MICROBIOLOGICAL QUALITY OF FRESH COCONUT MILK SAMPLED FROM SELECTED PROCESSED COCONUT VENDORS IN KOTA BHARU, KELANTAN

By

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LIST OF SYMBOLS & ABBREVIATION

%	Percentage
>	More than
<	Less than
≥	More than or equals to
/	Over to
°C	Degree Celcius
g	Gram
mL	Mililiter
µL	Microliter
CDC	Centre for Disease Control and Prevention
CFS	Centre for Food Safety
CFU	Colony Forming Units
EMB	Eosin Methylene Blue
E. coli	*Escherichia coli*
FCC	Faecal Coliform Count
FDA	Food and Drug Administration
K. pneumoniae	*Klebsiella pneumoniae*
MKKM	Makmal Keselamatan dan Kualiti Makanan
MOH	Ministry of Health
NSW	New South Wales
S. aureus	*Staphylococcus aureus*
TCC	Total Coliform Count
TPC	Total Plate Count
WHO	World Health Organization

ABSTRAK

KUALITI MIKROBIOLOGIKAL SANTAN SEGAR YANG DISAMPEL DARI PENJUAL KELAPA PARUT TERPILIH DI KOTA BHARU, KELANTAN

Santan segar digunakan secara meluas dalam masakan dan juga dalam makanan tersedia dimakan. Walau bagaimanapun, isu-isu yang berkaitan dengan kualiti mikrobiologi dan keselamatan santan segar masih diragui kerana ia mudah terdedah kepada pencemaran mikrob dan telah dikaitkan dengan penyakit bawaan makanan. Kajian ini telah dijalankan untuk menganalisis kualiti mikrobiologi iaitu jumlah kiraan plat (TPC), jumlah kiraan koliform (TCC) dan koliform najis (FC). Sebanyak 50 sampel santan segar diambil secara rawak daripada 10 penjual di pasar basah yang dipilih di Kota Bharu, Kelantan dari Oktober 2015 sehingga Februari 2016. Semua 50 (100%) sampel menunjukkan keputusan yang tidak memuaskan untuk TPC dan TCC kerana telah melebihi garis panduan yang dibenarkan untuk makanan sedia dimakan. Sebanyak 17 (34%) sampel daripada 8 penjual menunjukkan kehadiran FC dan *E.coli* telah dikesan dalam semua sampel ini. Oleh itu, dapat disimpulkan santan segar yang disampel di pasar yang dipilih mempunyai kualiti mikrobiologi tidak memuaskan dan tidak selamat untuk dimakan. Pengamalan kebersihan yang sesuai haruslah dipraktikkan oleh penjual untuk mengekalkan kualiti santan segar yang memuaskan.

ABSTRACT

MICROBIOLOGICAL QUALITY OF FRESH COCONUT MILK SAMPLED FROM SELECTED PROCESSED COCONUT VENDORS IN KOTA BHARU, KELANTAN

Fresh coconut milk is widely used in cooking and also in ready-to-eat food preparations. However, issues pertaining to its microbiological quality and safety for consumption remains doubtful as it is prone to microbial contamination and has been associated with foodborne diseases. This study was conducted to evaluate the microbiological quality viz. total plate count (TPC), total coliform count (TCC) and faecal coliform (FC) of 50 fresh coconut milk samples randomly obtained from 10 selected vendors in the wet markets, Kota Bharu, Kelantan wet markets from October 2015 until February 2016. All 50 (100%) samples showed unsatisfactory results for TPC and TCC which exceeded the proposed acceptable guidelines for ready-to-eat food. A total of 17 (34%) samples from 8 vendors showed presence of FC and *E.coli* was identified in all the samples. Therefore, we may conclude that the fresh coconut milk sampled from the selected markets have unsatisfactory microbiological quality and thus unsafe for consumption. Appropriate hygiene practices have to be followed by vendors to maintain satisfactory quality of fresh coconut milk.

Keywords: *Fresh coconut milk, Microbiological quality, Total plate count, Total coliform count, Faecal coliform count and E.coli*

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Ready-to-eat food is defined as food that is in a form which is edible without washing, cooking, or additional preparation by the food establishment or the consumer and that is reasonably expected to be consumed in that form (FDA, 2013). Ready-to-eat food includes washed raw whole or cut fruits and vegetables, cooked fruits and vegetables, raw animal food which is cooked or frozen and plant food presented for consumption for which further washing or cooking is not required and from which rinds, peels, husks, or shells are removed. (FDA, 2013). Besides, ready-to-eat food also includes plant-derived substances such as spices and seasonings, bakery items such as breads and cakes and potentially hazardous food that is unpackaged and cooked to the temperature and time required for the specific food (FDA, 2013).

Ready-to-eat foods can be hazardous as they can support the growth of pathogenic and spoilage microorganisms. They must be stored under ideal temperatures to minimize the growth of any pathogens which may be present in the foods. Besides, storing the foods in proper temperature prevents the formation of toxin which may harm health. NSW Food Authority (2009) stated that ready-to-eat foods includes a number of ingredients which may or may not be cooked, where processing method and individual components of the food must be taken into consideration for the interpretation of microbiological results obtained from the food testing.

Food storage condition is one of the key parameters in food quality and safety (Nerín *et al.*, 2016). Storing the food in proper temperature and humidity extends the shelf

life of food (Nerín *et al.*, 2016). Hence, food must be stored at ideal temperatures and humidity which prevents the growth of pathogens that causes contamination. Abdul-Mutalib *et al.* (2012) stated that food contamination can lead to food-borne disease if food handlers neglect proper food handling practices in their premises. To avoid this cross contamination, food handlers must practice hygiene sanitary steps such as proper hand-washing during the food preparation.

Foodborne diseases or known as foodborne illness which is a problem caused by consuming contaminated foods or beverages (CDC, 2015). There are many different contaminants of foods such as disease-causing microbes or pathogens, poisonous chemicals and other harmful substances which cause different type of foodborne diseases (CDC, 2015). Foodborne diseases which are caused by microorganisms can be classified as either intoxication or infection. Food infection occurs when food contaminated with live microorganisms such as bacteria or virus is consumed by a person. Food intoxication occurs if a person consumes food contaminated with microbial toxins of microorganisms which lead to disease (Miliotis & Bier, 2003).

Foodborne disease symptoms are different for each person. According to CDC (2015), the first common symptoms include nausea, vomiting, abdominal cramps and diarrhoea. Foodborne disease in Malaysia is very common and happens due to unhygienic equipment, contaminated water and improper food handling (Ministry of Health, 2006). Soon *et al.* (2011) stated that majority foodborne diseases in Malaysia are caused by unsanitary food handling procedures which accounted for more than 50% of the poisoning episodes as reported by the Ministry of Health (MOH) Malaysia in 2007. In Kelantan, the

Kelantan State Department reported that outbreaks due to food poisoning have increased for the past 10 years from the year 2000 to 2010.

Fresh coconut milk is one of the examples of ready-to-eat foods. Coconut milk is the liquid obtained by manual or mechanical extraction of the coconut endosperm. (Phattayakorn & Wanchaitanawong, 2009). Coconut milk is used as an ingredient for many traditional foods of Asian and Pacific regions such as curries and desserts (Peamprasart & Chiewchan, 2006). Coconut milk is perceived to be a healthy food as it is originated from a plant source (Marina & Nurul Azizah, 2014). Coconut milk is also used to replace dairy milk for people who are allergic or having lactose intolerance to dairy milk (Marina & Nurul Azizah, 2014).

However, fresh coconut milk allows the growth of microorganisms through poor handling, coconut shell contamination and the utensils used to extract coconut milk (Seow & Gwee, 1997). Coconut milk is extracted by breaking the coconut shell and removing the kernel via hands or machine. This may pose a risk of contamination due to direct handling if the coconut processing is not handled hygienically. Besides, fresh coconut milk has a short shelf life as it is prone to rapid microbiological spoilage because it supports the growth of microorganisms (Seow & Gwee 1997; Simuang *et al.*, 2004).

In the year 1991 in Thailand, there was an emergence of nalidixic acid resistant *Shigella dysenteriae* Type 1 in a local community and was traced to a point source outbreak at a local school (Hoge *et al.*, 1995). There was a foodborne disease outbreak due to the consumption of a coconut milk dessert prepared in an elementary school (Hoge *et al.*, 1995). The dessert preparation involved kneading the grated coconut by hand

without using plastic gloves, no handwashing policy before the dessert preparation by the food handler and the food handler also had diarrhoea just prior to the outbreak (Hoge *et al.*, 1995).

In Maryland, United States of America (USA) in August 1991, 3 cases of outbreak due to cholera were reported which were associated with imported commercial fresh frozen coconut milk from Thailand (Taylor *et al.*, 1993). This is because of the consumption of Thai-style rice pudding served with a topping of the fresh frozen coconut milk (Taylor *et al.*, 1993). *Vibrio cholerae* O1 biotype El Tor, serotype Ogawa was isolated from 4 out of 6 persons who consumed the food with coconut milk and the same bacteria strain was detected from the 1 out of 6 unopened bags of the fresh frozen coconut milk which were consumed by infected persons (Taylor *et al.*, 1993). Taylor *et al.*, (1993) also stated that investigation in Thailand has revealed that the coconut milk extraction has undergone several sanitary violations that suggest the contamination occurred during its preparation.

These poisoning incidences show that the safety of coconut milk is doubtful. Besides, there might be unreported cases, especially in Malaysia for food poisoning related with coconut milk. The processing of coconut milk by the food handlers influences the microbial quality of coconut milk, depending on the hygienic preparation. Thus, the research will conducted to analyse the microbiological quality of fresh coconut milk sold by vendors and to identify whether coconut milk is safe for consumption as well as free from contamination and foodborne pathogens. This will help to investigate whether the microbiological quality of coconut milk adheres to the Malaysian Food Regulations 1985 developed by the MOH.

1.2 Rationale of Study

This study seeks to determine the microbiological quality of fresh coconut milk sampled from selected vendors in Kota Bharu, Kelantan. Based on the cases reported, there were foodborne outbreaks caused by consumption of Thai-style rice pudding served with a topping made from frozen coconut milk.in Maryland, USA (Taylor *et al.*, 1993) and coconut milk dessert consumption in Thailand (Hoge *et al.*, 1995).

In this study, fresh coconut milk was chosen as it is widely used in food products of South East Asia countries such as Malaysia, Philippines and Thailand (Phattayakorn & Wanchaitanawong, 2009). In Malaysia, fresh coconut milk is commonly used in some of the local desserts such as '*cendol*', mango sticky rice and pudding, and in cooking such as curry and '*nasi lemak*'. If the microbial load is high in fresh coconut milk, this will cause serious health problem to the consumers.

The microbiological analyses chosen for this study were total plate count, total coliform count, faecal coliform count and determination of *E.coli*. This is because the presence of high count of either total plate count or total coliform count can be an immediate concern as there will be many foodborne diseases that can spread through the population. Total plate count or known as aerobic colony count is used to determine the sanitary quality of foods and the level of microorganisms present in the food (FDA, 2013). The high number of total plate count indicates low sanitary quality of the food.

Coliforms are bacteria that are found in the digestive systems of warm-blooded animals, in soil, on plants, and in surface water (CDC, 2010). Coliforms are usually a group of Gram-negative, facultative anaerobic rod-shaped bacteria (FDA, 2002).

Coliforms include thermotolerant coliforms and faecal coliforms. Total coliforms serves as a general indicator of sanitary condition in the food-processing environment and *E. coli* detection is used to indicate faecal contamination (FDA, 2002). Hence, it is essential to carry out the detection of the indicator organisms such as *E. coli* to inspect the level of microorganisms in fresh coconut milk and to ensure that fresh coconut milk is free from hazardous microorganisms and safe to consume.

1.3 Objectives

General Objective

To study the microbiological quality of fresh coconut milk sold by selected processed coconut vendors in Kota Bharu, Kelantan.

Specific Objectives

(a) To determine the total plate count and total coliform count in the fresh coconut milk samples.

(b) To determine the faecal coliform and the presence of *Escherichia coli* in the fresh coconut milk samples.

1.4 Research Conceptual Framework

The research conceptual framework is as shown in Figure 1.1. Fifty samples (n = 50) of fresh coconut milk were collected randomly over a period of three months from selected vendors in Kota Bharu, Kelantan. Microbiological analyses were conducted on the samples for total plate count (TPC), total coliform count (TCC), faecal coliform (FC) and *E.coli* detection. The results of microbiological analyses were compared in compliance to the Makmal Keselamatan dan Kualiti Makanan (MKKM) referring to Australian Standard: Methods for the Microbiological Examination of Food (1993). It also must comply with the Malaysian Food Act 1985 by the Ministry of Health, Malaysia. TPC should be below 100, 000 colonies count per gram of sample, TCC below 100 colonies counts per gram of sample and absence of *E.coli* for ready-to-eat foods to be satisfactory and safe for consumption.

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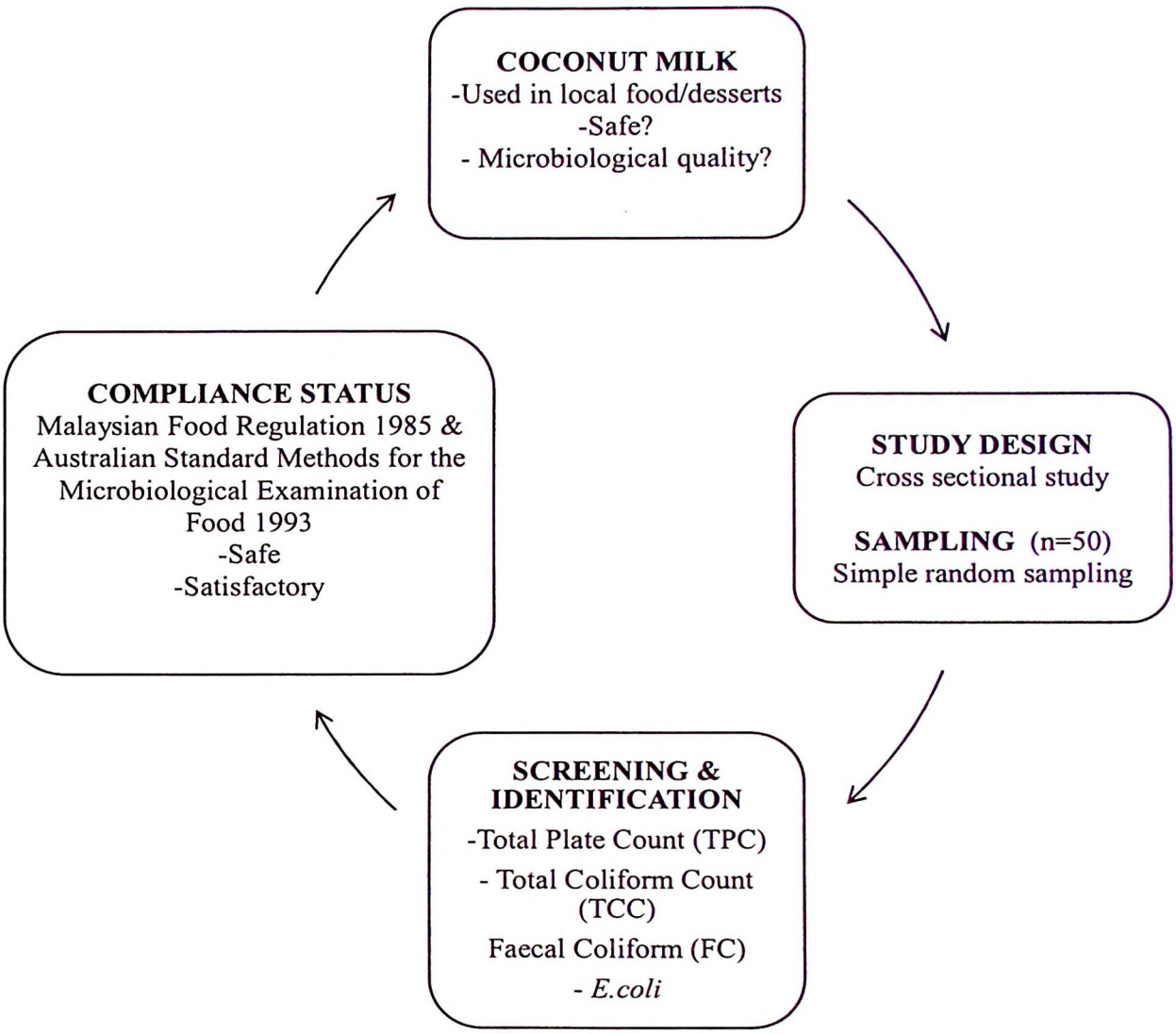

Figure 1.1 Research Conceptual Framework

CHAPTER 2

LITERATURE REVIEW

2.1 Foodborne Disease

Foodborne diseases are defined as those conditions that are commonly transmitted through ingested food (WHO, 2007). Foodborne diseases have increased in the recent years which impacts on both health and economy of developing countries (WHO, 2007). Foodborne diseases are also an important cause of morbidity and mortality worldwide (WHO, 2007). Foodborne diseases comprise a broad group of illnesses caused by enteric pathogens, parasites, chemical contaminants and bio-toxins (WHO, 2007). Foodborne disease can range from mild to serious especially for vulnerable groups such as children. Food poisoning is caused by the oral ingestion of viable or infectious microorganism or the toxins produced by the microorganisms in sufficient amounts which can develop the pathogenesis (Campos *et al.*, 2009).

The top five risk factors for food poisoning in food service operations include contaminated equipment, the improper temperature for storage, poor personal hygiene, inadequate cooking and purchase and receipt of food from unsafe sources (Roberts *et al.*, 2008). Saad *et al.* (2013) reported that food poisoning has been implicated with poor hygiene practices and microbial contamination of pathogens transmitted via food, water, air as well as well as contaminated utensils.

Food poisoning cases in Malaysia are common and have been gradually increasing. Zain & Naing (2002) stated that diseases spread through food still remain a common and persistent problems resulting in appreciable morbidity and occasional mortality. In 2012, food poisoning was the most critical factor in foodborne and

waterborne diseases which contributed to more than 56 incident rates per 100,000 populations of Malaysia (MOH, 2012). The data from MOH showed that there was a significant growth of incident rates for food poisoning cases from the year 2010 to 2011 which increased to 25 times (Saad *et al.,* 2013). MOH Malaysia stated that foodborne diseases in Malaysia were caused due to microbiological hazards and triggered by factors such as improper food handling training, usage of untreated water for non-drinking purposes and low sanitation and hygiene status (Saad *et al.,* 2013).

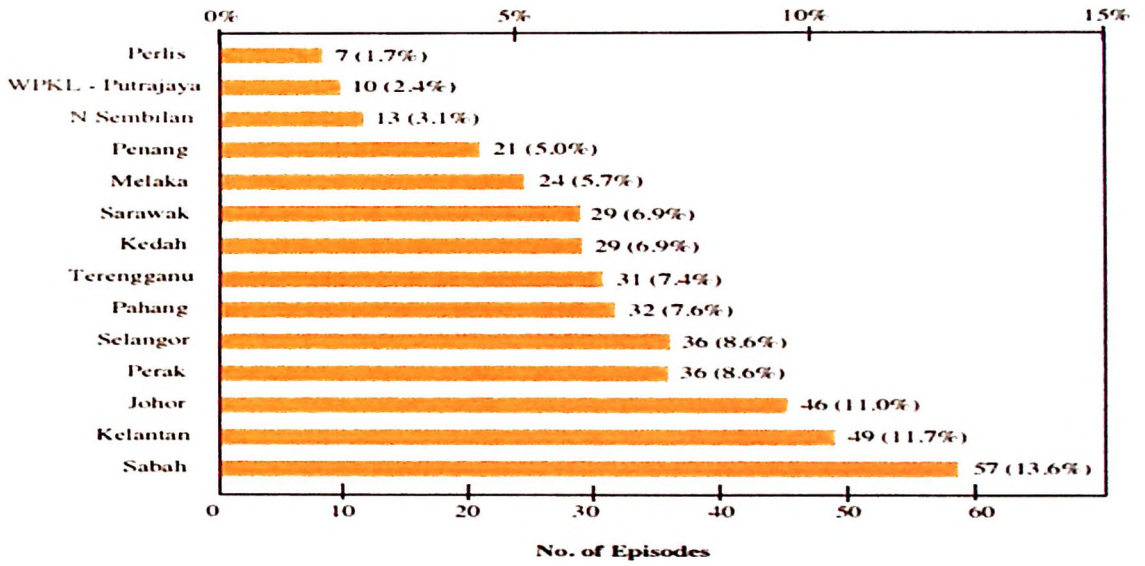

Figure 2.1 Food Poisoning Episode by Malaysia States, 2008

Source: MOH Malaysia (2008)

Data from MOH Malaysia showed that out of the 420 food poisoning episodes reported in 2008, Kelantan was one of the top five states with 49 food poisoning episodes (11.7%) and has the second highest number of cases reported (Figure 2.1). An unpublished data obtained from the Hospital Universiti Sains Malaysia (HUSM), Kubang Kerian, Kelantan showed that between the years 2004 to 2008, there were admission of 2457 patients due to foodborne and waterborne diseases (Noor Izani *et al.*, 2012).

2.2 Ready-To-Eat Food and Safety

Centre for Food Safety (CFS) (2007) defined ready-to-eat food as the food which is ready for an immediate consumption at the point of sale. Ready-to-eat food can be either raw or cooked, hot or chilled, and can be consumed without further heat-treatment including re-heating (CFS, 2007). As described by the FDA food code, ready-to-eat food is in an edible form without prior preparation such as washing or cooking by food handlers or consumers (FDA, 2013). Ready-to-eat foods are include chilled and frozen foods, whole or cut fruits and vegetables which can be raw or cooked and prepared beverages such as flavoured iced drinks, fruit juice and '*cendol*' by the street vendors.

Ready-to-eat foods have increased in terms of consumption regardless of any age number and profession due to their convenience and minimal preparation is required. Besides, ready-to-eat foods are affordable and can be obtained easily especially from food outlets or stalls. Various types of ready-to-eat foods are sold as street foods and include the community's staple food which can be nutritious and contributes to the elevation of health.

However, the safety of ready-to-eat food has become one of the major concerns for public health, since the potential for unsafe or unsanitary food handling by mobile food vendors is substantial (WHO, 2010). It was shown that raw ready-to-eat foods prepared or sold by street vendors have been recognized as potential vehicles of microbial foodborne bacteria such as *Salmonella* and entero-pathogenic *E.coli* (Campos *et al.*, 2015).

2.3 Contamination Sources of Ready-To-Eat Food

Potential sources of contamination of ready-to-eat food must be considered and investigated. Microbial cross-contamination is the transfer of direct or indirect, microorganisms from a contaminated item to a non-contaminated item (Minnesota Department of Health, 2007). Cross contamination of foodborne pathogens is a major concern as it increases the risk for humans to ingest contaminated food and become ill (Erickson *et al.*, 2015). Domestic food contact surfaces and utensils are also considered as contributing factors of transmission and the occurrence of sporadic foodborne disease (Erickson *et al.*, 2015).

According to Nerín *et al.*, (2016), the sources involved in food contamination include external raw food contamination due to environmental contamination, food conditioning which involves storage and cleaning and food packaging. Contamination of food can occur and consequently increase public health risks which include the improper storage of perishable foods, money and food handling by the same person, low frequency of hand hygiene, and lack of vendors' compliance with food safety training as well as scarcity of sanitary and proper waste disposal facilities (Cortese *et al.,* 2016).

Soon *et al.,* (2011) stated that unsanitary food handling process in Malaysia has caused more than 50% of poisoning episodes. Rosmawati *et al.,* (2015) stated that food stored under ambient temperature, wet utensils which can favour microbial growth. Dirty dish cloths used to dry utensils increases the cross contamination of food. In Asian countries including Malaysia, the sale of food in environments where live animals are exposed, such as wet markets, certainly increases the possibility of contamination (Barbosa *et al.*, 2016).

2.4 Coconut Milk

Coconut milk is an example of ready-to-eat food. Coconut milk is the liquid obtained by manual or mechanical extraction of the coconut endosperm (Phattayakorn & Wanchaitanawong, 2009). Narataruksa *et al.* (2010) stated that coconut milk is the liquid obtained by mechanical force of coconut flesh normally with added water. The percentage of fat in coconut milk is adjusted between 15% and 40% depending upon local requirement (Narataruksa *et al.*, 2010).

According to the Malaysian Food Act 1983 and Regulations 1985, coconut milk is categorised under nut and nut product; where it shall be the emulsion extracted from fresh, sound, the ripe kernel of the fruit of *Cocos nucifera*. Coconut milk shall contain not less than 30% of fat and 3% of protein (Malaysian Food Act 1983 and Regulations 1985). Malaysian Food Act 1983 and Regulations 1985 also stated that coconut milk shall not contain more than 55% of water and free from kernel residue and it may also contain permitted food conditioner and preservatives

Coconut milk is widely used in food products of Asian Pacific (Narataruksa *et al.*, 2010) and South East Asia countries including Indonesia, Malaysia, Philippines and Thailand (Phattayakorn & Wanchaitanawong, 2009). Coconut milk is known in Malaysia as '*santan*' and used as a main ingredient in many Malaysian food such as curry, noodles, '*nasi lemak*' and local desserts such as 'cendol', mango sticky rice and sago pudding. Coconut milk has been used in Malaysian cuisines for generations as thickener source and perfect harmonies blend of flavour and aroma (Marina & Nurul Azizah, 2014).

2.5 Coconut Milk Contamination Factors

According to Li & Schneider (2016) coconut is categorized under nut product, which is protected from microbial invasion due to the thick shell and sterile internal wall.

Schaffner *et al.* (1967) stated the processes of contamination of coconut milk starts from shelling out the coconut shells, removing the endosperm and coconut milk extraction. Ripe coconut which falls to the ground has the risk of shell contamination due to animal faeces as manures are used to fertilize the palm fields (Schaffner *et al.*, 1967). Schaffner *et al.*, (1967) also stated that coconut can support microbial growth when the cracked coconut shell causes the leaking of coconut water which can contaminate the coconut flesh.

Shelled coconuts contain many bacteria including coliforms and faecal coliforms (Schaffner *et al.*, 1967). Improper washing of the coconut flesh will cause the microbial increase. The microbial count also will increase during the coconut milk processing which includes contaminated equipment and utensils while preparation and if proper sanitary is not followed (Schaffner *et al.*, 1967).

The extraction of coconut milk involves the step removing the shells and paring of the mature coconuts and the extracted coconut flesh is washed, drained and grated by machine or manually (Seow & Gwee, 1997). After coconut milk is extracted, it must be stored at proper temperature which is at 4°C as room temperature can increase the microbial load of coconut milk. Coconut milk spoils rapidly even at cold temperatures, however, the microbial load is lesser than the storage at room temperature.

Coconut milk is a very rich medium which can support the growth of all the common spoilage microorganisms which are introduced via contaminated shells, processing equipment and utensils as well as handlers (Seow & Gwee, 1997). Mabesa & del Rosario (1979), found the common types of bacteria in coconut milk includes the genus *Bacilli* and coliform bacteria (Seow & Gwee, 1997).

2.5 Poisoning Related to Coconut Milk

In Malaysia, there aren't any cases of food poisoning reported regarding the consumption of coconut milk directly. There were cases with consumption of food cooked with coconut milk such as curry and '*nasi lemak*', however, the source of food poisoning or other factors were not specified. On April 2015 in a religious school in Klang Valley, 80 students, teachers and principal were hospitalized due to '*nasi lemak*' consumption. (My Metro, 2015). Recently, on April 2016 in a secondary school in Tapah, Perak, 43 students and a teacher were rushed admitted to hospital after they consumed chicken curry (My Metro, 2016). The curry was contaminated because unhygienic preparation and improperly stored according to the Perak State Health Director, (My Metro, 2016).

In 1991 at Thailand, there was an emergence of nalidixic acid resistant *Shigella dysenteriae* Type 1 in a local community and was traced to a point source outbreak at a local school (Hoge *et al.*, 1995). This is due to a foodborne outbreak caused by the consumption of coconut milk dessert in a local elementary school. Hoge *et al.*, (1995) stated that the dessert was prepared in an unhygienic way which involved kneading the

grated coconut by hand without wearing plastic gloves and there was no policy of handwashing practiced by the food handler.

In Maryland, USA on August 1991, there were 3 cholera cases outbreak which were associated with consumption of Thai-style rice pudding served with a topping of the fresh frozen coconut milk and the coconut milk was imported from Thailand (Taylor *et al.*, 1993). Taylor *et al.*, (1993) showed that the coconut milk extraction has violated the sanitary guidelines while the processing of the coconut.

CHAPTER 3

METHODOLOGY

3.1 Study Design

This is a cross-sectional study. Ten (10) coconut vendors were randomly selected from wet markets in Kota Bharu, Kelantan and five (5) samples from each vendors were used for analysis.

3.2 Materials and Equipment

The materials and equipment used are sterile plastic bags, petri dish, pipette tips (blue and yellow), universal container, biohazard bag, ice pack, Duran bottles (200mL, 500mL and 1000mL), measuring cylinder, beaker, test tubes, hockey stick, wire loop, Bunsen burner, pipette, ice box, stomacher, autoclave machine, Biological Safety Cabinet (BSC) Level II, incubator at various temperatures (32°C, 37°C and 44.5°C), water bath, electronic weighing balance, 70% alcohol, sterile distilled water,

3.3 Media and Chemical Preparations

The media used in this study were nutrient agar powder, MacConkey agar powder, Eosin Methylene Blue (EMB) agar powder, peptone water powder, tryptone water powder and Kovac's reagent. The preparation of the media were as described in Appendix A.

3.4 Microorganisms

The microorganisms required as control organisms in this study were *E.coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 33495 and *Staphylococcus aureus* ATCC 25923.

3.5 Sample Collection

The samples used in this study consisted of fresh coconut milk. 200 mL of samples were purchased from vendors in wet markets in Kota Bharu, Kelantan and placed into sterile plastic bags and sealed. They were stored and transported to the laboratory in an ice box at below 5°C for maintenance of samples condition and quality as well as to prevent spoilage. The samples must be transported to the laboratory within four hours and analysed immediately after reaching the laboratory.

3.6 Sample Preparation

Twenty-five gram (25 g) of each sample was weighed and transferred into sterile plastic bag which contains 225 mL of 0.1% peptone water as diluent. The plastic bag was placed into the stomacher. The sample was homogenized by using stomacher to produce the homogenized solution. Serial dilutions of 10^{-2} to 10^{-6} were prepared for the determination and enumeration of total plate count, total coliform count and faecal coliform count and *E.coli* determination.

3.7 Microbiological Analysis

Spread plate method was used for enumeration of microorganisms by counting the colonies which grew on the plate. Prior to culturing, the plates were labelled with sample code number and dilution factor. Then 100µL of diluted sample solution were pipetted onto triplicate plates on the culture medium for each sample; 10^{-2} to 10^{-6} dilutions. A hockey stick was used to evenly spread the diluted solution. The plates were then incubated at the specified temperatures. After incubation, all the colonies that grew on the plate were considered for the enumeration of microorganisms. Plates with less than 50 or more than 250 colonies will not be counted. Each colony forming units (CFU)

represents a bacterium that was present in the sample. The flow chart of the study is as shown in Figure 3.1.

3.71 Total Plate Count

The serial diluted samples were streaked on nutrient agar for the total plate count. For total plate count, nutrient agar was incubated at 32°C for 24-48 hours. Following incubation, all colonies which grew on the agar were counted for total plate count. The colonies count below 100 000 were classified as satisfactory and colonies count more than 100 000 were classified as unsatisfactory.

3.72 Total Coliform Count

The medium used promotes the growth of Gram-negative bacteria and able to distinguish lactose fermenter and non-lactose fermenter microorganisms. Incubation at 37°C is done as it is equivalent to human body temperature, thus, only human pathogenic bacteria can grow after 24 hours incubation. The colour of the growing colonies depends on their lactose fermenting properties. Strong lactose fermenter microorganisms will appear in red or pink colonies and non-lactose fermenter microorganisms will appear as pale yellow or colourless colonies.

For total coliform count, the serial diluted samples were streaked on MacConkey agar and incubated at 37°C for 24-48 hours. Any pink to red colonies observed were counted, indicating the total coliform count. The positive control and negative control used were *E.coli* ATCC 25922 and *S. aureus* ATCC 25923, respectively. The colonies count below 100 was classified as satisfactory and colonies count above 1000 was classified as unsatisfactory.

3.73 Faecal Coliform (*E.coli)*

E.coli is detected by isolation from MacConkey agar incubated at 44.5°C as *E.coli* is a strong lactose fermenting thermotolerant bacterium. *E.coli* grow as bright pink or red colonies and demonstrates strong lactose fermentation indicated by bright pink halo, bile precipitant around the colonies (Appendix B).

The serial diluted samples were streaked on MacConkey agar and incubated at 44.5°C for 24-48 hours. If there were any presence of pink or reddish colonies, the colonies will be counted. Then, at least three pink to red colonies will be streaked on EMB agar and incubated at 37°C for 18-24 hours for confirmation of *E.coli*. This step is essential to distinguish *E.coli* from other lactose fermenting microorganisms such as *K. pneumoniae*.

On the EMB agar, the presence of typical nucleated dark centered with green metallic sheen colonies indicates the presence of *E.coli*. However, other microorganisms such as *Citrobacter* and *Enterobacter* also displays the distinctive green metallic sheen properties. Hence, the biochemical test indole is conducted to distinguish the *E.coli* bacteria which is able to cleave indole from tryptophan.

For confirmation of *E.coli*, three typical green metallic sheen colonies were chosen and sub-cultured in tryptone water at 44.0-44.5°C for 24 hours before carrying out the indole test. For indole production, five drops of Kovac's reagent was added into the tube. A positive indole test is interpreted by the formation of cherry-red ring and negative indole is interpreted by yellow ring formation. Hence, positive indole test is confirmative of *E.coli*.

The estimated number of *E.coli* depends on the number of the green metallic sheen colonies shows the positive indole test for *E.coli*. For example, if 1 out of 3 colonies shows positive indole test and there were 9 faecal coliforms colonies counted, the estimated number of *E.coli* will be 3 colonies.

The positive control and negative control used were *E.coli* ATCC 25922 and *K. pneumoniae* ATCC 33495, respectively. The absence of *E.coli* indicates satisfactory microbiological quality and presence of *E.coli* indicates unacceptable microbiological quality.

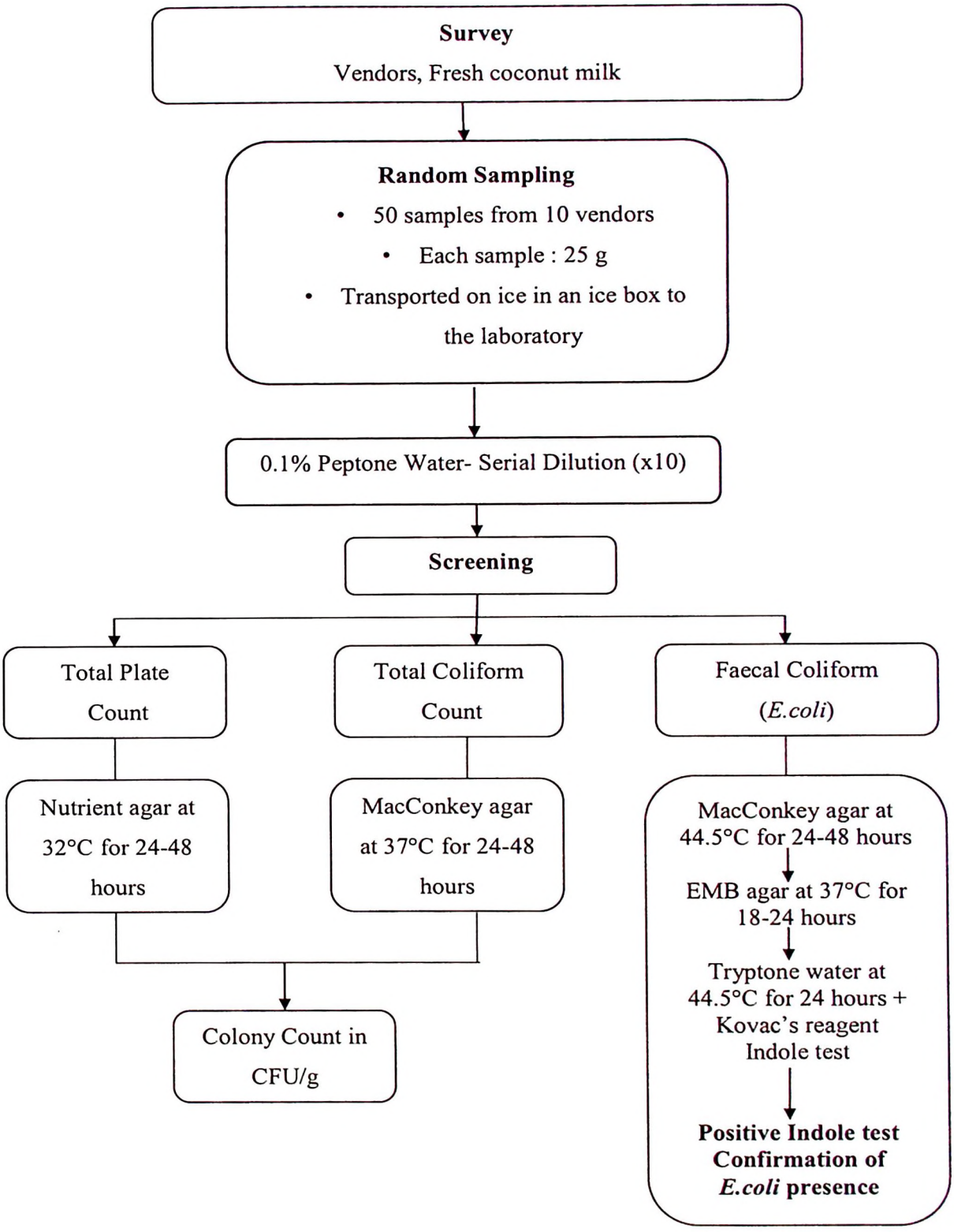

Figure 3.1 Flow Chart of the Study

3.8 Colony Counts

Colonies which grew after the incubation on nutrient agar at 32°C and MacConkey agar at 37°C were calculated as described below. It is expressed as number of bacteria per mL of serially diluted bacteria. The CFU/mL was converted into CFU per gram to determine the number of bacteria in one gram of the sample.

$$\text{CFU/g} = \frac{\text{Number of CFU} \times \text{Dilution Factor} \times \text{Volume Plated} \times \text{Total Volume}}{\text{Mass of Sample}}$$

3.9 Microbiological Quality Guidelines of Fresh Coconut Milk

The microbiological quality guideline of ready-to-eat foods was used to determine the satisfactory and unsatisfactory levels of fresh coconut milk. Compliance to the microbiological quality is provided by the Makmal Keselamatan dan Kualiti Makanan (MKKM), Kelantan based on the Australian Standard Methods for the Microbiological Examination of Food (1993). The guideline is tabulated in Table 3.1.

Two categories of microbiological quality which are satisfactory and unsatisfactory indicate the level of the coconut milk vendors' hygiene practices. Satisfactory results are indicated by hygiene and good microbiological quality and no action is required. Conversely, unsatisfactory results indicates that the microbiological quality falls outside the acceptable safety limits; which is suggestive of poor quality of hygiene practices among the coconut milk vendors'

Table 3.1 Guidelines of Microbiological Recommendation for Ready-to-Eat Foods

Indicator Test	Indicator Test Results (CFU/g)	
	Satisfactory	**Unsatisfactory**
Total Plate Count	< 100 000	≥ 100 000
Total Coliform Count	< 100	≥ 1000
E.coli	Absence	Presence

Source: Makmal Keselamatan dan Kualiti Makanan (MKKM), Kelantan (adapted from Australian Standard: Methods for the Microbiological Examination of Food, 1993)