Use of gases to improve survival of *Bifidobacterium bifidum* by modifying redox potential in fermented milk

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ABSTRACT

The aim of this work was to study the effect of the oxidoreduction potential, modified using gas, on the growth and survival of a probiotic strain, *Bifidobacterium bifidum*, and 2 yogurt strains, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. Three fermented milks were manufactured with an initial oxidoreduction potential value adjusted to +440 mV (control milk), +350 mV (milk gassed with N₂), and –300 mV [milk gassed with N₂ plus 4% (vol/vol) H₂ (N₂-H₂)]. Acidification profiles, growth during milk fermentation and survival during storage at 4°C for 28 d were determined. This study showed that fermented probiotic dairy products made from milk gassed with N₂ and, more particularly, those made from milk gassed with N₂H₂ were characterized by a significant increase in *B. bifidum* survival during storage without affecting the fermentation kinetics and the survival of *Strep. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*.

Key words: bifidobacteria, redox potential, survival, fermented milk

INTRODUCTION

Bifidobacteria are probiotic bacteria isolated from the human gastrointestinal tract. They are the most prevalent microbes in the large intestine but are also implanted throughout the distal jejunum and ileum in the small intestine (Kerckhoffs et al., 2006). The large intestine is mainly anaerobic with a low oxidoreduction (redox) potential (E_h), in the range of –200 to –300 mV in the colon lumen (Wilson, 2008). The E_h plays an essential role in microbial cell physiology (Pearson and Walker, 1976; Uden et al., 1990). Obligate aerobes are metabolically active in an environment with a positive E_h , whereas obligate anaerobes require a negative E_h . In the large intestine, many aerobes and

facultative anaerobes (enterobacteria, streptococci) are responsible for creating a reduced environment suitable for the establishment of obligate anaerobe bacteroides and bifidobacteria (Mackie et al., 1999).

Probiotic species that normally reside in the human gut (bifidobacteria, lactobacilli) have been associated with beneficial health properties, such as improving general gut health and positively influencing the development and activity of the immune system (Fuller, 1991; Kaur et al., 2002). To have any effect, probiotic strains must remain viable until they reach the intestinal tract. Bifidobacteria, classified as obligate anaerobes and implanted mostly throughout the colon, often present poor survival in fermented milk products and several authors have reported low viable counts of bifidobacteria in fermented milk (Jayamanne and Adams, 2009). The survival of probiotic organisms depends on various factors occurring during processing and formulation: pH, oxidoreducing environment, presence of other microbes, temperature and time of storage, and water activity (Mattila-Sandholm et al., 2002). Oxygen is a powerful oxidant; it has a dramatic effect on E_h value and on the viability of probiotic organisms during manufacturing and storage (Storz and Imlay, 1999; Talwalkar and Kailasapathy, 2004a,b).

Many techniques have been suggested to protect probiotic bacteria from oxygen toxicity in fermented milk. These include the following: use of special high-oxygen consuming strains (Lourens-Hattingh and Viljoen, 2001), use of ascorbate and L-cysteine as oxygen scavengers (Dave and Shah, 1997a,b), microencapsulation (Kailasapathy, 2002), generation of adaptation to oxidative stress (Rallu et al., 2000), or use of adequate packaging material (Champagne et al., 2005). Pretreatment of milk could also be employed to lower oxygen content or to modify the E_h , using electroreduction, experimented with in the manufacture of milk (Bolduc et al., 2006a), and milk enriched with probiotic organisms (Bolduc et al., 2006b), or specific heat treatment in the manufacture of yogurt enriched with probiotic organisms, allowing the depletion of molecular oxygen and the liberation of sulfur-containing AA (Mortazavian et

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al., 2006a). The use of gas might also be an alternative for degassing milk. Milk is an oxidizing medium when in contact with air; nitrogen, which is neutral, can be used to remove oxygen from milk. Even so, the milk E_h remains oxidizing, whereas hydrogen provides a reducing E_h (below 0 mV; Martin et al., 2010). The production of lyophilized probiotic bacteria (*Lactobacillus casei*) was previously tested with an increase in survival during storage (Cachon et al., 2008). Indeed, after 60 d of conservation at ambient temperature, twice as much biomass was obtained in reduced conditions than with the control. Besides, it has recently been shown that the use of gas is a possible way to improve the organoleptic quality of dairy products (Martin et al., 2010). But the use of gas to produce acidified probiotic-enriched dairy foods has never been investigated.

The objective of the present study was to determine if producing fermented probiotic dairy products using milk without oxygen (nitrogen degassing) or reduced milk (nitrogen-hydrogen degassing) affects the survival of a probiotic strain, *Bifidobacterium bifidum*, over 28 d of storage.

MATERIALS AND METHODS

Microorganisms and Culture Conditions

The strains *Lactobacillus delbrueckii* ssp. *bulgaricus* LB 340 and *Streptococcus thermophilus* TA 60 were obtained from the EA 4181 GPMA research unit (Université de Bourgogne, Dijon, France). The strain *B. bifidum* CIP56.7 was obtained from the Pasteur Institute collection (CIP), France. Stock cultures were prepared with subsequent storage at -80°C . Cells from thawed stock cultures were inoculated in 10 mL of de Man, Rogosa, and Sharpe (MRS) medium (Biokar Diagnostic, Beauvais, France) for *L. delbrueckii* ssp. *bulgaricus*, 10 mL of mMRS medium [MRS supplemented with 0.05% cysteine (wt/vol)] for *B. bifidum*, and 10 mL of M17 lactose (0.5%) medium (Difco Laboratories, Elancourt, France) for *Strep. thermophilus*. The cultures were incubated overnight and used to inoculate 100 mL of their respective media. The flasks were incubated in static conditions and the optical density at 600 nm was monitored with a Perkin Elmer LS 50B fluorescence spectrophotometer (Perkin Elmer, Shelton, CT).

Preparation of Fat-Free Fermented Milk Under Different E_h Conditions

For this study, organic skimmed ultra-high temperature (UHT) milk (Lactel, Laval, France) was used and 2% (wt/vol) of low-heat skim milk powder (Eurosérum, St. Martin Belle Roche, France) was added to fortify

the skim milk (corresponding to 33–35 g/L of protein; Martin et al., 2010). The fortified milk was stirred for 4 h after addition of the skim milk powder and heated to 45°C . It was then processed under different gaseous conditions. Different E_h conditions were applied in an anaerobic chamber (Bactron I, Sheldon Manufacturing, Inc., Cornelius, OR) by gas bubbling for 4 h with a gas flow of 20 mL/min [N_2 or N_2 plus 4% (vol/vol) H_2 ($\text{N}_2\text{-H}_2$)]. Experiments were also conducted without any gas bubbling (control milk) to obtain standard fermented milk. After the bubbling procedure, when applied, the mixture was fermented using 5×10^6 cfu/mL of each starter culture and 5×10^8 cfu/mL of the probiotic strain. The pH was recorded and when it reached 4.6 the fermented milks were cooled to 4°C for 1 h and stored at this temperature in Hungate tubes (Dutscher, Brumath, France) to maintain the intended E_h attained after bubbling various gases.

E_h and pH Measurements

The pH was monitored using a combined pH electrode (Inlab 405, Mettler-Toledo, Paris, France). The pH electrode was cleaned with a pepsin/HCl solution and was calibrated using 2 calibration buffers (pH 4 and 7; PolyLabo, Paris, France). The E_h was monitored using a combined platinum redox electrode (Pt4805, Mettler-Toledo) referred to as the Ag/AgCl system and cleaned by polishing with a fine alumina powder (Aluminum oxide; VWR Prolabo, Lyon, France) before use to restore the platinum surface (Jacob, 1970). Electrodes were controlled in tap water. Three measurements in tap water were compared and were within the confidence interval of their mean value (calculated at 20 mV, 95% confidence level) to ensure correct measurement (Abraham et al., 2007). For N_2 and $\text{N}_2\text{-H}_2$ conditions, pH and E_h measurements were carried out in the Bactron I anaerobic chamber (Sheldon Manufacturing, Inc.).

Data Acquisition of pH and E_h Profiles

The pH and E_h electrodes and the temperature sensor were connected to a controlled interface (ELIT multichannel pH meter/redox meter computer interface; Fisher Bioblock Scientific, Illkirch, France) that enabled real-time data acquisition on a computer. The pH and the measured redox potential (E_m ; mV) were recorded simultaneously. Based on the standard electrode value (E_r) at the temperature of fermentation ($E_r = 191$ mV), the E_m , expressed in comparison with the reference electrode Ag/AgCl, were converted into E_h values (redox potential expressed in comparison with the hydrogen electrode, $E_h = E_m + E_r$). The decrease

in the pH of the medium indirectly modifies the E_h (Nernstian response of the redox electrode). The E_h was then expressed as E_h at a given pH value, pH 7 (E_{h7}) according to the following equation $E_{h7} = E_h - \alpha \times (7 - \text{pHx})$, where α is the E_h – pH correlation factor and pHx is the pH of the medium. This factor α is determined experimentally by measuring the variation of E_h with pH using lactic acid. For the milk used in this study, a variation in E_h of 38 mV/pH unit at 45°C was measured. The E_{h7} values were +441 mV (± 4 mV) in the non-gassed milk, +391 mV (± 19 mV) in the milk gassed with N_2 , and –310 mV (± 17 mV) in the milk gassed with the N_2 - H_2 mixture.

Acidification Rate

The pH values were processed mathematically to calculate the acidification rate (dpH/dt, pH unit/h), allowing the maximum acidification rate (V_m^a ; pH unit/h), the time at which this rate occurred (T_m^a ; h), and the pH at which this maximum occurred (pH_m^a ; pH unit/h; Cachon et al., 2002) to be determined.

Bacterial Enumeration

Bacterial counts of *B. bifidum* and *L. delbrueckii* ssp. *bulgaricus* were obtained by spread-plating (0.5 mL) the appropriate peptone-water dilution using an Eddy Jet Spiral Plater (IUL SA, Barcelona, Spain) on mMRS-cysteine agar adapted according to Vinderola and Reinheimer (1999; MRS supplemented with 0.5 g of cysteine-HCl/L, 2.4 g of lithium chloride/L, 0.015 g of nalidixic acid/L, 0.01 g of neomycin sulfate/L, and 0.01 g of paromomycin sulfate/L; Sigma Chemical Co., Castle Hill, Australia) or MRS, respectively. Plates were then incubated in anaerobic conditions (Anaerocult A; Merck, Darmstadt, Germany) at 37°C for 48 h (*B. bifidum*) or 42°C for 24 h (*L. delbrueckii* ssp. *bulgaricus*). Bacterial counts of *Strep. thermophilus* were obtained by spread-plating (0.5 mL) the appropriate peptone-water dilution on *Strep. thermophilus* agar (ST agar) using an Eddy Jet Spiral Plater (IUL SA) and incubating the plates at 37°C for 24 h in aerobic conditions. All cells were plated on agar before and after milk fermentation, and during storage (d 1, 14, and 28). Colonies were counted with a CounterMat Flash Colony Counter (IUL SA, Barcelona, Spain).

Statistical Analysis

The experimental data were presented as mean values and variations with respect to mean values were pre-

sented as standard deviations. An ANOVA was carried out on the mean values using SigmaPlot Software v11.0 (SPSS Inc., Chicago, IL). The values were compared using the Tukey test at $P < 0.05$, and different letters were used to indicate statistically significant differences between values.

RESULTS AND DISCUSSION

Evolution of pH and E_{h7} During Fermentation

Evolution of E_{h7} . The evolution of E_{h7} measured in fermented milk as a function of time is presented in Figure 1A. The E_{h7} of control milk and milk gassed with N_2 decreased during fermentation (respectively around 100 and 20 mV), contrary to E_{h7} of milk gassed with N_2 - H_2 , which remained relatively stable (around –280 mV after about 3.1 h of fermentation). Furthermore, no significant differences were observed between milk fermented with or without *B. bifidum*. The E_{h7} decrease is most probably related to starter growth and fermentation, causing the reduction of the oxygen in milk to water (Jacob, 1971; Cachon et al., 2002; Martin et al., 2010). A recent study showed that for *Lactococcus lactis*, a lactic acid bacterium that reduces milk E_{h7} to –220 mV, thiol groups located on the bacterial cell surface appear to be the main components that are able to reduce milk after oxygen removal (Michelon et al.). Nevertheless, *L. delbrueckii* ssp. *bulgaricus* and *Strep. thermophilus* are weak reducing strains (Cachon et al., 2002; Brasca et al., 2007), and E_{h7} at the end of fermentation remained oxidant, except with N_2 - H_2 . *Bifidobacterium bifidum* is able to reduce milk to –100 mV (data not shown), but is a slow reducing strain and, in our study, the fermentation time was too short to reach such an E_{h7} . During storage, changes in E_{h7} were observed; on d 28 it reached +215 mV (± 13 mV), +178 mV (± 8 mV), and –187 mV (± 15 mV), respectively, for the control milk, the milk gassed with N_2 , and the milk gassed with N_2 - H_2 . At the end of storage, negative E_h values were still observed in the milk gassed with N_2 - H_2 .

Evolution of pH. Acidification profiles of fermented milk made under different gaseous conditions are presented in Figure 1B. After addition of the starter culture and the probiotic strain to milk, the pH changed slowly for 1 h, which was in agreement with previous results (Amice-Quemeneur et al., 1995). Thereafter, the pH decreased steadily until it reached pH 4.6 when the fermented milks were cooled to stop enzymatic and metabolic bacterial activity and to avoid post-acidification phenomena. The evolution of the pH during acidification was similar in the 3 conditions employed and the time to reach the final pH (T_{gel}) was not statisti-

Figure 1. Evolution of the oxidoreduction (redox) potential at pH 7 (E_{h7}; A) and pH (B) during fermentation of milk. Different gaseous conditions were applied to milk: control with probiotic strain (solid line), control without probiotic strain (dashed/dotted line), gassed with N₂ (dashed line), or gassed with N₂-H₂ (dotted line).

cally different (about 3.2 h). Moreover, the V_m^a , T_m^a , and the pH_m^a were analyzed (Table 1). In our experiments, no statistical differences between the acidification of the control milk, the milk gassed with N₂, or the milk gassed with N₂-H₂ were observed. Using gas allows the E_h to be manipulated without affecting the pH of the environment, contrary to cysteine or ascorbate, which have a negative effect on the textural and cul-

Figure 2. Evolution of a population of *Streptococcus thermophilus* (A), *Lactobacillus delbrueckii* ssp. *bulgaricus* (B), and *Bifidobacterium bifidum* (C) during fermentation and storage (at days 1, 14, and 28). Different gaseous conditions were applied to milk: control (solid line), gassed with N₂ (dashed line), or gassed with N₂-H₂ (dotted line).

Table 1. Mean values of the kinetic parameters calculated from acidification rates during fermentation of milk¹

Gas	T _{gel} (h)	V _m ^a (pH unit/h)	T _m ^a (h)
Control (+ <i>Bifidobacterium bifidum</i>)	3.14 (0.05) ^a	-1.35 (0.10) ^b	1.27 (0.07) ^c
Control (- <i>B. bifidum</i>)	3.20 (0.03) ^a	-1.25 (0.06) ^b	1.43 (0.11) ^c
N ₂	3.33 (0.28) ^a	-1.16 (0.08) ^b	1.17 (0.03) ^c
N ₂ -H ₂	3.22 (0.12) ^a	-1.17 (0.00) ^b	1.32 (0.13) ^c

^{a-c}Same superscript letters within a column indicate that groups were not significantly different at 5% α risk (ANOVA).

¹Different gaseous conditions were applied to milk: control (with or without probiotic strain), gassed with N₂, or gassed with N₂-H₂. T_{gel} = acidification time; V_m^a = maximum acidification rate; T_m^a = time to reach V_m^a.

tural properties of yogurt by suppressing growth of yogurt strains (Talwalkar and Kailasapathy, 2004a,b). Besides, as previously shown (Martin et al., 2010), no significant variation in pH during storage between d 1 and 28 occurred.

Bacterial Growth During Fermentation and Evolution of Survival During Storage

Evolution of Bacterial Counts During Milk Fermentation. As shown in Figure 2, only yogurt starters grew during fermentation. As expected, after analyzing the acidification profiles of fermented milk with or without a probiotic strain (Figure 1B and Table 1), the acidification activity of *B. bifidum* was lower than those of the yogurt bacteria. Moreover, no growth of *B. bifidum* occurred during the fermentation phase (Figure 2C). The use of gas did not enhance the growth of the 3 strains, which is again in correlation with the fact that gas bubbling has no effect on acidification profiles.

Evolution of Survival During Storage of the Fermented Dairy Product. It has been previously reported that incorporation of ascorbic acid (Dave and Shah, 1997b) or cysteine (Dave and Shah, 1997a), electroreduction (Bolduc et al., 2006b), or specific heat treatment (Mortazavian et al., 2006b) in fermented milk causes a decrease in oxygen content and an E_h which results in better survival of probiotic bacteria.

In aerobic conditions, a decrease in the bacterial count was observed for the 3 strains during storage. This decrease was moderate for *Strep. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, 0.66 log (Figure 2A) and 0.69 log (Figure 2B), respectively, after 28 d of storage at 4°C, and drastic for *B. bifidum*, with a decrease of 2.12 log after 28 d (Figure 2C). The difference in behavior between probiotic strains and starters could be explained by the fact that starters do not have the same sensitivity to oxygen and pH as do probiotic strains. Indeed, *B. bifidum* showed a moderate ability to deal with environmental stress, especially high

oxygen (Bolduc et al., 2006b) and acidic environments (Sun and Griffiths, 2000). Oxygen toxicity results from the effect of activated oxygen compounds, including superoxide, hydrogen peroxide, and hydroxyl radicals (Talwalkar and Kailasapathy, 2004a). Superoxide dismutase and catalase, the key enzymes that scavenge toxic oxygen metabolites, are absent in bifidobacteria, as is NADH peroxidase (which converts H₂O₂ into H₂O) in *B. bifidum* (Shimamura et al., 1992).

Gas bubbling before fermentation had a significant positive effect on the survival of *B. bifidum* during storage. Indeed, after 28 d of storage at 4°C, prior bubbling with N₂ or N₂-H₂ allowed greater survival of the probiotic strain with differences in bacterial counts of 1.18 and 1.46 log ($P < 0.05$), respectively, in comparison with the control (Figure 2C). This result is concordant with the literature describing the positive effect of the absence of oxygen on the survival of bifidobacteria (Dave and Shah, 1997a; Bolduc et al., 2006b). Because the increase in survival of *B. bifidum* during storage is not due to improved growth, we can suppose that the cells adapted during fermentation (Ouvry et al., 2002). Moreover, in all conditions (control, N₂, and N₂-H₂), the increase in E_{h7} during storage was accompanied by a decrease in survival of *B. bifidum*. But contrary to electroreduced milk, which showed positive E_h values on d 7 (Bolduc et al., 2006b), in our study, N₂-H₂ bubbling and storage in Hungate tubes allowed negative values to be maintained and better survival of *B. bifidum*. In contrast, gas bubbling had no influence on survival during storage of *Strep. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. Once again, this could be explained by the difference in sensitivity to oxygen and the combined effect of putative oxidative and pH stress on the 3 strains.

Further to the elimination of oxygen (due to N₂) and the addition of a reduction compound (N₂-H₂), the increase in survival in anaerobic and reduced conditions could be due to cellular compounds, such as thiol groups (-SH), being maintained in reduced form. Thiol groups are important compounds both for reducing activity

(Michelon et al., 2010) and for antioxidant properties and resistance to oxidative stress (Balcerczyk and Bartosz, 2003; Patsoukis and Georgiou, 2004). The effect of gases on lipid composition and membrane fluidity (Ouvry et al., 2002) could also contribute to enhanced survival.

CONCLUSIONS

This study shows that low E_h , modified using N_2 - H_2 gas, enhanced *B. bifidum* survival during storage without having a negative effect on acidification parameters because it did not affect growth or survival of yogurt strains. Furthermore, use of a gaseous environment similar to that in the large intestine (absence of oxygen and E_h below 0 mV due to the presence of H_2) further enhanced survival during storage. Preservation of reducing cell surface proteins or modification of lipids could be responsible for this phenomenon.

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