MULTI-LEVEL CHARACTERIZATION OF MICROBIAL CONSORTIA INVOLVED IN THE BIODETERIORATION OF WOODEN AND STONE ROMANIAN HERITAGE CHURCHES

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1. Introduction

According to several studies biodeterioration represents the process of modification of material properties due to the activity of organisms from different systematic groups: bacteria, archaea, actinomycetes, algae, fungi, lichens, magnoliophytes, and animals [1,2]. The present study aims to investigate, by qualitative and quantitative methods, the microbial communities from biodeteriorated surfaces of wooden and stone monument churches, most of them included in the list of Romanian cultural heritage of local (Class B) and national (Class A) importance.

The Romanian wooden churches are a category of monuments which belong to the great family of European wood architecture [3].

Placed since 1991 under the protection of the National Museum of the Romanian Peasant (MNȚR) of Bucharest, Arad and Hunedoara, the wooden churches represent monuments of national importance (Class A). Unfortunately, they are today in various stages of degradation, with minimum interventions being made as regards their state of conservation. In most cases, restoration has been carried out as an emergency in-

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tervention, as no national programs are dedicated to the restoration of monuments in critical situations requiring immediate restoration.

The cultural and historical importance for the village communities in the areas where these churches are located is a major one, given the multiethnic component specific to the region, which include small Orthodox communities. For the Romanians from the villages of Mureş, Arieş and Târnave (Transylvania region), the wooden churches bear witness to the serious consequences of denationalization, often accompanied by religious persecution. These abusive interventions, on both the local population and the churches, have strongly affected community life and the condition of church edifices up to the present. Some members of the religious communities even left the country or converted to other confessions, therefore the duty of preserving the monuments remained the concern of the parish or the state (Class A Monuments).

Thus, from a historical perspective, wooden churches have been a way for the Orthodox communities in the area to assert their ethnic and religious identity, whereas from a cultural perspective, the aesthetic, architectural and artistic value of the buildings demonstrates an obvious concern for the construction of sustainable religious establishments, marking the spiritual life of the practicing communities.

At present, these places of worship have a strictly heritage and historical value, and religious rites specific to the Orthodox cult are no longer practised. In all the villages where the churches, under the protection of MNȚR are located, new stone churches have been built to better withstand the climatic and geographical conditions specific to the area (i.e. low temperatures in cold seasons and high humidity, creating favorable conditions for severe wood damage and the development of various species of fungi, both outdoors and indoors).

Being an integral part of the peasant communities, these churches have a series of features in common with traditional houses: similar dimensions, similar raw materials (wood logs) and techniques (such as “blokbau” or interlocking) were used for their construction. The design of the wooden churches included: the narthex, the nave and the altar, arranged on the longitudinal axis of the construction to which, in some cases, the porch is added (as is found especially in Transylvania and sometimes in Oltenia, Muntenia or Moldova). These monuments have an obvious spatial architectural unity, as they are often small and measured from the entrance to the narthex to the altar, so the church is generally the size of a house.

The architecture, building techniques and decorations of the wooden churches are similar to those of the peasant houses. The main feature is represented by the austere style of the exterior, as the buildings were conceived to adapt to the landform and climate of the area. Massive wooden pillars whose tops are often shaped like a stylized horse’s head and the heads of the rafters that support the eaves of the roof, are also specific to these monuments.

Some aspects related to the execution technique or iconographic development, are imposed by the special form of the wooden architecture and the way this material is prepared. The painting registers (the series of iconografic images and their order in the church) generally include the temple, icons, altar, vaults and paintings of the founders. The latter are present in some of the churches, as the Byzantine painting *hermēneia* (manual) considers them optional. Also, it is important to mention that they cannot be attested in the case of monuments where the painting has been completely removed or destroyed, especially where there is no photographic evidence of the original painting. As an execution technique, tempera, frequently ap-

plied directly on wood, canvas, plastered wood, or even fresco on wood covered with plaster, are common.

The pictorial ensembles specific to the wooden churches from Arad (Groşii Noi, Juliţa and Troaş) and Hunedoara (Lunca Moţilor and Bejan) under the protection of MNȚR (The Romanian Peasant Museum – http://www.muzeultaranuluiroman.ro/museum.html), date from the 18th and the beginning of the 19th century and are the work of the peasant painters.

Of all the mentioned churches, the one in the MNȚR museum courtyard is in the most advanced stage of deterioration. Both the outside and inside bear evident signs caused by unfavorable weather conditions and the lack of emergency conservation and restoration interventions. Currently, all churches require restoration work and are inaccessible to the general public.

A number of studies around the world have documented the degradation of wooden cultural heritage objects [4-10]. However, there is still insufficient information regarding identification at species level and the enzymatic activity of the filamentous fungi species involved in the biodeterioration of cultural heritage. Molecular methods available to identify fungi, by sequencing conserved regions of rDNA, could facilitate identification and provide information on the diversity of fungi causing the degradation. Fungal agents are also involved in the deterioration of stone monuments [11-13], causing discoloration and degradation due to fungal growth on their stone surfaces, physical intrusion or penetration of fungal hyphae and production of pigments and organic acids (e.g. oxalate) [14-16], while bacterial colonization could lead to crust formation, exfoliation and color alteration [17,18]. It must also be taken into account that several environmental parameters, such as temperature, relative humidity or light intensity, have a great impact on fungal development [19-21].

Therefore, geographically located studies are required in order to elucidate the diversity of the bio-deteriorating species and to further develop efficient tools for reducing their negative impact. Moreover, the presence of filamentous fungi inside the heritage churches could be a major cause of respiratory diseases and allergies in humans [3,22].

2. Materials and methods

2.1. Phenotypic (macroscopic and microscopic) identification of microbial isolates

Based on the observed changes, samples were taken with cotton sterile swabs from a total number of thirteen churches of wooden (n=5) and stone (n=8) from Arad (AR), Hunedoara (HD) and Bucharest, between July and August 2018. Most of the investigated churches (Table 1) are included in the national cultural heritage of Romania.

The samples were taken from areas of about 10 cm^2 of the walls of the narthex, nave and altar, to which aero-microbiota samples were added. A microbial attack was suspected as the majority of the isolated points in the investigated churches presented visible alterations, colored spots, discolored areas, deposits or patina on the surfaces; for this reason, several samples were taken. Areas without visible changes were considered as negative controls.

Table 1. Name, code, location and geographical coordinates of the investigated churches.

Church	Village, county	Geographical coordinates	On the list of national cultural heritage of Romania
Wooden churches			
Saint Nicolae Hierarch	Bucharest	44.4364°N;26.08783°E	No
The wooden Church of the Three Holy Hierarchs	Troaș, Săvârșin, AR county	46.09050°N;22.29510°E	Yes, AR-II-m-A-00655
The wooden Church of the Lord's greeting	Groșii Noi, Bârzava, AR county	46.106532°N;21.993263°E	Yes, AR-II-m-A-00607
The wooden Church entrance of the Virgin Mary	Julița, Vărădia de Mureș, AR county	46.039275°N;22.134529°E	AR-II-m-A-00615
The wooden Church of the Assumption of the Virgin Mary	Lunca Moților, HD county	46.170306°N;22.68047°E	HD-II-m-A-03360
Stone churches			
The stone Church of the Assumption of the Virgin Mary	Strei, HD county	45.716650°N;22.988115°E	Yes, HD-II-m-A-03452
The stone Church Descent of the Holy Spirit	Ostrov, HD county	45.527907°N; 22.846613°E	Yes, HD-II-a-A-03400
The stone Church of Sântămărie	Orlea, HD county	45.590715°N; 22.969964°E	Yes, HD-II-m-A-03445.
Prislop monastery	Near to Silvașu de Sus, HD county	45.6317548°N;22.8503078°E	Yes, HD-II-m-A-03447
The stone Church of the Descent of the Holy Spirit	Paroș, HD county	45.495998°N; 22.969807°E	Yes, HD-II-m-A-03401
The St. Nicholas Church of Densuș	Densuș, HD county	45.582631°N; 22.805392°E	HD-II-m-A-03454
The Church of the Ascension of the Lord	Nucșoara, HD county	45.480841°N; 22.916338°E	HD-II-m-B-03370
The Church of Saint George	Sînpetru, HD county	45.551702°N; 22.912181°E	HD-II-m-A-03307

After sampling, cotton swabs were submerged in 1 mL of sterile distilled water solution for spore suspension, dilutions were performed and then plated onto two different media: PDA (Potato Dextrose Agar) and Rose Bengal (Liofilchem) incubated at 22 ± 1◦C for 5-7 days, allowing fungal growth and PCA (Plate Count Agar) for bacterial growth. After this period, colonies were then purified (using the same culture medium from where they were first isolated) and subcultures were prepared for morphological and automated identification. Fungal isolates were identified to genus or species level using biometric parameters (colony diameter) and microscopic features. Microscopic examination was performed using blue cotton lactophenol staining. Bacterial strains were preliminarily identified by Gram staining, catalase and oxidase tests.

2.2. Quantitative assessment of microbial contamination by cultivation dependent methods

In the next step, the number of colony-forming units (CFU/mL) of bacteria, yeasts, and filamentous fungi was determined, according to the following equation: CFU/m^2 = (number of the developed colonies x 100)/dilution.

2.3. Automated identification using MALDI-TOF system

The identification of microbial consortia (filamentous fungi and bacteria) by the MALDI-TOF system was based on a database that requires a permanent update for the most accurate identification.

2.4. Quantitative evaluation of the fungal adherence to inert substrata

Evaluation of biofilm development on the inert substrate was assessed by the microtiter broth method. Overnight cultures were grown in 96 multi-well plates containing Potato Dextrose Broth (PDB) medium for fungi and Muller Hinton Broth (MHB) supplemented with 2% glucose for bacteria at 22°C for 48-72 h, then the content was removed and washed three times with phosphate-buffered saline (PBS). The adherent cells were then fixed with cold methanol, stained with an alkaline 1 % violet crystal solution for 15 min., washed with water and resuspended in a 33 % acetic acid solution. The intensity of the suspension was spectrophotometrically assessed, the amount of adhered biomass being proportional to the absorbance value read at 492 nm [23].

2.2. Evaluation of capsule and spore forming bacteria

Staining of the spores with Malachite green according to the Benito Trujillo protocol was performed on all Gram-positive strains. The microbial strains were grown in Luria Broth (LB) medium supplemented with 0.5 M $CaCl_2$ and incubated at 30^0 C for up to 72 hours. After incubation, microbial cultures were stained according to protocol and spores were examined under the optical microscope. Depending on the stage of development, the endospores appeared colored in different shades of green and the vegetative cells in red.

2.3. Nucleic acid-based methods for fungal identification and diversity

The fungal strains not identified by MALDI analysis were taxonomically identified by sequencing the intergenic region of ribosomal genes. The genetic diversity of the fungal species was studied by the PCR method of the Internal Transcribed Spacer (ITS) region, a highly conserved rDNA region. The DNA was extracted by the classical phenol/chloroform method and subsequently amplified using the following primers: ITS-1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3'). 20ng of DNA and 0.5μM of each primer were introduced into the amplification reaction. The amplification program included: initial denaturation of 95°C, 5min and 35 cycles which included (95°C, 1 min; 55°C, 60 sec; 72°C, 8 min). The obtained amplicons were verified in 1.5% agarose gel and subsequently sequenced using the ABI 3730xl DNA sequencer – Applied Biosystems. The obtained sequences were analyzed using ChromasLite 2.1.1 (http://www.technelysium.com.au) and compared with the sequences from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program for species identification.

3. Results and discussion

3.1. Microbial load of the collected samples

The comparative study of the microbial load of the samples taken from six wooden (n=4) and stone (n=2) churches located in the counties of AR and HD (Bârzava, Lunca Moților, Troaș, Densuș, Julița and Strei churches) between July and August 2018 from different sampling points, revealed a two-log variation of the total microbial load, among different churches and sampling points inside each church, ranging from $2.18x10^7$ to $3x10^5$ CFU/mL (Table 2).

Table 2. Comparative representation of total microbial loads (log CFU/mL) in different churches and sampling points.

Church 1 Bârzava (AR county)				
Pronaos	Naos	Altar	Objects	Entrance door
$1x\ 10^5$ $3x\ 10^5$ $201x10^5$	$1x\ 10^5$ $3x\ 10^5$ $200x10^5$	$1x\ 10^5$ $2x\ 10^5$ $3x\ 10^6$	-	-
Church 2 Lunca Moților (HD county)				
Pronaos	Naos	Altar	Objects	Entrance door
$1x\ 10^5$ $3x\ 10^5$		$1x\ 10^5$ $2x\ 10^5$	-	-
Church 3 Troaș-Săvârșin (AR county)				
Pronaos	Naos	Altar	Objects	Entrance door
$8x\ 10^5$	$7x\ 10^5$	$2x\ 10^5$ $8x\ 10^5$ $12x\ 10^5$	$1x\ 10^5$	-

Church 4 Densuș (HD county)				
Pronaos	Naos	Altar	Objects	Entrance door
1x 10^5				3x 10^5
Church 5 Julița (AR county)				
Pronaos	Naos	Altar	Objects	Entrance door
1x 10^5 76x 10^5	3x 10^5 218x 10^5	3x 10^5 12x 10^5	141x 10^5	1x 10^5
Church 6 Strei (HD county)				
Pronaos	Naos	Altar	Objects	Entrance door
-	-	-	-	1x 10^5

3.2. Phenotypic characterization of filamentous fungi

One of the main goals of our study was to characterize the fungal strains isolated from the wooden and stone monument churches showing various degrees of biodeterioration and to investigate some features associated with their survival and resistance in different conditions, taking into account the results of many studies, showing that both microclimate and materials affect microbial density [24] (Fig. 1 A and B; 2 A and B; 3 A and B).

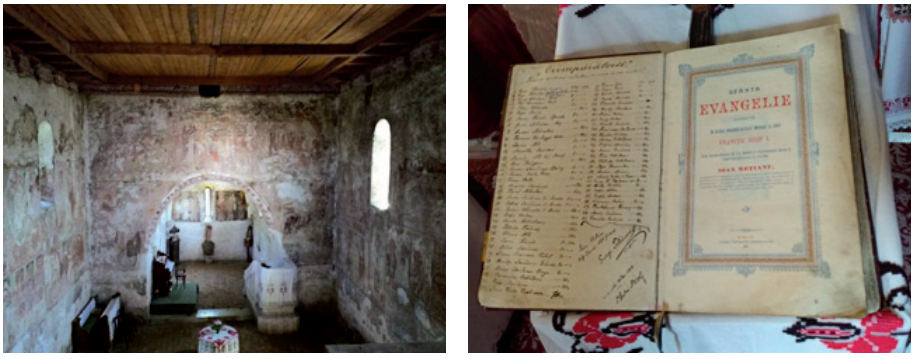
A B

Figure 1. Wooden churches: iconostasis of the Saint Hierarch Nicolae Church (A); and the Iconostasis of the wooden The Lord's greeting, Barzava (B).

It is well known that microorganisms, particularly fungi can inhabit, alter and degrade different types of materials [25]. Cotton can be degraded when it is exposed to ultraviolet light and chemical and biological attacks, as well as to contamination and mechanical stress [26]. These are all materials that support the growth of fungi throughout the years. Based on the literature data, the majority of fungi species involved in the deterioration of the cultural heritage belonged to *Ascomycota phylum*, and the occurrence of basidiomycetes restricted to wood degradation in churches or other protected historical monuments. Zygomycetes are frequently isolated from pieces of art, but in most cases their presence is only transitory [25].

A B

Figure 2. The naves. A: the wooden Church of the Assumption of the Virgin Mary in Lunca Motilor; and B, the stone Church of the Ascension of the Lord in Nucșoara.



A B

Figure 3. The stone Church of Sântămărie (A); an orthodox book from the stone Church of Saint George in Sînpetru (B).

Preliminary phenotypic identification of the filamentous fungi was performed based on the examination of macroscopic characters observable after 5-7 days of incubation at 22°C, analyzing the obverse of colonies and their reverse and taking into account the time of culture onset, the degree of development (biometric parameter), consistency of the culture, the pigmentation, the shape of the colonies, their profiles, etc. Microscopic examination allowed us to evidence the presence of vegetative and reproductive structures ensuring the correct interpretation of results, as there are many species which, though present on the surface of the heritage object or in the environment, might be dormant and thus not directly responsible for the decay of heritage objects (Fig. 4. A1-A8).

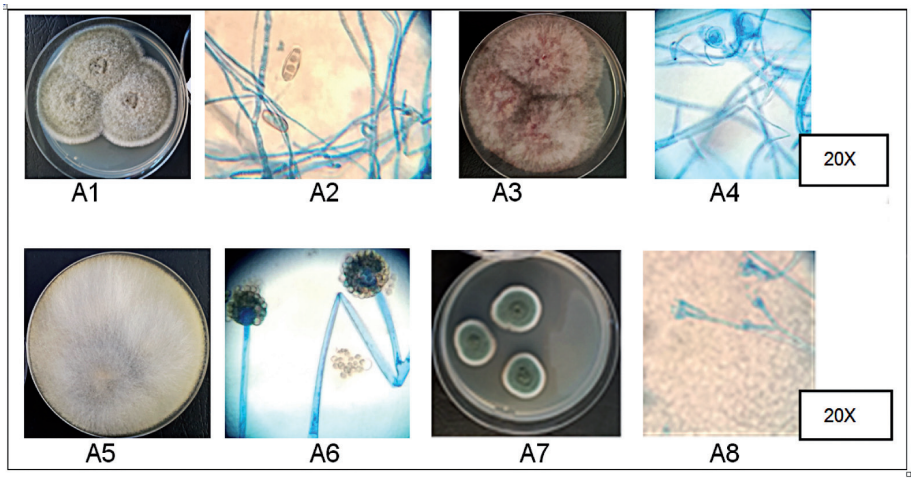


Figure 4. The macro- and microscopic aspects of some representative filamentous fungi genus isolated from the investigated churches. A1: A. alternata – obverse aspect of the colonies; A2: microscopic aspect of A. alternata – blue cotton staining; A3: Fusarium spp. – macroscopic aspects of the colonies on PDA culture media; A4: Fusarium spp. – clamidospores – microscopic aspect; A5: M. circinelloides – macroscopic aspects of the colonies on PDA culture media; A6: M. circinelloides – microscopic aspects of the sporangiospores; A7: P. corylophylum – macroscopic view; A8: P. corylophylum – microscopic aspects of the conidiophores and conidia.

3.3. Taxonomic assignment of the filamentous fungi isolated from wooden and stone churches

The sampling from the wooden church belonging to the Romanian Peasant Museum in Bucharest allowed the identification of filamentous fungi distributed on the following sources of isolation/species: ascomycetes belonging to the *Aspergillus terreus* and *Rhizopus oryzae* species, as well as two basidiomycetes belonging to the *Schizophyllum commune* species in the narthex area; *Penicillium digitatum* and *Alternaria alternata* predominated in the nave area, while the church altar was colonized with isolates belonging to *R. oryzae* and *Cladosporium* sp. In the aero-microbiota samples, the *R. oryzae* and *Trichoderma orientale* strains were dominant. In the wooden church, The Lord's greeting in Bârzava, Groșii Noi village, the nave was colonized by *Penicillium* spp., the narthex by *P. chrysogenum* and *A. alternata* and the altar by *R. stolonifer* and *Cladosporium* spp.

In the wooden church in Săvârșin village, the predominating species in the nave and altar was *P. corylophilum*.

The Julița village church was contaminated in the nave with *P. corylophilum* and *P. chrysogenum*, in the narthex and attic with *P. corylophilum* and *Mucor circinelloides*, while from the altar *P. corylophilum* and *P. expansum* were isolated. As regards the wooden church, The Assumption of the Virgin Mary Church in Lunca Moților, Hunedoara County, transferred to the Bucharest Romanian Peasant Museum, *P. Corylophilum* prevailed in all sampling points (Table 3).

Table 3. Comparative representation of the fungal species isolated from wooden churches and sampling points.

Church 1- Bucharest: Frequency of species with percentages				
Pronaos	Naos	Altar	Object	Others: aeromicrobiota
A. terreus, *R. oryzae*, *S. commune* (16.16% each)	*P. digitatum*, *A. alternata* (25% each)	*R. oryzae,* Cladosporium sp. (5.71% each)		*R. oryzae, T. orientale* (22.22% each)
Church 2 – Bârzava (AR): Frequency of species with percentages				
Pronaos	Naos	Altar	Attic	Others
P. chrysogenum, *A. alternata* (33.33% each)	*Penicillium* sp. (50%)	*R. stolonifer* and *Cladosporium* sp. (12.5% each)		
Church 3 – Troaș – Săvârșin (AR): Frequency of species with percentages				
Pronaos	Naos	Altar	Attic	Others
P. corylophilum (20%)	*P. corylophilum* (20%), *A. flavus* (10% each)	*P. corylophilum* (20%), *P. chrysogenum, R.* stolonifer (10% each)		
Church 4 – Julița (AR): Frequency of species with percentages				
Pronaos	Naos	Altar	Attic	Others: roof
P. corylophilum M. circinelloides (5.55% each)	*P. corylophilum (13.88%),*	*P. corylophilum, P. expansum (5.55% each)*	*P. corylophilum* (5.55%)	*A. flavus* (2.77%)
Church 5 – Lunca Moților (HD): Frequency of species with percentages				
Pronaos	Naos	Altar	Attic	Others: outer wall
P. corylophilum (13.88%)	*P. corylophilum (25%), A. niger (13.88%)*	*P. corylophilum (16.66%)*		*P. corylophilum (5.55%)*

As regards the stone churches from the Hunedoara county, the following genera and species were isolated:

– Nucșoara church - *Aspergillus versicolor* strains were dominant followed by *A. alternata*, *M. circinelloides*, *P. chrysogenum* and *Aspergillus sydowii*;

– the church of "Sfântul Mare Mucenic Gheorghe" in Sînpetru village - *Aspergillus niger*, *Penicillium* spp., *A. nidulans*, and *Purpureocillium lilacinum*;

– In the church in Ostrov village - *A. alternata* was the dominant species, followed in decreasing order by *Penicillium roqueforti*, *P. digitatum,* and *P. expansum*;

– in Prislop Monastery - the dominant species was A*. niger*, followed by *A. alternata*, *Trichoderma* spp., *Penicillium commune,* and *Mucor circinelloides*;

– in the Church of Strei – the majority of the isolates belonged to *A. alternata*, followed by *A. niger* and *A. arundinis*;

– in the Sânămarie Church, in Orlea village - *A. alternata* and *Fusarium cerealis culmorum* group strains were dominant, followed by *R. oryzae, Penicillium citrinum, Aspergillus flavus oryzae* group, and *P. corylophylum*;

– in the Church of Paroș village - *A. versicolor* and other species of the genus *Aspergillus* were dominant, followed by *Cladosporium* sp. and *A. alternata* strains (Table 4).

Table 4. Comparative representation of the fungal species isolated from stone churches.

	Identified species	**Frequency (%)**
Church 6 – Nucșoara (HD)	*A. versicolor*	32.55%
	A. alternata	9.30%
	M. circinelloides, P. chrysogenum. A. sydowii	6.97%each
Church 7– Sînpetru (HD)	*Aspergillus niger*	19%
	A. nidulans, P. lilacinum, Penicillium spp.	18% each
	Rhizopus spp.	9%
	Arthrinium phaeospermum, Aspergillus spp.	9% each
Church 8 Ostrov (HD)	*A. alternata*	40%
	P. roqueforti, P. digitatum, P. expansum	20% each
Church 9 – Prislop (HD)	*Aspergillus niger*	34%
	Trichoderma spp., A. alternata	22% each
	Penicillium commune, M. circinelloides	11% each
Church 10 – Strei (HD)	*A. alternata*	72%
	A. niger, Arthrinium arundinis	14% each
Church 11 – Sântămărie (HD)	*A. alternata, Fusarium cerealis culmorum group*	25%
	P. corylophylum, R. oryzae	13% each
	A. flavus oryzae group, P. citrinum	12% each
Church 12 – Paroș (HD)	*A. versicolor, Aspergillus sp.*	17% each
	Cladosporium sp., A. alternata, A. sydowii	12% each
	Aspergillus niger *Stachybotrys chartarum* *Penicillium expansum* *Penicillium spp.*	6% each

In Romania, only several studies have investigated the above-mentioned topic. In 2013, Dănească et al. investigated one wooden church from Hunedoara County (Boz village) included in the list of monuments of local importance (Class B). The study demonstrated that several parts of the investigated church were in an advanced state of degradation because of water infiltration through the highly damaged roof and required emergency interventions for its conservation, preservation and restoration [27]. In 2018, another group investigated the diversity of filamentous fungi and bacteria isolated from one of the wooden churches (Bihor County) included in Class B of the

national list of historical monuments and demonstrated – similarly to our study – the presence of *Aspergillus*, *Rhizopus*, *Penicillium* and *Mucor* genera; these fungi/bacteria are also associated with a major risk to human health and can cause infections, allergies, and toxicity [28]. The same group performed a similar study on the paintings found on the interior walls of the Orthodox wooden Church of Saint Martyrs Constantin Brâncoveanu and His Sons, on the University of Oradea campus, also a Class B historical monument; in this case, they identified *Penicillium*, *Aspergillus*, *Alternaria* or *Cladosporium* strains [29].

Using culture-based methods to determine the filamentous fungi involved in the biodeterioration of the mural paintings in the old orthodox church named "Annunciation", located in the Sibiu area, two species of the *Penicillium* genus (*P. rugulosum* and *P. chrysogenum*) were identified [30].

Several genera of filamentous fungi (*Alternaria*, *Penicillium*, *Aspergillus*, and *Cladosporium*), diverse *Bacillus* species, *Planomicrobium* and *Variovorax* genera, not previously reported in paintings or on wood, were identified in samples recovered from the wall and icon surfaces of a Class A monument, the seventeenth-century wooden church of Nicula, located in Cluj County, Romania [31].

The filamentous strains not identified by phenotypic approach to the species level were submitted to molecular analysis. The ITS (internal transcribed spacer) region was used as a target region in the phylogenetic analysis as it generally exhibits sequence variations between species, but only minor variations within strains of the same species. Molecular identification of the fungi up to species level was largely based on an analysis of the ITS regions including ITS1, 5.8S rDNA and ITS4. The DNA sequences in the ITS region are also highly variable and could serve as a marker of the phylogenetically distant taxonomic groups. The molecular investigation of the *Penicillium* spp., *Trichoderma* spp. *Cladosporium* spp. and *Alternaria* spp. strains allowed the identification of *P. chrysogenum*, *P. expansum*, *P. crustosum*, *P. colylophylum*, *T. longibrachyatum*, *C. cladosporoides,* and *A. alternata* species, with 100% identity (molecular identification presented 100% identity with similar species from NCBI database – those with 99% were excluded from the study).

3.4. Bacterial yeast associations present in the analyzed samples

There are few studies regarding bacterial diversity associated with the biodegradation of wooden and stone objects. Initial tests such as Gram staining revealed that Gram-positive bacilli are the predominant group, the results being confirmed by MALDI analysis. According to the MALDI results, most of the bacterial strains isolated from the wood churches belonged to the *Bacillus* genus.

A total number of 27 bacterial strains and 3 yeast strains belonging to the *Cryptococcus* genus were isolated from the wood churches included in the Bucharest Romanian Peasant Museum, the predominant species being *Bacillus pumilus*, followed by *B. megaterium* species and *B. subtilis*. From the less frequently isolated Gram-negative strains, the predominant genus was *Arthrobacter*. More than 50% of the bacterial strains not identified by using the MALDI-TOF technique, revealed the great diversity of the bacterial strains found in these samples, as compared, for example, with clinical samples. In addition, 28 bacterial strains and 5 yeast strains belonging to the *Candida* genus (3) and *Cryptococcus* (2) genera were isolated from different stone churches located in the Haţeg region. The predominant species was *B. megaterium* followed by *B. pumillus* and *B. thuringiensis* (Table 5).

Table 5. Comparative representation of the bacterial species isolated from wooden and stone churches.

Wood churches – AR county	Identified species	Frequency (%)
	Bacillus pumillus	11%
	Bacillus megaterium	7%
	Bacillus subtilis	3%
	Arthrobacter globiformis	4%
	Arthrobacter aurescens	4%
	Arthrobacter bergeri	4%
	Rhodococcus erythropolis	4%
	Ps. enthomophila	4%
	Lb. alimetarius	4%
	S. epidermidis	4%
Stone churches – HD county	Identified species	Frequency (%)
	Bacillus megaterium	36%
	Bacillus pumillus	14%
	Bacillus thuringiensis	4%
	P. koreensis	4%
	Penibacillus sp.	7%
	Candida sp.	11%

Some strains belonging to *Staphylococcus* genera were considered anthropic contaminants of objects without direct impact on their degradation.

In the wooden churches the bacterial diversity was higher compared to the stone churches, probably due to the higher humidity and the presence of cellulose, which is an excellent nutritional support for many microorganisms. However, similar to stone churches, Gram-positive bacteria were predominant, as expected, given the physiological conditions such as low humidity, pH variation, and the presence of mostly inorganic nutrients.

It was also noted that the bacterial community increased with the advancement of the deterioration process.

3.5. Feature related to microbial survival and resistance strategies

3.5.1. Biofilm development

The quantitative evaluation of the degree of biofilm development on the inert substrate was performed for 77 fungal strains isolated from the wooden churches located in Bucharest, Arad and Hunedoara, and 55 strains isolated from the stone churches located in Hunedoara County. Only 6 fungal strains out of 77 fungal strains from wooden churches in Bucharest, Arad and Hunedoara showed the capability to form biofilms.

The main species recurrently found to harbor this feature was *A. alternata*, followed by *P. corylophilum* and *Cladosporium* spp (Fig.5). The results of our study are in agreement with those reported in the scientific literature regarding the heterotrophic components of biofilms formed on wooden artifacts [32]. The capacity of the fungal strains isolated from wood and stone churches to form biofilms seems to be correlated with the high microbial load and the advanced degree of deterioration, an aspect highlighting the need for intervention measures based on anti-biofilm compounds for preventing the biodegradation of heritage objects.

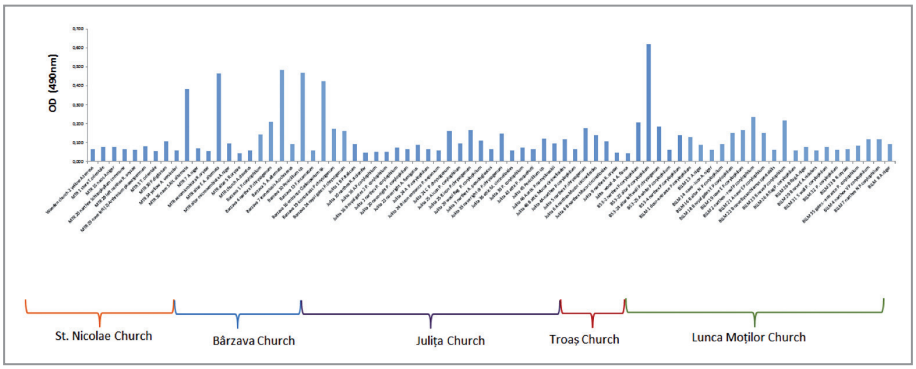


Figure 5. Graphic representation of the biofilm forming ability of the strains isolated from wooden churches.

In Serbia, a research group investigated the biofilm-forming community isolated from the stone Church of the Holy Ascension and revealed that the dominant genera were *Alternaria*, *Arthrinium* and *Cladosporium*, also isolated in our study both from wooden and stone churches. It must be mentioned that the *Cladosporium* species represents one of the main biological agents involved in the biodeterioration of the mural paintings [33].

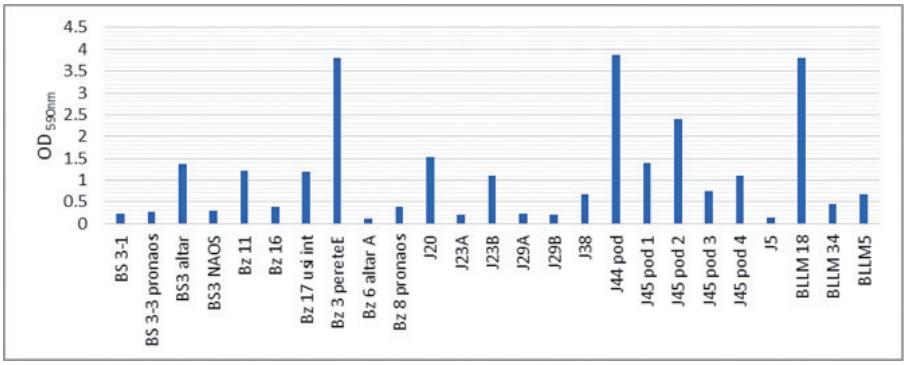


Figure 6. Graphic representation of the biofilm forming ability of the bacterial strains isolated from wooden churches.

A similar study performed in Italy, on deteriorated wall paintings from seven medieval churches revealed the presence of different species belonging to *Penicillium*, *Aspergillus*, *Fusarium*, and *Alternaria* genera [34].

Species such as *Aspergillus versicolor*, *Cladosporium*, or *Penicillium* spp. revealed by other research groups, such as Trovão et al., in 2019, demonstrated the presence of these species together with molds in samples taken from an old cathedral in Portugal [35].

As for wooden churches, the highest biofilm producers were *B. megaterium* and *B. pumillus* strains, followed by *Arthrobacter globiformis, Ps. entomophila* and *B. subtilis* (Fig. 6).

The bacterial strains isolated from stone churches exhibited a higher biofilm formation ability compared with strains isolated from wooden churches, half of them being intensive biofilm producers. The most prolific in biofilm formation were *Paenibacillus peoriae*, *B. megaterium* and *Ps. koreensis* strains.

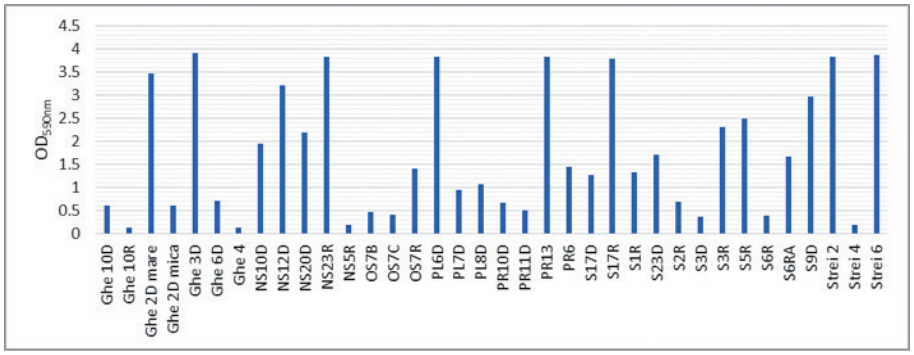


Figure 7. Graphic representation of the biofilm forming ability of the bacterial strains isolated from stone churches.

3.5.2. Capsule and spore formation

Spores, along with capsule, are considered additional mechanisms of resistance/ persistence of bacterial strains to extreme environmental conditions.

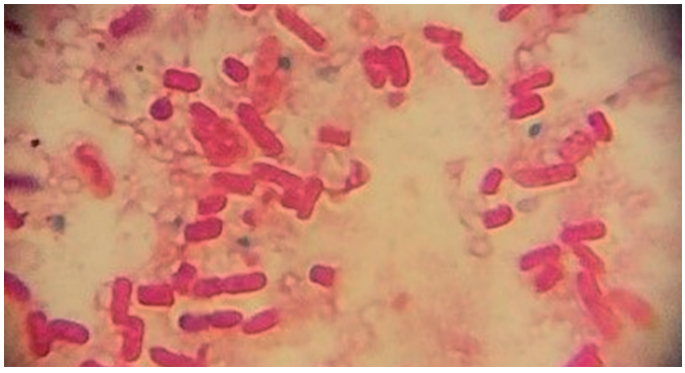


Figure 8. Microscopic aspect endospores – B. megaterium – Green Malachite staining; 1000X.

As stated above, the dominant bacteria isolated from wooden and stone churches were *B. subtilis, B. megaterium,* and *B. mojavensis*. All these species presented extremely resistant inert endospores in response to nutrient deficiency and other environmental stress conditions. All *B. megaterium* strains produced spores after 28 hours of incubation in medium supplemented with $CaCl_2$, while the other bacterial strains belonging to the *Bacillus* genus started the sporulation process later, i.e., after 48-72h of incubation (Fig. 8).

As expected, the presence of the capsule was correlated with the ability of the bacterial strains to form biofilm, the capsular polysaccharides facilitating the adhesion process.

Besides, the presence of different features acting as protecting factors against harmful external factors (capsule, spore, biofilm), half of the bacterial strains produce cellulases, that could be involved in the biodegradation process of wood objects.

4. Conclusion

This multi-level characterization of microbial consortia from the wooden and stone Romanian heritage churches was carried out by culture dependent (qualitative and quantitative assessment of the microbial contamination, direct microscopic examination, MALDI-TOF identification, quantitative evaluation of the fungal and bacterial adherence to inert substrata, evaluation of capsule and spore forming bacteria) and independent (PCR and sequencing of ITS intergenic regions in fungal strains) methods. The results of this study could improve the understanding of the microbial biodeterioration process of the heritage churches from Romania and allow reliable decontamination methods to be defined.

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Summary

The 17th to 19th century wooden and stone churches are an iconic symbol of Romanian national heritage. The present study investigates by qualitative and quantitative methods the microbial communities from the biodeteriorated surfaces of wooden and stone monument churches included in the cultural heritage list of local or national importance. From a total of twelve monuments, samples were taken with cotton sterile swabs, inoculated on specific culture and identified by classical, automated and molecular methods. A total of 133 strains belonging to *Ascomycota phylum* were identified and confirmed at species level from the wooden churches, amongst which, *Penicillium* spp. strains (mostly *P. corylophylum, P. chrysogenum*) were the most frequent, followed by *Alternaria alternata* and species of *Trichoderma*, *Aspergillus*, *Rhizopus*, *Mucor*, and *Fusarium* genera. From the stone churches a total of 100 strains belonging to *Aspergillus*, *Alternaria*, *Mucor*, *Penicillium*, *Aspergillus*, *Trichoderma*, *Fusarium* and *Rhizopus* genera were isolated.

A total of 55 bacterial strains were isolated and identified as *Bacillus*, *Artrobacter* and *Pseudomonas* species. The microbial load of the samples ranged between $2.18x10^7$ and $3x10^5$ CFU/mL. A very small number of fungal strains (6/77) isolated from wooden churches (mostly *A. alternata*, followed by *P. corylophilum* and one *Cladosporium* spp. strain) and from stone churches (5/55) (mostly *A. alternata*, followed by *A. versicolor*, *A. nidulans* strain) were involved in biofilm formation.

The results of this study can help to improve understanding of the microbial deterioration of Romanian heritage churches and allow more reliable decontamination, conservation and preservation tools to be defined.

Riassunto

Le chiese in legno e pietra dal XVII al XIX secolo sono un simbolo iconico del patrimonio nazionale rumeno. Il presente studio indaga con metodi qualitativi e quantitativi le comunità microbiche provenienti dalle superfici biodeteriorate di chiese monumentali in legno e pietra incluse nell'elenco dei beni culturali di importanza locale o nazionale. Da un totale di dodici monumenti, sono stati prelevati campioni con tamponi di cotone sterili, inoculati su colture specifiche e identificati con metodi classici, automatizzati e molecolari. Un totale di 133 ceppi appartenenti ad Ascomycota phylum sono stati identificati e confermati a livello di specie dalle chiese di legno, tra cui Penicillium spp. I ceppi (principalmente P. corylophylum, P. chrysogenum) sono i più frequenti, seguiti da Alternaria alternata e dalle specie dei generi Trichoderma, Aspergillus, Rhizopus, Mucor e Fusarium. Dalle chiese in pietra sono stati isolati in totale 100 ceppi appartenenti ai generi Aspergillus, Alternaria, Mucor, Penicillium, Aspergillus, Trichoderma, Fusarium e Rhizopus.

Un totale di 55 ceppi batterici è stato isolato e identificato come specie Bacillus, Artrobacter e Pseudomonas. La carica microbica dei campioni varia tra 2,18x107 e 3x105 CFU / mL. Un numero molto piccolo di ceppi fungini (6/77) isolati da chiese in legno (principalmente A. alternata, seguito da P. corylophilum e un ceppo Cladosporium spp.) e da chiese in pietra (5/55) (principalmente A. alternata, seguito di A. versicolor, ceppo di A. nidulans) sono stati coinvolti nella formazione del biofilm.

I risultati di questo studio possono aiutare a migliorare la comprensione del deterioramento microbico delle chiese del patrimonio rumeno e consentire la definizione di strumenti di decontaminazione, conservazione e conservazione più affidabili.