CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of lactic acid bacteria (LAB)

From the 100 faecal samples and 100 Thai fermented foods samples 1366 of gram positive and catalase negative bacterial isolates were obtained. A total of 803 and 563 strains were isolated from faecal samples and fermented food origins, respectively. Among these, 864 strains showed the typical characters of lactic acid bacteria, gram positive bacteria and catalase negative colony (**Table 5**).

Table 5 Number of strains obtained after purification and characterization (Gram staining and catalase production)

Bacterial origins	Number of isolates	
	Purification	Characterization(gram posive and catalase negative)
Feces	803	590
Fermented pork	237	169
Fermented fish	102	46
Fermented tea leaves	161	41
Pickled garlic	63	18
Total	1366	864

4.2 Screening for acid and bile tolerant

Microbial strains that are suitable for probiotics should be able to tolerate in acid media for at least 90 min since it is the food transit time through the human. stomach (Havenaar et al., 1992). Hence, this time period was used in this experiment. Of 864 isolates, only 218 strains could maintain their survival ability in the screening test acid media as shown **Table 6**. The isolates showed higher tolerant in the bile condition than in acid. Among 274 isolates, 119 strains could grow in MRS broth containing 0.3% bile salt as shown in **Table 6**. It was found that the 218 isolates that tolerated to the acid condition also exhibited the resistance to bile condition. These 218 isolates were considered to be the acid and bile tolerant strains and were used for further studies.

Table 6 Number of viable strains after acid and bile tolerant screening test

Bacterial origins	Acid tolerant (pH 2.5)	Bile tolerant (0.3% bile salt)
Feces	167	452
Fermented pork	31	66
Fermented fish	9	22
Fermented tea leaves	10	15
Pickled garlic	1	16
Total	218	571

4.3 Effect of pH

The effect of pH ranging from 2.0 to 7.0 on the survival rate of the 218 selected strains was studied. Resistant strains showed little decrease in viable cell numbers even after 2 h of incubation at pH 3.0. Their viability decreased only by 1 log cycle up to 2 h at pH 2.5. The strains showed a gradual decrease in viability within 2 h at pH 2.0. Nine strains showed the survival rates >50% after 2 hours of incubation at pH 2.0. The survival rates of strains 2311, 3007, 3010,6014, 8510, 9401, 9905, 9913 and 10101A were found to be higher than all isolates under this study. The strain no. 2311, isolated from fermented tea leaves showed the highest acid resistant at extremely low pH (pH 2.0)The data are presented in **Table 7**.

When the pH was up to 7.0, all isolates could survive. Mishra and Prasad (2005) reported that when pH was raised to pH 3.0, the test *Lactobacillus casei* showed higher survival than in pH 2.0. In our experiment, the results were in agreement that the higher the pH, the higher the percentage of lactobacilli viability.

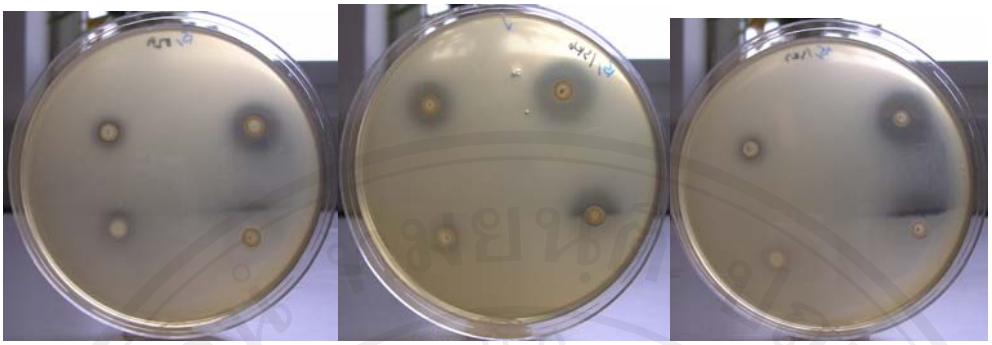
4.4 Effect of bile concentration

Besides being in the strong acid media in the stomach, the probiotic bacteria taken orally had to defense itself with the bile salt in the gastrointestinal tract. Hence, bile tolerance is considered to be one of the important properties required for good probiotic. There is no consensus about the precise concentration to which the selected strain should be tolerant. The physiological concentration of bile salts in the small intestine is between 5,000 and 20,000 µM, or 0.3% to 0.5% (Dunne et al., 2001). In our study, the concentration of 0.3%, 0.5%, and the extremely high concentration of 1.0% bile salt, equivalent to 8,000-24,000 µM of sodium deoxycholate were used.

The results revealed that all strains could survive more than 60% in 0.3%,0.5% , and 1.0% bile salt solutions as shown in **Table 7**. The isolates from our experiment showed stronger bile tolerant than those reported by other investigators (Papamanoli et al., 2003). The gradually decrease in viable cells was observed when the concentration of bile salt was increased up to 1.0%. This was considered that bile salt caused the increase in permeability of bacterial cell membranes, as the membrane composed of lipids and fatty acids. The most important probiotic property of desirable bacteria is dependent on their ability to remain viable in acid and bile in gastrointestinal tract ecosystem. These 9 strains; were selected for further studies.

4.5 Detection of antibacterial activity

The good probiotics should present their antimicrobial actions particularly to the pathogens in the GI system. In this study, *S. aureus*, *S. typhi*, and *E. coli* were used as the test bacteria because they are occasionally found as food borne that cause gastroenteritis. The result revealed that the antibacterial activity of the nine selected lactobacilli could inhibit all test pathogenic bacteria however in different inhibition level as shown in **Table 7.** and **Figure 5**. The production of organic acid and hydrogen peroxide by lactobacilli was reported to inhibit both gram positive and gram negative bacteria, whereas bacteriocin affects only the growth of gram positive bacteria (Schillinger and Lucke, 1989).



(a) (b) (c)

Figure 5 The inhibitory effects of LAB isolates on *Staphylococcus aureus* TISTR 029 (a), *Escherichia coli* TISTR 780 (b), and *Salnonella typhi* DMST 5784 (c).

4.6 Cell surface hydrophobicity

The ability to adhere can give information about the possibility of probiotics to colonize and modulate the host immune system. Several mechanisms were reported in the adhesion of microorganisms to intestinal epithelial cells (Savage, 1992). Cell hydrophobicity is one of factors that may contribute to adhesion of bacterial cells to host tissues (Ram and Chander, 2003). This property could confer a competitive advantage, important for bacterial maintenance in the human gastrointestinal tract (Naidu et al. 1999). In this study, the in vitro determination of microbial adhesion to hexadecane droplets was carried out. This method was reported to be qualitative valid to estimate the ability of a strain to adhere to epithelial cells (Kiely and Olson, 2000). The results revealed that the hydrophobicity in hexadecane and xylene were found in the strain no. 8510 at the highest value of 83.8% and 89.4 %, respectively. The data were shown in **Table 9.**

Table 7 Survival of selected acid- and bile-resistant strains in buffer pH 2, 2.5 and 3, and MRS containing bile salt at 0.3%, 0.5% and 1.0% of concentration.

Isolate no.	% survival							
	Varied pH				MRS containing bile salt			
	pH2	pH2.5	pH3.0	pH7	0%	0.3%	0.5%	1%
TISTR 892[a]	58.28	70.25	91.93	101.43	100	58.88	61.40	64.74
FTL 2311	62.09	81.10	99.93	96.68	100	97.92	100.88	95.47
FP 3007	57.69	86.76	98.81	97.70	100	93.22	92.17	93.97
FP 3010	56.28	84.93	93.36	85.36	100	99.20	96.83	97.83
FS 6014	60.43	92.50	98.81	99.45	100	82.04	91.32	84.87
FS 8510	55.31	74.07	90.65	96.59	100	72.48	77.37	74.69
FS 9401	59.63	82.01	94.28	99.39	100	68.98	75.31	75.89
FS 9905	53.01	54.34	93.01	97.66	100	92.81	89.49	90.54
FS 9913	49.96	51.11	97.70	99.98	100	94.24	92.49	78.50
FS 10101A	56.96	84.52	94.25	94.81	100	86.98	93.04	91.97

[a] *Lactobacillus delbrueckii* subsp.*bulgaricus* TISTR 892 (Thailand Institute of Scientific and Technological Reserch Culture Collection, Bangkok MIRCEN); FTL = fermented tea leaves; FP = fermented pork; FS = faecal sample

Table 8 Antimicrobial activity of the selected strains

Strain	Inhibition zone* (mm) of indicator strains		
	S. aureus TISTR 029	*E. coli* TISTR 780	*S. typhi* DMST 5784
FTL 2311	5.43 ± 2.15	7.30 ± 1.59	6.70 ± 1.60
FP 3007	4.67 ± 1.35	4.97 ± 1.31	5.90 ± 0.69
FP 3010	6.30 ± 0.83	6.87 ± 0.87	7.33 ± 2.31
FS 6014	14.3 ± 3.86	9.50 ± 1.04	7.50 ± 1.95
FS 8510	5.33 ± 0.77	6.2 ± 1.64	3.95 ± 1.76
FS 9401	9.35 ± 0.69	6.43 ± 0.42	9.83 ± 0.75
FS 9905	3.55 ± 1.63	4.30 ± 1.59	3.55 ± 1.37
FS 9913	5.20 ± 1.71	3.55 ± 0.55	4.28 ± 0.75
FS 10101A	3.35 ± 0.57	3.07 ± 0.7	4.05 ± 1.08

* : The diameter of inhibition was calculated as the difference between the total of inhibition zone and the diameter of selected strains spot. (mean ± standard deviation, n=4)

FTL = fermented tea leaves; FP = fermented pork; FS = faecal sample

Table 9 Hydrophobicity of the selected strains in hexadecane

Isolate no.	Food origins	Hydrophobicity[a] (%)	
		Hexadecane	xylene
FTL 2311	Fermented tea leaves	20.9 ± 1.6	1.6± 0.5
FP 3007	Fermented pork	68.7 ± 6.2	41.2 ± 3.9
FP 3010	Fermented pork	53.7 ± 8.2	58.2 ± 6.8
FS 6014	Feces	47.3 ± 2.9	30.3 ± 5.5
FS 8510	Feces	83.8 ± 6.4	89.4 ± 4.9
FS 9401	Feces	33.03± 6.1	88.1 ± 8.2
FS 9905	Feces	5.7 ± 2.9	71.9 ± 2.9
FS 9913	Feces	27.2 ± 4.4	68.5 ± 5.8
FS 10101A	Feces	1.8 ± 1.1	74.8 ± 1.9

[a]mean ± standard deviation., n=3

FTL = fermented tea leaves; FP = fermented pork; FS = faecal sample

4.7 Effect of inulin on selected *Lactobacillus* strains growth

Glucose is considered the main carbon source by all microorganisms due to its size, rapid uptake utilization and cellular energy conversion. However, carbon sources some bacteria have a complete enzymatic machine that allows them to use complex

carbohydrates. Inulin used in this experiment was the famous prebiotic. Based on the average for all 9 isolates, growth on inulin was better than that on Raftiline and banana, excepted for strain no. 8510 (data shown in **Figure 6**).

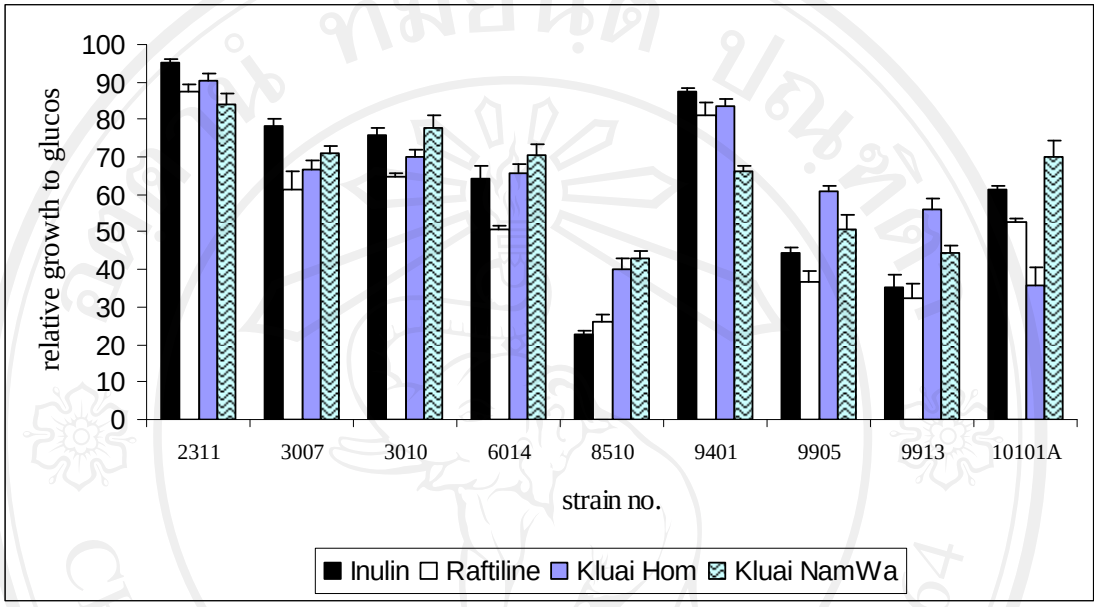


Figure 6 Comparison of maximum growth achived in 24 hours by nine selected strains on MRS broth containing glucose, or inulin, Raftiline, banana1 (Kloui Hom or Gros Michael or *Musa* AAA group), banana2 (Kloui Nam Wa or Pisang Awak or *Musa* ABB group)

Note : Growth on glucose was calculated as 100% Growth

4.8 Identification of bacterial isolates

The preliminary identification of nine selected LAB isolates were investigated by sugar fermentation pattern using API 50 CH system. As the results of API 50 CHL, strains FTL 3007, FP 3010, and FP 10101A were identified as *L. fermentum*, whereas FS 6014, FS 8510 and FS 9401 belonged to the species of *L. plantarum*. Strain FTL 2311 was *L.buchneri*. Strain FS 9905 and FS 9913 were identified as *L.cellobiosis*.

The selected isolates were further identified with Biolog AN Microplate test. A positive utilization reaction was indicated by the development of a purple color in the revelent well. Other wells remained colorless, as did the control well with no carbon source (**Figure 7**). The identification results of selected isolates by Biolog 's Microlog database are shown un **Table 10**. For the strain FS 6014 and FS 8510, they could not get any identification result from this method. Comparing to API 50 CH and PCR method, this system is not appropriate to identify lactic acid bacteria because of error that make difficult the bacterial identification

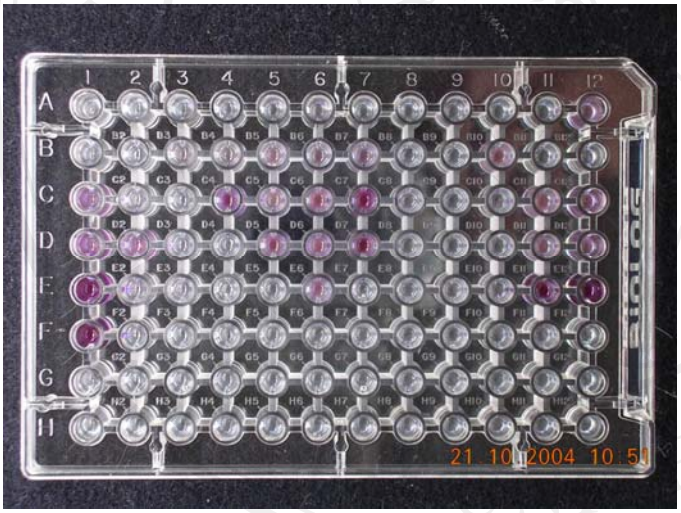


Figure 7 The identification of LAB isolate by Biolog AN Plate.

The results from gel electrophoresis of genus-specific PCR, multiple PCR, and species-specific PCR were used to identify the nine selected strains.

From genus-specific PCR, all of selected isolates were identical to *Lactobacillus* species. LbLMA1-rev and R16-1 were used to amplify DNA fragment for *Lactobacillus* genus specific level identification and *L. crispatus* Lb6 were used as

type strain. From gel electrophoresis result (**Figure 8**) showed that all of selected isolates were confirmed identified to belong to *Lactobacillus* species.

1 2 3 4 5 6 7 8 9 10 11 12 13

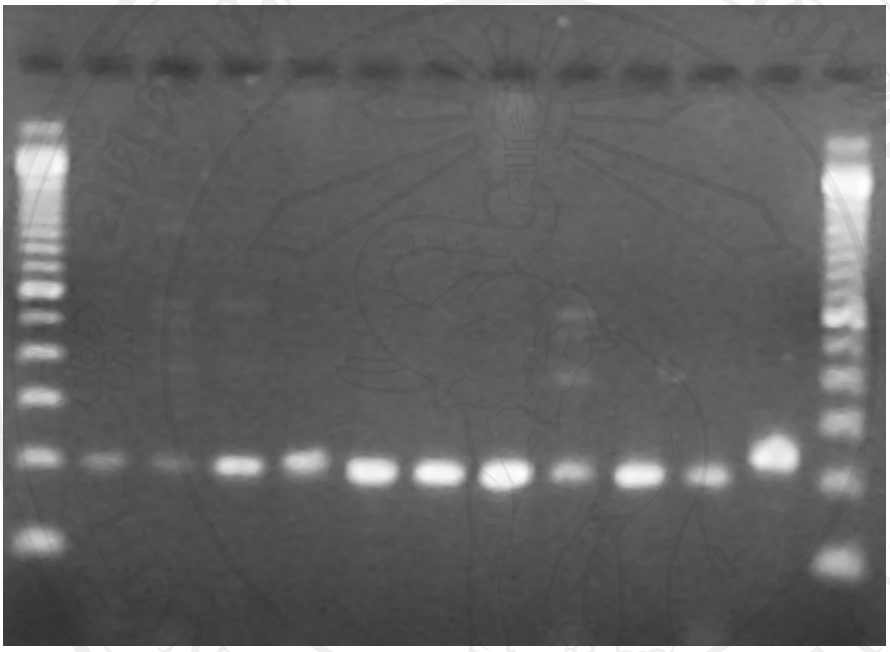


Figure 8 Genus-specific PCR of selected isolates. Lane no.: 1 and 13 = marker; 2 = FTL 2311; 3 = FP 3007; 4 = FP 3010; 6 = FS 6014; 7 = FS 8510; 8 = FS 9401; 9 = FS 9905, 10 = FS 9913, 11 = FS 10101A;12 = type strain Lb6)

For multiple PCR, the primer set comprising of five primers, Lac2, Ldel7, LU5, LU3 and LU1 were used. *L. crispatus* (Lb6) and *L. fermentum* (Lb95) were used to be type strains. DNA from tested isolates generated fragments corresponding to the 350 bp fragment of Lb95 (**Figure 9**.), a positive control for 350 bp group that comprise *L. curvatus, L. reuteri, L. plantarum, L. parapentarum, L. pentosus, L. keferi, L. fermentum, L. animalis, L. mucosae, L. aviaries ssp.aviaries, L. salivarius ssp.salicinus, L. salivarius ssp.salivarius, L. hilgardii*, and *L. panis.*

1 2 3 4 5 6 7 8 9 10 11 12 13 14

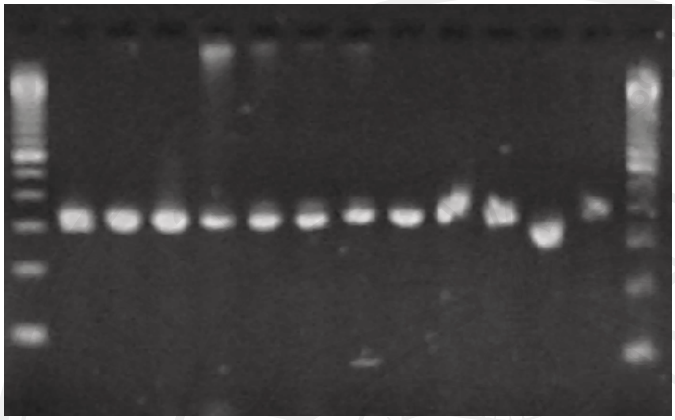


Figure 9 Gel electrophoresis of PCR products from multiplex PCR assays. Lane no.1 and 13 = marker; 2 = FTL 2311; 3 = FP 3007; 4 = FP 3010; 6 = FS 6014; 7 = FS 8510; 8 = FS 9401; 9 = FS 9905, 10 = FS 9913, 11 = FS 10101A; 12 = Lb6 (*L. crispatus*); 13 = Lb95 (*L. fermentum*)

The genotypic analysis by species specific assays of the strains FTL 2311, FP 3007, FP 3010, FS 9905, FS 9913 and FS 10101A, it was found that theirs relativity to *L. fermentum*, whereas the strains of FS 6014, FS 8510 and FS 9401 belonged to *L. plantarum* (**Figure 10 & Figure 11)**. The identification results of these bacterial strains were concluded in **Table 10**.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 10 Gel electrophoresis of PCR product from *L. fermentum* species specific PCR with primers Lferm3 and Lferm4. *L. fermentum* was used as positive control and *L. plantarumi* was to be negative control. Lane no.1 and 15 = marker; 2 = FTL 2311; 3 = FP 3007; 4 = FP 3010; 6 = FS 6014; 7 = FS 8510; 8 = FS 9401; 9 = FS 9905, 10 = FS 9913, 11 = FS 10101A;12 = type strain Lb23 (*L. fermentum*); 13 = *L. fermentum* ; 14 = type strain Lb23 (*L. plantarum*)

1 2 3 4 5 6 7 8 9 10 11 12 13 14

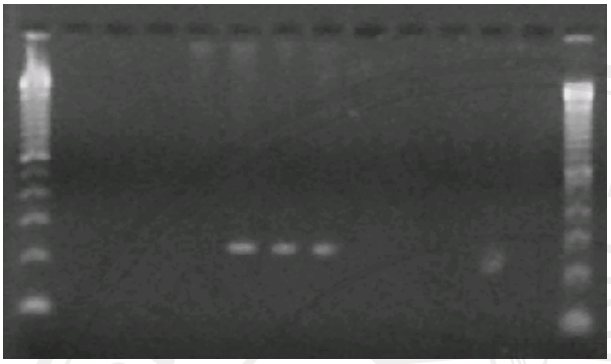


Figure 11 Gel electrophoresis of PCR product from *L. plantarum* species specific PCR with primers Lpla2 and Lpla3. *L. plantarum* was used as positive control and *L. reuteri* was to be negative control. Lane no.1 and 14 = marker; 2 = FTL 2311; 3 = FP 3007; 4 = FP 3010; 6 = FS 6014; 7 = FS 8510; 8 = FS 9401; 9 = FS 9905, 10 = FS 9913, 11 = FS 10101A; 12 = type strain Lb23 (*L. plantarum*); 13 = type strain Lb13 (*L. reuteri*)

Table 10 Identification of selected strains using API 50CHL, Biolog system and PCR method (species specific primer)

Isolated no.	API 50 CH	Biolog	PCR
FTL 2311	*L. buchneri*	*L. reuteri*	*L. fermentum*
FP 3007	*L. fermentum*	*L. fermentum*	*L. fermentum*
FP 3010	*L. fermentum*	*L. fermentum*	*L. fermentum*
FS 6014	*L. fermentum*	-	*L. plantarum*
FS 8510	*L. plantarum*	-	*L. plantarum*
FS 9401	*L. plantarum*	*Lactobacillus* sp.	*L. plantarum*
FS 9905	*L. cellulobiosus*	*L. reuteri*	*L. fermentum*
FS 9913	*L. cellulobiosus*	*L. reuteri*	*L. fermentum*
FS 10101A	*L. fermentum*	*L. fermentum*	*L. fermentum*

Furthermore, isolated no. FTL 2311, FP 3007, and FP 3010 were analyzed for 16S rRNA, The sequence of each of these three strains was used to search the GenBank and EMBL databases. They were distantly related to *Lactobacillus fermentum*. From the results presented here, it can be seen that the strains FTL 2311, FP 3007, and FP 3010 isolated from fermented tea leave and fermented pork can be identified as *L. fermentum* on the basis of their biochemical, physiological, (**Table 11**) and their 16S rDNA sequences (**Figure 12 and Table 12**).

Table 11 Physiological and biochemical characteristics of the selected strains

Characteristic	FTL 2311	FP 3007	FP 3010
Acid production from:			
L-Arabinose	-	-	-
D-Xylose	-	-	-
Galactose	+	+	+
D-Fructose	+	+	+
Lactose	+	+	+
Melibiose	+	+	+
Raffinose	+	+	+
Gluconate	+	+	+
Growth at 45 °C	+	+	+

Table 12 Sequencing of 16S rRNA of selected strains

Strains	Bacteria	Accession number	Identities (%)
FTL 2311	*L. fermentum*	AB362625.1	100
FP 3007	*L. fermentum*	AB362628.1	99.9
FP 3010	*L. fermentum*	AP008937.1	100

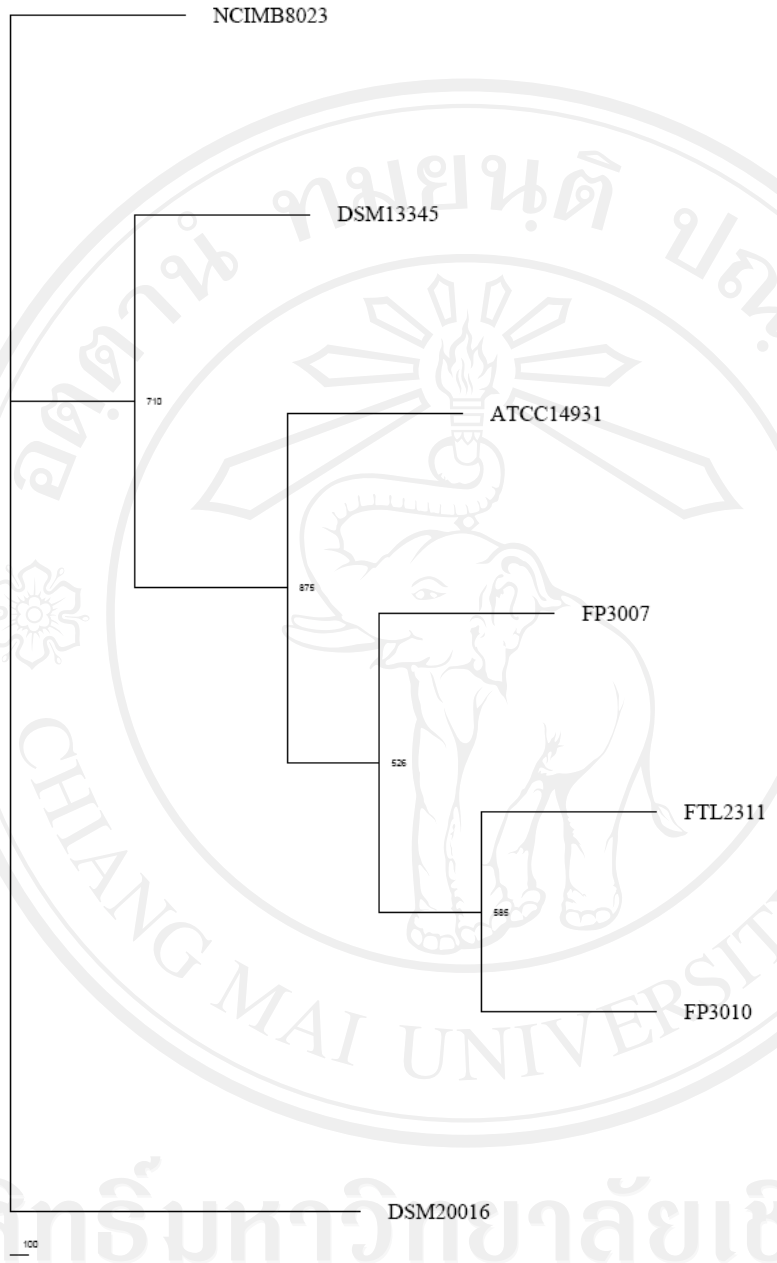


Figure 12 Phylogenetic tree showing the positions of strains FTL 2311, FP 3007, FP 3010 and some *Lactobacillus* species based on 16S rRNA

4.9 Antibiotic susceptibility

Antibiotic susceptibility of lactobacilli was one of the crucial criteria for the safety point of view of potential probiotics since bacteria used as probiotics may serve as host antibiotic resistant genes, which can be transferred to pathogenic bacteria. The isolates FTL 2311 and FP 3010 showed resistance to gentamycin and linezolid whereas isolate FP 3007 was susceptible to the drug as shown in **Table 13.** All tested isolates were susceptible to the protein synthesis inhibitors, chloramphenicol, quinupristin, erythromycin, kanamycin, rifampicin, streptomycin and tetracycline since the MIC to the antibiotics was lower than the breakpoints. Our results showed difference from some other works previously reported. Fons et al. (1997) reported the erythromycin resistance of *L. fermentum* isolated from human gut whereas Ahn et al. (1992) reported the resistance to chloramphenicol of *Lactobacillus* spp. isolated from dairy origin. The difference in antibiotic susceptibility of *L. fermentum* from different food origin indicated the role of bacterial sources which might influence the bacteria in genetic level of antibiotic resistance. For trimethoprim, the isolates no. 2311, 3010 and 10101A showed resistance, whereas the others were susceptible. Trimethoprim inhibits the synthesis of folic acid which is necessary for the synthesis of purines, essential substance in bacterial nucleic acid. Resistance of the strains FLT 2311, FP 3010 and FS 10101A to trimethoprim might be due to bacterial production of trimethoprim-insensitive dehydrofolate reductase. The results to a cell wall synthesis inhibitor showed that the strains FTL 2311, FP 3010, FS 9913, and FS 10101A were susceptible to ampicillin whereas all selected isolates were classified as resistance to ciprofloxin and vancomycin. Resistance to ampicillin, ciprofloxin, and vancomycin are commonly found in genus *Lactobacillus*. The high frequency of vancomycin

resistance found among lactobacilli might not pose a problem as this type of vancomycin resistance is different from the inducible, transferable mechanism observed in enterococci (Klein et al., 2000). Moreover, it was previously reported the absence of resistant transferable genes, *van A*, *van B*, or *van C1-3* in *L. fermentum*. Therefore, all three selected strains of *L. fermentum* in our study were considered as safe.

4.10 Haemolytic activity

None of the *Lactobacillus* strains examined exhibited α-haemolytic activity when grown in Columbia human blood agar. All of the selected strains isolated from fermented foods (3 strains) and two strains from fecal samples were *γ*-haemolytic (i.e. no haemolysis), while four strains from feces exhibited α-haemolysis (**Table 14**).

Table 13 Antibiotic susceptibility of the selected strains

Antibiotics	MIC (µg/ml)								
(breakpoint[a] µg/ml)	FTL 2311	FP 3007	FP 3010	FS 6014	FS 8510	FS 9401	FS 9905	FS 9913	FS 10101A
Ampicillin (2)	<1	2	<1	4	4	>4	2	<1	<1
Chloramphenicol (16)	<8	<8	<8	<8	<8	<8	<8	<8	<8
Ciprofloxin (4)	4	8	4	4	>8	4	4	4	4
Quinipristin (4)	<2	<2	<2	<2	<2	<2	<2	<2	<2
Erythromycin (4)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Gentamycin (1)	1	<0.5	1	2	<0.5	2	<0.5	1	<0.5
Kanamycin (32)	<16	<16	<16	<16	<16	<16	<16	<16	<16
Linezolid (4)	4	4	4	<2	<2	4	4	4	<2
Rifampicin (32)	1	1	1	2	2	2	1	1	1
Streptomycin (16)	<8	<8	<8	16	16	16	<8	<8	<8
Tetracycline (16)	<2	<2	<2	<8	4	<8	<2	8	<2
Trimethoprim (32)	32	<16	32	<16	<16	<16	<16	<16	32
Vancomycin (4)	>8	>8	8	>8	>8	>8	>8	>8	>8

[a] The breakpoints for *Lactobacillus* sp. used by SCAN categorizing bacterial species as resistant. Strains with MICs equal to or higher than the breakpoints are considered as resistant.

Table 14 Haemolytic activities of lactobacilli strains

Isolated no.	Haemolytic activity
FTL 2311	γ-haemolytic
FP 3007	γ-haemolytic
FP 3010	γ-haemolytic
FS 6014	β-haemolytic
FS 8510	β-haemolytic
FS 9401	γ-haemolytic
FS 9905	β-haemolytic
FS 9913	β-haemolytic
FS 10101A	γ-haemolytic

4.11 Safety of viable lactobacilli

Before a novel probiotic strain is introduced into the market the clarification of its safety is needed. Results of this study revealed no mortality was found in all diets. No noticeable abnormal behaviour change in activity, or decline in hair luster was detected in animals after 2 weeks feeding with lactobacilli. There was no significant difference observed in average daily weight gain between the feed with lactobacilli at a concentration 10^6 cfu/mouse/day and the control (data shown in **Table 15**). Several standards on safety assessment of probiotic strains have recently been recommended (Donohue et al., 1998; Zhou et al., 2000; Zhou et al., 2001). The isolated test

lactobacilli were considered to be not toxic according to those standards. Appetite, activity, and weight gain are the most general indicators on health status for animals. According to this, we used the average weight gain and behaviour as well as condition of the mice to evaluate the acute toxicity of test strains. A very high inoculation dosage (10^{10} cfu/mouse/day) of the test strains was used. All animals were healthy and survived after 14 days of the inoculation. Moreover, the mice treated with 10^{10} cfu/mouse/day of the isolated lactobacilli presented a significantly higher weight gain than those in the control group. Specifically, the percentage average weight gain of mice fed with *L. fermentum* FTL. 2311 at concentration 10^{10} cfu/day was the highest at 13.28%, whereas that of the control was 5.60%. This indicated the growth promotion activity of the test strains. Park et al. (2005) isolated *L. fermentum* PL 9005 from fecal origin. They reported no detectable changes in body weight and clinical signs between the bacteria-fed mice and the control groups. They concluded that this strain can be applied in the functional food. In our study, the selected strains FTL 2311, FP 3007, and FP 3010 showed not only non toxic but also served as the health promotion in the test animals. Therefore, these three strains could be promoted for further used as probiotic starter in neutraceutical market.

Table 15 Effect of viable lactobacilli on growth performance of Swiss albino mice

	control	FTL 2311		FP 3007		FP 3010		TISTR 892	
		10^6 cfu/d	10^{10} cfu/d	10^6 cfu/d	10^{10} cfu/d	10^6 cfu/d	10^{10} cfu/d	10^6 cfu/d	10^{10} cfu/d
Initial body weight (g)	35.00±2.1	34.75±1.67	31.63±2.2	35.75±1.58	36.63±1.19	36.63±1.06	34.25±1.49	33.88±1.64	36.88±2.85
Final body weight (g)	36.96±2.36	36.63±2.43	35.81±3.29	38.24±1.57	40.08±1.00	39.91±1.52	37.51±1.84	36.68±0.75	39.64±3.11
Average daily gain (g/d)	$0.14±0.12^{a}$	$0.13±0.08^{a}$	$0.30±0.11^{c}$	$0.18±0.05^{ab}$	$0.25±0.05^{bc}$	$0.23±0.07^{bc}$	$0.23±0.07^{bc}$	$0.15±0.06^{a}$	$0.20±0.03^{ab}$
Number of dead mouse	0	0	0	0	0	0	0	0	0

Value within a raw with different superscript are significantly ($P<0.05$) different and the levels containing a group of means within which there are no statistically significant differences.

4.12 Effect of cryoprotectants

The industrial exploitation of lactobacilli as starter and/or probiotic cultures relies heavily on the ability to concentrate and preserve them, so as to guarantee long-term delivery of viable and functional cultures. Maximum survival of bacteria during drying and subsequent storage is of vital importance technologically and economically. Dried cultures are commonly produced by lyophilization. This process is regarded as a gentle drying procedure to the cells in comparison with spray drying. In this study, the three selected strains were subjected to lyophilization process with various kinds and concentrations of cryoprotectant. *L. delbrueckii* ssp. *bulgaricus* TISTR 892 was used as standard strain. Skimmed milk at concentrations of 10% and 20% showed no significant difference in cryoprotective activity. All strains revealed the lowest percentage of survival cells in skimmed milk 10% + 4% of glycerol whereas the diluent comprised 10% skimmed milk + 4% sucrose gave the highest survival level. This result was similar to the other investigation which 5% sucrose was reported to protect concentrated *Lactococcus lactis* ssp. *lactis* better than 10% glycerol when stored at -20 °C to -70 °C (Chavarri et al., 1988). However, our results of *L. delbrueckii* ssp. *bulgaricus* was in contrast to the report of De Giulio et al. (2005) which no significant difference in viability after lyophilization were observed for this strain in the presence of sucrose, glucose, and lactose. In our study, the presence of 4% sucrose showed significantly higher viability than other additives as shown in **Table 16**. The results demonstrated the significant increase in bacterial survival rate of all three selected strains when concentration of sucrose was raised from 2% to 4%. This effect could also be seen in the standard strain but not obviously. The increase concentration of glycerol from 2% to 4% caused the slight decrease in

survival rate of all strains. The similar result was found in other cryoprotectants i.e., sodium glutamate, glucose, and lactose when different concentrations were compared. When compared with the use of skimmed milk 10%, the results indicated that the adding of glycerol or sodium glutamate, glucose, or lactose, or even 2% sucrose caused the decrease in survival rate of the bacteria. The result suggested that only when adding skimmed milk 10% with 4% sucrose could increase the survival rate of all test bacteria. This indicated the beneficial use of the cryoprotectant mixture on preservation of bacterial cells during lyophilization process.

From all previous results showed that *L. fermentum* FTL 2311 had the highest potential to be probiotic strain with the criteria which we used to investigate. Thus, this strain was selected for formulating probiotic tablets.

Table 16 Survival rate of *Lacobacillus* spp. strains after lyophilization

Cryoprotectants[a]	Survival rate[b]			
	FTL 2311	FP 3007	FP 3010	TISTR 892
SM 10%	73.9 ± 6.5	66.8 ± 5.1	67.5 ± 6.7	56.9 ± 1.9
SM 20%	71.7 ± 2.9	70.4 ± 5.9	68.2 ± 5.3	57.9 ± 0.9
SM 10% + glycerol 2%	44.3 ± 3.2	29.3 ± 2.6	28.6 ± 1.4	41.1 ± 1.5
SM 10% + glycerol 4%	36.4 ± 6.9	25.1 ± 6.3	25.0 ± 3.7	31.9 ± 5.9
SM 10% + sucrose 2%	42.1 ± 3.1	55.9 ± 4.5	56.1 ± 5.1	51.5 ± 4.1
SM 10% + sucrose 4%	75.4 ± 1.4	72.1 ± 5.6	71.5 ± 0.8	58.9 ± 1.7
SM 10% + Na glutamate 2%	41.2 ± 8.9	43.9 ± 1.3	45.0 ± 1.6	49.6 ± 2.6
SM 10% + Na glutamate 4%	57.7 ± 11.2	54.0 ± 9.1	54.1 ± 6.8	51.9 ± 2.2
SM 10% + glucose 2%	40.4 ± 5.1	41.9 ± 1.6	42.9 ± 1.6	40.1 ± 1.7
SM 10% + glucose 4%	40.6 ± 0.7	42.4 ± 3.5	43.1 ± 4.2	47.4 ± 1.2
SM 10% + lactose 2%	43.6 ± 4.4	42.3 ± 1.5	42.2 ± 2.8	40.9 ± 2.2
SM 10% + lactose 4%	45.6 ± 1.4	53.4 ± 4.8	52.9 ± 2.8	47.5 ± 2.4

[a]skimmed milk

[b]mean ± standard deviation, n=3

4.13 Tableting

4.13.1 Effect of compression force on probiotic powder

The effect of the compression force on the viability of *Lactobacillus acidophilus* La-5, *L. acidophilus* 72-4, and *L.fermentum* FTL 2311 was investigated. As can be seen in **Figure 12**, when the cell powders was compressed, the survival of LAB gradually decrease to less than 20% as a pressure of 20 kN was reached. Blair (1991) and Kalchayanand et al. (1998) have reported that bacteria could be injured or killed when subjected to high pressure. Another report of Chan and Zhang (2001) has shown that the decay in the viability of *L. acidophilus* ATTC 4356 cells became more distinct above 120 MPa (3.392 kN) where only about 30% of the cells survived after they were compressed at 180 MPa (5.936 kN).

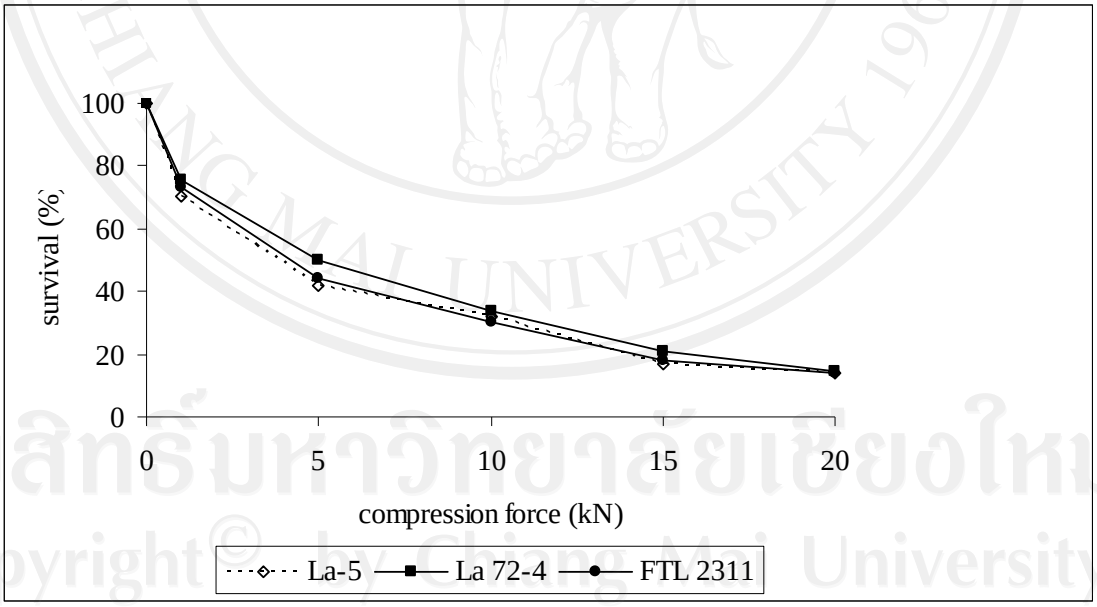


Figure 13 Effect of compression force on viability of LAB cell powder of *L. acidophilus* La-5 (La-5), *L. acidophilus* 72-4 (La 72-4), and *L. fermentum* (FTL 2311)

On the other hand, the tablets which pressed with various compression forces, were enhanced for all strains to survive in 0.1 N HCl. After 1 hour of acidic treatment, the number of viable LAB in tablet was higher than lyophilized powder as shown in **Figure 14.**

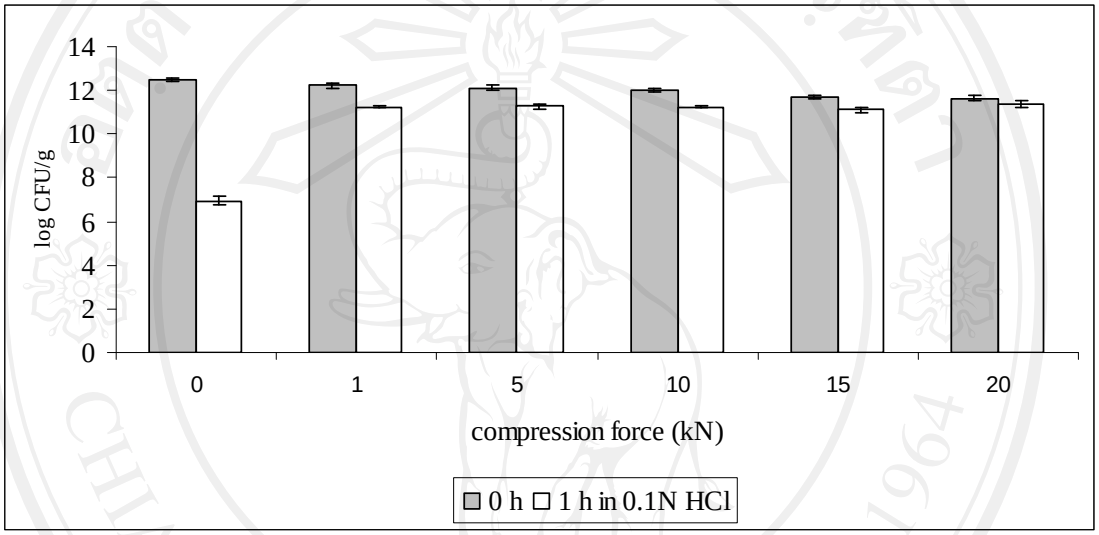


Figure 14 Survival of *L. fermentum* FTL 2311 in tablets compressed with various pressures.

4.13.2 Effect of hydroxypropylmethyl-cellulosephthalate (HPMCP 55)

The results showed that the survival rate in artificial gastric juice of *L. fermentum* FTL 2311 and *L. acidophilus* La-5 were much higher when tablets were prepared with high amount of lyophilized powder (**Figure15**).

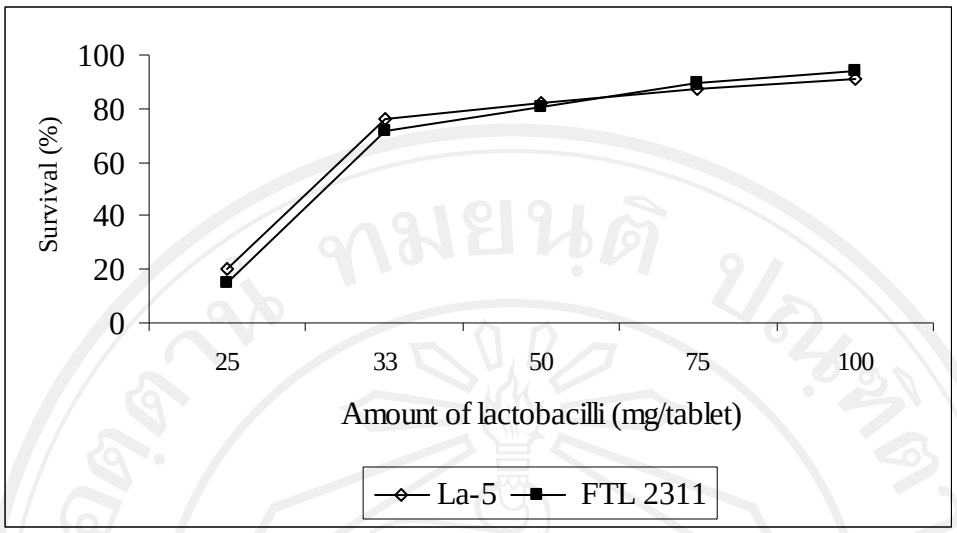


Figure 15 Effect of the amount of lactobacilli lyophilizate on survival rate of lactobacilli after exposure to 0.04 N HCl for 2 h

In case of *L. fermentum* FLT 2311, tablets with different ratios of bacteria to HPMCP 55 (formulation no. A1-A5) were prepared with compression force of 5 kN. Bacterial survival inside the tablets after exposure to an acid medium of pH 1.5 for 2 h was investigated according to Blanquet et al. (2004). The pH of the in stomach is acidic (pH 1.5-5.5) and transient time is about 2 h. Vizoso Pinto et al. (2006) showed that the viability of *L. johnsonii* LA1 in an artificial gastric electrolyte solution containing lysozyme and pepsin at pH 2.5 was 87.5% whereas that was <70% in the pH 2.5 without the enzymes. This means that pH had a stronger bactericidal effect than the enzymes. Thus in our study, we did not add enzymes to gastric mimicking solutions. Results indicated that the percentage of survived probiotic cells in the tablets was increased when the quantity of the bacteria in the tablet formulation was increased as shown in **Figure 16**.

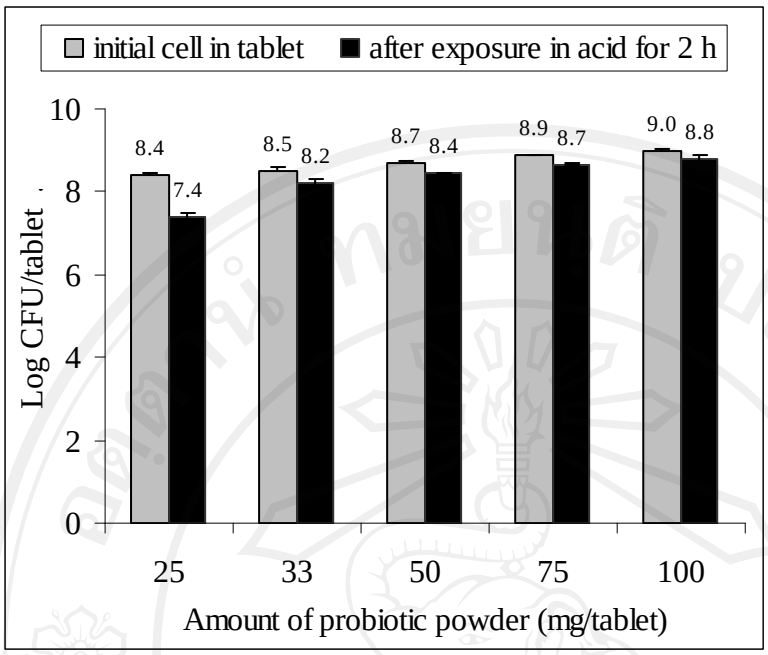


Figure 16 Effect of probiotic concentration in tablets on cell viability (n=3)

According to tablets containing a constant of lactobacilli (100 mg) and various amounts of HPMCP 55, gastric juice (0.04 N HCl) resistance decreased the by lowering HPMCP 55 content (**Table 1**7). For the tablets formulated with only LAB and HPMCP 55, the compaction force of 2 kN was too low to produce the tablets being stable in gastric juice. Concerning the disintegration time in phosphate buffer pH 6.8, only tablets with the compaction force of 5 kN disintegrated within one hour (**Table 17**).

When compare the tablet hardness of the same formulation at different compression force, it was found that increasing of compression force increased the hardness of probiotic tablets (**Table 17**). Because of the increase of the pressure, all pore space including both interparticulate voids were decreased and each particle was closer with more interparticulate attraction (Paronen and Ilkka, 1995)

Furthermore it was tried to increase the survival rate of LAB in acid medium. A partial of HPMCP 55 was took place by swelling excipients, sodium alginate, pectin, and Metolose® (formulation no. 29-46). In the formulations containing apple pectin and Metolose®with 2 kN of compaction force, the viability of bacterial cells were still too much lost during incubation in 0.04N HCl. Tablets with sodium alginate demonstrated the highest cell survival whereas apple pectin and Metolose® did not show cell protection effect. After 2 h immersing to the acid medium, the tablets were tested for disintegration property by using PBS (pH 6.8) as a medium. It was shown that the disintegration time of the tablets with apple pectin and Metolose® was slightly shorter than those of tablets without swelling agents (1.25 and 1 h, respectively). The disintegration time of the tablets containing sodium alginate was substantially longer (> 5 h). However, only small pieces of about less than 10% of these tablets were found to stick tightly to the stainless sieve after 5 h of the test. It was noted that more than 90 % of the tablet mass passed the sieve after 5 h of incubation. More interestingly, sodium alginate showed the strongest cell protection activity with more than 90% retention of cell viability, as compared to the other formulations (cell viability 50-70%). Stadler and Viernstein (2003) reported that probiotic tablets prepared from compaction of a mixture of *L. acidophilus* La-5, hydroxypropylmethylcellulose acetate succinate and sodium alginate was the best protective formulation against artificial gastric juice. This report along with our results supported the use of sodium alginate as one of the essential excipients in probiotic tablets. In general, sodium alginate hydrocolloids show good stability between pH 3 to 10 and they are rather resistant to bacterial and enzymatic degradation. The hydrogel barrier formed around the tablet containing sodium

alginate may retard the permeation of the acidic fluid into the tablets (Chan and Zhang, 2002; Calinescu et al, .2005). This could explain the observed excellent survival of bacteria in the alginate tablets after exposure to pH 1.5 for 2 h. Moreover, the transient time of the ingested food from the gastric to the intestinal tract is about 2-6 h (Blanquet et al., 2004). Therefore, the tablets should ideally protect probiotic bacteria until this time and then release viable bacteria in the intestinal tract. In this study, the tablets with sodium alginate as swelling agent showed the highest retention of cell viability with an acceptable disintegration time. In addition, an incubation in simulated intestinal fluid (phosphate buffer pH 6.8) for 5 hours lead to no cell viability reduction (**Figure 17**). Furthermore, acceptable values of friability (≤ 1%) were obtained for all batches of probiotic tablets with sodium alginate, indicating good mechanical properties which can withstand handling. The HPMCP 55 based probiotic tablet formulation with the addition of sodium alginate was considered to be the best formulation and selected for further study in stability test during storage.

Table 17 The hardness, survival of *L. fermentum* FTL 2311 and disintegration of tablets containing HPMCP and sodium alginate, apple-pectin, or Metolose®

Formulation no.	Hardness[a] (N)	Survival of LAB[b] (%) in 0.04 N HCl for 2 h	Disintegration (h)	Note
A1	67.50 ± 10.61	-	-	Disintegrated in 0.04 N HCl
A2	53.33 ± 7.77	-	-	Disintegrated in 0.04 N HCl
A3	47.67 ± 4.16	-	-	Disintegrated in 0.04 N HCl
A4	33.00± 7.00	-	-	Disintegrated in 0.04 N HCl
A5	23.00	65.49 ± 2.43	2	-
A6	43.00 ± 4.97	87.60 ± 4.40	2	-
A7	76.33 ± 7.51	85.90 ± 2.15	2.5	-
A8	95.00 ± 16.09	87.38 ± 1.75	2.5	-

Table 17 (continued)

Formulation no.	Hardness[a] (N)	Survival of LAB[b] (%) in 0.04 N HCl for 2 h	Disintegration (h)	Note
A9	102 ± 10.5	85.32 ± 2.90	2.5	-
A10	103.67 ± 10.97	89.29 ±3.06	3	-
A11	101.67 ± 6.35	83.76 ± 0.98	4	-
A12	22.00 ± 2.65	83.33 ± 2.10	2	-
A13	35.67 ± 5.69	83.34 ± 1.05	2	-
A14	54.25 ± 4.27	85.65 ± 3.04	2.5	-
A15	43.00 ± 1.00	84.26 ± 1.08	2.5	-
A16	43.00 ± 1.73	82.56 ± 1.04	2.5	-
A17	46.33 ± 2.52	82.78 ± 2.11	3.5	-

Table 17 (continued)

Formulation no.	Hardness[a] (N)	Survival of LAB[b] (%) in 0.04 N HCl for 2 h	Disintegration (h)	Note
A18	4.33 ± 1.15	64.50 ± 0.54	0.5	-
A19	16.25 ± 0.96	68.18 ± 1.88	1	-
A20	19.00 ± 1.00	72.73 ± 2.45	1.25	-
A21	17.00	86.38 ± 1.33	1.5	-
A22	14.67 ± 0.58	87.27 ± 1.24	2	-
A23	15.00	-	-	Disintegrated in 0.04 N HCl
A24	4.50 ± 0.34	-	-	Disintegrated in 0.04 N HCl
A25	4.3 0 ± 0.20	-	-	Disintegrated in 0.04 N HCl
A26	5.00 ± 0.30	-	-	Disintegrated in 0.04 N HCl

Table 17 (continued)

Formulation no.	Hardness[a] (N)	Survival of LAB[b] (%) in 0.04 N HCl for 2 h	Disintegration (h)	Note
A27	4.88 ± 1.25	-	-	Disintegrated in 0.04 N HCl
A28	4.33 ± 0.58	48.12 ± 0.99	0.25	-
A29	57.00 ± 1.20	89.77 ± 1.24	>5 h	-
A30	60.40 ± 2.46	89.22 ± 2.09	>5 h	-
A31	63.16 ± 3.55	90.19 ± 2.21	>5 h	-
A32	25.00 ± 1.20	59.56 ± 1.22	>5 h	-
A33	26.80 ± 0.50	65.12 ± 0.89	>5	-
A34	32.00 ± 6.4	70.18 ± 1.45	>5	-
A35	45.00 ± 2.45	40.58 ± 3.00	1	-

Table 17 (continued)

Formulation no.	Hardness[a] (N)	Survival of LAB[b] (%) in 0.04 N HCl for 2 h	Disintegration (h)	Note
A36	48.00 ± 1.50	45.22 ± 2.05	1	-
A37	53.50 ± 1.84	48.93 ± 0.98	1	-
A38	28.50 ± 1.20	20.88 ± 1.22	0.5	-
A39	26.40 ± 5.10	40.62 ± 0.54	0.5	-
A40	30.40 ± 2.45	56.08 ± 0.88	0.5	-
A41	72.80 ± 4.20	50.89 ± 1.06	1	-
A42	78.00 ± 3.50	54.65 ± 1.02	1	-
A43	82.14 ± 2.05	60.21 ± 0.89	1	-
A44	22.50 ± 3.40	-	-	Disintegrated in 0.04 N HCl

Table 17 (continued)

Formulation no.	Hardness[a] (N)	Survival of LAB[b] (%) in 0.04 N HCl for 2 h	Disintegration (h)	Note
A45	25.84 ± 4.25	-	-	Disintegrated in 0.04 N HCl
A46	32.56 ± 2.22	-	-	Disintegrated in 0.04 N HCl

[a]mean ± standard deviation., n=5; [b]mean ± standard deviation., n=3

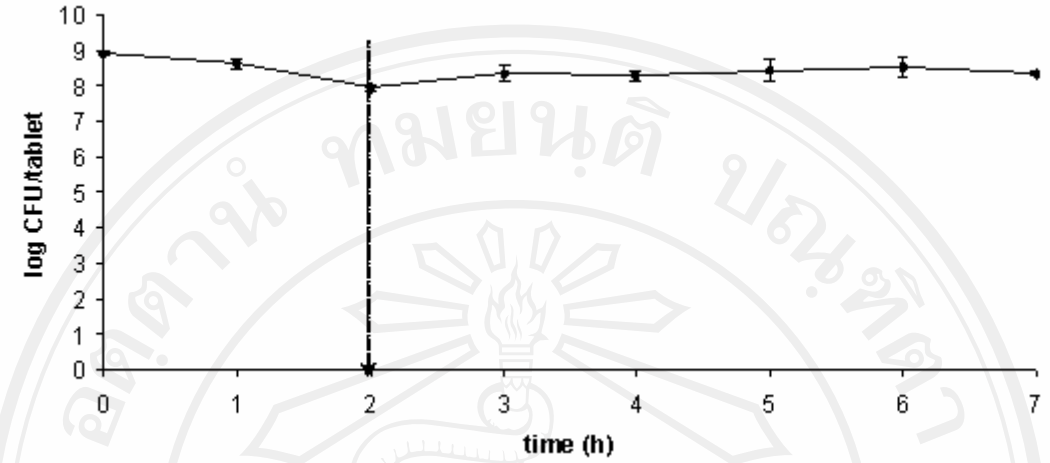


Figure 17 Survival of *L. fermentum* FTL 2311 in formulation containing HPMCP 55 and sodium alginate with tablet weight of 171 mg under gastric condition (0-2 h) in 0.04N HCl and followed by intestinal fluid (2-7 h) at pH 6.8.

4.13.3 Effect of inulin and banana powder

Relatively, new functional food ingredients are oligosaccharides which provide useful modifcations to food flavour and physicochemical characteristics (Crittenden and Playne, 1996). This is based on the ability of the probiotic bacteria to utilize fructooligosaccharides as a selective carbon and energy source while potentially harmful residents of the colon can not (Tomomatsu, 1994). Subsequently, oligosaccharides have been termed prebiotics and foods containing the combination of both probiotics and prebiotics are often referred to as synbiotics. The aim of the present study was to investigate the possibilities to add inulin and banana powder into probiotic tablet as synbiotic products.

The survival of LAB after incubation in 0.04 N HCl was showed in **Table 18** and **Table 19**. Compared to resistant to acidic condition of *L. acidophilus* 72-4 tablets

, *L. fermentum* FTL 2311 tablet formulations were selected for further formulation studies. It was found that LAB tested strains in five formulations showed different resistance to acidic conditions. Survival of LAB increased when tablet weight was increased. Lactose in formulations was used as diluent. The survival rate of FTL 2311 in tablets comprised of Kluai Hom (B) and Kluai Namwa with and without lactose were not different (**Table 18**). The addition of Avicel in formulation decreased cell viability of FTL 2311 during pass trough acid condition.

4.13.4 Tablets containing HPMCP 55, banana powder, and sodium alginate

The probiotic tablets containing HPMCP 55, banana powder and sodium alginate were formulate to combine the effect of banana powder as prebiotic substance and swelling agent as well as enteric polymer in each formulation. The aim of this formulated experiment was to find the symbiotic tablet with the high survival rate in gastric condition. In this study, we changed to fix the content of HPMCP 55 of 5 % according to manufacture manual. This amount of HPMCP 55 was recommend for enteric coating. Dibasic calcium phosphate ($CaHPO_4$) and lactose were used as diluent. It was found that most of tablet formulated with HPMCP 55, $CaHPO_4$, and Avicel (formulaion no. C1-C4) were disintegrated in acid medium. It same as formulation no. C7 - C10 that some part of tablets were broken in 0.04 N HCl. From the data in **Table 20**, the formulation no. C18 which comprised of, showed the highest survival of *L.fermentum* FTL2311 under the acidic condition of 0.04 N HCl for 2 hours. Furthermore, the effect of the tablet weight could be observed that the survival of LAB increases with increasing amounts of tablet weight.

Table 18 The hardness, survival of *L.acidophilus* 72-4 and *L. fermentum* FTL 2311 and disintegration of formulation tablets containing inulin and banana powder

Formulation no.	Hardness[a] (N)	Survival of LAB[b] in 0.04 N HCl		Disintegration (h)	Note
		1 h	2 h		
72-4 I	119.5 ± 3.50	75.97 ± 1.32	65.13 ± 0.94	1	-
72-4 B1	27.75 ± 5.68	72.99 ± 2.44	55.93 ± 1.04	>5	1 h in disintegration tester
72-4 N1	31.00 ± 3.37	75.00 ± 0.94	70.45 ± 1.52	>5	1 h in disintegration tester
2311 I	32.44 ± 4.20	84.23 ± 1.12	72.78 ± 2.78	2	-
2311B1	25.34 ± 1.60	95.26 ± 2.45	84.25 ± 2.32	5	-
2311N1	54.26 ± 2.60	94.67 ± 1.26	83.31 ± 1.15	5	-

[a]mean ± standard deviation., n=5; [b]mean ± standard deviation., n=3

Table 19 The hardness, survival of *L.fermentum* FTL 2311 and disintegration of formulation tablets containing inulin and banana powder

Formulation no.	Hardness[a] (N)	Survival of LAB[b] (%) in 0.04 N HCl		Disintegration (h)	Note
		1 h	2 h		
2311 I	32.44 ± 4.20	84.23 ± 1.12	72.78 ± 2.78	2	-
2311B1	25.34 ± 1.60	95.26 ± 2.45	84.25 ± 2.32	5	-
2311B2	14.25 ± 0.50	72.30 ± 0.88	63.84 ± 1.56	2	-
2311B3	23.75 ± 2.22	92.20 ± 1.96	81.40 ± 2.84	5	-
2311B4	20.25 ± 0.96	74.99 ± 0.44	58.03 ± 4.56	1	-
2311B5	31.0 ± 3.36	89.56 ± 1.95	70.43 ± 2.67	4	-
2311B6	32.02 ± 4.04	93.47 ± 1.22	81.92 ± 2.89	5	-
2311B7	18.25 ± 3.20	84.06 ± 1.56	40.83 ± 1.34	2	-
2311B8	43.50 ± 8.35	90.71 ± 0.83	79.29 ± 3.12	4	-
2311B9	47.25 ± 1.71	97.16 ± 1.25	86.35 ± 1.12	4	-

Table 19 (continued)

Formulation no.	Hardness[a] (N)	Survival of LAB[b] (%) in 0.04 N HCl		Disintegration (h)	Note
		1 h	2 h		
2311N1	54.26 ± 2.60	94.67 ± 1.26	83.31 ± 1.15	5	-
2311N2	23.25 ± 1.70	74.85 ± 0.67	-	-	Disintegrated in 0.04 N HCl
2311N3	35.25 ± 5.10	85.40 ± 2.48	50.85 ± 2.42	5	-
2311N4	121.75 ± 6.65	75.74 ± 1.22	-	-	Disintegrated in 0.04 N HCl
2311N5	156.5 ± 9.60	85.65 ± 1.87	71.80 ± 0.59	4	-
2311N6	53.50 ± 3.11	88.93 ± 2.09	78.35 ± 2.44	5	-
2311N7	50.75 ± 2.22	79.22 ± 0.46	-	-	Disintegrated in 0.04 N HCl
2311N8	54.25 ± 3.77	88.42 ± 2.11	82.73 ± 1.02	4	-
2311N9	87.75 ± 2.87	90.15 ± 0.23	86.53 ±	4	-

[a]mean ± standard deviation., n=5; [b]mean ± standard deviation., n=3

Table 20 The hardness, survival of *L.fermentum* FTL 2311 and disintegration of tablets containing HPMCP, banana, and sodium alginate.

Formulation no.	Hardness[a] (N)	Survival of LAB[b] (%) in 0.04 N HCl for 2 h	Disintegration (h)	Note
C1	20.05 ± 3.10	-	-	Disintegrated in 0.04 N HCl
C2	35.00 ± 5.4	-	-	Disintegrated in 0.04 N HCl
C3	25.18 ± 2.90	-	-	Disintegrated in 0.04 N HCl
C4	30.00	-	-	Disintegrated in 0.04 N HCl
C5	35.00 ± 1.2	50.89 ± 1.21	4	-
C6	54.00 ± 3.21	62.34 ± 0.83	4	-
C7	35.00± 4.00	62.84 ± 0.95	0.25	-
C8	42.80 ± 2.50	74.33 ± 1.29	0.25	-
C9	25.08 ± 3.00	50.16 ± 0.74	0.25	-
C10	31.50 ± 4.20	61.98 ± 0.76	0.25	-

Table 20 (continued)

Formulation no.	Hardness[a] (N)	Survival of LAB[b] (%) in 0.04 N HCl for 2 h	Disintegration (h)	Note
C11	71.65 ± 1.20	68.14 ± 1.63	4	-
C12	86.80± 2.10	80.25 ± 2.44	>5	-
C13	52.00 ± 4.50	71.11 ± 1.63	3	-
C14	68.00 ± 1.00	82.67 ± 0.46	3.5	-
C15	53.48 ± 2.52	60.20 ± 0.95	3.5	-
C16	68.12 ± 4.60	72.84 ± 0.97	3.5	-
C17	51.50 ± 0.50	84.11 ± 1.95	>5	-
C18	82.80 ± 1.50	94.55 ± 1.02	>5	-

[a]mean ± standard deviation., n=5; [b]mean ± standard deviation., n=3

4.14 Stability of probiotic tablets

Concerning the storage stability, it was found that storage at 30 °C caused gradually decrease in cell viability. After six months, the loss of viability was observed to be less than 1 log unit. Importantly, however, no decrease in viability was observed during storage of the tablets at 10 °C for six months. It has been reported that the storage stability of freeze-dried *Lactococcus lactis* encapsulated in a calcium alginate matrix was higher than that of the freeze-dried free cells. Chan and Zhang (2002) developed tablets with a core consisting of *L. acidophilus* ATCC 4356 cells surrounded with sodium alginate and claimed that they had 10 times higher stability than the free cell powders after 30 days storage at 25 °C. The results in our study indicated the effect of temperature on viability of the lyophilized probiotic cells distributed homogeneously inside the tablets. The 20 °C difference (between 30 °C to 10 °C) affected the cell stability of about 1 log CFU. The stability data of the probiotic tablet containing HPMCP 55 and sodium alginate with tablet weight of 171 mg were shown in **Figure 1**8.

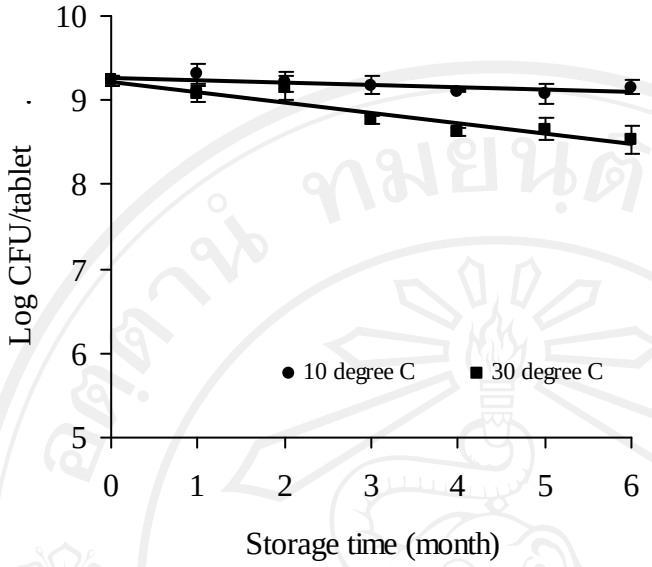


Figure 18 Stability of probiotic tablet containing HPMCP 55 and sodium alginate with 171 mg of tablet weight**.**

Stability at 10 °C of seven selected formulation which showed high survival rate in gastric condition were also investigated. Data of stability were shown in Figure . Most of selected formulation was decreased in number of bacteria about 1 log cycle after 6 month storage.

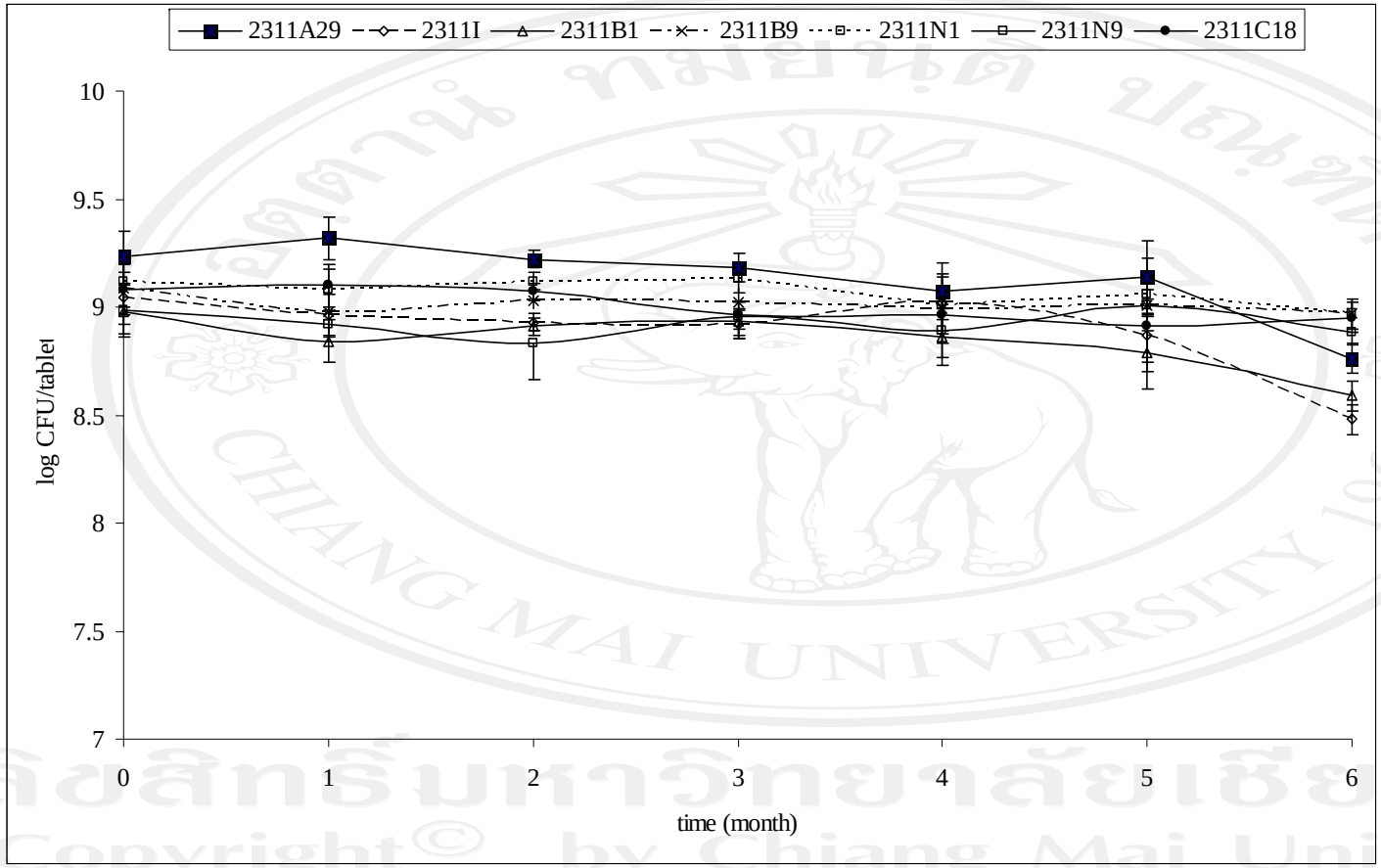


Figure 19 Stability of selected formulations of *L.fermentum* FTL 2311; The log CFU/tablet were shown as mean ± standard deviation (n=3)