_{157}:H_7$ and subsequent outbreaks. 24.6% of the raw milk samples showed E. coli counts above 5 x 104 cfu/ml **(ITC, 2004)**. The purpose of this study was to determine the effectiveness of lactic acid bacteria or probiotic bacteria added to refrigerated milk as an inhibitor of Coliform bacterial growth and to determine if Lactobacillus spp. can exert inhibitory action on E. coli $O_{157}:H_7$ at 7°C on sterilized milk.

MATERIAL AND METHODS

Milk:

Raw milk samples were collected from local cheese factories and examined by storch test to detect the heat-treated milk. In experiments involving inoculation with E. coli $O_{157}:H_7$, the sterilized milk purchased from local supermarket. Coliform counts on the sterilized milk were zero/ml.

Organisms:

Lactobacillus lactis cultures and E. coli $O_{157}:H_7$ strain were obtained from Microbiological Resources Center, Cairo Mireen-Egypt, the Egyptian Microbial Culture Collection (EMCC).

Preparation of microorganisms:

Frozen concentrated cultures of Lactobacillus lactis were prepared by inoculating by (0.1%, V/V) Man Rogosa Sharp (MRS) broth with milk grown cultures, incubating at 37°C for 24 hrs and centrifuging at 2500 x g for 10 min at 4°C. The cell pellet was resuspended in 20 ml. of cold sterile non fat milk to yield a population of approximately $1x10^9$ cfu/ml. The resulting concentrated culture was dispensed in 2 g portions into cryogenic vials and frozen at -196°C in liquid nitrogen until needed. E. coli $O_{157}:H_7$ cultures were maintained in culture agar at 5°C, then grown in nutrient broth at 37°C for 24 hrs to being used in the experiment. Twenty-four hour culture was sediment by centrifugation and pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.0). The cell suspensions were serially diluted to an approximate final concentration of 10^5 cfu/ml.

Inhibition of Coliform growth:

The cultures were adjusted to pH 6.7 with NaOH (5N) prior to addition to milk. Appropriate

amount of a concentrated culture of L. lactis were added to raw milk (200 ml) in sterile conical flasks (500 ml) to yield population of 1 x 10^6 cfu/ml, 1 x 10^7 cfu/ml and 1 x 10^8 cfu/ml of L. lactis. A sample without lactic acid bacteria was a control. The control and inoculated milks were incubated at 7°C for 48 hrs. Coliform counts were determined on days 0 and 2 by plating appropriate dilutions using the pour plate method on Violet Red Bile Agar (VRBA). VRBA plates were incubated at 37...C for 24 hrs. On day 0 the total number of lactobacillus present were determined by plating appropriate dilutions on MRS agar. MRS agar plates were incubated at 37...C for 48 hrs.

Inhibition of E. coli growth:

Appropriate amounts of concentrated cultures were added to sterilized milk (200 ml) in sterile conical flasks (500 ml) to yield population of 5 x 10^7 and 1 x 10^8 cfu/ml of L. lactis. Also, samples were inoculated with E. coli $O_{157}:H_7$ to yield initial population of 1 x 10^5 cfu/ml of E. coli strain. An uninoculated samples served as a negative control. The control and inoculated sterilized milks were incubated at 7°C. One flask from each treatment was analysed on days 0, 2 and 4. Numbers of lactobacillus was determined as described in the previous paragraph and counting of E. coli $O_{157}:H_7$ was achieved by surface direct plating of decimal dilutions of prepared samples (**APHA, 1992**) in which 0.1 ml of each serial dilution was surface plated into Sorbitol-MacConkey agar and incubated at 37°C for 24 hrs.

Determination of pH :

pH of milk samples were determined by using pH meter model SA720 (Orion, USA).

Statistical analysis :

Statistical analysis were done using SAS (**Littell et al., 1991**).

RESULTS

DISCUSSION

The results given in Tables (1 and 2) showed that on day 0 there were no significant changes or differences ($P > 0.05$) in the pH of both control and L. lactis inoculated samples. After 2 days of storage at 7°C there were significant declines in the pH samples inoculated with L. lactis. There was an additional significant decline ($P < 0.05$) in the pH values after 4 days of storage of sterilized milk at the same temperature. Addition of lactic acid bacteria to raw milk resulted in a 0.17 to 0.1 unit decrease in pH in 2 days at 7°C. Although a similar drop (0.17 to 0.1 unit) in pH was obtained in sterilized milk in 2 days at 7°C additional 0.36 to 0.31 unit decrease in 4 days at 7°C. Nearly similar findings were reported by **Griffiths et al. (1991) and Brashears and**

Duzre (1999). However, other workers have reported dramatically pH drop (6.5 to 4.01) of inoculated milk with lactic acid bacteria over 36 hrs at 30°C (**El-Gazzar et al., 1992 and Rossland et al., 2003**). There was no such pH drop during refrigerated storage of milk containing lactic acid bacteria, could be explained according to **Champagne et al. (1990)**, who reported that lactic acid bacteria did not show much growth at 7°C. L. lactis population of 1 x 10^6, 1 x 10^7 and 1 x 10^8 cfu/ml had no an inhibitory effect on total number of Coliform bacteria during storage of raw milk at 7°C. On day 0 and after 2 days there were no significant difference ($P > 0.05$) among the numbers of Coliform bacteria for the four treatments although, there were significant declines in the pH of samples inoculated with L. lactis after 2 days at the same temperature. On contrary, **Reinheimer et al. (1990)** found that the inhibitory activity of cell free filtrates of lactic cultures against a number of Coliform bacteria was inversely proportional to the pH. Experiments involving the direct application of L. lactis to other food, freshly slaughtered beef and pork carcass samples, resulted in significant reduction in the growth of coliforms at 5...C for 6 days (**Senne and Gilliland, 2003**). L. lactis populations of 5 x 10^7 and 1 x 10^8 cfu/ml had an inhibitory effect on total numbers of E. coli O_{157}:H_7 during storage in sterile milk at 7°C. On day 0 there were no significant differences ($P > 0.05$) among the initial populations of E. coli for the three treatment. After 2 days of storage at 7°C, there were significant declines in number of E. coli O_{157}:H_7 for treatments containing 5 x 10^7 and 1 x 10^8 cfu/ml of L. lactis. There were an additional significant decline ($P < 0.5$) in the numbers of cells of E. coli after 4 days of storage for the same two treatments. The number of cells of E. coli O_{157}:H_7 did not change ($P > 0.05$) for the control sample which contained no added lactobacilli (Table 2). The obtained results were in agreement with those reported by **Brashears et al. (1998), Caridi (2002), Senne and Gilliland (2003) and Wilderdyke et al. (2004)**. Inhibitory effect of L. lactis may not have resulted entirely form the low pH caused by acid production but several investigators reported that inhibitory substances other than organic acids i.e. hydrogen peroxide, bacteriocins and diacetyle, can be produced by LAB and are antagonistic toward microorganisms. Furthermore, under the right circumstances, hydrogen peroxide generated by the LAB can activate the lactoperoxidase system to generate hypothiocyanate, which can inactivate or temporary inhibit E. coli (**Ayno et al., 2003**). There is some evidence that lactobacillus may help to improve immunity by increasing the number of antibody secreting cells in the intestinal lining and it may help control blood ulcers in the intestines called Crohn's disease (**Rundles et al., 2000 and Schultz and Sartor 2000**).

Generally, it could be concluded that lactobacillus cultures have potential for use in an interventions technology for the control of food borne pathogens and an extension of shelf life of milk can result from the decrease growth of psychrotrophic spoilage microorganisms.

Table (1) Effect of the addition of lactic acid bacteria on growth of coliforms and pH value of raw milk stored at 7°C

Treatment	Lacto-bacilli/ml[1,3]	Day 0		Day 2	
		Coliforms/ml[1,2]	pH[1]	Coliforms/ml	pH[1]
1	$1x10^6$	a $12x10^2±10^2$	a 6.88	a $11x10^4±1x10^4$	d 6.78
2	$1x10^7$	a $12x10^2±10^2$	a 6.88	a $6x10^4±1x10^4$	c 6.73
3	$1x10^8$	a $11x10^2±5x10$	a 6.88	a $3x10^4±1x10^4$	b 6.71
Control	0	a $12x10^2±5x10$	a 6.88	a $15x10^4±5x10^3$	a 6.84

1) Each value represents the mean of three trials.

2) Reported as colony forming units on VRDA agar.

3) Reported as colony forming units/ml on MRS agar on day 0.

Means with different superscripts in each column are significantly differed at level ($P < 0.05$).

Table (2) pH value and *E. coli* $O_{157}:H_7$ population in treated sterilized milk at 7°C with lactic acid bacteria

Treatment	Lacto-bacilli/ml[1,3]	Day 0		Day 2		Day 4	
		E. coli/ml[1,2]	pH[1]	*E. coli*/ml[1,2]	pH[1]	*E. coli*/ml[1,2]	pH[1]
1	$5x10^7$	a $12x10^4±2x10^4$	a 6.70	b $6x10^3±4x10^2$	b 6.56	b $1x10^2±5$	c 6.39
2	$1x10^8$	a $5x10^4±5x10^3$	a 6.70	b $4x10^3±3x10^3$	b 6.52	b 7x10±2x10	b 6.32
Control	0	a $13x10^4±3x10^4$	a 6.70	a $9x10^5±1x10^5$	a 6.68	a $2x10^7±1x10^6$	a 6.65

1) Each value represents the mean of three trials.

2) Reported as colony forming units on Sorbitol-MacConkey agar.

3) Reported as colony forming units/ml MRS agar on day 0.

Means with different superscripts in each column are significantly differed at level ($P < 0.05$).

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