Effect of different preservatives on total bacteria count in raw milk for laboratory proficiency test

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Abstract

In 1999, Taiwan established a total bacterial count (<100,000 CFU/mL) as a standard for raw milk acceptance. However, total bacteria count in raw milk are influenced by series factors, such as climate, herd health, bed and milking hygiene, and the equipment performance of automatic milking system. Effective methods are essential for resolving controversies between dairy products factories and farmers when there is abnormal bacterial counts test. This study aims to evaluate the effect of two preservatives, Azidiol (AZ) and bronopol (BR), on total bacterial counts in raw milk stored at 4°C, 25°C, and 37°C. Bacterial counts were measured by using flow cytometry (FossBactoScan FC) and plate counting methods, then collected and stored separately the values of IBC (individual bacterial counts) and CFU (colony-forming unit) after the number of days (0 to 7 days). Results showed that both detection methods indicated that AZ was effective in maintain bacterial counts for 7 days at 4°C, while BR was effective for only 1 day at 25°C. At 37°C, neither preservative maintained bacterial counts beyond the same day. In conclusion, AZ is the optimal choice for maintaining bacterial counts at the stable temperature of 4°C. If the storage temperature is likely instable, the bacteria count test within one day is recommended. These results enhance dairy products factories' ability to evaluate bacterial counts, and also increase better collaboration with farmers.

Keywords: Total bacteria count, Azidiol, Bronopol.

Introduction

Since June 1999, Taiwan has used bacterial counts to grade raw milk quality. When bacterial counts exceed 300,000 CFU/mL, dairy farmers might face deductions of milk payment, and might further lead to termination of contract. High bacterial counts, particularly in summer, impact both dairy farmers' income and dairy plants' profitability. Factors such as udder hygiene, milking procedures, equipment, and air quality influence bacterial levels (Verdier Metz *et al.,* 2009). Automated milking systems has also been linked to temporary increases in bacterial counts (Castro *et al.,* 2017).

Preservatives like Azidiol (AZ) and Bronopol (BR) are used to maintain bacterial stability in raw milk. AZ, a mixture of sodium azide and chloramphenicol, inhibits bacterial growth, but there are some doubts on its environmental persistence (Chang and Lamm, 2003; Oong and Tadi, 2023; Russo *et al.,* 2007; Vigolo *et al.,* 2022). BR, a broad-spectrum preservative, disrupts microbial membranes, reducing bacterial survival (Bryce *et al.,* 1978; Shepherd *et al.,* 1988; Singh and Gandhi, 2015).

This study aims to standardize bacterial count testing in raw milk by evaluating the effects of AZ, BR, and different storage temperatures (4°C, 25°C, 37°C) on bacterial counts stability. Bacterial counts will be assessed using FOSS BactoScan FC (Foss Analytical, Hillerød, Denmark) and total plate count (TPC) methods across multiple storage periods to establish reliable preservation conditions while maintaining original bacterial levels for accurate industry testing.

Materials and methods

Sampling and drug preparation

AZ consists of sodium azide, chloramphenicol, ethanol, sodium citrate tribasic hydrate, and bromophenol blue, while BR is made from 2-bromo-2-nitropropane-1,3-diol. In this study, raw milk (8,640 mL) was collected from the Northern Branch of the Livestock Research Institute and divided into three groups (144 bottles per group, 20 mL each): no preservative (NP), 0.33% AZ, and 0.4% BR. Samples were stored at different temperatures (4°C, 25°C, and 37°C) and tested over 0 to 7 days to evaluate bacterial stability under varying conditions.

Data collection

During the experiment, one sample from each group (NP, AZ, BR) was analyzed daily using the FOSS BactoScan FC to measure individual bacterial counts (IBC). The output in thousand IBC was multiplied by 1,000 to obtain the original IBC value, which was then log-transformed $\log_{10}$(IBC). Then total plate count analysis was conducted by diluting and culturing one sample per group at four dilution levels (10^2–10^5), each with two replicates. After 48 hours, viable bacteria were counted following Ministry of Health and Welfare (Taiwan)(MOHW) guidelines (No. 1121900620), selecting plates with 25–250 colonies to determine colony-forming unit (CFU/mL), then log-transformed $\log_{10}$(CFU).

Statistical analysis

For statistical analysis, $\log_{10}$(CFU) and $\log_{10}$(IBC) were analyzed by R language, considering storage temperature, preservative type, and time. NP at 4°C on day 0 served as the baseline. ANOVA was performed to determine statistical differences between treatment groups.

Results

The purpose of the study is to ensure accurate bacterial count measurements, therefore, NP4-0 (raw milk at 4°C on day 0) was used as the control group. The study aimed to determine the optimal preservative and storage conditions. Results (Table 1) showed that preservative type, temperature, and storage duration significantly affected bacterial counts (P<0.001).

Effect of low-temperature storage (4°C)

FOSS BactoScan FC results (Table 1) showed no significant difference between NP4 0, AZ4-7, and BR4-7 (4.75±0.02, 4.75±0.01, and 4.52±0.06 log_{10}IBC/mL). However, traditional plate count (Table 2) indicated that only AZ4-7 maintained bacterial stability (NP4-0 vs. AZ4-7: 4.09±0.03 vs. 3.96±0.06 log_{10}CFU/mL), while BR4-7 significantly decreased bacterial count (3.24±0.05 log_{10}CFU/mL).

Effect of medium-temperature storage (25°C)

At 25°C, bacterial counts in AZ25-0 and BR25-0 remained stable on day 0 but increased significantly from day 1 (AZ25-1: 4.89±0.53 $_{10}$CFU/mL). FOSS BactoScan FC results showed AZ maintained stability for one day but increased significantly on day 2 (AZ25 2: 6.06±0.59 log_{10}IBC/mL). BR followed a similar trend, maintaining stability for one day in traditional TPC (4.04±0.06 log_{10}CFU/mL) and two days via FOSS BactoScan FC (5.03±0.07 log_{10}IBC/mL).

Effect of high-temperature storage (37°C)

On day 0, bacterial counts were stable in AZ37-0 and BR37-0 (4.19±0.05 and 3.83±0.03 log_{10}CFU/mL). From day 1, AZ counts increased significantly (AZ37-1: 7.21±0.14 log_{10}CFU/mL). BR remained stable via FOSS BactoScan FC but showed a significant decrease from day 2 in traditional TPC (BR37-2: 3.10±0.02 log_{10}CFU/mL), continuing to decline until day 7 (BR37-7: 1.70±0.00 log_{10}CFU/mL).

Discussion

Effects of low-temperature storage (4°C) with different preservatives

Table 1 shows that bacterial counts in the AZ4-7 and BR4-7 groups stored at 4°C for seven days were not significantly different from the control group when analyzed using the FOSS BactoScan FC. However, traditional TPC results (Table 2) indicated that only AZ4-7 maintained bacterial stability, while BR4-7 showed a significantly lower bacterial count than the control group (3.24±0.05 log_{10}CFU/mL). According to Sun *et al* (2023), raw milk stored at 5°C for seven days without preservatives had a bacterial count of approximately 4.8 log_{10}CFU/mL, which closely matches our NP4-7 group, supporting its validity as a reference. Sierra (2009) reported that milk preserved with AZ at 4°C showed no significant bacterial count change between days 0 and 7, which aligns with our findings that AZ effectively stabilizes bacterial counts under low-temperature storage. Additionally, Groxdanovska *et al* (2015) observed that BR reduced bacterial counts by approximately 45% after ten days of storage at 4°C. This trend is consistent with our study, suggesting that while BR initially stabilizes bacterial counts, its effectiveness may decline over time. These results indicate that for low-temperature storage, AZ is a reliable preservative for maintaining bacterial stability in raw milk for up to seven days.

Effects of medium-temperature storage (25°C) with different preservatives

At 25°C, both instrument and traditional culture results showed no significant difference between AZ25-0, BR25-0, and the control group on day 0. However, after one day at 25°C, the bacterial count in the AZ group became significantly higher than the control group when assessed using traditional culture, while BR25-1 remained stable across both methods. Souza *et al* (2012) found that storing raw milk at 20°C with BR for 1–3 days did not significantly affect bacterial counts when tested using automated instruments. This supports our observation that BR can maintain bacterial stability for at least one day at 25°C. However, beyond this point, bacterial counts in both AZ and

Table 1. Bacterial counts of raw milk samples in different temperatures with preservatives for 0–7 days, analyzed by FOSS BactoScan FC.

	Storage							
Treatments / Days	0	1	2	3	4	5	6	7
NP4	4.75 ± 0.02^{2}	4.75 ± 0.02^{3}	4.79 ± 0.02^{4}	4.77 ± 0.01^{3}	4.74 ± 0.07^{3}	4.75 ± 0.02^{3}	4.70 ± 0.01^{3}	4.75 ± 0.05^{3}
AZ4	4.74 ± 0.01^{2}	4.77 ± 0.08^{3}	4.77 ± 0.01^{4}	4.74 ± 0.03^{3}	4.58 ± 0.19^{3}	4.74 ± 0.01^{3}	4.77 ± 0.02^{3}	4.75 ± 0.01^{3}
BR4	4.52 ± 0.02^{2}	4.48 ± 0.04^{3}	4.52 ± 0.02^{4}	4.45 ± 0.04^{3}	4.23 ± 0.16^{3}	4.58 ± 0.13^{3}	4.47 ± 0.02^{3}	4.52 ± 0.06^{3}
NP25	5.04 ± 0.01^{2}	$8.16\pm0.03^{1*}$	T	T	T	T	T	T
AZ25	4.80 ± 0.04^{2}	4.79 ± 0.03^{3}	$6.06\pm0.59^{2*}$	$6.69\pm0.17^{*}$	$7.25\pm0.06^{1*}$	$7.86\pm0.01^{1*}$	$8.01\pm0.13^{1*}$	$8.06\pm0.02^{1*}$
BR25	4.48 ± 0.08^{2}	4.44 ± 0.02^{3}	5.03 ± 0.07^{3}	$6.06\pm0.04^{*}$	$6.52\pm0.02^{*}$	$6.74\pm0.03^{2*}$	$6.88\pm0.03^{2*}$	$7.11\pm0.05^{2*}$
NP37	$5.82\pm0.06^{1*}$	T	T	T	T	T	T	T
AZ37	4.79 ± 0.06^{2}	$6.79\pm0.02^{2*}$	$8.05\pm0.09^{1*}$	T	T	T	T	T
BR37	4.46 ± 0.03^{2}	4.30 ± 0.04^{3}	4.30 ± 0.07^{4}	4.29 ± 0.01^{3}	4.24 ± 0.05^{3}	4.34 ± 0.03^{3}	4.30 ± 0.04^{3}	4.34 ± 0.08^{3}

*Indicates a significant difference in bacterial count compared to NP at 4°C on day 0 (*P*<0.05).

^{123} Indicates a significant difference in bacterial count due to different preservatives and temperatures (*P*<0.05).

T Indicates too many colonies to count.

Table 2. Bacterial counts of raw milk samples in different temperatures with preservatives for 0–7 days, analyzed by TPC.

Treatments \ Days	Storage							
	0	1	2	3	4	5	6	7
NP4	4.09±0.03^{2}	3.73±0.56^{3}	4.00±0.04^{4}	4.01±0.08^{3}	3.94±0.01^{3}	4.03±0.05^{3}	3.91±0.05^{2}	3.85±0.07^{2}
AZ4	4.05±0.02^{2}	4.06±0.09^{3}	3.97±0.01^{3}	4.03±0.01^{3}	3.94±0.09^{3}	3.98±0.02^{3}	3.94±0.02^{2}	3.96±0.06^{2}
BR4	3.97±0.07^{2}	3.88±0.03^{3}	3.66±0.03^{3}	3.60±0.03^{3}	3.57±0.11^{3}	3.43±0.08^{3}	3.38±0.08^{2}	3.24±0.05^{2}
NP25	4.16±0.04^{2}	7.40±0.00^{1*}	T	T	T	T	T	T
AZ25	4.10±0.03^{2}	4.89±0.53^{2*}	6.62±0.75^{1*}	6.74±0.66^{1*}	7.13±0.65^{1*}	7.90±0.01^{1*}	T	T
BR25	3.82±0.03^{2}	4.04±0.06^{3}	5.46±0.03^{2*}	5.94±0.04^{2*}	6.31±0.05^{2*}	6.52±0.01^{1*}	6.77±0.49^{1*}	7.37±0.21^{1*}
NP37	5.88±0.10^{1*}	T	T	T	T	T	T	T
AZ37	4.19±0.05^{2}	7.21±0.14^{1*}	7.40±0.00^{1*}	T	T	T	T	T
BR37	3.83±0.03^{2}	3.10±0.02^{3*}	2.75±0.15^{4*}	1.80±0.17^{4*}	1.85±0.21^{4*}	1.70±0.00^{4*}	2.39±0.12^{3*}	1.70±0.00^{3*}

*Indicates a significant difference in bacterial count compared to NP at 4°C on day 0 (P<0.05).

^{123} Indicates a significant difference in bacterial count due to different preservatives and temperatures (P<0.05).

T Indicates too many colonies to count.

BR groups increased significantly, indicating that these preservatives are not effective for extended storage at moderate temperatures.

Effects of high-temperature storage (37°C) with different preservatives

At 37°C, both instrument and TPC results showed that bacterial counts in AZ and BR groups remained stable on day 0. However, after one day, bacterial counts in the AZ group (AZ37-1) increased significantly. While instrument-based analysis suggested BR preserved bacterial stability over seven days, traditional TPC results showed a significant decline in bacterial counts from day 2 onward, continuing until day 7. Sierra *et al* (2006) reported that BR preserved bacterial counts in raw goat milk stored at 37°C for four days without significant changes when analyzed using automated instruments, which partially aligns with our findings. This difference might be contributed to differences in detection methods. Automated bacterial counting with fluorescence staining detects both live and dead bacteria, while traditional TPC only measures viable bacteria (Gunasekera *et al.,* 2000). This likely explains why traditional TPC results showed a significant decrease in bacterial counts under high-temperature storage. Based on these findings, neither AZ nor BR could effectively preserve bacterial stability at 37°C for extended periods. However, both preservatives can maintain stability for up to one day under high-temperature storage conditions.

Conclusion

This study evaluated the effects of AZ and BR preservatives on bacterial stability in raw milk stored at 4°C, 25°C, and 37°C for 0–7 days using both automated and traditional culture methods. Results showed that AZ effectively maintained bacterial stability for seven days at 4°C, while BR preserved stability for one day at 25°C. At 37°C, both AZ and BR maintained stability only on day 0. In conclusion, maintaining raw milk at 4°C with AZ ensures bacterial stability for seven days, but if temperature fluctuations occur, testing should be completed within the same day or within 24 hours.

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