EFFECT OF HYDROGEN PEROXIDE, LACTOPEROXIDASE AND ULTRASOUND ON MICROBIOLOGICAL QUALITY AND SAFETY OF RAW MILK

by

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LIST OF ABBREVIATIONS

ALOA	Listeria Ottavani and Agosti Agar
Amp	Ampicillin
ANOVA	Analysis of Variance
BAM	Bacteriological Analytical Manual
BGLB	Brilliant Green Lactose Bile Broth
BHI	Brain Heart Infusion
BPA	Baird Parker Agar
BPW	Buffered peptone water
C	Chloramphenicol
CDC	Centers for Disease Control and Prevention
Cip	Ciprofloxacin
CLSI	Clinical and Laboratory Standard Institute guideline
Cn	Gentamycin
COB	Clot on boiling
DA	Clindamycin
DNA	Deoxyribonucleic Acid
DVS	Department of Veterinary Services Malaysia
E	Erythromycin
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
Fox	Cefoxitin
GAHP	Good Animal Husbandry Practices
H_2O_2	Hydrogen peroxide
ISO	International Organization of Standardization
K	Kanamycin
L-EMB	Levine - Eosin Methylene Blue
LP-s	Lactoperoxidase system
LST	Lauryl Tryptose broth
Lzd	Linezolid

MAR	Multiple Antibiotic Resistance
MHA	Muller Hinton Agar
MKTTn	Muller Kauffmann Tetrathionate-Novobiocin Broth
MOH	Ministry of Health, Malaysia
MPN	Most Probable Number
MRS	Man, Rogosa and Sharpe
NaSCN	Sodium thiocyanate
NCBI	National Center for Biotechnology Information
NPCB	National Pharmaceutical Control Bureau, Malaysia
OECD	Organization for Economic Co-operation and Development
OLA	Oxford Listeria Agar
P	Penicillin
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid
RVS	Rappaport Vassiliadis Broth with soya
SALT	Livestock Farm Practices Scheme
TA	Titratable acidity
Te	Tetracycline
TPC	Total Plate Count
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
USDA	United States Department of Agriculture
W	Trimethoprim
WHO	World Health Organization
XLDA	Xylose Lysine Deoxycholate Agar
XLT-4	Xylose Lysine Tergitol 4 Agar
UHT	Ultra High Temperature

KESAN HIDROGEN PEROKSIDA, LAKTOPEROKSIDA DAN ULTRABUNYI KE ATAS KUALITI MIKROBIOLOGI DAN KESELAMATAN SUSU MENTAH

ABSTRAK

Keselamatan dan kualiti susu mentah adalah kebimbangan utama di Malaysia kerana tahap kebersihan yang rendah di ladang-ladang dan kekurangan rantaian sejuk untuk penyimpanan dan pengangkutan susu mentah. Objektif kajian ini adalah untuk menentukan kualiti mikrobiologi dan keselamatan susu mentah yang dihasilkan di ladang tenusu berskala kecil di Pulau Pinang; menilai kesahihan ujian platform yang digunakan di pusat-pusat pengumpulan susu untuk penilaian kualiti dan potensi jangka hayat susu mentah; mengkaji kesan kepekatan hidrogen peroksida (H_2O_2), sistem laktoperoksida (nisbah NaSCN: H_2O_2) dan keberkesanan sonikasi terhadap bilangan mikroorganisma perosak dan patogen bawaan makanan dalam susu mentah. Kajian menunjukkan susu mentah yang diperoleh dari 5 ladang yang berbeza mempunyai jumlah kiraan plat (TPC) (7.77 ± 0.33 log cfu mL^{-1}), koliform (3.40 ± 0.22 log cfu mL^{-1}) dan *Staphylococcus aureus* (3.17 ± 0.14 log cfu mL^{-1}) yang tinggi. Sembilan puluh peratus daripada sampel yang diuji dicemari oleh *E. coli*. Serovar *Salmonella* dan *Listeria monocytogenes* masing-masing dikesan dalam 5 dan 3.33% daripada sampel susu mentah yang diuji. Kesemua 60 "strain" *S. aureus* yang dipencilkan dari sempel susu mentah rentan terhadap gentamisin, kanamisin, kloramfanikol dan ciprofloksin. Pencilan *S. aureus* didapati mempunyai kerintangan terhadap penisilin (23.3%), amfisilin (23.3%), trimethoprim (18.3%), cefoxitin (15.0%), linezolid (11.7%), clindamycin (10.0%), eritomisin (8.3%) dan tetraciklin (5.0%). Pengaktifan system laktoperoksida dan rawatan H_2O_2 dapat mengurangkan bilangan awal TPC, *S. aureus* dan koliform dalam susu mentah dan juga

meningkatkan jangka hayat susu mentah sebanyak 50% berbanding dengan susu kawalan. Masa penyimpanan dan kepekatan H_2O_2 atau nisbah NaSCN: H_2O_2 mempunyai kesan yang signifikan ($P<0.05$) terhadap bilangan TPC, koliform, *S. aureus,* keasidan tertitrat (TA) dan pH susu. Hasil kajian ini juga menunjukkan bahawa ujian alkohol, "clot-on-boiling" (COB), pH, TA dan resazurin bukan kaedah atau ujian yang sesuai digunakan untuk penilaian kualiti susu mentah. Kesan penyahaktifan mikroorganisma oleh ultrabunyi dipengaruhi oleh amplitud, masa sonikasi, jenis mikroorganisma dan kepekatan H_2O_2 atau nisbah NaSCN:H_2O_2. Pengurangan jumlah mikroorganisma yang lebih tinggi diperolehi pada amplitud yang lebih tinggi (125> 62.5 µm) dan masa pendedahan yang lebih lama (15> 10> 5 min). Bakteria Gram positif lebih rintang terhadap sonikasi berbanding bakteria Gram negatif. Bilangan mikroorganisma dalam susu mentah dan juga patogen dan mikroorganisma perosak yang diiokulasi ke dalam susu UHT dapat dikurangkan secara keseluruhan selepas disonikasi selama 10 dan 15 min pada 125 dan 62.5 µm amplitude masing-masing, dengan pengunaan bersama H_2O_2 atau NaSCN:H_2O_2. Kaedah ultrabunyi digabungkan dengan rawatan H_2O_2 atau NaSCN:H_2O_2 mempunyai potensi untuk memastikan keselamatan dan meningkatkan jangka hayat susu mentah.

EFFECT OF HYDROGEN PEROXIDE, LACTOPEROXIDASE AND ULTRASOUND ON MICROBIOLOGICAL QUALITY AND SAFETY OF RAW MILK

ABSTRACT

Safety and quality of raw milk is of major concern in Malaysia due to poor hygiene on the farms and the lack of cold chain for storage and during transportation of raw milk. The objectives of this study are to determine the microbiological quality and safety of raw milk produced by small dairy farms in Penang; to evaluate the reliability of platform tests used at milk collection centers for assessment of quality and shelf life potential of raw milk; to determine the effects of different concentration of hydrogen peroxide (H_2O_2), lactoperoxidase system (NaSCN: H_2O_2 ratio) and effect of ultrasound on selected spoilage organisms and food-borne pathogens in milk. Raw milk obtained from 5 different farms had high Total plate (TPC) (7.77 ± 0.33 log cfu mL^{-1}), coliforms (3.40 ± 0.22 log cfu mL^{-1}) and *Staphylococcus aureus* (3.17 ± 0.14 log cfu mL^{-1}) counts. Ninety percent of samples examined were positive for *E. coli*. *Salmonella* serovars and *Listeria monocytogenes* were detected in 5 and 3.33% of raw milk samples, respectively. All the 60 *S. aureus* isolates isolated from raw milk samples were susceptible to gentamycin, kanamycin, chloramphenicol and ciprofloxacin. *S. aureus* isolates were resistant to penicillin (23.3%), ampicillin (23.3%), trimethoprim (18.3%), cefoxitin (15.0%), linezolid (11.7%), clindamycin (10.0%), erythromycin (8.3%) and tetracycline (5.0%). Treatment of raw milk with activated lactoperoxidase system and H_2O_2 was able to reduce initial TPC, *S. aureus* and coliforms count in raw milk and also increase the shelf life of raw milk by 50% as compared to the control. Storage time and concentration of H_2O_2 or NaSCN: H_2O_2 had significant effects ($P<0.05$) on titratable acidity, pH, TPC, coliform and *S. aureus* count in milk. This study also revealed that alcohol, clot on boiling, pH, TA and resazurin tests are not reliable methods for the assessment of raw milk quality. Microbial inactivation by ultrasound was influenced by amplitude, sonication time, types of microorganism and concentration of either H_2O_2 or NaSCN: H_2O_2. Higher microbial reduction was obtained at higher amplitude (125 > 62.5 μm) and prolonged exposure time (15 > 10 > 5 min). Gram positive bacteria were more resistant to ultrasound as compared to Gram negative bacteria. In the presence of either H_2O_2 or different ratios of NaSCN:H_2O_2, microbial counts in raw milk, pathogens and spoilage microorganism inoculated in UHT milk were eliminated after 10 and 15 min of sonication at 125 and 62.5 μm amplitude, respectively. Ultrasound combined with H_2O_2 or NaSCN: H_2O_2 has the potential to ensure safety and improve the shelf life of raw milk.

CHAPTER 1

INTRODUCTION

1.1 Background

Dairy milk is a staple beverage consumed by humans. Milk and its by-products are an important part of our diet because milk is considered as a healthy and beneficial drink as it contains essential nutrients such as protein and calcium (FAO, 2013a). Over the years, consumption of milk and dairy products have increased in Malaysia, while the volume of milk produced from 2004 to 2013 is only 10% of total milk consumption and thus Malaysia depends heavily on imported milk (DVS, 2013). The microbiological quality, quality assessment methods used, hygiene, handling and storage of raw milk are serious issues of concern in Malaysia.

Quality and safety of raw milk is of serious concern as milk is a perishable food and its nutritional content provides a favorable environment for microbial growth. In Malaysia, many of the dairy farms, especially the small scale dairy farms still uses traditional milking and handling techniques which can increase the risk of microbiological contamination of raw milk. Chye *et al.* (2004) and Sim *et al.* (2012) reported that the Total Plate Count of raw milk in Malaysia exceeded 1.2×10^{7} cfu mL^{-1} and 10^{7} cfu mL^{-1}, respectively, and thus it is of unacceptable quality.

The milk collection centers under the Department of Veterinary Services (DVS), Malaysia, uses platform tests such as titratable acidity, clot on boiling and alcohol test to assess raw milk quality. Even though the platform tests are easy to perform, provides a rapid and cheap method for evaluation of milk quality, their reliability in grading and determining whether the raw milk should be accepted, segregated or rejected is of concern. It has been observed by farmers that there are occasions where milk of acceptable quality being rejected leading to lost and wastage of milk.

Preservation methods of raw milk are important to ensure good quality, prevent spoilage and prolong the shelf life of raw milk. Methods routinely used for preservation or shelf life extension of raw milk are refrigeration storage, heat treatment and microfiltration (Walstra *et al.*, 2006; Rahman 2007). However these methods may not be feasible for small scale dairy farmers due to financial constraints as various equipment are expensive. In Malaysia, majority of the small scales dairy farms do not have access to cold chain infrastructure for storage and transportation and as such milk is exposed to elevated temperatures for long period of time, often under unhygienic conditions. Moreover, as farms are located far away from processing facilities or milk collection centers this will contribute to increase in microbial counts and spoilage during transportation. In tropical countries, such as Malaysia, the weather is conducive for microbial growth leading to rapid spoilage of milk. These factors commonly lead to high post-harvest losses of raw milk in dairy industries, especially in developing countries. Alternative methods for preservation of raw milk which are economical and that can be applied directly at the farm need to be examined, as it could assist in improving keeping quality and prolong the shelf life of milk. Chemical preservation methods such as hydrogen peroxide treatment and activation of lactoperoxidase system should be considered and studied for the preservation of raw milk. These methods would be beneficial, especially for the small scale dairy farmers since it is economical and easy to use.

At milk collection centers, raw milk is stored in refrigerated bulk tank and only processed when sufficient quantities have been collected. It might take two to three days before sufficient milk have been collected. Even though refrigerated storage can help with the preservation of raw milk, the presence of psychotropic bacteria such as *Pseudomonas* spp can still cause deterioration of quality and milk spoilage (Champagne *et al.*, 1994). Pre- treatment of raw milk to minimize or inactivate microbial counts before bulk refrigerated storage will assist in prolonging shelf life of raw milk. Thus there is a need to identify and evaluate pre- treatment methods that are able to reduce microbial counts and prolonged the shelf life of raw milk. Ultrasound which is a non-thermal processing method has the potential for pre-treatment for raw milk. Ultrasound is capable of reducing microbial counts and the slight increase in temperature of raw milk during sonication will not drastically affect the chemical and nutritional properties of raw milk (Cameron *et al.*, 2009; Chemat *et al.*, 2011).

1.2 Objectives

The objectives of this present study were:

a) To determine the microbiological quality and safety of raw milk produced by dairy farms in Penang, Malaysia

b) To determine the reliability of different types of platform tests used in evaluation of raw milk quality.

c) To determine the effects of hydrogen peroxide, activation of lactoperoxidase system and ultrasound on microbial counts in raw milk.

d) To determine the antibiotic resistance of *Staphylococcus aureus* isolates obtained from raw milk.

CHAPTER 2

LITERATURE REVIEW

2.1 Milk

2.1.1 Properties of milk

Milk is defined as the liquid excreted by the mammary glands of both human females and mammals used as a source of food for the newly born (Singh and Bennett, 2002). In terms of dairy technology milk can be defined as product obtained from simple or multiple milking of mammals such as cows that are kept for the purpose of milk production (Bylund, 1995). Milk is a liquid which is white or sometimes yellowish in color depending on the carotene content and it is opaque due to suspended particles present in milk such as fat, proteins and certain minerals. Milk is an oil-in-water emulsion because the milk fat is present as small droplets dispersed in the milk serum. Milk normally is slightly sweet in taste due to presence of lactose. Density, pH and freezing point of bovine milk normally ranges between 1.028 and 1.038 g cm^3, 6.5 to 6.7 and –0.54 to – 0.59 °C, respectively, depending on the milk composition (Bylund, 1995; Singh and Bennett, 2002).

The major components in milk are water, fat, protein and lactose while the minor components found in milk are enzymes, vitamins, minerals and phospholipids. On average the composition of cow milk consists of 87.0% water, 4.0% fat, 3.4% total proteins, 4.8% lactose and 0.8% minerals. The protein in cow milk is of high quality as it contains a good balance of essential amino acids including lysine. Milk also contains natural minerals and vitamins such as calcium, magnesium, phosphorus, selenium, vitamin A (retinol and carotene), vitamin B2 (lactoflavin and riboflavin), vitamin B6 (adermin), vitamin D (cholecalciferol and ergosterol) and vitamin C. The composition of milk varies depending on factors such as cattle breed, age and health status, stages of lactation, feedstuff and environmental factors (Spreer, 1998; Singh and Bennett, 2002).

Malaysia food regulation described raw milk as normal, clean, fresh mammary secretion of healthy cow that is properly fed and kept, excluding the secretion obtained during the first four days following calving. Raw milk should contain more than 3.25 and 8.5% of milk fat and non-fat milk solid respectively and should not contain added water, food additive, antibiotic or other added substances. The milk may be cooled but should not have been subjected to other physical treatment (FoSIM, 2016).

2.1.2 Importance of milk

Milk is an important natural food resource, especially since milk and its products are part of the human diet. Milk is considered as healthy and beneficial food since it contains essential nutrients which are required for the development and maintenance of human life for example growth and maintenance of healthy bones and teeth. Consumption of milk has also been linked to reduced risk in cardiovascular disease, high blood pressure, type 2 diabetes and obesity (Spreer, 1998; FAO, 2013a). The Malaysian Food Pyramid suggests that humans should consume one to three servings of milk and milk products daily (Figure 2.1) (Nutrition Society of Malaysia, 2012).

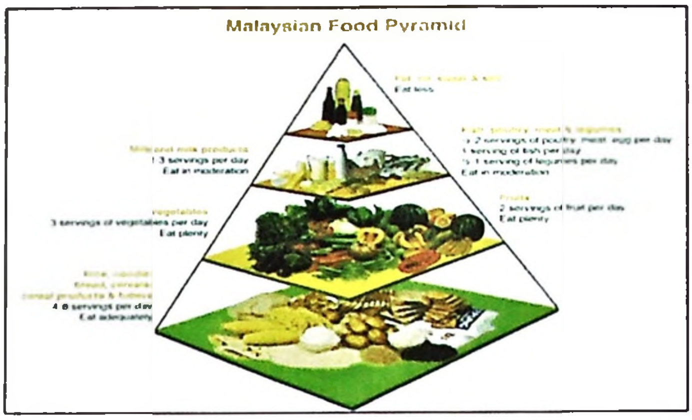

Figure 2.1 Malaysian Food Pyramid
(Source: Nutrition Society, Malaysia, 2013)

Raw milk is of importance to the food industry as it is used to produce a variety of products. The fat content of milk is adjusted to produce products such as whole milk, skimmed milk, semi-skimmed milk, low fat milk and standardized milk. Processing procedure such as pasteurization, ultra high temperature (UHT) treatment and spray drying is used to produce pasteurized milk, UHT milk and powdered milk products. Milk is also used for production of dairy product such as butter, ghee, cream, yoghurt, dadih and kefir. The multifunctional properties of milk make it a versatile ingredient and are used in a wide range of foods from dishes cooked in the kitchen to products manufactured on industrial scale (Early, 1997; FAO, 2013a).

2.1.3 Milk production, consumption and dairy industry

Globally, cow milk accounts for 83% of total milk output, while the rest (17%) is obtained from buffalo, goat, sheep and camel. According to the Organization for Economic Co-operation and Development (OECD) and Food and Agriculture Organization (FAO), milk and dairy sector will continue to be one of the fastest-growing agricultural subsectors in terms of production. The world milk production in 2013 was expected to grow by 1.9% to 780 million tons (FAO 2013a;

FAO, 2013b). Population growth, rising income and urbanization, especially in the developing countries are leading to overall increase in food consumption and changes in dietary pattern leaning toward consumption of food of animal origin such as livestock products. Over the period of 1961 till 2007, the consumption of milk had almost doubled. The increase in consumption and production of dairy products is essentially demand driven and is expected to remain strong (FAO, 2013a).

In developing countries, the increasing demand in milk and dairy products could not be met in some of the countries as most of the milk is still produced in traditional small-scale systems. Thus these countries have to rely on imported milk and dairy products (FAO, 2013a). Asia (Malaysia, Singapore, Indonesia, Japan, the Philippines, Thailand and China) is the major importer of dairy products accounting for 55% of world imports. Other countries such as Saudi Arabia and the United Arab Emirates also imports large quantities of dairy products. However, the world trade in dairy products is expected to decrease by 0.9 percent in 2013 to 53.0 million tons due to constraints in producers exporting as a result of changes in weather (FAO, 2013b).

In Malaysia the milk output is predominantly obtained from cattle with a small niche market for goat's milk. Table 2.1 shows the Livestock Population in Malaysia form year 2009 to 2013.

Table 2.1 Livestock population in Malaysia, 2009 - 2013

Livestock Type	2009	2010	2011	2012	2013*E
Buffalo	127,100	125,900	123,200	118,600	118,000
Cattle	860,400	836,900	768,400	742,500	751,700
Goat	514,200	496,100	476,400	458,600	482,200
Sheep	136,200	123,400	126,400	131,900	129,800

* E: Estimated

Source: Department of Veterinary Services Malaysia, 2013

The majority of local dairy farms are small scale farms with less than 30 cows. Boniface *et al.* (2010) observed that 57 of the 133 dairy farms examined in Malaysia were small scale dairy farms. Table 2.2 shows the number of dairy farms in Malaysia based on farm type. In Malaysia, dairy cattle are grazed on grass with poor nutrient content and the feed is supplemented with agro-waste such as rice bran, copra cake, palm kernel cake, oil palm frond, sago, tapioca and broken rice (Loh, 2002; DVS, 2003). Generally, cattle can provide 5 to 15 liters of milk output per day but this depends on various factors such as breed, feedstuff, lactation stage, weather condition or temperature and cattle's health (FAO, 2013a). Farmers normally sell the milk to milk collection centers owned by government, dairy processor, milk agents or restaurants. The milk collection centers and dairy processors buy milk at a predetermined price while milk agents and restaurants buy the milk based on market price (Boniface *et al.*, 2010).

Table 2.2 Number of dairy farm in Malaysia based on farm type, 2010

Farm Type	Number of Cattle	Number of Farm	Percentage (%)
Small scale	< 30	57	42.9
Semi commercial	31-50	25	18.8
Commercial	51-100	31	23.3
Large scale	> 100	20	15.0

Source: Boniface *et al.*, 2010

Similar to the global trend, increase in population, rising income and better socio-economic condition have led to an increase in the consumption of milk and dairy products in Malaysia. The dairy market in Malaysia is highly dependent on imported milk products (DVS, 2003). Table 2.3 shows the output, consumption and self-sufficiency of milk products in Malaysia from year 2004 to 2013. Even-though a continuous increase in local milk production was observed from year 2004 to 2013, the high demand for milk and dairy products leads to insufficient milk production to meet its demand. From the year 2004 up to 2013 the percentage of self-sufficiency in terms of milk production was always below 10%, with the exception for the year 2011 in which the self-sufficiency increased to 13.17% due to a decrease in milk consumption (DVS, 2013b).

Table 2.3 Malaysia: Output, consumption and self-sufficiency of milk products, 2004 - 2013

Year	Output (million liters)	Consumption (million liters)	Self-sufficiency (%)
2004	38.77	1300.47	2.98
2005	41.10	895.06	4.59
2006	45.45	975.81	4.66
2007	51.07	889.05	5.74
2008	56.49	650.83	8.68
2009	62.30	708.83	8.79
2010	67.00	789.23	8.49
2011	70.87	538.18	13.17
2012 *P	75.00	807.66	9.29
2013*E	79.35	852.89	9.30

* P : Provisional E : Estimated

Source: Department of Veterinary Services Malaysia, 2013

In Malaysia, the microbial quality, handling during milking, storage and transportation of milk and quality assessment methods used at milk collection centers are issues of concerns. The raw milk produced generally have high Total Plate Count and is of unacceptable quality since it exceeds 1×10^6 cfu mL^{-1} which is the limit set by Department of Veterinary Services, Malaysia (DVS) for milk price incentive (Chye *et al.*, 2004; Sim *et al.*, 2012) . Since majority of the farms are of small scale, they practice hand or manual milking technique, which lack proper hygiene practice, thus increasing the risk of contamination. There is also lack of cold chain facilities for storage and transportation of raw milk, allowing milk to be exposed often under unhygienic condition at ambient temperature. Furthermore the hot and humid climate in Malaysia facilitates microorganism growth, thus making milk more susceptible for microbial contamination and hence increasing the risk of spoilage. At milk collection centers in Malaysia, milk is often kept in tanks under refrigeration and is send to processing plants when sufficient quantities of milk have been collected. This commonly takes two to three days. During refrigerated storage, psychotropic microorganisms will grow albeit slowly and lead to spoilage of milk (Champagne *et al.*, 1994).

2.2 Microbiological quality and safety of raw milk

A major concern about raw milk is its quality and safety. This is due to the fact that milk in its natural state is a highly perishable material because it is susceptible to rapid spoilage by the action of contaminating microorganisms and naturally occurring enzymes (Chambers, 2002; Adam and Moss, 2008). Microbial load of freshly drawn milk from a healthy cow is usually less than 1000 cfu mL^{-1}. However, microbiological count of raw milk can increase to more than 10^{6} cfu mL^{-1} due to contamination (Ritchter *et al.*, 1992). Sources of microbial contamination in milk can be from the milk handlers, cow (udder), surrounding environment (air, water) and handling during processing (equipment, milking techniques) (Adams and Moss, 2008).

Most of the small scale dairy farms still use hand milking technique thus the milk handlers can be a source of microbial contamination in milk. Microorganisms are present on human bodies such as hands and nasal cavity and it can be transferred to the food if the food handlers do not apply proper hygienic steps such as wearing gloves, covering their hair, wearing an apron and washing their hands (Chambers, 2002; Adams and Moss, 2008). Poor personal hygiene by the food handlers can also allow contamination by foreign substances such as hair and nails into the food. When cows are hand milked it is possible that the microbial contamination can occur during milking and handling process by direct contact of milk handlers hands with milk, dislodging dirt particles from the udder into the milk and increasing aerial contamination through accelerated air movement (Forsythe and Hayes 1998; Chambers, 2002).

The water supply can also be a source of contamination; water used should be of potable water quality. Some farms use or depend on untreated water such as water from boreholes, wells, lakes and rivers which might be contaminated with microorganisms. The commonly found bacteria in untreated water are microbes of fecal origin, such as coliforms, fecal streptococci and clostridia. If untreated water is used for rinsing equipments and containers, the microbes present from water can contaminate the milk (Chambers, 2002)

Milking equipments and containers are also sources for microbial contamination. Milk contact surface such as bulk tank or milk cans need to be cleaned and sanitized properly to eliminate microorganisms. When milking machines are used, it is important that the machines are kept in clean condition and also well maintained to prevent microbial build up. Dairy farms that are not maintained and kept in a clean condition can be dangerous as dusts, dirt, metals and wood splinters can be the source of contamination (Chambers, 2002; Ray 2004).

The cattle can also be one of the sources for microbial contamination. Mastitis is an inflammation of the cattle's udder usually caused by bacterial infection due to *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Escherichia coli*. These microbes normally grow on the udder and teats of infected cattle and will be transferred into the milk during milking process. Cattle should be checked by veterinary professionals on regular basis and cattle's that are ill should be isolated from the healthy cattle herd to prevent infection (Lammers *et al.*, 2001; Bradley, 2002).

In addition to that, the surrounding environment such as soil and vegetation can also be a source for microorganisms, especially saprophytic microorganisms such as *Pseudomonas* spp and *Bacillus* spp. Pests such as insects and rodents present on the farm can also lead to microbial contamination since these pests usually carry bacteria (Chambers, 2002; Ray 2004).

2.2.1 Risks associated with consumption of raw milk

Consumption of raw milk and its products can pose a health risk to human, especially people with weakened immune systems, elderly people, pregnant women and children, due to the presence of pathogenic microorganisms which can cause illness and food borne outbreaks (FDA, 2011; Claeys *et al.*, 2013). The potential health risk from consumption of raw milk has caused certain countries for example Canada, Australia, New Zealand, Scotland and certain states in the United States of

America (example: Alabama, Colorado, Florida and Michigan) to prohibit sales of raw milk for direct human consumption and requires that milk intended for consumption must be at least pasteurized to ensure its safety (MPI, 2014). Consumption of raw milk can lead to food poisoning or intoxication and also serious complication such as stillbirth, miscarriage and death. The United States Centers for Disease Control and Prevention (CDC) reported that raw milk is 150 times more likely to cause food borne illness and 13 times more risk of hospitalization as compared to processed milk such as pasteurized milk (FDA, 2012a). It was reported that at least 80 people were ill with one dead and 20% of the victims were hospitalized in Utah, USA due to *Campylobacter* outbreak which was linked to raw milk consumption. In Australia, a 3 year old boy died while four other children were ill due to outbreak of *E. coli* and *Cryptosporidium* which were associated with raw milk consumption. UK Food Standard Agency have recalled raw milk product in Kentisbury due to potential link to two cases of *E. coli* O157:H7 infection (Food Safety News, 2014 a, b and c). Table 2.4 shows the extent of food borne illness due to consumption of raw milk and its products reported from year 1987 to September 2010 in United States of America (FDA, 2011).

Table 2.4 Food borne illness due to consumption of raw milk and its products from year 1987 to September 2010

Categories	Number
Outbreaks	133
Cases of illness	2659
Hospitalizations	269
Stillbirth	6
Miscarriages	2
Death	3

Source: United States Centers for Disease Control and Prevention, 2011

In Malaysia, although food poisoning has the highest incidence compared to other communicable diseases, the data on the type of food which is associated with food poisoning outbreaks are unavailable (MOH, 2012). There is no information on the prevalence of food borne illness due to consumption of raw milk and its products.

2.2.2 Microbiological standards for milk

In many countries, there are guidelines or standards to determine microbial quality of milk and to ensure only good quality raw milk is consumed or used for production. In Malaysia, the microbiological limit set by DVS on milk price incentive is that Total Plate Count (TPC) should be less than 10^{6} cfu mL^{-1}. United Kingdom has a more stringent standard, in which the microbiological limits set for the raw milk price incentive are as follows: grade A should be less than 2×10^{4} cfu mL^{-1} (bonus), grade B between 2×10^{4} cfu mL^{-1} to 1×10^{5} cfu mL^{-1} and grade C which is more than 1×10^{5} cfu mL^{-1} (penalized) (Chambers, 2002). In comparison, European Union (EU) divides the guidelines based on raw milk intended for direct human consumption or for processing in which TPC should be less than 2×10^{4} and 1×10^{5} cfu mL^{-1}, respectively (EU, 1992). The microbiological limit in UK and EU which is more strigent, can provide a better control to ensure safety and quality of raw milk as compared to limit set in Malaysia.

The concern on microbiological quality and safety of raw milk has lead to research being carried out in order to obtain information on microorganism present in raw milk. Table 2.5 shows the result of microbial quality of raw milk reported by various researchers.

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Table 2.5 Microbial quality of raw milk reported by various researchers.

Country	Raw Milk Samples	Microbiological Quality	Reference
Baleiric Island, Spain	990 samples from farms in Majorca.	TPC: 6.52 log cfu mL^{-1}, coliforms: 5.26 log cfu mL^{-1}, psychrotrophs: 5.55 log cfu $mL^{-1}$and thermoduric microorganisms: 4.47 log cfu mL^{-1}.	Soler *et al.*, 1995.
	114 samples from farms in Minorca.	TPC: 6.33 log cfu mL^{-1}, coliforms: 4.86 log cfu mL^{-1}, psychrotrophs: 5.98 log cfu $mL^{-1}$and thermoduric microorganisms: 4.60 log cfu mL^{-1}.	
Trinidad	287 samples from 8 collection centers.	TPC: 3.3×10^{6} to 9.8×10^{7} cfu mL^{-1}, *E. coli*: 4.2×10^{4} to 1.6×10^{6} cfu mL^{-1} and *S. aureus*: 1.4×10^{4} to 1.2×10^{5} cfu mL^{-1}.	Adesiyun *et al.*, 1995.
Malaysia (Peninsular Malaysia)	930 samples from 360 dairy farms.	TPC: 12×10^{6} cfu mL^{-1}, coliforms: 90%, *E. coli*: 65% positive, *E. coli* 0157:H7: 33.5% positive, *S. aureus*: 60% positive, *Salmonella* spp: 1.4% positive and *Listeria* spp: 4.4% positive.	Chye *et al.*, 2004.
Khartoum, Sudan	16 samples from 4 farms.	TPC: 9.65×10^{6} cfu mL^{-1}, coliforms: 5.27×10^{4} cfu mL^{-1}, fecal coliforms: 1.40×10^{4} cfu mL^{-1}, *S. aureus*: 6.00×10^{5} cfu mL^{-1}, yeast: 6.97×10^{5} cfu mL^{-1} and lactic acid bacteria: 6.70×10^{4} cfu mL^{-1}.	Asmahan, 2010.
Zimbabwe	120 samples from 3 small holder dairy schemes.	TPC: 6.4 log cfu mL^{-1}, coliforms: 6.4 log cfu mL^{-1}, *E. coli*: 6.2 log cfu mL^{-1} and *S. aureus*: 5.4 log cfu/m.	Mhone *et al.*, 2011.

Country	Raw Milk Samples	Microbiological Quality	Reference
Malaysia (Sabah)	150 samples from 11 dairy farms and 1 collection center.	TPC: $\geq 10^{7}$ cfu mL^{-1}, coliforms: 2.96 to 4.03 log cfu mL^{-1}, *E. coli*: 31.3% positive, *S. aureus*: 2.73 to 3.55 log cfu mL^{-1} and *Salmonella* spp: 11.3% positive	Sim *et al.*, 2012.
New Zealand	297 samples from 290 farm vats.	*E. coli*: 99.7% positive, S. *aureus*: 60.34% positive, *Salmonella* spp not detected *L. monocytogenes*: 0.68% positive, *L. innocua*: 4% positive and *Campylobacter* spp: 0.34% positive.	Hill *et al.*, 2012.
Cameroon (Maroua)	42 samples from milk churn.	TPC: 6.23 log cfu mL^{-1}, coliforms: 3.83 log cfu mL^{-1}, *E. coli:* 2.25 log cfu mL^{-1} and coagulase-positive Staphylococci: 2.65 log cfu mL^{-1}.	Belli *et al.*, 2013.
Morocco	80 samples from 3 rural communes.	TPC: 1.4×10^{6} cfu mL^{-1}, coliforms: 1.7×10^{3} cfu mL^{-1}, fecal coliforms: 1.9 ×x 10^{2} cfu mL^{-1}, *S. aureus*: 1.7×10^{3} cfu mL^{-1}, *Salmonella* spp: not detected and *L. monocytogenes*: 0.8% positive.	Belbachir *et al.*, 2015

2.2.2(a) Coliforms and *Escherichia coli*

Enterobacteriaceae is a family of Gram negative, facultative anaerobic, non spore forming and rod shaped microorganism. *E. coli*, *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter* and *Proteus* are among those categorized under the Enterobacteriaceae family. Within the Enterobacteriaceae family those which ferment lactose such as *E. coli*, *Enterobacter* and *Klebsiella* are grouped as coliforms bacteria. Coliforms are considered as indicator organism to indicate possible presence of pathogens and contamination such as from fecal matters (Blood and Curtis, 1995; Manafi, 2003).

E. coli is normally found in the intestinal tract of human and other warm-blooded animals. It is also widely distributed in the environment such as soil, water and vegetation (Blood and Curtis, 1995). *E. coli* have also been associated with cattle and it was reported that *E. coli* have been isolated from 0.9 to 8.2% of healthy cattle in the United Kingdom. Besides *E. coli* can also cause illness such as mastitis in cattle (Lammers *et al.*, 2001; Ray, 2004). *E. coli* has been isolated from different groups of foods such as vegetables, meat, poultry and dairy products. The presence of *E. coli* in food is not necessarily harmful for human since only certain serotype, such as *E. coli O157* can cause food poisoning. However, the presence of *E. coli* indicates poor hygiene and sanitation in production of the food (Ray, 2004).

2.2.2(b) *Staphylococcus aureus*

S. aureus is a coccus shaped, non-spore forming Gram positive bacteria which are classified under the Staphylococcaceae family. *S. aureus* is facultative anaerobe and mesophilic bacteria (Ray, 2004; Fernandes, 2009).

S. aureus is frequently found in human nose, throat and also as part of the skin micro flora. *S. aureus* contamination occurs as a result of poor handling of the food that leads to extensive human contact, or contaminated food surface contact (Ray, 2004). Animals can also be a source of *S. aureus* contamination since *S. aureus* can be present in unhealthy goat, sheep and cattle. *S. aureus* is commonly associated with mastitis which is an inflammation of the cattle's udder (Bradley, 2002; Walstra *et al.*, 2006).

S. aureus is harmful for human and can cause Staphylococcal food poisoning due to the toxins produced. *S. aureus* produces variety of toxins which includes enterotoxins (SEA, SEB, SEC. SED, SEE, SEG, SEH, SEI, SER, SES, and SET) and also staphylococcal-like proteins (SE/J, SE/K, SE/L, SE/M, SE/N, SE/O, SE/P, SE/Q, SE/U, SE/U2, and SE/V) (Argudin *et al.*, 2010). Although *S. aureus* cells are destroyed under normal heat treatment, the toxin produced is commonly heat stable. Symptoms of Staphylococcal intoxication include nausea, vomiting, diarrhea and abdominal cramping (Fernandes, 2009; Argudin *et al.*, 2010). In Japan a food poisoning outbreak which occurred in 2001 was due to consumption of milk product that was found to contain *Staphyloccocus* enterotoxins A (SEA) (Asao, 2003). While in 2007 an outbreak occurred in Austria which affected 40 children was attributed to *S. aureus* producing SEA and SED linked to bovine milk products (Schmid, 2009).

2.2.2(c) *Salmonella* spp

Salmonella is classified under the Enterobacteriaceae family and is a rod shaped Gram negative bacteria. *Salmonella* is a facultative anaerobe, non spore forming and generally motile microorganism (Ray, 2004; Fernandes, 2009). The genus *Salmonella* consists of two main species which are *Salmonella enterica* and *Salmonella bongori* (Ellermeier and Slaunch, 2006).

Salmonella is generally found in the intestinal tract of human and other warm blooded animals. They can also be found in soil, water, food and feed through contamination by fecal matter (Ray, 2004). Presence of *Salmonella* in food indicates poor food handling and processing such as cross contamination and inadequate heat treatment.

Salmonellosis is the food poisoning which occurs from ingestion of viable *Salmonella* cells. Transmission of *Salmonella* generally occurs by the fecal-oral route in which intestinal contents from animals infected with *Salmonella* contaminates food or water which is then consumed by humans. Common foods associated with the presence of *Salmonella* are poultry, eggs and meat. Symptoms of *Salmonella* food poisoning are such as fever, chills, diarrhea, nausea and vomiting, but the severity is dependent on virulence of the serovar (Leaver, 1997; Ray, 2004). In 2002, two children in Ohio was hospitalized due to consumption of raw milk that was found to contain S. Typhimurium (CDC, 2003). It was reported in Pennsylvania, USA that a food poisoning outbreak with 29 cases of diarrheal illness was due to presence of S. Typhimurium linked to consumption raw milk and its products (CDC, 2007).

2.2.2(d) *Listeria* spp

Listeria, classified under the Listeriaceae family, is a coccobacillus shaped Gram positive bacteria. The genus *Listeria* consists of six main species which are *Listeria monocytogenes*, *Listeria grayi*, *Listeria innocua*, *Listeria ivanovii, Listeria seeligeri and Listeria welshimeri*. These bacteria are facultative anaerobes, non spore forming and motile microorganisms (Ray, 2004; Fernandes, 2009).

Among the Listeria species, *L. monocytogenes* is considered to be the most important as it is a pathogen that causes Listeriosis which is a food borne illness due to consumption of viable *L. monocytogenes* cells. *L. monocytogenes* is ubiquitous in the environment as it has been found in soil, sewage sludge, decaying vegetation, silage, feces and from fresh and salt water. Presence *of L. monocytogenes* has been associated with wide range of foods from vegetables, meat, poultry and even milk (Ray, 2004; FDA, 2012b).

Listeriosis is harmful especially for pregnant women, young or elderly and immune compromised people as the symptoms can vary from mild flu-like illness to meningitis and meningoencephalitis. In more severe cases it can lead to stillbirth or premature labor for pregnant women. *L. monocytogenes* is also a disease causing agent in cattle's as it can cause meningoencephalitis (Ray, 2004; FDA, 2012b). In North Carolina an outbreak due to *L. monocytogenes* which occurred from 2000 to 2001 caused 12 illnesses, five still births, three premature deliveries and two infected newborn was linked to consumption of Mexican-style cheese made from raw milk (CDC, 2001). In 2007, three people died due to an outbreak associated with presence of *L. monocytogenes* from consumption of pasteurized milk and the local dairy and bottling facility in Massachusetts which produced the milk was shut down (CDC, 2008).

2.2.3 Antibiotics resistance microorganisms

Antibiotics are drugs which are commonly used in human and veterinary medicine to treat and prevent diseases and also to help growth promotion in livestock and poultry (FAO, 2012). In cattle, the main reason for administration of antibiotics is to treat mastitis which is caused by microorganisms such as *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, and *Streptococcus uberis* (Lammers *et al.*, 2001; Bradley, 2002; Jamali *et al.*, 2015). The most commonly used antibiotics for treatment of bovine mastitis are penicillin, tetracycline, oxacillin, erythromycin, cefazolin, clindamycin and tobramycin (Lammers *et al.*, 2001; Goa *et al.*, 2012; Jamali *et al.*, 2014). The continuous use of antibiotics has caused a steady upwards trend of antibiotics resistant microorganisms which is an issue of concern for both human and animal health (FAO, 2012). Once microbe develops resistance to a certain antibiotic, the microbe would be less responsive or have no effect to the antibiotic treatment thus it would be more difficult and expensive to cure the infection caused by this microbe.

It is reported by Sampimon *et al.* (2011) that *Staphyloccocus* isolates obtained from raw milk were resistant to penicillin, oxacillin, erythromycin, pirlimycin, and tetracycline. Research by Shitandi and Sternesjö (2004) also showed that *Staphyloccocus* isolates obtained from raw milk were resistant to penicillin, tetracycline, erythromycin, trimethoprim/sulfamethazine, and chloramphenicol.

While Moser *et al.* (2013) reported that *S. aureus* isolated from raw milk shows resistance towards ampicillin, kanamycin-cefalexin and penicillin.

Presence of antimicrobial resistance genes in *Staphylococcus* species can be transferred between staphylococcal species through lateral transfer and also transferred between human and animals through direct transmission of resistant pathogens (Walther and Perreten, 2007). Testing and monitoring on antimicrobial susceptibility of microorganism is important to provide information on the development of antimicrobial resistant microorganism and also treatment to be used for infection.

2.3 Platform tests

Milk testing and quality control are an important component of the milk processing and dairy industry, since it is an essential step in ensuring that milk and its product will have satisfactory quality and is safe for consumption. To produce dairy products with good quality, it is important that the raw milk used is of good quality since raw materials have an impact on the outcome of the final products. Ensuring good quality of milk begins at the farm and continues throughout processing, storage and transportation stages (Pande and Voskuil, 2011).

Platform tests or milk reception tests are rapid analysis methods which are used as quality control tools that help to determine if the raw milk should be accepted or rejected. These tests provide a quick, cheap and easy method for evaluation of milk quality in terms of milk organoleptic characteristic, composition (protein and fat), physical and chemical characteristic (specific density and freezing temperature) and hygiene level (microbiological) (Teuvo, 2000; Draaiyer *et al.*, 2009). The purposes of platform tests are for screening and grading of raw milk and also to provide a fair payment system between the milk producer and milk agent or processor. The platform tests are beneficial for both the milk producer and milk processor since milk producer will obtain a fair price according to the quality of the milk produced, while for the milk processor the test provides assurance that the milk produced is of acceptable composition and quality (FAO, 1996; Teuvo, 2000). The platform tests are normally carried out at the dairy farms, milk collection centre or the dairy plant. Some examples of the commonly used platform tests are organoleptic test, clot on boiling test (COB), alcohol test, titratable acidity (TA), resazurin test, Gerber test, Lactometer test and sediment test. Milk that passes the platform tests will be accepted for further use such as processing of dairy products, but milk that fails the test will be rejected since the milk is not within the accepted quality standard. For example, milk that fails the resazurin test means that the milk has unsatisfactory microbial count. When the quality of raw milk cannot be determined by the platform test, then the milk will be separated for further laboratory analysis (FAO, 1996; Teuvo, 2000; Draaiyer *et al.*, 2009). It is important that proper action is taken depending on the result of the milk testing in which milk should either be accepted, segregated or rejected since one single lot of poor quality milk can spoil the whole milk bulk.

2.3(a) Organoleptic test

Organoleptic test is carried out based on the judgment of the tester using his or her senses such as sight, smell and taste. It provides information about the milk general characteristic such as color, odor and taste. In general milk should be slightly