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Floristic Composition Mediates Change in Forage Nutritive Quality of Atlantic Mountain Grasslands after Experimental Grazing Exclusion

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Abstract: Simultaneous reduction of biodiversity and forage nutritive value after grazing abandonment represents a critical agroecological problem observed in temperate mountain grasslands. However, how both processes affect each other after the exclusion of large grazers is not well understood. To address this knowledge gap, we used four Atlantic grassland sites in the Aralar masif (northern Iberian Peninsula) to ask whether floristic composition mediates change in forage nutritive quality after grazing exclusion, and, if so, how much of the effect on forage quality is mediated. To examine the effects of grazing exclusion on forage quality and floristic composition a repeated-measures (2005–10) randomized complete block experiment was used. Then, the direct effects of grazing exclusion on forage quality were disentangled from the indirect effects mediated by concurrent change in floristic composition. Grazing exclusion deteriorated forage mineral content, phosphorus content, neutral detergent fiber and, especially, crude protein and enzymatic digestibility. Concurrent floristic change mediated change in crude protein (80%), enzymatic digestibility (55%) and forage content in calcium (31%). Our study shows that plant diversity and forage quality are intimately linked features of Atlantic mountain grasslands and highlights the importance of preserving leguminous and dicotyledonous species to maintain the nutritive value of these grasslands.

Keywords: Iberian Peninsula; mediation analysis; mixed modelling; repeated-measures randomized complete block experiment



Citation: García-Baquero, G.; Odriozola, I.; Aldezabal, A. Floristic Composition Mediates Change in Forage Nutritive Quality of Atlantic Mountain Grasslands after Experimental Grazing Exclusion. *Agronomy* **2021**, *11*, 25. <https://dx.doi.org/10.3390/agronomy11010025>

Received: 14 November 2020

Accepted: 22 December 2020

Published: 24 December 2020

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1. Introduction

Atlantic and Pyrenean mountain grasslands located below 2000 m.a.s.l. (meters above sea level), preserved by extensive multispecific (sheep, cattle, horses) livestock grazing in local transhumance, are among the most biodiverse habitats in Europe [1]. Decreasing plant diversity in these grasslands might cause negative consequences on ecosystem functioning, stability, and services [2,3]. For instance, plant diversity enhanced quality-adjusted yield and revenues, independently on management intensity, in a large-scale biodiversity experiment in semi-natural grasslands [4]. Many studies have reported that grazing contributes the maintenance of several ecosystem functions and services, especially in historically grazed temperate grasslands; among others, landscape heterogeneity [5], suitable habitat for wild vertebrates [6], plant diversity [7], forage quality [8–10], soil microbial activity [11], nutrient cycling [12–14], pollinator networks [15] and invertebrate diversity [16,17].

In addition to being a reservoir of biodiversity, semi-natural managed grasslands are also an essential food resource for herbivores and prevent the consumption of supplementary feed (and the consequent economic cost) during the growing season (which could last from four to six months), contributing to the sustainability of the grazing system [18]

and GHG mitigation [19]. However, the future of the traditional management of these ecosystems is under constant threat due to socio-economic and market changes [20]. Similar to most extensively managed European grassland systems [21], the use of mountain grasslands by domestic livestock has declined slowly in most of the Pyrenean-Basque-Cantabrian grasslands [22,23].

Grazing can influence forage quality (i.e., palatability and nutritional value) directly, for instance, by promoting the growth of younger plant tissues [24]. This is expected in historically grazed grasslands [10] with several livestock species exerting a non-selective grazing pressure, where plants respond with a tolerance strategy [25]. Grazing may also affect forage quality indirectly, through changes in floristic composition (e.g., [10,26,27]). Non-selective shoot herbivory exerted by mixed livestock equalizes the ability of plant species to compete for light [28], thus allowing the co-existence of short-statured highly palatable legumes and nutrient-rich forbs [29,30], together with tall competitive grasses [31]. After grazing abandonment tall grasses tend to out compete remaining short-statured species [7,32], reducing forage quality and plant diversity altogether. These effects vary both in time and in space, but long-term manipulative studies that explore them are rare, and the relative contribution of direct and indirect effects of grazing abandonment on forage nutritive value are hardly known.

This study aims at addressing this knowledge gap by using the results of a repeated-measurements (2005–2010) randomized complete block design experiment (RCBD) implemented to test for the effects of ungulate grazing exclusion, via experimental fencing, on both forage nutritive quality and species composition. After testing for the effects of grazing exclusion over time on forage nutritive quality and floristic composition, we asked whether simultaneous change in floristic composition mediates change in forage quality and, if so, how much of the effect of grazing exclusion on forage quality is direct and how much is mediated by concurrent change in floristic composition. For that purpose, we used mediation analysis [33] with the aim to disentangle the direct effects of grazing exclusion (X) on forage quality (Y) from those (indirect) effects mediated by change in floristic composition (M), as outlined in Figure 1. Thus, the direct effects of grazing exclusion on forage quality (i.e., the path XY), as they are here framed, include any effect that is not mediated by floristic composition but that may, in fact, be either truly direct or rather mediated by factors other than floristic composition, such as the accumulation of an increasingly thick organic layer that provides insulation to the soil [8]. Mediation analysis, though, must consider the possible existence and influence of variable(s) Z (Figure 1), sometimes called moderators, acting as potential confounders of the relationship between the mediator M and the outcome Y [34]. In this work, Z is represented by the physical and chemical properties of the topsoil. The importance of soil characteristics in determining species composition has been highlighted in previous studies [35–37], and although the influence of such characteristics on floristic composition is more pronounced in arid environments compared to temperate grasslands, where species richness and diversity strongly respond to grazing [38], it can also affect the floristic composition and should not be ignored.

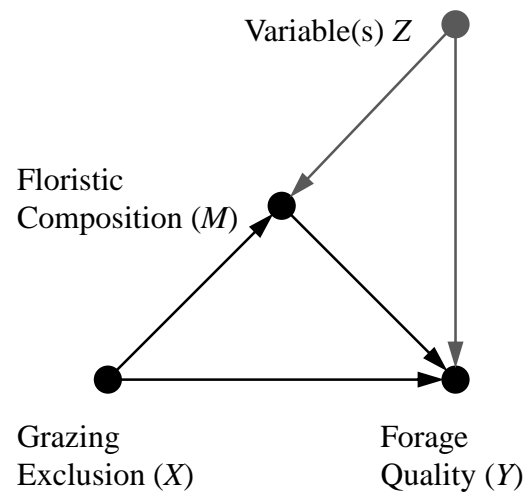


Figure 1. Directed acyclic graph representing direct and indirect (via concurrent change in floristic composition) effects of grazing exclusion (X) on forage nutritive quality (Y). Floristic composition acts as a mediating variable (M). The variable(s) Z denotes potential confounders of the mediator M and the outcome Y. Grazing exclusion (X) is a randomized experimental factor, and so stands as a parent node with no incoming arrows. The path from X to Y ($X \rightarrow Y$) represents the direct effects and the path from X to Y through M ($X \rightarrow M \rightarrow Y$) represents the indirect effects, once that the potential confounding effects of the Z variables have been controlled for.

2. Materials and Methods

2.1. Study Area

The study area is located in the Aralar Natural Park (42°59'48" N and 2°06'51" W), an 11,000-ha Special Area of Conservation (part of the European Natura 2000 network) located in the Atlantic Basque Country (Northern Iberian Peninsula, see Supplementary Figure S1). The area has oceanic climate, with mean annual temperature equal to 7 °C and annual precipitation of about 1330 mm. A mixture of calcareous and acid substrates is present in the area, but its oceanic climate with abundant precipitation throughout the growing season has led to the development of acidic soils. In these soils, the average of Kjeldahl total nitrogen is 6534 ppm, available phosphorus 8.3 ppm, mean soil water content at 10-cm depth 46.3%, and pH 4.8 ppm [7]. The vegetation in the park comprises a mosaic of gorse-heather shrublands and semi-natural grasslands, which are located between 800 m.a.s.l and 1400 m.a.s.l. This area is managed as a communal land, called Enirio-Aralar, and native grasslands included in the Habitat Directive are dominant: the most relevant is the *Jasiono-Danthonietum* grassland (code 6230, subtype a, 92/43/EEC, European Commission, 2013), located in relatively flat terrain (slope range: 0–20°) and primarily comprising perennial graminoids such as *Festuca nigrescens* subsp. *microphylla* (St-Yves) Markgr.-Dannenb. (chewings fescue), *Agrostis capillaris* L. (common bent), *Luzula campestris* (L.) DC. in Lam. & DC. (field woodrush) in Lam. & DC., and herbaceous dicotyledons such as *Galium saxatile* L. (heath bedstraw), *Trifolium repens* L. (white clover) and *Cerastium fontanum* Baumg. (starweed) [7,11]. The mean aboveground net primary production (ANPP) of *Jasiono-Danthonietum* grassland is 1.97 t dry mass ha⁻¹ year⁻¹ with standard error of 0.39 [39]. Livestock herds (sheep, cattle and horses) are managed in short transhumance, using the lowland farms (below 700 m.a.s.l.) in winter, and extensively using the upland grasslands (up to 1400 m.a.s.l.) from May to October–November. In Enirio-Aralar communal land, the number of grazing animals has remained almost stable, with 18,000 dairy ewes, 800 beef cattle and 700 mares [11]. The stocking rate varies from 2.3 to 4.5 LU ha⁻¹d⁻¹ along the growing season [8]. The most common size of the transhumance flocks is between 200 and 500 lactating ewes. Lactating ewes' milk is destined mainly to the production of the local Idiazabal Protected Denomination of Origin (PDO) cheese, awarded worldwide, and sold mainly in local market or directly on farms.

2.2. Experimental Design and Sampling Procedure

Experimental sites were selected using a GIS application, intersecting slope and vegetation maps and obtaining a new map with suitable areas for establishing exclusion fences. Four field sites were selected randomly into this map to conduct the experiment in the *Jasiono-Danthonietum* temperate grassland (Supplementary Figure S1): Oidui (42°59'16.6" N, 2°5'59.4" W; 876 m.a.s.l), Uzkuiti (43°0'50" N, 2°4'3" W; 1300 m.a.s.l); Igaratza (42°59'9.25" N, 2°2'9.7" W; 1247 m.a.s.l); and Alotza (43°0'10.6" N, 2°5'22" W; 1223 m. a.s.l). The minimum and maximum distance between any of these sites was 2175 m and 4720 m respectively and all sites were located on relatively flat terrain. In order to simulate grazing cessation, one permanent fenced plots (50 m × 50 m each) was installed in May 2005 at each experimental sites. Next to each fenced plot ('exclosure' level), we delineated a grazed plot ('grazing' level), where dairy sheep, beef cattle and horses were allowed to graze continuously during the vegetative period (from May–November). Additionally, 3–4 temporary moveable cages of 1.5 × 1.5 m were randomly allocated within the grazing plot at the beginning of each growing season. Each month, quadrats outside permanent fences were clipped inside temporary moveable cages that were randomly relocated in grazed plot after biomass measurements. This procedure was carried out every month. The sequential use of such temporary cages prevented that the plant biomass inside the cage was eaten by grazing animals, and allowed us to estimate accurately the nutritive quality of forage consumed by herbivores. Each month, three 1 m²-quadrats were clipped per site and treatment at ground level. Unfortunately, in some cases it was not possible to sample all quadrats due to problems with temporary cages and meteorological conditions, so the total number of samples per growing season varied from 46 to 71 quadrats.

The experiment was implemented according to a repeated-measurements randomized complete block (RCB) design [40]. The field sites acted as blocks (categorical random factor 'Site' with four levels). Grazing exclusion represented the experimental manipulation (fixed factor 'Treatment' with three levels: grazing, cage, and exclosure). The factor 'Time', understood as a fixed but otherwise quantitative variable, constitutes a second fixed factor. Time is indexed in months, starting in June 2005 (month number 1) and ending in October 2010 (month number 65). Thereby, the basic RCB design converts into a repeated-measurements RCB design. Therefore, the statistical model corresponding to this experimental design is (using 'effects' model parameterization):

$$y_{ijk} = \mu + \tau_i + b_{ij} + \delta_k + (\tau\delta)_{ik} + \varepsilon_{ijk} \quad (1)$$

where y_{ijk} corresponds to the response measured in treatment i in site j at time k ; μ is the level of reference (grazing, in this application); τ_i corresponds to the effect of treatment i (with respect to the level grazing); b_{ij} is the term for the random factor 'site', i.e., for site j under treatment i ; δ_k corresponds to the effect of time k ; $(\tau\delta)_{ik}$ is the interaction between the fixed effects; finally, ε_{ijk} corresponds to the error. b_{ij} is independently and identically distributed as normal $(0, \sigma_b^2)$; likewise, ε_{ijk} is independently and identically distributed as normal $(0, \sigma^2)$.

Vegetation sampling took place from June 2005 to October 2010, using the quadrat cover method [38,41]: once per month from May/June–October, floristic composition was estimated visually within three randomly placed, non-overlapping 1-m² quadrats per plot. For estimating species cover, we used the following semi-quantitative indices: + (<1%), 1 (1–10%), 2 (11–20%), 3 (21–30%), 4 (31–40%), 5 (41–50%), 6 (51–60%), 7 (61–70%), 8 (71–80%), 9 (81–90%), 10 (91–100%). All vascular plants present within each quadrat were identified at the species level. These semi-quantitative indices were transformed to quantitative ones, replacing each index by the middle value of the cover range (for instance: index 1 was replace by 5%). The index '+' was converted to 0.5%. After composition determination, standing plant biomass of the same 1-m² quadrat was clipped in order to measure forage quality variables. Each sample was stored in plastic bags, labelled, and processed in the Eskalmendi Agricultural Laboratory, drying them in an oven at 60 °C for

48 h. The nutritional variables thus measured were the following: macrominerals (calcium, Ca; phosphorus, P; potassium, K; and magnesium, Mg), crude protein (CP, calculated as nitrogen \times 6.25), neutral detergent fiber (NDF), enzymatic digestibility (NDF cellulase, and NDFcel). All analytical procedures follow standard protocols [42].

Topsoil properties were measured at the end of the experiment. Soil samples were collected from a depth of 0–10 cm, using a 3-cm-diameter soil core sampler. Each soil sample comprised a pool of 10 cores (subsamples) per 1-m² quadrat. The measured soil variables were as follows: pH, effective cation-exchange capacity (cmol_c kg⁻¹), effective aluminium cation-exchange capacity (%), oxidable organic matter (%), and macronutrients, including Kjeldahl total nitrogen (mg L⁻¹), available phosphorus (mg L⁻¹), available potassium (mg L⁻¹), available sodium (mg L⁻¹), available calcium (mg L⁻¹), and available magnesium (mg L⁻¹), and texture (percentages of gross and fine sand, silt, clay). All analytical procedures were conducted in Fraisoro Agricultural Laboratory following ADAS standard procedures [43].

2.3. Mixed Modelling

After exploratory data analysis [44], we applied mixed effects modeling [45] to test for the effects of the treatment and time factors. Mixed models were fitted with function `lme()` of package `nlme` [46] in R software [47]. Restricted maximum likelihood was employed for parameter estimation. Including a correlation structure was needed in some cases, albeit a simple structure of the type autoregressive process of order 1 (`corAR1`) sufficed. The packages `MuMIn` [48] and `sjPlot` [49] were used to obtain R^2 statistics and graphs, respectively.

2.4. Mediation Analysis

To disentangle the direct effects of grazing exclusion on forage quality from the effects mediated by concurrent change in floristic composition, mediation analysis was applied using the model-based approach implemented in the R package `mediation` [50]. We used data from only the end of the experiment (year 2010) to fit sets of linear models according to our experimental design; then mediation effects were estimated from these fitted models [51,52], via function `mediation`. Three variables derived from species composition (legume-to-grass ratio, proportion of dicots, and Simpson's index of diversity) were tried as mediators, simultaneously controlling for potential confounding due to topsoil properties (variables Z in Figure 1). Simpson's index of diversity [53], which indicates the probability that any two individuals in the sample will belong to different species, was computed as follows:

$$\text{Simpson's index of diversity} = 1 - \sum p_i^2 \quad (2)$$

where p_i represents the proportional abundance of species i in the sample.

The value of the Simpson's index ranges between 0 (minimum diversity) and 1 (maximum diversity). Observed Simpson's index of diversity and Shannon index (H') are often correlated, even though the latter is upper-unbounded. Both direct and mediation effects are defined according to [54]'s concept of potential outcomes (See also [55]). Significance of mediation effects was assessed via quasi-Bayesian Monte Carlo tests [51].

2.5. Data Availability

The dataset is available from figshare: ForageQuality.Dataset.xls. To access the dataset, go to <https://doi.org/10.6084/m9.figshare.13336877>.

3. Results

3.1. Forage Quality

Exploratory data analysis (Supplementary Tables S1 and S2 and Figure S2) showed that most forage quality variables were sufficiently symmetrical for linear modeling purposes; besides, they were significantly correlated among them, though, in general terms,

not strongly correlated (the exceptions are the pairs Mg-Ca and NFD-NDF_{cell}, whose correlations are close to or above |0.5|).

Except for ash and Ca, the interaction between treatment and time was significant in all cases (Tables 1 and 2), indicating that the effect of treatment on Mg, K, P, CP, NDF, and NDF_{cell} depends on Time (Figure 2 and Supplementary Figure S3); main tests for time are not significant in many cases, which is due to the observed crossover interactions (Figure 2a and Supplementary Figure S3). Tukey's post-hoc tests (Supplementary Table S3) indicate that the levels grazing-exclosure and cage-exclosure are significantly different in all cases but K, and also that the levels grazing-cage are significantly different only in the cases of Mg, K, and CP.

Table 1. Hypothesis testing results, after linear mixed modelling, for eight forage quality properties and three derived floristic composition variables measured in a repeated-measurements (2005–2010) RCB experiment, implemented to simulate the effects of grazing exclusion on forage quality. Columns 2–4 report *F*-values, together with degrees of freedom (among parentheses); *p*-values are indicated with asterisks. Degrees of freedom differ among variables because, in some cases, there are missing values. Parameter estimation is reported in Table 2 and illustrated in Figures 2 and 3, Figures S3 and S4. FF = Fixed Factors; RF = Random Factor.

	Treatment	Time	Treat. × Time	FF <i>R</i> ²	RF <i>R</i> ²
Ash	26.40 (2, 333) ***	0.39 (1, 333)	n.s.	0.107	0.211
Ca	18.44 (2, 331) ***	28.14 (1, 331) ***	n.s.	0.161	0.000
Mg	39.14 (2, 331) ***	0.54 (2, 331)	14.26 (2, 331) ***	0.210	0.130
K	18.77 (2, 331) ***	2.01 (2, 331)	16.49 (2, 331) ***	0.157	0.113
P	26.71 (2, 331) ***	9.45 (1, 331) **	8.64 (2, 331) ***	0.137	0.285
CP	88.62 (2, 331) ***	2.90 (1, 331)	17.83 (2, 331) ***	0.381	0.022
NDF	16.95 (2, 331) ***	0.35 (1, 331)	8.10 (2, 331) ***	0.128	0.014
NDF _{cell}	18.44 (2, 329) ***	1.59 (1, 329)	13.08 (2, 329) ***	0.156	0.030
Simpson's index	0.69 (2, 329)	22.84 (1, 329) ***	7.86 (2, 329) ***	0.091	0.140
Legume-to-grass ratio	20.78 (2, 329) ***	7.69 (1, 329) **	40.20 (2, 329) ***	0.228	0.182
Dicots proportion	21.60 (2, 329) ***	1.50 (1, 329)	27.81 (2, 329) ***	0.149	0.353

n.s., *p* > 0.05; **, *p* < 0.01; ***, *p* < 0.001.

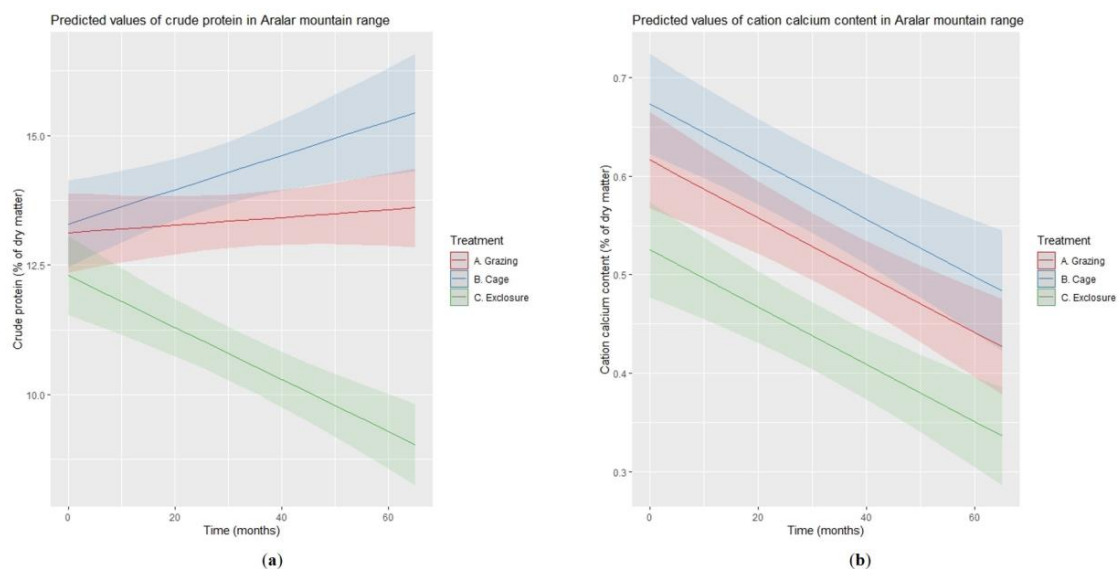


Figure 2. (a) Modeled change in crude protein; (b) Modeled change in cation calcium content. The patterns for the change in Mg, K, P and enzymatic digestibility are similar to that for crude protein, whereas the pattern for neutral detergent fiber is just the inverse of the pattern for enzymatic digestibility. Ash does not change over time (see Supplementary Figure S3). Time is indexed in months, starting in June 2005 (month number 1) and ending in October 2010 (month number 65). Restricted maximum likelihood estimates (ReML) for cation calcium content and crude protein at the end of each season (October) over the period 2005–2010 are presented in Supplementary Table S4.

Table 2. Restricted maximum likelihood parameter estimation for the forage quality properties and floristic composition measured in four field sites during 2005–2010. The parameter σ_b corresponds to the variability due to the random factor Site; the parameter σ corresponds to the residual variability. Cage vs. grazing has been omitted, since, in most cases, these effects are not significant (Supplementary Table S3). Hypothesis testing is reported in Table 1.

Variable	Level of Reference: Grazing (<intercept>)	Exclosure vs. Grazing	Time	Exclosure \times Time	σ_b	σ
Ash	7.96	−1.65	-	-	1.16	2.08
Ca	0.62	−0.09	−0.003	-	0.00	0.20
Mg	0.14	−0.01	0.000	−0.001	0.02	0.03
K	0.94	0.18	0.001	−0.008	0.11	0.29
P	0.24	−0.01	−0.0003	−0.0005	0.03	0.04
CP	13.12	−0.82	0.01	−0.06	0.38	2.02
NDF	67.55	0.24	−0.04	0.09	0.59	4.62
NDFcel	42.64	0.51	0.02	−0.11	0.92	4.77
Simpson’s index	0.71	0.05	0.001	−0.001	0.03	0.07
Legume-to-grass ratio	0.14	0.09	0.002	−0.005	0.06	0.11
Dicots proportion	0.31	0.04	0.001	−0.003	0.06	0.08

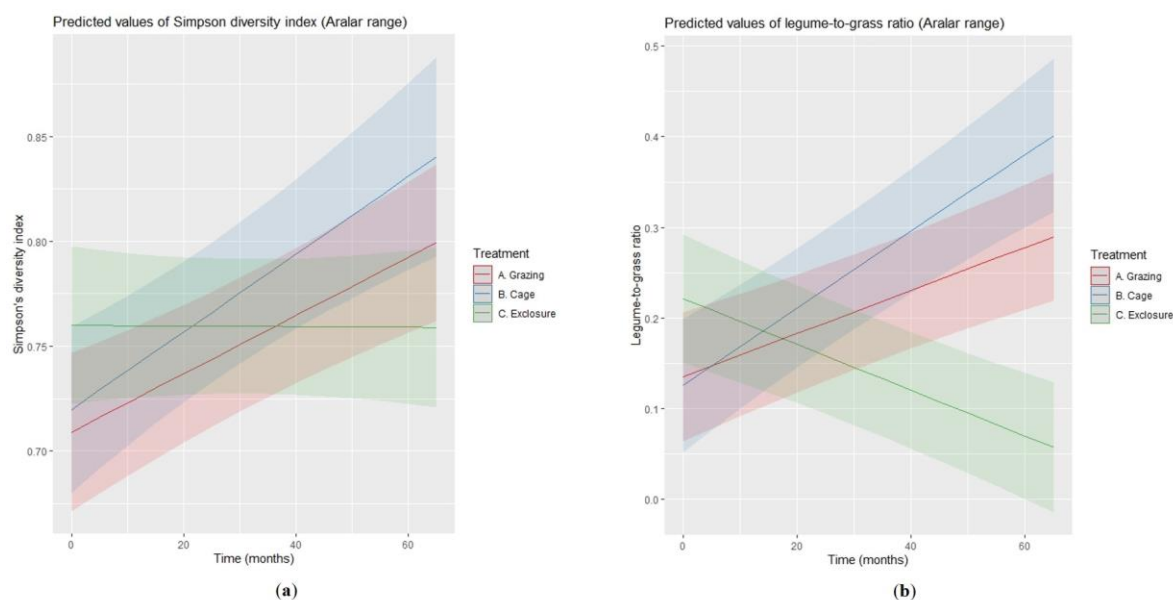


Figure 3. (a) Change in Simpson’s (plant) diversity index; (b) change in legume-to-grass ratio content. The observed patterns for the change in the proportion of dicots is similar to that for legume-to-grass ratio (see Supplementary Figure S4). Time is indexed in months, starting in June 2005 (month number 1) and ending in October 2010 (month number 65). Restricted maximum likelihood estimates (ReML) for Simpson’s index and legume-to-grass ratio at the end of each season (October) over the period 2005–2010 are presented in Supplementary Table S4.

In general, the indicators of forage nutritive quality Mg, K, P, CP, NDF, and NDFcel under the levels grazing and cage of the treatment factor tend to remain stable or improve over time, whereas they tend to deteriorate under the exclosure level of treatment. In the case of Ca (Figure 2b), only both main effects are significant; in the case of ash, only treatment is significant (Supplementary Figure S3). In summary, all quality descriptors except ash deteriorated quickly after grazing exclusion (Table 2, Supplementary Figure S3), with no generalized significant differences between grazing and cage levels of the treatment factor.

3.2. Floristic Composition

Simpson’s index of plant species diversity remained stable over time within the experimental exclosures, whereas it improved outside the exclosures (Figure 3a; Tables 1

and 2), i.e., under grazing and cage levels of the treatment factor. By contrast, whereas the legume-to-grass ratio and the proportion of dicots deteriorated over time within the experimental exclusions, they also tended to improve outside the exclusions (Figure 3b and Figure S4; Tables 1 and 2). The evidence for statistical difference between grazing and cage levels of the treatment factor, though significant for the legume-to-grass ratio, was, in general terms, weak (Supplementary Table S3).

3.3. Mediation Analysis

As shown in Figure 4, using only the data corresponding to the end of the experiment (i.e., after six years of grazing exclusion), either the legume-to-grass ratio or the proportion of dicots mediated change in crude protein ($p = 0.02$), enzymatic digestibility ($p = 0.02$) and forage content in cation calcium ($p = 0.05$). Further results (not detailed here) also hint at a possible mediation effect of species diversity on potassium forage content ($p = 0.09$), but no evidence of indirect effects was found regarding magnesium and phosphorus forage content. Since ash did not change over time, mediation analysis was not applied to this outcome variable.

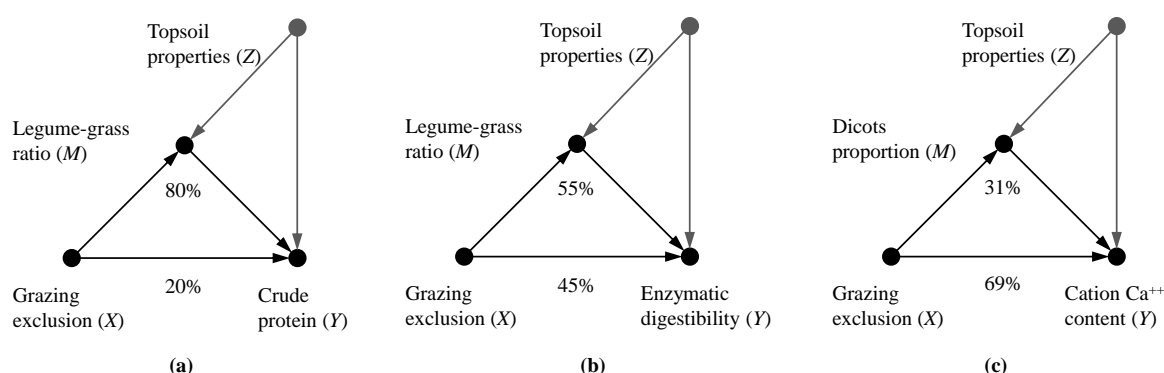


Figure 4. Direct (paths from X to Y) and indirect effects (paths from X to Y passing through M) of experimental grazing exclusion (X) on descriptors of forage nutritive quality (Y), once that topsoil properties (Z) have been taken into account. (a) Simultaneous change in the legume-to-grass ratio mediates 80% of change in crude protein content and, (b) 55% of change in enzymatic digestibility; (c) concurrent change in the proportion of dicots mediates 31% of change in cation calcium forage content.

4. Discussion

Discovering causal mechanisms is an essential objective of science [55], for which randomized experiments constitute a primary tool [56]. Very often, though, even randomized experiments may merely ascertain whether a given factor causes change in another (the response), but without unveiling how exactly this relationship takes place [57]. By means of linear mixed modelling [40,45], we found that experimental fencing to recreate exclusion of ungulate grazers in an Atlantic grassland system reduced forage nutritive quality indicators after just six years of experimental manipulation. These quality indicators were mineral content, crude protein, phosphorus content, neutral detergent fiber and enzymatic digestibility. Additionally, by means of mediation analysis [33,34] based on Robins and Greenland's [54] concept of potential outcomes, we found that concurrent floristic change at the end of our experiment mediates 80% of change in crude protein. Concurrent floristic change also mediates 55% of change in enzymatic digestibility and 31% of change in forage content in calcium. Thus, a substantial proportion of the decline in the said three forage quality descriptors is due to simultaneous change in floristic composition.

Cessation of grazing in productive long-history grasslands, where plant species are adapted to tolerate grazing, was expected to lead to reduction in forage nutrient content and palatability [24,58]. Similar results have been, in fact, reported in humid and sub-humid grasslands [10,26,59,60]. However, the positive temporal trends in forage quality

found in both grazed treatments were unexpected. In a previous study in these field sites, we reported an increasing trend of ANPP between 2005–2010 for both grazed and fenced treatments [39], concurrent with an increase in stocking rate (cattle and, especially, horses) [61]. Increased stocking rate increases defoliation intensity and fertilization by urine and feces [62], both of which are known to contribute to improve forage quality. Defoliation promotes the growth of younger, more palatable plant tissue [24] and fertilization favors a resource acquisition strategy and may also improve forage quality [63,64]. Nevertheless, we believe that the increasing trend in forage quality will probably be short-lived, since the observed increases in stocking rate and ANPP are likely local trends within longer-term oscillations.

We found significant, albeit small in size, differences between grazing and cage treatments for a few forage quality indicators. These differences might be due to the fact that plants collected from cages were complete fully-grown individuals with higher leaf:stem ratio, whereas the plant samples collected in the grazed plots were necessarily constituted by basal parts with lower leaf:stem ratio. In grasslands, foliar nitrogen allocation increases from the bottom to the top of the plant canopy [65]. Grasses contain the leaf sheath and other lignin-rich components in the basal area and dicots have the youngest (with less lignified structures) tissues in the aerial part, due to the apical growth meristems. Lignin, in turn, is the principal factor limiting forage digestibility [66]. Moreover, plant parts also differ in carbon and nitrogen allocation: leaves are often of a higher quality than stems in mature stage, due to lower C/N ratio and richer non-cell wall components (i.e., protein and soluble carbohydrates) [58], whereas the temporal cages represent the forage that was consumed by livestock in the previous time interval, the grazed plots represent what remained after the grazing activity. For this reason, we believe that the best measure of animal-consumed forage quality is the one measured inside the temporal cages.

That tall competitive grasses dominate after grazing exclusion, at the expense of smaller species (i.e., legumes and forbs), is something known since Tansley and Adamson [32] conducted the first-known grazing exclusion experiment. Our results also agree with the predictions of the general model of grazing for productive grasslands with a long evolutionary history of grazing [67,68]. However, plant diversity, as measured by Simpson's index, remained stable after grazing exclusion. The short time elapsed (6 years.) since the beginning of the experiment might explain the absence of diversity loss in the excluded plots. This explanation is based in that a previous study carried out in the same plots showed that the observed reduction of dicots proportion resulted in plant diversity loss only after 13 years of grazing exclusion [7]. Thus, the observed steady reduction in legumes and forbs after grazing exclusion may be an indication of posterior biodiversity loss.

Dicots proportion and plant species diversity also increased, unexpectedly, under grazing treatments. The aforementioned increase in stocking rate at the end of the experimental period might also explain floristic changes in grazed plots. Creation of colonization opportunities is an important mechanism by which herbivory promotes diversity [69]. Enhanced defoliation and trampling by herbivores opens new gaps in the vegetation and brings about opportunities for ruderal species to become established [70]. Our results support this hypothesis, since increased diversity is mainly explained by the appearance in grazed plots of several annual species of ruderal-nitrophile nature such as *Trifolium dubium* Sibth., *Taraxacum* gr. *officinale* Weber., *Sherardia arvensis* L., *Poa annua* L., *Malva neglecta* Wallr., *Medicago lupulina* L., and *Stellaria media* (L.) Vill.).

Changes in floristic composition mediated the effect of grazing on several forage quality indicators. In particular, the legume-to-grass ratio explained most of the variation in crude protein (80%) and digestibility (55%). These results indicate that preserving nitrogen-fixing legumes linked to extensive grazing is of paramount importance to preserve the nutritional quality of Atlantic mountain grasslands. This is so because reducing the proportion of fiber and increasing the proportion of protein of forage are key factors to increase livestock production [71,72]. Moreover, dicots proportion mediated a significant

part of the grazing effect on forage content in calcium (31%), but the significance of the effect of Simpson diversity index in potassium content was marginal. Our results agree with previous findings indicating that dicots are richer than grasses in minerals [29,30], which are essential to meet the nutritional requirements of grazing animals. The abundance of legumes and forbs also contributes to enhance the nutritive quality of milk produced by lactating ewes: it correlates with higher fat content, richer in α -linolenic acid and its biohydrogenation intermediates, as well as long-chain saturated branched-chain and cis-monounsaturated fatty acids [73,74].

Our results show that preserving plant diversity is required in order to maintain high forage nutritive value in Atlantic mountain grasslands. In a recent experiment on biodiversity and ecosystem function, carried out in semi-natural grasslands, increasing plant diversity promoted quality-adjusted yield [4]. However, opposite to our findings, found that only biomass yield increased, whereas forage quality remained stable [4]. However, whereas in our experiment plant diversity is correlated with the abundance of nutrient rich legumes and forbs, in Schaub et al.'s experiment, plant diversity was uncorrelated with the presence of specific functional groups. This was because diversity was experimentally augmented by randomly adding plant species. Thus, the link observed in our study between conservation of biodiversity and forage nutritive quality is likely applicable to only natural grassland systems where plant diversity correlates positively with the presence of nutrient-rich plant functional groups.

5. Conclusions

Grazing is a pivotal process for mountain grasslands that links economic use with emphasis on forage productivity and quality with conservation and management for maintaining biodiversity. By unveiling and quantifying mediation mechanisms by which changing plant diversity influences forage quality in Atlantic mountain grasslands, our findings shed light on the precise nature of that link. Thus, in terms of grassland management within the context of global change, our results underline the practical importance of preserving, by means of extensive grazing, high leguminous-to-grass ratio and proportion of dicotyledonous species to maintain the high nutritive value of these grasslands. Our findings indicate that the traditional management of Atlantic mountain grasslands, with livestock grazing in local transhumance regimes, promotes the sustainability of the grazing system (by reducing the need of supplemental feed and consequent reduction on GHG emissions), the production of high-quality local products with much-needed added economic value and the conservation of biodiversity with all the ecosystem services linked to this diversity. For Atlantic grasslands, and possibly for almost every grassland type, sustainable economic use and biodiversity conservation are not opposed management objectives.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2073-4395/11/1/25/s1>, Figure S1: Study area and experimental plots; Figure S2: Bivariate plots, histograms and Kendall correlations for forage nutritive quality descriptors; Figure S3: Change in descriptors of forage nutritive quality, Figure S4: change in dicots proportion, Table S1: Definition of forage nutritive quality and floristic composition descriptors; Table S2: descriptive statistics of forage quality properties and floristic composition; Table S3: Post-hoc (Tukey) tests; Table S4: Restricted maximum likelihood estimates (ReML) for cation calcium content (% of dry matter, DM), crude protein (% of dry matter, DM), Simpson's index and legume-to-grass ratio at the end of each season (October) over the period 2005–2010.

Author Contributions: Conceptualization: G.G.-B. and A.A.; methodology: G.G.-B. and A.A.; formal analysis: G.G.-B.; investigation: A.A.; resources: A.A.; data curation: G.G.-B. and I.O.; writing—original draft preparation: G.G.-B., I.O., and A.A.; writing—review and editing: G.G.-B., I.O., and A.A.; visualization: G.G.-B.; supervision: A.A.; project administration: A.A.; funding acquisition: A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Basque Government—Eusko Jaurlaritzza—(grant numbers: ETORTEK10/34, IT1022-16), by the UNESCO Cathedra of the University of the Basque Country—UPV/EHU—(grant number: UNESCO07/07), and by the Ministry of Economy and Competitiveness of the Spanish Government (grant number: AGL2013-48361-C2-1-R).

Acknowledgments: We are indebted to all those who helped us with fieldwork and provided logistical support. In particular, we are grateful to Ana Etxeberria (technical assistant), Jose Antonio Irastortza (guard of the Aralar Natural Park) and to the botanists of ARANZADI Scientific Association (especially to Mari Azpiroz and Lorena Uriarte).

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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