Kasetsart J. (Nat. Sci.) 42 : 277 - 284 (2008)

# Probiotic Production by Mixed Culture of Lactic Acid Bacteria and Yeast

**Ratchadaporn Thongheam, Aunchulee Thongjub, Wanna Malaphan and Patoomporn Chim–anage***


## ABSTRACT

Batch mixed culture of probiotic lactic acid bacteria (LAB), *Enterococcus faecium* PR–2 and/or *Lactobacillus plantarum* IG–3 and lactate assimilating yeast (MB–1 or KN–1 or KN–2) is a mean to maintain pH in culture broth suitable for the LAB growth. The result in this study showed that almost all the probiotic cell productions of mixed culture (i.e. mixed culture of LAB strains and mixed culture of LAB and lactate assimilating yeast) in MRS-sucrose medium were higher than those of pure culture. Among the combinations of these microorganisms, growth of the mixed culture of *Ent. faecium* PR–2 and MB–1 was the highest and the viable cell counts of LAB increased 2.58 log CFU/ml within 24 h. It was 2 times higher than the cell increment of only *Ent. faecium* PR–2 (1.27 log CFU/ml). The mixed culture and pure culture were compared for acid and bile tolerance under simulated conditions of broiler gastrointestinal (GI) tract. After 3h incubation at pH 2.5, the viable LAB cells of mixed culture of *Ent. faecium* PR–2 and *L. plantarum* IG–3 and KN–1 were the highest (100 % survival). On the other hand, the survival of LAB in mixed culture of *L. plantarum* IG–3 and KN–1, *L. plantarum* IG–3 and MB-1 were the highest tolerance towards 0.3% ox bile for 18h (94 % survival). Moreover, the survived cells of LAB of mixed culture after bile treatment were found to have antagonistic effect against indicator strains of food born pathogens.


**Key words:** probiotics, lactic acid bacteria, yeast, lactate, mixed culture, pure culture

## INTRODUCTION

Probiotics are a live microbial food and feed supplement, which beneficially affects host by improving the intestinal microbial balance. Fuller (1989) reported beneficial effects on cattle, pigs and chickens that included improvement of general health, more efficient feed utilization, faster growth rate and increase milk and egg production. Lactic acid bacteria (LAB) are the most commonly used as probiotics for animal feed. However, the most important problem in LAB production is the inhibition of growth due to increase of lactate accumulation and decrease of pH. To avoid these problems, pH control involving addition of alkali was introduced (Shimizu *et al.,* 1999). Although the addition of alkali as a mean of pH adjustment is possible, it sometimes inhibits cell growth due to osmotic pressure. Moreover consumers sensitive to environmental issue are presently increasing in numbers. They prefer to consume products without addition of artificial ingredients. Therefore, mixed culture system with lactate assimilation yeast was a possible alternative

Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
* Corresponding author, e-mail: fscippc@ ku.ac.th

to remove lactate from the medium. This approach was originally developed for nisin production (Shimizu *et al*., 1999). In this study, probiotic production was carried out by batch culture. *Enterococcus faecium* PR–2 and *Lactobacillus plantarum* IG-3 were used as probiotic microorganisms and lactate produced was assimilated by yeasts isolated from Pak-Kad-Dong (fermented vegetable) and kefir. Moreover probiotics from pure and mixed culture were evaluated for their acid and bile tolerances under simulated broiler digestive condition and consequently analyzed antagonistic activity against pathogenic bacteria.

## MATERIALS AND METHODS

### Microorganisms and their growth conditions

*Enterococcus faecium* PR–2 and *Lactobacillus plantarum* IG–3 are LAB**,** probiotic microorganisms, previously isolated from Thai fermented pork (Nham) and broiler caecum, respectively. Lactate assimilating yeasts were isolated from fermented vegetables (i.e. Pak-Kad-Dong

Sauerkraut, Pak-Sien-Dong and fermented mixed vegetables), fermented fruit (fermented mango), fresh fruits (watermelon and oranges) and kefir.

*Salmonella* Typhimurium DMST16809 and *Salmonella* Typhi DMST1328 were from Culture Collection of Department of Medical Sciences, Ministry of Public Health, Thailand.

### Medium

The medium used for growth of microorganisms are summarized as follows. Modified medium (Shimizu *et al*., 1999) contained (per liter): L– lactate (10 g); yeast extract (0.5 g); $K_2HPO_4$ (7 g); $KH_2PO_4$ (3 g); $(NH_4)_2 SO_4$ (2 g) adjusted pH to 4.5 with lactic acid. Modified medium agar for selection of lactate assimilated yeast contained agar (15 g/L) and 0.004% of bromcresol purple. De Man, Rogasa and Sharpe agar (MRS; Merck, Germany) with 0.5% $CaCO_3$, Potato Dextrose Agar (PDA; Merck, Germany: adjusted pH to 3.5 with 5 N HCl) for enumeration of LAB and yeast as the number of colony forming unit (CFU), respectively and Tryptic Soy Broth (TSB; Merck, Germany) for *Salmonella* growth.

### Lactate assimilated yeast selection method

Lactate assimilated yeast were isolated from fermented vegetables, fresh and fermented fruits and kefir obtained from local markets and preparation in Laboratory (Table 1). The sample were cross streaked on modified medium agar with 0.004% bromcresol purple, and only colonies that changed agar color from yellow to purple were isolated.

**Table 1** The samples for Lactate assimilated yeast selection used in this study.

| Samples | Source |
|---|---|
| Pak-Kad-Dong | Nonthaburi |
| Pak-Kad-Dong | Nonthaburi |
| Sauerkraut | Laboratory |
| Sauerkraut | Laboratory |
| Sauerkraut | Bangkok |
| Sauerkraut | Bangkok |
| Sauerkraut | Laboratory |
| Sauerkraut | Bangkok |
| Sauerkraut | Bangkok |
| Sauerkraut | Bangkok |
| Sauerkraut | Laboratory |
| Pak- Sien-Dong | Bangkok |
| Fermented mixed vegetables | Bangkok |
| Fermented mixed vegetables | Bangkok |
| Fermented mixed vegetables | Bangkok |
| Watermelon | Bangkok |
| Oranges | Bangkok |
| Oranges | Bangkok |
| Pak-Kad-Dong | Bangkok |
| Pak-Kad-Dong | Bangkok |
| Fermented mango | Nonthaburi |
| Kefir | Nakhon Ratchasima |

**Disaccharide assimilation of LAB and selected yeast strains**

One milliliter of starter of *Ent. faecium* PR–2, *L. plantarum* IG–3 or selected yeast strains were transferred to 9 ml MRS broth containing either 2% maltose, lactose or sucrose instead of glucose, incubated at 37°C for 48 h and compared the turbidity of each sample.

**Cultivation methods**

Every experiment of cultivation of LAB and yeast was carried out as follows:

**Starter culture** : Starter of lactate assimilating yeast was prepared in 250 ml Erlenmeyer flask containing 150 ml of modified medium broth. The flask was incubated on a rotary shaker at 150 rpm at 37°C for 18 h. Starter of LAB was performed in 250 ml Erlenmeyer flask containing 150 ml of MRS broth or MRS-sucrose broth if mentioned. The flasks were statically incubated at 37°C for 24 h.

**Cultivation**: Starter cultures (3%, v/v) of pure or mixed cultures of a LAB and a lactate assimilating yeast were inoculated in 250 ml Erlenmeyer flask containing 150 ml of MRS–sugar broth in which glucose was replaced by other sugars if mentioned. The initial concentration of the inoculum of starter culture for each treatment was similar, i.e.$10^7$ CFU/ml for LAB and $10^6$ CFU/ml for yeast. LAB or yeast starter were prior adjusted to 0.3 $OD_{600}$ and mixed at various combination as shown in Table 2. In case of treatment containing two LAB strains, they were mixed to obtain $10^7$ CFU/ml. Throughout this study, the flasks of pure culture and mixed culture were incubated on a rotary shaker at 100 rpm and 37°C for 30 h.

**Resistance of LAB and yeast to the simulating conditions of the broiler gastrointestinal tract**

Acid and bile tolerances of pure and mixed cultures under simulating conditions of the broiler gastrointestinal tract was modified from those of Tsai *et al*. (2005). One milliliter of pure and mixed cultures in MRS–sucrose (30h) were transferred to 9 ml sterile phosphate buffer saline (PBS, adjusted pH 2.5 with 0.1 N HCl) and incubated at 42°C in anaerobic jar for 3 h. The survived cells of LAB and yeast from acid were checked for viable cell counts. The culture broths were consequently centrifuged (10,000 xg, 10 min), washed with PBS buffer (pH 7.2) and then grown in 9 ml MRS broth with and without 0.3% (w/v) ox bile (Merck, Germany) for 18 and 36 h.

**Table 2** Initial ratio of LAB and lactate assimilating yeast.

| Treatments | % initial ratio(LAB : yeast) ml/ml |
|---|---|
| PR- 2 | 3 : 0 |
| PR–2 : MB-1 | 3 : 1 |
| PR–2 : KN-1 | 3 : 1 |
| PR–2 : KN–2 | 3 : 1 |
| IG–3 | 3 : 0 |
| IG–3 : MB-1 | 3 : 1 |
| IG–3 : KN-1 | 3 : 1 |
| IG–3 : KN–2 | 3 : 1 |
| PR-2 + IG- 3 | 1.5 : 1.5: 0 |
| PR-2 + IG- 3 : MB-1 | 1.5 : 1.5 : 1 |
| PR-2 + IG- 3 : KN-1 | 1.5 : 1.5 : 1 |
| PR-2 + IG-3 : KN-2 | 1.5 : 1.5 : 1 |

PR-2 = *Ent. faecium*, IG-3 = *L. plantarum* , MB-1 KN-1 and KN-2 = lactate assimilated yeasts

Bile tolerance of LAB from pure and mixed cultures were determined for viable cell counts and antibacterial activity assay.

**Analysis and assay**

The viable cell count of LAB was determined by plating serial dilutions of the culture in MRS agar with 0.5% $CaCO_3$ and incubated 37°C for 48 h anaerobically. In case of yeast, PDA (pH 3.5) were used. The culture was aerobically incubated at 37°C for 24 h.

Antibacterial activity assay was conducted using the agar well diffusion method modified from Bevilacqua *et al*. (2003).

## RESULTS AND DISCUSSION

**Selection of lactate assimilating yeast**

Lactate assimilating yeast of 18 isolates from fermented vegetable, fresh fruits and kefir samples were selected using modified medium agar containing 0.004% bromcresol purple. They were included 4 isolates from fermented Chinese vegetable, 4 isolates from sauerkrauts, 2 isolates from Pak–Sein-Dong, 3 isolates from fermented mixed vegetable, 1 isolate from watermelon, 2 isolates from oranges and 2 isolates from kefir. In this study, MB–1, KN–1 and KN–2 were the best yeast strains which capable to assimilate lactate within 2 days. They were selected to cultivate with *Ent. faecium* PR–2 and *L. plantarum* IG–3 for probiotic production.

**Disaccharide assimilation of LAB and selected yeast strains**

The main objective of this research is to produce probiotic cells (LAB cells) by mixed culture of LAB and lactate assimilating yeast. According to biochemical test of LAB and selected yeast strains, they fermented glucose well (under paper preparation). Thus, the competition of glucose assimilation could contribute to the growth limitation of both LAB and yeast strains in mixed culture. To prevent this problem, the selection of yeast strain which utilize lactate in preference to sugar is necessary. The preliminary study was carried out to check type of sugar which LAB was more fermentable (than yeast) by measuring the turbidity. The results revealed that *Ent. faecium* PR–2, *L. plantarum* IG–3 grew well in sucrose, lactose and maltose. The growth of yeast strains KN-1 and KN-2 were very low in MRS-sucrose while MB-1 was moderately assimilated. In order to emphasize the effect of sucrose on growth of pure cultures of LAB and yeast, further study was performed by determining the specific growth rates of each strain after cultivation in MRS-sucrose broth. The results in Table 3 showed that *Ent. faecium* PR–2 and *L. plantarum* IG–3 demonstrated the specific preference for sucrose indicated by high specific growth rates while the yeast MB-1 had the lowest value. This suggested that sucrose is a good carbon source for *Ent. faecium* PR–2 and *L. plantarum* IG–3 but not for yeast MB-1. Therefore, MRS-sucrose should be used for cultivation of the mixed culture in next experiments.

**Cells growth of probiotics (LAB) in MRS–sucrose broth**

Figure 1 shows the cell growth of LAB in terms of cell increment when pure culture of LAB or mixed culture of two LAB strains or the mixed culture of LAB and yeast were grown in MRS-sucrose medium. The slightly increase in cell population of each treatment at 30 h was observed

**Table 3** Specific growth rates (μ) of Lactic acid bacteria and yeasts in MRS- sucrose broth.

| Microorganisms | Specific growth rate, $\mu(h^{-1})$ |
|---|---|
| *Ent. faecium* PR-2 | 0.780 |
| *L. plantarum* IG-3 | 0.644 |
| Yeast (MB-1) | 0.206 |
| Yeast (KN-1) | 0.314 |
| Yeast (KN-2) | 0.313 |

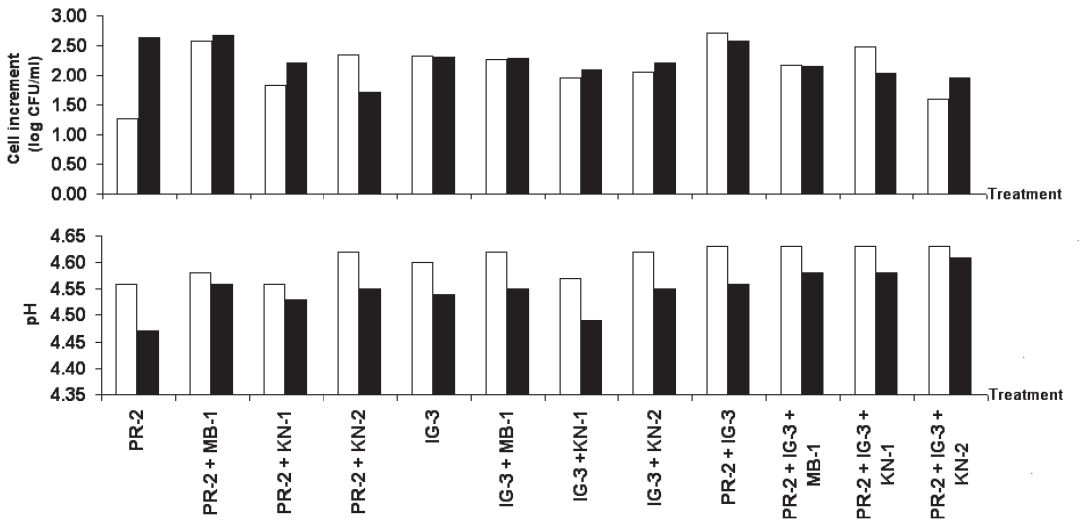


**Figure 1** Cell growth of probiotic in terms of cell increments and pH of pure culture (*Ent. faecium* PR-2 or *L. plantarum* IG-3 ) and mixed culture (two LAB strains or LAB combinations lactate assimilating yeast) in MRS-sucrose medium (initial pH 6.8), ( □ ) for 24 h and ( ■ ) 30 h.

**Note:** *Ent. faecium* PR-2 and *L. plantarum* IG-3 = probiotics
MB-1, KN-1 and KN-2 = lactate assimilated yeasts.

as compared to those of 24 h. However the growth reduction were detected in the treatments of *Ent. faecium* PR-2 + KN-2 and *Ent. faecium* PR-2 + *L. plantarum* IG-3 + KN-1. The growth inhibition at 30 h might cause by the accumulated acid production of these LABs which indicated by pH lower than 5. Therefore, the LAB growth at 24 h were used to compare the growth of mixed culture. The treatment of *Ent. faecium* PR-2 + *L. plantarum* IG-3 showed the highest cell increment of 2.72 log CFU/ml. While *Ent. faecium* PR-2 + MB-1 and *Ent. faecium* PR-2 + *L. plantarum* IG-3 +KN-1 were slightly lower i.e. 2.58 and 2.49 log CFU/ml, respectively. In addition, Figure 1 revealed that pH of culture broth of mixed cultures were mostly higher than that of pure culture. This can be explained as the yeasts consumed lactic acid produced by LAB and consequently increased the pH in the culture broth, It should be mentioned that the probiotic increments of mixed culture between *Ent. faecium* PR-2 and lactate assimilating yeast were higher than those of pure culture at 24 h fermentation and the combination of *Ent. faecium* PR–2 and yeast MB–1 was the highest. The probiotics increased about 2.58 log CFU/ml while cell production of *Ent. faecium* PR–2 alone increased only 1.27 log CFU/ml. Though the mixed culture of *Ent. faecium* PR-2 + *L. plantarum* IG-3 can grow best, it is difficult to control the cell number of each strain when it is propagated in a large scale. Hence growing single strain of LAB separately with lactate assimilating yeast such as *Ent. faecium* PR-2 + MB-1 is more practical.

## Survival under simulating conditions of the broiler gastrointestinal tract

### Acid conditions

Gastrointestinal tract is the major location to affect the viability of probiotics. Most bacteria do not survive well at low pH values (Lin *et al.*, 2006). According to Havenaar and Husis (1992), the stabilities of LAB cells obtained from either *in vivo* or *in vitro* study are similar. For acid

tolerance study, PBS or animal gastric liquid were used. The viable cell count of mixed culture in each treatment was determined after 3 h incubation in PBS buffer at pH 2.5 and 42°C in anaerobic condition. After 3 h exposure, the highest survival of LAB was observed in mixed culture of 3 strains i.e. *Ent. faecium* PR–2, *L. plantarum* IG–3 and KN–1 (8.45 log CFU/ml; 100 % ) as shown in Table 4. The other mixed culture displayed LAB cells survival ranging between; 93.17–98.08 %, while the pure culture of *Ent faecium* PR–2, *L. plantarum* IG–3 were 88.55 and 98.17 %, respectively. These results indicated that most of mixed cultures tended to be more acid tolerance than pure culture. However, *L.plantarum* IG–3 could be stable in acid condition much better than *Ent. faecium* PR–2. This was agree with some research works which reported that *Lactobacillus* strains were able to retain their viability when exposed to pH values of 2.5–4.0, but displayed loss of viability at lower pH value (Conway *et al*.,1987; Dunne and Mahony, 2001). It should be mentioned that when mixed strains of *Ent. faecium* PR–2, *L. plantarum* IG–3 were grown with or without yeasts, the viability of LAB were almost similar (97.14 %-100%). The effect of acid on yeasts are also reported in Table 5. The three yeast strains are very high acid tolerance when they grew in mixed culture (85.11–100 % survival) except the treatments of *L. plantarum* IG-3 grew with yeast strains KN-1 or KN-2 (62.40–69.58 % survival).

**Table 4** Effect of acid and bile on survival of lactic acid bacteria when grown in pure culture and mixed culture.

| Treatments | Initial cells* | LAB counts after incubation (log CFU/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | (log CFU/ml) | Acid (PBS , pH 2.5) | % survival** | MRS-bile 18 h | % survival*** | MRS-bile 36 h | % survival**** |
| PR-2 | 8.82 | 7.81 | 88.55 | 7.21 | 81.74 | 9.53 | 100.00 |
| PR-2 + MB-1 | 8.93 | 8.32 | 93.17 | 7.80 | 87.35 | 5.30 | 59.35 |
| PR-2 + KN-1 | 8.83 | 8.45 | 95.70 | 7.45 | 84.37 | 5.78 | 65.46 |
| PR-2 + KN-2 | 8.51 | 8.01 | 94.12 | 7.47 | 87.78 | 5.85 | 68.74 |
| IG–3 | 8.77 | 8.61 | 98.17 | 7.38 | 84.15 | 9.21 | 100.00 |
| IG–3 + MB-1 | 9.00 | 8.48 | 94.22 | 8.46 | 94.00 | 4.00 | 44.44 |
| IG–3 + KN-1 | 8.81 | 8.50 | 96.48 | 8.33 | 94.55 | 5.95 | 67.54 |
| IG–3 + KN-2 | 9.01 | 8.47 | 94.01 | 7.52 | 83.46 | 4.00 | 44.40 |
| PR-2 + IG–3 | 8.74 | 8.49 | 97.14 | 7.32 | 83.75 | 5.78 | 66.13 |
| PR-2 + IG–3 + MB-1 | 8.85 | 8.68 | 98.08 | 7.80 | 88.14 | 9.55 | 100.00 |
| PR-2 + IG–3 + KN-1 | 8.45 | 8.45 | 100.00 | 7.31 | 86.51 | 5.00 | 59.17 |
| PR-2 + IG–3 + KN-2 | 8.83 | 8.56 | 96.94 | 7.27 | 82.33 | 5.30 | 60.02 |

ND = Non Detected due to cell died, PR-2 = *Ent. faecium*, IG-3 = *L. plantarum* , MB-1 KN-1 and KN-2 = lactate assimilated yeasts

* **Initial cells** of LAB were obtained by measuring 10 fold diluted of LAB in MRS-sucrose broth after 30 h incubation.

** Percentage of survival cell from acid condition = (cell growth of LAB in PBS pH 2.5/Initial cells) × 100

*** Percentage of survival cell from bile condition (18 h) = (cell growth of LAB in MRS broth with 0.3 % ox bile (w/v) for 18 h/Initial cells) × 100

**** Percentage of survival cell from bile condition (36 h) = (cell growth of LAB in MRS broth with 0.3 % ox bile (w/v) for 36 h /Initial cells) × 100

100 % means that the growth rate of LAB were not affected by the incubated in PBS pH 2.5 or the added 0.3 % ox bile (w/v) at all.

**Bile conditions**

After incubation in acid buffer (pH 2.5), every treatment was transferred to MRS–bile medium aseptically and incubated at 42°C in anaerobic condition for 18 and 36 h. During 18 h incubation, LAB and yeast must adapt themselves to new environments. The results in Table 4 and 5 revealed that most of them could recover and cell viability of LAB in pure culture and mixed culture were high (81.74-94.55%). This condition might not be suitable for yeasts as a result KN-1 and KN-2 were not detected and about 60-68% of MB-1 retained in the broth. This result will provide the information of using probiotics produced by mixed culture of LAB and lactate assimilating yeasts (KN-1,KN-2 and MB-1) as feed for poultry safely. The yeast growth will be inhibited at 18 h incubation and low pH in gastrointestinal tract of poultry will be maintained. Moreover, the dead yeast cells will support the growth of probiotics as well. After incubated in MRS–bile for 36 h, pure culture was greater bile tolerance. This result suggests that probiotics to be added in poultry feed should be the mixture between *Ent. faecium* PR–2 and *L. plantarum* IG–3 and each of them should be grown by mixed culture with yeast MB-1 in MRS–sucrose broth.

**Antibacterial activity assay**

After incubation in acid (pH 2.5, 3h) and bile (18 and 36 h), the survival cells of pure and mixed cultures were evaluated for their antagonistic activities against food borne pathogenic bacteria i.e. *Salmonella* Typhimurium DMST16809 and *Salmonella* Typhi DMST1328. Culture broths of *Ent. faecium* PR–2 (18 h) and *L. plantarum* IG–3 (36 h) inhibited the growth of *Salmonella* Typhimurium DMST16809. The mixed culture of *L. plantarum* IG–3 and yeasts (36 h) and mixed culture of *Ent.faecium* PR–2 and yeasts (36 h) inhibited the growth of *Salmonella* Typhimurium DMST16809 and *Salmonella* Typhi DMST1328, respectively. Though the final pH of culture broth of some treatments were higher than

**Table 5** Effect of acid and bile on survival of yeasts in mixed culture.

| Treatments | Initial cells* | Yeast counts after incubation (log CFU/ml) | | | |
|---|---|---|---|---|---|
| | conc. | Acid | % survival** | MRS–bile | % survival*** |
| | | (PBS , pH 2.5) | | 18 h | |
| PR-2 + MB-1 | 6.08 | 6.40 | 100.00 | 4.17 | 68.59 |
| PR-2 + KN-1 | 6.25 | 6.18 | 98.88 | ND | ND |
| PR-2 + KN-2 | 6.28 | 7.00 | 100.00 | ND | ND |
| IG–3 + MB-1 | 6.28 | 7.05 | 100.00 | 3.82 | 60.83 |
| IG–3 + KN-1 | 6.41 | 4.00 | 62.40 | ND | ND |
| IG–3 + KN-2 | 6.18 | 4.30 | 69.58 | ND | ND |
| PR-2 + IG–3 + MB-1 | 5.63 | 6.52 | 100.00 | 3.84 | 68.21 |
| PR-2 + IG–3 + KN-1 | 6.66 | 7.20 | 100.00 | ND | ND |
| PR-2 + IG–3 + KN-2 | 7.05 | 6.00 | 85.11 | ND | ND |

ND = Non Detected due to cell died, PR-2 = *Ent. faecium*, IG-3 = *L. plantarum* , MB-1 KN-1 and KN-2 = lactate assimilated yeasts

* **Initial cells** of yeast were obtained by measuring 10 fold diluted of yeast in MRS-sucrose broth after 30 h incubation.

** Percentage of survival cell from acid condition = (cell growth of yeast in PBS pH 2.5/Initial cells) × 100

*** Percentage of survival cell from bile condition (18 h) = (cell growth of yeast in MRS broth with 0.3 % ox bile (w/v) for 18 h /Initial cells) × 100

100 % means that the growth rate of yeast were not affected by the incubated in PBS pH 2.5 or the added 0.3 % ox bile ( w/v) at all.

5 which should not inhibit *Salmonella* sp. (Fuller and Brooker,1974), the growth inhibition was detected. This inhibition therefore could be due to the production of other inhibitory compounds in addition to organic acids. Since probiotics can stay in the intestine longer than 36 h at the villi of broilers they may develop lower pH to prevent *Salmonella* as well**.**

## CONCLUSIONS

The aim of this study is to produce LAB as probiotics by mixed culture of LAB and lactate assimilating yeast. Consuming of lactate by yeast can prevent acid accumulation in the culture broth and raising pH to prolong the LAB growth. The results are summarized as follows: (i) mixed culture of *Ent. faecium* PR–2 and *L. plantarum* IG-3 showed the highest growth and the latter belonged to the co-culture of *Ent. faecium* PR–2 and MB-1. Though the mixed culture of two LAB strains (*Ent. faecium* PR–2 and *L. plantarum* IG-3) grew best in this experiment, it is difficult to control the growth performance of each strain during fermentation. Therefore co-culture of *Ent. faecium* PR–2 and MB-1 should be more suitable for probiotic production in a large scale. (ii) study on the survival of LAB cells under simulating conditions of the broiler gastrointestinal tract suggested that mixed culture of *Ent. faecium* PR–2 and *L. plantarum* IG–3 and yeast MB– 1, *Ent. faecium* PR–2 and *L. plantarum* IG–3 and KN-1 tended to be more stable in broiler intestine than the others. (iii) after acid and bile treatment, the final culture broths of mixed culture were found to have antagonistic effect to *Salmonella* spp. However, further study is required to emphasize this result.

## LITERATURE CITED

Bevilacqua, L., M. Ovidi, E.D. Mattia, L.D. Trovtelli and F. Canganella. 2003. Screening of *Bifidobacterium* strains isolated from human feces for antagonistic activities against potentially bacterial pathogens. **J. Microbiol. Res.** 158: 179-185.

Conway, P.L., S.L. Gorbatch and B.R. Goldin. 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. **J. Dairy Sci.** 70: 1-12.

Dunne, C.O. and L. Mahony. 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation *in vivo* findings. **Am. J. Clin. Nutri.** 73: 386-392.

Fuller, R. 1989. Probiotics in man and animals. **J. Appl. Bacteriol.** 66: 365-378.

_____ and B.E. Brooker. 1974. *Lactobacillus* which attach to the crop epithelium of the fowl. **Am. J. Clin. Nutri.** 27: 1305-1312.

Havenaar, R. and J.H.J. Huis In't Veld. 1992. Probiotics: A General View, pp. 151-170. *In* B. J. B. Wood (ed.). **The Lactic Acid Bacteria Vol. 1.** The Lactic Acid Bacteria in health and disease. Elsevier Science Publishers, London.

Lin, W.H., C.F. Hwang, L.W. Chen and H.Y. Tsen. 2006. Viable counts, characteristic evaluation for commercial lactic acid bacteria products. **Food Microbiol.** 23: 74-81.

Shimizu, H., T. Mizuguchi, E. Tanaka and S. Shioya. 1999. Nisin production by a mixed culture system consisting of *Lactococcus lactis* and *Kluyveromyces marxianus*. **Appl. Environ. Microbiol.** 65: 3134-3141.

Tsai, C. C., H.Y. Hsih, H.H. Chiu, Y.Y. Lai, J.H. Liu, B. Yu and H.Y. Tsen. 2005. Antagonistic activity against *Salmonella* infection *in vitro* and *in vivo* for two *Lactobacillus* strains from swine and poultry. **Int. J. Food Microbiol.** 102: 185-194.