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Research article

Survival of *Clostridium difficile* Spores at Low Temperatures

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Running Title: *C. difficile* spores survive low temperatures.

Key Words: *C. difficile*, spore, low temperature, storage spore resistance, meats

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Abstract

*Clostridium difficile´*s presence has been reported in meat products stored typically at low temperatures. This study evaluated the viability in phosphate buffer saline (PBS) of spores from epidemic *C. difficile* strain R20291 (4.6 log CFU/ml) and M120 (7.8 log CFU/ml). Viability was assessed during 4 months at -80°C, -20°C, 4°C (refrigeration), and 23°C (room temperature), and after 10 freeze (-20^{o}C)/thaw (+23^{o}C) cycles. Although spore viability decreased, significant viability was still observed after 4 months at -20°C, i.e., 3.5 and 3.9 log CFU/ml and -80°C, i.e., 6.0 and 6.1 log CFU/ml for strains R20291 and M120, respectively. The same trend was observed for M120 at 4°C and 23°C, while for R20291 the viability change was non-significant at 4°C but increased significantly at 23°C ($p>0.05$). After 10 freeze-thaw cycles, viability of both strains decreased but a significant fraction remained viable (4.3 and 6.3 log CFU/ml for strain R20291 and M120, respectively). Strikingly, both strains showed higher viability in a meat model than in PBS. A small but significant decrease ($p<0.05$) from 6.7 to 6.3 log CFU/ml in M120 viability was observed after 2-month storage in the meat model while the decrease from an initial 3.4 log CFU/ml observed for R20291 was non-significant ($p=0.12$). In summary, *C. difficile* spores can survive low-temperature conditions for up to 4 months.

1. **Introduction**

Clostridium difficile is a Gram-positive, spore-forming, anaerobic bacterium and a major causative agent of antibiotic-associated diarrhea (Ananthakrishnan, 2011), and pseudomembranous colitis (McFarland et al., 1989). Most episodes of *C. difficile* infections (CDI) are believed to be acquired from healthcare settings through person-to-person transmission or from the hospital environment (Rupnik et al., 2009). Recently, the whole-genome sequencing of 1250 *C. difficile* isolates obtained from patients with CDI revealed that there were considerable non-hospital reservoirs of *C. difficile* (Eyre et al., 2013). Community-acquired CDI (CA-CDI) has been recently suggested to represent ~32% of all CDI cases in the U.S.A. (Lessa, 2013) and strengthened the conclusion that non-hospital transmission sources might be involved in CDI. Since *C. difficile* is also a farm animal pathogen, meat products are potential reservoirs of *C. difficile* (Hoover and Rodriguez-Palacios, 2013). Several reports have demonstrated that *C. difficile* is present in meat products, including but not limited to ground beef and pork, turkey, vacuum-packed meat, and various meat sausages. Although convincing evidence is still lacking, the presence of *C. difficile* spores in foods suggests the foodborne transmission of *C. difficile* (Hoover and Rodriguez-Palacios, 2013). Therefore, *C. difficile* could be considered a zoonotic pathogen transmitted by farm animals, foods and water.

Food transmission of *C. difficile* implies that *C. difficile* spores survive common environmental stressors found in industrial food processing and handling steps. To date, only one study has addressed the ability of *C. difficile* spores of various strains to survive at the heating temperatures typically used in meat processing. Their results demonstrated that *C. difficile* spores are able to survive 2 h at 71°C, and that a 10-min treatment at 85°C inactivates only ~90% of the spore strains (Rodriguez-Palacios et al., 2010), suggesting that current commercial thermal

processing practices may be insufficient to reduce *C. difficile* spores to an acceptable level. Also, it has been suggested that the reduction of *C. difficile* spore recovery might be enhanced during storage at 23°C for 20 or 52 weeks (Rodriguez-Palacios and Lejeune, 2011).

Hughes and Nobbs (Hughes and Nobbs, 2004) studied fecal microorganisms in 30-40 year old human feces collected in an Antarctic Peninsula location with regular daily positive maximum of 0 to 5^{o}C, and negative minimum temperatures of -7 to -40°C in the summer and winter months, respectively. They found that spore-forming *Bacillus* and *Clostridium* species can survive these climatic conditions with *Clostridium perfringens* and *Bacillus* spp. spores in fecal samples reaching $5x10^{7}$ CFU/g and less than 10^{3} CFU/g, respectively. Another study indicated that the spores of *Clostridium welchii*, a major spoilage organisms of meat and meat product, can survive freezing storage at -5 and -20°C for up to 26 weeks (Barnes et al., 1963). Early work on food freezing cited by Georgala and Hurst (1963) showed that spores can remain viable more than 100 d at -2 to -20^{o}C. Reviewing freeze-thaw studies, Young et al. (1968) concluded that *Bacillus* and *Clostridium* spore-formers spp. would survive daily cycles of -70^{o}C in dry ice followed by 4.5 h thawing at 25^{o}C. A study on the effect of storing *Bacillus cereus* endospores at 4°C in PBS for up to 1 month showed a loss of viability (Cronin and Wilkinson, 2008). Another study on *C. perfringens* found less than 1.3 and 1.6 log reductions in the viability in sporulation medium of spores from some *C. perfringens* strains after storage for up to 6 months at 4 and -20°C, respectively (Li and McClane, 2006).

A review of published work showed that the effect of low temperatures on the survival of *C. difficile* spores has been reported only in human feces and for nosocomial strains p24 and B32 (clinical toxigenic isolates, PCR ribotype 1 and 78, respectively) and for strain E16 (environmental toxigenic isolate, PCR ribotype 44). Storage temperature (-20°C or 4°C) and

multiple of freeze/thaw cycles had minimal effects upon the viability of their spores (Freeman and Wilcox, 2003). Therefore, the aim of this work was to evaluate the effect of storage in PBS and in a meat model for up to 4 months at freezing, refrigeration and room temperature on the viability of *C. difficile* spores of strains associated with hospital acquired- and CA-CDI. The effect of freeze/thaw cycles commonly observed in commercial meat handling was also included.

2. Material and Methods

2.1 *C. difficile strains and spore preparation*

Two *C. difficile* strains were used in this study. *C. difficile* M120, kindly provided by Dr. Trevor Lawley (Wellcome Trust Sanger Institute, Hinxton, UK) is a PCR-ribotype 78 strain often detected in farm animals, and even more frequently found in CA-CDI (GOORHUIS et al., 2008). *C. difficile* R20291, kindly provided by Dr. Nigel Minton (University of Nottingham, UK), is a ribotype 27 strain that is positive for *tcdA* (*tcdA*$^+$), *tcdB* (*tcdB*$^+$) and *cdtB* (*cdtB*$^+$). *C. difficile* strains were plated and incubated on 1.5% tryptone-yeast extract (TY) agar (Difco, BD Diagnostic Systems, Sparks, MD) for 7 days at 37°C under anaerobic conditions (5% H_2, 5% CO_2 and 90% N_2) in a ShelLab Bactron III-2 chamber (Sheldon Manufacturing, Inc., Cornelius, OR). Plates were flooded with sterile distilled water to resuspend and collect sporulating cells which were washed by repeated centrifugation (10 times, 14000 g, 10 min) using sterile distilled water. Free spores were separated by density gradient centrifugation (14000 g, 45 min) using 50% Nycodenz (Sigma-Aldrich Corp., St. Louis, MO). After washing five times with PBS to eliminate Nycodenz, spores were enumerated using a microscope counting chamber. Spore suspensions of both strains ($5x10^9$ spores/ml, >99% free of vegetative cells, sporulating cells and

cell debris) were prepared in phosphate buffer saline (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4) as previously described (Sorg and Sonenshein, 2008) and stored for up to 3 months at 80°C until use. The same spore stock suspensions were used for all experiments conducted in this study.

2.2 *Low temperature storage*

Spore suspensions with counts diluted from $5x10^9$ to $1.7x10^8$ spores/ml, were prepared in PBS. Aliquots (50 µl) of triplicate spore suspensions were kept in Eppendorf tubes for up to 4 months at 23°C (room temperature), 4°C (refrigeration), -20°C, and -80°C. In addition to storage studies, spores were subjected to 10 cycles of freezing triplicate PBS suspensions at -20°C and then thawing them at room temperature (20 min at each cycle condition). For studies in meat, spore suspensions were mixed in triplicates with sterile ground beef at $2.5x10^7$ spores/g and then stored at -20°C for 2 months in 15 ml Falcon centrifuge tubes (VWR International LLC, Radnor, PA). Sample aliquots were collected every month for up to 2 and 4 months for storage tests in meat and PBS, respectively, and after 10 freeze-thaw cycles of the PBS spore suspensions, and then serially diluted before plating onto 1.5% Brain Heart Infusion (BHI) agar (Difco, BD Diagnostic Systems, Sparks, MD) supplemented with 0.5% yeast extract (Y) and 0.1% sodium taurocholate (ST, Himedia, Mumbai, India). BHI-YST plates were incubated at 37°C for 36 h under anaerobic conditions (5% H_2, 5% CO_2 and 90% N_2) in a ShelLab Bactron III-2 chamber (Sheldon Manufacturing, Inc., Cornelius, OR). Colony-forming units (CFU) were recorded and converted to log CFU/mL and log CFU/g values for tests in PBS and meat, respectively. All experiments were conducted with three biological replicates. Data was then analyzed by ANOVA and pairwise comparisons using Microsoft Excel 2013 (Redmond, WA).

3. Results and discussion

3.1 *Germination efficiency of* C. difficile *spores*

Prior to analyzing the survival ability of *C. difficile* spores at low temperature, a striking difference in the germination ability of *C. difficile* strain R20291 and M120 spores was observed. Estimations of the % of spores forming colonies (CFU/spore count x 100) showed that only 0.03% of R20291 spores formed viable colonies while the corresponding value for M120 was ~35% suggesting that most *C. difficile* spores were unable to germinate or form colonies. To confirm that the spore suspensions of both strains were viable, *C. difficile* spores were decoated and plated onto BHI agar plates containing lysozyme, which triggers germination by directly degrading the spore peptidoglycan cortex (Paredes-Sabja and Sarker, 2011; Paredes-Sabja et al., 2009). Spore colony-forming efficiency reached ~90% for both strains indicating that nearly all spores were fully viable (data not shown).

3.2 *Survival of* C. difficile *spores at low temperature*

An increasing body of work suggests that meat products may be an important reservoir of *C. difficile* spores (Rodriguez-Palacios et al., 2009; Rodriguez-Palacios et al., 2007; Rupnik, 2007; Songer et al., 2009; Weese et al., 2009). Although low temperatures are commonly used to increase their shelf-life, including freezing (i.e., < -20°C) and refrigeration temperatures (i.e., 4°C), the ability of *C. difficile* spores to survive these storage conditions is unclear. The initial colony forming units of the $1.7x10^8$ spores/ml of both strains in PBS buffer were 4.6 and 7.8 log

CFU/ml for R20291 and M120, respectively. Results showed that the 4-month storage at -80°C caused only 0.7 and 1.7 decimal reductions in *C. difficile* R20291 and M120 spore viability, respectively (Fig. 1A). After 4-month storage at -20°C, the reduction in spore viability was 1.2 and 1.7 log CFU/ml R20291 for M120, respectively (Fig. 1B). These results indicate that *C. difficile* spores, albeit being inactivated to some extent by 4-month storage at -80°C and -20°C, can persist at freezing storage temperatures in agreement with earlier observations by Freeman and Wilcox (2003) on *C. difficile* spores in fecal samples, and reports on the survival to low temperatures of *C. welchii, C. perfringens, and B. cereus* spores (Barnes et al., 1963; Cronin and Wilkinson, 2008; Li and McClane, 2006).

The effect of multiple freeze-thaw cycles on the *in vitro* viability of *C. difficile* spores was also evaluated in this study. *C. difficile* R20291 and M120 spores were frozen at -20°C and thawed at room temperature (23^{o}C) ten times. A significant 0.9 log CFU/ml spore viability reduction from 7.2 to 6.3 log CFU/mL ($p<0.01$) was observed for M120 spores, while a non-significant reduction from 4.6 to 4.3 log CFU/mL ($p=0.07$) was observed for R20291 spores (Fig. 2). These results indicate that despite the fact that multiple freeze-thaw cycles might inactivate some spores, many are likely to survive and persist in *C. difficile* contaminated meats.

Next, the survival ability of *C. difficile* spores stored at refrigeration and room temperature was studied. After 4-month storage at 4°C, the observed decrease in M120 spores viability from 7.8 to 6.2 log CFU/mL was significant ($p < 0.05$), whereas the reduction in R20291 spores viability from 4.6 to 4.7 log CFU/mL was not (Fig. 3A). A significant decrease of 1.2 log CFU/ml in spore viability of M120 spores 7.8 to 6.9 log CFU/mL was observed after 4-month storage at 23°C (Fig. 3B). However, there was a striking increase in the ability of R20291 spores to form colonies with an overall increase of 1.8 log CFU/ml from 4.6 to 6.4 log CFU/mL

(Fig. 3B). Indeed, after 4-month storage at 23°C, nearly 1 % of R20291 spores were able to form colonies as compared to ~ 0.03% when the spores were from the freshly prepared suspension (i.e., time zero, Fig. 3B). After 6-month storage at 4°C, *C. perfringens* spores showed a significant viability difference between chromosomal *cpe* and plasmid *cpe* isolates, the former showing a 0.3-log reduction while the latter showed a larger 1.3-log reduction (Li and McClane, 2006). Freeman and Wilcox (2003) found minor changes in the number of total viable counts of a multiple strains mixture of *C. difficile* spores stored at 4 and -20°C for up to 56 days while this study provides evidence that *C. difficile* strains have significant resistance differences to low temperature storage. Under refrigeration, strain R20291 spores showed a higher survival rate than strain M120. During prolonged room temperature storage, R20291 spores appear to be in a superdormant stage and undergo some unidentified maturation process, allowing them to germinate in the presence of taurocholate and nutrients. Maturation of bacterial spores has been well described for spores of *B. subtilis* species (Sanchez-Salas et al., 2011), but has not been reported for *C. difficile*. Another tentative explanation is that R20291 spores may gradually lose their exosporium structure, which has recently been suggested to have a role in *C. difficile* spore germination efficiency (Escobar-Cortes et al., 2013). These findings are notable because they suggest that *C. difficile* spores are not only able to survive at room and freezing temperatures, but also that spores of some *C. difficile* strains are able to recover from superdormancy and become viable after long periods of storage at room temperature. Further work is clearly needed to understand the molecular mechanisms underlying *C. difficile* spore resistance and ability to form superdormant spores.

A meat model system previously used to study *C. perfringens* spore survival (Akhtar et al., 2008; Akhtar et al., 2009) was used to validate *in vitro* results obtained in this study. Meat

was contaminated with *C. difficile* spores of strains M120 and R20291 and stored at -20°C for up to 2 months. A significant viability decrease of 0.4 log CFU/ml ($p < 0.05$) from 6.7 to 6.3 log CFU/ml was observed for M120 spores after 2 months (Fig. 1B). Strikingly, a non-significant viability decrease ($p=0.12$) from the initial 3.4 log CFU/mL count was observed for R20291 spores (Fig. 1B). These results indicate that the meat matrix provides low temperature protection to some *C. difficile* spores, facilitating their survival in meat products.

4. Conclusions

In summary, the main conclusions from this work are that *C. difficile* spores can survive at -80 and -20°C and that spores of at least some strains may increase their germination ability when stored at 23°C. Although long-term storage at freezing temperatures inactivates some spores, these reductions are negligible when analyzed from a food safety perspective. Further studies to understand the behavior of *C. difficile* spores to stressors found in food processing operations will lead to improved estimates of the foodborne transmission risk of *C. difficile*.

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294
295

Figure legends

Figure 1. Effect of long-term storage at freezing temperature on the viability of *C. difficile* spores. *C. difficile* spores of strains R20291 (filled squares) and M120 (filled triangles) suspended in PBS buffer were incubated at -80°C (A) and -20°C (B) during 4 months. *C. difficile* spores of strains R20291 (empty squares) and M120 (empty circles) in meat were incubated at -20°C during 2 months. Aliquots were plated onto BHI-YST agar plates, and colony forming units counted after anaerobic incubation for 36 h at 37°C. Results are the average of three independent experiments. Standard error was < 5% of the mean resulting in error bars too small to be visible for some data points.

Figure 2. Effect of multiple freeze-thaw cycles on the viability of *C. difficile* spores. The effect of multiple freeze-thaw cycles on the viability of *C. difficile* spores of strains M120 and R20291 was analyzed subjecting spores to 10 freeze (-20°C) and thaw cycles. Initial counts prior to freeze-thaw cycles (white bars) and final counts after 10 freeze-thaw cycles (black bars) were determined by plating aliquots onto BHI-YST agar plates. Results are the average of three independent experiments. Standard error was < 5% of the mean.

Figure 3. Effect of long-term storage at refrigerated temperature on the viability of *C. difficile* spores. *C. difficile* spores of strains R20291 (filled squares) and M120 (empty squares) in PBS buffer were incubated at 4°C (A) and 23°C (B) during 4 months. Monthly aliquots were plated onto BHI-YST agar plates, and colony forming units counted after anaerobic incubation for 36 h at 37°C. Results are the average of three independent experiments. Standard error was < 5% of the mean.

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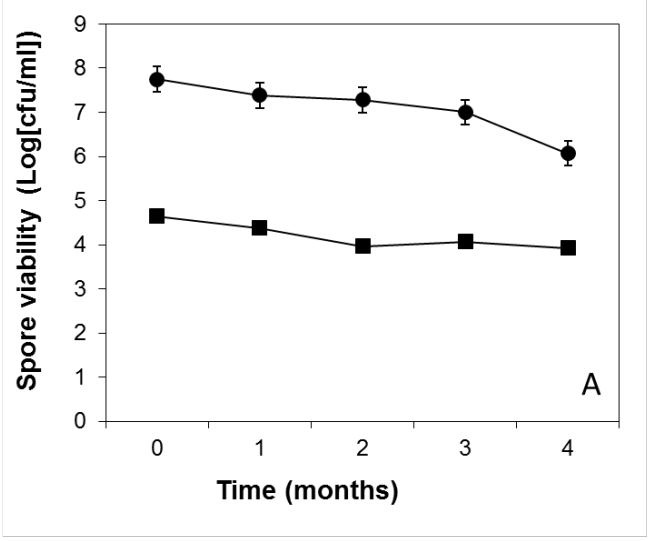
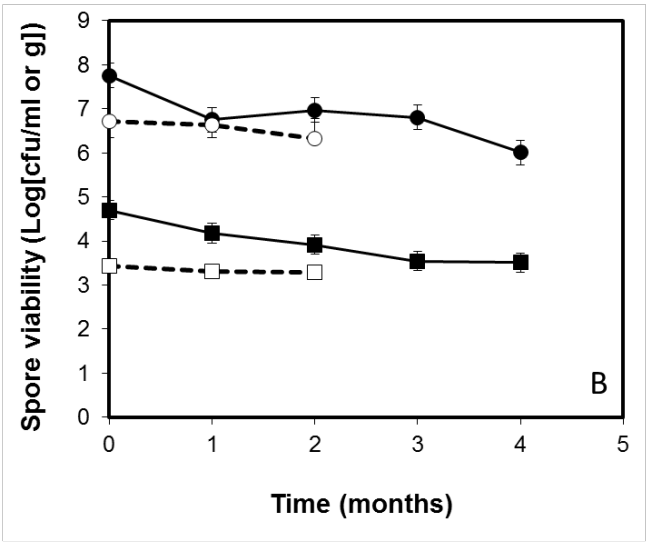
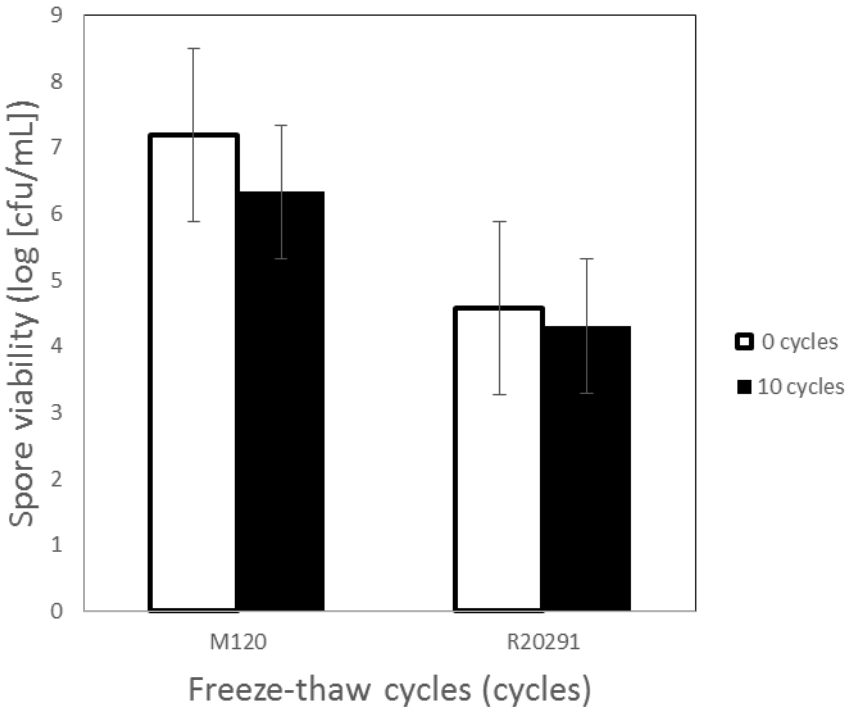

Spore viability (Log[cfu/ml])
Time (months)
A


Spore viability (Log[cfu/ml or g])
Time (months)
B

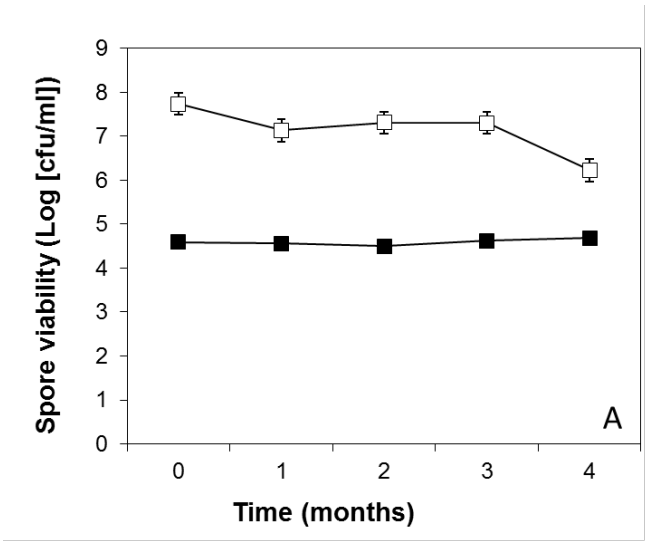
318

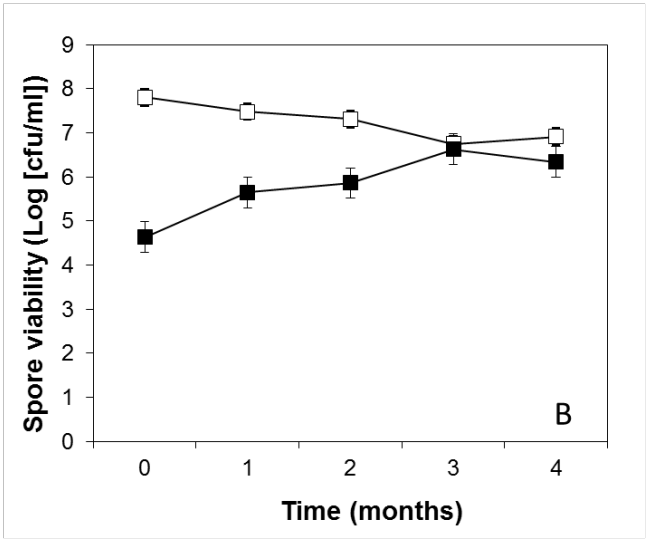
319


Spore viability (log [cfu/mL])
9
8
7
6
5
4
3
2
1
0
M120
R20291
Freeze-thaw cycles (cycles)
0 cycles
10 cycles

320

321


Spore viability (Log [cfu/ml])
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
Time (months)
A


Spore viability (Log [cfu/ml])
9
8
7
6
5
4
3
2
1
0
0
1
2
3
4
Time (months)
B

322