CHAPTER 3

RESULTS

3.1 Construction of plasmid for human lactoferrin expression

3.1.1 LF expressing plasmid construction

The PCR products were electrophoresed on agarose gel and bands were photographed under UV lamp. The PCR products before and after fusion are shown in Figure 3.1 A and 3.1 B. The size of lactoferrin fragment and *nanH* promoter are 2100 and 300 bp, respectively. The *nanH* promoter-*hLF* fusion fragments were generated by PCR during which *Bam*HI/*Sal*I sites were introduced at the 5' and 3' end, respectively. The size of fusion fragment was about 2.4 kb, while *Bam*HI/*Xho*I digested fragment from pSKLF was about 2.2 Kb. The DNA fragments were individually inserted to pVAL-1 at *Bam*HI/*Sal* I site, then transformed to *E. coli* HB101 to increase the number of recombinant plasmids. The amount plasmids extracted from *E. coli* strains HB101(pVLFK) and HB101 (pVLFNp) were subjected for DNA sequencing.

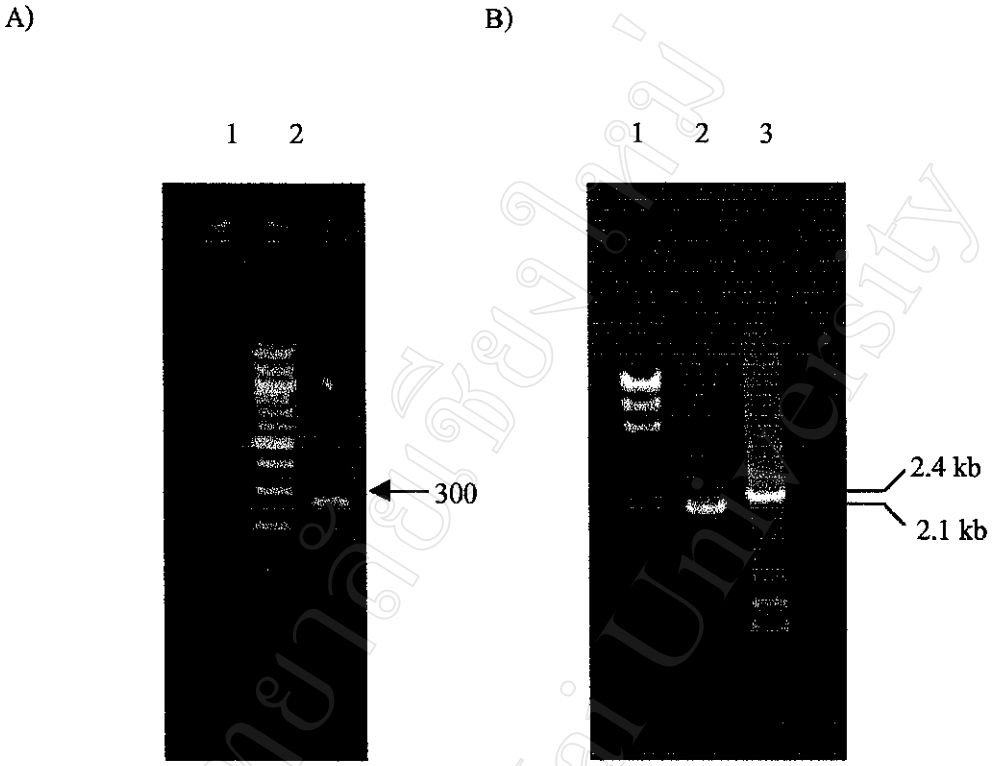


Figure 3.1 A) The molecular size of *nanH* promoter PCR product (lane 2) compared with DNA ladder 100 bp (lane 1), run on 3% agarose gel, B) PCR product amplified *hLF* (lane 2) and *nanH* promoter-*hLF* fusion product (lane 3) compared to λ-*Hind*III molecular weight marker (lane 1), run on 0.7% agarose gel

3.1.2 Nucleotide sequencing on subcloning site and promoter region

The sequences from 5'-end included subcloning site and open reading frame are shown in Figure 3.2. The subcloning site of these 2 plasmids from 5'-end was GGATCC, which is *Bam*HI site (red, bold). The pVLFNp open reading frame included *Bacteroides nanH* (neuraminidase gene) promoter (black small) and signal sequence (black capital), ribosomal binding site (red capital) and ATG for start codon. The translated amino acid sequences of strain pVLFK was automatically estimated by Genetic Mac software. The result showed that 5'-DNA sequences of pVLFK also presented promoter like region for bacteria transcription (underline), ribosome binding site (bold) and ATG of start codon for translation. There is no mutation on the sequence of the N-terminal of both *hLF* genes.

(A) pVLFK

BamH I -35 -10

5'-ggatccccgggctgcaggaattccggctagaaaatgaatatgtaagaaagcttatagtagttaaatcattgt

RB M K L V F L V L L F

agaaccagccgagttgtcaagtcgcctccAGaccgcagacATGAAACTTGTCTTCCTCGTCCTGCTGTTC

L G A L G L C L A G R R R R S V Q

CTCGGGGCCCTCGGACTGTGTCTGGCTGGCCGTAGGAGAAGGAGTGTTCAG

W C A V S Q P E A T K C F Q W Q R N

TGGTGCCGGGTATCCCAACCCGAGGCCACAAAATGCTTCCAATGGCAAAGGAAT

M R K S

ATGAGAAAAAGT

(B) pVLFNp

BamHI

tggtgggatcctcgagataacatccctctctttgtttttaaatacaatccaaatcctgattgatgggatagtcttcaatgaccgctttgaatatgttt

-35 -10

atttccgatggtttagtgcttttgcgtcgaatagtgtcttgtttattaaatcgactgtatataataaaatatttaattaacataaccgttatataataaaa

RB M K K A V I L F S L

aatactatctttgctcccgataacataaatattaattAGtcgataccATGAAAAAAGCCGTAATTCTATTTTCGCTT

F C F L C A I P V V Q A A G R R R R R

TTCTGTTTTCTGTGTGCTATACCGGTAGTTCAAGCAGCAGGCCGTCGTCGTCGT

S V Q W C A V S Q P E A T K C F Q W Q

AGTGTTCAGTGGTGCCGGGTATCCCAACCCGAGGCCACAAAATGCTTCCAATGGCAA

R N M R K S

AGGAATATGAGAAAAAGT

Figure 3.2 Sequence of subcloning site and 5'-end DNA sequences of *hLF* genein plasmids pVLFK (A) and pVLFNp (B)

3.2 Transconjugation, plasmid and *hLF* gene existence

The recombinant plasmids were transformed to *E.coli* HB101 (R751) and then transformed *E. coli* strains were used as donors for transconjugation plasmids to *B. uniformis* strain BU1001 by filter mating procedure (Valentine *et al.*, 1985). The *B. uniformis* harboring plasmid was resistant to erythromycin as an antibiotic resistant marker for pVAL-1 in *B. uniformis*. The ratio of recipient to donor 1:1 or 1:5 generated the same frequency of transconjugation that was about 10^{-4}.

Plasmid DNA isolated from transfected *B. uniformis* in each strain was electrophoresed on agarose gel to determine the presence of plasmid (Figure 3.3). All strains presented the plasmid with the original size. The recombinant plasmids were slightly larger than vector pVAL-1. Because pVAL-1 is the plasmid that has low copy number, isolated plasmid were not sufficient for determining the presence of *hLF*. They were subsequently transformed to *E. coli* to increase the number of plasmid and re-isolated.

Each plasmids extracted from *E. coli* were therefore checked for a presence of *hLF* cDNA. This was demonstrated by PCR products of the DNA isolated from bacterial cells using specific primers (Figure 3.4). *B. uniformis* transconjugated with *hLF* constructed in either orientation, produced a PCR product of 2.1 kb (pVLFK; lane 3, pVLFNp; lane 4), which was not evident in the control strain (pVAL-1; lane2).

The confirmed nucleotide sequences of N-lobe *hLF* is shown in Figure 3.5. The sequences showed no mutation on the N-terminal sequence of *hLF* gene in pVLFK. The result confirmed the stability of *hLF* in *B. uniformis* and the direct subcloning strategies.

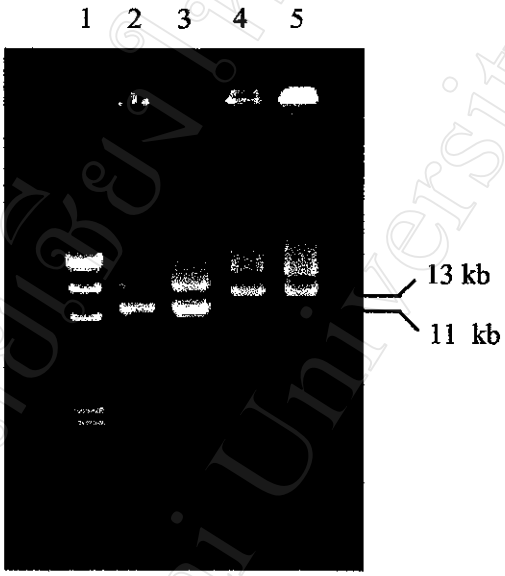


Figure 3.3 Plasmid DNA extracted from *B. uniformis* strains BU1001 (pVAL-1) (lane 3), BU1001 (pVLFK) (lane 4) and BU1001 (pVLFNp) (lane 5) compared to purified pVAL-1 (lane 2) and λ-*Hind*III molecular markers (lane 1), run on 0.7% agarose gel

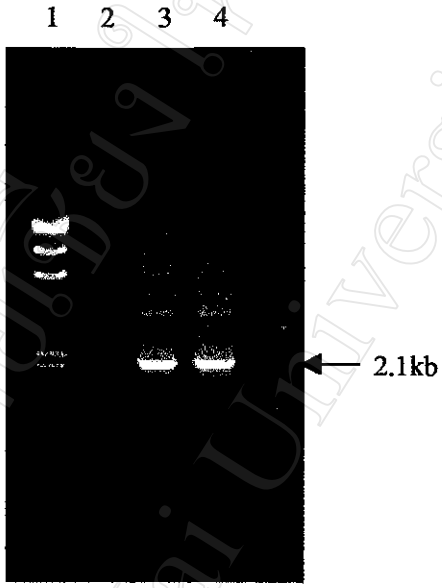


Figure 3.4 PCR products of *hLF* (2.1 kb) by using plasmid DNA template isolated from *B. uniformis* strains BU1001 (pVAL-1) (lane 2), BU1001 (pVLFK) (lane 3) and BU1001 (pVLFNp) (lane 4) compared to purified pVAL-1 (lane 2) and λ-*Hind*III molecular marker (lane 1), run on 0.7% agarose gel.

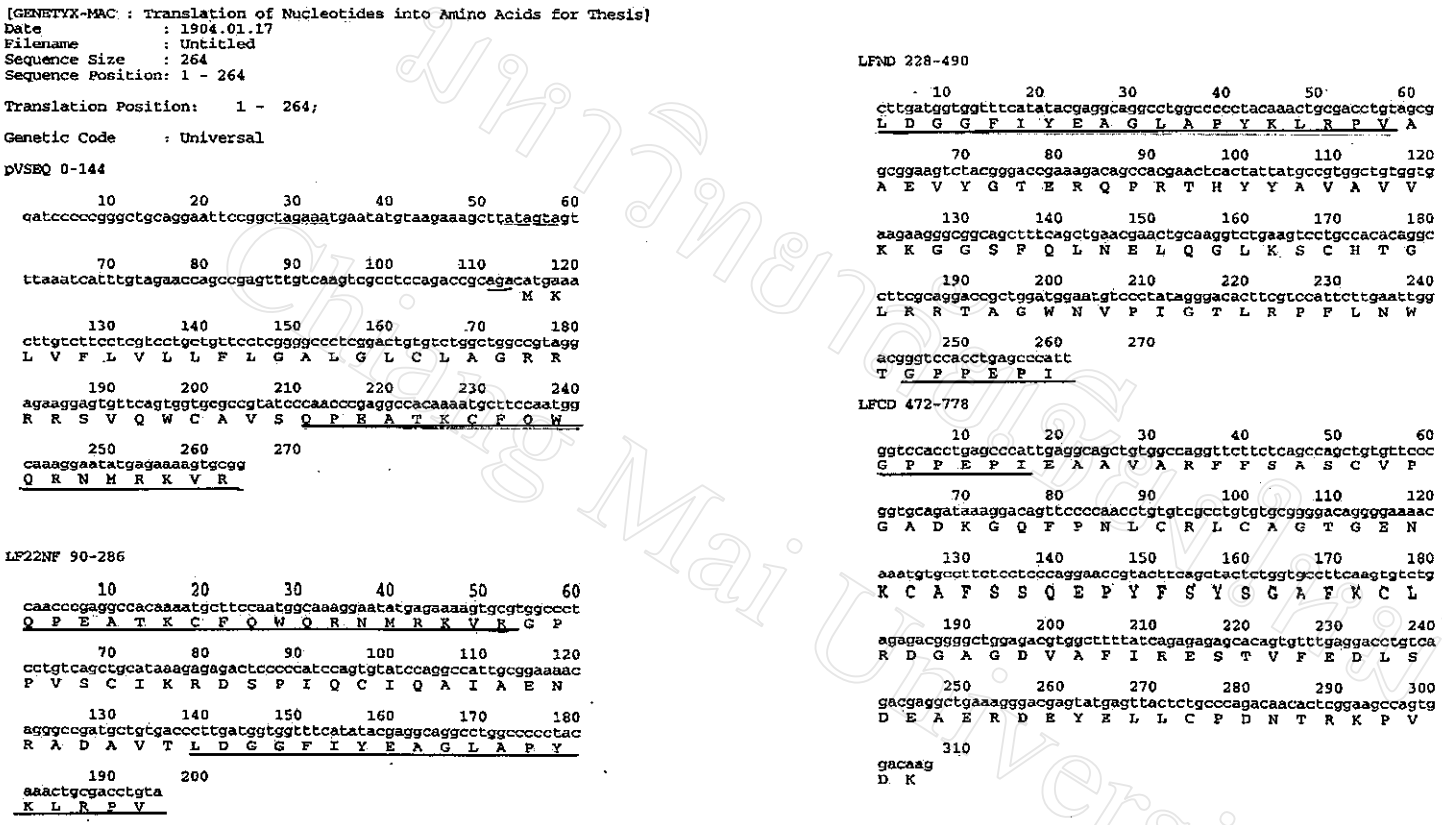


Figure 3.5 Nucleotide sequences of 5'-terminal region of *hLF* in pVLFK and deduced amino acid. Underlined letters are overlapping sequence of each sequent primer.

3.3. Expression of *hLF* gene in *B. uniformis*

3.3.1 Transcriptional level of lactoferin-producing *B. uniformis*

To assess the transcriptional level of *hLF* gene in *B. uniformis*, Northern blot hybridization using total RNA from *B. uniformis* BU1001 (pVAL-1) and *B. uniformis* BU1001 (pVLFK) was carried out. The concentration of total RNA extracted from *B. uniformis* strain pVAL-1 was 1.38 mg/ml that was higher than strains BU1001 (pVLFK) and BU1001 (pVLFNp) (1.10 and 0.76 mg/ml, respectively) and RNA concentration then were all adjusted to 1 μg/ml individually. Figure 3.6A showed the rRNA (16s and 23s) from *B. uniformis* electrophoresed on 1% formaldehyde agarose gel following ethidium bromide staining. Total 5.5 μg of total RNA showed the same intensity of bands, which could normalize the amount of RNA loading. Figure 3.6B and C showed the Northern blot hybridization using N-lobe probe (3.6 B) and C-lobe probe (3.6 C) of *hLF* cDNA as hybridization probes. The transcription with a length of 2.2 kb was present in *B. uniformis* strains BU1001 (pVLFK) and BU1001 (pVLFNp), while no mRNA was detected in *B. uniformis* strain BU1001 (pVAL-1). The mRNA intensity of *hLF* expressed in *B. uniformis* strain BU1001 (pVLFNp) was slightly higher than strain BU1001 (pVLFK).

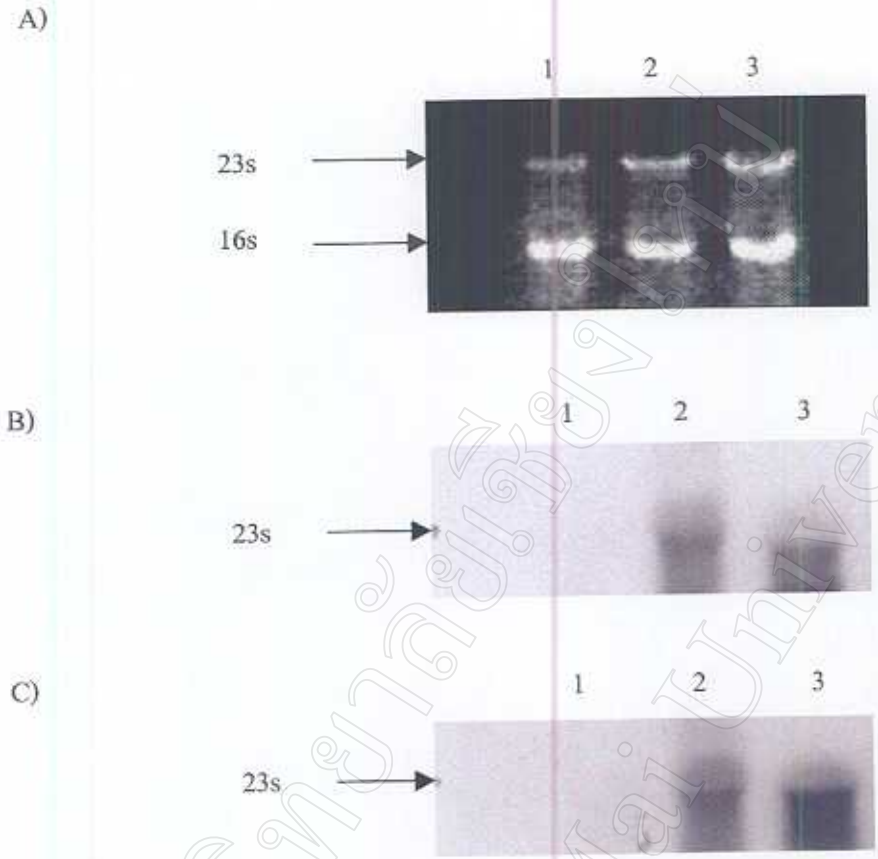


Figure 3.6 A) Five micrograms of RNA extracted from *B. uniformis* strain BU1001 (pVAL-1) (lane 1), BU1001 (pVLFK) (lane 2) and BU1001 (pVLFNp) (lane 3)stained by ethidium bromide for molecular weight markers (A) . Northern hybridization of *hLF* mRNA by using DIG-labeled *hLF* cDNA N-region (B) or C-region (C), as hybridization probe.

3.3.2 Recombinant lactoferrin determination

Total protein from cell homogenate (S9) from each strain was electrophoresed and blotted onto PVDF membrane. Western blot analysis using anti-human lactoferrin antibody revealed a specific gene product in homogenized cells of *B. uniformis* BU1001 (pVLFK) (Figure 3.7A, lane 3) with the same size as purified human lactoferrin from milk (80 kDa, Figure3.7A, lane1). This band was absent in cells carrying vector plasmid pVAL-1 alone or pVLFNp (Figure 3.7A, lanes 2 and lane 4, respectively). The specific product, human lactoferrin, also was not observed in culture supernatant (Figure3.6 lanes 5, 6 and 7, respectively), although the culture supernatant was 10 fold concentrated by Quigen® spin column. The results was confirmed by using CSPD as a substrate and exposure to X-ray film (Figure 3.7B) These result show that *hLF* gene was successfully transcribed and translated only in *B. uniformis* strain BU1001 (pVLFK) but that there was no secretory hLF product from this recombinant bacteria.

It was confirmed in Figure 3.8 that the rhLF was detected in cell homogenate (S9) (lane 3) of *B. uniformis* BU1001 (pVLFK). The rhLF was presented on the bacterial outer-membrane (P200, lane 7) that it was not presented on cytosolic fraction (S200, lane 5). The result indicated that the rhLF was expressed on outer-surface of recombinant bacteria.

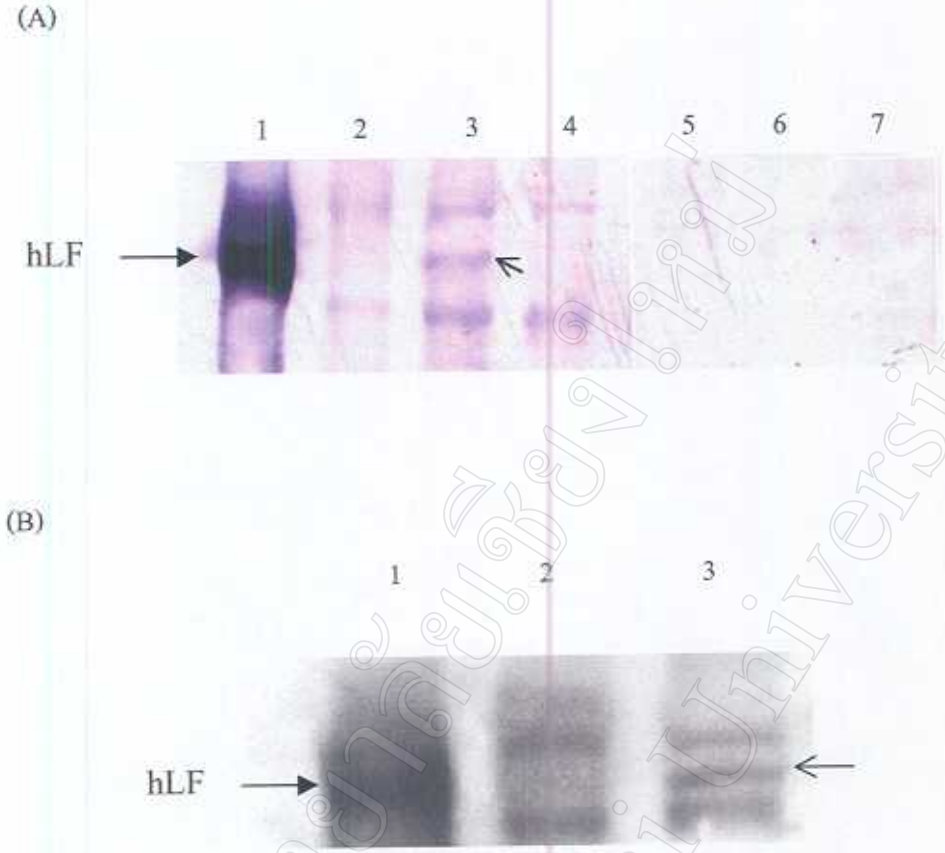


Figure 3.7 (A) Detection of rhLF in *B. uniformis* strains BU1001 (pVAL-1) (lanes 2, and 5), BU1001 (pVLFK) (lanes 3, and 6) and BU1001 (pVLFNp) (lane 4 and 8) by Western blot analysis with anti-hLF antibody. Two microgram cellular protein were electrophoresed on 8% SDS-PAGE and using NCMB color developing system. (B) Cell homogenate of *B. uniformis* strains BU1001 (pVAL-1) (lanes 2), BU1001 (pVLFK) (lanes 3) were electrophoresed on 10% SDS-PAGE and using chemiluminescence detection system. Black arrow indicates standard commercial human lactoferrin from Sigma-Aldich (80 kDa), open arrow points rhLF from *B. uniformis* strains BU1001 (pVLFK) .

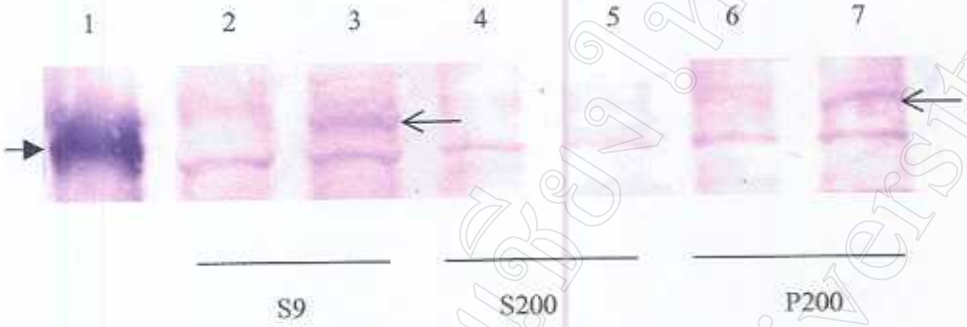


Figure 3.8 Detection of rhLF in *B. uniformis* strains BU1001 (pVAL-1) (lanes 2, 4 and 6) and BU1001 (pVLFK) (lane 3,5 and 7). Two micrograms of cell homogenate (lane 2 and 3), cytosol (lane 4 and 5) and outer membrane (lane 6 and 7) were electrophoresed on 10% SDS-PAGE and Western blot analysis with anti-hLF antibody. Black arrow indicates standard commercial human lactoferrin from Sigma-Aldich (80 kDa), open arrow points rhLF from *B. uniformis* strains BU1001 (pVLFK) .

3.4 The effect of *B. uniformis* cultures on growth of *E. coli* strain HB101

Bacterial numbers of one night culture of *B. uniformis* strains BU1001 (pVAL-) and BU1001 (pVLFK) were similar. Table 3.1 and Figure 3.9 show the growth curve of *E.coli* strain HB101 in co-cultivation with recombinant strains of *B. uniformis*. The number of *E. coli* cells at the start of culture after adding GAM broth, GAM broth with 1 mg/ml bovine lactoferrin (bLF), *B. uniformis* BU1001 (pVAL-1) or *B. uniformis* BU1001 (pVLFK) were 3.6×10^7, 4.6×10^7, 2.9×10^7 or 3.2×10^7 CFU/ml, respectively. The growth of *E. coli* was inhibited by addition of bLF as compared with that in broth added by GAM broth alone. The growth of *E.coli* mixed with *B. uniformis* was slower than that with GAM broth or bLF, probably due to competition of nutrients in the medium. Six hour after cultivation, the numbers of *E.coli* in GAM broth mixed with water, bLF, *B. uniformis* (pVAL-1) and *B. uniformis* (pVLFK) were 6.5×10^8, 5.6×10^8, 3.2×10^8 and 9.1×10^7 CFU/ml, respectively. This result indicates that the gene product in *B. uniformis* strain BU1001 (pVLFK) significantly inhibited the growth of *E. coli* strain HB101 compared to plasmid control strain BU1001 (pVAL-1) ($p<0.05$).

Table 3.1 The effect of *B. uniformis* cultures on the growth of *E. coli* strain HB101

	Number of bacteria (CFU/ml) [a] (log CFU/ml±SD)			
Time (hour)	0	1	3	6
Co-culture				
GAM	3.6×10^7 (7.63±0.02)	6.8×10^7 (7.81±0.09)	4.5×10^8 (8.40±0.28)	6.5×10^8 (8.90±0.16)
GAM+bLF [b]	4.6×10^7 (7.64+0.15)	5.8×10^7 (7.75+0.04)	2.3×10^8 (8.37+0.13)	5.6×10^8 (8.69+0.12)
B. uniformis BU1001				
pVAL-1	2.9×10^7 (7.64+0.35)	3.9×10^7 (7.66+0.08)	1.6×10^8 (8.12+0.31)	3.2×10^8 (8.33+0.32)
pVLFK	3.2×10^7 (7.55+0.13)	3.5×10^7 (7.75+0.04)	$6.6 \times 10^{7\,c)}$ (8.37+0.13)	$9.1 \times 10^{7\,d)}$ (8.69+0.12)

a) Average from triplicate plate

b) The concentration of bLF was 1 mg/ml

c) Significantly different from GAM , $p<0.0001$

d) Significantly different from pVAL-1 , $p<0.05$,

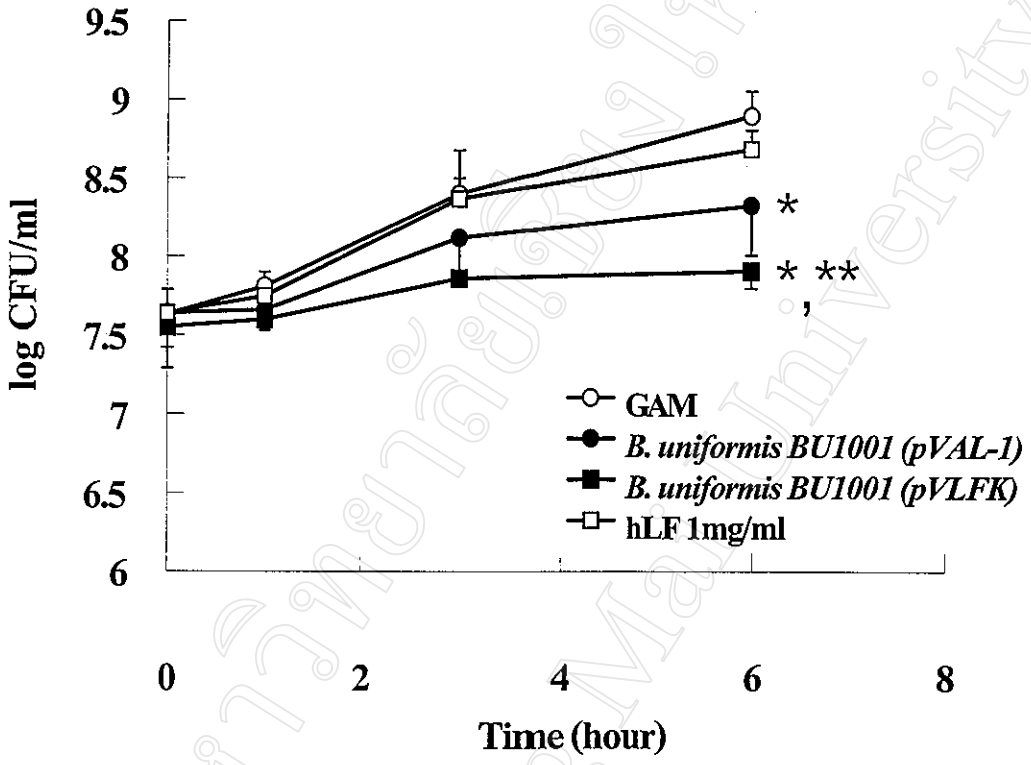


Figure 3.9 The growth of *E. coli* strain HB101 cultured with lactoferrin producing *B. uniformis*

* Significantly different from GAM group, $p<0.05$

** Significantly different from *B. uniformis* strain BU1001 (pVAL-1), $p<0.05$.

3.5 Modulating effect of hLF-producing *B. uniformis* on AOM-induced ACF formation

The mean body weight of rats treated with cultures of *B. uniformis* is shown in Figure 3.10. The body weight of rats was slightly different but not significant among the untreated group (238.0±10.5 g), *B. uniformis* BU1001 (pVAL-1) and *B. uniformis* BU1001 (pVLFK)-treated groups (242.9±4.3 g and 243.3±14.2 g, respectively) at the end of the experiment for five weeks. The number of erythromycin resistant anaerobic bacteria in feces of rats treated with cultures of *B. uniformis* BU1001 (pVAL-1) and *B. uniformis* BU1001 (pVLFK) for one week were 4.02±1.63 x 10^9 and 2.66±1.08 x 10^9 CFU/g feces, respectively. Number of ACF and multiplicity in the rat colon is summarized in Table 3.2. The result showed that treatment with overnight culture of *B. uniformis* BU1001 (pVAL-1) increased the number of ACF significantly (45% increase) compared with the water-treated group ($p<0.01$). In the group treated with culture of *B. uniformis* BU1001 (pVLFK), the numbers of ACF and ACF having more than three crypts per focus were significantly decreased (23% and 27% reduction, respectively) compared with those of the *B. uniformis* BU1001 (pVAL-1)-treated group ($p<0.05$). There is no significant difference when compared with the water-group. In the second experiment, *B. uniformis* BU1001 (pVLFK) clearly showed the inhibitory effect on the AOM-induced ACF formation (Table 3.6). The Bacterial culture decreased the number of ACF and ACF with more than 3 crypt/focus by about 58% and 64%, respectively from those of rats treated with *B. uniformis* strain BU1001 (pVAL-1). When combined with two independent experiments (Table 3.2 and Table 3.6 group 2-5), the result was clearly shown in Figure 3.11.

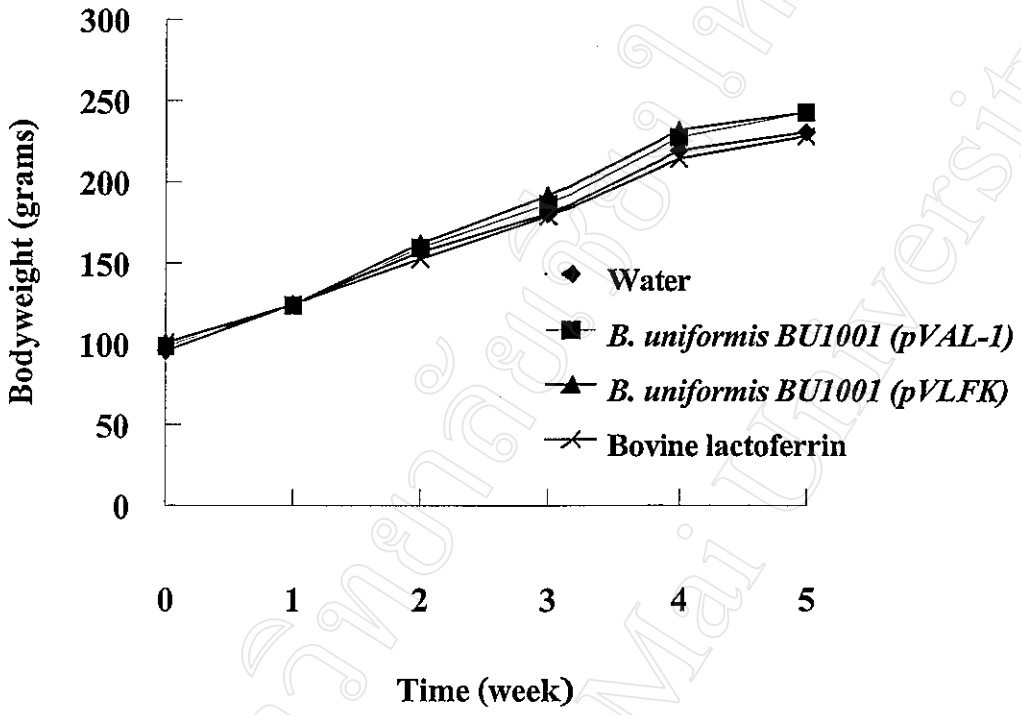


Figure 3.10 Body weight of rats treated with the culture of *B. uniformis,* Rats were weight once a week during administration of water, bacterial culture and bLF at a dose 200 mg/rat/day.

Table 3.2 Effect of lactoferrin-producing *B. uniformis* on AOM-induced ACF formation in the rat colon

Treatment	No	Number of ACF[a]		
		Total	ACF>3[b]	C/F
Water	6	197.7±34.5	21.0±11.8	2.27±0.12
B.uniformis BU1001 (pVAL-1)	5	285.2±15.8[c]	39.2±7.6[c]	2.36±0.10
B.uniformis BU1001 (pVLFK)	5	219.5±16.9[d]	28.7±4.5[d]	2.31±0.12
bLF (200 mg/rat/day)	5	191.0±71.1	21.0±11.8	2.32±0.11

a) mean±SD b) number of ACF which have more than 3 crypt/focus

c) significantly different compared to Water group; $p<0.01$

d) significantly different compared to strain BU1001 (pVAL-1) group; $p<0.05$

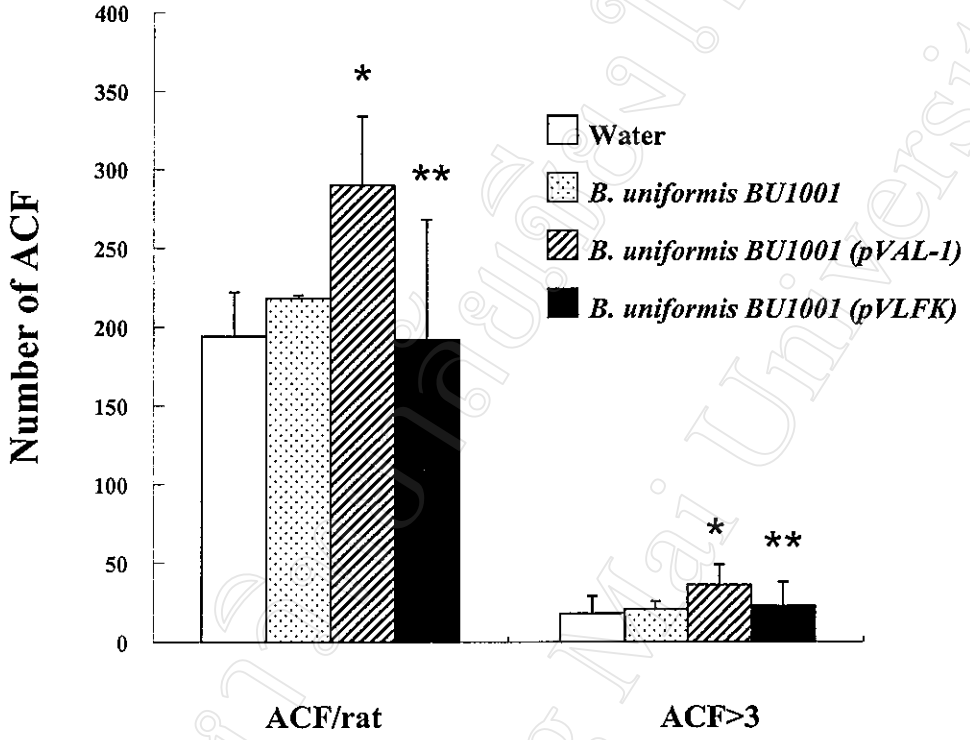


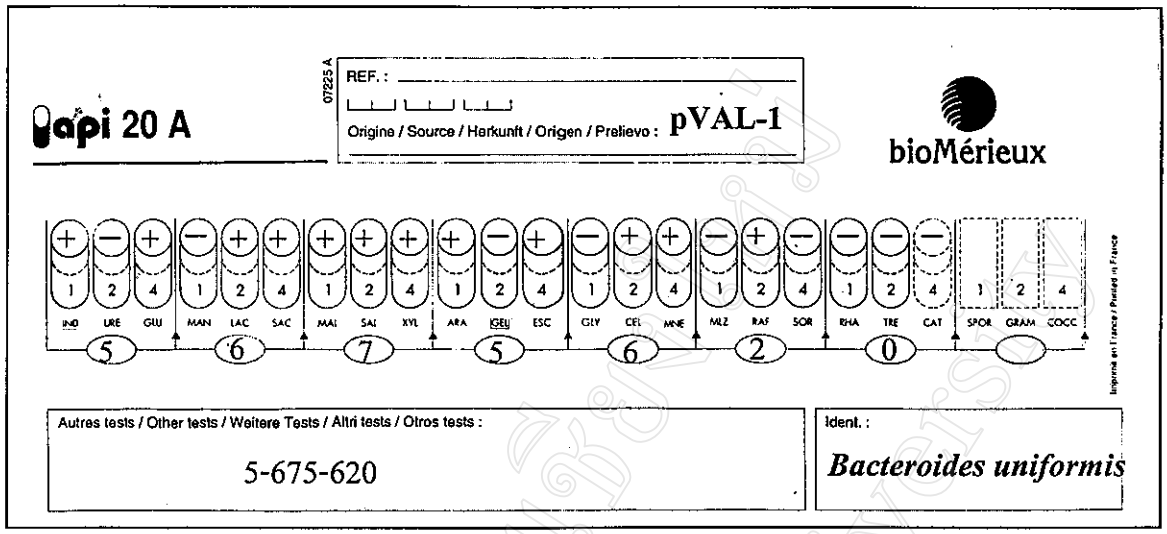
Figure 3.11 Number of ACF and multiple ACF combined two independent experimental data

* significantly different from water group, $p<0.01$

** significantly different from *B. uniformis* strain BU1001 (pVAL-1), $p<0.01$

3.6 The numbers of fecal bacteria in rats treated with cultures of recombinant *B. uniformis*

The numbers of aerobic, anaerobic and erythromycin-resistant anaerobic bacteria in rat feces from each group were determined weekly. The results summarized in Table 3.3. The numbers of aerobic bacteria in feces from rats treated with cultures of recombinant *B. uniformis* strains were lower than those in feces from the water-treated group, while the number of anaerobic bacteria was quite similar. The number of total bacteria in feces from the *B. uniformis* BU1001 (pVLFK)-treated group was slightly lower than that in *B. uniformis* BU1001 (pVAL-1)-treated group. These results suggest that treatment with *B. uniformis* BU1001 (pVLFK) might prevent the formation of ACF in the rat colon by changing the profile of intestinal microflora. The erythromycin-resistant bacteria in feces of rats treated with *B. uniformis* BU1001 (pVAL-1) or BU1001 (pVLFK) were (in triplicate) identified as *B. uniformis* by API A20® anaerobic identification kit as shown in examples of identification sheet in Figure 3.12.



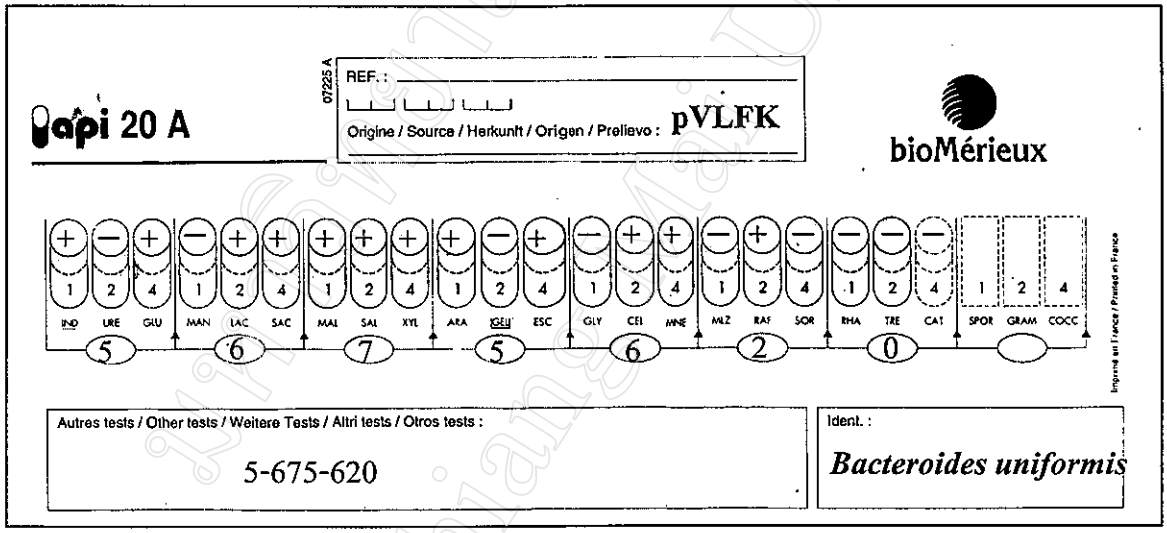


Figure 3.12 Api 20 ® Anaerobic bacteria identification sheets identified Em^r-anaerobic bacteria in feces of rats treated with *B. uniformis* strain A) BU1001 (pVAL-1) (group 2) and B) BU1001 (pVLFK) (Group 3)

Table 3.3 Number of fecal bacteria in rats treated with bacteria cultures

Treatment	No of bacteria (CFU/g feces) [a]		
	Water	BU1001 (pVAL-1)	BU1001 (pVLFK)
Aerobic bacteria ($x10^9$)			
Week 1	ND	ND	ND
Week 2	0.92±0.43	0.10±0.10[b]	0.06±0.01[b]
Week 3	1.48±0.29	0.61±0.33[b]	0.39±0.07
Week 4	1.63±0.55	2.71±1.11	1.54±0.6
Week 5	3.11±0.32	2.70±0.79	1.90±0.43
Anaerobic bacteria ($x10^9$)			
Week 1	6.20±5.10	6.80±3.10	3.90±1.30
Week 2	1.33±0.60	4.02±0.32[b]	0.58±0.13[e]
Week 3	1.74±0.25	4.69±0.25[d]	3.31±0.30[f,e]
Week 4	1.69±0.48	3.06±1.27	2.08±1.22
Week 5	4.03±0.47	7.79±1.68[b]	4.21±0.69[g]
Em^r- Anaerobic bacteria ($x10^9$)			
Week 1	ND	12.20±6.30	1.60±1.40
Week 2	ND	4.59±3.49	0.40±0.39[g]
Week 3	ND	4.85±0.72	2.79±0.42[g]
Week 4	ND	ND	ND
Week 5	ND	4.02±1.63	2.66±1.08

a) Mean±SD from duplicate plates of 3 feces samples.

b) Significant different from Water group, $p<0.05$, c) $p<0.01$, d) $p<0.0001$, f) $p<0.005$

e) Significant different from BU1001 (pVAL-1) group, $p<0.001$, g) $p<0.05$

ND= Not determined

3.7 Activity of β-glucuronidase in feces of treated rats

Activity of β-glucuronidase in cell free extracts and culture supernatant of *B. uniformis* in both strains BU1001 (pVAL-1) and BU1001 (pVLFK) was determined and shown in Table 3.4. The activity of β-glucuronidase in cell free extracts of *B. uniformis* strains BU1001 (pVAL-1) and BU1001 (pVLFK) was very low at 0.28 and 0.06 nmole/min/mg protein, respectively. The activity of β-glucuronidase in *C. perfingen* strain GAI0668 showed very high, 14.5 nmole/min/mg protein, which used as positive control. The activity of these enzymes was not observed in culture supernatants.

Activity of β-glucuronidase from feces of rats treated bacterial cultures for 2 week is shown in Figure 3.13. The activity of this enzyme was significantly decreased in rats treated with the cultures of *B. uniformis* strains BU1001 (pVAL-1) and BU1001 (pVLFK) which contained erythromycin. Activity of β-glucuronidase from feces of treated rats at before 3 hours and 3 days after second dose of AOM injection is summarized in Table 3.5 and Figure 3.14. The activity of β-glucuronidase in feces of non-treated rat was 4.25±1.36 before AOM injection. The activity then was slightly increased to 5.33±1.07 μmole/h/mg protein after injection of AOM, but not significantly. At 3 hour after AOM injection, the activity of fecal β-glucuronidase was clearly modulated by cultures of *B. uniformis*. In rats treated with culture of *B. uniformis* BU1001, the activity of fecal β-glucuronidase was lower than that in non-treated group, while the activity of rats treated with *B. uniformis* strains BU1001 (pVAL-1) and BU1001 (pVLFK) were strongly decreased to 1.19±0.25 (78%) and 0.51±0.11(90%) μmole/h/mg protein, respectively ($p<0.001$). The result also showed that rat treated with *B. uniformis* strain BU1001 (pVLFK) had lower activity of fecal β -glucuronidase than *B. uniformis* strain BU1001 (pVAL-1) about 57% ($p<0.05$).

Table 3.4 β-glucuronidase activity in bacterial cell free extract and culture supernatant

Bacteria	β -glucuronidase activity	
	Cell free extract (nmole/min/mg protein)	Supernatant (nmole/min/ml)
C. perfingen GAI0668	14.5	ND
B. uniformis BU1001 (pVAL-1)	0.28	ND
B. uniformis BU1001 (pVLFK)	0.06	ND

Table 3.5 β-glucuronidase activity in feces of rats treated with cultures of *B. uniformis*

Treatment	β -glucuronidase activity (μmole/h/mg protein)[a]		
	Before	After 3 hours	After 3 days
Water	4.25±1.36	5.33±1.07	5.13±0.86
B. uniformis BU1001	2.22±0.30[b1]	4.25±1.03	3.46±1.97
B. uniformis BU1001 (pVAL-1)	0.71±0.23[b2,c1]	1.19±0.25[b2,c2]	0.85±0.31[b1,c1]
B. uniformis BU1001 (pVALK)	0.61±0.29[b2,c1]	0.51±0.11[b2,c2,d]	0.44±0.18[b1,c1,]

a) Mean±SD

b) Significantly different compared to Water group, (1) $p<0.01$, (2) $p<0.001$

c) Significantly different compared to *B. uniformis* BU1001 , (1) $p<0.05$, (2) $p<0.01$

d) Significantly different compared to *B. uniformis* BU1001 (pVAL-1), $p<0.05$

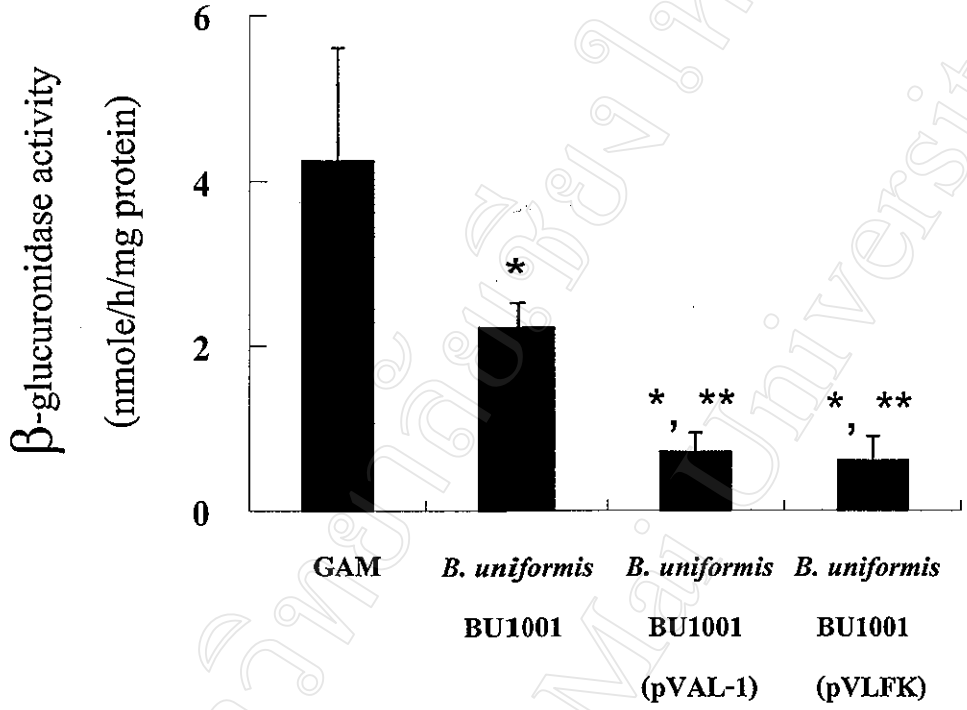


Figure 3.13 The activity of β-glucuronidase in feces of rats treated with the cultures of *B. uniformis* for 2 weeks. Fecal samples were randomly collected from 3 rats and duplicate determined for enzyme activity.

* significantly different from water group, $p<0.01$

** significantly different from *B. uniformis* BU1001, $p<0.05$

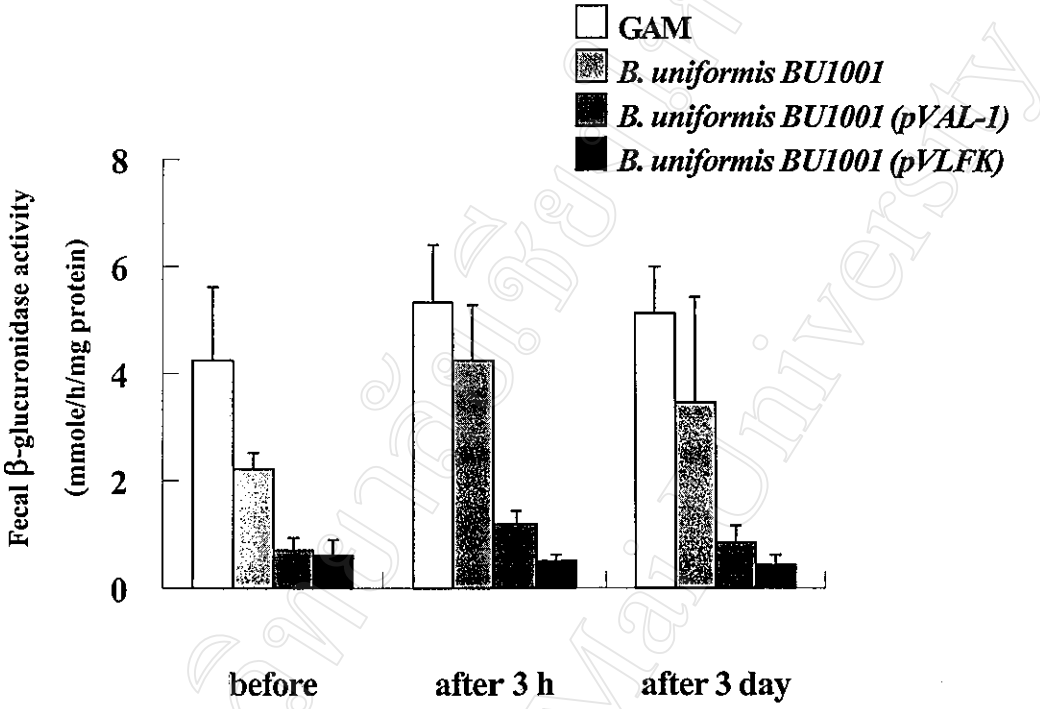


Figure 3.14 The activity of β-glucuronidase in feces of rats treated with the cultures of *B. uniformis* before, 3 hours or 3 days after AOM administration. The statistical different of fecal β-glucuronidase at 3 hour and 3 days after AOM administration were similar to before AOM administration

3.8 Effect of culture of various strains of *Bacteroides* on AOM-induced ACF formation in rat colon

Table 3.6 shows the numbers of ACF and crypt per focus (c/f) in the rats treated with AOM and cultures of various strains of *Bacteroides*. Table 3.7 also summarizes that strains of various species of Bacteroides which no effect on the number of ACF were *B. eggethrii* ATCC27754, *B. thetaiotaomicron* Werner E50, *B. uniformis* ATCC8492, *B. vlgatus* ATCC8482, *B. caccae* JMC9498 and *B. stercoris* JMC9496. *Bacteroides*, strains that enhanced the number of ACF were *B. uniformis* BU1001, *B. uniformis* BU1001 (pVAL-1), *B. distasonis* ATCC8503 and *B. ovatus* ATCC8483. *Bacteroides* strains which reduced the number of ACF in the rat colon were *B. thetaiotaomicron* KYU 1, *B. uniformis* KYU 2, *B. fragillis* KYU 3, *B. ovatus* KYU 4, *B. merdae* JMC9497 and lactoferrin producing *B. uniformis* BU1001 (pVLFK). The sizes of ACF, presented in crypt/focus, were quite similar in each group ranging from 2.16 to 2.31. Therefore *B. uniformis* strain KYU2 was selected for expressing human lactoferrin to investigate the effect on AOM-induced colon carcinogenesis.

Table 3.6 The number of ACF, multiple ACF and AC/focus in rat treated with various strains of *Bacteroides*

Treatment	No	No of ACF	Multiple ACF/rat [a]	AC/focus
Water	3	186 ± 8.19 [b]	12.0 ± 7.21	2.06 ± 0.23
B. uniformis BU1001	3	218 ± 2.08 [c2]	20.7 ± 5.13	2.30 ± 0.07
B. uniformis BU1001(pVAL-1)	3	298 ± 78.5 [c2]	30.7 ± 19.4	2.22 ± 0.18
***B. uniformis* BU1001(pVLFK)**	**4**	**126 ± 24.5 [c1, d2, e]**	**11.0 ± 3.56 [d1]**	**2.28 ± 0.08**
B. uniformis ATCC8492	3	180 ± 27.8	15.7 ± 6.03	2.18 ± 0.12
***B. thetaiotaomicron* KYU1**	**3**	**130 ± 25.2 [c1]**	**15.0 ± 5.29**	**2.28 ± 0.11**
***B. uniformis* KYU2**	**3**	**127 ± 47.4**	**15.0 ± 7.55**	**2.23 ± 0.19**
***B. fragilis* KYU3**	**3**	**137 ± 35.5**	**18.3 ± 9.02**	**2.41 ± 0.32**
***B. ovatus* KYU4**	**3**	**135 ± 4.51 [c2]**	**14.3 ± 4.62**	**2.22 ± 0.17**
B. ovatus ATCC8483	3	202 ± 44.1	14.0 ± 5.29	2.17 ± 0.13
B. distasonis ATCC8503	3	220 ± 63.5	20.3 ± 16.2	2.22 ± 0.17
B. eggerthii ATCC27754	3	175 ± 32.1	13.3 ± 0.58	2.16 ± 0.07
B. thetaiotaomicron Werner E-50	3	180 ± 28.9	13.7 ± 6.03	2.21 ± 0.07
B. vulgatus ATCC8482	3	187 ± 18.5	20.7 ± 3.51	2.31 ± 0.09
B. caccae JCM9498	3	163 ± 14.5	10.7 ± 3.22	2.17 ± 0.17
***B. merdae* JCM9497**	**3**	**139 ± 31.6**	**15.3 ± 6.51**	**2.30 ± 0.14**
B. stercoris JCM9496	3	164 ± 60.0	13.0 ± 2.65	2.18 ± 0.06

[a] Number of ACF which have more than 3 AC per 1 focus

[b] Mean±SD

[c] Significantly different from water group, [1] $p < 0.05$ or [2] $p < 0.01$

[d] Significantly different from the culture of *B. uniformis* BU1001 treatment, [1] $p < 0.05$ or [2] $p < 0.01$

[e] Significantly different from the culture of *B. uniformis* BU1001 (pVAL-1) treatment, $p < 0.01$

Table 3.7 Classification of Bacteroides strains by ACF formation in AOM-induced rats colon.

Increase ACF	**No effect**	**Decrease ACF**
B. distasonis ATCC8503	*B. caccae* JCM9498	***B. fragilis* KYU3**
B. ovatus ATCC8483	*B. eggerthii* ATCC27754	***B. merdae* JCM9497**
B. uniformis BU1001	*B. stercoris* JCM9496	***B. ovatus* KYU4**
B. uniformis	*B. thetaiotaomicron* Werner E-50	***B. thetaiotaomicron* KYU1**
BU1001 (pVAL-1)	*B. uniformis* ATCC8492	***B. uniformis* KYU2**
	B. vulgatus ATCC8482	***B. uniformis*** **BU1001(pVLFK)**

3.9 Effeect of *B. uniformis* KYU 2 harboring pVLFK on theAOM-induced ACF formation

The number of ACF formation in AOM-treated rat is shown in Table3.8. The number of ACF in rat with no treatment (water group) was 111±15 ACF/rat and showed the multiple crypt at 13±3.8 ACF/rat. Similar to *B. uniformis* BU1001, number of ACF in rats treated with *B. uniformis* KYU2 (pVAL-1) was increased compared to the water group. However the number of ACF in rats treated with the culture of *B. uniformis* KYU2 (pVLFK) was decreased from that KYU 2 (pVAL-1) group (16%), and the multiple crypt was also decreased (24%). The significant difference between groups was not observed. Additionally, other independent experiment also demonstrated that *B. uniformis* KYU 2 harboring pVLFK slightly inhibited the formation of ACF in the rat colon when compared with strain KYU 2 (pVAL-1) group (Table 3.9).

Table 3.8 Effects of *B. uniformis* KYU2 cultures on AOM-induced aberrant crypt focus formation in F344 rats (I)

Treatment	ACF/rat	Multiple ACF/rat [a]	AC/F
Water	111±15 [b]	13±3.8	2.3±0.2
B. uniformis KYU2 (pVAL-1)	122±85	13±20	2.0±0.4
B. uniformis KYU2 (pVLFK)	93±51	9.8±6.9	2.3±0.1

a Number of ACF with more than 3 AC per focus.

b Mean±SD.

Table 3.9 Effects of *B. uniformis* KYU2 cultures on AOM-induced aberrant crypt focus formation in F344 rats (II)

Treatment	ACF/rat	Multiple ACF/rat [a]	AC/F
Water	228.4±57.7 [b]	13.6±5.7	1.97±0.14
B. uniformis KYU2	222.4±29.8	7.5±2.3	1.95±0.07
B. uniformis KYU2 (pVAL-1)	282.4±48.4	17.6±7.7	2.00±0.07
B. uniformis KYU2 (pVLFK)	263.7±48.9	20.2±9.9	2.08±0.14

a Number of ACF with more than 3 AC per focus.

b Mean±SD.