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Growth and viability of *Lactobacillus acidophilus* NRRL B-4495, *Lactobacillus casei* NRRL B-1922 and *Lactobacillus plantarum* NRRL B-4496 in milk supplemented with cysteine, ascorbic acid and tocopherols

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ABSTRACT

The effect of three reducing agents (RAs), L-cysteine, ascorbic acid, and tocopherols, on the growth of *Lactobacillus acidophilus*, *Lactobacillus casei*, or *Lactobacillus plantarum* during milk fermentation was evaluated. pH, redox potential, and *Lactobacillus* counts were determined until pH ≈ 4.6. Further, the study aimed to optimise the concentration of the RAs by formulating the fermented milks with the same RA at different concentrations (0–250 mg L^{-1} for L-cysteine or ascorbic acid and 0–15 mg L^{-1} for tocopherols) using a Box–Behnken experimental design. After 45 days of refrigerated storage, the viability of each *Lactobacillus* species was maximised. We observed that the effect of RA on *Lactobacillus* is species dependent; ascorbic acid and tocopherols reduced the fermentation time (29%–43%), whereas L-cysteine enhanced the *Lactobacillus* counts (≥1 $\log_{10}$ cfu mL^{-1}). *Lactobacillus* species differ in terms of oxygen sensitivity.

1. Introduction

In dairy industries, lactic acid bacteria (LAB) are widely used as starter cultures and probiotics. LAB may use non-enzymatic (Mn^{2+}, ascorbate, tocopherols, and glutathione) and enzymatic (thioredoxin and thioredoxin reductase, catalase, NADH oxidases, NADH peroxidases, and superoxide dismutase systems) mechanisms for defence against reactive oxygen species (ROS; Calderini et al., 2017). *Lactobacillus casei* consumes oxygen (under aerobic growth conditions) through enzymes (Zotta et al., 2014). However, some LAB genomes do not encode enzymes for ROS elimination, which adversely affects cell performance by attacking proteins, lipids, and nucleic acids; the major causes of cell death (De Angelis & Gobbetti, 2004). For example, *Lactobacillus acidophilus* NCFM has genes associated with oxygen-consuming routes, disulphide-reducing pathways, and DNA repair (Altermann et al., 2005). The metabolic activity of LAB that involves a series of dehydrogenation (oxidation) and hydrogenation (reduction) reactions facilitates adaptation of LAB to different environmental conditions (Larsen et al., 2016). During anaerobic glycolysis in homofermentative LAB, glucose is oxidised to two moles of pyruvate with the formation of two moles of NADH, which subsequently reduces pyruvate to form lactic acid (Martin et al., 2013). LAB provided with NADH oxidase can reduce oxygen to water (promote intracellular and extracellular redox environment), which results in changes in metabolism and cell physiology. Oxidising or reducing environment can be monitored by measuring the redox potential (E_h); therefore, it can be a key parameter in assessing the quality of fermented dairy products. However, during manufacturing, redox potential is usually completely ignored because of its difficulty to be measured and controlled (Martin et al., 2013).

A positive E_h indicates an environment that favours oxidation reactions, whereas a negative E_h indicates a reducing environment. Because most LAB are anaerobic or facultative aerobic bacteria, they prefer reducing environments. Redox potential can be changed by pH adjustments, modifications in redox-active gas concentrations (O_2, H_2, and H_2S; Oktyabrskii & Smirnova, 2012), or addition of oxidising/reducing agents (RAs). Previous studies have shown that E_h has a direct relation with milk fermentation kinetics of starter LAB (Abraham et al., 2013) and with viability of probiotic LAB (Dave & Shah, 1997a,b; Sousa et al., 2012).

In starter LAB, the physicochemical factors such as E_h influence the fermentation of dairy products and contribute to create

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adequate conditions for flavour balance (Cachon, Jeanson, Aldarf, & Divies, 2002). The aroma profile of yoghurt depends on E_h, whereas gel structural properties (apparent viscosity and whey separation) are highly influenced by E_h (Martin et al., 2010, 2011). In probiotic dairy products, RAs increased the cell numbers during growth and maintained their viability during storage, as Dave and Shah (1997a,b) showed for *L. acidophilus* and *Bifidobacterium* counts from yoghurt supplemented with L-cysteine or ascorbic acid. To date, few reports included tocopherol as a reducing agent or *Lactobacillus* protective additive (Ying et al., 2011). In addition, the effect of RAs has not been evaluated in other *Lactobacillus* species. Therefore, the aim of this work was to evaluate the effect of three RAs (L-cysteine, ascorbic acid, or tocopherols) on the growth of *L. acidophilus*, *L. casei*, or *Lactobacillus plantarum* during milk fermentation, and evaluate the pH and E_h. In addition, optimisation of the concentration of tested RAs was performed, to maximise the viability of each *Lactobacillus* in fermented milks after 45 days of refrigerated storage using a Box–Behnken design.

2. Materials and methods

2.1. Strains, culture conditions, and materials

L. acidophilus NRRL B-4495, *L. casei* NRRL B-1922, and *L. plantarum* NRRL B-4496 were acquired in lyophilised form from the USDA Agricultural Research Service (Peoria, IL, USA). Lactobacilli were activated and routinely cultured in de Mann, Rogosa and Sharpe (MRS) broth (Difco™ BD, Sparks, MD, USA) in anaerobic conditions at 35 °C for 48 h. To generate anaerobic conditions, tubes were incubated in an AnaeroJar™ system (Oxoid Ltd., Hampshire, UK). L-Cysteine, ascorbic acid, and tocopherols were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Growth curves

To evaluate the effect of tested RAs on the survival and growth of *Lactobacillus* during fermentation of milk, commercial pasteurised whole milk samples (33 g of fat L^{-1}, 31 g of protein L^{-1}, 5 g of vitamin D L^{-1}, and 666 mg retinol equivalents L^{-1}) were supplemented with L-cysteine (50, 250, or 500 mg L^{-1}; Sigma Aldrich, St. Louis, MI), ascorbic acid (50, 250, or 500 mg L^{-1}; Sigma Aldrich), or tocopherols (5, 7.5, or 15 mg L^{-1}; Sigma Aldrich), inoculated with 10 mL of the corresponding MRS grown culture per liter, and incubated at 35 °C until a pH ≈ 4.6 was reached. Milk samples without RAs were used as controls. Every 4 h, 1 mL of inoculated milk was taken, and adequate 10-fold dilutions were performed and plated on MRS agar (Difco™ BD). Plates were incubated at 35 °C under anaerobic conditions. Anaerobic conditions were provided by the GasPak™ Pouch Anaerobic System (Difco™ BD) for 48 h for the three LAB and then counted.

2.3. Experimental design for optimisation

The response surface methodology, using a Box–Behnken experimental design (Table 1), was utilised to estimate the effects of three factors (tested RAs) and three levels (tested concentrations) on *Lactobacillus* viability (Log N), E_{h7}, and pH of fermented milks after 45 days of refrigerated storage. The design consisted of 17 sets of evaluated conditions where levels of each factor were set at high, central, and low levels, with additional five replicate centre points. The order of the experiments was randomised to avoid any bias. After the experiments had been performed, a second-order polynomial equation based on the obtained data was utilised to determine the effects and interactions of evaluated factors.

Table 1
Variables and levels utilised for the Box-Behnken design for each *Lactobacillus* species tested.[a]

Variable	Level		
	Low (−1)	Central (0)	High (+1)
Independent variables			
L-Cysteine (mg L^{-1})	0	125	250
Ascorbic acid (mg L^{-1})	0	125	250
Tocopherols (mg L^{-1})	0	7.5	15
Dependent variables			
Log_{10} N (cfu mL^{-1})			
Eh_7 (mV)			
pH			

[a] Log_{10} N, *Lactobacillus* viability; Eh_7, redox potential.

Equation (1) describes the effect of tested factors including linear, quadratic, and cross-product terms:

$$Y = C_0 + C_1X_C + C_2X_A + C_3X_T + C_{12}X_CX_A + C_{13}X_CX_T + C_{23}X_AX_T + C_{11}X_C^2 + C_{22}X_A^2 + C_{33}X_T^2 \quad (1)$$

where Y is the predicted response of the dependent variable (Log N, E_{h7}, or pH), and C_0 is the second-order constant term. The regression coefficients (C_i) represent linear (C_1, C_2, and C_3), interaction (C_{12}, C_{13}, and C_{23}), and quadratic (C_{11}, C_{22}, and C_{33}) effects of the model. X is the coded value of the factor indicated by the attached subscript (C, L-cysteine; A, ascorbic acid; T, tocopherols). Furthermore, an optimisation of RA concentration for the maximum *Lactobacillus* viability was performed.

Each fermented milk formulation was prepared as previously described in Section 2.2. After adequate milk batches were prepared and they reached a pH ≈ 4.6, every container was cooled to 5 °C for 24 h. After this cooling time, the storage time was considered equal to 0 day for pH, redox potential, and bacterial counts. At the beginning and after 45 days, bottles of each tested combination were taken from refrigerator and analysed as described in Section 2.4. For microbial counts, adequate 10-fold dilutions were plated on MRS agar, incubated at 35 °C for 48 h, and then counted. As quality control tests, additional analyses to search for microorganisms other than *Lactobacillus* were performed in fermented milks; hence, total coliforms, and moulds and yeasts were plated in violet red bile agar (VRBA, Difco™ BD, Sparks, MD) and acidified potato dextrose agar (PDA, Difco™ BD, Sparks, MD) (14 mL L^{-1} agar) with tartaric acid (100 g L^{-1}), respectively. Inoculated VRBA plates were incubated at 35 °C for 24 h, whereas inoculated PDA were incubated for 5 days at 25 °C. Counts for total coliforms and mould and yeast were <10 cfu mL^{-1} for every tested fermented milk at the times analysed (day 0 and day 45).

2.4. Redox potential and pH measurements

The pH was measured using electrode immersion with a pH meter (UB-10, Denver Instrument, Bohemia, NY, USA). For redox potential, a pH meter (CON 510 Series, Oakton, Singapore) was used with a platinum electrode referred to the Ag/AgCl (Conductronic HH6, Conductronic, Mexico); these measurements were also performed using electrode immersion and reported in mV. Platinum surface was polished with slightly humidified fine alumina powder before using. According to Abraham et al. (2013), redox potential (E_h) determined using Ag/AgCl electrode must be corrected to E_h by equation (2):

$$E_h = E_m + E_r \quad (2)$$

where E_h is the redox potential in relation to the normal hydrogen electrode (in mV), E_m is the redox potential measured with the redox sensor (in mV), and E_r is the redox potential of the reference electrode used for measurement (in mV). E_r value for Ag/AgCl (3 mol L^{-1} KCl) reference electrode at 35 °C is 199 mV according to the manufacturer. For pH 7.0, $E_{h7} = E_h - 39\,(7 - pH)$. The correcting value of 39 mV pH^{-1} unit was obtained from the measurement of redox potential of milk versus pH. For the redox potential of fermented milks stored at 5 °C, temperature correction was realised with equation (3):

$$E_r(mV) = 207 + 0.8*(25 - T) \quad (3)$$

2.5. Statistical analysis of the data

Statistical analysis was performed by ANOVA and Tukey's mean comparison tests ($p < 0.05$), using Minitab® Statistical Software (ver. 17, Minitab Inc., State College, PA, USA), to identify significant differences in microbial counts, pH, and redox potential (E_{h7}). Response surface design analysis of the Box–Behnken experimental design was performed using Minitab® Statistical Software (ver. 17). Backward elimination of terms was utilised while maintaining response surface model hierarchy. Models' significance and terms were evaluated maintaining those terms that were significant at a probability (p) value < 0.10. The adequacy of the polynomial equations was determined by regression coefficients and lack-of-fit tests.

3. Results and discussion

3.1. Effect of RAs on the growth of Lactobacillus

L-Cysteine and ascorbic acid concentrations were selected based on the previous reports of Dave and Shah (1997a,b); ascorbic acid was tested at higher concentrations than those reported to investigate its effect. Codex Standard 192–1995 (FAO/WHO, 1995) allows up to 500 mg L^{-1} tocopherols for dairy desserts (this category includes aromatised yoghurt) as antioxidant, whereas Kamal-Eldin and Appelqvist (1996) suggest that tocopherols should be used at the lowest concentrations possible to avoid pro-oxidant activity; therefore, relatively low amounts were selected for this antioxidant.

RAs had different effects on *Lactobacillus* growth that were species and reducing agent dependent. *L. acidophilus* required long incubation times to reach pH 4.6; 40–44 h was necessary for intermediate and high-tested cysteine concentrations; however, low concentrations of cysteine (50 mg L^{-1}) needed fermentation times (28 h) similar to controls to reach the desired pH (Figs. 1 and 2). At the end of incubation, *L. acidophilus* supplemented with cysteine (250 or 500 mg L^{-1}) had more viable counts ($p < 0.05$) than milk with 0 or 50 mg L^{-1} cysteine added.

These results are in accordance with those reported by Dave and Shah (1997b) who observed fermentation time increments of 15–30% or 40–60% in milks inoculated with four starter cultures (they contained *Streptococcus thermophilus*, *L. acidophilus*, and *Bifidobacterium* and two also contained *Lactobacillus delbrueckii* ssp. *bulgaricus*) when 250 or 500 mg cysteine L^{-1} was utilised, respectively. Prolonged times in our case were approximately 14% or 43% longer when utilising 250 or 500 mg cysteine L^{-1}, respectively, for *L. acidophilus*.

In the case of *L. casei*, 50 mg cysteine L^{-1} increased around 14% of the fermentation time to reach a pH ≈ 4.6, whereas 250 mg cysteine L^{-1} prolonged fermentation time close to 29% and 500 mg cysteine L^{-1} lengthened it up by nearly 43%. Cysteine delayed the fermentation time for *L. plantarum* around 14% when 50 or 250 mg cysteine L^{-1} was utilised and approximately 29% when utilising 500 mg cysteine L^{-1}. In the case of *L. casei* and *L. plantarum*, 50 mg cysteine L^{-1} showed prolonged fermentation times. When cysteine was supplemented at any of the tested concentrations, *L. casei* counts were higher than control (≈1.3 $\log_{10}$ cfu mL^{-1}) ($p < 0.05$). For *L. plantarum*, increments in cell counts were <1 $\log_{10}$ cfu mL^{-1} at any tested cysteine concentrations compared with controls, and therefore, no improvements were observed. To our knowledge, no previous reports about RAs' effects on *L. casei* and *L. plantarum* have been published.

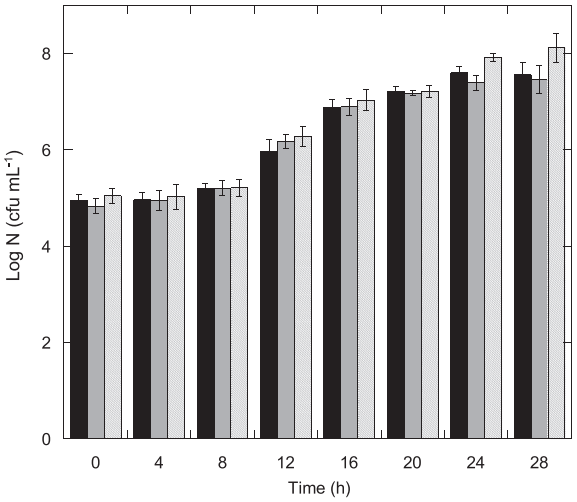

Fig. 1. Microbial counts of (■) *Lactobacillus acidophilus*, (▒) *Lactobacillus casei* and (▨) *Lactobacillus plantarum* during milk fermentation at 35 °C till a pH ≈ 4.6 is obtained without any reducing agent (0 mg L-cysteine, 0 mg ascorbic acid, and 0 mg tocopherols).

Ascorbic acid had variable effects on the growth of *Lactobacillus* (Figs. 1 and 3). For *L. acidophilus*, every tested concentration increased fermentation time (by around 29% and 43% for 50 and 250 or 500 mg ascorbic acid L^{-1}, respectively), without notable increments in cell counts (<1 $\log_{10}$ cfu mL^{-1}). *L. casei* and *L. plantarum* reduced their milk fermentation times at any ascorbic acid tested concentration by roughly 29%, despite no greater viable cell counts were observed (<1 $\log_{10}$ cfu mL^{-1}). Moreover, viable counts of *L. plantarum* were similar in milks with or without ascorbic acid ($p > 0.05$). However, *L. casei* viable counts in fermented milks supplemented with ascorbic acid were higher ($p < 0.05$) compared with control. Likewise, no previous reports were found about the effect of ascorbic acid on the fermentation time of *Lactobacillus*.

Fig. 4 presents the effect of tocopherols on the growth of *Lactobacillus* when the milk was supplemented. Tocopherols increased fermentation times of *L. acidophilus* by almost 43% at any tested concentration (5, 7.5 or 15 mg tocopherols L^{-1}); correspondingly, cell counts were increased, even though increments ≈1 $\log_{10}$ cfu mL^{-1} were observed when utilising 5 and 15 mg tocopherols L^{-1}. Despite no interesting increases of *L. acidophilus* counts, they were higher than the control milk ($p < 0.05$). Low and intermediate tested levels of tocopherols (5 and 7.5 mg tocopherols L^{-1}) reduced fermentation times for *L. casei* by approximately 43%, whereas 15 mg tocopherols L^{-1} shorten fermentation times by nearly 29%. Viable cell counts of *L. casei* were similar ($p > 0.05$) at any tested concentration of tocopherols and control milks. In the case of *L. plantarum*, tocopherols reduced close to 43% the fermentation time and similar viable counts ($p > 0.05$) were obtained for the control milk and those with the three tested levels of concentration of tocopherols. No previous studies were found regarding the effect of tocopherols on *Lactobacillus* fermentation. However, Ying et al. (2011) reported a protective

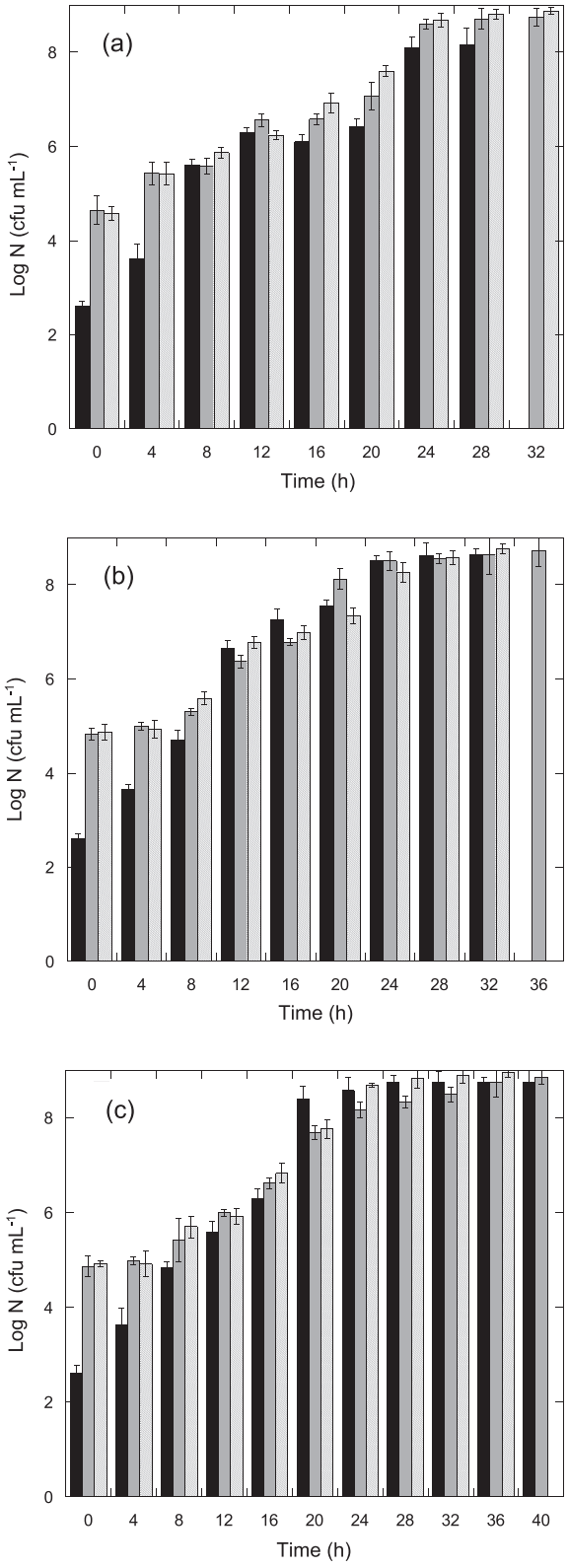

Fig. 2. Microbial counts of (■) *Lactobacillus acidophilus*, (▒) *Lactobacillus casei* and (▨) *Lactobacillus plantarum* at different concentrations of L-cysteine during milk fermentation at 35 °C until pH ≈ 4.6 was obtained: (a) 50 mg L-cysteine L^{-1}, (b) 250 mg L-cysteine L^{-1}, and (c) 500 mg L-cysteine.

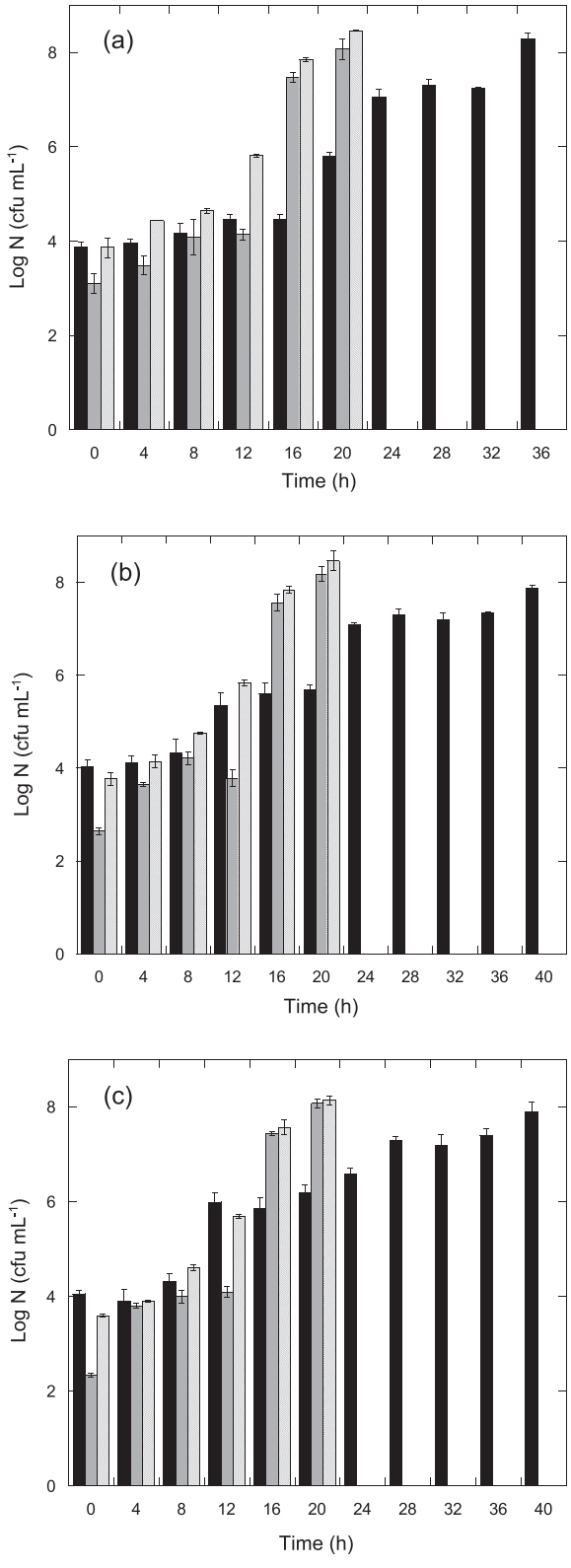

Fig. 3. Microbial counts of (■) *Lactobacillus acidophilus*, (▒) *Lactobacillus casei* and (▨) *Lactobacillus plantarum* at different concentrations of ascorbic acid during milk fermentation at 35 °C until pH ≈ 4.6 was obtained: (a) 50 mg ascorbic acid L^{-1}, (b) 250 mg ascorbic acid L^{-1}, and (c) 500 mg ascorbic acid L^{-1}.

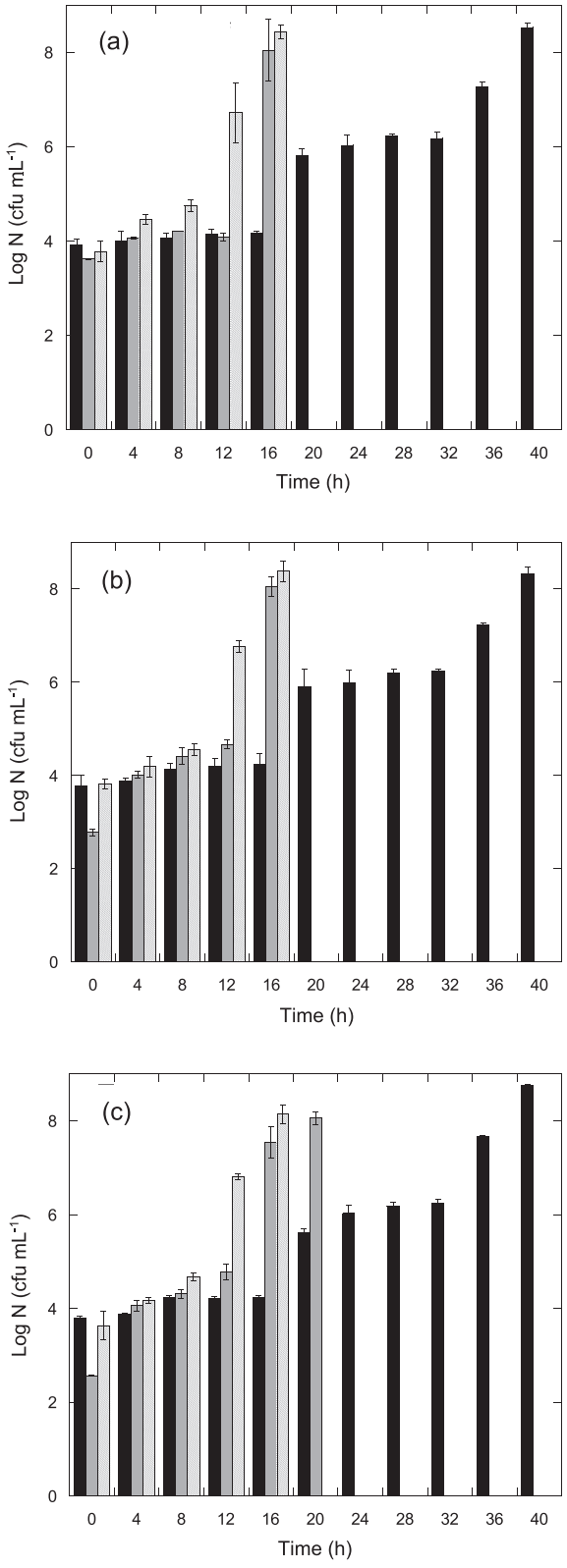

Fig. 4. Microbial counts of (■) *Lactobacillus acidophilus*, (▒) *Lactobacillus casei* and (▨) *Lactobacillus plantarum* at different concentrations of tocopherols during milk fermentation at 35 °C until pH ≈ 4.6 was obtained: (a) 5 mg tocopherols L^{-1}, (b) 7.5 mg tocopherols L^{-1}, and (c) 15 mg tocopherols L^{-1}.

effect of tocopherol in *Lactobacillus rhamnosus* GG encapsulated and stored for 6 months; Soghomonyan, Akopyan and Trehounian (2011) reported that the growth rate of *Lactobacillus salivarius* had decreased proportionally with increasing concentrations (1–5 mM) of reducing agent (DL-dithiothreitol), whereas the lag phase for *L. salivarius* increased. These results were attributed to the ability of the tested reducing agent to increase the H^+ and K^+ fluxes across the membrane of *L. salivarus*. Another report observed different acidification kinetics for different *Lactococcus lactis* strains, which were attributed to differences in metabolisms instead of growth rate fermentation processes under aerobic conditions (Jeanson et al., 2009).

3.2. Redox potential and pH during *Lactobacillus* growth

Figs. 5–7 display the E_{h7} and pH values obtained during the fermentation processes of milk inoculated with the *Lactobacillus* studied. At the beginning, E_{h7} value of pasteurised milk (+344 ± 26 mV) was lower than the value reported (+405 mV) previously by Martin et al. (2011) but higher than the value (230 ± 70 mV) reported by Brasca, Morandi, Lodi, and Tamburini (2007); these differences could be because of the different nature of the milk studied. At the beginning (t = 0), decline in the E_{h7} of the milk depended on the concentration and type of reducing agent. The milk had a pH of 6.6 as expected, and the milk supplemented with any reducing agent had a pH value between 6.2 and 6.5.

The highest E_{h7} reduction was observed for milk inoculated with *L. acidophilus* supplemented with L-cysteine, whereas ascorbic acid and tocopherols maintained positive values (Fig. 5). During fermentation, pH and E_{h7} had specific behaviour depending on each reducing agent. Milk without L-cysteine maintained higher pH and E_{h7} values (>6.0 and > +200 mV) up to 12 h of incubation ($p < 0.05$) and both dropped dramatically at 16 h when values of E_{h7} ~150 mV were reached, then constant and fast decreases were observed up to pH 4.6. On the other hand, milk with L-cysteine at any concentration had constant reductions of E_{h7}. At the beginning, and when values of E_{h7} were ~100 mV, pH decreased constantly until pH was 4.6. After 16 h of incubation, all fermented milks had the same pH and E_{h7}, then at 20 h the control milk reached the lowest E_{h7} value ($p < 0.05$) and then began to increase as the L-cysteine supplemented milks. Control milk sample and milk sample supplemented with 50 mg L^{-1} L-cysteine reached pH 4.6 at 28 h, whereas milk supplemented with 250 or 500 mg L^{-1} L-cysteine required 36 and 44 h, respectively, to reach the same pH value.

Ascorbic acid and tocopherols supplementation slightly reduced the redox potential of milk, and positive values (>100 mV) were maintained up to 8 h of incubation with pH values > 6 in the same time period. The addition of ascorbic acid or tocopherols dropped E_{h7} quickly after 12 h of incubation, whereas control milk dropped E_{h7} after 16 h of incubation. Likewise, milk supplemented with any concentration of ascorbic acid or tocopherols required more time ($p < 0.05$) to reach pH 4.6 than control milk, extending the incubation time up to 36 and 40 h, respectively. At the end of fermentation (pH ≈ 4.6), milk E_{h7} values between −47 and −98 mV were observed in those supplemented with 50, 250, or 500 mg cysteine L^{-1} or 7.5 or 15 mg tocopherols L^{-1}, whereas, when 50 mg ascorbic acid L^{-1} was utilised, milk E_{h7} value reached −22 mV; at the other tested concentrations of ascorbic acid, milks had positive values (>+91 mV). Control milk had the lowest value of E_{h7} (from −163 to −156 mV).

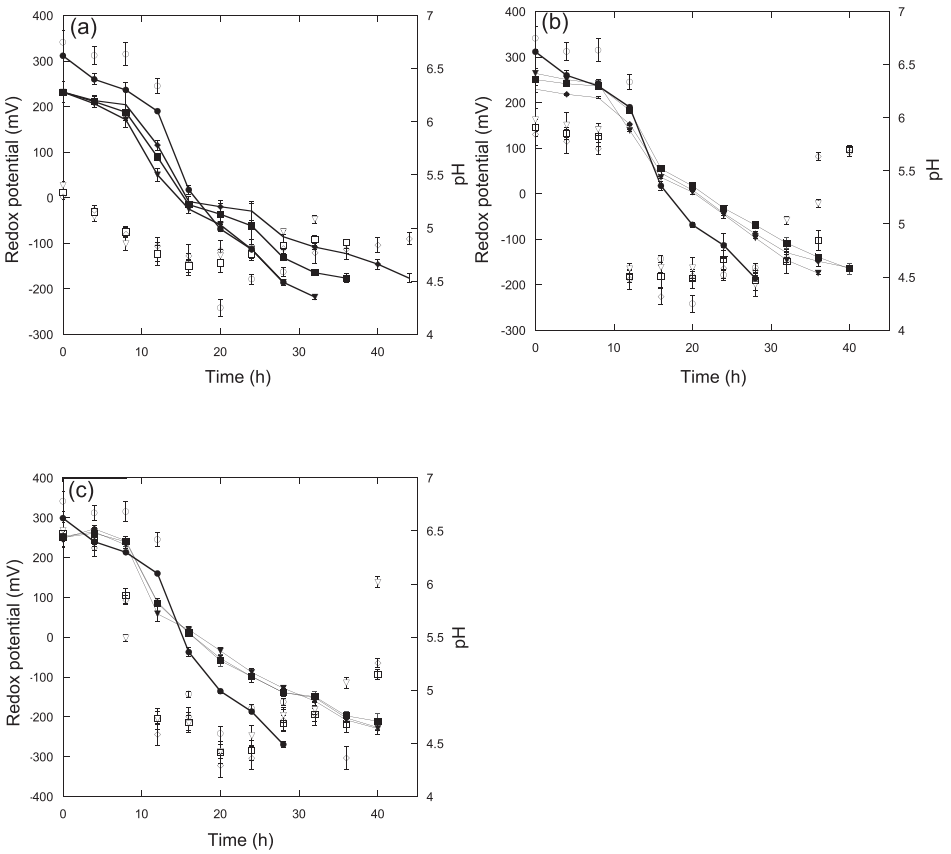

Fig. 5. Redox potential (E_{h7}) and pH values during milk fermentation with *Lactobacillus acidophilus* supplemented with three reducing agents at four levels: (a) L-cysteine (0 (○ E_{h7}, ● pH), 50 (▽ E_{h7}, ▼ pH), 250 (□ E_{h7}, ■ pH), and 500 (◇ E_{h7}, ◆ pH) mg L^{-1}), (b) ascorbic acid (0 (○ E_{h7}, ● pH), 50 (▽ E_{h7}, ▼ pH), 250 (□ E_{h7}, ■ pH), and 500 (◇ E_{h7}, ◆ pH) mg L^{-1}), and (c) tocopherols (0 (○ E_{h7}, ● pH), 5 (▽ E_{h7}, ▼ pH), 7.5 (□ E_{h7}, ■ pH), and 15 (◇ E_{h7}, ◆ pH) mg L^{-1}).

Fig. 6 shows E_{h7} and pH during milk fermentation inoculated with *L. casei* and supplemented or not with RAs. Initial E_{h7} values were ~0 mV when 250 or 500 mg L^{-1} L-cysteine was added, whereas milk supplemented with 50 mg L^{-1} L-cysteine had ~ +60 mV, and milk added with any concentration of ascorbic acid or tocopherols had values > +100 mV. The pH and E_{h7} had the same behaviour described previously for *L. acidophilus* with cysteine. However, pH and E_{h7} were not equal at 16 h of incubation, and pH 4.6 was reached after 32, 36, and 40 h for 50, 500, and 250 mg L^{-1} cysteine, respectively. Ascorbic acid maintained positive values of E_{h7} and pH > 6.0 after 8 h of incubation; at 4 and 8 h, pH values were similar ($p < 0.05$) among milk without ascorbic acid and milks supplemented with 50 and 250 mg L^{-1} ascorbic acid. After 12 h of incubation, E_{h7} values of milks supplemented with any concentration of ascorbic acid dropped from −250 to −310 mV and pH began its drop quickly reaching the pH of 4.6 at 20 h; in this period, the pH values of the three milks were equal ($p > 0.05$) and decreased at the same rate. The longest fermentation time was observed for milk without ascorbic acid. Relations of low E_{h7} values could be related with the decrease in pH as Michelon et al. (2010) observed for *Lac. lactis*. By contrast, tocopherols achieved reduction of pH without a negative E_{h7} value when milk was supplemented with 5 mg L^{-1}. However, rapid decreases of pH were obtained when milk had negative E_{h7} values (−30 to −150 mV, at 12 h incubation). For 5 and 7.5 mg L^{-1} tocopherols, the shortest fermentation times were obtained (16 h), followed by 15 mg L^{-1} tocopherols at 20 h. At the end of fermentation, lower values of E_{h7} were observed when utilising ascorbic acid (−248 to −319 mV) than tocopherols (−183 to −210 mV) and cysteine (−64 to −112 mV).

E_{h7} and pH during milk fermentation with *L. plantarum* are presented in Fig. 7. At time zero, values of E_{h7} and pH were similar to the values previously described for *L. acidophilus* and *L. casei* for the three RAs. In addition, pH and E_{h7} had the same behaviour during the first 16 h of incubation in comparison with that observed for *L. acidophilus*. However, only pH of milks added with 50 and 250 mg L^{-1} cysteine and 0 and 500 mg L^{-1} cysteine had similar pH ($p < 0.05$) at 16 h. Time to reach pH 4.6 was 32 h for 50 and 250 mg L^{-1} and 36 h for 500 mg L^{-1}. In milk supplemented with ascorbic acid, constant reductions of pH and E_{h7} were observed throughout fermentation up to reaching pH 4.6 at 20 h. E_{h7} values were negative after 12 h of incubation and diminished up to 16 h. Any ascorbic acid concentration provided the same pH value ($p < 0.05$) and dynamics in milk throughout the fermentation. Therefore, tested concentrations do no result in different fermentation rates. As observed for *L. casei*, tocopherols influenced the production of lactic acid of *L. plantarum* without observing negative E_{h7} values, because at the beginning and throughout the fermentation process, the pH and E_{h7} decreased more slowly up to reaching pH 4.6 at 16 h. As occurred for ascorbic acid, the tocopherols concentration did not affect the fermentation rate or the pH value because the same tendency and pH value were observed ($p > 0.05$); the pH value was only different at 8 h of incubation. Fermented milks had their lowest E_{h7} when utilising ascorbic acid (−211 to −227 mV), then tocopherols (≈−195 mV) and cysteine (−121 to −142 mV) at the end of the fermentation process. In the future, it will be interesting to investigate the mechanism by which the tocopherols act on the cells of *L. casei* or *L. plantarum* because without negative E_{h7} values their lactic acid production is stimulated.

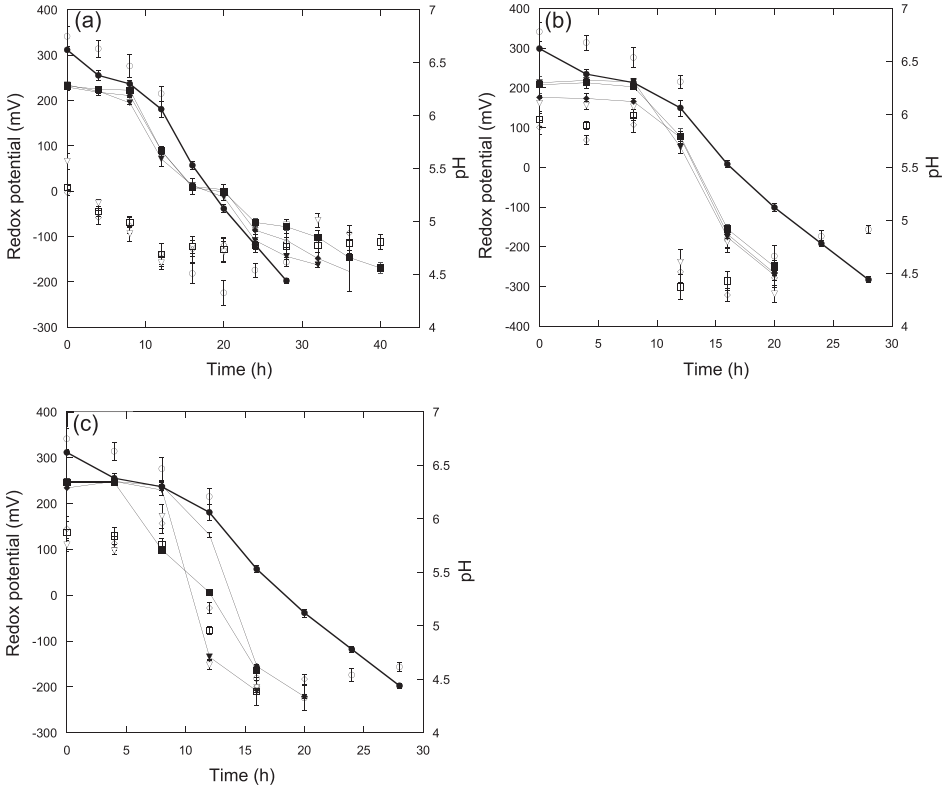

Fig. 6. Redox potential (E_{h7}) and pH values during milk fermentation with *Lactobacillus casei* supplemented with three reducing agents at four levels: (a) L-cysteine (0 (○ E_{h7}, ● pH), 50 (▽ E_{h7}, ▼ pH), 250 (□ E_{h7}, ■ pH), and 500 (◇ E_{h7}, ◆ pH) mg L^{-1}); (b) ascorbic acid (0 (○ E_{h7}, ● pH), 50 (▽ E_{h7}, ▼ pH), 250 (□ E_{h7}, ■ pH), and 500 (◇ E_{h7}, ◆ pH) mg L^{-1}), and (c) tocopherols (0 (○ E_{h7}, ● pH), 5 (▽ E_{h7}, ▼ pH), 7.5 (□ E_{h7}, ■ pH), and 15 (◇ E_{h7}, ◆ pH) mg L^{-1}).

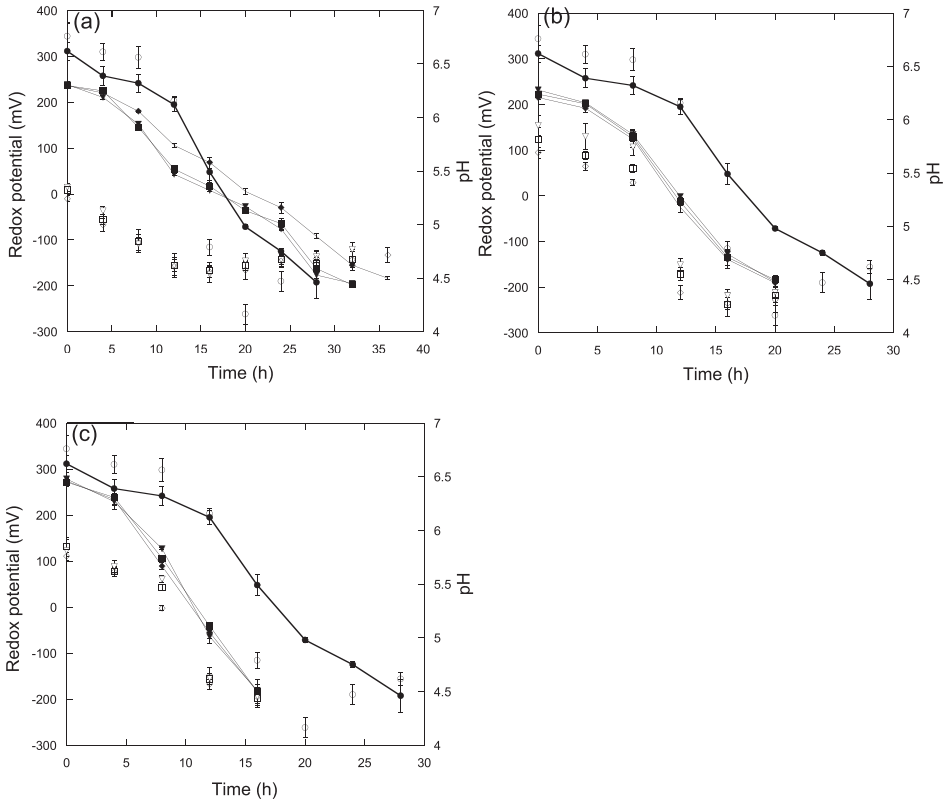

Fig. 7. Redox potential (E_{h7}) and pH values during milk fermentation with *Lactobacillus plantarum* supplemented with three reducing agents at four levels: (a) L-cysteine (0 (○ E_{h7}, ● pH), 50 (▽ E_{h7}, ▼ pH), 250 (□ E_{h7}, ■ pH), and 500 (◇ E_{h7}, ◆ pH) mg L^{-1}), (b) ascorbic acid (0 (○ E_{h7}, ● pH), 50 (▽ E_{h7}, ▼ pH), 250 (□ E_{h7}, ■ pH), and 500 (◇ E_{h7}, ◆ pH) mg L^{-1}), and (c) tocopherols (0 (○ E_{h7}, ● pH), 5 (▽ E_{h7}, ▼ pH), 7.5 (□ E_{h7}, ■ pH), and 15 (◇ E_{h7}, ◆ pH) mg L^{-1}).

Similar decreasing behaviour of E_{h7} was reported for five strains of *L. plantarum* and ten strains of *L. paracasei* ssp. *paracasei* (Brasca et al., 2007); however, in their case, reducing values were reached in very short times, close to 7.5 h. In our case, *L. casei* and *L. plantarum* supplemented with ascorbic acid and tocopherols showed faster acidification than other tested combinations; these higher reducing values were observed after nearly 16 h of fermentation.

In previously published reports, there is not a consensus about when the drop of redox potential occurs, because some state that it occurs at the transition from logarithmic to stationary phases; however, other authors mention that it can happen even after the log phase (Caldeo, 2015); in our case, the drop of redox potential occurred at the end of log phase. It has been well documented that variations in reducing capacity and the time required to reduce milk depend on microbial species, subspecies, and even strains (Brasca et al., 2007; Cachon et al., 2002; Caldeo, 2015).

Mechanisms by which the redox potential decreases in milk are still unclear because of unknown redox-couples involved in reactions, even more if redox-sensitive regulatory systems of cells respond to every oxidised/reduced form or to individual redox-active compounds (Oktyabrskii & Smirnova, 2012); however, it is strongly related to the presence of whole cells of lactobacilli (Caldeo, 2015). Michelon et al. (2010) demonstrated that changes of E_{h7} in MRS broth by *Lac. lactis* subsp. *cremoris* TIL46, which reached values ~200 mV, were mediated by exofacial thiol groups of the whole cells (localised on cysteine residues of exoproteins of the cell wall). Thiol groups not only protect cell against oxidative stress and contribute to detoxifying the ROS (Green & Paget, 2004) but also are responsible for the drop in the redox potential (Michelon et al., 2010) under anaerobic conditions. Furthermore, in *L. casei* the causes for redox homeostasis are cysteine/cysteine, glutathione/glutathione disulphide, and thioredoxin/thioredoxin disulphide, which depend on the cysteine external provision (Licandro-Seraut, Scornec, Pédron, Cavin, & San sonetti, 2014). It seems tenable that in our study, *L. acidophilus, L. casei* and *L. plantarum* whole cells could be contributed to redox potential because oxygen removal only reaches E_{h7} values around −100 to −150 mV (Michelon et al., 2010); however, the mechanism by which *Lactobacillus* species reduced the environment should be studied.

3.3. Response surface regression analysis

The design matrix and obtained results are exhibited in Table 2 for *Lactobacillus*. Responses were analysed using coded units to evaluate the significant effects of the studied factors. A factor was considered significant if it differs from 0 and the *p*-value was ≤0.10. Models' coefficients and corresponding p-values obtained in each case are presented in Table 3. Tested RAs notably affected the viability of *L. acidophilus* and *L. casei* after 45 days of storage, not so for *L. plantarum* ($p > 0.10$). L-Cysteine, ascorbic acid, and tocopherols affected *L. acidophilus* and *L. casei* viability, as was already observed during the fermentation tests; furthermore, each studied *Lactobacillus* had a specific behaviour with each tested reducing agent, as previously discussed.

The lack of significant interaction among RAs ($p > 0.10$) highlighted the lack of additive, synergistic, or antagonistic effects among them on their reducing capacity. In addition, it is possible that individually tested RAs have similar reducing pathways. Data from the Box–Behnken design analysis and models were analysed through the lack-of-fit test and the coefficient of determination, R^2. The R^2 of Log_{10} N for *L. acidophilus* was 0.63, whereas for *L. casei,* it was 0.74; models with R^2 value of ≈0.6 can be considered as valid models (Gong, Zhang, Xu, Wei, & Lee, 2007). For both models, the lack-of-fit test was significant ($p > 0.25$) demonstrating the adequacy of the reduced models to predict the response.

Previous studies that used cysteine or ascorbic acid as RAs to improve *L. acidophilus* viability in yoghurt during storage demonstrated high *L. acidophilus* survival (≈1 log_{10} cycle higher than control yoghurts) after 35 days (Dave & Shah, 1997a,b). In our case, similar results were observed; fermented milks without any reducing agent had 6.77 log_{10} cfu mL^{-1}, which was lower than 96% of the counts observed for the Box–Behnken design obtained responses. Rodrigues et al. (2011) tested the protective effect of cysteine during the storage of microencapsulated probiotics (*L. acidophilus* and *L. paracasei*) to improve their viability and observed that cysteine had different protective effects that depend on the assessed lactobacilli. In their case, cysteine enhanced the viability of *L. acidophilus* which was more sensible to oxygen than *L. paracasei*; however, the viability of *L. paracasei* did not improve with cysteine even after 180 days of storage at 5 °C or 22 °C (Rodrigues et al., 2011). Ying et al. (2011) studied the antioxidant effect of sodium ascorbate and tocopherol (alone or combined) on microencapsulated *L. rhamnosus* viability during their storage; they concluded that only tocopherol alone enhanced the viability of *L. rhamnosus,* whereas sodium ascorbate or the combination of sodium ascorbate and tocopherol had detrimental effects on probiotic survival. RAs may improve the viability of *Lactobacillus*

Table 2
Box-Behnken response surface design and dependent variables response values obtained for *Lactobacillus* strains studied.[a]

Run	C (mg L^{-1})	A (mg L^{-1})	T (mg L^{-1})	*L. acidophilus*			*L. casei*			*L. plantarum*		
				Log_{10} N	Eh_7	pH	Log_{10} N	Eh_7	pH	Log_{10} N	Eh_7	pH
1	250	0	7.5	7.60	22	3.91	7.70	29	3.65	7.79	15	4.22
2	125	125	7.5	7.61	−26	3.64	7.44	39	3.64	7.47	20	4.21
3	125	125	7.5	7.30	110	4.12	7.47	63	3.63	7.72	39	4.12
4	125	125	7.5	7.67	−33	3.62	7.61	65	3.63	7.55	56	4.10
5	250	125	15.0	7.57	42	3.93	6.63	−6	3.85	7.50	56	4.12
6	125	0	0.0	7.20	96	4.05	7.51	54	3.64	7.59	58	4.09
7	250	250	7.5	7.65	47	3.86	7.03	25	3.59	7.51	53	4.24
8	125	125	7.5	7.60	29	3.92	7.55	42	3.55	7.93	45	4.16
9	125	250	15.0	7.57	−61	3.91	7.14	65	3.56	7.85	57	4.23
10	250	125	0.0	7.46	29	3.94	6.56	16	3.58	7.77	94	4.20
11	125	0	15.0	6.65	−3	3.67	7.66	3	3.68	7.34	46	4.20
12	125	250	0.0	7.64	−78	3.60	7.17	58	3.65	7.69	70	4.21
13	0	250	7.5	7.66	−69	3.83	7.46	49	3.79	7.65	29	4.21
14	0	0	7.5	7.60	72	4.13	7.51	58	3.76	7.75	26	4.18
15	125	125	7.5	7.45	26	3.90	7.13	34	3.61	7.65	107	4.21
16	0	125	15.0	7.53	106	4.00	7.48	15	3.78	7.59	60	4.15
17	0	125	0.0	7.67	−66	3.60	7.57	45	3.68	7.76	45	4.15

[a] Abbreviations are: C, L-cysteine; A, ascorbic acid; T, tocopherols; Log_{10} N, *Lactobacillus* viability (cfu mL^{-1}); Eh_7, redox potential (mV).

Table 3
Regression coefficients and associated probability (p) of selected responses obtained for *Lactobacillus* studied.[a]

Term	*L. acidophilus*						*L. casei*					
	Log_{10} N		pH		Eh_7		Log_{10} N		pH		Eh_7	
	Coefficient	p	Coefficient	p	Coefficient	p	Coefficient	p	Coefficient	p	Coefficient	p
Constant	7.486	0.000	3.86	0.000	14.3	0.307	7.433	0.000	3.62	0.000	52.63	0.000
X_C	−0.021	0.773	–	–	–	–	−0.263	0.013	−0.042	0.090	−12.88	0.028
X_A	0.184	0.025	−0.070	0.230	−43.5	0.041	−0.195	0.051	–	–	6.62	0.217
X_T	−0.082	0.270	0.039	0.492	–	–	0.012	0.897	0.040	0.109	−12.00	0.038
$X_C{}^*X_C$	0.1919	0.075	–	–	–	–	–	–	0.089	0.015	−17.42	0.031
$X_A{}^*X_A$	–	–	–	–	–	–	–	–	–	–	–	–
$X_T{}^*X_T$	−0.170	0.109	–	–	–	–	−0.216	0.107	–	–	−12.67	0.097
$X_C{}^*X_A$	–	–	–	–	–	–	–	–	–	–	–	–
$X_C{}^*X_T$	–	–	–	–	–	–	–	–	–	–	–	–
$X_A{}^*X_T$	–	–	0.171	0.049	–	–	–	–	–	–	14.50	0.069

[a] Abbreviations are: C, L-cysteine; A, ascorbic acid; T, tocopherols; Log_{10} N, *Lactobacillus* viability (cfu mL^{-1}); Eh_7, redox potential (mV).

during their storage in different formulated preparations; however, each species and strain respond differently.

For milk pH response when utilising *L. acidophilus* only, the interaction between ascorbic acid and tocopherols was significant, whereas in the case of *L. casei,* cysteine, tocopherol, and the interaction cysteine–cysteine were significant. The R^2 for the pH model when utilising *L. acidophilus* was 0.64, and for *L. casei*, it was 0.72, being the lack-of-fit test non-significant ($p > 0.25$) in both cases. Neither milk pH nor E_{h7} values when utilising *L. plantarum* were affected by tested RAs after 45 days of storage. The redox potential of milk was influenced by cysteine, tocopherols, and the interactions cysteine–cysteine, tocopherols–tocopherols, and ascorbic acid–tocopherols in the case of *L. acidophilus*; R^2 was 0.74. When utilising *L. casei*, the E_{h7} of milk was significantly influenced only by ascorbic acid ($R^2 = 0.65$).

As previously mentioned, tested RAs had no synergistic or antagonistic effects among them. Dave and Shah (1997a,b) reported decline in pH after 35 days of storage when cysteine (250 or 500 mg L^{-1}) was added to yoghurt (their starter culture included *L. acidophilus*), whereas no changes in pH were observed in yoghurts fermented and stored (35 days) with ascorbic acid (50, 150, or 250 mg L^{-1}). Redox potential had an irregular behaviour when cysteine (250 mg L^{-1}) was supplemented to yoghurts prepared with four different starter cultures (that included *L. acidophilus*) and stored for 35 days; values oscillated between 20 and 80 mV (Dave & Shah, 1997b); values observed in our case were similar to those previously reported, when only L-cysteine was supplemented. To our knowledge, no previous reports about fermented milks or yoghurts formulated with *L. casei* and tested RAs, nor their effect on their pH or E_{h7}, had been published.

3.4. Optimisation

In our case, the goal chosen was to maximise the response of *Lactobacillus* viability (Log_{10} N cfu mL^{-1}), to determine the RAs combination and levels that improved the viabilities of tested *L. acidophilus* and *L. casei* at the end of the storage (45 days). Fig. 8 shows the optimum levels of the RAs to achieve the maximum

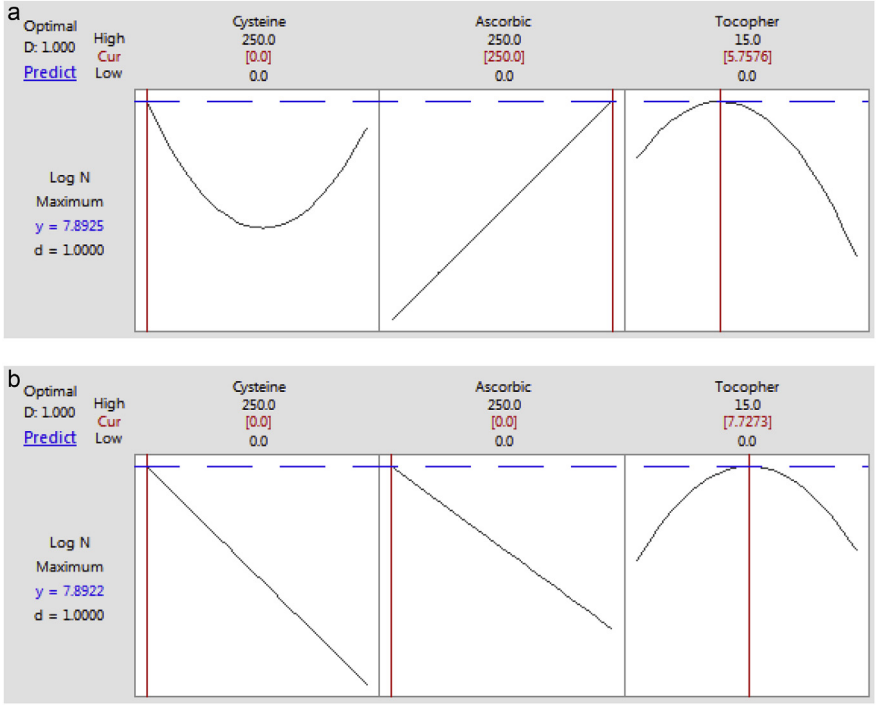

Fig. 8. Optimum levels of tested reducing agents (L-cysteine, ascorbic acid, and tocopherols) to achieve the maximum counts of *Lactobacillus acidophilus* (a) or *Lactobacillus casei* (b).

counts of *L. acidophilus* and *L. casei*. Optimum levels for maximum counts or *L. acidophilus* were 250 mg ascorbic acid L^{-1} and 5.75 mg tocopherols L^{-1}. Surprisingly the optimum level for cysteine was 0 mg mL^{-1}; this contrasted with previous reports that suggest cysteine as a protective or reducing agent that enhances probiotic or lactobacilli survival in yoghurts (Dave & Shah, 1997b) and when encapsulated (Rodrigues et al., 2011; Sousa et al., 2012). Differences between studied strain and those tested in previous reports could be the reason for the unfavourable results in our case. The optimum combination for *L. casei* was to add only 7.72 mg tocopherols L^{-1} without cysteine and ascorbic acid (Fig. 8); this result is in accordance with a previous report, which mentioned unfavourable counts of encapsulated *L. casei* when utilising cysteine (Sousa et al., 2012).

Values of milk pH and E_{h7} were predicted with the optimum levels of the RAs for maximum viability (7.89 $\log_{10}$ cfu mL^{-1}) of studied lactobacilli. Predicted pH for milk when utilising *L. acidophilus* (as well as 250 mg ascorbic acid L^{-1} and 5.75 mg tocopherols L^{-1}) was 3.74 and its predicted E_{h7} was −29 mV. *L. acidophilus* is an oxygen-sensitive bacterium, which prefers a reduced environment to be maintained alive, as previous studies reported it (Dave & Shah, 1997a,b). Predicted milk pH and E_{h7} when utilising *L. casei* (and 7.72 mg tocopherols L^{-1}) were 3.75 and 41 mV, respectively. *L. casei* is an aero-tolerant bacterium, which can survive well in low-oxidant environments.

4. Conclusions

RAs tested had a different effect on the growth of studied *Lactobacillus* during milk fermentation; ascorbic acid and tocopherols reduced fermentation times without important increments in cell counts, whereas L-cysteine and tocopherols enhanced viable counts ($\geq$1 $\log_{10}$ cfu mL^{-1}) instead of diminishing fermentation times. These changes on fermentation time or viability are related usually with E_{h7}, as can be seen in this study. It is very important because if a manufacturer prefers short times to produce a fermented dairy product, they could supplement with ascorbic acid or tocopherols for *L. casei* and *L. plantarum*. However, if viable counts are to be enhanced (a product design as a probiotic), the utilisation of L-cysteine or tocopherols on products containing *L. acidophilus* improves it.

Box–Behnken design allowed the generation of models that adequately described *Lactobacillus* viability and milk pH and E_{h7}, as well as to find optimum levels of tested RAs to maximise *Lactobacillus* viability at the end of studied storage (45 days) for *L. acidophilus* and *L. casei*; 250 mg ascorbic acid L^{-1} and 5.75 mg tocopherols L^{-1} were the optimum levels for *L. acidophilus*, whereas for *L. casei,* 7.72 mg tocopherols L^{-1} was enough to reach maximum viability. RAs tested had different effects under growth conditions (or at the end of storage); furthermore, these effects depended on *Lactobacillus* species. Hence, of the *Lactobacillus* species used in this study, *L. acidophilus* was the most sensitive to oxidising conditions; thus, RAs improved its viability. *L. casei* was aero-tolerant because the middle, tested concentration of tocopherol enhanced its viability. In addition, the viability of *L. plantarum* was not increased by the RAs tested; then, the studied reducing conditions did not affect it; or it may have effective enzymatic or non-enzymatic systems to eliminate ROS. Finally, the redox potential may not only result in better quality of the dairy product but also may contribute to improving viability.

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