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Fast molecular methods for the detection of spoilage fungi (in food products)

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DOCTORAL THESIS

FAST MOLECULAR METHODS FOR THE DETECTION OF SPOILAGE FUNGI (IN FOOD PRODUCTS)

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Fast molecular methods for the detection of spoilage fungi (in food products)

Dª. Marta Prado

D. Alejandro Garrido-Maestu

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Fast molecular methods for the detection of spoilage fungi (in food products)

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RESUMO

Unha das principais preocupacións para as autoridades gobernamentais a nivel mundial é a identificación de medidas para previr a perda de alimentos nos primeiros pasos da cadea de distribución pero tamén a nivel de venta ao por menor e de consumidor. Segundo a Comisión Europea (CE), anualmente xéranse 88 millóns de toneladas de residuos de alimentos na UE, cun custo estimado de 143 millóns de euros. Segundo a Organización das Nacions Unidas para a Alimentación e a Agricultura (FAO), globalmente un tercio de todos os alimentos producidos acaban en residuos ou perdas. Ademais, segundo o índice de perda de alimentos (FLI) da FAO, o 14 % dos alimentos producidos pérdese entre a post-colleita ata o comercio polo miúdo. Os residuos de alimentos non só teñen un impacto económico e ético, senón que leva ao esgotamento dos recursos naturais. Como resultado, a UE comprometeuse a reducir a perda de alimentos ao longo da cadea de produción e subministración e a reducir á metade os residuos de alimentos per cápita a nivel de venda polo miúdo e ao consumidor para o 2030. Neste sentido, o deterioro microbiolóxico xoga un papel importante na perda de alimentos e en particular, especialmente a causada por fungos. Ademais, os fungos toxigenicos, en condicións favorables, poden producir metabolitos secundarios denominados micotoxinas. Algunhas das micotoxinas poden ser extremadamente perigosas para a saúde humana e animal debido aos seus efectos cancerígenos e teratogénicos, pero tamén poden causar perdas económicas ao longo da cadea de subministración. Como resultado, a contaminación dos alimentos con fungos micotoxixénicos é de gran importancia debido aos problema que ocasiona tanto a seguridade alimentaria como ao comercio de alimentos.

A día de hoxe, a detección de levaduras e fungos realízase fundamentalmente empregando técnicas tradicionais de microbioloxía clásica. Estas técnicas son laboriosas, implican un elevado consumo de tempo e requieren un enorme gasto de reactivos. Así, o tempo global de análise pódese estender ata 7 días, facendo que estas técnicas non sexan adecuadas para alimentos cunha vida útil curta e os sistemas de produción intensiva que existen hoxe en día. Por conseguinte, os resultados obtidos só se poden usar retrospectivamente e por tanto teñen un valor limitado para a calidade e o control do proceso. Outra desvantaxe destes métodos é que non poden detectar microorganismos viables pero non cultivables (VBNC). Este tipo de vida microbiana pode ser o resultado dos procesos de limpeza e/ou actividades de desinfección que fan que as células entren en un estado de actividade metabólica moi baixa e por tanto non crecen empregando as técnicas tradicionais de microbioloxía., Pero aínda en ese estado metabólico, estas células xogan un papel importante nos estragos producidos por enzimas ou outros metabolitos de importancia. Como consecuencia, son necesario métodos máis rápidos e sensibles para a detección de fungos que reduzan os custos xerais da industria alimentaria debido as perdas de produto e ás longas análises.

Durante as últimas décadas xurdiron técnicas de amplificación baseadas no ADN co obxectivo último de superar algúns dos inconvenientes dos métodos tradicionais de microbioloxía. Estes métodos baseados na detección de ADN destacan pola súa alta sensibilidade e especificidade. En particular, a reacción en cadea da polimerasa (PCR) e a PCR cuantitativa (qPCR) son moi utilizados para a detección de levaduras e mofos alterantes nunha grande variedade de produtos alimentarios. Unha das principais limitacións da maioría dos ensaios de PCR/qPCR desenrolados ata o momento, é que non inclúen un control de amplificación interna (IAC). Básicamente, o IAC é un ADN non diana (é decir, un fragmento de ADN engadido artificialmente a mostra) implementado no ensaio que co-amplifica co xene obxectivo e sempre debe amplificar en mostras negativas. Neste sentido, pódense evitar falsos negativos por inhibición da reacción.

Ultimamente, alternativamente a PCR/ qPCR, apareceron técnicas de amplificación de ácido nucleico isotérmico permitindo a simplificación dos ensaios e os custos de redución. Estes ensaios só precisan un baño de auga ou un bloque de calor xa que funcionan a unha temperatura constante e, como resultado, non hai necesidade de equipos caros como un termociclador. Ademais, dado que os resultados na maioría dos casos poden visualizarse a simple vista, estes ensaios pódense implementar en dispositivos portátiles para aplicacións en puntos de interese. Entre as diferentes técnicas de amplificación isotérmica, a amplificación isotérmica mediada por bucle (LAMP) e a amplificación da polimerase de recombinase (RPA) son as máis populares para a detección de microorganismos. A pesar de que se informaron de moitos ensaios para a detección de bacterias e virus patóxenos, a aplicación destes métodos no campo dos fungos transmitidos por alimentos segue sendo limitada e está centrada principalmente na detección de fungos micotoxixénicos. Os resultados pódense visualizar de moitos xeitos diferentes segundo o ensaio, incluída a detección a simple vista do cambio de cor, electroforese en xel, tiras de fluxo lateral e fluorescencia en tempo real. Ademais, demostrouse que estas técnicas son máis robustas contra os inhibidores comúns de PCR que poden levar a falsos negativos.

Unha das principais limitacións de todos os métodos baseados na detección de ADN é que non poden diferenciar entre microorganismos vivos e mortos. Isto é debido a que o ADN é unha molécula estable e, polo tanto, pode persistir e estar presente durante algún tempo despois da morte do microorganismo. Un enfoque para a detección de microorganismos viables basease na detección de ARN por transcriptasa inversa (RT-PCR) ou RT-qPCR. Non obstante, a manipulación do ARN é máis difícil xa que debe ser manipulado con coidado durante a extracción por mor das ARNasas, as enzimas que dixeren o ARN, xa que están omnipresentes no ambiente. Ademais, a contaminación pode producirse debido a un procesamento de mostras inadecuado, condicións de almacenamento ou contaminación con encimas degradantes do ARN. Outro inconveniente deste enfoque é que é necesario un paso de retrotranscrición para converter o ARN en ADN complementario (ADNc). Outro enfoque que se explorou ultimamente, implica o uso de colorantes intercalantes como PMA e EMA, que poden penetrar en células mortas e despois da fotoactivación, unirse irreversiblemente ao ADN, bloqueando así a súa amplificación. Unha das principais vantaxes do emprego de estes reactivos químicos é que se poden implementar fácilmente nos protocolos de extracción de ADN e tamén son compatibles con moitas técnicas de amplificación. Ademais, con estes colorantes é posible a detección de microorganismos en estado VBNC.

O obxectivo desta tese foi o desenvolvemento e avaliación de diferentes métodos moleculares para a detección de fungos responsables do deterioro das froitas, así como de fungos micotoxixénicos, co obxectivo global de reducir o tempo total de análise, mantendo a sensibilidade en comparación cos métodos convencionais. Para conseguilo, propuxéronse diferentes obxectivos específicos.

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O primeiro obxectivo específico compredeu o desenrolo dun protocolo para o pre-enriquecemento, o tratamento de mostras e a extracción do ADN para a detección de fungos de interese nas matrices de alimentos seleccionados. Para lograr este, púxose a proba, de xeito que se puidese seleccionar o óptimo para o crecemento dos fungos de interese. Con base en estudos de cinética e nos protocolos existentes no sitio do noso socio industrial, MEB foi o caldo seleccionado para o paso previo ao enriquecemento. En continuación, avaliáronse diferentes volumes de mostra e condicións de incubación e cas condicións óptimas foron escollidas tendo en conta a experiencia anterior do socio industrial. Adicionalmente, o tratamento da mostra optimizouse con pasos de centrifugación diferencial, co fin de eliminar os restos de alimentos e concentrar os microorganismos antes da extracción do ADN. Finalmente, elixiuse un kit comercial para a extracción do ADN. Co protocolo de extracción optimizado, obtivose ADN de alta calidade.

O segundo obxectivo específico implicou o desenrolo e avaliación dun método de qPCR para a detección de fungos totales, "panfungal", cun NC-IAC, que despois podería servir como método de referencia para técnicas de amplificación isotérmicas do ADN. Neste sentido, seleccionouse un conxunto de cebadores universais dirixidos a unha rexión común dus fungos e unha sonda de hidrolise foi deseñada en este estudo. A especificidade do ensaio foi evaluada fronte a un panel de microorganismos que se atopan frecuentemente nos produtos alimentarios. Ademais, logrouse unha sensibilidade analítica de 10.4 fg/ µL de ADN puro. O último paso no desenvolvemento do ensaio panfungal qPCR foi a avaliación do método en mostras reais. Como resultado, as mostras de preparación de froitas foron inoculadas con diferente concentracións de levaduras e mofos, e determinouse o límite de detección (LOD) do ensaio. Os valores LOD_{95} obtidos foron 3.9 CFU/ 50 g para levaduras, 1.2×10^2 esporas/ 50 g e 3.7×10 esporas/ 50 g para mofos despois de 24 e 48 h de pre-enriquecemento, respectivamente. O LOD calculado para levaduras estaba dentro do rango de otros estudios realizados para a detección de levaduras alterantes dos alimentos, destacando así a alta sensibilidade do noso ensaio. A partir dos valores de LOD_{95} obtidos, calculáronse os parámetros de rendemento do método. Os valores obtidos foron superiores ao 85.0 % e o k de Cohen por encima de 0,86 para todos os ensaios, o que significa que o noso método estaba case en concordancia completa co método de referencia, neste caso os métodos baseados en microbioloxía clásica. En xeral, o método desenvolvido resultou moi sensible para a detección de fungos e, en consecuencia, serviu como método de referencia para as técnicas de amplificación isotérmica que se desenvolveron máis tarde.

O terceiro obxectivo específico desta tese implicou o desenvolvemento e avaliación de diferentes técnicas de amplificación isotérmicas xunto coa detección a simple vista para microorganismos alterantes e fungos micotoxixénicos. Para este fin, desenvolvéronse dous métodos panfúngicos, un ensaio de amplificación isotérmica mediada por bucle (LAMP) dirixido ao xene 18S rRNA e un ensaio RPA dirixido á rexión ITS. En máis detalles, no ensaio de LAMP visualizáronse os resultados con fluorescencia en tempo real, pero tamén a simple vista por cambio de cor, mentres que para as tiras de fluxo lateral RPA usáronse para a detección a simple vista. Ademais, desenvolveuse e avaliouse un ensaio RPA xunto con SYBR Green I para a detección de fungos produtores de patulina.

En canto ao ensaio panfungal baseado en LAMP, deseñouse un conxunto completo de cebadores co programa informático Primer Explorer v.4 e a súa inclusividade/ exclusividade foi valorada *in vitro* contra un panel de fungos e bacterias que se atopan habitualmente nos produtos alimentarios. Ademais, ao probar as dilucións seriadas obtivose unha sensibilidade analítica de 1.4 pg ADN levadura/ reacción foi alcanzada tanto polo ensayo fluorescente como polo colorimétrico. Ademais, cando se usou o ADN puro de *Neosartorya fischeri*, detectouse ata 17 pg ADN/ reacción e 170 pg ADN/ reacción co ensaio fluorescente e o colorimétrico, respectivamente. No último paso, a avaliación do método realizouse en preparados de amora e piña con diferentes concentracións de

levaduras. O método de referencia de comparación foi o ensaio Panfungal qPCR anteriormente desenvolvido e, ademais, os resultados foron comparados con un conxunto de cebadores de LAMP descritos previamente para a detección panfungal, dirixido ao mesmo xene. A detección realizouse tanto mediante observación do cambio de cor a simple vista, como a fluorescencia en tempo real para comparación directa. Así, en total avaliáronse catro metodoloxías diferentes. O LOD_{95} obtido foi de 3.1 CFU/ 50 g para os dous ensaios fluorescentes, polo que ambos os ensaios foron igualmente sensibles. En canto aos ensaios colorimétricos, o desenvolvido neste estudo resultou ser máis sensible cun LOD_{95} de 3.0 CFU/ 50 g, mentres que o usado para a comparación resultou nun LOD_{95} de 10.9 CFU/ 50 g. Ademais, o LOD obtido foi similar ao informado para a detección de levaduras por qPCR nesta tese (LOD_{95} 1.0 CFU/ 50 g). Por conseguinte, o ensaio de LAMP recentemente desenvolvido foi tan sensible como o método de referencia, pero coas vantaxes engadidas de que o ensaio colorimétrico pode usarse para a detección en punto de interese en lugar de en laboratorios especializados de bioloxía molecular, y tamén produce unha redución de custos xa que non hai necesidade de equipos caros como un termociclador de tempo real. Con base nos LODs obtidos, determináronse os parámetros de calidade e en todos os ensaios os valores obtidos foron por encima do 90 % con algunhas excepcións para cada ensaio que foi o resultado de poucos PDs e/ou ND_S. Isto pode ser o resultado da contaminación cruzada xa que nos ensaios de LAMP o risco de contaminación cruzada é moi elevado a través de produtos aerosolizados debido á alta produción de ADN que pode levar a resultados falsos positivos. Finalmente, baseándose nos valores k de Cohen, o recén desenvolvido ensaio colorimétrico estivo en "concordancia case completa" co método de referencia, é dicir, o ensaio panfugal qPCR, mentres que o fluorescente estivo en "acordo substancial".

En canto ao ensaio de RPA panfungal, os cebadores universais ITS3/ITS4 foron seleccionados e etiquetados con digoxigenina e biotina, respectivamente. Neste sentido, evitouse o uso dunha sonda *nfo*, especialmente tendo en conta que TwistDX (o distribuidor principal dos reactivos RPA) descontinuou recentemente o kit *nfo* para a detección de fluxo lateral. A especificidade dos cebadores probouse como se explicou anteriormente para os outros ensaios. Todos os cebadores presentaron unha boa inclusividade cara aos fungos seleccionados e a boa exclusividade xa que todas as bacterias seleccionadas non se ampliaron. Ademais, co ensaio desenvolvido, lograrouse unha sensibilidade analítica de 1.2 pg ADN/ µL, que abarca un rango dinámico de 4 logaritmos, tanto para levaduras como para mofos. A avaliación do método Panfungal RPA-LF realizouse tanto en levaduras como en mofos en mostras de marmelada de amoras. O LOD_{50} atopouse como 1.0 CFU/ 50 g para levaduras e 47.5 esporas/ 50 g para mofos, respectivamente. O LOD_{50} obtido para levaduras foi o mesmo que o informado para os ensaios de qPCR e LAMP Panfungal desenvolvidos nesta tese, o que indica que todos os métodos desenvolvidos durante este estudo foron igualmente sensibles para a detección de levaduras. Finalmente, todos os parámetros de calidade estaban por encima do 80 % e o k de Cohen foi 0.77 tanto para a detección de levaduras como para a detección de mofos, o que significa que os métodos desenvolvidos estaban de "acordo substancial" co método de referencia.

O último obxectivo específico desta tese foi o desenrolo dunha metodoloxía para a diferenciación de fungos viables e non viables empregando colorantes intercalantes combinados con métodos baseados en moleculares. Desenvolvéronse e evaluáronse dous métodos baseados en técnicas moleculares, concretamente un qPCR e un RPA xunto con SYBR Green I (RPA-SG), para a detección de fungos produtores de patulina en mazás e subprodutos. Ademais, a detección de só fungos viables lograrouse coa implementación de propidio monoazida (PMA) durante o tratamento da mostra.

En canto ao ensaio qPCR, elixiuse un conxunto de cebadores PCR publicados anteriormente dirixidos ao xene *idh* e unha sonda fluorescente foi deseñada específicamente para ese estudo. A inclusividade/ exclusividade dos cebadores avaliouse como se describiu antes e todos os

oligonucleótidos foron específicos. O ensaio de qPCR cubriu un rango dinámico de 5 logaritmos ata 1.25 pg/ µl de ADN puro *Penicillium expansum*. A sensibilidade analítica reportada caeu dentro do rango de valores informados anteriormente para a detección de fungos micotoxixénicos. A avaliación do método realizouse en mazás picadas, puré de mazá e zume de mazá e o LOD_{50} calculouse como 8.1×10^3 esporas/ 5 g. Finalmente, todos os parámetros de calidade estaban por encima do 90 % e o k de Cohen foi de 0.93, o que indica que o ensaio estaba "en concordancia case completa" co método de referencia, neste caso o método baseado na cultura.

En canto ao ensaio RPA-SG, deseñouse un novo conxunto de cebador co uso do programa informático PrimedRPA. O xene diana foi o mesmo que o empregado no ensaio da qPCR xa que está implicado na vía biosintética da patulina. Unha vez máis, a especificidade dos cebadores avaliouse *in vitro* fronte a unha selección de microorganismos. Co ensaio desenvolvido, cubriuse un rango dinámico de 4 logaritmos detectando así ata 23.8 pg/ µl de ADN puro *P. expansum*. A avaliación do método global foi realizada mediante as mazás e os produtos das mesmas e o qPCR desenvolvido anteriormente para a detección de fungos produtores de patulina serviu como método de referencia. O LOD_{50} calculouse como 5.8×10^4 esporas/ 5 g. Finalmente, en función dos valores LOD obtidos, os parámetros de calidade atopáronse por encima do 90 % e o k de Cohen foi 0,92, o que significa que o ensaio estaba "en concordancia case completa" co método de referencia.

Ademais, neste estudo demostramos que a PMA podería bloquear de xeito eficiente a amplificación do ADN orixinario de esporas mortas con concentracións de ata 10^7 esporas/ mL. Ademais, non se observaron efectos tóxicos nas esporas vivas. Ademais, avaliouse o efecto da PMA en mostras de mazá picadas e unha vez máis a PMA bloqueou a amplificación do ADN a partir de esporas mortas nos mesmos rango de concentracións. Tamén se avaliou o efecto da PMA en mazás inoculadas cunha mestura de esporas vivas e mortas en diferentes relacións. Observouse que cando as mazás foron picadas con 10^8 esporas mortas, a PMA non inhibiu plenamente a amplificación xa que unha das tres réplicas foi positiva. Estes resultados suxeriron que a PMA pode inhibir a amplificación do ADN procedente de esporas mortas con concentracións de ata 10^7, pero a presenza de concentracións máis altas de microorganismos mortos poden dificultar a capacidade de bloqueo do colorante.

En xeral, na presente tese desenrolaronse e avaliáronse diferentes ensaios baseados en técnicas moleculares. A detección de fungos alterantes e micotoxixénicos en diferentes matrices alimentarias conseguiuse en 24-48 h, proporcionando unha alternativa máis rápida ás técnicas convencionais baseadas na microbioloxía clásica que requiren ata 7 días desde a mostraxe ata o resultado. Polo tanto, os métodos desenvolvidos demostraron ser unha opción interesante para a industria alimentaria que contribúe á redución dos custos asociados a longas análises. Máis concretamente un ensaio qPCR cun IAC para a detección de fungos nos preparados de froitas foi evaluado por separado para levaduras e mofos e debido ao seu excelente rendemento foi seleccionado como método de referencia para os métodos que se desenvolveron máis tarde. Neste sentido, nun segundo paso desenvolveuse un ensaio de LAMP seleccionando dúas formas diferentes para a visualización de resultados, é dicir, o cambio de cor observable a simple vista e a fluorescencia en tempo real. O ensaio de LAMP resultou igualmente sensible co ensaio qPCR con parámetros de calidade notables. Posteriormente, probouse outra técnica isotérmica de amplificación de ADN, é dicir, un ensaio RPA xunto con LF para a detección de fungos universais. Neste ensaio, os cebadores foron etiquetados con digoxigenina e biotina e evitouse deste xeito o uso dunha sonda NFO, simplificando así o ensaio. De novo, o ensaio desenvolvido foi tan sensible como os ensaios de panfungal qPCR e LAMP. A vantaxe do ensaio de LAMP colorimétricas e o ensaio RPA-LF é que se poden usar para aplicación en puntos de interés para cribado precoz de froitas e produtos ao longo da cadea de distribución. Por conseguinte,

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desenrolaronse solucións analíticas altamente fiables e dependerá do usuario final para decidir que método é máis adecuado para a aplicación prevista. Finalmente, investigáronse métodos rápidos para a detección de fungos produtores de patulina, incluído un ensaio qPCR e máis tarde nun ensaio RPA xunto coa detección de cor verde a simple vista debido ao uso da molécula SYBR, que emite fluorescencia. Os dous ensaios, foron menos sensibles en comparación cos ensaios panfúngicos e, entre os dous, o qPCR foi lixeiramente máis sensible en comparación co RPA-SG. A diferenciación de células viables e non viables conseguiuse con éxito engadindo PMA antes da extracción do ADN. A adición da PMA na concentración seleccionada foi capaz de inhibir a amplificación orixinaria de células mortas sen causar ningún efecto tóxico nas células vivas.

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ABSTRACT

Identification of measures to prevent food losses in the primary steps of supply chain but also later on in order to prevent food waste at the retail and consumer level is of utter importance and still remains of major concern for the relevant authorities worldwide. According to the European Commission (EC), 88 million tons of food waste are generated annually in the EU, with an estimated cost of 143 billion euros. According to the Food and Agriculture Organization (FAO), globally 1/3 of all the food produced results in waste or losses. Furthermore, according to FAO's Food Loss Index (FLI), 14 % of the food produced is lost between post-harvest up to, but excluding, retail. Food waste does not only have an economical and ethical impact, but also leads to depletion of natural resources. As a result, the EU has committed to reduce food losses along production and supply chain, and to halve *per capita* food waste at the retail and consumer level by 2030. In this sense, microbiological spoilage plays an important role in food spoilage, and in particular, fungi are the most important microorganisms implicated in food losses. Additionally, toxigenic fungi under favorable conditions can produce mycotoxins which are secondary metabolites. Some of the mycotoxins can be extremely dangerous for human and animal health due to their carcinogenic and teratogenic effects but also they can cause economic losses along the supply chain. As a result, contamination of foods from mycotoxigenic fungi is of utter importance due to food safety as well as for food trade issues.

To this day, detection of yeasts and moulds is performed based on traditional culture-based methods. These techniques are laborious, time consuming and require huge amounts of reagents. In addition, the overall analysis time can be extended to up to 7 days, rendering these techniques not suitable for foods with short shelf-lives and also not suitable for the increased production systems that exist nowadays. Consequently, the obtained results can only be used retrospectively being of limited value for quality and process control. Another disadvantage of these methods is that they cannot detect Viable But Non-Culturable (VBNC) microorganisms, that can be the result of processing, cleaning and/or disinfection activities since at this state microorganisms cannot grow on media but they can still play an important role in spoilage by producing enzymes or other metabolites of importance. As a consequence, there is a need for faster and more sensitive methods for fungal detection that will reduce the overall costs of the food industry due to product recalls and long analyses.

In this sense, over the last decades DNA-based amplification techniques have emerged with the ultimate goal to overcome some of the drawbacks of the traditional methods due to their high sensitivity and specificity. In particular, PCR and qPCR have been widely used for the detection of spoilage yeasts and moulds in a variety of food products. One of the major drawbacks of most reported PCR/qPCR assays is that they do not include an Internal Amplification Control (IAC). Basically, the IAC is a non-target DNA, implemented in the assay that co-amplifies with the target gene and should always amplify in negative samples. In this sense, false negative results due to reaction inhibition can be avoided. Nevertheless, only few assays for fungal detection have reported using an IAC, which can result in false diagnosis especially in the case of pathogenic fungi.

Lately alternatively to PCR/qPCR, isothermal nucleic acid amplification techniques have appeared allowing the simplification of the assays and the reduction costs. These assays only require

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a water bath or heat block since they run at a constant temperature, and as a result there is no need for expensive equipment like a thermocycler. Furthermore, since the results in most of the cases can be visualized by naked-eye, these assays can be implemented in portable devices for Point of Care (POC) applications. Among the different isothermal amplification techniques, Loop-mediated isothermal amplification (LAMP) and Recombinase Polymerase Amplification (RPA) are the most popular for the detection of microorganisms. Even though, many assays have been reported for the detection of pathogenic bacteria and viruses, application of these methods in the field of foodborne fungi still remains limited and is mainly focused on the detection of mycotoxigenic fungi. The results can be visualized in many different ways depending on the assay, including naked-eye detection of colour change, gel electrophoresis, lateral flow strips and real-time fluorescence. Furthermore, it has been proven that these techniques are more robust against common PCR inhibitors that can lead to false negative results.

One of the major limitations of all DNA-based methods is that they cannot differentiate between live and dead microorganisms. This is because DNA is a stable molecule and thus can persist and be present for some time after the death of the microorganism. One approach for the detection of viable microorganisms relies on the detection RNA by RT-PCR or RT-qPCR. However, handling of RNA is more difficult and RNA should be carefully manipulated during extraction since RNase, the enzyme that digests RNA, is ubiquitous in the environment. In addition, contamination can occur due to improper sample processing, storage conditions or contamination with RNA-degrading enzymes. Another drawback of this approach is that a retrotranscription step is required in order to convert RNA to cDNA. Another approach that has been explored lately, involves the use of intercalating dyes such as PMA and EMA, which can penetrate dead cells, and upon photoactivation, irreversibly bind to the DNA, thus blocking its amplification. One of the main advantage of these assays is that they can easily be implemented in a DNA extraction protocol and also they are compatible with many amplification techniques. Furthermore, with these dyes detection of microorganism in the VBNC state is possible.

The objective of this thesis, was the development and evaluation, of different molecular-based methods for the detection of selected spoilage responsible, and mycotoxigenic fungi in fruits, and products thereof, with the overall goal of reducing the total time of analysis, while maintaining the sensitivity when compared to the conventional methods. In order to achieve this, different specific objectives were proposed.

The first specific objective included the development of a protocol for pre-enrichment, sample treatment, and DNA extraction for the fungi of interest in the selected food matrices. In order to achieve this, different media were tested, so that the optimal one for the growth of the fungi of interest could be selected. Based on kinetics studies, and on the protocols in place on our industrial partner's site, MEB was the selected broth for the pre-enrichment step. In continuation, different sample volumes, and incubation conditions, were evaluated and the optimal ones were chosen taking into account the previous experience from the industrial partner. Additionally, the sample treatment was optimized involving differential centrifugation steps, in order to remove food debris and to pellet the microorganisms before the DNA extraction. Finally, a commercial kit was chosen for DNA extraction. With the optimized protocol, extraction of DNA of high quality was achieved.

The second specific objective involved the development and evaluation of a panfungal qPCR method, with a NC-IAC, that could later on serve as the reference method for isothermal DNA amplification techniques. In this sense, a set of universal primers was selected targeting the ITS region and a TaqMan probe was designed *in house*. The assay was evaluated for its specificity against a panel of microorganisms frequently encountered in food products. In addition, an analytical

sensitivity of 10.4 fg/ μL of pure DNA was achieved. The final step in the development of the panfungal qPCR assay was the evaluation of the overall method in real samples. As a result, fruit preparation samples were spiked with different concentration of yeasts and moulds and the LOD of the assay was determined. The obtained LOD_{95} values were 3.9 CFU/ 50 g for yeasts, 1.2×10^2 spores/ 50 g and 3.7×10 spores/ 50 g for moulds after 24 and 48 h of pre-enrichment, respectively. The calculated LOD for yeasts was within the range of previously reported assays for the detection of spoilage yeasts; thus highlighting the high sensitivity of our assay. Based on the obtained LOD_{95} values, the performance parameters of the method were calculated. The obtained values were higher than 85.0 % and the Cohen's k above 0.86 for all the assays, meaning that our method was almost in complete concordance with the reference method, in this case culture-based methods. Overall, the developed method proved to be very sensitive for the detection of fungi and consequently served as the reference method for the isothermal amplification techniques that were developed later on.

The third specific objective of this thesis involved the development and evaluation of different isothermal based amplification techniques coupled with naked-eye detection for spoilage and mycotoxigenic fungi. To this end, two panfungal methods were developed, a LAMP assay targeting the 18S rRNA gene and a RPA assay targeting the ITS region. In more detail, in the LAMP assay the results were visualized with real-time fluorescence but also naked-eye by colour change, while for the RPA lateral flow strips were used for naked-eye detection. Furthermore, a RPA assay coupled with SYBR Green I for the detection of patulin-producing fungi was developed and evaluated.

Regarding the panfungal LAMP assay, a full set of primers was designed with Primer Explorer v.4 and their inclusivity/ exclusivity was assessed *in vitro* against a panel of fungi and bacteria commonly found in food products. In addition, by testing ten-fold serial dilutions an analytical sensitivity of 1.4 pg/ reaction of pure yeast's DNA was reached by both the fluorescent and the colorimetric assay. Furthermore, when pure DNA from *N. fischeri* was used, detection down to 17 pg/ reaction and 170 pg/ reaction were detected with the fluorescent and the colorimetric assay, respectively. In the last step, the evaluation of the method was performed in spiked blackberry and pineapple preparations with different concentrations of yeasts. The reference method for comparison was the previously developed panfungal qPCR assay and additionally the results were compared against a previously set of LAMP primers for panfungal detection, targeting the same gene. The detection was performed both by naked-eye observation of colour change, and real-time fluorescence for direct comparison. Thus in total four different methodologies were evaluated. The LOD_{95} obtained was 3.1 CFU/ 50 g for the two fluorescent assays, hence both assays were equally sensitive. Regarding the colorimetric assays, the one developed in this study proved to be more sensitive with a LOD_{95} of 3.0 CFU/ 50 g, whereas the one used for comparison resulted in a LOD_{95} of 10.9 CFU/ 50 g. Furthermore, the obtained LOD was similar to the one reported for the yeasts detection by qPCR in this thesis (LOD_{95} 1.0 CFU/ 50 g). Consequently, the newly developed LAMP assay was as sensitive as the reference method but with the added advantages that the colorimetric assay can be used for POC detection in decentralized settings and also result in costs reduction since there is no need for expensive equipment like a real-time thermocycler. Based on the obtained LODs the quality parameters were determined and in all the assays the values obtained were above 90 % with some exceptions for each assay that were the result of few PDs and/or NDs. This can be the result of cross-contamination since in LAMP assays the risk of cross-contamination is very high through aerosolized products due to the high production of DNA that can lead to false positive results. Finally, based on the Cohen's k values, the newly developed colorimetric assay was in "almost complete concordance" with the reference method, i.e. the panfugal qPCR assay, while the fluorescent one was in "substantial agreement".

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Regarding the panfungal RPA assay, the universal primers ITS3/ITS4 were selected and tagged with digoxigenin and biotin, respectively. In this sense, the use of a *nfo* probe was avoided especially taking into account that TwistDx (the main distributor of the RPA reagents) recently discontinued the *nfo* kit for lateral flow detection. The specificity of the primers was tested as previously explained for the other assays. All the primers presented good inclusivity towards the selected fungi and good exclusivity since all the selected bacteria failed to amplify. In addition, with the developed assay an analytical sensitivity of 1.2 pg/ μL, covering a 4-log dynamic range, was achieved for both yeasts and moulds. The evaluation of the panfungal RPA-LF method was performed both in yeasts and moulds in blackberry jam samples. The LOD_{50} was found to be 1.0 CFU/ 50 g for yeasts and 47.5 spores/ 50 g for moulds, respectively. The obtained LOD_{50} for yeasts was the same as the one reported for the developed panfungal qPCR and LAMP assays in this thesis, indicating that all the methods developed during this study were equally sensitive for the detection of yeasts. Finally, all the quality parameters were above 80 % and the Cohen's k was found to be 0.77 for both yeasts and moulds detection, meaning that the developed methods were in "substantial agreement" with the reference method.

The ultimate specific objective of this thesis concerned the development of a methodology for the differentiation of viable and non-viable fungi using intercalating dyes combined with molecular-based methods. Two molecular-based methods, namely a qPCR and a RPA coupled with SYBR Green I (RPA-SG), for the detection of patulin-producing fungi in apples and by-products were developed and evaluated. In addition, detection of only viable fungi was achieved with the implementation of propidium monoazide (PMA) during the sample treatment.

Regarding the qPCR assay, a previously published set of PCR primers was chosen that targeted the *idh* gene and a fluorescent probe was designed *in house.* The inclusivity/ exclusivity of the primers was evaluated as it was described before and all the oligonucleotides were found to be specific. The qPCR assay covered a 5-log dynamic range down to 1.25 pg/ μL of pure *P. expansum* DNA. The reported analytical sensitivity fell within the range of previously reported values for the detection of mycotoxigenic fungi. The evaluation of the method was performed in spiked apples, apple puree and apple juice and the LOD_{50} was calculated to be 8.1×10^3 spores/ 5 g. Finally, all the quality parameters were above 90 % and the Cohen´s k was 0.93, indicating that the assay was "in almost complete concordance" with the reference method, in this case the culture-based method.

Regarding the RPA- SG assay, a new set of forward and reverse primer were designed with the use of the PrimedRPA software. The target gene was the same as with the qPCR assay as it is involved in the biosynthetic pathway of patulin. Once more, the specificity of the primers was assessed *in vitro* against a selection of microorganisms. With the developed assay, a 4-log dynamic range was covered thus detecting down to 23.8 pg/ μL of pure *P. expansum* DNA. The evaluation of the overall method was performed by spiking apples and products thereof and the previously developed qPCR for the detection of patulin-producing fungi served as the reference method. The LOD_{50} was calculated to be 5.8×10^4 spores/ 5 g. Finally, based on the obtained LOD values, the quality parameters were found to be above 90 % and the Cohen´s k was 0.92, meaning that the assay was "in almost complete concordance" with the reference method.

Furthermore, in this study it was proved that the PMA could efficiently block the amplification of DNA originating from dead spores with concentrations up to 10^7 spores/ mL. Additionally, no toxic effects were observed on the live spores. In addition, the effect of the PMA was assessed in spiked apple samples and once again PMA successful blocked the amplification of DNA from dead spores in the same range of concentrations. The effect of PMA in apples inoculated with a mixture of live and dead spores in different ratios was also assessed. It was observed that when apples were spiked with 10^8 dead spores, the PMA did not fully inhibit the amplification as one of the three replicates

was positive. These results suggested that the PMA can inhibit the amplification of DNA coming from dead spores with concentrations of up to 10^7 but the presence of higher concentrations of dead microorganisms may hinder the blocking capacity of the dye.

Overall, in the present thesis different molecular-based assays were developed and evaluated. Detection of spoilage-responsible and mycotoxigenic fungi in different food matrices was achieved in 24-48 h, providing a faster alternative to the conventional culture-based techniques which require up to 7 days from sampling to result. Therefore, the developed methods have proven to be an interesting option for the food industry contributing to reduction of costs associated with lengthy analyses. More specifically one qPCR assay with an IAC for the detection of fungi in fruit preparations was evaluated separately for yeasts and moulds and due to its excellent performance it was selected as the reference method for the methods that were developed later on. In this sense, in a second step a LAMP assay was developed selecting two different ways for results visualization, namely naked-eye colour change and real-time fluorescence. The LAMP assay proved to be equally sensitive with the qPCR assay with remarkable quality parameters. Subsequently, another isothermal based DNA amplification technique was tested, namely a RPA assay coupled with LF for universal fungal detection. In this assay, the primers were tagged with digoxigenin and biotin and in this way the use of an *nfo* probe was avoided, thus simplifying the assay. Again, the developed assay was as sensitive as the panfungal qPCR and LAMP assays. The advantage of the colorimetric LAMP assay and the RPA-LF assay is that they can be used for POC applications for early screening of fruits and products along the supply chain. Consequently, highly reliable analytical solutions were developed, and it will depend on the final user to decide which method is more suitable for the intended application. Finally, rapid methods for the detection of patulin-producing fungi were investigated, including a qPCR assay and later on a RPA assay coupled with naked-eye SYBR Green detection by emission of fluorescence. Both assays, were less sensitive compared to the panfungal assays and between the two the qPCR was slightly more sensitive compared to the RPA-SG. Differentiation of viable and non-viable cells was successfully achieved by adding PMA before the extraction of the DNA. The addition of the PMA in the selected concentration was able to inhibit amplification originating from dead cells without causing any toxic effect in the live cells.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
AC	Relative Accuracy
ALT	Altenuene
AME	Alternariol monomethyl ether
AOH	Alternariol
ATCC	Americal Type Culture Collection
ATX-I/ATX-II	Altertoxins
AuNPs	Gold Nanoparticles
a_w	Water activity
B3	Backward Outer Primer
BEN	Balkan Endemic Nephropathy
BIP	Backward Inner Primer
BLAST	Basic Local Alignment Search Tool
bp	Base Pairs
CBS	Collection from the Westerdijk Fungal Biodiversity Institute
cDNA	Complementary DNA
CECT	Spanish Type Culture Collection
CFU	Colony Forming Unit
Cq	Quantification Cycle
CUP	Catholic University of Porto
ddPCR	Digital Droplet PCR
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
DON	Deoxynivalenol
DRBC	Dichloran Rose Bengal Chloramphenicol
dsDNA	Double-Stranded DNA
EAs	Ergot alkaloids
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ELISA	Enzyme Linked Immunosorbent Assay
EMA	Ethidium Monoazide
EU	European Union
F3	Forward Outer Primer
FAO	Food and Agriculture Organization

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FBs	Fumonisims belonging to B series
FIP	Forward Inner Primer
FLI	Food Loss Index
GI	Gastrointestinal
GMO	Genetically Modified Organism
gRNA	Guide RNA
HDA	Helicase-Dependent Amplification
HNB	Hydroxy Napthol Blue
HRM	High-Resolution Melting
IABkFQ	Iowa Black®FQ
IAC	Internal Amplification Control
IARC	International Organization for Research on Cancer
isoTeA	Isotenuazonic acid
ITS	Internal Transcribed Spacer
k	Cohen's Kappa Index
LAMP	Loop-Mediated Isothermal Amplification
LB primer	Loop Primer B
LF	Lateral Flow
LF primer	Loop Primer F
LOD	Limit of Detection
MEA	Malt Extract Agar
MEB	Malt Extract Broth
m-PCR	Multiplex PCR
mRNA	Messenger RNA
MUM	Micoteca da Universade do Minho
MY50G	Malt Yeast Extract 50% Glucose
NA	Negative Agreement
NASBA	Nucleic Acid Sequence-Based Amplification
NC-IAC	Non-Competitive IAC
ND	Negative Deviation
NPV	Negative Predicted Value
OGY	Oxytetracycline Glucose Yeast Extract
OTA	Ochratoxin A
PA	Positive Agreement
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PD	Positive Deviation
PDA	Potato Dextrose Agar
PMA	Propidium Monoazide
POC	Point-of-Care
POD	Probability Of Detection
PON	Point-of-Need

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PPV	Positive Predicted Value
qPCR	Real-time Polymerase Chain Reaction
RASFF	Rapid Alert System for Food and Feed
RCA	Rolling Circle Amplification
RNA	Ribonucleic Acid
RPA	Recombinase Polymerase Amplification
rRNA	Ribosomal RNA
RT	Reverse Transcriptase
RTE	Ready-to-Eat
RT-PCR	Reverse Transcription PCR
RT-qPCR	Real-Time Reverse Transcription PCR
rxn	Reaction
SB	Sodium Borate
SDB	Sabouraud Dextrose Broth
SE	Relative Sensitivity
SG	SYBR Green
SNPs	Single Nucleotide Polymorphisms
SP	Relative Specificity
ssDNA	Single-Stranded DNA
ssRNA	Single-Stranded RNA
TeA	Tenuazonic Acid
TGY	Tryptone Glucose Yeast Extract
Tm	Melting Temperature
tmRNA	Transfer-Messenger RNA
UDG	Uracil-DNA Glycosylase
UM	University of Minho Collection
US	United States
UV	Ultra-Violet
VBNC	Viable But Non-Culturable
WDCM	World Data Centre for Microorganisms
WHO	World Health Organization
YPD	Yeast Extract Peptone Dextrose
YY	Yakima Yellow
ZEN	Zearalenone

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CHAPTER 1.

INTRODUCTION

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1 INTRODUCTION

Food loss refers to the decrease in the quantity or quality of food resulting from decisions and actions by food suppliers in the chain, excluding retailers, food service providers and consumers while food waste refers to the decrease in the quantity or quality of food resulting from decisions and actions by retailers, food service providers and consumers [1]. According to the European Commission (EC), 88 million tons of food waste are generated annually in the EU, with an estimated cost of 143 billion euros [2]. According to the Food and Agriculture Organization (FAO), globally 1/3 of all the food produced results in waste or losses [3]. Furthermore, according to FAO's Food Loss Index (FLI), 14 % of the food produced is lost between post-harvest up to, but excluding, retail [1]. Food waste does not only have an economical and ethical impact, but also leads to depletion of natural resources. Consequently, based on the 2030 Agenda for Sustainable Development, the EU has committed to reduce food losses along production and supply chain, and to halve *per capita* food waste at the retail and consumer level by 2030 [4]. As a result, identification of measures to prevent food losses in the primary steps of supply chain but also later on preventing food waste in the retail and consumer level is of utter importance.

Food loss can be the result of insect or rodent damage, microbiological, physical and/ or chemical spoilage, losses during transportation and/ or further processing, and end of shelf-life. Microbiological spoilage plays an important role in food spoilage, and in particular, fungi are the most important microorganisms implicated in food losses. Contamination of food can occur in the pre-harvest stage from plants, animals, soil and water. Soil can be a source of contamination since it contains a large number of fungi, bacteria, etc. The contamination from soil can be direct during production, and harvesting, or indirect through deposition of wind-borne mould spores [5]. Regarding post-harvest contamination, a variety of raw materials and food have a structure integrity that protects most of their mass from microorganisms [6]. For example, the rind, or skin, of fruits and vegetables protects their interior from microbial contamination. However, upon processing (cutting, trimming, crushing, etc.) the interior of the fruit can get contaminated from the microorganisms that have been on the surface of the fruit.

Furthermore, contamination can occur during human handling. Dirty hands/ gloves, talking, sneezing/ coughing can be sources of contamination. In addition, cross-contamination can occur when food comes in contact with raw materials, and dirty equipment or utensils [5]. Finally, in a given food type, based on parameters, such as the pH, water activity (a_w) and storage temperature, only one or few microorganisms will manage to grow and cause spoilage [7]. Microbial spoilage occurs due to the biochemical activity of the microorganisms present in the food; frequently resulting in changes in their appearance, taste, smell and texture [5].

Fungi under favourable conditions can also produce secondary metabolites that are known as mycotoxins and can have an impact on human and animal health. In addition, they can result in economic losses along the supply chain. Consequently, the relevant authorities worldwide have set limits for the different mycotoxins in foodstuffs and the timely detection of mycotoxin-producing fungi is very important.

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1.1 FRUITS, BY-PRODUCTS AND SPOILAGE

1.1.1 Whole and fresh cut fruits

Over the last decades, consumption of fruits has risen due to the increased awareness in healthy eating habits, the increased availability of fresh produce, and the fast development of fresh-cut and ready-to-eat (RTE) products. According to the International Fresh-cut Product Association [8], fresh-cut fruit is defined as any fruit that has been physically altered from its original form but still remains in a fresh state. These type of products offer to consumers a RTE, convenient, nutritious and fresh-like alternative to whole fruits [5].

Contamination of whole fruits can occur in all the different stages of the supply chain including: cultivation, pre-harvesting and post-harvesting handling, storage and distribution. However, the majority of microorganisms that are found on the surface of whole fruits are soil inhabitants that are spread to fruits through airborne spores, irrigation water and soil particles. Fruits contain high amounts of sugars and other nutrients. This composition, combined with the high water activity that fruits possess, make them suitable for microbial growth. However, because of their low pH, bacterial spoilage in this food category is limited since bacteria typically grow at neutral pH [9]. Thus, spoilage is mainly associated with three acid-tolerant groups, namely: aciduric bacteria, moulds and yeasts. In particular, yeasts and moulds are the main type of spoilage microorganisms found in fresh fruits [10]. Yeasts of the genera *Saccharomyces*, *Candida*, *Torulopsis* and *Hansenula* have been isolated from fruits, and are associated with fruit fermentation, resulting in off-flavour and off-odour. Contamination and spoilage of fruits from moulds is mainly related with species belonging to the class of *Ascomycetes*. In particular, species of *Penicillium*, *Botrytis*, *Alternaria*, *Fusarium*, *Aspergillus*, *Cladosporium*, *Phytophthora*, *Phoma*, *Trichoderma*, *Rhizopus*, *Aureobasidium*, and *Colletotrichum* are commonly culprits of fruit spoilage. Symptoms include visible growth, rots (blue mould rot, gray mould rot, brown mould rot and botrytis rot) and discoloration [5]. A detailed list of the fungi affecting the different categories of fruits can be found in **Table 1.1**.

Regarding fresh-cut RTE fruits, contamination is often the result of the existing microorganisms on the surface of whole fruits. Other sources of contamination and subsequently spoilage include contact of the fruits with processing equipment and improper handling from workers. As a result, the same fungi that are encountered in whole fruits, can be also found in the fresh-cut ones. Furthermore, other yeasts related to quality problems including *Rhodotorula mucilaginosa*, *R. glutinis*, *Zygosacchramocyes baillii*, *Z. rouxii* and *Z. bisporus* have been isolated from a variety of fresh-cut fruits [5].

Table 1.1. Frequently encountered fungi in different fruit categories

Fruit	Common fungi
Citrus fruits	*Penicillium italicum* (blue rot), *P. digitatum* (green rot), *P. ulaiense* (whisker mould) *Geotrichum candidum* (sour rot) *Alternaria* spp. belonging to *A. alternata clade** (black rot) *Cladosporium sphaerospermum* (soft rot) *Colletotrichum gloeosporioides* (Anthracnose) *P. expansum**, *P. solitum* (blue rot)
Pome fruits	*Botrytis cinerea* (grey rot) *Rhizopus stolonifer* (transit rot) *Monilinia fructicola*, *M. fructigena* (brown rot)
Stone fruits	*R. stolonifer*, *R. oryzae* (transit rot)

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Tomatoes and other solanaceous fruits	*P. expansum** (blue rot) *Trichothecium* spp.*(pink rot) *Alternaria* spp.* (black/brown spots) *A. alternata* *(black rot), *A. solani* (early blight rot) *B. cinerea* (grey rot) *G. candidum* (sour rot) *R. stolonifer* (watery rot)
Melons and other Cucurbits	*C. lagenarium* (anthracnose) *A. alternata** (black rot) *Cladosporium* spp.* *Fusarium* spp.*
Grapes	*B. cinerea* (grey rot) *Penicillium* spp.* *Aspergillus niger**, *A. Carbonarius** *A. alternata**
Berries	*B. cinerea* (grey rot) *R. stolonifer* (leaking rot) *Mucor piriformis* (leaking rot)
Figs	*A. niger** (black rot) *Fusarium* spp.* (soft rot) *A. alternata**
Tropical fruit	*Hanseniospora uvarum* (souring) *Colletotrichum musae*, *C. gloeosporioides* (anthracnose) *Fusarium* spp.* (brown rot) *A. alternata** *R. oryzae*

The information of this table was retrieved from [11,12]. *Fungi that have been identified as mycotoxin producers.

1.1.2 Fruit juices

Fruit juices can be divided in two categories: chilled and ambient fruit juices. The former refers to a diverse category of products with juice contents between 20 to 100 %. The latter refers to products that have been pasteurized at temperatures of about 80- 90 °C and have been aseptically filled in bottles for storage at ambient temperature [5].

The main spoilage microflora of chilled fruit juices includes fermentative yeasts, moulds and few aciduric bacteria as a result of the low pH, reduced O_2 content and low protein and amino nitrogen content. The most dangerous microorganisms for this category are those with the ability to grow at temperatures of, or below, 5- 7 °C. The main source of contamination is assumed to be the fruits that are used for the juice production. Fruits can be contaminated with a variety of spoilage microorganisms at various stages such as cultivation, harvesting, handling, transportation, and storage. Nevertheless, soil appears to be the main source of microorganisms with a huge variety of soil-borne yeasts being able to contaminate fruits during growing and harvesting [5]. *Saccharomyces*, *Deberomyces*, *Hansenula*, *Pichia*, and *Kluyveromyces* are the main genera of concern. Results of spoilage from fermentative yeasts include off-flavours, turbidity, sliminess and bloated packages. Regarding contamination from moulds the most predominant species include *Penicillium* spp., *Cladosporium* spp., *Aspergillus niger* and *A. flavus*, *Botrytis cinerea*, and *Aureobasidium pullulans* [13,14]. However, these fungi are readily destroyed during heat treatment; hence their presence in the final product is the outcome of post-process contamination due to bad hygienic conditions, environmental air/ dust, and contaminated material [5]. Spoilage defects due to moulds, include off-flavours, visible mycelial fibres or mould "mats" in the surface of the juice or in the interior of the packaging [10,14].

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Ambient juices are also susceptible to spoilage by fermentative yeasts and environmental moulds that can enter during packaging or due to the loss of aseptic conditions. In addition, ambient juices can be spoiled by heat-resistant moulds and alicyclobacilli. Since these microorganisms do not produce CO_2 that could deform the bottle, spoilage is not apparent immediately and usually results in flavour, smell and visual defects. The principal heat-resistant moulds related to fruit juice spoilage include *Byssochlamys fulva*, *Neosartorya fischeri*, *Talaromyces macrosporus* and *Eupenicillium brefeldianum*. These microorganisms can survive juice pasteurization thanks to their ability to produce heat-resistant ascospores [5]. Since the source of the heat-resistant fungi is the soil, juices of pineapple, berries and passionfruit are more susceptible due to the contact of the fruit with the soil before or after harvesting [15].

1.1.3 Dried fruits

The process of making dried fruits includes an initial preservation step with SO_2 that inhibits microbial growth even during long storage. However, if the levels of SO_2 fall below 1 g/ kg the product is susceptible to spoilage by xerophilic yeasts like *Z. rouxii* and xerophilic moulds like *Eurotium* spp. and *Xeromyces bisporus* [11]. In addition, species belonging to *Aspergillus* section *Nigri* that are responsible for ochratoxin A production, including *A. niger*, *A. ochraceus* and *A. carbonarius*, have been isolated from a variety of dried vine fruits and figs [16–18]. Furthermore, species belonging to *Aspergillus* section *Flavi*, the main aflatoxin producers, have been also isolated [16,19]. Finally, in glazed fruits, like glazed pineapple slices, the SO_2 is added in the syrup that the fruit is infused in varying concentrations. In this case, *Schizosaccharomyces pombe* can be present that has the ability to resist the SO_2 and the low a_w and thus grow during the infusion process [11].

1.1.4 Jams

Jams and canned fruits, are traditionally prepared from fruits and sucrose followed by boiling or evaporation in order to reach a a_w of 0.75 or lower. Subsequently, they are hot filled into jars rendering them microbiologically stable. Spoilage incidents are often related with inadequate heating treatments that result in products with a_w between 0.80 and 0.82 that can support mould growth. Fungi related to the spoilage of jams most frequently include *Eurotium* spp. and *Aspergillus restrictus*; however, xerophilic *Penicilia*, in particular *P. corylophilum*, have also been isolated from this type of samples. Even though jams are hot filled, and the yeast *Z. rouxii* should not pose a risk, incidents of spoilage from this yeast have been reported and have been associated with contamination after opening of the product [11].

1.1.5 Fruit concentrates

Fruit concentrates are fruit juices with a Brix of 65-80°, low pH and a_w, that have been pasteurized, evaporated and hot filled. Hence, these type of products are also microbiologically stable. During the pasteurization process all the microorganisms are killed with the exception of heat-resistant fungi; however, this group of fungi cannot grow due to the low a_w that concentrates possess. In some instances, *Z. rouxii* a xerophilic yeast, can contaminate, and spoil, fruit concentrates through the filling system. This yeast, is able to grow in foods with a_w as low as 0.62, and produce CO_2 leading to swollen and exploded containers [11].

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1.2 MICROORGANISMS

In this section information is given about some of the most important fungal species (filamentous and unicellular fungi) related to food spoilage and mycotoxin production, with a special focus on those associated to fruits, and their by-products.

1.2.1 Filamentous fungi (moulds)

Moulds can grow in a wide range of temperatures and water activities, with specific genera being able to grow in products with a a_w as low as 0.62 and as high as nearly 1.0. They can grow in a pH range between 2 to 11, but they prefer acidic environments. They are obligate aerobes, with oxidative metabolism and they are the most common food spoilage microorganisms throughout the supply chain. Representative genera of food spoilage include *Penicillium*, *Aspergillus*, *Alternaria*, *Mucor*, *Fusarium*, *Byssochlamys*, *Cladosporium*, *Rhizopus*, *Geotricum*, and *Eurotium* [5].

1.2.1.1 Genus *Penicillium*

Penicillium species are ubiquitous, opportunistic saprophytic fungi. Some species like *P. expansum*, *P. digitatum* and *P. italicum* are fruit pathogens, and a few can grow at a_w below 0.80, for example *P. brevicompactum*, *P. chrysogenum* and *P. implicatum*. Furthermore, species like *P. roqueforti* are resistant to preservatives and can grow at low oxygen tension. In addition, many species being psychotrophic, meaning that they can grow at low temperatures but have optimal and maximal growth temperatures above 15 and 20 °C [20], can cause spoilage even at refrigeration temperatures. Species belonging to *Penicillium* subgenus *Penicillium* are the most important in terms of food spoilage. They can grow at low temperatures and a_w, and as a result they can be found in a variety of food products. Related to mycotoxin production, all of the species belonging to subgenus *Penicillium*, with the only exception of the fruit-rotting species, *P. italicum*, *P. digitatum*, *P. solitum* and *P. ulaiense*, are considered mycotoxin producers [11].

P. digitatum is the main cause of rot in citrus fruits [21]; however it has also been isolated from nuts, cereal crops, meat [22], and olives [23]. It is universally distributed but has a preference for warmer climates [24]. *P. digitatum* can grow in temperatures between 6-7 °C up to 37 °C [24]. Regarding the a_w, the minimum for growth is 0.90, 0.95 and 0.99 a_w at 25 °C, 30 °C and 5 °C, respectively. In addition, at 37 °C or at 0.87 a_w no germination occurs [25,26].

P. expansum is the main cause of rot in pomaceous fruits (apples and pears) but has been isolated from a variety of other fruits including tomatoes, strawberries, mangoes and grapes [21,22]. Frequently, it is also associated with spoilage of meat, and meat products [22,27]. Furthermore, even though less common, it has been encountered in vegetables, cereals [28], nuts [22,28] and cheese [29]. *P. expansum* can produce the mycotoxins patulin and citrinin [30,31]. This species is a psychrophile meaning that it can grow at temperatures as low as -6 °C [32]. It has been reported that the fungus can grow quite well at 0 °C [33]; however the optimal growth temperature is set at 25 °C [34]. The maximum temperature for growth is near 35 °C [34]. Regarding the a_w, the minimum requirement for germination is at 0.82-0.83 [26,35]. In addition, *P. expansum* can produce patulin in temperatures ranging between 0 to 25 °C at least, with the optimal temperature been 25 °C [36]. The minimum a_w value for patulin production is set at 0.95 at 25 °C [37].

P. griseofulvum is mainly encountered in cereals, bakery products and nuts [22,28]. Even though, it can create lesions when inoculated in pomaceous fruits, it is not considered as a common spoilage

fungi for this category [38]. *P. griseofulvum* can produce different mycotoxins, namely patulin, cyclopiazonic acid, roquefortin C and griseofulvin [39]. It can grow in a range of temperatures between 4 and 35 °C, with 23 °C considered to be the optimal one. Regarding the minimum a_w requirements, 0.81 and 0.83 are the minimum values for germination at 23 °C and at 16 or 30 °C, respectively [35].

P. italicum is also an important cause of spoilage in citrus fruits [21]. In few occasions it has also been isolated from other fruits [22], fruit juices [40], cheese, and meat and by-products [41,42]. The fungus can grow at temperatures ranging from -3 to 32-34 °C, with an optimum around 22-24 °C. It can also grow in a wide range of pH values, in particular from 1.6 to 9.8 [34]. Finally, 0.87 is the minimum a_w for growth at 10 and 25 °C [25,34].

P. solitum is a common spoilage agent of pomaceous fruits [38,43] and it has also been isolated from cheeses [44,45] and sausages [41]. Due to the fact that this species was only recently recognised, there is a lack of data related to its physiology; however since it belongs to the *Penicillium* subgenus *Penicillium,* it can grow at low temperatures and aw values but not at 37 °C [11].

P. verrucosum, is almost always encountered in cool temperate zones and in cereals. It is endemic in European and Canadian cereals, and has also been isolated from cheese. *P. verrucosum,* is the main source of ochratoxin A in this environment and food category. Additionally, some strains can also produce citrinin. Regarding growth requirements, between 0 and 31 °C growth can occur, with an optimum set at 20 °C. Also, it can grow in a wide pH range from 2.1 to 10.0 at least. Finally, the minimum a_w value for germination and growth is around 0.80 [46–49].

1.2.1.2 Genus *Aspergillus*

Aspergillus species are one of the most frequently encountered fungi in the world. They are of great economic importance, on the positive side due to their use for production of enzymes, synthesis of chemicals and biosynthetic transformation, but on the negative side due to their ability to spoil foods and produce aflatoxins [11].

A. carbonarius is typically found in grapes and vineyard environment. It has been isolated from dried vine fruits and figs [17,50,51], coffee beans [52,53] but also from other food products [54,55]. *A. carbonarius* can produce ochratoxin A and it is considered the main producer of this mycotoxin in grapes and by-products [50,56]. Since the drying of grapes for production of dried vine fruits occurs under the sun with no preservatives, *Aspergillus* can continue to grow and produce high levels of the toxin [57,58]. Also regarding the production of ochratoxin A in coffee beans, the contamination typically occurs post-harvest and the toxin can be introduced as a consequence of inadequate drying of the beans [52]. Regarding the physiology of the fungus, it can grow in a temperature range between 10 to 41 °C, with an optimum near 30 ° C. Furthermore, the optimum a_w for growth is between 0.96-0.98 and the minimum is set at 0.85 at 25-30 °C [59–63]. Finally, *A. carbonarius* can grow in a wide range of pH values between 2 to 10 and produce ochratoxin A [64]. The optimal conditions for ochratoxin A production are, cool temperatures around 15 °C, and 0.95-0.97 a_w or 20 °C and 0.98-0.99 a_w. Above 35 °C, or lower than 0.92 a_w, the mycotoxin cannot be produced [61–63,65,66].

A. flavus is a ubiquitous fungus that has been encountered in the majority of foodstuffs. It has a preference for tropical climates, and is mainly isolated from nuts and cereals [11]. Maize, and by-products, are of particular concern [22,67,68]; however it has been reported commonly in other cereals and products thereof [22,69–71]. In addition, it can be found in green coffee beans and spices [53,72]. Other sources of *A. flavus* include beans [73], processed meats [22] and dairy products [29].

Regarding fruits and vegetables, it can spoil a variety of them including citrus, tomatoes, pineapples and peaches among other, but it is not of great importance compared to other fungal species [21,74]. Furthermore, *A. flavus* is the main producer of aflatoxins. Some strains of the fungus can also produce cyclopiazonic acid [11]. Regarding its pathogenicity, *A. flavus* can cause human allergic bronchial aspergillosis and pulmonary infections in immunocompromised patients. In addition, it has been reported to cause ear infections [75]. *A. flavus* can grow in temperatures ranging from 10-12 °C up to 43-48 °C, with an optimum around 33 °C [22]. In addition, it can grow in a wide range of pH from 2.1 to 11.2 with an optimal growth reported in the range of 3.4 to 10 and a peak near 7.5. The pH range of growth was assessed in three different temperatures namely 25, 30 and 37 °C [46,76]. Regarding the minimum a_w values for growth, different values have been reported. For example, Pitt & Hocking [77] reported a minimum of 0.84 a_w at 25 °C while Pitt & Miscamble [78] reported a a_w of 0.82 at the same temperature. The latter, also reported minimum values of 0.80 and 0.81 a_w at 37 and 30 °C, respectively. Finally, regarding production of aflatoxins it appears that it can occur in a range of temperatures between 13-37 °C and above 0.82 a_w. Optimal conditions are regarded temperatures between 16 to 31 °C and 0.95-0.99 a_w [79].

A. niger is considered a ubiquitous fungus, like *A. flavus*, and it has been isolated from a variety of foodstuffs, mainly in warmer climates [11]. It is considered the most important *Aspergillus* spp. that can cause post-harvest decay in a variety of fresh fruits including apples, pears, figs, grapes and strawberries among other [21,22]. In addition, it frequently occurs in nuts [80,81], cereals [28,70,82], meat products [83,84] and sun dried products like dried vine fruits [17,85], dried, cured and smoked fish, cocoa beans [22] and spices [72]. Less frequent sources include vegetables [74,86,87] and cheeses that can cause thread lesions [45]. Regarding mycotoxins production, *A. niger* can produce ochratoxin A and fumonisin B2 [59,88,89].Regarding the growth conditions, *A. niger* is xerophile that can germinate in water activity as low as 0.77 a_w at 37 °C [90]. In addition, it can grow at low pH such as 2.0 when the water activity is high [91]. Finally, the growth temperatures range from 6-8 °C up to 45-47 °C, with an optimum around 35-37 °C [34,63]. However, Palacios-Cabrera et al. [92] reported no growth in three different media at 8 °C.

A. ochraceus is considered a great source of contamination for dried and stored products. In particular, it is frequently encountered in green coffee beans [52,93,94] and nuts [22,28]. Other sources, include pepper [95] and dried fruits [58] and less frequently cereals [71,82] and cheeses [22,96]. *A. ochraceus* can produce ochratoxin A as well as B and C, which are less toxic. However, it was discovered that only a few strains of this species are toxigenic, and that the main ochratoxin A producers are the closely related species *A. westerdijkiae* and *A. steynii* [97–99]. In addition, *A. westerdijkiae*, can also produce other toxins like penicillic acid, xanthomegnin, viomellein, and vioxanthin and consequently is of great importance in foods like coffee beans, cereals and beverages [11]. In addition, *A. ochraceus* also produces penicillic acid in temperatures ranging from 10 to 35 °C and minimum a_w of 0.81 [79,99]. *A. ochraceus* can grow optimally at 37 °C, with a maximum of 40 °C [11]. Optimal a_w, is reported between 0.95 to 0.99, with a minimum at 0.80 and 0.85 a_w at 20 or 30 °C and at 10 °C, respectively. Additionally, no germination occurs at 0.75 a_w [79,100–102]. The pH range for fungal growth is set between 3 and 10 [46]. Finally, regarding the optimal conditions for ochratoxin A production, in barley grains it is found to be 0.98-0.99 a_w and 25-30 °C. Similar conditions have been reported in coffee beans with the highest yield occurring at 20 °C. The lowest a_w for ochratoxin A production in coffee beans is set at 0.85; however no mycotoxin production occurs at this condition neither in barley grains, nor in grapes [100,103–105]. The pH range for ochratoxin production is from 5.5 to 8.5 [106].

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A. parasiticus is considered a tropical and subtropical species and the main food source is peanuts that according to Pitt & Hocking [11] the species is considered to be endemic. Other nuts have also been reported to be contaminated by *A. parasiticus* [22]. Less frequent sources include cereal grains [107], meat products [108], herbs and spices [22,72]. Along with *A. flavus* are considered the main aflatoxin producers, with only a few strains of *A. parasiticus* been non-toxigenic [109,110]. Regarding its physiology, growth is reported in the range from 12 to 42 °C, with an optimum at 32 °C [79]. Pitt & Miscamble [78] reported similar minimum a_w values to *A. flavus*. The optimal pH range for growth is considered to be 3.5 to 8, however growth can occur between pH 2.4 to 10 at 25, 30 and 37 °C [46]. Final optimal conditions for aflatoxins productions were found to be temperatures between 12-40 °C, water activity down to 0.86 a_w and pH values in the range of 3 to 8 or higher [79].

1.2.1.3 Genus *Neosartorya*

In terms of food spoilage, among the different *Neosartorya* spp., only *N. fischeri* is of interest to the food industry due to the heat resistance of its ascospores. *N. fischeri* has been isolated mainly from heat-treated fruit products like canned strawberries, fruit purees and pasteurised fruit juices. In rare occasions, it has also been isolated from non-heat treated or non-processed foods [11,22,111]. It has been reported that *N. fischeri* can produce mycotoxins such as fumitremorgens A and C and verruculogen that are highly toxic [112–114]. Ascospores of *N. fischeri*, along with those of *Byssochlamys*, are considered to be the most heat resistant fungal spores known, with the ascospores of *N. fischeri* being more resistant than those of *B. fulva* [11].

1.2.2 Unicellular fungi (yeasts)

Yeasts are typically mesophilic and they showcase optimal growth at a_w above 0.90. They can grow in a pH range from 3 to 10. They can be classified into two categories, namely fermentative and oxidative yeasts. Fermentative yeasts are the most common spoilage yeasts. They are facultative anaerobes that can produce ethanol and CO_2 from sugars [5]. Some yeasts species belonging to this category are characterized as the most known osmophilic microorganisms with the ability to grow at a_w as low as 0.60 [115]. Representative genera of this category include *Saccharomyces* and *Zygosaccharomyces*. Oxidative yeasts, that appear to be less common, can grow on fermented foods and metabolize alcohol and organic acids. These yeasts seem to possess the morphological characteristics of yeasts but the metabolic characteristics of moulds. Representative genera include *Candida*, *Pichia*, *Debaryomyces* and *Mycoderma* [5]. According to Pitt & Hocking [11], around 10 species of yeasts are mainly responsible for spoilage of food products. Below the most relevant for the spoilage of fruits and by-products will be introduced.

Candida krusei has been isolated from citrus and other fruit products, figs, grapes, fermenting mango, olives and soft drinks [22,116–118]. In addition, it has been found in other food products including dairy [119] and meat products [120]. *C. krusei* grows on the surface of the foods by forming films. It can grow at temperatures ranging from 8 to 47 °C and in very low pH [121,122]. Furthermore, it is quite resistant towards preservatives [123].

Debaryomyces hanseii has been isolated from a variety of fruits, fruit juices, soft drinks and canned fruits [22,115,116,124]. It has also been found in high numbers in yoghurts [125]. The yeast can grow at temperatures ranging from 2 to 33 °C however, the maximum temperature can be as high as 38 °C in products with 60 % w/w glucose. The optimum growth temperature is 24-25 °C in 10% w/w glucose or 27-30 °C in 60 % w/w glucose [126,127]. In addition, it can grow in a pH range from 2.0-2.5 up to at least 8.0 at 25 °C [128].

Pichia anomala has been reported to cause spoilage to fruits, fruit juices and beverages, wine and beer, meat, dairy products and low a_w products [116,129]. Furthermore, it has been isolated from yoghurts with fruit purees or fruit flavorings [11]. According to Deak & Bechaut [116], it is the third most common foodborne yeast after *S. cerevisiae* and *D. hanseii*. In addition, it can cause infections in immunocompromised and paediatric patients [75,130]. Regarding its physiology, it can grow in pH as low as 3.0 at 37 °C or 2.0 at 25 °C, while the highest pH value for growth is set at 12.4 [131]. It is also considered relatively resistant to preservatives.

Rhodotorula mucilaginosa is found in fresh fruits and vegetables but also in heat-treated products like apple sauce and strawberries [11,132–134]. It can grow at temperatures between 0.5-5 up to 35 °C and the minimum a_w for growth is set at 0.92 [11,122]. Finally, the minimum pH for growth is reported to be around 2.2 in the presence of HCl or organic acids [121].

Saccharomyces cerevisiae is a yeast widely spread in foods since it occurs naturally on the leaves of fruits and in nectars and exudates. It has been reported to cause spoilage in soft drinks, fruit juices, concentrates and purees [22,116,135,136]. The minimum growth temperature is set at 4 °C in the presence of 10 % glucose, or at 13 °C in 50 % glucose. The maximum growth temperature is between 38-39 °C with an optimal around 33-35 °C with10-30 % glucose [127]. Finally, growth of the yeast was reported in a_w as low as 0.89 in glucose media at neutral pH [137].

Zygosaccharomyces bailii has been reported to cause spoilage in products like fruit juices and concentrates [22,138,139], fruit syrups [132], sauces [140,141] and olives [142]. Since it is highly resistant to weak acid preservatives, like benzoic, sorbic, acetic, propionic acids and SO_2, the food products must be packed sterile or pasteurised in order to avoid spoilage by this yeast. The minimum temperature for growth is 6.5 °C in 10 and 30 % w/w glucose or 13 °C in 60 % glucose. Maximum growth temperatures were reported to be 40 °C in 60 % w/w glucose or 37 °C in the presence of lower glucose concentrations. Optimal temperatures range from 30-32 °C in 10% w/w glucose or 34-36 °C in 60% w/w glucose [127]. Furthermore, *Z. bailii* is a xerophile yeast with the ability to grow in products with water activity as low as 0.80 a_w at 25 °C. However, at 30 °C the minimum a_w was reported to be 0.86 in fructose [143,144].

Z. rouxii can grow in products with very low water activity and as a result it has been isolated from products like fruit concentrates, glazed fruits, honey and syrups, and sauces [140,145–147]. It is considered to be the second most xerophilic organism known with the ability to grow in water activities as low as 0.62 a_w in fructose solutions, or 0.65 a_w in sucrose/glycerol [143,145]. The optimal growth temperature varies from 24 °C in 10 % w/w glucose (0.99 a_w) to around 33 °C in 60 % w/w glucose (0.87 a_w). Similarly, maximum growth temperatures range from 37 °C in 10 % glucose and 42 °C in 60 % glucose. The minimum temperature was reported to be 4 °C in 10 % glucose or 7 °C in 60 % glucose [127].

1.3 MYCOTOXINS

Mycotoxins are secondary metabolites that are produced by several fungi under favourable conditions, and can cause adverse health effects to humans and animals. Favourable conditions for mycotoxin production include high temperatures, and humidity, as well as poor hygiene during transportation and storage. Mycotoxicoses, the diseases caused by the consumption of mycotoxins, can be acute or chronic. Chronic toxic effects can lead, amongst others, to cancer and immunosuppression. Up to date, approximately 400 different mycotoxins have been identified; however, the most frequently encountered in foods include: aflatoxins, ochratoxin A (OTA), patulin, *Fusarium* toxins (fumonisins, zearalenone, trichothecenes), ergot alkaloids, and *Alternaria* toxins

amongst others. The fungi that produce mycotoxins can be classified in two categories, namely field fungi and storage fungi. Field fungi contaminate the crops before harvest including plant pathogens, fungi that grow on stressed plants, and fungi that colonize the crops before harvest and contaminate after harvest. On the other hand, storage fungi contaminate the crops after harvest [148,149].

Apart from their importance for public health, mycotoxins can impact world trade and lead to economic losses. In particular, according to the annual report of 2020 published by the Rapid Alert System for Food and Feed (RASFF), mycotoxins were classified as the 2nd most frequently reported hazard for food products originating from non-member countries with a total of 400 notifications. Aflatoxins were the most reported mycotoxins and "nuts, nut products and seeds", "fruits and vegetables", and "herbs and spices" were the most affected food categories. Pesticide residues were at the top of the list due to the ethylene oxide incident that had a great impact on the reported notifications [150]. In addition, according to Eskola et al. [151], 60-80 % of the grains worldwide is contaminated by mycotoxins. For all the above mentioned reasons the European Food Safety Authority (EFSA) [152], the World Health Organisation (WHO) and the Food and Drug Administration (FDA) have set maximum limits for mycotoxins in foodstuff.

1.3.1 Patulin

Patulin was originally discovered in the 1940's as an antibiotic agent against Gram-positive and Gram-negative bacteria. Furthermore, it can act as a phytotoxin and is active against pathogenic fungi. Its use has also been suggested for treating the common cold, and as an antiviral agent. However, due to its toxicity, clinical trials on humans were paused [153,154]. Patulin (molecular formula $C_7H_6O_4$) is a crystalline, water-soluble and colourless compound that belongs to the group of toxic lactones [155]. The main fungal species that produce patulin belong to the genera of *Penicillium*, *Aspergillus*, *Paecilomyces* and *Byssochlamys*, being *P. expansum* the main patulin-producing mould [156]. It is frequently associated with fruits and fruit products, in particular apples and their by-products; however, it has been isolated from pears, figs, grapes and tomatoes. In addition, patulin has been reported in vegetables like bell peppers, grains including wheat, rice and corn and in some cheeses [149,157].

Different processing steps (clarification/filtration, heat treatment, fermentation) and reduction techniques (biological control agents, chemical additives, physical treatment) have been applied with the overall goal of eliminating patulin. However, with these techniques elimination of patulin was not complete. As a result, prevention for the presence of patulin-producing fungi is better [157].

Patulin toxicity can be divided into acute and sub-acute toxicity, and chronic toxicity. Symptoms of acute toxicity include gastrointestinal (GI) symptoms like nausea, vomiting and ulcers. Sub-acute toxicity in rats led in weight loss, GI disturbances and inhibition of several enzymes [149]. Related to long-term exposure to patulin, *in vitro* and *in vivo* studies in animals have shown that this mycotoxin can lead to carcinogenicity, immunotoxicity, teratogenicity, genotoxicity and embryogenicity [155]. However, due to inadequate data the International Agency for Research on Cancer (IARC) has classified patulin as a Group 3 carcinogen, meaning that it cannot be classified towards its carcinogenicity to humans [158]. Maximum patulin levels have been established by EFSA for apples and by-products, fruit juices and baby foods (**Table 1.2**) [152]. Furthermore, the WHO [159] and the FDA [160] have set limits of 50 μg/ L for patulin in foodstuff.

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The biosynthetic pathway of patulin production has been described and consists of about 10 steps as suggested by several biochemical studies and by the identification of several mutants that are blocked during the biosynthesis of patulin (**Figure 1.1**) [156].

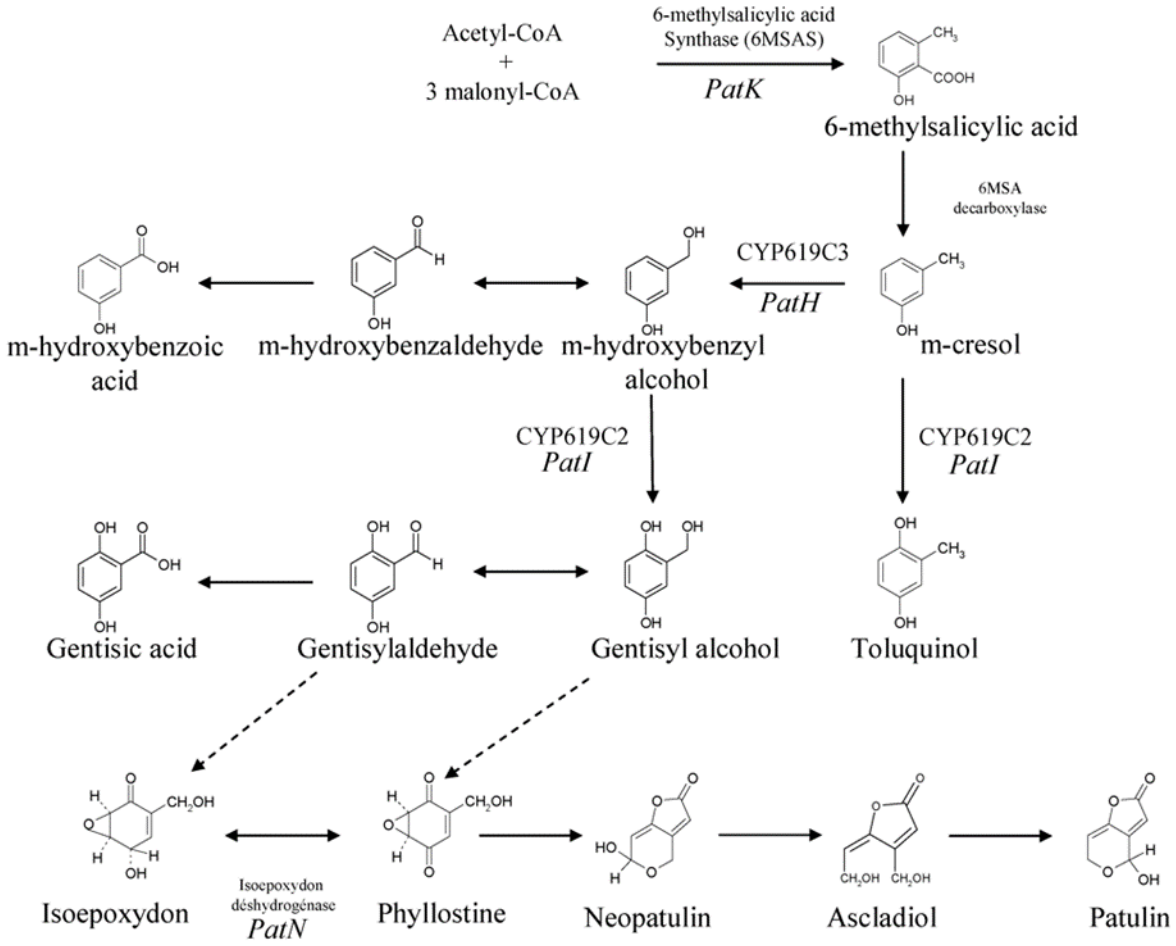

Figure 1.1. Biosynthetic pathway of patulin production (Image used with license from Creative Commons Attribution License 3.0 Unported [156]).

1.3.2 Aflatoxins

Aflatoxins were initially isolated in the 1960´s, they are the most studied mycotoxins and are produced by species belonging to the genus *Aspergillus* section *Flavi* [161,162]. There are six main aflatoxins, namely AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2, which are dihydrofuran or tetrahydrofurano moieties fused to a coumarin ring. Like many other heterocyclic compounds, aflatoxins have fluorescence properties. In particular, AFB_1 and AFB_2, exhibit blue fluorescence under UV, while AFG_1 and AFG_2 fluoresce green. Hence, the letters B and G. Furthermore, AFM_1 and AFM_2 which are metabolites in milk (hence the letter M) also fluoresce blue [162,163]. The main aflatoxin-producing fungi are *A. flavus* and *A. parasiticus* which are found to be pathogenic to both humans and animals [148]. Other species in this section, like *A. nomius*, *A. pseudotamarii* and *A.*

bombycis, have also been identified as aflatoxin producers [164]. *A. flavus* mainly produces AFB_1 (and its metabolite AFM_1) and AFB_2, while *A. parasiticus* can produce AFB_1, AFB_2, AFG_1 and AFG_2 [165].

Aflatoxins are frequently associated with cereal crops, where pre-harvest contamination can occur in temperate and tropical regions. Related to fruits, aflatoxins can grow in figs, dates and citrus fruits [166,167]. In addition to these, aflatoxin contamination has also been reported in spices [168] and nuts [169]. Factors contributing to fungal invasion and aflatoxin production include dry environmental conditions, insect damage and timing of irrigation. Post-harvest contamination is facilitated when storage conditions are optimal for production of the mycotoxin. Aflatoxins are very stable compounds and can be quite resistant against processing techniques like roasting, extrusion, cooking and baking. As a result, they can be a problem in foods like roasted nuts and baked goods [161].

Aflatoxins have been characterized as very potent carcinogens in all the tested animals as well as in some organs with the liver been the main target. In addition, they are genotoxic compounds and according to IARC the main aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) are classified as Group 1 carcinogens, while AFM_1 is classified as Group 2B meaning that is a possible carcinogen for humans [158,170]. Variations are observed in the toxicity of the different aflatoxins [148]. In particular, AFB_1 is the most toxic aflatoxin in both acute and chronic toxicity and has been characterised as both carcinogenic and cytotoxic, while AFM_1 can be equally hepatotoxic as AFB_1 but not carcinogenic [162]. Aflatoxicosis, the disease caused by aflatoxins, is an important health problem in poor areas with bad hygienic conditions and drought [171]. Symptoms of acute aflatoxicosis include vomiting, abdominal pain and pulmonary or cerebral oedemas. Acute toxicity is more common in animals since highly contaminated feed is more frequent. Symptoms of chronic toxicity to humans include liver cancer, problems with the reproductive and the immune system, interstitial fibrosis, and encephalopathy [161]. The EFSA has established maximum levels of aflatoxins (AFB_1, AFM_1 and sum of all) in foodstuff which can be found in **Table 1.2** [152]. In the US, the FDA has described aflatoxins as “adulterants”, and has set a limit of 20 ppb in food and feed, and 0.5 ppb in milk [162].

1.3.3 Ochratoxin A (OTA)

Ochratoxin A (OTA), originally isolated by *A. ochraceus* in 1965, is a phenylalanine derivative of a substituted isocoumarin and is mainly produced by species belonging to *Aspergillus* and *Penicillium* genera. The main producers are species belonging to *Aspergillus* section *Circumdati*, *Aspergillus* section *Nigri*, *P. verrucosum* and *P. nordicum* [161]. *Aspergillus* spp. typically produce OTA in humid and warm places, whereas *Penicillium* spp. can produce OTA in temperatures as low as 5 °C [148].

OTA is a very stable compound that can withstand common processing techniques. For example, heating for several minutes in temperatures above 250 °C are required to reduce OTA concentration [172]. Since removal of the mycotoxin is not feasible from food products, the main strategy of the food industry includes the inhibition of the growth of OTA-producing fungi [173]. OTA is mainly found in cereals, grapes/ raisins, wine, dried fruits, green coffee and spices. In addition, cocoa beans, as raw materials, are susceptible to OTA contamination [148,161]. *Penicillium* spp. have also been associated with production of OTA in fermented meats and cheeses [174].

Regarding its carcinogenic potency, OTA has been classified by IARC as a Group 2B, meaning that there is insufficient evidence for carcinogenicity in humans, but adequate data from studies in experimental animals [158]. OTA has been recognized as a potent nephrotoxin since kidneys are the

main target organ. In addition, OTA has proved to be teratogenic, genotoxic, mutagenic and immunotoxic. Symptoms of acute toxicity include multifocal haemorrhages in many organs and fibrin throbin in the spleen [161]. Regarding its chronic effects in humans, OTA has been associated with a typical kidney disease in the Balkans, namely Balkan Endemic Nephropathy (BEN). Symptoms of BEN typically include anaemia, proteinuria, jaundice, headache, anorexia and uraemia. Nevertheless, even though the exposure data of OTA seem to be consistent, they are insufficient to make an association with BEN [161,175]. EFSA has established maximum levels of OTA in foodstuffs that can be seen in **Table 1.2** [152].

The biosynthetic pathway of OTA has not been completely described even though some have been proposed [176]. Huff & Hamilton [177] proposed a biosynthetic pathway for OTA however, attending to the most recent results by Harris & Mantle [178] some discrepancies can be observed. In particular mellein, a precursor suggested by Huff & Hamilton [177], does not play a role in the biosynthesis of OTA, and the ubiquitous ochratoxin B is totally ignored.

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Table 1.2. Maximum levels allowed for mycotoxins as established by EFSA

	Foodstuffs	Maximum levels (µg/ kg)		
2.1	**Aflatoxins**	**B1**	**Sum of B1, B2, G1 & G2**	**M1**
2.1.1	Groundnuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	8.0	15.0	-
2.1.2	Nuts to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5.0	10.0	-
2.1.3	Groundnuts and nuts and processed products thereof, intended for direct human consumption or as an ingredient in foodstuffs	2.0	4.0	-
2.1.4	Dried fruits to be subjected to sorting, or other physical treatment, before human consumption or use as an ingredient in foodstuffs	5.0	10.0	-
2.1.5	Dried fruits and processed products thereof, intended for direct human consumption or as an ingredient in foodstuffs	2.0	4.0	-
2.1.6	All cereals and all products derived from cereals, including processed cereal products, with the exception of foodstuffs listed in 2.1.7, 2.1.10 and 2.1.12	2.0	4.0	-
2.1.7	Maize to be subjected to sorting or other physical treatment before human consumption or use as an ingredient in foodstuffs	5.0	10.0	-
2.1.8	Raw milk, heat-treated milk and milk for the manufacture of milk-based products	-	-	0.050
2.1.9	Following species of spices: *Capsicum* spp. (dried fruits thereof, whole or ground, including chillies, chilli powder, cayenne and paprika) *Piper* spp. (fruits thereof, including white and black pepper) *Myristica fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric)	5.0	10.0	-
2.1.10	Processed cereal-based foods and baby foods for infants and young children	0.10	-	-
2.1.11	Infant formulae and follow-on formulae, including infant milk and follow-on milk	-	-	0.025
2.1.12	Dietary foods for special medical purposes intended specifically for infants	0.10	-	0.025
2.2	**Ochratoxin A**			
2.2.1	Unprocessed cereal		5.0	
2.2.2	All products derived from unprocessed cereals, including processed cereal products and cereals intended for direct human consumption with the exception of foodstuffs listed in 2.2.9 and 2.2.10		3.0	

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2.2.3	Dried vine fruits (currants, raisins and sultanas)	10.0
2.2.4	Roasted coffee beans and ground roasted coffee, excluding soluble coffee	5.0
2.2.5	Soluble coffee (instant coffee)	10.0
2.2.6	Wine (including sparkling wine, excluding liqueur wine and wine with an alcoholic strength of not less than 15 % vol) and fruit wine	2.0
2.2.7	Aromatised wine, aromatised wine-based drinks and aromatised wine-product cocktails	2.0
2.2.8	Grape juice, concentrated grape juice as reconstituted, grape nectar, grape must and concentrated grape must as reconstituted, intended for direct human consumption	2.0
2.2.9	Processed cereal-based foods and baby foods for infants and young children	0.50
2.2.10	Dietary foods for special medical purposes intended specifically for infants	0.50
2.2.11	Green coffee, dried fruit other than dried vine fruit, beer, cocoa and cocoa products, liqueur wines, meat products, spices and liquorice	-
2.3	**Patulin**	
2.3.1	Fruit juices, concentrated fruit juices as reconstituted and fruit nectars	50
2.3.2	Spirit drinks, cider and other fermented drinks derived from apples or containing apple juice	50
2.3.3	Solid apple products, including apple compote, apple puree intended for direct consumption with the exception of foodstuffs listed in 2.3.4 and 2.3.5	25
2.3.4	Apple juice and solid apple products, including apple compote and apple puree, for infants and young children and labelled and sold as such	10.0
2.3.5	Baby foods other than processed cereal-based foods for infants and young children	10.0

Adapted from COMMISSION REGULATION (EC) No 1881/2006 [152].

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1.3.4 Other toxins

Ergot alkaloids (EAs) are classified as indole alkaloids and they are mainly produced as a toxic cocktail in the sclerotia of *Claviceps* spp., which are well-known pathogens of various grass species. Reports of diseases related to ingestion of contaminated cereals, flour and other by-products by these mycotoxins are dated back to the ancient years. *Claviceps purpurea* is the main species encountered in grains originating from Europe and a number of EAs including ergotamine, ergometrine, and ergosine amongst others are found in its sclerotia. The human disease is called ergotism of St. Anthony's fire and generally two forms exist the gangrenous and the convulsive. The former affects the blood supply while the latter one the central nervous system. Nevertheless, ergotism as a human disease is eliminated today thanks to the modern methods for grass cleaning. However, it still of great concern for animals like cattle, sheep, pigs, and chickens. Symptoms in animals include gangrene, abortion, convulsions, suppression of lactation, hypersensitivity and ataxia [161,179].

Fumonisins are a family of mycotoxins mainly produced from different *Fusarium* species belonging to the *Liseola* section, with *F. verticillioides* and *F. proliferatum* being the main producers. Fumonisins belonging to B series (FBs) namely B_1, B_2 and B_3, are the most important ones. Fumonisin B_1 is the most important toxin due to its toxic potency. *F. verticillioides* is of a great economic importance since it is a corn endophyte that typically does not cause plant disease; however, under the appropriate conditions and in the presence of the necessary fungal and plant genotype it can cause seedling blight, stalk rot, and ear rot. In addition, even if the fungus is visible present this does not mean that the toxins are produced since most of the strains are not capable of producing them. Regarding its carcinogenic potency IARC has classified fumonisins as a group 2B, meaning that they are probably carcinogenic to humans [161,179].

Trichothecenes is a family of more than 170 metabolites that are produced from various species including *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma* and *Trichodermium* amongst others. Based on their chemical structure they are classified in 4 types (A-D). The most important metabolites of type A are HT-2 and T-2 toxins, and for group B is deoxynivalenol (DON). Types C and D include trichothecenes of lesser importance. Fumonisins from type A and B are typically produced by different *Fusarium* spp. and in some cases from species of *Trichoderma*. DON is one of the toxins most frequently encountered in grains that upon ingestion can cause nausea, vomiting and diarrhoea in farm animals. Even though it less toxic compared to other toxins is of major importance due to its high prevalence in cereals like barley, wheat and corn. Furthermore, diacroxyscripenol and T-2 are considered to be cytotoxic and immunosuppressive. Acute effects include gastrointestinal, dermatological and neurologic symptoms. Finally, they have been associated with a human disease called alimentary toxic aleukia that causes inflammation of the skin, vomiting and damage to hematopoietic tissues [161,179].

Zearalenone (ZEN) is a secondary metabolite produced by different *Fusarium* spp., *F. graminearum* being the main producer of this toxin. All the species associated with ZEN production are common contaminants of cereal crops worldwide. *Fusarium* spp. related with ZEN production can also produce other metabolites like α-zearalenol and β-zearalenol. Even though, ZEN is classified as a mycotoxin the definition of mycoestrogen would be more accurate since this metabolite is hardly toxic, while resembling 17β-estradiol, the basic hormone produced by ovaries in order to allow estrogen receptors to bind in mammalian target cells. Among the different zearalenones, α-zearalenol exerts the highest estrogenic potential [161,179].

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Alternaria toxins are structurally divided in three groups namely the dibenzopyrone derivatives, the perylene derivatives and the tetramic acid derivatives. From the first group the most known toxins are alternariol (AOH), alternariol monomethyl ether (AME) and altenuene (ALT), from the second group the altertoxins (ATX-I and II) and from the third group the tenuazonic acid (TeA) and iso-tenuazonic acid (iso-TeA). *Alternaria alternata* is considered the most important producer of the *Alternaria* toxins. Regarding their toxic effects, AOH, AME, TeA and ATX have been found to teratogenic and fetotoxic in animals, while AOH and AME have been proved to be mutagenic and clastogenic in many *in vitro* studies [161].

EFSA has established maximum levels on foodstuffs for DON, ZEN, fumonisins B_1 and B_2, T-2 and HT-2 toxins but not for EAs and *Alternaria* toxins [152]. Since most of the above mentioned toxins and their related fungal producers are mainly encountered in cereal crops they remained out of the scope of this study. Regarding *Alternaria* toxins, even though they can contaminate fruits and products thereof, they are considered a relatively new group of mycotoxins and as a result up to date only a few studies have been focused on the detection of their respective producers.

1.4 METHODS FOR THE DETECTION OF YEASTS AND MOULDS IN FOOD PRODUCTS

1.4.1 Culture-based methods

Up to date, detection of yeasts and moulds is based on cultural procedures. Typically, the process starts with a 1:10 dilution of the sample (25- 50 g) with a buffer such as Phosphate-buffered Saline (PBS), Butterfield's buffer, or 1% buffered peptone water, in a stomacher bag followed by homogenization with a Stomacher or a Pulsifier. Blending can also be used but is generally less effective. In continuation, spread plating of serial dilutions on agar plates and incubation is performed. For liquid foods, like juices, pour plating can also be performed; however, with this technique fungi grow slower below the agar surface and might be hidden by faster growing colonies from surface spores. As a result, spread plating provides a more uniform colony development while improving the accuracy of the colonies enumeration. Typically, the plates are incubated for 5 days at 25 °C and the results are expressed as viable counts per gram or sample. If there is no growth after 5 days, incubation can be extended for another 48 h [5,11].

Selection of a suitable medium is of outmost importance for correctly enumerating the fungi of interest and some considerations must be taken into account before choosing. The most important factor to be considered is if the method is destined for foods with high or low a_w. Based on the different categories of dried foods, namely foods low in soluble solids, food high in sugars, and salty foods, the most adequate media can be selected. Additionally, attention should be paid to the targeted microorganism, i.e. moulds, yeasts or both as well as in the presence or absence of preservatives. In **Table 1.3** a list of media recommended for enumeration, detection and isolation of fungi from fruits and by-products can be found [11,180].

Another method for detection, enumeration and isolation of fungi is based on direct plating, serving as the method of preference for foods that can be handled with forceps like grains and nuts. With this approach, food particles are placed directly on solidified agar plates after an initial surface disinfection step, in order to remove dust and other particles that might hinder the recovery of the fungi. The plates are incubated for 5 days at 25 °C and at the end of the incubation period the number of contaminated particles are counted and the results are expressed as a percentage. If there is no growth after 5 days, incubation can be extended for another 48 h. For optimal results the selection of media, a stereomicroscope and laboratory experience are very important [11,180].

For heat-resistant moulds more elaborated procedures are required due to the low number of heat-resistant ascospores present in fruits and fruit juices. These procedures typically include the use of large amount of samples (often 100 mL or g), heat inactivation of the ascospores and plating at different agar plates followed by incubation at 30 °C for up to 30 days [5].

As a consequence, culture-based methods are time-consuming, extending the overall time of analysis up to 7 days, rendering them not suitable for products with short shelf-lives, and do not provide the short sample-to-result time in agreement with the needs of the food industry. Hence, the obtained results can only be used retrospectively being of limited value for quality and process control. In addition, they are laborious and require large amounts of media and reagents [181,182]. Another disadvantage of these methods is that they cannot detect Viable But Non-Culturable (VBNC) microorganisms, that can be generated during processing, cleaning and/or disinfection activities since at this state they cannot grow on media but they can still play an important role in spoilage by producing enzymes or other metabolites of importance [183].

Table 1.3. Recommended media for fungal detection, enumeration and isolation from fruits and by-products

Food	Target microorganism	Medium
Fruits	Moulds	DRBC
	Yeasts	TGY, MEA, OGY
Fresh fruit juices	General	DRBC
	Yeasts	TGY, MEA, OGY
Preserved fruit juices	Preservative resistant yeasts	TGYA, malt acetic agar
Fruit juices to be pasteurised, or pasteurised products	Heat resistant moulds	PDA, MEA
Fruit juice concentrates	Xerophilic yeasts	MY50G

DRBC: dichloran rose bengal chloramphenicol agar, TGY: tryptone glucose yeast extract agar, MEA: malt extract agar, OGY: oxytetracycline glucose yeast extract agar, PDA: potato dextrose agar, MY50G: malt extract yeast 50 % glucose agar. Adapted from Pitt & Hocking [11].

1.4.2 DNA-based methods

1.4.2.1 End-point Polymerase Chain Reaction (PCR)

PCR has been used for the detection of a number of microorganisms. A typical PCR reaction requires a forward and a reverse primer with complementing sequences to the target DNA sequence, the DNA template, a DNA polymerase, dNTPs, and salts. PCR consists of multiple cycles, typically between 30 and 35, and each cycle consists of 3 steps namely the denaturation, annealing and extension. The amplification occurs exponentially, and after some cycles enough copies of the target DNA sequence will have been accumulated enabling its detection. The PCR product is typically visualized by agarose gel electrophoresis. The detection is based on the size of the amplicon with the use of fluorescence dyes [184,185].

One of the main disadvantages of end-point PCR is that the amplification and the detection occurs separately, in a two-step process. Even though the amplification is "automatic" by using a thermocycler, the reading of the results still remains a manual process [185].

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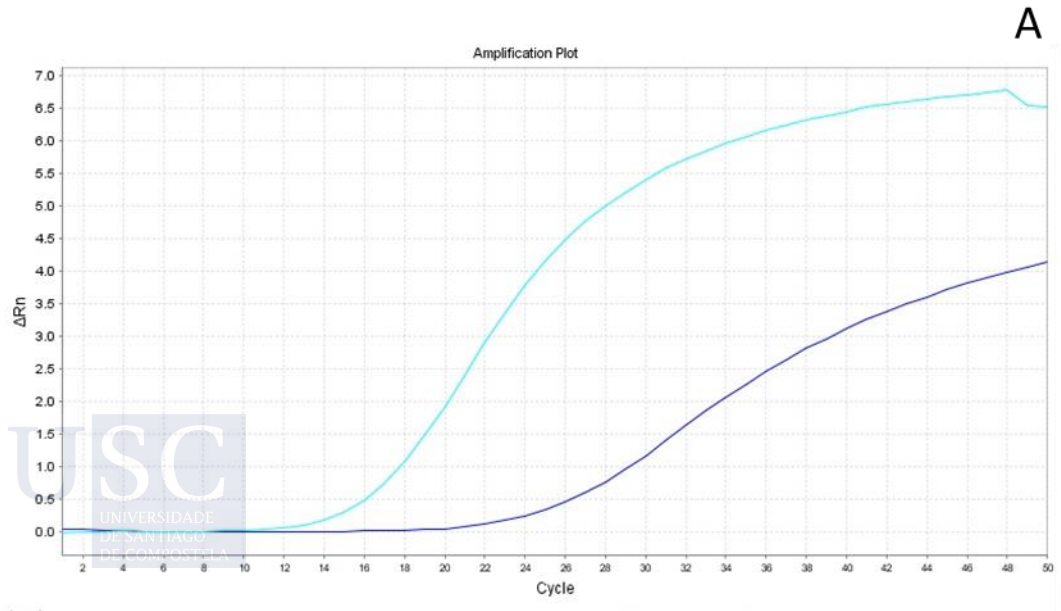
1.4.2.2 Real-time PCR (qPCR)

The difference between conventional or end-point PCR and qPCR is that the amplification and visualization of the results occurs simultaneously by measuring the fluorescence in real time. In particular, the fluorescence is measured after each cycle at the end of the extension step. The cycle at which the fluorescence intensity increases above the detectable level corresponds proportionally to the initial number of template DNA molecules in the sample is called the quantification cycle (Cq) [186,187]. The amplification curves obtained can be used for quantification purposes. Different chemistries can be applied being able of generating a fluorescence signal; however fluorescent dyes that are double-stranded DNA (dsDNA) specific or fluorescent hydrolysis probes that are sequence-specific, are the most common ones [184,188]. The first dye to be used was ethidium bromide [189,190] and since then others have been used such as YO-PRO-1 [191,192]; however the most frequently used is SYBR-Green I [190,193]. Other detection chemistries include the use of molecular beacons, scorpion and hybridization probes [184,188].

In qPCR assays implementing SYBR-Green I, the products can be differentiated by performing a melt-curve analysis after the amplification. A given amplicon will have a characteristic melting temperature (Tm) which is dependent on the GC content, size and sequence of the amplicons [185]. The melt-curve is obtained by measuring the gradual loss of fluorescence of the dye near the denaturation temperature of the PCR product [190]. T_m is the defined temperature at which the steepest decrease of signal occurs. Typically, amplicons generated due to non-specific amplification of primer dimers can be distinguished since they have lower T_m because of their small size [188]. An advantage of melt-curve analysis over gel electrophoresis is that it can distinguish products with the same size but different GC/AT ratios as well as amplicons with the same size and GC content but different distribution of the GC bases [185]. An example of typical amplification plots along with their corresponding melt-curve analysis can be seen in **Figure 1.2**. Furthermore, high-resolution melting (HRM) technology is a low cost approach for the detection of specific single nucleotide polymorphisms (SNPs), the discrimination of alleles defined by multiple SNPs or tandem-repeat number, and the determination of DNA methylation status. It can be performed directly in a real-time thermocycler after the qPCR assay with the use of specific dyes like LCGreen, SYTO9, and EVAGreen [194].



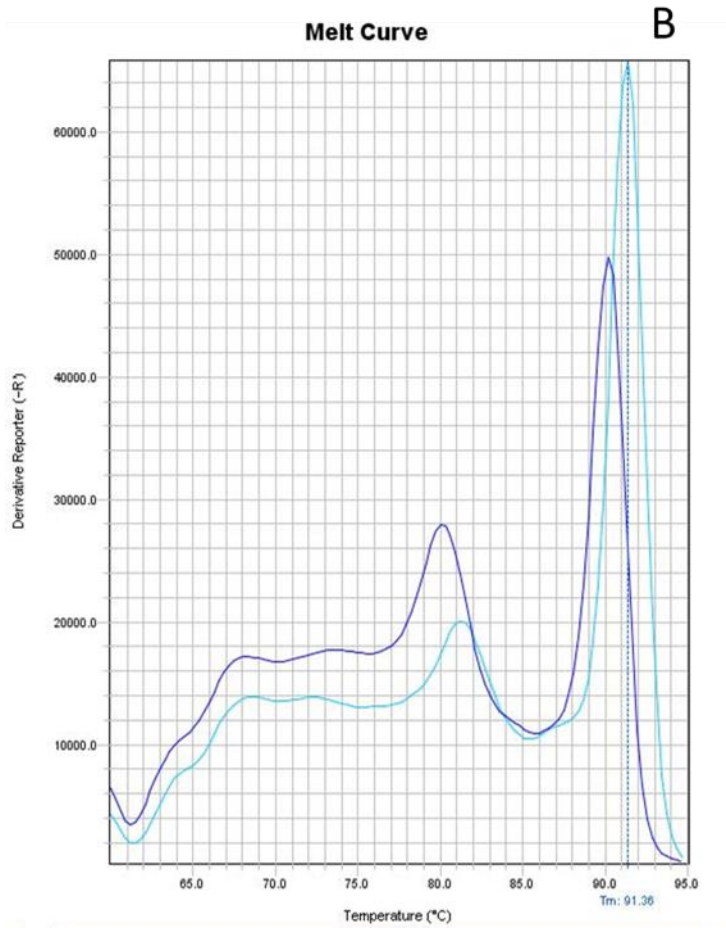


Figure 1.2. Typical amplification plots (A) and their corresponding melt-curve analysis (B).

The hydrolysis probes, such as TaqMan®, are sequence-specific oligonucleotide sequences which are labelled in the 5'-end with a fluorophore and in the 3'-end with a quencher. While the probe is intact, the quencher absorbs the fluorescence and no signal is detected however, upon proper hybridization of the primers and the probe, during the amplification, and when the DNA polymerase reaches the probe, due to its 5'→ 3' exonuclease activity, the polymerase degrades the probe physically separating the fluorophore from the quencher resulting in detectable emission of fluorescence [188]. One advantage of hydrolysis probes compared to SYBR-Green I is the increased specificity of the assay since the probe will selectively bind to the complementary sequence of the amplicon [184].

Among the advantages of the qPCR some include that it is a robust, fast and relatively easy to perform assay, and the risk of cross- contamination is minimal since it is performed in a close-tube format [188]. In addition, other advantages of the qPCR over end-point PCR is that it does not require post-amplification processing, that it has a wide dynamic range (with more than eight order of magnitude) [195] and its high reliability since amplification data are acquired in real-time. Furthermore, the ability to multiplex and detect more than one target simultaneously is possible with the use of hydrolysis probes tagged with different fluorophores, or by performing a melt-curve analysis [184]. Finally, qPCR contrary to the end-point PCR allows for quantification, since the Cq

value of a certain sample can be compared against a standard curve, generated from serial dilutions of known amounts of DNA, in order to define its initial DNA concentration [196].

End-point PCR and qPCR assays, can be both affected by the presence of compounds that inhibit the amplification of the DNA; thus there is a strong need for adequate purification protocols that can eliminate these compounds. Inhibiting substances may originate from the food matrices, the culture media, but they can also be introduced during sample processing and/ or the DNA extraction process [197,198]. Consequently, this can lead to reduced sensitivity, or false negative results, due to reaction inhibition. PCR inhibitors can be inorganic like calcium ions, but the majority are organic compounds, for example, polysaccharides, phenols, ethanol, proteins and proteinases [197,199]. In foods the main PCR inhibitors are fats, polysaccharides, glycogen and minerals [200,201]. Additionally, some compounds can be introduced during handling of the sample and DNA extraction process. This includes powder from gloves, salts like NaCl or KCl, and detergents or organic compounds like EDTA, ethanol, isopropyl alcohol or phenol [202–204]. Even though these compounds are necessary for cell lysis, cleaning and elution of the DNA during the extraction, they can also lead to PCR inhibition [198]. The mechanism of action of the PCR inhibitors can be one or more of the following: a) interference with the cell lysis, b) degradation or capturing of nucleic acids and c) inactivation of the DNA polymerase [205].

One way to identify problems related with false-negative results due to reaction inhibition is the addition of an amplification control in the PCR assay. An Internal Amplification Control (IAC) is analysed in the same tube as the target; while an external control is analysed separately [206]. Basically, IAC is a non-target DNA, present in the assay that co-amplifies with the target gene and should always amplify in negative sample. In this way, false negative results due to reaction inhibition can be identified. In addition, the IAC can be competitive or non-completive. A competitive IAC is amplified with the same primers of the target, and can be separated based on the size of the product or the sequence or with the use of a hydrolysis probe tagged with a different fluorophore of that of the target sequence. On the other hand, a non-competitive IAC is amplified with its own set of primers and thus, can be universal [198]. The different types of IAC and their application in PCR and qPCR assays have been extensively studied [207,208]. Furthermore, Paterson [209] highlighted the need for implementing an IAC in fungal PCR assays. In his review, only one group of researchers was identified that implemented an IAC in their assay for the detection of *Aspergillus* spp. responsible for invasive pulmonary aspergillosis [210]. Furthermore, in a few more studies for the detection of mycotoxin producing fungi the implementation of an IAC has been reported [211–213].

1.4.2.3 Isothermal amplification techniques

Isothermal amplification techniques have emerged over the last decades as an alternative to PCR and qPCR since they are usually faster than PCR and performed at constant temperature, and therefore with the potential to be used for point-of-care (POC) testing [214]. In **Table 1.4** a list of the main isothermal amplification tenchiques along with possible detection methods can be found. Additionally, the main advantages and disadvantages of each method are presented in the same table.

1.4.2.3.1 Loop-mediated isothermal amplification (LAMP)

LAMP is an isothermal amplification technique that was first described by Notomi et al. (2000) [215], claiming that can amplify few copies of DNA to 10^9 in less than an hour and with great specificity. Typically, a LAMP reaction uses two inner primers, namely FIP and BIP, and two outer

primers, namely F3 and B3, as well as a DNA polymerase with high strand displacement activity, the *Bst* DNA polymerase. In addition, one or two loop primers (LF/LB) that hybridize to the stem-loops can be added to accelerate the reaction. The reaction runs between 60-65 °C for up to 1 hour. The final LAMP products are a mixture of stem-loop DNAs with different stem lengths and cauliflower-like structures with multiple loops [215,216]. During the reaction large amounts of pyrophosphate are produced that bind with Mg^{2+} to form the insoluble magnesium pyrophosphate [217]. The mechanism of a LAMP reaction can be seen in **Figure 1.3**.

The amplified DNA can be visualized by naked-eye due to the formation of magnesium pyrophosphate that causes turbidity in the tube; thus a gel electrophoresis is not necessary. Even though results interpretation by naked-eye is the easiest, and more economic method, sometimes skills for assessing the results are needed. Since the turbidity of the reaction is related to the amount of the DNA produced, quantification can occur with the use of a real-time turbidimeter [218]. In order, to facilitate the naked-eye detection an intercalating dye like SYBR-Green [219,220], Picogreen [221,222] or propidium iodide [220] can be added at the end of the reaction, resulting in a colour change when the reaction is positive. However, the main disadvantage of this approach is that it requires opening of the reaction tubes that increases the risk of cross-contamination. Since the amount of Mg^{2+} is decreasing throughout the reaction, quantification can occur by measuring its concentration [223]. In this sense, colorimetric assays have been developed that utilize a fluorescence metal indicator like calcein [224], hydroxy napthol blue (HNB) [223] or malachite green [225,226]. In the case of LAMP assays with calcein and HNB the addition of $MnCl_2$ in the pre-mixture is essential for observing the colour change. Another possibility is the use of pH-sensitive dyes such as phenol red, cresol red, neutral red and/ or m-cresol purple for detection of DNA amplification, since the pH of the reaction shifts from alkaline to acidic [227]. The main advantage of these approaches compared to intercalating dyes is that they can be added before the reaction starts reducing the risk of cross-contamination. Another possibility for colorimetric detection of LAMP products is the use of carboxyl acid-functionalized gold nanoparticles (AuNPs). The colour change is generated upon aggregation-disaggregation of the AuNPs triggered by a complex mechanism that involves the carboxyl acid-contained molecules in the Au surface and the magnesium pyrophosphate that is generated from the reaction [228–230]. Finally, LAMP products can be detected in a real-time format with the use of a device that measures fluorescence and that can be combined with melt-curve analysis for discrimination of products [231,232].

Some of the advantages of the LAMP assay include its increased specificity due to the use of four primers that recognize six distinct regions in the initial steps of LAMP, and later on of two primers that recognize four independent regions on the target sequence, and sensitivity with a detection limit down to a few copies of DNA. Furthermore, LAMP can be easily used for the detection of RNA by adding in the reaction a reverse transcriptase (RT). In addition, LAMP based protocols are relatively easy to follow and there is no need for expensive equipment since the reaction only requires a water bath or a heat block [215]. Finally, this technique is less susceptible to common PCR inhibitors [233,234].

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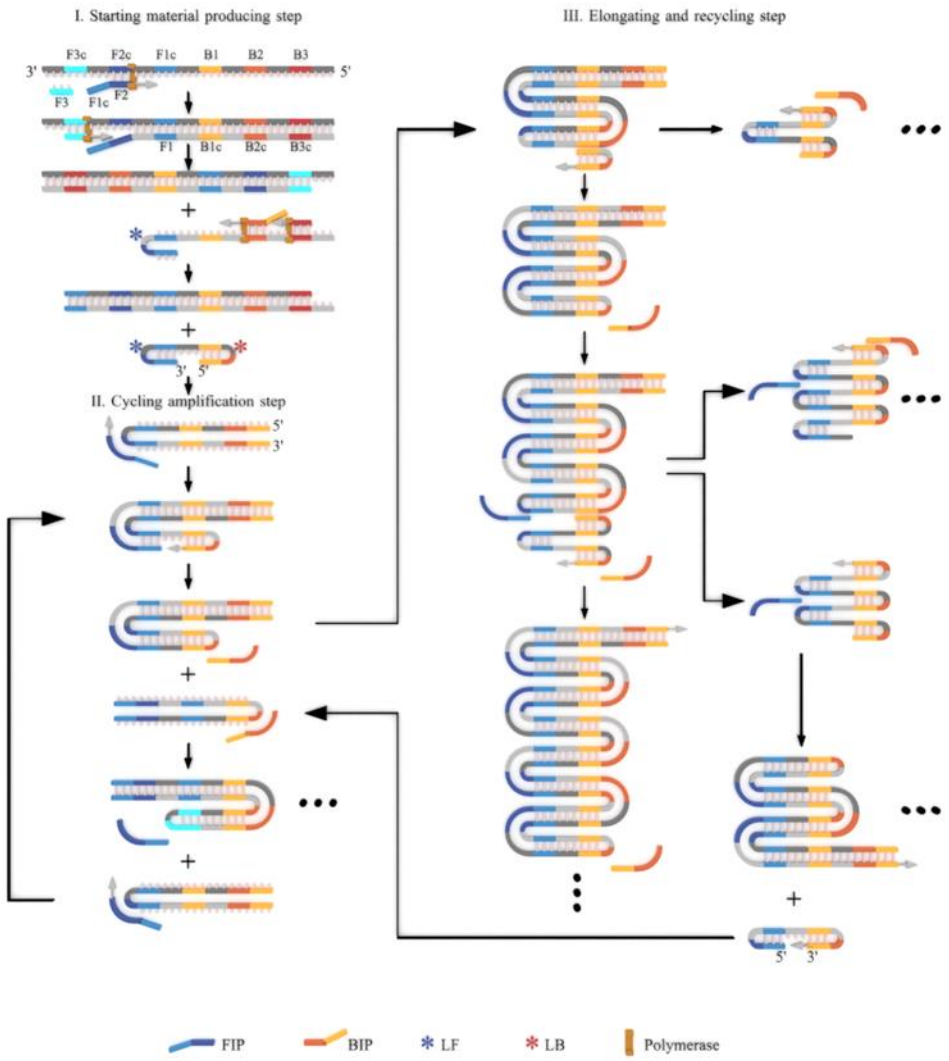

Figure 1.3. Mechanism of LAMP reaction (Image used with license from John Wiley and Sons License [235]).

1.4.2.3.2 Recombinase Polymerase Amplification (RPA)

RPA is an isothermal technique, first described by Piepenburg et al. [236], that instead of melting of the dsDNA, utilises recombinase-primer complexes that scan for homologous dsDNA followed by strand exchange and extension with the help of a polymerase with strand-displacement ability. Repetition of the process results in exponential amplification of the target DNA. The mechanism of the assay can be seen in **Figure 1.4**. Typically, RPA reactions run in a temperature ranging from 37 to 42 °C, and usually an incubation for 20–40 min is adequate for DNA amplification. Kits with freeze-dried reagents are commercially available in different formats (TwistAmp® basic, exo and nfo) as well as for RNA detection by TwistDx (TwistDX Limited, Maidenhead, UK). The amplified product can be visualized by gel electrophoresis, by measuring real-time fluorescence or by lateral flow (LF). For real-time detection, a RPA- exo, or fpg, probe is used, while for lateral-flow detection the design of a nfo probe is necessary [237].

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Some of the main advantages of RPA are that is highly sensitive and specific, simple and rapid due to the low and constant run temperature, without the need of a denaturation step or multiple primers (like in the case of LAMP). Furthermore, it presents multiplexing capabilities and although initially longer primers, 30–35 bp, were designed for RPA assays, it has been observed that PCR primers can be successfully used. Thus, the primer selection process, and design, can be the same of that of PCR. Finally, the RPA has proven to be less sensitive to common PCR inhibitors [237,238]. On the other hand, the conditions of RPA reactions are stringent and the commercial available kits are expensive [239].

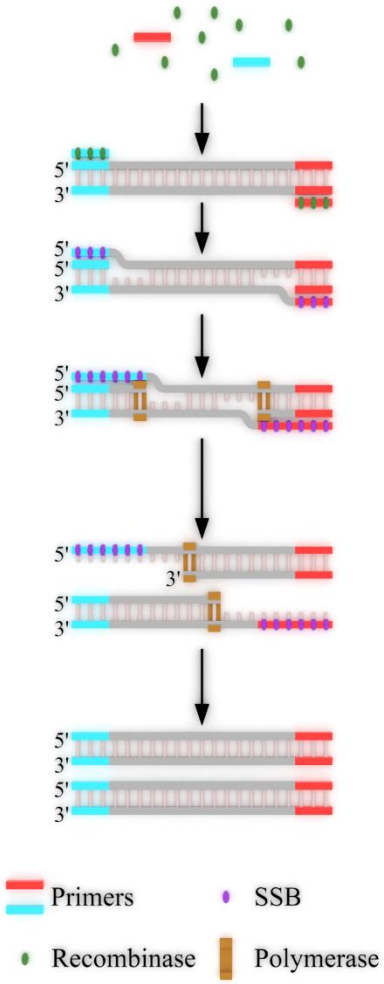

Figure 1.4. Mechanism of RPA reaction (Image used with license from John Wiley and Sons License [235]).

1.4.2.3.3 Other isothermal techniques

In this section other isothermal amplification techniques will be briefly explained due to the fact that even though they present the advantages of an isothermal method they have not been explored yet for the detection of fungi in food products.

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1.4.2.3.3.1 Helicase-Dependent Amplification (HDA)

HDA is an isothermal technique that utilizes DNA helicases for separating the complementary strands of dsDNA in order to create single strands for primer hybridization and extension with the aid of a DNA polymerase. Hence, the thermal dissociation step of a PCR is not necessary. This technique is very similar to the *in vivo* DNA replication. The helicase can act again on the newly synthesized dsDNA and the cycle repeats with kinetics similar to those of PCR, at 60-65 °C. The protocol requires 60- 120 min and the detection can be performed with any of the existing fluorescent chemistries that are used for PCR [240,241]. Some of the advantages of HDA include the simple primer design and the elimination of an initial denaturation step. On the other hand, main disadvantages of the assay include the high possibility of false positive results and its low specificity and sensitivity [239].

1.4.2.3.3.2 Rollicing Cycle Amplification (RCA)

RCA is an isothermal method that utilizes the continuous amplification of circular DNA template by a strand displacing DNA polymerase. The DNA polymerase displaces the synthesized strand and "rolls" with the DNA synthesis, resulting in long single-stranded amplicons with tandem repeats of circular template. Two types of RCAs have been developed, namely linear and exponential. The RCA requires small and circular ssDNA as template; however, many DNA targets relevant in diagnostics are linear dsDNA molecules. In order to overcome this shortcoming, oligonucleotides called padlock probes have been designed with the ability to transform dsDNA into circular ssDNA that can act as template. However, before the circularization process begins, a pre-treatment is required including digestion by restriction enzymes and degradation [240].

1.4.2.3.3.3 Nucleid Acid Sequence-Based Amplification (NASBA)

NASBA is an isothermal transcription-mediated amplification method for ssRNA or ssDNA sequences that is performed at 41 °C. The method is especially suited for RNAs (mRNA, rRNA, tmRNA, and gRNA) while dsDNA cannot be amplified without an initial denaturation step. The assay utilized two RNA primers and three enzymes and an initial heating step at 65 °C is required for removal of secondary structures before the addition of the enzymes. The ssRNA products can be detected with electroluminescence, LF and electrochemical detection upon implementation of sequence-specific probes. In addition, molecular beacons can be utilized as an alternative detection method [240,241].

Table 1.4. Main isothermal amplification techniques and their advantages/disdvantages

Technique	Detection methods	Advantages	Disadvantages
LAMP	Real-time fluorescence Real-time turbidity Turrbidimetric (naked-eye) Colorimetric (naked-eye)	Highly specific (4 to 6 primers for amplification) Tolerant to common PCR inhibitors RNA amplification with the addition of RT	Prone to cross-contamination Complex primer design Difficulty in performing multiplex amplification
RPA	Real-time fluorescence LF strips Gel electrophoresis	Low amplification temperature (37- 42 °C) Very fast (20- 40 min) Simple primer design Tolerant to common PCR inhibitors	Strict reaction conditions Expensive reagents

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HDA	Real-time fluorescence Gel electrophoresis ELISA	No need for need for an initial dissociation step Simple primer design	High chance of false positive results Not possible to perform multiplex amplification Low sensitivity and specificity
RCA	Gel electrophoresis	Low amplification temperature Simple primer design	Requires small and circular ssDNA as template Not possible to perform multiplex amplification
NASBA	Electroluminescence LF ELISA Real-time fluorescence	RNA amplification Low amplification temperature (41 °C)	Need for need for an initial dissociation step Less efficient for long RNA targets

ELISA: Enzyme Linked Immunosorbent Assay

1.5 DETECTION OF VIABLE MICROORGANISMS

One major limitation of all the DNA-based methods is that they cannot differentiate between viable and non-viable microorganisms. This is because DNA is a stable molecule and thus can persist and be present for some time after the death of the microorganism [184]. One approach to overcome this problem is the detection of RNA by RT-PCR/RT-qPCR, since certain types of RNA like mRNA and/ or rRNA, have short half-lives, or are present in high numbers. Since most mRNA molecules have a short lifespan, in the range of minutes, and do not amplify after a moderate heat treatment, they can be used as viability indicators [242,243]. On the other hand, rRNA can be used for detection of viable cells under extreme conditions, like autoclaving, since moderate heating might not lead to complete degradation [244]. However, since rRNA is a universal component of ribosomes, and some rRNA types are present in high amounts in cells, the use of this RNA can greatly improve the limit of detection [245].

Nevertheless, handling of RNA is more difficult. Great care should be taken while extracting the RNA since RNase, the enzyme that digests RNA, is ubiquitous in the environment, and RNA is prone to contamination due to improper sample processing, storage conditions or contamination with RNA-degrading enzymes. A second difficulty of this approach is that an extra step is required in order to convert RNA to cDNA, the reverse transcription. In addition, the mRNA expression level depends on the physiological status of the cell, rendering it difficult to accurately estimate the size of the microbial population which might lead to overestimation [184,246].

Another approach that has been investigated lately is the use of intercalating dyes like propidium monoazide (PMA) and ethidium monoazide (EMA) which can penetrate cells with compromised cell walls and membranes, and upon photoactivation, irreversibly bind to the DNA, thus blocking its amplification [247–249]. These dyes can be easily implemented in a conventional DNA extraction protocol and are compatible with different amplification techniques [232]. Furthermore, implementation of the dyes represents an added advantage since they can detect VBNC cells, that can be generated during processing, cleaning and/or disinfection activities [250–252].

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1.6 EXISTING LITERATURE ON THE DETECTION OF SPOILAGE AND MYCOTOXIGENIC FUNGI IN FOOD PRODUCTS

Over the last decades, DNA-based methods have emerged in order to overcome the disadvantages of traditional culture-based methodologies due to their high sensitivity and specificity. In this sense, different protocols have been developed for the detection of spoilage, and mycotoxigenic, fungi in a variety of food products. In addition, a variety of molecular-based methods have been utilized including PCR, qPCR, LAMP and RPA assays as well as different methodologies for results visualization. However, one of the main challenges while reviewing these assays is the discrepancies in the way the results are expressed that does not facilitate the comparison between studies. Below examples of already existing studies will be discussed, additionally a detailed list of the different assays available can be found in the **Tables 1.5 to 1.7.**

Different conventional PCR assays have been developed for the detection of spoilage yeasts in foods targeting the ITS region and 18S rRNA gene, that are considered universal for fungi detection. In particular, Mayoral et al. [253] and Garcia et al. [181] developed methods for the detection of yeasts in dairy products with a Limit of Detection (LOD) of 10 CFU/ mL. In addition, Ros-Chumillas et al. [254] reported a LOD of 10^3 CFU/ mL for yeasts detection in orange juice. Apart from studies for the detection of spoilage microorganisms, many methods have been published for the detection of mycotoxigenic fungi. Some studies are targeting genes that are involved in the biosynthetic pathways of the different mycotoxins while others are using universal primers. In this sense, Luque et al. [174,255,256] developed methodologies for the detection of patulin-, aflatoxin- and OTA-producing moulds targeting the *idh*, *omt-1* and *otanps*PN genes that are involved in the biosynthesis of the abovementioned mycotoxins, respectively. Different food matrices were selected for each toxin and LODs ranging from 10^2-10^4 CFU/ g, were reported. On the other hand, *A. flavus* and *A. parasiticus* were detected on wheat flour by targeting the ITS1-5.8S rRNA-ITS2 [257,258]. Furthermore, various multiplexed-PCR assays have been published for the detection of beer spoilage yeasts [259] or mycotoxigenic fungi [260,261]. Finally, a RT-PCR for the detection of viable fungi has been reported with a LOD of 10 CFU/ mL in milk [243] and another one for the detection of *K. marxianus* with a LOD of 10^2 CFU/ mL in yoghurt [245].

Regarding qPCR assays, many articles have been published for real-time detection of spoilage and mycotoxigenic fungi utilizing one of the two most common chemistries of SYBR-Green combined with melt-curve analysis or TaqMan® probes. For instance, two studies deal with the detection of spoilage yeasts in fruit juices by developing qPCR-SG assays with melt-curve analysis [262,263] and another one with the detection of spoilage moulds in fruit juices but this time a hydrolysis probe was used [264]. For the latter, a LOD of 10^3 CFU/ mL was achieved. Furthermore, as with the conventional PCR, many methods have been developed for the detection of mycotoxigenic fungi. In particular, patulin-producing fungi have been detected by targeting the *patF* or *idh* genes of the biosynthesis of patulin [176,265]. Rodriguez et al. [176] reported a LOD of 10 conidia/ g. Additionally, assays for the detection of *A. carbonarius* on grapes and wine have been developed targeting the *PKS* or the calmodulin genes [266–268]. Soares-Santos et al. [269,270] developed two cells-qPCR assays for yeasts, *B. bruxellensis*, and *S. cerevisiae* in grape must and wine.

Different multiplex qPCR assays have been developed for the simultaneous detection of mycotoxigenic fungi [271–274]. Furthermore, studies dealing with the detection of viable fungi either by RT-qPCR or with the use of intercalating dyes, have been published. In particular, Hierro et al. [275] developed a RT-qPCR assay for the detection of viable yeasts in wine targeting the 26S rRNA gene and with a LOD of 10 CFU/ mL. Pavon et al. [276] reported a RT-qPCR assay for the detection of viable *Alternaria* spp. with a LOD of 1 CFU/ mL in tomato pulp. On the other hand, Crespo-

Sempere et al. [277] developed a qPCR with PMA for the detection of viable *Alternaria* spp. with a LOD of 10^2 conidia/ g in tomatoes. Finally, other studies have been published for the detection of viable yeasts by qPCR coupled with PMA or EMA in beverages [183,278,279].

Compared to the vast amount of PCR/qPCR assays developed for fungi, a limited number of isothermal amplification assays have been published, most of them applying LAMP for the detection of mycotoxigenic fungi. Frisch & Niessen [280] developed a colorimetric LAMP for the detection of patulin-producing *Penicillium* spp. in grapes and apples targeting the *idh* gene. In the same way, Storari et al. [281] developed a methodology for OTA producing *A. carbonarius* and *A. niger* in grapes. Apart for the assays targeting mycotoxigenic fungi, Zhang et al. [282] developed a panfungal colorimetric LAMP in pepper and paprika powder with a LOD of 10^4 CFU/ g. In addition, Liu et al. [283] developed a LAMP assay coupled with LF dipstick for the detection of *Zygosaccharomyces* spp. Finally, a RPA assay coupled with gel-electrophoresis and naked-eye green fluorescence has been developed for the detection of *A. flavus* in corn and peanuts. The reported LOD was 10 conidia/ g for the naked-eye method and 10^2 conidia/ g when electrophoresis was used [284]. Unfortunately, we could not identify other RPA assays targeting spoilage and mycotoxigenic fungi in food products.

Lastly, one important issue of the molecular-based fungal assays is the absence of internal controls in order to eliminate problems related to false negative results due to reaction inhibition [209]. Indeed, we were able to identify only a few studies that implemented internal controls in their assays. Zur et al. [285] developed a PCR assay with an IAC for the detection of *Alternaria* spp. in grains. Contreras et al. [286] also developed a PCR assay for the detection of *B. bruxellensis* in wine. Diguta et al. [287] developed a qPCR assay with an IAC for the detection of *B. cinerea* in grapes and Rodriguez et al. [211] for the detection of cyclopiazonic-producing moulds. Additional assays that implemented an IAC can be found in **Tables 1.5 and 1.6** [212,261,265,271,277,288–292].

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Table 1.5. Published PCR methods for the detection of spoilage and mycotoxigenic fungi in foods

Microorganism	Assay	Target gene	Detection	Matrix	Sensitivity	LOD	Reference
Aflatoxigenic moulds	PCR	*ver-1* *omt-1* *apa-2*	Gel electrophoresis	Ground corn grains	-	10^2 spores/ g (*ver-1*)	[293]
Aflatoxigenic moulds	PCR	*nor-1* *ver-1* *omt-A*	Gel electrophoresis	Figs	25 pg DNA/ rxn 250 pg DNA/ rxn[b]	-	[167]
Penicillium spp.	PCR	ITS- 5.8S rRNA	Gel electrophoresis	Camembert Roquefort	-	-	[294]
Alternaria spp.	PCR	5.8S rDNA	Gel electrophoresis	Tomato sauce and tomato powder	-	-	[295]
Alternaria spp.[a]	PCR	5.8S rDNA	Gel electrophoresis	Grains	-	-	[285]
P. expansum	PCR	*pepg1*	Gel electrophoresis	-	25 spores	-	[296]
Yeasts	PCR	18S rRNA	Gel electrophoresis	Dairy products	-	10^5 CFU/ mL[c] 10 CFU/ mL[d]	[181]
A. carbonarius	PCR	-	Gel electrophoresis	Coffee beans	-	-	[297]
B. bruxellensis *B. anomalus*	PCR	D1/D2 loop of the 26S rRNA	Gel electrophoresis	Wine	-	10^4-10^5 CFU/ mL	[298]
Yeasts	PCR	18S rRNA	Gel electrophoresis	Vacuum packed ham	-	10^6 CFU/ cm^2 [c] 10^2 CFU/ cm^2 [e]	[299]
Yeasts	PCR	18S rRNA	Gel electrophoresis	Yoghurt	-	10^6 CFU/ mL[c] 10 CFU/ mL[e]	[253]
Aflatoxigenic moulds	PCR	*aflR*	Gel electrophoresis	Groundnuts Maize	10^2 spores	10^2 CFU/ g	[300]
Aspergillus spp. in the section *Nigri*	PCR	ITS	Gel electrophoresis	-	10 pg DNA	-	[301]
OTA producing *A. carbonarius* and *A. ochraceus*	PCR	ITS	Gel electrophoresis	-	1-10 pg DNA	-	[302]
OTA producing fungi	PCR	PKS	Gel electrophoresis	-	-	-	[303]
Yeasts	PCR	ITS- 5.8S rRNA	Gel electrophoresis	Orange juice	20 pg DNA/ rxn	10^3 CFU/ mL	[254]
A. niger *A. tubigensis*	PCR	calmodulin	Gel electrophoresis	-	10 pg DNA/ rxn	-	[304]
A. flavus	PCR	ITS1- 5.8S rRNA- ITS2	Gel electrophoresis	Wheat flour	-	10^2 spores/ g	[257]

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B. bruxellensis[a]	PCR	-	Gel electrophoresis	Wine	-	-	[286]
OTA producing *Aspergillus* section *Circumdati* spp.	PCR	ITS1- 5.8S rRNA- ITS2	Gel electrophoresis	-	-	-	[173]
A. parasiticus	PCR	ITS1- 5.8S rRNA- ITS2	Gel electrophoresis	Wheat flour	-	10^6 spores/ g[c] 10^4 spores/ g[f] 10^2 spores/ g[d]	[258]
Mycotoxigenic *Aspergillus spp.*	PCR	ITS1- 5.8S rRNA- ITS2	Gel electrophoresis	Paprika Chilli	5.5 pg DNA	-	[305]
Patulin producing moulds	PCR	*idh*	Gel electrophoresis	Cooked products Ripened foods Fruits	0.5 ng DNA 15 ng DNA[b]	1.8×10^2- 2.7×10^3 conidia/g for the different food products	[255]
Aflatoxigenic moulds	PCR	*omt-1*	Gel electrophoresis	Fruits Nuts Cooked products Ripened foods	15 pg DNA 25 pg DNA[b]	10^2- 10^3 CFU/g for the different food products	[256]
OTA producing moulds	PCR	*otanps*PN	Gel electrophoresis	Cooked products Ripened food Fruits Nuts Spices	25 pg DNA 50 pg DNA[b]	10^2- 10^4 CFU/ g for the different food products	[174]
B. bruxellensis	PCR	ITS1 ITS2	Gel electrophoresis	Red wine	10 ng of DNA/ mL	10 CFU/ mL	[306]
Aflatoxin producing moulds	PCR	*nor-1* *apa-2* *omt-1*	Gel electrophoresis	-	-	-	[307]
B. bruxellensis	PCR	*VPR1*	Gel electrophoresis	Wine	5 pg of DNA	10^2 CFU/ mL	[308]
Byssochlamys spp. *Hamigera* spp.	PCR nested PCR	ß-tubulin	Gel electrophoresis	-	1 ng of DNA/ µL (PCR) 10 pg of DNA/ µL (nested PCR)	-	[309]
Neosartorya spp.	PCR nested PCR	ß-tubulin calmodulin	Gel electrophoresis	Acidic beverages	40 pg of DNA/ µL (PCR) 4 pg of DNA/ µL (nested PCR)	-	[310]
Byssochlamys spp.	PCR nested PCR	ß-tubulin	Gel electrophoresis	-	0.1 ng of DNA/ µL (PCR) 10 pg of DNA/ µL (nested PCR)	-	[311]
C. globosum *C. funicola*	PCR nested PCR	ß-tubulin	Gel electrophoresis	-	10 pg DNA (PCR) 1 pg DNA (nested PCR)	-	[312]

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Thermoascus spp.	PCR nested PCR	RPB1	Gel electrophoresis	Beverages	100 pg of DNA (PCR) 10 pg of DNA (nested PCR)	-	[313]
Dekkera-Brettanomyces spp.	nested PCR	-	Gel electrophoresis	Sherry	10 CFU	10^4 CFU/ mL	[314]
Alternaria spp.[a]	duplex PCR seminested PCR	*Alt a 1*	Gel electrophoresis	Tomato products Cereal based infant food	10^2 CFU/ mL	10^2 CFU/ mL of tomato pulp	[288]
OTA producing *Aspergillus* spp.	PCR m-PCR	-	Gel electrophoresis	Coffee beans	-	-	[315]
Aflatoxigenic fungi	PCR m-PCR	*omt 1* *ver 1* *afl R*	Gel electrophoresis	Maize kernels	10 pg DNA (PCR) 500 pg DNA (mPCR)	100 pg of fungi/ g of maize kernels	[316]
Aflatoxin and sterigmatocystin producing fungi	m-PCR	*nor-1* *ver-1* *omt-A*	Gel electrophoresis	-	-	-	[317]
A. flavus group	m-PCR	*nor-1* *ver-1* *omt-A* *apa-2*	Gel electrophoresis	Peanut kernels	-	-	[318]
Aflatoxigenic moulds	m-PCR	*avfA* *omtA* *ver-1*	Gel electrophoresis	Korean fermented foods and grains	-	-	[260]
Mycotoxigenic fungi[a]	m-PCR	*nor1* *Tri6* *FUM13* *otanps*	Gel electrophoresis	Maize	100 pg of pure DNA	2×10^3 CFU/ g (*nor-1* positive *Aspergillus* spp. and *Tri6* and *FUM13*-positive *Fusarium* spp.) 2×10^4 CFU/ g (*otanps*-positive *Penicillium* spp.)	[261]
Patulin, OTA and aflatoxin producing moulds	m-PCR	*idh* *otanps*PN *omt-1*	Gel electrophoresis	Dry-fermented sausage Paprika Apple Wheat Peanuts	1 ng DNA	10^3 – 10^4 CFU/ g for the different food products	[319]
Beer-spoilage yeasts	m-PCR	26S rDNA ITS	Gel electrophoresis	Beer	10^3 CFU	-	[259]
Viable fungi	RT-PCR	EF-Tu EF-1α	Gel electrophoresis	Milk Yoghurt Beer	-	10 CFU/ mL	[243]

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Viable *K. marxianus*	RT-PCR	18S rRNA	Gel electrophoresis	Yoghurt	10^2 CFU/ mL	10^2 CFU/ mL	[245]

a: An IAC was implemented in the assay
b: Reported sensitivity when the target DNA was mixed with non-target DNA
c: Reported LOD without incubation
d: Reported LOD after 16 h of incubation
e: Reported LOD after 24 h of incubation
f: Reported LOD after 8 h of incubation

Table 1.6. Published qPCR methods for the detection of spoilage and mycotoxigenic fungi in foods

Microorganism	Assay	Target gene	Detection	Matrix	Sensitivity	LOD	Reference
A. flavus	qPCR	*nor1*	SYBR-Green	Black pepper	-	4.5×10^3 CFU/ g	[320]
D. bruxellensis	qPCR	26S rRNA	SYBR-Green	Wine	-	1 CFU/ mL	[321]
Alternaria spp.	qPCR	AM-toxin I	SYBR-Green	Apples	4 pg of DNA	2×10^2 spores	[322]
A. westerdijkiae	qPCR	ß-tubulin	SYBR-Green	Coffee beans	100 haploid genomes/ g of coffee beans	-	[323]
Saccharomyces spp. *Hanseniaspora* spp.[a]	qPCR	5.8S rRNA-ITS2	SYBR-Green	Wine Wine fermentations	10 cells/ mL	10^2 cells/ mL	[290]
A. carbonarius	qPCR	PKS	SYBR-Green	Grapes	-	-	[266]
B. bruxellensis	qPCR	*rad4*	SYBR-Green/ Melt curve analysis	Wine	10^3 CFU/ mL	10^4 CFU/ mL	[324]
S. cerevisiae	qPCR	-	SYBR-Green/Melt-curve analysis	Sweet wine Red wine	5.6 CFU/ mL	3.8 CFU/ mL of sweet wine 5.0 CFU/ mL of red wine	[325]
Z. bailii	qPCR	D1/D2 loop of the 26S rRNA subunit	SYBR-Green/ Melt curve analysis	Fruit juices Wine	11 CFU/ mL	2 CFU/ mL of cranberry raspberry and apple juices 22 CFU/ mL in grape juice 6 CFU/ mL in wine	[326]
Dekkera/ Brettanomyces bruxellensis[a]	qPCR	D1/D2 loop of the 26S rRNA *rad4*	SYBR-Green/Melt curve analysis	Grapes	-	10^3-10^4 cells/ mL	[327]
Spoilage yeasts	qPCR	5.8S rRNA ITS	SYBR-Green/ Melt curve analysis	Orange juice	-	-	[263]

A. ochraceus *A. westerdijkiae*	qPCR	ITS	SYBR-Green/ Melt curve analysis	Green coffee beans Grapes	2.5 pg DNA/ rxn	10^6 spore/ mL	[328]
Brettanomyces spp.	qPCR	*RAD4*	SYBR-Green/ Melt curve analysis	Wine	31 CFU/ mL	3 GU/ mL	[289]
B. cinerea	qPCR	RNA helicase	SYBR-Green/Melt curve analysis	Grapes	-	-	[329]
B. cinerea	qPCR	IGS	SYBR-Green/ Melt curve analysis	Grapes	6.3 pg DNA	-	[287]
A. flavus *A. parasiticus*	qPCR	ITS2	SYBR-Green/Melt curve analysis	Wheat flour	0.5 ng of DNA/ µL	10^6 spores/ mL (without enrichment) 10^2 spores/ mL (enrichment for 16 h)	[330]
Brettanomyces spp.	qPCR	26S rRNA	SYBR-Green/ Melt curve analysis	Red wine	10 CFU/ mL	10 CFU/ mL	[331]
Aflatoxin B1, OTA and patulin producing moulds[a]	qPCR	*omt-1* *otanps*PN *idh*	SYBR-Green/Melt curve analysis	Dry-cured ham	-	1 log CFU/ g	[291]
C. oxysporum BPS	qPCR	ß-tubulin	SYBR-Green/ Melt curve analysis	Dry-cured fermented sausages	-	1.4 log CFU/ cm^2	[332]
P. expansum[a]	qPCR	*patF*	SYBR-Green/ Melt curve analysis	Apples	0.1 ng DNA/ rxn	-	[265]
K. servazzii	qPCR	ITS1 5.8S rRNA ITS2	SYBR-Green/Melt curve analysis	Packaged fresh pizza	-	-	[333]
T. flavus	qPCR	*rlf*	SYBR-Green/ Melt curve analysis	Strawberries	200 fg of DNA/ rxn	640 ascospores/ g	[334]
A. flavus	qPCR	*nor*-1	TaqMan®	Maize Pepper Paprika	-	-	[335]
A. carbonarius[a]	qPCR	calmodulin	TaqMan®	Grapes	5×10^{-4} ng DNA/ rxn	-	[268]
Spoilage moulds	qPCR	18S rRNA	TaqMan®	Orange juice	-	10^3 CFU/ mL	[264]
A. flavus[a]	qPCR	5.8S rRNA 28S rRNA ITS2	TaqMan®	-	0.4 DNA templates/ rxn	-	[292]
Aspergillus section *Flavi* spp.	qPCR	*nor*-1	TaqMan®	Peanuts	125 pg of DNA/ µL	-	[336]

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Cyclopiazonic acid producing moulds[a]	qPCR	*dmaT*	TaqMan®	Dry-cured ham, Dry-ripened cheese Paprika Peanut Durum wheat semolina	-	1-2 log CFU/ g for the different food products	[211]
Z. rouxii	qPCR	5.8S rRNA 18S rRNA 28S rRNA ITS1 ITS2	TaqMan®	Sugars Dried fruits	10 fg of DNA / μL	-	[337]
K. servazzii *C. sake*	qPCR	ITS	TaqMan®	Kimchi	1.2 × 10^2 CFU/ mL (*K. servazzii*) 3.1 × 10^2 CFU/ mL (*C. sake*)	-	[338]
A. carbonarius	qPCR	PKS	SYBR-Green/ Melt curve analysis TaqMan®	Wine	2.4 genome equivalents/ rxn (SYBR-Green) 24 equivalents/ rxn (TaqMan)	5 × 10^2 conidia/ g (SYBR-Green) 5 × 10^3 conidia/ g (TaqMan)	[267]
A. carbonarius	qPCR	ITS2	SYBR-Green/Melt curve analysis TaqMan®	Grapes	2.5 × 10^{-5} ng DNA/ rxn	0.4 pg DNA/ g	[339]
OTA producing strains of the *A. niger* aggregate	qPCR	PKS	SYBR-Green/ Melt curve analysis TaqMan®	Corn kernels	10^4 copies	-	[340]
Patulin producing moulds	qPCR	*idh*	SYBR-Green/ Melt curve analysis TaqMan®	Cooked meat products Dry-cured meat products Fruits	-	10 conidia/ g	[176]
OTA producing moulds	qPCR	*otanps*PN	SYBR-Green/ Melt-curve analysis TaqMan®	Cooked meat products Ripened foods Fruits	0.01 pg of DNA	1-10 conidia/ g for the different food products	[341]
Aflatoxin producing moulds	qPCR	*omt-1*	SYBR-Green/ Melt curve analysis TaqMan®	Cereals Nuts Spices Ripened foods	-	1-2 log CFU/ g for the different food products	[342]
Yeasts	Cells- qPCR	26S rRNA	Eva-Green/ Melt curve analysis	Grape must Wine	10^2 CFU/ mL	-	[269]
Yeasts	Cells-qPCR	26S rRNA 5.8S rRNA-ITS2	Eva-Green/ Melt curve analysis	Grape must Wine	10^2 CFU/ mL	-	[270]

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Mycotoxigenic *Aspergillus*, *Penicillium* and *Fusarium* spp.	m-qPCR	ITS	TaqMan®	Distiller's grain	1 pg of DNA	-	[272]
Patulin, OTA and aflatoxin producing moulds	m-qPCR	*idh* *otanps*PN *omt-1*	TaqMan®	Fruits Nuts Cereals Dry-ripened meat and cheese	-	1- 3 log CFU/ g for the different food products	[274]
Verrucosidin producing moulds[a]	m-qPCR	-	TaqMan®	Dry-ripened foods	0.1 pg of DNA	1 log CFU / g	[212]
Mycotoxigenic *Aspergillus*, *Penicillium*, *Fusarium* spp.	m-qPCR	*Pks* 28S rRNA *Tri5*	TaqMan®	Barley	3 pg of DNA/ rxn	-	[273]
P. chrysogenum Aflatoxin producing moulds[a]	m-qPCR	*pgafp* *omt-1* *ß-tubulin*	TaqMan®	Dry-cured ham	0.01 pg of DNA (aflatoxin producers) 0.1 pg of DNA (P. chrysogenum)	2-3 log CFU/ cm^2	[271]
viable *Alternaria* spp.	RT-qPCR	ITS	TaqMan®	Fresh fruits and vegetables Processed foodstuffs	1 CFU/ mL	1 CFU/ mL in tomato pulp	[276]
Viable *Z. bailii*	qPCR with EMA	D1/D2 loop of the 26S rRNA subunit	SYBR-Green/ Melt curve analysis	Fruit juices	12.5 CFU/ mL (in the presence of 10^5 CFU/ mL dead cells)	-	[183]
Viable *Alternaria* spp.	qPCR with PMA	ITS	SYBR-Green/ Melt curve analysis	Tomatoes	-	10^2 conidia/ g of tomato	[277]
Viable wine yeasts	qPCR with PMA qPCR with EMA	26S rRNA 5.8S rRNA-ITS2	SYBR-Green	Grape must fermentation Ageing wines	-	-	[279]
A. niger aggregate species *A. carbonarius*	qPCR m-qPCR	PKS	SYBR-Green/Melt curve analysis TaqMan®	Grapes	30 genome equivalents/ rxn	-	[343]
(VBNC) *B. bruxellensis*	qPCR RT-qPCR	D1/D2 domain of the 26S rRNA *RAD4* actin	SYBR-Green	Wine	-	10^3 CFU/ mL (DNA *RAD4*) 10^2 CFU/ mL (26S rRNA, mRNA *RAD4*) 10 CFU/ mL (actin)	[344]
(Viable) yeasts	qPCR RT-qPCR	D1/D2 domain of the 26S rRNA	SYBR-Green/ Melt curve analysis	Wine	10^2 CFU/ mL (qPCR) 10 CFU/ mL (RT-qPCR)	10^3 CFU/mL (qPCR)	[275]

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Viable total yeasts *P. anomala* *P. guillermondii* *P. kluyveri*	qPCR RT-qPCR	ITS D1/D2 domain of the 26S rRNA	SYBR-Green/Melt curve analysis	Table olives Table olives brine	10 CFU/ mL (*P. anómala* with qPCR) 10^2 CFU/ mL (*P. kluyveri*, *P. guilliermondii* with qPCR) 10^3 CFU/ mL (RT-qPCR , total yeasts with qPCR)	-	[345]
viable *B. bruxellensis*	qPCR with PMA RT-qPCR	ITS	EvaGreen/ Melt curve analysis	Wine Beer	-	0.83 log CFU/ mL of red wine (qPCR with PMA) 0.63 log CFU/ mL of white wine (qPCR with PMA) 0.23 log CFU/ mL of beer (qPCR with PMA) 4 log CFU/ mL of wine (RT-qPCR) 7 log CFU/ mL of beer (RT-qPCR)	[278]
Total eumycetes *S. fibuligera* *W. anomalus*	qPCR ddPCR	26S rRNA *car1*	SYBR-Green/ Melt curve analysis	Bread	0.6 pg of DNA/ µL (total yeasts with qPCR and *W. anomalus* with qPCR and ddPCR) 0.06 pg/ µL (total yeasts with ddPCR) 6 pg of DNA/ µL (*S. fibuligera* with qPCR and ddPCR)	10^3 CFU/ mL (*S. fibuligera* with qPCR and ddPCR) 10^2 CFU/ mL (*W. anomalus* with qPCR) 10 CFU/ mL (*W. anomalus* with ddPCR)	[346]
Spoilage yeasts	PCR qPCR	5.8S rRNA ITS2 *cs1*	Gel electrophoresis SYBR-Green/ Melt curve analysis	Fruit juice samples	120 pg of C. *krusei* DNA	-	[262]
A. ochraceus	PCR qPCR	-	Gel electrophoresis SYBR-Green/ Melt curve analysis	Green coffee	-	0.4 ng DNA/ rxn (PCR) 4.7 pg DNA / rxn (qPCR)	[347]

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A. carbonarius *A. niger* aggregate strains	PCR qPCR	PKS	Gel electrophoresis SYBR-Green/ Melt curve analysis	Table grapes	-	2.37×10^{-2} ng/ µL	[348]
OTA producing moulds	PCR qPCR	*β-tubulin* *otanps*PN	Gel electrophoresis SYBR-Green/ Melt curve analysis	Ripened foods Nuts Grapes	-	-	[349]
OTA producing *Penicillium* spp.	PCR qPCR	*otapks*PN *otanps*PN	Gel electrophoresis TaqMan®	Cured ham	-	-	[350]
Viable fungi	RT-PCR RT-qPCR	*act*	Gel electrophoresis SYBRGreen	Yoghurts Pasteurized fruit-derived products	25 pg RNA/ µL (RT-PCR)	10^3 CFU/ g in yoghurt (RT-PCR) 10^2 CFU/ g in fruit juice and fruit preserves (RT-PCR)	[351]

a: An IAC was implemented in the assay

Table 1.7. Published isothermal amplification methods for the detection of spoilage and mycotoxigenic fungi in foods

Microorganism	Assay	Target gene	Detection	Matrix	Sensitivity	LOD	Reference
Brettanomyces/Dekkera spp.	LAMP	ITS	Real-time turbidity	Wine Beer	1×10^1 CFU/ mL	1.5×10^1 CFU/ mL of wine 5.4×10^1 CFU/ mL of beer	[352]
Aflatoxigenic moulds	LAMP	*acl1* *amy1*	Naked-eye calcein fluorescence	Brazil nuts Peanuts Green coffee beans	2.4 pg DNA/ rxn (*A. flavus*) 7.6 pg DNA/ rxn (*A. nomius*) 20 pg DNA/ rxn (*A. parasiticus*)	-	[353]
OTA producing *A. carbonarius* and *A. niger* clade	LAMP	PKS	Colorimetric (HNB)	Grapes	0.1 ng/ rxn	-	[281]
A. flavus *A. nomius* *A. caelatus*	LAMP	*acl*1 *amy1*	Naked-eye calcein fluorescence	Brazil nuts	10^1 conidia/ rxn for *A. flavus* 10^2 conidia/ rxn for *A. nomius*	-	[354]

Aflatoxin producing fungi	LAMP	*acl*-1 *amy*-1	Real-time turbidity	Shelled Brazil nuts Maize Peanuts	10 conidia/ rxn (*A. flavus*) 10^2 conidia/ rxn (*A. parasiticus*, *A. nomius*)	10 conidia/ g of Brazil nuts (*A. nomius*, *A. flavus*) 10^2 conidia/g of peanuts (*A. flavus*) 10^5 conidia/ g of peanuts (*A. parasiticus*) 10^4 conidia/ g of maize (*A. flavus*, *A. parasiticus*)	[355]
P. nordicum	LAMP	*otapksPN*	Colorimetric (HNB)	Dry-cured meat products	100 fg DNA/ rxn	-	[356]
Panfungal	LAMP	18S rRNA	Colorimetric (HNB)	Pepper Paprika powder Rice	100 copies/ rxn	1.1 10^4 CFU/ g	[357]
Aflatoxin producers within *Aspergillus* section *Flavi*	LAMP	*nor1*	Naked-eye calcein fluorescence Colorimetric (neutral red)	Nuts Raisins Dried figs Powdered spices	9.03 pg of DNA/ rxn	-	[358]
T. flavus	LAMP	*rlf*	Real-time fluorescence	Strawberries	1 fg of DNA/ µl	64 spores/ g	[359]
Patulin producing *Penicillium* spp.	LAMP	*idh*	Colorimetric (neutral red)	Grapes Apples	2.5 pg of DNA/ rxn	-	[280]
P. expansum	LAMP	coding sequence of gene PEX2_044840 gene	Colorimetric (neutral red)	Apples Grapes Apple juice Apple puree Grape juice	25 pg of DNA/ rxn	-	[360]
P. oxalicum	LAMP	coding for protein PDE_07106 of *P. oxalicum*	Naked-eye calcein fluorescence Colorimetric (neutral red)	Grapes	100 pg of DNA/ rxn	-	[361]
Zygosaccharomyces spp.	LAMP	ITS	Lateral flow dipstick	-	1.0×10^1 copies/ µL	-	[283]
Panfungal	LAMP	ITS	Colorimetric (HNB)	Dry food	9.6 copies	-	[362]

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A. flavus	RPA	ITS1 5.8S rRNA ITS2	Gel electrophoresis Naked-eye fluorescence (SYBR Green I, Celfinder™)	Corn Peanuts	-	10 conidia/ g (naked-eye detection) 10^2 conidia/ g (gel electrophoresis)	[284]

HNB: Hydroxyl Napthol Blue

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CHAPTER 2.

HYPOTHESIS & OBJECTIVES

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2 HYPOTHESIS & OBJECTIVES

Detection of fungi still relies on culture-based methods, in particular plating on selective or differential media. These techniques are laborious and usually require up to 7 days for results, making them non-suitable for products with short shelf-lives. As a consequence, there is a need for faster and more sensitive methods for fungal detection that will reduce the overall costs for the food industry due to product recalls and lengthy analyses. In this sense, DNA-based methods can help overcome some of these disadvantages due to their high sensitivity and specificity.

The **main objective** of this thesis was the development, and evaluation, of different molecular-based methods for the detection of selected spoilage responsible, and mycotoxigenic fungi in fruits, and products thereof, with the overall goal of reducing the total time of analysis, while maintaining the sensitivity when compared to the conventional methods. In order to achieve this, the following **specific objectives** were proposed:

- To develop a protocol for pre-enrichment, sample treatment, and DNA extraction for the fungi of interest in the selected food matrices. (Articles 1-4)
- To develop and evaluate a panfungal qPCR method, with an IAC, that will serve as the reference method for isothermal DNA amplification techniques. (Article 1)
- To develop and evaluate different isothermal amplification techniques combined with naked-eye detection of spoilage-responsible and mycotoxigenic fungi. (Articles 2-3)
- To develop a methodology for the differentiation of viable and non-viable fungi using intercalating dyes combined with molecular-based methods. (Article 4)

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CHAPTER 3.

METHODOLOGY

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3 METHODOLOGY

3.1 FUNGAL STRAINS AND CULTURE MEDIA

A detailed list of all the species used in each assay can be found in **Table 3.1**, including previously identified spoilage responsible fungi isolated from real fruit preparation samples and reported as problematic by our industrial collaborators. In addition, other strains responsible for spoilage as well as mycotoxin production (e.g. *Penicillium* spp.) were acquired from external sources. All the microorganisms used in this study were stored at -80 °C with 30 % glycerol. Fresh yeast cultures were prepared by inoculating a loopful of stock culture in 5 mL of Malt Extract Broth (MEB, Liofilchem S.r.l., Italy). The inoculum was incubated for 24 h at 30 °C. Fresh mould cultures were prepared by streaking a loopful of the stock culture in Potato Dextrose Agar (PDA, Biokar diagnostics S.A., France) followed by incubation for 7 days, either at 30 or 25 °C, depending on the fungus. In particular, only *N. fischeri*, *N. glabra* and *N. laciniosa* were incubated at 30 °C. After the incubation, fungal spores were harvested by adding 10 mL of Milli-Q water on the plate and scraping its surface. The recovered liquid was passed by a syringe filled with cotton in order to retain the hyphae. Viable reference values of each strain were calculated by making 100-fold serial dilution in MEB, or Milli-Q water, from the fresh cultures and plating on PDA. The plates were incubated at 25 °C or 30 °C for 24–96 h. In addition, Dichloran Rose Bengal Chloramphenicol agar (DRBC, Biokar diagnostics S.A., France) and PDA were used for confirmation of the molecular-based techniques. In particular, 100 µL of the spiked samples were plated on PDA and DRBC plates followed by incubation at either at 25 or 30 °C, depending of the microorganism, for up to 96 h. Finally, a panel of bacteria was selected for testing the inclusivity/ exclusivity of the assays.

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Table 3.1 List of species selected for the inclusivity/ exclusivity tests

Microorganism	Source	patulin production	panfungal qPCR	panfungal LAMP fluorescent (LAMP 18S)	panfungal LAMP colorimetric (LAMP 18S)	panfungal RPA (RPA-LF)	qPCR for patulin-producing fungi	RPA-SG for patulin-producing fungi
P. expansum	MUM 17.41	+	+	+	+	N/A	+	+
	MUM 17.69	+	+	+	+	N/A	+	+
	CECT 2278	+	+	+	+	+	+	+
	fruit preparation	N/D	+	+	+	N/A	N/A	N/A
P. griseofulvum	CECT 2919	+	+	+	+	+	+	+
P. tunisiense	MUM 17.62	-	+	+	+	+	-	-
Penicillium spp.	chestnuts	N/D	+	+	+	N/A	N/A	N/A
			+	+	+	N/A	N/A	N/A
N. fischeri	FRULACT S.A.	-	+	+	+	+	-	-
N. glabra	MUM 9836	-	+	+	+	+	-	-
N. delicata	CBS101754	-	N/A	+	+	+	-	-
N. coreana	CBS117059	-	N/A	+	+	+	-	-
N. laciniosa	CBS117721	-	N/A	+	+	+	-	N/A
C. albicans	ATCC 24433	-	+	+	+	+	-	N/A

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C. intermedia	CECT 1431	-	+	+	+	+	-	-
M. guilliermondii	fruit preparation	-	+	+	+	+	-	-
M. caribbica	fruit preparation	-	+	+	+	+	-	-
P. fermentans	fruit preparation	-	+	+	+	+	-	-
P. anomala	CECT 1113	-	+	+	+	+	-	-
Mucor spp.	chestnuts	-	+	+	+	N/A	N/A	-
T. rugulosus	-	-	N/A	N/A	N/A	N/A	N/A	-
Salmonella spp.	WDCM 00031	-	-	N/A	N/A	N/A	-	-
	AMC 82	-	-	-	-	-	N/A	N/A
L. monocytogenes	WDCM 00021	-	-	-	-	-	-	-
L. innocua	CUP 1325	-	-	-	-	N/A	-	N/A
L. ivanovii	WDCM 00018	-	N/A	-	-	N/A	N/A	N/A
Y. enterocolitica	WDCM 00038	-	-	-	-	-	-	N/A
C. difficile	CECT 531	-	N/A	N/A	N/A	N/A	-	N/A
S. aureus	WDCM 00033	-	N/A	-	-	-	-	N/A
	WDCM 00034	-	-	N/A	N/A	N/A	N/A	N/A
Staphylococcus coagulase +	interlaboratory test	-	N/A	N/A	N/A	N/A	N/A	-
C. jejuni	AMC	-	-	-	-	N/A	N/A	N/A
C. coli	UM	-	N/A	-	-	-	-	N/A

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	-	-	N/A	N/A	N/A	N/A	N/A	-
E. faecalis	WDCM 00009	-	-	-	-	-	N/A	-
E. coli	WDCM 00012	-	-	N/A	N/A	N/A	N/A	-
	WDCM 00014	-	-	-	-	-	N/A	N/A

MUM: Micoteca da Universidade do Minho, CECT: Spanish Type Culture Collection, CBS: Collection from the Westerdijk Fungal Biodiversity Institute, ATCC: American Type Culture Collection, WDCM: World Data Centre for Microorganisms, CUP: Catholic University of Porto, UM: University of Minho collection. N/D: not determined. N/A: not applicable

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3.2 SAMPLE TREATMENT

3.2.1 Fruit preparations and commercial blackberry jam

Blackberry and pineapple preparations were provided by FRULACT S.A. (FRULACT S.A., Maia, Portugal). In addition, blackberry jam was purchased from a local supermarket. These products were the selected food matrices for inoculation in the panfungal qPCR, LAMP and RPA assays. Samples were prepared by weighing 50 g of product and mixing with 50 mL of MEB in a stomacher bag with filter (pore size <250 μm). After spiking with the desired microorganism, the samples were homogenized for 30 s in a Stomacher 400 Circulator (Seward Limited, West Sussex, UK). In addition, non-spiked samples were also analysed and served as negative controls. The samples were incubated at 30 °C with constant agitation (150 rpm). In the case of samples spiked with yeasts, incubation was performed for 24 h. For samples spiked with moulds, two different incubation times were tested, namely 24 and 48 h. After the incubation, 10 mL of sample were recovered and transferred in a clean 15 mL tube. The tube was centrifuged at 400 × g for 2 min, to remove food debris, and the supernatant was transferred to a new tube and centrifuged at 9000 × g for 5 min. After the centrifugation, the supernatant was removed and the pellet was resuspended in 1 mL of PBS, following a centrifugation under the same conditions. The supernatant was removed and the resulting pellet was used for DNA extraction.

3.2.2 Apples, apple juice and apple puree

Apples, as well as commercial apple juice and apple puree, were purchased from a local supermarket, and were the selected food matrices for the inoculation experiments, in the study concerning the detection of viable patulin-producing fungi. Initially, 5 g of sample were mixed with 10 mL of MEB in a stomacher bag, followed by homogenization and incubation at 25 °C with constant agitation (150 rpm) at 25 °C for 24 h. Furthermore, as in the previous section, non-spiked samples were also analysed in parallel to serve as negative controls. After the incubation, all the liquid was recovered and the cleaning protocol previously described in section 3.2.1was followed.

3.3 INACTIVATION OF FUNGI AND VIABILITY TREATMENT

For the inactivation of fungi, 1 mL of a fresh spore culture was heated at 85 °C for 40 min. Following the heat treatment, a loopful was streaked on PDA, in order to ensure correct inactivation of the fungi. The plates were incubated at 25 °C for up to 96 h.

Regarding the viability treatment, the pure cultures and/ or the spiked samples were centrifuged at 9000 × g for 5 min and the supernatant was removed. The pellet was resuspended in 1 mL of PBS and 5 μL of Propidium Monoazide (PMA, Biotium, Hayward, CA, USA) were added. Following this procedure, the final PMA concentration was 100 μM. The tubes were flicked occasionally and incubated for 5 min at room temperature in the dark. The photoactivation of the dye was performed in a PMA-Lite™ LED Photolysis Device (Biotium) for 30 min. The tubes were centrifuged at 9000 × g for 5 min and the supernatant was removed. The remaining pellet was used for DNA extraction as described below. In the case of spiked samples, the inactivation was performed directly after the sample cleaning that was described in section 3.2.

In order to assess possible inhibitory effects of the dye, PMA, in live cells, as well as the efficiency of the inactivation protocol, live and dead spore suspensions of *P. expansum* were analysed

with and without the PMA treatment. The experiment was performed in biological triplicates meaning that three different samples were inoculated at the same time with the same number of microorganisms and later on they were processed and analysed in parallel. In addition, for determining the capacity of the PMA treatment to block the amplification of DNA only from dead cells, apples were inoculated with a mixture of live: dead cells in different ratios. The selected ratios were 10^7:10^7, 10^6:10^7, 10^5:10^7 spores/ mL, respectively. In addition, apples were spiked with only live or dead cells. These experiments were performed in biological duplicates.

3.4 DNA EXTRACTION

DNA was extracted using the DNeasy® PowerSoil® Pro kit (Qiagen, Dusseldorf, Germany) following the manufacturer's instructions. The pellet resulting from the initial treatments, i.e. the sample cleaning and the PMA treatment, was resuspended in 800 μL of the first buffer of the kit (CD1) and transferred to the bead tube provided for DNA extraction. DNA extracts were stored at -20 °C until they were analysed. DNA concentration was measured with a Qubit™ 4 Fluorometer (Invitrogen™, Carlsbad, CA, USA) with the use of the Qubit™ dsDNA HS Assay Kit.

3.5 GENE AND PRIMERS/PROBE DESIGN

The design of the primers/ probes for the molecular-based assays was performed as follows: in the first step sequences of the targeted gene were retrieved from the GenBank and aligned with the CLC Sequence Viewer 8 (CLC Bio, Qiagen, Aaarhus, Denmark). Next, the consensus that resulted from the alignment was used for the design of the desired oligonucleotides. For designing the qPCR oligonucleotides the online software Primer3Plus [363] was selected. The LAMP primers were designed with the assistance of the Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0/index.html) and the RPA primers with the PrimedRPA Software [364]. All the primers and probes were purchased from Integrated DNA Technologies Inc. (IDT, Leuven, Belgium). A detailed list of the sequences can be found in **Table 3.2**.

Table 3.2 Primers and probes list

Assay	Prime/Probe	Sequence (5'→3')	Modification	Reference
panfungal qPCR	ITS3 (F)	GCA TCG ATG AAG AAC GCA GC	-	[365]
	ITS4 (R)	TCC TCC GCT TAT TGA TAT GC	-	
	ITSP	TCC AGA GGG CAT GCC TGT TTG AGC	FAM/IABkFQ	[366]
	NC-IAC F	AGT TGC ACA CAG TTA GTT CGA G	-	[367]
	NC-IAC R	TGG AGT GCT GGA CGA TTT GAA G	-	
	IAC P	AGT GGC GGT//GAC ACT GTT GAC CT	YY/ZEN/ IABkFQ	[368]
panfungal LAMP (LAMP 18S)	F3 18S	ACG GGG AAT AAG GGT TCG A	-	[369]
	B3 18S	TTG GAG CTG GAA TTA CCG C	-	
	FIP 18S	ATT GGG TAA TTT GCG CGC CTG TTT TAG AGG GAG CCT GAG AAA CG	-	
	BIP 18S	TAC AGG GCC CTT TCG GGT CTT TTT CTT GCC CTC CAA TTG TTC CT	-	
	LF 18S	GCC TTC CTT GGA TGT GGT AGC	-	
LAMP POW	F3 POW	CAA AGT CTT TGG GTT CTG G	-	[357]
	B3 POW	CCA ACT AAG AAC GGC CAT	-	

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	FIP POW	CAA ATT AAG CCG CAG GCT CCT ATG GTC GCA AGG CTG AA	-	
	BIP POW	CTC AAC ACG GGG AAA CTC ACC CAC CAT CCA AAA GAT CAA GAA	-	
	LF POW	GCC CTT CCG TCA ATT TCT TTA AGT	-	
panfungal RPA-LF	ITS3-DIG (F)	GCA TCG ATG AAG AAC GCA GC	Digoxigenin	[365]
	ITS4-Bio (R)	TCC TCC GCT TAT TGA TAT GC	Biotin	
qPCR for patulin-producing fungi	idh2444 (F)	ATG CAC ATG GAA GGC GAG AC	-	[313]
	idh2778 (R)	CAA VGT GAA TTC CGC CAT CAA CCA AC	-	
	idhP2 (P)	TGG GAG GGC CGA CAG AGG TG	FAM/ZEN/IA BkFQ	[370]
RPA-SG for patulin-producing fungi	idhRPA F	ACG CGG GAA CTA GGA GGC ACG CGG GGT ATG	-	[370]
	idhRPA R	CGA TAA TCA CGT CAA TTC GTC CGA GTC GCT	-	

YY (Yakima Yellow), IABkFQ (Iowa Black®FQ) and ZEN (secondary, internal quencher) are trademarks from IDT.

3.5.1 Panfungal qPCR assay

For the panfungal qPCR assay, the fungi detection was achieved by targeting the internal transcribed spacer (ITS) region. In this sense, the universal primers ITS3 and ITS4 [365] were selected and a hydrolysis probe (ITSP) was designed *in house* for the assay. To this end, the following sequences of the ITS region were retrieved from the GenBank in order to design the hydrolysis probe: *Candida santamariae*: KJ707034, KY102388; *Meyerozyma guilliermondii*: MW217204; *Wickerhamomyces anomalus*: KJ70630, MT875243; *Aspergillus fischeri*: MH858698, MN121343; *Penicillium expansum*: MT872092, MT738604; *Penicillium griseofulvum*: MH866419; *Candida albicans*: MW335163, MT777659; *Pichia fermentans*: MT645427, MT136540; *Candida intermedia*: FJ515166, MT731452. In addition, a non-competitive Internal Amplification Control (NC-IAC) was included in each reaction to eliminate false negative results due to reaction inhibition [367].

3.5.2 Panfungal LAMP assay (LAMP 18S)

For the panfungal LAMP assay the 18S rRNA gene was targeted. In this sense, sequences for the 18S rRNA gene were retrieved from GenBank (*C. intermedia* EF142412, EF152415 and AB013571; *C. santamariae* NG_063403 and NG_063417; *P. fermentans* NG_063276, EF550372, AB053241, GQ458040, DQ489318, AY497743, KX150667 and KX150681; *P. anomala* NG_062034, MG712300, KU147479 and KU147484; *N. fischeri* NFU21299, EU278602 and GU733354; *S. cerevisiae* MF662289 to MF662299, MF662300 to MF662309, MF662311, MF662312, MF662313 and MF662314). Furthermore, a set of previously published LAMP primers, from now on LAMP POW, also targeting the 18S rRNA gene, was used for comparison purposes against the developed assay [357]. The previously developed panfungal qPCR assay (section 3.5.1) served as the confirmation method.

3.5.3 Panfungal RPA assay (RPA-LF)

As with the panfungal qPCR, the ITS region was selected for the development of this assay. In particular, the universal primers ITS3 and ITS4 [365] were chosen. Based on the study by Liu et al. [371], the forward primer was tagged with digoxigenin and the reverse primer with biotin, at their 5′ end for detection with the lateral flow (LF) strips (Abingdon Health, York, UK). Once more, the panfungal qPCR assay was used as the confirmation method.

3.5.4 qPCR assay for patulin-producing fungi

Detection of patulin-producing fungi was achieved by targeting the isoepoxydon dehydrogenase (*idh)* gene which is involved in the biosynthetic pathway of patulin production. To this end, the previously published primers idh2444 and idh2778 [313] were selected and a hydrolysis probe (idhP2) was designed *in house*. For this purpose, sequences of the *idh* gene were retrieved from GeneBank: *P. paneum* DQ343635, DQ343636; *P. carneum* DQ343637, DQ343638; *P. gladioli* DQ343625, DQ343626; *P. expansum* DQ084388, DQ343639, DQ343640, DQ343642, AY885568, AY885569, AY885570; *P. sclerotigenum* DQ343632, DQ343634; *P. clavigerum* AY885571, AY885572; *P. dipodomyicola* DQ343643; *P. glandicola* DQ343627, DQ343631.

3.5.5 RPA-SG assay for patulin-producing fungi

For the RPA assay the *idh* gene was targeted again. In this sense, a forward and a reverse primers were designed using the same consensus sequence as the one selected for the design of the probe of the qPCR assay. The results of the RPA-SG assay were compared against the results of the qPCR assay for the patulin-producing fungi.

3.6 ASSAYS OPTIMIZATION

All the real-time assays (i.e. panfungal qPCR, panfungal LAMP with fluorescence detection, and qPCR for patulin-producing moulds) were performed in a StepOne Plus™ Real-Time PCR system (Applied Biosystems™) with StepOne™ Software v2.3. In addition, the end-point assays that were coupled afterwards with naked-eye detection (i.e. colorimetric LAMP, RPA-LF, and RPA-SG) were performed in a Veriti Thermal Cycler (Applied Biosystems™).

3.6.1 Panfungal qPCR assay

For the optimization, the following parameters were considered: primers and probe concentration, amplification temperature and template volume. The qPCR amplification reactions were carried out in a final volume of 20 μL out of which 10 μL corresponded to TaqMan® Fast Advanced Master Mix (Applied Biosystems™, Foster City, CA, USA). For the NC-IAC, 100 nM of primers and probe were added along with 1 μL of NC-IAC DNA which corresponded to 926 copies/ μL. Initially, the following thermal profile was used: 2 min at 50 °C for Uracil-DNA Glycosylase (UDG) treatment, 2 min at 95 °C for Hot-Start polymerase activation and 40 cycles of denaturation at 95 °C for 15 s followed by annealing at 58 °C for 1 min and extension at 72 °C for 20 s. To determine the optimal primer concentration, the range between 300 to 700 nM were tested. Subsequently, the probe concentration was optimized by testing 250, 350 and 500 nM. Thirdly, 3 to 5 μL of template DNA were evaluated. Finally, the optimal amplification temperature was determined in a gradient from 55 to 60 °C.

3.6.2 Panfungal LAMP assay (LAMP 18S)

Regarding the optimization of the panfungal LAMP assay the following parameters were evaluated: ratio of outer and inner primers, loop primer concentration, amplification temperature, and template volume. The evaluation was performed by real-time fluorescence detection coupled with melt curve analysis. The optimal conditions were adopted for the colorimetric assay. The experiments were performed in a final reaction volume of 20 μL out of which 12 μL corresponded to GspSSD

Isothermal Mastermix (Optigene Ltd., Horsham, UK). For the colorimetric assay the Visual detection RT Isothermal Mastermix (Optigene Ltd.), was selected. Initially, the optimal ratio of outer and inner primers was optimized. In this sense, the concentration of the F3/B3 primers was kept constant at 200 nM and concentrations of 800, 1000 and 1200 nM of FIP/BIP primers were tested. This corresponded to 1:4, 1:5 and 1:6 of outer: inner primers ratio, respectively. Secondly, the loop primer concentration was optimized by testing 300, 400 and 500 nM. Subsequently, the template volume was evaluated between 3 to 6 µL of DNA. Finally, the optimal amplification temperature was determined in a gradient from 61 to 66 °C. During the optimization process, experiments were run at 65 °C for 30 min with fluorescence detection every 30 s. To perform the melt curve analysis, once the amplification step was completed, the samples were heated at 95 °C for 15 s, 80 °C for 60 s and heating up to 95 °C with temperature increments of 0.2 °C, and fluorescence acquisition after each increment. The colorimetric LAMP experiments were performed at 66 °C for 30 min. For interpretation of the results, tubes with a blue/turquoise colour were considered positive while colourless tubes were negative. In addition, the positive tubes were incubated for extra 10 min to generate a more intense colour change.

3.6.3 qPCR assay for patulin-producing fungi

For this assay the same conditions as for the panfungal qPCR assay were optimized. In addition, the time of annealing/ extension was evaluated. Initially, the primers concentration was optimized in a range from 200 to 500 nM, and the probe between 150 and 350 nM. The optimal amplification temperature was determined in a gradient from 57 to 62 °C. Subsequently, the thermal profile was assessed by reducing the annealing time and elimination of the extension step in order to reduce the time of the assay without compromising the sensitivity. Finally, 2 to 5 µL of template were evaluated. During the optimization experiments, the following thermal profile was used: 2 min at 50 °C 2 min at 95 °C and 40 cycles of denaturation at 95 °C for 15 s followed by annealing at 59 °C for 1 min and extension at 72 °C for 1 min.

3.7 RPA ASSAYS

3.7.1 Panfungal RPA assay (RPA-LF)

The panfungal RPA was performed with the TwistAmp® basic kit (TwistDX Limited, Maidenhead, UK) in a final volume of 50 µL. The primer concentration was set at 400 nM, and the DNA template was 2 µL. The volumes of the rehydration buffer (29.5 µL) and the magnesium acetate (2.5 µL) were the ones recommended by the manufacturer. All the experiments were run at 39 °C for 30 min. Five microliters of the amplified product were mixed with 1 µL of 6 × NZYDNA loading dye (NZYTech, Lisbon, Portugal) and subsequently separated in a 2 % agarose gel electrophoresis in sodium borate buffer (SB) [372] containing 4 µL of GreenSafe (NZYTech). The electrophoresis was run for 30 min at 300 V, and the gel was visualized in a Gel Doc EZ Imager (Bio-Rad laboratories, Inc., USA). The NZYDNA Ladder VI (NZYTech) was included to verify the size of the amplicons. For the LF detection, the PCRD FLEX strips (Abingdon Health, York, UK) were used. Five microliters of the amplified product were mixed with 145 µL of PCRD FLEX Extraction Buffer, and the LF strip was immersed in the tube. The results were visualized after 2 min of incubation at room temperature.

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3.7.2 RPA-SG assay for patulin-producing fungi

The assay was performed with the TwistAmp® Basic kit (TwistDx Limited) in a final volume of 25 µL out of which 14.75 µL corresponded to rehydration buffer and 1.25 µL to magnesium acetate. The primer concentration was 400 nM and the template volume was set at 2 µL. All the experiments ran for 20 min at 39 °C. At the end of the experiment the amplified product was mixed with 1 µL of 1000X SYBR Green I (SG, Invitrogen™, Carlsbad, CA, USA) and fluorescence was observed naked-eye with a help of a handheld UV Lamp in the "short wave" mode which corresponded to 254 nm (Analytik Jena, Upland CA, USA). In addition, the fluorescence was also visualized in the Gel Doc EZ Imager (Bio-Rad laboratories, Inc.).

3.8 EVALUATION OF THE ASSAYS

3.8.1 Inclusivity/ exclusivity

The inclusivity/ exclusivity of all the primers and probes was tested *in silico* using the Basic Local Alignment Search Tool (BLAST®, https://blast.ncbi.nlm.nih.gov/Blast.cgi). In continuation, confirmation was performed *in vitro* against a selected panel of yeasts, moulds and bacteria, relevant to food quality and safety. In **Table 3.1**, a detailed list of the microorganisms tested in each assay with the obtained results can be found.

3.8.2 qPCR efficiency and dynamic range

The efficiency of the qPCR assays, as well as the dynamic range, was determined by testing 10-fold serial dilutions of pure fungal DNA in sterile Milli-Q water. The efficiency was calculated based on the equation [186]:

$$e = 10^{1/s} - 1 \qquad \textbf{Equation 1}$$

where "s" corresponds to the slope of the standard curve. The determination of the efficiency was performed in biological triplicates. All experiments were performed in technical triplicates, providing a total of 9 data points per concentration level. The determination of the dynamic range was performed in biological triplicates using one technical replicate.

3.9 EVALUATION OF THE METHODS

3.9.1 Determination of the Limit of Detection (LOD) in food samples

The LOD_{50} and LOD_{95}, meaning the LOD with 50 % and 95 % probability of providing a positive result, were calculated based on the PODLOD calculation program described by Wilrich & Wilrich [373]. Food samples were spiked with decreasing concentrations of fungi in biological quadruplicates. The number of positive and negative samples for each spiking level were inputted to the model to calculate the LOD. Simultaneously, non-spiked samples were tested to confirm that the original sample was not previously spoiled or contaminated.

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3.9.2 Fitness-for-purpose

For the evaluation of the fitness for purpose additional samples were spiked with different concentrations of fungi. Each spiking experiment was performed in biological duplicates. The samples were classified as Positive/Negative Agreements (PA/NA), if the results obtained were in accordance with the reference method or as Positive/Negative Deviations (PD/ND) if the results did not match. All the parameters were calculated attending to the equations and definitions previously described [374,375]. In particular, the following quality parameters were calculated: relative sensitivity (SE), relative specificity (SP), accuracy (AC), positive and negative predictive values (PPV/NPV) and the Cohen's kappa (k).

- SE: percentage of positive samples giving a correct positive signal.

$$SE = \frac{PA}{(PA + ND)} \times 100$$ **Equation 2**

- SP: percentage of negative samples giving a correct negative signal.

$$SP = \frac{NA}{(PD + NA)} \times 100$$ **Equation 3**

- AC: degree of correspondence between the response obtained by the expected result and the method on identical samples.

$$AC = \frac{(PA + NA)}{N} \times 100$$ **Equation 4**

N= total number of samples analysed.

- PPV and NPV: measure the performance of the method by calculating the probability of a sample being truly positive or negative when the method results in a positive or negative result.

$$PPV = \frac{PA}{(PA + PD)} \times 100$$ **Equation 5**

$$NPV = \frac{NA}{(NA + ND)} \times 100$$ **Equation 6**

- The Cohen's kappa (k): degree of concordance between the method and the expected result.

$$k = 2\,\mathrm{x}\,\frac{(\mathrm{PA\ x\ NA}) - (\mathrm{ND\ x\ PD})}{(\mathrm{PA} + \mathrm{PD})\,\mathrm{x}\,(\mathrm{PD} + \mathrm{NA}) + (\mathrm{PA} + \mathrm{ND})\,\mathrm{x}\,(\mathrm{ND} + \mathrm{NA})}$$ **Equation 7**

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CHAPTER 4.

RESULTS

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4 RESULTS

4.1 ARTICLE 1

Roumani, F., Azinheiro, S., Rodrigues, C., Barros-Velázquez, J., Garrido-Maestu, A., & Prado, M. (2022). Development of a real-time PCR assay with an internal amplification control for the detection of spoilage fungi in fruit preparations. *Food Control*, *135*, 108783.

https://doi.org/10.1016/j.foodcont.2021.108783

4.2 ARTICLE 2

Roumani, F., Gómez, S., Rodrigues, C., Barros-Velázquez, J., Garrido-Maestu, A., & Prado, M. (2022). Development and evaluation of a real-time fluorescence, and naked-eye colorimetric, loop-mediated isothermal amplification-based method for the rapid detection of spoilage fungi in fruit preparations. *Food Control*, *135*, 108784.

https://doi.org/10.1016/j.foodcont.2021.108784

4.3 ARTICLE 3

Roumani, F., Rodrigues, C., Barros-Velázquez, J., Garrido-Maestu, A., & Prado, M. (2022). Development of a Panfungal Recombinase Polymerase Amplification (RPA) Method Coupled with Lateral Flow Strips for the Detection of Spoilage Fungi. *Food Analytical Methods*, 1-10.

https://doi.org/10.1007/s12161-022-02242-1

4.4 ARTICLE 4

Roumani, F., Barros-Velázquez, J., Garrido-Maestu, A., Prado, M. (2022). Real-time PCR, and Recombinase Polymerase Amplification combined with SYBR Green I for naked-eye detection, along with Propidium Monoazide (PMA) for the detection of viable patulin-producing fungi in apples and by-products. *Food Control*, *144*, 109347.

https://doi.org/10.1016/j.foodcont.2022.109347

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CHAPTER 5.

DISCUSSION

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5 DISCUSSION

Fast and sensitive methods for the detection of fungi is of utter importance for the food industry, particularly the detection of spoilage-responsible fungi in order to reduce costs related with product recalls and lengthy analyses, and to prevent food loss and waste [376]. Furthermore, these methods can be an important tool for food safety and quality control of mycotoxin-producing fungi due to the adverse health effects that mycotoxins represent to human and animal health (https://food.ec.europa.eu/safety/chemical-safety/contaminants/catalogue/mycotoxins_en). In this sense, DNA-based amplification techniques have been utilised in the last years for the detection of spoilage-responsible, and mycotoxigenic fungi in a variety of food products. In particular, many different PCR/ qPCR assays have been reported for fungal detection due to their high sensitivity and specificity [176,253,256,262,332]. However, over the last years alternatives to PCR/ qPCR based on isothermal nucleic acid amplification techniques, like LAMP and RPA, have been explored. These techniques do not require expensive equipment since they run at a constant temperature, and consequently only a thermal block or water bath is needed. In addition, visualisation of the results can be performed by naked-eye observation of colour change [282], fluorescence [284], turbidity [377] or even combined with lateral-flow dipsticks [283]. This represents an added advantage of these techniques since they can be implemented in POC (Point-Of-Care) or PON (Point-Of-Need) devices for on-site testing as a tool for rapid screening of raw materials and foods. In this thesis, different molecular-based amplification methods for panfungal detection as well as for the detection of patulin-producing fungi in fruits and products thereof were developed and evaluated.

Initially, different media were tested, including YPD, MEB and SDB, in order to select the optimal one for the growth of the fungi of interest. Based on kinetics studies, and on the protocols in place on our industrial partner's site, MEB was the selected broth for the pre-enrichment step. In continuation, the sample size, and incubation conditions, were determined based on previous studies and the previous experience from the industrial partner. Additionally, the sample treatment was optimized according to previous experiments performed in our laboratory. Finally, regarding the DNA extraction, two commercial kits were tested, being the PowerSoil Pro kit from Qiagen the one providing the best results, and thus it was the selected one for the experiments to follow.

Regarding the molecular methods, in the first step a qPCR assay was developed and evaluated. The method targeted both, yeasts and moulds, and was evaluated in different fruit preparations. In this sense, the universal primers ITS3 and ITS4, which have been previously described by White et al. [365], were selected. To further increase the specificity of the assay a fluorescent hydrolysis probe was designed and evaluated *in house*. The selectivity of the primers and probe was evaluated both *in silico* by running a BLAST® analysis as well as *in vitro* against a panel of 18 fungal strains and 8 bacterial species, typically found in food products.

Furthermore, up to date, only a few assays for the detection of fungi in food products, have been published that include an IAC [211,212,265,271]. Implementation of an IAC in the assay provides increased confidence against false negative results that are the outcome of reaction inhibition. In

particular, Paterson [209] stressed out the importance of including an IAC in fungal PCRs, especially in the case of pathogenic fungi that false negative results can have a strong effect on human's health as well as due to the fact that many fungi can produce secondary metabolites that might have inhibitory properties. In this sense, a NC-IAC was implemented in our assay that had been previously reported by Garrido-Maestu et al. [367,368].

The analytical sensitivity of the qPCR assay was assessed by testing ten-fold serial dilutions of *C. intermedia*'s pure DNA and building a standard curve. Although, no guidelines have been established for fungal qPCR assays, Fredlund et al. [378] suggested that the criteria that have been set for GMO can be used. According to those, the slope of the standard curve typically lies between -3.1 to -3.6, the efficiency between 90 and 110 % and the R^2 should be equal or above 0.98. In our assay, the efficiency was calculated to be 83 %, which was below the aforementioned range. However, since the aim of the developed assay was the detection of the presence of a wide range of fungi, rather than the quantification of a specific species, the decreased efficiency was considered as no problematic. This was confirmed later on during the evaluation of the method. In addition, the reported efficiency was in the range previously reported by Op De Beeck et al. [379] that used the same set of primers. Additionally, the size of the amplification product is 337 bp, which is considerable larger than the recommended amplicon length for qPCR assays that should not exceed the 150 bp [380]. This might explain the lower efficiency that was obtained and consequently in this case was considered optimal.

With the optimized assay, detection of down to 10.4 fg/ μL of pure DNA could be detected. Our reported analytical sensitivity fell within the range of previously reported assays. In particular, Syromyatnikov et al. [337] were able to detect down to 10 fg/ μL of pure *Z. rouxii* DNA. The same analytical sensitivity was reported by Rodríguez et al. [341] for the detection of OTA-producing moulds. Additionally, González-Salgado et al. [339] reported an analytical sensitivity of 25 fg/ μL for *A. carbonarius*. Bernáldez et al. [271] developed a multiplex qPCR for the detection of aflatoxin-producing moulds and *P. chrysogenum*, being able to detect down to 10 fg/ μL and 100 fg/ μL of pure DNA, for each target microorganism respectively.

The final step in the development of the panfungal qPCR assay was the evaluation of the overall method in real samples. In this sense the evaluation was performed separately for yeasts and moulds in fruit preparations. In addition, for moulds two different pre-enrichment times were selected, namely 24 and 48 h; while samples inoculated with yeasts were incubated only for 24 h. The obtained LOD_{95} values were 3.9 CFU/ 50 g for yeasts, 1.2×10^2 spores/ 50 g and 3.7×10 spores/ 50 g for moulds after 24 and 48 h of pre-enrichment, respectively. The calculated LOD for yeasts was within the range of previously reported assays for the detection of spoilage yeasts; thus highlighting the high sensitivity of our assay. In particular, Phister & Mills [321] reported a LOD of 1 CFU/ mL for the detection of *D. bruxellensis* in wine. Additionally, Martorell et al. [325] were able to detect *S. cerevisiae* with a LOD of 3.8 and 5.0 CFU/ mL in sweet and red wine, respectively. Rawsthorne & Phister [326] developed a qPCR assay for the detection of *Z. baillii* in fruit juices and wine with an estimated LOD ranging from 2- 22 CFU/ mL for the different food products. Finally, Tofalo et al. [331] reported a LOD of 10 CFU/ mL for the detection of *Brettanomyces* spp. in red wine. Regarding, the LOD of moulds, once more the obtained values fell within the range of previously reported assays. In a similar study, Sardiñas et al. [330] reported LODs of 10^6 spores/ mL and 10^2 spores/ mL without enrichment, and after a 16 h enrichment for the detection of *A. flavus* and *A. parasiticus* in wheat flour; thus highlighting the importance of a pre-enrichment step in order to decrease the LOD of the assay. In addition, Rodríguez et al. [211,342] reported LODs ranging from 1 to 2 log CFU/ g for the different food products tested in two assays developed for the detection of cyclopiazonic acid-

producing and aflatoxin-producing moulds. Based on the obtained LOD_{95} values, the performance parameters of the method were calculated. The obtained values were higher than 85.0 % and the Cohen's k above 0.86 for all the assays, meaning that our method was almost in complete concordance with the reference method [374,381], i.e. traditional culture-based plating. In total 8 deviations were observed that corresponded to 4 PDs of non-spiked samples, with a Cq value close to the cut-off value of the qPCR assay, and 4 NDs. Two of the samples that were classified as NDs showcased reaction inhibition since the NC-IAC did not amplify either. Consequently, a 1:10 dilution was tested in order to remove possible qPCR inhibitors; however, the samples resulted again negative for our target but positive this time for the NC-IAC. Nevertheless, we cannot discard the fact that due to the low initial concentration, by performing the dilution in order to remove the inhibitors the fungal DNA might have also been diluted in excess resulting in a negative result. Furthermore, the third sample that gave a false negative result corresponded to a spiked sample close to the LOD, which can explain the deviation. Finally, the fourth sample that deviated was a DNA extract obtained more than a year ago prior to the analysis with the final qPCR assay, that was frozen and thawed several times, that might have resulted in a decrease of the quantity and quality of the extracted DNA; thus explaining the false negative result. Overall, the developed method was sensitive for the detection of both yeasts and moulds and consequently served as the reference method for the isothermal amplification techniques that were developed later on.

After the development of the qPCR assay with a NC-IAC for panfungal detection, two isothermal amplification techniques were developed. First, a LAMP assay targeting the 18S rRNA gene and in continuation a RPA assay coupled with LF detection that targeted the ITS region. In more detail, a newly designed set of primers for the LAMP assay was optimized and evaluated. Two different detection strategies were selected, namely real-time fluorescence and colour change. Regarding the RPA assay, the universal primers ITS3/ ITS4 [365] were selected and tagged with digoxigenin and biotin, respectively. This approach was adapted by Liu et al. [371] with the aim of simplifying the assay by avoiding the use of a *nfo* probe. As with the qPCR, the specificity of the primers for both assays was assessed *in silico* as well as *in vitro* against a selection of microorganisms. In particular, 20 fungal strains, including 6 yeasts and 14 moulds, and 10 bacterial species, including 5 Gram-positive and 5 Gram-negative bacteria, were selected for testing the specificity of the assay. The bacteria that were tested for the exclusivity are commonly encountered in food samples. All the primers presented good inclusivity towards the selected fungi and good exclusivity since all the selected bacteria failed to amplify.

Subsequently, the analytical sensitivity of the assays was evaluated by testing ten-fold serial dilutions of *M. caribbica* and *N. fischeri* pure DNA. Both the colorimetric and the fluorescent LAMP assays were able to cover a 5-log dynamic range down to 1.4 pg/ reaction when pure DNA from *M. caribbica* was used. When pure DNA from *N. fischeri* was used, detection down to 17 pg/ reaction and 170 pg/ reaction were detected with the fluorescent and the colorimetric assay, respectively. The reported results were in the range of previously published studies. In particular, Luo et al. [353] reported an analytical sensitivity of 2.4 pg/ reaction for *A. flavus* in their LAMP assay combined with calcein fluorescence detection by naked-eye. In another study, Niessen et al. [358] were able to detect down to 9.03 pg/ reaction of pure DNA from aflatoxin-producing moulds belonging to the *Aspergillus* section *Flavi*. Vogt et al. [361] developed a LAMP assay coupled with naked-eye detection of colour change or clacein fluorescence for *P. oxalicum* with an analytical sensitivity of 100 pg/ reaction. Frisch et al. [360] detected down to 25 pg/ reaction of pure DNA from *P. expansum* with their developed naked-eye colorimetric LAMP. Even though, in some of the studies the reported sensitivity was higher it should be noted that the amplification time was increased, in some instances even up to 60 min [280,382–384]. In this sense, Shan et al. [385] reported different analytical sensitivities after

incubating at different times for the detection of *F. temperatum* using SYBR Green and gel electrophoresis as the detection methods. In particular, after 60 min of incubation up to 10 pg/ reaction were detected, after 45 min 100 pg and after 30 min 100 ng could be successfully detected. Similarly, Panek & Fraç [359] reported values ranging from 100 to 1 fg/ μL after 30 and 55 min, for the detection of *T. flavus* in a real-time fluorescence LAMP assay. However, it should be taken into account that extended incubation times can lead to false positive results [386,387]. Consequently, optimization of the amplification time is of utter importance in LAMP assays in order to avoid these type of issues.

Regarding, the RPA-LF assay an analytical sensitivity of 1.2 pg/ μL, covering a 4-log dynamic range, was achieved for both yeasts and moulds. Since detection of fungi frequently encountered in foods has still not been explored with RPA, the results were compared against assays for the detection of fungal pathogens. In this sense, different groups have reported an analytical sensitivity of 10 pg/ μL of pure fungal DNA for *Phytophthora* spp. and *Verticillium dahliae* [388–390]. In another study, Dai et al. [391] reported an analytical sensitivity of 0.1 ng/ μL for *P. hybernalis* pure DNA. In other studies, more sensitive assays have been developed like Lu et al. [392] who were able to detect down to 0.5 pg/ μL of pure *P. infestans* DNA and Karakkat et al. [393] that reported values ranging from 1-100 fg/ μL for the detection of different root-infecting fungi.

The final step in the development of the panfungal isothermal assays consisted on their evaluation in food samples. The quality parameters of both the colorimetric and the fluorescent LAMP were assessed by spiking fruit preparations with different yeasts. In addition, the quality parameters were evaluated with a previously published set of LAMP primers [282] and the detection was performed both by naked-eye observation of colour change, and real-time fluorescence for direct comparison. Thus in total four different methodologies were evaluated. The LOD_{95} obtained was 3.1 CFU/ 50 g for the two fluorescent assays, hence both assays were equally sensitive. Regarding the colorimetric assays, the one developed in this study proved to be more sensitive with a LOD_{95} of 3.0 CFU/ 50 g, whereas the one used for comparison resulted in a LOD_{95} of 10.9 CFU/ 50 g. Furthermore, the obtained LOD was similar to the one reported for the yeasts detection by qPCR in this thesis (LOD_{95} 1.0 CFU/ 50 g). As a result, the newly developed LAMP assay was as sensitive as the reference method but with the added advantages that the colorimetric assay can be used for POC detection and additionally reduce the cost of analyses, since only a thermal block for heating is needed. Furthermore, the reported LOD was similar with a previously reported study by Hayashi et al. [352] for the detection of *Brettanomyces*/ *Dekkera* spp. in wine and beer. Finally, Zhang et al. [282] who developed the comparative LAMP assay selected for this study, reported a LOD of 1.1×10^3 CFU/mL for *A. niger* in spiked paprika samples without an enrichment step. However, we were able to reduce this limit down to 10.9 CFU/ 50 g and 3.1 CFU/ 50 g for the colorimetric and fluorescent assay by implementing a pre-enrichment step. Even though the inclusion of a pre-enrichment step delays the results by 24 h, it allows to highly improve the LOD, and when compared with the lengthy culture-based methods it still represents a significant time reduction from 7 days to next-day detection that can be a great advantage for the food industry. Regarding the determination of the quality parameters, in all the assays the values obtained were above 90 % with some exceptions for each assay that were the result of few PDs and/or NDs. This can be probably explained by the fact that one of the disadvantages of the LAMP assays is the risk of cross contamination through aerosolized products due to the high production of DNA that can lead to false positive results [394]. Finally, based on the Cohen's k values, the newly developed colorimetric assay was in "almost complete concordance" with the reference method, i.e. the panfugal qPCR assay, while the fluorescent one was in " substantial agreement" [374,381].

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The evaluation of the panfungal RPA-LF method was performed both in yeasts and moulds in blackberry jam samples. The LOD_{50} was found to be 1.0 CFU/ 50 g for yeasts and 47.5 spores/ 50 g for moulds, respectively. The obtained LOD_{50} for yeasts was the same as the one reported for the developed panfungal qPCR and LAMP assays in this thesis, indicating that all the methods developed during this study were equally sensitive for the detection of yeasts. On the other hand, the reported LOD_{50} for moulds was higher compared to the one of the panfungal qPCR assay which was found to be 8.6 spores/ 50 g. Nevertheless, our assay was more sensitive compared to the one of Ju et al. [389] that reported a LOD of 10^3 spores/ g for the detection of *V. dahliae* in soil samples and it was in the same range with the study of Liu et al. [284] for the detection of *A. flavus* in grains. Regarding the quality parameters, all of them were above 80 % and the Cohen's k was found to be 0.77 for both yeasts and moulds detection, meaning that the developed methods were in "substantial agreement" with the reference method [374,381]. Regarding the recorded deviations, two samples spiked with 1.0 CFU of yeast were found to be positive for the RPA-LF but negative for the qPCR; thus, they were classified as PDs. However, the inoculation level of these samples was equal to the LOD_{50} of both assays, meaning that there is a 50 % possibility of getting a positive result at this spiking level. This can explain the fact that one assay resulted positive while the other was negative. Furthermore, one sample spiked with mould spores was classified as PD. This specific sample should have been positive for both assays; however, a positive result was only obtained for the RPA-LF whilst the qPCR was negative, and additionally a reaction inhibition was observed since the IAC did not amplify as well. As a result, a 1:10 dilution of the sample was tested, but again the qPCR was negative for the ITS target but positive this time for the IAC. These results can be explained by the fact that RPA has reported to be more robust against common PCR inhibitors [237]. In addition, 2 samples spiked with yeasts were classified as NDs; however, the inoculation level was low.

Lastly, we developed two molecular-based methods, namely a qPCR and a RPA coupled with SYBR Green I, for the detection of patulin-producing fungi. Apples and products thereof were the selected food matrices, since *P. expansum*, which is the main patulin producer, is also responsible for food spoilage incidents in this fruit that can lead to huge economic losses. In addition, detection of only viable fungi was achieved with the implementation of an intercalating dye (PMA) during the sample treatment. In this sense, a previously published set of PCR primers was chosen [313] that targeted the *idh* gene and a fluorescent probe was designed *in house*. For the RPA assay a new set of forward and reverse primer were designed with the use of the PrimedRPA software [364]. The selected gene, *idh*, is involved in the biosynthetic pathway of patulin by encoding for an enzyme that catalyses the conversion of isoepoxydon to phyllosistine [156]. The inclusivity/ exclusivity of the primers was evaluated as it was described before and all the oligonucleotides were found to be specific.

In continuation, the analytical sensitivity of both assays was assessed by testing ten-fold serial dilutions of *P. expansum* DNA. The amplification efficiency of the qPCR assay was calculated to be 89 %, which is slightly lower from the recommended minimum value of 90 % [378]. Nevertheless, as with the panfungal qPCR assay, this assay was intended for detection rather than quantification. Furthermore, the assay covered a 5-log dynamic range down to 1.25 pg/ μL of pure *P. expansum* DNA. The reported analytical sensitivity fell within the range of previously reported values for the detection of mycotoxigenic fungi. In particular, Tannous et al. [265] reported a value of 0.1 ng of pure *P. expansum* DNA. Passone et al. [336] reached an analytical sensitivity of 125 pg/ μL and Sardiñas et al. [330] of 0.5 ng/ μL both for the detection of aflatoxin-producing moulds. Furthermore, Gil-Serna et al. [328] detected down to 2.5 pg/ reaction for OTA-producing fungi. Finally, Vegi et al. [273] were able to detect down to 3 pg/ μL in a multiplex PCR for the simultaneous detection of mycotoxigenic fungi. Regarding the RPA-SG assay, a 4-log dynamic range was achieved thus

detecting down to 23.8 pg/ μL. The reported value is similar to the one reported by other studies (10 ng/ μL) for the detection of fungal pathogens [388–390]. However, the panfungal RPA assay that was developed during this thesis was more sensitive compared to this assay, with the ability to detect down to 1.2 pg/ μL. Nevertheless, it should be taken into account that in all the above mentioned assays, detection with lateral flow was performed, while in this particular study emission of fluorescence was observed.

One of the main limitations of DNA-based amplification techniques is that they cannot differentiate between viable and dead cells. In order to overcome this problem, PMA was included in the assays, which is an intercalating dye with the ability to penetrate dead cells, bind to their DNA and, upon photoactivation, block its amplification. Consequently, in this study we proved that the PMA could efficiently block the amplification of DNA originating from dead spores with concentrations up to 10^7 spores/ mL. Additionally, no toxic effects were observed on the live spores. Furthermore, the effect of the PMA was assessed in spiked apple samples and once again PMA successful blocked the amplification of DNA from dead spores in the same range of concentrations. The effect of PMA in apples inoculated with a mixture of live and dead spores in different ratios was also assessed. It was observed that when apples were spiked with 10^8 dead spores, the PMA did not fully inhibit the amplification as one of the three replicates was positive. These results suggested that the PMA can inhibit the amplification of DNA coming from dead spores with concentrations of up to 10^7 but the presence of higher concentrations of dead microorganisms may hinder the blocking capacity of the dye. These results are in line with previously reported assays for the detection of viable fungi [183,277–279].

In the final step the evaluation of both methods was performed in spiked apples, apple puree and apple juice. The LOD_{50} was calculated to be 8.1×10^3 spores/ 5 g and 5.8×10^4 spores/ 5 g, for the qPCR and the RPA-SG method, respectively. Even though, the calculated LOD for the qPCR was higher compared to the one of the panfungal qPCR assay (LOD_{50} 2.9×10 spores/ 50 g) that was developed during this thesis, it was within the range of other assays for detection of fungi. More specifically, Bagnara et al. [320] reported a LOD of 4.5×10^3 CFU/ g for the detection of *A. flavus*. Gil-Serna et al. [328] were able to detect OTA-producing fungi in green coffee beans and grapes with a LOD of 10^6 spores/ mL. Wan et al. [264] and Hierro et al. [275] both reported a LOD of 10^3 CFU/ mL for the detection of spoilage moulds and yeasts present in wine, respectively. In addition, Selma et al. [267] achieved a LOD of 5×10^3 conidia/ g for the detection of *A. carbonarius* in wine. In relation to the LOD of the RPA-SG assay, this was also higher compared to the LODs of the RPA-LF as well as the panfungal qPCR, which were evaluated during this thesis. However, the limit of detection was comparable with the one previously reported by Ju et al. [389] for the detection of *V. dahliae* in artificially contaminated soil samples. Finally, based on the obtained LOD_{50} values the quality parameters were evaluated. All the qPCR tests were performed using the original DNA extracts as well as a 10-fold dilution to exclude the possibility of false negative results due to reaction inhibition since an IAC was not implemented in this assay. Indeed, inhibitors were present in some occasions but this inhibition could be avoided with the additional dilution. In the case of the RPA-SG assay that was not considered problematic since it has been proved to be more robust to inhibitors compared to qPCR [237]. Even though in this specific assay an IAC was not included due to time restrictions, it should be highlighted its importance in order to overcome problems related to false negative samples due to PCR inhibitors present in the samples [209]. For both developed methods the quality parameters were above 90 % and the Cohen´s k was 0.93 and 0.92, for the qPCR and the RPA-SG, indicating that both assays were “in almost complete concordance” with the reference method [374,381]. During the evaluation of the qPCR assay two deviations were recorded, 1 PD and 1 ND. The ND corresponded to an apple sample spiked with a mix of live: dead spores. Even though

this sample was expected to be positive, no amplification was observed. This can be the result of reaction inhibition since it was correctly identified as positive by the RPA-SG assay. Additionally, it has been previously reported that the RPA is more robust against common PCR inhibitors [237]. The PD corresponded to a sample spiked with 2.1×10^8 dead spores and as a result it was expected to be negative. Nevertheless, a positive result was recorded that can be the outcome of the inability of the PMA to block the DNA amplification from such a high number of dead microorganisms. In particular, previous studies have reported that the capacity of PMA is limited [232,395]. Furthermore, the same sample was classified as PD by the RPA-SG assay, further enhancing our theory about the limited capacity of the PMA to block the amplification of DNA from high concentrations of dead cells. Finally, during the evaluation of the RPA-SG another sample was classified as PD.

Overall, during this thesis different molecular-based assays were developed and evaluated. More specifically one qPCR assay with an IAC for the detection of fungi in fruit preparations was evaluated separately for yeasts and moulds and due to its excellent performance it was selected as the reference method for the methods that were developed later on. In this sense, in a second step a LAMP assay was developed selecting two different ways for results visualization, namely naked-eye colour change and real-time fluorescence. The LAMP assay proved to be equally sensitive with the qPCR assay with remarkable quality parameters. The advantage of the colorimetric assay is that it can be used for POC applications; however extreme care should be taken when handling the LAMP reactions to avoid problems related to cross-contamination through aerosolized products [394]. Subsequently, another isothermal based DNA amplification technique was tested, namely a RPA assay coupled with LF for universal fungal detection. In this assay, the primers were tagged with digoxigenin and biotin and in this way the use of an *nfo* probe was avoided, thus simplifying the assay and taking into account that the *nfo* kit from TwistDx was discontinued recently. Again, the developed assay was as sensitive as the panfungal qPCR and LAMP assays. In the same way as with the LAMP assay, the added advantage of the RPA is that it can be used for POC applications and on-site testing for rapid screening of raw materials and products along the food value chain. Consequently, highly reliable analytical solutions were developed, and it will depend on the final user to decide which method is more suitable for the intended application. After the development of the panfungal assays, it was decided to investigate rapid methods for the detection of patulin-producing fungi. In this sense, initially a qPCR assay was developed and later on a RPA assay coupled with naked-eye SYBR Green detection by emission of fluorescence. Both assays, were less sensitive compared to the panfungal assays and between the two the qPCR was slightly more sensitive compared to the RPA-SG. The reasons for the decreased analytical sensitivity and increased LODs can be the use of different matrix, apples instead of fruit preparations, as well as the different target fungi since in this case it was *Penicillium* spp., while in the panfungal assays it was mainly *N. fischeri*. By optimizing the sample size and/ or the incubation time and temperature, can result in increased sensitivity.

Finally, even though traditional culture-based methods remain the selected techniques for detection of yeast and moulds by the food industry they present many disadvantages. In particular, they are laborious, time consuming, and needing up to 7 days for results thus rendering them unsuitable for products with short-shelf lives [181,182]. In this sense, DNA-based methods can overcome some of these disadvantages due to their high sensitivity and sepcificity. As a consequence, during this thesis, it was successfully managed the decrease of the analysis time from 7 days to a next-day or two-day detection with the optimization of a protocol including, pre-enrichment, sample treatment and DNA extraction and ultimately a molecular-based assay for detection with high specificity and sensitivity. In addition, with the simple implementation of an intercalating dye before the DNA extraction, detection of only viable cells can be easily achieved. Consequently, the developed methods are representing an interesting alternative for food industry that can result in faster

and more economic analyses. In addition, they can be implemented for POC applications in order to perform early-screening and control of raw ingredients and/ or foods across the supply chain.

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CHAPTER 6.

CONCLUSIONS

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6 CONCLUSIONS

In the present thesis, the detection of spoilage-responsible and mycotoxigenic fungi in different food matrices was achieved in 24-48 h, providing a faster alternative to the conventional culture-based techniques which require up to 7 days from sampling to result. Therefore, the developed methods have proven to be an interesting option for the food industry contributing to reduction of costs associated with lengthy analyses. Small modifications of the proposed methodology including enrichment time and temperature as well as sample size have proven to contribute to higher sensitivity and/or specificity, which gives the developed methodology the required flexibility to fit the needs of the food industry. Furthermore, the different methods showcased different advantages. In particular, the real-time PCR assays were found to be more sensitive; however, they require a real-time thermocycler in order to be performed. On the other hand, the isothermal amplification techniques coupled with naked-eye detection could be used for POC testing and early screening since they only require a heating device; providing in many cases a comparable sensitivity to the qPCR assays.

The main achievements of the study are summarized below:

- A protocol for pre-enrichment, sample treatment, and DNA extraction and purification was developed in order to increase the sensitivity of the assays by removing possible inhibitors from the food matrices in the most efficient possible manner. In particular, a pre-enrichment step of 24-48 h was included to enhance the sensitivity of the method. Furthermore, differential centrifugation and cleaning steps followed by DNA extraction and purification with a commercial kit were adopted resulting in fungal DNA extracts of good quality and yield as well as free of DNA inhibitors.

- In the first step, a panfungal qPCR assay targeting the universal ITS region was developed and evaluated. The universal primes ITS3/ITS4 were used and a hydrolysis probe was designed in order to increase the specificity of the assay. The implementation of a NC-IAC in the assay resulted in higher confidence by elimination of false negative results due to reaction inhibition. This assay served as the reference method for the isothermal amplification techniques that were designed later on.

- Two isothermal DNA amplification techniques were developed, and evaluated, combined with different detection strategies for panfungal detection. First, a LAMP assay was designed based on the 18S rRNA gene and coupled with either real-time fluorescence or naked-eye detection by colour change. In addition to this, a RPA assay targeting the ITS region using the universal primers ITS3/ITS4 was evaluated, and upon modification of the primers with digoxigenin and biotin, naked-eye detection with LF strips was possible. The colorimetric LAMP and the RPA-LF have the potential to be used for POC testing throughout the food chain since they do not require expensive equipment.

- Two molecular assays were developed and evaluated, namely a qPCR and a RPA, for the detection of patulin-producing fungi in apples and apple-based products. Both assays targeted the *idh* gene. Results visualization of the RPA assay was performed by naked-eye after the addition of SYBR Green in the amplified product. The developed qPCR assay was proved to be more sensitive compared to the RPA-SG; however, the proposed RPA assay had the

advantage of enabling naked-eye detection of the results and consequently, could be used for on-site testing and be a helpful tool for an early screening of the fruits.

- Differentiation of viable and non-viable cells was successfully achieved by adding PMA before the extraction of the DNA. The addition of the PMA in the selected concentration was able to inhibit amplification originating from dead cells without causing any toxic effect in the live cells.

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LIST OF FIGURES

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LIST OF EQUATIONS

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APPENDIX

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APPENDIX

APPENDIX I– PUBLICATIONS

PUBLICATION 1

Roumani, F.[1,2], Azinheiro, S.[1,2], Rodrigues, C.[3], Barros-Velázquez, J.[2], Garrido-Maestu, A.[1], & Prado, M.[1] (2022). Development of a real-time PCR assay with an internal amplification control for the detection of spoilage fungi in fruit preparations. *Food Control*, *135*, 108783.

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PUBLICATION 2

Roumani, F.[1,2], Gómez, S.[2], Rodrigues, C.[3], Barros-Velázquez, J.[2], Garrido-Maestu, A.[1], & Prado, M.[1] (2022). Development and evaluation of a real-time fluorescence, and naked-eye colorimetric, loop-mediated isothermal amplification-based method for the rapid detection of spoilage fungi in fruit preparations. *Food Control*, *135*, 108784.

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PUBLICATION 3

Roumani, F.[1,2], Rodrigues, C.[3], Barros-Velázquez, J.[2], Garrido-Maestu, A.[1], & Prado, M.[1] (2022). Development of a Panfungal Recombinase Polymerase Amplification (RPA) Method Coupled with Lateral Flow Strips for the Detection of Spoilage Fungi. *Food Analytical Methods*, 1-10.

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PUBLICATION 4

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In the present thesis, the detection of spoilage and mycotoxigenic fungi in different food matrices was achieved in 24-48 h,
providing a faster alternative to the conventional culture-based techniques which require up to 7 days from sampling to result. Consequently, the developed methods present an interesting alternative for the food industry as each method showcased different advantages. In particular, the real-time PCR assays have proven to be more sensitive; however, they require a real-time thermocycler in order to be performed. On the other hand, the isothermal amplification techniques coupled with naked-eye detection could be used for POC testing and early screening since they only require a heating device; providing in many cases a comparable sensitivity to the qPCR assays.