Biological risk assessment in the History and Historical Documentation Library of the University of Milan

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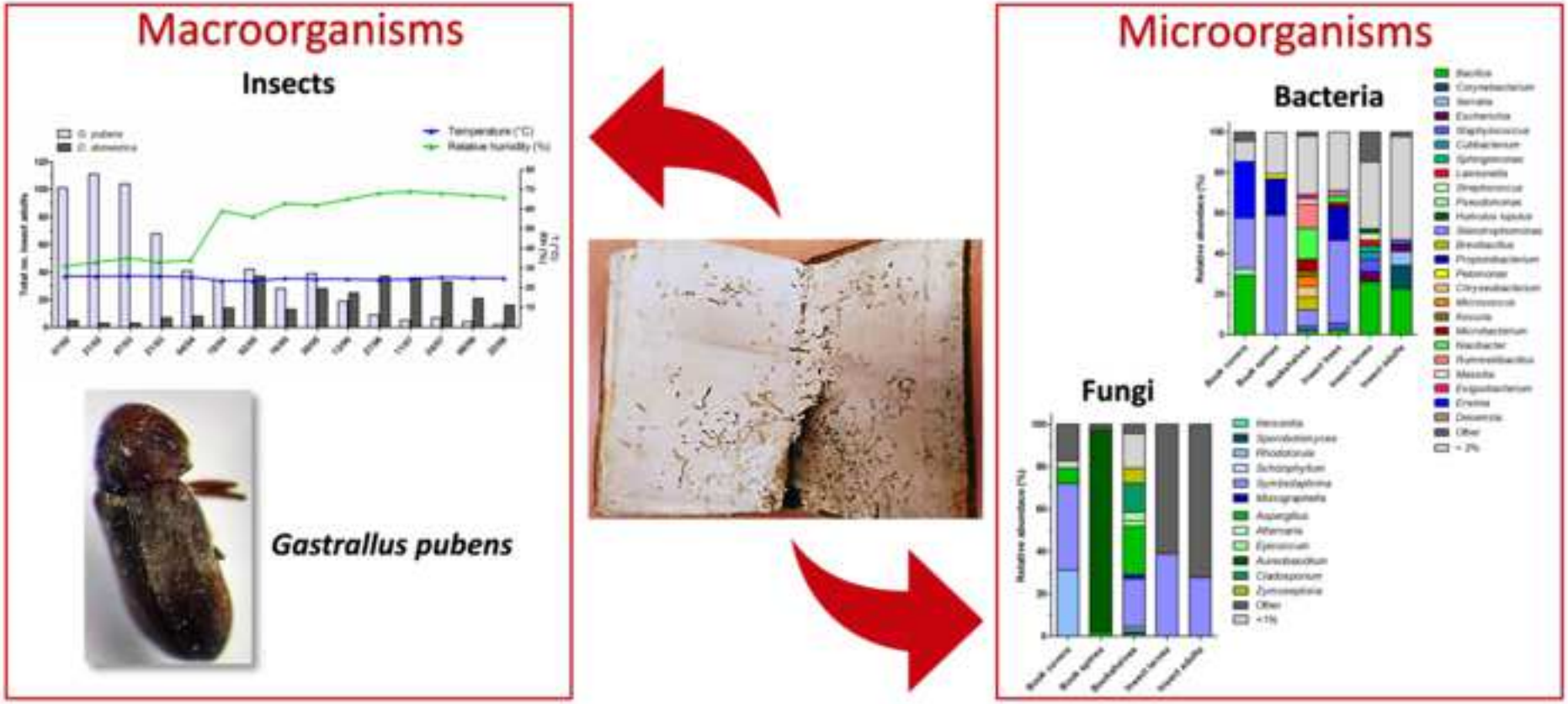
Abstract

Belonging to the anthroposphere there are billions of books that in recent and in ancient times have been produced by the human race containing evidence of its intellectual and cultural efforts. Even when stored in libraries, not all these books survive over time undamaged, because in the biosphere their materials are potential nutrients. This is the unfortunate case of the History and Historical Documentation Library of the University of Milan, where biological agents have badly affected rare and valuable old books. An entomological monitoring was carried out using sticky traps and collecting insects during inspections. The beetle *Gastrallus pubens* Fairmaire, rarely identified in European libraries so far, was the main biological agent responsible for the book damage, since several tunnels due to larval activity and holes made by adults were observed. Using the Illumina MiSeq sequencing technology, Proteobacteria, Firmicutes and Actinobacteria were found to be the most abundant phyla. Ascomycota was the dominant phylum among three fungal phyla. As bacteria and fungi spread by the insects are primary indications of the insect presence in the library, in this paper a potential biomarker able to detect the *G. pubens* presence before visible infestation was searched for among the bacterial and fungal community peculiar in the insect frass and gut, but also found on books and the surfaces of shelves. *Symbiotaphrina*, an ascomycete fungus described as one of the symbiotic levuliform fungi, present in the anobiid beetles' gut, was the only one found in all samples analyzed and has therefore been proposed as a valid biomarker for the presence of the insect in libraries, since its early stages of life.

Keywords: *Gastrallus pubens*, *Symbiotaphrina*, monitoring, Illumina MiSeq, *Humulus lupulus*

Graphical Abstract



Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Highlights

- *Gastrallus pubens* was identified as the main insect responsible of book damage
- Illumina MiSeq sequencing used to identify bacteria and fungi of larvae
- *Symbiotaphrina* might be a valid biomarker for the presence of *G. pubens*
- For the first time *G. pubens* biology and developmental time are reported
- Material from the hop (*Humulus lupulus*) plant was also used to produce the books

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Abstract

Belonging to the anthroposphere there are billions of books that in recent and in ancient times have been produced by the human race containing evidence of its intellectual and cultural efforts. Even when stored in libraries, not all these books survive over time undamaged, because in the biosphere their materials are potential nutrients. This is the unfortunate case of the History and Historical Documentation Library of the University of Milan, where biological agents have badly affected rare and valuable old books. An entomological monitoring was carried out using sticky traps and collecting insects during inspections. The beetle *Gastrallus pubens* Fairmaire, rarely identified in European libraries so far, was the main biological agent responsible for the book damage, since several tunnels due to larval activity and holes made by adults were observed. Using the Illumina MiSeq sequencing technology, Proteobacteria, Firmicutes and Actinobacteria were found to be the most abundant phyla. Ascomycota was the dominant phylum among three fungal phyla. As bacteria and fungi spread by the insects are primary indications of the insect presence in the library, in this paper a potential biomarker able to detect the *G. pubens* presence before visible infestation was searched for among the bacterial and fungal community peculiar in the insect frass and gut, but also found on books and the surfaces of shelves. *Symbiotaphrina*, an ascomycete fungus described as one of the symbiotic levuliform fungi, present in the anobiid beetles' gut, was the only one found in all samples analyzed and has therefore been proposed as a valid biomarker for the presence of the insect in libraries, since its early stages of life.

Keywords: *Gastrallus pubens*, *Symbiotaphrina*, monitoring, Illumina MiSeq, *Humulus lupulus*

1. Introduction

Books represent a vital part of our cultural heritage and libraries are responsible for collecting, organizing and preserving them and for providing access to knowledge and information. Books are subject to biodeterioration processes and biological attacks by insects, bacteria and fungi (Querner, 2015; Polo et al., 2017; Okpalanozie et al., 2018). Additional materials used in papermaking, like adhesives, are often supplementary nutrients, i.e. several old books with a high content of gelatine or starch, used as glue, can be susceptible to insect and microbial attack (Trematerra and Pinniger, 2018). In historic libraries, the paper, book covers (bindings) using leather, parchment, cardboard, wood or wooden shelves can be infested by a few species of insect pests, but this can result in severe damage if the infestation is not promptly found (Querner, 2015).

In the past, the correct identification of insects was not part of the pest management concept in libraries and biocides were used to treat infestations when they were obvious, without an appropriate taxonomic identification. Today it is known that insect pest monitoring and identification are important parts of managing active infestations. Indeed, collecting specimens helps to identify the species involved and to relocate the infested objects or problems within the building (Child et al., 2011).

Prevention of pest infestations by the application of Integrated Pest Management (IPM) procedures in libraries and archives is the most relevant approach for safeguarding book collections (Querner, 2015). Indeed, the sealing of the library room against pest entry, the control of micro-climate, the maintaining of high hygienic standards, the quarantining all new and incoming objects and the monitoring of pest infestations with traps are, among others, all valuable approaches against the spread of insect populations in libraries (Querner, 2015). However, the infestation is often not found at the beginning and, when it becomes visible, the insect spread is already extensive and severe damage has already occurred. Notably, IPM is a recent approach, and prevention measures were not totally applied in the past. In some cases, the use of chemicals is the way followed to manage insect populations inside libraries and archives (Nicosia, 2011). Disinfestation treatments have been applied to book collections and indoor environments. However, the use of most of these products is restricted by the European Union's Biocidal Products Directives (BPD, 2016), because of their hazards for human health, considering that certain insecticide formulations, when sprayed on surfaces, can release molecules of insecticide and solvents into the air. In addition, only a small number of agents have been tested with respect to their compatibility with historic materials and only few studies have dealt with the temporal efficiency of these practices (Odegaard, 2019).

The case study we dealt with in this work relates to the History and Historical Documentation Library of the University of Milan where several old and rare books are located in an non-conditioned environment that is exposed to biodeterioration agents (Figure 1A). In this study, selected books showed obvious crater-shaped holes and irregularly shaped tunnels which extended from the book binding to the book center and contained a large amount of insect frass (Figure 1B). They were also characterized by dark or light brown/yellow stains on the front covers (Figure 1C) and little black stains on the text blocks of the volumes (Figure 1D). Consequently, an entomological monitoring was carried out to verify the presence of insects which could cause direct damage to the books, and a microbiological sampling was made to identify microorganisms potentially harmful to the library heritage. Moreover, the microbiota of damaging insects was characterized to estimate the role of insects as potential spreaders of harmful microorganisms and biomarkers, verifying whether the microorganisms found on the book materials were also present in the insects.

2. Materials and methods

2.1 Case study

The History and Historical Documentation Library, University of Milan, houses approximately 1800 rare and valuable books, published from 1570 to the present (Figure 1A). The volumes are arranged by thematic sections, in accordance with the library's classification scheme, and they are stored in 13 metal cabinets with a lockable wire mesh door. The room is also designed as a reading room for students, professors and external users who are interested in the collections. The room (13 x 6 x 3.5 m) is located in the basement with a window overlooking the internal courtyard.

In 2004, for the first time, the librarians noticed damage potentially caused by an unknown insect attack. In 2016, during a visual inspection by the authors of this paper, most of the books appeared deteriorated and discoloured with evident damage from biological agents.

2.2 Environmental monitoring

During each inspection the room temperature and humidity were measured with a thermohygrometer (Avidsen 107240, Tours, France).

2.3 Insect monitoring

From February to August 2018 a fortnightly insect monitoring was carried out in the room of the History and Historical Documentation Library, University of Milan, where the fine editions are kept. Two sticky traps for crawling insects,

baited with a multi-species food tablet (3-way trap, GEA S.r.l., Settimo Milanese, Milan, Italy), were placed in the room and replaced at each monitoring. During entomological inspections, each metal cabinet was carefully inspected and all insects (dead or alive) present on the shelves or books were collected with entomological forceps and placed in plastic containers (Ø= 5 cm), closed with a lid and transported to the entomology laboratory of University of Milan for identification. Collected insects were examined using a stereomicroscope (Leica MZ12, Leica Microsystems GmbH, Wetzlar, Germany) and classified according to morphological and anatomical characteristics (Español, 1992; Kučerová, 1997**;** Toskina, 2003). To evaluate how the insect population was affected by sampling time and cabinet/trap position a two-way ANOVA was performed by using the XLSTAT package (XLSTAT 2019.3.2). In addition, a correlation test between the total number of insects and humidity was carried out using the same software package. Statistically significant results were defined by p-values ≤ 0.05.

2.4 Microbiological sampling

A culturally-independent approach was used to investigate the relationship between the book surface-associated microbial communities (book covers and book spines), the microbial communities in the dust on the shelves near the volumes and the gastro-intestinal tract microbiota of insects, both adults and larvae, as well the microbiota inhabiting the insect frass. Sampling procedures were performed on May 2018.

2.4.1 Sampling of book spines and book covers

Nitrocellulose membrane filters were used as a non-invasive technique for collecting samples from book spines and book covers corresponding with the discolorations putatively caused by a microbial attack on books (Polo et al., 2017). Briefly, nitrocellulose membranes (Sartorius AG, Göttingen, Germany), 47 mm in diameter, were handled with sterile tweezers and, using a sterile swab, were gently pressed onto the surface of the volume for 30 s. The membranes were then immediately transferred into tubes containing phosphate buffered saline (PBS, Sigma-Aldrich, Milan, Italy) and transported to the laboratory for processing. The cells collected on the nitrocellulose membrane filters were recovered by vortex mixing for 15 min and centrifugation at 9000 g for 30 min. Then the pellet was resuspended in 1 ml of lysis buffer (EDTA 40 mM, Tris HCl 50 mM pH 8, sucrose 0.75 M) and the DNA was extracted.

116 2.4.2 Sampling of dust on the book shelves and insect frass

117 The dust on the shelves where the books were exhibited as well as the insect frass between the book pages were collected
118 by using sterile soft brushes and transferred into sterile tubes through little pieces of sterile laboratory greaseproof paper.
119 Samples were transferred to the laboratory and resuspended in 1 ml of lysis buffer and the DNA was extracted. A fraction
120 of the frass was observed using a stereomicroscope (Leica MZ12, Leica Microsystems GmbH, Wetzlar, Germany).

121

122 2.4.3 Sampling of insect larvae and adults

123 A total of 9 larvae and 30 adults were collected using sterile forceps from the selected book and were transferred
124 immediately to the laboratory in a sterile tube for their processing.
125 In order to remove any microorganisms different from those hosted in the gut, both larvae and adults were first surface-
126 sterilized for 30 s with 70% ethanol and 30 s with 0.5% sodium hypochlorite. Insects were then washed three times for
127 30 s with ddH_2O. The third washing water was collected and plated in standard tryptic soy agar in order to confirm the
128 completion of sterilization.
129 Three larvae and 10 adults from different books werepooled in one tube as one replicate.
130 800 µL of lysis buffer and approximately 100 µL of glass beads (diameter <106 µm and between 425–600 µm, Sigma
131 Aldrich, USA) were added to insect tubes. Insect were subjected to a mechanical disruption using a Precellys bead beater
132 (Bertin Instrument, Montigny-le-Bretonneux, France) performing six cycles of 30 s at 6500 rpm, with a 30-s period of
133 cooling between cycles. Then, each sample was centrifuged at 7,000 g for 15 min. The supernatants were recovered,
134 and DNA extracted.

135

136 2.5 DNA extraction, Illumina MiSeq DNA sequencing and statistical analysis

137 DNA was extracted by using an enzymatic step followed by a modified phenol-chloroform protocol, as previously described
138 by Polo et al. (2017). The quantity and the quality of extracted nucleic acids were measured by a NanoDrop ND-1000
139 Spectrophotometer (Thermo Fisher Scientific Inc., MI, Italy). The extracted DNA from three replicates was combined.
140 High-throughput sequencing analysis of the V3–V4 region of the bacterial 16S rRNA gene (primers CS1_341F/CS2_806R)
141 and the internal transcribed spacer 1 (ITS1) region of the fungal rRNA cistron (primers ITS1F-ITS2aR) were performed by
142 using a MiSeq platform (Illumina) with v3 chemistry providing 2x300 paired-end reads.
143 Raw data were pre-processed, quality filtered, trimmed, de-noised, paired, and modeled via QIIME2 (Bolyen et al., 2019)
144 and DADA2. Chimeras were detected using DADA2 according to the “consensus” method (Callahan et al., 2016). Sequences

were clustered into Amplicon Sequences Variants (ASV). ASVs were assigned using a Naïve-Bayes classifier trained on the SILVA database. To estimate microbial community dissimilarity between samples, Bray-Curtis distance scores were obtained using the XLSTAT package (XLSTAT 2019.3.2). Bray-Curtis distances were projected onto Principal Coordinate Analysis (PCoA) and MultiDimensional Scaling (MDS) sample spaces.

3. Results

3.1 Environmental monitoring

Temperature and relative humidity data are reported in Figure 2A. The average temperature in the library was 24.9 °C, ranged from a minimum of 23.4 °C to a maximum of 26.0 °C. The average relative humidity was 53.8%, ranging from 31% to 69%.

3.2 Insect monitoring

Insects collected on the shelves of cabinets and within books were identified as *Gastrallus pubens* Fairmaire (Coleoptera, Ptinidae, Anobiinae) after verifying that they did not match with the beetles commonly found infesting books (Savoldelli et al., 2018). *G. pubens* adults present a body of dark brown color, more or less dark, completely covered with short pubescence. The adults are 2 to 3.5 mm long (Figure 2B), and the last three antennomeres of the antenna are enlarged. The larvae are whitish and have a length of about 5 mm at the end of the larval stage (Figure 2C). Species identification was based on morphological and anatomical examination in accordance with Español (1992) and Toskina (2003). The Anobiidae family has recently changed hierarchical classification, and it is now considered as a subfamily of Ptinidae (Anobiinae) (Bouchar et al., 2011).

Results of fortnightly monitoring are reported in Figure 2A. A total number of 614 *G. pubens* insect adults were collected during the monitoring time. The average number of collected insects each time was 41, ranging from 2 to 111. The two-way ANOVA statistical analysis showed that cabinets contributed 40.5 % ($P<0.0001$) to the variability of the *G. pubens* adult number. Indeed, cabinets 4, 5, 3, and 8 were the main outbreaks of infestation as more than 97% of the insects were collected there. The greatest numbers of beetles were found in cabinets 4 and 3, with respectively 37.8% and 33.1% of the total collected specimens during all the monitoring time, followed by cabinet 2 (16.8%) and 8 (9.6%). Statistical analysis also revealed that the variability of the *G. pubens* total adult number was affected up to 13.0% ($P<0.0003$) by sampling time. Accordingly, 51.5% of specimens were collected during the first four samplings, with an average amount of 105 adults between February and the beginning of March. From the end of March, a progressive reduction in the number of adults

was recorded. Correlation analysis revealed that humidity negatively affected the number of *G. pubens* adults as a significant correlation between the two variables was found (Pearson r: -0.8960; P<0.0001) (Figure S1A). Additionally, although with a minor impact, a positive correlation was found between the number of *G. pubens* insect adults and the temperature (Pearson r: 0.6019; P=0.0176) (Figure S1B).

In addition to the adults' emergence holes and larval tunnels, a large amount of insect frass was observed (Figure 2D). Frass was made up of eaten material passed through the digestive system and it is a considered as a visible sign of an infestation and larval activity. Frass produced by the larvae that dig the tunnels was formed by fragments of material mixed with the excrements that derive from the activity of nourishment. The stereomicroscope image highlighted the presence of pellets of different sizes depending on the age of the larvae which testify their trophic activity (Figure 2D).

The entomological analysis of insects collected in sticky traps, revealed a major presence of the psocid *Dorypteryx domestica* (Smithers) (Psocoptera, Psyllipsocidae) (97.2% of the total captures). In addition, mosquitoes, carabid beetles, springtails and pill bugs were occasionally found in sticky traps, representing 2.8% of the total captures in sticky traps in all the monitoring period.

A total number of 286 psocids were collected during the whole of the monitoring time (Figure 2A). The average number of collected insects each time was 9.5, ranging from 1.5 to 18.5.

The two-way ANOVA statistical analysis showed that trap position slightly affected the distribution of collected psocids (9.84 % of the data variability, P=0.0007). However, sampling time contributed 77.84% (P<0.0001) to the variability of the psocid adult number. The major fraction of insects (73.1% of total psocids) was collected from May to July, with an average amount of 14.9 specimens each time. From February to the end of April and in August, only an average number of 3.3s insect were collected each time. Correlation analysis revealed that humidity positively affected the number of *D. domestica* as a significant correlation between the two variables was found (Pearson r: 0.7955; P=0.0004) (Figure S1A). Additionally, a negative correlation was found between the number of psocids and the temperature (Pearson r: -0.7360; P=0.0018) (Figure S1B).

3.3 Illumina MiSeq sequencing dataset

A detailed description of the Illumina MiSeq sequencing dataset is shown in Table 1. Illumina MiSeq sequencing generated a total of 252,922 paired-end reads for the bacterial 16S rRNA gene and a total of 204,911 paired-end reads for the fungal ITS region. The average number of bacterial sequences per sample was 42,154, ranging from 21,343 of insect larvae to 82,524 of book covers. The average number of fungal sequences per sample was 40,982, ranging from 32,062 of book

covers to 42,697 of insect larvae. Illumina miSeq sequencing generated any reads of the fungal ITS intergene region for the insect frass sample.

3.4 OTU Richness

Based on a 97% sequence similarity cutoff, a total of 1542 OTUs were identified for bacteria and 447 OTUs for fungi (Table 1). The average number of bacterial OTUs per sample was 257, ranging from 136 of book spines to 393 of insect adults. For fungi, the average number of OTUs per sample was 89, ranging from 7 of insect adults to 307 of book shelves.

Rarefaction curves of both bacteria (Figure 3A) and fungi (Figure 3B) approached a plateau which suggests that sequencing efforts captured the bacterial and fungal community at each site to saturation.

Intersection analysis of OTU lists showed that overlapped OTUs were only 6 in bacteria, accounting for 1.5 % (insect adults) and 4.4 % (book spines) of overall OTUs of each investigated sample. In fungi, the number of OTUs shared to all samples were 4, accounting for 1.3 % (book shelves) and 57.1 % (insect adults) of the overall OTUs in each samples.

3.5 Bacterial community taxonomy overview

Bacterial sequences were classified into 37 phyla (Table S1). Proteobacteria, Firmicutes and Actinobacteria were the most abundant phyla and together accounted for over 86.0% of all detected OTUs in all samples. Indeed, Proteobacteria were the dominant phylum in book spines and covers as well as in the insect frass, with a relative abundance of respectively 66.5%, 62.2% and 47.9% of the entire bacterial community. In the book spines and in the insect frass, Actinobacteria was the second most represented phylum, followed by Firmicutes, while in the book covers it was the opposite. Firmicutes was the dominant phylum in insect larvae and adults as well as in the book shelves, with a frequency of respectively 43.5%, 35.5% and 35.9% of all detected bacterial OTUs. In the same samples, Proteobacteria and Actinobacteria accounted for the second and third most represented phyla.

The relative abundance of the most relevant genera is reported in Figure 3C. Interestingly, an average of 21.0% of genera in all samples was present with a frequency lower than 1%, ranging from 7.4% in the book covers to the 38.1% in insect adults.

Bacillus (Firmicutes phylum) was the most represented bacterial genus in insect adults and larvae as well as in the book covers, accounting respectively for 22.7%, 26.2% and 29.4% of all detected OTUs. *Stenotrophomonas* (Proteobacteria phylum) was the most represented bacterial genus in the book spines and in the insect frass accounting respectively for 59.0% and 40.1% of all detected OTUs. This genus was also the third in abundance in the book covers (25.2% of detected

OTUs). Notably, both *Bacillus* and *Stenotrophomonas* together with *Staphylococcus*, *Pseudomonas*, *Paenibacillus*, *Brevibacillus* were the only genera present in all samples. *Naxibacter* (Proteobacteria phylum) was the most represented in the book shelves, with a frequency of 15.4% of the entire bacterial community. Other relevant genera include: *Erwinia* (Proteobacteria phylum), the second most represented genus in the book covers, with a frequency of 27.8% of the entire bacterial community; and *Propionibacterium* (Actinobacteria phylum), the second genus most represented in both book spines and insect frass, with a frequency of respectively 18.1% and 16.3% of the entire bacterial community.

3.6 Fungal community taxonomy overview

Fungal sequences were classified into 3 phyla (Table S1). Ascomycota was the dominant phylum and accounted for more than 99.6% of all detected OTUs in both insect larvae and adults, as well as in the book spines.

Ascomycota were also well represented in the book shelves and book covers, where they accounted respectively for the 88.5% and 68.1% of the entire fungal community. Among the Ascomycota, 65.2% and 57.6% of all detected OTUs in insects, respectively adults and in insect larvae, could not be classified beyond the phylum, which may indicate the presence of novel uncharacterized classes.

Basidiomycota was the second dominant phylum, accounting for 32.0% and 11.3% of all detected OTUs respectively in the book covers and book shelves. However, this phylum was present with a percentage lower than 0.003% in insect larvae and adults and in the book spines. The phylum Chytridiomycota was also present in the book shelf samples at a very low frequency (0.0003%).

The relative abundance of the most relevant genera is reported in Figure 3D. The *Symbiotaphrina* genus (Ascomycota phylum) was the only one present in all samples, with a dominant frequency in all samples exceptfor that in the book spines (0.2% of all detected OTUs). Indeed, the *Symbiotaphrina* frequency ranged from 22.3% of all detected OTUs in the book shelves to 40.5% of all detected OTUs in the book cover samples. However, in the book spines, the most abundant genus was *Aureobasidium* (Ascomycota phylum), with a frequency of 95.1% of all detected OTUs. Among the Ascomycota phylum, the genus *Aspergillus* and the family Aspergillaceae were well represented among all samples, especially in the book shelves and the book covers, where they accounted together for respectively 27.0% and 23.0% of all detected OTUs. In the remaining samples, these fungi were present with a frequency higher than 3.4%. In the book shelves, 13.5 % and 7.9% of all detected OTUs were classified as respectively *Cladosporium* and *Zymoseptoria*, both belonging to the Ascomycota phylum.

Rhodotorula was the only genus among Basidiomycota with a frequency higher 1.5% and accounted for 31.4% of all detected OTUs in the book covers.

3.7 PCoA and MDS analysis

PCoA and MDS analyses based on Bray-Curtis distances were performed to compare the overall bacterial and fungal community structure.

As regarding the bacterial community, the highest values of Bray-Curtis distance (Figure 4A), i.e. the highest dissimilarity, were recorded between insect adults and book spines and insect larvae and book spines (0.97 and 0.96 distance score respectively), whereas the lowest values were found between the book spines and insect frass (0.33 distance score) and between insect adults and larvae (0.45 distance score). Bray-Curtis distances were projected onto PCoA (Figure 4C) and MDS (Figure 4D) sample spaces. Bacterial PCoA results were totally in agreement with the MDS analysis and displayed a similar distribution of samples in the graph space. Insect adults and larvae were clustered tightly, indicating a very similar bacterial community. Similarly, book spines and insect frass displayed a short distance in the representation space, confirming the presence of few differences in their bacterial community. On the contrary, book shelves and book covers did not cluster with other samples and were diametrically opposed, suggesting a specific bacterial pattern.

As regards the fungal community, the highest values of Bray-Curtis distance (Figure 4B) were recorded between insect adults and book spines (0.98 distance score), larvae and book spines (0.98 distance score), book spines and book shelves (0.95 distance score) and the book covers and book shelves (0.95 distance score). On the contrary, the lowest values were found between insect adults and larvae (0.11 distance score). Distribution of samples in the graph space was similar for PCoA (Figure 4E) and MDS (Figure 4F) analysis. Insect adults and larvae were clustered tightly, indicating a similar fungal community, whereas book shelves, book covers and book spines did not cluster, suggesting a specific fungal pattern.

4. Discussion

The results of this study gave evidence of an infestation in progress in the History and Historical Documentation Library of the University of Milan and the beetle *G. pubens* was identified as the organism mainly responsible of the library book damage, while sticky traps also revealed the presence of psocids.

As reported by Poggi (2007), *G. pubens* was first described by Leon Fairmaire on the base of two specimens collected by Abdul Kerim in Nafta, Tunisia, on 10/05/1873 (Fairmaire, 1875). It was considered synonymous with *Gastrallus sericatus* (Laporte de Castelnau), but then reconsidered as a valid species by Pic (1912). Español (1963a) described for the first

289 time the male aedeagus characteristics and reported the discovery of this insect in several locations in central-eastern
290 Africa and in a library in Barcelona, Spain (Español, 1963a,b; 1972).
291 Several synonyms of this species are known, but in some cases there are different opinions among the authors. In
292 particular *Gastrallus bollei*, erroneously reported due to a printing error as *G. rollei* (Reitter, 1912a), is not unanimously
293 regarded as a synonym of *G. pubens*, but Poggi (2007) explained that *G. bollei*, found in a museum in Perugia (Italy) in
294 1912 (Reitter, 1912b), was actually *G. pubens,* since *G. bollei* is to be considered a synonym of *G. pubens*. Considering
295 the different synonyms (including *G. bollei*), *G. pubens* is spread in west and central-east Africa, in various areas of the
296 mediterranean basin such as Spain, Tunisia, Egypt, Israel and Lebanon up to the southern Caucasus. Halperin and
297 Español (1978) reported *G. pubens*' presence among insects collected from books in Israel; in 1990 *G. pubens* was
298 identified at Berkeley Law Library, University of California, in books coming from Italy (Boal, 1990). In Italy it was
299 collected in Perugia in 1912, but called *G. bollei* (Reitter 1912a,b), and more recently on ancient books in San Bernardino
300 convent in Genoa (Poggi, 2007), and in 2009 in Sicily, in books of the Regional Library in Catania (Not et al., 2008). In this
301 paper we report that this species is also present in the Lombardy region and for the first time we report information
302 about population density variation during spring and summer.
303 Damage was characterized by larval activity, mainly on bookbinding materials, so that evidently the larvae can integrate
304 into their diets the substances of animal or vegetal origin present in the glues (Not et al., 2008). The distribution of *G.*
305 *pubens* population between the cabinets was not uniform. Several factors can influence beetle preferences for certain
306 cellulosic or additive materials, such as a higher starch or sugar content, which are more attractive than cellulose alone,
307 which is not enough for the development of anobiid larvae (Moşneagu, 2012; Silva et al., 2013). For example, for
308 *Tricorynus herbarius* (Gorham) (Coleoptera, Ptinidae), a pest of historical books in United States, clay-filled and
309 chemically treated papers were less attractive (White, 1963). Notably, a large amount of debris frass was found on
310 shelves and inside tunnels in the oldest books, with covers made of paper/cardboard or parchment.
311 No data are available in the literature on *G. pubens* biology and developmental time. Monitoring data indicate an adults'
312 peak in February and early March, with a decrease in the following months to a few, sporadic individuals in the summer
313 months. Usually, the factors influencing the development cycle include environmental ones: in the library, the
314 temperature and humidity recorded are certainly within suitable values to allow the development of insects.
315 Psocids are small, soft-bodied insects, herbivores or detritivores, feeding on microflora and organic debris on the surface
316 of vegetation and other substrates. They are also known as booklice since they can feed on mold that develops on old
317 books and papers (Baz, 2008). Indeed, they are one of the most frequent group found in the libraries (Fizialetti et al., 2017).

318 *Dorypteryx domestica* was originally described in dwellings (Smithers, 1958), and was reported present in Italy since 1986
319 (Locatelli and Ottoboni, 1986). Sporadic records were reported in the food industries, stored grain and in open country
320 (Kalinović et al., 1981; Kučerová, 1992). Recently they have been found in the Angelica Library in Rome (Fizialetti et al.,
321 2017).
322 The high humidity registered in the investigated library in spring and summer and the direct contact with the outside, has
323 helped to create an ideal place for psocid refuge. Similarly to the Angelica Library in Rome, the population of *D. domestica*
324 increased in the summer months, when the climatic conditions are characterized by high relative humidity. Especially, the
325 lack of air circulation and the particularly high relative humidity in the room can create favorable environmental conditions
326 that promote infestations by these organisms. Additionally, data analysis showed an influence of trap position in *D.*
327 *domestica* catches. The trap near the basement window, overlooking the internal courtyard, caught the highest number
328 of specimens together with several other occasional pests. The presence of mosquitoes, carabid beetles, springtails and
329 pill bugs, that usually develop and live outside, indicates the possibility for pests, included *D. domestica*, to enter the library
330 through the basement window. Therefore, their presence could simply be due to an accidental introduction into the room
331 through the open entrances rather than to an infestation from the inside.
332 The presence of *D. domestica* does not represent a problem or a danger to the stored volumes. These insects are not
333 mentioned in the literature as biological agents of deterioration inside conservation areas (Fizialetti et al., 2017).
334 Accordingly, no damage attributed to psocids was found on the books during the inspections. As they have a life cycle
335 strongly influenced by environmental conditions, they can be easily kept under control by managing the environmental
336 parameter, e.g. by restoring suitable thermo-hygrometric values for the conservation of library artifacts in the deposits
337 (Fizialetti et al., 2017). Indeed, without a suitable environment for their life cycles the populations of these species will
338 quickly decline.
339 In comparison to *D. domestica*, the presence of *G. pubens* can be regarded as particularly harmful and dangerous. Indeed,
340 its irreversible action on books together with the lack of biological information make this insect a matter of severe
341 concern in the library investigated, highlighting the need for a finely targeted intervention rather than a unique and
342 simple control of environmental parameters to reduce the population.
343 Recalling that protection and preservation of cultural heritage is a priority, in this study an advanced and holistic
344 approach has been proposed, i.e. finding a *G. pubens* biomarker, able to detect the insect presence before the
345 infestation becomes too extensive. Therefore, it could be possible to isolate objects as fast as possible and in time to
346 avoid extended damage. Indeed, larvae and insect adults act as bacterial and fungi diffusers in the environment and

directly on and in books. In this paper, bacteria and fungi spread by *G. pubens* have been proposed as primary indications of the insect presence in the library, before the infestation becomes visible. Indeed, a *G. pubens* biomarker was searched for among the bacterial and fungal community peculiar to the insect (insect frass and gut), but also found on books and shelves' surfaces at the same time.

Bacteria and fungi can attack polymers through a variety of enzymes (Lynd et al., 2002) that lead to depolymerisation and material embrittlement (Dunca et al., 2014). As for fungi, several genera are able to grow even in conditions of low availability of water and nutrients (Zyska, 1997). When favorable conditions for development occur, fungal spores have rapid growth cycles and their colonization of substrates can proceed quickly. The presence of microorganisms and especially of fungi can also promote the development of mycophagous insects that feed on fungal mycelia, such as psocids or booklice (Trematerra and Pinniger, 2018). It is therefore essential to take measures for the development of appropriate programs for active and passive conservation, necessary to avoid the deterioration of the books over time.

As regards bacteria, in this study the genera *Bacillus* and *Stenotrophomonas* together with *Staphylococcus*, *Pseudomonas*, *Paenibacillus*, *Brevibacillus* were found in all samples. However, beside these genera which have been previously reported associated with insects (Yun et al., 2014; Rojas-Jimene and Hernandez, 2015), they are also widespread in the environment and potentially may have been carried by dust particles, people and air ventilation systems into the library (Cha et al., 2017; Brągoszewska et al., 2018), independently of the presence of insects. Therefore, they are not suitable to be considered valuable *G. pubens* biomarkers.

Among fungi, microbiological investigations showed that *Symbiotaphrina* were the only genus found in all types of materials analysed, i.e. insect, book and shelves samples. *Symbiotaphrina* is an ascomycete fungus described among the symbiotic levuliform fungi (YLS - Yeast Like Symbionts) present in the anobiid beetles' gut. In particular, *Symbiotaphrina kochii* and *Symbiotaphrina buchneri* were found to be intracellular symbionts of *Stegobium paniceum* (L.) and *Lasioderma serricorne* (F.) (Coleoptera, Ptinidae) (Blackwell, 2017; Noda and Kodama, 1996). They are housed in caeca at the anterior end of the insect midgut, and the female transmits yeast cells to the next generation by smearing them on the eggshell, which is consumed by the hatching larva (Blackwell, 2017). These yeast-like symbionts are important in providing nutrients for the host insects and also in detoxifying plant toxins (Vega and Dowd, 2005).

As far as we know, no studies have been conducted on the microbiome of *G. pubens* and consequently, there are no reports on the relationship between this insect and the fungi of the *Symbiotaphrina* genus.

The direct relationship of this fungal genus with living plant tissues, which are generically the basis of the raw materials with which the volumes examined were manufactured, has however been ascertained, although in sporadic cases. *S.*

376 *kochii*, *S. desertorum*, *S. larreae* were also found on tissues of *Dracaena*, on branches of *Krascheninnikovia lanata* and
377 *Larrea tridentata* (both cases in Arizona) (Baral et al., 2018).
378 Fungal species recently transferred to the *Symbiotaphrina* genus, such as *S. lignicola*, *S. microtheca* and *S. sanguinea* -
379 previously respectively afferent to the genera *Hyphozima*, *Tromera* and *Sporotrichum* (Baral et al., 2018) -, have also
380 been isolated from plants, such as galls on poplar and cortical cancers (Hutchison et al., 1993), fir wood (Saccardo, 1889)
381 and from bark of *Quercus* and *Castanea* (Baral et al., 2018). The presence of *Symbiotaphrina* on the different materials
382 analyzed (book spines, covers, sheets of paper) seems to be correlated to the trophic action of *G. pubens* that leaves its
383 excrements on books, rather than to a direct growth of the fungus on the materials themselves. In fact, *Symbiotaphrina*
384 requires very different substrate conditions compared to the paper material kept in the library, especially as regards
385 their free water and nutrient content.
386 In light of all these considerations, *Symbiotaphrina* can be totally ascribable to *G. pubens*. MiSeq sequencing analysis
387 highlighted that bacterial and fungal communities were very close in insect larvae and adults, and therefore no
388 differences in the *G. pubens* microbiota were found depending on its vital stage (See PCoA and MDS analysis). This is of
389 added value as it means that *Symbiotaphrina* might be a valid biomarker for the presence of *G. pubens* right from its
390 early stages of life and thus its early presence in the library. However, new studies in other libraries may help to validate
391 this finding.
392 When formulating effective conservation strategies, the knowledge of which microorganisms are colonizing the
393 substrate and the different energy sources they consume to sustain themselves is a must to understand the process of
394 biodeterioration (Negi and Sarethy, 2019). In this research, the Illumina technology helped to identify the bacterial and
395 fungal community inhabiting book surfaces and capable of degrading library material. For example *Bacillus* and related
396 species, here found with the highest frequency in book covers and insect guts, have already been isolated from paper
397 affected by foxing as well as from wooden art objects in museum environments (Michaelsen et al., 2010; Lavin et al.,
398 2014; Kalaskar and Zodpe, 2016, Okpalanozie et al., 2018). They are cellulolytic bacteria and potentially play an active
399 role in the deterioration processes. Accordingly, *Bacillus* spp. have been found as the predominant cellulolytic group of
400 bacteria in landfill, where cellulose accounts for 40% to 50% of the municipal solid waste, and in paper mill environments
401 (Ameen et al., 2016). In addition, they form a significant proportion of the intestinal microbial community of soil
402 invertebrates (Konig, 2006).
403 The resuspension of dust and direct human emissions seems to be coupled with the insect presence as a significant
404 sources of microorganisms in the studied indoor spaces. *Stenotrophomonas*, the most represented bacterial genus in

the book spines and in the insect frass, has been found throughout the environment, particularly in close association with soil and plants (Ryan et al., 2009), and therefore is probably carried by dust particles. Notably, members of this genus produce extracellular enzymes such as proteases and chitinases, of which synergistic effects on the degradation of cellulose have been observed (Kobayashi et al., 2002). Similarly, *Aspergillus* spp., well represented among all samples, are slow-growing xerophilic fungi and occur very frequently in biodeteriorated books and manuscripts (Micheluz et al., 2015). They produce numerous spores and conidia that are easily dispersed by air. Indeed, the longevity of their spores has been reported as ranging from 2 to 20 years (Paulussen et al., 2017). These fungi are a potential risk of causing biodeterioration due to their ability to dissolve cellulose fibers, with the action of cellulolytic enzymes, as well as glues, inks or oil binders (Sterflinger et al., 2018). Notably, some *Aspergillus* species are considered hazardous to human health as they cause mycotoxicosis and allergies (Raduli et al., 2020). *Rhodotorula*, abundant in the book covers and book shelves, is a common indoor yeast, frequently encountered in water tanks and/or humidifiers. Some species are also known to be harmful to human health (Wirth and Goldani, 2012). The presence of yeasts has been reported in similar studies, both from air samples and on contaminated art objects (Sterflinger, 2010). Indeed, this genus was noticed after renovation and mechanical cleaning performed inside a library's storeroom (Karbowska-Berent et al., 2012). *Rhodotorula* produces pink to red colonies that can be responsible for book discoloration (Wirth and Goldani, 2012). *Propionibacterium*, the second genus most represented in both book spines and insect frass, is from the human microbiome, i.e. it is a commensal of human skin (Brüggemann, 2016). The genus has already been identified on samples from museums (Piñar et al., 2015) and its presence on documentary heritage can be attributed to direct inoculation by human handling.

Our results also show the presence of some microorganisms belonging to genera that have never been reported in such environments, such as the bacterium *Naxibacter* and the fungi *Itersonilia*, *Zymoseptoria* and the already described *Symbiotaphrina.* Indeed, the application of Illumina on library environments has shown that new unsuspected microbial consortia could be involved in the biodeterioration processes within the library.

Chloroplast sequences among bacterial 16S ribosomal RNA gene were detected in both larvae and adult gut samples. Chloroplasts are evolutionarily descended from bacteria, so it is not surprising that the 16S genes are nearly homologous (Hanshew et al., 2013). Indeed, unintended chloroplast contamination often occurs when a microbial community in phytophagous insects that consume plant-related substrates has been investigated (Hanshew et al., 2013). Beside this being a major methodological obstacle for projects studying these systems, here the finding of chloroplast sequencing within the insect gut added interesting information about the *G. pubens* diet in the library. In this research, a chloroplast

sequence belonging to *Humulus lupulus* was found in the gut of both larvae and adults with a frequency of respectively 2.0 and 0.8% of all OTUs detected with the 16S ribosomal primer, suggesting that this plant was present in the substrate eaten by the insect. *H. lupulus*, the hop, in the family of Cannabaceae, is a perennial herbaceous plant up to 10 meters tall, widely cultivated throughout the temperate regions of the world. Hops are collected and used as major additives and preservatives in beer (Zanoli and Zavatti, 2009). Indeed, the variety lupulus, which was found in the investigated samples, is native to Europe and western Asia (Natsume et al., 2015). However, this plant is not present in the surrounding area close to the library. Therefore, the only way of ingestion by insects is through the feeding of a substrate present in the library made with this plant, likely the paper of which books are made. Indeed, in the 19th century, manufacturing of paper from fresh or spent hops, alone or combined with other material was quite usual (Bickerdyke, 2017). In addition to *H. lupulus*, traces of other plants not belonging to the autochthonous flora were found in the gut of the insect, i.e. *Capsicum annuum*, *Cercis gigantea*, *Gossypium arboretum*, *Nicotiana otophora*, *Trachelomonas ellipsoidalis*, and *Trachelomonas oblonga*. Notably, several books in the library contained between pages the evidence of dried plants, likely ingested by insects and thus found in their gut.

In conclusion, despite some pesticides used to combat the entomological attack to the books and in the air of the History and Historical Documentation Library of the University of Milan in the past, the library is still under active attack. With information on the biology and developmental stages of *G. pubens* provided in this study, the infestation might be solved in a definitive way. Future research will address the establishment of *Symbiotaphrina* search by molecular techniques as a tool to prevent severe damage to the volumes at the time of monitoring or when *G. pubens* infestation is suspected in libraries.

Acknowledgements

The authors wish to thank the librarian Alessandra Carta for access to the library and her help throughout this research. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Declaration of competing interest

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

Table legend

Table 1. Number of reads and number of OTUs of different samples based on a 97% sequence similarity cut off; na stands for “not available”.

Figure legends

Figure 1. Image of the Library of the History Department in the University of Milan building (Panel A). Selected books for the study presented obvious crater-shaped holes and irregularly shaped tunnels which extended from the book binding to the book center and a large amount of insect frass (Panel B). Dark or light brown/yellow discoloration on the front covers (Panel C) and little black stains on the text blocks of the volumes (Panel D) were present in the selected books.

Figure 2. Total number of *Gastrallus pubens* adults (light grey) collected in book cabinets; total number of *Dorypteryx domestica* collected in sticky traps (dark grey); temperature (°C) (blue line) and relative humidity (%) (green line) values, measured during each inspection at the History and Historical Documentation Library, University of Milan, from February

662 to August 2018 (Panel A). *Gastrallus pubens* adult (Panel B) and larva (Panel C). *Gastrallus pubens* frass. Bar corresponds
663 to 1 mm (Panel D).
664
665 Figure 3. Sample coverage-based rarefaction curves of bacterial (Panel A) and fungal (Panel B) richness of each sample
666 calculated at the 97% similarity level cut off. Relative abundance of the most relevant bacterial (Panel C) and fungal
667 (Panel D) genera across samples.
668
669 Figure 4. Bray-Curtis proximity matrix (A and B), PCoA (C and E) and MDS (D and F) analysis showing similarities between
670 bacterial (A, C and D) and fungal (B, E and F) communities of each samples. Panel A and B: white:<0.25, light blue: 0.25-
671 0.50; blue: 0.50-0.75; dark blue:>0.75. Panel C: principal component F1 and F2 of bacterial PCoA explained 87.0% and
672 36.6% of the variance respectively. Panel E: principal component F1 and F2 of fungal PCoA explained 65.2% and 35.9%
673 of the variance respectively. The more similar the microbial community, the closer the distance in the score matrix and
674 scatter plots.
675
676 **Supplementary information**
677 Figure S1. Correlation between the number of collected *D. domestica* (black circle) and *G. pubens* (red square)
678 specimens and the relative humidity (Panel A) and temperature (Panel B).
679
680 Table S1. Average of relative abundance of bacteria and fungi in the samples.
681

	Bacteria		Fungi	
Sample	**No. reads**	**No. OTUs**	**No. reads**	**No. OTUs**
Book covers	82524	199	38062	105
Book spines	25462	136	42336	19
Book shelves	56384	339	39239	307
Insect frass	40774	138	na	na
Insect larvae	21343	337	42697	9
Insect adults	26435	393	42576	7

Table 1. Number of reads and number of OTUs of different samples based on a 97% sequence similarity cut off; na stands for “not available”.

Figure 1

Click here to access/download;Figure;Figure 1.tif

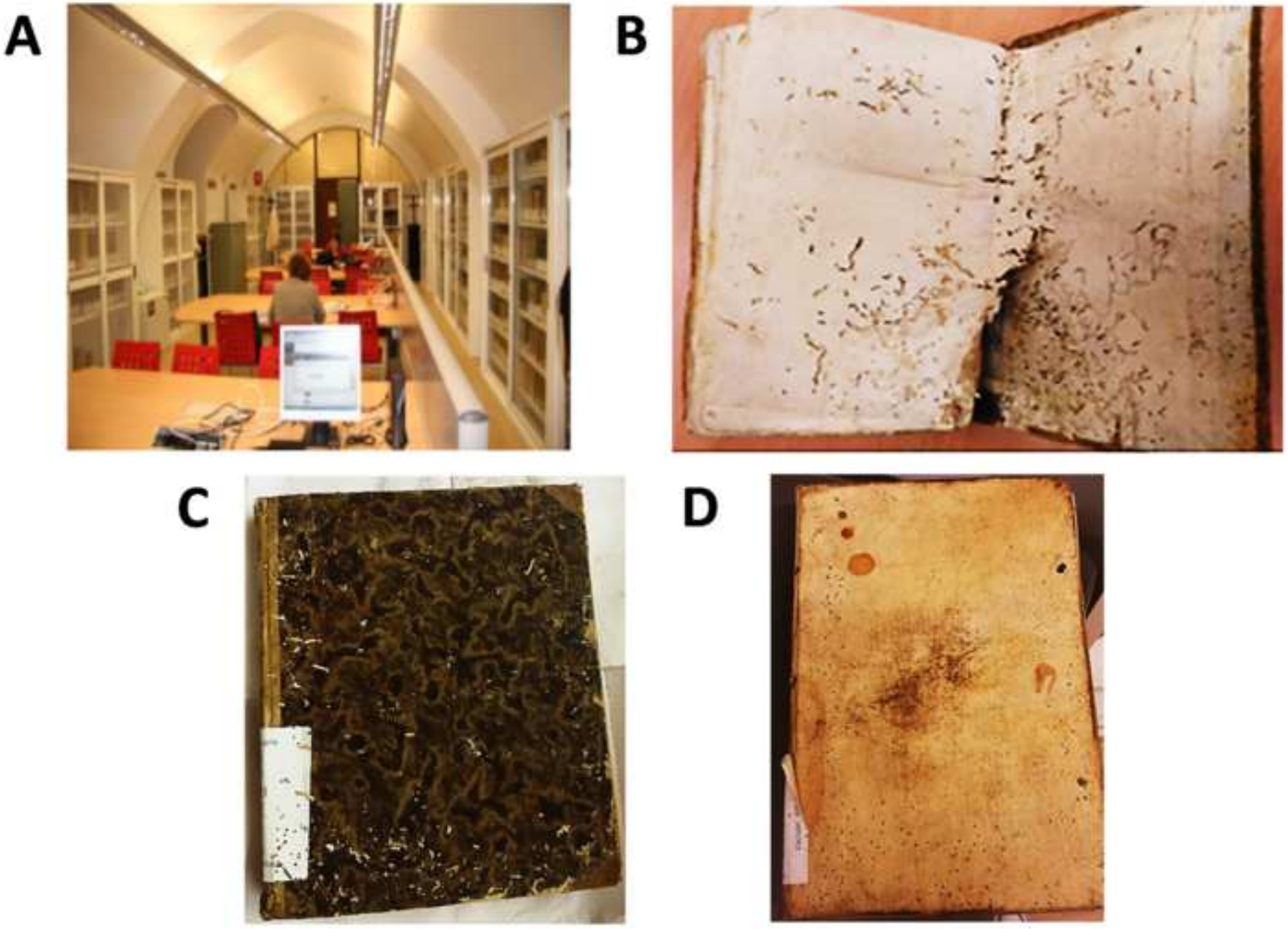


Figure 2

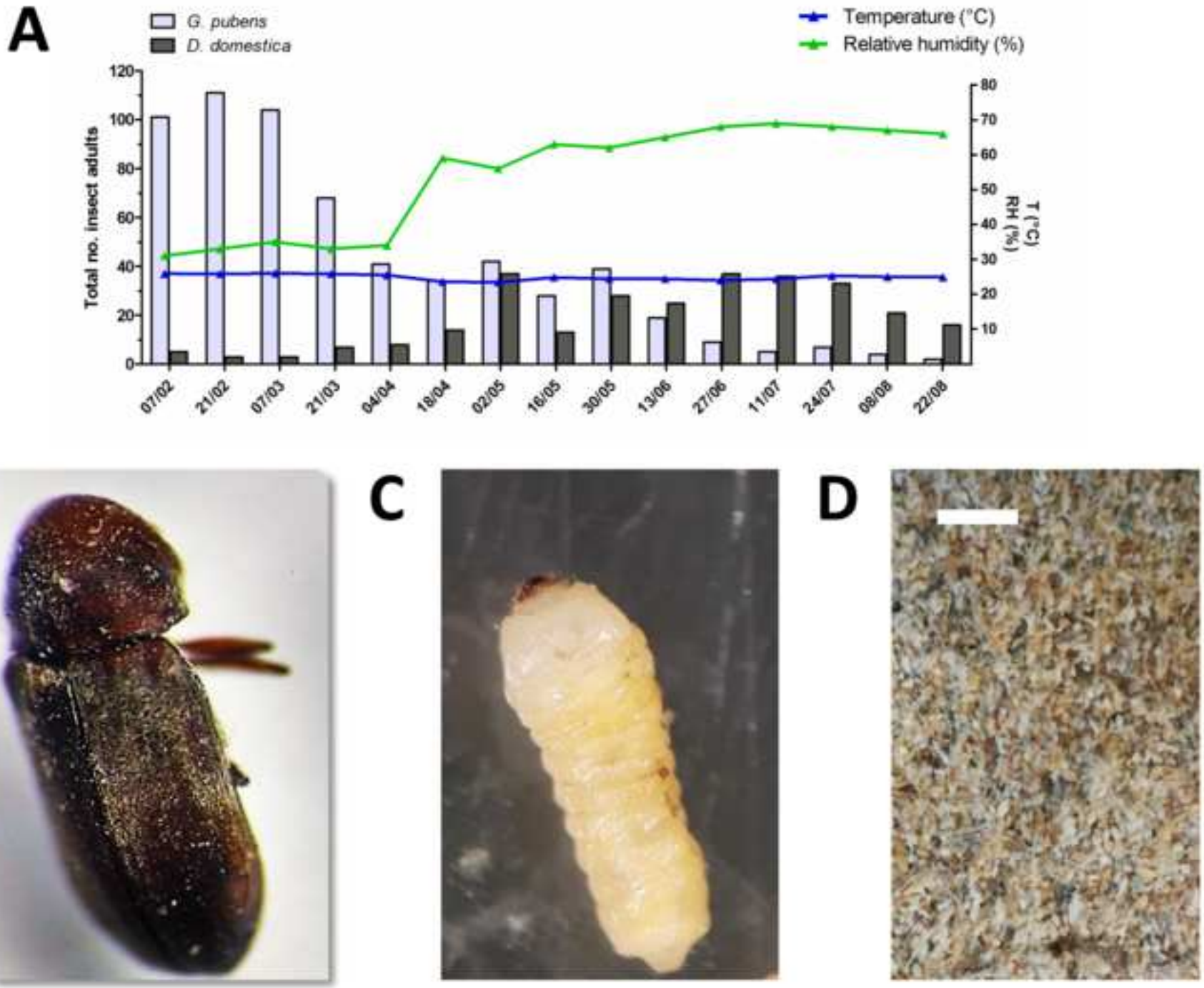


Figure 3

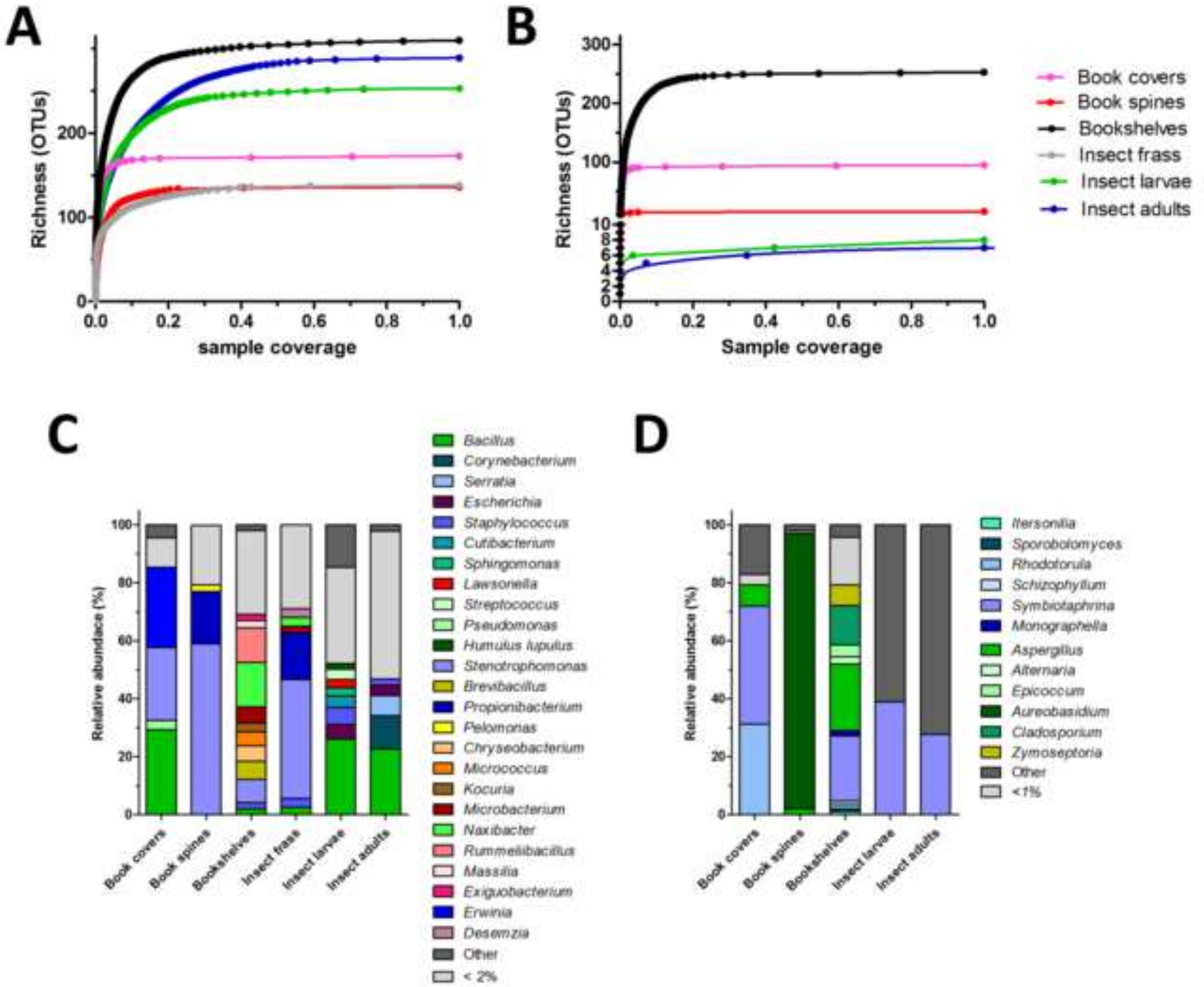


Figure 4

Click here to access/download;Figure;Figure 4.tif

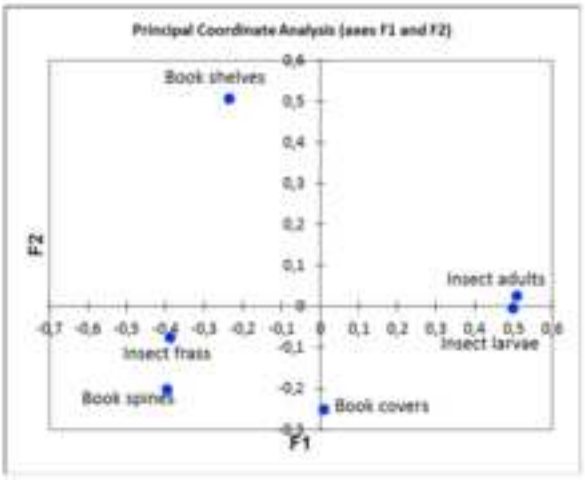
A

	Book covers	Book spines	Book shelves	Insect frass	Insect larvae	Insect adults
Book covers	0.00	0.66	0.83	0.64	0.72	0.76
Book spines	0.66	0.00	0.78	0.33	0.96	0.97
Book shelves	0.83	0.78	0.00	0.66	0.93	0.93
Insect frass	0.64	0.33	0.66	0.00	0.93	0.94
Insect larvae	0.72	0.96	0.93	0.93	0.00	0.45
Insect adults	0.76	0.97	0.93	0.94	0.45	0.00

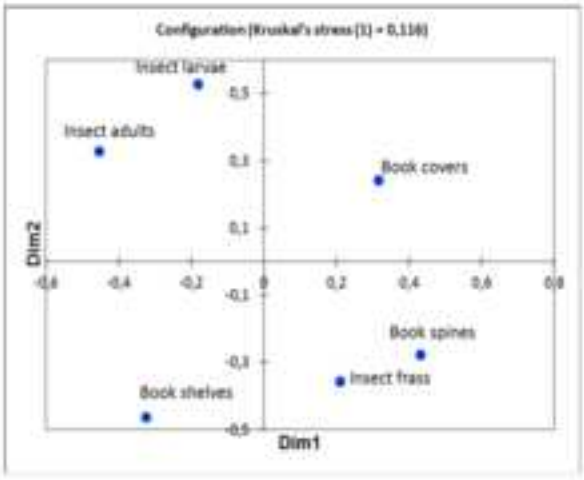
B

	Book covers	Book spines	Book shelves	Insect larvae	Insect adults
Book covers	0.00	0.95	0.62	0.56	0.64
Book spines	0.95	0.00	0.95	0.98	0.98
Book shelves	0.62	0.95	0.00	0.74	0.73
Insect larvae	0.56	0.98	0.74	0.00	0.11
Insect adults	0.64	0.98	0.73	0.11	0.00

C



D



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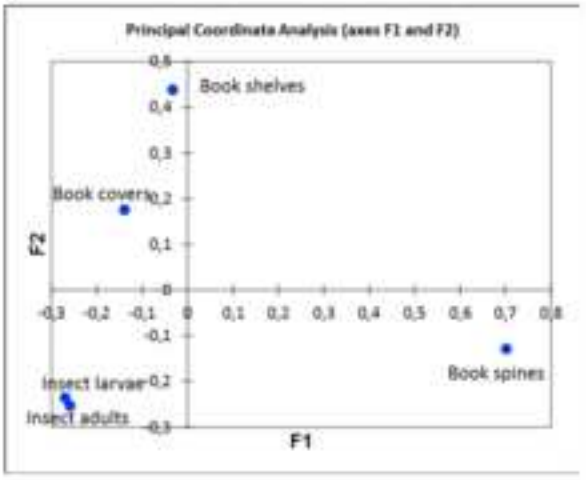

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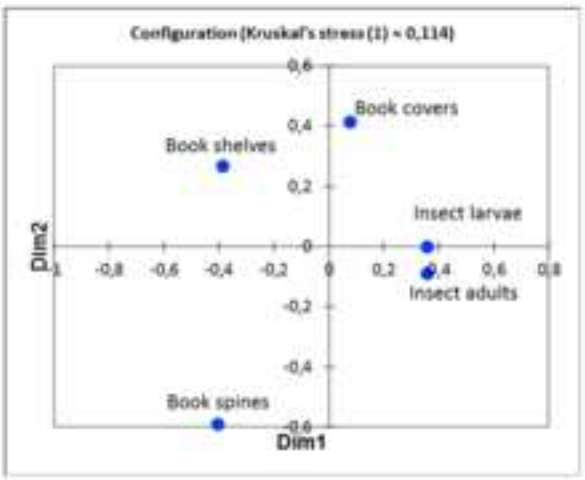

Figure S1

Table S1

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Conception and design of study: S.S., C.C., F.V., M.S., F.T., P.C., F.C.

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Drafting the manuscript: S.S., C.C., M.S.

Revising the manuscript critically for important intellectual content: P.C, F.C.

Approval of the version of the manuscript to be published: S.S., C.C., F.V., M.S., F.T., P.C., F.C.

Declaration of interests

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: