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In Vitro Semi-Solid Fermentation of Two Prickly Pear (*Opuntia* sp.) Cultivars as Food Supplement for Ruminants

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ABSTRACT

Objective: To increase the protein content of two commercial prickly pear cultivars, fermenting and adding some components, in order to complement the diet of ruminants in areas whose conditions impose limitations upon agriculture.

Design/Methodology/Approach: A randomized complete bock design and a factorial treatment arrangement were used to test two prickly pears cultivars (Cristalino (*Opuntia albicarpa*) and Rojo Pelón (*Opuntia ficus-indica*)), two particle sizes (chopped and blended), and two non-protein nitrogen (NPN) sources and their combination (1% urea, 0.1% ammonium sulfate, and urea + ammonium sulfate). The substrates were fermented for 9 h. One percent yeast (*Saccharomyces cerevisiae*) and 0.25% *Saccharum* spp. treacle were added to the substrates.

Results: The levels of the tested factors recorded significant differences ($p<0.05$). The "Cristalino" cv, the "blended" particle size, and the "urea plus ammonium sulfate" NPN had the highest protein content (CP): 29.9%, 33.5%, and 37.7%, respectively. The treatment with the highest CP (46.1%) used the Cristalino cv, blended particles, and urea plus ammonium sulfate.

Study Limitations/Implications: The study faced no limitations.

Findings/Conclusions: Fermenting prickly pears is a nutritious option to feed ruminants.

Keywords: Prickly pear; fermentation; forage supplement; crude protein.

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INTRODUCTION

Prickly pears (*Opuntia* sp. (Caryophyllales: Cactaceae)) are native to North America (Griffith, 2004) and South America (Majure *et al.*, 2012). They are distributed in several arid, semi-arid, and temperate regions of Europe, Africa, Asia, Australia, and the Americas. Several species are grown in over 2.6 million ha for different purposes (Nefzaoui, 2018).

It is a major natural resource for these areas, as a result of its socioeconomic, agricultural, and environmental benefits, as well as its functional, nutraceutical, and biological properties

(Abbas *et al.*, 2022; Stavi, 2022). Since the climate change vulnerability of traditional crops has reduced their productivity and quality, prickly pear cultivation has intensified in the last years, given its morphological and physiological characteristics, its scalability, and its adaptability to scarce and erratic precipitation and soil degradation. Prickly pear survives as a consequence of its resilience, efficient water use, resistance to heat stress, low input requirement, high productivity, and other characteristics (Horibe, 2021; Jorge *et al.*, 2023; Sipango *et al.*, 2022; Thakuria *et al.*, 2020).

For a long time, prickly pear has been used as a complementary and emergency forage during droughts and winter, when forage and grain production becomes scarce (Fuentes-Rodríguez *et al.*, 2017). It has increasingly been used to feed animals in arid and semi-arid zones, because it is a promising forage option that provides energy, minerals, and water for several species, guaranteeing food safety and reducing hunger and poverty in those areas (Dubeux Jr. *et al.*, 2021). However, cladodes have a low fiber and crude protein content and must be complemented with other crops or otherwise enriched (Torres-Ponce *et al.*, 2015). This procedure makes up for the lack of such components and might also slow down its laxative effect (Gusha *et al.*, 2015). In this context, prickly pear could be a fundamentally strategic forage with high productive potential for various productive systems located in the semi-arid regions of the world (Rocha *et al.*, 2021).

Studies about the nutritional content of prickly pears show that they provide little protein; therefore, ingredients that add protein and energy are required to increase in their quality (Fuentes-Rodríguez *et al.*, 2017). Consequently, technologies must be implemented to increase their nutritional value through such biotechnological processes as silage (Veleta *et al.*, 2024). Silage is an anaerobic process that preserves wet crops through fermentation; under optimum silage conditions, bacteria mainly ferment soluble carbohydrates, preserving the nutrients of the forage (El Hajji *et al.*, 2022). Fermentation in containers (bioreactors) is another option that can improve the protein content of the substrate, increasing the unicellular protein in the cell wall of fermenting microorganisms (González *et al.*, 2019).

Solid state fermentation is a process that produces microbial protein degrading the glucose of the cladodes. This process is supported by the microorganisms that grow in the insoluble substrate, in the absence of added water. The said fermentation principle is based on the metabolization of glucose by yeast; therefore, an effective process requires the release of glucose (Torres-Ponce *et al.*, 2015).

The first studies about the enrichening of prickly pear were carried out in northeastern Brazil at the end of the past century, as a strategy to tackle the lack of quality forage, resulting from the low and erratic precipitation and the high costs of protein supplements. Oliveira (2001) recorded that crude protein increased by 12.8% when prickly pear was fermented with *Aspergillus niger*. Likewise, Araújo *et al.* (2005) reported a 26% increase in crude protein as a result of the use of *Saccharomyces cerevisiae*. In Mexico, Díaz-Placencia *et al.* (2012) increased protein content from 9.3 to 19.3% through *in vitro* fermentation of prickly pear, inoculated with a *Kluyveromyces lactis* yeast, obtained from waste apples. For their part, Flores-Hernández *et al.* (2021) reported that protein increased from 10.4 to 36.5%, when *S. cerevisiae* was applied to the *O. megacantha* cultivar. Likewise, Castro *et al.*

(2022) used the same yeast and increased protein from 3.9 to 27.5 %. Finally, Veleta *et al.* (2024) reported a similar increase in protein (6.6 to 32.7 %), 72 h after fermentation.

Considering that prickly pears have a high palatability, are easy to reproduce, grow fast, can recover after they are cut, have a high productivity per surface unit, and can be produced at a low cost, this species could withstand the challenges of global climate change. However, since several research teams have reported high variation in the crude protein content of different species and cultivars, the aim of this study was to determine the crude protein content of two prickly pear (*Opuntia* sp.) cultivars grown in the region, using two particle sizes and two sources of non-protein nitrogen, in a fermentation process, in order to increase the nutritional value of its forage. The purpose was to corroborate its potential as a forage alternative for livestock producers in central and northern Mexico.

MATERIALS AND METHODS

Study Site

The study was carried out under lab conditions, in the Water, Soil, and Plant Lab of the Colegio de Postgraduados - Campus San Luis Potosí, located in Salinas de Hidalgo, San Luis Potosí.

Preliminary Test

During the initial test, the effect of adding a 1% yeast or 1% yeast plus 2% fermented agave juice inoculum, to the prickly pear-based substrate on the crude protein content was evaluated. In addition, the substrate was fermented for 6, 8, 10, and 12 h to determine its effect on the crude protein content. In the absence of any significant differences, these factors were not included in the final test.

Final Test

This test used a factorial design to evaluate the effect of the cultivar, particle size, and non-protein nitrogen (NPN) on the crude protein content of fermented prickly pear. In the case of the cultivar (cv), mature cladodes from Rojo Pelón (*Opuntia ficus-indica*) without prickles and Cristalino (*O. albicarpa*) with prickles were used. The Rojo Pelón cv came from a germplasm collection located in Salinas de Hidalgo, San Luis Potosí (22° 37' 33.6" N, 101° 42' 39.4" W, at 2,075 m.a.s.l.); meanwhile, the Cristalino cv was collected at the community of La Victoria, Pinos, Zacatecas, México (22° 15' 27" N, 101° 37' 48" W, at 2,308 m.a.s.l.). The 10- to 12-month-old cladodes were collected in February 2024. Regarding particle size, the cladodes were prepared in one of two ways: 1) blended in a standard Oster® blender (BLSTBPST013), unwashed and without removing the prickles; and 2) cut with a knife into approximately 1.0 cm^2 squares. Finally, in terms of non-protein nitrogen (NPN), Flores *et al.* (2019) recommend adding 1% urea and 0.1% ammonium sulfate. Therefore, the effect of each recommended dose was evaluated separately and combined. Therefore, the following levels were added: 1% urea, 0.1% ammonium sulfate, and the same ratio of urea + ammonium sulfate. The factorial design resulted in 12 treatments (Table 1).

Table 1. Treatment and factors evaluated during the fermentation of two prickly pear (*Opuntia* spp.) cultivars.

Treatment	Cultivar (cv)	Particle size	Non-protein nitrogen source
CrLiUr	*Cristalino*	Blended	Urea
CrLiSa	*Cristalino*	Blended	Ammonium sulfate
CrLiUS	*Cristalino*	Blended	Urea + ammonium sulfate
CrPiUr	*Cristalino*	Chopped	Urea
CrPiSa	*Cristalino*	Chopped	Ammonium sulfate
CrPiUS	*Cristalino*	Chopped	Urea + ammonium sulfate
RpLiUr	*Rojo pelón*	Blended	Urea
RpLiSa	*Rojo pelón*	Blended	Ammonium sulfate
RpLiUS	*Rojo pelón*	Blended	Urea + ammonium sulfate
RpPiUr	*Rojo pelón*	Chopped	Urea
RpPiSa	*Rojo pelón*	Chopped	Ammonium sulfate
RpPiUS	*Rojo pelón*	Chopped	Urea + ammonium sulfate

Substrate Preparation

To prepare the substrates for fermentation, 250 g of each prickly pear cultivar and each size were initially put into 600-mL beakers. Subsequently, urea, ammonium sulfate, or both were added, depending on the treatment. In all cases, 1% yeast was added as inoculum, along with 0.25% molasses to provide energy and to facilitate fermentation.

Both the NPN and yeast sources were added in the order suggested by Flores *et al.* (2019): first, urea, followed by ammonium sulfate, yeast, and finally molasses. Additionally, 140 mL of deionized water were added to the cut pricky pear at 35 °C, to achieve an even distribution of yeast in the surface of the prickly pear.

Fermentation Process

The fermentation process was carried with a 45-kg Eberbach™ 5900 reciprocal shaker (Científica Senna, Mexico). The beakers with the substrates were placed in 18×4×12 cm box carriers and shaken at ≈60 oscillations min^{-1}. The substrates were continuously shaken during the 9-h fermentation. Additionally, the cut prickly pear substrate was subsequently shaken by hand for 1 min every hour, because yeast settled during the shaking, preventing its interaction with the whole substrate. The substrate temperature was measured with a mercury thermometer, ranging from 17 °C (at the start of the fermentation process, in the morning) to 26 °C (at the end of the fermentation process, in the evening).

To stop the fermentation of the substrate at the end of the process and to determine the crude protein content afterwards, samples were taken from each fermented substrate and placed in 50-mL Eppendorf centrifuge tubes®. Following the suggestions of Díaz-Plascencia *et al.* (2012), two drops of 86.5% phosphoric acid (orthophosphoric acid) were added. The samples were placed in a Thermo Scientific™ flask at −5 °C awaiting their analysis.

Protein Content

The samples were defrosted in water at room temperature and dehydrated in a FELISA® FE-292D oven (Monterrey and Mexico City), at 60 °C for 48 h, following the guidelines of Díaz-Plascencia *et al.* (2012). They were subsequently crushed in a mortar and kept in a 10-mL centrifuge tube. Finally, Parafilm was placed around the lid, awaiting analysis.

The Dumas method used to determine total nitrogen complies with the regulations of the Association of Official Analytical Chemists (AOAC). This method was employed to estimate total nitrogen, placing 0.1 g of the sample in a nitrogen-free tin foil cup. The cups were subsequently placed in each of the slots of the carousel of a Truspec N 4334 elemental determinator (LECO, USA). The Total Crude Protein (TCP) was determined based on the total nitrogen value and a 6.25 protein conversion factor. This determination process was also applied to the Cristalino and Rojo Pelón cultivar substrate controls (without any addition or fermentation).

Other Bromatological Analyses

In addition to their crude protein content, both the treatments and the controls were analyzed to determine their ash content, ether extract, humidity, total solids, and pH. Likewise, in terms of protein content, the Neutral Detergent Fiber of the best treatment in each cultivar and the controls was determined, following the official methods established by the AOAC (1975 and 1990). The ether extract, humidity, ash, and neutral detergent content were quantified using the AOAC method 945.39 (with a Soxhlet extractor), the AOAC method 966.02, the AOAC method 923.03, and the ANKOM method (1999), respectively. Total solids were quantified based on the humidity content. Finally, pH was measured with an Apera Instruments® PH700 pH meter.

Statistical Analysis

A completely randomized experimental design with a factorial arrangement was used to generate 12 treatments, with different numbers of repetitions. The data were subjected to an analysis of variance with a factorial design and means were compared with Tukey's test ($p<0.05$), using version 9.4 of the SAS statistical analysis system (SAS® 9.4). Finally, the 12 treatments with the factorial design plus two controls (prickly pear cultivars without treatments) were subject to an analysis of variance.

RESULTS AND DISCUSSION

Preliminary Test (Fermentation Time and Inoculum)

During the preliminary test, the fermentation time of the prickly pear substrates did not record significant differences in crude protein (CP) content ($p=0.57$). The highest CP content (30.0%) was recorded after a 10 h fermentation, while the lowest content (28.2%) was recorded after 8 h. Intermediate contents were reported at 6 h (29.6%) and 12 h (29.7%). Likewise, no significant differences ($p=0.34$) were found in CP content, when yeast or yeast plus fermented agave juice were added to the substrate. However, when yeast plus fermented agave juice was added, the CP content (29.8%) was higher than with yeast alone (29.0%).

Final Test

Table 2 shows the results of the factorial ANOVA for the crude protein (CP) content, especially in the three main factors (cultivar, particle size, and source of non-protein nitrogen). All interactions showed significant effects (cultivar*particle size) or highly significant effects (the rest).

Cultivar

ANOVA pointed out highly significant differences ($p<0.0001$) in CP percentage between both prickly pear cultivars (Rojo Pelón and Cristalino). Figure 1 shows that the Cristalino cv (29.9 %) had a higher CP content (Tukey $p<0.05$) than the Rojo Pelón cv (27.3 %).

Particle Size

The ANOVA showed highly significant differences ($p<0.0001$) in CP content, depending on the preparation of the substrates (cut or blended cladodes). Figure 2 shows that the CP value recorded after the fermentation of the blended cladodes (33.5%) was higher (Tukey $p<0.05$) than that of fermented cut cladodes (21.5%).

Source of Non-Protein Nitrogen (NPN)

The ANOVA also indicated highly significant differences ($p<0.0001$) in CP content, when different sources of nitrogen were added to the substrates for their fermentation.

Table 2. Effect of main factors and their interactions on the crude protein content of fermented prickly pear.

Source	F	p
Cultivar	25.63	<0.0001
Particle size	549.38	<0.0001
Nitrogen source	981.00	<0.0001
Cultivar*Particle size	3.90	0.0494
Cultivar*Nitrogen source	14.23	<0.0001
Particle size*Nitrogen source	115.60	<0.0001
Cultivar*Particle size*Nitrogen source	8.00	0.0004

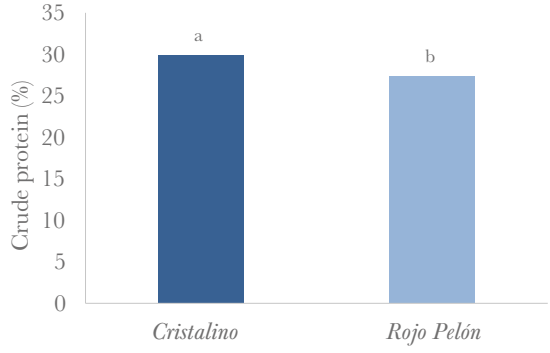

Figure 1. Effect of cultivar on the CP content (%) of fermented prickly pear substrates.

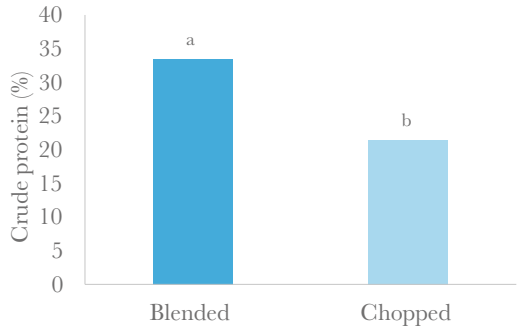

Figure 2. Effect of particle size on the CP content (%) of fermented prickly pear substrates.

Figure 3 shows statistical differences (Tukey $p<0.05$) between the three levels in this study: a higher PC content (37.7 %) was recorded with the addition of urea + ammonium sulfate. Meanwhile, the lowest CP value (13.2%) was reported when ammonium sulfate was added and an intermediate CP value (35.2%) was observed with the addition of urea.

CP Content per Treatment

ANOVA showed highly significant differences ($p<0.0001$) in CP content between the treatments evaluated. Figure 4 shows that the CrLiUS (*Cristalino*, blended, and urea + sulphate) treatment recorded the highest CP content (46.1%). However, it was not statistically different (Tukey $p<0.05$) from the RpLiUS (*Rojo Pelón*, blended, and urea + sulphate) and CrLiUr (*Cristalino*, blended, and urea) treatments, which recorded a 45.4 % and 43.7 % CP content, respectively. Nevertheless, its CP content was different from the other treatments. Finally, the controls (prickly pear cultivars without treatment) recorded the lowest CP values. Particularly, the *Rojo Pelón* cv had the lowest value (4.8 %) and was statistically different from the rest of the treatments, except for the *Cristalino* control.

Table 3 includes both the abovementioned CP results and the values of other determinations analyzed, based on the levels of the evaluated factors. The values of these variables are also shown for the controls (prickly pear without treatment).

Regarding the CP content, the highest Neutral Detergent Fibre (NDF) content (20.5%) in the substrate was recorded by the *Cristalino* cv (CrLiUS: *Cristalino*, blended, and urea + ammonium sulfate.). Meanwhile, the best treatment for the *Rojo Pelón* cv reached 18.0%

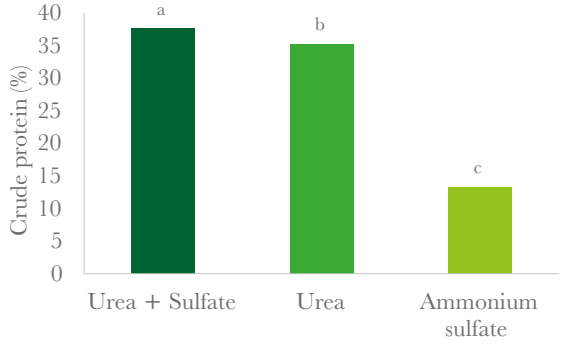

Figure 3. Effect of the nitrogen source on the CP content (%) of the fermented prickly pear substrates.

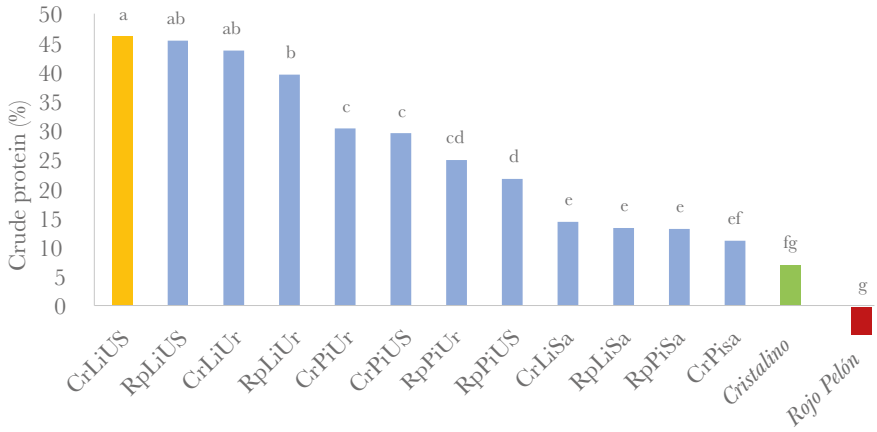

Figure 4. Crude protein content (%) per treatment. Cr=*Cristalino*, Rp=*Rojo Pelón*, Li=blended, Pi=cut, Ur=urea, Sa=ammonium sulfate, US=urea + ammonium sulfate.

Table 3. Bromatological characteristics and pH of the fermented prickly pear substrates and the controls.

Factor	Level	PC (%)	C (%)	EE (%)	H (%)	ST (%)	pH
Cultivar	Rojo pelón	27.3 b*	15.3 a	0.44 a	92.1 b	7.9 a	5.7 a
	Cristalino	29.9 a	15.0 b	0.36 a	92.3 a	7.7 b	5.2 b
Size	Blended	33.5 a	15.3 a	0.35 b	91.8 b	8.2 a	5.4 a
	Chopped	21.5 b	15.1 a	0.45 a	92.6 a	7.4 b	5.4 a
Source	Ammonium sulfate	13.2 c	16.4 a	0.44 a	92.5 a	7.6 c	4.8 c
	Urea	35.2 b	15.3 b	0.39 a	92.0 c	7.9 a	5.8 a
	Urea + Ammonium sulfate	37.7 a	13.9 c	0.37 a	92.2 ab	7.8 ab	5.6 b
Controls	Rojo pelón	4.8	9.6	0.16	91.2	8.8	4.7
	Cristalino	7.0	11.4	0.11	94.2	5.9	4.9

* Different letters between factor levels indicate significant differences (Tukey $p<0.05$). CP (PC)=crude protein, A(C)=ashes, EE=ether extract, H=humidity, TS (ST)=total solids.

(RpLiUS: *Rojo Pelón*, blended, and urea + ammonium sulfate). Finally, the NDF content of the controls (prickly pear without treatment) reached 17.2% and 19.0% in the *Rojo Pelón* and *Cristalino* cv, respectively.

The results of this study confirm that fermentation can improve the nutritional value of prickly pears (*Opuntia* spp.), making it a useful supplement for the diet of confined livestock, in association with other inputs. These findings support the results obtained in Brazil (Araújo *et al.*, 2005; Araújo *et al.*, 2008) and Mexico (Castro *et al.*, 2022; Díaz-Plascencia *et al.*, 2012; Flores-Hernández *et al.*, 2017; Flores *et al.*, 2019; Herrera-Torres *et al.*, 2014; Herrera *et al.*, 2017), the main countries in which this biotechnological process has been successfully tested. All those studies were conducted under various conditions, including different prickly pear cultivars, incubation methods, fermentation time, type of microorganism, NPN, and sugar sources. However, they all increased microbial protein from 200 to 400%, indicating their high potential as an alternative forage source for ruminants in the arid and semi-arid zones of the Mexican territory.

Few studies that explore the fortification of prickly pear in Mexico specify the species or cultivar used. Only a few point out that they are defenseless forage, including the AT-TV6 (Díaz-Plascencia *et al.*, 2012), AV6 (Herrera-Torres *et al.*, 2014), and one unspecified (Castro *et al.*, 2022) varieties. Only two researches involved wild varieties: *Opuntia leucotricha* (prickly) (Maldonado-Quiñones *et al.*, 2022) and *O. rastrera* (Fuentes-Rodríguez *et al.*, 2017). However, as far as this research team was able to determine, no work has used commercial cultivars to produce fruit. This study provides regional producers of prickly pear fruits an alternative for the exploitation of pruning waste and a source of high-quality forage supplement during summer and winter.

The CP content of the prickly pear cultivars used in this study showed statistical differences: the *Cristalino* cv (*O. albicarpa*) had a 29.9 % value, while the *Rojo Pelón* cv (*O. ficus-indica*) reached 27.3 %. Under similar *in vitro* conditions, Herrera-Torres *et al.* (2014) and Herrera *et al.* (2017) increased protein content from 14 to 16.31%, respectively. Under different fermentation and microorganism conditions, the values recorded in this study using a higher volume of prickly pear were higher than those obtained in Brazil by Araújo *et al.* (2005) and Araújo *et al.* (2008), who reported 26 and 10.4% values, respectively. Meanwhile, in Mexico, Díaz-Plascencia *et al.* (2012), Herrera-Torres *et al.* (2014), Herrera *et al.* (2017), and Maldonado-Quiñones *et al.* (2022) reported 19.4, 14, 16.3, and 22.7% values, respectively. Meanwhile, similar results were recorded by Flores-Hernández *et al.* (2017) (29.8%) and Castro *et al.* (2022) (27.5%), but Flores-Hernández *et al.* (2019) obtained better results (33.5%). This wide variability could be linked to the concentration levels of the substrate (Díaz-Plascencia *et al.*, 2012), because non-fibrous carbohydrates favor external fermentation (fortification) and internal fermentation (rumen fermentation) (Flores-Hernández *et al.*, 2017).

The two sizes tested had statistical differences. The blended prickly pear substrate recorded higher CP values (33.5%) than the cut prickly pears (21.5%). In most researches about the fortification of prickly pear the substrate has been cut (Castro *et al.*, 2022; Flores-Hernández *et al.*, 2017; Flores *et al.*, 2019), mainly due to its practicality (saving in labor and mechanization of the process) and technological issues (high humidity content), which hinder the reduction of the particle size. However, the results of this research indicate that the reduction in particle size can favor the development of the yeast microorganisms found in the substrate and the effect of the addition of non-protein nitrogen (NPN).

With regards to the NPN source, the tested levels had statistical differences. Adding both urea and ammonium sulfate increased the protein level to 37%, while separately adding each source caused a decreasing trend, both for urea (35.2%) and ammonium sulfate (13.2%). Herrera *et al.* (2017) recorded less protein when no nitrogen sources were added; they attributed the protein increase (from 5.12 to 16.31%) to the presence of native microbes in the substrate. According to Díaz-Plasencia *et al.* (2012) and Herrera-Torres *et al.* (2014), adding a combination of NPN and microorganisms to the substrate during the fermentation process facilitates the increase of protein levels, because they activate, accelerate, and make fermentation more efficient. For their part, Maldonado-Quiñones *et al.* (2022) mentioned that adding NPN to the prickly pear, through the urea and ammonium sulfate (which is not lost as ammonia), is a source of degradable protein for the rumen; however, it must

be synchronized with the addition of a source of rapidly degrading carbohydrates (grains and molasses), to avoid an increase in potentially toxic ammoniacal nitrogen. Therefore, detoxifying ammonia is particularly important.

Therefore, in terms of CP content, the best treatments were those to which urea and ammonium sulfate were added and in which cladodes were blended. The cultivar factor was less important, because the best treatments alternated between both cultivars. Therefore, the treatment with the highest CP content (46.1%) was based on blended *Cristalino* cv, to which urea plus ammonium sulfate (CrLiUS) was added, because its values were higher than those of cultivars without treatment: 4.8% for Rojo Pelón and 7.0% for *Cristalino*. The best treatment (CrLiUS) had a 46.1% CP content —higher than the reports of any of the works about the fortification of protein through prickly pear fermentation quoted in this study.

CONCLUSIONS

This study proved that fermentation increases the protein content of prickly pear and therefore, its quality as forage. The levels of the factors evaluated (prickly pear cultivar, particle size, and source of non-protein nitrogen) had significant differences ($p<0.05$). The highest CP values were recorded by *Cristalino* cv (Cr) on its own (29.9%), blended (Li, 33.5%), and with the addition of urea plus ammonium sulfate (US, 37.7%). The combination of these factors (CrLiUS) resulted in the treatment with the highest CP content (46.1%). The fermentation times used in this study (6, 8, 10, and 12 h) did not cause any significant differences, just like the inoculation with yeast only or with yeast plus fermented agave juice. Prickly pear fermentation is a nutritious and feasible option to feed ruminants. However, further research that resulted in the standardization and optimization of its processes would provide products with better dietary evenness, which could be used in livestock production at a low cost.

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