Development and Effect of Two Different Packaging on Stability of Probiotic Drink

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DECLARATION

I hereby declare that the work embodied in this report is the result of the original research and has not been submitted for a higher degree to any universities or institutions.

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Date :

I certify that the report of this final year project entitled "**Development and Effect of Two Different Packaging On Stability of Probiotic Drinks**" by **Alya Athirah Binti Ruziman**, matric number **F15A0011** has been examined and all the correction recommended by examiners have been done for the degree of Bachelor of Applied Science (Product Development Technology) with Honours, Faculty of Agro-Based Industry, Universiti Malaysia Kelantan.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

CMC	Carboxymethylcellulose
EM	Evaporated milk
FM	Fresh milk
GA	Gum Arabic
ANOVA	Analysis of Variance
CFU	Colony Forming Units
FAO	Food and Agricultural Organization
FDA	Food and Drug Administration
HDPE	High Density Polyethylene
PS	Polystyrene
HPP	High Pressure Processing
PET	Polyethylene Terephthalate
TiO_2	Titanium dioxide
mL	milliliter
N	Normality
NaOH	Sodium Hydroxide
HCl	Hydrochloric acid
H_2SO_4	Sulphuric acid
rpm	revolutions per minute
SPSS	Statistical Package for Social Sciences
%	per cent
°C	Degree Celsius

spp.	Species
ssp.	Subspecies

LIST OF APPENDICES

Pembangunan Dan Kesan Dua Pembungkusan yang Berlainan Keatas Kestabilan Minuman Probiotik

ABSTRAK

Minuman probiotik mengandungi bakteria aktif yang menjadi salah satu sumber probiotik yang biasanya yang dapat memberi kesan yang bermanfaat kepada manusia. Probiotik kebiasaannya diaplikasikan ke dalam makanan dan minuman seperti makanan yang ditapai dan susu kultur. Kajian ini memfokuskan perkembangan minuman probiotik daripada dua formulasi, susu segar dengan carboxymethylcellulose serta susu sejat dengan karboksimetilselulosa. Kajian kestabilan dilakukan untuk menilai kesan dua bungkusan minuman probiotik yang berbeza iaitu pembungkusan HDPE dan pembungkus 'pouch' (bahan PET). Selain itu, kajian kestabilan yang dijalankan adalah analisis kimia yang merangkumi ujian pH dan analisis mikrobiologi yang merujuk kepada jumlah kiraan plat. Selain dripada itu, analisis fizikokimia bagi minuman probiotik ditentukan oleh penentuan lemak oleh kaedah Soxhlet, penentuan protein menggunakan kaedah Kjeldahl dan jumlah kandungan gula oleh analisis Brix. Keputusan yang diperoleh menunjukkan bahawa kestabilan minuman probiotik mempunyai perbezaan yang signifikan (p<0.05) dengan pH sementara tiada perbezaan signifikan (p>0.05) dengan koloni kiraan probiotik. Pada minggu keenam penyimpanan, minuman probiotik menunjukkan pH yang lebih rendah daripada 3.0 manakala kiraan koloni menunjukkan kiraan jumlah yang lebih tinggi antara 10^3 hingga 10^5 CFU / mL yang lebih tinggi selepas 8 minggu penyimpanan. Analisis fizikokimia minuman probiotik menunjukkan perbezaan yang signifikan (p <0.05). Jumlah kandungan lemak menunjukkan nilai dalam lingkungan antara 2.0 % hingga 4.0 % manakala jumlah kandungan gula di antara 8.0 % hingga 14 %. Jumlah kandungan protein menunjukkan nilai rendah yang berada dalam lingkungan 0.1 % hingga 0.8%. Berdasarkan hasil analisis, minuman probiotik menunjukkan anggaran jangka hayat penyimpanan 4-5 minggu. Minuman probiotik dianggap boleh diterima untuk dimakan hanya selepas 6 minggu penyimpanan kerana pH mula mencatat bacaan yang lebih rendah daripada 3.0 yang terlalu berasid untuk diminum.

Kata kunci: Minuman probiotik, homogenisasi, ujian kestabilan, pembungkusan, analisis fizikokimia

Development and Effect of Two Different Packaging On Stability of Probiotic Drink

ABSTRACT

Probiotic drinks contain active bacterial cultures which become one of the most common sources of probiotics that can confer beneficial effect to human. Probiotics usually being incorporated into food and drinks like fermented foods and cultured milk. This study highlights the development of probiotic drinks of two formulations of fresh milk with carboxymethylcellulose as well as the evaporated milk with carboxymethylcellulose. The stability study was conducted to evaluate the effect of two different packaging of the probiotic drinks which were HDPE packaging and pouch packaging (PET materials). Furthermore, the stability study conducted were chemical analysis that include pH testing and microbiological analysis which refer to the total plate count. The physicochemical analysis of probiotic drinks determined were fat determination by Soxhlet method, protein determination using Kjeldahl method and total sugar content by Brix analysis. The result obtained shows that the stability of the probiotics drinks has significant difference ($p<0.05$) with pH while not significantly difference ($p>0.05$) with colony of count of the probiotics. At week 3 of storage, the probiotic drinks show pH of lower than 3.5 while colony count shows higher total viable count of between 10^3 to 10^5 CFU/mL after 8 weeks of storage. The physicochemical analysis of the probiotic drinks shows significant ($p<0.05$) different in value. The total fat content shows value in range of between 2.0 % to 4.0 % with total sugar content in between 8.0 % to 14 %. Total protein content shows low in value which was in range of 0.1 % to 0.8 %. Based on the analytical results, the probiotic drinks show shelf life estimation of 4 to 5 weeks of storage. The probiotic drinks were considered acceptable to be consumed only after 6 weeks of storage as the pH started to record pH of lower than 3.0 which was too acidic for probiotic drinks to be consumed.

Keywords : Probiotic drinks, homogenization, stability test, packaging, physicochemical analysis

CHAPTER 1

INTRODUCTION

1.1 Research Background

Probiotic as defined by World Health Organization, can be categorized as beneficial microorganisms which when consumed in adequate amount, can give health benefits to the host (Shi, Balakrishnan, Thiagarajah et al., 2016). Probiotic help in enhancing intestinal health, improve the accessibility of nutrients in the body as well as reducing the lactose tolerance in certain people who are allergic with milk (Parvez, Malik, Ah Kang et al., 2006). The usage of probiotic was widely used in producing a lot of cultured products like probiotics milk, yogurt and kefir. The most common products in the market were probiotic milk like Vitagen, Yakult, Soltivite and Nestle Bliss. All of these probiotics milk were made from lactic acid bacteria through fermentation which will produce lactic acid as the main products (Shi et al., 2016). The main lactic acid bacteria that were used as starter culture were from the Gram-positive bacteria like *Lactobacillus* and *Bifidobacterium* genera which had been reported to be the advantageous probiotics to consumers (Shi et al., 2016). Moreover, these types of probiotics strain also do help in giving great therapeutic benefits on gastrointestinal

tracts. Gastrointestinal tracts problem may lead to diarrheal disease which might cause spread in antibiotic resistance among the microbes inside the body (Culligan, Hill, & Sleator, 2009). Besides, according to (Brown & Valiere, 2004), some of products made from strains, *L. plantarum* and *L. rhamnosus,* could be the effective treatment in balancing microbes inside the colon and preserving nutrients released by the bacteria that could benefit the health of gastrointestinal tract.

In addition, probiotic also played important role in giving protection from any pathogen, toxins, infections and cancer. Besides, probiotic also could be functional in treating bile creation and gastric secretion which act as gatekeeper of what's allowed in blood stream as well as becoming the immune function. On the other hands, probiotic do help in giving a lot of benefits to people. Its ability to help in treating most internal sickness like modulating the development of the immune system and frequently regulate shifting immune responds toward regulatory and anti-inflammatory conditions had given such an impact towards human health. Besides, according to Kober & Bowe (2015), chronic inflammatory conditions like bowel disease, acne, rosacea, atopic dermatitis and photo aging could be treated with probiotics by modification of the immune system that help human body to be tolerance from all of these diseases.

Moreover, in this research, the main starter culture used that were chosen to used were *Lactobacillus, Bifidobacterium* and *Streptococcus*. This study was performed to obtain a better understanding on stability study that could affected by a number of different factors including the ingredients employed, the properties of the packaging material used and the storage conditions (Granato, Masson, & Freitas, 2010). Product quality was often mathematically modeled around multiple parameters such as physicochemical and microbiological which was essential to the industry once it can generate. In a more accurate

way, the information related with the preservation conditions of the products, and thus it is a crucial step in developing and testing new products (Manzocco & Lagazio, 2009).

Besides, in this study, products from two formulations were studied in order to test on stability of probiotic the products originated from Biotech Era Sdn. Bhd. during their designated shelf-life. Chemical analysis on pH testing able to give comprehensive understanding on properties and qualities of probiotic drink products. Additionally, the viability of bacteria inside the products could be calculated using selective agars. In addition, physicochemical properties could be performed to determine the total fat content, total sugar content and protein content of the new developed products in order to evaluate their nutritional information related to the products.

1.2 Problem Statement

Homemade probiotics products were now being produced by people due to high cost of the probiotic drinks sold in market. In production of probiotic milk, homogenization was important to prevent cream and milk from separating during incubation. However, in homemade probiotic drink, the inconsistency of the milk sometimes happens due to a few problems. Homogenization problem occur when milk was not being homogenize by using homogenizer which could lead to separation or sedimentation in the milk. Besides, homemade products usually were not being incorporated with any additional preservatives to help in retaining longer shelf life of the product. In addition, the suitability of the existing packaging was related to the short shelf life of the packed product as the packaging the material was important in keeping the products from affected by the environment or external

factors with the aid of suitable storage temperature. Moreover, no nutritional information was performed as this probiotic drinks product was a new developed product.

1.3 Hypothesis

Null Hypothesis, H_0

- The development of probiotic drinks formula supplied from Biotech Era Sdn. Bhd. could be improved

Alternative Hypothesis, H_1

- The development of probiotic drinks formula supplied from Biotech Era Sdn. Bhd. could not be improved

Null Hypothesis, H_0

- The stability of probiotic drinks supplied from Biotech Era Sdn. Bhd. based on effect of different packaging in different storage condition could be evaluated

Alternative Hypothesis, H_1

- The stability of probiotic drinks supplied from Biotech Era Sdn. Bhd. based on effect of different packaging in different storage condition could not be evaluated

Null Hypothesis, H_0

- The physico-chemical properties of probiotic drinks supplied from Biotech Era Sdn. Bhd. could be determined

Alternative Hypothesis, H_1

- The physico-chemical properties of probiotic drinks supplied from Biotech Era Sdn. Bhd. could not be determined

1.3 Objectives

1. To improved formulation of probiotic drinks from BioTech Era Sdn. Bhd.

2. To determine the stability of probiotic drinks supplied from Biotech era Sdn. Bhd. based on the effect of different packaging in different storage condition

3. To evaluate the physico-chemical properties of probiotic drinks supplied from BioTech Era Sdn. Bhd.

1.4 Significance of Study

Homogenization issue was not common in huge scale production of milk as their production depends upon the usage of homogenizers, which function to give pressure to the product during entering the pump block of the machines. As many of the people started to practice of having probiotic milk in their daily lives for their health, many new manufacturers also begin to create their own probiotic milk in order to save more money. From the customer's overview, the sedimentation in milk was associated with stale milk which was

already expired and cannot be consumed. Besides, the packaging stability for probiotics milk in plastic packaging also had been questioned either it is safe or not to be stored for a long time at the market.

1.5 Scope of Study

In assessing the homogenization inside the probiotic milk from Bio Tech Era Sdn. Bhd., improvement in the development of formulation was needed by substituting the ingredients with new formula. Moreover, for stability assessment, microbiological analysis and chemical analysis were carried out. Microbiological analysis was tested to determine quantitative estimations of specific number of microorganisms inside the probiotic drinks. One of the example of microbiological analysis that was carried out include the Total Plate Count method which was meant to define the amount of present microorganism. Besides, the physico-chemical analysis tested refer to protein and fat determination as well as total sugar in probiotic drinks in order to evaluate the protein, fat and sugar content inside the products.

1.6 Limitation of Study

The limitation of this study in order to conduct this experiment is the homogenizer. Homogenizer machine is very hard to be found as it is expensive and rarely being used especially by homemade milk entrepreneur. This machine is used to avoid the separation

occur in milk after storage. In large milk production, homogenizer has functional properties which have the ability to load large amount of milk to homogenize. Besides, as this experiment take up the used of milk, there will be some limitations to perform this experiment as milk has short shelf life and need to be consume in one go to avoid contamination. In addition, the probiotic that is needed to make this probiotic drinks will be supplied from BioTech Era Sdn. Bhd. which is located in Perak. During the distribution of the probiotic, it needs to be stored under specific condition to maintain their functional activity. Other than that, restricted time was the number one limitation as most of the tests conducted need huge time allocation. Besides, most of the machines are limited to be used as many of the other students used the same machine to conduct their test.

RESEARCH ACTIVITIES

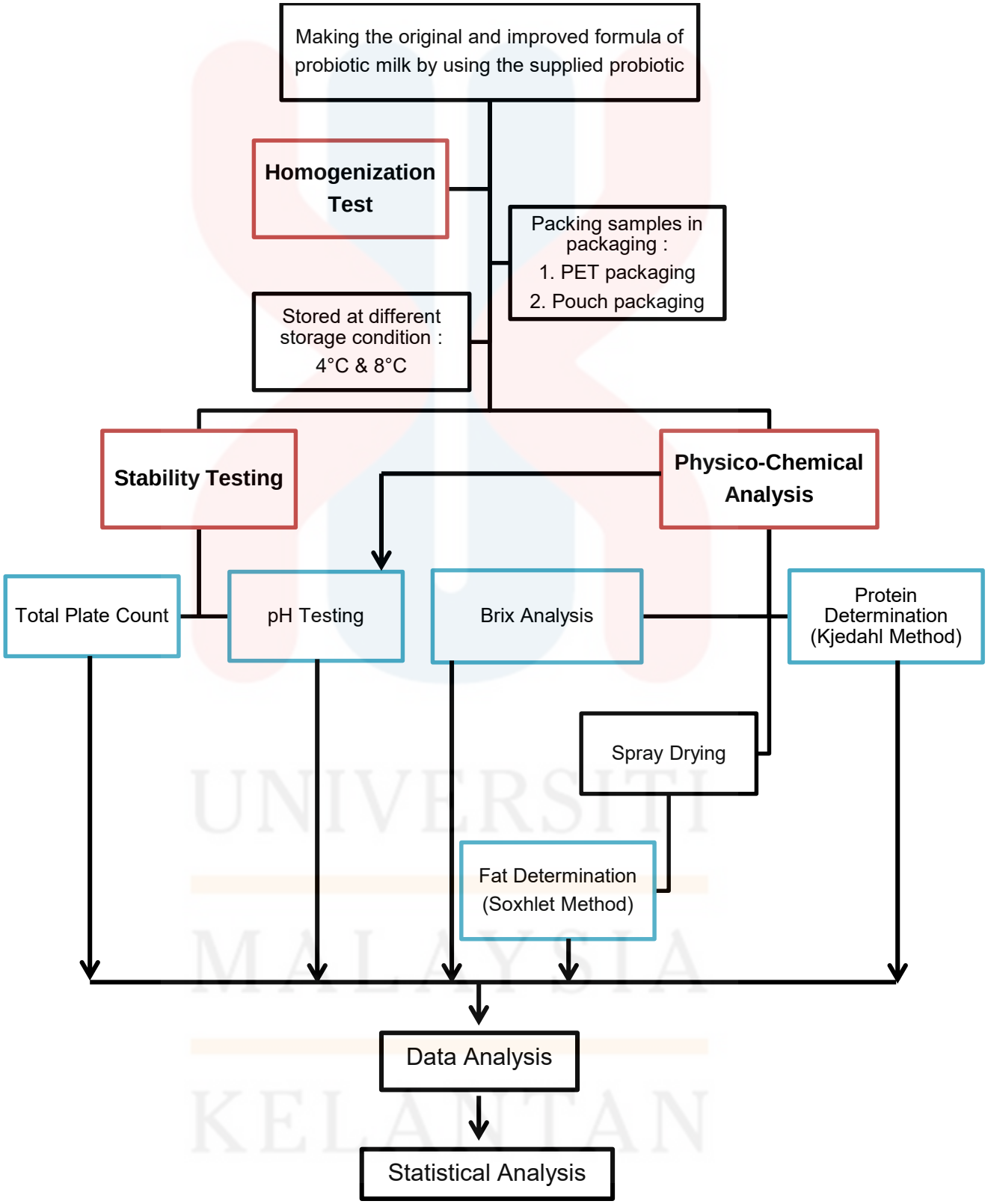


Figure 1.0 : The steps in conducting this study

CHAPTER 2

LITERATURE REVIEW

2.1. Milk

In making probiotic milk, milk was the important ingredients as it contains all sorts of nutrients like proteins, carbohydrates, lipids, minerals as well as other minor substances such as vitamins and hormones. Milk, which was the liquid matrix of water was highly perishable and was a great source of calcium and phosphorus (Adolphi et al., 2008). Most of the milk consumed by people was from cow. Other important source of milk are goat, buffalo, sheep and camel (The Editors of Encyclopedia Britannica, 2018). The average composition of milk were '87.5% water, 3.9% milk fat, 3.4% protein, 4.8% carbohydrates and 0.80% minerals' (Bylund, 1995).

Moreover, milk came as whole milk, reduced fat milk, low fat milk and fat free milk. All of these milks were different in term of their milk fat content. Whole milk contains 3.5% fat, reduced fat milk contains 2% and low fat milk contains 1% fat. Besides, milk could produce a lot of product like cream, butter, yogurt, kefir, and cheese (Anonymous, 2018).

Milk proteins had diversity in their ‘construction, structure, dimensions (molecular weight), amino acid composition, origin and also by their nutritional value and functional properties (Bonczar, Walczycka, & Duda, 2016; Hurley et al., 2012; Szwajkowska, Wolanciuk, Barlowska et al., 2011). Milk proteins could be the main subject for hydrolysis process which was one of the sources of biologically active peptides with variety of health properties (Bonczar et al., 2016; Szwajkowska et al., 2011). It was already proven that protein hydrolysis together with lactic acid milk fermentation and the help of starter cultures may reduce the allergy reaction of milk. The examples of most allergenic protein were casein, α-lactoalbumin and β-lactoglobulin. Kefir was the most applicable fermented drinks that could help in lactose intolerance (Bonczar et al., 2016)

In production of probiotic milk by Bio Tech Era Sdn. Bhd. evaporated milk had been used in the formulation. Evaporated milk was originated from fresh cow milk with 60% of the water content inside the milk was extracted out. The manufacturing of evaporated milk includes few steps like evaporation, concentration, homogenization, cooling, packaging, and sterilization. Evaporated milk commonly being packed using cans that was ‘coated with a polymeric layer to prevent tin or iron from leaching into the product and compromising the nutritional quality or sensory characteristics of the milk’ (Karaman, Özer, Pascall et al., 2015).

Furthermore, raw milk also had been used in producing the probiotic milk. Raw milk also called ‘pure milk’ was the milk that was not being pasteurized either at 72 ℃ or 90 ℃. According to (Lucey, 2015), ‘raw milk had been identified to cause food borne illness outbreak’. In US, there were about 73 out of 121 dairy products outbreak that involved the uses of unpasteurized raw milk. However, the outbreak only represents small proportion which had been well investigated (Lucey, 2015). A few years back, there were some claims

regarding with raw milk that was associated with lessen the lactose intolerance in certain people. According to (Hogan, Maryland, Industry et al., 2015), pasteurize milk also cannot substantially change lactose content in milk. A study had been conducted to obtain the result of raw milk effect to people. 16 students had been served with three milk samples organic raw whole milk, organic pasteurized whole milk and plain soymilk. From the result, the data shows unexpected outcome with higher lactose malabsorption from students who drank raw milk (Hogan et al., 2015).

2.2 Fermentation

Fermentation could be defined as metabolic process converting sugar, acid, gases and/or alcohol (OMICS International, 2008). Fermentation shows the growth of microorganism on its medium for bulk range production. Fermentation of microorganisms could be in aerobic and anaerobic environment (Anon., 2014). Fermentation in industrial production usually associated by using fermentation tanks which was done after incubation to ease the draining of the viscous fluid using with cone bottom designed tank. Fermented dairy beverages contained proteins that had high biological value which was derived from whey. This was the reason why fermented milk was categorized as drinks that have high nutritional value (Sanmartín, Díaz, Rodríguez-Turienzo et al., 2012).

Moreover, inoculation was the process involve in making probiotic milk which was done before fermentation starts. Based on (Abbasi et al., 2009) it was already well established that inappropriate inoculation could influence the fermentation time as well as the product quality. The fermentation of milk could be carried out within 42-43 ℃ in range of

temperature. Some bacteria like *Bifidobacterium* were suitable for low growth temperature. During fermentation, milk was pumped into a stainless steel fermentation tank (Paskov, Karsheva, & Pentchev, 2010).

2.3 Probiotics

Probiotic could be defined as the microbial supply with beneficial effect on human. *Lactobacillus* and *Bifidobacterium* were the example of genera that contribute to health effect on human which was mostly categorized as lactic acid bacteria. Lactic acid bacteria were capable of producing different inhibitory substances which help in producing longer shelf life products especially in fermented products (Geilman et al.,1992). Both Lactobacillus and *Bifidobacterium* bacteria were commonly used to produced probiotic based products (Kailasapathy & Chin, 2000). The main functions of probiotics were improving the health gastrointestinal tract and enhance the immune system towards more tolerance to health issues (Saarela, Mogensen, Fondén et al., 2000).

Additionally, the common probiotics species available in probiotic based product were *Lactobacillus*: *acidophilus, sporogenes, plantarum, rhamnosum, delbrueck, reuteri, fermentum, lactus, cellobiosus, brevis, casei, farciminis, paracasei, gasseri, crispatus, Bifidobacterium*: *bifidum, infantis, adolescentis, longum, thermophilum, breve, lactis, animalis, Streptococcus*: *lactis, cremoris, alivarius, intermedius, thermophilis, diacetylactis*.

Based on the probiotic milk manufacturer in Malaysia like Vitagen, Yakult and Nestle Bliss, each of the probiotics content were different. Vitagen contain *L. acidophilus*, and *L.*

casei which were imported from Chr. Hansen Laboratory in Denmark while Yakult composed of *L. casei* Shirota stain. Yakult had patented their formulation of probiotic milk in order to protect their product from being copyright by the others (Shi et al., 2016). Besides, Nestle Bliss comprised of five combinations of live bacteria named *S. thermophillus, B. lactis, and L. acidophilus* (Nestle Malaysia, 2018)

2.3.1 Genus *Lactobacillus*

This genus of probiotic was gram-positive and was non-sporing rod. *Lactobacillus* comprise of high number of GRAS (Generally Recognized As Safe) species that were usually among the most important bacteria in human nutrition and food microbiology like fermented products. This type of lactic acid bacteria could withstand high acidic environment with pH of 4-5. About more than 100 species of *Lactobacillus* had been described and 25% of *Lactobacillus* were all intestinal micro-flora which was good for human gut (Felis, Dellaglio, Scientifico et al., 2007). *L. acidophilus* also plays an important role as component of normal intestinal flora for human health. They were typically non-motile, non-spore forming and their growth was enhanced by anaerobic conditions which was either grows with or without oxygen. The optimum growth temperature of *L. acidophilus* is between 35- 40 ℃ while its optimum pH value was between 5.5-6.0 (Shah, 2000)

Furthermore, *L. rhamnosus* was one of the *Lactobacillus* species can also grow in the presence of carbon dioxide (CO_2). It was the most clinically studied probiotic bacterium and does not ferment any sucrose or lactose (Goldin et al., 1992). This was due to *L. rhamnosus*

did not grow in fermented milk stored at 4 ℃. However, it can barely survive for certain of period. (Salvetti, Torriani, & Felis, 2012). Besides, *L. casei* was the most common isolates of nonstarter lactic acid bacteria which were functional to confer health benefits as probiotics. *L. casei* was imply in various environmental conditions applications like low pH (Hossein Nezhad, Stenzel et al., 2010). It appeared that *L. casei* had survived and grows in such conditions. The optimum growth temperature for *L. casei* was 37 ℃ (Tan, Budinich, Ward et al., 2012).

Moreover, *L. plantarum* which belongs to phylum *Firmicutes*, was one of the major phyla that can dominate intestinal mucosa. It has been proven to benefit human gastrointestinal tract from gastric transit (Siezen et al., 2010). *L. plantarum* was frequently used as starter culture in food and pharmaceutical industries due to its health benefit to the host. Besides, *L. plantarum* also help in promote health effect like treatment for irritable bowel syndrome, gastrointestinal problems as well as cancer (Le & Yang, 2018).

Additionally, *L. delbrueckii subsp. bulgaricus* was one of the *Lactobacillus* species which has very slender and long rods. The optimum growth rate of *L. delbrueckii subsp*. *Bulgaricus* is at 42 ℃ and best grow at pH 4.6-5.4 under anaerobic condition which need lack free oxygen. Besides, *L. delbrueckii subsp.* Contribute in final stage of fermentation and its metabolic activities under low pH led to the post acidification of fermented product like cultured drink (Liu, 2011).

Moreover, for *S. thermophilus* strains, it was one of the most consumed probiotics as it was widely used in production of cheese and yoghurt (Uriot et al., 2017). *Streptococcus* strains able to provide flavor compound (acetaldehyde) when it produces formic acid that promotes the growth of *Lactobacillus*. In addition, the proteolytic activity that occurs able to help keeping the Streptococcus strains growing in milk. Besides, *S. thermophilus* had the

ability to inhibit some effect of intestinal pathogenic organisms when in combination with other probiotic bacteria. A study to determine probiotic drinks efficiency from (Hickson et al., 2007) had stated that *S. thermophilus* together with *L. casei* as well *as L. bulgaricus* could help in prevention of diarrhea in association with antibiotics (Sharma et al., 2014).

2.3.2 Genus *Bifidobacterium*

Bifidobacterium was incorporated as active ingredients into many functional foods. It was among the first microbes that were believed to exert positive benefits for human gastrointestinal tract. *Bifidobacterium* was a Gram-positive probiotic that was non-motile rods and often was in Y-shape or clubbed at the end. Besides, this genus lac lactase and strictly anaerobic which was free from oxygen. Furthermore, *bifidobacterium* optimum growth temperature was about 37 ℃ to 40 ℃ while its optimum pH was 6.5 to 7.0 (Shah, 2000). As *bifidobacterium spp*. requires very rigorous growth conditions, a studied by (Shah, et al) had observed that there was a slight declining of *bifidobacterium* numbers in commercial probiotic fermented products (Moriya, Fachin, Lourdes et al., 2006).

Moreover, the intrinsic properties of probiotics that need to be considered include the toxicity and metabolic activity of the microorganism during the processing step (Ishibashi & Yamazaki, 2001). Other than that, robust technology was chosen in order to select probiotic strains which were widely used approach in nowadays common practice. This technology had the ability to maintain good viability throughout the shelf life even if setup under normal commercial manufacturing and storage conditions (Robertson, 2009). The viability of the probiotics inside the product needs to be high in number which should not be less than 10^6

CFU/mL. The recommended intake which was effective to be taken in a day is between 10^8 to 10^9 CFU/mL (Yildiz, 2010).

In addition, the sensitivity of probiotics was varied towards oxygen (Talwalkar & Kailasapathy, 2004). This shows that the importance of selecting the right probiotics in order for them to survive for a long period inside the packaging. Different formulation as well as processing system also can give impact on probiotics. As instants, study from showed that *L. acidophilus* strain 2409 and *B. infantis* strain 1912 had maintained its viability after over 42 days of storage. Besides, 'the great selection of probiotic strains like dealing with robust technological properties, could give the ability to maintain good viability of the probiotics under normal commercial manufacturing and storage conditions for a greater product shelf life' (Robertson, 2009).

Other than that, after a long storage, bacterial action also might cause deterioration in the product, due to the continuous action of the bacteria culture and spore-forming bacteria that was high tolerance to heat treatment. The factors leading to deterioration include the pH of the products. pH testing need to be carried on to 'stimulated research aimed at elucidating the effect of oxygen on survival of probiotics during shelf life and on approaches to reduce the oxygen content of probiotic milk, including approaches involving packaging' (Macbean, 2010).

2.4 Probiotics Milk

Probiotic milk was originated from fermented milk which was produced with the combination of lactic acid bacteria or beneficial bacteria. 'The production of fermented milk does not require special equipment apart from a thermometer. Thus, the combination of the generic probiotic starter culture with a simple production protocol allows for the reproducible, controlled and safe production of probiotic fermented milk by almost anybody at any place, including production at the household and cottage industry levels in rural areas in resource-poor countries' (Westerik, Wacoo, Sybesma et al., 2016).

In probiotic milk, stabilizer was present in the formulation in order to produce smooth beverage and free of any graininess in texture. 'The primary purpose of using a stabilizer in was to produce smoothness in body and texture, impart gel structure, and reduce wheying off (or syneresis). The stabilizer increases shelf life and provides a reasonable degree of uniformity of the product. A good stabilizer should not impart any flavor, should be effective at low pH values, and should be easily dispersed in the normal working temperatures in a food plant. The stabilizers that were generally used in yogurt were modified starch, gelatin, whey protein concentrates, and pectin. The stabilizer system used in yogurt mix preparations was generally a combination of starch and gelatin. Whey protein concentrates at 0.5–1% level were also widely used for their water-binding attributes' (Chandan, 2014).

2.5 Homogenization

Homogenization was considered a suitable alternative to thermal processes due to its function that give lack of thermal damage towards the products. It was also one of the innovative technologies with a positive change in milk particles which leads to enhance the quality, shelf life and popularity of product. Besides, homogenization also benefits 'to improve the mouth-feel of the product as well as enhancing the water holding capacity of milk protein in order to reduce the probability of the product to syneresis' (Massoud, Belgheisi, & Massoud, 2016).

Homogenization was categorized as 'mechanical process which reduces the size of the fat globules via pressure and decreases the separation of the creamy part of the product' (Massoud et al., 2016). The homogenization decreases the size of milk fat globules by pumping milk at high pressure through a valve (Massoud et al., 2016). The purpose of homogenization was to finely distribute fat in milk to avoid fat globules become clumping which was done by 'causing disruption of the fat globules into much smaller particles' (Bylund, 1995). Besides, homogenization also does help in stabilizing 'the fat emulsion against gravity separation' (Bylund, 1995).

In addition, homogenization could result in having whiter color of milk because the high number of fat globules inside the milk scatters light more efficiently. Moreover, homogenization also function in keeping the milk become off-flavor as well as forming soft curd when the fermented milk enters human guts (Lalita, 2012)**.** During homogenization, the temperature of the milk must be over 45 °C (113°F) because inactivation of milk lipase and many microbial lipases were rendered at this temperature (Chandan & Kilara, 2011; Massoud

et al., 2016). The effect of pressure on the size and distribution of fat globules starts at 50 ℃. From here, it was understandable that below within this temperature, there would be no changes of milk due to the stability of the fat particles membrane (Sfakianakis & Tzia, 2014).

2.6 Packaging of Probiotics Milk

Packaging material and its type was the crucial element in packaging products especially for products that were related with food and drinks. The packaging materials characteristics of the probiotics milk need to be less permeable to oxygen (Miller, 2003). Oxygen scavenging packaging materials (Miller, 2003) was one of the examples. The packaging also needs to be equipped with encapsulation of oxygen sensitive probiotic cultures which were important to protect oxygen from penetrating through the product (Kailasapathy & Chin, 2000). Variations of production methods did help in reducing the oxygen level inside the products (Macbean, 2010).

The common probiotic milk that was sold in the market mostly was made from polystyrene (PS), which was a great invention in term of packaging materials. Polystyrene packaging was integrated after the usage of polyethylene terephthalate (PET) bottles. Polystyrene was more lightweight and suitable for dairy packaging production due to its gas and water vapor permeability (Schade et al., 2008). The longest duration storage for probiotic milk that was packed inside polystyrene packaging is 42 days as stated by (Miller, Nguyen, Rooney et al., 2002). Besides polystyrene, high impact polystyrene (HIPS) had been recognized which was the improved plastic packaging from polystyrene. High impact

polystyrene had the characteristics of less brittle than polystyrene (Bhunia, Sablani, Tang et al., 2013). In addition, high impact polystyrene also provides cushion and splitting protection during transportation (Macbean, 2010).

Moreover, high impact polystyrene packaging was sealed with aluminium foil or paper/plastic laminate heat-seal lid closure that helps to protect against oxygen permeation. Titanium dioxide (TiO2) white pigment chemical, was added together to the high impact polystyrene for opaque or semi-opaque type packaging, to improve packaging barrier to light as well as improving the appearance of the packaging (Robertson, 2009). Clear high impact polystyrene packaging also available which need overwraps layer on the packaging in order to reduce light penetration which would affect the quality of the probiotic milk (Macbean, 2010). Great 'oxygen barrier would help to protect the products from oxidation while good light barrier packaging would benefit in delaying fading of light sensitive colors as well as avoiding light-induced oxidation' (Macbean, 2010).

Furthermore, high density polyethylene (HDPE), as shown in Figure 2.1, was the other type of packaging that was usually used to pack milk. High density polyethylene was a kind food contact material that does not transmit any chemicals into foods and drinks as well as safe to be used (Bond, 2017). Besides, high density polyethylene also was recyclable and had down-gauging properties that allow the plastics manufacturers to use less material. high density polyethylene packaging was efficient as it uses low sources of the material and could help in reducing the product costing. Other than that, high density polyethylene was also known to be stiffer than other polyethylene films which aids in maintaining its shape to pack products. As high density polyethylene packaging had good moisture barrier properties, it

could be functional to maintain the suitable conditions inside the packaging especially for dairy products packaging (DuPont, 1996).

Figure 2.1 : Example of HDPE packaging

Other than working on packaging to improve shelf life of the product, high pressure processing (HPP) techniques was also required which aimed for yeasts and molds destruction at post packaging without affecting the probiotics. As this cold pasteurization had already sealed final product before undergo pasteurization, suitable closure of high pressure processing need to be selected that will withstand high pressure. During this cold pasteurization, the packed product would be submerged in water and would help to avoid decontamination through open fillers system. As a result, the products would last about 6 weeks and would become longer with suitable environment during processing (Macbean, 2010).

Additionally, in order to retard microbial growth and prolong the shelf life of the product, modified atmosphere packaging was also preferable due to its function (Lewis and Dale, 2000). Nitrogen and carbon dioxide become the selected gases used to retard microbial

growth. Studies by (da Cruz, Faria, & Van Dender, 2007)., had evaluate that the uses of probiotic packaging were restricted to ‘active’ packaging with oxygen absorb in order ‘to avoid toxicity and death of the microorganism and the consequent loss of functionality of the product’ (da Cruz et al., 2007).

Based on the packaging availability to pack probiotic milk other than polystyrene, pouch packaging from Figure 2.2, can be the other alternatives as it was more eco-friendly and convenience towards children. The pouch bag that could be found was spouted pouch packaging that would be better alternative to glass container due to its recyclable characteristics (Nader Brittany, 2018). Besides, pouch packaging has less carbon footprint than glass and polyethylene terephthalate packaging (Eagle, 2016). However, as homemade probiotic milk does not contain preservatives, its probiotic availability inside the packaging and the shelf life of the product need to be known to make sure the effectiveness of taking probiotic products to our body.

Figure 2.2 : Example of pouch packaging

(Topsimages.com, 2018)

2.7 Stability Study

Stability study refers to the evaluation on degradation of product characteristics which is measured over time. Stability study could be applied using appropriate statistical technique in order to determine the relation of time with the value of characteristic. Besides, in stability study, failure time was not directly observable as shelf life study. However, the determination of failures could be estimated from the data by defining appropriate cutoff value (Guillet & Rodrigue, 2010).

Moreover, stability studies also provide quality product information which varies over time and under physical conditions such as humidity and temperature and/or light. Stability study was evaluated based on the effect of storage conditions like material degradation, consumer product active ingredient stability, shelf life evaluation and raw material and chemical stability (Guillet & Rodrigue, 2010)

2.7.1 Stability Testing

2.7.1 (a) Chemical Analysis - pH Analysis

pH analysis was a method to determine the quantitative measure of acidity and basicity of a products in the range of pH between 0 to 14. 'The U.S. National Bureau of Standards had defined pH values in terms of the electromotive force existing between certain standard electrodes in specified solutions' (Lotha, 2018).

pH meter was usually being measured using pH meter which 'consists of a voltmeter that was attached to a pH-responsive electrode and a reference (unvarying) electrode. The pH-responsive electrode was usually from glass, and the reference was usually a mercury-mercurous chloride (calomel) electrode, although a silver-silver chloride electrode' (Lotha, 2018). In the dairy industry for example, incoming raw milk will be the first drinks that will be checked in order to assure freshness. For cultures that are already converted (milk sugar) to lactic acid, there will be drop in pH number from 6.6 to 4.8. From here, it clearly shows that pH does plays a critical role throughout the processing stages in the food industry (Lotha, 2018).

2.7.1 (b) Microbiological Analysis

Microbial Analysis had a wide scope of study. Total Plate Count was selected to determine the viable count inside probiotic drink. The present of microorganisms require the employment of special techniques that could help in the microbes to multiply grow while avoiding any contamination in order to attain a reasonable count of microorganisms in the samples. Besides, the correct aseptic technique was crucial in obtaining the great result which was calculated as colony forming units (CFU) that refer to scientific estimation of viable number in the samples. Additionally, aseptic technique refers to the practices and procedures that could help in preventing any contaminations from pathogens which include usage of sterilized apparatus that were in contact with the samples (Edward & May, 2014).

2.7.1 (b) (i) Determination Total Plate Count

Total Plate Count function in determining the concentration in milk especially fermented milk which comprises of several lactic acid bacteria that are beneficial for health. This method helps in to find out the colonies formed after incubation process ± 37 ℃ for ± 48 hours. Later, the result would be collected which was given in CFU/g or ml (colony forming units per gram or millimeter). As reported by (Edward & May, 2014) CFUs was not meant to define the quality of bacteria, but merely the amount of present microorganisms in the samples. Moreover, this method begun with the dilution of the samples, which was the probiotic milk. The dilution was made using potassium dihydrogen phosphate anhydrous to dilute the samples before continued to spread on the agar plate.

Total plate count method was vital in determining the exact value of microorganism present especially inside what people consume in their everyday life. Standard plate method was conducted using Plate Count Agar. As stated by (Atlas, 2010), Plate Count Agar was used for the cultivation and enumeration of microorganism from food as well as to grow the microorganisms contain inside the tested sample for Total Plate Count method. Besides, Plate Count Agar had its own compositions which consist of agar, glucose, pancreatic digest of casein and yeast extract.

In addition, based on (Macbean, 2010), the Codex Alimentarius (2003) had prepared guidelines which stated that the minimum viability of microorganism in probiotics drink were 10^7 colony-forming units CFU/mL constitute with a minimum of 10^6 CFU/mL starter culture for any microorganism present that was labeled on the packaging. Besides, the number of

probiotic organisms that was effective for gut health is 10^6 CFU/mL or more (Shah, 2000; Talwalkar and Kailasapathy, 2004; Vasiljevic and Shah, 2008).

2.7.2 Physico-chemical Analysis

Physico-chemical analysis refers to the methods to make possible the nature interaction between system physical properties and composition. Preset composition and structure were the basic problems of physicochemical analysis that investigate the interrelation between structure, composition and properties as well as determination of synthetic conditions of various products (Dhami, Reddy, & Mukherjee, 2012).

2.7.2 (b) Brix Analysis

Brix value was a specification parameter for food and beverages using refractometer. Brix analysis was the measurement by weight of sucrose in pure water solution which is valued in percentage. Brix degrees was only valid for pure sucrose solutions that was extracted from sugar-beet or sugar-cane (Robert, 2014). It was obtained by conducting Brix test include the total sugar content and total soluble solid in a sample (Wilberforce, 2016). Besides, the refractometer also had been valuable in determining the sugar content in fruits and fruit products mostly. For manual refractometer, it was determined when the refraction of the light that was seen on the prism which is passed from one medium to another with the

different density. The banding of the light beam shows the refractive index or value for the measurement. In addition, refractive index was related to the speed light travels through it and become a fundamental physical property of a solution. Both the Brix and refractive index scales could be used to measure a nearly unlimited number of fluids. (Magwaza & Opara, 2015).

2.7.2 (b) Protein Determination

Proteins consist of nitrogen-containing substances which are formed by amino acids (Hoffman & Falvo, 2004). Protein, or so called as amino acids, was made up of hundreds or thousands of smaller units that are linked to one another in long chains. Protein in the diet and body were associated with a number of vitamins and minerals and were more complex and variable than other energy sources such as fat and carbohydrate. In order to label protein on product packaging, there were limit requirement for protein intake that need to be followed. In addition, the composition of protein value in fermented milk should be in range of 1.7 ± 0.01 g/100g of milk origin according to (Dias et al., 2013). On the other hand, the recommended nutrient intake of protein from food and drinks for Malaysian adult is 55.3 g per day which was based on a 2,000 calorie diet and about 10%-15% protein contribution to total energy intake (FDA, 2018b; Mohd Yusop et al., 2017).

2.7.2 (c) Total Fat Determination

Fat was crucial in human diet due to its energy density and its essentiality for growth and development as well as for physical function. Additionally, dietary fat aids in absorption, digestion and transportation of fat soluble vitamins and fat soluble phytochemicals (Mohd Yusop et al., 2017). Fat could be divided into two types, saturated and unsaturated fat. In determining fat content inside the products, total fat need to be obtained. Total fat refers to the sum of saturated, monounsaturated and polyunsaturated fats inside food or drinks products. The obtained total fat value was method-dependent. The method includes continuous extraction to perform which is time consuming and need long period to extract lipids. In extracting the lipids, there would be the implementation of mixed polar and non-polar solvents for most food and drinks. In addition, fat was tested in food and drinks to gain information related to food and drinks performance, measure degree of deterioration and against changes in fat stability. According to (Dias et al., 2013) the composition of fat value in fermented milk should be in range of 0.5 ± 0.01 g/100g. Reasonable amount of fat intake should be considered as lipid content in milk contain saturated fatty as well as cholesterol that might be harmful if consumed in high doses (Mourad & Bettache, 2014). Moreover, based on the Dietary Guidelines for Americans, they recommended that total fat consumption should be less than 10% of calories to consume per day especially for saturated fat (FDA, 2018a).

In summary, the consistency of probiotic drinks from BioTech Era Sdn. Bhd could be improvised with the use of homogenizer during processing. New formulation also might affect the consistency of the probiotic drink with the new stabilizer like gum Arabic which

was not a common ingredient to be used in the formulation of probiotic drinks. Moreover, for the stability study, 2 possible packaging would be used to determine the relation of time with value characteristic of the products which include several stability testing like the total plate count, enumeration of yeast and mold as well as pH test. The microbiological affect and pH of the products played an important part in determining the rate of deterioration of probiotic drinks as it becomes the indicator for the probiotic drink to keep the freshness of the products.

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Chemical and Reagents

The materials involved when conducting this experiment were water, salt, sugar, carboxy methyl cellulose (supplied from BioTech Era Sdn. Bhd), gum Arabic, citric acid (supplied from BioTech Era Sdn.Bhd), evaporated milk, fruit cordial, fresh milk (FarmFresh brand), probiotics (supplied from BioTech Era Sdn. Bhd). The chemicals that will be used in this research are potassium dihydrogen phosphate anhydrous (Bendosen), distilled water, ethanol (70%), nutrient agar (Oxoid), buffer solution (Bendosen); for calibration of the pH meter at pH 4.0 and pH 7.0, phosphate-buffered saline (Merck KGaA), sodium carbonate (Friendemann Schmidt), Sulphuric acid (Merck KGaA), sodium hydroxide - 40% (R&M Chemicals), 0.1 M hydrochloric acid (HmbG Chemicals), boric acid 4% (Merck KGaA), methyl red (Bendosen), Kjeldahl catalyst tablets Copper (Fisher Chemical), bromocresol green indicator solution (Bendosen), petroleum ether (R&M Chemicals).

3.1.2 Equipment

The equipment used in conducting this experiment includes the homogenizer (Blender-Philips 220-240V, ~50-60Hz, 350W), wooden spatula, seiver, 15 liters water container, HDPE packaging, pouch packaging, aluminium foil, analytical balance, dropper, glass rod, beaker (50mL, 250mL, 500mL,1000mL), sterile glass petri dishes (≥15mm X 90mm), test tubes with lids, pipette (1000 µL, 5mL and 10mL), sterile pipette tips (1000 µL, 5mL and 10mL), dilution bottles (100mL), hocking stick, Bunsen burner, parafilm, incubator (37 ℃ ± 1 ℃), pH meter (Hanna Instruments Checker pH meter), magnetic stirrer, beaker, Kjeldahl assembly, Erlenmeyer flask, volumetric flask (1000mL), reagent bottle, (50mL and 1000mL), measuring cylinder (50mL, 100mL and 500mL), retort stand, burette (50mL), round bottle flask 29/32, round bottle flask 24/29, soxhlet extractor, condenser, thimble, heating mantle, filter funnel, spatula, desiccator, hot plate, forced air oven, sterile container (50mL), Spray dry machine (YKNTECH Double Nozzle Ultrasonic Spray Dryer), refractometer (Atago Pocket Digital Condiment Refractometer PAL-98S Brix Meter)

3.2 Methods

3.2.1 Experimental Design

This experiment begun with the improvement on formulation of this probiotic drink recipe. The formulation of the recipe was done 2 weeks before the stability study begun. During this time, one formulation of probiotic drink had been made in order to compare the homogenization with the other product that was formulated by BioTech Era Sdn Bhd. A few tests were conducted after the formulation testing finished. Besides, the experiment was continued with the stability test which will be conducted in 2 months of period starting with week 0 to week 8. This stability test consists of 2 analysis test which are microbiological analysis and chemical analysis. The samples were prepared based on the experimental design of 2 x 3 x 2 x 8 which refers to two samples of formulation that will be done triplicate and stored in two suitable conditions at 4 ℃ and 8 ℃ in two different packaging types for 8 times of observations. Two samples of formulation refer to the original and improved recipe of the probiotic drinks while it was done triplicate to verify the results become more reliable.

3.2.2 Sample Preparation

3.2.2 a) Preparation of Probiotic Drink

1/3 spoon of salt, 1 kilogram of sugar, 2 spoon of carboxyl methyl cellulose or gum Arabic, 1 ½ spoon of citric acid, 2 tins of evaporated milk or 780 mL of fresh milk, 400 mL of fruit flavored cordial as well as 200 mL of probiotic (in liquid form) supplied from

BioTech Era Sdn Bhd will be mixed with 11 liters of water in 15 liters water container. A blender (Philips, 220-240V,~50-60Hz,350 W) was used as alternative to homogenizer to homogenize all of the ingredients inside the mixture. The blended probiotic drinks mixture was chilled under 4 ℃ and 8 ℃ inside 250 mL of two different packaging, HDPE and pouch packaging (PET packaging materials). Further testing was conducted to test the products.

3.2.2 b) Preparation of Agar

3.2.2 (b) (i) Nutrient Agar

Pancreatic digest of casein, yeast extract, agar and glucose were added to distilled water and brought its volume to 1.0L. The mixture was mixed thoroughly. Later, the mixture was gently heat and boiled. Then, the mixture was distributed into bottles and the bottles were autoclave for 15 minutes at 1 atm (15psi) pressure at 121 ℃. The sterile mixture then was poured into sterile petri dishes and waited to solidify before being used to spread the samples for total plate count.

3.2.3 Development of Probiotic Drinks

Four formulation of probiotic drinks were prepared. The first formulation was the formulation that was formulated by Biotech Era Sdn Bhd which was evaporated milk with carboxymethycellulose (EM/CMC). This formula would be compared for sedimentation

settling of the drinks with the new formulation which were fresh milk with carboxymethycellulose, fresh milk with gum Arabic and evaporated milk with gum Arabic. The observation of all the formulation were recorded.

3.2.4 Stability Testing

3.2.4 (a) Determination of pH value

3.2.4 (a) (i) Buffer Calibration (4.00 and 7.00 buffer solutions)

First, two separate 50 mL beakers will be prepared and marked with pH 4.00 and pH 7.00 which indicates each buffer solution to be filled. Later, both beakers were filled with 30 mL of the buffer solutions. Then, one 100 mL beaker was taken to fully fill distilled water for rinsing purpose. The electrode of the pH meter later was rinsed with the distilled water using a dropper and then rinse again with a small amount of 7.00 buffer solution to avoid diluting the buffer solution. The calibration menu of the pH meter was entered by following the instruction to select the 7.00 buffer. After that, the pH electrode was submersed in the 7.00 buffer solution beaker and waited for the meter to indicate that it was stable. The calibration need to be confirmed on the meter. (Granato, Branco, Cruz et al., 2010)

Then, the electrode was first rinsed with the distilled water using a dropper and later rinsed again with a small amount of 4.00 buffer solution to avoid diluting the buffer solution. Next, pH electrode was submersed in the 4.00 buffer solution and waited for the meter to indicate that it was stable. The calibration need to be confirmed on the meter. Next, the

electrode was rinsed with the distilled water to clean the electrode before tested for samples. (FDA, 2017).

3.2.4 (a) (ii) Determination

50 mL of prepared probiotic drinks was poured into 100 ml beaker. Later, the sample was stirred using magnetic stirrer and the pH electrodes was immersed in the samples to read the pH value. The data pH value data was recorded.

3.2.4 (b) Microbiological Analysis

3.2.4 (b) (i) Determination of Total Plate Count

10 mL of probiotic milk sample was pipetted using 10 mL pipette and placed into 90 mL of potassium dihydrogen phosphate anhydrous in 100 mL dilution bottle to dilute. Later, the dilution bottle was shake for 25 times within 7 seconds to homogenized the samples. Next, decimal dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} in 6 different 10 mL test tubes were prepared. Then, 9 mL of distilled water was pipetted into all six test tubes of decimal dilution. All the petri dishes were prepared triplicate for each decimal dilutions and marked clearly with samples name and dates.

Later, the lower end of a glass hocking stick was dipped into 70% ethanol. Then, the hocking stick was sterilized by quick pass through Bunsen burner flame to ignite the alcohol.

After that, the hocking stick was removed from the flame and allowed to be cool. Next, the lids of the bottle containing liquid bacteria culture must first be loosen and flamed neck of the tube. By using a pipette, 1 mL of broth was removed from the dilution bottle and placed into 10^{-1} test tubes of decimal dilutions. The test tube was shake to homogenized the sample mixture. The neck of the dilution bottle was flamed again and replaced back the bottle's cap. 1 mL of previous dilution starting from the decimal dilution of 10^{-1} was transferred to 9 mL of distilled water in the next decimal dilution test tube until decimal dilution of 10^{-6}.

Then, 1 mL of pipetted solutions of the culture in each decimal dilution was placed onto the agar surface and the lid of the Petri dish will be replaced. The used tips will immediately be discarded in a container. After that, the lid of the Petri dish will be lifted to allow entry of sterilized glass spreader and place the spreader on the surface of the inoculated agar. The dish will be rotated with the left hand while spreading by moving the spreader in a top-to-bottom or side-to-side motion to spread the inoculum all over the surface of the agar. Finally, the petri dishes will be seal and incubate at 37 ℃ ± 1 ℃ in inverted position promptly for 48 hours ± 2 hours. (Maturin & Peeler, 2001)

3.2.4 (b) (ii) Counting of colonies

Counting colonies were done for both total plate count and enumeration of yeast and mold testing. After incubation period, colonies will grow in all petri dish. Normal plates can contain 25 colonies to 250 colonies. Spreader-free plates were selected and all colony forming units (CFU) were counted, including those of pinpoint size, on selected plates. The

dilutions used were recorded and total number of colonies were counted. Plates with more than 250 colonies, when the number of CFU per plate exceeds 250 colonies, all dilutions were recorded the counts as too numerous to count (TNTC). The colony count was calculated using formula (3.1) (Burton, 2018). Plates with no CFU: When plated from all dilutions have no colonies, TPC would reported as less than 1 X 10^1 the corresponding lowest dilution used. (Tournas, Stack, Mislivec et al., 2001).

$$\mathbf{CFU/mL} = \frac{\text{Colonies formed}}{\text{Dilution} \times \text{Volume plated (ml)}} \quad (3.1)$$

3.2.5 Physico-chemical Analysis

3.2.5 (a) Total Sugar Content by Brix Analysis

Probiotic samples were prepared. Refractometer need to be calibrated with distilled water first before taking measurements. Firstly, wiped off the prism surface. Next, by using a dropper, 2 or 3 drops of samples were dropped onto the prism surface. Later, READ key was pressed on the digital refractometer to get the reading. The measurement displayed in units of % BRIX was recorded. The tested sample on the prism was then removed by absorbing with soft tissue. After that, the prism was rinsed with distilled water by using a dropper. The prism then was wiped with soft tissue to dry (Dalgleish et al., 2007).

3.2.5 (b) Determination of Total Fat using Semi Continuous Solvent Extraction Method : Soxhlet Method

3.2.5 (b) (i) Preparation of Sample

Three samples of prepared probiotic drinks, sample 1, sample 2 and sample 3 were measured by 250 mL in volume for each samples. Each samples were added by 12.5% gum Arabic as wall materials with addition of 200 mL of water to dilute the solution which give in total of 450 mL of solution for each samples to spray dried. The spray-drying process was conducted using a laboratory-scale spray drier (YKNTECH Double Nozzle Ultrasonic Spray Dryer). For each of the infeed solutions, 450 mL was sprayed through nozzle tip by the co-current flow atomizer into the in drying chamber with the same direction as the drying air flow. Two different ultrasonic frequencies were utilized for the atomizer which was 40kHz and 120 kHz. The inlet temperature was set at 100 ± 5 ℃ while the outlet temperature was set at 80 ± 5 ℃ at flow rate of 1000mL/hour. The outlet temperature was controlled by the flow rate. Spray drying was done for 2 hours for each sample. The powdered form of samples collected after spray dried was kept inside sterile container to keep the powder dry before being used for fat determination by soxhlet extraction (Mohtar, Mohamed & Hamdan et al., 2014; Tan, Kha & Parks et al., 2015).

3.2.5 (b) (ii) Procedure

The predried samples were weighted about 2 grams into extraction thimble, with porosity permitting a rapid flow of petroleum ether. Later, predried round bottom flask was

weighted. The petroleum ether then was added into the round bottom flask. Next, the round bottom flask, Soxhlet extractor and condenser were assemble. Extraction started in the Soxhlet extractor at rate of 5 or 6 drops per second condensation for about 4 hours by heating the solvent in the round bottom flask. After that, the boiling flask with the extracted fat was dried in oven at 100 ℃ for 30 minutes and cooled in a desiccator. The extracted fat later was weighted and calculated (Bradley, Pegg, & Landen, 2010).

3.2.5 (b) (iii) Calculation

$$\% \textbf{ Fat on dry weight basis } = \frac{(\text{ g of fat in sample})}{(\text{ g of dried sample})} \times 100 \qquad (3.2)$$

3.2.5 (c) Determination of Proteins using Kjeldahl Method

3.2.5 (c) (i) Procedure

The sample was prepared by blending until homogeneous. The sample needs to be kept in screw-cap bottle if the samples cannot be analyzed on the same day. Cool sample was first being thaw out at room temperature and the samples was mixed thoroughly. 1 mL of sample was weighted and placed into the digestion tube. 1 gram of catalyst copper tablets and 12 mL of sulphuric acid were added to prevent the solution from bumping.

Later, the digestion tube was placed in the digester. The mixture was digested initially at low temperature to prevent frothing and boil briskly until the solution was clear and free of carbon or until oxidation was complete. Digestion was continued until a clear digest was

obtained. After the liquid had become clear, the sample was heated for another hour to complete breakdown of all organic matter.

Then, 250 mL Erlenmeyer flask containing 50 mL of 4% boric acid was placed with indicator as receiver on the distillation unit. 80 mL of water and 50 mL of 40% sodium hydroxide were added to the digests and start the distillation. The sample was distilled until all ammonia has been released or approximately >or equal to 150mL distilled was obtained.

Next, the receiver flask was lowered so that the delivery tube was placed above the liquid surface and distillation will be continued for 2 minutes. The delivery tube later was rinsed with water and allow the washing to drain into the flask. Finally, the distillate was titrated with standardized 0.1 N hydrochloric acid until first appearance of pink color. The volume of the acid used will be recorded to the nearest 0.05 mL.

3.2.5 (c) (ii) Calculation

% Protein = % nitrogen × factor (based on sample matrix)

$$\% \text{ Nitrogen} = \frac{(\text{mL standard acid} - \text{mL blank}) \times \text{N of acid} \times 1.4007}{\text{Weight of sample in grams}} \qquad (3.3)$$

Table 3.1 : The protein factor for conversion of nitrogen to protein

Food items	Factor
Meat and Fish	6.25
Casein	6.40

Milk and milk products		**6.38**
Gelatine		5.55
Human milk		6.37
Eggs	Whole	6.25
	Albumin Vitelline	6.32
	Vitelline	6.12
Wheat	Whole	5.83
	Embryo	6.31
	Bran	5.80
	Endosperm	5.70
Rice flour and rice		5.95
Rye, barley, oats, and flour		5.83
Millet		6.31
Maize/Corn, beans		6.25
Castor bean		5.30
Soybean		5.71
Brazil nuts, peanuts and groundnuts		5.46
Coconuts, cashew nuts and other nuts		5.30
Sunflower seeds, sesame seeds and all other seeds		5.30
All other Foods		6.25

3.3 Shelf Life Estimation

Shelf life was a period of time during which food will remain safe and in a suitable quality for consumption while being stored as instructed (Hancock, 2017). Prediction on critical quality parameters of chemical analysis which was pH testing was conducted to determine the effect towards this stability study. Shelf life estimation was calculated using formula : $\theta = \frac{B - B_0}{k}$ (3.4)

θ = Estimated shelf life

B = deterioration rate at day final

B_0 = deterioration rate at day 0

k = rate constant to deteriorate

Rate constant of deterioration was calculate by :

$$k = \frac{B - B_0}{\theta}$$

B = pH of probiotic drinks started to deteriorate (pH of lower than 3)

B_0 = initial pH of probiotic drinks being tested (week 0)

θ = The number of weeks tested

3.4 Statistical Analysis

Two-way ANOVA with SPSS Version 20.0 was performed to compare differences of means of stability testing and physicochemical analysis of probiotic drinks. This was followed by using Tukey's Honest Significant Difference (HSD) parametric test at 95% confidence level ($p<0.05$) to determine in more detail how different the sample differed.

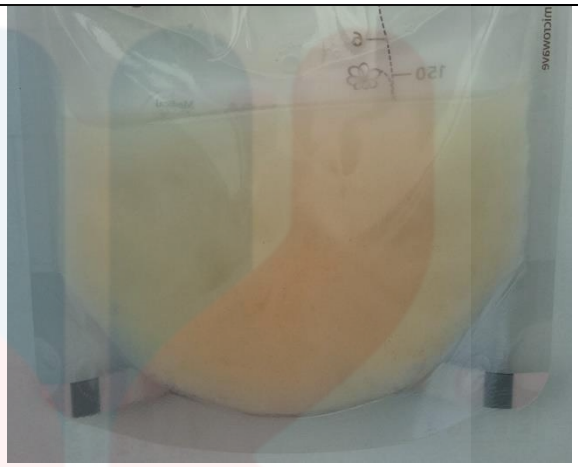
CHAPTER 4

RESULTS AND DISCUSSION

4.1 Development of Probiotic Drinks

Development of probiotic drinks from Biotech Era Sdn. Bhd. would have discussed based on the sedimentation properties of the samples. Based on Figure 4.1, this Figure refers to the original formulation of probiotic drinks with formulation of evaporated milk and carboxymethylcellulose (CMC). This formula was not being reformulated as this sample would be compared in terms of its sedimentation properties. Figure 4.2, 4.3 and 4.4 was derived from the original formulation of probiotic drink.

Figure 4.2 shows formulation of fresh milk with carboxymethylcellulose (Fm/Cmc). Fresh milk was substituted from evaporated milk to compare its sedimentation properties. Besides, Figure 4.3 shows the formulation of evaporated milk with gum Arabic (Em/Ga). Gum Arabic was substituted from carboxymethylcellulose to observed the sedimentation properties. Furthermore, Figure 4.4 shows the formulation of fresh milk with gum Arabic (Fm/Ga). Gum Arabic and fresh milk were substitute to observed the sedimentation properties of the sample.

SAMPLES	OBSERVATION
EM/CMC (Original Formulation)	 **Figure 4.1 : The sedimentation in milk with formulation of evaporated milk and carboxymethylcellulose**
FM/CMC	 **Figure 4.2 shows the sedimentation occur in milk with formulation fresh milk and carboxymethylcellulose**
EM/AG	 **Figure 4.3 : The sedimentation in milk with formulation of evaporated milk and Arabic gum**

FM/AG	 **Figure 4.4 : The sedimentation in milk with formulation of fresh milk and Arabic gum**

Formulation of fresh milk with carboxymethylcellulose was chosen to be compared with the original formulation. This was due to its properties which result in more homogenized sample mixture than the other two samples, Em/Ga and Fm/Ga. The observation from Figure 4.3 shows that Em/Ga result in two layers of sediment. The first layer shows a clear solution while the second layer shows cloudy solution. There were two separations of layers due to incomplete homogenization. As this formulation was prepared using evaporated milk, there would be high in concentration of the milk because evaporated milk was obtained by partially remove of water (Nouh et al., 2017). High in concentration can cause sedimentation due to its high density. Besides, it can also be observed that the Em/Ga sample shows high in milk clumping on the second layer of the sediments. Additionally, based on Figure 4.4, Fm/Ga shows two separation of layers which was same as the Em/Ga sample. However, there was no clear solution observed at the first layer. This is due to the usage of fresh milk which have low in density that can cause less in sedimentation inside the sample.

Based on Figure 4.2, the sedimentation inside formulation Fm/Cmc cannot be achieved as the homogenizers used was not compatible to homogenized the milk well. Blender or blade type homogenizer was used to blend the formulation which only applied approximately 3500 rpm for 1 hour. According to Phungamngoen, Asawajinda & Santad (2016) the usage of homogenizers at 15,000 rpm for 15 minutes can be effective to homogenize milk sample. Besides, the application of pressure towards milk sample also functional to homogenize the milk. Milk normally being homogenized at 15 to 20 MPa based on Massoud et al. (2015) and also supported by (Bensmira, Nsabimana, & Jiang, 2010).

Additionally, the uses of stabilizer inside fermented milk product also crucial which act as food additives that function to homogenized mixture of immiscible materials as well as smoothen the texture of the samples (Tasneem et al., 2014). The stabilizer that was used in the development of this probiotic drinks was carboxymethylcellulose (CMC). According to (Tasneem et al., 2014), carboxymethylcellulose has extensive application in dairy product especially which help in exhibiting the range of low and high viscosities at specific concentration in dairy products. From the original formulation of the probiotics drink, the mass fraction of the stabilizer used is 0.25%. Based on Tasneem et al. (2014), the recommended usage of CMC in dairy products especially for yoghurts is approximately 0.5%. Besides, it was also supported that the usage of stabilizers especially CMC must be at least 0.5% as lower percentage than this value may lead to have low viscosity of drinks and separation inside the product after leaving to settle for some time (Akkarachaneeyakorn & Tinrat, 2015). This could be one of the reason why development of the probiotic drinks cannot be achieved.

Moreover, fat content can also affect the rate of homogenization as high fat content can lead to less efficient in homogenization of milk. High fat content can help in raising the

temperature which improve homogenization technology. However, this situation may create large total fat globule that will led the plasma protein become insufficient in forming new membranes on fat (Sabikhi & Reddy, 2012).

Furthermore, this formulation also includes the use of fruit cordial. Fruit cordial may contain specific stabilizers in order to get the gelling texture as well as high in concentration. According to (Blendhub Corp., 2015), the common stabilizers used was guar gum, xanthan gum, cellulosic gum and pectin. There were specific combinations of stabilizers with specific amount were needed in order to create great texture of cordial. As instance, based on (Blendhub Corp., 2015), the combination of xanthan gum with CMC would result in having high viscosity cordial. This findings also being supported by (Akkarachaneeyakorn & Tinrat, 2015). As the cordial used do not specifies what types of stabilizers were used. This could lead to give unsuitable combination of stabilizers that would affect the homogenization in the probiotic drinks.

4.2 Stability of Probiotic Drinks

4.2.1 pH Analysis

In conducting this studies, a positive control was chosen in order to test whether the experiment was against something where you know what the effects would be. The sample of Vitagen was chosen as standard positive control in order to compare with the formulated probiotic drinks. According to (Wan, Sahar, & Zainal, 2011), the standard pH of Vitagen drinks was 4.00 which was applicable to be consumed by gastric patients. Besides, this positive control sample has a shelf life of about ±6 weeks after production which can be seen

at the packaging of the products. This Vitagen control was incorporated with two types of strains which were *Lactobacillus acidophilus* and *Lactobacillus casei* that do functioning in helping digestion problems as well as boosting immune systems. Additionally, this positive control was advisable to be stored at temperature of ±4°C and can only last for two to three hours in room temperature to avoid contamination (Wan et al., 2011).

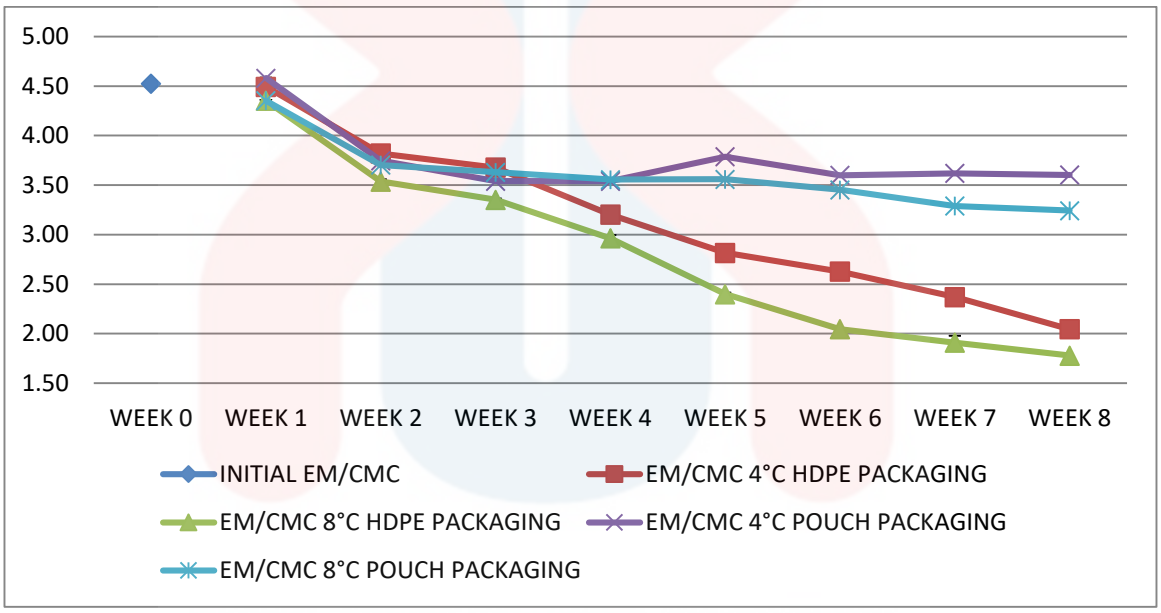


Figure 4.5 : pH of evaporated milk with carboxymethylcellulose (CMC) formulation at 4°C and 8°C of storage condition.

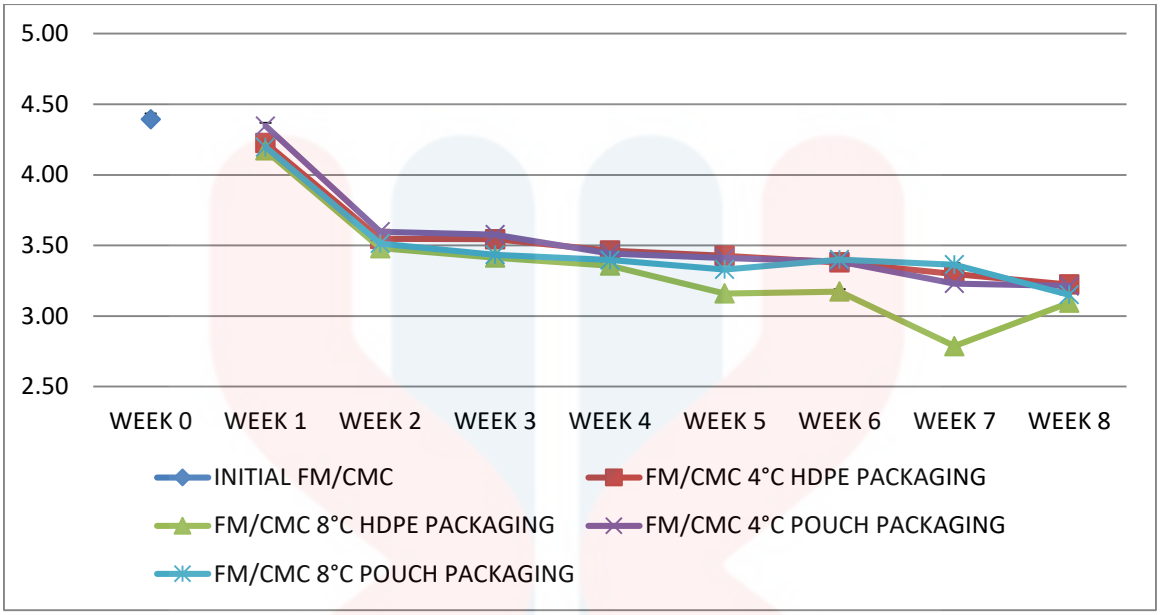


Figure 4.6 : pH of fresh milk with carboxymethylcellulose (CMC) formulation at 4°C and 8°C of storage condition

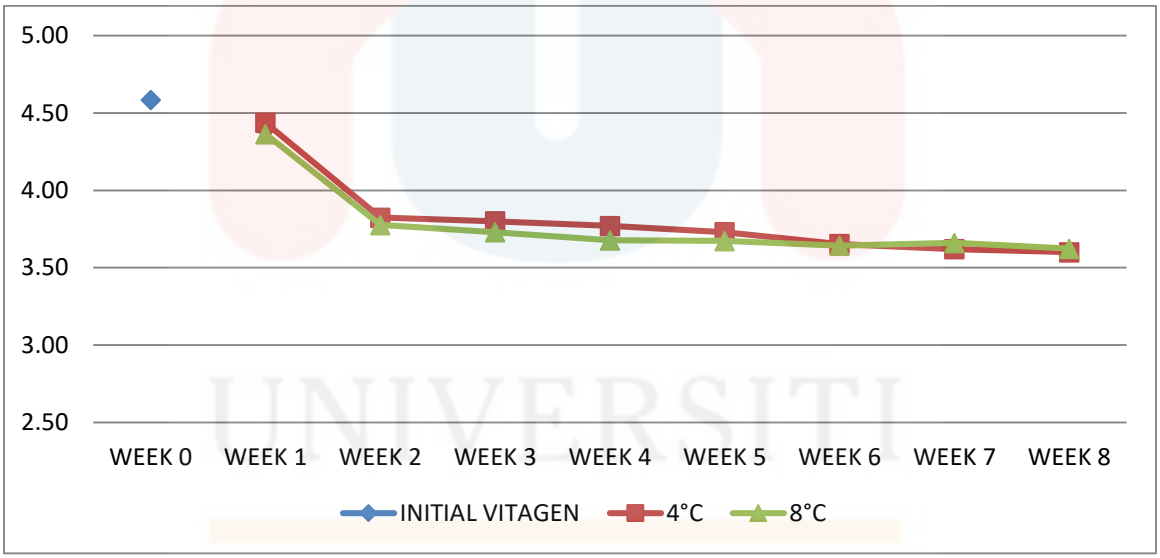


Figure 4.7 : pH of Vitagen at 4°C and 8°C of storage condition

The results of pH evaluation of probiotic drinks were presented in Figure 4.5, 4.6 and 4.7. During fermentation period, at pH 4.50, casein micelles with negative charged had started to neutralized the positive charged that result in the accumulation and formation of

coagulum. Therefore, incubation for fermentation should be stopped when pH had started to reach pH of 4.5. (Arioui, Saada, & Cheriguene, 2017). From the Figure 4.5, 4.6 and 4.7, at week 0, the pH shows high in values than pH of 4.5, which is the correct pH to stop incubation before being distributed in packaging for stability study.

On the other hands, based on Figure 4.5 and 4.6, the differences in pH value was recorded in different storage conditions. At 4°C and 8°C, from Figure 4.5, pH of Em/Cmc shows low in pH value in HDPE packaging compared to pouch packaging. Besides, based on Figure 4.6, all of Fm/Cmc sample in different packaging shows no significant difference in pH reading except for HDPE packaging at 8°C that depicts the lowest pH value starting on week 4 of storage. Overall, at storage condition of 4°C for both samples Em/Cmc and Fm/Cmc in HDPE and pouch packaging shows a great retain in pH of samples until week 3. Starting from week 4, the pH would record a steady decrease in pH as the samples started to deteriorate after long storage.

Based on Table 4.1 there were significant differences ($p \leq 0.05$) in pH values of all probiotic drinks samples based on weekly observation. From Figure 4.5, 4.6, 4.7, at week 0, which refer to the fermentation phase of the samples, pH value recorded the highest reading which was 4.40. During this time, according to (Dias et al., 2013) when pH begin to reach pH of lower than pH 5.0, the consumption of lactose by high metabolic activity of lactic acid bacteria had result in production of lactic acid with storage condition of between ±0°C to ±5°C. Besides, the slow and progressive decrease in pH reading starting from week 2 shows from Figure 4.5, 4.6, 4.7, attribute to the limited growth of different starter cultures which refer to the probiotics as well as the slow fermentation process of the residual lactose inside the samples (Barrantes et al., 1994; Hussein, 2011; Nassar, Shamsia, & Attia, 2016).

Table 4.1 : pH of probiotic drinks from week 0 to week 8.

Week	pH
Week 0	4.46[a]
Week 1	4.40[b]
Week 2	3.69[c]
Week 3	3.64[d]
Week 4	3.43[e]
Week 5	3.31[f]
Week 6	3.27[g]
Week 7	2.97[h]
Week 8	2.65[i]

* a, b, c represents the homogenous subset of probiotic drinks samples

Furthermore, according to Figure 4.5 starting at week 2, the pH value had been decreasing dramatically to pH of below than 4.00. The reduction of pH occur due to the transformation of lactose to lactic acid that result in accumulation of lactic acid which help to increase the acidity (Gamage et al., 2016). Besides, the dropped in pH also attributed to the limited growth of different bacterial starter cultures and the slow fermentation of residual lactose (Nassar et al., 2016). In addition, according to (Cais-Sokolińska, Michalski, & Pikul, 2004) pH of milk under processing which starting from when bacterial cultures was first being inoculated would be decreasing until reaching of about approximately pH of 4.34 after 21st day of storage. However, from the result, at day 21st which refer to week 3, from Figure 4.5, 4.6, 4.7 , the pH of all the probiotic drinks result at pH of below than pH 4.00 which were slightly acidic than what had been stated by (Cais-Sokolińska et al., 2004).

Table 4.2 shows that the pH value have significant difference with all of the samples of probiotic drinks, Em/Cmc and Fm/Cmc stored inside different types of packaging. pH of sample Em/Cmc shows higher in pH than the sample for Fm/Cmc sample. This might be due to the type of milk used to prepared these two samples which were evaporated milk and fresh milk. Fresh milk tends to promotes acidic pH faster than evaporated milk due to its short shelf life. According to (Department of Food Science, 2007), pasteurized milk was likely to withstand for only 2 to 5 days after selling it. Thus, the likelihood of Fm/Cmc to deteriorate faster was high and would cause decrease in pH after a long storage.

Table 4.2 : pH reading of probiotic drinks based on sample and packaging of the products

Sample and Packaging	**pH**
Em/cmc	4.52[a]
Fm/cmc	4.39[b]
Em/cmc – Pouch packaging	3.65[c]
Fm/cmc – Pouch packaging	3.52[d]
Fm/cmc – HDPE packaging	3.46[e]
Em/cmc – HDPE packaging	2.87[f]

* a, b, c represents the homogenous subset of probiotic drinks samples

Both of samples in pouch packaging shows only a slight different in pH due to preservation method applied which refer to the sterilized pouch packaging used. Both of the samples Em/Cmc and Fm/Cmc are more suitable to be stored in pouch packaging rather than in HDPE packaging which shows a steep decrease in pH value. According to Ansari & Datta, (2003) sterilized pouch packaging is maintained by sterile air under positive pressure and

also applicable to be used to packed acidic products with pH of less than 4.6. This application can lead with retaining longer shelf life in products which is approximately 3 weeks from table 4.1 that recorded pH of higher than 3.5.

This application can lead with retaining longer shelf life in products which is approximately 3 weeks from Table 4.1 that recorded pH of higher than 3.5. Based on the standard control, the pH of the products was higher than pH 3.5 as what had recorded on Graph 4.3. Based on Figure 4.5 and 4.6, the other two samples, Fm/Cmc and Em/Cmc recorded pH of lower than 3.0 after 6 weeks of storage. At pH 3.0, a study by Sahadeva et al. (2011) stated that, too acidic pH could cause complete destruction of all cells which outnumbers the rate of cells dying inside the probiotic drinks. Besides, this product might not be consumable after 6 weeks which shows directly proportional with the estimated shelf life based on Figure 4.7.

4.2.2 Microbiological Analysis

Microbial analysis of this study was carried out by using Total Plate Count method to determine the total viable count of probiotics inside all of the samples, Em/Cmc and Fm/Cmc including the control, Vitagen. Based on the (Atlas, 2010), Total Plate Count test was susceptible to be conducted as it was suitable for the 'cultivation and maintenance of a wide variety of microorganisms' (Atlas, 2010). Nutrient agar was selected as the media for growth as this agar inhibit the result obtained from all sorts of bacteria including the *Lactobacillus*, *Bifidobacteria* as well as the *Streptococcus* species. Moreover, this method includes the use of spread plate method as this method was compatible to be carried out than pour plate method. Spread plate method results in providing higher colony count compare to the pour plate methods. Besides, it was also accurate as well as improve the colony development and appearance of the bacteria to be observed (Taylor, Allen, & Geldreich, 2014).

Based on Table 4.2, the total viable count of probiotics for sample Em/Cmc in initial condition and Em/Cmc sample in HDPE packaging shows an increase in value from 10^3 to 10^4 CFU/mL. Besides, for sample Fm/Cmc sample in initial condition and in both HDPE and pouch packaging also shows high in colony count which was from 10^4 to 10^5 CFU/mL. As for the control sample, there was a slight increase in total viable count from week 0 to week 8. According to (Khodke, Shinde, & Yenge, 2015), increasing in storage period would increase the total viable count. However, based on study from (Dobrucka, 2013; Hsiao, Lian, & Chou, 2004), the colony count should have lower in number count when high permeability of packaging materials were used as pouch packaging (PET materials) and HDPE packaging (Siracusa, 2012).

Table 4.3 : Colony count of probiotic drinks at week 0 and week 8.

SAMPLES/TREATEMENT	WEEK	COLONY COUNT (CFU/mL)
Em/Cmc	0	$9.50 \times 10^3 \pm 3.54 \times 10^3$
Fm/Cmc	0	$1.15 \times 10^4 \pm 2.21\ 10^3$
Vitagen (Control)	0	$1.30 \times 10^4 \pm 5.66 \times 10^3$
Em/Cmc - Hdpe Packaging	8	$1.10 \times 10^4 \pm 3.30 \times 10^3$
Em/Cmc - Pouch Packaging	8	$1.55 \times 10^5 \pm 6.58 \times 10^4$
Fm/Cmc - Hdpe Packaging	8	$9.42 \times 10^4 \pm 1.09 \times 10^5$
Fm/Cmc - Pouch Packaging	8	$1.80 \times 10^5 \pm 5.67 \times 10^4$
Vitagen (Control)	8	$1.40 \times 10^4 \pm 1.41 \times 10^3$

* a, b, c represents the homogenous subset of probiotic drinks samples

The decrease in number of colony should be the cause from the *Bifidobacteria* species which was more sensitive than lactic acid and strictly anaerobic. In relation with decrease in pH during storage period might be the reason why the decrease in number of colony count due to its sensitive towards acidic pH especially for *Bifidobacteria* species. The colony count of the probiotic drinks should be retain or decrease in value, which was not compatible with the result obtained. Besides, as the colony count from the result were increasing based on Table 4.2, this might be due to the contamination occurred during conducting the experiment. The sample also might be contaminated after long storage in different packaging.

Based on Table 4.4, there are significantly difference colony count for different samples in different packaging conditions. The highest value of colony count shows in Fm/Cmc samples inside pouch packaging while the lowest colony count was calculated in Em/Cmc samples in HDPE packaging. This result was comparable with what had been

obtained by (Khodke et al., 2015) which shows increase in colony count after storage. Based on the result in Table 4.3, Em/Cmc in HDPE packaging give the best result after storage packaging as it shows the lowest bacteria count after 8 week of storage. According to (Zygoura et al., 2004), HDPE packaging can preserve packed products by retaining the nutrients inside the products compare to other types of packaging. However, it might need the implementation of additional packaging materials like monolayer and multilayer properties in order to retain longer shelf life of products.

Table 4.4 : Colony count of probiotic drinks based on samples and packaging of the products.

Samples and Packaging	Colony Count (CFU/mL)
Em/Cmc	9.50×10^{3} f
Fm/Cmc	1.15×10^{4} d
Em/Cmc – HDPE packaging	1.08×10^{4} e
Em/Cmc – Pouch packaging	1.55×10^{5} b
Fm/Cmc – HDPE packaging	9.42×10^{4} c
Fm/Cmc – Pouch packaging	1.78×10^{5} a

* a, b, c represents the homogenous subset of probiotic drinks samples

Moreover, the increase in microbial load of the samples in Em/Cmc and Fm/Cmc might be due to the susceptibility of protein and fat content of the samples which was a perfect medium for microbial growth (Khodke et al., 2015). Additionally, based on (Kregiel, 2015), pouch packaging, which was made out of PET packaging materials also can be susceptible to absorption of organic matters when long storage condition was applied which

can cause adhesion of microbes to bottle surfaces. This condition can provide microhabitat which was suitable for microflora and thus lead to contamination (Kregiel, 2015).

On the other hands, as stated by Guevarra & Virginia (2016), the minimum viable count of probiotic drinks should be 10^6 CFU/mL and some had stated the recommended viable count of probiotics of between 10^8 to 10^9 CFU/mL for therapeutic merit, in order to confer health benefits from the product. High number in viable count of probiotic was needed as viable cells will decline after consumed. The probiotic need to survived the acidity inside stomach as well as the enzymes and bile salts in the intestine. Based on the result, there were less number of viable count obtained from all of the samples. The need to incorporate high number of viable count was crucial to confer ideal microbial activity of probiotics at the consumption time.

4.3 Physico-chemical Properties of Probiotic Drinks

4.3.1 Fat Content

From Table 4.5, fat content of all the probiotic drinks samples, Fm/Cmc, Em/Cmc as well as the Vitagen samples were tested in order to evaluate their fat content. The determination of fat content was done using Soxhlet method. This method was chosen because this method was a comprehensive lipid analysis that had been used for most food and drinks (Vishnuraj, Gurunathan, Shukla et al., 2016). Furthermore, the samples were extracted using petroleum ether solvents. This solvent was chosen due its non-polar, low

boiling, cheaper and non-flammable properties. The suitability of solvents used is crucial as solvents function to trapped lipid that will help in breaking up the polar barriers. The solvents also functional to let reach the non-polar compound and extract the fat (Bergeron & Benning, 2002).

Next, as most of this method was uses for solid food, the need of drying the samples was also crucial. Same goes to liquid products. Liquid samples need first to be dried by spray drying process. The reason for drying was to make sure that the solvents can easily penetrates into foods that would lead to have an efficient fat extraction. Besides, as the sample was drier, it was also finely ground in order to produce more homogenous sample as well as accelerating the surface area of lipid to be exposed to the solvent. Additionally, the grinding process of the sample also important to be carried out at low temperature in order to reduce the probability of lipid oxidation that might effected the lipid extraction later (McClements, 2018). In addition, fat also can confer important sensory properties like creamy and smooth texture which intervenes the intensity, balance and gave great after effect (Mourad & Bettache, 2014).

Moreover, Table 4.5 shows that fat content of Em/Cmc sample was higher compare to the Fm/Cmc and Vitagen Samples which is 3.9 %. This value attainable with what had stated by (Brasileiro, 2014) that the value of fat content inside probiotic drinks should be above 3 % which also supported by (Irigoyen, Arana, Castiella et al., 2005). Besides, high fat content in Em/Cmc also relatable with the sources of milk used to prepare this sample which was evaporated milk. Based on (Nouh et al., 2017), evaporated milk involve the evaporation process of removing 60% of water content inside the milk which would led in high concentration of milk product. A study from Nouh et al. (2017), the legislation stated

from (Helrich, 1990) had confirmed that, the level of fat content to be declare for evaporated milk was 9 %. As it was part of a mixture from the formulation of probiotic drinks for sample Em/Cmc, the level of fat content should be lower than 9 % which was correspond with the result obtained.

Table 4.5 : Fat content of probiotic drinks

	FAT CONTENT
Fresh Milk + Carboxymethylcellulose (Fm/Cmc)	2.300 ± 0.050^{b}
Evaporated Milk + Carboxymethlcellulose (Em/Cmc)	3.913 ± 0.180^{b}
Vitagen (Control)	2.547 ± 0.176^{a}

* a, b, c represents the homogenous subset of probiotic drinks samples

Additionally, based on Table 4.5, the fat content of Vitagen is significantly different in Fm/Cmc and Em/Cmc. As what had been stated on Vitagen packaging, the fat content inside this drink should be 0 %. However, based on the result obtained, the fat content of this control was contrary with what had been stated. The level of fat content inside this sample was higher than in Fm/Cmc sample. The level of fat content in Fm/Cmc sample should be low as it was prepared with fresh milk ingredients which has significantly low level of fat than in evaporated milk. This indicates why the fat content in Fm/Cmc has low level of fat content. From here, it shows that, different types of milk sources would lead to different in fat content value.

4.3.2 Total Sugar Content (Brix Analysis)

Based on Table 4.6, it shows that the total sugar content by Brix analysis was determined to estimate the sugar content inside all the probiotic samples. Total sugar content was determined by total soluble solid method, using a digital refractometer. A digital refractometer was used instead of a hand held refractometer as it is chemical resistant and sealed against moisture ingress. Besides, the traditional handheld refractometer also in need of light to be transmitted by the sample in order to see the measurement (Crawford, 2011).

Table 4.6 : Total sugar content (Brix Analysis) of probiotic drinks samples.

BRIX ANALYSIS (TOTAL SUGAR CONTENT)	
Fresh Milk + Carboxymethylcellulose (Fm/Cmc)	8.033 ± 0.058^{c}
Evaporated Milk + Carboxymethlcellulose (Em/Cmc)	9.267 ± 0.058^{b}
Vitagen (Control)	14.033 ± 0.058^{a}

* a, b, c represents the homogenous subset of probiotic drinks samples

Moreover, this type of refractometer also cannot control the temperature of the surrounding during measurement that would affect the reading result. Additionally, temperature was also important in determining the brix degree of a sample. Variation in temperature reading the sample may lead to inaccuracy of reading measurement. Thus, it was vital to stabilize the sample temperature. Based on (Pratt & Scientific, 2011), in order to

stabilize temperature control of the sample, the usage of refrigerated bath circulator or laboratory chiller. Furthermore, liquid food products were usually being measured using brix scale. Thus, it was suitable to be measured using digital refractometer rather than handheld refractometer that was measured using refractive index (RI) (Crawford, 2011).

In addition, Vitagen sample result shows high reading of Brix % which indicates that this sample was high in sugar content. As stated by (Wan et al., 2011), the Vitagen product can last for 6 weeks. The incorporation of sugar like natural sweeteners inside the products would confer sweet taste as well as act as a preservative for the products in order to accelerating the shelf life of the product. Natural sweeteners like glucose was a natural carbohydrates sweetener which was the primary source of energy (Kregiel, 2015).

On the other hands, based on Table 4.6, there were significantly different ($p \leq 0.05$) in sugar content for all samples, Em/Cmc, Fm/Cmc and Vitagen. Vitagen result in having the highest level of sugar which is attainable with what had stated by (Prajapati, Reddy, & Sreeja, 2012). In comparison with the other two formulations, the total sugar content was lower due to its formulation which had been reduced in the amount of sugar as cordial was added in order to improve the taste of the formulation, Em/Cmc and Fm/Cmc.

4.3.3 Protein Content

Based on the Table 4.7, protein content was determined for all of the probiotic drinks samples. Protein determination was carried out using Kjeldahl method. Kjeldahl method was an indirect protein determination as it inferred the 'determination of nitrogen content or chemical reaction with functional group within the protein' (Mæhre, Dalheim, Edvinsen, Elvevoll, & Jensen, 2018). This method was chosen as it was already being recognized as standard method for protein determination by AOAC international (Mæhre et al., 2018).

Table 4.7 : Protein content of probiotic drinks samples.

	PROTEIN CONTENT
Fresh Milk + Carboxymethylcellulose (Fm/Cmc)	0.196 ± 0.049^{b}
Evaporated Milk + Carboxymethlcellulose (Em/Cmc)	0.308 ± 0.049^{b}
Vitagen (Control)	0.756 ± 0.084^{a}

* a, b, c represents the homogenous subset of probiotic drinks samples

Moreover, according to (Sáez-Plaza, Michałowski, Navas et al., 2013), Kjeldahl method actually lack of the analytical selectivity to distinguish protein based nitrogen from non-protein nitrogen. However, this method actually overestimates the true protein content which also refer to the other form of organic and inorganic nitrogen inside a sample which made protein determination by Kjeldahl method result in having high yield in protein content

(Mæhre et al., 2018; Moore, DeVries, Lipp et al., 2010; Sáez-Plaza et al., 2013).. Moreover, this indirect method that does not calculate protein content directly need a conversion factor of nitrogen concentration to a protein concentration. As for protein determination for dairy based products, a conversion factor of 6.38 (constant) was used to be multiplied by nitrogen concentration that was obtained, according to (Sehgal, 2016).

Furthermore, this method would involve three main steps which were digestion, distillation and titration. Digestion starts by converting nitrogen inside the sample into ammonium ions. A clear liquid solution indicates the completion of the reactions (Unckell, 2018). The obtained result from Kjeldahl method would confer great result by boiling samples with catalyst and diluting it with sulfuric acid in digestion process. Moreover, distillation was continued in order to distillated the added alkaline liquid into standard acid which convert the ammonium ions into ammonia. The last steps include the titration of the excess acid by standardized hydrochloric acid for the detection of end point to obtained nitrogen concentration (Sáez-Plaza et al., 2013; Unckell, 2018).

In addition, Vitagen sample exhibit the highest protein content than Em/Cmc and Fm/Cmc samples. In comparison with the standard, the Em/Cmc shows a slight increase in protein content which was about 0.3 %. As milk was categorized as most complete food which was rich in protein, this would be the cause of high in protein value of the sample. According to (Dias et al., 2013) the suggested protein content that should be comprise inside probiotic drinks was 1.7 %. This study was not attainable with what had been collected based on the Table 4.7. However, a study from Yerlikaya, Ender, Torunoglu et al., (2015) had stated that the protein content inside probiotic drinks should be in range of 2.7 to 2.9 % which also

supported by (Brasileiro, 2014), as incorporation of added ingredients inside the probiotic drinks may be varied for different drinks.

Besides, based on Table 4.7 the protein content of Vitagen sample do have a significant difference, ($p \leq 0.05$) with the Em/Cmc and Fm/Cmc samples. This might be due to the heat application during homogenizing the sample for Em/Cmc and Fm/Cmc during sample preparation as it might alter solubility as well as the measurement of the protein content (Moore et al., 2010). Homogenization of the sample include the application of heat when blending time of the sample was more than 3 minutes due to the friction of the blender's blade during mixing.

4.4 Shelf Life Estimation

Based on Figure 4.8, Em/Cmc in pouch packaging at 4°C result in having longer shelf life which is 5 weeks. This sample have longer shelf life due to suitability of storage condition at 4°C based on what had stated by (Gamage et al., 2016; Khodke et al., 2015). Furthermore, this type of packaging also had been sterilized which can be one of the reason why there were extension of shelf life than other samples in different packaging. Overall shelf life estimation of Fm/Cmc in HDPE packaging, Fm/Cmc in pouch packaging as well as Vitagen sample shows approximately same shelf life which is 4 weeks. Besides, Em/Cmc sample in HDPE packaging stored both at 4°C and 8°C shows the shortest shelf life due to its high acidity of probiotic drinks which was not consumable anymore to be drinks.

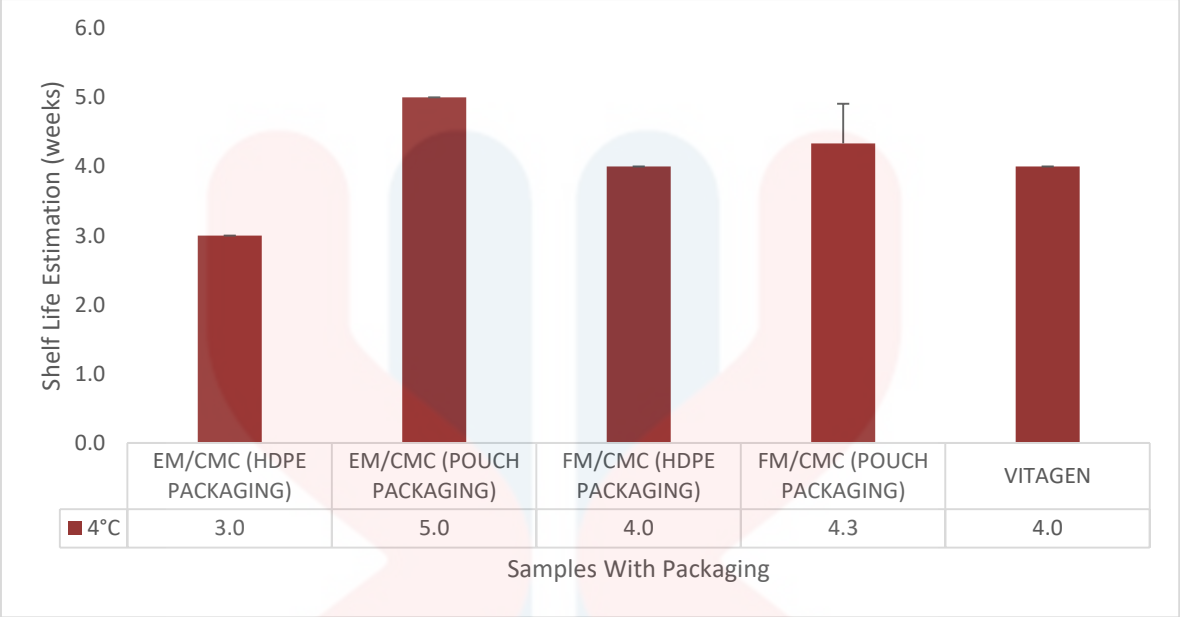


Figure 4.8 : Shelf life estimation of all probiotic drinks in different packaging

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The development of probiotic drinks could not be determined as the sedimentation of the probiotic drink with formulation of fresh milk with carboxymethylcellulose (CMC) still result in having sedimentation. Homogenization process by blade type homogenizer would not be an effective method to prevent sedimentation. On the other hands, the stability of the probiotic drinks evaluated in different packaging types could be determined by chemical and microbiological analysis via pH analysis as well as the total plate count determination. pH analysis result in having significant difference (p<0.05) which rejecting H_0. Besides, colony count of probiotics result in having no significant difference (p>0.05) which accepting H_0. All probiotic drink samples were best stored in pouch packaging in order to obtained longer shelf life. The shelf life estimation for the probiotic drinks result in only ±5 weeks of storage period. Besides, chemical analysis of all of the probiotic drinks samples also had been

evaluated. All of the samples result in having high fat content within range of 0.3 % to 0.8 % with high sugar content within range of 9.0& to 14 %. Protein content also depicts low in value ranging in between 2.0 % to 4.0 %. All physicochemical analysis of the probiotic drinks does have significant difference which results in rejecting $H_{0.}$

5.2 Recommendation

From this study, it can be concluded that further investigations must be performed in order to developed the sedimentation issues of the probiotic drinks. High technology homogenizer is needed in order to prevent separation in probiotic formulation.

On the other hands, full determination of proximate analysis can help in evaluation of nutritional information for the developed product. Proximate analysis function in partitioning of compounds in a feed into six categories based on the chemical properties of the compounds. This include the analysis of moisture, ash, crude protein, crude lipid, carbohydrates and crude fiber.

Besides, the need to prevent pathogenic microorganism to grow in the samples are also vital in order to avoid any probability of contamination before the expiry date. As instance, yeast and mold analysis could be done using potato dextrose agar (PDA). Additionally, Lactobacillus count and Streptococcus count using De Man, Rogosa, and Sharp agar (MRS) and Lee's agar medium respectively. Certain microbes can withstand different rate of incubation for fermentation, thus, it is important to determine what type of strain could resist in low pH condition in order to confer health benefits for human consumption. This

tests also important to be conducted as it help to determine the viability of specific probiotics available in the samples to retain the maximum viable count as recommended which in range of 10^8 to 10^9 CFU/mL.

Moreover, it is important to retain anaerobic condition during the incubation of the plates. The used of anaerobic jar help in retarding any oxygen available that might affect the results. The anaerobic jar also helps by evacuating and replacing the atmosphere with an oxygen-free gas mixture.

In addition, sensory evaluation also crucial to be determined as it determines the level of acceptance from consumers as well as the organoleptic properties of the products itself. More improvement like texture and taste and color can be made based on sensory evaluation.

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APPENDICES

Example of probiotic drinks packed in pouch packaging (PET materials).

The example of probiotic drinks packed in HDPE packaging bottles.

The mean ± standard deviation of pH analysis for all probiotic samples in different packaging at different storage condition.

pH Value									
Samples	Average								
	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Em/Cmc	4.5233		-	-	-	-	-	-	-
Fm/Cmc	4.3933		-	-	-	-	-	-	-
Vitagen	4.5833	-	-	-	-	-	-	-	-
Em/Cmc 4 ℃ Hdpe Packaging	-	4.49	3.82	3.68	3.20	2.82	2.63	2.37	2.05
Em/Cmc 4 ℃ Pouch Packaging	-	4.58	3.75	3.54	3.54	3.79	3.60	3.62	3.60
Fm/Cmc 4 ℃ Hdpe Packaging	-	4.23	3.55	3.54	3.46	3.43	3.38	3.30	3.22
Fm/Cmc 4 ℃ Pouch Packaging	-	4.35	3.60	3.58	3.44	3.41	3.39	3.23	3.21
Vitagen 4 ℃	-	4.44	3.82	3.80	3.77	3.73	3.65	3.62	3.60
Em/Cmc 8 ℃ Hdpe Packaging	-	4.35	3.54	3.35	2.96	2.40	2.05	1.91	1.78
Em/Cmc 8 ℃ Pouch Packaging	-	4.35	3.70	3.63	3.56	3.56	3.45	3.29	3.24

Continue….

Fm/Cmc 8 ℃ Hdpe Packaging	-	4.17	3.48	3.41	3.36	3.16	3.17	2.79	3.09
Fm/Cmc 8 ℃ Pouch Packaging	-	4.20	3.51	3.43	3.40	3.33	3.40	3.36	3.15
Vitagen 8 ℃	-	4.36	3.78	3.73	3.68	3.67	3.64	3.66	3.62

Continue….

pH Value									
	Standard Deviation								
Samples	Week 0	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Em/Cmc	0.0252	-	-	-	-	-	-	-	-
Fm/Cmc	0.0416	-	-	-	-	-	-	-	-
Vitagen	0.0208	-	-	-	-	-	-	-	-
Em/Cmc 4 ℃ Hdpe Packaging	-	0.03	0.03	0.05	0.02	0.02	0.06	0.06	0.01
Em/Cmc 4 ℃ Pouch Packaging	-	0.05	0.01	0.05	0.01	0.01	0.01	0.01	0.01
Fm/Cmc 4 ℃ Hdpe Packaging	-	0.01	0.02	0.01	0.06	0.02	0.01	0.03	0.02

Fm/Cmc 4 ℃ Pouch Packaging	-	0.03	0.02	0.01	0.01	0.01	0.02	0.03	0.01
Vitagen 4 ℃	-	0.03	0.01	0.03	0.01	0.02	0.02	0.02	0.01
Em/Cmc 8 ℃ Hdpe Packaging	-	0.01	0.01	0.02	0.01	0.03	0.02	0.01	0.07
Em/Cmc 8 ℃ Pouch Packaging	-	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Fm/Cmc 8 ℃ Hdpe Packaging	-	0.01	0.03	0.01	0.01	0.01	0.01	0.02	0.01
Fm/Cmc 8 ℃ Pouch Packaging	-	0.02	0.01	0.02	0.01	0.02	0.01	0.01	0.01
Vitagen 8 ℃	-	0.01	0.01	0.01	0.03	0.01	0.01	0.01	0.01

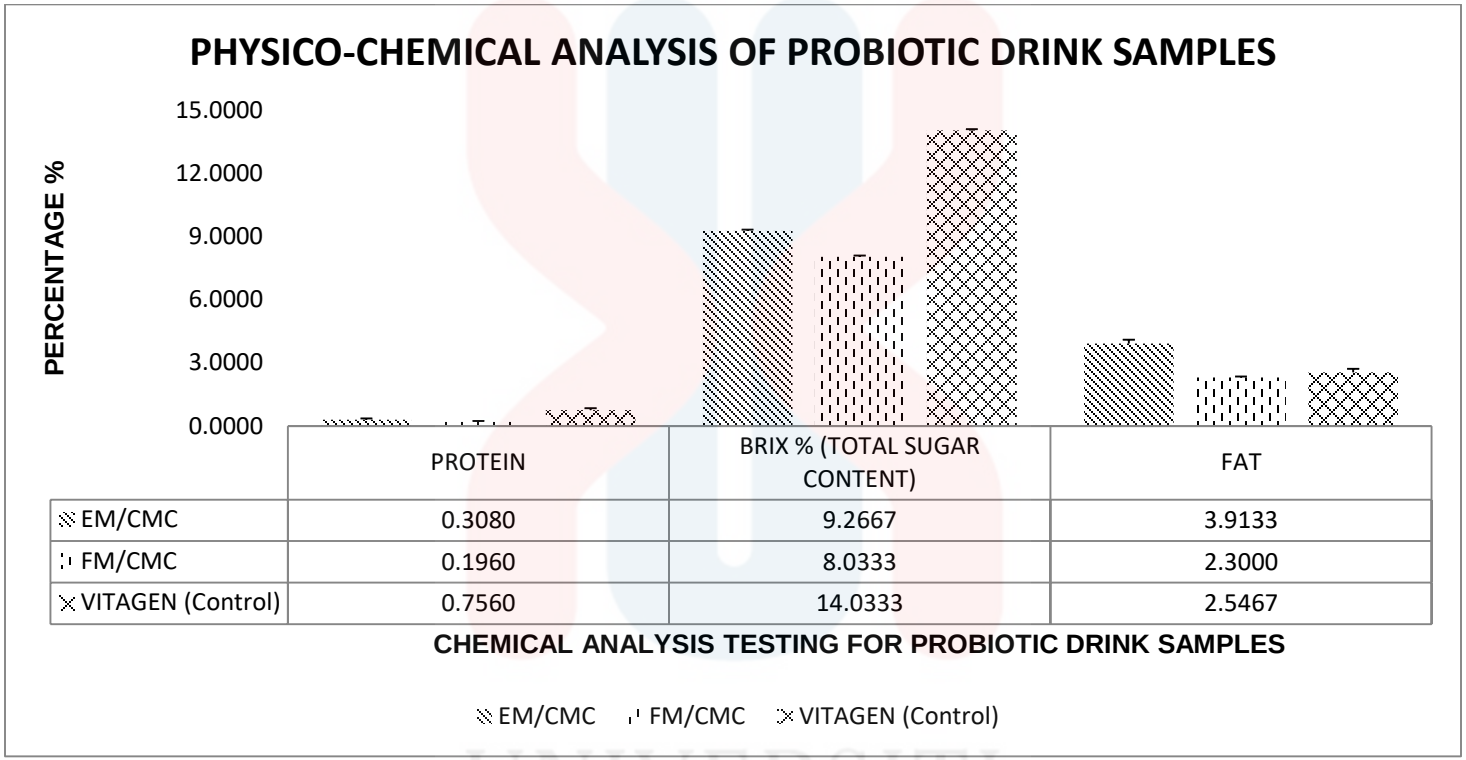
The mean ± standard deviation of total plate count for all probiotic samples in different packaging at different storage condition at week 0.

0 WEEK (Based On Dilution of 10^{-3})		
Samples	Average	Standard Deviation
Em/Cmc	9.50E+03	3.54E+03
Fm/Cmc	1.15E+04	2.12E+03
Vitagen	1.30E+04	5.66E+03

The mean ± standard deviation of total plate count for all probiotic samples in different packaging at different storage condition at week 8.

Week 8 (Based On Dilution Of 10^{-3})								
Samples /Packaging	HDPE Packaging 4 ℃		HDPE Packaging 8 ℃		Pouch Packaging 4 ℃		Pouch Packaging 8 ℃	
	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation	Average	Standard Deviation
Em/Cmc	1.10E+04	5.66E+03	1.05E+04	7.07E+02	1.55E+05	4.81E+04	1.56E+05	1.03E+05
Fm/Cmc	1.84E+05	6.08E+04	4.50E+03	3.54E+03	1.80E+05	5.16E+04	1.76E+05	8.34E+04
Vitagen (Initial Condition)	4 ℃		8 ℃					
	Average	Standard Deviation	Average	Standard Deviation				
	1.40E+04	1.41E+03	1.30E+05	3.75E+04				

Percentage of physicochemical analysis of probiotic drinks.



	PROTEIN	BRIX % (TOTAL SUGAR CONTENT)	FAT
EM/CMC	0.3080	9.2667	3.9133
FM/CMC	0.1960	8.0333	2.3000
VITAGEN (Control)	0.7560	14.0333	2.5467

The mean ± standard deviation of physicochemical analysis of probiotic drinks samples.

PHYSICOCHEMICAL ANALYSIS						
Samples	**Protein (%)**		**Brix % (Total Sugar)**		**Fat (%)**	
	Average	**Standard Deviation**	**Average**	**Standard Deviation**	**Average**	**Standard Deviation**
EM/CMC	0.3080	0.0485	9.2667	0.0577	3.9133	0.1804
FM/CMC	0.1960	0.0485	8.0333	0.0577	2.3000	0.0500
VITAGEN	0.7560	0.0842	14.0333	0.0577	2.5467	0.1762

The spray drying yield of probiotic drinks samples.

Samples	**Powder Quantity Collected (g)**	**Quantity Introduced In Feed Solution (mL)**	**Spray Drying Yield (%)**
EM/CMC	9.11	450	2.7652
FM/CMC	12.44	450	2.0240
VITAGEN	12.26	450	2.7243

$$\text{Yield of Spray Drying} = \frac{\text{Powder quantity collected}}{\text{Quantity introduced in feed solution}} \times 100$$ (Tan et al., 2015)