INSTITUTO UNIVERSITÁRIO EGAS MONIZ

MESTRADO EM TECNOLOGIAS LABORATORIAIS EM CIÊNCIAS FORENSES

USING FORENSIC MICROBIOLOGY TO ASSOCIATE VEHICLES AND THEIR COMPONENTS

Trabalho submetido por
Madalena Véstia Antunes
para a obtenção do grau de Mestre em Tecnologias Laboratoriais em Ciências Forenses

novembro de 2025

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Trabalho orientado por
Prof. Doutora Madalena Oom

e coorientado por
Prof. Doutora Zoé Vaz da Silva e Prof. Doutor Paulo Mascarenhas

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Dedicatória

Primeiramente dedico esta tese a mim mesma, à Joana, aos meus pais e irmã, à minha restante família e amigos, e sobretudo, a quem partiu cedo demais e não viu este momento chegar, sei que estão tão orgulhosos quanto eu neste momento.

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"Tenho em mim todos os sonhos do Mundo"

-Fernando Pessoa

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Resumo

A microbiologia forense aplica a caracterização de comunidades microbianas a contextos investigativos. Este estudo avaliou se os fungos presentes no pó recolhido de vários veículos de passageiros podem contribuir como marcadores para a sua identificação e diferenciação. Foram recolhidas amostras das superfícies interiores e exteriores de oito veículos de três sub-regiões portuguesas. Os fungos presentes nessas amostras foram isolados em meios seletivos. Os fungos foram identificados morfologicamente e o seu ácido desoxirribonucleico (ADN) foi amplificado por PCR com *primers* para as regiões ITS. Os produtos resultantes foram enviados para sequenciação Sanger. Foram identificados um total de 18 taxas fúngicas, das quais *Sporobolomyces roseus*, *Cladosporium spp*. e *Penicillium expansum* foram as mais prevalentes. As superfícies interiores apresentaram maior homogeneidade, possivelmente devido à presença de fungos associados ao contacto humano, enquanto as superfícies exteriores apresentaram maior variabilidade associada à exposição ambiental. Embora tenham sido observadas algumas diferenças entre os grupos analisados, o método utilizado para identificar o veículo de origem teve um desempenho limitado. A taxa de identificação correta a partir de uma peça externa foi de apenas 12%, um resultado que não é melhor que o obtido por acaso. Quando se consideraram as três ou cinco hipóteses mais prováveis, as taxas de identificação correta aumentaram para 54% e 67%, respetivamente. Embora já ultrapasse as chances do acaso, continua a ser insuficiente para uma identificação fiável da viatura, podendo contudo contribuir para a eliminação de alguns dos veiculos suspeitos. Embora alguns tipos de microrganismos pareçam estar mais presentes em determinados veículos, essas associações deixaram de ser estatisticamente significativas após uma análise mais rigorosa. Por conseguinte, estes padrões devem ser interpretados apenas como indícios preliminares e não como evidências forenses consistentes. O estudo exploratório confirma o potencial da análise de comunidades fúngicas como ferramenta complementar na investigação forense, mas demonstra igualmente que o método, na sua forma atual, carece de precisão discriminatória para uma aplicação operacional. Estudos futuros deverão incluir dados de abundância, amostragens mais extensas, controlo de variáveis ambientais e integração com métodos forenses tradicionais, como o ácido desoxirribonucleico (DNA) as impressões digitais.

Palavras-chave: microbiologia forense; micologia; veículos; bioindicadores; fungos; investigação criminal.

Abstract

Forensic microbiology applies the characterisation of microbial communities to investigative contexts. This study assessed whether the fungi present in the dust collected from various passenger vehicles can contribute as markers for their identification and differentiation. Samples were taken from the interior and exterior surfaces of eight vehicles from three Portuguese sub-regions. The fungi present in these samples were isolated on selective media.

The fungi were identified morphologically and their deoxyribonucleic acid (DNA) was amplified by PCR with the primers for the ITS regions. The resulting products were sent for Sanger sequencing. A total of 18 fungal taxa were identified, of which *Sporobolomyces roseus*, *Cladosporium spp*. and *Penicillium expansum* were the most prevalent. Interior surfaces showed greater homogeneity, possibly due to the presence of fungi associated with human contact, while exterior surfaces showed greater variability associated with environmental exposure. Although some differences were observed between the groups analysed, the method used to identify the vehicle of origin had limited performance. The correct identification rate from an external part was only 12 per cent, a result that is no better than that obtained by chance. When the three or five most likely hypotheses were considered, the correct identification rates increased to 54 per cent and 67 per cent respectively. Although this already exceeds the chances of chance, it is still insufficient to reliably identify the vehicle, but it could help to eliminate some of the suspect vehicles. Although some types of microorganisms appear to be more present in certain vehicles, these associations are no longer statistically significant after a more rigorous analysis. Therefore, these patterns should only be interpreted as preliminary indications and not as consistent forensic evidence. The exploratory study confirms the potential of fungal community analysis as a complementary tool in forensic investigation, but also demonstrates that the method, in its current form, lacks the discriminatory precision for operational application. Future studies should include abundance data, more extensive sampling, control of environmental variables and integration with traditional forensic methods, such as deoxyribonucleic acid (DNA) and fingerprints.

Keywords: forensic microbiology; mycology; vehicles; bioindicators; fungi; criminal investigation.

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ADB:	Agarose Dissolving Buffer
DNA:	Deoxyribonucleic Acid
dNTP:	Phosphate Deoxyribonucleotides
EDTA:	Ethylenediaminetetraacetic acid
IQR:	Interquartile Range
ITS:	Internal Transcribed Spacer
LOOCV:	Leave-One-Out Cross-Validation
MALDI-TOF:	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
NGS:	Next Generation Sequencing
PCR:	Polymerase Chain Reaction
PERMANOVA:	Permutational Multivariate Analysis of Variance
R^2:	Coefficient of Determination
SD:	Standard Deviation
SDA:	Sabouraud Dextrose Agar
TAE:	Tris-Acetate-EDTA

1. Introduction

1.1. General Background

In recent decades, forensic science has firmly established itself as a vital part of the criminal justice system, characterised by the continuous adoption of scientific and technological advances from various fields of study, such as DNA analysis and fingerprinting (1,2). Traditionally, forensic methods have relied on well-established techniques such as fingerprint analysis, fibre examination, ballistics and genetic identification using deoxyribonucleic acid (DNA) (3). These methodologies play a fundamental role in identifying individuals, reconstructing events, and establishing links between suspects, victims, and crime scenes (4). However, the effectiveness of these techniques often depends on the preservation, availability and quality of the evidence collected (5). Often, the absence of DNA, degraded fingerprints or contaminated samples makes it impossible to apply traditional approaches, necessitating complementary and innovative solutions (3,4).

In this context, forensic microbiology has emerged as a promising scientific field, the relevance of which has been progressively recognised by the academic and forensic communities (6). This discipline is dedicated to studying microbial communities - consisting of bacteria, fungi, viruses, and other microorganisms - present in biological, environmental or physical remains of criminal interest (6,7). These microorganisms are widespread in practically all natural and artificial substrates, and their distribution patterns are influenced by ecological, geographical, and anthropogenic variables (6). These characteristics give microbiomes potential evidential value, as they can act as biological markers that characterise certain individuals, objects, places and/or circumstances (6,7).

Significant progress has been made in characterising complex microbiomes from small, often degraded samples thanks to the development of molecular biology techniques, particularly Next Generation Sequencing (NGS) (8). These technologies allow microbial communities to be analysed in terms of both taxonomy and function with a high degree of precision, even in a challenging forensic context (8). Several studies have demonstrated the applicability of forensic microbiology in areas such as estimating the post-mortem interval, identifying the geographical origin of corpses, tracing human activity on contact surfaces and associating environmental traces with suspects (6,7).

Despite progress having been made, the forensic application of microbiology is still in its infancy and faces multiple methodological and operational challenges (9). One of the least explored contexts is the vehicle environment, which, due to its unique characteristics, is of great interest to criminal investigators (10,11). Motor vehicles function as dynamic micro-environments, simultaneously influenced by the external environment (climate, geography and environmental dust) and human actions (presence of occupants, usage habits and ventilation (11). The dust that accumulates on different vehicle components - both inside and outside the passenger compartment - can harbour diverse microbial communities whose composition may reflect usage patterns, routes travelled, geographical areas crossed, and human contact profiles (12,13). Therefore, vehicles and their components can provide valuable information for obtaining forensic microbiological profiles, particularly in cases where traditional evidence, such as DNA or fingerprints, is unavailable or inconclusive (12,13).

In this context, it is important to investigate the potential of applying forensic microbiology to the field of vehicle analysis, with a particular focus on characterising the fungal communities present in dust particles collected from car components. Fungi are particularly relevant in this type of study due to their ubiquity, resilience, and ability to persist in dust particles for long periods of time, acting as stable biological recorders of environmental exposure. Moreover, fungal communities show considerable diversity and geographical specificity, making them valuable potential markers for linking vehicles or their fragments (14).

1.2. Forensic Microbiology and the Role of Fungi

Forensic microbiology is a subdiscipline of forensic science which seeks to apply microbiological knowledge to the resolution of criminal cases by analysing microbial communities in material and biological remains. This approach assumes that, due to their ubiquity, environmental specificity and adaptability, microorganisms are a highly informative form of evidence that can be used to reconstruct events, identify contact sites and establish links between individuals and objects. The microbiomes that can be detected on different substrates, such as soil, dust, surfaces, fabrics or personal objects, can vary significantly depending on ecological, geographical and behavioural factors. For this reason, they are considered indirect biological markers with evidential potential (15).

Of the various microbial groups of forensic interest, fungi are particularly relevant due to their unique characteristics. These eukaryotic organisms belong to the Fungi kingdom and demonstrate remarkable morphological, ecological, and metabolic diversity (16). They are also adapted to a wide range of environments, including extreme habitats. Many species of fungi have the ability to produce resistant spores that can persist for long periods in adverse environmental conditions, facilitating their detection even in old or degraded samples (17). Additionally, fungi demonstrate high ecological specificity, with characteristic communities associated with particular soils, vegetation, climatic conditions, and human activities (18). This ecological specificity makes them valuable for identifying specific environmental or geographical contexts (18).

Although forensic fungal analysis is still relatively unexplored compared to other biological traces, it has gained recognition as a valuable addition to criminal investigations. Recent studies have demonstrated the potential of using fungal profiles to determine the origin of objects, differentiate between indoor and outdoor environments, and identify the routes travelled by individuals or vehicles (19,20). Dust particles, which often accumulate on frequently used or difficult-to-clean surfaces, are an important reservoir of fungal spores and can represent the environmental and human history of a given object (21,22).

However, the forensic application of mycology still faces several challenges, particularly concerning standardising sampling methods, DNA extraction protocols and taxonomic analyses (22). One key difficulty is that fungal communities detected on vehicle surfaces may include taxa derived from the environment, which can indicate geographical origin, as well as taxa associated with humans resulting from skin or respiratory shedding (21). The latter do not necessarily reflect the surrounding environment and can complicate the interpretation of fungal profiles in a forensic context. This issue is particularly relevant for frequently handled areas such as steering wheels, door handles, and seats, where fungi of human origin tend to accumulate (23). Therefore, it is essential to carefully differentiate between these sources to ensure that the recovered fungal signatures provide reliable information about environmental exposure and geographical context. The relevance of fungi in forensic microbiology is particularly evident when analysing vehicle remains. The dust that accumulates on the exterior and interior of vehicles, for example, on side mirrors, rims, carpets or ventilation grilles, tends to incorporate fungal communities that reflect the vehicle's geographical exposure, environmental conditions and usage habits (24). Identifying these communities could therefore help link vehicle fragments, which are often found at crime scenes in cases such as hit-and-runs, to the vehicle of origin. This would strengthen the body of evidence available for criminal investigations.

In this sense, fungi appear to have high evidential value due to their ubiquity, resistance and ability to reflect specific environmental contexts (5). This research, therefore, aims to expand knowledge of the role of fungi in forensic microbiology, particularly in relation to analysing traces associated with motor vehicles, from an innovative perspective that remains largely unexplored in Portugal.

1.3. Portugal's Geographical and Environmental Diversity: Microbiological Relevance

Despite its small size, Portugal boasts great geographical and climatic diversity. This is the result of various factors, including latitude, topography, proximity to the Atlantic Ocean and the combined influence of the Mediterranean and Atlantic (25). This diversity is reflected in a variety of ecosystems, ranging from humid coastal areas to more arid inland regions, including mountainous areas, agricultural plains, and urban environments (25). This ecological diversity directly impacts the composition and dynamics of microbial communities, particularly fungal communities, which adapt to the specific environmental conditions of each region.

Since the 14th century, Portugal has traditionally been divided into provinces (26). These evolved into a more detailed territorial organisation that is currently subdivided into regions and sub-regions with specific boundaries. These administrative divisions serve as management tools and reflect the different environmental, social and economic realities that influence local biological patterns. In this context, the focus of this research is on three sub-regions with contrasting environmental and climatic characteristics: Grande Lisboa, Península de Setúbal and Alto Alentejo (26).

The climate classification is based on Köppen's system, which divides Portugal into two regions. One has a temperate climate with rainy winters and dry, hot summers (designated Csa), while the other has a temperate climate with rainy winters and dry, slightly hot summers (designated Csb) (27). Due to their proximity to the Tagus River, the Grande Lisboa and Península de Setúbal areas have altitudes close to zero compared to the Alto Alentejo area. Specifically, the area where samples were collected has a higher altitude due to its proximity to the São Mamede Mountain range (27,28).

The figure below presents two instances of the map of Portugal, where on the left (a) it represents the differences between the climate regions, according to Koppen's classification and on the right (b) it represents the altitude ranges across the country map.

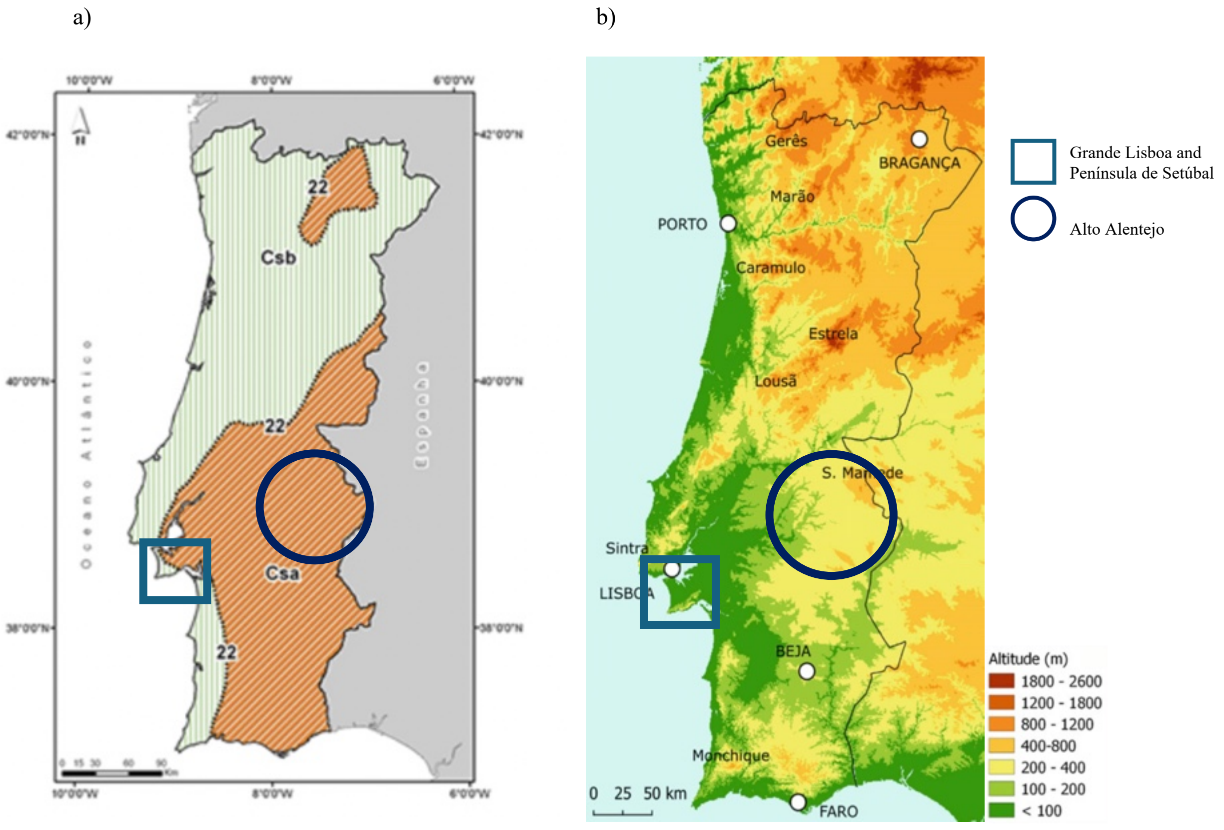

Figure 1. Map of Portugal representing, on the left (a) the two different climate regions (27) and on the right (b) the difference of altitude across the country (28). Additionally, it is marked with a square the sub-regions of Grande Lisboa and Península de Setúbal, and with the circle the sub-region of Alto Alentejo.

The metropolitan area of Grande Lisboa, which includes the country's capital, is characterised by a highly urbanised environment with high population density, intense industrial and economic activity, and heavy road traffic, as well as the Península de

Setúbal (28,29). The proximity to the Tagus River and low altitude promote a Mediterranean temperate climate with rainy winters and dry, hot summers. This generates a microbiome that is heavily influenced by human activity, with fungi that are common in indoor and urban environments predominating, such as species from the genera *Aspergillus*, *Penicillium* and *Cladosporium* (29). These species have adapted to environments altered by urbanisation, atmospheric pollution, and the frequent presence of artificial surfaces. They form fungal communities that reflect the region's lifestyle and materials (29).

On the other hand, Alto Alentejo has a continental Mediterranean climate, characterised by hot, dry summers and relatively cold, dry winters (28,30). The region is mostly rural, dominated by agricultural areas, forests and sparsely populated areas. These conditions favour the prevalence of fungal communities typical of natural and semi-natural environments, with specimens from genera such as *Fusarium*, *Alternaria* and *Mucor* associated with soils, organic matter and native vegetation (31). Less human intervention and the frequent contact of vehicles with natural soils and dust promote the accumulation of fungal microbiomes that reflect the local ecology, contributing to the biogeographical differentiation of these environments.

These climatic and geographical differences between the two regions result in ecological gradients that shape the composition of the fungal communities present in the dust accumulated on vehicles and their components. Factors such as temperature, humidity, soil type, predominant vegetation and degree of urbanisation influence the diversity, abundance and resistance of detectable fungi, making it possible to identify distinctive regional microbial profiles (31).

This spatial variation in fungal communities has important implications for forensic microbiology, since characterising and comparing microbial profiles can help to link vehicles or vehicle fragments to specific geographical areas. In criminal investigations, this ability can be crucial in reconstructing routes, locating crime scenes, and identifying the origin of vehicle traces (32).

1.4. Vehicular Environments as Microbial Microecosystems

Motor vehicles represent unique micro-ecological environments where various environmental and human factors interact to create complex microbial communities. Unlike other natural or urban environments, vehicles have diverse surfaces, such as metal, plastic, textiles and glass that are exposed to external conditions, such as dust, humidity and pollution (33). They are also in direct contact with occupants, creating specific and differentiated microbial niches.

The dust that accumulates on vehicle components such as rear-view mirrors, wheels, tyres, carpets, and upholstery acts as a natural reservoir for microorganisms, including bacteria, viruses, and fungi (34). The composition of these communities reflects a combination of factors, including the geographical and climatic environment in which the vehicle circulates, interactions with occupants (e.g. skin and respiratory microbiomes), and maintenance and cleaning habits (35). Fungi are particularly notable for their high resilience and ability to colonise varied environments, resisting adverse conditions such as temperature, humidity and ultraviolet radiation, making them less susceptible to being altered after the crime is committed, enhancing their value as a potential biomarker (36).

The accumulation of these fungi in vehicles can be influenced by various environmental factors, such as ambient temperature, relative air humidity, journey frequency and duration, and the surface on which the vehicle is parked (37). Vehicles parked in rural areas, for instance, may harbour fungal communities with greater diversity of soil and vegetation species, whereas those in urban areas tend to accumulate microorganisms associated with pollutants and human-altered environments (37,38). Furthermore, the distinct characteristics of each vehicle component influence fungal colonisation. For instance, exterior surfaces such as side mirrors and rims are exposed to the external environment, accumulating microbiomes that reflect local conditions such as soil type, pollution and surrounding vegetation. In contrast, the interior of the vehicle, including the upholstery and panelling, harbours a microbiota that is more influenced by human contact and interior environmental factors, such as ventilation and internal humidity.

Most of the studies conducted so far have focused on analysing airborne fungi in different types of vehicles, such as private cars, buses and trains . The authors aimed to identify fungal communities from a public health perspective rather than a forensic one. Although the samples consisted of air particles, many fungi were identified, and despite the studies having different sampling locations and being conducted in different seasons and years, many studies had common fungi.

For example, a 2005 study by (37) analysed the indoor and outdoor air of several vehicles and concluded that the most prevalent genera of fungi were *Cladosporium, Penicillium, Aspergillus* and *Alternaria.* More recently, a study by Vasconcelos et al. (2023) identified the same fungi genera in air particles originating from air conditioners, as well as many others, including *Rhodotorula, Fusarium* and *Curvularia,* in samples collected from light and heavy vehicles in Brazil (39).

In 2021, Parsay et al. characterised the airborne fungi originating from air conditioners in 138 cars. Once again, the genera *Cladosporium spp., Penicillium spp.* and *Aspergillus niger* were the most common identified genera (40).

Overall, these studies demonstrate that certain fungal genera, notably *Cladosporium*, *Penicillium* and *Aspergillus*, are consistently prevalent in vehicle environments, irrespective of location. At the same time, the detection of additional taxa in different regions suggests that fungal profiles may reflect environmental conditions and vehicle use. However, most research so far has focused on airborne fungi and air conditioning systems, while other potential substrates, such as dust, upholstery, and frequently touched surfaces, have been largely overlooked.

From a forensic perspective, the microbiological analysis of vehicles and their components is a promising method of obtaining information to complement traditional techniques. The diversity and specificity of vehicle microbiomes can be used to establish links between vehicles and specific locations, identify routes travelled and provide evidence in cases where other forms of proof, such as human DNA or fingerprints, are unavailable or insufficient.

However, applying these principles in a forensic context presents significant methodological challenges. These include the need for rigorous protocols for collecting, preserving, and analysing microbiological samples, as well as careful data interpretation, given the complexity and natural variability of vehicle microbiomes. Distinguishing between microorganisms from the external environment and those originating from vehicle occupants is essential for ensuring the reliability of the results.

1.5. Application of Forensic Microbiology in the Association of Vehicle Components

In criminal investigations, motor vehicles often take centre stage, either as a means of transport for illicit activities or as an element directly involved in crimes such as hit-and-runs, theft, kidnapping or abandoning corpses. In many of these situations, traditional forms of evidence, such as fingerprints, biological residues or visual records, may be absent, contaminated or damaged (41). In such cases, microbiological analysis, particularly forensic fungal microbiology, can provide valuable clues in establishing links between vehicle fragments and places where the vehicle was driven or parked (41).

Fragments such as broken wing mirrors, rims, bodywork, bumpers or debris adhering to the underside of the vehicle can retain microbial traces reflecting the environment to which they were exposed. The fungi present on these fragments tend to persist over time and retain a relatively stable ecological signature when protected from solar radiation or frequent washing, acting as biogeographical markers (42).

Vehicle dust is an effective means of transporting and preserving fungal communities. Made up of soil particles, organic matter, spores, plant residues and atmospheric contaminants, this dust accumulates in different parts of the vehicle, forming a kind of 'environmental record' of the places it has been (43,44). Analysing the fungal composition present in this dust using culture methods or genetic sequencing makes it possible to compare samples collected from suspect vehicles with those found at specific locations or crime scenes to look for matches that support their association (43).

This approach is particularly relevant in situations involving vehicle fragmentation, such as collisions or hit-and-runs, where parts come loose and are left at the scene. Forensic analysis of these fragments can identify the vehicle of origin, even if there are no direct identifying features, such as licence plates or chassis numbers, present. If the fungi present on a fragment match the microbial profile of a suspect vehicle, this can support the hypothesis that the vehicle is linked to the event under investigation.

However, the effectiveness of this method depends heavily on various factors, including the robustness of the sampling and preservation protocols, the existence of regional fungal reference databases that allow ecological contextualisation, and the control of external variables that could introduce interpretative noise, such as fungi introduced by human contact or artificial environments.

The practical application of this methodology requires the standardisation of comparison criteria and the quantification of similarity between microbiological profiles. It also requires the definition of scientific interpretation thresholds that support the validity of the expert analysis.

Although this is still a developing field, recent research has shown that analysing microbiomes - particularly fungal communities - can achieve high levels of spatial and environmental resolution. This makes it a progressively more reliable complementary tool for forensic identification. The methodology's value lies not only in directly identifying sources, but also in excluding alternative hypotheses or reconstructing the routes travelled by a particular vehicle.

1.6. Aim

This study aims to analyse the relationship between fungal communities present on a vehicle's exterior and interior components. The study also seeks to develop and test a standardised protocol for use in real forensic contexts. This work aims to contribute to the use of fungi as bioindicators in the forensic analysis of vehicles by offering the forensic community practical tools and methodological guidelines to strengthen the reliability and applicability of microbiological evidence.

2. Materials And Methods

2.1. Materials

2.1.1. Sampling, inoculation and incubation

Sterile Sabouraud Dextrose Agar (SDA) medium with antibiotic was prepared before sampling. Samples were collected using sterile swabs and Falcon tubes containing sterile peptone water. The medium preparation, transfer of peptone water to Falcon tubes, inoculation of samples and re-sowing of colonies were all performed in a laminar flow chamber to guarantee sterilisation.

2.1.2. Molecular analysis

The following reagents were for DNA extraction: lysis buffer (p:p:p:p 1:2,8:1,46:6,3) 2-mercaptoethanol, phenol:chloroform:isoamyl alcohol (v:v:v 25:24:1) and isopropanol. The enzyme used for PCR reactions was the GoTaq® DNA Polymerase Enzyme (ref. M7808), and the primers were purchased from STAB VIDA, Lda, FCT/UNL Ed. Departamental (Química/Ambiente), Lab 007, with its sequencing listed on the following table:

Table 1. Primer sequences for the ITS regions.

Primer	Sequence	Reference	Acquired from:
ITS1	5- TCC GTA GGT GAA CCT GCG G -3	(44–46)	STAB VIDA, Lda, FCT/UNL Ed. Departamental (Química/Ambiente), Lab 007 2829-516 Caparica
ITS2	5- GCT GCG TTC TTC ATC GAT GC -3		
ITS3	5- GCA TCG ATG AAG AAC GCA GC -3		
ITS4	TCC TCC GCT TAT TGA TAT GC -3		

The agarose gel for the electrophoresis run was made using the Agar Cat. 604-005 (GeneON GmbH Bioscience) and TAE Buffer 1x (Tris-Acetate 0.4M, EDTA 0.01M and ultrapure water). In order to visualise the DNA bands, the GreenSafe Premium (NZYTECH ref. MB13201) was also used, and the marker was the Biotin Hyperladder 100bp.

The following reagents for DNA purification were used: ADB (Agarose Dissolving Buffer), Isopropanol, DNA washing PE buffer, and the column used for the process was a silica column (ref. grisp GLC. PN59).

2.2. Methods

2.2.1. Selection and sampling of cars

To establish a basis for comparison between different geographical and environmental contexts, eight passenger cars from three sub-regions of mainland Portugal – Grande Lisboa, Península de Setúbal and Alto Alentejo - were sampled. The sample was selected based on pre-established inclusion and exclusion criteria. This included selecting vehicles for regular personal use, while excluding commercial vehicles that had recently been sanitised or had not been used in the seven days prior to sample collection.

Following a detailed analysis, six sites of representative dust accumulation were identified on each vehicle: the driver's mat, the driver's seat, the steering wheel, the front grille, the side mirror and the wheel. Sampling was carried out in triplicate at each location, with two negative controls (one internal and one external) also collected from each vehicle. A total of 160 dust samples were collected.

The samples were collected using sterile swabs that had been soaked in sterile peptone water.

2.2.2. Inoculation and incubation

In the laboratory, the swabs were vortexed for one minute to release the particulate material into the transport liquid. An aliquot of the contents of each tube was then spread onto Petri dishes containing Sabouraud Dextrose Agar (SDA) medium, which is suitable for growing filamentous fungi and yeasts. The plates were then incubated at 25 °C for five to seven days, with observations made daily to record any microbial growth.

Figure 2 represents a schematic illustration of the sampling, inoculation and incubation process.

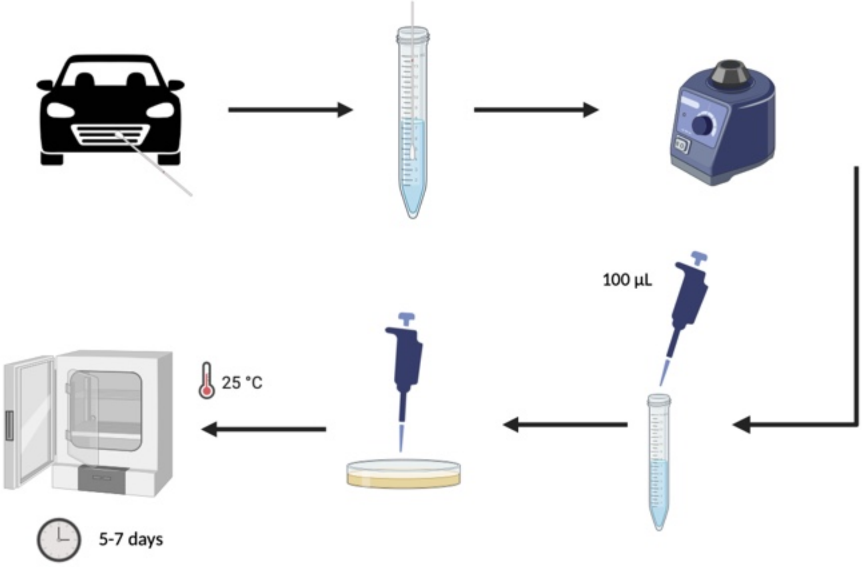

Figure 2. Schematic illustration of the sampling, inoculation and incubation process, created with BioRender.

2.2.3. Morphological Identification

The obtained colonies were assessed macroscopically using a stereoscopic magnifying glass from Leica EZ4. Characteristics such as shape, colour, texture, pigmentation and margin morphology were recorded. Based on these criteria, colonies with differentiated morphology were selected for microscopic analysis, using the Olympus Microscope.

For microscopic observation, the specimens were mounted on a slide in lactophenol cotton blue to identify mycelial and reproductive structures (e.g. hyphae, conidiophores, conidia or spores). This analysis enabled preliminary identification of the fungus based on morphology, following (Ellis, 2007) and (Käärik et al., 1983) (45,46).

The colonies with different morphological characteristics were then replated on new SDA plates to obtain the single cultures needed for molecular analysis.

2.2.4. Re-inoculation of isolates

For colony replating, serial dilutions from the initial colony were performed, ranging from 1:10 to 1:1 000 000. Negative control consisting exclusively of peptone water was also prepared everytime an isolate was re-inoculated. Figure 3 illustrates the method described.

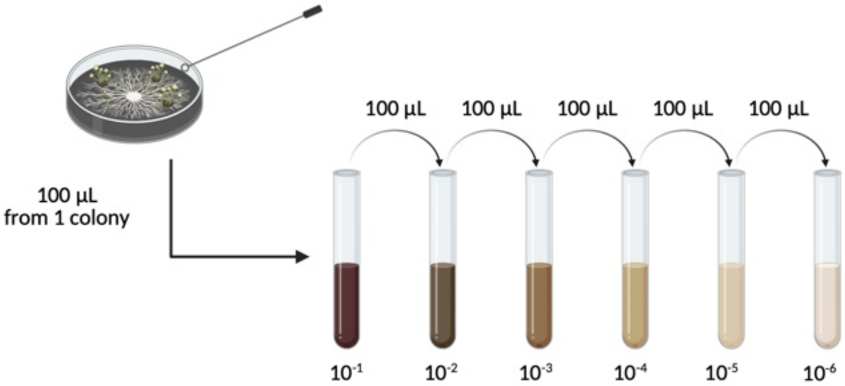

Figure 3. Schematic illustration of colony repicking and its serial dilution created with BioRender.

2.2.5. Molecular Analysis

The molecular analysis was divided into three phases. The first phase was DNA extraction, which was carried out carefully to prevent loss of material. Following extraction, a polymerase chain reaction (PCR) was performed, followed by agarose gel electrophoresis to visualise the PCR products. The DNA bands were then cut from the gel and submitted to DNA purification. The final step was Sanger sequencing.

2.2.5.1. DNA extraction

Genomic DNA was extracted from pure cultures according to a protocol adapted from (47), with modifications to the centrifugation and precipitation conditions.

A portion of the colony was transferred to a 2 ml tube, frozen in liquid nitrogen to promote cell rupture by thermal shock and then mechanically fragmented. Lysis buffer and 2-mercaptoethanol were then added to rupture the membrane, inactivate the nuclease and stabilise the DNA. After vortexing, the samples were incubated at 65 °C for one hour.

Next, a phenol:chloroform:isoamyl alcohol mixture (25:24:1, v/v/v) was added to remove proteins and separate the phases, followed by a two-minute vortex agitation. The samples were then centrifuged at 13,000 rpm for 20 minutes. The aqueous phase was transferred to a new tube, mixed with an equal volume of isopropanol and stored at -80°C for 15 minutes to precipitate the DNA. Subsequently, the samples were centrifuged at 13,000 rpm at 4°C for 20 minutes.

The pellet was then washed with 70% ethanol, centrifuged again, air-dried, and finally resuspended in sterile distilled water. DNA quantification was performed by spectrophotometry, after which the samples were stored at -20°C until use in PCR.

2.2.5.2. PCR and Electrophoresis

The ITS (internal transcribed spacer) region of ribosomal DNA was amplified using the above-described primers, which are widely recognised for molecular fungal identification (44). PCR reactions were performed using the GoTaq® DNA Polymerase from Promega following the manufacturer's recommendation, and thermocycling conditions optimised for each primer pair. The thermocycler used was the (MJ Mini PTC-1148, Bio-Rad Laboratories Inc., Hercules, Canada)

The ITS1 and ITS2 primer protocol began with a denaturation step at 95 °C for two minutes, followed by 34 amplification cycles consisting of denaturation at 95 °C for 30 seconds, annealing at 53 °C for 30 seconds, and extension at 72 °C for 30 seconds. The final extension step was then performed at 72 °C for seven minutes.

The procedure for the ITS3 and ITS4 primer pair was identical, except that the annealing temperature was set at 56 °C for 30 seconds and the extension step was carried out at 72°C for 45 seconds.

For the ITS1 and ITS4 primer pair, the protocol included an initial denaturation step at 95°C for two minutes, followed by 34 amplification cycles at 95 °C for 30 seconds, 53°C for 30 seconds, and 72 °C for 90 seconds. The final extension was performed at 72 °C for 10 minutes.

The amplified products were analysed by electrophoresis on a 2.0% agarose gel stained with the GreenSafe intercalating agent and visualised under ultraviolet (UV) radiation.

2.2.5.3. DNA purification and Sequencing

The bands corresponding to the fragments of interest were then purified and sequenced using the Sanger method.

The band of interest was carefully cut out and transferred to a suitable tube. Next, an ADB buffer solution was added in a proportion of 3:1 according to the size of the DNA band, and the mixture was incubated at 50°C for 10 minutes until the agarose had completely dissolved. Then, isopropanol was added in a proportion of 3:1 according to the ADB volume, and the mixture was gently homogenised. The contents were then transferred to a purification column. The column was centrifuged at 13,000 rpm for one minute, after which the resulting eluate was discarded. The PE washing buffer was then added and left to act for five minutes. The column was then centrifuged again at 13,000 rpm for one minute, after which the eluate was discarded. A final centrifugation was performed under the same conditions for three minutes to ensure complete buffer removal. The column was transferred to a new tube, to which pre-heated ultra-pure water was added. It was left to act for two minutes. Finally, the column was centrifuged at 13,000 rpm for two minutes. The resulting eluate corresponded to the purified sample, ready for Sanger sequencing at STAB VIDA, FCT/UNL Ed. Departamental (Química/Ambiente), Caparica.

The obtained sequences were compared with those deposited in the GenBank (NCBI) database using the BLAST algorithm to assign taxonomic identification to the fungal isolate.

2.3. Study Design and Statistical Analysis

The statistical analysis was performed on the R Project for Statistical Computing. Statistical analyses were performed in R version 4.4.1 (R Core Team, 2024)[1] using RStudio (RStudio Team, 2024), as well as in Python 3.11 using pandas, NumPy, SciPy,

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[1] R Core Team. (2024). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.r-project.org/

scikit-learn, and Matplotlib within a fully scripted workflow in Spyder (Spyder Development Team, 2024)[2].

2.3.1. Study Design and Sampling Framework

This pilot study employed a cross-sectional comparative design to evaluate the discriminatory potential of fungal communities associated with vehicle parts for forensic identification.

Eight privately owned passenger vehicles were recruited from distinct Portuguese regions, encompassing varied models (*Citroën Xsara, Renault Grand Scenic, Renault Kangoo Compac, Opel Corsa, Volkswagen Golf, Peugeot 308, Peugeot Jumpy,* and *Opel Combo*) with differing occupancy levels (1–5 occupants) and maintenance histories.

From each vehicle, six sampling sites were standardised: three exterior (front grille, wheel cap, side mirror) and three interiors (steering wheel, seat upholstery, car mat). This approach captured both environmentally exposed and occupant-associated fungal signatures, providing a holistic within-vehicle profile.

2.3.2. Fungal Identification

Across all samples, 18 distinct fungal taxa were identified. A binary presence/absence matrix was constructed, coding taxa as 1 = present or 0 = absent. Although abundance data were not retained, this binary design provides robust information for comparative community-level analyses.

2.3.3. Descriptive and Exploratory Analyses

Vehicle- and sample-level attributes (occupancy, part type, circulation area, and fungal richness) were summarised using mean ± SD, median [IQR], and percentages where applicable.

Species prevalence (percentage of samples containing each taxon) was categorised as highly frequent (> 25%), moderately frequent (10–25%), or rare (< 10%).

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[2] Spyder Development Team. (2024). *Spyder: The Scientific Python Development Environment* (Version X.X) [Computer software]. https://www.spyder-ide.org/

Fungal distributions were visualised as a bar plot ranking species prevalence (Fig. 7), and a heatmap of species occurrence by vehicle part (Fig. 8).

2.3.4. Community Structure and Dissimilarity Analysis

Community composition was examined at three aggregation levels. The first level was the fused vehicle profiles: six parts combined per vehicle; the second level was the interior-only profiles: steering wheel, seat upholstery, car mat; finally, the third level was the exterior-only profiles: front grille, wheel cap, side mirror.

Pairwise dissimilarities between profiles were computed using two binary indices:

- (1) Jaccard dissimilarity
- (2) Sørensen–Dice dissimilarity (Bray–Curtis binary form)

where a = shared taxa, b = taxa unique to vehicle i, and c = taxa unique to vehicle j.

Both range from 0 (identical) to 1 (completely distinct). Mean, SD, and range were calculated separately for each index and area type. Heatmaps were generated to visualise pairwise distances among vehicles. All computations used *scipy.spatial.distance* within fully scripted Python workflows.

2.3.5. Cluster Analysis

To visualise relationships among parts and vehicles, hierarchical agglomerative clustering (HAC) was performed using the unweighted pair-group method with arithmetic mean (UPGMA**).**

Clustering was applied to Jaccard and Sørensen–Dice distance matrices at the vehicle level (interior vs exterior), and the surface level (six-part types merged across all vehicles).

Dendrograms were produced in *matplotlib 3.9*, with x-labels rotated 35° and colour-coded (blue = exterior, orange = interior) to enhance legibility. Cluster stability and interpretability were verified by comparing tree topologies across indices.

2.3.6. Exterior-Only Statistical Analyses

All subsequent inferential analyses were restricted to the 24 exterior samples (three parts × eight cars).

2.3.6.1. Multivariate Community Analysis (PERMANOVA)

A Permutational Multivariate Analysis of Variance (PERMANOVA) was performed on the Jaccard dissimilarity matrix using 9,999 permutations. The model tested whether fungal community centroids differed between vehicles. The pseudo-F statistic, R^2, and permutation-based p-value were reported.

2.3.6.2. Machine Learning Classification

A Random Forest model (scikit-learn, 500 trees, maximum depth = 4) evaluated whether exterior fungal profiles could predict the correct vehicle ID. Model performance was estimated using leave-one-out cross-validation (LOOCV), and feature-importance scores were extracted to identify the most discriminative taxa.

2.3.6.3. Statistical Significance Testing

Pairwise Jaccard dissimilarities were separated into within-vehicle and between-vehicle sets.

Distributions were compared using the Mann–Whitney U test, and Cohen's d (computed on $1 - $ Jaccard similarities) quantified the magnitude of effect.

2.3.6.4. Indicator Species Analysis

For each taxon, Fisher´s exact tests examined associations between species presence and individual vehicles. P-values were adjusted using the Benjamini–Hochberg FDR procedure to control type-I error.

No taxa remained significant after correction, but several (Allophoma labilis, Penicillium brevicompactum) showed weak uncorrected enrichment.

2.3.7. Vehicle-Level Classification and Top-k Analysis

To evaluate the capacity of exterior fungal profiles to discriminate between vehicles, a Random Forest classifier was applied to the 24 exterior samples (three parts × eight vehicles). Binary presence/absence data for 18 fungal taxa served as predictors, and the categorical variable Vehicle_ID as the response. Models were trained using 500 trees with a maximum depth of four, limiting model complexity relative to sample size. Performance was estimated via leave-one-out cross-validation (LOOCV), in which each sample was iteratively excluded from training and predicted using the remaining 23.

For each hold-out sample, posterior probabilities for the eight vehicles were extracted to compute top-k hit rates: (i) Top-1 accuracy = percentage of samples where the highest-probability class equalled the true car; (ii) Top-3 and Top-5 hit rates = percentage where the true vehicle appeared among the three or five highest-probability candidates. Chance levels (12.5%, 37.5%, 62.5%) correspond to random guessing among eight vehicles.

Classification performance was also examined by vehicle (3 samples per car) and by exterior part type (front grille, side mirror, wheel cap) to identify components contributing most to discriminative accuracy. The same probability matrices were used to construct a confusion matrix, quantifying recurrent misclassification patterns.

3. Results

3.1. Selection of vehicles

Crucial information about each car was gathered in order to understand the differences among the vehicles and how it could affect the expected results (Table2). It is important not only to understand the environment to which these vehicles are normally exposed, but also to acknowledge the impact that a recent car wash can have.

Table 2. Information collected about each vehicle.

Vehicle	Car ID	Year	Type of use	Last wash	Collection Date	Passengers	Sub-Region
Renault Kangoo Compac	1	2014	Urban/Rural Coastal	May de 2024	April 2025	2	Península de Setúbal
Peugeot 308	2	2010	Coastal City	December 2024	April 2025	2	Península de Setúbal
Opel Corsa	3	2014	Rural Inland/Urban Coastal	August 2024	March 2025	2	Alto Alentejo/ Península de Setúbal
Renault Grand Scenic	4	2012	Inland Rural	September 2024	March 2025	3	Alto Alentejo
Peugeot Jumpy	5	2010	Inland Rural	October de 2024	April 2025	2	Alto Alentejo
Wolkswagen Golf	6	2014	Inland Rural	Septmber de 2024	April 2025	5	Alto Alentejo
Citroen Xsara	7	2000	Coastal City	March 2025	March 2025	1-4	Grande Lisboa
Opel Combo	8	2019	Inland Rural	February 2025	April 2025	2	Alto Alentejo

Further information was also collected regarding the types of flora to which vehicles are exposed daily. As previously mentioned, vehicles in Alto Alentejo encounter more flora than those in Grande Lisboa and Península de Setúbal. This reinforces the idea that a lack of urbanisation and contact with nature can impact the results obtained.

Table 3. Flora to which the vehicles are exposed daily.

Vehicle	Car ID	Flora
Renault Kangoo Compac	1	Mulberry tree, pepper plant, grapevine
Peugeot 308	2	Stone pine, eucalyptus
Opel Corsa	3	Eucalyptus, stone pine, cork oak, olive tree, holm oak
Renault Grand Scenic	4	Eucalyptus, cork oak, olive tree, holm oak
Peugeot Jumpy	5	Eucalyptus, cork oak, olive tree, holm oak
Wolkswagen Golf	6	Eucalyptus, cork oak, olive tree, holm oak
Citroen Xsara	7	Stone pine, eucalyptus
Opel Combo	8	Eucalyptus, cork oak, olive tree, holm oak

Further information was gathered to understand which fungi could be associated with the two regions, depending on differences in various factors such as elevation, climate, precipitation, soil types and dominant flora (28).

Table 4. Summary about the information gathered from each sub-region, and the fungi possibly associated with them.

Factors	**Alto Alentejo**	**Península de Setúbal/Grande Lisboa**
Elevation	200-400 meters	0-50 meters
Climate	Continental Mediterranean: hot, dry summers, cold, dry winters	Maritime Mediterranean: mild summers, mild and wet winters
Precipitation	400-600 mm/year	600-800 mm/year
Soils	Calcareous, sandy, poor in organic matter.	Urban, riparian soils, influenced by the Tagus River, rich in organic matter
Dominant flora	Cork oak, Holm oak, Olive tree, Eucalyptus	Stone pine, Poplar, Willow
Pathogenic fungi	*Fusarium, Allophoma, Phytophthora, Botrytis*	*Alternaria*, *Fusarium*, *Allophoma*, *Phytophthora*
Saprophytic Fungi	*Trichoderma*, *Xylaria*, *Sordaria*, *Penicillium*, *Aspergillus*, *Cladosporium*	*Penicillium*, *Aspergillus*, *Xylaria*, *Sordaria*, *Mucor*
Mycorrhizal fungi	*Tuber*, *Boletus*, *Rhizophagus*, *Scleroderma*, *Rhizopogon*	*Rhizopogon*, *Scleroderma*, *Suillus*, *Tuber*

3.2. Morphological Identification

All colonies were stained and observed for morphological identification. Some of the colonies observed are listed below in Figures 4, 5, 6, 7 and 8.

Figure 4 shows the colonies that have grown in the culture plate from the sample collected from the steering wheel of Car 7. Two morphologically distinct fungal colonies were observed: a white and pink colony, and three green and yellow colonies. The pink colony was difficult to analyse microscopically, due to lack of reproductive structures easy to identify, so it was directly identified by Sanger sequencing, which revealed it to be *Aspergillus versicolor*. The green colonies were identified as the genus *Aspergillus*.

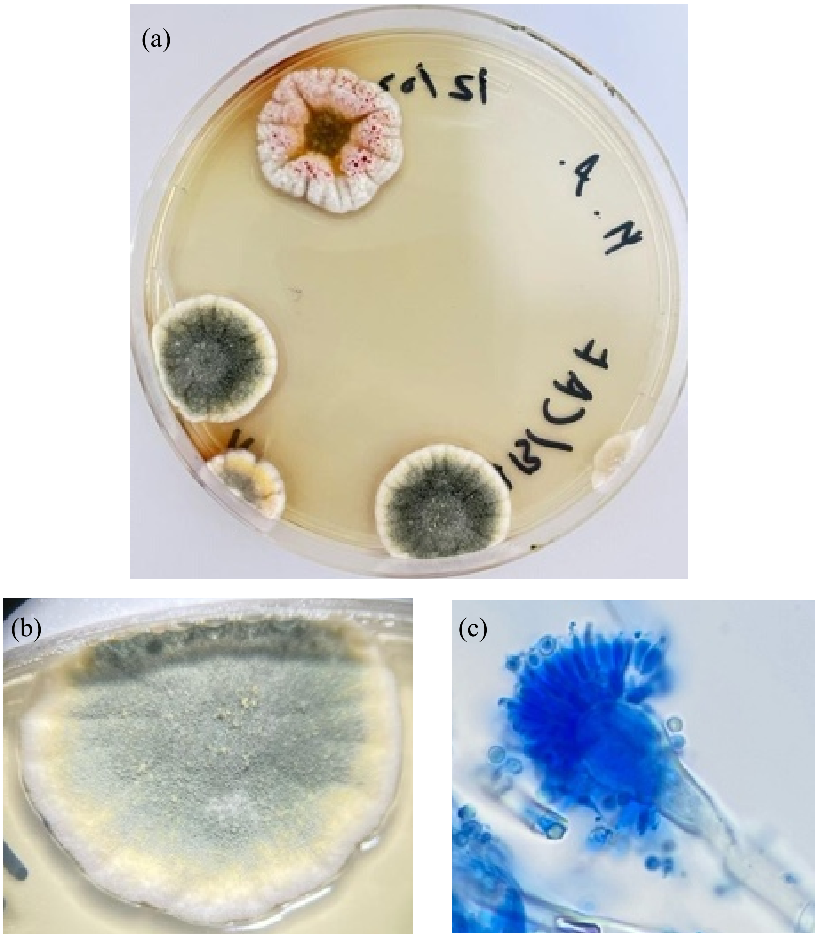


Figure 4. Colonies obtained from the steeringwheel sample collected from Car 7, where (a) shows the macroscopic view of the plate, (b) shows the colony macroscopically, and (c) shows the microscopic morphology of the genus *Aspergillus*, observed at 400x magnification.

Close observation of the microscopic structures, allowed to identify the stipe, a biseriate conidial head with a vesicle, followed by the metulae and phialides. It is also possible to see some conidia in Figure 5, as well as other structures.

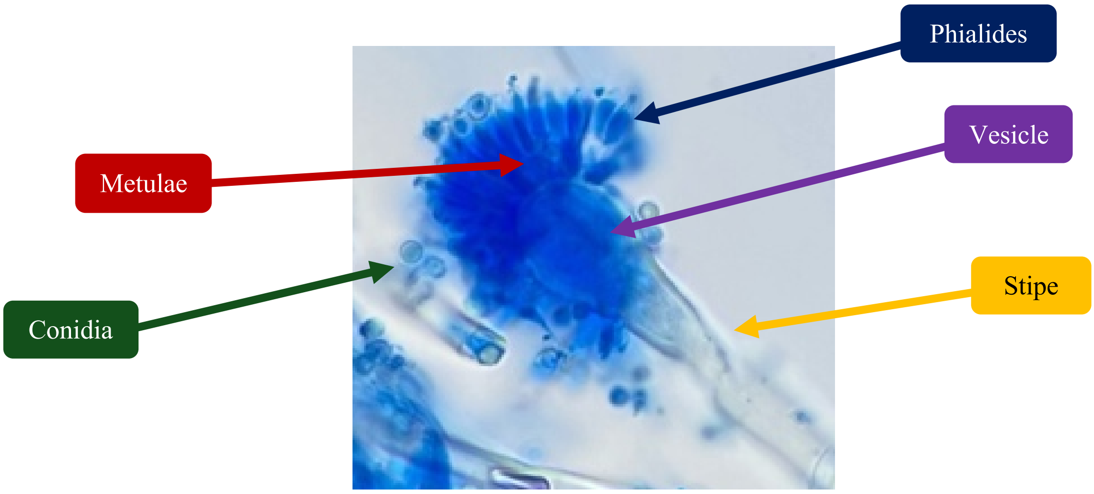


Figure 5. Morphological structures of *Aspergillus*, showing the stipe, vesicle, metulae, phialides and conidia, observed at 400x magnification.

Figure 6 shows colonies from the mat sample from Car 7. It was possible to differentiate two types of colonies: a green one, in more abundance, and a small pink spot. The green colonies were identified as fungi from the genus *Cladosporium*. On the other hand, the pink spot was revealed to be a yeast from the genus *Sporobolomyces.*

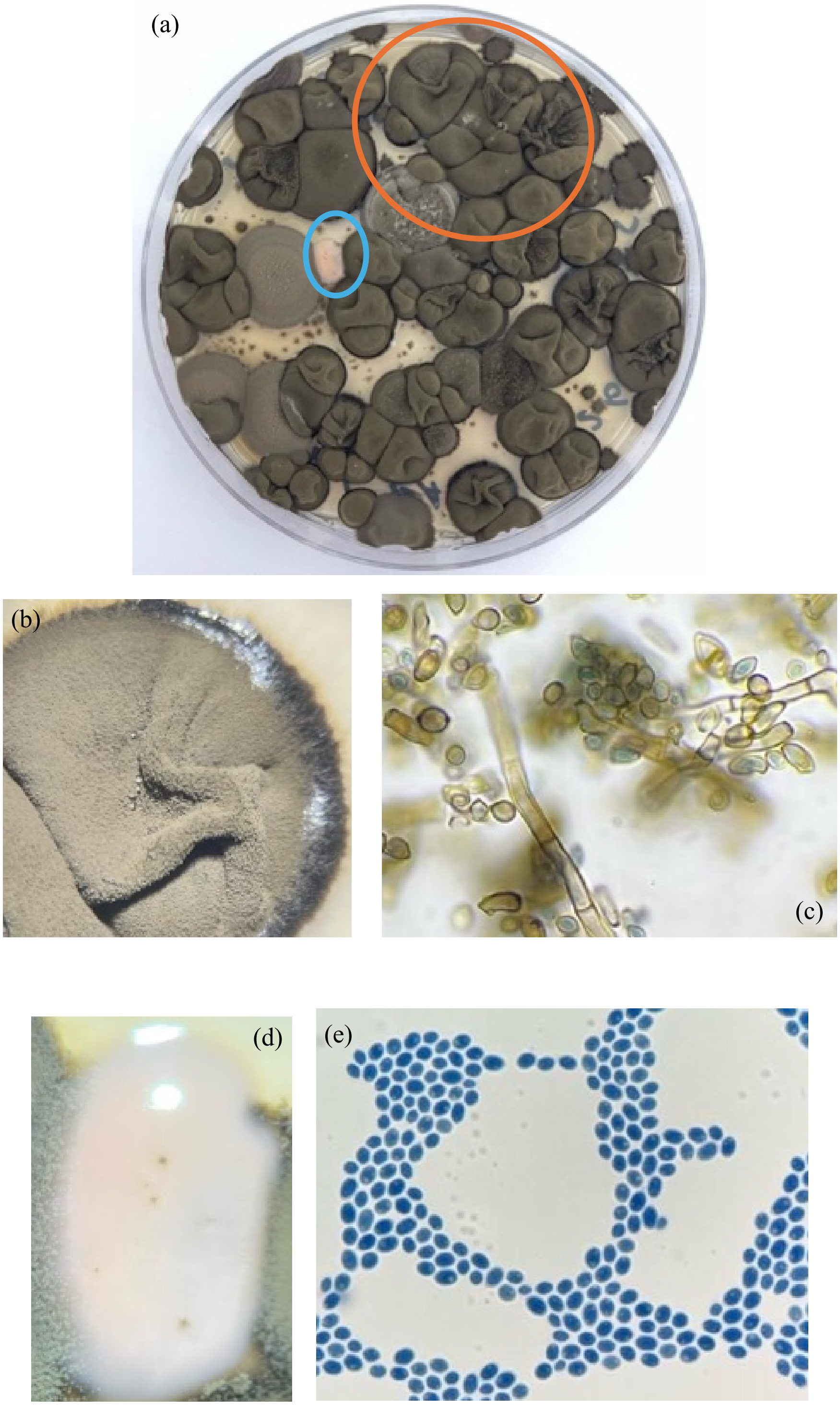


Figure 6. Colonies obtained from the mat sample collected from Car 7, where (a) shows the macroscopic view from the plate, (b) shows the green colony macroscopically, (c) shows the structure from genus *Cladosporium*, the microscopic structure observed under the microscope (400x) (d) shows the pink colony macroscopically and (e) shows the structure from genus *Sporobolomyces,* observed under the microscope (400x).

The conidia and conidiophore are described in detail in Figure 7.

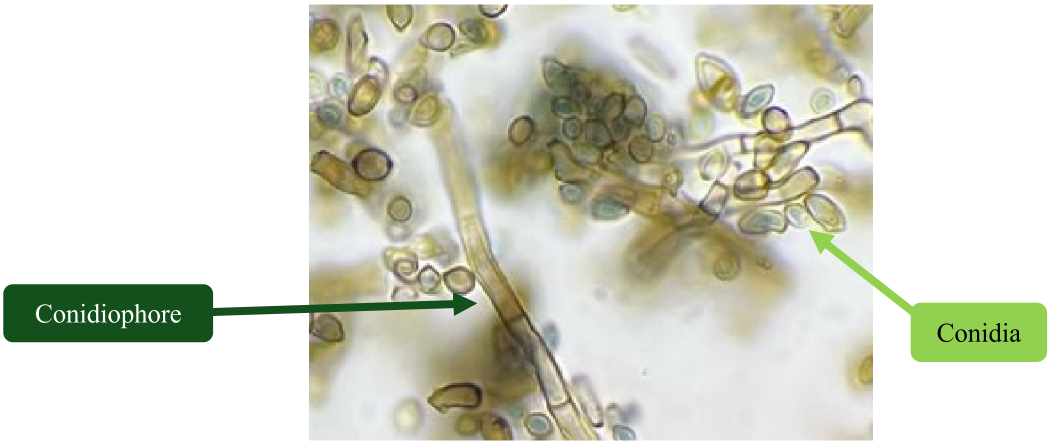


Figure 7. Morphological structures of *Cladosporium*, showing the conidia and conidiophores, observed at 400x magnification.

On the other hand, many of the colonies observed were difficult to identify macroscopically. One of the examples is the fungi *Allophoma labilis*, presented in Figure 8. The samle was collected from the mat of Car 4. At first sight, it appeared to be two different colonies; however, macroscopically and microscopically different. However, the results from Sanger sequencing revealed that it was *Allophoma labilis*. The Figure 8 shows this fungus´s macroscopic and microscopic structures. The sample was collected from the mat of Car 4.

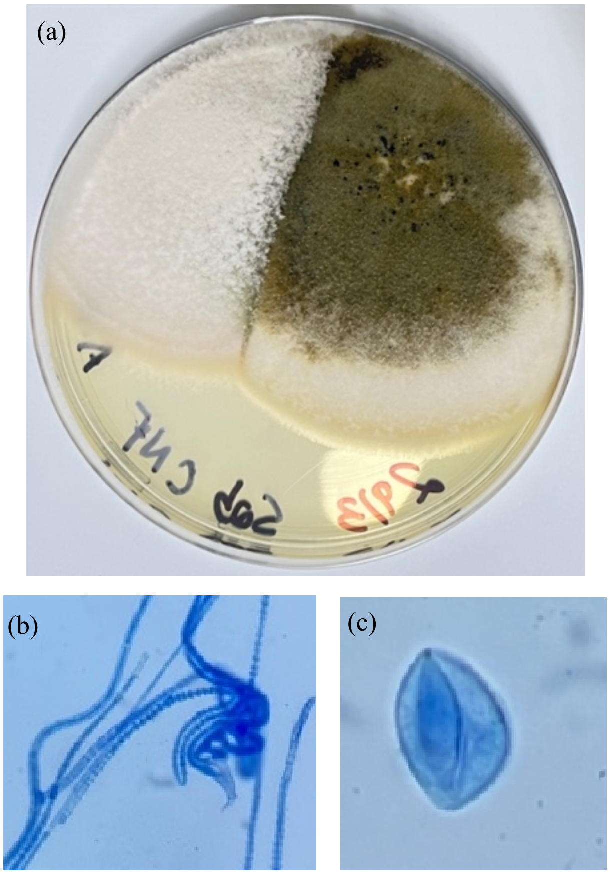


Figure 8. Colonies obtained from the mat sample collected from Car 4, where (a) shows the macroscopic view from the plate, (b) shows the colony macroscopically, (c) shows the structure view microscopically (x400).

3.3. Descriptive Statistic: Vehicle and Sample Characteristics

Although the dust samples were collected in triplicate, the results were analysed as a single sample, considering the area from which the dust was collected.

Therefore, the dataset comprises 8 vehicles and 48 samples from interior and exterior parts. Mean number of occupants: 2.50 (SD 1.34); median 2.00 [IQR 1.75–3.25]. Fungal richness (number of fungi) per sample: mean 1.73 (SD 1.22), median 2.00 [IQR 1.00–2.25].

Table 5. Descriptive analysis, showing vehicle and sample characteristics.

Domain	Variable / Category	Value
Study Scope	Vehicles (unique), n	8
	Samples, n	48
Area Type	Levels	Exterior, Interior
Part Type	Levels	Front grille, Wheel, Side mirror, Steering wheel , Seat upholstery, Car mat
Circulation Area	Levels	Alto Alentejo, Península de Setúbal, Grande Lisboa, Península de Setúbal/Alto Alentejo
	Alto Alentejo	24 (50.0%)
	Península de Setúbal	12 (25.0%)
	Grande Lisboa	6 (12.5%)
	Península de Setúbal/Alto Alentejo	6 (12.5%)
Car Occupants	n (non-missing)	48
	Mean (SD)	2.50 (1.34)
	Median [IQR]	2.00 [1.75–3.25]
	Range	1.00–5.00
Fungal Richness (per sample)	Mean (SD)	1.73 (1.22)
	Median [IQR]	2.00 [1.00–2.25]
	Range	0–4
Fungal Species (global prevalence)	N species	18
	Sporobolomyces roseus	60.4%
	Cladosporium spp.	22.9%
	Penicillium expansum	16.7%
	Mucor fragilis	10.4%
	Penicillium brevicompactum	
	Allophoma labilis	
	Diaporthe foeniculina	8.3%
	Aureobasidium melanogenum	6.2%
	Aspergillus versicolor	4.2%
	Alternaria infectoria	
	Aspergillus spp.	
	Xylaria spp.	2.1%
	Penicillium restrictum/melinii	
	Aspergillus hiratsukae	
	Sordaria fimicola	
	Penicillium velutinum	
	Fusarium spp.	
	Aspergillus Ibericus	

Fungal species were grouped into three frequency tiers based on overall detection rates across all samples: species present in more than 25% of samples were classified as highly frequent, those between 10-25% as moderately frequent, and those below 10% as less frequent. Group-level prevalence was calculated as the proportion of samples in which at least one species from that tier was detected.

Table 6. Frequency statistics for each species identified.

Frequency group	**N species**	**Species list**	**Group frequency (%)**
Highly frequent (>25%)	1	*Sporobolomyces roseus*	60.4%
Moderately frequent (10–25%)	5	*Cladosporium spp., Penicillium expansum, Penicillium brevicompactum, Allophoma labilis, Mucor fragilis*	52.1%
Rare (<10%)	12	*Aspergillus versicolor, Fusarium spp., Aureobasidium melanogenum, Diaporthe foeniculina, Aspergillus spp., Penicillium restrictum/melinii, Xylaria spp., Alternaria infectoria, Aspergillus hiratsukae, Sordaria fimicola, Penicillium velutinum, Aspergillus Ibericus*	29.2%

Figure 9 illustrates in a graph the information listed in Table 5.

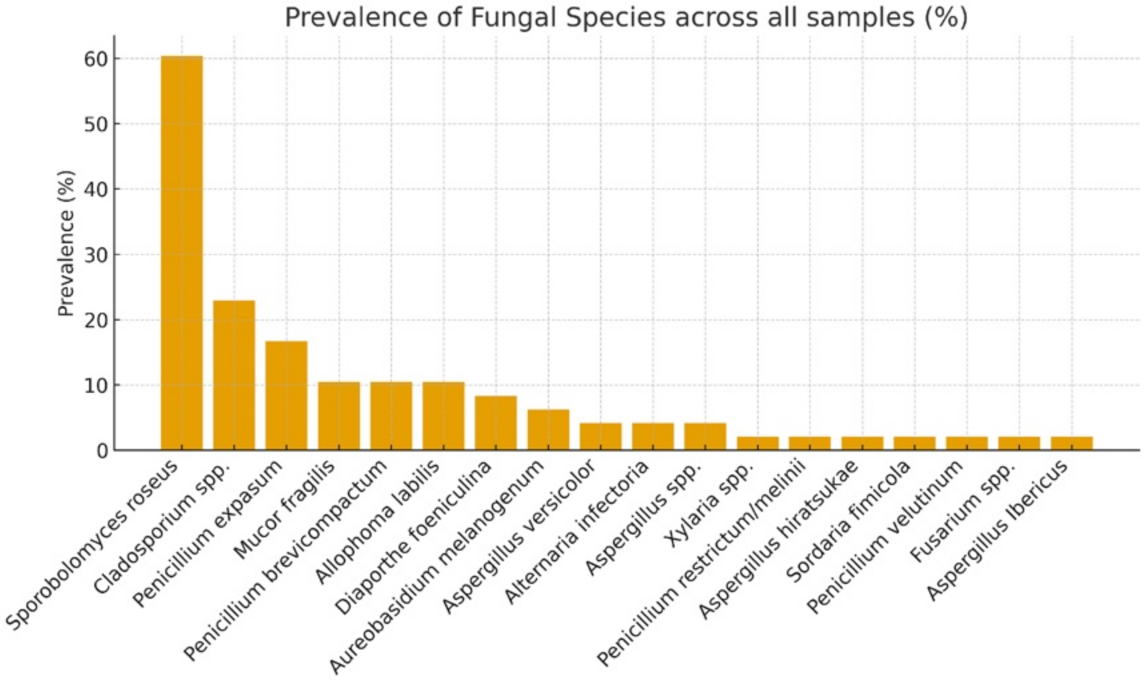


Figure 9. Prevalence of Fungal Species across all samples, expressed as percentage.

Although describing the prevalence of fungal species was important, understanding how these species were distributed among the different vehicle components was more important (Figure 10). From that distribution, it is possible to conclude that *Sporobolomyces roseus* had a high prevalence in side mirrors and front grille parts. On the other hand, other fungi as *Penicillium velutinum*, Fusarium spp.,

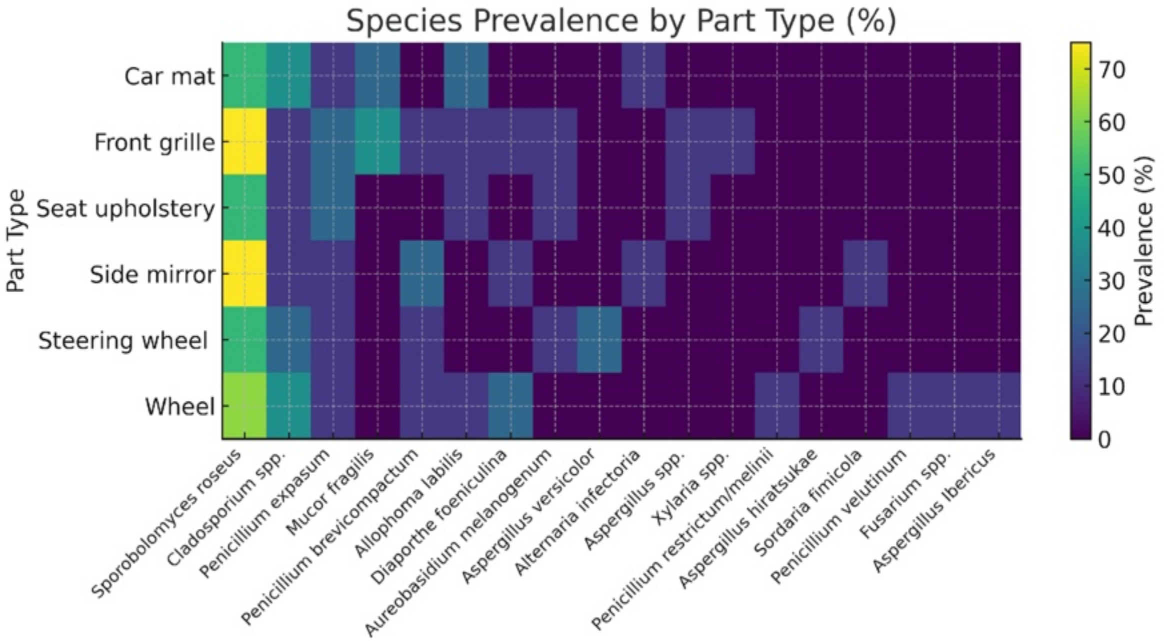


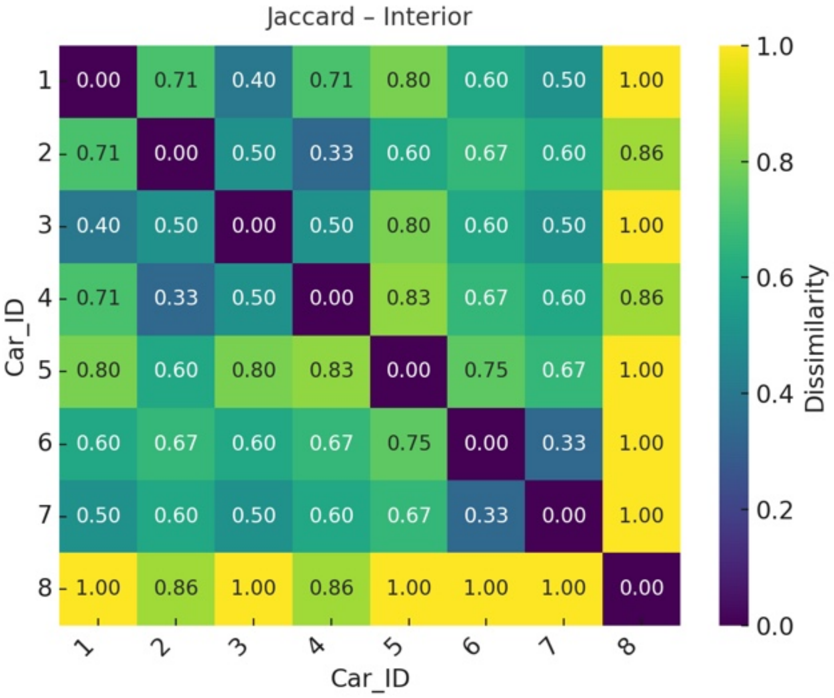
Figure 10. Species prevalence by vehicle part.

Aspergillus ibericus, Xylarias pp., Aspergillus hiratsukae had a lower prevalence, less than 30%, across all parts.

The Jaccard and Sørensen-Dice dissimilarity indices measure the difference between two fungal communities, with values ranging from 0 (identical composition) to 1 (completely different communities). Figures 11 and 12 show the application of these indices to the fungal communities found on the interior surfaces of eight vehicles. Each value corresponds to a comparison between two vehicles, indicating the extent to which their internal communities are similar or different. Values closer to 1 show that the vehicles have very different internal fungal compositions, while lower values indicate greater similarity in species composition.

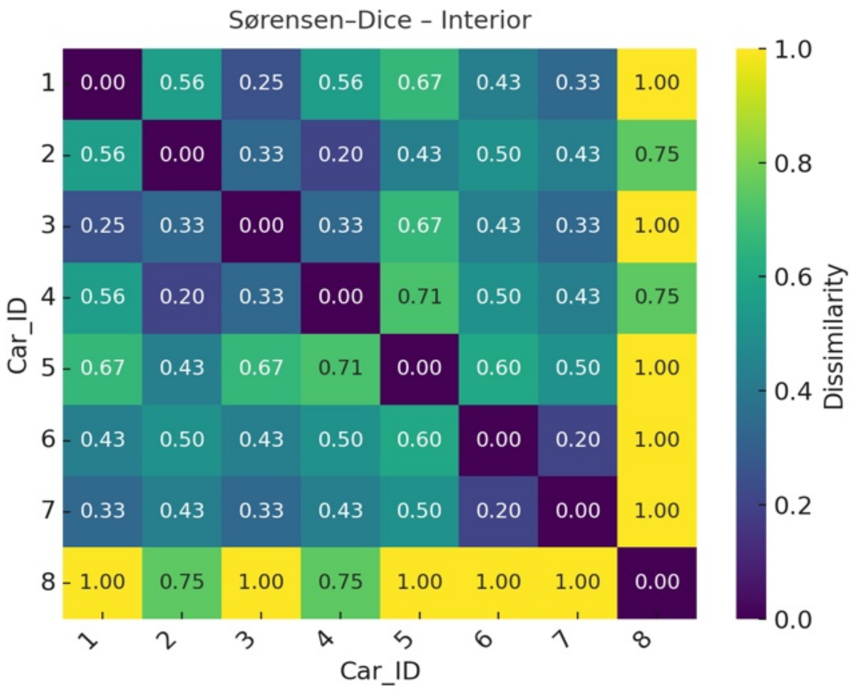
Figures 13 and 14 show the same type of analysis applied to the fungal communities on the exterior surfaces of the vehicles. As with the interior analysis, each value represents a comparison between pairs of vehicles, enabling us to assess whether the exterior surfaces share a similar set of fungi or exhibit very different profiles.

Figures 11 and 12 show the pairwise dissimilarities, both on Jaccard and Sørensen-Dice, between the fungal communities detected on the interior vehicle surfaces.



Car_ID	1	2	3	4	5	6	7	8
1	0.00	0.71	0.40	0.71	0.80	0.60	0.50	1.00
2	0.71	0.00	0.50	0.33	0.60	0.67	0.60	0.86
3	0.40	0.50	0.00	0.50	0.80	0.60	0.50	1.00
4	0.71	0.33	0.50	0.00	0.83	0.67	0.60	0.86
5	0.80	0.60	0.80	0.83	0.00	0.75	0.67	1.00
6	0.60	0.67	0.60	0.67	0.75	0.00	0.33	1.00
7	0.50	0.60	0.50	0.60	0.67	0.33	0.00	1.00
8	1.00	0.86	1.00	0.86	1.00	1.00	1.00	0.00

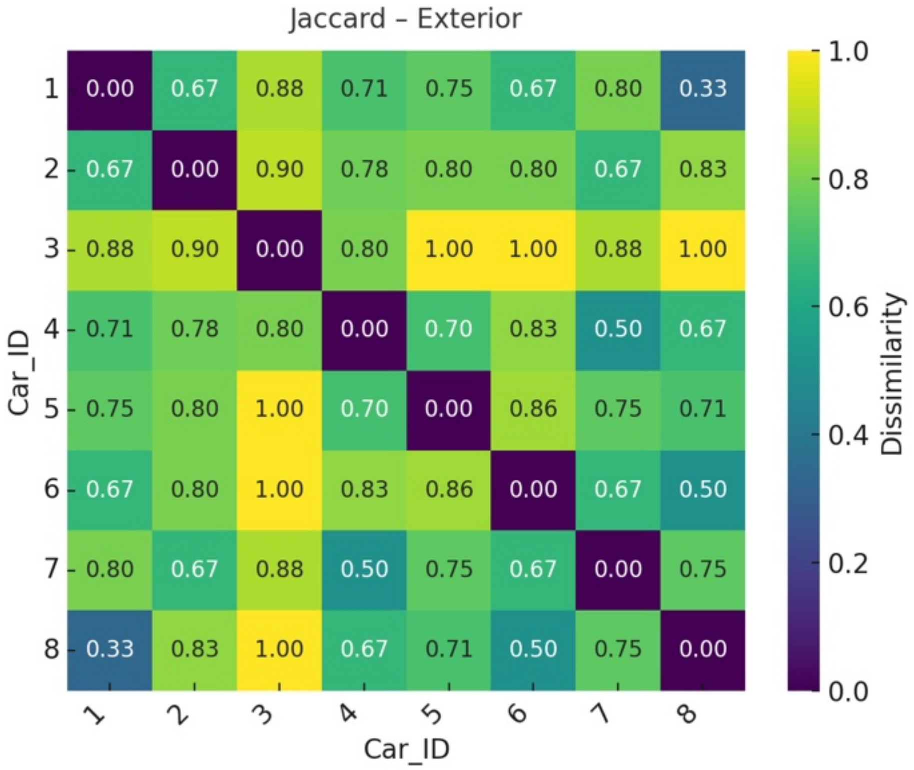
Figure 11. Pairwise Jaccard dissimilarity indices between fungal communities detected on interior vehicle surfaces.



Car_ID	1	2	3	4	5	6	7	8
1	0.00	0.56	0.25	0.56	0.67	0.43	0.33	1.00
2	0.56	0.00	0.33	0.20	0.43	0.50	0.43	0.75
3	0.25	0.33	0.00	0.33	0.67	0.43	0.33	1.00
4	0.56	0.20	0.33	0.00	0.71	0.50	0.43	0.75
5	0.67	0.43	0.67	0.71	0.00	0.60	0.50	1.00
6	0.43	0.50	0.43	0.50	0.60	0.00	0.20	1.00
7	0.33	0.43	0.33	0.43	0.50	0.20	0.00	1.00
8	1.00	0.75	1.00	0.75	1.00	1.00	1.00	0.00

Figure 12. Pairwise Sørensen–Dice dissimilarity indices between fungal communities detected on interior vehicle surfaces.

On the other hand, Figures 13 and 14 show the same indices, but applied to the fungal communities detected on the exterior surfaces.



Car_ID	1	2	3	4	5	6	7	8
1	0.00	0.67	0.88	0.71	0.75	0.67	0.80	0.33
2	0.67	0.00	0.90	0.78	0.80	0.80	0.67	0.83
3	0.88	0.90	0.00	0.80	1.00	1.00	0.88	1.00
4	0.71	0.78	0.80	0.00	0.70	0.83	0.50	0.67
5	0.75	0.80	1.00	0.70	0.00	0.86	0.75	0.71
6	0.67	0.80	1.00	0.83	0.86	0.00	0.67	0.50
7	0.80	0.67	0.88	0.50	0.75	0.67	0.00	0.75
8	0.33	0.83	1.00	0.67	0.71	0.50	0.75	0.00

Figure 13. Pairwise Jaccard dissimilarity indices between fungal communities detected on exterior vehicle surfaces.

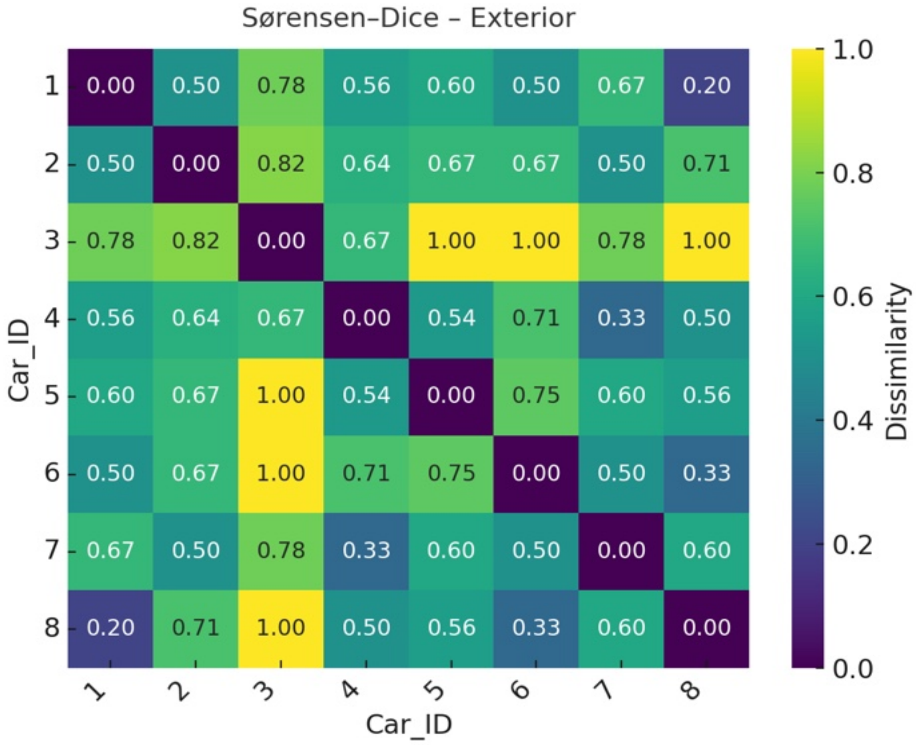


Figure 14. Pairwise Sørensen–Dice dissimilarity indices between fungal communities detected on exterior vehicle surfaces.

All informations presented in the graphs are summarized in Table 7.

Table 7. Summary of the Jaccard and Sørrensen-Dice indexes analysis, for interior and exterior, and its interpretation.

Index	Area Type	Mean dissimilarity ± SD	Range	Interpretation
Jaccard	Interior	0.69 ± 0.20	0.33–1.00	Moderate to high heterogeneity; several shared taxa between vehicles.
Sørensen–Dice		0.57 ± 0.25	0.20–1.00	Slightly lower than Jaccard due to the weighting of shared species; indicates partial overlap of common fungi.
Jaccard	Exterior	0.76 ± 0.15	0.33–1.00	Generally higher dissimilarity; strong turnover between exterior surfaces.
Sørensen–Dice		0.63 ± 0.19	0.20–1.00	Confirms greater heterogeneity across external vehicle parts compared to interiors.

Quantitatively, the exterior mycobiomes exhibit higher dissimilarity (≈0.7) than interiors (≈0.6), underscoring the influence of environmental exposure and spatial variability on external colonization. In contrast, interior samples show reduced dissimilarity, suggesting the presence of recurrent taxa across vehicles that may represent a shared "vehicular indoor mycobiome."

The difference between the two indices (Jaccard > Sørensen–Dice) reflects the greater sensitivity of the latter to shared taxa, confirming that some fungal species (notably *Sporobolomyces roseus* and *Cladosporium spp.*) recur across vehicles even when overall assemblages differ.

3.4. Interpretation of Dissimilarity Patterns

Pairwise dissimilarity indices (Jaccard and Sørensen–Dice) provide quantitative estimates of how distinct the fungal communities are between vehicles. Both indices range from 0 (identical composition) to 1 (completely different communities).

3.4.1. Overall structure

Across all eight vehicles, mean dissimilarities were high (≈0.6-0.8) for both indices, indicating substantial heterogeneity of fungal assemblages among vehicles. This suggests that each car supports a partially unique microbial signature, reflecting differences in exposure, use history, and local environment.

3.4.2. Exterior vs Interior

Exterior parts showed slightly higher dissimilarities on average. These surfaces are more environmentally exposed (airborne spores, rain splash, soil particles), leading to more transient and variable fungal colonisation (Figures 13 and 14).

In the heatmaps, exterior profiles rarely cluster tightly together, supporting a pattern of high turnover between vehicles.

Interior parts exhibited somewhat lower dissimilarities, implying partial overlap in common indoor or occupant-related fungi (e.g. *Sporobolomyces roseus, Cladosporium spp.*). Interiors are also more micro-climatically stable (temperature, humidity) and subject to human contact, which can foster consistent taxa across vehicles (Figures 11 and 12).

3.4.3. Ecological and forensic implications

High between-car dissimilarity values (≥0.7) demonstrate strong individuality of fungal profiles, supporting their potential as trace evidence markers for vehicle attribution.

Moderate within-habitat similarities (especially among interiors) suggest the existence of recurrent “core taxa” that dominate enclosed automotive microenvironments, possibly serving as ecological baselines.

Sørensen–Dice values are slightly lower than Jaccard because the index double-weights shared taxa, thus highlighting biologically meaningful overlaps even among largely distinct communities.

3.4.4. Summary interpretation

In summary, fungal communities are more homogeneous within interiors and more heterogeneous across exteriors. The pattern indicates that vehicle interiors retain persistent, human- and environment-derived fungal signals, whereas exterior surfaces accumulate more stochastic environmental spores**.**

3.5. Cluster Analysis of Car Surface Fungal Communities

Hierarchical agglomerative clustering was performed on the six car surface types (three interior and three exterior), using the binary presence/absence matrix of all detected fungi. Blue labels correspond to exterior parts, and orange labels to interior parts.

The Jaccard dendrogram (Figure 15), illustrates clustering of the six surface types based on shared presence of fungal taxa. Labels are rotated to avoid overlap and colour-coded by area type (interior vs exterior).

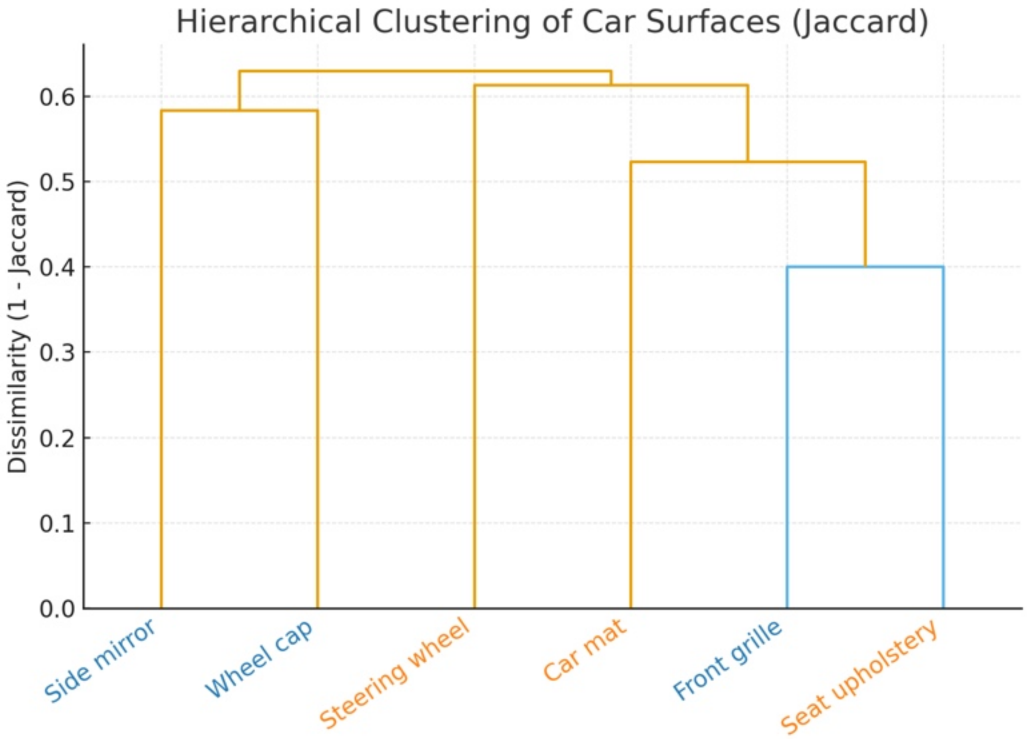


Figure 15. Clustering of Surfaces according to Jaccard Index, divided by area type: exterior, in blue, and interior, in orange.

The Sørensen–Dice dendrogram, in Figure 16, which gives double weight to shared taxa, shows a similar grouping pattern, confirming that the clustering structure reflects real compositional differences between interior and exterior surfaces.

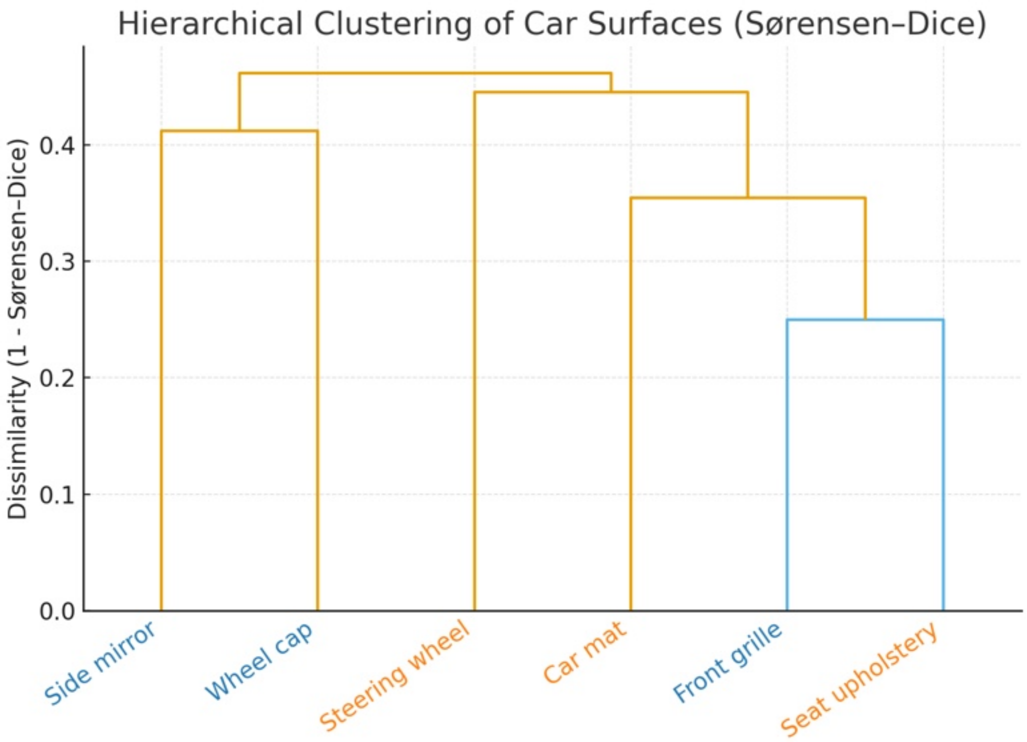


Figure 16. Clustering of Surfaces according to Sørensen–Dice Index, divided by area type: exterior, in blue, and interior, in orange.

Hierarchical clustering of the six car surfaces revealed a clear bifurcation between interior and exterior parts, consistent across both dissimilarity indices (Figures 13-14).

In both the Jaccard and Sørensen–Dice dendrograms, the three interior parts (steering wheel, seat upholstery, and car mat) clustered tightly, merging at lower dissimilarity heights (≈ 0.35-0.45). This indicates substantial community overlap and suggests that these enclosed components share a relatively stable set of occupant-associated and microclimate-tolerant fungi, including *Sporobolomyces roseus*, *Cladosporium spp.*, and *Penicillium expansum*.

In contrast, exterior parts (front grille, side mirror, and wheel cap) formed a separate, more diffuse cluster, with higher linkage distances (≈ 0.6–0.8). These surfaces exhibit greater heterogeneity, reflecting their direct exposure to fluctuating environmental conditions and airborne fungal sources.

The Sørensen–Dice dendrogram produced slightly shorter branch lengths overall, highlighting the contribution of a few recurrent taxa across interior and exterior microhabitats. Nevertheless, the topological consistency between both indices confirms that the clustering pattern is biologically robust and index-independent.

Overall, the hierarchical structure supports the presence of two primary ecological assemblages within vehicles: (1) a stable, occupant-mediated interior mycobiome, and (2) a variable, environmentally influenced exterior mycobiome. This distinction provides a strong foundation for using fungal community composition as a forensic biomarker to associate detached car parts with their likely origin (internal vs external).

3.6. Exterior Fungal Community Analysis

Permutational multivariate analysis of variance (PERMANOVA) based on Jaccard dissimilarities revealed a significant vehicle effect on exterior fungal community composition (pseudo-F = 5.93, df = 7,16, $R^2 = 0.72$, p = 0.0084). Approximately 72% of the variance in community structure was attributable to differences among vehicles, indicating distinct exterior mycobiomes. Despite this, Random Forest classification using 500 trees (max depth = 4) achieved only 12.5% overall accuracy under leave-one-out cross-validation, showing that single exterior samples cannot yet be reliably assigned to their source vehicle. The most influential taxa were *Diaporthe foeniculina*, *Sporobolomyces roseus*, *Penicillium brevicompactum*, *Allophoma labilis*, and *Cladosporium spp*. Table 8 reveals the rank of taxon importance.

Table 8. Top fungal taxa contributing to Random Forest discrimination.

Rank	Taxon	Importance
1	*Diaporthe foeniculina*	0.137
2	*Sporobolomyces roseus*	0.124
3	*Penicillium brevicompactum*	0.117
4	*Allophoma labilis*	0.109
5	*Cladosporium spp.*	0.101
6	*Mucor fragilis*	0.061
7	*Penicillium expansum*	0.057
8	*Alternaria infectoria*	0.047

Pairwise Jaccard distances supported moderate within-car similarity: mean within-vehicle = 0.62 ± 0.39 vs between-vehicle = 0.78 ± 0.27 (Mann–Whitney U = 2374, p = 0.0338), corresponding to a medium effect size (Cohen's d = 0.56). No individual taxon–vehicle association remained significant after FDR correction, although *Allophoma labilis* (Car 4) and *Penicillium brevicompactum* (Car 1) showed weak, uncorrected enrichment. Overall, exterior fungal communities display significant multivariate differentiation between cars, but high variability limits predictive accuracy, highlighting the need for larger datasets to stabilise species-level forensic signals. The summary of this statistical approach is listed in Table 9.

Table 9. Summary of exterior-community statistical analyses.

Analysis	Statistic / Metric	Value	Interpretation
PERMANOVA (Jaccard)	pseudo-F (7,16)	5.93	Significant among-vehicle differentiation
	R^2	0.72	72% of variance explained by car identity
	p (9,999 permutations)	0.0084	Reject null of identical centroids
Random Forest (LOOCV)	Accuracy	0.125 (3 / 24)	Low individual-sample predictability
	Trees / Depth	500 / 4	Controlled overfitting
Jaccard dissimilarity	Within-vehicle mean ± SD	0.62 ± 0.39	Moderate similarity within cars
	Between-vehicle mean ± SD	0.78 ± 0.27	Higher turnover across cars
Mann–Whitney U	U / p	2374 / 0.034	Within < Between (significant)
Cohen's d (similarity)	d	0.56	Medium effect size
Indicator species (FDR)	Significant taxa	None	No robust species–vehicle links

3.7. Manuscript-style Results: Random Forest, top-k performance, and part-type comparison

Random Forest classification of exterior fungal profiles to vehicle identity showed limited top-1 accuracy but moderate gains when broader candidate sets were considered. Using 500 trees (max depth = 4) and leave-one-out cross-validation, top-1 accuracy was 12.5% (3/24), essentially equivalent to random guessing among eight vehicles (12.5%). However, when allowing the correct vehicle to appear among the model's three most probable candidates (top-3), performance increased to 54.2% (13/24), exceeding the theoretical chance level of 37.5%. Allowing top-5 candidates yielded a 66.7% hit rate (16/24), slightly above the 62.5% expected by chance. Thus, while single exterior samples cannot yet support reliable one-to-one attribution, they do provide modest narrowing of the pool of candidate vehicles, particularly under a "shortlist" (top-3 / top-5) framework.

The results are more noticeable in the following table. The Top-k hit rate = proportion of samples for which the true vehicle is included among the k highest-probability predictions.

Table 10. Global Random Forest performance for exterior fungal profiles (8 vehicles, 24 samples).

Metric	Value	Chance level (8 cars)	Notes
Top-1 accuracy	12.5% (3/24)	12.5%	Essentially random
Top-3 hit rate	54.2% (13/24)	37.5%	True car in 3 most probable
Top-5 hit rate	66.7% (16/24)	62.5%	True car in 5 most probable

Performance varied substantially between vehicles and between exterior part types. For top-1 accuracy, the Citroën Xsara (Car 1) was the most distinctive, with 2 of 3 exterior samples correctly classified (66.7%), followed by the Opel Corsa (Car 4) with 1 of 3 (33.3%). All other vehicles showed 0% direct match accuracy. At the component level, wheel caps performed best for top-1 identification (25%, 2/8 correct), followed by side mirrors (12.5%, 1/8), whereas front grilles achieved 0% top-1 accuracy but the highest top-5 hit rate (75%, 6/8 with the true car in the top five). These results indicate that an exterior part left behind at a scene could, in principle, reduce but not pinpoint vehicle identity, especially when wheel caps or front grilles are available.

Table 11. Performance by exterior part type: top-1, top-3, top-5.

Exterior part	**n samples**	**Top-1 accuracy**	**Top-3 hit rate**	**Top-5 hit rate**	**Approx. counts (top-1, top-3, top-5)**
Front grille	8	0.0 (0/8)	0.50 (4/8)	0.75 (6/8)	0 / 4 / 6
Side mirror	8	0.125 (1/8)	0.50 (4/8)	0.625 (5/8)	1 / 4 / 5
Wheel cap	8	0.25 (2/8)	0.625 (5/8)	0.625 (5/8)	2 / 5 / 5

3.7.1. Performance of Exterior Components

Analysis of different exterior vehicle components revealed significant differences in their ability to support identification based on fungal profiling. In terms of top-1 accuracy (the strict assignment of a sample to the correct vehicle), wheel caps performed best, with a success rate of 25%. Side mirrors followed with 12.5%, while front grilles yielded no direct matches (0%).

However, when a more flexible evaluation approach was adopted, considering the Top-3 and Top-5 metrics (which reflect the use of samples to create a shortlist of potential matches), both wheel caps and front grilles proved useful. In particular, front grilles provided the greatest reduction in the candidate pool: in 75% of cases, the true vehicle appeared within the top five predictions generated by the model.

3.7.2. Pre-Vehicle Accuracy and Confusion Patterns

The per-vehicle top-1 accuracy, calculated using three exterior samples per vehicle, showed considerable variation between models. The Citroën Xsara (Car 1) displayed the highest rate of correct identification (66.7%, with two out of three samples correctly classified), suggesting that it has a particularly distinctive exterior profile. The Opel Corsa (Car 4) achieved intermediate accuracy (33.3%), while the remaining vehicles (Cars 2, 3, 5, 6, 7 and 8) showed no correct matches (0% Top-1 accuracy).

Confusion patterns derived from the confusion matrix highlight substantial overlaps between the exterior 'fungal signatures' of certain vehicle models. The Peugeot 308 (Car 6) was misclassified as a Peugeot Jumpy (Car 7) in all three samples (3/3 misclassifications), suggesting near-complete similarity in their exterior fungal signatures. The Opel Combo (Car 8) was frequently misclassified as either the Renault Grand Scénic (Car 2) or the Volkswagen Golf (Car 5), and the Renault Kangoo Compact (Car 3) was often misclassified as an Opel Corsa (Car 4).

3.7.3. Forensic Implications

The results indicate that it is currently not possible to reliably attribute a specific exterior fungal sample to a single vehicle using the available dataset. Nevertheless, the findings reveal partial forensic potential for using fungal profiles to narrow down suspects in investigative contexts. In practice, the correct vehicle was among the top three candidates for around half of the test samples, and among the top five for around two-thirds of the cases.

Performance notably improves when shortlist-based metrics (Top-3 or Top-5) are considered and/or the analysed samples originate from wheel caps or front grilles. I can also improve when the fungal evidence is integrated with complementary forensic information, such as DNA, trace evidence, or CCTV footage, performance improves further.

3.8. Predictive and Forensic Implications

The Random Forest analysis confirmed that exterior fungal communities, though individually noisy, contain measurable discriminatory information across vehicles. Strict one-to-one attribution remained unreliable (top-1 = 12.5%), yet allowing broader candidate sets produced substantial narrowing of the suspect pool: the correct vehicle appeared among the top-3 predictions in 54.2% of samples and within the top-5 in 66.7%, both exceeding chance expectations. This suggests that, in a forensic context, a single exterior trace (e.g. detached wheel cap or mirror) could probabilistically reduce a set of eight possible vehicles to a smaller, higher-likelihood subset, assisting investigative prioritisation rather than providing absolute identification.

Performance heterogeneity across vehicle parts offers practical guidance. Wheel caps yielded the highest top-1 accuracy (25%) and shared high top-3/top-5 hit rates with front grilles, whereas side mirrors performed marginally less well. These differences likely reflect distinct exposure histories: wheel caps and grilles experience consistent environmental deposition, favouring more vehicle-specific colonisation patterns, whereas mirrors capture more transient airborne spores. At the vehicle level, the Citroën Xsara exhibited the most distinctive profile (67% accuracy), while several models with low fungal richness (e.g. Peugeot 308, Opel Combo) were consistently misclassified.

Although current accuracy falls short of evidentiary standards, these findings demonstrate quantifiable, non-random vehicle-specific signals in exterior mycobiomes. Future datasets incorporating higher taxonomic resolution (e.g. metabarcoding) and increased sample replication could elevate fungal community profiling from exploratory discrimination toward a probabilistic trace-evidence framework applicable to real-world accident or abandonment scenarios.

4. Discussion

4.1. Forensic Potential and Limitations

The present study explored whether profiling fungal communities could provide information that would help to link vehicles and their components in a forensic context. Statistical analysis confirmed significant differences in fungal assemblages across the sampled vehicles, demonstrating measurable compositional structure between them. This finding provides empirical evidence that vehicle-associated micobiomes are not random, but are instead partially shaped by environmental, geographical, and usage-related factors. However, the forensic potential of these differences should be interpreted with caution (5,48).

Although the presence of differences between vehicles suggests that distinct fungal communities can form under different conditions, these profiles proved to have minimal predictive capacity for individual attribution. Random Forest classification achieved a Top-1 accuracy of 12.5%, which is equivalent to random guessing among eight vehicles, and only moderate improvement at the Top-3 (54.2%) and Top-5 (66.7%) thresholds. These results suggest that although measurable microbial variation exists, it is insufficient for reliable vehicle identification (49,50). Therefore, the forensic relevance of these data lies primarily in their exploratory and contextual value rather than their diagnostic utility.

As previously mentioned, fungal assemblages were described as “unique microbial signatures”, implying a degree of individualisation comparable to that of established forensic markers. However, such a designation is not supported by the results. The observed differences reflect ecological heterogeneity rather than singular identity, and variability among exterior surfaces further undermines the idea of stable, vehicle-specific 'signatures'. Therefore, the current findings should be interpreted as evidence of microbial diversity and environmental differentiation rather than forensic exclusivity.

4.2. Methodological Considerations and Ecological Insights

The methodological design of this research provided the first comparative framework for analysing fungi in vehicular environments, but it also imposed several critical constraints.

Firstly, the analytical dataset was binary, recording only the presence or absence of taxa. The absence of abundance data substantially reduced the discriminatory resolution of the analysis by preventing the quantification of dominance patterns that might otherwise have revealed more distinct ecological gradients between vehicles. Future investigations should therefore incorporate quantitative sequencing or colony count-based approaches to capture finer community structure.

Secondly, the small sample size (N = 8 vehicles) limits the study's statistical power and generalisability. While this may be sufficient for an exploratory pilot study, such a small dataset is more susceptible to stochastic variation and environmental noise. Furthermore, environmental conditions, including climate, altitude, urbanisation and vegetation, were not experimentally controlled, but rather inferred from regional classification. These uncontrolled confounders likely contributed to the observed heterogeneity in vehicle exteriors and the low predictive accuracy of the classification models.

Thirdly, the lack of replication in independent datasets prevents the stability of the models from being evaluated. Cross-validation within the same dataset provides internal consistency, but not external validity. Ideally, future research should aim to reproduce these findings under different geographical and environmental conditions using a larger and more diverse vehicle pool.

Finally, the present study did not integrate fungal data with other forensic techniques, such as DNA profiling, analysis of trace materials or particulate composition. This limits the ability to evaluate how microbial evidence could supplement conventional methods. Although the potential for integration is strong in theory, particularly in cases lacking DNA or fingerprint evidence, there has been little practical demonstration of this, which is a crucial area for further study.

The superior performance of wheels compared to other exterior parts (25% top-1 accuracy for wheel caps versus 12.5% for side mirrors and 0% for front grilles) likely reflects their unique ecological position. Wheels experience direct contact with diverse substrates, including soil, vegetation, and road surfaces, facilitating acquisition of location-specific fungal propagules (51,52). This finding suggests that crime scene applications should prioritise wheel-associated evidence when available, particularly wheel arch deposits and tyre treads that accumulate environmental material. The distinct patterns between airborne and soil mycobiomes documented in recent biogeographic studies underscore how vehicle location and movement history systematically shape external fungal signatures (53).

4.3. Interior and Exterior Fungal Community Patterns

The results clearly distinguished between the fungal community structures of the interior and exterior. Interior surfaces exhibited greater homogeneity and were dominated by taxa such as *Sporobolomyces roseus and Cladosporium spp*., which are often found on human skin or in the respiratory tract, or in ubiquitous indoor environments (16). This shared 'vehicular indoor mycobiome' reflects common human occupancy patterns rather than vehicle-specific signatures, thereby limiting its discriminatory value.

In contrast, exterior surfaces displayed greater variability, influenced by environmental exposure, climate, and regional vegetation. The sub-regional distinction between the Setúbal Peninsula, Greater Lisbon, and Alto Alentejo was evident in the presence of taxa such as *Fusarium spp.*, *Trichoderma*, and *Allophoma labilis*, which are associated with the soil and plant matter found in rural or agricultural areas(54). While this ecological differentiation produced statistically measurable dissimilarity, however, it did not translate into reliable predictive classification.

This dichotomy, homogeneity within interiors and high heterogeneity across exteriors, highlights the complexity of microbial transfer and persistence on vehicle surfaces. It suggests that, although fungal communities record environmental exposure, their variability and transient nature limit their forensic reproducibility. Consequently, the concept of 'vehicle-specific microbial signatures' should be replaced by the more precise term 'context-dependent microbial assemblages'.

4.4. Indicator Taxa and Interpretive Validity

An exploratory analysis of indicator species suggested that *Allophoma labilis*, *Penicillium brevicompactum* and *Diaporthe foeniculina* were enriched in certain vehicles. However, none of these associations remained statistically significant following correction for multiple testing using the Benjamini-Hochberg method. Therefore, the initial interpretation that these taxa represented 'vehicle-specific indicators' has been revised.

Rather than providing conclusive forensic markers, these findings highlight ecological tendencies that may correspond to environmental or usage-related exposure. For instance, *A. labilis* and *D. foeniculina* are common plant-associated fungi that are often found in rural or agricultural dust, whereas *P. brevicompactum* is present in both indoor and outdoor environments and is resilient to cleaning processes. Therefore, their detection in certain vehicles may reflect the vehicles' usage environments (e.g. rural vs urban circulation) rather than their unique microbial identity.

It is also important to note that the statistical significance of these associations is greatly affected by the small sample size and binary nature of the dataset. Consequently, such species should be interpreted as ecological indicators of exposure rather than as diagnostic markers of the source. Therefore, the term 'indicator' must be used with care in forensic reporting: ecological enrichment does not imply forensic exclusivity.

4.5. Temporal and Environmental Stability

A critical limitation of this study is the single-time-point sampling design, which provides no information on the temporal stability of fungal communities. Vehicle washing, seasonal changes, and geographic movement likely influence community composition, potentially affecting the reliability of fungal evidence over time. Recent studies of decomposer fungal succession demonstrate rapid community turnover under changing environmental conditions (55), suggesting that fungal evidence may have limited temporal persistence. However, emerging research on bacterial community stability in other forensic contexts provides cautious optimism: while microbial communities undergo shifts in abundance following removal from their natural environment, community composition can remain sufficiently stable for reliable classification when samples are appropriately stored (56,57). Whether similar patterns apply to vehicle-associated fungal communities requires dedicated longitudinal investigation.

The influence of vehicle washing history (Table 2) was evident in our dataset, with recently washed vehicles exhibiting reduced fungal diversity and altered community composition. This finding has important implications for casework, as suspects may deliberately wash vehicles to eliminate trace evidence. However, certain taxa may persist in protected microenvironments (e.g. wheel wells, door seals, underbody crevices), warranting targeted sampling strategies (51). Forensic studies of microbial persistence on personal objects demonstrate that microbiota can persist in sheltered environments under standard indoor storage conditions for extended periods (58). Analogously, interior fungal communities in vehicles may demonstrate greater temporal stability than exterior profiles, though this requires empirical validation.

Humidity and temperature fluctuations have documented impacts on microbial community stability. Controlled storage of vehicle trace evidence at cooler temperatures may delay compositional changes and enhance the utility of fungal profiling for delayed casework (59). Furthermore, environmental factors such as precipitation and air quality have been shown to influence fungal composition in vehicular and roadside environments (60), suggesting that seasonal and meteorological data could serve as important contextual variables in future forensic applications.

4.6. Integration with Existing Forensic Frameworks

Fungal community profiling should be integrated within existing trace evidence workflows rather than deployed as an isolated technique. The approach demonstrates particular synergy with soil evidence analysis, as many vehicle-associated fungi originate from environmental substrates (61,62). Combined analysis of soil particles, pollen, and fungal spores could provide comprehensive environmental signatures linking vehicles to specific locations. Pioneering casework demonstrates the utility of integrated mycological and palynological evidence: a homicide investigation in Argentina combined fungal and pollen analysis to establish a link between a suspect's clothing and a coastal burial site through detection of marine-origin diatoms and species-specific fungi not found in the suspect's home (63)

The statistical framework developed here, utilising PERMANOVA and machine learning approaches, provides objective measures of evidence strength suitable for expert testimony. The probabilistic interpretation of results (top-3 accuracy, similarity rankings) aligns with contemporary forensic science practices emphasising quantitative rather than categorical conclusions. PERMANOVA offers advantages over traditional univariate approaches by accommodating the multivariate nature of community data and providing permutation-based p-values robust to heterogeneous dispersions. The R^2 statistic (0.72 for vehicle effect) quantifies the proportion of community variance explained by vehicle identity, a measure suitable for communicating evidence strength to legal stakeholders (64,65).

The forensic value of fungal community profiling currently lies in its ability to provide contextual or corroborative evidence rather than definitive identification. In cases where traditional biological traces are absent or degraded, microbial patterns may provide insight into environmental exposure or contact histories. However, the present data indicate that this approach alone cannot meet evidentiary standards for source attribution.

The observed classification accuracy (12.5% Top-1) demonstrates that attribution based on a single sample is unreliable. Even the moderate improvements seen in Top-3 and Top-5 predictions are close to chance levels, so their practical contribution to narrowing suspect pools should be considered indicative rather than probative. Describing these results as demonstrating 'genuine forensic promise' would overstate their evidentiary weight. Instead, they reveal conceptual promise under controlled conditions.

To progress towards forensic integration, future work must pursue cross-validation across laboratories, establish reference databases of environmental fungal diversity and develop probabilistic frameworks analogous to those used in DNA mixture interpretation. Only through such standardisation and validation can microbial evidence progress from exploratory research to being admissible in a court of law.

4.7. Technological and Analytical Advances

Future implementations should incorporate quantitative approaches (qPCR, DNA metabarcoding) to capture abundance information alongside presence-absence data (66,67). Abundance patterns may provide additional discriminatory power, as rare taxa can dominate communities under specific environmental conditions. High-throughput sequencing would also enable the detection of unculturable taxa, expanding the taxonomic resolution available for forensic applications. Next-generation sequencing technologies have demonstrated substantially improved resolution compared to culture-based identification, enabling the detection of rare community members and uncovering taxa composition previously undetectable by conventional mycology.

Machine learning approaches show promise for pattern recognition in complex fungal datasets. The Random Forest analysis successfully identified key discriminative taxa with importance scores ranging from 0.101–0.137 for the top five taxa. More sophisticated algorithms (support vector machines, neural networks, deep learning) may enhance classification accuracy, particularly when applied to larger training datasets encompassing broader geographic and temporal variation (68). Recent applications of Shapley Additive exPlanations (SHAP) values to forensic microbiome data provide interpretable frameworks for understanding model predictions, enhancing transparency and legal defensibility (69). Ensemble methods combining multiple distance metrics (Jaccard, Sørensen-Dice, UniFrac-based measures) have been shown to achieve robust discrimination regardless of underlying association patterns (70).

Incorporation of bacterial community analysis alongside fungal profiling represents a promising avenue for enhanced discriminatory power. Soil microbiome studies demonstrate that combined bacterial-fungal analyses can differentiate geographic origins with >95% accuracy when appropriately trained (71), suggesting potential for improved vehicle discrimination.

In addition to these analytical advances, future studies should employ more sophisticated identification technologies, such as matrix-assisted laser desorption/ionisation time-of-flight (MALDI-ToF) mass spectrometry and next-generation sequencing (NGS). MALDI-ToF enables rapid, high-confidence identification at the species level based on protein spectral profiles, while NGS provides comprehensive coverage of both culturable and non-culturable taxa, substantially improving taxonomic resolution (8,72). Integrating these high-throughput methods would enhance the precision and reproducibility of fungal profiling, enabling more detailed discrimination between vehicle-associated communities and environmental sources.

4.8. Legal and Evidentiary Considerations

The evidentiary framework for fungal community profiling requires careful calibration. Current accuracy levels (top-1 = 12.5%, top-3 = 54.2%) support use in an investigative context for hypothesis generation and suspect prioritisation rather than definitive identification. Expert testimony should articulate the probabilistic nature of findings, employing established likelihood-ratio frameworks to communicate the relative support for defence versus prosecution propositions (49,50). Database development represents a critical infrastructure need. Establishing reference collections of fungal communities from vehicles across diverse geographic regions, vehicle types, and maintenance histories would enable more sophisticated probabilistic comparisons (73). Such databases should document metadata including geographic coordinates, seasonal context, and vehicle characteristics. Privacy-protective approaches to database construction, modelled on existing DNA and microbial databases, would be essential for judicial acceptance (74).

4.9. Future Research Directions

This study presents a preliminary framework that focuses exclusively on the exterior surfaces of vehicles. These surfaces are directly exposed to environmental factors and therefore reflect transient and spatially variable fungal assemblages. Future research should extend this work to interior environments, where microbial communities may be more stable and persistent, particularly on enclosed surfaces such as dashboards, steering wheels and seat fabrics. Sampling the steering wheel presented a significant challenge during this study due to the low fungal biomass and frequent cleaning of this surface, highlighting the necessity for enhanced low-biomass collection and DNA recovery protocols.

Further work should also explore the potential for geographic inference (geolocation) based on fungal community signatures. Correlating community composition with environmental context, such as soil type, vegetation or regional climatic factors, could probabilistically associate vehicles with particular geographic areas. In addition, it may be important to compare the fungal prevalence deposited in dust on a seasonal basis between summer and winter. This would enhance the evidentiary value of fungal profiles in forensic investigations.

To increase the robustness and generalisability of the findings, future sampling campaigns should analyse a greater number of vehicles and cover a wider geographic area. Sampling different regions of Portugal and incorporating vehicles with diverse usage patterns, maintenance histories, and exposure conditions would improve statistical power and ecological representativeness. Similarly, sampling additional zones within each vehicle (e.g. door seals, undercarriage areas and boot linings) would provide a more comprehensive view of heterogeneity within vehicles and enhance classification performanc

5. Conclusion

This study investigated the potential of forensic mycology to distinguish between vehicles using fungal community profiling. Statistical analysis revealed measurable differences between vehicles, confirming that vehicle surfaces harbour distinct yet overlapping mycobiomes.

Despite this measurable structure, the predictive accuracy remained limited. Random Forest classification achieved only 12.5% top-one accuracy, which is equivalent to randomly guessing among eight vehicles. However, broader likelihood-based metrics revealed substantially greater discriminatory value; the correct vehicle appeared in the top three predictions in 54.2% of samples, and in the top five predictions in 66.7% of samples. These results suggest that, while 1:1 attribution is currently unfeasible, fungal community signatures can significantly narrow down the pool of potential vehicles.

The findings suggest that, while heterogeneous and partially structured, fungal community signatures do not possess sufficient individuality for forensic attribution. Interior surfaces shared consistent human-derived taxa, whereas exterior surfaces exhibited variable, environmentally driven assemblages. This variability, coupled with the small sample size and the binary treatment of the data, restricts the discriminative power.

This shortlist-based capacity is of practical forensic interest. Rather than identifying a single vehicle, the method supports exclusionary reasoning, enabling investigators to eliminate vehicles with a low probability and prioritise those that are most compatible with the fungal evidence. When combined with other evidence, such as DNA samples, trace particles or witness testimony, fungal profiling can help to narrow down the number of suspect vehicles rather than providing definitive identification.

Interior surfaces displayed consistent homogeneity, reflecting shared human-derived taxa. In contrast, exterior surfaces showed high variability, driven by environmental deposition. This ecological instability, combined with the small sample size and the use of presence/absence rather than abundance data, limits the robustness of predictions. After multiple-testing correction, no single taxon remained significantly associated with any vehicle, indicating that discriminatory power lies in multi-taxon community patterns rather than species-specific markers.

Therefore, fungal profiling should presently be viewed as an exploratory, supportive approach rather than a definitive forensic method. Its value lies in revealing ecological differentiation that may assist broader investigative analyses, rather than in providing independent proof of origin. Substantial methodological refinement would be required for practical application, including the incorporation of quantitative abundance data, validation across larger datasets, control for environmental confounders and integration with established evidence types, such as DNA or fingerprint analysis.

Future progress will depend on methodological expansion, including incorporating quantitative abundance data, high-throughput sequencing and larger, more geographically diverse datasets, as well as controlling for environmental covariates. The most realistic path towards operational utility is integration with established forensic techniques, particularly soil analysis, DNA profiling and mineralogical or palynological evidence.

In conclusion, this work contributes novel empirical data on vehicular mycobiomes and their variability across Portuguese regions, offering a foundation for future standardisation efforts. However, its current performance does not justify its deployment in operational forensics. Nevertheless, the approach remains promising as a research avenue for developing complementary microbial trace evidence frameworks, pending further methodological and validation studies.

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Attachment


[redacted]

Figure A 1. Vehicle 1:Renault Kangoo Compac.

Figure A 2. Vehicle 2: Peugeot 308.


[redacted]

Figure A 3. Vehicle 3: Opel Corsa

[redacted]

Figure A 4. Vehicle 4: Renault Grand Scenic.


[redacted]

Figure A 5. Vehicle 5: Peugeot Jumpy.

[redacted]

Figure A 6. Vehicle 6: Wolkswagen Golf .

Figure A 7. Vehicle 7: Citroen Xsara.

Figure A 8. Vehicle 8: Opel Combo.

Table A 1. Pairwise Jaccard dissimilarity indices between fungal communities detected on interior vehicle surfaces.

Car ID	1	2	3	4	5	6	7	8
1	0.000	0.714	0.400	0.714	0.800	0.600	0.500	1.000
2	0.714	0.000	0.500	0.333	0.600	0.667	0.600	0.857
3	0.400	0.500	0.000	0.500	0.800	0.600	0.500	1.000
4	0.714	0.333	0.500	0.000	0.833	0.667	0.600	0.857
5	0.800	0.600	0.800	0.833	0.000	0.750	0.667	1.000
6	0.600	0.667	0.600	0.667	0.750	0.000	0.333	1.000
7	0.500	0.600	0.500	0.600	0.667	0.333	0.000	1.000
8	1.000	0.857	1.000	0.857	1.000	1.000	1.000	0.000

Table A 2. Pairwise Sørensen–Dice dissimilarity indices between fungal communities detected on interior vehicle surfaces.

Car ID	1	2	3	4	5	6	7	8
1	0.000	0.556	0.250	0.556	0.667	0.429	0.333	1.000
2	0.556	0.000	0.333	0.200	0.429	0.500	0.429	0.750
3	0.250	0.333	0.000	0.333	0.667	0.429	0.333	1.000
4	0.556	0.200	0.333	0.000	0.714	0.500	0.429	0.750
5	0.667	0.429	0.667	0.714	0.000	0.600	0.500	1.000
6	0.429	0.500	0.429	0.500	0.600	0.000	0.200	1.000
7	0.333	0.429	0.333	0.429	0.500	0.200	0.000	1.000
8	1.000	0.750	1.000	0.750	1.000	1.000	1.000	0.000

Table A 3. Pairwise Jaccard dissimilarity indices between fungal communities detected on exterior vehicle surfaces.

Car ID	1	2	3	4	5	6	7	8
1	0.000	0.667	0.875	0.714	0.750	0.667	0.800	0.333
2	0.667	0.000	0.900	0.778	0.800	0.800	0.667	0.833
3	0.875	0.900	0.000	0.800	1.000	1.000	0.875	1.000
4	0.714	0.778	0.800	0.000	0.700	0.833	0.500	0.667
5	0.750	0.800	1.000	0.700	0.000	0.857	0.750	0.714
6	0.667	0.800	1.000	0.833	0.857	0.000	0.667	0.500
7	0.800	0.667	0.875	0.500	0.750	0.667	0.000	0.750
8	0.333	0.833	1.000	0.667	0.714	0.500	0.750	0.000

Table A 4. Pairwise Sørensen–Dice dissimilarity indices between fungal communities detected on exterior vehicle surfaces.

Car ID	1	2	3	4	5	6	7	8
1	0.000	0.500	0.778	0.556	0.600	0.500	0.667	0.200
2	0.500	0.000	0.818	0.636	0.667	0.667	0.500	0.714
3	0.778	0.818	0.000	0.667	1.000	1.000	0.778	1.000
4	0.556	0.636	0.667	0.000	0.538	0.714	0.333	0.500
5	0.600	0.667	1.000	0.538	0.000	0.750	0.600	0.556
6	0.500	0.667	1.000	0.714	0.750	0.000	0.500	0.333
7	0.667	0.500	0.778	0.333	0.600	0.500	0.000	0.600
8	0.200	0.714	1.000	0.500	0.556	0.333	0.600	0.000

Informed Consent

Monte de Caparica, de ano 2025

Exmo.(a) Sr.(a),

No âmbito do Mestrado em Tecnologias Laboratoriais em Ciências Forenses na Unidade Curricular de Projeto em Ciências Forenses/Dissertação do Instituto Universitário Egas Moniz, sob a orientação da Prof. Doutora Madalena Oom, solicita-se autorização para a participação no estudo "Using Forensic Microbiology to Associate Vehicles and Their Components", com o objetivo de fazer associação entre veículos e as suas partes, bem como entre diferentes veículos, através de um estudo microbiológico, que consiste na seguinte participação: recolha de poeira do veículo pessoal.

A participação neste estudo é voluntária. A sua não participação não lhe trará qualquer prejuízo.

Este estudo pode trazer benefícios tais como progresso na ciência, ao progresso do conhecimento.

A informação recolhida destina-se unicamente a tratamento estatístico e/ou publicação e será tratada pelo(s) orientador(es) e/ou pelos seus mandatados. A sua recolha é anónima e confidencial.

(Riscar o que não interessa)

ACEITO/NÃO ACEITO participar neste estudo, confirmando que fui esclarecido sobre as condições do mesmo e que não tenho dúvidas.

____

(Assinatura do participante ou, no caso de menores, do pai/mãe ou tutor legal)