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Survival of *Brucella abortus* in milk fermented with a yoghurt starter culture

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ABSTRACT. In countries such as Mexico, brucellosis is still an important public health problem due to the consumption of non-pasteurized milk and dairy products, contaminated with *Brucella* spp. The aim of this study was to look into the survival of *Brucella abortus* during fermentation of milk with a yoghurt starter culture and storage at refrigeration temperature. Sterile skim milk was inoculated with *B. abortus* at two concentrations, 10^5 and 10^8 CFU/ml simultaneously with a yoghurt starter culture of lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subspecies *bulgaricus*). Inoculated flasks were incubated at 42°C, followed by refrigeration at 4°C. Samples were taken during fermentation and during storage and viable count of *B. abortus* and lactic acid bacteria and pH were determined. Results showed that after 10 days of storage at 4°C, *B. abortus* was recovered in fermented milk at a level of 10^5 CFU/ml, despite the low pH below 4.0. Therefore *B. abortus* is able to survive in fermented milk. This finding may imply that non-pasteurized fermented milk contaminated with *Brucella abortus* could be a means of transmission of these bacteria.

Key words: *Brucella* spp, *Brucella abortus*, fermented milk, survival, yoghurt.

INTRODUCTION

Brucellosis is a zoonotic disease of worldwide distribution that mainly affects people in contact with domestic animals and animal products. Although the disease is being controlled in many developed countries, it still remains endemic in many parts of the world, including Latin America, the Middle East, Spain, Africa, and western Asia.^{4,5,14,15,22,24} The disease is mainly transmitted to humans through the ingestion of raw milk or non-pasteurized cheese contaminated with one of the four *Brucella* species pathogenic to humans.^{6,19,20} Brucellosis in dairy cattle is very important in countries such as Mexico, where the incidence has fluctuated between 4-11% during the last years.¹¹⁻¹⁴

Local food habits, like the consumption of unpasteurized dairy products, have led to epidemic outbreaks of hu-

RESUMEN. En países como México la brucelosis representa un importante problema de Salud Pública debido principalmente al consumo de leche y productos lácteos no pasteurizados contaminados con *Brucella* spp. El objetivo de este estudio fue determinar la sobrevivencia de *Brucella abortus* en leche inoculada con un cultivo iniciador para elaborar yogur, fermentada a 42°C y posteriormente refrigerada. La leche descremada estéril se inoculó con el cultivo iniciador para elaborar yogur formado por *Streptococcus thermophilus* y *Lactobacillus delbrueckii* subespecie *bulgaricus*, simultáneamente con *B. abortus* a dos diferentes concentraciones, 10^5 and 10^8 UFC/ml. Se tomaron muestras durante la fermentación y durante el almacenamiento, en las cuales se determinó cuenta viable de *B. abortus*, así como de *S. thermophilus* y *L. delbrueckii*; también se determinó el pH. Los resultados mostraron que *B. abortus* fue capaz de sobrevivir hasta 10 días en leche fermentada y almacenada a 4°C, a pesar de que el pH fue de alrededor de 4.0. Por tanto, la leche fermentada no pasteurizada y contaminada con *Brucella abortus* puede ser vehículo para la transmisión de esta bacteria.

Palabras clave: *Brucella* spp, *Brucella abortus*, leche fermentada, sobrevivencia, yogur.

man illness.^{1-3,5,12,16,18,23} In 1997, dairy products accounted for 84% of the sources of infection of human brucellosis (40% milk, 40% cheese, 4% other dairy products, and 5% were occupational cases in Mexico.¹¹

The survival of *Brucella* in non fermented dairy products has been investigated by some authors, who have demonstrated that *Brucella* spp. was able to survive for periods as long as 18 months; however there is little information published about the survival of *B. abortus* in fermented milk food.^{7,10,21}

To understand the role of yoghurt as a vehicle for transmitting *B. abortus*, a research study was carried out on the behavior of this pathogen during sterilized skim milk fermentation with a yoghurt starter culture and subsequent storage at refrigeration temperature.

MATERIAL AND METHODS

Strains. *B. abortus* biovar 1 strain 544 ATCC 23448, NCTC 10093 was grown in sterilized skim milk at 12% (SSM, Difco) and incubated 48 hours at 37°C under a 6% CO₂ atmosphere. Culture counts were performed on Farrell's agar plates (Oxoid) using tryptose soy broth as diluent.

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Starter culture. A lyophilized yoghurt starter culture obtained in our laboratory from natural fermented dairy products, composed of a 1:1 mix of *Streptococcus salivarius* subsp. *thermophilus* and *Lactobacillus delbrueckii* subspecies *bulgaricus* was used. Working lactic cultures were prepared mixing 0.1 g of the starter culture and 10 ml of SSM and incubated 8 hours at 42°C. Therefore 2% (v/v) inoculum was mixed with 200 ml of SSM and incubated under the same conditions. This procedure was performed twice to obtain the activated working culture.

Microbiological assays. Assays were performed in duplicate with concentrations of 1×10^5 or 1×10^8 CFU of *B. abortus* per ml of milk. One set of five flasks containing 200 ml of SSM was used for each concentration of the pathogen. Three flasks were inoculated with a *B. abortus* suspension to obtain the desired concentration; two of these flasks were also inoculated with the yoghurt starter culture (10^6 CFU of lactic acid bacteria/ml of milk); the one that only contained *B. abortus* was considered the pathogen control. A fourth flask was inoculated only with the yoghurt starter culture (starter culture control) and the fifth one was not inoculated (negative control).

All flasks were incubated at 42°C for 8 h; thereafter they were stored at 4°C. Samples were taken at 2 h intervals during fermentation and every 2 days during storage; they were diluted in peptone water (Oxoid) and plated in duplicate on Lactobacillus-Streptococcus Differential agar (LSD) for colony counting of lactic acid bacteria and on Farrell's agar (Oxoid) for counts of *B. abortus*. LSD agar is a selective medium which provides good growth and the differentiation of thermophilic lactobacilli and streptococci in yoghurt products; it was prepared according to the formulation of Eloy and Lacrosse.⁹ All plates were incubated 48 h at 37°C under a 6% CO₂ atmosphere. Samples were taken until the pathogen was not detected in two consecutive samplings. The pH of the milk samples was measured with a pH meter equipped with a combined electrode.

RESULTS

Figure 1 shows the behavior of *B. abortus* in milk inoculated with 1×10^5 CFU per ml during fermentation at 42°C. Growth of the lactic culture was similar in the absence or presence of the pathogen. For this reason, in Figures 1 to 4, only growth of *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subspecies *bulgaricus* in the starter culture control flask is shown. After 8 h of incubation, a slight growth of *B. abortus* close to one logarithm of magnitude, in the pathogen control flask was observed, whereas in the flask containing *B. abortus* and the starter culture, no growth of the former was observed.

After the fermentation phase, the *B. abortus* population decreased after the first day of storage at 4°C; and after 10 days no colony on agar plates was observed (Fig. 2). On the other hand, *B. abortus* population in the pathogen control flask did not show any variation during 12 days. Lactic acid bacteria counts did not change with storage.

The behavior of *B. abortus* inoculated at 1×10^8 CFU/ml of milk during the fermentation phase is shown in Figure 3. When *B. abortus* was incubated together with the starter culture, its population did not change after 8 hours of incubation; whereas in the absence of the starter culture, a slight increase in *B. abortus* population was observed.

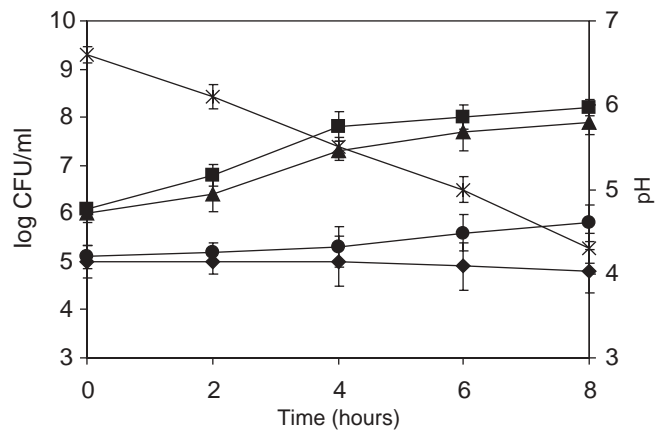


Figure 1. Counts of *S. salivarius* subsp. *thermophilus* (■), *L. delbrueckii* subsp. *bulgaricus* (▲), *B. abortus* when incubated with the starter culture (◆), *B. abortus* in the pathogen control (●) and pH values (×) during milk fermentation at 42°C with an initial concentration of *B. abortus* of 10^5 CFU/ml.

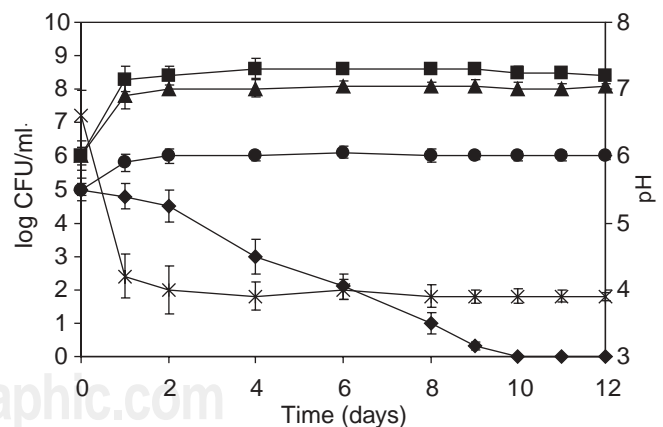


Figure 2. Counts of *S. salivarius* subsp. *thermophilus* (■), *L. delbrueckii* subsp. *bulgaricus* (▲), *B. abortus* when incubated with the starter culture (◆), *B. abortus* in the pathogen control (●) and pH values (×) during storage at 4°C with an initial concentration of *B. abortus* of 10^5 CFU/ml.

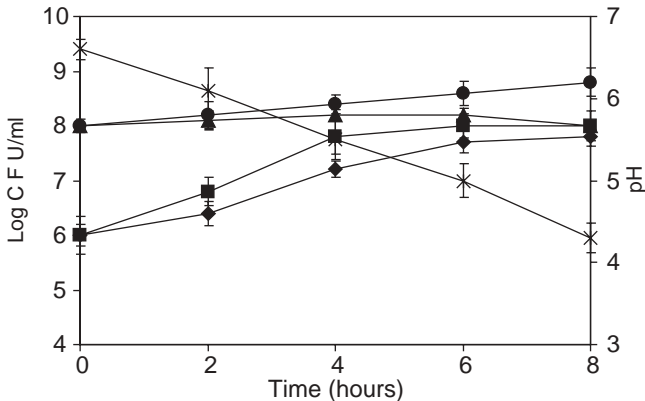


Figure 3. Counts of *S. salivarius* subsp. thermophilus (■), *L. delbrueckii* subsp. bulgaricus (▲), *B. abortus* when incubated with the starter culture (◆), *B. abortus* in the pathogen control (●) and pH values (×) during milk fermentation at 42°C with an initial concentration of *B. abortus* of 10^8 CFU/ml.

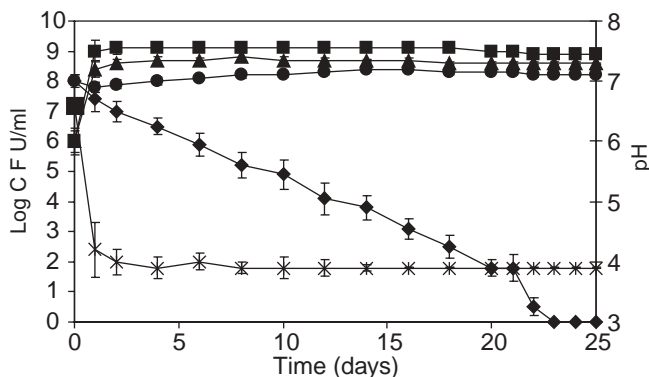


Figure 4. Counts of *S. salivarius* subsp. thermophilus (■), *L. delbrueckii* subsp. bulgaricus (▲), *B. abortus* when incubated with the starter culture (◆), *B. abortus* in the pathogen control (●) and pH values (×) during storage at 4°C with an initial concentration of *B. abortus* of 10^8 CFU/ml.

B. abortus was able to survive for up to 22 days in the presence of the starter culture during storage (Fig. 4), in spite of the low pH values (3.8 to 4), but it died after 23 days. In the pathogen control flask, the population of *B. abortus* was kept close to 10^8 CFU/ml throughout 25 days of storage.

In all flasks the initial pH value of the milk was 6.4, but in the presence of the starter culture it dropped to less than 5 after 8 h, and to less than 4 after 24 h.

DISCUSSION

Lactic acid bacteria are used in the manufacture of fermented foods. They contribute to the development of fla-

avour as well as to the preservation of raw milk, mainly due to the presence of lactic acid. In the present study, *B. abortus* viability was slightly reduced after 8 h in the presence of the starter culture, at a concentration of 10^5 CFU of *B. abortus* per ml. It could be due to a bacteriostatic effect observed by the drastic decrease of pH in the presence of lactic acid bacteria and maybe due to the production of other antimicrobial compounds. Reports have been made by other researchers of the influence of some factors, such as bacteriocins, hydrogen peroxide, volatile compounds, and other metabolites produced by lactic acid bacteria, which have proven their efficacy by inhibiting the growth of pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes*, both of which could be present in milk used in the manufacture of dairy products.^{17,21}

Previous studies have shown that the pH of dairy products played a critical role in the survival and growth of *Brucella* spp and they have argued as well that the role of dairy products as a vehicle for transmitting several pathogens could be predicted by determining the pH values of the products. Davies and Casey⁷ showed the effect of the change of pH on the survival of *B. abortus* in milk and milk products, suggesting a direct correlation between the survival of the microorganism and the pH. According to this study *B. abortus* did not survive when the pH was lower than 4.0.

Nevertheless, in this study, we have shown that even at pH 4.0, the starter culture did not inhibit the growth of *B. abortus* in associative culture in milk and did not die as quickly as expected. Rather, the organism was able to survive several days and even weeks in the fermented milk. The survival time of *B. abortus* in fermented milk observed in this study was longer than the time reported in previous researches.^{8,10,21} Apparently this was due to differences in the selected strain of *Brucella*, size of inoculum, different conditions in experimental assays, inoculation of the pathogen before or after the fermentation, type and size of the starter culture, etc.

This study demonstrated that *B. abortus* was able to survive 22 days in milk fermented with a yoghurt starter culture stored at 4°C when the pH was around 3.8. Certainly, this unexpected survival represents a risk for the health of the consumers. In our country, where traditional hand-made techniques are still used to manufacture dairy products like yoghurt, the consumption of contaminated products could generate isolated cases or outbreaks of human brucellosis.

Finally, it is important to point out that non-pasteurized fermented dairy products, including yoghurt, cannot be considered *Brucella*-free after a fermentation process and subsequent storage, especially if milk comes from cattle bred in endemic areas. Therefore, milk must be obtained and handled with high standards of hygiene followed by a proper heat treatment to prevent brucellosis.

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