

## CHAPTER 4

### RESULTS AND DISCUSSION

#### 4.1 Isolation lactic acid bacteria

Lactic acid bacteria are responsible for contributing to the flavor and preservation of fermented foods by producing antagonistic substances such as lactic acid, acetic acid, hydrogen peroxide, diacetyl, antibiotic and bacteriocin (Stile and Hastings, 1991, Ray 1992). LAB are commonly isolated from meats and fermented meat products (Berry *et al*, 1990; Schillinger *et al*, 1987, 1989). A total of 50 different fermented meat products sample were analyses (Appendix B), 66 isolates that were found to observe as presumptive lactic acid bacteria. Those isolates showed clear zone around the paper disc after tested with MRS agar plus CaCO<sub>3</sub>. If is it not LAB it not show clear zone in plate because they can not produce acid to digest CaCO<sub>3</sub>.

Many studies have screened LAB isolates from fermented meat; for exsample, in fresh meat (Nielsen *et al*, 1990; Winkowski *et al*, 1993), minced meat (Skytta *et al*, 1991; Schillinger *et al*, 1991), German style fermented sausages (Schillinger *et al*, 1991), Amerian-style fermented sausages (Berry *et al*, 1990; Foegeding *et al*, 1992), Italian salami (Campanini *et al*, 1993), frankfurter sausages (Berry *et al*, 1991), vacuum-packaged wieners (Degnan *et al*, 1992) and Spanish- style fermented sauges (Hugas *et al*, 1995), hoi drong, plaa som, som fak (Phithakpol *et al*, 1995; Østergard *et al*, 1998), Nham (Noonpakdee *et al*, 2002; Swetwivatthana *et al*, 1999) and fermented sausages (Cocolin *et al*, 2001). These organisms can inhibit the natural microflora of meat, which include spoilage bacteria and, if present, pathogens such as *L. monocytogenes* and *S. aureus* (Bacus, 1994) also reported similar results for against *L. monocytogenes*, two strains of *Aeromonas hydrophila* and two strains of *S. aureus* (Lewus *et al*, 1991). Pathogenic and food spoilage microorganism such as

*Salmonella*, *E. coli*, *B. cereus* and *S. aureus* are problem in various food and cause diseases in human, other animal and also in plants. Such as *S.aureus* capable of producing a large number of extracellular enzyme and toxin such as enterotoxin it highly stable to heat, so it is problem canned food. In the case of *B. cereus* it capable to produced a wide variety of extracellular toxic, enzyme and *B. cereus* can cause two distinct type of illness. A diarrhea syndrome and emetic syndrome (Reinheimer *et al*, 1988, El- Gazzar and Marth, 1992, Vignolo *et al*, 1993, Chiang *et al*, 2000). So this thesis were use as 4 indicator microorganisms in experiment.

#### 4.2 Screening of antimicrobial substance producing LAB

Sixty-six isolates of presumptive LAB that were isolate from 50 samples of fermented meat products were used for screening of antimicrobial substance producing LAB base on paper disc diffusion method. Thirty eight of 66 isolates were examined as LAB that produced potential inhibitory substance against indicator microorganisms, *E. coli* TISTR73, *B. cereus*, *S. aureus* and *S. enteritidis*. These 38 isolates were selected as antimicrobial substance producing LAB because they showed the clear inhibition zones around the paper disc on test medium. The results shown that 20 isolates were inhibited all of four indicator microorganisms as show in number 1 to 20 of Table 4.1, while other 13 isolates and 5 isolates inhibited only 3 and 2 of indicator microorganisms as show in Table 4.2 and 4.3, respectively.

Table 4.1 showed 4 isolates on the inhibition of four indicator microorganisms higher than other isolates on *E. coli* TISTR 73, *S. enteritidis*, *S. aureus* and *B. cereus*. Isolates FA6-01/3 and FS7-01/8 able to inhibit *E. coli* TISTR73 by shown clear zone size 9.5 mm. and one isolate FE7-01/9 inhibited *S. enteritidis* by show clear zone size 11 mm. Isolate of FE7-01/1 could inhibit the growth of *B. cereus* by show clear zone size 11 mm. and FE7-01/1, FE7-01/9 capable to inhibited *S. aureus* by shown clear zone size 11 mm., respectively.

The lactic acid bacteria which can inhibit of 3 indicator microorganisms are presented in Table 4.2. Antimicrobial substance produce by isolates FF4-01/1 and

FP5-01/2 showed inhibition against *B. cereus* and *S. aureus* with showed clear zone size 9.1 and 10 mm, respectively. Isolate FE6-01/2 showed inhibitory spectrum against indicator microorganisms, *E. coli* TISTR 73, by showed clear zone size 10.5 mm, while isolate FE6-01/5 and FF4-01/1 inhibit *S. enteritidis* by showed clear zone size 10 mm.

**Table 4.1** Inhibition of four indicator microorganisms by antimicrobial substance producing lactic acid bacteria compare with clear zone on MRS agar plus CaCO<sub>3</sub>

Number	Isolates	Clear zone size (mm.)				
		<i>E. coli</i>	<i>S. enteritidis</i>	<i>B. cereus</i>	<i>S. aureus</i>	CaCO <sub>3</sub>
1	FS7-01/8	9.5	8.0	9.0	6.0	7.0
2	FA6-01/3	9.5	7.2	9.1	8.0	7.0
3	FE7-01/9	7.0	11	6.0	11	7.0
4	FE7-01/1	7.0	10	11	11	7.0
5	FF3-01/3	9.3	9.0	9.0	8.6	7.0
6	FP5-01/6	9.3	10	10.3	9.0	5.0
7	FP5-01/4	9.0	10	10.3	9.0	5.0
8	FA6-01/1	8.0	7.0	9.5	7.7	6.3
9	FF2-02/3	7.0	7.1	7.5	7.2	6.3
10	FF3-01/4	9.0	8.0	9.2	7.5	8.0
11	FF3-01/10	8.5	7.8	9.0	7.5	7.0
12	FF4-01/8	7.5	7.5	9.0	9.0	9.0
13	FE6-01/1	9.0	7.6	9.5	7.7	7.0
14	FF6-01/4	9.4	7.3	7.3	10	7.0
15	FF6-01/1	8.7	6.3	8.3	7.5	7.5
16	FE7-01/2	7.0	7.0	9.0	9.0	5.0
17	FE7-01/4	7.0	8.0	8.0	7.0	5.5
18	FE7-01/7	7.0	9.0	9.0	8.0	7.0
19	FS7-01/3	5.0	7.0	5.0	6.0	6.3
20	FS7-01/6	9.4	8.0	10	8.0	7.0

**Table 4.2** Inhibition of three indicator microorganisms by antimicrobial substance producing lactic acid bacteria compare with clear zone on MRS agar plus CaCO<sub>3</sub>

Number	Isolates	Clear zone size (mm.)				
		<i>E. coli</i>	<i>S. enteritidis</i>	<i>B. cereus</i>	<i>S. aureus</i>	CaCO <sub>3</sub>
1	FE6-01/2	<b>10.5</b>	9.6	8.7	-	7.0
2	FF4-01/1	8.0	<b>10</b>	<b>9.1</b>	-	7.0
3	FE6-01/5	8.7	<b>10</b>	-	5.0	8.7
4	FP5-01/2	8.7	-	8.7	<b>10</b>	5.0
5	FP5-01/3	8.5	7.0	9.0	-	7.0
6	FF4-01/5	8.5	-	8.8	8.5	8.0
7	FP5-01/5	7.6	8.2	8.3	-	6.0
8	FF5-01/2	8.0	7.0	7.2	-	-
9	FF2-01/8	6.7	6.5	7.2	-	7.0
10	FF3-01/5	6.5	-	7.3	7.0	7.0
11	FA6-01/4	7.8	8.5	8.5	-	8.0
12	FB5-01/2	-	6.9	7.0	7.0	6.5
13	FF3-01/6	-	7.0	8.0	7.0	6.0

-, no inhibited

The antimicrobial substance producing LAB which able to inhibited two indicator microorganisms show in Table 4.3. Three isolates, FA6-01/2, FB5-01/1 and FE6-01/3, showed inhibitory clear zone with *E. coli* TISTR 73, *S. enteritidis* and *B. cereus* by size 8.7, 8.8 and 8.0 mm, respectively. Chen *et al.* (2006) used 1% CaCO<sub>3</sub> add to the MRS agar plate to distinguish acid producing bacteria from other bacteria. While, Ketkaew *et al.* (2005) used 0.5% add to MRS-BPC agar plate. However, some of isolate have clear zone on CaCO<sub>3</sub>, higher than the clear zone detect on indicator microorganisms plate. This result it may be is effect of other antimicrobial such as organic acid or hydrogen peroxide. Conner *et al.* (1990) report that tryptic soy broth with yeast extract (TSBY) acidified to pH 4.5 with 0.4% lactic acid inhibited *L. monocytogenes*. In ATP the hexose content is sufficient for LAB to

produce lactic acid at a theoretical maximum concentrate of 1% and an average pH value of 4.2. Base on that principle we applied it to select the strains which could produced other antimicrobial substances except organic acid by compared the clear zone size on the MRS agar plus indicator microorganisms with those on the MRS agar plus CaCO<sub>3</sub>.

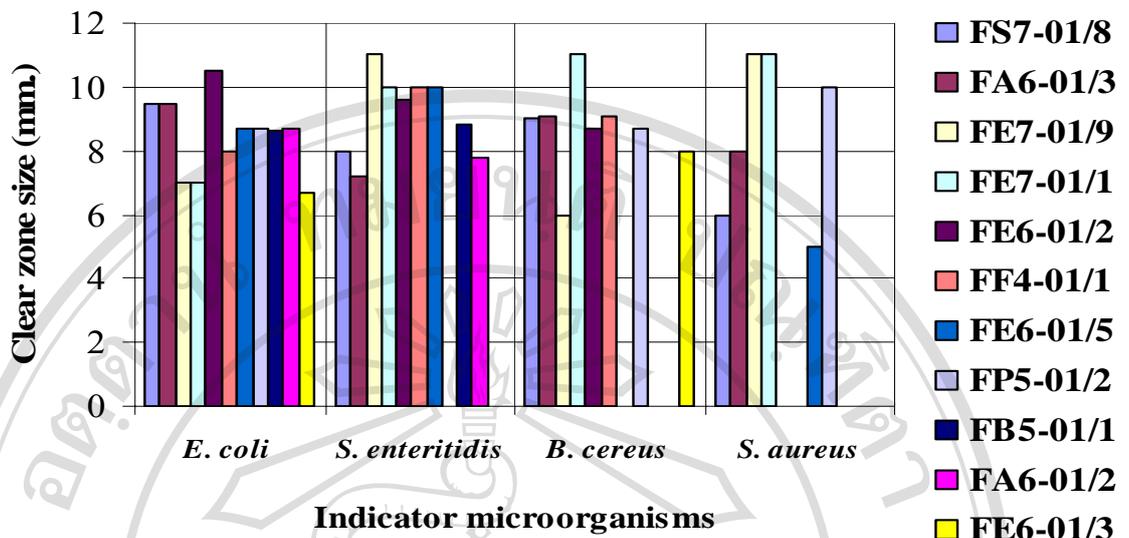
**Table 4.3** Inhibition of two indicator microorganisms by antimicrobial substance producing lactic acid bacteria compare with clear zone on MRS agar plus CaCO<sub>3</sub>

Number	Isolates	Clear zone size (mm.)				
		<i>E. coli</i>	<i>S. enteritidis</i>	<i>B. cereus</i>	<i>S. aureus</i>	CaCO <sub>3</sub>
1	FB5-01/1	8.6	<b>8.8</b>	-	-	5.5
2	FA6-01/2	<b>8.7</b>	7.8	-	-	7.5
3	FE6-01/3	6.7	-	<b>8.0</b>	-	6.0
4	FF6-01/2	8.2	-	-	-	5.0
5	FE6-01/4	-	-	7.8	-	6.0

-, no inhibited

It was found that clear zone of those 38 isolates on MRS agar plus indicator microorganisms are bigger than the clear zone in MRS agar plus CaCO<sub>3</sub> that mean it should have other of antimicrobial substances produce by LAB not only acid alone. However, some of isolate have clear zone on CaCO<sub>3</sub>, higher than the clear zone detect on indicator microorganisms plate. This result it may be is effect of organic acid.

In conclusion, 11 LAB isolates from Table 4.1, 4.2 and 4.3, capable of good inhibit indicator microorganism. The results obtained are shown in figure 4.1. The isolate FE6-01/2 able to inhibit *E. coli* TISTR73 higher than other isolates, while isolates FE7-01/9 and FE7-01/1 showed inhibition against *S. enteritidis* and *B. cereus*, respectively and also both of isolates able to inhibit *S. aureus* by showed clear zone size 11 mm.



**Figure 4.1** The ability of eleven isolate on the Inhibition of indicator microorganisms by antimicrobial substance producing lactic acid bacteria

#### 4.3 Selection of antimicrobial substance producing LAB

After screening of antimicrobial, 38 isolates as positive isolates, the concentrated supernatant of those isolates were checked by paper disc diffusion assay. To find out other antimicrobial substance in the supernatant of culture broth, we designed to separate the concentrated supernatant in to two parts, non-adjusted pH and adjusted to pH 7 with 3 N NaOH, for eliminate the effect of acid before apply to paper disc. It was found that the non-adjust pH concentrated from 38 isolates are able to inhibit all of indicator microorganisms by showed the clear zone appear on MRS agar plate plus with each indicator microorganism, *E. coli* TISTR73, *S. enteritidis*, *B. cereus* and *S. aureus*. But the concentrated adjust to pH 7.0 did not showed the clear zone on MRS agar plus indicator microorganism. So we predicted that the antimicrobial substance that produce by those 38 isolates may need low pH condition for working as the previous papers that the bacteriocin was active over a wide range between 2 and 10 (Noonpakdee *et al*, 2002; Oh *et al*, 2000). Millette (2006) Report that *P. acidilactici* MM33, are surviving and remained antimicrobial activity at pH 4.0 and 5.0, but showed decreased activity at pH 3 and below, and pH 6. Michael *et al*. (1988) the result presented activity of a bacteriocin produce by *Pediococcus*

*acidilactici* PAC 1.0 strains could grow at pH 5.5 in ATP broth. Thus, we need other method to determine the type of antimicrobial substance of these 38 isolates.

#### 4.4 Determination of antimicrobial substance

Over the past few years, the study concerning bacteriocin produced by LAB have received an increasing because of the potential use of bacteriocins as food preservative (Cleveland *et al.*, 2001; O'Sullivan *et al.*, 2002). De Martinis *et al.* (2002) screened twenty samples of Brazilian meat and meat products and isolated four bacteriocin-producing LAB that presented antilisterial activity. Bromberg *et al.* (2004) isolated 285 samples of meat and meat products were evaluated for the presence of bacteriocin-producing LAB that inhibit the growth of spoilage bacteria and food borne pathogens. During the past several years, the isolation of bacteriocinogenic strains from fermented meat has been frequently reported.

Most studies of anti-bacterial LAB have tested pure cultures in well-or disc-diffusion assays (Jeppesen and Huss, 1993a) or by overlay on replica plates (Vaughan *et al.*, 1994). Bromberg *et al.* (2004) checked for bacteriocin production using the well-diffusion assay. Similar to report Ø stergaard *et al.* (1998) that screened LAB capable of inhibiting *Listeria* sp. using well diffusion assay. However, the results of experimented depend on each method for detect antimicrobial. In this study, the results of the paper disc diffusion assay showed that 38 isolates produce antimicrobial substances. All 38 isolates were chosen for determination of antimicrobial substance study. Agar well-diffusion techniques were used to determination the substance from those isolates. The results of well-diffusion assay showed that the concentrated supernatant from 24 isolates were depressed with 0.5 g/ml (w/v)  $\beta$ -glycerophosphate, while 14 isolates were not depressed with 0.5 g/ml (w/v)  $\beta$ -glycerophosphate and showed the clear zone around the well. That means the potential to inhibit the indicator microorganisms of those 24 isolates are the result of lactic acid. Thus, it was possible that the others 14 isolates are bacteriocins producing microorganism as shown in Table 4.4. Similar to previous reports, Michael *et al.* (1988) use 2% sodium  $\beta$ -glycerophosphate for determination of bacteriocin by add 2% sodium

$\beta$ -glycerophosphate in ATP agar to rule out acidic inhibition. According to method of Bromberg *et al.* (2004) to use the same method in MRS medium for isolation of bacteriocin producing LAB from meat products. In this study sodium  $\beta$ -glycerophosphate used for inhibition due to pH reduction caused by organic acid production. After to 0.5 g/ml (w/v)  $\beta$ -glycerophosphate added to the 10 fold - concentrated of supernatants it was observed that some of 14 isolated still appear ability to inhibit indicator microorganisms. Thus, the antimicrobial substances which produce by these 14 isolated are not acid.

**Table 4.4** Elimination of acids effect using  $\beta$ -glycerophosphate

Isolates	Clear zone size (mm.)							
	<i>E. coli</i>		<i>S. aureus</i>		<i>B.cereus</i>		<i>S. enteritidis</i>	
	S + H <sub>2</sub> O	S+ $\beta$	S + H <sub>2</sub> O	S+ $\beta$	S + H <sub>2</sub> O	S+ $\beta$	S + H <sub>2</sub> O	S+ $\beta$
FF2-02/3	8.0	5.0	9.8	7.7	8.0	5.3	9.5	9.3
FF3-01/3	7.3	4.3	9.8	6.7	6.3	4.7	8.3	7.5
FF3-01/4	7.8	5.0	9.3	6.0	6.5	4.3	8.0	7.5
FF3-01/5	7.5	4.5	9.3	6.0	9.0	4.3	9.5	8.5
FF3-01/10	8.3	5.0	9.0	6.0	9.0	4.3	10.3	6.7
FF4-01/1	9.3	5.3	8.5	5.7	8.5	4.7	9.8	7.3
FF4-01/5	8.8	6.0	8.5	7.3	11.3	7.7	7.5	6.7
FF4-01/8	7.5	4.0	7.8	6.3	11	5.0	8.0	5.0
FP5-01/4	7.0	5.7	10	8.5	11	4.3	7.7	4.7
FA6-01/2	7.3	4.5	9.0	6.5	8.5	4.8	7.5	7.0
FE6-01/2	7.3	5.3	9.0	5.7	9.0	5.3	9.3	6.0
FE6-01/5	7.0	5.5	8.7	5.7	9.5	5.8	9.5	7.3
FF6-01/1	7.3	5.0	8.3	8.0	9.8	5.0	8.8	5.0
FF6-01/4	8.0	4.5	8.0	8.0	10	4.8	7.8	4.5

S+H<sub>2</sub>O; Sample+H<sub>2</sub>O, S+ $\beta$ ; Sample+  $\beta$ -glycerophosphate

#### 4.5 Determination of bacteriocin producing LAB

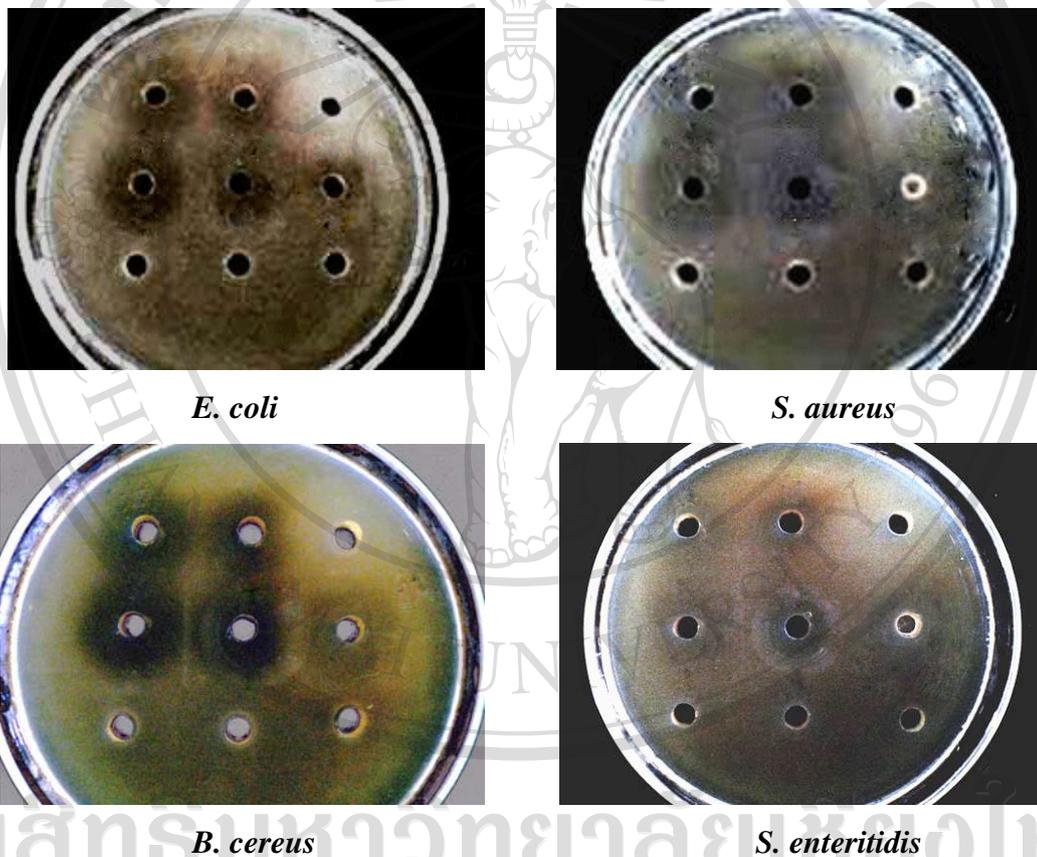
Then proteinase K and  $\beta$ -glycerophosphate were added to the reaction. It was found that 3 samples from isolate FF2-02/3, FF4-01/5 and FP5-01/4 of 14 isolates loss the ability to inhibit against indicator microorganisms because the bacteriocin which produced by these three LAB was degraded by proteinase K and the effect of lactic acid was omitted  $\beta$ -glycerophosphate (Table 4.5).

**Table 4.5** Screening of bacteriocin from antimicrobial substances producing LAB

Isolates	Clear zone on MRS agar plus indicator strain											
	<i>E. coli</i>			<i>S. aureus</i>			<i>B. cereus</i>			<i>S. enteritidis</i>		
	S+H <sub>2</sub> O	S+ $\beta$	S+ $\beta$ +Pro K	S+H <sub>2</sub> O	S+ $\beta$	S+ $\beta$ +Pro K	S+H <sub>2</sub> O	S+ $\beta$	S+ $\beta$ +Pro K	S+H <sub>2</sub> O	S+ $\beta$	S+ $\beta$ +Pro K
FF2-02/3	+	+	-	+	+	-	+	+	-	+	+	-
FF4-01/5	+	+	-	+	+	-	+	+	-	+	+	+
FP5-01/4	+	+	-	+	+	-	+	+	+	+	+	-
FF3-01/3	+	+	+	+	+	+	+	+	+	+	+	+
FF3-01/4	+	+	+	+	+	+	+	+	+	+	+	+
FF3-01/5	+	+	+	+	+	+	+	+	+	+	+	+
FF3-01/10	+	+	+	+	+	+	+	+	+	+	+	+
FF4-01/1	+	+	+	+	+	+	+	+	+	+	+	+
FF4-01/8	+	+	+	+	+	+	+	+	+	+	+	+
FA6-01/2	+	+	+	+	+	+	+	+	+	+	+	+
FE6-01/2	+	+	+	+	+	+	+	+	+	+	+	+
FE6-01/5	+	+	+	+	+	+	+	+	+	+	+	+
FF6-01/1	+	+	+	+	+	+	+	+	+	+	+	+
FF6-01/4	+	+	+	+	+	+	+	+	+	+	+	+

<sup>a</sup> +, positive; -, negative; S+H<sub>2</sub>O; Sample+H<sub>2</sub>O, S+ $\beta$ ; Sample+  $\beta$ -glycerophosphate, S+ $\beta$ +Pro K; Sample+  $\beta$ -glycerophosphate+Proteinase

Similar to Peeva *et al.* (2006) report that the protein nature of the antibacterial agent in the producer strain cultures was demonstrated by treatment of 500  $\mu$ l prepared culture supernatant with 25  $\mu$ l proteinase K solution (20 mg/ml) at 37°C for 2 h. It was found that protein nature of the bacteriocin-like substance loss of inhibitory activity after treatment with proteinase K. Therefore 3 of the 14 isolates are bacteriocin-producing isolates originated from fermented meat products. Isolate FF2-02/3 had inhibitory activity against all four indicator microorganisms (Figure 4.2).

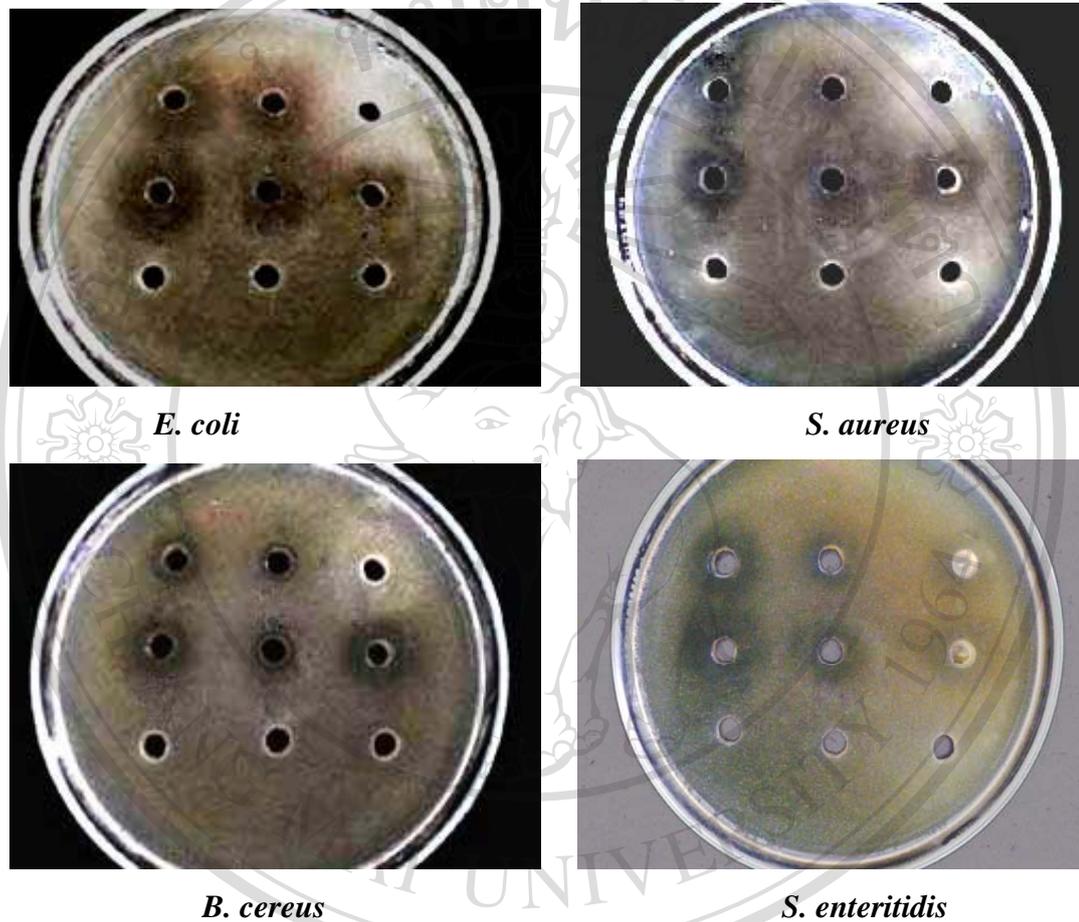


**Figure 4.2** The effect of bacteriocin from FF2-02/3 on the inhibition of indicator microorganisms.

A	B	C
D	E	F
G	H	I

**A,D:** sample + H<sub>2</sub>O; **B,E(boil):** sample +  $\beta$ -glycerophosphate; **C:** sample +  $\beta$ -glycerophosphate + proteinase K; **F:** sample + H<sub>2</sub>O + proteinase K; **G:** H<sub>2</sub>O + proteinase K (control); **H:** H<sub>2</sub>O +  $\beta$ -glycerophosphate (control); **I:** H<sub>2</sub>O +  $\beta$ -glycerophosphate + proteinase K (control).

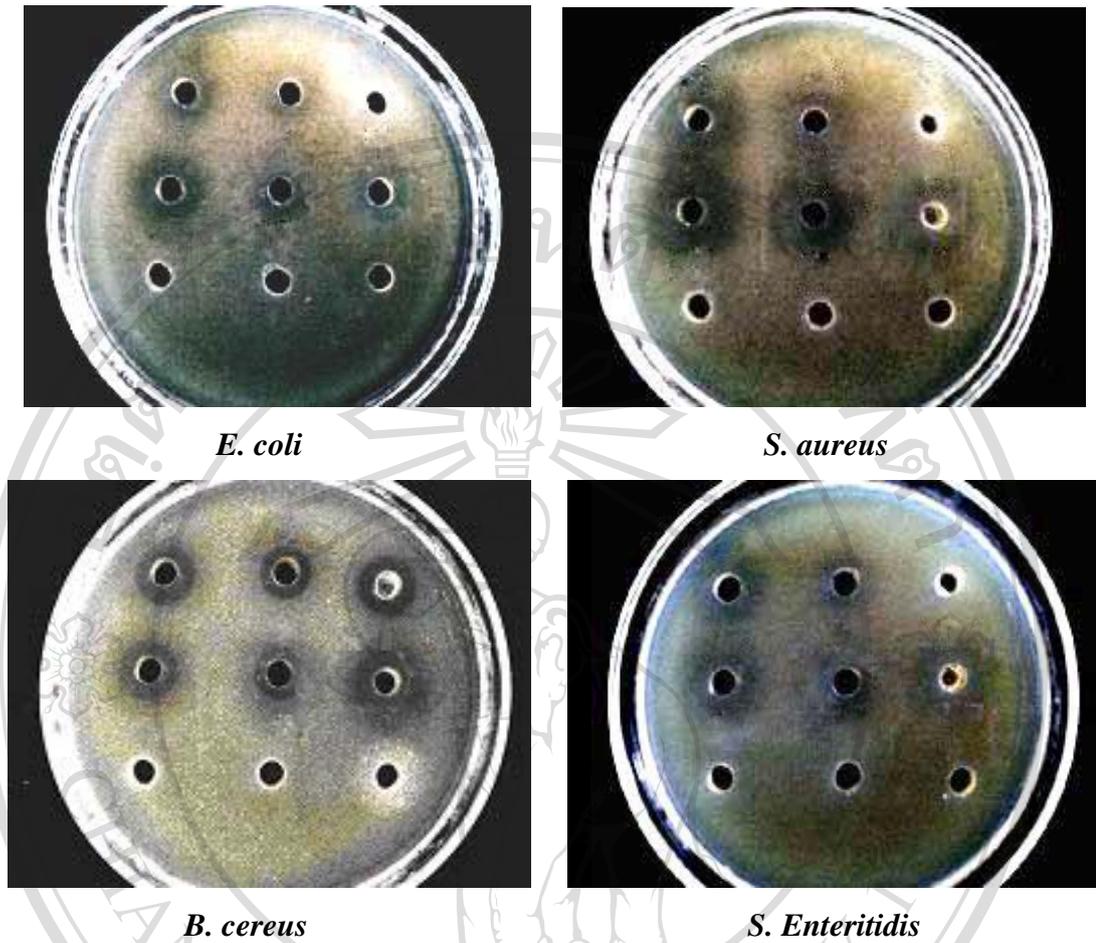
Isolate FF4-01/5 which inhibited *E. coli* TISTR73, *S. aureus* and *B. cereus* (Figure 4.3). While isolate FP5-01/4 inhibited *E. coli* TISTR73, *S. aureus* and *S. enteritidis* (Figure 4.4).



**Figure 4.3** The effect of bacteriocin from FF4-01/5 on the inhibition of indicator microorganisms

A	B	C
D	E	F
G	H	I

**A,D:** sample + H<sub>2</sub>O; **B,E(boil):** sample + β-glycerophosphate; **C:** sample +β-glycerophosphate + proteinase K; **F:** sample + H<sub>2</sub>O + proteinase K; **G:** H<sub>2</sub>O + proteinase K (control); **H:** H<sub>2</sub>O + β-glycerophosphate (control); **I:** H<sub>2</sub>O + β-glycerophosphate + proteinase K (control).



**Figure 4.4** The effect of bacteriocin from FP5-01/4 on the inhibition of indicator microorganisms

A	B	C
D	E	F
G	H	I

**A,D:** sample + H<sub>2</sub>O; **B,E(boil):** sample +  $\beta$ -glycerophosphate; **C:** sample +  $\beta$ -glycerophosphate + proteinase K; **F:** sample + H<sub>2</sub>O + proteinase K; **G:** H<sub>2</sub>O + proteinase K (control); **H:** H<sub>2</sub>O +  $\beta$ -glycerophosphate (control); **I:** H<sub>2</sub>O +  $\beta$ -glycerophosphate + proteinase K (control).

From figure 4.2, the clear zone of isolate FF2-02/3 detects on each plate show the antimicrobial ability on the inhibition of 4 indicator microorganisms. The clear zone not appear when test with sodium  $\beta$ -glycerophosphate and proteinase K (C). While, it was found that clear zone still detected on B, E and F. That mean isolate

FF2-02/3 capable to produce both of bacteriocin and other antimicrobial because after add proteinase K clear zone still appear on plate. Therefore, the clear zone detected on plate as result of other antimicrobial not action of bacteriocin. However, isolate FF2-02/3 still gave activity when test with  $\beta$ -glycerophosphate that mean clear zone appear is not effect of organic acid. Not only isolate FF2-02/3 produce antimicrobial like-bacteriocin but also found that some of antimicrobial were able to inhibit indicator microorganisms.

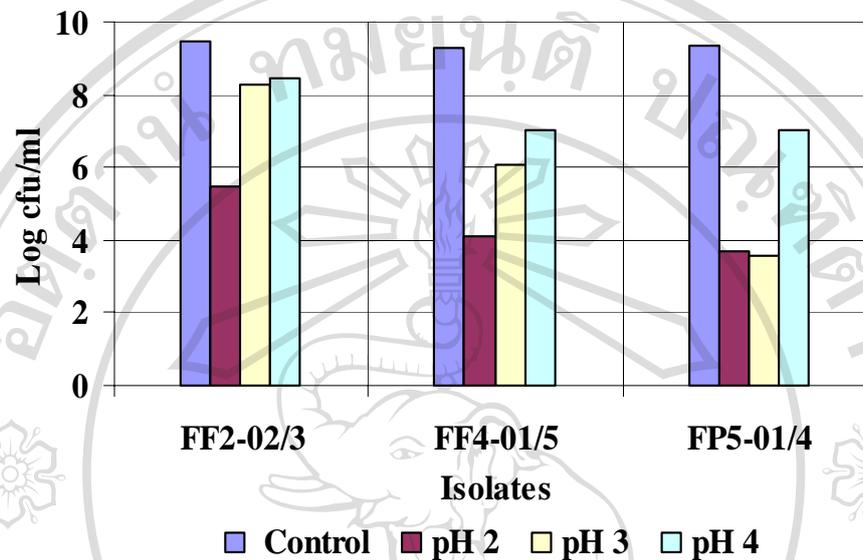
In the case of isolate FF4-01/5 on the inhibition of 3 indicator microorganisms, similar result were obtained by describe above. But this isolate, it was not produce bacteriocin active against *S. enteritidis*. Because clear zone detect on plate of *S. enteritidis* when test with  $\beta$ -glycerophosphate and proteinase K (C). While, isolate FP5-01/4 from Nham inhibit only *E. coli*, *S. aureus* and *S. enteritidis* do not produce bacteriocin inhibit *B. cereus*. In addition, clearing zone were observed in plate when test with  $\beta$ -glycerophosphate and proteinase K.

#### 4.6 Acid tolerance

The three isolates bacteriocin producing LAB that have been isolate from fermented meat products, were tested for acid and bile tolerance. The acid tolerance of LAB is dependent upon the pH profile of  $H^+$ -ATPase and the composition of the cytoplasmic membrane, which is largely influenced by the type of bacteria, type of growth media, and the incubation condition (Havenaar *et al*, 1992; Hood and Zottola, 1998). Acid tolerance is a fundamental property that indicates the ability of probiotic microorganism to survive passage through the stomach (Prasad *et al*, 1998; Park *et al*, 2002).

The result on acid tolerance as survival at various pH levels showed that all of the isolates were tolerance to acid. The isolate FF2-02/3 showed better tolerance to acid than other two isolates. Generally, survival was low at pH 0.5, 1 and 2, moderate at pH 3 and good at pH 4 and 5 (Jin *et al*, 1998). However, the results on acid tolerance showed that most of the isolate FF2-02/3 were tolerance to acid and had a moderate survival rate at pH 2 and good survival rate at pH 3 and 4. The isolate

FF4-01/5 had a moderate survival rate at pH 3, 4 and low survival rate at pH 2, while isolate FP5-01/4 had a moderate survival rate at pH 4 and low survival rate at pH 2 and 3. Survival of these isolates is presented in figure 4.5.

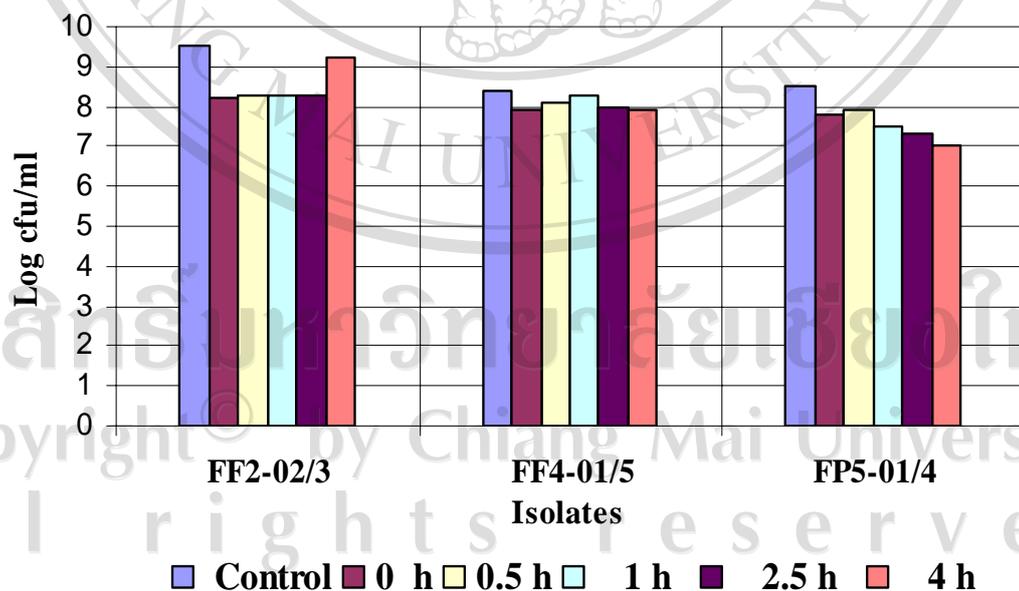


**Figure 4.5** Surviving of three isolates, FF2-02/3, FF4-01/5 and FP5-01/4 at pH 2, 3 and 4

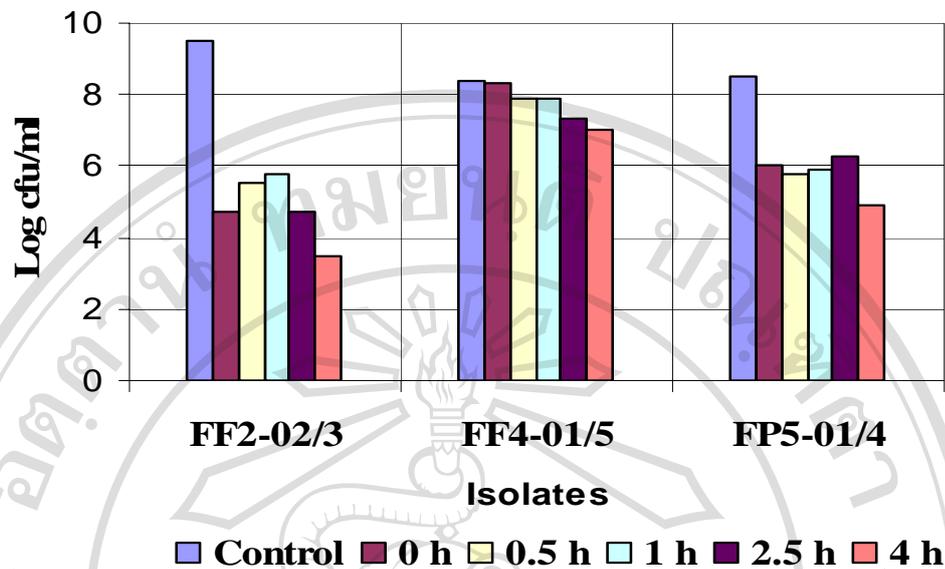
The some of isolate proved to be most acid tolerance. Little was seen at pH 2 with these isolate. This similar to the result obtained by Goldin and Gorbach (1992) with *Lactobacillus* GG which is regarded as a probiotic. Erkkila and Petaja (2000) found one *Lactobacillus curvatus* and two *Pediococcus* spp. From commercial meat starter cultures, were resistant to pH 3. According to Prasad *et al.* (1998) were selection *Lactobacillus*, *Bifidobacterim* spp. and was found two strains were able to survive at low pH 1-3. This experiment was replicated four times, and each viable cell count was performed in MRS plate. Isolate FF2-02/3 was found to be the best acid tolerant than other isolates, with a final population of 5.5, 8.26 and 8.45 log cfu/ml in pH 2, 3 and 4, respectively. The population of LAB of isolate FF4-01/4 show final population of 4.1, 6.06 and 7.0 log cfu/ml in pH 2, 3 and 4 respectively, whereas isolate FP5-01/4 was found to be the least acid tolerant at pH 2 and 3, with a final population of 3.7 and 3.6 log cfu/ml respectively but this isolate showed enhanced growth in pH 4, with a final population of 7.0 cfu/ml.

#### 4.7 Bile salts

Bile tolerance is one of the most essential criteria for a strain to be used as a probiotic culture (Gilliland, 1979). Bile salts are surface active chemicals produce in the liver from the catabolism of cholesterol (Brandt and Bernstein, 1976). In this study the results indicated that at concentration 0.15%, bile salts had no affect the numbers of LAB (figure 4.6). It was also found that at 0.15% bile salt, the number of lactic acid bacteria of each isolate activity still remained after incubated at 37°C for 0.5, 1.0, 2.5 and 4.0 hours. Isolate FF2-02/3 exhibited good bile tolerance. Isolate FF2-02/3 showed enhanced growth in media containing 0.15% bile acids after 4 h of incubation, while isolate FF4-01/5 and FP5-01/4 decreased after 4 h of incubation. However, 0.30% bile salts there were differences in the survival capacity of the tested isolates (Figure 4.7). As show in Figure 4.7, all three strains were found to be the least 0.3% bile tolerant after 4 h of incubation, isolate FF2-02/3 a high population of 5.8 log cfu/ml at 1 hours, whereas the population of isolate FF4-01/5 and FP5-01/4 a high population of 8.3 and 6.3 log cfu/ml at 0 and 2.5 hours, respectively.

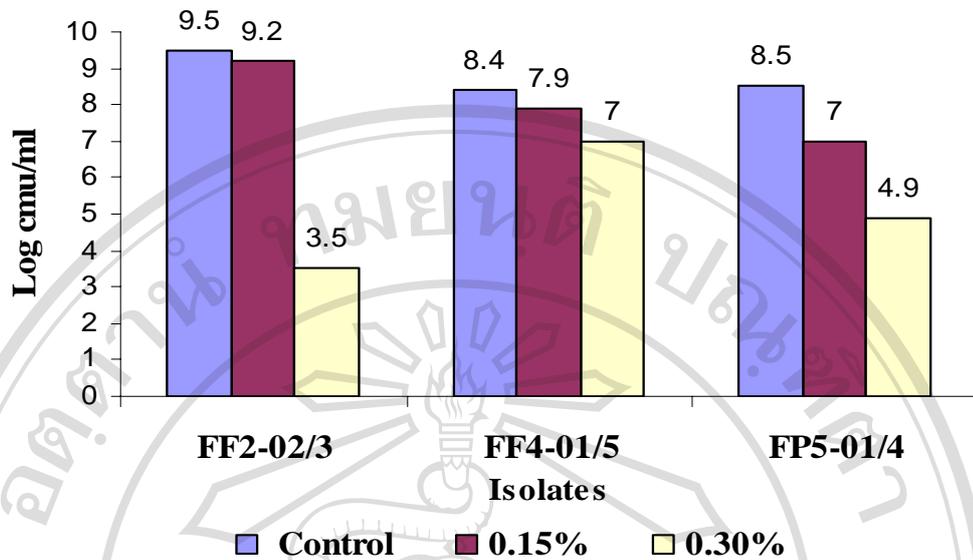


**Figure 4.6** Effect of 0.15% bile salt on survivability of three isolates, FF2-02/3, FF4-01/5 and FP5-01/4



**Figure 4.7** Effect of 0.3% bile salt on survivability of three isolates, FF2-02/3, FF4-01/5 and FP5-01/4

After compare with the results of 0.15 and 0.3% bile of 3 isolates after incubated at 37°C for 4.0 h. It was also found that at 0.15% bile salt, the number of lactic acid bacteria of each isolate activity still remained after incubated at 37°C for 4.0 hours. Isolate FF2-02/3 and FF4-01/5 were exhibited excellent at 0.15% bile tolerance, while isolate FP5-01/4 decreased after 4 h of incubation. However, 0.30% bile salts there were differences in the survival capacity of the tested. All 3 isolates were found to be the least 0.3% bile tolerant after 4 h of incubation, isolate FF2-02/3 the population of 3.5 log cfu/ml, whereas the population of isolate FF4-01/5 and FP5-01/4 a high population of 7.0 and 4.9 log cfu/ml, respectively. The survivals of all isolates were found to decrease with an increase in bile salt concentration from 0.15–0.3% at 4 h after incubation. According to Chateau *et al*, (1994) reported that there was extreme variability of resistance to 0.3 % bile salts in the *Lactobacillus* isolates and all the strains tested showed a delayed growth when compared to a reference culture with bile salts.



**Figure 4.8** Effect of bile concentration (0.15 and 0.3%) on survivability of three isolates, FF2-02/3, FF4-01/5 and FP5-01/4 in MRS medium for 4 h

#### 4.4 physiological characterizations of bacteriocin-producing strains

Three isolates, FF2-02/3, FF4-01/5 and FP5-01/4 that are bacteriocin producing lactic acid bacteria were identified to the genus level based on the following key biochemical characteristics (Axelsson, 1998). The phenotypic characterization from biochemical test of 3 isolates of bacteriocin producing lactic acid bacteria showed in table 4.6. All 3 isolates were Gram-positive and catalase negative. They grew at 6.5% NaCl, at pH 4.4 and pH 9.6. But these isolate not growth at 18% NaCl. Two isolates, FF4-01/5 and FP5-01/4 grew at 10°C. Only isolates FF2-02/3 showed the tetrad form arrangement.

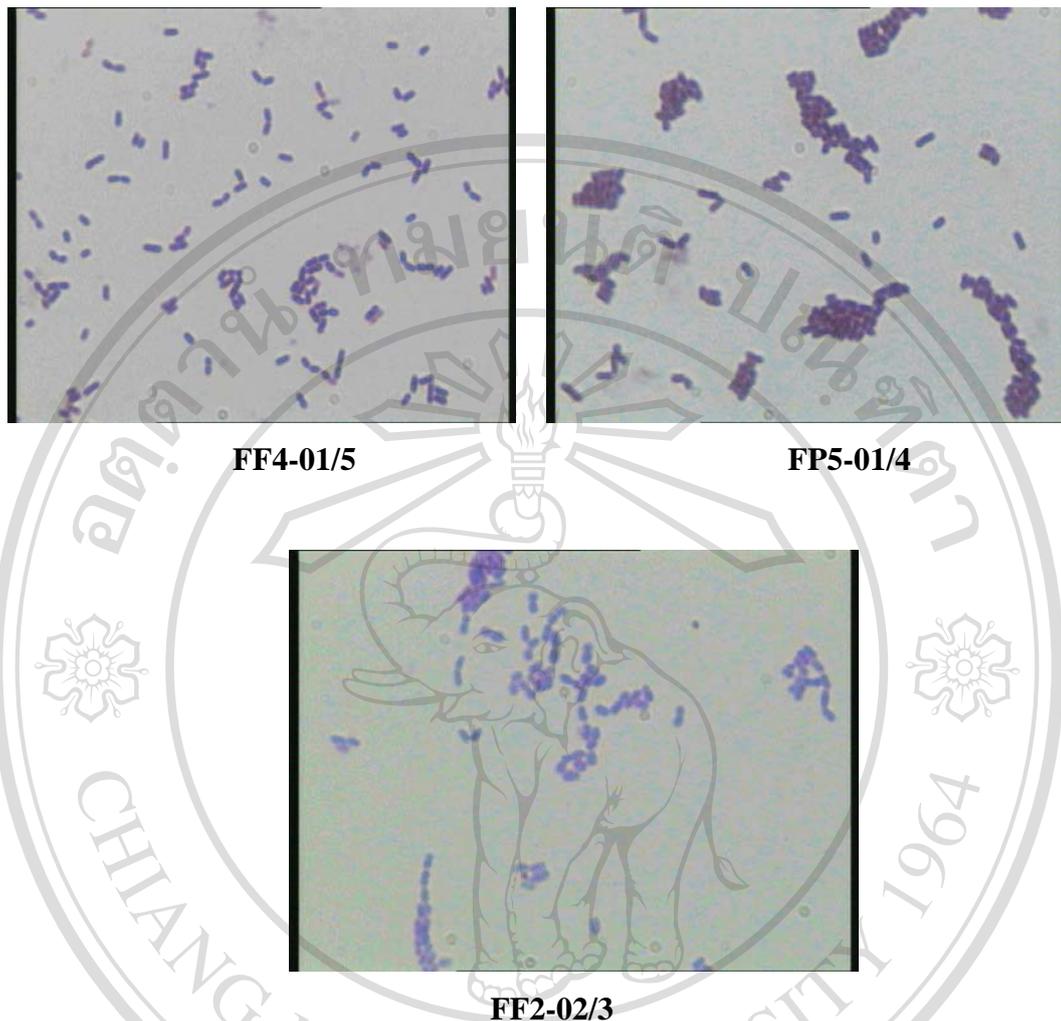
Biochemical test data are shown in Table 4.6. Three isolates, FF4-01/5, FP5-01/4 and FF2-02/3 were rod physiological characteristics (Figure 4.9) and identified as *Lactobacillus* sp. Normally, the genus *Lactobacillus* is by far the largest of the genera included in LAB. It is also very heterogeneous, encompassing species with a large variety of phenotypic, biochemical, and physiological properties.

**Table 4.6** The phenotype from biochemical test of 3 isolates properties of bacteriocin producing LAB

Tests	Isolates		
	FF2-02/3	FF4-01/5	FP5-01/4
<b>Gram staining</b>	+	+	+
<b>Cell</b>	<b>rod</b>	<b>rod</b>	<b>rod</b>
<b>Catalase test</b>	-	-	-
<b>CO<sub>2</sub> production</b>	-	-	+
<b>Growth</b>			
- 10°C	-	+	+
- 45°C	-	-	+
- 6.5% NaCl	+	+	+
- 18% NaCl	-	-	-
- pH 4.4	+	+	+
- pH 9.6	+	+	+
<b>Lactate</b>	<b>ND</b>	<b>ND</b>	<b>ND</b>

ND, not determine

Hugas *et al.* (1993) reported all isolates belonging to the species *L. sakei*, *L. curvatus* and *L. plantarum* grew at 15°C, whereas the percentage of the strains which grew at 45°C was 20, 33 and 64%, respectively. Schillinger and Lücke (1987) in meat and meat products detected 87% of *L. sakei*, 77% *L. curvatus* and 100% *L. plantarum* grew at 45°C. All *L. plantarum* strains grew at 6.5-10% NaCl. Hugas *et al.* (1993) suggested that growth at different temperature and salt concentrations might be strain dependent.



**Figure 4.9** Cell arrangements of isolate FF4-01/5, FP5-01/4 and FF2-02/3 under light microscope

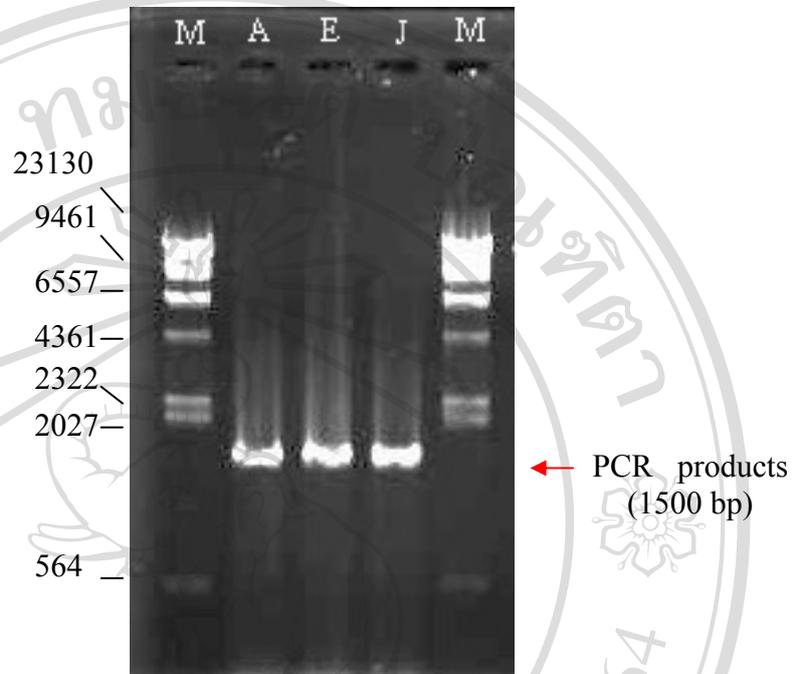
#### 4.5 Characterization of three isolates by 16S-rRNA Gene Sequence Analysis

Traditional classification methods based on morphology, physiology, and biochemical tests may not show the clear result. In recent years, several changes have occurred in the classification of LAB, and phylogenetic approaches to identification have become a major part of taxonomic studies (Stiles and Holzapfel, 1997). Automated nucleic acid sequencing methods and the availability of computational tools and publicly accessible genomic databases have accelerated the development of genetic based methods for the identification of microorganisms. The identification of different species of LAB using the polymerase chain reaction (PCR) has been

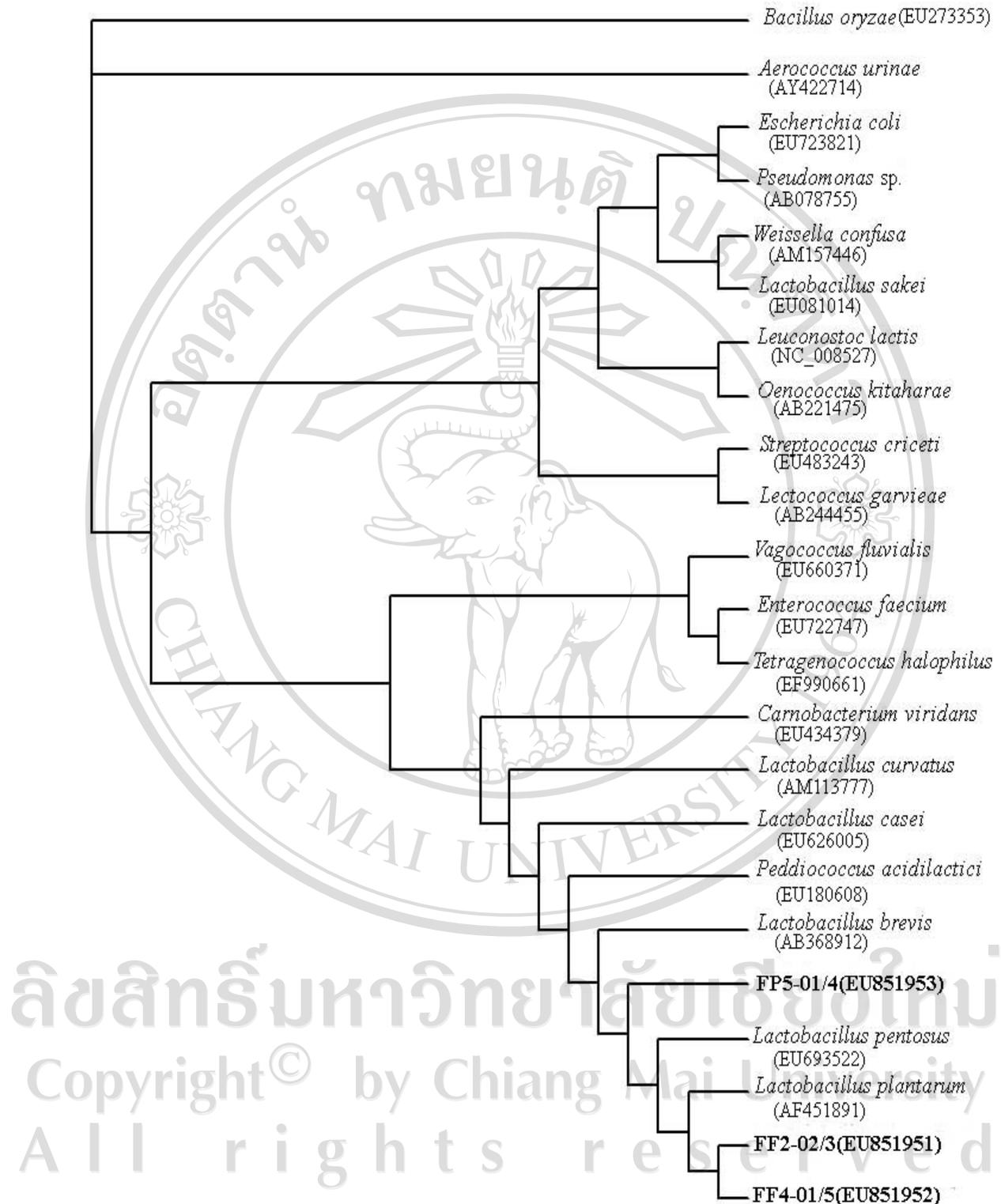
reported by several research groups (Klijn *et al*, 1991; Brooks *et al*, 1992; Betzl *et al*, 1990; Ehrmann *et al*, 1994; Hertel *et al*, 1991, 1993; Nissen *et al*, 1994; Tilsala-Timisjärvi and Alatossava, 1997). The standard approach has been to amplify ribosomal RNA sequences and then to confirm with species-specific DNA probes targeted to the 16S, 23S, or the 16S to 23S rRNA internal transcribed spacer regions.

In the present work, we use 16S-rRNA gene sequencing for identified three isolates LAB from fermented meat products. The Three isolates, pre-selected on the basis of biochemical properties, were characterized to species level in a taxonomy approach involving both phenotypic and genotypic method. Of these 3 isolates, isolates FF4-01/5, FP5-01/4 and FF2-02/3 could classified as *Lactobacillus* sp. by phenotypic methods. To confirm the phenotypic method of the three isolates, the near full length nucleotide sequences of the 16S-rRNA gene were determined. As a result of PCR amplification using two primers 27F and 1492R, PCR products showed similar electrophoresis patterns in each of *Lactobacillus* sp. (figure 4.10), expected sizes of the PCR products were 15 kb. The sequences of PCR products were aligned and compared with corresponding ones retrieved from DDBJ nucleotide database using BLAST system. The phylogenetic tree was drawn using the rectangular-cladogram method (Figure 4.11), the 16S- rRNA gene of isolates FF4-01/5(EU851952) and FP5-01/4(EU851953) closes to *Lactobacillus pentosus* with showed 99 % (FF4-01/5) and 90% (FP5-01/4) similarity. Isolate FF2-02/3(EU851951) was identified as *Lactobacillus plantarum* with 100% similarity. However, *Lactobacillus pentosus* and *Lactobacillus plantarum* are genotypically closely related, as indicated by 16S-rRNA sequence similarity of >99% (Collins *et al*, 1991). In cases where the species are very closely related, rRNA sequencing may not be sufficiently discriminative and do not allow a ready distinction between these species (Quere *et al*, 1997; Kostinek *et al*, 2005b). Thus, may be need others method such as rep-PCR, RAPD and API to confirm these isolates too.

DNA size  
(bp)



**Figure 4.10** Ethidium bromide stained 1% agarose gel of PCR products obtained from *Lactobacillus* sp. M: Lambda DNA/*HindIII* used as molecular marker, A: FF2-02/3 EU851951 (*Lactobacillus plantarum*), E: FP5-01/4 EU851953 (*Lactobacillus pentosus*), J: FF4-01/5 EU851953 (*Lactobacillus pentosus*)



**Figure 4.11** Phylogenetic relationships of representative strains of LAB isolate from fermented meat products base on 16S-rRNA gene sequences analysis. The phylogenetic tree constructed using the rectangular-cladogram method