UNIVERSIDADE DE LISBOA

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FUNGAL ISOLATES FROM THE ARCHIVE OF THE UNIVERSITY OF COIMBRA: IONIZING RADIATION RESPONSE AND GENOTYPIC FINGERPRINTING

Inês Marques Nunes

MESTRADO EM MICROBIOLOGIA APLICADA

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Dissertação orientada pelo Prof. Dr. António Portugal (FCTUC-DCV)

e pela Prof. Dra. Manuela Carolino (FCUL-CBA)

Inês Marques Nunes

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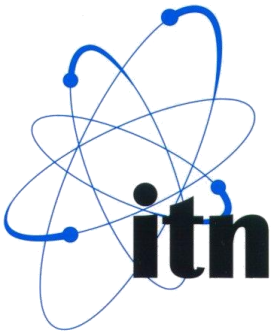

Centre for Functional Ecology

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Inês Marques Nunes

MASTER THESIS

2011

This thesis was performed at Center for Functional Ecology, Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra under the direct supervision of Prof. Dr. António Portugal and at Unit of Physics and Accelerators, Nuclear and Technological Institute under the direct supervision of Dr. Sandra Cabo Verde.

Prof. Dr. Manuela Carolino was the internal designated supervisor in the scope of the *Master in Applied Microbiology* of the Faculty of Sciences of the University of Lisbon

Ao meu Pai

Acknowledgements

This thesis results from the collaboration of different institutions, groups and persons which cannot be forgotten. In this way, I have to thank to:

My supervisors, Prof. António Portugal and Prof. Manuela Carolino, for accepted this orientation work and for all the availability, support and friendship during this road.

Dr. Maria Luísa Botelho for the kindles reception and acceptance in Technologies of Radiation Processes and Products Group (GTRPP), in Technological and Nuclear Institute (ITN), for all the personal development opportunities that had provided to me and for teach me that gamma radiation is much more than Nuclear Catastrophes.

Sandra Cabo Verde for the tireless co-supervising work, for all the patience when “There are more unexpected than expected situations” and for teach me to “Go with the flow”.

All my GTRPP mates, not only for the indisputable scientific availability and help, but also for the complicity and union at lab and for all the friendship.

Nuno Mesquita for the collaboration, help and availability in the Center for Functional Ecology laboratory in Coimbra.

Finally, to the FCT, for founding the scientific project PTDC/HAH/65262/2006.

If, on the one hand, this work could not be accomplished without a multidisciplinary scientific support, every person needs to have their own “roots and pillars” of support. I can never forget to thank to my “foundations”, without which, surely, I could not be succeed:

My Mother, for the strength she is.

Mário, to be my safe haven in stormy days.

João Ferreira, for the unconditional ability to make me laugh and think about different things.

To all my family: Brother, Sister in law, Nieces, Uncles, Cousins and Grandparents for support, union, care and help.

To all my friends, especially to João Carvalho, Inês Martins, Ricardo Fernandes and Inês Brito for always be there.

I also thanks to:

Filipa Xavier, not only for the long friendship but also for the bed in *Rás* during my stays in Coimbra and to Mrs. Olinda Brandão for the stay in the last work week in Coimbra.

For All (and in Portuguese), Um Grande Bem-Haja

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Communications in Scientific Meetings

"Behind the Air we Breathe: Characterization of the Microbial Population from the Indoor Air of the Archive of the University of Coimbra". Nunes I., Mesquita N., Videira S., Cabo Verde S., Carolino M. M., Portugal A., Botelho M. L.. Poster presented at the *XXXVI Jornadas Portuguesas de Genética*, 2011; Coimbra – Portugal.

"Fungal growth and inactivation patterns in parchment documents from the Archive of the University of Coimbra". Nunes I., Mesquita N., Videira S., Cabo Verde S., Carolino M. M., Portugal A., Botelho M. L.. Poster presented at the *4th FEMS meeting*, 2011; Geneva - Switzerland

Resumo

A problemática da biodeterioração nos diferentes tipos de obras de arte tem vindo a tornar-se um dos pontos centrais nas áreas da conservação e restauro. Entende-se por biodeterioração, a degradação causada nos materiais por acção directa de organismos vivos ou por consequência de produtos das suas actividades vitais. Entre os materiais mais afectados encontramos o património escrito devido à diversidade de materiais orgânicos com que é constituído. A evolução dos suportes de escrita ao longo da História (e.g. Papiro, Pergaminho, Papel de fibras de trapo e Papel com origem na madeira), levou à presença de diferentes biopolímeros, como a celulose, a hemicelulose, o colagénio ou a elastina nas obras mais diversas e preciosas para o Conhecimento e Cultura das nossas Sociedades. Sendo estes polímeros descritos como substratos nutricionais para algumas formas de vida, tais como bactérias, fungos e insectos, a colonização/contaminação e consequente degradação destes documentos torna-se um passo simples, caso não sejam tomadas as medidas adequadas.

Os fungos são biocontaminantes problemáticos uma vez que, possuem uma morfofisiologia que lhes permite boa penetração em diferentes substratos (hifas), bem como a capacidade de utilizar polímeros complexos como fonte nutricional (e. g. produção de enzimas hidrolíticas que lhes permite degradar moléculas complexas e usar a absorção como modo de nutrição).

Nos dias de hoje, ciências como a conservação e restauro têm que ultrapassar as barreiras da revitalização pós-degradação e actuar na prevenção da contaminação (pré-degradação) sendo imperativo a detecção e identificação de contaminantes bem como de possíveis fontes e vectores de contaminação.

Neste sentido, diversas medidas preventivas têm sido testadas em arquivos e bibliotecas. No entanto, a maioria apresenta “contra-indicações” tais como toxicidade para os trabalhadores e utilizadores dos diferentes espaços (ex. uso de pesticidas ou atmosferas controladas) ou a degradação dos materiais (ex. tratamentos a baixa e a alta temperatura).

Tendo em conta as suas características (alta capacidade de penetração e poder letal sobre os biocontaminantes) bem como o balanço das vantagens vs. desvantagens da sua utilização, a radiação gama aparenta ser um tratamento alternativo. O seu uso como agente descontaminante em materiais de arquivos e bibliotecas tem sido testado e descrito desde o início dos anos 60, sendo que baixas doses (<10 kGy) estão descritas como suficientes para descontaminar os materiais não causando danos nas suas características. A maior parte dos estudos têm sido efectuados em documentos e livros cujo principal constituinte é o papel.

O pergaminho foi inventado no século II antes de Cristo numa cidade Grega de nome *Pergamo*. A sua origem animal (pele de ovelha, vaca ou cabra) torna-o um material

complexo cujas características estruturais são extremamente variáveis inter e intra documento, tanto devido ao processo do seu fabrico como à diferente origem animal ou às diferentes condições ambientais a que é exposto ao longo do tempo. Devido à sua resistência, foi o principal suporte de escrita durante a Idade Média tendo sido muito usado por monges copistas e em documentos religiosos. É assim, um importante ancestral do Papel que nos permite conhecer melhor o passado de diferentes sociedades. Apesar da sua importância cultural e científica bem como da sua beleza, o Pergaminho nem sempre é tratado com o devido respeito sendo cada vez mais necessário entender a sua estrutura intrínseca para melhor desenvolver medidas de conservação e protecção.

Mergulhando nesta problemática o Arquivo da Universidade de Coimbra (AUC), responsável pela preservação do património cultural da Universidade e do Distrito de Coimbra, tem-se envolvido activamente em diferentes projectos científicos no sentido de encontrar soluções para os problemas de biodeterioração e de conservação que tem identificado no seu espólio. Esta tese enquadra-se num desses projectos (*Mycoarchive*) constituindo uma parceria entre o Instituto Tecnológico e Nuclear (ITN), a Faculdade de Ciências e Tecnologia da Universidade de Coimbra e o próprio AUC. Sendo assim, os objectivos do trabalho desenvolvido nesta dissertação foram i) Caracterizar a comunidade microbiana do ar interior do AUC, identificando possíveis factores de contaminação cruzada com os documentos contidos no espaço; ii) Caracterizar a contaminação natural de pergaminhos do AUC; iii) Estudar os padrões de inactivação por radiação gama da população microbiana dos pergaminhos a fim de propor uma dose mínima ($D_{min.}$), para uso futuro desta tecnologia como tratamento de descontaminação; iv) Determinar potenciais alterações induzidas pela radiação gama nas propriedades físicas do pergaminho a fim de estimar uma dose máxima ($D_{máx.}$) à qual o material pode ser exposto e v) Analisar alterações nos perfis genéticos provocadas pela exposição à radiação gama de isolados fúngicos do ar e de pergaminho do AUC, usando técnicas de PCR *fingerprinting*.

Assim sendo, o primeiro artigo contido nesta tese descreve os trabalhos efectuados no sentido de caracterizar a comunidade microbiana do ar do interior do AUC. Os resultados apontam para uma baixa contaminação microbiana (<200 CFU/m^3), sendo os tipos morfológicos mais frequentes os cocos gram positivos, catalase positivos e os fungos filamentosos.

No sentido de identificar correlações entre a comunidade microbiana do ar interior do AUC e a contaminação de pergaminhos presentes no mesmo espaço, validou-se uma metodologia de determinação da carga microbiana de pergaminho. Os resultados apontam para uma baixa contaminação microbiana (<10^2 CFU/cm^2) sendo esta essencialmente constituída pelos mesmos tipos morfológicos anteriormente

encontrados no ar interior do AUC. No entanto, a identificação das espécies fúngicas isoladas destes dois ambientes, não permitiu avaliar o papel do ar como vector de contaminação de forma conclusiva. A aplicação da radiação gama como tratamento de descontaminação deste material foi igualmente avaliada. Os resultados demonstraram que a população microbiana deste tipo de documentos não apresenta uma cinética de inactivação exponencial, porém foi obtida uma eficiência de inactivação maior que 99% a partir dos 5 kGy. Assim, propõe-se uma dose de 5 kGy como dose mínima ($D_{min.}$) de radiação gama a aplicar como tratamento de descontaminação do pergaminho.

Num tratamento de esterilização ou de descontaminação há que ter em conta, não só a inactivação dos contaminantes mas também os efeitos do agente esterilizante/descontaminante no material. Em arte antiga, o problema agrava-se uma vez que estamos a falar de obras únicas e irreprodutíveis. O terceiro artigo desta tese pretende, deste modo, averiguar os efeitos da radiação gama na textura e cor de pergaminhos, de forma a determinar uma dose máxima para os tratamentos de descontaminação deste material. Os resultados indicam que, até aos 30 kGy parece não existir qualquer efeito da radiação gama nas características estruturais do pergaminho.

A radiação gama é um conhecido agente mutagénico estando descrito que os principais efeitos nos sobreviventes dos processos de irradiação são deleções no material genético. O quarto artigo contido nesta dissertação aborda o tema da alteração genética provocada pela radiação gama. Foram obtidos perfis genéticos de quatro isolados fúngicos antes e após exposição a várias doses de radiação gama. Dois dos isolados pertencem à população microbiana do ar interior do AUC (*Penicillium griseofluvum* e *Neosartorya fumigata*) e os restantes às amostras de pergaminho (*Cladosporium* sp. e *Epicoccum nigrum*). A técnica de PCR *fingerprinting* mostrou ser adequada na detecção de alterações genéticas induzidas por baixas doses de radiação gama (2 kGy) em fungos, sendo ainda possível relacionar a resistência descrita para cada um dos géneros fúngicos com as alterações observadas. No entanto, os resultados obtidos não apresentam um padrão que relacione as alterações genéticas detectadas e o aumento da dose de radiação.

Em conclusão, os resultados apontam para a aplicabilidade de doses de radiação gama entre os 5 e os 10 kGy, como tratamento de descontaminação de materiais de Arquivo.

Palavras-chave:

Biodeterioração; Descontaminação; Pergaminho; Inactivação por radiação gama; PCR *fingerprinting*

Abstract

Archive and library materials are composed by complex organic materials which could be metabolized by some living beings, turning the biodeterioration into one concerning issue in conservation and restoration areas.

The Archive of the University of Coimbra (AUC) fight to find solutions to the biodeterioration problems that had been detected.

This thesis objectives were: i) Characterize the AUC indoor air microbial population in order to identify its role in documents contamination; ii) Characterize the AUC parchments natural contamination; iii) Study the parchment bioburden gamma radiation inactivation patterns in order to propose a minimal dose ($D_{min.}$); iv) Evaluate the gamma radiation effects in parchment physic characteristics in order to estimate a maximum gamma radiation dose ($D_{max.}$) and v) Estimate the genetic profile alterations induced by gamma radiation in AUC air and parchment fungal isolates, using PCR *fingerprinting*.

AUC indoor air microbiota was characterized and results point out to a low microbial contamination (<200 CFU/m^3) being the most frequent morphological types the gram positive, catalase positive cocci and the filamentous fungi. A parchment bioburden assessment methodology was evaluated and results indicated a low bioburden (<10^2 CFU/cm^2), mainly constituted by the same morphological types found in AUC indoor air. However, molecular identification of air and parchment fungal species was not able to correlate the air as a contamination vector. Gamma radiation was evaluated as parchment decontamination treatment using microbial inactivation studies. Results indicated that parchment microbial population did not follow an exponential inactivation kinetics, however a 99% inactivation efficiency was achieved for 5 kGy. This dose is proposed as D_{min} for parchment decontamination treatment and doses up to 30 kGy seems to have no effects on parchment texture and colour.

PCR *fingerprinting* techniques were used to detect gamma radiation effects on AUC fungal isolates genetic profiles. No patterns were identified to correlate the observed genetic alterations and gamma radiation dose. Although, differences in gamma radiation resistance were detected for the different isolates.

Results indicate a 5-10 kGy range as effective for Archive materials decontamination.

Keywords:

Biodeterioration; Decontamination; Parchment; Gamma Radiation Inactivation; PCR *fingerprinting*

Introduction

1. Written Cultural Heritage: Degradation and Conservation

Art is an important part of our cultural heritage. In a simple way, art is a documentation technique that could report simple quotidian actions or central changes in our society, humanity and world. Since the early beginning of humankind, man wants to know what happened before his arrival. The increasing interest in techniques to improve artefacts preservation, conservation and decontamination emerged from this demand. Unfortunately, despite its historical and cultural interests, these artefacts are not always treated with the due respect and are exposured to different chemical (e.g. chemical reactions with natural or exterior causes), physical (e.g. ripping), biological (e.g. fungi, molds, yeasts, bacteria, algae and insects) and environmental (e.g. humidity, temperature variation) factors which leads to their degradation (Maggi *et al*, 2000; Katusin-Razem *et al*, 2009; Guiamet *et al*, 2011).

Biodeterioration is one of the most actual issues in conservation and restoration areas. It is itself, a natural process resulting from the physiological organisms activities such as nutrition (e.g. hydrolytic enzymes production), secretion (e.g. toxic secondary metabolites) or substrate aggregation (e.g. fungal hyphae penetration). Biodeterioration origins are related to the biological contaminants type and to the environmental conditions to which they are exposured. Different contamination vectors such as air, water (rain) or wind were related with artefacts biocontamination (Gallo, 1993; Górny *et al*, 2002; Stryjakowska-Sekulska *et al*, 2007; Borrego *et al*, 2008; Borrego *et al*, 2010; Guiamet *et al*, 2011).

Between the different artefacts types, some of the most biodegradable are written materials. Our written culture heritage is vast and diverse. Different materials were used for writing along time and each culture and epoch has their own written traditions. Papyrus is a natural vegetal material consisting in *Cyperus papyrus* pith. It was the first written material. Created in Egypt since the third millennium Before Christ, it was used until the 2nd century BC (Bar-Ilan, 1995, <http://faculty.biu.ac.il/~barilm/papyrus.html>; Bernhardt, 2000, <http://papyri.tripod.com/texts/papyrus.html>; Nicholson, 2006) when a new material was created: the parchment (Cappitelli and Sorlini, 2005). Parchment replaced almost completely papyrus in Third/Fourth century AD. This new material is obtained from animal (e.g. goat, cow or sheep) skin that is reduced to its dermis by physical and chemical treatments (Curci, 2003, <http://www.sca.org.au/scribe/articles/parchment.htm>; Briscoe, <http://www.ehow.com/facts_5272725_history-parchment-paper.html>). Due to its animal origin, parchment is rich in complex biopolymers such as collagen and elastin which turns it into a good substrate for different life forms (Tiano, 2002; Cappitelli and Sorlini, 2005). Parchment name had derived from the Greek city of Pergamum where its creation had been described (Cappitelli and Sorlini, 2005). Because of its resistance and fashionable

potentialities Christian writers had gradually adopted it as main written support until paper's introduction in occident through the "Silk Rote". In the Middle Age parchment was the principal written support in Europe. It only was slowly substituted in XIV century with the Johannes Gutenberg discover: the printer, which brought the paper to the first plan of the written materials (Flieder and Duchein, 1993; Ferreira, 2010).

2. The Archive of the University of Coimbra (AUC)

The Archive of the University of Coimbra (AUC) was created in 1290 by the King D. Dinis and consists in the repository of all the produced and received University documentation and of all the Coimbra district archive documents. Its mission was focuses in three main objectives: i) The preservation, enrichment and treatment of its documental and bibliographic patrimony; ii) The education and investigation support and iii) The development of autonomous editorial activities (<http://www.uc.pt/en/auc>). Due to their first main objective, AUC is continuously searching new techniques to preserve their written materials participating in different scientific projects such as *Mycoarchive* (PTDC/HAH/65262/2006).

3. Fighting Biodeterioration

To avoid contamination and consequently biodeterioration, nowadays, conservation and restoration areas exceed the direct intervention in artefacts associating preventive technics. Many methodologies had been applied to eliminate and prevent biocontamination in art, especially in archives and libraries (Tiano, 2002; Teygeler *et al*, 2001). Two main treatment types had been developed: chemical and non-chemical treatments.

3.1. Chemical Treatments

The chemical treatments consist of exposing the contaminated material to fumigants/pesticides in order to reduce plagues invasion, specially the insect ones. Among these products, there are: Ethylene Oxide (ETO), Methyl Bromide (also known as Bromo-Gas, Celfume, Embofume, MB, MeBr, Methogas, Profume, Terr-o-gas and Zytox) and Sulphuryl Fluoride (commercially known by Vikane). For MB, there is no evidence of archive/library material degradation. The ETO seems to induce some chemical and physical alterations in paper, parchment and leather. The Vikane degrades resins, some pigments and metals. Nevertheless its preservation potentiality, for all these fumigants the toxicity levels could be high and a prolonged exposure can cause health problems such as nausea, headaches or even respiratory problems and cancer. (Bond, 1998)

3.2. Non-chemical Treatments

The non-chemical treatments are obtained by changing the indoor atmosphere, the environmental temperature, or through the application of radiation such as microwaves or gamma rays. Controlled atmospheres, like low oxygen (Kaplan and Schulte, 1996) and high carbon dioxide (or other inert gases) seems to be effective against insects and less efficient against microorganisms (Magaudda, 2004). This treatment had been used in other indoor environments beyond archives such as fruit and vegetable storages. However, some measures implied in these treatments could be toxic to people (Saltveit, 2003), including archive and library's staffs. Freezing and other cold treatments have to deal with humidity problems in treatment chambers and, as heating treatments, it cannot be used in many materials (Ketcham, 1984). High humidity levels can damage the materials, improve contamination probabilities or act as contamination vector. Exposure to high temperatures has further problems like oxidation and aging, which could drastically alter the documents characteristics (Teygeler *et al*, 2001).

The use of microwaves was reported (Brezner and Luner, 1989), however it was detected a reduced penetration capacity being ineffective for thick books conservation (Teygeler *et al*, 2001). Gamma radiation seems to be an alternative conservation treatment for some archive materials (Sinco, 2000).

3.3. Gamma Radiation

The use of gamma radiation for artefacts, such as archive and library materials, decontamination has been studied since the early 60's (Sinco, 2000; Magaudda, 2004; Katusin-Razem *et al*, 2009).

Gamma rays consist in photons released from nuclear spontaneous decay of radioactive materials such as cobalt-60 and cesium-137. They have high penetration capacity and could interact with matter changing that by energy deposition, similarly to thermal and chemical processes (IAEA, 1973). The effects in Non-Biological and Biological materials are different and depend on their specific constitution.

3.3.1. Effects in Non-biological Materials

Non-biological materials could be differently affected by the direct effect of gamma radiation and by the indirect effect of free radicals release during irradiation. Gorna and Gogolewski (2003) had reported that aromatic polymers are more resistant to gamma radiation than aliphatic ones. In other hand, impurities and additives could enhance polymer degradation and/or chain crosslinking. The main polymer changes induced by gamma

radiation are: main-chain scission (leading to mechanical properties loss), crosslinking, end-linking (leading to initial increase in tensile strength) and production of volatile degradation products (Goldman *et al*, 1996; Gorna and Gogolewski, 2003; Maxwell *et al*, 2003). Grant *et al* (1973) had described yellowing, tensile strength decrease, crystallinity loss, hydrogen bounds break and chain scission as gamma irradiation effects in collagen fibres.

Due to the reported gamma radiation effects in non-biological materials, different technics were developed in order to understand the gamma radiation impact in different material characteristics such as texture and colour. Material texture was studied since the late 19th/ early 20th centuries (Bourne, 1978; Szczesniak, 2002). Starting with sensorial analysis to understand the consumers' interests in food area, nowadays, texture is an important physical parameter to determine material structural alterations promoted by gamma radiation treatments (IAEA, 2001; Pang *et al*, 2007; Araújo *et al*, 2007; Landfeld *et al*, 2010; Rosidah *et al*, 2010). Texture measurement technics are based on texture analysers which could evaluate different sub-parameters such as hardness, springiness, brittleness, adhesiveness and cohesiveness (Wilkinson *et al*, 2000; Bourne, 2002; Szczesniak, 2002).

Colour is other important physical parameter to understand material alterations resulting from different decontamination procedures. The majority of the studies were performed in food area using electronic colorimeters (Landfeld *et al*, 2010; Pipek *et al*, 2005; Wagenaar and Snijders, 2004; Zaied *et al*, 2007). Nevertheless, Araújo *et al* (2007) noticed that increasing gamma radiation doses resulted in an increase of rice paper sheet yellowness.

The maximum gamma radiation dose that could be applied to a material without visible alterations in its structural characteristics is known as Maximum Dose ($D_{max.}$)

3.3.2. Effects in Biological Materials

Interaction between the released photons and molecules can cause direct or indirect damages in biological materials (IAEA, 1973). Direct effects results from biomolecules ionization (atoms' electrons ejection with consequent photon energy absorption) (Holm and Berry, 1970) leading to cell miss-function and death. In spore and spore-forming microorganisms' irradiation, these are the predominant gamma radiation effects (Block, 1983).

Vegetative cells are mainly constituted by water (since 80%), a molecule that could interact with gamma rays originating free radicals such as hydrogen atoms (H_x), hydroxyl radicals (OH_x) or solvated electrons (e_s^-). These reactive intermediates could diffuse through the cell and interact with the biomolecules causing the gamma radiation indirect effects (Von Sonntag, 1992). Furthermore, oxygen (often present during irradiation processes) can react with free radicals converting them into the corresponding peroxyl (e.g. H_2O_2), a toxic substance to the cell (Von Sonntag, 1992).

Although the chemical bonds breakage, the molecules oxidation or the decreasing enzyme activity, the critical gamma radiation biological target is the deoxyribonucleic acid (DNA), which could present single-strand breaks in the phosphodiester backbone or lesser extend (5-10%) double strands breaks (Moseley, 1989).

Early in history of radiation biology, studies had been performed in order to understand the relation between the number of inactivated microorganisms and the received gamma radiation dose. In 1924, Crowther had presented the original target theory which reports an exponential loss of biological activity with ionizing radiation. Radiation survival follows, in the most cases, the same exponential kinetics allowing the use of D_{10}-value to characterize microorganisms' radiation sensitivity. D_{10} represent the dose required to inactive 90% of a population or the irradiation dose needed to produce a 10-fold reduction in the population. Mathematically, the survival curve can be represented by: $logN = -{}^{1}/_{D_{10}}\, D + logN_0$, were N_0 represents the initial organisms number, N consists in the number of microorganisms surviving the radiation dose D and D_{10} is the decimal reduction dose. D_{10} can be achieved by calculating the inverse of the obtained regression line slope. However, inactivation curves do not always shows a straight line (exponential curves). Curvilinear survival plots are also often found and can present an initial shoulder (e.g. convex and sigmoidal curves) or an ending tail (e.g. concave curve). The shoulder effect could be explained by multiple targets and/or certain repair processes (Davies *et al*, 1973). It is observed at low doses and an exponential phase is achieved for higher doses. The ending tails could be related to the microbial population gamma radiation resistance heterogeneity, being the less resistant cells the first to be inactivated leaving the more resistant to tail out (Cerf and Metro, 1977).

Microorganisms have different gamma radiation resistances both due to their intrinsic biological characteristics and to extrinsic irradiation conditions. The resistance can differ from species to species and even between strains from the same species. Spores have been described as high gamma radiation tolerant structures (Morgan and Reed, 1954; Blank and Corrigan, 1995; Nicholson *et al*, 2000; Rainey *et al*, 2005). *Dinococcus radiodurans* was described as the most radiotolerant bacteria (Battista, 1997; Battista *et al*, 1999; Lipton *et al*, 2002). It has been reported that some fungi can resist to doses higher than 5 kGy (Dadachova and Casadevall, 2008).

In other hand, gamma radiation resistance can also be related to irradiation parameters such as temperature, gaseous environment (e.g. oxygen presence), water activity, substrate pH and components and dose rate (Block, 1983).

Considering its biological effects, gamma radiation has been described as a powerful sterilization and decontamination technique, namely in medical (White *et al*, 1994; Stafford *et al*, 2003) and food areas (Farkas, 1998; Bidawid *et al*, 2000; Adamo

et al, 2004; Farkas, 2006; Ferreira-Castro *et al*, 2007; Zaied *et al*, 2007). However, this still faces problems, especially due to the people's opinion and fears related to the biological gamma radiation effects resulting from the direct human body exposure to high doses. In other hand, a gamma radiation decontamination or sterilization treatment did not promote materials radioactivity and, consequently, resulting products/materials are innocuous to consumers.

In sterilization and decontamination assessment studies two sides have to be addressed: the inactivation of the biocontaminants ($D_{min.}$) to a level that assures the objective of the treatment (e.g. decontamination, sterilization) and the potential material degradation ($D_{max.}$), having in account all the vantages and advantages in a premium-price game.

4. Decontamination Target: Fungi

In this study, the targets were microorganisms, namely the fungi. Fungi are eukaryotic sessile (mainly) organisms which had aroused the human interest since the early beginning of its discovery, both for positive and negative reasons. Initially associated to the Plant (until 1969), nowadays they have their own specific kingdom, The Fungi (Whittaker classification), due to their unique morph-physiological characteristics such as chitin cell wall and specific heterotrophic nutrition type (absorption) (Whittaker, 1969). Fungal diversity is huge: 5 million fungal species were estimated to exist in the Nature (Blackwell, 2011) but only about 100 000 were identified until now (Kirk *et al*, 2008). Fungi are very variable organisms turning possible to find them both at microscopic (molds and yeasts) and at macroscopic (mushrooms) levels, and in different extreme environmental conditions such as high and low temperatures (deserts and ocean deep sediments), high salt concentrations or high UV and cosmic radiation (aeronautic experiments).

Sharing the enzymatic biopolymer degradation capacity with animals, fungi use absorption as nutrition achievement mode. Fungal enzymes production potentiality has been explored by man since the beginning of our societies, turning the food fermentation procedures usual in all cultures in the world (e.g. bread, wine, beer, chucrute) (Caplice and Fitzgerald, 1999). The penicillin discover in 1928 by Fleming open minds to new possibilities and, nowadays, fungi are used and improved in order to obtain different substances such as antibiotics, specific enzymes (e.g. cellulase), pesticides and antifungals in industrial scale (Arnstein and Cook, 1947; Youssef *et al*, 1978; Krings and Berger, 1998; Haggag and Mohamed, 2002; Etschmann and Schrader, 2006). However neither all fungal produced substances are innocuous to human. Fungal toxins (mycotoxins) could produce serious health consequences. As example, the aflatoxin B1 is one of the most carcinogenic known substances (Aguilar *et al*, 1993; Mcglynn *et al*, 1995; Lunn *et al*, 1999; Smela *et al*, 2001).

Behind all the economical interesting characteristics, enzymes production turns fungi in high specified degradation organisms. Different material types, especially organic ones, are colonized and degraded by fungi (Botelho *et al*, 1988; Szczepanowska and Cavaliere, 2000; Abrusci *et al* 2005; Michaelsen *et al*, 2006; Silva *et al*, 2006). These biodeterioration potential leads to the necessity of eliminate these microorganisms from materials, especially from art (decontamination). However, this seems to be a "hard task" considering the reported fungal resistance to the different lethal agents (Block, 1983).

5. Objectives and Outline of the Thesis

This dissertation had Parchment documents as study subject, and was applied the following methodology: i) Study the impact of AUC indoor air in written documents biocontamination (particularly in parchment documents); ii) Characterize the typical parchment bioburden and outline its gamma radiation inactivation pattern ($D_{min.}$ assessment); iii) Evaluate the gamma radiation effects in parchment structure ($D_{max.}$ assessment) and iv) Estimate fungal genetic patterns variations promoted by gamma radiation exposure. In short, it was intended to determine possible contamination factors/vectors in the Archive of the University of Coimbra, and to evaluate the potentiality of gamma radiation as a decontamination treatment of parchment documents. In this way, this dissertation is divided in four chapters each one corresponding to a scientific paper. The first one consists in an AUC indoor air analysis. The second one presents the results from the parchment bioburden characterization and gamma radiation inactivation pattern. The third consists in the analysis of the gamma radiation effects in parchment structure characteristics and the last one documents the gamma radiation genetic effects in four different fungi isolated from the AUC indoor air and from parchment samples.

Behind the Air we Breathe: Characterization of indoor air bioburden of the Archive of the University of Coimbra

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Keywords

Indoor Air; Airborne population; Diversity

Abstract

Aims: This study aimed to characterize the indoor air microbial population of the Archive of the University of Coimbra (AUC), in order to identify control/preventive measures for the biodeterioration of written heritage. Airborne microbial and fungal diversity were assessed and compared with previous samplings.

Methods and Results: Indoor air samples were collected in AUC using an air sampler. Air bioburden was determined by conventional culture methods and isolates were typified using morphological techniques. Fungal isolates were further identified by ITS-DNA sequencing. Results indicated a low microbial air contamination (107±12 CFU/m^3), particularly for fungal propagules (6±1 CFU/m^3). Among fungal isolates, *Penicillium* was the most frequent genus, and *P. griseofluvum* was the predominant species. Simpson diversity index (1-D) was applied to phenotypic and genotypic typification results.

Conclusions: The microbial phenotypic diversity (Simpson Index) varied from 0.4 to 0.8. Regarding fungal species, it was obtained a diversity greater than 0.6. Results suggest the influence of climatic conditions in the AUC microbial indoor air populations.

Significance and Impact of Study: Understand the impact of airborne microbial populations in the contamination of documents, in order to optimize their conservation and disinfection procedures.

Introduction

Since the beginning of civilization that humankind looks to leave its own mark in the world. In fact, technology, knowledge and History were born out of this desire. However we wouldn't know much about our past if there were no available records. That is a reason why documentation and techniques such as restoration and conservation are so important in our society (Bonacker, 2011, <http://articles.sfgate.com/2011-06-26/opinion/29701737_1_historic-preservation-commission-art-subject-diversity>).

Written documents are direct and indirect sources of information of our past. If the words can tell us the facts, the materials used to support them (such as parchment, papyrus or paper) can show us behaviors, traditions or even the environment of the past epochs (Ebron, <http://nationalhumanitiescenter.org/tserve/freedom/1609-1865/essays/aaculture.htm>). However, how could these documents resist to the time effects?

Assuming its role on the conservation of Coimbra's cultural heritage, the Archive of the University of Coimbra (AUC) is responsible for the preservation and divulgation of a vast written patrimony in different forms (books, parchments, papers, etc). On a daily basis, the AUC fights against our history deterioration (<http://www.uc.pt/en/auc>).

Our cultural heritage is mainly made of natural organic materials that are influenced and modified, not only by chemical and physical factors, but also by biological ones. Organic compounds in these materials (such as cellulose, collagen, keratin, and elastin) turn them into an excellent biological substrate (Tiano, 2002).

Biodeterioration is itself a natural process resulting of organisms vital activities, and one of the main problems for the documents conservation (Guiamet *et al*, 2011). Its direct relation to climatic fluctuations (e.g. humidity, temperature) increases the interest in study and control the environmental conditions to which the materials are exposed to, specially the indoor ones (Gallo, 1993; Górny *et al*, 2002; Stryjakowska-Sekulska *et al*, 2007; Borrego *et al*, 2008; Borrego *et al*, 2010; Guiamet *et al*, 2011). Contamination sources, such as air or water, also contributed to the materials biodeterioration. These sources appear with different forms and could be related to climatic conditions (e.g. rain or sun) and different propagation vectors such as insects (Trovão Lima, 2011), flows or staff/visitors movements (Borrego *et al*, 2010).

Air, as water, is a source of life on Earth and the main component of our atmosphere. It is constituted by a large number of different particles with different sizes, shapes and origins (Guiamet *et al*, 2011). However, in spite of its benefits, there is a world of microbial life that can be a source of contamination, disease and material biodeterioration.

Fungi are especially problematic biocontaminants due to its morphological (e.g. hyphae penetration capacity) and physiological characteristics (e.g. enzymes production and secretion) (Szczepanowska and Cavaliere, 2000; Sterflinger, 2010). These

microorganisms could be introduced in documents environment by different vectors from different origins such as air. Comparative analysis between air fungal isolates of archives and libraries and documents fungal isolates indicated the existence of a correlation (Nugari *et al*, 1993; Borrego *et al*, 2010; Niesler *et al*, 2010).

Nowadays, documents preservation/conservation consists, beyond the direct intervention on degraded materials, on contamination prevention. For that, it is crucial to identify possible vectors and transmission routes. The effect of climatic fluctuations and, consequently of seasons on microbial population can be a significant issue. The monitorization of indoor air bacterial and fungal communities could help on the prevention of documents biodeterioration.

This study aims to characterize the AUC indoor air microbial population, in order to identify the fungal airborne typical propagules and to evaluate the air microbial population impact on documents biodeterioration.

Material and Methods

Indoor Air Sampling Plan

AUC indoor air Samples (0.5 m^3; 100L/min) were collected by an air sampler (MAS-100, MERCK) directly through Triptic Soy Agar (TSA) petri dishes. Four different AUC floors (II, III, IV and V) where sampled, and three samples were collected by floor (n=3/floor; total n=12). Samples were collected in April 2010.

Air Bioburden Characterization

Petri dishes were incubated at 30 ± 2 ºC during 7 days. Colony forming units per m^3 (CFU/m^3) were estimated for each AUC floor.

AUC indoor air isolates were macroscopically characterized (e.g. pigmentation, texture, shape) in order to select a representative population for morphological (e.g. Bacteria - cellular morphology, presence/absence of endospores; Fungi – reproductive and vegetative structures morphology) and biochemical characterization (e.g. Bacteria - gram staining, catalase and oxidase activities). For morphological characterization, bacteria were isolated in TSA medium and fungi were isolated in MEA (Malt Extract Agar) medium. Isolates were divided in eleven morphological types based on the different characteristics detected. The definition of the eleven morphological types was based on the Bergey's Manual of Determinative Bacteriology (Holt *et al*, 1994). The frequency of each morphological type was calculated based on the number of isolates and its characterization.

Fungal Molecular Identification

Fungal isolates' total genomic DNA was extracted using the extractor ABI Prism 6100 total genomic DNA extraction protocol. The total ITS sequence of each isolate was amplified by PCR using fungal specific primers (Manter and Vivanco, 2007). After purification, amplification products were sequenced and compared with NCBI Blast – GenBank database to identify fungal isolates at species level. Isolates identification were also confirmed by morphological analysis.

Diversity Assessment

Simpson diversity index (1-D) (Simpson, 1949 cited by Dvoretsky and Dvoretsky, 2011; Magurran, 1988) was applied to morphological and fungal isolates' identification results.

$$1\text{-}D = 1 - \sum (n/N)^2$$

n – Number of colonies per morphological type/fungal species (or genus)

N – Total colonies number in the population

Results

Air Bioburden Characterization

The AUC indoor air average bioburden values per floor are shown in table 1. Floors II and IV presented the highest bioburden (133±36 and 129±54 CFU/m^3, respectively) in comparison with floors III and V, although there were no significant differences ($P>0.05$) between AUC floors air bioburden average values. Fungal contamination was low for all the AUC floors ranging from 3 CFU/m^3 (floor II) to 11 CFU/m^3 (floor V), and also did not present significant differences ($P>0.05$) between floors.

Table 1 – Total and Fungal AUC indoor air average bioburden values (CFU/m^3) per floor and respective standard error.

Floor	CFU/m^3	
	Total	Fungal
II	133±36	3±1
III	83±17	5±2
IV	129±54	4±2
V	81±30	11±5
Total	107±12	6±1

Bioburden values point out to a homogenous AUC indoor air microbial population. Furthermore, the isolates were morphologically characterized to evaluate air contamination pattern and diversity.

Eight distinct morphological types were identified among the isolates (n=72) and their distribution was uniform in the four AUC floors, with exceptions for non-spore forming gram positive rods in floor IV and spore forming rods in floors IV and V (figure 1).

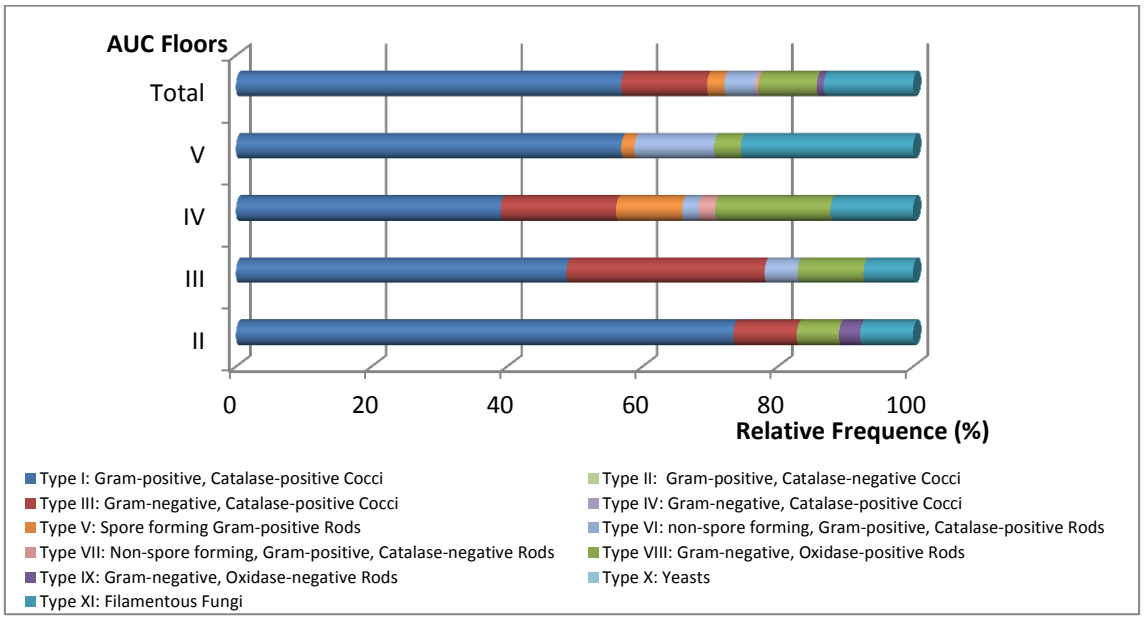

Figure 1 – Frequency (%) of morphological types of AUC's indoor air isolates

The most frequent morphological types in AUC air were the gram positive, catalase positive cocci (57%), filamentous fungi (16%) and gram negative, catalase positive cocci (13%).

Fungal Species Identification

The phenotypic and genotypic characterization of AUC indoor air fungal isolates (n=21) revealed the presence of eight different fungal genera (table 2).

Table 2 – Genus and Species identification of the 21 fungal isolates of the AUC's indoor air.

Floor	Relative Frequency (%)	Genus	Species	Similarity (%)
II	0.11	*Aspergillus*	*Aspergillus versicolor*	87.2
II	0.51	*Fusarium*	*Fusarium solani*	96.5
II	0.51	*Aspergillus*	*Aspergillus ochraceus*	87.5
II	0.51	*Fusarium*	*Fusarium proliferatum*	97.7
II	0.51	*Neosartorya*	*Neosartorya fumigata*	96.0
III	0.51	*Penicillium*	*Penicillium* sp.	93.4
III	0.51	*Alternaria*	*Alternaria* sp.	99.5
III	0.51	*Acremonium*	*Acremonium* sp.	82.9
IV	0.51	*Chaetomium*	*Chaetomium nigricolor*	82.5
IV	1.02	*Penicillium*	*Penicillium oxalicum*	94.2
IV	1.02	*Fusarium*	*Fusarium* sp.	95.9
V	1.02	*Penicillium*	*Penicillium griseofulvum*	99.1
V	0.51	*Penicillium*	*Penicillium griseofulvum*	99.1
V	0.51	*Paecilomyces*	*Paecilomyces lilacinus*	82.0
V	0.51	*Neosartorya*	*Neosartorya fumigata*	96.0
V	0.51	*Penicillium*	*Penicillium griseofulvum*	99.1
V	0.51	*Penicillium*	*Penicillium griseofulvum*	99.1
V	1.52	*Penicillium*	*Penicillium griseofulvum*	99.1
V	0.51	*Paecilomyces*	*Paecilomyces lilacinus*	82.0
V	0.51	*Chaetomium*	*Chaetomium globosum*	82.9
V	0.51	*Penicillium*	*Penicillium griseofulvum*	99.1

Penicillium was the most frequent genus (6.11%) with the higher representation on floor V (4.58%). The genera *Fusarium*, *Neosartorya*, *Chaetomium* and *Paecilomyces* were also isolated, although with lower frequencies (2.04% for the first one and 1.02% for the other three).

Species classification indicated the presence of 14 fungal species. *Penicillium griseofluvum* was the most frequent (5%) fungal species and was isolated from floor V. *Fusarium solani*, *Fusarium sp*., *Neosartorya fumigata*, *Penicullium oxalicum* and *Paecilomyces lilacinus* were identified with relative frequencies higher than 1%. The remaining AUC indoor air fungal species have a low representation with frequencies varying between 0.11% and 0.51%.

Diversity Evaluation

Diversity indexes such as Simpson Diversity Index are ecological measures/parameters to understand the diversity level in different systems. Its high potential comes from the parameters analyzed: beyond the number of types, Simpson Diversity Index (1-D) also considers their relative frequencies (Simpson, 1949 cited by Dvoretsky and Dvoretsky, 2011; Magurran, 1988).

The diversity of total microbiota varied between 0.4 and 0.8, based on the performed phenotypic characterization. The higher diversity for the total population was found in the floor IV (table 3).

Table 3 – Simpson diversity indexes of total and fungal microbial air communities in each AUC floor

Floor	Total Population	Fungal Population	
		Genera	Species
II	0.4	0.6	0.8
III	0.7	0.7	0.7
IV	0.8	0.6	0.6
V	0.6	0.5	0.5
TOTAL	0.6	0.7	0.8

For fungal isolates, the diversity varied between 0.5 and 0.8 for both, genera and species analysis. Results are identical for both genera and species diversity analysis, with exception for floor II where there were some species belonging to the same genus (e.g. *Aspergillus versicolor* and *Aspergillus ochraceus*; *Fusarium solani* and *Fusarium proliferatum*).

Microbial airborne monitorization: 2008 to 2010

The same air sampling procedure described in this study (2010) was performed previously in 2008 and 2009. The air bioburden values obtained in the three consecutive years were graphically compared (figure 2), in order to find a possible pattern. The 2009 sampling presented higher bioburden values (CFU/m^3) in both total and fungal indoor air populations. However, in all samplings, fungal contamination was

lower than < 50 CFU/m^3. Quantitatively, similarities were found between the 2008 and 2010 samplings (different months), while 2009 and 2010 (same month) air samples were distinct. These unexpected results can be possibly explained by local abnormal weather conditions that occurred during the sampling period.

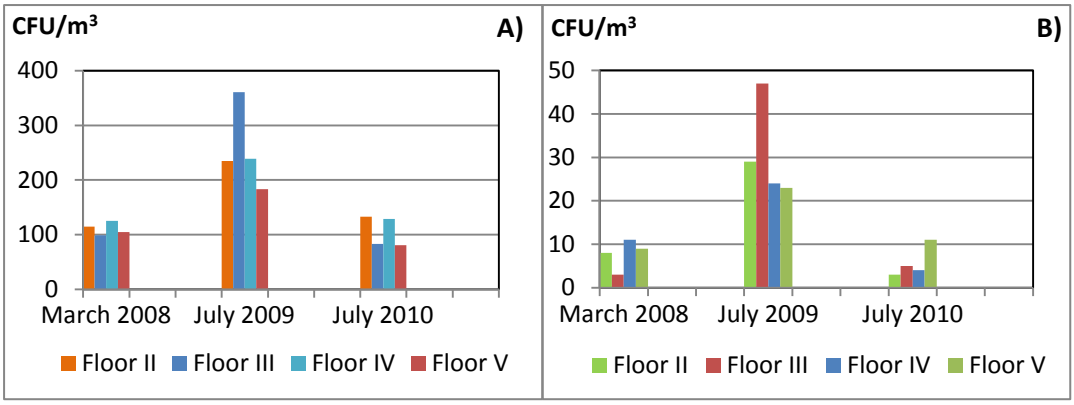

Figure 2 – AUC Indoor air bioburden (CFU/m^3) for the three consecutive years samplings (2008, 2009 and 2010): Total (A) and Fungal (B) microbial indoor air populations.

The same morphological types were found in the three samplings, although showing different relative frequencies (figure 3).

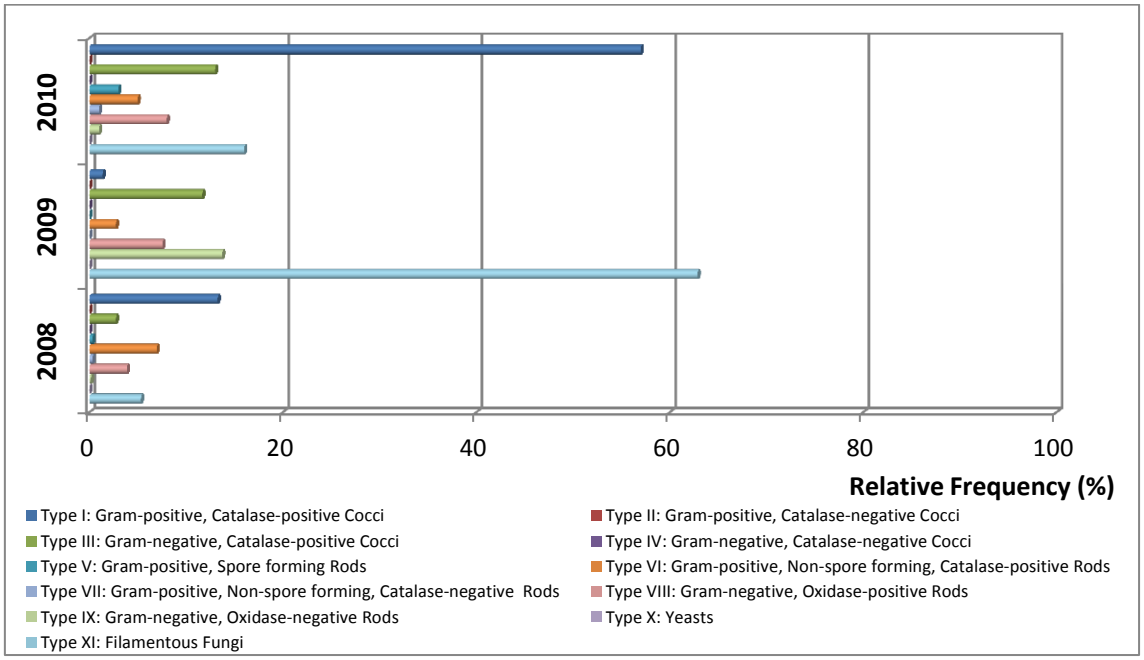

Figure 3 – Morphological types frequency for 2008-2010 AUC indoor air samplings.

Like quantitative results, the microbiota typification point out to a similarity between 2008 and 2010 samplings: i) the most frequent morphological type in these two years were the gram positive, catalase positive cocci (13% and 57%, respectively); ii) the absent morphological types in 2008 (types II, IV, V, VII, IX e X) were also absent, or emerged with very low frequencies, in 2010 (3% for type V e 1% for type IX). In contrast, for 2009 sampling the most frequent type was the filamentous fungi group (63%).

Despite these differences, the Simpson Diversity Index was similar among all the three samplings (table 4).

Table 4 –Simpson diversity indexes for total microbial AUC indoor air communities in 2008, 2009 and 2010

Year	2008	2009	2010
Simpson Diversity Index (1-D)	0.7	0.6	0.6

Discussion

AUC indoor air bioburden presented low values (< 200 CFU/m^3), particularly for fungal population (< 20 CFU/m^3). According to a National Technical Note (NT-SCE-02) that guides the methodology to audit Indoor Air Quality in buildings, the bacterial and fungal concentrations in AUC indoor air are below the critical limits (bacteria and fungi: 500 CFU/m^3). Moreover, the obtained results indicate a microbial conformity in indoor air according to international literature that establishes maximal bioburden values of 700 CFU/m^3 (Neto and Siqueira, 2000) and 1000 CFU/m^3 (Wonder Makers Environmental, Inc., 2001 and Eagle Industrial Hygiene Associates, 2004 both cited by Borrego *et al*, 2010). The achieved AUC indoor air bioburden values are also lower than cited results for similar environments (Stryjakowska-Sekulska *et al*, 2007; Borrego *et al*, 2008; Borrego *et al,* 2010; Guiamet *et al*, 2011; Karbowska-Berent *et al*, 2011). Temperature, ventilation and relative humidity control inside AUC seems to be effective. It has been reported that air movements caused by ventilation could avoid high microbial concentrations in the air (Vaillant, 1996 cited by Guiamet *et al*, 2011). Factors such as documents type (e.g. paper, parchment or papyrus), origin and age in each floor, as well as different floor height seems to have no influence in microbial floor air contamination, since no significant differences were found for air bioburden values between AUC floors.

The indoor air isolates characterization indicated the Gram positive cocci as most prevalent bacterial type in AUC. For fungi, the most frequent genus was *Penicillium* followed by *Fusarium*, *Neosartorya*, *Chaetomium* and *Paecilomyces*. The molecular identification of airborne fungal isolates detected the presence of potential pathogenic and toxicogenic fungi, such as *Fusarium* sp., *Aspergillus versicolor* or *Neosartorya fumigata*. In agreement with the guidelines of NT-SCE-02, the presence of these fungi could indicate a non-compliance environment, if fungal indoor air concentration is higher than exterior. Both bacterial and fungal communities were similar to previous reports for indoor environments (Borrego *et al,* 2008; Bogomolova and Kirtsideli, 2009; Borrego *et al*, 2010; Guiamet *et al*, 2011).

The total microbial population diversity (Simpson Index) varied from 0.4 (floor II) to 0.8 (floor IV), according to the performed morphological characterization. These

diversity differences between floors could be justified by diverse indoor environmental conditions (e.g. temperature fluctuations, humidity, height, present substrates or its different constitution, staff movements), which could lead to the development of different microbial communities according to its requirements and capabilities. It has been reported that indoor fungal contamination could be highly dependent of relative humidity and indoor bacteria contamination may be strongly affected by occupancy levels (Rajasekar and Balasubramanian, 2011). A recent study (Ponizovskaya *et al*, 2011) indicated that low relative humidity variations were required to alter the germination and growth of some fungi.

Results suggested the influence of climatic conditions in the AUC's microbial indoor air populations, namely high outdoor humidity levels (associated to rainy climatic conditions). Dryer conditions may potentiate lower bioburden values with predominance of bacteria. The presence of these communities can be influenced by short-term environmental conditions and not only by seasons (Ponizovskaya *et al*, 2011; Rajasekar and Balasubramanian, 2011).

AUC indoor air could be a potential documents contamination vector, considering its microbial constitution and its similarities to archive and library materials contamination described in literature (Szczepanowska and Cavaliere, 2000; Tiano 2002; Borrego *et al,* 2010; Sterflinger, 2010; Guiamet *et al*, 2011). Nevertheless, studies are being carried out to characterize AUC documents natural microbiota in order to establish contamination links.

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Bioburden Assessment and Gamma Radiation Inactivation Patterns in Parchment documents

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Keywords

Parchment; Fungi; D_{min}; Gamma Radiation; Conservation

Abstract

Aims: The aim of this study is to validate an appropriate methodology to characterize parchment bioburden, in order to evaluate the growth and inactivation patterns of parchment microbiota. As a final goal, a minimal dose (D_{min}) is proposed to be applied in parchment decontamination treatment by gamma radiation as an alternative conservation method.

Methods and Results: Two methodologies were evaluated to assess parchment bioburden: swab method (SM) and destructive method (DM). The recovery efficiency of each method was estimated by artificial contamination, using a *Cladosporium cladosporioides* spore suspension. Parchment microbiota was typified by morphological methods and ITS-DNA sequencing was used for fungal isolates identification. Parchment microbial inactivation pattern was evaluated using DM after exposure to different gamma radiation doses, and using *C. cladosporioides* as reference. Based on the applied methodology, parchment samples presented bioburden values $<5x10^3$ CFU/cm^2 for total microbiota, and <10 CFU/cm^2 for fungal propagules. The results indicated that parchment microbial population did not follow an exponential inactivation pattern.

Conclusions: A minimal gamma radiation dose ($D_{min.}$) of 5 kGy is proposed for parchment decontamination treatment.

Significance and Impact of Study: The establishment of a parchment minimal decontamination dose is essential for the application of gamma radiation as an alternative treatment method for this type of documents.

Introduction

Art, in its different forms such as paintings, sculptures or written documents, can fill human beauty and knowledge necessities. However, these ancient artefacts are not always treated with the due respect and, many times, they are exposed to physical, chemical and biological deterioration factors. Man forgot that behind the importance for our History, art is mainly made from degradable raw materials that are modified and destroyed according to their intrinsic characteristics and structure, and due to the effects of environmental conditions such as humidity and temperature. Raw materials, specially the organic ones, can be good substrates for insects, bacteria, fungi, molds and other life forms, making biodeterioration an increasing problem for art curators (Katusin-Razema *et al*, 2009; Guiamet *et al*, 2011). This leads to the necessity of developing new approaches in restoration, preservation, conservation and decontamination areas.

Microorganisms such as bacteria have been described as important archive documents biocontaminants and degrading agents. Bacteria due to their small size can penetrate between polymers fibres leading to strong substrate aggregation and colonization. Some bacteria can even accelerate the deterioration due to the excretion of specific substances, such as, cellulolytic enzymes or organic acids, which can reduce the pH, promoting new microorganisms attacks (Tiano, 2002). Fungi can play an important role in deterioration of cultural heritage (Sterflinger, 2010). Apart the problems caused by the production of some toxic and pigmented secondary metabolites, fungal biocontaminants are especially problematic due to their morphological characteristics (e.g. hyphae penetration capacity) and their nutrition by absorption (enzyme secretion) (Szczepanowska and Cavaliere, 2000).

Archive materials present different susceptibility to microorganisms according to their diverse constitution (e.g. paper is mainly attacked by fungi - mainly constituted by cellulose, and parchment under aerobic conditions by bacteria – it's mainly constituted by collagen) (Tiano, 2002).

Parchment was, along with papyrus, one of the most important paper ancestors. It is a natural and organic material consisting of animal (goat, cow or sheep) skin with a special treatment. It has been used in Europe as write support especially during the Middle Age (Curci, 2003, <http://www.sca.org.au/scribe/articles/parchment.htm>; Briscoe, <http://www.ehow.com/facts_5272725_history-parchment-paper.html>; Ferreira, 2010; Flieder and Duchein, 1993). Thanks to its animal origin, parchment is rich in compounds such as collagen, keratin, elastin, albumin and globulin that turn it into an excellent biological substrate, especially for fungal communities (Tiano, 2002).

Research in biodeterioration of old historical documents is important for the development and optimization of methodologies that help to prevent their degradation. Many chemical (Bond, 1998) and non-chemical methodologies have been applied in order to decontaminate and protect different art forms, especially archive documents. Between the chemical ones, we can find fumigants such as Ethylene Oxide (ETO) (Hengemihle *et al*, 1995), Methyl Bromide or Sulphuryl Fluoride. Their decontamination potential is real but, unfortunately, fumigants and pesticides are toxic to human.

Non-chemical treatments such as modifying indoor atmospheres (Kaplan and Schulte, 1996) (they act by increasing the carbon dioxide and/or other inert gas level and decreasing oxygen level) could help on preventing contamination but, the toxicity to archives and libraries staffs is still high. Other non-chemical treatments outweigh the toxicity problems. Gamma radiation has been applied and tested in cultural heritage since early 60's (Sinco, 2000; Magaudda, 2004). This is a clean energy with high penetration capacity. Low doses seem to be effective on archive documents decontamination with no evidence for materials alterations (Adamo *et al*, 1998). However, the most studied documents are mainly constituted by paper (Adamo *et al*, 2001; Gonzalez *et al*, 2002; Magaudda, 2004) and few studies were made about radiation effects on parchment.

In this way our studies aimed: i) The validation of a methodology to characterize the AUC parchment bioburden; ii) The microbiota characterization to identify parchment contamination patterns iii) The evaluation of the growth and inactivation patterns of parchment microbiota, in order iv) to propose a minimal dose (D_{min}) to be applied in parchment decontamination treatment by gamma radiation, as an alternative conservation method.

Material and Methods

Sample Identification

Four different parchment samples from the Archive of the University of Coimbra (AUC) (n=4) were used: "1-Cabido da Sé de Coimbra Séc. XV (1472)" (1CSC); "2 – Livro de Baptismos de Alvarenga (1719-1751)" (2LBA); "5 – Colações - Fig. Foz" (5CFF) and "6 – Séc. XVI?" (6SXVI).

Sampling Plan

Each parchment sample was divided by the middle: one half was used to validate and estimate bioburden characterization methodology (1.); and the other one was used in the gamma radiation inactivation studies (3.). For (1.) experimental procedure each parchment half (1CSC; 2LBA; 5CFF and 6SXVI) was subdivided into twelve similar subsamples (≈ 11.1 cm x 4.8 cm). Six were used for determination and characterization of parchment bioburden using SM and DM (n=3 subsamples per method) and the other six were used for evaluation of these methods recovery efficiencies.

For minimal dose determination studies, the remaining four parchment halves were cut by the middle in order to obtain two quarters per parchment: one remains complete and the other one was divided in 10 similar subsamples (≈7.2 cm x 4.7 cm). Eight of these were separated in 3 replicates (n=24) to be used in the inactivation studies.

1. Validation of a Bioburden Determination Method

The validation of parchment bioburden determination method was performed using artificial contamination of parchment subsamples with a known concentration suspension of *Cladosporium cladosporioides* (AUC indoor air's isolate – 2008 sampling). Two spore suspensions had been prepared from *Cladosporium cladosporioides* seven days pure cultures (Malt Extract Agar medium), using saline solution (0.9% NaCl) with 0.1% of Tween 80 as suspension medium. Concentration was obtained by counting in a Neubauer chamber (>10^7 spores/mL). Six subsamples of each parchment (n=6) were artificially contaminated: 250 µL (5 sub inoculums of 50 µL/subsample) of *Cladosporium cladosporioides* spore suspension (8x10^7 spores/mL). Spiked parchments were dried overnight in the laminar flow chamber.

Artificial contaminated subsamples were analysed using two different bioburden determination methods: Swab method (SM) and Destructive method (DM).

For SM three artificially contaminated subsamples per parchment were sampled using a swab for each one. Each swab was placed in a tube containing 5 mL of washing solution (0.9% NaCl with 0.1% of Tween 80) and homogenized by vortex. Aliquots of 0.1 mL of serial decimal dilutions were inoculated, in triplicate, in Triptic Soy Agar (TSA) petri dishes. For DM, three artificially contaminated subsamples (per parchment) were individually placed into sterile bags containing 100 mL of washing solution (0.9% NaCl with 0.1% of Tween 80), and homogenized during 15 min in a stomacher blender equipment (Stomacher 3500; Seaward, UK). Aliquots of 0.1 mL of serial decimal dilutions were inoculated, in triplicate, in Triptic Soy Agar (TSA) petri dishes.

Non spiked samples were also tested by the two methods, in order to evaluate their potentialities to remove and quantify parchment natural microbiota.

Inoculated Petri dishes were incubated during 5 days at 25-28ºC and then were transferred to 30 ± 2 ºC until the seventh day. Colony Forming Units per cm^2 (CFU/cm^2) were estimated for each parchment sample.

Considering the initial *C. cladosporioides* spore suspension concentration and the determined CFU/cm^2, the recovery efficiency was calculated for each method.

Furthermore, a representative population of parchment natural microbiota was selected based on macroscopic characteristics (e.g. pigmentation, texture, shape). For further characterization, bacteria were isolated in TSA medium and fungi were isolated in MEA (Malt Extract Agar) medium. Morphological (e.g. Bacteria - cellular morphology,

endospores presence/absence; Fungi – reproductive and vegetative structures morphology) and biochemical characterization (e.g. Bacteria - gram staining, catalase and oxidase activities) results divided the representative isolates (n=93) in eleven morphological types (definition based on the Bergey's Manual of Determinative Bacteriology) (Holt *et al*, 1994). The frequency of each morphological type was calculated based on the number of isolates and its characterization.

Total genomic DNA from fungal isolates was extract using the extractor ABI Prism 6100's total genomic DNA extraction protocol. A PCR amplification of total ITS region was performed for each fungal isolate using fungal specific primers (Manter and Vivanco, 2007). Amplification products were sequenced after purification and compared with NCBI Blast – GenBank data base to identify fungal isolates at the species level. Isolates identification was also confirmed by morphological analysis.

2. Irradiation

Twelve replicas of each parchment (n=4) were spiked with 100 µL of a *C. cladosporioides* spore suspension ($1x10^7$ spores/mL) as previously described.

The subsamples were packed into sterile plastic and irradiated in a cobalto-60 experimental source (Precisa22) located in the Nuclear and Technological Institute *campus* (Sacavém, Lisbon). The absorbed dose was monitored by calibrated routine dosimeters (maximum range variation dose of ± 2.5%; Perspex, Harwell) to identify the highest and lowest doses absorbed by the product.

The spiked replicas (12 per parchment sample) were exposed to gamma radiation doses between 2 kGy and 15 kGy at dose rates from 0.9 kGy/h to 2.3 kGy/h. Naturally contaminated replicas (12 per parchment sample) were subjected to absorbed doses between 2 kGy and 12 kGy (dose rates 1.2 – 2.3 kGy/h).

3. Inactivation studies

Parchment bioburden was estimated before and after irradiation using the previously validated Destructive Method (DM). For irradiated non-spiked samples, 10 mL aliquots of parchment washing solution were filtrated, in triplicate, through cellulose nitrate membrane (0.45 µm; Advantec®; Japan) and placed into TSA petri dishes. The quantification and characterization of the surviving microorganisms was performed as described in *Validation of Bioburden Determination Method*.

4. Post Irradiation Fungal Growth Monitorization

After *Inactivation studies*, samples stomacher bags were stored at 4 ± 2ºC. The viability of parchment fungal microbiota was evaluated after 37 and 58 days of storage. Briefly, 0.1 mL aliquots of each parchment washing solution were inoculated in MEA

medium petri dishes and incubated at 30 ± 2ºC during 7 days. Fungal colonies were isolated, morphologically characterized (e.g. pigmentation, texture, reproductive and vegetative structures morphology) and identified by molecular methods.

5. Diversity Assessment

Simpson diversity index (1-D) (Simpson, 1949 cited by Dvoretsky and Dvoretsky, 2011; Magurran, 1988) was applied to morphological types and fungal molecular identification results (before and after storage), using the following equation:

$$1\text{-}D = 1 - \sum (n/N)^2$$

n – Number of colonies; N – Total colonies number in the population

Results

1. Validation of a Bioburden Determination Method

Two methods were evaluated in order to select one to be used in the parchment samples bioburden estimation. The intent was to validate a non-destructive method (swab method - SM) that could be applied hereafter to monitor and control the microbial growth in written documents. As so, the swab method was compared with a washing destructive method (DM), using *C. cladosporioides* artificial contamination as reference. The recovery efficiencies are presented in Table 1.

Table 1 – Recovery efficiency of swab (SM) and destructive (DM) methods in parchment samples

Parchment	Method	Efficiency (%)
1CSC	SM	6.3
	DM	>100
2LBA	SM	8.9
	DM	>100
5CFF	SM	1.0
	DM	>100
6SXVI	SM	2.6
	DM	66.1
TOTAL	SM	4,7
	DM	>100

Results point out to a low recovery efficiency of Swab method (<10%), probably due to its superficial character. Comparatively, the destructive method presented higher recovery efficiencies (>100%) for all the parchments with the exception of 6SXVI. The natural microbiota of parchment samples was also evaluated by both methods and the bioburden values are presented in Table 2. The natural contamination values justify the recovery efficiencies above 100% obtained for the destructive method.

Table 2 – Average values and respective standard errors of Parchment bioburden evaluated by swab (SM) and destructive (DM) methods. [(a)] Method detection limit

Parchment	Method	CFU/cm^2	
		Total	Fungal
1CSC	SM	10±3	10±3
	DM	101±73	17±8
2LBA	SM	16±3	10±3
	DM	5137±997	<2[(a)]
5CFF	SM	4±2	1±0.3
	DM	63±27	21±12
6SXVI	SM	11±2	10±2
	DM	55±33	32±14

Considering the low efficiency of the swab method, also visible in the evaluation of natural contamination, the destructive method was selected to further assess the bioburden of parchment samples, although its destructive character.

Results also indicate a non-uniform contamination of parchment, intra and inter samples. In order to outline the contamination pattern of parchment samples, a representative microbial population (n=93) was morphologically characterized. The isolates were subdivided in morphological types according to their phenotypic characteristics (Figure 1). The most frequent morphological types among parchment samples isolates were the non-spore forming (72.7%) and the spore forming (11.6%) gram-positive rods. Filamentous fungi seemed to be mainly present in 1CSC, 5CFF and 6SXVI parchments (20.8%, 19.7% and 39%, respectively), presenting a total frequency of 6.1%.

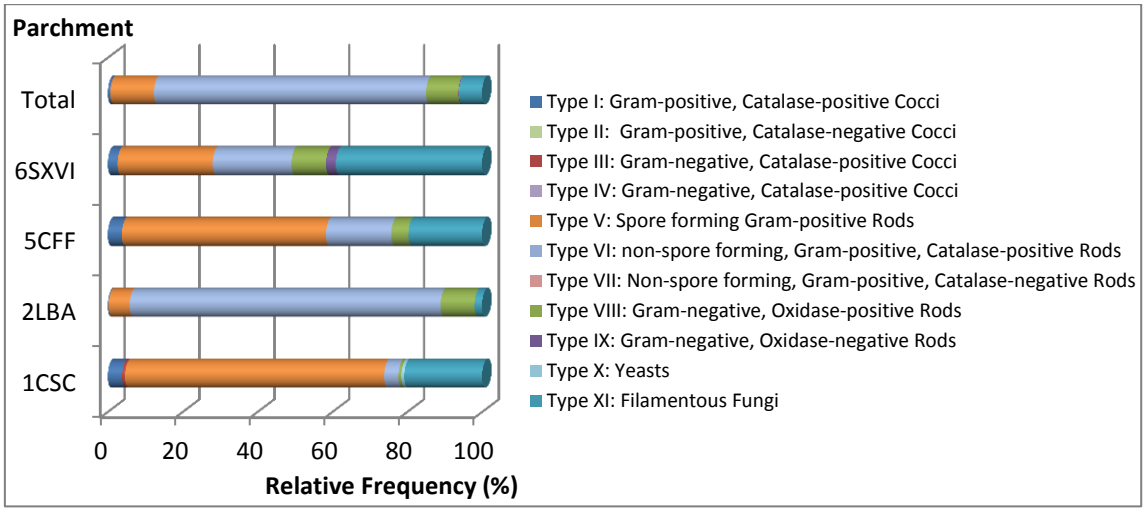

Figure 1 – Relative frequency (%) of morphological types of the isolates (n = 93) of each and total parchment samples

Overall, the parchment microbiota seemed to have a homogeneous constitution, presenting the same morphological types' pattern among samples, although with different frequencies.

The fungal molecular identification (n=9 isolates) indicated the presence of, at least, four species belonging to three genera, in parchment samples (Table 3).

Table 3 – Molecular identification of parchment fungal isolates (n=9)

Parchment	Species	Genus	Frequency (%)
1CSC	*Cladosporium* sp.	*Cladosporium*	0.53
1CSC	*Cladosporium cucumerinum*	*Cladosporium*	0.28
2LBA	*Cladosporium cucumerinium*	*Cladosporium*	1.34
2LBA	*Cladosporium cladosporioides*	*Cladosporium*	0.06
5CFF	*Cladosporium cladosporioides*	*Cladosporium*	0.16
5CFF	*Cladosporium cucumerinium*	*Cladosporium*	0.03
5CFF	*Aspergillus fumigatus*	*Aspergillus*	0.28
6SXVI	*Cladosporium cucumerinium*	*Cladosporium*	0.78
6SXVI	*Penicillium* sp.	*Penicillium*	0.03

The most frequent genus in parchment was *Cladosporium* (3.18%) followed by *Aspergillus* (0.28%). *Cladosporium* genus was represented by, at least, two species, *Cladosporium cucumerinium*, a plant pathogen (Kwon *et al*, 1999; Kwon *et al*, 2000), and *Cladosporium cladosporioides*, described as one of the most common fungi (Zyska, 1997). The genus *Aspergillus* was represented by only one species: *Aspergillus fumigatus*, a known human pathogen responsible for the major aspergilosis in immunocompromised individuals (Latgé, 1999; Nierman *et al*, 2005; Pasqualotto, 2008).

The diversity of parchment samples microbial population was estimated based on Simpson Diversity Index, which was applied to total phenotypic characterization and to fungal molecular identification (Table 4).

Table 4 - Simpson Diversity Indexes for total and fungal parchment populations

Parchment	Simpson Diversity Index (1-D)	
	Total	Fungal
1-CSC	0.48	0.45
2-LBA	0.30	0.08
5-CFF	0.63	0.52
6-SXVI	0.73	0.07
TOTAL	0.45	0.57

The obtained results for total microbial population indicated that the most diverse parchment sample was 6SXVI and the less was 2LBA. Total Simpson diversity index was 0.45. The 2LBA and 6SXVI parchment samples presented a low diversity regarding fungal population, whereas 1CSC and 5CFF presented medium diversity values. However, in a total analysis the fungal population revealed a medium diversity.

2. Inactivation studies

Parchment samples were irradiated at sub-lethal gamma radiation doses to study the inactivation pattern of its microbial population. As mentioned before, the destructive method was applied to assess samples bioburden before and after irradiation. The initial parchment samples bioburden ranged between 17±4 CFU/cm^2 and 107±31 CFU/cm^2 for total

microbiota, and was <6 CFU/cm^2 for fungal population. The obtained bioburden results indicated that 75% (3/4) of the samples could be included in a contamination class of 10-100 CFU/cm^2. Comparatively, in the previous bioburden evaluation, 50% of samples belong to this class, but it was found a contamination peak (>5000 CFU/cm^2). These outcomes point out to the non-uniformity of parchment microbial population inter e intra samples.

Considering samples low initial contamination, it was not evident any inactivation trend. However, the inactivation efficiencies for each parchment microbiota are presented in Table 5.

Table 5 - Parchment bioburden inactivation efficiencies

Parchment	Inactivation Efficiency (%)					
	2 kGy	3 kGy	4 kGy	5 kGy	6 kGy	12 kGy
1-CSC	40.0	----	92.2	----	100.0	----
2-LBA	86.9	----	99.3	----	100.0	----
5-CFF	----	98.8	----	99.8	----	99.8
6-SXVI	----	63.9	----	99.0	----	99.4

Inactivation efficiencies higher than 90% were obtained for all parchment samples, for doses higher than 4 kGy. That corresponds to a microbial population decrease of one decimal logarithm.

In order to propose a minimal decontamination gamma radiation dose, it was used parchment artificial contamination with *Cladosporium cladosporioides*, since the natural contamination was not able to define a clear response to gamma radiation. The selection of this fungus was related to its presence in parchment samples and its documented resistance to gamma radiation (Dadachova and Casadeval, 2008). The obtained inactivation survival curve for *C. cladosporioides* is presented in Figure 2.

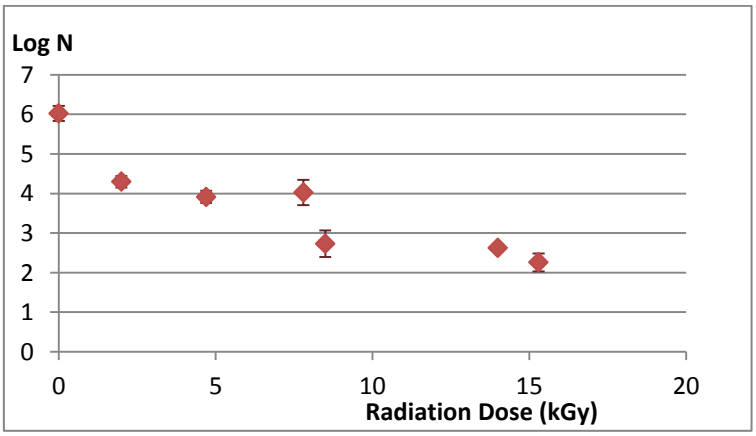

Figure 2 - *Cladosporium cladosporioides* survival curve after gamma irradiation in Parchment (1<n<50; α=0.05)

Cladosporium cladosporioides seems to follow an exponential inactivation kinetics in parchment. The same strain of *C. cladosporioides* spiked in paper had presented a sigmoidal survival curve to gamma radiation (Portugal *et al*, 2010). These results highlight the influence of the substrate in the response of microorganisms to gamma radiation. A decrease of two decimal logarithms was obtained for *C. cladosporioides* at 5 kGy, corresponding to an inactivation efficiency of 99%. Obtained

inactivation results for natural microbiota and *C. cladosporioides* suggest that a minimal dose of 5 kGy could be adequate for parchment decontamination treatment.

Considering microbial population dynamics with gamma radiation doses, eight different morphological types were found among the four parchment samples (n=131 isolates). The most frequent surviving types were the Filamentous Fungi (36%); the Gram-positive, catalase-positive cocci (24%); the Gram-negative, oxidase-positive rods (19%) and the Spore forming gram-positive rods (12%) (figure 3). The higher dose survivors belong mainly to the groups of Spore forming gram-positive rods and Filamentous fungi. The patterns of morphological types varied with the applied radiation dose, and a decrease of the number of morphological types was verified with the increase of the absorbed dose (figure 3).

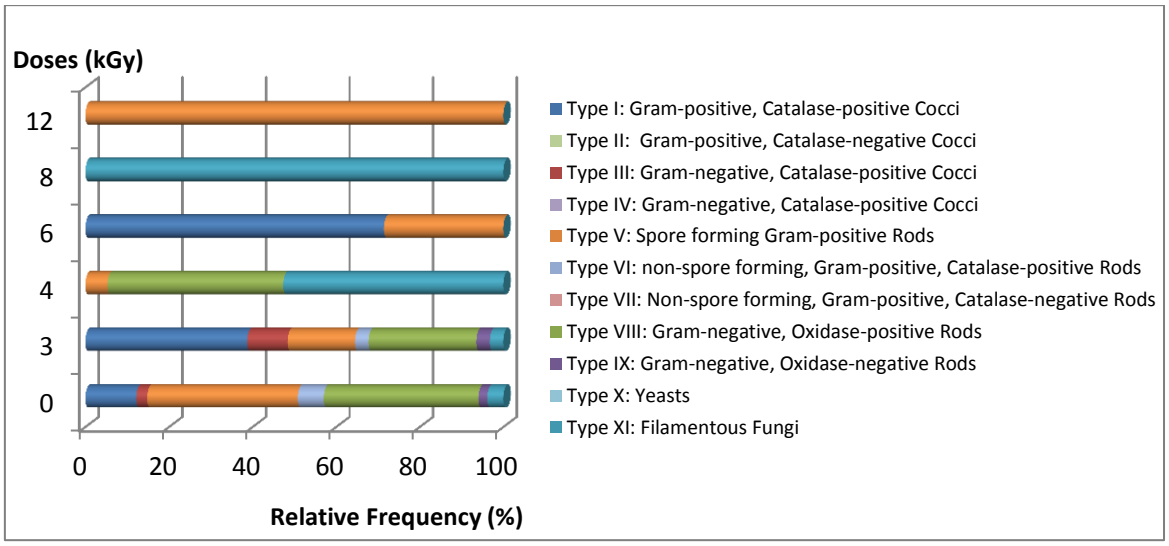

Figure 3 - Frequency (%) of parchment's morphological types after irradiation (0-12 kGy)

As expected, the diversity seems to decrease with the increase of gamma radiation dose (table 6).

Table 6 - Simpson Diversity Indexes for total parchment bioburden in each radiation dose

Dose (kGy)	Simpson Diversity Index (1-D)
0	0.7
3	0.7
4	0.5
6	0.4
8	0.0
12	0.0

Surviving fungal isolates (n=22) were identified after inactivation studies (table 7) and after storage (table 8). The first results pointed out for the presence of two fungal genera among the four different parchment samples: *Penicillium* and *Aspergillus*. For the higher doses (4 and 8 kGy) only the genus *Aspergillus* was found.

After storage, the major fungal genus identified was *Penicillium*, although *Cladosporium* and *Aspergillus* were also found. Similarly, the genus *Aspergillus* was found after storage at the higher doses.

Table 7 - Molecular identification of the parchment fungal isolates (n = 9) after inactivation studies

Dose (kGy)	Genus	Species
0	*Penicillium*	*Penicillium commune*
0	*Penicillium*	*Penicillium commune*
0	*Penicillium*	*Penicillium commune*
0	*Penicillium*	*Penicillium commune*
2	*Penicillium*	*Penicillium spinulosum*
2	*Penicillium*	*Penicillium commune*
3	*Alternaria*	*Alternaria* sp.
4	*Aspergillus*	*Aspergillus fumigatus*
8	*Aspergillus*	*Aspergillus fumigatus*

Table 8 - Post irradiation results: Molecular identification of the parchment fungal isolates (n=13) after storage

Storage (days)	Dose (kGy)	Genus	Species
58	0	*Penicillium*	*Penicillium spinulosum*
58	0	*Penicillium*	*Penicillium commune*
37	0	*Penicillium*	*Penicillium commune*
37	0	*Cladosporium*	*Cladosporium phaenocomae*
37	0	*Penicillium*	*Penicillium commune*
58	0	*Chaetomium*	*Chaetomium murorum*
58	0	*Epicoccum*	*Epicoccum nigrum*
58	0	*Coprinelus*	*Coprinelus* sp.
37	0	*Cladosporium*	*Cladosporium* sp.
37	3	*Phoma*	*Phoma glomerata*
37	3	*Penicillium*	*Penicillium commune*
37	3	*Penicillium*	*Penicillium commune*
58	6	*Aspergillus*	*Aspergillus fumigatus*

These results suggest the gamma radiation resistance and persistence of some fungal genus in parchment samples.

Discussion and Conclusions

The development of a nondestructive methodology to evaluate parchment bioburden was not attained. The use of swabs has been reported as a good method for superficial analysis especially for human pathogen determination (Sheen *et al*, 1998; Erol *et al*, 2004; Frank *et al*, 2003). Comparing the achieved efficiencies for SM and DM we could inferred that parchment microbiota could be present not only in the superficial layers but also in the deep fiber tram. SM low efficiencies values range according to the parchment sampled which could indicate that each parchment sample has specific fiber structure characteristics, such as different porosities. Parchment is a complex and heterogeneous ancient material which characteristics could vary with the production method, the animal origin or the different environmental exposures (Možir *et al*, 2011). The destructive method was selected to remove the microorganisms present in parchment samples, to prevent the underestimation of samples bioburden. A similar destructive method had already been described to determine biomaterials bioburden and to establish sterilization doses (Cabo Verde *et al*, 2010).

Concerning parchment microbiota characterization, the four samples presented a bioburden lower than $5x10^3$ CFU/cm^2, mainly composed by the same morphological types,

with different frequencies. The most frequent morphological types found were the Non-spore forming (72.2%) and Spore forming (11.3%) Gram-positive rods. Endospores are resistant forms which allows this type of organisms to resist in inhospitable environments (e.g. low nutrients concentration, high temperatures, high saline concentrations or some chemical concentrations) (Block, 1983; Huemer *et al*, 1998; Nicholson *et al*, 2000; Amoozegar *et al*, 2005; Lima *et al*, 2010). Despite its organic composition, parchment could be an unfavourable environment for bacteria growth. Results indicated that parchment microbiota was different in the four parchment samples, either quantitatively or qualitatively. These differences could be related to the variety of parchment constitution or to the different physical, chemical and environmental factors to which each sample was exposed before (Možir *et al*, 2011).

Molecular fungal identification showed the presence of three fungal genera (*Cladosporium*, *Aspergillus* and *Penicillium*) represented by, at least, four species, being the most frequent the *Cladosporium cucumerinium* (16.7%). The three fungal genera are commonly found in indoor and outdoor environments and have been described as archive materials and indoor air contaminants (Maggi *et al*, 2000; Szczepanowska and Cavaliere, 2000; Tiano 2002; Cappitelli and Solini, 2005; Borrego *et al*, 2008; Borrego *et al*, 2010; Sterflinger, 2010; Guiamet *el al*, 2011).

The statistical evaluation of diversity by Simpson diversity index also demonstrate the heterogeneity, inter and intra samples, of parchment microbiota.

The parchment microbial population seems to follow a decreasing tendency with the increase of the gamma radiation dose. Although, the results were inconclusive since the low bioburden values do not allow to fit any typical survival curve (Block, 1983). Apparently, the most gamma radiation resistant morphological types were the Spore forming gram-positive rods and Filamentous fungi. Results are in agreement with previous studies which indicate these types of microorganisms as the most gamma radiation resistant (Morgan and Reed, 1954; Blank and Corrigan, 1995; Nicholson *et al*, 2000; Rainey *et al*, 2005).

Fungal molecular identification was performed after storage in order to understand if the initial fungal isolation procedures had detected all the present fungal genera/species.

Fungal genera identified before storage were also present after storage with exception for *Alternaria* (only present before storage). However, five new genera were detected after storage (*Cladosporium*, *Chaetomium, Epicoccum, Coprinelus* and *Phoma*) which indicates that first analysis (before storage) didn't detected all fungal genera present in the parchment samples. Visual fungal growth was also detected in the storage stomacher sacs both after 37 and 58 days at 4ºC. Considering all fungal isolates, the most frequent identified genus was *Penicillium* followed by *Aspergillus*.

Studies had been made using gamma radiation to decontaminate paper and other archive materials. Doses ranging from 3 to 10 kGy seemed to be effective in decontamination of microfungi with no significant material alterations (Adamo and Magaudda, 2003). Higher

doses (near 15 kGy) have been described for archive paper materials disinfection without damages in the initial material structure (Sinco, 2000; Gonzalez *et al*, 2002; Rizzo *et al*, 2002; Rocchetti *et al*, 2002). Nevertheless, to our knowledge there was no literature for gamma radiation effects in parchment documents. Based on the inactivation efficiencies achieved for parchment natural microbiota, and inactivation response of *Cladosporium cladosporioides* we propose a dose of 5 kGy as a minimal decontamination dose, considering that in this study, our objective is to decontaminate and not sterilize the parchment documents. These results are according to the achieved gamma radiation minimal dose range (3 – 8 kGy) determined to microfungal decontamination in paper by Magaudda (2004).

Furthermore, studies are being performed in order to understand and quantify the gamma radiation effects on parchment documents.

Acknowledgements

We would like to tank to the Archive of the University of Coimbra for their collaboration in these studies and, especially, for the kindles samples assignment.

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Gamma Radiation Effects on Physical Properties of Parchment Documents: Assessment of D_{max}

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Keywords

Parchment; Gamma Radiation; Texture; Colour; $D_{max.}$

Abstract

Aims: The aim of this study was to evaluate the gamma radiation effects on the colour and texture of parchment documents. The assessment of these effects will be used to estimate a maximum gamma radiation dose ($D_{max.}$) that could guarantee a decontamination treatment of parchment without significant alteration of its physical properties.

Methods and Results: Parchment samples were exposed to gamma radiation doses from 10 kGy up to 30 kGy. Texture and colour were analysed before and after irradiation of parchment subsamples using a texture analyser and an electronic colorimeter. Hardness and elasticity were determined based on texture spectrums. Lightness (L*), chromaticity (C), greenness vs. redness (a*) and yellowness vs. blueness (b*) were obtained from colorimetric measures. Results point out to no significant effect on texture and colour of parchment for the applied radiation doses.

Conclusions: A maximum gamma radiation dose of 30 kGy suggests no significant modifications in parchment texture and colour characteristics, based on the evaluated parameters and their conditions.

Significance and Impact of Study: The assessment of gamma radiation effects on parchment physical properties is required for the evaluation of gamma radiation as an alternative decontamination treatment.

Introduction

Behind their role in our cultural heritage, written documents are Art manifestations that should be preserved. Nowadays, conservation and restoration are increasing areas that have always new challenges to solve. Biodeterioration is one of the most problematic issues that conservers have to deal. It consists in material degradation as result of organisms metabolic activities, and can be associated to different chemical, physical, climatic and environmental factors to which materials are exposed (Katusin-Razema *et al*, 2009; Guiamet *et al*, 2011). Preventive approaches have been developed in order to decontaminate and preserve the archive and library materials. One of the used methods is chemical and comprises the use of pesticides and fumigants (Bond, 1998). Despite their indisputably decontamination potential, these chemicals (e.g. Ethylene Oxide, Methyl Bromide and Sulphuryl Fluoride) are toxic to human. Non-chemical treatments consists in modified atmospheres (Kaplan and Shulte, 1996) (e.g. low oxygen), temperature alterations (e.g. cold and high temperature treatments) or in exposition to ionizing radiation (e.g. Gamma radiation). However, modified atmospheres could be still toxic to the archives and libraries staffs, cold treatments have to deal with chambers humidity problems and high temperature could lead to oxidation and aging. Gamma radiation seems to be a clean and safe alternative treatment. Its high penetration capacity and the possibility to be used in a large range of materials than temperature treatments, turn it in a developing issue in conservation, art decontamination and preservation (Adamo *et al*, 2001; Gonzalez *et al*, 2002; Magaudda, 2004). Despite its already long history in archive and library materials decontamination (since the 60's) (Sinco, 2000; Magaudda, 2004), there are some gaps especially for complex materials such as parchment. Parchment is an ancient written material created in the second century BC in Pergamum, Greece, and consists of animal skin (Flieder and Duchein, 1993; Curci, 2003, <http://www.sca.org.au/scribe/articles/parchment.htm>; Cappitelli and Sorlini, 2005; Briscoe, <http://www.ehow.com/facts_5272725_history-parchment-paper.html>; Ferreira, 2010). It is composed by organic compounds such as collagen, turning it into a potential microbial substrate (Tiano, 2002). Parchment structure characteristics as well as its production techniques are still in study, and to our knowledge there is no literature for parchment texture or colour analyses influenced by gamma radiation.

In this study, the objectives were to assess texture and colour properties of parchment in order to evaluate the gamma radiation effects on its physical properties. Based on that, it is proposed a maximum radiation dose ($D_{max.}$) to which this material could be exposed without significant alteration of its structure.

Material and Methods

Sampling Plan

It was used a quarter of four parchment samples (n=4): "1-Cabido da Sé de Coimbra Séc. XV (1472)" (1CSC); "2 – Livro de Baptismos de Alvarenga (1719-1751)" (2LBA); "5 – Colações - Fig. Foz" (5CFF) and "6 – Séc. XVI?" (6SXVI). Twelve subsamples with 2cm x 7cm were obtained from each parchment quarter. For each parchment sample it was used three replicates (three subsamples) per irradiation dose. Subsamples were packed using sterile amilon plastic bags. Non-irradiated subsamples (n=3) were used as control.

A micrometer (Mitutoyo 7360, precision = 0.01 mm) was used to evaluate the parchment subsamples thickness.

Irradiation Procedures

The samples were irradiated in a cobalto-60 experimental source (Precisa22) located in the Nuclear and Technological Institute campus (Sacavém, Lisbon). The applied doses were 10 kGy; 19 kGy; 22 kGy; 28 kGy; 30 kGy at a maximum dose rate of 3.1 kGy/h. The absorbed dose was monitored by calibrated routine dosimeters (maximum range variation dose of ± 2.5%; Perspex, Harwell) to identify the highest and lowest doses absorbed by the material.

Texture – TPA (Texture Profile Analysis)

Texture tests were performed by compression of parchment subsamples *loops* using a texture analyser (TA-Hdi, Stadle Microsystems) using a 50 Kg capacity compression load cell – *loop* test (figure 1).

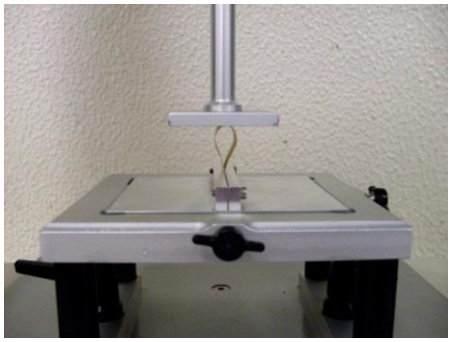

Figure 1 – Texture Analyser compressing a parchment sample

For each subsample, a two cycle assay was made using a 10 mm/s speed for pre and post-tests, a 1.7 mm/s test speed, a compression time of 5 seconds and a 12 mm compression distance. Results were obtained using the XTRA Dimension software. Hardness corresponds to the maximal force (Newton (N)) used in first compression cycle. Springiness consists in the material recuperation capacity and was calculated as the ratio between the two deformation times (t_2/t_1).

Colour analysis

Superficial parchment colour was analysed using an electronic Minolta colorimeter with an 8 mm opening and a C illuminant. Results were registered using CIE L*a*b* coordinate system. For each subsample (non-irradiated and irradiated) it

were performed four randomly measurements (n=288). Saturation (Chromaticity – C) was calculated according to the formula: $C = \sqrt{a^2 + b^2}$.

Statistical Analyses

ANOVA statistical tests were applied to texture and colour results using Origin 7.5 software. Non-parametric Kruskal-Wallis statistical test was used when homogeneity of variance was not verified.

Results

Texture – TPA (Texture Profile Analysis)

Considering the parchment origin (animal skin) an approach was made based on leather used technics. The texture analyser loop test was published as means of quantify leather softness in 1993 (Alexander and Stosic, 1993). It allows determining the compression and decompression energy, the peak force and the springiness. For this, a specific aluminium sample support had been design to apply in a TA-Hdi, Stadle Microsystems texture analyser and used in texture analyses. Parchment samples hardness and springiness were evaluated by the *loop* test using a texture Analyser and the obtained results are represented in Figure 2.

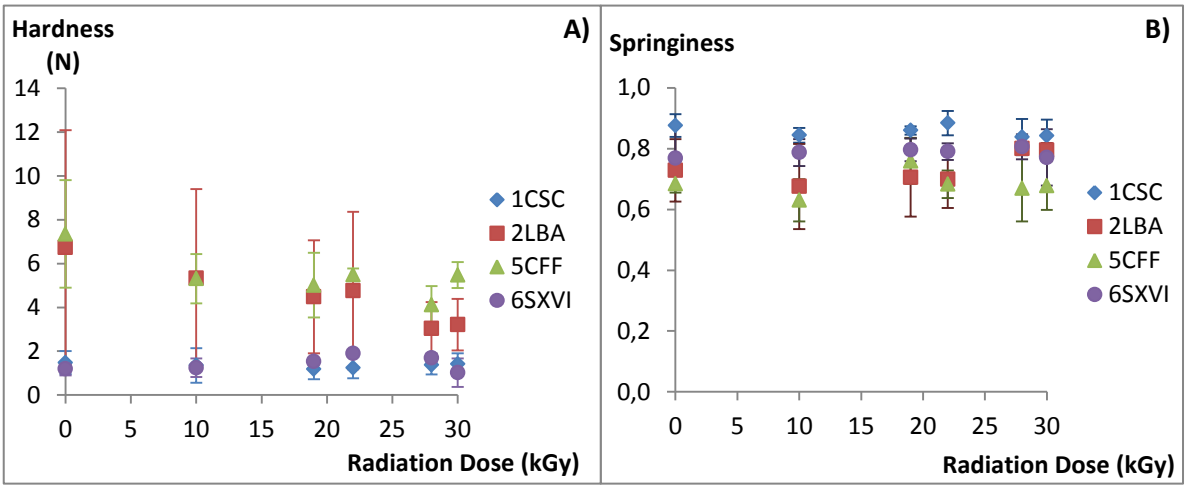

Figure 2 – Hardness (A) and Springiness (B) of parchment samples with increasing gamma radiation doses (n=3/dose/parchment; α=0.05)

The initial sample hardness average values were significantly different (*p*-value = 0.0496), which could indicate that each parchment present specific hardness characteristics. Moreover, subsamples showed significantly inter and intra thickness differences. For parchment samples, the *loop* test texture assay was destructive and it was not possible to analyse successively the same subsample, which may have introduced some variations in the measurements. Two distinct hardness patterns were verified for the four parchment samples (Figure 2-A): 2LBA and 5CFF parchments (thicker samples) presented higher

hardness than 1CSC and 6SXVI parchments. Gamma radiation doses seemed to have no significant (*p*-value > 0.05) effect on parchment hardness namely for 1CSC and 6SXVI samples. For 2LBA and 5CFF parchments, hardness seemed to decrease with the increase of gamma radiation dose. However, ANOVA analysis showed that the 2LBA and 5CFF hardness average values were not significantly different between doses (*p*-value > 0.05).

Based on the obtained results, each parchment sample appeared to present a specific springiness, being the initial springiness inter samples mean values significantly different (*p*-value=0.043) (figure 2-B). No significant intra samples differences were found for springiness with gamma radiation dose (*p*-value > 0.05).

Colour Analysis

L* coordinate characterizes the sample lightness and range between 0% (Black) and 100% (white), a* coordinate represents redness (+a) or greenness (-a) and b* coordinate denotes yellowness (+b) or blueness (-b) (Loughrey, 2002).

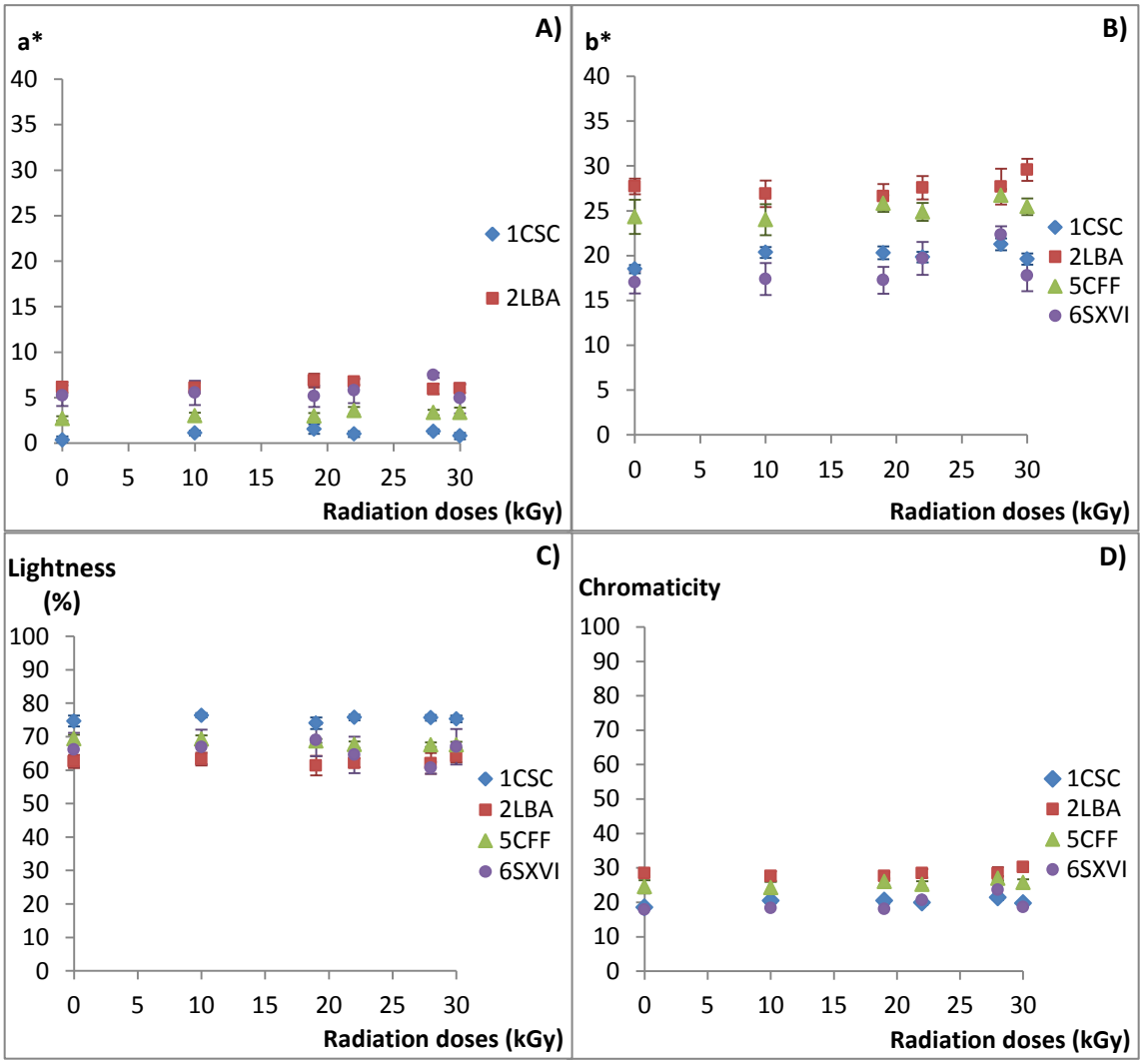

Figure 3 – Parchment colour parameters variation with gamma radiation dose: a* parameter (A); b* parameter (B); Lightness (C) and Chromaticity (D) (n=4/dose/parchment; α=0.05)

The analysed colour parameters (a*, b*, Lightness, and Chromaticity) presented significantly different values (*p*-value < 0.05), between parchment samples (figure 3). As for springiness, each parchment sample seemed to have a characteristic colour pattern.

No gamma radiation doses effect in parchment colour parameters was verified (figure 3). Significant differences (*p*-value < 0.05) observed between doses for 1CSC, 6SXVI and 5CFF parchments could be related to sample intrinsic colour variability.

Discussion

Texture is a multi-parameter attribute which study had started in the late 19th and early 20th centuries (Bourne, 1978; Szczesniak, 2002). The interest for this issue has begun in food area (e.g. bread, meat, fruit and vegetables) from the necessity to understand and improve structural characteristics responsible for specific sensorial sensations, increasing the consumers interest in products. Nowadays, texture investigation exceeds the sensorial analysis, associating measurement techniques to study texture attributes, such as hardness, cohesiveness, adhesiveness or springiness (Wilkinson *et al*, 2000; Bourne, 2002; Szczesniak, 2002). Texture became an import parameter to analyse the alterations in materials by decontamination processes (IAEA, 2001; Pang *et al*, 2007; Araújo *et al*, 2007; Landfeld *et al*, 2010; Rosidah *et al*, 2010).The results presented in this study indicate that the tested gamma radiation doses did not have an effect on parchment hardness and springiness. The apparently hardness decrease tendency observed for 2LBA and 5CFF samples was probably related with the parchment intrinsic variations, associated with different production methods, animal origins and environmental conditions exposure (Možir *et al*, 2011). To deeper understand these facts, non-destructive texture analysing methods should be tested in order to turn possible the use of the same subsamples in irradiation-texture analysis procedures. Non-destructive technics such as near infrared reflectance (NIR), Nuclear Magnetic Resonance Spectroscopy (NMR), environmental scanning electron microscopy (ESEM) and confocal scanning laser microscopy (CSLM) had already been applied in food texture analysis (Pawley, 1989; Kalab *et al*, 1995; Kjolstad, 1990, Dantilos, 1991, Hemming, 1992 and Robertson, 1992 all cited by Wilkinson *et al*, 2000).

Colour, as texture, is a physical parameter that had been used in decontamination studies in order to understand the lethal agent effects on materials properties (Wagenaar and Snijders, 2004; Pipek *et al*, 2005; Araújo *et al*, 2007; Zaied *et al*, 2007; Landfeld *et al*, 2010). According to the obtained results, tested gamma radiation doses seemed to have no effect in colour parchment characteristics. In conclusion, a maximum radiation dose (D_{max}) of 30 kGy shows no significant modification of parchment texture and colour characteristics, based on the performed measures and conditions.

However, having in account that there is a large variation in parchment texture and colour characteristics (Možir *et al*, 2011), further work must be performed using more and diverse parchment samples, in order to understand if all parchment documents types have the similar D_{max}. Furthermore, the parchment samples used in these tests didn't have any pigments. Some gamma irradiation pigments alterations had been reported in literature (Belyakova, 1961 and Tomazello, 1994 both cited by Rizzo *et al*, 2002).

Nevertheless, this study highlights the use of gamma radiation in parchment decontamination treatment, without severely affect its physical proprieties.

Acknowledgements

We would like to thank Prof. Gabriela Lima from Superior Agriculture School, Polytechnic Institute of Santarém, for her availability and help in preliminary parchment texture tests. We are also grateful to Prof. Maria João Trigo and to Engineer Armando Ferreira from INIA-INRB, who kindly collaborate with us in parchment texture and colour analysis during $D_{max.}$ determination approach.

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Assessment of Genetic Gamma Radiation Effects on Genetic profiles of Fungal Isolates from an Archive

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Keywords

Fungi; Gamma Radiation; Fingerprinting

Abstract

Aims: This study aims to: i) Estimate the genetic profile alterations induced by gamma radiation in four fungal isolates: *Penicillium griseofulvum*, *Neosartorya fumigata*, *Cladosporium* sp. and *Epicoccum nigrum*, using PCR *fingerprinting* techniques, and ii) Evaluate the relation between the applied gamma radiation doses and the observed genetic alterations.

Methods and Results: Spore suspensions (10^7 spores/mL) were obtained from four fungal isolates. Suspensions were exposure to three gamma radiation doses (2 kGy, 6 kGy and 10 kGy). Total genome DNA was extracted from the non-irradiated and irradiated spore suspensions and genetic PCR *fingerprintings* were obtained using two different primers: M13 and P888. Genetic profile alterations were visible in PCR *fingerprintings* since 2 kGy. Results also point out to a relation between the observed genetic alterations and the reported fungal species gamma radiation resistance.

Conclusions: The PCR genetic *fingerprinting* technique was able to detect fungal genetic profile alterations induced by the applied gamma radiation doses. Fungal DNA *fingerprintings* suggested no tendency with the increase of gamma radiation dose.

Significance and Impact of Study: Evaluate the gamma radiation doses effects on the genetic profile of environmental fungi.

Introduction

The mutation phenomenon can result from natural cellular processes (e.g. replication errors) or can be induced by external agents. Between these agents we can find chemicals (e.g. ethidium bromide, acridine orange, bromouracil or nitrosguanidine), radiation (e.g. UV radiation, cosmic radiation, ionizing radiation) and viral infections (Griffiths *et al*, 2008). Gamma radiation is reported as a random mutations inducer agent (Gardner & Snustad, 1987 cited by Cordeiro *et al*, 1995). Exposure to sub-lethal gamma radiation doses could change fungal characteristics, decreasing their virulence or promoting the secretion of some commercial interesting products (e.g. antifungals) (Zeinab *et al*, 2001; Aziz and Moussa, 2002; Haggag and Mohamed, 2002; Prado *et al*, 2003; Jitareerat *et al*, 2005). It had been described that the most typical gamma radiation damages in surviving cells are DNA segments deletions (Casarett, 1968).

Genetic mutations are divided in different type groups according to different classification criteria. Considering the number of DNA bases changes, mutations can be divided in point mutations (only one DNA base is changed) or mutations which DNA sequences are altered in several points leading to more extend alterations. According to the mutation functionality, point mutations can be classified as silent mutations (no function alterations were detected), nonsense mutations (no protein production), missense mutations (new proteins with new functions were obtained) or frameshift mutations (codon reading alteration resulting in a completely different translation). Mutations can also be classified in deletion mutations (when there is DNA missing from a gene sequence), insertion mutations (when some DNA bases are introduced in a gene sequence), duplication mutations (occurs when a specific DNA block is duplicate), inversion mutations (when there is gene inversion in the DNA sequence) and translocation mutations (resulting from chromosome translocation – chromosome blocks shift) (Griffiths *et al*, 2008).

Genetic *fingerprinting* is a molecular technique that can be used to detect genetic alterations by comparing the initial (without mutation agent) and final obtained *fingerprintings* (after exposure to mutation agent). Differences achieved consist in number or molecular weight alterations in the *fingerprinting* bands. Multi-bands *fingerprintings* allows simple and reproducible genotype discrimination (Paiva de Carvalho, 2010). Many studies had been performed using these potentiality to species and strains differentiation both in fungal and bacteria communities (Guimarães *et al*, 2011; Akoijam and Singh, 2011; Nolan *et al*, 2011; Bastardo *et al*, 2011; Singh and Kumar, 2010; Aggarwal *et al*, 2010). However, few studies were developed using this technique for detection of genetic gamma radiation induced mutations and the majority of them uses macroorganisms (Kubota *et al*, 1995; Lee *et al*, 2000; Lu *et al*, 2007).

The aims of this study were: i) To estimate the genetic profile alterations induced by gamma radiation in four fungal isolates: *Penicillium griseofulvum*,

Neosartorya fumigata, *Cladosporium* sp. and *Epicoccum nigrum*, using PCR fingerprinting techniques, and ii) To evaluate the relation between the applied gamma radiation doses and the observed genetic alterations.

Material and Methods

Fungal Isolates

Four fungal isolates from the Archive of the University of Coimbra (AUC) were used. *Penicillium griseofulvum* and *Neosartorya fumigata* were isolate in April 2010 from AUC indoor air using an air sampler (MAS-100, MERCK). *Cladosporium* sp. and *Epicoccum nigrum* were isolated from AUC parchment documents using a washing method.

Spore Suspension Preparation

A 6 mL spore suspension was prepared for each AUC fungal isolate (n=4), using sterile water as suspension medium. Concentrations were estimated in a Neubauer chamber and homogenised to 10^7 spores/mL. Each suspension was divided in four 1.5 mL micro tubes (one per fungus and per gamma radiation dose).

Irradiation Procedures

Samples micro tubes were irradiated in a cobalto-60 experimental source (Precisa22) located in the Nuclear and Technological Institute *campus* (Sacavém, Lisbon). Three gamma radiation doses were applied: 2 kGy, 6 kGy and 10 kGy, using a dose rate ranging between 2.45 kGy/h and 2.87kGy/h. Absorbed doses were monitored using calibrated routine dosimeters (maximum range variation dose of ± 2.5%; Perspex, Harwell).

DNA Extraction

The 16 spore suspensions were centrifuged, suspension medium was eliminated and the pellet was macerated with a lysis buffer overnight. Total genomic DNA was extracted using the extractor ABI Prism 6100 according to manufacturer instructions.

PCR Fingerprinting

Genetic *fingerprintings* for each dose and for the four fungal isolates (n=16 micro tubes) were obtained using PCR amplification with two different *primers* M13 (5'-GAGGGTGGCGGTTCT-3') and p888 (5'-DBDCACACACACACACACA-3', D is A, G or T and B is C, G or T) in separate reactions, followed by electrophoresis.

ITS fungal specific region *primers* (ITS1F and ITS4) were used as positive DNA extraction controls.

PCR mixtures reactions contained 4 µL of template DNA and 12.5 µL of Jump Start DNA polymerase with $MgCl_2$ (Sigma D 9307). According to each PCR

fingerprinting, it was used a 0.93µM concentration of M13 primer, 0.36µM for primer p888 and ITS primers, and sterile distilled water to a total volume of 28 µL.

The PCR termocycling conditions (Gene Amp PCR System 9700, Applied Biosystems (USA)) are described in table 1.

Table 1 – PCR termocycling conditions using p888, M13 and ITS *primers*

PCR Amplification steps	**p888 / ITS1F+ITS4**		**M13**	
	Temp. (ºC)	Time	Temp. (ºC)	Time
Initial Denaturation	95	2 min	95	2 min
Cycles	**35**		**40**	
Denaturation	95	1 min	95	45 s
Annealing	53	1 min	50	1 min
Extension	72	1 min	72	1.5 min
Final extension	72	5 min	72	6 min

PCR products were analysed by electrophoresis on a 1% agarose gel under non-denaturing conditions at 90 V for 90 min. HyperLadder II (50-2000 bp; Bioline) DNA ladder was used as molecular size marker. The gels were stained using Gel Red (Biotium) and documented by Molecular Imager Doc XR system 170-8170 EDU image acquisition system (BioRad).

Fingerprinting images were analysed using Gel doc XR+ 2.0.1 software.

Results

PCR genetic fingerprintings

Four fungi were used in these studies: *Penicillium griseofulvum*, *Neosartorya fumigata*, *Cladosporium* sp. and *Epicoccum nigrum*. The first two were isolated from AUC indoor air and the last two from AUC parchment documents. *P. griseofulvum* and *N. fumigata* were the most frequent fungi isolated from AUC indoor air in 2010. *Cladosporium* sp. was selected considering its common presence in archive and library documents (Mesquita, 2009) and its reported high gamma radiation resistance (Dadachova and Casadevall, 2008). *E. nigrum* was selected due to its unusual visible characteristics (e. g. pigment production).

PCR *fingerprintigs* were obtained for non-irradiated and irradiated fungal suspension using two *primers*: the universal minisatellite *primer* M13 and the degenerate *primer* p888, used in fungal microsatellite *fingerprinting* techniques. These *primers* link to the genome regions which present *tandem* repeated sequences, allowing the multi-bands *fingerprintings* achievement (Bachmann, 1992; Karp *et al*, 1996). Figure 1 shows the positive controls for fungal DNA extraction (ITS primers), and the obtained fungal PCR fingerprintingings are presented in Figure 2. An attempt was made to use RAPD fingerprinting technique (*primers* OPC15 and OPC19) however no genetic profiles were obtained for the four fungal isolates (data not shown).

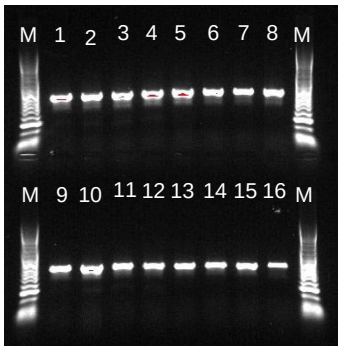

Figure 1 – ITS amplification results: Positive control for DNA extraction. M – DNA Leader; 1 – 0kGy-*P. griseofulvum*; 2 – 2kGy- *P. griseofulvum*; 3 – 6kGy-*P. griseofulvum*; 4 – 10kGy-*P.griseofulvum*; 5 – 0kGy-*N. fumigata*; 6 – 2kGy-*N. fumigata*; 7 – 6kGy-*N. fumigata*; 8 – 10kGy-*N.fumigata*; 9 – 0kGy-*Cladoporium* sp.; 10 – 2kGy-*Cladosporium* sp.; 11 – 6kGy-*Cladosporium* sp.; 12 – 10kGy-*Cladosporium* sp.; 13 – 0kGy-*E. nigrum*; 14 – 2kGy-*E.nigrum*; 15 – 6kGy-*E.nigrum*; 16 – 10kGy-*E. nigrum*

The ITS region PCR amplification results (figure 1) show that total DNA was extracted for all the spore suspensions (4 fungi and 4 gamma radiation doses).

In a global analysis, M13 *fingerprintings* presented higher number of bands than p888 genomic profiles. Higher differences were notice for higher doses in almost all fungal isolates especially using M13 *primer*. *Penicillium griseofulvum* and *Epicoccum nigrum* pointed out to be the most sensitive fungal isolates using both *primers*. Both fungal isolates presented higher alterations for irradiated genomic profiles. *P. griseofulvum* almost double the initial genomic profile bands number in 10 kGy *fingerprinting* for both M13 and p888 *primers*. Also, for 10 kGy *E. nigrum* M13 genomic profile did not presented any band and the p888 *fingerprinting* has undergone several alterations. *Cladosporium* sp. and *Neosartorya fumigata* seemed to have fewer bands alterations in both M13 and p888 *fingerprintings,* which could indicate higher gamma radiation resistance (figure 2).

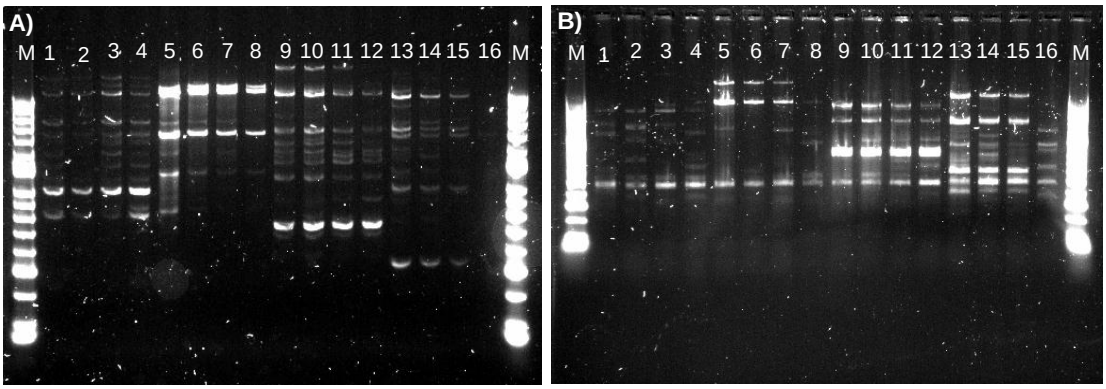

Figure 2 – M13 (A)) and p888 (B)) *fingerprinting* results. M – DNA Leader; 1 – 0kGy-*P. griseofulvum*; 2 – 2kGy-*P. griseofulvum*; 3 – 6kGy-*P. griseofulvum*; 4 – 10kGy-*P.griseofulvum*; 5 – 0kGy-*N. fumigata*; 6 – 2kGy-*N. fumigata*; 7 – 6kGy-*N. fumigata*; 8 – 10kGy-*N.fumigata*; 9 – 0kGy-*Cladoporium* sp.; 10 – 2kGy-*Cladosporium* sp.; 11 – 6kGy-*Cladosporium* sp.; 12 – 10kGy-*Cladosporium* sp.; 13 – 0kGy-*E. nigrum*; 14 – 2kGy-*E.nigrum*; 15 – 6kGy-*E.nigrum*; 16 – 10kGy-*E. nigrum*

The genomic profile analysis was performed considering the number of bands and the corresponding molecular weight. Tables 2 and 3 present the achieved results.

Table 2 results showed a decrease in the number of bands with the increase of gamma radiation dose for *Cladosporium* sp., *E. nigrum* and *N. fumigata*.

Table 2 – M13 *fingerprinting*: Bands number and molecular weight analysis. A) *P. griseofulfum*; B) *N. fumigata;* C) *Cladosporium* sp. and D) *E. nigrum.*

A) *Penicillium griseofulvum*				
Bands Molecular Weight (bp)	Gamma Radiation Doses (kGy)			
	0	2	6	10
4758	-	-	+	+
4256	-	-	+	+
3751	+	+	+	+
2376	+	+	+	+
2001	+	+	+	+
1779	-	-	+	+
1461	+	-	+	-
1230	+	-	+	+
993	-	-	-	+
828	+	+	+	+
654	-	-	-	+
596	+	+	+	+
Total	7	5	10	11

B) *Neosartorya fumigate*				
Bands Molecular Weight (bp)	Gamma Radiation Doses (kGy)			
	0	2	6	10
3798	+	+	+	+
2413	+	+	+	-
2006	+	+	+	+
1514	+	-	-	-
1116	+	+	+	+
609	+	-	-	-
Total	6	4	4	3

C) *Cladosporium* sp.				
Bands Molecular Weight (bp)	Gamma Radiation Doses (kGy)			
	0	2	6	10
5653	+	+	+	-
3695	+	+	+	+
2649	+	-	-	-
2118	+	+	+	+
1880	-	+	-	-
1749	+	+	+	-
1581	+	-	-	+
1509	-	+	-	+
1433	+	-	+	-
1306	+	+	+	+
1031	+	+	+	+
737	-	-	-	+
563	-	+	-	-
484	+	+	+	+
427	+	+	+	+
Total	11	11	9	9

D) *Epicoccum nigrum*				
Bands Molecular Weight (bp)	Gamma Radiation Doses (kGy)			
	0	2	6	10
3892	+	-	-	-
3554	+	+	+	-
3068	+	-	-	-
2367	+	+	-	-
2157	+	+	+	-
1959	+	+	+	-
1591	+	-	-	-
1421	-	+	-	-
1144	+	+	-	-
883	+	+	+	-
274	+	+	+	-
Total	10	8	5	0

Table 3 – p888 *fingerprinting*: Bands number and molecular weight analysis. A) *P. griseofulfum*; B) *N. fumigata;* C) *Cladosporium* sp. and D) *E. nigrum.*

A) *Penicillium griseofulvum*				
Bands Molecular Weight (bp)	Gamma Radiation Doses (kGy)			
	0	2	6	10
3874	-	-	+	-
3271	+	-	-	-
3052	-	+	+	+
2075	+	+	+	+
1696	+	-	-	+
1386	-	+	-	-
845	-	+	-	-
705	-	-	-	+
520	-	+	+	+
437	+	+	+	+
327	-	-	-	+
Total	4	6	5	7

B) *Neosartorya fumigata*				
Bands Molecular Weight (bp)	Gamma Radiation Doses (kGy)			
	0	2	6	10
6323	+	+	+	-
3787	+	+	+	+
1882	+	-	+	-
1299	-	-	-	+
635	-	+	-	-
558	-	-	-	+
459	+	+	+	+
247	+	-	+	-
Total	5	4	5	4

C) *Cladosporium sp.*				
Bands Molecular Weight (bp)	Gamma Radiation Doses (kGy)			
	0	2	6	10
3457	+	+	+	+
2332	+	+	+	+
1423	-	+	-	+
1041	+	+	+	+
705	-	-	+	+
572	-	+	-	-
471	+	+	+	+
354	-	-	-	+
Total	4	6	5	7

D) *Epicoccum nigrum*				
Bands Molecular Weight (bp)	Gamma Radiation Doses (kGy)			
	0	2	6	10
4489	+	+	+	-
2332	+	+	+	-
1845	-	-	-	+
1579	-	+	-	-
1253	+	+	+	+
1097	-	-	-	+
1001	+	-	-	-
963	-	+	-	-
835	+	-	-	-
705	-	-	-	+
638	+	+	+	-
483	+	+	+	+
381	+	+	+	-
336	-	-	-	+
Total	8	8	6	6

Simultaneously to band number decrease, *Cladosporium* sp. seems to suffer alteration in band molecular weight (Table 2-C). Some of these alterations could result from initial bands shortening (e.g. 1749bp initial band and 1509bp 10 kGy band). *E. nigrum* seems to present the same effect. The initial 1591bp band could result in 1421bp 2kGy band (Table 2-D)). *N. fumigata* genomic profiles only show band number alteration (Table 2-B)). *P. griseofulvum* present a decrease in band number for 2 kGy. For 6 kGy and 10 kGy the initial band number and corresponding molecular weights are maintained but new bands were observed.

Overall, for p888 *fingerprinting* no tendencies in the band number was noticed with gamma radiation doses (table 4). *E. nigrum* seems to have a decrease in the band number with gamma radiation dose increase (table 3-D). In other hand, *P. griseofulvum* and *Cladosporium* sp. present a higher band number for irradiated *fingerprintings* than for non-irradiated (table 3-A,C). As in M13 *fingerprintings*, p888 genomic profiles show alterations in band molecular weight for irradiated samples. Some “new” bands in irradiated samples fingerprinting also seems to result from initial band shortening.

N. fumigata presented identical p888 *fingerprintings* for 0 kGy and 6 kGy (Table 3-B). However, for 2 kGy both band number and molecular weight were altered.

Conclusions

Gamma radiation is a known random mutation inducer agent (Gardner & Snustad, 1987 cited by Cordeiro *et al*, 1995; Griffiths *et al*, 2008). This potentiality had been used in industry to achieve new products or reduce contaminants (Zeinab *et al*, 2001; Aziz and Moussa, 2002; Haggag and Mohamed, 2002; Prado *et al*, 2003; Jitareerat *et al*, 2005). Deletions were reported as the most frequent genetic alterations in gamma irradiated survivor cells (Casarett, 1968). Considering these mutagenic gamma radiation characteristics and PCR *fingerprinting* concepts, it should be possible to detect genetic alterations between individuals exposed to different gamma radiation doses using PCR *fingerprinting* techniques. In this way, both *fingerprintings* bands number and molecular weight alterations were expected. Schlick *et al* (1994) had detected genetic alterations in *Trichoderma harzianum*, induced by a 64 kGy gamma radiation dose, using PCR *fingerprinting* techniques. In our work we had tested this PCR *fingerprinting* potentiality using lower gamma radiation doses.

As expected, alteration in band number and weight were detected resulting from gamma radiation exposure. However, a single alteration pattern was not detected as a direct result from increasing gamma radiation doses, for both PCR *fingerprintings*. In some cases, a decrease in band number was achieved and in other cases the opposite was observed. Some bands detected in irradiated samples have lower molecular weight than initial profile bands which could result from terminal segments deletion. However, deletion effect could alter fungal *fingerprintings* in different ways: i) Altering primers ligation sequence blocking band detecting (missing bands), ii) Extending an initial primer ligation sequence allowing the

detection of higher molecular weight bands, or iii) Changing the initial non-recognised sequence allowing primers ligation (new bands).

Based on the obtained results, *fingerprinting b*and alterations were visible even for 2 kGy. However, the applied technique did not allow the mutation type characterization. More studies, using other methods, are needed to understand the detected mutations function: Was it a silent mutation? Were Some organism's characteristics missed (non-sense mutation)? Or a new function was achieved (missense mutation)?

Furthermore, if gamma radiation is known for random genetic mutation induction, the reproducibility of the results must be evaluated to verify if there is a pattern. Otherwise, the replication of this preliminary study is significant to understand *N. fumigata* p888 genomic profile for 6 kGy (0 kGy and 6kGy *fingerprintings* are identical but alterations were verified in 2 kGy *fingerprinting*). Moreover, the absence of *E. nigrum* M13 fingerprinting for 10 kGy must be verified, if is an effective outcome or is resulting from some problems in PCR amplification and/or electrophoresis (ITS DNA extraction positive control did not present any problem).

Our results point out to a possible gamma radiation resistance differentiation. The most resistant fungal isolates presented few *fingerprintin*g bands alterations. To our knowledge no references were found for gamma radiation resistances of *E. nigrum* and *N. fumigata*. However, some *Cladosporium* species have been reported as highly resistant to gamma radiation. Some species present D-values higher than 5 kGy (Silva *et al*, 2006; Dadachova and Casadevall, 2008). Yun *et al* (2008) had described the D-value of *P. griseofulvum* ranging from 0.28 kGy to 0.48 kGy, depending to the used substrate (water or apple model system, respectively). According to the presented analysis, *E. nigrum* and *P. griseofulvum* seems to present lower gamma radiation resistance whereas *Cladosporium* sp. and *Neosartorya fumigata* seems to present higher gamma radiation resistance.

Future work is needed to optimize (e.g. other *primers*) the applied methodology to become more discriminative and specific in the detection of genetic mutation, in order to understand the gamma radiation effects in fungal species.

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Final Considerations

The four studies presented in paper form in this thesis were made in order to achieve one global goal: the AUC Parchment documents preservation using Gamma Radiation. Sub-objectives, such as understanding the air role in documents contamination or studying the gamma radiation treatment feasibility had been defined and some conclusions can only be achieved with a transversal analysis relating the four different articles results. The conclusions present below result from this correlation analysis.

AUC Indoor Air and AUC Parchment documents microbiota was characterized (Paper I and II). Both morphological types and fungal genera results are according to the previously describe in literature for biocontamination in similar environments and documents (Maggi *et al*, 2000; Szczepanowska and Cavaliere, 2000; Tiano 2002; Cappitelli and Sorlini, 2005; Borrego *et al* 2008; Bogomolova and Kirtsideli, 2009; Mesquita *et al*., 2009; Borrego *et al*, 2010; Sterflinger, 2010; Guiamet *et al*, 2011).

Results achieved for AUC Indoor Air (Paper I) and AUC Parchment Documents (Paper II) microbiota characterization are compared in table 1.

Table 5 – Morphological types of the isolates from AUC Indoor air and AUC Parchment documents.

Morphological type	Indoor Air	Parchment Documents	
		B.I.	A.I
Gram-positive, Catalase-positive Cocci	+	+	+
Gram-positive, Catalase-negative Cocci	-	-	-
Gram-negative, Catalase-positive Cocci	+	+	+
Gram-negative, Catalase-negative Cocci	-	-	-
Spore forming Gram-positive Rods	+	+	+
Non-Spore forming, Gram-positive, Catalase-positive Rods	+	-	-
Non-Spore forming, Gram-positive, Catalase-negative Rods	+	+	+
Gram-negative, Oxidase-positive Rods	+	+	+
Gram-negative, Oxidase-negative Rods	+	+	+
Yeasts	-	+	-
Filamentous Fungi	+	+	+

B.I. – Before Irradiation; A.I. – After Irradiation

As we could verify in table 1, all the morphological types present in Parchment documents are also present in AUC indoor air, with exception for Yeasts. However, this morphological type presents a low relative frequency (0.03%). The obtained morphological type correspondence could indicate the AUC indoor air as a possible parchment documents contamination vector. To further analyse this relation, fungal molecular identification for AUC indoor air and AUC Parchment documents isolates was also likened (table 2).

Table 6 – Fungal genera isolated from AUC Indoor Air and AUC Parchment Documents

Fungal Species	Indoor Air	Parchment Documents	
		B.I.	A.I
Aspergillus versicolor	**+**	**-**	**-**
Aspergillus ochraceus	**+**	**-**	**-**
Aspergillus fumigatus	-	+	+
Fusarium solani	+	-	-
Fusarium proliferatum	+	-	-
Fusarium sp.	+	-	-
Penicillium sp.	+	+	-
Penicillium oxalicum	+	-	-
Penicillium griseofulvum	+	-	-
Penicillium commune	-	+	+
Penicillium spinulosum	-	+	-
Neosartorya fumigata	+	-	-
Alternaria sp.	+	+	-
Acremonium sp.	+	-	-
Chaetomium nigricolor	+	-	-
Chaetomium globosum	+	-	-
Chaetomium murorum	-	-	-
Paecilomyces lilacinus	+	-	-
Cladosporium cucumerinum	-	+	-
Cladosporium cladosporioides	-	+	-
Cladosporium phaenocomae	-	+	-
Cladosporium sp.	-	+	-
Epicoccum nigrum	-	+	-
Phoma glomerata	-	-	+
Coprinelos sp.	-	+	-

B.I. – Before irradiation; B.I. – After Irradiation

Previously studies reports the existence of a relation between Air and libraries/archives documents fungal contaminants (Nugari, 1993; Borrego *et al*, 2010; Niesler, 2010). The number of fungal genera detected is equal for the two studied environments (n=8). Four fungal genera were only present in AUC Parchment documents and another 4 fungal genera were only present in AUC indoor air. *Aspergillus, Penicillium, Alternaria* and *Chaetomium* genera were present in both air and parchment documents. Regarding species (table 2) only two correspondences were found between the two different environments: *Penicillium* sp. and *Alternaria* sp. The obtained results were inconclusive relatively to the air role as a parchment contamination vector. More studies are needed in order to understand which factors (e.g. seasonality) could alter the fungal AUC airborne population, promoting the *Cladosporium*, *Epicoccum*, *Phoma* and *Coprinelos* genera as well as different parchment fungal species proliferation. Studies performed previously (2008) had detected *Cladosporium cladosporioides*. in AUC indoor air.

Studies in archive and library materials decontamination using gamma radiation had been performed since the early 60's (Sinco, 2000; Magaudda, 2004). Among material types, the majority of the studies were conducted in paper documents (Adamo *et al*, 2001; Gonzalez *et al*, 2002; Magaudda, 2004). In this work, gamma radiation was evaluated in

order to decontaminate parchment documents (Paper II), as well as the potential structural damages induced by the treatment in this complex ancient material (Paper III).

Considering the natural contamination and *Cladosporium cladosporioides* inactivation studies (Paper II), along with the Parchment texture and colour response to gamma radiation (Paper III), it is proposed a minimal dose of 5 kGy as decontamination treatment, being aware that 30 kGy does not seems to change colour and texture parchment characteristics. Although the absence of information about decontamination studies in parchment, our results seems to be according to the determined gamma radiation doses applied in paper materials decontamination. Adamo and Magaudda (2003) report gamma radiation doses ranging from 3 to 10 kGy as effective in microfungal decontamination without significant material alterations. Higher doses (around 15 kGy) have been described for archive and library paper materials disinfection with no visible alterations in initial material characteristics (Sinco, 2000; Gonzalez *et al*, 2002; Rizzo *et al*, 2002; Rocchetti *et al*, 2002).

Considering our results, a “study case” decontamination treatment was applied to a recently restored book "Livro de Registos da Chancelaria da Sede Vacante (1647-49)" (LRCSV), a manuscript document dated from XVII century and currently in the safekeeping of the AUC. LRCSV is constituted by a parchment cover and ninety three “rag fibre” paper sheets (Ferreira, 2010). During 2010, the book was restored according to the minimal intervention and material reversibility principles by Sandra Ferreira, as part of her master degree in Restoration and Conservation (Ferreira, 2010).

Figure 1 – LRCSV book

The LRCSV study case consisted in the evaluation of the inactivation efficiency of book microbial population using incremental doses of gamma radiation. The higher dose applied was 3 kGy, and it was not visible any alterations in documents colour and texture (documented by photography). The 3 kGy dose is lower than the proposed D_{min} of 5 kGy. However, this adjustment had in account that LRCSV is constituted by two different material types (Parchment and paper), and that this book is not a sample that could be destroyed. The obtained results indicated that 3 kGy decrease the initial LRCSV contamination ($< 10^2$ CFU/sheet), resulting in an inactivation efficiency of 44% for fungal population.

Furthermore, the obtained results suggest that the application of gamma radiation as decontamination treatment for ancient art should be a case specific considering each artefact characteristics. Rather than a unique D_{min} proposal it must be referred a dose range for radiation treatment, as mentioned by Magaudda (2004) which suggest a dose range between 3 kGy and 8 kGy as secure in paper decontamination treatments. We propose a 5-10kGy range for the same treatments in parchment documents.

Four AUC Fungal isolates spore suspensions were irradiated at three different gamma radiation doses (Paper IV). Genetic alterations were detected using PCR *fingerprinting* techniques with M13 and p888 *primers*. Results point out that genetic profile variations were visible since 2 kGy. For 6kGy (a gamma radiation dose near the proposed 5kGy parchment decontamination dose), alterations were more visible. There are few studies using PCR *fingerprinting* as detection method for gamma radiation induced mutations. Schlick *et al* (1994) used similar PCR techniques to detected gamma radiation induced mutations in fungi for 64 kGy. In this preliminary study it was observed that this technique was efficient to detect fungal genetic profile alterations for lower doses, but its reproducibility must be evaluated. More studies are needed using higher doses (until the described 30 kGy maximal decontamination treatment dose) in order to understand if there is a pattern between genetic alterations and gamma radiation dose increase.

Future Work

In this kind of work, new topics are always found. The initial project diverge in different directions and areas, and new issues were faced in order to achieve better or new solutions. The preservation of Archive documents using gamma radiation is an open subject in which much more work must be performed.

Considering our particular results for AUC case, future work has to be developed in different fields. In first place, new studies are needed in order to understand the seasonality effects in biological indoor air contamination, as well as to understand if fungal communities' constitution could be associated to specifically seasons. Is there an indoor air contamination pattern for each season? Moreover, more studies must be performed to evaluate other contamination vectors such as insects.

All the inactivation studies were made using a destructive bioburden determination method. A non-destructive methodology should be developed to assess parchment bioburden. Furthermore, more samplings are required which includes other parchment types in order to recognize if there is a pattern between its origin (animal type and production date) and its biocontamination type, especially its fungal contamination. It is also essential to verify the biocontamination inactivation patterns as well as the gamma radiation effects in colour and texture characteristics in pigmented parchment samples.

At last, for the characterization of gamma radiation induced mutations in decontamination treatments, more fungal isolates and other primers should be tested.

Papers I and II will be submitted respectively to *International Biodeterioration and Biodegradation* and *Radiation, Physics and Chemistry* journals. The last two papers were preliminary approaches and need more studies for publication.

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