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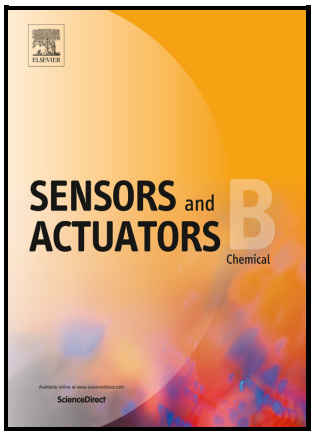


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PII: S0925-4005(22)01560-X

DOI: https://doi.org/10.1016/j.snb.2022.132917

Reference: SNB132917

To appear in: *Sensors and Actuators: B. Chemical*

Received date: 26 July 2022
Revised date: 4 October 2022
Accepted date: 26 October 2022

Please cite this article as: Susana P. Costa, Catarina R.F. Caneira, Virginia Chu, Paulo P. Freitas, João P. Conde and Carla M. Carvalho, A microfluidic platform combined with bacteriophage receptor binding proteins for multiplex detection of *Escherichia coli* and *Pseudomonas aeruginosa* in blood, *Sensors and Actuators: B. Chemical,* (2022) doi:https://doi.org/10.1016/j.snb.2022.132917

# A microfluidic platform combined with bacteriophage receptor binding proteins for multiplex detection of *Escherichia coli* and *Pseudomonas aeruginosa* in blood

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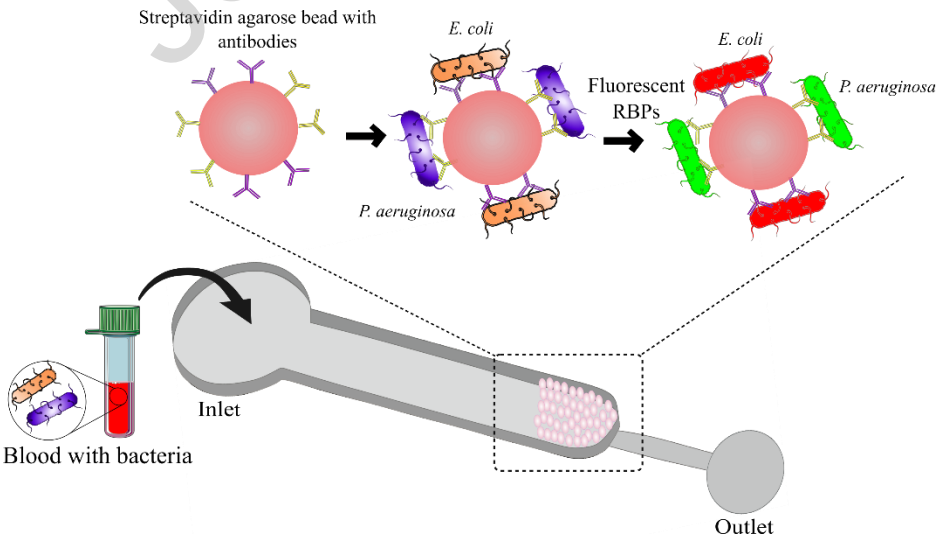
Graphical Abstract



**Abstract**

Bloodstream infections (BSIs) are triggered by the existence of pathogens in blood and are considered a major health burden worldwide, especially when they result in sepsis and septic shock. Common diagnostic methods are time-consuming, present low specificity, or suffer from interference of blood components, which hampers a timely and effective treatment of BSIs.

In this work, a novel microfluidic assay was developed combining a bead-based chip and bacteriophage receptor binding proteins (RBPs) as extremely specific and sensitive recognition molecules for the multiplex concentration and detection of *Escherichia coli* and *Pseudomonas aeruginosa*, which are highly prevalent bacteria in BSIs. The device comprises a microcolumn in which antibody-functionalized agarose beads were packed allowing the entrapment of the target bacterium from blood, providing its concentration and separation. For bacterial detection, two recombinant RBPs (Gp54 and Gp17) were fused with different fluorescent proteins and used for the identification of *P. aeruginosa* and *E. coli* by the measurement of the distinct fluorescent signals obtained. The developed microfluidic-based assay enabled a fast (70 min) and highly specific multiplex detection of both pathogens in whole blood, achieving a detection limit of around $10^3$ CFU, without requiring any time-consuming bacterial pre-enrichment step. Furthermore, it provided a quantitative assessment of bacterial loads present in blood. Noteworthy, this miniaturized and inexpensive device presents simple fabrication and operation, showing great potential to be fully automated, demonstrating to be ideal in point-of-care settings.

**Keywords**:

Receptor binding proteins; microfluidic devices; bacterial separation from blood; fluorescence detection**;** *Pseudomonas aeruginosa*; *Escherichia coli*

## 1. Introduction

Bloodstream infections (BSIs) are a consequence of the presence of infectious agents in the blood, which can trigger life-threatening conditions like sepsis and septic shock [1]. Sepsis is characterized by severe organ dysfunction resulting from dysregulation in the host's response to an infection [2]. The sepsis burden is estimated to affect 49 million people every year and is responsible for 11 million deaths [3]. Moreover, the financial impact of sepsis is high as it represents a major source of expenditure on the total healthcare budget in some European countries [4,5]. This condition can be a result of several other infections, being a remarkable threat nowadays since it is reported as the main cause of death for COVID-19 patients [6]. Sepsis is often associated with hospital-acquired infections (HAI), although most cases occur in the community environment [7].

*Escherichia coli* and *Pseudomonas aeruginosa* are Gram-negative bacteria recognized as highly resistant to antibiotics and represent two of the most common pathogens isolated from BSIs and sepsis [8–10]. Conventional culture methodologies for bacterial detection and identification in blood are laborious, time-consuming, and require specialized laboratory equipment [11]. For this reason, delayed administration of effective antibiotics is common, which contributes to the increase in mortality risk of BSIs [12,13]. Hence, there is a need for the development of novel methods providing a faster, high-throughput, and preferentially point-of-care (PoC) detection of bacterial pathogens. Notably, the implementation of rapid diagnostic tests for sepsis has shown a positive effect in reducing the time to appropriate therapy, healthcare costs, and hospital length of stay [14–16]. Moreover, their use is related to lower mortality risk when combined with antibiotic susceptibility tests (AST) [14]. Rapid diagnostics tests include methodologies based on Polymerase Chain Reaction (PCR) [17,18], Matrix-Assisted Laser Desorption Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF-MS) [19], or Fluorescent *in situ* Hybridization (FISH) [20,21]. Nevertheless, these technologies present some disadvantages such as being expensive, requiring laborious

protocols, frequently suffering from issues related to nucleic acids amplification accuracy or inhibition, and requiring prior knowledge of the target DNA/RNA sequence or mass spectrometry profile of the microorganisms [22–24]. An important constraint is the applicability of these methods in complex matrices such as blood samples which, due to their complexity, can hamper bacterial detection by the interference of blood cells or human DNA, causing inhibitory or competitive issues [25]. Moreover, the number of bacterial cells present in blood that trigger BSIs can be low as 5–200 CFU/mL [26], which are greatly outnumbered by blood cells like erythrocytes. This is likely the largest challenge regarding bacterial separation from blood. Several studies have described the use of centrifugation [27] or filtration [28] techniques to tackle this constraint. Nevertheless, the small size difference between some blood components and bacteria and the occurrence of filter clogging due to red blood cells represent key limitations of these approaches. Moreover, some of these methods require specific equipment and are time-consuming [29].

Microfluidic approaches have emerged as a way to fulfill a demand for portable and more affordable systems for biomedical purposes [30,31], offering high speed and accurate analysis, while enabling the use of small sample volumes [32]. Furthermore, they improve the target capture/detection as a result of the smaller diffusion distances and high surface-to-volume ratios, contributing to greater sensitivities [33]. These systems can integrate a biosensor to accomplish the sample preparation and detection of the target in the same device [34,35].

Microfluidics-based techniques have been widely used for pre-concentration of bacteria cells, taking advantage of magnetic properties [36,37], or acoustophoresis [38–40], among others [41–43]. Nevertheless, these methods entail certain drawbacks, such as a large amount of sample input, expensive reagents, and/or can cause cell damage [36]. Moreover, some of the existing methods lack application in human whole blood [36,44], do not allow species identification [43], and/or do not afford on-chip detection [36,39,40].

Another way to accomplish sample preparation is by using nanoporous beads, which offer increased surface-to-volume ratios, potentially improving the sensitivity [45]. These beads have

been employed in microfluidics for the detection of toxins [46] or nucleic acids [47,48]. Therefore, present the potential for replacing the magnetic beads-based approaches for bacterial sample preparation. Moreover, bead-based microfluidic systems can be more affordable and reliable since do not need extra equipment such as permanent magnets or magnetic coils, which simplify the system.

To develop a biosensing microfluidic device it is important to choose the most appropriate recognition molecule for the target analyte [33]. Commonly used probes include antibodies [37,49], however, are expensive, and have low specificity and stability [50–52]. Some emerging alternatives include bacterio(phages), which normally present high affinity and specificity towards different bacterial species. Some studies have used wild-type or engineered phages in microfluidic devices, providing bacterial sorting [53], bacterial detection [54,55], or allowing both steps [56], however, lack applicability in blood matrices. Conversely, other phage-based microfluidic systems that were tested in blood are not fully integrated, such as the study reported by Dow *et al.* [39]. Phage proteins, in particular, receptor binding proteins (RBPs) have been proven as valuable recognition elements in a variety of bacterial detection systems [57–59] due to their outstanding specificity, stability, and feasibility for the detection of numerous pathogens in different types of samples [58,60]. In biosensing systems, phage proteins can be more reliable due to their small size reducing distances between probe and analyte, which is important in some sensors whose sensitivity depends upon the distance to the analyte [61,62]. Moreover, as recombinant proteins, RBPs can be easily produced at a low cost and can be fused with different tags such as fluorescent proteins [63]. RBPs have been used as probes in some biosensors, such as magnetoresistive [64] or surface plasmon resonance (SPR) [65]. However, their application in microfluidic devices for bacterial detection in whole blood, to the best of our knowledge, has not been described in the literature.

In this work, we coupled the advantages of RBPs as recognition molecules with a bead-based microfluidic system accomplishing in a single device bacterial concentration and multiplex detection of *P. aeruginosa* and *E. coli* directly from the whole blood. For this, the microfluidic device comprised

a microcolumn in which agarose beads were stacked and functionalized with specific antibodies allowing the entrapping of bacterial cells. The target bacteria were then recognized by recombinant fluorescent RBPs. For the detection of *P. aeruginosa,* a novel RBP (Gp54) was designed and characterized in terms of recognition capabilities and together with the previously described *E. coli* RBP Gp17 [66] assisted in the development of the novel microfluidic-based method. Overall, our developed assay revealed to be very specific and sensitive, can be completed in 70 minutes, and achieved a detection limit of around $10^3$ bacterial cells in blood. Moreover, it is simple to perform and presents the prospect for automation if coupled with optical sensors and used as PoC. Overall, it presents superior characteristics when compared with the conventional methods for BSIs diagnosis, which represents a step further in overcoming the negative impact associated with this type of infection.

## 2. Experimental Section

### 2.1. Bacterial growth conditions and human blood samples

All the clinical bacterial strains (Supplementary Material - Table S1) reported in this research were gently provided by the Hospital of Braga, Portugal. *E. coli* BL21 was purchased from Invitrogen. *P. aeruginosa* HB10 was utilized as the target bacteria for mAmetrine-Gp54 and as the negative control for mCherry-Gp17 [66]. *E. coli* HB106 was used as the target bacteria for mCherry-Gp17 protein and as the negative control for experiments using mAmetrine-Gp54. All bacterial strains were inoculated in Tryptic Soy Broth (TSB, Liofilchem). For growth on solid plates, the same media was used adding 12 g/L of agar (Liofilchem). To perform the experiments, bacterial suspensions were prepared after an overnight growth at 37°C, 120 rpm, followed by centrifugation (6,000 × g 10 min), and the pellet was diluted in Phosphate buffer 0.1 M pH 7.2 (PB) until the $OD_{600nm}$ equaled 0.5.

Blood samples were collected from healthy volunteers, upon informed written consent, in EDTA Blood collection tubes (BD Vacutainer). The samples were mixed and processed de-identified,

being the samples and related data fully anonymized. The suspensions of bacteria were centrifuged (6,000 × g 10 min) and resuspended in 1:5 (v/v) diluted blood in PB. Serial dilutions of bacterial cells ($10^3$-$10^8$ Colony Forming Units (CFU) per mL) were performed in PB or diluted blood.

### 2.2. Bioinformatic analysis, synthesis, and production of the RBPs

The gene encoding for a potential tail fiber protein was identified in the *Pseudomonas* phage TL (NC_023583.1) [67] which belongs to the PYO97_15 phage cocktail (Georgian Eliava Institute of Bacteriophage, Microbiology, and Virology) [68]. The encoded protein sequence (Gp54) was screened in Protein Basic Local Alignment Search Tool (BLASTp) database to find similar proteins and possible functional domains were searched using Pfam [69] and InterProScan [70]. The gene *gp54* which encodes a potential RBP was synthesized and fused with the *mAmetrine* gene from *Aequorea victoria,* an enhanced fluorescent protein derived from the green fluorescent protein (GFP). These genes were then cloned into the expression vector pHTP1 (NZYTech). The pHTP1-mAmetrine-Gp54 vector DNA was then used for the chemical transformation of *E. coli* BL21 (DE3) cells (Invitrogen). The *E. coli* RBP (Gp17) used in this study was previously identified and synthesized fused with mCherry (mCherry-Gp17) [66]. The proteins were expressed and purified according to Costa *et al*. [66].

### 2.3. Functional analysis of the RBPs

The protein mAmetrine-Gp54 was evaluated in terms of binding affinity against *P. aeruginosa* cells and against other bacterial species to assess its specificity through fluorescence microscopy analysis and spectrofluorimetry assays. Briefly, for microscopy experiments, *P. aeruginosa* HB10 was used as the target bacteria and *E. coli* HB106 as the negative control bacteria, and the procedure described previously was conducted [66]. After, bacterial samples were then examined in a confocal microscope LSM780 (Zeiss) using the brightfield 5 mW 488-645 nm light source or under a laser at 405 nm (DPSS 405-10) for mAmetrine excitation and setting a bandpass

filter (500-625 nm) in the Zeiss ZEN 2010 software. Experimental control samples were prepared simultaneously, namely bacterial cells without recombinant protein and mAmetrine alone. For binding spectrum assessment, analysis through spectrofluorimetry was performed. In these experiments, all bacteria cells listed in Table S1 (Supplementary Material) were prepared as described previously [71] and were measured in a dark 96-well microplate on a BioTek™ Synergy H1 Hybrid Multi-Mode Microplate Reader with the BioTek Gen5 software, setting 420/526 nm as excitation/emission wavelengths (gain 80). The functional analysis of the *E. coli* RBP mCherry-Gp17 was previously performed as reported in Costa *et al.* [66].

### 2.4. Microfluidic microcolumn fabrication

The microcolumn design comprises two channel heights to facilitate the physical entrapment of the agarose beads [72]. To obtain these devices, polydimethylsiloxane (PDMS) was employed by replicating a two-level SU-8 master, as previously described elsewhere [73]. Briefly, these include the fabrication of two aluminum masks by direct-write optical lithography (DWL) and wet etching of aluminum. To fabricate the SU-8 mold, first, the 20 µm height layer was defined on a silicon substrate using SU-8 2015 (Microchem Corp., Newton, USA), by spin coating. Then, the mask was put on top of the substrate and exposed to UV light, and to develop propylene glycol monomethyl ether acetate (PGMEA) (Sigma-Aldrich) was applied. The second layer with 100 µm was defined with SU-8 50 on the top of the first layer, by spin coating. Before UV light exposure, the second hard mask was manually aligned with the 20 µm features. Afterward, the development was done using PGMEA and the mold was hard-baked at 150°C for 15 min. The PDMS structures were manufactured by mixing PDMS (Sylgard 184 silicone elastomer kit, Dow Corning, USA) with the curing agent in a 10:1 (w/w) ratio. The PDMS was degassed, then poured on top of the master mold and cured at 70°C for 2 h. After curing, the PDMS was removed from the master mold, and inlet and outlet holes were punched. The device was sealed in a 500 µm PDMS pellicule after activation using oxygen plasma (PDC-002-CE, Harrick Plasma, USA).

### 2.5. Microfluidic-based assay

All fluids were inserted into the PDMS microcolumn by applying negative pressure at the outlet using a syringe pump (model 4000, New Era Pump Systems, Inc.). Streptavidin functionalized agarose beads with an average diameter of 90 µm (69203-3, Merck Millipore) (5 µL) were mixed with 20 µL of 30% (w/w) Polyethylene Glycol 8000 (PEG) (Fisher Scientific) and injected on the microcolumn at a flow rate of 5 µL/min. After packing about half of the column, the beads were rinsed with 10 µL PB at 5 µL/min and were functionalized with 50 µL of target-specific biotinylated antibodies at 1 mg/mL, namely the anti-*E. coli* polyclonal (LS-C56164, LSBio) or the anti-*Pseudomonas* polyclonal antibody (PA1-73116, Thermo Fisher Scientific). In the multiplex assays, both antibodies were mixed to a final volume of 50 µL, before inserting into the microcolumn. After antibody immobilization, 25 µL of BSA 5% (w/v) prepared in PB was inserted at 5 µL/min for blocking the beads. Individual or mixed bacterial suspensions of *E. coli* and *P. aeruginosa* at different concentrations ($10^3$-$10^8$ CFU/mL) were introduced into the microcolumn at 5 µL/min for 30 min. A 50 µL volume of the fluorescent proteins (mCherry-Gp17 or mAmetrine-Gp54) at 20 µM was inserted into the column and then two washes of 5 min at 5 µL/min and 10 µL/min, respectively, with PB, were performed. In the multiplex assays, both proteins were mixed to a final volume of 50 µL before introducing into the channel.

#### 2.6.1. Monitoring and analysis of fluorescence

Fluorescence signals were continuously monitored during bacterial recognition by the RBPs mCherry-Gp17 and mAmetrine-Gp54 and washing steps and recorded using an inverted Nikon Eclipse Ti-E fluorescence microscope equipped with a Neo sCMOS color camera (Andor Technology). The filter settings for mAmetrine protein were 380/10 nm as excitation filter and 531/40 nm as emission filter. For mCherry protein, an excitation filter of 560/25 nm was used coupled with an emission 620/60 nm filter. The fluorescence signals from both proteins were acquired with an

exposure time of 800 ms, and 10 × magnification. A specific region of interest (ROI) corresponding to the entire end section of the microcolumn (as represented in the Supplementary Material - Figure S1) was defined and the fluorescence of this area was evaluated and quantified using the ImageJ software (National Institutes of Health, U.S.A.). Images obtained in three independent experiments were used for the data analysis.

### 2.6. Bacteria cell quantification and plating

Bacterial samples were collected from the microdevice through the inlet into microcentrifuge tubes, then were serially diluted in PB, plated on TSB agar plates, and further incubated overnight at 37°C. The bacterial trapping efficiency was defined as the ratio between the number of bacterial cells obtained in each sample after the experiment and the number of cells initially inserted in the microfluidic chip and expressed as CFU.

### 2.7. Statistical data analysis

All data are represented as mean ± standard deviation (SD) of at least 3 independent assays. In Figure 4, the Ordinary One-way ANOVA test was applied followed by Tukey's multiple comparisons test (p-value<0.05). For Figure 5, multiple comparisons of means were performed using Two-way ANOVA followed by Tukey's multiple comparisons test (p-value<0.05).

## 3. Results and discussion

### 3.1. Bioinformatics and functional characterization of the *P. aeruginosa* RBP mAmetrine-Gp54

The *gp54* was identified in the genome of the *Pseudomonas* phage TL (NC_023583.1) as the gene encoding for a tail fiber protein [67], which indicates that it is a possible RBP since most of the

RBPs are correlated with tail proteins such as tail fibers and other phage tails [74]. Accordingly, the sequence of the encoded protein was analyzed and compared against the data available at the National Center for Biotechnology Information (NCBI). The results from the BLASTp revealed that Gp54 presents homology with several other *Pseudomonas* phage tail fibers deposited, showing the highest similarities with the RBPs from the *Pseudomonas* phage Pa223 (96.3%), the *Pseudomonas* phage PSA13 (96.3%), the *Pseudomonas* phage PSA31 (95.8%) and the *Pseudomonas* phage SaPL (95.8%). The conducted search to find possible matches with recognized domains and families retrieved a Tail Collar Domain Receptor-binding domain (at position 86-107 aa) and a Phage tail fiber repeat (at position 140-153 aa). Also, it predicted a Receptor-binding domain of the short tail fiber protein superfamily at the C-terminal.

After the bioinformatics analysis, the Gp54 protein was synthesized fused with a gene encoding a fluorescent protein (mAmetrine), resulting in the recombinant protein mAmetrine-Gp54. The next step was to characterize by fluorescence microscopy this potential novel RBP in terms of binding affinity to the target and non-target bacterial species. The results indicate that the RBP mAmetrine-Gp54 displayed effective recognition and binding to *P. aeruginosa* HB10 cells (Figure 1-A) and did not label the negative control *E. coli* HB106 (Figure 1-B). To further characterize its binding spectrum and specificity, we investigate its binding activity towards clinical isolates of *P. aeruginosa* and other species by spectrofluorometric measurements. The results (Supplementary Material - Table S1) indicate that Gp54 bound to 83.8% of *P. aeruginosa* strains tested and presented 100% specificity, since it did not depict binding to any other bacterial species, including *Staphylococcus aureus*, *Klebsiella pneumoniae,* or *Citrobacter freundii.*

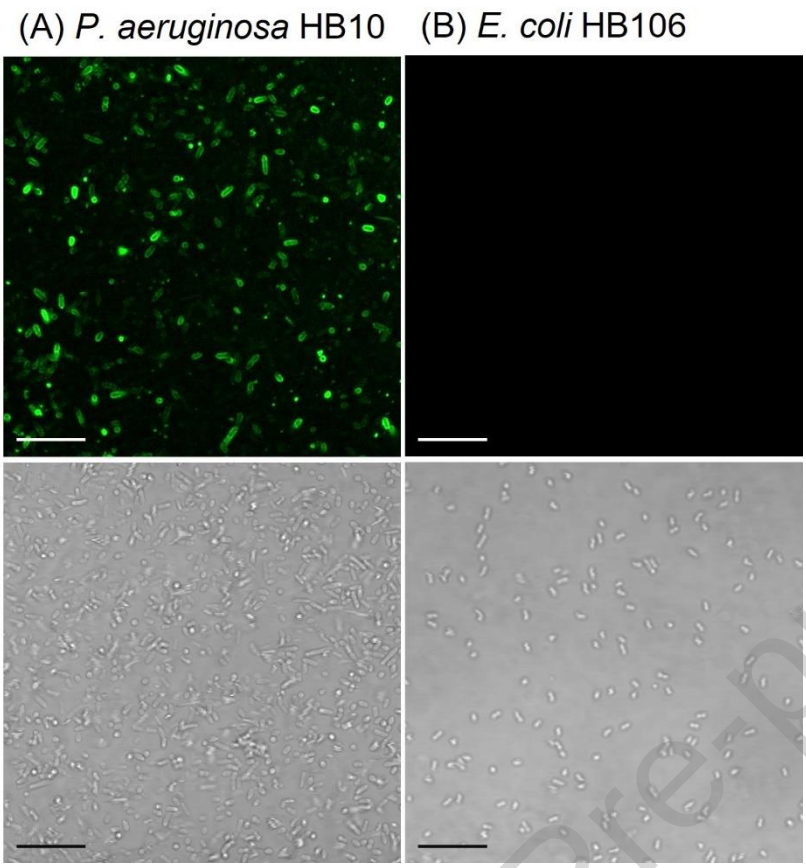



**Figure 1 –** Functional analysis of the RBP mAmetrine-Gp54 by fluorescence microscopy. Resulting images after analysis of the labeling of *P. aeruginosa* HB10 (A), and *E. coli* HB106 (B), as the negative control, with mAmetrine-Gp54. Scale bar corresponds to 10 µm.

According to these results, Gp54 demonstrated to be species-specific, which is an advantage as a probe for clinical diagnostics. These results are in agreement with previous studies characterizing *P. aeruginosa* RBPs [60,75,76], although the number of studies describing these RBPs is very scarce. He *et al*. found that the recombinant *P. aeruginosa* RBP P069 retained the capacity for recognizing only *P. aeruginosa* strains, without showing any binding affinity towards bacteria belonging to the same or other genera [75]. The phage RBPs commonly target the bacterial lipopolysaccharide (LPS) as receptors on the host cell [77,78] and it was found that *P. aeruginosa* phages target the O-antigen of LPS [79,80]. Moreover, previous studies indicated that the C-terminal portion of the encoded tail fibers of the *P. aeruginosa* phages JG004 and PaP1 (P069) were the determinants of the host specificity [80]. Among Gram-negative bacteria, the most diverse

component of LPS is the O-antigen [81,82]. Its structure and composition differ between species or even within a species and some Gram-negative bacteria are not able to synthesize this component [82]. Thus, the recognition of the O-antigen on the bacterial LPS by the *P. aeruginosa* RBPs may explain the species specificity. The fact that Gp54 did not show sequence similarity to any previously characterized *P. aeruginosa* RBP makes this an interesting RBP to be further exploited to understand the receptors involved in its binding and host-specificity.

The *E. coli* RBP Gp17 bioinformatic analysis and functional characterization were previously described by our group [66]. The protein showed high specificity against *E. coli* bacterial species and when combined with a spectrofluorometry assay revealed great potential for the detection of *E. coli* in different human biological specimens, such as blood, urine, and saliva.

### 3.2. Testing the RBP-based microfluidic device for individual and multiplex bacterial detection in buffer samples

The microfluidic device used herein allows nanoporous beads to be stacked into a microcolumn due to the different heights of the microchannels. This concept was previously described and adapted for the detection of mycotoxins [46] and DNA [47]. The device comprises two channels of different heights (as illustrated in Figure 2), a taller channel (100 µm) for packing agarose microbeads which creates a bead bed, and a lower channel (20 µm) preventing the passage of the beads downstream during the microfluidic assay. The principle for the biological recognition event is similar to a sandwich assay (Figure 2). This makes use of streptavidin functionalized agarose beads which bind to biotinylated antibodies, specific for *E. coli* and *P. aeruginosa* (Figure 2) for capturing the bacterial cells (in buffer or blood) inside the microchannel. Then, the fluorescent RBPs mCherry-Gp17 [66] and the novel RBP mAmetrine-Gp54 were used as probing elements for fluorescent detection of both bacterial species.

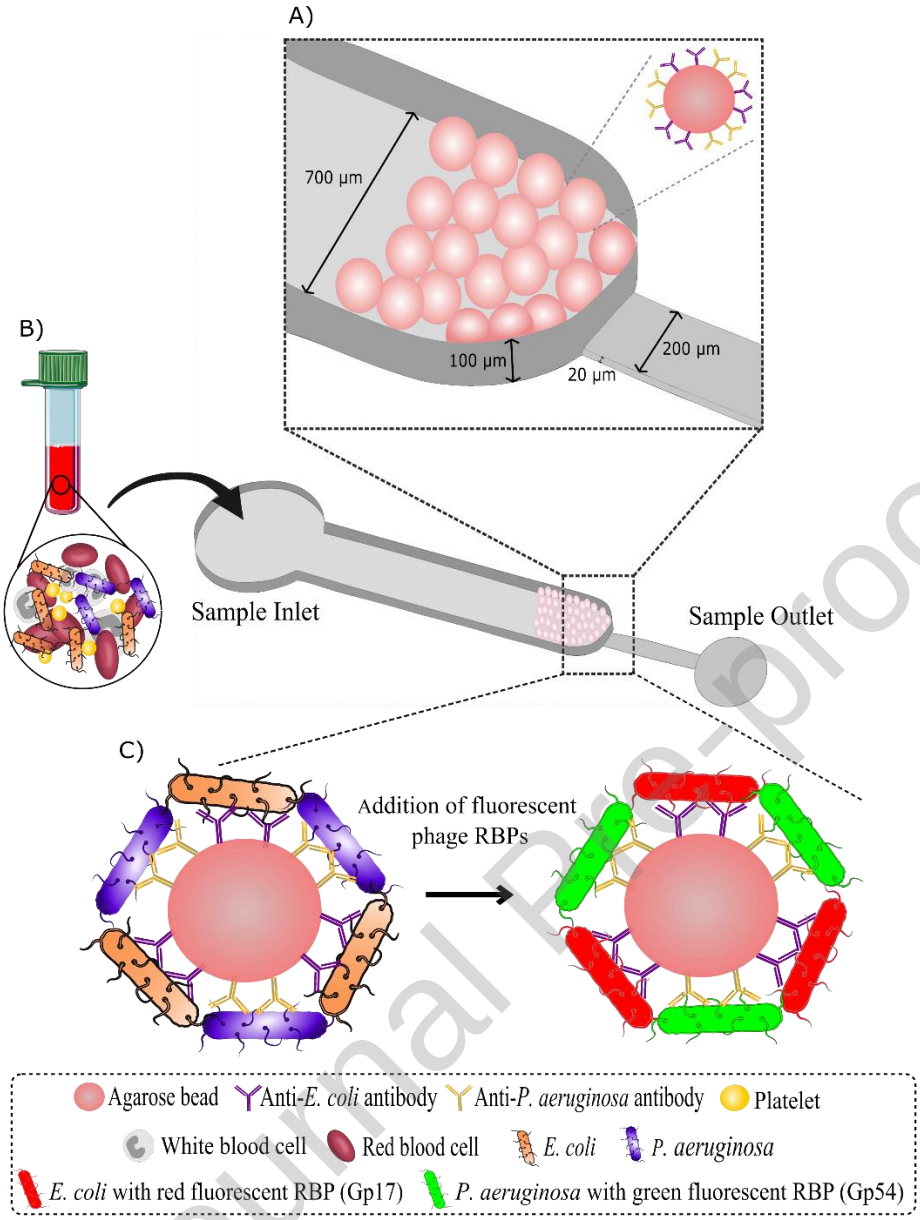



**Figure 2 –** Schematic illustration of the microfluidic microcolumn developed where agarose beads are stacked to enable bacterial trapping and subsequent detection. (A) After packing streptavidin agarose beads (average diameter of 90 µm) inside the microcolumn, these are functionalized with biotinylated antibodies specific for *E. coli* and *P. aeruginosa*. (B) Individual or mixed bacterial cell populations prepared in human blood (or buffer) are introduced inside the channel and remain trapped due to the affinity with antibody-functionalized beads. (C) After bacterial trapping, mAmetrine-Gp54 and mCherry-Gp17 fluorescent proteins are added, specifically recognizing their host receptors and upon binding to the target, the *E. coli* cells become red fluorescent and *P. aeruginosa* green fluorescent.

In the microfluidic experiments, the bacterial cells were successfully entrapped inside the channels with high recoveries efficiencies (Supplementary Material – Figure S2) and then labeled

with the fluorescent-RBPs specific to each bacterium. In order to test the feasibility of the assay, we first started by testing individual and mixed populations of *E. coli* and *P. aeruginosa* at concentrations of $10^6$ and $10^8$ CFU/mL in buffer samples and monitoring the fluorescent signals obtained from the mCherry-Gp17 (red) and the mAmterine-gp54 (green). The results illustrated in Figure 3 (Samples A to D) showed that both bacterial cells originate a significant signal during the individual detection assay performed on the microcolumn. *P. aeruginosa* cells displayed higher signals for both concentrations tested in comparison with *E. coli* at the same concentrations. *P. aeruginosa* mean fluorescence signals were 12.5±1.1 a.u. for $10^8$ and 5.2±0.6 a.u. for $10^6$ CFU/mL against the 10.0±1.2 a.u. and 4.2±1.1 a.u. for *E. coli* at the same concentrations, respectively. This could be explained by the intrinsic fluorescence of the fluorescent protein used in each construction since mAmetrine (fused with Gp54) is reported as having a higher brightness and quantum yield than mCherry [83]. The multiplex detection of these two pathogens was evaluated for the aforementioned concentrations. As shown in Figure 3 (Samples E and F), it was possible to successfully recognize both bacterial species in a mixed population in buffer. The green fluorescent mean signals in mixed populations were consistent with the ones obtained for individual *P. aeruginosa* cells (12.2±0.8 and 6.3±0.9 a.u. for $10^8$ and $10^6$ CFU/mL, respectively), and the red fluorescent signals were slightly lower in the case of *E. coli* cells in the multiplex in comparison with the individual detection (7.1±0.3 and 3.5±0.8 a.u. for $10^8$ and $10^6$ CFU/mL, respectively). All the individual and multiplex fluorescent mean signals were significantly different from the negative controls, which were conducted by testing the fluorescent RBP against the non-target bacteria (Figure 3 Samples G and H). These results show that the assay is specific for both individual and multiplex detection of *P. aeruginosa* and *E. coli.*

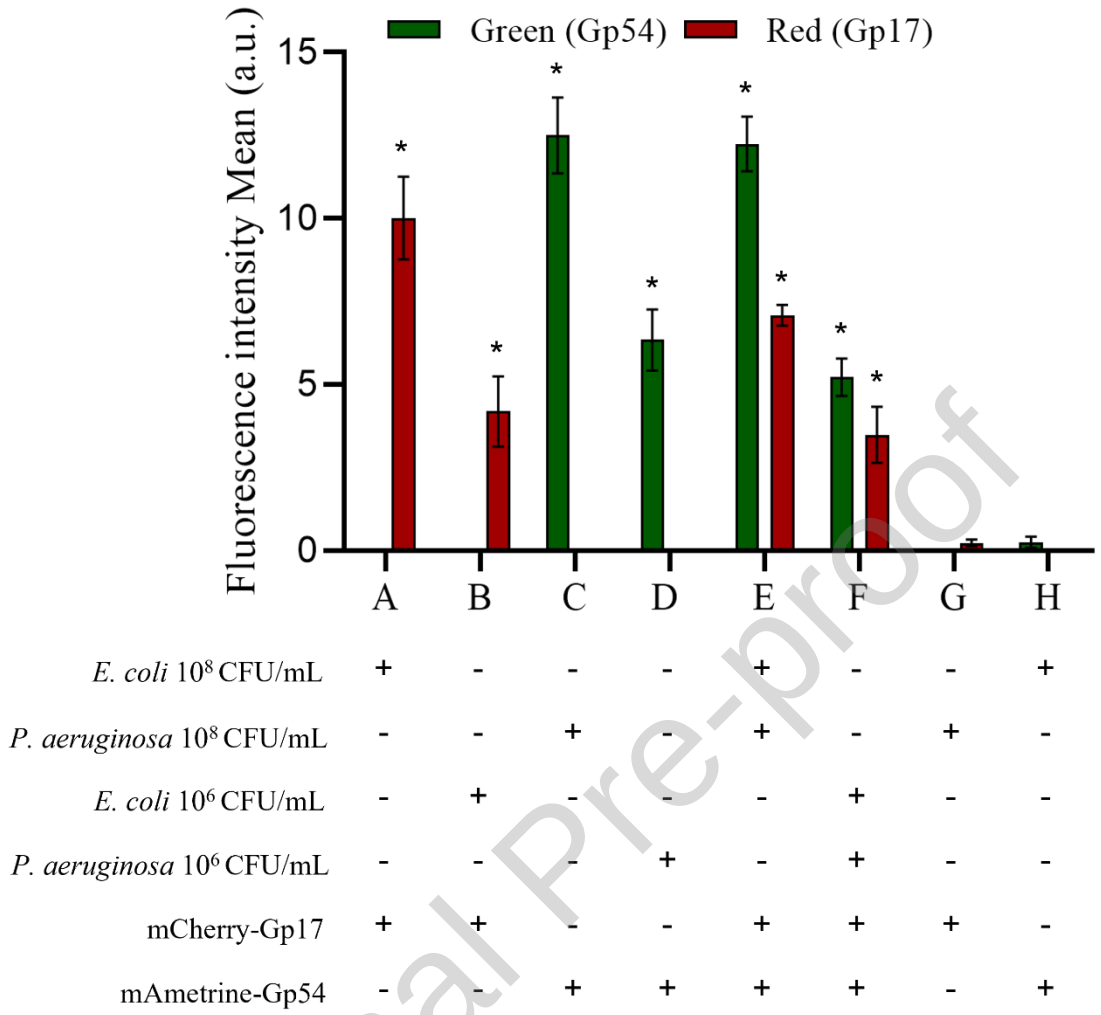



**Figure 3 –** Mean Fluorescence Intensity in arbitrary units (a.u.) obtained in the assays performed on the beads microcolumn with buffer samples for multiplex or individual bacterial cells. (A) and (B) corresponded to individual *E. coli* HB106 cells labeled with mCherry-Gp17 at $10^8$ and $10^6$ CFU/mL, respectively; (C) and (D) correspond to individual *P. aeruginosa* HB10 cells labeled with mAmetrine-Gp54 at $10^8$ and $10^6$ CFU/mL, respectively; (E) and (F) represent the multiplex in buffer for $10^8$ and $10^6$ CFU/mL, respectively; (G) represents the negative control for mCherry-Gp17 protein incubated with $10^8$ CFU/mL *P. aeruginosa* HB10 cells; and (H) the negative control for mAmetrine-Gp54 incubated with $10^8$ CFU/mL *E. coli* HB106 cells. Error bars represent the standard deviation of measurements performed in three independent experiments (n=3). *Statistical analysis was performed using Ordinary One-way ANOVA with Tukey's multiple comparisons test ($p<0.05$) comparing each sample with the respective control with non-target bacteria (Samples G and H).

### 3.3. Exploitation of the RBP-based microfluidic device for multiplex bacterial detection in blood

### 3.3.1. Analysis of the fluorescence signals acquired in mixed bacterial populations in blood

To test the efficiency of the developed device for bacterial detection in complex matrices, human blood samples were simultaneously spiked with *E. coli* and *P. aeruginosa,* and the two RBPs were used to label and detect these pathogens**.** The signals were recorded during the addition of the fluorescent proteins, as well as during the washing steps, to determine which signals effectively resulted from the binding of bacterial cells to the correspondent RBP used as the recognition molecules.

The results of the fluorescence monitoring during the microfluidic experiments are depicted in Figure 4 for the multiplex detection of *E. coli* and *P. aeruginosa* at $10^4$ and $10^8$ CFU/mL in blood and the respective negative controls. As can be observed in the graphic, the red and green fluorescence signals after the addition of the probing fluorescent RBPs for labeling the *E. coli* and *P. aeruginosa* cells, respectively, increased over the assay time after the protein incubation for both bacterial concentrations, reaching a plateau which was higher in the case of mAmetrine-Gp54 signals (green) in comparison with mCherry-Gp17 (red). Representative images of these signals are presented in Figures 4 A1-A2 and B1-B2. In the two controls, corresponding to bacterial cells at a concentration of $10^8$ CFU/mL labeled with the non-target RBPs, the plateau reached is slightly below the ones obtained for the target bacterial cells. A reasonable explanation is that since the target cells are not present, the RBPs' binding epitopes are unavailable for the protein to attach to cells. The resulting signals may be originated from proteins that stayed trapped non-specifically on the channel or the beads before the washing step was conducted. During the washing step, all signals dropped in consequence of the removal of the excess RBPs that were present, and only the proteins that effectively remained attached to the specific bacterial epitopes recognized were quantified. Accordingly, the mean signals were lower for the $10^4$ CFU/mL concentration (Figure 4 C1-C2) and

higher for the $10^8$ CFU/mL (Figure 4 D1-D2). The negative controls mean signals were residual and possibly resulted from some proteins that remained attached to the beads or the channels' walls.

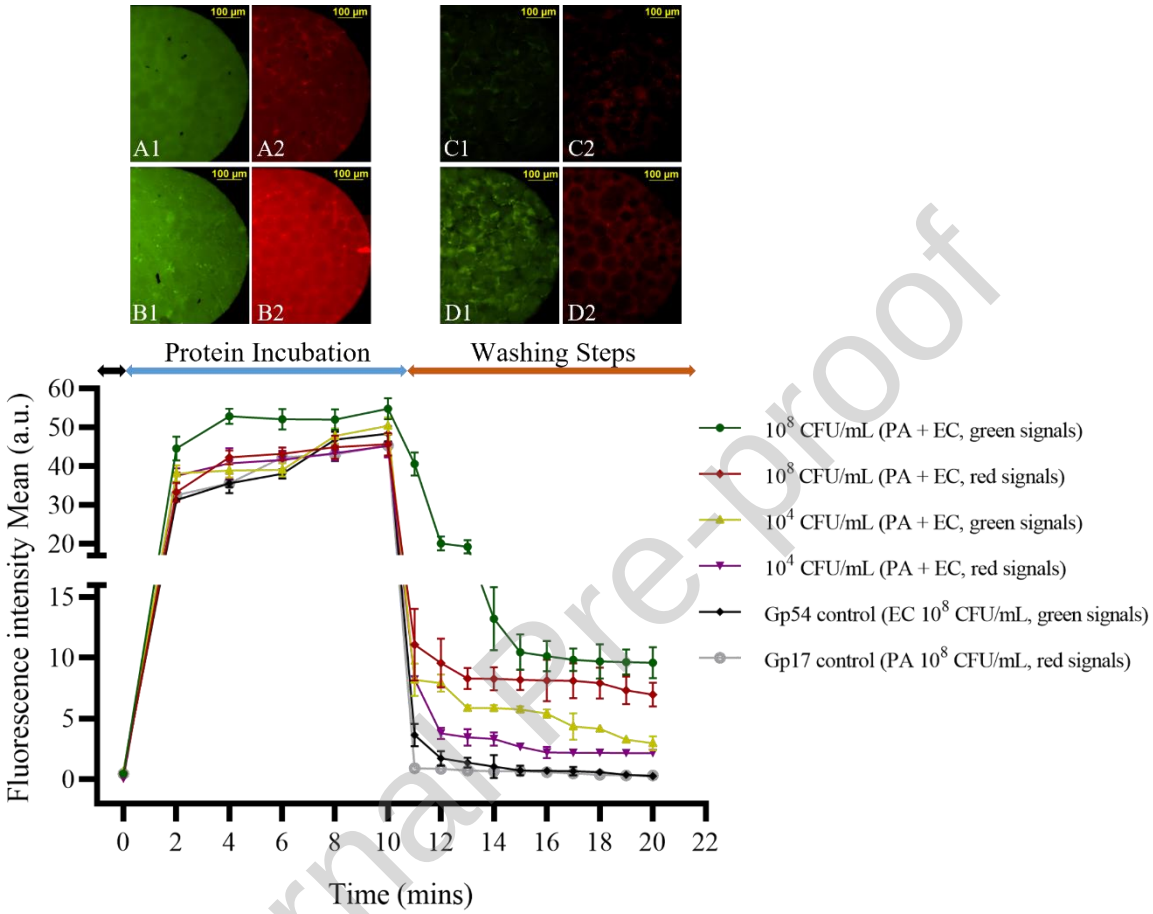



**Figure 4 –** Graphical representation of the fluorescence monitoring during the microfluidic experiments performed for multiplex detection of *E. coli* (EC) and *P. aeruginosa* (PA) in blood. Error bars represent the standard deviation of three independent measurements (n=3). The images shown are representatives of the signals recorded for each bacteria, *E. coli* (Red) and *P. aeruginosa* (green), immediately after the protein incubation and before washing for $10^4$ (A1-A2) and $10^8$ CFU/mL (B1-B2) and after the washing steps for $10^4$ (C1-C2) and $10^8$ CFU/mL (D1-D2). All images were acquired in the fluorescence microscope and all measurements were obtained after Image J analysis.

### 3.3.2. Detection limit assessment in blood samples

Another important step for characterizing the developed assay concerns the assessment of its detection limit. For this, suspensions with both *E. coli* and *P. aeruginosa* ranging from $10^3$ to $10^8$ CFU/mL were used to artificially contaminate human blood samples. As illustrated in Figure 5-A,

the mean fluorescence signals obtained in all concentrations were lower than the ones acquired in buffer (Figure 3), but the complexity of blood samples can explain the lower signals by interfering in the binding between RBPs and their epitopes on the bacterial cell membrane [25,84]. The signals acquired for *P. aeruginosa* with the mAmetrine-Gp54 (green) were higher than for *E. coli* with mCherry-Gp17 (red), which is in accordance with the results obtained in buffer experiments.

The fluorescence mean values for the negative controls, which corresponds to the incubation of each RBP against the non-target bacteria (Figure 5-A, "NC1" and "NC2" samples), were similar for both proteins and revealed to be residual (0.3±0.3 a.u). As for the blood samples with the two fluorescent RBPs and without bacterial cells (Figure 5A – "SC" samples), the signals obtained were very low (0.4±0.3 and 0.3±0.3 a.u. for blood with mCherry-Gp17 and mAmetrine-Gp54, respectively), indicating that the background derived from the sample matrix was minimal

The lowest fluorescence mean values possible to be detected showing statistically significant differences when compared to the negative controls were acquired for $10^4$ CFU/mL of both pathogens (Figure 5A). Even in the case of *E. coli* (red signals) at this concentration, the signal obtained in the multiplex was seven times higher than the negative control (2.1±0.2 a.u. against the 0.3±0.3 of the negative control). This bacterial concentration corresponds to $1.5 \times 10^3$ cells inside the microcolumn, and it is not so distant to the range of bacterial concentration found in peripheral blood of septic patients, which is estimated to be 5–200 CFU/mL [26]. Therefore, our assay will enable to shorten (approximately by half) [85] the time required for the enrichment step in comparison with standard methods that require a bacterial concentration of $10^8$ CFU/mL for blood culture positivity. This can take around 13 hours for *E. coli* and 21 hours for *P. aeruginosa* [85] plus 12-36 hours for bacterial identification [11].

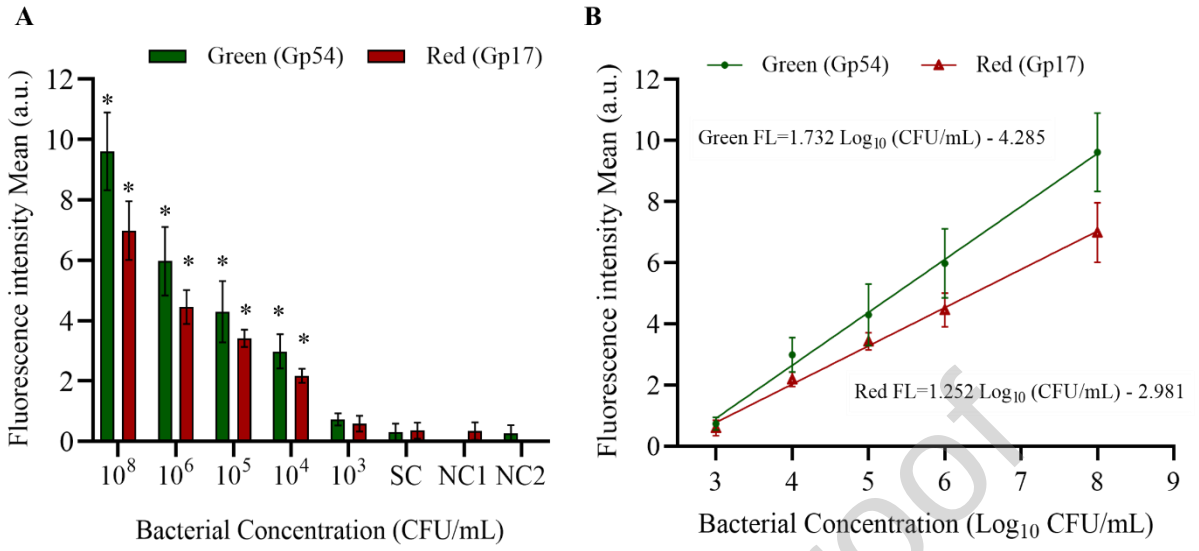



**Figure 5 –** Assessment of the detection limit in multiplex assays performed in blood samples (A) and trendline determination (B). Mixed populations of *E. coli* HB106 and *P. aeruginosa* HB10 at concentrations ranging from $10^3$ to $10^8$ CFU/mL were incubated with both RBPs (Gp54 and Gp17) and the fluorescent signals (red and green) were measured. Error bars represent the standard deviation of three independent measurements (n=3). Shown in (A) are the results of fluorescence mean signals of the blood samples with different bacterial concentrations and the respective control samples: "SC" (sample controls) corresponds to blood samples without bacteria incubated with RBPs mCherry-Gp17 and mAmetrine-Gp54; "NC" represents negative controls, where each protein was incubated with the non-target bacteria at $10^8$ CFU/mL being NC1 the *E. coli* RBP (mCherry-Gp17) against *P. aeruginosa* and NC2 the *P. aeruginosa* RBP (mAmetrine-Gp54) against *E. coli*. *Statistically significant difference ($p<0.05$) of each sample relative to the respective control samples (NC1, NC2, and SC for the different fluorescence). In (B) the linear trendline of fluorescence signals versus bacterial load is shown.

Furthermore, in the developed assay, it was possible to observe a linear trend of the fluorescent signals for both RBPs that increased proportionally to the bacterial concentrations of both pathogens initially added to the microbeads column (Figure 5-B). These results show that the developed method can provide a quantitative assessment of bacterial loads present in blood from $10^4$ to $10^8$ CFU/mL. In Figure 6 are represented images of the microcolumn when different concentrations of mixed populations of *E. coli* and *P. aeruginosa* were tested with both RBPs (Figure 6A-D for *E. coli*, Figure 6F-I for *P. aeruginosa*). The respective negative control (RBP with the non-

target bacteria) with *P. aeruginosa* is presented in Figure 6-E and with *E. coli* in Figure 6-J. As observed, the fluorescent signals decrease according to the reduction in the concentration of bacterial cells, corroborating the data shown in Figure 5. This phenomenon is expected since a lower number of bacterial cells is present, thus the RBPs have fewer binding receptors available for their recognition. Moreover, it is possible to verify the difference between the lowest concentration detected ($10^4$ CFU/mL) for *E. coli* (Figure 6 - D) and *P. aeruginosa (*Figure 6 - I) and the respective negative controls (Figure 6-E and Figure 6 - J, respectively).

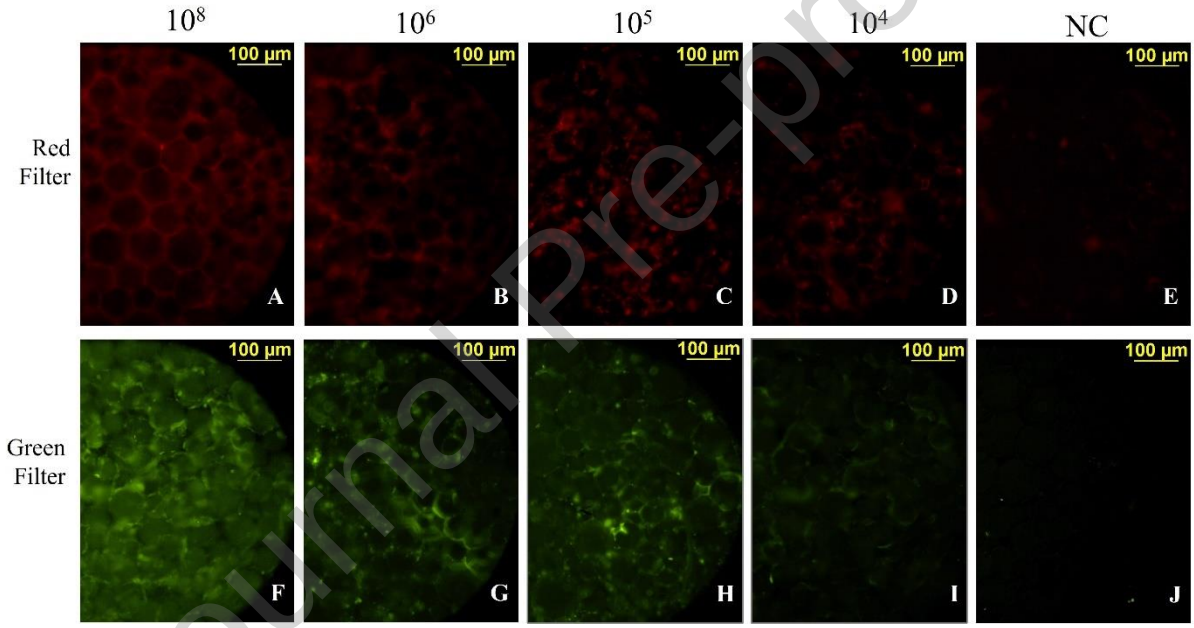



**Figure 6 –** Representative fluorescence microscopy images of part of the end section of the microcolumn (ROI) for the different concentrations of bacterial cells tested (between $10^4$ to $10^8$ CFU/mL) in the assays performed in blood. The results from the red fluorescence resulting from the labeling of cells with mCherry-Gp17 are displayed in (A) to (D) and the negative control (NC) for mCherry-Gp17 against *P. aeruginosa* HB10 cells is depicted in (E); Figures (F) to (I) show the green fluorescence signals acquired after mAmetrine-Gp54 cells staining and (J) display the results of the NC which is *E. coli* HB106 labeled with mAmetrine-Gp54.

The developed bead-based assay assisted in the bacterial concentration directly from blood. The fluorescent signals obtained allowed to conclude that using the bead microcolumn, the blood

sample matrices were eliminated by the fluidic flow, which enabled the removal of the possible interferents and components that usually display autofluorescence, such as the red blood cells [86]. In fact, the recorded signals in samples with blood and fluorescent proteins, although showed some background, could be neglected. The assay provided a specific and simultaneous detection of *E. coli* and *P. aeruginosa*, presenting advantages over traditional methods since it can detect rapidly, without requiring lengthy enrichment steps, laborious protocols, and specialized personnel [11,22–25] and shows great potential to be fully automated and extended to further bacterial species.

To date, several authors reported the isolation of bacterial cells from whole blood using microfluidic approaches leveraged by size-exclusion [43], acoustophoresis [38,39], dielectrophoresis [41,42], magnetic-based methods [36,37], among others [34,87]. Some of these methods provide higher sensitivity and/or less analysis time in comparison with the method developed herein, however, most of the systems do not afford both sample preparation and detection on-chip, lack testing in whole blood matrices, demand complex microfluidic designs or instrumentation or imply the use of more expensive probes like antibodies (as detailed in Table 1). Additionally, the developed assay is simple and versatile, allowing the simultaneous detection of two bacterial species relevant for BSIs, which is not a feature displayed by most microfluidic detection systems reported to date (Table 1).

The detection based on fluorescence affords the possibility of extending the multiplex detection through the use of different fluorescent proteins, which constitutes an advantage over other methods such as the ones based on luminescence [39,56] (Table 1). Nevertheless, our system lacks high-throughput screening due to the space limitation of the microcolumn and the possibility of spectral crosstalk when using several fluorescent proteins. To enable the detection of more than two bacteria in our device, it would be necessary to use other RBPs that recognize different bacterial species and fuse them with fluorescent proteins that do not present spectral overlapping. Moreover, the agarose beads should be functionalized with antibodies that capture both Gram-negative and Gram-positive bacteria. Another option would be to immobilize on the beads non-fluorescent phage

RBPs with lower specificity such as the Gp12, described by Ongwae *et al*. [59], that recognized both *E. coli* and *P. aeruginosa*. This strategy would contribute to decreasing the total assay cost and capturing different bacterial species with the same probe, thus not compromising the bead surface area availability. In principle, these modifications would not impact the overall specificity of the assay since this is provided by the fluorescent RBPs probes that are added after the bacterial capture on the packed beads.

Although RBPs have been applied for diagnostic purposes in clinical samples [58,60,75,76], studies regarding their use in human whole blood are scarce [66,71]. Also, very few studies have employed RBPs probes in microfluidic devices [64,88], and to the best of our knowledge, none performs on-chip bacterial detection in whole blood. Some authors have reported the use of engineered phages as recognition molecules [39,56] although these systems imply demanding genetic engineering approaches, which are time-consuming and laborious. Furthermore, they require that the phage infection process occurs before the detection assay execution which increases the total assay time, is dependent on the bacterial growth state, and demands the use of incubators. On the contrary, the fusing of phage RBPs with fluorescent proteins can be effortlessly done by cloning procedures or DNA synthesis, simplifying the process. Moreover, the binding of phage proteins to their receptors is fast and temperature independent [71,84].

Overall, the developed microfluidic device is innovative and reliable, enabling to perform on-chip both the isolation and bacterial detection in whole blood samples with a simple process, taking just 70 minutes for the assay to be completed. Moreover, our designed method showed promising quantification capabilities allowing the differentiation of $10^4$ to $10^8$ CFU/mL of *P. aeruginosa* and *E. coli* by measuring the fluorescent signals retrieved.

A portable and miniaturized fluorescence detection system could be easily combined with this microfluidic chip such as an array of photodiodes [46,89], replacing the necessity of a fluorescence microscope. This would greatly increase the reliability of the method and improve the overall sensitivity. Combining these optical sensors with the exceptional high specificity and stability

of RBPs [90,91], along with the cheap and easy production of these probes and microfluidics fabrication [92], make the developed microdevice ideal for PoC detection of BSI.

**Table 1 –** Critical comparison of similar microfluidic-based methodologies for bacterial detection.

*On-chip detection (PCR-chip) conducted in an external chip.

| **Target pathogen** | **Separation and detection Method** | **Recognition molecule** | **Analysis Time** | **Detection limit** | **Sample Matrix** | **On-chip detection?** | **Ref.** |
|---|---|---|---|---|---|---|---|
| *P. aeruginosa* and *E. coli* | Fluorescence detection in a bead-based microcolumn | RBPs | 70 min | $10^4$ CFU/mL | Whole blood | Yes | This study |
| *E. coli* | Cross-flow filtration and fluorescence | Not applicable | 8 min | $2.2 \times 10^7$ CFU/mL | Whole blood | No | [43] |
| *E. coli* | Magnetic trapping and Fluorescence | Not applicable | Not reported | $1.11 \times 10^7$ CFU/mL | Plasma | No | [36] |
| *E. coli* | Magnetic trapping and ATP luminometer | Antibodies | 60 min | 10 CFU/mL | Whole blood | No | [37] |
| *E. coli* | Acoustophoresis and luminescence measurements | Reporter Phage | >60 min | 6 cells | Whole blood | No | [39] |
| *P. aeruginosa* and *S. aureus* | Filtration with immunoaffinity and electrochemical detection | Antibodies | 30 min | 10 CFU/mL | Plasma | Yes | [44] |
| *Pseudomonas putida* | Acoustophoresis and PCR-chip | Not applicable | 2 h | $10^3$ CFU/mL | Whole blood | Yes* | [38] |
| *E. coli* and *S. aureus* | Membraneless dialysis, dielectrophoresis and culture and counting | Not applicable | 5-12 h | $10^3$ CFU/mL | Whole blood | No | [41] |
| *S. aureus, K. pneumoniae, P. aeruginosa, E. coli,* and *E. faecalis* | Inertial and PCR | Not applicable | 9 h | 100 cells/mL | Whole blood | No | [93] |
| *P. aeruginosa, E. coli* and *S. aureus* | Dielectrophoresis and PCR | Not applicable | 3 h | $10^3$ CFU/mL | Whole blood | Yes | [94] |
| *E. coli* | Immunoaffinity and Fluorescence microscopy | Antibodies | 30 min | 50 CFU/mL | Whole blood | Yes | [95] |

## 4. Conclusions

In summary, we have demonstrated for the first time that combining a microfluidic bead-based microcolumn with phage RBPs can result in a fruitful and useful method for multiplex bacterial detection in blood samples. A novel RBP targeting *P. aeruginosa* (Gp54) was described, which along with the *E. coli* RBP Gp17 revealed to be valuable probing elements in the developed detection methodology. The microfluidic assay can be concluded in 70 minutes, using just a small volume of blood, and presented a detection limit of $10^4$ CFU/mL which corresponds to $1.5 \times 10^3$ cells detected by each microcolumn device. Both the duration of the assay and the sensitivity are susceptible to be improved with further optimization. This platform can be integrated to originate a PoC or point-of-need biosensing system allowing portable and miniaturized bacterial isolation and detection on a single device. The concept of this assay and the device architecture is amenable to be extended to other bacterial species or sample matrices for other biomedical applications or even food safety.

**Funding**

The authors acknowledge the funding from the Portuguese Foundation for Science and Technology (FCT) under the scope of the project “Phages-on-chip” PTDC/BTM-SAL/32442/2017 (POCI-01-0145-FEDER-032442) and the strategic funding of the research units CEB (UIDB/04469/2020) and INESC MN (UID/05367/2020) through the pluriannual BASE and PROGRAMATICO financing and BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 - Programa Operacional Regional do Norte. S.P.C. and C.R.F.C. acknowledge the FCT for the grants SFRH/BD/130098/2017 and PD/BD/135274/2017, respectively.

**Declaration of interests**

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

**Acknowledgments**

We would like to thank Centro Clinico Académico (2CA-Braga) and Doctor Alberta Faustino from the Hospital of Braga for gently providing the clinical bacterial isolates used in this study. We thank the INL Nanophotonics & Bioimaging Facility for the help in microscopy image acquisition.

**Authors contributions**

S.P.C.: Methodology, Investigation, Formal analysis, Writing – original draft. C.R.F.C.: Methodology, Investigation. V. C: Conceptualization, Funding acquisition. P.P. F.: Conceptualization, Writing – review & editing, Supervision, Funding acquisition. J.P.C.: Conceptualization, Methodology, Writing – review& editing, Supervision, Funding acquisition. C.M.C.: Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

**Competing interests**

The authors declare no conflict of interest.

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## Highlights

- **Microfluidic device for multiplex bacterial separation and detection in blood**
- **Phage receptor binding proteins provided specificy for two bacterial species**
- **Fluorescent detection** of $10^4$ CFU/mL bacteria in less than 1.5 h
- Possible integration of photodetectors for a portable/integrated system