Antagonism against *Helicobacter Pylori* and Proteolysis of *Lactobacillus Helveticus* CU631 and Strain Identification

Y. H. Yoon* and B. R. Won

Department of Animal Science, Chung-Ang University, 40-1 Neri, Daedukmyun, Ansungsi, kyunggido 456-756, Korea

ABSTRACT : The antagonistic activities of 30 strains of lactobacilli against *Helicobacter pylori* were determined and *Lactobacillus helveticus* CU631 has been selected as the strain which possesses the strongest inhibitory effect in the disc diffusion assay showing inhibition zone diameter of 10±1.5 mm, whereas those of *L. plantarum* and *L. fermentum* have been shown to be 4.0±0.6 mm. *H. pylori* G88016 revealed the highest vacuolating toxin producing activity among the 8 strains, the inhibitory activity of *L. helveticus* CU631 in vacuolating toxin producing activity of *H. pylori* manifested in the co-culture of two strains and in the 5:5 mixture of supernatant of the two strains. Both *L. helveticus* CU631 and cell free culture supernatant had a strong inhibitory activities in urease and cytotoxin producing activities of *H. pylori* NCTC11637 and CJH12. An accelerated proteolytic activity of water soluble peptides by *L. helveticus* CU631 during the refrigeration storage has been manifested in the cream cheese. DNA seqences of 16S-23S ribosomal RNA spacer region showed typical pattern among the various strains of *L. helveticus,* which could be used in the identification of *L. helveticus* CU 631. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 7 : 1057-1065)***

Key Words : Antagonistic Activity, Lactobacilli, *Helicobacter Pyroli,* Urease, Vacuolatijg Toxicity, 16S-23S R RNA Spacer Gene Sequencing

INTRODUCTION

Lactobacilli have been used since decades against infectious diseases, these bacteria are supposed to compete with other microorganism on mucosal surfaces, a process named bacterial interference. In order to colonize mucosal epitherial surface, lactobacilli need to have some abilities to adhere to epitherial cells, to exclude competitive strains and to produce inhibitory substances. Antagonistic or inhibitory effect of lactic acid bacteria termed as probiotic effects, the term probiotic first used to describe organisms and substances added to animal feed to promote growth (Parker, 1974). This definition has since been broadened to include health promoting actions of microorganisms, possible mechanism of action include acid production (Wendakoon et al., 1998; Fuller, 1991) and other by-products of bacterial metabolism (Streekumar and Hosono, 2001). It has been proposed that lactic acid production by these organisms, unrelated to pH, is responsible for inhibition of *Helicobacter pylori,* the curved Gram negative bacterium that can cause peptic ulcer disease in man (Cover and Blaser, 1992). Considering high rate of infection of approximately 80% with this pathogenic organism in the oriental countries, it is a major public health concern in many part of world (Gill and Desai, 1993; Graham et al., 1991; Midolo et al., 1995). Treatment of *H. pylori* infection with antibiotics does not always eradicate the organism, and antibiotic therapy frequently produces adverse effect (NIH, 1994). *H, pylori* is becoming resistant to a number of antibiotics, particularly to metronidazole and clarythromycin which are currently being used to treat patients with gastric ulcers (Heatley, 1995). alternative forms of effective and simple therpeutical regimens are needed.

It is needed to screen lactobacilli probiotic strains which is antagonistic to *H. pylori* to characterize and identify the strains, the results of *in vitro* inhibitory activity of vacuolating cytotoxin and urease activity by lactobacilli could provide some clues that probiotic organisms may have a role in *H. pylori* treatment both through direct action against the organism and in the lessening of clinical side effects associated with antibiotics. Those informations on proteolysis of water soluble peptides could be applied in utilyzing them as a starter organism in the preparation of fermented dairy products.

Several lactobacilli strains have been identified by DNA sequencing of 16S-23S ribosomal RNA spacer region, this study was conducted to screen strains which have a strong inhibitory effects on *H. pyroli* and to characterize and identify probiotic effects of the strain.

MATERIALS AND METHODS

Organisms and propagation

H. pylori and *Lactobacillus spp.* strains used in this study have been shown in table 1. *Lactobacillus* spp. used in this study were maintained at -80°C in skim

* Address reprint request to Y. H. Yoon. Tel: +82-31-670-3027, Fax: +82-31-675-5216, E-mail: yungh310@post.cau.ac.kr

Received September 13, 2001; Accepted February 15, 2002

milk/glycerol medium, an aliquot of each lactic strain from the stock at -80°C was grown at 37°C in DeMan, Rogosa, Sharpe (MRS) (Difco, Detroit, Mich.) in aerobic condition. Washed cells were prepared from the MRS broth cultures by centrifuging 10,000×g for 2 min and washing the pellet with phosphate buffered saline (PBS pH 7.2) 3 times. The stock cultures of *H. pylori* NCTC 11637, CH7-1, CH333-1, CH578, CJH 12, CJH57, GB8016, GB8037-1 were kept at -80°C in peptone glycerol medium. *H. pylori* were cultured at 37°C in Brucella media (Difco) added with 10% horse serum in the jar with CampyloPak Plus (BBL, USA).

Determination of inhibitory activity of *Lactobacillus spp.* by agar diffusion

Agar diffusion method (Cooper, 1964) were used to determine the antagonistic activity of *lactobacilli* aginst *H. pylori*, washed cells of lactobacilli were diluted with PBS and plated on MRS plates and individual colonies of lactobacilli were appeared on MRS agar plate by incubation at 37°C for 48 h.

The plates with the colonies were allowed to dry, 2 ml of Brucella media added with 10% horse serum containing 0.75% soft agar with *Helicobacter pylori* NCTC11637 was spreaded over the plate and incubated 10% CO_2 incubator at 37°C for 24 h and the diameter of inhibition zone was measured in mm.

Inhibition of urease activity of *H. pylori*

H. pylori was broth cultured for 48 h at 37°C and pelletized at 16,000×g for 5 min. 3 ml of Christensen urea broth containing 0.012 g/L of phenol red was added with 30 μl *H. pylori* in destilled water as a urease control, another cuvette contains 30 μl *H. pylori* and 30 μl of *L. helveticus* CU 631 cell suspension in 3ml of Christensen urea broth containing 0.012 g/L of phenol red, optical density of the media determined at 550 nm wavelenth every 10 min for 3 h by spectrophotometer based on the method described by Coconnier and Lievin (1998). *L. helveticus* CU 631 was cultured in modified MRS broth and pelletized at 16,000×g for 5 min.

Cell culture and Inhibition of vacuolating cytotoxin of *H. pylori* by *L. helveticus* CU 631

RK-13 cells were cultured in RPMI1640 (GIBCO BRL,USA) supplemented with 10% fetal bovine serum in 10% CO2/ 90% air atmosphere at 37°C. *H. pylori* were inoculated in Brucella broth added with 10% horse serum for 7 days at 37°C in microaerobic atmosphere. Vacuolating cytotoxin (VT) activity was determined by Kamiya et al. (1994). 100 μl of RK-13 cell suspension of 3.42×104/ml was put in the each wells of 96 well plate cultured 24 h at under 10% CO_2 atmosphere. On the next day 100 μl of *H. pylori* culture supernatant diluted to 2X, 4X, 8X, 16X, 32X, 64X, 128X were added to each well and incubated 24 h at 37°C. VT titer was determined by the observation of vacuole in the RK cell by invert microscope, and VT titer stands the largest dilution factor which shows vacuole. Inhibitory activity by *L. helveticus* CU 631 was determined by the addition of mixture of *H. pylori* culture supernatant and *L. helveticus* CU 631 supernatant, the ratio of the mixture was 5:5, 6:4, 7:3, 8:2, 9:1.

Proteolytic activity of *L. helveticus* CU 631 by HPLC

Ten grams of cheese slurry were mixed in 20 ml of deionized destilled water in a mixer for 30s, resulting mixtures were tempered in a bath 40°C for 20 min., the extracts were centrifuged at 10,200×g for 35 min (SR20.22, Jouan INC. France) at 9°C. The solidified fat layer was removed and aqueous layer was filtered through glass wool into a clean container. The remaining cheese pellet was reextracted with 20 ml of deionized destilled water and the aquous portin was combined with the previous extract. Peptide profiles of slurries were obtaineed using the following procedure. Ten milligram of freeze dried water soluble extract from each slurry was reconstututed in 1 ml of solvent A and filtered through a 0.45 μm filter. A 50 μl loop was used to introduce the sample onto the HPLC (Model 305;Gilson Co., France). Gradient elution was used in a C 18 analytical column (Lichrospher 100 RP-18, 5 μm (Merck, USA) for which solvent A was 0.1% trifluoroacetic acid in 99.9% HPLC grade water and solvent B was 0.1% trifluoroacetic acid 90% acetonitrile, 9.9% HPLC water.

Peptides were detected using a UV detedctor (UV 119; Gilson, France) at 220 nm, the peptide profile data was processed and stored on a computer.

Identification of the *L. helveticus* CU 631 strain by sequencing of 16S-23S spacer ribosomal RNA and alignment comparison

Overnight culture of *L. helveticus* CU 631 was pelleted and washed twice with 50 mM EDTA, chromosomal DNA was isolated by using the Wizard genomic DNA purification kit (Promega USA), described by Alatossava and Timiskjarvi (1997).

First used to PCR was performed in a DNA thermal cycler 480 (Perkin Elmer, Norwalk) with a AccuPower PCR premix (Bioneer, Korea). Oligonucleotide primer used to amplifying the 16S-23S ribosomal RNA gene spacer region were Hel I 5'-GAAGTGATGGAGAGTAGAGTAG AGATA-3' Hel II 5'-CTCTTCTCGGTCGCCTTG-3' a specific primer for *L. helveticus* used (Alatossava and

Timiskjarvi, 1997). A reaction mixture (50 μl) for PCR of the 16S-23S ribosomal RNA gene spacer region consisted of reaction buffer (end concentration 1.5 mM $MgCl_2$), 200 μM each dNTP, 1 μM of Hel I and Hel II primer, 50 ng of bacterial DNA and 0.6 U of Bioneer DNA polymerase. The amplification profile was at 92°C for 30s, 62°C for 30s, 72°C for 30s. This is repeated for 30 cycles. The program also included a preincubation at 92°C for 2 min before the first cycle and an incubation at 72°C followed by a cooling step down to 4°C after last cycle. Amplification products were analyzed with an agarose gel electrophoresis. Amplified DNA products were then purified free from primers and nucleotide with a AccuPrep PCR purification kit (Bioneer, Korea).

PCR products from the spacer regions were sequenced directly by cycle sequencing method using Automatic seqencer (New England Biolab. Beverly) and radioactive labeling.

Sequences were read and analyzed with Generunner software and compared with those of Genebank.

Statistical analysis

Within the same treatment groupe. for the comparison of the inhibitory halo diameter by lactobacilli strains and comparison of peak number and peak area between the treatments values were compared using SAS Duncan's multiple-range test.

RESULTS AND DISCUSSION

Antagonistic activity of Lactobacilli against *H. pylori*

A total of 30 dairy cultures obtained from either cultured dairy products or various culture collections were screened against 8 strains of *H. pylori*. In the screenig the organism, which showed a clear halo around the lactobacillus colony (figure 1) were taken as positive cultures. The extent of the inhibitory effect was found to be strain dependent. table 1 shows the average radius of inhibition zone of *H. pylori* by *L. helveticus* CU631, a strain *L. acidophilus* CU 620 did not make any inhibiotion zone of *H. pylori*, all the other lactobacilli revealed positive results; made a halo ranged from 3.0 mm to 10.0 mm. *L. helveticus* CU631, *L. helveticus* CU632, *L. paracasei* CU 480 were found to have higher inhibitory action on *H. pylori* NCTC1637. the largest halo diameter was appeared by *L. helveticus* CU631 averaging 10.04±1.51. However one strain of *L. acidophilus* CU 620 did not revealed inhibitory action on *H. pylori* NCTC 1637, *L. delbrueckii*CU 693, *L. fermentum* ATCC 14931 and *L. plantarum* CU722 showed low degree of inhibitory activity of under 4.0 mm. Of the ten strains of *L. acidophilus* tested, nine were found to be inhibitory to *H. pylori* NCTC 1637. Fermented milks are claimed to contain a number of biologically active compounds which may contrubute to the inhibition of bacterial growth. These include diacetyl, organic acids. peptides. bacteriocins and some low molecular weight compounds (Bernet-Camard et al., 1997). The effect of probiotic organism such as *L. acidophilus* on *H. pylori* in the gastric mucosa remains to be elucidated. The acid resistance of *Lactobacillus* helveticus CU 631 demonstrated by the survival in skim milk broth below pH 3.5. and production of organic acid could be the the base of antagonism.

Inhibitin of urease and vacuolating cytotoxin production activity of *L. helveticus* CU 631

The effects of *L. helveticus* CU631 culture and cell free culture supernatant on the urease activity of *H. pylori* NCTC11637 were measured as shown in figure 2A.

Urease activity is proportional to the red color intensity (550 nm), urea of the Christensen urea broth is turned into ammonia by the urease on culture.

A prominent urease activity was shown in *H. pylori* NCTC11637 and CJH12. the activity was strain dependent, hence the two strains were used to the urease inhibition test by *L. helveticus* CU631. The urease activity of *H. pylori* NCTC11637 was inhibited prominently by both *L. helveticus* CU631 culture and culture supernatant of *L. helveticus* CU631 (figure 2B). The inhibitory effect of *helveticus* CU631 on *H. pylori* NCTC11637 urease activity was heat stable. Coconier and Lievin (1998) claimed that lactic acid does not participate in the action of *L. acidophilus* against *H. pylori* urease. The urease of *H.*

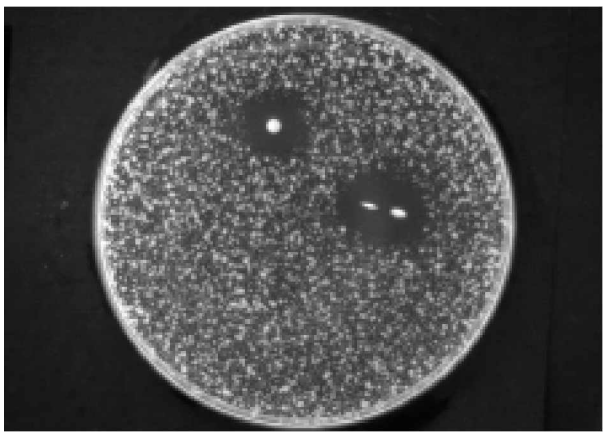


Figure 1. Inhibition zone surrounding *L. helveticus* CU631 against *H. pylori* NCTC11637.

Table 1. Inhibition of *H. pylori* NCTC11637 by *Lactobacillus* spp. and source of strains (diameter:mm)

Species	Strain	Inbibition zone (mm)		Source	Origin
		SM*	SD**		
L. acidophilus	CU620	Non-inhibition		Lab. Dairy biol. Chung-Ang Univ.	Biograde Yogur (Netherland)
L. acidophilus	CU681	6.15^{de}	0.91	"	Denliska Kefir (Sweden)
L. acidophilus	CU674	5.16^{fghij}	0.40	"	Yosa (Finland)
L. acidophilus	CU460	4.5^{jkl}	0.57	"	Joghurt (Germany)
L. acidophilus	CU711	4.72^{ijk}	0.44	"	Actimel (Spain)
L. acidophilus	CU683	5.3^{fghi}	0.61	"	Yoggit acidophilus (Sweden)
L. acidophilus	CU721	6.16^{de}	0.95	"	Biogurt PUR (Germany)
L. acidophilus	CU677	5.57^{efgh}	0.60	"	Valiogurtii (Finland)
L. acidophilus	CU723	6.87^{e}	0.83	"	Joghurt (Germany)
L. acidophilus	KCTC2182	4.4^{jkl}	0.31	Korea Food Research Institute	
L. brevis	ATCC8287	4.41^{jkl}	0.54	"	
L. casei	IFO3533	4.85^{hijk}	0.35	"	
L. casei	KCTC2680	4.44^{jkl}	0.38	"	
L. dekbruekii subsp. delbruekii	ATCC9469	5.1^{ghil}	0.22	"	
L. delbruekii	CU693	3.0^{m}	0.49	Lab. Dairy Microbiol. Chung-Ang Univ.	Biola (Norway)
L. delbruekii	CU632	8.3^{b}	0.53	"	Kefir (Netherland)
L. delbruekii	CU660	5.16^{fghij}	0.49	"	Yoggurt (Netherland)
L. delbruekii	NRRLB763	4.80^{ijk}	0.55	Korea Food Research Institute	
L. fermentum	ATCC14931	3.91	0.62	"	
L. helveticus	CU631	10.04^{a}	1.51	Lab. Dairy Microbiol. Chung-Ang Univ.	Kefir (Netherland)
L. paracasei	CU580	6.76^{cd}	1.12	"	La laitiere (France)
L. paracasei	CU702	5.76^{efg}	0.56	"	Yogurt (Denmark)
L. paracasei	CU480	8.55^{b}	0.76	"	Biogurt (Austria)
L. plantarum	KCTC1048	5^{hij}	0.37	Korea Food Research Institute	
L. plantarum	CU685	5.87^{ef}	0.58	Lab. Dairy Microbiol. Chung-Ang Univ.	Filmjolk (Sweden)
L. plantarum	CU722	4.21^{kl}	0.39	"	Almighurt (Germany)
L. plantarum	CU731	4.43^{jkl}	0.62	"	B10 cottage cheese (Egland)

* SM: Sample mean.

** SD: Standard deviation.

[a-m] Values with different superscripts differ at $p<0.05$.

pylori is a surface protein component of *H. pylori* producing ammonia which allow survival by neutralizing the acidic environment of stomach. (Arthurton et al.. 1995).

Vacuolating cytotoxin production is encoded by 259 bp *VacA* gene and the presence of the gene in all the test strains was evidenced by the PCR amplification results (Atherton et al.. 1995). VT titer was determined by the observation of vacuole in the RK cell by invert microscope. figure 3A

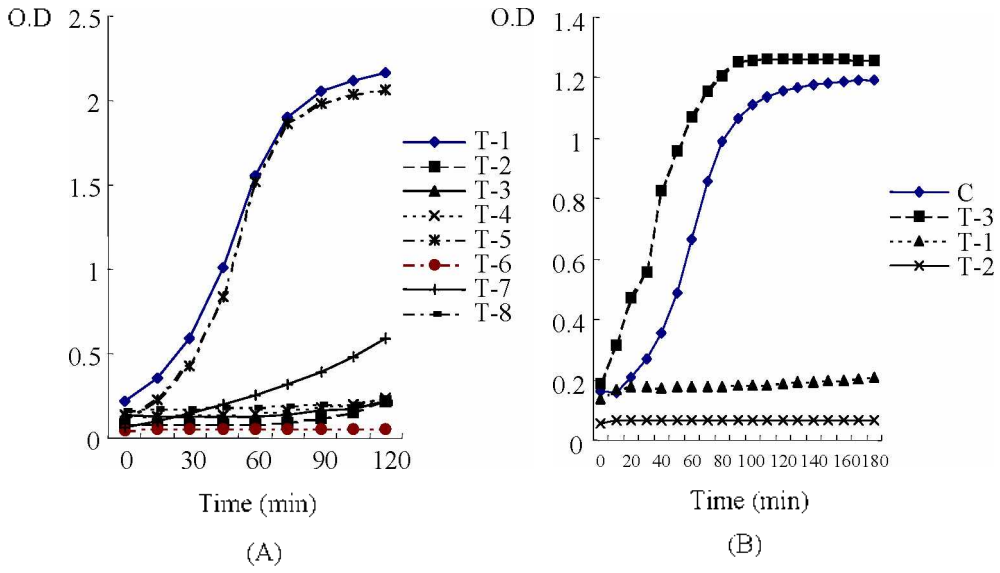


Figure 2(A). Urease activity of *H.pylori* strains.
T-1: *H. pylori* NCTC11637, T-2: *H. pylori* CH7-1, T-3: *H. pylori* CH333-1, T-4: *H. pylori* CH578, T-5: *H. pylori* CJH12, T-6: *H. pylori* CJH57, T-7: *H. pylori* G88016, T-8: *H. pylori* G88037-1
(B). *In vitro* activity of *L. helveticus* CU631 and *L. helveticus* CU631 culture supernatant against urease activity of *H. pylori* NCTC1163.
C: *H. pylori* NCTC11637, T-1: *H. pylori* NCTC 11637 and *L. helveticus* CU631, T-2: *H. pylori* NCTC 11637 and *L. helveticus* CU631 culture supernatant, T-3: Modified MRS.

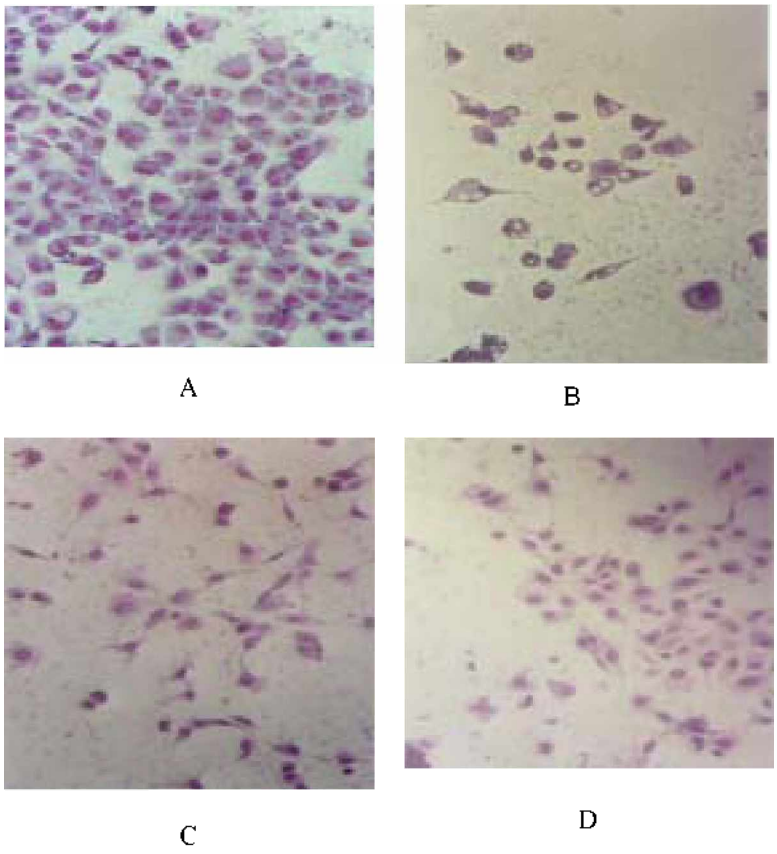
Table 2. Effect of *L.helveticus* CU631 and culture supernatatnt on the Vacuolating Toxin (VT)titer of *H. pylori* G88016 *in vitro*

Treat	Vacuolating cytotoxin (VT) titer					
no*.	1X	2X	4X	8X	16X	32X
1	+	+	+	+	-	-
2	+	+	-	-	-	-
3	+	+	+	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	+	+	+	-	-	-
7	+	+	+	-	-	-
8	+	+	+	-	-	-

Legend*
1: Control.
2: Control+*L.helveticus* CU631.
3: Contorl+*L. helveticus* CU631 culture supernatant.
4: *H. pylori* G88016 supernatant : *L. helveticus* CU631 culture supernatant=5:5.
5: *H.pylori* G88016 supernatant : *L. helveticus* CU631 culture supernatantt=6:4.
6: *H. pylori* G88016 supernatant : *L. helveticus* CU631 culture supernatant=7:3.
7: H. *pylori* G88016 supernatant : *L. helveticus* CU631 culture supernatant=8:2.
8: *H. pylori* G88016 supernatant : *L. helveticus* CU631 culture supernatant=9:1.

shows the RK cell which reveals no vacuolation in the cell when *H. pyroli* culture supernatant was not added. in figure 3B a prominant vacuole formation are found in the case *H. pyroli* culture supernatant was added, figure 3C and D shows the inhibitory activity of *L. helveticus* to form the vacuoles in RK cell by the addition of *H. pyroli* culture supernatant with the disappearance of vacuoles. and VT titer stands the largest dilution factor which shows vacuole. VT titer of *H. pylori* G88016 have been shown to be 8x. all the other strains showed 2x (table 2). the VT titer of *H. pylori* strains was strain dependent. *H. pylori* G88016 were selected to be used for the determination of inhibitory activity by *L. helveticus* CU 631.

Using RK -13 cell lines, the inhibitory activity of *L. helveticus* CU631 and *L.helveticus* CU631 culture supernatant on the vacuolating cytotoxin activity of *H.pylori* G88016 *in vitro* was shown in table 2. Inhibitory activity by *L. helveticus* CU 631 was determined by the addition of *H. pylori* culture supernatant and *L. helveticus* CU 631 supernatant, a prominent inhibition of VT of *H. pylori* by *L. helveticus* CU 631 has been revealed; an additon of 10% *L. helveticus* CU 631 supernatant to *H. pylori* culture supernatant inhibited the vacuole formation and caused to decrease VT titer from 8x to 4x which stands 50% inhibition (table 2). Vacuole in the cytoplasm of RK



A: Vacuolating toxin Negative (*H. pylori* supernatant not added)
B: Vacuolating toxin Positive (*H. pylori* supernatant 100 μl added)
C: *H. pylori* supernatant: *L. helveticus* CU631 supernatant=5:5
D: *H. pylori* supernatant: *L. helveticus* CU631 supernatant=6:4

Figure 3. Vacuolation of RK-13 cell by Vacuolating toxin produced by *H. pylori* G88016 and inhibitory effect of vacuolationg toxin activity of *H. pylori* G88016 by *L. helveticus* CU631 culture supernatant. (Giemsa-staining: 10 × 10).

cell formed due to the presence of approxomately 500,000 dalton protein (Leunk et al., 1988). RK cell line is very sensitive to vacuole formation which forms vacuole under the induction of both cytotoxin m1 and m2, as the cytotoxin known as vacuolating toxin is produced *in vivo* by the presence of antibody to vaculoating toxin in the sera of patients infected with *H. pylori*, this toxin has been proposed as a new virulence factor of *H. pylori*. (Pagliaccia et al., 1998).

Proteolytic activities of *L. helveticus* CU 631

Water soluble peptide pattern of cream cheese and *L. helveticus* CU631 culture added probiotic cream cheese has been determined by HPLC and the results were shown in figure 4 and in table 3, we evaluated total peaks and total peak areas of the chromatograms to determine whether *L. helveticus* CU 631 had an impact on these parameter. The HPLC analysis of water-soluble extracts showed some differences between with and without *L. helveticus* CU 631 products. In all of the chromatograms, there are noticeable differences in the heights of peaks and differences in the number of small peaks collected between 20 to 50 min. Comparions of the water soluble peptide pattern between cream cheese with *L. helveticus* CU 631 resulted the number of peptide peaks and peak area from the cream

Table 3. Total number of peaks and total peak areas of peptides determined by HPLC for probiotic cream cheeses from 1 to 4 weeks of ripening at 4°C.

Number	Ripening time (week)	Total peaks[2]	Total peak areas[1, 2]
C-1	1	80[a]	2.0[a]
	4	86[a]	2.9[a]
C-3	1	85[a]	2.9[a]
	4	100[b]	4.5[b]

[1] Arbitrary units (×10).
[2] Mean values in column with differnet superscript letters differ ($p<0.05$).

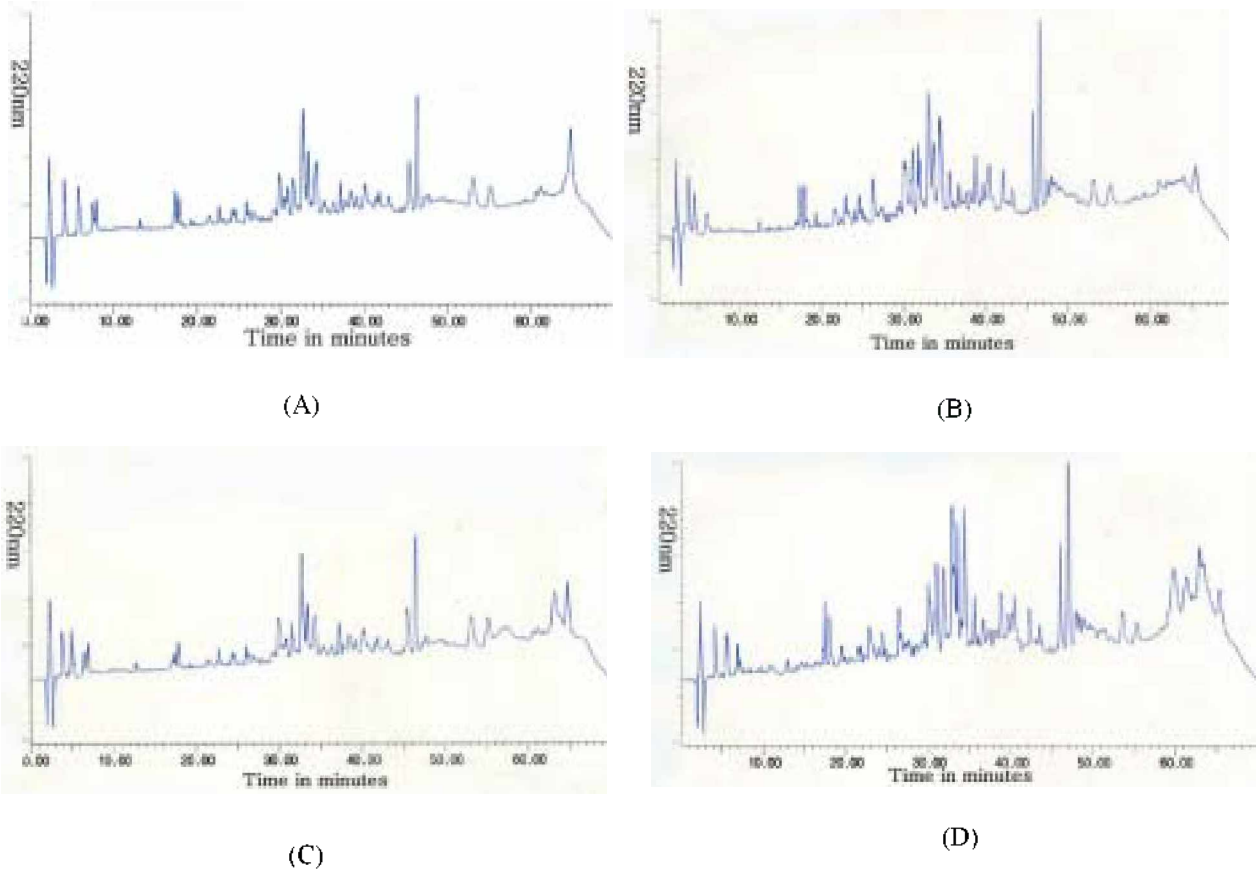


Figure 4. HPLC chromatograms of peptide profiles (Water-soluble extract) from C-3 probiotic cream cheese ripened for at 4°C for 4week. (A) C-1 (1week) (B) C-1 (4week) (C) C-3 (1week) (D) C-3 (4week).

cheese without *L. helveticus* CU 631 were 80 and 2.0 arbitrary unit whereas those with *L. helveticus* CU 631 were 85 and 2.9 respectively, which indicates peptidases originated from *L. helveticus* CU 631 proceeded intensive proteolysis. During the ripening of 4 weeks at 4°C the viable counts increased to 3.3×107 cfu/g of *L. helveticus* CU 631, the number of peptide peaks and peak area from the probiotic cream cheese were 100 and 4.5. arbitrary unit respectively ($p<0.05$) whereas those without *L. helveticus* CU 631 were 86 and 2.9 respectively. Statistically significant differences were observed in total peak areas and total number of peaks in terms of ripening. Table 3 shows that as the sllurries ripened, and as the *L. helveticus* CU 631 added to the slurries, the total peak area increased, and the total number of peaks increased, indicating a greater quantity of peptides or compounds being absorbed at 220 nm. The height differnces indicate variation in the quantity of specific peptides and represent the proteolytic ability to release greater amounts of specific peptides from protein (Muehlenkamp-Ulate; 1999). Each peak may represent more than one peptide because of coelution, and other methods could be needed to separate these compounds, the identities of the peaks were not determined in this study. The results of this studies are in agreement with Muehlenkamp-ulate (1999) and Fenelon (2000).

Identification of the *L. helveticus* CU 631 strain by sequencing of 16S-23S spacer ribosomal RNA

DNA seqences of 16S-23S ribosomal RNA spacer region of *L. helveticus* CU 631 and three other *L. helveticus* strains from gene bank and accession number have been shown in figure 5. The size of 16S-23S ribosomal RNA spacer region of *L. helveticus* CU 631 was shown to be 253 bp, those of *L. helveticus* AF182726, U32970, and Z75483 were 660 bp, 205 bp and 204 bp respectively. The heterogeneity in the sequences was typical: there was no conserved region. It is reported that spacer sequences of lactic acid bacterial strains contain three conserved region and variable region (Alatossava and Timiskajarvi, 1997). Hence for the identification of *L. helveticus* strain, determination of DNA seqences of 16S-23S ribosomal RNA spacer region could provide an evidence.

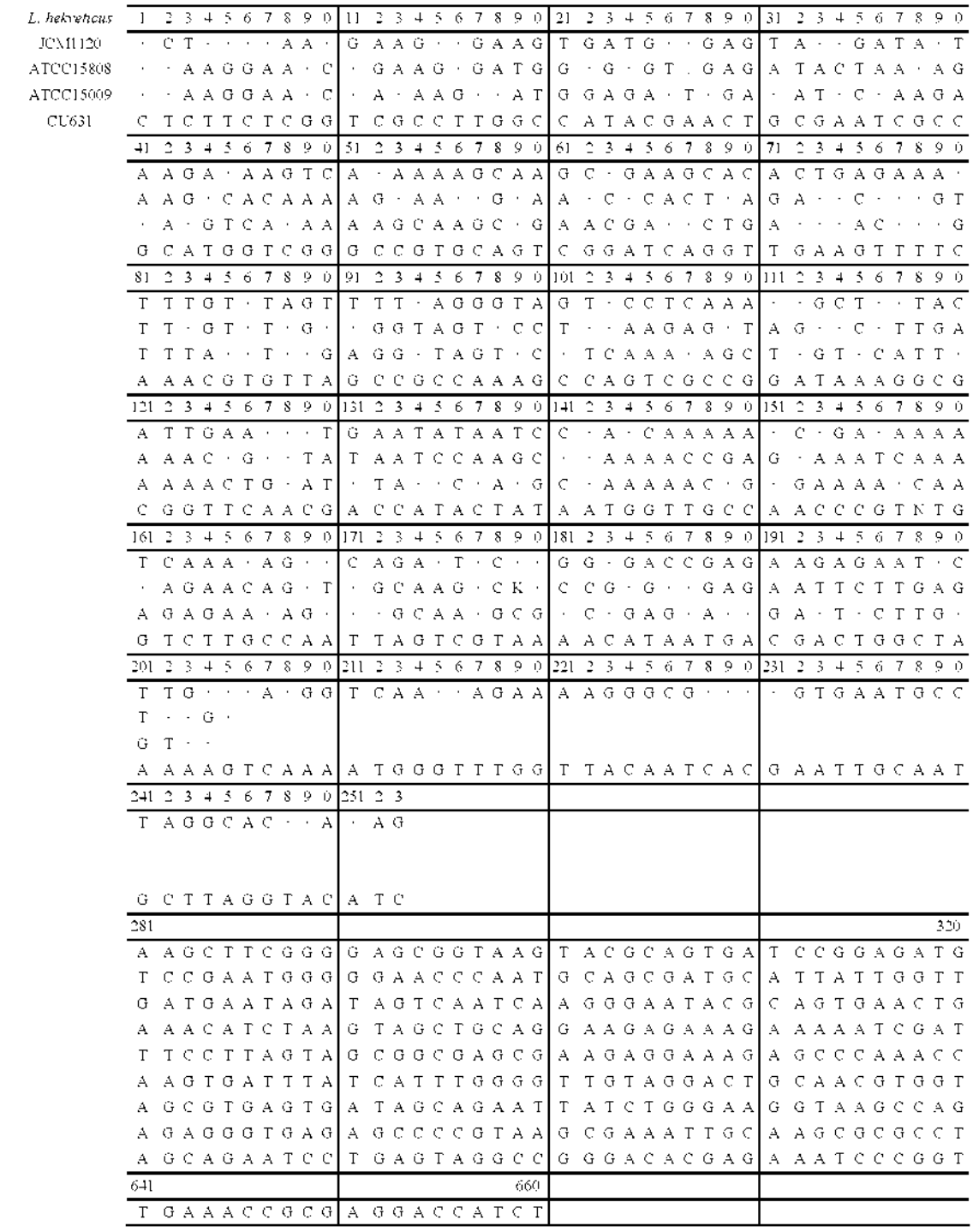


Figure 5. Alignment of nucleotide sequence of 16S-23S r RNA spacer region of *L.helveticus* CU 631 with JCM1120 (Songa et al., 2000), ATCC15808 (Alatossava and Timiskajarvi, 1997) and ATCC15009 (ATCC15009).

REFERENCE

Alatossava, T. and A. T. Timiskajarvi. 1997. Development of oligonucleotide, primers from the 16S-23S r RNA intergenic sequences for identifying different dairy and probiotic lactic acid bacteria by PCR. Intrnational J. Food Microbiol. 35:49-56.

Atherton, J. C., P. Cao, R. M. Peek, Jr., M. K. Tummuru, M. J. Blaserand and T. L. Cover. 1995. Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific VacA types with cytotoxin pro duction and peptic ulceration. J. Biol. Chem. 270:17771-17777.

Bernet-Camard, M., V. Lievin, J. N. Brassart and A. Servin. 1997. The human *Lactobacillus acidophilus* strain LA1 secrets a nonbacteriocin antibacterial substance(s) active *in vitro* and

in vivo. Appli. Environ. Microbiol. 63(7):2747-2753.

Coconnier, M. H. and V. Lievin. 1998. Antagonistic activity against *Helicobacter pylori* infection *in vitro* and *in vivo* by the human *Lactobacillus acidophilus* strain LB. Appl. Enviromental Microbiol. 64(11):4573-4580.

Cooper, K. E. The theory of antibiotic inhibition zone . 1964. In "Analytical Microbiology" (Ed. F. Kavanaugh). Academic Press, Inc. New York. pp. 1-86.

Cover, T. L. and M. J. Blaser. 1992. *Helicobacter pylori* and gastroduodenal disease. Ann. Rev. Med. 43:135-145.

Fuller, R. 1991. Probiotics in human medicine. 32:439-442.

Gill, H. H. and H. G. Desai. 1993. J. Clinical Enterol. 16(1):6-9.

Graham, D. Y., H. M. Malaty, D. G. Evans, D. J. Evans, P. D. Klein and E. Adam. 1991. Gastroenterol. 100:1495-1501.

Heatly, R. V. 1995. The Helicobacter Handbook, Blackwell Science Ltd.

Kamiya, S., M. Kai, A. Ozawa, H. Kobayashi, T. Shirai, S. Harasawa and T. Miwa. 1994. Characteristics of vacuolating toxin produced by *Helicobacter pylori*. European J. Gastroenterology & Hepatology. 6(suppl. 1):S23-27.

Leunk, R. D., P. T. Johnson and B. C. David. 1988. Cytotoxin activity in broth-culture filtrates of *Campylobacter pylori*. J. Med Microviol. 26(2):93-94.

Midolo, P. D., J. R. Lambert, R. Hull, F. Luo and M. L. Grayson. 1995. *In vitro* inhibition of *Helicobacter pylori* NCTC11637 by organic acid and lactic acid bacteria. J. applied bacteriol. (79):475-479.

Muehlenkamp-ulate, M. R. and J. J. Warthesen. 1999. Evaluation of Several Nonstarter *Lactobacilli* for Their Influence on Cheddar Cheese Slurry Proteolysis. J. Dairy Sci. 82:1370-1378.

National Institute of Health. 1994. NIH Consensus Statement 12(1)

Parker, R. B. 1974. Probiotics: The other half of the antibiotics story. Animal nutrition and Health. (29):4-8.

Pagliaccia, C., de M. Bernard, P. Luoetti, X. Ji, D. Burroni, T. L. Cover, E. Papini, R. Rappuoli, J. L. Telford and J. M. Reyrat. 1998. The m2 form of *Helicobacter pylori* cytotoxin has cell type-specific vacuolating activity. Proc. Natl Acad. Sci. USA. 95:10212-102278.

Songa, Y., N. Kato, C. Liua, Y. Matsumiyaa H. Katob and K. Watanabe. 2000. Rapid identification of 11 human intestinal lactobacillus species by multiplex PCR assays using groupe - and species -specific primers derived from the 16S-23S r RNA intergenic spacer region and its flanking 23S rRNA. FEMS Micrbiol. Lett. 187(2):167-173.

Streekumar, O. and A. Hosono. 2001. Antimutagenic Properties fo Fermented Milk. Asian-Aust. J. Anim. Sci.14:218-231.

Wendakoon, C. N., W. Fedio, A. Macleod and L. Ozimek. 1998. *In vitro* inhibition of *Helicobacter pylori* by dairy stater cultures. Milchwissenschaft. Milk science international. 53(9):499-502.