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Evaluation of two microbial inoculants as fermentation activators in silages of *Tithonia diversifolia* (Hemsl.) A. Gray

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Abstract

Objective: To evaluate the effect of two microbial inoculants as fermentation activators in silages of *Tithonia diversifolia* (Hemsl.) A. Gray.

Materials and Methods: *T. diversifolia* forage, with 90 days of regrowth, was obtained from a plot fertilized with 20 t of organic matter/ha. Collection was manual and the plants were processed in a stationary chopper (1-2 cm). They were subject to pre-drying, under roof, for 24 hours. The treatments were: a) *T. diversifolia* without inoculant, b) *T. diversifolia* with whey and c) *T. diversifolia* with PROBIOLACTIL®. The evaluations were carried out after 15, 30, 45 and 60 days of storage. The inoculants had a concentration of lactic bacteria, CFU mL^{-1}, of 10^6 for the whey and 10^9 for PROBIOLACTIL®. The indicators dry matter, pH, soluble protein, lactic acid bacteria count and organoleptic indicators (smell, color, texture and moisture) were evaluated. The data were processed using a simple variance analysis and the difference among means according to Duncan, with the program Statgraphic Plus, version 5.0.

Results: The average dry matter percentage was adequate for conservation (35,2 %) and showed a trend to increase, as the sampling time elapsed, just like soluble protein (0,77 mg mL^{-1}) on average. The counts of viable microorganisms showed that the inclusion of biological additives facilitated the predominance of lactic acid bacteria. During storage, pH values tended to decrease among treatments, although without responses.

Conclusions: The results of pH, dry matter, protein, lactic acid bacteria count and organoleptic characteristics proved that microbial inoculants activate the fermentation process in *T. diversifolia* silages. PROBIOLACTIL® was better than whey.

Keywords: silage additives, ruminant feeding, bacteria

Introduction

Promoting the development of science towards new production approaches, which guarantee higher efficiency to face the growing problems of food security, has favored the search for sustainable alternatives to provide animal feedstuffs at lower cost and higher productivity. In Cuba, due to the limitations faced by cattle with forage availability in the dry season, to diversify forage supply, in quantity and quality, in animal husbandry systems, constitutes an essential need (Ontiveros-Vasallo, 2021).

In recent years, the inclusion of forage protein plants, such as *Tithonia diversifolia* (Hemsl.) A. Gray, in cattle diets has reduced production costs and the incidence of metabolic diseases, besides increasing the productive and reproductive performance of the animals. The use of this plant as a resource for animal feeding is increasingly frequent, due to its good nutritional value, rusticity and high biomass production rate (Galindo-Blanco *et al*., 2018).

There are experiences with the use of *T. diversifolia* in feeding systems, even in silvopastoral ones (Galindo-Blanco *et al*., 2018). However, its conservation in the form of silage has been less studied, despite the fact that this procedure, in addition to ensuring its availability throughout the year, allows to retain the nutritional qualities of the original grass much better than haymaking (Rodríguez *et al*., 2019). Currently, inoculants with lactic acid bacteria (LAB) are becoming the most frequent type of silage additive (Tingo-Jácome, 2020).

Under normal conditions, conservation-promoting fermentations develop from native bacteria present in plants. They are highly variable because they depend not only on forage, but also on environmental conditions (Ojeda-García *et al*., 2020). That is why the introduction of LAB-based biological additives is promoted, to confer a numerical advantage to these microorganisms. Thus, through the rapid reduction of pH, the inhibition of other microorganisms that

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deteriorate the preserved material is guaranteed and, above all, the conservation of the original carbohydrates and proteins present in the forages is favored (Castro-Rincón *et al*., 2020).

From this perspective, the University of Matanzas has implemented the use of two microbial inoculants to improve the fermentative and nutritional quality of silages: a probiotic based on lactic acid bacteria, which induces benefits in productive, morphometric, immunological and health indicators, of different species and animal category, which is PROBIOLACTIL® (Rondón-Castillo *et al*., 2018; 2020), and whey, a byproduct of the cheese industry, in whose composition there are high concentrations of LAB, which reveals its potential to be used as a biological inoculant in forage conservation.

The objective of this work was to evaluate the effect of these microbial inoculants as fermentation activators in *T. diversifolia* silages.

Materials and Methods

Location. The research was conducted at the School of Agricultural Sciences of the University of Matanzas (UM) and the Pastures and Forages Research Station Indio Hatuey (EEPFIH, for its initials in Spanish). Two microbial inoculants (whey and PROBIOLACTIL®) were used as fermentation activators in *T. diversifolia* silages.

Origin and composition of the microbial inoculants. The whey came from the cheese factory, belonging to the Provincial Enterprise of Dairy Products, in Matanzas, Cuba. It is a byproduct of the dairy industry, which has a LAB concentration of 10^6 CFU mL^{-1} in its composition. PROBIOLACTIL® came from the Center for Biotechnological Studies (CEBIO) of the UM. This is a probiotic biopreparation, based on *Lactobacillus salivarius*, strain C65, with a concentration of 10^9 CFU mL^{-1}, previously isolated from the cecum mucosa of broiler chicken and identified by molecular biology techniques. The additive was elaborated according to the methodology proposed by Rondón-Castillo (2009).

Selection and treatment of the plant material used for silage. The *T. diversifolia* forage was obtained from a plot that had been established for four years. The area received a homogenization cut and organic fertilization equivalent to 20 t ha^{-1}. The age at regrowth was 90 days and the plants were not flowering. The collection was done manually and the plant was processed in a stationary chopper (1-2 cm). The material was spread on a plastic blanket and subject to a pre-drying treatment, under roof, for 24 h.

Experimental procedure. The silages were packed in four-micron thick polyethylene bags, 12 cm wide by 24 cm long, at a rate of eight bags per treatment, for a total of 24 bags. The material (400 g per bag) was manually introduced and compacted, taking care not to puncture the bags. In the treatments with inoculant, the corresponding 3 200 g of forage were placed on a tray. Ten mL of inoculant per 100 g of plant material were manually and homogeneously incorporated. After being filled, the bags were hermetically sealed by rolling adhesive tape over the polyethylene. To reinforce the anaerobic conditions, they were placed in another bag, which was also wrapped with adhesive tape. Each bag constituted an experimental unit. Conservation was carried out under ambient conditions, on a shelf protected from sunlight. The opening times were pre-fixed at 15, 30, 45 and 60 days. Two open bags per treatment were established each sampling day, to evaluate the indicators pH, soluble protein (Lowry *et al*., 1951) and dry matter (DM) (AOAC, 2010), LAB count and organoleptic indicators, (smell, color, texture and moisture) of the ensiled material, according to Ojeda (2018). A complete randomized design was applied. Table 1 shows the evaluated treatments.

Statistical processing. The evaluated indicators (pH, DM and protein) were subject to simple variance analysis, after testing the normal distribution of the data and variance homogeneity. Differences among means were determined using Duncan's multiple range comparison test. The program Statgraphic plus, version 5.0, was used for this analysis. The counts of viable microorganisms were transformed according to Log N, to guarantee the conditions of normality in the growth curve.

For the analysis, the formula (K+N). 10 x was applied, where:

K - constant representing the logarithm of the dilution where the microorganism was inoculated.

N - number of CFU

10 - basis of logarithms

X - dilution at which inoculation was carried out

Results and Discussion

DM content of the forage is the most important indicator to be considered before starting the ensiling process. Its optimum value should vary between 30 and 35 %, to achieve adequate fermentation and minimize losses of the final product. According

to Sánchez-Ledezma (2018), when the forages to be ensiled do not meet this requirement, pre-drying is recommended before making the silages. In this research, *T. diversifolia* forage showed initial DM and protein values, similar to those found by Londoño *et al*. (2019). However, Table 2 shows the modification of these values due to the effect of pre-drying.

In the conservation process, after 15 days, DM showed a trend to increase in the three silages (table 3). There was significant interaction ($p < 0,05$) between time and the treatments. The control showed lower values compared with the inoculated treatments. Of these, PROBIOLACTIL® showed the highest percentage. Although there were differences between the silages that used microbial additives, at 60 days both showed values higher than or equal to 35 %. However, the DM of the non-inoculated silage (control) was below this value.

It should be noted that the final DM content of the inoculated silages (whey and PROBIOLACTIL®) did not vary practically with regards to the pre-dried fresh forage (table 3). There was a trend for it to recover its initial value (35,2 %). This performance is related to that reported by Castro-Rincón *et al*. (2020), who state that the presence of inoculants with LAB improves DM stability in silages. According to these authors, the use of LAB reduces DM loss and thus, the quality of the ensiled plant is preserved as close as possible to its original state. Kung *et al*. (2018) claim that the objective of making silage is to produce a stable feedstuff, with high recovery of DM, energy and nutrients, highly digestible, similar to the fresh crop.

In correspondence with the results of this study, Castro-Rincón *et al*. (2020) obtained higher DM content, when using LAB to improve the quality of silages of *Zea mays* L. Therefore, the use of additives is considered an alternative to optimize the ensiling process and maintain the nutritive value of the plant, without affecting fermentation parameters (Muck *et al*., 2018).

Table 4 shows the soluble protein content, when there was significant interaction ($p < 0,05$) between the treatments and storage time. The highest values were found in the silages in which LAB were inoculated. Of these, the silage treated with PROBIOLACTIL® showed no differences between 15 and 30 days, but it did show differences between 45 and 60 days, the latter being the one with the highest value (0,99 mg mL^{-1}). This result may be due to the effect of LAB on the silages.

Table 1. Evaluated treatments.

Treatment	Plant material	Inoculant	Dose mL/100 g^{-1} forage
Control	Pre-dried *T. diversifolia*	-	0
Whey	Pre-dried *T. diversifolia*	Whey	10
PROBIOLACTIL®	Pre-dried *T. diversifolia*	PROBIOLACTIL®	10

Table 2. DM and soluble protein percentages of *T. diversifolia*.

Indicator	Unit	Initial	Pre-dried
DM	%	22,6	35,2
Protein	%	12,7	11,9

Table 3. Time and treatment interaction for DM during silage conservation.

Treatment	Dry matter, %			
	15 days	30 days	45 days	60 days
Control	$33,3^{h}$	$34,0^{g}$	$34,2^{fg}$	$34,4^{ef}$
Whey	$34,0^{g}$	$34,3^{efg}$	$34,7^{cd}$	$35,0^{bc}$
PROBIOLACTIL®	$33,0^{h}$	$34,6^{de}$	$35,1^{b}$	$35,5^{a}$
SE ±	0,168	0,095	0,141	0,161
P - value	0,000			

a, b, c, d, e, f, f, g, and h: Means with different letters differ for $p < 0,05$** (Duncan, 1955)

The results of this study are in correspondence with the criteria expressed by authors who state that the use of lactobacilli accelerates the initial lactic acid fermentation rate, decreases pH and generates reduction in degradation (proteolysis) and protein loss during the conservation process (Ertekin and Kızılşimşek, 2019). The most common bacterial inoculants, found in the market, are made up of homofermentative LAB. These bacteria manage to preserve the quality of the ensiled plant, reduce DM losses to a minimum, and decrease protein denaturation (Tingo-Jácome, 2020).

It was found that *L. salivarius* C-65, used to produce PROBIOLACTIL®, is a homofermentative bacterium, capable of utilizing carbohydrates present in the diet (Rondón-Castillo *et al*., 2020). This characteristic can be related to what happened in this experiment, since it is known that *T. diversifolia* forage has significant amounts of protein and soluble carbohydrates (Gallego-Castro *et al*., 2017; Londoño *et al*., 2019), so once this inoculant (PROBIOLACTIL®) is supplied to the silage, these lactobacilli must participate in the degradation of sugars present in the plant to produce, fundamentally, lactic acid, and contribute to avoid further loss of protein. Therefore, the highest protein values reported in this study are in correspondence with this treatment.

Gutiérrez *et al*. (2014) ensiled *T. diversifolia* with a mixture of *Cenchrus purpureus* (Schumach.) Morrone cv. Cuba CT-169 in different proportions and inoculated with the biological product VITAFERT, which contains yeasts and lactobacilli. The best results, in terms of protein, were obtained with the addition of 4,5 to 6 % of the commercial inoculant. Dávila-Hidalgo *et al*. (2016) evaluated the nutritional usefulness of the silage of *T. diversifolia* and *Sorghum bicolor* (L.) Moench. These studies indicated that as the proportion of *T. diversifolia* in the mixture increased, the amount of protein in the silage significantly increased. However, it has not been possible to compare the results of this indicator in the ensiled plant, because most of the studies published in national and international literature are not conducted under similar conditions.

In addition to the reduction in proteolysis, in this work it was observed that the addition of PROBIOLACTIL® should increase the population of LAB in the processed silages. Table 5 shows the concentration of this microbial genus, according to treatment and sampling time. At 15 days, the silages did not differ from each other. At time 30, the PROBIOLACTIL® treatment showed higher values compared with the control, but not with regards to the silages inoculated with whey, which did not differ from the control either. At 45 days there were no differences among them, but at 60 days there were differences between the inoculated treatments and the control, which showed the lowest values in the evaluation.

The inclusion of biological inoculants (whey and PROBIOLACTIL®) showed that both facilitate the population predominance of LAB during the fermentation process with regards to forage ensiled in natural form (control). In addition, these bacteria use soluble carbohydrates (SC) for their growth, as the main source of energy to form lactic acid and favor the decrease of pH. This is one of the most relevant indicators in silage elaboration. It is used as a reference or indicator of the fermentative quality of forage, because it is one of the most radical transformations that occur in forage, and because it has great relationship with degradative processes during conservation (Kung *et al*., 2018).

Figure 1 shows that as the conservation process elapsed, pH values tended to decrease. However, although from a statistical point of view, the treatments did not show responses during the sampling times, there were variations from one

Table 4. Time and treatment interaction for soluble protein during silage conservation.

Treatment	Soluble protein, mg mL^{-1}			
	15 days	30 days	45 days	60 days
Control	$0,64^{j}$	$0,66^{i}$	$0,70^{h}$	$0,78^{e}$
Whey	$0,72^{g}$	$0,77^{e}$	$0,79^{d}$	$0,81^{c}$
PROBIOLACTIL®	$0,73^{f}$	$0,73^{f}$	$0,93^{b}$	$0,99^{a}$
SE ±	0,015	0,016	0,032	0,033
P - value	0,000			

a, b, c, d, e, f, f, g, h, i and j: Means with different letters differ according to Duncan for $p < 0,05$

Table 5. Growth of lactic acid bacteria during the conservation process.

Treatment	Sampling time, days			
	15	30	45	60
	Unit, Log CFU mL^{-1}			
Control	$8,18^{b}$ ($15,10x10^{7}$)	$7,75^{bc}$ ($97,05x10^{7}$)	$8,69^{ab}$ ($19,25x10^{7}$)	$6,99^{c}$ ($51,66x10^{7}$)
Whey	$8,89^{b}$ ($24,46\ x10^{7}$)	$8,80^{ab}$ ($10,50\ x\ 10^{8}$)	$9,34^{a}$ ($24,58\ x\ 10^{8}$)	$8,63^{ab}$ ($69,22\ x\ 10^{7}$)
PROBIOLACTIL®	$8,56^{ab}$ ($32,95x10^{7}$)	$9,06^{a}$ ($19,40x10^{8}$)	$9,64^{a}$ ($58,39x10^{8}$)	$9,02^{a}$ ($19,01x10^{8}$)
SE ±	0,017	0,039	0,040	0,069
P - value	0,000			

a, b and c: Means with different letters differ according to Duncan for $p < 0,05$. Original data ( )

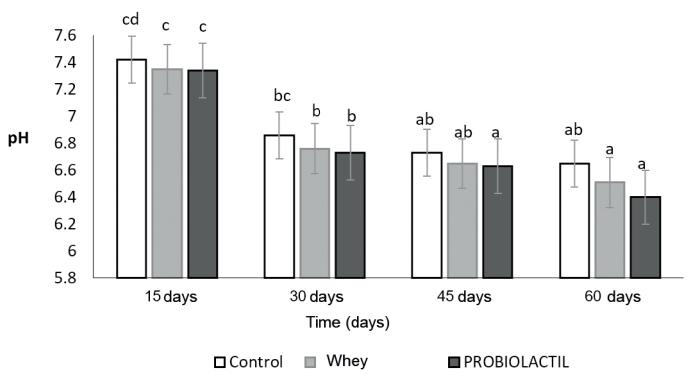

Figure 1. Performance of pH during the preservation process.

time to another. To analyze the favorable action of the inoculants on this indicator, the evolution of pH should be examined individually, and the values found in the control treatment should be considered as reference results.

The non-inoculated silage (control) led to a slow decrease in pH, which only showed differences with regards to the first sampling from day 45. The treatments inoculated with LAB showed a decline since 30 days. After this time, pH remained stable for the whey inoculant. However, PROBIOLACTIL® continued to decline until 45 days. The final pH values, although not differing among them, showed figures of 6,6 (control); 6,5 (whey) and 6,4 (PROBIOLACTIL®), values that, according to the quality criteria established for tropical silages, are inadequate (Demanet-Filippi, 2017).

The acidity reached by the silages during conservation is the result of the interaction of three indicators present in the forage: DM percentage, SC concentration and buffering capacity. Optimal conditions for their values to control fermentative processes are achieved when the percentages of the first two indicators (DM, SC) are high, and those of the third one, low (Sánchez-Ledezma, 2018). That is why legumes and forages with high protein contents are considered difficult forages to preserve because they show low SC concentrations and the nitrogenous compounds they generate during fermentations promote higher values in buffering capacity compared with grasses (Ojeda-García *et al*., 2020).

In evaluations carried out by Holguín-Castaño (2016) with Mexican sunflower (*T. diversifolia*), difficulties were observed in obtaining adequate pH in the silages, when the plant was kept alone or with LAB-based additives, results that the above-cited au-

thor ascribed to its high buffering capacity values. To counteract this limitation, she elaborated silages with different proportions of *C. purpureum* and found the best results when she incorporated it at 67 %. Erazo-Leyton (2018) reported that when ensiling Mexican sunflower with sugarcane vinasse, there was no variation in pH levels compared with the control.

Kung *et al.* (2018) stated that some legume and grass silages, with values of 30 to 35 % DM, have higher pH than normal. They also add that low SC content in forage can limit fermentation conditions, thus failing to reduce pH to optimal conditions. Sánchez-Ledezma (2018) points out that the final pH of silage increases as DM content increases, because bacteria activity is limited due to the lack of water for their vital functions.

The pH results in this research could be a direct consequence of the pre-drying treatment (24 h), with its subsequent loss of water, the increase in DM as the storage time progressed and the high LAB concentration, which could quickly deplete all the silage substrate. Although this result (pH) does not meet the expectations of this study, the organoleptic analyses were favorable for the use of these bacterial inoculants (table 6).

The color characterization of silages from predried forages, should be evaluated with moderation because the original pigments change their tonality due to oxidations that occur during the dehydration process (Kung *et al*., 2018). These transformations were observed during the pretreatment performed with the forage used in the research and induced no contrasts among treatments, except at 60 days,

Table 6. Evaluation of the silages in terms of organoleptic parameters.

Indicator	Treatment	Sampling time, days			
		15	30	45	60
Color	Control	Dark green (3)	Dark green (3)	Dark green (3)	Dark green (3)
	Whey	Dark green (3)	Dark green (3)	Dark green (3)	Olive green (5)
	PROBIOLACTIL®	Dark green (3)	Dark green (3)	Dark green (3)	Light coffee (4)
Smell	Control	Moist forage (5)	Acetic acid (4)	Strong acetic acid (3)	Putrid Unpleasant smell on hands (2)
	Whey	Forage and milk (5)	Slight acetic acid smell (4)	Ripe fruit (5)	Ripe fruit (5)
	PROBIOLACTIL®	Forage and sugars (5)	Strong sugar smell (5)	Slight ripe fruit smell (5)	Ripe fruit (5)
Texture	Control	Defined contours. Non-transparent leaves (4)	Defined contours. Non-transparent leaves (4)	Defined contours. Non-transparent leaves (4)	Little-defined contours. Transparent leaves(3)
	Whey	Defined contours. Non-transparent leaves (4)	Defined contours. Non-transparent leaves (4)	Defined contours. Non-transparent leaves (4)	Defined contours. Non-transparent leaves (4)
	PROBIOLACTIL®	Defined contours. Non-transparent leaves (4)	Defined contours. Non-transparent leaves (4)	Defined contours. Non-transparent leaves (4)	Defined contours. Non-transparent leaves (4)
Moisture	Control	High moisture (2)	Moisturizes hands (2)	Moisturizes hands (2)	Moisturizes hands (2)
	Whey	Moisturizes hands little (4)	Moisturizes hands little (4)	Moisturizes hands little (4)	Does not moisturize hands (4)
	PROBIOLACTIL®	Moisturizes hands little (4)	Moisturizes hands little (4)	Moisturizes hands little (4)	Does not moisturize hands (4)

Individual rating: 5= Excellent, 4= Good, 3= Fair, 2= Poor, 1= Not Classifiable

when they were inoculated. This improved the scores for coloration, as they changed to less intense tones.

Smell is the organoleptic property that best allows us to perceive how the preservation process developed, because the organic acids and nitrogen compounds generated during fermentations show peculiar aromas, which facilitate their identification or predominance, and both, in silages. However, when the prevailing smell is acetic acid, its origin can come from several sources, mainly from lactic heterofermentative bacteria and enterobacteria, as a result of high pH that allows their development; by fermentations of *Clostridium* bacteria, which in addition to increasing their concentrations, generate butyric acid and amines, providing the silages with smells of rancid fats and putrefying organic matter (Sánchez-Ledezma, 2018).

Mexican sunflower, preserved without inoculant, showed since the second sampling moment a degradative evolution in its smells until ending in a decomposing organic product. This result is in agreement with the poor values found in the evaluated biochemical and microbiological indicators. The introduction of whey into the silages reversed this performance, because after 30 days of conservation it was evident that lactic acid bacteria began to dominate the fermentations, and remained so throughout the experimental period. The responses with PROBIOLACTIL® were more categorical and stable, which confirms that, in this inoculant, LAB have higher colonization potential than those present in whey. PROBIOLACTIL® is known to have a concentration (10^9 CFU) of *L. salivarius*, a strain recognized for its ability to grow in harsh environments and generate antagonisms with other microorganisms (Sayan *et al*., 2018; Seo *et al*., 2019). Its probiotic condition provides *T. diversifolia* silage with an added value, which has favorable repercussion on the animal.

The changes in texture of the silages compared with the forages that originated them are linked to the quality with which the conservation was carried out. The closer it was to the initial one, the better the process developed. This indicator did not show variations among treatments until the 60-day sampling moment, when the control showed degradative structural changes.

In pre-dried silages, the impression of moisture to the touch comes from the compounds generated by fermentations, and increases when the organic matter is decomposing, as was the case in the control treatment. The organoleptic evaluations integrated and complemented the findings in the other indicators, and allowed ratifying the deficient capacity of the Mexican sunflower to be preserved individually, the effectiveness of the inoculants to improve the fermentative development of the silages and the superiority of PROBIOLACTIL® compared with whey.

Conclusions

The results of pH, DM, protein, lactic acid bacteria count and organoleptic characteristics showed that microbial inoculants activate the fermentation process in *T. diversifolia* silages. The superiority of PROBIOLACTIL® over whey was proven.

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Conflict of interests

The authors declare that there is no conflict of interests among them.

Authors' contributions

- Marlen Rodríguez-Oliva. Planning and execution of the research, analysis of results, paper writing.
- Felix Ojeda-Garcia. Execution of the research, analysis of results and final paper drafting.
- Yaimara Pozo-Pérez. Execution of research and analysis of results.
- Ana Julia Rondón-Castillo. Research planning, analysis of results, paper writing and final revision.
- Grethel Milián-Florido. Analysis of results, paper writing and final revision.

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