CHAPTER 4

RESULTS AND DISCUSSION

Study of lactobacilli strains

4.1 Fermented foods collection and isolation of LAB

Total 928 strains of lactobacilli were isolated from 480 fermented food samples collection. Total of 227 strains were isolated from 133 samples of Thai fermented soybean foods such as pickled of soybean, soybean paste, soy sauce and northern Thailand soybean food (Thua nao). The 484 strains were isolated from 254 fermented food or beverages containing other plants, which were excepted foods from soybean, such as fruit or vegetable pickles (Som pak), fermented plant beverages (FPBs) and fermented tea leaves (Miang). Moreover, 93 samples of fermented food from pork (such as Nham moo and Moo som) or fish (such as Nham pla, Pla som, Pla ra and Pla jom) were collected and isolated lactobacilli with total amount as 217 strains. Each lactobacilli isolate was selected by LAB characteristics with yellowish colony growth on MRS agar plus bromocresol purple, Gram-positive, non-spore forming rods and catalase-negative. These strains of lactic acid bacteria (LAB) are microorganisms that may play an important role in further lactic acid fermentation.

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4.2 Study for key functional properties of isolated LAB strains

4.2.1 Bile salt and pH tolerance

The basically probiotic characteristics are bile and acid tolerance (Brink et al., 2006). The strains which could tolerate bile and acidic conditions indicated the existence in stomach, intestinal juice and fermented food products. This may increase shelf life throughout the pressured conditions in gastrointestinal tract (GI). Among total 928 isolates of lactobacilli, the results of growth ability on MRS agar supplemented with bile salt agar and survived in various pH environment was shown in Table 4.1. All strains with both 0.15% and 0.30% (w/v) of bile salt tolerance (535 isolates) were continually determined for the survival in various pH. From the results, all tested strains could survive at pH 4 with survival rate more than 90% for 2 h incubation. In contrast, none of isolates was able to initiate survive at pH 9. These criteria are prerequisite for the existence and functional activity in the acidic environment of the stomach with pH between 1.5 and 3.0 (Corzo and Gilliland, 1999) and bile in the small intestine with concentration ranged 0.15-0.30% (w/v) of the host (Šušković et al., 2000). The bile salt with concentration 0.30% (w/v) of the host is also a critical concentration of probiotic selection for human (Gilliland et al., 1985; Chou and Weimer, 1999).

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Table 4.1 Bile and pH tolerance of LAB isolated from fermented foods and beverages

Profile		Number of isolates and/or percent of positive tests from each sample source		
		Fermented plant (not included from soybean)	Fermented soybean	Fermented pork or fish
Sample collection (total 480 samples)		254	133	93
Isolated lactobacilli (total 928 isolates)		484	227	217
Tolerance to bile salt	0.15% (w/v)	246 (50.82%)	197 (86.78%)	188 (86.64%)
	0.15% and 0.30% (w/v)	238 (49.17%)	183 (80.62%)	114 (52.53%)
Survive in various pH 2, 3, 4, 5, and 8 (2 h incubation)	>50% of growth survival rate	181/238 (76.05%)	119/183 (65.03%)	27/114 (23.68%)
	>90% of growth survival rate	135/238 (56.72%)	91/183 (49.73%)	21/114 (18.42%)

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The tolerance to acid-base environments, which encountered in food and in the GI tract, provides the survival of LAB. As a consequence, the lactobacilli strains with surviving in bile and acid-base ecosystem may bring about transition of the gastrointestinal tract of the host and to face stress from the processing of functional foods and beverages (Chiu et al., 2008) due to the tolerance the acidic pH and bile salt, which may account for increase in its survival. The pH of gastric juice which was secreted into gastrointestinal tract of host will be static between 3 and 5 (Cotter and Hill, 2003). The acid adaptation of selected lactobacilli strains may be due to the strain isolated from the acid fermented foods and this was in agreement with the previous study of pickled vegetable by Chiu et al. (2008).

4.2.2 Starch, protein and lipid utilization

Total 327 strains with tolerance to bile salt condition and survival more than 50% in various pH conditions from section 4.2.1 were further determined for nutrient utilization. The ability to hydrolyze both starch and protein, and only lipid was expressed by 63 and 24 lactobacilli strains out of 327 strains, respectively. From the results, there were few strains that can digest protein and lipid. This corresponds with the report of Essid et al. (2009) that LAB is known to have weak proteolytic and lipolytic activity. However, there was reported that lactobacilli can utilize starch, protein and lipid such as the report of Giruad and Cuny (1997). *L. plantarum* and *L. amylovorous* can hydrolyze starch via α-amylase extracellular enzyme. *L. delbruekii* subsp. *bulgaricus* exhibited the protease activity to hydrolyze protein (Kawai et al., 1998). Gobbetti et al. (1997) reported that the lipid

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tributyrin was hydrolyzed by intracellular tributyrin esterase of *L. plantarum* 2739. These nutrient utilized activities indicated that these bacteria may provide application to the host's digestive system and nutrient degradation during food processing. Total 87 of lactobacilli isolates from the results (from 63 strains and 24 strains had ability to utilize both starch and protein, and only lipid, respectively) were selected for further study.

4.2.3 Antimicrobial activity to tested microorganisms

The antimicrobial activity is one of the most important functional properties. Total 87 strains with bile and acid-base tolerance, hydrolyze starch, protein and lipid activity were investigated for antimicrobial activity. All 3 parts of supernatants (original supernatant, pH neutralized part and H_2O_2 neutralized part) from 16 isolates displayed strong antimicrobial activity against all target microorganisms such as *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhi*, *Shigella sonnei* and *Candida albicans* ATCC 90028 (Table 4.2). Among these parts, cells supernatant pH 7 and cell supernatant pH 7 containing catalase enzyme showed lower antimicrobial activities than normal cell supernatant part. The strong activity of the normal supernatant against microbial might be represent the synergistic activity of their LAB metabolic compounds. From the hexose fermentation of LAB, it provides organic acids with powerful antimicrobial activity such as lactic acid (homofermentation) or equimolar amounts of lactic acid, acetic acid, ethanol, and CO_2 (heterofermentation) (Ouwehand and Vesterlund, 2004).

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Furthermore, phenyllactic acid (PLA) may one of the bioactive compound which produced by these lactobacilli with its ability to against all microbial tests. In Kantachote et al. (2010) work, *L. plantarum* DW3 produced PLA that inhibited the growth of contaminating yeast in FPBs. PLA is produced by some LAB and is an efficient antimicrobial substance to control the growth of Gram positive and Gram negative bacteria and some mycotoxigenic species (Kantachote et al., 2010).

The antimicrobial activities of these 16 lactobacilli were broad inhibitory spectrum, against yeast and bacteria both of gram-negative and gram-positive. The normal supernatant of these isolates showed strong activity, while supernatant pH 7.0 and supernatant pH 7.0 containing catalase enzyme showed weak antagonistic activities. The purposes of pH adjustment and catalase addition were to eliminate activity from acid and hydrogen peroxide, respectively. From the results, it could be explained for the potential antagonistic activity from 16 lactobacilli tests which eliminated the effects of organic acids and hydrogen peroxide according to report of Jacobsen et al. (1999). Although, supernatant without H_2O_2 and acid showed weak activity, it was realized that 16 strains can produce other effective metabolites except acid and hydrogen peroxide to inhibit target organisms. It is interesting that the SC 359 strain show strong activity in all 3 parts of supernatant against the growth of *C. albicans*. *Candida* species are the leading cause of nosocomial bloodstream infection and has become a major health problem as an opportunistic infection such as oral candidiasis of HIV/AIDS (Pfaller and Diekema, 2007; Sesthathri, 2012). In this result indicate that SC 359 is a potential strain with an ability to control opportunistic fungal *Candida albicans* by producing a number of naturally antimicrobial compounds.

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The antimicrobial activity might be owing to their activities related to the amount of bacteriocin from LAB that are active against a number of microorganisms at the optimum pH (De Waard et al., 2002). In general, the antimicrobial activity of lactobacilli may be due to several metabolic compounds include organic acids (Kuwaki et al., 2002), hydrogen peroxide (Caplice and Fitzgerald, 1999), bacteriocins or proteinaceous substances (Testa et al., 2003), fatty acids, diacetyl (Dunne and others, 2001) or other inhibitory substances from metabolites (Caplice and Fitzgerald, 1999). From these results indicate that 16 lactobacilli strains are potential strains to be used as biopreservative organisms against food-borne pathogens, spoilage microorganisms and yeasts. Moreover, the SC 359 strain was likely to show strong activity to against *C. albicans*, could be used as the promising strain for further produce the antifungal nature product against oral candidiasis. The incidence and mortality rates associated with invasive candidiasis have remained high largely public health problem due to delays in the administration of appropriate antifungal therapy despite major advances in the field of antifungal therapy (Pfaller and Diekema, 2007). This finding may benefits all those who suffer from candidiasis or opportunistic *Candida* infection. The results of this study show that 16 lactobacilli strains play an important role in the yeast and bacterial inhibition. However, the certain antimicrobial mechanisms or compounds should be in progress to determine for using these promising strains as biopreservative microorganisms.

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Table 4.2 The antimicrobial activity of lactobacilli strains against 7 microbial indicators by using agar well diffusion technique

Strain	Part	Antimicrobial activity						
		E. coli ATCC 25922	*S. aureus* ATCC 25923	*B. cereus* ATCC 11778	*Ps. aeruginosa* ATCC 27853	*S. typhi*	*S. sonnei*	*C. albicans* ATCC 90028
TGCM 15	1	++	+++	+++	++	++	+++	+++
	2	++	++	++	++	++	++	++
	3	+	++	++	+	+	+	++
TGCM 26	1	++	++	++	++	++	++	++
	2	+	++	++	++	++	++	++
	3	+	+	+	+	+	+	+
TGCM 33	1	+++	+++	++	+++	++	++	+++
	2	++	++	++	++	++	++	+++
	3	+	++	++	+	+	++	++
TGCM 128	1	++	++	+++	+++	++	++	+++
	2	+	++	++	++	++	++	++
	3	+	+	+	+	+	+	++
SC 72	1	+	+++	++	+	+	+	+
	2	+	++	++	+	+	+	+
	3	+	++	++	+	+	+	+
SC 111	1	+	+++	++	+	+	+	++
	2	+	++	++	+	+	+	+
	3	++	++	++	++	++	++	++

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Table 4.2 (continued)

Strain	Part	Antimicrobial activity						
		E. coli ATCC 25922	*S. aureus* ATCC 25923	*B. cereus* ATCC 11778	*Ps. aeruginosa* ATCC 27853	*S. typhi*	*S. sonnei*	*C. albicans* ATCC 90028
SC 159	1	++	+++	+++	++	+	++	+++
	2	++	++	++	++	++	++	++
	3	++	+++	+++	++	++	++	++
SC 359	1	++	+++	++	++	++	++	+++
	2	+	++	++	++	++	++	++
	3	++	++	++	++	++	++	+++
SC 435	1	++	++	++	++	+	+	++
	2	++	++	++	++	++	++	+++
	3	++	++	++	++	++	++	++
LCC 67	1	++	+++	+++	++	++	++	++
	2	++	++	++	++	++	++	++
	3	+	+	+	+	++	++	+
LCC 150	1	++	+++	++	+++	++	++	+++
	2	++	++	++	++	++	++	++
	3	+	++	++	+	+	+	+
LCC 252	1	++	+++	++	++	++	++	+++
	2	++	++	++	++	++	++	++
	3	++	+	+	+	++	++	+

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Table 4.2 (continued)

Strain	Part	Antimicrobial activity						
		E. coli ATCC 25922	*S. aureus* ATCC 25923	*B. cereus* ATCC 11778	*Ps. aeruginosa* ATCC 27853	*S. typhi*	*S. sonnei*	*C. albicans* ATCC 90028
LCC 274	1	+++	+++	++	+++	++	++	+++
	2	++	++	++	++	++	++	+++
	3	+	++	++	+	+	++	++
LCC 339	1	++	++	++	++	++	++	++
	2	++	++	++	++	++	++	++
	3	++	+	+	+	++	++	+
LCC 392	1	++	++	++	++	++	++	++
	2	++	++	++	++	++	++	++
	3	+	++	++	+	+	+	++
LCC 397	1	+++	+++	++	+++	++	++	+++
	2	++	++	++	++	++	++	+++
	3	++	++	++	++	++	++	++

Part 1: Cell supernatant, Part 2: Cell supernatant pH 7.0, and Part 3: Cell supernatant pH 7.0 containing catalase. +++: zone of inhibition larger than 6 mm diameter (strong); ++: zone of inhibition between 3 and 6 mm (medium); +: zone of inhibition less than 3 mm (weak); no inhibition data were not shown. Inhibition zone not included diameter of wells (7 mm).

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4.2.4 Sensitivity to antibiotics of selected lactobacilli strains

The antibiotic susceptibility is the one criteria of safety evaluations in probiotic claimed. This study concerned that potential probiotics, which used living organisms, does not deliver a host of antibiotic resistant genes with the risk of transferring the genes in many probiotic bacteria and other pathogenic bacteria. The 16 selected lactobacilli strains were evaluated for susceptibility to 9 antibiotics by using agar dilution. The evaluation was displayed as MICs of antibiotics against 16 tested strains. The MICs values of lactobacilli tested strains demonstrated variability (Table 4.3). All 16 lactobacilli tested strains were susceptible to the lower concentration breakpoints of antibiotics namely ampicillin, chloramphenicol, erythromycin, gentamicin, kanamycin, rifampicin, streptomycin and tetracycline. The strains SC 435, LCC 67 and LCC 392 were found to be resistant to plentifully higher concentration breakpoints of vancomycin with the MICs breakpoints more than 4 µg/ml. From the results, the lactobacilli strains were susceptible to all the tested antibiotics. Although, some tested strains showed vancomycin resistance, this result was similar to the reports of Klein et al. (2000) and Zhou et al. (2005). In recognition of the importance of assuring safety, the guidelines for the evaluation of probiotics in food joint FAO/WHO recommends that probiotic strains be characterized antibiotic resistance patterns. Although the antibiotic resistant strains were recommended to discard, the beneficial antibiotic resistances of *Lactobacillus* strains support the balancing of normal intestinal microbiota in antibiotic-associated diarrheal patients (Salminen et al., 1998). In addition, another report demonstrated that some species of *Lactobacillus* such as *L. casei* or *L. paracase*i, *L. rhamnosus*, *L.*

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plantarum, *L. brevis* and *L. vaginalis* displayed resistance at much higher concentration than MICs breakpoints (≥256 µg/ml) (Delgado et al., 2005). As a result of the vancomycin resistance being likewise a natural trait of lactobacilli due to the special structure of their cell wall peptidoglycan precursors, the resistance does not seem to possess any problem (Delgado et al., 2005). Furthermore, it also defined as the transferrable mechanism and the inducible pattern differs from those of other bacteria (Delgado et al., 2005). Besides, Klein et al. (2000) reported that the vancomycin resistance transfer genes, which were *van*A, *van*B and *van*C genes, were not presented in *Lactobacillus* strains. From this study, the 13 selected lactobacilli strains displayed low opportunity to transfer antibiotic resistance phenotype since the strain did not express resistance to all tested antibiotics.

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Table 4.3 Minimum inhibitory concentrations (MICs) of antibiotics to selected lactobacilli strains

Antibiotics	The MICs (µg/ml) of lactobacilli strains								SCAN[a] Break points
	TGCM 15	TGCM 26	TGCM 33	TGCM 128	SC 72	SC 111	SC 159	SC 359	
Ampicillin	0.25	0.5	0.12	0.5	0.12	0.25	0.25	0.25	2
Chloramphenicol	4	2	2	2	2	2	4	2	16
Erythromycin	0.25	0.25	0.25	0.5	0.25	0.5	0.5	0.25	4
Gentamicin	0.5	0.5	0.12	0.25	0.06	0.5	0.5	0.5	1
Kanamycin	16	16	4	16	4	4	16	16	32
Rifampicin	2	2	0.5	1	1	0.5	0.25	1	32
Streptomycin	8	2	8	4	2	4	8	8	16
Tetracycline	4	4	0.12	0.12	2	0.25	4	4	16
Vancomycin	2	2	1	2	0.12	0.5	2	1	4

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Table 4.3 (Continued)

Antibiotics	The MICs (μg/ml) of lactobacilli strains								SCAN[a] Break points
	SC 435	LCC 67	LCC 150	LCC 252	LCC 274	LCC 339	LCC 392	LCC 397	
Ampicillin	1	0.25	0.5	0.25	0.25	0.25	0.25	1	2
Chloramphenicol	8	8	0.12	2	2	2	2	8	16
Erythromycin	1	0.5	0.12	0.5	0.25	0.5	0.5	0.5	4
Gentamicin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1
Kanamycin	16	16	8	16	16	16	16	8	32
Rifampicin	2	2	1	4	1	4	4	4	32
Streptomycin	8	8	4	8	8	8	8	8	16
Tetracycline	8	4	4	4	4	4	4	8	16
Vancomycin	≥256	64	1	2	1	2	128	2	4

[a]The breakpoints for *Lactobacillus* strains by SCAN category. Strain with MIC equal to or higher than the SCAN breakpoint is evaluated as resistance

4.2.5 Haemolytic activity

All 16 lactobacilli strains showed γ-haemolytic activity or no haemolysis on human blood TSA plates. The haemolytic activity is one recommended safety criteria of probiotic evaluation for human or animal host. The lactobacilli strain with no exhibition of the antibiotic resistance and haemolytic activity are recommended safety for host (Uymaz et al., 2009).

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4.2.6 Bile salt hydrolase (BSH) activity

In this study, four strains of isolated lactobacilli displayed the BSH enzyme activity. Total 4 strains (TGCM 15, TGCM 33, SC 359 and LCC 150) out of 16 lactobacilli strains, which were selected from potential probiotic screen, exhibited BSH activity by providing the precipitation zone around colonies on plate assay. The cholesterol-lowering effect of lactobacilli strain is by several means. One of mechanisms through bile salt hydrolase (BSH) activity is reported that associated reduction of cholesterol (De Smet et al., 1995; Corzo and Gilliland, 1999; Lim et al., 2004; Liong and Shah, 2005a; Begley et al., 2006). BSH is the enzyme responsible for bile salt deconjugation during enterohepatic circulation (Pereira et al., 2003; Moser and Savage, 2001). Liong and Shah (2005a) explained that BSH secreted from *Lactobacillus* strain, which able to catalyze the hydrolysis of glycine- or taurine-conjuagted bile salts into amino acid residues and free bile salts. Free bile salts are less soluble than conjugated bile salts providing lower absorption in the intestinal lumen. Thus, deconjugation of bile acids can reduce serum cholesterol level by increasing the formation of new bile acids instead of those free bile acids flee from the enterohepatic circulation. Four lactobacilli strains from these results were potentially expected to reduce cholesterol. Thus, they were further studied for cholesterol-lowering property in *vitro*.

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4.2.7 *In vitro* cholesterol-lowering property of active cell-free broth

Total 6 lactobacilli strains, which were potent probiotic, both with BSH activity and without, had ability to decrease cholesterol concentration in culture broth. Total 6 strains out of 16 strains that exhibited potent functional properties provided the ability to decrease cholesterol concentration in culture broth. These 6 strains were TGCM 15, TGCM 26, TGCM 33, TGCM 128, SC 359 and LCC 150. Among these strains, TGCM 26 and TGCM 128 did not show BSH activity. Cholesterol reduction of 6 strains in active free broth ranged between 11.81µg/ml and 31.24 µg/ml. Cholesterol augmentation of active cell pellet of 6 strains ranged 2.07-4.77 µg/ml. Cholesterol reduction of resting cell free broth ranged 7.55-11.07 µg/ml. Cholesterol augmentation of resting cell pellet ranged 0.96–2.02 µg/ml. Cholesterol reduction of dead cell free broth ranged 6.96-9.77 µg/ml. Cholesterol augmentation of dead cell pellet ranged 0.89-1.81µg/ml. Among the tested strains, TGCM 15 had the significantly highest cholesterol-lowering property in cell-free broth ($P<0.05$). TGCM 15 also had the significantly highest cholesterol-increasing activity in cell pellet ($P<0.05$). Cholesterol in TGCM 15 pellet had the significantly highest increase ($P<0.05$) among all strains. On the contrary, TGCM 26 was the strain with the significantly lowest activity ($P<0.05$) (Table 4.4).

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Table 4.4 Change of cholesterol content of cell-free broth and cell pellet by lactobacilli during a 24-h incubation of culture

Strain	pH of culture	Cholesterol lowering in cell-free broth and increasing in cell pellet (µg/ml)					
		Active cells		Resting cells		Dead cells	
		broth	pellet	broth	pellet	broth	pellet
TGCM 15	4.37	31.24±0.52 ^{A,a}	4.77±0.46 ^{A,a}	11.07±0.22^{B,a}	2.02±0.06^{B,a}	9.77±0.35^{C,a}	1.81±0.22^{B,a}
TGCM 26	5.46	11.81±0.71 ^{A,d}	2.49±0.32^{A,c}	9.05±0.84^{B,b}	1.05±0.05^{B,c}	7.84±0.61^{B,b}	0.96±0.14^{B,b}
TGCM 33	4.22	29.70±0.91 ^{A,b}	3.32±0.40^{A,b}	10.15±0.17^{B,a}	1.77±0.13^{B,b}	8.98±0.87^{B,a}	1.48±0.10^{B,a}
TGCM 128	4.88	14.36±0.64 ^{A,c}	2.07±0.18^{A,c}	7.55±0.64^{B,c}	0.96±0.12^{B,c}	6.96±0.14^{B,b}	0.89±0.17^{B,b}
SC 359	4.43	29.42±0.23 ^{A,b}	3.30±0.22 ^{A,b}	10.56±0.66 ^{B,a}	1.69±0.07 ^{B,b}	9.34±0.61 ^{C,a}	0.97±0.17 ^{C,ab}
LCC 150	4.14	30.66±0.09 ^{A,ab}	4.32±0.53 ^{A,b}	11.01±0.68 ^{B,a}	1.83±0.12 ^{B,b}	9.70±0.70 ^{B,a}	1.74±0.22 ^{C,a}

^{ABC} Means of individual trials (broth or pellet) within a row with different superscript letters are significantly different (P<0.05).

^{abcd} Means of individual trials (broth or pellet) within a column with different superscript letters are significantly different (P<0.05).

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4.2.8 *In vitro* cholesterol-lowering property of inactive (dead and resting) cell free broth

The cholesterol reduction levels from resting and dead cell free broth of all 6 strains ranged between 13.11-23.28% and 11.44-19.53%, respectively (Figure 4.1). The cholesterol reduction levels from resting cell free broth of lactobacilli strain TGCM 15, TGCM 26, TGCM 33, TGCM 128, SC 359 and LCC 150 were 23.28%, 17.46%, 20.61%, 13.11%, 21.81% and 23.11%, respectively. The cholesterol reduction levels from dead cell free broth of lactobacilli TGCM 15, TGCM 26, TGCM 33 and TGCM 128 were 19.53%, 13.97%, 17.26%, 11.44%, 18.29% and 19.31%, respectively.

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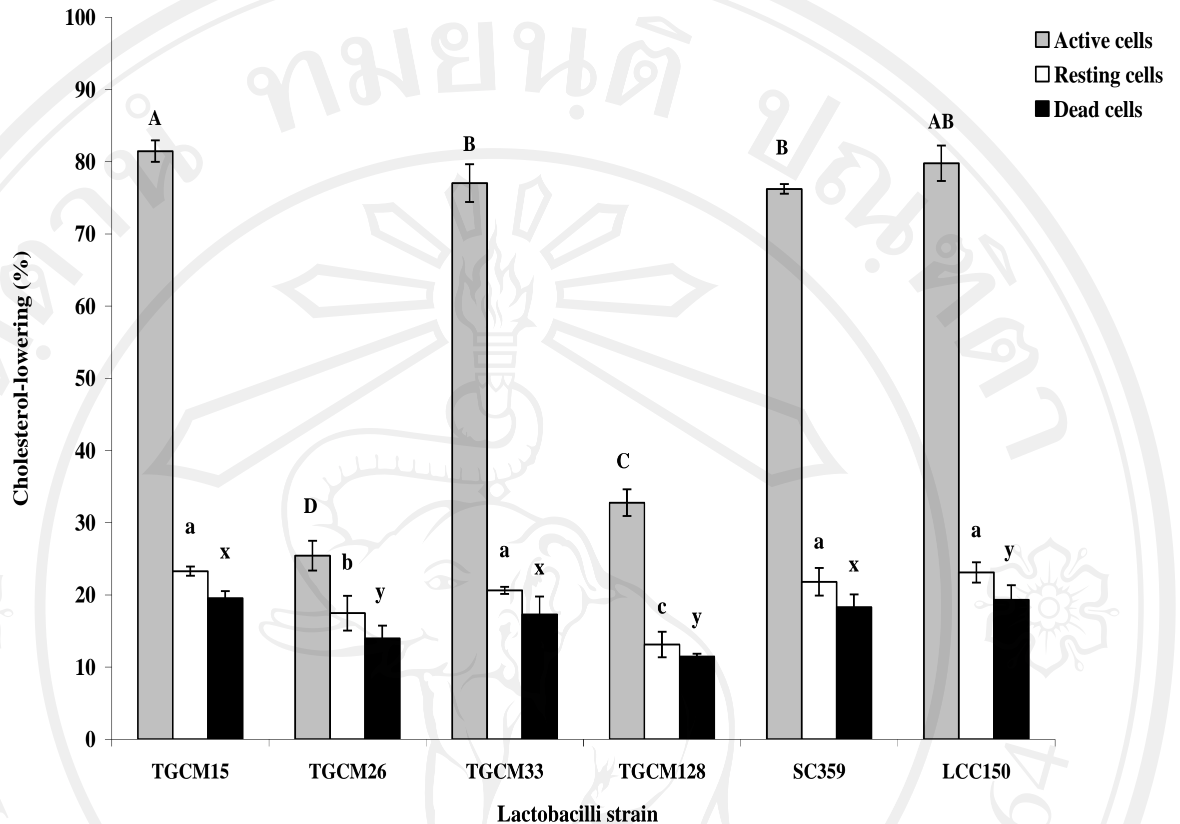

Figure 4.1 Percentage of cholesterol-lowering content of cell-free broth by active, resting, and dead cells of lactobacilli after 24 h of growth. The error bars indicate the standard deviation (SD) between individual trials, and different superscript letters are significantly different (P< 0.05) (*n*=3).

Several studies indicated that lactobacilli strains were able to reduce cholesterol via several mechanisms (Gilliland et al., 1985; Brashears et al., 1998; Liong and Shah, 2005a; Liong and Shah, 2005b). The cholesterol-lowering effect of *Lactobacillus* spp. is by several means through bile salt hydrolase (BSH) activity (De Smet et al., 1995; Corzo and Gilliland, 1999; Lim et al., 2004; Liong and Shah,

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2005a; Begley et al., 2006). BSH is the enzyme responsible for bile salt deconjugation during enterohepatic circulation (Pereira et al., 2003; Moser and Savage, 2001). In this study, the isolated lactobacilli strains were evaluated for the cholesterol-lowering activity via BSH enzyme activity and the capability of bacterial cell to remove cholesterol from culture broth. Among the six lactobacilli strains, TGCM 15, TGCM 26, TGCM 33, TGCM 128, SC 359 and LCC 150, BSH activity was observed in four strains which were TGCM 15, TGCM 33, SC 359 and LCC 150. Furthermore, cholesterol in broth of these four strains in all forms decreased, while in pellet increased. These results might be due to lactobacilli cells bind and absorb cholesterol. The active cells of these four strains exhibited significantly the highest ability ($P<0.05$) to decrease cholesterol from broth and increase in pellet. The TGCM 15, TGCM 33, SC 359 and LCC 150 exhibited cholesterol-lowering property higher than the other two strains without BSH activity. These results suggested that the BSH ability supported the mechanism for the in *vitro* lowering of cholesterol of the cells (Parvez et al., 2006; Kim et al., 2008).

Despite that, the TGCM 26 and TGCM 128 strains did not have BSH activity but still had the activity to reduce cholesterol from cell-free broth by active cells with the percentages of 25.41 and 32.76, respectively. This suggests that the reason for cholesterol-lowering activity of strains without BSH activity may be due to the acid produced from natural lactic acid fermentation of these lactobacilli strains (total acid of lactobacilli culture broth were detected but the data were not shown). The precipitation of cholesterol in supernatant appears to be related to the deconjugation of bile salts and their subsequent precipitation at low pH which ranged from 4.22 to 5.46. The pH of the culture broth decreased due to the organic acid

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production by the bacteria. Bile acids are less soluble and are more likely to precipitate at pH lower than 6.0 (Klaver and Van der Meer, 1993; Brashears et al., 1998).

Total six strains showed significant change of cholesterol contents by active cells or viable cells, but not a significant difference by resting and dead cells. Cholesterol-lowering by active cells was significantly higher ($P<0.05$) than resting and dead cells. Among six strains, the TGCM 15, TGCM 33, SC 359 and LCC 150 had the higher cholesterol-lowering in cell-free broth with the percentages more than 50. In particular, the TGCM 15 exhibited the significant highest cholesterol-lowering activity ($P<0.05$) at 81.46%. Conversely, the strain TGCM 26 was determined to have significantly the lowest cholesterol-lowering activity ($P<0.05$) at 25.41%. These were supported by the results of experiments that the percentage of cholesterol-lowering by resting cells and dead cells (static broth condition at pH 7.0) showed lower activity than by active cells with acidic pH. This corresponded to the report that deconjugated bile salts can co-precipitate in acidic environment at pH lower than 5.5 (Klaver and Van der Meer, 1993; Mathara et al., 2008). This is similar to the study of Brashears et al. (1998) which reported that some lactobacilli can remove cholesterol from suspension in culture broth during growth. The greater reduction of cholesterol by active cells corresponded to the growth of cells (Liong and Shah, 2005a). Therefore, the greatest cholesterol-lowering activities were found by active cells with BSH activity. Furthermore, the lactobacilli cells were known to assimilate the cholesterol which was associated and incorporated in the cells during growth (Gilliland et al., 1985). Consistently, this study showed the increasing cholesterol in the cell pellets of all strains. Also, active cells of these BSH absent strains had the

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ability to associate and incorporate cholesterol in the cells during growth (Walker and Gilliland, 1993; Noh et al., 1997). The lowering of cholesterol by resting and dead cells was slightly exhibited. This indicated that cholesterol might be attached via binding to cells (Liong and Shah, 2005a).

Thus, the 4 strains of lactobacilli were considered as provisional probiotic strains due to their superiority on probiotic properties, BSH activity (TGCM 15, TGCM 33, SC 359 and LCC 150) and cholesterol-lowering property were also investigated. From present results, the 4 lactobacilli isolated from food origins were considered as the effective probiotics with cholesterol-lowering property. These lactobacilli strains will be used as functional or bio-therapeutic agents. The authors will further study the mechanisms and activity in *in vivo*.

4.2.9 Assay for growth curve and β-glucosidase activity

Total 4 strains of lactobacilli, TGCM 15, TGCM 33, SC 359 and LCC 150, were selected to screen growth curve OD at 620 nm and β-glucosidase enzyme activity. The growth profile of 4 lactobacilli strains was determined (Figure 4.2). From the study, the growth profile of 4 lactobacilli strains in MRS broth were similar growing pattern. The growth curves of TGCM 15, TGCM 33, SC 359 and LCC 150 in MRS broth showed short period of lag phase and then growing entered the exponential phase in the 12th hour of growth. The stationary phase of each strain started at the twenty-fourth hour of growth with the quantity of TGCM 15, TGCM 33, SC 359 and LCC 150 were $4.38x10^8$, $7.22x10^6$, $5.14x10^8$, $6.87x10^8$ cfu/ml, respectively.

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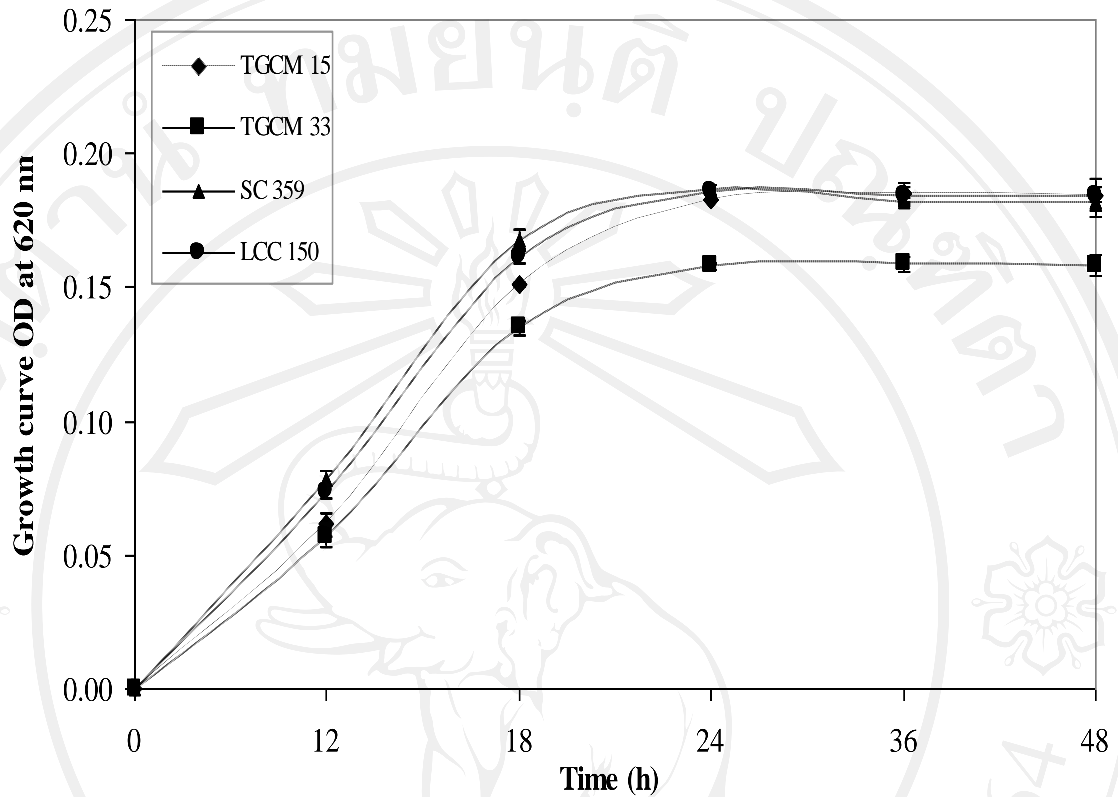

Figure 4.2 Growth profile OD at 620 nm of lactobacilli strains.

The results showed as mean ± standard deviation (SD) of enzyme units, individual data point is average of 5 repeated measurements from 3 independently triplicate experiments ($n = 3$).

The β-glucosidase activity was detected in two lactobacilli tested strains during culture in the medium for 12, 18, 24, 36 and 48 h at 37ºC. One unit of enzyme activity is the amount of β-glucosidase that released 1 μmol of ρ-nitrophenol from the substrate ρNPG/ml/min at 37ºC. The maximum enzyme activities among the different lactobacilli strains and incubation time were found to be varied (Figure 4.3).

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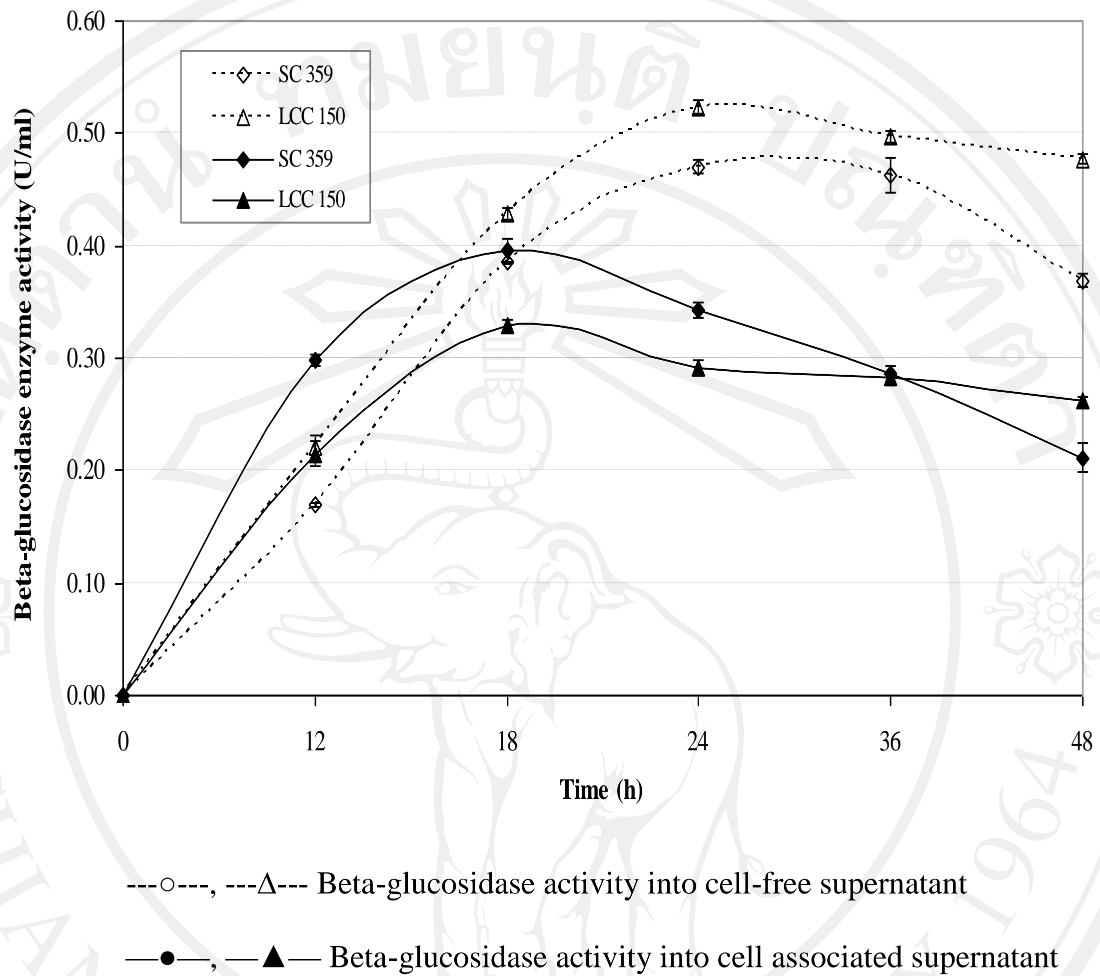

---○---, ---Δ--- Beta-glucosidase activity into cell-free supernatant

—●—, —▲— Beta-glucosidase activity into cell associated supernatant

Figure 4.3 β-glucosidase activity (U/ml) of 4 lactobacilli strains cultured in MRS broth along incubation time.

Data showed as mean ± standard deviation (SD) of enzyme units, individual data point is average of 5 repeated measurements from 3 independently triplicate experiments (*n* = 3).

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The enzyme activities among the different lactobacilli strains were shown at the incubation time within two parts, cell-free supernatant and cell-associated supernatant. Two strains, TGCM 15 and TGCM 33 were not detected for the activity of enzyme. On the contrary, SC 359 and LCC 150 showed β-glucosidase activities in both cell-free and cell-associated supernatant. The enzyme activity of SC 359 and LCC 150 culture were detected in cell-free supernatant for 48 h of incubation time in a range of 0.169±0.002 and 0.470±0.006 U/ml and 0.222±0.010 and 0.524±0.007 U/ml, respectively. While, the enzyme activity of cell-associated supernatant of SC 359 was determined ranging from 0.211±0.013 to 0.470±0.006 U/ml. The enzyme activity of LCC 150 ranged 0.215±0.010-0.329±0.006 U/ml.

The enzyme activities of these lactobacilli were detected as the maximum level in cell-free form. For cell-free form, enzyme activity of LCC 150 showed significantly ($P<0.05$) stronger activity than of SC 359. LCC 150 showed maximum enzyme activity into cell-free form at 24 h with enzyme activity of 0.524±0.007 U/ml. Whilst SC 359 showed maximum activity of 0.470±0.006 U/ml at 24 h. On the other hand, SC 359 showed significantly ($P<0.05$) stronger enzyme activity than of LCC 150 in cell associated form at 18 h with the maximum enzyme activity of 0.470±0.006 U/ml and 0.329±0.006 U/ml, respectively. Almost two strains expressed the maximum enzyme activities at the same time of culture. Therefore, both strains were chosen for further investigation for its ability to inhibit adherence of pathogens in *vitro*.

There were several studies reported that *Lactobacillus* spp. is the one group of LAB with the highest efficient β-glucosidase producing (Ciafardini et al., 1994; Leal-Sanchez, 2003) due to the members of this bacterium being well recognized to

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hydrolyze various β-glucoside of plant origin (Ciafardini et al., 1994). In our study, the strain SC 359 and LCC 150 were isolated from a pickle of soybean and pickle vegetable or som pak (cabbage and leek mixed pork skin), respectively which may be the good sources of substrate for β-glucosidase enzyme. The β-glucosidase enzyme produced by these lactobacilli strains is responsible for the growth of cell biomass OD_{620nm} in MRS medium (Figure 4.2). The pattern change of β-glucosidase enzyme activity of two forms is related to the growth period, in which the highest activity of the β-glucosidase enzyme was detected during the logarithmic phase of growth (during 18-24 h of incubation). The enzyme activity of two strains in cell-free and cell associated form increased to the maximum level at the 24 h and 18 h of growth, respectively. Then, it was declined until 48 h of incubation. This might be due to the metabolic repression from the amounts of nutrient sources that are terminated (Mahajan et al., 2010). Mahajan et al. (2010) reported that carbon sources and nitrogen sources of MRS medium can support the β-glucosidase enzyme production of *Lactobacillus*, while glucose, which is the carbon source of MRS medium, can induce the maximum β-glucosidase activity. The enzyme activity and property, which exhibited in each part from different strains of LAB could display specie-dependent according to the characterization of Michlmayr et al. (2010) that *L. brevis* SK3, *L. casei* and *L. mesenteroides* exhibit the intracellular located β-glucosidase, while, *L. plantarum* exhibit the extracellular located β-glucosidase.

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4.2.10 *In vitro* adherence inhibition assay

The *E. coli*, *Salmonella* spp. and *Shigella* spp. are the leading cause pathogenesis of diarrheal diseases (Hütt et al., 2006). One important strategy of severe infection of these enteropathogens is tight adherence to host epithelial cells (Reis and Horn, 2010). The authors emphasized to defend the pathogenesis of these pathogens by the adherence resistance of the selected lactobacilli strain (Figure 4.4, 4.5 and 4.6). The effect of adherence inhibition on pathogenic bacteria should be the way to prevent or against the intestinal infection caused by colonization and invasion of those pathogens (Collado et al., 2006; Gueimonde et al., 2006). From the results, the adherence of three pathogens in the presence of two lactobacilli strains were described that each pathogen incubated in the presence of SC 359 and LCC 150 expressed the lower adherent efficacy, as compared to incubation with only the pathogen. The SC 359 and LCC 150 strains expressed the ability to obstruct the adherence of three pathogens approximately 33.50±0.56 to 74.39±2.42 %inhibition (Figure 4.6). The inhibition activities against three pathogens adherence by the SC 359 and LCC 150 strains in three ways namely elimination (lactobacilli pre-inoculation), competition (co-culture) and displacement (lactobacilli post-inoculation). For the results of SC 359 strain, the remaining adherent cells of pathogens were displayed in the way of pre-inoculation, co-culture and post-inoculation with the percentage ranged 33.62-53.92%, 26.63-59.23% and 49.41-66.50%, respectively (Figure 4.4). In case of LCC 150 strain in the way of pre-inoculation, co-culture and post-inoculation, the remaining adherent cells of pathogens were exhibited with the percentage ranged 30.80-45.23%, 25.61-39.84%

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and 42.79-47.87%, respectively (Figure 4.5). The eliminated inhibition and competitive inhibition were significantly (P<0.05) higher inhibition than the displaced inhibition.

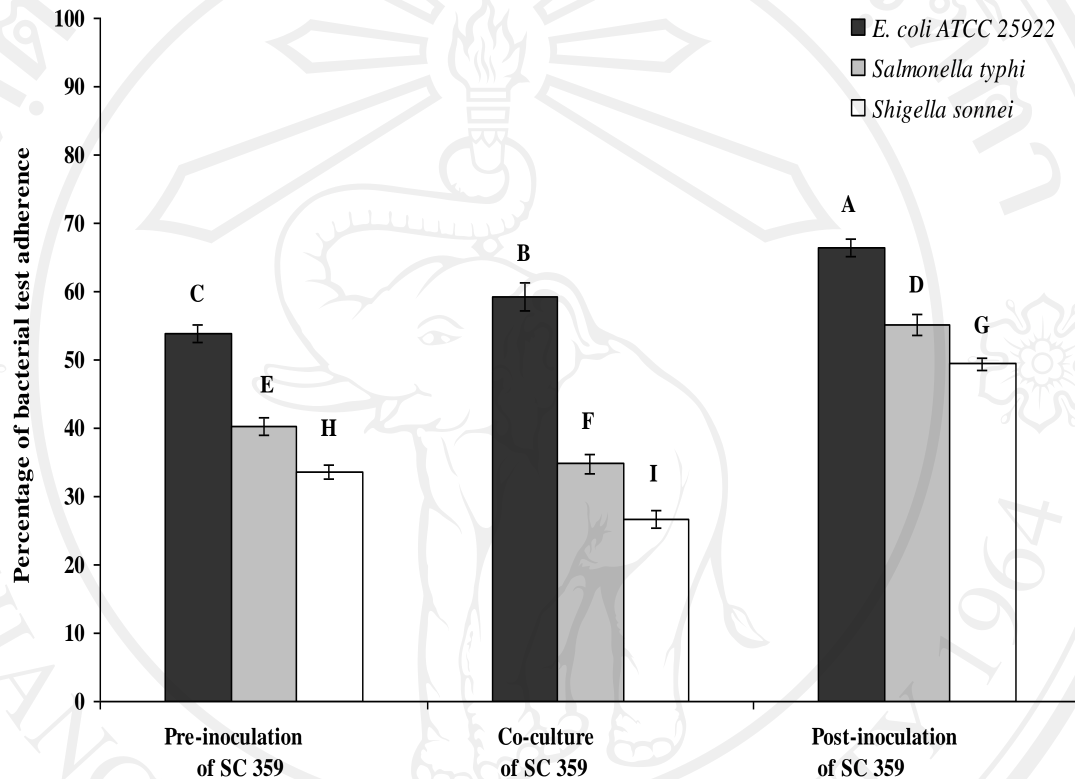

Figure 4.4 Percentage of *E. coli* ATCC 25922, *S. typhi* and *S. sonnei* adhere to Caco-2 cells in the presence of lactobacilli SC 359 strain. The error bars indicate the standard deviation (SD) and different superscript letters are significantly different (P< 0.05) (*n*=3). The adherence inhibitions of SC 359 against three bacterial tests were evaluated with three ways as elimination (lactobacilli SC 359 pre-inoculation), competition (co-culture of lactobacilli SC 359 and the pathogen) and displacement (lactobacilli SC 359 post-inoculation)

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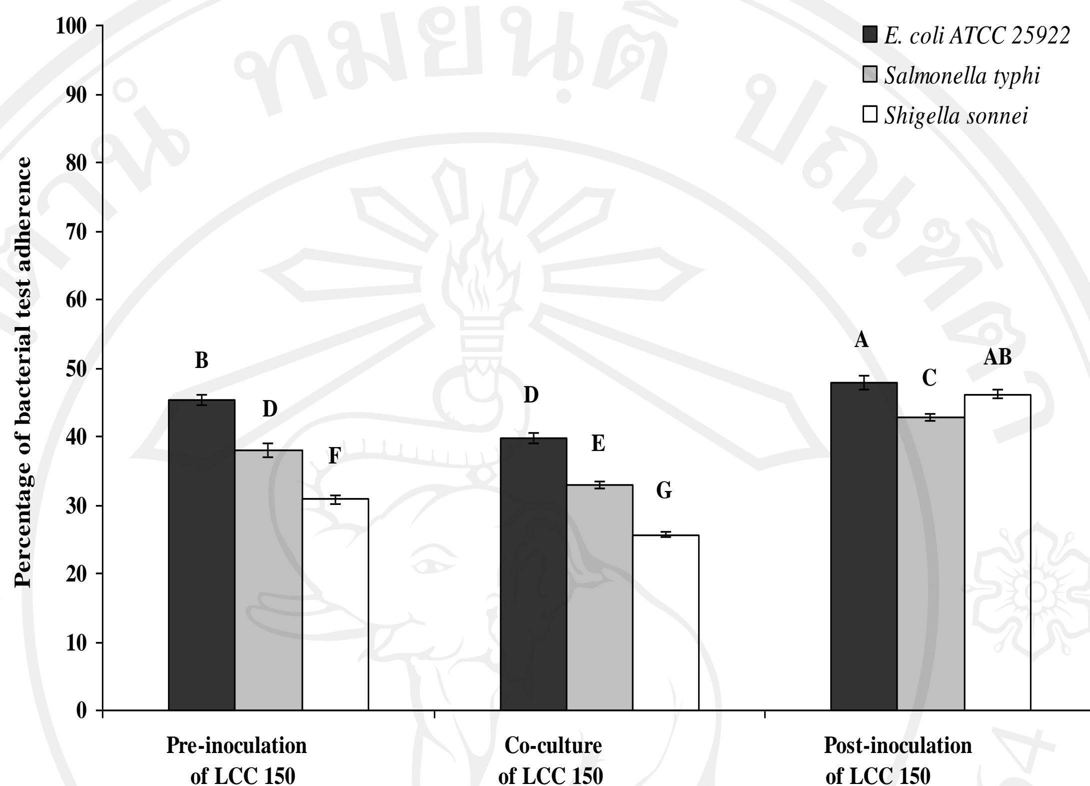

Figure 4.5 Percentage of *E. coli* ATCC 25922, *S. typhi* and *S. sonnei* adhere to Caco-2 cells in the presence of lactobacilli LCC 150 strain. The error bars indicate the standard deviation (SD) and different superscript letters are significantly different ($P < 0.05$) ($n=3$). The adherence inhibitions of LCC 150 against three bacterial tests were evaluated with three ways as elimination (lactobacilli LCC 150 pre-inoculation), competition (co-culture of lactobacilli LCC 150 and the pathogen) and displacement (lactobacilli LCC 150 post-inoculation)

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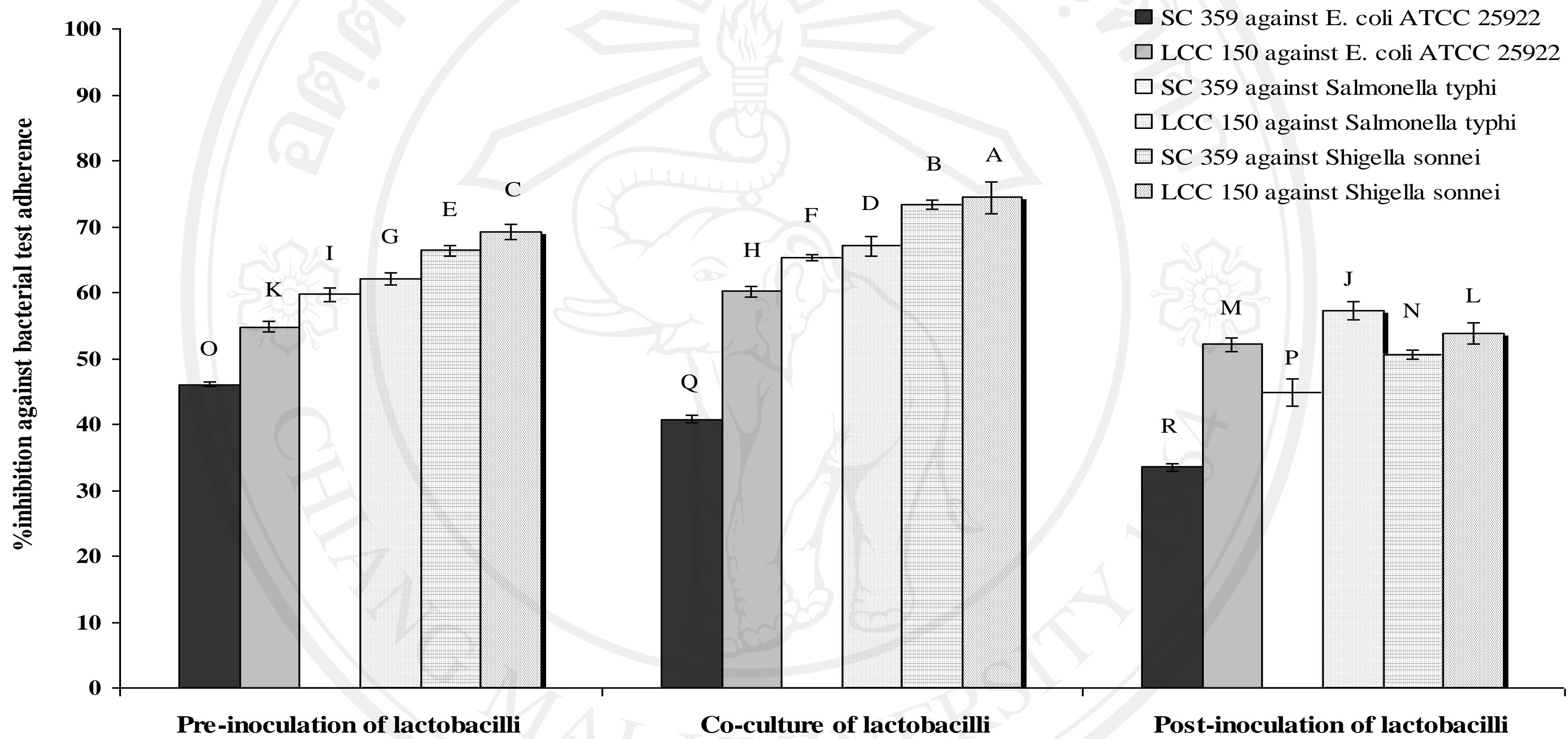

Figure 4.6 Percentage of adherent inhibition to Caco-2 cells of lactobacilli against *E. coli* ATCC 25922, *S. typhi* and *S. sonnei* in the presence of lactobacilli strain. The error bars indicate the standard deviation (SD) and different superscript letters are significantly different (P< 0.05) (n=3). The percentage of adherent inhibition of lactobacilli against three bacterial tests were evaluated with three ways as elimination (lactobacilli pre-inoculation), competition (co-culture of lactobacilli and the pathogen) and displacement (lactobacilli post-inoculation)

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The pathogen adherent inhibitions of the strain SC 359 and LCC 150 were demonstrated by the decreasing number of pathogens via the approaches of inhibition such as elimination, competition and displacement. The decreasing number of bound pathogens in the lactobacilli strain pre-incubation could be related to the activity of lactobacilli strain adhere to Caco-2 cells, which obstructs the adherent sites or receptors for pathogens adhesion (Lee et al., 2003). In addition, the case of pathogen decrement in the co-culture of lactobacilli strain and pathogens could suggest that the SC 359 and LCC 150 strain have the ability to interfere with the pathogens to adhere to receptor of Caco-2 cells. Furthermore, the adherence inhibition can be caused by the production of some antibacterial substances such as lactic acid, bacteriocin or other inhibitory metabolites from metabolism of lactic acid bacteria including the strain SC 359 and LCC 150 against pathogens and pathogenic adhesion (Forestier et al., 2001; Lee at al., 2003; Gueimonde et al., 2006) (Figure 4.4, 4.5 and 4.6; Table 4.2). This is similar to the antagonistic activity of sterilized supernatant of lactobacilli strains against pathogenic microorganisms of the present study. The adherent inhibition activity indicated that metabolite of the SC 359 and LCC 150 strain have an effect on the growth of microbial pathogens or representative intestinal bacteria such as *E. coli*, *S. typhi* and *S. sonnei*. In the case of post-inoculation of the SC 359 or LCC 150 to pathogens, this inhibition indicated the ability to displace the tested pathogens by SC 359 and LCC 150. It is possible that the displacement of three pathogens by SC 359 or LCC 150 is similar to the pattern of co-culture inhibition. The mechanism may be due to the producing of some antibacterial substances against the pathogens existence and this result was corresponded with this study (Table 4.2) and the report of Forestier et al. (2001). Based on the results, the displacement of

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lactobacilli strains on three pathogens showed the lowest percentage inhibition than other inhibitions. This might be due to the 1 h incubation being such a short term. As Lee et al. (2003) suggested the displacement for pathogens by lactobacilli is a slow process and thus the incubation time should be extended more than 1 h for the displacement by those species of lactobacilli. However, the SC 359 and LCC 150 strains investigated in this study expressed efficient interference of the pathogens adherence by three assayed mechanisms. Hence, the both strains may be the selected bio-agent to counteract the gastrointestinal problems caused from these pathogens by defense or therapy.

4.2.11 Dead cell lactobacilli affected adhesion on Caco-2 cells and induced interleukin-6, interleukin-10 and interleukin-12 production

The highest efficient strain LCC 150, which was isolated from a pickle vegetable or som pak (mixed of cabbage, leek and pork skin), was selected from functional properties as a representative for starter culture of soybean fermentation. The LCC 150 strain was investigated the adherence and cytokine-induction of the heat-killed form. The effects of heat-killed LCC 150 (HK-LCC150) on adhesion to Caco-2 and the immune response via observation of IL-6, IL-10 and IL-12 were shown in Tables 4.5 and 4.6, respectively. The growth profile of each microorganism was investigated such as optical density and growth number, which were investigated every hour (data not shown). Specific growth rate of 5 lactobacilli strains and *E. coli* ATCC 25922 were obtained by growth profile study as shown in Table 4.5.

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Table 4.5 Specific growth rate, number of cells and percent adhesion of HK-LA450, HK-LC047, HK-LCC1, HK-LCC2, HK-LCC150 and HK-EC to Caco-2 cells

Strains	**Specific growth rate:μ (h^{-1})**	**Number of bacterial cells (Log cfu/ml) at OD_{600} = 0. 5**	**Percent adhesion (mean±SD)**
L. acidophilus TISTR 450	0.75^{D}	$9.92±0.24^{A}$	$6.68±0.49^{d}$
L. casei sub. *rhamnosus* TISTR 047	0.77^{CD}	$8.74±0.13^{B}$	$7.41±0.14^{c}$
LCC 1	0.83^{AB}	$8.48±0.11^{D}$	$7.95±0.60^{a}$
LCC 2	0.81^{BC}	$8.52±0.18^{D}$	$7.52±0.05^{c}$
LCC 150	0.80^{BC}	$8.67±0.21^{C}$	$7.74±0.23^{b}$
E. coli ATCC 25922	0.87^{A}	$8.23±0.20^{E}$	$5.68±0.05^{e}$

Specific growth rate (μ) with different big letters indicated significant differences ($P<0.05$) between strains.

Number of bacterial cells (Log cfu/ml) at OD_{600} = 0. 5 with different italic big letter indicated significant difference ($P<0.05$) between strains.

Percent adhesion with different small letters indicated significant differences ($P<0.05$) between strains.

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E. coli ATCC 25922 had the significantly ($P<0.05$) highest specific growth rate of 0.87 h^{-1} with the number of cells (OD_{600}=0.5) 8.23 ± 0.20 log cfu/ml or had the lowest generation time. *L. acidophilus* TISTR 450 had the significantly ($P<0.05$) lowest specific growth rate of 0.75 h^{-1} with the number of cells (OD_{600}=0.5) 9.22 ± 0.24 log cfu/ml or highest generation time.

The percent adhesion to Caco-2 cells varied among the tested bacteria in strain-dependent manner ($P<0.05$) (Table 4.5). The percentage of adhesion was between 5.68±0.05 and 7.95±0.60%. HK-LCC1 was the most adhesive strain in this study since approximate 7.95±0.60% of the added bacteria bound to Caco-2 cell cultures. HK-EC was the least adhesive strain. The adhesion of HK-LCC150 was significantly different ($P<0.05$) from all bacterial tests. Nevertheless, all heat-killed lactobacilli adhered better than HK-EC.

L. acidophilus TISTR 450 and *L. casei* sub. *rhamnosus* TISTR 047 were used as standard strains because of well-known properties. LCC1 and LCC2 with the cytokine-induced property were recieved from Health Product Research Unit, Chiang Mai University. *E.coli* ATCC 25922 was used as a control because it is gram-negative bacteria and some strains of *E.coli* are pathogen. The specific growth rate (h^{-1}) of five lactobacilli tested strains were not significanlty ($P\geq0.05$) different in all strains. The number of cells and specific growth rate is used to evaluate the time of cultured bacteria for preparation of dead cell. Growth profile was used to calculate the cell number of bacteria before process to dead cell. Bacterial adhesion is considered important as one of the selection criteria for probiotic strains. So, the Caco-2 adhesion was important for this study. HK-LCC1 had the best adhesion property when compared with standard strains. Besides, HK-LCC150 adhered better

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than HK-LC047, which is standard strain. The adhesion of heat-killed lactobacilli may be caused by several mechanisms such as 1) strains bound specific interactions mediated by adhesin (Beachey, 1981; Busscher and Weerkamp, 1987), 2) carbohydrates on the bacterial cell wall appeared to be partly responsible for the interaction between the bacteria and the extracellular adhesion-promoting factor (Coconnier et al., 1992). Furthermore, Schillinger et al. (2005) reported that the hydrophobic potential of strains differed considerably. Hydrophobicity plays a key role in first contact between a bacterial cell and mucus or epithelial cells.

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Table 4.6 Production of cytokines by Caco-2 when stimulated by HK-LA450, HK-LC047, HK-LCC1, HK-LCC2, HK-LCC150 and HK-EC

Strains	**IL-6 (pg/ml)**	**IL-10 (pg/ml)**	**IL-12(pg/ml)**
L. acidophilus TISTR 450	3.17±0.36 ^{e}	171.33±1.28 ^{C}	ND
L. casei sub. *rhamnosus* TISTR 047	4.03±0.49 ^{b}	147.04±0.63 ^{D}	ND
LCC 1	4.63±0.70 ^{a}	255.59±1.22 ^{A}	ND
LCC 2	3.86±0.37 ^{c}	241.77±1.56 ^{B}	ND
LCC 150	3.64±0.58 ^{d}	248.43±1.62 ^{AB}	ND
E. coli ATCC 25922	3.83±0.41 ^{c}	143.81±0.63 ^{D}	2.45±0.11

Data are shown as means ± SD of triplicate experiments; ND= Not detected

Means of individual trials within a column with different small letters (IL-6) and capital letters (IL-10) are significantly different (P<0.05) with each column.

The ability of the immune cells associated with the Caco-2 to respond to different heat-killed lactobacilli was assessed by the cytokine release. The ability of the HK-LA450, HK-LC047, HK-LCC1, HK-LCC2, HK-LCC150 and HK-EC to induce IL-6, IL-10 and IL-12 were investigated. Table 4.6 showed the productions of IL-6, IL-10 and IL-12, which were 3.17±0.36 - 4.63±0.7, 143.81±0.63 - 255.59±1.22 and ND - 2.45±0.11 pg/ml, respectively. All heat-killed lactobacilli strongly enhanced IL-10 when compared to HK-EC. HK-LCC1 induced the significantly

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(P<0.05) highest IL-10 production in Caco-2. Heat-killed lactobacilli did not stimulate IL-12 production. Whereas, the HK-EC induced IL-12 production.

Intestinal inflammation is one potential target for probiotic therapy; clinical improvement and protective effects have been shown in food alergies and atopic dermatitis with probiotic (Kalliomaki et al., 2001). Cytokine induction is the determinant important probiotic activity such as immune modulation, pathogen exclusion, enhanced healing of damaged mucosa and prolonged transient colonization (Alander et al., 1999), it is thought to be important for modulating the immune system (O'Halloran et al., 1998). The proinflammatory cytokines secreted by the epithelium, such as tumor necrosis factor (TNF)-α, Interleukin (IL)-1, IL-6, IL-8 and IL-12 (Isolauri, 1999). The inhibition of proinflammatory cytokines and the supplementation of anti-inflammatory cytokines reduced inflammation. For instance, IL-12 is a proinflammatory cytokine and a modulator of cell-mediated immunity, which is mainly produced by macrophage, dendritic and B cell. Lipopolysaccharide (LPS) from gram negtive bacteria induced the production IL-12. IL-6 is a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cell and macrophage. IL-10 is a cytokine that regulates immune-mediated inflammation. It appears having two major functions: to inhibit cytokine (i.e., TNF, IL-1, chemokine, and IL-12) production by macrophages and inhibit the accessory functions of macrophages in T cell activation. The effects of these actions cause IL-10 to play mainly an anti-inflammatory role in the immune system. Bacterial DNA and cell walls, such as capsular polysaccharides, peptidoglycans, lipoteichoic acids and lipopolysaccharides (LPS), can stimulate Caco-2 cells to produce IL- 6 and IL-10. Nevertheless, the heat-killing method used in this study denatured DNA. Therefore,

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bacterial DNA did not take into account for the IL-10 induction in this study. The production of IL-6, IL-10 and IL12 of HK-LCC150 were observed. It was found that HK-LCC1 can stimulate highest IL-6 and IL-10. Besides, it was found significant (P<0.05) difference that all HK-lactobacilli could stimulate IL-6. All heat-killed lactobacilli could not induce IL-12, which promote chronic inflammation when compare with HK-EC, probably due to LPS present in the cell wall. Mechanism of cytokine induction by these heat-killed lactobacilli is being undertaken.

4.3 Identification of selected lactobacilli strain with functional properties

The LCC 150 strain showed the morphology with string of rods by scanning electron microscope (Figure 4.7) and was identified to the *Lactobacillus* genus level according to the phenotype such as gram-positive lactobacilli, non-spore forming, catalase-negative and following *Lactobacillus* carbohydrate fermentation profiles (Table 4.7). Moreover, the partial 16S rDNA sequences of SC 359 and LCC 150 strains expressed that this strain has 100% homology with *L. plantarum* strain L550 (which data of SC 359 was not shown). The strain was submitted to the NCBI GenBank database with the accession number of GQ421852. The phylogenic tree of the selected strain LCC 150 is presented in Figure 4.8. Although the species *L. plantarum* is not as frequently reported as predominant lactobacilli in human and used for human as much as *L. acidophilus* and *L. casei* (Shortt, 1999; Byun et al., 2004), *L. plantarum* has been used in the manufacture of various food products manufacture such as kimchi, soybean paste and several cheeses (Jeun et al., 2010). It is used for

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human consumption (Mogensen et al., 2002) and involves in many lactic acid fermented vegetable foods and beverages (Leal-Sanchez, 2003).

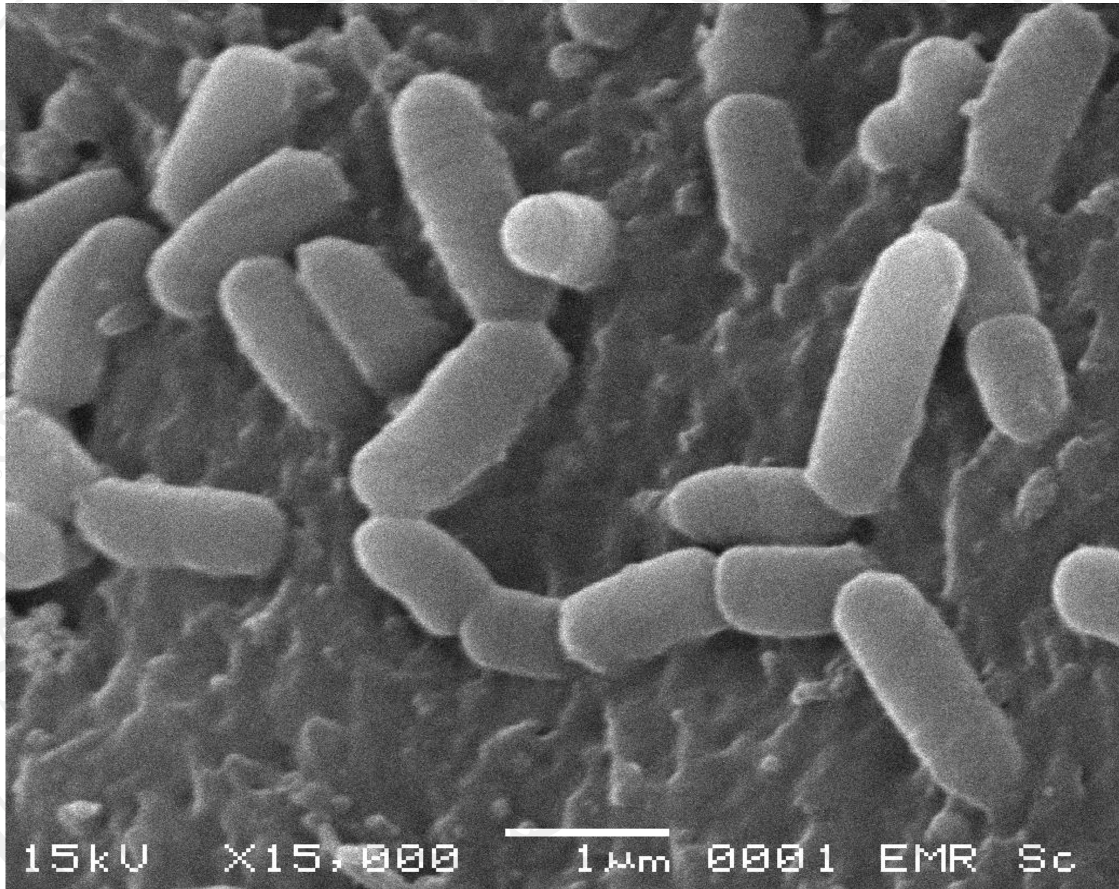

Figure 4.7 Scanning electron micrograph showing morphology of *L. plantarum* LCC 150 (x15,000)

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Table 4.7 Biochemical reactions produced by selected lactobacilli strain LCC 150 in API 50CHL system

Identification : *Lactobacillus plantarum*

0	Control		20	α Methyl-D-mannoside	+	40	D-Turanose	
1	Glycerol		21	α Methyl-D-glucoside		41	D-Lyxose	
2	Erythritol		22	N Acetyl glucosamine		42	D-Tegatose	
3	D-Arabinose	+	23	Amygdalin	+	43	D-Fructose	
4	L-Arabinose	+	24	Arbutine		44	L-Fucose	
5	Ribose	+	25	Esculine	+	45	D-Arabitol	
6	D-Xylose		26	Salicine		46	L-Arabiol	
7	L-Xylose		27	Cellobiose	+	47	Gluconate	+
8	Adonitol		28	Maltose	+	48	2- ceto-gluconate	
9	B Methyl-xyloside		29	Lactose	+	49	5- ceto-gluconate	
10	Galactose	+	30	Mellibiose	+			
11	D-glucose	+	31	Saccharose	+			
12	D-Fructose	+	32	Trehalose	+			
13	D-Mannose	+	33	Inulin				
14	L-Sorbose		34	Melezitose	+			
15	Rhamnose	+	35	D-Raffinose	+			
16	Dulcitol		36	Amidon				
17	Inositol		37	Glycogene				
18	Mannitol	+	38	Xylitol				
19	Sorbitol	+	39	β Genitobiose	+			

blank : negative

+ : positive

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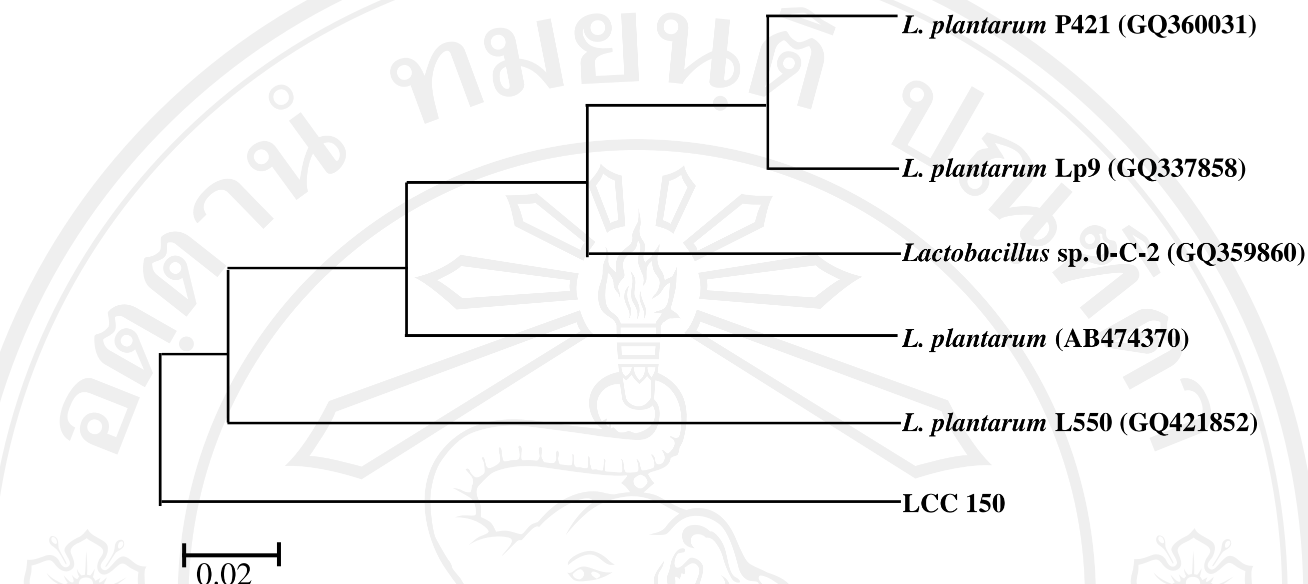

Figure 4.8 Phylogenetic tree of the selected lactobacilli LCC 150 and related known bacterial *Lactobacillus* species based on the nucleotide sequences of 16S rDNA sequence. Accession numbers and bacterial species are presented. The scale bar represents the calculated distances between the sequences.

L. plantarum has a long history of natural occurence and safe use in variuos kinds of food product. De Vries et al. (2006) reported that a few *Lactobacillus* species, which included *L. crispatus*, *L. gasseri*, and *L. plantarum*. *L. plantarum*, exhibit representatives that are both involved in traditional and industrial food fermentations and survive gastric transit to colonize exist in the gastrointestinal tract of human and other mammals. *L. plantarum* is a versatile LAB which encounter in a range of environment including dairy (various cheese), meat (various sausage) and several vegetable fermentations (olives, cocoa beans, cassava, sauerkraut). Furthermore, *L. plantarum* is described for the effects of consumption on human

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physiology. A variety of *L plantarum* are recently marketed as probiotics for human health in various form such as capsule (IFlora, Acidophilus Formula, Probiotic Eleven, Plantadophilus, FloraFood, Super Detox System, Udo's Choise), fruit drink (Provita), drink (Lactovitale), and powder or gel (ProBios)(De Vries et al., 2006). According to the assured results of benefits and activities of *L. plantarum* LCC 150, it may provide the potential probiotic effect on human host. All this will lead to select the LCC 150 strain as the starter culture of functional foods from soybean milk fermentation.

Study of fermented soybean milk by using a selected *Lactobacillus* strain as functional starter

4.4 Study the fermented soybean milk

4.4.1 Physicochemical analysis of soybean milk

In this study, the basic formula of fermented soybean milk during fermentation time on 0 h, 24 h, 72 h, 3 d, 5 d, 7 d, 14 d and 21 d of incubation were taken and analyzed for %ash, %moisture, %protein, %fat, %carbohydrate and calories showed in Table 4.6. After fermentation period for 21 days, the physicochemical composition including %ash, %fat, %protein, %carbohydrate and energy were significantly increased ($P<0.05$) from 0-21 day of fermentation with the quantity ranged 0.2472-0.2887%, 1.2643- 2.2647%, 0.0767-0.1432%, 10.1741-12.6262% and 56.4991-59.3130 Kcal, respectively. In contrast, %moisture was significantly

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(P<0.05) decreased with the amount of the compositions ranged 84.6782-87.7476%. Results of this analysis displayed that the nutritional compositions, such as ash, carbohydrate and energy, in which fermented soybean milk were detected same in range of the commercial soy milk. In contrast, the protein and fat contents of fermented soy milk were found lower than in those commercial non-fermented soy milks. This finding suggested that the low protein and fat contents in which soybean milk may due to the different preparation of soy milk such as soaking soybean seeds and preheating at 121ºC for 15 min, filtration through cheesecloth and also the ration of ingredients. Although the nutritional contents such as protein and fat were detected in low quantity, the benefits in which functional properties, bioactive compounds and bioactivities of fermented soybean milk were compensated. The autoclaved soybean seeds at 121ºC for 15 min was prepared due to destroyed the antinutritional factors such as trypsin inhibitor, lipoxygenase, saponins and phytic acids which are destroyed or inactivated at a high temperature in agreement with Saidu (2005). The inactivation of antinutritional factors to the acceptable levels for soymilk in the high temperature, made it possible to also destroy harmful microorganisms. The soybean pretreatment received from preliminary fermentation study of untreated and unfermented soybean milk, made non acceptance characteristics and absence fermentation (data was not shown). A development the nutrition of fermented soy milk products according to Thai dairy recommended intake (DRI) may be further carried out, especially for the functional foods and beverages industries.

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Table 4.8 Physicochemical composition of fermented soybean milk during the fermentation time for 21 days

Fermentation time	Physicochemical composition of fermented soybean milk					
	%Ash	**%Moisture**	**%Protein**	**%Fat**	**%Soluble Carbohydrate**	**Energy (Kcal)**
0 d	0.2472 ±0.1821[g]	87.7476 ±0.4625[a]	0.0767 ±0.1122[h]	1.2643 ±0.1437[h]	10.6642 ±0.4022[c]	56.4991 ±0.4527[h]
1 d	0.2502 ±0.2165[f]	87.7322 ±0.3210[b]	0.0878 ±0.2076[g]	1.3766 ±0.1465[g]	10.5532 ±0.3714[e]	56.7892 ±0.1417[g]
3 d	0.2578 ±0.0045[e]	87.6804 ±0.2183[c]	0.0922 ±0.2082[f]	1.5432 ±0.0067[f]	10.4264 ±0.2521[f]	57.2133 ±0.2894[f]
5 d	0.2674 ±0.1576[d]	87.6764 ±0.4110[d]	0.0955 ±0.1640[e]	1.7866 ±0.0321[e]	10.1741 ±0.3276[h]	58.1273 ±0.2753[e]
7 d	0.2754 ±0.3245[c]	87.2084 ±0.2363[e]	0.1106 ±0.0348[d]	2.1460 ±0.0052[d]	10.2596 ±0.0054[g]	58.6475 ±0.2749[d]
10 d	0.2785 ±0.2642[bc]	86.7826 ±0.1473[f]	0.1178 ±0.1329[c]	2.1778 ±0.1023[c]	10.6433 ±0.3211[d]	58.7021 ±0.4612[c]
14 d	0.2812 ±0.0087[b]	85.5748 ±0.1211[g]	0.1287 ±0.1823[b]	2.2104 ±0.0046[b]	11.8049 ±0.2703[b]	58.8350 ±0.1624[b]

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Table 4.8 (Continued)

Fermentation time	**Physicochemical composition of fermented soybean milk**					
	%Ash	**%Moisture**	**%Protein**	**%Fat**	**%Soluble Carbohydrate**	**Energy (Kcal)**
21 d	0.2887 ±0.0583[a]	84.6782 ±0.2311[h]	0.1432 ±0.0749[a]	2.2647 ±0.2477[a]	12.6252 ±0.3763[a]	59.3130 ±0.2434[a]
Nutrition labeling of commercial soy milk in Thailand**	0.14-1.0	-	2-3	13-17	9-13	10-100

Values are means of three determination (*n*=3) ± standard deviation (SD)

Different letters that followed numbers within the same column indicated significantly different ($P<0.05$) between the treatments

To determine differences between treatment means with DMRT were employed

**means the range of nutrient information labeling of Thai soybean milk products

4.4.2 Growth curve of starter lactobacilli in fermented soybean milk

This study was carried out to evaluate the growth profiles of *L. plantarum* LCC 150 starter during fermentation of soybean milk. The growth profile of starter were determined at 0 h, 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, 5 d, 7 d, 14 d and 21 d of

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fermentation by pour plating into the modified molten MRS agar. The growth curve of LCC 150 starter was shown in Figure 4.9.

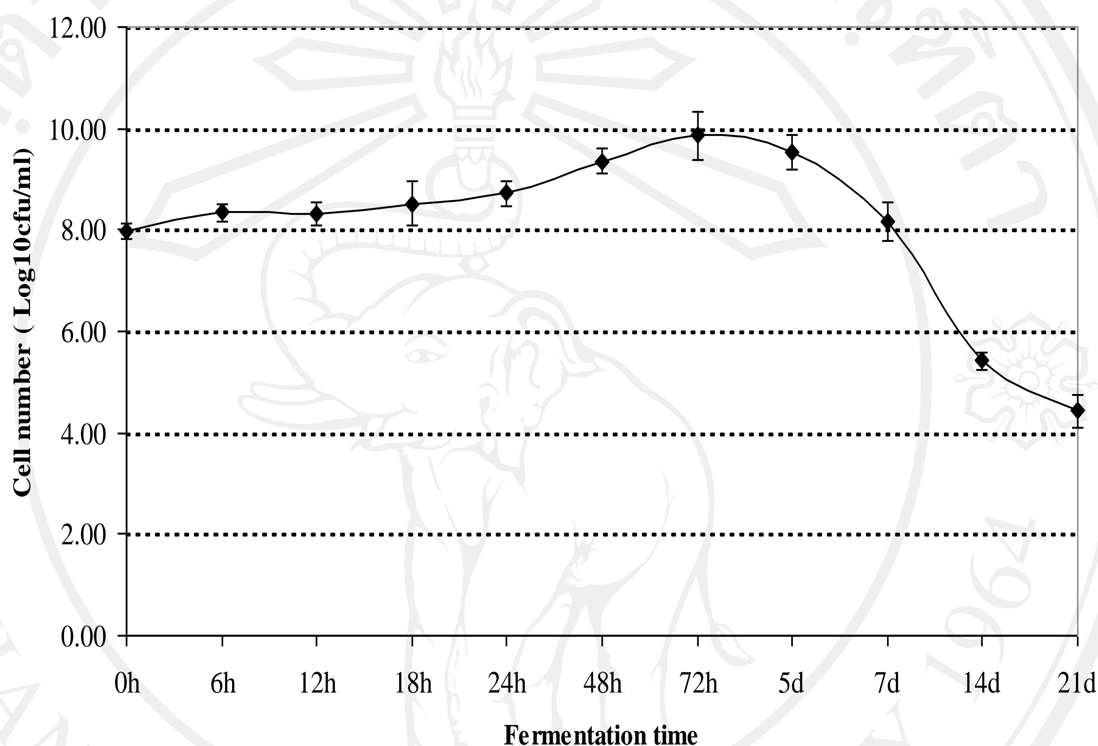

Figure 4.9 Growth curve of *L. plantarum* LCC 150 starter in the soybean milk during fermentation period at 37ºC for 21 days

From Figure 4.9, the cell number of LCC 150 at the initial inoculation time (0 h) into soybean milk was enumerated at 7.98±0.14 log cfu/ml and was found at 4.42±0.32 log cfu/ml for 21 d of fermentation. The growth profile of LCC 150 strain in soymilk fermentation showed the exponential phase after 24 h of growth and the maximum growth was significantly ($P<0.05$) exhibited at 3 d of fermentation. The cell number was slightly decreased after 3 to 7 d, and was then rapidly decreased after

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7 d with the viable cell less than 8 log cfu/ml through 21 d of fermentation. There was an approximately 5 log cycles decrease in the viable count of strains after 21 d of fermentation at 37ºC. The points corresponding to the late exponential growth phase fell between 48-72 h during fermentation. From the result, the appropriated fermentation time of soybean milk with *L. plantarum* LCC 150 starter that provide high amount of cell number was ranged between 24 h and 72 h (1-3 d) of fermentation period, with the highest ($P<0.05$) growth approximately amount at 9.86±0.47 log cfu/ml. *L. plantarum* LCC 150 was normally culture in MRS broth which containing the complete nutrient. This study was to investigate the profile of LCC 150 growth when cultured into the soybean milk which was different nutrient source from MRS medium. Thus, from the result, LCC 150 was able to grow with amount of viable count greater than 8 log cfu/ml during 7 d of fermentation. Moreover, the viable cell LCC 150 in 3-7 d fermented soybean milk being stable the quantity at $\geq 10^6$ cfu/ml after 1 month of cold storage or at 4 ºC (the stability tests was not shown), therefore, the probiotic property of fermented soybean milk could be claimed due to the viable cells still more than 6 log cfu/ml.

4.4.3 Change of pH and titratable acidity during fermentation

The decrement in pH and the change of total acid of the fermented soybean milk at 0 h, 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, 5 d, 7 d, 14 d and 21 d of fermentation period were displayed in Figure 4.10. The acid titration was calculated as % lactic acid (w/v) of fermented soybean milk.

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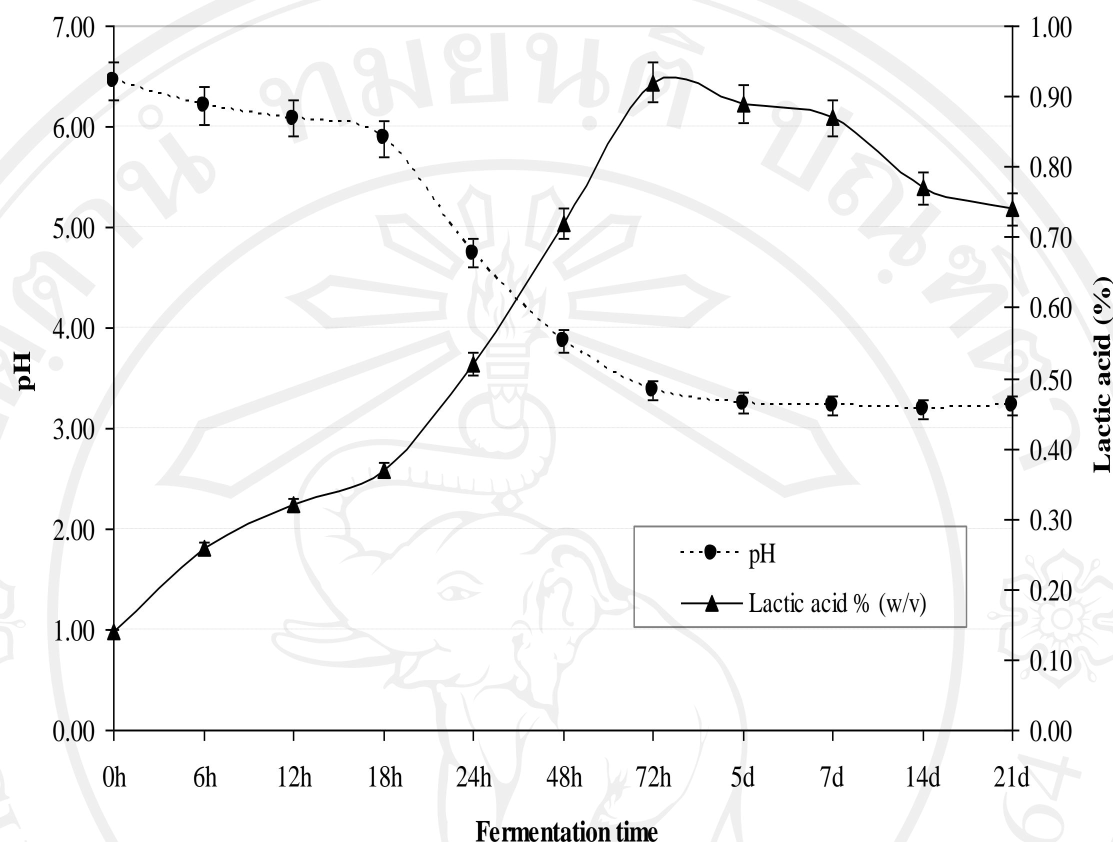

Figure 4.10 The change of pH values and %lactic acid (w/v) in fermented soybean milk with *L. plantarum* LCC 150 starter during fermentation period at 37ºC for 21 days

The result from the Figure 4.10 showed that the change of pH and total acidity (%lactic acid) in soybean milk fermentation. The pH decrement and lactic acid increment were noted in soybean milk through the fermentation period (0 d -21 d). At the 21st day of soybean milk fermentation, the pH declined from 6.45 to 3.22, while lactic acid increased from 0.14% to 0.74% (w/v). Lactic acid reached the maximum amount at 72 h (3 d) of fermentation with the value 0.92% (w/v). The

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change of pH and lactic acid were related to the growth profile with maximum changeable cell number, pH and total acidity between 24 h and 72 h of fermentation period. The lowering pH of soybean milk and the production of lactic acid are essential for manufacturing the fermented foods and beverages. The nutrient sources belongs to soybean milk are not only soluble carbohydrate same as other plants but also including sugar, soluble protein, amino acid and oligosaccharides (Bordignon et al., 2004) which provide to LAB growth. Strains of LAB mainly study as starter culture for dairy and milk from soy belonged to bifidobacteria, *L. acidophilus* and *L. casei* (Otieno and Shah, 2007; Cagno et al., 2010). Most LAB strains could grow with lower pH and produce lactic acid in soybean milk including *L. casei*, *L. lactis*, *L. delbrueckii*, *S. thermophilus*, *B. bifidum* and *B. infantis* (Bordignon et al., 2004). From this study, *L. plantarum* LCC 150 has potential to grow, lower the pH and increase the lactic acid during soybean milk fermentation according to the study of Cagno et al. (2010) that investigated the soy milk by using *L. plantarum*.

4.4.4 Study β-glucosidase activity in fermented soybean milk

The *β*-glucosidase enzyme activity in soybean milk during fermentation with *L. plantarum* LCC 150 at 37ºC for at 0 h, 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, 5 d, 7 d, 10 d, 14 d and 21 d of fermentation was determined. The results of enzyme activity were displayed in Figure 4.11.

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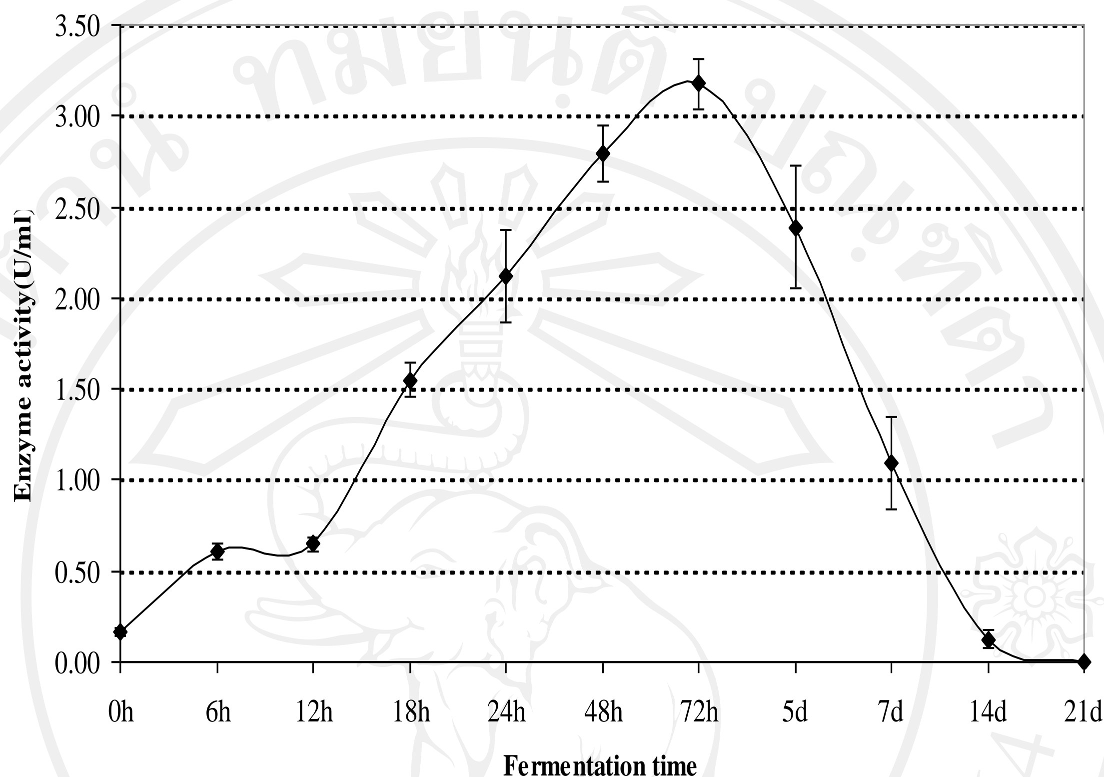

Figure 4.11 *β*-glucosidase enzyme activity in soybean milk during fermentation with *L. plantarum* LCC 150 at 37ºC for 21 days.

The enzyme activities belonged to soybean milk during the 21 days of fermentation period with LCC 150 showed in a range of 0.123±0.051 and 3.174±0.138 U/ml, while the activity was not able to detect at day 21 of fermentation. The enzyme activity was significantly ($P<0.05$) highest at 72 h during fermentation with the value of 3.174±0.138 U/ml. The profile of enzyme activity was similar to profile of growth curve and lactic acid production with was significant ($P<0.05$) gradually increased between 24 h and 72 h during fermentation period, which was the same as described in the report of Otieno and Shah (2007). Otieno and Shah (2007)

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described that trend of β-glucosidase enzyme activity appeared to correlate with the growth profile of *Bifidobacterium* and *Lactobacillus*. The maximum enzyme activity detected from soybean milk was greater than from MRS medium approximately 5 to 6 times. The β-glucosidase-producing LAB play major roles in the hydrolysis of numerous plant β-glucosides including soybean β-glucosides. The β-glucosidase enzymes producing *L. plantarum* LCC 150 involved the conversion of soybean isoflavones from glucosides to aglycones, which improve health protective effects of the consumers (Yin et al., 2005). Furthermore, the β-glucosidase enzymes might be corresponded to the organoleptic properties and nutritive value improvement of fermented plant foods and beverages especially soy-derived fermented food products (Pyo et al., 2005; Yin et al., 2005). Consequently, this study supports that the *L. plantarum* LCC 150 strain with potential activity of β-glucosidase enzyme producing strain should be further applied as a functional starter culture of vegetables, medicinal plant and soy food fermentation.

4.4.5 The isoflavone content in fermented soybean milk

The isoflavone content in soybean milk during fermentation with *L. plantarum* LCC 150 at 37ºC at 0 h, 6 h, 12 h, 18 h, 24 h, 48 h, 72 h, 5 d, 7 d, 10 d, 14 d and 21 d of fermentation was exhibited in Figure 4.13. The reverse-phase HPLC chromatogram of soybean milk isoflavones were shown in Figure 4.12 and the limitation of detection (LOD) and limitation of quantity (LOQ) of isoflavone contents were displayed in Table 4.9.

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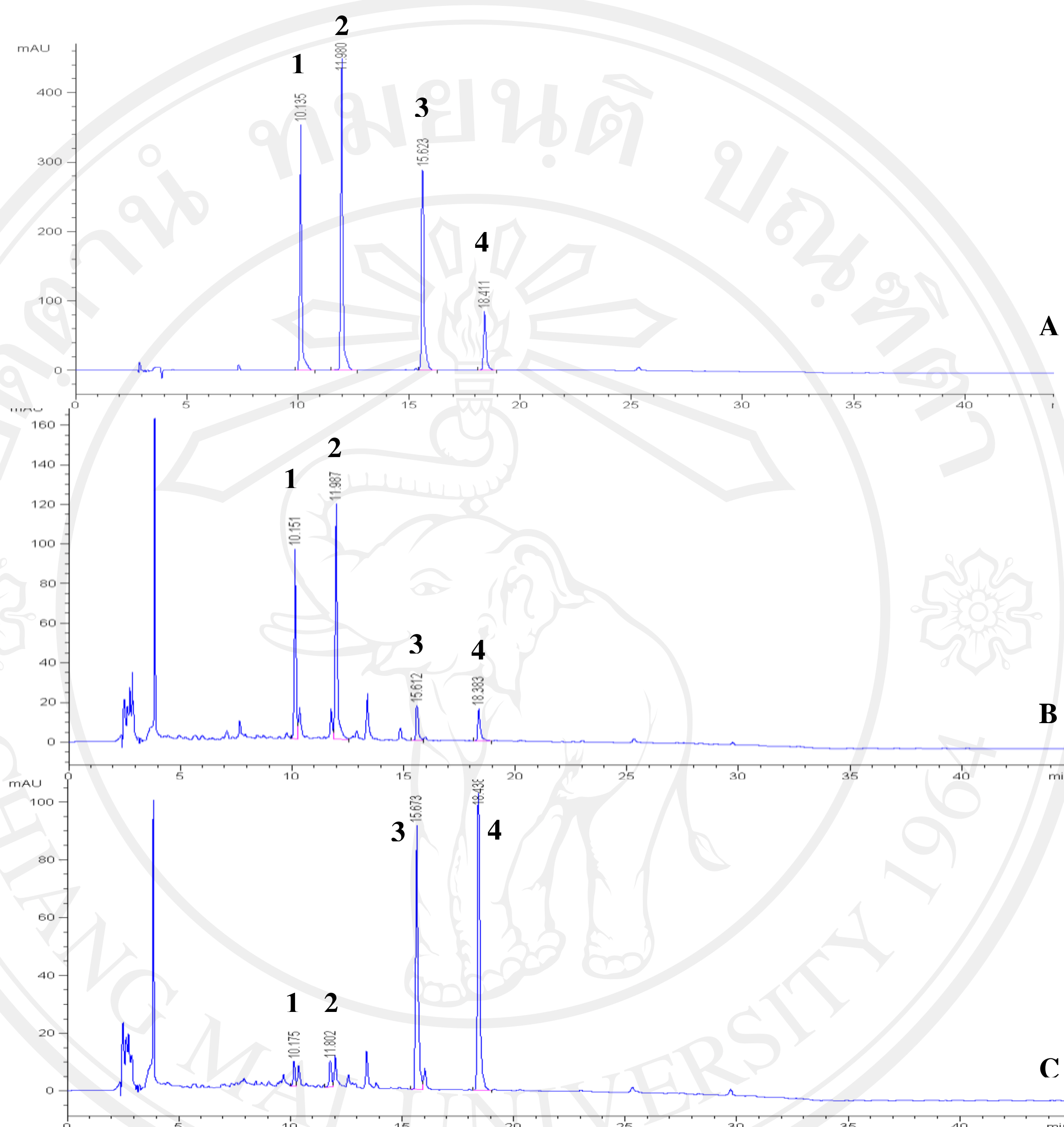

Figure 4.12 HPLC chromatogram displaying approximate retention time of soybean isoflavones detected in soybean milk during fermentation at 37ºC. **(A)** Standard isoflavones**:** peak 1. Daidzin (RT=10.135 min), peak 2. Genistin (RT=11.980 min), peak 3. Daidzein (RT=15.623 min), peak 4. Genistein (RT=18.411 min), **(B)** Peaks of isoflavone content which found in soybean milk during 0 d of fermentation, **(C)** Peaks of isoflavone content which found in soybean milk during 3 d of fermentation

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Table 4.9 Limit of detection (LOD) and limit of quantity (LOQ) of isoflavone contents by HPLC analysis

Isoflavone content	LOD (mg/100ml)	LOQ (mg/100ml)
Daidzin	0.172	0.429
Genistin	0.204	0.511
Daidzein	0.213	0.533
Genistein	0.145	0.362

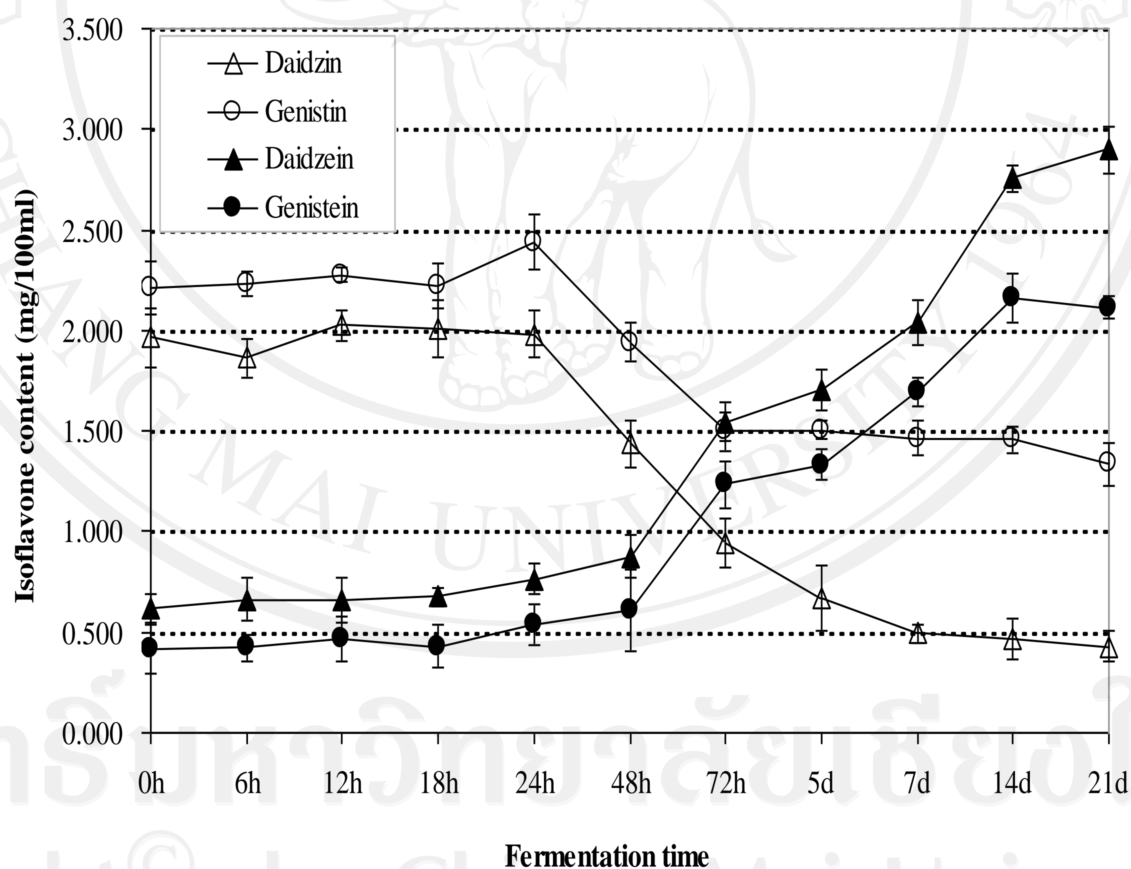

Figure 4.13 Isoflavone content of soybean milk during fermentation with *L. plantarum* LCC 150 at 37ºC for 21 days.

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The isoflavone contents including daidzin and genistin (isoflavone glucosides), daidzein and genistein (isoflavone aglycones) were detected (the values were higher than the limitation of detection (LOD)) till the last day during fermentation period for 21 days. The daidzin and genistin were determined greater level than the daidzein and genistein for 48 h during fermentation. The content of daidzin, genistin, daidzein and genistein were detected prolong concentration at the first period of fermentation from 0 h to 24 h. After 24 h fermentation period, daidzin and genistin were gradually decreased, while the content of daidzein and genistein increased rapidly, reaching to the maximum concentration at the end of fermentation (21 d).

The daidzin and genistin were detected from the initial time and decreased until the day 21st of fermentation with the amount ranged from 1.967±0.147 to 0.431±0.077 mg/100ml and 2.213±0.133 to 1.335±0.104 mg/100ml, respectively. In contrast, the content of daidzein and genistein was rapidly increased after 24 h fermentation. Daidzein and genistein content changed from 0.619±0.067 to 2.897±0.113 mg/100ml and from 0.145±0.123 to 2.114±0.059 mg/100ml, respectively. From the results, it was concluded that any increase or decrease patterns in isoflavone content was largely corresponding to the growth curve of starter LCC 150, pH reduction, total acid increment and β-glucosidase activity. The results corresponded to the suggestion of Otieno et al. (2006a, b). The ability to release isoflavone aglycone specifically depends on β-glucosidase (Yin et al., 2005). The augmentation or declination of β-glucosidase activity was largely corresponded to the appearance of isoflavone aglycone form, which related to the ability of β-glucosidase

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enzyme on hydrolyzing the glycosidic bond. This altered isoflavone glucosides into the bioactive aglycone (Otieno et al., 2006a).

The hydrolysis of daidzin and genistin releases daidzein and genistein, respectively, which can undergo the growth-associated of *L. plantarum* LCC 150 and β-glucosidase activity profile. These results are in agreement with those observed by Raimondi et al. (2009), which suggested that the disappearance of daidzin and the increment of daidzein were growth-associated and occurred mostly during the exponential growth phase. The isoflavone bioactive compounds are significant impact on bioavailability and functional properties of soybean milk or soybean foods. Isoflavones, with a structure homology to human estrogens belong to a group of plant-derived phenolic compounds that exhibit estrogenic activity (Zhang et al., 2004). Several reports attended the health protective effects of soy isoflavones including the preventing development of heart disease and cancers (Chien et al., 2006), relief inflammatory symptom, lowering rates of prostate, breast and colon cancer, improving bone health (Yin et al., 2005). The abundant of isoflavone aglycone form in soybean products have a significant correspondence to health effects of consumer. It was due to the aglycones were absorbed faster and in greater amounts than their glucoside form in human (Chien et al., 2006). Furthermore, Zhang et al. (2004) suggested that isoflavone glycosides were not directly transported across the membrane of absorptive epithelial cells in the host gastrointestinal tract. From this investigation, it was implied that probiotic *L. plantarum* LCC 150 is the potential starter of soybean milk fermentation which can generate the increasing isoflavone aglycone during the fermentation.

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4.5 Study the optimum levels of the fermented soybean milk factors to find the optimum formula that could support the probiotic culture growth profile and biological activity in fermented soybean milk at day 3 of fermentation

4.5.1 Study the chemical composition of fermented soybean milk, growth profile of starter lactobacilli, the β-glucosidase activity and isoflavone content in fermented soybean milk

From the previous investigations of this experiment, the levels for the three factors of soybean milk fermentation with lactobacilli starter in the 2^3 factorial experiments in the central composite design for 3 days of fermentation. The increasing number of starter LCC 150, pH, total acidity, β-glucosidase activity and isoflavone contents of 11 experimental treatments (runs) of soybean milk during fermentation for 3 days Tables 4.10 and Table 4.11 showed that the treatment of soybean milk with significantly ($P<0.05$) maximum increasing number of starter LCC 150 after 3 days of fermentation were found in formula with initial inoculum at 6 log cfu/ml (treatment sets 2, 4 and 6) approximately enhanced at 3.92±0.77-3.97±1.12 log cfu/ml. Most pH values were not significantly ($P\geq0.05$) different in each fermented soymilk treatment. From the statistical results in Table 4.10 and 4.11 for appropriate levels of soybean milk ingredients (water extraction ratios per 1 part of soybean (w/w), initial inoculum of LCC 150 starter (log cfu/ml) and sucrose ratios added per 1 part of soybean milk (w/w)), it could be concluded that the appropriate ingredient that would support the increasing total acidity, viability of LCC 150 starter, β-glucosidase enzyme activity and isoflavone aglycones (daidzein and genistein) contents was

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exhibited in Table 4.12. From the conclusion of appropriate levels of soybean milk ingredients, water extraction ratios at 11 per 1 part of soybean (w/w), initial inoculum of LCC 150 starter at 6 log cfu/ml and sucrose added to soybean milk at 10% (w/v) was the significantly ($P<0.05$) respond the maximum enhancing of total acidity, viable cell of starter, β-glucosidase enzyme activity and isoflavone aglycones contents. Furthermore, LCC 150 can increase growth ability at 3 days fermented soybean milk in this experiment greater than 9 log cfu/ml, which high amount of viable cells relate to the suitable dosage used of probiotic microbes recommended at least 6 log cycles of viable cells.

From the results, the water extraction ratios and sucrose added ratios might be not direct effect on enzyme activity and isoflavone aglycone enhancing, but these might effect on growth ability of *L. plantarum* LCC 150 which utilized sucrose and grew well in agreement with Chen et al. (2011). Chen et al. (2011) reported that *L. plantarum* utilize sucrose and grow well with large amount acid producing. Furthermore, Ewe et al. (2010) reported that sucrose in soymilk could also be the main disaccharide which utilized by probiotic LAB as a carbon source. According to the previous investigation of this study, β-glucosidase enzyme activity related to growth-associated of starter culture. However, Otieno and Shah (2007) suggested that it is possible that the occurrence of a higher amount of glucose (glucose and fructose which are products of sucrose hydrolysis) could stimulate higher β-glucosidase activity.

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Table 4.10 Microbial cell number in soybean milk during fermentation with *L. plantarum* LCC 150 for 3 days of fermentation with the optimum levels of the fermented soybean milk factors (sugar added, water solute and cell concentration of starter)

Run	Soymilk formula			Cell number of LCC 150 (log cfu/ml) at day 3	Increasing number of LCC 150 from day 0 (log cfu/ml)
	Ratio of water extraction per 1 part of soybean	Cell number of Starter (log cfu/ml)	Sucrose added to soybean milk (%w/v)		
1	-1(5)	-1(6)	-1(2)	9.79±1.12	3.84±0.86[b]
2	1(11)	-1(6)	-1(2)	9.77±1.21	3.92±0.77[a]
3	-1(5)	1(10)	-1(2)	10.46±0.65	0.59±0.07[e]
4	-1(5)	-1(6)	1(10)	9.84±0.89	3.95±1.02[a]
5	1(11)	1(10)	-1(2)	10.13±0.87	0.45±0.11[d]
6	1(11)	-1(6)	1(10)	9.84±0.89	3.97±1.12[a]
7	-1(5)	1(10)	1(10)	9.87±1.12	0.13±0.02[f]
8	1(11)	1(10)	1(10)	10.44±1.47	0.51±0.02[e]
9	0(8)	0(8)	0(6)	9.94±0.68	2.11±0.32[c]
10	0(8)	0(8)	0(6)	9.79±1.03	2.13±0.07[c]
11	0(8)	0(8)	0(6)	9.82±0.77	2.08±0.21[c]

Different letters that followed numbers within the same column indicated significantly different ($P<0.05$) between the treatments

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Table 4.11 Chemical and isoflavone content of soybean milk during fermentation with *L. plantarum* LCC 150 for 3 days of fermentation with the optimum levels of the fermented soybean milk factors (sugar added, water solute and cell concentration of starter)

Run	**Soymilk formula**			**pH**	**%TA (w/v)**	**β-glucosidase enzyme (U/ml)** (n=5)	**Isoflavone content (mg/100ml)** (n=3)			
	Ratio of water per 1 part of soybean	**Cell number of Starter (log cfu/ml)**	**Sucrose added to soybean milk (%w/v)**				**Daidzin**	**Genistin**	**Daidzein**	**Genistein**
1	-1(5)	-1(6)	-1(2)	3.32±0.08^{bc}	0.85±0.24^{b}	3.248±0.104^{b}	0.946±0.086^{ef}	1.567±0.061^{a}	1.512±0.027^{c}	1.287±0.076^{c}
2	1(11)	-1(6)	-1(2)	3.24±0.11 ^{c}	0.83±0.17^{bc}	2.764±0.0.63^{e}	0.932±0.114^{gh}	1.534±0.111^{b}	1.506±0.122^{c}	1.278±0.064^{cd}
3	-1(5)	1(10)	-1(2)	3.48±0.33^{abc}	0.65±0.28^{f}	1.656±0.071^{g}	0.927±0.053^{h}	1.363±0.071^{f}	1.157±0.053^{g}	0.834±0.071^{i}
4	-1(5)	-1(6)	1(10)	3.22±0.07^{c}	0.79±0.21^{cd}	2.751±0.073^{e}	0.958±0.047^{d}	1.442±0.041^{e}	1.529±0.044^{b}	1.278±0.064^{d}
5	1(11)	1(10)	-1(2)	3.42±0.33 ^{a}	0.76±0.23^{de}	2.887±0.103^{d}	0.941±0.069^{fg}	1.494±0.077^{d}	1.252±0.116^{f}	0.898±0.123^{h}

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Table 4.11 (Continued)

Run	Soymilk formula			pH	%TA (w/v)	β-glucosidase enzyme (U/ml) (*n*=5)	Isoflavone content (mg/100ml) (*n*=3)			
	Ratio of water per 1 part of soybean	Cell number of Starter (log cfu/ml)	Sucrose added to soybean milk (%w/v)				Daidzin	Genistin	Daidzein	Genistein
6	1(11)	-1(6)	1(10)	3.34 ± 0.05^{bc}	0.97 ± 0.13^{a}	3.362 ± 0.057^{a}	0.958 ± 0.047^{de}	1.442 ± 0.041^{e}	1.582 ± 0.124^{a}	1.324 ± 0.066^{a}
7	-1(5)	1(10)	1(10)	3.64 ± 0.52^{ab}	0.48 ± 0.21^{g}	1.126 ± 0.087^{h}	0.822 ± 0.232^{i}	1.114 ± 0.135^{g}	0.976 ± 0.113^{i}	0.893 ± 0.212^{h}
8	1(11)	1(10)	1(10)	3.32 ± 0.33^{bc}	0.73 ± 0.23^{e}	1.874 ± 0.210^{f}	0.973 ± 0.344^{bc}	1.503 ± 0.221^{c}	1.084 ± 0.204^{h}	0.946 ± 0.127^{g}
9	0(8)	0(8)	0(6)	3.27 ± 0.12^{c}	0.86 ± 0.13^{b}	3.153 ± 0.122^{c}	0.987 ± 0.207^{a}	1.367 ± 0.142^{f}	1.477 ± 0.113^{e}	1.314 ± 0.103^{b}
10	0(8)	0(8)	0(6)	3.33 ± 0.27^{bc}	0.87 ± 0.13^{b}	3.148 ± 0.065^{c}	0.979 ± 0.144^{ab}	1.484 ± 0.089^{d}	1.529 ± 0.044^{b}	1.267 ± 0.132^{e}
11	0(8)	0(8)	0(6)	3.36 ± 0.14^{bc}	0.84 ± 0.08^{b}	3.157 ± 0.117^{c}	0.966 ± 0.095^{cd}	1.522 ± 0.201^{bc}	1.494 ± 0.227^{d}	1.218 ± 0.083^{f}

Different letters that followed numbers within the same column indicated significantly different ($P<0.05$) between the treatments

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Table 4.12 Appropriate levels of ingredient added for fermented soybean milk with LCC 150 for 3 days of fermentation using 2^3 factorial experiments in the center point

Variable parameter	Ingredients	level
%Total acid (w/v)	Water extraction ratios	11
	Initial inoculum	6
	Sucrose added	10
Increasing viable cell number of LCC 150 starter (log cfu/ml)	Water extraction ratios	11, 5, 11
	Initial inoculum	6, 6, 6
	Sucrose added	2,10, 10
β-glucosidase enzyme activity (U/ml)	Water extraction ratios	11
	Initial inoculum	6
	Sucrose added	10
Daidzein (mg/100ml)	Water extraction ratios	11
	Initial inoculum	6
	Sucrose added	10
Genistein (mg/100ml)	Water extraction ratios	11
	Initial inoculum	6
	Sucrose added	10

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4.5.2 Sensory evaluation

All the accepted products were investigated for sensory attributes by Hedonic scale numerical values ranging from 1-9 where represented dislike extremely and 9 like extremely in Table 4.13 showed the response to fermented soybean milk products.

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Table 4.13 Responses sensory evaluation for 20 volunteer consumers' perception of the most important sensory attributes of the 11 treatments of 3 days fermented soybean milk by 9-point hedonic scale

Attributes	**Average liking score***										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Overall appearance	5.55±1.15^{c}	5.70±0.80^{bc}	3.85±0.59^{d}	7.30±0.62^{a}	4.05±0.60^{d}	6.05±0.60^{bc}	3.90±0.64^{d}	4.30±0.84^{d}	6.10±0.72^{b}	6.10±0.15^{b}	6.05±0.76^{bc}
Color	7.70±0.66^{a}	7.65±0.75^{a}	6.05±0.69^{c}	6.25±0.79^{c}	6.15±0.75^{c}	6.85±0.67^{b}	6.35±0.67^{c}	6.05±0.54^{c}	6.85±0.67^{b}	7.10±0.55^{b}	8.00±0.72^{b}
Overall flavor	6.20±0.83^{b}	6.35±0.88^{b}	6.20±0.70^{b}	6.85±0.81^{a}	3.95±0.69^{c}	6.95±0.69^{a}	3.85±0.78^{c}	4.00±1.03^{c}	6.15±0.59^{b}	6.25±0.79^{b}	6.90±0.72^{a}
Aroma	7.80±0.77^{a}	7.65±0.75^{a}	7.85±0.81^{a}	7.00±0.65^{b}	7.00±0.97^{b}	7.00±0.65^{b}	7.00±0.76^{b}	8.15±0.55^{a}	7.10±0.72^{b}	7.05±0.89^{b}	7.75±0.79^{a}
Sourness	5.20±0.95^{c}	5.25±1.02^{c}	6.30±0.92^{a}	5.10±0.97^{c}	5.95±1.05^{ab}	6.40±1.05^{a}	5.55±1.05^{bc}	6.40±0.99^{a}	6.45±0.94^{a}	6.15±0.85^{ab}	6.15±0.75^{ab}
Overall liking	6.05±0.60^{b}	6.00±0.86^{b}	4.15±0.94^{c}	6.10±0.85^{b}	4.25±0.90^{c}	7.00±0.83^{a}	4.20±1.06^{c}	6.10±0.55^{b}	6.10±0.72^{b}	6.00±0.79^{b}	6.20±0.70^{b}

*Values are the means±SD of 20 consumer responses. 9 = like extremely and 1 = dislike extremely

Different letters that followed numbers within the same row indicate significantly different ($P<0.05$) between the treatments

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The sensory evaluation was displayed in Table 4.13. The mean scores of sensory evaluation for all attributes evaluated on the 9-point hedonic scale were between 3.85±0.78 and 8.15±0.55 representing the low-range to almost high range of the scale. Overall appearance were scored between 3 and 4 (dislike slightly) for treatment sets 3, 5, 7 and 8 while the treatment 4 was significantly ($P<0.05$) highest scored at 7.30±0.62 (like moderately) and the others were scored between 5 (neither like nor dislike) and 6 (like slightly). Color and aroma were scored almost high-range of the scale were between 6 (like slightly) and 8 (like very much) for all treatments of soybean milk. Sourness were scored between 5 (neither like nor dislike) and 6 (like slightly). Overall flavor were scored at 4 (dislike slightly) for treatment sets 5, 7, 8 while the treatment sets 4, 6 and 11 were scored at 7 (like moderately) and the others were scored at 6 (like slightly). Overall liking was scored at 4.15-4.25 (dislike slightly) for treatment sets 3, 5, 7 while the treatment 6 was significantly ($P<0.05$) highest scored at 7 (like moderately) and the others were scored about 6 (like slightly). All appearance for the treatment sets 6, 9, 10 and 11 were scored almost high-range between 6 and 8.

4.5.3 In *vivo* cholesterol profile changes of selected lactobacilli strain and fermented soybean milk with lactobacilli strain

In this study, the administration of *L. plantarum* LCC 150 and 3 days fermented soybean milk with LCC 150 (ingredient preparation selected from section 4.5.1) to rats within 6 treatment groups at a dosage of LCC 150 10^9 cells/rat for 12 weeks. The weight increment or weight gain of all groups was exhibited in Figure

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4.14. Moreover, cholesterol, triglycerides, HDL and LDL cholesterol were investigated and shown in Figure 4.15

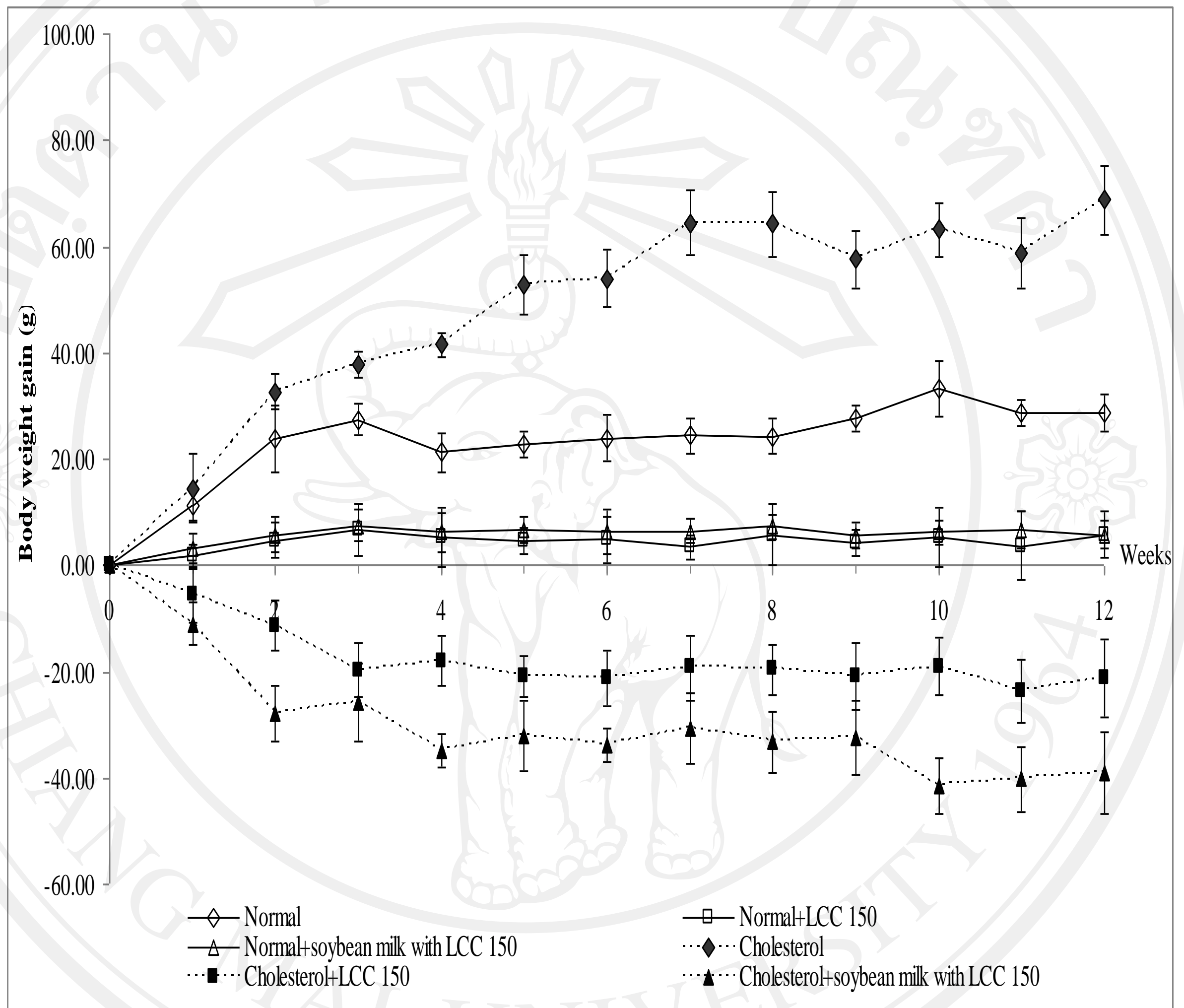

Figure 4.14 Body weight gains of rats within 6 groups; normal or cholesterol supplemented group, normal or cholesterol challenged group treated with LCC 150, normal or cholesterol supplemented group treated with fermented soybean milk using LCC 150 as a probiotic starter

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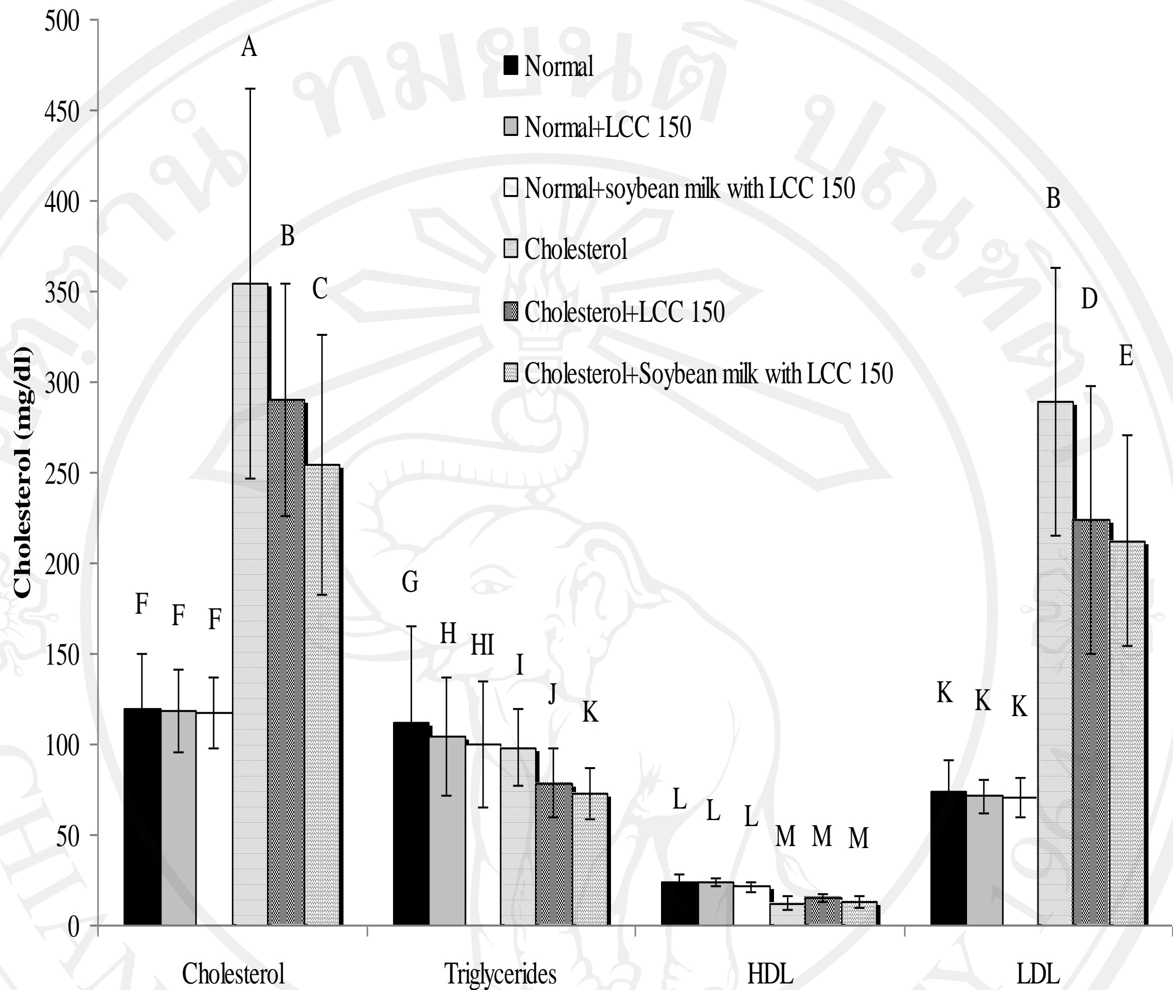

Figure 4.15 Serum cholesterol change profiles of rats within 6 groups (*n*=10/group); normal or cholesterol supplemented group, normal or cholesterol challenged group treated with LCC 150, normal or cholesterol supplemented group treated with fermented soybean milk using LCC 150 as a probiotic starter

The data were analyzed statically using Analysis of Variance by applying a factorial experiment in randomized complete block design (RCBD)

DMRT was applied to determine differences between treatments means (P<0.05)

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From Figure 4.14, the body weight gain of rats within normal and cholesterol supplemented group were observed for high body weight increment. The group supplemented with cholesterol was observed for significantly (P<0.05) highest gain of body weight. The rats within normal group treated with LCC 150 and group treated with fermented soybean milk with LCC 150 were observed no significant (P≥0.05) differences in body weight gain throughout the experiment. Also, these two groups were detected for slightly higher body weights. Otherwise, the rats within two high cholesterol groups which treated with LCC 150 and fermented soybean milk with LCC 150 were observed for the body weight decrement. Furthermore, the high cholesterol group treated with fermented soybean milk with LCC 150 was observed for the significantly (P<0.05) highest decreased of body weight throughout the experiment. The potential decrease of body weight, which was observed in animal group administration with LCC 150 and soybean milk containing LCC 150, may be due to the weight control efficacy of these strain and its product according to the report of Duangjitcharoen et al. (2009). The probiotics support the weight control by reducing glucose absorption from the intestine and also enhance the metabolic use of glucose into the body (Duangjitcharoen et al., 2009).

The concentration of serum cholesterol, triglyceride, HDL and LDL cholesterol in rats within normal group were observed with no significant (P≥0.05) difference of cholesterol change profiles. In contrast, the rats within cholesterol supplemented group were observed with significant (P<0.05) difference in concentration of serum cholesterol, triglyceride and LDL cholesterol. The LCC 150 and fermented soybean milk containing LCC 150 were implied as the high efficacy treatment to reduce cholesterol concentration in such animal within cholesterol

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supplemented group. Furthermore, fermented soybean milk with LCC 150 was observed for the significantly ($P<0.05$) highest activity to reduce serum cholesterol, triglyceride and LDL cholesterol. *L. plantarum* LCC 150 and its soybean milk product were observed for the slightly effect on HDL cholesterol in both normal and high cholesterol group. The serum cholesterol, triglyceride, HDL and LDL cholesterol within rat normal group treated with LCC 150 and its fermented soybean milk were detected with concentration ranged 117.35±19.52-118.68±22.50, 100.24±34.55-104.32±32.23, 21.58±2.85-23.57±2.02 and 70.43±11.05-71.23±9.05 mg/dl, respectively. In case of rats within cholesterol supplemented group treated with LCC 150 and its fermented soybean milk, serum cholesterol, triglyceride, HDL and LDL cholesterol were detected ranged 254.32±72.11-290.44±64.41, 72.54±14.18-78.79±19.26, 13.44±3.17-15.13±2.45 and 212.344±58.45-223.65±74.12 mg/dl, respectively. In case of control groups, normal rats were detected for serum cholesterol, triglyceride, HDL and LDL cholesterol as 120.018±30.05, 111.57±53.15, 24.14±4.02 and 73.43±18.05 mg/dl, respectively. The rats within control group with cholesterol supplemented were detected for serum cholesterol, triglyceride, HDL and LDL cholesterol as 354.67±97.44, 98.03±21.28, 12.40±3.82 and 289.14±74.24 mg/dl, respectively.

From the results, it was shown that selected probiotic strain LCC 150 reduced rat serum cholesterol, triglyceride and LDL cholesterol within cholesterol supplemented group. Furthermore, the fermented soybean milk product containing this strain LCC 150 was detected for greater activity of cholesterol lowering than only LCC 150 strain administration. It might be due to the effects of both LCC 150 strain and soybean bioactive compounds on cholesterol lowering levels in rat. In case of

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soybean isoflavone bioactivity on cholesterol, it was the same as described by Kawakami et al. (2005) that the dietary isoflavones, particularly isoflavone aglycone, have regulative function on cholesterol and fatty acid metabolism in rats fed cholesterol. Moreover, these regulative functions were more effective in isoflavone aglycones than in isoflavone glucosides. Therefore, the positive effect of soybean isoflavone aglycones consumption may reduce the risk of some cardiovascular diseases (Kawakami et al., 2005). The LCC 150 strain was identified as *L. plantarum*. *L. plantarum* have been indicated as potential probiotics with cholesterol-lowering property. This is similar to the report of Nguyen et al. (2007), who suggested that isolated *L. plantarum* from infant feces was a potential probiotic with cholesterol-lowering effects. The strain exhibits the potential to reduce mice serum cholesterol and triglyceride levels through high activity of bile salt hydrolase (Nguyen et al., 2007). The cholesterol lowering properties by *Lactobacillus* spp. were explained for several possible mechanisms (Gilliland et al., 1985; Brashears et al., 1998; Liong and Shah, 2005a; Liong and Shah, 2005b). The first way of which cholesterol lowering property through bile salt hydrolase (BSH) activity (De Smet et al., 1995; Corzo and Gilliland, 1999; Lim et al., 2004; Liong and Shah, 2005a; Begley et al., 2006) according to our study. In this study, the selected LCC 150 strain was detected for the cholesterol-lowering activity via BSH enzyme activity and the capability of bacterial cell to remove cholesterol from culture broth. Furthermore, cholesterol in broth of these 2 strains in all forms decreased, while in pellet increased. The active cells of these 2 strains exhibited significantly the highest ability ($P<0.05$) to decrease cholesterol from broth and increase in pellet. The lactobacilli strain with BSH activity exhibited cholesterol-lowering property higher than the strains without

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BSH activity. These results suggest that the BSH ability supported the mechanism for the in *vitro* lowering of cholesterol of the cells (Parvez et al., 2006; Kim et al., 2008).

The precipitation of cholesterol at low pH can cause less solubility of bile acids and likely to precipitate at pH lower than 6.0 (Klaver and Van der Meer, 1993; Brashears et al., 1998). Brashears et al. (1998) reported that some lactobacilli can remove cholesterol from suspension in culture broth during growth. The greater reduction of cholesterol by active cells corresponded to the growth of cells (Liong and Shah, 2005a). Therefore, the greatest high cholesterol-lowering activities were found by active cells with BSH activity. Furthermore, the *Lactobacillus* cells were known to assimilate the cholesterol which was associated and incorporated in the cells during growth (Gilliland et al., 1985).

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