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SURVIVAL STUDY AND HAEMOLYSIN ACTIVITY OF *Escherichia coli* IN RAW AND PASTEURIZED MILK PRODUCED IN NEGERI SEMBILAN

Farra Amira Mohamed, Aimi Nadia Ramli, Noorlis Ahmad

Faculty of Applied Sciences, Universiti Teknologi MARA, Cawangan Negeri Sembilan, Kampus Kuala Pilah, 72000, Kuala Pilah, Malaysia

Email: farraamiramohamed@gmail.com

Abstract

Demand for milk has increased in Malaysia due to the increased in awareness of healthy foods consumption. Hence, research of milk is crucial to ensure that it is not contaminated with *Escherichia coli*. This study evaluated the survival of *Escherichia coli* at different temperature and haemolysin activity of *Escherichia coli* on blood agar. A total of 8 samples of raw fresh and pasteurized milk were collected from nearby farm and market in Negeri Sembilan, Malaysia. After an overnight exposure to four different temperatures of 0^0C, 28^0C, 35^0C and 45^0C, the bacteriological test of milk was evaluated for the presence of *Escherichia coli*. Overall, all raw fresh milk sampled exceeded the acceptable limit of bacterial count of 1 x 10^5 CFU/ml. Raw fresh milk recorded the highest count at 35^{o}C with 4.4 x 10^7 CFU/ml and the lowest at 0^{o}C with 8.3 x 10^4 CFU/ml. The presence of *Escherichia coli* was detected in 7/20(35%) of the total raw fresh milk samples. All pasteurized milk showed no presence of *Escherichia coli* due to the effectiveness of heat treatment. Haemolysin test showed no haemolytic activity. Milk contaminated with *Escherichia coli* can cause diarrheal, gastrointestinal diseases and urinary infection. Hence, it is important to study the survival rate of *Escherichia coli* and its pathogenicity in milk to ensure public safety.

Keywords: *Raw milk, Survival, Haemolysin, Escherichia coli, temperature, Total Plate Count*

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Introduction

Milk is the fluid secreted from the female's mammary gland (Grosch, 2008). It is a whitish liquid that serves as food for the mammal's young (Mourad *et al.*, 2014). Every mammalian has unique composition of milk (Kumar *et al*., 2014) as it has a high amount of water content at approximate 88.6% (Mourad *et al.*, 2014) and is one of the most perfect foods with protein, fats, sugars, vitamin and minerals (De Silva *et al*., 2016). However, raw milk is also favorable for the multiplication of microorganisms due to its physicochemical environment (Oliveira *et al*., 2015). The bacterial growth rate is affected by the temperatures (De Silva *et al*., 2016) and it can cause spoilage of the milk (Magan *et al.*, 2001)

Milk has been used and consumed by human ever since ancient ages (Pal *et al*, 2014). Due to globalization, people tend to be more aware of their health and the foods they consume. Generally, raw fresh milk is thought to be a better choice of milk for most people. This is because they are naturally made and contain no preservatives or processed chemicals. The interest in consuming raw milk is related to the enhanced in nutritional qualities, better taste and health benefits (Oliver *et al*., 2009).

Raw or processed milk is known for their capability of causing infections (intoxications) to the consumers and spoilage of products as it supports the growth of several microbes (Shunda *et al.*, 2013). In fact, pathogenic bacteria such as *Escherichia coli* can be life threatening to human who consume them. *Escherichia coli* is one of the most common contaminants that can cause gastrointestinal infection including foodborne illnesses and food poisoning (Kumar & Prasad, 2010).

The bacteriological safety of milk is a major concern to the public health community (Pervin *et al*, 2016). Consumers are not aware of the food safety level when the milk is not handled properly and exposed to different temperature before drinking it. Therefore, further research regarding the outbreak of food pathogen caused by milk need to be done to ensure public awareness. On top of that, it is also to create awareness on the public regarding milk safety on the exact temperature to store milk upon drinking it.

Literature review

Raw milk is dairy product specifically milk from cow, sheep and goat or any other animals (FDA, 2018) that has not received any heat treatment to destroy pathogens or spoilage organisms. It is also known as real milk, which indicates organic, non-homogenized and 'grass fed' as its criteria. According to Mourad *et al*. (2014), milk is the product of the total, full and uninterrupted milking of a dairy female in good health condition. It has not undergone heat treatment beyond 40ºC or any treatment that contributes to the equivalent effects (FAO/WHO, 2004). It contains wide spectrum of microorganisms (Vacheyrou *et al*., 2011) that is almost impossible to be controlled precisely (Dunge, 2016). They consist of Gram negative Psychrotrophs, Coliforms and other pathogenic bacteria such as *Escherichia coli* and *Staphylococcus aureus* that can also be found in the milk (Syed *et al*., 2014).

Pasteurization is the process of killing harmful bacteria by heating it at specific temperature (FDA, 2018) in a corresponding specific time continuously. It is done to kill microbes in foods and drinks but not all microorganisms are intended to be killed. This is because the purpose of pasteurization is to decrease the number of viable pathogens so it will not cause any diseases. Pasteurization is also a microbial heat treatment that is used to reduce the number of pathogens in milk and liquid milk products to a level where they contribute no significant health hazard (FAO/WHO, 2004) producing a safe pasteurized milk. Pasteurization is not designed to sterilize milk as the microbiological quality of pasteurized milk is governed by the flora of raw milk, processing conditions and post-heat treatment contamination (Sarkar, 2015).

Foodborne illnesses associated with the consumption of dairy products are mainly caused by *Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, and Salmonella entertica* (Pal *et al.*, 2016). On top of that, raw milk is said to be an ideal growth medium for microorganisms (Kumar & Prasad, 2010). Therefore, consumption of raw milk is discouraged as many epidemiology outbreaks and even death have been recorded (Sarkar, 2015).

Escherichia coli is a common gastrointestinal tract of humans and animals (Welch, 2006). Most of the *Escherichia coli* are non-pathogenic and harmless (CDC, 2017) but some of them can be pathogenic if they are opportunistic (Akond *et al*., 2009) and commonly affect those individuals of immune-challenged (Frederick, 2011). Pathogenic *Escherichia coli* is usually the types that can cause diarrhea when transmitted through contaminated water and foods (Matthew *et al.*, 2013). *Escherichia coli* can be found in varieties of foods of animal products, water, soil and on plants and intestinal tracts of animals. It has its own range of temperature of which it can live. *Escherichia coli* can live in a range of 7ºC to 50ºC with 37ºC as its optimum temperature (WHO, 2018). This is similar to the finding made by Noor *et al*. (2013) as maximal growth of

Escherichia coli was found in different media at 37ºC. Pathogenic *Escherichia coli* can live and grow in at 7ºC or warmer and will decline gradually when the raw fresh milk is held at 5ºC or colder but can still survive in frozen milk (King *et al*., 2014). This is because of the preexisting bacteria in the raw milk. According to Wang *et al*. (1996), *E. coli* in raw fresh milk could not survive at the temperature of 5ºC as its population decreased after being incubated for 28 days. The population was at peak at 22ºC as *Escherichia coli* increased dramatically after first day of incubation. *Escherichia coli* also increased rapidly in raw fresh milk at 20ºC on the first day of incubation (Alhelfi *et al*., 2012). *Escherichia coli* colonies were not found in water at 50ºC and above after overnight (Than, 2011). Heat treatment can affect the milk in terms of activation of bacteria and spores (Hanson *et al*., 2005). Hence, the aim of this study is to determine the survival rate of *Escherichia coli* presence at different temperature and to determine the haemolysin activity of *Escherichia coli* from cow raw fresh milk.

Methods

Sample Collection and Preparation

A total of four cow raw milk samples were collected from nearby farms and markets in Kuala Pilah, Negeri Sembilan, Malaysia. As for the pasteurized milk, the samples were purchased from several selected wet markets in Kuala Pilah. Random samplings were applied to both samples. The samples obtained were labeled and placed in a sterile container to prevent contamination from happening. The milk was carried in a sterile labelled ice box and immediately analyzed on the day of sampling.

Survival Test

All of the samples obtained were kept at four different storage temperatures of 0ºC, 28ºC (room temperature, RT), 35ºC and 45ºC. The triplicate samples were incubated overnight at the respective temperatures (Than, 2011). Temperature of 0ºC is to simulate the milk being placed in household refrigeration. At RT (28ºC), the temperature is to simulate the room temperature condition in which the milk is being displayed at air-conditioned retail stores. While at 35 and 45ºC, were set to simulate when the milk is placed under air-conditioned storage where the milk could be subjected to temperature abuse.

Serial Dilution Method

In serial dilution, it was carried out up to 10^{-5}. A 0.1mL of each dilution was transferred to Eosin Methylene Blue Agar (EMBA) plates and a sterile glass spreader was used to spread the diluted broth on the agar surface. Then, the EMBA plates were incubated for 18-24 hours at 35ºC (Bakr *et al.*, 2011).

Total Plate Count

In this method, colony counter was used to count the number of microbial colonies that grow on the selective agar. The number of bacterial colonies that grow on the surface was counted and converted to CFU/ml (Sieuwerts *et al.*, 2008). Colonies that were counted below 30 on the plate at the final count were too few to count (TFTC) while colonies above 300 were known as too many to count (TMTC). According to Syed *et al*. (2014), if there is presence of *Escherichia coli*, the colony will appear in metallic green sheen (Asmahan & Warda, 2011) color on the EMBA.

Biochemical Test

IMViC test was used as the biochemical test. The IMViC test stands for Indole test, Methyl-Red test, Voges-Proskauer test and Citrate Utilization test. These tests are crucial in order to confirm the presence of *Escherichia coli* (Asmahan & Warda, 2011). Indole test, Methyl-Red test, Voges-Proskauer test and Citrate Utilization test were carried out following Hemraj *et al*. (2013).

Haemolysin Test

The haemolysin test was carried out to observe the haemolysin production of *Escherichia coli*. Haemolysin production in *Escherichia coli* has been associated with extra-intestinal diseases in human (Short & Kurtz, 1971). A loopful suspected *Escherichia coli* growth was streaked on blood agar (nutrient agar with sterile blood) and incubated at 37°C for 24 hours. The streaked *Escherichia coli* on plates was observed for the pattern of haemolysis around the isolated suspected colonies. There are several types of haemolysin, including an alpha haemolysin, beta haemolysin and a gamma haemolysin (Cavalieri *et al*., 1984). Clear zone around the growth of *Escherichia coli* signified beta haemolytic activity (Aghemwenhio *et al*., 2017). Alpha haemolysin is recorded when there is incomplete clearing and gamma haemolysin for no evidence of haemolysis (Adesiyun & Shehu, 1990).

Results and Discussion

Survival Test

Samples of raw fresh milk and pasteurized milk were obtained from markets and nearby farms in Kuala Pilah, Negeri Sembilan. The samples were kept in separate containers with ice during transportation directly to the laboratories for testing where the milk samples were exposed to different temperatures. A serial dilution was carried out up to 10^{-5}. Each fold was conducted in triplicate and were plated on EMB agar for 24 hours. As shown in Table 1.1, the overall prevalence of *Escherichia coli* in pasteurized and raw fresh milk samples after exposing it to different temperatures for overnight was varied from 8.3×10^4 to 4.4×10^7 CFU/ml.

Table 1.1 Microbial load (CFU/ml) of *Escherichia coli* in Pasteurised and Raw Milk Fresh Milk

Samples	Temperature (^{O}C)	No. Of Colony (CFU/ml)				
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Pasteurized milk	RT	0	0	0	0	0
	0	0	0	0	0	0
	35	0	0	0	0	0
	45	0	0	0	0	0
Raw Fresh Milk	RT	TMTC	$117(1.2 \times 10^5)$	$94(9.4 \times 10^5)$	$51(5.1 \times 10^6)$	$12(1.2 \times 10^7)$
	0	TMTC	$83(8.3 \times 10^4)$	$49(4.9 \times 10^5)$	$26(2.6 \times 10^6)$	$25(2.5 \times 10^7)$
	35	TMTC	TMTC	$55(5.5 \times 10^5)$	$51(5.1 \times 10^6)$	$44(4.4 \times 10^7)$
	45	TMTC	$105(1.1 \times 10^5)$	$67(6.7 \times 10^5)$	$39(3.9 \times 10^6)$	$20(2.0 \times 10^7)$

There was no contamination in all pasteurized milk as it showed an absence of *Escherichia coli*. This is because of the heat treatment applied to it during pasteurization. Ambient temperature where the pasteurized milk is stored can still affect the shelf life of the milk depending on the pasteurization efficiency. As the samples showed colony on EMB agar, it can be confirmed that the pasteurization method for the samples

were reliable enough for the milk to be consumed. Effectiveness of pasteurization can minimize the chance of postpasteurization contamination from occurring (Garedew *et al*., 2012). Hence, it was proven that pasteurization is a must-have step in order to produce a consumable milk (Nordin *et al*., 2018).

Our results showed that, all raw fresh milk samples examined were heavily contaminated with *Escherichia coli* and according to O'Connor (1994), the limit of bacteria in milk should not contain more than 10^4-10^5 CFU/ml. This is supported by Nordin *et al*. (2018) which reported that a good quality of milk should contain less than 10^5 CFU/ml for the total bacteria count. All raw fresh milk samples were non-consumable as the number of colonies found exceeded the limit stated by Food Regulation 1985.

From our observations, the raw fresh milk samples produced metallic green sheen colonies on EMB agar indicating the presence of *Escherichia coli*. This is approved by Antony *et al*. (2016) that metallic green sheen colonies indicate positive growth of *Escherichia coli*. The highest count of *E. coli* was 4.4 x 10^7 CFU/ml from the milk sample exposed overnight at 35ºC. A high bacterial count might be due to the temperature abuse applied to as it increased the number of coliforms. This was also confirmed in the Food Standards Australia New Zealand (2009), as the temperature of raw fresh milk is over 30ºC after leaving the udder during milking, bacteria will grow rapidly. Other than that, unhygienic condition of manufacturing unit, poor quality of material used and water supplied for washing the utensils might contribute to the bacterial counts in the milk product (Soomro *et al*., 2002). It could also come from an unhealthy animal, improper cleaning of its udder and teats before milking and poor hygienic practice (El nahas *et al*., 2015). This is supported by Dissasa *et al.* (2017) which reported that milk can be easily contaminated from many different sources including the contaminated udder, poor personal hygiene, poor water quality, and unsanitized containers.

In addition, temperature can also affect the microbial count in milk. This is because the optimum temperature of *Escherichia coli* is in the range of 30-42ºC. Notably, temperature of 35ºC falls within the range and is near the optimum temperature of *Escherichia coli*. According to Doyle and Schoeni (1984), *Escherichia coli* grows well at that range with 37ºC as its optimum temperature. This is supported by Than (2011) as total bacterial count recorded at 37ºC was the highest when compared to other temperatures. According to Noor *et al*. (2013) the growth of *Escherichia coli* is confluent and colony forming unit is constant at 37ºC.

The lowest microbial count recorded was 8.3 x 10^3 CFU/ml at 0ºC. This finding is in parallel with Arias *et al*. (2001) as the number of *Escherichia coli* declined significantly and was at the lowest when stored at 0ºC. This might be due to inhibition of bacterial growth below 4ºC. According to O'Connell *et al*. (2016), the total bacterial count of raw milk was low when stored at temperature of 4ºC and below. This result is similar with the finding made by Wiking *et al*. (2002) as the number of bacteria was suppressed in milk stored at 4ºC. This is also supported by Vithanage *et a*l. (2017) as milk stored at 2ºC and 4ºC have longer storage life for quality and safety as compared to higher storage temperature.

At dilution 10^{-4}, the result showed that the number of colony increased gradually from 2.6 x 10^6 CFU/ml at 0ºC to 5.1 x 10^6 CFU/ml at room temperature (28ºC), remain constant at 37ºC and decrease when incubated at 45ºC to 3.9 x 10^6 CFU/ml. The number of colonies were at the highest at RT and 35ºC because *Escherichia coli* can grow well at 7ºC and warmer than that (King *et al*., 2014). This is supported by Nguyen (2006) as the best temperature for *Escherichia coli* to grow is in the range of 25-35ºC. The decline in the

number of colony at 45°C is in accordance with Doyle and Schoeni (1984) findings that the growth of *Escherichia coli* was slower at 44-45°C. According to Palumbo *et al*. (1994), *Escherichia coli* strains showed viable count less than 21 colonies at 45°C.

Confirmation of *Escherichia coli*

The green metallic sheen colonies on EMB agar were further tested with IMViC bacteriological test. The result in Table 1.2 showed that the three potential bacteria found were all coliform bacilli and Gram negative bacteria which consist of *Escherichia coli*, *Citrobacter freundii* and *Citrobacter koseri.* This is supported by Wardhan & Nain, (2017) which reported that these bacteria can be found in raw milk. Only raw milk samples were proceeded with IMViC test as pasteurized milk showed no presence of any colony on EMB agar after heat exposure and serial dilution.

Table 1.2 Confirmation of *Escherichia coli* in raw milk through IMViC Test

Sample	Dilution plated	Indole test	Methyl-red test	Voges Proskauer test	Citrate test	Potential bacteria
Escherichia coli		+	+	–	–	
Raw milk RT	10^{-1}	+	+	–	–	*Escherichia coli*
	10^{-2}	–	+	–	+	*Citrobacter freundii*
	10^{-3}	–	+	–	–	X
	10^{-4}	–	+	–	–	X
	10^{-5}	–	+	–	+	*Citrobacter freundii*
Raw milk 0°C	10^{-1}	+	+	–	–	*Escherichia coli*
	10^{-2}	–	+	–	–	X
	10^{-3}	–	+	–	+	*Citrobacter freundii*
	10^{-4}	+	+	–	–	*Escherichia coli*
	10^{-5}	–	+	–	–	X
Raw milk 35°C	10^{-1}	–	+	–	–	X
	10^{-2}	+	+	–	+	*Citrobacter koseri*
	10^{-3}	+	+	–	–	*Escherichia coli*
	10^{-4}	+	+	–	–	*Escherichia coli*
	10^{-5}	–	+	–	+	*Citrobacter freundii*
Raw milk 45°C	10^{-1}	+	+	–	–	*Escherichia coli*
	10^{-2}	+	+	–	+	*Escherichia coli*
	10^{-3}	+	+	–	+	*Citrobacter koseri*
	10^{-4}	–	+	–	+	*Citrobacter freundii*
	10^{-5}	–	+	–	–	X

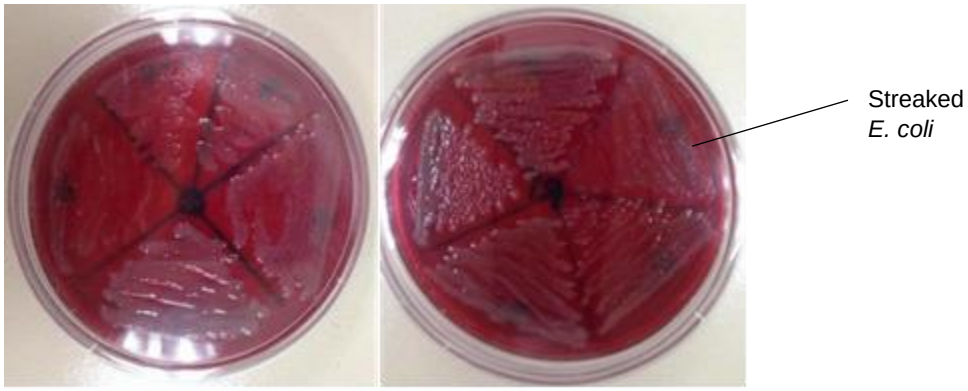
'+' change colour, '-' no change of colour

Overall prevalence of *Escherichia coli* isolated in raw fresh milk after exposing it to different temperature, 7/20(35%) were identified as *Escherichia coli* in the raw fresh milk sample. Raw milk at 0ºC, 35ºC and 45ºC showed similar presence of *Escherichia coli*, 2/20(40%) while raw milk at room temperature only showed 1/20(20%) of *Escherichia coli* presence. *Escherichia coli* is one of the most common bacteria still of concern today in raw milk and dairy products (Disassa *et al*., 2017). *Escherichia coli* can cause severe diarrhea and vomiting particularly in infants and children (Soomro *et al*., 2002). Gastroenteritis can also be caused by *Escherichia coli* strains through the consumption of raw milk (Ombarak *et al*., 2016).

Citrobacter freundii were identified in 5/20(25%) respectively. While 2/20(10%) of the samples were identified as *Citrobacter koseri*. The pathogenicity of *Citrobacter* spp. includes urinary tract infections, blood stream infections, intra-abdominal sepsis, brain abscesses, and pneumonia and other neonatal infection (Pepperell *et al*., 2002). Moreover, it can also affect infants of two months old or younger through central nervous system infection. Both *Citrobacter freundii* and *Citrobacter koseri* have the potential in causing brain abscesses and deaths.

Haemolysin Test

A total of 20 *Escherichia coli* isolates were streaked on the blood agar. From our observation, the colonies on the blood agar showed gamma haemolytic activities (Figure 1). There are several types of haemolysin, including an extracellular protein (alpha-haemolysin), a cell-bound protein (beta-haemolysin) and a haemolysin expressed by nalidixic acid-resistant mutants (gamma-haemolysin) (Cavalieri *et al*., 1984). Gamma haemolysis means that there is no haemolysis reactions present on the blood agar. Besides, gamma-haemolysin cannot haemolyse rabbit or human blood as the way alpha and beta-haemolysin do (Fernandez *et al*., 1999). The result was as expected for *Escherichia coli* as it has various kinds of strains that can contribute to different haemolysin results. However, in this study, the colonies appeared to be grey white moist, glistening and opaque which depicted the reactions as gamma-haemolysin. The results were in accordance with the previous findings reported by Soomro *et al*. (2002). According to Aghemwenhio *et al*. (2017), beta-haemolysin is the outcome of pathogenic strains of *Escherichia coli* while alpha-haemolysin is caused by virulence factor producing extra-intestinal infection. These results were also supported by Hossain *et al*. (2017) as the presence of *Citrobacter freundii* and *Citrobacter koseri* showed no differences from gamma- haemolysin.



(a) **(b)**

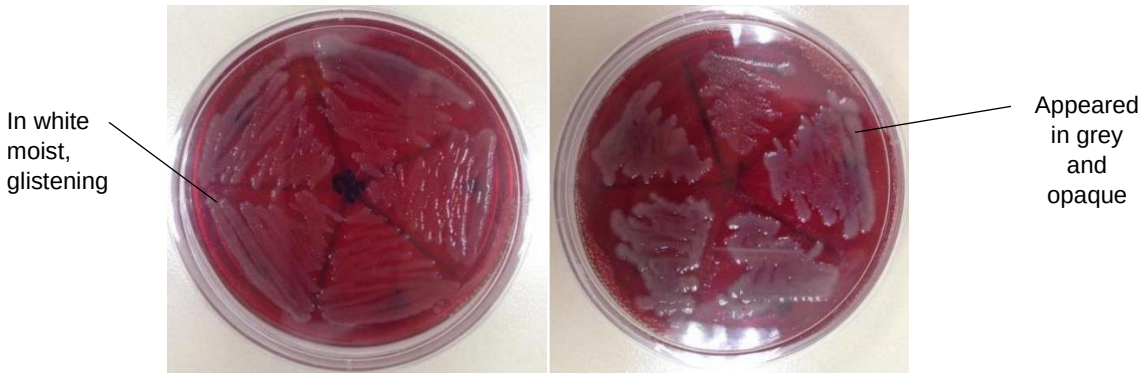


Fig 1 Appearance of *Escherichia coli* on Blood Agar for Haemolysin Test (a) At 0ºC, (b) At 28ºC (Room Temperature), (c) At 35ºC and (d) At 45ºC.

Conclusion

In conclusion, the highest bacterial count in raw fresh milk was recorded at 4.4 x 10^7 CFU/ml at 35ºC while the lowest was 8.3 x 10^4 CFU/ml at 0ºC. This result showed that the survival of *Escherichia coli* was at peak at 35ºC and was almost inactive at 0ºC. All samples of raw fresh milk showed contamination as the total bacterial count exceeded the safe limit which is 1 x 10^5 CFU/ml. Pasteurized milk samples showed no presence of *Escherichia coli* colony on EMB agar even after heat exposure and serial dilution process. This indicates that the milk is safe to consume. Besides, there are 7/20(35%) of raw fresh milk samples that were identified as *Escherichia coli.* Furthermore, the haemolysin activity of *Escherichia coli* on the blood agar was identified as gamma- haemolysin for all raw fresh milk samples due to its non-haemolytic properties.

It is recommended that the raw fresh milk obtained is taken from sources with a distinguished health condition. This is because it can affect the presence of bacteria in the milk. Besides, the different temperature treatment applied to the milk should be carried out in a longer period to confirm the presence of *Escherichia coli* in milk after days in ambient temperature. Finally, the milk can also be obtained from different farms in particular places.

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