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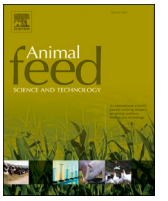

# Influence of heat stimulus temperature and ensiling temperature on growth and performance of silage inoculants

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ABSTRACT

The main objective of this research was to observe patterns of fermentation between silages inoculated with lactic acid bacteria exposed to high, or low, levels of heat stimulus and corresponding ensiling temperatures. Commercial inoculants in liquid media were exposed to heat stimulus for 24 h at 30°C (low heat stimulus: LHS) and 40°C (high heat stimulus: HHS) to test growth. All inoculants showed significant inhibition of growth in liquid culture at 40℃ compared to 30℃. Heat-challenged inoculants were then applied to mini silos of chopped, whole-plant corn incubated at 30°C and 45°C. Mini silos containing Inoculant 11 (*Pediococcus pentosaceus* 12455 and *Lentilactobacillus buchneri* (formerly designated *Lactobacillus buchneri*) 40788 had the lowest pH when ensiled at 45ºC regardless of prior heat stimulus level but was significantly lower in the HHS group. Despite their poor performance in liquid culture prior to ensiling, inoculants 6, 7, 10 and 11 all showed significant improvement in silage pH after high-heat stimulus. In conclusion, prior exposure to heat stimulus produced varied effects on the performance of silage inoculants in liquid culture and during ensiling. Exposure to HHS resulted in lower silage pH values and varied fermentation profiles for some inoculants when compared to LHS or uninoculated controls. The results of the current study provide initial evidence that heat acclimatization of silage inoculants warrants further investigation for industrial and scientific agricultural applications.

## 1. Introduction

Ensiling is the process of preserving forage by the conversion of carbohydrates into organic acids. Successful ensiling depends primarily on the epiphytic lactic acid bacteria (LAB) present on the crop (Pahlow et al., 2003). However, it is possible to improve fermentation through the addition of microbial inoculants. Inoculants facilitate the ensiling process by increasing the likelihood or degree of conservation, accelerating fermentation, increasing nutrient availability to the animal, and improving silage hygiene (Muck et al., 2018; Wilkinson and Muck, 2018).

In the 20th century, silage additives were used largely to ensure a fermentation dominated by LAB and/or improve aerobic stability. The functional metabolic groups of LAB are subject to some debate among microbiologists (Muck et al., 2018). But, for practical purposes, the two main types of silage inoculants include traditional homo-fermenters, such as *Pediococcus* species and *Enterococcus faecium*, that convert 6-carbon sugars into a single product, lactic acid. In contrast, hetero-fermentative bacteria, such as

*Abbreviations:* LHS, low heat stimulus; HHS, high heat stimulus; LAB, lactic acid bacteria; MRS, De Man–Rogosa–Sharpe agar; DM, dry matter.

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*Lentilactobacillus buchneri and* facultatively heterofermentative *Lactiplantibacillus plantarum* produce multiple products from the metabolism of 6-carbon sugars.

Most LAB species found in silages have optimal growth temperatures near 30ºC and do not grow above 45ºC (McDonald et al., 1991). Common anaerobic spoilage organisms like clostridia generally have higher optimal growth temperatures and may grow at temperatures greater than 45ºC. The disparity between conducive growth conditions for the desirable LAB and undesirable clostridia raise concerns regarding high ambient temperatures and potential effects on ensiling. Higher ensiling temperatures typically lead to a shift from homolactic to heterolactic microbial populations, but the majority of silage microbiological knowledge focuses on optimal fermentation conditions, rather than optimal outcomes under environmental extremes (Muck et al., 2018).

Microorganisms grown under heat stimulus, temperatures higher than their optimal, can become acclimated and more thermotolerant (Mulrooney and Kung, 2008). At the same time, higher temperatures may also induce significant cell wall damage and denaturation of ribosomes and proteins (Teixeira et al., 1997). Heat acclimatization and heat stimulus are not mutually exclusive, and with higher temperatures, diminishing returns from acclimatization are expected as temperatures approach the limit of an organism's heat tolerance plasticity.

High temperatures and unexpected severe weather events impact industries that rely on forage preservation. Solutions to this challenge will likely include novel inoculants and procedures. Characterizing the heat tolerance of available commercial inoculants is a logical first step in developing best practices for a changing climate. We hypothesize that heat-acclimated inoculants will better conserve forages ensiled at high temperatures when compared to inoculants grown with lower levels of heat stimulus.

The objective of this study was to expose common silage inoculants to heat stimulus and then introduce them to whole plant corn silage in order to estimate their acclimatization and fermentation potential after stimulus. Freeze-dried LAB were tested for growth at 30 ºC, 35 ºC, 40 ºC, and 45 ºC, to measure baseline plasticity. Surviving cultures were subjected to heat stimulus and silage inoculation. Surviving cultures were exposed to 30 ºC and 40 ºC for heat stimulus and then used to inoculate silage corn incubated at 30 ºC and 45 ºC throughout ensiling. Other objectives of this study investigated the patterns of fermentation by estimating pH, dry matter loss, and lactic acid content between silages inoculated with LAB exposed to high- and low- levels of heat stimulus and corresponding ensiling temperatures.

## 2. Materials and methods

### 2.1. Inoculant cultivation

The commercial silage inoculants used in this study are described in Table 1. All inoculants were purchased independently and kept frozen at −20ºC.

Freeze-dried inoculants were aseptically rehydrated in 4 ml of ultra-pure dH20, following the concentration recommendations of each manufacturer (Table 1), and vortexed to mix. After 30 min, 100 µl were aseptically transferred to 2 ml of autoclaved MRS broth in quadruplicates. Inoculants composed of more than one species of LAB were treated identically to single-species inoculants and grown in co-culture. The 27 ml glass tubes were incubated at 25C on a shaker at 150 rpm overnight. The cultures were serially diluted $10^4$-fold, and an MRS agar plate was made for each tube. The plates were incubated at 25C, and after 72 h, the colony morphology observed was identical to those reported for LAB, and then they were streaked for isolation on subsequent plates.

**Table 1**
Description of inoculants used in the study.

| Legend | Strain | Manufacturer Location | Concentration (cfu/g) |
|---|---|---|---|
| Inoc 1 | *Lentilactobacillus buchneri* LN4637 | Johnston, IA, USA | 10 × 1010 |
| Inoc 2 | *Lactiplantibacillus plantarum* (No strain specified)<br>*Enterococcus faecium* (No strain specified) | Johnston, IA, USA | 9.0 × 1010 |
| Inoc 3 | *Lactiplantibacillus plantarum* CH6072 & LSI<br>*Pediococcus pentosaceus* P6 | Milwaukee, WI, USA | 4.55 × 1010 |
| Inoc 4 | *Lactiplantibacillus plantarum* MTD/1 NCIMB 40027 | North Yorkshire, UK | 9.1 × 1010 |
| Inoc 6 | *Lentilactobacillus buchneri* ATCC PTA−2494<br>*Enterococcus faecium* ATCC 55593<br>*Lactiplantibacillus plantarum* ATCC 53187 & ATCC 55942 | Johnston, IA, USA | 11 × 1010 |
| Inoc 7 | *Lactiplantibacillus plantarum* (No strain specified)<br>*Enterococcus faecium* (No strain specified) | Johnston, IA, USA | 12.5 × 1010 |
| Inoc 8 | *Lactobacillus delbrueckii subsp. Lactis* (No strain specified)<br>*Lactiplantibacillus plantarum (Aber F−1 & L−54)* | Deforest, WI, USA | 6.0 × 1010 |
| Inoc 9 | *Lactiplantibacillus plantarum* MA18/5U<br>*Lentilactobacillus buchneri* NCIMB 40788 | Blagnac, France | > 2.10 × 1010<br>> 3.10 × 1010 |
| Inoc 10 | *Pediococcus pentosaceus* NCIMB 12455<br>*Lactiplantibacillus plantarum* NCIMB 12422<br>*Propionobacterium freudenreicheii* NCIMB R2453 | Milwaukee, WI, USA | > 4.99 × 1010 |
| Inoc 11 | *Pediococcus pentosaceus* NCIMB 12455<br>*Lentilactobacillus buchneri* NCIMB 40788 | Milwaukee, WI, USA | 1.25 × 1010<br>9.08 × 1010 |
| Inoc 12 | *Pediococcus pentosaceus* NCIMB 12455<br>*Propionibacterium fruedenreichii* NCIMB R2453 | Milwaukee, WI, USA | > 5.45 × 1010 |

Single colonies from each isolation plate, or a 1 cm streak for multi-species inoculants, were picked and placed in 15 ml MRS broth and incubated for 72 h at temperatures of 30℃, 35℃, 40℃, and 45℃ and pH values of 6 and 4. Inoculants selected were those that grew well at 40℃. No inoculants grew at 45℃ without prior exposure to heat stimulus.

### 2.2. Heat stimulus procedures

Selected colonies or consortia were grown in six replicate MRS broth tubes and incubated at 25℃ with 150 rpm shaking for 72 h. A hundred microliters of each culture was transferred to 15 ml of MRS broth in preparation for the heat stimulus segment of the experiment. Cultures were exposed to two heat stimulus temperature regimes for 24 h before application to silage. Three tubes of each culture were heat-acclimated at 30℃ for the low heat stimulus (LHS) treatment. The remaining three tubes of each culture were heat-acclimated at 40℃ for the high heat stimulus (HHS) treatment. Cultures were incubated for 24 h at their respective temperatures in an orbital shaker at 150 rpm. It is important to note that this approach serves as a proof-of-concept, demonstrating the potential acclimatization of inoculants to short exposures of heat. Another shortcoming of the approach is that it also does not guarantee the growth of each strain of multi-strain inoculants under these conditions.

Following heat stimulus incubation, 200 µl of each culture were divided into duplicate 10 ml tubes of MRS broth to test growth of both LHS and HHS cultures at higher and lower temperatures. All cultures were incubated at both 30℃ and 40℃ for 24 h in orbital shakers at 150 rpm. Growth was measured via optical density at 600 nm (OD600), determined via tandem spectrophotometry (Spectronic 21, Bausch & Lomb, Canada) and MRS plate count to estimate bacterial cell density. The relationship between optical density and bacterial cell density is described by the equation [y (cfu/ml) = 5,053x + 2915] where x: OD at 600 nm (Trabelsi et al., 2013)

### 2.3. Effects of cryopreservation on high heat stimulus culture performance

To ascertain the effects of cryopreservation on any potential heat acclimatization of HHS cultures, one milliliter of each culture was collected and preserved in 20 % glycerol and stored at −80℃. After 30 days at −80℃, cultures were revived in 10 ml MRS media broth and incubated at 40℃. And using a spectrophotometer (Spectronic 200E, Thermo Fisher Scientific, Waltham, MA) the optical density at 600 nm (OD 600) was determined from each tube after 24 h for comparison to growth kinetics prior to cryopreservation. Prior to measuring samples, a tube containing only MRS media broth was used to calibrate the machine to 0 absorbance at 600 nm i.e., blank.

### 2.4. Ensiling

Silage corn (Dairyland 3508RA) used for ensiling was grown at the United States Dairy Forage Research Center (USDFRC) Research Farm in Prairie du Sac, WI and was harvested at 38 % dry matter. Fresh corn, chopped to a 25.4 mm theoretical length of chop, was inoculated with LHS and HHS inoculant via pipette and mixed by hand. Laboratory silos were prepared in duplicates, one stored at 30℃ and the other at 45℃. Silos were prepared in 20 ml screw top vials packed by hand with 20 g of chopped corn, a target density of ~368 $Kg/m^3$, and inoculated with either 100 µl of LHS or HHS inoculants and ensiled for 30 days. Ensiling treatments were: 1) LHS inoculant ensiled at 30℃, 2) HHS inoculant ensiled at 30℃, 3) LHS inoculant ensiled at 45℃, 4) HHS inoculant ensiled at 45℃, with three replicates per treatment. In addition to the commercial inoculants exposed to heat stimulus, a control silage was prepared with sterile water added in place of inoculant. All vials were weighed before and after storage to calculate dry matter loss by the weight difference.

After 30 days, silos were opened and a subsample was collected and dried in a forced-air oven for 72 h at 55℃ to measure dry matter on an analytical balance scale with sensitivity — 0.001 g up to few micrograms (AOAC, 1990; method 934.01). Dry matter was estimated as the total mass lost upon drying, as is consistent with commercial laboratory testing methods. No correction for volatile compounds was applied because of the complexities of pH's effects on volatility and the use of a unique ensiling protocol. Five grams of subsample were weighed, added to 55 g of deionized water, and mixed for 1 min in a blender. The resulting water extracts were filtered with a Whatman P8 filter and the pH was measured on an Orion economy pH meter (Thermofisher, Waltham, MA, USA). Water extracts (1500 µl) were also prepared for (HPLC) High-Performance Liquid Chromatography (Shimadzu, Kyoto, Kyoto, Japan) to determine fermentation end products. HPLC analysis was used to quantify lactic acid, acetic acid, propionic acid, butyric acid, ethanol, 1,2-propanediol, and isobutyrate (modified from Siegfried et al., 1984). To determine the degree of protein hydrolysis, the ninhydrin colorimetric method was employed to quantify amines in conjunction with a leucine standard (Winters et al., 2002). The ninhydrin assay was used to estimate proteolysis due to its specificity in detecting free amino acids, which are products of proteolysis. In contrast, the Ammonia Nitrogen (NH3-N) method measures total nitrogen, including components that are not specific to proteolysis. Degree of proteolysis was further calculated from the difference in detected amines before and after ensiling.

### 2.5. Statistical analyses

Inoculant growth as a response variable was evaluated via ANOVA, with three factors (heat stimulus temperature: 30℃ or 40℃ (S); incubation temperature: 30℃ or 45℃ (IT); inoculants (I)) using R (RStudio, version 1.3.959, agricolae package). The cryopreservation for treatment HHS incubated at 45℃ was included in the model considering a heat stimulus factor. The statistical analyses for ensiling parameters were evaluated via ANOVA with three factors (heat stimulus temperature: 30℃ or 40℃ (S); ensiling temperature: 30℃ or 45℃ (ET); and inoculants (I)). Data were evaluated for violations of the assumption of normal distribution and the presence of outliers within the samples were assessed. Post-hoc analysis using Tukey's honest significant difference (HSD) was conducted to assess the

significant differences in the means. Significance was considered at an α-level of 0.05 i.e., significant differences were accepted if $P < 0.05$. The analyses and figures were created using the ggplot2 package in R and Microsoft Excel.

## 3. Results

The eight inoculants selected to test acclimatization were determined based on inoculants that grew strongly at 40℃ (Table 2).

### 3.1. Growth response of stimulus-acclimatized inoculants

As expected, heat-stimulused inoculants grew better when incubated at 30℃ than when incubated at 45℃ (Fig. 1). Cell density of LAB after 24 h growth among treatments and following cryopreservation of HHS cultures are compared in Fig. 1. All inoculants showed significant inhibition of growth in liquid culture at 45℃ compared to growth at 30℃ (Table 2). However, compared to LHS exposure and incubation at 30 ℃, a slight numerical trend of increased growth was observed for inoculants 1, 3, 4, and 11 after HHS exposure and incubation at the same temperature (Fig. 1). Growth patterns for heat stimulus by growth temperature interactions are more complex. Inoculants 2, 3, 4, 6, 7, and 10 grew poorly at 45℃ regardless of prior heat stimulus treatments. Inoculant 1 tolerated 45℃ cultivation with higher growth than previously mentioned inoculants, but no difference between its exposure to HHS and LHS treatments (Fig. 1). Inoculant 11 adapted better to growing in 45℃ after HHS as it displayed growth at 45℃ similar to its performance at 30℃ when acclimatized to LHS (Fig. 1). HHS culture samples cryopreserved in 20 % glycerol at −80℃ revived and cultured in MRS for 24 h at 45℃ performed similarly to HHS cultures prior to cryopreservation, including Inoculant 11 (Fig. 1).

### 3.2. Silage quality after ensiling for 30 days at 30°C and 45°C

Silage samples showed clear differences in quality between ensiling temperatures of 30°C and 45°C. Silos ensiled at 45°C showed a nearly uniform response of significant increases in final pH and DM loss during ensiling (Fig. 2). Notably, samples inoculated with HHS Inoculant 11 and ensiled at 45°C showed equivalent pH and DM loss values as seen when ensiled at 30°C. Silage pH values between HHS and LHS inoculants were largely indistinguishable at 30°C, but at 45°C HHS inoculants 6, 7, 10, and 11 silages had significantly lower pH values. Dry matter loss among samples showed the most variability from inoculant to inoculant, with some non-systematic differences by ensiling temperature (Fig. 2). However, the range of dry matter loss between HHS and LHS inoculants was moderately similar irrespective of ensiling temperatures. HHS inoculants 1, 4, and 7 produced significantly improved DM recovery in silage at 45°C when compared to LHS inoculated silage samples. LHS inoculants 1 and 7, when ensiled with silage at 45 ℃, showed a significant increase in DM loss when compared to ensiling at 30 °C (Fig. 2).

### 3.3. Fermentation profile of inoculated silage samples

Fermentation profiles of inoculated silages revealed significant shifts across heat stimulus treatment of inoculants, ensiling temperature, and inoculants (Table 3). Overall, ensiling at 45°C uniformly decreased concentrations of lactic acid, acetic acid, and ethanol as determined by HPLC. The ratios of lactic acid to acetic acid were variable in the study, with variation driven by changes in both lactic and acetic acid concentrations (Fig. 3). Contributing to the variation is the presence of homofermentative and heterofermentative species within the inoculants, especially for inoculants with more than one species. HHS inoculants 1, 7, and 11 revealed estimates of protein degradation via ninhydrin assay were increased over time point 0, T0 control values. Overall, patterns of difference across ensiling temperatures and heat stimulus treatments were not uniform. In general, LHS and HHS inoculants produced similar amine values at 45°C, but HHS inoculants 1, 2, 3, 6, and 7 all displayed significantly lower amine concentration values at 30°C.

## 4. Discussion

High temperature is an important factor limiting the benefits of silage LAB inoculation in tropical and subtropical locations due to heat stimulus inhibition (McDonald et al., 1991; Ohmomo et al., 1995). Successful use of inoculants is dependent on the species/strain chosen and dosage of viable bacteria (Mulrooney and Kung, 2008). The temperature within a silage pile, particularly in the tropics and

**Table 2**
Evaluation of growth at different temperatures and pH.

| Inoculant | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Control |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 30℃ | + | + | + | + | + | + | + | + | + | + | + | + | - |
| 35℃ | + | + | + | + | + | + | + | + | + | + | + | + | - |
| 40℃ | + | + | + | + | W | + | + | W | W | + | + | W | - |
| 45℃ | + | W | W | W | - | W | W | - | - | W | + | - | - |
| 30℃ @ pH 4 | + | + | + | + | + | + | + | + | + | + | + | + | - |
| 40℃ @ pH 4 | + | + | + | W | W | + | + | W | W | + | + | W | - |

+ : 90 % or more of the strains positive
-: 90 % or more of the strains negative
W: weakly positive.

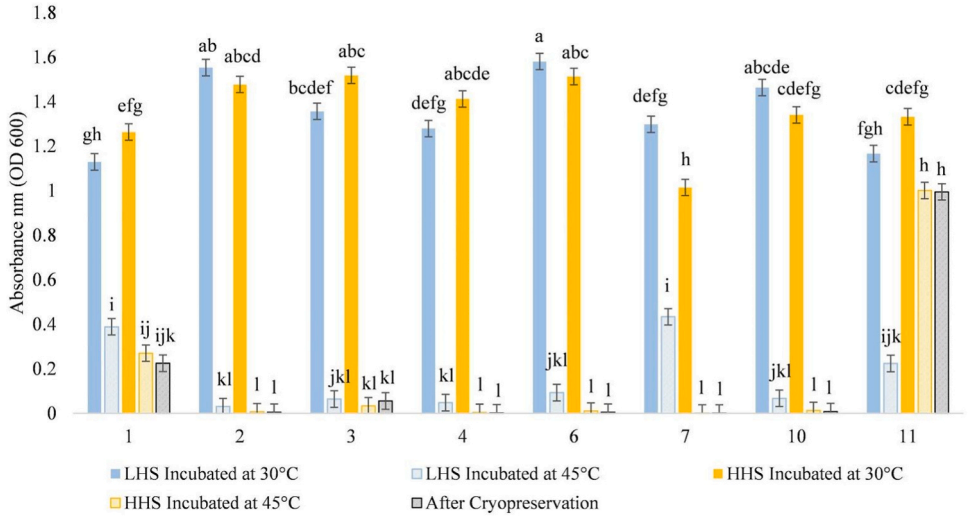


**Fig. 1.** Effects of low (30°C) and high (40°C) heat stimulus on microbial cell density as determined by absorbance of inoculant cultures at 600 nm (OD600) following incubation at 30°C or 45°C after 24 h. [Gray bars represent the cell density of freeze-dried HHS samples revived and cultured directly into 45°C MRS broth; bars that share at least one letter (from pairwise comparisons of means using Tukey's honest significant difference) are not significantly different; *p < 0.01 for inoculant × heat stimulus temperature x incubation temperature interaction. Standard error of the mean = 0.11.].

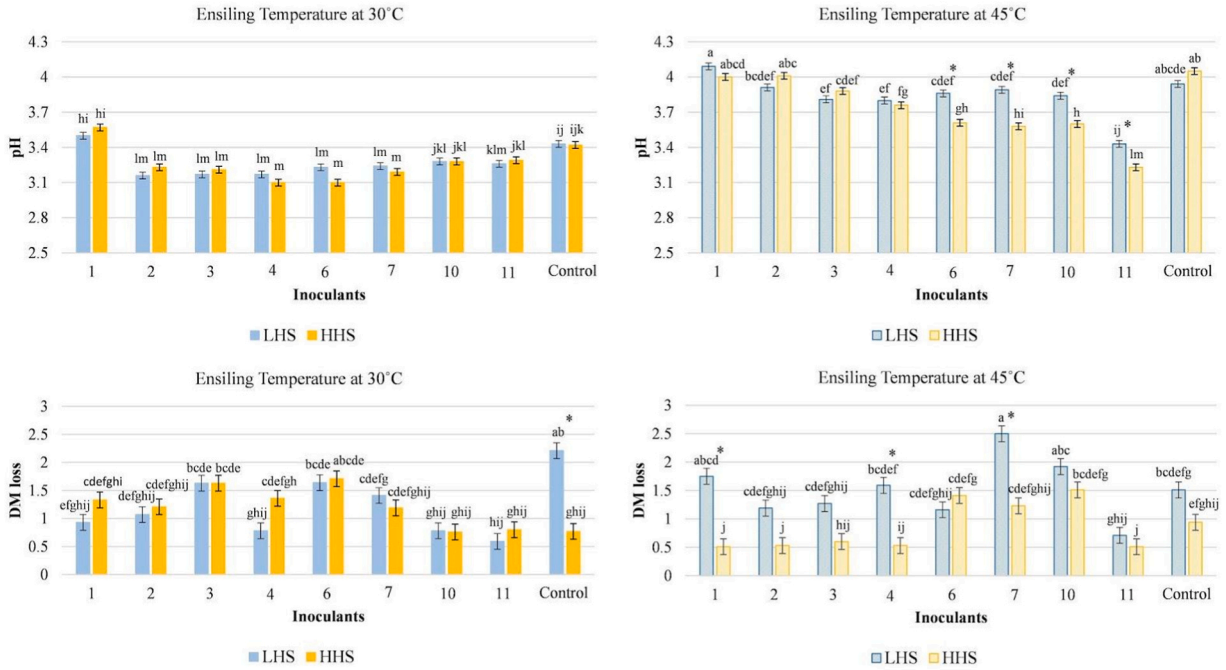


**Fig. 2.** Effects of low (30°C) and high (40°C) heat stimulus on silage pH and DM loss when ensiled at 30°C or 45°C after 30 days. [pH: p < 0.01 for inoculant × heat stimulus temperature x ensiling temperature interaction. Standard error of the mean = 0.03. DM loss: p < 0.01 for inoculant × heat stimulus temperature x ensiling temperature interaction. Standard error of the mean = 0.14. Asterisks denote statistical differences between LHS and HHS responses for each inoculant, and bars that share at least one letter (from pairwise comparisons of means using Tukey's honest significant difference) are not significantly different].

subtropics, may rise to more than 40°C at the start of ensiling due to oxygen contained within the forage matrix supporting plant and microbial respiration (Bernardes et al., 2018). Higher ensiling temperatures decrease silage quality, typically by limiting acidification through decreased organic acid production and increased volatilization (Kim and Adesogan, 2006). These conditions promote spoilage losses and are particularly favorable to clostridial growth (McDonald et al., 1966), which impact animal health, production, and

**Table 3**
Effects of low (30C) and high (45) heat stimulus on silage quality when inoculants were ensiled at 30C or 45C after 30 days. *LHS = low heat stimulus (30C). HHS = high heat stimulus (40C).

| Inoculant | Ensiling Temperature | Heat stimulus | [b]Lactic acid, %DM | [a]Acetic acid, %DM | Propionic acid, %DM | [a]Isobutyrate, g/kg DM | [a]Ethanol, % DM | [bΔ]Ninhydrin, g/kg DM |
|---|---|---|---|---|---|---|---|---|
| 1 | 30C | LHS | 2.95j | 5.81a | 0.10 | 0.03 f | 0.12efgh | 2.69abc |
| | | HHS | 2.85j | 4.30b | 0.03 | 0.01jklm | 0.12efgh | 1.13defg |
| | 45C | LHS | 0.72k | 0.63efgh | 0.13 | 0.02hijkl | 0.03hf | 1.36defg |
| | | HHS | 1.02k | 0.48efghijk | 0.02 | 0.05e | 0.04gh | 1.04defg |
| 2 | 30C | LHS | 6.13[a] | 0.63efg | 0.15 | 0.01lmn | 0.46b | 3.05[a] |
| | | HHS | 5.13bcdef | 0.63efgh | 0.10 | 0.03fgh | 0.33bcd | 0.70fg |
| | 45C | LHS | 1.05k | 0.35jk | 0.13 | 0.01lmn | 0.04gh | 1.64bcdef |
| | | HHS | 1.06k | 0.35jk | 0.04 | 0.05de | 0.04gh | 1.33defg |
| 3 | 30C | LHS | 5.18abcdef | 0.66ef | 0.26 | 0.03fghi | 0.62a | 2.72ab |
| | | HHS | 4.24fgh | 0.66ef | 0.07 | 0.02ghij | 0.34bcd | 0.77efg |
| | 45C | LHS | 1.29k | 0.33jk | 0.14 | 0 n | 0.03 h | 1.32defg |
| | | HHS | 1.06k | 0.24jk | 0.05 | 0.06 cd | 0.04gh | 1.06defg |
| 4 | 30C | LHS | 6.04ab | 0.57efghij | 0.13 | 0.01jklm | 0.44b | 0.65fg |
| | | HHS | 5.32abcde | 0.71e | 0.07 | 0.01jklm | 0.38bc | 0.87efg |
| | 45C | LHS | 1.29k | 0.32jk | 0.14 | 0.02ghijk | 0.03 h | 0.90efg |
| | | HHS | 0.75k | 0.45fghijk | 0.07 | 0.02ghijkl | 0.02 h | 1.02defg |
| 6 | 30C | LHS | 5.57abc | 0.54efghijk | 0.18 | 0.03 fghi | 0.45b | 1.88bcde |
| | | HHS | 5.66abc | 0.61efghi | 0.03 | 0.02ghijk | 0.26cde | 0.61fg |
| | 45C | LHS | 1.04k | 0.33jk | 0.15 | 0.03 f | 0.03 h | 0.89efg |
| | | HHS | 1.33k | 0.33jk | 0.03 | 0.05de | 0.03 h | 1.08defg |
| 7 | 30C | LHS | 4.57defgh | 0.50efghijk | 0.11 | 0.01jklm | 0.42b | 1.62bcdef |
| | | HHS | 4.82cdefgh | 0.51efghijk | 0.13 | 0.03 f | 0.46b | 0.31 g |
| | 45C | LHS | 0.82k | 0.40ghijk | 0.08 | 0.02ijkl | 0.05fgh | 1.57cdef |
| | | HHS | 1.30k | 0.31k | 0.12 | 0.06bc | 0.04gh | 0.81efg |
| 10 | 30C | LHS | 5.50abcd | 0.66ef | 0.07 | 0.01mn | 0.37bc | 1.64bcdef |
| | | HHS | 4.11gh | 0.67ef | 0.08 | 0.03 fg | 0.15efgh | 0.61fg |
| | 45C | LHS | 1.23k | 0.42fghijk | 0.11 | 0.02hijkl | 0.04fgh | 1.17defg |
| | | HHS | 1.16k | 0.39ghijk | 0.03 | 0.06bcd | 0.03gh | 1.25defg |
| 11 | 30C | LHS | 5.06cdefg | 2.02c | 0.11 | <0.01mn | 0.26cde | 2.10abcd |
| | | HHS | 4.23fgh | 0.73e | 0.04 | 0.01jklm | 0.20def | 1.19defg |
| | 45C | LHS | 2.51ij | 0.48efghijk | 0.11 | 0.03 f | 0.05fgh | 0.84efg |
| | | HHS | 3.13j | 0.38hijk | 0.03 | 0.07ab | 0.03 h | 1.52def |
| Control | 30C | LHS | 4.50efgh | 1.30d | 0.05 | 0.01klmn | 0.19defg | 0.87efg |
| | | HHS | 4.06hi | 1.14d | 0.03 | 0.01jklm | 0.15efgh | 0.90efg |
| | 45C | LHS | 0.86k | 0.37ijk | 0.10 | 0.03 f | 0.04gh | 0.97defg |
| | | HHS | 0.80k | 0.43fghijk | 0.03 | 0.08a | 0.03 h | 1.26defg |
| SEM | | | 0.173 | 0.140 | 0.030 | 0.002 | 0.028 | 0.205 |
| *P*-value | | I x S | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| | | I x ET | <0.01 | <0.01 | 0.43 | <0.01 | <0.01 | <0.01 |
| | | S x ET | <0.01 | <0.01 | 0.62 | <0.01 | <0.01 | <0.01 |
| | | I x S x ET | 0.07 | <0.01 | 0.59 | <0.01 | <0.01 | 0.06 |

[a] Data are means of three samples, means in the same column that share letters are not significantly different ($P < 0.05$) for interaction of inoculant, heat stimulus temperature and ensiling temperature.

[b] Data are means of three samples, means in the same column that share letters are not significantly different (lactic acid: $p = 0.07$; Ninhydrin difference: $p = 0.06$)) for the interaction of inoculant (I), heat stimulus temperature (S) and ensiling temperature (ET).

human food safety. Ohmomo et al. (1995) suggested that poor silage quality, even after LAB inoculation, may be due to high temperatures (42°C or above) reached during the early stages of ensiling since rapid early fermentation and acidification is critical to final silage quality (Muck et al., 2003).

Heat-resistant LAB have been proposed as a method to enhance silage fermentation in warmer climates due to the impaired growth of many LAB at higher temperatures (Chen et al.,2013; Gulfam et al., 2017). Acclimatization, or prior exposure to stimulus, can change the response of microorganisms to subsequent stimulus events (Mulrooney and Kung, 2008). The mechanism of induced thermotolerance is unknown, but growth conditions, such as pH play a role in determining response to heat stimulus (Ahmad et al., 2002). At temperatures above optimum, bacteria respond to thermal stimulus by rapid induction of heat-shock proteins to help with adaptation (Gould, 1989). Despite many lactic acid bacteria having optimal growth temperatures between 25°C and 40°C (Pahlow et al., 2003), specific data on thermotolerance of silage inoculant bacteria are lacking. While some LAB species may benefit from oxygen (potentially introduced during agitation) via NAD+ regeneration (Gupta et al., 2011), it can inhibit growth in others, like *Lactococcus lactis* (Duwat et al., 1995). This concern is specific to the heat stimulus segment of the experiment as the anerobic segment excluded oxygen, aligning with industrial conditions (Othman et al., 2017). Potential oxidative stimulus may have contributed to observations; however heat stimulus was a major driver of acclimatization.

LAB grown at higher temperatures in the present study showed generally lower growth in MRS broth, likely due to heat-induced cell death or injury, but the extent varied by inoculant. The effects of prior heat stimulus exposure and subsequent growth in liquid

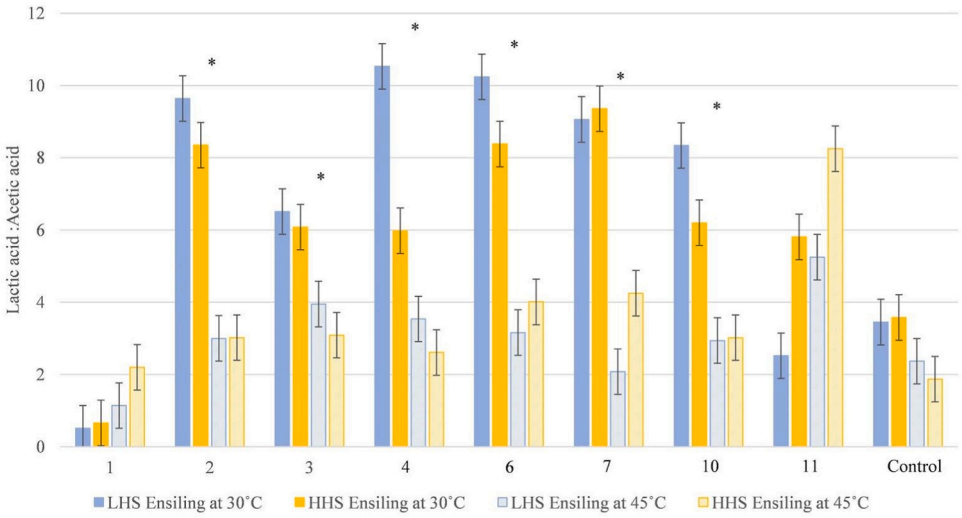


**Fig. 3.** Effects of low (30°C) and high (40°C) heat stimulus on the ratio of lactic acid to acetic acid in silage when ensiled at 30°C or 45°C after 30 days. [Asterisks denote statistical significance for interaction Inoculants x Ensiling temperature ($p < 0.01$). p-values were $p = 0.21$ for Inoculant x heat stimulus temperature x Ensiling temperature interaction; $p < 0.01$ for Inoculant x heat stimulus temperature interaction; $p < 0.01$ for Inoculant x Ensiling temperature interaction; $p < 0.01$ for heat stimulus temperature x Ensiling temperature interaction].

culture reveal complex interactions with temperature. Acclimatization over a period of 24 hrs leverages plastic flexibility in response to environmental changes and is distinct from adaptive evolution, which results from population-level adaptation. While both processes are necessary for bacterial success in dynamic environments, acclimatization is a comparatively rapid and simple process. For most inoculants, growth at 45℃ was poor regardless of prior heat stimulus, but inoculants 1, 7, and 11 were notable exceptions. Inoculant 7 showed somewhat improved performance at 45℃ in the LHS treatment over HHS. In contrast, inoculant 11 appeared to benefit strongly from prior HHS exposure with significantly higher growth than LHS cultures at either 30℃ or 45℃. These shifts may be illustrative of differences in heat acclimatization and tolerance strategies of the cultured organisms. However, in addition to the complex interaction of growth at different temperatures and exposure to heat stimulus, differential survival of strains within inoculants containing multi-strain consortia may play a role in observations.

Inoculants 1, 7, and 11 showed a higher tolerance for growth at 45℃. Inoculants 1 and 11 share *L. buchneri* as a component species, however, inoculant 6 also contains *L. buchneri* and did not show a similar effect. Chen et al. (2013) observed higher tolerance to high temperature in heterofermentative strains, like *L. buchneri*, but this does not explain the discrepancies of inoculants 6 and 7, which contains no heterofermenters. Mulrooney and Kung (2008) found both *L. plantarum* (MTD/1) and *L. buchneri* 40788 appeared to have better heat tolerance after exposure heat stimulus (45℃) than the other organisms, but similar results were not seen uniformly in this study.

Interestingly, HHS inoculants performed identically prior to and following cryopreservation. In the case of inoculant 11, this is particularly significant due to its unique performance after HHS treatment. The industry standard for inoculant processing and storage is freeze-drying. The response of inoculant 11 following glycerol cryopreservation provides initial evidence that the effects of heat acclimatization may be transferable after processing and storage. Therefore, acclimated traits may be preserved following freeze-drying. The effects of freeze-drying and time in storage on these effects should be the subject of future work.

Small decreases in viability of LAB would most likely result in the inability of the added LAB to dominate a silage fermentation process (Mulrooney and Kung, 2008). Growth in liquid culture did not appear to be predictive of poor performance of silage fermentation. In particular, even with a decrease in cell density for HHS cultures incubated at 45°C, Inoculant 7 was able to promote good fermentation with lower pH and DM loss than LHS cultures incubated at 45°C. The lactic acid:acetic acid ratio observed suggests heterolactic fermentation (Zhou et al., 2016). But the lower ratio for silages ensiled at 45°C in this study were associated with decreased lactic acid production. Adesogan (2006) also reported that corn silage stored at 40°C underwent a restricted fermentation with more proteolysis and lower lactate:acetate ratio than silage stored at 20°C. Inoculant 11 was an exception to this, as the amount of lactic acid decreased less than other inoculants, similar to performance at 30°C. It must be noted that the experiments carried out and the data presented do not assess dynamics of the species and strains that make up the inoculants. Additionally, a limitation of the methodology used in this study is that there is a probability that the consortia of bacteria in some of the inoculants have differing growth rates. Ergo, no definitive or conclusive statements can be made about specific impacts of species or strains on fermentation parameters or quality.

Temperature can play an important role in the fermentation profile of silages (Bernardes et al., 2018). In the literature, high ensiling temperatures have been shown to limit fermentation (Zhang et al., 2000, Zhou et al., 2016, Guan et al., 2020). These results were consistent with the present study, in which the 45°C silage samples produced less lactic acid, resulting in a higher pH compared to 30°C silages. In addition, lesser lactic acid could result from lactic acid breakdown by clostridia bacteria in sub-optimal silage

conditions. High ensiling temperatures can also result in butyric acid fermentation, which is also an indicator of clostridial fermentation, and increased proteolysis in silage (Wieringa, 1960; Rooke and Hatfield., 2003; Zhang et al., 2010; Liu et al., 2011). Clostridial activity in silage is not desired in silage because it aids spoilage which leads to reduced feed quality and potential health risks for animals and a well-adapted inoculant could help mitigate clostridial fermentation in hot environments (Kung et al., 2018). Levels of isobutyrate measured in this present study were used as a proxy for clostridial fermentation. Decreased ethanol in warmer silages could represent lower yeast content but also may be associated with volatilization or drier environment conditions that were not appropriate for yeasts (Pahlow et al., 2003).

## 5. Conclusions

Overall, prior exposure to heat stimulus of silage inoculant cultures produced varied effects on the performance of inoculants in culture and in the silo. Prior exposure to high heat stimulus resulted in lower silage pH values at high temperatures of incubation and varied fermentation profiles when compared to LHS or uninoculated controls. Induction of heat tolerance is a largely unexplored facet for silage inoculant optimization, which calls for the investigation of potential methodologies for LAB strains. The results of the current study provide initial evidence for the potential efficacy of heat acclimatization. Further work is warranted and is of value to both research and agricultural industries. Limitations in the current study that should be addressed in future work include the use of either isolates only or genetic identification of consortia members and further evaluation of the effects of cryopreservation of inoculants on subsequent ensiling.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2025.116446.

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