

Full Length Research Paper

Elimination of acidic or oxidative stress for four probiotics with some chemicals *in vitro*

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Accepted 28 May, 2013

Lactobacillus bulgaricus or *Lactobacillus helveticus* was incubated in skim milk with addition of sodium citrate, calcium carbonate or sodium carbonate at 37°C for 24 h, whereas *Bifidobacterium animalis* or *Bifidobacterium infantis* was incubated in skim milk with addition of sodium D-isoascorbate, sodium L-ascorbate or L-cysteine at 37°C for 24 h in an anaerobic chamber. Viable count of *L. bulgaricus*, *L. helveticus*, *B. animalis* or *B. infantis* in culture was enumerated by standard plate counting technique. The result showed that the viable count of *L. bulgaricus* or *L. helveticus* had an increase about 150 ~ 220%, *B. animalis* or *B. infantis* in culture had an increase about 150 ~ 190%. The effects of these chemicals that could eliminate acidic or oxidative stress for four probiotics during incubation *in vitro* were validated thereby, which implies that such technique might be a simple and effective approach for probiotic production in higher density.

Key words: *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, *Bifidobacterium animalis*, *Bifidobacterium infantis*, chemical stressing.

INTRODUCTION

The gut microflora is composed of microorganisms that are classified as pathogenic, neutral or beneficial to the host. The latter group of bacteria, also known as probiotic bacteria, generally predominates in the healthy gut. Probiotics have been defined as "living microorganisms that contribute to intestinal microbial balance and have the potential to improve the health of their human host" (Fuller, 1991). Probiotics, being used in large amounts in the preparation of foods and dairy products, are able to survive the passage through the upper digestive tract as well as to adhere to intestinal cells, providing beneficial effects to the intestine (Gilliland, 1989). Recent clinical trials have shown their efficacy for treatment of allergic rhinitis (Sunada et al., 2007; Peng and Hsu 2005). The role of probiotics in prevention of atopic conditions such as eczema, food allergy and asthma also were reported (Kalliomäki, 2001; Lodinová- Zádňíková, 2003; Kopp, 2008). Probiotics are commonly included in dairy foods, such as yoghurt (Gilliland et al., 2002), ice cream (Godward and Kailasapathy, 2003) and cheese (Corbo et al., 2001), but are also available in other forms. Fermented dairy pro-

duct including cheese and yoghurt, have been widely accepted as vehicles for transmission of probiotics to consumers (Adhikari et al., 2003). Lactic acid bacteria isolated from human and animal gastrointestinal tract, when largely consumed are well known to be beneficial to the intestinal balance (Ferreira, 2003). Lactic acid bacteria (LAB) products have been one of the major health-related foods in the world (Khali, 2009). *Lactobacillus* and *Bifido-bacterium* species are especially among the most commonly used probiotics and have a long history of being securely consumed (Stanton et al., 2001, 2003).

Although there is overwhelming evidence that consumption of certain specific strains exert a number of beneficial health effects (Salminen et al., 2005), the large-scale cultivation and subsequent storage of probiotics in high concentration often present a major bottleneck to the realization of their commercial potential. In order to impact the desired health benefits, probiotic bacteria should be present in the product in higher viable count ($7-9 \text{ Log CFU mL}^{-1}$) during their whole shelf life (Kurmman and Rasic, 1991), which is required to successfully develop foods (Heller, 2001). As higher count of viable cells is needed to develop functional foods, the most common technique for increasing count of probiotics in cultures now is relied on continuous culture to eliminate the stress of some disadvantage effects occurred to probiotics, such

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as detrimental effects of acidity, oxygen (Reilly and Gilliland, 1999; Desmond et al., 2002). Our previous work had confirmed that some chemicals, alkaline salts or antioxidants, could be applied in culture to eliminate acidic or oxidative stress of two probiotics (*Lactobacillus acidophilus* and *Bifidobacterium bifidum*) and to provide favorable condition for probiotics growth; there was a significant increase of the viable count of cells of probiotics, and the related chemical mechanism had been proposed (Zhao and Li, 2008). Whether this new approach might be available to other probiotics needs to be validated.

In the presented study, four probiotics, *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, *Bifidobacterium animalis* and *Bifidobacterium infantis*, were incubated in skim milk. The acidic or oxidative stress produced during incubation was eliminated by adding some chemicals to culture. The chemicals used for the elimination of acidic stress were three alkaline agents (sodium citrate, calcium carbonate and sodium carbonate), and these used for the elimination of oxidative stress were three antioxidants (D-isoascorbate, L-ascorbate and L-cysteine). The results showed that such treatments were also effectively to increase the viable count of cells of four probiotics in culture.

MATERIALS AND METHODS

Strains and their activation

Four probiotic species, *L. bulgaricus*, *L. helveticus*, *B. animalis* and *B. infantis* were from the Key Lab of Dairy Science of Ministry of Education, Northeast Agricultural University. Lyophilized *L. bulgaricus* and *L. helveticus* was rehydrated in 1 ml of a sterilized rehydration medium (12% skim milk) with subsequent 5% (v/v) inoculation into normal lactobacilli MRS medium (Difco, Detroit, MI, USA), then incubated at 37°C for 24 h. Lyophilized *B. animalis* and *B. infantis* was rehydrated in 1 ml of a sterilized rehydration medium (12% skim milk) with 10% (v/v) inoculation into TPY medium, then incubated at 37°C for 24 h in an anaerobic chamber (Beckman Coulter, USA) with atmosphere composition of 85% N₂, 5% CO₂ and 10% H₂.

Elimination of acidic stress of *L. bulgaricus* and *L. helveticus*

The ultra-high temperature (UHT) skim milk was used as the culture in a 100 ml glass bottle. *L. bulgaricus* or *L. helveticus* was incubated in 25 ml 12% (w/v) skim milk at 37°C for 24 h, in which one of sterilized alkaline salt solutions, sodium citrate, sodium carbonate or calcium carbonate suspension, was added with different ways before inoculation. The viable count of *L. bulgaricus* or *L. helveticus* in culture was determined with standard plate counting technique as below. UHT skim milk without alkaline salt addition was served as control.

Alternatively, *L. bulgaricus* or *L. helveticus* was also incubated in 25 ml 12% (w/v) skim milk at 37°C for 6 h. Sterilized sodium citrate solution, sodium carbonate solution or calcium carbonate suspension was added to culture. Then skim milk was incubated continuously at 37°C for 18 h. The viable count of *L. bulgaricus* or *L. helveticus* in culture was determined. UHT skim milk without alkaline salt addition was served as control.

Elimination of oxidative stress of *B. animalis* and *B. infantis*

UHT skim milk was used as culture and one of three antioxidants, sodium D-isoascorbate, sodium L-ascorbate or L-cysteine, was added di-

rectly to culture at different levels. After inoculation, *B. animalis* or *B. infantis* was incubated at 37°C for 24 h in an anaerobic chamber. The viable count of *B. animalis* or *B. infantis* in culture was determined with standard plate counting technique as below. UHT skim milk without antioxidant addition was served as control.

Counting method of viable cells

Standard plate counting technique was utilized to count the viable cells (Maus and Ingham, 2003). Each sample was swirling mixed to get the content uniform. Then 0.5 ml was transferred to 4.5 ml of axenic 0.9% NaCl solution. Further decimal dilutions were made. The viable cells in 0.1 ml volumes were enumerated by pour-plating using MRS agar medium or TPY agar medium. The plates were incubated at 37°C for 48 h for *L. bulgaricus* and *L. helveticus*, or at an anaerobic chamber (85% N₂, 5% CO₂ and 10% H₂) for *B. animalis* and *B. infantis*. All pure colonies were counted and expressed as cfu·mL⁻¹.

Statistical analysis

All data are expressed as means ± SE from at least three independent experiments. Differences between the mean values of multiple groups were analyzed by one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Elimination of acidic stress of *L. bulgaricus* and *L. helveticus*

Lactic acid produced during culture had clear stress on probiotics, such as *L. bulgaricus*. When *L. bulgaricus* was incubated at 37°C for 24 h in culture, and pH of culture was intended pre-adjusted from 4.5 to 3.5 with lactic acid solution, the viable cells of *L. bulgaricus* in culture obtained decreased significantly comparing with those in control treatment (Figure 1). It suggests that lactic acid is unfavorable to the growth of *L. bulgaricus*.

The effects of three alkaline salts (sodium citrate, sodium carbonate or calcium carbonate) to eliminate acidic stress for *L. bulgaricus* in culture were significant. When skim milk culture was added with alkaline salts by different ways (before inoculation or during incubation), the viable count of *L. bulgaricus* in culture increased clearly comparing to that in control. If the original pH of culture was adjusted with sodium citrate solution to 6.8 before inoculation, the count of viable cells of *L. bulgaricus* increased about 150%. Alternatively, sodium citrate could be added to culture during incubation. After culture was incubated for 6 h, then sodium citrate solution was added to adjust culture pH to 6.0 to neutralize lactic acid produced, the count of viable cells of *L. bulgaricus* increased about 210%. Calcium or sodium carbonate also could eliminate the acidic stress of lactic acid. Calcium carbonate added at level of 10 g·L⁻¹ had similar eliminating effect as addition of sodium citrate to culture before inoculation. The eliminating effectiveness of sodium carbonate for *L. bulgaricus* was superior that of sodium citrate at same addition level and addition ways because more cells were obtained with sodium carbonate addition (cells increased 160 or 220%). The eliminating effects on acidic stress of these salts for *L. bulgaricus* are presented in Figure 2.

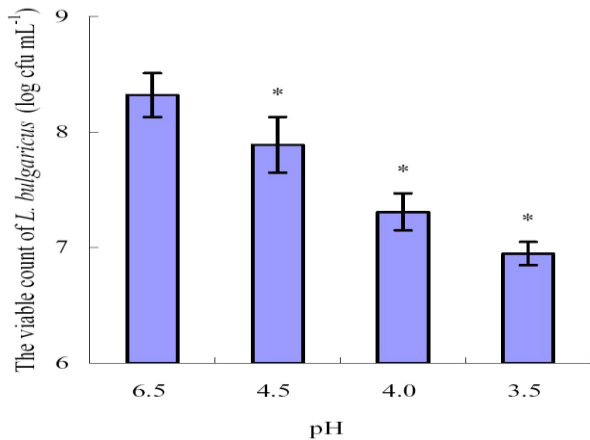


Figure 1. Effect of citric acid on *L. bulgaricus* growth in culture. The pH of culture was adjusted with lactic acid to pH 3.5, 4.0 or 4.5, compared with control treatment pH 6.5, the viable cells of *L. bulgaricus* in culture showed a decrease of 96%, 90%, and 64% respectively. The stars indicate that the values are significantly different from the control treatment of pH 6.5 ($P < 0.01$, $n = 3$).

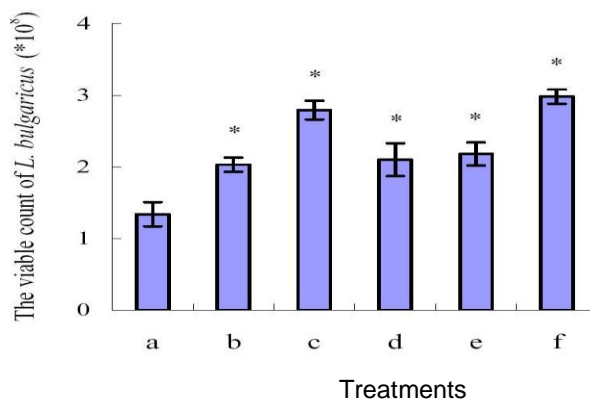


Figure 2. Eliminating effects of three alkaline salts on *L. Bulgaricus*. a: UHT skim milk without alkaline salt addition served as control; b: culture pH was adjusted to 6.8 with sodium citrate before inoculation; c: sodium citrate was added to culture after incubation of 6 h to give pH 6.0 and then incubation continued; d: calcium carbonate suspension was added to culture at 10 g·L⁻¹; e: culture pH was adjusted to 6.8 with sodium carbonate before inoculation; f: sodium carbonate was added to culture after incubation of 6 h to give pH 6.0 and then incubation continued. The stars indicate that the values are significantly different from the control treatment ($P < 0.01$, $n = 3$).

All alkaline salts also showed eliminating effect on acidic stress for *L. helveticus* in culture significantly. When culture pH was adjusted to 6.9 with sodium citrate solution before inoculation, the count of viable cells of *L. helveticus* increased about 180%. If fermentation progressed for 6 h and sodium citrate solution was added to adjust pH to 6.0, the count of viable cells of *L. helveticus* in culture in-

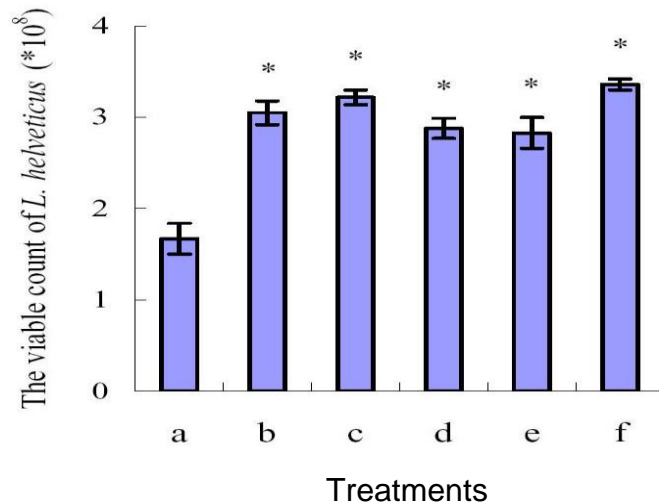


Figure 3. Eliminating effects of three alkaline salts on *L. helveticus*. a: UHT skim milk without alkaline salt addition served as control; b: culture pH was adjusted to 6.9 with sodium citrate before inoculation; c: sodium citrate was added to culture after incubation of 6 h to give pH 6.0, and then incubation continued; d: calcium carbonate suspension was added to culture at 5 g·L⁻¹; e: culture pH was adjusted to 6.9 with sodium carbonate before inoculation; f: sodium carbonate was added to culture after incubation of 6 h to give pH 6.0 and then incubation continued. The stars indicate that the values are significantly different from the control treatment ($P < 0.01$, $n = 3$).

creased about 190%. Calcium carbonate added at level of 5 g L⁻¹ had eliminating effect on acidic stress but its effect was less than that of direct addition of sodium citrate to culture before inoculation. Expectedly, more cells could be obtained when skim milk culture was added with sodium carbonate solution by different ways. The eliminating effects on acidic stress of these salts for *L. helveticus* are presented in Figure 3.

Elimination of acidic stress with alkaline agents had been used in other bacterium. Beom et al. (2004) used NaOH in culture to balance pH of *Escherichia coli* and cut down the density of acetic acid, and found that the survival of *E. coli* was increased nearly 150%. In our previous study, addition of sodium citrate or calcium carbonate to culture of *L. acidophilus* had been proven to be a good approach to increase the viable count of cells in culture two-fold (Zhao and Li, 2008). Lactic acid produced by *L. bulgaricus* or *L. helveticus* can be neutralized by alkaline agents. Sodium citrate, calcium carbonate or sodium carbonate added to culture at different ways (before inoculation or during incubation). They were used to adjust pH of culture or to neutralize lactic acid produced during incubation in our study. The production of citric acid (when sodium citrate is added) might also lead to acidic stress on *L. bulgaricus* or *L. helveticus*, but citric acid could be metabolized through the tricarboxylic acid cycle (Krebs, 1970), then transformed to nontoxic compounds in cells. To eliminate acidic stressing of lactic acid in culture for *L. bulgaricus* or *L. helveticus*, the related reactions occurred in culture are as follow.

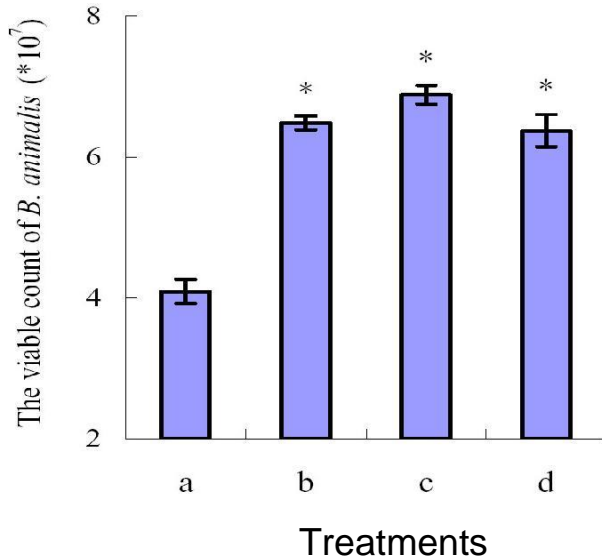


Figure 4. Eliminating effects of three antioxidants on *B. animalis*; a: UHT skim milk medium without antioxidant addition served as control; b: addition of D-isoascorbate to culture at 1.2 g·L⁻¹; c: addition of L-ascorbate to culture at 1.6 g·L⁻¹; d: addition of L-cysteine to culture at 2.4 g·L⁻¹. The stars indicate that the values are significantly different from the control treatment (P < 0.01, n = 3).

Reaction I: Lactic acid + Sodium Citrate → Sodium Lactate + Citric acid (metabolized later).

Reaction II: Lactic acid + Calcium carbonate → Calcium Lactate + H₂O + CO₂

Reaction III: Lactic acid + Sodium carbonate → Sodium lactate + H₂O + CO₂

The logarithmic phase of *L. bulgaricus* or *L. helveticus* is about at 6 ~ 8 h. We added some alkaline salts to culture when fermentation progressed about 6 h to adjust pH of culture and extend the logarithmic phase of *L. bulgaricus* or *L. helveticus*. The final results showed that this treatment was superior to addition of alkaline salts before inoculation, which is shown in Figures 2 and 3 (see the difference between column b and c, or column e and f).

Elimination of oxidative stress of *B. animalis* and *B. infantis*

Three antioxidants, sodium D-isoascorbate, sodium ascorbate or L- cysteine, as oxygen scavengers that might eliminate the oxidative stress of oxygen on *B. animalis* and *B. infantis*, were evaluated. When D-isoascorbate was added to culture before inoculation at level of 1.2 g L⁻¹, or sodium ascorbate at level of 1.2 g·L⁻¹, or L-cysteine at level of 2.4 g L⁻¹, the viable cells of *B. animalis* in culture increased about 160, 170 and 150% respectively compared with control treatment (Figure 4). If sodium iso-

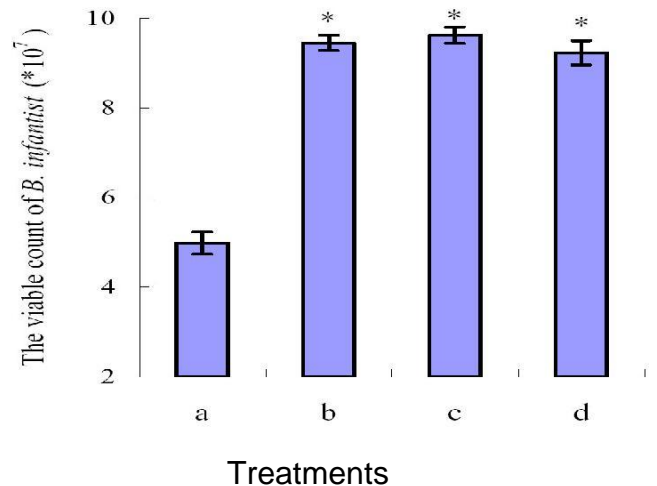


Figure 5. Eliminating effects of three antioxidants on *B. infantis*. a: UHT skim milk medium without antioxidant addition served as control; b: addition of D-isoascorbate to culture at 1.2 g·L⁻¹; c: addition of L-ascorbate to culture at 1.2 g·L⁻¹; d: addition of L-cysteine to culture at 2.4 g·L⁻¹. The stars indicate that the values are significantly different from the control treatment (P < 0.01, n = 3).

ascorbate or ascorbate or cysteine was added at same level in culture for *B. infantis*, the viable cells of *B. infantis* increased about 190% compared with control treatment (Figure 5). These results showed that three antioxidants selected had clear eliminating effect on oxidative stress for *B. animalis* and *B. infantis*.

Oxygen is toxic to anaerobic bacterium. When anaerobic bacterium is exposed to oxygen condition, superoxide and H₂O₂ are produced, which can be transformed to non-toxic compounds by superoxide dismutase and catalase. These two enzymes are absent in anaerobic bacterium unfortunately. If anaerobic bacterium lives in oxygen condition, these toxic metabolically compounds would also lead to the formation of oxidative stress, and the death of anaerobic bacterium. Marie-Pierre et al. (2006) added some reductants to culture of *Bifidobacterium* strains, and found that this treatment had a positive impact on the survival of strains tested. Using a similar approach, our previous study also confirmed that two antioxidants, sodium D-isoascorbate or sodium L-ascorbate, could be used to eliminate oxidative stress of *B. bifidum* and to develop the viable count of cells (Zhao and Li, 2008). D-isoascorbate, L-ascorbate and L-cysteine all belong to reductant and have antioxidative property, which make them have the ability to scavenge oxygen or oxidants in culture. The addition of these antioxidants to culture before inoculation eliminated the oxidative stress and provided a more favorable condition for *B. animalis* or *B. infantis* to grow; hence the viable count of cells increased. It also could be found that D-isoascorbate and L-ascorbate had stronger eliminating effect on oxidative stress for *B. animalis* or *B. infantis* than L-cysteine had, as shown in Figure 4 and Figure 5.

Conclusions

Elimination of acidic or oxidative stress for four strains of probiotics in vitro with some chemicals was confirmed by addition of these chemical to culture before inoculation or during incubation. Sodium citrate, calcium carbonate, sodium carbonate could be added to culture before inoculation or during incubation to eliminate acidic stress for *L. bulgaricus* and *L. helveticus*, while sodium D-isoascorbate, sodium ascorbate and L-cysteine added to culture before inoculation had the ability to eliminate oxidative stress for *B. animalis* and *B. infantis*. These chemicals might transform toxic compounds (lactic acid, oxygen etc) with chemical reactions and provide a favorable condition for probiotics to grow, which led to increase in viable cells about 150% ~ 220%. Treatment of culture for four probiotics with these chemicals showed that this approach might be a simple and effective ways to increase the viable count of cells, implying that it might also have potential application to produce other probiotics or microorganism in higher density.

ACKNOWLEDGMENTS

This work was supported by the Research Plans of Elite Teachers of High Education of Heilongjiang Province (No. 1055G006).

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