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Rapid detection of contaminant microorganisms in food containers

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Abstract

One issue food industry faces is the contamination and consequent spoilage of food products due to the presence of undesired microorganisms. In the food industry, yeasts are associated with beneficial properties, such as baker's yeast that fermenters the product in order to have the desired characteristics; and negative proprieties such as food spoiling. The latter is a source of major concern by the food industries since it causes quality and health problems.

Spoilage of the product causes economic and commercial losses in companies. Detection in real operation time is of extreme importance to avoid food spoilage and most importantly avoid sending a poor-quality product. For companies, this rapid detection of contaminant microorganisms is essential. However, current methods employed are time-consuming and labour-intensive, taking up to 2-3 days to obtain results. This time delay also represents an economic loss, since sending of the product is delayed to ensure there is no contamination.

A major priority is to find a rapid detection method that could provide results in a couple hours and allow a fast intervention if a contaminant is present. To attend these needs, peptide nucleic acid (PNA) probes were tested for two microorganisms of interest, *Candida spp*. and *Pichia spp*. using fluorescence *in situ* hybridization (FISH).

Testing of the probe with food isolates confirmed that *Candida* probe worked for *Candida spp.*, but also for other non-*Candida spp*. *Pichia* probe showed positive results by only identifying *Pichia sp*. but more strains are needed to consolidate these results. An optimum hybridization temperature was found for *Candida* probe taking in consideration the mean fluorescence intensity of *Candida spp*. and non-*Candida spp*. performances. The optimum hybridization temperature found was 53ºC to work in multiplex.

The miniaturization and semi-automation of this method was attempted with microfluidic devices. The combination of the FISH procedures with microfluidics devices was successfully achieved and with further developments could be in the future implemented in food industry.

Keywords: fluorescence *in situ* hybridization; FISH; peptide nucleic acid; PNA; *Candida spp.; Pichia spp.*

Resumo

Um dos problemas que a indústria alimentar encontra é a contaminação e subsequente desperdício de produtos alimentares devido à presença de micro-organismos indesejáveis. Na indústria alimentar, as leveduras estão associadas a propriedades benéficas, como a levedura de panificação que fermenta o produto para ter as características desejadas; e propriedades negativas, como a deterioração de alimentos. Esta última é uma importante fonte de preocupações para a indústria alimentar, pois provoca problemas de qualidade e saúde.

A deterioração de produtos provoca prejuízos económicos e comerciais às empresas. A deteção em tempo real das operações é de extrema importância para evitar o desperdício de alimentos e, sobretudo, evitar enviar produto de baixa qualidade. Para as empresas, é essencial uma rápida deteção de micro-organismos contaminantes. No entanto, os métodos utilizados atualmente são demorados e trabalhosos, levando 2 a 3 dias até obter resultados. Este tempo de demora é, também, um prejuízo económico, uma vez que é necessária a retenção do producto para verificar se há ou não contaminação.

Uma das principais prioridades é encontrar um método de deteção rápida que possa apresentar resultados num par de horas e permitir uma intervenção rápida, se houver um contaminante presente. Para responder a estas necessidade foi testada uma sonda de ácido nucleico peptídico (PNA) para dois micro-organismos de interesse, *Candida spp*. e *Pichia spp.*, utilizando o método de hibridização fluorescente *in situ* (FISH).

Testes realizados as estirpes dadas pela empresa confirmaram que a sonda da *Candida spp*. hibridou com ambas espécies de *Candida* e não-*Candida*. Os resultados da sonda da *Pichia* foram positivos uma vez que é possível identificar só *Pichia sp*. mas são necessárias mais estirpes para fumentar estes resultados. Mesmo assim, uma temperatura optima de hibridação foi determinada com as estirpes e resultados existentes. A temperatura ideal de hibridização determinada é 53 ºC para trabalhar em multiplex.

Foi realizada a introdução deste método com dispositivos microfluidicos para avaliar se uma combinação de ambos poderia funcionar. Esta combinação foi aplicada com sucesso e resultou num metodo semi automatico que com continuação de trabalho pode ser implementada na indústria alimentar.

Palavras-chave: hibridização fluorescente *in situ*; FISH; ácido nucleico peptídico; PNA; *Candida spp.; Pichia spp.*

Declaration

I hereby declare, on my word of honor, that this work is original and that all non-original contributions were properly referenced with source identification.

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Glossary

List of Acronyms

ANOVA	Analysis of variance
AUF	Arbitrary units of fluorescence
bp	base pair
C.	*Candida*
CFU	Colony forming unity
Cs	*Candida* strains
DAPI	4′,6-diamidino-2-phenylindole
DEFT	direct epifluorescent filter technique
DNA	Deoxyribonucleic
ELISA	enzyme-linked immunosorbent assay
EPS	extracellular polymeric product
ε	Épsilon
FCM	Flow cytometry
FISH	Fluorescence *in situ* hybridization
H.	*Hanseniospora*
ISH	*in situ* hybridization
Ku	Kurtosis
LNA	Locked nucleic acid
LSD	Least significant difference
mRNA	messeger ribonucleic acid
nCs	Non *Candida* species
OD	Optical density
P.	*Pichia*
PCR	Polymerase Chain Reaction
PDMS	Polydimethylsiloxane
PNA	Peptid nucleic acid
RNA	Ribonucleic acid
rRNA	Ribossomal ribonucleic acid
RT	reverse transcriptase
S/N	signal-to-noise
sig.	significance
Sk	Skewness
sp.	specie
spp.	species
T.	*Torulospora*
TCs	total number of *Candida* strains
Th	Hybridization temperature
Tm	Melting temperature
TnC	total of non-*Candida* strains
W.	*Wickerhamomyces*
YEPD	Yeast Extract Peptone Dextrose
ΔG°	Gibbs free energy change

1 Introduction

1.1 Framing and presentation of the work

In the food industry, yeasts can be associated with both beneficial and negative proprieties. Despite being very widely used by the food industry for their beneficial proprieties, they also represent a major concern for food companies because of their negative effects, one of them food spoilage. Food spoilage happens due to an overgrowth of unwanted yeast, that ferments the product causing visible alterations to the food and also causing quality and possible health problems (Fleet and Balia 2006; Lelieveld et al. 2005).

Foods that have a high sugar content and anaerobic conditions are susceptible to contamination with yeasts since they have the requirements for fermentation to occur. For this reason, certain foods such as syrups, jams, fruit, vegetables and yogurts are more susceptible to contamination. (Betts 2013).

To prevent and control the contaminant it is necessary to assess the quality of the product. Currently, the most common processes used to assess this quality are plating and Polymerase Chain Reaction (PCR)-based methods. Plating methods are time-consuming and labour-intensive. PCR is faster but still needs a pre-enrichment step that can take up to 2 days (Law et al. 2014). This would mean a hold back of the product or sending the product without knowing whether it is contaminated or not.

As a case study for these thesis, strains from *Candida spp*. and *Pichia spp*. were selected for being a particular problem in fruit preparations (Lelieveld et al. 2005). The main goal of this work is to develop and optimize a rapid method to detect these contaminant microorganisms in order to ensure a timely response to these contaminations. For this reason, PNA-FISH was selected since it is an established and rapid reporting method already used in microbial analysis. (Forrest 2007).

To apply this method, two different PNA probes were developed, one for each of the two genera of microorganism that are known to cause food spoilage in food processing units, *Candida spp*. and *Pichia spp*. For *Candida spp*. the probe was designed to target the 18S ribosomal RNA sequences and for *Pichia spp*. the probe was designed to target the 26S ribosomal RNA sequences.

Once the probes were developed, optimization of the hybridization procedure was performed. Optimization consisted in changing different parameters such as temperature,

hybridization solution, incubation time, to have a faster process and the highest fluorescence signal intensity.

Although PNA-FISH allows a rapid detection, it is not a fully automated process. The introduction of this method to the microfluidic devices was tested to verify if it was feasible. This combination would allow the company a continuous vigilance of the food containers as well as a rapid action in the case of a contamination.

1.2 Presentation of the company

Frulact SA is a Portuguese food industry company specialized in fruit processing. One of the main issues this company faces during production is the overgrowth of microorganism in the food containers. This becomes a major problem due to the fact that the company sends this food containers to other countries in order to make yogurts. To ensure the product does not have contamination, it has to be hold-back for 2-3 days to obtain the results, as such it causes company economical losses due to the retention of the product.

1.3 Contributions of the Work

The work presented in this thesis was mainly done by me, except the design of probes and microfluidic devices. Andreia Azevedo contributed to the work by designing and testing theoretical parameters and thermodynamics of the probe. André M. Ferreira and Daniela Cruz-Moreira contributed with design of the microfluidic devices used in this work.

1.4 Organization of the Thesis

This thesis presents six chapters. In chapter one an introduction to the purpose and motivations of the work are presented, along with the main goals of the work.

Chapter two provides a review of the state of art. It focusses on role of yeast in food contamination, particularly the ones studied in this work, and the current detection methods available to prevent and control that contamination. In this work a fluorescence in situ hybridization technique using a peptic nucleic probe was used. For this reason, both fluorescence in situ hybridization and peptide nucleic acid probes are described in more detail. Because microfluidic devices were combined with FISH, a review of microfluidics was also included.

Chapter three describes materials and methods used in this work. Culture maintenance, probe design and hybridization both in glass slides and microchips are described.

Chapter four presents the results obtained during the work. Aspects such as identification of microorganism, protocol optimization and FISH in microfluidic devices are

discussed. Hybridization temperature for both probes is selected and confirmation that the procedure can be introduce to microfluid devices technique is performed.

Chapter five presents main conclusions taken from this work as well as limitations. In chapter six an analysis of the goals achieved, and future work is described.

2 Context and State of the art

2.1 Yeasts morphology and classification

Yeasts are eukaryotic fungi that grow as single cell microorganisms and reproduce asexually by budding or sexually by spores (Baron 1996). While yeasts are commonly characterized as unicellular, several species are dimorphic, meaning that both unicellular growth and pseudohyphae and hyphae are present (Figure 1).

Pseudohyphae and hyphae cells are elongated filamentous forms. In the pseudohyphae, the daughter buds elongate and remain attached to the mother cell, even after septum formation, resulting in constraining chains (figure 1b). Meanwhile, the hyphae form does not present the constriction of the walls, displaying parallel-sided walls (figure 1c). (Mukaremera et al. 2017; Sudbery et al. 2004).

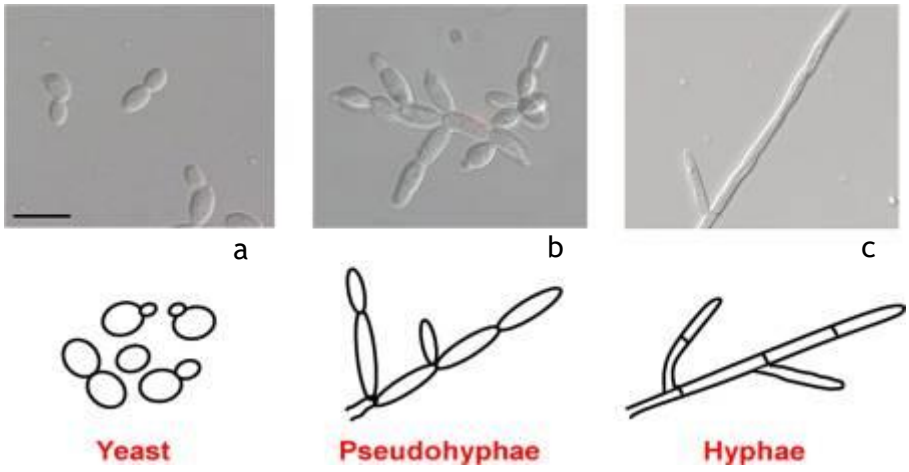


Figure 1. Types of morphologies in Candida spp. Top images are microscopy visualizations by differential interference contrast (bar=10 µm). Bottom images are schematics of these states (Thompson et al. 2011)

Yeasts that reproduce sexually induce an alteration of generations due to the formation of cells where meiosis happens and ascospores could be formed (Boekhout and Phaff 2003). Asexual reproductive yeasts are called anamorphs while sexual reproductive yeasts are called teleomorphs. Very often, these two states represent different names, both valid, being one based on the sexual state and the other based on the asexual state (Kurtzman et al. 2011). Yeasts nomenclature has undergone several changes due to phylogenetic studies using gene sequencing and genome comparison (Kurtzman 2014). The combination of the two above-mentioned factors resulted in taxonomic rearrangements and, consequently, the appearance of synonyms and teleomorph-anamorph pairs (Deak 2007b). The two-different name given to sexual and asexual is due to species being named at different times by different groups and phylogenetic analyses being performed later (Cannon and Kirk 2000; Taylor 2011). To people

not familiarized, these nomenclatures have caused misinterpretation since it is difficult to know both names refer to one single microorganism (Brandt and Lockhart 2012). On July 30, 2011, the XVIII International Botanical Congress decided to end "Two names-One fungus" practice and proposed the "One fungus=one name" which is the renaming of the anamorph to the teleomorph genus (Brandt and Lockhart 2012; Cannon and Kirk 2000; Taylor 2011). Although, rules for how the nomenclature will change are still being formulated, in the cases where the anamorph name is more commonly known than the teleomorph it is being considered the anamorph naming the species instead of teleomorph.

The genus *Candida* is anamorphic, belongs to the Ascomycota division and includes around 200 different species of yeast. As mentioned above, *Candida spp*. has known teleomorphs, some of them being of the genus *Pichia* or *Torulopsis* (Deak 2007b; Diezmann et al. 2004; Segal and Baum 1994). *Pichia* is a teleomorphic genus, belongs to the Ascomycota division and comprises around 20 species of yeasts (Kurtzman 2011).

Both genera are considered polyphyletic (Boekhout and Phaff 2003), with species from each genera belonging to several main clades (Deák 2006). The genus *Pichia* has undergone numerous changes in their taxonomy throughout time, one of them has been the inclusion of another genus (*Hansenula*) based on a study by Kurtzman (1984) that showed a 68-75% shared ancestry between *Hansenula spp*. and *Pichia* spp. However, Kurtzman et al. (2008) proposed a reassignment of several *Pichia (Hansenula) spp*. including the creation of the new genus *Wickerhamomyces*. An example of this reassignment was *Pichia anomala*, now *Wickerhamomyces anomalus*. This reduced the number of species from over 100 to around 20 (Kurtzman 2011). In the same study, it was also established that the *Pichia anomala* clade included several *Pichia* and *Candida* species. Studies by Kurtzman and Robnett (1998) and Villa-Carvajal et al. (2006) demonstrated that *Pichia* and *Candida* genus are very closely-related species based on the analysis of their 26S rRNA sequences, given identical profiles for teleomorphic and anamorphic states of several *Pichia* and *Candida* species. Also, in the Kurtzman and Robnett (1998) study, a comparison between *Pichia spp* 18S rRNA was performed where it was concluded that they are phylogenetically different, further confirming the widely distribution of *Pichia* through the other species. Table 1 shows several anamorph-teleomorph relations between *Candida* and Pichia species, as well as, synonyms.

Table 1. Anamorph-teleomorph relations between Pichia and Candida species and their synonyms.(Kurtzman 1998; Romi et al. 2014; Vaughan-Martini et al. 2005)

Teleomorph	***Anamorph***	***Synonym***
Pichia anomala	*Candida pelliculosa*	*Hansenula anomala*
Pichia fermentans	*Candida lambica*	*Candida lambica*
Meyerozyma caribbica	*Candida fermentati*	*Pichia caribbica*
Torulaspora delbrueckii	*Candida colliculosa*	*Saccharomyces delbrueckii*

2.2 Role of yeast in food contamination

Although not always associated with spoilage, yeasts are one of the main responsibles for it. In most cases, the presence of yeast in foodstuff is unlikely to reflect in a health problem, with only a few cases of foodborne yeasts isolated from clinical cases, although it is hard to detect from the food that cause it. Cases of infection by yeasts are rare with only a few cases of gastroenteritis and allergies and these symptoms could be because spoilage causes the formation of allergic compounds or deterioration of preservatives in food. (Deák 2006; Fleet and Balia 2006; Lamps et al. 2014). Even without causing health problems, a control over this contaminant microorganism in food is needed (Fleet 2007; Lelieveld et al. 2005).

For companies, yeast contamination causes organoleptic changes due to the growth and development of contaminant microorganism leading to commercial and economic losses because of product spoiling. These organoleptic changes, such as gas production, off-flavour and texture changes, affect the smell and/or aspect of the product causing quality problems that harm the company financially and commercially. (Leyva Salas et al. 2017; Stratford 2006).

Most of the time these changes happen because yeast cause the fermentation of the product. Since fermentation is more likely to happen under anaerobic conditions, products with a high water activity are more likely to suffer fermentation from yeast since liquid media tends to form anaerobic conditions and, also yeast being single cell microorganism, they tend to disperse easily in liquid (Pitt and Hocking 1997; USDA 2012). As opposite to bacteria, yeast can grow out of their optimum conditions, meaning that even with a slower grown rate when in conditions with low pH, temperature, water activity (high sugar content) and high nutrient content, yeast survive and grow. It is in these conditions that yeast play a major role in spoilage since they don't have competition from bacteria and are able to grow and spoil the product. (Stratford 2006; Viljoen 2001).

For all the above-mentioned reasons, certain foods such as syrups, jam, fruit, vegetables and yogurts which have more adverse conditions for bacteria are more susceptible to

contamination by yeasts (Betts 2013). Processed fruits are easy targets for yeast since during processing the natural integrity and biological structure is lost making sugar substrates easily available for fermentation to happen (Fleet 2003b). In these cases, some of the predominant yeasts that are responsible for fermentation are *Candida* and *Pichia,* but there are also other types such as *Saccharomyces, Zygosaccharomyces, Hanseniaspora* and *Rhodotorula* (Cletus Kurtzman 2011). Processed fruits are, in most cases, used for preparation of yogurt, syrups, cakes, etc, being a primary source for yeast contamination and spoilage of the product (Fleet 2011).

2.2.1 Genus *Candida*

As mentioned above, *Candida spp*. is one of the yeast associated with fruit processed products (Fleet 2003a), however in an environment such as processed fruits it is not possible to distinguish *Candida* spoilage from the general spoilage done by other contaminant microorganisms. Nevertheless, several *Candida spp*. produce pseudohyphae which leads to the development of biofilms (Deák 2006). In table 2 it is possible to see frequencies of the more common *Candida* species and their respective morphology in fruits and low water activity products.

Table 2. Frequencies of common Candida spp. in fruit products and products with low water content (aw). Both frequencies are analysed since contamination in fruit process is not the same as in fruits, because processed fruit has a high sugar content, meaning a low water activity. Frequencies were calculated based on the number of occurrences, type of food and isolates found, and about 100 species were considerate for each column. (Deák 2006; Thompson et al. 2011)

Species	Fruits	Low a_w	Morphology
C. albicans	0.37	-	Yeast, Pseudohyphae, Hyphae
C. colliculosa	4.68	7.53	
C. glabrata	0.86	0.7	Yeast, Pseudohyphae
C. intermedia	0.74	0.35	
C. lambrica	1.6	1.88	
C. tropicalis	1.85	1.41	Yeast, Pseudohyphae, Hyphae
C. parapsilosis	1.38	1.12	Yeast, Pseudohyphae
C. pelliculosa	4.25	3.53	

Within the genus *Candida*, the most important species are: *C. albicans, C. tropicalis, C. glabrata* and *C. parapsilosis,* due to their frequency and pathogenesis (Segal and Baum 1994).

Based on a study by Pfaller et al. (2010) these four species represented 92% of all the *Candida* isolates. These species are the cause of most invasive infection associated to *Candida* in humans with around 90% belonging to them (Spampinato and Leonardi 2013). Although they are extremely frequent, when it comes to fruits and products with low water activity their frequency does not exceed 1.5%. The low frequency of the most pathogenic species in this genus, as well as infections commonly happening in immunocompromised patients (Spampinato and Leonardi 2013), could be one of the reason why foodborne infections derived from this species are not common.

2.2.2 Genus *Pichia*

The yeast in the genus *Pichia* are known contaminants for several foods and beverages with a high sugar content (low aw activity) and low pH products (Passoth et al. 2006). The ability for *Pichia* to produce ethyl acetate make it highly associated with wine, being a contribution in the first stages of fermentation (Villa-Carvajal et al. 2006). Within this genus, some species such as *Pichia anomala* (Bakir et al. 2004) and *Pichia ohmeri* (Otag et al. 2005) have been associated as causes of human infections in immunocompromised patients and paediatric intensive care units. Although, out of the two reported, pathogens *Pichia anomala* is the most important since Deak and Beuchat (1996) identify it as the third most frequent foodborne yeast.

2.3 Detection of microorganisms

In food industry, detection of contaminant microorganism in time is an asset to prevent economic losses. The current methods available to detect yeast are conventional methods, such as culture-based methods (White et al. 2009), and "rapid" methods such as accelerated conventional methods, optical techniques, biochemical methods, electrometric methods, immunological techniques and molecular techniques (Deak 2001). Fluorescence *in situ* hybridization (FISH), a molecular technique, is one of the most relevant techniques, and for that reason it is going to be discussed in the next section. Table 3 presents an overview of the methods that are going to be mentioned more thoroughly in the sub-chapters below.

Table 3. Overview of some of the most used detection methods and important characteristics that define them such as time-to-react and specificity. (Deak 2007a; Mandal et al. 2011)

Method	*Detection limit/cfu•mL^{-1}*	*Time before result/h*	*Specificity*
Plating technique	1	24-72	Good
Pretifilm	10^2-10^3	24-48 h	Good
Flow cytometry	10^2-10^3	0.5 h	Good
DEFT	10^3-10^4	0.5 h	Average
Immunological methods	10^4	1-2 h	Moderate/good
Nucleic acid-based assays	10^3	6-12 h	Excellent

2.3.1 Culture-based methods

Culture-based methods use a variety of media to detect, isolate and enumerate yeasts. These selective media are selected in accordance to the target microorganism. For example, to separate yeast from bacteria normally low pH mediums are used, such as potato dextrose or malt extract agar since yeast can grow in these and most bacteria cannot. For *Candida spp*. there is a chromogenic media that differentiates different *Candida* species based on colour (Deak 2003; Safavieh et al. 2017). Although these are simple methods, they have low reproducibility and are time consuming; the results are only obtained after several days and require a lot of procedures, such as sample and media preparation, dilution, inoculation, incubation, counting and isolation (Deak 2001; Mangal et al. 2016).

The accelerated process for culture-based methods are devices that help with preparation of samples and dilution, making the process faster while also eliminating error. Some of these commercialized products are Petrifilm® and Iso-Grid systems. 3M™ Petrifilm™ Rapid Yeast and Mold Count Plates is a dry medium composed with nutrients, selective agents, indicators and water-soluble gel (3M™ 2004) prepared on a support membrane and covered in a film (Deak 2003). The dry media is rehydrated when applying 1 ml of sample and then, incubated for at least 48 h. The Iso-grid® is a filter technique with hydrophobic grid membrane that captures the isolated microorganism within in the squares. The membrane is then transferred to a agar plate and incubated and counted (Deak 2007a; Neogen). Both systems are approved methods for the enumeration of total yeasts (Entis 1996; Knight et al. 1997).

2.3.2 Direct counting techniques

The two-major direct counting techniques used are: direct epifluorescent filter technique (DEFT) and flow cytometry (FCM). DEFT method comprises the passage of food samples through a membrane, fluorescence straining of the microorganism and visualization in a epifluorescent microscope (Deak 2001; Mandal et al. 2011). In flow cytometry, cells in suspension are carried by laminar flow until they are intersected by a beam of light, most commonly a laser. The scattered light is collected by lenses and analysed, providing information on the cells size, structure and shape (Brown and Wittwer 2000). This technique can be coupled with dyes and when passing through the beam of light, this is going to be of the same wavelength as the respective absorption spectrum of the dye (Mandal et al. 2011). It is a highly sensitive and rapid method capable of measuring several particle/cell characteristics such as structural properties and biological activity, allowing determination of viability and testing vitality (Brown and Wittwer 2000; Deak 2001, 2007a; Deere et al. 1998; Malacrinò et al. 2001). However, it is a labour-intensive technique and most of the times it does not allow specific identification of certain microorganisms since many microbial cells are not distinguishable from each other (Crook 1996).

2.3.3 Immunological techniques

Immunological techniques are widely used for the detection of bacterial pathogens in food products and there is a lot of know-how as well as development of the assays. However, application for yeast lack information and results (García et al. 2004). These methods use antigen-antibody reactions to be able to detect microorganism. The antibody has to bind to only a specific antigen even in a complex food matrix with other microorganism (Mangal et al. 2016). Within the immunological techniques, sandwich enzyme-linked immunosorbent assay (ELISA) is one of the most used (Law et al. 2014; Priyanka et al. 2016).

Most yeasts produce thermostable extracellular polymeric product (EPS), yet the excretion of EPS is affected by growth conditions. In ascomycetous, the antibodies directed against their EPS could detect viable and dead yeast cells because the antigens of ascomycetous are species and genus specific (Middelhoven and Notermans 1993).

Detection of *Candida spp*. and *Pichia spp*. in food spoilage through sandwich ELISA technique with polyclonal antibodies has been demonstrated on works by García et al. (2004) and Yoshida et al. (1991). At the time, these results are of limited value, because of the different specificities that different species have for antisera (García et al. 2004).

Immune assays could be, in the future, of great interest for the detection of yeast in food because of the high sensitivity and specificity they demonstrate for bacteria, as well as cost-effective but at the moment it is necessary to deepen the knowledge (Deak 2007a).

2.3.4 PCR-based methods

PCR methods are based on the amplification of nucleic acids using single pair of primers to detect one microorganism at a time (Mustapha and Li 2006). The detection is performed by gel electrophoresis, ethidium bromide staining and visual examination using UV light (Scheu et al. 1998). When compared to culture-based methods, PCR is faster, although sensitivity and specificity depend on several factors, such as food matrix (Leonard et al. 2003; Scheu et al. 1998). Furthermore, it has limitations based on the occurrence of false negatives and false positives. False positives could happen because PCR is not able to distinguish viable cells from non-viable which means that DNA from dead microorganism would be detected. False negatives happen due to the presence of inhibitors compounds that could be present within the food matrix (Mandal et al. 2011; Scheu et al. 1998). Some of the limitations associated with PCR method can be prevented with enrichment steps that reduce the interferences. However, these enrichments can take up to 48 h (López-Campos et al. 2012; White et al. 2009).

Several commercial PCR systems have been developed, such as The BAX® and GeneDisc®, systems that allow a standardized and automated method for the detection and identification of contaminant microorganism in food samples (Corporation 2017; Hygiena 2018). However, the major advantage of these systems is the elimination of the laborious work performed by the technique since the enrichment steps are still necessary and can also take around 2 days to show results.

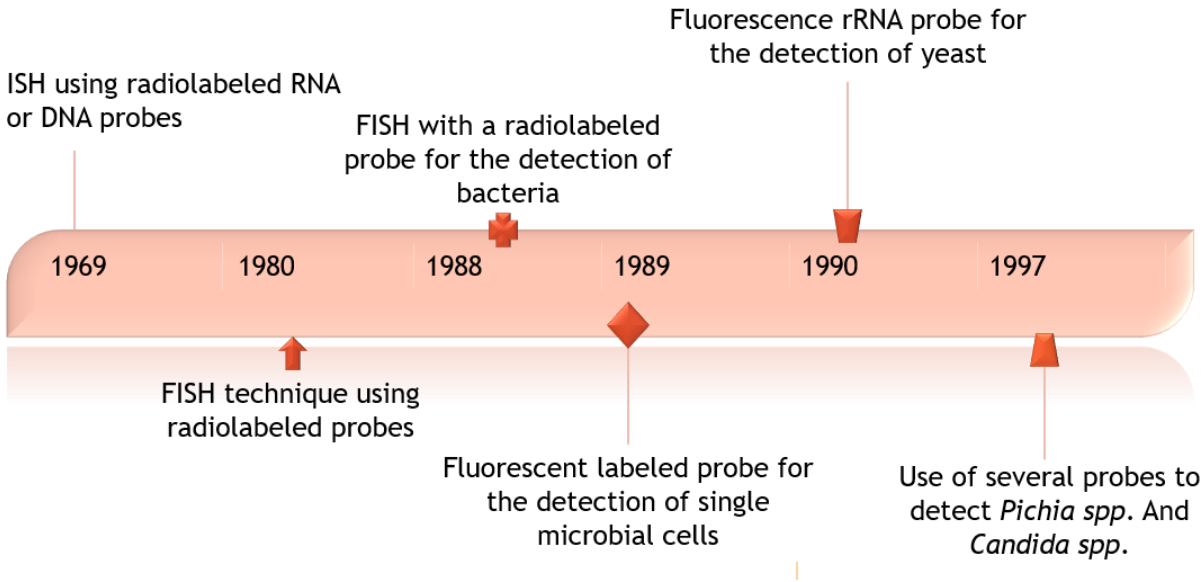
Modifications to the conventional PCR method have been performed to reduce time and decrease the false positive results. Some of these modified protocols are reverse transcription (RT)-PCR and real-TIME PCR. RT-PCR instead of detecting DNA, detects mRNA which is an indicator of viability. However, mRNA is unstable and difficult to extract in complex food matrices without degradation (Mustapha and Li 2006). Real-time PCR is a powerful technique because it can quantify the target DNA with higher precision as well as detect multiple species at the same time when combined with multiplex PCR. The current limitation for the use of this method is the expensive cost of the equipment and reagents. (Law et al. 2014; Mustapha and Li 2006).

2.4 Fluorescence *in situ* Hybridization

FISH is whole-cell procedure that consists in the hybridization of fluorescence labelled oligonucleotide probes to its complementary target sequence and, once, hybridization occurs cells are detected using epifluorescence microscopy (Moter and Göbel 2000; Wagner et al. 2003). Being a whole-cell procedure is already an advantage when compared to PCR because it does not require the extraction of nucleic acids from cell. It is a powerful and rapid method for the identification, visualization and quantification of microorganism with a widespread field

of applications (Almeida et al. 2010; Amann et al. 1990; Lischewski et al. 1997; Wagner et al. 2003). A timeline of the evolution of this technique is shown in Figure 2.

Figure 2. Timeline of the evolution of the FISH procedure, starting with the in situ hybridization method until the fluorescence in situ hybridization capable of detecting Candida spp. and Pichia spp.



This method started as only *in situ* hybridization (ISH) with reports from around 1960s, where Pardue and Gall (1969b), John et al. (1969) and Pardue and Gall (1969a) demonstrated the hybridization of radioactive labelled RNA and DNA probes to the target DNA sequences in mammalian cells. The detection of the resulting hybrids was obtained by autoradiography. However, this method has some drawback, mainly: the instability of the labelled isotypes, and consequently of the probe; resolution in autoradiography depends of the type of radioactive isotopes used; and the need for long-time exposure in order to obtained detection signal, as well as the problems associated with long-time exposure to radioactivity (Abraham 2001; Aquino de Muro 2005; Hyypia 1985).

Bauman et al. (1980) was the first to describe a method that was capable of detecting specifically a DNA target sequence through the hybridization with a fluorochrome labelled probe. Later, Giovannoni et al. (1988) described the detection of bacteria using radiolabelled oligonucleotide probes. With the evolution of fluorescent labelled probes and the limitations presented by radiolabelled probes, DeLong et al. (1989) described a fluorescent labelled probe capable of detecting single cells. Bertin et al. (1990) described the detection of yeast trough flow cytometry by a fluorescent rRNA probe and Kosse et al. (1997) described various probes that detected several microorganism including *Pichia spp.* and *Candida spp.* There are numerous descriptions of the use of FISH procedures for detection of *Candida spp.* and *Pichia spp.* (Frickmann et al. 2012; Lakner et al. 2012).

Important parameters for the probe in the FISH procedure are specificity and sensitivity. Specificity is predicted through the analysis of numerous sequences to determine if cross-hybridization happens, meaning if species that are not the target one react with the probe. Sensitivity is described as the percentage of target strains that are identified by the probe sequence comparing to the total strains of the target microorganism (M. D. Kane et al. 2000). Sensitivity lower than 100% indicates that not all strains of the microorganism of interest react with the probe.

2.4.1 Probe design

Probes are nucleic acid molecules of single-stranded DNA or RNA that exhibit a strong affinity to a target DNA or RNA sequence. Designing of the probe is strongly influenced by sensitivity and specificity since the goal is to select a specific sequence that has the higher specificity and sensibility for the target microorganisms.

A common target of these probes is rRNA. The advantages for choosing rRNA are related with its abundance in cells which improves the chances of hybridization and being very conserved regions within the species. (Lischewski et al. 1996) For microorganism of the Eukaryote domain, most common used sequence is 18S rRNA but 26S is also used for some microorganism (Cerqueira et al. 2008; Froehlich et al. 2009).

Labelling of the probe can be performed in two ways: direct and indirect. Direct labelling it is a fast and cheap method because it does not require further steps after hybridization. In direct labelling, fluorophores are associated with probes by chemical conjugation to the nucleic acid, while in indirect labelling the chemical conjugation of the nucleic acid is to a non-fluorescent molecule that can bind to the fluorophore after hybridization (Morrison et al. 2002; Moter and Göbel 2000).

There are two types of probes used in FISH procedure: DNA/RNA probes and nucleic acid mimics. DNA/RNA probes range from 20-50 bp while nucleic acid mimic probes are smaller, ranging from 13-18 nucleotides. The latter have proven to have advantages when compared to DNA/RNA probes. Examples of the nucleic acid mimic probes are peptide nucleic acid (PNA) and locked nucleic acids (LNA) (Aquino de Muro 2005; Beliveau et al. 2012; Cerqueira et al. 2011).

PNA molecules are synthetic DNA analogues, where the negatively charged backbone sugar-phosphate is replaced by neutral polyamide backbone formed by repetitive N-(2-aminoethyl) glycine units. LNA molecules are synthetic RNA where the ribose is linked to a methylene bridge between 2'-oxigen and 4'-carbon atoms (Figure 3) (Cerqueira et al. 2008). For the interest of these thesis, PNA probes are going to be discussed in the chapters ahead.

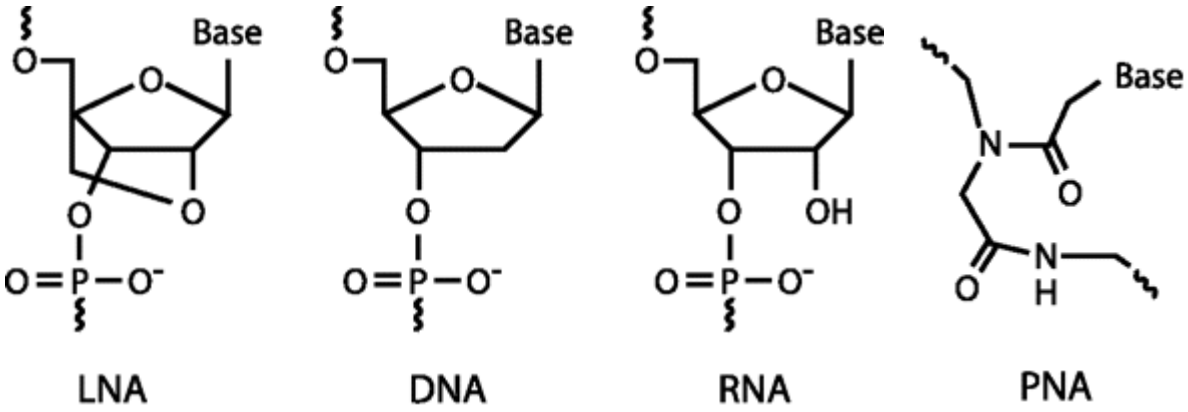


Figure 3. Structures of DNA, RNA, PNA and LNA. Adapted from Kubota et al. (2006)

2.4.2 FISH Protocol

FISH protocol encompasses 5 steps: fixation, permeabilization, hybridization, washing and visualization (Figure 4). Fixation and permeabilization steps are important to maintain cell and RNA integrity, as well as, prepare the cells for probe diffusion by permeabilizing cell wall. Agents used for these steps can be ethanol or methanol, paraformaldehyde or formaldehyde (Moter and Göbel 2000; Rocha et al. 2018). In the hybridization step, probe and target cells are placed in contact, if the probe sequence is complementary to the target sequence, annealing will happen. Washing step guarantees that any loosely bound or free residues from probe do not influence the results by being removed. Hybridization step can be highly influenced by conditions in which it occurs. For this reason, temperature, pH and solution concentrations, in particular hybridization solution, must be optimized to ensure hybridization (Cerqueira et al. 2008; Moter and Göbel 2000; Rocha et al. 2018). Visualization can happen both by fluorescence microscopy or by flow cytometry.

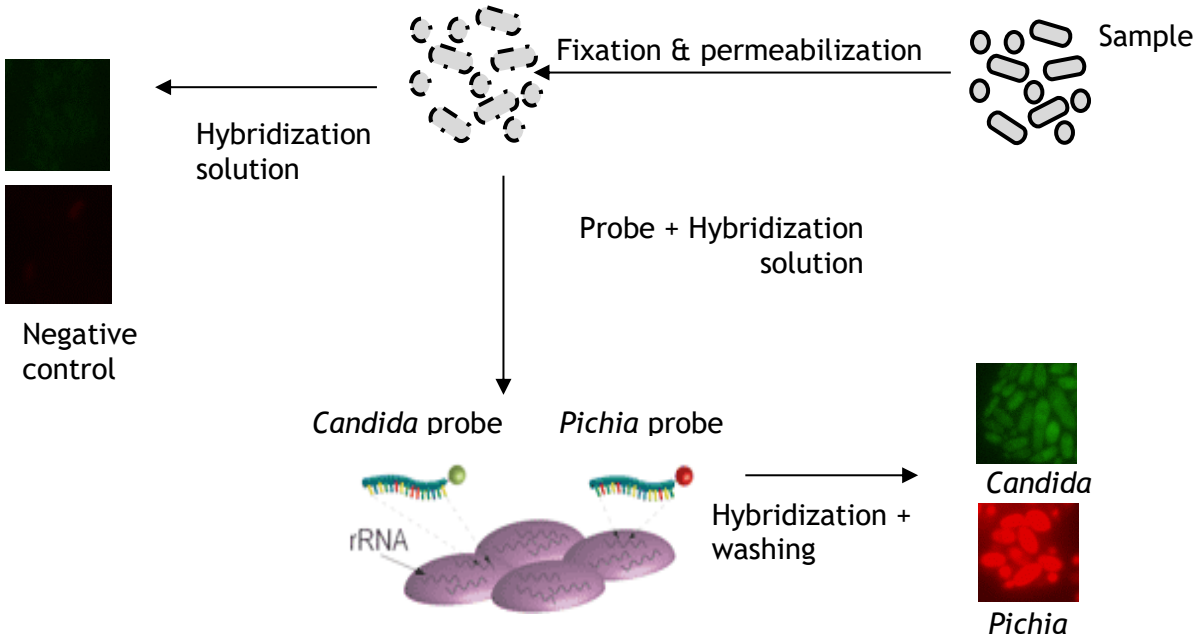


Figure 4. Schematic FISH protocol where the five steps of FISH protocol are present: i) fixation and permeabilization of the sample to allow probe penetration; ii) hybridization of the probe with target sequence; iii) washing of the unbounded probe; iv) visualization through microscope or flow cytometry. Adaptation from Moter and Göbel (2000)

Alike the other methods discussed above, FISH also presents some limitations based on the false positive and false negative results. The review of these limitations is based on Moter and Göbel (2000)

False positive results happen due to autofluorescence of the microorganism and lack of specificity.

Autofluorescence is a major concern in FISH and the main reason why controls need to be done in order to assess if the microorganism has or not autofluorescence. Margo and Bombardier (1985) and Graham (1983) have reported this problem in fungi.

Lack of specificity depends on the design of the probe. Accuracy and reliability of this method depends on if the probe is going to hybridize with closely related sequences to the probe and/or if the sequence picked was the correct one. Probe design is usually done by recurring to sequencing databases, if designing is performed using a database that is not the most recent update, (Bhatia et al. 1997), it could lead to a mistake in the sequence selected for the probe, and consequently a non-specific sequence for the selected microorganism(s). Therefore, regular analysis of sequencing databases is necessary to minimize these risks.

A common cause of a false negative is the probes inability to penetrate the cell wall. Other possible false negative result could be due to low rRNA content. In most cases rRNA

content is high within cell but it is also dependent on growth rate. In these cases, the results obtained would have very low signal intensity or false negatives.

With DNA probes, false negatives or low signal intensities are more likely to happen due to the lack of hybridization efficiency DNA probes have to hybridize with target rRNA molecules. The low efficiency is mainly due to accessibility of the probe to the target site. Overall Gibbs free energy change is the indicator of affinity and although the ideal is a negative value, it cannot be too negative because otherwise annealing would be too strong and more cross-hybridization would happen because washing solution would not be able to remove the probe in those cases. In DNA probes for a ΔG° negative enough that would allow a good hybridization, longer DNA probe sequences were proposed although this causes two problems: low flexibility and more possible mismatches (Kubota et al. 2006).

2.5 Peptide Nucleic Acid (PNA) Fluorescence *in situ* Hybridization

As briefly mentioned above, PNA are synthetic nucleic acid DNA mimics where the neutral backbone composed of N-(2-aminoethyl) glycine units is replacing the negative charged backbone (Cerqueira et al. 2008). The development of PNA probes by Nielsen et al. (1991) was due to the problems DNA probes exhibited.

PNA are capable of hybridize to its complementary DNA and RNA sequences according to Watson and Crick base-paring rules (Guimarães et al. 2007). The affinity for the complementary sequences is much higher when compared to DNA probes since there is no electrostatic repulsion between PNA backbone and natural nucleic acids. This higher stability with PNA/DNA duplex increases the melting temperature (Tm). The increase in melting temperature affects some characteristics of the probes, such as allowing the use of shorter sequences for PNA probes because a mismatch of a single-base in higher Tm has a much bigger impact. This gives PNA probes higher specificity when compared to DNA probes. The optimal sequence of a PNA probe is 15 bp (Briones and Moreno 2012; Cerqueira et al. 2008; Cerqueira et al. 2011; Stender et al. 2002). The Tm of a probe is frequently used as a reference for the hybridization temperature (Th) although a relationship between the two has not been confirmed in literature. (S. Fontenete et al. 2016a)

Furthermore, the synthetic backbone makes PNA more resistant to nucleases and proteases which can be a factor for better probe stability (Briones and Moreno 2012; Cerqueira et al. 2008; Cerqueira et al. 2011; Stender et al. 2002). Their accessibility to rRNA is also improved due to the fact that hybridization can occur in conditions that destabilize rRNA secondary structure, such as low salt concentration and higher temperatures (Wagner et al. 2003).

PNA probes for the detection of *Candida* species have been previously describe (Kim and Brehm-Stecher 2015; Rigby et al. 2002; Shepard et al. 2008) although these probes are usually one or multiple species specific but until date not for the whole genus.

2.6 FISH-ON-CHIPS

In the chapter above, limitations of the performance of FISH procedure were presented, but there is one limitation when it comes to the application of this method in industry. FISH procedure as described above is not an automated system, requiring all the steps presented in Figure 4 to be performed manually (Huber et al. 2016). It is also not prepared for a high-throughput-analysis as necessary for the food industry and requires sample preparation because of the food matrix (Kant et al. 2018; Liu et al. 2013; Rohde et al. 2015). In addition to being a time-consuming process, it is an expensive process in particular due to the expensive cost of the probes (Huber et al. 2016).

Development of the microfabrication technology has allowed the creation of microfluidic devices or as usually called, labs-on-chip. These devices are networks that have fluidic channels of the micrometre scale, capable of a high surface-to-volume ratio and mass transport. High surface to volume ratio enables the concentration of the microorganism in a pre-determined location, eliminating the enrichment step that increases the time in standard procedures (Ferreira et al. 2017; Heo and Hua 2009; Jiang and Korivi 2014).

There are five major types of microfluidic platforms: capillary, pressure driven, centrifugal, electrokinetics and acoustic. For the purpose of this thesis we are going to focus on pressure driven. A pressure driven laminar flow is based on pressure gradient to transport liquids, leading to hydrodynamically stable laminar flow within the microchannels. In this platforms, the reagents and samples are introduced through the inlet whether in continuous or batch flow, and a pressure source such as a syringe enables the transport through the microchannels (Mark et al. 2010). It is the possibility for continuous sample processing that is an important feature for testing in the food industry.

2.6.1 Sample preparation

The main challenge on the application of standard procedures to microfluidic devices is sample preparation. Sample preparation comprises enrichment from complex food matrices (Kant et al. 2018; Mairhofer et al. 2009) and retaining of the cells within the chip (Perez-Toralla et al. 2015). Sample preparation needs to be carefully performed since performance of the microfluidic devices will depend on it (Chung et al. 2010). Sample preparation from complex food matrices is done by performing a pre-treatment that separates the desired microorganism from the unnecessary compounds (Chung et al. 2010). The goal is to obtain high cell density,

but avoiding the over accumulation because otherwise the probe is not capable to hybridize all the cells, and preventing disruption of the cells in the microchannels due to high shear stress (Perez-Toralla et al. 2015).

Sample pre-treatment comprises filtration, purification and pre-concentration (Chung et al. 2010) and it is commonly performed off the chip (Thorslund et al. 2006). The filtration techniques can consist of packed beads (Andersson et al. 2000; Mulvaney et al. 2007), porous membranes (Thorslund et al. 2006; Wei et al. 2011) and filtering pillars (Zhang et al. 2006). In filtering pillars, gaps between pair of pillars need to be smaller than the size of the cell desired and several rows are positioned in order to improve entrapment efficiency (Ferreira et al. 2017; Zhang et al. 2006). The filtration process can be compromised if the size of the gap between pillars is too big, because collapsing of the channels can happen (Andersson et al. 2000).

2.6.2 Microfabrication

The design and fabrication of the microfluidic platform depends on the performance required (mixing, transport, valving, separation, incubation and others) (Mark et al. 2010). For the FISH procedure, microchips must be adequate to perform the delivery of complex mixtures with different viscosities in the microchannel, without cross contamination and precise temperature control, mostly because of the hybridization step in the FISH procedure. Furthermore, it needs to be prepared for fluorescence imaging to obtain the results of signal detection and quantification (Perez-Toralla et al. 2015).

Fabrication materials include silicon, glass, soft or hard polymers and biomaterials (Dutse and Yusof 2011) although, when it comes to silicon and glass-based microdevices, polymeric ones are preferred because of the possibility for high-volume and low-cost fabrication using soft lithography, high chemical resistance and good optical transparency (Calaon et al. 2015).

The fabrication of a microdevice consists of three phases: creation of the pattern by photolithography, replication of the mold by soft lithography and binding of the elastomer to the coverslip (Figure 5).

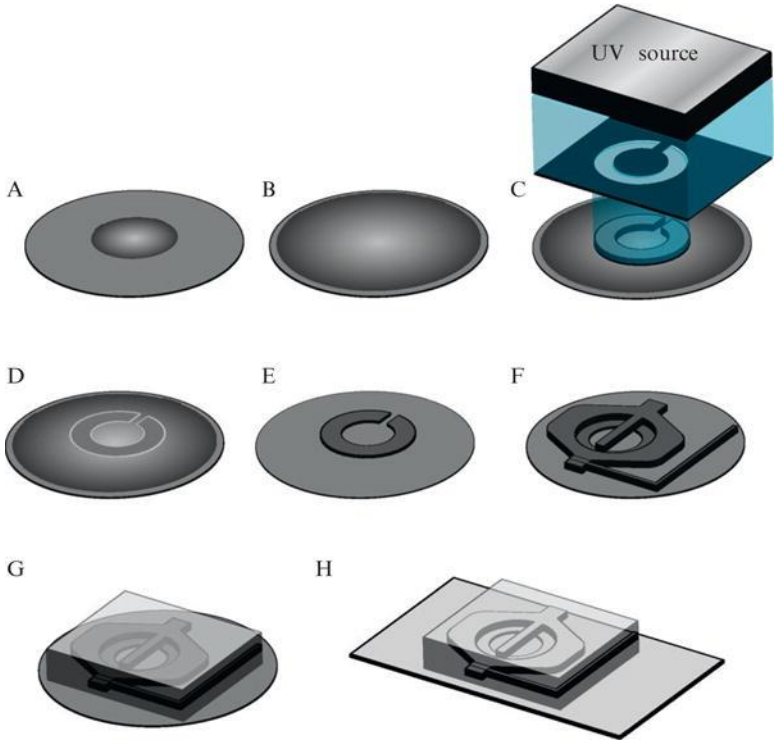


Figure 5.Schematic procedure for fabrication of a microfluidic device. A-E represent photolithography for the creation of patterns in wafers; G is the soft lithography technique for the creation of a mold with the patterns imprinted; H represents the bonding of the polymeric mold to the coverslip. (Ferry et al. 2011)

2.6.2.1 Photolithography (SU-8)

Photolithography is a technique that fabricates geometrical patterns via light radiation. The patterns are transferred from a mask to a photosensitive layer (Ferry et al. 2011). The patterns are created on SU-8 photoresist that is spin-coated on the wafer (Figure 5, A-B) and then exposed to UV light through a photomask (Figure 5, C) that will transfer the patterns for the photoresist layer (Figure 5, D) (Ferry et al. 2011). The areas not exposed to the UV light will be washed away after immersion in solvent, resulting in the pattern in the wafer (Figure 5, E) (Ferry et al. 2011). The wafer should be thoroughly cleaned before starting the process to guaranty that no impurities will be incorporated in the final design (Ferry et al. 2011).

2.6.2.2 Soft lithography

Soft lithography is a technique that produces micropatterns. It uses an elastomeric stamp or mold to replicate patterns by molding. A two-part liquid polymer is casted on the pattern wafer, and once cured it is separated from the wafer (Figure 5, G) (Xia and Whitesides 1998). After the curing process, the polymer is flexible, and an easy demoulding is possible (Ferry et al. 2011). Furthermore, this process could be improved using a removing agent, that also serves to preserve the patterns in the waffle (Duffy et al. 1998). Finally, the separated

chip is bonded to coverslips (Figure 5, H). It is important that coverslips are thoroughly cleaned to avoid any impurities that could affect the performance of the device (Ferry et al. 2011).

Several elastomeric polymers can be used, such as polymethylmethacrylate (PMMA), polydimethylsiloxane (PDMS), polyvinylchloride (PVC), polyethylene (PE) and polycarbonate (PC) (Xia and Whitesides 1998), although PDMS is the more commonly-used among researches (R. S. Kane et al. 1999). PDMS is a silicone elastomer, inexpensive, biocompatible, durable and can be used for cell cultures (R. S. Kane et al. 1999; Mark et al. 2010).

2.6.3 Applications of microfluidics to FISH

The use of microfluidics devices to perform FISH analyses has gained a lot of attention in part due to the advantages mentioned above, but also because it offers an automated and low-cost procedure, and reduces the quantity of reagents needed, such as probe. Furthermore, being able to perform sample preparation and a complete FISH procedure inside the chips reduces the possibility of contamination (Perez-Toralla et al. 2015; Sato 2015).

When applying standard FISH procedure there are some factors to take in consideration such as melting and hybridization temperature, hybridization time, buffers composition and probes, that can influence the outcome of the test. Using microfluidic devices, besides the factors mentioned above other such as flow rate and chamber might need to be taken in consideration (Nguyen et al. 2016) for retaining and concentration of the cells occur most effectively (Ferreira et al. 2017). Figure 6 presents a schematic illustration of the PNA-FISH procedure in the microchannels with an array of pillars for both concentration and retaining of the cells.

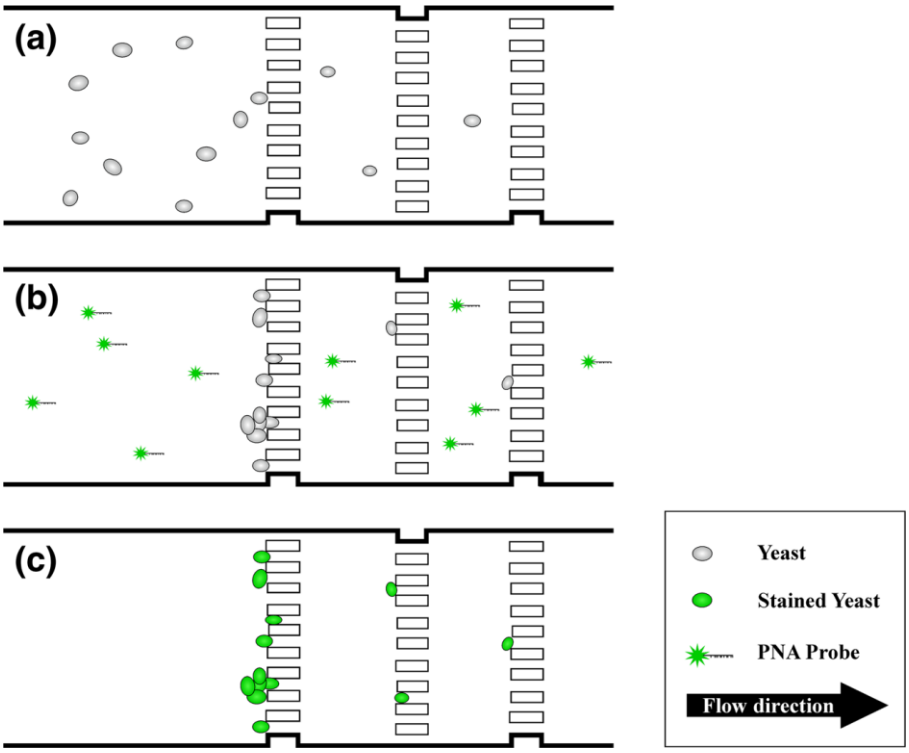


Figure 6. Schematic illustration of FISH procedure performed in a microchannel with an array of pillars. a) Trapping of the yeast cells by the array of pillars; b) pumping of the PNA probe solution at a defined flow rate, to hybridize with the trapped cells; c) introduction of the washing solution, post-hybridization to remove the unbound probe (Ferreira et al. 2017).

3 Materials and Methods

3.1 Culture maintenance

Eleven different strains obtained from food containers were given to us by the company and four *Candida* reference species were gently provided by Mariana Henriques from Minho University. All microorganisms were grown in agar plates containing the YEPD medium (1% (wt/vol) yeast extract, 2% (wt/vol) peptone, 2% (wt/vol) dextrose) and placed in an incubator at 30 ºC for 24h.

Identification of the strains obtained from the company was performed by sequencing at STABVIDA. Extraction of DNA and use of adequate primers to obtain a segment of fungal ribosomal DNA of approximate 1600 bp containing the complete ITS and D1/D2 regions and partially 26S rRNA sequence.

Reference species were *Candida albicans, Candida tropicalis, Candida parapsilopis* and *Candida glabrata*. Company species were *Wickerhamomyces anomalus, Candida santamariae, Pichia fermentans, Rhodototula sp., Hanseniaspora osmophila, Meyorozyma caribbica, Pichia caribbica, Torulaspora delbrueckii, Hanseniaspora opuntiae*, FRU14 and FRU61.

3.2 PNA probe design

Two PNA-probes previously design for detection of *Candida spp*. and *Pichia spp*. were used. Briefly, the identification of potential oligonucleotide sequences for the probe was performed by selecting several 18S rRNA and 26S rRNA gene sequences, for respective probes, from the BLAST databases available at NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Using Clustal W program available at the European Bioinformatics Institute website (https://www.ebi.ac.uk/Tools/msa/clustalo/), alignment of these gene sequences was performed to identify the potential sequences of interest. These sequences were then tested in NCBI website to find which had the highest number of target microorganism sequences detected and the lowest non-target microorganism detected. Theoretical specificity (equation 1) and sensitivity (equation 2) were calculated based on formulas from Almeida et al. (2010).

$$Tspecificity = \frac{nCs}{(TnC)} x100 \qquad \text{(equation 1)}$$

where nCs stands for the number of non-*Candida* strains that did not react with the probe and TnC is the total of non-*Candida* strains examined.

$$Tsensibility = \frac{Cs}{(TCs)} x100 \qquad \text{(equation 2)}$$

where Cs stands for the number of *Candida* strains detected by the probe and TCs is the total number of *Candida* strains present in the databases. The PNA probes features and thermodynamic parameters are shown in table 4.

Table 4. PNA probes features and respective thermodynamic parameters

	Candida spp.	***Pichia spp.***
Target rRNA	18S	26S
Probe sequence	5'-Alexa488-OO-CACCCACAAAATCAA-3'	5'-Alexa594-OO-CACGTGCTGTTTCAC-3'
Target sequence	5'- TTGATTTTGTGGGTG-3'	5' –GTGAAACAGCACGTG- 3'
Tm / ºC	75.69	73.72
ΔG/ kcal.mol^{-1}	-16.37	-18.45
Specificity/ %	96.04	99.90
Sensibility/ %	84.79	80.65

3.3 Determination of cell concentration by DAPI staining

Cell concentration was determined using 4′,6-diamidino-2-phenylindole (DAPI) staining followed by microscopy visualization. For all strains, cells from 1-day-old cultures were harvested from YEPD plates, suspended in sterile water, and homogenized by vortexing. Five different optical densities (OD) between 0.1 and 1.0 were prepared for three *Candida* strains. Smears of each strain and OD were placed in glass slides and immersed with 4% (wt/vol) paraformaldehyde for 10 min, then they were washed with sterile water and stained with 4′,6-diamidino-2-phenylindole and covered with coverslip for 10 min. Subsequently, coverslips were removed, and glass slide were washed with distilled water to remove the excess of DAPI. Samples were dried and taken to the microscope for visualization.

The smears were mounted with one drop of non-fluorescent immersion oil (Merck) and cells were analysed using a Leica DM LB2 epifluorescence microscope connected to a Leica DFC300 FX camera (Leica Microsystems, Wetzlar, Germany). The optical filter used combination for optimal viewing of stained preparations (Chroma 61,000-V2), consisted of a 545/30 nm excitation filter combined with a dichromatic mirror at 565 nm and suppression filter 610/75. For image capture, Leica Application Suite (LAS) v4.2. using a 100x magnification objective.

For each sample, a total of 15 fields with an area of 6.03×10^{-5} cm^2 were counted and the average was used to calculate the total cells per cm^2.To determine the relation between cell concentration and OD, the number of cells per area was counted and knowing the area of the glass slide, the concentration of cells is determined. This relation was assumed to be the same for the microorganisms used in this work.

The OD used to perform the following tests was between 0.550 and 0.7, which corresponds to a cell concentration between $2.5x10^5$ and $3.5x10^5$ cells/µl, as assess by DAPI (Appendix 1).

3.4 PNA probe optimization

3.4.1 Hybridization on glass slides

To perform the optimization of the PNA probes, hybridization in glass slides procedure was performed according to Guimarães et al. (2007) with some modifications. To optimize the probes, five different temperatures ranging from 51ºC to 59ºC were tested. For all strains, cells from 1-day-old cultures were harvested from YEPD plates, suspended in sterile water, and homogenized by vortexing. Subsequently, smears of each strain were placed in glass slides and immersed in 4% (wt/vol) of paraformaldehyde followed by 50% (vol/vol) ethanol for 10 minutes each and allowed to air-dry. The smears were then covered with 20 µl of hybridization solution containing 200 nM of the PNA probe, 10% (wt/vol) dextran sulfate (Sigma), 10 mM NaCl (Sigma), 30% (vol/vol) formamide (Sigma), 0.1% (wt/vol) sodium pyrophosphate (Sigma), 0.2% (wt/vol) polyvinypyrrolidone (Sigma), 0.2% (wt/vol) Ficol (Sigma), 5 mM disodium EDTA (Sigma), 0.1% (vol/vol) Triton X-100 (Sigma), 50 mM Tris-HCl (pH 7.5, Sigma). Samples were covered with coverslips, placed in moist chambers, and incubated for 1h, for the temperatures mentioned above. Subsequently, the coverslips were removed, and the slides were submerged in a pre-warmed washing solution containing 5 mM Tris base (Sigma), 15 mM NaCl (Sigma), and 1% (vol/vol) Triton X (pH 10; Sigma) and incubated for 30 min. Washing was performed at the hybridization temperature for 30 min, and then samples were dried at the incubator in the dark before being observed under the microscope.

To assess if the cells had autofluorescence, a control test similar to the one described above was performed with hybridization solution without the probe. This assessment is important to confirm that the positive results obtained are due to probe hybridization.

3.4.2 Microscopy visualization

The PNA *Candida* probe has an Alexa488 fluorochrome with maximum excitation/emission wavelength at 490/525 nm; meanwhile *Pichia* probe has an Alexa594 fluorochrome that has a maximum excitation/emission wavelength at 590/617 nm.

The smears were mounted with one drop on non-fluorescent immersion oil (Merck) and visualized using Nikon eclipse Ci with a fluorescent camera DS-Qi2. The filters used were B-2A (excitation: 450-490 nm; emission 505-525 nm) for Alexa488 fluorochrome and TRITC (excitation: 525-555 nm; emission 570-615 nm). The program used to acquire images was Nis-Elements BR V5.02.00, using Nikon E plan 100x/1.25 oil objective.

The parameters used for each probe are specified in table 5 and were maintained constant for all the results.

Table 5. Microscope parameters used for each probe.

	Exposure	***Gain***
Candida probe	200 ms	31.4x
Pichia *probe*	300 ms	57.7x

3.4.3 Quantification of the fluorescence

To measure the fluorescence intensity, ImageJ software was used. The quantification was obtained using an adaptation of a macro called FISHji5, previously published by Sílvia Fontenete et al. (2016b). The adapted macros used to measure fluorescence intensity for *Candida* probe and *Pichia* probe shown in Appendix 2.

3.5 Fluorescent *in situ* hybridization developed on a chip

3.5.1 Microchip design

The design of these microchannels was previously performed and is describe by Ferreira et al. (2017). The microchannels had different geometries and wides. The depth of the channels used were 30 µm. The molds containing the microchannels imprinting were gently provided by Professor João Mário from Porto University.

3.5.2 Fabrication of microchannels

Microchannels fabrication was performed as described by Ferreira et al. (2017) with some modifications. A two-part PDMS kit (Sylgard, USA) was used to create the liquid polymer, the microchips consisted of a PDMS block with the microchannels imprinted on to it and a cover glass to seal the microchannels. The PDMS block consisted in a 1:5 ratio of PDMS with curing agent. To remove air bubbles formed during the mixing, the liquid polymer was placed in a desiccator connected to a vacuum pump until no air bubbles were observed. The liquid PDMS

was poured over the prefabricated mold at room temperature and pre-cured for 20 min at 80 °C. The PDMS blocks were stored at room temperature in a plastic petri dish. Then, the PDMS block with imprinted microchannels was removed from the mold and access points were created using a needle. The bound of the cover glass to the PDMS block was done through oxygen plasma treatment since studies performed by Ferreira et al. (2017) prove it to be more effective.

3.5.3 PNA-FISH in microchannels

Hybridization in glass slides was used to perform all optimization tests. However, to perform the microfluidics experiment the fixation step has to be done in suspension. Hence, the procedure in suspension was performed for three strains, CI, CP and CT, as previously described by Almeida et al. (2010), with some modifications. Briefly, cells from 1-day-old cultures were harvested from YEPD plates, suspended in sterile water, and homogenized by vortexing. Subsequently, 1 ml of cell suspension was pelleted by centrifugation at 10,000 × *g* for 5 min, resuspended in 500 µl of 4% (wt/vol) paraformaldehyde (Sigma), and fixed for 1 h. The fixed cells were rinsed in autoclaved water, resuspended in 500 µl of 50% (vol/vol) ethanol, and incubated for 30 min at −20°C.

The introduction of fluid in the microchannel was performed by a widen inlet hole that allow the introduction of fluids using a micropipette and an outlet connected to a syringe that at a controlled flow rate would pull the fluid. The system used was a neMESYS low pressure syringe pump. One hundred µl of cell suspensions prepared above, followed by 20 µl of hybridization solution with 200 nM PNA probe, where passed through the microchannels at a flow rate of 1 µl/min. Chips where placed in moist chambers, and incubated for 1h at 55ºC. Afterwards, 100 µl of prewarmed washing solution containing 5 mM Tris base (Sigma), 15 mM NaCl (Sigma), and 1% (vol/vol) Triton X (pH 10; Sigma), was passed through the chips and incubated for 30 min. Chips were left to dry in the dark before being visualized under the microscope. The negative control was performed by repeating the procedure above but only passing hybridization solution.

3.5.4 Microscopy visualization

When performing microfluidics, two types of microscopy analyses were done:

1) When cell suspension was being passed through the microchannels to asses if the cells were being retained. The microscope used was Leica DMI 5000M, using 20x and 40x magnificence. Images were acquired using Leica Application Suite (LAS) v4.2.
2) After PNA-FISH procedure to assess if hybridization within the microchannels occurred or not. The microscopy visualization for fluorescent labelled cells was equal to the one described in 3.4.2.

3.6 Statistical analyses

Statistical tests were performed using SPSS. In order to evaluate if the results obtained were significantly different from each other, two tests were used:

- Student's T test: to evaluate if there are significant differences between the stained cells and the control samples (without the probe) for each temperature;
- ANOVA test: to evaluate if differences between the fluorescence intensities obtained for the different hybridization temperatures tested in the samples were statistically significant for each strain.

The statistical analysis results were evaluated based on confidence levels instead of a p-value since it gives more information about the magnitude of the difference and it is also more accepted nowadays (O'Brien and Yi 2016; Rothman 1978). The confidence interval used was 95%. In this case, if the difference between two means is calculated for this CI and zero is within the range, then the $p \geq 0.05$ – not significant (Beukelman and Brunner 2016). Significance is usually divided in four levels Miller (1966):

- $p > 0.05$ – not significant
- $0.05 \geq p < 0.01$ – significant *
- $0.01 \geq p < 0.001$ – very significant **
- $p \leq 0.001$ – extremely significant ***

Both the T-test and ANOVA assume normality of the data. In order to draw reliable and accurate conclusions from data it is important to assure that it follows normal distribution (Ghasemi and Zahediasl 2012). ANOVA, besides assuming normality, it also assumes sphericity. Sphericity, using the Mauchly's Test of Sphericity, needs to be higher than 0.05, otherwise the test will not be considered accurate. If this does not happen, a correction factor needs to be applied. There are two types of correction factors: Greenhouse-Geisser and Huynh-Feldt. If epsilon (ε) is lower than 0.75, Greenhouse-Geisser correction if used , it is higher, Huynh-Feldt correction factor is applied (Laerd 2018).

T-test assumes normality of the data when $p \geq 0.05$ (Ghasemi and Zahediasl 2012). According to Kline (2005) when $p < 0.05$ the data can still be considered normal if the absolute value of Skewness (Sk) and Kurtosis (Ku) are: $Sk < 3$ and $Ku < 8$.

4 Results and discussion

4.1 Optimization of the probes

The starting temperature was selected based on the Tm of the probes which was of around 75 ºC. In a study by S. Fontenete et al. (2016a), the relation between predicted Tm and Th was studied for PNA probes. It concluded that the difference between Tm and Th is around 15 ± 5 ºC. This result does not take into account a correction factor for the formamide concentration, because at that point no correction was available for PNA probes. Formamide is a reagent used in the hybridization solution that acts as a denaturant used to lower the stability of DNA and increasing the exposure surface of the molecule (S. Fontenete et al. 2016a; Genet et al. 2013). For this reason, the starting Th selected was 55 ºC, around 20 ºC lower than the predicted Tm.

4.1.1 *Candida* probe

The initial parameter that was used to compare temperatures was the signal-to-noise ratio. The noise is related with cells autofluorescence that could interfere with the results. To minimize these interferences, a S/N ratio was calculated with the mean fluorescence intensity of positive and negative control results (table 6).

Table 6. Signal-to-noise values of the Candida probe for all five temperatures tested. Highlighted in green is the temperature for which each Candida strain has the highest signal-to-noise ratio.

	51 ºC	53 ºC	55 ºC	57 ºC	59 ºC
C. santamariae	4.795	6.208	3.595	1.511	2.252
C. albicans	2.241	12.065	11.436	1.766	2.117
C. glabrata	9.587	8.011	3.723	1.095	3.124
C. tropicalis	28.413	5.388	3.354	1.351	1.038
C. parapsilosis	6.165	13.688	12.773	6.678	1.272
T. delbrueckii	10.530	10.404	6.169	1.151	1.533
M. caribicca	3.736	2.687	1.813	1.765	1.400
P. caribicca	3.865	3.954	1.437	1.772	1.282
W. anomalus	6.679	2.137	3.725	3.119	3.031
H. osmophila	7.815	12.709	3.138	3.216	2.547
H. opuntiae	5.392	3.833	4.745	4.729	1.969

For every *Candida* strain, the highest signal to noise ratios occurs at temperatures lower than 55 ºC. When the signal-to-noise ratio is higher than three that negative control fluorescence intensity can be easily discriminated from the positive control (Figure 7). A statistics T-test was applied to evaluate if results are statistically significant (appendix 3). As mentioned before, due to the assumptions T-test makes, normality must first be calculated

(Appendix 3). Since data followed normality for all strains, test results can be considered accurate. For the *Candida* strains tested with the *Candida* probe, all strains had statistically significant differences ($p<0.05$) for 51 ºC and extremely significant differences ($p<0.001$) for 53 ºC.

Signal-to-noise ratio above 3 meant that autofluorescence is not relevant, as it can be seen by figure 7.

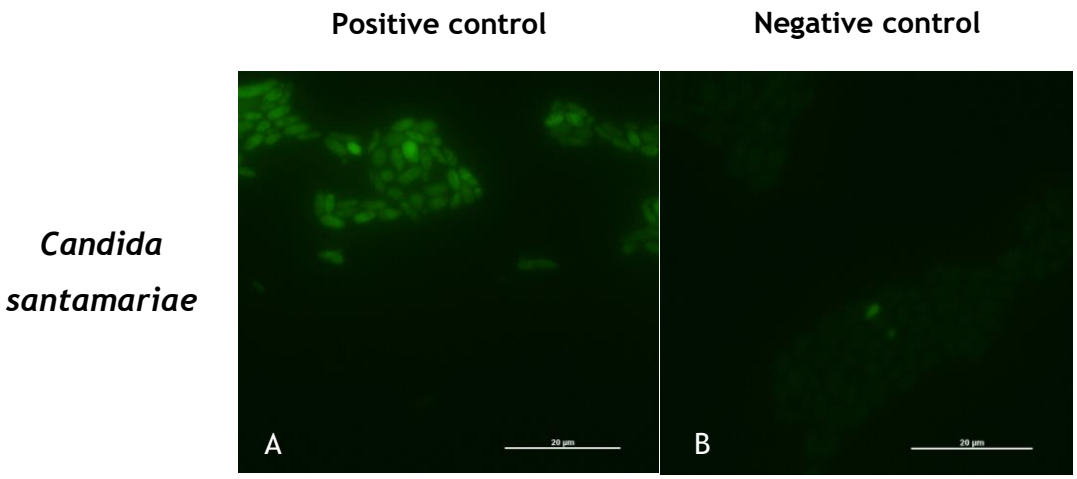


Figure 7. Positive and negative control for Candida santamariae. The autofluorescence signal intensity in the negative control is much lower than in the positive control.

While the *Candida* probe actually detected all *Candida* strains, it also detected non-*Candida* species. In fact, taking into consideration the results in Table 6, 2 out of the 11 strains were not in any way related to *Candida spp.*, *H. osmophila* and *H. opuntiae,* 4 out of 11 were non-*Candida spp.* but related to them, and 5 out of the 11 tested strains are *Candida spp.* The optimum hybridization temperature should be selected in order to decrease the hybridization of the non-*Candida spp.* and increase the hybridization of *Candida spp.* However, analysing exclusively the S/N ratio, there is not one temperature between 51 ºC and 53 ºC that stands out. For 51 ºC, six strains including two *Candida spp.* have the higher S/N while for 53 ºC it is five strains being three of them *Candida spp.*

Being the future application of this probe in an industrial context, a separation between species of *Candida* is not problematic since the main goal is the *Candida* genus. For this reason, *Candida* strains and non-*Candida* strains fluorescence intensity results were combined for each temperature and an overall statistical analysis was calculated with the goal of trying to find if there was a significant difference between the two groups at different temperatures or not. The statistical test used was ANOVA and the data fulfilled all the assumptions of the test. The detailed ANOVA test results $F(3.955)=14.390$, $p=0.000$ for *Candida spp.* are shown in chapter

4.1, appendix 4 and the test results for non-*Candida spp.*, F(2.491)=115.769, p=0.000 are shown in chapter 4.2, Appendix 4.

For *Candida spp.*, the relation between all temperatures, except 51 ºC and 53 ºC, have at least a very significant difference (p<0.01). Comparing the medium and associating these significant differences, 51 ºC and 53 ºC have higher fluorescence intensities confirming what was assessed earlier: optimum hybridization temperature is between 51 ºC and 53 ºC. Between 51ºC and 53ºC, there are no significant differences (p=0.593). For non-*Candida spp.*, temperatures have extremely significant different (p<0.001), except between 51 ºC and 53 ºC (p=0.490).

The selected temperature is 53 ºC because there are no significant differences with 51 ºC and it is the temperature with the highest average. It is also at the temperature non-*Candida spp*. have the lowest fluorescence intensity between 51 ºC and 53 ºC.

As mentioned in chapter 2.1 some yeasts are capable of forming pseudohypae and hyphae. Pseudohyphae and hyphae cells are usually not as permeable as ovulated cells (Romanelli and Wickes 2015). Consequently, in species with formation of these states, penetration of the probe in these cells will not be as good resulting in a lower fluorescence intensity (figure 8). Of the strains tested, the ones exhibiting pseudohyphae or hyphae states are: *Candida santamariae*, *Hanseniaspora osmophila*, *Hanseniaspora opuntiae* and *Candida tropicalis*. The differences in fluorescence intensity for strains with hyphae and pseudohyphae states cause a higher standard deviation as seen in figure 8.

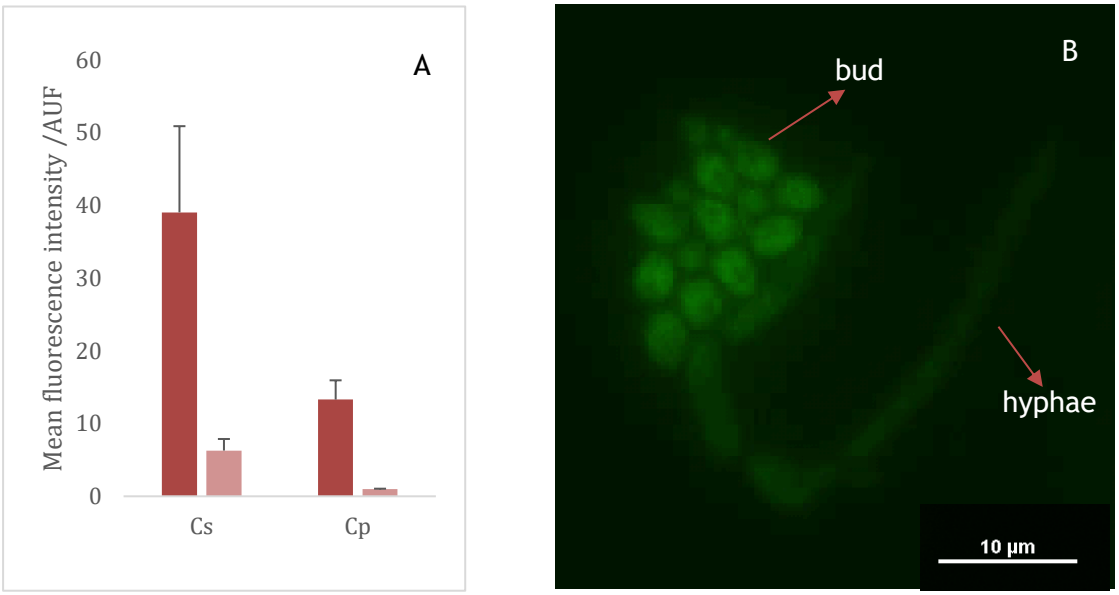


Figure 8. Influence of pseudohuphae/hyphae states in fluorescence hybridization. In figure 7A, mean fluorescence intensity and respective standard deviations at 53ºC for Candida santamariae (Cs) and Candida parapsilopsis (Cp) are plotted. Dark red bar represents probe testing and light red bar represents negative control. Figure 7B is a microscopy image obtained during the work that shows clearly the difference in fluorescence intensity for the two different states.

4.1.2 *Pichia* probe

As in the chapter above, to compare the temperatures the parameter used was signal-to-noise ratio. Results obtained for the quotient between fluorescence intensity of positive and negative controls are shown in table 8.

Table 7. Signal-to-noise values obtained with the Pichia probe for all five temperatures. Highlighted in green is the temperature for which each strain has the highest signal to noise ratio.

	51 ºC	53 ºC	55 ºC	57 ºC	59 ºC
P. fermentans	11.194	11.521	7.581	10.395	10.380
W. anomalus	1.002	0.340	0.497	0.797	1.133
Rhodotorula sp	1.531	0.683	3.928	1.394	0.978

The *P. fermentans* S/N is high and all temperatures had extremely significant differences ($p<0.001$) between positive and negative controls, indicating that autofluorescence is not relevant (figure 9). For *W. anomalus*, $S/N \leq 1$ and there are no significant differences between positive and control results, indicating that fluorescence intensity is similar (figure 9).

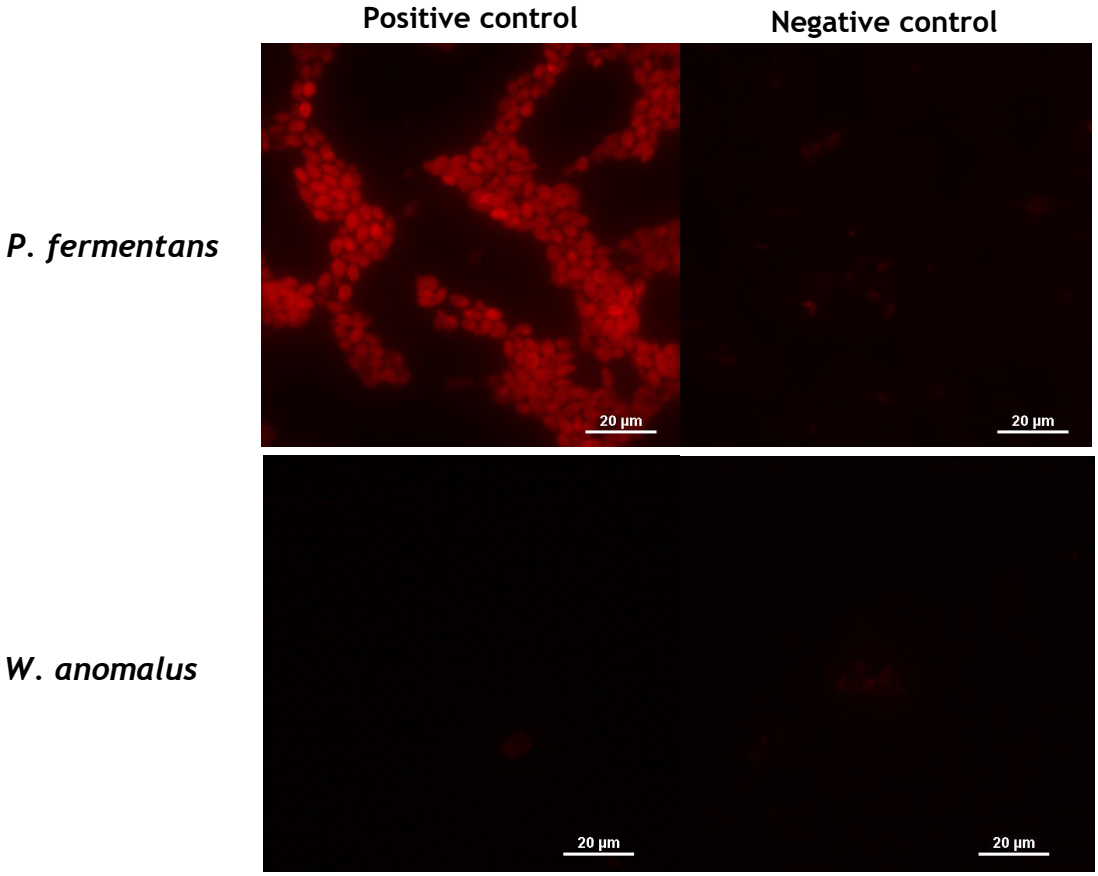


Figure 9. Positive and negative control for P. fermentans and W. anomalus for temperature of 55 ºC. There is difference between intensity of positive and negative control (upper); There is no fluorescence intensity and no difference between positive and negative control (lower).

For the *Pichia* probe, *W. anomalus* was renamed and does not belong to the *Pichia spp*. and it is not detected by the probe as seen in table 7. On the other hand, *Rhodoturola sp*. only has significant differences ($p<0.01$) for two temperatures, 51 ºC and 55 ºC meaning these temperatures should not be used. Meanwhile, *P. fermentans* has for significant differences for all, meaning that a temperature minimizing *Rhodotorula sp*. hybridization would be possible. To avoid detection of *Rhodotorula sp.,* temperature 55 ºC should not be selected, since it is the one with the highest S/N. *Pichia fermentans* has a high S/N for every temperature tested, being the highest for 53 ºC.

In the present case, few strains are available for testing an optimum hybridization temperature. However, since the probe works very well for *Pichia fermentans* an assessment using ANOVA statistics was performed to select the optimum temperature for this strain. All normality assumptions were met and ANOVA $F(1.934)=14.425$, $p=0.000$ results are presented in chapter 4.3, Appendix 4.

For the temperature of 53 ºC there are significant differences ($p<0.05$) with the other temperatures due to its overall fluorescence intensity being lower than the other temperatures. Although it is still a very high fluorescence intensity (38.947 AUF) and it is the one with the lowest interference of autofluorescence. Since *Rhodotorula sp*. does not have significant difference for this temperature and S/N is lower than 1, this temperature was selected since it has the advantage that it could be applied to both probes by the company.

4.2 Identification and complementary of isolates

Identification of the strains performed an important role in this work since optimization and specificity of the probe would be dependent on if the strains represented the microorganism the probes were targeting for. The theoretical specificity and sensitivity of the probes is not 100% meaning that there is a possibility for species that are not the target ones hybridizing with the probes (specificity) and, also, species of interest not hybridizing with the probe (sensitivity).

Identification was performed later in the work. Due to time schedule it was not possible to evaluate all strains for both probes for the optimum temperatures of the probes. For that reason, the results of the species that did not hybridize with either of the probes are for the starting temperature of 55 ºC.

The identification of the strains as well as the result for the probe testing for the starting temperature, are shown in table 8. After identification of the strains, an analysis using BLAST database (NCBI) was performed to several strains (at least 3) of each microorganism to verify

if 18S and 26S rRNA sequences of the identified microorganisms would be complementary to the probes sequences (Table 8).

Table 8. Identification and test results with the probes. (+): hybridization occur, (-): hybridization did not occur; (*): at some temperatures it had fluorescence but not for the optimum one.

Strain	Identification	*Candida* probe	*Pichia* Probe	Complementary sequence to:
SC5414	*Candida albicans*	+	-	*Candida* probe
S34784	*Candida glabrata*	+	-	*Candida* probe
IGG30975	*Candida tropicalis*	+	-	*Candida* probe
571324	*Candida parapsilosis*	+	-	*Candida* probe
CS	*Candida santamariae*	+	-	*Candida* probe
PA	*Wickerhamomyces anomalus*	+	*	Both probes
PF	*Pichia fermentans*	-	+	*Pichia* probe
CI	*Pichia caribbica*	+	-	*Candida* probe
MC	*Meyerozyma caribbica*	+	-	*Candida* probe
TD	*Torulaspora delbrueckii*	+	-	*Candida* probe
R	*Rhodotorula sp.*	-	*	*Pichia* probe
HOP	*Hanseniaspora opuntiae*	+	-	*Candida* probe
HOS	*Hanseniaspora osmophila*	+	-	*Candida* probe
14	FRU14	-	-	-
61	FRU61	-	-	-

Going to the 18S rRNA sequences of the species hybridizing with the *Candida* probe a comparison between these and the probe sequence was performed to potentially determine why the hybridization occurred. For all the strains, the sequences were proven to be complementary. As mentioned in table 1 and described in chapter 2.1, most strains hybridizing with *Candida* probe have an anamorph that belongs to the *Candida spp.*, with only two species of the *Hanseniaspora* genus not having any documented relationship with *Candida spp*. As established above, the anamorph-teleomorph relationship is the same microorganism with different names given to it. For this reason, an evaluation of the database used would be the

first step, to verify if this anamorph-teleomorph relationship were taken in consideration. Moreover, since there is a specificity problem due to hybridization with *Hanseniaspora* a mixture of probes could be used to avoid this, or a new evaluation of the databases could also be performed to redesign the probe this time taken in consideration the anamorph-teleomorph relations.

Pichia probe provided a strong signal with *Pichia fermentans* however, with *Wickerhamomyces anomalus* fluorescence intensity could be seen but with a very low intensity, also creating doubts if it was because of autofluorescence. For *Pichia caribbica* no fluorescence intensity, what would be expected since there is no complementary sequence. This species was also reassigned to a different genus (table 1) what could be the explanation for not having complementary sequence. Besides *P. fermentans,* this probe is expected to work with *Wickerhamomyces anomalus* and *Rhodotorula sp.* since they both have complementary sequences. With *Rhodotorula sp.* is was shown in the chapter above that it is not identified at the optimum temperature 53 ºC, but the strains available in the database it does shown complementary sequence. This could be due to the existence of multiple 18S rRNA copies. Since we do not have the 18S rRNA sequence of the strain tested, it could be different from the ones in the database, not having a complementary sequence and not being so frequently expressed. On the other hand, having complementary sequence is a case of specificity of the probe being lower than 100%.

For the Pichia probe, before deciding for the redesign or mixture of probes more strains have to be tested in order to take more meaningful conclusions. Based on the Villa-Carvajal et al. (2006) study, that show a genetic compatibility on the 26S rRNA, a probe capable of detection both *Pichia spp.* and *Candida spp.* from 26S rRNA could also be investigated.

4.2.1 Current database

From probe check program and using SILVA database v132 (https://www.arb-silva.de/documentation/release-132/) an analysis of current databases with mRNA sequences here tested was performed. Recalculating the specificity with an updated database, it lowered to 94.4%. Although the problem of nomenclature remained. Several non-*Candida spp.* that had complementary sequences with the probe were *Candida* teleomorphs. One solution for this would also pass by redefining what the probe target microorganisms are. Since databases are not yet updated with the “one name=one fungus” guidelines the use of the probe here described could be a possibility if applied to *Candida* and related genera. Moreover, if the probes were to be redesign the guidelines would have to be very defined knowing these relations and a special attention to the databases would be necessary. One problem that was maintained in

the databased used was the fact that over 90% of *Hanseniaspora* strains present in the databased hybridized with the *Candida* probe.

4.3 FISH-on-chip

The introduction of microfluidics in the FISH procedure had two main goals: test if the microchannels were able to retain the yeast cells, and test if the FISH procedure was able to be performed inside the microchannels.

As mentioned above, one of the main advantages of this procedure is the high surface-to-volume ratio that occurs because cells are concentrated in a defined location. If retention is not efficient then the FISH procedure will be affected since the probe and the cells are barely in contact. Microchannels available had different geometries. Geometries of the channels is important for an efficient cell retention since gaps between array of pillars and different pillars conformation influences the retention. Since the microchannels had been previously designed, it was necessary to verify if cells used in this work would be retained and concentrated and for which pillars. Efficiency of cells retention can only be verified after FISH procedure was performed. As seen in figure 10, cells are retained and appear to be concentrated enough for the FISH procedure to occur. Although due to the conformity of the pillars the cells are not retained as they are adhered by the hydrophobic treatment. With the passing of fluids in the microchannel the cells lose adherence and detach, resulting in no bounding of the probe and consequently no fluorescence.

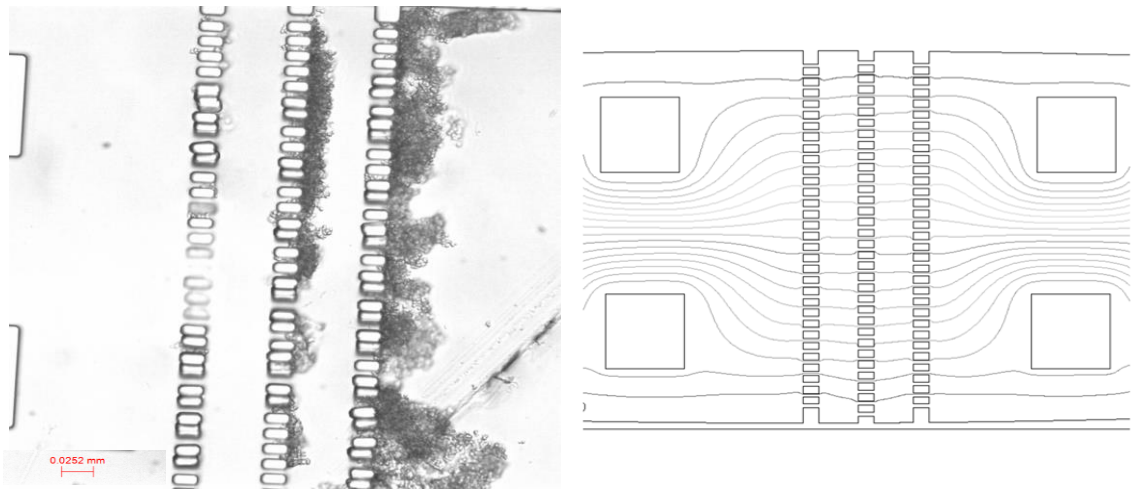


Figure 10. Entrapment of Candida tropicalis cells inside the microchannels. Figure 10A presents the concentration of cells within the microchannels visualized with microscope, while figure 10B is a schematic representation of the channels showing the streamlines (Ferreira et al. 2017).

Also, as mentioned in chapter 2.6.3, flow rate is very important to make sure cells stay retained inside the microchannels. When a flow rate is too high, cells might start passing through the microchannels due to the pressure driven forces being too strong. It is also important to note that cells are not subjected to this flow rate only once but at least three times, to allow all the FISH step procedures: introduction of cells, PNA-probe and washing solution. This means that the flow rate needs to be efficient for trapping to happen but not too high for cells to be able to handle these three steps without escaping. In figure 11 it is possible to see that at higher flow rates, cells do not concentrate and the few that do are able to escape if the flow rate keeps being applied.

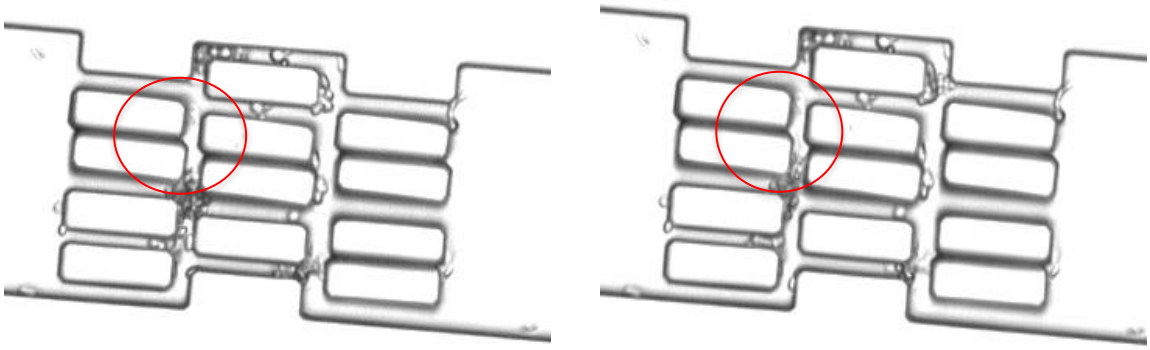


Figure 11. Influence of the flow rate in the retention of cells. Higher flow rates cause the cells to not concentrate and stay retained (left) and the few that do are able to escape with time (right). These images were taken 3 seconds apart using back light microscopy with a 40x magnification objective.

Out of the channels tested the efficiency to retained cells and obtain hybridization was the one shown in figure 11 (right) and this could be because contrary to the one in figure 9, the array of pillars are intercalated between rows which helps entrapment. When the FISH procedure was applied to this microchannels, hybridization with the *Candida* probe occurs, as shown in figure 12 (left). The strains tested were *Candida parapsilopis* and *Candida tropicalis*.

Flow rate used for the cells to stay retained within the microchannels was 1 µL/min during 5 min and it was kept constant for all the steps. Cells concentration was around $2.5x10^5$ cells/µL.

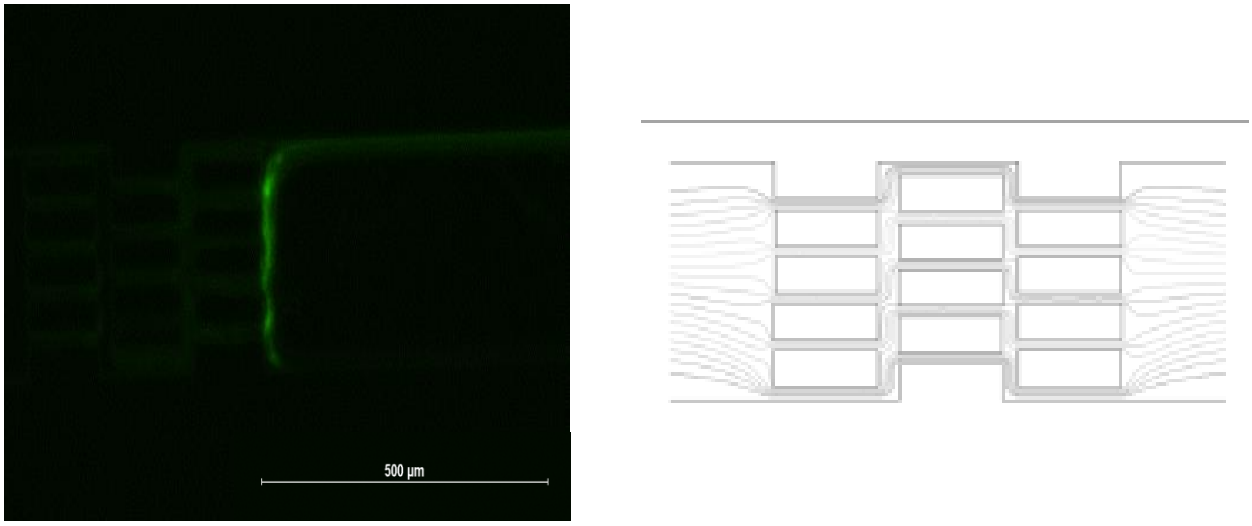


Figure 12. Result obtained after hybridization procedure was performed. Epifluorescence microscopy image where a green line can be seen that represents the hybridized cells retained in the microchannel (Left). Schematic image of the microchannel structure used and streamlines (Ferreira et al. 2017) (Right).

At this temperature, and as shown in chapter 4.2.1, the cells have no autofluorescence meaning that it is indeed fluorescence signal due to hybridization with the probe. To further confirm this, negative controls were performed, and no fluorescence intensity was observed.

One downside observed is that the majority of the cells were being retained before the first array of pillars, and this could be a problem since cell detachment could occur when flow rate is stopped. If cells were within the second or third row, that would be unlikely to happen. Although there were other geometries available, such as the one shown in figure 9, in all cells were retained within the first array of pillars and since retention but in these geometries, cells were not actually get retained since geometry was not the adequate. The cells retention seen in figure 9 is due to the hydrophilic treatment. However, as fluids were pass they lost adhesion and detached from the microchannels being washed away with flow. The channel that proved to be more efficient was the one shown in figure 11.

This problem was encountered during the work being one of the reasons the disconnection of the syringe to be able to visualize cells in an epifluorescence microscope. This change in pressure could lead to cells detachment that consequently lead to no cells being visualized under the microscope. A possible solution for this problem would be the use of an inverted epifluorescence microscope that would allow visualization with the syringe still connected.

The main goal of using microdevices to perform the FISH procedure was to improve the standard method for it to be a viable possibility for the detection of microorganisms in the food industry. With the FISH-on-chips procedure, results were obtained within 3-4h being still the incubation and washing steps the ones taking the longest, 1h30min overall. Not only it was a faster method but also a simpler one and less labour-intensive. Solving the specificity issues with the probes, introduction of a more automated process using microfluidics is possible.

5 Conclusions

The work of this thesis aims at test and optimize probes for the detection of *Candida spp*. and *Pichia spp*. During the work several problems have arisen related with the specificity and sensitivity of the probes due to confusion going on in yeasts taxonomy, in particular caused by anamorph-teleomorph nomenclature. *Candida* probe worked successfully with *Candida spp*. although it also had complementary sequences to several other microorganisms. *Pichia* probe successfully detected *Pichia sp*. although it is necessary more strains.

Concerning the optimization of the FISH procedure, *Candida spp*. had extremely significant differences ($p<0.001$) between positive and negative controls, while *Pichia spp*. had mixed values with *P. fermentans* having extremely significant differences but the other two strains that hybridize with the Pichia probe having very low fluorescence intensity and consequently the results having no significant differences. For temperature optimization, since *Candida* probe hybridized with non-*Candida spp*., an ANOVA statistic was applied for both groups and the temperature more suitable was found to be between 51ºC and 53ºC. The selection of 53ºC as the optimum hybridization temperature was due to mean fluorescence intensity being the highest for *Candida spp*. and the lowest for non-*Candida spp*, as well as having higher S/N ratio within *Candida spp*. For *Pichia* probe, the optimum temperature was based on a *Pichia* strain and a non-*Pichia* strain. Taking into account the results of the non-*Pichia* strain the temperature excluded was 55ºC and using the statistical analyses and S/N results of *Pichia* strain the temperature of 53 ºC was selected since it has a higher S/N ratio, and although it has a lower fluorescence intensity it is still a very higher one and with advantage that does not have as much influence of autofluorescence. It also has the advantage that 53 ºC allows the company to apply both probes at the same time. Because the number of tested microorganism was low (n=3), for the result to be accurate more strains are needed. For the use of these probes the parameter that should be assessed is S/N to minimize detection of undesired microorganisms.

Overall, the FISH method was a rapid method to detect contaminants, in which results were obtained within a working day. Surpassing the adversities with the probe either by redesigning it or mixing several probes to increase specificity, FISH would be a reliable and efficient technique that allowed results in real time operations.

However, the procedure as it is performed is not automated, which is something that would be of great value in a company. The combination of microfluidic devices with FISH procedures allowed for a more automated work with the purpose of being implemented in industry in the future. It was effective both because a sufficient concentration of cells was retained within the microchannels and a successful hybridization was achieved.

6 Assessment of the work done

6.1 Objectives Achieved

The main goal of this work was to test and optimize two PNA probes for the detection of two contaminant microorganisms: *Candida spp.* and *Pichia spp*. For none of the probes a complete optimization was possible due to non-specificity of the probe design for *Candida* probe or due to lack of strains that could validate the results obtained, as it was the case of the *Pichia* probe. Nevertheless, an optimum hybridization temperature was selected for the both probes taking into consideration the limitations they both had.

6.2 Other works carried out

For the context these probes were designed for - application in food industry - it was important to automatize the FISH procedure making it non-dependent of the operator. To achieve this the combination of the FISH procedure with microfluidics devices was attempted. In the first stage of this testing, the operator is still needed but with further work it would be possible to be completely automatized.

6.3 Limitations and future work

Limitation of these works was related with the late identification of the microorganisms which caused a delay in any conclusions that could be taken from the results obtained. Apart from this, no other problems arisen in the course of the work except for the probes designed not being very specific. This was partly related to the anamorph-teleomorph relationship and the interference they might have in the design of the probes as well as their performance. This leads to the future work to be developed. A more thorough assessment of the relationships between *Candida spp.* and *Pichia spp.* is needed, even to understand if the need for two probes is real or one could be the solution to reduce the specificity problem. The use of a single probe could be considered based on phylogenetic studies of the 26S rRNA sequence. Moreover, a simple redesign now taking into consideration these relationships could also help increase the target sequence specificity, or a mixture of probes to help with this issue. Testing of Pichia probe for with other *Pichia spp.* would also be important to verify the performance of the probe.

6.4 Final Assessment

Realization of this work in the university laboratory gave me a different vision of what working in a laboratory really was. The preparation it takes for performing a day of work, materials necessary included, as well as coordination with other people was a different reality from what had been used until now. It also gave me a lot of autonomy and experience on how to find a solution for the small adversities encountered when working in a laboratory. Research skills and fast learning were some of the skills improved with this project, since I had not used a lot of equipment necessary to perform this project. Furthermore, being able to associate this project with a company gave me an idea of how laboratory techniques can be implemented in companies at perspective of larger scale.

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Appendix 1 Cells concentration

Determination of cells concentration using DO was performed using equation below:

$$\text{Cells/cm}^2 = \frac{number\ of\ cells\ x\ glass\ slide\ area\ (cm2)}{microscope\ area\ (cm2) x\ volume\ (\mu l)}$$

Glass slide area = 1,54 cm^2

Microscope area = $6{,}03x10^{-05}$

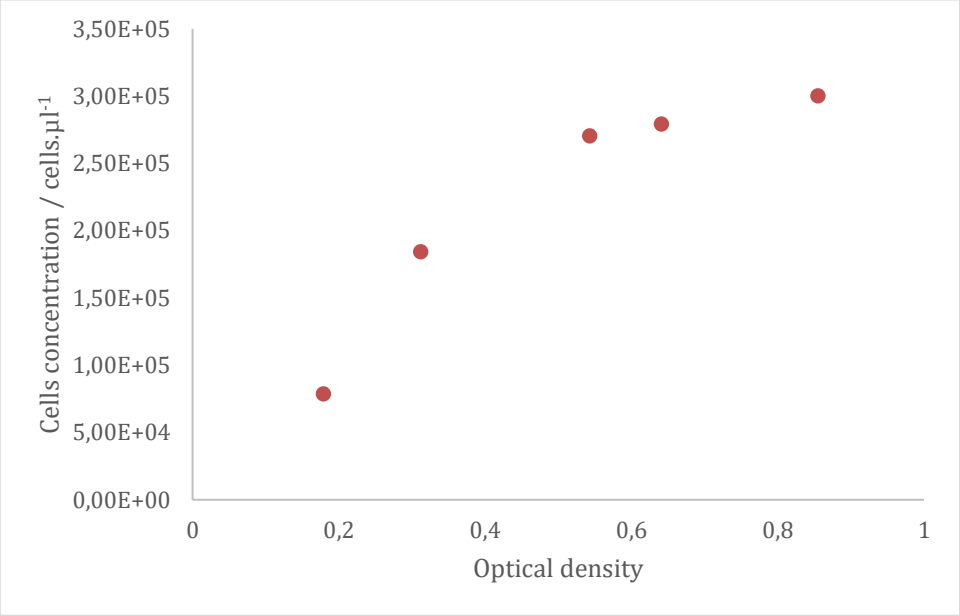

Figure 13. Relation between cells concentration and optical density for three *Candida* spp.

Appendix 2 Macros

2.1 *Candida* probe macro

The macro used for determining mean fluorescence intensity with *Candida* probe was as described below.

```
title=getTitle();
run("Duplicate...", "title=2");
selectImage(title);
run("RGB Split");
selectImage(title+" (blue)");
close();
selectImage(title+" (red)");
close();
selectImage(title+" (green)");
run("Subtract Background...", "rolling=600");
t=getTitle();
run("Duplicate...", "title=1");
 AUTO_THRESHOLD = 5000;
 getRawStatistics(pixcount);
 limit = pixcount/10;
 threshold = pixcount/AUTO_THRESHOLD;
 nBins = 256;
 getHistogram(values, histA, nBins);
 i = -1;
 found = false;
 do {
        counts = histA[++i];
        if (counts > limit) counts = 0;
```

```
        found = counts > threshold;
} while ((!found) && (i < histA.length-1))
hmin = values[i];
i = histA.length;
do {
        counts = histA[--i];
        if (counts > limit) counts = 0;
        found = counts > threshold;
} while ((!found) && (i > 0))
hmax = values[i];
setMinAndMax(hmin, hmax);
//print(hmin, hmax);
run("Apply LUT");
// Segmentation
run("Convolve...", "text1=[0 0 0 -1 -1 -1 0 0 0\n0 -1 -1 -3 -3 -3 -1 -1 0\n0 -1 -3 -3 -1 -3 -3 -1 0\n-1 -3 -3 6 13 6 -3 -3 -1\n-1 -3 -1 13 24 13 -1 -3 -1\n-1 -3 -3 6 13 6 -3 -3 -1\n0 -1 -3 -3 -1 -3 -3 -1 0\n0 -1 -1 -3 -3 -3 -1 -1 0\n0 0 0 -1 -1 -1 0 0 0\n] normalize"); // Convolution Laplacian of Guassian kernel 9x9
run("Make Binary");
run("Fill Holes");
// Analysis
run("Set Measurements...", "  mean redirect=["+t+"] decimal=0");
run("Analyze Particles...", "size=260-infinity show=Nothing add");
selectImage(title+" (green)");
close();
// Statistics
statist=newArray("Mean");
            rf = newArray(statist.length*nResults);
            for (j=0; j<statist.length; j++){
```

```
            for (i=0;i<nResults; i++){
                rf[j+i*statist.length] = getResult(statist[j],i);
            }
        }
// Statistics output
Array.getStatistics(rf, min, max, mean, stdDev);
print("Image: " + title);
print("Mean: " + mean);
print("STD: " + stdDev);
run("Clear Results");
// Overlay output
selectWindow(1);
run("Add Image...", "image=2 x=0 y=0 opacity=100");
selectWindow(2);
close();
```

2.2 *Pichia* probe macro

The macro used for determining mean fluorescence intensity with *Candida* probe was as described below.

```
title=getTitle();
run("Duplicate...", "title=2");
selectImage(title);
run("RGB Split");
selectImage(title+" (blue)");
close();
selectImage(title+" (green)");
close();
selectImage(title+" (red)");
```

```
run("Subtract Background...", "rolling=30");
t=getTitle();
run("Duplicate...", "title=1");
// Auto Threshold Brigthness and Contrast Treatment
 run("Threshold...");
        wait(1000);
        setAutoThreshold("Default dark");
        waitForUser("Threshold", "Please adjust threshold and then click OK")
// Segmentation
run("Convolve...", "text1=[0 0 0 -1 -1 -1 0 0 0\n0 -1 -1 -3 -3 -3 -1 -1 0\n0 -1 -3 -3 -1 -3 -3 -1 0\n-1 -3 -3 6 13 6 -3 -3 -1\n-1 -3 -1 13 24 13 -1 -3 -1\n-1 -3 -3 6 13 6 -3 -3 -1\n0 -1 -3 -3 -1 -3 -3 -1 0\n0 -1 -1 -3 -3 -3 -1 -1 0\n0 0 0 -1 -1 -1 0 0 0\n] normalize"); // Convolution Laplacian of Guassian kernel 9x9
run("Make Binary");
run("Fill Holes");
// Analysis
run("Set Measurements...", "  mean redirect=["+t+"] decimal=0");
run("Analyze Particles...", "size=35-infinity show=Nothing add"); //
selectImage(title+" (red)");
close();
// Statistics
statist=newArray("Mean");
        rf = newArray(statist.length*nResults);
        for (j=0; j<statist.length; j++){
            for (i=0;i<nResults; i++){
                rf[j+i*statist.length] = getResult(statist[j],i);
            }
        }
```

```
// Statistics output
Array.getStatistics(rf, min, max, mean, stdDev);
print("Image: " + title);
print("Mean: " + mean);
print("STD: " + stdDev);
run("Clear Results");
// Overlay output
selectWindow(1);
run("Add Image...", "image=2 x=0 y=0 opacity=100");
selectWindow(2);
close();
```

Appendix 3 T-test statistics

3.1 *Candida* spp.

Candida albicans

Table 9. Normality test for strain Candida albicans. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 °C	Control	0.263	5	.200*	0.828	5	0.133
	Probe	0.208	5	.200*	0.946	5	0.711
53 °C	Control	0.473	5	0.001	0.552	5	0.000
	Probe	0.338	5	0.064	0.801	5	0.083
55 °C	Control	0.473	5	0.001	0.552	5	0.000
	Probe	0.346	5	0.050	0.849	5	0.191
57 °C	Control	0.367	5	0.026	0.694	5	0.008
	Probe	0.287	5	.200*	0.877	5	0.296
59 °C	Control	0.324	5	0.094	0.811	5	0.099
	Probe	0.182	5	.200*	0.962	5	0.822
	a. Lilliefors Significance Correction						

Table 10. Descriptive of Sk and Ku parameters for strain Candida albicans to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 °C	Control	-0.398	0.913	-3.005	2.000
	Probe	0.691	0.913	-0.854	2.000
53 °C	Control	2.236	0.913	5.000	2.000
	Probe	-1.127	0.913	-0.366	2.000
55 °C	Control	2.236	0.913	5.000	2.000
	Probe	-1.549	0.913	3.109	2.000
57 °C	Control	0.610	0.913	-3.323	2.000
	Probe	-1.154	0.913	0.280	2.000
59 °C	Control	-1.797	0.913	3.501	2.000
	Probe	0.859	0.913	0.915	2.000

Candida glabrata

Table 11. Normality test for strain Candida glabrata. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.492	6	0.000	0.496	6	0.000
	Probe	0.281	6	0.152	0.778	6	0.037
53 ºC	Control	0.492	6	0.000	0.496	6	0.000
	Probe	0.301	6	0.096	0.854	6	0.170
55 ºC	Control	0.406	6	0.003	0.681	6	0.004
	Probe	0.244	6	.200*	0.875	6	0.247
57 ºC	Control	0.296	6	0.108	0.764	6	0.027
	Probe	0.342	6	0.027	0.816	6	0.082
59 ºC	Control	0.492	6	0.000	0.496	6	0.000
	Probe	0.281	6	0.152	0.778	6	0.037
	a. Lilliefors Significance Correction						

Table 12. Descriptive of Sk and Ku parameters for strain Candida glabrata to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	2.449	0.845	6.000	1.741
	Probe	0.837	0.845	-1.884	1.741
53 ºC	Control	2.449	0.845	6.000	1.741
	Probe	1.026	0.845	-0.621	1.741
55 ºC	Control	1.031	0.845	-1.500	1.741
	Probe	-1.096	0.845	0.293	1.741
57 ºC	Control	-1.977	0.845	4.100	1.741
	Probe	-1.780	0.845	3.841	1.741
59 ºC	Control	0.167	0.845	-2.850	1.741
	Probe	0.127	0.845	1.774	1.741

Candida tropicalis

Table 13. Normality test for strain Candida tropicalis. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.504	13	0.000	0.464	13	0.000
	Probe	0.224	13	0.073	0.878	13	0.067
53 ºC	Control	0.390	13	0.000	0.676	13	0.000
	Probe	0.322	13	0.001	0.784	13	0.004
55 ºC	Control	0.356	13	0.000	0.617	13	0.000
	Probe	0.140	13	0.200*	0.924	13	0.283
57 ºC	Control	0.292	13	0.003	0.783	13	0.004
	Probe	0.116	13	0.200*	0.959	13	0.740
59 ºC	Control	0.214	13	0.107	0.796	13	0.006
	Probe	0.210	13	0.120	0.934	13	0.382
	a. Lilliefors Significance Correction						

Table 14. Descriptive of Sk and Ku parameters for strain Candida glabrata to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	2.276	0.616	3.960	1.191
	Probe	0.199	0.616	-1.817	1.191
53 ºC	Control	0.592	0.616	-1.897	1.191
	Probe	-1.588	0.616	1.955	1.191
55 ºC	Control	-2.018	0.616	2.784	1.191
	Probe	0.212	0.616	-1.429	1.191
57 ºC	Control	-1.979	0.616	6.046	1.191
	Probe	0.096	0.616	-1.018	1.191
59 ºC	Control	-1.048	0.616	-0.497	1.191
	Probe	-0.353	0.616	-0.861	1.191

Candida parapsilopis

Table 15. Normality test for strain Candida parapsilopis. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.473	5	0.001	0.552	5	0.000
	Probe	0.215	5	.200*	0.934	5	0.627
53 ºC	Control	0.473	5	0.001	0.552	5	0.000
	Probe	0.192	5	.200*	0.900	5	0.409
55 ºC	Control	0.473	5	0.001	0.552	5	0.000
	Probe	0.246	5	.200*	0.810	5	0.097
57 ºC	Control	0.473	5	0.001	0.552	5	0.000
	Probe	0.151	5	.200*	0.973	5	0.893
59 ºC	Control	0.257	5	.200*	0.825	5	0.127
	Probe	0.224	5	.200*	0.929	5	0.587
	a. Lilliefors Significance Correction						

Table 16. Descriptive of Sk and Ku parameters for strain Candida parapsilopis to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	2.236	0.913	5.000	2.000
	Probe	0.019	0.913	-2.107	2.000
53 ºC	Control	2.236	0.913	5.000	2.000
	Probe	1.044	0.913	0.502	2.000
55 ºC	Control	2.236	0.913	5.000	2.000
	Probe	-0.436	0.913	-3.113	2.000
57 ºC	Control	2.236	0.913	5.000	2.000
	Probe	-0.602	0.913	-0.189	2.000
59 ºC	Control	-0.379	0.913	-3.057	2.000
	Probe	-0.954	0.913	1.750	2.000

Candida santamariae

Table 17. Normality test for strain Candida santamariae. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.298	7	0.061	0.858	7	0.144
	Probe	0.195	7	.200*	0.936	7	0.603
53 ºC	Control	0.301	7	0.055	0.756	7	0.015
	Probe	0.288	7	0.082	0.794	7	0.035
55 ºC	Control	0.376	7	0.003	0.674	7	0.002
	Probe	0.249	7	.200*	0.878	7	0.219
57 ºC	Control	0.254	7	0.192	0.877	7	0.215
	Probe	0.225	7	.200*	0.831	7	0.081
59 ºC	Control	0.292	7	0.071	0.722	7	0.006
	Probe	0.171	7	.200*	0.926	7	0.520
	a. Lilliefors Significance Correction						

Table 18. Descriptive of Sk and Ku parameters for strain Candida santamariae to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	-1.475	0.794	2.124	1.587
	Probe	-0.368	0.794	-1.492	1.587
53 ºC	Control	-1.985	0.794	4.395	1.587
	Probe	1.195	0.794	-0.296	1.587
55 ºC	Control	-2.355	0.794	5.859	1.587
	Probe	-0.918	0.794	-0.444	1.587
57 ºC	Control	1.130	0.794	0.513	1.587
	Probe	0.333	0.794	-2.399	1.587
59 ºC	Control	2.186	0.794	5.074	1.587
	Probe	-0.059	0.794	-1.560	1.587

Meyerozyma caribbica

Table 19. Normality test for strain Meyerozyma caribbica. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.167	14	0.200*	0.958	14	0.698
	Probe	0.348	14	0.000	0.728	14	0.001
53 ºC	Control	0.170	14	0.200*	0.915	14	0.184
	Probe	0.123	14	0.200*	0.963	14	0.768
55 ºC	Control	0.231	14	0.042	0.911	14	0.162
	Probe	0.336	14	0.000	0.676	14	0.000
57 ºC	Control	0.298	14	0.001	0.781	14	0.003
	Probe	0.152	14	0.200*	0.950	14	0.560
59 ºC	Control	0.207	14	0.106	0.944	14	0.474
	Probe	0.315	14	0.001	0.825	14	0.010
	a. Lilliefors Significance Correction						

Table 20. Descriptive of Sk and Ku parameters for strain Meyerozyma caribbica to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	-1.016	0.597	-0.959	1.154
	Probe	-0.137	0.597	-0.894	1.154
53 ºC	Control	-0.547	0.597	-0.448	1.154
	Probe	0.482	0.597	0.175	1.154
55 ºC	Control	0.841	0.597	-0.098	1.154
	Probe	2.662	0.597	7.992	1.154
57 ºC	Control	1.576	0.597	1.821	1.154
	Probe	-0.070	0.597	1.179	1.154
59 ºC	Control	-0.701	0.597	0.781	1.154
	Probe	1.477	0.597	2.118	1.154

Wickerhamomyces anomalus

Table 21. Normality test for strain Wickerhamomyces anomalus. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.135	13	.200*	0.943	13	0.493
	Probe	0.128	13	.200*	0.941	13	0.472
53 ºC	Control	0.165	13	.200*	0.948	13	0.571
	Probe	0.157	13	.200*	0.966	13	0.838
55 ºC	Control	0.127	13	.200*	0.958	13	0.721
	Probe	0.117	13	.200*	0.979	13	0.972
57 ºC	Control	0.222	13	0.080	0.750	13	0.002
	Probe	0.155	13	.200*	0.953	13	0.649
59 ºC	Control	0.121	13	.200*	0.968	13	0.874
	Probe	0.127	13	.200*	0.978	13	0.966
	a. Lilliefors Significance Correction						

Table 22. Descriptive of Sk and Ku parameters for strain Wickerhamomyces anomalus to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	-0.068	0.616	-1.346	1.191
	Probe	0.446	0.616	-0.798	1.191
53 ºC	Control	0.170	0.616	1.986	1.191
	Probe	0.346	0.616	-0.372	1.191
55 ºC	Control	0.614	0.616	0.144	1.191
	Probe	-0.008	0.616	0.428	1.191
57 ºC	Control	2.380	0.616	6.955	1.191
	Probe	0.490	0.616	0.211	1.191
59 ºC	Control	0.475	0.616	0.634	1.191
	Probe	0.235	0.616	0.705	1.191

Pichia caribbica

Table 23. Normality test for strain Pichia caribbica. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0,208	12	0,162	0,814	12	0,013
	Probe	0,206	12	0,169	0,873	12	0,071
53 ºC	Control	0,401	12	0,000	0,703	12	0,001
	Probe	0,233	12	0,071	0,851	12	0,038
55 ºC	Control	0,155	12	,200*	0,960	12	0,781
	Probe	0,147	12	,200*	0,932	12	0,397
57 ºC	Control	0,320	12	0,001	0,600	12	0,000
	Probe	0,213	12	0,138	0,904	12	0,179
59 ºC	Control	0,159	12	,200*	0,925	12	0,327
	Probe	0,158	12	,200*	0,941	12	0,505
	a. Lilliefors Significance Correction						

Table 24. Descriptive of Sk and Ku parameters for strain Pichia caribbica to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	-1.947	0.637	4.794	1.232
	Probe	-1.497	0.637	2.569	1.232
53 ºC	Control	-1.729	0.637	1.986	1.232
	Probe	0.598	0.637	-1.279	1.232
55 ºC	Control	-0.056	0.637	-0.229	1.232
	Probe	0.854	0.637	0.311	1.232
57 ºC	Control	-1.947	0.637	4.794	1.232
	Probe	-1.497	0.637	2.569	1.232
59 ºC	Control	-1.729	0.637	1.986	1.232
	Probe	0.598	0.637	-1.279	1.232

Hanseniaspora osmophila

Table 25. Normality test for strain Hanseniaspora osmophila. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.347	12	0.000	0.692	12	0.001
	Probe	0.149	12	0.200*	0.898	12	0.151
53 ºC	Control	0.180	12	0.200*	0.910	12	0.215
	Probe	0.205	12	0.176	0.925	12	0.326
55 ºC	Control	0.212	12	0.143	0.898	12	0.151
	Probe	0.192	12	0.200*	0.947	12	0.591
57 ºC	Control	0.185	12	0.200*	0.904	12	0.180
	Probe	0.158	12	0.200*	0.966	12	0.861
59 ºC	Control	0.166	13	0.200*	0.955	13	0.681
	Probe	0.187	13	0.200*	0.928	13	0.317
	a. Lilliefors Significance Correction						

Table 26. Descriptive of Sk and Ku parameters for strain Hanseniaspora osmophila to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	1.302	0.637	-0.169	1.232
	Probe	-0.374	0.637	-1.522	1.232
53 ºC	Control	0.440	0.637	-1.238	1.232
	Probe	-0.652	0.637	2.093	1.232
55 ºC	Control	-1.277	0.637	1.893	1.232
	Probe	0.613	0.637	-0.510	1.232
57 ºC	Control	1.074	0.637	0.732	1.232
	Probe	-0.392	0.637	-0.423	1.232
59 ºC	Control	1.302	0.637	-0.169	1.232
	Probe	-0.374	0.637	-1.522	1.232

Torulaspora delbrueckii

Table 27. Normality test for strain Torulasppra delbrueckii. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.420	13	0.000	0.613	13	0.000
	Probe	0.120	13	0.200*	0.983	13	0.990
53 ºC	Control	0.388	13	0.000	0.687	13	0.000
	Probe	0.166	13	0.200*	0.906	13	0.160
55 ºC	Control	0.320	13	0.001	0.740	13	0.001
	Probe	0.149	13	0.200*	0.956	13	0.686
57 ºC	Control	0.173	13	0.200*	0.872	13	0.055
	Probe	0.213	13	0.112	0.884	13	0.082
59 ºC	Control	0.166	13	0.200*	0.955	13	0.681
	Probe	0.187	13	0.200*	0.928	13	0.317
	a. Lilliefors Significance Correction						

Table 28. Descriptive of Sk and Ku parameters for strain Torulaspora delbrueckii to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	1.450	0.616	0.168	1.191
	Probe	0.272	0.616	-0.062	1.191
53 ºC	Control	0.952	0.616	-1.229	1.191
	Probe	0.273	0.616	-1.492	1.191
55 ºC	Control	0.722	0.616	-1.405	1.191
	Probe	-0.001	0.616	-1.158	1.191
57 ºC	Control	0.862	0.616	-0.617	1.191
	Probe	0.780	0.616	-0.321	1.191
59 ºC	Control	0.504	0.616	0.634	1.191
	Probe	-1.111	0.616	1.759	1.191

Hanseniaspora opuntiae

Table 29. Normality test for strain Hanseniaspora opuntiae. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.242	10	0.099	0.870	10	0.101
	Probe	0.145	10	0.200*	0.930	10	0.452
53 ºC	Control	0.155	10	0.200*	0.963	10	0.819
	Probe	0.256	10	0.063	0.881	10	0.133
55 ºC	Control	0.259	10	0.057	0.881	10	0.133
	Probe	0.122	10	0.200*	0.983	10	0.981
57 ºC	Control	0.392	10	0.000	0.722	10	0.002
	Probe	0.215	10	0.200*	0.939	10	0.541
59 ºC	Control	0.219	10	0.192	0.869	10	0.097
	Probe	0.149	10	0.200*	0.978	10	0.954
	a. Lilliefors Significance Correction						

Table 30. Descriptive of Sk and Ku parameters for strain Hanseniaspora opuntiae to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	-1.290	0.687	1.568	1.334
	Probe	1.083	0.687	1.515	1.334
53 ºC	Control	-0.435	0.687	0.118	1.334
	Probe	1.350	0.687	2.231	1.334
55 ºC	Control	1.327	0.687	3.538	1.334
	Probe	0.344	0.687	0.090	1.334
57 ºC	Control	1.255	0.687	1.255	0.687
	Probe	-0.235	0.687	-1.048	1.334
59 ºC	Control	1.055	0.687	0.215	1.334
	Probe	0.073	0.687	1.092	1.334

Table 31. P-values from T-test statistical analyses to compare between with and without the probe fluorescence intensities for the Candida spp. for the five temperatures, for all the strains. P-value are according to least significant difference (LSD). Values need to be below 0.05 to be consider significant.

	51 ºC	53 ºC	55 ºC	57 ºC	59 ºC
C. santamariae	0,000***	0,000***	0,000***	0,000***	0,000***
C. albicans	0,000***	0,000***	0,000***	0,038*	0,000***
C. glabrata	0,000***	0,000***	0,000***	0,000***	0,000***
C. tropicalis	0,000***	0,000***	0,000***	0,000***	0,028*
C. parapsilosis	0,000***	0,000***	0,004**	0,000***	0,014*
T. delbrueckii	0,000***	0,000***	0,000***	0,000***	0,000***
H. osmophila	0,000***	0,000***	0,000***	0,000***	0,000***
H. opuntiae	0,036*	0,001**	0,002**	0,001**	0,002**
M. caribicca	0,005**	0,006**	0,000***	0,304	0,000***
P. caribicca	0,000***	0,000***	0,000***	0,000***	0,562
W. anomalus	0,000***	0,001**	0,000***	0,001**	0,102

3.2 *Pichia spp.*

Pichia fermentans

Table 32. Normality test for strain Pichia fermentans. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.309	12	0.002	0.665	12	0.000
	Probe	0.179	12	0.200*	0.900	12	0.161
53 ºC	Control	0.303	12	0.003	0.752	12	0.003
	Probe	0.170	12	0.200*	0.964	12	0.833
55 ºC	Control	0.250	12	0.037	0.779	12	0.006
	Probe	0.249	12	0.038	0.865	12	0.057
57 ºC	Control	0.200	12	0.200*	0.936	12	0.454
	Probe	0.214	12	0.134	0.905	12	0.182
59 ºC	Control	0.169	12	0.200*	0.934	12	0.422
	Probe	0.276	12	0.012	0.725	12	0.001
	a. Lilliefors Significance Correction						

Table 33. Descriptive of Sk and Ku parameters for strain Pichia fermentans to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	2.475	0.637	6.664	1.232
	Probe	-0.776	0.637	-0.664	1.232
53 ºC	Control	-0.719	0.637	-1.620	1.232
	Probe	-0.017	0.637	-1.080	1.232
55 ºC	Control	1.861	0.637	4.105	1.232
	Probe	-0.821	0.637	-0.660	1.232
57 ºC	Control	0.498	0.637	0.919	1.232
	Probe	-1.074	0.637	1.298	1.232
59 ºC	Control	0.857	0.637	1.165	1.232
	Probe	-2.459	0.637	7.205	1.232

Wickerhamomyces anomalus

Table 34. Normality test for strain *Wickerhamomyces anomalus*. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.333	6	0.036	0.792	6	0.049
	Probe	0.424	6	0.001	0.644	6	0.001
53 ºC	Control	0.194	6	0.200*	0.923	6	0.530
	Probe	0.432	6	0.001	0.589	6	0.000
55 ºC	Control	0.267	6	0.200*	0.870	6	0.225
	Probe	0.227	6	0.200*	0.872	6	0.232
57 ºC	Control	0.232	6	0.200*	0.894	6	0.342
	Probe	0.368	6	0.011	0.739	6	0.015
59 ºC	Control	0.204	6	0.200*	0.921	6	0.512
	Probe	0.259	6	0.200*	0.901	6	0.380
	a. Lilliefors Significance Correction						

Table 35. Descriptive of Sk and Ku parameters for strain Wickerhamomyces anomalus to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

		Sk	Std. error	Ku	Std. error
51 ºC	Control	0.982	0.845	-1.370	1.741
	Probe	2.324	0.845	5.544	1.741
53 ºC	Control	-0.214	0.845	-1.318	1.741
	Probe	-2.398	0.845	5.801	1.741
55 ºC	Control	0.541	0.845	-1.365	1.741
	Probe	1.465	0.845	2.209	1.741
57 ºC	Control	0.534	0.845	-1.580	1.741
	Probe	1.395	0.845	0.673	1.741
59 ºC	Control	0.881	0.845	0.425	1.741
	Probe	0.572	0.845	-1.424	1.741

Rhodotorula sp.

Table 36. Normality test for strain *Rhodotorula sp.* Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistics	df	Sig.	Statistics	df	Sig.
51 ºC	Control	0.262	12	0.022	0.807	12	0.011
	Probe	0.192	12	.200*	0.911	12	0.219
53 ºC	Control	0.407	12	0.000	0.563	12	0.000
	Probe	0.275	12	0.013	0.763	12	0.004
55 ºC	Control	0.449	12	0.000	0.456	12	0.000
	Probe	0.243	12	0.048	0.777	12	0.005
57 ºC	Control	0.327	12	0.001	0.681	12	0.001
	Probe	0.515	12	0.000	0.359	12	0.000
59 ºC	Control	0.107	12	.200*	0.945	12	0.565
	Probe	0.134	12	.200*	0.980	12	0.983
	a. Lilliefors Significance Correction						

Table 37. Descriptive of Sk and Ku parameters for strain Rhodotorula sp. *to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.*

		Sk	Std. error	Ku	Std. error
51 ºC	Control	-1.707	0.647	5.421	1.232
	Probe	0.666	0.647	0.478	1.232
53 ºC	Control	3.062	0.647	7.135	1.232
	Probe	-2.269	0.647	6.701	1.232
55 ºC	Control	3.349	0.647	7.427	1.232
	Probe	1.554	0.647	1.543	1.232
57 ºC	Control	1.928	0.647	2.696	1.232
	Probe	3.456	0.647	6.959	1.232
59 ºC	Control	0.473	0.647	-0.045	1.232
	Probe	0.104	0.647	-0.520	1.232

Table 38. P-values from T-test statistical analyses to compare between with and without the probe fluorescence intensities for strains hybridizing with Pichia probe for the five temperatures. P-value are according to least significant difference (LSD). Values need to be below 0.05 to be consider significant.

	51 ºC	**53 ºC**	**55 ºC**	**57 ºC**	**59 ºC**
P. fermentans	0.000***	0.000***	0.000***	0.000***	0.000***
Rhodotorula sp.	0.001**	0.384	0.003**	0.671	0.707
W. anomalus	0.781	0.000***	0.065	0.540	0.709

Appendix 4 ANOVA statistics

4.1 Candida spp.

Table 39. Mean fluorescence intensities for positive controls of Candida spp. for each temperature.

Temperature	Mean	Std. Deviation	Lower bound	Upper bound
51 ºC	16.107	1.191	13.731	18.484
53 ºC	16.824	1.650	13.531	20.118
55 ºC	11.703	0.588	10.529	12.877
57 ºC	6.307	0.285	5.738	6.876
59º C	7.503	0.466	6.572	8.434

Table 40. Normality test for Candida spp. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistics	df	Sig.	Statistics	df	Sig.
51ºC	0.309	12	0.002	0.665	12	0.000
53ºC	0.303	12	0.003	0.752	12	0.003
55ºC	0.250	12	0.037	0.779	12	0.006
57ºC	0.200	12	0.200*	0.936	12	0.454
59ºC	0.169	12	0.200*	0.934	12	0.422

a. Lilliefors Significance Correction

Table 41. Descriptive of Sk and Ku parameters for Candida spp. to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

	Sk	Std. error	Ku	Std. error
51ºC	**0.449**	0.291	**-1.322**	0.574
53ºC	**1.258**	0.291	**0.088**	0.574
55ºC	**1.099**	0.291	**0.966**	0.574
57ºC	0.574	0.291	2.194	0.574
59ºC	0.785	0.291	-0.073	0.574

Table 42. Sphericity calculated by Mauchly's W test for Candida spp. Sig. needs to be above 0.05 or epsilon needs to be below 0.75 for Greenhouse-Geisser correction to be used.

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b]		
					Greenhouse-Geisser	Huynh-Feldt	Lower bound
Temperature	0.057	187.223	9	0.000	0.519	0.536	0.250

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.

a. Design: Intercept Within Subjects Design: Temperature

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Table 43. Corrections factors results for ANOVA statistcs for Candida spp.

Source		Type III Sum of Squares	Df	Mean Square	F	Sig.
Temperature	Sphericity Assumed	16552.610	5	3310.522	14.390	0.000
	Greenhouse-Geisser	**16552.610**	**3.955**	**4185.686**	**14.390**	**0.000**
	Huynh-Feldt	16552.610	4.329	3824.064	14.390	0.000
	Lower-bound	16552.610	1.000	16552.610	14.390	0.000

Table 44. ANOVA test for the comparison between different temperatures of hybridization for Candida strains. Mean (μ), standard deviation (σ) and p values according to the Least Significant Difference (LSD). Values need to be below 0.05 to be consider significant.

	51ºC μ=16.107 σ=1.191	53ºC μ=16.824 σ =1.650	55ºC μ=11.703 σ =0.588	57ºC μ=6.307 σ=0.285	59ºC μ=7.503 σ=0.466
51ºC					
53ºC	0.593				
55ºC	0.000***	0.001**			
57ºC	0.000***	0.000***	0.000***		
59ºC	0.000***	0.000***	0.000***	0.001**	

4.2 Non-Candida spp.

Table 45. Mean fluorescence intensities for positive controls of non-Candida spp. for each temperature.

Temperature	Mean	Std. Deviation	Lower bound	Upper bound
51 ºC	31.014	1.203	28.625	33.403
53 ºC	27.912	1.477	24.980	30.845
55 ºC	13.251	0.736	11.790	14.712
57 ºC	13.157	0.535	12.095	14.220
59º C	10.428	0.425	9.583	11.272

Table 46. Normality test for Candida spp. Sig. in Shapiro-Wilk test. Sig. needs to be above 0.05 for the data to be consider normal. Highlight in red are the values that do not follow this requisite.

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistics	df	Sig.	Statistics	df	Sig.
51ºC	0,131	96	0,000	0,872	96	0,000
53ºC	0,126	96	0,001	0,936	96	0,000
55ºC	0,167	96	0,000	0,865	96	0,000
57ºC	0,111	96	0,006	0,958	96	0,003
59ºC	0,145	96	0,000	0,911	96	0,000

a. Lilliefors Significance Correction

Table 47. Descriptive of Sk and Ku parameters for non-Candida spp. to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

	Sk	Std. error	Ku	Std. error
51ºC	1.667	0.246	4.460	0.488
53ºC	0.573	0.246	-0.631	0.488
55ºC	1.440	0.246	2.277	0.488
57ºC	0.303	0.246	-0.924	0.488
59ºC	1.028	0.246	0.544	0.488

Table 48. Sphericity calculated by Mauchly's W test for non-Candida spp. Sig. needs to be above 0.05 or epsilon needs to be below 0.75 for Greenhouse-Geisser correction to be used.

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b]		
					Greenhouse-Geisser	Huynh-Feldt	Lower bound
Temperature	0.166	167.572	9	0.000	0.623	0.641	

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.

a. Design: Intercept Within Subjects Design: Temperature

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Table 49. Corrections factors results for ANOVA statistcs for non-Candida spp.

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Temperature	Sphericity Assumed	34974.338	4	8743.584	115.769	0.000
	Greenhouse-Geisser	34974.338	2.491	14042.311	115.769	0.000
	Huynh-Feldt	34974.338	2.563	13645.863	115.769	0.000
	Lower-bound	34974.338	1.000	34974.338	115.769	0.000

Table 50. ANOVA test for the comparison between different temperatures of hybridization for non-Candida strains. Mean (μ), standard deviation (σ) and p values according to the Least Significant Difference (LSD). For significant differences values need to be below 0.05.

	51ºC μ=31.0.14 δ=1.203	53ºC μ=27.912 δ=1.477	55ºC μ=13.251 δ=0.736	57ºC μ=13.157 δ=0.535	59ºC μ=10.428 δ=0.425
51ºC					
53ºC	0.490				
55ºC	0.000***	0.000***			
57ºC	0.000***	0.000***	0.903		
59ºC	0.000***	0.000***	0.000***	0.000***	

4.3 Pichia spp.

Table 51. Mean fluorescence intensities for positive controls of P. fermentans for each temperature.

Temperature	Mean	Std. Deviation	Lower bound	Upper bound
51 ºC	51.809	1.825	13.731	18.484
53 ºC	38.947	1.520	13.531	20.118
55 ºC	42.505	4.603	10.529	12.877
57 ºC	53.715	2.042	5.738	6.876
59º C	53.993	1.152	6.572	8.434

Table 52. Normality test for Candida spp. Sig. in Shapiro-Wilk test needs to be above 0.05 for the data to be consider normal. Sig. needs to be above 0.05 to be consider significant. Highlight in red are the values that do not follow this requisite.

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistics	df	Sig.	Statistics	df	Sig.
51ºC	0.309	12	0.002	0.665	12	0.000
53ºC	0.179	12	0.200*	0.900	12	0.161
55ºC	0.303	12	0.003	0.752	12	0.003
57ºC	0.170	12	0.200*	0.964	12	0.833
59ºC	0.250	12	0.037	0.779	12	0.006

a. Lilliefors Significance Correction

Table 53. Descriptive of Sk and Ku parameters for P. fermentans to evaluate if data assumes normality. Sk needs to be below 3 and Ku needs to be below 8.

	Sk	Std. error	Ku	Std. error
51ºC	-0.776	0.637	-0.644	1.232
53ºC	-0.170	0.637	-1.080	1.232
55ºC	-0.821	0.637	-0.660	1.232
57ºC	-1.074	0.637	1.298	1.232
59ºC	-2.459	0.637	7.205	1.232

Table 54. Sphericity calculated by Mauchly's W test for Pichia fermentans. Sig. needs to be above 0.05 or epsilon needs to be below 0.75 for Greenhouse-Geisser correction to be used.

Within Subjects Effect	Mauchly's W	Approx. Chi-Square	df	Sig.	Epsilon[b]		
					Greenhouse-Geisser	Huynh-Feldt	Lower bound
Temperature	0.100	26.297	9	0.002	0.484	0.567	0.250

Tests the null hypothesis that the error covariance matrix of the orthonormalized transformed dependent variables is proportional to an identity matrix.

a. Design: Intercept Within Subjects Design: Temperature

b. May be used to adjust the degrees of freedom for the averaged tests of significance. Corrected tests are displayed in the Tests of Within-Subjects Effects table.

Table 55. Corrections factors results for ANOVA statistcs for Pichia fermentans.

Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Temperature	Sphericity Assumed	5977.995	4	1494.499	14.425	0.000
	Greenhouse-Geisser	5977.995	1.934	3090.563	14.425	0.000
	Huynh-Feldt	5977.995	2.266	2637.611	14.425	0.000
	Lower-bound	5977.995	1.000	5977.995	14.425	0.002

Table 56. ANOVA test for the comparison between different temperatures of hybridization for Pichia strains. Mean (μ), standard deviation (σ) and p values according to the Least Significant Difference (LSD). For significant differences values need to be below 0.05.

	51ºC μ=51.809 δ=1.825	53ºC μ=38.947 δ=1.520	55ºC μ=42.505 δ=4.603	57ºC μ=53.715 δ=2.042	59ºC μ=53.993 δ=1.152
51 ºC					
53 ºC	0,000***				
55 ºC	0,115	0,011*			
57 ºC	0,478	0.000***	0,053		
59 ºC	0.376	0,000***	0,025*	0,913	