IN VITRO CHOLESTEROL-LOWERING MECHANISMS OF SELECTED *LACTOBACILLUS* AND *BIFIDOBACTERIUM* SPECIES AND EFFECTS OF PHYSICAL TREATMENT

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by

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List of Abbreviations

Abbreviations	Caption
ANS	8-anilino-1-napthalenesulfonic acid
BSA	Bovine serum albumin
BSH	Bile salt hydrolase
C:P	Cholesterol: phospholipids
DNA	Deoxyribonucleic acid
DPH	1,6-diphenyl-1,3,5-hexatriene
E.	*Escherichia*
Fan	Fluorescence anisotropy
GIT	Gastrointestinal tract
L.	*Lactobacillus*
LAB	Lactic acid bacteria
LC	L-cysteine. hydrochloride
MDA	Malondialdehyde
MRS	de Mann Rogosa Sharpe
NADH	Nicotinamide adenine dinucleotide-reduced form
PBS	Phosphate buffer saline
PI	Propidium iodide
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
TMA-DPH	1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene
UV	Ultraviolet

MEKANISME MERENDAHKAN KOLESTEROL *IN VITRO* OLEH SPESIES *LACTOBACILUS* DAN *BIFIDOBACTERIUM* YANG TERPILIH DAN KESAN RAWATAN FIZIKAL

ABSTRAK

Lima belas spesies (*Lactobacillus* dan *Bifidobacterium*) disaring melalui ciri-ciri pelekatan mereka. *Lactobacillus acidophilus* BT 1088, *L. acidophilus* FTCC 0291, *L. bulgaricus* FTCC 0411, *L. bulgaricus* FTDC 1311, dan *L. casei* BT 1268 menunjukkan keupayaan pelekatan yang lebih tinggi di dalam media dan justeru itu dipilih untuk analisis keupayaan penyingkiran kolesterol. Keupayaan penyingkiran kolesterol dilaksanakan secara *in vitro* dalam keadaan yang menyerupai saluran usus manusia (pH 8.0). Kajian ini memberikan bukti yang mengukuhkan hipotesis, yakni lactobacilli dapat menyingkirkan kolesterol melalui pelbagai mekanisme, antaranya termasuklah asimilasi kolesterol semasa pertumbuhan, pelekatan kolesterol pada membran sel, perencatan pembentukan misel kolesterol, penggabungan kolesterol ke dalam membran sel, dekonjugasi garam hempedu, aktiviti hidrolase garam hempedu (BSH), dan penukaran kolesterol kepada koprostanol.

Antara mekanisme yang dikaji, mekanisme penggabungan kolesterol adalah lebih menonjol dan lokasi-lokasi kolesterol yang digabungkan juga telah dikenal pasti. Oleh sebab penggabungan kolesterol ke dalam membran sel melibatkan kebolehtelapan membran, jadi rawatan-rawatan fizikal submaut turut seperti ultrasonik (20-100 W; 1-3 min), elektroporasi (2.5-7.5 kV cm^{-1}; 3-4 ms), dan sinaran UV (UVA-UVC, 30-90 J m^{-2}) diaplikasikan untuk meningkatkan penyingkiran kolesterol oleh spesies *Lactobacillus*. Rawatan-rawatan fizikal ini meningkatkan pertumbuhan sel-sel lactobacilli semasa fermentasi ($P < 0.05$) dan juga meningkatkan

keupayaan penyingkiran kolesterol oleh lactobacilli dengan ketara ($P < 0.05$) melalui asimilasi and penggabungan kolesterol ke dalam membran sel yang disebabkan oleh peningkatan kebolehtelapan membran lactobacilli.

Antara parameter yang dikaji, ultrasonik (100 W; 2 min), elektroporasi (7.5 kV cm^{-1} selama 3.5 ms) dan sinaran UV (UVB; 60 J m^{-2}) adalah lebih menonjol dalam peningkatan ($P < 0.05$) asimilasi kolesterol bagi sel-sel yang dirawat berbanding dengan kawalan. Memandangkan kesan-kesan positif rawatan fizikal, kajian ini seterusnya bertujuan untuk menilai kemungkinan pewarisan ciri-ciri penyingkiran kolesterol dan probiotik (keupayaan pelekatan pada usus) oleh sel-sel pada fasa yang berikutnya. Rawatan-rawatan fizikal ini meningkatan ($P < 0.05$) asimilasi kolesterol bagi sel-sel induk yang dirawat berbanding dengan kawalan, diiringi oleh peningkatan ($P < 0.05$; > 73.1%) penggabungan kolesterol ke dalam membran sel. Tambahan pula, peningkatan (>21.7%) asimilasi kolesterol juga diperhati dalam sel-sel pada fasa pertama. Walau bagaimanapun, ciri-ciri itu tidak diwarisi oleh sel-sel yang dirawat oleh ultrasonic and sinaran UV pada ketiga-tiga fasa yang berikutnya.

IN VITRO CHOLESTEROL-LOWERING MECHANISMS OF SELECTED *LACTOBACILLUS* AND *BIFIDOBACTERIUM* SPECIES AND EFFECTS OF PHYSICAL TREATMENTS

ABSTRACT

Fifteen strains of *Lactobacillus* and *Bifidobacterium* were screened based on their adherence property. *Lactobacillus acidophilus* BT 1088, *L. acidophilus* FTCC 0291, *L. bulgaricus* FTCC 0411, *L. bulgaricus* FTDC 1311, and *L. casei* BT 1268 showed higher adherence property compared to other strains studied and were thus selected for examination on cholesterol removal. Cholesterol removal ability was conducted *in vitro*, under conditions that mimic the human gastrointestinal tract (pH 8.0). This study provided experimental evidence to strengthen the hypothesis that lactobacilli could remove cholesterol via different mechanisms, namely assimilation of cholesterol during growth, binding of cholesterol to cellular surface, disruption of cholesterol micelle, incorporation of cholesterol into the cellular membrane, deconjugation of bile salt, bile salt hydrolase (BSH) activity, and conversion of cholesterol to coprostanol.

Among the mechanisms studied, cholesterol incorporation mechanism was more prominent and the locations of incorporated cholesterol have also been identified. Considering that incorporation of cholesterol into the cellular membrane involves membrane permeability, thus sub-lethal physical treatments such as ultrasound (20-100 W; 1-3 min), electroporation (2.5-7.5 kV cm^{-1}; 3-4 ms), and UV radiation (UVA-UVC, 30-90 J m^{-2}) were applied with the objective to further increase cholesterol removal by *Lactobacillus* species. These physical treatments increased ($P < 0.05$) viability of lactobacilli cells during fermentation and also

improved the ability of lactobacilli cells to remove cholesterol significantly ($P < 0.05$) via assimilation and membrane incorporation of cholesterol due to improved membrane permeability.

Among the parameters studied, ultrasound (100 W; 2 min), electroporation (7.5 kV cm^{-1} for 3.5), and UV radiation (UVB; 60 J m^{-2}) showed the most prominent effect in increased cholesterol assimilation of treated lactobacilli cells. Considering the positive effects of physical treatment, this study further aimed to evaluate the possible inheritance of cholesterol removal and probiotic properties (intestinal adherence ability) by subsequent passages of treated lactobacilli cells. These physical treatments affected the intestinal adherence ability of treated lactobacilli cells but increased ($P < 0.05$) their cholesterol assimilation compared to that of the control, accompanied by increased ($P < 0.05$) incorporation of cholesterol into the cellular membrane. Additionally, an increase (> 21.7%) in assimilation of cholesterol was also observed for the first passage of electroporated lactobacilli cells. However, such traits were not inherited by the subsequent three passages of ultrasound- and UV-treated lactobacilli cells.

1.0 Introduction to Thesis

1.1 Background

1.1.1 Probiotics and general benefits

Lactobacilli and bifidobacteria are the most common adjunct cultures in fermented dairy products and have been widely associated with probiotic properties. Probiotics have been defined by the FAO/WHO as "viable microorganisms which when administered in adequate amounts confer a health benefit to the host" (FAO/WHO, 2001). The interest in lactobacilli and bifidobacteria has consistently increased due to awareness of the benefits for gut health and disease prevention and therapy. In addition to gut health, strains of lactobacilli and bifidobacteria have also been reported to reduce hypertension, stimulate immune response, suppress traveler's diarrhea, and alleviate postmenopausal disorders (Liong, 2007).

1.1.2 Cardiovascular disease and hypocholesterolemic effect of *Lactobacillus* and *Bifidobacterium*

A high level of cholesterol in the blood has been associated with increased risks of cardiovascular diseases such as coronary heart disease and stroke. Cardiovascular disease is one of the most common diseases found in developing countries and there is an increased urgency for its management due to the increased occurrences annually (Ooi *et al.*, 2010). Various dietary means have been implemented to reduce blood cholesterol levels, such as the reduction of high-cholesterol foods, increasing intake of fibers, and drug interventions.

Recent studies have also found that the consumption of lactobacilli could reduce blood cholesterol levels and improve blood lipoprotein profiles (Ooi *et al.*, 2010; Liong *et al.*, 2007). A lowering effect on serum cholesterol by lactobacilli was

also observed when mini pig was fed with meal supplemented with lactobacilli cells (du Toit *et al.*, 1998). In another study, Xiao *et al.* (2003) found that milk fermented by *Bifidobacterium* (*B.*) *longum* BL1 reduced serum total cholesterol, LDL cholesterol, and triglycerides not only reduced in rats, but also in hypercholesterolemia patients and healthy adults. This reduction of cholesterol *in vivo* and removal of cholesterol *in vitro* by lactobacilli could play an important role in modulating the human cardiovascular disease, where a 1% reduction in serum cholesterol could reduce the risk of coronary heart disease by 2-3% (Ooi and Liong, 2010).

Although positive clinical and experimental evidences have shown that lactobacilli and bifidobacteria could reduce serum cholesterol levels, the exact mechanisms still remained unclear and thus are increasingly investigated. It has been reported that this cholesterol removal ability might be attributed to either the assimilation of cholesterol by growing cells (Pigeon *et al.*, 2002) and binding of cholesterol to cellular surface (Kimoto *et al.*, 2002). Cholesterol adhered to bacteria cells would be less available for absorption, leading to reduced serum cholesterol levels. However, the *in vivo* occurrence of cholesterol assimilation and binding would involve the presence of bile and till now, no study has evaluated the effects of bile on the adherence of cholesterol onto cellular surface of lactobacilli cells. In addition, cholesterol bound onto cellular surface could also exert an inhibitory effect on cholesterol micelle formation in the intestines. The disruption of micelle formation would be another possible mechanism for reducing cholesterol levels, as cholesterol micelles are required to transport fatty acids through an aqueous medium to the intestinal mucosal surface for absorption (Cheeke, 2000). It has been

hypothesize that when bile salts are bound to the bacterial cell, less bile would be available for the formation of cholesterol micelle. To my knowledge, there has been no study evaluating on the disruption of cholesterol micelle by lactobacilli.

Previous studies also reported that incorporation of cholesterol into the cellular membrane, deconjugation of bile via bile salt hydrolase (BSH; Liong *et al.*, 2007), and conversion of cholesterol to coprostanol (Ma, 2006) could be the mechanisms involved in cholesterol removal by lactobacilli. Cholesterol incorporated into the cellular envelope of lactobacilli was postulated to alter the cellular membrane of the organism and produced lactobacilli cells that were more robust towards sonication death, cellular lysis, and exhibited changes in the membrane fatty acid profiles (Liong and Shah, 2005a). However, to my knowledge, little information is available on the incorporation of cholesterol into cellular membrane of lactobacilli. Also, the location of the incorporated cholesterol has never been evaluated and we would be the first to do so.

1.1.3 Effects of physical treatments on membrane permeability and cell growth

In addition, physical treatments such as ultrasound, electroporation, and ultraviolet (UV) radiation have been shown to influence the permeability and diffusion across membranes of bacterial cells. Pitt and Ross (2003) and Ananta *et al.* (2005) reported that the low intensity and sub-lethal ultrasound treatment increased bacterial membrane permeability, leading to enhanced uptake of molecules and nutrients across the cytoplasmic membrane. In addition to ultrasound treatment, Loghavi *et al.* (2007) also found that temporary pore formations on bacteria cells upon electroporation could enhance nutrient diffusion and acceleration of microbial growth by increase cell membrane permeability. Similar observation was also

demonstrated by Bose and Chatterjee (1995), where the liposomal membrane becomes more permeabilized that subsequently increase the transportation of substrates in and/or out from the membrane upon UV treatment. Hortnag *et al.* (2010) also reported that the growth of *Acinetobacter lwoffii* increased upon UV exposure and has effective repair mechanisms that maintained their cell production. Thus, we hypothesized that physical treatments could induce permeabilization of cell membrane that not only increases the removal of cholesterol from medium by lactobacilli but also maintain the cell growth. To date, no attempt has been made to utilize such treatments to enhance and/or evaluate the cholesterol removal properties of lactobacilli, either via assimilation of cholesterol during growth or incorporation of cholesterol in bacterial membrane. Additionally, there has been no information on the sustainability of such treatments in affecting the growth, intestinal adherence, and cholesterol removal abilities for subsequent three passages.

1.2 Aims and objectives of research

This research aims to have a better understanding on the possible mechanisms of cholesterol removal by lactobacilli and the evaluation of physical treatments on such removal properties, to enhance and justify the cholesterol removal ability of lactobacilli. The sustainability of the treatments on the treated bacteria cells in affecting the growth, intestinal adherence and cholesterol removal abilities for three passages was also examined. The specific objectives of this study were:

1) To screen and select strains of lactobacilli and bifidobacteria based on their adherence property.

2) To evaluate the *in vitro* mechanisms involved on the removal of cholesterol by lactobacilli.

3) To examine the effects of physical treatments on cell viability, membrane properties, and possible increase in cholesterol removal ability of lactobacilli.

4) To investigate the possible inheritance effect of physical treatments on lactobacilli based on their growth, intestinal adherence, and cholesterol removal abilities for the subsequent three passages.

2.0 Literature Review

2.1 Probiotic

The human gastrointestinal tract (GIT) is a complex ecosystem that harbors a rich pool of diverse microflora (Goel *et al.,* 2006). These indigenous bacteria are known to be potentially harmful or health promoting. The strains with beneficial properties are classified as probiotics (Isolauri *et al.*, 2004). Probiotics are defined as "live microorganisms that confer a health benefit to the host once administered in adequate amounts" (FAO/WHO, 2001). Lactic acid bacteria (LAB) are among the most important probiotic microorganisms typically associated with the human GIT (Holzapfel *et al.*, 2001). Some species of LAB, such as *Lactobacillus (L.) acidophilus, L. delbrueckii* subsp. *bulgaricus, L. casei, L. fermentum, L. plantarum,* and *L. reuteri,* and *Bifidobacterium* species have been reported as probiotics (Oh *et al.*, 2000).

Several traits have been identified for probiotics, namely safe for consumption, resistant towards acid and bile of the gastrointestinal environment, able to adhere to the intestinal epithelium of the hosts, possess antagonistic activity against pathogenic bacteria, and are able to maintain viability during storage (Lin *et al.*, 2007). Many LAB could adapt and grow in the gut environment, and subsequently produce antimicrobial substances such as organic acids and bacteriocins (Guerra *et al.,* 2007). Although the minimum effective dose to exert a health beneficial effect is not precisely known, it is commonly recommended that oral administration should exceed 10^9 CFU per day (De Champs *et al.,* 2003). Additionally, some beneficial effects such as the improvement of lactose intolerance, modulation of immune system, and antihypertensive effects have also been reported

to depend upon enzyme activities and fermentation products of probiotics (Vinderola and Reinheimer, 2003).

2.1.1 *Lactobacillus*

Lactobacilli are generally characterized as gram-positive, non-sporeforming, and non-flagellated rods or coccobacilli (Gomes and Malcata, 1999). They are commonly found in habitats such as the mucosal membranes of humans and animals (oral cavity, intestine, and vagina), on plants and material of plant origin, in manure and man-made habitats such as sewage and fermenting or spoiling food where these habitats are rich with carbohydrate-containing substances. In healthy humans, lactobacilli are normally present in the oral cavity (10^3-10^4 CFU g^{-1}), the ileum (10^3-10^7 CFU g^{-1}), and the colon (10^4-10^8 CFU g^{-1}), and dominate in the vagina (Bernardeau *et al.*, 2008). The most common *Lactobacillus* species isolated from the human intestine included *L. acidophilus*, *L. salivarius*, *L. casei*, *L. plantarum*, *L. fermentum*, *L. reuteri*, and *L. brevis* (Saito, 2004). The optimum growth temperature of lactobacilli is 35-40°C, however, the growth of this bacteria may also occur at higher temperature such as 45°C (Gomes and Malcata, 1999).

2.1.2 *Bifidobacterium*

Bifidobacteria are gram-positive, non-motile, non-sporeforming, and strictly anaerobic bacteria (Delcenserie *et al.*, 2007). They may differ in shapes and occur as short, curved rods, club-shaped rods, and bifurcated Y-shaped rods (Gomes and Malcata, 1999). In addition, they are generally nonpathogenic (Delcenserie *et al.*, 2007). They have been isolated from a number of environments such as sewage, anaerobic digester, and fermented milk (Hoyles *et al.*, 2002), but they are the

predominant bacterial group in the normal intestinal flora of healthy breast-fed newborns, in which it constitutes more than 95% of the total population. However, this population of organisms gradually decreases in number with increasing age of the individual and may account for 25% of the total intestinal flora in healthy adults (Kheadr *et al.*, 2007). The main species present in humans are *B. adolescentis*, *B. bifidum*, *B. infantis*, *B. breve*, and *B. longum* in the colon (Roy, 2001). Their optimum temperature for growth is 37-41°C, with maximum growth at 43-45°C and virtually no growth at 25-28°C or below (Gomes and Malcata, 1999).

2.1.3 General benefits

The presence of probiotics in the human intestine is almost universally accepted to be a contributing factor to a healthy well being (Roy, 2001). By improving the intestinal microbial balance, probiotics have been reported to exert beneficial physiological effects, such as inhibiting the growth of pathogenic microorganisms, reducing colon cancer, improving lactose intolerance, preventing gut inflammation, enhancing natural immunity, and reducing serum cholesterol on the host of all age groups (Liu *et al.*, 2007). Therefore, many health care professionals such as holistic practitioners, naturopaths, chiropractors, and herbalists routinely use products containing lactobacilli, bifidobacteria, and other possible probiotics to enhance health properties (Reid *et al.*, 2003).

There are various evidences to support the verification of such effects, from *in vitro*, animal and human studies. Intestinal mucus could act as initial binding site, nutrient source, and matrix for the proliferation of bacteria and protecting the mucosa from certain microorganisms. Adherence to the intestinal epithelium and mucus is

closely related to the stimulation of the immune system, and adhesion to the intestinal mucosa is also crucial for transient colonization, an important prerequisite for probiotics to control the balance of the intestinal microflora (Juntunen *et al.*, 2001). Probiotics are reported to be able to adhere to the small intestine and colonize it upon oral administration with the production of surface-active components which can inhibit the adhesion of other pathogenic bacteria (Pereira *et al.*, 2003). Probiotics have also been found to inhibit the growth of bacterial pathogens by producing antimicrobial metabolites such as organic acids (acetic and lactic acids), hydrogen peroxide (in anaerobic environment), β-hydroxypropionaldehyde, and bacteriocins. The production of organic acids by probiotics subsequently reduces the pH of the medium and further inhibiting the growth of pathogens (Fayol-Messaoudi *et al.*, 2005). Bifidobacteria strains have been reported to inhibit Shiga toxin-producing *Escherichia* (*E.*) *coli* growth *in vitro* by producing a high concentration of acetic acid (56 mM) that lowered the pH of the intestine (to pH 6.75) (Asahara *et al.*, 2004).

Probiotic also serves as a source of lactase in the small intestine. Upon consuming products containing lactose, lactase-deficient individuals could experience abdominal pain, bloating, and diarrhea due to the colonic fermentation of lactose in the intestines that produce acid and gas. Dairy products containing probiotic strains have been found to enhance tolerance to lactose as compared to unfermented dairy products (Sander and Klaenhammer, 2001). This is due to the ability of probiotic strains to produce lactase or β-galactosidase, which digests the lactose in dairy products to glucose and galactose, thus facilitating digestion and alleviating intolerance (Harish and Varghese, 2006).

Moreover, probiotics have been found to be effective in the prevention of colon cancer. Several mechanisms have been postulated, namely the alteration of the metabolic activities of intestinal microflora, alteration of physicochemical conditions in the colon, binding and degradation of potential carcinogens, quantitative and/or qualitative alterations in the intestinal microflora incriminated in the production of carcinogens, production of antitumorigenic or antimutagenic compounds, and enhancing the immune response of the host (Harish and Varghese, 2006). In an *in vitro* study, Oatley *et al.* (2000) found that bifidobacteria were able to bind carcinogenic aflatoxin, thus rendering it unavailable for absorption in the intestinal tract. The consumption of diets containing viable probiotic has been also reported to reduce faecal β-glucuronidase activity in human (Goldin *et al.*, 1980). β-glucuronidase is often produced by intestinal bacteria and could hydrolyze glucuronide, a compound that is required to detoxify foreign compounds (Fotiadis *et al.*, 2008).

2.2 *In vitro* cholesterol removal by lactobacilli and bifidobacteria[1]

Cholesterol plays a major role in human heart health. High cholesterol in serum could lead to increase the risk factor for human cardiovascular disease. Lactobacilli and bifidobacteria have been used as potential cholesterol lowering milk additives. Past studies have indicated that the consumption of lactobacilli and bifidobacteria resulted in the reduction of serum cholesterol levels in humans and animals (Psomas *et al.*, 2003). Several *in vitro* mechanisms of cholesterol removal by lactobacilli and bifidobacteria have been proposed and investigated. However, controversial results have been raised, where numerous studies have showed insignificant hypocholesterolaemic effects. Thus, more studies are needed to better understand the mechanisms involved.

2.2.1 Assimilation of cholesterol by lactobacilli and bifidobacteria

The assimilation of cholesterol by lactobacilli and bifidobacteria in the small intestine could reduce intestinal absorption of cholesterol (Pigeon *et al.*, 2002). Lactobacilli and bifidobacteria must be viable and growing, in order to be able to remove or assimilate cholesterol (Piston and Gilliland, 1994). In an *in vitro* study, Tahri *et al.* (1995) reported that the growing cells of *Bifidobacterium* sp. were able to remove cholesterol from the fermentation broth via assimilation of cholesterol. The assimilation of cholesterol may be affected by the growth of bacteria in medium containing bile salts that are similar to those of the gastrointestinal conditions. However, little information is available on the effects of bile on cholesterol assimilation. Additionally, past studies have also shown controversial results, where some researches has exhibited insignificant results. Brashears *et al.* (1998) reported the both *L. casei* E5 and N19 showed insignificant effects on the removal of

[1] Parts of section 2.2 have been published in: Lye, H. S., Kuan, C. Y., Ewe, J. A., *et al.* (2009) *International Journal of Molecular Science*, 10, 3755-3775.

cholesterol compared to the control. Similarly, Walker and Gilliland (1993) also found that the cultures of *L. acidophilus* ATCC 4356 could not remove cholesterol from the medium. Therefore, further assessment of this mechanism is needed to better understand the pathways involved and to justify the positive results obtained.

2.2.2 Binding of cholesterol to bacterial cells

Using *in vitro* experiments, Liong and Shah (2005a) reported that cholesterol could be removed from medium by lactobacilli and bifidobacteria not only through assimilation during growth, but also through binding of cholesterol to the cellular surface. This mechanism was proposed when non-growing cells and dead cells were found to remove cholesterol. In addition, Taranto *et al.* (1997) showed that about 20% of cholesterol that was removed and bound to the cellular surface. Meanwhile, Dambekodi and Gilliland (1998) also reported that cholesterol was attached to the cell surface, upon determination of cholesterol content of the membrane fraction of *B. longum*. This mechanism is important as some products such as yoghurt and lassi may not contain viable cells, unless labeled otherwise.

2.2.3 Disruption of micelle formation

Formation of micelle is essential in the digestion and absorption of lipids. Bile salts are amphiphatic molecules with a planar hydrophobic moiety enabling them to penetrate into bile bilayers. Cholesterol is solubilized in the presence of bile and phopholipids, forming micelle in aqueous solutions (Van de Heijning *et al.*, 1994). Micelles are cylindrical in shape, with bile salts on the outside with their hydrophilic portions oriented outward while various insoluble molecules form the inner part (Johnson and Byrne, 2003). Disruption of micelle formation may play an

important role in the removal of intestinal cholesterol. Micelles are the reservoir and transport vehicles for cholesterol across the small intestines, and facilitate the uptake of monomeric cholesterol by enterocyte (Wang, 2003). The formation of micelle may be affected by different bile salts as bile salts are essential for the formation of micelle. However, up to date, little information is available on the effect of bile and lactobacilli towards the disruption of cholesterol micelles. Therefore, further studies are required to elucidate this matter.

2.2.4 Incorporation of cholesterol into the cellular membrane

Other researchers have suggested that the incorporation of cholesterol into cellular membrane could be another mechanism to reduce cholesterol from medium. Razin (1975) found that most of the cholesterol from the medium was incorporated into the cytoplasmic membrane, however, the outer membranes of the intact cells are more easily accessed by cholesterol. There was twice the amount of cholesterol in the protoplast membranes than that of intact cells. This indicated cholesterol could be preferentially bound to the cytoplasmic membranes (Tani *et al.*, 1993). In a previous study, Noh *et al.* (1997) found that cholesterol uptake by *L. acidophilus* ATCC 43121 occurred during growth. However, most assimilated cholesterol recovered from the cells was not metabolically degraded. Therefore, the authors suggested that the removal of cholesterol may also due to the ability of *L. acidophilus* ATCC 43121 to incorporate cholesterol into cellular membranes during growth. In addition, Liong and Shah *et al.* (2005b) reported differences in fatty acid distribution patterns for cells grown with or without cholesterol. The authors found that lower amount of total saturated fatty acids and higher amount of total unsaturated fatty acids were recovered from cells grown in medium containing cholesterol compared to those in

the absence of cholesterol. This was attributed to the incorporation of cholesterol into the membrane rather than cellular synthesis because LAB living under fatty conditions might lose their ability to synthesize lipids or fatty acids (Kiatpapan *et al.*, 2001). Cholesterol that is incorporated into bacterial cells during growth in the small intestine is less absorbed into the enterohepatic circulation, thus could lead to reduced serum cholesterol in humans. We postulate that the cholesterol incorporated into cell membrane may affect the lipid order in lactobacilli membrane. Thus, further evaluation is required to support this hypothesis.

2.2.5 Enzymatic deconjugation of bile salts

Another hypocholesterolaemic mechanism that was postulated involved the ability of certain lactobacilli and bifidobacteria to deconjugate bile acids enzymatically. Deconjugation of conjugated bile salt to deconjugated bile salt is catalyzed by BSH. BSH (or known as cholyglycine hydrolase; EC 3.5.1.24) is the enzyme that catalyzes the hydrolysis of glycine- and/or taurine-conjugated bile salts into amino acid residues and free bile acids (Kim *et al.*, 2004). Deconjugation of bile salts mainly occurs in the small and large intestines of mammalian host. However, the exact location of this metabolite activity is dependent on the distribution of the host species. For example, bile salt deconjugation starts in the small intestine of mice whereas a significant deconjugation in humans begins at the end of the ileum and completed in the large bowel (Tanaka *et al.*, 2000). BSH activity has been detected in intestinal bacteria such as *Lactobacillus* and *Bifidobacterium* sp. (Liong and Shah, 2005c). It has been demonstrated previously that the removal of cholesterol by *L. reuteri* CRL 1098 was closely related to the BSH activity of the cells which hydrolysed the amide bond of bile salts releasing free bile acids (Taranto *et al.*,

1999). The mechanism of actions of BSH on bile is shown in Figure 2.1A. Conjugated bile salts are readily to be absorbed into gastrointestinal tract due to higher hydrophilicity, while free bile acids are less soluble and thus less efficiently reabsorbed into the intestines compared to conjugated bile salts, and thus are more prone to be excreted into faeces. This will increase the need for the synthesis of new bile to replace the loss. Cholesterol is the precursor for the *de novo* synthesis of new bile acids (Kumar *et al.*, 2012; Figure 2.1B). The use of cholesterol to synthesis new bile would lead to a decrease concentration of cholesterol in blood. Considering that this mechanism involves physiological assessments, the applicability of *in vitro* experimental data to the *in vivo* systems needs to be justified.

(A) (B)

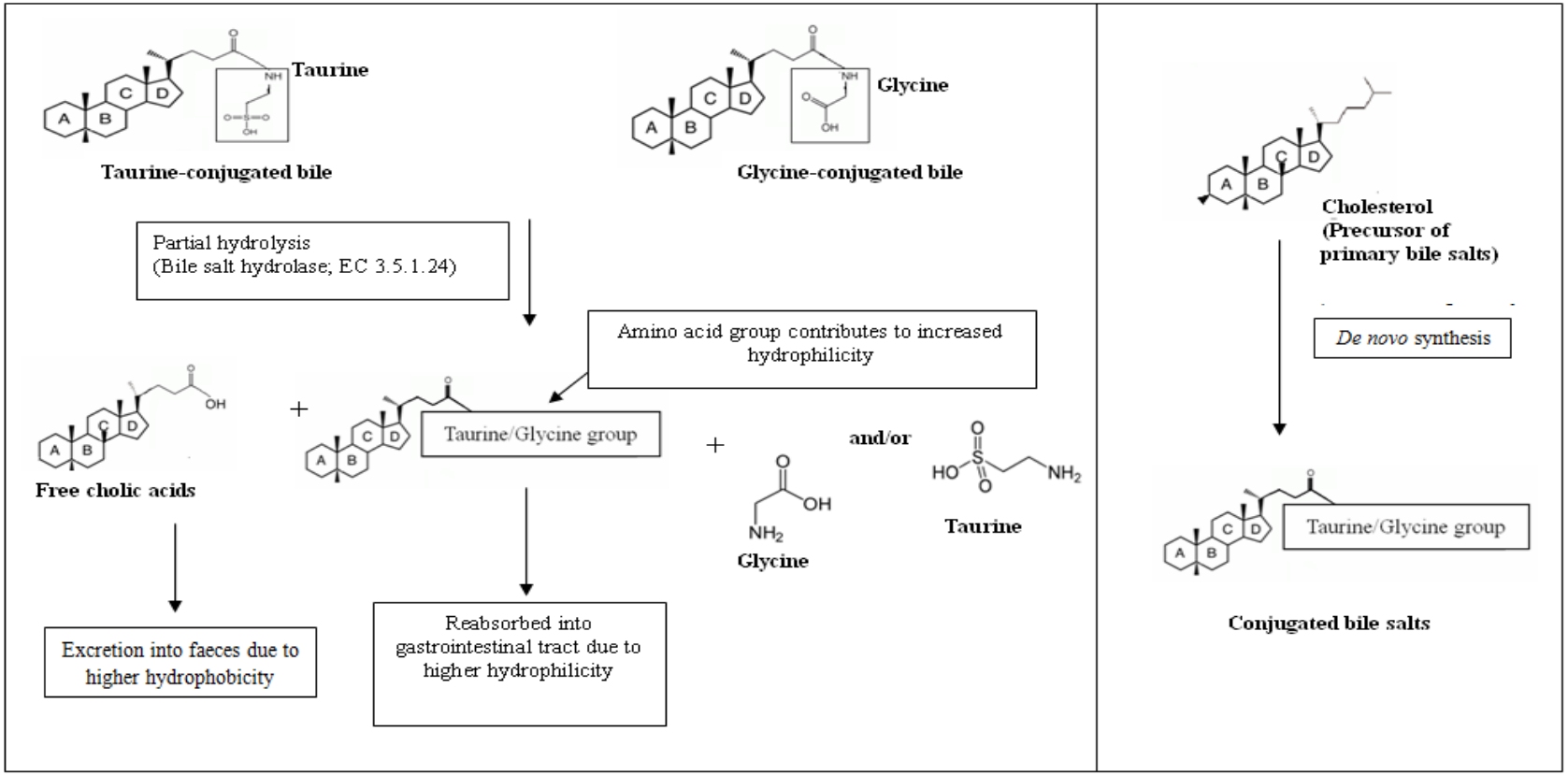

Figure 2.1: Postulated mechanism of BSH on bile (A) and the role of cholesterol as the precursor for synthesis of new bile acids (B)

2.2.6 Conversion of cholesterol to coprostanol

The microbial transformation of cholesterol to coprostanol mainly occurs in the large intestine of many species (Ma, 2006). It has been found that *Bifidobacterium* sp., *Clostridium* sp., and *Bacteroides* spp. were the common intestinal bacteria responsible for the transformation of cholesterol to coprostanol. Reduction of cholesterol to coprostanol is catalyzed by cholesterol reductase. The main metabolite of cholesterol conversion is coprostanol (Benno *et al.*, 2005), which is rarely absorbed in the intestine and is often excreted into the faeces. In addition, an inverse relationship has been observed between serum cholesterol levels and the coprostanol/cholesterol ratio in faeces. Thus, the conversion of cholesterol to coprostanol has been considered a natural means of lowering serum cholesterol levels in humans (Gerard *et al.*, 2007). Viega *et al.* (2005) reported that intestinal bacteria almost completely converted cholesterol to coprostanol in the intestines. However, little information is available on such an occurrence on lactobacilli and bifidobacteria, and the ability of lactobacilli strains to produce cholesterol reductase is yet to be elucidated.

2.3 *In vivo* studies on hypocholesterolaemic effect of lactobacilli and bifidobacteria[2]

Past *in vivo* trials have provided the experimental evidence to support the roles of lactobacilli and bifidobacteria in lowering serum cholesterol and improving lipid profiles. Kieling *et al.* (2002) used a randomized, crossover, and placebo-controlled design trial involving 29 women to evaluate the hypocholesterolaemic effect of yoghurt supplemented with *L. acidophilus* 145 and *B. longum* 913. The crossover study of 21 weeks involved the administration of 300g per day yoghurt, and the results obtained

[2] Parts of section 2.3 have been published in: Lye, H. S., Kuan, C. Y., Ewe, J. A., *et al.* (2009) *International Journal of Molecular Science*, 10, 3755-3775.

showed that HDL-cholesterol was increased significantly ($P < 0.05$) by 0.3 mmol L^{-1} and the ratio of LDL/HDL cholesterol decreased from 3.24 to 2.48. Sindhu and Khetarpaul (2003) conducted another placebo-controlled study to evaluate the effects of a probiotic fermented food on serum cholesterol levels in 20 young Swiss mice. The experimental group was fed a food mixture containing probiotics and 1% cholesterol while the control group was fed food without probiotics but containing 1% cholesterol for 42 days. The authors reported that the feeding of *L. casei* NCDC-19 (10^9 CFU) and *Saccharomyces boulardii* (10^9 CFU) caused a 19% reduction in the total serum cholesterol while LDL cholesterol levels was reduced by 37% upon 42 days of the feeding trial. In another study, De Rodas *et al.* (1996) used a placebo-controlled design trial that involved 33 hypercholesterolaemia-induced pigs (Yorkshire barrows) to examine the hypocholesterolaemic effect of probiotic. The authors reported that pig fed with *L. acidophilus* ATCC 43121 (2.5×10^{11} cells per feeding) for 15 days showed a reduced total blood cholesterol by 11.8% compared to the control that was not fed the probiotic. These positive clinical and experimental evidences have shown that probiotics could reduce serum cholesterol levels. Thus, further evaluation is needed to better comprehend the mechanisms involved.

2.4 Other compounds/ dietary substances that reduce cholesterol

Past *in vivo* studies have reported that other compounds/dietary substances were able to reduce cholesterol levels in human and animal models. Pande *et al.* (2012) used a placebo-controlled study to evaluate the effects of dietary cluster beans (*Cyamopsis tetragonoloba*) on serum cholesterol levels in 60 male Wistar rats. The authors reported that rats fed with 12.5% and 25% freeze dried cluster beans either on high cholesterol

diet (0.5%) or basal control diet for 8 weeks showed an increase in HDL cholesterol level compared to the control that was not fed the freeze dried cluster beans. These data showed that cluster beans have a cholesterol lowering potential in both normal and hypercholesterolaemic conditions. Kristensen *et al.* (2012) conducted another randomized, crossover, and double blind design trial that involved 17 subjects (10 women and 7 men) to examine the effect of flaxseed dietary fibers in different food matrices on lipid profile. Subjects consumed with flaxseed fibre drink (3/day; 19.3% dietary fiber) showed a decreased in total cholesterol and LDL cholesterol by 12% and 15%, respectively compared to the control while a reduction of 7% in total cholesterol and 9% in LDL cholesterol were observed with subjects consumed flaxseed fiber bread (3/day; 19.3% dietary fiber). In another study, Matsui *et al.* (2006) used a placebo-controlled study involving 21 male Wistar rats to evaluate the hypocholesterolaemic effect of catechin-free saponin-rich extract from green tea leaves (TE). The results obtained showed that TE supplementation for 28 days reduced the serum cholesterol level of hypercholesterolaemia-induced rats and enhanced their faecal cholesterol excretion. The authors also found that TE was able to inhibit the incorporation of cholesterol into micelles in *in vitro* experiment. Thus, TE might be exhibited the hypocholesterolaemic effect probably through inhibiting cholesterol micelles formation by preventing cholesterol incorporation and subsequently reduces its absorption from the intestine. These positive clinical and experimental evidences have shown that dietary substances could have the ability to reduce serum cholesterol levels, however, they do not possess additional “live” benefits as those exerted by probiotics. Other “live” benefits of probiotics that have been reported include alleviation of lactose intolerance,

antibacterial, preventing gut inflammation, and enhancing natural immunity (Liu *et al.*, 2007).

2.5 Administration of physical treatments

Cell poration and cell fusion are important biological methods which have a wide variety of applications, including gene transfection, drug delivery, antibody production, and cell hybridization (Chang, 1989). Physical treatments such as ultrasound, electroporation, and UV radiation have been applied to increase permeability and diffusion across membrane of bacterial cells. These have been found to increase the uptake of nutrients and excretion of waste that further increase the growth of bacteria in medium. Physical methods are advantageous as they could prevent biological and chemical damages that occur in conventional treatments. The utilization of physical treatment has been found to increase fusion and transfection yield compared to treatment using chemical and biological methods (Chang, 1989). Thus, many studies have been done to evaluate the effect of physical treatment on cells and tissues, laboratory animals, and human subjects. The overall effects of physical treatments on bacterial cells are shown in Figure 2.2. However, no attempt has been made to utilize such treatments to enhance and/or evaluate the cholesterol removal properties of lactobacilli, either via assimilation of cholesterol during growth or incorporation of cholesterol into the bacterial membrane.

2.5.1 Introduction

2.5.1.1 Ultrasound treatment

Ultrasound can be divided into two categories based on frequency levels, namely high frequency-low power ultrasound (2-10 MHz) and low frequency-high power ultrasound (20-100 kHz). Ultrasound is often applied to increase membrane permeability in order to release intracellular enzymes and organelles that are used in industry and medicine (Rokhina *et al.*, 2009). The effect of ultrasound on cells is dependent on strength and frequency of waves, cell wall structure, and treatment environment (Tabatabaie and Mortazavi, 2008).

Cavitation or oscillations of bubbles is a principal effect of low frequency ultrasound and has been reported to be responsible for many biophysical effects on cells (Richardson *et al.*, 2007). Cavitation involves a series of dynamic courses such as vibration, enlargement, shrinking, and even collapse of bubbles occurring during ultrasound treatment (Yang *et al.*, 2010). Repeatable oscillation of bubbles without implosion at low intensity forms stable cavitation (Runyan *et al.*, 2006) and is often harmless. However, when the intensity of ultrasound increases, the size of bubbles near the resonant size for the applied frequency starts to oscillate nonlinearly and eventually collapse (Richardson *et al.*, 2007). The collapse of bubbles results in a violent implosion that produces a shock wave that breaks cellular and membrane chemical bonds. This membrane structural alteration of cells subsequently affects the functionality of the cell membrane and metabolism activities (Tabatabaie and Mortazavi, 2008).

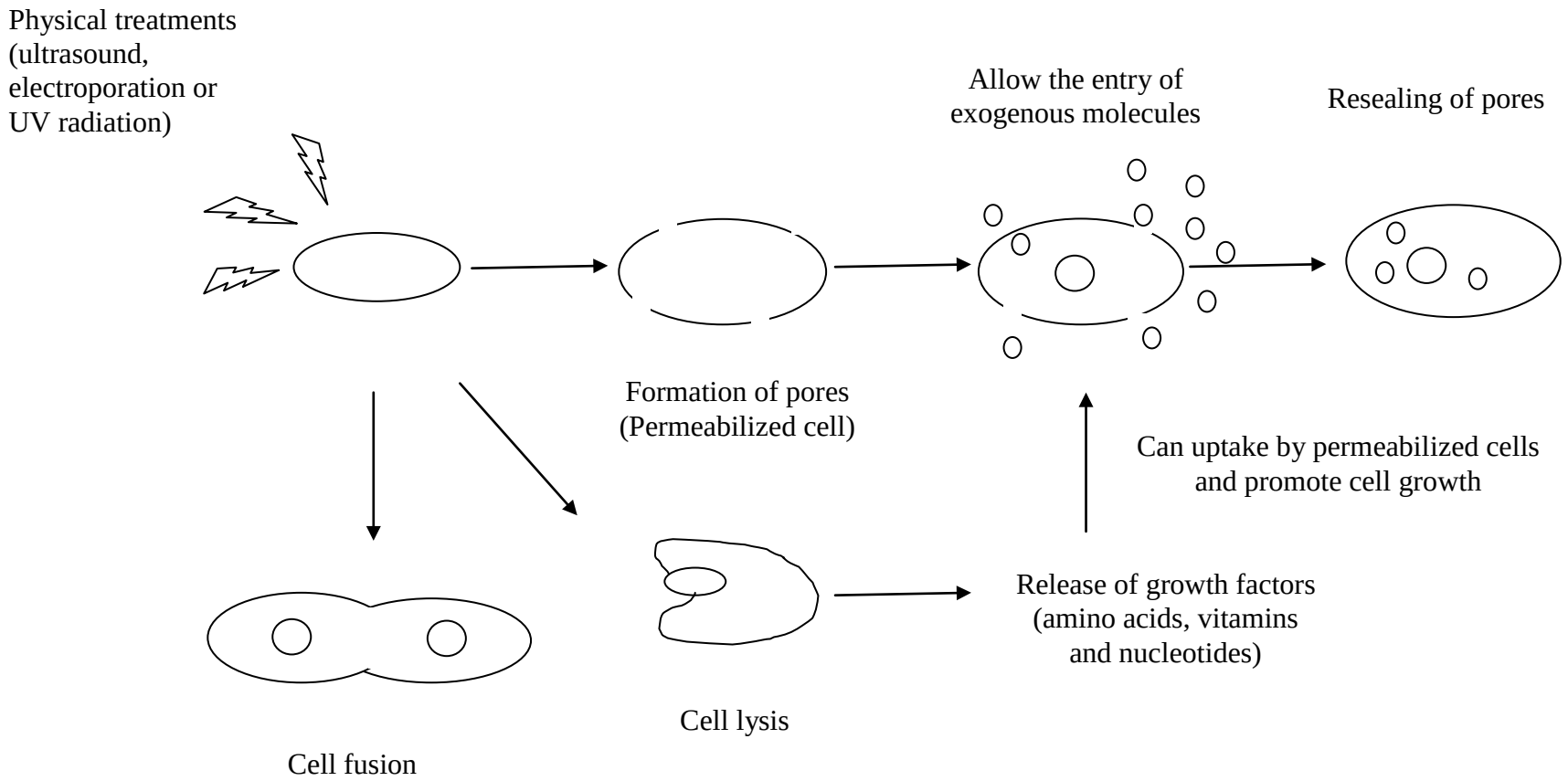

Figure 2.2: Postulated effects of physical treatments on bacteria cell.