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Original Articles

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ABSTRACT

Grasslands represent key functional ecosystems due to their global contribution to macronutrients cycling and their role as reservoirs of microbial diversity. The strategic importance of these habitats rests on their involvement in carbon and nitrogen fluxes from the atmosphere to the soil, while at the same time offering extensive sites for livestock rearing. In this study the management type, differentiated in pasture or meadow, was investigated as a variable for its possible effects on overall bacterial diversity and specific genes related to functional guilds. Its contribution was compared to that of other variables such as region, soil pH, and soil organic carbon, to rank their respective hierarchies in shaping microbial community structure. A latitudinal gradient across the European continent was studied, with three sampling groups located in Norway, France, and Northern Italy. The applied methods involved 16S DNA metabarcoding for taxonomic classification and determination of the relative abundance of the bacterial component, and quantitative PCR for the genetic determinants of bacterial and archaeal nitrification, intermediate or terminal denitrification, and nitrogen fixation. Results indicated that soil pH exerted the dominant role, affecting high taxonomy ranks and functions, along with organic carbon and region, with whom it partly covaried. In contrast, management type had no significant influence on microbial community structure and quantitative counts of functional genes. This suggests an ecological equivalence between the impacts of pasture and meadow practices, which are both perturbations that share the aspect of vegetation withdrawal by browsing or cutting, respectively.

1. Introduction

Grassland ecosystems cover over 37 % of the planet's terrestrial areas (Zhong et al., 2015; Bai and Cotrufo, 2022) and represent 70 % of global agricultural land (Mencel et al., 2022), with 34 % of Europe's agricultural area being comprised of grasslands (Schils et al., 2022). Grasslands contribute to approximately 34 % of terrestrial organic carbon stock, storing carbon as root biomass and soil organic carbon (SOC) (Bai and Cotrufo, 2022). They play a fundamental role in conserving biodiversity and landscape diversity, offering multiple ecosystem services such as carbon storage, erosion control, and water management (Burczyk et al., 2018; Schils et al., 2022). Moreover, grasslands are vital for livestock systems as they provide feed for ruminants and other herbivores, serving as meadows for hay/silage production or as pastures for animal grazing (Bunce et al., 2004; Mencel et al., 2022). Due to their significance as ecosystems and their role in low-intensity agriculture, grasslands are considered High Nature Value (HNV) farmland in Europe (Lomba et al., 2014). Despite their importance, grasslands remain poorly understood regarding their soil microbial communities and the potential impacts of different human uses, such as pastures or meadows, on these communities. Various grassland management practices can transform these ecosystems into either sinks or sources of greenhouse gases, such as

methane (CH_4) or nitrous oxide (N_2O), with implications for climate change related to processes directly mediated by the microbial community (Chadwick et al., 2018; Chang et al., 2021). Soil microbial communities are the primary drivers of biogeochemical cycles (Crowther et al. 2019), including those of nitrogen and carbon, through enzyme-catalyzed reactions within metabolic pathways (Cavicchioli et al., 2019; Dong et al., 2020; Louca et al., 2018; Mencel et al., 2022; Rocca et al., 2015; Zhou et al., 2012). Diversity and functional redundancy within microbial communities are essential for sustaining ecological functions and resilience in biogeochemical processes (Louca et al., 2018; Maron et al., 2018). Therefore, microbial communities can be considered as functional groups in which species richness is less relevant than specific phenotypic traits for specific reactions, which define the functional richness of ecosystem processes (Bahram et al. 2018; Louca et al. 2018; Moonen and Bàrberi, 2008). In grassland ecosystems, the composition of microbial communities is influenced by pedological conditions, such as pH (Tripathi et al., 2018; Yang et al., 2022a,b) and organic carbon content (Smith et al. 2021), and local disturbances, such as animal grazing (Wang et al., 2022), mowing (Mencel et al., 2022), and fertilization (Goulding and de Varennes, 2016; Liu et al., 2014), which alter soil conditions. Among pedological factors, pH (Fierer, 2017; Bahram et al., 2018), moisture (Li et al. 2017), soil organic carbon (Tripathi et al., 2018; Yang et al., 2022a,b), and nitrogen (N) content (Kuypers et al., 2018) are considered the most important drivers of microbial community structure. pH can directly affect microbial enzyme activity (Luan et al. 2023; Yang et al., 2022a,b) and indirectly affect microbial community structure by altering other significant factors, such as plant cover (Lammel et al. 2018). Soil moisture determines the saturation level, causing more anaerobic conditions, which can limit the activity of soil microbial activities (Li et al. 2017). Organic Carbon and Nitrogen are essential nutrients for microorganisms, so variation in their content can change microbial diversity and activity (Bahram et al., 2018; Fierer, 2017). Meanwhile, in terms of local disturbance, different intensities of animal grazing can directly and indirectly affect soil conditions through trampling-induced asphyxia (Mencel et al., 2022; Wang et al., 2022; Yin et al., 2020), selective removal of vegetation (Mencel et al., 2022; Wang et al., 2022), and deposition of urine and feces (Du et al., 2019; Wang et al., 2022). Animal trampling can induce soil compaction, altering oxygen concentrations and soil water potential, thereby directly affecting microbial composition (Chroňáková et al., 2009; Mencel et al., 2022; Wang et al., 2022; Yin et al., 2020). Vegetation browsing by animal grazing can change its composition as regards primary production, litter, and root exudates, which are part of the C inputs to the soil, and these changes have direct effects on microbial communities (Mencel et al., 2022; Mueller et al., 2017; Qu et al., 2016; Wang et al., 2022). Additionally, the deposition of animal excreta directly increases the content and availability of nutrients, such as carbon and nitrogen, in the soil (Kohler et al., 2005; Wang et al., 2022). However, the microbial communities of grasslands have shown diverse responses to grazing: one study found variations in microbial β-diversity (community composition) but no changes in α-diversity (species richness within a community) (Qin et al., 2021), another study observed a significant decrease in microbial diversity with an increase in grazing intensity (Yang et al., 2023). Moreover, mowing and fertilization practices can induce changes in soil microbial communities in grasslands used as meadows (Cui et al. 2020; Wang et al. 2021). Mowing can partially simulate plant consumption that would occur during grazing, inducing the stimulation of root exudation and shifts in N and C cycles (Mencel et al., 2022). Liming or fertilization using animal dung or chemical compounds can also alter nutrient content and availability, thus affecting soil conditions such as pH (Goulding and de Varennes, 2016; Liu et al., 2014; Schroder et al., 2011). Variations of nutrient profile and pH can change the relative abundances of bacterial phyla, such as Proteobacteria, Firmicutes, and Bacteroidetes changing soil microbial communities (Stoian et al. 2022). As a result, similarities between local disturbances exerted by animals or humans, along with

common pedoclimatic conditions due to spatial proximity, can lead to similar microbial communities in grasslands used as pastures or meadows. However, certain fractions of microbial communities may resist local disturbances with respect to their taxonomic and functional profiles. Several studies have investigated the effects of management type and pedological factors on soil microbial communities within limited geographic areas (Degrune et al. 2019; Su et al. 2023). Thus, the relative importance of geographic patterns, different management types, and pedological factors in driving grassland soil microbial communities, both in terms of structure and functions, remains poorly understood. Starting from these considerations, we compared the microbial communities of multiple grasslands managed by several different farms, dominated by either pasture or meadow use, and fertilized with animal dung. The comparison was conducted across three European countries (France, Italy, and Norway) in terms of taxonomic and functional profiles and pools of specific genes, combining two molecular methods, 16S rDNA metabarcoding and qPCR analysis, to gain a comprehensive view of the associated microbial communities. 16S rDNA metabarcoding is a sequencing-based method that provides information about the taxonomic composition and diversity of microbial communities (Beckers et al. 2016), while qPCR is a quantitative technique used to measure the abundance of specific microbial taxa or genes (Smith and Osborn 2009). The aim of these analyses was to verify: 1) a presence of similar microbial communities in pastures and meadows across different latitudes in terms of both taxonomic and functional profiles, in relation to the common aspect of being fertilized with animal dung. We wished to verify whether this fertilization could contribute to a homogenization of soil microbial assemblages between pastures and meadows upon the delivery of dung-carried microbiota, which have a certain degree of conserved taxa in man-managed herbivores' guts; 2) the hierarchical rank and relevance of soil chemical drivers (pH and organic carbon) in comparison to the management type in determining microbial community structure and its functional traits in grasslands. Specifically, we expected pH and organic carbon to have a more significant effect on microbial communities due to their direct effects on physiology and metabolism, thus indirectly leading to effects of the management type through variations in pedological features.

The innovative aspects of the present approach in comparison to existing studies are: (a) the focus on a direct comparison between pastures and meadows, and (b) targeting a set of reporter genes spanning across the whole nitrogen cycle from N_2 fixation to N_2 reemission as bio-indicators of the ecosystem services integrity.

Understanding the structure and main environmental drivers of soil microbial communities in agricultural context can help to interpret ongoing phenomena to foster the preservation and enhancement of the ecosystem services of pastures and meadows. This knowledge lays the groundwork to identify indicators to assess the extent of sustainable equilibria in pastures and meadows. The ensuing results provide insights for management improvement, offering a clearer view of the hitherto overlooked importance of microbial communities in maintaining and enhancing ecosystem services in the grassland environment.

2. Materials and methods

2.1. Site location

This study was part of the European Project "Highlands.3" and involved 16 farms in three European mountain areas with a historical presence of agricultural systems (Fig. 1): the Massif Central in France (FR – 6 farms), the Alpago-Cansiglio in Italy (IT – 5 farms), and the Vestvågøy area in Norway (NR – 5 farms). For each farm, representative permanent pastures and meadows were sampled for a total of 34 areas (FR: 6 meadows and 6 pastures; IT: 5 meadows and 5 pastures; NR: 5 meadows and 7 pastures). Pastures coincided with areas used only for livestock grazing, while most meadows were managed with one or two cuts, to produce hay and/or silage during summer, and grazed for short
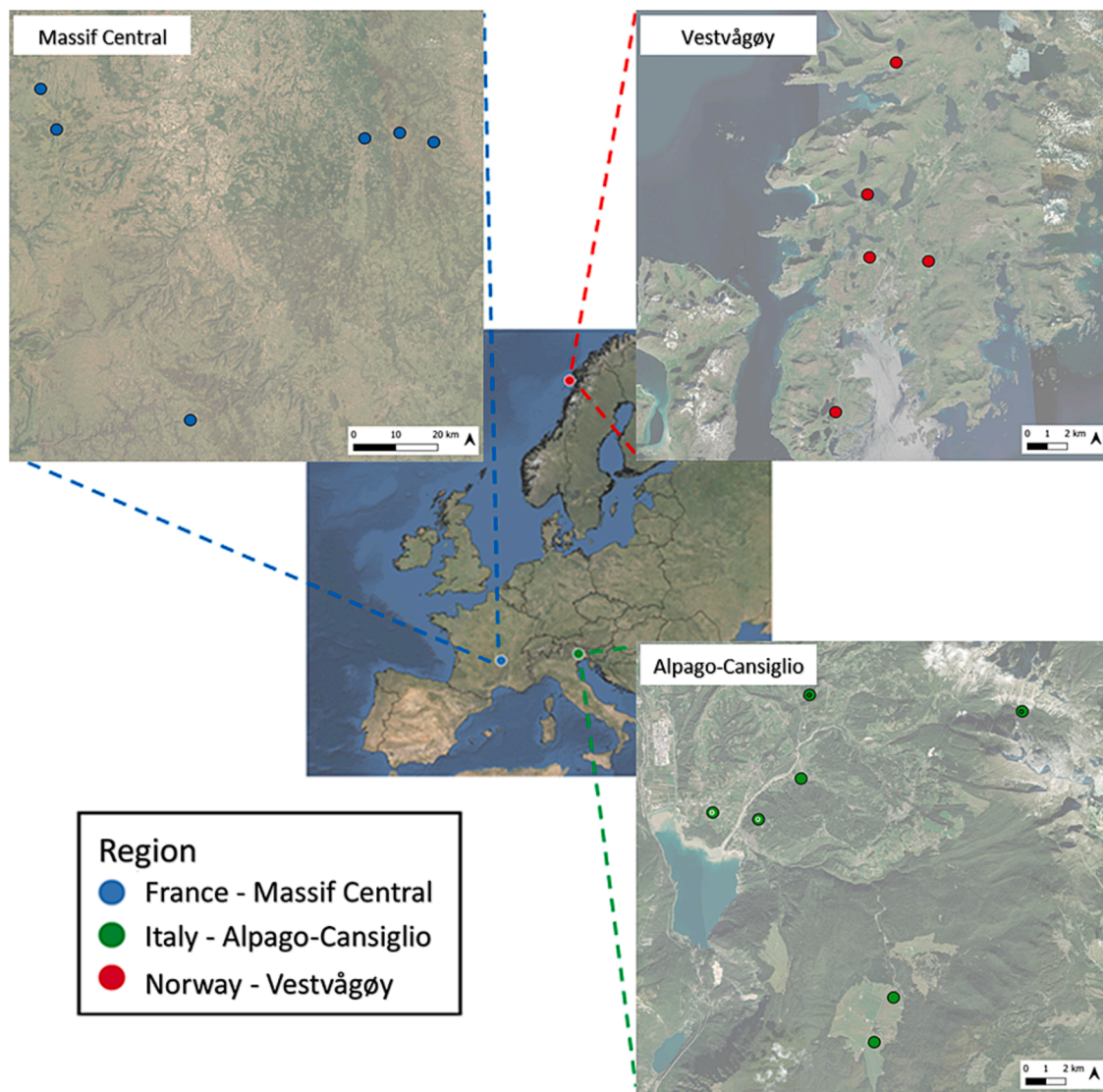
Fig. 1. Area locations: Sampling points in France (Massif Central) are marked in blue, in Italy (Alpago-Cansiglio) in green, and in Norway (Vestvågøy) in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

periods in autumn. All selected pastures and meadows were fertilized by animal dung without inorganic fertilizers, except for three meadow areas in Norway where farmers used both fertilizer types. For each area, 12 topsoil samples (the top 15 cm of soil) were taken randomly and then used to form a representative bulk per area, whose a part was used for the chemical analyses and another for the molecular ones. Soil samples were air-dried before being pooled and subsequently analysed.

The three highland regions presented different extensions and pedoclimatic conditions according to the FAO soil map (https://www.fao.org/soils-portal/soil-survey/soil-maps-and-databases/faounesco-soil-map-of-the-world/en/) and the Köppen climate classification (Rubel and Kottek, 2010). The largest area, with the consequent highest number of farms, was the north of the Massif Central, located in the centre of France. This region is characterised by a temperate climate and features andosols. The Italian region is in the eastern Alps and presents an alpine climate and eutric cambisols. Instead, the Norwegian region is in the Lofoten archipelago, above the Arctic Circle, and is characterised by a subartic climate, mitigated by the presence of the ocean (Uleber et al., 2014), and orthic podzols. Sampling took place during July 2021, at which time the three regions (countries) featured different temperatures and rainfall levels (France: mean temperature = 16.0 ± 1.1 °C, mean rainfall = 2.2 ± 0.5 mm; Italy: mean temperature = 17.8 ± 0.7 °C, mean

rainfall = 2.5 ± 0.1 mm; Norway: mean temperature = 11.3 ± 0.2 °C, mean rainfall = 1.7 ± 0.07 mm - Muñoz Sabater, 2019).

2.2. Chemical and molecular analyses

Soil chemical analyses included quantification of pH by soil suspension in water (ISO 10390) and organic C assessment by high-temperature dry combustion (ISO 10694).

Total soil DNA was extracted from an amount of 0.25 g of dried soil using the Qiagen DNeasy PowerSoil kit (Qiagen, Germany) as described by the manufacturer's protocol. The DNA extracted was quantified with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, US-CA) using the Qubit™ DNA HS Assay Kit (Thermo Fisher Scientific, US-CA) and stored at −20 °C.

The extracted DNA was sequenced upon 16S rDNA metabarcoding on an Illumina MiSeq sequencer in the paired-end 2x300 bp format by BMR Genomics s.r.l., Padova, Italy, targeting the V4 region using the following universal primers: Modified 515f: GTGYCAGCMGCCGCGGTAA, (Parada et al., 2015), and Modified 806r: GGACTACNVGGGTWTCTAAT (Apprill et al., 2015).

RealTime qPCR was performed using a QuantStudio 5 system (Thermo Fisher Scientific, US-CA). The volume of the qPCR reaction was

equal to 5 μL, 1 μL of template DNA, and 4 μL of reaction mix, composed of 1.2 μL PCR-grade water, 0.15 μL each of the F and R primers (Table 1) and 2.5 μL Power SYBR Green PCR Master Mix containing Taq polymerase (Thermo Fisher Scientific, US-CA). The thermal cycle conditions were set to a pre-denaturation stage at 95° C for 10 min, followed by 40 cycles with a denaturation step at 95° C for 15 sec, an annealing step at 57° C for 60 sec and an extension at 72° C for 60 sec. For each amplification, a negative control of ultra-pure PCR-grade water was analyzed in triplicates. The Ct threshold cycles were transformed into gene copies using the equation of Dong et al. (2020). The undetermined Ct (cycle threshold) was set 40 to equal 0 genes' copies by the transformation.

The bioinformatics processing of the sequencing data was based on QIIME (Caporaso et al., 2010) using the similarity threshold of 97 % to cluster the single ASV (Amplicon Sequence Variant - Callahan et al., 2017). The ASV profiles were analysed using FAPROTAX 1.2.4 (Louca et al., 2016) to extract functional profiles, which correspond to lists of ecological functions performed by the taxa identified. The DNA sequences have been deposited in the GenBank repository SRA Archive, under the project code PRJEB56444, https://www.ebi.ac.uk/ena/browser/view/PRJEB56444.

2.3. Statistical analysis

Statistical analyses were performed in R 4.2.0 (R Core Team, 2016).

We preliminarily verified that pH and C org were not correlated (N = 34; r = -0.21; p-value > 0.05). Then, to detect possible nested features of the data, we analysed pH and C org as a function of the main effects and the two-way interaction of region (France – FR, Italy – IT, Norway – NR) and management type (meadow – M or pasture – P) through a permutation ANOVA using the 'aovp' function of the "lmperm" library 'lmperm' (Bates et al., 2015). The results indicated a highly significant effect of the region on pH (p < 0.001; Supplementary Table S1). Therefore, in the subsequent analyses, we included C org and pH as explanatory variables in the same models without considering their 2-way interaction due to the absence of all possible combinations (pH class "1" – C org class "1": NA; pH class "3" – C org class "3": NA). For these analyses, we categorized pH and C org values into four classes based on quartiles: (pH class: 4.5<"1"≤5.4; 5.4<"2" ≤5.8; 5.8<"3" ≤6.2; 6.2<"4" ≤7.6 – C org class: 3 % <"1"≤4.7 %; 4.7 %<"2" ≤7.5 %; 7.5 %<"3" ≤10.1 %; 10.1 %<"4" ≤35.9 %).

2.4. Diversity indices of microbial communities

We calculated the Shannon and Simpson alpha diversity indices (Jost, 2007) and the Pielou evenness index (Pielou, 1966) for the microbial communities of each sample at the ASV and phylum levels using the 'vegan' library (Dixon, 2003). To detect the effects of regional, management type and soil characteristics on the diversity, we analysed the indices using ANOVA with permutation test (5000 permutations) with the 'aovp' function of the library 'lmperm' (Bates et al., 2015) with two models. The first model included the main effects of the region (FR, IT, NR) and management type (M or P) and their 2-way interaction. The second model included the main effects of pH and C org class. Finally, we calculate Kendall's rank correlations of the percentage of ASVs assigned by FAPROTAX ("perc. ASVs assigned") and the number of functions detected ("n.functions") with pH, C org, alpha diversity indices, and Pielou indices at the ASV and phylum levels, and the numbers of both ASVs and phyla.

2.5. Taxonomic and functional profiles of microbial communities

Firstly, we used Venn diagrams (Shade and Handelsman, 2012) to identify the suites of ASVs, phyla, and functions shared by the microbial communities defined by region, management type, pH, and C org. Venn diagrams were constructed with the 'venn.diagram' function of the 'VennDiagram' package (Chen and Boutros, 2011). Then, we calculated the dissimilarity matrices, based on the Bray-Curtis distance, of the taxonomic and functional profiles to allow multivariate approaches (Anderson and Walsh, 2013) to the analysis of taxonomic compositions and ecological functions within and between microbial communities. We analysed dissimilarity matrices with ANOSIM (Analysis of Similarity – Clarke, 1993) with the 'anosim' function of the 'vegan' library (Dixon, 2003), PERMANOVA (Permutational Multivariate Analysis of Variance – Anderson, 2001) with the 'adonis2' function of the "vegan" library, and the Mantel test (Mantel, 1967) with the 'mantel' function of the "vegan" library. In all the analyses, we used 9999 permutations.

In the ANOSIM, we tested in separate models whether the similarity (expressed as rank distances) between the levels of each factor (region, three levels: FR, IT, NR; management type, two levels: M and P; pH class, four class: 4.5–5.4; 5.4–5.8; 5.8–6.2; 6.2–7.6; C org class, four classes: 3 %–4.7 %; 4.7 %–7.5 %; 7.5 %–10.1 %; 10.1 %–35.9 %) was greater than within the levels. The ANOSIM provides for each factor an R statistic constrained between −1 and +1, where a value close to 0 indicates a strong similarity between levels, while the positive/negative limit indicates a strong dissimilarity. For the factors whose levels differed significantly, we used NMDS (Nonmetric Multidimensional Scaling) to plot a 2D representation of the similarities (Kenkel and Orlóci, 1986; Kruskal, 1964). The 'goodness of fit' of each NMDS was verified by extracting the 'stress value', which should be below 0.2 (Dexter et al., 2018). We then identified the ASV, phyla, and functions associated with each factor level with an indicator species analysis using the 'multipatt' function of the 'indicspecies' library (De Cáceres and Legendre, 2009). This analysis allows us to measure the association of each factor's level with each phylum, ASV, and function through an Indicator Value (stat) constrained from 0 to 1, where higher values correspond to stronger associations (Dufrêne and Legendre, 1997).

In the PERMANOVA, we tested whether the variability, expressed as a spatial distance, differed between factors. We used two models, one testing the main effects of region, management type and their 2-way interaction, and the other testing the main effects of pH and C org. We used the 'betadisper' function of the "vegan" library to test the homogeneity of factor dispersion, in terms of beta diversity (Anderson et al.,

Table 1
List of primers used for qPCR with associated functions and references.

Primer	Function	Sequence	Amplicon length	References
16S F	–	GGGTTGCGCTCGTTGC	60 bp	Johnson et al. (2016)
16S R	–	ATGGYTGTCGTCAGCTCGTG		
Archaeal amoA - AOA F	Ammonia oxidation	STAATGGTCTGGCTTAGACG	635 bp	Francis et al. (2005)
Archaeal amoA - AOA R	Ammonia oxidation	GCGGCCATCCATCTGTATGT		
Bacterial amoA - AOB F	Ammonia oxidation	GGGGTTTCTACTGGTGGT	500 bp	Rotthauwe et al. (1997)
Bacterial amoA - AOB R	Ammonia oxidation	CCCCTCKGSAAAGCCTTCTTC		
nifH F	Nitrogen-fixation	AAAGGYGGWATCGGYAARTCCACCAC	432 bp	Rösch et al. (2002)
nifH R	Nitrogen-fixation	TTGTTSGCSGCRTACATSGCCATCAT		
nosZ F	Nitrous Oxide reduction	CGYTGTTCMTCGACAGCCAG	706 bp	Rösch et al. (2002)
nosZ R	Nitrous Oxide reduction	CATGTGCAGNGCRTGGCAGAA		
nirK F	Nitrite reduction	ATYGGCGGVCAYGGCAG	160 bp	Henry et al. (2004)
nirK R	Nitrite reduction	RGCCTCGATCAGRTTRTGGTT		

2006). When the dispersion was significantly heterogeneous for a factor, we analysed the distances of the samples from the centroid of their factor using a one-way ANOVA to assess the dispersion within factors.

For the Mantel test (Mantel, 1967), we calculated the dissimilarity matrices on Euclidean distances for pH and C org and assessed the Spearman correlations with taxonomic and functional dissimilarities.

2.6. Functional profiles of genes

To compare the potentials of specific functions of microbial communities, we analysed the abundances of qPCR gene copies (log-transformed) first with Kendall's correlations between genes and then with an ANOVA based on permutation tests, using the 'lmp' function from the 'lmPerm' library (Wheeler and Torchiano, 2010). We used two models, one including the main effects of the region (FR, IT, NR), management type (M or P), and their 2-way interaction, and the other including the main effects of pH and C org class. Moreover, to assess possible niche differences between archaeal and bacterial amoA genes (AOA and AOB, respectively), we calculated the ratio between AOB and the total ammonia-oxidation guild (AOA + AOB). The ratio was analysed with a generalised additive model based on beta distribution and log link function, using the function 'gam' of the package 'mgcv' (Wood, 2017). We used the two same models as described above.

3. Results

3.1. Dominant taxa and functions

The 16S metabarcoding analysis generated 8,059,837 paired-end reads, with an average of 98,291 ± 21,214 reads per sample. A total of 2,846 different ASVs were identified, with 0.6 % remaining unassigned. On average, each sample contained 134 ± 33 ASVs, with 4 ± 1 % being unassigned. The identified ASVs were classified into 830 taxa. Most of the annotated sequences were classified at the phylum (94.9 %), class (93.9 %), order (92.7 %), family (90.1 %), and genus (81.7 %) levels. The most abundant phyla were Firmicutes (31.5 ± 12.6 %), Proteobacteria (20.6 ± 5.8 %), and Actinobacteriota (13.9 ± 6.6 %) (Fig. 3 A). Function prediction tool FAPROTAX assigned an average of 24 ± 5 % ASVs per sample, inferring 42 functions in total, with an average of 13 ± 4 functions per sample. The most abundant functions were chemoheterotrophy (28.4 ± 7.6 %), aerobic chemoheterotrophy (24.5 ± 5.8 %), aerobic ammonia oxidation (10.3 ± 8.5 %), and Nitrification (10.3 ± 8.5 % - Fig. 3 B).

3.2. Alpha-Diversity and evenness indices

The ANOVA on the permutation test of the alpha-diversity and evenness indices revealed a significant effect of the region only at the ASV level, with no significant effect of management type at both ASV and phylum levels (Supplementary Material Table S2). Regarding
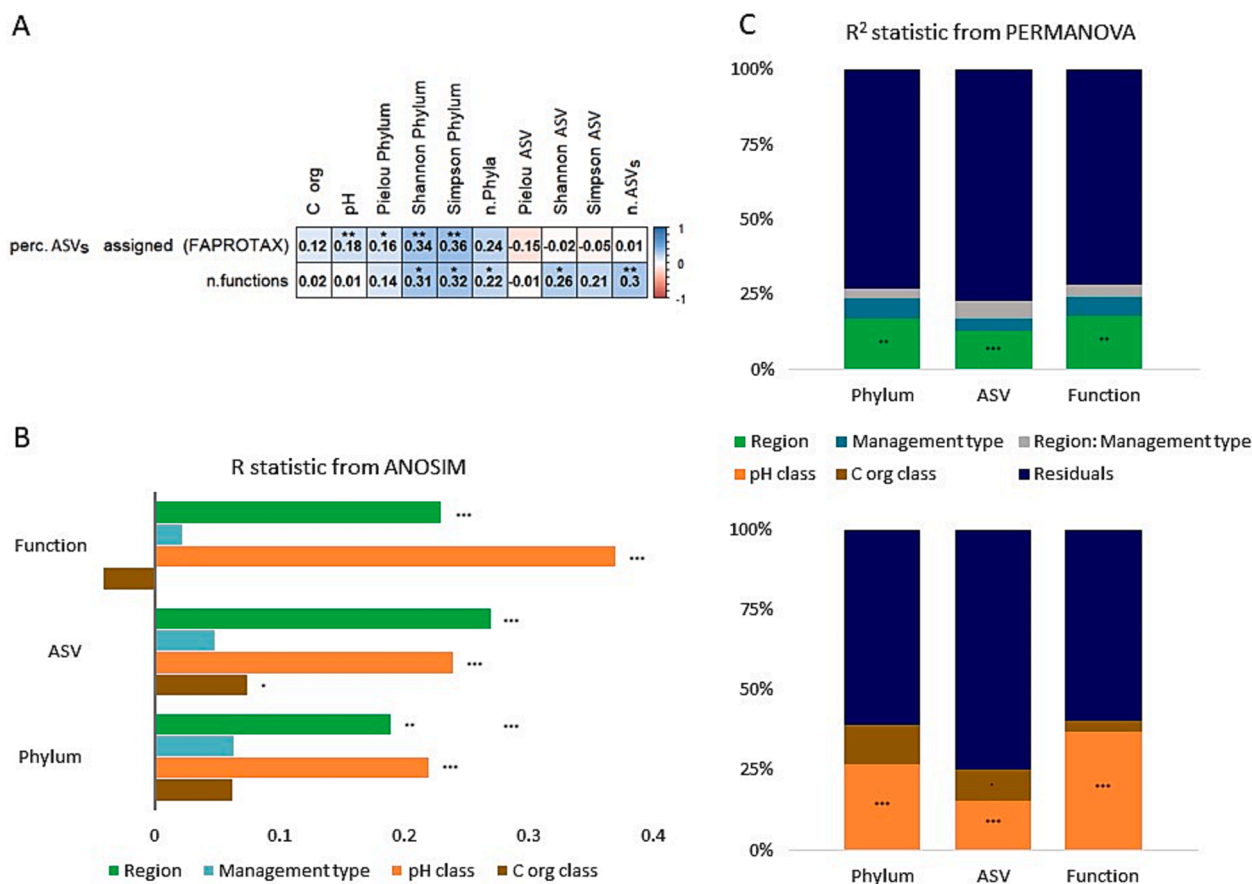
Fig. 2. Panel A: plot of Kendall rank correlations between the percentage of ASVs assigned by FAPROTAX (perc.ASVs assigned) or the number of functions (n. functions), with pH, C org, index of alpha-diversity and evenness at phylum and ASV rank level, number of phyla and ASVs. Panel B: barplot of R statistics from ANOSIM based on ASV, Phylum and Function Bray-Curtsi dissimilarity matrix, as a function of "region", "management type", "pH class" and "C org class". Panel C: R^2 statistics from two PERMANOVA models (first model: region*management type - first row; second model: pH class + C org class – second row) based on ASV, Phylum and Function Bray-Curtis dissimilarity matrix. Significant effects are reported in table S3 and S6 and are represented by: * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).
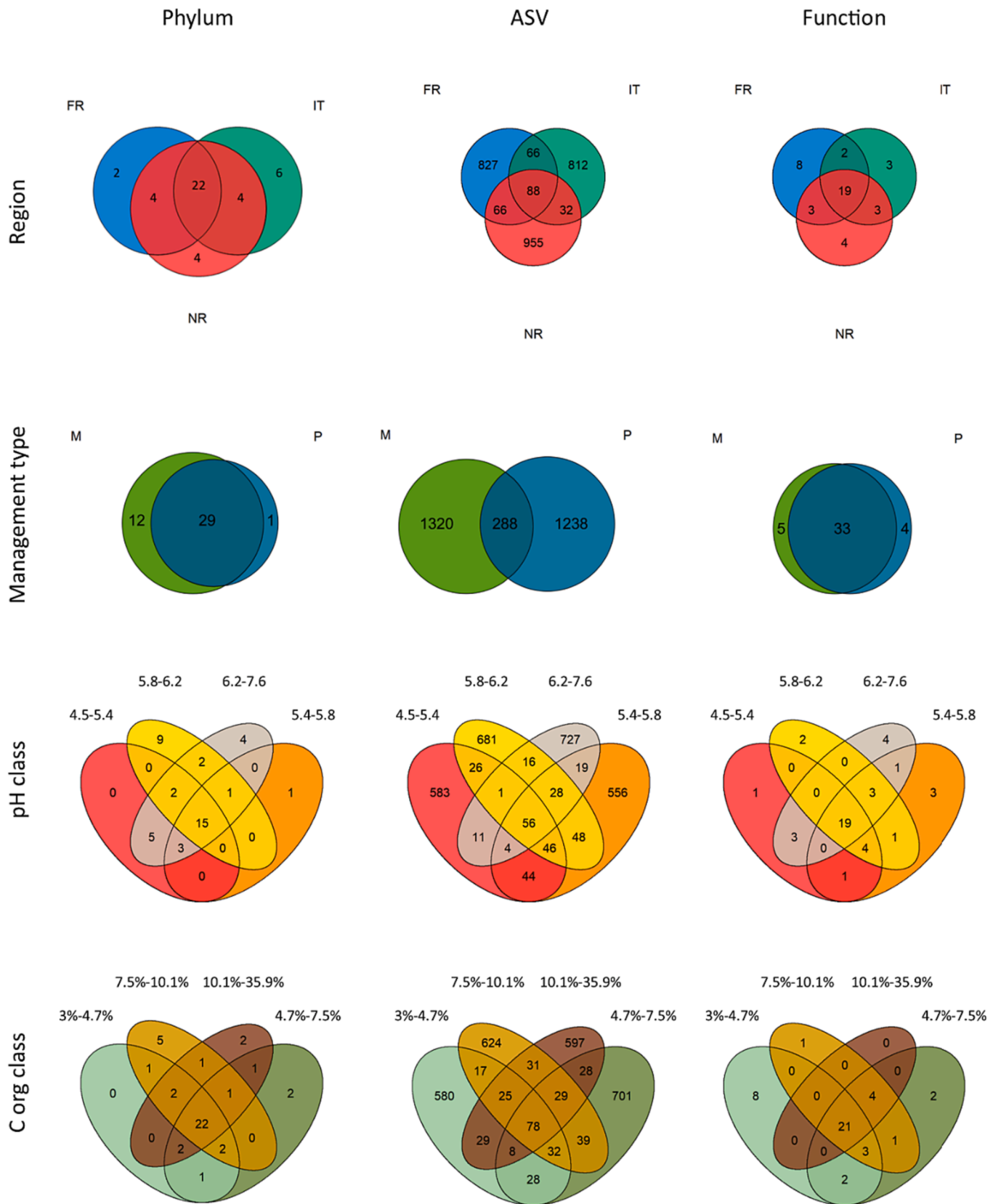
Fig. 3. Venn diagrams of phyla, ASVs and function distinguished by region (row A – "FR" France, "IT" Italy and "NR" Norway), pH class (row B), C org class (row C) and management type (row D – "M" meadow and "P" pasture). The pH class and the C org class present four levels in the quartiles.
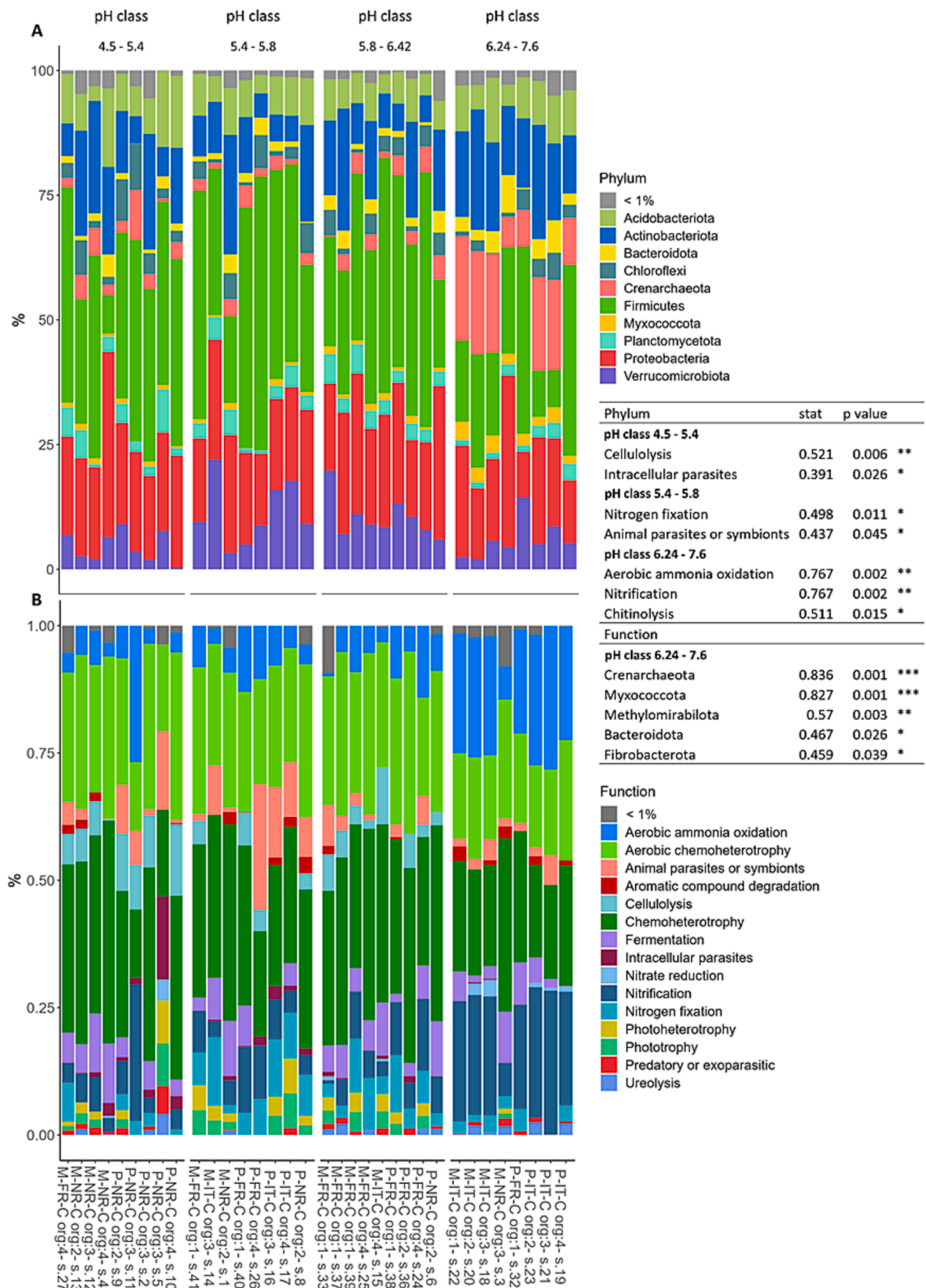
Fig. 4. Barplot chart of the taxonomy results for single samples at phylum rank level (panel A) and functions identified by FAPROTAX (panel B), distinguished by pH class and ordered by management type, region and organic C class. Each sample is labelled reporting the management type (M or P), region (FR, IT, NR), and the C org class (1, 2, 3, 4) followed by the sample number. The phyla characterized by relative abundance below 1 % were pooled as a single group. The pH class and the C org class present 4 levels in the quartiles (C org class: 3 %<"1"≤4.7 %; 4.7 %<"2"≤7.5 %; 7.5 %<"3" ≤10.1 %; 10.1 %<"4"≤35.9 %). The phyla and functions for pH class were reported.

pedological conditions, significant differences were observed with respect to pH and C org classes at the phylum level (Supplementary Material Table S2). Alpha diversity indices of phyla were significantly and positively correlated with the pH class (Shannon of phylum: p-value < 0.01; Simpson of phylum: p-value < 0.05) and significantly and negatively correlated with the C org class (Shannon of phylum: p-value < 0.05; Simpson of phylum: p-value < 0.05 - Supplementary Material Table S2). The Pielou index, indicating evenness, was significantly affected by the region at the ASV level (p < 0.05 - Supplementary Material Table S2) and by the pH class at both phylum and ASV levels (phylum: p-value < 0.05; ASV: p-value < 0.01- Supplementary Material Table S2).

The analysis of correlations with the percentage of ASVs assigned to a known function by FAPROTAX or with the n. of functions revealed significant and positive relations for alpha-diversity indices at the phylum rank level (Shannon Phyla - perc.ASV assigned: $\tau = 0.34$, p-value < 0.01; Simpson Phyla – perc.ASVs assigned: $\tau = 0.36$, p-value < 0.01; Shannon Phyla – n.functions: $\tau = 0.31$, p-value < 0.05; Simpson Phyla – n.functions: $\tau = 0.32$, p-value < 0.05; Fig. 2 A). The percentage of ASVs assigned by FAPROTAX was significantly and positively correlated with pH ($\tau = 0.18$ – p-value < 0.01; Fig. 2 A) and Pielou index at the phylum level ($\tau = 0.16$ – p-value < 0.05; Fig. 2 A), while the number of functions was significantly and positively correlated with the number of phyla ($\tau = 0.22$ – p-value < 0.05; Fig. 2 A), Shannon index at the ASV rank level ($\tau = 0.26$ – p-value < 0.05; Fig. 2 A), and the number of ASVs ($\tau = 0.3$ – p-value < 0.01; Fig. 2 A).

3.3. Taxonomic profiles

The Venn diagrams of taxonomic profiles (Fig. 3) revealed different patterns between the phylum and ASV levels concerning management type, region, pH class, and C org class. The phylum level presented more

Region ● FR ● IT ● NR

Phylum	stat	p value	
FR			
Firmicutes	0.423	0.021	*
IT			
Crenarchaeota	0.569	0.004	**
Myxococcota	0.541	0.004	**
Methylomirabilota	0.491	0.005	**
Fibrobacterota	0.394	0.043	*
NR			
Dependentiae	0.547	0.002	**
Patescibacteria	0.508	0.006	**
Proteobacteria	0.429	0.022	*
RCP2.54	0.382	0.047	*

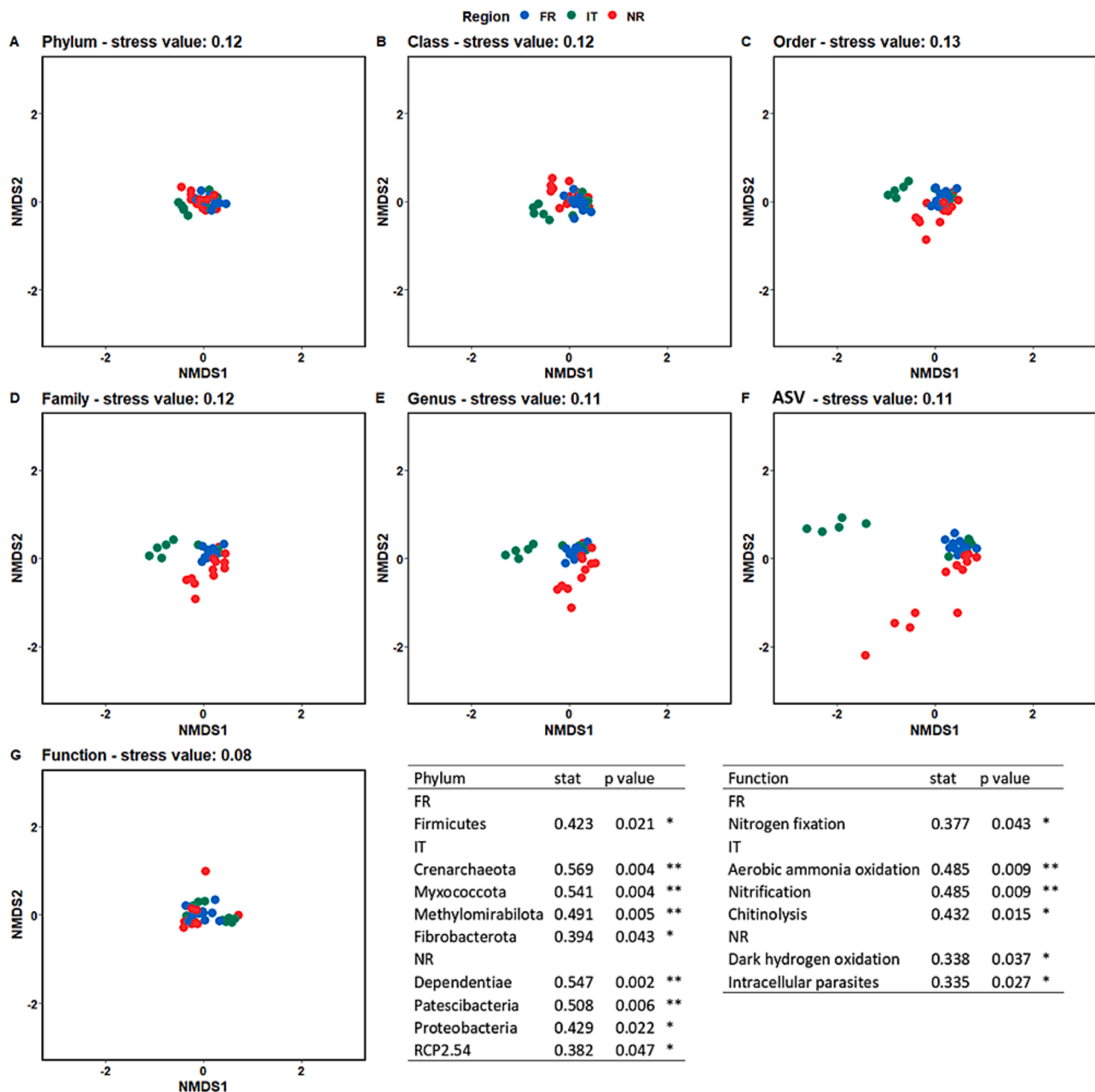| Function	stat	p value	
FR			
Nitrogen fixation	0.377	0.043	*
IT			
Aerobic ammonia oxidation	0.485	0.009	**
Nitrification	0.485	0.009	**
Chitinolysis	0.432	0.015	*
NR			
Dark hydrogen oxidation	0.338	0.037	*
Intracellular parasites	0.335	0.027	*

Fig. 5. NMDS (Non-metric Multidimensional Scaling) of taxonomic profiles at different rank levels and functions (panel A-G), and the most associated phyla and functions (panel H) with respect to the region. The stress value (goodness of ordination) is reported for each rank level.

abundant shared cores for all factors considered than the ASV level, which was characterized by more abundant cores for single-factor levels. The ANOSIM (Fig. 2 B; Supplementary Materials Table S3) highlighted significant differences in the microbial taxonomy profiles at both the ASV and phylum levels, with greater dissimilarity for the former than the latter. The region significantly explained the differences between the samples at both the ASV and phylum levels (ASV region: p-value < 0.001 – R = 0.27; phylum region: p-value < 0.001 – R = 0.19), while the management type did not have significant effects at all considered (Supplementary Materials Table S3). Regarding soil characteristics, pH significantly affected both ASV and phylum levels (ASV pH: p-value < 0.001 – R = 0.24; phylum pH: p-value < 0.001 – R = 0.22), while organic C significantly affected only at the ASV level (ASV organic C: p-value < 0.05- R = 0.075). The stress value of NMDS based on two dimensions was rather constant from phylum to ASV level, passing from 0.11 to 0.13 (Fig. 5, Supplementary Materials Fig. S2). The PERMANOVA analysis of the ASV profiles confirmed the ANOSIM results (Fig. 2 C), showing a significant effect of the region (p-value < 0.01) and the absence of an effect of the management type. Despite these differences, there were no effects of the interaction (Supplementary Materials Table S6). Regarding soil characteristics, PERMANOVA confirmed a stronger effect of the pH class (p-value < 0.01) than the effect of the C org class (p-value < 0.05) to explain the variance of the ASV profiles. In particular, pairwise comparisons of PERMANOVA at the ASV level revealed the strongest differences between FR and NR (p-value < 0.001), pH classes 1 and 4 (p-value < 0.001), pH classes 3 and 4 (p-value < 0.001), and organic C class 1 and 4 (p-value < 0.001 - Supplementary Materials Table S6). The dispersion analysis revealed heterogeneous communities as a function of the region (p-value < 0.01), showing significant differences in beta diversity. France presented significant differences in the distances from the centroid compared to Italy and Norway (Supplementary Materials Fig. S4 and Table S7). The PERMANOVA analysis of the phyla profiles also confirmed in part the results of the ANOSIM, showing a strong significant effect of the region (p-value < 0.01) and pH class (p-value < 0.001) but without an effect of management type, the 2-way interaction, and C org class (Supplementary Materials Table S3). The dispersion analysis confirmed the presence of heterogeneous communities according to the region (p-value < 0.01). As for the ASV, France presented significant differences in terms of distance from the centroids compared to Italy and Norway (Supplementary Materials Fig. S4 and Table S7). The Mantel test confirmed the significant effect of pH on the levels of ASV and phylum with positive linear trends (ASV: p-value < 0.001 – r = 0.53; phylum: p-value < 0.001 – r = 0.45) but not for the C org, which presented positive but weak trends (ASV: r = 0.039; phylum: r = 0.054 - Fig. 2 B; Supplementary Materials Fig. S6 and Table S8).

The Indicator Species Analysis of differentially featured taxa revealed significant different associations in terms of ASV and phylum abundances among the three countries (Fig. 3 and Fig. 4). In particular, France showed significant associations for 29 ASVs, Italy for 25 and Norway for 14, while in terms of phyla, France presented 1 significant association, Italy and Norway both presented 4 significant associations. Regarding the management type, these taxonomic differences involved only 13 different ASVs, 10 of which were enhanced in the pasture cases and 3 in the meadow, while in terms of phyla, there was only 1 case for the meadow (Supplementary Materials Table S4 and Table S5). The Indicator Species Analysis of differentially featured taxa in relation to the variables also confirmed that pH explained more dissimilarities between taxonomic levels and their abundances (53 ASVs, 5 phyla) than organic C (24 ASVs, 2 phyla), where the class 4 of pH presented the most dissimilar taxonomic profiles with the greatest number of associations (Supplementary Materials Table S4 and S5). At the ASV level, pH class 1 presented 8 associated ASVs, class 2 had 5 ASVs, class 3 13 and class 4 27 (Supplementary Materials Table S4 and S5), while at phylum level, there were significant phyla only at class 4 with 5 associated phyla (Fig. 3). Organic Carbon significantly affected the ASVs occurrences and

abundances, particularly in class 1 with 12 significant ASVs and in class 4 with 11 (Supplementary Materials Table S4 and S5).

3.4. Functional profiles

The Venn diagram of function profiles (Fig. 3) reflected the patterns found at the phylum level, with a high number of units shared between levels of management type, region, pH class, and C org class. The functional profiles reflected the significant differences found at the phylum level through the ANOSIM, PERMANOVA, and Mantel test (Fig. 2 B, 2C, 4 and 5; Supplementary Materials Figures S2, S4, and Tables S3, S6, S8). The most significant factor for the functional profile was the pH class (ANOSIM p-value < 0.001 – R = 0.37; PERMANOVA p-value < 0.001; Mantel test p-value < 0.001 – r = 0.43 - Fig. 2 B), followed by the region (ANOSIM p-value < 0.001 – R = 0.23; PERMANOVA p-value < 0.01). The interaction between region and management type was not significant in terms of variance (PERMANOVA p-value > 0.05). No effects of management type and C org were detected for the functional profiles (Supplementary Materials Table S6). A pairwise comparison of PERMANOVA revealed the strongest differences between FR and IT, and NR and IT (p-value < 0.05), and between pH class 4 and 1, 2 and 3 (p-value < 0.01). The dispersion test detected a homogeneous distribution of samples around the centroids for all factors. The NMDS of function profiles presented a good fit according to the stress value equal to 0.08 (Fig. 5).

The Indicator Species Analysis of differentially featured functions in relation to the variables also confirmed that region and pH explained the greatest dissimilarities between functional profiles and their abundances (region: 6 associated functions; pH class: 7 associated functions), while no associations were detected in functions of the C org class (0 associated functions - Supplementary Materials Table S4 and S5). In terms of the region, IT presented 3 associated functions, NR 2, and FR 1, while in terms of pH classes, classes 1 and 2 presented 2 associated functions, and class 4 presented 3 associated functions (Supplementary Materials Table S4 and S5). As a function of management type, only 3 functions were significantly associated with meadow, while 0 with pasture (Supplementary Materials Table S4 and S5).

3.5. Gene indicators

Real-time PCR-quantified gene copies for nitrogen-cycling reactions showed different correlations and patterns (Fig. 6 A; Supplementary Materials Fig. S5 and Tables S9): *nosZ*, *nifH*, AOA *amoA*, and AOB *amoA* showed significant and positive correlations between them (*nosZ-nifH*: τ = 0.30 – p-value < 0.01, *nosZ*-AOA *amoA* a: τ = 0.5 – p-value < 0.001, *nosZ*-AOB *amoA*: τ = 0.25 – p-value < 0.001, AOB *amoA-nifH*: τ = 0.16 – p-value < 0.01), while AOA *amoA* and AOB *amoA* were significantly correlated only between them (AOA *amoA*-AOB *amoA*: τ = 0.17 – p-value < 0.05).

ANOVA of gene copies revealed a significant effect of the region for almost all genes (*nosZ*: p-value < 0.05, *nirK*: p-value < 0.05, *nifH* < 0.05, AOA *amoA*: p-value = 0.05), except for 16S and AOB *amoA* (Fig. 6 B; Supplementary Materials Fig. S5 and Table S9). No significant effects of management type and the 2-way interaction were detected (Fig. 6 B; Supplementary Materials Fig. S5 and Table S9). The ANOVA showed a generally stronger effect of the C org class than the pH class on gene copies, except for *nirK*, which was more shaped by pH (Fig. 6 B; Supplementary Material Fig. S5 and Table S9). The two pedological variables significantly affected the *nosZ* (pH class: p-value < 0.05; C org class: p-value < 0.05) and AOA (pH class: p-value ~ 0.05; C org class: p-value < 0.05), while *nirK* was only affected by pH (p-value < 0.01- Fig. 6 B; Supplementary Material Fig. S5 and Table S9). In general, the pH class and the C org class presented opposite trends, where for the former the trend tended to be positive while for the latter it tended to be negative (Supplementary Materials Fig. S5). No effects of both pH and C org class on 16S were found (Fig. 6 B; Supplementary Material Fig. S5 and
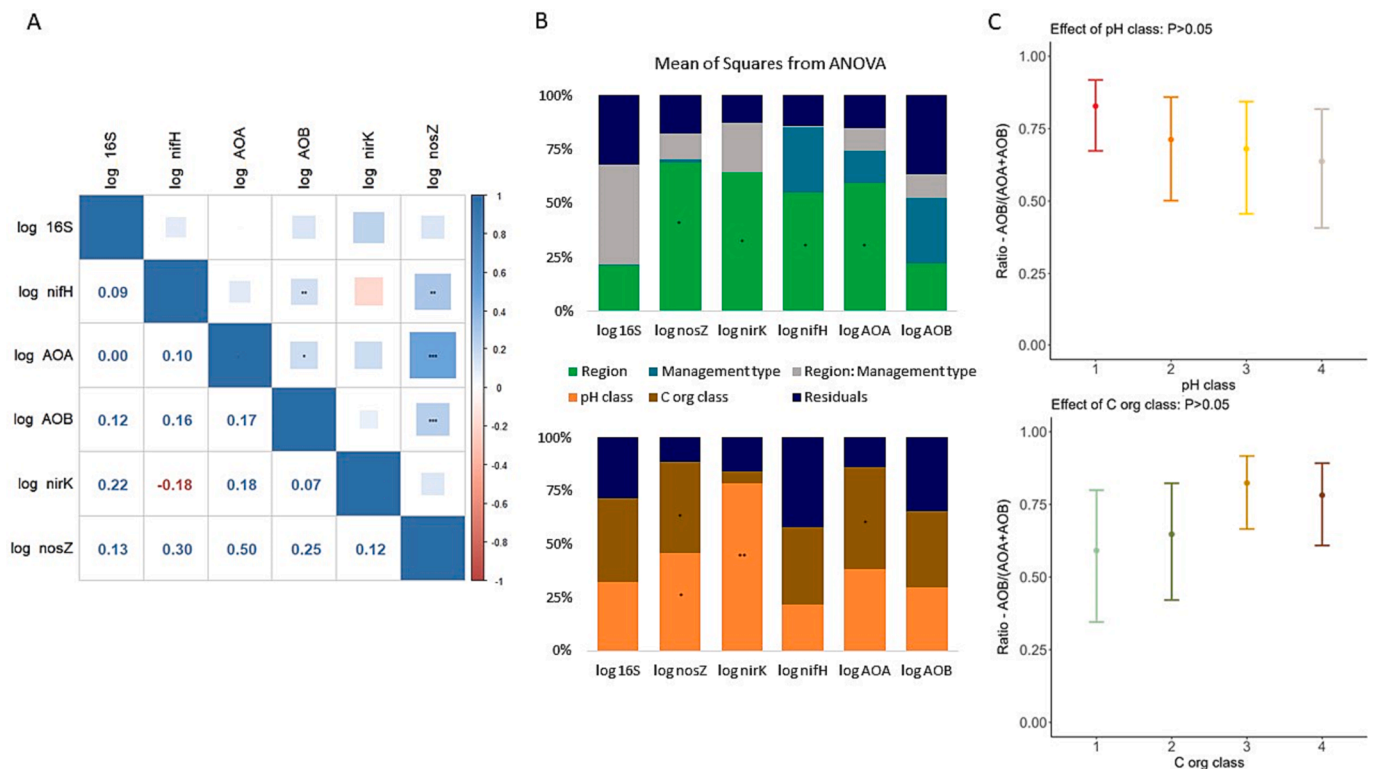
Fig. 5. Panel A: Kendall rank correlations among log-transformed gene abundances (16S, *nifH*, AOA *amoA*, AOB *amoA*, *nirK* and nosZ). Panel B: Mean of Squares of log-transformed gene abundances (16S, *nifH*, AOA *amoA*, AOB *amoA*, *nirK* and *nosZ*) from the two ANOVA models based on permutation test (first model: region*management type – left side; second model: pH class + C org class – right side) expressed as percentage. Panel C: least square means of ratio AOB *amoA*/(AOA *amoA* + AOB *amoA* log-transformed gene abundances) as a function of pH class and C org class. Significant effects were estimated using the GAM model based on the beta distribution with log link function. Significant effects are reported in Tables S9–S10 and are represented with: * (p < 0.05), **.

Table S9).

The ratio between AOB *amoA* and the sum of AOA *amoA* and AOB *amoA* did not present a significant effect for both region and management type, and their interaction (Fig. 6 C - Supplementary Table S10). Also, in terms of pH and C org classes, there were no significant effects on the ratio. However, the two factors presented two opposite trends, where the former presented a negative trend and the latter a positive one (Fig. 6 C – Supplementary Materials Table S10). The effect of organic carbon was stronger than pH (F-ratio C org class: 1.4; F-ratio pH class: 1.1 - Supplementary Materials Table S10).

4. Discussion

4.1. Taxonomic diversity

The investigated permanent grasslands presented appreciably different microbial communities in terms of taxonomic profiles. The communities shared a common core of phyla (Fig. 3), consisting mainly of Firmicutes, Proteobacteria, and Actinobacteriota (Fig. 3), which are typically among the most abundant in both soil and water environments (Bahram et al., 2018). The taxonomic differences increased as we descended from phylum to ASV rank (Fig. 4, Supplementary Materials Fig. S2). At the ASV rank, the number of common cases was drastically reduced (Fig. 3), indicating a common phyletic starting point and subsequent time-related dispersion. Considering the metrics used to assess microbial identity and phylogeny, such as the 16S ribosomal RNA gene (Woese, 1987), the estimated rate of change suggests that phyla started branching around 1–2 billion years ago (Clark et al., 1999), while species originated on average over 100 million years ago. In practical terms, considering the timing of tectonic motions and land emersion that determined the current position of European regions, and the relatively

short duration of soil formation (centuries to thousands of years), the contribution of in situ bacterial speciation can be regarded as practically irrelevant in this type of survey. Therefore, when interpreting the data, differences in composition should be seen as partly resulting from deterministic forces, such as local environmental selection, and partly from stochastic forces, like dispersal drifts. The existence of a common core composed of the most abundant phyla indicates a shared origin among microbial communities, which subsequently diversified due to environmental conditions. Despite the common core, the diversity of phyla showed a significant tendency to be grouped by the 'region' variable. However, 'region' should not be interpreted solely in terms of straight geography; it also encompasses concurrent differences in pedoclimatic conditions, such as pH and organic C, in addition to latitude. At the phylum level, the beta diversity of microbial communities seems to have been influenced by the region factor in its variability (Supplementary Material Fig. S3 and Table S7), while the alpha diversity of phyla appears to be primarily regulated by pH and organic C (Supplementary Material Table S4). These factors also affected evenness, confirming the role of local pedoclimatic conditions. Interestingly, even at the ASV level, the beta diversity of microbial communities was driven by the region, showing higher variability within Norway and Italy compared to France (Supplementary Material Fig. S3 and Material Table S7), while the alpha diversity of ASVs appeared to be relatively constant and only the evenness was governed by pH (Supplementary Material Table S4). This difference between phylum and ASV diversity shows how environmental conditions change their influence on the microbial communities at different levels. The difference among communities was driven by region at both phylum and ASV level but the absence of significant effect of pH and C org on beta diversity suggests the dispersion is driven by different environmental factors which were not explored in this study. Interestingly, the internal geographical

distance appeared to marginally account for differences among microbial communities, as evidenced by France exhibiting the least dispersion in both phylum and ASV levels despite greater sample distances. The lowest dispersion of France is possibly attributable to a higher overall homogenization of environmental conditions in the study area. Geographical distances seem to explain community dissimilarities, which are shaped by local conditions, as previous studies have already confirmed (Fierer et al. 2006; Louca et al. 2016; Thompson et al. 2017). When inspecting the differences between microbial communities concerning the uniqueness or differential abundance of the highest ranks, i. e., phyla, such differences are mainly attributed to the rarest phyla and those with the lowest abundance of sequence reads. The effect of pH and C org on phylum alpha diversity and evenness can reveal physiological adaptations, such as the variation of membrane protein activity (Luan et al. 2023; Müller and Engel, 1999), which can induce changing in relative abundance, according to Bartram et al. (2014). In particular, the pH has a positive effect on the alpha diversity, confirming the tendency of microbial communities to reach the maximum diversity at neutral condition, which tends to reduce the impact on microbial metabolism with positive effect on survival, growth rate and diversity (Bahram et al. 2018; Fierer et al. 2006; Thompson et al. 2017).

4.2. Functional diversity

The functional profiles presented patterns similar to the taxonomy-related ones at the phylum level concerning region, management type, and pH (Fig. 3). There was also a common and persistent core for the functions (Fig. 3), which is not unusual since a large fraction of metabolic genes encoding functions appeared early in Earth's history and propagated into multiple clades (David and Alm, 2011; Falkowski et al., 2008). This common functional core may be affected not only by the adaptive loss of functions due to environmental conditions (Morris et al., 2012) but also by horizontal gene transfer (David and Alm, 2011; Falkowski et al., 2008). Horizontal gene transfer is independent of the 16S-based phylogeny on which bacterial taxonomy and metabarcoding assignments are based, suggesting that bacteria may possess actual functional traits that are not detectable through current ribosomal database-dependent annotation methods, which account for the sharing of traits among ASVs. In our case, the common functional core consists of chemoheterotrophy and aerobic chemoheterotrophy, representing two general metabolisms in microbial communities. The abundance of chemoheterotrophy and aerobic chemoheterotrophy can be attributed to the high quantity of organic C in grasslands, revealing the microbial communities' preference for obtaining energy through oxidation rather than carbon fixation, which might lead to the emission of greenhouse gases such as CO_2 (Yu et al., 2021; Zhang et al., 2018). Thus, permanent grasslands used by humans as pastures or meadows seem to present a high potential for carbon cycling, potentially leading to CO_2 emissions. Interestingly, chemoheterotrophy was significantly associated with the meadow (Supplementary Material Table S7), suggesting a possible contribution of fertilization or cutting in supporting C cycling, despite the absence of general significant effects of management type and organic C amount. The autotrophic activity of nitrification was negatively correlated with the heterotrophic activities of chemoheterotrophy (τ = -0.49 – p-value < 0.001), in either aerobic or anaerobic conditions. This indicates that the presence of organic substrates, not needed by the autotrophic activity of nitrification, has a higher hierarchical effect than the presence of oxygen, which is required only by one of the heterotrophic nitrification and chemoheterotrophy. The uneven oxygen requirements, with respect to respiration and ammonia oxidation, contribute to explaining this difference. The neutral pH class was associated with a significantly higher presence of nitrification and ammonia oxidation, whose larger bars' width is also appreciable in the graph, while the more acid pH class was associated with cellulolysis, animal parasites, and symbionts (Fig. 4). The inferred higher levels of these ammonium metabolism-related traits are confirmed by the

representation of the source data at the taxonomy level (Fig. 4), as these functions can be performed by Crenarchaeota and Myxococcota (Langwig et al., 2022; Weidler et al., 2008), and we can observe the evidence of their higher occurrence at the corresponding pH class, particularly for Crenarcheota, at the expense of the Firmicutes share. Thus, our results confirm the nitrification is favored by neutral pH, according to the preference of nitrifying bacteria to neutral to slightly alkaline conditions thanks to more abundant NH3 substrate (Ayiti and Babalola 2022; DeForest and Otuya 2020).

4.3. Gene indicators

Gene pools analyzed by quantitative PCR confirmed functional and phylum-level effects but with an added resolution level, in that only a part of the N cycle genes, in particular *nifH* (nitrogen fixation), *nirK* and *nosZ* (intermediate and terminal denitrification steps, respectively) were significantly affected by the region factor. Of these three genes, only *nirK* and *nosZ* were significantly affected by the pH along with the AOA *amoA* (archaeal nitrification). *nirK* was more abundant in France, while *nifH* and *nosZ* were more abundant in Italy, which presented the highest pH. Thus, denitrification, represented by *nirK* and *nosZ*, appears to be favoured by neutral soils rather than acidic ones, according with the results of the analysis of functional profiles. The two denitrification genes seem to be enhanced by different soil pH conditions, since *nirK* was more abundant with pH between 5.8 and 6.4 while *nosZ* with pH between 6.4 and 7.6, suggesting a possible environmental allocation of the two denitrification stages. Nitrification, represented by AOA and AOB, showed a similar preference despite the absence of a significant regional effect. AOA showed greater variability than AOB, which were more constant as a function of region, pH, and organic Carbon. This may be due to the different niches between archaea (AOA) and bacteria (AOB), where the former prefers environments more limited by nutrients than the latter, as the analysis of the ratio between AOB and (AOA + AOB) confirmed in agreement with the positive trend of organic C (Baolan et al., 2014; Sun et al., 2019). Moreover, the negative trend of pH on the AOB proportion seems to indicate that AOA are more adapted to neutral soil. AOA are part of Crenarchaeota, which were however reported as present in acid pH condition according to the preference of archaea nitrifiers when comparing neutral vs acidic soil (Lehtovirta et al., 2009; Prosser and Nicol, 2012). Our results show a different scenario with a consistent presence of Crenarchaeota and AOA under neutral pH conditions, revealing the possible presence of this phylum in a wide variety of pH conditions (Fig. 4). Thus, the niche diversification between AOA and AOB would not be universal, and AOA has indeed been found at both low and neutral pH (Sun et al., 2019). The common trend of AOA and *nosZ* in relation to pH may reveal a likely interdependency between the two genes, also highlighted by the moderate and positive relation between *nosZ* and AOA (Fig. 6 A). The high potential of ammonia oxidation may support high rates of nitrification and then complete denitrification. *nifH* is instead an indicator for nitrogen fixation, either free-living or symbiotic (Shaffer et al., 2000) and it showed an interesting behaviour as it was significantly affected by region but not by pH and C org. The absence of a significant effect of pH, which was significantly related to the region (Supplementary Material Fig. S1 panel A), suggests different environmental conditions able to modify its abundance as possibly vegetation and fertilisation, irrespective of the hierarchically dominant ones. *nifH* was influenced by plant cover during field restoration (Wang et al., 2017) as a possible consequence of interactions between the diazotrophic community, i.e., bacteria able to fix N_2, and plant species. Within this context, typical of pastures and meadows, acid soil with high amounts of organic carbon can favor functions different from those related to N cycling, even though general microbial communities are not influenced by pH and organic carbon, as the constant abundance of 16S genes reveals.

4.4. Effect of management type, region, pH, and C org

The investigated permanent grasslands presented interesting patterns of microbial community responses to the selected factors. The constant absence of significant effects of management type on both phyla and functions, as well as genes' abundances (Fig. 2, Supplementary Material Tables S3 and S6), suggests that the dominant common cores of microbial community structures can resist disturbances derived from pastures and meadows. This may be due to the grazing-like mowing practice applied to both types, which simulates the disturbance resulting from animal presence and leads to similar community cores (Liu et al., 2014; Mencel et al., 2022; Schroder et al., 2011). Additionally, the return of animal excreta as fertilizer may have limited direct effects on community composition, as the excreta also contain DNA from transient taxa that are abundant in the soil and browsed vegetation where animals spend their time (NandaKafle et al., 2017). Furthermore, persistent management practices can alter soil conditions, such as pH and organic carbon, which are known to directly influence microbial communities (Lauber et al., 2009; Ni et al., 2021). The constant absence of effect of management type can be due to its absorption by the effects of pH and organic carbon. Thus, the effect of the management type can be considered more indirect than direct since it can act on the microbial community by altering soil conditions. The investigated permanent grasslands showed a significant tendency for microbial communities' taxonomic and functional diversity to be grouped by the 'region', indicating distinct microbial compositions across different geographical areas, which are likely influenced by local environmental conditions, such as pH and C org. The strongest driver for differentiating microbial communities at the ASV level was region, while at the phylum and function levels, it was the pH, according to ANOSIM analysis. This difference highlights the possibility of a stronger local environmental influence than pH in shaping microbial ASVs, such as vegetation or other factors not considered in this study. The pH and organic carbon were confirmed as direct drivers for the diversification or adaptation of microbial communities of soil (Rousk et al., 2010; Tripathi et al., 2018), especially at ASV level. Among these factors, pH showed the strongest influence on microbial communities from our comparison., This result aligns with Yang et al. (2022a,b), which suggest that pH serves as a primary driver due to its significant impact on a wide range of biogeochemical conditions, thereby affecting microbial growth and survival through the modulation of extracellular enzyme activity. The variable effects of pH observed on certain genes related to the N cycle further support its influence on extracellular enzyme activity. Consequently, the effect of pH on extracellular enzyme activity can influence various metabolic pathways and functional profiles. C org seemed to be a significant, yet not constant, driver for soil microbial communities, as it significantly affected ASVs but not phyla and functional profiles (Ni et al., 2021; Žifčáková et al., 2017). The weaker effect of C org compared to pH may be attributed to the differences in their mechanisms of influence: C org can influence microbial activity by altering the availability of resources (Eisenhauer et al. 2010; Smith et al. 2021), whereas pH directly affects the physiology of microorganisms (Lauber et al., 2009; Jin and Kirk, 2018). Thus, our results showed a hierarchy among pedological drivers.

4.5. Phylum diversity as an Indicator of functional potential

The coherence between phylum-level taxonomy and function, strengthens the concept that functional profiles of ecosystems are mainly defined by the phyla. Therefore, the diversity of the phylum can be regarded as a better index of the functional potential compared to ASV diversity, despite the positive and significant correlation between the number of total ASVs and functions (Fig. 5 A). A high number of ASVs appears to increase the number of both functions and phyla, but only marginally considering the whole community. The marginal contribution of ASV diversity to functional profiles may be explained, since most

functions are not monophyletic and multiple, coexisting distinct ASVs can perform common functions (Aguilar et al., 2004; Louca et al., 2018; Martiny et al., 2015). The presence of distinct ASVs capable of performing shared functions provides an ecosystem buffer against taxonomic diversity variation due to local disturbances, making the entire community performance resistant to impacts of a given extent (Jurburg and Salles, 2015; Louca et al., 2018). Thus, it is reasonable to uncouple considerations on ASV diversity from microbial functional diversity thanks to the existence of functional redundancy across taxonomy (Louca et al., 2018). It can also be postulated that the current databases used to extract the functional profiles could likely be more influenced by high taxonomy levels, such as phylum, during the assignment. This could also be partly due to the presence of ASVs that lack lineage annotation, as their individuation is based only on a concept of sequence uniqueness, but not on taxonomical recognition, as revealed by the negative correlation between function assignment and total ASVs number (Fig. 5 A). The functional profiles and the abundance of target genes were not always coherent. The nitrogen fixation was found significantly associated to intermediate pH and the France region, but the *nifH* presented the highest average abundance in Italy and no significant influence of pH. Instead, the nitrification and the oxidation of ammonium presented coherent results between functional profiles and gene abundances. Both the processes were significantly associated to the Italian sites and the most alkaline class according to the genes *amoA*. Different results between inferred functions from sequencing analysis and real-time PCR were found in previous studies (Yang et al., 2022). The different patterns between functional profiles derived from sequencing and real-time PCR can rely on the difference between the two molecular techniques. The sequencing provides a comprehensive view of the microbial community targeting specific region of the 16S rRNA while the real-time PCR amplifies and detects specific DNA sequences. Both techniques present limitations that can partially explain the different results. Sequencing can be affected by biases due to the difficult distinction of similar ASVs with high-sequence similarity of partial 16S variable regions (Gao et al. 2017; Jeong et al. 2021) with consequent limitations to infer the functional profiles using reference database, such as FAPROTAX. Real-time PCR provides quantitative information about a target gene, without distinguishing whether the gene belongs to live microorganisms or is a cell-free relic that has been preserved and accumulated in the soil (Kralik and Ricchi 2017; Smith and Osborn 2009). Thus, both methods can overestimate and underestimate the abundance of a target function but the combination of them can provide mutually compensative and consequently more robust information about the functional potentialities of an environment.

In terms of innovation level and field advancement over previously acquired notions, the present report addressed, from different standpoints, including whole bacterial community (NGS metabarcoding) and single genes abundance (by qPCR), a hitherto unexplored direct comparative assessment between pastures and meadows. Such analysis allowed to trace an ecological equivalence between the impacts of these two alternative management practices. This evidence pointed out how two types of perturbation that periodically affect the plant biomass cover concur to similar outcomes in soil community composition and consequent physiology.

5. Conclusions

Our study across a latitude gradient in Europe provides valuable insights into the status of soil microbial communities in permanent grasslands, despite the limited sample size. Although these communities shared a common core of phyla, their deep-level variability was mainly influenced by pedological conditions, particularly pH, which also significantly impacted taxonomy-inferred functions. The coherence between phylum-level taxonomy and function suggests that phylum diversity could be a more reliable indicator of functional potential compared to ASV diversity, owing to the presence of functional

redundancy across taxonomy. Regarding management type, whether pasture or meadow, we observed only marginal effects on ASV-level diversity, with no significant shifts in higher taxonomic levels and functional profiles. This indicates that, at the examined level of intensification, the use of grasslands for pasture or meadow may have minor effects on functional biodiversity, as microbial communities exhibit high resistance to local disturbances. Thus, the management type had a marginal effect compared to the geographical region and pedological drivers. Among the pedological drivers, pH emerged as the strongest direct influencer of microbial communities, with organic carbon also exerting a significant but less pronounced effect compared to pH. The hierarchical effect of these drivers underscores the intricate interplay between environmental factors and microbial communities in permanent grasslands. Our findings verified our starting hypotheses providing valuable guidance for future studies aiming to investigate microbial patterns and identify different drivers, enabling the development of more sustainable management strategies for permanent grasslands. Understanding the functional diversity of the soil microbial community in these grasslands plays a crucial role in enhancing management practices to maximize ecosystem services, such as carbon sequestration and nitrogen fixation, while minimizing ecosystem disservices, such as greenhouse gas emissions. This knowledge is crucial for advancing agricultural sustainability and ecosystem conservation efforts. Further prospects to enhance this knowledge include increasing the sample size to cover an intensity gradient of different management types across a broad geographical region and considering more microbial drivers, such as local vegetation, soil moisture, and nitrogen. These efforts aim to improve the understanding of hierarchical effects on soil microbial communities.

CRediT authorship contribution statement

S. Raniolo: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **L. Maretto:** Formal analysis, Methodology, Writing – review & editing. **E. Benedetti del Rio:** Investigation, Writing – review & editing. **S. Cournut:** Writing – review & editing. **M. Cremilleux:** Conceptualization, Investigation, Writing – review & editing. **B. Nowak:** Conceptualization, Investigation, Writing – review & editing. **A. Michaud:** Conceptualization, Funding acquisition, Investigation, Writing – review & editing. **V. Lind:** Conceptualization, Investigation, Writing – review & editing. **G. Concheri:** Writing – review & editing. **P. Stevanato:** Writing – review & editing. **A. Squartini:** Conceptualization, Formal analysis, Methodology, Writing – review & editing. **M. Ramanzin:** Conceptualization, Formal analysis, Methodology, Writing – review & editing. **E. Sturaro:** Conceptualization, Funding acquisition, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2023.111063.

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