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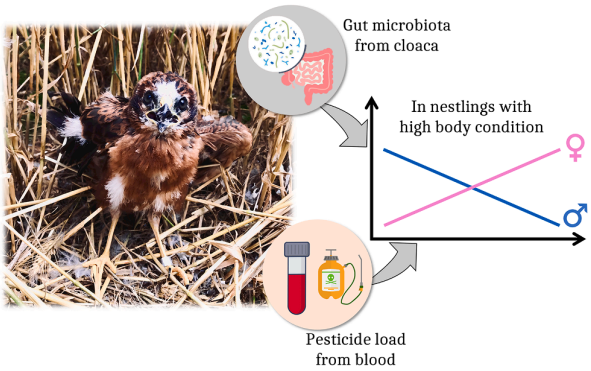
Léa Bariod [a,*], Elva Fuentes [a], Maurice Millet [b], Samuel Jacquiod [c], Joël White [d,e], Jérôme Moreau [a,f], Karine Monceau [a,g]

[a] UMR 7372, Centre d'Études Biologiques de Chizé, La Rochelle Université & CNRS, Villiers en Bois 79360, France
[b] Université de Strasbourg, CNRS-UMR 7515, ICPEES, 67087 Strasbourg cedex 2, France
[c] Agroécologie, Institut Agro Dijon, INRAE, Université Bourgogne, Université Bourgogne Franche-Comté, Dijon, France
[d] Centre de Recherche sur la Biodiversité et l'Environnement, UMR 5300, CNRS-IRD-UT3-INPT 5174, 118 Route de Narbonne, Toulouse F-31062, France
[e] Ecole Nationale Supérieure de Formation de l'Enseignement Agricole, Castanet-Tolosan, 31320, France
[f] Biogéosciences, UMR 6282 CNRS, Université de Bourgogne, 6 Boulevard Gabriel, Dijon 21000, France
[g] LTSER "Zone Atelier Plaine & Val de Sèvre", CNRS, Villiers-en-Bois 79360, France

HIGHLIGHTS

- Pesticides may impact gut microbiota structure.
- 36 compounds of pesticides were detected in blood of Montagu's harrier chicks.
- Microbial diversity shifts were related to pesticide load, sex and body condition.
- Proteobacteria were linked to lower pesticide load, Bacteroidota to higher levels.
- Farmland birds may serve as bio-sentinels in ecosystem health monitoring.

GRAPHICAL ABSTRACT



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ABSTRACT

Recent studies in humans have shown that certain pesticides could affect the composition and functions of the gut microbiota, an essential modulator of vertebrate physiology, leading to potential dysbiosis. However, this relationship remains largely unknown in wild birds despite the implications of pesticides in the current decline of farmland species. The present study sought to fill this gap by providing data on the association between pesticide concentrations in blood and gut microbiota characteristics in relation to individual traits in a farmland raptor, the Montagu's harrier (*Circus pygargus*). Results showed that females with higher body condition and higher pesticide load exhibited greater gut bacterial richness and diversity, while the relationship was opposite in males with higher body condition. In terms of taxonomic composition, Proteobacteria emerged as the dominant phylum across all nestlings. Differences in the abundance of specific phyla and genera were observed according to

* Corresponding author.
E-mail addresses: lea.bariod@cebc.cnrs.fr, bariod.lea@orange.fr (L. Bariod), elva.fuentes@cebc.cnrs.fr (E. Fuentes), mmillet@unistra.fr (M. Millet), samuel.jacquiod@u-bourgogne.fr (S. Jacquiod), joel.white@univ-tlse3.fr (J. White), jerome.moreau@univ-lr.fr (J. Moreau), karine.monceau@univ-lr.fr (K. Monceau).

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pesticide load, with higher levels of Bacteroidota and *Leifsonia*, but lower levels of *Bulkholderia*, in nestlings with higher pesticide concentrations in their blood. This study highlights differences in microbiota and contamination by several pesticides according to the phenotypic characteristics of a wild raptor, and shows that farmland birds can represent relevant biosentinels for assessing the health/proper functioning of ecosystems (One Health approach).

1. Introduction

The gut microbiota plays a central role in host metabolism and health [1]. It contributes to several physiological functions, such as energy harvest and storage [2], detoxification of trace metal elements [3], regulation of immunity and protection against pathogens [4,5]. However, this host-microbiota association can be disturbed by extrinsic factors such as antibiotic treatments, infections, changes in dietary habits, and other changes to the environment [6]. This disturbance, called dysbiosis, is often characterised by a reduction in beneficial microorganisms, concomitant with an increase in the number of potentially pathogenic microorganisms, leading to physiological disorders (e.g., digestive disorders, inflammation [7,8]. Among the disruptive factors, exposure to pesticides is likely to have a substantial impact on the structure of these microbial communities [9,10]. Indeed, pesticides are becoming a major health concern, especially in humans, because they can induce diverse pathologies such as metabolic diseases [11], dysregulation of the immune system [12,13], endocrine disruption [14] or gut dysbiosis leading to inflammatory damages and degenerative diseases [9,15]. Current research on the toxicity of pesticides on gut microbiota is thus carried out in humans [16-18], although recent studies in model species such as *Apis mellifera*, *Mus musculus*, and *Coturnix japonica* have demonstrated that increasing concentrations of some pesticides such as thiacloprid or trichlorfon in organisms decreased gut bacterial species diversity [19-21]. The disruption of the composition and, therefore, the function of this complex system can lead to significant metabolic imbalances, especially in glycolipid metabolism, as demonstrated in the gut microbiota of *Danio rerio* following exposure to carbendazim [22].

In birds, contaminant effects on gut microbial communities remain largely ignored, except for a few studies on experimental model species in controlled conditions (i.e., poultry, [23]; Japanese quails *Coturnix japonica*, [24,20,25]). However, captivity significantly alters the gut microbiota, likely due to the dietary, social and housing conditions, thus limiting extrapolations to wild species [26]. Wild birds remain understudied despite current concerns about their decline particularly that of farmland bird specialists due to the intensification of agriculture [27, 28]. Indeed, across Europe, these avian populations are declining, and one of the causes behind this decline is the use of pesticides and their toxic effects [29]; however, empirical studies on the eco-evolutionary consequences of the long-term use of agrochemicals remain lacking [30]. As they are pervasive in soil, water and air, pesticides are found in non-target organisms, even several years after banning (e.g., in mammals, [31,32]; in birds, [33], 2024; in insects, [34,35]. In the literature, the privileged exposition pathway for birds is through food and/or water ingestion [36]. Active chemical molecules pass through the digestive tract, are absorbed and then spread to various tissues *via* the bloodstream [19]. The gastrointestinal system, particularly the gut microbiota dwelling in this habitat, is the main interface between such synthetic chemical compounds and the organism's tissues. Given its involvement in processes linked to nutrient acquisition, the immune system and detoxification [37-40], it seems essential to integrate the gut microbiota into a global "One Health" approach to better understand the mechanisms linking pesticide exposure and avian population declines in rural areas [41]. In this context, farmland birds might represent valuable biosentinels, as the link between pesticide exposure, the gut microbiota, immunity and health can be transposed to humans [41,42].

The Montagu's harrier (*Circus pygargus*) is a migratory raptor species inhabiting agricultural lands, whose French populations have decreased by 10–30 % over the last 30 years [43,44], mainly due to agricultural intensification [45-47]. This bird species nests on the ground in cereal crops where nestlings dwell for approximately 35 days [48]. Consequently, they might be directly exposed to local pesticide contamination throughout their growth period, through direct spray on eggs, contact with chemicals on the soil and crop or especially *via* their diet (i.e., contaminated prey, see Fuentes et al., 2024). As a top predator, this species constitutes a relevant biological model, especially by being at the top of the food web with potentially higher exposure due to bioaccumulation/biomagnification [49]. Given that microbial colonisation of bird guts occurs after hatching [37,50] and that developing organisms are generally more vulnerable to environmental stressors and pollutants [25], gut microbial communities in Montagu's harrier nestlings could, therefore substantially reflect the level of contamination of the habitat in which they are raised, and highlight the effects of these pollutants on the gut microbiota.

To our knowledge, this study is the first to investigate the relationships between the gut microbiota and pesticide load in a non-target organism, the Montagu's harrier, at the crucial stage of fledging. Contrary to other publications considering a single molecule (e.g., [24,20, 25,23]), we capitalised on our ability to detect several pesticide molecules in bird blood [51] to assess the degree of exposure of wild populations and to examine the link between this diversity of molecules (i.e., the so-called cocktail effect) and microbiota characteristics. Blood has been chosen as a preferred matrix for pesticide titration because it is supposed to reflect recent pesticide exposure [52]. We hypothesised gut bacterial communities to be modified in diversity and composition in relation to pesticide load in the blood [19,21]. Given the deleterious effects of pesticides, we expected that nestlings with higher concentrations of pesticides detected in their blood would host gut bacterial communities with lower species diversity [53,54]. Pesticide load may reduce intra-specific variability in gut composition if deterministic homogenising processes are at play (e.g., [55] or, on the contrary, increase intra-specific variability if the stressor induces stochastic changes in microbiota composition [56]. Regarding specific taxa, we could expect a positive correlation between pesticide load and the relative abundance of phyla such as Bacteroides and Proteobacteria (e.g., *Coturnix japonica,* [20,24]).

2. Material and methods

2.1. Study site and biological model

Data on Montagu's harrier nestlings were collected in the Long-Term Social-Ecological Research (LTSER) platform Zone Atelier Plaine & Val de Sèvre (ZAPVS), a study site covering 450 km², located in the *Deux-Sèvres* department (southwestern France; 46°11′N, 0°28′W). This area contains open landscapes where agricultural activity is intense: cereal crops are the predominant cultures (ca. 41.5 % of the area between 2009 and 2016), followed by sunflowers and oilseed rape, with only a few urbanised areas and natural or semi-natural habitats [57].

In this area, Montagu's harrier population has been monitored since 1994 [57]. This raptor nests on the ground, mainly in cereal crops and lays up to eight eggs [58], which are incubated by females for 29 days. Nestlings then remain for approximately 35 days in the nest before fledging [48]. Female nestlings are, on average, larger than male nestlings in this species [45]. In the study site, the mean productivity of this species is reported to be 2.05 fledglings per breeding attempt [59]. The

breeding success depends mainly on food availability (i.e., mainly voles and orthopterans if voles are scarce, [46,60]). These prey are mainly provided by males to females and nestlings by hunting further away from the nest, i.e., the home range of males observed in the study area being ~14 km^2 [61]. Females may also bring food items to nestlings by hunting in the vicinity of the nest (<1 km) before fledging [62].

2.2. Sampling

The reproduction of Montagu's harriers was monitored from late April to late July 2022. Twenty-two nestlings (11 males, 11 females) from 13 different nests were studied. The nests were visited twice before the eggs hatched and every week subsequently. After hatching, nestlings were head-marked with a water-based coloured pen to allow individual identification. At 15 days, they were ringed with a unique coded aluminium ring provided by Museum National d'Histoire Naturelle de Paris (France) and sexed according to their iris colour (i.e., brown for females, grey for males; [63]). Finally, when nestlings were 26 ± 2 days old, they were carefully handled in a short time frame (see different steps in [64]) to weigh them with a Pesola 500 g spring scale (accuracy 5 g), to measure their left and right tarsus twice with a digital calliper (accuracy 0.1 mm), and to collect blood and microbiota samples. Based on tarsus length and body mass, we estimated the body condition of each nestling by using the scaled mass index (SMI) following Peig and Green [65]. In this study, males and females had an average (mean ± se) body condition of 294.22 ± 10.17 g and 320.63 ± 8.70 g, respectively. To assess pesticide load, 50 µL of blood was collected by puncturing the brachial vein using a sterile needle and heparinised capillaries and then placed in Eppendorf tubes. Gastrointestinal bacterial communities were sampled in the cloaca of nestlings. Cloacal bacterial communities have been shown to reflect bacterial communities in the higher intestine [66] and have successfully been used to study the microbiota of wild birds, representing a reliable non-invasive technique to assess inter-individual variability in gut communities [67,68]. Bacterial sampling was performed by washing the cloaca with 1 mL of sterile Phosphate-Buffered Saline (PBS) and drawing out approximately 1.5 mL of the faeces-PBS mixture. A negative control was performed for each sample. All samples (i.e., blood, cloacal solution) were frozen at −20°C for further analyses.

2.3. Microbiota analysis

2.3.1. PCR amplification and sequencing

Although samples were conserved in Phosphate Buffered Saline (PBS), they all underwent an extra rinsing with sterile x1 PBS after thawing and before extraction to reduce the impact of urea as a potential PCR inhibitor. Bacterial DNA was extracted following the method of Qiagen DNeasy® PowerSoil Pro Kit (Qiagen, Venlo, Netherlands). Bacterial DNA was amplified by PCR targeting the V3-V4 hypervariable regions of the 16 s rRNA, prepared in a sterile room dedicated solely to metabarcoding procedures. The 20 µL PCR solution contained 2X Master Mix AmpliTaq Gold (Fisher), 0.008X BSA, 0.25 µM forward primer (GTGYCAGCMGCCGCGGTAA) and 0.25 µM reverse primer (GGACTACNVGGGTWTCTAAT) and 2 µL DNA extract. The amplification profile used the following conditions: initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min 35 s and a final elongation step at 72°C for 10 min. Three PCR replicates identified by three different libraries were carried out to detect possible faulty PCRs [69]. To monitor potential contaminants at each step of the lab work, extraction controls and negative PCR controls were included in all amplifications. Positive PCR controls were included to monitor the amplification performance. To obtain a unique tag combination for each PCR product, the primers were tagged using a system of 36 octamers with at least five differences between them. Sequencing controls (non-existent tag combinations) were included to detect potential tag-jumps. The library construction (PCR-free Bioscientific library preparation kit) and the sequencing (Illumina MiSeq 250 bp paired-end v3 chemistry) were performed at the Genopole of Toulouse (France).

2.3.2. Bioinformatics and data curation

Illumina sequencing data were processed and filtered using the "OBITools" package [70]. Forward and reverse reads were aligned with the *illuminapairedend* command. Alignments with a score below or equal to 40 were filtered out [71]. Sequences were then assigned to samples using the *ngsfilter* command, allowing 0 and 2 errors on the tags and primers, respectively. Identical sequences were merged with the *obiuniq* command. Next, low-quality sequences were filtered out, i.e., sequences with less than one read in the whole dataset, sequences containing ambiguous bases or that were shorter than expected (<80 bp). Then, sequences were grouped into MOTUs (Molecular Taxonomic Operational Units) with *Sumaclust* using a similarity threshold of 97 %. The taxonomic assignment for each MOTU was carried out with the SilvaNGS pipeline v.1.9.10 and the SILVA SSU database v.138.1. Data curation was carried out using the R package "MetabaR" [72]. More specifically, PCR/sequencing errors, contaminant sequences, tag-jumps and dysfunctional PCRs were minimised. MOTUs with maximum abundance in the negative controls were considered contaminants, and those with a similarity to the reference sequence below 80 % were considered degraded sequences or chimaeras and were discarded. MOTUs that did not belong to the targeted clade (i.e., Bacteria) were also excluded. The noise produced by tag-jumps was reduced by removing a MOTU in a given PCR product when its relative abundance represented < 0.03 % of the total MOTU abundance in the entire dataset. Finally, PCR replicates with a sequencing depth below a given threshold, and those that did not show good reproducibility were removed. The final MOTU table was produced by summing the reads of the PCR replicates originating from the same sample. To standardise differences in sequencing depth among samples (range: 4387–31100 reads per sample with an average of 17684.8 reads per sample), we rarefied all samples to a 4300 reads (Fig. S1) for the alpha diversity, beta diversity and the taxonomic composition analyses.

2.3.3. Metrics studied

Several alpha and beta diversity metrics were chosen to characterise the gut microbial communities. Alpha diversity was estimated using i) the number of different MOTUs present in each sample as a measure of specific richness and ii) the Shannon diversity index (H'), which considers the relative abundance of each MOTU within samples [73]. Beta diversity [74] was estimated using i) the Bray-Curtis index (based on a relative abundance matrix, [75] and ii) the Jaccard dissimilarity index (based on presence/absence matrix, [76]. Both indices vary between 0 and 1, with a high beta diversity index indicating a low level of similarity, while a low beta diversity index shows a high level of similarity. The matrices of the two indices were noticeably distinct (Fig. S2). Thus, we chose to use both indices to measure beta diversity.

2.4. Pesticide analysis

2.4.1. Laboratory analyses

Pesticide is the term that often refers to plant protection products (PPP). These chemicals are composed of at least one active ingredient (herbicide, fungicide or insecticide) mixed with synergists (increasing the action of pesticides) or phytoprotectors (improving the selectivity of herbicides towards weeds rather than cultivated plants). In this study, a multiresidue pesticide analysis was carried out from blood samples following the method developed by Rodrigues et al. [51]. This method allowed the detection and quantification of 116 compounds, mainly active molecules (i.e., 38 fungicides, 43 herbicides, 33 insecticides), among the most used in the ZAPVS, as well as a synergist (piperonyl butoxide) and a phytoprotector (benoxacor). Subsequently, we will use the term "pesticides", which designates all the compounds sought,

including the phytoprotector and synergist.

Pesticide extraction was performed by concentrating samples and then subjecting them to multiresidue analysis using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and automated thermal desorption gas chromatography coupled to tandem mass spectrometry (ATD-GC-MS/MS) (see [51] for more details on methodology, with information on the quality assurance - QA - and quality control - QC). Briefly, QA and QC were determined for both LC and GC methods and each sample by repeating and analysing three times all calibration levels of all pesticide compounds from spiked samples on the same day (repeatability) and on different days (reproducibility). The limits of detection (LOD) and quantification (LOQ) values of detected pesticides are provided in Supplementary material (Table S1).

2.4.2. Metrics studied

In addition to the concentration of each pesticide, the contamination level is commonly assessed in ecotoxicology studies by the number of pesticides detected and the total sum of pesticide concentrations [31, 77]. Furthermore, as for the microbiota, we calculated a Shannon index per individual by transforming the concentrations to the nearest integer to represent the abundances of each pesticide, then, we studied beta diversity to assess the change in the diversity of pesticides from one profile to another. Thereafter, "pesticide load" will be used to refer generally to the level of pesticide contamination of individuals but will not target a metric unless it is specified.

2.5. Statistical analysis

Alpha (MOTU richness and Shannon index) and beta (Bray-Curtis and Jaccard indices) diversity indices were calculated with the R package "phyloseq" [78]. The beta diversity analyses were performed with R using the "vegan" package [79]. All the statistical procedures were performed using R v.4.3.2. [80].

To evaluate the possible links between body condition and the different metrics of microbiota or pesticide load, we performed bootstrap-based Spearman's rank correlation tests to obtain 95 % confidence intervals (95 % CI). We also used this test to determine which pesticide load metrics to use for studying the link between microbiota and pesticides, i.e., the number of pesticides or the sum of the concentrations. Then, to study the change in microbiota alpha diversity (MOTU richness, Shannon index) according to the contamination status, we used generalised linear mixed effects models (GLMMs), including the sex, the body condition and the pesticide load in interaction as fixed effects and nest as random effect. MOTU richness followed a Poisson distribution, and the Shannon index was log-transformed to fit a Gamma distribution. The distributions were adjusted based on the *descdist* function from the "fitdistrplus" package [81].

For microbiota beta diversity, we analysed the variance partitioning due to the interaction between sex, body condition, and pesticide load on the distribution of MOTUs based on dissimilarity matrices using PERMANOVA, a permutational multivariate analysis of variance (ADONIS function; 1000 permutations). We also used Mantel tests to investigate correlations between the microbiota dissimilarity matrix (Bray Curtis and Jaccard) and pesticide concentration dissimilarity matrix (Euclidean distance). Then, an EnvFit analysis (1000 permutations) was performed to determine whether each pesticide affects microbial composition distribution (Holm-Bonferroni adjusted p-value < 0.05).

To study microbiota taxonomic composition and differential taxonomic abundances according to pesticide load, we used the ANCOM-BC (analysis of the compositions of microbiomes with bias correction; [82]) function. We tested differential abundances of bacterial phyla and genera according to sex, body condition and pesticide load. All other parameters of the function were left on the default settings. Taxa with a Log Fold Change (LFC) value beyond $> |1.5|$ and an adjusted p-value (Holm-Bonferroni) below 0.05 were considered differentially abundant.

The pesticide molecules detected in nestling blood had a very different range of concentrations (see Table S2 and Fig. S3). Moreover, some individuals had a very high sum of concentrations (see below in Table 1). So, we chose to centre and scale the concentrations by molecule that refers to relative concentrations before summing them to obtain an index of pesticide load. This index was then used as the pesticide load variable in statistical analyses (i.e., GLMM, PERMANOVA, ANCOM-BC), hereafter referred to as the "sum of standardised concentrations" in the results section. The range of this variable varied between –13.12 and 20.73, corresponding to a variation from a low pesticide load to a high pesticide load respectively. Moreover, this index was strongly correlated with the sum of raw concentrations, the number of pesticides and the Shannon index on pesticide diversity (see Fig. S4), reflecting the level of contamination of individuals.

3. Results

3.1. Microbiota diversity and composition in nestling cloaca

From the 22 nestlings, we identified 139 distinct MOTUs, with on average (mean ± se) 26 ± 2 MOTUs per individual cloacal sample (26 ± 3 MOTUs per male and 26 ± 3 MOTUs per female). For the Shannon index, we had an average index of 1.93 ± 0.05 per individual cloacal sample (1.92 ± 0.09 per male and 1.94 ± 0.06 per female). Cloacal microbiota MOTU richness and Shannon index were not different among the 13 nests (Kruskall-Wallis tests: MOTU richness, $Khi^2 = 14.16$, df = 12, *p-value* = 0.29 and Shannon index, $Khi^2 = 8.72$, df = 12, *p-value* = 0.73). The relationship between individuals' body condition and microbiota metrics showed inverse trends depending on their sex (Fig. S4). Indeed, females with higher body condition harboured a higher MOTU richness and Shannon diversity of bacteria (Spearman's rank correlation test: $\rho_{\text{MOTU richness}} = 0.31$, 95 % CI = [-0.42;0.91], n = 11; $\rho_{\text{Shannon index}} = 0.61$, 95 % CI = [-0.07;0.92], n = 11), unlike males for whom the relationship seemed to be inverse ($\rho_{\text{MOTU richness}} = -0.51$, 95 % CI = [-0.85; 0.14], n = 11; $\rho_{\text{Shannon index}} = -0.49$, 95 % CI = [-0.88;0.15], n = 11).

Overall, three phyla (Proteobacteria, Actinobacteria & Firmicutes) were detected in all individuals, and to a lesser extent, the phylum Bacteroidota was present in more than 50 % of the nestlings in five males and seven females (Fig. S5). The phylum Proteobacteria was the most prevalent and abundant in the cloacal microbiota of our individuals, representing more than 75 % of the bacteria in all nestlings (Fig. S5). At the genus level, 90 genera were identified, with *Pseudomonas*, *Alcaligenes*, *Stenotrophomonas* and *Acinetobacter* being the most abundant and found in all nestlings (Fig. S6).

3.2. Pesticides detected in nestling blood

Overall, 36 of the 116 pesticide molecules were detected in the blood of the 22 nestlings, including 12 fungicides, 17 herbicides and seven insecticides (Fig. S3). Of the molecules found, 11 are supposed to be prohibited for use in France (Table S1). We identified on average (mean ± se) 11 ± 0.68 molecules per individual, with an average sum of the

Table 1
Pesticide load description in the 22 nestlings according to their sex. The sum of concentrations is in pg/mg.

		Mean ± SE	[Min; Max]
Males (n = 11)			
	Number of pesticides	10.64 ± 0.90	[5; 15]
	Sum of concentrations	2717.61 ± 641.32	[551.87; 6314.26]
	Shannon index	1.86 ± 0.09	[1.33; 2.24]
Females (n = 11)			
	Number of pesticides	11 ± 1.06	[6; 16]
	Sum of concentrations	4271.16 ± 879.43	[1510.30; 11772.86]
	Shannon index	1.96 ± 0.12	[1.15; 2.41]

concentrations of 3494.38 ± 557.49 pg/mg and an average Shannon index of 1.91 ± 0.08 (see Table 1 for the description of contamination profiles by sex). Five pesticides were present in half or more of the nestlings: ethofumesate (n = 19), tebufenpyrad (n = 16), propiconazole (n = 14), lenacil (n = 11) and S-metolachlor (n = 11). Ethofumesate was present in all males and 8 of the 11 females. The number of pesticides, the sum of pesticide concentrations (raw concentration values) and the Shannon index on pesticide diversity in individuals were not different among the 13 nests (Kruskal-Wallis tests: Number of pesticides, Khi^2 = 11.20, df = 12, *p-value* = 0.51; Sum of concentrations, Khi^2 = 13.26, df = 12, *p-value* = 0.35; Shannon index, Khi^2 = 13.69, df = 12, *p-value* = 0.32). Body condition did not appear to be correlated with pesticide metrics (Fig. S4). Only one trend really seemed to appear in males, with a positive relationship between their body condition and their pesticide diversity estimated by the Shannon index (ρ = 0.47, 95 % CI = [-0.35;0.92], n = 11), although this was not significant (ρ = 0.47, *p-value* = 0.15). For the other relationships, Spearman's rank correlation tests did not show any significant correlations between body condition and a) number of pesticides (males: ρ = 0.17, 95 % CI = [-0.55;0.81], n = 11; females: ρ = 0.15, 95 % CI = [-0.50;0.78], n = 11), b) sum of pesticide concentrations (males: ρ = −0.28, 95 % CI = [-0.80;0.46], n = 11; females: ρ = −0.04, 95 % CI = [-0.74;0.75], n = 11), c) Shannon index (females: ρ = −0.29, 95 % CI = [-0.85;0.44], n = 11).

3.3. Relationship between the gut microbiota and pesticide contamination

Cloacal microbiota MOTU richness was significantly affected by the sum of the standardised concentrations in interaction with the sex and the body condition (GLMM: z value = −3.16, *p-value* = 0.002; Fig. 1, and see the summary of GLMM in Table S3, Supplementary Material). Indeed, the MOTU richness of the microbiota increased with the sum of standardised concentrations of pesticides only in females with a high body condition (group "High" in Fig. 1). Conversely, in males with a high body condition (group "High" on Fig. 1), microbiota richness decreased with increasing pesticide load. The result was the same with the Shannon diversity index, which was also influenced by the triple interaction between sex, body condition and the sum of standardised concentrations (GLMM: t value = −2.77, *p-value* = 0.006; see the summary in Table S3). The Shannon index representing the microbiota diversity increased with the sum of standardised concentrations of pesticides in females with a high body condition, while in males with a high body condition, it decreased.

Regarding the beta diversity, we did not find any effect of the interaction between sex, body condition and the sum of standardised concentrations of pesticides on microbial community structure (PERMANOVA: Bray-Curtis distances, F = 0.23, *p-value* = 0.94; Jaccard distances, F = 1.13, *p-value* = 0.27). The microbiota dissimilarity matrices (Bray-Curtis and Jaccard, respectively) were not correlated with the pesticide concentration dissimilarity matrix (Mantel: r = -0.14, *p-value* = 0.79 and r = 0.03, *p-value* = 0.41). The EnvFit analysis did not demonstrate any effects of specific pesticides on the microbial structure (Table S4).

Finally, regarding taxonomic composition, we separated our individuals into two groups of differences in pesticide load: low sum of standardised concentrations (Low pesticide load) and high sum of standardised concentrations (High pesticide load), ranked according to the median to have the same number of samples in each group. At the phylum scale, we only found a significant difference in the relative abundance of Actinobacteria between these two groups of individuals (Table S5), with more of this phylum in nestlings with a high pesticide load in their blood (nestlings with high pesticide load: median = 4 % of the microbiota; nestlings with high pesticide load: median = 2 % of the microbiota). However, the ANCOM analysis highlighted a significant effect of the sum of the standardised concentrations on one phylum, Bacteroidota (lfc = 0.09 ± 0.02, W = 4.06, *q-value* = 0.018). No effects of sex and body condition were found (Table S6). At the genus level, no effect of the variables tested (sex, body condition, sum of the standardised concentrations) was found with the ANCOM analysis on the ten main genera (Table S6). However, focusing on the ten most common genera (out of 90 detected), we found that individuals who had a lower pesticide load (on the left in Fig. 2), had a higher relative abundance of *Burkholderia* (in light green, median = 4 % of the microbiota; 0 % for the

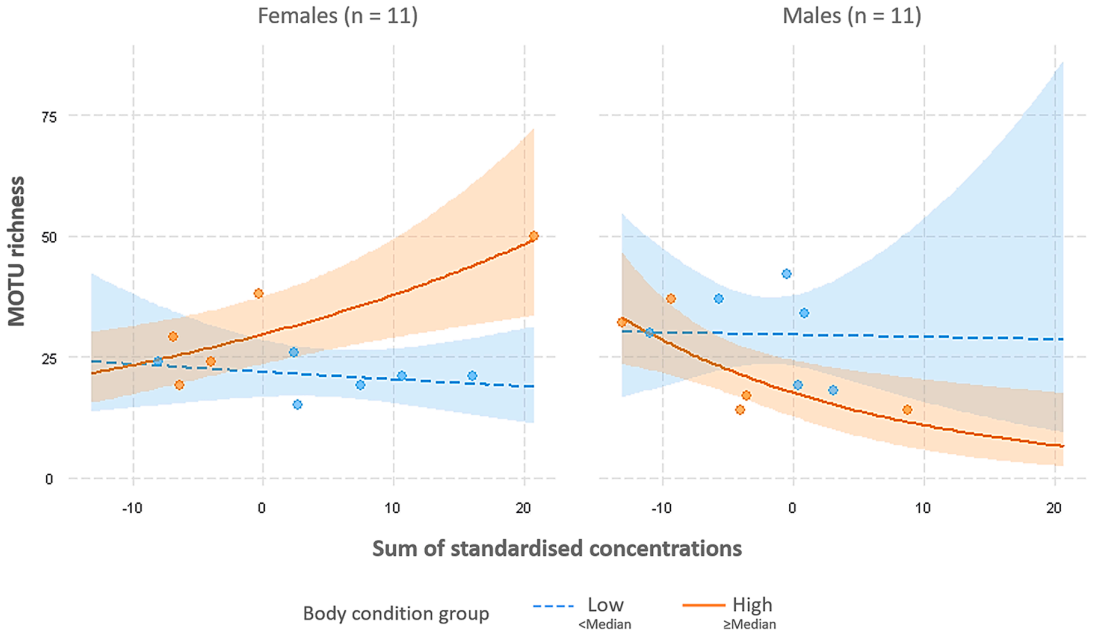

Fig. 1. Relation between MOTU richness and the sum of standardised concentrations according to the body condition and by sex. For greater visibility of the results, we divided our individuals into two classes of body conditions established by sex around their median condition: Group "Low" in blue, below the median corresponding to a low body condition (males: [245.26 g; 288.12 g], females: [283.25 g; 313.07 g]); Group "High" in orange, above the median corresponding to a high body condition (males: [288.12 g; 358.03 g], females: [313.07 g; 372.51 g]).

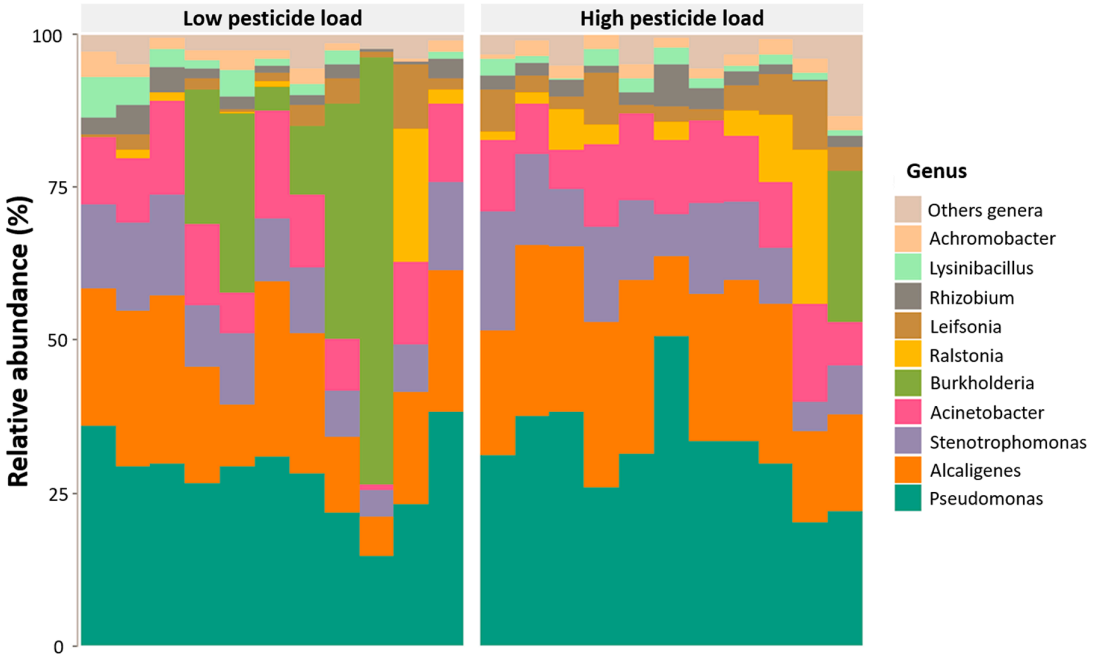

Fig. 2. Relative abundance of the ten most common genera found in the microbiota of Montagu's harrier nestlings according to their pesticide load. "Low pesticide load" corresponds to the load below the sum of the standardised concentrations median, and "High pesticide load" corresponds to the load above the sum of the standardised concentrations median.

"High pesticide load" group) and a lower relative abundance of *Leifsonia* (in brown, median = 2 % of the microbiota; 4 % for the "High pesticide load" group) in their microbiota (Mann-Whitney U tests: *Bulkholderia*, W = 88, *p-value* = 0.03; *Leifsonia*, W = 27, *p-value* = 0.03). We did not find any differences for other genera (Table S5).

4. Discussion

To our knowledge, this study is the first to examine Montagu's harrier gut microbiota and provides evidence for the effect of exposure to a cocktail of pesticides on the gut microbiota of farmland bird according to their sex and body condition. Briefly, at a high phylogenetic level, the global composition of the gut microbiota is congruent with what is typically found in birds, featuring the four main phyla: Actinobacteria, Bacteroidota, Firmicutes and Proteobacteria. This last phylum was found predominantly in all nestlings; nevertheless, the genus *Burkholderia* detected more in individuals who had a lower pesticide load in their blood. Another phylum, Bacteroidota, was significantly more present in nestlings with a higher concentration of pesticides in their blood. Regarding the characteristics of nestlings, in high-body condition females, there was a positive correlation between pesticide load and gut microbiota diversity, while the relationship was the opposite in high-body condition males, with a lower microbial diversity when pesticide load was high. These results suggest that body condition exacerbates the effect that pesticides have on the microbiota richness and diversity in a sex-dependent manner.

4.1. Microbiota structure

The microbiota composition we found is common to those described in wild birds [39], with the main represented phyla being Proteobacteria, Actinobacteria, Firmicutes and Bacteroidota, in decreasing order of relative abundance. Firmicutes are often the phylum that dominates the avian cloaca microbiota [37,83,84], whereas in Montagu's harrier nestlings, Proteobacteria were the most present. However, this may depend on many factors and should not be generalised for all bird species. For instance, a study on another raptor, the Eurasian Kestrel (*Falco tinnunculus*), showed that the same four major phyla (Proteobacteria, Actinobacteria, Firmicutes and Bacteroidota) were found in their microbiota (investigated in faeces) but adults harboured higher abundances of Proteobacteria while nestlings exhibited higher abundances of Firmicutes and Actinobacteria [85]. Another study showed that the cloaca microbiota of the insectivorous barn swallow (*Hirundo rustica*) contained Proteobacteria outnumbering Firmicutes by a 2:1 ratio [86]. Thus, the composition of the microbiota is dependent on factors related to the sampling site, the stage of host development and also the bird species studied, making general rules difficult to raise.

In the Montagu's harrier nestlings, Proteobacteria were the dominant phylum, and this result is reflected at the genera scale. Four genera were found in all nestlings, all belonging to the Proteobacteria phylum: *Acinetobacter*, *Alcaligenes*, *Pseudomonas*, and *Stenotrophomonas*, from which very few are currently known in the literature, especially in bird species. *Acinetobacter* or *Alcaligenes* species are usually commensal organisms, but they occasionally cause infections, as in Humans [87,88]. *Stenotrophomonas* spp. are often associated with soil and plants [89], this genus containing opportunistic bacteria that are widespread within the environment [90]. Likewise, *Pseudomonas* includes bacteria widely distributed in the environment, particularly in soil and water, and thus commonly found in plants and animals. This genus includes bacteria with numerous functional and environmental implications (see [91], and some of them are associated with health issues, such as *Pseudomonas aeruginosa*, which is the most documented and important species affecting humans, responsible for serious life-threatening infections [92,93]. The increase of these pathogen bacteria in hosts could reflect host responses to environmental stressors, such as contamination by pesticides.

4.2. Pesticide contamination patterns

A wide range of pesticides was present in the studied nestlings, highlighting the agricultural context in which the nestlings are raised. The pesticide loads found in nestlings were variable, with some

individuals being strongly contaminated (e.g., 15 pesticides in their blood and 11772.86 pg/mg) and others having lower contamination levels (e.g., five compounds and 551.87 pg/mg). Herbicides were most abundant in nestlings (17 of 36 compounds), with ethofumesate present in 19 of 22 individuals and dicamba present at the highest concentrations (Table S2, Fig. S3). All pesticide molecules found in individuals were considered non-toxic based on acute toxicity hazard classification, even for molecules that were found at high concentrations, such as dicamba, whose highest concentration (4272.08 ± 217.84 pg/mg) found in all individuals did not exceed 4 % of the LD50 value, the median lethal dose (Table S1).

Montagu's harriers nest on the ground in cereal crops, which are dominant in the study area. Our results highlighted that naïve individuals such as nestlings might be contaminated by pesticide mixtures after only four weeks of life in crop plots. The quantities of substances applied on the study site and around the nests may vary depending on several parameters: quantity of pesticide to be applied per hectare, concentrations of active ingredients in the products, and type of crop. For example, a beet crop can lead to higher use of metamitron, while a corn crop will be treated with other herbicides such as dicamba. Overall, concentrations of different pesticides found in nestlings reflected the general pattern of pesticide use in the study area [94,95] and would involve the persistence of substances for several weeks or months in crop plots, i.e., given that the treatments are mainly done before the reproduction of the Montagu's harrier, starting in winter.

Moreover, the variability of pesticide load in nestlings could come from diverse exposure routes: ingestion of contaminated prey (e.g., voles), breathing and/or skin contact with contaminated soil, rain or crops [94-96]. These diverse routes can be a source of variability in the contamination of nestlings, for example, with individuals that eat more and ingest more pesticides than others. Among the molecules detected in the blood of nestlings, metamitron and carbendazim are examples of molecules that had also been detected in micromammals in the study area, possible sources of food contamination in the Montagu's harrier [31]. Pesticide ingestion can occur differently among individuals, depending on the type of prey ingested, for instance. Although we did not evaluate the pesticide content of the soil or crop at the nest in this study, it would be interesting to do so in the future to assess the influence of these possible sources of contamination. It would also be interesting to carry out pesticide assays in the blood of voles, the Montagu's harrier preferred prey, in the areas where the parents hunt. The analysis of hairs contained in the pellets of rejection found in the nests could also provide insight into the contamination by pesticides of part of their ingested diet, although the pellets of diurnal raptors such as Montagu's harriers are well-digested and difficult to analyse precisely.

Finally, the global contamination of all nestlings is also worrying because we observed that several molecules found in the blood of chicks have been banned from use for several years, e.g., acetochlor, bifenthrin, quinoxyfen, terbutryn. This may suggest either fraudulent use, strong persistence in soils treated before the ban, or the detoxification process of females exposed during their wintering in Africa to pesticides, which are still authorised for use [96]. Our results, therefore, highlight the ubiquity of pesticides in agroecosystems, including some that have been banned for many years.

4.3. Potential role of pesticide contamination on gut microbial community shifts

Regarding the microbial composition of the nestlings, this seems to reflect their contamination by pesticides. Bacteroidota increased in the presence of a higher pesticide load in nestlings seems in agreement with the result of a study on Japanese quail, which had shown a positive relationship between Bacteroidota and pesticide load [24]. Other experimental studies on Japanese quails have shown that the ingestion of trichlorfon, an organophosphate insecticide, induces an overall increase in Proteobacteria in microbiota, which can also be identified as an indicator of microbial dysbiosis in mammals [97-99]. Thus, the high presence of Proteobacteria in the nestling microbiota could be the consequence of the disturbance by all the pesticides and could become deleterious for the host. The high presence of this phylum could also reflect the plasticity/acclimatisation/adaptation of the host to the disturbance due to pesticides [7,100]. Among different phyla, Proteobacteria are known to harbour many pesticides degrading bacteria [101], but Actinobacteria are also known to remove xenobiotics such as pesticides [102], which could also explain the higher abundance of this phylum in nestlings with a higher pesticide load in their blood. The high abundance of Actinobacteria is mainly explained by the members of the genus *Leifsonia*. Thus, the strong presence of Proteobacteria and Actinobacteria in nestlings could be an early adaptation of these individuals to an agricultural context where a wide range of pesticides is used.

Another genus caught our attention, *Burkholderia* (Proteobacteria), which was significantly more abundant in nestlings that had a lower concentration of pesticides in their blood. Commonly found in soil and associated with plants, *Burkholderia* includes certain bacterial strains such as *Burkholderia cenocepacia*, which are known to degrade methylparathion, an organophosphate pesticide, or lindane, and organochlorine pesticide, in soils [103-105]. In animals, *Burkholderia* spp. are gut bacteria common to a wide range of heteropteran insects, including major pests in agriculture (e.g., *Riptortus pedestris*) [106]. In these hosts, studies have highlighted the involvement of these bacteria in the degradation of pesticides, such as fenitrothion in stinkbugs, imparting protection against organophosphorous pesticides to these organisms as a symbiont [107,108]. Insects are part of the diet of Montagu's harriers through direct (mainly orthopteran insects) or indirect consumption through voles, which are insectivorous [109,110]. Studying the diet of the Montagu's harrier nestlings through the composition of the rejection pellets to identify the different items and then the study of some prey (e.g., insects) could be the first step to verify if the prey that can be ingested by nestlings contains high abundances of *Burkholderia*.

4.4. Sex differences in gut microbiota responses to pesticide contamination

Male nestlings with a high body condition had lower microbial richness and diversity when they had a higher pesticide load, whereas for high body condition females, the relationship was reversed. This was not the case for nestling with a low body condition, regardless of their sex. Pesticides such as neonicotinoids or organophosphates have already been associated with a reduction in microbiota diversity [20,24], which agrees with the observed pattern in males. In females, the opposite trend could be explained by physiological and particularly endocrine processes, which are different from those of males.

Given that Montagu's harrier is an altricial species, juveniles depend on food provided by their parents, and the larger sex, i.e., female [45], is usually more expensive to raise and has overall higher nutritional requirements, also showing higher growth and metabolic rates [111]. Females with a higher body condition could thus modulate the toxic effect of pesticides on the microbiota and reduce these impacts by dilution or retention effect in adipose tissues [112]. This would suggest a lesser impact of pesticides on the microbiota of females thanks to the presence of more adipose tissues, compared to males, which are generally smaller and have a less advantageous metabolism to reduce the effects of pesticides. Moreover, males would have not the advantage over females to acquire more food in the nest [45]. This priority effect in resource acquisition could leave the males with fewer choices, with items rejected by females or with less food available, particularly when food is not abundant, and parents are taking smaller prey, such as insects instead of micromammals. This could thus suggest a difference in the sources of microbial colonisation between the sexes, i.e., females with a higher body condition ingesting more varied food items, which would increase their microbial diversity. While males would have fewer food choices and, therefore, less microbial diversity ingested, being more

reflected in larger males.

5. Conclusion

This study highlights for the first time a relationship between the exposure to a cocktail of pesticides and the variation in the composition of the gut microbiota in a farmland raptor, although correlative and obtained on a few individuals (due to the conservation status of Montagu's harriers). Given the crucial role of the microbiota have on its host health and physiology, such as vitamin synthesis, host metabolism, nutrient absorption, immune function, and organ development [50, 113], it is mandatory to further investigate how pesticide cocktails act on this major physiological function. Our results thus open new perspectives to understand the contribution of pesticides to farmland bird health and, to a greater extent, to the health of non-target organisms.

Environmental implication

While the role of the gut microbiota in vertebrate health is increasingly recognised, no study to date has examined changes in the composition of the microbiota in relation to exposure to pesticides under natural conditions. We took advantage of the monitoring of a wild raptor species, which can be considered a biosentinel of our environment, in a "One Health" context. We measured more than a hundred pesticide molecules in the chicks' blood and the bacterial composition of their gut flora. This significant advance improves our understanding of the complex effects of pesticides on this fundamental physiological function.

Ethical consideration

This work has received approval for research ethics from the "Centre de Recherches sur la Biologie des Populations d'Oiseaux" (CRBPO; Museum National d'Histoire Naturelle; licence #1308) and a proof of approval is available upon request. Bird captures, and experimental procedures conformed to the French guidelines for the ethical use of animals in research (APAFIS #36725–2022041910139000 v6).

CRediT authorship contribution statement

Léa Bariod: Writing – original draft, Formal analysis, Conceptualization. **Elva Fuentes:** Writing – review & editing, Conceptualization. **Maurice Millet:** Writing – review & editing, Formal analysis. **Samuel Jacquiod:** Writing – review & editing. **Joël White:** Writing – review & editing, Formal analysis, Conceptualization. **Jérôme Moreau:** Writing – review & editing, Supervision, Conceptualization. **Karine Monceau:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors have nothing to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2024.136857.

Data availability

The raw sequencing data are deposited on Dryad (datadryad.org) with the following accession number: DOI:10.5061/dryad.r7sqv9snf. The rest of the data will be made available on request.

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