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CHOLESTEROL ASSIMILATION OF *Lactobacillus plantarum* L8 AND *Lactobacillus pentosus* S1 THROUGH IN-VITRO CHOLESTEROL LOWERING ACTIVITY

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Abstract

Probiotics gives a new insight in treating hypercholesterolemia. Recent research found that probiotics such as *Lactobacillus* possess multiple health benefits including cholesterol assimilation in human body. Bile salt deconjugation and cellular cell binding were proposed as underlying mechanisms for cholesterol lowering activity. *Lactobacillus plantarum* L8 and *Lactobacillus pentosus* S1 isolated from fermented fish food known as pekasam were assessed for their ability to deconjugate bile salt such as Taurodeoxycholic acid (TDCA). To achieve this, *Lactobacillus plantarum* L8 and *Lactobacillus pentosus* S1 were tested for bile salt de-conjugation through direct plating assay on de Mann, Rogosa, Sharpe (MRS) agar supplemented with TDCA. Different stages of cell growths of these strains were also tested for their ability to reduce cholesterol in MRS broth supplemented with cholesterol. Bacterial growth was also observed to identify whether incorporation of cholesterol in MRS broth would affect the growth pattern of *L. plantarum* L8 and *L. pentosus* S1 and their correlation with cholesterol reduction. The result showed that both *L. plantarum* L8 and *L, pentosus* S1 did not deconjugate bile salt on selective agar. However, only the growing, resting, and dead cells of *L. plantarum* L8 were able to assimilate cholesterol by 33%, 8% and 1%, respectively, while those of *L. pentosus* S1 did not show any activity which might be due to species specificity. Besides, *L. plantarum* L8 has a faster doubling time and a higher growth rate as compared to *L. pentosus* S1. This explains the cholesterol removal of *L. plantarum* L8 being higher than *L. pentosus* S1. Besides, after 18 h of incubation, *L. plantarum* L8 supplemented with cholesterol had a maintained growth up to 24 h as compared to its control (absence of cholesterol). In conclusion. *L. plantarum* L8 has the potential to act as cholesterol lowering probiotics.

Keywords: Hypercholesterolemia, Lactobacillus, Cholesterol assimilation, bile salt, fermented fish

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Introduction

Dyslipidemia or high levels of lipid in the blood contribute to detrimental cardiovascular diseases including coronary artery diseases (CAD), angina, and myocardial infarction which also known as a heart attack (Kunnen & Van Eck, 2012; Jeong et al., 2018). It is a worrying situation where almost 96.8% patients suffered from cardiovascular disease (CVD) including high blood pressure, diabetes and dyslipidaemia (The Star Malaysia, 2018). Even worrying, leading death cases were coming from ischaemic heart disease (IHD) which corresponded to 17 % of 109,155 certified deaths in 2020 (Department of Statistics Malaysia, 2022). Normally, health expert would suggest a healthy life style such as balance diet and exercise as preventive care (Tomaro-Duchesneau et al., 2015; American Heart Association, 2020). However, for

treatment purpose, drug medication has been implemented for century. Most common drugs used was statin (O'Morain & Ramji, 2019). It was confirmed that statin is able to hijack cholesterol synthesis pathway through HMG-CoA reductase, the rate limiting enzyme in mevalonate pathway (Ramkumar et al., 2016). Statin is also combined with ezetimibe, a lipid lowering agent, which further enhances low-density lipoprotein-cholesterol (LDL-C) reduction in the human body (Lamb, 2020). However, this medication comes with a pricy cost and long-term aftermaths such as rhabdomyolysis, myalgia, liver damage, kidney damage and several drug interactions (Jia et al., 2011; Benjamin et al., 2018; Jansen et al., 2020).

Currently, researchers from health and food industries considered probiotics as additional natural biotherapeutic to fight hypercholesterolemia (Majeed et al., 2019). Despite easy production and low cost, probiotics advocate for health benefits such as improved digestive system by modifying inflammatory response, balanced gut microflora, increased barrier function (Wieers et al., 2020) and hypocholesterolemic potential (Markowiak & Ślizewska, 2017; Bhat, & Bajaj, 2019). Probiotics are mostly lactic acid bacteria (LAB) such as *Lactobacillus* spp. and *Bifidobacteria* spp. (Ooi & Liong, 2010; Kumar et al., 2012; *de Melo* Pereira et al., 2018). The exact principles for cholesterol lowering activity by lactic acid bacteria are still unclear, however, several mechanisms were proposed. Studies have proven the ability of *Lactobacillus* spp. to reduce hypercholesterolemia by bile salt deconjugation (Miremadi et al., 2014; Shokryazdan et al., 2017; Singhal et al., 2021). There are several types of bile salts in human including glycocholic acid (GCA), glycodeoxycholic acid (GDCA), Taurocholic acid (TCA) and Taurodeoxycholic acid (TDCA). The ability to deconjugate bile salts is due to bile salt hydrolase (BSH) enzyme expressed by certain probiotics. High BSH activity in our gut promotes the conversion of cholesterol into bile, thus reducing cholesterol level. There is evidence where cholesterol can bind to all state of bacterial cells which further enhance cholesterol reduction in respective medium. BSH-producing probiotics deconjugate these bile salts into its free form (Cholic or deoxycholic acid) which is then excreted into the feces due to its low solubility (Pereira & Gibson, 2002; Kriaa et al., 2019). This action triggered *de-novo* synthesis of bile salt which required cholesterol to be converted into bile salts. High conversion of cholesterol promotes cholesterol reduction activity (Vinderola & Reinheimer, 2003; Miremadi et al., 2014). Another proposed mechanism is cholesterol assimilation through cellular cholesterol-binding, which was proved to reduce cholesterol (Miremadi et al., 2014; Sivamaruthi et al., 2019). Since bacteria produce exopolysaccharides, it was hypothesized that this glue-stick constituent can bind cholesterol to cell membrane forming biomatrix (Oleksy & Klewicka, 2017). Scanning electron image (SEM) by Shehata et al., (2019) showed exopolysaccharide produced around cells bound cholesterol to its membrane. Cholesterol accumulation of growing bacterial cells inhibit cholesterol absorption into blood serum thus allow cholesterol reduction (Miremadi et al., 2014; Bhat & Bajaj, 2020). Non-growing cells such as resting and dead phase of cell growth were also proven to reduce cholesterol in vitro (Tjandrawinata et al., 2022). The ability of bacteria to bind cholesterol at all stages of growth might further enhance cholesterol reduction and promotes Lactobacilli as remarkable cholesterol reducing agent (Shehata et al., 2019). A study by Choi & Chang (2015) shows a high cholesterol reduction by growing, resting and dead cells of *L. plantarum*. However, these properties are strain specific.

The selection of probiotics is dependent on its source. Lactobacilli are usually harbored from fermented products since their preservation requires lactic acid fermentation (Taylor et al., 2020). Lactobacillus has been isolated from various fermented products globally including kefir (Huang et al., 2013); Kimchi (Huang et al., 2019); Kalarei milk (Bhat & Bajaj, 2019); and Tangerine vinegar (Sui et al., 2021). This study intends to promote fermented local products which might consist of good probiotics. Besides, these probiotics might become one of the future supplements to treat hypercholesterolemia. Malaysia is rich with fermented fish products and one of the most common local products is pekasam (Huda, 2012). Pekasam consists of fermented fish made from freshwater fish (Ezzat et al., 2015). However, there is still limited research has been done on its health benefits. *L. plantarum* L8 and *L. pentosus* S1 were isolated from pekasam where they showed good characteristics as probiotics (Ida Muryany et al., 2017). A recent study

of these probiotics reported strong adhesion ability towards cell membranes and that they possess zero toxicity (Ida Muryany et al., 2018). These significant characteristics allow further understanding of bacterial-cell interaction and their health benefits towards host (Alves et al., 2020). With these properties, this research focuses on investigating the cholesterol assimilation effect by *L. plantarum* L8 and *L. pentosus* S1.

Method

Bacterial strain and growth condition

Two bacterial strains *L. plantarum* L8 and *L. pentosus* S1, are previously isolated from the Malaysian fermented fish, pekasam with accession numbers KT591875 and KT920464 (Ida Muryany et al., 2017). Bacterial cultures were revived and grown at 37°C for 48 h in MRS agar under aerobic condition. Activated culture was then transferred to MRS broth and grow overnight.

Bile salt deconjugation

Bile salt deconjugation ability was tested by direct plating assay on MRS agar supplemented with taurodeoxycholic acid (TDCA) following Vinderola & Reinheimer (2003) with slight modification by Gorenjak et al., (2014). Approximately 0.5% (w/v) of sodium salts of TDCA (Merck) and 0.32 µg calcium chloride ($CaCl_2$) were added to MRS agar and then autoclaved (121°C, 15 min). Subsequently, 10^8 CFU/ml bacterial cultures, *L. plantarum* L8 and *L. pentosus* S1 were spotted on the selective agar plates then incubated overnight at 37°C. Bacterial cultures grown on MRS agar only were used as negative control. Positive results were indicated by the formation of halo zone around spotted bacterial cultures.

Cholesterol assimilation by growing, resting and dead cells in MRS broth

Cholesterol assimilation in MRS broth was performed following method by Miremadi et al., (2014). For growing cells, 10^8 CFU/ml overnight cultures of *L. plantarum* L8 and *L. pentosus* S1 were inoculated into sterile MRS broth supplemented with 100 µg/mL cholesterol (Sigma-Aldrich). Cultures inoculated in 1 ml MRS broth only were used as negative control. For resting cells, overnight cultures were centrifuged at 4000 x g for 10 minutes and washed with sterile distilled water. Cell pellet was added with Phosphate Buffer Saline (PBS) was then vortexed for homogenous mixing. 10^8 CFU/mL of cultures from mixture were inoculated into 1 mL PBS supplemented with 100 µg/mL cholesterol. Negative control for resting cells were 10^8 CFU/mL of cultures added into 1 ml PBS only. To prepare dead cells, overnight cultures were autoclaved at 121°C for 15 min. Subsequently, 10^8 CFU/ml from autoclaved cultures were inoculated in MRS broth supplemented with 100 µg/mL cholesterol while the negative control was devoid of cholesterol. All prepared cells including the growing, resting and dead cells were incubated overnight at 37°C. Cholesterol assimilation for growing, resting and dead cells were quantified using cholesterol quantification kit, MAK034 (Sigma-Aldrich). A standard curve was plotted following manufacturer's instruction to determine the amount of cholesterol present in the samples. Cholesterol assimilation was calculated in percentage as follows:

$$\frac{100 - amount\ of\ cholesterol\ left\ in\ media}{100} \times 100\%$$

Bacterial growth

L. plantarum L8 and *L. pentosus* S1 growth were observed at different time intervals in the presence of cholesterol. Following the method by Majeed et al., (2019), 10^8 CFU/ml from overnight cultures were inoculated into MRS broth added with 100 µg/mL cholesterol. Cell densities were recorded using UV-VIS spectrophotometer at 600 nm starting from initial inoculation of bacterial cultures from 0h, 12h, 18h up to 24h at 37°C. For negative control, *L. plantarum* L8 and *L. pentosus* S1 were inoculated in the same condition with absence of cholesterol. Measurements were taken in triplicates and repeated twice. Bacterial growth rate (µ) and generation time (g) were calculated between two data points in plotted bacterial growth (Zannini et al., 2016). The respective equations are as follow:

$$\mu\ (Growth\ rate) = \frac{2.303(\log(OD_2) - \log\ (OD_1)}{T_2\ -\ T_1}$$

$$g\ (Generation\ time) = \frac{\log\ (OD_2) - \log\ (OD_1)}{log2}$$

Statistical analysis

One-way ANOVA were used in comparing means of growing, resting and dead cells of Lactobacilli in cholesterol removal followed by post-hoc Tukey test to indicates significant difference between means. All data were analysed using SPSS 27.

Result and Discussion

The ability to deconjugate bile salt became one of the desirable criteria for potential probiotics selection as cholesterol reducing agents (Begley et al., 2006). Bacteria with bile salt hydrolase activity will promote cholesterol assimilation by deconjugating bile salt, releasing glycine and taurine from its primary form thus producing non-soluble bile salts that can be easily excreted through feces (Ooi & Liong, 2010; Chiang & Ferrel, 2020). Most probiotics including Lactobacillus have shown their tolerance and ability to hydrolyze bile salts (Gorenjak, 2014; Allain et al, 2018; Huang et al, 2019). However, this cholesterol lowering activity was proposed to be strain specific where only certain Lactobacillus strain is able to deconjugate bile acid while others are not (Vinderola & Reinheimer, 2003; Prete et al., 2020). Figures 1(a) and 1(b) show direct plating assay of bile salt hydrolase activity of *L. plantarum* L8 *and L. pentosus* S1.

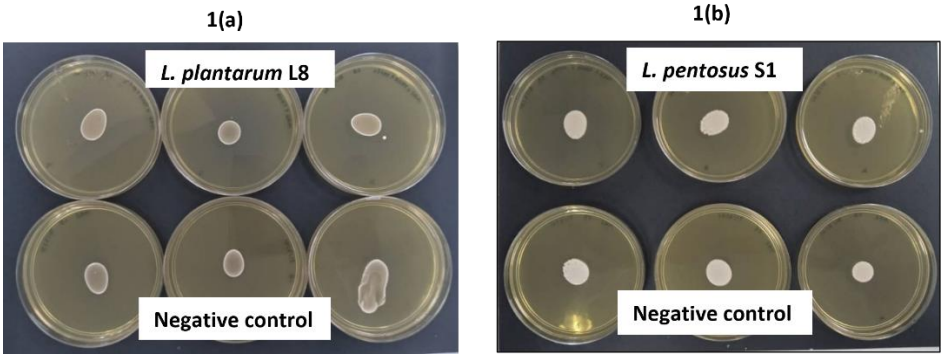

Figure 1. (a) *L. plantarum* L8 overnight cultures spotted on selective MRS agar added with 0.5% (w/v) TDCA and 0.32 µg $CaCl_2$ while negative control served as overnight cultures of *L. plantarum* L8 spotted on MRS agar only, (b) *L. pentosus* S1 with the same conditions as (a).

Based on bile salt hydrolase activity shown in Figure 1(a) and 1(b), there were no opaque halo formation on grown cultures for *L. plantarum* L8 and *L. pentosus* S1 as compared to the negative control. This indicates that there is no BSH activity by both cultures towards TDCA. *L. plantarum* L8 and *L. pentosus* S1 might lack bile salt hydrolase (BSH) gene towards TDCA. A study by Miremadi et al., (2014) reported 14 probiotic strains with the ability to deconjugate sodium glycocholate rather than taurocholate acid. This might be due to high abundance of glycocholate in human body where the ratio of glycine to taurine is 3:1 (Kriaa et al., 2019; Miyazaki et al., 2020). Acquired high BSH probiotics are important to improve lipid profile in the blood serum since mass conversion of bile salts into free bile salts is required for high demand of cholesterol for *de novo* synthesis (Tsai, 2014; Bhat & Bajaj, 2020). However, bacteria without the ability to deconjugate bile salt in the absence of BSH could still assimilate cholesterol (Sirilun et al., 2010). This was supported by Singhal et al., (2019) where *E. faecium,* which was unable to deconjugate bile salt in direct plating assay, still possessed the ability to assimilate cholesterol in MRS broth. This is in

conformance with our findings where *L. plantarum* L8 portrays cholesterol reduction in MRS broth (Figure 2) even though showing no BSH activity.

Cholesterol entrapment as mentioned by Kriaa et al., (2019) has been studied and shown in several strain of Lactobacilli where cholesterol assimilation in media has a relationship with the incorporation of molecules into the cell membrane. Besides, interaction of bacterial cell walls to cholesterol contributed to reducing cholesterol where not only growing cell possess this ability, but also resting and dead cells (Liong & Shah, 2005; Miremadi et al., 2014; Shehata et al., 2019).

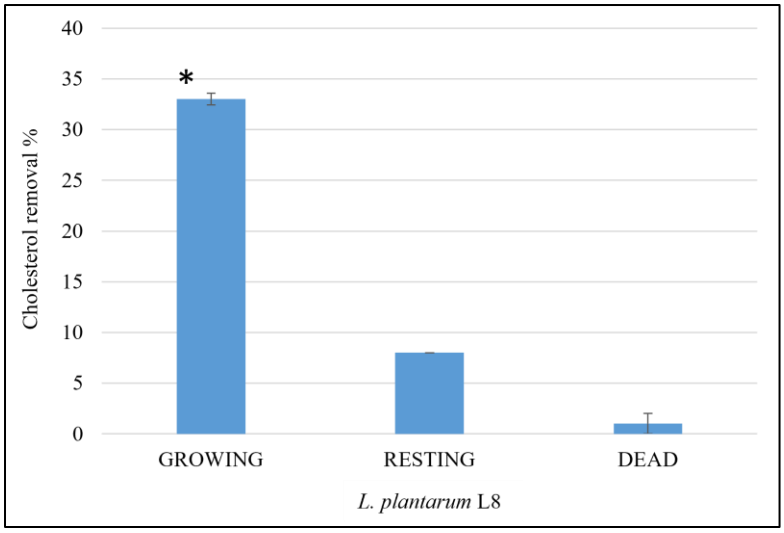

Figure 2. Cholesterol assimilation (%) of growing (G), resting (R) and dead (D) cells f *L. plantarum* L8 incubated in MRS supplemented with 100µg/mL cholesterol at 37°C. *L. pentosus* S1 did not show any cholesterol lowering activity. Data presented as mean ± standard error (SE). Cholesterol removal by growing, resting and dead cells for both probionts varied significantly ($P < 0.05$). * indicates significant difference.

Based on several studies, *L. plantarum* strain has shown the highest cholesterol assimilation ability among other strains (Hojjati et al., 2020; Singhal et al., 2021). This is in conformance with this study where *L. plantarum* L8 present higher cholesterol removal in MRS broth as compared to *L. pentosus* S1. Growing cells of *plantarum* L8 exhibit 33 % reduction, followed by 8% by resting cells and 1 % by dead cells. *L. plantarum* TGCM 26 and *L. plantarum* TGCM 128 exert the same cholesterol reduction by 25% and 32%, respectively (Sirilun et al., 2010). The findings are in agreement with a previous study whereby cholesterol reduction was shown to be dependent on the growing phase of bacteria (Majeed et al., 2019). However, non-growing cells such as resting and dead cells also show slight reduction of cholesterol which might be due to cholesterol attachment towards bacterial cell wall (Miremadi et al., 2014; Sukarno et al., 2021). Recent findings reported that growing bacteria changed in shape after incubation with cholesterol indicating cholesterol incorporation into bacterial cell membrane (Tjandrawinata et al., 2022). Meanwhile, *L. pentosus* S1 did not show any cholesterol reduction. This might be due to species specific or strain specific capability to reduce cholesterol (Tjandrawinata et al., 2022). Bacterial growth in the presence of cholesterol has been identified in this study where *L. plantarum* L8 showed distinct growth capacity as compared to *L. pentosus* S1 as shown in Figure 3. At 12 h of incubation, *L. plantarum* L8 had already reached its stationary phase while *L. pentosus* S1 was still in exponential state. The growth rate of *L. plantarum* L8 and *L. pentosus* S1 at 12 h is 0.41 h^{-1} and 0.20 h^{-1}, respectively. *L. plantarum* L8 also showed higher growth rate as compared to *L. pentosus* S1. This is in conformance with its generation time where L8 was able to divide after 1.69 h

which is faster than S1 that divided every 3.50 h. Growth rate and generation time are shown in table 1. These findings are supported by Matejcekova et al., (2016) where *L. plantarum* L8 portrayed enhanced growth in suitable condition. Since high percentage of cholesterol reduction was caused by growing cells as stated by Vourakis et al., (2021), this explains why *L. plantarum* L8 could assimilate cholesterol but not *L. pentosus* S1 due to its slow growth rate within 24 h.

Table 1: Growth rate and generation time of *L. plantarum* L8 and *L. pentosus* S1

Bacteria	Growth rate	Generation time
L. plantarum L8	0.41 h^{-1}	1.69 h / 101 min
L. pentosus S1	0.20 h^{-1}	3.50 h / 210 min

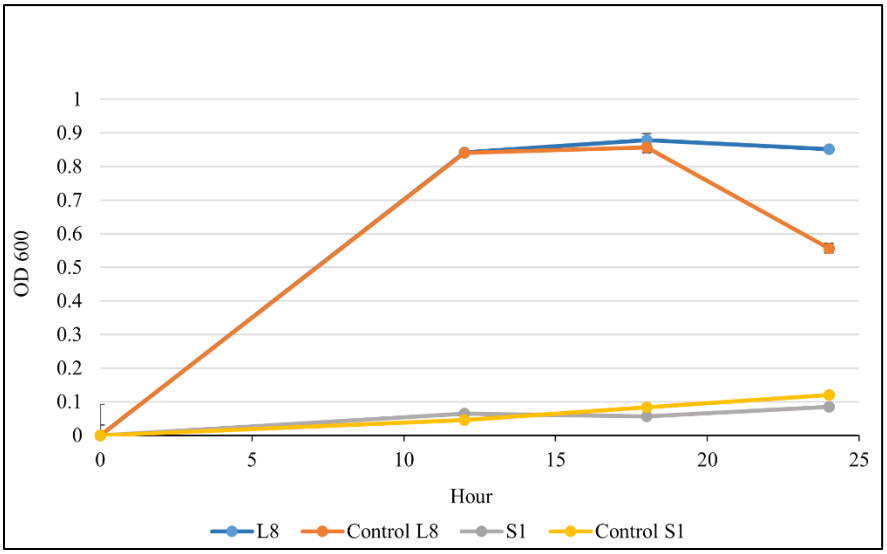

Figure 3: Growth (OD at 600 nm) of *L. plantarum* L8 and *L. pentosus* S1 in presence of 100 µg/mLcholesterol in MRS broth for 0h, 12h, 18h, 24h time interval. The control of *L. plantarum* L8 and *L. pentosus* S1 were grown on MRS broth with absence of cholesterol.

Besides, Figure 3 shows a maintained growth of *L. plantarum* L8 after 18h of incubation in the presence of cholesterol compared to its control. After the stationary phase, cells density usually depletes due to unfavorable niche of bacteria to survive (Bailey & Regina, 2021). *L. plantarum* L8 was able to maintain its growth up to 24h and this might be due to cholesterol incorporation into the cell membrane. Cholesterol stabilizes the structure of cell membrane by changing the behavior of membrane lipid bilayer (Yang et al., 2017). Some bacteria acquired cholesterol from its host for nutrients and membrane components (Samanta et al., 2017). It can be concluded that manipulation cholesterol into cell membrane assists in maintaining the growth of *L. plantarum* L8.

Conclusion

Based on the results, *L. plantarum* L8 exerts the ability to reduce cholesterol in MRS broth. On the other hand, it acquired faster doubling time (1.69 h) compared to *L. pentosus* S1 (3.5 h). *L. plantarum* L8 also shows higher growth rate (0.41 $^{h-1}$) than *L. pentosus* S1 (0.20 $^{h-}$1). Furthermore, L8 portrays an ability to maintain growth after 18 h of incubation up to 24 h. This promotes *L. plantarum* L8 as a potential candidate for cholesterol lowering probiotics. Nevertheless, further studies including molecular experiment such as gene modulation for cholesterol transporter genes by *L. plantarum* L8 and *L. pentosus* S1 against human intestinal cells are required.

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Author Contribution

A Ismail - collecting data, data processing and analysis, manuscript writing; Ilyanie HY – analysis, experimental design and supervision, IM Md Yasin - conceptualization, supervision, manuscript writing, review and editing.

Conflict of Interest

The authors declare no competing interest.

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