

## Survival of *Borrelia burgdorferi* in Whole Milk, Low Fat Milk, and Skim Milk at 34°C and in Skim Milk at 5°C

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### ABSTRACT

Autoclaved whole milk, low-fat milk, protein-fortified skim milk and regular skim milk were inoculated to contain ca.  $10^5$  to  $10^6$  *Borrelia burgdorferi* strains 35210, 35211, or EBNI/ml and stored at 34°C for 16 d. Similarly inoculated skim milk also was held at 5°C for 46 d. Numbers of survivors were estimated by the Most Probable Number (MPN) technique. In all instances, numbers of *B. burgdorferi* decreased over the storage period. At 34°C, no strain of *B. burgdorferi* was detected after day 12. The mean D-values, at 34°C, for strains 35210, 35211, and EBNI were 2.2, 2.4, and 2.2 d, respectively. The mean D-values, at 34°C, for all strains in whole milk, low-fat milk, protein-fortified skim milk, and regular skim milk were 2.4, 2.3, 1.9, and 2.4 d, respectively. At 5°C, spirochete numbers in regular skim milk decreased, but all three strains remained at a detectable level for 46 d. The mean D-values, at 5°C, for strains 35210, 35211, and EBNI were 12, 15, and 12 d, respectively.

*Borreliae* were discovered in the 1870s during the early days of bacteriology. Since the 1950s the number of scientific articles on borreliae averaged less than 10 a year. However, in the 1980s there was a renewed interest in the genus and especially in the species *Borrelia burgdorferi* (12).

*B. burgdorferi* is the etiologic agent of Lyme disease (18). The name "Lyme disease" was coined by Steere et al. (51) when they reported on a cluster of cases of arthritis in patients residing in Lyme, Connecticut. Many components of the Lyme complex of disorders have been recognized for several decades in Europe (1), but their appearance in North America is a more recent phenomenon (10) and one of increasing frequency and concern. Figures for the U.S. for 1982-1988 show that there was a ninefold increase (492 to 4572) in reported cases of the disease (7).

In humans, the symptoms of Lyme disease may be brief and inconsequential or chronic and severely disabling (10). The first stage is generally characterized by a rash, erythema chronicum migrans (ECM), which develops after 3 to 4 d at the site of the bite by a tick carrying the spirochete. Subsequent manifestations may involve the skin, joints, nervous system, or cardiac muscle. These symptoms may appear months to years after the onset of the infection (52). *B. burgdorferi* has been obtained from the skin (14), blood (41), spleen, kidney, bone marrow (44),

joint fluid (46), cerebral cortex (35), and cerebrospinal fluid (31) of patients and from fetal tissue (39).

Lyme disease is transmitted primarily by ticks (50); *Ixodes dammini* and *Ixodes pacificus* are the recognized vectors in the U.S., whereas in Europe *Ixodes ricinus* has been identified as the vector (45). The principal natural reservoirs of *B. burgdorferi* are the white-tailed deer (*Odocoileus virginianus*) and the white-footed mouse (*Peromyscus leucopus*); these are the principal hosts for *I. dammini* (16,22). However, as indicated by Anderson and Magnarelli (3), other hosts can be colonized by the developing tick. The Lyme disease spirochete has been recovered from several types of animals including white-footed mouse (6,37), raccoon (4), Eastern chipmunk (5), woodland-jumping mouse, white tailed deer, meadow vole, bird (2), horse (20), cat (20), and dog (27). *B. burgdorferi* has been isolated from the spleen, kidneys, blood (48), and urine (15) of infected animals.

In recent years, several reports have cited the isolation of *B. burgdorferi* and/or antibodies to this organism from cows/cattle. Occurrence of *Borrelia* spirochetes in cattle in the Netherlands, Germany (53), Sweden (29), and Australia (43) has been reported. Burgess, in a study on cows in Wisconsin, found that colostrum, synovial fluid, and serum samples were antibody-positive for *B. burgdorferi* (19). She isolated the pathogen from blood, urine, synovial fluid, and colostrum of infected cows. The pathogen was not isolated from milk of normal composition. Barbour and Hayes (12), citing Sergent, mentioned the presence of borreliae in milk. Many of the animals from which *B. burgdorferi* have been isolated are in close association with humans and can bring the spirochete into the human environment by transporting ticks and by direct shedding of the spirochetes (20). The latter occurrence raises the question of transmission by direct contact, without an arthropod vector.

Oral, nasal, or aerosol infection with the spirochete may be possible (21). Laboratory experiments have provided evidence for contact transmission (23). A study by Burgess and Patrican (21) indicated that *B. burgdorferi* infection can occur via the oral route. It also has been demonstrated that rats and dogs can be infected through consumption of infected organs (33). Sergent (49) reported that a small proportion of guinea pigs that consumed milk

of an infected female became infected themselves. Post et al. (40) noted that a mature cat fed 4 ml of milk from an affected cow, seroconverted from a negative *B. burgdorferi* antibody titer to a positive titer. In addition, five negative *P. leucopus* all developed positive titers when inoculated subcutaneously with 0.1 ml of milk or with 0.1 ml of urine from the infected cow. These authors suggested that infectious spirochetes may be transmitted in urine and in milk (40).

For a foodborne illness to occur several events must happen sequentially: (a) the pathogen must reach the food, (b) it must survive in the food until ingested, (c) often it must multiply to infectious levels, and (d) the person who ingests the food must be susceptible at the levels ingested (17).

The purpose of this research was to determine the answers to some of these questions in relation to *B. burgdorferi*, namely, can the pathogen grow or survive in milk?

## MATERIALS AND METHODS

### Microorganism

Three strains of *B. burgdorferi* were used in these experiments. Two of the strains, 35210 and 35211, were obtained from ATCC. The third strain, EBNI, came from E.C. Burgess, School of Veterinary Medicine, University of Wisconsin-Madison. Stock cultures, which were stored at  $5 \pm 1^\circ\text{C}$ , were maintained by regular transfers into Barbour-Stoenner-Kelly (BSK) medium.

### BSK medium

Glassware used to prepare the medium was free from detergent. The BSK medium had the following constituents: 400 ml  $\text{H}_2\text{O}$  (glass distilled), 50 ml of 10 x concentrate of CMRL (Connaught Medical Research Laboratories) 1066 without glutamine (Gibco Laboratories, Grand Island, NY), 1.0 g yeastolate (Difco, Detroit, MI), 3.0 g HEPES (N-2-hydroxyethylpiperazine-N-2-ethane-sulfonic acid) (Sigma Chemical Co., St. Louis, MO), 2.5 g D (+) glucose, 0.35 g sodium citrate, 0.4 g sodium pyruvate, 0.2 g N-acetyl glucosamine, 1.1 g sodium bicarbonate, 50 mg L-glutamine (Sigma), 2.5 g neopeptone (Difco) dissolved by heating in 50 ml  $\text{H}_2\text{O}$  and then cooled and 25 g bovine serum albumin (Fraction V, Sigma, A4503). CMRL 1066 (10x) contains 56 individual components, all in mg/L. Included are five inorganic salts, 20 individual amino acids, 14 individual vitamins, and 17 other organic compounds. The aforementioned ingredients, which were combined in the order listed, were stirred for a minimum of 5 h. The pH was then adjusted to 7.6, at 20 to  $25^\circ\text{C}$ , with 1 N NaOH (Sigma), and the resulting solution was filtered through 0.22- $\mu\text{m}$  pore-size membranes at atmospheric pressure at  $5^\circ\text{C}$ . The filtered medium was supplemented with 100 ml of a sterile (autoclaved) solution containing 7 g of gelatin (Difco) and 0.5 g of agarose (SeaKem LE, low electroendosmosis, Polysciences, Inc. Warrington, PA). The resulting medium was aseptically dispensed in 4.5-ml portions into sterile screw-capped test tubes.

### Enumeration of *B. burgdorferi*

The Most Probable Number (MPN) technique (54) was used to enumerate *B. burgdorferi*. Cultures were serially diluted in BSK medium, then three fresh tubes of BSK medium were inoculated from each of three dilutions. Tubes of medium were then incubated for 30 d at  $34 \pm 1^\circ\text{C}$ . This temperature was chosen as it is in the optimum range for *B. burgdorferi* (9). The incubation time was selected based on results of preliminary trials.

Tubes were considered positive when the color of the pH indicator, phenol red, in the BSK medium changed to yellow as a result of growth of *B. burgdorferi*. Presence of *B. burgdorferi* was confirmed in a minimum of 30% of positive tubes by microscopic examination of preparations stained with carbol fuchsin. The MPN of viable cells was determined with a 3-tube MPN table (54).

### Milk samples

Pasteurized borreliae-free (36) whole milk, low fat milk, protein-fortified skim milk, and regular skim milk were obtained at a local retail outlet. Milks were dispensed in 20-ml portions into screw-cap universal containers (40-ml capacity) and sterilized by autoclaving. Each sample was inoculated with 0.5 ml of a culture of *B. burgdorferi* grown in BSK at  $34 \pm 1^\circ\text{C}$  and serially diluted in BSK to give an inoculum level of  $\geq 10^6/\text{ml}$  milk. In all instances, the number of passages of the bacterium in BSK was less than ten. Inoculated milk samples were stored at  $34 \pm 1^\circ\text{C}$  or at  $5 \pm 1^\circ\text{C}$ . Samples stored at  $34 \pm 1^\circ\text{C}$  were tested on days 0, 1, 3, 5, 7, 9, 12, and 16, whereas those stored at  $5 \pm 1^\circ\text{C}$  were tested on days 0, 4, 8, 12, 15, 23, 27, 32, and 46. When incubation was at  $34 \pm 1^\circ\text{C}$ , experiments were done with each strain of *B. burgdorferi* in each of the four milk products. When storage was at  $5 \pm 1^\circ\text{C}$ , only skim milk was used. In all instances, the experiments were done in triplicate.

### Statistical analysis

Data for log MPN/ml were analyzed by regression to obtain the line of best fit. D-values (the negative reciprocal of the slope) were determined from the resulting regression equations. The contributory effect of product, strain of organism, temperature, and the interactions of these parameters was analyzed by split plot using SAS Institute (Cary, NC) statistical software. Individual means from the split-plot analysis were compared by the methods outlined by Cochran and Cox (25).

## RESULTS AND DISCUSSION

Few scientific papers focus on enumeration of *B. burgdorferi*. An important reason for lack of information on this topic is the difficulty in growing this pathogen in vitro (11). The medium currently employed, Barbour-Stoenner-Kelly (BSK), is time consuming and costly to prepare. In addition, a recent report indicates that morphological characteristics of *B. burgdorferi* varied with different lots of BSK, even though the medium was prepared by the same procedure (24). The source of bovine serum albumin (Fraction V) was a significant factor affecting the ability of BSK to grow *B. burgdorferi* (24). Despite these obstacles, the increasing impact of Lyme disease in North America, Europe, and Asia is a compelling reason for continued and sustained efforts to develop improved methods to cultivate this bacterium.

Viable bacterial counts may be determined using a plate count procedure. The ability of *B. burgdorferi* to form colonies on plates has been reported, but the plating efficiency is variable (34,38). The MPN technique has been used for enumeration of *B. burgdorferi* (36) and was employed in the work reported here. Low passage cultures ( $N \leq 9$ ) of *B. burgdorferi* were used as it has been reported that once borreliae are in culture they undergo change in one or more traits. With continuous in vitro passage the organism can lose the ability to infect laboratory animals (30). Such

changes have been reported after as few as 15 passages (47) or as many as 150 passages (32). Any interpretation of results from experiments with *B. burgdorferi* must consider these changes.

Three different strains were employed in the work reported here as differences between isolates of *B. burgdorferi* have been noted in the literature (55). The ATCC 35210 culture was isolated from *I. dammini* collected from Shelter Island, New York (U.S.) (9). The ATCC strain 35211 is a European isolate from *I. ricinus* in Switzerland. The third isolate, EBNI, also is a U.S. isolate from *I. dammini*.

The milk products, held at  $34 \pm 1^\circ\text{C}$  and at  $5 \pm 1^\circ\text{C}$ , did not support the growth of *B. burgdorferi*. In all instances, numbers decreased during the storage period (Fig. 1 to 5). These results differ from the findings for most food pathogens. For example, *Listeria monocytogenes* increased in number at  $4^\circ\text{C}$  and at  $35^\circ\text{C}$  in skim, whole, or chocolate milk (42). Test conditions reported here were not favorable for proliferation of *B. burgdorferi*. Observations made during the microscopic examination of positive cultures indicated variation in the length, spirality of the organism, and presence of blebs. Blebs are produced under conditions of stress (13), by chemical means, or on aging (41). It has been speculated that if bleb formation occurs in vivo, these structures may contain poorly degradable components that could continue to act as antigens long after the live spirochetes have disappeared (13). At  $34 \pm 1^\circ\text{C}$  all the strains of *B. burgdorferi* were detected in all milk samples until day 9. However, the Lyme pathogen was not detected in any sample after day 12 (Fig. 1 to 4). Felsenfeld (26) showed that borreliae can remain viable for 4 d at  $37^\circ\text{C}$ .

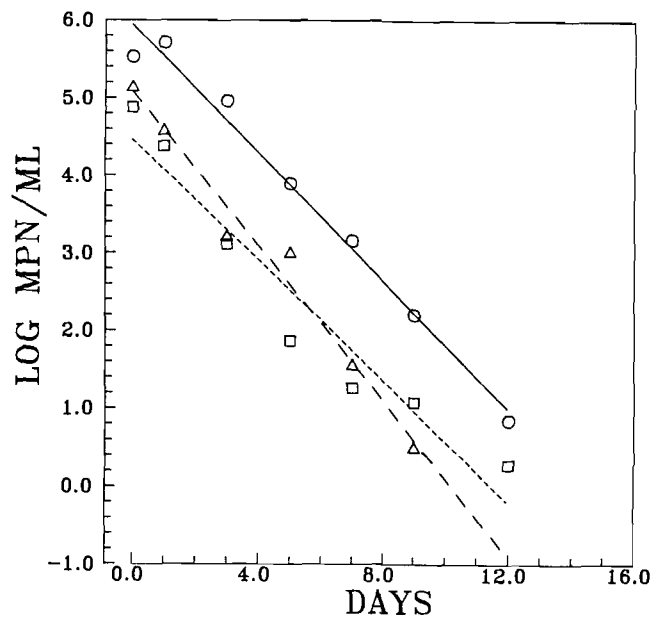


Figure 1. Survival of *B. burgdorferi* strains in whole milk when stored at  $34^\circ\text{C}$ . Key: ●—● = 35210, □—□ = 35211, ▲—▲ = EBNI.

Using split-plot analysis, we found that D-values for the pathogen were not significantly affected by strain, product, or strain-product interaction ( $p > 0.05$ ). Analysis with Fishers least significant differences (LSD) test, at

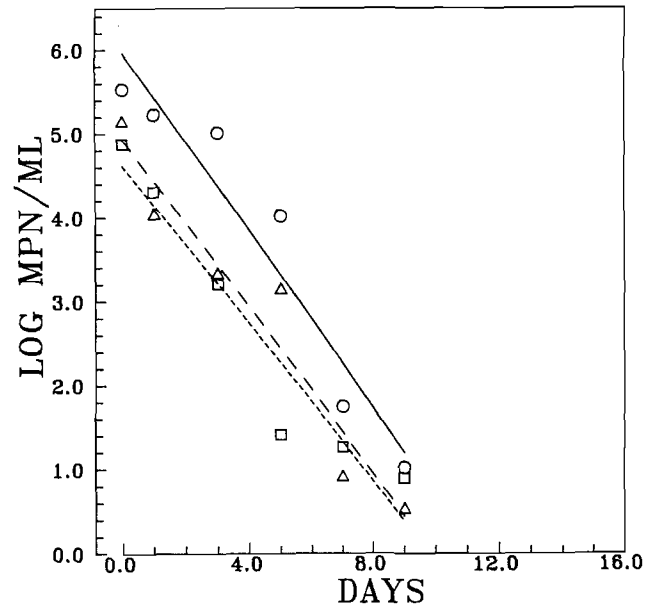


Figure 2. Survival of *B. burgdorferi* strains in low fat milk stored at  $34^\circ\text{C}$ . For key, see Fig. 1.

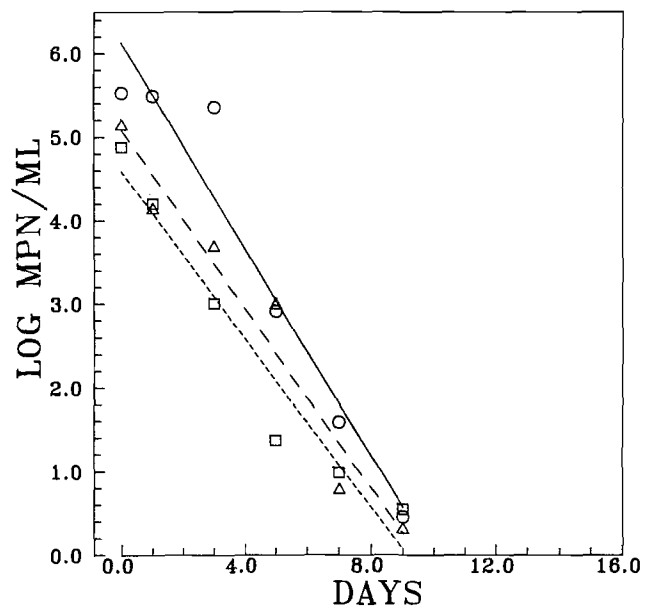


Figure 3. Survival of *B. burgdorferi* strains in protein-fortified skim milk stored at  $34^\circ\text{C}$ . For key, see Fig. 1.

$p=0.05$ , indicated that the D-values for individual strains of *B. burgdorferi* were not significantly different. However, *B. burgdorferi* in protein-fortified skim milk samples had a mean D-value (1.9 d) which was significantly less ( $p < 0.05$ ) than that for the bacterium in whole and skim milk products (2.4 and 2.4 d, respectively) (Table 1). The D-values for strain 35210 in protein-fortified skim milk and regular skim milk samples were significantly different ( $p < 0.05$ ).

When the strains of *B. burgdorferi* in skim milk were stored at  $5 \pm 1^\circ\text{C}$ , numbers decreased, but, in all instances, remained at a detectable level for 46 d. Work with *Borrelia persica* indicated this organism was viable for 19 d at  $4^\circ\text{C}$  and for 7 d at  $11$  to  $15^\circ\text{C}$  (26). Baranton and Saint-Girons

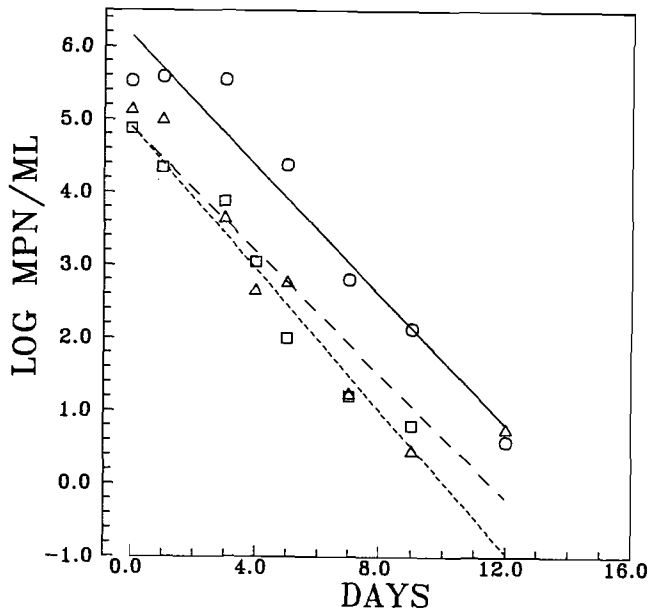


Figure 4. Survival of *B. burgdorferi* strains in regular skim milk stored at 34°C. For key, see Fig. 1.

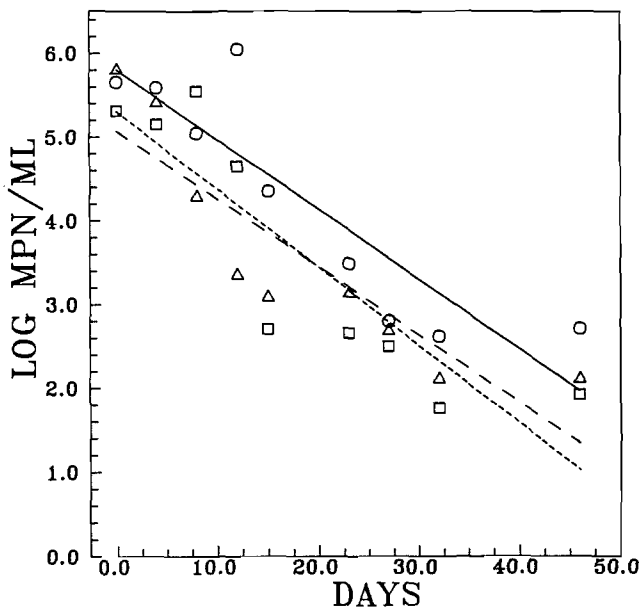


Figure 5. Survival of *B. burgdorferi* strains in regular skim milk stored at 5°C. For key, see Fig. 1.

(8) reported that *B. burgdorferi* inoculated into blood, at a level of  $10^6$ /ml, remained viable for 60 d, when stored at 4°C.

The mean D-values for strain 35210, 35211, and EBNI in skim milk at  $5 \pm 1^\circ\text{C}$  were 12, 15, and 12 d, respectively (Table 2). *B. burgdorferi* had a significantly greater ( $p < 0.05$ ) mean D-value when stored at  $5 \pm 1^\circ\text{C}$  rather than at  $34 \pm 1^\circ\text{C}$  (13 vs 2.4 d). The effect of strain and the interaction of strain and product were nonsignificant but the effect of temperature was significant ( $p < 0.01$ ).

TABLE 1. Decimal reduction time (D-value), in days, for three strains of *Borrelia burgdorferi* in milk products stored at 34°C.

Product	Strain		
	35210	35211	EBNI
Whole milk	2.3	2.4	2.5
Low fat milk	2.3	2.6	2.1
Protein-fortified skim milk	1.7	2.1	2.0
Regular skim milk	2.6	2.5	2.2

TABLE 2. Decimal reduction time (D-value), in days, for three strains of *Borrelia burgdorferi* in skim milk, stored at 5°C.

Strain	D-value
35210	12
35211	15
EBNI	12

It may be concluded from the results reported here that *B. burgdorferi* did not grow in whole milk, low fat milk, protein-fortified skim milk, or skim milk when stored at  $34 \pm 1^\circ\text{C}$  or in skim milk at  $5 \pm 1^\circ\text{C}$ . The spirochete, if present at  $\geq 10^4$ /ml, may survive for at least 9 d at  $34 \pm 1^\circ\text{C}$  and for at least 46 d at  $5 \pm 1^\circ\text{C}$ . Literature reports on the presence of *B. burgdorferi* in animal tissues do not give any indication of the numbers/g or ml. It is unlikely that natural contamination would achieve the numbers used as the inoculum level in this study. However, evidence indicates that a relatively few spirochetes in the infected host can produce chronic systemic disease (28).

Any interpretation of the findings reported here must consider a number of factors which affect the viability and recovery of *B. burgdorferi*: (a) reliability of BSK medium in the recovery of borreliae cells, especially those stressed by the environment; (b) variations in composition of medium and milk over a given time period; and (c) changes which occur on subculturing the organism.

The longevity of the pathogen suggests that raw milk, if infected, could be a source of viable *B. burgdorferi* cells and highlights the need for further research in this area.

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