

## Effect of Feed Supplementation of Lactic Acid Bacteria on Microbial Changes in Broiler Intestine

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### ABSTRACT

Four isolates of lactic acid bacteria were examined as good probiotics. They were *Enterococcus faecium* PR-2, Rumen bacterium MG-2, *Lactobacillus plantarum* IFS-1 and *L. plantarum* IG-3. These strains survived well at pH (2 to 3 and 8 to 9) and bile of broiler intestinal conditions. The four lactic acid bacterial strains were supplemented to the broiler feed and examined for the microbial changes in ileum and cecum of chickens. The results revealed that these probiotics influenced the population of lactic acid bacteria and *E. coli* in broiler intestines. In comparison with control group, the cell counts of lactic acid bacteria in the ileums of broilers consuming commercial probiotics, single culture of those four strains and mixed culture during rearing period were higher ( $p < 0.01$ ) while *E. coli* in ceca of the same treatments were lower ( $p < 0.01$ ). However, the population of lactic acid bacteria was higher than *E. coli* in both ileums and ceca of chickens. Moreover, *Salmonella* sp. was not detected in both control and treated broiler intestines.

**Key words:** probiotics, lactic acid bacteria, broilers

### INTRODUCTION

Lactic acid bacteria (LAB) are regarded as a major group of probiotic bacteria for human and animals (Brashears *et al.*, 2003; Chen *et al.*, 2005). This is due to many reasons such as they are recognized as safe and can tolerate acid and bile. They adhere to the intestinal epithelium of the hosts and can inhibit the growth of pathogenic bacteria such as *Escherichia coli* and *Salmonella* which are the major pathogenic microorganisms that colonize the intestinal tract of chicken (Murry *et al.*, 2004). They should be able to keep their

viability during animal feed processing and storage (Lin *et al.*, 2007). Therefore, the attempt to reduce antibiotic in livestock production by using probiotic feed additives is attracting increased attention as a cost-effective alternative to controlling animal disease and improving breeding performance (Reuter, 2001).

The objective of this study was to investigate the selected LAB strains suitable for feed additives of poultry and the changes of viable counts of LAB and *E. coli* in treated broiler intestines including the antagonistic effect of these LAB strains against *Salmonella* sp.

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## MATERIALS AND METHODS

### Microorganisms

The four strains of Lactic acid bacteria (LAB) identified as *Enterococcus faecium* PR-2, Rumen bacterium MG-2, *Lactobacillus plantarum* IFS-1 and *L. plantarum* IG-3 were isolated from Thai fermented pork (Nham), Pla-som, intestinal tracts of fish and chicken, respectively. They had been checked for antagonistic effect for some pathogens such as *Salmonella* sp., *Staphylococcus aureus*, *Enterococcus faecalis* etc. (data not shown). For strain MG-2, it was partially identified by 16s rDNA sequence analysis to belong to Rumen bacterium and must be completely checked later on. In this study, MG-2 was called as Rumen bacterium. These bacteria were used as probiotics in this study.

For bacteria i.e. *Clostridium acetobutyricum*, *Bacillus megaterium*. and yeast, i.e. *Candida tropicalis*, were stocked cultures of Department of Microbiology, Faculty of Science, Kasetsart University. These strains were grouped as probiotics.

### Media and culture conditions

The four selected strains of LAB were cultured in de Man Rogosa and Sharpe (MRS) medium. The other bacteria and yeast were cultured in nutrient broth and potato dextrose broth, respectively. The seed cultures of LAB were prepared by growing in the 250 ml Erlenmeyer flasks containing 50 ml of MRS medium and incubated at 37°C for 18 h. For cultivation of LAB cells, one percent of seed culture was inoculated into the 500 ml Erlenmeyer flask containing 100 ml of appropriate medium and incubated at 37°C for 24 h.

To test the effect of the initial pH on growth of selected LAB strains, MRS media were adjusted to different four pH values (2.0, 3.0, 8.0 and 9.0) by either 8 N HCl or 5 N NaOH. Addition of 1 to 5% of chicken bile to MRS media were

made to study the effect of bile on LAB growth.

### Broiler feeding trials

The experiment was done by the completely randomized design using 680 broiler chicks. They were divided into 8 treatments with 4 replicates. Each replicate had 20 broilers. These treatments were composed of T1 or control: commercial diet (without antibiotics and probiotics), T2: commercial diet supplemented with avilamycin, while T3 with commercial probiotic (mixed culture of *L. acidophilus*, *Ent. faecium* and *Saccharomyces cerevisiae*). T4 to T8 were fed by commercial diet supplemented with *Ent. faecium* PR-2 (T4), Rumen bacterium MG-2 (T5), *L. plantarum* IFS-1 (T6), *L. plantarum* IG-3 (T7) and mixed culture of probiotics (i.e. *B. megaterium*., *C. acetobutyricum*, *C. tropicalis*, *Ent. faecium* PR-2 and *L. plantarum* IFS-1, at the ratio of 1:1:1:1:1, (T8) via drinking water at  $1 \times 10^6$  cfu/ml, respectively. The broilers were reared at Suwanvajokkasikij poultry farm, Department of Animal Science, Faculty of Agriculture, Kasetsart University, Bangkok.

### Enumeration of microorganisms in cecum and ileum

Viable bacterial counts in the cecum and ileum of broiler were determined after feeding for 7, 21, 28, 35 and 42 days. Each week, 64 broilers were killed and aseptically removed of ceca and ileums. Samples with suitable dilution were either spread on Eosin methylene blue agar (EMB, Merck) or poured plates on MRS agar containing 0.5%  $\text{CaCO}_3$  to enumerate *E. coli* and LAB, respectively. In each experiment, the ileum and cecum samples were tested for the presence of *Salmonella* by Bacteriological Analytical Manual, BAM (AOAC, 1998).

### Statistical analyses

The data were analyzed by a completely randomized design using the ANOVA procedure

of Statistical Analysis System (SAS, 1996). The significant difference among treatment mean values ( $p < 0.01$ ) were analyzed by Duncan's new multiple range test (Duncan, 1995).

## RESULTS AND DISCUSSION

It is speculated that the benefit derived from probiotics is a result of the organisms growing and contributing some beneficial function in the intestinal tract. Therefore, one of most important considerations in achieving the desired effect from using probiotics as growth promoter is to ensure that the organisms survive passage through the stomach and proliferate in the intestinal tract. To establish successfully in the intestine, bacterial strains must be able to survive in acidic and bile conditions *in vitro* (Jin *et al.*, 1998).

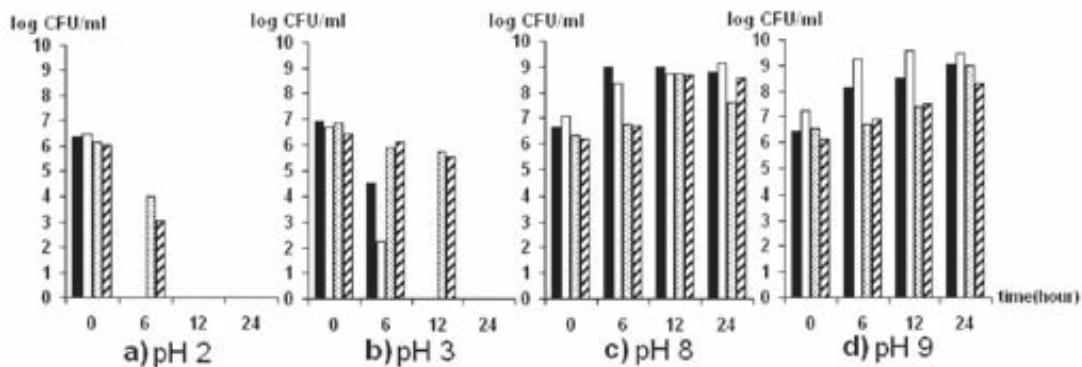
### Effect of acid and bile on selected Lactic acid bacteria strains

In this study, the viable LAB counts were determined after 6, 12 and 24 hours incubation in MRS broth (pH 2, 3, 8 and 9). Results from Figure 1 showed that viable LAB counts were affected by the acid and alkaline. *L. plantarum* IFS-1 and *L. plantarum* IG-3 could survive at pH 2 for 6 hours incubation. Viable cell counts of these

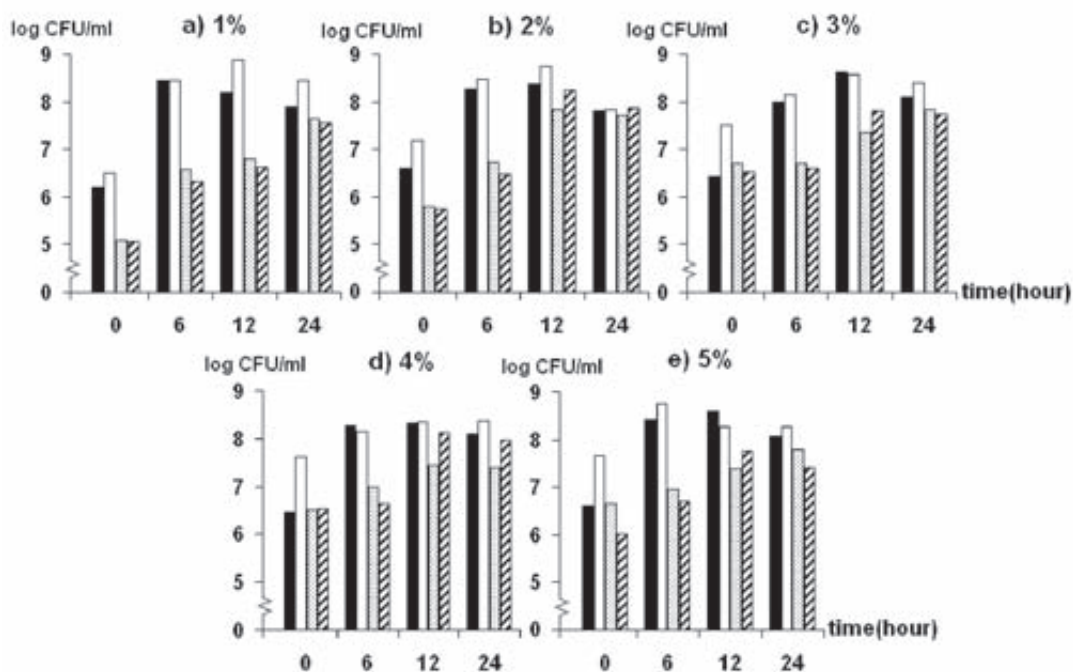
strains decreased by 2.08 and 2.97 orders, respectively. The growth of *Ent. faecium* PR-2 and Rumen bacterium MG-2 were inhibited. When the same experiment was repeated with shorter sampling interval, the latter strains were able to withstand at pH 2 for 4 hours at 62.70 and 33.48% survival, respectively (data not shown). After incubation in MRS broth at pH 3 for 6 and 12 hours, viable LAB counts from *L. plantarum* IFS-1 and *L. plantarum* IG-3 exhibited good growth (about 86 – 96.9% survival), while *Ent. faecium* PR-2 and Rumen bacterium MG-2 had fairly low survival rate (65.2 and 33.3%, respectively) at 6 hours. Since the time required for poultry feed to pass through the entire alimentary tract of chicken was as short as 2.5 to 3 hours (O'Sullivan, 2001), these four LAB strains were possible to survive passage through the stomach of chicken.

On the contrary, the pH of ceca and cloaca of chicken are about 5.4 to 8.4 (Sturkie, 1976), the LAB as probiotics must grow under this condition too. After incubation in pH 8 and 9 MRS broth for 24 hours. *Ent. faecium* PR-2, Rumen bacterium MG-2, *L. plantarum* IFS-1 and *L. plantarum* IG-3 were able to grow well and the cell counts were higher than the initial cell concentration (Figure 1).

To study the bile resistance, total LAB



**Figure 1** Effect of acid on the survival of Lactic acid bacteria cells, PR-2 (■), MG-2 (□), IFS-1 (▤) and IG-3(▨)



**Figure 2** Effect of 1-5% chicken bile on growth of Lactic acid bacteria.

PR-2 (■), MG-2 (□), IFS-1 (▤) and IG-3 (▨)

counts were determined every 6 hours interval for 24 hours incubation in 1-5% (v/v) chicken bile MRS broth. Results presented in Figure 2 showed that viable LAB counts were affected. Population of *Ent. faecium* PR-2 and Rumen bacterium MG-2 gradually increased within 12 hours and began to decrease later on. However, the growth of *L. plantarum* IFS-1 and *L. plantarum* IG-3 were comparatively low. The results on bile tolerance showed that *Ent. faecium* PR-2 and Rumen bacterium MG-2 were more bile resistant than *L. plantarum* IFS-1 and *L. plantarum* IG-3. Though gram-positive bacteria are found to be more sensitive to bile than gram-negative bacteria, bile acids are secreted into the duodenum in the conjugated form which are less inhibitory than the deconjugated form (Dunne and Mahony, 2001). Moreover, exposing organisms to mild stresses (e.g. higher pH) can induce tolerances such as acid and bile conditions and become a potential

strain (Jin *et al.*, 1998; O'Sullivan, 2001). As a result, these LAB strains could survive in the presence of bile.

#### Effect of probiotics on population of Lactic acid bacteria in broiler intestine

The effect of the probiotic strains was investigated based on the changes of viable counts of LAB and *E. coli* in treated chicken intestine (ileum and cecum). The results in Table 1 indicated that after seven days of feeding trials, the counts of LAB in ileums of T6 (*L. plantarum* IFS-1) was higher ( $p < 0.01$ ) than those of control group (T1) and commercial group (T3). At feeding forty two days, the counts of LAB in ileums of T3 to T8 were higher than that of control treatment (T1). Especially, T7 (*L. plantarum* IG-3) and T8 (mixed strains) showed the highest LAB cell counts. Considering the cell numbers of LAB in ileum at feeding from seven to forty two days, T7 was better

**Table 1** Effect of probiotics on population of Lactic acid bacteria in broiler intestine.

| Treatment | Ileum (log CFU/ml) <sup>1/</sup> |                           | Cecum (log CFU/ml) <sup>1/</sup> |                            |
|-----------|----------------------------------|---------------------------|----------------------------------|----------------------------|
|           | 7 days                           | 42 days                   | 7 days                           | 42 days                    |
| T1        | 7.44 <sup>bc</sup> ± 0.67        | 7.83 <sup>c</sup> ± 0.06  | 7.77 <sup>bc</sup> ± 0.78        | 8.63 <sup>ab</sup> ± 0.02  |
| T2        | ND                               | 7.85 <sup>c</sup> ± 0.02  | ND                               | 8.12 <sup>cd</sup> ± 0.02  |
| T3        | 7.20 <sup>c</sup> ± 0.41         | 8.07 <sup>cd</sup> ± 0.05 | 7.91 <sup>abc</sup> ± 0.40       | 8.16 <sup>bc</sup> ± 0.08  |
| T4        | 8.06 <sup>ab</sup> ± 0.10        | 8.35 <sup>b</sup> ± 0.06  | 7.98 <sup>abc</sup> ± 0.08       | 7.84 <sup>cde</sup> ± 0.04 |
| T5        | 7.94 <sup>ab</sup> ± 0.23        | 8.13 <sup>c</sup> ± 0.04  | 7.92 <sup>abc</sup> ± 0.52       | 7.61 <sup>de</sup> ± 0.11  |
| T6        | 8.28 <sup>a</sup> ± 0.43         | 8.03 <sup>d</sup> ± 0.04  | 7.37 <sup>c</sup> ± 0.36         | 7.87 <sup>cde</sup> ± 0.11 |
| T7        | 7.87 <sup>ab</sup> ± 0.42        | 8.60 <sup>a</sup> ± 0.01  | 8.22 <sup>ab</sup> ± 0.19        | 7.53 <sup>e</sup> ± 0.01   |
| T8        | 7.17 <sup>c</sup> ± 0.29         | 8.57 <sup>a</sup> ± 0.01  | 8.61 <sup>a</sup> ± 0.29         | 8.94 <sup>a</sup> ± 0.54   |

<sup>1/</sup> mean values in the same column with the different letter superscripts are highly significant difference ( $p < 0.01$ )

ND: Not determined

T1 : control or commercial diet (CD), T2: CD + avilamycin, T3 :CD + commercial probiotics,

T4 : CD + *Ent. faecium* PR-2 , T5 : CD + Rumen bacterium MG-2 , T6 : CD + *L. plantarum* IFS-1,

T7 : CD + *L. plantarum* IG-3 and T8 : CD + mixed culture of probiotics.

than T8 and T3, ( $p < 0.01$ ). However, LAB cell counts of T6 was significantly high only at seven day feeding but tended to decrease at forty two days. Therefore, T7 (diet supplemented with *L. plantarum* IG-3) revealed the highest LAB counts throughout the feeding trials (7 and 42 days).

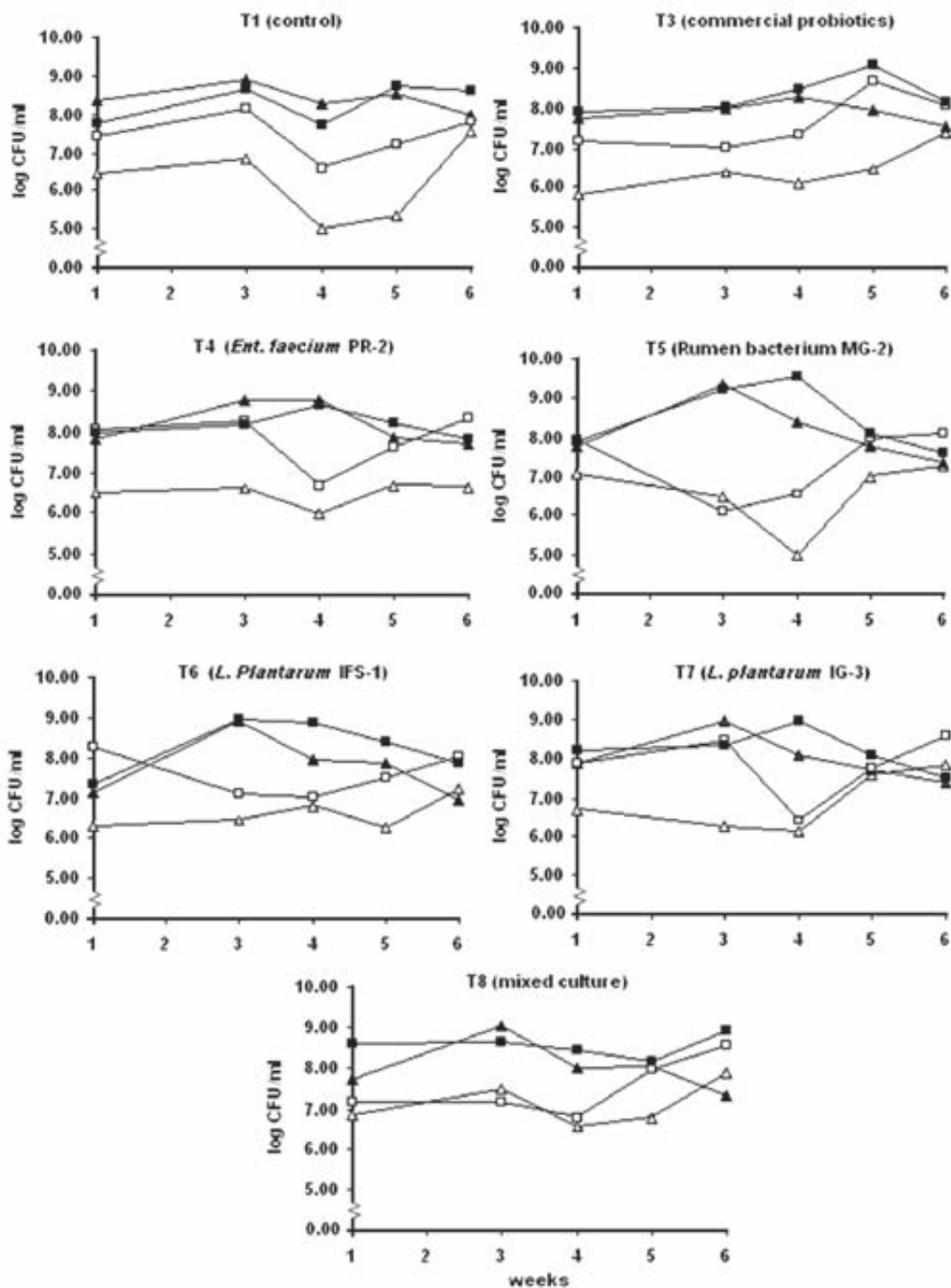
When consider the growth performance of LAB (log cfu/ml) in ceca of broilers after feeding for seven days, T8 was better than control, T1, ( $p < 0.01$ ) and significantly different to T6 ( $p < 0.01$ ). However, there were no difference ( $p > 0.01$ ) among T3, T4, T5, T7 and T8. After feeding for 42 days, the growth of LAB in ceca of broilers treated by T8 was highest as compared to those of T2 and T3 ( $p < 0.01$ ). Hence the overall picture with respect to the growth of LAB in ileum and cecum of the broiler, T8 (mixed strains treatment) was the most suitable combination of probiotics enhanced the LAB growth in broiler's intestine. The proper ratio of each LAB strain in the mixed culture should be studied in order to improve LAB growth.

The influence of probiotics on changes of the number of *E. coli* in ileum after feeding for 42 days was observed, it tended to increase while the reduction of *E. coli* counts were found in

cecum. The benefit of adding the selected LAB strains as feed additives for broilers were shown in Figure 3. The LAB counts of both chicken ileum and cecum of every treatment were higher than those of *E. coli*. However, this evident was much clearer in mixed strain (T8) than the single strain (T4 to T7), commercial feed and control. The reduction of *E. coli* probably due to antimicrobial activity of short chain fatty acids and lactic acid which are the major metabolites of LAB (Sinha, 1986). Moreover, *Salmonella* sp. was not detected in every treatment including control which were coincident with the report of Pascual *et al.* (1999). This may be due to good sanitation and management of the poultry farm as well.

## CONCLUSION

*Enterococcus faecium* PR-2, Rumen bacterium MG-2, *Lactobacillus plantarum* IFS-1 and *L. plantarum* IG-3 were the probiotic Lactic acid bacteria. They possessed acid and bile tolerances as follows: *L. plantarum* IFS-1 and *L. plantarum* IG-3 survived at pH 2 for 6 hours incubation. Viable cell counts of these strains decreased by 2.08 and 2.97 orders, respectively. The results on bile tolerance show that



**Figure 3** Effect of probiotics on the changes of viable counts of Lactic acid bacteria and *E. coli* in treated chicken intestine (□) LAB in ileum, (■) LAB in cecum, (△) *E. coli* in ileum and (▲) *E. coli* in cecum. (T2 was not determined).

*Ent. faecium* PR-2 and Rumen bacterium MG-2 were more bile resistant than *L. plantarum* IFS-1 and *L. plantarum* IG-3. Supplementation of these 4 probiotic strains in drinking water affected the viability of LAB and *E. coli* in treated chicken intestine (ileum and cecum). In comparison with control group, the cell counts of lactic acid bacteria in the ileums of broilers consuming commercial probiotics, single culture (4 strains) and mixed culture during rearing period were higher ( $P < 0.01$ ) while *E. coli* in ceca of the same treatments were lower ( $p < 0.01$ ). However, the population of lactic acid bacteria was higher than *E. coli* in both ileum and cecum of chickens. Moreover, *Salmonella* sp. was not detected in the treated broiler intestines. Chitanont *et al.* (2007) reported that optimal supplementation of probiotic content with these LAB strains in drinking water could reduce fat content level and cholesterol level in broiler meats. Therefore, application of the four LAB strains as feed additives for broilers is possible.

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