Safe processing method and storage time threshold for consuming of powdered-infant formula based on total plate count test

S Amelia[1 *], NDA Lubis[2], MF Rozi[3], and IFF Nababan[3]

[1]Department of Microbiology, Faculty of Medicine, UniversitasSumatera Utara, Indonesia
[2]Department of Nutrition and Dietetics, Faculty of Medicine, Universitas Sumatera Utara, Indonesia
[3]Faculty of Medicine, Universitas Sumatera Utara, Indonesia

[*]Email: sriamelia@usu.ac.id, doktermely@yahoo.com

Abstract. Powdered-infant formula (PIF) is theoretically suggested as a substitution for breast-milk. The presence of PIF has brought several problems related to its contamination, handling, and processing. Our study aimed to provide the evidence related to determine the safe processing method, between using hot water (HW) or boiled water (BW), and storage time threshold after PIF was left at room temperature for acertain period of time. We obtained PIF, cow milk-based, from supermarket and made it as suspension. Bacterial growth assessment was performedusing total plate agar, total plate count test, while several tests including the use of agar, blood and McConkey agar, and biochemical reactions were used to determine the bacterial species. We provided formula suspension in different amount of dilution (10^{-1}, 10^{2},10^{3}, and 10^{-4}), furthermore observation of bacterial colonies for 3-8 hours was done. The study was carried out at Department of Microbiology, Faculty of Medicine, Universitas Sumatera Utara in October 2017. We obtained a significant number of bacterial colonies at the first time of observation in HW and using BW was safer than HW regardless of its nutritional value, based on total plate count tests. Meanwhile, safe storage time threshold for consuming after it was left at room temperature was no more than one hour. We identified *Bacillus subtilis* and *Klebsiella sp.* as contaminants during the observation. Thus, less contamination also reduces infection rate among infantsby performing appropriate handling.

1. Introduction

Infant formula has its own long journey, Hippocrates also stated that introduction of solid food should occur as soon as the children emerged their first teeth while Aristotle had talked about animal and human milk was nutritious for infant, it was all written in 'Historiaanimalium'. He also defined "milk consists of whey and curds, and more curds make it more nutritious". In 70-130 AD, Soranus' and Galen's dietary prescriptions for infants, not in Avicenna's prescription (980-1036 AD), described using cow's milk combined with honey as the first food and initiated breastfeeding on the second day of life [1].

Since, its introduction in 70-130 AD for infants, infant formula manufacturers have evolved into larger industry. Infant formula is well-described as not a pharmaceutical product, if it becomes a pharmaceutical, under US law, it should be included in safety and effectiveness study which is not

performed for milk product. US has been established the Infant Formula Act of 1980 as revised in 1986 containing about nutritional adequacy and its no-guarantee statement of PIF free from pathogenic organisms [2] Meanwhile, according to the Codex Alimentarius Commission states that "infant formula is defined as breast-milk substitution, tailor-made, manufactured to meet the infant's nutrients during the first months of life until introduction of appropriate complementary feeding"(Codex Alimentarius Commission 2007, Section 2.1.1) [3].

Because of its high birth rate, Indonesia is one of the major markets for milk product. Statistically, it was proved that infant formula consumption increase by 37 % according to the annual report in 2008-2013 worldwide. Therefore, Ministry of Agriculture (MOA) has prepared several plans to boost domestic milk industries since the country still imported 2,536 tonnes of milk and milk products while only 1,490 tonnes of the product domestically produced, the data was based on annual report from FAO in 2016 [4-5].

Several bad health implications associated with PIF consuming are emphasized by Dr.Cicely Williams through an article entitled "Milk and Murder" which talks about inappropriate handling of PIF in low-income countries, this brings evidence that contamination and improper handling can cause some impacts to infant itself. Several studies proved diverse bacteria, including *Enterobactersakazakii, Salmonella spp., Pantoeaagglomerans, Escherichia vulneris, Hafniaalvei, Klebsiella spp., Citrobacter spp., Enterobacter cloacae, Bacillus cereus, Clostridium spp., Staphylococcus aureus,* and *Listeria mono cytogenes*, could cause contamination [6-8]. Poor hygiene and handling related to inadequate PIF bottle cleaning and its poor storage will cause contamination. PIF components are very supportive for bacterial proliferation, when stored in improper method, bacterial proliferation will occur when the infectious dose for causing a disease has been fulfilled [9]. Based on study conducted in developing country, half of the mothers admitted that storing PIF that were not consumed within two hours and then consumed again for their infants [10].

Thus, our study aimed to provide the evidence related to bacterial growth and proliferation based on total plate count test results according to different processing methods, using hot water and boiled water, and safe storage time threshold of PIF, lastly, we obtained a conclusion not to debate several recommendation but the evidence will be useful for increasing awareness of bacterial contamination in PIF.

2. Materials and methods

We designed a descriptive study using powdered-infant formula milk (PIF) which is commonly utilized by parents at home and is cow milk-based, without describing its brand. We provided the data including bacterial growth based on total plate count test results after diluted-PIF was left at room temperature for a certain period of times. Safe storage time is defined as the time when the level of bacterial colonies is significantly higher in formula and did not meet eligible criteria based on Indonesia National Standards, it also reflected safe times for parents who always have behavior of storing diluted-PIF inside its bag or storage box when they are outside. Meanwhile, comparison of PIF processing method between using hot water (HW) and boiled water (BW) was also performed, the indicator was also bacterial colonies based on total plate count test result. The entirety process of the study was carried out in Bacteriology Laboratory, Department of Microbiology, Faculty of Medicine, Universitas Sumatera Utara between October and November 2017. In order to get minimal contamination, we prepared the samples under biosafety cabinet.

2.1 Formula milk-powdered processing

We obtained PIF from the supermarket and noted some specifications from the packaging, it was also stated that the manufacturer has carried out good farming practice (GFP) and good handling practices (GHP), therefore, there was no baseline bacterial identification in the initiation of the study. The two different processing methods were used in our study, using HW and BW to dilute PIF.

There were ten volumetric flasks labeled with different amount of dilution, HW referred to hot water with a temperature of 70^0C measured using digital water temperature thermometer (HM digital®Applied

Membranes Inc.) and BW referred to boiled water with a temperature of 100^0C. Five volumetric flasks for HW and labeled as HW, HW 10^{-1}, HW 10^{-2}, HW 10^{-3}, and HW 10^{-4} respectively. The rest, other five, were also encoded for similar fashion as BW, BW 10^{-1}, BW 10^{-2}, BW 10^{-3}, and BW 10^{-4}.First suspension, HW, was processed by pouring 180cc of hot water into first volumetric flask (labeled HW) containing three tablespoons of PIF and make it as suspension. Secondly, as much as 10cc of HW, first volumetric flask, was obtained and poured into second volumetric flask (HW 10^{-1}), after dilution process, it became 10^{-1}dilution. Thirdly, in the next volumetric flask, labeled HW 10^{-2}, next suspension was made by diluting 10cc of PIF from HW10^{-1} into HW 10^{-2} to make 10^{-2}dilution, and so on, until the last amount of dilution, HW 10^{-4}. We also used the similar manner for diluting PIF with BW and make it into four distinct amount of dilution. Every half hour, samples from each volumetric flask were obtained to assess and identify its bacterial growth during three hours, total plate count tests and several tests described in the next section were used for identification of the colonies.

In addition, safe storage time threshold for consuming of PIF was determined by using similar method, yet, in different manner. We provided two volumetric flasks, first was uncovered (labeled OC or open container) while the second one was closed (labeled CC or closed container), contained eight tablespoons of formula powder. Thus, the formula suspension was made by diluting it with 240 cc of boiled water. Lastly, the total plate count tests and bacterial identification were performed every hour for the next eight hours.

2.2 Total plate count tests and bacterial identification

We obtained 1cc of formula from four different dilutions in each group, HW and BW, and poured it into petri dish. Pour plate method was used in the study to mix the inoculum into the plate count agar (Oxoid™, Thermo Fisher Scientific Inc.), afterward the plate was rotated gently to made the agar unification with inoculum. Thus, incubation was carried out for 24-48 hours at 37^0C. Meanwhile, bacterial assessment for safe storage time threshold also used total plate count test, however it was checked for every hour in the next eight hours. Interpretation of TPC would be defined as colony forming unit per milliliter or per gram (cfu/ml or cfu/g).

Bacterial identification was performed after incubation for 24-48 hours using blood agar (Oxoid™, Thermo Fisher Scientific Inc.) and McConkey agar (Oxoid™, Thermo Fisher Scientific Inc.). Initially, bacterial identification involved multiple steps consisting of microscopic assessment including gram staining, catalase test, coagulase test, and several biochemical reaction tests based on sugar fermentation, indol production, methyl red test, motility test, oxidase test, citrate test, triple-sugar iron (TSI) test, urease and VogesProskauer test. All procedure and tests amentioned above were established as basic identification test for bacteria in accordance with manual of basic laboratory procedure in clinical bacteriology, 2nd edition, 2003.

We provided the data in descriptive manner, therefore, the data was presented in table to reveal the total plate count test results univariately.

3. Results and Discussions

Primary data was obtained from total plate count test results. First observation, which aimed to determine the bacterial growth difference between PIF using HW and BW, samples were cultured every half hour for three hours. It showed that there was no significant finding of bacterial growth in BW until third observation, at the 60th minutes. In addition, bacterial colonies emerged slower in thinner suspension. At the 60th minute, the bacteria had appeared 10 cfu/ml in BW (no dilution) while in another amount of dilution there were no significant bacterial colonies at the same time. Meanwhile, since the first beginning of observation HW were positively evident with significant colonies of bacteria (>$100x10^4$cfu/gram) in every amount of dilution. Increasing of bacterial growth was evident along with addition of time and the less level of solvent or water. The data was provided in Table 1 for each time series.

We also presented of safe storage time threshold for consuming after PIF was left at room temperature in different table, Table 2. It showed a significant number of bacteria emerged in 2nd

observation, at first hour. In addition, bacterial identification exhibited *Bacillus subtilis* and *Klebsiellapneumoniae,* it was obtained from colonies produced in the media.

Table 1. Avarage of colony forming unit in formula both in HW and BW groups.

Colonies in both groups (cfu/gram)	**Observation time (minutes)**						
	0’	30’	60’	90’	120’	150’	180’
Boiled water (BW)	<1	<1	$0,06\times10^4$	$0,05\times10^4$	$1,2\times10^4$	$2,2\times10^4$	$5,1\times10^4$
Hot water (HW)	$6,75\times10^4$	16×10^4	74×10^4	$>100\times10^4$	$>100\times10^4$	$>100\times10^4$	$>100\times10^4$

Table 2. Colony forming units/mililiter based on abandonment time of formula in room temperature

Observation time (hours)	**Groups (cfu/ml) x 10^4**	
	Open container (OC)	Closed container (CC)
0	0	0
1	10	4
2	23	11
3	45	30
4	97	48
5	>100	87
6	>100	>100
7	>100	>100
8	>100	>100

Our study provided the evidence of the two distinct processing methods, using HW or BW, could produce a significant difference in bacterial contamination. It also proved that higher concentration of PIF made it more prone to contamination. Contamination in infant formula is still problematic as the infant, mainly neonates, fragility of certain pathogenic bacteria is evident, as we identified *Klebsiella sp.* and *Bacillus subtilis* as contaminant in the samples. In addition, PIF needs clean water to dilute it into a proper suspension, excessive amount of water can lead several disadvantages, nutritionally inadequate and gastrointestinal problems [11]. Contamination usually occurs along the handling including not using clean water. Moreover, it also clearly explained that PIF is not a sterile product albeit the manufacturer has performed current hygiene standards, it reflects that the presence of contaminant may also be positive since in the very beginning [12].

Comparison between two groups, HW and BW, revealed the colonies in HW is significantly higher and its proliferation rapidly emerged at the first time of observation. Based on World Health Organization (WHO), water or solvent used to dilute PIF should no less than 70^0C. Meanwhile, our study proved the temperature must higher than 70^0C to prevent bacterial proliferation and it also showed that the absence of bacterial growth found in BW until the 60^{th} minutes of observation. Rosset et al. proved that combination of different parameters including high initial PIF temperature, high storages temperatures, and long reheating time could affect the bacterial potential growth [13]. British National Health System also stated similar rules, yet with additional recommendation consisting of using freshly boiled drinking water or it must be boiled previously but not in the kettle for no more than 30 minutes to prevent contamination [14]. WHO recommendation about water temperature to dilute PIF remains controversial, in practice only 22% of parents use water with temperature of $\geq70^0$C. In addition, using BW as solvent to dilute PIF also remains debatable as certain nutritional value degrades by the presence of high temperature, mainly vitamin [15].

The relationship between time after diluted-PIF was left at room temperature and bacterial growth descriptively presented in Table 2. It showed a different number of bacterial colonies emerged in OC and CC, as much as 10 cfu/ml (OC) and 4 cfu/ml (CC). According to Indonesia Nasional Standard No.01-6366-2000, the minimum bacterial colonies contained in milk product must not exceed 11 cfu/ml. Consequently, based on our study, one hour is suggested as safe storage time threshold of diluted-PIF to re-consumes it again. WHO/FAO recommends that in casediluted-PIF will not be consumed within two hours, it must becooled and stored in refrigerator as soon as possible (at maximum temperature of 5^0C). After 24 hours in refrigerator, diluted-PIF should be consumed because the bacterial proliferation and change on nutritious value will ensue by its prolonged storage. A cohort study revealed that 6% of mothers still kept PIF at room temperature for >2 hours while in different study, 50% of mothers and caregivers did not discard unconsumed-PIF within two hours [10-11]. Big gap of understanding about storage and processing of PIF can establish because of the lack of education of the handlers to read written protocols or inaccessible way to ask health-professional [16].

4. Conclusion

PIF is substitution for breast-milk and it still needs further attention, as its form in unsterile condition in the first handling, caregivers and mothers should perform safe preparation, storage and handling of PIF according to WHO guidelines both in care and home setting. We provide some evidence that precise amount of dilution and water temperature used to dilute PIF are essential to reduce or increase risk of bacterial growth potential. In addition, safe storage time after diluted-PIF was left at room temperature was commonly neglected by mothers or caregivers, it results in risky condition for infants who may get food-borne disease. Furthermore, an understanding of all aspects related to safe handling and processing of PIF are mandatory.

References

[1] Castilho SD and Filho AAB 2010 *Jornal de Pediatria* **86** (3) 179-188
[2] Kent G 2012 *Clinical Lactation* **3** 21-5
[3] World Health Organization (WHO)/ Food and Agricultural Organization of the United Nations (FAO) 2007 *Codex Alimentarius 2007 Standard for infant formula and formulas for special medical purposes intented for infants (CODEX STAN 72-1981)*
[4] Darmawan B 2017 Indonesia 2017 dairy and products annual report Global Agricultural Information Network
[5] Griffin M 2016 *Milk and milk products*Food Outlook 111
[6] Knight-Jones TJD, Hang'ombe MB, Songe MM, Sinkala Y, Grace D 2016 *International Journal of Environmental Research and Public Health* **13** 737
[7] Sospedra I, Rubert JV, Soler C, Soriano JM, Manes J 2009 *Foodborne Pathogens and Disease* **6** (10) 1269-72
[8] Ali A, Akhtar N, Bashir U, Hafeez R, Haider MS 2015 *Biologia (Pakistan)* **61** (2) 271-7
[9] Gribble KD and Hausman BL 2012 *Australian Medical Journal* **5** (5) 275-83
[10] Usai T, Mutonhodza B, Makamure C, Tshalibe RS, Chinofunga D 2013 *International Journal of Scientific & Technology Research* **2** (9)
[11] Labiner-Wolfe J, Fein SB, Shealy KR 2008 *Pediatric s***122** (2) 85-90
[12] Kent G 2015 *International Breastfeeding Journal***10** (6)
[13] Rosset P, Noel V, Morelli E 2007 *Food Control* **18** (11) 1331-476
[14] Silano M, Paganin P, Davanzo R 2016 *Italian Journal of Pediatrics* **42** (17)
[15] World Health Organization (WHO) 2007 *Safe preparation, storage and handling of powdered infant formula*
[16] Martin CR, Ling P, Blackburn GL 2016 *Nutrients* **8**