Short Communication: The Effect of Water Temperature on the Viability of Silage Inoculants

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ABSTRACT

The objective of this study was to determine if exposure to high temperatures in water affects the viability of various silage inoculants. Inoculants were enumerated on De Man, Rogosa, Sharpe agar to standardize a final count (colony-forming units) in water such that about 500 mL added to 1 tonne of wet forage would achieve a recommended application rate of about 100,000 cfu of lactic acid bacteria (LAB) per gram of wet forage. Testing was done in 4 sequences (SEQ). For each SEQ, inoculants were mixed in deionized water for 45 min at 30°C followed by incubation for 6 h at 30°C (SEQ 1), 35°C (SEQ 2), 40°C (SEQ 3), or 45°C (SEQ 4) in duplicate 125-mL flasks rotating at 125 rpm. After 6 h, rotation was stopped and the temperature was lowered to 30°C for the next 18 h for all SEQ. Numbers of LAB were enumerated at 0, 3, 6, and 24 h. Each sequence was repeated twice. Incubation at a moderate temperature (SEQ 1) did not affect the viability of the microbial inoculants. The viability of the inoculants declined with increasing temperature (SEQ 2 to 4) but the effect varied by inoculant. For some inoculants exposure to 35°C resulted in substantial decreases in viable cells (loss of 0.5 to 1 log cfu/mL). Incubation at higher temperatures resulted in even greater losses in viability for some inoculants. Losses of more than 0.5 log cfu/mL would most likely make the application of these inoculants ineffective in the field. *Lactobacillus plantarum* MTD/1 was the most thermotolerant organism tested, because it was unaffected by all temperatures (30 to 45°C) after 3 h of incubation. *Lactobacillus plantarum* MTD/1 and *Lactobacillus buchneri* 40788 also appeared to have better thermotolerance as their numbers substantially increased between 6 and 24 h in SEQ 4. These data show that some silage inoculants are more thermotolerant than others and that precautions should be taken to ensure that microbial inoculants that are ap-

plied to forage do not reach elevated temperatures during use.

Key words: silage, inoculant, forage

Lactic acid bacteria (**LAB**) are added to forages at the time of ensiling with the goal of improving the fermentation process. These inoculants are applied in a dry form or they are commonly mixed in water and held in applicator tanks (Kung et al., 2003). Success in using these inoculants is highly dependent on the specific inoculant used and adding sufficient numbers of viable bacteria. One place where the viability of these organisms might be affected is in the applicator tanks. For example, forages are often harvested on warm summer days when ambient temperatures may exceed 38°C and some applicator tanks are subject to intense solar exposure and are in close proximity to tractor exhausts or engines that could cause the temperature of the water in applicator tanks to reach detrimental levels. Even moderately high temperatures (40 to 45°C) may result in a heat-shock response in various bacteria (Lindquist, 1986; Pagan et al., 1997; Arsene et al., 2000). If heat shock decreases the number of viable organisms in applicator tanks, the application rates of added microbial inoculants might be less than recommended. Thus, the objective of this study was to evaluate to viability of various microbial silage inoculants when subjected to a range of water temperatures.

The classical acid silage inoculants used in this study were: MTD/1 (**Eco**; *Lactobacillus plantarum*, strain MTD/1, Ecosyl Products Ltd., Stokesley, UK; lot number 610101), Pioneer 1127 Grass/Legume Silage Inoculant (**P1127**; *L. plantarum* and *Enterococcus faecium*, Pioneer Hi-bred Intl., Johnston, IA; lot number 368711A), Biotal Plus II (**BP11**; *Pediococcus pentosaceus*, *Propionibacteria freudenreichii*, β -glucanase (3,908 IU/g), xylanase (3,554 IU/g), and galactomannanase (1,776 IU/g), Lallemand Animal Nutrition, Milwaukee, WI; lot number 6041801), Biomax MP Multipurpose Microbial Silage Inoculant (**BMP**; *L. plantarum* and *P. pentosaceus*, Chr. Hansens Biosystems, Milwaukee, WI; lot number 2463867) and Biomax 5

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(**BM5**; *L. plantarum*, strains PA-28 and K-270, Chr. Hansens Biosystems; lot number 2487305). A heterolactic acid-based inoculant, *L. buchneri* 40788 (**LB**; *L. buchneri* 40788, Lallemand; lot number 5010731) was also tested. All inoculants were bought commercially within the year of the experiment with the exception of Eco, which was from a bottle that had been kept under refrigeration for 2 yr.

For all inoculants, sterile 10-fold serial dilutions in quarter-strength Ringer's solution (made with Oxoid BR0052, Unipath, Basingstoke, UK) were pour plated in De Man, Rogosa, Sharpe (**MRS**) agar (Oxoid CM361). Counts of LAB obtained from each inoculant were used to equilibrate the starting count (cfu/mL).

This study was conducted in 4 temperature sequences (**SEQ**). Preparation for each SEQ involved mixing each inoculant in deionized water for 45 min at 30°C to achieve a theoretical starting concentration of about 8.3 log cfu/mL (except for LB). This concentration was chosen because addition of 500 mL of this mixture to 909 kg of wet silage would theoretically result in silage with about 109,461 cfu of LAB/g and is close to the minimum recommended application rate for classical (homolactic acid) bacterial inoculants (Kung et al., 2003). For LB, a theoretical starting concentration was targeted at 8.8 log cfu/mL because the recommended final application rate of this inoculant is 400,000 cfu/g of forage. A 75-mL sample of each inoculant mix was placed in duplicate 125-mL Erlenmeyer flasks with cotton plugs and set in an incubated orbital shaker set at 125 rpm (model C24, New Brunswick Scientific Co. Inc., Edison, NJ). The first 6 h with shaking was used to simulate movement of the liquid inoculant in a tank mounted on a chopper in the field during harvest. The first 6 h for each SEQ was as follows: a) SEQ 1: 30°C, b) SEQ 2: 35°C, c) SEQ 3: 40°C, and d) SEQ 4: 45°C. It took about 20 min for flasks to reach the target incubation temperature after being placed in the incubator. After 6 h, the incubator temperature was reduced to 30°C for an additional 18 h without shaking. This period was meant to simulate unused inoculant left over in a tank. Each SEQ was run twice. Samples were taken from each flask at 0, 3, 6, and 24 h and enumerated on MRS agar to determine the numbers of LAB (as previously described).

Numbers of LAB were converted to log₁₀ scale. Data from each sequence were analyzed separately with main effects of inoculant, sampling time, and inoculant × time interaction and analyzed as repeated measures using the PROC MIXED procedure of SAS (SAS Institute, 1999). Significance was declared at $P < 0.05$. Data are reported as least squares means with SEM. When significant effects were detected among treatments,

Table 1. Effect of incubating silage inoculants in water at 30°C for 24 h on numbers of lactic acid bacteria (log₁₀ cfu/mL; SEM = 0.12)

Inoculant ¹	Incubation time, h			
	0	3	6	24
Eco	8.22	8.20	8.46	8.27
LB	8.62 ^a	8.61 ^a	8.68 ^a	8.11 ^b
P1127	8.23 ^a	8.20 ^a	7.96 ^a	7.39 ^b
BP11	8.44 ^a	7.96 ^b	8.12 ^{ab}	7.10 ^c
BMP	8.06	8.02	8.13	7.92
BM5	8.44 ^a	8.26 ^a	8.36 ^a	7.86 ^b

^{a-c}Means in rows with unlike superscripts differ, $P < 0.05$.

¹Eco = *Lactobacillus plantarum*, strain MTD/1, Ecosyl Products Ltd., Stokesely, UK; LB = *L. buchneri* 40788, Lallemand, Milwaukee, WI; P1127 = Pioneer 1127 Grass/Legume Silage Inoculant (*L. plantarum* and *Enterococcus faecium*) Pioneer Hi-bred Intl., Johnston, IA, lot number 368711A); BP11 = Biotol Plus II (*Pediococcus pentosaceus*, *Propionibacteria freudenreichii* and enzymes) Lallemand; BMP = Biomax MP Multipurpose Microbial Silage Inoculant (*L. plantarum* and *P. pentosaceus*) Chr. Hansens Biosystems, Milwaukee, WI; and BM5 = Biomax 5 (*L. plantarum*, strains PA-28 and K-270) Chr. Hansens Biosystems.

mean separation was tested using the PDIFF option in SAS.

The initial average starting concentration of the classical bacteria at time 0 was 8.2 log cfu/mL across all SEQ (Tables 1 to 4). For LB, the initial average starting concentration of bacteria at time 0 was 8.6 log cfu/mL across all SEQ (Tables 1 to 4). The results of SEQ 1 are shown in Table 1. Through 6 h of incubation at 30°C, the viability of LAB for Eco, LB, P1127, BMP, and BM5 in water remained constant when compared with their numbers at time 0. Numbers of LAB were lower after 3 h of incubation for BP11 but not at 6 h, suggesting that this might have been an aberration

Table 2. Effect of incubating silage inoculants in water at 35°C (0 to 6 h) followed by incubation for an additional 18 h at 30°C on numbers of lactic acid bacteria (log₁₀ cfu/mL; SEM = 0.10)

Inoculant ¹	Incubation time, h			
	0	3	6	24
Eco	8.20	8.12	8.41	8.25
LB	8.44 ^a	8.41 ^a	8.45 ^a	8.01 ^b
P1127	8.10 ^a	7.96 ^a	7.84 ^a	7.24 ^b
BP11	8.42 ^a	7.95 ^b	7.17 ^c	6.96 ^c
BMP	7.96	8.00	8.02	7.96
BM5	8.46 ^a	8.20 ^b	7.87 ^b	7.01 ^c

^{a-c}Means in rows with unlike superscript differ, $P < 0.05$.

¹Eco = *Lactobacillus plantarum*, strain MTD/1, Ecosyl Products Ltd., Stokesely, UK; LB = *L. buchneri* 40788, Lallemand, Milwaukee, WI; P1127 = Pioneer 1127 Grass/Legume Silage Inoculant (*L. plantarum* and *Enterococcus faecium*) Pioneer Hi-bred Intl., Johnston, IA, lot number 368711A); BP11 = Biotol Plus II (*Pediococcus pentosaceus*, *Propionibacteria freudenreichii* and enzymes) Lallemand; BMP = Biomax MP Multipurpose Microbial Silage Inoculant (*L. plantarum* and *P. pentosaceus*) Chr. Hansens Biosystems, Milwaukee, WI; and BM5 = Biomax 5 (*L. plantarum*, strains PA-28 and K-270) Chr. Hansens Biosystems.

from sampling. After 24 h, numbers of LAB for Eco and BMP were not different from those at time 0, but were lower in LB (decrease of 0.51 log cfu/mL from time 0), P1127 (decrease of 0.84 log cfu/mL from time 0), BP11 (decrease of 1.34 log cfu/mL from time 0), and BM5 (decrease of 0.58 log cfu/mL from time 0).

Initial incubation at 35°C for 3 h resulted in findings that were similar to those found in SEQ 1 for all inoculants (Table 2). Numbers of LAB in Eco and BMP mixes were stable throughout the entire 24 h of SEQ 2. However, there were substantial decreases in LAB numbers for BP11 (decrease of 1.25 log cfu/mL from time 0) and BM5 (decrease of 0.59 log cfu/mL from time 0) inoculants after 6 h. The most marked declines in number of LAB over 24 h occurred for BP11 and BM5. The average numbers of LAB for these inoculants at time 0 was 8.44 log cfu/mL. However, the final concentration of these LAB averaged 6.99 log cfu/mL.

Only LAB counts in Eco and BMP were unaffected by the initial incubation temperature of 40°C and viability was maintained for these inoculants throughout the 24 h of incubation (Table 3). After only 3 h of incubation at this temperature, decreases in viable counts from time 0 were noted for LB (−0.54 log cfu/mL), P1127 (−1.00 log cfu/mL), BP11 (−0.63 log cfu/mL), and BM5 (−0.49 log cfu/mL). Further reductions in cell numbers were not observed for LB and P1127 after 6 h of incubation. Cell numbers continued to decrease between 3 and 6 h for BP11 and BM5. The greatest reduction in viable LAB after 24 h of incubation was noted for BM5 (−2.28 log cfu/mL decrease from time 0).

When incubated at 45°C the counts of viable bacteria decreased after 3 h of incubation for all inoculants except for Eco (Table 4). At the 6-h sampling point, declines in viable counts of bacteria were noted for all treatments, with the greatest decreases for BP11, BMP, and BM5. After an additional 18 h of incubation at 30°C, numbers of viable bacteria remained the same for P1127, BP11, and BM5 but declined further for BMP. In contrast, viable bacteria increased from the 6-h to the 24-h sampling point for Eco and LB. The counts of viable bacteria for Eco at 24 h were the same as at time 0.

It is well known that temperatures above the growth optimum for bacteria have detrimental effects on their metabolism and affect their viability (Moats, 1971). At temperatures slightly above that required for optimal growth, bacteria respond to thermal stress by rapid induction of heat-shock proteins meant to help with adaptation (Gould, 1989). Microbes grown at temperatures higher than that required for optimal growth often become more thermotolerant. However, at high temperatures, significant changes in cell physiology

Table 3. Effect of incubating silage inoculants in water at 40°C (0 to 6 h) followed by incubation for an additional 18 h at 30°C on numbers of lactic acid bacteria (log₁₀ cfu/mL; SEM = 0.14)

Inoculant ¹	Incubation time, h			
	0	3	6	24
Eco	8.06	8.08	8.24	8.16
LB	8.68 ^a	8.14 ^b	8.12 ^b	7.84 ^b
P1127	8.28 ^a	7.28 ^b	7.08 ^b	7.07 ^b
BP11	8.09 ^a	7.46 ^b	6.58 ^c	6.26 ^c
BMP	8.00	7.98	7.82	7.69
BM5	8.16 ^a	7.67 ^b	6.66 ^c	5.88 ^d

^{a-d}Means in rows with unlike superscript differ, $P < 0.05$.

¹Eco = *Lactobacillus plantarum*, strain MTD/1, Ecosyl Products Ltd., Stokesely, UK; LB = *L. buchneri* 40788, Lallemand, Milwaukee, WI; P1127 = Pioneer 1127 Grass/Legume Silage Inoculant (*L. plantarum* and *Enterococcus faecium*) Pioneer Hi-bred Intl., Johnston, IA, lot number 368711A); BP11 = Biotol Plus II (*Pediococcus pentosaceus*, *Propionibacteria freudenreichii* and enzymes) Lallemand; BMP = Biomax MP Multipurpose Microbial Silage Inoculant (*L. plantarum* and *P. pentosaceus*) Chr. Hansens Biosystems, Milwaukee, WI; and BM5 = Biomax 5 (*L. plantarum*, strains PA-28 and K-270) Chr. Hansens Biosystems.

occur. For example, Teixeira et al. (1997) reported that for *Lactobacillus bulgaricus*, initial heating to 62°C resulted in cell wall damage and further heating (65°C and above) resulted in denaturation of ribosomes and proteins.

Exposing LAB in our study to heat resulted in some cell death or injury that prevented their subsequent growth in MRS agar. Although the temperatures tested in this study were not extremely high (30 to 45°C), bacteria were also probably subjected to nutrient starvation during incubation because we did not add any fermentable substrate to the flasks. Lack of fermentable substrate was supported by the fact that there was no growth during the incubation periods at the lower temperatures. Spinks et al. (2006) reported that some bacteria are more susceptible to heat stress when they are prestarved of nutrients. Thus, in the current study, a lack of nutrients more than likely added another major stress to the organisms that reduced their viability when they were exposed to the higher temperatures (40 and 45°C). The effect varied with the specific inoculant and the temperatures to which they were exposed. Exposure of applicator tanks to solar radiation and heat from engines on tractors could result in the water and inoculant mixes to reach stressful temperatures. If an applicator tank contained an inoculant mix with an initial concentration of 8.3 log cfu/mL, applying 500 mL to 1 t of forage would supply 109,750 cfu of LAB/g of forage. A decrease in the concentration of LAB in the tank (due to excessive heat) of 0.5, 1, or 2 log cfu/mL would result in theoretical final application rates of 34,706, 10,975, and 1,098 cfu/g of silage, respectively. These values are well be-

Table 4. Effect of incubating silage inoculants in water at 45°C (0 to 6 h) followed by incubation for an additional 18 h at 30°C on numbers of lactic acid bacteria (log₁₀ cfu/mL; SEM = 0.19)

Inoculant ¹	Incubation time, h			
	0	3	6	24
Eco	8.01 ^a	7.68 ^a	7.25 ^b	8.01 ^a
LB	8.71 ^a	7.85 ^b	7.06 ^c	8.03 ^b
P1127	8.30 ^a	7.16 ^b	6.82 ^{bc}	6.77 ^c
BP11	8.00 ^a	6.06 ^b	5.24 ^c	5.68 ^c
BMP	8.06 ^a	7.52 ^b	5.96 ^c	5.50 ^d
BM5	8.26 ^a	4.82 ^b	4.41 ^b	4.70 ^b

^{a-d}Means in rows with unlike superscript differ, $P < 0.05$.

¹Eco = *Lactobacillus plantarum*, strain MTD/1, Ecosyl Products Ltd., Stokesely, UK; LB = *L. buchneri* 40788, Lallemand, Milwaukee, WI; P1127 = Pioneer 1127 Grass/Legume Silage Inoculant (*L. plantarum* and *Enterococcus faecium*) Pioneer Hi-bred Intl., Johnston, IA, lot number 368711A); BP11 = Biotol Plus II (*Pediococcus pentosaceus*, *Propionibacteria freudenreichii* and enzymes) Lallemand; BMP = Biomax MP Multipurpose Microbial Silage Inoculant (*L. plantarum* and *P. pentosaceus*) Chr. Hansens Biosystems, Milwaukee, WI; and BM5 = Biomax 5 (*L. plantarum*, strains PA-28 and K-270) Chr. Hansens Biosystems.

low the recommended concentration of 100,000 bacteria per g of silage for many inoculants (Kung et al., 2003). (The recommended rate of *L. buchneri* 40788 used in this study is 400,000 cfu/g for silages and 600,000 cfu/g for high-moisture corn.) Thus, even small decreases in viability of LAB would most likely result in the inability of the added LAB to dominate a silage fermentation process.

Based on the results of our study, it is evident that the thermotolerance of LAB is an important factor that could affect the efficacy of the organisms used as silage inoculants because their viability can be affected before application. Thermotolerant LAB may also be more likely to survive elevated temperatures that could be reached during ensiling. Pahlow et al. (2003) stated that many lactic acid bacteria have growth temperature optimums between 25 and 40°C but specific data on thermotolerance of bacteria used in silage inoculants are lacking. Zhang et al. (2000) reported that *L. plantarum* CA 28 found in grass silage did not grow in pure culture when incubated at a temperature of 40°C or above. Muck et al. (2003) stated that higher temperatures increase the rate of silage fermentation as long as these temperatures do not exceed the optimum for LAB in the forage mass. Ohmomo et al. (1996) suggested that poor silage quality and the inability of commercial inoculants of LAB to be effective may be due to high temperatures (45°C or above) attained during the early stages of ensiling. We have measured temperatures in the top 20 cm of forages during the early stages of fermentation that have reached as high as 48°C (L. Kung Jr., personal observation). Kim and Adesogan (2006) reported that corn silage stored at

40°C underwent a restricted fermentation with more proteolysis and lower lactic:acetic ratio than silage stored at 20°C. Similar results have been reported by Weinberg et al. (1998, 2001). Selection of LAB that are thermotolerant could overcome this problem. For example, Zhang et al. (2000) isolated 8 strains of epiphytic LAB from forage crops and although all strains improved silage fermentation at 25°C, only one strain was capable of this when the temperature was 45°C.

Lactobacillus plantarum (MTD/1) in Eco was the most thermotolerant of the inoculants tested in our study. It was the least affected by incubation at 45°C compared with the other inoculants. Both *L. plantarum* (MTD/1) from Eco and *L. buchneri* 40788 from LB appeared to have better heat tolerance after heat shock than the other organisms, because their numbers increased substantially between 6 and 24 h but only *L. plantarum* (MTD/1) fully recovered to numbers of bacteria that were present at time 0. *Lactobacillus plantarum* MTD/1 and *L. buchneri* 40788 appear to be organisms that would also be able to withstand moderately high temperatures (40 to 45°C) during ensiling. Thermotolerance of *L. plantarum* MTD/1 in Eco may be one reason that this inoculant has been extremely successful (Gordon, 1989; Heron, 1991; Kung et al., 1991). The specific reasons for better thermotolerance in *L. plantarum* MTD/1 and *L. buchneri* 40788 are unknown, but adaptation of bacteria at moderately high temperatures (40 to 50°C) during growth has resulted in bacteria with more thermotolerance when they are heat shocked (55 to 65°C; Mushtaq et al., 2002; De Angelis et al., 2004).

We did not determine the possibility of long-term cell recovery, which has been shown in some bacteria. For example, Dabbah et al. (1969) reported that although *Pseudomonas* spp. were exposed to heat that prevented growth on agar plates, some cells were able to recover and grow in liquid medium 10 d after the initial exposure to heat. Because a rapid drop in pH at the start of ensiling is an important factor for a good fermentation, even if some heat-shocked LAB are able to recover days later, their numbers would probably be considerably lower and the lag in their recovery would most likely make them less competitive in a silage environment.

We tested the viability of several commercial silage inoculants when they were exposed to water of different temperatures. The effects were variable depending on temperature and inoculant. Most inoculants were relatively stable when exposed to 30 and 35°C for 3 to 6 h. However, exposure to 40 and 45°C resulted in marked reductions in viable cells within 3 h for some inoculants. *Lactobacillus plantarum* MTD/1 was the most thermotolerant bacteria tested. We suggest using

microbial inoculants that are thermotolerant and for most inoculants, care should be taken to ensure that the temperature of the water they are in does not exceed 35 to 40°C. In addition, the use of insulated applicator tanks and the practice of putting ice packs in the tanks may help to keep water temperatures cool.

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