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Spontaneous *Lactobacillus delbrueckii* phage-resistant mutants with acquired bile tolerance

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Abstract

Three commercial phage-sensitive strains of *Lactobacillus delbrueckii* (strains Ab_1, YSD V and Ib_3) and four spontaneous phage-resistant mutants (strains A_7, A_{17}, V_2 and I_{39}) isolated from them, all with a probiotic potential previously demonstrated were studied for their tolerance of bile salts (ox gall). Minimal Inhibitory Concentrations (MICs) ranged from 0.30% to 0.35% (w/v) of ox gall. These strains were exposed to gradually increasing concentrations of ox gall with the aim of isolating bile resistant derivatives. Stable derivatives able to tolerate up to 0.9% of ox gall were obtained from *L. delbrueckii* Ab_1, as well as from its spontaneous phage-resistant mutants A_7 and A_{17}. Random Amplified Polymorphic DNA (RAPD-PCR) analysis revealed a strong genetic homology between the ox gall-tolerant derivatives and their respective non-adapted original strains. These derivatives maintained, in general, the phage resistance phenotype of the non-adapted strains, with only one exception (phage-resistant mutant A_7). After progressive ox gall adaptation, the phage-resistant mutant A_7 also exhibited progressive reversion of the phage resistance phenotype. The derivative with the highest ox gall-acquired tolerance ($A_7^{0.9}$) became sensitive to the phage, but derivatives with low ($A_7^{0.3}$) and intermediate ($A_7^{0.6}$) ox gall-acquired tolerance retained phage resistance. The technological properties of ox gall derivatives were comparable to those of their respective parent strains. However, the cells of the former were smaller than those of the original strains. Finally, the tolerant derivatives grew faster in the presence of ox gall than the parent strains. Our results demonstrated that it was possible to obtain, by a natural selection strategy, probiotic strains with acquired ox gall-tolerance from three (*L. delbrueckii* Ab_1 and their phage-resistant mutants A_7 and A_{17}) of seven tested strains. Since such derivatives keep both phage resistance and other useful technological properties, they could be used for production of functional foods.
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Keywords: *Lactobacillus delbrueckii*; Bile resistance; Phage resistance; Probiotics

1. Introduction

Probiotics are defined as live microorganisms that when administered in adequate amounts confer a health benefit on the host. It is well recognized that yogurt consumption can improve lactose digestion and eliminate symptoms of lactose intolerance, as many *Lactobacillus delbrueckii* and *Streptococcus thermophilus* strains have a high lactase activity (Sanders et al., 1996). Thus, these cultures clearly fulfill the current concept of probiotics. Recently, de Moreno de LeBlanc and Perdigón (2005) reported that yogurt prepared from cultures of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* (strain pool from culture collection of the Centro de Referencia para Lactobacilos-CERELA, Argentina) inhibited tumor progression and promotion. Additionally, *L. delbrueckii* given orally may be able to enhance host immune response (Perdigón et al., 1999).

Technologically, *L. delbrueckii* contributes to fast lactic acid development, and also to flavor and texture modifications in fermented milks (Curry and Crow, 2003). This starter activity of *L. delbrueckii* can be seriously affected when phage infection occurs (Zago et al., 2006). In previous studies (Guglielmotti et al., 2006) we demonstrated the isolation of *L. delbrueckii* spontaneous phage-resistant mutants from a sensitive strain. This approach is a convenient, simple and 'natural' strategy for replacing phage-sensitive strains, since there are no regulatory restrictions on it

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(Moineau and Lévesque, 2005). Phage-resistant variants retained the probiotic properties of parent strains. However, the potential probiotic role of *L. delbrueckii* is still questionable because of its sensitivity to the high bile concentrations and acid conditions that occur in the gut (Marteau et al., 1997; Vinderola and Reinheimer, 2003, Vinderola et al., 2007). A number of approaches to improving the survival of probiotic organisms after ingestion have been explored including encapsulation (Hou et al., 2003), immobilization on alginate beads (Lamboley et al., 2003) and progressively adaptation to increasing concentrations of bile salts (Margolles et al., 2003).

There are, however, no data to show how bile adaptation would affect other desirable properties of *L. delbrueckii* phage-resistant mutants. Thus, the aim of the present work was to induce ox gall-tolerance in *L. delbrueckii* spontaneous phage-resistant mutants, and to investigate the possible changes in the phage resistance phenotype and other technologically desirable properties, such as acidifying and proteolytic activities, during the adaptation process.

2. Materials and methods

2.1. Bacterial strains, bacteriophages and culture conditions

Three commercial strains (identified as Ab_1, YSD V and Ib_3) of *L. delbrueckii* were used. They were isolated from commercial starters used in Argentinean milk fermentation processes and were sensitive to phages YAB, BYM and Ib_3, respectively. These phages were isolated from yogurt samples that showed slow acidification (Quiberoni et al., 2004). Additionally, four spontaneous phage-resistant mutants (strains A_7, A_{17}, V_2 and I_{39}) previously isolated (Guglielmotti et al., 2006) from the sensitive strains by using the respective phages, were included in this study. *L. delbrueckii* strains were routinely grown in de Man, Rogosa and Sharpe (MRS) broth (Biokar, Beauvais, France) or agar (Biokar) at 42 °C, and stored at −80 °C in MRS broth supplemented with glycerol (15%, v/v). Phage enumerations were carried out by the double-layer plaque titration method (Svensson and Christiansson, 1991).

2.2. Sensitivity to ox gall of L. delbrueckii strains and phage-resistant mutants, and isolation of tolerant derivatives

Minimal Inhibitory Concentrations (MICs) of ox gall for both phage-sensitive and phage-resistant strains were determined in MRS broth supplemented with ox gall (Sigma, St. Louis, MO, USA) at concentrations of 0.10%, 0.15%, 0.20%, 0.25%, 0.30%, 0.35%, 0.40%, 0.45% or 0.50% (w/v), inoculated (2% v/v) with each *L. delbrueckii* culture and incubated at 37 °C for 48 h. Growth rates for each culture at every ox gall concentration, were determined from increases in the optical densities at 560 nm (OD_{560nm}) (Margolles et al., 2003). If OD_{560nm} values higher than 0.8 were recorded, cultures were diluted to obtain an OD_{560nm} reading for each between 0.2 and 0.8, and the resulting OD_{560nm} was corrected for the dilution factor. The MIC was defined as the lowest ox gall concentration that gives an OD_{560nm} value (as bacterial growth measurement) lower than 0.2 units after 48 h of incubation at 37 °C (Loessner et al., 1997).

Both phage-sensitive and phage-resistant *L. delbrueckii* strains were tested to obtain derivatives tolerant to higher ox gall concentrations, according to Margolles et al. (2003). With this aim, each *L. delbrueckii* strain was inoculated (2% v/v) in MRS broth supplemented with 0.10% (w/v) ox gall and incubated at 37 °C for 48 h. Growth rates were assessed by spectrophotometric determinations (OD_{560nm}), and cultures with OD_{560nm} values higher than 0.8 were treated as detailed above. After incubation, grown cultures were used for subculture (2% v/v) into MRS broth supplemented with 0.15% (w/v) ox gall and once more incubated at 37 °C. This procedure was repeated for each culture using gradually increasing concentrations of ox gall, i.e. 0.20%, 0.25%, 0.30%, 0.35%, 0.40%, 0.45%, 0.50%, 0.55%, 0.60%, 0.65%, 0.70%, 0.75%, 0.80%, 0.85%, 0.90%, 0.95%, and 1.0% (w/v).

2.3. Stability of the induced tolerance to ox gall

The stability of the induced tolerance to ox gall was studied according to Margolles et al. (2003) with slight modifications. After six successive subcultures in MRS broth at 37 °C for 48 h, one additional subculture in MRS broth with or without ox gall at different concentrations (0.3%, 0.6% or 0.9%, w/v) was performed and incubated (37 °C, 48 h). Growth rates were determined from increases in the OD_{560nm} values. Cultures with OD_{560nm} values higher than 0.8 were treated as detailed above (Section 2.2). The final numbers of bacteria in each culture were determined by spreading suitable dilutions on plates of MRS agar and incubated at 42 °C for 48 h.

2.4. Genetic analysis

Total DNA of sensitive strains, their respective phage-resistant mutants and the ox gall-resistant derivatives were obtained by phenol–chloroform extractions as was previously described (Quiberoni et al., 1998) and quantified by electrophoresis on 0.8% (w/v) agarose gels.

Random amplification of polymorphic DNA (RAPD-PCR) was applied to bile-salt sensitive strains, their respective phage-resistant mutants and the ox gall resistant derivatives, to determine their genetic similarity. Oligonucleotide primers M13 (Huey and Hall, 1989; Stendid et al., 1994) and 1254 (Akopyanz et al., 1992), 5′-GAGGGTGGCGGTTCT-3′ and 5′-CCGCAGCCAA-3′, respectively, were used in separate amplification assays. PCR was performed in a volume of 25 μl containing 2.5 μl of 10× PCR buffer (Perkin Elmer), 200 μM of each dNTP, 0.6 IU of *Taq* DNA polymerase (Perkin Elmer), 3.0 mM $MgCl_2$, 2.0 μM primer M13 or 0.8 μM primer 1254, and 1.5 μl of template DNA. The PCR program used for primer M13 was carried out according to Giraffa et al. (2000). A first denaturing step of 94 °C for 2 min was followed by 40 cycles of 94 °C for 1 min (denaturation), 45 °C for 20 s (annealing) and 72 °C for 2 min (extension), and final extension at 72 °C for 10 min. For primer 1254 the following program was used: four cycles of 94 °C for 5 min, 36 °C for 5 min and 72 °C for 5 min; followed by 30 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min; and final extension at 72 °C

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for 10 min. The amplification products were visualized by electrophoresis in 1.5% agarose gels, and staining with ethidium bromide. The DNA molecular weight marker 1 Kb DNA Marker (Amersham Biosciences, Buckinghamshire, United Kingdom) was used as a standard. The amplification pattern images of the gels were analyzed as previously described (Quiberoni et al., 1998). Calculation of similarity of band profiles was based on the Pearson correlation coefficient *r*, and strains were grouped using the Unweighted Pair Group Method with Arithmetic Averages (UPGMA) (Vauterin and Vauterin, 1992).

2.5. Characterization of *L. delbrueckii* ox gall-tolerant derivatives

Phage resistance was confirmed by challenging each stable ox gall-tolerant derivative, cultivated in MRS-Ca broth, with the corresponding lytic phage. Phage resistance was considered to be maintained when derivatives were able to grow in the presence of phages, after three consecutive subcultures, at the same rates as the control cultures (Reinheimer et al., 1993). Phage resistance stability, efficiency of plaquing (EOP) (Carminati et al., 1993) and adsorption rates (Neviani et al., 1992) were also determined, to compare the phage resistance phenotype of the stable ox gall-tolerant derivatives isolated with that of non-ox gall adapted *L. delbrueckii* strains.

Stationary phase cells of stable ox gall-tolerant derivatives and original *L. delbrueckii* strains were examined microscopically, under phase contrast illumination using a Jenamed 2 microscope (Carl Zeiss, Jena, Germany).

Milk acidification kinetics, as well as proteolytic and acidifying activities were determined for each non-adapted and stable ox gall-tolerant *L. delbrueckii* derivatives. Acidification kinetics, proteolytic and acidifying activities were studied by inoculation (2%, v/v) of the strains in sterile, reconstituted (10%, w/v), commercial, dry skim milk (RSM) and incubation for 24 h at 42 °C. pH values were measured with a model SA 720 pH meter (Orion, Beverly, Massachusetts, USA) and plotted against time. Proteolytic activity was determined by the *o*-phthaldialdehyde spectrophotometric assay (OPA Test) (Church et al., 1983) and values were expressed as the difference in absorbance at 340 nm (A_{340}) between strain cultures and a control of non-inoculated milk. The acidity developed, measured by titration with 0.1 N NaOH to pH 8.4, was expressed as % lactic acid. The 'fast' or 'slow' character of the strains was determined according to their growth in milk (Reinheimer et al., 1995), by measurement of pH values reached, after 24 h at 42 °C, by each culture inoculated at 2% (v/v) in RSM, RSM supplemented with 1% glucose or with 0.25% casein hydrolysate, and RSM supplemented with both 1% glucose and 0.25% casein hydrolysate (Mc Kay and Baldwin, 1974; Efstathio and Mc Kay, 1976).

2.6. Growth of ox gall-tolerant derivatives in the presence of ox gall

The ability of each ox gall-tolerant derivative and parent *L. delbrueckii* strain to grow in the presence of ox gall was determined. Exponentially growing cultures in MRS broth were inoculated (2%, v/v) into MRS broth and MRS broth supplemented with ox gall at 0.3% and 0.9% (w/v). The inoculated broths were then incubated at 37 °C. The OD_{560nm} was measured during incubation of each culture.

3. Results

3.1. Growth in the presence of ox gall

Ox gall MICs for *L. delbrueckii* phage-sensitive strains and phage-resistant mutants were 0.30% or 0.35% (w/v). Bile tolerant derivatives with tolerance to 0.3%, 0.6% or 0.9% (w/v) ox gall were obtained. They were identified as $Ab_1^{0.3}$, $Ab_1^{0.6}$ and $Ab_1^{0.9}$, from *L. delbrueckii* Ab_1; $A_7^{0.3}$, $A_7^{0.6}$ and $A_7^{0.9}$, from phage-resistant mutant A_7; $A_{17}^{0.3}$, $A_{17}^{0.6}$ and $A_{17}^{0.9}$, from phage-resistant mutant A_{17}; YSD $V^{0.3}$, YSD $V^{0.6}$ and YSD $V^{0.9}$, from *L. delbrueckii* YSD V; and $V_2^{0.3}$, $V_2^{0.6}$ and $V_2^{0.9}$, from phage-resistant mutant V_2. Ox gall adaptation was not achievable with *L. delbrueckii* Ib_3 and its phage-resistant mutant I_{39}.

3.2. Stability of the induced tolerance to ox gall

After six subcultures in MRS broth and an additional, seventh, subculture in MRS broth supplemented or not with 0.3%, 0.6% or 0.9% (w/v) ox gall, the derivatives of *L.*

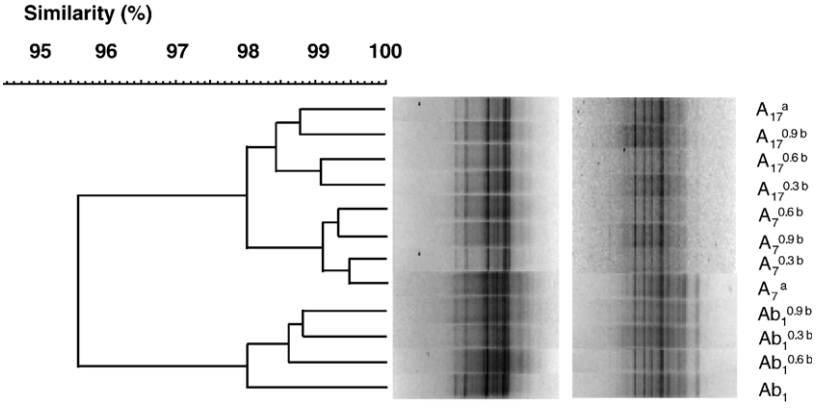

Fig. 1. RAPD-PCR profiles obtained from total DNA from *Lactobacillus delbrueckii* Ab_1, its phage-resistant mutants ([a]) and the ox gall-tolerant derivatives isolated from them ([b]).

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Table 1
Phage sensitivity, adsorption rates and technological properties of *Lactobacillus delbrueckii* Ab_1, its phage-resistant mutants (A_7 and A_{17}) and the ox gall-tolerant derivatives isolated from them

Strain	Phage sensitivity	Phage adsorption (%) [a]	pH [a, b]	Acidifying activity [a, b] (% lactic acid)	Proteolytic activity [a, b] (OPA Test, A_{340})
Ab_1	+	99.30±0.50	3.98±0.10	1.76±0.09	0.56±0.04
$Ab_1^{0.3}$ [c]	+	99.20±0.53	4.02±0.03	1.56±0.10	0.49±0.03
$Ab_1^{0.6}$ [c]	+	99.15±0.350	4.05±0.05	1.69±0.06	0.53±0.02
$Ab_1^{0.9}$ [c]	+	99.70±0.60	4.06±0.02	1.75±0.04	0.52±0.04
A_7 [d]	−	0	3.92±0.10	1.72±0.07	0.53±0.05
$A_7^{0.3}$ [c]	−	0	3.95±0.05	1.62±0.05	0.45±0.01
$A_7^{0.6}$ [c]	−	0	4.10±0.01	1.68±0.02	0.50±0.03
$A_7^{0.9}$ [c]	+	99.20±0.20	4.03±0.10	1.54±0.09	0.48±0.06
A_{17} [d]	−	0	5.66±0.15	0.36±0.01	0.08±0.01
$A_{17}^{0.3}$ [c]	−	0	5.72±0.10	0.37±0.02	0.09±0.05
$A_{17}^{0.6}$ [c]	−	0	5.77±0.06	0.35±0.03	0.10±0.01
$A_{17}^{0.9}$ [c]	−	0	5.78±0.03	0.34±0.03	0.10±0.02

[a] Values are the mean of three determinations±standard deviation.
[b] Values reached in RSM (42 °C, 24 h).
[c] Ox gall-tolerant derivatives.
[d] Spontaneous *L. delbrueckii* phage-resistant mutants isolated in a previous work (Guglielmotti et al., 2006).

delbrueckii Ab_1, A_7 and A_{17} were able to grow to similar levels in both conditions, evidenced by similar ($P>0.05$) OD_{560nm} values. The numbers of bacteria in ox gall–MRS cultures were similar ($P>0.05$) to those reached in the absence of ox gall. In contrast, a lack of ox gall-tolerance was observed when the derivatives of *L. delbrueckii* YSD V and V_2 were assayed. These strains attained very low OD_{560nm} values in ox gall–MRS broth, which were significantly different ($P<0.05$) from the values attained in MRS broth. The numbers of bacteria in ox gall–MRS cultures decreased significantly ($P<0.05$) (data not shown).

3.3. Genetic analysis

The combined RAPD-PCR profiles for primers M13 and 1254 are reported for *L. delbrueckii* Ab_1, its phage-resistant mutants (A_7 and A_{17}) and the ox gall-tolerant derivatives isolated from them (Fig. 1). Similarity coefficients were greater than 97%.

3.4. Characterization of L. delbrueckii ox gall-tolerant derivatives

Table 1 summarizes the results obtained when the phage resistance of stable ox gall-tolerant *L. delbrueckii* derivatives and non-adapted strains were compared. In general, a high level of phage resistance was exhibited by the derivatives isolated from the spontaneous phage-resistant mutants, as quantified by high EOP values ($<5.5\cdot10^{-8}$) and stability of the phenotype. Adsorption interference was responsible for phage resistance. Besides, ox gall-tolerant derivatives isolated from phage-sensitive strain *L. delbrueckii* Ab_1 remained sensitive to phage YAB. These results indicated that the ox gall-tolerant derivatives preserved, in general, the phage resistance phenotype of the corresponding non-adapted strains, with only one exception. After progressive ox gall adaptation, phage-resistant mutant A_7 also exhibited a progressive reversion of the phage resistance phenotype, since derivative with the highest ox gall-acquired tolerance ($A_7^{0.9}$) reverted to the sensitivity to phage YAB; but derivatives with low and intermediate ox gall-acquired tolerance ($A_7^{0.3}$ and $A_7^{0.6}$) retained their phage resistance. The ox gall-tolerant derivative $A_7^{0.9}$ had the same very high rate of adsorption of phage YAB as the parent strain. However, $A_7^{0.3}$ and $A_7^{0.6}$ did not adsorb phage YAB.

When the cell morphology of stationary phase stable ox gall-tolerant derivatives and the original *L. delbrueckii* strains were examined, the derivative strains were smaller cells (Fig. 2).

Milk acidification kinetics are shown in Fig. 3. Ox gall-tolerant derivatives had the same behaviors as the corresponding non-adapted strains. Thus, derivatives obtained from *L. delbrueckii* Ab_1 and A_7 showed fast milk acidification kinetics, while slow and poor acidification was exhibited by the ox gall-tolerant derivatives of A_{17}. Fig. 3 shows only the behavior of $Ab_1^{0.9}$ and $A_{17}^{0.9}$, in comparison with the corresponding non-adapted strains. Performance of $Ab_1^{0.3}$ and $Ab_1^{0.6}$ was similar to that exhibited by $Ab_1^{0.9}$, while performance of $A_{17}^{0.3}$ and $A_{17}^{0.6}$ was similar to that showed by $A_{17}^{0.9}$. The behavior

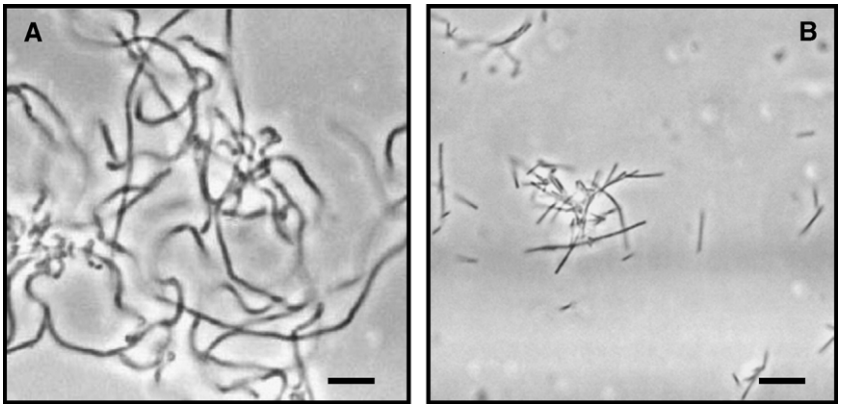

Fig. 2. Cell morphologies of stationary phase cultures of the original strain *L. delbrueckii* Ab_1 (A) and its ox gall-tolerant derivative *L. delbrueckii* $Ab_1^{0.9}$ (B). Bars represent 10 μm.

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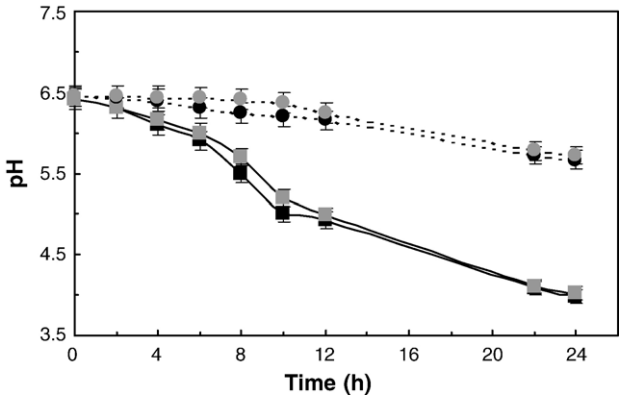

Fig. 3. Milk acidification kinetics of *L. delbrueckii* Ab_1 (■), the phage-resistant mutants A_{17} (●), and its ox gall-tolerant derivatives $Ab_1^{0.9}$ (▒) and $A_{17}^{0.9}$ (◍). Error bars represent the SD of three independent experiments.

of $A_7^{0.3}$, $A_7^{0.6}$, $A_7^{0.9}$ was comparable to those of the ox gall-tolerant derivatives of Ab_1.

Acidifying and proteolytic activities of stable ox gall-tolerant derivatives were comparable to those of the corresponding parent strains (Table 1). Ox gall-tolerant derivatives of *L. delbrueckii* Ab_1 and A_7 were classified as 'fast', according to the pH values reached in RSM cultures after 24 h at 42 °C (pH 4.04±0.04, $n=6$), while ox gall-tolerant derivatives of A_{17} were classified as 'slow' (pH 5.76±0.06, $n=3$). The 'fast' derivatives exhibited acidifying (1.64±0.06% lactic acid, $n=6$) and proteolytic (A_{340} 0.50±0.03, $n=6$) activities similar to those shown by the parent strain (1.76±0.09% lactic acid, A_{340} 0.56±0.04). The 'slow' variants had lower values of acidifying (0.35±0.03 % lactic acid, $n=3$) and proteolytic (A_{340} 0.10±0.08, $n=3$) activities. When the 'slow' variants were cultured in RSM supplemented with casein hydrolysate, they were able to coagulate milk and produced a significant ($P<0.05$) decrease of pH, similar to 'fast' variants.

3.5. Growth of ox gall-tolerant derivatives in the presence of ox gall

Fig. 4 shows growth kinetics for *L. delbrueckii* Ab_1 strain and its ox gall-tolerant derivatives $Ab_1^{0.3}$ and $Ab_1^{0.9}$ in MRS supplemented or not with ox gall. Their behavior is representative of all derivatives. No differences in the growth in MRS broth for ox gall-tolerant derivatives and non-adapted *L. delbrueckii* strains were observed (Fig. 4A). In ox gall-supplemented MRS broth, derivatives exhibited higher rates of growth than the non-adapted *L. delbrueckii* strains (Fig. 4B and C). Additionally, the inhibitory effect of increasing ox gall concentrations (0.3% and 0.9%) on growth was significantly higher ($P<0.05$) for the non-adapted strain than for the ox gall-tolerant derivatives.

4. Discussion

Even though the most important characteristics of probiotic bacteria are their positive effects on the health of the host, the evaluation of technological behavior should be an important consideration for the selection of strains for food applications (Stanton et al., 2003). The ability to resist bacteriophage infection is, perhaps, one of the most important characteristics sought in starter strains, because phage attack is the main cause of partial delay or failure of starter activity during industrial fermentations (Moineau et al., 2002). The isolation of spontaneous phage-resistant mutants was the first approach used to obtain phage-resistant derivatives from phage-sensitive strains. This technique has the convenience of simplicity and it is considered a 'natural' strategy to improve strains, since there are no regulatory restrictions for the use of resistant strains in industrial environments (Sing and Klaenhammer, 1993; Weimer et al., 1993; Moineau et al., 2002). Our recent studies provided evidence that several *L. delbrueckii* strains and their spontaneous phage-resistant variants exhibit characteristics generally considered essential for probiotic organisms (Guglielmotti et al., 2007), such as antibacterial activity displayed toward pathogens, adherence to Caco-2/TC-7 monolayers and significant inhibition of the invasion of *Salmonella enterica* serovar

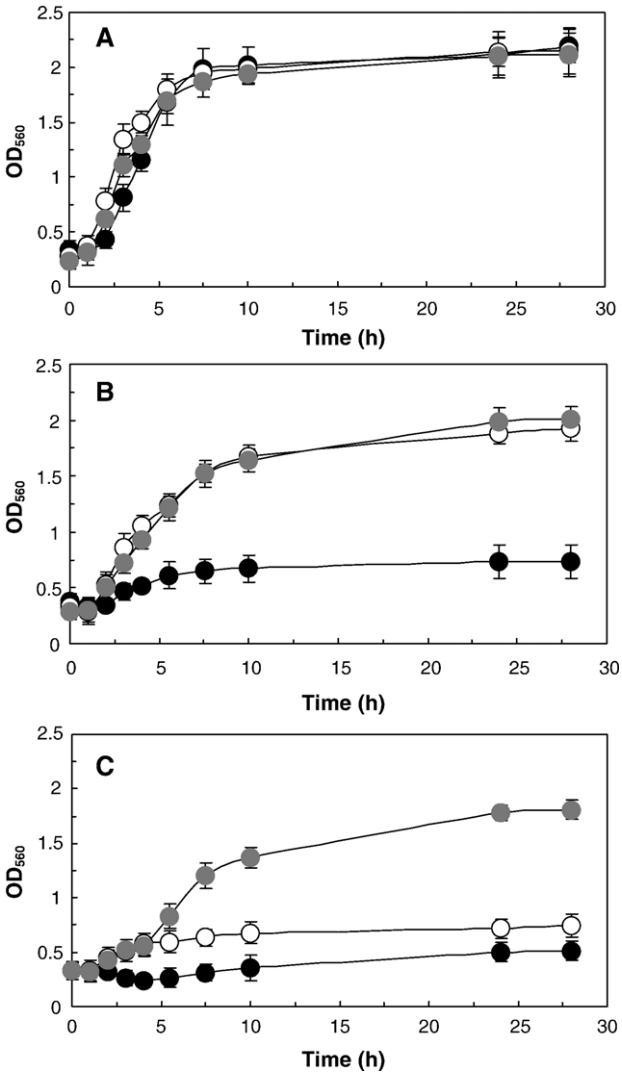

Fig. 4. Growth for *L. delbrueckii* Ab_1 (●) and its ox gall-tolerant derivatives, $Ab_1^{0.3}$ (○) and $Ab_1^{0.9}$ (◍), in MRS broth with 0% (A), 0.3% (B), and 0.9% (C) ox gall. Error bars represent the SD of three independent experiments.

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enteritidis into Caco-2/TC-7 cells. Also, the ability of the parent strains to activate the small intestine immune response in mice was preserved or enhanced in phage-resistant mutants (Vinderola et al., 2007). However, the potential probiotic role of *L. delbrueckii* is still a controversial issue.

Bile salts are toxic for living cells, since they disorganize the structure of the cell membrane (Margolles et al., 2003). There has been little investigation of the effect of bile salts on the viability and functionality of *L. delbrueckii*. Noh and Gilliland (1994) demonstrated that low concentrations of ox gall increased the permeability of *L. delbrueckii* subsp. *bulgaricus* cell membranes to allow more substrate to enter the cells and increase β-galactosidase activity, but at higher concentrations it was inhibitory to β-galactosidase activity. Similarly, Aslim et al. (2007) reported that autoaggregation and hydrophobicity of *L. delbrueckii* subsp. *bulgaricus* strains decreased after treatment with bile.

It is well known that bile-salt concentration in the gut is not static, ranging from 1.5% to 2% w/v in the first hour of digestion, while levels decrease afterwards to around 0.3% w/v (Noriega et al., 2004). Therefore to simulate the small intestine conditions, a broader range of bile concentrations was tested in our work.

This study involved the adaptation to high ox gall concentrations of *L. delbrueckii* strains with acquired phage resistance. Despite the simplicity of the technique, only some of the strains tested were able to acquire ox gall-tolerance. It has been suggested that changes in *Bifidobacterium* adherence as a consequence of a brief treatment with bile may be related to metabolic changes that lead to modifications of the cell membrane (Gómez-Zavaglia et al., 2002; Margolles et al., 2003; Noriega et al., 2004). In this sense, Gueimonde et al. (2005) hypothesized that changes in the surface composition may also have been induced in the bile-resistant variants, leading to a modification of mucus adhesion properties and to changes in the hydrophobicity of these strains. A relationship between phage resistance and point mutations in the genome was hypothesized in our previous works to explain the isolation of spontaneous phage-resistant mutants (Quiberoni et al., 1998; Guglielmotti et al., 2006). Moreover, the production of a masking substance could reduce the availability of phage receptors, causing a decrease in adsorption efficiency (Moineau and Lévesque, 2005). Additionally, it could be suggested that these modifications in the cell surface of spontaneous phage-resistant mutants are responsible for the changes in technological properties (Quiberoni et al., 1998; Guglielmotti et al., 2006).

The present study is the first work to focus on the exploitation of 'natural' strategies to sequentially improve *L. delbrueckii* strains. These strains, with an improved phage resistance, adequate technological properties, and acquired ox gall-tolerance could be excellent candidates for industrial applications in functional foods. However, further research is needed to elucidate the mechanism involved in the acquisition of ox gall-tolerance in these *L. delbrueckii* spontaneous phage-resistant mutants.

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