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Collection methods of wild barn owl pellets at low environmental contamination and proposals of microbiological and ecological investigations

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Abstract

Background The barn owl *Tyto alba* is a medium-sized nocturnal raptor, predator of small mammals, birds, reptiles, and insects, distributed all over the world. Environmentally uncontaminated wild barn owl pellets are hard and rare to find and provide a natural matrix for biological investigations.

Results Different solutions for collecting wild barn owl pellets at low environmental contamination are proposed. These solutions are based on a daily sanitized surface for sample retrieval, followed by rapid analysis. As an example of the possible investigations that can be carried out on these matrices, with the aim to highlight the relevance of the obtained results also from a biotechnological perspective. Two rare pellet samples from wild barn owl *T. alba*, appropriately collected in a rural area of central Italy, were analyzed using culture-dependent, molecular (Next-Generation Sequencing), and enzyme profile analysis techniques. The osteological observations of the bone remains provided useful information for identifying the prey, mainly rodents (*Apodemus* sp.). Under our experimental conditions, the results revealed both a wide heterogeneity between the pellet microbiota and a great percentage of uncultured bacteria not classified at the species level. Furthermore, microbial cultures of *Malbranchea albolutea*, *Debaryomyces hansenii*, and *Lactobacillus* sp. were isolated and studied.

Finally, we quantified the environmental impact of our experimental work in terms of CO_2 equivalent release. To compensate for the release of 300 kg/CO_2 eq., three *Taxus. baccata* L. were planted.

Conclusions This work provides a starting point for developing an effective strategy to study and characterize wild barn owl pellets at low environmental contamination. It presents a simple and easy technique/protocol for collecting the pellets. The microbiota heterogeneity found in the two analyzed samples suggests that barn owl pellets can represent a potential natural reservoir for the discovery of new microorganisms to be used in various biotechnological applications. This could open the way for further large-scale studies on a greater number of samples and populations.

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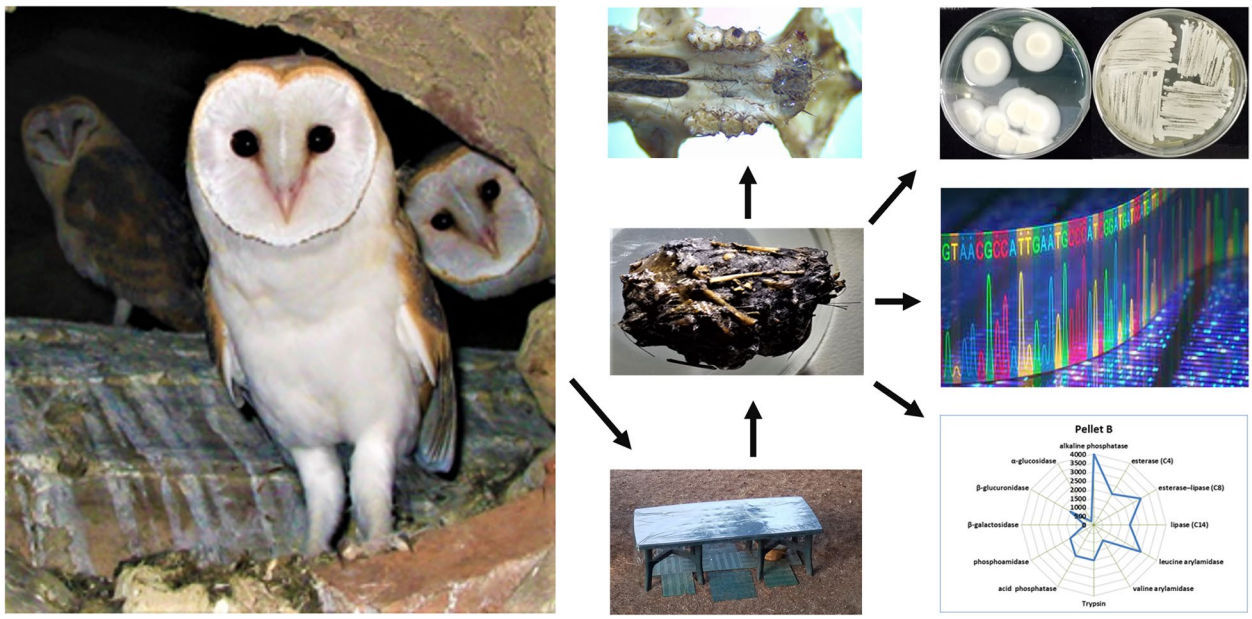
Graphical Abstract



Introduction

The barn owl (*Tyto alba*) is a medium-sized nocturnal raptor representing the most widespread owl species on Earth (Kross et al. 2016). It is distributed in Europe, Africa, Southern Asia, Oceania, North and South America, both on mainland and on islands (BirdLife International 2019; Viganò et al. 2020). This raptor plays a very important role in the ecological balance of a territory. Beyond its ecological importance as a top predator, *T. alba* may be considered a biological control agent of rodent pests in agriculture (Marti et al. 1979; Wood and Fee 2003; Whelan et al. 2008; Meyrom et al. 2009; Kross et al. 2016, 2018).

Barn owls readily occupy human-made structures, persist in agricultural settings better than many bird species and, with enough nests, several breeding pairs may be present in one km^2 (Taylor 2003; Kasprzykowski and Goławski 2006; Meyrom et al. 2009; Roulin 2020). These factors, together with their ability to remove large quantities of rodents and the abundance of rodent pests within agroecosystems, make them an attractive option for farmers to control pests (Durant et al. 2004; Marti 2010; Kross et al. 2016; Johnson et al. 2018; St. George and Johnson 2021).

Raptors have a varied diet, some rely on insects, others on small birds, many on small vertebrates (Teta et al. 2012; Roulin and Dubey 2012; Roulin and Christe 2013). In the raptor stomach, the small mammal is subjected to the action of digestive juices that destroy the tissue leaving the skin and skeleton intact. These residues, under the action of a rotational movement, aggregate together until they gradually form an ovoid mass, which the bird of prey will regurgitate after a few hours. This small elongated greyish ball, often pointed at its extremities, is called "pellet" or "rejection ball". The formation of pellets avoids a long and difficult digestion of the unassimilated elements by the raptor (mammal hair and bones, insect chitin, insect larvae hair, etc.). Raptors usually reject one pellet per day, rarely two or three, which may contain the remains of several small mammals. The pellets accumulate at the foot of nocturnal raptor nests, in church steeples, in isolated attics, in hollow trees, on cliffs and in cave entrances (Chaline et al. 1974). Since the analysis of pellet content is relatively easy and cheap, barn owl diet has been thoroughly investigated, especially in Europe and America (Jaksic et al. 1982; Bellocq 2000; Love et al. 2000; Cagnacci et al. 2012; Milana et al. 2016, 2018; Romano et al. 2020). Barn owl diet can offer a source of information on the state of conservation of the environment surrounding their settlement (Chaline et al. 1974). However, despite the several studies carried out on barn owl pellets, almost no one has focused on the analysis of pellet associated microbial communities. The study of microbial community dynamics is important for many aspects, including ecological and scientific discovery, biotechnological development, sustainable agriculture, environmental protection, and human health (Bucci et al. 2017).

It is well known that nature offers great potential for the study of both microbial biodiversity and functional ecology. The study of animals living in captive conditions are quick, convenient, easy, and more easily attributable to a specific individual (e.g. age, sex, etc.). Indeed, the collection of residues and wastes (feces, urine, litter, etc.) for example from cages and aviaries is a usual practice. However, the condition of captive life (e.g., feeding regimen, instinctive behavior, etc.) induces profound alterations in animals compared to the wildlife (Rawat and Agarwal 2015; Feckler et al. 2023).

Obtaining "genuine" samples of residues and wastes from animals in the wild free from environmental contamination is very difficult and complex. In fact, wild animals release their residues randomly and very unpredictably. Therefore, the collection of samples from free-living animals in natural matrices (e.g., soil, plant surfaces, water, etc.) is tedious, time-consuming, and often fails to yield useful results. The environment rapidly induces contamination of free-living animal residues altering the genuineness of the samples (Holt and Miller 2010). In view of this, how to collect wad samples at low environmental contamination? What kind of useful information could be derived from samples obtained from wild raptors? (Hegedus et al. 2023).

To address these questions we propose: (I) a feasible solution for recovering residual pellet samples from wild nocturnal raptors; (II) microbiological investigations to characterize *T. alba* wad microbiota and to unravel the potential presence of novel and uncharacterized strains of biotechnological interest; III) a method to acquire information on the barn owl diet through wad osteological observations; IV) finally, an evaluation of the environmental impact of our experiments, in terms of CO_2 equivalent release, and how to compensate it.

Materials and methods

Experimental design

The experimental design adopted in this research (Fig. S1) has been developed with the aim to provide an effective strategy to collect and study from a microbiological and ecological perspective wild barn owl pellets, at low environmental contamination.

Sampling site

The sampling site of this study is located at a rural area (42° 20′ 59.23″ N 14° 01′ 55.38″ E) of Rosciano municipality, Abruzzo region, (Central Italy). The area is at 20 km from Adriatic Sea, where the territory is mainly hilly (150–300 m altitude) at a transitional zone from Mediterranean to temperate bioclimate (Blasi et al. 2014). The area is characterized by i) a mosaic of croplands, mainly herbaceous (cereals) and horticultural crops used in succession as sheep pasture; ii) uncultured areas with Mediterranean and sub-Mediterranean arboreal and shrubby vegetation, hedges and coppices composed of mixed *Quercus pubescens* Willd. and riparian vegetation of the valley floor; iii) large area with destination both to traditional and specialized orchards (olive cultures) and vineyards (Montepulciano d'Abruzzo cultivar). The landscape composition was confirmed using Google Earth (®) (last accessed July 2023) and landscape elements were analyzed within a 1 km radius around the nest site of barn owls (Fig. S2a and b).

Pellet collecting systems

Based on previous long-term observations of the area where the active wild barn owls' nest was located (less than 20 m), we noticed that adults released their pellets by night. It was noticed that remains of pellets were sometimes found in a narrow area, under the tree canopy, in the garden. Therefore, four different sample collecting systems were proposed, tested, and compared (Fig. 1a and b).

System 1. A hard, flat surface of about 2.5 m^2, like a Table (2.40 m × 1.0 m; height from the ground, 90 cm) in PVC, green coloured, was adopted. A daily checked table surface covered with a transparent plastic sheet was cleaned before nightfall by a hygienic water solution of alkyldimethylbenzylammonium 10% w/v (Neo-Desogen®, Teleflex Medical S.r.l., Varedo, Italy) and rinsed with sterile water. The two main reasons for the operating conditions described above are: (a) to avoid contamination by soil microorganisms and, (b) to define the date of recovery of samples.

System 2. A perforated (1.0 × 1.0 cm), rigid PVC net suspended two meters above the ground; tension is provided by tie rods on 4 posts driven into the ground, placed at the corners of the rectangular-shaped net (2.0 m × 5.0 m). Every day, the surface of the net is inspected and cleaned of plant residues that may have settled inside, fallen by gravity or atmospheric events (rain, wind, etc.). Before sunset, the upper surface of the net is washed and disinfected from above with a spray bar containing a 2% benzalkonium chloride solution in water, followed by a final rinse.

System 3. A perforated (1.0 × 1.0 cm), roll-up net in nylon, suspended two meters above the ground; tension is provided by tie rods on 4 posts driven into the ground, placed at the corners of the rectangular-shaped net, followed by sanitisation phase, as above (system 2);

Fig. 1 Images of wild barn owls *T. alba* living under a roof of Villa Vanda house, at Rosciano, Italy: **a** System 1; **b** System 2, at low environmental contamination. **c** One individual, in the position of alarm and/or fear; **d** Three individuals living in the same nest, in rest position

System 4. A vegetable roll-up fabric net (natural hemp, jute, cotton, etc.), adopted like the above system 2 and 3.

Samples and sampling

Two pellet samples (henceforth reported as A and B) were collected in Rosciano municipality. After sampling, A and B pellets were quickly subjected to the following laboratory activities: partial sterile dissection for optical observation and morphological analyses; inoculation in different media, for microbial growth, isolation, and characterization of isolates; DNA extraction for molecular investigations of the microbial communities. The pellets A and B from wild barn owls (*T. alba*) were freshly collected in the same site on 15th and 30th December 2022, respectively. Both pellets were collected under a 40-year-old tree (*Pinus nigra* J.F. Arnold). Then, the pellets were handled with latex gloves, immediately placed in sterile conical centrifuge tubes (volume 50 mL) (Corning Pharmaceutical Glass, SPA, Pisa, Italy), and transferred to the laboratory under refrigerated conditions for subsequent analyses, as quickly as possible, and no later than 48 h.

The collection surface is located less than 20 m from an active nest located under the roof of a historic building dating from the late 18th century (Villa Vanda, Rosciano, Italy) (Fig. 1a and b). The nest location allowed us to know the beginning and end of nocturnal predation activity. Therefore, images were acquired manually with a digital camera (Nikon, Coolpix L19 model) at first light when the raptors returned to the nest (Fig. 1c and d).

Pellet analyses and morphological observations

At laboratory scale, sterile conditions were adopted to avoid contaminations during the main phases of pellet analyses. Preliminary, pellets were weighted by a lab balance (Extend mod., Sartorius, Goettingen, Germany), under a bunsen flame. Then, the pellets were processed and disassembled, under sterile cabinet, to obtain subsamples (in small pieces) both for microbial cultivation and molecular investigations of bacterial and fungal communities. Measures of pH and conductivity were performed with HI 763100 and HI11310 probes, respectively (Edge mod., Hanna Instruments, Woonsocket, RI, USA). The pH values were determined in ratio 1:10 w/v a physiological solution (0.9 M NaCl).

The remaining, almost complete pellets were submitted to optical observation by stereomicroscope (Axioplan mod., Zeiss, Germany) at 10x, 20x and 35x magnifications to identify barn owl prey. Records of images and morphological data (shape and dimension) with bar scale were acquired (Fig. 2a and b). The osteological parts were observed, and the identification was performed at the lowest possible taxonomic level (species level) comparing the undigested bone rests with the reference literature (Chaline et al. 1974; Di Palma and Massa 1981; Erome 1982; Brom 1986; Nappi 2001; Paolucci and Bon 2022). According to McDowell and Medlin 2009, the minimum number of individuals obtained by counting the most common diagnostic osteological remnants (element) of each species of small mammal in each pellet determined the prey number (McDowell and Medlin 2009).

Media and cultural techniques

The microbial growth tests were performed under sterile conditions. From both A and B pellets, several sub-samples were taken separately, cutting each pellet with a thin metal blade, previously sterilized under the flam.

Each pellet sample was cut into small pieces, randomly collected, and weighed into a sterile test tube. Then, 1.0 g of pellet was added to 9.0 mL of sterile saline (0.9% NaCl w/v). Samples ranged from 10 to diluted to 1.0×10^{-8} were inoculated into agar culture media: TSA (Tryptic Soya Agar -Biolife Italiana, Milano, Italy) for bacteria determination and PDA (Potato Dextrose Agar -BD Difco™, Milano, Italy) added with 100 µg/mL ampicillin (Fisher BioReagents) for fungi. The plates were incubated at 37 °C for 24–72 h to allow the growth of bacteria, and at 28 °C for 4–5 days for fungi.

C-EC agar (Biolife Italiana, Milano, Italy) was used for discriminating between total and fecal coliforms and *Escherichia coli* (Aquilano et al. 2022).

Bacillus spp. determination was performed by a total spore count. The saline sample mixture was heat shocked at 80 °C for ten minutes on a water bath. Then, 0.1 mL of the sample mixture was plated onto TSA and incubated overnight at 37 °C (Murray and American Society for Microbiology 1995). *Lactobacillus* spp. determination was carried out on MRS medium, incubation at 30 °C for 72 h (de Man et al. 1960; DSMZ 2023). All microbial counts were expressed as Colony Forming Unit (CFU) per 1.0 g of fresh weight (f.w.). The statistical data were expressed in terms of mean ± Standard Error (SE).

Microbial isolation

Morphology of individual colonies supported us in the strain isolation. Colonies with different color, size, border shape, and texture were chosen and inoculated in TSA and PDA. Then, the purity of each isolate was confirmed, and the isolated strains were observed under optical microscope.

In addition, preliminary identification tests were performed, including morphological properties under microscopic observations and physiological and biochemical tests for bacteria by a Gram reaction and API-Systems (Biomerieux Italia, Firenze), according to Bergey's Manual (Bergey et al. 2000).

To confirm the identity of microbial isolates, the yeast and fungal strains were sequenced for the ITS rDNA (ITS1, 5.8S, and ITS2 28S rRNA gene) (Raja et al. 2017) whereas bacterial strains were subjected to the partial sequencing (500 bp) of the 16S rRNA gene (Drancourt et al. 2000). Sequencing was performed by BMR Genomics (Padua, Italy). The obtained sequences were submitted to GenBank in the National Center for Biotechnology Information (Zhang et al. 2000; Morgulis et al. 2008). Bacteria isolated from barn owl pellets were stored at − 80 °C in cryovials (Microbank), whereas fungi and yeasts were preserved at 4 °C on PDA slants (Jurado et al. 2021).

Fig. 2 Imagine of wild barn owl pellet: **a** shape and dimension of an analyzed pellet with bar scale; **b** View entire pellet under stereomicroscope

Enzymatic activities

Multiple enzymatic activities of pellet as a whole and of isolated microorganisms were determined by the API-ZYM system (bio-Merieux Italia, Rome, Italy). A semi-quantitative evaluation of the activities of 19 hydrolytic enzymes [alkaline phosphatase, esterase (C4), esterase–lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cistine arylamidase, trypsin, α-chymotripsin, acid phosphatase, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetil-b-glucosamidase, α-mannosidase, and α-fucosidase] was performed (Ranalli et al. 2001; Viti et al. 2006). All determinations were made on three replicates. The reproducibility was > 95%. Results are reported in figures by MS Excel, radar function.

16S rRNA gene and ITS2 region amplicon library preparation and sequencing

Next Generation Sequencing analysis was performed at BMR Genomics company (Padova, Italy), following the protocol previously used by Monaco and colleagues (Monaco et al. 2020, 2021) for bacterial community investigation. The V3–V4 regions of the 16S rRNA gene were amplified using the primer pair 16S_univFor and 16S_univRev, modified with universal tails (Nadkarni et al. 2002).

The ITS2 regions of fungi were amplified using the modified primers ITS3_KYO2 (Toju et al. 2012) and ITS4r (White et al. 1990). PCR products were purified with Thermolabile Exonuclease I (NEB) and amplified with Nextera XT Index (Illumina) on a second PCR step. Amplicons were then normalized with SequalPrep (Thermo Fisher) and multiplexed. The pool was purified with Agencourt XP 1X magnetic beads. The library was run on the Illumina MiSeq and sequenced with V3 chemistry – 300PE strategy. Bioinformatics analysis was performed using QIIME2 tools version 2021.4 (Caporaso et al. 2010; Bokulich et al. 2018).

The obtained reads were cleaned of primers by using the Cutadapt software (v. 2021.4) and then processed with the denoised paired plugin of the DADA2 software (Callahan et al. 2016). Briefly, sequences have been trimmed at the 3′ end and filtered by quality and length. Then, they were de-replicated and merged to obtain unique sequences, and chimeras were eliminated. The Amplicon Sequence Variants (ASVs) with a frequency < 0.01% were not considered. All reads from bacterial communities were classified to the lowest possible taxonomic rank using a reference dataset from the SILVA database (version 138). On the other hand, all reads obtained from fungal communities were classified to the lowest possible taxonomic rank using a reference dataset from the UNITE database (version 8.3). Sequences generated in the present study were deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA957615.

Alpha-diversity was calculated by using the Shannon index whereas similarity between the investigated bacterial and fungal communities (beta-diversity) was calculated by using Bray–Curtis dissimilarity index (Ärje et al. 2016; Caprari et al. 2023).

Predictive functional analysis on sequencing data

The predictive functional profiling/analysis of microbial communities (taxa bacteria) responds to the question: who does what! For the functional ecological rules of microbial communities 16S rRNA marker gene sequences were analyzed (Langille et al. 2013; Xia et al. 2019) by the python script "collapse_table.py", Python 3 environment by FAPROTAX database (version 1.2.6, Available online:https://pages.uoregon.edu/slouca/LoucaLab/archive/FAPROTAX/lib/php/index.php, accessed on 13 December 2023) (Zhang et al. 2023; Liu et al. 2023). Such a script works in two steps. Firstly, it maps the list of prokaryotic clades detected in the previously mentioned NGS analysis to the respective metabolic or ecologically relevant functions. To do this, it relies on the FAPROTAX database, which consolidates information from the literature by explicitly providing such mappings. Second, it counts the occurrences of the functions and reports. As a result, we obtained a list of functions with the respective number of occurrences. We executed the script for our two samples (A and B). We plot the obtained results using a heatmap in which the rows represent the detected functions, the columns represent the samples (A and B), and each cell contains a circle sized proportionally to the number of prokaryotic clades detected in the sample (column) for the function (row). We only report functions for which we observed at least one occurrence.

Environmental impact, CO_2 equivalent emission and mitigation actions

In order to assess, reduce and mitigate the greenhouse gas generated by the laboratory activities originating from these researches, we adopted the protocol and indicators (Table 1) of a previous work (Palmieri et al. 2023).

Results

Comparison methods for collecting wild raptor pellets, at low environmental contamination.

The results among the four tested systems to collect wild raptors pellets, comparing the advantages and limits, are described in Table 2.

Because of the detailed notes above reported, method 1 was evaluated positively and adopted. Therefore, the

Table 1 Indicators selected to calculate the environmental footprint originated by a research activity, impacts and green mitigation acts. Amount x K conversion = CO_2 eq. Data by Palmieri et al. 2023 modified (Palmieri et al. 2023)

Type Consumptions	Evaluation of CO_2 eq. emission				
	Parameter	Unit	Amount	K conversion to CO_2	CO_2 yield (kg)
Energy (electric power by fossil)	Equipments (instruments, steril hoods, fans, cooling, sterilization, others).	kWh		0.224	
	Tap water	m^3		0.32	
Chemical products	Reagents (dried cultural media; acid and basic solutions, others).	kg, liter		1.47	
	Toxic, hazard (solvents).	kg		0.62	
Wastes	Plastic	kg		1.74	
	Glass	kg		0.85	
	Paper	kg		2.42	
	Effluents	m^3		0.29	
Transports personnel and samples/materials		km		0.121	
CO_2 eq. total emissions (kg)					
Social Costs (€)					
Mitigation Action	N° of plants to be planted K conversion kg CO_2 eq. to number of plant, 100:1	Which type; when, where; how; who provide; control and guarantee in the time.			

Table 2 Advantages and limits of methods for collecting and sanitizing wild raptor pellets, at low environmental contamination. - not easy, not fast; +/- easy, not fast; + easy and fast; + + very easy and fast; + + + very easy and very fast

N°	Methods	Set-up	Recovery	Sanitisation
1	Hard, flat surface (like table)	Easy to assemble and transport; cumbersome. Only for flat land	+ + +	+ + +
2	Perforated roll-up net in PVC + 4 poles for land support	Easy to fix; less complex to move, cumbersome. Even for non-flat land	+ +	+ +
3	Perforated roll-up net in nylon + 4 poles for land support	Easy to fix; less complex to move, not cumbersome. Even for non-flat land	+ +	+
4	Vegetable fabric net roll-up + 4 poles for land support (like hammock)	Easy to fix and to move, not cumbersome. Even for non-flat land	+/-	-

collection of wild barn owl pellets was carried out using a table surface (about 2.5 m^2), daily sanitized. It can be considered useful and adequate for sampling recovery on the site selected.

Pellet characteristics

Results of preliminary analyses on *T. alba* pellets are shown in Table 3.

Both samples appeared fresh, intact, in good condition, of brown color and shiny appearance with some bony elements on the surface alongside, others deepening. The two pellet samples were characterized by small size (< 40 mm) and weight less than 3.0 g each.

Morphological observations

Results of the prey identification at the lowest possible taxonomic level (Fig. 3a and b) were: *Apodemus sylvaticus* and *Apodemus* spp. for pellet A, and *Apodemus flavicollis* and *Rattus rattus* for pellet B.

Table 3 Main characteristics of the analyzed A and B pellets

Main Characteristics	A	B
Weight (g)	2.16	2.14
Dimension (length, mm)	33	37
Color	Brown with bone remains	
Appearance	Translucent on surface	
pH	7.3	7.5
Conductivity (µS)	16.5	12.0

Microbial count and isolated microorganisms

Regarding bacterial viable counts (Table 4), high values were obtained for both pellet samples, with average

Fig. 3 Example of undigested bones used as diagnostic elements to identify *T. alba* preys: lower jaw (**a**) and skull (**b**) of a mouse belonging to *Apodemus* spp

Table 4 Microbial counts on analyzed pellet samples (data expressed as log CFU/g ± SE)

Microbial group	Pellet A	Pellet B
Total viable aerobic heterotrophic bacteria	6.5 ± 0.5	6.4 ± 0.4
Fungi & Yeasts	5.1 ± 0.3	4.6 ± 0.2
	0.2 ± 0.05	nd[a]
Enterobacteriaceae	2.0 ± 0.2	2.9 ± 0.4
E. coli	1.5 ± 0.2	1.8 ± 0.3
Spore-forming bacteria	3.5 ± 0.1	3.0 ± 0.2
Lactobacillus spp.	2.2 ± 0.3	3.1 ± 0.5

[a] *nd* not detected

values of 10^6—10^7 CFU/g f.w. At the same time, spore-forming bacteria showed a colony amount ranging from 10^3 to 10^4 CFU/g f.w. Results of fungal counts varied from 10^4 to 10^5 CFU/g f.w. The presence of yeasts was observed only in pellet A. In both pellets, similar amounts of *Enterobacteriaceae* colonies, including, *E. coli* were detected.

In Fig. 4a and b, one yeast colony and one fungal colony were reported, respectively. Table 5 shows the identification of microorganisms isolated from the analyzed pellets based on the comparison with DNA sequences available in reference databases.

Information databases using the internal transcribed spacer region from the fungi type and reference material showed that isolate strain 3B_1 had 100% identity with *Malbranchea albolutea* and the isolate strain AS4 had 100% identity with *Penicillium* sp. However, the ITS marker alone for identification does not discriminate at the species level in certain fungal genera such as *Penicillium* sp., and it may be necessary to sequence one or more protein-coding genes to obtain a more precise identification at the species level (Raja et al. 2017).

The isolate A5 showed an identity of 100% with both *Debaryomyces* sp. isolate FBFY22 and *Debaryomyces hansenii* clone Turkey strain M 6.3.

Analogs analyses with bacterial strains revealed that the strain N2 showed 100% of identity with *E. coli* strain LWY24; NB1 strain showed 100% of identity with *E. coli*

Fig. 4 Microbial growth of *D. hansenii* (**a**) and *M. albolutea* (**b**) on PDA medium, respectively

Table 5 Molecular identification of some of the strains isolated from the investigated wild barn owl pellets

Strains	Accession number	Closest affiliations	Identification and Accession Number (% Identity)
Bacteria			
N2	OR044424	*E. coli* strain LWY24	CP054556.1 (100)
NB1	OR044425	*E. coli* strain AVS0973	CP124471.1 (100)
NB2	OR044426	*E. ludwigii* strain RHB47	CP099325.1 (100)
NB3	OR044427	*P. fragi* strain NMC25	CP021132.1 (100)
Fungi			
3B_1	OR044530	*M. albolutea*	OW988116.1 (100)
AS4	OR044532	*Penicillium* sp.	MW662654.1 (100)
Yeast			
A5	OR044531	*D. hansenii* clone Turkey strain M6.3	MT004747.1 (100)

strain AVS0973; NB2 strain showed 100% identity with *Enterobacter ludwigii* strain RHB47; NB3 strain showed 100% of identity with *Pseudomonas fragi* strain NMC25.

Enzymatic profiles

Figure 5a and b show two enzymatic profiles obtained by the API ZYM assay on both pellet A and B. As expected, pellet A showed many enzyme activities (15 out of 19), while a lower number we recorded in pellet B (12 out 19) reflecting differences in microbial communities.

Figure 5c and d show the enzymatic profiles obtained by isolated yeast *D. hansenii* and bacterial strain *P. fragi*, respectively. The yeast *D. hansenii* shows 7 out of 19 profiles, with the peculiar presence of esterase-lipase (C8) and α-galactosidase enzymes, although with a low activity.

In contrast, the enzyme profile of the isolated bacterial strain of *P. fragi* reported here shows a greater number of enzymes (13 out of 19), with the peculiar presence of trypsin and β-glucuronidase enzymes.

Microbial community composition

NGS analysis provided interesting information about the composition of microbial communities associated with barn owl pellets. With regards to *Bacteria*, sequencing of the V3–V4 regions of the 16S rDNA resulted in 106 ASVs (Amplicon Sequence Variants) for sample A and in 104 ASVs for sample B, with a total number of 33,211 and 27,396 final reads, respectively. For fungal communities, the ITS2 region sequencing allowed 39,942 final reads and 33 ASVs for pellet A, and 37,963 final reads and 51 ASVs in the case of pellet B.

Overall, the microbial communities of the two wild barn owl pellets consisted of a few dominant phyla: *Firmicutes*, *Proteobacteria*, *Actinobacteriota*, *Bacteroidota*, and *Desulfobacterota* for Bacteria and *Ascomycota*, *Mucoromycota*, *Basidiomycota*, and *Mortierellomycota* for Fungi (Fig. 6). In more detail, the analysis of the bacterial community structure revealed that *Firmicutes* were the main phylum in both wild pellets, with a relative abundance of 61.3% in sample A and 90.8% in sample B.

Proteobacteria showed a percentage of 30.4% in pellet A (with *Gammaproteobacteria* class particularly abundant), but they were poorly represented in pellet B (0.2%). The relative abundance of *Actinobacteriota* varied from 5.5 to 8.1%, whereas *Bacteroidota* ranged between 0.2 and 2.4%. *Desulfobacterota*, not found in pellet A, was among the top phyla of pellet B (0.9%).

Regarding mycobiota composition, *Ascomycota* phylum was the most represented in both investigated wads (relative abundance 78.4% in sample A and 83.1% in sample B), followed by *Mucoromycota*, which showed percentages between 14.7 (sample B) and 21.4% (sample A), and *Basidiomycota* (0.1% sample A; 2.1% sample B).

Fungi belonging to *Mortierellomycota* phylum were not retrieved in wad A, whereas they were found in pellet B even if in a very low percentage (0.1%).

At genus taxonomic level (Fig. 7), the similarity based on Bray–Curtis index between the investigated bacterial communities was 43.5%. *Lactobacillus* was the most abundant genus in both samples but in the wad B it was predominant accounting nearly 90% of the total reads, with *L. oris* (26.5%) and *L. gasseri* (15.3%) among the most represented species. *Enterorhabdus* (3.3%), *Muribaculaceae* members (2.3%), *Desulfovibrio* (0.9%), *Corynebacterium* (0.9%), and few other bacterial genera with relative abundance values ≤ 0.5% completed the composition of the microbiota B.

In pellet A, unclassified species of *Lactobacillus* genus accounted for 38.8%, followed by species belonging to the genera *Yersinia* (21.0%), mainly *Y. enterocolitica* (20.1%), *Streptococcus* (15.8%), *Escherichia-Shigella*

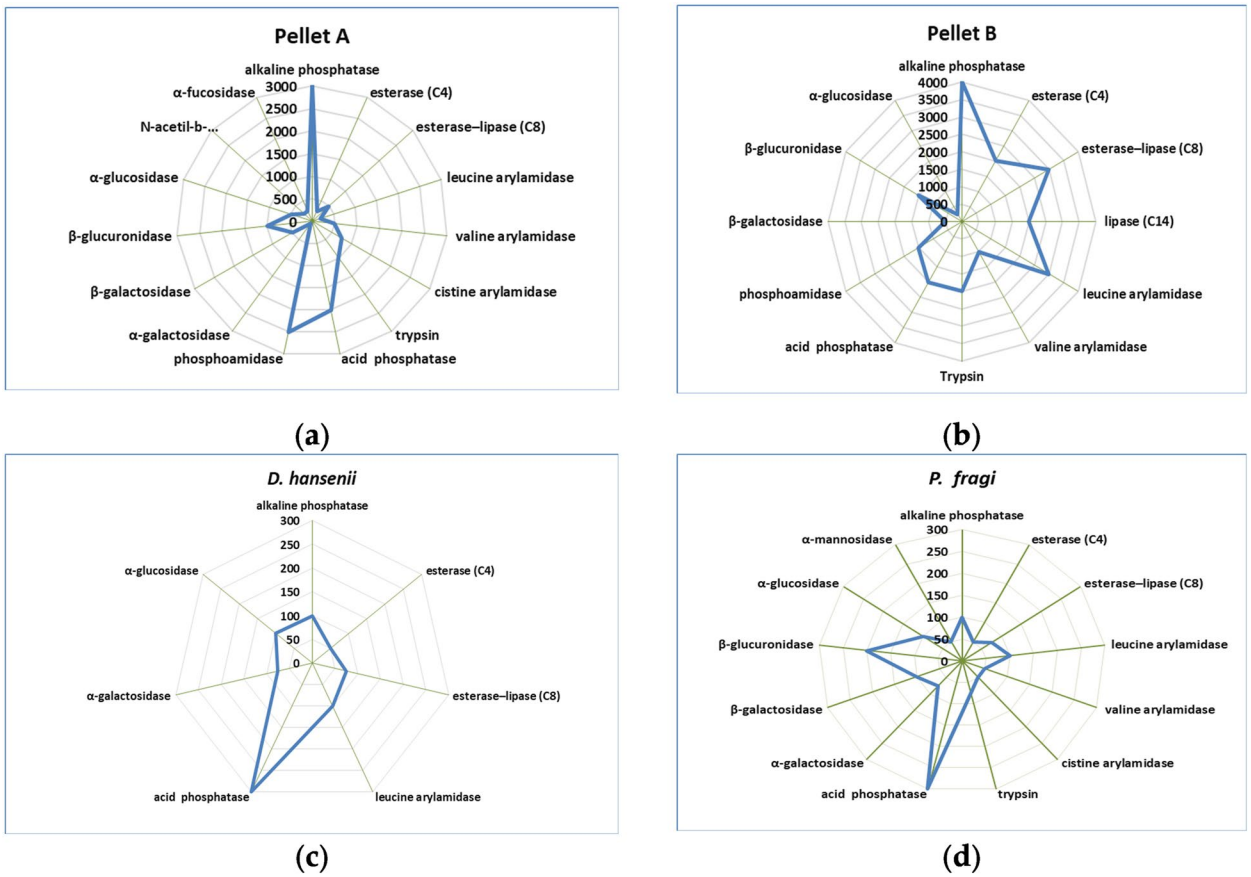


Fig. 5 Enzymatic profiles (in nanomoles) on pellets A (**a**) and B (**b**); *D. hansenii* isolate (**c**) and *P. fragi* isolate (**d**)

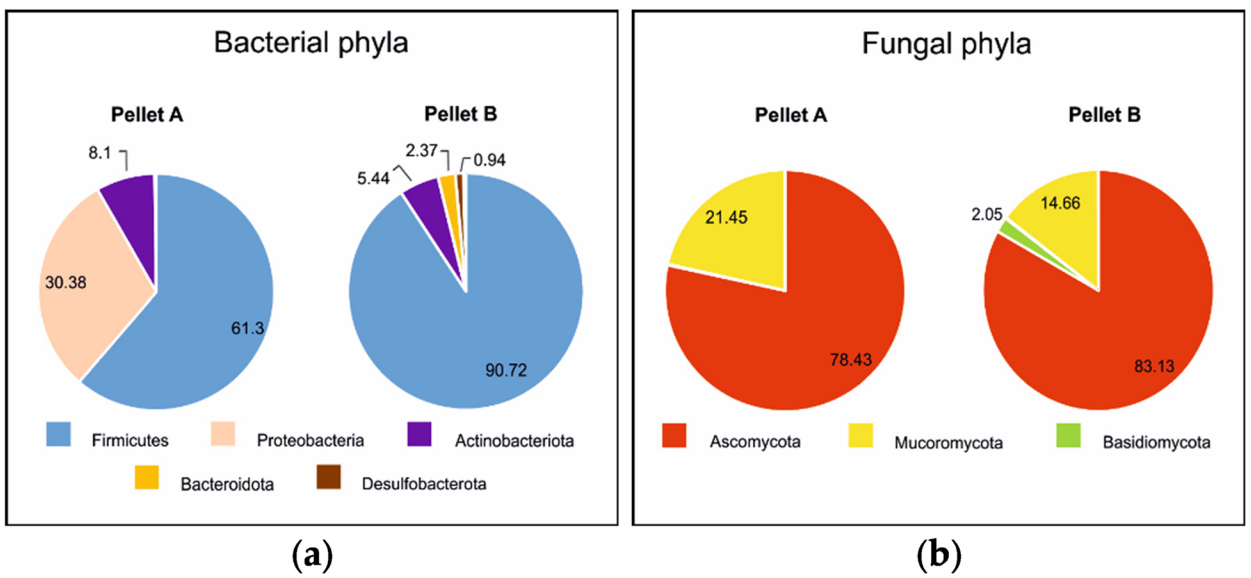


Fig. 6 Microbial communities of barn owl pellets at Phylum level; bacterial phyla (**a**) and fungal phyla (**b**)

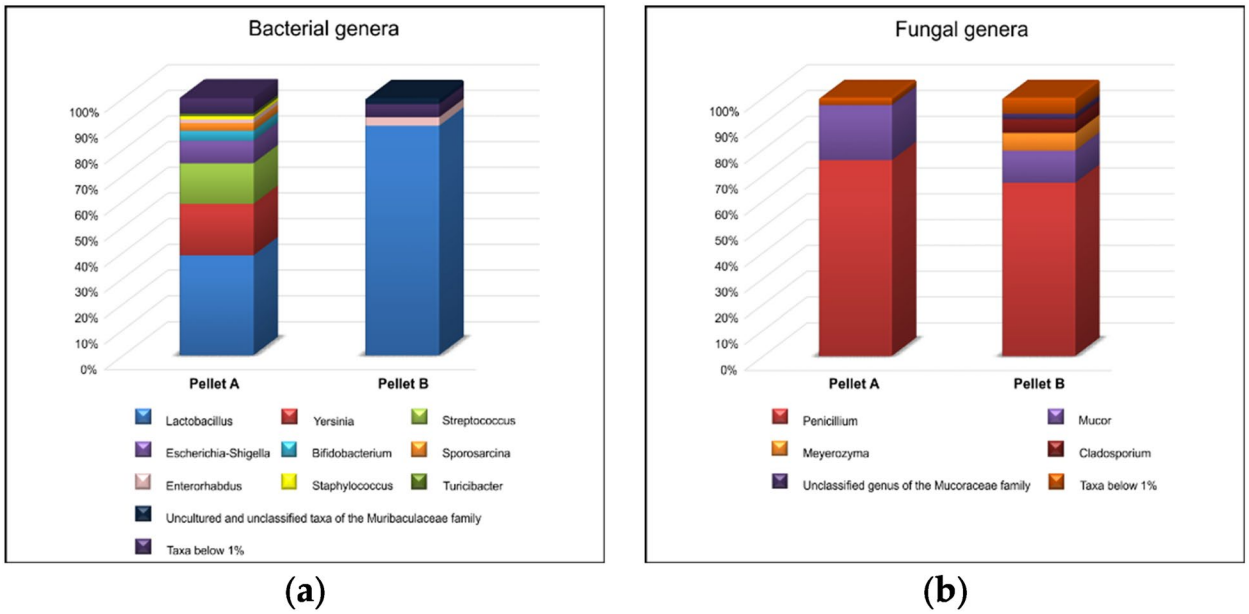


Fig. 7 Microbial communities of barn owl pellets at Genus level; bacterial genera (**a**) and fungal genera (**b**)

(8.8%), *Bifidobacterium* (3.9%), *Sporosarcina* (3.0%), *Enterorhabdus* (1.9%), *Staphylococcus* (1.2%), *Turicibacter* (1.1%), and *Corynebacterium* (0.8%).

Overall, a high percentage of uncultured and unclassified bacteria at species level was detected both in sample A (77.1%) and in sample B (56.0%).

As regards the mycobiota, the similarity percentage at genus level was 79.7%. In sample A, *Penicillium* and *Mucor* dominated fungal communities, with percentages of 75.9 and 21.4%, respectively. In pellet B, in addition to these two genera that represented 79.6% of the mycobiota, other fungal genera such as the yeast *Meyerozyma* (6.8%) and the mold *Cladosporium* (5.4%) showed high relative abundance values (Fig. 7).

The alpha diversity was estimated through the Shannon relative index (h). The obtained results for bacterial communities were 0.59 (pellet A) and 0.47 (pellet B) whereas for fungal communities the obtained values were 0.49 (pellet A) and 0.44 (pellet B).

Predictive functional analysis

The predictive ecological and functional data of microbial communities and microorganisms (bacteria) found on two pellets are reported in Fig. 8. The identified bacterial functional groups were 17, both for pellet A and pellet B. In the two samples, great amounts of bacterial sequences were assigned to fermentation and chemoheterotrophy (anaerobic predominance). Furthermore, in pellet A, invertebrate parasites and animal parasites or symbionts were detected. A low number of bacterial sequences were assigned to aerobic chemoheterotrophy, human pathogens, nitrate reduction and ureolysis.

In pellet B, different predictive ecological-functional characteristics were noticed with abundance assigned to human gut, human associated, mammal gut and animal parasites or symbionts. The function of sulfate respiration and respiration of sulfur compounds were detected only in pellet B. The functions of human pathogens pneumonia, plant pathogen, and aromatic compounds degradation were rare in both pellets.

Environmental impact, CO_2 eq. emission and mitigation actions

Here we report the evaluation of environmental impact related to this study, the CO_2 equivalent emission, and mitigation actions by new plantations, according to the Mat e Meth section. The evaluation of the environmental impact of this manuscript, in terms of social cost and CO_2 equivalent emission, resulted in 12.0 euro and about 300 kg CO_2 eq., respectively. This bring us to provide a new plantation by tree *Taxus baccata* L. plants for mitigation actions and a QRcode tag linked to this manuscript was added to each tree planted in the Green Campus – Unimol (41° 60′ 76″ N 14° 26′ 50″ E).

Discussion

Concerning the daily observations and collection of fresh pellets when present, our experimental protocol guarantees the rapid analyses in the lab. The sanitized table represents a valid and simple solution to study wild barn

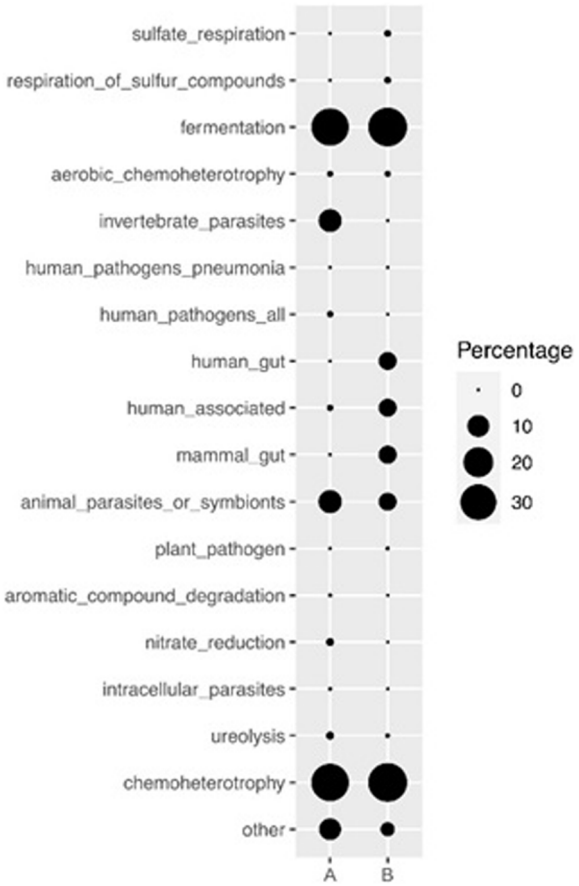


Fig. 8 Predictive functional profiling of bacteria (bacterial communities) present in pellets **A** and **B**

owl pellets, and minimizes potential environmental contamination. Since the rarity of the phenomenon, in four months we could observe only two samples, therefore our protocol should be considered as a valid model to collect and analyze wild barn owl pellets.

The determined pH values of these pellets were not acidic as expected but slightly above neutral. The data appeared to be markedly different from values reported in previous research, where pellets were characterized by acidic pH values. A large amount of reflux of alkaline pancreatic or intestinal fluid into the stomach could explain our observations (Leprince et al. 1979).

Ecology of barn owl prey

Based on the remains found in the analyzed pellets, barn owl prey can be fitted in two main groups of species. The first comprised *A. sylvaticus* and *R. rattus* that are adapted to almost all habitat types in agroecosystems due to their generalist behavior and their characteristics that allow them to exploit the spatial niches provided by ecotone-rich landscapes (Tattersall et al. 2001; Amori et al. 2008; Loy et al. 2017). The second group included *A. flavicollis* which is mainly related to highly heterogeneous landscapes in which the presence of natural patches of shrub or forest habitats is remarkable (Paniccia et al. 2022). This ecological information on the prey species is consistent with the environmental features of the territory in which pellets were collected. A mosaic of wide agricultural areas surrounded by native oak edges can host a diverse micromammal community. The different pellet composition we observed is related to different hunting events conducted by barn owls in the habitats of the study area. Indeed, in agro-forestal environments barn owls have home ranges not very vast (average 6.6 km^2) and tend to prey in habitats located not so far from their nest (Séchaud et al. 2021).We believe our pellet are composed by preys hunted on agricultural patches and on woody edges, where different mice species live.

Wild barn owl pellet microbial communities and predictive functional analysis

To date, little is known about microbiological aspects of wild barn owl pellets (Orosz-Coghlan et al. 2022). Consequently, we have examined the microbiota of the two wild *T. alba* pellets collected on a sanitized surface. Information on the structure and composition of the bacterial and fungal community is just one example of information that can be obtained from samples at low environmental contamination. To achieve this goal, both traditional cultivation techniques (culture-dependent methods) and molecular investigations (Next-Generation Sequencing) have been employed.

NGS analyses of the two wild barn owl samples revealed differences in the structure and composition of microbial communities and the predominance of a limited number of bacterial and fungal taxa. Regarding the bacterial communities, most of the sequences were assigned to enteric bacteria, first *Lactobacillus* species (*Firmicutes* phylum), which are considered the most important probiotic bacteria of the gut microbiota (Afrin et al. 2021; Rastogi and Singh 2022). Moreover, pellet A contained a relatively high percentage of bacteria belonging to the genus *Bifidobacterium*, gut colonization obligate anaerobes widely used as probiotics (Sharma et al. 2021). However, in addition to lactobacilli and bifidobacteria, pathogenic bacteria such as *Yersinia enterocolitica* have also been found in the analyzed pellets. This species, which in pellet A reached a relative abundance of 21.0%, is widely distributed in nature and it can be isolated from terrestrial and aquatic habitats, as well as from the gut of numerous mammals, avian species, and cold-blooded animals. The presence of fecal indicator bacteria and enteric organisms implicated in human infections within barn owl pellets was also reported by Orosz-Coghlan and

colleagues, who therefore recommended handling with caution pellets used for educational purposes (Orosz-Coghlan et al. 2022). It is likely that the enteric bacteria found in the examined pellets may derive from *T. alba* prey (mice and rats), rather than from the barn owl itself. This is further supported by the presence of bacteria typically associated with mouse gut, including species belonging to *Enterorhabdus* genus and *Muribaculaceae* family (Clavel et al. 2009, 2010; Lagkouvardos et al. 2019). Moreover, it seems that the abundance of some taxa, such as *Turicibacter*, one of the main bacterial genera of mammalian gut microbiota, that in sample A showed a percentage of 1.1%, may vary in relation to host sex and age (Zhou et al. 2019; Wu et al. 2022). Consequently, the investigation of pellet microbial communities, especially if extended to a larger number of samples, could indirectly provide information about raptor diet and prey, useful to integrate morphological analysis data.

With regards to mycobiota analysis, *Penicillium* and *Mucor* were the main genera shared between the two *T. alba* pellets. *Penicillium* fungi are ubiquitous ascomycetes commonly found in soil, air, as well as in extreme environments, in certain food products, or associated with plants.

It is noteworthy that a high percentage of unclassified and/or uncultured microorganisms (including *Bifidobacterium*, *Enterorhabdus*, *Lactobacillus*, and *Muribaculaceae* members for bacteria and *Penicillium* species for fungi) was detected in both *T. alba* pellets. Consequently, barn owl pellets could also be an important source of new, yet un-described microorganisms with further interesting potential applications (probiotic strains, biocontrol agents, producers of biologically active compounds, food-associated molds, etc.).

Microbial ecological functions refer to the natural processes carried out by microorganisms in the environment (Zhao et al. 2023). The preliminary results of our predictive functional analysis suggested an involvement of the pellet microbiota both on *T. alba* diet and its digestive processes.

Potentiality of isolated strains

The aim of isolating microorganisms potentially useful in the field of biotechnology was largely achieved. Among the isolated strains, there was *D. hansenii*, a halotolerant yeast, which showed esterase-lipase (C8) and α-galactosidase activities (Nguyen et al. 2009). Due to their enzymatic activities, *D. hansenii* strains could be not only of considerable biotechnological and commercial importance (in food, dairy, detergent, and pharmaceutical industries), but they could also be used in biorestoration field to remove unwanted residues like fats and lipids on altered artworks (Banik 2003; Papagora et al. 2013; Ranalli and Zanardini 2021).

Based on the enzyme profile in Fig. 5d and the metabolic versatility of the genus *Pseudomonas*, *P. fragi* could be adopted in various biotechnological applications like bioremediation and degradation of various xenobiotic compounds in soil and polluted wastewater (Viti et al. 2006; Bosch-Roig et al. 2016). The peculiar presence of trypsin enzyme could be associated with a potential use in new strategies to pest control (Pilon et al. 2017). Furthermore, β-glucuronidase activity can lead to a reduction in glucuronide conjugation, which causes xenobiotic-induced toxicity in humans (Dashnyam et al. 2018).

Among fungi, *M. albolutea* was also isolated. As reported by (Díaz-Rojas et al. 2021) *M. albolutea* Sigler & Carmich [*Onygenaceae*; syn. *Auxarthron alboluteum* Sigler & Hambleton] is a keratinophilic fungus isolated from soil worldwide (Sigler and Carmichael 1976). Recently, the same authors affirmed that no chemical and pharmacological investigations on *M. albolutea* were carried out to biosynthesize different classes of bioactive compounds. For these purposes, a first report of a great interest were received on *M. albolutea* for the ability to produce alboluteins A-C (1–3), ardeemins and sartoryglabrins analogs; these compounds are involved in the PTP1B inhibitions, a negative modulator of insulin and leptin signaling, highly validated pharmacological target against insulin resistance and obesity (Kerru et al. 2018). For these reasons, the importance to future deep study and comparison among several *M. albolutea* strains, on the possible effects that alboluteins A-C have antitumoral potential since PTP1B is like a positive regulator of tumor progression (Díaz-Rojas et al. 2021).

Conclusions

T. alba pellets represent a natural matrix for analyzing wild barn owl diet and associated microbial communities. For this reason, we developed a strategy to properly collect and analyze these rare and precious matrices. The main conclusions of this paper can be summarized as follows:

1) Under our experimental conditions, the collection system 1 proved to be a viable and simple solution for both recovering wild barn owl pellets and minimizing potential environmental contamination.
2) Microbiological analyses can unravel the biodiversity existing in these poorly investigated matrices and lead to the discovery of microorganisms that could play a key role in biotechnological processes. Moreover, the results obtained can be considered as a starting point to develop more effective cultivation- based techniques for the isolation, the growth, and the characterization of a larger number of microorganisms still unidentified in barn owl pellets of wildlife specimens.

3) Osteological analyses can be considered a useful tool to identify the barn owl preys, and when coupled with microbiological investigations, may provide more detailed information on their diet.
4) The environmental impact in terms of CO_2 eq. was evaluated and mitigation actions were performed by new tree plantation.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13213-024-01759-6.

Additional file 1: Fig. S1. Schematic protocol adopted in this work.

Additional file 2: Fig. S2. a View of the area around the nest; b QR code for panoramic view by drone

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Data availability statement
Sequences deposited in the NCBI GenBank database on Sequence Read Archive (SRA), accession number PRJNA957615.

Authors' contributions
Investigation: C.C. and G.R.; data curation: A.B., C.C., F.D., S.G., E.M., P.M., G.P., L.Q., S.S. and G.R.; writing—original draft preparation: A.B., C.C., M.P., and G.R.; writing—review and editing: A.B., C.C., M.P., and G.R.; funding acquisition: C.C. and G.R. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials
The numerical data used to support the findings of this study are included in the article. The datasets generated and analyzed during the current study are available in the NCBI repository, the link used for data upload: https://www.ncbi.nlm.nih.gov/, and accession numbers are: OR044424; OR044425; OR044426; OR044427; OR044530; OR044531; OR044532.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interest.

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