grasses

MDPI

Article

Overcoming Forage Challenges in Mesophytic Grasslands—The Advantages of *Lotus tenuis*

María Elena Vago [1,2,*,†], Paula Virginia Fernández [2,3,†], Juan Pedro Ezquiaga [4], Santiago Javier Maiale [5], Andrés Alberto Rodriguez [5], Juan Manuel Acosta [6,7], Maximiliano Gortari [5], Oscar Adolfo Ruiz [4,5,‡] and Marina Ciancia [2,3,‡]

1 Laboratorio de Evaluación de Alimentos para Uso Animal, Facultad de Ingeniería y Ciencias Agrarias, Pontificia Universidad Católica Argentina, 1680 Alicia Moreau de Justo Ave., Ciudad Autónoma de Buenos Aires C1107AFH, Argentina
2 Cátedra de Química de Biomoléculas, Departamento de Biología Aplicada y Alimentos, Facultad de Agronomía, Universidad de Buenos Aires, 4453 San Martín Ave., Ciudad Autónoma de Buenos Aires C1417DSE, Argentina; vfernand@agro.uba.ar (P.V.F.); ciancia@agro.uba.ar (M.C.)
3 Centro de Investigación de Hidratos de Carbono (CIHIDECAR), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad de Buenos Aires, 4453 San Martín Ave., Ciudad Autónoma de Buenos Aires C1417DSE, Argentina
4 Chacra Experimental Integrada de Chascomús (CEICh), Chascomús B2700XAC, Provincia de Buenos Aires, Argentina; jpezk@yahoo.com.ar (J.P.E.); ruiz@intech.gov.ar (O.A.R.)
5 Instituto Tecnológico de Chascomús (INTECH), Escuela de Nano y Biotecnología, Universidad Nacional de San Martín (UNSAM), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Km 8.2 Intendente Marino Ave., Provincia de Buenos Aires B7130, Argentina; smaiale@intech.gov.ar (S.J.M.); andres.a.rodriguez@gmail.com (A.A.R.); maxigortari@hotmail.com (M.G.)
6 Instituto de Botánica Darwinion, 200 Labardén St., San Isidro B1642HYD, Provincia de Buenos Aires, Argentina; jacosta@darwin.edu.ar
7 Cátedra de Botánica Sistemática y Vegetación, Facultad de Ingeniería y Ciencias Agrarias, Pontificia Universidad Católica Argentina, 1680 Alicia Moreau de Justo Ave., Ciudad Autónoma de Buenos Aires C1107AFH, Argentina
* Correspondence: mevago@uca.edu.ar
† These authors contributed equally to this work and share the first authorship.
‡ These authors should be considered senior contributors.

Academic Editor: Fabio Gresta

Received: 14 March 2025
Revised: 11 April 2025
Accepted: 23 April 2025
Published: 7 May 2025

Citation: Vago, M.E.; Fernández, P.V.; Ezquiaga, J.P.; Maiale, S.J.; Rodriguez, A.A.; Acosta, J.M.; Gortari, M.; Ruiz, O.A.; Ciancia, M. Overcoming Forage Challenges in Mesophytic Grasslands—The Advantages of *Lotus tenuis*. *Grasses* **2025**, *4*, 19. https://doi.org/10.3390/grasses4020019

Abstract: Previous studies in the Salado River Basin (Argentina) demonstrated that the introduced forage species, *Lotus tenuis* Waldst. & Kit. ex Wild. (Fabaceae), possesses high tolerance to abiotic stresses—including flooding, alkalinity, salinity, and drought. The efficient biological fixation of nitrogen in a region with a scarce presence of native legumes is one of its advantages. Despite these qualities, a year-long characterization of cell wall (CW) polysaccharides in *Lotus tenuis* and their relationship with the high nutritional quality is missing. In this study, seasonal parametric investigations of *L. tenuis*, regarding its photosynthetic and ionic status, modifications in CW composition, and concomitant nutritional quality, were performed. Our results demonstrate the high plant digestibility and protein content of this legume, even in summer, when most accompanying species reduce their forage quality. Regarding gas production kinetics (in vitro production is a proxy for the animal rumen's output), spring biomass had the highest values. The CW material yields are similar throughout the year, but with differences in polysaccharide composition. In summer and winter, pectins predominate, while in the regrowth periods (spring and autumn), pectins and β-glucans are found in similar amounts. This work confirms that *Lotus tenuis* can help optimize grassland productivity in challenging mesophytic terrains to increase livestock productivity through environmentally friendly services.

Keywords: *Lotus tenuis*; forage quality; cell wall composition; mesophytic meadow

1. Introduction

The extensive plain in central–eastern Argentina, known as the "Flooding Pampas" or Salado River Basin, which includes the area surrounding the largest river in the region, is mainly used for cattle raising, an activity that occupies 90,000 km^2 [1,2]. Different native grass communities, plus some exotic species such as *Lotus tenuis Waldst. & Kit*. ex Wild (narrowleaf trefoil, narrow-leaved bird's-foot trefoil, slender trefoil, creeping trefoil, or prostrate trefoil), provide the main sources of forage [3,4]. This species of glycophytic Fabaceae (legume), native to the Mediterranean area and naturalized in some regions of Argentina, has adaptations that enable it to grow in this kind of environment under simultaneous or consecutive abiotic stresses, such as alternating periods of drought and flooding, and the consequent salinity [5]. *Lotus tenuis* is a perennial, yellow-flowered, herbaceous leguminous species that was introduced into the Salado River Basin in Argentina to provide forage for livestock, predominately cattle (for more detailed morphological, ecological, and distributional descriptions, see the *L. tenuis* treatments in the Jepson eFlora https://ucjeps.berkeley.edu/eflora/eflora_display.php?tid=31609, Kew Royal Botanic Garden Plants of the World Online https://powo.science.kew.org/taxon/urn:lsid:ipni.org:names:503917-1 "URL (accessed on 20 May 2024" and the Global Biodiversity Information Facility https://www.gbif.org/species/7515045 "URL (accessed on 24 June 2024)").

The introduction or promotion of *L. tenuis* has thus made an interesting contribution due to its persistence in these difficult environments and protein content [5,6]. Recently, it has been shown that its implementation and persistence establish continuous improvements in the nitrogen cycle through efficient biological fixation in symbiosis with native rhizobia [7]. In addition, a significant contribution has been evidenced in the quality of the forage offered, especially in the summer months, the season of the year when graminoid species increase their lignin content, reducing their nutritional quality [3]. The correlation with the structural elements of the aerial parts of *L. tenuis* was never evaluated despite its significant impact on the great economic activity of the Salado River Basin.

Moreover, when it grows with other plant species that thrive in these sites, such as *Paspalidium paludivagum*, *Echinochloa helodes*, *Leersia hexandra*, *Glyceria multiflora*, *Phalaris angusta*, *Setaria parviflora*, *Sporobolus indicus*, or *Distichlis* sp. [8–10], it can enhance forage performance due to its good digestibility, improve soil nutrients due to its high capacity for phosphorus metabolization and biological nitrogen fixation through interaction with mycorrhizae and rhizobium, and promote bacterial growth [7,11,12]. The limitations are inherent to the soil types in the area characterized by low organic matter content and a natric horizon [13,14].

In extensive production, where animals graze on natural grasslands, the primary nutritional objective for ruminants is to feed the ruminal microbiota with forage cell wall polysaccharides that contribute efficiently to building up microbial protein and thus cover the requirements of the animals [15,16]. The characteristics of cell carbohydrates in the forage, which comprises soluble carbohydrates, starch, and especially the cell walls given their high proportion of the total plant biomass, are closely related to nutritional quality [17,18].

As a legume, *L. tenuis* has type I primary cell walls, with cellulose microfibrils, xyloglucans as the major hemicellulose, and large quantities of pectins concentrated in the middle lamella (like a special adhesive between adjacent cells), forming a hydrated gel matrix throughout the cell wall. Pectins have dynamic structures, and they are the most soluble of the polysaccharides in legume cell walls, impacting porosity, cell communication, ion exchange, and electrostatic capacity [19,20]. The development of a secondary cell wall in this species provides glucuronoxylans and mannans (hemicelluloses) and higher quantities

of cellulose and lignin. A mixture of cell walls containing different combinations of these polymers could be found in the aerial part of the plant [21,22].

This study aims to determine the seasonal contribution of *L. tenuis* in the mesophytic meadows of the Salado River basin, considering nutritional and photosynthetic performances, and cell wall composition parameters, providing evidence that justifies the mention of *L. tenuis* as a key species for the economic sustainability of the region [3,14]. A year-long characterization of its cell wall structures and their relationship with the high nutritional quality of *Lotus tenuis* was performed, confirming that *Lotus tenuis* can help optimize grassland productivity in challenging mesophytic terrains.

2. Materials and Methods

2.1. Plant Sampling and Experimental Farm Description

Plants of *Lotus tenuis* were collected at Instituto Tecnológico de Chascomús (INTECH), Buenos Aires Province, Argentina (35°37′26″ S, 57°59′50″ W). Edaphically, the sampling area was defined as a mesophytic meadow (35°37′24,96″ S, 57°59′52,08″ W) based on the contrasting characteristics of the soil [13] (Figure 1).

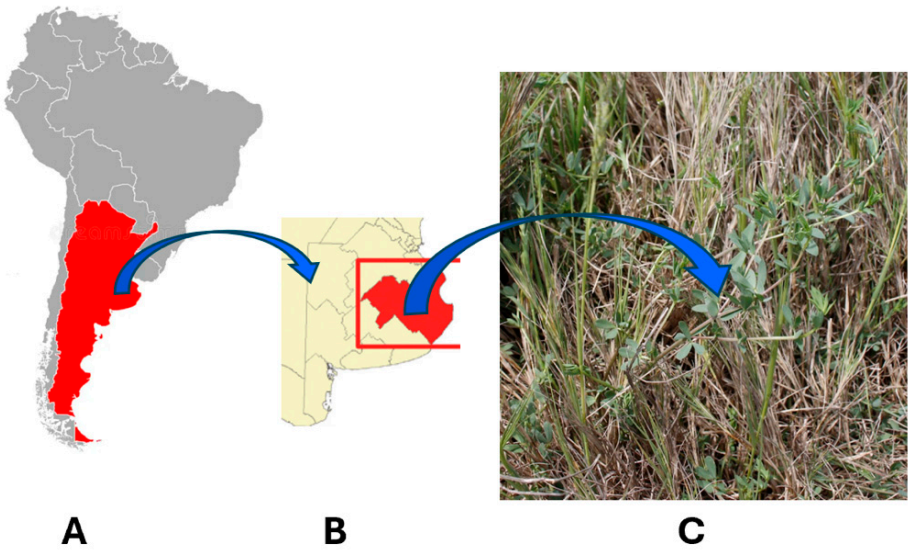

Figure 1. (**A**,**B**): Study location area in South America, Argentina country highlighted in red (**A**): the Flooding Pampas area in the province of Buenos Aires (**B**) (red zone). *Lotus tenuis* growing with *Distichlis* sp. (halophytic grass) in the mesophytic meadow sampling site of the Flooding Pampas (**C**) (INTECh, Chascomús, Buenos Aires, Argentina).

The average annual climatic characteristics of the region are attached as Supplementary Figure S1. Species were determined based on vegetative morphological characteristics. The sampling dates were chosen in four-year seasons. Triplicate samples of *L. tenuis* plant biomass (leaves and shoots) were collected from three neighboring paddocks (100 × 100 cm, approximately) to determine the nutritional and K^+/Na^+ contents (as described below). For cell walls, the chemical analysis of the seasonally collected material was pooled.

2.2. Soil Analysis

Three soil replicates were collected during the summer. With the help of a Procheck GS3 (Decagon Devices Inc., Pullman, WA, USA) device, electrical conductivity was measured where the material was collected. Each replicate consisted of a mixture of 20 pooled and thoroughly mixed soil subsamples. Soil subsamples were collected from four neighboring paddocks (100 × 50 cm, approximately). Soil cores were taken with a borer

(20 cm length × 7 cm diameter) every 10 m. Soil replicates were introduced into individual plastic bags and immediately transported to the laboratory, where they were sieved through a 2 mm mesh and used for further analysis. There, aliquots of soil samples were analyzed for pH (paste) and the sodium adsorption ratio (SAR). Measurements were performed at the Soil Laboratory of Facultad de Agronomia facilities, Universidad de Buenos Aires (Argentina), according to IRAM-SAGyP norms [10,23].

2.3. Analysis of Sodium and Potassium Contents

Sodium (Na^+) and potassium (K^+) contents were extracted from ground shoot and leaf material samples with 100 mM HCl and determined using a flame photometer (Cole-Palmer Instrument Co., Chicago, IL, USA) [24], as described by Campestre et al. [25].

2.4. Photosynthetic Activity Analysis

In situ, a HANDY PEA fluorometer (Hansatech Instruments® Ltd., King's Lynn, Norfolk, UK) was used according to the manufacturer's instructions to record the chlorophyll "a" fluorescence emission kinetics and OJIP (fast chlorophyll a fluorescence induction) parameters, as described by Bolhar-Nordenkampf et al. [26].

2.5. Nutritional Parameters and Digestibility Using the In Vitro Gas Production Technique

Dry matter (DM) was determined after drying samples of the aerial portions of *L. tenuis* (shoots + leaves) at 65 °C. Nutritional parameters were determined in ground material: ash, using 2 mg at 600 °C for 2 h; crude protein (CP), using 0.5 g via the Kjeldhal method; and neutral detergent fiber (aNDF) with α-amylase and acid detergent fiber (ADF), using an ANKOM® device (Model 220, ANKOM™ technology, Fairport, NY, USA). Additionally, lignin content was determined via sulfuric acid treatment (Lignin (sa)) [16,27]. Plain abbreviations were used: g/kg DM, CP, aNDF, ADF, and Lignin (sa).

To assess digestibility without losing the fermentation kinetics, the in vitro cumulative gas production technique was used on dried and ground samples comprising shoots and leaves. Samples were incubated with ruminal liquor, as described by Theodorou et al. [28] and modified by Wawrzkiewicz and Danelón [29]. The pressure in the incubation bottles was measured with a pressure transducer (T443A, Bailey and Mackey Ltd., Birmingham, England). Cumulative gas production (NCGP) data were expressed per gram of incubated DM (mL/g DM), and values were corrected via gas blanks. Dry matter digestibility (*iv*DMD) was evaluated in two bottles of each sample/treatment, with the addition of 2–3 drops of a saturated thymol solution. The reaction was stopped after 48 h of incubation. The incubation of the remaining bottles continued until 72 h, and the samples were filtered through fiber filter bags (ANKOM #F57) before being sealed. The evaluation of *iv*DMD was calculated from the residues of the filtrate and before the treatment with the neutral detergent solution. In addition, neutral detergent fiber digestibility (NDFD) was also analyzed [26]. The true DMD at the two incubation times (48 and 72 h) was calculated as follows: *iv*DMD (%) = 100 − aNDF residue × 100/DM incubated.

2.6. Alcohol-Insoluble and Hot-Water-Insoluble Residue Determination

Alcohol-insoluble and hot-water-insoluble residues were obtained from the ground biomass (shoots and leaves) dried at 65 °C until a constant weight. The dry material (100 mg/mL) was extracted sequentially for 1 h with organic solvents: twice with ethanol (Porta, Córdoba, Argentina), twice with acetone (Sintorgan, Buenos Aires, Argentina), and once with ethyl ether (Sintorgan, Buenos Aires, Argentina). Finally, the residue was dried to obtain the alcohol-insoluble residue (AIR). Then, the hot-water-insoluble residue (HWIR) was obtained from AIR via two sequential extractions with H_2O for 3 h at 90 °C (~50 mg/mL). The final residue was centrifuged, washed, and finally freeze-dried [30].

2.7. Analysis of Cell Wall Chemical Composition

Total carbohydrates and uronic acids sample concentrations were determined colorimetrically [31]. The treatment of insoluble material was carried out following the method of Ahmed and Labavitch [32]. After saponification, the phenolic contents were estimated via a Folin–Ciocalteu colorimetric assay, using gallic acid as a standard and expressing the results as gallic acid equivalents (GAEs) [33]. For the determination of neutral monosaccharide compositions, gas chromatography (GC) was used. Alditol acetates were obtained in triplicate throughout hydrolysis with trifluoroacetic acid (TFA) (Sintorgan, Buenos Aires, Argentina) 13 M (37 °C, 1 h), acid dilution to 11.5 M, heating at 100 °C for 1 h, and further dilution to 2 M, achieving the hydrolysis conditions necessary for water-insoluble polysaccharides [34]. After hydrolysis, the samples were derivatized relative to the corresponding alditol acetates, and the GC technique was used on an Agilent 7890 A gas–liquid chromatograph (Agilent, Avondale, PA, USA) according to the protocols previously described [10].

2.8. Spectroscopy ATR-FTIR Analysis

The infrared spectra from 4000 to 400 cm^{-1} were obtained with an iS50 Nicolet FT-IR spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA) equipped with a DTGS Attenuated Total Reflectance (ATR) detector. A resolution of 4 cm^{-1} was obtained using 32 scans. Subsequently, the spectra were processed using Spectragryph optical spectroscopy software, version 1.2.15, 2020 http://www.effemm2.de/spectragryph/ "URL (accessed on 3 December 2024)".

2.9. Statistical Analysis

Differences in photosynthetic, digestibility, and nutritional parameters; sodium and potassium levels; hot-water-insoluble and alcohol-insoluble residues; and cell wall material chemical analyses were determined using parametric tests (Student's *t*-test; statistical significance, $p \leq 0.05$) after normal distribution assessment (Shapiro–Wilk's test; *p*-values > 0.05) and homoscedasticity evaluations (Levene's test; *p*-values > 0.05). The means and standard errors of the data distribution were reported for comparative purposes. The Infostat version 2020p software (Universidad Nacional de Córdoba, Córdoba, Argentina) was used for analyses and graphs.

3. Results

3.1. Characterization of Soil and Plants in the Areas of This Study Using Physicochemical and Physiological Parameters

The first step was to perform a parametric analysis of the area previously described as a mesophytic meadow (M) [5,32] in terms of soil and the overall condition of the plants from a physicochemical and physiological standpoint. The soil samples (collected in summer) had a pH of 7.57, electrical conductivity (EC) of 3.62 dS/m, and sodium adsorption ratio (SAR) of 7.25 meq/L.

Plants were initially characterized from a physiological standpoint. Photosynthetic activity, described through F_V/F_M, remained mostly below optimal values (0.75–0.85) for spring and autumn (Figure 2A), while in summer and winter, optimal F_V/F_M values were registered. Lower values of the photosynthetic performance index (PI_{ABS}) in spring and autumn evidence lower photosynthetic activity. However, higher values were found in winter and especially in summer (Figure 2B).

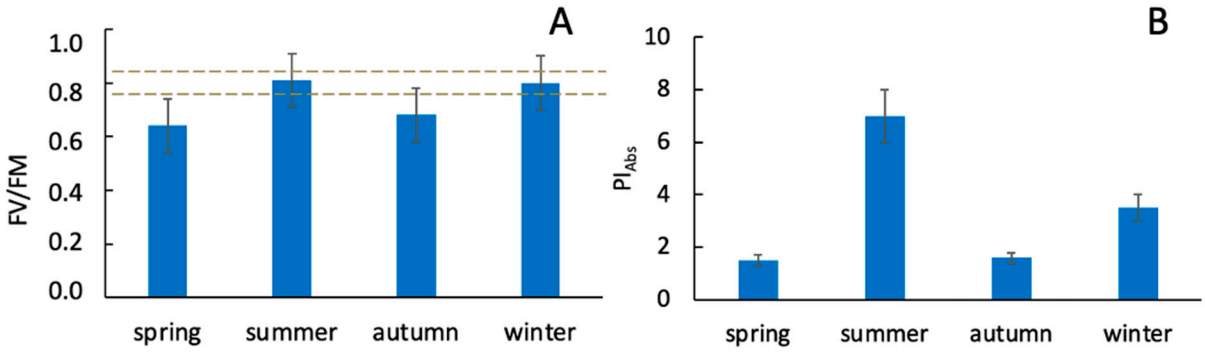

Figure 2. *Lotus tenuis* Photosystem II (PSII) state determination via the OJIP test technique. Evaluation of the maximum quantum efficiency of the PSII (FV/FM) (**A**) and the performance index based on equal absorption (PIabs) (**B**) of *L. tenuis* during the year. Dashed lines represent the maximum (0.85) and minimum (0.75) FV/FM optimum ratios. Data represent mean values ± S.E. between seasons from plants growing on a mesophytic meadow.

Simultaneously, the overall condition of the plants was characterized using the K^+/Na^+ ratio, which was high for all four sampling dates (Figure 3). By increasing K^+ uptake and accumulation, plants in marginal environments reduce ionic phase stress and thrive in these soil conditions [35,36], reflecting a higher ratio as a general indicator of greater plant salt tolerance.

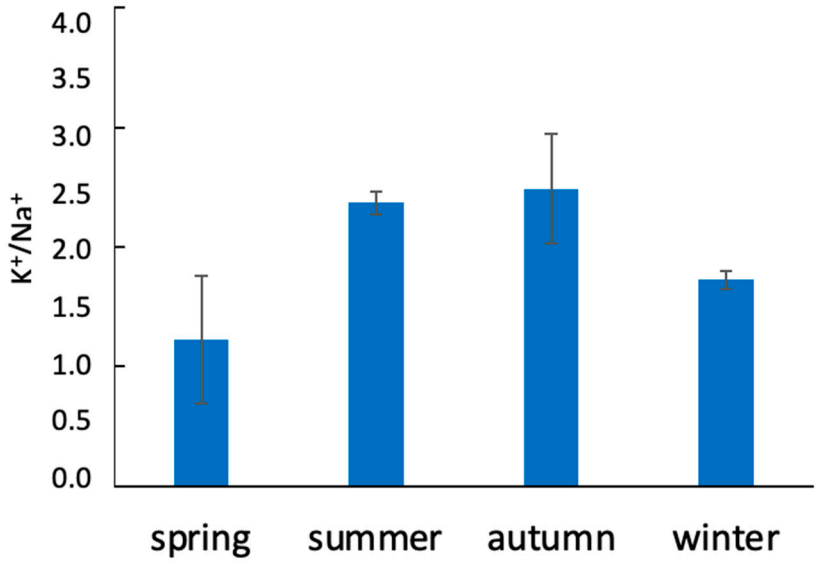

Figure 3. Values of the K^+/Na^+ ratio in *L. tenuis* aerial parts. Data represent K^+/Na^+ mean ± S.E. in grams.

3.2. Nutritional Parameters and Characterization of Cell Wall Material

3.2.1. Nutritional Characterization

Lotus tenuis exhibited significant differences among seasons for DM, aNDF, ADF, hemicellulose, and lignin. Interestingly, non-significant differences were found for crude protein, *iv*DMD, and ash content (Figure 4A–C). DM was lower in spring and autumn, following the regrowth cycle (mean values: 211.4 ± 2.1 and 203.4 ± 16.0 g/kg DM, respectively) (Figure 4A). Spring and winter samples presented higher aNDF contents. Concerning ADF, significant differences were found between spring (mean: 216.33 ± 30.3 g/kg DM), summer (mean: 173.9 ± 7.8 g/kg DM), and autumn (mean: 152.2 ± 5.8 g/kg DM). A significant reduction in hemicellulose concentration was detected only in summer (Figure 4C). Lignin contents differed between spring (mean: 106 g/kg DM), summer (177.7 g/kg DM), and

winter (165.8 g/kg DM). Ash contents ranged from 81.6 to 110.8 g/kg DM. High crude protein levels ranged from 193.40 to 269.20 g/kg DM without noticeable differences between seasons. In vitro digestibility differed slightly between seasons: spring: 805 g/kg DM; summer: 856.5 g/kg DM; autumn: 775 g/kg DM; winter: 791.5 g/kg DM (Figure 4B).

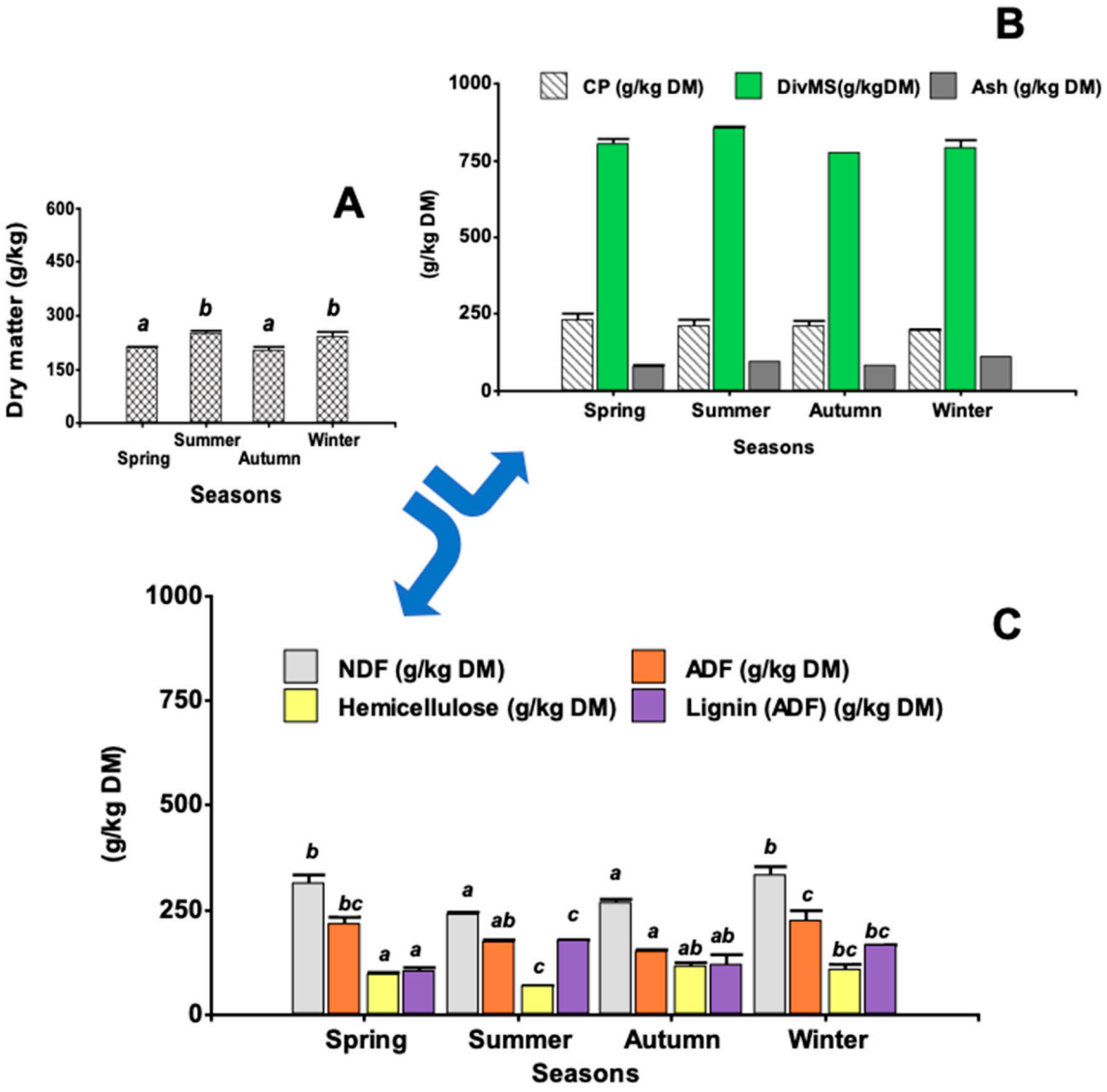

Figure 4. The nutritional contribution of *L. tenuis* to a marginal environment (**A**–**C**): (**A**): dry matter content (DM); (**B**): crude protein (CP); (**C**): in vitro dry matter digestibility (DivMS) and ash content. C: neutral detergent fiber (aNDF), acid detergent fiber (ADF), and hemicellulose and lignin (Lig(sa)) content. Dry matter data are expressed as g/kg fresh matter ± SD; the rest of the parameters are expressed as g/kg of dry matter ± SD. Different letters for each parameter denote significant differences between samples.

3.2.2. Gas Production Kinetics

Gas production kinetics differed noticeably between seasons in the range of 2 to 18 h. Gas production had the highest values in spring samples, followed by summer samples and then winter samples, and the lowest was observed in autumn samples.

A difference of nearly 100% was found in the first 5 h of gas production between spring and summer samples, and an increase of 25% was observed up to 15 h (Figure 5A). Considering the previously reported results for NDF and ADF, gas production for spring is consistent with more soluble materials in neutral detergent solution, less DM, and seasons in which *L. tenuis* has a strong regrowth.

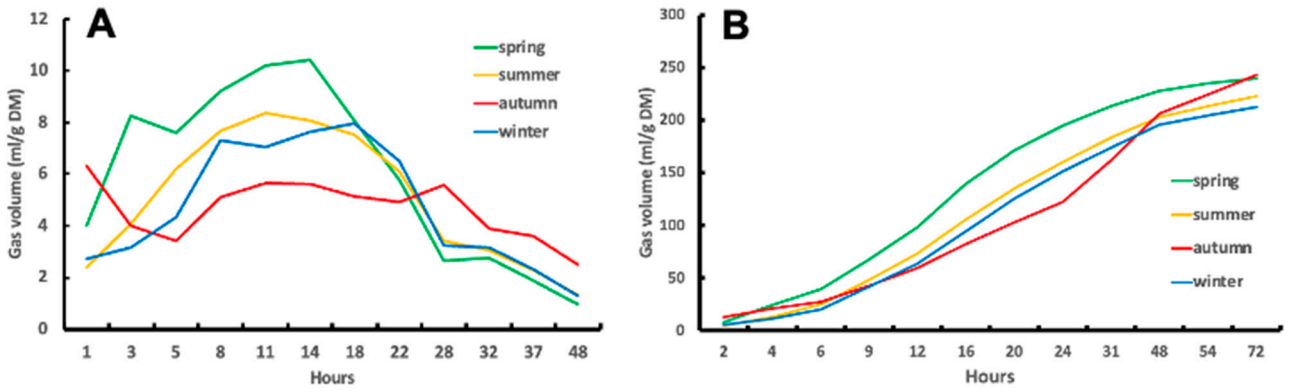

Figure 5. (**A**) In vitro gas production kinetics (ml/g DM). (**B**) Net gas production (ml/g DM) of shoots + leaves from *L. tenuis* for spring, summer, autumn, and winter.

The total cumulative gas production agrees with the digestibility behavior, without significant differences between seasons. The final gas production is similar, but the gradient is different, suggesting different chemical composition (Figure 5B).

3.2.3. Characterization of Cell Wall Materials

The hot-water-insoluble residues (HWIR) and the alcohol-insoluble residues (AIR) did not vary significantly over the seasons. However, HWIR presented greater dispersions, though not enough to establish significant differences. The total phenolic content was in the range of 2–8 g/kg HWIR during different seasons. Cell wall monosaccharide compositions in HWIR were determined over the year (Figure 6A). Based on these results, the different cell wall polysaccharide contributions to dry matter were estimated (Figure 6C).

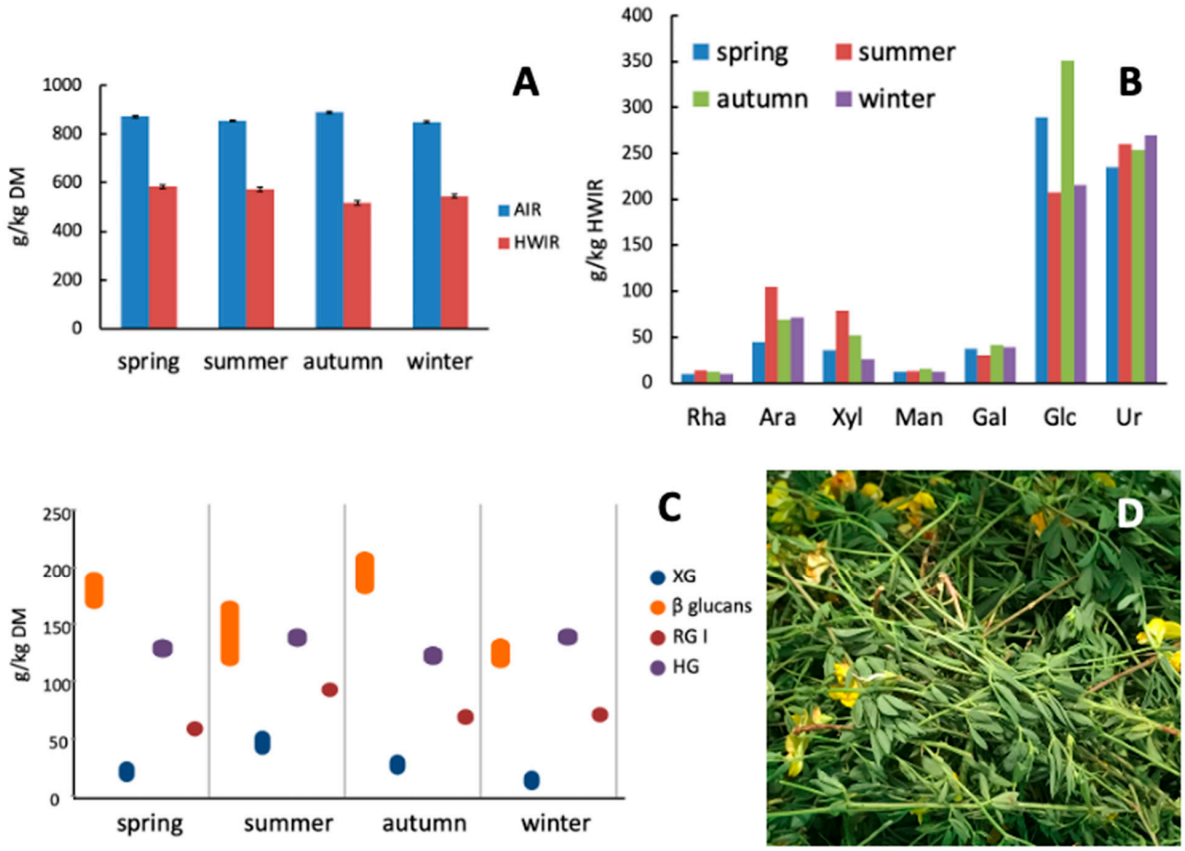

Figure 6. (**A**) Yields of alcohol-insoluble residue (AIR) and hot-water-insoluble residue (HWIR), expressed as g/kg of dry matter ± SD. (**B**) Monosaccharide composition of the hot-water-insoluble

residue (HWIR) from aerial parts of *L. tenuis* between seasons. Rha: Rhamnose; Ara: arabinose; Xyl: xylose; Man: mannose; Gal: galactose; Glc: glucose; Ur: uronic acids. (**C**) Estimated contribution of different cell wall polysaccharides to the dry matter of the aerial parts of *L. tenuis*. XG = Xyloglucans; β-glucans: ranges estimated considering glucose as part of cellulose and xyloglucans (together with xylose). GX = glucuronoxylans; ranges correspond to the cases in which xylose corresponds to GX and to the assumption of 10% maximum substitution with GlcA. HG = Homogalacturonan, calculated from the total amount of UA minus the amount of Rha, the range corresponds to the possible contribution of GlcA to the total UA content. RGI = rhamnogalacturonan I, calculated as the amount of 2 × Rha + Ara + Gal. Small amounts of mannans were also detected (7–8 g/kg DM), but they were not included. (**D**) *Lotus tenuis* summer sample growing in the mesophytic site.

3.2.4. ATR-FTIR Spectroscopy

The spectra of the samples of HWIR corresponding to different seasons provided very similar results (Figure 7).

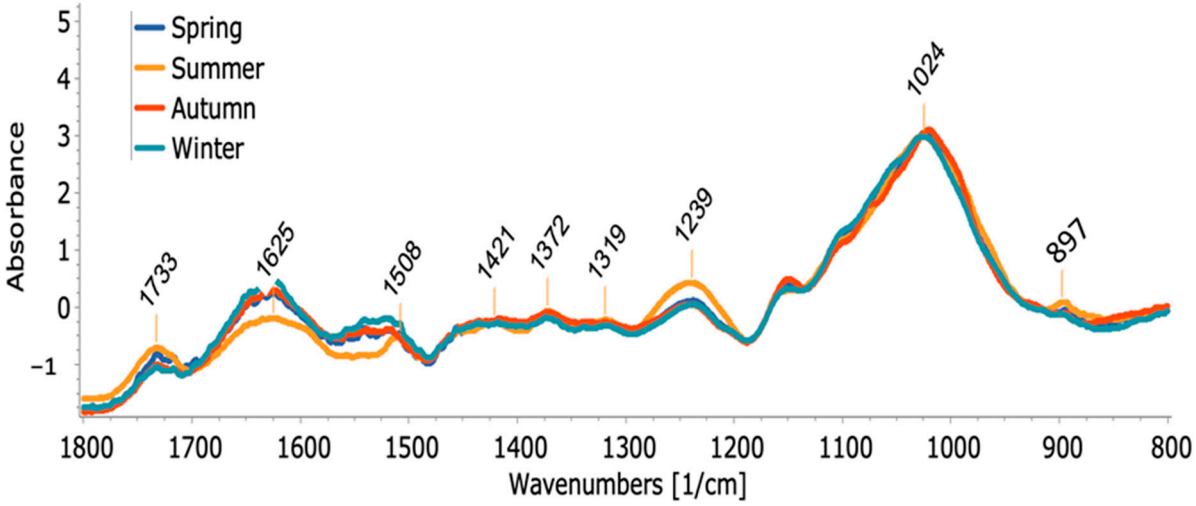

Figure 7. ATR-FTIR spectra of hot-water-insoluble residue (HWIR) from the aerial parts of *Lotus tenuis* in the different seasons.

The analysis was focused on the range of 1800–800 cm^{-1}, as is usually carried out for cell wall polysaccharides. The spectra show a major peak originating from the high absorbance in the region of 1100–950 cm^{-1}, with the maximum intensity at around 1020–1035 cm^{-1}, corresponding to samples rich in cellulose and hemicellulose [37]. The peaks at around 1733, 1625, and 1239 cm^{-1} are associated with pectins, those at 1421 cm^{-1} are related to cellulose, and those at 896 cm^{-1} correspond to β-glycosidic bonds [37]. These results agree with the estimated contribution of different cell wall polysaccharides to the dry matter of the aerial parts of *L. tenuis* given the seasons of the year, which show major amounts of cellulose and pectins (especially HG) (Figure 6C).

The nutritional quality of a forage species is largely determined by the characteristics of its cell walls because of its substantial contribution to the total dry matter. The presence and quantity of different polysaccharides, polyphenols, and structural proteins; their interactions; and their chemical structures are determinants of dry matter intake and digestibility.

4. Discussion

The importance of Argentina in the production of quality beef is recognized worldwide.

This situation justifies the deepening of studies that improve the environmental sustainability of the Salado River Basin region, especially the supply and quality of forage. Its

better understanding promotes the study of native [10] and introduced [3,5] plant species. Within the latter, *L. tenuis* constitutes a very successful example of naturalization, which allows an improvement in the qualities of the soil and the supply and nutritional quality of feed for livestock. However, studies of its variations throughout the year to support better livestock management and prevent overgrazing are scarce.

L. tenuis nutritional values are comparable in protein content and high digestibility to those of *Lotus corniculatus* and *Trifolium repens*, which grow in better soil conditions [3,4], but only a few legume species are adapted to growing in mesophytic or saline meadows [1,11,38]. For feeding grazing ruminants, the availability of energy, protein, and other nutrients is affected by the concentration of the fraction commonly called "fiber", the less digestible components of the plant cell wall, which exclude pectins and are rapidly used by the rumen microbiota. Several aspects of fiber contents affect the time that feed remains in the rumen (until its particle size is reduced and it can continue through the digestive tract), and the following are some of the most important: the degree of digestibility (ratio between the components of cell wall polysaccharides and soluble components), protein availability, arrangement and amount of lignin, and shoot–leave ratio [39,40].

As expected for a legume, *L. tenuis* showed high CP levels (Figure 4A), as a consequence of the use of nitrogen fixed by rhizobium and phosphorus nutrition throughout mycorrhizae symbiosis [7]. In addition, this legume improves the N cycle in this environment, with a positive impact on the growth of associated plant species [3,5,41]. There are periods when the protein content of the natural species of grassland in these marginal environments is critical. C4 grasses present good protein content and fiber quality in their vegetative state and lose their quality very quickly in the reproductive state, making a large contribution of fibrous dry matter in feed rations with low protein content (</= 50 g/1000 g). *L. tenuis* protein could be an essential asset because rumen microbiota has a minimum protein requirement of 7% for cattle during maintenance, but this need doubles in animals that are growing or producing milk such that microbial protein synthesis, added to by-pass proteins, can cover animal protein requirements [1,3,18,42].

The energy contribution to the diet of carbohydrates contained in the biomass of *L. tenuis*, as a proportion of that provided by other species growing in this marginal environment, warrants a more detailed description. Its contribution in dry matter is lower than that of grasses, but its nutritional contribution is better, with less cell wall fibrillar material [3,18,42,43]. Moreover, the types of cell wall polysaccharides (higher concentration of pectins), different from those of grasses, should be related to the better nutritional performance. NDF contents were lower in spring, summer, and autumn than in winter in concordance with their growth cycle, and there was consequently a higher portion of material that was soluble in neutral detergent solutions (mainly starch and pectins, as well as low-molecular-weight carbohydrates). Pectins should be present in minimal amounts or completely absent in the neutral detergent-insoluble fraction (NDF). NDF was in the range of 238–370 g/kg, which predicted the better voluntary intake of *L. tenuis* than that of grasses, such as *Paspalidium paludivagum*, *Phalaris angusta, Echinochloa helodes*, *Leersia hexandra*, *Glyceria multiflora*, and *Distichlis* sp., which are characteristic of this area [5,16]. The fraction of cell wall materials that remain in the acid detergent fiber, mainly cellulose, lignin, and insoluble protein, was in the range of 193–270 g/kg, in agreement with the high values of in vitro digestibility found for *L. tenuis,* like other legumes that cannot grow on this site (i.e., *Medicago*). Moreover, it maintained high values of in vitro digestibility throughout the year, without differences among seasons (average: 812 g/kg DM; SD: 36.3) (Figure 4B) and with values 100% higher or even more than: for example, *Distichlis* sp., which is a predominant grass species in this area [10,44].

Moreover, the results from in vitro digestibility mirror in vivo digestion kinetics [43]. As was described previously, the nutritional composition correlates with the gas produced in vitro [45,46]. Cell wall polysaccharides and reserve α-glucans have different digestion rates, and a strongly negative correlation between NDF content and gas production rate was described [47]. There is a relationship between large quantities of gas production between 3 and 10.3 h and starch and pectin compositions [16]. This is consistent with results from the current study, especially for spring samples, in which high in vitro gas production matches the exponential plant growth [29]. The total cumulative gas production rate differed between seasons, which was also noticeable in the lower values of NDF (240–340 g/kg), while the HWIR values were above 500 g/kg.

Lignin content is usually considered a negative nutritional aspect of feed because it reduces the microbial degradation. The samples of *L. tenuis* analyzed in this work presented low values of this phenolic biopolymer throughout the year (106–177 g/kg DM), with significant differences only between, on one hand, the spring sample and, on the other, the winter and summer samples. Moreover, legumes do not contain lignin cross-linked by ferulate bridges to the polysaccharides of the cell wall, reducing ruminal degradation, as is the case with grasses [48].

The results from nutritional parameters, plus the information from gas production kinetics and cumulative gas production, provided useful but incomplete information. To obtain more in-depth knowledge of cell wall components, the fractions insoluble in hot water (HWIR) and alcohol (AIR) were obtained, and the HWIR was characterized. The main monosaccharides of the cell walls were glucose (Glc; 207–350 g/kg HWIR) and uronic acids (UA; 235–270 g/kg HWIR), with the most notable differences being observed in the former (Figure 6B). Based on the composition of monosaccharides and the knowledge of this type of cell wall, it was possible to estimate the contribution of the different families of polysaccharides to the cell wall of the material [22,27,49].

For the purposes of this analysis, it was assumed that glucose corresponds to cellulose and other minor β-glucans, such as xyloglucans (XG), because the α-glucan content derived from starch that could remain in these residues should be of little relevance. Xylose derives mostly from glucuronoxylans (GX; a minor component limited to secondary walls); meanwhile, arabinose can be attributed to the side chains of rhamnogalacturonan I (rhamnose, arabinose, and galactose-rich pectins). Uronic acids, including glucuronic and galacturonic acid, can be part of both GX and pectins, respectively [50]. Based on these results, Figure 6C shows that the major cell wall polysaccharides in the aerial parts of *L. tenuis* are β-glucans (cellulose/XG), followed by HG. But if pectins are taken as a whole (HG + RGI), this family of polysaccharides predominates in summer (Figure 6D) and winter. On the other hand, in spring and autumn, the total amount of pectins is lower and overlaps with that of β-glucan.

There is a strong relationship between feed digestibility and animal ruminant intake, the time spent in the rumen, and the energy available to the animal. Grass with a high percentage of cell wall components of low digestibility, as happens in the growth cycle of some species adapted to mesophytic environments, limits voluntary intake. Moreover, as mentioned previously, a low percentage of crude protein reduces the normal rate of the reproduction of ruminal microbiota and therefore restricts the digestion of cell wall components (minimum CP level of 7%) [22,51].

Recent studies suggest the importance of legumes and grasses growing together in marginal environments for grazing [3,52]. Greater plant diversity provides better access to nitrogen (N), more efficient use of N, and more soil C and N sinks than in areas with only one species (monoculture) [53,54].

Furthermore, it is a well-known fact that increasing the diet protein concentration simultaneously satisfies the microbiota requirements not covered by grasses and may leave

a surplus that could enhance digestibility, intake, and energy consumption, impacting the grazing ruminant depending on sex, live weight, and type of production (dry–lactating–fattening periods) [15,18].

Temperate rangelands in South America consist mainly of a few C3 grass and legume species, offering fairly good biomass production in winter and providing forage for cow-calf operations in a season when the forage quality of native grasslands is scarce due to the lignification process of certain species, which contrasts with what was observed for *L. tenuis*. Thus, the presence of C4 species in these environments provides a chance to improve biomass stability, ensuring year-round productivity, particularly in the Flooding Pampas, where specific edaphic limitations such as high levels of halo-hydromorphism are very common [3]. This statement is based on the fact that the spring–summer–autumn growth cycle of the C4 species in the ecosystem (*Paspalum dilatatum*, *Sporobolus indicus,* and *Cynodon dactylon)* can improve biomass stability. Therefore, they complement the forage gap left by the C3 species (*Festuca arundinacea* and *Thinopyrum ponticum*) with an autumn–spring–summer growth cycle. In this sense, *L. tenuis*, a species with a spring–summer–autumn growth cycle, would complement the aforementioned C4 species, improving the productivity and nutritional quality of the forage [3].

In this study, various methodologies were used to evaluate the contribution of *L. tenuis* throughout its growth cycle in a mesophytic site. The multidisciplinary perspective enabled the observation of certain changes that would otherwise have been overlooked.

5. Conclusions

When assembling rations for grazing animals in unrestricted environments, it is common practice to use the Van Soest detergent system (NDF and ADF) to evaluate the diet's fiber input. However, in marginal environments, such as mesophytic lowlands, this is not enough. For accurate determination, additional factors must also be evaluated, such as cell wall compositions, photosynthetic and ionic activities, and in vitro digestibility with gas production.

In this paper, following a year-long study, our work confirms that, although *L. tenuis* contributes a limited quantity of dry matter, it provides high, stable digestibility and high protein contents for the entire year.

To guarantee sufficient forage for grazing animals in this type of restrictive ecosystem, it is imperative to successfully manage different complementary species. *L. tenuis* can improve the environment's protein content by capitalizing on the nitrogen fixation afforded by rhizobia. *L. tenuis* is a keystone species for the Salado River Basin. Its introduction may be well worth considering in other comparable edaphic environments.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/grasses4020019/s1, Figure S1: Climate in Chascomús, Buenos Aires Province, Argentina: weather by month.

Author Contributions: All authors have read and approved this manuscript and have contributed to its preparation as follows: Ms design and conception: M.E.V., P.V.F., O.A.R. and M.C.; data collection: M.E.V., P.V.F., J.P.E., S.J.M., A.A.R., J.M.A. and M.G.; analysis, interpretation, and discussion of results: M.E.V., P.V.F., S.J.M., A.A.R., J.M.A., P.V.F., O.A.R. and M.C.; draft manuscript preparation: M.E.V., P.V.F., O.A.R. and M.C. All authors have read and agreed to the published version of the manuscript.

Funding: ANPCYT-Argentina [grant number PICT 2018-3723 and PICT 2020-2023]; Universidad de Buenos Aires [grant number UBACYT 20020190200352BA]; the European project: "Raising the bio-based industrial feedstock capacity of marginal Lands (Margin Up)", by CONICET-Argentina [grant numbers PIP 2022-2024, 11220210100584, and PIP 11220200102126CO]; and Laboratorio de Evaluación de Alimentos para Uso Animal (from Pontificia Universidad Católica Argentina). This

study is based only on the authors' views and opinions. The European Union nor the European Commission cannot be considered responsible for them.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author(s).

Conflicts of Interest: The authors declare that there are no financial interests or personal relationships that have influenced the results and opinions included in this manuscript.

References

1. Rodriguez, A.; Jacobo, E.; Preliasco, P.; Roitman, G.; Miñarro, F. Pastoreo controlado: Una herramienta para el manejo de los pastizales naturales en sistemas ganaderos extensivos. In *Buenas prácticas para una ganadería sustentable de pastizal: Kit de extensión para las pampas y campos*; Fundación Vida Silvestre Argentina, Aves argentinas/AOP; FAUBA: Buenos Aires, Argentina, 2012.
2. Imbellone, P.A.; Taboada, M.A.; Damiano, F.; Lavado, R.S. Genesis, Properties and Management of Salt-Affected Soils in the Flooding Pampas, Argentina. In *Saline and Alkaline Soils in Latin America*; Taleisnik, E., Lavado, R.S., Eds.; Springer International Publishing: Cham, Switzerland, 2021; pp. 191–208. ISBN 978-3-030-52591-0.
3. Campestre, M.P.; Antonelli, C.J.; Bailleres, M.A.; Gortari, M.; Maguire, V.G.; Ezquiaga, J.P.; Taboada, M.A.; Ruiz, O.A. An Efficiently Biological Nitrogen Fixation of Non-Native Lotus Tenuis Justifies Its Key Role in the Flooding Pampas (Argentina). *Farming Syst.* **2025**, *3*, 100122. [CrossRef]
4. Cid, M.S.; Grecco, R.C.F.; Oesterheld, M.; Paruelo, J.M.; Cibils, A.F.; Brizuela, M.A. Grass-Fed Beef Production Systems of Argentina's Flooding Pampas: Understanding Ecosystem Heterogeneity to Improve Livestock Production. *Outlook Agric.* **2011**, *40*, 181–189. [CrossRef]
5. Nieva, A.S.; Ruiz, O.A. Lotus spp.: A Foreigner That Came to Stay Forever: Economic and Environmental Changes Caused by Its Naturalization in the Salado River Basin (Argentina). In *Saline and Alkaline Soils in Latin America*; Taleisnik, E., Lavado, R.S., Eds.; Springer International Publishing: Cham, Switzerland, 2021; pp. 431–446. ISBN 978-3-030-52591-0.
6. Teakle, N.L.; Snell, A.; Real, D.; Barrett-Lennard, E.G.; Colmer, T.D. Variation in Salinity Tolerance, Early Shoot Mass and Shoot Ion Concentrations within Lotus Tenuis: Towards a Perennial Pasture Legume for Saline Land. *Crop Pasture Sci.* **2010**, *61*, 379. [CrossRef]
7. García, I.V.; Covacevich, F.; Fernández-López, C.; Cabello, M.N. Lotus Tenuis Maintains High Arbuscular Mycorrhizal Diversity in Grasslands Regardless of Soil Properties or Management. *Rhizosphere* **2023**, *27*, 100754. [CrossRef]
8. Vignolio, O.R.; Fernandez, O.N. Bioecología de Lotus glaber Mill. (Fabaceae) en la Pampa Deprimida (provincia de Buenos Aires, Argentina). In *Revista Argentina de Producción Animal*; Asociación Argentina de Producción Animal: Balcarce, Argentina, 2006; pp. 113–130.
9. Veblen, T.; Young, K.; Orme, A. *The Physical Geography of South America*; Oxford University Press: Oxford, UK, 2007; ISBN 978-0-19-531341-3.
10. Fernández, P.V.; Vago, M.E.; Ezquiaga, J.P.; Maiale, S.; Rodriguez, A.; Acosta, J.M.; Gortari, M.; Ruiz, O.A.; Ciancia, M. Cell Wall Composition and Nutritional Quality through Seasons of the Saltgrass Distichlis Laxiflora Growing in Halophytic and Mesophytic Meadows. *Plant Stress* **2024**, *13*, 100519. [CrossRef]
11. García, I.; Mendoza, R.; Pomar, M.C. Deficit and Excess of Soil Water Impact on Plant Growth of Lotus Tenuis by Affecting Nutrient Uptake and Arbuscular Mycorrhizal Symbiosis. *Plant Soil* **2008**, *304*, 117–131. [CrossRef]
12. Mendoza, R.; Escudero, V.; García, I. Plant Growth, Nutrient Acquisition and Mycorrhizal Symbioses of a Waterlogging Tolerant Legume (Lotus Glaber Mill.) in a Saline-Sodic Soil. *Plant Soil* **2005**, *275*, 305–315. [CrossRef]
13. Lavado, R.S.; Taboada, M.A. Water, Salt and Sodium Dynamics in a Natraquoll in Argentina. *CATENA* **1988**, *15*, 577–594. [CrossRef]
14. Perelman, S.B.; León, R.J.C.; Oesterheld, M. Cross-scale Vegetation Patterns of Flooding Pampa Grasslands. *J. Ecol.* **2001**, *89*, 562–577. [CrossRef]
15. Bondi, A. *Animal Nutrition*; John Wiley and Sons: West Sussex, UK, 1987.
16. Vago, M.E.; Jaurena, G.; Estevez, J.M.; Castro, M.A.; Zavala, J.A.; Ciancia, M. Salt Stress on Lotus Tenuis Triggers Cell Wall Polysaccharide Changes Affecting Their Digestibility by Ruminants. *Plant Physiol. Biochem.* **2021**, *166*, 405–415. [CrossRef]
17. Buxton, D.R.; Casler, M.D. Environmental and Genetic Effects on Cell Wall Composition and Digestibility. In *ASA, CSSA, and SSSA Books*; Jung, H.G., Buxton, D.R., Hatfield, R.D., Ralph, J., Eds.; American Society of Agronomy, Crop Science Society of America, Soil Science Society of America: Madison, WI, USA, 2015; pp. 685–714. ISBN 978-0-89118-238-2.

18. McDonald, P.; Edwards, R.A.; Greenhalgh, J.F.D.; Morgan, C.A.; Sinclair, L.A.; Wilkinson, R.G. *Animal Nutrition*, 8th ed.; Pearson Education Limited: Essex, UK, 2011; ISBN 978-1-292-25166-0.
19. Cosgrove, D.J. Growth of the Plant Cell Wall. *Nat. Rev. Mol. Cell Biol.* **2005**, *6*, 850–861. [CrossRef]
20. Cosgrove, D.J. Structure and Growth of Plant Cell Walls. *Nat. Rev. Mol. Cell Biol.* **2024**, *25*, 340–358. [CrossRef] [PubMed]
21. Somerville, C.; Bauer, S.; Brininstool, G.; Facette, M.; Hamann, T.; Milne, J.; Osborne, E.; Paredez, A.; Persson, S.; Raab, T.; et al. Toward a Systems Approach to Understanding Plant Cell Walls. *Science* **2004**, *306*, 2206–2211. [CrossRef] [PubMed]
22. Albersheim, P.; Darvill, A.; Roberts, K.; Sederoff, R.; Staehelin, F.; Fry, S.C. Plant Cell Walls. From Chemistry to Biology. *Ann. Bot.* **2011**, *108*, viii–ix. [CrossRef]
23. Begenisic, F. Recopilación de las técnicas de Laboratorio Vigentes y Reconocidas Por El SAMLA, En Proceso de Revisión y Actualización En El Marco Del Convenio IRAM-SAGPyA. Resolución Nro. 478/98 y 238/03. 2004. [Cd- Rom].
24. Chen, S.; Li, J.; Wang, S.; Hüttermann, A.; Altman, A. Salt, Nutrient Uptake and Transport, and ABA of Populus Euphratica; a Hybrid in Response to Increasing Soil NaCl. *Trees* **2001**, *15*, 186–194. [CrossRef]
25. Campestre, M.P.; Antonelli, C.J.; Castagno, N.L.; Maguire, V.G.; Ruiz, O.A. Interspecific Hybridization and Inoculation with *Pantoea Eucalypti* Improve Forage Performance of *Lotus* Crop Species under Alkaline Stress. *Plant Biol.* **2024**, *26*, 245–256. [CrossRef]
26. Bolhar-Nordenkampf, H.R.; Long, S.P.; Baker, N.R.; Oquist, G.; Schreiber, U.; Lechner, E.G. Chlorophyll Fluorescence as a Probe of the Photosynthetic Competence of Leaves in the Field: A Review of Current Instrumentation. *Funct. Ecol.* **1989**, *3*, 497. [CrossRef]
27. Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597. [CrossRef]
28. Theodorou, M.K.; Williams, B.A.; Dhanoa, M.S.; McAllan, A.B.; France, J. A Simple Gas Production Method Using a Pressure Transducer to Determine the Fermentation Kinetics of Ruminant Feeds. *Anim. Feed. Sci. Technol.* **1994**, *48*, 185–197. [CrossRef]
29. Wawrzkiewicz, M.; Danelón, J.L. Mejoramiento de la técnica de producción de gas in vitro para evaluar alimentos para rumiantes. In *Revista Argentina de Producción Animal*; Asociación Argentina de Producción Animal: Balcarce, Argentina, 2004; pp. 187–197.
30. Fry, S.C. *The Growing Plant Cell Wall: Chemical and Metabolic Analysis*; Longman Scientific & Technical: Harlow, UK, 1988.
31. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]
32. Ahmed, A.E.R.; Labavitch, J.M. A Simplified Method for Accurate Determinationof Cell Wall Uronide Content. *J. Food Biochem.* **1978**, *1*, 361–365. [CrossRef]
33. Shui, G.; Leong, L.P. Residue from Star Fruit as Valuable Source for Functional Food Ingredients and Antioxidant Nutraceuticals. *Food Chem.* **2006**, *97*, 277–284. [CrossRef]
34. Morrison, I.M.; Stewart, D. Plant Cell Wall Fragments Released on Solubilisation in Trifluoroacetic Acid. *Phytochemistry* **1998**, *49*, 1555–1563. [CrossRef] [PubMed]
35. Munns, R.; Tester, M. Mechanisms of Salinity Tolerance. *Annu. Rev. Plant Biol.* **2008**, *59*, 651–681. [CrossRef]
36. Munns, R.; Gilliham, M. Salinity Tolerance of Crops–What Is the Cost? *New Phytol.* **2015**, *208*, 668–673. [CrossRef]
37. Liu, X.; Renard, C.M.G.C.; Bureau, S.; Le Bourvellec, C. Revisiting the Contribution of ATR-FTIR Spectroscopy to Characterize Plant Cell Wall Polysaccharides. *Carbohydr. Polym.* **2021**, *262*, 117935. [CrossRef]
38. Di Bella, C.E.; Kotula, L.; Striker, G.G.; Colmer, T.D. Submergence Tolerance and Recovery in Lotus: Variation among Fifteen Accessions in Response to Partial and Complete Submergence. *J. Plant Physiol.* **2020**, *249*, 153180. [CrossRef]
39. Van Soest, P.J. *Nutritional Ecology of the Ruminant*; Cornell University Press: Ithaca, NY, USA, 1994.
40. Buxton, D.R.; Redfearn, D.D. Plant Limitations to Fiber Digestion and Utilization. *J. Nutr.* **1997**, *127*, 814S–818S. [CrossRef]
41. González, F.A.; Noemí Cosentino, V.R.; Loza, C.; Cerón-Cucchi, M.E.; Williams, K.E.; Bualó, R.; Costantini, A.; Gere, J.I. Inclusion of *Lotus Tenuis* in Beef Cattle Systems in the Argentinian Flooding Pampa as an Enteric Methane Mitigation Strategy. *N. Z. J. Agric. Res.* **2024**, 1–12. [CrossRef]
42. Bailey, R.W.; Ulyatt, M.J. Pasture Quality and Ruminant Nutrition: II. Carbohydrate and Lignin Composition of Detergent-Extracted Residues from Pasture Grasses and Legumes. *N. Z. J. Agric. Res.* **1970**, *13*, 591–604. [CrossRef]
43. Vignolio, O.R.; Cambareri, G.; Maceira, N. Lotus tenuis (Fabaceae). Productividad y manejo agronómico. *Rev. Argent. De Prod. Animal* **2013**, *30*, 97–116.
44. Bustan, A.; Pasternak, D.; Pirogova, I.; Durikov, M.; Devries, T.T.; El-Meccawi, S.; Degen, A.A. Evaluation of Saltgrass as a Fodder Crop for Livestock. *J. Sci. Food Agric.* **2005**, *85*, 2077–2084. [CrossRef]
45. Wangui, J.C.; Millner, J.P.; Kenyon, P.R.; Tozer, P.R.; Morel, P.C.H.; Pain, S.J. In Vitro Fermentation of Browsable Native Shrubs in New Zealand. *Plants* **2022**, *11*, 2085. [CrossRef]
46. Boga, M.; Yurtseven, S.; Kilic, U.; Aydemir, S.; Polat, T. Determination of Nutrient Contents and In Vitro Gas Production Values of Some Legume Forages Grown in the Harran Plain Saline Soils. *Asian Australas J. Anim. Sci.* **2014**, *27*, 825–831. [CrossRef] [PubMed]

47. Rymer, C.; Huntington, J.A.; Williams, B.A.; Givens, D.I. In Vitro Cumulative Gas Production Techniques: History, Methodological Considerations and Challenges. *Anim. Feed. Sci. Technol.* **2005**, *123–124*, 9–30. [CrossRef]
48. Jung, H.G.; Allen, M.S. Characteristics of Plant Cell Walls Affecting Intake and Digestibility of Forages by Ruminants. *J. Anim. Sci.* **1995**, *73*, 2774. [CrossRef]
49. Carpita, N.C.; Gibeaut, D.M. Structural Models of Primary Cell Walls in Flowering Plants: Consistency of Molecular Structure with the Physical Properties of the Walls during Growth. *Plant J.* **1993**, *3*, 1–30. [CrossRef]
50. Caffall, K.H.; Mohnen, D. The Structure, Function, and Biosynthesis of Plant Cell Wall Pectic Polysaccharides. *Carbohydr. Res.* **2009**, *344*, 1879–1900. [CrossRef]
51. CSIRO. *Nutrient Requirements of Domesticated Ruminants*; CSIRO Publishing: Melbourne, Australia, 2007.
52. Wei, Z.; Maxwell, T.M.R.; Robinson, B.; Dickinson, N. Legume Nutrition Is Improved by Neighbouring Grasses. *Plant Soil* **2022**, *475*, 443–455. [CrossRef]
53. Fornara, D.A.; Tilman, D. Plant Functional Composition Influences Rates of Soil Carbon and Nitrogen Accumulation. *J. Ecol.* **2008**, *96*, 314–322. [CrossRef]
54. Gliessman, S.R. *Package Price Agroecology: The Ecology of Sustainable Food Systems*; CRC Press: Boca Raton, FL, USA, 2021.