Journal of Food and Nutrition Research, 2023, Vol. 11, No. 3, 185-192
Available online at http://pubs.sciepub.com/jfnr/11/3/3
Published by Science and Education Publishing
DOI:10.12691/jfnr-11-3-3

Microbiological Assessment of Pasteurized Milk in Japan by Different Testing Methods

Hodaka Suzuki[1,*], Amalia Widya Rizky Sahoed[1], Rikako Midoh[1], Yumiko Okada[2]

[1]College of Agriculture, Ibaraki University, Ami, Ibaraki, Japan
[2]Division of Biomedical Food Research, National Institute of Health Sciences, Kawasaki, Kanagawa, Japan
*Corresponding author: hodaka.suzuki.food@vc.ibaraki.ac.jp

Received February 02, 2023; Revised March 04, 2023; Accepted March 10, 2023

Abstract A total of 73 samples of pasteurized milk retailed in Japan, which comprised 47 low-temperature long-time pasteurized samples (LTLT; 63–66°C, 30 min), 13 high-temperature short-time pasteurized samples (HTST; 72–79°C, 15 sec), and 13 high-temperature long-time pasteurized samples (HTLT; 75–85°C, 15–30 min), were analyzed for hygiene indicator microorganisms (standard plate counts [SPCs], coliforms, Enterobacteriaceae, *Escherichia coli*, and coagulase-positive *Staphylococcus aureus*) to assess microbiological quality using the Japanese Official Method (JOM), International Standard Organization Methods (ISO), and commercial dehydrated medium sheets. Of the 73 milk samples, one LTLT milk sample was positive for both coliforms and Enterobacteriaceae, and another LTLT milk sample exceeded the Japanese microbiological criterion for the SPC (≤50,000 colony forming units (CFU)/mL). All the samples tested were negative for *E. coli* and *S. aureus*. The SPC values obtained using ISO 4833-1:2013 (incubation at 30°C for 72 h) were significantly higher than those obtained using JOM (incubation at 32 °C for 48 h); however, a high correlation was observed (R^2 = 0.7130). In contrast, the correlations between SPCs based on JOM and the two types of dehydrated medium sheets were relatively low (R^2 = 0.1023 and 0.4046, respectively) although these products obtained third-party certification for analyzing many food products. Our results indicate the importance of verifying alternative methods when new methods are introduced for testing new types or categories of food samples.

Keywords: *dehydrated medium sheet, hygiene indicator, method comparison, pasteurized milk*

Cite This Article: Hodaka Suzuki, Amalia Widya Rizky Sahoed, Rikako Midoh, and Yumiko Okada, "Microbiological Assessment of Pasteurized Milk in Japan by Different Testing Methods." *Journal of Food and Nutrition Research*, vol. 11, no. 3 (2023): 185-192. doi: 10.12691/jfnr-11-3-3.

1. Introduction

Foodborne disease outbreaks related to contaminated milk and dairy products are frequently reported [1-3]. Contaminants of milk and dairy products may include harmful microorganisms, such as *Salmonella* spp., Shiga-toxin-producing *Escherichia coli*, *Campylobacter* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, *Yersinia* spp., *Mycobacterium* spp., and *Brucella* spp. However, the initial cell concentration of these pathogens is generally low [4]. Therefore, hygiene indicators, such as standard plate counts (SPCs) and/or presence of Enterobacteriaceae and/or coliforms, are used in many countries to control and evaluate the microbial qualities of milk and dairy products instead of directly detecting pathogens. These hygiene indicator bacteria may also indicate the occurrence of milk spoilage [5].

The microbiological testing methods for milk and dairy products in Japan are described in the "Ministerial Ordinance concerning the Ingredient Standards for Milk and Dairy Products (Order of the Ministry of Health and Welfare No. 52 of 1951)" which was first established by the Ministry of Health and Welfare (presently the Ministry of Health, Labour and Welfare) of Japan in 1951 [6]. SPCs and coliforms have been used as microbiological criteria for evaluating pasteurized milk and certain dairy products to maintain hygiene level of these products. According to Japanese criteria, the SPC is limited to no more than 50,000 colony forming unit (CFU)/mL and coliforms must not be detected in pasteurized milk at the end of its shelf life [6].

In the United States, the SPC must not exceed 20,000 CFU/mL and coliform counts must be limited to no more than 10 CFU/mL in grade A pasteurized milk [7]. In the European Union, Enterobacteriaceae counts must be ≤10 CFU/mL in all five samples in one lot of pasteurized milk [8]; however, this criterion is applied for process hygiene. In the food safety criterion, *L. monocytogenes* should not be detected in five samples (25 mL each) of the product [8].

Dehydrated medium sheets are widely used for microbiological testing instead of the conventional plating method used in the food industry, as their use can save time, labor, and space [9]. A few of these products have been validated as alternatives to conventional methods, such as the Food and Drug Administration's Bacteriological Analytical Manual (BAM) and/or the International Standard Organization Methods (ISO) by third-party

organizations such as AOAC International and French Standardization Association (AFNOR).

In the present study, we aimed to examine the microbiological quality of retail pasteurized milk in Japan. Additionally, to verify the use of commercial dehydrated medium sheets as an alternative method for testing pasteurized milk samples, the results were compared between the official and the alternative methods.

2. Materials and Methods

2.1. Samples

A total of 73 samples of pasteurized milk retailed in Japan were mainly purchased from the Kanto area of Japan (Tokyo, Kanagawa, Chiba, and Ibaraki Prefectures) and stored at below 10°C until further examination. The examination was performed within the shelf-life range of each sample. The milk samples consisted of low-temperature long-time pasteurized milk (LTLT; 63–66 °C, 30 min; n = 47), high-temperature short-time pasteurized milk (HTST; 72-79°C, 15 sec; n = 13), and high-temperature long-time pasteurized milk (HTLT; 75–85°C, 15–30 min; n = 13). Ultra-high temperature sterilized milk (UHT; 120–150°C, 1–3 sec) was not included in this study because few bacteria could be detected in the preliminary study, although more than 90% of milk sold in the Japanese market represents UHT-sterilized milk [10]. All examinations were performed between July 2018 and June 2019.

2.2. Microbiological Analysis

2.2.1. Determination of SPC

The SPC was determined based on the Japanese Official Methods (JOM) described in the Ministerial Ordinance on Milk and Milk Products Concerning Compositional Standards, etc. [6], ISO 4833-1:2013 [11], a modified method of ISO 4833-1, and by using the alternative methods, Petrifilm™ Rapid Aerobic Count (RAC) plates and Aerobic Count (AC) plates (3M Japan Limited, Tokyo, Japan).

Thirty-five milliliter of milk samples were aseptically transferred to 50 mL conical tubes and serially diluted 10-fold with sterilized buffered peptone water (Merck KGaK, Darmstadt, Germany). For JOM and ISO 4833-1, 1 mL each of the original milk samples and their dilutions were plated into two Petri dishes, followed by pouring standard method agar (Nissui Pharmaceutical, Tokyo, Japan), which was held at 47°C and mixed. The plates were incubated at 32 °C for 48 h in JOM and at 30 °C for 72 h in ISO 4833-1. After incubation, the CFUs were counted, and the average count of the two plates in each dilution was calculated. For the modified ISO 4833-1, 0.1 mL of each of the original milk samples and their dilutions were plated onto the surface of two standard method agar plates and incubated at 30 °C for 72 h. Petrifilm™ RAC plates and AC plates were used as the alternative methods to JOM and ISO 4833-1, according to the manufacturer's instructions. The media, sample volumes, and incubation conditions are summarized in Table 1.1. A CFU count below the detection limit of each method was considered to be 0 CFU/mL for further calculations.

2.2.2. Examination of Coliforms

Coliform detection was performed using the JOM [6]. Briefly, 1 mL each of the original milk samples and their dilutions (1/10 and 1/100) were inoculated into two fermentation tubes containing brilliant green lactose bile broth (Eiken Chemical, Tochigi, Japan) and incubated at 35°C for 48 h. A single loop of the samples in which gas production was detected was used to inoculate eosin methylene blue agar plate (EMB; Eiken Chemical) and incubated at 35°C for 24 h. In cases where typical colonies were observed, confirmation tests (lactose broth fermentation and Gram staining) were performed. Colonies that were positive for gas production in the lactose broth fermentation tubes, gram-negative, and non-spore-forming were identified as coliforms. For enumeration of coliforms, Petrifilm™ Coliform Count (CC) plates (3M Japan Limited) were used according to the manufacturer's instructions. CC plates were incubated at 35°C for 24 h. Typical colonies were subjected to the aforementioned confirmation tests after purification. The media, sample volumes, and incubation conditions are summarized in Table 1.2.

Table 1.1. Methods for standard plate counts

	Japanese Official Method	ISO 4833-1 Pour plating	ISO 4833-1 Surface plating	Alternative methods for JOM	Alternative methods for JOM	Alternative methods for ISO	Alternative methods for ISO
Medium	Standard Method Agar	Standard Method Agar	Standard Method Agar	Petrifilm™ RAC Plate	Petrifilm™ AC Plate	Petrifilm™ RAC Plate	Petrifilm™ AC Plate
Sample volume	1 mL x 2 plate	1 mL x 2 plates	0.1 mL x 2 plates	1 mL x 1 plate	1 mL x 1 plate	1 mL x 1 plate	1 mL x 1 plate
Method	Pour plating	Pour plating	Surface plating	Inoculation	Inoculation	Inoculation	Inoculation
Incubation temperature	32°C ± 1°C	30°C ± 1°C	30°C ± 1°C	32 °C± 1°C	32°C ± 1°C	30°C ± 1°C	30°C ± 1°C
Incubation period	48 h ± 3 h	72 h ± 3 h	72 h ± 3 h	24 h ± 2h	48 h ± 3 h	60 h ± 3 h	72 h ± 3 h

Table 1.2. Methods for coliforms

	Japanese Official Method	Alternative method
Medium	Brilliant Green Lactose Bile broth	Petrifilm™ CC Plate
Sample volume	1 mL of original milk sample x 2 tubes, 1 mL of 1/10 dilution x 2 tubes, 1 mL of 1/100 dilution x 2 tubes	1 mL x 1 plate
Method	Inoculation (qualitative)	Inoculation (quantitative)
Incubation temperature	35°C ± 1°C	35°C ± 1°C
Incubation period	48 h ± 3 h	24 h ± 2 h

Table 1.3. Methods for Enterobacteriaceae

	ISO 21528-1	ISO 21528-2	Alternative method
Medium	Plating on VRBG agar plate after enrichment	VRBG Agar plates	Petrifilm™ EB Plate
Sample volume	25 mL	1 mL x 2 plates	1 mL x 1 plate
Method	Inoculation (quantitative)	Pour plating (quantitative)	Inoculation (quantitative)
Incubation temperature	37°C ± 1°C	37°C ± 1°C	37°C ± 1°C
Incubation period	Enrichment: 18 h ± 2 h Plating: 24 h ± 2 h	24 h ± 2 h	24 h ± 2 h

2.2.3. Examination of Enterobacteriaceae

Detection and enumeration of Enterobacteriaceae were performed according to ISO 21528-1:2017 [12] and ISO 21528-2:2017 [13], respectively. Petrifilm™ Enterobacteriaceae Count (EB) plates (3M Japan Limited) were additionally used according to the manufacturer's instructions as an alternative method for enumeration of Enterobacteriaceae. The media, sample volumes, and incubation conditions are summarized in Table 1.3.

2.2.4. Examination of *E. coli*

E. coli was detected using JOM [14]. Briefly, 1 mL of the original milk samples was inoculated into three fermentation tubes of EC Broth (Thermo Fisher Scientific, Basingstoke, UK) and incubated at 44.5°C for 24 h. The samples that tested positive for gas production were used to inoculate EMB agar plates and incubated at 35°C for 24 h. In cases where typical colonies were observed, confirmation tests (lactose broth fermentation and Gram staining) were performed. Colonies that tested positive for gas production in lactose broth fermentation tubes were subjected to confirmation tests (indole production, Voges-Proskauer (VP) test, methyl red reaction, and citrate utilization). Colonies that exhibited indole production, a positive methyl red reaction, a negative VP result, and negative citrate utilization were identified as *E. coli*. The enumeration of β-glucuronidase-positive *E. coli* was performed according to ISO 16649-2:2001 [15]. Briefly, 1 mL of the original milk samples was plated in a Petri dish, followed by pouring sterilized tryptone bile X-glucuronide agar (Merck KGaK), which was then held at 47°C and mixed. The plates were incubated at 44°C for 24 h. Typical colonies were considered to be beta-glucuronidase-positive *E. coli*. Petrifilm™ Select E. coli Count (SEC) plates (3M Japan Limited) were used according to the manufacturer's instructions for *E. coli* enumeration. The SEC plates were incubated at 44°C for 24 h.

2.2.5. Examination of Coagulase-positive *S. aureus*

Coagulase-positive *S. aureus* colonies were enumerated using ISO 6888-1:1999 [16]. Briefly, 0.1 mL of each of the original milk samples were inoculated on two plates of Baird-Parker Agar (BP; Merck KGaK) and incubated at 37°C for 48 h. Typical colonies were purified on tryptic soy agar plates at 37°C for 24 h and subjected to coagulase test using rabbit plasma for confirmation. Petrifilm™ Staph Express Count (STX) plates (3M Japan Limited) were used according to the manufacturer's instructions for *S. aureus* enumeration. STX plates were incubated at 37°C for 24 h. Staph Express Disk (3M Japan Limited) was used according to the manufacturer's instructions to identify *S. aureus*.

2.3. Statistical Analyses

One-way analysis of variance followed by Tukey's post-hoc test was used to compare the SPCs among the different milk samples. The linear regression formula and coefficient of determination were calculated to evaluate the correlation between methods. The normality of the data set was confirmed using the Kolmogorov-Smirnov test, and then the Bland-Altman analysis was performed to compare the details of the methods. A one-sample t-test or Wilcoxon signed-rank test was performed for fixed bias, in which one method provided values that are higher (or lower) than those from the other by a constant amount. Pearson's or Spearman's correlation was calculated for proportional bias, in which one method provided values that are higher (or lower) than those from the other by an amount that is proportional to the level of the measured variable [17]. Statistical analyses were performed using GraphPad Instat® Version 3.10 (GraphPad Software, San Diego, CA, USA), R (version 4.1.2; R Core Team, 2021) [18], or Excel (Microsoft Japan, Tokyo, Japan).

3. Results

Of the 73 milk samples, one LTLT milk sample was positive for both coliforms and Enterobacteriaceae, and another LTLT milk sample exceeded the Japanese microbiological criterion for the SPC (≤50,000 CFU/mL). All the samples tested negative for *E. coli* and coagulase-positive *S. aureus*.

3.1. SPCs of LTLT, HTST, and HTLT Pasteurized Milk Samples

The SPCs in 73 milk samples, including 47 LTLT, 13 HTST, and 13 HTLT milk samples, were determined using the JOM and ISO methods. The SPC ranges based on JOM were 0–5.30 log CFU/mL in LTLT, 0–3.50 log CFU/mL in HTST, and 0–1.74 log CFU/mL in HTLT, respectively (Table 2). The average SPC ± standard deviation based on JOM of the LTLT milk samples was 1.94 ± 0.89 log CFU/mL, and those of HTST milk and HTLT milk samples were 1.61 ± 1.02 log CFU/mL and 0.65 ± 0.74 log CFU/mL, respectively. In contrast, the SPC ranges based on ISO were 0.60–5.42 log CFU/mL in LTLT, 0–3.61 log CFU/mL in HTST, and 0–2.08 log CFU/mL in HTLT, respectively. The average SPC ± standard deviation based on ISO of the LTLT milk samples was 2.28 ± 0.86 log CFU/mL, and those of the HTST and HTLT milk samples were 1.89 ± 1.22 log CFU/mL and 0.88 ± 0.77 log CFU/mL, respectively.

Table 2. Standard plate count of LTLT, HTST, and HTLT pasteurized milk by Japanese Official Method (JOM) and ISO

Methods	Sample	No. of samples	Range (log CFU / mL)	Average ± S.D. (log CFU / mL)
Japanese Official Method	LTLT	47	0 - 5.30	1.94 ± 0.89 [a, b]
	HTST	13	ND - 3.50	1.61 ± 1.02 [a, c]
	HTLT	13	ND - 1.74	0.65 ± 0.74 [d]
ISO	LTLT	47	0.60 - 5.42	2.28 ± 0.86 [a, b]
	HTST	13	ND - 3.61	1.89 ± 1.22 [a, c]
	HTLT	13	ND - 2.08	0.88 ± 0.77 [d]

ND: not detected, $p<0.001$ between b and d, $p<0.05$ between c and d.

When comparing SPCs among LTLT, HTST, and HTLT milk samples determined using both the JOM and ISO, significant differences ($p < 0.05$) were found between LTLT and HTLT milk samples and between HTST and HTLT milk samples, but not between LTLT and HTST milk samples. This is quite reasonable because the pasteurization condition of HTLT milk (75–85°C, 15–30 min) is a higher temperature and requires a longer period than LTLT (63–66°C, 30 min) and HTST (72–79°C, 15 sec) milk samples.

3.2. Comparison and Correlation of Viable Bacterial Counts among the Different Methods

Nine different methods were used to determine the SPC in this study: JOM (pour plating, 32°C, 48 h incubation), ISO (pour plating, 30°C, 72 h incubation), modified ISO (surface plating, 30°C, 72 h incubation), RAC plates (32°C, 24 h incubation; 30°C, 72 h incubation), and AC plates (32°C, 48 h incubation; 30°C, 72 h incubation). The linear regression formula and coefficient of determination among the methods and the mean difference bias (average ± standard deviation), 95% limit of agreement, and p-values for fixed and proportional biases are summarized in Table 3.

3.2.1. Comparison of SPCs between JOM and ISO

The linear regression formula and coefficient of determination between the JOM and ISO were $y = 0.8797x + 0.5057$ and $R^2 = 0.7130$, respectively (Figure 1a). A high correlation exists between JOM and ISO. The Bland-Altman analysis revealed that the CFUs obtained using ISO were 0.31 log higher than those obtained using JOM on average, and a fixed bias existed ($p < 0.001$); however, no proportional bias existed between JOM and ISO ($p > 0.05$) (Figure 1b).

Table 3. Summary of the statistical analysis results

Method A	Method B	Regression analysis		Bland-Altman analysis				
		linear regression formula	coefficient of determination	Kolmogorov-Smirnov test	mean difference bias (S.D.)	95% limit of agreement	fixed bias	proportional bias
JOM	ISO	y = 0.8797x + 0.5057	R^2 = 0.7130	p > 0.05	0.307 (0.575)	-0.820 to 1.434	p < 0.001	p > 0.05
ISO	RAC	y = 0.8812x - 0.3582	R^2 = 0.7566	p > 0.05	-0.594 (0.536)	-1.645 to 0.457	p < 0.001	p > 0.05
ISO	AC	y = 0.9302x - 0.2821	R^2 = 0.7631	p > 0.05	-0.407 (0.542)	-1.469 to 0.655	p < 0.001	p > 0.05
JOM	RAC	y = 0.2065x + 0.1808	R^2 = 0.1023	p > 0.05	-1.131 (1.009)	-3.109 to 0.847	p < 0.001	p < 0.001
JOM	AC	y = 0.5146x + 0.0326	R^2 = 0.4046	p > 0.05	-0.770 (0.797)	-2.331 to 0.791	p < 0.001	p > 0.05
ISO	ISO modified	y = 0.8379x + 0.3419	R^2 = 0.7428	p > 0.05	0.020 (0.476)	-0.913 to 0.954	p > 0.05	p > 0.05

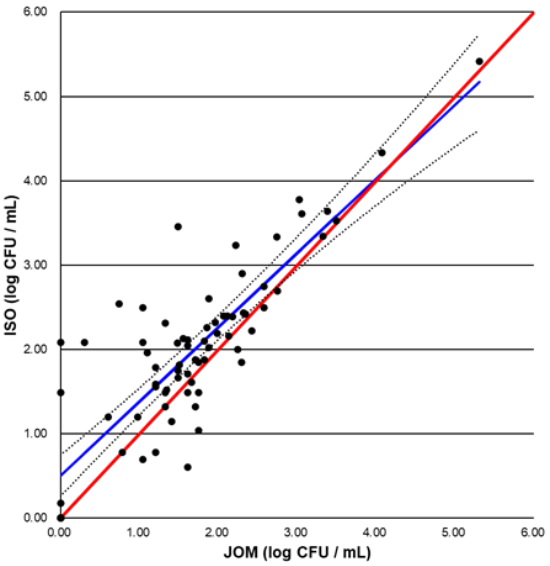

Figure 1a. Correlation between JOM and ISO, Solid blue line: the linear regression best fit line, Dashed black line: 95% confidence interval, Solid red line: ideal correlation line

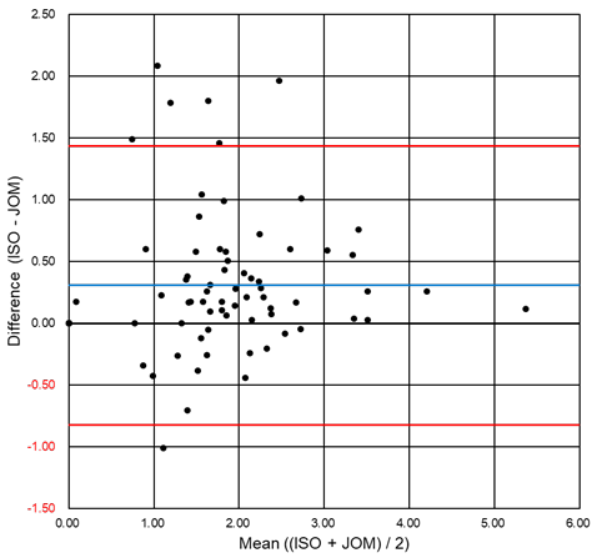

Figure 1b. Bland-Altman plot between JOM and ISO, Solid blue line: the mean difference, Solid red line: upper and lower limits of agreement (mean ±1.96 standard deviations)

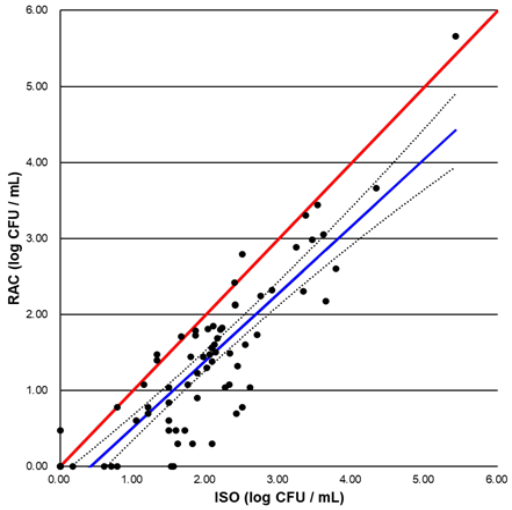

Figure 2a. Correlation between ISO and RAC, Solid blue line: the linear regression best fit line, Dashed black line: 95% confidence interval, Solid red line: ideal correlation line

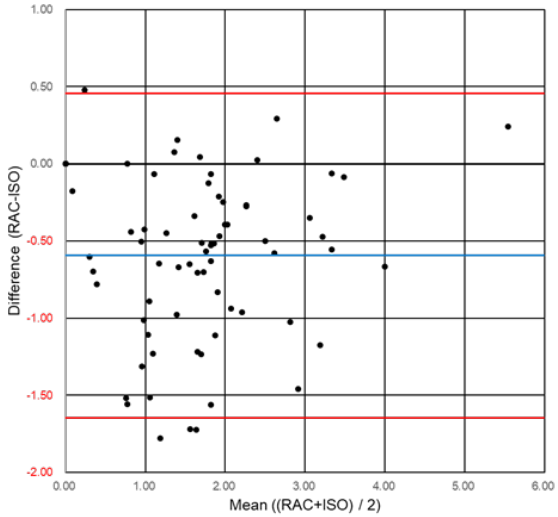

Figure 2b. Bland-Altman plot between ISO and RAC, Solid blue line: the mean difference, Solid red line: upper and lower limits of agreement (mean ±1.96 standard deviations)

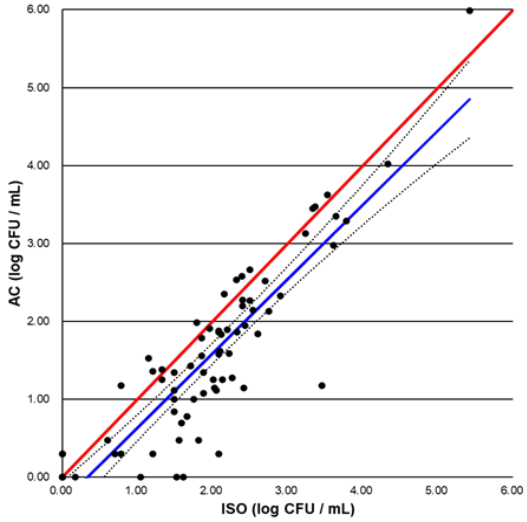

Figure 2c. Correlation between ISO and AC, Solid blue line: the linear regression best fit line, Dashed black line: 95% confidence interval, Solid red line: ideal correlation line

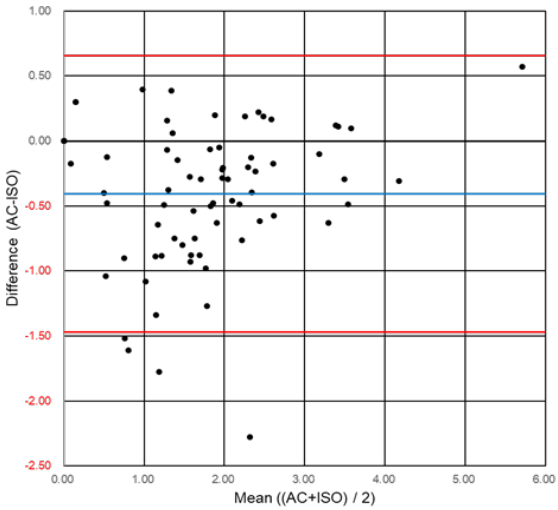

Figure 2d. Bland-Altman plot between ISO and AC, Solid blue line: the mean difference, Solid red line: upper and lower limits of agreement (mean ±1.96 standard deviations)

3.2.2. Comparison of SPCs between ISO and RAC or AC

High correlations were observed between ISO and RAC and between ISO and AC. The linear regression formula and coefficient of determination were y = 0.8812x - 0.3582 and R^2 = 0.7566 between ISO and RAC (Figure 2a), and y = 0.9302x - 0.2821 and R^2 = 0.7631 between ISO and AC (Figure 2c). The Bland-Altman analysis revealed that the CFUs obtained using RAC and AC were 0.59 and 0.41 log lower than those obtained using ISO on average, and fixed biases existed ($p < 0.001$); however, proportional biases did not exist between ISO and RAC and between ISO and AC ($p > 0.05$) (Figure 2b and Figure 2d).

3.2.3. Comparison of SPCs between JOM and RAC or AC

In contrast to the aforementioned results, the correlations between JOM and RAC and between JOM and AC were considered to be low to moderate. The linear regression formulas and coefficients of determination were y = 0.2065x + 0.1808 and R^2 = 0.1023 between JOM and RAC (Figure 3a) and were y = 0.5146x + 0.0326 and R^2 = 0.4046 between JOM and AC (Figure 3c), respectively. The Bland-Altman analysis revealed that the CFUs obtained using RAC and AC were 1.13 and 0.77 log lower than those obtained using JOM on average, and both fixed and proportional biases existed between JOM and RAC ($p < 0.001$); however, only a fixed bias existed between JOM and AC ($p < 0.001$) (Figure 3b and Figure 3d).

3.2.4. Comparison of SPCs between Pour Plating and Surface Plating Methods in ISO 4833-1

Despite the different sample volumes and methods, a high correlation was observed between pour plating and surface plating in ISO 4833-1. The linear regression formula and coefficient of determination were y = 0.8379x + 0.3419 and R^2 = 0.7428, respectively (Figure 4a). The Bland-Altman analysis revealed that the CFUs obtained using surface plating were 0.02 log higher than those obtained using pour plating on average, and no fixed and proportional biases existed between pour plating and surface plating ($p > 0.05$) (Figure 4b).

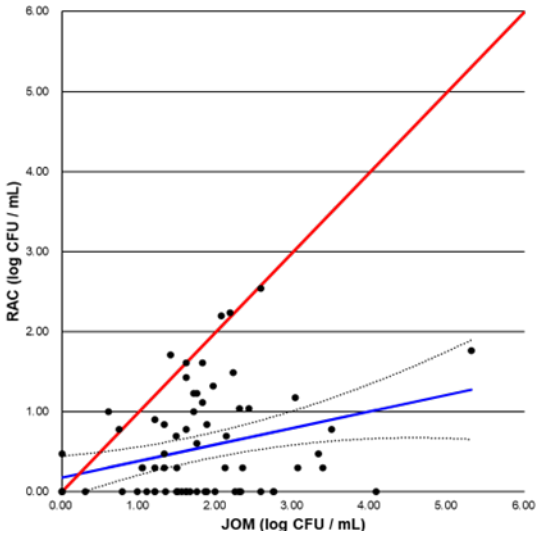

Figure 3a. Correlation between JOM and RAC, Solid blue line: the linear regression best fit line, Dashed black line: 95% confidence interval, Solid red line: ideal correlation line

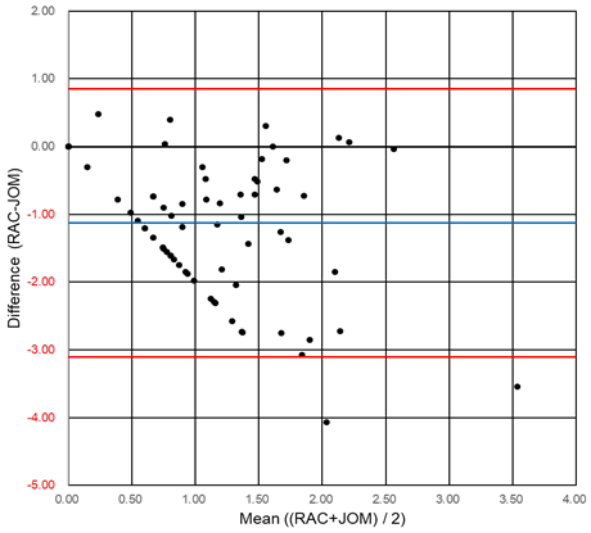

Figure 3b. Bland-Altman plot between JOM and RAC, Solid blue line: the mean difference, Solid red line: upper and lower limits of agreement (mean ±1.96 standard deviations)

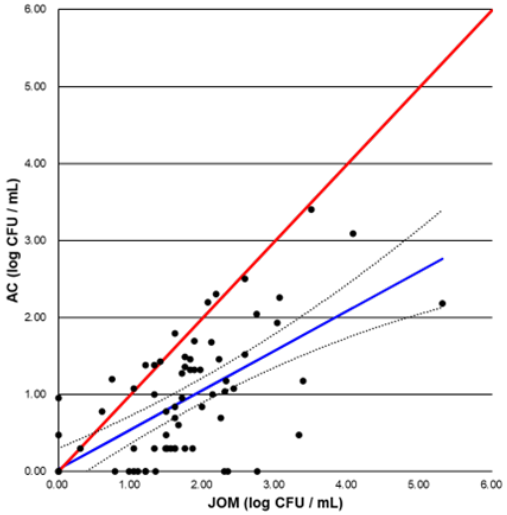

Figure 3c. Correlation between JOM and AC, Solid blue line: the linear regression best fit line, Dashed black line: 95% confidence interval, Solid red line: ideal correlation line

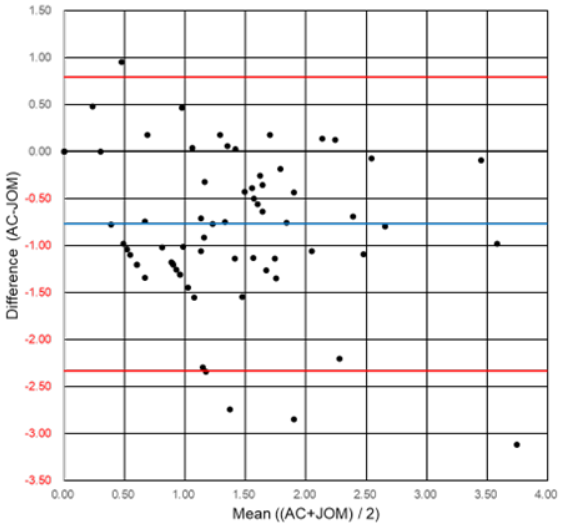

Figure 3d. Bland-Altman plot between JOM and AC, Solid blue line: the mean difference, Solid red line: upper and lower limits of agreement (mean ±1.96 standard deviations)

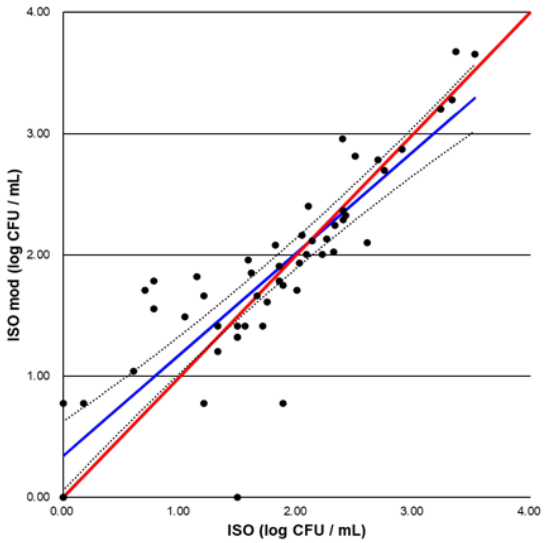

Figure 4a. Correlation between ISO and ISO modified, Solid blue line: the linear regression best fit line, Dashed black line: 95% confidence interval, Solid red line: ideal correlation line

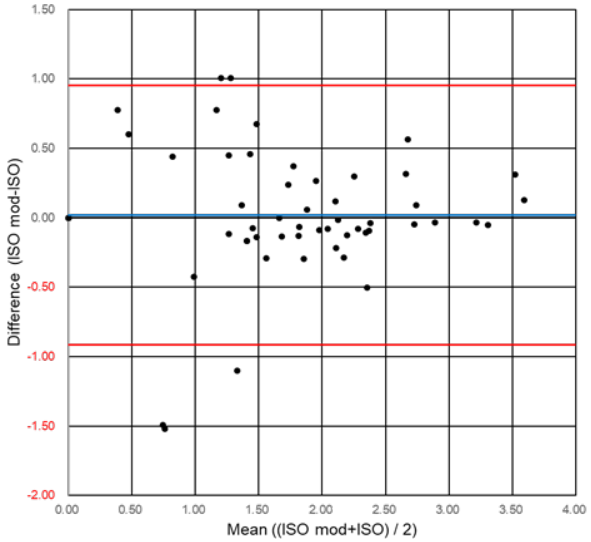

Figure 4b. Bland-Altman plot between ISO and ISO modified, Solid blue line: the mean difference, Solid red line: upper and lower limits of agreement (mean ±1.96 standard deviations)

4. Discussion

Regarding the SPCs of the three types of pasteurized milk, a significantly lower count of viable bacteria was detected for HTLT milk samples than those of LTLT and HTST milk samples. This is reasonable because the pasteurization condition of HTLT milk involves a higher temperature and a longer period than those used in LTLT and HTST processes. In this study, the SPCs determined were almost comparable to that reported by Angelidis et al. [19]; however, seven LTLT milk samples had SPCs of $>10^3$ CFU/mL.

In Japan, the shelf life of pasteurized milk is set to a maximum of 1 week, and temperature of refrigeration in households is set to a maximum of 10 °C. Angelidis et al. showed that SPCs of pasteurized milk samples are increased by approximately 1 log CFU/mL at 4 °C and by more than 2 log CFU/mL at 8 °C within their shelf lives after 3 days [19]. Valik et al. reported that the growth of inoculated *Bacillus cereus* and naturally contaminated SPC in pasteurized milk is largely dependent on the storage temperature of milk, and the growth rates of these bacteria at 9 °C are higher than those at 7 °C [20]. Since the temperature of refrigerators in households is usually set to 10 °C in Japan, the SPCs of LTLT milk products may exceed the regulation (≤50,000 CFU/mL) within their shelf lives. As shown in the present study, LTLT milk products may contain higher SPCs than those of other types of milk. This suggests the importance of strict temperature control before consumption and determination of an adequate period of shelf life of LTLT milk.

Coliforms and Enterobacteriaceae were detected in only one of the 73 milk samples using JOM for coliforms and ISO 21528-1 and 2; they were not detected using the alternative methods Petrifilm™ CC plates and EB plates. The detection limit of JOM for coliforms is 1 CFU/2.22 mL of sample, and those of ISO 21528-1 and ISO 21528-2 are 1 CFU/25 mL and 0.5 CFU/mL, respectively. In contrast, the detection limit of the alternative methods used in this study was 1 CFU/mL. The level of contamination of this sample was 2.5 CFU/mL according to ISO 21528-2, suggesting that the level of contamination of the sample was too low to be evaluated using the alternative methods.

Upon comparing SPCs among the methods, a relatively high correlation was observed between JOM and ISO, although a significantly higher number of colonies was obtained using ISO than that obtained using JOM. Regarding the SPC protocol, ISO and JOM designate the use of the same medium; however, ISO designates the use of a longer incubation period and a lower incubation temperature. Mesophilic bacteria are often present in milk samples; thus, SPCs after incubation at 30°C for 72 h are usually higher than those obtained after incubation at 32°C for 48 h [21]. The countries that determine SPCs at 32°C for 48 h for dairy products coupled with ISO 4833-1 must review their standards of SPCs.

A high correlation was obtained by comparing the pour plating and surface plating methods according to ISO 4833-1. These methods describe the use of the same medium and incubation at the same temperature for the same period; however, they involve the use of different volumes (1 mL vs. 100 μL). The limit of detection of the pour plating method (1 CFU/mL) is theoretically 10 times lower than that of surface plating (10 CFU/mL); however, in the case of milk samples, it is difficult to count colonies after performing pour plating of non-diluted samples because of the cloudiness. Therefore, dilution of the sample is necessary for pour plating, and the limits of detection are almost the same for both methods. Other factors that may affect colony formation include mechanical injury caused to bacterial cells by spreading the inoculum on the surface of the agar or heat injury caused to bacterial cells by high temperature of the pour-plating agar [22]. The results of the spiral plate count method, a type of surface plating method, are comparable to those of the standard plate count method for raw and pasteurized milk [23] and for goat milk cheese [24]. Alonso-Calleja et al. suggested that the suitability of the spiral plate count method may depend on the microbial group examined [24].

The number of colonies obtained through the alternative methods used in this study, Petrifilm™ RAC and AC, showed a significantly lower but relatively higher correlation with that obtained using ISO. In contrast, the number of colonies obtained using RAC and AC was significantly lower and had low-to-moderate correlations with those obtained using JOM. This indicates that these alternative methods, even though validated as alternative methods for ISO by third-party organizations, may be challenging to perform as alternative methods for JOM, at least for testing pasteurized milk. However, this may be mainly attributed to the longer incubation periods in ISO (72 h) than in JOM (48 h). When the incubation periods were longer than those recommended by the manufacturer, better correlations were obtained using JOM. Freitas et al. reported similar results regarding the prevalence of poor dye-reducing bacteria in pasteurized milk [21]. The official Japanese method for SPC determination is similar to BAM Chapter 3. Few of the alternative methods used in the present study are certified by AOAC for analyzing pasteurized milk. However, the pasteurized milk products retailed in United States are usually sterilized using HTST at 72 °C for more than 15 sec [7]; milk products sold in Japan, including the samples used in the present study, are sterilized via LTLT pasteurization. The processing conditions for milk pasteurization differ among countries and they cause differences in the microbiological qualities of the products. The AOAC does not permit matrix extension in their certification, and the results of this study support its reason.

In previous studies, the results obtained using alternative methods have been reported to be comparable to those obtained using conventional methods for testing non-pasteurized raw milk (without heat-injured cells) [25] and/or UHT-sterilized milk (mostly heat-resistant cells) [26]. This suggests that longer incubation periods might be required for facilitating colony formation on the alternative film media by cells injured by pasteurization at 63–85°C. However, many bacterial cell populations in LTLT milk are sublethally injured. This may explain the difference between the results of the pour-plating method and the dehydrated medium sheets obtained in this study.

In pasteurized milk samples that contained heat-injured bacterial cells, the SPCs obtained using dehydrated medium sheets were significantly lower than those obtained using the pour plate method. Sublethal injuries

caused by other food-processing techniques, such as high hydrostatic pressure, dehydration, freezing, and sanitization, may cause similar phenomena, although further studies are necessary. The results in this study suggest that the microbiological characteristics of food processes, such as temperature, have a strong influence on the results obtained using alternative methods. It is also suggested the importance of method verification described in the guideline ISO 16140-3 published in 2021 [27].

Acknowledgements

This work was supported in part by the Ministry of Health, Labour, and Welfare under the Health and Labour Science Research Grant No. H30-shokuhin-ippan-009 and 20KA1007.

References

[1] Asao, T., Kumeda, Y., Kawai, T., Shibata T, Oda, H., Haruki, K., Nakazawa, H. and Kozaki, S. "An extensive outbreak of staphylococcal food poisoning due to low-fat milk in Japan: estimation of enterotoxin A in the incriminated milk and powdered skim milk," *Epidemiology and Infection*, 130(1). 33-40. 2003.

[2] De Buyser, M.L., Dufour, B., Maire, M. and Lafarge, V. "Implication of milk and milk products in food-borne diseases in France and in different industrialised countries," *International Journal of Food Microbiology*, 67(1-2), 1-17. 2001.

[3] Gruber, J.F., Morris, S., Warren, K. A., Kline, K. E., Schroeder, B., Dettinger, L., Husband, B., Pollard, K., Davis, C., Miller, J., Weltman, A., Mattioli, M., Ray, L., Tarr, C. and Longenberger, A.H, "Yersinia enterocolitica outbreak associated with pasteurized milk," *Foodborne Pathogens and Disease*, 18(7), 448-454. 2021.

[4] Heidinger, J.C., Winter, C.K. and Cullor, J.S. "Quantitative microbial risk assessment for Staphylococcus aureus and Staphylococcus enterotoxin A in raw milk," *Journal of Food Protection*, 72(8), 1641-1653. 2009.

[5] Masiello, S.N., Martin, N.H., Trmčić, A., Wiedmann, M. and Boor, K.J. "Identification and characterization of psychrotolerant coliform bacteria isolated from pasteurized fluid milk," *Journal of Dairy Science*, 99(1), 130-140. 2016.

[6] Ministry of Health and Welfare, Ministry of Health and Welfare Ordinance No. 52, "*Ministerial Ordinance on Milk and Milk products Concerning Compositional Standards, etc*." 1951. (in Japanese)

[7] Food and Drug Administration (FDA), *Grade "A" Pasteurized Milk Ordinance 2019 Revision*. 2020. https://www.fda.gov/media/140394/download [accessed on December 25, 2022].

[8] European Union (EU), *COMMISSION REGULATION (EU) No 365/2010 amending Regulation (EC) No. 2073/2005 on microbiological criteria for foodstuffs as regards Enterobacteriaceae in pasteurised milk and other pasteurised liquid dairy products and Listeria monocytogenes in food grade salt*. 2010. https://eur-lex.europa.eu/eli/reg/2010/365/oj [accessed on December 25, 2022].

[9] Linton, R.H., Eisel, W.G. and Muriana, P.M. "Comparison of conventional plating methods and Petrifilm for the recovery of microorganisms in a ground beef processing facility," *Journal of Food Protection*, 60(9), 1084-1088. 1997.

[10] Takagi, K., Yoda, K., Miyazawa, K., Harata, G., He, F., Hiramatsu, M. "Physicochemical properties and sensory attributes of LTLT milk and UHT milk," *Japanese Journal of Sensory Evaluation*, 20: 10-15. 2016.

[11] International Organization for Standardization. ISO 4833-1:2013. Microbiology of the food chain-Horizontal method for the enumeration of microorganisms-Part 1：*Colony count at 30°C by the pour plate technique*. 2013.

[12] International Organization for Standardization. ISO 21528-1:2017. Microbiology of the food chain-Horizontal method for the detection and enumeration of Enterobacteriaceae-Part 1: *Detection of Enterobacteriaceae*. 2017.

[13] International Organization for Standardization. ISO 21528-2:2017. Microbiology of the food chain-Horizontal method for the detection and enumeration of Enterobacteriaceae-Part 2：*Colony-count technique*. 2017.

[14] Ministry of Health and Welfare, *Standards set by the Ministry of Health notice No. 370*. 1959.

[15] International Organization for Standardization. ISO 16649-2:2001. Microbiology of food and animal feeding stuffs-Horizontal method for the enumeration of b -glucuronidase-positive Escherichia coli-Part 2: *Colony-count technique at 44 using 5-bromo-4-chloro-3-indolyo b-D-glucuronidase*. 2001.

[16] International Organization for Standardization. ISO 6888-1:1999. Microbiology of food and animal feeding stuffs-Horizontal method for the enumeration of coagulase-positive staphylococci (Staphylococcus aureus and other species)-Part 1: *Technique using Baird-Parker agar medium*. 1999.

[17] Ludbrook, J. "Comparing methods of measurements," *Clinical and Experimental Pharmacology and Physiology*, 24(2), 198-203. 1997.

[18] R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2021. URL https://www.R-project.org/.

[19] Angelidis, A.S., Tsiota, S., Pexara, A. and Govaris, A. "The microbiological quality of pasteurized milk sold by automatic vending machines," *Letters in Applied Microbiology*, 62(6), 472-479. 2016.

[20] Valík, Ľ., Görner, F. and Laukova, D. "Growth dynamics of Bacillus cereus and shelf-life of pasteurised milk," *Czech Journal of Food Sciences*, 21(6), 195-202. 2003.

[21] Freitas, R., Nero, L. A. and Carvalho, A. F. "Enumeration of mesophilic aerobes in milk: Evaluation of standard official protocols and Petrifilm aerobic count plates," *Journal of Dairy Science*, 92(7), 3069-3073. 2009

[22] Thomas, P., Mujawar, M. M., Sekhar, A. C. and Upreti, R. "Physical impaction injury effects on bacterial cells during spread plating influenced by cell characteristics of the organisms," *Journal of Applied Microbiology*, 116(4), 911-922. 2014.

[23] Donnelly, C.B., Gilchrist, J.E., Peeler, J.T. and Campbell, J.E. "Spiral plate count method for the examination of raw and pasteurized milk," *Applied and Environmental Microbiology*, 32(1), 21-27. 1976.

[24] Alonso-Calleja, C., Carballo, J., Capita, R., Bernardo, A. and García-López, M.L. "Evaluation of the spiral plating method for the enumeration of microorganisms throughout the manufacturing and ripening of a raw goat's milk cheese," *Journal of Food Protection*, 65(2), 339-344. 2002.

[25] Ginn, R.E., Packard, V.S. and Fox, T.L. "Evaluation of the 3M dry medium culture plate (Petrifilm™ SM) method for determining numbers of bacteria in raw milk," *Journal of Food Protection*, 47(10), 753-756. 1984.

[26] Casillas-Buenrostro, R.M., Heredia, N.L., Benesh, D.L. and García, S. "Efficacy of 3M™ Petrifilm™ aerobic count plates for enumerating Bacillus sporothermodurans and Geobacillus stearothermophilus in UHT milk," *International Dairy Journal*, 25(2), 147-149. 2012.

[27] International Organization for Standardization. ISO 16140-3. Microbiology of the food chain-Method validation-Part 3: *Protocol for the verification of reference methods and validated alternative methods in a single laboratory*. 2021.