CHAPTER 2

LITERATURE REVIEW

2.1 Cardiovascular Disease and Hypercholesterolemia

Cardiovascular disease, or CVD, is the leading cause of death in the world, accounting for approximately 17.9 million fatalities in 2019, with 85% of the cases being due to heart attack and stroke (World Health Organization, 2021). Death often occurs as a result of complications of atherosclerosis, thrombosis, or hypertension, all of which are usually closely related to each other (Solomon et al., 2011). Roughly 25% of deaths among adults in industrialised countries are due to blockage of arteries instigated by atherosclerotic plaque, leading to heart attacks (Goldstein and Brown, 2015).

Indeed, CVD is not only responsible as a major contributor to the world's cause of death, but also produces considerable disability and reduced comfort amongst surviving patients (Ahmed et al., 2017), as well as a significant economic burden (O'Morain and Ramji, 2019). The current report from the Malaysian Ministry of Health (MOH) and the World Health Organization (WHO) discloses that noncommunicable diseases, principally CVD, diabetes and cancer, have cost nearly RM 9 billion in productivity losses due to absenteeism in the workplace and premature death among the working age population in Malaysia. Apart from this value, the combination of diseases also causes approximately RM 100.79 billion of the burden of disease cost due to disability and loss of healthy life years (World Health Organization, 2020a).

Generally, CVD refers to any disorders that affect the heart and blood vessels. It comprises various types of conditions that either develop at the same time or lead to the development of other ailments, such as:

i) coronary heart disease, which affects the vessels supplying blood to the heart muscle;
ii) cerebrovascular disease, which relates to vessels supplying blood to the brain;
iii) peripheral arterial disease, which affects blood vessels located outside the heart and brain;
iv) rheumatic heart disease, which is a condition of permanent damage to the heart valve secondary to rheumatic fever;
v) congenital heart disease, which is a malformation that is present at birth and could affect the structure development and function of the baby's heart;
vi) deep vein thrombosis, which is a blood clot that forms in the deep veins and could dislodge and move to other parts of the body, such as the lungs and cause pulmonary embolism (World Health Organization, 2021).

Numerous studies have been done over the 20th century to prove the link between hypercholesterolemia and CVD (Goldstein and Brown, 2015; Pang et al., 2015; Perak et al., 2016). People with hypercholesterolemia have roughly three times the risk of getting a heart attack when compared to those with normal ranges of blood lipids (Kumar et al., 2012). For each 1 mmol increment of cholesterol above the normal level, a person has approximately a 35% and 45% higher risk of CVD and death, respectively (Wang et al., 2014), while a 1% serum cholesterol drop is correlated with a 2 to 3% decline in the risk of some CVD conditions (Liu et al., 2017). The analysis of acute heart attack cases incidences in Iraq reported that improved cholesterol control can reduce about 20% of cardiovascular complications (Ahmed et al., 2017). Both heart

attacks and strokes are principally caused by prevention of blood flow to the heart or brain, most commonly due to blockage by fatty deposits or cholesterol on the endothelial wall. Apart from this, strokes may also happen due to bleeding from blood vessels (World Health Organization, 2021).

Cholesterol is a waxy substance that is important to animal cells. The normal supply of cholesterol in the body is crucial as cells use cholesterol to make up the cell membrane, certain hormones, bile acids, and certain vitamins (Solomon et al., 2011; Yu et al., 2014). However, an increase in cholesterol above the normal range can cause a variety of pathological problems. This condition is further distinguished by several lipid levels which consist of serum total cholesterol (TC) (> 5.2 mmol/L), triglycerides (TG) (> 1.7 mmol/L), high-density lipoprotein cholesterol (HDL-C) (< 1.0 mmol/L for males and < 1.2 mmol/L for females), and low-density lipoprotein cholesterol (LDL-C) (multiple values depending on individual's health condition (Management of Dyslipidaemia, 2017). LDL-C is called "bad" cholesterol as it carries cholesterol to tissues, including the arteries. The higher the level of LDL-C in the blood, the greater the risk of CVD. While HDL-C is also known as "good" cholesterol because it takes cholesterol from tissues to the liver, which removes it from the body (Solomon et al., 2011). High levels of cholesterol or hypercholesterolemia often show no symptoms while slowly causing the formation of clogged arteries known as atherosclerosis before the clinical manifestations become evident.

Atherosclerosis, which originated from the Greek word *atheros* (gruel), describes the cheesy materials exuded from the sectioning of plaques (Goldstein and Brown, 2015). The development of atherosclerosis is affected by multiple risk factors that can be altered, such as smoking, obesity, and dyslipidemia, or unaltered factors such as age, gender and genetic factors, for example, familial hypercholesterolemia (FH) and

Tangier disease (O'Morain and Ramji, 2019). The term atherosclerosis means thickening of the intimal layer of arteries and accumulation of fat, and it consists of two parts: the first is atherosis, or the accumulation of fat accompanied by various macrophages; and the second is sclerosis, which is the fibrosis layer encompassing smooth muscle cells, leukocytes, and connective tissue (Rafieian-Kopaei et al., 2014). Being the underlying cause of multiple CVD, atherosclerosis is a disorder of chronic inflammation of the blood vessels with a slow onset but that has increased substantially among the elderly. The most common pathogenicity of atherosclerosis involves the increase in LDL-C levels in conjunction with a decrease in HDL-C levels (Yang et al., 2021).

The excess LDL-C in the blood enters the inner layer of blood vessels that is made up of endothelial cells, hence becomes oxidised by free radicals and accumulates between and beneath the endothelial cells (Solomon et al., 2011). The accumulation of LDL-C causes phagocytosis by macrophages, and the macrophages will be transformed into foam cells (Yu et al., 2013). Collective volumes of foam cells lead to fatty streaks development that are not clinically significant but are the precursor of more lesions in the progression of atherosclerosis (Lusis, 2000). The accumulation of fatty substances, smooth muscle fibers, collagen, macrophages, and other cells will gradually form the atherosclerotic plaque. As plaque forms, the blood vessels eventually lose their ability to stretch, the diameter of the lumen declines, and it gradually becomes obstructed (Solomon et al., 2011). Hence, the maintenance of cholesterol within the normal range is necessary to avoid its overload, which could lead to the development of atherosclerosis.

2.2 Homeostasis of Cholesterol

Although cholesterol is synthesised by most cells, the amount is not sufficient for their needs. Therefore, the cells must rely on additional cholesterol delivered by the blood, which is derived either endogenously or exogenously from the diet. Homeostasis of cholesterol in the body necessitates precise metabolic interactions between *de novo* cholesterol synthesis in the liver, the highly fluctuating dietary cholesterol ingestion, the reabsorption back of cholesterol into the circulation from the intestines, as well as biliary and faecal excretion (Kruit et al., 2006; Yu et al., 2014). It involves a compensatory mechanism that shifts the pathways in reverse directions, so when the cholesterol synthesis escalates, the cholesterol absorption will decline, and the other way around (Afonso et al., 2018). A disruption in homeostasis can cause not only CVD but also an increase in the number of cancers and neurodegenerative diseases (Luo et al., 2019).

The liver is the main site of various mechanisms involving cholesterol homeostasis (Trapani et al., 2012). This includes the biosynthesis of cholesterol, which starts with acetylCoA, the major precursor that gives rise to hydroxyl methylglutaryl-CoA (HMG-CoA). Then, HMG-CoA is catalysed by HMG-CoA reductase to mevalonate, the key organic compound in cholesterol synthesis. The cholesterol undergoes an esterification process by Acyl-CoA cholesterol acyltransferase before it is transported to the gallbladder and secreted into the small intestine together with bile salts (Rogers et al., 2015).

The amount of cholesterol in the intestine is greatly influenced by endogenous sources between 800 and 1000 mg, compared to an exogenous source from the daily diet of about 300 to 400 mg (Afonso et al., 2018). The absorption rates vary substantially between 20% and 80% (Paalvast et al., 2017), proving the impact of both metabolic and

genetic factors in the regulation of cholesterol absorption (Cohn et al., 2010). As it is minimally soluble, the unesterified or free cholesterol is packed in the form of mixed micelles together with bile salt, triglycerides, and phospholipids and is absorbed into the enterocytes (Iqbal and Hussain, 2009). Within the enterocyte, the esterification of the absorbed cholesterol molecule with fatty acid will occur at the endoplasmic reticulum before it is packed into chylomicrons or HDL-C and is secreted into the lymph and consequently enters the blood vessels (Chen and Davidson, 2012). The reesterification process is an important regulator of intestinal cholesterol absorption because it increases the diffusion gradient to facilitate the entry of intraluminal cholesterol into the enterocytes (Iqbal and Hussain, 2009). The cholesterol uptake into the enterocyte is mediated by passive diffusion, and the uptake volume increases following the concentration of unesterified cholesterol in the lumen (Nakano et al., 2019).

The balance of cholesterol absorption and secretion in the gastrointestinal tract is modulated by various proteins (Figure 2.1) Niemann-Pick C1-Like 1 (NPC1L1) is a transmembrane protein responsible for dietary cholesterol and biliary cholesterol absorption in the intestine, which is located in the brush border of the enterocytes and is endocytosed upon exposure to cholesterol on the apical membrane (Nakano et al., 2019). Mice that were deficient in or inhibited by NPC1L1 showed a decrease in cholesterol absorption of more than 70% (Paalvast et al., 2017). Another study on the impact of diosgenin, a steroidal saponin found in tubers of wild yam, demonstrated that diosgenin significantly decreased the expression of NPC1L1, causing a reduction in the serum total cholesterol, triglycerides, and LDL-C in hypercholesterolemic Sprague-Dawley rats compared to the control group (Li et al., 2019b).

Scavenger Receptor Class B Member 1 (SCARB1) is the protein transporter responsible for HDL-C uptake and is most abundantly found in the liver (Shen et al., 2018). Besides the liver, SCARB1 is also expressed in intestinal cells, adipose tissue, vascular endothelial cells, smooth muscle cells, and some immune cells (Wang et al., 2020). SCARB1 contributed to approximately 25% cholesterol absorption (Lim et al., 2017). The cholesterol absorption of transgenic mice with overexpression of SCARB1 was significantly higher than of wild-type mice, suggesting that this protein could act as a cholesterol transporter in the small intestine (Yamanashi et al., 2017). The study on the effects of pemafibrate, the lipid-lowering drug, on intestinal cholesterol transporter regulation using Sprague-Dawley rats concluded that the drug may inhibit intestinal cholesterol absorption by downregulating the expression of both SCARB1 and NPC1L1 (Tanaka and Kamisako, 2021).

Meanwhile, Cluster of Differentiation 36 (CD36) gene is expressed in various cells, for example platelets, smooth muscle cells, adipose tissues, and intestinal epithelial cells. The expression of this transmembrane protein is abundant on the brush border membrane of the enterocytes (Zhao et al., 2021). CD36 plays an important role in regulating the transport of sterols and fatty acids across the intestinal cells (Lim et al., 2017). Moreover, CD36 may regulate the homeostasis of cholesterol by being involved in the synthesis of cholesterol, the formation of lipoprotein and reverse cholesterol transport (Ulug and Nergiz-Unal, 2020). It was reported that CD36-knockout mice retained more cholesterol in the intestinal lumen, suggesting its contribution to cholesterol absorption into the enterocytes (Nassir et al., 2007).

Liver X receptor (LXR) α, also known as Nuclear Receptor Subfamily 1 Group H Member 3 (NR1H3), are nuclear receptor proteins expressed in a variety of organs including the liver, intestine, adrenal glands, lungs, and kidney (Colin et al., 2008). The

activation of the NR1H3 gene indirectly affected the cholesterol modulation by downregulating NPC1L1 expression and upregulating the expression of ATP-binding cassette transporters sub-family G member 5 (ABCG5) and member G 8 (ABCG8) in the small intestine. Both ABCG5 and ABCG8 are the apical transporters that work antagonistically with NPC1L1, excreting cholesterol and non-cholesterol sterols from enterocytes back into the intestinal lumen (Chen and Davidson, 2012). As this transporter is heteromeric and both genes generate half of the proteins, the absence of either gene will not form a functional transporter.

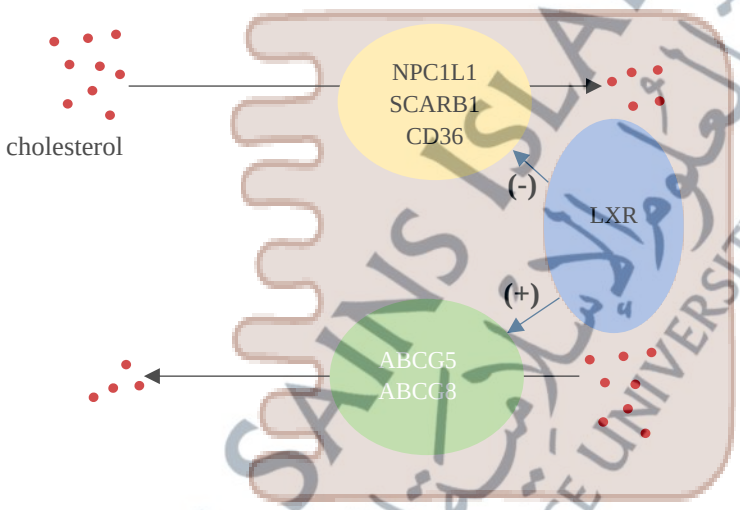


Figure 2.1: Regulation of Cholesterol Absorption and Excretion by Proteins in The Enterocytes

Approximately 30% of cholesterol is excreted from the total cholesterol synthesised per day in healthy adults (Ishimwe et al., 2015), either by the hepatobiliary pathway or the transintestinal cholesterol excretion (TICE) pathway. The prior pathway involves HDL-C, which mediates the transport of cholesterol from peripheral tissues to the liver, where the cholesterol will be converted into bile salt or secreted as unesterified cholesterol into bile before it is excreted via faeces (Stieger, 2003). Meanwhile, the

TICE pathway started with cholesterol uptake at the basolateral membrane of the enterocyte and translocation of cholesterol to the apical side to be excreted into the intestinal lumen with partial reabsorption of the secreted cholesterol by NPC1L1 (Grefhorst et al., 2019). The cholesterol excretion from the body through epithelial cell sloughing or shedding is also included as a non-regulatory fraction of TICE (Nakano et al., 2019).

As the intestine participates actively in cholesterol excretion and is an important source of HDL-C, the intestine becomes one of the strategic targets in the manufacture of therapeutic agents to lower cholesterol and the risk of CVD (Kruit et al., 2006). The inhibition of cholesterol absorption and the excretion of cholesterol through the TICE pathway have always been studied separately in the findings of hypercholesterolemia treatment (Nakano et al., 2019). However, since these two events are interconnected to one another, research considering both mechanisms is needed when aiming at the small intestine for prevention or treatment.

2.3 Hypercholesterolemia Treatment

The interesting fact about hypercholesteremia is that this condition can be overcome using a variety of approaches. Preferably, cholesterol-lowering therapy should be commenced before the development of atherosclerotic plaques or at least before the developed plaques become threatening (Goldstein and Brown, 2015). Often, lifestyle changes are the first line of defence which can help lower the cholesterol back to a normal level, such as dietary intervention, behaviour modification, and frequent physical activity. However, high blood cholesterol could also be treated using therapeutic approaches (Bhat and Bajaj, 2020).

Modification of diet is an example of non-pharmacologic intervention that can be done to reduce blood cholesterol concentrations, for example, incorporating fatty acids, phytosterols, vitamin C, soy, non-soy legumes, and nicotinic acid into the diet (Guo et al., 2011; Barkas et al., 2020). For example, tempeh can minimise the risk of plaque development due to its constituents that can inhibit the enzyme responsible for cholesterol biosynthesis and prevent LDL-C oxidation (Harmayani et al., 2019). The consumption of fermented milk in large amounts also provides a factor that impairs cholesterol synthesis and thus leads to a hypocholesterolemic effect (Hasan et al., 2014). Besides, the intake of complex carbohydrates, fruits, and vegetables can also reduce hypercholesterolemia and decrease the risk of CVDs as it promotes satiation, hence it could lower the consumption of foods high in refined carbohydrates, fat, and cholesterol (Ishimwe et al., 2015).

Another example of health intervention to treat hypercholesterolemia that is not based on medication is behaviour adjustment. Smoking cessation is significant as this behaviour has been linked to plaque vulnerability and carotid artery atherosclerosis, as well as the development of multiple CVD (Kiriyama et al., 2019). Meanwhile, regular aerobic exercise for at least 2 h per week can increase HDL-C levels up to 0.06 mmol/L while decreasing total cholesterol, LDL-C and triglyceride levels approximately by 0.08 to 0.10 mmol/L (Kelly, 2010). Although exercise training appears to be beneficial in preventing hypercholesterolemia (Lavie et al., 2019), it does not reduce CVD incidents or mortality in the elderly or people with chronic conditions (Ballin and Nordström, 2021).

Besides therapeutic lifestyle changes, treatments using drugs with various modes of action may be necessary to achieve the target cholesterol level (Figure 2.2). Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)

reductase, the rate-controlling enzyme in the hepatic cholesterol biosynthesis pathway (Kruit et al., 2006). The use of these drugs results in upregulation and expression of LDL-C receptors on the hepatocytes and better clearance of LDL-C from the blood, which leads to a decline in LDL-C and triglyceride levels (Sirtori, 2014). Its multiple benefits include the direct effect on atherosclerosis, such as stabilising the plaque, reducing the plaque proliferation and inflammation, as well as reducing platelet activation, and other beneficial properties including anticoagulation, vasodilation, antioxidant effects, and a decrease in inflammation mediators (Trentman et al., 2017).

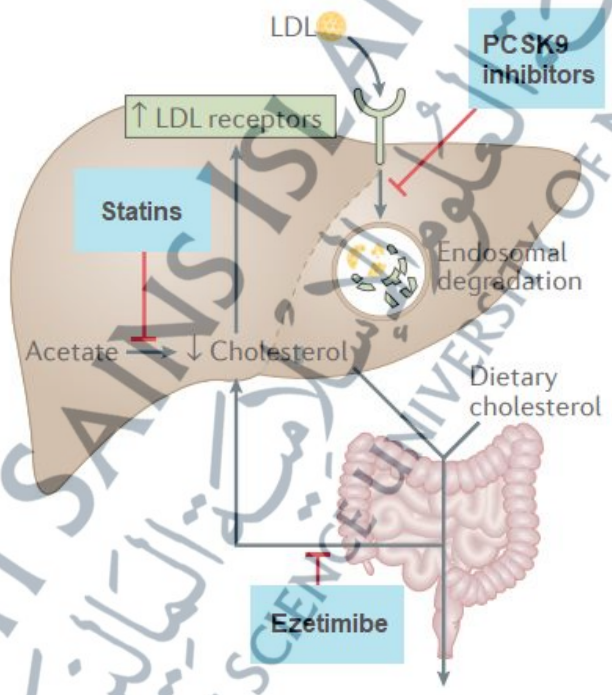


Figure 2.2: Schematic Overview of Drugs (Statins, Ezetimibe and PCSK9 Inhibitors) Used to Treat Hypercholesterolemia and Its Mode of Action (Grundy, 2016).

Almost similar to statins, proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors also lower cholesterol levels by affecting the LDL-C receptors. PCSK9 is a protein involved in the degradation of LDL-C receptors in the liver, thus blocking the binding of PCSK9 to the receptors could decrease the degradation of the receptors

(Grundy, 2016). The higher density of LDL-C receptors at the hepatocyte cell surface leads to increased clearance of LDL-C, resulting in a reduction of cholesterol levels (Management of Dyslipidaemia, 2017). In a clinical programme, the monoclonal antibodies that act as inhibitors against PCSK9 were shown to show up to a 60% reduction in LDL-C levels, including patients prescribed with the maximum dose of statin (Sabatine, 2019).

The next example of drug to treat hypercholesterolemia is ezetimibe, which reduces the intestinal absorption of both dietary and biliary cholesterol (Kosoglou et al., 2005). Ezetimibe functions by blocking the vesicular endocytosis of NPC1L1, the protein responsible for mediating cholesterol uptake into enterocytes and causing their retention in the plasma membrane (Xie et al., 2012). This action also keeps the cholesterol in the intestinal lumen for elimination through faeces and causes a significant decrease in LDL-C. This drug, which can be prescribed as monotherapy or in combination with a statin, has a positive influence on the development of atherosclerosis and reduction of CVD (Phan et al., 2012). A combination of ezetimibe and statins was significantly more effective in managing hypercholesterolemia compared to statin monotherapy (Lamb, 2020).

However, treatments for CVD using drugs are often discontinued or missed for several reasons such as cost of medication, affordability, unclear label instructions, and patient perception of having to take medication (Management of Dyslipidaemia, 2017). According to World Health Organization (2021), low- and middle-income countries accounted for more than three-fourths of CVD deaths. The use of statins and ezetimide is considered quite expensive; it requires around USD $250 per month (Trentman et al., 2017). Moreover, although statin are the main drugs used to lower cholesterol, significant numbers of patients who receive these drugs do not reach their target

cholesterol levels or have intolerance to the drug (Phan et al., 2012). The use of drugs is also associated with multiple side effects such as myopathy, increased liver enzymes, diarrhea, headache, nasopharyngitis, as well as abdominal and limb pain (Management of Dyslipidaemia, 2017).

These situations prompted scientists to continuously find alternative ways to treat CVD, which includes nutraceutical therapies that help to support pharmacological drugs. Nutraceuticals are defined as foods comprised of molecular complexes with medicinal effects on human health and include food supplements, products derived from herbs, medical foods, prebiotics, and probiotics (Shinde et al., 2014; Piemontese, 2015). For instance, ayurvedic physicians believe that eating a small amount of ginger and turmeric every day can lower cholesterol and prevent blood clots, thus lowering the risk of heart attack (Ahmed et al., 2017). However, most plant-based medicines have little scientific or clinical data to support their effectiveness in therapeutic use.

Lowering cholesterol using microorganisms, for example, probiotics, seems to be effective due to multiple reports of its effectiveness. The investigation into the potential cholesterol lowering ability of *Lactobacillus plantarum* DMDL 9010, a novel bacterial strain isolated from naturally fermented mustard, showed that the strain could significantly lower serum total cholesterol, LDL-C and atherosclerosis index, although it did not display a significant effect on serum triglycerides reduction and HDL-C increase, thus making it prospective bacteria to reduce CVD risk (Liu et al., 2016). Probiotic supplements can also significantly reduce serum total cholesterol, with longer intervention time and consumption of probiotic capsules contributing to a better therapeutic outcome (Wang et al., 2018).

2.4 Probiotics

The human gastrointestinal tract comprises numerous bacterial populations known as gut microbiota (Thursby and Juge, 2017). The microbiota is distributed along three main locations, which are in the stomach with a bacterial population of approximately 10^2 colony-forming units (cfu)/mL, the ileum with a bacterial population of approximately 10^2 to 10^3 cfu/mL and the large intestine with a bacterial population of approximately 10^{10} to 10^{12} cfu/mL (Behnsen et al., 2013). The diverse bacteria along this system includes *Lactobacillus, Veillonella, Helicobacter* in proximal gut, Streptococcaceae, Actinomycinaeae and Corynebacteriaceae in duodenum and small intestines, as well as higher ratio of Enterobacteriales, Lachnospiraceae and Bacteroidetes in the colon (Dieterich et al., 2018; Villmones et al., 2020). These microorganisms are interdependent with the host and play roles such as the development of the intestinal immune system, digestion of food, and prevention of pathogen colonisation (Behnsen et al., 2013). In addition to genetic factors, age, personal hygiene, infections, and medication (Dieterich et al., 2018), diet is considered the main influence in shaping the diversity of the microbiota in each individual across their lifetime (Thursby and Juge, 2017), one of which is probiotic (Maukonen and Saarela, 2015).

The term "probiotic" was introduced in 1953 by the German scientist Werner Kollath with the definition of "active substances that are essential for a healthy development of life." Later, in 1965, Lilly and Stillwell redefined the word as "substances secreted by one organism which stimulate the growth of another." In 1992, Fuller constructed a more accurate definition for probiotics, which is "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Gasbarinni et al., 2016). In 2001, due to emerging research in the field of probiotics, the Food and Agriculture Organization of the United Nations (FAO)

and the WHO working group agreed to redefine probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). The definition was refined again in 2013 with a slight grammatical adjustment as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014).

All the above definitions indirectly highlight the benefits of probiotics to health. In the human gastrointestinal tract, probiotics encourage the host to secrete β-defensin and IgA to suppress the growth of pathogens. In addition, probiotics help to maintain the tight junctions and induced the production of mucus, hence strengthening the intestinal barrier. Probiotics also mediate the secretions of cytokine which influence the proliferation and differentiation of intestinal epithelium and the immune cells, such as T cells (Hemarajata and Versalovic, 2013). Disruption of the microbial niches in the gastrointestinal tract can increase the host's tendency to get several different diseases. This was due to the fact that genes expressed by the gut flora may complement the genes needed for important biological pathways in the human gut that are missing or incompletely encoded in the human genome (Hemarajata and Versalovic, 2013). For example, studies on the influence of microbiota on hosts utilising germ-free animal models showed weakened capacity to combat the pathogens due to immature intestinal mucosal immunity, smaller lymph nodes, and decreased numbers of immune cells such as plasma cells and T-cells (Takiishi et al., 2017).

As probiotics can improve the condition of a disease or prevent its occurrence by restoring the composition of the microbiome in the gastrointestinal tract, modulation of the gut microbiota using probiotic approach has caught the attention of researchers to find a cure for these diseases (Azad et al., 2018). The analysis of genes in samples obtained from healthy volunteers subjected to six-week treatment with probiotic

bacteria, namely *Lactobacillus acidophilus* Lafti L10, *L. casei* CRL-431, and *L. rhamnosus* GG, revealed changes in gene expression profiles of the mucosa of the small intestine (Hemarajata and Versalovic, 2013). Probiotics could also reduce the inflammatory response, for example by reducing C-reactive protein (Valdes et al., 2018), minimise oxidative stress, as well as reduce the permeability of the intestine by increasing the expression of adhesion proteins within the intestinal epithelium (Gomes et al., 2014). These synergistic effects enhance insulin sensitivity and decrease autoimmune responses, thus leading to effective prevention and management of Type 2 and Type 1 diabetes (Gomes et al., 2014). *Lactobacillus acidophilus* KFRI342, *Bifidobacterium longum*, *Lactobacillus gasseri*, *Lactobacillus salivarius*, and *Lactobacillus casei* are considered a novel therapeutic approach as they either prevent the development of colorectal cancer or reduce the adverse effects caused by chemotherapy treatment (Elsalem et al., 2020).

The most investigated probiotic groups are LAB and Bifidobacteria (Azad et al., 2018). Generally, LAB is classified as generally recognised as safe (GRAS) due to the fact that it has been involved in food processing for centuries and the consumption of food containing live or dead bacteria and their metabolites does not result in any reports of any unfavourable effects (Awaisheh, 2012). LAB are suitable as sources of exogenous probiotics because these microorganisms are also important components of the endogenous microbiota in the human ileum, jejunum, and colon (Behnsen et al., 2013).

The selection criteria of a potential probiotic comprise its multiple underlying mechanisms, which fall into three categories:

i) Widespread mechanisms (among studied probiotics): colonisation resistance, acid and short-chain fatty acid production, intestinal transit regulation,

normalisation of disturbed microbiota, increased turnover of enterocytes, and competitive pathogen elimination;

ii) Frequent mechanisms (species-level effects): synthesis of vitamins, antagonism ability, gut barrier reinforcement, bile salt metabolism, enzymatic activity, and carcinogens neutralisation;

iii) Rare mechanisms (strain-specific effects): neurological, immunological, and endocrinological effects, as well as the production of specific bioactive compounds (Hill et al., 2014).

The greatest effect of probiotics can be achieved if the probiotics withstand the gastric acidity and bile salt action and can attach to the cells of the intestinal mucosa. However, prolonged host colonisation by the bacteria will be detrimental as it may cause excessive stimulation of the targeted pathway (Behnsen et al., 2013). It is also reported that probiotics seem to pass into the faeces without adhering to the enterocytes or proliferating (Bezkorovainy, 2001). Although there are concerns that most microbial supplements are unable to inhabit the gut and become the resident community (Bezkorovainy, 2001), probiotics, including bacteria isolated from food, impact the host by sharing their genes and metabolites, supporting threatened gut flora, and affecting epithelial and immune cells directly (Wieërs et al., 2020). Therefore, in order to obtain a sustained exogenous probiotic effect, probiotic cultures could be ingested continuously.

Probiotics and fermented foods are closely related to each other, and probiotics from those foods are the most widely reported and researched. Fermented food products which contain probiotics such as yoghurt, kefir, beer and wine have long been consumed in various parts of the world and were considered as nutritious foods with therapeutic values even before the scientific studies on these foods were conducted (Bhat and Bajaj,

2019). In fact, previous literature concluded that the efficacy of taking probiotics alone was reported to be lower than when taken in the form of fermented food (Bell et al., 2017). Since probiotics are reported to show weak colonisation and adaptation in subjects originating from different regions of the world compared to where the original source of the probiotic was found, research is continually being done to find the potential probiotics from indigenous samples (Bhat and Bajaj, 2020).

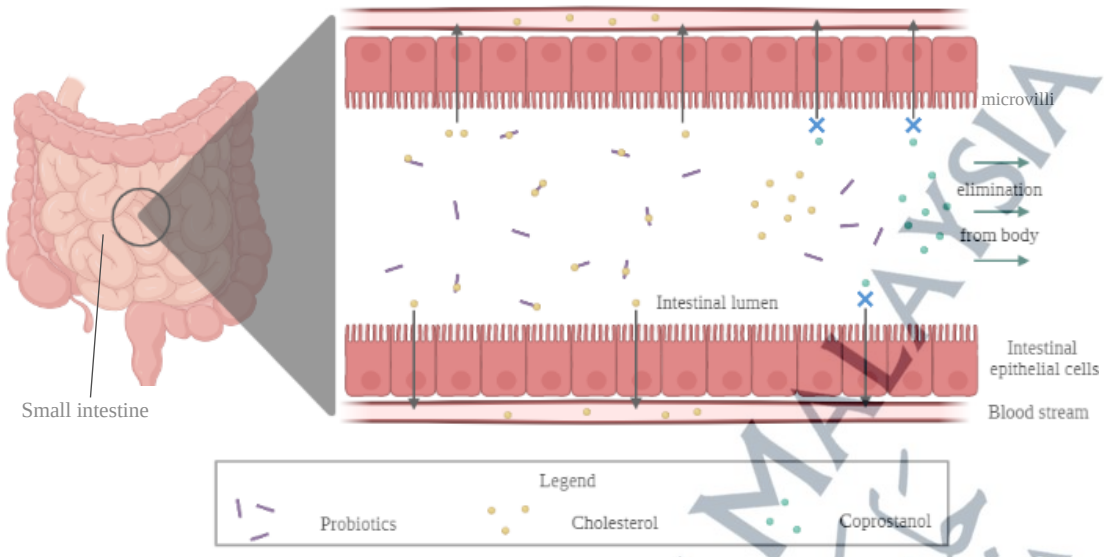
Due to increased research in the field of probiotics, more and more products containing probiotics have been introduced on the market, which include probiotic drugs; probiotics that are genetically modified; probiotics for animal use; and probiotic foods such as various dairy and non-dairy products; dietary supplements; and food ingredients (Awaisheh, 2012; Arora et al., 2013). Probiotics are also grouped differently around the globe following the regulations of each country. For example, they are categorised as food supplements in Denmark, Sweden, and Finland, biotherapeutic agents in European countries, Belgium, and Germany, and functional foods in Japan, India, China, and Malaysia (Arora et al., 2013).

The use of probiotics has also been extended to critically ill patients but limited to strains and indications that have been proven for their effectiveness (Awaisheh, 2012), as well as their safety profiles. This could be done by performing several tests, which include determination of antibiotic resistance profiles, hemolytic activity and toxin production, evaluation of certain metabolic activities such as bile salt deconjugation and D-lactate production, assessment of side-effects during human studies, epidemiological surveillance of adverse incidents in consumers (post-market), and assessment of probiotic infectivity in immuno-compromised animals (FAO/WHO, 2002).

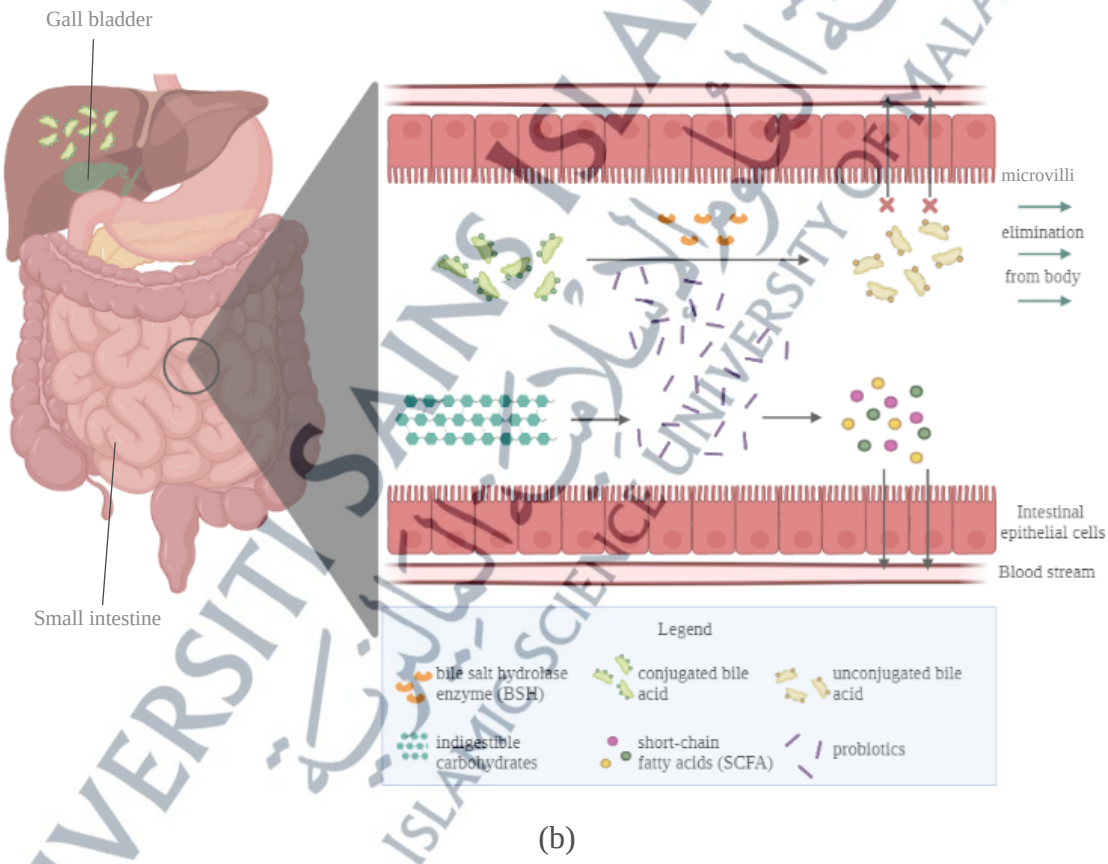
2.5 Hypocholesterolemic Effect of Probiotics

The small intestine participates significantly in maintaining the appropriate amount of cholesterol in the human body, which is influenced by both dietary and host-derived cholesterol (Kenny et al., 2020). The small intestine is the only organ that is able to regulate the amount of exogenous dietary and endogenous biliary cholesterol absorbed into the body and excreted out of the body on a daily basis, which brings the idea that any metabolic changes that take place in the intestine will alter the plasma cholesterol levels and affect the whole body (Field et al., 1990). Besides the metabolism by host cells, the regulation of cholesterol levels by microorganisms in the digestive tract may also serve to decrease cholesterol absorption in the intestine and increase cholesterol excretion through the faeces, resulting in lower plasma cholesterol levels.

In the past few decades, the use of probiotics with cholesterol-lowering properties has become more and more prominent due to their classification as GRAS and their benefits to human health. There have been numerous in-vitro and in-vivo studies to investigate the hypocholesterolemic effects of probiotics with proven cholesterol lowering ability. For example, there is evidence on the ability of a combination of fermented ginseng, *Bifidobacterium longum* BORI, and *Lactobacillus paracasei* CH88 supplemented to the diet of mice to increase the cholesterol excretion into faeces (Kang et al., 2018). The potential probiotics could be isolated from multiple sources such as feces, fermented food, dairy products, and pickles (Yang et al., 2021), and involve a variety of different mechanisms which are specific to different strains (Bhat and Bajaj, 2019). Direct action by microorganisms or indirect action by producing metabolites and influencing the host cell to modulate cholesterol (Figure 2.3) are two possible mechanisms by which microorganisms' lower cholesterol.



(a)



(b)

Figure 2.3: Schematic Representation of Cholesterol Lowering Mechanism Through a) Direct Action by Probiotics (Cholesterol Assimilation and Conversion of Cholesterol into Coprostanol) and b) Indirect Action by Probiotics (Secretion of BSH and Production of SCFA)

Microorganisms could directly be involved in lowering the serum cholesterol by assimilating the cholesterol, either by incorporating the cholesterol into cellular membranes during their growth or by binding the cholesterol onto their cell surface (Liong and Shah, 2005a). Probiotic lactobacilli strains showed better growth as well as an increase in the amount of total fatty acids in the presence of cholesterol, indicating that cholesterol stimulates the growth of microorganisms. The integration of cholesterol could also increase the concentration of saturated and unsaturated fatty acids, resulting in increased membrane strength and better cellular resistance (Ooi and Liong, 2010). Meanwhile, the attachment of cholesterol to the lactobacilli cell surface has been proved qualitatively by using a scanning electron microscope (Lye et al., 2010b). This physical interaction between cholesterol and bacterial cells could be affected by the presence of amino acids on the cell wall peptidoglycans. The attached cholesterol was removed together with the bacterial cells from the media after the centrifugation process. Although the cholesterol assimilation by growing cells is higher, the resting and dead cells also have the capacity to eliminate cholesterol and prove the attachment ability. They could therefore contribute as cholesterol-reducing vehicles in the digestive tract (Miremadi et al., 2014).

Another direct mechanism produced by the microorganisms is the ability of probiotics to convert cholesterol into coprostanol, a sterol that cannot be absorbed by the intestine, hence it is excreted through faeces. *Lactobacilli* strains were able to lower the cholesterol concentration and increase the coprostanol concentration following the fermentation period (Lye et al., 2010a). The cholesterol absorption takes place mainly in the small intestine, which also harbours lactic acid bacteria that are able to significantly modulate cholesterol conversion to coprostanol (Kriaa et al., 2019). Recent research has validated a group of microbial cholesterol dehydrogenases encoded by the

intestinal sterol metabolism A genes (*ismA*) which are involved in the coprostanol conversion (Kenny et al., 2020). As cholesterol-coprostanol conversion is varied and associated with the composition of the gut microbiota (Kriaa et al., 2019), the introduction of microorganisms with this ability into the human gut could help to modulate the intestinal and plasma cholesterol levels, thus impacting the human health.

Besides direct mechanisms, probiotics could also reduce the cholesterol level indirectly; for example by deconjugating the bile salt by bile salt hydrolase activity. The resultant unconjugated bile acids are less soluble and less absorbed by the intestines, and they are thus eliminated from the gastrointestinal tract through faeces. A minimum volume of cholesterol loss could be contributed by the co-precipitation with the free bile acids, although it would not be a key factor in regulating the serum cholesterol (Liong and Shah, 2005b). Since cholesterol is needed as a precursor to synthesise new bile salts, this homeostatic reaction contributes to a higher decrease in cholesterol in the blood (Ooi and Liong, 2010). Indeed, three *Lactiplantibacillus plantarum* strains isolated from rhizospheric soil collected from different parts of central India exhibited probiotic potential and hypocholesterolemic abilities. The NGG, NS14, and NS16 strains also harboured bsh enzymes and assimilated 48.84%, 60.52%, and 60.40% cholesterol from the medium, respectively (Singhal et al., 2021). Though the capability to produce the bile salt hydrolase (BSH) enzyme has been listed as one of the potential probiotic criteria (FAO/WHO, 2020), it is still uncertain whether its existence is necessary or not, as BSH activity is also thought to be potentially harmful to the host (Begley et al., 2006). This is due to the fact that excessive BSH could cause impaired digestion of lipids DNA damage, activation of carcinogens, and the formation of gallstones.

One more possible mechanism is the production of short-chain fatty acids (SCFA) that is strain-dependent (Kahouli et al., 2015), upon fermentation of food-derived indigestible carbohydrates by probiotics in the gastrointestinal tract (Pavlovic et al., 2012). For instance, probiotic *Lactobacillus plantarum* IS 10506 supplementation for 21 days was strongly associated with the increase of SCFA metabolites in respondents in the tested group compared to the respondents in the placebo group (Kusumo et al., 2019). The relative ratios of SCFA, for example, butyrate, propionate, and acetate, depend on the availability of the substrate. Butyrate and propionate have been shown to lower cholesterol by inhibiting cholesterol and fatty acids synthesis in the liver (Ooi and Liong, 2010). Moreover, SCFA could also redirect the plasma cholesterol towards the liver and modulate the food consumption and energy sensing processes in the brain, which indirectly leads to cholesterol metabolism reduction (Kumar et al., 2012). Many of the SCFA modulation activities are mediated by binding to membrane receptors and the production of signalling pathways in target cells (O'Morain and Ramji, 2019).

Since several earlier studies have shown the positive effects of probiotics in lowering cholesterol levels, more studies are currently being conducted to investigate the effects of probiotics in more detail. A number of authors have recognised the impact of probiotics on genes involved in cholesterol modulation. For example, Lim et al. (2017) conducted a comprehensive exploration of the treatment effects of probiotic LAB *Pediococcus acidilactici* LAB4 and *Lactobacillus plantarum* LAB12 on experimental cells prior to and following exposure to cholesterol. Experimental cells that were pre-treated with the bacteria showed reduced cholesterol levels, together with down-regulation of ABCA1, CD36, and SCARB1. Meanwhile, treatment with the LAB following cholesterol exposure resulted in the up-regulation of ABCA1, restoration of CD36 to basal level, and down-regulation of NPC1L1.

The screening of probiotic characteristics and cholesterol-lowering properties of LAB isolated from Korean salted fermented shrimp has also been conducted. It was found that out of 191 LAB strains, *L. plantarum* FB003 assimilated the highest amount of cholesterol. The gene modulation study further suggested that *L. plantarum* FB003 inhibits uptake of cholesterol by experimental Caco-2 cells through an upregulation of PPARα to inhibit NPC1L1 mRNA expression (Le and Yang, 2019a).

In fact, the exploration of cholesterol metabolism of potential probiotic strains isolated from multiple sources such as breast milk, infant stool, and fermented products of Yunnan, Tibet, and Gansu provinces of China has also been done. The evaluations include the screening for BSH activity qualitatively and quantitatively, cholesterol removal, and expression of genes involved in cholesterol metabolism. Out of 54 strains, 24 strains showed BSH activity, and 17 strains were able to remove cholesterol. The selected six strains were able to cause down regulation of NPC1L1 and FXR genes and up regulation of LXR genes (Liang et al., 2020).

2.6 Fermented Food

The growing world population should be directly proportional to food availability, as food is a basic need for every human being. Apart from focusing on food production, the process that needs to be considered is to ensure food supply is continuous, diverse, and safe for human consumption (Lee, 1997; Rahman, 2007). One of the ways to meet these three requirements is through the process of food fermentation. The fermentation process that has been practised by humans since the Neolithic period was described by Louis Pasteur as "*La vie sans l'air*" or life without water (Bourdichon et al., 2012). Fermentation is an effective process to extend the shelf life of food and can be done with inexpensive ingredients and basic equipment. This process remains relevant for

application by people around the world, especially in developing countries and rural communities with limited facilities (Rahman, 2007). The fermentation process could occur naturally or could be controlled by adding starter culture to the raw materials (Leroy and De Vuyst, 2004). In fact, the microorganisms isolated from fermented food could be utilised as starter cultures in the development of food products if they meet certain conditions such as not enhancing biogenic amines production, being susceptible to antibiotics, and/or exhibiting antagonism properties against some target pathogenic microorganisms (Martínez-Álvarez et al., 2017).

Although numerous microorganisms are found in various fermented foods, they normally fall under the LAB group. Most of the LAB, such as *Lactobacillaceae, Pediococcus*, some *Stretococcus, Weissella*, and *Enterococcus*, are harmless to humans, making them an ideal agent for food preservation (Martínez-Álvarez et al., 2017). LAB consists of bacteria that are Gram-positive, non-spore forming, coccus- or rod-shaped, and are mostly tolerant to low pH values. Based on the production of by-products during fermentation, LAB may be classified as homofermentative (fermenting carbohydrate to almost completely lactic acid) or heterofermentative (ferment carbohydrate to a combination of lactic acid, carbon dioxide, acetic acid, and/or ethanol). During the fermentation process, the microorganism will also release various metabolites, including protease, which leads to the major biochemical event, which is proteolysis of the raw materials (Kilinc et al., 2005). The production of these compounds lowers the pH and contributes to the distinct texture and flavour of the fermented food and also their ability to inhibit the growth of pathogens (Nuraida, 2015).

As it is a common practise to preserve food, fermentation plays a role in improving the nutritional and functional properties of foods by synthesising large quantities of peptides and amino acids (Hajeb and Jinap, 2012). Recent research on the

nutritional and biochemical qualities of Puthi shidal, the traditional fermented fish product in the north-eastern states of India, indicated that the product could serve as a significant source of essential amino acids such as lysine and also contains a considerable amount of eicosapetanoic acid (EPA) and docosahex-anoic acid (DHA) (Kakati et al., 2018). The degradation of raw materials into amino acids and peptides also causes a considerable effect on the taste and aroma of the fermented foods, which makes them popular as condiments and side dishes (Kilinc et al., 2005).

Nowadays, consumers are more interested in choosing healthier diets with functional products that are increasingly becoming more influential in the food industry. Functional food could be described as foods or food components that offer not only basic nutrition but also may function in lessening the risk of certain diseases and health conditions (Wells et al., 2017). For example, the presence of probiotics that are mostly reported and researched in fermented foods (Bell et al., 2017). Of relevance here is the research of the association of fermented food consumption with a systematic signal in the gut microbiome and metabolome using 6,811 respondents (Taylor et al., 2020). The findings showed a statistically significant difference in microbiome between fermented food consumers and non-consumers, with enrichment of conjugated linoleic acid, a health-promoting molecule, in the metabolome of fermented food consumers.

The fermentation process also provides an advantage in terms of controlling the growth of spoilage microorganisms and reducing the risk of foodborne illness, besides proper hygiene and good manufacturing practises during the processing of food. Therefore, the fermentation process is able to make an important contribution to the food industry, particularly on the issue of food safety, which has become a problem in developing countries due to economic problems (Mohamed and Mustafa, 2021). The microbial studies of Hout-Kasef, the traditional salted fermented fish product from

Saudi Arabia, reported the coliform count below 1 Log10 CFU/g and the absence of pathogenic bacteria such as *Listeria monocytogenes*, *Vibrio* spp., *Campylobacter* spp., and *Yersinia* sp. (Gassem, 2019).

Some fermented foods are consumed around the world, for example cheese, soy sauce, and yoghurt (Chilton et al., 2015), whilst the remaining are still restricted in the countries or ethnic groups that originally formulated them. In certain places, fermented products are used to replace the use of salt in cooking. For example, *Myeolchi-Aekjeot*, a traditional Korean salted-fermented anchovy sauce, is widely used as a seasoning, including in the making of kimchi (Lee et al., 2016). The sauce, which contains a high content of amino acids and fatty acids, as well as a rich umami flavour, could bring not only a distinct taste, but also strengthen the nutritional values of the food.

2.6.1 Fermented Food in Malaysia

Since the fifteenth century, people in Southeast Asia included fermented fishery products in their daily diets as staples, side dishes, or seasoning (Hajeb and Jinap, 2012). This situation may be influenced by geographical factors of countries in Southeast Asia that have rivers and are surrounded by the sea, including east and west Malaysia. The importance and significance of the Malaysian fishing sub-sector was proved by the contribution of this sector amounting to RM2226 million to the value of gross output, as well as 15,690 vacancies with salaries and wages paid equal to RM315 million in 2015 (Department of Statistics Malaysia, 2021). The fermented fishery products are also gazetted as Malaysian national heritage under the National Heritage Act 2005 (Act 265) (Som et al., 2020).

Fermentation of shrimp and fish involves various methods such as salting, ripening, drying, and marinating. The variation of methods along with the quality of

raw materials and the ratio of salt to raw materials contributes to the variety of products' quality, including their biochemical characteristics (Mueda, 2015). The fermentation period required is also different to produce different product categories, whether sauce, paste, or solid, as well as products with different flavours and colours (Mohamed and Mustafa, 2021). Figure 2.4 shows several types of fermented shrimp and fish products in Malaysia.

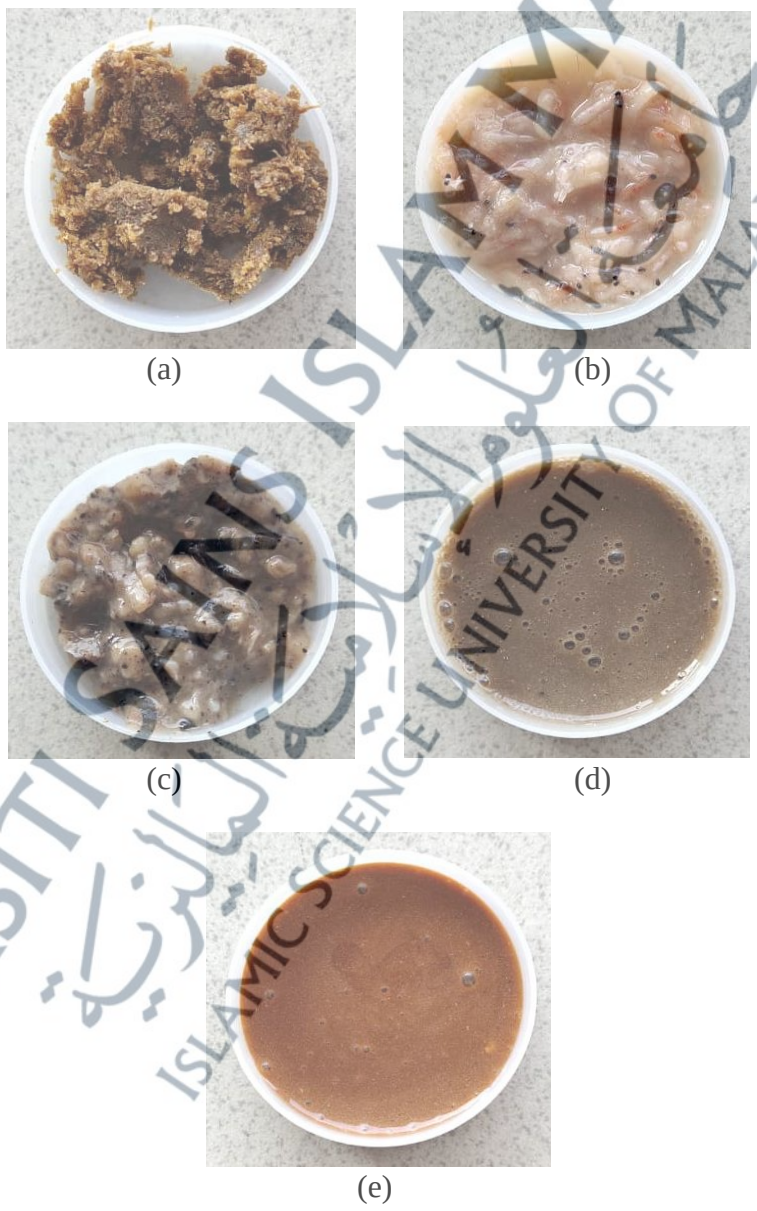


Figure 2.4: Fermented Food Products in Malaysia. a) Belacan b) Cincaluk c) Bosou d) Non-cooked Budu and e) Cooked Budu

Belacan is a thick paste of fermented shrimp with a pungent flavour and aroma that is produced in all Malaysian states (Huda, 2012). To produce belacan, the small shrimps such as *Acetes* sp. or mysids are washed and mixed with salt in a certain ratio before being sun-dried and crushed to form a paste-like structure. The paste is stored in a container to start the fermentation. The process would occur repeatedly until the desired texture is achieved. Belacan is frequently added as a flavouring ingredient in various Malaysian dishes, such as shrimp paste fried rice (nasi goreng belacan), spicy tamarind fish (asam pedas ikan) and rice noodle served in spicy soup (laksa). Besides that, sambal belacan is a Malaysian raw hot and spicy condiment made by pounding a mixture of belacan, chilies, lime juice, and salt by hand to form the desired texture. In the context of sensory attributes of dishes comprising shrimp paste, a positive correlation between belacan content and the sensory characteristics of final foods was reported, with dishes containing belacan having significant meaty and salty tastes (Jinap et al., 2010). The synergistic interaction between glutamate and 5'-nucleotide content consequently produces an intense umami taste of belacan, thus making belacan one of the most important umami contributors in Malaysian gastronomies (Hajeb and Jinap, 2012).

Meanwhile, different methods and additional materials are involved in the making of cincaluk. The small shrimp will be washed before they are combined with salt and cooked rice. The mixture will then be inserted into a suitable container for a fermentation process (Huda, 2012). However, in the industrial production of cincaluk, the small shrimp are mixed with salt and sugar in a particular proportion before the container is covered to allow the fermentation within a certain range of time. Cincaluk has a mixture of pungent, sour, and salty tastes and is served as an accompaniment besides the main side dishes (Ng and Karim, 2016). It is mixed with lime juice, chillies,

and shallots, and is commonly eaten with rice and fried fish. The rich umami flavour is reflected by its content of free glutamic acid, which is approximately 864 mg/100 g (Hajeb and Jinap, 2012).

Meanwhile, bosou (also known as noonsom sada) is an ethnic food of the Kadazandusun tribes in Sabah. It is almost similar to pekasam, which is widely consumed in Peninsular Malaysia. The differences are in terms of the raw materials used to prepare bosou and the way to consume it. The main ingredient for the preparation of bosou is fresh small freshwater fish. The fish were gutted, scaled, and washed with water. The cleaned fish were inserted into a small container and were mixed with the required amount of salt, cold cooked rice, and fermented pangi (*Pangium edule*) seeds. Before mixing, the black kernel inside the pangi seed was ground or blended to allow homogenous dissemination. The container was closed tightly and was kept for up to two weeks to allow complete fermentation (Lajius, 2014). Bosou could be served raw or cooked prior to consumption. Due to its strong odor, bosou is always kept in an airtight container.

Budu is a fish sauce widely produced in Kelantan and Terengganu, the states located in the east coast region of West Malaysia. The process of producing budu began with a collection of fresh anchovies caught along the nearby coast of Malaysia. The fresh anchovies are transferred to a container and mixed with salt according to the required quantity. The container is covered and incubated for six months. Hydrolysis of anchovies' protein into small peptides and free amino acids improves the content of total soluble peptides, thereby affecting the distinctive sensory properties of budu (Sim et al., 2015). Following the incubation phase, the product obtained is processed to obtain two different products, which are cooked budu and non-cooked budu. The broth was cooked for 4 h in temperature of 100^{o}C before it was filtered to remove the fish bones

and allowed to cool for three days. A certain amount of sugar, colouring, and seasoning were added to alter the final taste of cooked budu. Finally, the product is packed into plastic or glass bottles before it is distributed to the market. Meanwhile, the non-cooked budu was immediately filtered after the incubation period to remove the non-digestive fish bones and was packed without altering the final taste. The glutamic acid production by LAB strains isolated from budu was reported to be in the range of 22 to 106 μmol/L (Zareian et al., 2012). The presence of glutamic acid in budu could have a positive impact in terms of improving taste perception and synaptic transmission, as well as enhancing nourishment in geriatrics and in patients with poor nutrition. Two novel peptides were also successfully isolated from budu extract and showed high antioxidant activity, allowing budu to become a potential source of novel peptides for natural antioxidants (Najafian and Babji, 2018).

2.6.2 Isolation of Beneficial Microorganisms from Fermented Food

Varieties of fermented foods have been continuously consumed by billions of people around the world. As many microorganisms involved in food fermentation offer various beneficial impacts, research is continuously done to isolate, identify, and characterise the properties of each of the microorganisms. Not only do microorganisms appear as a result of the fermentation process, but they are also naturally present in the living cells of the animals and their environment (Gómez-Sala et al., 2015).

A number of hypotheses concerning the nature of cholesterol degradation by microorganisms isolated from fermented foods have been described by various researchers. For example, the investigation into cholesterol degradation and cholesterol oxidase production by *Bacillus subtilis* SFF34 isolated from Korean traditional fermented flatfish demonstrated that the residual cholesterol content cultivated with the

strain was reduced and 4-cholesten-3-one was detected as the reaction product of cholesterol oxidation activities (Kim et al., 2002). In another study, Rapsang and Joshi (2013) observed the cholesterol utilization of multiple *Lactobacillus* strains isolated from tungtap, the traditionally fermented fish of Meghalaya, India.

Besides the ability of microorganisms to degrade cholesterol, research attention is being devoted to isolating and characterisation of bacteriocin-producing microorganisms from fermented food products that have the potential to be developed as probiotics or biopreservatives to control pathogens in food. This is due to the fact that bacteriocins produced by LAB during fermentations are GRAS (Hwanhlem et al., 2011). For example, several LAB strains have been isolated from Philippine fermented shrimp and fish products, namely *burong tilapia* (fermented rice-tilapia mixture) and *balao-balao* (fermented rice-shrimp mixture) (Elegado et al., 2016). *Pediococcus acidilactiti, Pediococcus lolii*, and *Lactobacillus brevis* were identified through 16S rRNA sequencing and shown to be bacteriocinogenic towards the indicator organisms that are *Enterococcus faecium 79* and *Listeria monocytogenes.*

Research on the production of enzymes by microorganisms and their benefits is also being done by many researchers. One of the examples is the screening of fermented food products for the presence of bile salt hydrolase (BSH). The ability of microorganisms to produce BSH not only allows protection of bacterial communities in the gastrointestinal tract against the toxicity of conjugated bile salts but also may help to reduce cholesterol levels (Zago et al., 2011). For instance, the *Lactobacillus* strain isolated from boza and rayeb, the fermented foods in Egypt, showed BSH enzyme activity with the presence of precipitation zones around the colonies and was able to survive in the presence of 0.3% Ox-bile after a 3 h incubation period (Shehata et al., 2019).

Research done on fermented food products also found numerous species, some of which exert promising probiotic potential. The *Lactobacillus brevis* strain LAP2 isolated from fermented fish originated from Manipur, India called *Hentak*, showed potential probiotic characteristics including resistance to pH 3 and 0.3% bile salt, susceptible to antibiotics, as well as a good percentage (35%) of autoaggregation and hydrophobicity (Aarti et al., 2017). Of specific concern are the LAB, which exert multiple beneficial effects in the gastrointestinal tract, for example, prevention of pathogen adherence and replication via their antagonism mechanisms (Ida Muryany et al., 2017; Ida Muryany et al., 2018), as demonstrated by *Lactobacillus plantarum* (strains L8 and L20) and *Lactobacillus pentosus* (strain S1) isolated from pekasam, the Malaysian fermented fish.

The isolation of these strains concluded that fermented foods are undeniably a rich source of microbial diversity with numerous health advantages. Therefore, more studies should be done on these microorganisms to investigate their potential to be developed as functional foods or health products.