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Effects of Lactic Acid Bacteria Inoculants and Stage-Increased Storage Temperature on Silage Fermentation of Oat on the Qinghai–Tibet Plateau

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Abstract: Ensiling is a simple and effective method of alleviating a shortage of forage for ruminants. This study aimed to investigate the effects of lactic acid bacteria (LAB) inoculants and stage-increased temperature on the fermentation characteristics and chemical composition of oat silage on the Qinghai–Tibet Plateau. The silage was treated with local laboratory inoculant (I) and commercial inoculant (S) and stored at ambient temperature (<10 °C) or stage-increased (5, 10 and 15 days) temperatures of 10 °C and 15 °C for 60 days. The results showed that stage-increased storage temperature can improve silage fermentation. Compared with 10 °C, a stage-increased storage temperature of 15 °C effectively ($p < 0.05$) promoted the fermentation rate of silage by increasing the dominance of *Lactiplantibacillus plantarum*, with higher lactic, acetic and propionic acid contents and a lower ammonia-N ratio of the total N and final pH value. Compared with S, treatment with I increased the water-soluble carbohydrate and lactic acid contents and decreased the ammonia-N ratio of the total N and final pH value. This work demonstrated that increasing the storage temperature in stages using a warming infrastructure facilitates the preservation of oat silage in cold regions, and the inoculation of lactic acid bacteria could advance silage fermentation on the Qinghai–Tibet Plateau.

Keywords: oat silage; lactic acid bacteria; stage-increased storage temperature; fermentation parameters; Qinghai–Tibet Plateau

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1. Introduction

The Qinghai–Tibet Plateau is one of the key pastoral areas in China, accounting for about one-third of the total grassland area in China [1]. Its unique geographical location has created specific climatic conditions, especially its intensive radiation and low temperature. The unique climate and landforms have severely restricted the development of animal husbandry on the Qinghai–Tibet Plateau. In addition, unreasonable grazing has resulted in an imbalance of grass and livestock and the serious degradation of pastures, which directly affects the living income of residents. Importantly, the shortage of forage in winter and early spring is harmful for ruminants. Oat, as the main forage with high nutritional value, has been cultivated intensively for ruminants on the Qinghai–Tibet Plateau. However, determining how to preserve the harvested forage is still a concern in autumn due to the sharp decline in ambient temperature.

The production process of ensilage is simple, the capital investment is low, and it can be stored for a long time [2], which can improve the quality and utilization rate of forage and alleviate the difficulty of forage shortage in the cold season. Lactic acid bacteria (LAB) play a key role during ensiling. They can inhibit harmful spoilage of the remaining microbial community by producing organic acids so that the feed can be stored for a long time [3]. In low-temperature areas, LAB also showed good performance in promoting silage fermentation [4]. In addition, LAB can convert water-soluble carbohydrates (WSC)

into lactic acid and other organic acids, which can reduce pH and inhibit the activity of harmful microorganisms so that the fermentation quality of silage is better [5].

Temperature is an important factor affecting silage quality, especially in cold or tropical regions [4]. The low temperature in cold areas exhibits negative effects on silage preservation. In particular, low temperature will inhibit the decrease in pH [6,7], which makes the fermentation of silage worse. Bai et al. [8] reported that the fermentation quality of silage was better and the nitrate degradation rate was higher at 25 °C than 10 °C. Zhou et al. [9] showed that the temperature during fermentation was 10 °C, which restricted silage fermentation compared with the fermentation temperature of 20 °C. Most of these articles studied the effect of constant temperature during storage on the fermentation quality of silage, but the operability of this method was poor. Ferrero et al. [10] showed that the environmental temperature's influence on the temperature of silage was very important for the fermentation and subsequent quality of silage. Therefore, we assume that in cold regions, if the silage is warmed in stages at the early stage of fermentation, will it bring the same effect as the whole stage warming? This phased approach is highly operable and easy to achieve in cold regions. At present, there are few studies on the effects of stage-increased storage temperature on the nutritional quality and chemical composition of silage. It is not known whether this warming method will have the same effect as the whole warming stage.

Therefore, in this experiment, a phased increased by 10 °C and 15 °C was used during storage to explore the impact of a phased increase on silage. The purpose of this study was to explore the effects of inoculants and stage-increased temperature on oat fermentation quality and chemical components. We speculated that: (1) stage-increased temperature can effectively improve the fermentation quality and chemical composition of silage, and (2) LAB inoculants can effectively improve the fermentation quality and chemical composition of oat.

2. Materials and Methods

2.1. Silage Preparation

The experiment was conducted at the experimental base (Hongyuan; N31°51′–33°33′, E101°51′–103°22′; altitude 3500 m) of the Sichuan Academy of Grassland Sciences, China. The oat at the flowering stage was harvested, air-dried for two hours in sunny conditions, chopped manually to a length of 1–3 cm, and then, randomly divided into two equal fractions for the following treatments: (1) local laboratory inoculant was added (I: *Lactiplantibacillus plantarum* BP18, *Pediococcus pentosaceus* HS1 and *Lentilactobacillus buchneri* LP22; isolated from natural fermented silage on the Qinghai–Tibet Plateau; applied at 10^6 cfu/g FM; recommend by Chen et al. [11]); (2) commercial inoculant (S: *L. plantarum* and *L. buchneri*, each 10^6 cfu/g FM; provided by Gaofuji Biotechnology Co., Ltd., Chengdu, China). All of the chopped grasses were well-mixed additives that were manually placed into plastic bags (300 g in each bag), degassed using a vacuum sealer, and stored in incubators (at 10 °C and 15 °C) for 5, 10 and 15 days. Then, the silos were stored at ambient temperature (2–20 °C), and triplicate silos per treatment were sampled on days 5, 10, 15, 30 and 60 of ensiling, respectively.

2.2. Chemical Analysis

Fresh samples (20 g) from each bagged silo were mixed with 180 mL distilled water in a reciprocal mixer for 3 min. The pH of the filtrate was measured using a pH meter (PHSJ-4 F; Shanghai INESA Scientific Instrument Co., Ltd., Shanghai, China). About 5 mL of the filtrate was centrifuged (4500× *g*, 15 min, 4 °C), and lactic acid, acetic acid, propionic acid and butyric acid in the supernatant were analyzed using HPLC [12]. Ammonia-N was determined according to Broderick and Kang [13]. WSC was determined according to the method of McDonald et al. [5].

2.3. Microbial Analysis

10 g of fresh silage sample was mixed with 90 mL of distilled water and fully shaken for half an hour in a laboratory blender (LB20ES, Shanghai Prim Technology Co., Ltd., Shanghai, China). Serial dilutions were then carried out. LAB cultures were grown on MRS agar (GCM188, Beijing Luqiao Technology Co., Ltd., Beijing, China) and placed in an incubator at 37 °C for 48 h.

The extraction of bacterial DNA from fresh samples was conducted using the method of Li et al. [12]. Phusion® High-Fidelity PCR Master Mix (New England Biolabs) was used to carry out PCR reactions, following the manufacturer's instructions. The primers 515 F and 907 R were chosen to amplify the V4–V5 region of the 16S rRNA gene. The PCR amplicons were then sequenced using the Illumina MiSeq PE2500 platform at Novogene Company (Beijing, China). After sequencing, paired reads were merged using FLASH (Version 1.2.7) and filtered using QIIME. The UPARSE method was employed to assign operational taxonomic units (OTUs) to the 16S rRNA at a cutoff level of 3% using the Usearch software platform (Version 7.1). Based on OTU results, the alpha indices were calculated using QIIME (Version 1.7.0) and displayed using R software (Version 2.15.3).

2.4. Statistical Analysis

In SPSS, a general linear model (SPSS 19.0 program SPSS Inc.) was used for variance analysis of the data, and GraphPad Prism 8 was used for graphical analysis.

3. Results and Discussion

The pH change of ensiling oats is shown in Figure 1. Silage is a complex bacterial fermentation process that accumulates organic acids and decreases pH [14,15]. The stage-increased storage temperature affected ($p < 0.05$) the fermentation quality of silage. The final pH value of S + ambient treatment was greater than 5.5. Compared with 10 °C, the decline rate of 15 °C was faster, and the final pH was lower. The pH value and decline rate of silage are important indicators reflecting the silage fermentation quality and microbial activity. A pH value of 4.2 or lower indicates that the silage is well fermented [5,7]. A pH value that is too high will lead to the growth of acid-intolerant and harmful microorganisms [3], which will lead to inadequate silage fermentation and a decline in quality. The pH decline rate at low temperature (10 °C) was slowed and had a certain delay compared with 15 °C. Therefore, we believe that low temperature limits the fermentation of silage. Li et al. [7] and Zhou et al. [9] also obtained similar results. Their research results confirmed that low temperature limited the fermentation of silage. A suitable inoculum of LAB becomes the dominant bacterium after the fermentation of silage, which can rapidly reduce the pH value and help the silage achieve better fermentation [16]. Compared with commercial inoculant, local laboratory inoculant can reduce pH more rapidly. Using this method, the final pH of I + 15 °C was stabilized below 4, compared with the conventional temperature-raising silage test; for example, the final pH value of the silage of Zhou et al. [9] and Bai et al. [8] was about 4, and the short-term temperature increase in the early stage of silage can also have the same effect. This could bring new hope and development potential to winter forage in cold regions.

The changes in the content of lactic acid, acetic acid, propionic acid and butyric acid in oat silage are shown in Figure 2. Silage is also a process of organic acid accumulation. Lactic acid is the most important acid, and can rapidly reduce pH and inhibit the activities of harmful microorganisms, making the surrounding environment more conducive to the storage and fermentation of forage grass. The lactic acid content was the highest at 15 °C. Lactic acid was produced rapidly and massively in the first 15 days of fermentation; compared with 10 °C, 15 °C effectively ($p < 0.05$) increased the production of lactic acid, and 15 °C had a faster acid production rate and higher lactic acid content. The lactic acid content of the 15 °C local laboratory inoculant treatment was stable at about 2.2 (% DM), which was much higher than that of the other treatments. Bernardes et al. [17] found that at a higher temperature (15–25 °C), there was a higher level of acetic acid content and

a higher degree of acidification. Our research obtained similar results. Bad butyric acid fermentation will make the quality of the silage worse, so the production of butyric acid should be avoided as much as possible during the silage process. The fermentation effect of oat silage with low butyric acid content or without butyric acid is the best. The butyric acid content at I + 15 °C + 15 d is about 0.1 (% DM), which indicates that the feed can be better fermented when the temperature is increased and local laboratory inoculants are added.

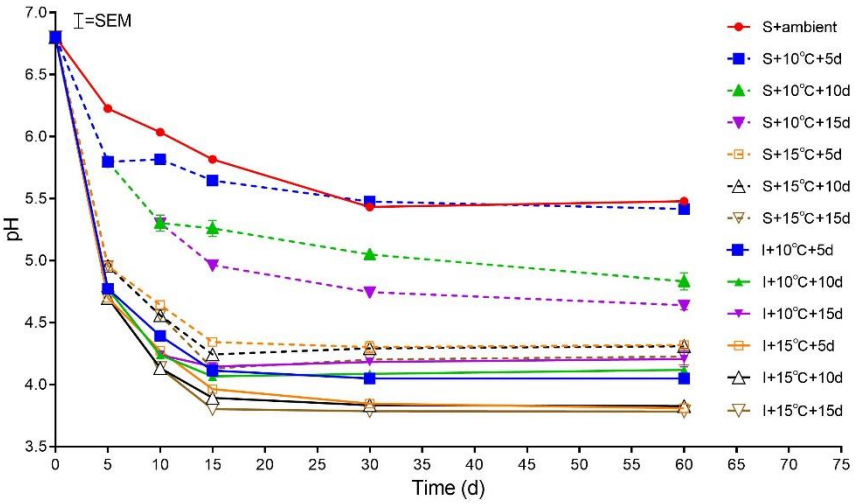

Figure 1. Dynamics of pH of silage inoculated with inherent LAB (I) and commercial LAB (S) and stored at ambient temperature and stage-increased temperatures of 10 °C and 15 °C for 5, 10 and 15 days, respectively.

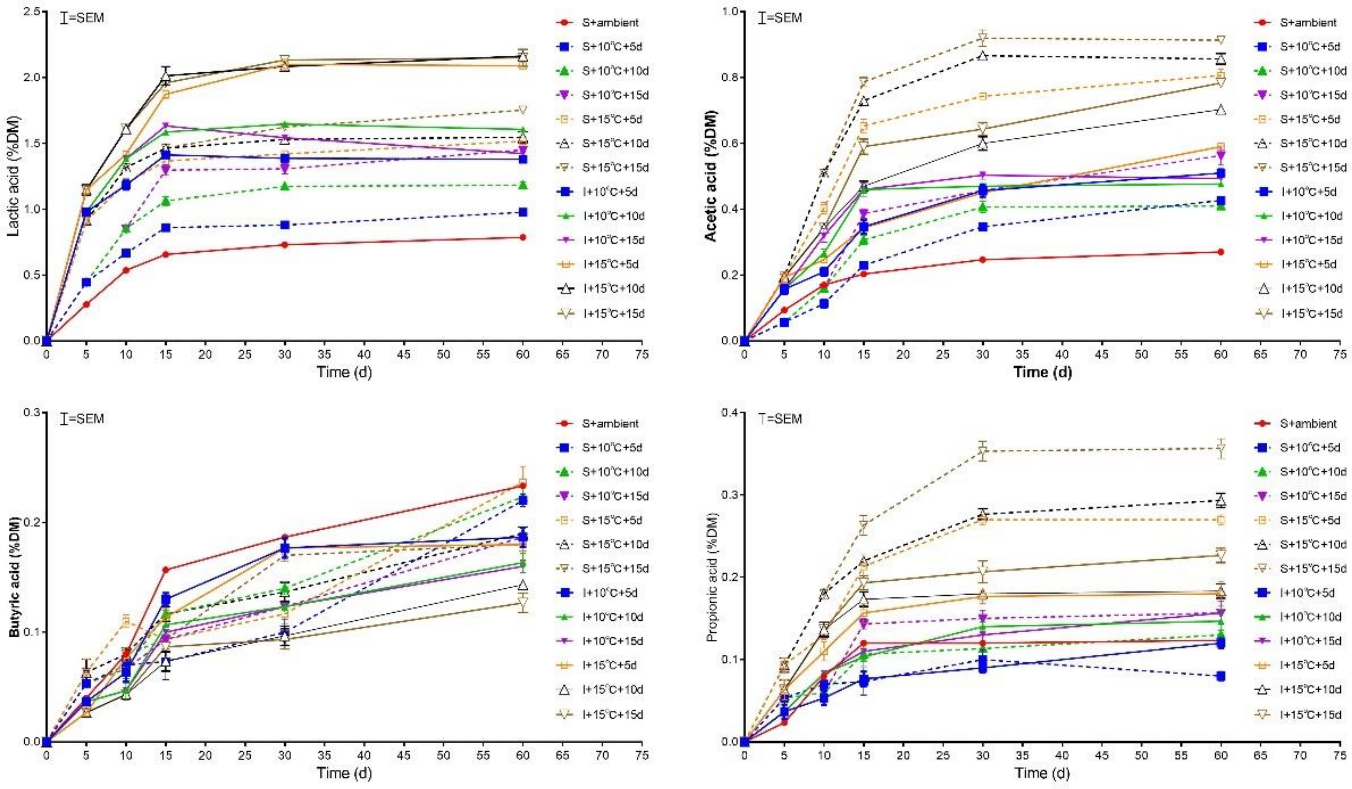

Figure 2. Dynamics of lactic acid, acetic acid, propionic acid and butyric acid in silage inoculated with inherent LAB (I) and commercial LAB (S) and stored at ambient temperature and stage-increased temperatures of 10 °C and 15 °C for 5, 10 and 15 days, respectively.

The change in ammonia nitrogen content in oat silage is shown in Figure 3. Silage with high ammonia nitrogen content will produce a pungent smell, and the palatability will also become worse [18]. When the pH value is higher than 4.5, clostridium will ferment protein and WSC into butyric acid [16]. At the same time, the decrease in pH can inhibit the growth of pathogenic microorganisms and prevent protein from being degraded into ammonia nitrogen [19]. A temperature of 15 °C contained lower ammonia nitrogen content, which may be due to the low pH at 15 °C, which inhibits protein degradation. Good silage can prevent the growth of harmful microorganisms, reduce protein hydrolysis to reduce the content of soluble nitrogen, and prevent protein degradation to ammonia [9], thus reducing the content of ammonia nitrogen. It is an effective and convenient way to improve the quality of silage by briefly raising the temperature in the early stage of silage. At the same time, adding LAB inoculants can improve the quality of low-temperature silage to a certain extent. Among the two additives in this experiment, local laboratory inoculant additives can better promote fermentation.

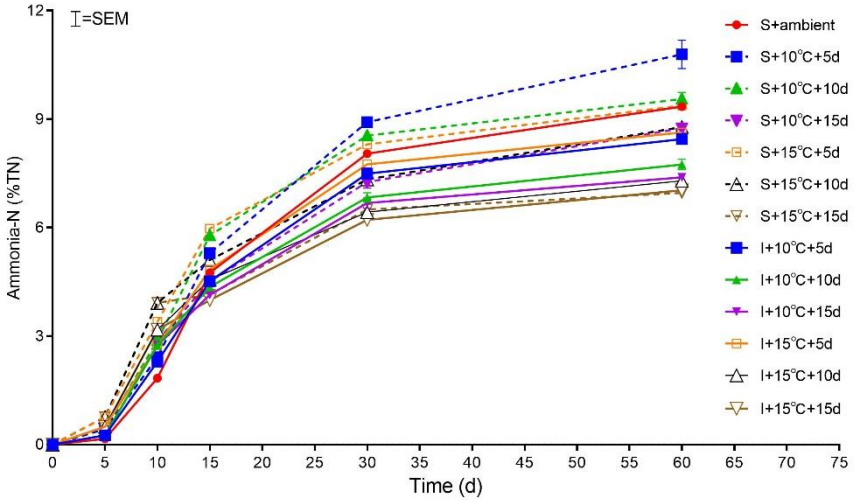

Figure 3. Dynamics of ammonia-N in silage inoculated with inherent LAB (I) and commercial LAB (S) and stored at ambient temperature and stage-increased temperatures of 10 °C and 15 °C for 5, 10 and 15 days, respectively.

The change of WSC content in oat silage is shown in Figure 4. WSC is an important factor affecting the fermentation of silage. By converting WSC into ideal fermentation acid, LAB can rapidly reduce the pH value of silage [20] and inhibit the growth of putrefactive microorganisms, so that the silage can be fermented well. Therefore, sufficient WSC content can better promote the production of good acids. The WSC content of the silage material (8.91% DM) is sufficient for full fermentation of the material during silage [21]. Zhou et al. [6] found that the residual WSC content of silage fermented at low temperature (10 °C) was higher. The results of the 10 °C staged increase showed similar results. Our research results showed that under the same temperature treatment, commercial inoculant contained higher WSC content. The reason may be that local laboratory inoculant and higher temperature (15 °C) can convert more WSC into lactic acid, so these treatments all contained higher lactic acid content and a lower pH value.

The changes in the microbial content of oat silage are shown in Figure 5. In this study, in the most important early stage of silage fermentation (0–15 d), the content of LAB increased rapidly. With the extension of fermentation time, the number of LAB and *L. plantarum* decreased gradually, which showed that the silage can achieve better fermentation. It is well known that the content of LAB in silage materials is the key factor for successful silage nutrition preservation, and it plays an important role in it. When the epiphytic LAB count in the silage material is more than 10^5 cfu/g FM, the silage can be better preserved [22]. The content of WSC and the number of naturally attached LAB in

the material together determine the increase rate of lactic acid and the decrease rate of pH in the early stage of silage, which is very important for stabilizing the production of silage [23]. Microbes play an important role in the preservation of silage. Inappropriate silage will contain a large number of harmful microorganisms, which play a negative role in silage [3]. *L. plantarum* is a facultative aerobic bacteria with a high number of living bacteria, which can produce a large amount of acid to improve silage fermentation. In our research results, we found that a stage-increased storage temperature of 10 °C had a better promotion effect on the production of LAB than that of 15 °C, but treatment with local laboratory inoculant rapidly increased the number of LAB. Compared with commercial inoculant, local laboratory inoculant had a better promotion effect on LAB and *l. plantarum*, which showed that adding additives is also an effective way to promote silage fermentation. However, some research results showed that LAB inoculants could not increase the number of other microorganisms [24]. This also showed that when temperature conditions are not suitable for silage fermentation, adding additives can improve this situation, which is similar to the experimental result of Muck et al. [25].

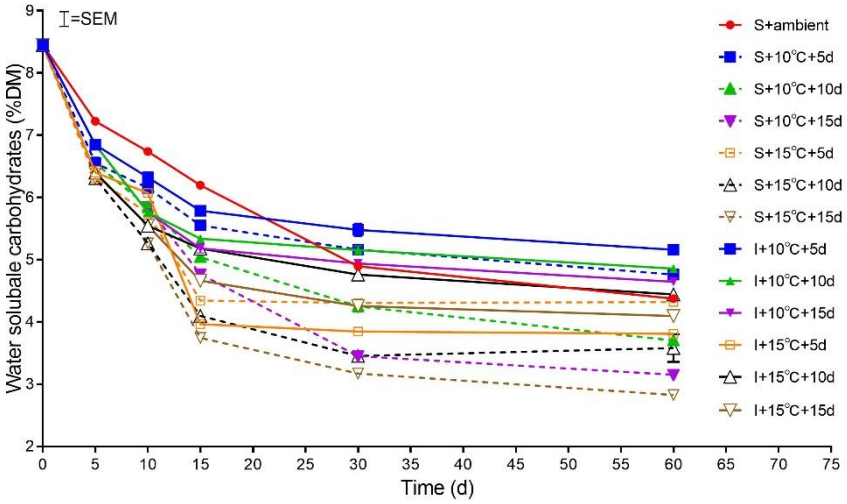

Figure 4. Dynamics of WSC in silage inoculated with inherent LAB (I) and commercial LAB (S) and stored at ambient temperature and stage-increased temperatures of 10 °C and 15 °C for 5, 10 and 15 days, respectively.

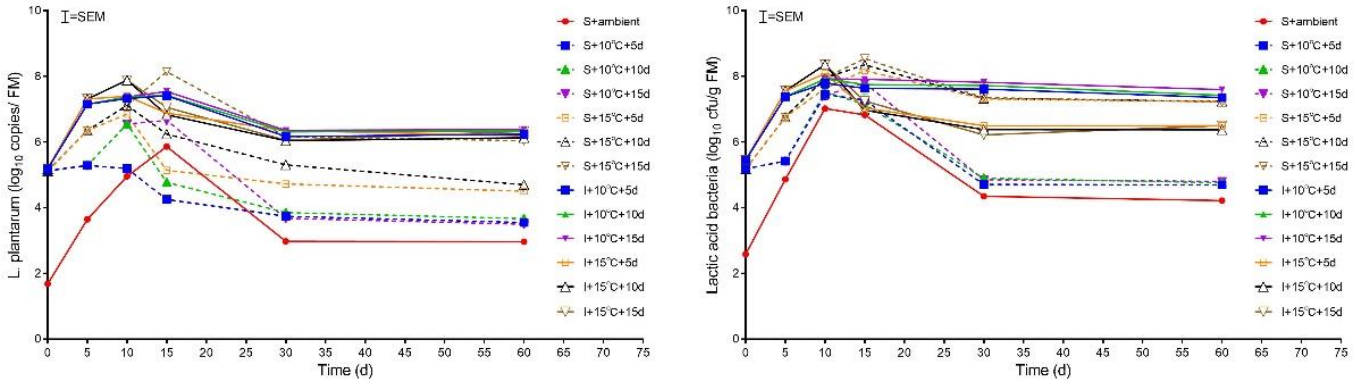

Figure 5. Dynamics of *L. plantarum* and LAB in silage inoculated with inherent LAB (I) and commercial LAB (S) and stored at ambient temperature and stage-increased temperatures of 10 °C and 15 °C for 5, 10 and 15 days, respectively.

4. Conclusions

Low temperature will limit the fermentation of silage. Raising the temperature can help silage achieve better fermentation. In this experiment, better silage quality was

obtained at a stage-increased storage temperature of 15 °C for 15 days, with prevalence of *L. plantarum*, lactic acid, acetic acid and propionic acid, and low levels of pH and ammonia nitrogen. At low storage temperature, application of LAB inoculants enhanced the silage fermentation of oat, with local laboratory inoculant showing a better effect. This work enabled us to understand the fermentation profile during ensiling at a stage-increased storage temperature using a mathematical model.

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