

## Full Length Research Paper

# Effect of L-cysteine and ascorbic acid on the propagation of *Lactobacillus casei* in milk

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### Abstract

Today's consumers can select functional foods from a wide variety of these products with components such as proteins, carotenes, dietary fiber or microorganisms known as "probiotics", among which are those belonging to the *Lactobacillus* and *Bifidobacterium* genera. This work evaluates the effect of the addition of ascorbic acid and L-cysteine as redox potential reducing agents (Eh) on the propagation of a probiotic microorganism, *Lactobacillus casei*, in milk. The reducing agents are added to the milk individually or combined in concentrations of 0, 100 and 200 ppm, in order to obtain the combination that would allow the maximum growth of the probiotic culture. The different combinations of ascorbic acid and L-cysteine were evaluated on the number of colony forming units of *L. casei* before and after 5 days of incubation in milk. The propagation of the microorganism was analyzed by the count method on MRS agar plates incubated anaerobically at  $35 \pm 1^\circ\text{C}$  for 72 hours. The combination of the higher concentrations of ascorbic acid (200 ppm) and L-cysteine (100 or 200 ppm) gave the maximum count of *L. casei*, with a population higher than  $10^{10}$  CFU/mL. These additives proved to be effective in the multiplication of probiotic microorganisms to generate new healthy products in the food industry.

**Keywords:** ascorbic acid, *L. casei*, L-cysteine, probiotics, reducing agents.

## INTRODUCTION

In recent years, interest in probiotics among researchers and consumers alike has been growing. Probiotics are microorganisms which, when administered in appropriate amounts, travel through the gastrointestinal tract and bring health benefits to consumers (Williams, 2010). The species most used as probiotics are *L. acidophilus* and species belong to the *L. casei* group (*L. casei*, *L. paracasei* and *L. rhamnosus*) (Curry and Crow, 2003b, Gopal, 2003).

Probiotic bacteria such as *L. acidophilus* and *Bifidobacterium spp.* are known for the important health benefits they provide, helping to maintain the intestinal flora, regulating the immune system, reducing the risk of cancer, preventing diarrhea in children and lowering cholesterol levels. In countries around the world there is a variety of products containing probiotic bacteria: capsules, tablets, powders, fermented foods and,

particularly, yoghurt (Williams, 2010). Live probiotic bacteria is one of the most important things the consumer looks for when purchasing a probiotic-enriched dairy product.

The greatest challenge associated with the incorporation of probiotic microorganisms in the development of dairy foods, is to retain their viability during processing and storage and while passing through the gastrointestinal system. For these microorganisms to be viable when they reach the intestine, a sufficient number of the probiotic bacteria must survive in the product, so that it contains  $10^6$  CFU/mL when consumed (Blanchette *et al.*, 1996). It is well known that a probiotic bacterium cannot work its beneficial effects on the organism unless in very high concentrations, for which there are no exact figures (Charteris *et al.* 1998).

In general, a concentration of  $10^7$  CFU per g or mL of

product at the time of ingestion is accepted as the minimum probiotic population needed to produce a positive effect on health. Similarly, ingesting  $10^8$ - $10^9$  CFU a day has been indicated as the minimum therapeutic dose, which would be achieved by consuming 100 g or mL of product (De Vuyst, 2000, Salminen and Ouwehand, 2003), however, these figures should not be taken as absolute values since much depends on the food in which the bacteria were ingested; the food itself may exhibit a protective action, and on the strain used, which may have a different sensitivity to biological barriers (Stanton *et al.* 2001).

Some environmental conditions enhance the viability of microorganisms. Talwalkar and Kailasapathy (2004) mention, for example, that hydrogen peroxide production and acidity are factors that improve the survival rate of *L. acidophilus* and *Bifidobacterium* in yoghurt. The reduction of oxygen is fundamental to the survival of these microorganisms in fermented dairy products.

Talwalkar & Kailasapathy, 2004, have reported that probiotic bacteria such as *L. acidophilus* and *Bifidobacterium spp.* are unable to synthesize enzymes such as cytochrome and other heme enzymes that are important for electron transport and, therefore, cannot synthesize ATP via the respiratory chain, using instead the fermentative pathway in their metabolism. In the case of anaerobic microorganisms, the organic substrate undergoes a series of redox reactions through pyridine nucleotides such as NADH.

Some authors (Dave and Shah, 1997 a, b, Shishata and Shah, 1999; Kim, *et al.*, 2002) have reported on the importance of the change of redox potential which, at higher concentrations, inhibits the growth of anaerobic bacteria due to the oxygen dissolved in the medium. The redox potential is defined as the measure of the tendency of a substrate to lose or gain electrons. When an element loses electrons, it is said to have been oxidized, and when it gains electrons, reduced; the more oxidized a substance is, the more positive its redox potential (Eh) and vice versa for reduced substances (Díaz *et al.*, 2002). Milk products contain important scavenger compounds, one of which is *L*-cysteine, which acts as an oxygen scavenger, keeping the redox potential low and increasing the viability of the probiotic bacteria. Similarly, it has been reported that the presence of ascorbic acid and *L*-cysteine have a synergic effect on the reduction of redox potential and therefore increase the viability of the probiotic bacteria.

Due to the above, the food and the dairy industry in particular have explored different procedures to help probiotics reach their sites of action while still viable and in sufficient quantities, using yoghurt and fermented milks as the delivery vehicles of probiotic bacteria (Dave and Shah, 1997c, Nighswonger *et al.* 1996, De Vuyst, 2000, Heller, 2001, Coeuret *et al.* 2004, Awaishah *et al.* 2005).

Many *Lactobacillus* can grow reasonably well in milk reaching maximum concentrations of around  $10^8$ - $10^9$

CFU/ mL after 24 h of incubation at 37°C (Stanton *et al.* 2003). This optimum cell development has been attributed to the ability of the said bacteria to degrade casein thanks to its complex proteolytic system, although this activity depends on the species and the strain (Grippon, 1994, Vassal, 1996).

The spread and survival of lactic acid bacteria can be enhanced by using reducing compounds that generate negative Eh values and are associated with reducing conditions. The aim of this work was to evaluate the effect of two reducing agents, ascorbic acid and *L*-cysteine, both individually and combined, in the propagation of probiotic *Lactobacillus casei* in milk.

## MATERIALS AND METHODS

### Raw material

We used whole, ultra-pasteurized milk of the "Lala" brand (Lala, S.A de C.V. México) bought from self-service stores in the city of Puebla and reagent-grade ascorbic acid and *L*-cysteine (Sigma Aldrich Chemicals, U.S.A.). *Lactobacillus casei* was isolated from the Yakult (Mexico) brand fermented milk product. 100 mL was centrifuged at 13,000 rpm for 10 minutes. A cell count was made of the *L. casei* in the sediment obtaining a population of approximately  $10^{10}$  CFU/mL. The cell suspension was used to inoculate the study systems.

## METHODOLOGY

### Effect of reducing agents on the propagation of *Lactobacillus casei*

Different concentrations of ascorbic acid and *L*-cysteine were used as reducing agents to enhance the viability of probiotic bacteria. The concentrations studied are shown in Table 1. Three repetitions were made of each treatment.

An aliquot of the *L. casei* cell suspension was inoculated into an Erlenmeyer flask containing 100 mL of the milk to reach a concentration in each of the systems of  $10^6$  CFU/mL, and the *L*-cysteine and ascorbic acid was then added.

Once the systems were inoculated, they were kept in an incubator (Imperial Lab Line I, U.S.A.) at  $35 \pm 1^\circ\text{C}$  to encourage anaerobiosis. A count of the microorganisms in each of the systems was made at 0 and 5 days of incubation.

In order to perform the counts, consecutive dilutions were prepared from  $10^{-1}$  to

$10^{-6}$  by placing 9 mL of sterile isotonic saline solution and 1 mL of the milk prepared with the culture previously incubated at  $35 \pm 1^\circ\text{C}$ , which contained an approximate population of  $10^6$  CFU/mL. The mixture was placed in 13

**Table 1.** Propose systems for the ascorbic acid and *L-cysteine* addition

System	Ascorbic Acid (ppm)	<i>L-Cysteine</i> (ppm)
1 (control)	0	0
2	0	100
3	0	200
4	100	0
5	100	100
6	100	200
7	200	0
8	200	100
9	200	200

x100 mm glass tubes. One mL of each of the prepared serial dilutions was placed in a Petri dish, seeding in triplicate, by the pour-plate method onto Mann Rogosa Sharp (MRS) agar (Difco, MI, U.S.A.) and incubated anaerobically for 72 h at  $35 \pm 1^\circ\text{C}$  (Song *et al.*, 2003). After the incubation period, the procedure was repeated for the prepared systems at day 5 of incubation. An incubation period of 5 days was chosen because studies conducted by Cerón (2008) and Blanchette *et al.* (1996) showed that this time frame ensured good viability of the probiotics. The counts were made with the help of a Quebec colony counter and the results of the counts were multiplied by the inverse of the dilution factor.

### Statistical analysis

A variance analysis was made for the treatments at the start ( $t = 0$ ) and at 5 days to evaluate the effect of the reducing agents and their combinations. A Tukey multiple comparison test with 95% confidence interval was performed to determine which of the treatments were significantly different. The statistics program used was the Minitab version 15 (licensed to BUAP).

## RESULTS AND DISCUSSION

The addition of growth-promoting substances such as *L-cysteine* and ascorbic acid, used in this investigation, is another strategy for increasing the development and survival of probiotic bacteria in the products into which they are incorporated, above all because of the weak growth of some of them in milk (Lourens-Hattingh and Viljoen, 2001).

The results of the microbial counts for the systems formulated with different concentrations of *L-cysteine* and ascorbic acid are shown in Table 2. In all cases, the number of viable cells increased, although not in the same proportion. Similarly, adding the reducing agents individually or in combination produced greater propagation of the microorganism, above all in the latter case (Table 2).

For this reason, the outcome of adding *L-cysteine* individually in this study (Table 2) showed an important effect on the propagation of *L-casei*, most notably when 200 ppm of the amino acid were added. Significant differences are appreciated in higher concentrations of the amino acid in comparison to the ascorbic acid. These differences suggest *L-cysteine* has a reducing power for the development of *L. casei* since it promotes a decrease in the amount of oxygen in the medium, having the same effect when used in combination with ascorbic acid or individually.

Following the 5-day incubation period, there was an increase in the number of *L. casei* cells, as shown in Table 2, compared with the control (0 ppm ascorbic acid, 0 ppm *L-cysteine*). These results match those reported by Cerón, 2008, who determined the viability of *L. casei* in milk, and those of Blanchette *et al.* (1996) who determined a good viability of *B. infantis* for 5 days, which was negligible at 28 days of fermentation.

A similar result was reported by Dave and Shah (1997c), and by Talwalkar and Kailasapathy (2004) who showed that the addition of ascorbic acid to probiotic yoghurt helped the survival of lactic acid bacteria and attributed the beneficial effects to the reduction in dissolved oxygen content and the decreased redox potential. These authors suggest using 250 ppm of ascorbic acid in probiotic yoghurt to maintain low levels of redox potential during at least 20 days of storage and ensure the survival of *L. acidophilus*.

This result can be compared to that obtained by Dave and Shah (1997b), who showed that concentrations of 250 and 500 ppm of *L-cysteine* enhanced the growth of *L. acidophilus*, in contrast to those systems to which lower (50 ppm) or zero concentrations of the amino acid were added.

The same observation has been made by other investigators. Kim *et al.* (2002), reported similar results to those obtained in this study, adding *L-cysteine* as a redox potential reducing agent to four different brands of commercial yoghurt, and evaluated the survival of *L. casei* as a probiotic. These authors observed that the medium produced the optimum environment for the growth and development of this kind of microorganism,

**Table 2.** Average Log CFU/mL of *L. casei* in milk with different concentrations of *L*-cysteine and ascorbic acid

<i>L</i> -cysteine	Ascorbic Acid	Day 0 (average CFU/mL)*	Day 5 (average CFU/mL)*
n=3	n=3		
0	0	6.11 ± 0.78 <sup>a</sup>	8.14 ± 0.91 <sup>a</sup>
0	100	6.23 ± 0.79 <sup>a</sup>	8.19 ± 0.91 <sup>a</sup>
0	200	6.20 ± 0.79 <sup>a</sup>	8.19 ± 0.91 <sup>a</sup>
100	0	6.23 ± 0.79 <sup>a</sup>	8.19 ± 0.91 <sup>a</sup>
100	100	6.14 ± 0.79 <sup>a</sup>	8.19 ± 0.91 <sup>a</sup>
100	200	6.20 ± 0.79 <sup>a</sup>	8.25 ± 0.92 <sup>a</sup>
200	0	6.14 ± 0.78 <sup>a</sup>	9.20 ± 0.96 <sup>a,b</sup>
200	100	6.20 ± 0.79 <sup>a</sup>	10.20 ± 1.01 <sup>c</sup>
200	200	6.23 ± 0.79 <sup>a</sup>	10.18 ± 1.01 <sup>c</sup>

<sup>a</sup> Averages with different letters are significant (p<0.05)

\*Log of CFU/mL

when the yoghurts were kept in refrigerated storage for 10 days.

In another similar study conducted by Dave and Shah (1998), the addition of cysteine, concentrated serum proteins, casein hydrolyzates and tryptone were effective in enhancing the viability of bifidobacteria in yoghurt also containing *L. acidophilus* and *S. thermophilus*, while the viability of *L. acidophilus* was only increased by the addition of cysteine.

In addition, *L*-cysteine is an amino acid containing sulfur, which administers amino nitrogen as a growth factor for lactic acid bacteria and at the same time reduces the redox potential favoring the growth of these anaerobic bacteria (Dave and Shah, 1997b). Talwalkar and Kailasapathy (2004), mention that a property of ascorbic acid and *L*-cysteine is that they act as oxygen scavengers and maintain a low redox potential so that appropriate conditions are provided for the viability of the probiotic bacteria.

Along the same lines, one of the compounds that acts as an oxygen scavenger is ascorbic acid, reducing the redox potential and increasing the propagation of lactic acid bacteria, such as in the case of *L. casei* (Brunner *et al.*, 1993 a, b). Talwalkar and Kailasapathy, in a 2004 study, considered oxygen toxicity to be responsible for the cell death of lactic bacteria.

These authors report that ascorbic acid also reduces redox potential, observing that in many cases it is most effective when used in combination with *L*-cysteine.

Furthermore, a pH reduction in the milk was observed from 6.7 to 4.0-4.7, due to the production of lactic acid during fermentation, and in some cases the acidification increases while the product is in storage since *L. casei* is a homofermentative bacterium that produces the said acid. The acidity of these products is one of the most influential factors in maintaining the viability of probiotic bacteria (Heller, 2001, Lourens-Hattingh and Viljoen, 2001).

Donkor *et al.* (2006) have observed a greater survival rate of *L. acidophilus* L10 and *L. paracasei* L26 in the

acid conditions of yoghurt, and attribute this quality to the susceptibility of the microorganisms to organic acids and the pH reduction during storage of the product. However, resistance to the acid medium is strain-dependent (Shah, 2000).

In another study, Schillinger *et al.* (2005) observed no reduction in the population of *L. rhamnosus* GG and two strains of the *L. casei* group when exposed to a simulated gastric fluid (pH 2 and pepsin) with the addition of milk, while the same strains had shown an almost total loss of viability in the same experiment, but without the addition of milk. Likewise, Vinderola *et al.* (2000b) showed that the viability of different probiotic bacteria was better sustained in an acid medium (pH 2 and 3) when incorporated into a cheese homogenate than when used in lyophilized form.

Moreover, Gardiner *et al.* (1999b) also found a protective effect of yoghurt on the viability of a probiotic strain of *Enterococcus* in gastric fluid at pH 2.0, which increased to 3.65 after the addition of the food. These authors suggest that this buffer effect was not solely responsible for the protection, but that other factors also contributed, such as the presence of extracellular polysaccharides, due to a greater reduction in viability being observed when the microorganisms were directly exposed to a gastric fluid at pH 3.65.

In another study, Shishata and Shah (1999), demonstrated that reducing the pH in ultra-pasteurized milk to values around 4.6 (optimal pH for the growth of lactic acid bacteria), produced the propagation of *L. acidophilus*.

Furthermore, Shah (2000) proved that when the pH of a real system (yoghurt) drops to values less than or close to 3.0, the propagation of *L. casei* is reduced due to the high concentration of lactic acid. This effect was corroborated in this study, which used milk as the model system. However, adding *L*-cysteine to reduce the redox potential increased the survival rate of the probiotic, reaffirming that the amino acid is a powerful reducing agent, enhancing conditions for the removal of dissolved

oxygen, which favors the development of *L. casei*. It is also known that lactic acid bacteria (LAB) can produce sulfur-containing compounds that are responsible for the distinctive aroma of cheese, due to the contribution of SH groups and the redox potential, which gives flavor to dairy products (Brasca *et al.*, 2007).

There are reports on the effect of adding different growth promoting substances to milk in combination with proteins, enzymes and other substances, however, the majority of the works published do not give an individual evaluation or in combination with ascorbic acid and L-cysteine, the central theme of this investigation.

There is insufficient data in the bibliography on the interactions of these two reducing agents and their effect on probiotic bacterial growth, which is important to know for maintaining probiotic viability in milk.

In the context presented here, the development of milk with probiotic bacteria and in combination with reducing substances provides an excellent opportunity to launch a new functional food.

In recent years in Mexico, as in the rest of the world, a large number of yoghurts and milks with added probiotic bacteria have appeared on the market, due to the relative novelty of this type of food. The results obtained in this study provide the dairy industry with an opportunity for economic spread, as well as to maintain the viability and probiotic characteristics of the products into which they are introduced throughout the preparation process and storage, when the microorganisms may be subject to different stress conditions. Today it is well known that maintaining a healthy life requires a good diet containing nutritious food, such as that developed in this investigation.

## CONCLUSIONS

This study showed that the individual use of the reducing agents L-cysteine and ascorbic acid is effective for the propagation of *L. casei*. The reduction of the redox potential, adding ascorbic acid and L-cysteine could be used to select, adapt and propagate the *L. casei* strain for use in fermented milk products. This work demonstrated that the use of reducing agents increases the viability of *L. casei* in milk products since, when used individually or in combination, they remove oxygen from the medium reducing the redox potential (Eh).

For future works the authors recommend the analysis of the effect of reducing the redox potential on the pH and acidity of fermented milk products with added probiotics.

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