# Effect of temperature-time combination and carbon dioxide concentration on inactivation of microorganisms in chilled pasteurized cow milk.

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**Abstract**
*The main objective of this study was to find out the effect of temperature – time (TT) combination and carbon dioxide concentration on inactivation of thermoduric and psychrotrophic microorganisms in chilled pasteurized cow milk. Raw cow milk obtained from the university farm was standardized to 3.25% fat, homogenized and HTST pasteurized using different TT combinations such as 72°C/15 sec ($TT_1$), 74.5°C/13 sec ($TT_2$), 77°C/12 sec ($TT_3$), 79.5°C/10 sec ($TT_4$), 82°C/8 sec ($TT_5$). Pasteurized milk was bottled and chilled at 4 °C. Phosphatase test was conducted to detect the efficiency of pasteurization. In a separate study pasteurized milk was treated with carbon dioxide and sensory threshold was determined using ASTM (American Society for Testing and Materials) E 679 Ascending Concentration Series Method of Limits to select the suitable carbon dioxide level that can be added into milk. Pasteurized bottled milk was treated with 3 levels of carbon dioxide (0, 7 and 15 mM), chilled at 4 °C and analyzed using standard microbiological methods for thermoduric and psychrotrophic counts. Sensory threshold level of carbon dioxide was 15.61 mM. A significant ($P< 0.05$) TT combination x dissolved carbon dioxide concentration x storage time interaction was detected for psychrotrophic and thermoduric counts (log cfu/ml) in pasteurized carbon dioxide added to cow milk. Each TT combination had different psychrotrophic killing effects. $TT_5$ showed significantly higher ($P< 0.05$) psychrotrophic count on day 1. Therefore, this TT combination was not effective in killing psychrotrophs when compared with other TT combinations. A significant reduction of psychrotrophic counts was observed with the increase of carbon dioxide concentration in most of the TT combinations. It clearly indicated that the carbon dioxide has an effect on the destruction of psychrotrophs. $TT_1$ showed the highest increase of psychrotrophic count with the storage period involving the lowest temperature and highest time. There was no clear relationship between each TT combination and between each carbon dioxide concentration for thermoduric counts. Thermodurics increased with storage period in all treatment combinations. In most of the cases, this increment was significant. The above study revealed that the application of small amounts of carbon dioxide could effectively control the growth of psychrotrophic microorganisms in refrigerated pasteurized cow milk without detrimental effects to the product quality.*
**Key words:** psychrotrophs, thermodurics, carbon dioxide

## Introduction

Milk is a complete source of food and hence is a favourable medium for the growth of microbes. In order to destroy the microorganisms and to improve the quality, safety and storage life, application of heat is widely practised in the milk industry. Among the different types of heat treatments, pasteurization is the commonest method of heat processing milk for safety and in most industrialized countries pasteurized milk contributes the largest share of the liquid milk market. Pasteurization is a method of heat treatment that aims at killing pathogenic microorganisms. In addition, pasteurization kills most of the spoilage microorganisms as well. Different temperature time combinations can be applied to get different end products of pasteurization. Refrigerated pasteurized milk

has a limited shelf life due to the presence of Gram-negative psychrotrophic bacteria and thermoduric psychrotrophs. Combination of heat treatment with other processing alternatives can be applied to control the growth of such organisms.

Therefore, a study was conducted to find out the effect of temperature-time combination and carbon dioxide concentration on inactivation of thermoduric and psychrotrophic microorganisms in chilled pasteurized cow milk.

**Materials and methods**

Raw cow milk obtained from the university farm was standardized to 3.25% fat, homogenized in a two stage homogenizer (at 1500 and 2500 p.s.i.) and High Temperature Short Time (HTST) pasteurized using different temperature-time (TT) combinations such as 72°C/15 sec ($TT_1$), 74.5°C/13 sec ($TT_2$), 77°C/12 sec ($TT_3$), 79.5°C/10 sec ($TT_4$), 82°C/8 sec ($TT_5$). Pasteurized milk was bottled and chilled at 4°C. Phosphatase test (SLS C.S. 181:1972) was conducted to detect the efficiency of pasteurization.

In a separate study, pasteurized milk was treated with carbon dioxide (food grade $CO_2$ cylinder with a pressure gauge and a regulator-soft drink dispensing unit was used to introduce $CO_2$) and sensory threshold was determined using ASTM (American Society for Testing and Materials) E 679 Ascending Concentration Series Method of Limits (Meilgaard *et al.*, 1999) to select the suitable $CO_2$ level that can be added into milk. Dissolved $CO_2$ was measured using a gas chromatograph set (Shimadzu, model GC-14B, Tokyo, Japan). Pasteurized bottled milk was treated with 3 levels of $CO_2$ (0, 7 and 15 mM), chilled at 4°C. Thermoduric and psychrotrophic counts were determined using pour plate technique. One ml of each inoculate was pipetted out into sterile Petri dishes aseptically. Standard Plate Count Agar (SPCA) that is cooled to 45°C was carefully poured in to Petri dishes and rotated gently in order to mix and spread the solution evenly. Inoculated Petri dishes were incubated at 32°C for 48 hours and 5-7°C for 10 days for thermodurics and psychrotrophs respectively. A day x temperature-time combination x $CO_2$ concentration (6 x 5 x 3) factorial arrangement of treatments in a complete randomized design with repeated measures was utilized. Data were taken at 6 time periods for the analysis. Differences among treatments were evaluated by analysis of variance. Analysis was performed using SAS (Version 8.1) computer package. Duncan's Multiple Range Test was used for mean comparisons.

**Results and discussion**

Sensory threshold level of $CO_2$ was 15.61mM. Determination of sensory threshold level of $CO_2$ is important because addition of $CO_2$ should be carefully controlled in order to determine that sufficient amount required to minimize or retard the growth of microorganisms is added without being detectable by the consumers. A significant ($p< 0.05$) TT combination x dissolved $CO_2$ concentration x storage time interaction was detected for psychrotrophic count (log cfu/ml) in pasteurized $CO_2$ added cow milk.

Except $TT_5$ (where highest temperature of 82°C and lowest time of 8 sec was applied), at each level of $CO_2$ concentration, there were no significant differences between each TT combination on day 1 (Tables 1a, b and c). $TT_5$ showed significantly higher psychrotrophic count (log cfu/ml) on day 1. Therefore, this TT combination was not effective in killing psychrotrophs when compared with other TT combinations. On day 6 at 15 mM $CO_2$ concentration (Table 1c), the same pattern was observed. However, on the other days, significantly different ($p< 0.05$) psychrotrophic counts were observed in some TT combinations except day 10 at 7 mM $CO_2$ concentration and day 13 at 15 mM $CO_2$ concentration. Therefore, it can be stated that TT combinations had different psychrotrophic killing effects. Psychrotrophic bacteria (eg. *Pseudomonas* spp., *Flavobacterium* etc.) which get access to pasteurized milk due to post pasteurization

contamination, will survive refrigeration temperatures and lead to spoilage of pasteurized milk. Effective pasteurization should destroy all the psychrotrophic microorganisms present in milk. However, keeping quality of correctly pasteurized milk may be severely reduced by post pasteurization contamination; hence, it is important to check that milk has not been contaminated with Gram-negative bacteria after pasteurization and prior to bottling since very low levels of contamination can ultimately lead to high bacterial numbers if strict temperature control is not practiced (Harding, 1997). According to Vernam and Southerland (1994), a number of surveys in the UK, the US and elsewhere, have shown that the level of such contamination is often unacceptably high.

**Table 1. Effect of dissolved $CO_2$ and temperature-time (TT) combination on mean psychrotrophic count (log cfu/ml) of pasteurized milk during 21 days of storage at 4°C**

(a)

| Days of storage | 0 mM $CO_2$ | | | | |
|---|---|---|---|---|---|
| | $TT_1$ | $TT_2$ | $TT_3$ | $TT_4$ | $TT_5$ |
| 1 | $1.49^{cD}$ | $1.33^{abcCD}$ | $1.26^{abcD}$ | $1.5^{cD}$ | $2.16^{dA}$ |
| 6 | $1.78^{fC}$ | $1.53^{eC}$ | $1.25^{bcdD}$ | $1.78^{fC}$ | $2.31^{gAB}$ |
| 10 | $1.99^{eC}$ | $1.46^{cdCD}$ | $1.84^{eC}$ | $1.85^{eC}$ | $2.35^{fAB}$ |
| 13 | $2.33^{cB}$ | $1.23^{abD}$ | $2.18^{cB}$ | $2.19^{cB}$ | $2.18^{cB}$ |
| 17 | $4.25^{fA}$ | $2.31^{dB}$ | $2.23^{dB}$ | $2.31^{dB}$ | $2.3^{dAB}$ |
| 21 | $4.36^{dA}$ | $2.7^{bA}$ | $2.5^{bA}$ | $2.66^{bA}$ | $2.51^{bA}$ |

(b)

| Days of storage | 7 mM $CO_2$ | | | | |
|---|---|---|---|---|---|
| | $TT_1$ | $TT_2$ | $TT_3$ | $TT_4$ | $TT_5$ |
| 1 | $1.12^{abE}$ | $1.26^{abcA}$ | $1.1^{abB}$ | $1.38^{bcB}$ | $2.28^{dA}$ |
| 6 | $1.28^{bcdDE}$ | $1.23^{bcdA}$ | $1.13^{abB}$ | $1.45^{cdeB}$ | $1.62^{efB}$ |
| 10 | $1.45^{cdD}$ | $1.25^{abcA}$ | $1.23^{abcAB}$ | $1.45^{cdB}$ | $1.57^{dB}$ |
| 13 | $2.25^{cC}$ | $1.28^{abA}$ | $1.28^{abAB}$ | $2.14^{cA}$ | $1.38^{bB}$ |
| 17 | $3.8^{eB}$ | $1.43^{cA}$ | $1.05^{aB}$ | $2.13^{dA}$ | $1.33^{bcB}$ |
| 21 | $4.08^{dA}$ | $1.54^{aA}$ | $1.45^{aA}$ | $2.35^{bA}$ | $1.5^{aB}$ |

(c)

| Days of storage | 15mM $CO_2$ | | | | |
|---|---|---|---|---|---|
| | $TT_1$ | $TT_2$ | $TT_3$ | $TT_4$ | $TT_5$ |
| 1 | $1.07^{aC}$ | $1.12^{abAB}$ | $1.09^{abB}$ | $1.06^{aB}$ | $1.44^{cA}$ |
| 6 | $1.05^{abC}$ | $1.06^{abB}$ | $1.02^{aB}$ | $1.07^{abB}$ | $1.48^{deA}$ |
| 10 | $1.07^{abC}$ | $1.05^{aB}$ | $1.1^{abB}$ | $1.27^{bcdAB}$ | $1.33^{bcdA}$ |
| 13 | $1.23^{abC}$ | $1.18^{abAB}$ | $1.13^{abB}$ | $1.07^{aB}$ | $1.15^{abA}$ |
| 17 | $2.38^{cB}$ | $1.1^{abAB}$ | $1.06^{aB}$ | $1.06^{aB}$ | $1.18^{abA}$ |
| 21 | $3.23^{cA}$ | $1.28^{aA}$ | $1.36^{aA}$ | $1.41^{aA}$ | $1.45^{aA}$ |

(n=6)

$^{a, b, c, d, e, f, g}$ Means within the same row without a common superscript differ significantly ($p<0.05$)

$^{A, B, C, D, E}$ Means within the same column without a common superscript differ significantly ($p<0.05$)

($TT_1$=72°C 15 sec., $TT_2$=74.5°C 13 sec., TT3=77 °C12 sec., $TT_4$=79.5°C10 sec., $TT_5$=82°C 8 sec.)

A significant ($p < 0.05$) reduction of psychrotrophs was observed with the increase of $CO_2$ concentration in most of the TT combinations (Table 1 a, b and c). When each of the TT combinations was considered separately with each level of $CO_2$ concentration, the above trend can be observed very clearly indicating that $CO_2$ reduced the growth of psychrotrophs in chilled pasteurized milk even though the survival of those microbes was higher in $TT_5$ in each $CO_2$ level on the 1st day of storage period. $TT_1$ showed the highest increase of psychrotrophic count with the storage period where the lowest temperature and the highest time (72°C/15 sec.) were used. Methods for controlling psychrotrophic bacteria are receiving attention, since psychrotrophic bacterial growth and multiplication in refrigerated milk create quality problems for the dairy industry. Roberts and Torrey (1988) studied the generation times for five psychrotrophic pseudomonads in sterile milk treated with $CO_2$ and ungassed sterile milk and observed that the generation time increases significantly ($p < 0.05$) in $CO_2$ treated milk. Hotchkiss *et al* (1999) demonstrated that the $CO_2$ could effectively inhibit the growth of many food spoilage microorganisms, especially Gram-negative psychrotrophic bacteria. The current study also clearly demonstrates that the $CO_2$ can effectively inhibit the growth of psychrotrophs.

Psychrotrophs increased significantly with the storage period in some TT combinations at each level of $CO_2$ concentration. As indicated in Table 1, in some TT combinations this increment was not significant between two time periods (e.g. day 6 and day 10 of $TT_1$ at 0 level of $CO_2$, day 13 and 17 of $TT_3$ at 0 mM $CO_2$). Psychrotrophic microorganisms are capable of growth at refrigeration temperatures and ultimately cause spoilage of the refrigerated dairy products.

**Table 2. Effect of dissolved $CO_2$ and temperature-time (TT) combination on mean thermoduric count (log cfu/ml) of pasteurized milk during 21 days of storage at 4°C**

(a)

| Days of storage | 0 mM $CO_2$ | | | | |
|---|---|---|---|---|---|
| | $TT_1$ | $TT_2$ | $TT_3$ | $TT_4$ | $TT_5$ |
| 1 | $3.47^{bE}$ | $3.49^{bD}$ | $3.24^{bD}$ | $3.46^{bD}$ | $3.42^{bC}$ |
| 6 | $3.59^{abE}$ | $3.49^{abD}$ | $3.96^{cC}$ | $3.4^{abD}$ | $4.37^{dB}$ |
| 10 | $3.98^{bD}$ | $3.64^{aDC}$ | $4.5^{cC}$ | $3.95^{bC}$ | $4.43^{cB}$ |
| 13 | $4.23^{defC}$ | $3.79^{abcC}$ | $4.24^{defBC}$ | $4.45^{efB}$ | $4.28^{defB}$ |
| 17 | $5.09^{eB}$ | $4.5^{cdB}$ | $4.11^{aB}$ | $4.43^{abcdB}$ | $4.48^{bcdB}$ |
| 21 | $5.36^{bA}$ | $5.18^{bA}$ | $5.12^{bA}$ | $5.18^{bA}$ | $5.23^{bA}$ |

(b)

| Days of storage | 7 mM $CO_2$ | | | | |
|---|---|---|---|---|---|
| | $TT_1$ | $TT_2$ | $TT_3$ | $TT_4$ | $TT_5$ |
| 1 | $3.48^{bC}$ | $3.53^{bC}$ | $2.59^{aE}$ | $3.39^{bD}$ | $3.45^{bD}$ |
| 6 | $3.47^{abC}$ | $3.56^{abC}$ | $3.39^{abD}$ | $3.44^{abD}$ | $3.49^{abD}$ |
| 10 | $4.25^{bcB}$ | $3.48^{aC}$ | $4.23^{bcC}$ | $3.96^{bC}$ | $3.41^{aD}$ |
| 13 | $4.57^{fAB}$ | $3.51^{aC}$ | $4.19^{defC}$ | $4.35^{efB}$ | $3.9^{bcdC}$ |
| 17 | $4.43^{abcdA}$ | $4.45^{abcdB}$ | $4.59^{dB}$ | $4.54^{cbB}$ | $4.4^{abcdB}$ |
| 21 | $4.42^{aA}$ | $5.22^{bA}$ | $5.32^{bA}$ | $5.22^{bA}$ | $5.23^{bA}$ |

(c)

| Days of storage | 15mM $CO_2$ | | | | |
|---|---|---|---|---|---|
| | $TT_1$ | $TT_2$ | $TT_3$ | $TT_4$ | $TT_5$ |
| 1 | $3.5^{bD}$ | $3.36^{bD}$ | $2.69^{aD}$ | $3.4^{bD}$ | $2.65^{aF}$ |
| 6 | $3.71^{bcD}$ | $3.54^{abDC}$ | $3.49^{abC}$ | $3.55^{abCD}$ | $3.3^{aE}$ |
| 10 | $4.04^{bC}$ | $3.48^{aDC}$ | $4.03^{bB}$ | $3.57^{aCD}$ | $3.64^{aD}$ |
| 13 | $4.3^{defBC}$ | $3.74^{abC}$ | $4.15^{cdeB}$ | $3.92^{bcdBC}$ | $4.05^{bcdeC}$ |
| 17 | $4.5^{cdAB}$ | $4.13^{abB}$ | $4.31^{abcdB}$ | $4.23^{abcB}$ | $4.33^{abcdB}$ |
| 21 | $4.73^{aA}$ | $5.16^{bA}$ | $5.31^{bA}$ | $5.18^{bA}$ | $5.09^{bA}$ |

(n=6)

$^{a, b, c, d, e, f,}$ Means within the same row without a common superscript differ significantly ($p<0.05$)
$^{A, B, C, D, E, F}$ Means within the same column without a common superscript differ significantly ($p<0.05$)
($TT_1$=72°C 15 sec, $TT_2$=74.5°C 13 sec, TT3=77°C12 sec, $TT_4$=79.5°C10sec, $TT_5$=82°C 8 sec.)

There was no clear relationship between each temperature time combination used and each $CO_2$ level for thermoduric (including the genera such as *Microbacterium, Micrococcus, Enterococcus, Lactobacillus*) counts. Furthermore, it is very clear that the thermodurics increased with storage period in all treatment combinations. In most of the cases the increment was significant (Table 2 a, b and c). According to Harding (1997), some spore forming bacteria (usually *Bacillus spp.*) which are also psychrotrophic, can survive pasteurization and germinate in the pasteurized milk, ultimately causing spoilage of the finished product. An improvement in shelf life of pasteurized milk may be obtained if the milk is micro filtered to remove thermoduric organisms.

Psychrotrophic species of the genus *Bacillus* become the dominant spoilage organisms at storage temperatures below 5°C when competitive Gram negative micro flora are present only in low numbers, possibly as a result of the imposition of severe precautions against post process contamination in attempts to extend storage life (Vernam and Southerland, 1994). They also observed that freshly pasteurized milk in Scotland should have the thermoduric count of less than $1 \times 10^4$ cfu/ml (less than 4 log cfu/ml). In this experiment the values obtained at day 1 were less than the above in all the treatment combinations of pasteurized $CO_2$ added cow milk. In addition, on day 6 also the counts were less than $1 \times 10^4$ cfu/ml except $TT_5$ at 0 level of dissolved $CO_2$ concentration.

## Conclusions

The above study revealed that the application of small amounts (less than 15.61 mM) of carbon dioxide could effectively control the growth of psychrotrophic microorganisms in refrigerated pasteurized cow milk without affecting the product quality. There was no clear relationship between each TT combination used and each $CO_2$ level for thermoduric counts.

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