ORIGINAL ARTICLE

Effect of Shifting Temperatures on the Growth and Survival of *Klesiella pneumoniae* in Selected Milks and Their Substitutes

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ABSTRACT

Introduction: Milk is one of the good sources for bacterial growth. Poor consumer handling may result in temperature abuse of milk and aid the growth of bacteria. *Klebsiella pneumoniae* is one of the predominant bacteria present in milk that can cause foodborne diseases. The main objective of this study was to determine the adaptation and survival curves of *K. pneumoniae* ATCC 13883 shifted from 37°C to various stress temperatures in fresh raw coconut milk, pasteurised cow's milk, and Ultra-High Temperature (UHT) coconut milk. **Methods:** The first part of the study was aimed to determine the microbiological quality of milks and constitutes using Tryptone Soy Agar (TSA) and Eosin Methylene Blue (EMB) Agar. Secondly, the growth curve for *K. pneumonia* at 37°C in Tryptone Soy Broth (TSB) was established. In the third part of study, a stationary phase culture of *K. pneumoniae* was grown in TSB for 24 hours before shifting to coconut milk, pasteurised cow milk and UHT milk at different temperatures: 7°C, 27°C, 55°C and 65°C. **Results:** *K. pneumoniae* shifted from 37°C to 7°C showed bacteriostatic effects, while shifting *K. pneumoniae* from 37°C to at 27°C did not affect the growth potential in any of the samples. By contrast, *K. pneumoniae* shifted to 55°C only exhibited thermotolerance in fresh raw coconut milk, while survival curves of *K. pneumoniae* exhibited straight-line death kinetics when shifted to 65°C in all kinds of milk. **Conclusion:** The growth and survival of *K. pneumoniae* depend on the temperature stress conditions and types of media used.

Keywords: *Klebsiella pneumoniae*, Temperature abuse, Temperature stress, Milk, Survival curve

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INTRODUCTION

Foodborne disease remains a major public health hazard because some countries in the Asian region have not fully developed their national food control systems (1). Many factors influence the epidemiology of foodborne illnesses (2) including the adaptation of microorganism to environmental conditions. Toxigenic *K. pneumoniae* is an important antimicrobial-resistant bacterium, which is considered devastating diseases of global prominence (3). *K. pneumoniae*, a type species of the family Enterobacteriaceae, is the causative agent of pneumonia (characterised by the emission of bloody sputum) outbreaks and urinary tract infections in humans (4).

Various biochemical properties and antigenic types are used to characterize the different types of diseases caused by *Klebsiella* spp. This organism can be distinguished based on two important biochemical tests (5). One study stated that *K. pneumoniae* is normally associated with hospitalisation, as it can easily people with serious chronic patients, such as diabetes mellitus patients (6). Another study describes *K. pneumoniae* as a particularly harmful bacterium to humans, relative to other members of the *Klebsiella* genus.

Microorganisms' heat resistance is linked to their growth temperatures, and various bacteria require varied temperature ranges to grow (7). Psychrophilic microbes are the most heat-sensitive, followed by mesophiles, while thermophiles are the most heat resistant (8). Gram-positive bacteria are more heat resistant than

Gram-negative bacteria, owing to thick peptidoglycan covering that protects them from external conditions, while spore-forming bacteria are more heat resistant than non-spore formers. However, the temperature characteristics of the organism are also influenced by pH, nutrient availability, and other environmental factors. The threats of these bacteria are from direct and indirect contact into food chain results from improper treated water usage in preparation, contaminated soil for planting, unhygienic practice by food handlers, or improper quality assurance policy (7).

Milk provides an ideal nutrient medium for microbial growth. One example of milk that is widely used during food preparation is coconut milk. It is an essential ingredient in commercial kitchens (9). However, coconut milk can easily deteriorate if it is not subjected to heat treatment (10). The second, Ultra-High Temperature (UHT) milk, is commonly purchased for large groups of consumers. This milk is subjected to UHT treatment (above 135°C for 2–5 seconds), which is an effective method of destroying many bacterial endospores while extending the shelf life (11). The third, pasteurised milk, is treated with mild heat (71.5°C for 15 seconds) to ensure its safety by reducing to microbial load. This preservation method provides a refrigerated shelf life of about 2 weeks (12) and, at the same time, has a negligible effect on the taste and nutritive characteristics of the milk (13).

Microbiological analysis is an important method to determine the level of microbial contamination by foodborne pathogens. In previous works, we have reported on the microbiological quality of fermented cassava ice cream (14), stingless bee honey (15), "kerabu mangga" (16), keropok lekor (17), and fresh ulam (18). However, limited studies are available on the microbiological quality of different types of milk, particularly the occurrence of *K. pneumoniae* from local foods (19).

Foodborne illness is almost always a result of temperature abuse (20), and how slight temperature abuse affects the survival of bacteria is poorly understood. Previously, Migeemanathan et al. (21) has studied the growth, survival and inactivation of *Salmonella* Typhimurium as affected by temperature stress in goat's milk. In addition, Lani et al. reported the effect of sublethal temperature stresses on the cultivability and percentage injury of *Escherichia coli* grown in laboratory medium (22). In their study, sublethal temperature stresses (45°C, 40°C, and 20°C) did not effectively kill the organism, but the organism was injured. However, the effects of temperature stresses on *K. pneumoniae* are poorly understood.

To determine the effect of sudden temperature shifts, *K. pneumoniae* was initially grown at 37°C before being subjected to 7°C, 27°C, 55°C, and 65°C, reflecting the temperature fluctuations possible during food processing. This study investigated the growth and survival of *K. pneumoniae* grown at 37°C and shifted to four different temperatures; 7°C, 27°C, 55°C, and 65°C in coconut milk, pasteurised milk, and UHT milk. This study may help to provide an understanding of the adaptation and survival of *K. pneumoniae* at shifting temperatures, which may be one of the causes of foodborne illness.

MATERIALS AND METHODS

Source of the microorganism

K. pneumoniae ATCC 13883 used in this study was purchased from the American Type Culture Collection (ATCC), and this culture was maintained on slant agar at 4°C. This strain culture is recommended by ATCC for use in the test described in ASTM Standard Test Methods D4783-89 and E979-91. The viability of the culture was regularly maintained by the technical assistant, Faculty of Fisheries and Food Science, Universiti Malaysia Terengganu.

Culture media

The microbiological media used in this study were Tryptone Soy Broth (TSB; CM0129), Tryptone Soy Agar (TSA; CM0131), and Eosin Methylene Blue Agar (Levine) (CM0069). The diluent and solution used in this study were Buffered Peptone Water (CM1049) and Saline Tablets (BR0053) for 0.85% saline solution. All media and diluent were purchased from Oxoid, England. The media were prepared and sterilised using an autoclave as specified in the manufacturer's instructions.

Microbiological examination of milk samples

A total of 9 samples of each fresh raw coconut milk, UHT packs of milk, and pasteurised cow's milk were purchased at local supermarkets in Kuala Terengganu, Terengganu, Malaysia. The fresh raw coconut milk samples were pressed from the fresh grated coconut prior to sampling. UHT milk samples were obtained from supermarket, which was always available on the shelf and maintained at room temperature. The fresh pasteurised cow's milk sample was taken from milking process in the early morning prior to exposure to heat treatment at 70.5°C for 15 seconds. During the process of pasteurisation, no additional water or treatment was given to the cow's milk and this pasteurisation was done at the farm in Kuala Terengganu. After pasteurisation, the owner distributed the milk to different stalls for customers to purchase.

The samples were collected in sterile packaging by using sterile plastic bag and brought back aseptically in an icebox containing ice at temperature between 0° C to 4°C immediately upon purchased. Experiment was conducted at room temperature within 2 hours upon arrival in the laboratory. Aseptic technique was applied where all the equipment sterilised prior to experimentation. Firstly, 25 mL of each sample were

added with 225 mL buffed peptone water in stomacher bags and homogenised using a stomacher for 5 seconds. 1 mL of each homogenised sample (10^{-1}) was transferred into 9 mL of 0.1% buffered peptone water (buffered) for serial dilution until dilution of 10^{-8} prior to plating onto Tryptone Soy Agar (TSA) and Eosin Methylene Blue (EMB) Agar using the spread plate method. Then, all TSA and EMB plates were incubated at 37°C for 24 hours. After 24 hours of incubation at 37°C, the serial dilutions with colonies between 30-300 colonies were enumerated and the colony forming unit (CFU/mL) were determined following standard microbiological enumeration procedures (16, 22).

Growth curve of *K. pneumoniae* in Tryptone Soy Broth at 37°C

The method described by Lani et al. (22) was used in order to establish the growth curve of this microorganism. Using an inoculation loop, one loopful of an overnight pure culture of *K. pneumoniae* ATCC 13883 from TSA slant was aseptically transferred into 100 mL of TSB (Oxoid, UK) and incubated at 37°C for 30 minutes in an agitated shaker at 150 rpm. After 30 minutes, 1 mL of inoculum was transferred into 300 mL of TSB and grown at 37°C in an agitated shaker for 24 hours. In plotting the growth curve, 1 mL sample was taken out from the flask at 3-hour intervals, subjected to serial dilution using 0.85% saline, plated onto TSA, and incubated at 37°C for 24 hours. The observation of colonies grown on the plates was done after 24 hours and the serial dilution representing colonies between 30 to 300 colonies per CFU/mL were enumerated. The standard growth curve was performed using Microsoft Excel, Version 2013.

Survival curve of *K. pneumoniae* in different milk samples

After *K. pneumoniae* ATCC 13883 was grown in 300 mL of TSB at 37°C in a shaking incubator for 24 hours (stationary phase), it had reached 9.13 $\log_{10}$ CFU/mL. Immediately, 1 mL of the *K. pneumoniae* grown in TSB was transferred into four different flasks containing 100 mL of fresh TSB for the survival study under different temperature stress conditions, which were 7°C, 27°C, 55°C, and 65°C while the control broth had no bacterial culture added to it. These temperatures were chosen to represent various temperature stress conditions that may occur in milk samples. The sampling of bacterial cultures shifted from 37°C to different temperature stresses was carried out at different time intervals of 0, 1, 2, 3, 6, 9, 12, 24 h of growth.

Viable cells of *K. pneumoniae* were measured on TSA and EMB agar using the spread plate method. TSA is a non-selective medium that supports the growth of both and injured and uninjured *K. pneumoniae*, whereas EMB is a selective medium where uninjured cells of *K. pneumoniae* can grow. All plates were incubated at 37°C for 24 hours. The microbial count (CFU/mL) was determined as described earlier. The survival curve was plotted using Microsoft Excel, Version 2013.

Statistical analysis

MINITAB version 14 was used to transform the raw microbial count data from different microbiological analyses into $\log_{10}$ CFU/mL. The differences between group means were compared using One-way of variance (ANOVA) at the 95% confidence level ($P < 0.05$).

RESULTS

Microbiological quality in different milk samples

The microbiological quality of milk is expressed in terms of Total Plate Count which was based on the enumeration of colonies grown on TSA and *Klebsiella* count based on enumeration of colonies grown on EMB agar. Table I shows the mean *Klebsiella* count in coconut milk, $\log_{10}$ 8.06 ± 0.03 (SD) CFU/mL, was the highest count among all three samples, while the lowest count was observed in UHT milk, where there was no growth of *Klebsiella*. Meanwhile, the mean value of 4.77 ± 0.17 (SD) in pasteurised cow's milk was the second-highest count among all three samples. The lowest count was observed in UHT milk because there was no growth on the media plate. Similar trend was observed for Total Plate Count (TPC) where the highest count was reported in the coconut milk ($\log_{10}$ 8.52 ±0.11), followed by pasteurised cow's milk ($\log_{10}$ 4.95 ±0.28) and the lowest count was observed in the UHT milk (no growth). There was a significant difference ($P < 0.05$) between the coconut milk and pasteurised milk, which showed that both kinds of milk favor the growth of *K. pneumoniae*.

Table I: Microbiological quality in terms of Total Plate Count (TSA media) and *Klebsiella* count (EMB Agar) in coconut milk (CM), pasteurised cow's milk (PM) and ultra-high temperature (UHT) samples

Microbiological quality	Types of media	Number of samples	Sample	Mean and standard deviation
Total Plate Count	TSA	9	CM	8.52 ± 0.11^{a}
		9	PM	4.95 ± 0.28^{b}
		9	UHT	0.0 ± 0^{c}
Klebsiella Count	EMB Agar	9	CM	8.06 ± 0.30^{a}
		9	PM	4.77 ± 0.17^{b}
		9	UHT	0.0 ± 0^{c}

Growth curve of *K. pneumoniae* in TSB at 37°C

Fig. 1 shows the growth curve of *K. pneumoniae* at 37°C in TSB plotted as a logarithmic function of the bacterial population against time. From the growth curve, there was an initial slow rate of increase in cell density in the first 3 hours of growth, which continued up to 6 hours of growth. The result shows that the time taken for *K. pneumoniae* to reach the exponential and stationary phases was between 3 and 9 hours and between 12 and 24 hours, respectively. The accumulation of waste metabolites within the environment becomes sufficiently inhibitory to cause a measurable reduction in the rate of the cell number increase. This phenomenon is called the stationary phase, where this organism remained

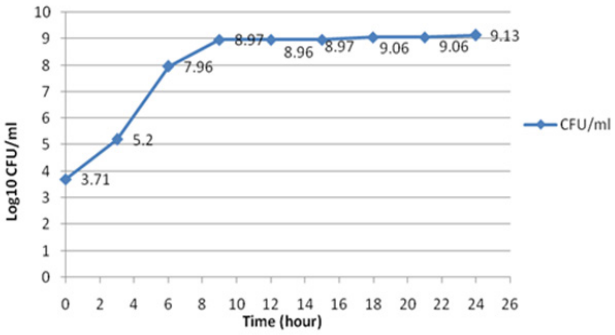

Figure 1: Growth curve of *Klebsiella pneumonia* using plate count on TSA

even after 24 hours of culture. During this phase, conditions may also become sufficiently inhibitory to lead to cell death, lysis, and further reduction in the net rate of population increase. Eventually, the rate of cell death and lysis exceeds the capacity of the environment to support cell division, leading to a decline in growth after 24 hours of incubation.

Survival curve of *K. pneumoniae* in different types of milk at different stress temperatures

The initial inoculums from the stationary phase of *K. pneumoniae*, 9.13 $\log_{10}$ CFU/mL, were grown at 37°C and exposed to temperature stresses, 7°C, 27°C, 55°C, and 65°C. Fig. 2 clearly shows a prolonged lag phase when the temperature of this organism was shifted from 37°C to 7°C throughout 12 hours of stress adaptation. This prolonged lag phase demonstrates the bacteriostatic effects caused by refrigeration. Next, when shifted from 37°C to 27°C, *K. pneumoniae* grew faster in UHT milk, followed by coconut milk, and the slowest growth was observed in pasteurised milk in both TSA and EMB (Fig. 3). When *K. pneumoniae* was shifted from 37°C to 55°C, only coconut milk showed resistance to 55°C, whereas the organism continued to grow gradually at this temperature on both TSA and EMB agars (Fig. 4). This result demonstrates that *K. pneumoniae* grown in coconut milk has the ability to resist thermal temperatures, but *K. pneumoniae* grown in pasteurised cow's milk and UHT milk did not possess this heat-resistant characteristic. Shifting of *K. pneumoniae* from 37°C to 65°C exhibited that thermal inactivation only occurs in coconut milk, where complete destruction of *K. pneumoniae* occurred after 6 hours of stress adaptation compared with UHT milk and pasteurised milk, in which *K. pneumoniae* was completely destroyed after 2–3 hours of stress adaptation (Fig. 5).

Table II shows that the growth rate of *K. pneumoniae* in UHT milk was greater than that in coconut milk and pasteurised milk when the stress temperature was shifted from 37°C to 7°C on EMB agar, with no significant difference ($P > 0.05$) between all three milk types. A similar growth pattern was obtained when the bacterium was shifted from 37°C to 7°C on TSA,

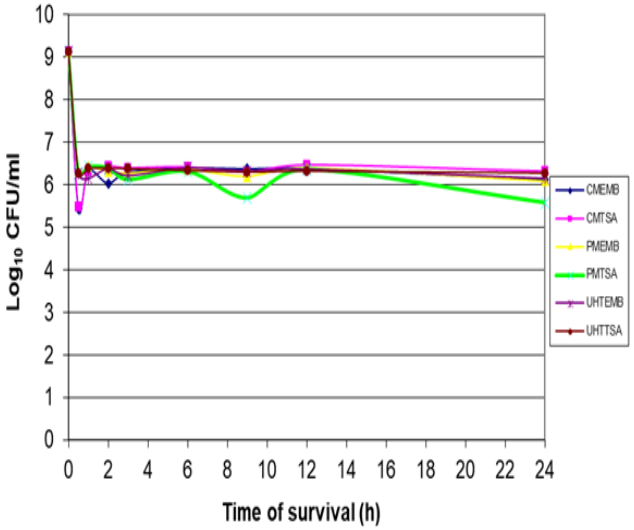

Figure 2: Survival curve of shifting temperature stress of *K. pneumoniae* from 37°C to 7°C in coconut milk (CM), pasteurised cow's milk (PM) and UHT on TSA and EMB Agar

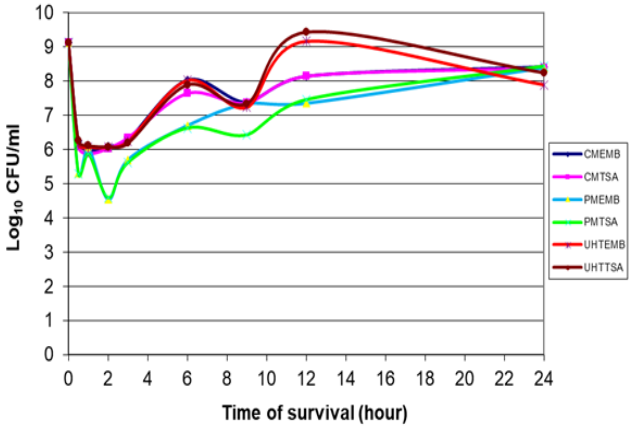

Figure 3: Survival curve of shifting temperature stress of *K. pneumoniae* from 37°C to 27°C in coconut milk (CM), pasteurised cow's milk (PM) and UHT on TSA and EMB Agar

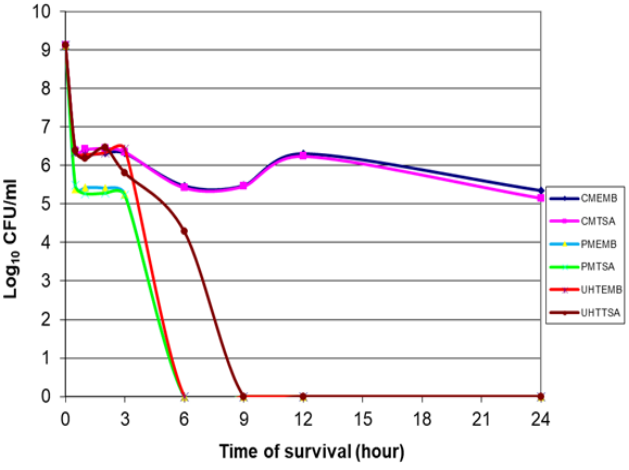

Figure 4: Survival curve of shifting temperature stress of *K. pneumoniae* from 37°C to 55°C in coconut milk (CM), pasteurised cow's milk (PM) and UHT on TSA and EMB Agar

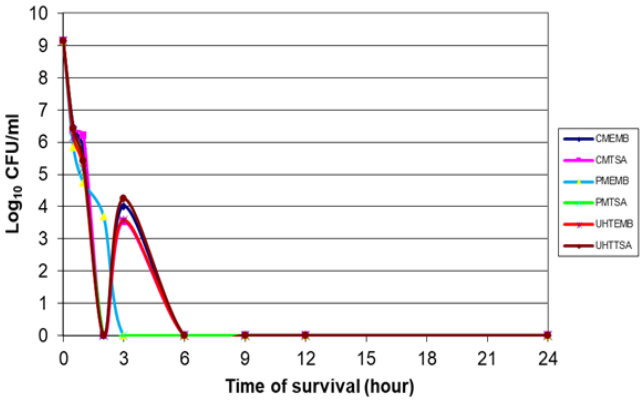

Figure 5: Survival curve of shifting temperature stress of *K. pneumoniae* from 37°C to 65°C in coconut milk (CM), pasteurised cow's milk (PM) and UHT on TSA and EMB Agar

where the growth of this bacterium was inhibited at 7°C regardless of milk type. Not surprisingly, *K. pneumoniae* shifted from 37°C grew well at 27°C in all types of milk. The growth rate of *K. pneumoniae* in UHT milk was greater than that in coconut milk and pasteurised milk. No significant difference ($P > 0.05$) in microbial counts of *K. pneumoniae* in TSA. At the same time, significant differences ($P < 0.05$) of microbial counts of *K. pneumoniae* in EMB agar between all types of milk samples, as shown in Table II.

The microbial growth in three types of milk increased with adaptation time as the organism would not be under stress because 27°C is still in the ideal range for *K. pneumoniae* growth. Next, the growth rate of *K. pneumoniae* in coconut milk was greater than that in UHT milk and pasteurised cow's milk when the stress temperature was shifted from 37°C to 55°C on EMB and TSA agar. The significant differences ($P < 0.05$) of microbial counts were observed between all three types of milk samples on EMB and TSA agar. The growth rate of *K. pneumoniae* in coconut milk was greater than that in UHT milk and pasteurised milk when the stress temperature was shifted from 37°C to 65°C on EMB and TSA agar and showed no significant difference in growth ($P > 0.05$) between all three milk samples on TSA.

DISCUSSION

The microbiological quality of the Total Plate Count and Klebsiella count in coconut milk was the highest, followed by pasteurised milk, and there was no growth of *K. pneumoniae* in UHT milk. The highest count of TPC and *Klebsiella* count in coconut milk is definitely because the coconut milk did not accept any preservative treatment. Other studies showed that from a total of 78 of the most popular Malaysian samples examined, 25 samples (32%) were positive for *K. pneumoniae* where the incidence of *K. pneumoniae* was found to be 100% (19). A study by (23) showed that *K. pneumoniae* was present in ready-to-eat items sold in a cafeteria, whereas on a dairy farm, the source of *Klebsiella* in milk samples was the wood materials used in milk handling. Therefore, to control *Klebsiella mastitis*, the authors suggested that environmental hygiene and the use of inorganic bedding materials such as sand to be used (24-25). A study in Malaysia showed the presence of *K. pneumoniae* in street foods while other reports showed that contamination occurs via the fecal–oral

Table II: Mean count and standard deviation of *K. pneumoniae* growth in coconut milk (CM), pasteurised cow's milk (PM) and UHT after shifting temperature from 37°C to 7°C, 27°C, 55°C and 65°C as measured on TSA and EMB Agar

Types of media	Sample	Shifted temperature	Mean & standard deviation	Decreasing order of microbial count due to shifting temperature	*P* value
EMB Agar	CM	7°C	6.16 ± 0.37	UHT > CM> PM	0.120
	PM		6.10 ± 0.09		
	UHT		6.28 ± 0.10		
TSA	CM	7°C	6.26 ± 0.34	UHT > CM > PM	0.548
	PM		6.15 ± 0.25		
	UHT		6.32 ± 0.08		
EMB Agar	CM	27°C	7.07 ± 0.91	UHT > CM > PM	0.076
	PM		6.43 ± 1.01		
	UHT		7.10 ± 1.17		
TSA	CM	27°C	6.98 ± 0.87	UHT > CM > PM	0.024
	PM		6.29 ± 0.91		
	UHT		7.19 ± 1.22		
EMB Agar	CM	55°C	5.99 ± 0.32	CM > UHT > PM	0.005
	PM		2.69 ± 1.76		
	UHT		3.17 ± 1.26		
TSA	CM	55°C	5.99 ± 0.41	CM > UHT > PM	0.004
	PM		2.66 ± 1.36		
	UHT		3.64 ± 1.82		
EMB Agar	CM	65°C	1.81 ± 2.89	CM > UHT > PM	0.994
	PM		1.59 ± 2.72		
	UHT		1.66 ± 2.89		
TSA	CM	65°C	1.79 ± 2.96	CM > UHT > PM	0.999
	PM		1.27 ± 2.91		
	UHT		1.79 ± 2.50		

route, which is possible through cross-contamination between food handlers, during the preparation process, by surface-to-food contact, or by contact with the food itself (24). The findings of (26-27) indicated that food handlers must follow standard sanitation procedures to prevent contamination with pathogenic microbes and to produce safe food for the consumer.

Using TSB as media for microbial growth, the growth curve of *K. pneumoniae* at 37°C was plotted. At 37°C, the lag phase of *K. pneumoniae* was short and occurred in the first hour of growth and it was similar to the optimal growth temperature for *Listeria monocytogenes* in TSB at 37°C as reported by Lani (30). The lag phase is a time during which the cells are adjusting their physiology and biochemistry to exploit their environment. The present study shows that the organism entered the log phase after 3 hours of growth until it reached its maximum growth rate, which was at 9 hours of growth (28). A similar study reported that the log phase of *Klebsiella* can be achieved after 1 to 2.5 hours when the organism was grown in Luria Bertani (LB) broth at 37°C, shaking at 150–200 rpm, while the optical density at 600 nm of 1.0 was equivalent to 4.8 x 10^6 CFU/mL of *K. pneumoniae* Xen39.

During the log phase, cells exhibit balanced growth where their entire metabolic effort is directed to reproduction (29-30). In the present study, the stationary phase of *K. pneumoniae* was from 10 to 24 hours of growth. A study stated that the stationary phase is a steady-state where cells are not multiplying, nutrients have been taken up, and the toxins have accumulated. Another study on the growth curve of *K. pneumoniae* on biofilm with modified conditions showed that the stationary state was achieved by 36 hours of growth with an initial cell density of 10^6 CFU/mL (31). When bacteria at the stationary phase, they adapt to the situation for their survival while in a remarkably inactive state.

The nature and concentration of the chemicals present in the culture media, the pH of the medium, the physiological status and age of the cell, and the temperature at which the cell is cultured are all factors that influence the heat resistance of microorganisms (30). In the present study, four different temperatures; 7°C, 27°C, 55°C, and 65°C were selected to study temperature stress in *K. pneumoniae*. These four temperatures were selected to represent four conditions to which foods are always exposed - high (55°C and 65°C), reduced (7°C), and ambient temperature (27°C), which are common during different stages of food processing and in foodservice environments. The inoculum size of *K. pneumoniae* shifted from 37°C to 7°C, 27°C, 55°C, and 65°C was about 9.13 $\log_{10}$ CFU/mL where the organism was in the stationary phase. Bacteria in the stationary phase of growth are more thermotolerant, resistant to oxidative stress, acid-resistant, and better able to withstand osmotic stress and famine phenotypically (29, 32).

The present study showed that adaptation curves of *K. pneumoniae* shifted from 37°C to 27°C were biphasic curves where a distinct lag phase was observed for the first 3 hours of growth, and then the organism started to multiply after a 3-hour lag phase. The generation time for pasteurised milk on both selective and non-selective agar showed that *K. pneumoniae* can grow well in pasteurised milk other than UHT milk and coconut milk. Meanwhile, the survival curve for 55°C and 65°C showed a straight-line death kinetic, which followed a first-order death kinetic as these temperatures were sufficient to destroy *K. pneumoniae* except in coconut milk. The thermotolerance effects showed by the organism in coconut milk at these temperatures may be due to the synergistic effects of the presence of *K. pneumoniae* with the inoculated *K. pneumoniae* in coconut milk. However, poor synergistic effects were observed in UHT milk and pasteurised milk.

The cells were grown at 37°C until they reached the stationary phase before shifting to 7°C, 27°C, 55°C, and 65°C showed biphasic survivor curves. The occurrence of biphasic curves has proven the microbial injury phenomenon in *L. monocytogenes* at sublethal stress temperatures (30). The generation time of *K. pneumoniae* was determined by using both plating media (TSA and EMB agar) using an Excel graph. The value of generation time during shifting temperatures indicated the time required for the growth of *K. pneumoniae* under temperature stress conditions.

The present study showed the survival of *K. pneumoniae* shifted from 37°C to different temperatures; 7°C, 27°C, 55°C, and 65°C. The occurrence of the lag phase was clearly observed for all the survival curves and adaptation curves in all types of milk samples. Based on the hypothesis by (33), a lag time is apparent where cells require adjustment to new environmental conditions. Prolong lag time showed there was no cell division during the adaptation.

The *K. pneumoniae* strain used throughout this study (ATCC13883) was clinically isolated from human fecal infection. Survival studies of *K. pneumoniae* generally involve the use of pure culture of organism in TSB as growth medium for survival studies (34). From the survival curve in this study, instantaneously decreasing the temperature from 37°C to 7°C showed that the growth of *K. pneumoniae* was greatly inactivated in all types of milk, where a prolonged lag phase duration exhibited bacteriostatic effects due to low-temperature stress. Another study performed by (35) stated that at low temperatures between 0°C and 5°C, the growth of bacteria is limited.

When the bacterium was grown at stressing temperature of 7°C, the microbial population decreased in the

first hours and then continued in stasis, but no death occurred. Both selective agar (EMB) and non-selective agar (TSA) exhibited similar behaviours, which showed that this organism has psychotropic properties, whereby it can survive refrigerated temperatures, thus imposing a serious public threat to consumers. Untreated coconut milk degraded quickly in previous tests, even when stored at a cool temperature. The time it takes for bacteria to multiply in coconut milk has been observed to drop from 232 minutes at 10°C to 44 minutes at 30°C (36). The storage of coconut milk at low storage temperature (4°C) had a shelf-life of 7.01 days, while storage temperature at (12°C) had a shelf-life of 6.00 days, which showed that an increase in storage temperature would reduce the lag phase of the bacteria and increase the growth rate of the microorganism (37-38).

Transient shifts in the growth period to or from the low range showed cultures transferred from the normal to the low-temperature range showed biphasic curves, consisting of a lag phase followed by a period of aberrant growth before the characteristic steady-state rate proceeded. Contaminating organisms occur during food processing and handling may arise from temperatures within the normal physiological temperature range or the low-temperature range (39).

When the stressing temperature was shifted from 37°C to ambient temperature, 27°C, *K. pneumoniae* grew well, especially in UHT milk throughout a 12-hour period. However, the other two samples also favored the growth of *K. pneumoniae* during the stress condition. The milk processing method that will completely sterilise all the present microbes and the short exposure to high temperature minimizes the impact of the nutritional composition and characteristics of the milk (14). Therefore, this might be among the factors that contributed to the rapid growth of *K. pneumoniae* in the pasteurised cow's milk samples, relative to that in UHT milk and coconut milk. The nutritional composition of cow's milk enriched in such a way as to enhance the growth of *K. pneumoniae* in pasteurised cow's milk (40). Generally, the presence of dipotassium hydrogen phosphate in the agar plating system would also increase the heat resistance of *K. pneumoniae* and indirectly enhance the organism's growth.

Meanwhile, coconut milk also has a rich nutrient composition; however, the physico-chemical properties of the food itself, conditions of the storage environment, and processing methods are among the factors affecting the growth of *K. pneumoniae* in the sample (38-40). Past studies on storage temperature showed that when milk was stored at 21°C, the shelf-life of the milk was 2.71, and when stored at 24°C, the shelf-life of coconut milk was 2.21 days (41).

When *K. pneumoniae* was shifted to a stress temperature of 55°C, the bacterium was exposed to the temperature danger zone, which defined as the temperature range that supports the bacterial growth (26). *K. pneumoniae* was grown in pasteurised milk, and UHT reached complete destruction after 6 and 9 hours of stress adaptation, respectively. However, the growth of *K. pneumoniae* in coconut milk increased gradually, even after 12 hours of stress adaptation. This indicates that the thermal inactivation of *K. pneumoniae* in coconut milk is higher than that of *K. pneumoniae* in pasteurised milk and UHT milk. A temperature of 55 C is sufficient to inactivate the growth of *K. pneumoniae* in pasteurised milk and UHT milk. The increase in the bacterial count in coconut milk showed that the organism may be adapted to thermotolerance conditions. A similar phenomenon has been observed during the adaptation of *Listeria monocytogenes* shifted from 37°C to 55°C (30), while the volatile compound changes in coconut milk due to various treatments increase the storage shelf-life of coconut milk products (42).

When the temperature was shifted from 37°C to 65°C, *K. pneumoniae* growth in all types of milk was completely arrested after less than 6 hours of stress adaptation. *K. pneumoniae* showed the fastest mortality in UHT milk, followed by pasteurised milk and lastly, coconut milk. The temperature of 65°C is above the temperature danger zone, and the result could explain the effects of *K. pneumoniae* growth beyond this zone (26, 43). In a related study, of the 41 isolates identified in Mozzarella cheese from a local dairy, where 37 were *K. pneumoniae* and 2 were *K. oxytoca*, exposure to 63°C for 15 minutes caused the death of all total and fecal coliforms (44).

The medium used for this research was the milk medium itself as it was in liquid form. No enriched medium such as broth or peptone water was used to grow *K. pneumoniae* in different milk samples during stress conditions. Meanwhile, the water activity concentration, soluble carbohydrates, (mainly sucrose), salts, pH, fats, proteins, and heating medium are among the related factors affecting the thermal resistance of the bacteria (42).

It is known that microorganisms exposed to sublethal temperatures respond by synthesizing heat shock protein. Heat shock proteins are a protein family expressed at a high level (5%–10%), which is significantly increased when a cell is subjected to various stress conditions (45-46). The introduction of the heat shock response is a kind of stress, such as temperature stress, which enables cells to develop thermotolerance. Once the protein is exposed to temperature stress conditions, cellular proteins become partially or completely denatured. Therefore, the tendency of the protein to refold prevents bacterial cell motility (30, 47).

There is no reported study on the generation time available for the growth of *K. pneumoniae* at stress temperatures in coconut milk, pasteurised cow's milk,

and UHT milk. However, there are studies conducted on *L. monocytogenes* by (48) showing that the generation times at 35ºC, 13ºC, and 4ºC have been determined in autoclaved skim milk, chocolate milk, and whipping cream. At 35ºC, they reported generation times of 0.65 hours in chocolate milk, 0.67 hours in cream, and 0.69 hours in skim milk and whole milk.

K. pneumoniae is a Gram-negative bacterium, which can survive under stress conditions; however, the growth of this bacterium was limited to certain types of stress adaptation. Moreover, *K. pneumoniae* can survive in a variety of conditions, including high salt levels, low pH, low temperature, and the very hostile environment of the human immune system (49). In normal cases, growth conditions influence the composition of the bacterial cells, and the physiological state of the microorganism may be affected by its susceptibility to damage by subsequent exposure to stress.

Foodborne disease is caused by temperature abuse, and monitoring temperature history throughout food production, distribution, and storage are some easy, efficient ways to reduce the risk of food poisoning. The common relationship of *K. pneumoniae* with survival or growth in cooked and refrigerated milk indicates the possibility for microbiological risks and suggests a strategy for controlling the pathogen if faulty processing, food contamination, or temperature abuse occurs. To reduce the microbial load in milk, one option is the use of a microwave. This idea is supported by a study on *K. pneumoniae* in infant milk, which showed that the bacterium can be destroyed by microwave heating (50). Therefore, other new techniques or technologies, such as risk assessment in the food industry, are being studied to minimize foodborne illness.

Besides the type of temperature stress applied, it is believed that the ingredients and production treatment of the milk also influence the thermal resistance of *K. pneumoniae*. The prevalence of microorganisms in coconut milk and pasteurised milk was higher than allowed by the Malaysia Food Law 1983 and Regulation 1985, which indicated that milk was contaminated during handling or processing. Therefore, food must be kept under the correct temperature and storage conditions to avoid food contamination through temperature abuse. Although many other factors contribute to foodborne illness, temperature abuse is the most important factor to consider in food industry, as the food must be heat treated before consumption in many instances.

CONCLUSION

In conclusion, the exponential and stationary of the growth curve of *K. pneumoniae* was observed after 3–9 hours and 10–24 hours, respectively. The cultivability of the stationary phase of *K. pneumoniae* subjected to sublethal temperature stress (7°C, 27°C, 55°C, and 65°C) showed biphasic curves for shifted temperatures at 7°C and 27°C, and a straight-line death kinetic (55°C and 65°C), except in coconut milk. The presence of a stress temperature at 27°C indicated that the milk samples were abused at ambient temperature, and the growth of *K. pneumoniae* was increased as the stress adaptation increased in both selective and non-selective agars. Throughout the study, survival in coconut milk was higher than in pasteurised milk and UHT milk. At low temperature (7°C) stress exposure, the growth and survival of *K. pneumoniae* became bacteriostatic; *K. pneumoniae* exposed to low temperature were inhibited but did not die off. The survival curves of *K. pneumoniae* stressed from 37°C to high temperatures (55°C and 65°C) followed first-order death kinetics except for *K. pneumoniae* grown in coconut milk. The organism grown in UHT milk was killed the fastest, followed by that grown in pasteurised cow's milk and lastly, coconut milk. By understanding the survival of *K. pneumoniae* in milk and its substitutes, it is advised to avoid from keeping or handling milk at room temperature for too long and immediately maintained the storage of milk and substitutes prior to storing (below 5°C). For UHT milk, it should be immediately consumed or if it is left for later consumption, it should be kept at refrigerated temperature. For coconut milk and pasteurised cow's milk, it should be heated above 65°C to ensure Klebsiella and other foodborne bacteria are destroyed. This study concludes that the growth and survival of *K. pneumoniae* depend on the types of temperature stress and types of media used. This safety alert may help public and food operators to reduce the occurrence of food poisoning cases during handling of milks and its substitutes.

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