
# Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 2 (8): 619-624, 2007
ISSN 1816-4935


# Effects of Temperature and Days of Storage on the Survival and Spoilage Rate of *Bacillus subtilis* and *B. cereus* in Sterilized Milk

[1]Fardous M. Bellow, [1]Sanaa O. Yagoub and [2]Ibtisam E.M. El Zubeir
[1]Department of Microbiology and Molecular Biology,
Faculty of Science and Technology, El Neelain University,
P.O. Box 12702 Khartoum, Sudan
[2]Department of Dairy Production, Faculty of Animal Production,
University of Khartoum, Postal Code 13314, Khartoum North, Sudan

**Abstract:** In the present study the effect and survival of *Bacillus* spp. (*Bacillus subtilis* and *B. cereus*) in milk samples that were collected from Khartoum, Khartoum North and Omduraman were studied. In order to evaluate the degree of spoilage by those organisms, sterile milk sample were inoculated by $3.1\times10^3$ cfu $mL^{-1}$ of *B. cereus* and $3.5\times10^3$ cfu $mL^{-1}$ *B. subtilis* and stored at 7, 12, 21, 37 and 45°C. The milk samples were investigated daily for total bacterial counts, fat %, protein % and acidity. The present results revealed that *Bacillus* spp. inoculated in milk samples showed significant differences for protein, fat, acidity and bacterial count ($p<0.05$) at different storage periods and temperatures. Moreover the milk samples showed a shelf life of more than 5 days at 7 and 12°C. However, the milk samples that stored at 21, 37 and 45°C recorded a shelf life of 1-4 days.

**Key words:** *Bacillus subtilis*, *B. cereus*, milk, storage, temperature, shelf life

## INTRODUCTION

Milk is a good medium for bacteria so it cannot be kept for long time; it spoils and smells very bad (Doreene and Hyatt, 2000). From the time milk leaves the udder till its consumption could become contaminated with many different kinds of bacteria (IDF, 1994). *Bacillus* spp. are quite common in the agricultural environment and many contaminate milk from various sources during the production, storage and processing (Janštová and Lukasora, 2001).

The strain of *Bacillus* isolated from milk has the ability to produce acid and grows fermentatively and most were denitrified in milk (Ternstrom *et al*., 1993). Other species of *Bacillus* such as *B. subtilis* generally affect products that not stored at low temperatures and defects include gas production, acid and sweet coagulation (IDF, 1994).

*Bacillus cereus* is of great significance among *Bacillus* spp. because it can grow quickly, therefore the species may grow at low temperatures and produce enzymes which result in sweet curdling (IDF, 1994). Also *B. cereus* is generally only mildly heat resistant but may occasionally produce highly resistant spores, which can even affect UHT milk. *Bacillus cereus* and *Bacillus subtilis* are both food poisoning and food spoilage organisms (Lechner *et al*., 1998). Similarly Brown (2000) reported that the most common food poisoning from a spore-former is caused by *C. perfringens* and the other food poisoning spore-formers include *Bacillus cereus*, *B. subtilis* and *B. licheniformis*.

Lindsay *et al*. (2000) reported that none of the isolates of *Bacillus* spp. grew below 11°C or above 56°C and optimum growth temperatures were in the high mesophilic range (36-44°C). Hence it is suggested that milk should be stored at 5°C or less to avoid growth of *B. cereus* and *B. subtilis* (Ternstrom *et al*., 1993).

**Corresponding Author:** Ibtisam E.M. El Zubeir, Department of Dairy Production, Faculty of Animal Production, University of Khartoum, Postal Code 13314, Khartoum North, Sudan Fax: +249 85 321246

In Sudan most of the milk offer for sale is raw, so there is great chance to be contaminated by bacteria, which might brought unacceptable changes. Hence The present study was a contribution to evaluate the effect of *Bacillus* species (*B. cereus* and *B. subtilis*) on raw milk and their effect on some constituents of milk during different storage temperatures and periods.

## MATERIALS AND METHODS

### Milk Samples

Raw milk was brought from University of Khartoum farm and examined for microbiological and chemical properties. About 70 mL milk were poured into sterilized glass bottles and plugged with foil paper. The milk samples were sterilized using the autoclave for 30 sec in 120°C.

### Preparation of Inoculum of Organism

Known *Bacillus subtilis* and *B. cereus* that were isolated from raw milk samples were inoculated into nutrient broth medium and incubated at 37°C overnight. After that it was serially diluted (Richardson, 1985) and from the third dilution 0.5 mL were taken and cultured in nutrient agar plates and incubated at 37°C overnight to know the number of organism which inoculated in each milk sample.

### Effect of the Inoculated Bacteria on the Keeping Quality of Milk

The milk samples were inoculated by $3.1\times10^3$ cfu $mL^{-1}$ of *B. cereus* or $3.5\times10^3$ cfu $mL^{-1}$ *B. subtilis* and both stored at different temperatures (7, 12, 21, 37 and 45°C). Then the samples were examined daily for up to 10 days to estimate the effect of the inoculated bacteria on milk protein, fat, acidity and total counts. The fat content was determined by Gerber method and the protein was determined by Kjeldahl method (AOAC, 1990). Similarly the acidity of the samples was determined according to the method described in AOAC (1990). Total viable count was done according to standard methods described by Richardson (1985).

Data were analyzed using Analysis of Variance (ANOVA). Mean separation was also carried out using Duncan's Multiple Range Test.

## RESULTS

As shown in Table 1 the fat content (3.85 and 3.85%) was not significantly ($p>0.05$) affected by the type of *Bacillus* spp. Moreover Table 2 showed that fat content of milk samples inoculated with both *B. cereus* and *B. subtilis* decreased with increasing storage periods and storage temperature.

The protein content showed variation between milk samples inoculated with *B. cereus* (3.431%) *B. subtilis* (3.001%) as shown in Table 1. The protein contents of milk samples inoculated with both *B. cereus* and *B. subtilis* decreased significantly ($p<0.05$) with increasing storage periods and storage temperature. The protein in milk samples inoculated with *B. subtilis* was 6.2% in the first day at 7°C and started to decrease till it reached 1.1% on the ninth day of storage. Moreover it was found that milk samples began to spoil on second and third days at 45 and 37°C, respectively. From day six until day ten, milk samples were spoiled when stored over 7°C (Table 3). The same table showed that the protein content of milk samples inoculated with *B. cereus* at the first day, decreased from 6.7%

Table 1: Variation of milk constituent as affected by type of *Bacillus* species inoculated into milk

| Type of bacteria | Fat (%) | Protein (%) | Acidity (%) | Total count (cfu $mL^{-1}$) |
|---|---|---|---|---|
| *B. cereus* | 3.850$^a$ | 3.431$^a$ | 0.475$^b$ | 8.9814$^c$ |
| *B. subtilis* | 3.850$^a$ | 3.001$^c$ | 0.544$^c$ | 9.2966$^b$ |

Different superscript letter(s) on the same raw are significantly different ($p\leq0.05$)

Table 2: Effect of storage periods on fat content of milk samples inoculated by *Bacillus* species at different temperature

| | | *B. cereus* | *B. subtilis* |
|---|---|---|---|
| Period (days) | Temperature (°C) | Mean±SD | Mean±SD |
| 1 | 7 | 5.40±0.141 | 5.40±0.141 |
| | 12 | 5.20±0.141 | 5.20±0.141 |
| | 21 | 4.50±0.000 | 4.50±0.000 |
| | 37 | 3.80±0.141 | 3.80±0.141 |
| | 45 | 2.60±0.141 | 2.70±0.000 |
| 2 | 7 | 5.20±0.141 | 5.30±0.212 |
| | 12 | 4.90±0.071 | 5.05±0.071 |
| | 21 | 4.05±0.071 | 4.30±0.071 |
| | 37 | - | 3.10±0.071 |
| 3 | 7 | 5.01±0.071 | 5.10±0.212 |
| | 12 | 4.05±0.495 | 4.50±0.000 |
| | 21 | 3.03±0.212 | 4.30±0.071 |
| 4 | 7 | 4.06±0.141 | 5.10±0.141 |
| | 12 | 2.06±0.141 | 3.90±0.071 |
| | 21 | - | 2.40±0.141 |
| 5 | 7 | 4.04±0.141 | 4.20±0.141 |
| | 12 | 1.06±0.071 | 2.40±0.141 |
| 6 | 7 | 1.95±0.071 | 4.00±0.071 |
| 7 | 7 | 4.02±0.212 | 3.30±0.212 |
| 8 | 7 | 3.05±0.071 | 2.60±0.141 |
| 9 | 7 | 3.05±0.071 | 2.20±0.141 |
| 10 | 7 | 2.05±0.071 | 1.80±0.212 |

Table 3: Effect of storage period on protein content of milk samples inoculated by *Bacillus* species at different temperature

| | | *B. cereus* | *B. subtilis* |
|---|---|---|---|
| Period (days) | Temperature (°C) | Mean±SD | Mean±SD |
| 1 | 7 | 6.70±0.000 | 6.20±0.0354 |
| | 12 | 5.30±0.000 | 5.30±0.0212 |
| | 21 | 4.10±0.710 | 3.90±0.000 |
| | 37 | 3.80±0.071 | 3.03±0.004 |
| | 45 | 3.40±0.000 | 2.50±0.000 |
| 2 | 7 | 5.10±0.000 | 5.10±0.071 |
| | 12 | 5.05±0.071 | 4.20±0.000 |
| | 21 | 3.30±0.000 | 2.90±0.0212 |
| | 37 | 2.50±0.071 | 1.90±0.000 |
| 3 | 7 | 5.90±0.000 | 4.80±0.000 |
| | 12 | 4.70±0.071 | 3.10±0.000 |
| | 21 | 2.30±0.000 | 2.10±0.0212 |
| 4 | 7 | 3.60±0.071 | 3.20±0.000 |
| | 12 | 2.10±0.000 | 2.40±0.0212 |
| | 21 | - | 1.90±0.000 |
| 5 | 7 | 2.80±0.000 | 2.90±0.000 |
| | 12 | 1.40±0.071 | 1.20±0.000 |
| 6 | 7 | 2.01±0.000 | 2.30±0.071 |
| 7 | 7 | 1.80±0.000 | 2.05±0.071 |
| 8 | 7 | 1.50±0.071 | 1.90±0.000 |
| 9 | 7 | 1.50±0.000 | 1.30±0.000 |
| 10 | 7 | 1.25±0.071 | 1.10±0.0141 |

at 7°C to 3.4% at 45°C. At the second day, protein content decreased from 5.1% at 7°C and continued to decrease to 2.5±0.08 at 37°C. At the third day, protein content decreased from 5.9% at 7°C to 2.3% at 21°C. At days four and five, milk samples stored at temperature above 12°C were spoiled. The acidity of milk samples revealed significant variations due to increase of both temperature and storage periods in milk inoculated with *B. subtilis* and *B. cereus*. At days four and five milk spoiled at temperature above 12°C and from day six to the end of storage period milk samples were spoiled when stored above 7°C (Table 4).

Table 4: Effect of storage period on acidity of milk samples inoculated by *Bacillus* species at different temperature

| | | *B. cereus* | *B. subtilis* |
|---|---|---|---|
| Period (days) | Temperature (°C) | Mean±SD | Mean±SD |
| 1 | 7 | 0.0372±0.124 | 0.2440±0.000 |
| | 12 | 0.0606±0.267 | 0.2660±0.000 |
| | 21 | 0.0574±0.084 | 0.4720±0.008 |
| | 37 | 0.0854±0.192 | 0.7000±0.016 |
| | 45 | 1.0025±0.071 | 1.0230±0.0141 |
| 2 | 7 | 0.0261±0.008 | 0.0244±0.000 |
| | 12 | 0.0594±0.009 | 0.2660±0.000 |
| | 21 | 0.0617±0.008 | 0.3220±0.000 |
| | 37 | 1.0002±0.000 | 1.0010±0.0141 |
| 3 | 7 | 0.2715±0.008 | 0.2660±0.016 |
| | 12 | 0.0422±0.016 | 0.3330±0.000 |
| | 21 | 0.0639±0.008 | 0.3830±0.008 |
| 4 | 7 | 0.0003±0.000 | 0.2940±0.024 |
| | 12 | 0.0073±0.008 | 0.3940±0.009 |
| | 21 | - | 0.5660±0.016 |
| 5 | 7 | 0.0326±0.008 | 0.3550±0.016 |
| | 12 | 1.0001±0.0141 | 0.4830±0.024 |
| 6 | 7 | 0.0355±0.016 | 0.0040±0.016 |
| 7 | 7 | 0.0377±0.016 | 0.4330±0.062 |
| 8 | 7 | 0.0428±0.008 | 0.4890±0.016 |
| 10 | 7 | 0.0064±0.016 | 0.7500±0.040 |

Table 5: Effect of storage period on total bacterial count of milk samples inoculated by *Bacillus* species at different temperature

| | | *B. cereus* | *B. subtilis* |
|---|---|---|---|
| Period (days) | Temperature (°C) | Mean±SD | Mean±SD |
| 1 | 7 | 8.919±0.00 | 9.919±0.00 |
| | 12 | 9.342±0.00 | 10.340±0.00 |
| | 21 | 9.623±0.00 | 10.624±0.00 |
| | 37 | 10.690±0.00 | 10.708±0.00 |
| | 45 | 9.914±0.00 | 10.914±0.00 |
| 2 | 7 | 8.079±0.00 | 9.079±0.00 |
| | 12 | 8.591±0.00 | 10.597±0.00 |
| | 21 | 8.130±0.00 | 10.701±0.00 |
| | 37 | 9.857±0.00 | 10.862±0.00 |
| 3 | 7 | 8.230±0.00 | 9.230±0.00 |
| | 12 | 8.591±0.00 | 10.713±0.00 |
| | 21 | 8.653±0.00 | 10.838±0.00 |
| 4 | 7 | 8.322±0.00 | 9.322±0.00 |
| | 12 | 8.613±0.00 | 10.725±0.00 |
| | 21 | 9.890±0.00 | 10.899±0.00 |
| 5 | 7 | 8.447±0.00 | 10.449±0.00 |
| | 12 | 9.857±0.00 | 10.862±0.00 |
| 6 | 7 | 8.531±0.00 | 10.535±0.00 |
| 7 | 7 | 8.556±0.00 | 10.531±0.00 |
| 8 | 7 | 8.602±0.00 | 10.605±0.00 |
| 9 | 7 | 8.691±0.00 | 10.743±0.00 |
| 10 | 7 | 8.892±0.00 | 10.834±0.00 |

As shown in Table 5, the total bacterial count of milk samples inoculated with *B. cereus* increased with increasing storage temperature. At day one the total count increased from 8.919 at 7°C to 10.690 at 37°C and then decreased to 9.914 at 45°C. At day four an increase in the bacterial count was noticed from 8.322 at 7°C to 9.890 at 21°C. At day five milk samples stored at temperature above 12°C were spoiled, while those stored above 7°C were spoiled at day six to day ten. Moreover at day four an increase in the bacterial count was noticed at both 7 and 21°C (9.322 and 10.899%, respectively). At day five the milk samples stored at temperature above 12°C were spoiled and at days six to ten the milk samples stored above 7°C were spoiled.

## DISCUSSION

The study revealed that both storage temperature and period have reduced fat and protein content of the inoculated milk. This result supported Labuza and Schmidl (1988) who reported that *B. cereus* and *B. subtilis* reduced fat and protein significantly at different temperatures. From the data on Table 5, it can be deduced that although *B. cereus* and *B. subtilis* were found to show growth at different temperature (7-45°C), the growth was noticed at a higher temperature (45°C). This might be because the heat resistance of the spores and ability of the vegetative cells to produce extra cellular enzymes that might cause the deterioration of milk and milk products (IDF, 1994). Although the storage of milk below 7°C is recommended as a method of preserving milk as stated by Ternstrom *et al.* (1993), the present study revealed growth of *Bacillus* spp. at 7°C. This supported the fact that some *Bacillus* spp. is known to be psychrophilic and able to grow in milk and milk products at cold storage temperature. Further the prolonged storage of the inoculated milk resulted in reduction of the fat content due to *B. cereus* and *B. subtilis* and increased in the total bacterial count, which supported the findings of Janzen (1980).

The spoilage of the milk samples was due to the proteolytic enzymes (IDF, 1994). Also *Bacillus* spp. preferred high temperature (45 and 37°C) to grow well and to spoil milk (Ternstrom *et al.*, 1993). Moreover the higher count of bacteria at higher temperature and prolonged storage period was correlated with the end shelf life and the sensory quality of milk. These were due to proteases which attacked protein causing putrid and off-flavour in milk (Ternstrom *et al.*, 1993).

The acidity was found to be affected by the inoculated bacterial types (*B. cereus* and *B. subtilis*), temperature and storage period. Moreover the increase of acidity of the milk inoculated with *B. subtilis* supported the fact that *B. cereus* attacks sugar slowly to produce acid and returned back to consumption of the fat and protein, when the acidity increased due to lactic acid production and gel formation (Stone, 1952). Moreover Ternstrom *et al.* (1993) found that *Bacillus* spp. produced acid in milk and grew fermentatively and most was denitrified, as the consumption of sugar, fat and protein by *Bacillus* spp. changes the milk flavour resulting in spoilage of milk (IDF, 1994).

It was observed that the number of organisms increased gradually for all inoculated milk samples leading to their spoilage. The deterioration of the milk inoculated by *B. cereus* was more pronounced, which showed lower count than that of *B. subtilis*, although the initial inoculation of the milk samples was relatively the same. Similarly it was observed that *B. cereus* and *B. subtilis* growth was influenced by the increasing temperature, which lead to the increasing acidity and consumption of the remaining milk constituents (Labuza, 1982).

The present study concluded that the studied bacteria were associated with milk spoilage as was shown in the change of milk constituents and shelf life. Similarly the effect was different according to species of bacteria. Moreover, the effect of these bacteria is influenced by both variation of the temperature and the storage periods.

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