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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 Bifidobacteri umgenera. 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 were added to 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 milk incubation. The propagation of the microorganism was analyzed by counting colonies on Mann Rogosa Sharp (MRS) agar plates incubated anaerobically at 35 ± 1°C for 72 h. The comb ination of the higher concentration 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 the probiotic bacteria to generate new healthy products in the food industry.

Key words: ascorbic acid, *Lactobacillus casei*, L-cysteine, probiotics, reducing agents.

INTRODUCTION

In recent years, interest in probiotics among researchers and consumers has been growing. Probiotics are microorganisms which, when administered in appropriate amounts, travel through the gastrointestinal tract and bring health benefits to consumers[1,2].The species most used as probiotics are *L. acidophilus* and species belong to the *L. Casei* group *(L. casei*, *L. paracasei*and *L. rhamnosus)* [2-4].

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 [2].In several countries around the world there is a variety of products containing probiotic bacteria: capsules, tablets, powders, fermented foods and, particularly,

yoghurt[1,5,6,7].Live probiotic bacteria is one of the most important characteristic that consumers 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 tract. 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 must contain at least 10^6 CFU/mL when consumed[8].It is well known that probiotic bacteria cannot exert its beneficial effects on the organism unless it is consumed in very high concentrations, therefore there are not recommend exact numbers[9].In general, a concentration of 10^7CFUper 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 10^8-10^9CFU per day has been indicated as the minimum therapeutic dose, which would be achieved by consuming 100 g or mL of product[10,11,12];however, these figures should not be taken as absolute values since depends on the food in which the bacteria were ingested; the food itself may exhibit a protective action, and also depends on the strain used, which may have a different sensitivity to the biological barriers[13].

Some environmental conditions enhance the viability of microorganisms. Talwalkar and Kailasapathy (2004) [14]mention, for example, that hydrogen peroxide production and acidity are factors that improve the survival rate of *L. acidophilus* and bifidobacteriain yogurt. The reduction of oxygen is fundamental to the survival of these microorganisms in fermented dairy products.

Talwalkar and Kailasapathy (2004) [14] 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 reactionsthrough pyridine nucleotides such as NADH.

Some authors [15-18],have reported the importance of the change of redox potential which, at higher values, inhibits the growth of anaerobic bacteria due to the dissolved oxygen 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 [19]. 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 has a synergic effect on the reduction of redox potential and therefore increase the viability of probiotic bacteria.

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 yogurt and fermented milks as the delivery vehicles of probiotic bacteria [20-24].

Many lactobacilli can grow reasonably well in milk reaching maximum concentrations of around 10^8-10^9CFU/mL after 24 h of incubation at 37ºC [25]. This optimum cell development has been attributed to the ability of the bacteria to degrade casein thanks to its complex proteolytic system, although this activity depends on the species and the strain[26,27].

The growth and survival of lactic acid bacteria can be enhanced by using reducing compounds that generate negative Eh values. 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 (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 provided by the Food Microbiology Laboratory from Universidad de las Américas Puebla. A loopful of the strain was inoculated in100 mL of Mann Rogosa Sharp (MRS) broth (Difco, MI, U.S.A.), incubated 18 h at 35°C, centrifuged at 13,000 rpm for 10 minutes and re-suspended in 1 mL of milk. Viable cell count was of approximately 10^{10} CFU/mL. The cell suspension was used to inoculate the experimental systems.

Methodology

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

Different concentrations of ascorbic acid and *L*-cysteinewere 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 every systems of approximately 10^6CFU/mL, and then *L*-cysteineand/or ascorbic acid were added.

Once the systems were inoculated, they were kept in an incubator (Imperial Lab Line I, U.S.A.) at 35 ± 1°C in an aerobiosis. A count of the microorganisms in each of the systems was made after 0 and 5 days of incubation.

In order to perform the viable *L. casei* counts, 1 mL of the inoculated milk was consecutive diluted in sterile isotonic saline solution, platted on MRS agar (Difco, MI, U.S.A.), and incubated anaerobically for 72 h at 35 ± 1°C [28]. 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) [29] and Blanchette *et al*. (1996) [8]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 were expressed as CFU/ml.

Statistical analysis

Analysis variance was made, in Minitab v. 15.0, between the viable *L. casei* counts obtained treatments at time zero and after 5 days to evaluate the effect of the reducing agents and their combinations. A Tukey multiple comparison test with a 95% confidence interval was performed to determine which of the treatments were significantly different.

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, due to the weak growth of some of them in milk[30].

The results of the microbial counts for the systems formulated with different concentrations of *L*-cysteine and ascorbic acid are shown in Table 1. 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 (Table 1).

Table 1 Average Log CFU/mL of *Lactobacillus casei* in milk with different concentrations of *L*-cysteine and/or ascorbic acid

System	Ascorbic Acid (ppm)	*L-Cysteine* (ppm)	Day 0 (Log of CFU/mL)	Day 5 (Log of CFU/mL)
1 (control)	0	0	6.11 ± 0.78 [a]	8.14 ± 0.91 [a]
2	0	100	6.23 ± 0.79 [a]	8.19 ± 0.91 [a]
3	0	200	6.20 ± 0.79 [a]	8.19 ± 0.91 [a]
4	100	0	6.23 ± 0.79 [a]	8.19± 0.91 [a]
5	100	100	6.14 ± 0.79 [a]	8.19± 0.91 [a]

The effect of adding *L*-cysteine individually in this study (Table 1) showed an important effect on the propagation of *L. casei*, most notably when 200 ppm of the amino acid were added. Significant differences are appreciatedin higher concentrations of the amino acid in comparison to use of ascorbic acid. These differences suggest that *L*-cysteine facilitates 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.

After the 5-day incubation period, there was an increase in the number of *L. casei* cells, as shown in Table 1, compared with the control (0 ppm ascorbic acid, 0 ppm*L*- cysteine). These results are similar to those reported by

Cerón, 2008 [29], who determined the viability of *L. casei* in milk, and those of Blanchette *et al*. (1996) [8]who determined the viability of *B. infantis*and found acceptable counts after 5 days, but negligible counts after 28 days of storage. A similar result was reported by Dave and Shah (1997c) [20], and by Talwalkar and Kailasapathy (2004) [14]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 of dissolved oxygen content and to 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*.

Our results can be compared to those obtained by Dave and Shah (1997b) [16],who showed that concentrations of 250 and 500 ppm of *L*-cysteine enhanced the growth of *L. acidophilus*,in contrast to those systems with lower (50 ppm) or zero concentrations of the amino acid. The same observation has been made by other researchers. Kim *et al*. (2002) [18] reported similar results to those obtained in this study, adding *L*- cysteine as a redox potential reducing agentto 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 microorganism, when the yoghurts were kept in refrigerated storage for 10 days. In another similar study conducted by Dave and Shah (1998) [31], the addition of cysteine, concentrated serum proteins, casein hydrolyzates and tryptone were effective in enhancing the viability of bifidobacteria in yoghurt containing also *L. acidophilus* and *S. thermophilus*, while the viability of *L. acidophilus*was only increased by the addition of cysteine.

In addition, *L*-cysteineis an amino acid containing sulfur, whichadministers 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 [16]. Talwalkar and Kailasapathy (2004) [14], mention that a property of ascorbic acid and *L*-cysteineis 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* [32]. Talwalkar and Kailasapathy (2004) [14]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 along incubation time storage, probably due to *L. casei*as a homofermentative bacterium produces lactic acid. The acidity of these products is one of the most influential factors in maintaining the viability of probiotic bacteria[22, 30].

Donkor*et al.* (2006) [33]have observed a greater survival rate of *L. acidophilus L10* and *L. paracasei L26*in the acid conditions of yogurt, and attribute this quality to the susceptibility of the microorganisms to organic acids and the pH reduction during product storage. However, resistance to the acid medium is strain-dependent[34].

In another study, Schillinger *et al*. (2005) [35]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*.[37] 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. Gardiner *et al*. (1999) [36]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) [17], 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) [34]proved that when the pH of a real system (yogurt) 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*-cysteineto reduce the redox potential increased the survival 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 [38].

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. 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 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 yogurts 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.

Table1Average Log CFU/mL of *Lactobacilluscasei* in milk with different concentrations of *L*-cysteine and/or ascorbic acid

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3	0	200	6.20 ± 0.79 [a]	8.19 ± 0.91 [a]
4	100	0	6.23 ± 0.79 [a]	8.19± 0.91 [a]
5	100	100	6.14 ± 0.79 [a]	8.19± 0.91 [a]
6	100	200	6.20 ± 0.79 [a]	8.25 ± 0.92[a]
7	200	0	6.14 ± 0.78 [a]	9.20 ± 0.96[a,b]
8	200	100	6.20 ± 0.79 [a]	10.20 ± 1.01[c]
9	200	200	6.23 ± 0.79 [a]	10.18 ± 1.01[c]

[a] *Averages with different letters in columns are significantly different (p<0.05)*

CONCLUSION

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. For future works we 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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