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Microbial Safety of Local Dried Fruit, *Garcinia atroviridis* (Asam Keping)

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DECLARATION

I hereby declare this thesis entitled "Microbial Safety of Local Dried Fruit, *Garcinia atroviridis* (Asam Keping)" is the results of the original research except that I have cited in the references.

hawa sukri

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Microbial Safety of Local Dried Fruit, *Garcinia atroviridis* (Asam Keping)

ABSTRACT

Food safety was a global issue that affects food production, processing and human consumption. Given that the drying process of foods enables to prohibit microbial growth. However, the spike number of foodborne cases worldwide that related to low moisture food caused by pathogens presence recently have proved that pathogenic microorganisms can persist for long periods in dried foods even under low water activity conditions. The study assessed the microbiological safety of local dried fruit that is frequently used for human consumption which is *Garcinia atroviridis* or locally known as asam keping (AK). This product may be subjected to a wide spectrum of microbial contamination during pre-and post-harvest and they can display high microbial counts due to conventional production. Four different media growth were used for microbial evaluation on the samples, to determine the presence of total aerobic count (NA), total *Salmonella spp*. count (SSA), *B. cereus* (BCA) and *E. coli* (EMBA). The analysis has shown all three AK sample has exceeded the limit of microbial content for total aerobic count (TAC) (< 10^6 CFU/mL) and *B. cereus* counts (< 10^4 CFU/mL) where AK 3 shows the highest contamination rate with 24.5×10^7 CFU/mL for TAC and 5.00×10^5 CFU/mL for *B. cereus* count. While there were no detection of *Salmonella spp.* and *E. coli* presence for all AK samples. The presence of focused pathogenic organisms in these dried fruit products indicates that consumers may be exposed to a public health risk. Hence, proper implementation of GMPs and personal hygiene are in need of improvement, particularly for conventional or home-based food production.

Keyword: Food safety, low water activity, microbiological safety, asam keping, media growth

Keselamatan Mikrob Buah Kering Tempatan, *Garcinia atroviridis* (Asam Keping)

ABSTRAK

Keselamatan makanan adalah isu global yang menjejaskan pengeluaran makanan, pemprosesan dan penggunaan manusia. Memandangkan proses pengeringan makanan membolehkan untuk melarang pertumbuhan mikrob. Walau bagaimanapun, peningkatan bilangan kes bawaan makanan di seluruh dunia yang berkaitan dengan makanan lembapan rendah yang disebabkan oleh kehadiran patogen baru-baru ini telah membuktikan bahawa mikroorganisma patogen boleh bertahan untuk tempoh yang lama dalam makanan kering walaupun dalam keadaan aktiviti air rendah. Kajian ini menilai keselamatan mikrobiologi buah kering tempatan yang kerap digunakan untuk kegunaan manusia iaitu *Garcinia atroviridis* atau tempatan dikenali sebagai asam keping (AK). Produk ini mungkin tertakluk kepada spektrum luas pencemaran mikrob semasa pra-dan selepas tuaian dan ia boleh memaparkan jumlah mikrob yang tinggi disebabkan oleh pengeluaran konvensional. Empat pertumbuhan media yang berbeza digunakan untuk penilaian mikrob pada sampel, untuk melihat kehadiran jumlah kiraan aerobik (NA), jumlah *Salmonella spp.* kiraan (SSA), *B. cereus* (BCA) dan E. coli (EMBA). Analisis menunjukkan ketiga-tiga sampel AK telah melebihi had kandungan mikrob bagi jumlah kiraan aerobik (TAC) ($< 10^6$ CFU/mL) dan kiraan *B. cereus* ($< 10^4$ CFU/mL) di mana AK 3 menunjukkan kadar pencemaran tertinggi dengan 24.5×10^7 CFU/mL untuk TAC dan 5.00×10^5 CFU/mL untuk kiraan *B. cereus*. Sementara itu, tiada pengesanan *Salmonella spp*. dan kehadiran *E. coli* untuk semua sampel AK. Kehadiran organisma berpotensi patogen dalam produk makanan kering ini menunjukkan bahawa pengguna mungkin terdedah kepada risiko kesihatan awam. Oleh itu, pelaksanaan GMP dan kebersihan diri yang betul memerlukan penambahbaikan, terutamanya untuk pengeluaran makanan konvensional atau berasaskan rumah.

Kata kunci: Keselamatan makanan, aktiviti air rendah, keselamatan mikrobiologi, asam keping, pertumbuhan media

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LIST OF ABBREVIATION

No	Abbreviation	Meaning
1.	AK	Asam keping
2.	AOAC	Association of Analytical Communities
3.	BCA	*Bacillus cereus* Agar
4.	BGA	Brilliant Green Agar
5.	BWP	Buffered Peptone Water
6.	CD	Convective Drying
7.	CFU	Colony Forming Unit
8.	DNA	Deoxyribonucleic acid
9.	DOA	Department of Agriculture Malaysia
10.	EMBA	Eosin methylene Blue Agar
11.	FAO	Food and agriculture Organization
12.	FCA	Faecal Coliform Agar
13.	FD	Freeze Drying
14.	GHP	Good Hygienic Practices
15.	GMPs	Good Manufacturing Practices
16.	H_2S	Hydrogen sulfide
17.	HACCP	Hazard Analysis Critical Control Point
18.	HCA	Hydroxyl Citric Acid
19.	HP	Heat Pump Drying
20.	LDL	Low Density Lipoprotein
21.	LMF	Low Moisture Food

22.	OD	Osmotic Drying
23.	PCA	Plate Count Agar
24.	PDA	Potato Dextrose Agar
25.	PIF	Powdered Infant Formula
26.	RCA	Reinforced Clostridial Agar
27.	RNA	Ribonucleic Acid
28.	RTE	Ready-To-Eat
29.	SD	Spray Drying
30.	SD	Standard deviation
31.	SE	Staphylococcal enterotoxin
32.	SFD	Staphylococcal food borne disease
33.	SPC	Standard Plate Count
34.	SSA	*Salmonella-Shigella* Agar
35.	TAC	Total aerobic counts
36.	TNTC	Too numerous to Count
37.	VRBA	Violet Red Blue Agar
38.	WHO	World Health Organization

LIST OF SYMBOLS

No	Symbol	Meaning
1.	g	Gram
2.	ml	Millilitre
3.	%	Percentage
4.	^{o}C	Degree Celsius
5.	≤	Less than or equal to
6.	>	Greater than
7.	μL	Microlitre
8.	±	Plus/minus
9.	×	Times

CHAPTER 1

1.0 INTRODUCTION

Dehydration or drying is the process to preserve food by lowering and removing water content from food products by evaporation in order to produce a final product in solid form. According to Codex Alimentarius International Food Standards, dehydration refer to low moisture food (LMF) that contain 0.85 a_w of water content or below. This method enables to cease the microbial growth because biological reaction cannot take place in low water conditions. This category encompasses a broad variety of food product such as dried fruits and vegetables, animal feeds, pet foods, cereal, flour, egg powder, powdered infant formula, herbs, spices and condiments (Beauchat, 2016). Despite the fact that low moisture foods have bring advantages in terms of food safety, but still there are some major concerns. Recently, foods product and food ingredients with low water activity (a_w) have been reported involved as a vehicle for pathogens that have triggered illness outbreaks (Beuchat, 2013).

Besides, food processing environment of dried foods production also play a major cause in inhibit microbial growth especially in cleaning and sanitizing process that often conducted using a wet procedure. Next, is consumer assumption on low moisture food is been sterile during handling process. Thus, this issue can increase mishandling or poor practices such as temperature abuse at any stage between final retail or consumption that may contribute to contamination (FAO/WHO, 2014). For example, storage of infant formula at ambient temperature for long periods of time, allowing pathogens like *Bacillus cereus* and *Cronobacter* species to grow (Beuchat, 2013). According to previous research by Byry-Bredbenner (2013), it stated that the primary locations where most foodborne illness cases began to occur is home. Although, still many did not realize that this place as a major factor for foodborne illness.

As issues mentioned above, food safety has become a global concern. In recent years, the presence of food-borne pathogens in low water activity (a_w) foods has caused spike in number of outbreaks (Ijabadeniyi, 2017). As a consequence of the increased outbreaks, there is a sparked interest and urgency to identify the possible of pathogens growth in LMF. Thus, it can enable producer and consumer to understand the possible causes of pathogen presence in low moisture foods.

Low moisture food consists of ≤0.85 of water activity (a_w). As for asam keping, the water content is traditionally lowered by evaporation (sun drying) that may give a negative impact on low quality products where a significant number of microorganisms may be able to survive during the dehydration process. It can contribute as a threat to consumer when the pathogenic microorganism has proliferated in sufficient number and persist after drying process. Moreover, the growth of surviving organisms can be promoted and increase the risk of human infection if low water activity food products is used to prepare food with a high final water activity (Bourdoux, 2016).

According to Code of Hygiene Practice for Low Moisture Foods, 2015 has stated that *Salmonella spp*. and *Bacillus cereus* are the two most common pathogens known as illness contributor that related to low moisture foods and has been reported, *Salmonella spp* has identified as major causes of outbreak-related illnesses related to LMF. According to previous research related to microbiological quality of selected dried fruits and vegetables in Maseru, Lesotho, pathogens from the genera *Salmonella, Shigella, Bacillus* and other *Enterobacteriaceae* were determine as possible pathogenic organisms that presence and isolated from home dried fruits and vegetables (Victor, 2016).

Dried food products like fruits, vegetables, herbs and spices are widely produced in and sourced worldwide, but the presence of foodborne pathogens like *Salmonella spp.* has triggered to outbreaks. In Malaysia, *Garcinia atroviridis* or locally known as asam keping are mainly produced conventionally where the sliced fruits have been dried in open environment and directly under the sunlight.

It is widely used by Malaysian for culinary purposes as a seasoning or a sour relish. But it still lacks of commercial processing where the majority of asam keping production is still depends or dehydrated using product sun drying method. In this research, asam keping samples from three different location of street market were taken around Jeli, Kelantan and were analyzed in terms of the microbial load of *Salmonella spp.*, *Bacillus cereus* and *Escherichia coli* in the sample by isolate and enumerate using selective media. The research was conducted at University Malaysia Kelantan, Jeli Campus.

1.1 PROBLEM STATEMENT

Traditional sun drying is a time-consuming process that can lead to microbial contamination on dry food products that caused by haze, high humidity (rain), wind-blown debris, weather uncertainty, insect, rodent and bird infestations. This can contribute to poor quality of final dry food product in terms of color, nutritional composition, biological reaction and hygiene (Karam, 2016). Sunlight and wind worked as a convective agent to supply heat to the material. Its reliance on the climate, the time it takes to dry and the poor quality of the final products has become the key disadvantages of sun drying process (Bourdoux, 2016). The final quality of asam keping is difficult to control because the thermal processing was proceeding in open environment (Hanim, 2020).

Next, it has been reported in previous study that foodborne outbreaks associated to dried fruits and vegetables are mostly happens in North American and Europe (Bourdoux, 2016). *Salmonella spp.*, *Bacillus cereus*, *Clostridium perfringens* and *Hepatitis A virus* (for semidried tomatoes only) have been reported as the most common foodborne pathogens associated with these dried foods because they be able to survive and grow in low-water activity food products. Proper hygiene practices are essential when dehydrating foods to reduce the risk of pathogens and spoilage microorganisms contaminating the product.

Furthermore, dried food has been desirable and demandable for the consumer due to their extended shelf life**.** Low a_w in dried food has led to consumer perception that microorganism cannot proliferate in that conditions. However, previous study has proved that although microorganisms cannot growth in low a_w but it still can survive for longer

time (Ijabadeniyi, 2017). As mentioned above, pathogenic microorganism like *Salmonella spp*. is responsible and known as major contributor of illness outbreaks linked to LMF products (finn, 2013). It can persist for long periods of time in harsh and dry conditions and regularly found in raw ingredients (Podolak, 2010).

1.2 HYPOTHESIS

H_0: The pathogen presence in asam keping samples does exceed the limit of food standard.

H_1: The pathogen presence in asam keping samples does not exceed the limit of food standard.

1.3 OBJECTIVE

1) To determine the presence of foodborne pathogen of *Salmonella spp., Bacillus cereus* and *E. coli* in asam keping samples.
2) To determine the microbial safety in asam keping samples has not exceed the limit of food standard.

1.4 SCOPE OF THE STUDY

The following are the scopes of this research to support the above-mentioned objectives:

Asam keping is traditionally produced and widely used in Malaysian cooking. However, quite number of reported cases related to foodborne outbreaks by pathogenic organism associated with low moisture food is worrisome since it can affect consumer health. Therefore, in this study determination of focused pathogenic bacteria in asam keping samples will be determine by isolation and enumeration using different selective media depend on organism tested. Where the finding of pathogen presence in this research can improve the further production of asam keping and food safety in our country. Based on objective one, the total of microbial presence (CFU/mL) in asam keping samples that taken from street market were identified either amount of microorganism presence

complies with the limit specification or not. Meanwhile, the time and duration for this research were conducted within 3 months started from the collection of samples.

1.5 LIMITATIONS OF STUDY

The study aims to determine the microbial safety and the presence of pathogenic bacteria of *Salmonella spp, Bacillus cereus* and *E. coli* in asam keping samples. However, there was area limitation occur in collective the sample due to Covid-19 outbreaks that limiting the area for sample collection activities from actual place where the study will be conducted. Also, fruit season constraint for *Garcinia atroviridis.*

1.6 SIGNIFICANCE OF THE STUDY

According to Food and Agriculture Organization (FAO), food safety refers to how we handle, store and prepare food in order to avoid contamination and to ensure that our food contains enough nutrients that required for our healthy diet. In recent year, we can see significant report regarding foodborne infection of pathogenic microorganism towards low moisture foods (LMF) worldwide. Therefore, it proves that consumer

perception towards food products with low water activity (a_w) are able to inhibit the proliferation of microorganism due to their extended shelf life is deniable. Whereas, there are various of study has stated that microorganism is still capable to survive in lack of water conditions (Pillay, 2017). Although, in this type of foods characteristic, pathogen microorganism cannot growth but there is a possibility they will remain around for a long time.

This research was to determine the focused pathogenic bacteria that presence in Malaysian common foods ingredient which is asam keping and determine either the microbial safety in this product has meet the limit of food standard or not. Thus, the finding from this research are able to emphasize the significance and requirement of proper processing, food safety and hygiene practices especially for home-based producer of dried fruits and vegetables to hinder the foodborne outbreaks associated by pathogenic infection.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 *Garcinia atroviridis*

Asam keping or its botanical name is *Garcinia atroviridis*. The etymology of this genus name of Garcinia is generated from French botanist, Laurent Garcin. Meanwhile, the species name is a combination of Latin adjectives which is Atroviridis that means very dark color of leaves. This plant is belonging to Clusiaceae family. It is shown in Table 2.1 the scientific classification of *Garcinia atroviridis*. This plant can be found abundance in the Southeast Asia countries such as Malaysia, Thailand, Indonesia, India and Philippines. It is also cultivated in other regions of the world. According to herbs and spices statistic issued by Department of Agriculture Malaysia (DOA) in year 2019, only certain state in Malaysia that known as producer of asam keping such as Johor, Kedah, Kelantan, Pahang and Perak.

Table 2.1 shows the scientific classification of *Garcinia atroviridis.*

Kingdom	**Plantae**
Order	Malpighiales
Family	Clusiaceae
Genus	Garcinia
Species	*G. atroviridis*

Table 2.2 shows hectarage, production and value of production asam keping by state.

State	**Asam gelugor**		
	Planted area (Ha)	Harvested area (Ha)	Production
Johor	1.40	1.40	0.82
Kedah	18.36	18.36	80.73
Kelantan	14.58	12.42	56.82
Pahang	2.08	1.98	6.71
Perak	73.09	55.09	212.86

Source: Department of Agriculture (2019).

The common name used for this species also differ based on countries such as in Malaysia, its known as asam keping, assam gelugor or asam gelugo. In Indonesia, commonly called as asam potong or asam keping. Meanwhile, in Thailand known as som khaek, ma kham khaek or som pha ngun. The genus garcinia is a genus of the family Clusiaceae that comprises about 250 species in the tropics that commonly found in Indo-Malesia such as *G. mangostana* and *G. intermedia. G. atroviridis* is a fruit-bearing tree that is both ornamental and has perennial lifespan. This species is easy to adapt to climate in subtropical or temperate country that have temperature range of 33-45°C and the plant, however, can withstand cool nights for a short period of time to produce edible fruits in favourable conditions. Prolonged cold weather is not suitable for this species because it can affect to stunted growth, slows the level of maturity and affect the flavour of fruit too acidic.

The asam keping tree is a single-leaved, large, elongated oval, dark green tree with a shiny top surface that grows to a maximum height of 20 meters (Halim et al., 2018). The young shoots or leaves of asam keping is pinkish in colour and will turn to a dark green colour when it reaches to maturity level. Meanwhile, the colour of cultivated fruit is bright green and turns yellowish yellow when it ripens with normal size of 6.5–10 cm wide and the seeds is strong flattened. The shape of asam keping fruit differs depending on the variety, which can be round, oval, or oblong. Its fruits are 100–700 g in weight and have 12–16 fruit curves.

Figure 2.1 shows the leaves, flower and fruit of *Garcinia atroviridis.*

2.1.1 Processing of asam keping

In Malaysia, asam keping is widely used especially in culinary and traditional medicinal. However, it is still less commercially produced especially in rural areas. This drying process of asam keping is depending a lot on nature which is heat from sunlight. If the scorching heat persists, wet slices of asam keping will only dry out within 7-12 days but if the weather is not good, it will take longer for asam keping to dry out and reach specific amount of moisture content. Though, in this traditional drying method, the actual moisture content in asam keping is difficult to control due to uncontrolled environment.

Figure 2.2 shows the slices of asam keping before going through the drying process

To perform the drying process, only ripe asam keping fruit with yellow colour will be selected. The selection of young fruit that is green in colour should be avoided to keep and control good quality of the finished asam keping. Selected asam keping fruit must be washed first until clean and all the impurities attached were removed and tossed. The clean fruit will be sliced using a knife with a slice thickness between 2.0-3.0 cm as shown in Figure 2.2 above. Each slice should be done uniformly. The seed, stalk and lower part of the fruit should be removed. The sliced fruit will be arranged on a flat surface for the drying process. The arrangement of the fruit should be avoided from overlapping to ensure that each slice gets enough heat from sunlight to dry. Slices of asam keping that are arranged can be dried in the sun until there is a change in colour such as dark yellow, brown or somewhat blackish according to the needs and desires of the market. The dried

slices of asam keping need to be monitor and flipped several times to ensure that all parts dry evenly. Asam keping that has been fully dried should be collected to check the quality level before it is sent to the market. Quality inspection for this low moisture food product is important because the drying process is carried out under uncontrolled environment.

2.1.2 Traditional medicinal Uses

The fruit and leaves are the edible parts of this plant for a variety of uses such as in food, beverages and medicinal. Traditionally, *G. atroviridis* leaves and roots are used to overcome dandruff, cough and leaves also used to make a juice for woman after childbirth (Lim, 2015). It is also used as pre or postpartum cure for stomach-ache because of pregnancy and used as a lotion after confinement to rub it over abdomen (Hamidon et al., 2017). Meanwhile, fresh fruit or dried one are widely used in culinary as a seasoning or sour relish. Furthermore, the consumption of fruit extract has been used to promote health by lowering blood cholesterol levels and expanding blood vessels, as well as reducing stress and increasing bowel movements.

2.1.3 Natural product of antiobesity activities

This plant contains a number of phytochemicals, including flavonoids and organic acids. Hydroxy citric acid, or more specifically (-)-hydroxy citric acid, has been described as a possible supplement for weight loss and as an antiobesity agent among all organic acids in this plant due to the availability of hypolipidemic activities (Chuah et al., 2013). Refer to Figure 2.3 shows the compound of hydroxycitric acid that originated from this plant genus and widely used in weight lose management (Hamidon et al., 2017). This HCA enables to lower the appetite by reducing de novo lipogenesis and increasing glycogen development. The potassium hydroxy citrate from *G. atroviridis* fruit juice was once used for lowering body weight and cholesterol level in rats. The low-density lipoprotein (LDL) or bad cholesterol level as well as body weight were successfully lowered as a result of the treatment. Meanwhile, high density lipoprotein (HDL) or good cholesterol levels, on the other hand, were increased. Thus, the result showed and agreed that extract from *G. atroviridis* fruit had lipid modulating properties on the animal model used. According to previous study conducted in Thailand on 25 obese women has proved that the consumption of water-soluble calcium hydroxy citrate (HCA) from *Garcinia atroviridis* resulting to significant weight loss within two months. HCA is a non-toxic substance that enables to help reduce and regulate appetite as well as decompose fat cells in the body.

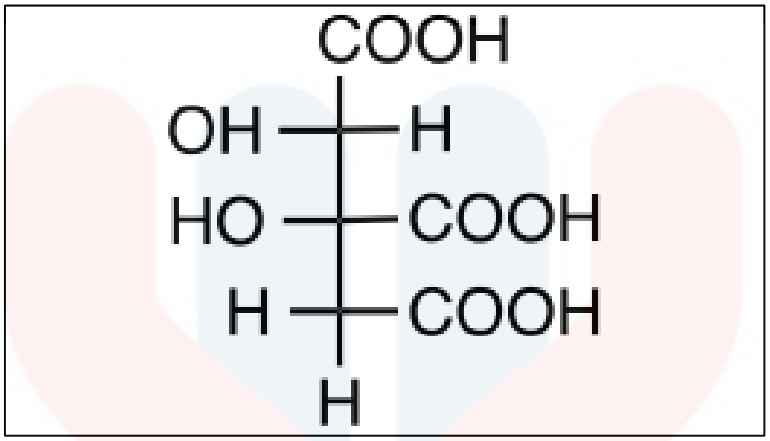

Figure 2.3 shows the chemical structures of Hydroxy citric acid compounds in *G. atroviridis* (Sources: Hamidon et al., 2017).

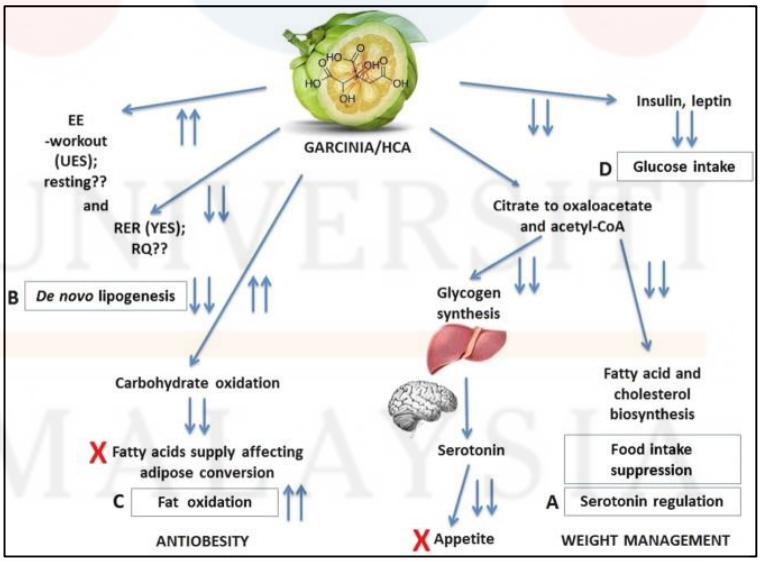

Figure 2.4 shows the mechanism to antiobesity effect of *G. atroviridis*/ HCA (Sources: Chuah et al., 2013).

2.2 Low moisture foods (LMF)

According to Codex Alimentarius International Food Standard (2015), low moisture foods are referring to final food product that contain 0.85 and below of water activity (a_w). The common food product under this category are dried fruits and vegetables, mushroom, fish and meat dried products, whole or powder form of herbs and spices, egg powder, grain products like cereal and flours, dried nuts, legumes and pulses, tea, coffee, powdered milk products and powdered infant formula (PIF). These LMF product can be derived high moisture foods that have been dehydrated or desiccated to achieve their required of water content, or they can be naturally low in moisture. It important to identify the a_w content in food product because it is one of the factors that can be use in identify of rate at which deterioration reactions occur and guarantee the food safety of dried food products (Ijabadeniyi & Pillay, 2017). Low moisture foods become desirable among the consumer because of its advantages of extended shelf life. Thus, this has attributed to consumer perceptions that not concern in terms of microbiological or food safety aspect because they think that food product with low a_w is long-lasting safe for them to consume (Byrd-Bredbenner, Berning, Martin-Biggers & Quick, 2013). This perception results because they assume that microorganisms could not survive or proliferate in a dry environment. However, a number of studies published have discovered that some pathogenic microorganisms have potential to survive in LMF. Although microorganisms cannot grow in such conditions of food product, but they are able to persist for long periods of time. (Beuchat et al., 2013; Finn et al., 2013).

This has proved by the recent spike in number of food-borne illness outbreaks associated with LMF. One of the cases refer to Table 2.3, reported that shorter curing time, low a_w and a high pH of the salami product are the main reason contribute to *Salmonella spp.* outbreaks in Europe associated to salami and fermented sausage product (Gieraltowski et al., (2012). These conditions encourage the *Salmonella spp*. pathogen to grow and survive. The illness causes by the microorganism is depends on its type. According to Bozoğlu & Erkmen, (2016), 0.60 is the minimum water activity level (a_w) conditions for all microbial proliferation in dried food products. Therefore, food spoilage below will not be a microbiological sort and Gram-negative bacteria adapted to low a_w conditions more sensitively compared to Gram-positive bacteria.

Table 2.3 shows the foodborne outbreak that related to low moisture food.

Pathogen	**Food vehicles**	**Year**	**Country**	**Number of affected**	**Reference**
Salmonella Montevideo	Salami (fermented and dried meat)	2009-2010	USA	272	Gieraltowski et al, (2012)
Bacillus cereus	White pepper	2012	Denmark	12	Eliasson et al, (2014)
Salmonella	Organic sprouted chia powder	2014	US	31	Rachon & Gibbs, (2015)
Bacillus cereus	Spices and herbs	2017	France	146	Eliasson et al, (2014)
Salmonella	Salami and other fermented sausages products	Not reported	Europe	Not reported	Gieraltowski et al, (2012)

Based on these foodborne outbreaks related to dried foods, cross-contamination can be the risk factor contribute to pathogen outbreaks in low moisture foods. This cross-contamination may occur as a consequence of poor sanitation, contaminated equipment, a lack of basic hygiene knowledge among food handlers, or inappropriate storage conditions. Meanwhile, cross-contamination also can occur when food producer packs their dried food products with loose packaging and letting customer to handle or touch the product with bare hands. This directly can cause pathogen outbreaks of dried food.

2.3 The survival mechanism of microorganisms in low moisture foods

Even though, bacteria cannot proliferate in low-moisture conditions, but they still can thrive well in this kind of situation. However, several studies published that microorganisms can persist in foods with low water activity (Beuchat et al., 2013; Finn et al., 2013). The longer period regarding the survival of vegetative cells and spores in the low water activity conditions is for months or even years compared to high-moisture foods and wet environments (Beuchat et al., 2011). Cell metabolism in bacteria is dramatically slowed and proteins conferring heat resistance are developed so that dehydrated cells are more heat tolerant and have a better ability to survive in these conditions (Rachon & Gibbs, 2015). For food in category of low moisture, it is difficult to destroy the presence of pathogen by only using the application of mild heat treatment such as pasteurization which is the temperature apply is below 100 °C. Bacteria can cause illness to LMF by enter these food products in several routes. For dried agriculturally

based food products, the contamination can occur during agricultural process which is from soil, non-potable water, organic fertilizers, wild animals and personnel or machinery that been used during harvesting.

Storing conditions of low activity food products can trigger the survival mechanism of pathogen. Two conditions that determine the growth rate of the pathogen is conditions that favouring growth is high moisture environment with increasing temperatures, all of which contribute to the optimum for growth kinetics. Meanwhile, conditions that do not support growth, is low moisture environment with high temperatures, that contribute to opposite effect. Chemical reactions and cell metabolism are significantly slowed at lower storage temperatures which is below than 16°C. Thus, when the rate of desiccation is slowed, it minimizes the physical changes within the bacterial cell and at the cell wall. While the opposite occurs at higher temperature and lack of water activity which disrupt the growth of bacteria. In this condition, the desiccation process of cell progresses is faster causing the bacterial cell wall damage and cytoplasm leakage (Rachon & Gibbs, 2015).

Besides, the mechanism of microbial survival in desiccated and low water activity are more depends on bacteria species, water activity level, temperature and food composition. In food processing, high temperature of heat treatment was used to preserve food by lowering the number of bacteria present in the product. High temperature in food microbiology refers to any temperature that is higher than the ambient temperature. This improves a product's shelf life and strengthens food safety (Ashenafi, 2012). One of the heat treatments used in food in low moisture foods industry is freeze drying. Through this treatment, it allows vegetative microbial cells and spores to be kept in a severely desiccated condition for several years. It is because there is no water available when cells are freeze dried, thus bacterial cell metabolism stops completely. Although, the growth is

prevented. The presence of *Salmonella spp.* in LMF even in low numbers still can poses a threat to consumer (Finn et al., 2013).

In food industry, *Salmonella spp.* known as a challenging pathogenic microorganism to handle with. It cannot presence in food product even in low infective dose. This pathogen is found in raw materials and can persist in harsh and dry environments for long periods of time (Doren et al., 2013). Other than presence from low a_w, deficiencies that occur in the production site such as poor hygiene and sanitation practices are among the main causes of *Salmonella spp.* contamination in LMF (Carrasco, Rueda, & Gimeno, 2012).

Table 2.4 shows microbial characteristic in low-moisture environment.

Pathogen	Aerobic/ anaerobic	Survival in low moisture foods
Bacillus cereus	Facultative anaerobe	Spore can survive for months or year in dry environment
Campylobacter species	microaerophilic	Does not survive in dry environment
Clostridium botulinum	Aerobic	Spores survive in dusty and dry environment
Clostridium perfringens	aerobic	Spores survive in dry environment
Cronobacter spp. (*formerly *Enterobacter sakazakii)	Facultative anaerobe	Can survive in dry foods (evidence of survival of bacteria is powdered infant formula for up to two years)
Escherichia coli 0157:H7	Facultative anaerobe	Can survive in dry food (e.g. dry fermented meats)
Listeria monocytogenes	Facultative anaerobe	Can survive in dry foods (e.g. dry fermented meats and peanut butter)
Salmonella spp	Facultative anaerobe	Can survive for weeks, months or years in low moisture food (> 0.30 a_w)
Staphylococcus aureus	Facultative anaerobe	Can survive for a month in dry foods

Source: Parto (2016).

The survival mechanism in low moisture foods are also include osmoprotectants, filamentation and biofilm formation which are shortly review in the next section.

2.3.1 Osmoprotectants

Bacteria have osmoprotectants that help them survive in low water activity conditions. Osmoprotectants is to balance the osmolarity within the bacterial cell with to the external environment. This is to prevent water losses (Ijabadeniyi & Pillay, 2017; Finn et al., 2013). Numerous cellular mechanisms in bacteria are involved in the osmoregulation process. The accumulation of electrically neutral, low molecular weight compatible solutes (osmoprotectants) including proline, glycine-betaine and ectoine, for example, can aid the bacterial cell limit the water loss. Due to low water content, it was hypothesized that the degree and strength of water molecule vibrations in dry bacteria are severely restricted. Although at very high temperatures, it prevents denaturation of cytoplasmic and membrane proteins. This mechanism can develop to high levels within the bacterial cell without interfering with enzyme function.

2.3.2 Filamentation

Filament formation can occur as a result of osmotic stress inhibiting cell division proteins. According to Finn, Condell, McClure, Amézquita, & Fanning (2013), the development of filaments increases total biomass without increasing the number of cells. This, of course, creates a challenge for food manufacturers. If bacterial filamentation occurs in a food product, the amount of possible cells present may be underestimated. It is because when tested using conventional microbiological methods, the formation of long filaments will not increase the colony forming unit (CFU). However, by using enrichment step, can allow for cell septation, resulting in a higher number of cells that can be enumerate (Burgess et al., 2016). In contrast to non-filamentous cells on a stainless-steel surface, the formation of filaments prior to entering a dry state has been shown to improve desiccation tolerance (Stackhouse et al., 2012).

2.3.3 Biofilm formation

Biofilm formation is the irreversible attachment and growth of microorganisms on a surface, as well as the production of extracellular polymers that aid attachment and matrix formation, resulting a change in the phenotype of the organisms in terms of growth rate and gene transcription. When stressed bacterial cells attach to a surface and secrete a

protective layer made up of extracellular polysaccharides (EPS), proteins and nucleic acids, it can contribute to biofilms formation. The bacterial population is encapsulated in this layer, which provides both protection and a way for them to interact with their environment. Reported by Rachon & Gibbs (2015), that *Salmonella spp.* is the most common pathogen associated with low moisture foods. *Salmonella spp.* is well known as pathogen that be able to form biofilms under a variety of conditions, including starvation stress. Thus, biofilm formation can play a role in *Salmonella spp.* persistence to desiccation and low-aw stress. Curli fimbriae production is a major component of biofilms and cellulose have also been shown to improve long-term desiccation survival.

2.4 Foodborne pathogen

Food-borne pathogens have emerged as a major risk concern that associated to public health around the world in recent years and their impact on public health and the economy is increasingly recognized due to high in morbidity and mortality rates (Barberena, 2016). Meanwhile, for food industry, will incur substantial financial costs in the implementation and continuous oversight of food hygiene and safety systems, product recalls and potential action taken if outbreaks occur. Food-borne microorganisms are major pathogens that disturb food safety and affect to human illness as a result from consuming certain foods. The stated human illness is foodborne disease outbreak or also called as foodborne infection or food poisoning can be admissible when a group of people are consuming contaminated food with pathogen, chemical or toxin (U.S Food & Drugs

Administration (FDA), 2020). Then, two or more of them comes with the symptoms of foodborne illness (Bintsis, 2017). The severity of the symptoms varies depending on the agent that caused the illness. Upset stomach, abdominal pains, nausea, vomiting, diarrhea, fever, headache and dehydration are the most common symptoms of foodborne illness. According to Yunus (2019), foodborne pathogens are generally found in a very small quantities which is <100 CFU/g. Bacteria, viruses, or parasites are the key of foodborne pathogens that usually found in food and may cause severe illnesses including food poisoning (Safavieh, Nahar, Zourob, & Ahmed, 2015).

2.4.1 Outbreaks associated with dried fruits, vegetables, herbs and spices

Dried food products like fruits and vegetables, herbs and spices are increasingly being related to foodborne illness outbreaks. Reported by Bourdoux, Li, Rajkovic, Devlieghere, & Uyttendaele (2016), *Salmonella spp.*, *Bacillus cereus* and *Clostridium perfringens* are the most common foodborne pathogens linked to these dried products. These pathogens microorganism have been shown to survive in low water activity food products and the conditions that surrounds them (Farakos, & France, 2014). Despite the fact that low water activity less than 0.7 is known can impede the microbial from keep proliferate. However, pathogens that presence in raw materials can withstand the drying process which using heat application and removing a_w. Thus, they can remain survive during storage afterward. Thus, it posing a risk of foodborne illness if consumed alone or being used in ready-to-eat (RTE) foods as an ingredient.

A number of outbreaks have been linked to contaminated spices, dried herbs and other LMF that linked to the mentioned pathogen above. Reported by Doren et al. (2013), one case was reported related to *Salmonella spp.* outbreaks that associated to the potato chips that have been seasoned with paprika. This was identified in food manufacturing, after the final pathogen reduction step. This resulted to 1,000 cases of *Salmonella spp.* illness. Besides, there is 20 cases reported of foodborne illness that associate to others low moisture foods such as cereal product like grain, rice and seeds/pulses that happen in France in year 2011 that caused by *Bacillus cereus* (Parto, 2016). Diarrhoea and vomiting are the symptom that last a few days (up to 24 hours) of spore-forming bacterium that produces toxins. *B. Cereus* not only can be present in foods but also in environment such as soil (cultivation environment). Spores have the ability to withstand harsh conditions. Rice, potatoes, beans and spices are among the raw agricultural food products that commonly associated to *B. cereus* infection through soil. *B. cereus* outbreaks reported in processed foods are due to contamination of raw materials which leads spore resistance to thermal and other processing processes. Then, in next process which is cooling it will allow the spore to germinates and multiply in the food or can produce emetic toxin cereulide based on the strain present.

Next, outbreaks that associate to LMF is from *Clostridium perfringens* that contaminate on products of barbecue spices, red pepper spices and red chilies that been reported in Denmark in year 2011. Meanwhile in U.S, *C. perfringens* is the second most common pathogen linked to foodborne outbreaks and a million of cases reported each year (Bintsis, 2017). These outbreaks are due to slow cooling processing or temperature abuse that allowing spores that have survived to germinate, resulting in vegetative cell proliferation (Grass, Gould, & Mahon, 2013). The symptoms that usually involve is cramping and abdominal pain after ingestion and a 7–30-hour incubation period, while

nausea and vomiting can also occur, lasting 24–48 hours. They are nonmotile, encased rod-shaped cells that produce protein toxins and produce spores that are resistant to radiation, desiccation and heat processing.

2.4.2 Outbreaks associated with powdered infant formula (PIF)

PIF and cereal products has been reported as the most related to foodborne outbreaks out of all food and ingredient categories. This may be due to the background of its main target consumer which is infant, who is easier to become affected and sick, or sensitivity of product properties, which can be rehydrated by absorbing moisture and then stored before consumption (Beuchat et al., 2013). Therefore, it is allowing the pathogens to develop and thereby raising the risk of illness. Despite being mistakenly thought to be low risk due to their inability to support microbial development of these food products, but still susceptible for microbial contamination to occur and may pose a threat to public health. Beuchat et al. (2011) stated that, this confusion can result in manufacturers to keep distributing unsafe products to the market which is totally not safe for human consumption due to not satisfying preparation practices that can promotes microbial proliferation. Prolonged periods of time in storing PIF at room temperature is an example.

Cronobacter sakazakii, *Salmonella enterica* and *Staphylococcus aureus* are proved as microbial pathogen that associated to foodborne illness in PIF (Cho et al., 2019; Wang et al., 2012). Reported that there are previous cases of pathogen outbreaks that

related to dry infant foods such as in Wyeth, almost 80% of fatality rates among infected children due to consumption of contaminated powdered milk with *Cronobacter sakazakii* (Wang et al., 2012). Meanwhile in France in year 2005, more than 141 children were infected by a *Salmonella spp.* outbreak associated to contaminated powdered infant formula. There also reported outbreaks of PIF with *Staphylococcus aureus* in China and approximately 150 babies and young children were affected.

Other than intrinsic causes (manufacturing or raw material), bacterial infections in infants are also believed to be caused by extrinsic contamination during the handling of PIF. Extrinsic contamination is thought to be a result of caregivers' unhygienic practices during the preparation of PIF. This contamination is due to attitude of caregivers that often mishandle PIF product, fail to follow hand sanitization guidelines such as keeping the used spoon that in contact with bare hands in the PIF container back without washing them. Thus, resulting in PIF as bacterial reservoir (Cho et al. (2019).

According to FAO/WHO (2007) stated that, *Cronobacter sakazakii* and *Salmonella enterica* is a category A which is clear evidence of causality since they are both well-known causes of infant illness and have been detected in powdered infant formula. The presence of *S. aureus* also has a significant risk of cross-contaminating powdered infant formula (Wang et al., 2012).

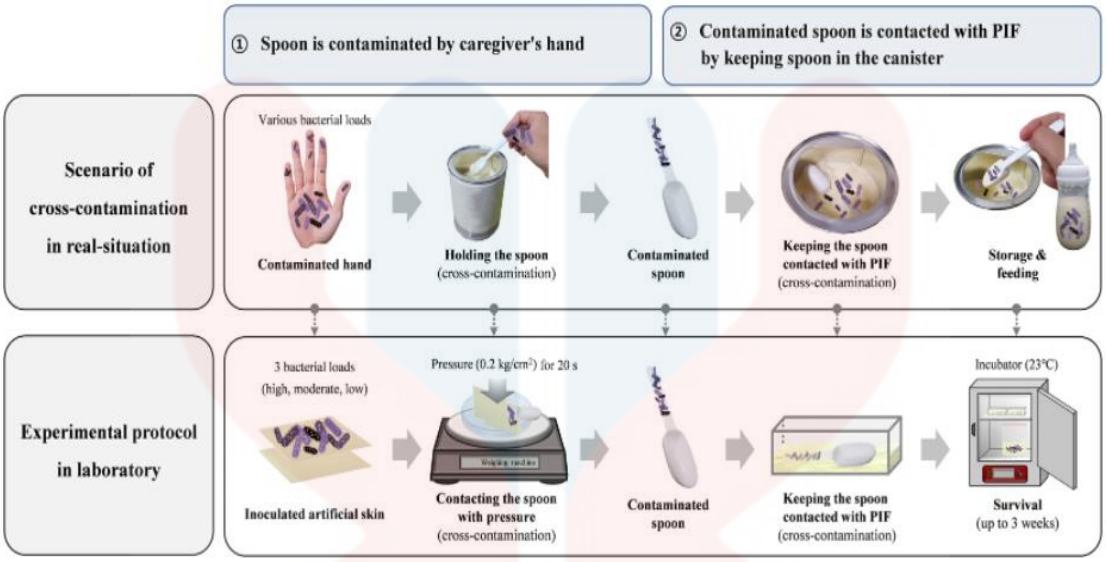

Figure 2.5 shows the schematic of cross-contamination scenarios during PIF reconstitution and storage (Source: Cho et al., 2019).

2.4.3 Selective media used to isolate microorganism in fruits and vegetables

Selective and differential growth media are used to isolate or identify specific organisms' presence in foods samples. It works by promoting the growth of certain types of organisms while preventing the growth of others.

Table 2.5 shows the media used for the isolation of microorganisms in fruits and vegetables.

Media	Organism	Temperature and time of incubation
Potato dextrose agar (PDA)	Fungi	25 ˚C for five days
Plate count agar (PCA)	Total aerobic counts	37 ˚C for 24 h
Violet red bile agar (VRBA)	Total coliform	37 ˚C for 24 h
Faecal coliform agar (FCA)	Faecal coliform	44 ˚C for 24 h
Eosin methylene blue agar (EMBA)	*E. coli*	37˚C for 24 h
Reinforced clostridial agar (RCA)	*Clostridium spp.*	37˚C for 24 h
Bacillus cereus agar (BCA)	*Bacillus spp.*	37˚C for 48 h
Brilliant green agar (BGA)	*Salmonella spp.*	37˚C for 24 h
Salmonella–Shigella agar (SSA)	*Salmonella* and *Shigella spp.*	37˚C for 24 h

Sources: Victor (2016).

2.5 Drying and dehydration process

Dehydration and drying are not synonymous. However, often used interchangeably. Drying is one of the methods used for preserving food. The concept of this process is to reduce the amount of water activity of microorganisms and enzymes in food. Drying requires heat to vaporize moisture and remove the water vapor after it has separated from the food. It is a complex process which involves the simultaneous of heat transfer that frequently used to eliminate water activity from high a_w food products resulting in solid-dried products. The activity of enzymes, bacteria, yeasts and molds is slowed when the moisture content of a food product is reduced to a certain amount and enables the producer to prolong the durability of their product's shelf life. Properties of foods also can change such as product's texture becomes harder and lowering the product's mass-to-volume ratio (Prabhakar, & Mallika, 2014).

Drying or reducing moisture method is widely used in the food industry due to numerous benefits such as easier to handle during transportation and distribution, cost efficient, reduce bulk and space for storage. It is also a suitable alternative for perishable crops such as fruits and vegetables to increase the shelf life throughout the distribution line and promote food security. Food drying time varies greatly depending on the process used as well as the size and moisture content of the food pieces.

Traditionally, sun drying is the oldest method of preserving food that using sun radiation to remove excess water from food (Michailidis, & Krokida, 2014). Exposing fresh foods to sunlight and spreading the product directly under the sun on one platform with no cover or protection for 7 to 10 days or until the desirable of moisture content is achieve. The temperature required for sun drying is >30°C and the best humidity suit for

sun drying is <60% (Ahmed, Singh, Chauhan, Anjum, & Kour, 2013). However, reported by Sontakke, & Salve (2015) there is limitation occur in this conventional method such as dry products will suffer negative effects due to their exposure to uncontrollable weather, dust, dirt, atmospheric pollution, insect and rodent attacks. Thus, these constraints cause the quality of the final product will degrade and often to the point of being unsafe for human consumption.

Due to these drawbacks, a number of drying techniques and novel drying technologies have been introduced and widely used to dehydrate foods such as convective drying (CD), spray drying (SD), freeze-drying (FD) and osmotic dehydration (OD). While for novel drying technologies is heat pump drying (HP), microwave drying (MD), infrared drying (ID), radio frequency drying (FR), refractance window drying (RW), explosion puffing drying (EPD), microwave-assisted convective drying (CD-MD) and vacuum-microwave drying (VMD) (Sánchez et al., 2020). Novel drying technologies are accepted in food industry because it is energy saving which consists of heat pump dryers, a combination of existing technology.

2.6 Effect of drying to microorganism

Drying is preserving process that involved a water transfer from a liquid state to a gaseous state through evaporation. a_w in one product will be reduce to inhibit microbial growth. It is enabling the transition of liquid products from to solid dried product. microorganism is much more resistant to dry heat compared to wet heat. However, these numbers can vary for example, halophilic bacteria can grow at 0.75 a_w and most bacteria grow at 0.87 a_w (Alp & Bulantekin, 2021). Thus, drying process must be done at sufficient degree of moisture to avoid reaching a minimum value for microorganism growth. *Staphylococcus aureus* can survive longer in dry foods and grow during storage, transportation and distribution and can resulting to spoilage (Chitrakar, Zhang & Adhikari, 2017).

Microbial inactivation through drying process is influenced by method of drying applied and conditions which can affect the heat resistance of microorganism and their toxins. Reported by Ghandi, Powell, Howes, Chen & Adhikari (2012), the flow of air temperatures which is inlet and outlet used during the drying process and composition of feed suspension are the major factors influencing the persistence of bacterial biomass during spray drying and their subsequent biological activity in spray dried powder. Microorganisms appear to be inactivated by oxidation in dry heat treatment, while protein denaturation and membrane damage appear to be essential in wet heat inactivation (Smelt & Brul, 2014). According to Phungamngoen, Chiewchan & Devahastin (2013), state that superheated steam drying is more potent in microbial decontamination and mycotoxin compared to hot air drying that commonly used in low moisture foods industry. Based on previous study from Nygaard (2008), regarding microbial inactivation of fish meal

through superheated steam drying found that D-value of *C. sporogenes* (spores) and *E. coli* through superheated steam drying at 300°C were less than 0.10 and 0.33 min. Meanwhile, D-value for the same organisms and temperature in hot air drying were 1.12 and 54 min.

DNA and RNA breakdown can happen during reducing water content in substances, as well as protein denaturation, cytoplasmic membrane modifications and damaging cell wall. It is believed that microorganism can increase its ability to survive during dehydration according to the compounds found in fruits and vegetables such as sucrose and other sugars, polypeptides, amino acids, glycerol and carboxylic acids that can promotes and improve bacterial survivability during drying process of pure cultures (Bourdoux et al., 2016). Microbial inactivation in drying process is not only due to stress applied that caused by water activity reduction. However, temperature and pressure changes, increased CO_2 or N_2 concentrations in environmental and electromagnetic waves also can affect microorganism inactivation. If the multiple stresses presence at one time, the inactivation results it can behave synergistically or antagonistically. The drying process of acidic fruits, such as berries that has lower pH can be harmful to microorganisms. Their complex structures and compositions again led to misperception among consumer regarding the survival of microorganisms during drying process.

2.7 Microorganism of concern in food safety

According to Codex Alimentarius International Food Standard (2015), *Salmonella spp.* and *Bacillus cereus* are the most common pathogens that linked to LMF and *Salmonella spp.* has been commonly found in food-borne outbreaks. As mentioned above, this pathogenic microorganism does not proliferate under this condition of < 0.85 aw but it has the capability to persist for prolong period. Capability of *Salmonella spp.* like their survivability in dry environment and in LMF products for longer periods of time can be a challenge for food industry to control in a low-moisture food processing atmosphere. Microorganisms in food with low water activity are more tendency towards heat resistant. Even though they cannot proliferate well in LMF conditions, but still their existence poses a risk.

The risk factors for *Salmonella spp.* infectious in dry processing environments could be increase if there a presence of water, which can promote growth rate of organism and contribute to higher risk to product contamination. This pathogen is frequently found in raw materials and can survive for prolong period even in harsh and low a_w environments (Doren et al., 2013). This bacterium's survival is influenced by various of factors, including matrix composition and storage temperature, as well as the aw of the environment. In food industry, lack of hygiene and sanitation practices in production environment or among the personnel are among the causes of *Salmonella spp.* contamination in LMF (Carrasco et al., 2012).

According to Chitrakar et al. (2017), desiccated *salmonella spp.* can survive at high temperature when subjected to dry heat and inactivation of *Salmonella Tennessee*

was found when the product of peanut butter being heated to 90 °C for 120 minutes. Related to dried product which is chocolate, it has a low moisture content with below 8% and high fat content. Although, *Salmonella spp.* cannot develop in chocolate until it reaches the finished product. *Salmonella spp.* cells can be sourced in raw cocoa beans or powdered milk. Thus, it increases the pathogen's thermal tolerance so that even high temperatures are needed to eliminate *Salmonella spp.* This pathogen can survive for up to 9 months in chocolate products (Ferrigno, Murino, Romano & Akkerman, 2013). Roasting step of raw cocoa bean is the only process in chocolate production have lethal effect on this pathogen if done correctly at proper temperature. During the roasting of cocoa beans, it found that *Salmonella spp.* are capable survive at 130°C for almost 5 minutes (Chitrakar et al., 2017; Nascimento et al., 2012). Dried food products like cornflour and egg powder also been reported regarding *Salmonella spp.* contamination.

CHAPTER 3

3.0 MATERIAL AND METHOD

3.1 Chemical and reagents

Distilled water, Buffered peptone water (BPW), crystal violet, Gram's iodine, acetone and safranin were used in this experiment. While, Nutrient agar (M001), *Salmonella Shigella* Agar (M108), *Bacillus cereus* selective agar (M833), Polymyxin B Selective Supplement (FD003) and Egg Yolk Emulsion (FD045) were supplied by HiMedia laboratories (Mumbai, India). The Eosin Methylene Blue Agar (CM0069) were supplied by Oxoid Ltd (Cheshire, England). All the chemical used were analytical grade.

3.2 Equipment and apparatus

Measuring cylinder (graduated 50ml, 250ml and 500ml), media bottle (graduated 1000ml and 500ml), bunsen burner, colony counter, conical flask, pipette tips box, yellow tip, blue tip, beaker, distilled water bottle, spatula, stirring rod, magnetic stirrer, funnel disposable plastic pipettes, hot plate, test tube, L- shaped hockey stick, petri dishes, parafilm, sterilized stomacher bag, zip lock plastic bag, glass slides, inoculating loop, immersion oil, cover slip were used in this experiment. The analytical balance was supplied by Saffron (Gujarat, India). The laminar flow was obtained from Camfil (Stockholm, Sweden). The autoclave HVE-50 was obtained from Hirayama (Tokyo, Japan). The incubator was obtained from Jeio tech (Daejeon, Korea). The bag mixer 400P was obtained from Interscience (France). The microscope Y100 was obtained from Raxvision (Florida, USA). The chiller was obtained from Puncak stainless steel (Kota Bharu, Kelantan). The micro pipette of 100 μL was obtained from Brandtech scientific (Essex, United States) and for micropipette 1000 μL was obtained from Thermo fisher scientific (Massachusetts, United States).

3.3 Collection of samples

The sample of dried *Garcinia atroviridis* or asam keping were collected from local street market in Jeli, Kelantan. This study was focused on three different samples of dried *G. atroviridis* (asam keping) from different location (refer to Table 3.1) which is widely used for human consumption such as in cooking, weight loss supplement and traditional medicinal. The samples were collected and stored in the zip lock plastic bag and subject to further processing in laboratory.

Figure 3.1 shows the samples of asam keping which taken from street market.

Table 3.1 shows the location of asam keping (AK) is collected.

Sample	Location
AK 1	Pasar pagi, Jeli, Kelantan
AK 2	Pasar Tani Ayer Lanas, Kelantan
AK 3	Bukit Bunga, Kelantan

3.4 Preparation of media growth

Twenty-eight grams of nutrient agar powder was suspended into 1 litre of distilled water. The dilution was heated with frequent agitation to boiling until it completely dissolves by using hot plate. Then, the solution was sterilized using autoclave at 121°C for 15 minutes. The solution was cooled at 45-50°C. Next, 15-20 ml of prepared media was poured into standard size of sterile petri dish and it becomes solidify and turned opaque. Each of the petri dish was labelled with the date of preparation and batch number. Then, the finished culture media was sealed using parafilm and store at 2-8°C. The plates were stored upside down to reduce chances of contamination and prevent the moisture from condensing on the agar surface.

The step above was repeated respectively for different type of selective media where, sixty grams of *Salmonella-Shigella* powder, forty-one grams of *Bacillus cereus* agar base and thirty-seven grams of EMB powder was suspended into 1 litre of distilled

water separately. The solution was heated to boil with frequent agitation for one minute to let the medium dissolve completely by using bunsen burner. Then, autoclave the solution at 121°C for 15 minutes except for *Salmonella-Shigella* agar to prevent it from overheat because it can destroy the selectivity of the medium as referring to the technical data (HiMedia Labs, 2011). Then, the solution was cooled at 45-50°C. For BCA, aseptically add the rehydrated contents of 1 vial of Polymyxin B Selective Supplement and 25 ml of sterile Egg Yolk Emulsion. While, for EMBA, the medium was shaken in order to oxidize the methylene blue and suspend the flocculent precipitate (Oxoid, 2010). Next, all the medium was mixed well respectively and 15-20 ml poured into sterile petri dishes and leave the medium standing to solidify. Each of the plates was labelled at the bottom with date of preparation and batch number. The prepared medium was stored upside down at 2-8°C.

3.5 Preparation of peptone water

Fifteen grams of peptone water powder (1.5% w/v) was suspended into 1 litre of distilled water. The dilution was boiled to let the medium dissolve completely. Then, the dilution was sterilized using autoclave at 121℃ for 15 minutes.

3.6 Preparation of samples and total microbial counts

Twenty-five grams of asam keping samples was weighed into stomacher bag and then mixed with 225 ml of peptone water to blend it for 60 seconds using bag mixer. Then, 10 ml from each asam keping sample was transferred to 90 ml sterile peptone water (0.1%) and thoroughly mixed. While, 1 ml of each sample was transferred using 1000 µL micropipette into test tube that contained 9 ml of sterile peptone water as a first dilution 10^{-1}, serial dilutions were made up to 10^{-6}. Next, 0.1 ml of enrichments was plated out aseptically in triplicate into the centre of petri-dishes for all media growth separately using 100 µL micropipette and spread out using hockey stick. Then, each plate was placed inverted and incubate respectively as required temperature and times for each media growth as for NA, SSA and EMBA was incubated at 35°C for 24 hours and at 30˚C - 32˚C for 48 hours for BCA. According to technical data by HiMedia (2011), colourless colony with black centre showed the presence of *Salmonella spp*. The turquoise to peacock blue colour colonies surrounded by zone of same colour on (BCA) plates was presumed as *Bacillus cereus*. While, colonies with green metallic sheen were counted as *E. coli*.

3.7 Gram staining method

Clean glass slides were smeared with the suspension of the sample using inoculation loop. Then, let it dry by air dry. Few drops of crystal violet were poured on the slide and kept it dry for about 30 seconds to 1 minutes and rinsed with distilled water. Next, the Gram's iodine was flooded on the slide and let for 1 minutes and rinsed again with distilled water. The glass was washed with acetone for about 10 – 20 seconds to get rid of excess dye and rinsed with distilled water. Then, safranin was added for about 1 minute and washed again with distilled water. Then, the glass slide was set to air dry and blot dry and continue with microscope observation. Gram-negative bacteria will stain pink/red, while gram-positive bacteria will stain blue/purple at the end of the Gram Stain (Raheem, 2021; Bishop & McCue, 2021).

3.8 Enumeration of standard plate count

The standard plate count (SPC) was conducted to enumerate the total visible count in asam keping sample using plate count agar according to the standard methods of AOAC. 0.1 ml amount of each dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) were transferred into Petri dishes of prepared standard plate agar plate by using micropipette (dried at 50˚C for 30 minutes) and spread thoroughly to ensure uniform dispersion of the microbial cell

by using hockey stick. The plates were incubated in an inverted position at 37˚C for 48 hours. This is to lessen contamination risks from airborne particles landing on them and prevent the accumulation of water condensation that could disturb or compromise a culture. The number of colonies on the plates was counted.

Colony counts made for plate that has 30 to 300 colonies. All the bacteria grow was recorded. If the number of colonies are more than 300 then recorded as too numerous to count (TNTC). The result has to multiply by dilution factor in order to determine the CFU/mL in the original sample (Chouhan, 2015).

$$CFU/mL = \frac{Number\ of\ colonies \times Dilution\ factor}{Volume\ of\ culture\ plate}$$

CHAPTER 4

4.0 RESULT AND DISCUSSION

A dilution series of each asam keping sample was carried out from 10^{-1} to 10^{-6} and it were plated in duplicate into each medium and the enumeration method of 30-300 colonies has done to calculate the microbial content (CFU/mL) by using the given formulation in chapter 3. The result of microbial content in three sample of asam keping (AK) are given in Table 4.1.

Table 4.1 shows the microbial content (CFU/mL) in three sample of asam keping.

Samples	NA ($\times 10^7$)	SSA	BCA ($\times 10^5$)	EMBA
AK 1	6.05 ± 2.47	n.d	4.45 ± 1.20	n.d
AK 2	4.85 ± 1.63	n.d	2.20 ± 0	n.d
AK 3	24.5 ± 9.19	n.d	5.00 ± 1.84	n.d

* NA: nutrient agar (total aerobic count); SSA: *Salmonella-Shigella* agar; BCA: *Bacillus cereus* agar; EMBA: Eosin methylene blue agar; n.d: not detected
* All parameter was conducted in replicates and values are expressed as mean ± SD
* *Salmonella spp.* and *E. coli* were completely absent from all samples.

Table 4.1 presents the microbial content (CFU/mL) in three sample of asam keping that were collected from different street market in area Jeli, Kelantan. Based on the results obtained, majority of the samples only has colony count on nutrient agar (NA) for total viable aerobic bacteria and on selective medium of *Bacillus cereus* agar (BCA) for *Bacillus cereus* bacteria. While no value of CFU/mL for *Salmonella spp.* and *E. coli* bacteria observed on sample of AK 1, AK 2 and AK 3.

The range of total viable counts (NA) for all asam keping samples were from $4.85 \pm 1.63\times10^7$ to $24.5 \pm 9.19\times10^7$. The sample of AK 3 shows the highest total aerobic counts (NA) of $24.5 \pm 9.19\times10^7$ followed by the sample of AK 1 with $6.05 \pm 2.47\times10^7$ CFU/mL and AK 2 with $4.85 \pm 1.63\times10^7$ CFU/mL. Similarly, Table 4.1 also indicates that *Bacillus spp.* was present in all three sample of asam keping. Sample of AK 3 shows the highest reading of microbial content of *B. cereus* with $5.00 \pm 1.84\times10^5$ and followed by AK 1 ($4.45 \pm 1.20\times10^5$) and sample of AK 2 ($2.20 \pm 0\times10^5$). As mentioned above, there was no *Salmonella spp.* detected on *Salmonella-Shigella* agar (SSA) and *E. coli spp.* presence on EMB agar for all these three asam keping sample.

The high frequency of contaminated samples suggests contamination from the environment or improper hygienic handling, filthy circumstances and prolonged air exposure. This is usually happening in sample bought at street market. Furthermore, asam keping is obtained from natural sources and, according to research, it may have been contaminated by the natural environment. It demonstrates a high frequency of *Bacillus cereus spp.* (pathogenic microorganism) in all three samples studied, which can pose health issues for consumers.

4.1 The microbial content in asam keping samples

4.1.1 Total microbial content in asam keping samples

According to Food Safety and Authority of Ireland (2020), value of total aerobic counts (TAC) with < 10^6 CFU/mL was acceptable and in satisfactory condition. Based on the Table 4.1, the aerobic bacteria were found in all AK samples examined, with the range of 4.85×10^7 CFU/mL to 24.5×10^7 CFU/mL where the high counts were detected on NA from the dilution series of AK 3. These finding shows that all AK sample has exceeded the limit of microbial safety for TAC. The number of aerobic bacteria was knowing as the real organism indicator to implies that food producer has practiced poor hygiene, sanitary conditions during the processing and compliance with good manufacturing practices (GMPs). The colonies from NA plate were also microscopically examined and the organism were found to be aerobic non spore-forming bacteria of the genus *Staphylococcus*. This is due to interpreted of Gram staining shape of cluster (refer to *Staphylococcus*) and the cells on slide is remained purple which considered to be gram-positive bacteria even after rinsed with decolorizer and acetone.

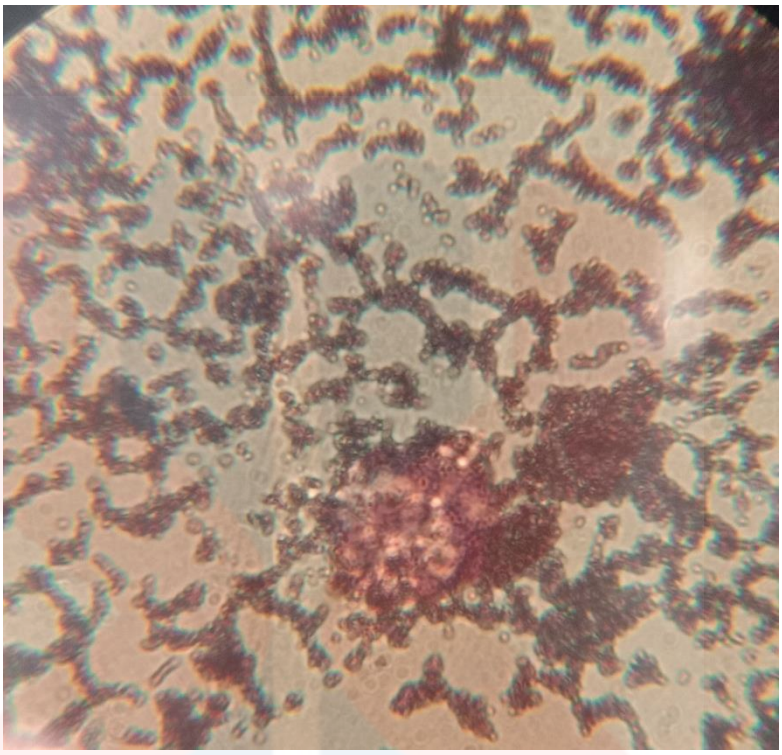

Figure 4.1 shows the imaged obtained from microscope which represented as *Staphylococcus.*

These *Staphylococci* bacteria do grow in foods and can cause food poisoning which can be hazardous to consumer. It is because this pathogen does produce toxin (staphylococcal enterotoxins) which is liable for nearly all staphylococcal food poisoning (FDA, 2021). At worst, the bacteria also can be found on the skin, there is a greater danger of an outbreak of food handlers do not thoroughly wash their hands before handling food (Salgado-Pabon, 2021). In fact, *Staphylococcus* can survive longer in dry foods (osmotic and pH stress condition) and proliferate during storage, transit and distribution until they are sold, causing spoiling (Alp, 2021). Their capability to survive in low water activity shows that this pathogen can thriving in wide variety of foods including cured meat.

In year 2000, has been reported foodborne outbreak associated with *S. aureus* and its staphylococcal enterotoxin (SE) on low moisture food (LMF) which is powdered skim milk in Japan. This staphylococcal food borne disease (SFD) has impacted thousands of people with total 13,420 cases (Kadariya et al., 2014). While reported by Ntuli et al. (2017), once there a presence of pathogenic microorganism and their toxin in dried fruit in South Africa which *Staphylococcus* and SE is one of the identified species. The symptoms can occur from consuming contaminated food with toxin produced by this pathogen is diarrhea, nausea, abdominal cramp and vomiting. While, headache, muscle cramping and transient changes in blood pressure and pulse rate may occur in severe cases. Their presence is one of the food safety concern.

4.1.2 Microbial content of *Salmonella spp.* in asam keping samples

In this study, the presence of *Salmonella spp.* also has been observed using selective media growth which is SSA. Generally, if there is detected with *Salmonell*a *spp.* in every 25 g food sample it will be considered as unsatisfactory for human consumption because it can cause hazardous to health. However, through the result obtained (refer to Table 4.1) in all asam keping samples shows there is no detection of colorless colony with black centre due to production of H_2S gas which represent as *Salmonella spp.* referring to technical sheet (HiMedia, 2011). There was a factor to support this finding which is according to Ehuwa et al. (2021), *Salmonella spp.* contamination is most commonly associated with product derived from poultry, cattle and their feeds. While according to

Munck et al. (2020), *Salmonella spp.* strains recovered from an avian, companion animal, biosolids-soil-compost, equine, porcine, poultry, reptile, ruminant and wildlife were all considered potential sources. However, dried food also important to control as it can be spread by food handler during processing and this bacteria capability itself which can survive for long periods of time in low-moisture food that can interrupt worldwide trade of dried food products as the bacteria have the ability to migrate and cross geographical boundaries (Ehuwa, 2021; Zwietering, 2016). Poor sanitation practices, inadequate equipment design and poor ingredient management have all been linked to *Salmonella spp.* cross-contamination in low-moisture foods (Podolak, 2010).

However, during the enumeration process, there were a colorless colony detected on SSA which may be considered as *Shigella spp.* This is because the media growth of SSA are enables the grow of some *Shigella* strains. The colorless colony presence on the medium was due to no H_2S gas produced. This species known can survive better in low moisture food and it is most acid resistant among others foodborne pathogen which some strains can withstand a pH of 2.5 or 3.0 for 2 hours (Al-Masaudi, 2020). There has been reported by Sospedra et al. (2010), there a high frequency of *Shigella spp.* on sample spices and herbs where 11% were found on bay leaf sample and it can cause health problem to the consumer. Besides, *Shigella spp* was also known as human pathogen that is spread by the faecal–oral route which is the ingestion of contaminated food, inadequate sanitation, or direct person-to-person contact are all ways for the bacteria to spread. The symptoms can occur when consuming food that contaminated with *Shigella spp* are abdominal pain, diarrhea, fatigue and fever (Bintsis, 2017). It can affect people of all ages, but the very young, the elderly and people with compromised immune systems are at a higher risk. Thus, to prevent this foodborne pathogen disease occur, food producer

must test to ensure there is no detection of microbial load from this species in every 25g of food samples.

4.1.3 Microbial content of *Bacillus cereus* in asam keping samples

The third media growth used in this study was BCA which to determine the presence of *Bacillus cereus* bacteria in asam keping samples. Based on the results obtained, all these sample does show positive result on this pathogen, where all the AK sample has been in unsatisfactory condition because has exceeded 10^4 CFU/mL in each sample with the range of 2.20×10^5 to 5.00×10^5, where the BCA plate for each AK sample has grown turquoise to peacock blue color of colonies which presumed as *B. cereus* referring to technical sheet (HiMedia, 2019). This result has been supported by previous study by Ntuli et al. (2017), that *Bacillus spp.* do also presence in home dried sample of peeled peaches, apples, spinach and pumpkin leaves with the range of 2.0×10^1, 4.5×10^1, 6.1×10^1 and 5.7×10^1 CFU g^{-1}. This finding has proved that aerobic spore forming and gram-positive bacteria like *B. cereus* are able to survive in food with low water activity ($< 0.8\ a_w$).

The ability to survive in stressed and harsh environment is might be due to formation of spores, since this bacterium is known as spore-forming bacteria. There is two type of toxin produced from this bacterium which is emetic (vomiting) and diarrheal. Vomiting syndrome is produce during the growth phase in food containing pre-formed

cereulide toxin. While, diarrheal syndrome is produced during growth phase when the organism producing toxin in small intestines (the host). These type cause differ illness. Vomiting syndrome has short incubation period and recovery time. The symptoms of disease are nausea, vomiting and abdominal cramp that usually occur after 1-5 hours of ingestion (Senesi, 2010). While, the symptoms for diarrheal syndrome are mild with nausea, abdominal cramps and watery diarrhea. The incubation period is within 8-16 hours of ingestion and the illness lasts for 12-14 hours or it might be continuing for several days.

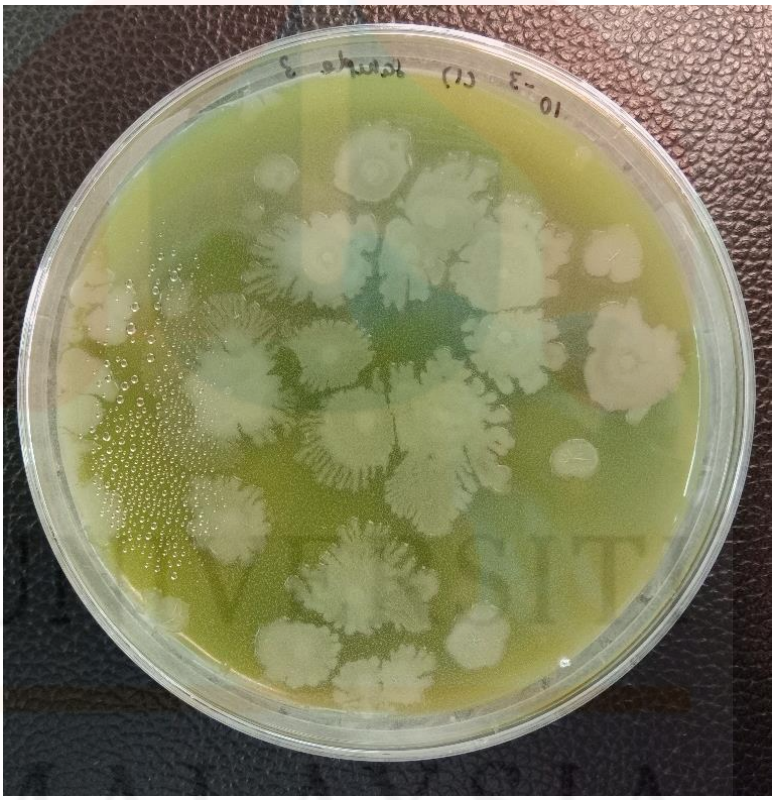

Figure 4.2 shows the colonies presence on BCA

Literally, the main source of *B. cereus* was from soil and it has been reported that 1,000 to 100,000 spores per gram can be found in soil. As a result, this bacterium is

commonly found in food, particularly raw agricultural produce such as raw fruits and vegetables, as well as raw herbs. These foods normally have less than 100 spores per gram, however, some herbs and spices may have a higher than that. Spores' subsequent thermal resistance which enable the spores to germinate and continued to multiply in foods. It has been reported in one study undertaken in the US associated with dried and processed food which 93.3% of *B. cereus* strains was isolated from retail uncooked rice sample which representing for diarrheal strains. Also, according to Rodrigo et al. (2021), the microbial content is differ depending on rice form, which brown rice is said to have high concentration of microorganism compared to white rice with the range of 2.5×10^3 to 2.5×10^1 CFU/g. This is due to the processing that dried seed (raw rice) receives during processing.

4.1.4 Microbial content of *Escherichia coli* in asam keping samples

Based on the result obtained (refer Table 4.1), all AK sample shows there is no detection of purple with black centre and green metallic sheen colony on EMBA media growth which will consider as the presence of *Escherichia coli* referring to technical sheet (HiMedia, 2020). This shows a negative result on asam keping sample because according to the food safety guideline, has stated that the presence of this foodborne pathogen must not be detected in every 25g of food samples or otherwise, the food product will be in unsatisfactory condition for human consumption. These findings were in line with a previous study by Ntuli et al. (2017), that also no presence of *E. coli* on their home-dried

fruits samples such as peeled peaches and apples tested. However, the author has reported that dried leafy vegetables such as spinach, *Wahlenbergia androsacea* (traditional vegetables) and pumpkin leaves shows the detection of *E. coli* bacteria with the microbial concentration of 9.0×10^1, 3.2×10^2 and 2.9×10^2.

The source of *E. coli* contamination in food is start from pre and post-harvest which include the soil, faeces or contaminated water that used for irrigation and in further processing. Significantly, leafy vegetables grow closer to the ground, making them more susceptible to soil microorganism contamination, as compared to apples and peaches, or even asam gelugor, which grow high up in the tree bushes, far away from soil microorganisms. Moreover, vegetables are more likely to be affected if the soil has been treated with low-quality animal manure as fertilizer or irrigated with contaminated water. Besides, poor hygienic practice and improper sanitation like washing hand using contaminated water among food handler also, one of the significant relationships with contamination levels, particularly *E. coli*, implying that there is a risk of contamination if the seller made direct contact with the food.

Based on the result analyzed, all three sample of asam keping that was collected from different street market in Jeli, Kelantan has a high frequency of microbial content for TAC and pathogenic bacteria of *B. cereus* which both microbial count (CFU/mL) has exceeded the permissible limit of food safety. When food samples fail any of the microbiological criteria and are deemed unsatisfactory, it means the food is potentially harmful to public health. Thus, it makes this dried fruit unsuitable or inedible for human consumption. This outcome can be attributed to some elements contributing to cross-contamination such as unsanitary conditions combined with lack of GMPs such as poor equipment design, poor ingredient control, lack of effective pest control, poor hygienic practices by food producers or lack of sanitary environments surrounding of asam keping

processing area. As for agricultural food product like asam keping, pathogenic contamination can occur starting from soil itself (Rachon, 2015) and known to carry both non-pathogenic and pathogenic microorganisms and they can become contaminated started from the growing and harvesting stages, as well as during processing and distribution, along with improper handling (Berthold-Pluta et al., 2021; Santo, Graca, Nunes & Quintas, 2018). Therefore, efficient cleaning is required to minimize the microbial load in further processing. As for low moisture food like asam keping, the raw products have not been treated under typical circumstances require careful attention. Otherwise, the sliced fruit which is frequently done in the open and with bare hands will be dried using conventional method which is direct full sun in the open environment. Hence, it can increase risk of microbial contamination in dried food products since the temperature imposed through this drying method is not high enough to inhibit the growth of pathogenic bacteria in foods.

Moreover, based on the result obtained it does show there is no detection of pathogenic bacteria like *Salmonella spp.* and *E. coli*. There is an element that can linked to these results obtained such as fruit has naturally low pH (below than pH 4.6). Generally, the level of fruit acidity increases as they ripen. Thus, most of the disease causing-bacteria cannot thrive in this harsh environment or they just proliferate very slowly. This is because when the cells are placed in acidic environment, the cells need to divert energy to stress resistance mechanisms which can cause cells death. As for *Salmonella spp*. and *E. coli,* they known as neutrophils bacteria which grow well at pH range of 5.5 to 8.5. While, fruits are frequently undergoing yeast and mold spoilage. This is because yeast and mold are more resilient to acidic pH (4.0). In most cases, pathogens and other organisms can be inhibited from growing when pH interacts with other factors such as a_w, salt, temperature, redox potential and preservatives.

Next important element that relates to microbial presence in dried food is water activity (a_w). In general, dried fruit has around 0.55-0.80 a_w. Water activity in foods has been reduce during drying process. The reduction of a_w will has a detrimental impact on the growth and survival of certain bacteria in foods, thus its slowing food deterioration and increases product shelf life. This is because according to FAO, at a_w 0.86, harmful microorganisms cannot grow because bacteria require a high level of moisture to keep growing. At that level of a_w, many pathogens cannot develop, which contributes to the LMFs' microbiological safety. Although bacteria from the Enterobacteriaceae family such as *Escherichia coli* and *Salmonella spp*. cannot reproduce on low-a_w plant products but they can survive for a long period (up to a year) on/in such products, especially when they are kept cold (Berthold-pluta et al., 2021). However, it been identified that mold and yeast can keep proliferate at that level of a_w but not at water activity below than 0.62. While, it's still depending on other growth elements in their surroundings, microorganisms have optimum and minimal levels of a_w for growth. Taxonomic classification is one indicator of microbial response. Gram (-) bacteria, for example, are more susceptible to low a_w than Gram (+) bacteria.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In conclusion, all three sample of asam keping shows a high frequency of microbial load exceeded the limit approved for human consumption according to food safety guidelines. As mentioned above, AK 3 was the most contaminated sample for both total aerobic counts and selective pathogen bacteria for *Bacillus cereus* with $24.5 \pm 9.19\times10^5$ and $5.00 \pm 1.84\times10^7$. This finding has proved that dried fruits product also can pose low risk to a public health. Even though, it has been classified into low water activity food products which possibility enables to inhibit the growth of microorganisms. As a consequence, the findings of this study emphasize the significance and necessity of adequate processing area dried fruits. Besides, consumer itself must be aware even the nature of dried food is known can inhibit the proliferation of microbial but there is a huge number of foodborne pathogen outbreak which associated with dried foods globally.

5.2 Recommendation

As for the recommendation, a further comprehensive study of microbial safety in *Garcinia atroviridis* (asam keping) is preferable to gain a better understanding since asam keping is one of the dried fruits that widely used by Malaysian for culinary purposes as a seasoning or a sour relish; and its mainly produced conventionally in our country where the sliced of asam keping has been dried directly to the sunlight. For further research, another microbial analysis can be explored to determine the presence of yeast and mold in this local dried fruit. This is because although dried fruits are not perishable, it is susceptible to mold growth and some of them can produce toxic metabolites such as mycotoxins due to their high sugar content, harvesting method and drying conditions. The presence of toxigenic molds and mycotoxins on these dried fruits can be a problem to agricultural sector, economic and posing a health risk to the population which they can cause acute or chronic toxic effects in humans, such as carcinogenic, mutagenic, teratogenic, atherogenic and oestrogenic effects.

Besides, the producer of asam keping must subject the fruits to a variety of pretreatment method prior to drying process. This alternative also can help in prolonging the shelf life of the product by inactivate the microbial and enzymes activity. As microorganisms can be altered before drying by proper selecting and sorting, cleaning process and blanching also enables to reduce the initial microbial load and simultaneously, can improve the product appearance. Also, blanching softens the structure of foods, allowing more water to be removed from them and so increasing the drying rate.

Furthermore, government or authorities must play a pivotal part in developing proper food safety control regulations and developing rigorous quality control measures for such products on the market and in stores. In addition, appropriate food safety training like GMP, HACCP, GHP must be held for local food producers particularly in remote locations. This is to reduce the risk of pathogenic contamination in foods and the risk assessment resulting from these groups of consumers' uses of RTE plant products must be examined by corresponding epidemiological monitoring authorities. As refer to the results obtained, both TAC and foodborne pathogen of *B. cereus* count has exceeded the limit of microbial load in AK samples. To put it another way, the contaminated products should not be released for human consumption. In such circumstances, proper actions should be done, such as an urgent inquiry and instruction to all parties concerned to stop the sale of the food product in issue, investigate immediately to determine the cause and take steps to remedy the problem. Take samples for investigation. Warning letters, source tracing and other forms of enforcement should also be considered.

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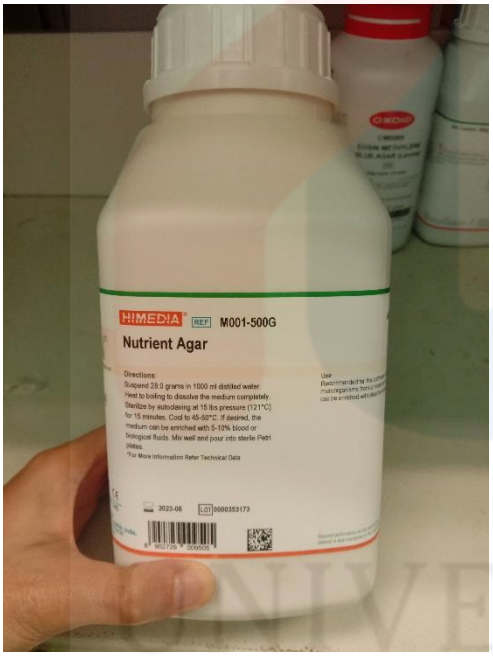
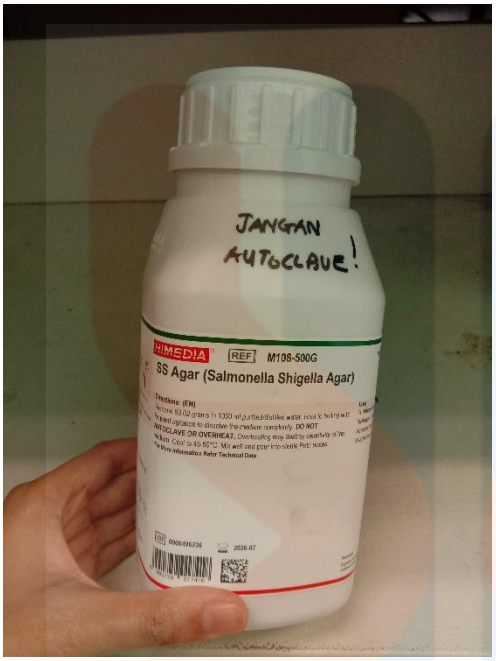
APPENDIX A

Figure	Description
	The sample of AK 1 taken from Pasar Pagi Jeli
	The sample of AK 2 taken from Pasar Tani Ayer Lanas

The sample of AK 3 taken from Bukit Bunga

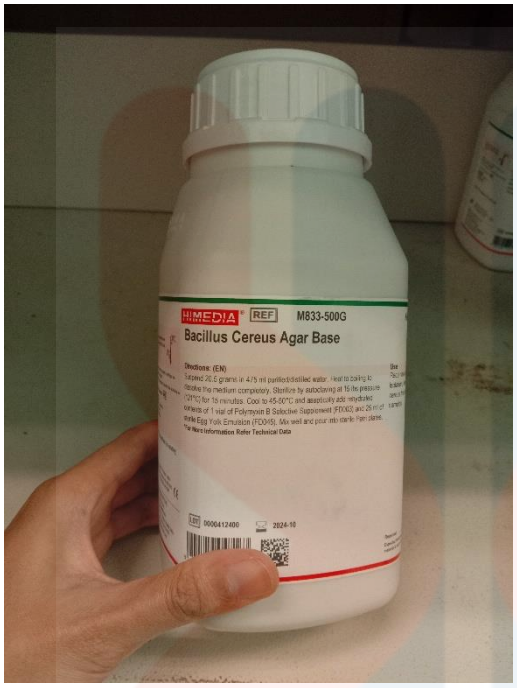


The media growth of nutrient agar (NA)



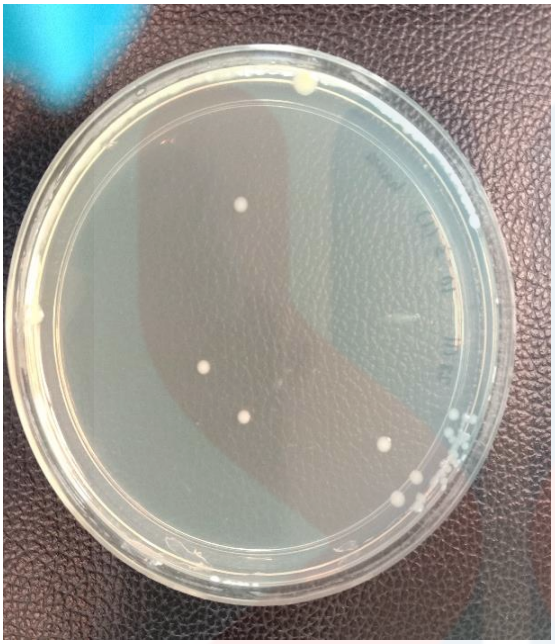
The selective medium of *Salmonella-Shigella* agar (SSA)

The selective medium of Eosin Methylene Blue Agar (EMBA)

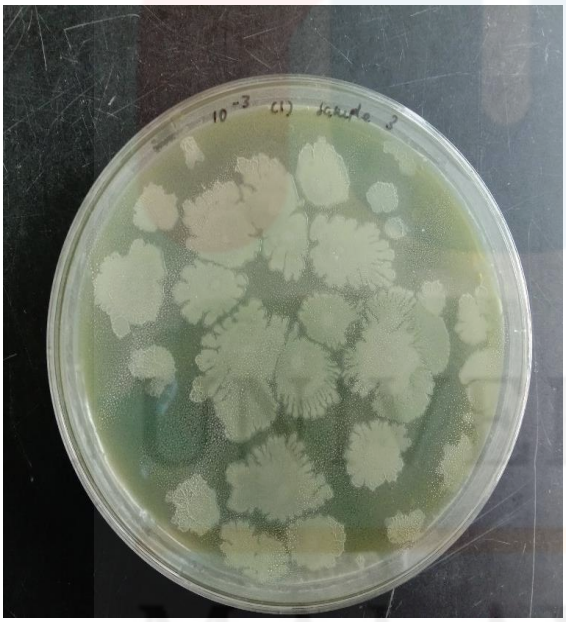


The selective medium of *Bacillus cereus* agar (BCA)

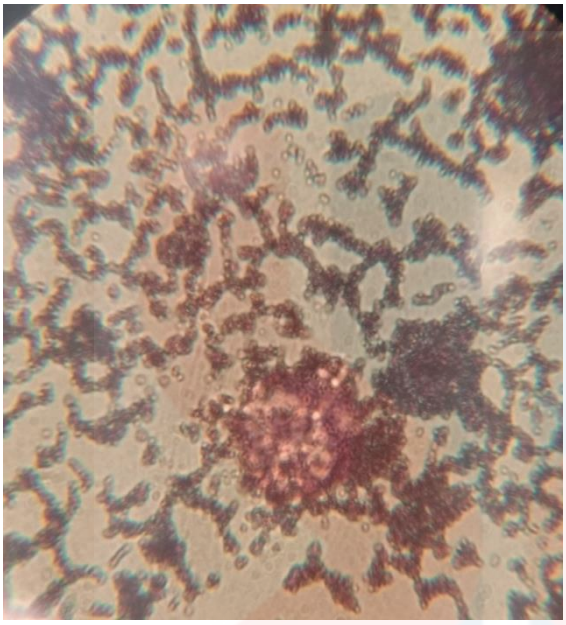
The BCA with egg yolk emulsion and 1 vial of Polymyxin B Selective Supplement



The colony growth on NA



The colony growth on BCA represent pathogenic bacteria of *B. cereus*



The imaged obtained from microscope which represented as *Staphylococcus*

The bag mixer used in preparation of asam keping samples

The laminar flow used in preparation of media and serial dilution in aseptically technique

The autoclave used for apparatus and media sterilization

The incubator used to growth the cell cultures

APPENDIX B

Microbiological guidelines in different country

		Satisfactory	Borderline	Unsatisfactory
Food standards Australia New Zealand (updated 2018)	Aerobic colony count	$<10^4$	$10^4 – <10^7$	$\geq 10^7$
	Salmonella spp.	Not detected in 25g	N/A	Detected in 25g
	Bacillus cereus	$<10^2$	$10^2 – <10^3$	$10^3 – \leq 10^5$
	E. coli	Not detected in 25g	N/A	Detected in 25g
Food safety and authority of Ireland (Revision) 2020	Aerobic colony count	$<10^6$	$10^6 – <10^7$	$\geq 10^7$
	Salmonella spp.	Not detected in 25g	N/A	Detected in 25g
	Bacillus cereus	$<10^3$	$10^3 – <10^5$	$>10^5$
	E. coli	Not detected in 25g	N/A	Detected in 25g
Microbiological Guidelines for Food (Hong Kong) 2014	Aerobic colony count	$<10^6$	$10^6 – <10^7$	$\geq 10^7$
	Salmonella spp.	Not detected in 25g	N/A	Detected in 25g
	Bacillus cereus	$<10^3$	$10^3 – <10^5$	$>10^5$
	E. coli	Not detected in 25g	N/A	Detected in 25g