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DEVELOPMENT OF NAM-FISH METHOD FOR THE DETECTION OF *CITROBACTER SPP.*

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Abstract

Citrobacter is a genus of Gram-negative bacteria that belongs to the *Enterobacteriaceae* family. Even though the prevalence of Urinary Tract Infections (UTI) caused by *Citrobacter spp.* is low compared to other *Enterobacteriaceae* species, it is still important to implement and optimize methods that help clinically diagnose UTIs caused by this species in an easy and fast manner. Fluorescence *in situ* hybridization (FISH) is a molecular biology technique that uses nucleotide probes to detect specific DNA or RNA sequences within microorganisms of interest. This technique can be faster and simpler than the current techniques applied in clinical trials, while still providing results with high sensitivity and specificity.

The objective of this dissertation is to develop NAM-FISH probes for the detection of *Citrobacter spp.* in UTIs. This involved the development of two LNA/2'OMe-FISH probes, named AM1 and AM2, targeting the 16S rRNA of Citrobacter *spp*. The obtained results show that the designed probes are theoretically very sensitive and specific towards their targets. Moreover, it was determined that both probes can be used in a multiplex assay for the detection of *C. freundii* SGSC 5345, *C. koseri* SGSC 4696 and *C. amalonaticus* SA5615 at a temperature range of 58-64ºC. The specificity tests detected the occurrence of cross-hybridization for 7 species at 60ºC and 64ºC, while the sensitivity tests generally confirmed the results previously obtained during the optimal hybridization temperature tests.

The method described in this dissertation can be applied to detect *Citrobacter spp.* in urine samples in less than 2 hours, hence assisting clinical personnel during UTI treatment.

Keywords (theme): *Citrobacter*; Urinary Tract Infection; UTI; Fluorescence *in situ* hybridization; FISH.

Resumo

A *Citrobacter* é um género de bactéria Gram-negativa que pertence à família *Enterobacteriaceae*. Ainda que a prevalência de Infeções do Trato Urinário (ITU) causadas por *Citrobacter spp.* seja baixo em comparação com outras espécies de *Enterobacteriaceae*, é importante implementar e otimizar métodos que ajudem a diagnosticar clinicamente as ITUs causadas por essa espécie de maneira fácil e rápida. A hibridização fluorescente *in situ* (FISH) é uma técnica de biologia molecular que utiliza sondas de nucleótidos para detetar sequências específicas de DNA ou RNA em microrganismos de interesse. Esta técnica consegue ser mais rápida e simples do que as técnicas atualmente aplicadas em ensaios clínicos, fornecendo resultados com alta sensibilidade e especificidade.

O objetivo desta dissertação é desenvolver sondas NAM-FISH para a deteção de *Citrobacter spp.* em ITUs. Para isso, foram desenhadas duas sondas LNA/2'OMe-FISH, denominadas AM1 e AM2, tendo como alvo o 16S rRNA de *Citrobacter spp*. Os resultados obtidos mostram que as sondas projetadas são teoricamente muito sensíveis e específicas para os seus alvos. Além disso, determinou-se que ambas as sondas podem ser usadas num ensaio multiplex para a deteção de *C. freundii* SGSC 5345, *C. koseri* SGSC 4696 e *C. amalonaticus* SA5615 num intervalo de temperatura de 58-64ºC. Os testes de especificidade detetaram a ocorrência de hibridização cruzada para 7 espécies a 60ºC e 64ºC, enquanto os testes de sensibilidade em geral confirmaram os resultados obtidos anteriormente durante os testes de temperatura ótima de hibridização.

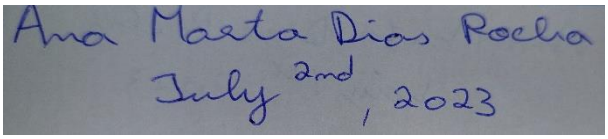
O método descrito nesta dissertação pode ser aplicado para detetar *Citrobacter spp.* em amostras de urina em menos de 2 horas, auxiliando assim o pessoal clínico durante o tratamento de ITUs.

Palavras-chave (tema): *Citrobacter*; Infeção do trato urinário; ITU; Hibridação fluorescente *in situ*; FISH.

Declaration

I hereby declare, under word of honor, that this work is original and that all non-original contributions is indicated, and due reference is given to the author and source.

Sign and date



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Notation and Glossary

2’OMe	2′-O-Methyl-RNA-based oligoribonucleotides
ΔH	Enthalpy variation
ΔS	Entropy variation
A	Adenine
Abs	Absorbance
AFU	Arbitrary Fluorescence Units
bp	Base pair
C	Cytosine
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FISH	Fluorescence *in situ* Hybridization
G	Guanine
K	Equilibrium constant
LFA	Lateral Flow Assays
LNA	Locked Nucleic Acid
M	Mean
NAM	Nucleic Acid Mimics
NCBI	National Center for Biotechnology Information
NN	Nearest Neighborhood
PCR	Polymerase Chain Reaction
PNA	Peptide Nucleic Acid
R	Molar gas constant
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SD	Standard Deviation
T	Thymine
T_m	Melting Temperature
TSA	Tryptic Soy Agar
UTI	Urinary Tract Infection

1 Introduction

1.1 Framing and presentation of the work

Citrobacter is a genus of Gram-negative bacteria that belongs to the *Enterobacteriaceae* family and can be found in the intestinal tract of humans and animals. However, some *Citrobacter* strains can cause urinary tract infections (UTIs), especially in infants and immune-compromised patients (Oliveira, Pinto et al. 2016). The most common species that cause this type of infection are *Citrobacter freundii* and *Citrobacter koseri* (Gajdács et al. 2019).

Most frequently used UTI detection methods display low sensitivities and can take up to 2 days to provide results (Kumar et al. 2016). Molecular methods (such as Polymerase Chain Reaction) that target nucleic acids are able to identify multiple infection-causing microorganisms (Wu et al. 2010). Nevertheless, these methods require correct handling and maintenance of complex and expensive equipment (Samonis et al. 2009, Gajdács et al. 2019).

To overcome such limitations, fluorescence *in situ* hybridization (FISH) was developed. FISH is a molecular biology technique that uses fluorescent probes to detect specific RNA or DNA sequences within microorganisms of interest. A probe is a DNA or RNA single strand that is complementary to a nucleotide sequence of interest. When optimized to the targeted species, this technique can display high sensitivity and specificity and can be faster and simpler than the current techniques applied in clinical diagnosis (Nácher-Vázquez et al. 2022).

This dissertation's original aim was to develop a nucleic acid mimic-fluorescence *in situ* hybridization (NAM-FISH) probe targeting *C. freundii* that could be used to detect this species in urine samples. To accomplish this goal, specific aims were established:

1. *In-silico* probe design: Initially, 16S rRNA sequences of *C. freundii* from arb-silva's database were aligned in the MEGA11 software. Other *Citrobacter* species and other *Enterobacteriaceae* family members were also included as a control. However, the selected sequences targeting *C. freundii* could also detect several other *Citrobacter* species. Therefore, the development of locked nucleic acid/2′-O-Methyl rRNA (LNA/2'OMe) probes targeting the *Citrobacter* genus became this dissertation's aim

from then on. Other criteria, such as melting temperature, pyrimidine content and Gibbs free energy were also considered during probe design.

2. Optimization of FISH protocol: Different hybridization temperatures were tested to establish the optimal hybridization temperature of the designed probes. The fluorescence signal intensity was measured using ImageJ for the selection of the optimal hybridization temperature.
3. Specificity and sensitivity tests: To further study the performance of the designed probes, non-target species and *Citrobacter* strains were used to study the specificity and sensitivity of the probes, respectively.

1.2 Contribution of the author to the work

This dissertation was mainly done by the author. Nevertheless, some laboratory activities required the help and involvement of other people. The initial dilution of the probes was carried out by Andreia Azevedo and Sónia Miranda. The cultivation and fixation of the strains used for the specificity and sensitivity tests was done by Sónia Miranda.

1.3 Organization of the dissertation

This dissertation is developed within 6 chapters. The first chapter serves as an introduction and briefly describes the main themes and motivations.

Chapter 2 provides an in-depth description of the themes at study in this dissertation. Firstly, it describes *Citrobacter* and its pathogenic relevance. Secondly, an overview of Urinary Tract Infections, including their worldwide prevalence and an analysis of the methods currently used to detect UTIs, is presented. Lastly, there is an extensive description of fluorescence *in situ* hybridization and the theory behind the probe design process, which are the main focuses of this dissertation.

Chapter 3 describes materials and methods such as growth medium, *in-silico* probe design, optimization of the NAM-FISH technique on glass slides, epifluorescence microscopy visualization, quantification of the fluorescence intensity signal and growth curves of strains *C. freundii* SGSC 5345, *C. koseri* SGSC 4696 and *C. amalonaticus* SA5615.

In Chapter 4 the results from the probe design process, the optimal hybridization temperature tests, the growth curves and the specificity and sensitivity tests are presented, analyzed, and discussed.

Chapter 5 summarizes the main conclusions, while chapter 6 displays suggestions of works than can be carried out in the future.

2 Context and State of the Art

2.1 *Citrobacter*: origins and pathogenic relevance

Citrobacter is a genus of Gram-negative bacteria that belongs to the *Enterobacteriaceae* family. The species that are part of this genus are slow lactose fermenters and facultative anaerobes, and can be found in food, wastewater, soil, and in the intestinal tract of not only humans, but also animals. Werkman and Gillen first described this genus in 1932 (Werkman et al. 1932). Before the 1990s, only 3 *Citrobacter* species were known to the scientific community: *C. amalonaticus*, *C. freundii* and *C. koseri* (previously named *C. diversus*) (Werkman et al. 1932, Frederiksen 1970, Borenshtein et al. 2006). In 1993, 8 other *Citrobacter* species were described: *C. braakii*, *C. farmeri*, *C. sedlakii*, *C. werkmanii*, C. *youngae*, and 3 other *Citrobacter* species which remained unnamed by then (Brenner et al. 1993). Between 1995 and 1999, the previously unnamed species were named *C. gillenii*, *C. murliniae* and *C. rodentium* (Schauer et al. 1995, Brenner et al. 1999). In more recent years, 5 other *Citrobacter* species have been described: *C. pasteuri* in 2015, *C. europaeus* and *C. portucalensis* in 2017, *C. cronae* in 2020 and *C. telavivensis* in 2021 (Clermont et al. 2015, Ribeiro et al. 2017, Ribeiro et al. 2017, Oberhettinger et al. 2020, Ribeiro et al. 2021).

Citrobacter are rare opportunistic nosocomial pathogens, and the prevalence of infections caused by species of this genus in humans is 3-6 %, among all the other *Enterobacteriaceae* species (Samonis et al. 2009). However, neonates and immunocompromised people of any age present a higher risk for clinical disease and consequent health complications if infected by these species (Oliveira et al. 2016). The mode of transmission is usually by person-to-person (mother-to-child transmission and intra-hospital infections are very common), but transmission through ingestion of infected food can also occur (Plakkal et al. 2013, Liu et al. 2017).

An infection by *Citrobacter* species can cause various diseases, such as pneumonia, meningitis, sepsis, diarrhea, and urinary tract infections (UTIs) (Oliveira et al. 2016).

2.2 Urinary Tract Infections

A UTI happens when bacteria are present in freshly-voided urine at 10^5 bacteria/mL, though symptoms can occur with 10^3 bacteria/mL (Lee et al. 2007). UTIs can also develop after urinary catheters are inserted into the bladder via the urethra. This procedure is performed in order to treat urinary incontinence or urinary retention. Nevertheless, multiple microorganisms can adhere to the catheter's surface and lead to the formation of biofilms, an assembly of microbial cells wrapped in an extracellular polymeric matrix. These biofilms are phenotype resistant and create an altered microenvironment that enhances antimicrobial resistance that is ultimately followed by serious, difficult-to-treat and often-recurrent infections (Sabir et al. 2017).

UTIs constitute one of the most frequently diagnosed infections worldwide, with an increasing incidence rate over the last 30 years. In 2019, Latin America, Africa and the Middle East were the regions with the most UTI cases, whereas, in the Australasian regions, very few cases were registered. In North America and Europe, the number of registered UTI cases in 2019 were similar to the cases registered in 1990, representing 40% of the registered cases worldwide (Yang et al. 2022). Figure 1 shows the most frequent nosocomial infections treated in a surgical intensive care unit in 2014 (Custovic et al. 2014). In this study, UTIs are the second most common nosocomial infection, only behind respiratory tract infections. Other common nosocomial infections include, for example, gastrointestinal tract infections and blood stream infections (Custovic et al. 2014).

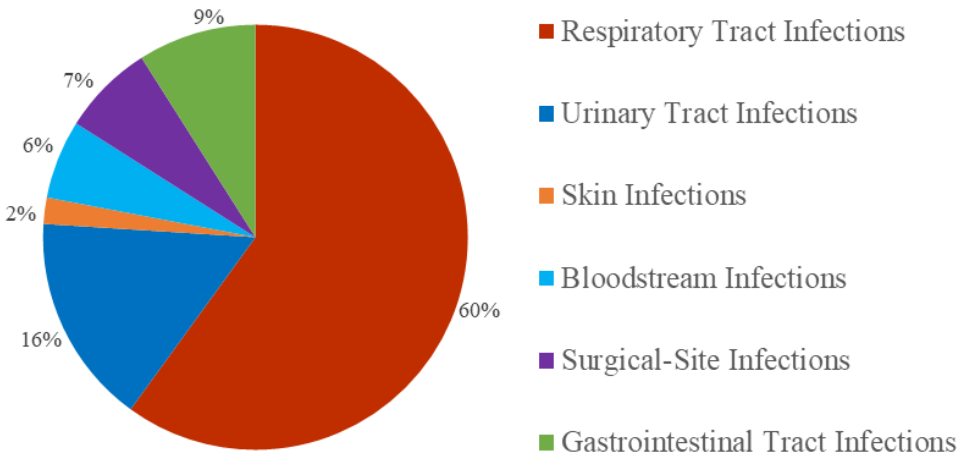


Figure 1 – Most common nosocomial infections in a surgical intensive unit (reprinted and adapted from Custovic et al. 2014).

Most of the UTIs are easily treatable and mainly caused by *Escherichia coli* (Lee et al. 2007). A rapid diagnosis of this infections should take place ideally within 12 to 24 hours since the first symptoms; otherwise, the infection may spread to the kidneys and ultimately lead to urinary tract obstruction and renal failure. (Kumar et al. 2016). Besides *E. coli*, other *Enterobacteriaceae* species (such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) are associated with complicated UTI cases, where the innate host defenses are disabled and lead to tissue injury (Lee et al. 2007). Figure 2 displays the distribution of UTI pathogens isolated from urine cultures.

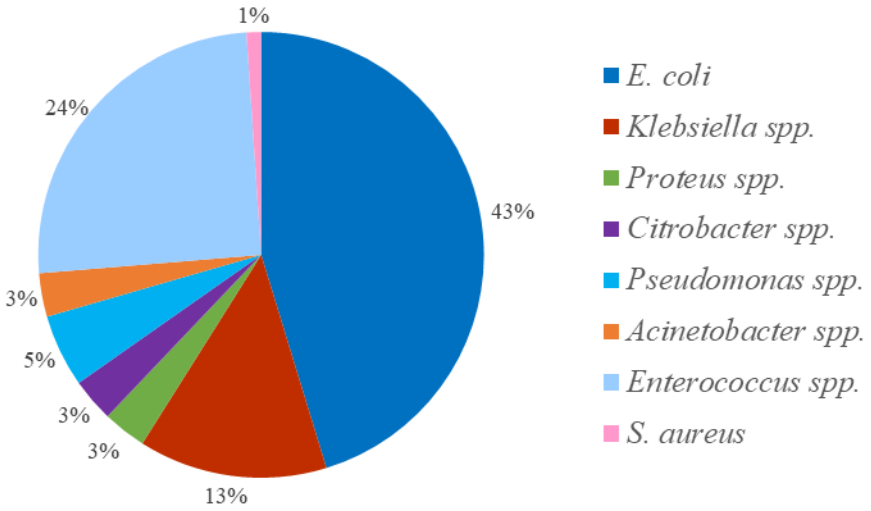


Figure 2 - Community-acquired bacteria causing UTIs (reprinted and adapted from Dr. P. Sneka, et al. 2019).

A study done over a period of 11.5 years (from June 1994 to January 2006) aimed to analyze every case of *Citrobacter* infection that was treated in a tertiary care university hospital. During this study, 78 patients with a *Citrobacter* infection were identified, 52.5 % of which had a UTI (Samonis et al. 2009). In another study, in which 4126 urine samples were analyzed, *Citrobacter* was the 3rd most common species causing UTIs in hospitalized patients (Metri et al. 2013). Later, a study with thousands of urine samples analyzed during a 10-year period (from January 2008 to December 2017), found that *C. koseri* and *C. freundii* were the most recurrent species of the *Citrobacter* genus to cause UTIs (Gajdács et al. 2019).

These studies demonstrate that the prevalence of *Citrobacter* infections is low but not uncommon, and the urinary tract tends to be the most targeted body region. Considering *Citrobacter*'s opportunistic nature to the young and vulnerable populations,

it is important to implement and optimize methods that help clinically diagnose this type of infections in an easy and fast manner.

2.3 UTI Detection Methods

The UTI detection methods can be divided into 2 categories (Figure 3): laboratory detection methods (which involve bacteria isolation and identification) and on-site detection methods (which give a sign that is visible to naked eye and can be applied as a point-of-care[1] diagnostic mechanism) (Santos et al. 2022).

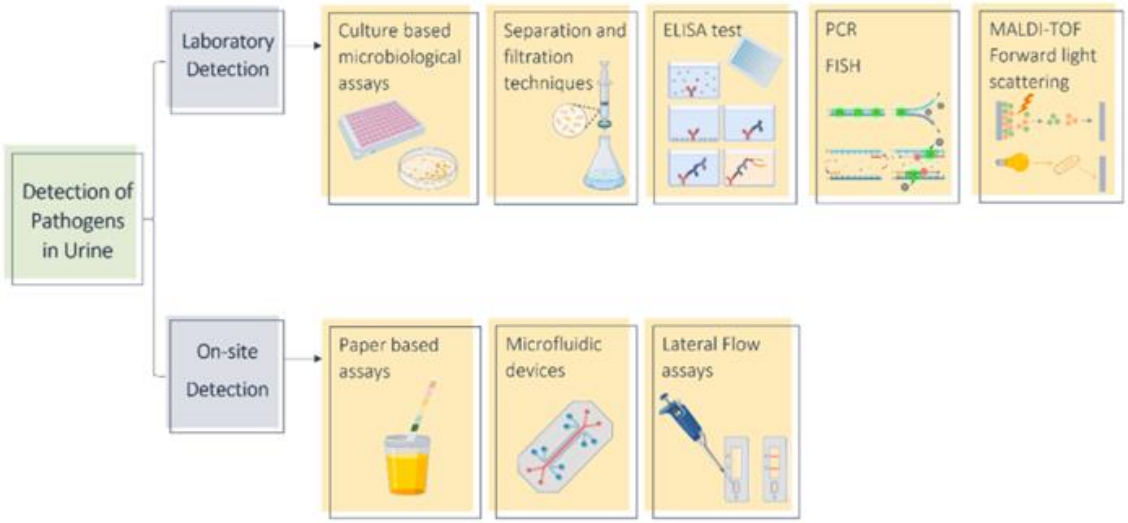


Figure 3 – UTI detection methods (reprinted and adapted from Santos et al. 2022)

One of the easiest and most widely-used laboratory detection methods is the culture-based microbiological assay. This method provides pathogen isolation, identification, and antimicrobial susceptibility testing. However, the diagnosis takes up to 2 or 3 days, allowing the infection to spread in the meantime (Pirkani et al. 2020). Chemical separation techniques (such as extraction and chromatography) can be performed to reduce this testing period; however, these methods display a major drawback, as the usage of toxic solvents can alter the cells' surface properties, making it harder to detect the microorganism (Santos et al. 2022). Another laboratory UTI detection method is the Enzyme-Linked Immunosorbent Assay (ELISA). ELISA is a biochemical test in which an enzyme reaction produces a color that indicates the presence of proteins,

[1] Point-of-care tests are medical examinations carried out in the same place and time in which the patient is being treated.

antigens, and antibodies. It displays high sensitivity and can quantify antigen content in a matter of hours. However, this is an expensive method and requires intensive labor (Wang et al. 2021). Polymerase chain reaction (PCR) is a molecular method that exhibits higher specificity and sensitivity than culture-based assays and has a significantly reduced detection time (Pirkani et al. 2020). The PCR procedure involves the amplification of specific genes from the genomic DNA found in urine specimens; nonetheless, it requires very expensive reagents and equipment (Santos et al. 2022). In addition, the traditional PCR methods cannot distinguish between alive and dead bacteria, even though this limitation can be overcome by using Viability-PCR (Nkuipou-Kenfack et al. 2013).

On-site detection methods (also known as paper-based methods) have emerged in recent years as a way of achieving simple and rapid detection of pathogens without requiring expensive and over-the-top equipment. One of the most well-known paper-based methods is the paper matrix test, where the paper strips check for nitrites and leukocytes that can be found in most UTIs. This method is easy to use, cheap, and can be performed by the patients themselves (Ghaderinezhad et al. 2020). However, it has limited quantitative precision and can't identify the pathogen that's causing the UTI, as it is only able to acknowledge its presence (Santos et al. 2022). The Lateral Flow Assay (LFA) is another type of paper-based test that operates with the same principles as the ELISA laboratory assay and can overcome some of the ELISA drawbacks (Wang et al. 2021). LFA faces similar drawbacks as the paper matrix test, but its sensitivity can be improved by employing laboratory methods such as PCR (Santos et al. 2022).

PCR high sensitivity and specificity and reduced detection time makes it arguably the best UTI detection method of all the methods previously described. However, more recent, promising methods like fluorescence *in situ* hybridization (FISH) have the ability to overcome some of PCR's limitations. For example, FISH does not require nucleic acid extraction, which is an expensive PCR step (Shakoori 2017). In addition, FISH allows the detection and quantification of biomass cells *in situ*. In other words, FISH can be applied directly where the biofilm cells are growing, while, in PCR, the spatial organization of the biofilm has to be destroyed (Azevedo et al. 2015).

2.4 Fluorescence *in situ* Hybridization

Hybridization is a genomic process where 2 complementary RNA or DNA molecules bond and subsequently produce a double-stranded molecule. *In situ*

hybridization, a technique first described in the late 1960s, takes this concept forward and aims to bond a previously labeled nucleotide probe with a complementary RNA or DNA target sequence to detect, quantify and localize it (Shakoori 2017).

FISH is one of the most well-known molecular biology assays. It can be applied to a comprehensive list of microbiology-related areas (such as pathogen detection in clinical settings and gene presence determination) and has the capability to obtain results in 1-2 hours (Oliveira et al. 2021).

In FISH, the targeted rRNA molecules bind with a fluorescently-labeled probe. After being excited by light at a specific wavelength, the fluorochrome-probe complex emits a signal that enables the detection of the hybridized cells, as the cells own enough copies of the targeted rRNA (Azevedo et al. 2022). In addition to its abundant concentration in the cells, the rRNA molecules display relatively high stability and regions with high and low sequence conservation, allowing a dissimilarity between particular microorganisms. Furthermore, the existence of many ribosomes within each cell leads to a higher fluorescence intensity (Teixeira et al. 2021).

The FISH protocol involves a total of 4 steps (Figure 4). The first step is permeabilization and fixation. This step allows the probes to go through the cellular membrane in order to reach the rRNA. Fixatives such as paraformaldehyde and ethanol are applied to the samples to allow the hybridization to happen by permeabilizing the cell membranes.

The second step, hybridization, is the most critical step of this protocol, and it must occur under rigorous conditions to grant a correct sequence-probe bonding. Therefore, the determination of the optimal hybridization conditions (such as hybridization time, temperature and agent denaturant) is the core aspect of the FISH technique. Hybridization time influences the piercing of the probe inside the cell membrane, the sequence-probe bond, and secondary and tertiary targeted structures unfolding. Hybridization temperature regulates probe affinity and can be theoretically determined by the hybridization reaction's Gibbs free energy change. (Oliveira et al. 2021). The denaturant agent reduces polynucleotides' thermal stability, allowing the probes to reach their target. Formamide is the most frequently used denaturant agent, but has been replaced in recent years by urea, a much less hazardous denaturant. Urea is a chaotropic agent that helps the probe to easily access its target. A study done by Azevedo, et al. (2019) demonstrated that

hybridization solutions that contain urea led to higher fluorescence values than the hybridization solutions that contain formamide instead (Azevedo et al. 2019). The probe's high theoretical sensitivity and specificity is also critical to the success of this step (Nácher-Vázquez et al. 2022)

After hybridization, a washing step is done to remove the probe quantity that remained unbonded. This step is usually performed under the same temperature as the one applied during hybridization. The washing solution consists of a high-concentration salt and a detergent (for example, Triton X-100). This detergent is used to increase the permeability of this process, which consequently increases its efficiency (Oliveira et al. 2021).

The final step, the visualization of the fluorescence, is performed using a flow cytometer or an epifluorescence microscope. The microscope images can be quantified using software programs such as ImageJ (Nácher-Vázquez et al. 2022).

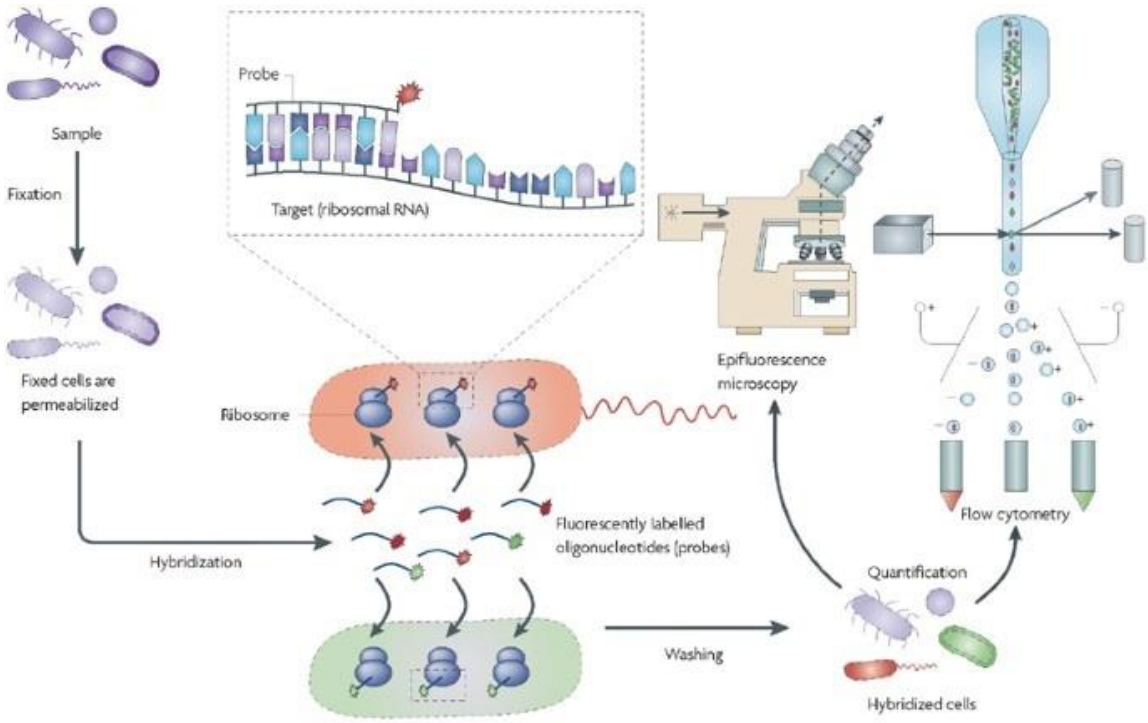


Figure 4 – Schematic demonstration of the FISH protocol (reprinted from Amann and Fuchs 2008).

2.4.1 Nucleic acid mimics used on FISH

Among the drawbacks associated with FISH are the ribosomal secondary structure (which hinders the accessibility to the rRNA molecules), the low permeability of cell membranes and probe's degradation by proteases or endonucleases from living cells. Such limitations affect the robustness of this method. To overcome this, artificially modified RNA and DNA probes, known as Nucleic Acid Mimics (NAMs), have been used as a replacement to natural nucleic acids (Karkare et al. 2006).

NAMs contain non-natural components (alternative nucleobases, sugars, or connecting backbones), which confer a different chemical structure and binding characteristics. These modifications lead to higher melting temperatures and single-base mismatches become easier to discriminate, since there is a greater difference between the melting temperatures of a duplex with a single mismatch and a totally-matched duplex. Furthermore, NAM probes require a lower optimal length (allowing a better permeabilization) and are more resistant to enzymatic degradation (Guimarães et al. 2021)

Peptide Nucleic Acid (PNA) is a type of NAM that was first described in 1991 and first used for the detection of microorganisms in 1999 (Figure 5) (Nácher-Vázquez et al. 2022). PNA probes are shorter than DNA probes, usually displaying 15 base pairs (while DNA probes have between 20 and 24 base pairs). PNAs are also neutrally charged and can provide consistent hybridization performances because of their diminished electrostatic repulsion between the charged RNA or DNA phosphodiester backbone and the non-charged polyamide backbone (Cerqueira et al. 2008).

Locked nucleic acid (LNA) is another type of NAM that was first described in the late 1990s. LNAs have a methylene group between 2'-O and 4'-C, causing the locking of the ribose ring (Figure 5). This change decreases the molecule susceptibility to nuclease degradation by improving the stability of the RNA duplex (Robertson et al. 2009). The 2'-O-Methyl-RNA based oligoribonucleotides (also known as 2′OMe) are another type of NAMs and have a C3'-endo furanose ring conformation, making them resistant to nuclease degradation (Figure 5) (Fontenete et al. 2016). If used in combination with LNA, they display a much better design flexibility compared to the DNA and PNA probes (Azevedo et al. 2015). The LNA/2'OMe probes showcase slight adjustments in the melting temperature by alternating between LNA and 2'OMe monomers. This means that

probes with identical melting temperatures can be designed, facilitating the detection of multiple targets at the same time (Azevedo et al. 2019).

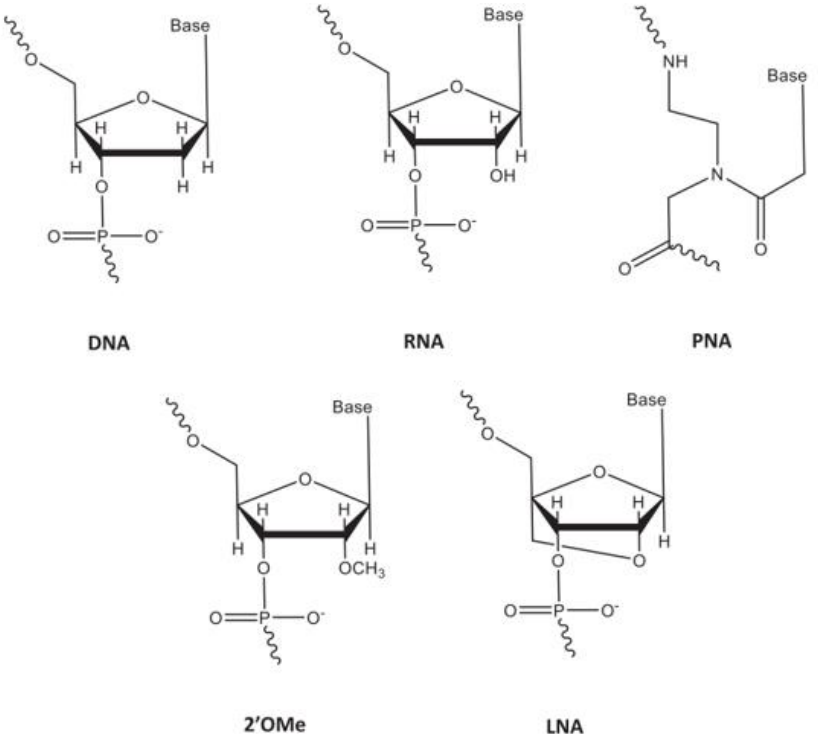


Figure 5 – Representation of the chemical structures of DNA, RNA, PNA, 2´OMe and LNA (reprinted from Nácher-Vázquez et al. 2022)

2.4.2 *In-silico* probe design process

The *in-silico* probe design process is crucially important for the success of FISH (Oliveira et al. 2021). A thorough theoretical evaluation should be carried out to determine if the designed probe complies with the required optimal ranges (Teixeira et al. 2021). The following parameters should be considered:

Probe length: This is the first parameter to be considered and has a direct effect on the probe's specificity and on the hybridization time. If the probe is too short (meaning it only displays a few base pairs), it is likely to have a low specificity, as its sequence has a high probability of being present in a large range of species. On the other hand, if the probe has too many base pairs, it can lead to longer hybridization times and greater probe synthesis costs (Prudent et al. 2018). A DNA probe should always have at least 18 nucleotides. However, NAM probes can display fewer nucleotides: PNA probes

usually contain between 13 and 18 base pairs, while LNA probes generally display a length of between 10 and 15 base pairs (Teixeira et al. 2021).

Overall Gibbs free energy change (ΔG^0 overall): The overall Gibbs free energy change indicates if a reaction is thermodynamically favorable. The reactions are more favorable the more negative the ΔG^0 value is (Teixeira et al. 2021). However, if it is too negative, the probes can hybridize with not-fully-complementary sequences (and, subsequently, compromise specificity in single-mismatch cases) since Gibbs free energy is calculated for duplex formation in the probe design process (Yilmaz et al. 2004). A temperature of 37 ºC, the standard human body temperature, is generally used to predict an oligonucleotide free energy change. For PNA probes, ΔG^0_{37} values must be between -13 and -20 kcal mol^{-1} (Yilmaz et al. 2004).

Melting temperature (T_m): The temperature at which 50% of nucleic acid strands establish a double-helix bond (while the other 50% remain single-stranded) is called melting temperature (Aquino de Muro 2008). To theoretically predict the melting temperature, the Nearest Neighbor (NN) model is generally used (Xia et al. 1998). The first step of the NN model is the determination of the enthalpy and entropy of the duplex formation. It is presumed that free energy, entropy, and enthalpy are all state functions, meaning the process can be subdivided in discrete steps. Furthermore, enthalpy and entropy are assumed to be independent. Two fundamental thermodynamic principles are considered in this step: the relation between free energy change and consequent enthalpy and entropy changes (Equation 1) and free energy on standard conditions to the equilibrium constant of the system (Equation 2)

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

$$\Delta G° = - RT \ln (K) \quad (2)$$

where ΔH is the enthalpy variation (cal mol^{-1}), ΔS is the entropy variation (cal mol^{-1} K^{-1}), is R the molar gas constant (1.9872 cal mol^{-1} K^{-1}) and K is the equilibrium constant (Xia et al. 1998). The second step involves the determination of T_m. Equations 1 and 2 are rearranged to form Equation 3, where T_m can be directly calculated (Xia et al. 1998).

$$T_m = \frac{\Delta H}{\Delta S - R \ln (K)} \quad (3)$$

These formulas are used for DNA:DNA homoduplexes, meaning the model must be adapted to theoretically evaluate NAM heteroduplexes. This also applies to the NN Watson-Crick interactions. The NN model is based on the influence of the base pair immediately before the one that is forming. Only 10 NN Watson-Crick interactions (out of 16) are considered when analyzing homoduplexes, since some combinations are thermodynamically equivalent as a consequence for being geometrically alike (Xia et al. 1998). These interactions are present in Table 1.

Table 1 - Nearest Neighbor Watson-Crick interactions (reprinted from Xia et al. 1998)

NN Watson-Crick interaction	**Geometrically alike interaction**
AA/TT	TT/AA
AT/TA	-
TA/AT	-
CA/CT	TG/CA
GT/CA	AC/TG
CT/GA	AG/TC
GA/CT	TC/AG
CG/GC	-
GC/CG	-
GG/CC	CC/GG

Pyrimidine content (%GC): The pyrimidine content relates to the percentage of cytosine and guanine present in the designed probe (Teixeira et al. 2021). The DNA strands contain 4 nucleobases: adenine (A), thymine (T), cytosine (C) and guanine (G). The base-pairing process occurs between a purine (A or G) and a pyrimidine (T or C). A and T bond with 2 hydrogen bridges, while G and C bond with 3 hydrogen bridges (Lunell et al. 2004). This bonding scheme can be observed in Figure 6.

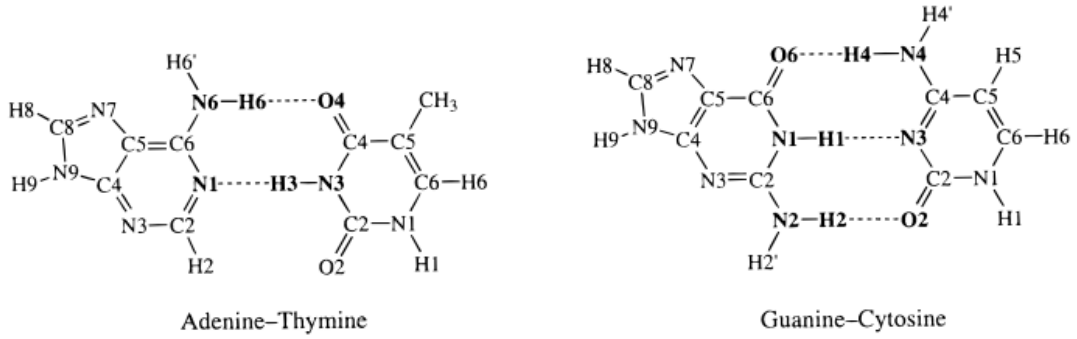


Figure 6 – Hydrogen bridges of A/T and G/C (reprinted and adapted from Mo 2006)

The fact that G and C bond with an extra bridge (compared to the bonding of A and T) means the GC base pairs are more stable and, therefore, display better thermodynamics than the AT base pairs. However, if the %GC is too high, secondary structures and self-annealing may occur. The optimal %GC value for a probe is between 50% and 60%. It is also important to consider that the higher the %GC value is, the higher the T_m (Aquino de Muro 2008).

Specificity and sensitivity: The determination of the probe's specificity and sensitivity is a very important step, as these two parameters have a direct impact on the success of hybridization (Rocha et al. 2019). The specificity is related to the probe's capability to detect the target for which it was designed. The higher the specificity, the fewer non-targets the probe can detect. The sensitivity represents the probe's capability to detect target taxa strains. The higher the sensitivity, the more target taxa strains the probe can detect (Rocha et al. 2019).

Complementary probe sequences and secondary structure: The presence of secondary structures considerably decreases the quantity of nucleotides that are available for binding, and consequently reduces FISH efficiency (Teixeira et al. 2021). Designing a probe without one of the 4 bases can avoid the occurrence of this issue (Apte et al. 2009).

3 Materials and Methods

3.1 Strains and growth conditions

The bacterial strains were grown at 37ºC on tryptic soy agar (TSA) [3% (w/v) tryptic soy broth and 1.5% (w/v) agar; Liofilchem, Italy] and subsequently streaked onto fresh TSA plates every 24 hours. Table 2 lists all the bacterial strains used in this dissertation.

Table 2 – Bacterial strains used in this study.

Microorganism	**Strain**	**Origin**
Citrobacter freundii	SGSC 5345	LEPABE's stock
Citrobacter freundii	CF#4	Clinical isolate from urine
Citrobacter freundii	CF#5	Clinical isolate from urine
Citrobacter freundii	CF#9	Clinical isolate from urine
Citrobacter freundii	CF#11	Clinical isolate from urine
Citrobacter koseri	SGSC 4696	LEPABE's stock
Citrobacter koseri	SGSC 5610	*Salmonella* Genetic Stock centre
Citrobacter braakii	CB	Unknown
Citrobacter braakii	CB8	Unknown
Citrobacter amalonaticus	SA5615	LEPABE's stock
Campylobacter coli	CNET 19	LEPABE's stock
Fusobacterium necrophorum	DSM21784	LEPABE's stock
Clostridium perfringes	ATCC 13124	LEPABE's stock
Pseudomonas fluorescens	CECT 378	LEPABE's stock
Pseudomonas aeruginosa	PAO1	LEPABE's stock
Escherichia coli	ATCC 25922	LEPABE's stock
Klebsiella pneumoniae	ATCC 43816	LEPABE's stock
Staphylococcus epidermidis	RP62A CECT 4184	LEPABE's stock
Salmonella typhimurium	SGSC 2523	LEPABE's stock
Acinetobacter baumannii	NCTC 13423	LEPABE's stock
Enterococcus faecalis	CECT 184	LEPABE's stock
Listeria monocytogenes	ATCC 7644	LEPABE's stock
Enterococcus faecium	CECT 410	LEPABE's stock
Proteus mirabilis	CECT 4101	LEPABE's stock
Streptococcus mitis	ATCC15919	LEPABE's stock
Lactobacillus casei	LMG 6964	LEPABE's stock
Helicobacter pylori	26695	LEPABE's stock

3.2 *In-silico* probe design for the specific detection of *Citrobacter*

To design the probes, an alignment software (MEGA11, Pennsylvania State University, United States of America) and a 16S rRNA database (arb-silva, http://www.arb-silva.de/) were used in accordance with the methodology previously described by Teixeira et al. (2021). The accession numbers of all 16S rRNA sequences used in this dissertation are present in Table 12 in Appendix A. An example of a partial alignment made in MEGA11 can be observed in Figure 7.

Figure 7 - Example of a partial alignment made in MEGA11 with the reverse complement sequence of AM1 probe.

In the first stage, 2 alignments were made. For these alignments, 10 different *C. freundii* strains, 7 different strains from other *Citrobacter* species and 7 different strains from other *Enterobacteriaceae* species were included. For the selection of the sequences/regions of interest, the following criteria were used:

1. All sequences were either 14 or 15 bp long and showcased high specificity (≥ 99.99%) and high sensitivity (≥ 90.00%). Equations 4 and 5 were used to calculate the theoretical specificity and sensitivity of the probes, respectively (Teixeira et al. 2021).

$$\text{Specificity} = \frac{\text{Database bacteria - Database targets - Target strains detected}}{\text{Database bacteria - Targets in database}} \times 100 \quad (4)$$

$$\text{Sensitivity} = \frac{\text{Target strains detected}}{\text{Database targets}} \times 100 \quad (5)$$

2. The Gibbs free energy of all sequences was between -26 and -42 kcal mol^{-1}, according to Teixeira et al. (2021);
3. The pyrimidine content of all sequences was superior to 40% (Teixeira et al. 2021);
4. Self-complementary structures were avoided (Teixeira et al. 2021);
5. In a multiplex assay, to increase the probability of the probes to hybridize under equal or similar conditions, the melting temperatures of the probes should be similar (Azevedo et al. 2019). The adjustment of the DNA length is the only way to reach different probes with equal or similar thermodynamic parameters (Azevedo et al. 2019); however, LNA and 2'OMe-RNA might be inserted in the sequences chosen to design probes with similar thermodynamic parameters. A combination of the NN set for isolate or consecutive LNA monomers within a DNA probe targeting a DNA sequence and a combination of the NN set for 2'OMe:RNA was used to predict the melting temperatures of the LNA/2'OMe:RNA duplexes (Fontenete et al. 2016). For the prediction of the LNA/2'OMe:RNA free energy change, the NN thermodynamic set for 2'OMe:RNA was used (Fontenete et al. 2016).

Both designed probes have Alexa Fluor 594 fluorochromes (Eurogentec, Belgium) attached to them in the 5' terminus.

3.3 Hybridization conditions

The hybridization temperatures of the designed LNA/2'OMe probes were optimized on pure samples of *C. freundii* SGSC 5345, *C. koseri* SGSC 4696 *and C. amalonaticus* SA5615, according to the FISH protocol described by Azevedo et al. (2019). This protocol was performed twice for each tested hybridization temperature (50ºC, 52ºC, 54ºC, 56ºC, 58ºC, 60ºC, 62ºC, 64ºC, 66ºC, 68ºC and 70ºC). Briefly, the *Citrobacter* bacterial cells were first harvested from the TSA plates and suspended in sterile water. Then, a volume of 30 µL of the suspended solution was dispensed in 14 mm well slides (Marienfeld, Lauda-Königshofen, Germany) that were left drying in an incubator for 10 minutes. For fixation/permeabilization, a volume of 40 µL of 4% (w/v) paraformaldehyde and 40 µL of 50% ethanol were dispensed on the wells at room temperature. Then, the slides were left drying in an incubator for 10 minutes. A volume

of 40 µL of hybridization solution containing 200 nM of the LNA/2'OMe probes AM1 or AM2 (Eurogentec, Belgium), 50 mM Tris-HCl (pH 7.5), 2 M Urea and 4 M NaCl were added to the wells. The samples were then incubated for an hour in an oven (FD 23, Binder, Germany) at each hybridization temperature (50ºC, 52ºC, 54ºC, 56ºC, 58ºC, 60ºC, 62ºC, 64ºC, 66ºC, 68ºC and 70ºC) under study. Afterwards, the samples were placed in coplin jars with washing solution comprised of 5 mM Tris-base, 15 mM NaCl and 0.1% (v/v) Triton X-100 and incubated for 30 minutes at the same temperature used in the previous hybridization step. The samples were then left drying in the dark before going into microscopy analysis (or stored at 4ºC in the dark for a maximum of 24 hours). After the optimization of the hybridization temperatures, the same protocol was used for the specificity and sensitivity tests.

3.4 Epifluorescence microscopy visualization

The microscope used in this study was a Leica DM LB2 epifluorescence microscope with a BP 515–550 excitation filter, a FT 580 beam splitter, and an LP 590 emission. The analyzed images were acquired using Leica Application Suite (LAS v4.2.) with a magnification of 100x. A drop of immersion oil was added to slide wells before observation. Five microscope pictures were taken from each of the 3 slide wells. Since every temperature was tested twice, a total of 30 pictures (15 photos from each test) were analyzed to determine the Average Fluorescence Units (AFU) for each temperature. For that, the quantification of fluorescence intensity of each image obtained by epifluorescence microscopy was done with the ImageJ software (National Institutes of Health Software, http://rsbweb.nih.gov/ij/index.html) (Azevedo et al. 2015). The images acquired from the microscope were first split into 3 grayscale images consisting of the green, blue and red components of the original image. Since the probes used in this thesis display red fluorescent light, the green and blue images were discarded. A threshold was applied after duplicating the grayscale image of the red components. After that, the fluorescence of the particles was analyzed by applying a mean grey value to the original grayscale image.

3.5 Planktonic growth curves and specific growth rate

To plot the planktonic growth curves and determine the specific growth rates of *C. freundii* SGSC 5345, *C. koseri* SGSC 4696 and *C. amalonaticus* SA5615, colonies from these species were used to inoculate a volume of 75 mL of 3% (w/v) tryptic soy broth

(TSB) [Liofilchem, Italy]. The cultures were incubated overnight (for 16-18 hours) under agitation (120 rpm) and at 37ºC. Subsequently, the cells were diluted 1:100 in fresh TSB and incubated at 37ºC and 120 rpm. The optical density at 620 nm was measured every 10 minutes until the cells reached the stationary state. After plotting the growth curves, the specific growth rate was calculated by determining the linear slope of the curves.

3.6 Statistical Analysis

To determine what temperatures could be used in a multiplex assay to detect *C. freundii* SGSC 5345, *C. koseri* SGSC 4696 and *C. amalonaticus* SA5615 with AM1 and AM2 probes, a paired-samples t-test was performed to compare the fluorescence values at all tested temperatures. For the sensitivity test, a paired-samples t-test was performed to compare the fluorescence values between the *C. freundii* strains and the *C. koseri* strain at 60ºC. For both tests, the level of significance was $P<0.005$ and the GraphPad Prism 9.5.1 software (San Diego, United States of America) was used.

4 Results and Discussion

A study done by Brenner at al. in the late 1990's showed promising results regarding the use of DNA hybridization to identify several *Citrobacter* strains. In fact, 119 strains out of 136 (88%) were successfully identified (Brenner et al. 1999). Nevertheless, this hybridization process involved the extraction and purification of DNA, which is a time-consuming process that requires the usage of toxic chemicals. Having these drawbacks in mind, this dissertation aims to develop an alternative hybridization assay by not only designing rRNA-targeted probes, but also assessing the NAM-FISH method performance regarding specificity and sensitivity. Although it is intended to detect UTIs, it can be applied to other usages if the hybridization conditions are optimized to obtain the highest possible specificity and sensitivity for the intended target (Azevedo et al. 2019).

4.1 Probe design and theoretical evaluation

In the first stage, 2 alignments were made, and 5 sequences of interest were selected for the detection of *Citrobacter freundii*. The selected sequences and their respective specificity and sensitivity values are presented in Table 3.

Table 3 – Theoretical evaluation of the selected sequences for the detection of *C. freundii*, with data retrieved from arb-silva's database.

Sequence number	Microorganism	Target gene	Probe sequence (5'-3')	bp	%GC	Specificity (%)	Sensitivity (%)
1	*C. freundii*	16S rRNA	CCAGTTTCGGATGC	14	57.14	99.99	94.24
2	*C. freundii*	16S rRNA	CCAGTTTCGGATGCA	15	53.33	99.99	94.24
3	*C. freundii*	16S rRNA	GCCAGTTTCGGATGC	15	60.00	99.99	93.88
4	*C. freundii*	16S rRNA	CTGGGCACATCCGAT	15	60.00	99.90	97.48
5	*C. freundii*	16S rRNA	CGTGGCATTCTGATC	15	53.33	99.79	94.60

As observed in Table 3, the theoretical specificity values were ~ 99.90%. This means that the remaining ~ 0.1% represents non-C. *freundii* strains that the probe can theoretically detect. In order to determine which non-target species are these, the TestProbe feature in the arb-silva's database website was used. Regarding *Citrobacter spp.*, sequences 1, 2 and 3 could detect strains of *C. braakii*, *C. europaeus*, *C. gillenii*, *C.*

murliniae, *C. pasteurii*, *C. portucalensis*, *C. werkmanii* and *C. youngae*. Sequence 4 could also detect strains of *C. amalonaticus* and *C. farmeri*. Sequence 5 could detect strains of all *Citrobacter* species available in the database.

To sum up, these results showed that, even though all these sequences display high sensitivity (> 90%) towards *C. freundii*, they were also able to detect other *Citrobacter* species. Given this, the theoretical sensitivity and specificity values of these sequences were determined assuming that the target would be the *Citrobacter* genus (Table 4).

Table 4 – Theoretical evaluation of the chosen sequences for the detection of *Citrobacter spp.*, with data retrieved from arb-silva's database.

Sequence number	Microorganism	Target gene	Probe sequence (5'-3')	bp	%GC	Specificity (%)	Sensitivity (%)
1	*Citrobacter*	16S rRNA	CCAGTTTCGGATGC	14	57.14	99.99	66.50
2	*Citrobacter*	16S rRNA	CCAGTTTCGGATGCA	15	53.33	99.99	67.83
3	*Citrobacter*	16S rRNA	GCCAGTTTCGGATGC	15	60.00	99.99	67.50
4	*Citrobacter*	16S rRNA	CTGGGCACATCCGAT	15	60.00	99.91	84.41
5	*Citrobacter*	16S rRNA	CGTGGCATTCTGATC	15	53.33	99.79	89.88

The results in Table 4 showed that all these sequences display low sensitivity (< 90%) towards the *Citrobacter* genus. Having these results in mind, a new alignment was made to evaluate the possibility of using 2 probes for multiplex detection of the *Citrobacter* genus. This new selected sequence is present in Table 5.

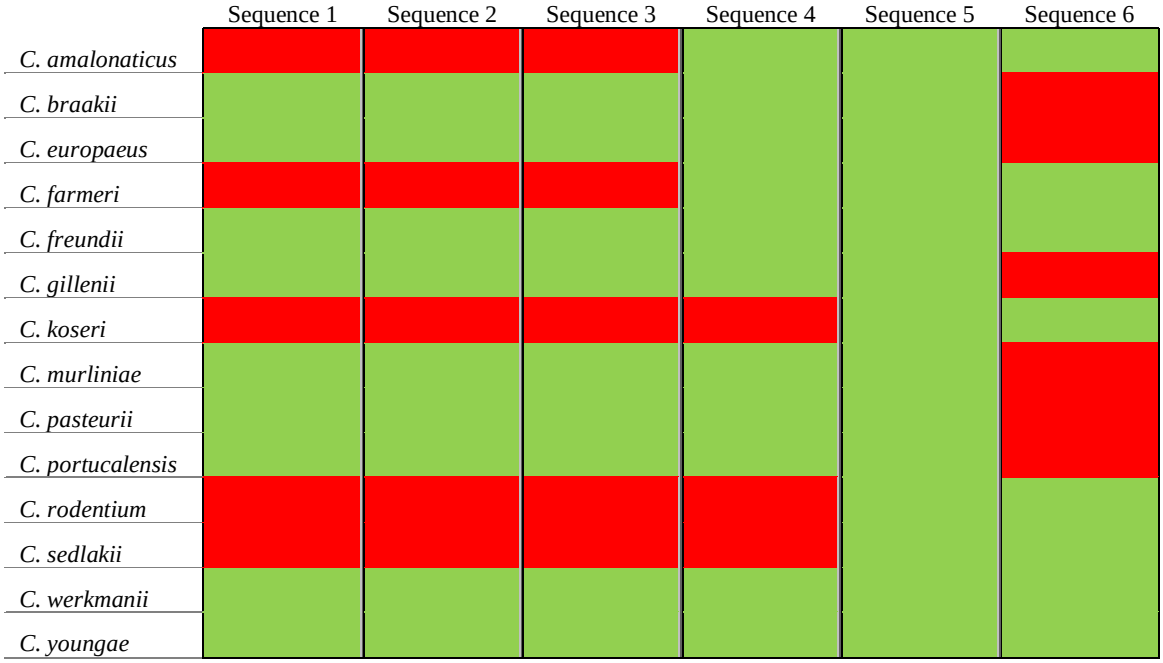
Table 5 - Theoretical specificity and sensitivity for sequence 6, with data retrieved from arb-silva's database.

Sequence number	Microorganism	Target gene	Probe sequence (5'-3')	bp	%GC	Specificity (%)	Sensitivity (%)
6	*Citrobacter*	16S rRNA	GAGACTCAAGCCTGC	15	60.00	99.99	24.05

This new alignment aimed to find a sequence that was capable of detecting *Citrobacter* species that sequences 1, 2 and 3 could not detect. By using the TestProbe feature in the arb-silva's database website, it was determined that this new sequence is

able to detect *C. amalonaticus*, *C. farmeri*, *C. freundii*, *C. koseri*, *C. rodentium*, *C. sedlaki*, *C. werkmanii* and *C. youngae*. Table 6 establishes a comparison between all selected sequences regarding their abilities to detect *Citrobacter* species.

Table 6 - Comparison between all selected sequences regarding their abilities to detect *Citrobacter* species according to the TestProbe feature in the arb-silva's database.



The green boxes mean that the sequence can detect the respective species. The red boxes mean that the sequence is not able to detect the species.

The results in Table 6 suggest that a multiplex detection of *Citrobacter spp.* could be possible using a two-probe combination of sequence 6 with sequences 1, 2 or 3, as this combination is able to theoretically detect strains of all *Citrobacter spp.* present in the database. However, and despite having a sensitivity lower than 90% (Table 4), sequence 5 alone can also detect strains of all *Citrobacter spp.*

Based on this analysis, using a single probe made of sequence 5 could be a better solution than using a multiplex assay with 2 probes. To further study both options, the percentage of non-targets that each sequence can detect was evaluated (Figure 8).

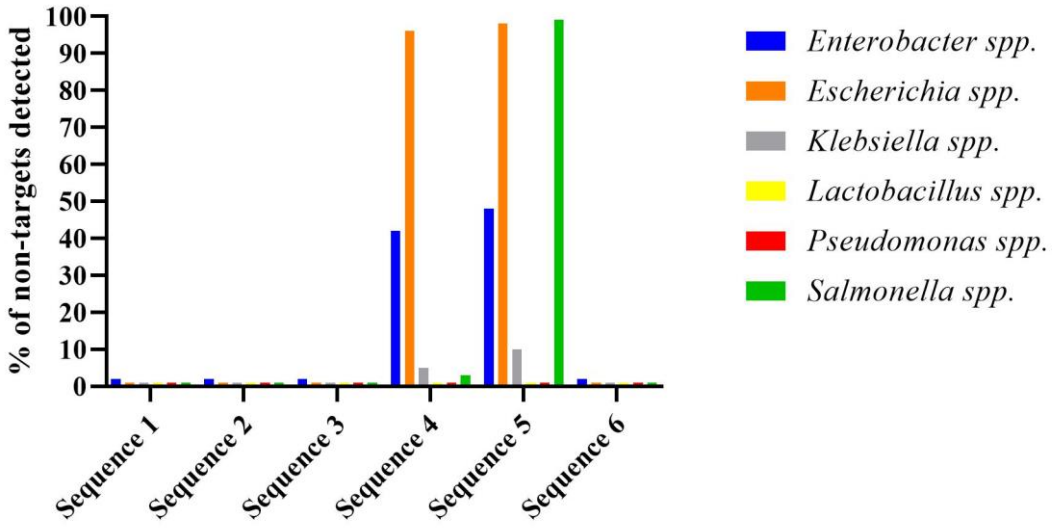


Figure 8 – Percentage of non-targets detected by sequences 1 to 6 according to the TestProbe feature in the arb-silva's database.

Figure 8 shows that sequences 4 and 5 could detect high percentages of non-targets: sequence 4 could detect 96.47% of *Escherichia spp.*, while sequence 5 could detect 98.68% of *Escherichia spp.* and 99.19% of *Salmonella spp.* On the other hand, the percentages of non-targets detected by sequences 1, 2, 3 and 6 were relatively low, with the highest being 2.73% (sequences 1, 2, 3) and 2.31% (sequence 6) for *Enterobacter spp.* These results show that sequences 1, 2, 3 and 6 remain suitable for multiplex detection of the *Citrobacter* genus, while sequences 4 and 5 should be discarded.

Given the similarities between sequences 1, 2 and 3, a closer look at their sensitivities values was done in order to select which sequence is more suitable to establish a multiplex assay with sequence 6. Table 3 shows that sequence 3 had a slightly lower sensitivity towards *C. freundii* (63.88%) than sequences 1 and 2 (94.24% for both sequences). Based on this, sequence 3 can be discarded. Although sequences 1 and 2 had the same sensitivity towards *C. freundii*, Table 4 shows that sequence 2 had a slightly higher sensitivity towards the *Citrobacter* genus (67.83%) than sequence 1 (66.50%). For this reason, sequence 1 was discarded and sequence 2 was chosen to be combined with sequence 6 for *Citrobacter* genus detection. Figure 9 shows the sensitivity percentages for each *Citrobacter* species for sequences 2 and 6.

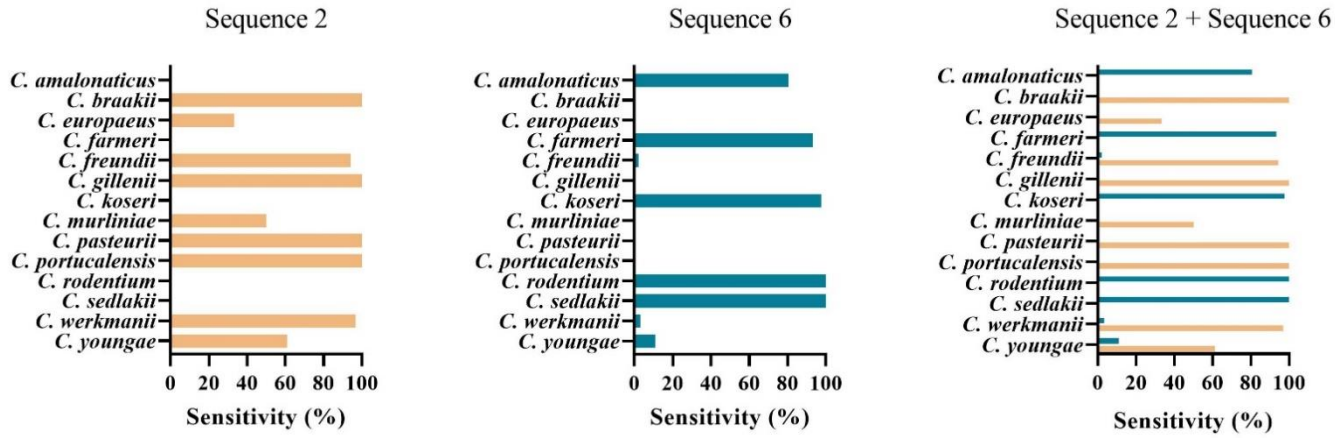


Figure 9 - Percentage of *Citrobacter spp.* detected by sequences 2 and 6, according to the TestProbe feature in the arb-silva's database.

The last step in the probe design process was to determine the T_m and the ΔG^o_{37} the for the chosen sequences. After testing different NN set combinations, a combination of one LNA monomer followed by two 2'OMe monomers for sequence 2 (after removing a bp at the end of the original sequence) and a combination of one LNA monomer followed by two 2'OMe monomers for sequence 6 were chosen. The LNA/2'OMe probes, their respective T_m and ΔG^o_{37} values and the probes' names can be seen in Table 7.

Table 7 - LNA/2'OMe probes and their respective T_m and ΔG^o_{37} values.

Sequence number	Probe name	Probe sequence (5'-3')	LNA/2'OMe probe sequence (5'-3')	bp	Tm (◦C)	ΔG^o_{37} (kcal mol^{-1})
2	AM1	GCCAGTTTCGGATGC	cCAgTTtCGgATgC	14	82.44	-30.22
6	AM2	GAGACTCAAGCCTGC	gAGaCTcAAgCCtGC	15	82.89	-33.15

4.2. NAM-FISH Method Performance

The FISH protocol is very simple and not very demanding; however, the hybridization process must be optimized in order to determine the optimal performing conditions of AM1 and AM2 probes. Several hybridization temperatures were tested in this dissertation, but the hybridization time and the concentration of the denaturant agent remained unaltered throughout the following tests.

4.2.1 Hybridization Temperature Optimization

To determine the temperature range to be tested, the previously predicted melting temperatures of AM1 and AM2 probes were considered. A study performed by Fontenete et al. (2015) indicated that, for LNA probes, the optimal hybridization temperature is 7ºC to 37ºC lower than the predicted T_m (Fontenete et al. 2015), meaning that, for AM1 and AM2 probes, it is between 45ºC and 75ºC.

Given this, the tested temperature range for AM1 probe was between 50ºC and 70ºC at 2 ºC intervals, while, for AM2 probe, it was between 58ºC and 66ºC (58ºC, 60ºC, 62ºC, 64ºC and 66ºC). For the AM1 probe, the *Citrobacter* strain tested to determine the optimal hybridization temperature was *C. freundii* SGSC 5345. For AM2 probe, two different *Citrobacter* strains were tested: *C. koseri* SGSC 4696 and *C. amalonaticus* SA5615. A large temperature range was tested for AM1 probe with *C. freundii* because this *Citrobacter* species was originally the main focus of this dissertation. Results are graphically represented in Figure 10.

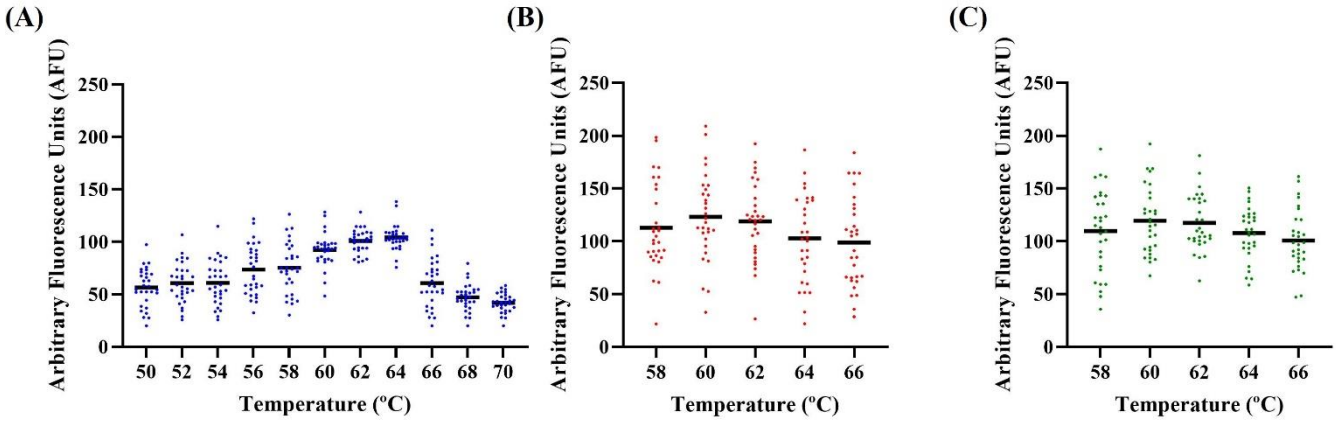


Figure 10 - Fluorescence intensity (AFU) for each tested hybridization temperature for AM1 probe with *C. freundii* SGSC 5345 (A) and for AM2 probe with *C. koseri* SGSC 4696 (B) and *C. amalonaticus* SA5615 (C).

As shown in Figure 10, the optimal hybridization temperature for *C. freundii* SGSC 5345 was 64ºC. For *C. koseri* SGSC 4696 and *C. amalonaticus* SA5615, the optimal hybridization temperature was 60ºC. These results are in agreement with the relation between the predicted melting temperatures and the optimal hybridization temperatures established by Fontenete et al. (2015) and mentioned above.

Figure 11 displays an epifluorescence image taken for each of these three *Citrobacter* strains at 60ºC and 64ºC. The images for all the other non-optimal temperatures can be seen in Figures 15, 16 and 17 in Appendix B.

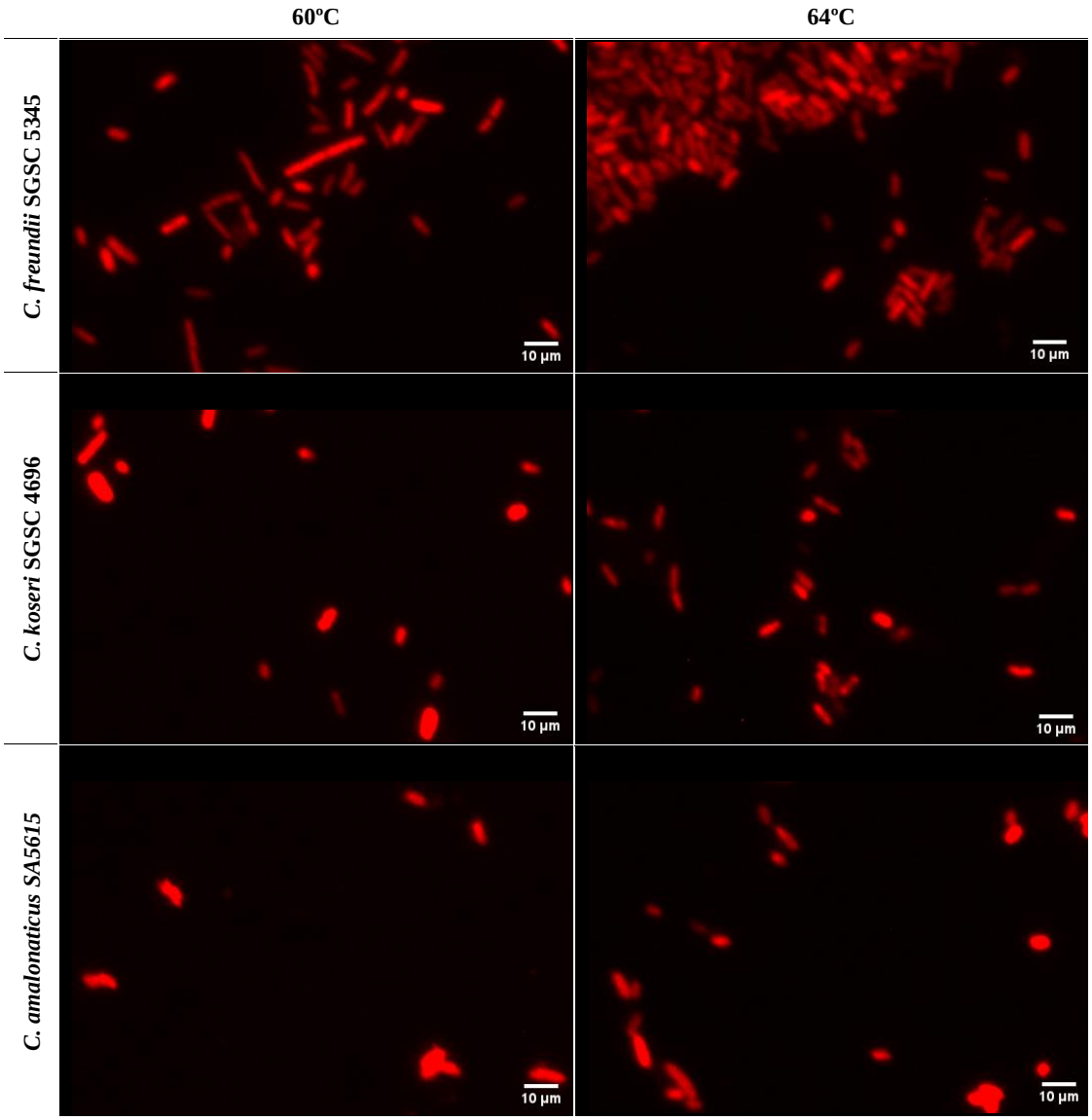


Figure 11 – Epifluorescence detection of *C. freundii SGSC 5345*, *C. koseri SGSC 4696* and *C. amalonaticus SA5615* using AM1 and AM2 probes at 60ºC and 64ºC.

In Table 8, the fluorescence values for both probes at both optimal hybridization temperatures are displayed.

Table 8 - Fluorescence values for both probes at 60ºC and 64ºC

Probe name	Strain tested	Temperature (ºC)	
		60ºC	64ºC
AM1	*C. freundii* SGSC 5345	92.3 ± 15.7	104.2 ± 11.9
AM2	*C. koseri* SGSC 4696	123.2 ± 40.4	102.7 ± 40.7
	C. amalonaticus SA5615	119.5 ± 31.0	107.7 ± 24.0

Even though the images in Figure 11 did not exhibit a visual significant difference in fluorescence between these strains at both temperatures, Table 8 shows that the fluorescence values were, in fact, different.

A paired-samples t-test was done to compare the fluorescence values at all tested temperatures for each strain. For *C. freundii* SGSC 5345, there was not a significant difference between the fluorescence result at 64ºC and the fluorescence results at 56ºC, 58ºC, 60ºC and 62ºC (M= 18.71, SD = 13.26); t (3) = 2.821, p = 0.0667. Regarding *C. koseri* SGSC 4696, there was not a significant difference between the fluorescence result at 60ºC and the fluorescence results at 58ºC, 62ºC and 64ºC (M= 11.74, SD = 8.113); t (2) = 2.507, p = 0.129. Finally, for *C. amalonaticus* SA5615, there was not a significant difference between the fluorescence result at 60ºC and the fluorescence results at 58ºC, 62ºC, 64ºC and 66ºC (M = 10.63, SD = 6.843); t (3) = 3.107, p = 0.053. These results show that a multiplex assay with both designed probes for the detection of these 3 *Citrobacter* species can be performed at either 58ºC, 60ºC, 62ºC or 64ºC, as the paired-samples t-test proved that the fluorescence values displayed by the probes were not significantly different at any of these temperatures.

Regardless of these statistical results, it is important to determine what can explain the different fluorescence values displayed by the *Citrobacter* species. Table 8 shows that, at 60ºC, *C. koseri* SGSC 4696 presented higher fluorescence values than *C. freundii* SGSC 5345 and *C. amalonaticus* SA5615 and, at 64ºC, *C. amalonaticus* SA5615 presented higher fluorescence values than *C. freundii* SGSC 5345 and *C. koseri* SGSC 4696. When bacterial cells are in exponential growth in steady-state conditions, their volume and macromolecular content increase exponentially at a similar rate. Their specific growth rate is determined by the rate at which cells can produce new proteins. This synthetization capability, on the other hand, depends not only on the ribosome

concentration in the cells, but also on how efficient the ribosomes are used (Bosdriesz et al. 2015).

However, there is an optimal ribosome concentration that must not be exceeded, as the protein concentration of a bacterial cell remains constant over time. This means that the production of more ribosomal proteins leads to reduction of the metabolic proteins' concentration. Since these metabolic proteins are the ones responsible for synthesizing amino acids (who increase ribosome's efficiency), a balance must be established between the amino acids' supply and protein synthesis to guarantee a proper function of the bacterial cells (Bosdriesz et al. 2015).

To sum up, a correlation between RNA content and the specific growth rate of a bacterial cell can be established in the exponential growth phase, where a higher specific growth rate value equals more ribosomal content (Hidalgo et al. 2022). Moreover, the higher the ribosomal content is in a cell, the higher is the number of probes that are capable of binding to their target sequence, ultimately leading to a higher signal intensity (Tang et al. 2005).

Taking this into account, the growth curves of *C. freundii* SGSC 5345, *C. koseri* SGSC 4696 and *C. amalonaticus* SA5615 were plotted and can be observed in Figure 12.

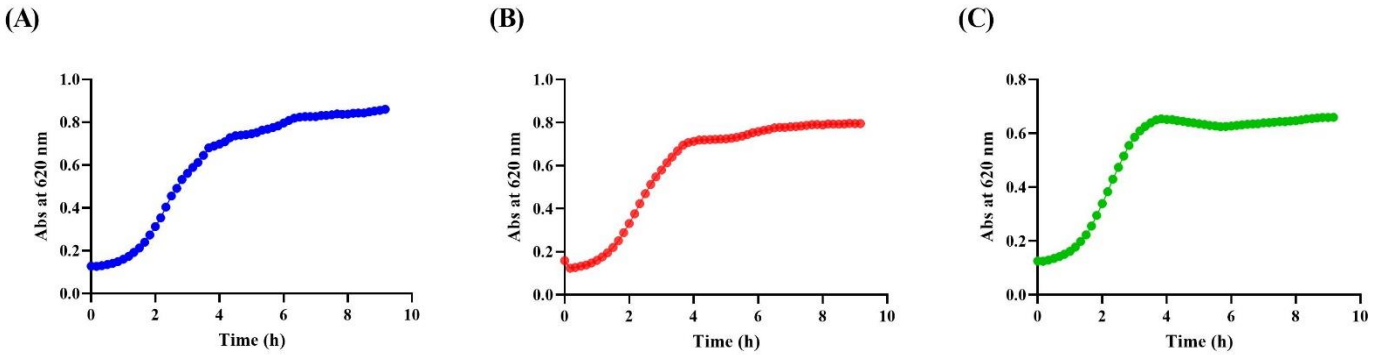


Figure 12 - Growth curves of *C. freundii* SGSC 5345 (A), *C. koseri* SGSC 4696 (B) and *C. amalonaticus* SA5615 (C) at 37ºC and 120 rpm.

The specific growth rate was calculated from the linear slope of the curves present in Figure 12. The values are present in Table 9.

Table 9 – Specific growth rate of each *Citrobacter* strain

Strain	**Specific growth rate (h^{-1})**
C. freundii SGSC 5345	0.520 ± 0.007
C. koseri SGSC 4696	0.530 ± 0.006
C. amalonaticus SA5615	0.600 ± 0.011

As can be seen in Table 9, the specific growth rates of these *Citrobacter* strains are very similar. Given these results, the previously-mentioned relation between ribosome content and specific growth rate in bacterial cells is deemed unconfirmed for these 3 *Citrobacter* strains.

Another alternative to partly help explain the discrepant fluorescence values is the thermodynamic approach (Yilmaz et al. 2004). As previously mentioned in Chapter 2 of this dissertation, the ΔG^{o} overall is the parameter that indicates the thermodynamic favorability of a reaction (Yilmaz et al. 2004). Reactions are more favorable for more negative ΔG^{o} overall values, but very negative ΔG^{o} values lead to hybridization with sequences that are not fully complementary (Yilmaz et al. 2004). A study done by Azevedo et al. (2015) suggests that ΔG^{o} overall values between -26 and -42 kcal mol^{-1} seem to lead towards higher affinity for LNA/2'OMe probes with a length between 14 and 16 base pairs (Azevedo et al. 2015). In fact, the theoretically-determined ΔG^{o} values of AM1 and AM2 probes are within this interval range. Furthermore, a study done by Liu et al. (2007) concluded that lower ΔG^{0} overall values not only increase probe-RNA duplex formation probability, but also lead to higher fluorescence values (Liu et al. 2007). AM2 probe has a lower ΔG^{0} overall value (-33.15 kcal mol^{-1}) than AM1 probe (-30.22 kcal mol^{-1}) (Table 7), and both *Citrobacter* strains that hybridized with AM2 probe in this dissertation presented higher fluorescence values than the *Citrobacter* strain that hybridized with AM1 probe, with the only exception being *C. koseri* SGSC 4696 with AM2 probe at 64ºC having a slightly lower fluorescence value that *C. freundii* SGSC 5345 with AM1 probe for that same temperature (Table 8). In part, these observations may explain the discrepancy of fluorescence values displayed by the probes involved in the present study.

4.2.2 Specificity

In a study done by Kuo et al. (2020), a 20-bp probe was developed for the detection of *C. freundii* in water samples (Kuo et al. 2020). However, a theoretical analysis of this probe (supported with data from arb-silva's database) revealed that, despite having high specificity, the sensitivity of this probe is lower than 50%. These parameters are crucially important, as they have a direct impact on FISH's most important step: hybridization (Oliveira et al. 2021).

For the specificity test of AM1 and AM2 probes, 17 non-targets species were tested to determine the occurrence of cross-hybridization, that is, the tendency of the probes to hybridize with non-targets species. The hybridization conditions were the same as previously used to determine the optimal hybridization temperature. The strains that were considered to have had cross-hybridization were the ones where a signal could be detected in the epifluorescence microscopy images. If the signal was too weak and cells could not be seen clearly, or if there was no signal at all, no cross-hybridization was considered. The images can be consulted in Figures 18 and 19 in Appendix C. The cross-hybridization results are represented in Table 10.

Table 10 – Specificity test at 60ºC and 64ºC for AM1 and AM2 probes based on epifluorescence microscopy images.

	Temperature (ºC)			
	60ºC		**64ºC**	
	AM1 Probe	**AM2 Probe**	**AM1 Probe**	**AM2 Probe**
Campylobacter coli CNET 19	+	+	+	+
Fusobacterium necrophorum DSM21784	+	+	+	+
Clostridium perfringes ATCC 13124	+	+	+	+
Pseudomonas fluorescens CECT 378	-	-	-	-
Pseudomonas aeruginosa PAO1	-	-	-	-
Escherichia coli ATCC 25922	-	-	-	-
Klebsiella pneumoniae ATCC 43816	-	-	-	-
Staphylococcus epidermidis RP62A CECT 4184	-	-	-	-
Salmonella typhimurium SGSC 2523	+	+	+	+
Acinetobacter baumannii NCTC 13423	-	+	-	+
Enterococcus faecalis CECT 184	-	-	-	-
Listeria monocytogenes ATCC 7644	-	-	-	-
Enterococcus faecium CECT 410	-	-	-	-
Proteus mirabilis CECT 4101	+	-	-	-
Streptococcus mitis ATCC15919	-	-	-	-

Lactobacillus casei LMG 6964	-	-	-	-
Helicobacter pylori 26695	+	-	-	-

The + symbol means the occurrence of cross-hybridization. The - symbol means that no cross-hybridization occurred.

As seen in Table 10, AM1 probe had cross-hybridization with C. *coli* CNET 19, *F. necrophorum* DSM21784, *C. perfringes* ATCC 13124, *S. typhimurium* SGSC 2523, *P. mirabilis* CECT 4101 and *H. pylori* 26695 at 60ºC and had cross-hybridization with these same strains (except *P. mirabilis* CECT 4101 and *H. pylori* 26695) and *Acinetobacter baumannii* NCTC 13423 at 64ºC. On the other hand, AM2 probe had cross-hybridization with *C. coli* CNET 19, *F. necrophorum* DSM21784, *C. perfringes* ATCC 13124, *S. typhimurium* SGSC 2523 and *A. baumannii* NCTC 13423 at both 60ºC and 64ºC.

To effectively understand and determine if the probes could be completely complementary with these strains, 16S rRNA sequences of these strains (or of different strains, but the same species) were aligned with the reverse complement sequence of AM1 and AM2 probes. The partial alignments for both probes are present in Figure 13.

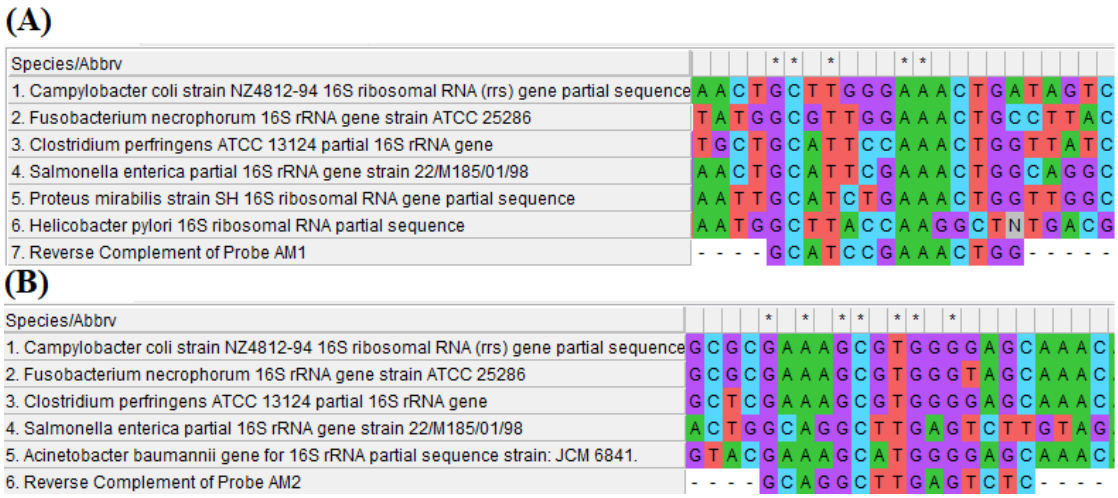


Figure 13 - Partial alignment of the reverse complement sequence of AM1 probe (A) and AM2 probe (B) with the species that were considered to have had cross-hybridization in the specificity test.

As observed in Figure 13, *C. coli*, *F. necrophorum*, *S. enterica* (also known as *S. typhimurium*) and *H. pylori* have several mismatches compared to the reverse complement of AM1 probe. On the other hand, *P. mirabilis* and *C. perfringens* have 1 and 2 mismatches, respectively. All the species that had cross-hybridization with AM2 probe have several mismatches with the reverse complement sequence of this probe.

To sum up, no species showed full complementarity with any of the probes. This means that further tests should be carried out in order to find the hybridization conditions that don't allow cross-hybridization to occur by any chance for both probes.

4.2.3 Sensitivity

For the sensitivity tests of AM1 and AM2 probes, different *Citrobacter* strains from different origins were hybridized with the respective probes. The strains used are detailed in Table 11 in Annex A. The hybridization conditions were the same used to determine the optimal hybridization temperature. As previously discussed in chapter 4.2.1, both probes can be used at a hybridization temperature of either 58ºC, 60ºC, 62ºC or 64ºC in a multiplex assay. Given this, the hybridization temperature chosen for the sensitivity test was 60ºC. The results are present in Figure 14.

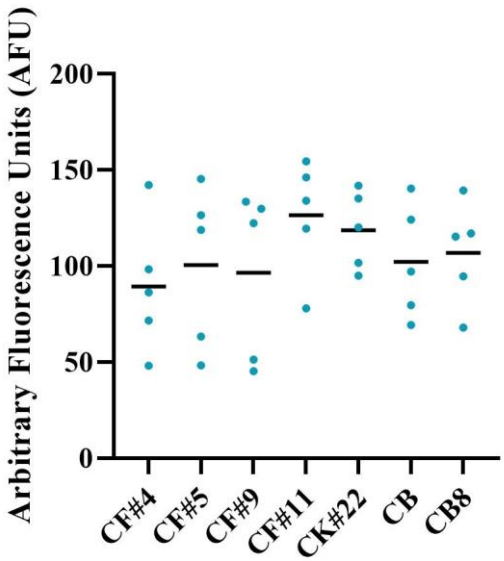


Figure 14 - Sensitivity test at 60ºC for AM1 and AM2 probes.

As seen in Figure 14, the strains with both the highest and lowest fluorescence values are *C. freundii* species (CF#11 and CF#4, respectively), while *C. koseri* strain CK#22 had the second highest fluorescence value, only behind the *C. freundii* strain CF#11. A paired-samples t-test was done to compare the fluorescence values of the *C. freundii* strains CF#4, CF#5, CF#9, and CF#11 with *C. koseri* strain CK#22. The results showed that there was not a significant difference between the fluorescence value of CK#22 and the fluorescence values of the forementioned *C. freundii* strains (M= 15.56, SD = 16.17); t

(3) = 1.925, p = 0.1499, meaning that these results are in agreement with the results previously obtained in the optimal hybridization temperature tests.

The average fluorescence values and the epifluorescence of the sensitivity test can be seen in Table 13 and Figure 20 in Appendix D, respectively.

5 Conclusion

For the detection of *Citrobacter spp.* in UTIs, two LNA/2'OMe-FISH probes, named AM1 and AM2, targeting the 16S rRNA structures of these species, were designed. The results imply that FISH is theoretically a valid option over traditional methods, as the probes were found to be theoretically very specific and sensitive for the detection of *Citrobacter* strains.

Despite the probes exhibiting different optimal hybridization temperatures (64ºC for AM1 probe and 60ºC for AM2 probe), a paired-samples t-test showed that the fluorescence values displayed by both probes at 58ºC, 60ºC, 62ºC and 64ºC are not significantly different, meaning that any of these temperatures can be used in a multiplex assay for the detection of *C. freundii* SGSC 5345, *C. koseri* SGSC 4696 and *C. amalonaticus* SA5615.

Moreover, a discrepancy of fluorescence values between *Citrobacter* species was observed. To further explain this, a relation between the specific growth rate and the fluorescence intensity signal of strains *C. freundii* SGSC 5345, *C. koseri* SGSC 4696 and *C. amalonaticus* SA5615 was suggested, but was unconfirmed. These differences were instead attributed to the different ΔG^0 overall values displayed by both probes.

The specificity test showed that there was cross-hybridization for both probes at 60ºC and 64ºC for the hybridization conditions used throughout this study. For the sensitivity test, a paired-samples t-test showed that the fluorescence values between the *C. freundii* strains (hybridized with AM1 probe) and the *C. koseri* strain (hybridized with AM2 probe) at 60ºC were not significantly different, confirming the results previously obtained during the optimal hybridization temperature tests.

6 Assessment of the work done

6.1 Objectives Achieved

The original aim of this dissertation, which was the development of a NAM-FISH probe for the detection of *Citrobacter freundii*, was achieved, but in a different manner than originally intended. The objective change that occurred during the course of this dissertation led to the development of two LNA/2'OMe-FISH probes (AM1 and AM2) targeting the *Citrobacter* genus. Since one of these probes (AM1) can detect *C. freundii* with a theoretical high sensitivity (>90%), it can be said that the original objective of this dissertation was somehow achieved.

6.3 Final Assessment

Given the subjective nature of epifluorescence microscopy (as different thresholds can be applied by different people for the same images), it is suggested to repeat the optimal hybridization temperature tests using flow cytometry, to determine if the results lead to the same conclusions presented in this dissertation.

In an attempt to improve the specificity results, it is suggested to repeat these tests with the same strains using different hybridization conditions, such as hybridization time and denaturant type and/or concentration. Besides that, a boarder range of species should be tested for both the specificity and sensitivity tests.

To better understand the differences in fluorescence intensity of both probes, it is suggested to determine the 16S rRNA copy number of strains *C. freundii* SGSC 5345, *C. koseri* SGSC 4696 and *C. amalonaticus* SA5615 by applying a quantitative real-time PCR assay (Liu et al. 2012).

Finally, to validate this method, it is suggested to test the AM1 and AM2 probes in urine samples from patients displaying UTI symptoms.

7 References

Apte, A. & Daniel, S. (2009). PCR primer design. Cold Spring Harbor protocols, 2009(3), pdb.ip65. https://doi.org/10.1101/pdb.ip65

Aquino de Muro, Marilena. (2008). Probe Design, Production, and Applications. 10.1007/978-1-60327-375-6_4

Azevedo, A. S., Almeida, C., Pereira, B., Madureira, P., Wengel, J., & Azevedo, N. F. (2015). Detection and discrimination of biofilm populations using locked nucleic acid/2 -O-methyl-RNA fluorescence *in situ* hybridization (LNA/2'OMe-FISH). Biochemical Engineering Journal. 104, 64-73. doi:10.1016/j.bej.2015.04.024

Azevedo, A. S., Sousa, I. M., Fernandes, R. M., Azevedo, N. F., Almeida, C. (2019) Optimizing locked nucleic acid/2'-O-methyl-RNA fluorescence *in situ* hybridization (LNA/2'OMe-FISH) procedure for bacterial detection. PLoS ONE 14(5): e0217689. https://doi.org/10.1371/journal.pone.0217689

Azevedo, A. S., Fernandes, R. M., Faria, A. R., Silvestre, O. F., Nieder, J. B., Lou, C., Wengel, J., Almeida, C., & Azevedo, N. F. (2022). Spectral imaging and nucleic acid mimics fluorescence *in situ* hybridization (SI-NAM-FISH) for multiplex detection of clinical pathogens. Frontiers in microbiology, 13, 976639. https://doi.org/10.3389/fmicb.2022.976639

Borenshtein, D. & Schauer, D.B. (2006). The Genus *Citrobacter*. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, KH., Stackebrandt, E. (eds) The Prokaryotes. Springer, New York, NY. https://doi.org/10.1007/0-387-30746-X_5

Brenner, D. J., Grimont, P. A., Steigerwalt, A. G., Fanning, G. R., Ageron, E., & Riddle, C. F. (1993). Classification of citrobacteria by DNA hybridization: designation of *Citrobacter farmeri sp.* nov., *Citrobacter youngae sp.* nov., *Citrobacter* braakii sp. nov., *Citrobacter werkmanii sp*. nov., *Citrobacter sedlakii sp.* nov., and three unnamed *Citrobacter* genomospecies. International journal of systematic bacteriology, 43(4), 645–658. https://doi.org/10.1099/00207713-43-4-645

Brenner, D. J., O'Hara, C. M., Grimont, P. A., Janda, J. M., Falsen, E., Aldova, E., Ageron, E., Schindler, J., Abbott, S. L., & Steigerwalt, A. G. (1999). Biochemical identification of *Citrobacter* species defined by DNA hybridization and description of

Citrobacter gillenii sp. nov. (formerly *Citrobacter genomospecies* 10) and *Citrobacter murliniae sp.* nov. (formerly *Citrobacter genomospecies* 11). Journal of clinical microbiology, 37(8), 2619–2624. https://doi.org/10.1128/JCM.37.8.2619-2624.1999

Bosdriesz, E., Molenaar, D., Teusink, B., & Bruggeman, F. J. (2015). How fast-growing bacteria robustly tune their ribosome concentration to approximate growth-rate maximization. The FEBS Journal, (282), 2029-2044. [10]. https://doi.org/10.1111/febs.13258

Clermont, D., Motreff, L., Passet, V., Fernandez, J. C., Bizet, C., & Brisse, S. (2015). Multilocus sequence analysis of the genus *Citrobacter* and description of *Citrobacter pasteurii sp.* nov. International journal of systematic and evolutionary microbiology, 65(Pt 5), 1486–1490. https://doi.org/10.1099/ijs.0.000122

Custovic, A., Smajlovic, J., Hadzic, S., Ahmetagic, S., Tihic, N., & Hadzagic, H. (2014). Epidemiological surveillance of bacterial nosocomial infections in the surgical intensive care unit. Materia socio-medica, 26(1), 7–11. https://doi.org/10.5455/msm.2014.26.7-11

Dr. P. Sneka, & Dr. V. Mangayarkarasi. (2019). Bacterial pathogens causing UTI and their antibiotic sensitivity pattern: a study from a tertiary care hospital from South India. Tropical Journal of Pathology and Microbiology, 5(6), 379-385. https://doi.org/10.17511/jopm.2019.i06.08

Fontenete, S., Guimarães, N., Wengel, J., & Azevedo, N. F. (2016). Prediction of melting temperatures in fluorescence *in situ* hybridization (FISH) procedures using thermodynamic models. Critical reviews in biotechnology, 36(3), 566–577. https://doi.org/10.3109/07388551.2014.993589

Fontenete, S., Carvalho, D. R., Guimarães, N., Madureira, P., Figueiredo, C., Wengel, J., & Azevedo, N. F. (2016). Application of locked nucleic acid-based probes in fluorescence *in situ* hybridization. Applied Microbiology and Biotechnology, 100(13), 5897-5906. https://doi.org/10.1007/s00253-016-7429-4

Frederiksen, W. (1970). "*Citrobacter koseri* (n. sp.) a new species within the genus *Citrobacter*, with a comment on the taxonomic position of *Citrobacter intermedium* (Werkman and Gillen)." Publ. Fac. Sci. Univ. JE Purkyne Brno 47: 89-94.

Gajdács, M., & Urbán, E. (2019). Resistance Trends and Epidemiology of *Citrobacter-Enterobacter-Serratia* in Urinary Tract Infections of Inpatients and Outpatients (RECESUTI): A 10-Year Survey. Medicina (Kaunas, Lithuania), 55(6), 285. https://doi.org/10.3390/medicina55060285

Ghaderinezhad, F., Ceylan Koydemir, H., Tseng, D., Karinca, D., Liang, K., Ozcan, A., & Tasoglu, S. (2020). Sensing of electrolytes in urine using a miniaturized paper-based device. Scientific reports, 10(1), 13620. https://doi.org/10.1038/s41598-020-70456-6

Guimarães, N.M., Azevedo, N.F., Almeida, C. (2021). FISH Variants. In: Azevedo, N.F., Almeida, C. (eds) Fluorescence *In-Situ* Hybridization (FISH) for Microbial Cells. Methods in Molecular Biology, vol 2246. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1115-9_2

Hasandka, A., Singh, A.R., Prabhu, A. et al. Paper and thread as media for the frugal detection of urinary tract infections (UTIs). Anal Bioanal Chem 414, 847–865 (2022). https://doi.org/10.1007/s00216-021-03671-3

Hidalgo, D., Martínez-Ortiz, C. A., Palsson, B. O., Jiménez, J. I., & Utrilla, J. (2022). Regulatory perturbations of ribosome allocation in bacteria reshape the growth proteome with a trade-off in adaptation capacity. iScience, 25(3), 103879. https://doi.org/10.1016/j.isci.2022.103879

Karkare, S., & Bhatnagar, D. (2006). Promising nucleic acid analogs and mimics: characteristic features and applications of PNA, LNA, and morpholino. Applied microbiology and biotechnology, 71(5), 575–586. https://doi.org/10.1007/s00253-006-0434-2

Kumar, M. S., Ghosh, S., Nayak, S., & Das, A. P. (2016). Recent advances in biosensor based diagnosis of urinary tract infection. Biosensors & bioelectronics, 80, 497–510. https://doi.org/10.1016/j.bios.2016.02.023

Kuo, J. T., Chang, L. L., Yen, C. Y., Tsai, T. H., Chang, Y. C., Huang, Y. T., & Chung, Y. C. (2020). Development of Fluorescence *In Situ* Hybridization as a Rapid, Accurate Method for Detecting Coliforms in Water Samples. Biosensors, 11(1), 8. https://doi.org/10.3390/bios11010008

Lee, Jasmine & Neild, Guy. (2007). Urinary Tract Infection. Medicine. 35. 423-428. 10.1016/j.mpmed.2007.05.009.

Liu, W. T., Guo, H., & Wu, J. H. (2007). Effects of target length on the hybridization efficiency and specificity of rRNA-based oligonucleotide microarrays. Applied and environmental microbiology, 73(1), 73–82. https://doi.org/10.1128/AEM.01468-06

Liu, L., Lan, R., Liu, L., Wang, Y., Zhang, Y., Wang, Y., & Xu, J. (2017). Antimicrobial Resistance and Cytotoxicity of *Citrobacter spp.* in Maanshan Anhui Province, China. Frontiers in microbiology, 8, 1357. https://doi.org/10.3389/fmicb.2017.01357

Lunell, S., & Sperber, G. (1967). Study of the hydrogen bonding in the adenine-thymine, adenine-cytosine, and guanine-thymine base pairs. The Journal of chemical physics, 46(6), 2119–2124. https://doi.org/10.1063/1.1841009

Metri, B. C., Jyothi, P., & Peerapur, B. V. (2013). Antibiotic resistance in *Citrobacter spp.* isolated from urinary tract infection. Urology annals, 5(4), 312–313. https://doi.org/10.4103/0974-7796.120295

Mo Y. (2006). Probing the nature of hydrogen bonds in DNA base pairs. Journal of molecular modeling, 12(5), 665–672. https://doi.org/10.1007/s00894-005-0021-y

Nácher-Vázquez, M., Santos, B., Azevedo, N. F., & Cerqueira, L. (2022). The role of Nucleic Acid Mimics (NAMs) on FISH-based techniques and applications for microbial detection. Microbiological research, 262, 127086. https://doi.org/10.1016/j.micres.2022.127086

Nkuipou-Kenfack, E., Engel, H., Fakih, S., & Nocker, A. (2013). Improving efficiency of viability-PCR for selective detection of live cells. Journal of microbiological methods, 93(1), 20–24. https://doi.org/10.1016/j.mimet.2013.01.018

Oberhettinger, P., Schüle, L., Marschal, M., Bezdan, D., Ossowski, S., Dörfel, D., Vogel, W., Rossen, J. W., Willmann, M., & Peter, S. (2020). Description of *Citrobacter cronae sp.* nov., isolated from human rectal swabs and stool samples. International journal of systematic and evolutionary microbiology, 70(5), 2998–3003. https://doi.org/10.1099/ijsem.0.004100

Oliveira, H., Pinto, G., Oliveira, A., Oliveira, C., Faustino, M. A., Briers, Y., Domingues, L., & Azeredo, J. (2016). Characterization and genome sequencing of a *Citrobacter freundii* phage CfP1 harboring a lysin active against multidrug-resistant isolates. Applied microbiology and biotechnology, 100(24), 10543–10553. https://doi.org/10.1007/s00253-016-7858-0

Oliveira, R., Azevedo, A. S., & Mendes, L. (2021). Application of Nucleic Acid Mimics in Fluorescence *In Situ* Hybridization. Methods in molecular biology (Clifton, N.J.), 2246, 69–86. https://doi.org/10.1007/978-1-0716-1115-9_5

Pirkani, G. S., Awan, M. A., Abbas, F., & Din, M. (2020). Culture and PCR based detection of bacteria causing urinary tract infection in urine specimen. Pakistan journal of medical sciences, 36(3), 391–395. https://doi.org/10.12669/pjms.36.3.1577

Plakkal, N., Soraisham, A. S., & Amin, H. (2013). *Citrobacter freundii* brain abscess in a preterm infant: a case report and literature review. Pediatrics and neonatology, 54(2), 137–140. https://doi.org/10.1016/j.pedneo.2012.10.004

Prudent, E., & Raoult, D. (2019). Fluorescence *in situ* hybridization, a complementary molecular tool for the clinical diagnosis of infectious diseases by intracellular and fastidious bacteria. FEMS microbiology reviews, 43(1), 88–107. https://doi.org/10.1093/femsre/fuy040

Ribeiro, T. G., Clermont, D., Branquinho, R., Machado, E., Peixe, L., & Brisse, S. (2017). *Citrobacter europaeus sp.* nov., isolated from water and human faecal samples. International journal of systematic and evolutionary microbiology, 67(1), 170–173. https://doi.org/10.1099/ijsem.0.001606

Ribeiro, T. G., Gonçalves, B. R., da Silva, M. S., Novais, Â., Machado, E., Carriço, J. A., & Peixe, L. (2017). *Citrobacter portucalensis sp.* nov., isolated from an aquatic sample. International journal of systematic and evolutionary microbiology, 67(9), 3513–3517. https://doi.org/10.1099/ijsem.0.002154

Ribeiro, T. G., Izdebski, R., Urbanowicz, P., Carmeli, Y., Gniadkowski, M., & Peixe, L. (2021). *Citrobacter telavivum sp*. nov. with chromosomal mcr-9 from hospitalized patients. European journal of clinical microbiology & infectious diseases: official publication of the European Society of Clinical Microbiology, 40(1), 123–131. https://doi.org/10.1007/s10096-020-04003-6

Robertson, K. L., & Thach, D. C. (2009). LNA flow-FISH: a flow cytometry-fluorescence *in situ* hybridization method to detect messenger RNA using locked nucleic acid probes. Analytical biochemistry, 390(2), 109–114. https://doi.org/10.1016/j.ab.2009.04.026

Rocha, R., Sousa, J. M., Cerqueira, L., Vieira, M. J., Almeida, C., & Azevedo, N. F. (2019). Development and application of Peptide Nucleic Acid Fluorescence *in situ* Hybridization for the specific detection of *Listeria monocytogenes*. Food microbiology, 80, 1–8. https://doi.org/10.1016/j.fm.2018.12.009

Sabir, N., Ikram, A., Zaman, G., Satti, L., Gardezi, A., Ahmed, A., & Ahmed, P. (2017). Bacterial biofilm-based catheter-associated urinary tract infections: Causative pathogens and antibiotic resistance. American journal of infection control, 45(10), 1101–1105. https://doi.org/10.1016/j.ajic.2017.05.009

Samonis, G., Karageorgopoulos, D. E., Kofteridis, D. P., Matthaiou, D. K., Sidiropoulou, V., Maraki, S., & Falagas, M. E. (2009). *Citrobacter* infections in a general hospital: characteristics and outcomes. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology, 28(1), 61–68. https://doi.org/10.1007/s10096-008-0598-z

Santos, M., Mariz, M., Tiago, I., Martins, J., Alarico, S., & Ferreira, P. (2022). A review on urinary tract infections diagnostic methods: Laboratory-based and point-of-care approaches. Journal of pharmaceutical and biomedical analysis, 219, 114889. https://doi.org/10.1016/j.jpba.2022.114889

Schauer, D. B., Zabel, B. A., Pedraza, I. F., O'Hara, C. M., Steigerwalt, A. G., & Brenner, D. J. (1995). Genetic and biochemical characterization of *Citrobacter rodentium sp*. nov. Journal of clinical microbiology, 33(8), 2064–2068. https://doi.org/10.1128/jcm.33.8.2064-2068.1995

Shakoori A. R. (2017). Fluorescence *In Situ* Hybridization (FISH) and Its Applications. Chromosome Structure and Aberrations, 343–367. https://doi.org/10.1007/978-81-322-3673-3_16

Tang, Y. Z., Gin, K. Y., & Lim, T. H. (2005). High-temperature fluorescent *in situ* hybridization for detecting *Escherichia coli* in seawater samples, using rRNA-targeted

oligonucleotide probes and flow cytometry. Applied and environmental microbiology, 71(12), 8157–8164. https://doi.org/10.1128/AEM.71.12.8157-8164.2005

Teixeira, H., Sousa, A. L., & Azevedo, A. S. (2021). Bioinformatic Tools and Guidelines for the Design of Fluorescence *In Situ* Hybridization Probes. Methods in molecular biology (Clifton, N.J.), 2246, 35–50. https://doi.org/10.1007/978-1-0716-1115-9_3

Wang, Y. C., Tsai, Y. H., Shen, C. F., He, M. Y., Fu, Y. C., Sang, C. Y., Lee, Y. T., & Cheng, C. M. (2021). Turntable Paper-Based Device to Detect *Escherichia coli*. Micromachines, 12(2), 194. https://doi.org/10.3390/mi12020194

Werkman, C. H., & Gillen, G. F. (1932). Bacteria Producing Trimethylene Glycol. Journal of bacteriology, 23(2), 167–182. https://doi.org/10.1128/jb.23.2.167-182.1932

Wu, Q., Li, Y., Wang, M., Pan, X. P., & Tang, Y. F. (2010). Fluorescence *in situ* hybridization rapidly detects three different pathogenic bacteria in urinary tract infection samples. Journal of microbiological methods, 83(2), 175–178. https://doi.org/10.1016/j.mimet.2010.08.015

Xia, T., SantaLucia, J., Jr, Burkard, M. E., Kierzek, R., Schroeder, S. J., Jiao, X., Cox, C., & Turner, D. H. (1998). Thermodynamic parameters for an expanded nearest-neighbor model for formation of RNA duplexes with Watson-Crick base pairs. Biochemistry, 37(42), 14719–14735. https://doi.org/10.1021/bi9809425

Yang, X., Chen, H., Zheng, Y., Qu, S., Wang, H., & Yi, F. (2022). Disease burden and long-term trends of urinary tract infections: A worldwide report. Frontiers in public health, 10, 888205. https://doi.org/10.3389/fpubh.2022.888205

Yilmaz, L. S., & Noguera, D. R. (2004). Mechanistic approach to the problem of hybridization efficiency in fluorescent *in situ* hybridization. Applied and environmental microbiology, 70(12), 7126–7139. https://doi.org/10.1128/AEM.70.12.7126-7139.2004

Annex & Appendix

Annex A – Origin of the *Citrobacter* strains used in the sensitivity tests

Table 11 - Origin of the *Citrobacter* species used in the sensitivity tests.

Species	Strain	Origin	Patient Gender
C. freundii	CF#4	Urine	Male
	CF#5	Urine	Female
	CF#9	Urine	Female
	CF#11	Urine	Male
C. koseri	CK#22	SGSC 5610 (from *Salmonella* Genetic Stock center)	-
C. braakii	CB	Unknown	-
	CB8	Unknown	-

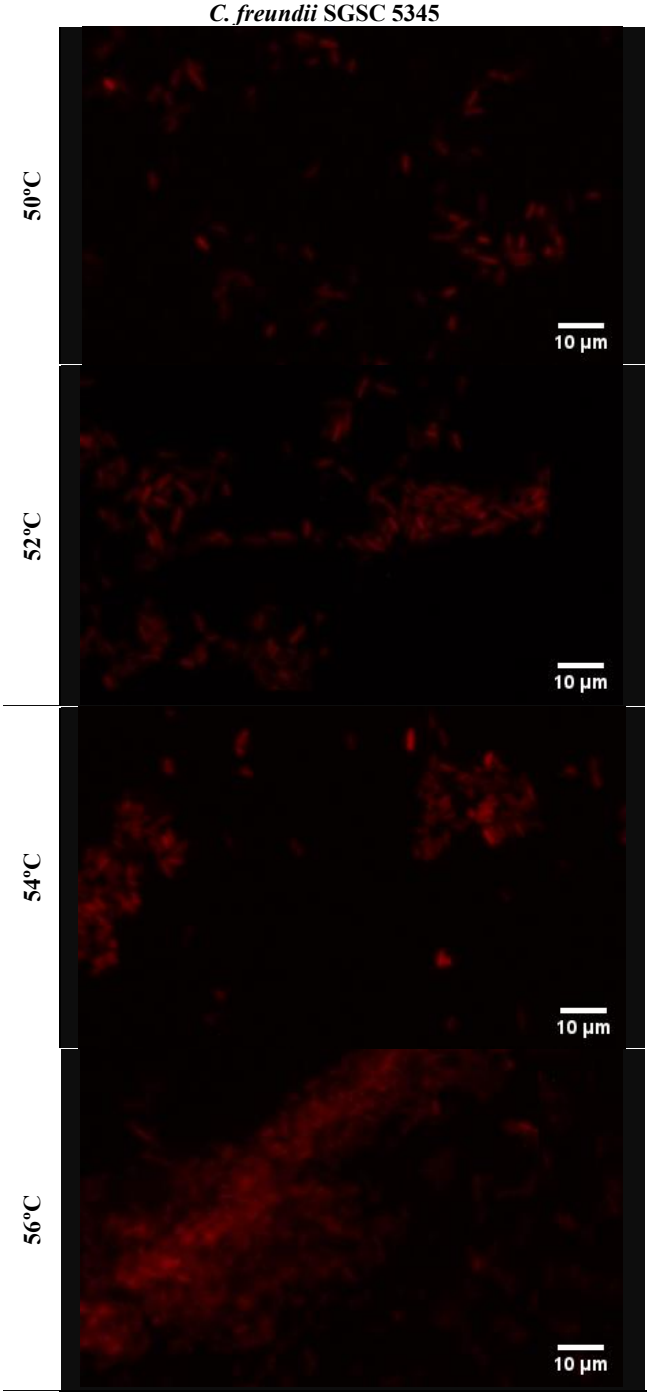
Appendix A – Alignment sets

Table 12 - Species used in the 3 alignments made to design the probe and their respective accession numbers.

1st alignment	2nd alignment	3rd alignment
10 *C. freundii* stains:	10 *C. freundii* stains:	
MF116254	MF116254	
AJ233408	AJ233408	
AB741696	AB741696	
AB680314	AB680314	
FJ768455	FJ768455	
DQ299179	DQ299179	
FJ581026	FJ581026	5 *Citrobacter* species, 2 strains each:
EU420928	EU420928	*C. amalonaticus* (AB680436 and AB680436)
DQ379504	DQ379504	*C. farmeri* (AB504755 and AB741662)
EF371903	EF371903	*C. koseri* (AF025366 and MF185380)
7 *Citrobacter* species:	7 *Citrobacter* species:	*C. rodentium* (AB045737 and AF025363)
C. amalonaticus (AB680436)	*C. gillenii* (DQ223881)	*C. sedlakii* (HM756471 and KM042416)
C. braakii (AB741663)	*C. koseri* (MF185380)	
C. europaeus (LT615140)	*C. murliniae* (KY436219)	
C. farmeri (AB504755)	*C. rodendium* (AF025363)	
C. koseri (AF025366)	*C. sedlakii* (HM756471)	
C. pasteurii (KP057683)	*C. werkmanii* (AF025373)	
C. rodentium (AB045737)	*C. youngae* (AB273741)	
7 *Enterobacteriaceae* species:	7 *Enterobacteriaceae* species:	10 *Enterobacteriaceae* species:
Escherichia coli (AB594752)	*Acinetobacter albensis* (KR611794)	*Enterobacter cloacae* (AB680341)
Morganella morganii (AB089243)	*Enterobacter cloacae* (AB680341)	*Enterobacter hormaechei* (AJ508302)
Plesiomonas shigelloides (FJ796245)	*Klebsiella pneumoniae* (LC335882)	*Echerichia albertii* (AJ508775)
Proteus cibarius (DQ885261)	*Pseudomonas aeruginosa* (AB305018)	*Escherichia coli* (AB594752)
Providencia alcalifaciens (AM407893)	*Salmonella enterica* (AB680018)	*Klebsiella pneumoniae* (LC335882)
Salmonella bongori (AF029227)	*Serratia marcescens* (AB030921)	*Klebsiella oxytoca* (AB004754)
Shigella boydii (AB273731)	*Yersinia aldovae* (AF366376)	*Pseudomonas acephalitica* (AM407893)
		Pseudomonas aeruginosa (AB305018)
		Salmonella bongori **(**AF029227**)**
		Salmonella enterica (AB680018)

Appendix B – Epifluorescence of non-optimal hybridization temperatures

- *Citrobacter freundii* SGSC 5345:



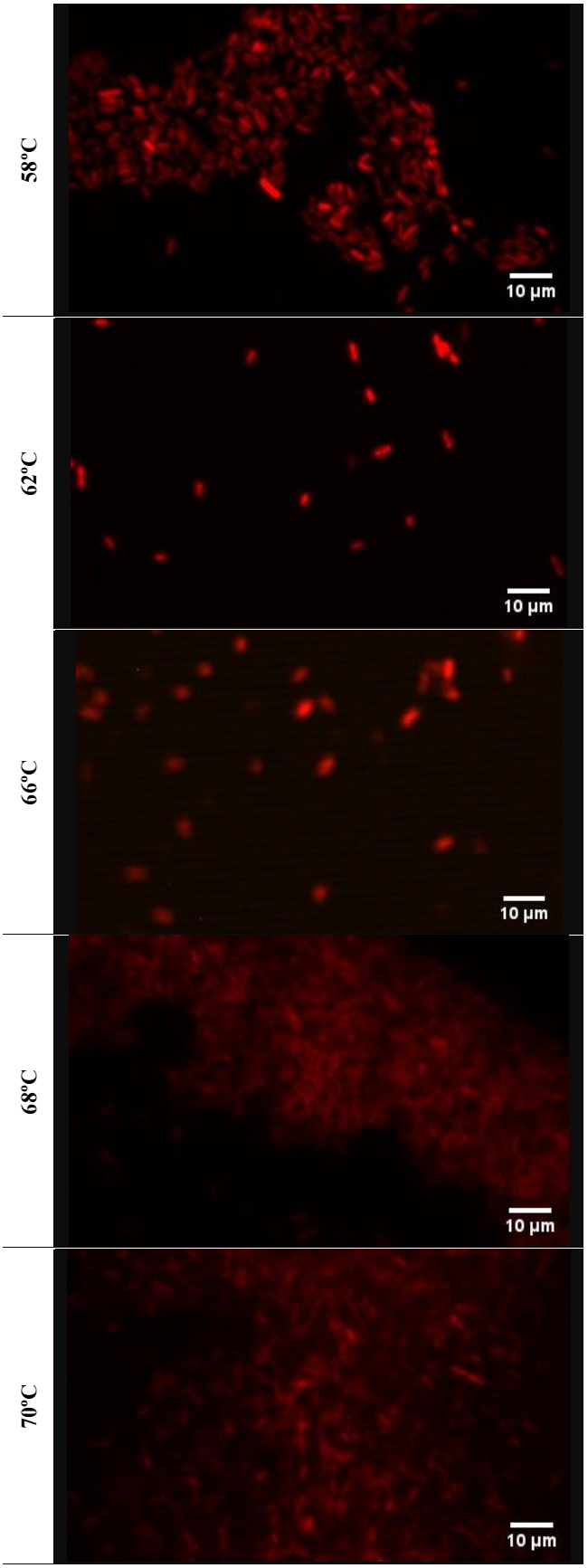


Figure 15 – Epifluorescence detection of all the non-optimal hybridization temperatures tested for *C. freundii* SGSC 5345.

- *Citrobacter koseri* SGSC 4696:

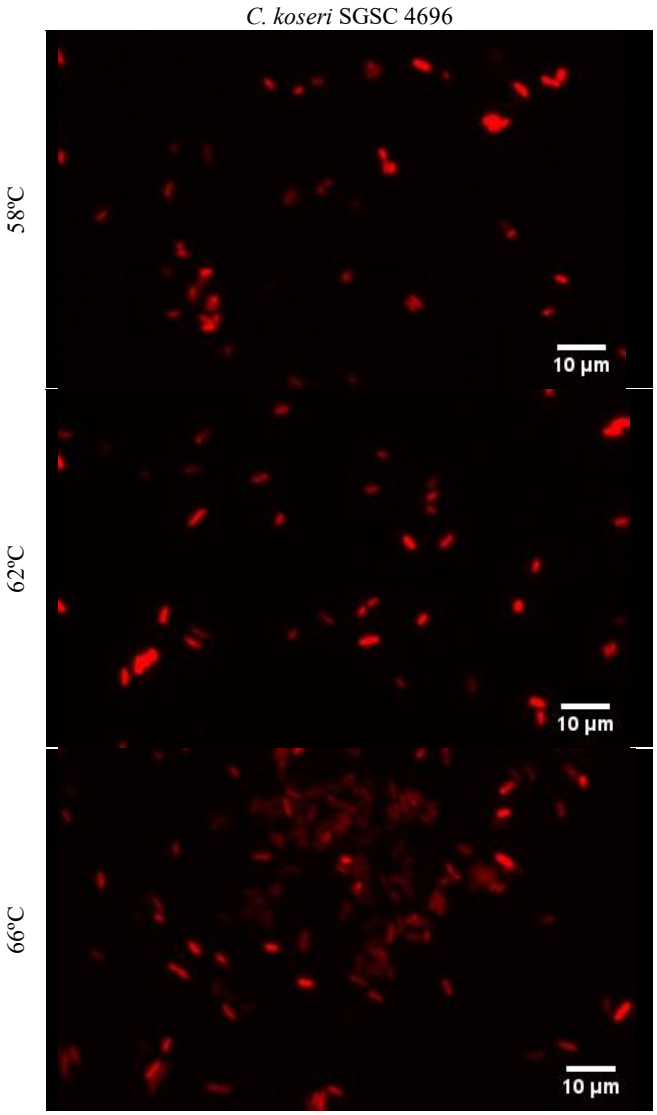


Figure 16 - Epifluorescence detection of all the non-optimal hybridization temperatures tested for *C. koseri* SGSC 4696

- *Citrobacter amalonaticus* SA5615:

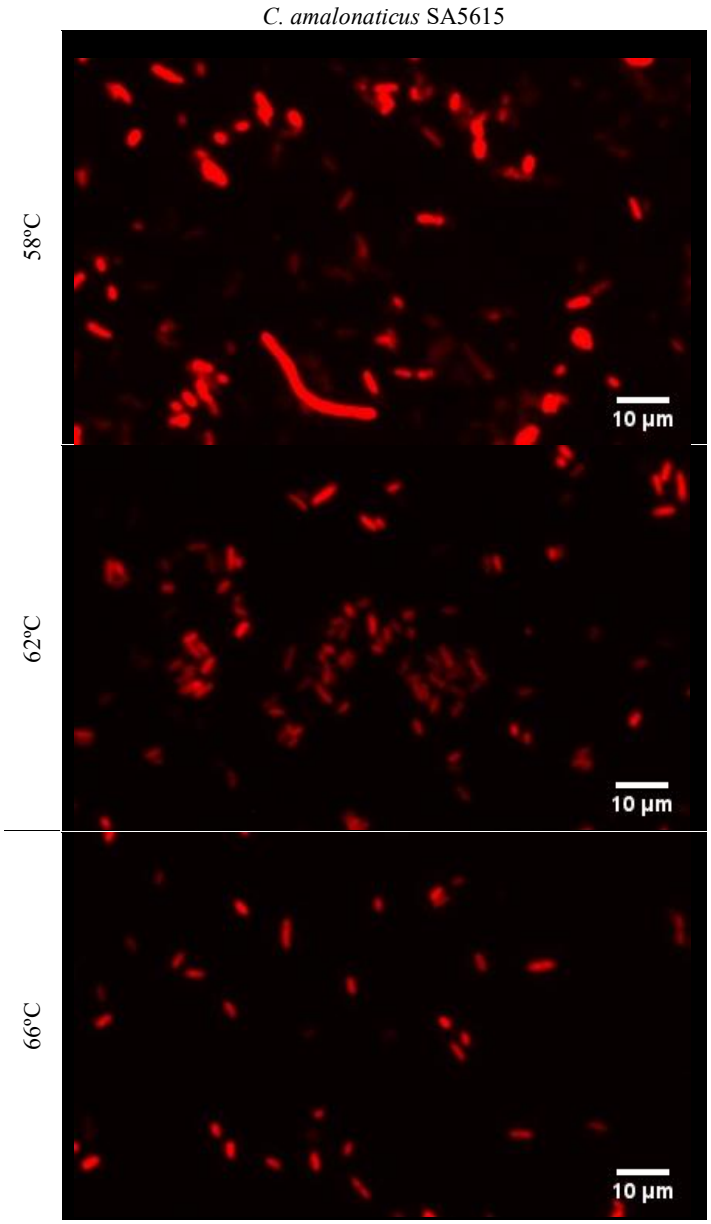


Figure 17 - Epifluorescence detection of all the non-optimal hybridization temperatures tested for *C. amalonaticus* SA5615

Appendix C – Specificity tests results

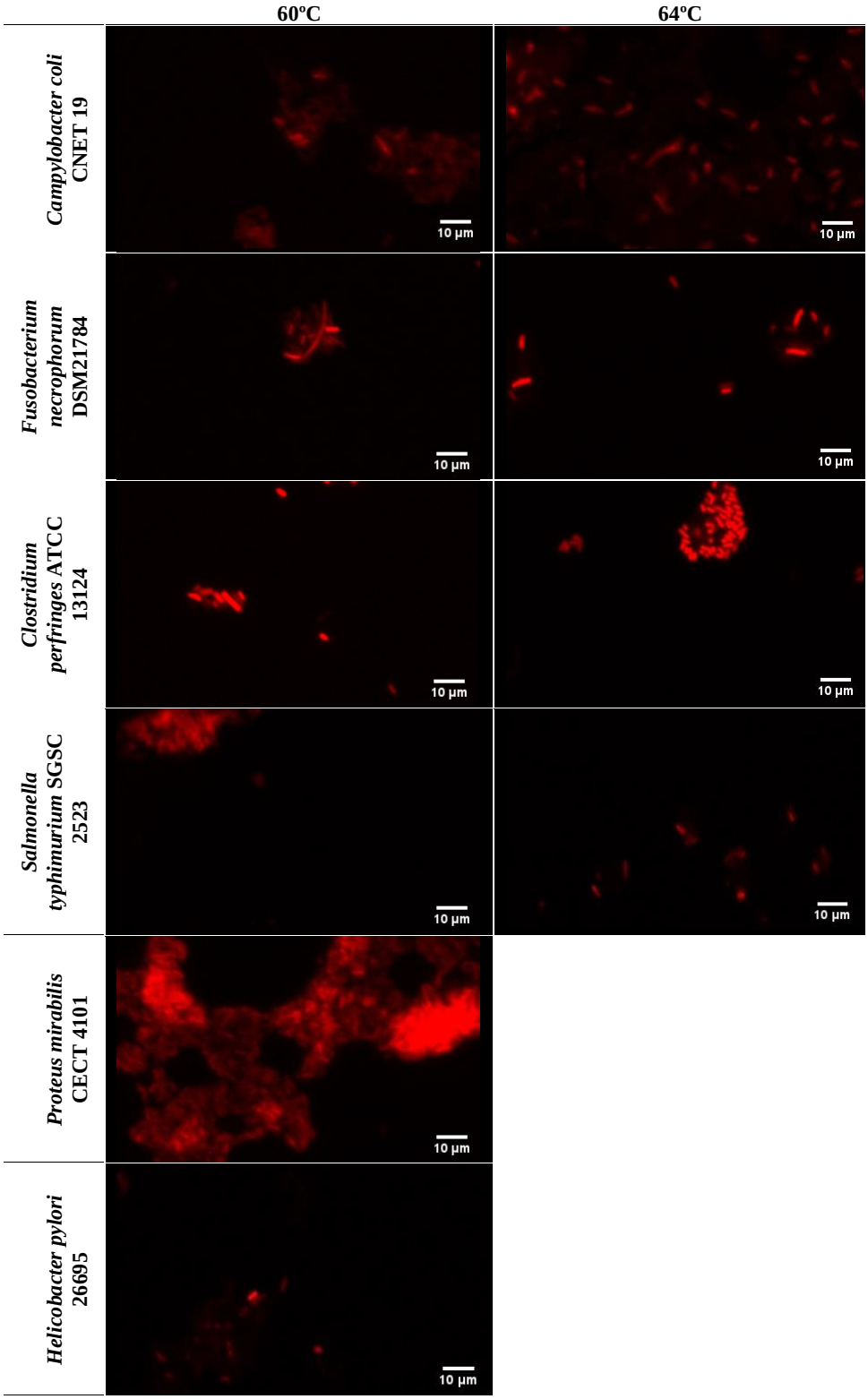


Figure 18 – Epifluorescence detection of the strains that were considered to have had cross-hybridization using AM1 probe at 60ºC and 64ºC.

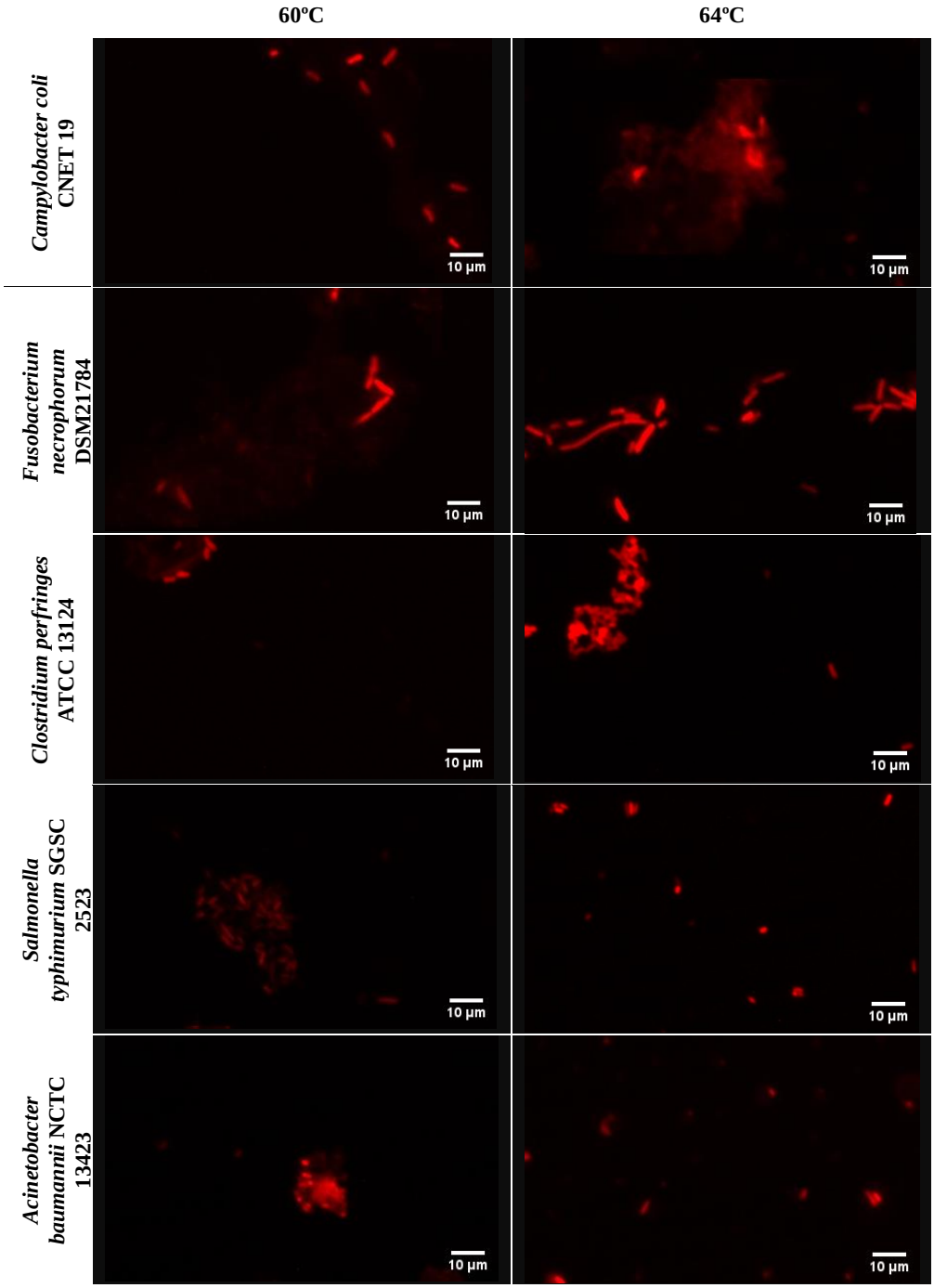
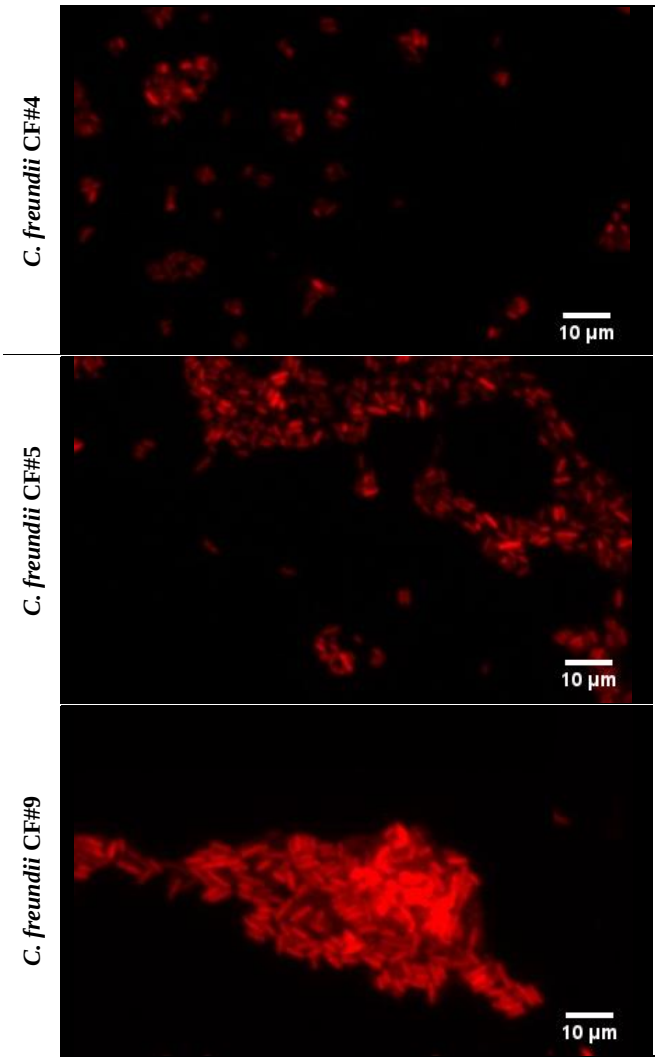


Figure 19 - Epifluorescence detection of the strains that were considered to have had cross-hybridization using AM2 probe at 60ºC and 64ºC.

Appendix D – Sensitivity tests results

Table 13 – AFU values for the sensitivity test at 60ºC for AM1 and AM2 probes

Strains	Probe	AFU
C. freundii CF#4	AM1	89.3 ± 31.2
C. freundii CF#5	AM1	100.4 ± 37.8
C. freundii CF#9	AM1	96.4 ± 39.5
C. freundii CF#11	AM1	126.4 ± 26.9
C. koseri CK#22	AM2	118.7 ± 18.2
C. braakii CB	AM1	102.0 ± 26.7
C. braakii CB8	AM1	106.8 ± 24.0



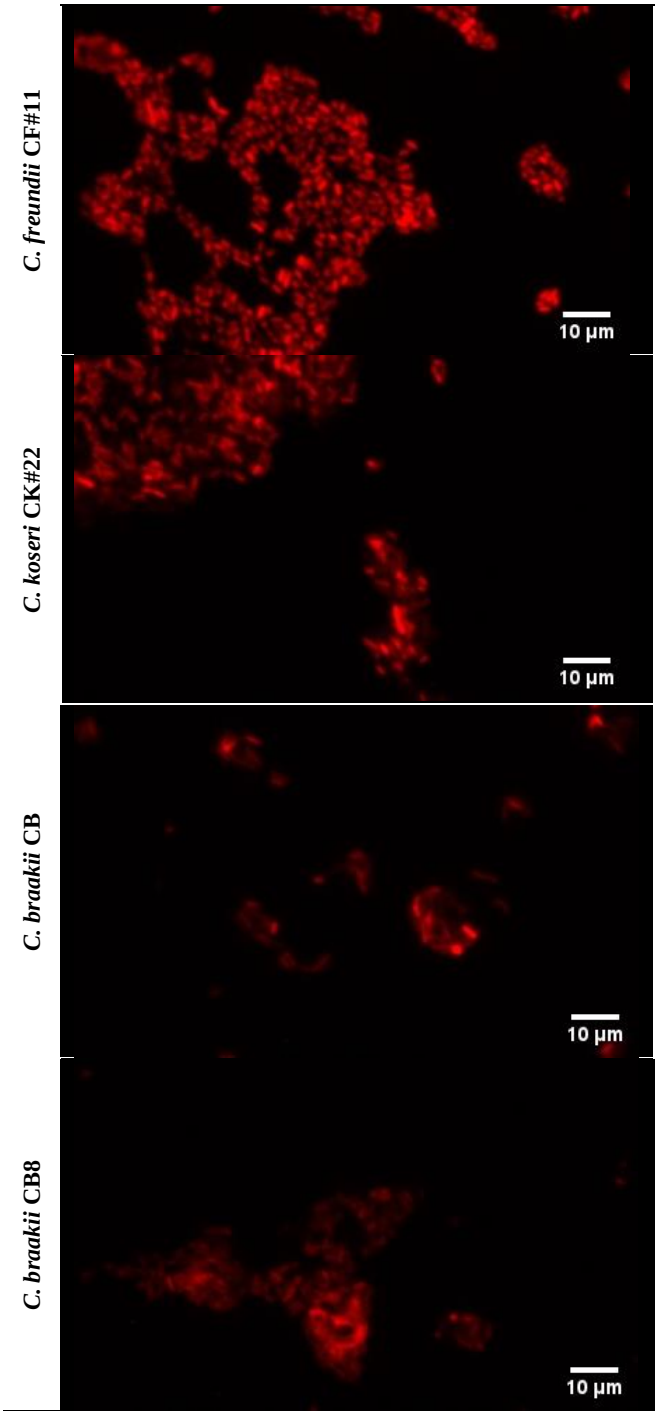


Figure 20 – Epifluorescence detection at 60ºC for AM1 and AM2 probes with the strains tested in the sensitivity tests.