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Effectivesness of Thermal Treatment on the Bacterial Spore Counts in Milk

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***Abstract*—**Untreated milk cannot be kept for long time due to microbial spoilage. The spore-formers are major contaminants as it significantly affects the shelf-life, quality, and wholesomeness of milk, which is treated with heat at different levels. Mesophilic and thermophilic spores can survive even post treatment. The comparison between the spore counts under different heating methods was performed on samples of different suppliers. 110 samples from six points were collected to analyze the effect of thermized and ultra-high temperature (UHT) on the spores. Samples were heated at 80 0C for 10 minutes to inactivate the vegetative cells. Plate Count Agar (PCA) was formulated and mesophilic spores were incubated at 30°C for 48 hours, while the Petri dishes for counting thermophilic spores were incubated at 55°C for 48 hours. Incubated culture plates were enumerated for spore-forming bacterial colonies. High heat resistant spores (Sporothermodurans) were not detected on milk of four suppliers. A significant difference ($p<.0.05$) was found to be among the spore counts of both thermophilic and mesophilic of raw, thermized and UHT milk. The reduction of spore-formers after thermization was higher on thermophilic than mesophilic. The study revealed that the most effective method for reduction of spore-forming bacteria was UHT.

***Keywords*—**Thermization, UHT, spore-forming bacteria, mesophiles, thermophiles

I. Introduction

Young mammals are nurtured only by milk during the initial period of their life. It is a widely consumed beverage that is essential to people's diets worldwide due to its high concentration of important macro and micronutrients (Visioli Strata, 2014). Molds, yeast, and bacteria can grow in foods and cause spoilage, while mostly bacteria cause foodborne illness (USDA, 2012). Bacteria are the most troublesome and important microorganisms for food processors. Therefore, chemical preservatives, irradiation, high-pressure processing, pulsed electric field, dehydration, modified atmosphere, low temperature, and high-temperature processing are commonly used to preserve foods in the food industry (James, 2000). An endospore is a dormant, tough, and non-reproductive structure produced by certain species of bacteria. Spores are resistant to heat, UV radiation, desiccation, and other environmental stresses that typical vegetative cells cannot withstand (Nicholson *et al*, 2000). The endospores are usually produced by G+ microorganisms such as Bacillus spp. and Clostridium spp. due to stress conditions like exhaustion of available nutrients or an essential nutrient (Kenneth, 2008). Endospore formation is an extreme survival strategy that allows the bacterium to produce a dormant and high resistant structure to preserve cell's genetic material. The four primary spore formers threatening the food industry are Clostridium botulinum, Bacillus cereus, Bacillus substilis, and Bacillus anthracis (USDA, 2012). Since endospore forming bacteria (vegetative cells) are dispersed everywhere it is important to identify environmental conditions needed for their growth (USDA, 2012). It has been found that there are six basic environmental conditions; nutrient, pH, oxygen, temperature, moisture, and time that are essential for the growth of bacteria (Errington, 2014; USDA, 2012; Hawthorn, 1969).

Bacillus spp is the most common spore-forming bacteria found in raw milk. Bacillus contamination levels, while variable, can reach 105 CFU/ml (Tamime, 2009). Aerobic and anaerobic bacterial species produce spores that can affect dairy products. They necessitate a wide range of optimal growth temperatures and growth requirements (Sadiq *et al*, 2016). Some vegetative cells are converted into endospores during the heat treatment, which are highly resistant to most treatments that destroy vegetative cells quickly. Toxins are often linked to spores, which are present either within the spore covering or released during germination or sporulation (USDA, 2012). Many experiments indicate that spores have extreme resistance due to the accumulation of a large depot of calcium dipicolinate and the encasement of the spore within multiple highly stable coat proteins (Setlow *et al*, 2012). Spores and other gram-positive bacteria are metabolically dormant and extremely resistant to host environmental stress (USDA, 2012). Mesophilic spore-forming microorganisms,

which grow in this mesophilic temperature range (30°C - 40°C) and have an impact on food safety. Thermophilic spore formers are more resistant to stress than mesophilic spore formers among spore forming bacteria (James, 2000).

Heat treatment is the most widely and commonly used processing technology in the dairy industry. The main purpose is to eliminate both pathogenic and spoilage microorganisms to ensure that the milk is safe for consumption and has a reasonable shelf-life (International Dairy Federation, 2018). Thermization is a subpasteurization operation that is sometimes used on raw milk that is intended to be refrigerated after consumption for extended periods of time before being used to make certain products. (Chen Sun, 2006). In thermization process, milk is heated to 57°C to 68°C for 15 seconds, which inactivates the psychrotrophic bacteria by prevent enzyme production (Rukke *et al*, 2011). UHT is a technique for preserving liquid food products by revealing them to short, intensive heating, which destroys the microorganisms in the product. Low-acid liquid products are usually treated at 135 – 150°C for a few seconds, by either indirect heating, direct steam injection, or infusion (Bylund, 1995). Howerver, microbial spore-formers are important contaminants in the dairy industry as they can significantly affect the quality and safety of food. So, effective controlling of these bacteria in milk products and the processing environment is still challenging, due to limited knowledge of their origin and characteristics related to food quality, such as thermoresistance or spoilage and their toxic potential. Untreated milk is spoiled by the growth of mesophilic and thermophilic spores. The milk is heated as a solution to preserve it. Even after the heat treatments, mesophilic and thermophilic spores can survive on milk. So far, no data on the spore count of mesophilic and thermophilic bacterial spores on thermized and UHT treated milk from various suppliers are available for public use. Therefore, a necessity arose to compare the mesophilic and thermophilic spore count under different heating applications. Therefore, the present study was carried out to compare the spore counts of mesophilic and thermophilic bacteria and to find an effective thermal application for milk.

II. METHODOLOGY

A. Sample Collection

The study was conducted on the milk of four major suppliers. Milk was delivered using bowsers in three forms such as raw, post-thermized, and post-pasteurized milk. They are further processed and delivered to the market as UHT-treated tetra packs. Before collecting samples, sample collecting glass bottles were sterilized with an autoclave (ST-85G, Korea) at 121°C for 30 minutes by saturated steam under 15 psi of pressure. Dairy milk samples were collected from four different suppliers. The samples were hygienically collected into pre-sterilized sample bottles for 30 days from each sampling point with two replicates. Collected samples were kept in a milk chiller (Hisense FC-27DD4HA, China) at approximately 4°C to prevent heat abuse. The different types of milk samples that were obtained from four suppliers is shown in Table I

Table I: Different samples of milk collected from different suppliers

Supplier	Type of milk sample collected
A	Post pasteurized milk, before UHT (Balance tank) and post UHT (Tetra pack)
B	Post pasteurized milk, Before UHT (Balance tank) and post UHT (Tetra pack)
C	Raw milk (Before thermized), post thermized milk, before pasteurized, post pasteurized milk, before UHT (Balance tank) and post UHT (Tetra pack)
D	Post thermized milk, before pasteurized, post pasteurized milk, before UHT (Balance tank) and Post UHT (Tetra pack)

B. Media Preparation

Plate Count Agar (PCA) was formulated according to the ISO 4833-1: 2013 Microbiology of food and animal feeding stuffs Horizontal method, for the enumeration of microorganisms' colony count at 30°C by the pour plate technique. The milk samples were diluted with peptone water accordingly before culturing to avoid colony overgrowth and overlapping to reduce inaccurate results.

C. Culturing and Enumeration

A portion of 1ml of milk was pipetted into a test tube containing 9ml of prepared peptone water, and it was mixed thoroughly using the advanced vortex mixture (ZX3, Italy). Afterward, the test tubes were kept in a water bath at 80°C for 10 minutes. Then, 1 ml from each sample was transferred into a Petri dish with pre-sterilized pipettes. A Portion of 15ml of PCA was poured into the petri dishes containing the samples. Then the plates were labelled separately for mesophilic and thermophilic spore-forming bacteria, and then the analyzed date was labelled. The Petri dishes intended for counting mesophilic spores were incubated at 30°C for 48 hours, while the Petri dishes intended for counting thermophilic spores were incubated at 55°C for 48 hours as per the guideline of Kent *et al*. (2016). After the period of incubation, the plates with colonies were counted separately with colony counter. Colony-forming units (CFU) were calculated per 1 ml of test samples. The ability of bacterial endospores to recover and forming of colonies were considered as a measure to determine the effectiveness of the heat treatment. Spreading colonies were considered as single colony. If less than one-quarter of the dish was overgrown by spreading, count the colonies on the unaffected part of the dish and calculate the corresponding number of the entire dish. Once the count was discarded, where more than one quarter of the dish was overgrown by spreading colonies.

D. Statistical Analysis of data

The data were analyzed using t-tests with 95% confidence level. All the tests were done by using SPSS (SPSS 20.0, IBM, New York, USA)

Table II: The mesophilic and thermophilic spore counts of raw, thermized, before pasteurized, post pasteurized, before UHT, and post UHT milk samples of different suppliers

Sample type	Industry-A		Industry-B		Industry-C		Industry-D	
	Mesophilic spores (CFU/ml)	Thermophilic spores (CFU/ml)	Mesophilic spores (CFU/ml)	Thermophilic spores (CFU/ml)	Mesophilic spores (CFU/ml)	Thermophilic spores (CFU/ml)	Mesophilic spores (CFU/ml)	Thermophilic spores (CFU/ml)
Raw milk	-	-	-	-	3.58×10^{5} $\pm1.68\times10^{4}$	3.41×10^{5} $\pm12.2\times10^{4}$	-	-
Post Thermized	-	-	-	-	$1.96\times10^{4*}$ $\pm2.02\times10^{3}$	1.56×10^{4} $\pm9.3\times10^{2*}$	2.99×10^{4} $\pm5.25\times10^{3*}$	1.28×10^{4} $\pm2.08\times103$
Before Pasteurized	-	-	-	-	2.39×10^{4} $\pm5.53\times10^{3}$	1.75×10^{4} $\pm7.63\times10^{2}$	3.61×10^{4} $\pm9.24\times10^{3}$	1.87×10^{4} $\pm1.42\times10^{3}$
Post Pasteurized	7.43×10^{2} $\pm2.16\times10^{2}$	3.81×10^{2} $\pm2.04\times10^{2*}$	3.61×10^{2} $\pm1.57\times10^{2*}$	2.07×10^{2} $\pm4.05\times10^{2*}$	2.06×10^{2} $\pm1.3\times10^{1}$	1.76×10^{2} $\pm1.2\times10^{1}$	2.09×10^{2} $\pm2\times10^{1*}$	2.47×10^{2} $\pm5.1\times10^{1*}$
Before UHT	9.17×10^{2} $\pm2.86\times10^{2}$	4.66×10^{2} $\pm2.56\times10^{2}$	3.81×10^{2} $\pm2.69\times10^{2}$	2.39×10^{2} $\pm3.57\times10^{2}$	4.44×10^{2} $\pm1.03\times10^{2}$	3.97×10^{2} $\pm1.23\times10^{2}$	3.3×10^{2} $\pm1\times10^{1}$	2.65×10^{2} $\pm5.6\times10^{1}$
Post UHT	0	0	0	0	0	0	0	0

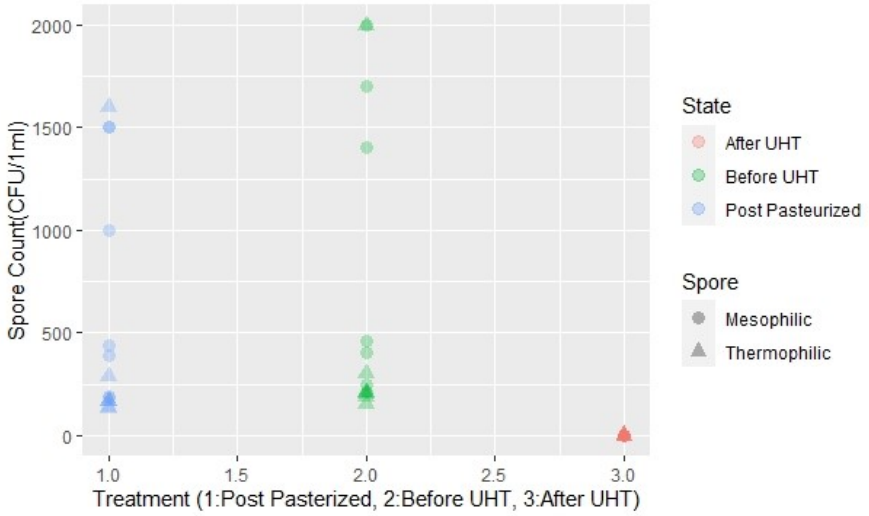

Figure 1: Mesophilic and thermophilic spore counts under different heat treatment of industry-A

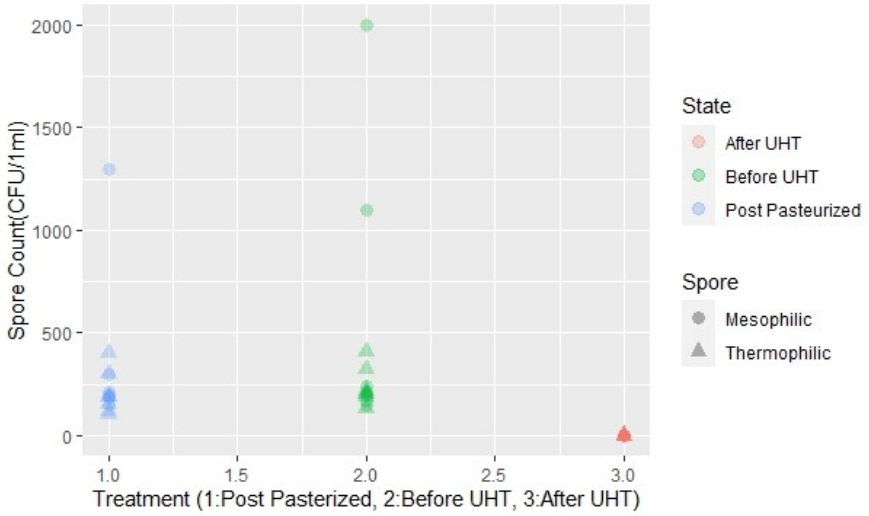

Figure 2: Mesophilic and thermophilic spore counts under different heat treatment of industry-B

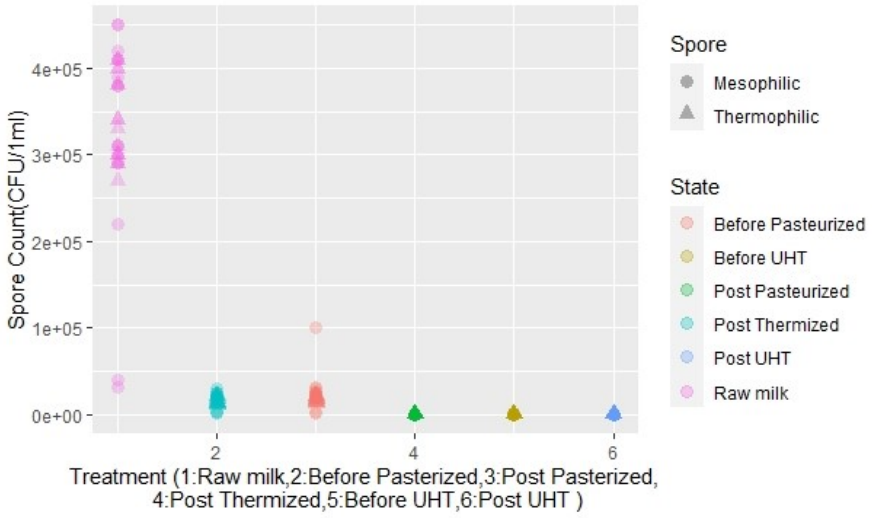

Figure 3: Mesophilic and thermophilic spore counts under different heat treatment of industry-C

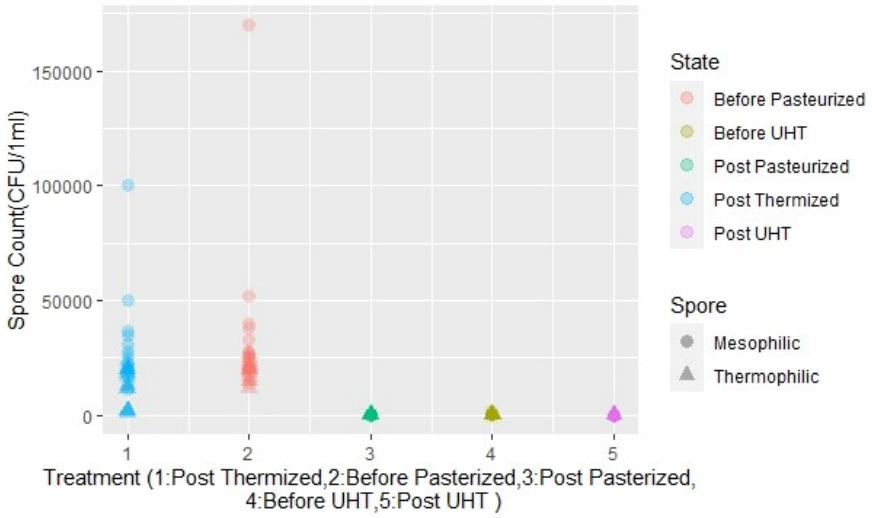

Figure 4: Mesophilic and thermophilic spore counts under different heat treatment of industry-D

III. RESULTS AND DISCUSSION

A. Industry-A

In post-pasteurized milk, both mesophilic and thermophilic spores counts were found to be ranged between 130 – 1500 CFU/ml. The milk prior to the treatment of UHT was found to be ranged between 140 – 2000 CFU/ml. However, most counts were concentrated in the range between 140 – 500 CFU/ml. Here, no mesophilic and thermophilic colonies were found to be observed after treatment with UHT. A significant difference ($p<0.05$) was found to be observed on both the mesophilic and thermophilic spore counts within the samples of prior UHT treatment. The post-pasteurized milk was not found to be shown any significant ($p>0.05$) difference among the samples for both mesophilic spore count and thermophilic spore counts as shown in Table II. According to the results obtained, the mesophilic colonies were found to be considerably higher than thermophilic colonies on both post-pasteurized and prior-UHT treated milk samples. During various heat treatments, spores were found to be destroyed by the heat, as being indicated by decreasing spore counts. Anyway, no high heat resistant spores were found to be seen.

B. Industry-B

Here, both the mesophilic and thermophilic spore counts were found to be ranged between 130 and 1500 CFU/1ml in post-pasteurized milk. Before UHT treatment, thermophilic spore counts were found to be less than 500 CFU/1ml, but mesophilic spore counts were found to be higher, being recorded with the value of 2000 CFU/1ml. A significant difference ($p<0.05$) was found to be observed within the samples of pre-UHT treatment on both mesophilic and thermophilic spore counts. The post-pasteurized milk was not found to be shown any significant ($p>0.05$) variation among the samples for both spore counts as shown in Table II. No colonies of mesophilic and thermophilic were found to be

observed post UHT treatments. As per the results obtained, the mesophilic colonies were found to be considerably higher than thermophilic colonies for both post-pasteurized and pre-UHT treated samples.

C. Industry-C

High counts of mesophilic and thermophilic spores were found to be in raw milk samples. Both mesophilic and thermophilic spore counts were found to be ranged between 130 and 1500 CFU/1ml in post-pasteurized milk. Pre-UHT treatment, thermophilic spore counts were found to be less than 500 CFU/1ml, whereas, mesophilic spore counts were found to be recorded at a higher value of 2000 CFU/1ml. Both the mesophilic and thermophilic colonies counts were found to be recorded at zero in post-UHT samples. As far as the industry-C was concerned raw, post-thermized, pre-pasteurized, and post-pasteurized milk samples for both mesophilic and thermophilic spore counts were found to be shown significant difference ($p<0.05$). But, no significant variation ($p>0.05$) between the post-thermized and pre-pasteurized samples for both the mesophilic and thermophilic spore counts were found to be observed as given in Table II. As per the results obtained, the mesophilic colonies were found to be considerably higher than that of thermophilic colonies for all milk samples.

D. Industry-D

Both mesophilic and thermophilic spore counts were found to be higher in post-thermized and pre-pasteurized milk samples. The mesophilic spore counts, on the other hand, were found to be higher in both cases. Although spore counts were found to be lower in post-pasteurized, pre-UHT, and post-UHT milk, the spore counts of both mesophilic and thermophilic were found to be reached to zero in post UHT milk. As far as the industry-D was concerned, a significant difference ($p<0.05$) was found to be observed within the samples of pre-pasteurized and post-pasteurized milk samples for both the mesophilic and thermophilic spore counts. At the same time, there was no significant difference observed for mesophilic spore counts. No significant difference ($p>0.05$) was found to be seen between post-pasteurized and pre-UHT milk for both mesophilic and thermophilic spore counts as shown in Table II. Based on the results obtained, the mesophilic colonies were found to be considerably higher than that of thermophilic colonies for all milk samples.

E. Comparison between different suppliers

According to the results of industry-C and A, the quality of milk C was found to be better than that of industry-A. But the milk sample of industry-A was brought as post-pasteurized. Therefore, there would be a possibility of the pasteurized milk being contaminated the pasteurized milk during transportation and being transferred into the tanks. But the milk of industry-C was brought as raw and pasteurized at the plant. Therefore, it is evident that the pasteurized milk was less contaminated. It could be observed that the hygienic condition of the industry is still under the standard level and this could be the possible reason for the high count of spores. When the post-UHT milk of the four industries was compared, no mesophilic and thermophilic colonies were observed. Regardless of the initial conditions, the final milk output was microbially hygienic. It was crystal clear that the raw milk samples had a higher microbial count since no any treatment was applied. When the spore count between the raw milk and thermized milk was compared, the thermophilic spore count was found to be decreased than that of mesophilic spore count. But it was observed that the mesophilic spore count in raw milk was found to be higher. Hence, it could be assumed that high heat resistant mesophilic spores were present in raw milk delivered by industry-C. According to this present study, no microbial colonies were observed in UHT treated milk samples and it could be concluded that the most effective method of heat treatment is UHT treatment. The temperature between 1380C-1500C was applied during UHT treatment, which showed that there were thermophilic and mesophilic spores observed that were resistant to this high temperature. Klijn *et al* (1997) found that Bacillus Sporothermodurans spores were able to survive from the UHT-treatments, but it could be observed that during this study, no such high heat resistant species were found to be seen.

IV. CONCLUSION

Based on the results obtained, it could be concluded that the best milk was supplied by industry-C, whereas the least quality milk was supplied by industry-A. Mesophilic spore counts were higher in all scenarios than that of thermophilic spore counts. However, no high heat resistant spores (Sporethermodurance) were found to be detected during this study among different milk suppliers in Sri Lanka. It was clearly indicated that the zero-valued spore counts in post-UHT milk for both thermophilic and mesophilic were observed. The reduction in spore count after thermization was found to be higher for thermophilic than that of mesophilic This present study divulged that the most effective method of thermal treatment for spore forming bacteria was UHT method.

REFERENCES

Bylund, G. (1995). *Dairy processing handbook*. Tetra Pak Processing Systems AB. Sweden.

Chen, X., Sun, D. (2006). Thermal food processing: new technologies and quality issues.Tamime, A.Y. ed. (2009). *Milk processing and quality management*. John Wiley Sons. pp 1-324.

Errington, J. (2014). Regulation of endospore formation in Bacillus subtilis. *Nature Reviews Microbiology, 1*(2), 117-126.

Hawthorn, J. (1969). Organisms in Foods. *Nature, 224*(5215), 196-197.

International Dairy Federation, (2018). "Heat Treatment of Milk-Overview", *IDF Factsheet 001/2018-02*, p. 4.

James, J. M. (2000). Modern food microbiology. Sixth Edition.

Kenneth, T. (2008). Pathogenic E. coli. Todars Online *Textbook of Bacteriology*.[Online], 2012. Available at: http://textbookofbacteriology.net/

Kent, D. J., Chauhan, K., Boor, K. J., Wiedmann, M., Martin, N. H. (2016). Spore test parameters matter: Mesophilic and thermophilic spore counts detected in raw milk and dairy powders differ significantly by test method. *Journal of dairy science, 99*(7), 5180-5191.

Klijn, N., Herman, L., Langeveld, L., Vaerewijck, M., Wagendorp, A. A., Huemer, I., Weerkamp, A. H. (1997). Genotypical and phenotypical characterization of Bacillus sporothermodurans strains, surviving UHT sterilisation. *International Dairy Journal*, 7(6-7), 421-428.

Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J., Setlow, P. (2000). Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. *Microbiology and molecular biology reviews, 64*(3), 548-572.

Rukke, E.O., Sørhaug, T. and Stepaniak, L. (2011). Heat Treatment of Milk. *Encyclopedia of Dairy Sciences, 4*,pp.693-698.

Sadiq, F. A., Li, Y., Liu, T., Flint, S., Zhang, G., Yuan, L., ... He, G. (2016). The heat resistance and spoilage potential of aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders. *International journal of food microbiology, 238*, 193-201.

Setlow, P., Liu, J. Faeder, J.R. (2012). Heterogeneity in bacterial spore populations, pp 201–216. InAbel Santos E (ed), Bacterial spores: current research and applications, Caister Academic Press, U.K.

United State Department of Agriculture (USDA), (2012). Introduction to the microbiology of food processing, Washington: US Department of Agriculture. Available at https://fstdesk.com/uploads/short-url/r1gAKLsqGSZGqiHm042q8qJNc8C.pdf (Accessed: 27 May 2021).

Visioli, F., Strata, A. (2014). Milk, dairy products, and their functional effects in humans: a narrative review of recent evidence. *Advances in nutrition, 5*(2), 131-143.