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RESEARCH ARTICLE

Species-rich old grasslands have beneficial effects on the health and gut microbiome of bumblebees

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Abstract

1. Recent studies have shown that old, traditionally managed semi-natural grasslands (SNGs) harbour specific plant assemblages characterized by high species richness, diversity, evenness and a great abundance and diversity of floral resources.
2. As nectar and pollen from many of these plants contain a multitude of favourable phytochemical compounds, we hypothesized that pollinator health and survival would improve in old, species-rich hay SNGs compared to younger SNGs.
3. We monitored experimental colonies of bumblebees placed in hay SNGs of different ages.
4. The survival of bumblebees increased with grassland age, thus they lived longer and produced more workers in the old SNGs. The abundance of actinomorphic flowers (with radial symmetry) increased with the age of the SNGs and was positively correlated with the body size, body mass and lipid reserves of the bumblebees.
5. The taxonomic composition of gut microbiota changed during the experiment, with a significant decrease of core taxa *Bombiscardovia* and *Bifidobacterium* in young SNGs, while the abundance of *Bombilactobacillus* increased in old SNGs. At the end of the experiment, the bumblebees of young SNGs hosted lower abundances of *Gilliamella* than those of old SNGs. In old SNGs, there was a positive relationship between floral richness and the abundance of six taxa, including the three core taxa *Bombiscardovia*, *Bifidobacterium* and *Snodgrassella*. Microbiota α-diversity decreased and microbiota β-diversity increased over time in young SNGs, while both remained stable in old SNGs. Both deterministic and stochastic processes acting simultaneously in bumblebee microbiomes within young SNGs seemed to explain these changes.
6. Compared to old forests, very little attention has been paid to old, species-rich grasslands. Considering their importance for pollinator health, as well as their high ecological and cultural values, it is crucial that the rare and endangered old, species-rich hay SNGs are protected.

KEYWORDS
Bombus terrestris, bumblebee, colony development, grassland age, gut microbiota, old species-rich grasslands, plant diversity

1 | INTRODUCTION

Permanent grasslands, defined as land containing herbaceous vegetation that has not been included in a crop rotation for a period of 5 years or longer (EU, 2004), cover approximately one third of the agricultural area of the European Union (Eurostat, 2020). For centuries, these grasslands were an integral part of every farm and were the foundation for grazing of livestock and haymaking (Dengler et al., 2014). This continuous low-intensity traditional land use, characterized by the absence of mineral fertilization, yearly mowing and often the grazing of regrowth, has been the main driver of remarkably high specific and genetic diversities in these semi-natural ecosystems (Cousins et al., 2009; Pärtel et al., 2007). Some old semi-natural grasslands (SNGs hereafter) are among the biotopes with the highest small-scale plant diversity in temperate areas (Wilson et al., 2012). However, over the past century, agricultural intensification and forest expansion have caused a dramatic decrease in SNGs (Peterken, 2013). For instance, between 1967 and 2007, losses in six western European countries (Belgium, France, former West Germany, Italy, Luxemburg and The Netherlands) have been estimated at around 30% (Huyghe et al., 2014). In addition, mineral fertilization, increased mowing frequency or overstocking have impoverished most of the species-rich remnants (Hejcman et al., 2013). As a result, old species-rich SNGs are becoming extremely scarce in Europe (Peterken, 2013).

According to recent studies, old SNGs that have only been traditionally extensively managed have a specific floristic composition, higher species richness, diversity and evenness (Inoue et al., 2021; Nerlekar & Veldman, 2020) and greater floral abundance (Pornon & Andalo, 2023) than young SNGs under similar management. However, the influence of the age of the SNGs and the importance of old SNGs on pollinator health is virtually unexplored. The abundance and composition of floral resources are main determinants of larval diet, adult body size (Chole et al., 2019) and individual lipid reserves (Arrese & Soulages, 2010; Hoover et al., 2006), which are essential for growth, survival during periods of starvation, reproduction and immunity of insects (Hoover et al., 2006; Tasei & Aupinel, 2008; Wright et al., 2018). Furthermore, nectar and pollen from Eudicot species contain many phytochemicals (Palmer-Young et al., 2017) that can support pollinator health through: (i) preservation of nectar quality with antibacterial and antifungal properties (Pozo et al., 2014; Schoonhoven et al., 2005); (ii) enhancement of insect immunity (Fitch et al., 2022; Richardson et al., 2015); and (iii) stimulation of feeding (De Boer & Hanson, 1987) or foraging (Barberis et al., 2023).

Another important component of bee health is the diversity and composition of its intestinal microbiota. There is in fact increasing evidence that gut bacteria play a crucial role in host metabolism, immunity, nutrition absorption, growth and development (Raymann & Moran, 2018). In addition, the composition and diversity of the gut microbiota of social bees are known to be influenced by the surrounding landscape. Zhao et al. (2018), for instance, found that Chinese black honeybees (*Apis mellifera* subspecies) from national nature reserves harboured richer gut microbiomes than those collected in nonprotected regions. Likewise, bumblebees (*Bombus terrestris*) collected in forests had more diverse gut microbiomes compared to those collected in oilseed rape fields and apple orchards (Krams et al., 2022), while the gut microbiome composition of *B. terrestris* queens from forests and urbanized habitats differed strongly (Bosmans et al., 2018). Environmental and anthropogenic changes, such as the presence of mass flowering crops on farmlands (Jones et al., 2018) and the use of pesticides such as glyphosate (Motta et al., 2018), can also alter the relative abundance of key microbial taxa. Changes in abundance, composition and diversity of the gut microbial community occur through shifts in flower availability, pollen and nectar composition (Billiet et al., 2016), local environmental conditions and the pool of environmental bacteria that may invade and colonize the insect gut (Bosmans et al., 2018). In most bee species, gut communities typically harbour dominant core taxa and more sporadic non-core taxa. Core taxa usually consist of *Snodgrassella*, *Gilliamella*, *Schmidhempelia*, *Bifidobacterium*, *Bombiscardovia* and *Lactobacillus* (Hammer et al., 2021; Krams et al., 2022). The abundance of core taxa may fluctuate between sites (Dew et al., 2020; Koch & Schmid-Hempel, 2011a) or with diet composition (Billiet et al., 2016), while the composition of non-core communities varies among individuals with developmental and environmental factors, including climate and forage (Newbold et al., 2015; Raymann & Moran, 2018; Regan et al., 2018).

Here, we test whether the age and age-associated characteristics of extensively managed mesophile hay SNGs affect the health and gut microbiota of pollinators. We set up 16 distinct colonies (one colony per SNG) of the buff-tailed bumblebee (*Bombus terrestris*), a common European pollinator (Garibaldi et al., 2013), in young and old SNGs for 15 days. We monitored the health of bumblebees through colony and individual metrics. At the colony level, we measured the survival of bumblebees and the emergence of new individuals during the experiment. At the individual level, we measured body size, body mass and lipid reserves, and examined the diversity and composition of the gut microbiota.

Due to the more diverse, more abundant and specific floral resources in old SNGs compared to young SNGs (Pornon & Andalo, 2023), we expected bumblebees in old SNGs to: (i) have higher survival, (ii) be larger and heavier and (iii) have higher lipid reserves. Furthermore, we expected (iv) significant differences in the structure of the gut microbial community of bumblebees and

the relative abundance of specific taxa according to grassland age (Bosmans et al., 2018; Dew et al., 2020).

2 | MATERIALS AND METHODS

2.1 | Study design and field data collecting

The study site is a hilly mollassic region that feature a mosaic of crop fields, small woods and grasslands near the city of Toulouse in south-west France. The climate is sub-Atlantic with sub-Mediterranean and mountain influences. The mean annual precipitation is 750 mm, and the mean annual temperature is 12.5°C. The altitude ranges from 187 to 326 m a.s.l.

To assess whether the age of semi-natural grasslands (SNGs) affected bumblebee health, colonies were placed (that which requires neither an administrative licence nor ethical approval) in traditionally managed (non-fertilized and mown once a year) old SNGs (>80 ± 8.8 years) and young SNGs (18.5 ± 11.2 years) in eight distinct geographical sites (Table S1). Each site included an old SNG and a young SNG. The distance between the two SNGs within each site was up to 1000 m (1000.25 ± 1131.7 m, SD). This block design aims to minimize variability and mitigate the influence of abiotic factors between SNGs within each site (Gotelli & Ellison, 2004). Selected paired SNGs were as similar as possible in terms of abiotic factors (such as slope, aspect, altitude, soil humidity, fertility and soil pH) and management practices (historical or current use as hayfields or fallows, presence or absence and intensity of mineral fertilization or manure, and whether they were originally seeded or not). A previous study that included all studied SNGs (Pornon & Andalo, 2023) demonstrated that among all these factors, age and, to a lesser extent, soil fertility (which exhibited little variation among SNGs) were the only factors contributing floristic differences between old and young SNGs.

In mid-May 2021 (D0), a bumblebee colony (Koppert, The Netherlands) was introduced in each SNG and placed in a cage (4 m long, 2 m wide and 1.5 m high; Tissue F 1032 CRISTAL, 100% PEHD, Anti UV; Mesh size: 3 × 2 mm; commercialized by Diatex SAS) for 15 days. The cages were moved every 2 days (six times during the experiment) to allow the colony to explore the SNG and renew the available floral resources. To minimize the risk that individuals would escape during cage movements, the hives were closed the evening before, ensuring that the bumblebees were enclosed when the cage was moved the next morning. Therefore, the entrance door to the cage was exceptionally opened when the bumblebees were foraging. To prevent individuals from escaping underneath the cage, the netting was wrapped around a horizontal PVC bar placed on the ground after carefully clearing the grass.

Before the experiment, all colonies were standardized to one queen and 30 workers (see Figure S1 for a detailed description of the experimental design and sampling). All individuals were painted with a green dot on the thorax. Extra workers were killed by freezing for further microbiome analyses (see below). Commercial food stores were removed and the nest (including the nest structure and larvae) was weighed.

Floral resources within each cage were assessed on each date by counting the number of flowers or flower heads (in the case of plants with compact inflorescences, for example, *Trifolium* spp., *Centaurea* spp.) in two 1-m^2 quadrats (a total of 192 quadrats). Additional rare species with flowers within the cages were also recorded.

2.2 | Colony demography

The effects of SNG age on the demography of bumblebee colonies were investigated by assessing the survival of the 30 marked bumblebees and the colony's potential to produce new individuals between D0 (start of the experiment) and D15 (end of the experiment; D0 + 15 days). The number of unmarked bumblebees at D15 could not be used as a proxy for the colony potential, since it might reflect more the number of larvae at D0 than the effects of SNGs per se. Therefore, we calculated the colony emergence potential, that is, the potential of the colony to produce new individuals during the experiment, as follows: $WN_{DO} - WN_{D15}$, where WN_{DO} and WN_{D15} were the weight (g) of the nest (nest structure and larvae) at D0 and D15, respectively, estimated with a precision balance (±0.001 g) ME103T (Mettler-Toledo GmbH, Greifensee, Switzerland). For a given nest, the weight should increase with the production of new larvae and resource storage and decrease with the emergence of new individuals. Since there were no larvae and no storage (except for three cells in a single hive containing negligible amounts of honey) in any nests at D15, the colony emergence potential could only be attributed to the emergence of new individuals.

We expected that the abundant and diverse resources of old SNGs would allow the majority of emerging individuals to survive until the end of the experiment, thus allowing the colony emergence potential to be expressed. Consequently, the total mass or number (given the extreme variability in individual mass) of new individuals would reflect the colony emergence potential, resulting in a positive correlation between the two. On the contrary, lower individual survival in young SNGs due to less abundant and diverse resources would disrupt the relationship between the colony's ability to produce new individuals (i.e. the colony emergence potential) and the mass or number of surviving individuals at D15. For each colony, the total dry mass of new individuals was calculated by multiplying the mean individual dry weight (obtained from 10 unmarked individuals randomly selected and weighted with an AP series Analytical + Ohaus balance; ±0.01 mg) and the number of unmarked individuals at D15.

2.3 | Body size, mass and lipid reserves

Ten newly emerged unmarked bumblebees per colony (160 bumblebees in total) were sampled and killed by freezing at D15 to investigate the effects of SNG age and floral characteristics on body size,

dry mass and lipid reserves. Body size was estimated by measuring the femur length of the right hind leg (Lihoreau et al., 2007) to the nearest 0.01 mm using a Nikon SMZ 745T dissecting scope (objective × 0.67) with a Toupcam camera model U3CMOS coupled to the ToupView software. Chloroform extraction was used to quantify body lipid reserves (Kraus et al., 2019). The bumblebees were dried at 65°C for 48 h, weighed, soaked in chloroform (changed every 24 h) for 3 days to extract the total body fat content, dried and again weighed to obtain lean mass (body mass without lipids).

2.4 | Gut microbiota

2.4.1 | Gut sample and DNA extraction

A total of 256 bumblebees (eight marked individuals at D0 and eight unmarked individuals at D15 from each colony) were dissected under a *binocular magnifying glass*. Hindgut parts were collected with sterile instruments for DNA extraction of the bacterial gut community. The individuals sampled at D0, not exposed to the SNGs, served as controls, whereas the individuals sampled at D15 were exposed to the SNGs. DNA was extracted using the Blood and Tissue Kit (Qiagen). The samples were incubated overnight in a lysis buffer (Tris-EdTA, Triton and lysozyme) and proteinase K in a 100 µL elution. Nine extraction controls were included with the bumblebee samples to monitor contamination.

2.4.2 | 16S rRNA gene amplification and sequencing

Hypervariable regions V3 and V4 of the 16S rRNA gene were amplified using universal primers, following the protocol detailed in the supplementary materials and methods. Libraries were prepared with the TruSeq DNA Nano kit (Illumina) according to the manufacturer's instructions and sequenced on a MiSeq Illumina, 2 × 250 pair-end using the NGS core facility at the Génopole Toulouse Midi-Pyrénées (www.get.genotoul.fr).

Sequence analysis was performed using OBITools v1.2.11 (Boyer et al., 2016) and sumaclust (Mercier et al., 2013) softwares, through the Snakemake pipeline (Mölder et al., 2021) of Benoiston (2022). Detailed procedures and dataset characteristics at each step of the sequence analysis are provided in the supplementary materials and methods and Table S2.

2.5 | Statistical analysis

2.5.1 | Floral resource analysis

We calculated the total mean flower abundance in the cages based on the counts of floral units in $2 \times 1\,m^2$ quadrats per cage and per date. Given the preference of bumblebees for zygomorphic flowers (Willmer, 2011), we also analysed the mean abundance of both zygomorphic and actinomorphic plant species (single flowers or inflorescences).

At each date and cage, we calculated three indices of α-diversity: Floral richness *S* (number of flowering plant species per cage at a given date), Shannon floral diversity index (*H′*) and floral evenness (*E*). See supplementary materials and methods for the formulas.

To investigate relationships between SNG age and floral characteristics, we used nonparametric Spearman correlation tests for species richness, evenness, total flower abundance and abundance of zygomorphic or actinomorphic flowers/inflorescences and a linear mixed effect model for *H′*, with SNG identity as a random variable.

2.5.2 | Bumblebee demography and characteristics

We used linear models to explore: (i) the effect of the age of the SNGs on the survival at D15 of the marked bumblebees; (ii) the effects of the colony emergence potential (one data point per SNG), the age of the SNGs (two categories: young and old SNG) and their interaction on either the number or the total mass of new individuals. The effect of the colony emergence potential on the number or total mass of new individuals was tested in young SNGs versus old SNGs using separate simple linear models.

We tested the effect of age of SNG alone or in interaction with floral characteristics on body size, body mass and lipid reserves using linear models. The nlme R package (Pinheiro et al., 2021) was used for all tests.

2.5.3 | Gut microbiota diversity and composition

We used the same three indices as for floral diversity to investigate the α-diversity of the gut microbiota: *S* (number of MOTUs per sample), *H′* and *E*. The effects of SNG age (young vs old), time (D0 vs D15) and their interaction, as well as SNG floral and colony characteristics (total number of bumblebees), on gut microbiota α-diversity indices were tested using linear mixed models, with SNG identity as a random variable. β-diversity of the gut microbiota was measured using both the Jaccard dissimilarity index (based on the presence/absences matrices) and the Bray–Curtis dissimilarity index (based on the relative abundance matrices). The effect of SNG age, time and their interaction on these dissimilarity indices was analysed using permutational multivariate analysis of variance (PERMANOVA, *Adonis* function, vegan package, R) with 1000 permutations and the margin option. We evaluated the homogeneity of dispersion (variance) according to SNG age and/or time using the Betadisper function (Vegan, R). Both Jaccard and Bray–Curtis distances provided similar results, but since the Jaccard index explained more of the variation in β-diversity, we chose to present only the results of the Jaccard index in this study.

To study the taxonomic composition of the microbiota and differential taxonomic abundances between SNGs and time, we used

ANCOM-BC function (analysis of microbiome compositions of with bias correction; Lin & Peddada, 2020). We tested the differential abundances of bacterial genera according to the age of the SNGs, accounting for the size of the colony (the total number of bumblebees) and the floral diversity of the SNGs (*H′*) in the model. All other parameters of the function were left in the default settings. Taxa with a log fold change (LFC) value beyond >|1.5| and an adjusted *p*-value (Holm–Bonferroni) less than 0.05 were considered differentially abundant. To standardize the differences in sequencing depth between samples (range: 110–46,815 reads per sample with an average of 14,132 reads per sample), we rarefied all samples to 1000 reads for the β-diversity and taxonomic composition analyses. Five samples were removed during rarefaction because they contained fewer than 1000 reads.

3 | RESULTS

3.1 | Characteristics of semi-natural grasslands

We found 46 species of flowering plant in the cages across all SNGs, including 26 actinomorphic and 20 zygomorphic species, and only two rare monocotyledons represented by very few individuals. On average, there were 5.7 (±6.5) flowering plant species per cage. The mean Shannon index (*H′*) was 1.01 (±0.47 SD) and the mean evenness (*E*) was 0.61 (±0.22 SD). The richness of flowering plant was positively correlated (Figure 1a) and the evenness marginally positively correlated (Figure 1c) with the age of the SNGs. *H′* increased with the age of SNGs (Figure 1b). We found a positive relationship between actinomorphic flowers/inflorescences abundance (Spearman rho = 0.52; $R^2 = 0.27$; $p < 0.0001$) and the age of SNGs and a marginally positive relationship between the latter and total flower abundance (Spearman rho = 0.19; $R^2 = 0.035$; $p = 0.069$). No correlation was observed between the abundance of zygomorphic flowers and the age of the SNGs (Spearman rho = −0.11; $R^2 = 0.011$; $p = 0.30$). Thus, in general, old SNGs were characterized by higher floral richness, diversity and a greater abundance of actinomorphic flowers/inflorescences than young SNGs.

3.2 | Demography of bumblebee colonies

Approximately half of the bumblebees marked at D0 (13.4 ± 6.2 SD out of 30) were still alive at D15 and each colony produced an average of 47.6 (± 18.6 SD) new individuals. Survival of the marked bumblebees was positively related to the age of the SNGs (Figure 2a). Neither the colony emergence potential, the age of SNGs nor their interaction had significant effects on the number of new individuals that emerged during the experiment (Adjusted R^2 of the model = 0.03, $p = 0.37$). However, the total mass of new individuals was positively related to the colony emergence potential (SLM = 0.04; Adjusted $R^2 = 0.17$; $p < 0.05$). Furthermore, linear models analysing young and old SNGs separately revealed positive relationships between the colony emergence potential and the number (marginally positive; Figure 2b) or the total mass of new individuals in old SNGs only (Figure 2c). Therefore, the majority of emerging individuals survived until the end of the experiment only in old SNGs.

3.3 | Body size, body mass and body lipid reserves of bumblebees

We observed weak but positive relationships between the abundance of actinomorphic flowers and the body size (Slope of the linear mixed model, SLMM = 0.002; Adjusted $R^2 = 0.036$; $p = 0.072$), body mass (Slope = 1.58e-04; Adjusted $R^2 = 0.043$; $p < 0.005$) and body lipid reserves (Slope 259 8.312e-06; Adjusted $R^2 = 0.054$;

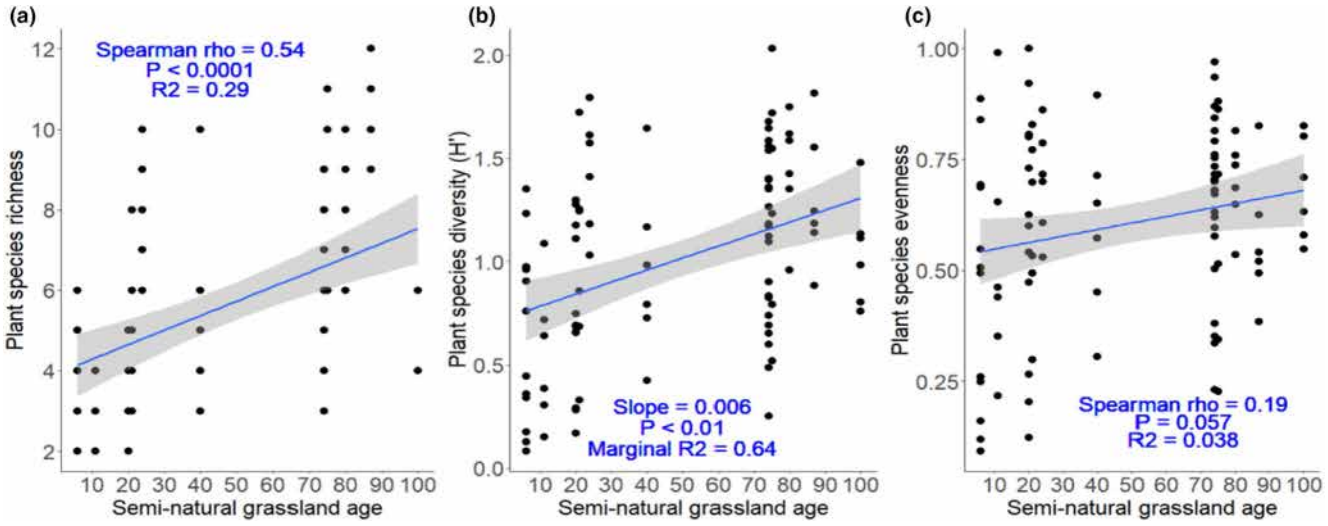

FIGURE 1 Characteristics of semi-natural grasslands. Relationships between the age of semi-natural grasslands (SNGs) and (a) plant species richness, (b) plant species diversity and (c) plant species evenness.

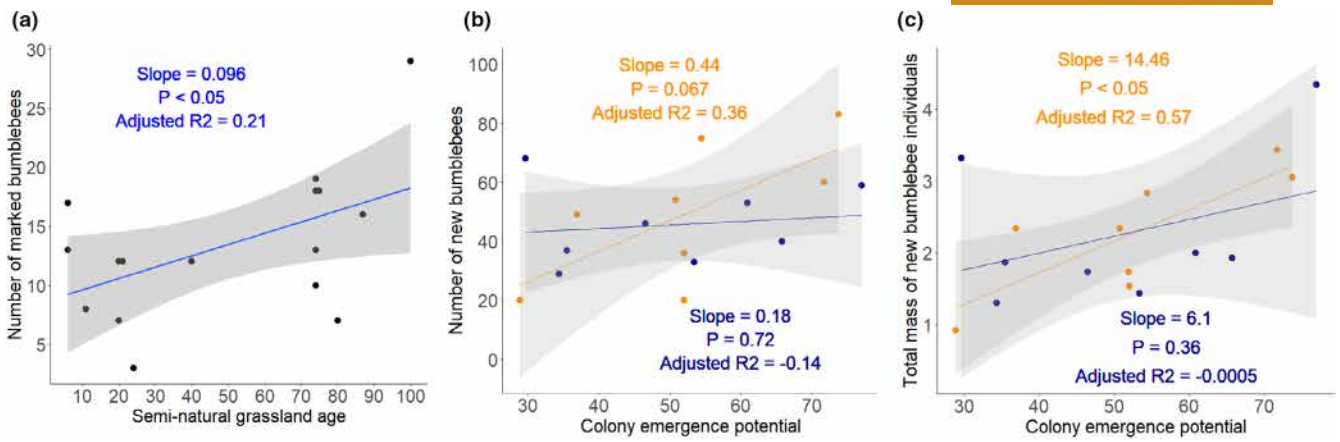

FIGURE 2 Demography of bumblebee colonies. (a) Relationship between the age of semi-natural grasslands (SNGs) and the number of individuals marked at the beginning of the experiment (D0) and still alive at its end (D15); relationship between the colony emergence potential (in g) and either the number (b) or the total mass (c) of new unmarked bumblebees that emerged during the experiment. Orange: Old SNGs; dark purple: Young SNGs.

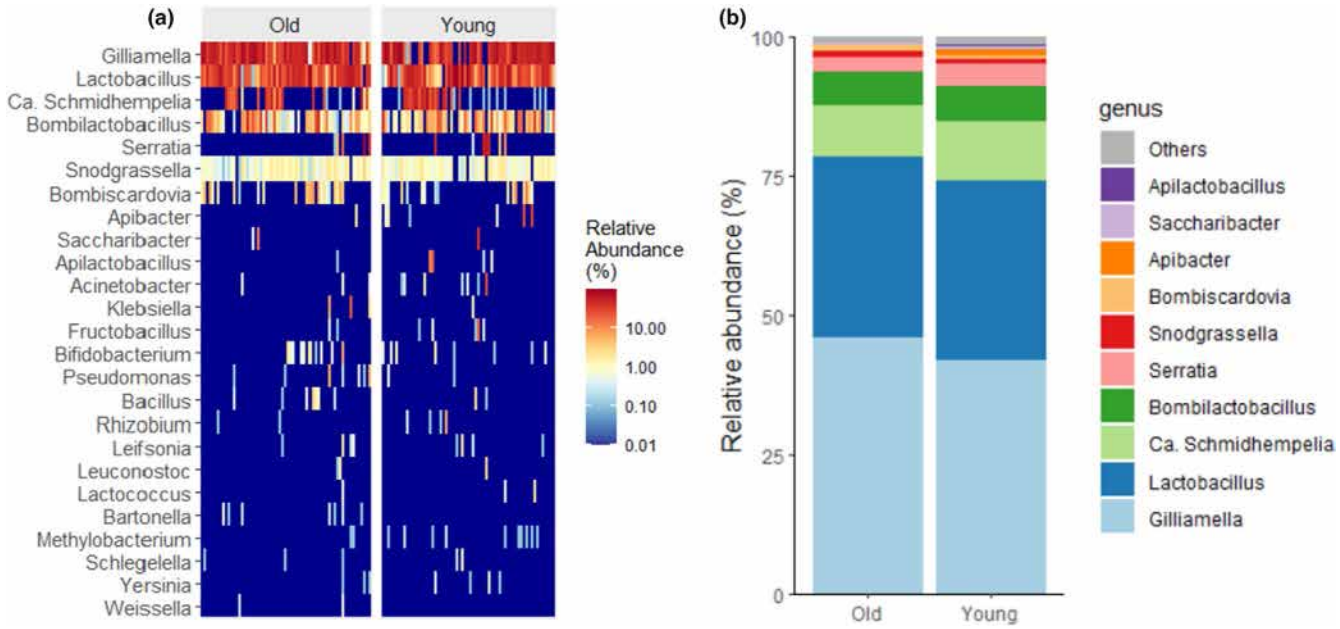

FIGURE 3 Taxonomic composition of bumblebee gut microbiota of young and old SNGs at the end of the experiment (D15). (a) Relative abundance of the 25 most abundant genera (each vertical bar corresponds to an individual); (b) mean relative abundance of the 10 most abundant genera. SNG, semi-natural grasslands.

$p < 0.001$) of individual bumblebees. The relationships were primarily explained by the abundance of *Centaurea decipiens* (mass: $p = 0.0017$; lipid reserves: $p = 0.0022$), *Leucanthemun vulgare* (mass: $p = 0.0004$; lipid reserves: $p = 0.02$) and *Jacobaea vulgaris* (size: $p = 0.023$; Lipid reserves: $p = 0.009$). Despite the increase in actinomorphic flower abundance with SNG age ($R^2 = 0.27$; $p < 0.0001$), no relationship was observed in the mixed linear model between the above three variables (size: $p = 0.10$; mass: $p = 0.31$; lipid reserves: $p = 0.12$) and age of SNGs alone or in interaction with plant diversity or abundance. Furthermore, there was no significant relationship between flowering plant richness or diversity and individual body size, mass and lipid reserves (all p-values >0.16).

3.4 | Bumblebee gut microbiota

3.4.1 | General gut microbiota features

From the 251 bumblebee gut samples, we identified 334 distinct molecular operational taxonomic units (MOTUs) with an average of 15.9 ± 0.36 SE MOTUs per bumblebee gut sample. Overall, three taxa (*Snodgrassella*, *Lactobacillus* and *Gilliamella*) found in more than 95% of individuals, and to a lesser extent *Bombilactobacillus* (more than 70% prevalence), could be categorized as core taxa (Figure 3a; Figure S2). In terms of relative abundance (Figure 3b; Figure S3), *Gilliamella and Lactobacillus* strongly dominated the microbial

communities, accounting for 45% and 34% of the total number of reads per sample, respectively. *Bombilactobacillus* and *Snodgrassella* were much less abundant, representing only 5% and 1%, respectively. *Candidatus schmidhempelia* was relatively abundant (9%), although found in just under 45% of samples.

3.4.2 | Change in gut microbiota taxonomic composition with grassland age and floral diversity

At D15, young SNG bumblebees hosted significantly lower abundances of *Gilliamella* compared to those of old SNGs (ANCOM-BC: LFC: −1.71, $W=-3.27$, $q=0.027$; Figure 3; Table S3). *Gilliamella* was completely absent in many bumblebees of three young SNGs at D15, compared to only one bumblebee at D0. We also observed temporal taxonomic shifts (D15 vs D0) according to the age of the SNGs and found a strong decrease in the relative abundance of the core taxa *Bombiscardovia* and *Bifidobacterium* in young SNGs (LFC: −3.25 and −2.2, respectively; $q \ll 0.0001$ for both taxa; Figure S4b; Table S4), while this decrease was much less pronounced in old SNGs (LFC: −1.61 and −1.38, respectively; $q=0.019$ for both taxa; Figure S4a; Table S5). Furthermore, we observed a significant increase in *Bombilactobacillus* in old SNGs (LFC: +1.59, $q=0.027$; Figure S4a; Table S5), but not in young SNGs (LFC: +1.54, $q=0.08$; Figure S4b; Table S4). Interestingly, in old SNGs, we also found a strong positive correlation between the floral diversity of the SNGs and the relative abundance of *Bombiscardovia* (LFC: +7.26, $q=0.0009$), *Bifidobacterium* (LFC: +4.97, $q=0.02$), *Snodgrassella* (LFC: +2.66, $q=0.0008$) and to a lesser extent *Burkholderia*, *Leifsonia* and *Apibacter* (Table S5). These positive correlations were absent in young SNGs (Table S4).

3.4.3 | α-diversity of the gut microbiota

In young SNGs, we observed that the individuals sampled at the end of the experiment (D15) exhibited lower Shannon diversity (time×age: $F_{1,233}=3.12$; $p=0.07$) and evenness (time×age: $F_{1,233}=4.89$; $p=0.03$), but not the richness of the OTU (time×age: $F_{1,233}=0.0003$; $p=0.98$) compared to the individuals sampled before the experiment (D0), while this trend was not observed in the old SNGs (Figure 4). This reveals a change in the relative abundance of taxa in microbial communities, specifically in young SNGs, as mentioned earlier regarding taxonomic shifts.

3.4.4 | β-diversity of the gut microbiota

β-diversity analyses revealed that the structure of the microbial communities was significantly, albeit weakly, affected by the interaction between SNG age and time (PERMANOVA, age×time, $F_{1,250}=2.12$; $R^2=0.008$; $p=0.002$). Specifically, the gut microbiota of bumblebees from young SNGs sampled at D15 underwent changes compared to those sampled at D0, whereas no change was observed in bumblebees from old SNGs (Figure 5). This difference resulted in a distinction in microbiome composition between bumblebees inhabiting young versus old SNGs at the end of the experiment (PERMANOVA: $F_{1,126}=1.84$; $R^2=0.015$; $p=0.003$). Furthermore, we observed a clear and significant increase in heterogeneity of gut composition (dispersion) over time in young SNGs (Betadisper; $F_{1,126}=16.11$; $p=0.0001$), contrasting with stable dispersion in old SNGs ($F_{1,121}=0.04$; $p=0.83$; Figure 5).

4 | DISCUSSION

Our study reveals significant impacts of grassland age and floral diversity on bumblebee survival and the diversity of their gut microbiota. Although the diversity and abundance of plant species with actinomorphic flowers or inflorescences increased with grassland age, only the latter had a weak but significant positive effect on bumblebee body size, body mass and lipid reserves.

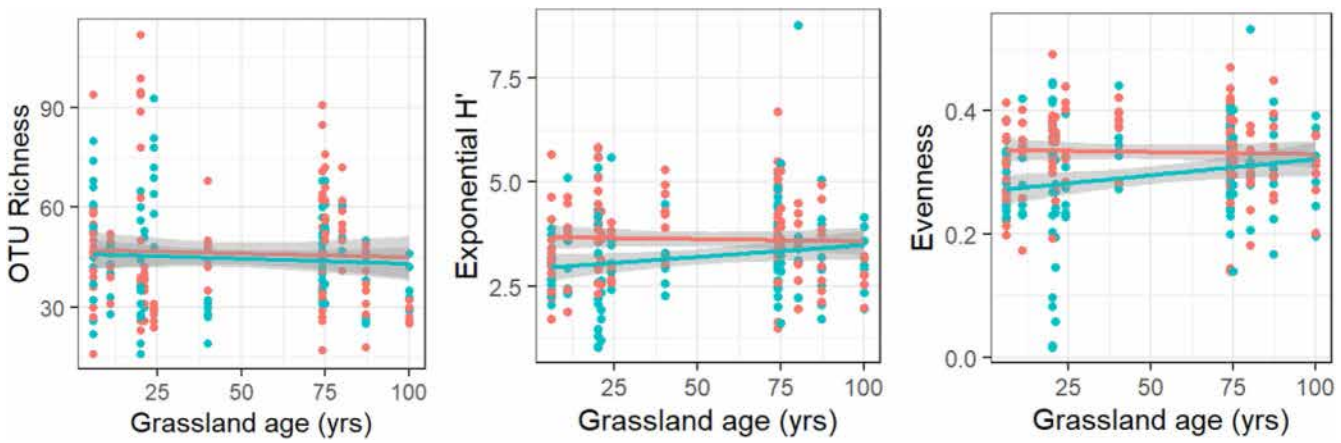

FIGURE 4 Taxon richness, diversity (exponential *H′*) and evenness (Pielou's *J*) of the gut microbiota of bumblebees according to the age of the SNGs (years) at D0 (orange; before the experiment) and at D15 (blue; end of the experiment).

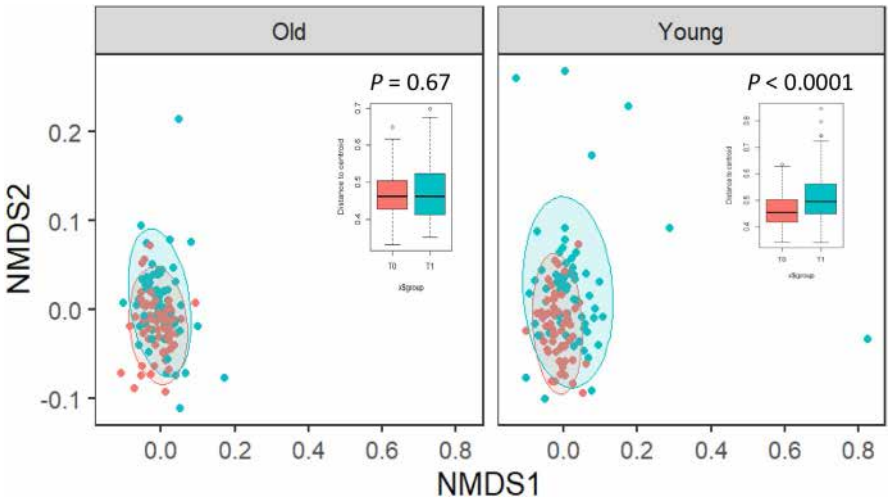

FIGURE 5 Nonmetric multidimensional scaling (NMDS) and multivariate dispersion tests (box plots) based on Jaccard dissimilarity from individual bumblebees at D0 (orange; before experiment) and D15 (blue; end of experiment) in old (left) and young (right) SNGs. Ellipses correspond to the 95% confidence interval.

4.1 | Positive impacts of the age of the grassland on the characteristics and demography of bumblebee

Consistent with our hypothesis, the survival of bumblebees increased with the age of SNGs for both adults present at the beginning of the experiment and those that emerged within the 15-day period. Although flower richness and diversity increased with SNG age, we did not observe a direct relationship between floral diversity and bumblebee survival. However, body size, mass and lipid reserves were all significantly, though relatively weakly, related to the abundance of actinomorphic species, particularly *Centaurea decipiens*, *Leucanthemum vulgare* and *Jacobaea vulgaris*, which increased along the SNG age gradient. *J. vulgaris* and *Centaurea* spp. are known to attract bees by providing large amounts of nectar (Hicks et al., 2016; Lundgren et al., 2013). These species may have influenced body size (Chole et al., 2019) and lipid reserves, which are crucial for insect survival, reproduction and immunity (Alaux et al., 2010; Arrese & Soulages, 2010), as well as the composition of the gut microbiota (Jones et al., 2018).

4.2 | Positive impacts of grassland age on the gut microbiota of bumblebees

In our study, *Snodgrassella* (Neisseriaceae), *Gilliamella* (Orbaceae), *Lactobacillus*, and *Bombilactobacillus* (Lactobacillaceae) comprised the core gut microbiota of workers of *B. terrestris*, consistent with previous findings (Billiet et al., 2016; Wang et al., 2019).

We found that grasslands age and floral diversity had several important consequences for the composition, α-diversity and β-diversity of bumblebee gut microbiota, contrary to previous studies that found changes in relative abundance but not in taxonomic diversity (Jones et al., 2018). α-diversity was higher and remained stable during the experiment period in old SNGs compared to young SNGs, mainly due to more balanced relative taxa abundances (higher evenness). Homeostasis in the gut microbiota has already been reported for well-fed bumblebees kept under relatively constant conditions (Hammer et al., 2021). This suggests that the movement of indoor-reared colonies to outdoor old SNGs did not result in a significant decline in the quality and amount of available resources. The abundance and diversity of eudicots in old SNGs, along with the numerous phytochemicals they contain (Palmer-Young et al., 2017), probably contributed to the stability of the gut microbiota and the higher survival of bumblebees observed in old SNG habitats. Indeed, in old SNGs but not in the young SNGs, we observed a positive effect of floral diversity on several taxa, including core taxa such as *Bombiscardovia*, *Bifidobacterium* and *Snodgrassella*, illustrating the importance of plant diversity for gut microbiota diversity. The decrease in α-diversity in young SNGs during the experiment was associated with a significant reduction in the frequency and abundance of the core symbionts *Bombiscardovia* and *Bifidobacterium*. *Bombiscardovia*, known for its adaptations to low temperatures (Killer et al., 2010), may have been somewhat buffered by dense vegetation shade in the SNGs during the hot May 2022 weather in the region. *Bifidobacterium* can metabolize a diverse array of plant-produced carbohydrates (Raymann & Moran, 2018), including hemicellulose (Zheng et al., 2019), supporting host nutrition.

Another notable finding was the lower abundance of the prominent *Gilliamella* taxon in young SNG bumblebees. This core taxon, together with *Snodgrassella*, forms a dense hindgut biofilm that acts as a protective barrier against pathogen invasion (Cariveau et al., 2014). *Gilliamella* is also the primary degrader of pectin in the pollen wall (Regan et al., 2018), facilitating pollen breakdown and the release of nutrient-rich content, and can metabolize the toxic sugars present in pollen (Zheng et al., 2016). Recent research suggests that *Gilliamella* and *Lactobacillus* are involved in the regulation of neurotransmitters that modulate bee behaviours through olfactory sensitivity (Zhang et al., 2022). Overall, the decline in beneficial taxa such as *Bombiscardovia*, *Bifidobacterium* and *Gilliamella* in young SNGs may have negative implications for bumblebee fitness.

β-diversity analyses revealed two main patterns. Firstly, similar to α-diversity, there was a weak but significant change in the

composition of the gut microbiota of bumblebees within young SNGs during the experiment but not in old SNGs. This could be due to the specific characteristics of young SNGs in terms of plant species composition or stress level that induces deterministic changes in the composition of the gut microbiota of bumblebees. The fact that, in some young SNGs, microbial losses (*Gilliamella*, *Bombiscardovia* and *Bifidobacterium*) affected the majority of individuals and that young SNG vegetation (including those in this study) harboured higher β-diversity (Pornon & Andalo, 2023), supports this deterministic hypothesis. There is increasing evidence that environmental factors (food choice, changes in land use, presence of mass flowering crops in close vicinity, etc.) may alter the composition and activity of the gut microbiota (Bosmans et al., 2018; Jones et al., 2018; Shell & Rehan, 2022; Zhao et al., 2018). The second observed pattern was that, contrary to old SNGs, microbial β-diversity clearly increased in young SNGs over the course of the experiment, indicating that at the end of the experiment, the gut microbiota of bumblebees were more dissimilar from each other than at the beginning. This higher heterogeneity could be explained by stress and/or perturbations in young SNGs that induce stochastic changes in the microbiota. Numerous studies have shown that, under stress, the microbiota of many animal lineages exhibits increased dispersion of the community composition (Zaneveld et al., 2017), a phenomenon referred to as the 'Anna Karenina principle' from Tolstoy's eponym novel: 'All happy families are all alike; each unhappy family is unhappy in its own way'. Therefore, it is likely that both deterministic and stochastic processes occur simultaneously in the bumblebee microbiomes within young SNGs, although the stochastic processes appear relatively more prominent given the moderate compositional change shown in Figure 4.

In general, the decrease in the evenness and diversity of the gut microbiota, the decline in the abundance of key beneficial taxa and the stochastic changes in gut composition in young SNGs suggest that they induce dysbiotic alterations in the gut microbiota (Stothart et al., 2016). Such dysbiosis can be detrimental to bumblebees, as gut bacteria, especially core symbionts, play crucial roles in host metabolism, immunity, nutrition absorption, growth and development and defence against pathogens (2021; Koch & Schmid-Hempel, 2011b; Raymann & Moran, 2018; Tauber et al., 2020). Therefore, the decline or loss of *Bombiscardovia*, *Bifidobacterium* and *Gilliamella* taxa could explain the lower survival of bumblebees in young SNGs and the change in α- and β-diversities. Given the positive relationship observed in old SNGs between floral diversity and abundance of certain core taxa such as *Bombiscardovia* and *Bifidobacterium*, it can be inferred that the low diversity and therefore the narrow range of pollen and nectar types, and the scarcity of flowers in young SNGs were partly responsible for the observed dysbiosis (Newbold et al., 2015). It is important to note that many other factors can potentially influence the microbiota, including the transfer of bacteria from vegetative organs or flowers, the assembly of which depends on local climate (Aydogan et al., 2018), as well as interactions with other pollinators or herbivorous insects (McFrederick et al., 2017).

In summary, our findings underline, the crucial role of grassland age on: (i) pollinator survival, (ii) the abundance of some essential core symbionts which appeared to be, to some extent, under the influence of floral diversity, (iii) the α-diversity and (iv) the interindividual similarity of gut microbiota. All these parameters significantly increased with grassland age which highlights the vital ecological role of old species-rich hay SNGs for pollinators.

On the other hand, flower diversity had no apparent effect, and floral abundance had limited positive effects on the body characteristics of bumblebees. These results are quite unexpected given the well-established importance of floral resource diversity and abundance for pollinators (Wright et al., 2018). This could be due to: (i) the influence of specific floral resource availability over richness per se on worker survival (Di Pasquale et al., 2016); (ii) the inaccessibility or unattractiveness of certain species to bumblebees, such as *Polygala vulgaris* (Pornon et al., 2019), *Achillea millefolium* (Larue-Kontić & Junker, 2016), *Agrimonia eupatoria* (King et al., 2013) or *Hypochaeris radicata* (Albrecht et al., 2007), (iii) the fact that a 15-day experimental period was possibly too short (due to an unusually early start of haymaking that year) to significantly alter the physical structure of insects, such as their body size and mass. Furthermore, (iv) it is possible that the individuals who died in greater numbers during the experiment in the young SNGs had fewer lipid reserves, were smaller and had a more disrupted microbiome than the individuals who survived. If this was the case, then the fact that we did not include the deceased individuals in our analysis has led to an underestimation of the negative effects of young SNGs on the health of the bumblebees.

Our study provides novel insights into the importance of the grassland age on pollinator health and opens new avenues of research on grasslands. Further studies will be needed to confirm our findings. Ideally, they should span the entire flowering period of the SNGs (i.e. 3 weeks to a month) and, consider, as much as possible, the individuals that died during the experiment. Our findings call for urgent attention and protection of old grasslands. Indeed, unlike old-growth forests (Gilg, 2015), these habitats have been overlooked in research and conservation efforts, despite their increasing rarity in many regions and their substantial ecological and cultural value.

AUTHOR CONTRIBUTIONS

A. Pornon supervised the study. A. Pornon and N. Escaravage performed field works. M. Lihoreau and B. Mahot-Castaing prepared the bumblebees hives. L. Moreau performed sample preparation and DNA laboratory work. A.-S. Benoiston performed the bioinformatics and data curation. G. Martin and J. White performed the statistical analyses. A. Pornon wrote the manuscript with contributions of all co-authors. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository: https://doi.org/doi:10.5061/dryad.x0k6djhv7.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1: Materials and methods.

Figure S1: Experimental design and sampling scheme. D0: start of the experiment (Day 0); D15: end of the experiment (Day 15). Unmarked individuals are individuals produced during the experiment.

Figure S2: Heatmap showing the prevalence and relative abundance of the 25 most abundant genera in the gut microbiota of the bumblebees used in the experiment ($n = 251$). Each vertical bar corresponds to an individual.

Figure S3: Relative abundance (%) of the 10 most abundant bacterial genera in the gut microbiota of the bumblebees used the experiment ($n = 251$). Each vertical bar corresponds to an individual.

Figure S4: Relative abundance of the 25 most abundant genera during the course of experiment (D0 vs D15) in (a) Old SNGs and (b) Young SNGs. Red asterisks indicate genera with a significant decrease in relative abundance over time and blue asterisk genera with a significant increase (ANCOM-BC analysis). The number of asterisks indicate the level of significance ($^{*}q < 0.05$; $^{****}q < 0.0001$; ANCOM-BC). Each vertical bar corresponds to an individual.

Table S1: Location (Lambert II coordinates) and age of the semi-natural grasslands investigated.

Table S2: Dataset characteristics at each step of the sequence analysis.

Table S3: Results of the differential abundance analysis (ANCOM-BC) of taxa at D15 according to SNG age, floral diversity (Shannon index) and colony size (total number of individuals). Taxa with significant differential abundances (Log Fold Change $> \pm 1.5$ and $q < 0.05$) are indicated in bold with corresponding LFC, SE, W test statistic, p-value and corrected q-value. Blue font colour indicates taxa with significant differential abundance according to SNG age.

Table S4: Results of the differential abundance analysis (ANCOM-BC) of taxa in young SNGs according to time (D0 vs D15), floral diversity (Shannon index) and colony size (total number of individuals). Taxa with significant differential abundances (Log Fold Change $> \pm 1.5$ and $q < 0.05$) are indicated in bold with corresponding LFC, SE, W test statistic, p-value and corrected q-value. Font colour indicates taxa with significant differential abundance according to time (Blue) and Floral diversity (Green).

Table S5: Results of the differential abundance analysis (ANCOM-BC) of taxa in old SNGs according to time (D0 vs D15), floral diversity (Shannon index) and colony size (total number of individuals). Taxa with significant differential abundances (Log Fold Change $> \pm 1.5$ and $q < 0.05$) are indicated in bold with corresponding LFC, SE, W test statistic, p-value and corrected q-value. Font colour indicates taxa with significant differential abundance according to time (Blue) and floral diversity (Green).

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