Rapid Microbial Detection Model System in UHT Milk Products using Poly(L-Lactic Acid) (PLLA) Thin Film
(Sistem Pengesanan Mikrob Model Pantas di dalam Produk Susu UHT menggunakan Filem Nipis (PLLA) Poli (Asid L-Laktik))

NURUL HIDAYAH YUSOF, NORRAKIAH ABDULLAH SANI, FARAH HANNAH ANUAR, MOHD SUZEREN JAMIL & SAIFUL IRWAN ZUBAIRI*

ABSTRACT
Ultra-high temperature is a process that involves heating of milk to a very high temperature to produce sterile milk products. However, food poisoning due to consumption of UHT milk still happen in Malaysia. This study was done to develop a film that is made by poly(L-lactic acid) (PLLA) to detect the presence of microorganisms in UHT milk products. UHT milk that was used in this study was full cream milk. Contaminated milk that contained Bacillus cereus was made to conduct a model system on the relationship between colony forming unit of microorganisms and contact angle. Contaminated milk was also used as a control sample to study the difference of milk properties between fresh and contaminated milk. Physicochemical analysis (Brix, colour, pH and contact angle) and microbiological analysis (total plate count) were done to UHT milk sample as soon as the packaging of the milk was unsealed. Analysis was done with 30 min time interval until 4 h and 30 min since the unsealing of packaging. The results showed that presence of microorganisms in UHT milk was detected after the milk product was unsealed and exposed to environment for 3 h and 30 min. Contact angle resulted from the presence of microorganisms in UHT milk was 64.34 - 65.44° with its colony forming unit, 2.1 – 3.9 cfu/mL. Therefore, the potential usage of contact angle on PLLA thin film with respect to colony forming unit (cfu) in detecting the presence of microorganisms in UHT milk product was attained and well modelled.

Keywords: Contact angle; microorganisms; PLLA; spoilage indicator; UHT milk

INTRODUCTION
According to Food Act 1983 (2015), ultra-high temperature (UHT) milk is milk that has been heated with temperature not less than 135°C for at least 2 s to make it commercially sterile. Tamime (2009) stated that contamination in UHT milk may happen due to two factors which are survival of heat resistant spore-forming bacteria and contamination after processing. In 2011, there were 97 food poisoning cases that was said due to UHT milk consumption from Program Susu 1Malaysia (PS1M). The milk was supplied by Dutch Lady Milk Industries Berhad (KKM 2012). Based on the laboratory test, there was B. cereus and its toxins in the milk (Yusmawati 2011). This is not surprising since Bacillus spp. is a pathogenic bacteria species that has been always related to contamination of UHT milk (Forschino et al. 1990; Luck et al. 1978; Skladal
et al. 1993). Following this incidence, rapid detection is needed to prevent this problem in the future. Rapid detection should take less than 8 h (Cox et al. 1978) or enable to give results as soon as the test is done (Wang 2015). Moreover, rapid detection should be sensitive to ensure low count of microorganisms can be detected as well (Wang 2015). Poly(L-lactic acid) (PLLA) is a polymer that is commonly used to produce hydrophobic surfaces and has the potential in substrate cell growth and cell engineering (Sousa et al. 2011; Tsuji et al. 1988). Based on the past studies, bacterial strains were able to colonize on PLLA surface (Sousa et al. 2011). Hence, a study on rapid microbial detection in UHT milk using thin film PLLA was carried out to indicate food spoilage on UHT milk via rapid detection. In this study, simplified experimental setup was used to measure contact angle of UHT milk sample on PLLA thin film surfaces. In addition, contact angle measurement was used to study the wetting properties in solid (PLLA)/liquid (milk) interaction whereby the components in microorganisms contributes to hydrophobicity of a cell surface thus plays an important role in the attachment to, or detachment from a surface (Che Johari et al. 2017; Krasowska & Sigler 2014; Zubairi et al. 2016). For that reason, the presence of microorganisms in milk are somehow affected the surface tension of the milk and hence, may cause changes in its contact angle. This study had two objectives which were to study the profile of colony forming unit of microorganisms in UHT milk with different time interval and to study the relationship between wetting of thin film PLLA and colony forming unit of microorganisms.

**MATERIALS AND METHODS**

**MAKING OF CONTAMINATED UHT MILK**

MIC (Minimal Inhibitory Concentration) test (Coyle 2015) was used to make contaminated milk. *Bacillus cereus* strain used in this study was ATCC11778. One loop of the strain was added into tryptone soya broth (TSB) and incubated at 30°C for 18 to 24 h. One loop of the bacterial culture was then streaked onto a nutrient agar to obtain a single colony of *B. cereus*. The single colony was then transferred into 10 mL of distilled water and compared with McFarland 0.5 standard. Microplate reading was also done on the *B. cereus* solution produced. This was to ensure that the colony forming unit of the bacterial solution was 1.5 cfu/mL and hence, complied with the standard. To make the contaminated UHT milk, 1 mL of *B. cereus* solution was added into 9 mL of milk in test tube. The colony forming unit of the bacterial solution was 1.5 cfu/mL and hence, complied with the standard. To make the colony forming unit of microorganisms in UHT milk was transferred into five 100 mL Schott bottles with equal volume which represented five different analyses. The bottles were left uncapped for 4 h and 30 min.

**FABRICATION OF THIN FILM PLLA**

The method used was adopted from a research by Ab Kadir et al. (2017, 2016) and Ili Afiqa (2017). 1 g of PLLA powder was weighed and added into 100 mL chloroform in a Schott bottle. The solution was then heated at 60°C and stirred by using magnetic stirrer with speed 100 rpm/min until PLLA was fully dissolved. PLLA solution was then transferred into two Petri dishes with equal volume. Petri dishes were then left in desiccator overnight to let it dry. PLLA film was then formed and cut into micro slide size. The film was then glued onto micro slide.

**MODEL SYSTEM**

Serial dilution was done on contaminated milk which was originally had 10^7 cfu/mL of *B. cereus* until dilution 10^7 to produce 1.5 × 10^4 cfu/mL colony counts of bacteria at the end of dilution. Contact angle of milk for each dilution was then measured by using Simplified Experimental Setup.

**PHYSICOCHEMICAL ANALYSIS**

Physicochemical analysis was done on both fresh and contaminated milk. Analyses that were done in this study were Brix, colour, pH and contact angle (Ramlan et al. 2018, 2017). Brix analysis was done by using Master refractometer (Master - 53α, Japan). Minolta Colorimeter, chromameter model (CR 400, Japan) was used for colour analysis by using Hunter Lab method. pH was measured using professional benchtop pH meter (BP3001). Calibration was done beforehand by measuring solution of pH4 (acid), pH7 (neutral) and pH9 (alkaline). Contact angle was measured by using Simplified Experimental Setup (Zubairi et al. 2015a, 2015b). All analyses were done with 30 min time interval which was as soon as the packaging of the milk was unsealed until the milk product was unsealed and exposed to environment for 4 h and 30 min.

**MICROBIOLOGICAL ANALYSIS**

Microbiological analysis was done on fresh milk with 30 min time interval which was as soon as the packaging of the milk was unsealed until the milk product was unsealed and exposed to environment for 4 h and 30 min. Total plate count was done using 3M™ Petrifilm™ Aerobic Count Plates. Serial dilution of milk was done until dilution of 10^-2. 1 mL of milk for each dilution was inoculated on petrifilm and incubated at 30 ± 1°C for 48 h.

**STATISTICAL ANALYSIS**

Minitab version 17 (Minitab Inc., Sydney, Australia) and IBM SPSS Statistics version 23 (IBM Corporation, New York, United States) were used to analyse data. One-way ANOVA and Fischer test were done to compare the mean difference
with confidence interval 95% (p<0.05). Coefficient of
determination, R² and Pearson correlation, r were done to
measure the strength of relationship between variables in
this study.

RESULTS AND DISCUSSION

Table 1 shows the result for model system. Model system
was done to study the relationship between colony forming
unit of microorganisms and contact angle. For the first
three dilutions, $10^7$ to $10^5$, contact angle of milk was
found to be decreased without any significant difference
(p>0.05). This shows that reduction of colony forming
unit of microorganisms did not give a huge impact on
contact angle of milk on PLLA film. Jones et al. (1996)
stated that contact angle depends on bacterial attachment
to substratum. Hence, reduction of bacterial attachment on
PLLA from dilution until had happened due to reduction
of colony forming unit of microorganisms. Less bacterial
attachment caused the contact angle to decrease as well.

As shown in Figure 1 and Table 2, regression analysis
was showing a R² of 0.74 in which about 74% of variance
in contact angle was explained by serial dilution. Also,
Pearson’s r for the correlation between contact angle and
serial dilution was 0.80 which was very close to 1. Thus,
there is a strong positive affiliation between contact angle
and serial dilution.

Contact angle of UHT milk with 30 min time interval
is shown in Table 3. There was significant difference
between contact angle of control sample and fresh milk
at the beginning of experiment, 0:00 (p<0.05). However,
at 1:30 and 2:30, no significant differences were detected
between contact angle of control sample and fresh milk.
Moreover, starting from 1:30, the contact angle of fresh
milk began to exceed the control sample of control sample
which was contaminated milk. Hence, based on this
analysis, probably UHT milk began to experience spoilage
after 1 h and 30 min since its packaging was unsealed and
exposed to environment. Microbiological analysis was
done to ensure whether the spoilage was caused by the
presence of microorganisms in milk sample.

<table>
<thead>
<tr>
<th>Dilution/colony forming unit (cfu/mL)</th>
<th>Contact angle, $\theta$ (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^7$</td>
<td>59.60 ± 121bc</td>
</tr>
<tr>
<td>$10^6$</td>
<td>59.28 ± 231bc</td>
</tr>
<tr>
<td>$10^5$</td>
<td>54.79 ± 6.37c</td>
</tr>
<tr>
<td>$10^4$</td>
<td>62.60 ± 1.07b</td>
</tr>
<tr>
<td>$10^3$</td>
<td>70.66 ± 2.94a</td>
</tr>
<tr>
<td>$10^2$</td>
<td>71.71 ± 4.23c</td>
</tr>
<tr>
<td>$10^1$</td>
<td>70.94 ± 0.57b</td>
</tr>
<tr>
<td>$10^0$</td>
<td>72.05 ± 0.86a</td>
</tr>
</tbody>
</table>

Results shown were means ± S.D. in triplicate (n=3). ** Mean with different letters indicate significant difference (p<0.05)

<table>
<thead>
<tr>
<th>Serial dilution</th>
<th>Coefficient of determination, R²</th>
<th>Contact angle, $\phi$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.75</td>
<td>0.80</td>
</tr>
</tbody>
</table>

Table 2. Coefficient of determination, R² and Pearson correlation on strength of the linear relationship between serial dilution and contact angle

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Contact angle (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:00 (control)</td>
<td>59.60 ± 1.21c</td>
</tr>
<tr>
<td>0:00</td>
<td>53.19 ± 0.88c</td>
</tr>
<tr>
<td>0:30</td>
<td>53.27 ± 1.11c</td>
</tr>
<tr>
<td>1:00</td>
<td>56.12 ± 1.26d</td>
</tr>
<tr>
<td>1:30</td>
<td>60.49 ± 0.44c</td>
</tr>
<tr>
<td>2:00</td>
<td>60.71 ± 0.24c</td>
</tr>
<tr>
<td>2:30</td>
<td>60.84 ± 0.21c</td>
</tr>
<tr>
<td>3:00</td>
<td>62.43 ± 0.91b</td>
</tr>
<tr>
<td>3:30</td>
<td>64.34 ± 0.47c</td>
</tr>
<tr>
<td>4:00</td>
<td>64.20 ± 0.42c</td>
</tr>
<tr>
<td>4:30</td>
<td>65.44 ± 1.10c</td>
</tr>
</tbody>
</table>

Results shown were means ± S.D. in triplicate (n=3). ** Mean with different letters indicate significant difference (p<0.05)

Figure 2 shows Brix measurement of UHT milk with 30 min time interval. It was found that the Brix values of UHT milk and control sample was significant
(p<0.05). However, Brix values of UHT milk were constant
throughout the experiment. Hence, it could be assumed that
there were no changes of total solids in UHT milk.

Next, Figure 3 shows the results for colour measurement
in terms of lightness (L*) of UHT milk with 30 min time interval. The lightness of fresh UHT milk was increasing significantly across time except at time 2:30 and 3:00
(p>0.05) and all L* values of fresh milk were significant
with control sample which had the lowest L* value
(p<0.05). The results for colour measurement in terms of
yellow intensity are shown in Figure 4. It was found that the yellow intensity of fresh UHT milk at all times were significant with control sample (p<0.05). The \(b^*\) values of fresh milk kept increasing until 2:30 and it began to decrease at 3:00 and 3:30 significantly (p<0.05), though the \(b^*\) values were found to increase again at 4:00 till the end of experiment. Kneifel et al. (1992) stated that an increase in psychometric chrome (\(b^*\)) can be as an indicator for milk spoilage during storage, especially spoilage which is not caused by enzyme. This kind of spoilage is known as Maillard reaction.

Figure 5 shows the pH values of fresh UHT milk with 30 min time interval. Results shown were means ± S.D. in triplicate (n = 3). *Mean with different letters indicate significant difference (p<0.05)
The wettability analysis of the contaminated milk on PLLA thin film and indicator of contamination by microorganisms in UHT milk are shown in Table 6 and Figure 6A, respectively. The images of milk droplets on PLLA film at 0:00 and 3:30 as well as the contaminated milk are shown in Figure 6B(i), Figure 6B(ii) and Figure 6B(iii), respectively.

**Conclusion**

The presence of microorganisms in UHT milk had caused a few physicochemical changes in the milk which were an increase in lightness, a decrease in yellow intensity and an increase in contact angle significantly. Colony forming unit of microorganisms with 30 min time interval had been studied. Presence of microorganisms in milk happened after the milk was unsealed and exposed to environment with temperature $27 \pm 0.3^\circ C$ and relative humidity as much as $73 \pm 1\%$ for 3 h and 30 min. The colony forming unit of microorganisms at that time was $2.1 \text{ cfu/mL}$ and increased significantly across time. The relationship between wetting of thin film PLLA and colony forming unit of microorganisms had been studied as well. The presence of microorganisms in milk at 3:30 with its colony forming unit, $2.1 \text{ cfu/mL}$ had caused the contact angle of milk on PLLA film to be $64.34^\circ$. Based on regression analyses, colony forming unit of microorganisms is correlated to contact angle. However, it was found that there was no significant difference of contact angle from 3:30 till 4:30 although the colony forming unit of microorganisms in milk was found to be increased significantly across those times. This shows that PLLA was less sensitive to colony count of microorganisms. As a conclusion, PLLA thin film can be used to detect the presence of microorganisms in UHT milk product, though it is less sensitive to the colony count of the microorganisms.

**Table 4. Microbiological analysis of UHT milk with 30 min time interval**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Colony count (cfu/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:00 - 3:00</td>
<td>$&lt;1.0 \times 10^6$</td>
</tr>
<tr>
<td>3:30</td>
<td>$2.1 \times 10^6$</td>
</tr>
<tr>
<td>4:00</td>
<td>$3.0 \times 10^6$</td>
</tr>
<tr>
<td>4:30</td>
<td>$3.9 \times 10^6$</td>
</tr>
</tbody>
</table>

Results shown were means ± S.D. in triplicate ($n = 3$). *Mean with different letters indicate significant difference ($p<0.05$)

**Table 5. Coefficient of determination and Pearson correlation on strength of relationship between Brix, colour (L*), colour (b*), pH, colony forming unit of microorganisms and contact angle**

<table>
<thead>
<tr>
<th>Coefficient of determination, $R^2$</th>
<th>Contact angle</th>
<th>Brix</th>
<th>Colour (L*)</th>
<th>Colour (b*)</th>
<th>pH</th>
<th>Colony forming unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact angle</td>
<td>0.001</td>
<td>0.33</td>
<td>0.11</td>
<td>0.11</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td>Pearson correlation, r</td>
<td></td>
<td>0.036</td>
<td>0.57</td>
<td>0.33</td>
<td>0.30</td>
<td>0.62</td>
</tr>
</tbody>
</table>

**Table 6. Contact angle, output and wetting of UHT milk on PLLA film**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Range of contact angle ($\theta$)</th>
<th>Range of Brix (°Bx)</th>
<th>Range of Colour (L*)</th>
<th>Range of Colour (b*)</th>
<th>Range of pH</th>
<th>Colony count (cfu/mL)</th>
<th>Diameter of circle indicator (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0:00 – 3:00</td>
<td>53.19 – 62.43°</td>
<td>12.5</td>
<td>83.76 – 84.17</td>
<td>8.52 – 8.92</td>
<td>6.82 – 6.84</td>
<td>$&lt;1.0 \times 10^6$</td>
<td>$0.7 \pm 0.05^a$</td>
</tr>
<tr>
<td>3:30 – 4:30</td>
<td>64.34 – 65.44°</td>
<td>12.5</td>
<td>84.27 – 84.39</td>
<td>8.77 – 8.99</td>
<td>6.83 – 6.84</td>
<td>$2.1 \times 10^1 – 3.9 \times 10^4$</td>
<td>$0.5 \pm 0.05^b$</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

All thanks are due to the Centre for Biotechnology & Functional Food, Universiti Kebangsaan Malaysia (UKM) and Smart Material & Food Engineering Group (SMAFEG) for the financial assistance providing the research facilities throughout the study.

REFERENCES


FIGURE 6. A) Indicator of contamination in UHT milk based on PLLA polymer thin film. B) Fresh UHT milk droplet on PLLA film at: i) time 0:00 with its contact angle, 50.70°; ii) time 3:30 with its contact angle, 64.20°; iii) its contact angle, 60.30°. Contact angle was obtained using simplified experimental setup.


Centre for Biotechnology & Functional Food
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
43600 UKM Bangi, Selangor Darul Ehsan
Malaysia

*Corresponding author; email: saiful-z@ukm.edu.my

Received: 9 February 2018
Accepted: 17 July 2018