Characterization of *Lactobacillus acidophilus* Isolated from Piglets and Chicken

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ABSTRACT : Lactic acid bacteria were isolated from piglets and chicken and characterized. Lactic acid bacteria showing resistance to low pH and bile, adhesion to intestinal epithelium cells, and the inhibition of *Escherichia coli* and *Salmonella* spp. were identified as *Lactobacillus acidophilus*. *L. acidophilus* PF01 survived for 2 h in MRS broth adjusted to pH 2. *L. acidophilus* CF07 was less resistant than *L. acidophilus* PF01 to pH 2, but survived at pH 2.5 for 2 h. Both of isolates were able to grow in MRS broth containing 0.3% (w/v) bile, with *L. acidophilus* CF07 being more tolerant to bile than *L. acidophilus* PF01. *L. acidophilus* PF01 and CF07 adhered specifically to the duodenal and jejunal epithelium cells of piglet, and the cecal and duodenal epithelium cells of chicken, respectively. Both of isolates did not adhere to the epithelium cells of the various animal intestines from which they were isolated. When *L. acidophilus* was cultured with *E. coli* and *Salmonella* spp. in MRS broth, MRS broth containing 2% skim milk powder or modified tryptic soy broth at 37°C, *L. acidophilus* PF01 and CF07 inhibited the growths of *E. coli* K88 and K99, and *S. enteritidis* and *S. typhimurium*, respectively. Both of isolates were found to possess the essential characteristics of probiotic lactic acid bacteria for piglet and chicken. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 12 :1790-1797)***

Key Words : Adhesion, Acid tolerance, Bile tolerance, Lactic acid bacteria, *Lactobacillus acidophilus*, Probiotics

INTRODUCTION

Probiotic lactic acid bacteria are presently the only choice available for replacing the antibiotics used universally by the feed industries (Jin et al., 1996a). They enhance the growth and health of animals, and maintain normal intestinal microflora through competitive exclusion and antagonistic action against pathogens in the intestines of animals (Fuller, 1989). The inhibitory activity against intestinal pathogens is mostly due to the metabolites, such as organic acids and hydrogen peroxide, and other inhibitory substances such as bacteriocins produced by the probiotic bacteria (Gilliland and Speck, 1977; Fuller, 1989). Lactic acid bacteria are commonly used in most probiotics preparations due to the historical belief that they are desirable members of the intestinal microflora and are thus generally regarded as safe (Tannock, 1997). The species currently being used in probiotic preparations are *Lactobacillus bulgaricus*, *L. acidophilus*, *L. casei*, *L. helveticus*, *L. lactis*, *L. salivarius*, *L. plantarum*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Ent. faecalis*, and *Bifidobacterium* spp. (Fuller, 1989). However, not all lactic acid bacteria have the probiotic function, and the primary characteristics required for the candidate probiotics are the ability to survive in the acidic stomach and establish in the digestive tract of the host (Conway et al., 1987; Lee and Salminen, 1995). In this study, we studied the characteristics of several *L. acidophilus* isolates from piglet and chicken in an attempt to discover promising lactic acid bacterial candidates for preparation of animal probiotics.

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MATERIALS AND METHODS

The bacteria

Lactic acid bacteria were isolated from the fecal samples of piglets and chicken aseptically collected from farms in Suwon, Kyounggi-do using sterile swabs. Selective medium for lactic acid bacteria was prepared by adding 0.02% (w/v) sodium azide into MRS broth (Difco Laboratories, Detroit, MI) (De Man, Rogosa, and Sharpe, 1960) adjusted to pH 5.5 using 1 N HCl. The sterile swabs with fecal samples were aseptically put into the acidified MRS broth kept in an ice box and transported to the laboratory. The sample tubes were incubated at 37°C for 24 h. Each sample culture was streaked on MRS agar (Difco) plates containing 0.02% (w/v) sodium azide and incubated at 37°C for 48 h. Typical colonies grown on MRS-azide agar plates were picked and checked for purity on the same medium. Subsequently, the isolates were inoculated into MRS broth and incubated at 37°C for 24 h. The culture broth was centrifuged at 7,000 rpm for 20 min, and the cells were suspended in 10% (w/v) skim milk (2.5 ml) and MRS broth (2.5 ml), and kept frozen at -60°C. All cultures were maintained by subculturing the isolates from piglet and chicken twice a month in MRS broth at 37

and 42°C, respectively, for 18 h before experimental use.

Escherichia coli K88 (F4) and K99 (F5), *Salmonella enteritidis* ATCC 13076, and *S. typhimurium* ATCC 14028 were donated by Lab. of Veterinary Pathology, College of Veterinary Medicine, Seoul National University. These cultures were maintained by subculturing in tryptic soy broth (TSB) (Difco) at 37°C.

Identification of lactic acid bacterial isolates

Lactic acid bacteria growing well in MRS broth adjusted to pH 4.0 as well as containing 0.2% (w/v) porcine bile extract (Sigma Chemical co., St. Louis, MO) or 0.2% (w/v) oxgall (Difco) were selected and used for further study. Identification of lactic acid bacterial isolates was based upon the species description in the Bergey's Manual (Kandler and Weiss, 1986). Utilization of carbohydrates by lactic acid bacteria was assayed using API kit (API 50 CHL, bioMèrieux, France), and the results were analyzed using the APILAB Plus software (Ver. 3.3.3; bioMèrieux, France).

Characteristics of lactic acid bacteria

Each overnight culture (10^4 CFU/ml) of lactic acid bacteria in MRS broth was inoculated into new MRS broth, incubated at 32, 37, 42, and 47°C, and plate-counted on MRS agar. Growth of lactic acid bacteria at various temperatures was analyzed using IFR MicroFit software (Ver. 1.0; UK Ministry of Agriculture, Fisheries and Foods) based on the model of Baranyi and Roberts (1994).

Survival of lactic acid bacteria isolated from piglets in MRS broth adjusted to pH 2 with 1 N HCl at 37°C was studied. Culture samples were taken every 20 min up to 2 h of incubation to count surviving cells. The culture samples were diluted serially in 0.1% (w/v) peptone solution, and 0.1 ml aliquots of appropriate dilutions were spread on MRS agar, which were then incubated at 37°C for 48 h. Survival of chicken isolates in MRS broth adjusted between pH 2 to 4 with 1 N HCl was determined. The surviving cells were counted after grown on MRS agar at 42°C, as described for lactic acid bacteria from piglets.

Lactic acid bacteria from piglets were inoculated into MRS broth containing 0.1, 0.3, and 0.5% (w/v) bile extracts (Sigma), and incubated at 37°C for 24 h. The surviving cells after bile challenge were tested for bile tolerance as described above for the acid tolerance. Bile tolerance of lactic acid bacteria from chicken was tested in MRS broth containing 0.3, 0.4, and 0.5% (w/v) oxgall (Difco) at 42°C using the same procedures as those for piglets. To estimate the growth inhibition of lactic acid bacterial isolates by bile, the inhibition coefficient (C-Inh) was calculated using the modified method of Gopal et al. (1996). Log_{10} numbers of viable cells of lactic acid bacterial isolates were calculated at the end of the exponential growth phase, and C-Inh was calculated according to the following formula.

C-Inh=(A–B):A

where, A is Log_{10} number of viable cells without bile, and B is Log_{10} number of viable cells with bile

Adhesions of lactic acid bacterial cells to the intestinal epithelium cells

The adhesions of lactic acid bacterial cells to the intestinal epithelium cells of piglets and chicken were examined *in vitro*. Suspensions of the intestinal epithelium cells of piglets and chicken were prepared using the method of Mäyrä-Mäkinen et al. (1983). The duodenum and jejunum of piglets were cut into 2×4 cm pieces, and the cecum and duodenum of chicken into 1×4 cm pieces. The epithelium tissue samples were held in buffered phosphate saline (PBS) at 4°C for 30 min to loosen the surface mucus, and washed seven times with PBS. Surfaces of epithelium tissues were scraped off with the edge of a sterile microscope slide, and the epithelium cells were suspended in PBS. All cell suspensions were examined microscopically to verify the removal of adherent bacteria. The adhesion of the lactic acid bacterial cells was tested using the method of Fuller et al. (1978). Each overnight culture broth of *L. acidophilus* cell was centrifuged at 6,000 rpm for 15 min at 4°C, and the cells (10^8 CFU/ml) were suspended in PBS. The epithelium cell suspension (1 ml) was then mixed with the lactic acid bacterial cell suspension (1ml), and the tubes containing the cell mixture were rotated at 20 rpm/min at 37°C for 30 min. Adhesion of the bacterial cells to the epithelium cells was examined using light microscopy (×1.500) (AXIOPHOT; Zeiss, Germany) after Gram-staining.

Inhibitions of *E. coli* and *Salmonella* spp. by lactic acid bacteria

Inhibitions of *E. coli* and *Salmonella* spp. by lactic acid bacteria were examined using the method of Ahn et al. (1997). MRS broth containing lactic acid bacteria (10^5 CFU/ml) were incubated with the same number of *E. coli* or *Salmonella* spp. cells at 37 and 42°C for isolates from piglet and chicken, respectively. Viable cells of lactic acid bacteria, *E. coli*, and *Salmonella* spp. on MRS agar containing 0.02% (w/v) sodium azide, EMB agar (Difco), and bismuth sulfite agar (Difco), respectively, were determined as follows. Serial dilutions of the bacterial suspensions were prepared in 0.1% (w/v) peptone water. Aliquots of the dilutions were plated out on each selective medium mentioned above and were incubated at 37 or 42°C for 48 h before counting typical colonies.

RESULTS AND DISCUSSION

Identification of lactic acid bacterial isolates

Among the lactic acid bacterial isolates from piglets and chicken, two strains able to grow well in MRS broth adjusted to pH 4.0 as well as containing 0.2% (w/v) bile extract or 0.2% (w/v) oxgall were chosen for further study. Based upon the Bergey's Manual (Kandler and Weiss, 1986) and the utilization of carbohydrates, they were identified as *Lactobacillus acidophilus*: *L. acidophilus* PF01 and CF07 from piglet and chicken. Both of *L. acidophilus* strains were Gram positive, homofermentative, catalase-negative, and non-sporeforming rods. Their characteristics and fermentation of carbohydrates are listed in Tables 1 and 2, respectively. Their carbohydrates fermentation gave the same patterns with the exception of mannose, salicin and trehalose: *L. acidophilus* PF01 fermented trehalose but did not ferment mannose and salicin, and *L. acidophilus* CF07 fermented mannose and salicin but not trehalose.

Growth of *L. acidophilus*

In order to survive and thrive in the gastrointestinal tract of the host, a short generation time is of utmost importance; otherwise the bacteria are washed off by the contractive motion of the gut (Havenaar et al., 1992). The doubling times of *L. acidophilus* PF01 and CF07 were estimated to be 1.71 and 1.07 (32°C), 0.52 and 0.57 (37°C), 0.44 and 0.47 (42°C), and 1.46 and 0.74 h (47°C), respectively. The two isolates generally grew slightly faster at 42°C than at 37°C. *L. acidophilus* PF01 isolate grew better near its optimum temperature (between 37 and 42°C), whereas *L. acidophilus* CF07 was able to grow well at broader range of temperatures and grew slightly better at above the temperature considered to be the optimum for *L. acidophilus* (Figure 1). This difference in growth temperatures is probably due to the adaptation of the bacteria to the body temperature of the host. That is, *L. acidophilus* CF07 isolate from chicken grew better at the body temperature of chicken.

Table 1. Characteristics of *L. acidophilus* from piglet and chicken

	Lactobacillus strains	
	L. acidophilus PF01[a]	*L. acidophilus* CF07[b]
Growth at 45°C	+++[c]	+++[c]
Growth at 15°C	-[d]	-[d]
Growth at pH 9.6	+++[c]	+++[c]
Growth at pH 4.0	+++[c]	+++[c]
Gas from glucose	-[e]	-[e]
Catalase production	-[e]	-[e]

L. acidophilus PF01[a], isolated from piglet; *L. acidophilus* CF07[b], isolated from chicken; +~+[c], heavy growth; -[d], no growth; -[e], not produced.

Table 2. Utilization of carbohydrates by *L. acidophilus* from piglet and chicken

Carbohydrates	*L. acidophilus* PF01	*L. acidophilus* CF07
Amygdalin	-	-
Arabinose	-	-
Cellobiose	+	+
Esculin	+	+
Fructose	+	+
Galactose	-	-
Glucose	+	+
Gluconate	-	-
Lactose	+	+
Maltose	+	+
Mannitol	-	-
Mannose	-	+
Melezitose	-	-
Melibiose	-	-
Raffinose	+	+
Rhamnose	-	-
Ribose	-	-
Salicin	-	+
Sorbitol	-	-
Sucrose	+	+
Trehalose	+	-
Xylose	-	-

+: Fermented, -: Not fermented.

Acid tolerance of *L. acidophilus*

L. acidophilus PF01 from piglet survived for 2 h in MRS broth acidified to pH 2.0 (Figure 2a), while *L. acidophilus* CF07 survived at pH 2.5 for 2 h (Figure 2b). The microorganisms in the stomach are generally inhibited by the gastric acid, which contains hydrochloric acid (Gilliland, 1979). Conway et al. (1987) claimed that microbial cultures for use as probiotics should be screened for their resistance to strong acidity. *L. acidophilus* is generally more resistant to low pH than other lactobacilli (Pettersson et al., 1983; Ahn et al., 1999). Ahn et al. (1999) reported that most *L. acidophilus* isolated from fermented milk products on Korean markets survived well (>90%) for 2 h in 12% (w/v) skim milk adjusted to pH 2.5 except *L. casei* ssp. *rhamnosus*. However, Jin et al. (1998) reported variations in the survival of the lactic acid bacteria at low pH depended on the origin of *L. acidophilus* strains, and those living in the cecum showed better survival rate in acidic environment than those from the ileum.

Bile tolerance of *L. acidophilus*

Bile tolerances of *L. acidophilus* PF01 and CF07 were investigated, and the results are shown in Figure 3. Although the growths of *L. acidophilus* PF01 and CF07 decreased with increasing concentration of bile extract or oxgall, both isolates grew well in the presence of 0.3% (w/v) bile in MRS broth. The inhibition coefficients (C-Inh) of *L. acidophilus* PF01 in the presence of bile extract were 0.11 (0.1%), 0.25 (0.3%), and 0.39 (0.5%), whereas those

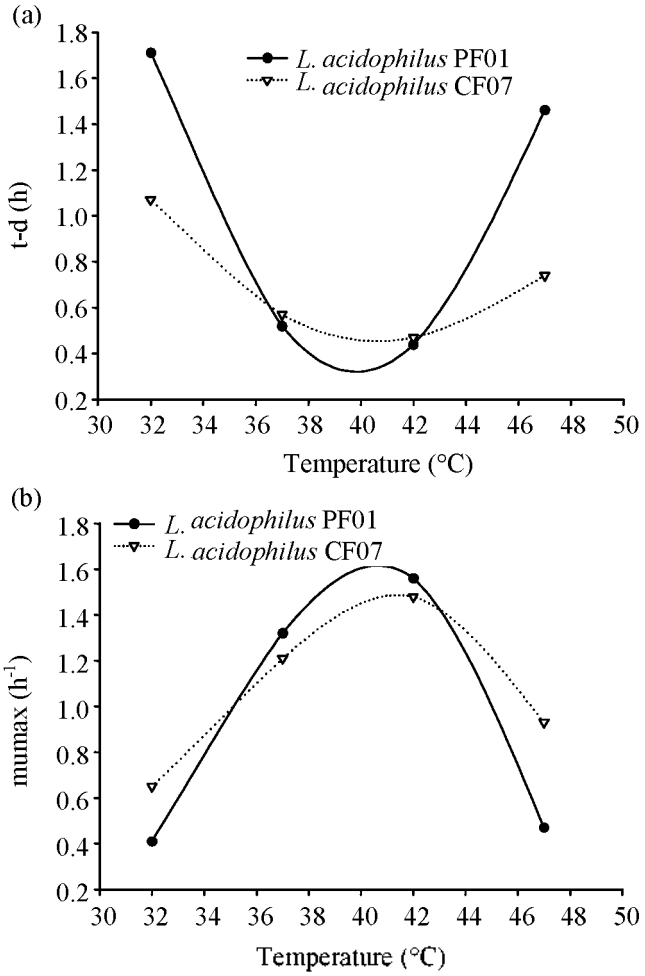

Figure 1. Doubling time (a) and maximum specific growth rate (b) of *L. acidophilus* PF01 and CF07 at various temperatures in MRS broth.
t-d, doubling time (h); mumax, maximum specific growth rate (h^{-1}).

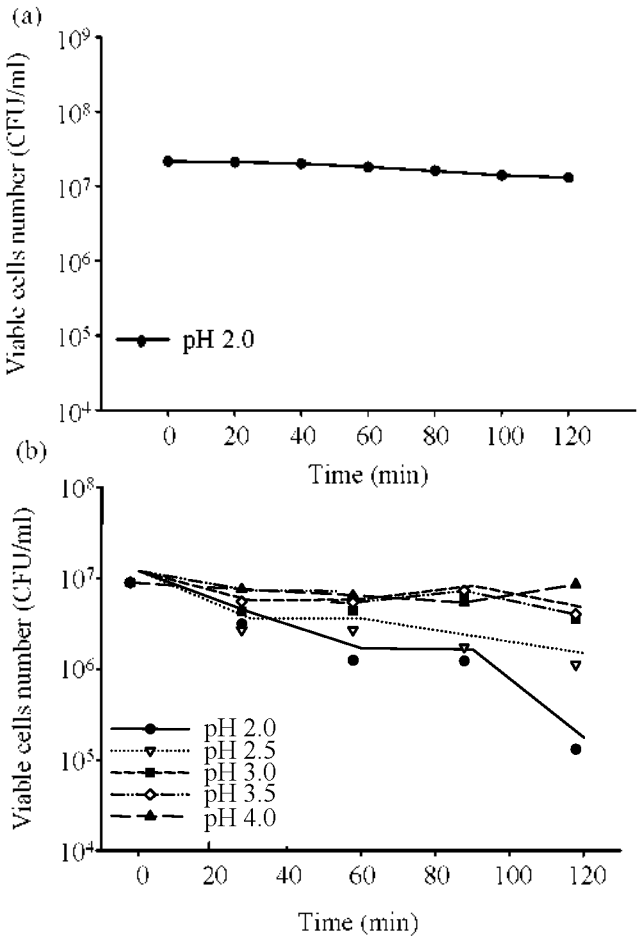

Figure 2. Resistance of *L. acidophilus* PF01 (a) and CF07 (b) to low pH in MRS broth.

of *L. acidophilus* CF07 in the presence of oxgall were 0.13 (0.3%), 0.19 (0.4%), and 0.28 (0.5%) (Table 3). *L. acidophilus* CF07 was more tolerant to bile than *L. acidophilus* PF01. The bile tolerance of *L. acidophilus* is an important characteristic for the survival and growth of the bacteria in the intestinal tract (Gilliland, 1979; Gilliland et al., 1984).

Adhesion of *L. acidophilus* to the intestinal epithelium cells

Attachment of the lactic acid bacterial cells to the intestinal mucosa of the host is one of the main selection criteria for probiotic bacteria and is considered as a pre-requisite for colonization (Ouwehand et al., 1999). *L. acidophilus* PF01 cells from piglet adhered heavily to the jejunal epithelium cells, whereas less to the duodenal epithelium cells of piglet (Figure 4). On the other hand, *L. acidophilus* CF07 cells from chicken adhered well to the cecal and duodenal epithelium cells of chicken (Figure 5). However, *L. acidophilus* cells did not adhere to the intestinal epithelium cells of the various hosts from which they were isolated (Figures 4 and 5). These results confirmed the host-specific adherence of the lactobacilli tested, as reported by many other researchers (Fuller, 1975; Suegara et al., 1975; Barrow et al., 1980; Mäyrä-Mäkinen et al., 1983).

Inhibition of *E. coli* and *Salmonella* spp. by *L. acidophilus*

Growth retardations of *E. coli* and *Salmonella* spp. cells were evident when these pathogens were challenged by *L. acidophilus* cells in MRS broth. *L. acidophilus* PF01 co-cultured with the same number of *E. coli* K88 or K99 cells in MRS broth reached the stationary growth phase after about 10 h of incubation, and the number of viable cells reached above 10^9 CFU/ml (Figure 6a and b). However, the growths of *E. coli* K88 (Figure 6a) and K99 (Figure 6b) were retarded considerably at the same growth phase,

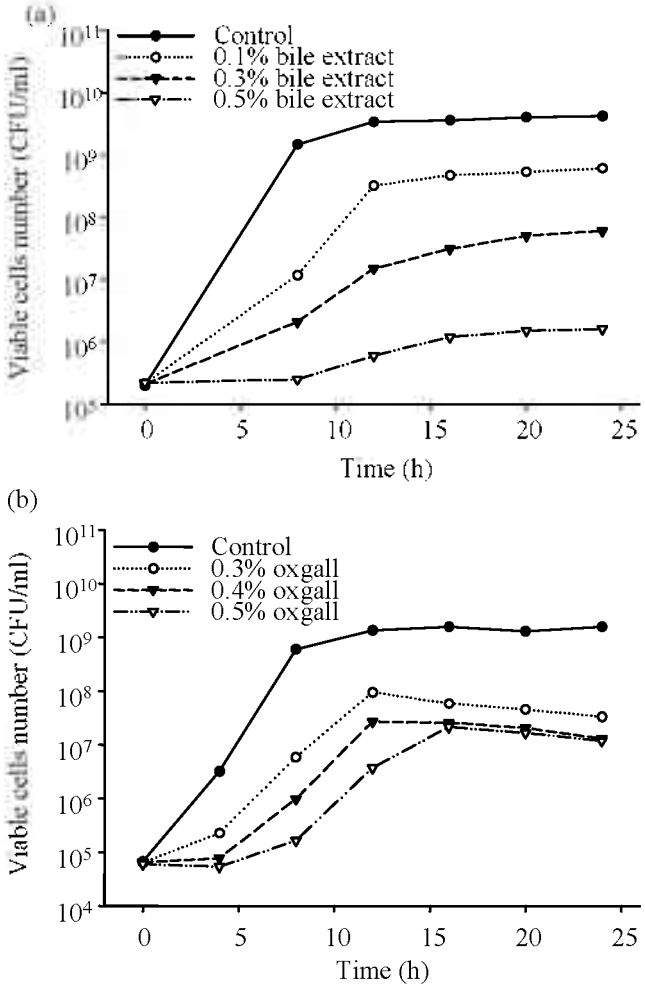

Figure 3. Bile tolerance of *L. acidophilus* PF01 (a) and CF07 (b) in MRS broth

during which the culture broths exhibited pH 4.45 and 4.46, respectively. Ahn et al. (1997) reported that the growths of *E. coli* O157 and *S. typhimurium* were considerably inhibited when *L. acidophilus* NCFM reached the stationary growth phase. Co-culturing with *L. acidophilus* CF07 resulted in the growth of *S. enteritidis* and *S. typhimurium* up to 6 h of incubation in MRS broth; however, the number of viable pathogens decreased when pH of the culture broth reached 5.73 and 5.56, respectively, and the growth of *L. acidophilus* CF07 reached the mid-exponential phase (Figure 7). *E. coli* survived better than *Salmonella* spp. in

Table 3. Growth inhibition coefficients (C-Inh)[a] of *L. acidophilus* PF01 and CF07 in MRS broth containing bile extract or oxgall

	Bile extract or oxgall			
	0.1%	0.3%	0.4%	0.5%
L. acidophilus PF01	0.11	0.25	nd[b]	0.39
L. acidophilus CF07	nd[b]	0.13	0.19	0.28

C-Inh[a]=(Log_{10} number of viable cells without bile -Log_{10} number of viable cells with bile); Log_{10} number of viable cells without bile; nd[b], not determined.

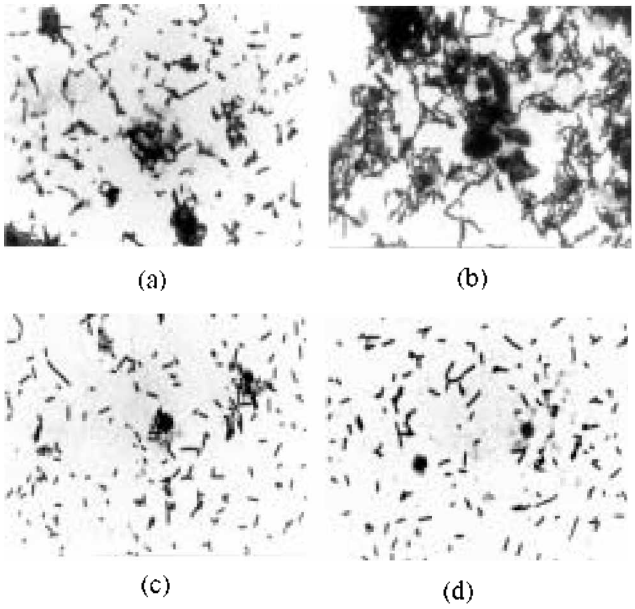

Figure 4. Adhesion of *L. acidophilus* PF01 to the duodenal (a) and jejunal (b) epithelium cells of piglets, and the cecal (c) and duodenal (d) epithelium cells of chicken.

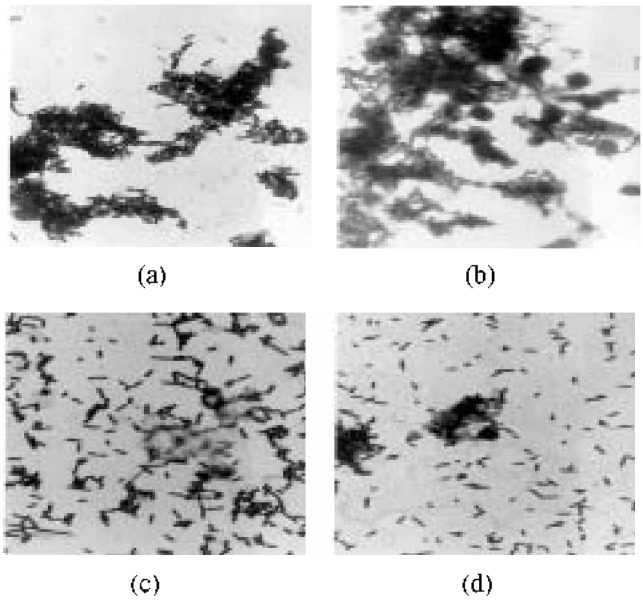

Figure 5. Adhesion of *L. acidophilus* CF07 to the cecal (a) and duodenal (b) epithelium cells of chicken, and the duodenal (c) and jejunal (d) epithelium cells of piglet.

MRS broth when co-cultured with *L. acidophilus*. Since MRS broth is not a good medium for both *E. coli* and *Salmonella*, 0.2% (w/v) skim milk powder was added into the MRS broth to determine its effect on the growth of *E. coli*. Effect of modified TSB containing 1% (w/v) dextrose and 0.1% (w/v) yeast extract on the growth of *Salmonella*

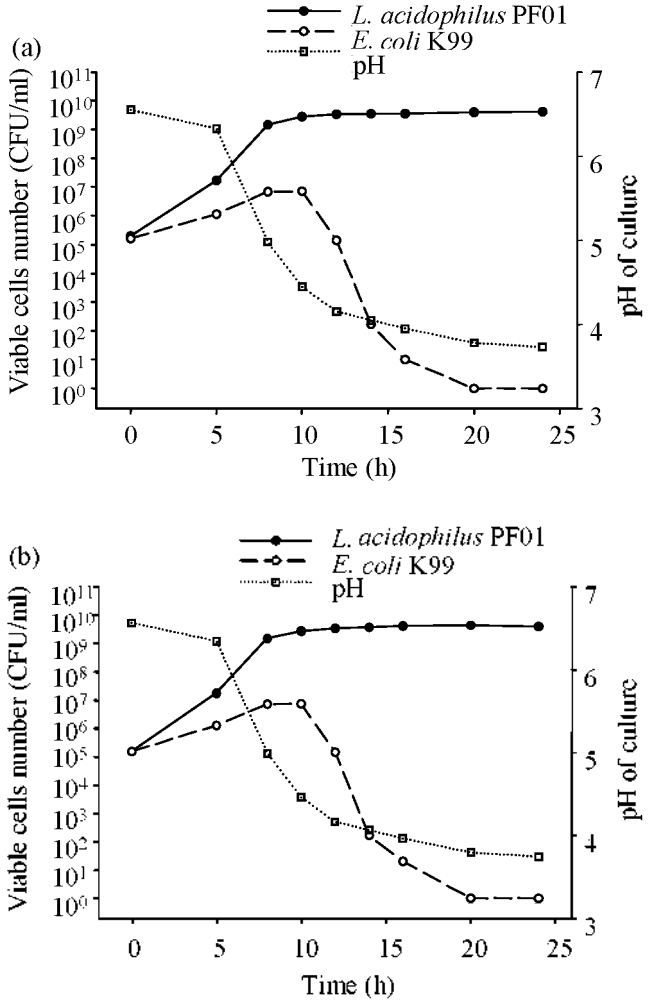

Figure 6. Inhibition of *E. coli* K88 (a) and K99 (b) by *L. acidophilus* PF01 in MRS broth.

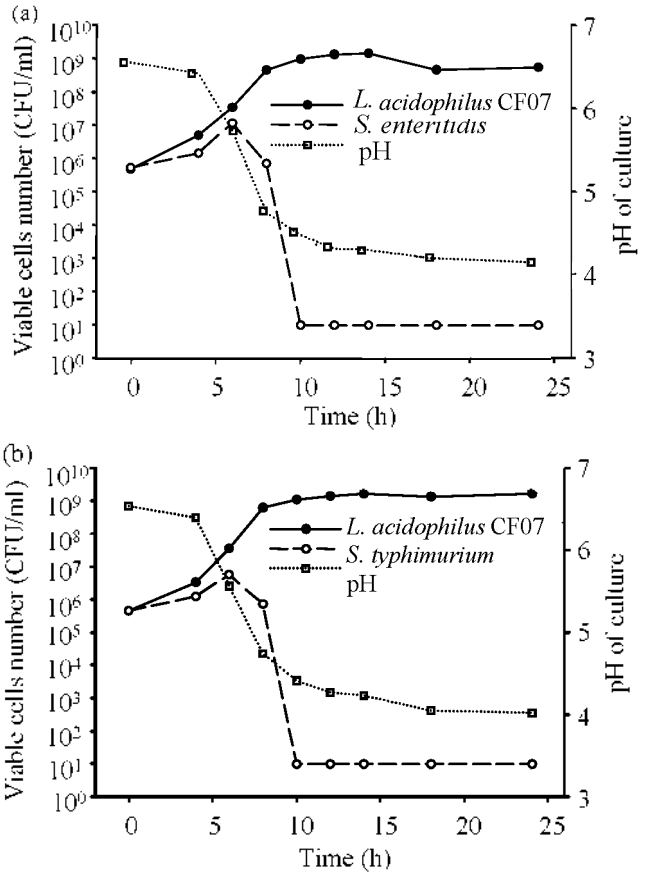

Figure 7. Inhibition of *S. enteritidis* (a) and *S. typhimurium* (b) by *L. acidophilus* CF07 in MRS broth.

spp. was determined. When *L. acidophilus* PF01 was co-cultured with *E. coli* K88 or K99 in MRS broth containing 0.2% (w/v) skim milk powder, *L. acidophilus* PF01 grew slightly slower, and the growths of *E. coli* K88 and K99 were inhibited when the culture exhibited pH 4.45 (Figure 8a and b). On the other hand, *S. enteritidis* and *S. typhimurium* outgrew *L. acidophilus* CF07 in modified TSB during 14 h of incubation. The growths of *S. enteritidis* and *S. typhimurium* were retarded at pH 4.62 and 5.08, respectively (Figure 9). Although some differences were observed depending on the nutritional environments, *Salmonella* spp. was more easily inhibited by *L. acidophilus* than *E. coli*, which is slightly more resistant to low pH than *Salmonella* spp.. *L. acidophilus* has antagonistic activities against *Staphylococcus aureus*, *S. typhimurium*, enterophothogenic *E. coli*, and *Clostridium perfringens* (Gilliland and Speck, 1977), mainly due to low pH and organic acids, particularly lactic acid (Ahn et al., 1997; Jin et al., 1996b; Hechard et al., 1990; Reinheimer et al., 1990). The organic acids produced by *L. acidophilus* PF01 and CF07 appeared to be the main factors for the antagonistic activities of *L. acidophilus* against *E. coli* and *Salmonella* spp. tested.

In conclusion, *L. acidophilus* PF01 and CF07 were confirmed to have most of the required characteristics, including acid and bile tolerances, specific adhesion to the host cells, and inhibition of pathogens. Furthermore, they are found in large numbers in animal feces (unpublished data), an indication that they survive and grow well in the gastrointestinal tracts of piglet and chicken.

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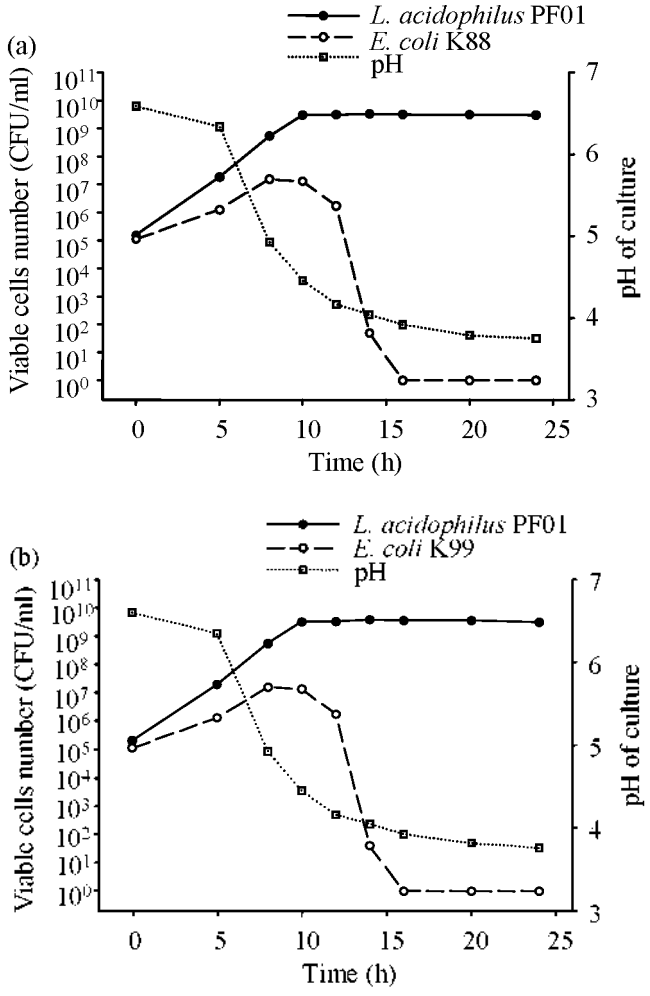

Figure 8. Inhibition of *E. coli* K88 (a) and *E. coli* K99 (b) by *L. acidophilus* PF01 in MRS broth containing 2% (w/v) skim milk powder

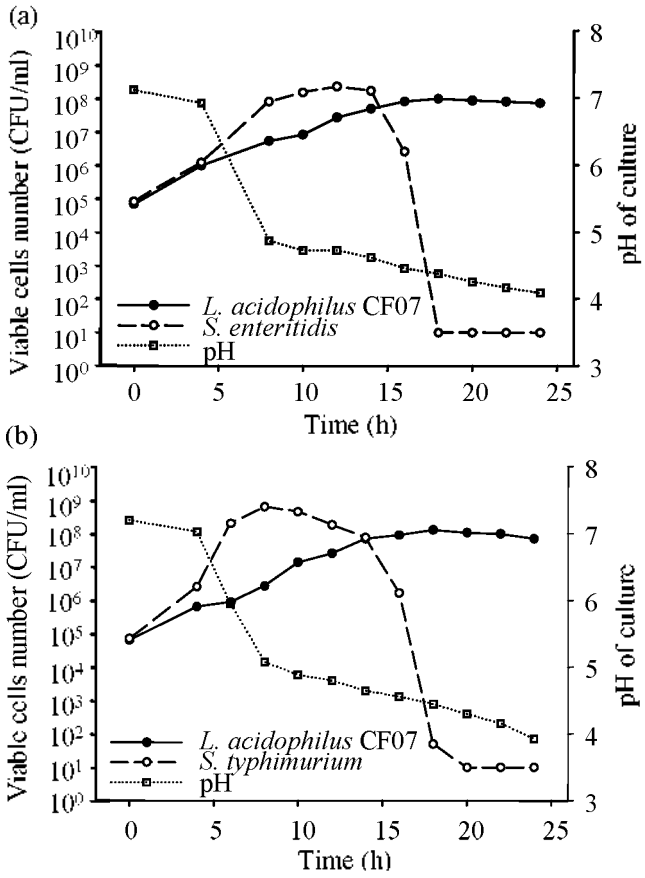

Figure 9. Inhibition of *S. enteritidis* (a) and *S. typhimurium* (b) by *L. acidophilus* CF07 in modified tryptic soy broth containing 1% (w/v) dextrose and 0.1% (w/v) yeast extract.

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