RESEARCH
www.commprac.com
ISSN 1462 2815

ISOLATION, CHARACTERIZATION, AND MOLECULAR PHYLOGENY OF BACTERIA ISOLATES FROM COMMERCIAL PASTEURISED MILK IN CHIANG MAI, THAILAND AT THE LAST DAY OF EXPIRED DATE

Nadya Ramadhani Susilo [1] and Tri Indrarini Wirjantoro [2]

[1,2] Department of Food Science and Technology, Faculty of Agro-Industry, Chiang Mai University, Thailand. Email: [2]triindrarini.w@cmu.ac.th

DOI: 10.5281/zenodo.11184258

Abstract

The purpose of this study were to evaluate the shelf-life of commercial pasteurised milk in Chiang Mai, Thailand at the last day of expired date and to evaluate its physicochemical and microbiological stability. The results of chemical and microbiological tests of samples, which were bought on the last day of expired date, indicated that the characteristics were affected during the storage period, and the results were not required with the standard specifications of pasteurised milk. According to total plate counts, psychrotrophic, and lactic acid bacteria results, both of the samples indicates that the quality of the pasteurised milk at the last day of expired date wasn't good as it slightly exceeded the standard. However, the result is significantly different at $p<0.05$ in terms of coliform counts between samples with brand A were exceeded the standard and brand B were still below the maximum standard. The bacteria isolation results represent that the isolates could be subdivided into gram-positive bacteria such as *Exiguobacterium* group, *Bacillus* group, *Paenibacillus polymyxa*, *Kurthia gibsonii*, and *Limosilactobacillus fermentum*, also gram-negative bacteria such as *Pseudomonas* group, *Aeromonas* group, *Massilia timonae*, *Stenotrophomonas maltophilia*, and *Escherichia hermannii.* Some of the bacteria were found to be heat-resistant and psychrotrophic bacteria that mostly found in the post-pasteurisation process, however there also found some bacteria that can be found in the pre-pasteurisation process. It indicates that there was violation in terms of providing the condition for storing pasteurised milk during the distribution process. Furthermore, control of post pasteurisation process is necessary as standard procedure.

Keywords: Pasteurized Milk, Shelf-Life, Abuse Temperature.

INTRODUCTION

The International Dairy Federation (IDF) defines pasteurization as a process applied to the milk product with the object of minimizing the conceivable health risks emerging from pathogenic microorganisms related by heat treatment, which is consistent with minimal chemical, physical, and organoleptic changes in the product (Minj, 2020). Heat is lethal to microorganisms, but heat tolerance in each species can be different. There are numerous temperature and time combinations for heat treatment to improve the quality by obtaining required microbial or chemical effects like the pathogens with a minimal nutritional loss, but it cannot inactivate thermoresistant spores, including pasteurization between 63 ^{o}C for 30 min (Low Temperature Long Time-LTLT) or 72 ^{o}C for 15 s (High Temperature Short Time-HTST) and cooled immediately to a temperature not less than 4 ^{o}C (Minj, 2020).

Pasteurised milk is more preferable than UHT (Ultra High Temperature) milk in some countries like China and Australia (Liam *et al.,* 2015). Based on the data from (Liem et al., 2016), Australian people prefer pasteurised milk due to some reasons such as UHT milk has been defined as having "cooked" and "flat" flavors that make it have a noticeable different flavor profile if compared to pasteurised milk. The "cooked" flavor, however, can dissipate after several weeks and replaced to an off flavor or "stale". This flavor partly happened because of the increasing of various sulfur-containing compounds such as methyl ketones and aliphatic aldehydes (Zabbia et al., 2012).

Moreover, the Maillard reaction which is induced in the UHT process increases various flavor compounds that give its distinct “off-flavors” (Liem et al., 2016; Zabbia et al., 2012). Because of milk is naturally mild, slightly sweet flavour, the development of any off-flavours is particularly noticeable in the product (Gandy et al., 2008). The “off-flavors” that presence in the UHT milk has been associated with consumer rejection in countries where consumers mainly drink pasteurised milk (Liem et al., 2016; Zabbia et al., 2012).

Despite of UHT and pasteurised milk difference in taste, the milk itself contains approximately the same amount of calories, fat, protein, fat-soluble vitamins, and minerals such as calcium, potassium, and phosphorus (Liem et al., 2016). However, some micronutrients such vitamin B_{12} and B_6 might be lost or lowered during process and storage of UHT milk (Barraquio, 2014). Some volatile flavour compounds such as hydroxylamine, phenol, and buranoic acid may also destroyed under high temperature process (Gandy et al., 2008).

However, the Gulf standard “Expiration date for food products” (150-1, 2013) and the (984, 2015) have specified the shelf-life of pasteurised milk should not exceed 5 days from the date of production. The optimum conditions of processing and storage of pasteurised milk, and the quality of raw milk, can extend shelf-life to 3 weeks. Additionally, the Gulf standard (984, 2015) also specifies that pasteurised milk should be transported and stored at a temperature not exceeding 5^{o}C until consumption (Al-Farsi et al., 2021). This limited shelf-life consumed during distribution which leaving no time for selling the product before its expiry date leads to serious challenge for the dairy industry in Thailand. Therefore, extending the shelf-life of pasteurised milk will solve these problems and enable companies to improve their competency in the dairy industry.

The high concern of milk quality in different countries are including low-quality raw material, contaminant after pasteurization, improper refrigeration temperature, man-handling during distribution, and inadequate system of packaging which lead to limited shelf-life (Angel Sinaja et al., 2023). Spoilage microorganism is one of the main factor that reduce the pasteurised milk shelf-life by producing adverse changes in taste and aroma that affect consumer acceptability (Al-Farsi et al., 2021). Thus, the key to control the shelf-life of pasteurised milk lies in manipulation of the microbial content. (Miller, 2006) stated that milk spoilage caused by psychrotrophic bacteria and their heat-stable enzymes is a serious challenge for the dairy industry. Not only pathogens must be excluded but spoilage organisms should be removed, destroyed or inactivated. Successful strategies to achieve this end include high hygienic processing by heat treatment, spore removal and stringent control of the cold chain (Muir, 2011). Therefore, the aim of the study is to observe the quality of pasteurised milk in Thailand market by evaluating the chemical and microbiological stability of local commercial brand of pasteurised milk at local market.

MATERIALS AND METHODS

Milk Collection

Commercial plain whole pasteurised milks of two local brands (3 samples of each brand) were purchased from a local market. Milk samples were examined for chemical properties such as pH, titratable acidity, total soluble solids, and color values (Gemechu et al., 2015) and microbiological characteristics such as total microbial count, psychrotrophic count, lactic acid bacteria (LAB), and coliform count on the final day of the milk shelf life (Montebello et al., 2018). The number of each microbial group was computed for the microbial enumeration (Montebello et al., 2018).

Chemical Analysis

pH values. The pH of the milk samples were determined in the laboratory using a digital pH-meter accordance with the protocol given by (Gemechu et al., 2015).

Total Titratable Acidity. Titratable acidity was determined by titrating pasteurised milk against standard solution (0.1 M) of NaOH using phenolphthalein as an indicator. In terms of % lactic acid/100 ml of pasteurized milk, titratable acidity was expressed (Gull et al., 2021). The analysis was carried out in triplicate. The analysis was performed in triplicate. Percentage of titratable acidity was determined using Equation 1.

$$\text{TA (\% lactic acid)} = \frac{\frac{N}{10}\,\text{NaOH (ml) x } 0{,}009}{\text{Weight of milk sample}} \text{x} 100$$

(Gemechu et al., 2015).

Total soluble solids. About 10 ml commercial pasteurised milk was used for the test. Total soluble solids represented as ºBrix was assayed using a refractometer. The analysis was done in triplicate (Gull et al., 2021).

Colour values. Using a lab colorimeter, the L*, a*, and b* values for milk color characteristics were assessed directly. Triplicate measurements were performed (Gull et al., 2021). The Lab colorimeter is used to visually compare colors based on variations in lightness (L*), red/green value (a*), and blue/yellow value (b*) (Chudy et al., 2020).

Microbiological Analysis

Total Microbial Count. Bacteria were injected on Plate Count Agar to measure the total plate count. Incubation of the cultures lasted between 24 and 48 hours at 37 °C. The findings were computed in log cfu/ml of milk (Kondratowicz et al., 2006). To effectively get the total microbial count in the pasteurised milk on the final day of its shelf life, serial dilutions for 10^{-3}, 10^{-4} and 10^{-5} were carried out. After that, 1 ml dilution of the sample from appropriate dilutions were transferred into 2 petri dishes (Anderson et al., 2011). An amount of 12-15 ml of plate count agar (cooled to 45ºC) is added to each plate within 15 min of original dilution. The agar was poured and mixed immediately and thoroughly with the sample dilutions by alternate rotation. The agar is let on laboratory bench to be solidified. Solidified petri dishes are inverted and incubated promptly at 37ºC for up to 48 h. After incubation, the results of total microbial in each sample was counted (Anderson et al., 2011).

Psychrotrophic Bacteria Count. After the determination of total microbial count, further observation was done to measure the number of psychrotrophic bacteria in milk samples. Each sample were prepared in the same solution and dilutions are going to be plated in the following media: PCA (Plate Count Agar) with an incubation at 7ºC for 10 days for aerobic psychrotrophic bacteria (Ercolini et al., 2009).

Lactic Acid Bacteria Count. For lactic acid bacteria enumeration and isolation, appropriate dilution of each sample was plated on MRS (deMan Rogosa Sharpe) culture agar plates, and then incubated microaerophilic at 30°C for 48-72 h for further counting the growth colonies (Bao et al., 2012).

Coliform Count. According to (Wong et al., 2010), a pasteurization failure, secondary contamination, and packing type can all lead to faecal contamination as indicated by a high coliform count and *E. coli* contamination. The process for the coliform plate count was the same as that for the total microbiological count. Nonetheless, the milk samples or the dilution were carefully and evenly mixed with the agar after about 15 ml of violet red bile salt agar

(VRBA) was added to the labelled sterile plate. The agar is allowed to be solidified and an additional 5 ml of VRBA was poured over the surface of the solidified agar mixture. Next, the agar was allowed to set and was incubated. It is possible to count the number of bacterial colonies following the incubation process (Anderson et al., 2011).

Morphological and Biochemical Identification Method

Morphological Characterization

Colour, shape, transparency and margin were examined and recorded as colony morphological characteristics according to (Al-Dhabaan, 2019). Microscopic features were recorded for all isolates via Gram stain protocol.

Biochemical Characterization

Gram Staining. Tested bacterial colony was previously smeared on the filter paper. Crystal violet stain was added over the fixed culture. After 10 to 60 seconds, the stain was poured off, and the excess stain was rinsed with water. Iodine solution was then used to cover the smear for 10 to 60 seconds. Iodine solution was poured off, and the slide was rinsed with running water. Excess water from the surface was shaken off. A few drops of decolorizer was then added to the slide. Decolorizers were often the mixed solvent of ethanol and acetone. The slide was rinsed again with water in 5 seconds. To prevent excess decolorization in the gram-positive cells, stop adding decolorizer as soon as the solvent was not colored as it flows over the slide. The smear was counterstained with basic fuchsin solution for 40 to 60 seconds. The fuchsin solution was washed off with water, and excess water was blotted with the bibulous paper. The slide can also be air-dried after shaking off excess water (Tripathi et al., 2023).

Catalase Test. Gas bubbles detecting within 10 s after added purified bacterial culture to 5 ml of hydrogen peroxide solution, considered as a positive catalase test (Al-Dhabaan, 2019).

Motility Test. One colony of bacteria was inserted into sulphide, indole and motility (SIM) media in a test tube. The test tube was incubated for 48 h at 37±1ºC. The observation of motility test was the growth of bacteria on the media. The bacteria that only grew around inserted location showed a negative result, while the bacteria that grew on the media surface or spread in the media showed positive result (Ismail et al., 2018).

Indole Test. Appearance of bright red and yellow color which composed after added 0.5 ml of Kovac's reagent to incubated bacterial culture at 35 C for 24 h on SIM media indicated a positive and negative results respectively (Al-Dhabaan, 2019).

Simmons Citrate Test. Simmons Citrate test was performed via inculcate Simmons Citrate Agar plates (TSBA, Himedia) surface with bacterial cultures then incubated at 37 C up to 48 h. Changing media colour from green to bright blue indicate positive reaction (Al-Dhabaan, 2019).

Methyl Red (MR) Test. After adding methyl red indicator solution (TSBA, Himedia) to inoculated culturing media and incubation at 35 C for up to 4 days, changing color to red indicate MR test positive- appearance of tested bacteria (Yamashoji et al., 2020).

Voges Proskauer (VP) Test. The test organism is cultured at 30 C for five days (at least 48 hours) after being injected into glucose phosphate (MR-VP) broth. A good response is indicated by the color red's appearance. The VP test was altered in a number of ways to speed up the acetoin oxidation process (Shewmaker et al., 2019).

Nitrate Test. The inoculated culture was then incubated at the optimal temperature of 37±1ºC for 24 h. If gas was occurred in a durham tube, it was showed as a positive result. If there was no gas production, 6 to 8 drops of nitrite reagent A and 6 to 8 drops of nitrite reagent B were added the test tube. The reaction in the tube was observed and positive result was seen as red colour whilst negative result was seen if no colour change was occurred (Shen & Zhang, 2022).

Citrate Test. Simmon's citrate agar slant was streaked back and forth with a light inoculum picked from the isolated colonies. The inoculated culture then was incubated aerobically at 37±1ºC for 24 to 48 h. The growth with colour change from green to intense blue would show positive reaction in citrate utilisation test (Shen & Zhang, 2022).

DNA-Based Identification Method

Pure strains from the culture were reactivated by streaking on PCA and MRSA, followed by a 48-hour incubation period at 32°C. After being incubated, the strains were transferred to TSB (Tryptone Soya Broth) and MRSB (deMan Rogosa Sharpe Broth). A thorough explanation of the techniques used for DNA extraction and analysis can be found in the sections that follow.

Extraction and Preparation of DNA. Overnight cultures 1 ml was transferred to sterile eppendorf tubes and were placed on ice. For three minutes at room temperature and 10,000 rpm, centrifugation was utilized to recover the bacterial cells. The supernatant was discarded and cleansed using 500 µl of TE buffer, followed by a vortex separator mix and a minute-long centrifugation at 10,000 rpm. Once the supernatant was disposed of, the cells were carefully reconstituted in 480 microliters of 50 milligram EDTA and 60 microliters of the appropriate lytic enzyme. Following a vortex, the cell pellets were incubated at 37°C for 60 minutes. To extract the supernatant, the cell pellets were centrifuged for 5 minutes at 13,000 rpm. Then 300 µl of Nuclei Lysis Solution was added and carefully stirred (Sambrook, 2001).

After incubating the cell pellets for a further five minutes at 80°C, it were cooled to room temperature. The cell lysate was mixed with 3 µl of RNAse solution, and the tubes were inverted two to five times to mix. This was followed by further incubation for 60 minutes at 37°C. After adding 100 µl of protein precipitation solution, vigorous vortexing was performed for 20 seconds. Following a five-minute period of cold incubation, the cell pellets underwent a three-minute centrifugation at 13,000 rpm. After adding 400 µl of isopropanol to a sterilized 1.5 µl eppendorf tube containing the DNA-containing supernatan, the tube was gently inverted until the thread-like DNA was visible (Sambrook, 2001)

The samples were centrifuged once again for 30 seconds at 5,000 rpm, 30 seconds at 10,000 rpm, and 2 minutes at 13,000 rpm. After the tubes were drained on clean absorbent paper and the supernatant was carefully disposed of, 500 µl of 70% ethanol was added to them. The tubes were then gently inverted several times to wash the DNA pellet. A second centrifugation was run for thirty seconds at 5,000 rpm, for thirty seconds at 10,000 rpm, and for two minutes at 13,000 rpm. The ethanol was gently aspirated, and the tubes were then emptied and given fifteen minutes to air dry. The DNA was rehydrated by incubating it at 65°C for one hour after adding the DNA rehydration solution to the tube (Sambrook, 2001).

Gel electrophoresis Electrophoresis in 1.65% agarose gel was used to identify the DNA quantity, and safe stain was used for staining (Invitrogen bioservices Ltd, India).

PCR Reaction. After the DNA had been extracted, a PCR method was used to multiply the 16s gene. A DNA fragment as described by (Huck, Hammond, et al., 2007; Huck, Woodcock, et al., 2007) was amplified using the primers described by (Drancourt et al.,

2004) and PCR conditions by (Durak et al., 2006). A final concentration of 50 µl/reaction was achieved by diluting the PCR reagents, which included 10x buffer (5 µl), dNTP (5 µl), MgCl2¬ (2 µl), 16s forward primer (2 µl), 16s reverse primer (2 µl), DNA template (2 µl), and Taq polymerase 5 U/µl, to dH2O. All the components were mixed together in a PCR tube, and then each reaction was performed using a thermocycler. The PCR products were identified using agarose gel electrophoresis (1.65% agarose, 100 V, 500 mA for 25 min). For further analysis, the outcomes of PCR reactions that yielded appropriate DNA products (with the right base pair size) were utilized, and the reactions were deemed successful (Huck, Woodcock, et al., 2007)

Purification of PCR Products. For the PCR purification, a Purification Kit was utilized in accordance with the manufacturer's recommendations. As part of this process, a column was used to purify the PCR products and eliminate any leftover PCR pair bases. This purification was necessary to gurantee that the sequencing data were of the highest quality (Sambrook, 2001).

DNA Quantification. Amplified DNA quantification was required to verify and adjust the material to the appropriate concentration for performing the sequencing process. A spectrophotometer was used to precisely measure the amount of amplified DNA after the purification step. The concentration was adjusted to 40 ng/µL in accordance with the size of the DNA fragment and the requirements of the sequencing technique (Sambrook, 2001).

DNA Sequencing. First BASE DNA sequencing services (Malaysia) received a purified DNA sample for sequencing. In order to generate high-quality data, DNA sequences were trimmed and aligned in Unipro UGen version 7.1.11 to 632 nt rpoB segments based on the findings, as explained by (Durak et al., 2006). By examining the acquired chromatogram or using a different molecular technique (i.e., the 16S part), ambiguities were cleared up. The strain was identified using this technique. For additional examination, only high-quality, double-stranded sequences were utilized (Huck, Hammond, et al., 2007; Huck, Woodcock, et al., 2007)

Strain Identification and Construction of Phylogenetic Trees. To identify the strains, the 16S section of the DNA was amplified. Identification entailed matching the sequences to information stored in two databases: Food Microbe Tracker, which was mainly focused on Bacillus (species and related groupings) linked to dairy products, and Basic Local Alignment Search Tool (BLAST, Ezbiocloud Database) (Vangay et al., 2013).

Phylogenetic trees were constructed using the sequences from every isolate that was gathered. To build the tree, DNA sequences from the 16s DNA segment amplification were aligned using MEGA11's Muscle program. The neighbor-joining approach in MEGA11 was used to align DNA sequences from the 16s DNA segment amplification in order to create the phylogenetic tree (Tamura et al., 2013). The 16S phylogenetic trees were used to classify isolates into clades according to their genetic relatedness. The neighbor-joining method's bootstrap values were utilized to set cutoff values throughout the phylogenic tree construction process, which helped define those clades. Every discrete clade is discernible when a bootstrap value of at least 70 is assigned. Isolates that represented each branch of the tree were described using information from existing research.

Statistical Analysis

Analysis of variance (ANOVA) was applied to the analytical method results using the Excel program and SPSS version 25.0 software. The means were described using Duncan's multiple range test with a 95% (P≤0.05) confidence level (Gull et al., 2021).

RESULTS AND DISCUSSION

Milk Collection

Six milk samples from two different local brands were collected from the domestic market in Chiang Mai, Thailand at the last day of expired date for brand A and brand B. Table 1 showed the chemical qualities of 2 different local brands of pasteurised milk including pH, total acidity (TA), and total soluble solid (TSS).

Chemical Analysis

The findings showed that pasteurized milk's color was enterely normal. The product was not affected by the pasteurization procedure because on the last day of its expiration date, it was still a brilliant white color. The results made the GSO standard necessary (984, 2015) (Al-Farsi et al., 2021).

The results, which are shown in Table 1, show that the samples' pH was within acceptable ranges for the world. As stated in Kenya Standard (2191, 2015), the ideal pH range for pasteurized milk is 6.5–6.8. There was no statistically significant difference between the sample pH differences for brands A and B ($p<0.05$) (Al-Farsi et al., 2021).

According to the GSO (984, 2015) standard, pasteurized milk's total acidity shouldn't be more than 0.18%. No discernible variations were found between any of the samples. Nevertheless, brand A's overall acidity was higher than the international standard. The total acidity of pasteurized milk can be used to determine the milk's age and microbiological condition. The increase in overall acidity that results from the milk's breakdown of lactose into lactic acid indicates the bacteria load and milk age (Al-Farsi et al., 2021).

The total soluble solid in pasteurized milk, as determined by Brix refractometry, ranged from 13.26 to 26.30, according to (Cavalcanti et al., 2006). The results did not significantly differ between the two samples ($p<0.05$). Still, sample A's outcome was lower than what was reported in the literature. The acidity and pH of the milk product had an impact on the TSS results (Cavalcanti et al., 2006).

Table 1: Chemical Qualities of 2 Commercial Pasteurised Milk in the Last Day of Expired Date

Colour	Average	Standard
A	L: 87.20 ± 0.02^{a} a*: -2.01 ± 0.20^{ab} b*: 8.62 ± 0.10^{a}	L: 86.70 ± 0.20 a*: -1.70 ± 0.10 b*: 9.80 ± 0.10
B	L: 86.19 ± 0.02^{a} a*: -1.55 ± 0.10^{ab} b*: 9.76 ± 0.01^{b}	Bright white – white bone (Milovanovic et al., 2021)
pH	Average	Standard
A	6.46 ± 0.02^{a}	6.50-6.80 (Al-Farsi et al., 2021)
B	6.62 ± 0.01^{a}	
TA	Average	Standard
A	0.20 ± 0.01^{a} %	Should not exceed 0.18% (Al-Farsi et al., 2021)
B	0.16 ± 0.10^{a} %	
TSS	Average	Standard
A	11.5 ± 0.33^{ab} ^{o}Brix	13.26-26.30 ^{o}Brix (Cavalcanti et al., 2006).
B	13.33 ± 0.13^{a} ^{o}Brix	

*log cfu/ml

*^{ab} superscripts that different at the same row show significant different ($p<0.05$).

Microbiological Analysis

The shelf life of pasteurized milk is largely influenced by its microbial content. Thus, heat treatment, spore removal, and strict control over the storage chain must be carefully considered in order to achieve a high standard for pasteurized milk ((Muir, 2011). As shown in Table 2, the microbiological content of pasteurized milk was tested on the final day of its expiration date in order to regulate the pasteurization process.

Table 2: Microbiological Tests of 2 Commercial Pasteurised Milk at the Last Day of Expired Date

Brand	TPC	Psychrotrophic	LAB	Coliform
A	5.4 ± 0.3[a]	5.6 ± 0.5[a]	2.55 ± 0.1[a]	1.5 ± 0.6[a]
B	5.3 ± 0.7[a]	5.4 ± 0.2[a]	2.53 ± 0.0[a]	<1[b]
Standard	≤5	≤5	≤2.48	≤1
		(ISO 6730:2005) (Angelidis et al., 2016)		

*log cfu/ml

*[ab] superscripts that different at the same row show significant different ($p<0.05$).

The results demonstrated that while there were minor deviations from the highest international standard, there were no appreciable differences in total plate counts (TPC), psychrotrophic bacteria, or lactic acid bacteria (LAB) between samples for various brands. Table 2, however, demonstrates that there is a substantial difference ($p<0.05$) in the coliform counts between samples A and B, with brand A surpassing the maximum limit and brand B staying below it.

The most precise way to count living microorganisms in raw and heat-treated milk is to use the total plate count method. The results of the total plate count exceeded the upper limit set by the Ministry of Public Health in Thailand (350, 2013), which stipulates that, following manufacturing and before the label's expiration date, the maximum number of bacteria that can be found in pasteurized cow's milk is 100,000 cfu/ml, or 5 log cfu/ml. These findings might indicate that the pasteurized milk in both samples was not at its optimal condition the day before it expired. Sample A was purchased from a local brand in a separate province, while Sample B was acquired from a local market company in Chiang Mai, Thailand (Limbo et al., 2020).

Nonetheless, some bacteria may still make it through the pasteurization process, such as those that are heat-resistant. Certain naturally occurring plant survivors can promote spoiling in the right circumstances. *Bacillus spp.* and *Corynebacteria* are usually the only microorganisms found in any appreciable quantity, although thermoduric *micrococci* and *lactococci* are sporadically recovered. Pasteurized products usually prevent *coryneforms*, *micrococci*, and *lactococci* from growing further if the temperature is maintained below 6 ^{o}C (Muir, 2011).

Another concern is psychrotrophic bacteria. Despite the fact that the psychrotrophic bacteria are killed by a modest heat treatment (pasteurization at 72 ^{o}C/15 s). Due to its adaptation to a low temperature environment, the Gram-negative pschrotrophic bacteria was found to be the most common post-contaminant of pasteurized milk (Al-Farsi et al., 2021). Psychrotrophic bacteria grow best at temperatures between 20 and 30 °C. Despite the fact that they can grow at refrigeration temperature as well, albeit more slowly (Muir, 2011). Thermoduric and psychrotrophic floras in milk have the extracellular enzyme activity necessary to cause spoiling, can multiply in the product, and can withstand pasteurization.

They therefore seriously jeopardize the shelf life of the pasteurized product. The results of the psychrotrophic test exceeded the Malaysian Food Act standard, which specified that pasteurized milk should not contain psychrotrophic content exceeding 100,000 cfu/ml, or equivalent to 5 log cfu/ml. According to the standard, on the day before it expired, the pasteurized milk in neither sample was of a quality that could be considered acceptable. The handling of pasteurized milk by humans during cold storage, distribution, and packaging affects its shelf life according to (Limbo et al., 2020).

Pasteurized milk frequently contains lactic acid bacteria (LAB). Certain lactic acid bacteria groups, such as *Lactococcus*, *Lactobacillus*, *Propionibacterium*, and *Leuconostoc*, provide beneficial purpose in the fermentation process (Islam et al., 2021; Perin et al., 2017). Its antimicrobial ingredients support probiotic qualities and can help extend the shelf life of milk while preserving its nutritional value. But since certain lactic acid bacteria are thought to be lethal and lost competition at higher temperatures, their presence in pasteurized milk can also be a sign of post-contamination (Malik et al., 2019). The result of Table 2 showed that both samples were exceeded the result from (Malik et al., 2019) with 2.48 log cfu/ml. The lactic acid bacteria results for both samples show that the pasteurized milk was not of good quality on the last day of it expired date as it slightly exceeding the standard. Inadequate storage conditions during distribution can shorten pasteurized milk's shelf life (Limbo et al., 2020).

Due to the bacteria's ease of removal through heat treatment, the presence of coliform bacteria in pasteurized milk usually indicates contamination during pasteurization. Gram-negative bacteria like *Escherichia coli* are part of the coliform bacteria group, which can be used as a hygiene indicator for pasteurized milk (Al-Farsi et al., 2021; Masiello et al., 2016). The dairy industry is particularly concerned about coliform bacteria that are psychrotolerant and able to grow at refrigerated storage temperatures. These bacteria can grow with or without oxygen, and they can ferment lactose to produce gas and acid in 48 h at 35 ^{o}C (Al-Farsi et al., 2021; Masiello et al., 2016).

The Ministry of Public Health Standard in Thailand (350, 2013) states that pasteurized cow's milk from a manufacturing facility should contain no more than 100 cfu/ml of coliform bacteria. The findings showed that there is a significant difference in coliform bacteria at $p<0.05$, with brand A exceeding the standard and brand B remaining below the maximum standard. Based on this finding, it's possible that contamination happened when sample A was distributed from the origin province to Chiang Mai.

Table 3: Biochemical Tests Results of Bacterial Isolates

Code	Isolates	Gram stain	Catalase test	Motility test	Indole test	MR / VP test	Nitrate test	Citrate test	Glucose/ Fructose/ Glucose
TK	*Bacillus subtilis*	+	+	+	-	- / +	+	+	+ / + / +
TH	*Bacillus subtilis*	+	+	+	-	- / +	+	+	+ / + / +
TM	*Bacillus tequilensis*	+	+	+	+	- / -	-	-	+ / + / +
T6	*Exiguobacterium indicum*	+	+	+	-	+ / +	+	+	+ / - / -
T1	*Kurthia gibsonii*	+	+	+	-	+ / -	+	-	+ / + / -
T7	*Exiguobacterium indicum*	+	+	+	-	+ / +	+	+	+ / - / -

T3	*Exiguobactericum acetylicum*	+	+	+	-	- / +	-	+	+ / + / -
TI	*Aeromonas salmonicida*	-	+	+	+	+ / +	+	-	- / - / -
PO	*Exiguobacterium acetylicum*	+	+	+	-	- / +	-	+	+ / + / -
PQ	*Exiguobacterium acetylicum*	+	+	+	-	- / +	-	+	+ / + / -
PF	*Pseudomonas aeruginosa*	-	+	+	-	- / -	+	+	- / - / -
PN	*Pseudomonas gessardii*	-	+	+	-	- / -	+	+	- / - / -
PT	*Exiguobacterium acetylicum*	+	+	+	-	- / +	-	+	+ / + / -
PZ	*Exiguobacterium acetylicum*	+	+	+	-	- / +	-	+	+ / + / -
PC	*Exiguobacterium indicum*	+	+	+	-	+ / +	+	+	+ / - / -
PV	*Exiguobacterium acetylicum*	+	+	+	-	- / +	-	+	+ / + / -
PX	*Exiguobacterium indicum*	+	+	+	-	+ / +	+	+	+ / - / -
PU	*Aeromonas veronii*	-	+	+	+	+ / +	+	+	+ / + / -
PW	*Exiguobacterium indicum*	+	+	+	-	+ / +	+	+	+ / - / -
PP	*Exiguobacterium acetylicum*	+	+	+	-	- / +	-	+	+ / + / -
PE	*Stenotrophomonas maltophilia*	-	-	+	-	- / +	-	-	+ / + / +
PY	*Massilia timonae*	-	+	+	-	- / +	-	+	+ / - / -
LK	*Bacillus cereus*	+	+	+	-	- / +	+	+	+ / + / -
LG	*Paenibacillus polymyxa*	+	+	+	-	- / +	+	-	+ / + / +
LA	*Lacticaseibacillus paracasei subsp. tolerans*	+	-	-	-	- / -	-	+	+ / + / +
LI	*Lacticaseibacillus paracasei subsp. tolerans*	+	-	-	-	- / -	-	+	+ / + / +
L1	*Lacticaseibacillus zeae*	+	-	-	-	- / -	-	+	+ / + / +
LC	*Lacticaseibacillus rhamnosus*	+	-	-	-	- / -	-	+	+ / + / +
LB	*Limosilactobacillus fermentum*	+	-	-	-	- / -	-	+	+ / + / +
CC	*Escherichia hermannii*	-	+	+	+	+ / -	-	-	+ / - / -
CK	*Bacillus paranthracis*	+	+	-	+	+ / +	+	-	+ / + / -
CD	*Bacillus cereus*	+	+	+	-	- / +	+	+	+ / + / -
CDI	*Bacillus cereus*	+	+	+	-	- / +	+	+	+ / + / -
CJI	*Bacillus paranthracis*	+	+	-	+	+ / +	+	-	+ / + / -
CJ	*Bacillus cereus*	+	+	+	-	- / +	+	+	+ / + / -

DNA-Based Identification

Pure strains collected from DNA sequencing-based 16s rRNA profiling led to the construction of a phylogenetic tree. Samples were collected from two local brands bought at the domestic market in Chiang Mai, Thailand. A wide diversity of bacteria can be identified from the samples (35 sequences). The phylogenetic tree in Figure 1 represents that the isolates could be subdivided into gram-positive bacteria such as

Exiguobacterium group, *Bacillus* group, *Paenibacillus polymyxa*, *Kurthia gibsonii*, and *Limosilactobacillus fermentum*, also gram-negative bacteria such as *Pseudomonas* group*, Aeromonas* group, *Massilia timonae*, *Stenotrophomonas maltophilia*, and *Escherichia hermannii.*

There are 12 samples identified as *Exiguobacterium* group with 7 samples of *Exiguobacterium acetylicum* and 5 samples of *Exiguobacterium indicum. Bacillus* group consists of 2 *B. subtilis,* 1 *B. teuilensis,* 4 *B. cereus,* and 2 *B. paranthracis.* There are 4 samples of *Lacticaseibacillus* group with 2 samples identified as *Lacticaseibacillus paracasei subsp. Tolerans,* 1 sample of *Lacticaseibacillus zeae,* and 1 sample of *Lacticaseibacillus rhamnosus.* Furthermore, there are 1 sample of *Paenibacillus polymyxa*, *Kurthia gibsonii*, and *Limosilactobacillus fermentum* each in the group of gram-positive bacteria.

In the group of gram-negative bacteria, there are 2 samples of *Pseudomonas* group consists of *Pseudomonas aeruginosa* and *Pseudomonas gessardii,* 2 samples of *Aeromonas* group identified as *Aeromonas salmonicida* and *Aeromonas veronii,* 1 sample of *Massilia timonae*, *Stenotrophomonas maltophilia*, and *Escherichia hermannii.*

Although many of the bacteria detected are those previously associated with milk products, lactic acid bacteria such as *Lacticaseibacillus* and *Limosilactobacillus* group as well as *Pseudomonas* and *Stenotrophomonas* group were supposed to be dominated after pasteurisation process (Quigley, McCarthy, et al., 2013). Another study from (Martinez et al., 2017) had found *Bacillus* and *Paenibacillus* group in both raw and concentrated milk. Since the primary spore-forming bacteria are thought to have survived the heat treatments, thermophilic strains of bacteria are present in pasteurized milk.

However, there are also some isolates that normaly found in the raw milk product such as *Exiguobacterium* group (Quigley, O'Sullivan, et al., 2013), *Aeromonas* group (Tahoun et al., 2016), *Kurthia gibsonii* (Ribeiro et al., 2019), *Massilia timonae* (Kim et al., 2017), and *Escherichia hermannii* (Saad et al., 2012). Among *Bacillus* group, *B. subtilis* has been previously reported as capable of producing gas and reducing nitrate in the dairy product, which can reduce the protection provided by this compound against *Clostridium spp. (Martinez et al., 2017).* Apart from the easily obtainable heat-resistant bacteria in pasteurized milk, the most prevalent psychrotolerant post-pasteurization contaminants are members of the Pseudomonas group. Numerous enzymes that this type of bacteria can produce cause sensorial defects in fluid milk. Pseudomonas produces lipases and proteases that degrade components of milk; as these activities have been reported (Martin et al., 2018).

This research revealed that some local pasteurised milk products in Chiang Mai, Thailand are still susceptible to bacterial spoilage. The study's findings also emphasize the importance of understanding heat-resistant and psychrotrophic bacteria associated with the processing of pasteurised milk, which then allowed for specific interventions to be applied to control these microorganisms in the processing chain. Such interventions may include strict supervision in the good man-handling and storage application during distribution as it plays an important role to maintain the product's integrity until further use.

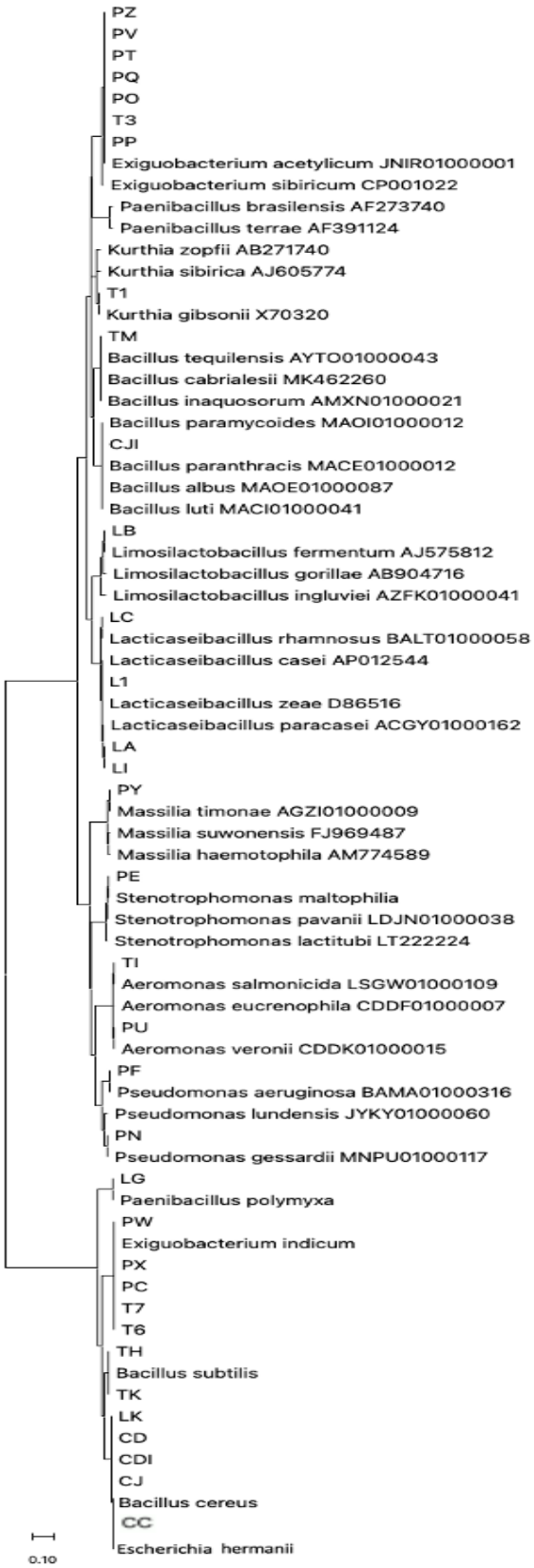

Figure 1: RpoB Phylogenetic Tree based on Isolate Sequence Obtained from Pasteurised Milk (brand A and B). A suffix at the 1st alphabet was added to indicate the type of isolation (T = TPC; P = Psychrotroph; L = LAB; C = Coliform)

CONCLUSION

The results of chemical and microbial quality of local commercial pasteurised milk at the last day of pasteurised day in Chiang Mai indicated that the characteristics were affected during storage period, and the results were not required with the standard specifications of pasteurised milk. Total plate counts, psychrotrophic, and lactic acid bacteria results for both samples show that the pasteurized milk's quality was not optimal as it slightly above standard on the final day before its expiration. Regarding coliform counts, the results show a significant difference ($p<0.05$) between samples A and B, with brand A exceeding the standard and brand B remaining below the maximum standard.

The bacteria isolation results represent that the isolates could be subdivided into gram-positive bacteria such as *Exiguobacterium* group, *Bacillus* group, *Paenibacillus polymyxa*, *Kurthia gibsonii*, and *Limosilactobacillus fermentum*, also gram-negative bacteria such as *Pseudomonas* group*, Aeromonas* group, *Massilia timonae*, *Stenotrophomonas maltophilia*, and *Escherichia hermannii.* Some of the bacteria were found to be heat-resistant and psychrotrophic bacteria that mostly found in the post-pasteurisation process, however there also found some bacteria that can be found in the pre-pasteurisation process. It indicates that there were violation in terms of providing the condition for storing pasteurised milk during distribution process. Furthermore, control of post pasteurisation process is necessary as standard procedure.

Acknowledgements

This project was supported by Chiang Mai University. The authors also thank the laboratory staff which helped during the research and provided valuable information for this research.

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