Comparative Study Between Conventional Preservatives and Methylene Blue on Chemical and Bacteriological Composition of Raw Milk

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INTRODUCTION

Several types of milk preservatives are used throughout the world. However, each has its specificities and advantages and disadvantages (Barbano et al., 2010). The first dairy chemical preservatives used in Europe were potassium dichromate (PD), mercuric chloride, boric acid, or combinations thereof (Mabon and Brechany, 1983). In the late 1960s, a preservative such as bronopol was also recommended (Grappin and Jeunet, 1970). Historically, there was a preference for potassium dichromate, not only because of its bactericidal power but also because of its specificity, since it does not absorb infrared light in the wavelength band used for milk analysis (Barbano et al., 2010). Sesh et al. (2012) found that PD was suitable to store ass’s and camel’s milk. According to Ng-Kwai-Hang and Hayes (1982), the use of potassium dichromate at an appropriate dose allows milk samples to be stored for 10 days at 4°C, without any significant effect on the chemical composition. However, the presence of potassium dichromate in raw milk samples prevents the determination of acidity, freezing point, and refractive index. In addition, this preservative causes the formation in the milk of an aldehyde-functional compound that may give the false impression of the presence of formalin (Upadhyay et al., 2014). The biocidal power of potassium dichromate can have negative effects on the environment, especially when it is disposed of directly in sewers. That’s why milk samples containing potassium dichromate should be emptied into a specific pipe (Bertand, 1996). According to the New Jersey Department Health (2010), potassium dichromate is very dangerous for human health. It has been declared corrosive and irritating to the skin. Prolonged exposure may cause skin burns and inflammation, blisters, itching, redness, and peeling. Inhalation of potassium dichromate by laboratory technicians may cause pulmonary irritation and even severe overexposure by inhalation may result in death. Currently, the use of potassium dichromate is prohibited in Europe. According to Institut D’Elevage France (2015), the only chemical preservative authorized for raw milk samples intended for performance control is bronopol. However, according to Barbano et al. (2010), bronopol affects milk composition and especially protein content more than fat content. One of the disadvantages of using bronopol is that at high storage temperatures, the product becomes unstable (Upadhyay et al., 2014). At high doses, bronopol can have a bactericidal effect. According to Bumbadiya et al. (2017), a 10% dose of bronopol has proved to be effective in destroying all milk flora and increasing the shelf life of samples even at room temperature. Monardes et al. (1995) suggested that the use of bronopol without yeast and mold inhibitors would not be the ideal storage agent for unrefrigerated sampling in very hot weather conditions. Bronopol is a product that has been declared very toxic when handled incorrectly. In fact, Bronopol is specifically toxic for the liver and stomach. It also

ABSTRACT

The objectives of this study were to compare conventional preservatives used in milk analysis, including potassium dichromate and bronopol with a non-toxic and biodegradable preservative such as methylene blue, to verify their effectiveness and limitations of use. This was achieved by comparing their effects on raw milk composition at different storage temperatures. The best performing preservative doses were 0.1, 0.036 and 0.01% for potassium dichromate, bronopol, and methylene blue respectively. Raw milk composition was significantly affected (p < 0.05) during the storage period at 25°C for all tested preservatives. However, at 4°C, potassium dichromate and bronopol preservatives maintained stable milk composition during 10 days of storage, whereas methylene blue maintained stable milk composition for three days. Methylene blue can be considered as an environmentally friendly and safe alternative for short-term preservation of refrigerated raw milk samples.

Keywords: Methylene blue, Preservatives, Raw milk composition, Storage time, Storage temperature.

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has a negative effect on the kidneys, thyroid, and adrenal glands. Direct exposure to bronopol may also compromise eye, skin, and respiratory health (Marina et al., 2001). The majority of reported toxicities involved people who were working in laboratories (Chang and Lamm, 2003). Until now, no dairy preservative without any effect on handler health has been used. No studies in the literature have shown the use of methylene blue (MB) as a preservative for raw milk samples. Methylene blue was the first synthetic compound ever used as an antiseptic in clinical therapy, and the first antiseptic dye to be used therapeutically (Oz et al., 2011). Methylene blue is an effective photosensitizing agent for the inactivation of pathogenic organisms, including viruses, bacteria, and yeast (Marina et al., 2001). It has also been a lead compound in drug research against various bacterial and viral infections and has been used safely for well over a hundred years (Wainwright and Crossley, 2002).

The objective of this study was to compare conventional preservatives used in milk analysis, notably potassium dichromate and bronopol with a non-toxic and biodegradable preservative which is methylene blue and to study their effects on the chemical and bacteriological composition of raw milk samples stored for 10 days at different temperatures, and to try to determine the optimal dose to use for each preservative.

Materials and Methods

Milk samples were collected from the dairy herd of the Superior Agronomic Institute of Chott-Meriam Susa Tunisia. Samples were quickly taken to the laboratory of animal production in a refrigerated container and were analyzed immediately for total aerobic mesophilic flora (TAMF) according to ISO 4833 (2013) and then stored at 4°C. Physicochemical analyses were carried out using a Lactoscan FTR 120, previously calibrated with guaranteed raw milk samples obtained from SECALAIT France according to ISO 9622 (2013). Freezing point (FP) was determined using an automatic Cryoscope, Cryostar I (Funke Gerber) according to ISO 5764 (2009). Electrical conductivity (EC) was measured using a HUMEAU COND7 conductivity meter according to Hamann and Zeconci (1998).

The optimal dose allowing stable results of chemical and bacteriological composition was determined by varying gradually the quantity of each preservative in raw milk. Each sample was subjected successively to a bacteriological and a physicochemical analysis for each dose of preservative (Table 1).

| Table 1: Preservative dose variations in 100 mL of raw milk |
|-------------------------------------------------------------|
| Dose (g 100 mL-1) | Dose 1 | Dose 2 | Dose 3 |
| Potassium dichromate | 0.1 | 0.2 | 0.3 |
| Bronopol | 0.036 | 0.072 | 0.0108 |
| Methylene blue | 0.005 | 0.01 | 0.015 |

After selecting the dose allowing the physicochemical and bacteriological results closest to the control, the effect of storage time and temperature on the stability of the analytical results for each preservative was tested. For this purpose, a volume of 5 L of raw milk, previously homogenized, was aseptically divided into 10 aliquots (500 mL each). Four aliquots were used to test the effect of each preservative on the physicochemical and bacteriological composition of raw milk, according to ISO 9622 (2013) and ISO 4833-1 (2013) respectively, during 10 days of storage. Two aliquots were kept preservative free as a control. Tested storage temperatures were 4°C and 25°C. Bacteriological and physicochemical analyses of all samples were carried out in 4 stages for both storage temperatures; directly after collection, day (D0), after 3 days (D3), after 7 days (D7) and after 10 days (D10).

Analysis of variance (ANOVA) was performed according to the general linear model (GLM SAS 9.3) procedure, to study the effect of different factors such as preservative type [potassium dichromate (PC), bronopol and methylene blue (MB)], preservative dose, storage time and storage temperature on chemical and bacteriological composition of raw milk samples. Significantly different means were identified using Duncan’s test. All statements of significance were based on a 5% probability. All analyses were carried out in duplicate.

Results and Discussion

Potassium Dichromate

The use of various doses of potassium dichromate (PD) in raw milk did not affect the levels of fat content (FC) and protein content (PC) in raw milk samples. However, it induced a significant increase ($p < 0.05$) in freezing point (FP) and electrical conductivity EC and a significant decrease ($p < 0.05$) in total aerobic mesophilic flora (TAMF). Used at a dose of 0.1%, PD gave results closest to the control for most of the measured parameters. The recorded differences from the control were about 0.03% FC, 0.05% PC, 0.08% Lactose, 0.009°C FP and 0.82 mv EC. The TAMF was significantly reduced to 44 cfu/mL compared to 692,000 cfu mL$^{-1}$ for the control (Table 2).

Effect of Storage Temperature on Potassium Dichromate Samples

Raw milk samples preserved with potassium dichromate (PD) showed significant variation ($p < 0.05$) in all measured parameters during the 10 days of storage at 25°C (Table 3). Starting from the 3rd day of storage, these variations become significant ($p < 0.05$) and characterized by a decrease in FC, FP, and EC, with an increase in PC and lactose content (Table 3). When stored at 4°C, all measured parameters became stable until the 10th day of storage without significant changes ($p > 0.05$), while chemical parameters (FC, PC, Lactose) showed an increasing trend compared to a decreasing trend of the FP (Table 3). Kroger (1985) reported a decrease in the fat content...
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Monardes et al. (1995) indicated a decrease in milk fat content of samples stored with PD at 25°C from 0.013–0.131% after 3 days and to more than 0.1% after 5 days. An experiment conducted by Ng-Kwai-Hang and Hayes (1982) revealed that samples stored for 10 days at 4°C with PD, showed a slight decrease (0.05%) in casein and protein from day 1 to day 4, followed by an increase (0.05%) from day 4 to day 10. For every 2 mg of PD rise per milliliter of raw milk, a 0.07% increase in protein content was observed. However, fat content was not modified. According to Chalermsan et al. (2004), PD does not affect the fat content of milk samples tested within 24 hours of the collection but decreased protein content. Bumbadiya et al. (2017) reported that the problems associated with PD are generally due to an increase of acidity in samples containing this preservative leading to coagulation with time storage progress at room temperature.

Bronopol

The use of bronopol as a preservative at varying doses did not have any effect on the chemical composition (FC, PC, and Lactose) or freezing point of raw milk. However, bronopol significantly affected the EC and TAMF (Table 4). An upward trend was observed for parameters PC, Lactose and CE, compared to a downward trend for FC, FP, and TAMF. The dose 0.036% of bronopol allowed to have chemical composition

**Table 2: Effect of potassium dichromate on physicochemical and bacteriological composition of raw milk**

| Dose  | FC (%) | PC (%) | Lact (%) | FP (°C) | EC (mv) | TAMF (cfu mL⁻¹) |
|-------|--------|--------|----------|---------|---------|----------------|
| 0.1   | 3.21   | 3.19   | 4.86b    | −0.548b | 6.52c   | 44b            |
| 0.2   | 3.28   | 3.24   | 4.94a    | −0.558b | 6.92b   | 38b            |
| 0.3   | 3.29   | 3.31   | 5.04a    | −0.571a | 7.79a   | 35b            |
| Control | 3.24   | 3.14   | 4.78b    | −0.539b | 5.70d   | 692000a        |

abValues assigned with different letters on the same column differ significantly (p<0.05)
FC, fat content; PC, protein content; FP, freezing point; EC, electrical conductivity; TAMF, total aerobic mesophilic flora
Values not assigned by letters do not differ significantly (p>0.05)

**Table 3: Effect of potassium dichromate on milk composition during storage at variable temperatures**

| Storage day | T = 25°C | T = 4°C |
|-------------|----------|---------|
|             | FC       | PC      | Lact    | FP       | EC      | TAMF   |
|             |          |         |         |          |         |        |
| D₀          | 3.21a    | 3.19b   | 4.86b   | −0.548b  | 6.52a   | 44     |
| D₃          | 3.21a    | 3.21b   | 4.88b   | −0.551b  | 6.45b   | 28     |
| D₇          | 3.15b    | 3.25a   | 4.95a   | −0.560a  | 6.42b   | 26     |
| D₁₀         | 3.18b    | 3.29a   | 5.01a   | −0.568a  | 6.40b   | 23     |

| Storage day | T = 25°C | T = 4°C |
|-------------|----------|---------|
|             | FC       | PC      | Lact    | FP       | EC      | TAMF   |
|             |          |         |         |          |         |        |
| D₀          | 3.21     | 3.19    | 4.88    | −0.548   | 6.52    | 44     |
| D₃          | 3.23     | 3.19    | 4.84    | −0.547   | 6.31    | 19     |
| D₇          | 3.24     | 3.20    | 4.88    | −0.551   | 6.30    | 20     |
| D₁₀         | 3.24     | 3.20    | 4.88    | −0.550   | 6.29    | 21     |

abValues assigned with different letters on the same column differ significantly (p<0.05)
FC, fat content (%); PC, protein content (%); FP, freezing point (°C); EC, electrical conductivity (mv); TAMF, total aerobic mesophilic flora (cfu/mL)
Values not assigned by letters do not differ significantly (p>0.05)

**Table 4: Effect of bronopol on physicochemical and bacteriological composition of raw milk**

| Dose  | FC%   | PC%   | Lact%  | FP (°C) | EC (mv) | TAMF (cfu mL⁻¹) |
|-------|-------|-------|--------|---------|---------|----------------|
| 0.036 | 3.25  | 3.15  | 4.81   | −0.542  | 5.91a   | 2b             |
| 0.072 | 3.27  | 3.18  | 4.84   | −0.546  | 6.10a   | 1b             |
| 0.108 | 3.20  | 3.20  | 4.87   | −0.549  | 6.29a   | 0b             |
| Control | 3.24  | 3.14  | 4.78   | −0.539  | 5.70b   | 692000a        |

abValues assigned with different letters on the same column differ significantly (p<0.05)
FC, fat content; PC, protein content; FP, freezing point; EC, electrical conductivity; TAMF, total aerobic mesophilic flora
Values not assigned by letters do not differ significantly (p>0.05)
Comparative Study Between Conventional Preservatives and Methylene Blue on Chemical results (FC, PC, and Lactose), FP and EC closest to those of the control milk with differences about 0.01% FC, 0.01% PC, 0.03% Lactose, 0.003°C FP and 0.21 mv EC. The bactericidal effect of bronopol was more pronounced than that of the other tested preservatives. The TAMF was significantly reduced to 2 cfu mL\(^{-1}\) compared to 692 000 cfu mL\(^{-1}\) of control milk (Table 4).

**Effect of Storage Temperature on Bronopol Samples:**
Samples preserved by bronopol showed significant variation (p < 0.05) in measured parameters throughout the storage period at 25°C (Table 5). This variation was more pronounced from the 3rd day of storage at 25°C. An upward trend was recorded for parameters FC, PC, Lactose, EC, and TAMF against a downward trend for the freezing point. It should be pointed out that on the 10th day of storage at 25°C samples coagulated. The 4°C storage of samples preserved by bronopol allowed the stability of all measured parameters for 10 days (Table 5). Upadhyay et al. (2014) reported that one of the disadvantages of using bronopol is its instability when used in samples stored at high temperatures. To this end, they recommended refrigeration of samples to obtain more reliable analytical results over time. According to Barbano et al. (2010), bronopol has a greater effect on the protein content of raw milk than on fat content. One of the disadvantages of using bronopol is that, at high temperatures, the product becomes unstable, and that is why the sample must be properly refrigerated (Upadhyay et al., 2014). Sanchez et al. (2005) found no significant difference in fat or protein content in goat’s milk samples stored with bronopol for 25 days at 4°C.

**Methylene Blue**
The use of methylene blue (MB) as a preservative in raw milk had no significant effect (p > 0.05) on chemical composition (FC, PC, Lactose), FP and EC. On the other hand, MB significantly affected (p < 0.05) the TAMF (Table 6). The use of MB as a preservative at the dose 0.01% allowed identical results to those obtained with the control milk for the PC, Lactose and FP parameters, while for the FC and EC parameters the differences compared to the control were about 0.02% and 0.02 mv respectively (Table 6).

**Effect of Storage Temperature on Methylene Blue Samples**
The use of methylene blue (MB) as a preservative in raw milk has shown that when samples are stored at 25°C, within three days, they coagulate and become unusable (Table 7). When samples preserved by MB are stored at 4°C, their physicochemical and bacteriological composition remained perfectly stable for 3 days. However, after 7 days of storage at 4°C, the levels of FC, PC, Lactose, and EC increased significantly (p >0.05), while FP and TAMF remained stable (Table 7). The results obtained by using MB as a preservative of raw milk are very encouraging, especially when samples are stored at 4°C for no longer than 3 days. At present, there are no studies in the literature showing the use of MB as a preservative for raw milk samples. Methylene blue was reported to be an effective agent for the inactivation of pathogenic organisms, including viruses, bacteria, and yeast (Marina et al., 2001). Methylene blue has also been a lead compound in drug research against various bacterial and viral infections and has been used safely for well over a hundred years (Wainwright and Crossley, 2002). Perhaps these antiseptic characteristics have conferred to MB blue its role as a preservative. In fact, MB is not harmful to human health and has been used in many areas of clinical medicine due to its therapeutic nature (Wainwright and Crossley, 2002). The use of MB as a preservative in refrigerated storage of raw milk samples can reduce the handling of toxic chemicals having negative effects on the environment, especially when disposed of directly in sewers. Methylene blue can also be a great advantage in preserving the laboratory staff’s health.

| Table 5: Effect of bronopol on milk composition during storage at variable temperature |
|-----------------------------------|-------|-------|-------|-------|-------|-------|
| **Storage day**                  | **T = 25°C** |       |       |       |       |       |
| D\(_0\)                          | 3.25  | 3.15  | 4.81  | –0.542\(^b\) | 5.91\(^a\) | 2\(^c\) |
| D\(_3\)                          | 3.24\(^b\) | 3.14\(^b\) | 4.78\(^b\) | –0.539\(^b\) | 5.86\(^b\) | 10\(^b\) |
| D\(_7\)                          | 3.86\(^a\) | 3.26\(^a\) | 4.96\(^a\) | –0.559\(^a\) | 6.48\(^a\) | 65\(^a\) |
| D\(_{10}\)                       | Coagulated | Coagulated | Coagulated | Coagulated | Coagulated | Coagulated |
| **Storage day**                  | **T = 4°C** |       |       |       |       |       |
| D\(_0\)                          | 3.25  | 3.15  | 4.81  | –0.542 | 5.91  | 2    |
| D\(_3\)                          | 3.24  | 3.15  | 4.78  | –0.539 | 5.85  | 8    |
| D\(_7\)                          | 3.24  | 3.15  | 4.79  | –0.541 | 5.90  | 6    |
| D\(_{10}\)                       | 3.25  | 3.16  | 4.81  | –0.546 | 5.88  | 8    |

(a,b) Values assigned with different letters on the same column differ significantly (p <0.05)
FC, fat content (%); PC, protein content (%); FP, freezing point (°C); EC, electrical conductivity (mv); TAMF, total aerobic mesophilic flora (cfu/mL)
Values not assigned by letters do not differ significantly (p >0.05)
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**Conclusion**

Potassium dichromate and bronopol can be used to store raw milk samples for physicochemical analyses for up to 3 days at 25°C temperature, without any significant changes in chemical composition, freezing point, electrical conductivity, and total aerobic mesophilic flora. However, using methylene blue as a preservative is not possible when raw milk samples are stored at 25°C. Refrigerated storage at 4°C of milk samples preserved respectively by potassium dichromate and bronopol allowed to maintain the stability of all measured parameters for 10 days. On the other hand, samples containing methylene blue could be stored for 3 days under refrigeration, without any change of milk composition. Analytical results obtained by methylene blue as a preservative are very encouraging. Methylene blue can be an efficient alternative solution for short-term refrigerated storage of raw milk samples. In addition, it’s very safe for the handler and can reduce the use of toxic chemicals having negative effects on the environment.

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### Table 6: Effect of methylene blue on physicochemical and bacteriological composition of raw milk

| Dose   | g/100 mL | FC % | PC % | Lact % | FP (°C) | EC (mv) | TAMF (cfu mL⁻¹) |
|--------|----------|------|------|--------|---------|---------|-----------------|
| Dose 1 | 0.005    | 3.08 | 3.15 | 4.80   | −0.540  | 5.75    | 54^b            |
| Dose 2 | 0.010    | 3.26 | 3.14 | 4.78   | −0.539  | 5.72    | 41^b            |
| Dose 3 | 0.015    | 3.38 | 3.15 | 4.80   | −0.541  | 5.75    | 46^b            |
| Control| 0        | 3.24 | 3.14 | 4.78   | −0.539  | 5.70    | 692000^a        |

^abValues assigned with different letters on the same column differ significantly (p <0.05)

FC, fat content; PC, protein content; FP, freezing point; EC, electrical conductivity; TAMF, total aerobic mesophilic flora

Values not assigned by letters do not differ significantly (p >0.05)

### Table 7: Effect of methylene blue on milk composition during storage at variable temperatures

| Storage day | FC   | PC   | Lact | FP   | EC   | TAMF |
|-------------|------|------|------|------|------|------|
| T = 25°C    |      |      |      |      |      |      |
| D0          | 3.26 | 3.14 | 4.78 | −0.539 | 5.72 | 41^b |
| D3          | Coagulated | Coagulated | Coagulated | Coagulated | Coagulated | Coagulated |
| T = 4°C     |      |      |      |      |      |      |
| D0          | 3.26^b | 3.14^b | 4.78^b | −0.540 | 5.74^b | 57   |
| D3          | 3.26^b | 3.14^b | 4.79^b | −0.539 | 5.90^a | 35   |
| D7          | 3.32^a | 3.25^a | 4.94^a | −0.541 | 5.90^a | 36   |
| D10         | 3.36^a | 3.23^a | 4.91^a | −0.546 | 5.90^a | 36   |

^abValues assigned with different letters on the same column differ significantly (p <0.05)

FC, fat content (%); PC, protein content (%); FP, freezing point (°C); EC, electrical conductivity (mv); TAMF, total aerobic mesophilic flora (cfu/mL)

Values not assigned by letters do not differ significantly (p >0.05)
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