

## INHIBITION OF SOME PATHOGENS IN FERMENTED MILK BY ZABADY STARTER

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### ABSTRACT

The inhibition of some undesirable microorganisms namely *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Listeria monocytogenes* and *Bacillus cereus* in fermented milk (Zabady) by mixed cultures of yoghurt (*Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbreuckii* subsp. *bulgaricus*) was studied. Results of this study revealed that acidity of Zabady treatments increased, while pH values decreased throughout storage period. The rates of increasing acidity and decreasing pH values were higher in treatments stored at room temperature than those stored at 5-7°C. Counts of target organisms declined sharply in the presence of lactic starter. The rate of disappearance at room temperature was higher than that at 5-7°C. The inhibition zone varied with the different target organisms. Supernatant obtained from the harvest of lactic culture incubated at room temperature had higher inhibitory effect than that at 5-7°C. Non boiled fluid had greatest effect than boiled extracts.

**Keywords:** Lactic acid bacteria (LAB), inhibition, target, yoghurt, *Listeria*, *Escherichia*, *Staphylococcus*, *Salmonella*, *Bacillus*.

### INTRODUCTION

Fermented dairy products have been a major part of the diet around the world. In Egypt, European and Asian countries, there is a long tradition of consumption of fermented milks (Zabady, Yoghurt-types, Kefir, Dahi...etc.), its production and consumption is growing continuously probably because of its therapeutic properties besides its high nutritive value. In Egypt, lactic acid bacteria of Zabady consist mainly of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbreuckii* subsp. *bulgaricus*. Other types of microorganisms including aerobic sporeforming, *Micrococcus* and coliform bacteria have been found, which are considered contaminants in some market Zabady samples (Abou-Donia *et al.*, 1975; Hargrove & Alford, 1978; El-Gendy, 1983, Mohran & Said, 1990 and Zin El-Din & El-Sawah, 1997).

*Listeria monocytogenes* is a Gram positive pathogenic bacterium causing the disease listeriosis, which have been isolated from raw milk, pasteurized milk and other dairy products. This organism survives pasteurization of milk (71.5°C for 15 S). Also, it has the ability to grow at refrigeration temperatures and hence could reach high numbers during storage of dairy products (Fleming *et al.*, 1985; Doyle, 1987; Khattab *et al.*, 1993 and El-Sayed *et al.*, 1998). Also, *Escherichia coli* is considered the main cause of haemorrhagic colitis and of haemolytic uraemic syndrome, which is a leading cause of acute renal failure in children and its course is

fatal in 5% of cases (Massa *et al.*, 1997; Abd El-Ghani & Hosny, 1998 and Shady *et al.*, 1999b). *Staphylococcus aureus* causes gastroenteritis and inflammation of the lining of the stomach and intestine (Zaki *et al.*, 1998 and Shady *et al.*, 1999b). Thus, controlling pathogenic bacteria could reduce food-borne outbreaks and assure consumers a safe, wholesome and nutritive food supply (Kubo *et al.*, 1993; Gomaa & El-Shawaf, 1998 and El-Sawah, 1999).

Lactic acid bacteria are widely used as starters in the manufacture of various dairy products, because of their ability to ferment lactose and produce acid. They also have inhibitory effects on the germination and growth of sporeforming bacteria and other pathogenic bacteria. These useful effects have been reported by several investigators (Driessen & Stadhouders, 1982; Sultan *et al.*, 1988; Mohran & Said, 1990; Smart *et al.*, 1993 and Shady *et al.*, 1999 a& b). Inhibition of food pathogens was attributed to combined effects of acidity, bacteriocins, hydrogen peroxide, high incubation temperatures and other factors. The predominant mechanism depends on conditions of testing, time, organisms used as inhibitors and the pathogenic organisms (Attaie *et al.*, 1987; Otero *et al.*, 1988; Mohammed & Younis, 1990; El-Sawah, 1999; Shady *et al.*, 1999 a&b and Shuhaimi *et al.*, 1999).

This study aimed to investigate the antibacterial effect of mixed culture of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbreuckii* subsp. *bulgaricus* against some spoilage and pathogenic bacteria in fermented milk.

## **MATERIALS AND METHODS**

### **Microorganisms:**

*Streptococcus salivarius* subsp. *thermophilus* STR DRI-VAC 0012209 and *Lactobacillus delbreuckii* subsp. *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 5-7°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 (10% w/v) solids, which was then incubated at 37°C for 18 h. Inocula were prepared from the second culture.

*Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria monocytogenes* as pathogenic organisms were obtained from Dairy Dept., Fac. of Agric., Mansoura Univ., Mansoura, Egypt.

*Bacillus cereus* 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 (Oxoid) and the activating inoculum was prepared after two successive 24-h cultures in TSB incubated at 37°C.

**Media:**

Lactose-M 17 (0.5% w/v) was used for lactic acid bacteria propagation, which consisted of (g/l): peptone from soy meal 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  $\beta$ -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 at pH  $7.2 \pm 0.1$  (Brinchman *et al.*, 1983).

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

The coliform group was counted on violet red bile agar (VRBA), (American Public Health Association, APHA, 1972).

The cfu ml<sup>-1</sup> of *Staphylococcus aureus* was obtained by plating on Baird-Parker medium (Oxoid, 1982). The plates were incubated at 37°C for 48 h, then counted (Otero *et al.*, 1988).

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

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

**Antagonism in contaminated Zabady milk:**

The standardized buffalo milk was boiled for 10-15 min, cooled to room temperature immediately and inoculated with 2.0% (V/V) Zabady starter (*Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbreuckii* subsp. *bulgaricus* equally). The cultured milk was divided into 6 portions; the first part served as a control and a sufficient amount (0.2%) of a 18 hrs-old nutrient broth culture of each contaminant organism was separately added to one portion of the other 5 Zabady milk portions. Contaminant cultures were also individually cultured in milk without Zabady starter. The 0.2% inoculum of spoilage microorganism was selected in order to provide concentration level of approximately 10<sup>5</sup> c.f.u./ml which represent a high level of contamination and to provide strong competition for the starter cultures. The other five portions were inoculated with spoilage microorganism with the present of lactic starter. The above milk portions were separately distributed into 150 ml plastic cubs and incubated at 42°C until complete coagulation for about 4 hrs. The control and contaminated Zabady as well as the milk cultures of contaminant organisms were divided into two groups for storage, one group was stored at 5-7°C (refrigerator) and the other group was stored at 25-30°C (room temperature) for 3 days. Samples were taken and tested for the count of each spoilage microorganism. The inhibition of each spoilage microorganism was obtained by plating the appropriate dilutions on appropriate medium for each pathogen and calculated using the formula of Gilliland & Speck (1977) and El-Sawah (1999).

Percentage of inhibition =

$$\frac{(\text{cfu / ml in control}) - (\text{cfu / ml in associative culture})}{(\text{cfu / ml in control})} \times 100$$

**Detection of antagonistic activity of lactic acid bacteria by the agar diffusion technique:**

The mixed culture of lactic acid bacteria (*Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbreuckii* subsp. *bulgaricus*) which was grown in Zabady (as control) for 48 and 72 hrs at 37°C was vigorously blended and centrifuged. The supernatant culture was collected by centrifugation (10 000 rpm for 10 min at 5-7°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). Two Petri dishes were filled with 15 ml of nutrient agar medium and inoculated with 0.1 ml of the target organisms to provide approximately 10<sup>4</sup> cells/ml. 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 each pathogenic organism. Then petri dishes were kept in the refrigerator for 2h for diffusion then incubated at 37°C for 24 h before examination for inhibition zones.

**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 (Knick-Digital-pH meter 646). The determination was carried out according to the standard method reported by Ling (1963).

## **RESULTS AND DISCUSSION**

Results in Table (1) show the changes in titratable acidity of Zabady produced by lactic starter (*Streptococcus thermophilus* and *Lactobacillus delbreuckii*), which was incubated at 5-7°C and at room temperature. It could be observed that, organic acids (as lactic acid %) produced by Zabady cultures were increased sharply during the incubation period with the fermentation of milk lactose. Acidity of Zabady treatments stored at room temperature were higher than those stored at 5-7°C. Results in Table (3) and Fig. (1) revealed that incubation at room temperature caused more pronounced reduction in counts of pathogenic bacteria, in comparison with

that caused by incubation at 5-7°C. Therefore, zabady became non cohesive and coloured, due to some fungal growth appeared particularly during storage at room temperature. It could be concluded that, organic acids are important for inhibition of foodborne pathogens, which express one of the agents that could reduce foodborne outbreaks. Which, these acids are generally recognized as safe (GRAS) bio-preservative agents. Recent research also revealed that some peptides produced by lactic acid bacteria act as antibiotics on pathogenic bacteria. Therefore, the consumption of fermented foods especially fermented milk (Zabady) is useful from hygienic and nutritional points of view. These observation and results are similar to that reported by Mohran & Said (1990); El-Sawah (1999) and Shady *et al.* (1999 a&b).

**Table (1): Evaluation of acidity in Zabady inoculated with lactic starter and some pathogenic bacteria.**

Time after inoculation (hrs)	Control		Acidity (% as lactic acid)										
			<i>Listeria monocytogenes</i>		<i>Escherichia coli</i>		<i>Staphylococcus aureus</i>		<i>Salmonella typhimurium</i>		<i>Bacillus Cereus</i>		
	1	2	A	B	A	B	A	B	A	B	A	B	
0	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
12	1.5	1.9	1.1	1.7	1.2	1.6	1.2	1.6	1.2	1.4	1.2	1.4	1.4
24	1.8	2.5	1.4	2.1	1.5	2.3	1.6	2.2	1.5	1.9	1.5	1.8	1.8
36	2.2	2.8	1.7	2.5	1.8	2.6	1.9	2.6	1.8	2.2	1.9	2.1	2.1
48	2.4	3.0	2.0	2.7	2.1	2.8	2.1	2.9	2.1	2.8	2.1	2.5	2.5
72	2.6	3.3	2.3	3.0	2.4	3.0	2.3	3.2	2.4	2.9	2.3	3.0	3.0

A= Zabady milk inoculated with lactic starter and pathogenic bacteria and incubated at 5-7°C.

B= Zabady milk inoculated with lactic starter and pathogenic bacteria and incubated at room temperature.

Control 1 = Zabady milk inoculated with lactic starter and incubated at 5-7°C.

Control 2 = Zabady milk inoculated with lactic starter and incubated at room temperature.

Data presented in Table (2) show the reduction in the pH value during storage. This reduction of pH level reached to 3.5 and 4.0. and this level of pH was sufficient to prevent or inhibit the growth of all pathogens in Zabady milk (Table 3 and Fig. 1). Results in Tables 1, 2 and 3 show that there is direct relationship between titratable acidity, pH and the disappearance of any pathogens through the fermentation process. These results are in harmony with those obtained by Thylin *et al.* (1995) and Gonzales de Liano *et al.* (1996) and Zin El-Din & Nasr (1999).

Counts of all pathogens were decreased sharply in the presence of lactic starter (Table 3 and Fig. 1). They were completely inhibited after 3 days, at room temperature. But, the rate of disappearance at room temperature was higher at any time compared with that at 5-7°C. On the other hand, counts of target organisms were increased in the absence of lactic starter. This means that, lactic acid bacteria (LAB) produced organic acids, reduced the pH of the fermented milk (Tables 1 and 2) and produced other substances, all these mechanisms inhibited the pathogenic organisms

and made the fermented food safe. These observations and findings are in agreement with those obtained by Mohammed & Younis (1990); Mohran & Said (1990); Thylin *et al.* (1995); Santos *et al.* (1996); Shady *et al.* (1999 a&b) and Zin El-Din & Nasr (1999).

**Table (2): Change in pH level of Zabady inoculated with lactic starter and some pathogens.**

Time after inoculation (hrs)	Control		pH value									
			Listeria monocytogenes		Escherichia coli		Staphylococcus aureus		Salmonella typhimurium		Bacillus cereus	
	1	2	A	B	A	B	A	B	A	B	A	B
0	6.6	6.6	6.6	6.6	6.6	6.6	6.8	6.8	6.7	6.7	6.6	6.6
12	4.7	4.4	5.0	4.8	5.1	5.0	5.0	4.9	5.2	5.0	5.2	5.0
24	4.6	4.2	4.8	4.6	4.9	4.6	4.7	4.6	4.8	4.6	4.8	4.7
36	4.4	3.9	4.6	4.3	4.7	4.4	4.4	4.4	4.3	4.2	4.6	4.3
48	4.2	3.5	4.4	4.0	4.4	4.2	4.1	4.0	4.1	3.8	4.2	4.0
72	4.0	3.4	4.2	3.5	4.2	3.6	4.0	3.5	4.0	3.6	4.0	3.7

A= Zabady milk inoculated with lactic starter and pathogenic bacteria and incubated at 5-7°C.

B= Zabady milk inoculated with lactic starter and pathogenic bacteria and incubated at room temperature.

Control 1 = Zabady milk inoculated with lactic starter and incubated at 5-7°C.

Control 2 = Zabady milk inoculated with lactic starter and incubated at room temperature.

Table (4) shows the inhibitory activity of Zabady starter fluid against some foodborne pathogenic bacteria, which can cause outbreak for human and animals. These fluids were able to inhibit all tested organisms. The inhibition zone varied between these tested organisms. Also, the cell-free extract obtained from the harvested lactic culture incubated at room temperature had higher inhibitory effect compared with that incubated at 5-7°C. The non-boiled fluid had greatest effect than the boiled ones. This suggests that the antibacterial substances present in the supernatant were affected with the boiling. This supports the idea that these antibacterial substances are of protein nature.

**Table (3): Counts of pathogens (x 10<sup>5</sup> cfu/g) in Zabady inoculated with lactic starter and pathogens.**

Time after inoculation (hrs)	Listeria monocytogenes				Escherichia coli				Staphylococcus aureus				Salmonella typhimurium				Bacillus cereus			
	5-7°C		R		5-7°C		R		5-7°C		R		5-7°C		R		5-7°C		R	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
0	41	40	42	41	29	28	30	31	26	25	27	28	32	30	31	30	53	52	50	51
12	24	43	19	47	17	33	11	37	15	35	11	39	21	35	13	38	37	56	27	58
24	14	50	9	59	11	37	7	42	9	43	6	48	11	40	6	43	17	60	11	62
36	5	52	3	56	5	45	3	49	4	47	2	51	4	44	1	49	11	63	6	65
48	3	54	1	57	2	49	0	55	1	49	0	58	1	46	0	53	6	67	2	70
72	0	51	0	55	1	48	0	51	0	51	0	54	0	45	0	50	2	62	0	66

A= Zabady milk inoculated with lactic starter and pathogenic bacteria.

B= Zabady milk inoculated with pathogenic bacteria only (control).

R= Room temperatures.

**Fig. 1: Inhibition (%) of pathogenic bacteria with lactic starter during 72 hrs as incubation period. = Room temperature, o = 4°C.**

**Table (4): Diameter of inhibition zones (mm) of culture Zabady fluids inoculated with lactic starter against some pathogens.**

Tested Organisms	Diameter of inhibition zone (mm)							
	Non boiled Zabady fluid				Boiled Zabady fluid			
	A		B		A		B	
	5-7°C	R	5-7°C	R	5-7°C	R	5-7°C	R
<i>Listeria monocytogenes</i>	1.6	1.8	1.9	2.1	1.5	1.6	1.7	1.9
<i>Escherichia coli</i>	1.8	2.0	2.0	2.3	1.6	1.7	1.8	2.0
<i>Staphylococcus aureus</i>	1.8	2.3	2.0	2.5	1.6	2.1	1.8	2.2
<i>Salmonella typhimurum</i>	2.0	2.5	2.3	3.0	1.7	1.9	2.1	2.3
<i>Bacillus cereus</i>	1.6	1.9	1.7	2.3	1.3	1.6	1.5	2.0

Disc diameter of 15 mm was excluded.

A= Zabady fluid was obtained after 48 hrs of incubation.

B= Zabady fluid was obtained after 72 hrs of incubation.

R= Room temperature.

The results in Table (4) also show that lactic acid bacteria had an inhibitory effect against spore forming bacilli and cocci as well as Gram negative and Gram positive bacteria. This means that, they have wide inhibition spectrum against a wide range of pathogens. Abd El-Ghani & Hosny (1998); El-Sawah (1999); Shuhaimi *et al.* (1999); and Zin El-Din & Nasr (1999) reported similar results and observations.

As a result of this work, it could be concluded that a mixed culture of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* fermented milks had greatest inhibitory effect on all pathogenic organisms. This could be attributed to the relatively high acidity produced during fermentation and/or the other substances produced by lactic starter that led to earlier disappearance of the pathogens. From the standpoint of the safety of these fermented products, it could be also concluded that the pathogenic microorganisms involved can survive for at least 2-4 days in all products, therefore, strict precaution must be taken to prevent contamination during milk processing and the manufacturing of the fermented products.

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## القضاء على التأثير السمي لبعض الممرضات فى اللبن المتخمّر باستخدام بادي الزبادى

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السموم البكتيرية والتي تفرز من البكتيريا المرضية قد تسبب حالات مرضية عديدة مثل الإسهال والقئ وغيرها ومن الممكن إحداث حالات وبائية كما يحدث فى المدارس والمستشفيات وبعض التجمعات العمرانية الأخرى فى بعض الأوقات ، ولكن وجد لبكتيريا حامض اللاكتيك والتي تستخدم كبادئ فى صناعة بعض منتجات الألبان وبصفة خاصة الزبادى المصرى كمثال للألبان المتخمرة دور حيوى هام فى منع نمو وتثبيط هذه الميكروبات المرضية وبالتالي منع وصول سمومها إلى المستهلكين مما يؤكد أهمية إستهلاك مثل هذه المنتجات ولذلك فقد هدفت هذه الدراسة إلى توضيح بعض العوامل التى تمتلكها هذه البكتيريا فى القضاء على الميكروبات المرضية وخلصت النتائج إلى : إزدادت الحموضة المتكونة تدريجياً مع زيادة فترة التخزين مما نتج عنه نقص مستوى الـ pH فى هذا المنتج إلى الحد الذى أدى إلى نقص أعداد البكتيريات المرضية المختبرة فى هذه الدراسة وكان التخزين على درجة حرارة الغرفة أكثر تأثيراً فى زيادة الحموضة ونقص الـ pH عنه عند التخزين على درجة حرارة الثلاجة . تناقصت أعداد البكتيريات المرضية فى وجود بكتيريا حامض اللاكتيك حتى إختفت بعد 3 أيام تخزين وكان النقص أعلى عند التخزين على درجة حرارة الغرفة . كان لمستخلص الزبادى دوراً فعالاً فى منع وتثبيط البكتيريات المرضية مما يؤكد دور بكتيريا حامض اللاكتيك فى تثبيط بكتيريات الغذاء المرضية وإن قل هذا التأثير بغليان المستخلص ولكنه كان أكثر فى المستخلص المأخوذ من زبادى محضن على درجة حرارة الغرفة

وخلصت هذه الدراسة تؤكد أهمية تناول مثل هذه المنتجات المتخمرة لأهميتها الصحية

والغذائية العالية