

# Effects of Anti-Caries Antibodies on *Lactobacillus GG* in Its Fermentation and Storage Periods<sup>1</sup>

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**Objective** To investigate how antibodies influence the fermentation of *Lactobacillus GG* and how *Lactobacillus GG* influences the biological properties of antibodies during the fermentation and storage periods. **Methods** Anti-caries immune colostrum powder (IP) and control colostrum powder (CP), skimmed milk powder (SP) at concentrations of 1%, 2.5%, 5% and 10% (w/v) were added to MRS and 50mM Hepes buffer system was used in the milk, the growth curves of *Lactobacillus GG* including viable cells, lactic acid concentration and pH, and also the titer of specific antibodies were determined during the fermentation and storage periods. **Results** In MRS, SP could improve the growth of *Lactobacillus GG* in all periods of fermentation, especially at the concentrations of 5% and 10%. CP at the concentrations of 1% and 2.5% had a good initial velocity at the beginning and CP (1%, 2.5%, 5%, 10%) in all the groups could reach a high viable cell concentration at the end of fermentation, which suggested that there were some growth factors for *Lactobacillus GG* in CP overcoming the inhibition of unspecific antibodies. IP at 1%, 2.5%, 5% and 10% could inhibit the growth of *Lactobacillus GG* in all the fermentation periods, the critical concentration point was between 2.5%-5%, and there was a competition between growth factors and the inhibition of specific antibodies. In pasteurized milk, the influence of Hepes could help the fermentation start quickly and reach the log phase earlier than in the control group; however, when combined with 5% IP, the growth of *Lactobacillus GG* was strongly inhibited during all the fermentation periods. The fermentation of *Lactobacillus GG* had no significant effect on the titer change of anti-caries antibodies during the fermentation and storage periods. **Conclusion** SP and CP is beneficial to the growth of *Lactobacillus GG* in MRS, whereas there is a competition between growth factors and the inhibition of specific antibodies to the growth of *Lactobacillus GG*, the critical concentration of IP is 2.5%-5%. Hepes could influence the titer of antibodies through buffering Ph, thus influence the fermentation of *Lactobacillus GG*. The fermentation of *Lactobacillus GG* with IP or CP could be used for the development of functionally fermented immune milk in the future.

**Key words:** Antibody; *Lactobacillus GG*; Fermentation

## INTRODUCTION

Probiotics in human nutrition is a live microbial food ingredient that is beneficial to health; it can improve health of the host by affecting immune system, inhibiting the growth

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Biographical note of the first author: Wei Hua, male, born in November, 1996, Master Degree, Associate Pro-fessor, research fields: food microbiology, won 3rd Grade Prize of Jiangxi Science and Technology Improvement for 2 times, published 30 papers in scientific journals.

of harmful bacteria and enhancing nutrient availability<sup>[1]</sup>.

A large number of publications have documented that probiotics plays an important part in prevention and treatment of gastrointestinal diseases<sup>[1, 2]</sup>. The bacterial genera often used as probiotics are lactobacilli and bifidobacteria. *Lactobacillus GG*, originally isolated from human intestine, has been proved as an important probiotics functioning in human body<sup>[3]</sup>. S. Elo and his colleagues showed that *Lactobacillus GG* could attach to human intestinal cell line Caco-2 *in vitro*, survive well at pH3.0 in human gastric juice *in vitro* and modulate colon microecology *in vivo* by decreasing the glucuronidase activity<sup>[4]</sup>. Alander *et al.* also showed that *Lactobacillus GG* is able to adhere *in vivo* to the colon<sup>[5]</sup>. Ruseler-van Embden *et al.* tested that *Lactobacillus GG* did not break down intestinal mucus glycoprotein and thus was safe to use for therapy<sup>[6]</sup>. Majamaa H concluded that certain strains of lactic acid bacteria, especially *Lactobacillus GG*, promoted serum and intestinal immune responses to rotavirus, and thus might be important in establishing immunity against rotavirus reinfections<sup>[7]</sup>. M. Malin *et al.* (1997) proved that orally administrated *Lactobacillus GG* had a potential to reinforce the mucosal barrier mechanism in juvenile chronic arthritis<sup>[8]</sup>.

The concentration of immunoglobulins in cow's colostrum is 50-100 times higher than that in milk. Specific colostrum antibodies could be got by systemic hyperimmunization of cows with specific microbial antigen<sup>[9]</sup>. More and more clinical studies have demonstrated the efficacy of immune milk preparations in the prophylaxis or therapy of human gastrointestinal diseases<sup>[10-16]</sup>. Roos *et al.* (1995) proved that the immunoglobulins from bovine colostrum retain at least partially biological activities in the human GI tract<sup>[17]</sup>.

Although great success has been reported in functional studies on the bovine colostrum antibodies and probiotics respectively, few studies were focused on the co-existence of antibodies and lactic acid bacteria (LAB), especially on *Lactobacillus GG*. A member of questions need to be answered, such as the cooperative function of antibodies and probiotics in the prevention and treatment of GI disease either *in vitro* or *in vivo*, the main contributory factors in the actions and the mechanism for adherence of epithelium of the mucus. The aim of this study is to investigate how antibodies influence the fermentation of *Lactobacillus GG* and how *Lactobacillus GG* influences the biological properties of antibodies during the fermentation and storage periods.

## MATERIAL AND METHODS

### *Bacterial Strain*

Strain of *Lactobacillus GG* was provided by Food Research Institute of Agricultural Center of Finland. Strains stored at -70°C was activated in 10% sterilized skim milk at 37°C for 12 h, and then was transplanted to MRS solid medium and incubated in the anaerobic environment at 37°C for 36 h; after 3 generation transplant on the MRS solid medium, single colony of *Lactobacillus GG* was inoculated to broth of MRS and incubated at 37°C for 10 h under anaerobic conditions. Cells were harvested by centrifugation at 3000g for 10 min, the supernatant was thrown away and pellet was washed two times by sterile 0.7% saline, and the pellet of viable cell was resuspended in saline as starter for fermentation.

### *Medium*

MRS liquid medium was prepared according to the formula and sterilized under 115°C for 15 min.

Pasteurized milk: fresh milk was centrifuged under 25 000 rpm for 15 min to remove the fat and casein, and then pasteurized at 85°C for 1 h.

Skim milk powder (SP) was provided by Valio Company.

Anti-caries immune colostrum powder (IP) and control colostrum powder (CP) were obtained according to the methods described<sup>[18]</sup>. 20% IP and 20% CP were dissolved into deionized water, and then sterilized by microfiltration (0.22 $\mu$ m). SP, CP and IP at concentrations of 1%, 2.5%, 5% and 10% (w/v) were added to MRS.

Hepes of 50 mmol/L was also used in the pasteurized milk before the milk was sterilized, and 5% IP was added according to the experiment plan.

### *Viable Cell Counts*

Viable cells were estimated by 10 fold dilution in 0.7% sterile saline. 100  $\mu$  L samples of 10<sup>-5</sup>, 10<sup>-6</sup>, 10<sup>-7</sup> dilution were plated on MRS agar, and each dilution was with 2 parallels. After incubation at 37°C for 48 h, colonies were counted to determine the viable cells of each group.

### *pH Determination*

pH meter.

### *Lactic Acid Determination*

1mL sample was diluted with sterilized water to 5 mL and kept in the boiling state; 15  $\mu$ L phenolphthalein (0.5% in 95% ethanol) was added, and then titrated with 0.1N NaOH by mixing the sample all the time. When the sample became pink and stable for 30 seconds, the final degree of lactic acid could be calculated as 1mL 0.1N NaOH equivalent to 0.0090 lactic acid.

### *ELISA*

The wells of polystyrene microtitre plates (Labsystems, Finland) were coated with a 100  $\mu$ L (10<sup>9</sup> CFU/mL) suspension of heat killed *S. mutans* in phosphate buffered saline (PBS, pH 7.3). After incubation at 4°C overnight, the wells were washed three times with PBS containing 0.05% Tween 20 (PBST) and rinsed three times with ionized water by the Mutiwash system (Labsystems, Finland). IP samples were tested in duplicate at six different dilutions (1:1000, 1:3000, 1:9000, 1:27000, 1:81000, 1:24300). An aliquot of 100  $\mu$  L of each dilution per well was added to the antigen coated plates. After incubation at 37°C for 90 min, the wells were washed as before and 150  $\mu$  L of alkaline phosphatase conjugated anti-bovine IgG (Sigma, St. Louis, MO, USA) was added (dilution 1:12000) at 37°C for 90 min. After washing the plates were developed for 30 min with 150  $\mu$  L of p-nitrophenylphosphate in diethanolamine-MgCl<sub>2</sub> buffer (2mg /mL). The absorbance at 405 nm was measured very quickly.

### *Lactobacillus GG Fermentation in MRS and Fresh Milk*

Activated viable cells in log phase were harvested and washed 3 times with 0.85% sterile saline, and then were suspended in saline and vortex to make it in an uniform state. Aliquot of 100  $\mu$  L was used to determine the viable cells in the suspension. 200  $\mu$ L (A), 1mL (B) and 2mL (C) of suspension were inoculated in 100mL MRS medium, and the pH, lactic acid and viable cells were determined during the fermentation time.

Two different inoculations were also performed in pasteurized milk, and the pH, lactic acid and viable cells were monitored during the fermentation time.

### *Influence of SP, IP and CP on the Growth of Lactobacillus GG in MRS*

IP and CP at 1%, 2.5%, 5% and 10% were added to MRS as test groups, while Sp at 1%, 2, 5%, 5% and 10% was added to MRS liquid medium as control groups. *Lactobacillus GG* saline suspension ( $3 \times 10^7$ CFU) of 1.0 mL was inoculated into 100mL medium of each group and incubated at 37°C until the log period was reached. During the fermentation period, an aliquot of 4mL was drawn out several times from each group to test the pH, lactic acid and viable cell.

### *Effects of Hepes System on the Growth of Lactobacillus GG in Milk*

50mmol/L Hepes was added to the pasteurized milk before sterilization to form hepes group, and the pasteurized milk was assigned as the control group. Suspension of viable cells was inoculated and incubated at 37°C for 48 h, and the pH, lactic acid, titres and viable cells were followed up. After 48 h, the groups were stored under 4°C, and all the indexes as above were checked once for several days.

### *Effects of IP on the Growth of Lactobacillus GG in Milk With Hepes Buffer System*

5% IP was added to the groups mentioned above, and all the procedure was the same as above.

### *Storage Experiments of Lactobacillus GG*

The same as mentioned above.

## RESULTS

### *Lactobacillus GG Fermentation in MRS and Fresh Milk*

Three different amounts of viable cells were inoculated in MRS and two different inoculation amounts were introduced in pasteurized fresh milk. The indexes of viable cells, lactic acid and pH were checked from time to time to determine the log phase of fermentation. Apparently, there was a big difference between MRS medium and FM for the growth of *Lactobacillus GG*. The log phase of *Lactobacillus GG* in MRS was about 9 h, no matter what kind of inoculation amount of viable cells was from the beginning; there was no significant difference between pH change, lactic acid metabolism and viable cells in all the fermentation periods (Fig. 1). However, different inoculation amounts of viable cells could significantly influence the pH change, lactic acid metabolism and viable cells, and the platform of log phase was about 36-48 h (Fig. 2).

### *Effects of SP, CP and IP on the Growth of Lactobacillus GG in MRS*

Skim milk powder at 1%, 2.5%, 5%, 10% was put into MRS medium and inoculated at 37°C, and the pH and viable cell were determined several times within 24 h. The results showed that 1%, 2.5%, 5%, 10% SP could significantly improve the growth of *Lactobacillus GG*, and that the log phase did not change much compared with the control group, but it was abnormal in the fermentation of 2.5% SP group at 8-12 hours (Fig. 3). It was also very interesting that the pH of 1%, 2.5%, 5% SP rose slightly around 8-10 hours, because some of the protein was probably broken and the amino groups were exposed to neutralize the acid. In the CP groups, 1%, 2.5%, 5%, 10% CP could also improve the growth of *Lactobacillus*

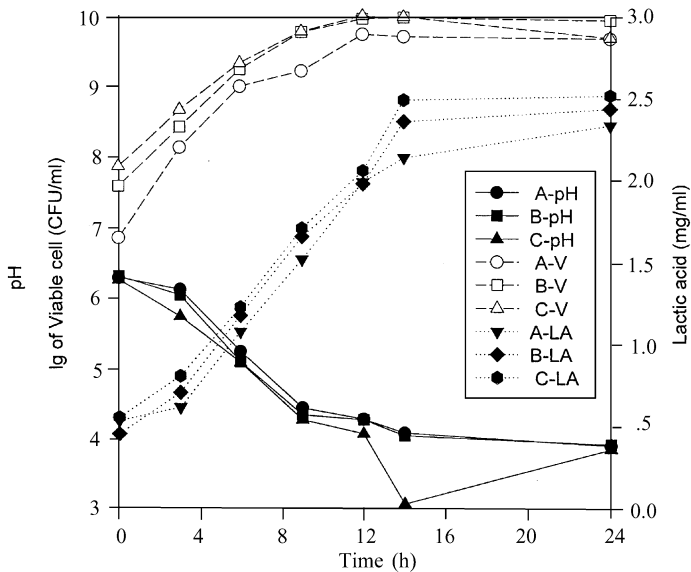


FIG. 1. *Lactobacillus GG* fermentation in MRS.

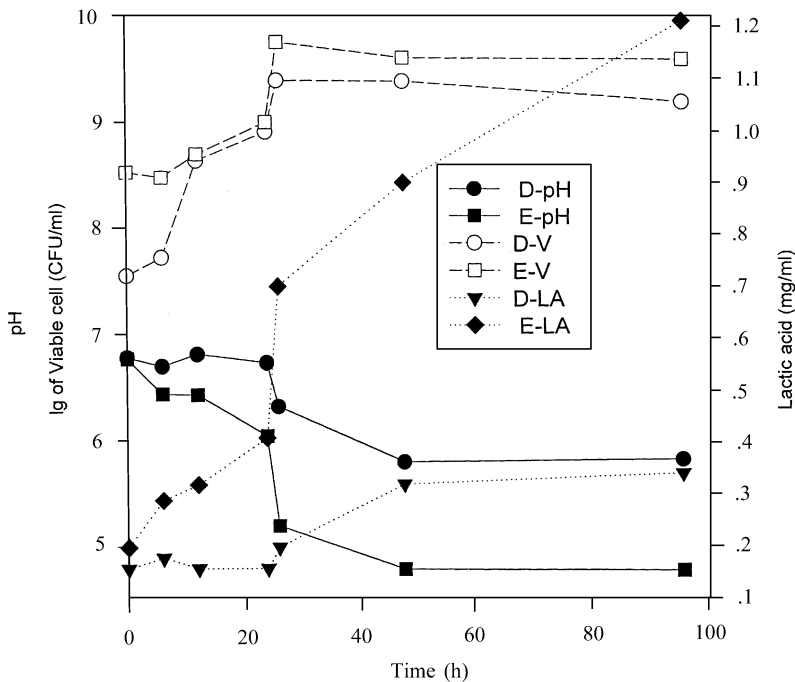
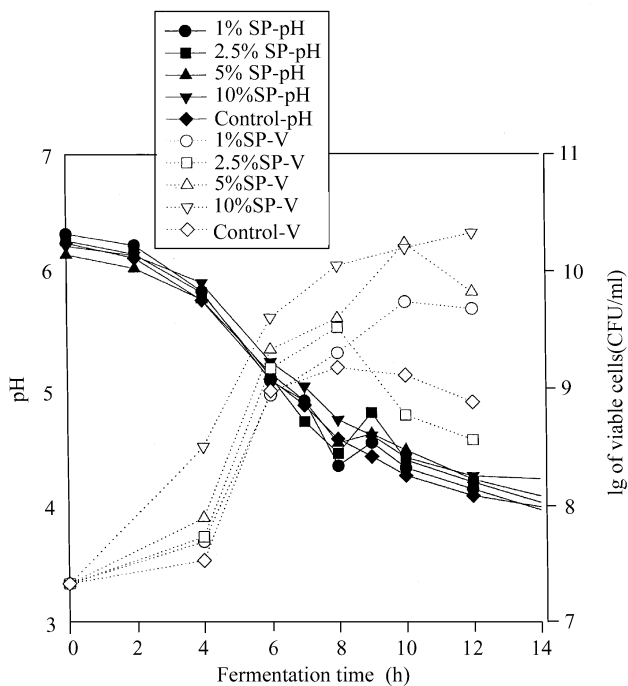
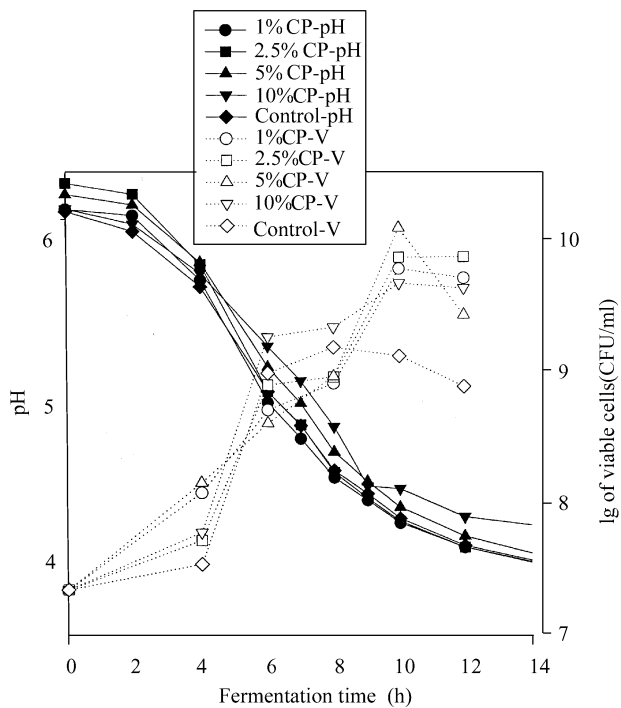


FIG. 2. *Lactobacillus GG* fermentation in fresh milk.

Fig. 3. Influence of SP to the fermentation of *L. GG*.Fig. 4. Influence of CP to the fermentation of *L. GG*.

*GG* and the log phase was delayed a little about 2 h, compared with the SP group, and the download of pH was very slow and smooth in 8-10 h (Fig. 4). In the IP groups, all the test concentrations had an inhibitory function on the growth of *Lactobacillus GG* and the critical point for strong inhibition was seen between 2.5% and 5% (Fig. 5).

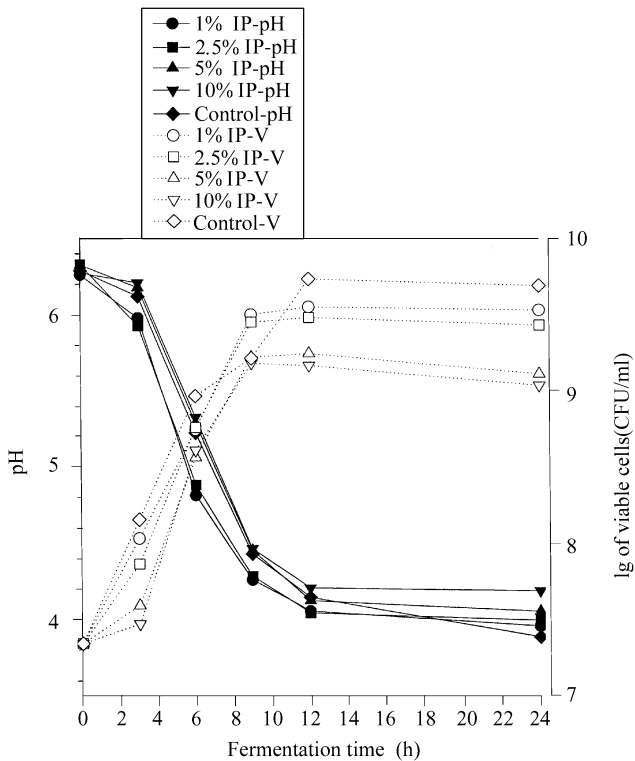


FIG. 5. Influence of IP to the fermentation of *L. GG*.

#### Effects of Hepes Buffer System on the Growth of *Lactobacillus GG* in Milk

50mmol/L Hepes was produced in the fresh milk and *Lactobacillus GG* was inoculated into it as was done with the control group. After incubated at 37°C for 48 h, samples were picked up at the time point of 6, 12, 24, 36 and 48 h, and the pH, lactic acid and viable cells were determined (Fig. 6).

Apparently, Hepes increased the growth of *Lactobacillus GG* at the beginning and its log phase was a little earlier than in the control group. The amount of lactic acid was a little higher than in control group before 24 h, but after 24 h the lactic acid increased very slowly, whereas that in the control group increased very fast which coincides with the viable counted results, suggesting that the log phase in control group was later than in the Hepes group.

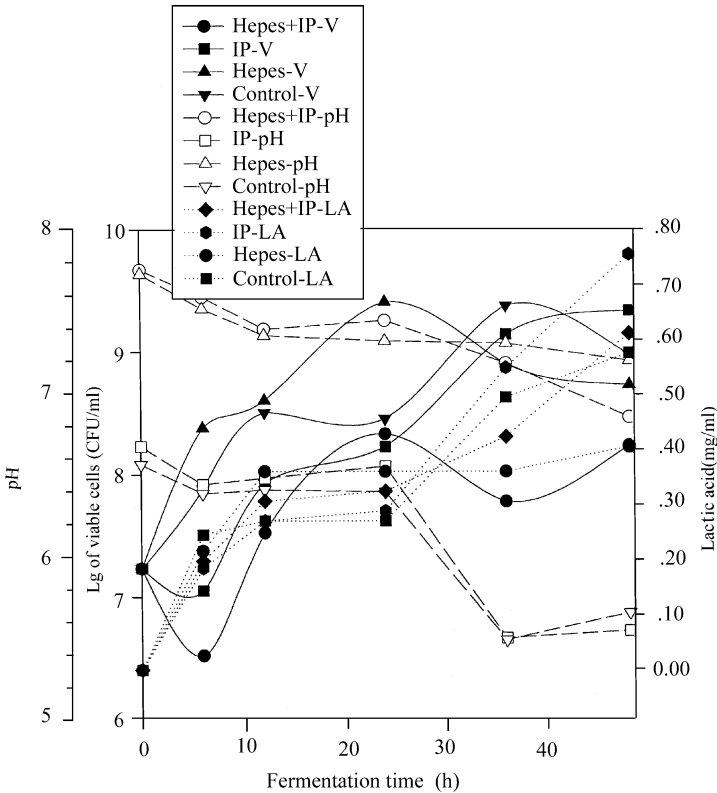


FIG. 6. Influence of HEPES & IP to the fermentation of *L. GG*.

### Effects of IP on the Growth of *Lactobacillus GG* in Milk With HEPES Buffer System

IP at 5% was added to the fresh milk combined with 50mmol/L HEPES, and the test results were shown in Fig. 6. From 0 to 6 h, IP had a strong inhibition of the growth of *Lactobacillus GG*, either in the group with or without HEPES; from 6 to 24 h, both of the IP group entered the log phase of growth. It seemed that the group with HEPES entered the platform of log phase earlier than the IP group without HEPES, as the neutral pH helped antibodies to inhibit the growth at a certain concentration. It was worthy noticing that the IP group without HEPES continued its log phase after 24 h, and entered platform slowly after 48 h, a little later compared with the HEPES group and the control group.

Although the lactic acid increased very fast at the period from 36 h to 48 h, the pH was maintained at the same level or even a little higher. It was possibly relevant with the cleavage of protein of milk, and the alkalinescence group was exposed and neutralized the lactic acid. It was very similar with the results in Fig. 3.

It seemed that HEPES and IP had different effects on the growth of *Lactobacillus GG*, and that HEPES could increase the growth of *Lactobacillus GG*, thus the log phase was a little earlier than control group, since it maintained a neutral environment to reduce the suppression of lactic acid to its growth at its buffer extent, although its ion concentration could have some influence on the fermentation. On the contrary, IP inhibited the growth of *Lactobacillus GG* at the beginning of fermentation. Its log phase was a little later compared



with the control group, however, it could reach at least the same level of viable cells and its lactic acid was higher than in the group with HEPES and the control group. When IP and HEPES combined with each other, the situation was different. At the beginning of fermentation, the growth of *Lactobacillus GG* was also strongly inhibited, and with the production of lactic acid, the growth of *Lactobacillus GG* surpassed the inhibition of IP, but when it reached a certain concentration, the growth and inhibition seemed to reach a balance. Therefore, HEPES strengthened the functions of antibodies of IP, while the lactic acid reduced the inhibition of IP to the growth of *Lactobacillus GG* by changing its pH.

It was noticed that at 6 h the titers of IP were higher than any other time point; at this time point the *Lactobacillus GG* was also heavily inhibited, thus some positive correlation or mechanism may exist between the titer and growth of *Lactobacillus GG*. The titer of IP+*L.GG* was significantly lower than that of the HEPES+IP+*L.GG*, HEPES+IP at 6 h. Therefore, HEPES played an important role in maintaining the pH at neutral, which was beneficial for the immunoglobulins to reach its maximum activities. Compared with IP group, the titer of IP+*L.GG* group was also remarkably lower at 6 h although the difference of pH was insignificant (data not shown in the Figure), thus the lactic acid could restrain the activities of immunoglobulin in some extent (Fig. 7).

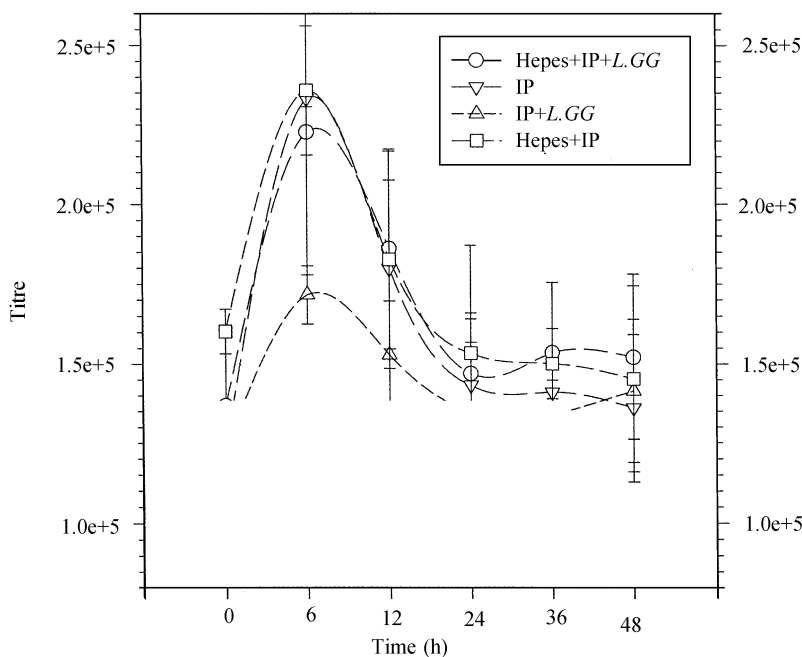


FIG. 7. Titre change of IP in the fermentation of *L. GG*.

#### Storage Experiments of *Lactobacillus GG*

When the growth of *Lactobacillus GG* reached the platform of Log phase, it was saved at 4°C and the indexes of viable cells, pH, lactic acid and titer were checked from time to time (Figs. 8 and 9).

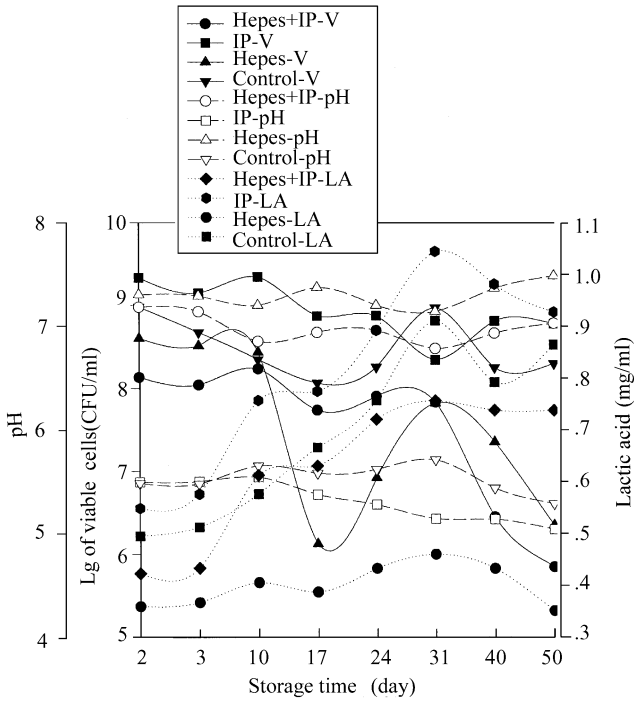


FIG. 8. Storage of *L. GG* under 4°C.

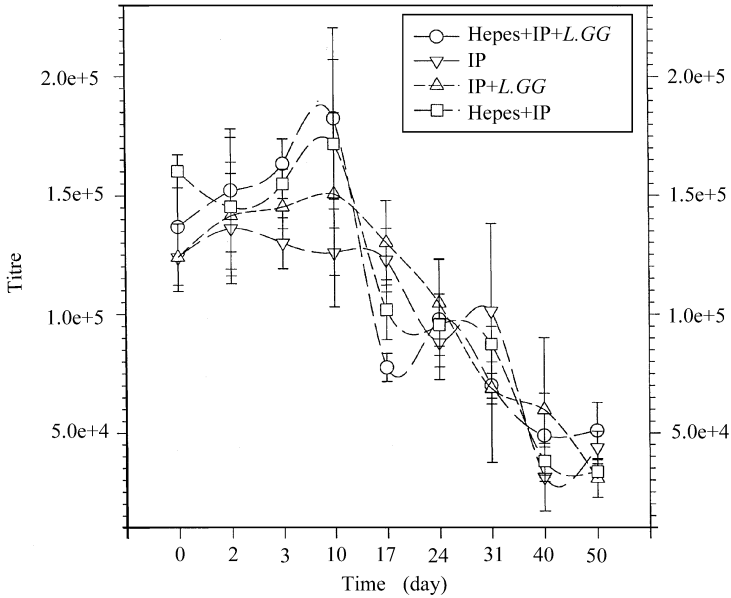


FIG. 9. Titre change of IP under 4°C in storage period.

Hepes is a main factor to influence the survival of *Lactobacillus GG*. In the groups without Hepes, IP and the control group could maintain nearly the same viable cell concentration during 50 days storage at 4°C. However, the groups with Hepes could only

keep the same viable cell level before 31 days.

From Fig. 8, we can also see that lactic acid concentration in the non-Hepes groups kept a higher level compared with the Hepes groups, suggesting that the metabolism of *Lactobacillus GG* was stronger in the none Hepes groups.

From Fig. 8, after 31 days storage, pH in the none-Hepes groups was maintained at the same level or continued to fall down, however pH in the Hepes groups rose slowly because of the death and lysis of cells.

As for titers, it seemed there was no significant difference between the Hepes groups and the non-Hepes groups. *L.GG* had little influence on the titer of immunoglobulin during the 50- day storage period.

## DISCUSSION

Probiotics in general is characterized by its resistance to acid and bile, adherence to intestinal epithelial cells and colonization in the intestine. Lactobacilli are widely used as probiotic bacteria for human health. *Lactobacillus GG* originally separated from the human gastrointestinal tract has been indicated as an acid-resistant and bile-resistant strain<sup>[19]</sup>. It could survive and colonize the human gastrointestinal tract temporarily, produce an antimicrobial substance and affect the metabolic activity of the resident microflora<sup>[19, 20]</sup>. It also colonizes the intestinal tract during oral antibiotic administration<sup>[21]</sup>.

In this study *Lactobacillus GG* had the same fermentation period, similar pH and lactic acid curves in MRS no matter what amount of viable cells were inoculated in the medium of MRS. Nevertheless, *Lactobacillus GG* has a long fermentation period in pasteurized milk, and very few publications have reported single use of *Lactobacillus GG* as starters. The inoculation amount and log phase state of *Lactobacillus GG* are critical to its growth in milk. It is suggested that the minimum oral dose of *Lactobacillus GG* for beneficial health function is  $10^{10}$ - $10^{11}$  CFU/day as a freeze-dried powder, fermented milk or as a fermented whey drink<sup>[22, 23]</sup>. In fermented milks the number of viable cells is often about  $10^8$  CFU/mL and thus 100-200mL of fermented milk will supply  $10^{10}$ - $10^{11}$  CFU<sup>[24]</sup>. Our test has also shown that *Lactobacillus GG* could reach above  $10^9$  CFU/mL although single strain was used as a starter.

In order to observe the effect of immune colostrum powder (IP) and control colostrum powder (CP) on the growth of *Lactobacillus GG* in MRS, different concentrations were added to the MRS medium and skim milk powder (SP) was used as the control.

All the SP groups could improve the growth of *Lactobacillus GG* in all periods of fermentation, especially at 5% and 10% concentration. Obviously, the skim milk powder played a buffer function and supplied some special carbonhydrates and nitrogen for the fast multiplication of cells. Between 8 and 10 h pH curves of the SP group were slightly higher than those of the control group, probably because of outburst of some cleavage of protein.

CP had a good start velocity for cells multiplication from 0 to 4 h, suggesting that CP may contain some growth factors for *Lactobacillus GG* and thus overcome the refresh period in common bacteria growth model. While entering the log phase (6-8 h), cell growth of the CP group was slower than that of the control group, suggesting an inhibition by some unspecific antibodies. From 8-12 h, the cells overcame the inhibition by antibodies and again reached a level higher than in the control group. It is most likely that the growth factor in CP plays a main role in overcoming the inhibition by antibodies.

As for the IP group, it seems that antibodies in IP play a main role in the fermentation period. They inhibited the multiplication of the cells of *Lactobacillus GG* during all the fermentation period, especially at the beginning. There was a difference between 1% and

2.5% IP group and 5% and 10% IP group, suggesting that the extent between 2.5% -5% is the critical point for weak or strong inhibition. The higher the concentration of IP, the stronger the inhibition, and the poorer the growth of *Lactobacillus*. From the pH change curves, pH of the 1.0%, 2.5% IP group fell very fast compared with the control group, which was also markedly different from the CP group.

Overall, SP, CP and IP have different functions for the growth of *Lactobacillus GG* since their compositions are different. SP owns common carbohydrates and nitrogen which are favorable for the growth of *Lactobacillus GG*, while CP contains unspecific antibodies with limited ability to inhibit cell growth and growth factors for *Lactobacillus GG*. As a result, growth of *Lactobacillus GG* is much improved in both cases. However, the situation for IP is quite different, in which there exist some specific antibodies to *streptococcus mutans* which may strongly inhibit the growth of *Lactobacillus GG* and also some growth factors of *Lactobacillus GG*, leading to a poor growth of *Lactobacillus GG*. The competition between the inhibition of antibodies and the growth factors of IP is also influenced by the trace trypsin or chymotrypsin in IP. Both enzymes could reduce the activities of antibodies. However, our results showed that the titers of antibodies experiences little change during the fermentation period (data are not shown).

Hepes buffer system is used in the pasteurized milk to probe the IP function in the milk fermentation of *Lactobacillus GG*. Hepes has two different functions for the growth of *Lactobacillus GG*. In one aspect, Hepes could reduce the refrain of accumulation of lactic acid for the growth of *Lactobacillus GG*, but in the other aspect, it could also help the antibody maintain a stable state of function by keeping pH neutral to some extent. Our test showed that Hepes could help the fermentation start quickly and reach the log phase earlier. However, when combined with IP, the results were opposite; the growth of *Lactobacillus GG* was strongly inhibited during all the fermentation period, the IP group was only slightly inhibited, and although the log phase came later, it could reach the same viable cells as the control group. This results coincide with the results of IP in the fermentation in MRS.

The shelf life of *Lactobacillus GG* and the titre of antibodies were critical in our test. The storage test showed that the viable cells of all the fermented milk groups could maintain the same level during the 31 day storage at 4°C. After 31 days, the groups with Hepes dropped faster, while the groups without Hepes continued to keep the same level. It seems that IP has little influence on the shelf life of *Lactobacillus GG* during the storage period. The pH change had little difference, while the lactic acid metabolism of the non-Hepes groups was more active than that of the Hepes groups, which coincides with the results of viable cell storage. The titre change in both Hepes groups and non-Hepes groups had no difference, the fermented milk could keep the same titer level in 10 days, could reserve 50% in 31 days and more than 30% in 50 days under 4°C. Further more, our experiments have shown that the functional ability of anti-caries antibodies in fermented immune milk remains intact after 50 day storage at 4°C (data not shown).

Using probiotic bacteria in immune milk fermentation is a new area to develop functional food for human health. Different strains and specific antibodies should be tested to get more information about the general mechanism of antibodies to probiotics' fermentation, and functional experiments should also be conducted *in vitro* or *in vivo*. Since there are some common antigens of probiotic bacteria that could react with antibodies and agglutination in the fermentation process, some probiotic bacteria should be chosen to avoid such agglutination or unspecific antibodies to probiotic bacteria should be used to constitute immune milk. Although fermentation of immune milk is a feasible way to develop functional food, how to keep the activity of antibodies and sterilize the immune milk in the same time before fermentation is still a problem, and some specific processing techniques should be developed.

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