Wild *Lactobacillus hilgardii* (CCMA 0170) strain modifies the fermentation profile and aerobic stability of corn silage

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**ABSTRACT**

The wild *Lactobacillus hilgardii* (CCMA 0170) strain isolated from sugarcane silage showed good results as an inoculant in this silage, so the aim of this study was to evaluate its effect on corn silage. After harvested, three replicates for each fermentation time of corn silage: 19, 60 and 103 days were prepared with *L. hilgardii* or uninoculated (control silage). Experimental silos were opened and their chemical composition was determined. The silages opened after 103 days were also assessed for aerobic stability and microbial population after aerobic exposure. The new inoculant reduced the dry matter losses and conserved more water-soluble carbohydrates. The inoculated silage showed higher lactic acid bacteria populations and lower yeasts and filamentous fungi than the control silage. The concentration of lactic and acetic acid and 1,2-propanediol increased in inoculated silage. The population of filamentous fungi and yeasts after aerobic exposure in the inoculated silage was less than the minimum detectable (\(<2.0 \text{ log CFU g}^{-1}\)). *Lactobacillus hilgardii* CCMA 0170 presents qualities for a good microbial inoculant for corn silage, more research is needed to evaluate their performance in large-scale silos.

**1. Introduction**

Corn (*Zea mays* L.) is the forage most widely used in the production of silage worldwide (Wilkinson and Toivonen 2003; Bernardes and do Rêgo 2014). Corn has a good standard of fermentation when ensiled; however, this silage is more prone to aerobic deterioration after opening of silo (Filya and Sucu 2010). The forage conservation process occurs through fermentation by epiphytic lactic acid bacteria (LAB) or resulting from the addition of inoculants. A bacterial strain to be effective as an inoculant must be compatible with the forage to be ensiled, or compete with the epiphytic microbiota, by promoting a rapid drop in pH, survive during fermentation and increase aerobic stability (Saarisalo et al. 2007; Ávila et al. 2014).

LAB are classified into three groups according to carbohydrate metabolism: obligatory homofermentative, obligatory heterofermentative and facultative heterofermentative. The first group metabolizes hexoses by glycolysis pathway, yielding lactic acid only. The second group metabolizes hexoses and pentoses by phosphoketolase pathway and forms lactate and ethanol or acetate. The third group metabolizes hexoses via glycolysis and pentoses through the phosphoketolase pathway. Many species of LAB have been evaluated as inoculants for corn silage. The most common are *Lactobacillus plantarum* (facultative heterofermentative) and *L. buchneri* (obligatory heterofermentative), the latter being more efficient in increasing aerobic stability of silage (Oude Elferink et al. 2001). The species *Lactobacillus hilgardii* has been little studied as silage inoculant. This species, previously classified as *L. vermiforme*, belonging to the obligatory heterofermentative LAB clade, a rod-shaped, Gram-positive, anaerobic acidotolerant, producer of propionic, acetic and lactic acids, ethanol and CO\(_2\), tolerant to acidic pH, high concentrations of ethanol (20%) and environments with diluted nutrient concentrations (Dicks and Endo 2009). To our knowledge, these *Lactobacillus* species are very similar in genetic and physiological profile. Phylogenetic analysis based on sequence comparison of the conserved marker gene rpoA revealed that *L. buchneri* CD034 was more closely related to *L. hilgardii* strains than to *L. brevis* and *L. plantarum* strains (Heinl et al. 2012). The *L. hilgardii* strain used in this study was isolated from sugar cane silage and initially evaluated as an inoculant in this silage (Ávila et al. 2014; Carvalho et al. 2014). In the evaluation of the fermentation profile using API 50 CHL Kit (Biomerieux), this strain had a fermentative profile similar to *L. buchneri* (Carvalho et al. 2014). The sugar cane silage evaluation resulted in silages with high propionic and acetic acid concentration, with consequent low loss of dry matter (DM) and great aerobic stability.

Ávila et al. (2014) and Carvalho et al. (2015) isolated and evaluated the effect of different strains of LAB in sugarcane silages. These authors found that among the studied strains, *L. hilgardii* CCMA 0170 was noted for providing silage with higher concentrations of acetic acid, propionic acid and 1,2-propanediol, lower yeast population and lower content of ethanol, which resulted in a higher aerobic stability of the silage. Assis et al. (2014) evaluated the effect of *L. hilgardii* CCMA 0170 in corn silage and observed that this strain was among the strains...
that improved the characteristics of the silages, such as high content of acetic acid and 1,2-propanediol. These authors concluded that some characteristics favouring silage as the largest population of LAB or reduction of filamentous fungi were dependent on the time of fermentation. Some questions related to the performance of these strains along the fermentative profile still were not answered. Based on this context and taking into account that this species has not been assessed as an inoculant in silage of different forage crops and that only one work is not enough to conclude about the effectiveness of an inoculant, the aim of this study was to evaluate the effects of *L. hilgardii* CCMA 0170 inoculation in corn silage during the whole fermentation period.

2. Materials and methods

2.1. Inoculant

The strain used was *L. hilgardii* CCMA 0170 which was isolated from sugarcane silage (Ávila et al. 2014). The inoculant was reactive and prepared according to Ávila et al. (2009), mixed with 80 mL of distilled water and uniformly mixed in 3 kg of forage to be ensiled, resulted in a theoretical application rate of 6 log CFU g⁻¹ of forage. In the control treatment, only distilled water (80 mL) was added to the forage.

2.2. Ensiling and bacterial inoculants

Corn plants, aged approximately 110 days, were harvested from a half line [302.0 g kg⁻¹ of DM, on the same day, using a forage harvester (Model JF 120, JF machines, São Paulo, Brazil). Nine experimental silos were prepared for each treatment (with and without inoculant); three were opened after 19 days, another three after 60 days and the last three after 103 days. Samples prior to ensiling (i.e. fresh forage) were also collected for analysis, totaling 24 experimental units. The ensilage process in experimental silos was according to Carvalho et al. (2014). The forage was ensiled in experimental silos (PVC tubes, 10 cm wide and 60 cm high; volume of 0.0047 m⁻³), fitted with Bunsen-type valves, and with an approximate density of 628 kg m⁻³ of fresh matter (approximately 2950 g of fresh forage/experimental silo). The forage was manually compacted in the silos, which were sealed, weighed and stored in a covered environment protected from sunlight and rain. DM loss was calculated as the difference between the weight of DM placed in each experimental silo at ensiling and the DM removed at the end of storage.

2.3. Chemical analyses

Part of each sample was weighed and dried in a fan-assisted oven (Model 320 E, Fanem, São Paulo, Brazil) at 55°C for 72 h. The dried samples were ground in a Willey-type grinder (MA-340, SP Labor, São Paulo, Brazil) using a 1-mm sieve and stored in labelled plastic containers. The samples were analysed for DM content (AOAC 1996), water-soluble carbohydrates (WSC) using the phenol method with a glucose standard curve (Dubois et al. 1956) and crude protein (CP) by the Kjeldahl method (AOAC 1996). The neutral detergent fibre (NDF) was analysed according to Pell and Schofield (1993), using heat-stable α-amylase.

Another part of samples was used to make a water extract to determine the pH value, evaluate the microbial population and detect the fermentation end products. To prepare water extracts, a 25-g sample of fresh forage or corn silage was blended in 225 mL of 0.1% sterile peptone water and homogenized in an orbital mixer (Model 430, Nova Ética, São Paulo, Brazil) for 20 min at 20°g. Then, the pH of each sample was determined. Water extracts (2 mL) were acidified with 10 μL of 50% (vol/vol) H₂SO₄ and frozen prior to analysis for the fermentation end products. The water acidified extracts were analysed for lactic acid, acetic acid, propionic acid, butyric acid, ethanol and 1,2-propanediol by high-performance liquid chromatography (Shimadzu model LC-10Ai; Shimadzu Corp., Tokyo, Japan) according to Santos et al. (2014).

2.4. Microbiological analyses

Water extracts were used for enumeration of the microorganisms. Sequential 10-fold dilutions were prepared to quantify the microbial groups. Yeasts and filamentous fungi were enumerated by Dichloran Rose Bengal Chloramphenicol Medium (DRBC, Difco; Becton Dickinson, Sparks, MD, USA). The plates were incubated at 28°C for 72 h. The yeasts were distinguished from the filamentous fungi by colony appearance and cell morphology. For LAB enumeration, pour plate onto De Man-Rogosa-Sharpe agar (M641I, HiMedia; Mumbai, India) plus nystatin (4 mL L⁻³) was used. The plates were incubated at 35°C for 48 h. The colonies were counted on plates containing a minimum of 30 CFU and a maximum of 300 CFU (Larry and Peeler 2001).

2.5. Aerobic stability

After 103 days of ensiling, the silos were opened and samples of approximately 2 kg were removed from each mini-silo and placed in plastic buckets to assess the aerobic stability during 6 days. Temperatures were measured every 30 min using Data Loggers (Impac, model MI-IN-D-2-L; São Paulo, Brazil) inserted into the silage mass at a depth of 10 cm. The aerobic stability was defined as the number of hours the silage remained stable before rising more than 2°C above the ambient temperature (25.6°C) (Kung et al. 2003). At the end of the air exposure period (7 days), the LAB, yeasts and filamentous fungi were enumerated according to item microbiological analyses.

2.6. Statistical analyses

The data obtained from the evaluation of the fermentation profile of the silages were analysed by ANOVA in a completely randomized design in a 2 × 4 factorial arrangement (2 silages (1 control without inoculants and inoculated silage) during four times of fermentation (0, 19, 60 and 103 days), with three replicates). The data on anaerobic fermentation were analysed using the model: \(Y_{ij} = \mu + S_i + T_j + (S \times T)_{ij} + e_{ij}\), where \(\mu\) is the overall mean; \(S_i\) is the silage effect \((i = 1–2)\); \(T_j\) is the time of
fermentation effect ($j = 1–4$); $(S \times T)_{jk}$ is the effect of interaction between silage and times of fermentation; and $e_{ij}$ is the experimental error, assumed independently and identically distributed in a normal distribution with average zero and variance $\sigma^2$. The data referring to the microbial population after opening the silos were analysed using the same model, but without the time of fermentation effect. Before the statistical analysis, all microbial data were transformed to log10 and presented on an FM basis. With respect to temperature data, only the control silage lost aerobic stability, so it was not possible to perform statistical analysis of this parameter, showing changes only on the temperature of the silage during the time of aerobic exposure. The treatments’ means were compared using the Tukey test at 5% probability. All the data were analysed using SISVAR® software, version 4.5.

3. Results and discussion

3.1. Corn plant characteristics

The characteristics of corn plant before ensiling are shown in Table 1. DM, CP, NDF and WSC contents are within the range recommended for ensilage. The count of LAB, yeasts and filamentous fungi of the epiphytic microbiota was 5.95, 6.02 and 3.85 log CFU g$^{-1}$, respectively. The content of the metabolites analysed in fresh forage was low and close to the values found in the literature (Krooneman et al. 2002; Assis et al. 2014).

| Variable                        | Average | Standard Error |
|---------------------------------|---------|----------------|
| pH                              | 5.46    | (0.04)         |
| Dry matter (g kg$^{-1}$)        | 302.5   | (0.39)         |
| Concentration (g kg$^{-1}$ DM)  |         |                |
| Crude protein                   | 69.2    | (3.99)         |
| Water-soluble carbohydrates     | 90.3    | (3.52)         |
| Neutral detergent fibre         | 594.3   | (11.61)        |
| Lactic acid                     | 14.8    | (1.22)         |
| Acetic acid                     | 1.7     | (0.61)         |
| Propionic acid                  | 1.4     | (0.97)         |
| Butyric acid                    | 0.2     | (0.01)         |
| Population (log CFU g$^{-1}$):  |         |                |
| Yeast                           | 6.02    | (0.32)         |
| Filamentous fungi               | 3.85    | (0.35)         |
| Lactic acid bacteria            | 5.95    | (0.45)         |

3.2. Fermentation profile of silages

The DM levels changed ($P < 0.01$) during the fermentation time; however, the treatments showed the same pattern of variation over the fermentation times (Table 2). Although there have been variations in DM concentrations, those were without any practical relevance. The levels of NDF and DM loss were influenced by the inoculant addition ($P < 0.05$) and fermentation time ($P < 0.01$) (Table 2). The NDF mean and DM loss were 594.4 g kg$^{-1}$ DM and 1.8% in the control silage, differing from the values for the inoculated silage that were 577.8 g kg$^{-1}$ DM and 1.5%, respectively. This difference possibly shows that the inoculant reduced the DM loss and conserved more WSC. Despite the differences in mean values between the inoculated silage and the control treatment, over time changes in NDF were similar between the two silages. Up to 60 days of fermentation, the values of NDF were similar to that found in the fresh forage. After 103 days of fermentation, reductions were observed ($P < 0.05$), reaching 559.9 g kg$^{-1}$ DM. Reducing the NDF can be explained by respiration losses or degradation of hemicellulose by the enzymatic action of the plant itself or due to acid action (Jones et al. 1992). DM loss increased up to 60 days of fermentation, reaching 2.2%. At day 103, there was a high loss reaching 2.5% (Table 2). The loss observed in this study was smaller when compared to other evaluations of corn silage (Santos et al. 2013; Assis et al. 2014). The CP contents were not influenced by the studied factors ($P > 0.05$), with mean values of 68.9 g kg$^{-1}$ DM.

The WSC content and pH values were influenced ($P < 0.01$) by an interaction between the inoculant and fermentation time factors (Table 3). The soluble carbohydrate substrates are essential for the growth of LAB, which hydrolyse to sugars, releasing organic acids, especially lactic acid and acetic acid, reducing the pH (Cheng et al. 2013). A reduction ($P < 0.05$) in the WSC content was observed during the fermentation process. During 60 days of fermentation, silage inoculated with *L. hilgardii* (CCMA 0170) had a higher content of WSC than the control silage. At 103 days of fermentation, the WSC content was not different ($P > 0.05$) between the two silages, with an average content of 3.4 g kg$^{-1}$ DM. Through evaluation, the fermentation profile was observed that WSC content in inoculated silage was reduced more slowly than in the control treatment. The reduction in WSC levels in control silage was more intense at the beginning of the fermentation. Silages inoculated with the *L. hilgardii* CCMA 0170 strain showed a lower reduction in WSC and values close to those found by Filia and Sucu (2010) in corn silage with LAB.

The pH values showed a distinct pattern among the treatments evaluated throughout the fermentation period. A reduction was observed along the 19 days of fermentation, where in the inoculated silage showed a higher pH value than the control silage (Table 3). However, at the end of the fermentation process, it was observed that the pH of the inoculated silage was lower than that observed in the control silage (3.74 and 3.84, respectively). The slower decrease in pH at 19 days of fermentation in inoculated silage may be due to the action of the inoculant strain that has a heterofermentative metabolism producing a greater amount of acetic acid, which is less effective in reducing the pH than the lactic acid. The pH value for both silages is within that recommended for corn silage, being at most 4.20, in order to inhibit the growth of spoilage microorganisms (Kleinschmit and Kung 2006).

An interaction between fermentation time and the application or not of *L. hilgardii* CCMA 0170 was observed for the populations of LAB ($P = 0.02$), yeasts ($P < 0.01$) and filamentous fungi ($P < 0.01$) (Table 3). The LAB population in inoculated silage was only higher than that observed in control silages for fresh forage (time 0), soon after inoculation (Table 3). An increase in the LAB population in inoculated silage was observed only after 60 days when stabilized at 8.61 log CFU g$^{-1}$ up to 103 days of fermentation. In the control silage, however, this increase was observed at 19 days of fermentation, when the LAB population increased to 6.80 log CFU g$^{-1}$ and then to 8.44 after 60 days when stabilized (Table 3). A rapid increase in the LAB population in silage is common and
happens due to the conditions inside the silo that provide the LAB domain. The increase was more intense in the control silage because the initial population was lower. In the inoculated silage, however, despite the increase being slower, the population was probably dominated or had a greater domain of silage. Although the increase was more intense in the control silage because the initial population was lower, the population of filamentous fungi at the end of the fermentation process, probably due to substrate deterioration or metabolites toxic to growth present in the silage.

The population of yeast in inoculated and control silage oscillated similarly until the 60 days of evaluation. After 103 days of fermentation, the inoculated silages showed counts below the minimum evaluation limit (2.00 log CFU g⁻¹). The initial population of filamentous fungi detected on the forage was 3.88 log CFU g⁻¹ (Table 2). After 19 days of fermentation, the population of filamentous fungi reduced to values below the minimum detectable. After 60 days, an increase in the population of that microorganism group was observed, wherein the control silage did not reduce at 60 days of fermentation but increased and remained stable until the end of the fermentation process, probably due to substrate deterioration or metabolites toxic to growth present in the silage.

Table 2. Effects of *Lactobacillus hilgardii* CCMA 0170 inoculation and different days of ensilage on dry matter (DM) (g kg⁻¹), DM loss (%), neutral detergent fibre (NDF), lactic, propionic and butyric acids (g kg⁻¹ DM) in fresh corn and corn silages.

| Variable            | 0            | 19           | 60           | 103          | Treatment | Control | Treatment | Days (D) | T × D |
|---------------------|--------------|--------------|--------------|--------------|-----------|---------|-----------|----------|------|
| DM                  | 302.0B       | 313.0AB      | 321.1A       | 303.3B       |           |         | 0.14      | <0.01   | 0.22 |
| DM loss             | 0C           | 2.2AB        | 1.9B         | 2.5A         |           |         | 0.01      | <0.01   | 0.21 |
| NDF                 | 595.3A       | 610.0A       | 579.2A       | 599.9B       |           |         | 0.04      | <0.01   | 0.62 |
| Lactic acid         | 14.8B        | 61.9A        | 69.2A        | 72.5A        |           |         | <0.01     |         |      |
| Propionic acid      | 1.4B         | 2.3AB        | 2.7A         | 2.8A         |           |         | <0.01     |         |      |
| Butyric acid        | 0.2B         | 0.9AB        | 1.2AB        | 1.4A         |           |         | 0.38      | 0.04    | 0.97 |

Note: Lower case letters comparing the two silages in each time and capital letters comparing the times in each silage.

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Table 3. Effects of *Lactobacillus hilgardii* CCMA 0170 inoculation and different days of ensilation on water-soluble carbohydrates (g kg⁻¹ DM) (WSC), pH, acetic acid (g kg⁻¹ DM), ratio of lactic/acetic acid, ethanol (g kg⁻¹ DM), 1,2-propanediol (g kg⁻¹ DM) and microbial population (log CFU g⁻¹) in fresh corn and corn silage.

| Variable                       | L. hilgardii | Control | Treatment | Days (D) | T × D |
|--------------------------------|--------------|---------|-----------|----------|------|
| WSC                            | 96.7aA       | 90.3bA  |           | <0.01    | <0.01 |
| pH                             | 5.38aA       | 5.46aA  |           | 0.44     | <0.01 |
| Acetic acid                    | 1.7aC        | 2.8aA   |           | <0.01    | <0.01 |
| Ratio of lactic/acetic acid    | 14.5aA       | 1.7bB   |           | <0.01    | <0.01 |
| Ethanol                        | 0.0bB        | 0.0bB   |           | <0.01    | <0.01 |
| 1,2-propanediol                | 0.00aA       | 0.00aA  |           | <0.01    | <0.01 |
| LABa                           | 6.79aA       | 5.9bC   |           | 0.12     | 0.01  |
| Yeast                          | 6.60aA       | 6.02aA  |           | <0.01    | <0.01 |
| Filamentous fungi              | 3.91aA       | 3.85aA  |           | <0.01    | <0.01 |

Note: Lower case letters comparing the two silages in each time and capital letters compare the times in each silage.

*LAB*: lactic acid bacteria.
greater concentrations after 60 days when the concentration of this compound has stabilized, reaching 2.7 g kg\(^{-1}\) DM after 103 days of fermentation (Table 2). The values were lower than that found by Santos et al. (2013). With the fermentation process, an increase in the butyric acid concentration was observed, with a higher concentration observed after 103 days of fermentation (1.4 g kg\(^{-1}\) DM). The butyric acid is mainly related to contamination by clostridia, making feed unpalatable and its rejection by the animal, with the maximum acceptable limit for silage at 0.1 g kg\(^{-1}\) DM (Oude Elferink et al. 2001; Rossi and Drellagio 2007).

There was an interaction between the factors studied for concentrations of acetic acid, ethanol and 1,2-propanediol (Table 3). The concentration of acetic acid increased until 60 days of fermentation in the inoculated silage reaching 13.6 g kg\(^{-1}\) DM. From the 60th day until the end of fermentation, the concentration of this metabolite has stabilized and reached 12.93 g kg\(^{-1}\) DM. In the control silage, acetic acid levels were similar (\(P > 0.05\)) throughout the fermentation period (Table 3), with an average value of 4.1 g kg\(^{-1}\) DM. The acetate content in inoculated silage was higher than the control silage from 19 days of fermentation. The inoculated strain \(L.\) hilgardii CCMA 0170 influenced the production of acetate, and is able to reduce lactic acid to acetic acid and 1,2-propanediol (Krooneman et al. 2002; Heinl et al. 2012).

The ethanol concentration in the silages was increased by the 19th day of fermentation (Table 3). At 60 and 103 days, an increase in ethanol concentration was observed; however, this increase was not statistically significant (\(P > 0.05\)) for this metabolite. This increase, however, occurred in different proportions for the two silages. The final ethanol concentration was 17.3 g kg\(^{-1}\) DM in inoculated silage and 20.4 g kg\(^{-1}\) DM in control silages. These values are within those normally observed for corn silage, being higher to that found by Li and Nishino (2011) and Reich and Kung (2010) and lower than the values observed by Santos et al. (2013).

The concentration of 1,2-propanediol increased in inoculated silage reaching 0.01 g kg\(^{-1}\) (Table 3), while the control silage showed no production of this metabolite. The 1,2-propanediol is a product from degrading lactic acid along with ethanol and acetic acid, by heterofermentative bacteria, which can be metabolized by some bacteria to 1-propanol and propionic acid (Oude Elferink et al. 2001; Nishino et al. 2003).

The addition of inoculants has reduced the population of filamentous fungi in silages after 103 days of fermentation. One of the major inhibiting factors was probably due to the inhibitory effect of acids produced during fermentation (Li and Nishino 2011). The minimum inhibitory concentration of organic acids may have been affected due to the synergistic effect of lactic, acetic and propionic acids very common in the ensiling process, and pronounced in inoculated silage with microbial strains producing these acids in silage (Moon 1983; Driehuis and Oude Elferink 2000).

### 3.3. Aerobic stability of silage

The temperatures of the silage inoculated with \(L.\) hilgardii CCMA 0170 remained stable throughout the time exposed to air (Figure 1). The control silage remained stable for 72 h. The control silage reached a maximum temperature of 27°C after 144 h. The growth of spoilage microorganisms in silages increases the temperature of the mass; thus, the silage temperature is an indirect measure of evaluating the deterioration (Kung et al. 2003). The reason for the inoculated silage maintaining stability during the 7-day trial can be explained by standard fermentation of the inoculated silage. Although the inoculated silages have higher concentrations of residual sugars and lactic acid, inoculation of \(L.\) hilgardii CCMA 0170 resulted in silage with a higher concentration of acetic acid, which has an antifungal effect. Furthermore, the inoculated silages showed a smaller number of yeasts and filamentous fungi that are the main microorganism responsible for aerobic deterioration of silage (Figure 2). Studies using heterofermentative strains, alone or in combination, showed a period of greater stability (Reich and Kung 2010; Carvalho et al. 2015). The addition of inoculants reduced (\(P < 0.05\)) the population of yeast and filamentous fungi in silages after 7 days, both silages showed a similar LAB population. These results indicate that the inoculated silage, even when close to the control population, had microorganisms capable of producing inhibitory metabolites of yeast and filamentous fungi, providing greater aerobic stability of silage.

![Figure 1. Temperature variation during the aerobic exposition of control and inoculated corn silages at 103 days of conservation.](image-url)
Figure 2. Lactic acid bacteria, yeast and filamentous fungi population on control and inoculated corn silages after 7 days of aerobic exposition. Means with different letters in the columns differ (P < 0.05).

4. Conclusions

The inoculation of *L. hilgardii* CCMA 0170 improved the quality of corn silage due to the lower metabolism of soluble carbohydrates to achieve a pH more acidic than the control silage. Besides, the production of acetic and propionic acid synergistically contribute to the inhibition of yeasts and filamentous fungi, providing higher aerobic stability than the control silage. More research is needed to evaluate their performance in large-scale silos.

Disclosure statement

No potential conflict of interest was reported by the authors.

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