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# Spectrophotometricaly Evaluation of Probiotic Growth in Liquid Media

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A completely randomized design experiment was applied in triplicates to evaluate the survival of four probiotic strains in liquid media. The growth and survival rate of these probiotic strains (*Lactobacillus acidophilus, Lactobacillus casei*, *Bifidobacterium lactis* and *Bifidobacterium longum*) in varying amount of sucrose (10, 15, 20 and 25 %), oxygen scavengering components (0.05 % L-cysteine and 0.05 % L-ascorbate) and temperatures (4 and -20 °C) during different periods of time (1, 2 and 3 months) were evaluated in MRS-broth medium. Optical density at 580 nm wavelength was used to measure growth. *Lactobacilli* strains proved to be highly resistant in comparison with *Biffidobacteria* strains. Growth and survival rate of *Lactobacillus casei* showed to be the highest. Spectrophotometricaly measurement of optical density at 580 nm was showed to be the simple, rapid and inexpensive method for evaluation of probiotic growth.

**Key Words: Probiotic, *Lactobacillus*, *Bifidobacterium*, Growth, Spectrophotometry.**

## INTRODUCTION

*Lactobacillus* and *Bifidobacterium* are the most common species of bacteria used as probiotics for the production of fermented dairy products[1]. Several studies have shown that the therapeutic value of live probiotic bacteria is more than unviable cells[2]. Although the researches about therapeutic value of dead probiotic cells and their metabolites are not sufficient, most researchers believe that the survival of probiotic bacteria is very important related to their therapeutic values[1]. Some studies have been done to evaluate the tolerance of probiotics against low pH and high bile such as gastro-intestinal tract conditions. More investigations are required to study the

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survival of probiotics during processing and storage of the relating products. The conditions of dairy foods such as type, presence of air and low temperature can affect the viability of probiotics. Factors affect probiotic survival have been studied in yoghurt, but for other dairy products such as ice cream more studies are required. International Dairy Federation (IDF) suggests that a minimum of $10^7$ probiotic bacteria cells should be alive at the time of consumption per gram of product[2]. In order for these bacteria to exert positive health effects, they have to reach their site of action alive and establish themselves in certain numbers[3]. However, studies indicate that probiotics may not survive in high required numbers when incorporated into dairy products[4]. Many studies have also focused on the survival of these bacteria in dairy products under different production and storage conditions[5].

The method of increasing probiotic survival depends to the type of food products[6]. Selection of resistant probiotic strains to tolerate production, storage and gastrointestinal tract conditions, is one of the important methods. Another way is to adjust the conditions of production and storage for more survival rates. The physical protection of probiotics by microencapsulation is a new method to increasing the survival of probiotics[7]. The addition of growth promoting factors or prebiotics, such as starch and oligosaccharides[8], buffering of yoghurt mixes with whey proteins[9] and modulating packaging conditions have improved the survival of bacteria[10]. In this study, the growth and survival rate of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Bifidobacterium lactis* and *Bifidobacterium longum* as probiotics in ice cream was investigated.

## EXPERIMENTAL

Pure cultures of four probiotic strains were obtained from CHR-Hansen (Denmark) and were activated by inoculating in the MRS-broth (de Man-Rogasa-Sharpe) at 37 °C for 24 h. For simulating the ice cream conditions, the MRS-broth medium (Merck) was used. The initial pH of MRS-broth medium was 5.72 units.

**Effect of sucrose on growth rate:** The primary amount of probiotic bacteria in culturing medium were 2 (g/L). After 24 h incubation at 37 °C a small droplet of inoculated medium was added to MRS-broth that had been supplied with 10, 15, 20 and 25 % sucrose in 10 mL test tubes by using sterile insulin syringe. The samples were shaked by tube-shaker and then incubated in 37 °C for 24 h. After 24 h optical density of all samples were determined by spectrophotometer (Novaspec® II, Pharmacia Biotech) at $\lambda_{max}$ = 580 nm.

**Effect of oxygen scavenger on growth rate:** The probiotic bacteria were added to MRS-broth which had been supplied with 0.05 % L-cysteine and L-ascorbate in 10 mL test tubes. Samples were prepared in two series. The first groups were deaerated by boiling water before inoculation, while the second groups of samples were inoculated with a small droplet of inoculated medium and incubated in 37 °C for 24 h. The next day optical density of all samples was determined by spectrophotometer at $\lambda_{max}$ = 580 nm.

**Effect of low temperature on survival rate:** The probiotic bacteria were added to MRS-broth in 40 mL vials. The vials were incubated in 37 °C for 24 h. Samples were taken after 24 h and placed at 4 °C and -20 °C for 3 months. After 1, 2 and 3 months 1 mL of each vial were added to 10 mL MRS-broth under the sterile conditions and shaked using tube-shaker. The tubes were incubated at 37 °C for 24 h. The next day optical density of samples were determined by spectrophotometer at $\lambda_{max}$ = 580 nm.

**Statistical analysis:** Factorial experiment based on completely randomized design (CRD) was used for data analysis. The mean values and the standard deviation were calculated from the data obtained with triplicate trials. These data were then compared by the Duncan's multiple range method[11].

## RESULTS AND DISCUSSION

**Growth rate of probiotic bacteria in high sucrose concentration:** Because of sucrose importance at different concentrations in the formulation of ice cream, the survival of probiotic strains used in this product may be affected by osmotic pressure associated with sucrose[12,13]. Four strains used in this study showed different responses in 10, 15, 20 and 25 % sucrose concentrations, respectively. *Lactobacillus casei* (Lc01) showed the highest growth rate in all sucrose concentrations (Table-1). The number of viable *Lactobacillus casei* cells at maximum absorbance (1.800) was $1 \times 10^{10}$ (CFU/mL).

In general, *Lactobacillus* genera grow better than *Bifidobacteria* in different sucrose concentrations. It was demonstrated[14] that reducing sugars such as maltose can increase the growth rate of *L. acidophilus, L. reuteri (fermentum)* and *L. plantarum*. Although sucrose is not a reducing sugar. The present results showed that it can increase the growth rate of *L. acidophilus* and *L. casei*. Resistance to osmotic pressure of sucrose is a strain dependent factor. The number of viable *Lactobacillus casei* cells at maximum absorbance (1.800) was $1 \times 10^{10}$ (CFU/mL). *Lactobacillus* genera resist better than *Biffidobacteria* in different sucrose concentrations. The latter was in contrast with results of Hekmat and McMahon[15].

TABLE-1
PROBIOTIC TOLERANCE TO OSMOTIC PRESSURE, OXYGEN AND COLD

| Probiotic strains | | | *L. acidophilus (La5)* | *L. casei (Lc01)* | *B. lactis (Bb12)* | *B. longum (Bb46)* |
|---|---|---|---|---|---|---|
| Mean growth ± SD ($O.D._{580}$ after 24 hour incubation at 37°C) at different sucrose concentrations (%) | | 10 | $0.561 \pm 0.018^{ns}$ | $1.614 \pm 0.164^{ns}$ | $0.383 \pm 0.091^{ns}$ | $0.117 \pm 0.072^{ns}$ |
| | | 15 | $0.569 \pm 0.057^{ns}$ | $1.574 \pm 0.108^{ns}$ | $0.260 \pm 0.126^{ns}$ | $0.058 \pm 0.014^{ns}$ |
| | | 20 | $0.635 \pm 0.037^{ns}$ | $1.541 \pm 0.160^{ns}$ | $0.204 \pm 0.037^{ns}$ | $0.039 \pm 0.010^{ns}$ |
| | | 25 | $0.687 \pm 0.014^{ns}$ | $1.339 \pm 0.040^{*}$ | $0.178 \pm 0.017^{ns}$ | $0.023 \pm 0.018^{ns}$ |
| Mean growth ± SD ($O.D._{580}$ after 24 hour incubation at 37°C) at different oxygen scavengering components | 0.05 % Cysteine | Aerobic | $1.039 \pm 0.057^{ns}$ | $1.495 \pm 0.056^{*}$ | $0.829 \pm 0.084^{*}$ | $0.005 \pm 0.003^{ns}$ |
| | | Anaerobic | $1.013 \pm 0.049^{ns}$ | $1.622 \pm 0.058^{*}$ | $0.675 \pm 0.014^{*}$ | $0.010 \pm 0.007^{ns}$ |
| | 0.05 % Vit.C | Aerobic | $1.063 \pm 0.015^{ns}$ | $1.402 \pm 0.032^{*}$ | $0.396 \pm 0.036^{*}$ | $0.032 \pm 0.005^{ns}$ |
| | | Anaerobic | $1.037 \pm 0.025^{ns}$ | $1.483 \pm 0.057^{*}$ | $0.374 \pm 0.068^{*}$ | $0.016 \pm 0.002^{ns}$ |
| Mean survival ± SD ($O.D._{580}$ after 24 hour incubation at 37°C) at different temperatures | 4 °C | 1 Month | $0.054 \pm 0.001^{ns}$ | $1.379 \pm 0.056^{*}$ | $1.001 \pm 0.015^{*}$ | $0.012 \pm 0.012^{ns}$ |
| | | 2 Months | $0.053 \pm 0.001^{ns}$ | $1.06 \pm 0.011^{ns}$ | $0.817 \pm 0.033^{*}$ | $0.04 \pm 0.011^{ns}$ |
| | | 3 Months | $0.061 \pm 0.002^{ns}$ | $1.06 \pm 0.057^{ns}$ | $0.729 \pm 0.073^{*}$ | $0.04 \pm 0.011^{ns}$ |
| | -20 °C | 1 Month | $0.106 \pm 0.011^{*}$ | $1.155 \pm 0.003^{ns}$ | $0.697 \pm 0.027^{ns}$ | $0.011 \pm 0.010^{ns}$ |
| | | 2 Months | $0.088 \pm 0.003^{*}$ | $1.145 \pm 0.004^{ns}$ | $0.593 \pm 0.009^{ns}$ | $0.000 \pm 0.000^{ns}$ |
| | | 3 Months | $0.029 \pm 0.018^{*}$ | $1.151 \pm 0.001^{ns}$ | $0.585 \pm 0.005^{ns}$ | $0.000 \pm 0.000^{ns}$ |
| Resistance to osmotic pressure of sucrose | | | High | High | Medium | Low |
| Resistance to oxygen | | | High | High | Medium | Low |
| Resistance to low temperatures | | | Low | High | High | Low |

Resistance to osmotic pressure, Oxygen and cold (compared with $O.D._{580}$; high > 0.8, 0.8 > medium > 0.2, low < 0.2); ns, *represents the statistically no significant and significant ($p \leq 0.05$) differences in the same column, respectively according to Duncan's multiple range test.

**Effect of oxygen scavengering components on the growth rate of bacteria:** During freezing of ice cream mix, air is incorporated into final product. Approximate overrun of ice cream is 100 %. It is clear that 21 % (v/v) of air is oxygen. On the other hand, probiotic bacteria are microaerophill to anaerobic bacteria and molecular oxygen has toxic effect on these bacteria. Application of oxygen scavengering components such as L-ascorbic acid and L-cysteine in dairy products and use of impermeable packaging material against oxygen permeation can reduce the amount of oxygen in dairy products[10,16,17]. L-Ascorbic acid has not considerable effect on pH of dairy products, but it decreases the oxidation/reduction potential of product and makes it suitable for probiotic survival[16]. Also hydrogen peroxide produced with lactic acid bacteria can decrease the oxidation/reduction potential of product by absorbing of molecular oxygen and changing of hydrogen peroxide to water[18]. Moreover, L-cysteine can be used by probiotic bacteria as an essential amino acid[17,19]. It is recommended to use 50 mg/kg of L-cysteine in probiotic dairy products[16,20]. The detrimental effect of oxygen on lactic acid bacteria may be indirect, as a result of toxic level of hydrogen peroxide accumulation, which is produced by some flavoprotein oxidases of lactic acid bacteria[21]. Effect of oxygen scavengering components on the growth rate of probiotic bacteria is strain dependant. Table-1 reports the results in terms of growth rate of four probiotic bacteria after plating fresh cells with 0.05 % of L-cysteine and L-ascorbic acid as oxygen scavengering components. A 0.05 % concentration of L-cysteine causes a significant ($p < 0.05$) increase in growth rate of *L. casei*. The number of viable *Lactobacillus casei* cells at maximum absorbance (1.600) was $2 \times 10^8$ (CFU/mL). The present results showed that the addition of L-cysteine increases the biomass production of *L. casei* better than L-ascorbic acid.

**Survival rate of bacteria in low temperatures:** Probiotics as dairy food additives must be able to survive at low storage temperatures of the refrigeration and freezing. In order to screening of probiotic bacteria strains for addition to long shelf life foods, we studied the effect of low temperatures (4 and -20 °C) on the survival rate during three months in MRS-broth medium. Table-1 represents the effect of low temperatures (4 and -20°C) on the survival rate of four probiotic bacteria after 1, 2 and 3 months. Results showed that low temperatures have significant ($p < 0.05$) effect on survival rate of these bacteria. The present results showed that *L. casei* has the highest survival rate at -20 °C after three months treatment. The number of viable *Lactobacillus casei* cells at maximum absorbance (1.400) was $5 \times 10^7$ (CFU/mL). In general, *Biffidobacteria* displayed low resistance than *Lactobacillus* genera at low temperatures as was seen by Gomes and Malkata[22].

## Conclusion

Spectrophotometricaly measurement of optical density at 580 nm was showed to be the simple, rapid and inexpensive method for evaluation of probiotic growth. All of the stress factors investigated such as sucrose concentrations, oxygen and low temperatures have been able to affect the growth and survival of *Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium lactis* and *Bifidobacterium longum*. This study has proved that it is possible to select the appropriate probiotic strains for use in ice cream with a simple, rapid and inexpensive method. In conclusion, a comparison with other probiotic strains (Table-2) revealed that *Lactobacillus casei* (Lc01) and *Bifidobacterium lactis* (Bb12) had the highest resistance to simulated ice cream conditions, making them suitable probiotic strains for use in ice cream. In summary, *Lactobacillus* genera showed higher growth and survival rate than *Bifidobacteria* in the presence of sucrose, oxygen and low temperature treatments.

TABLE-2
MICROBIAL RESPONSE FOR THE ENVIRONMENTAL CONDITIONS IN FOODS

| Microorganism | Resistance to sucrose | Resistance to oxygen | Resistance to low temp. | Ref. |
|---|---|---|---|---|
| *B. lactis (Bb12)*[a] | Resistant | Resistant | Resistant | PS |
| *B. infantis (1912)* | - | - | Resistant | 3 |
| *B. lactis (BLC-1)*[b] | - | Resistant | Resistant | 23 |
| *B. lactis (DD920)* | - | - | Resistant | 24 |
| *B. longum (Bb46)*[a] | Sensitive | Sensitive | Sensitive | PS |
| *L. acidophilus (2401)* | - | - | Resistant | 3 |
| *L. acidophilus (DD910)* | - | - | Resistant | 24 |
| *L. acidophilus (La5)*[a] | Resistant | Resistant | Sensitive | PS |
| *L. acidophilus (Lafti L10)* | - | - | Resistant | 23 |
| *L. casei (Lc01)*[a] | Resistant | Resistant | Resistant | PS |
| *L. johnsonii (La1)* | Resistant | - | Resistant | 25 |
| *L. paracasei subsp. Paracasei (LCS1)* | - | - | Resistant | 23 |

[a]Resistance to oxygen was evaluated in simulated ice cream conditions.
[b]Resistance to oxygen was evaluated in low fat ice cream conditions.
PS = Present study.

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## REFERENCES


1. L.V. McFarland and G.W. Elmer, in eds.: I. Goktepe, V.K. Juneja and M. Ahmedna, Properties of Evidence-Based Probiotics for Human Health, In: Probiotics in Food Safety and Human Health, Taylor & Francis, New York, pp. 109-138 (2006).
2. A.C. Ouwehand and S.J. Salminen, *Int. Dairy J.*, **8**, 749 (1998).
3. K. Sultana, G. Godward, N. Reynolds, R. Arumugaswamy, P. Peiris and K. Kailasaphathy, *Int. J. Food Microbiol.*, **62**, 47 (2000).
4. K. Kailasapathy and S. Rybka, *Aust. J. Dairy Tech.*, **52**, 28 (1997).
5. C. Beal, F. Fonseca and G. Corrieu, *J. Dairy Sci.*, **84**, 2347 (2001).
6. A. Homayouni, M.R. Ehsani, A. Azizi, M.S. Yarmand and S.H. Razavi, *Iranian Polym. J.*, **16**, (2007) (In Press).
7. P.A. Wojtas, L.T. Hansen and A.T. Paulson, *LWT-Food Sci. Tech.*, (2007) (in press).
8. R.G. Crittenden, L.F. Morris, M.L. Harvey, L.T. Tran, H.L. Mitchell and M.J. Playne, *J. Appl. Microbiol.*, **90**, 268 (2001).
9. R.R. Ravula and N.P. Shah, *Aust. J. Dairy Tech.*, **53**, 174 (1998).
10. C.W. Miller, M.H. Nguyen, M. Rooney and K. Kailasapathy, The Control of Dissolved Oxygen Content in Probiotic Yoghurt Using Active Packaging Technology, AIFST Annual Convention, Melbourne, August 24-27 (2003).
11. SAS, SAS User's Guide: Statistics, Version 6.12 edn. SAS Institute, Gary, NC (1996).
12. C.J. Ziemer and G.R. Gibson, *Int. Dairy J.*, **8**, 473 (1998).
13. M. Medici, C.G. Vinderola and G. Perdigon, *Int. Dairy J.*, **14**, 611 (2004).
14. D. Charalampopoulos, S.S. Pandiella and C. Webb, *Int. J. Food Microbiol.*, **82**, 133 (2003).
15. S. Hekmat and D.J. McMahon, *J. Dairy Sci.*, **75**, 1415 (1992).
16. R.I. Dave and N.P. Shah, *Int. Dairy J.*, **7**, 537 (1997).
17. N.P. Shah, *J. Dairy Sci.*, **83**, 894 (2000).
18. A.H. Varnam and J.P. Sutherland, Milk and Milk Products: Technology, Chemistry and Microbiology, Chapman and Hall, London (1994).
19. R.I. Dave and N.P. Shah, *J. Dairy Sci.*, **81**, 2804 (1998).
20. N. Ishibashi and S. Shimamura, *Food Tech.*, **47**, 29 (1993).
21. S. Shimamura, F. Abe, N. Ishibashi, H. Miyakawa, T. Yaeshima, T. Araya and M. Tomita, *J. Dairy Sci.*, **75**, 3296 (1992).
22. A.M.P. Gomes and F.X. Malcata, *Trend Food Sci. Tech.*, **10**, 139 (1999).
23. I.N. Haynes and M.J. Playne, *Aust. J. Dairy Tech.*, **57**, 10 (2002).
24. K. Kailasapathy, *LWT-Food Sci. Tech.*, **39**, 1221 (2006).
25. C. Alamprese, R. Foschino, M. Rossi, C. Pompei and L. Savani, *Int. Dairy J.*, **12**, 201 (2002).


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