Diversity and functions of soil microbial communities in the Trans-Himalayan grazing ecosystem: influence of herbivores and abiotic factors

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Originality-Significance statement

Diversity-function relationships in soil microbial decomposers are an important aspect of global biogeochemical processes. We study how microbial communities and functions respond to large mammalian herbivores and seasonality, as well as the underlying roles of abiotic conditions that link to functional redundancy.

Highlights

- Diversity-function relationships for microbial decomposers remain uncertain.
- Microbial diversity is positively related to functions
- This relationship is robust to grazer-exclusion.
- Microbial diversity-function relationship can be susceptible to altered precipitation

Summary

1. Diversity-function relationships are well established for producers and their productivity. They are also evident among consumers. However, these are not well known for microbial decomposers and decomposition processes in soil. Further, it also remains unknown whether and how consumers, such as large mammalian herbivores who are a major feature across more than one-third of the world's terrestrial realm, influence microbial decomposer diversity-function relationships.
2. We used a14-year old long-term herbivore-exclusion experiment to answer two questions: (a) whether microbial functions vary with microbial diversity (both species richness and composition), and (b) whether herbivores alter the diversity-function relationships among microbial decomposers. We measured functional richness, functional dispersion, and multifunction from the utilization profiles of 30 metabolic substrates. Alongside, we also measured species richness and species composition of soil microbes.
3. Data were from 60 16S rDNA gene amplicon sequences with 7.6 million reads covering 1937 potential species across 47 phyla, and 1800 catabolic profiles.
4. We found microbial functional diversity in soil was positively related (i.e., coupled) to community composition, but not to species richness. This challenges the prevailing paradigm of functional redundancy in hyper-diverse soil microbial communities where changes in species richness is expected to not affect functional diversity. Instead, our results indicate that certain combinations of species can outperform others. Therefore,

global change factors that alter microbial communities can also impact decomposition processes and services. This coupling between diversity and functions was unaffected by experimental herbivore-exclusion, indicating resilience and resistance among decomposers.

5. Structural equation models suggested that the strength of this relationship is favoured by availability of soil moisture. These also showed microbial functions varied more strongly with temporal variables (e.g., seasonality) than with spatial variables (e.g., edaphic factors such as soil texture and pH).
6. While ecosystem functions and services derived from microbial decomposers have intrinsic resilience and resistance, they respond strongly to variability in water availability. Decomposition in grazing ecosystems may be particularly susceptible to how soil microbes respond to increased precipitation variability under ongoing and projected climate change.

KEYWORDS:

Biodiversity; Ecosystem function; Functional redundancy; Microbial decomposers; Drylands; Species richness; Distance-based structural equation model

Introduction

Species diversity begets ecological functions, and high taxonomic diversity promotes functional diversity as well. Oppositely, depending on ecological resilience and resistance, loss of diversity can deplete functions. The empirical evidence for such diversity-function relationships mostly come from producers and their production (Schulze and Mooney, 1993; Loreau et al., 2001; Hooper et al., 2005; Allison and Martiny, 2008; Reich et al., 2012; Tilman et al., 2014). In comparison, diversity-function relationships for consumers are more complex and less generalizable due to variability in nutrient sources across space and trophic levels (Olff and Ritchie, 1998; Duffy, 2002; Duffy et al., 2007; Cardinale et al., 2011). It is pertinent to ask whether the patterns and processes seen in producers and consumers can be extrapolated to decomposers and decomposition processes. But the answer seems to be contentious as both ‘yes’ and ‘no’ have been reported by studies examining diversity-function relationships for decomposers (Wagg et al., 2014, 2019; Delgado-Baquerizo et al., 2016a; Louca et al., 2016; Chen et al., 2022). Generally, experimental manipulations tend to find positive diversity-function relationships in decomposers (Wagg et al., 2014, 2019; Delgado-Baquerizo et al., 2016a). In contrast, when natural variation in species richness in ecosystems is weighed against functions, studies tend to find no relationship due to functional redundancy in decomposers (Louca et al., 2016; Chen et al., 2022). Such contradictory evidence could be due to few species in experimental studies (c. 10^1 to 10^2 species) (Wagg et al., 2014, 2019; Delgado-Baquerizo et al., 2016a) and many species in observational studies (c. 10^3 species) (Louca et al., 2016; Chen et al., 2022). Increasingly, in natural communities across marine and terrestrial ecosystems, microbial community composition—but not their species richness—is increasingly seen to influence decomposition functions (Fierer et al., 2013; Delgado-Baquerizo et al., 2017; Galand et al., 2018). Therefore, the question of whether, or not, microbial decomposition process in soil is related (i.e., coupled) to

community composition and identity of the species, warrants further investigation. This is critical for knowing whether diversity can buffer and ameliorate the effect of stressors that act on decomposition functions and services.

Decomposition processes by microbes do not occur in isolation of other trophic levels. As consumers of plant production, large mammalian herbivores simultaneously alter both the quantity and quality of biomass-input to soil. Grazing can influence microbial community composition and regulate taxonomic profiles of the decomposer community in soil (Bardgett et al., 2001; Yang et al., 2019; Ylänne et al., 2021). Simultaneously, grazing can also influence functional attributes of soil microbes (Bagchi et al., 2017; Roy and Bagchi, 2022; Roy et al., 2023). These effects are known to be highly variable across ecosystems, and can be positive, negative, or neutral (Tanentzap and Coomes, 2012; McSherry and Ritchie, 2013; Wang and Tang, 2019; Eldridge et al., 2020; Roy and Bagchi, 2022; Roy et al., 2023). Given such heterogeneity, it remains poorly understood whether grazer influence on microbial functions in soil is mediated through their effects on microbial community composition, or not. The role of large mammalian herbivores in mediating inter-dependency between the taxonomic and functional aspects of soil microbial diversity has broad implications for management and sustainability of grazing in dryland ecosystems.

Grazer effects on soil functions arise from their interactions with microbial decomposers that can have far-reaching consequences for local, regional, and global biogeochemical cycles (Bardgett and Wardle, 2003; Bagchi et al., 2017; Naidu et al., 2022; Roy and Bagchi, 2022; Roy et al., 2023). In addition to grazers, underlying edaphic conditions such as moisture availability (Bagchi et al., 2017; Zhang et al., 2022), soil pH (Fierer et al., 2006; Delgado-Baquerizo et al., 2016b), and texture (Girvan et al., 2003) can also affect microbial decomposers. Moreover, soil microbial communities and functions can be influenced by seasonality as they undergo high degree of temporal change as well

(Frossard et al., 2012; Bagchi et al., 2017; Roy et al., 2023). Therefore, one can expect that microbial diversity-function relationship can also vary temporally (Wang et al., 2023).

Here we assess microbial diversity-function relationship by measuring variation in microbial diversity and functions at regular intervals through the growing season in the Trans-Himalayan ecosystem of northern India. The specific questions addressed were: (1) whether microbial diversity (both species richness and composition) and functions vary with grazing and across the growing season (3 levels: early, mid, late); (2) whether microbial community composition influences decomposition functions; (3) whether herbivores alter the relationship between functions and species composition that is otherwise mediated by edaphic conditions and seasonality.

Methods

Study site and sampling

We used a long-term replicated grazer-exclusion experiment started in 2005-06 in Spiti region of Himachal Pradesh in northern India (32° N, 78° E) (Bagchi and Ritchie, 2010a). We set up replicated herbivore exclosures (ungrazed plot), each 10×10 m^2, with adjacent paired control grazed plots to understand the effect of large mammalian herbivores on the ecosystem. Spiti region is a part of the larger Trans-Himalayan drylands across Central Asia (Fig. A.1). This high-altitude grazing ecosystem (elevation 4400-4800 m) experiences cold and semiarid climate with temperature dropping below -30 °C in winters. Vegetation growing-season starts in May, peaks in July-August, and senescence begins in September. Large mammalian herbivores comprise of native species (bharal *Pseudois nayaur*, ibex *Capra sibirica*, and yak *Bos grunniens*) and livestock (goat, sheep, donkey, horse, cattle, and yak-cattle hybrids) (Bagchi and Ritchie, 2010a, 2010b).

After 14 years of the grazer-exclusion experiment, in 2019, we sampled soil from *n*=10 paired-adjacent plots, in May (early-season), July (peak-season), and September (end-season). We used a 2.5 cm diameter and 20 cm depth corer for soils meant for microbial functions and edaphic variables. A smaller sample was packaged in sterilized vials for DNA analysis.

Soil microbial functions

We measured soil microbial functions and functional diversity using n = 30 metabolic substrates (Degens and Harris, 1997; Degens, 1998). In this way, metabolic profiles were obtained from 30×10×3×2=1800 measurements, plus corresponding controls (Fig. 2). We used substrates from different chemical groups: amino acids (Alanine, Arginine, Serine, Asparagine, Glutamine, Glutamate, Histidine), amines (Glucosamine, Putrescine), alcohols (Xylitol, Mannitol, Sorbitol, Glycerol), carbohydrates (D-Glucose, Xylose, Mannose, Sucrose, Inositol), carboxylic acids (α-Ketoglutaric acid, Fumaric acid, Succinic acid, Tartaric acid, Oxalic acid, Malic acid, Ascorbic acid, Citric acid, Malonic acid), an aromatic compound (Inosine), a phenolic compound (Gallic acid), and a polymer (Polysorbate). We measured soil respiration after addition of substrate (substrate induced respiration, SIR) compared to controls (basal respiration) (Degens and Harris, 1997). A substrate was judged to be catabolized if the corresponding SIR was above the 95% confidence-interval of basal respiration (i.e., treatment > control). We used the substrates concentrations such that equal amounts of C was added across all treatments (Degens and Harris, 1997): 100 mM for carboxylic acids and alcohols; 75 mM for carbohydrates; 40 mM for polymers; 15 mM for aromatic, amino acids, amines, and phenols. We pre-incubated 5 g soil at 60% water holding capacity at 28 °C for 24 h. Next, we added 1 ml of substrate solution and incubated soils in air-tight containers along with of 1.5 ml Cresol Red indicator dye (prepared from 2.5 mM

$NaHCO_3$, 150 mM KCl, 12.5 μg ml^{-1} Cresol Red dye) at 28 °C for 15 h (Rowell, 1995). Next, we estimated substrate utilization (mg C g^{-1} soil day^{-1} respired as CO_2) from colorimetric measurement of Cresol Red indicator at 570 nm wavelength, alongside corresponding controls and standards (Rowell, 1995).

Soil DNA extraction, amplification, and sequencing

We estimated microbial taxonomic diversity from Operational Taxonomic Units (OTUs) in DNA extracted from soil. Taxonomic profiles were obtained from 10×3×2=60 samples, plus corresponding controls (Fig. 2). We used MOBIO Power Soil DNA Extraction Kit (Netherlands) following manufacturer instructions. DNA extracts were stored at -20 °C until further molecular analysis. We performed amplification for 16S rRNA hyper-variable region V3-V4 using 25 ng DNA extract, KAPA HiFi HotStart Ready Mix, and 100 nM of 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3') primers (Klindworth et al., 2013). Initial denaturation was at 95 °C for 5 min followed by 25 cycles of 95 °C for 30 s, 55 °C for 45 s and 72 °C for 30 s with a final extension at 72 °C for 7 min. Amplicons were sequenced using Illumina MiSeq platform and data were processed using Mothur (Schloss et al., 2009) to obtain microbial taxonomy data. The raw sequences were aligned to form contigs, and only contigs with length between 300 bp and 532 bp were retained because they cover the targeted error-free sequence of the 16S rRNA region with high confidence. Contigs were then checked for chimeras, and removed, using reference-based chimera checking following UCHIME algorithm (Edgar et al., 2011). The contigs were then categorized into OTUs with 97% sequence similarity using SILVA_v138 database (Quast et al., 2013). As an additional level of quality filtering, OTUs with cumulative abundance of less than 20 across all samples in the data set were removed. Controls showed

minimal absorbance at 260 nm (<1 ng/μl of DNA) compared to samples (24 to 70 ng/μl of DNA), and we did not sequence them since they did not show any amplification.

Edaphic variables

We measured three key edaphic variables (Fig. A.2), namely soil moisture, pH, and texture following standard procedures (Robertson et al., 1999) as these can influence variation in microbial communities and in functions (Fierer and Jackson, 2006; Sinsabaugh et al., 2008). Briefly, soil moisture was measured as volumetric water content with a time domain reflectometry probe (Spectrum Technologies, USA), pH was measured from 1:2.5 (v/w) aqueous suspension using a table-top pH meter, and soil texture was measured from 50 g soil in 1000 ml of $(NaPO_3)_6$ solution using a Bouyoucos hydrometer.

Statistical analyses

We summarized microbial functions with three indices **a) Functional richness:** calculated as the number of substrates metabolized. b) **Dispersion:** multivariate measure of functional heterogeneity (dispersion) estimated as the distance of each sample relative to their respective group centroid (Anderson et al., 2006). We used 'vegan' library in R 3.6.3 to estimate richness and dispersion (Oksanen et al., 2019). **c) Multifunction:** measured as sum of SIR across 30 catabolites (mg C g^{-1} soil $hour^{-1}$). This represents the decomposition potential of the microbial communities. Multifunctionality is the ability of an ecosystem to perform multiple functions, or provide several services, simultaneously (Hector and Bagchi, 2007).

We estimated species richness as the number of OTUs in a sample. We used Generalized Linear Models (GLM) with grazing (grazed/ungrazed) and season (early, mid, and late) as fixed-effects, alongside plot-identity as the random-effect to assess the effects of grazing and season on species richness and functional diversity. This GLM accounts for the pair-wise

spatial structure in the data (Pinheiro and Bates, 2000), and we used ‘nlme’ library in R 3.6.3. We verified whether the data met the assumptions of GLMs using theoretical and observed quantiles of residuals, and the data did not require any transformations.

Microbial composition and function

We used ordination with principal coordinates analysis (PCoA) of pair-wise Bray-Curtis dissimilarity to visualise species composition and decomposer functions across seasons and grazing treatment in a multivariate space summarized as two axes (Legendre and Legendre, 2012).

We used kriging to visualize the relationship between species composition and microbial functions. Kriging is an interpolation method that helps estimate the value of a variable (e.g., functional richness) over a continuous space of microbial species composition using an already existing but a limited set of sampled data points (Dale and Fortin, 2014; Li et al., 2023). We used PCoA axes 1 and 2 as a measure of species composition to interpolate the values of functional indices for unmeasured values of species composition. Kriging is a two-step process: first, a variogram is fitted to determine the spatial (species composition space) covariance structure of the sampled data points; second, interpolate values for the unsampled locations across a given species composition space from the weights derived from the spatial covariance structure of the known data points (Dale and Fortin, 2014; Li et al., 2023). For this, various variogram models were fitted by changing the trend removal parameter (no trend removal, 1st order, 2nd order), model function (exponential, spherical, gaussian), and nugget (fixed, unfixed). First step in fitting the variogram model is trend removal. Trend removal is done by fitting a polynomial equation to the predictor values (PCoA axes 1 and 2) using ordinary least squares, and therefore the variograms are computed using the residuals. 1st order trend removal will remove linear trends, whereas 2nd order will remove quadratic

trends. Trend removal is followed by fitting a model to the empirically determined semivariogram using any of the three model functions where nugget (y-intercept value) is either fixed at zero or unfixed (variable). For each microbial functional index there were 18 different combinations (3 trend removal parameters × 3 model function × 2 nugget) to build a variogram model. We selected the final combination for each functional index where the resulting model had the least sum of squares (Table A.1). For all the functional indices we selected 2nd order trend removal with exponential function and nugget fixed at zero. We implemented kriging with "geoR" package in R (Justiniano {Ribeiro Jr} et al., 2022).

Distance-based structural equation models

We used distance-based structural equation models (dbSEM) to evaluate the relationship between species composition and function, and whether grazers affect the relationship directly or indirectly through edaphic factors. dbSEM uses pairwise-dissimilarity matrix for both dependant and predictor variable to disentangle direct and indirect hypothesized causal relationships (Fourtune et al., 2018; Jabot et al., 2020). We used permutation based dbSEM where SEM is performed on the input matrices of variables where rows and columns are randomly permuted to obtain an unbiased P-value for each hypothesized path (Fourtune et al., 2018; Jabot et al., 2020). P-value is computed as the proportion where permuted path coefficient values are less than or equal to the observed path coefficient value. The edaphic variables were soil moisture, pH and texture. For soil texture we only included sand content in the analysis because of autocorrelation with clay and silt content. The paths in the dbSEM were motivated by important processes that shapes up microbial communities and known *a-priori*. a) Large mammalian herbivore alter microbial communities (Grazing → Species composition) (Sankaran and Augustine, 2004; Bagchi et al., 2017). b) Herbivore can regulate decomposition process directly (Grazing → Function (functional richness, functional

composition, multifunction)), or indirectly through species composition (Grazing → Species composition → Function) (Bagchi et al., 2017; Naidu et al., 2022; Roy and Bagchi, 2022). c) Background edaphic variables can shape up the microbial communities (Edaphic (pH, sand, moisture) → Species composition) (Girvan et al., 2003; Fierer and Jackson, 2006; Bagchi et al., 2017). d) Variation in background edaphic variables can alter function directly (Edaphic → Function), or indirectly through changes in species composition (Edaphic → Species composition → Function) (Girvan et al., 2003; Delgado-Baquerizo et al., 2016b; Bagchi et al., 2017). e) both microbial community and function can vary temporally (Season → Species composition, Season → Function) (Bardgett et al., 2005; Frossard et al., 2012; Delgado-Baquerizo et al., 2013; Bagchi et al., 2017). f) Grazing can also influence soil moisture (Grazing → Moisture) (Bagchi et al., 2017), that can in-turn alter pH (Moisture → pH). g) Sand content can also alter moisture (Sand → Moisture) and pH (Sand → pH). We assessed goodness of fit between the model and data with χ^2 test, comparative fit index (CFI), and Standardized Root Mean Square Error of Approximation (RMSEA). We accepted the model $P(\chi^2) > 0.05$, CFI ~ 1, and RMSEA < 0.05. We implemented paths for dbSEM using 'lavaan' library in R (Rosseel, 2012).

Results

Microbial communities and functions –

We recorded 1937 unique OTUs across 47 known phyla and 7650894 reads. Actinobacteriota, Acidobacteriota, Planctomycetota, Proteobacteria and Verrucomicrobiota were most common (Fig. 1, A.3.). Species richness of OTUs ranged between 494 and 866 per sample (mean 713±80 SD). Microbial activity was highest for carboxylic acids such as α-Ketoglutaric acid, Malic acid, Citric acid, Succinic acid, and Malonic acid, Similarly,

functional richness (i.e., number of substrates metabolized) ranged between 6 and 30 (mean 21±7 SD, Fig. 1, A.3.).

Seasonality and grazer-effects on microbial species and functions –

Variation in species (OTUs) richness was not explained by season (Fig. 2a; $F_{2,45}$=0.02, *P*=0.98), and by grazing (Fig. 2a; $F_{1,45}$=1.61, *P*=0.21). Microbial functional richness varied with season (Fig. 2b; $F_{2,45}$=38.96, *P*<0.001) but did not vary with grazing (Fig. 2b; $F_{1,45}$=0.01, *P*=0.98). Similarly, microbial functional dispersion varied with season (Fig. 2c; $F_{2,45}$=48.89, *P*<0.001) but did not vary with grazing (Fig. 2c; $F_{1,45}$=0.01, *P*=0.92). Multifunction also varied with season (Fig. 2d; $F_{2,45}$=10.94, *P*<0.001), but not with grazing (Fig. 2d; $F_{1,45}$=0.07, *P*=0.79).

In multivariate space summarized with PCoA ordination on Bray-Curtis dissimilarity matrix, microbial species composition varied with season (Fig. 3; $F_{2,54}$=4.70, *P*=0.001), but not with grazing (Fig. 3; $F_{1,54}$=1.33, *P*=0.206). functional composition also varied with season (Fig. 3; $F_{2,54}$=4.83, *P*=0.001), but not with grazing (Fig. 3; $F_{1,54}$=0.87, *P*=0.555).

Relationship between species composition and functions –

The relationship between species composition and microbial functions was visualized using kriging (Fig. 4). Kriged maps of microbial functions indicates a dependence on microbial species composition where different community composition support different magnitude of functions.

Relationship between species composition and microbial functions were further assessed using dbSEM. Overall, dbSEM for functional richness (Fig. 5), functional dispersion (Fig. 5), and multifunction (Fig. 5) indicated good match between the data and hypothesized paths (SRMR=0.005, RMSEA<0.001, P-value (RMSEA)=0.982, CFI>0.999, χ^2=1.34, P-

value (χ^2)=0.512). Path coefficients suggested a significant positive relationship between species composition and functional richness (Species composition → Functional richness; path coefficient=0.08, *P*=0.06; Fig. 5), and with functional dispersion (Species composition → Dispersion; path coefficient=0.08, *P*=0.10; Fig. 5), but not with multifunction (Species composition → Multifunction; path coefficient=-0.06, *P*=0.13; Fig. 5). Sand content had a positive effect on species composition (Sand → Species composition; path coefficient=0.14, *P*=0.001; Fig. 5), and a negative effect on multifunction (Sand → Multifunction; path coefficient=-0.06, *P*=0.04; Fig. 5). Seasonality had positive relationship with species composition (Season → Species composition; path coefficient=0.14, *P*=0.001; Fig. 5), and soil moisture (Season → Moisture; path coefficient=0.29, *P*<0.001; Fig. 5), such that as the season progresses from early to mid to late, species composition and soil moisture increases. Moisture in turn had a positive effect on species composition (Moisture → Species composition; path coefficient=0.12, *P*=0.02; Fig. 5). In this way seasonality had a direct (Season → Species composition → Function), as well as indirect effect through moisture (Season → Moisture → Species composition → Function), on the species composition-function relationship. Grazing did not influence this relationship directly (Grazing → Species composition → Function), or indirectly through edaphic factors (Grazing → Edaphic → Species composition → Function).

Permutation based dbSEM for individual functions indicated good match between the data and hypothesized paths (SRMR=0.005, RMSEA<0.001, P-value (RMSEA)=0.982, CFI>0.999, χ^2=1.34, P-value (χ^2)=0.512). Significant species composition and function relationship was found for 6 out of 30 functions (Species composition → Function, Fig. A.1). The relationship was positive for 5 out of 6 functions, and negative for only Malic acid. For all these 6 functions season had a significant effect on the relationship (Season → Species

composition → Function, $P<0.05$). Season also had an indirect effect on 5 out of 6 significant species composition-function relationship through soil moisture (Season → Moisture → Species composition → Function, $P<0.05$). There was significant temporal variation in 28 out of 30 functions (Season → Function, $P<0.05$). Soil moisture and sand content also had positive effect on the relationship for all 6 functions. Soil moisture had positive effect on 17 out of 30 functions and negative effect on 3 out of 30 functions (Moisture → Function, $P<0.05$). Sand content had positive effect on 3 out of 30 functions and negative effect on only 1 function (Sand → Function, $P<0.05$). While grazing had a negative effect on 4 out of 30 functions (Grazing → Function, $P<0.05$), but grazing did not influence species composition-function relationship directly (Grazing → Species composition → Function, $P>0.05$), or indirectly through edaphic factors (Grazing → Edaphic → Species composition → Function, $P>0.05$).

Discussion

Diversity-function relationship is known for producers and consumers but remains uncertain for microbial decomposers and decomposition process. Here we investigate whether, or not, microbial taxonomic diversity (structure) is related to the microbial decomposition process (function) in soil. In the Trans-Himalayan drylands, we find three key results: (i) soil microbial communities and functions are more variable across time than over space (Fig. 1, 2, 3), (ii) microbial species composition affects functional richness, but not functional composition and multifunction (Fig. 4, 5), and (iii) large mammalian herbivores did not affect the diversity-function relationship for microbes, whereas background edaphic variables and season does affect the relationship (Fig. 5).

Taxonomy~Function relationship

A general paradigm in ecology is that taxonomy determines functions, such that greater taxonomic diversity will favor functional diversity. This paradigm is supported for producers and consumers, at both local (α-diversity) as well as landscape levels (β-diversity) (Loreau et al., 2001; Hooper et al., 2005; Reich et al., 2012; Hautier et al., 2018), and can influence the provisioning of ecosystem services. Unlike producers and consumers, soil microbial decomposers are hyper-diverse. As a case in point, we recorded more than 700 OTUs on average per sample, and 1937 OTUs overall across 7.6 million reads. Therefore, and understandably, the data did not show any support for the relationship between species richness and functions (Fig. A.5). For instance, where local richness is below 200 OTUs per sample, there is evidence of coupling and therefore a lack of redundancy (Delgado-Baquerizo et al., 2016a). But, further along at 700 OTUs per sample (Fig. 3-4) microbial diversity can appear de-coupled from functions (Peter et al., 2011; Louca et al., 2016; Chen et al., 2022). Thus, not surprisingly, different studies have encountered coupled as well as de-coupled relationships between microbial species richness and functions (Peter et al., 2011; Delgado-Baquerizo et al., 2016a; Louca et al., 2016; Chen et al., 2022), and our data emphasize how it is important to accommodate species composition (Hooper et al., 2005).

Our results show that taxonomically dissimilar community can perform more functions (richness) but has no effect on the magnitude (composition and multifunction) of the functions. Although this result seems counterintuitive but is not trivial. The ability to perform any function is tightly linked with the identity and relative abundance of each species in the community, but which functions will operate and at what rate depends on the environment. The interdependency between taxonomic composition and functions seems to challenge “functional redundancy” or “functional equivalence” hypothesis. It proposes that changes in microbial community composition exercise only proximate controls on functions whereas the current environment ultimately determines functions. Alternatively, “functional

dissimilarity" hypothesis proposes that not 'only' environment, but a 'combination' of environment and microbial community composition ultimately determine microbial functions (Strickland et al., 2009). Indeed, we find that while seasonality and edaphic factors (moisture and texture) together with community composition exerts strong control over microbial functions.

Seasonality

We find temporal variation in microbial community structure and functions to be more pronounced than spatial heterogeneity (Fig 2, 3, 5). This is consistent with other studies where fluctuations in resource availability explained why microbes respond to time more than space (Frossard et al., 2012; Bagchi et al., 2017; Roy and Bagchi, 2022; Roy et al., 2023). Vegetation at our study site follows a unimodal distribution in the growing season and the underlying temperature regime can cause a seasonal variation in resource availability for microbes to metabolize (Millard and Singh, 2010; Roy et al., 2023). Such a stark difference between temporal and spatial responsiveness of microbes lends further support to adage "everything is everywhere but the environment selects" (Bass-Becking, 1934), and encourages further discussion on the temporal aspect of soil microbes in soil and its functional implications. Our study also highlights why biodiversity-ecosystem function (BEF) studies, particularly for microbes, should incorporate temporal dynamics (days, months, years) rather than restricting to a snapshot in time.

Edaphic factors

We find a consistent effect of soil moisture and texture on the decomposer diversity-function relationship (Fig. 5). Moisture and texture can play an important role in habitat-filtering (Bass-Becking, 1934) as they promote functions through taxonomic composition

(Fig. 5), resulting in the loss of functional redundancies among decomposers. On average, the Trans-Himalayan drylands are getting wetter over decadal time-scales while experiencing increased inter-annual variability (Murthy and Bagchi, 2018; Hu and Zhou, 2021). Under such ongoing and projected precipitation regimes, the stability of ecosystem functions will depend on how changing moisture availability affects coupling of taxonomy and function. Further, strong association of texture with the functions can exacerbate the negative effects of habitat filtering by altered precipitation regimes on decomposition. Variation in soil texture had positive effect functional richness (Fig. 5), that invariably also reduces heterogeneity in multifunction across the landscape (Fig. 5), leading to effective homogenization. Soil texture can cause homogenization in decomposition process by altering soil water availability, pore size distribution, surface area, and ultimately nutrient availability that in turn opens new niches (increases access to newer substrates) for microbes to proliferate and reduces heterogeneity in decomposition. While the effect of moisture and texture (Fig. 4) is consistent with other studies across ecosystems (Girvan et al., 2003; Delgado-Baquerizo et al., 2016c), unlike other studies (Fierer and Jackson, 2006; Sinsabaugh et al., 2008) we find no effect of soil pH. This could be due to the invariability in soil pH across the landscape (space), and season (time), but it can vary between different landscapes and ecosystems.

Large mammalian herbivores

We find that the relationship between microbial taxonomy and function is robust to the presence or absence of large mammalian herbivores. This observation is in line with patterns seen for producers and their production systems where herbivore removal did not impact producer diversity and ecosystem stability relationship (Kohli et al., 2019). However, contrary to the direct effect of consumers on producers, the mechanisms operating for

decomposers might be different (Bagchi et al., 2017; Naidu et al., 2022; Roy and Bagchi, 2022). There could be multiple interdependent paths that could have led to this observation:

1. Balance between direct and indirect herbivore effects: Net effect, or a lack thereof, on the microbial decomposer diversity-function relationship can be a balance between the direct and indirect effects of large-mammalian herbivores (Bagchi and Ritchie, 2010a; Cherif and Loreau, 2013; Naidu et al., 2022; Roy and Bagchi, 2022; Roy et al., 2023). Direct effect of herbivores, through the quantity of nutrient input, on decomposers is exerted by consumption of plant productions that diverts nutrient away from the soil towards secondary production (Bagchi and Ritchie, 2010b). On the other hand, indirect effect of herbivores, through the quality of nutrient input, on decomposers is exerted by converting plant materials into dung and urine, and altering plant community composition, that change the proportion of labile to recalcitrant substrates (Bagchi et al., 2017; Naidu et al., 2022; Roy and Bagchi, 2022). Equilibrium in the relative strengths of the direct and indirect influence of herbivores can therefore manifest as no net effect on the decomposer diversity-function relationship (Hooper et al., 2000). This can also explain the variability in microbial responses to herbivores, with neutral, positive, negative effects in different ecosystems (Tanentzap and Coomes, 2012; McSherry and Ritchie, 2013; Wang and Tang, 2019; Eldridge et al., 2020).
2. Historical and evolutionary adaptations: The tight coupling between microbial diversity and function, could have established before the start of the decadal-scale grazer-exclusion experiment due to competition-cooperation interactions (Morrissey et al., 2019; Lechón-Alonso et al., 2021). Once established, the interactions among microbial decomposers may persist for long periods (Morrissey et al., 2019; López

Zieher et al., 2020), and can only respond to the prevailing edaphic constraints (moisture, texture) (Fig. 5).

3. Effect on other groups of microbial decomposers: We recognize that we cannot exclude the effect of herbivores on fungi or archaea that are also responsible for the decomposition process. Indeed, previous studies have shown that grazers shift fungal:bacterial ratio towards dominance by fungi that ultimately regulates soil-C dynamics (Bagchi et al., 2017). Therefore, this would need further research where we incorporate the fungal taxonomic information as well. Nonetheless, our study suggests that spatiotemporal variability in the soil microclimate had a stronger effect on the diversity-function relationship of microbial decomposers, than herbivory *per se* (Hooper et al., 2000). This is in line with the studies that suggests high altitude ecosystems to be more sensitive to variability in climate parameters than ecosystems at lower elevations (Ohmura, 2012; Lamprecht et al., 2018; Li et al., 2018).

Overall, we find grazer-effect microbial functions are mediated through changes in microbial community composition. Soil moisture controls most dryland ecosystem processes (Millard and Singh, 2010; Delgado-Baquerizo et al., 2013; Maestre et al., 2016), and future changes in soil moisture availability in drylands (Schlaepfer et al., 2017; Konapala et al., 2020), particularly in the Trans-Himalaya and Central Asia's high-elevation drylands, might challenge their conservation and sustainable management for ecosystem functions and services.

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FIGURES

Fig. 1

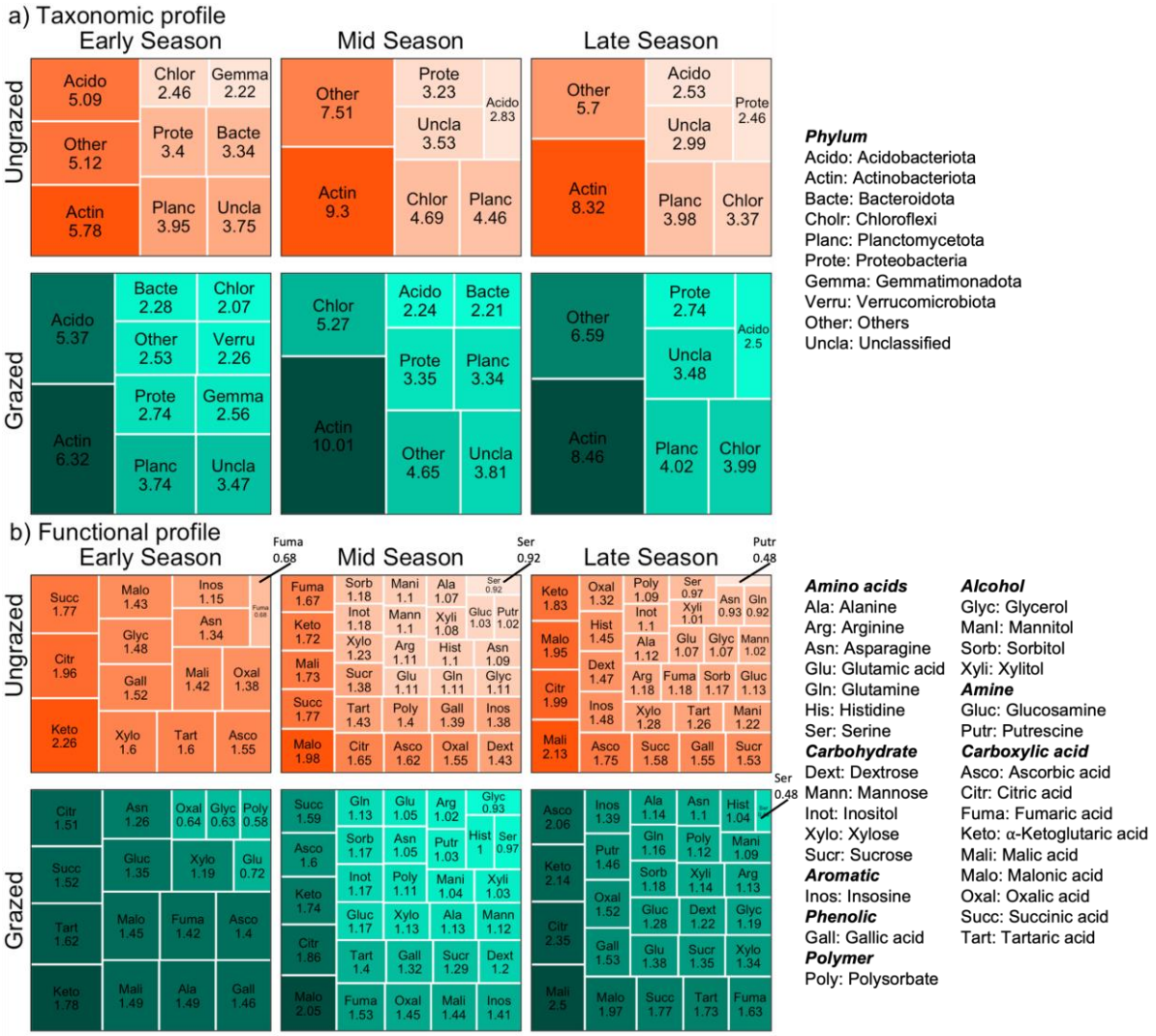

Figure 1. Treemaps summarizing the distribution of microbial taxonomic profile (phylum level) and microbial functional profile (n=30 substrates) in the presence and absence of large mammalian grazers (n=10 paired plots) averaged for all plots and across the growing-season (Early, Mid, and Late). Area of each cell under taxonomy and function is proportional to the total abundance of each phylum and microbial activity for each substrate, respectively. Number denotes the percentage of the relative abundance (taxonomy) and relative microbial activity (function).

Fig. 2

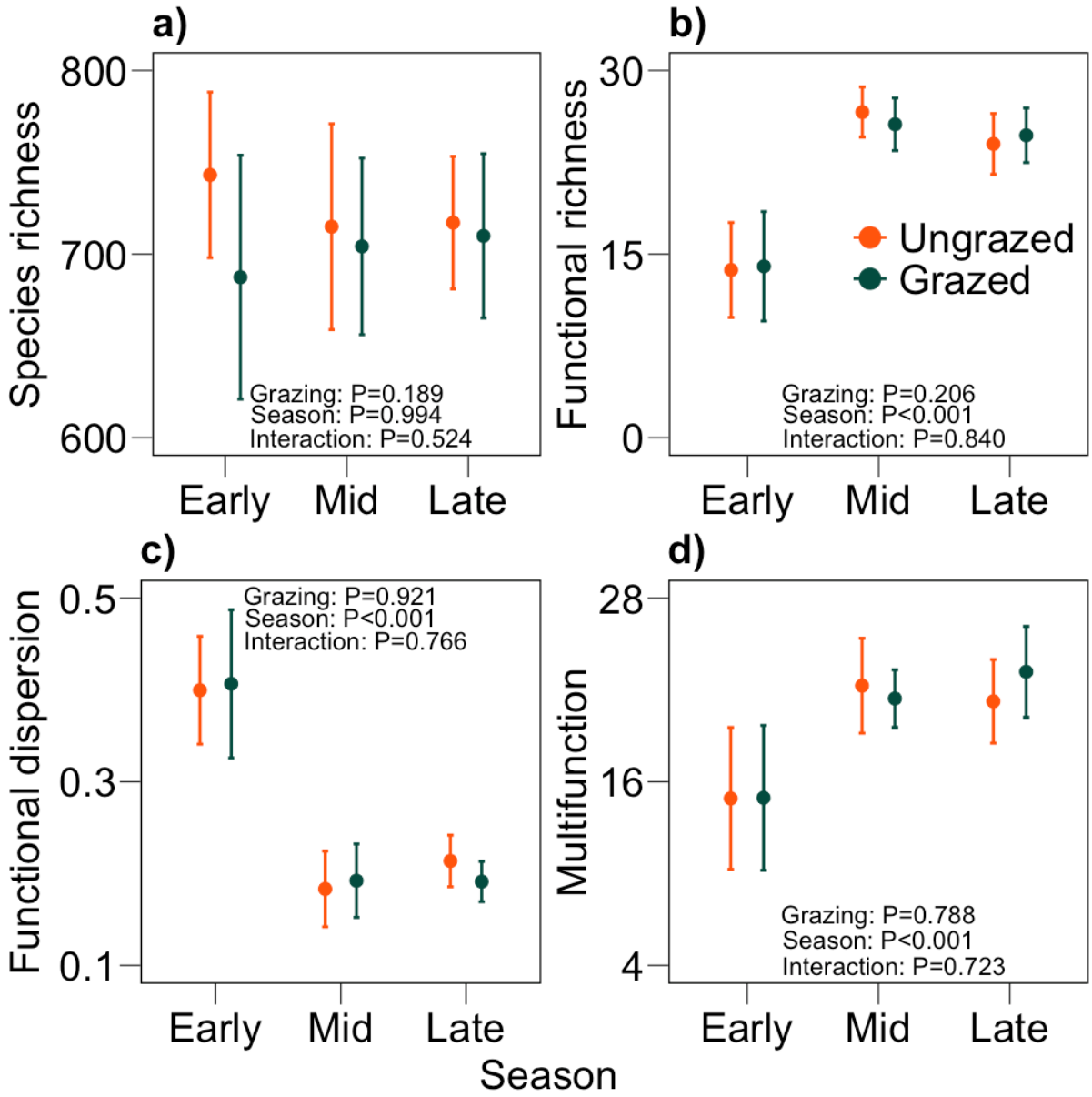

Figure 2. Species richness of microbial taxonomy (a), functional richness (b), functional dispersion (c), and multifunction (mg C g^{-1} soil $hour^{-1}$; d) in the presence and absence of large mammalian grazers across the growing-season. Species richness (mean ± 95% CI) did not vary between grazed and ungrazed plots, and with the growing-season. Functional richness, functional dispersion, and multifunction did not vary between grazed and ungrazed plots, but varied across the growing-season. Data from n=10 plots.

Fig. 3

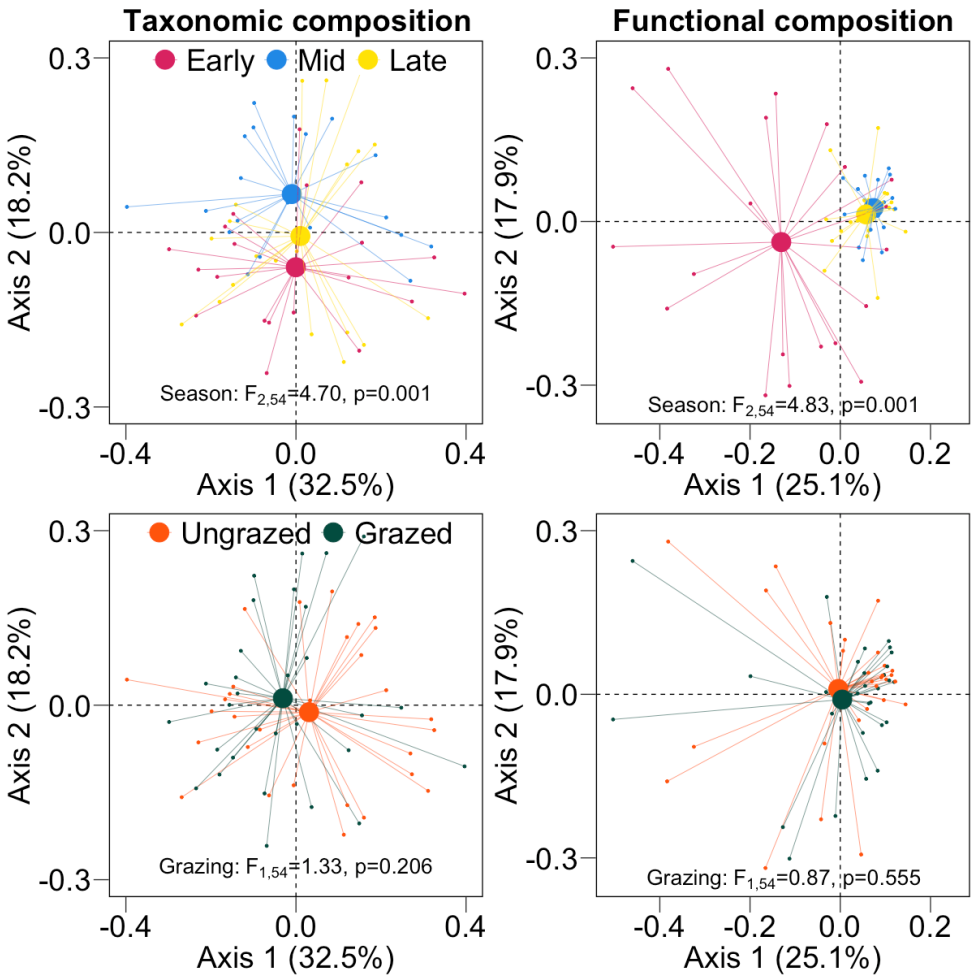

Figure 3. Principal coordinate analysis (PCoA) plot for microbial communities and functions in the presence and absence of large mammalian grazers, and at different points in the growing-season. The scatter plot shows principal coordinate 1 (Axis 1) versus principal coordinate 2 (Axis 2). Percentage of variation explained by each axis for each plot is shown in the axis labels.

Fig. 4

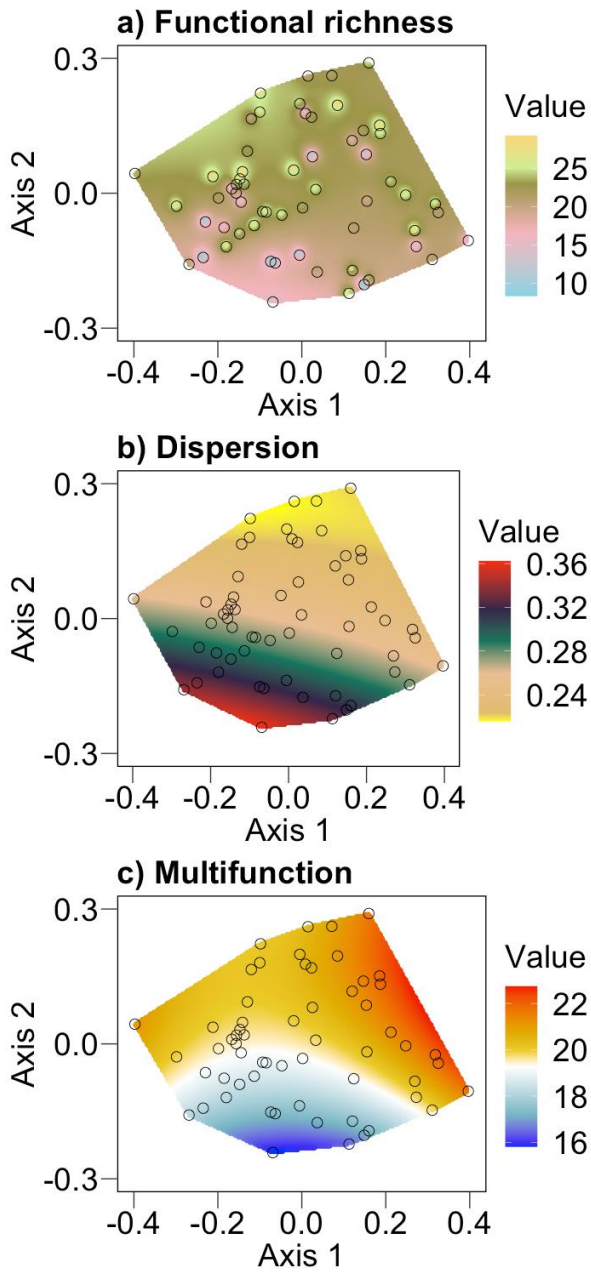

Figure 4. Kriged maps summarizing the relationship of microbial functional richness (a), functional dispersion (b), and multifunction (mg C g-1 soil hour-1; c) with microbial species composition. The colored space highlights the interpolation of microbial functions across the extent of microbial species composition from the study site measured at different points in the growing-season. Species composition is represented as axis 1 and axis 2 of principal coordinate analysis (PCoA). Hollow circles denotes the sampled data points for species

composition. Value in the legend indicates the range of interpolated values of respective functional index. Overall, microbial decomposer functions can vary with species composition.

Fig. 5

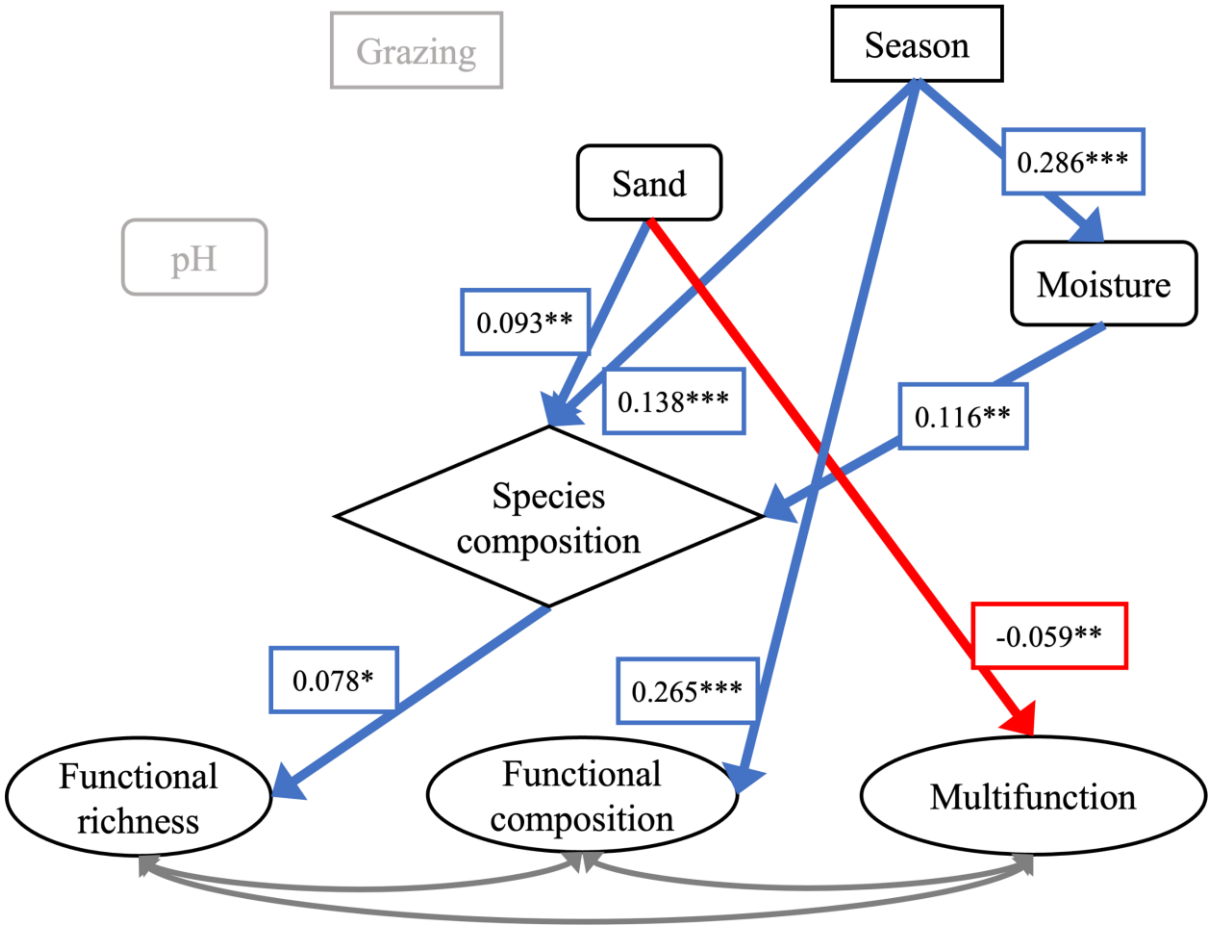

Figure 5. Models representing the distance-based path analyses results that evaluates the effects of grazing and season on species composition and function relationship via its influence in soil abiotic properties. Thick arrows are statistically significant paths ($\alpha = 0.10$), where blue arrows represent positive path coefficient and red arrow represent negative path coefficient. Value above every significant path represents standardized path estimates. Modelled paths from grazing and pH were statistically non-significant. Diagnostics for all the models were SRMR=0.004, RMSEA<0.001, P-value (RMSEA)=0.982, CFI>0.999, χ^2=1.34, df=2, P-value (χ^2)=0.512. Asterix represent statistical significance (***P≤0.01, **P≤0.05, and *P≤0.10). Overall, there was a positive relationship between microbial functions and species composition.

APPENDIX

Diversity and functions of soil microbial communities in the Trans-Himalayan grazing ecosystem: influence of herbivores and abiotic factors

Shamik Roy, Pronoy Baidya, Jalmesh Karapurkar, Jose Mathew, Sumanta Bagchi*

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Additional Introduction

Structure-function relationship documented from across a wide range of producers and consumers (Hooper et al., 2005; Reich et al., 2012; Grman et al., 2018; Hautier et al., 2018) have established a positive yet decelerating relationship (Fig. A.5.). This is also expected for microbial decomposers in soil (Coleman and Whitman, 2005; Delgado-Baquerizo et al., 2016a). From the curvilinear shape of the relationship, it follows that numbers, quantity and diversity of functions initially rise with taxonomic diversity and saturate thereafter (Fig. A.5.). Over the initial ascending part (Fig. A.5.), the structure-function relationship is coupled. Any increase in the taxonomic diversity will increase functional diversity, albeit at different rates in different microbial communities, and vice-versa. For the asymptotic region at high diversity (Fig. A.5.), one expects no relationship between the taxonomic and functional aspects. Here, these two aspects may appear decoupled (Bell et al., 2009; Frossard et al., 2012; Purahong et al., 2014) because microbial decomposer assemblages in soil have high levels of redundancy in their functions (Allison and Martiny, 2008; Wagg et al., 2019). We know that order and magnitude of this curvilinear relationship will vary with ecosystems as scale for taxonomic and functional axes changes (Delgado-Baquerizo et al., 2016a). This is because different ecosystems provision for different ecological functions and services.

Using experimental herbivore removal (see Methods), we study whether grazer effects on microbial functions are mediated through changes in microbial community structure. First, we determine whether grazer-exclusion alters soil microbial community structure and functions. Next, we evaluate the empirical support for alternative competing hypotheses on how grazer-exclusion may influence the degree of coupling between the taxonomic and functional aspects of soil microbial decomposers (Fig. A.5.). Grazer impacts

on functions can differ along a gradient from low to high taxonomic diversity, and this leads to three competing hypotheses:

i) Sensitivity Hypothesis (H_S): Over the initial ascending part, grazers can alter the slope of the relationship (i.e., sensitivity, Fig. A.5.). When grazer-exclusion increases the slope, it raises the sensitivity of functions to taxonomic diversity; loss of taxonomic diversity will lead to strong declines in functions. This outcome amounts to large decline in resilience under loss of herbivores. Otherwise, when grazers decrease the slope of this relationship, then grazer-exclusion will lower the sensitivity of functions to taxonomic diversity; loss of taxonomic diversity will lead to proportionately less reduction in functions. This outcome amounts to greater resilience in absence of grazing (Fig. A.5.).

ii) Magnitude Hypothesis (H_M): Any response in functions does not result from changes in slope of the relationship, but rather from change in the intercept (i.e., magnitude, Fig. A.5.). If grazing favors functions, then grazer-exclusion will lower the intercept such that lower levels of taxonomic diversity are needed to maintain functions under grazing than under grazer-exclusion. However, if grazing lowers functions, then grazer-exclusion will raise the intercept such that a higher taxonomic diversity is needed to maintain functions under grazing than under grazer-exclusion. In the asymptotic region, grazer-exclusion can either increase or decrease microbial functions (Sankaran and Augustine, 2004; Bagchi et al., 2017), but this can be independent of any response in taxonomic diversity.

iii) Sensitivity-Magnitude Hypothesis (H_{SM}): This is intermediate between H_S and H_M whereby the grazer-exclusion will not only change the slope of the relationship (sensitivity) but also the intercept (magnitude) (Fig. A.5.). This outcome has

consequences for both resilience and functional repertoire of microbial communities in the absence of grazing.

The null hypothesis against H_S, H_M, and H_{SM} expects no difference in either slope or intercept under grazing and under grazer-exclusion (Bengtsson, 1998).

Additional methods

Microbial richness and function: model comparison and SEM

We used a model selection and simplification approach with Akaike Information Criterion (AIC, with $\Delta AIC \leq -2$ between competing models for parsimony) to compare GLMs and assess support for the alternative hypotheses (Fig. A.5.; H_S, H_{M1}, H_{M2}, H_{SM}) in the data. The omnibus model contained a 3-way interaction between species richness, grazing, and season (m_0: Functions ~ Taxonomy × Grazing × Season) alongside the random-effect due to plot-identity. We checked whether season affects the richness-function relation through 2-way (Richness×Season) or 3-way (Richness×Grazing×Season) interaction. If season had an effect, then we evaluated simplified GLMs for each season separately, otherwise we evaluated GLMs on the full dataset. For these, we tested whether grazers altered the slope or intercept following a three-step approach.

Step 1: We tested for sensitivity (H_S, H_{SM}) by comparing a model with interactive effects between taxonomic diversity and grazing (m_1: Functions ~ Species richness × Grazing) against a model with additive effects between them (m_2: Functions ~ Species richness + Grazing). Support for m_1 in the data would suggest that H_S, H_{SM} prevails over H_{M1}, H_{M2}, and over the null hypothesis (Fig. A.5.). To understand whether the data supported H_S or H_{SM} we used analysis of variance (ANOVA) on the m_1 model. We checked whether grazing had a statistically significant effect (α=0.05) on functions, either as main-effect or through two-way interaction (Species richness × Grazing). If grazing had a statistically significant effect

(α=0.05) on functions through both main-effect and two-way interaction, then data indicates H_{SM}. Else, if grazing affects function only through two-way interaction, then data indicates H_S.

Step 2: If m_2 is supported over m_1 in step 1, then we tested for magnitude by comparing m_2 (Functions ~ Species richness + Grazing) and an intercept-only GLM (m_3: Functions ~ Grazing). Model m_2 indicates that both species richness and grazing affects functions, whereas model m_3 indicates that grazer-effects on functions are independent of species richness. Here, support for m_2 over m_3 would suggest that grazing-exclusion can alter magnitude (H_{M1}) without alteration of positive function-species richness relationship.

Step 3: In m_3 is supported over m_2 in step 2, we compared m_3 (Functions ~ Grazing) and null model (m_4: Functions ~ 1). Null model indicates that variation in microbial functions is independent of grazing and microbial species richness. Here, support for m_3 would suggest that grazing can alter magnitude (H_{M2}) without any function-species richness relationship. GLMs were evaluated for all functional indices (Cardinale et al., 2006; Grman et al., 2018).

Additional results

Relationship between species richness and functions –

The omnibus models for all aspects of microbial functions showed there was a lack of 3-way interaction as well as no 2-way interactions involving season (Table A.2.). So, we report results on effects of grazer-exclusion on the relationship between microbial functions and species richness for all seasons together (Fig. A.6.). Model comparison for all functional indices (m_1, m_2, m_3, m_4) showed no relationship between species richness and functions across all seasons, and this was not altered by grazing (Fig. A.6; Table A.2., A.3.). Since grazer-exclusion neither altered the slope nor the intercept of the taxonomy-function relationship (Fig. A.6; Table A.2., A.3.), the null hypothesis was supported over H_S, H_{SM},

H_{M1}, and H_{M2}. Model comparison of individual functions suggests a significant relationship between species richness and 8 out of 30 functions (Table A.4., A.5.). For alanine, inosine, malic acid, and mannitol, the data supports H_S such that grazer-exclusion reduces the sensitivity (slope) of the relationship. Data for glucosamine supports H_{M2} where there is no relationship between species richness and the function, but grazer-exclusion increases the magnitude of glucosamine. Data for mannose supports H_{M1} where grazer-exclusion increased the magnitude with no effect on the positive relationship between species richness and the function. For polysorbate and putrescine, the relationship varied with season due to significant two-way interaction (Richness×Season, $P<0.05$; Table A.5.). Model comparison for polysorbate find support for H_{M2} hypothesis in the mid-season and H_{M1} hypothesis in the late-season (Table A.5.). Model comparison for putrescine find support for H_{M1} hypothesis in the mid-season (Table A.5.).

Figure A.1. Map of sampling locations

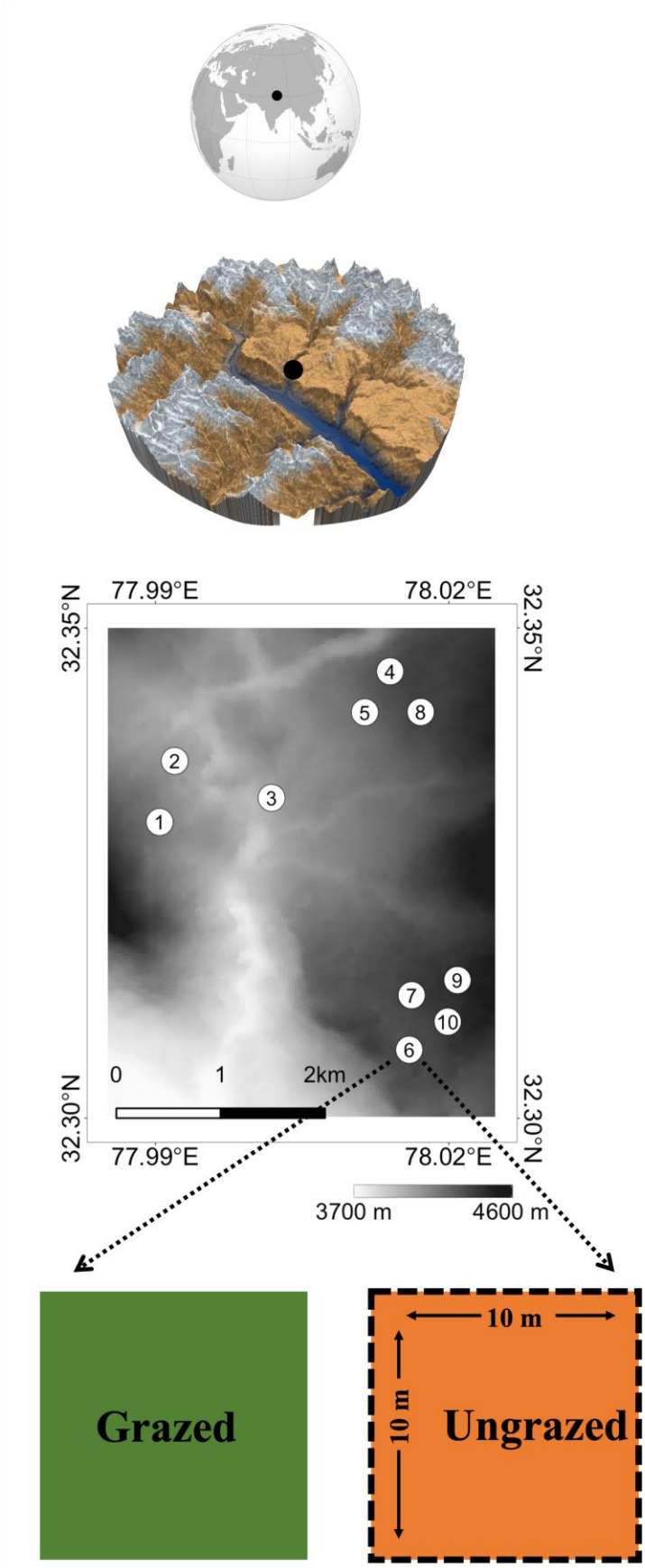

Figure A.2. Edaphic conditions soil pH, soil moisture, and soil texture in in the presence and absence of large mammalian grazers (n=10 paired plots). Soil moisture is measured as volumetric water content (VWC, %) across the growing-season.

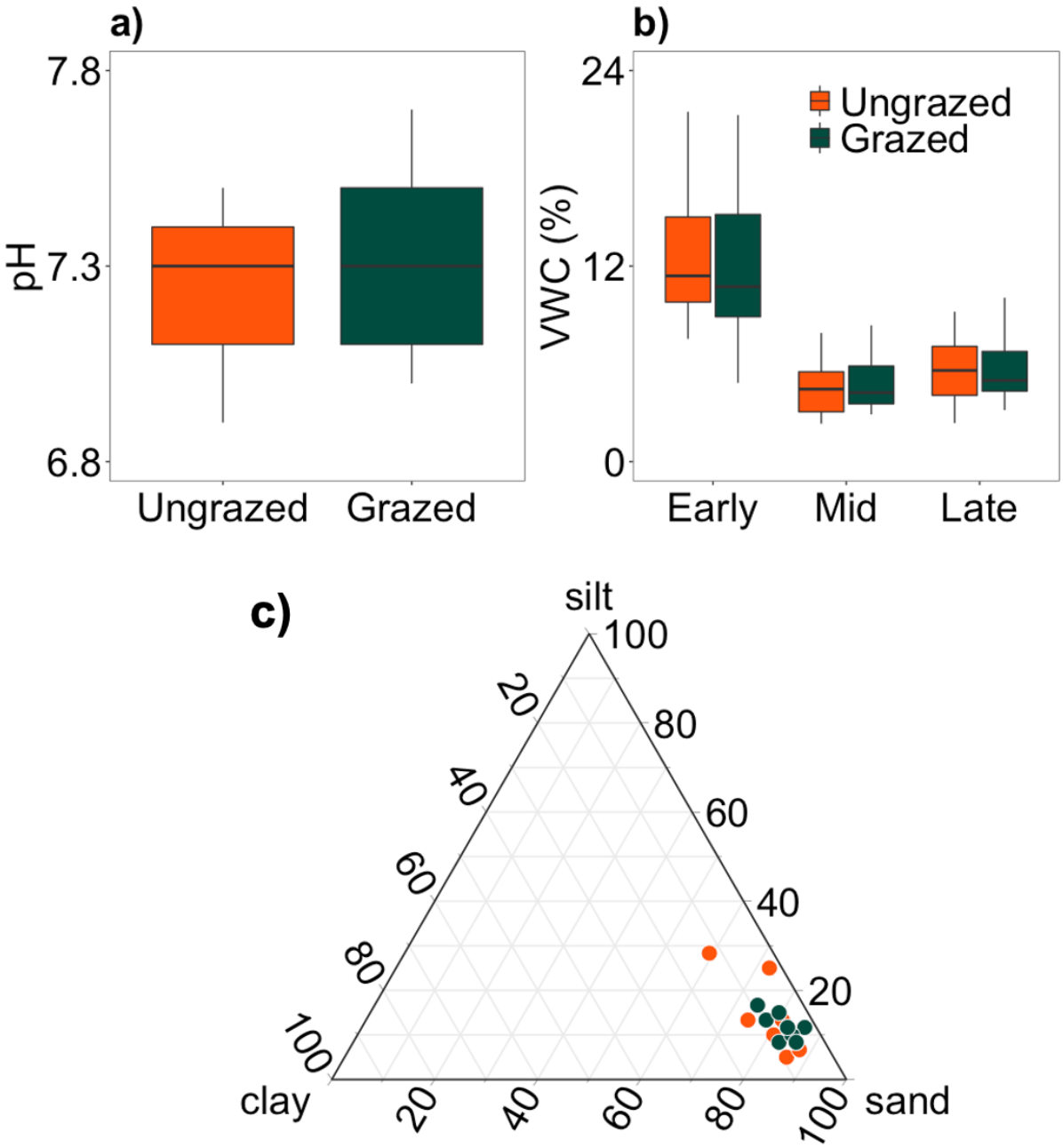

Figure A.3. Soil microbial communities at the phylum level (covering >=90% of the total abundance) and relative microbial functional profile (n=30 substrates) in the presence and absence of large mammalian grazers (n=10 paired plots) across the growing-season.

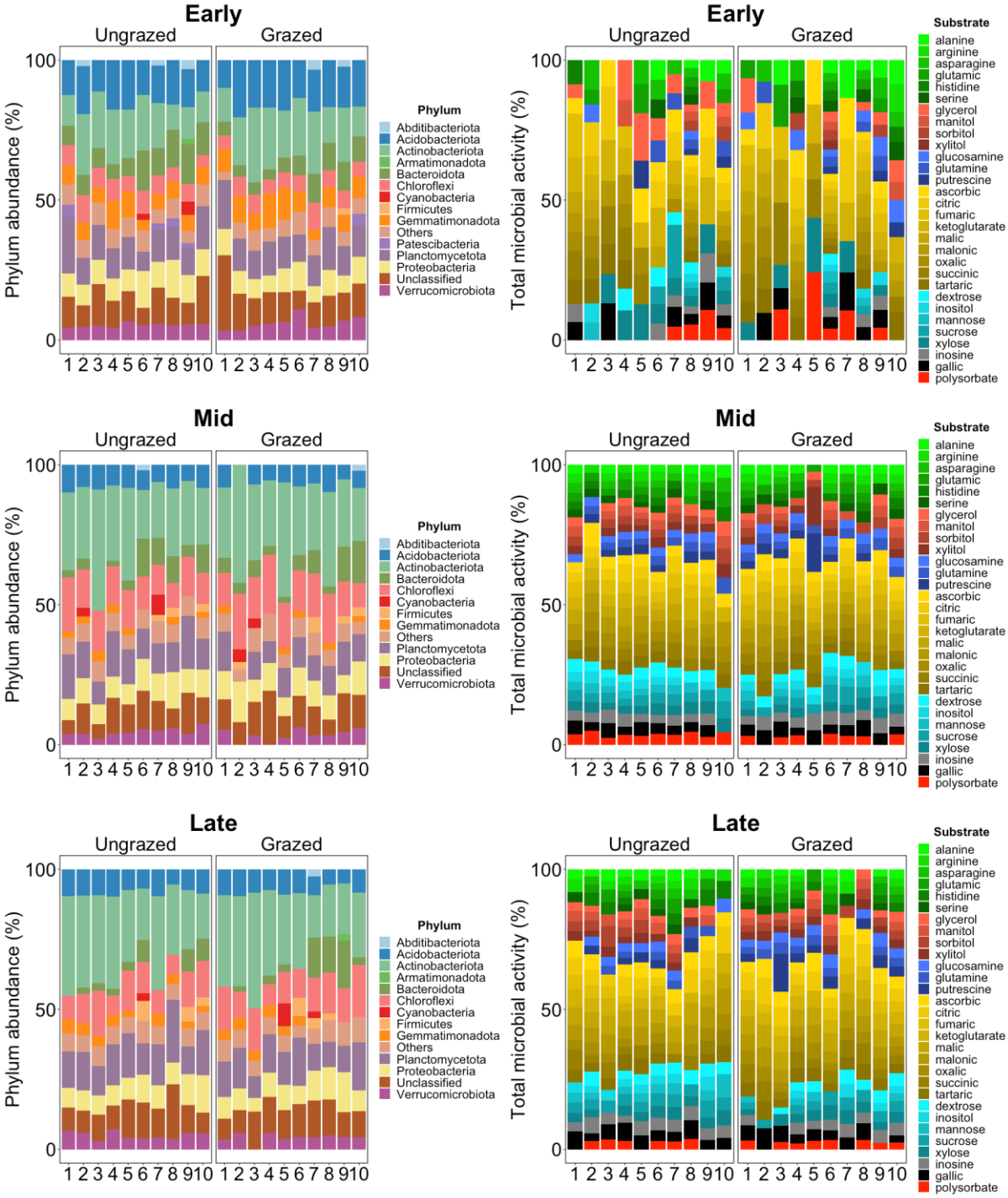

Figure A.4. dbSEM for all functions

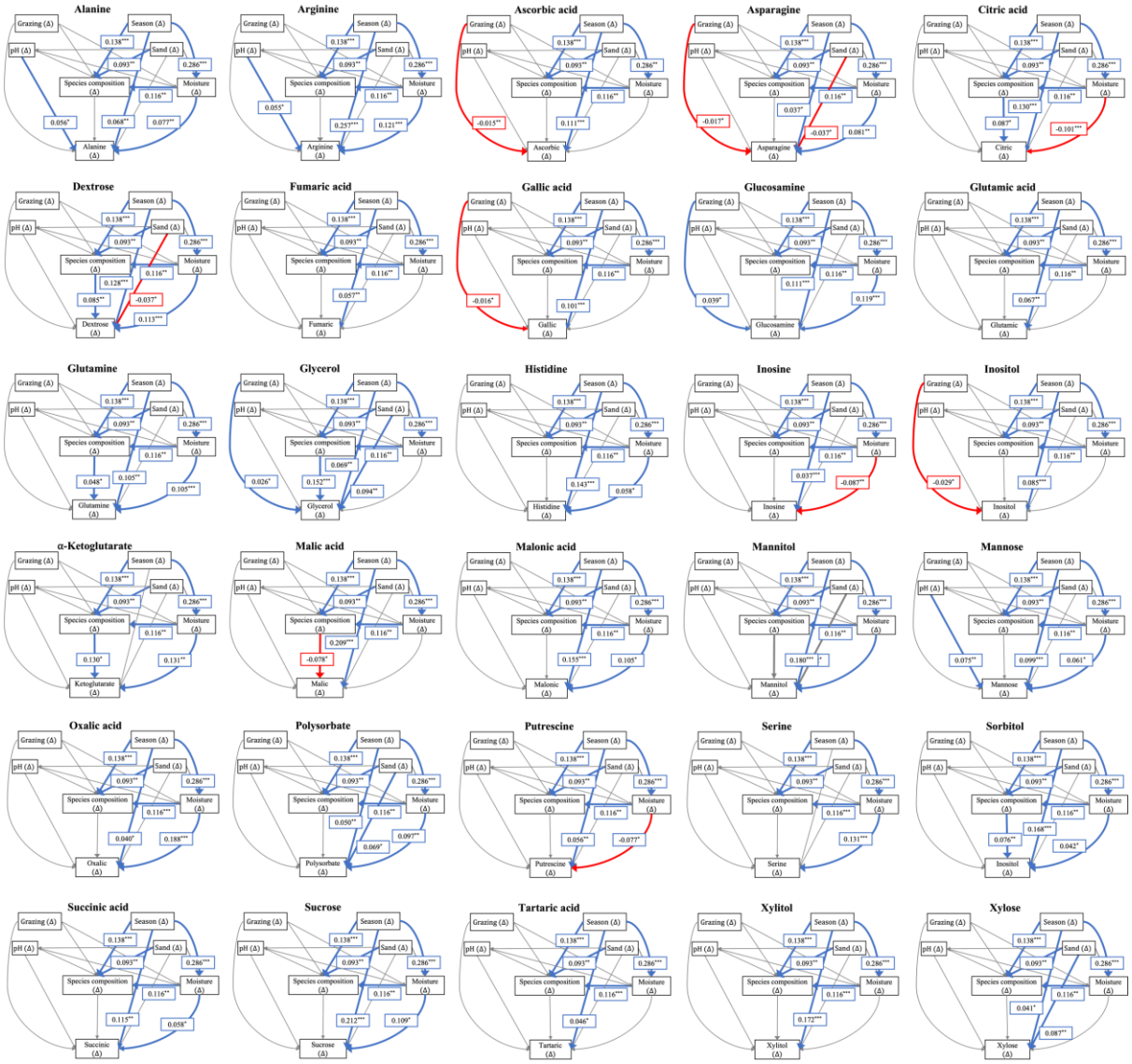

Figure A.5. Concept diagram to summarize hypothesized effects of microbial species richness on microbial functions in the presence (solid line) or absence (dashed line) of large mammalian grazers. Exclusion of grazers can alter the sensitivity of the functions to species diversity by either increasing or decreasing the slope (Sensitivity hypothesis; a). Alternatively, grazer-exclusion can alter the intercept (Magnitude hypothesis; c, d). Grazer-exclusion can also alter slope and magnitude of the relationship simultaneously (Sensitivity-Magnitude hypothesis; e).

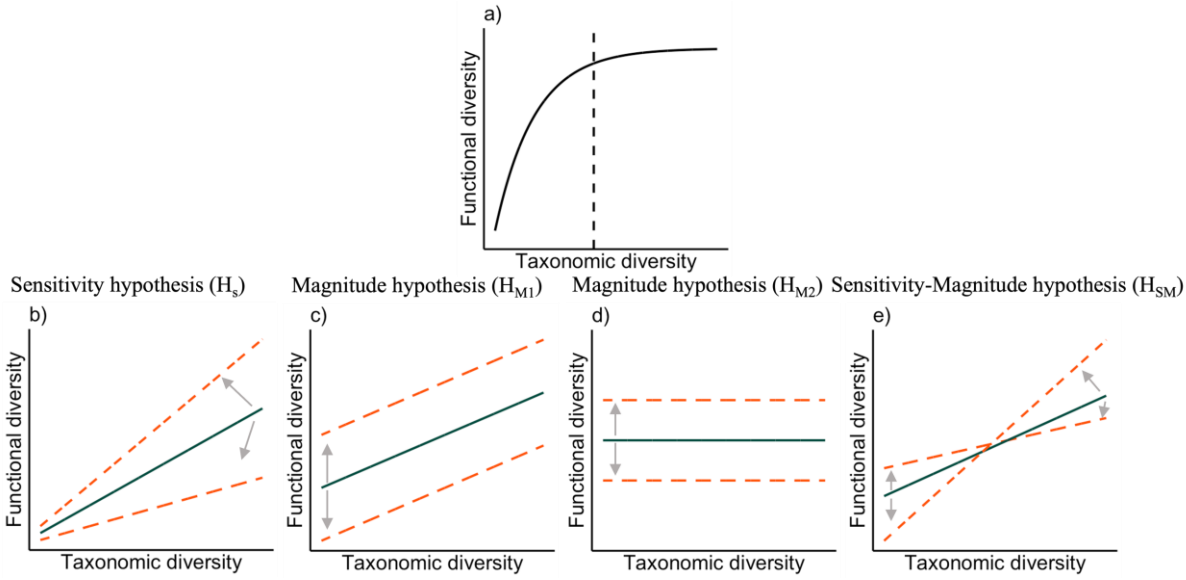

Figure A.6. Relationship between species richness and functional richness (a), functional dispersion (b), and multifunction (c) in the presence and absence of large mammalian grazers across the growing-season. Species richness was not related to microbial functions in both grazed and ungrazed plots across the growing-season. Units for 'Multifunction' was mg C g^{-1} soil $hour^{-1}$.

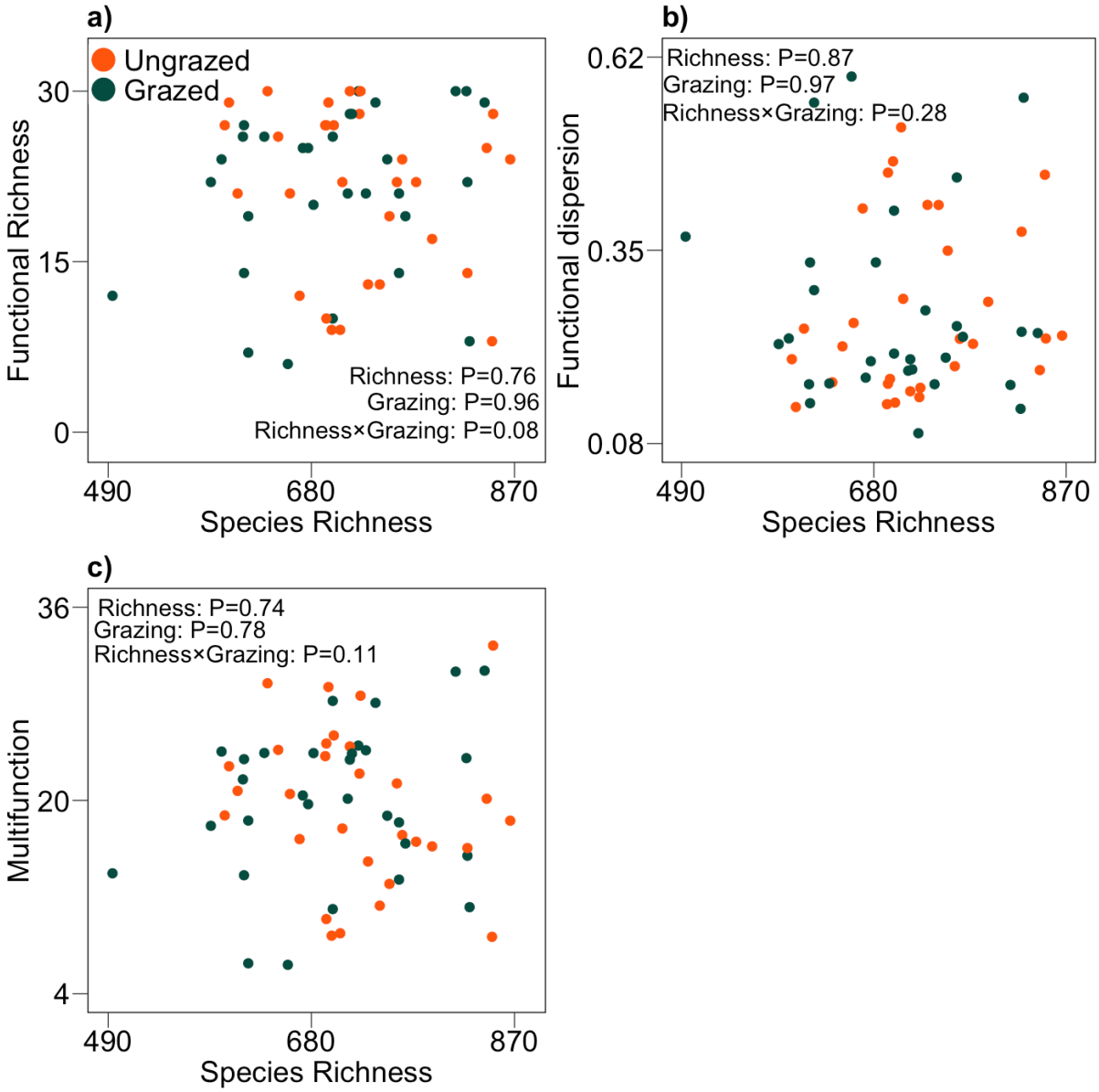

Table A.1. Best model for kriging using sum of squares (SSQ)

	Trend	Nugget	Exponential (SSQ)	Spherical (SSQ)	Gaussian (SSQ)
Functional richness	No	Fixed	52551.11	59125.98	59125.98
		Estimated	52745.91	51583.67	59125.98
	1st	Fixed	33433.22	39481.94	39481.94
		Estimated	33433.24	39481.94	39481.94
	2nd	Fixed	**29508.12**	34531.09	34531.09
		Estimated	29508.12	34531.09	34531.09
Functional dispersion	No	Fixed	0.007484251	0.007484251	0.007484251
		Estimated	0.007484251	0.007484251	0.007484251
	1st	Fixed	0.005320591	0.005320591	0.005320591
		Estimated	0.005320591	0.005320591	0.005320591
	2nd	Fixed	**0.004776054**	0.004776054	0.004776054
		Estimated	0.004776054	0.004776054	0.004776054
Multifunction	No	Fixed	40569.47	40569.47	40569.47
		Estimated	40569.47	40569.47	40569.47
	1st	Fixed	29273.22	29273.22	29273.22
		Estimated	29273.22	29273.22	29273.22
	2nd	Fixed	**22815.05**	22815.05	22815.05
		Estimated	22815.05	22815.05	22815.05

Table A.2. Summary of omnibus GLM model: Functions ~ Species richness * Grazing * Season

y-variable	x-variable	F-value	P-value
Functional richness	Species Richness	$F_{1,48}$=0.20	0.653
	Grazing	$F_{1,48}$=0.01	0.944
	Season	$F_{2,48}$=36.82	<0.001
	Taxonomy × Grazing	$F_{1,48}$=1.94	0.169
	Taxonomy × Season	$F_{2,48}$=1.25	0.296
	Grazing × Season	$F_{2,48}$=0.28	0.754
	Taxonomy × Grazing × Season	$F_{2,48}$=0.34	0.716
Functional dispersion	Species Richness	$F_{1,48}$=0.07	0.796
	Grazing	$F_{1,48}$=0.01	0.952
	Season	$F_{2,48}$=42.18	<0.001
	Taxonomy × Grazing	$F_{1,48}$=0.11	0.737
	Taxonomy × Season	$F_{2,48}$=0.27	0.767
	Grazing × Season	$F_{2,48}$=0.18	0.825
	Taxonomy × Grazing × Season	$F_{2,48}$=0.51	0.604
Multifunction	Species Richness	$F_{1,48}$=0.15	0.701
	Grazing	$F_{1,48}$=0.11	0.747
	Season	$F_{2,48}$=10.80	<0.001
	Taxonomy × Grazing	$F_{1,48}$=1.41	0.241
	Taxonomy × Season	$F_{2,48}$=2.19	0.122

	Grazing × Season	$F_{2,48}$=0.34	0.716
	Taxonomy × Grazing × Season	$F_{2,48}$=0.31	0.738

Table A.3. Summary of the GLM model comparison to test for alternative hypothesis (H_S, H_{M1}, H_{M2}, H_{SM}).

	Models	F-value	P-value	ΔAIC
Functional richness	1, 2	$F_{56,57}$=3.17	0.080	-1.31
	2, 3	$F_{57,58}$=0.09	0.760	1.90
	3, 4	$F_{58,59}$=0.01	>0.999	2
Functional dispersion	1, 2	$F_{56,57}$=1.19	0.28	0.74
	2, 3	$F_{57,58}$=0.03	0.873	1.97
	3, 4	$F_{58,59}$=0.01	0.948	1.99
Multifunction	1, 2	$F_{56,57}$=2.63	0.111	-0.75
	2, 3	$F_{57,58}$=0.14	0.711	1.85
	3, 4	$F_{58,59}$=0.05	0.822	1.95

Table A.4. Summary of omnibus GLM model for 30 functions: Functions ~ Species richness * Grazing * Season. R: Species richness, Grazing: Grazing, S: Season

Functions ↓	R		G		S		R:G		R:S		G:S		R:G:S	
	$F_{1,48}$	*P*	$F_{1,48}$	*P*	$F_{2,48}$	*P*	$F_{1,48}$	*P*	$F_{2,48}$	*P*	$F_{2,48}$	*P*	$F_{2,48}$	*P*
Alanine	0.01	0.924	1.72	0.195	0.07	0.931	**6.05**	**0.018**	2.29	0.112	2.1	0.133	0.23	0.792
Arginine	0.01	0.977	0.2	0.657	**15.31**	**0.001**	1.81	0.185	0.52	0.597	1.25	0.296	0.64	0.533
Ascorbic acid	0.34	0.562	0.08	0.779	**3.86**	**0.028**	2.71	0.106	0.05	0.954	1.37	0.263	2.39	0.102
Asparagine	0.01	0.951	0.07	0.793	0.69	0.505	1.01	0.32	0.86	0.431	0.1	0.906	0.77	0.471
Citric acid	0.05	0.825	0.16	0.688	**4.09**	**0.023**	0.47	0.497	0.96	0.39	0.51	0.607	1.91	0.159
Dextrose	1.5	0.226	1.81	0.185	**9.87**	**0.001**	0.97	0.33	1.98	0.149	0.01	0.997	2.43	0.099
Fumaric acid	1.57	0.217	0.17	0.685	1.35	0.269	0.79	0.379	0.45	0.638	0.71	0.495	0.91	0.408
Gallic acid	0.12	0.734	0.01	0.993	3.18	0.051	1.41	0.241	**3.34**	**0.044**	0.32	0.728	0.72	0.49
Glucosamine	0.77	0.384	**3.78**	**0.058**	2.68	0.079	0.46	0.499	0.2	0.816	1.35	0.27	0.52	0.596
Glutamic acid	0.01	0.912	0.32	0.575	0.61	0.547	1.04	0.314	1.1	0.341	0.53	0.593	0.07	0.933
Glutamine	0.27	0.603	0.62	0.435	2.44	0.098	0.01	0.946	**3.36**	**0.043**	0.08	0.92	0.85	0.435
Glycerol	0.92	0.343	**3.02**	**0.089**	1.23	0.3	0.66	0.421	0.21	0.815	0.93	0.403	0.08	0.924
Histidine	0.09	0.764	0.26	0.616	**3.86**	**0.028**	0.23	0.634	0.45	0.638	0.28	0.756	0.49	0.614
Inosine	0.01	0.982	0.35	0.555	**8.09**	**0.001**	3.2	0.08	0.74	0.48	1.13	0.332	0.57	0.569
Inositol	0.08	0.772	0.76	0.387	**5.37**	**0.008**	1.04	0.313	2.61	0.084	1.09	0.343	0.33	0.722
Ketoglutarate	1.21	0.277	0.12	0.725	0.79	0.462	0.79	0.38	0.49	0.615	0.83	0.441	0.65	0.526
Malic acid	0.45	0.505	2.35	0.132	**9.79**	**0.001**	3.56	0.065	1.22	0.304	1.42	0.251	0.29	0.746
Malonic acid	0.59	0.446	0.01	0.966	**11.14**	**0.001**	0.13	0.719	0.49	0.615	0.33	0.724	0.4	0.672
Mannitol	0.03	0.858	0.24	0.63	**6.65**	**0.003**	2.94	0.093	1.5	0.234	0.01	0.989	0.69	0.506
Mannose	**5.85**	**0.019**	1.67	0.202	**6.23**	**0.004**	0.06	0.815	0.64	0.531	2.19	0.123	0.23	0.792
Oxalic acid	0.08	0.778	0.4	0.53	0.91	0.409	0.77	0.384	0.12	0.89	0.8	0.456	0.47	0.63
Polysorbate	0.39	0.535	0.02	0.892	1.09	0.344	0.2	0.657	**4.35**	**0.018**	1.93	0.157	0.68	0.51
Putrescine	0.9	0.348	2.18	0.146	3.03	0.057	0.07	0.791	**4.5**	**0.016**	0.85	0.432	0.53	0.593
Serine	0.95	0.336	0.53	0.469	1.11	0.339	3.23	0.078	1.19	0.312	1.41	0.254	0.83	0.443
Sorbitol	0.02	0.898	0.56	0.459	**4.88**	**0.012**	0.82	0.37	0.12	0.891	0.18	0.835	0.38	0.688
Succinic acid	0.53	0.471	0.84	0.363	0.21	0.809	0.77	0.384	0.36	0.699	0.31	0.732	0.24	0.786
Sucrose	3.03	0.088	1.26	0.267	**6.72**	**0.003**	0.04	0.843	**3.23**	**0.048**	0.26	0.768	1.39	0.259
Tartaric acid	0.59	0.447	0.7	0.407	0.64	0.53	0.17	0.679	1.03	0.363	0.66	0.522	0.65	0.525
Xylitol	0.13	0.719	0.5	0.485	**9.14**	**0.001**	1.26	0.268	0.59	0.558	0.05	0.952	0.1	0.907
Xylose	0.11	0.742	1.43	0.238	0.25	0.78	0.42	0.521	1.04	0.362	0.63	0.538	0.12	0.89

Table A.5. Summary of the GLM model comparison to test for alternative hypotheses (H_S, H_{M1}, H_{M2}, H_{SM}) in individual functions. For few functions the GLMs were evaluated for each season separately due to a significant effect of season as identified from the omnibus model summarized in Table A.4.

Function	Season	Models	F-value	P-value	ΔAIC
Alanine		**1, 2**	**$F_{56,57}$=6.04**	**0.017**	**-4.15**
		2, 3	$F_{57,58}$=0.08	0.778	1.92
		3, 4	$F_{58,59}$=1.50	0.226	0.47
Arginine		1, 2	$F_{56,57}$=3.16	0.081	-1.29
		2, 3	$F_{57,58}$=0.01	0.973	1.99
		3, 4	$F_{58,59}$=0.13	0.715	1.86
Ascorbic acid		1, 2	$F_{56,57}$=1.34	0.252	0.58
		2, 3	$F_{57,58}$=0.33	0.566	1.65
		3, 4	$F_{58,59}$=0.03	0.861	1.96
Asparagine		1, 2	$F_{56,57}$=0.74	0.395	1.21
		2, 3	$F_{57,58}$=0.01	0.917	1.99
		3, 4	$F_{58,59}$=0.07	0.796	1.93
Citric acid		1, 2	$F_{56,57}$=0.11	0.740	1.88
		2, 3	$F_{57,58}$=0.07	0.790	1.92
		3, 4	$F_{58,59}$=0.12	0.729	1.87
Dextrose		1, 2	$F_{56,57}$=1.94	0.169	-0.04
		2, 3	$F_{57,58}$=0.74	0.394	1.23
		3, 4	$F_{58,59}$=1.70	0.197	0.26
Fumaric acid		1, 2	$F_{56,57}$=1.09	0.301	0.84
		2, 3	$F_{57,58}$=1.41	0.240	0.54
		3, 4	$F_{58,59}$=0.36	0.549	1.62
Gallic acid	Early	1, 2	$F_{16,17}$=0.09	0.773	1.89
		2, 3	$F_{17,18}$=3.67	0.072	-1.91
		3, 4	$F_{18,19}$=0.01	0.992	1.99
	Mid	1, 2	$F_{16,17}$=3.01	0.102	-1.45
		2, 3	$F_{17,18}$=1.69	0.211	0.10
		3, 4	$F_{18,19}$=0.15	0.699	1.83
	Late	1, 2	$F_{16,17}$=0.11	0.744	1.86
		2, 3	$F_{17,18}$=1.78	0.199	0.01
		3, 4	$F_{18,19}$=0.19	0.669	1.79

Glucosamine		1, 2	$F_{56,57}$=0.11	0.741	1.88
		2, 3	$F_{57,58}$=0.31	0.578	1.67
		3, 4	**$F_{58,59}$=4.22**	**0.045**	**-2.21**
Glutamic acid		1, 2	$F_{56,57}$=1.46	0.232	0.45
		2, 3	$F_{57,58}$=0.04	0.839	1.96
		3, 4	$F_{58,59}$=0.32	0.576	1.67
Glutamine	Early	1, 2	$F_{16,17}$=0.06	0.806	1.92
		2, 3	$F_{17,18}$=1.69	0.211	0.11
		3, 4	$F_{18,19}$=0.03	0.874	1.97
	Mid	1, 2	$F_{16,17}$=1.23	0.283	0.51
		2, 3	$F_{17,18}$=1.03	0.324	0.82
		3, 4	$F_{18,19}$=0.73	0.404	1.20
	Late	1, 2	$F_{16,17}$=1.55	0.231	0.15
		2, 3	$F_{17,18}$=3.88	0.065	-2.11
		3, 4	$F_{18,19}$=1.02	0.325	0.89
Glycerol		1, 2	$F_{56,57}$=0.35	0.554	1.62
		2, 3	$F_{57,58}$=1.58	0.215	0.36
		3, 4	$F_{58,59}$=2.59	0.113	-0.62
Histidine		1, 2	$F_{56,57}$=0.73	0.398	1.23
		2, 3	$F_{57,58}$=0.05	0.829	1.95
		3, 4	$F_{58,59}$=0.29	0.589	1.69
Inosine		**1, 2**	**$F_{56,57}$=4.66**	**0.035**	**-2.79**
		2, 3	$F_{57,58}$=0.01	0.950	1.99
		3, 4	$F_{58,59}$=0.28	0.599	1.71
Inositol		1, 2	$F_{56,57}$=1.71	0.196	0.19
		2, 3	$F_{57,58}$=0.02	0.890	1.98
		3, 4	$F_{58,59}$=0.70	0.405	1.28
α-Ketoglutarate		1, 2	$F_{56,57}$=1.06	0.301	0.87
		2, 3	$F_{57,58}$=1.37	0.247	0.58
		3, 4	$F_{58,59}$=0.03	0.856	1.97
Malic acid		**1, 2**	**$F_{56,57}$=4.35**	**0.041**	**-2.49**
		2, 3	$F_{57,58}$=0.13	0.716	1.86
		3, 4	$F_{58,59}$=1.97	0.1666	-0.002
Malonic acid		1, 2	$F_{56,57}$=0.08	0.773	1.91
		2, 3	$F_{57,58}$=0.459	0.501	1.52
		3, 4	$F_{58,59}$=0.01	0.945	1.99
Mannitol		**1, 2**	**$F_{56,57}$=4.37**	**0.041**	**-2.51**

		2, 3	$F_{57,58}$=0.01	0.926	1.99
		3, 4	$F_{58,59}$=0.217	0.643	1.78
Mannose		1, 2	$F_{56,57}$=0.44	0.508	1.53
		2, 3	**$F_{57,58}$=4.09**	**0.047**	**-2.16**
		3, 4	$F_{58,59}$=2.22	0.141	-0.25
Oxalic acid		1, 2	$F_{56,57}$=0.48	0.491	1.49
		2, 3	$F_{57,58}$=0.15	0.697	1.84
		3, 4	$F_{58,59}$=0.37	0.547	1.62
Polysorbate	Early	1, 2	$F_{16,17}$=0.07	0.798	1.91
		2, 3	$F_{17,18}$=2.41	0.139	-0.65
		3, 4	$F_{18,19}$=0.15	0.699	1.83
	Mid	1, 2	$F_{16,17}$=0.02	0.886	1.97
		2, 3	$F_{17,18}$=0.69	0.417	1.20
		3, 4	**$F_{18,19}$=5.18**	**0.035**	**-3.06**
	Late	1, 2	$F_{16,17}$=3.69	0.073	-2.15
		2, 3	**$F_{17,18}$=12.32**	**0.003**	**-8.90**
		3, 4	$F_{18,19}$=0.48	0.495	1.47
Putrescine	Early	1, 2	$F_{16,17}$=0.69	0.416	1.15
		2, 3	$F_{17,18}$=0.47	0.504	1.46
		3, 4	$F_{18,19}$=0.03	0.875	1.97
	Mid	1, 2	$F_{16,17}$=0.01	0.947	1.99
		2, 3	$F_{17,18}$=0.09	0.767	1.89
		3, 4	$F_{18,19}$=0.43	0.520	1.53
	Late	1, 2	$F_{16,17}$=0.79	0.388	1.04
		2, 3	**$F_{17,18}$=8.55**	**0.009**	**-6.14**
		3, 4	$F_{18,19}$=3.14	0.093	-1.22
Serine		1, 2	$F_{56,57}$=3.75	0.058	-1.89
		2, 3	$F_{57,58}$=1.09	0.301	0.86
		3, 4	$F_{58,59}$=31	0.582	1.68
Sorbitol		1, 2	$F_{56,57}$=1.76	0.190	0.14
		2, 3	$F_{57,58}$=0.06	0.813	1.94
		3, 4	$F_{58,59}$=0.49	0.484	1.49
Succinic acid		1, 2	$F_{56,57}$=1.04	0.312	0.89
		2, 3	$F_{57,58}$=0.83	0.367	1.14
		3, 4	$F_{58,59}$=0.71	0.404	1.27
Sucrose	Early	1, 2	$F_{16,17}$=0.79	0.386	1.03
		2, 3	$F_{17,18}$=3.25	0.089	-1.50

		3, 4	$F_{18,19}$=1.22	0.284	0.69
	Mid	1, 2	$F_{16,17}$=0.05	0.832	1.94
		2, 3	$F_{17,18}$=1.63	0.218	0.16
		3, 4	$F_{18,19}$=0.07	0.796	1.92
	Late	1, 2	$F_{16,17}$=2.41	0.140	-0.81
		2, 3	$F_{17,18}$=0.21	0.652	1.75
		3, 4	$F_{18,19}$=1.37	0.26	0.53
Tartaric acid		1, 2	$F_{56,57}$=0.33	0.571	1.65
		2, 3	$F_{57,58}$=0.83	0.366	1.13
		3, 4	$F_{58,59}$=0.53	0.471	1.46
Xylitol		1, 2	$F_{56,57}$=2.53	0.117	-0.66
		2, 3	$F_{57,58}$=0.05	0.822	1.95
		3, 4	$F_{58,59}$=0.47	0.494	1.51
Xylose		1, 2	$F_{56,57}$=0.39	0.535	1.58
		2, 3	$F_{57,58}$=0.02	0.884	1.98
		3, 4	$F_{58,59}$=1.68	0.200	0.29

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