

Selection and characterization of probiotic lactic acid bacteria with heterocyclic amine binding and nitrosamine degradation properties

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ABSTRACT

Potential probiotic strain for being health protectant especially intestinal illness is strain specific. This study investigated the selection of a new strain of probiotic of non-human origin and of human origin with the properties of intestinal protection against cancer. From the primary screening results, the human feces origin strains showed more bile salt tolerance than the fermented food origin strains. Whereas none of the human feces origin isolates could grow well in the acid condition. *Lactobacillus plantarum* CM4 was the new probiotic of non-human origin strain for this study. CM4 cells is said to tolerate and grow in 0.3% bile salt after 5 hours of incubation, at pH3 after 6 hours of incubation. This is in agreement with *in vivo* study for intestinal adherence ability of probiotic, a live CM4 cells was able to persist in mice small intestine and colon for 5 days. Live CM4 cells showed most effectiveness to bind 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) mutagen after 24 hours of incubation with 46.32% of binding ability while 144 hours of incubation with 85.34% of binding ability was the most effective for 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) mutagen. The significant difference ($p < 0.05$) was found at all those time points. Moreover, the CM4 strain could degrade diphenylnitrosamine (DPN) better than 1-nitrosopyrrolidine (NPR) with dose response relationship activity. These imply that the CM4 strain could be the value added for the consuming pharmaceutical probiotic product based on scientific proof of its role in intestinal survival properties and cancer prevention through binding PhIP and IQ mutagen as well as degrading nitrosamine.

INTRODUCTION

Élie Metchnikoff (1907) recognized the influence on longevity of the daily consumption of lactobacilli fermented product, and thus stated that lactobacilli could “arrest intestinal putrefaction and must at the same time postpone and ameliorate old age”. This recognition was considered as the birth of probiotic (Fuller, 1992) and theories of Metchnikoff remain very significant (Walter, 2008). The Food and Agriculture Organization of the United Nations and the World Health Organization (2001), present a definition of, probiotic as “Live microorganisms which

when administered in adequate amounts confer a health benefit on the host”. The completion of individual microbial community is after weaning (Mountzouris *et al.*, 2002; Dethlefsen *et al.*, 2006) that is influenced by genetic factors, diet, and environments (Mountzouris *et al.*, 2002). Without disturbance, the success of a microflora community is expected to last for whole life since the intestine is a stable microbial community (Curtis and Sloan, 2004). However, age (He, 2006), long-term diet (McCartney and Gibson, 2006), antimicrobial treatment, stress, malnutrition, surgery, and immune deficiencies are factors that can alter the shape of intestinal microflora (Waij, 1999). Among these factors, the use of probiotics is looking for restoring the balancing of intestinal microflora (Fuller, 1992). While the balance of intestinal microflora is critical for intestinal development, homeostasis, protective effect

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against pathogenic microorganisms, metabolic reactions, salvaging energy from fermentation of non-digestible dietary residues and controlling proliferation and differentiation of epithelial cell, and certain pathological disorders. Multisystem organ failure, colon cancer, and inflammatory bowel diseases are harmful effects related to the microflora which is an essential factor (Montalto *et al.*, 2009) since their shaping was badly altered. Moreover, heterocyclic aromatic amine (HCAs) and *N*-nitrosamines are toxic substances formed during food processing or preparations.

The epidemiological studies revealed that people who eat well cooked meat containing HCAs may elevate the risks for colon, breast, and prostate carcinogenesis (Turesky, 2007; Snyderwine, 2002; Ito *et al.*, 1997). Toxicity of HCAs is related to intestinal microbial metabolism and is a risk to human health via their bioactive compounds (Chung, 1983). Thus, as HCAs derivatives from the metabolic activation process in the liver and colon could form DNA-adducts (de Kok and van Maanen, 2000), DNA damage could be affected by HCA biotransformation through the intestinal bacteria as *Bacteroides*, *Clostridium*, *Eubacterium*, and *E.coli* (Nowak and Libudzisz, 2009). In addition, nitrosamines are nitroso compounds that have been pinpointed as having carcinogen and genotoxic potential since the metabolic activities via electrophilic intermediates could react with the cellular proteins, RNA, and DNA (Habermeyer and Eisenbrand, 2009). Research showed that dimethylnitrosamine could possibly be produced when rat intestinal bacteria appears with the substrates (Klubes *et al.*, 1972) including *E.coli* (Suzuki and Mitsuoka, 1984). Moreover, it has been reported that dietary *N*-nitrosamines may contribute to lung cancer (Rogers *et al.*, 1995; De Stefani *et al.*, 1996). It has also been observed that lungs, liver, kidneys, mammary glands, stomach, pancreas, bladder, or esophagus may be target organs in humans for carcinogenesis (Lijinsky, 1990). However, researches showed that probiotics could rebuild the microbial composition which include protectant illness of the gastrointestinal tract (Khedkar and Ouwehand, 2006), and thus there is evidences that *Bifidobacterium* and *Lactobacilli* contributed to individual physiological well-being (Salminen *et al.*, 1996).

In this study, the desirable characteristics of the new probiotic strain were identified. The most important characteristic of the probiotic is to pass through the intestinal tract in a viable state. Resistances to bile, low pH value of the stomach, and *in vivo* intestinal survival are preferable properties of the selected probiotic strain in this study. Furthermore, this research studied the properties of HCAs binding and nitrosamine degradation of a new probiotic strain *in vitro* since both toxic substances could be found in daily human consumption and thus have a harmful effect.

MATERIALS AND METHODS

Isolation of lactic acid bacteria (LAB) and primary probiotic screening

The tested LAB strains were isolated from 24 samples of traditionally Thai fermented food products and 10 unidentified

samples of human feces which included 5 samples of newborn infants and five samples from women in a maternity ward. Five strains of probiotic LAB and seven LAB strains from a culture collection center in Thailand (Thailand Institute of Scientific and Technology Research; TISTR) were provided by the Health Product Research and Development Center, Faculty of Pharmacy, Chiang Mai University. All of the selected strains showed good growth of MRS (de Man Rogosa and Sharpe), and were Gram-positive and catalase-negative.

Primary screening was conducted for mass LAB isolated strains by observing the strains that could survive and grow in 0.15 and 0.30% bile salt and pH 2-5 at 37°C (Conway *et al.*, 1987; Duangjitcharoen *et al.*, 2008). Those biological barriers were simulated by inoculation of the overnight culture (18-24hrs) of each strain by streak on bile salt agar plates while pH 2-5 tolerance ability was tested in pH-modified MRS broth. Before selection of probiotic LAB strain to test the intestinal persistent ability in animals, all the above studies were repeated by bacterial cell count.

0.3% bile salt tolerance was the determined cell survival at the time of 15 minutes, 1.5 hours, and 5 hours (Kim, 1968; Read, Cammack *et al.*, 1982). 10⁹ CFU/ml of each of the tested strains were suspended in phosphate buffer saline (PBS) with 0.3% bile salt while pH 1.5, 3 and 5 (Bennink *et al.*, 1999; Cotter and Hill, 2003; Erkkilä and Petäjä, 2000; Sherwood, 2010) were adjusted to the buffer to study the acid tolerance test. At pH 1.5, the viable cell count was performed at 15 and 30 minutes, whereas, at pH 3, the cell enumeration was carried out after the incubation time of 6 hours. pH 5.0 was the control condition.

Live cell binding PhIP and IQ (Nowak and Libudzisz, 2009)

10⁹ cell/ml of each selected strains were adjusted and suspended in appropriate buffer (pH 6.2-6.3) with 50µg/ml of PhIP or IQ and were incubated for 144 hrs at 37°C. The HCA residues in the supernatant were determined at the beginning, 20 minutes, 1.5, 24, 72, and 144 hours. The positive control was the buffer with 50µg/ml of each of the tested substances while the negative control was the cell suspension without the tested substances.

The filtrated supernatant was used to determine the quantity of unbound (uninhibited) tested substance with a reverse-phase high-performance liquid chromatography (HPLC) system that was fitted with a UV/VIS detector. A mobile phase of acetonitrile : water (50:50) was used, and the absorbance was measured at 254 nm. The amount of the PhIP or IQ in the supernatant was calculated as the percent binding on each LAB strain. The assays were repeated three times.

The binding rate was calculated as 1-(peak area of substrate from bacterial supernatant/peak area of substrate solution) x 100 (Turbic *et al.*, 2002). Statistical analysis of the results was performed using one-way analysis of variance (ANOVA).

Nitrosamine degrading ability (Grill *et al.*, 1995; Rowland and Grasso, 1975)

After 24 hours of culture in MRS broth, a bacterial cell suspension that was adjusted to 10^9 cells/ml in buffer was tested for the ability of degrading diphenylnitrosamine (DPN) and 1-nitrosopyrrolidine (NPYR). The final concentration of nitrosamine was 2-200 $\mu\text{g/ml}$. Nitrite released by the breakdown of nitrosamine was assayed colorimetrically at 540 nm after 20 hours of incubation time. Nitrosamine degrading activity of each LAB was calculated.

Persistence ability of probiotic selected strain in murine gastrointestinal tract

Gastrointestinal attachment by probiotic-selected strain was conducted as described by Lee *et al.* (2004) and Duangjitcharoen *et al.* (2009) by staining 10^9 bacterial cells with fluorescent dye cFDA-SE, 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester, MW = 557.47 (Molecular Probe), and then orally administered by orogastric intubation to mice. After dosing, three mice were sacrificed at each time point on days 1, 2, 3, 5 and 7. The fluorogenic dyed cells attached to the gastrointestinal wall were detected by flow cytometry (Becton Dickinson FACSCalibur) upon excitation at 488 nm and detecting the emission signal as a green color at 518 nm. Cells producing the signal from the cFDA-SE were assumed to have persisted gastrointestinal tract. The results were compared with the control mice that had been administered only PBS.

All animal experiments were approved by the Ratchathani University-Animal Care and Use Committee ACUC) at Ubon Ratchathani University, Thailand (protoc 24/2552/Research).

RESULTS AND DISCUSSION

Primary probiotic LAB screening

Eventually, isolate CM4 was one probiotic *Lactobacillus plantarum* for human use that was selected from 152 LAB in this study. The strain identification showed that the relation result between the conventional identification method 16S rRNA gene analysis. CM4 strain showed Gram-positive shape, lack of catalase enzyme, and produced acid amygdalin, arabinose, cellobiose, esculin, fructose, galactose, glucose, lactose, maltose, mannitol, raffinose, ribose, sucrose, trehalose. In addition, 16S rRNA sequences were determined using Blast (NCBI Basic Local Alignment Search Tools) program demonstrated the highest homology with other 16SrRNA genes in the GenBank. CM4 strain showed 99% similarity with *L. plantarum* WCFS1 (Siezen *et al.*, 2012) (Fig 1). As shown in Table 1, among the selected-strains included 103 isolates from traditional Thai fermented food products, 37 strains from human feces, 7 strains from TISTR (Thailand Institute of Scientific and Technological Research) lactic acid bacteria collection, and 5 probiotic LAB strains from the Health Product Research and Development Center, Faculty of Pharmacy, Chiang Mai

University. *L. plantarum* CM4 was the probiotic LAB that was provided by the Health Product Research and Development Center. It was a strain that was isolated from fermented *Morinda coreia* Ham. beverage (Duangchitchareon, 2006; Duangjitcharoen *et al.*, 2008, 2009).

Table 1: Summary of results of probiotic LAB primary screening.

Samples		No. of collected samples	No. of isolated LAB	No. of growth strains in 0.15/0.30% bile salt	No. of acid tolerance selected strain
Fermented meat	Mum Neur	2	13	6	1
	Sai Krog Esan	2	11	4	2
	Nam Neur	3	16	0	0
	Nam Moo	4	25	0	0
	Poo Dong	2	4	0	0
Fermented Fish	Pla Jom	4	10	2	3
	Pla Som	1	16	4	0
	Khai Pla Mag	1	6	0	0
	Phoong Pla Mag	5	2	1	0
Human feces	Female (Mother)	5	14	12	0
	Newborn Infant	5	23	8	0
TISTR strain			7	2	1
Probiotic strain			5	5	5
TOTAL			152	44	12

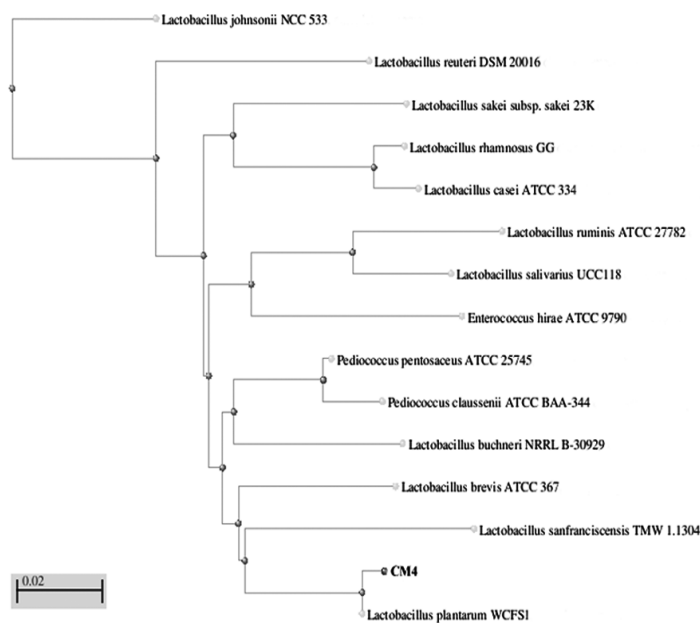


Fig. 1: Phylogenetic tree constructed using the neighbor joining method showing CM4 position and related species.

Although general criteria on the origin of probiotic strain suggested that if colonization is the goal of probiotic application, it is necessary to define species and particular location (Havenaar *et al.*, 1992; Salminen *et al.*, 1996). However, that is not meant to be impossible in case the origin of strain is not related to the place or host species to which the selected probiotic would be applied. Many researchers succeeded in screening probiotic strain from a non-human (host) origin. Fermented plant (Duangjitcharoen *et al.*, 2008, 2009; Kantachote *et al.*, 2010; Park *et al.*, 2002), fermented

meat (Sirilun *et al.*, 2010; Klingberg *et al.*, 2005), and fermented milk (Pinto *et al.*, 2006) were such sample sources of lactic acid fermentation that were collected for probiotic screening. Nevertheless, researchers have studied about the entero-mammary pathway that the dendritic cell would have the ability to take up non-invasive bacterial from mother intestinal lumen to colonize distant mucosal location through a circulation of lymphocyte in the mucosal-associated lymphoid tissue (Nasiraii *et al.*, 2011). Live bacteria including LAB (Nasiraii *et al.*, 2011, Martín *et al.*, 2007, 2009) could be directly sent from a mother's intestine to her baby via breast fed milk and; this may imply the possibility of finding human-origin LAB that may possess probiotic properties in a breast-feeding baby's intestine.

Bile salt and acid tolerance

The inherent bile and acid tolerance of bacteria appear to be a strain specific nature (Corcoran *et al.*, 2005; Tannock, 2004; Burns *et al.*, 2010; Guijie, 2012) and could not indicate which species is more resistant than the others (Guijie, 2012; Begley *et al.*, 2005). Since the genome (Kleerebezem *et al.*, 2003) and proteome (Koistinen *et al.*, 2007; Cohen *et al.*, 2006) of *L. plantarum* strain has been widely studied; apparently, strain-to-strain variation on genes involving in environmental adaptations has been found (Kleerebezem *et al.*, 2003; Zhang *et al.*, 2009; Molenaar *et al.*, 2005; Siezen *et al.*, 2010). That is the genomic diversity, therefore, *L. plantarum* strains could show different phenotypes (Siezen *et al.*, 2010; Molenaar *et al.*, 2005; Meijerink *et al.*, 2010)

In this study, the primary probiotic LAB screening showed that the isolated-strain from human feces origin was more relatively tolerant in 0.15 % and 0.30 % bile salt appearance condition than the isolate from fermented food origin (Table 1). 54.05% (20/37) of a total isolated strain from human feces were the tolerant strains to bile salt while 16.5% (17/103) were the selected strain from fermented foods. Furthermore, the isolated strains from adult human feces origin showed a higher number of tolerance strains than newborn infant feces origin (85.17% (12/14): 33.33% (8/23)). The result were totally different in the primary screening of acid tolerance condition in which none of human origin isolates could grow well at pH 2, 3, 4, 5 (Table 1). After this ordering step, 12 strains were selected for further study. They included 6 strains from fermented animal meat origin, 5 probiotic LAB strains from Thai lactic acid fermented plant beverage, and a strain from TISTR lactic acid bacteria collection.

By the bacterial cell count method in a repeated study for 0.3% bile salt tolerance and acid tolerance condition, three selected strains showed better results than the others while CM4 strain was the new probiotic LAB strain in this study. There was no significant difference about its growth in the presence of 0.3% bile salt condition during 15 minutes, 1.5 hours, and 5 hours of study (Fig 2). This simulated condition was done along with the transit time of a normal small bowel study (Kim, 1968; Read *et al.*, 1982) that ranged from 15 minutes to 5 hours and the mean transit time was 84 minutes (Kim, 1968). Bile salt is secreted into the

duodenum with bile. It is a highly toxic detergent for microorganisms since it can quickly dissolve membrane lipids resulting in leakage of cell content (Begley *et al.*, 2005). Therefore, the selected probiotic strain to be used as a food supplement for gastrointestinal and related health benefits should have viable ability in the small intestine area. Mechanism to tolerate bile salts by *Lactobacillus* usually found is active efflux of bile (Pfeiler and Klaenhammer, 2009; Bustos *et al.*, 2011), bile salt hydrolase enzyme (Lam-bertal *et al.*, 2008), and alteration of cell membrane and cell wall structure (Taranto *et al.*, 2003). With comparative proteomics study, *L. plantarum* has been proven to have those mechanisms to tolerate bile (Hamon *et al.*, 2011).

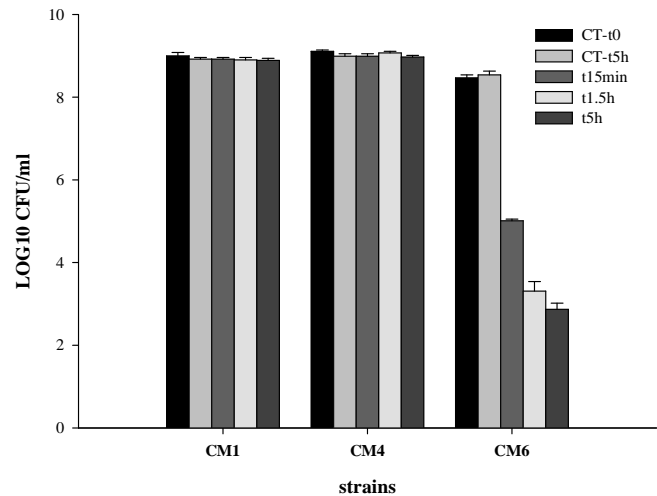


Fig. 2: Survival ability of selected LAB strains exposed to 0.3% bile salt in phosphate buffer saline solution. The results were the mean of the triplicate experiment. The bars were expressed as mean±standard deviation. (CT: buffer containing no bile salt; t: buffer+0.3% bile salt).

Tolerance of the acid condition in the stomach is a desirable characteristic that most research has proposed. Based on the relationship between normal gastric transit time and gastric pH, survival of tested-bacteria at pH 1.5 for 15, and 30 minutes were a simulated-condition since the fasting period of the stomach has pH 1.5 (Cotter and Hill, 2003) and liquid food that has the shortest transit time showed approximately 30-50% gastric emptying in 10-30 minutes after moving into the stomach (Bennink *et al.*, 1999). However, when there is presence of food, the pH raises to the level of pH 3 (Erkkilä and Petäjä, 2000). In addition, a viability study at pH 3.0 for 6 hours simulated the condition of increasing pH during feeding (Cotter and Hill, 2003) whereas 6 hours of testing is gastric emptying rate of taking a high fat meal since fat digests is slower than other nutrients (Sherwood, 2010).

It was found that three LAB strains had better growth than the other strains and the most resistant strain was strain CM4. Although this probiotic strain showed poor viability at pH 1.5 for 15-30 minutes of incubation, it was more tolerant at pH 3 for 6 hours (Table 2). Tannock (1983) suggested that daily consumption of probiotic is necessary to maintain the probiotic level in the body. As a result of the acid condition in the stomach, the acid tolerant microorganism that is Generally Recognized As Safe

(GRAS) are lactobacilli and bifidobacteria which are potential microorganisms to be considered as probiotic strains.

Table 2: Survival of selected LAB strains at pH 1.5 and 3.

Strains	pH5		pH1.5		pH3
	Control (log CFU/ml)		15min.	30min.	6 hrs.
	0 min	5hrs.	(CFU/ml)	(CFU/ml)	
CM1	8.75±0.04	8.79±0.16	0.67±0.58	0.67±0.58	2.18±0.78
CM4	8.81±0.09	8.99±0.01	1.67±0.58	1.67±0.58	3.89±0.53
CM6	8.43±0.12	7.91±0.48	ND ^a	ND	ND

^aND = not detectable. The values were expressed as mean±sd (n=3)

Since acid resistant ability of *Lactobacillus* is general recognized (Tannock, 2004), the proton motive force is the acts to response the acid stress condition (Rollan *et al.*, 2003). Based on the proton motive force generation, researchers have demonstrated the intracellular phenomenon of *L. plantarum* to contribute to such action include a proton-translocating FOF1-ATPase (Cotter and Hill, 2003), sodium-proton antiporters (Cotter *et al.*, 2001), enzymes contribute to pH homeostasis (e.g. glutamate decarboxylase, arginine deiminase) (Arena *et al.*, 1999; Siragusa *et al.*, 2007).

On the other hand, through suspending cells into the modified buffer in this study the environmental component of the probiotic vehicle is a necessity to aid gastrointestinal survival (Cotter and Hill, 2003; van Bokhorst-van de Veen *et al.*, 2012). Amino acid as arginine and aspartate (Zhang *et al.*, 2012), glucose (Shabala *et al.*, 2002), fatty acid as oleic acid (Corcoran *et al.*, 2007) are such the medium compositions that have been investigated to enhance acid tolerance of probiotic lactobacilli, while an addition of meat (Gänzle *et al.*, 1999) or soy protein (Shimakawa *et al.*, 2003) to the medium could also increase viability of bacterial cells in the intestinal bile salt releasing site.

Gastrointestinal persistence ability of an orally administered *L. plantarum* CM4

In this study, the goal application of probiotic selection was persistence in the host and, as a result, adherence in the animals was considered. By flow cytometry and, detection of live CM4 cells labeled with 5 (and 6)-carboxyfluorescein diacetate, succinimidyl ester (cFDA-SE) fluorescent molecules were found until day 5 in the animal gut wall including the small intestine and colon after the animals were orally administered a single dose of 10^9 labeled cell/mouse on the first day (Fig 3).

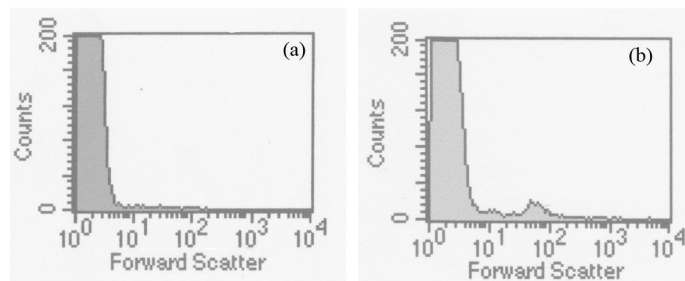


Fig. 3: Fluorescence intensity difference between control group (a) and tested-mice group (b).

However, this study could not define the number of persistence lactobacilli in the animal intestine. The finding of the intestinal persistence ability of CM4 strain in this study could indicate the possible adhesion of a non-human origin probiotic LAB strain. Probiotic is a supplement for repairing microbial defects as modern life-style including modern perinatal care provides unnatural foods and unnatural environment conditions. Actually, after weaning, the intestinal bacterial community was assumed to have the superior competitor in each intestinal compartment with individual difference (Vaahtovuori *et al.*, 2003). Without perturbation, it was expected to persist throughout the host's life since naturally the intestinal bacterial community appeared stable (Curtis and Sloan, 2004). Presently, it is difficult to escape the factors that affect the intestinal bacteria community. Factors that could disturb the composition of gastrointestinal microbiota, leading to health effects include the habitation of pathogenic bacteria and, overgrowth of commensal bacteria (Nord *et al.*, 1986). For this reason, probiotic use is a possible way to repair this defect. Again, the adherence of commensal bacteria to the intestinal mucosa depends on their role in the maintenance of normal gut function, the immune system, and inhibition of the growth of pathogenic bacteria by competitive exclusion (Hooper and Gordon, 2001). Similarly, adherence ability study of probiotic bacteria for human use is necessary. This study has found gastrointestinal tract persistence ability of probiotic *L. plantarum* CM4 strain in animals' small intestines and colons. Although genetic background is an influence that seems to be a natural selection for commensal bacteria in the human gastrointestinal tract (Hoskins, 1992), lactobacilli showed possible structures that offer their attachment to the home layer in the gastrointestinal tract. The adhesion of bacteria to the mucus layer is limited by two main factors: 1) the concentration of bacteria nearby the epithelial cell receptor 2) bacterial affinity to the epithelial cell receptor (Chow and Lee, 2006). That is why the administration of adequate amounts of probiotic is necessary in case of food supplements (Food and Agriculture Organization of United Nations and World Health Organization, 2001). Lactobacilli, including *L. plantarum* (Pretzer *et al.*, 2005), was identified as having the ability to adhere to mucosal surfaces with compatibility between the intestinal epithel cell receptor and lactobacilli adhesion. Boekhorst and colleagues (2006) showed that *Pediococcus pentosaceus* ATCC25745, a plant origin strain, could have this adherence ability. This finding suggested that the study of probiotic adherence properties may not be necessary to find new potential ones only from human or animal origins. From this study, a new probiotic, *L. plantarum* CM4, was isolated from the fermented-plant beverage. Molecular microbiology about adherence properties may be a possible way for future study in place of animal use.

Heterocyclic amine binding ability

Heterocyclic aromatic amines (HCAs) are considered carcinogenic from cooking (frying, grilling, baking, roasting, and smoking) protein-rich food such as muscle meat and fish at high

temperatures from 150 to 300 °C (Turesky, 2007). Several studies indicated that HCAs are involved in the cause of human colon cancer, (Rohrmann *et al.*, 2009), the well-done meat eaters in particular. HCAs are also associated with the risks of breast, lung, and gastric cancer (Terry *et al.*, 2003; Wu *et al.*, 2006). Their carcinogenicity requires metabolic activation through a drug metabolizing enzyme (Kato and Yamazoe, 1987). There is a wide range of HCAs, a human cancer risk factors, and thus one cancer case per 1000 individuals being the upper limit of their risk (Gaylor and Kadlubar, 1991), and 50 cases per million individuals being the lower limit (Lutz and Schlatter, 1992).

Because HCAs are found in foodstuffs and are risk factors for human cancers, the potential removal or binding carcinogen properties of new probiotic strain was the focus of this study since researchers reported the ability of LAB on binding HCAs (Nowak and Zibudzisz, 2009; Lankaputhra and Shah, 1998; Sreekumar and Hosono, 1998; Caldini *et al.*, 2005).

At the beginning of this test, the sudden binding of all tested strains was clearly shown (Fig 4).

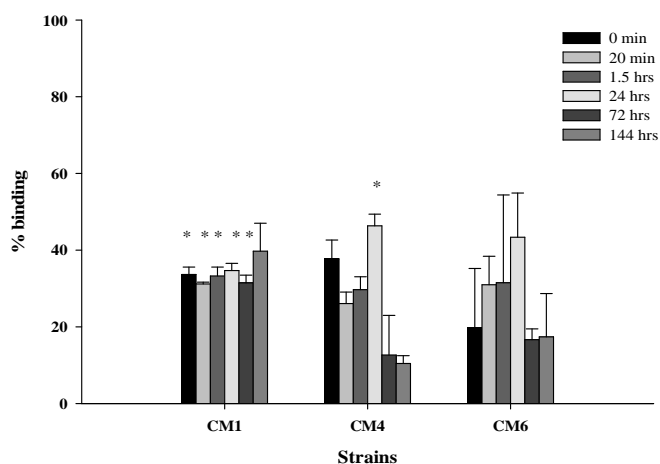


Fig. 4: PhIP binding ability of LAB tested strain. The bars were expressed as mean±standard error of mean (n=3). *Indicated statistical significance ($p<0.05$; one-way ANOVA) compared with the binding ability in absence of LAB tested strains.

At the beginning (0 minute) of the PhIP binding test, CM4 strain showed the 37.73% binding and the statistically significant difference ($p<0.05$) occurred at 24 hours. It was the CM4's most effective binding of 46.32%. All the tested strains showed binding IQ at the beginning (0 minute) (Fig 5). Throughout this study, CM4 strain was the most effective to bind IQ (62.47-85.34%). Its significant ability ($p<0.05$) was found at all tested time points in comparison to the condition of absence of LAB tested strains. However, in the cases of IQ binding ability at day 3 and 6, the derivatives were observed as a right shift of the chromatogram occurred.

Nevertheless, binding ability to the IQ-derivatives of LAB-tested strain was found. In addition, at the end of this study (144 hours) all tested strains showed that IQ is better removed than PhIP with 44.23-85.34%, whereas the most binding ability of PhIP was 45.55%. The binding capacity of LAB was proved as a

physical occurrence at the bacterial cell wall since the freeze-dried cell was studied. The bacterial cell wall was the cell structure that demonstrated the responsibility of binding/removal HCA carcinogen. Cation exchange, hydrophobic bonds of cell wall and the carbohydrate moieties of bacterial cell have been reported to influence binding modes (Tsuda *et al.*, 2007; Bolobnani *et al.*, 1997). Metabolic activity of HCAs via cytochrome P450-mediated-*N*-oxidation is the beginning of carcinogenicity/mutagenicity potency of HCAs since *N*-hydroxy-HCA could react with DNA to form adduct in both the liver and extra-hepatic tissues.

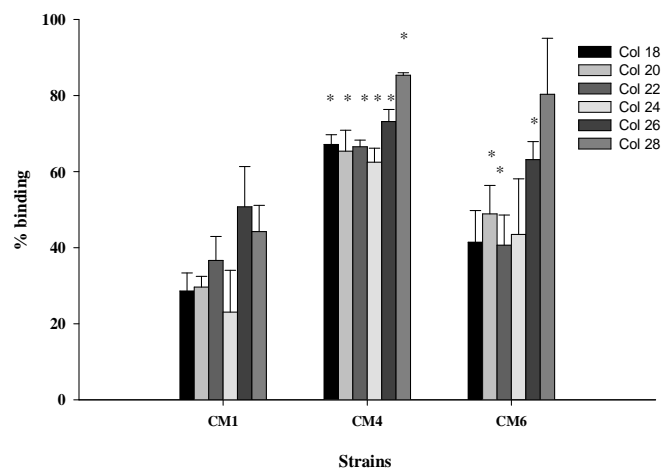


Fig. 5: IQ binding ability of LAB tested strain. The bars were expressed as mean±standard error of mean (n=3). * indicated statistical significance ($p<0.05$; one-way ANOVA) compared with the binding ability in absence of LAB tested strains.

Acetylation or sulfation is also the subsequently continuous bioactivation of *N*-hydroxy-HCA to form other products that react with DNA. Even if, the detoxification enzyme could reform those products to be parent HCAs or stable substances for excretion from the body, glucuronide conjugates could release toxic metabolite again via bacterial enzyme in the colon. Thus, this case was discussed by Zsivkovits and colleagues (2003) who showed that the direct binding effects of lactobacillus strain affected reduction in DNA migration in the colon and liver from *in vivo* study. In addition, the absorption of HCA was studied *in situ* (Terahara *et al.*, 1998).

It was found that parent form of HCAs was absorbed by the small intestine and the reduction of HCAs absorption decreased when LAB was present. This study found that the CM4 probiotic strain showed the ability of small intestinal as well as colon adherence and *in vitro* binding HCAs as PhIP and IQ. These may be the possible means of the CM4 probiotic strain to prevent carcinogenesis by binding/absorption HCAs in the small intestine before absorption of the toxic compounds to the liver or release to the colon.

Moreover, with an expectancy of modifying the microbial metabolism of probiotic, the decreasing activity of intestinal microflora enzyme that could hydrolyze the glucuronides conjugate to form a HCA-metabolite (Nagao *et al.*, 1977) may be a

possible help of a new probiotic LAB in the case of preventing the formation of toxic metabolite of HCA in the colon.

Nitrosamine degrading ability

More than 80 different nitroso compounds were identified as cancer-causing agents. The formation of nitrosamines results from the reaction of secondary amines that are in animal meat with nitrite at acid pH. Nitrite is commonly added to preserve meat and fish, and so nitroso compounds were measured in these foods (Ender and Ceh, 1968). Reduction of nitrate could be done by bacteria to produce nitrite. Leafy vegetables often have high levels of nitrate that the oral microbial flora of humans can reduce to form nitrite. This reaction can promote nitrite levels in saliva of between 6 and 10 ppm (Tannerbaum *et al.*, 1974).

It was found that the formation of *N*-nitrosodimethylamine (dimethylnitrosamine) could occur under anaerobic conditions at pH 7.0 since dimethylamine and sodium nitrite was incubated together with rat intestinal microflora (Klubes *et al.*, 1972). These findings indicated that nitrosamines could be generated in the intestine, where the pH is nearly neutral. In addition, the consumption of more red meat led to increased production of fecal nitroso compound, and this may possibly be related to the etiology of colorectal cancer (Bingham *et al.*, 1996). Suzuki and Mitsuoka (1984) have reported the ability of *E.coli* to form *N*-nitrosodimethylamine.

Moreover, if the nitroso compounds were absorbed from the small intestine to the liver, their bioactivation via human cytochrome P450 activity would generate the intermediate that may form DNA-adduct (Guttenplan, 1987). As a result, these compounds were studied in the role in other human cancers such as gastric, esophageal, nasopharyngeal, and bladder (Mirvish, 1995). However, bifidobacteria and lactobacilli could reduce nitrosamine quantities (Ayanaba and Alexander, 1973). This relates to the possible mechanism to prevent cancer (Hirayama and Rafter, 2000). Even though the breaking down of nitrosamines could reproduce nitrite ion, the substrate to produce the *N*-nitrosamine, acid production during the growth of lactic acid bacteria could decrease the concentration of nitrite (Walker, 1990).

The CM4 new probiotic selected strain in this study showed the highest activity of degradation DPN by dose response relationship (Fig 6). Moreover, the NPR breakdown of activity was weaker than DPN by all tested strains (Fig 7), and it was not dose response relationship activity. This study also found that all tested strains had a faster breakdown rate of DPN than the rate of breakdown in NPR. CM4 showed the highest activity of degradation, 100 μ g/ml (0.51 μ mol/ml) of DPN by releasing 11.10 μ mole of nitrite per ml at 20 hours of incubation time (200 μ g/ml was excluded from the test since the finding of the flocculation in the test tube).

Similarly, Rowland, & Grasso (1975) have reported that lactobacilli showed DPN degrading activity of 2.5 μ mol/ml of DPN by releasing 0.12-0.35 μ mole of nitrite per ml at 20 hours. Although the CM4 strain showed better activity than Rowland's

study, both studies suggested the use of the concentration of DPN during 1-100 μ g/ml. Better activities for testing nitrosamine degrading were observed. Rowland & Grasso (1975) reported that the DPN concentrations of less than 1 μ mol/ml and *Lactobacillus* in their study had the maximum rate of nitrosamine degrading activity. Nitrosamines such as *N*-nitrosodimethylamine, *N*-nitrosopyrrolidine, and *N*-nitrosopiperidine were found in food at concentrations of up to 10 μ g/ml (Grill *et al.*, 1995). Less than 0.005 μ mole of nitrite per ml of incubation time was the highest activity of breaking down of NPR, less than the breaking down activity of DPN by all tested strains, and none of the tested strain had the dose response relationship activity. Therefore, the rate of NPR and DPN breakdown varied considerably depending on the strains.

Thus, the enzymes for biotransformation of *N*-nitrosamine could be found in the liver, intestines, kidneys, lungs, brain, skin, and placenta (Hinuma *et al.*, 1990). By the oral route, however, probiotic CM4 that could colonize throughout the gastrointestinal tract may feasibly degrade nitrosamine and reduce the absorption of nitrosamine through the small intestine before producing reactive metabolites in the liver.

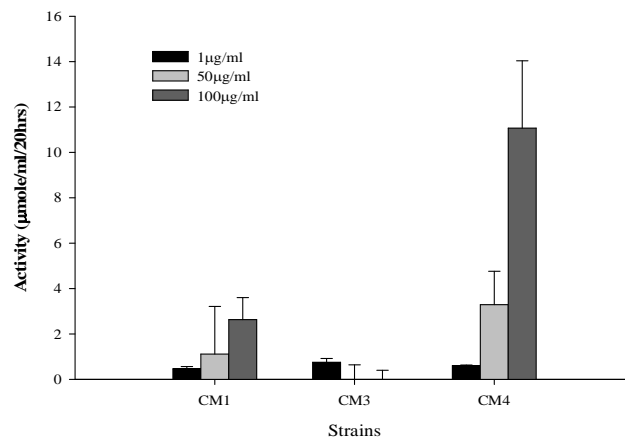


Fig. 6: The activity of breakdown of DPN over 20hours by the tested strains. The bars were expressed as mean \pm standard error of mean (n=3).

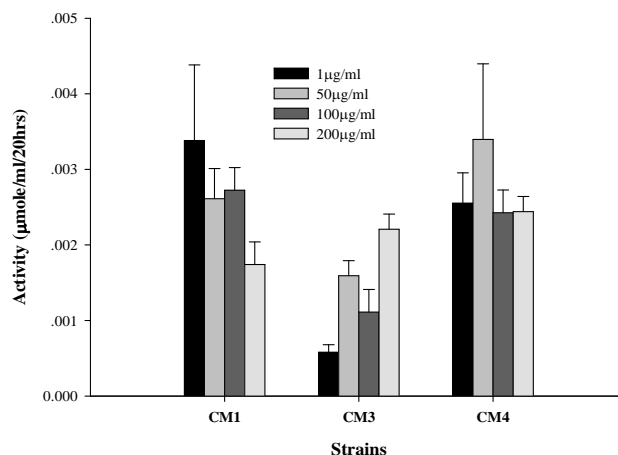


Fig. 7: The activity of breakdown of NPR over 20hours by the tested strains. The bars were expressed as mean \pm standard error of mean (n=3).

CONCLUSIONS

In conclusion, this study found that *L. plantarum* CM4 was the successful probiotic strain for human use based on evaluation through the *in vitro* study of bile salt, acid tolerance. Its *in vitro* survival relates to the persistence ability of live CM4 cells in animals small intestine and colon. Therefore, this finding may imply the heterocyclic amine binding and *N*-nitrosamine degrading ability of CM4 strain in the gastrointestinal tract. This simulated condition could be used to predict the health protection of a new probiotic strain from the bioactivation of HCA and *N*-nitrosamine since they could play a role in human cancer.

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