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Bacteriophage-receptor binding proteins for multiplex detection of *Staphylococcus* and *Enterococcus* in blood

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Abstract

Healthcare-associated infections (HCAIs) affect hundreds of millions of patients, representing a significant burden for public health. They are usually associated to multidrug resistant bacteria, which increases their incidence and severity. Bloodstream infections are among the most frequent and life-threatening HCAIs, with *Enterococcus* and *Staphylococcus* among the most common isolated pathogens. The correct and fast identification of the etiological agents is crucial for clinical decision-making, allowing to rapidly select the appropriate antimicrobial and to prevent from overuse and misuse of antibiotics and the consequent increase in antimicrobial resistance. Conventional culture methods are still the gold standard to identify these pathogens, however, are time-consuming and may lead to erroneous diagnosis, which compromises an efficient treatment. (Bacterio)phage receptor binding proteins (RBPs) are the structures responsible for the high specificity conferred to phages against bacteria and thus are very attractive biorecognition elements with high potential for specific detection and identification of pathogens. Taking into account all these facts, we have designed and developed a new, fast, accurate, reliable and unskilled diagnostic method based on newly identified phage RBPs and spectrofluorometric techniques that allows the multiplex detection of *Enterococcus* and *Staphylococcus* in blood samples in less than 1.5 hr after an enrichment step.

KEYWORDS

bacteria, bacteriophages, bloodstream infections, diagnostic method, receptor binding proteins

1 | INTRODUCTION

Healthcare-associated infections (HCAIs), also known as "nosocomial" or "hospital-acquired," are infections that were not present at the time of admission and appear 48 hr or more after hospitalization or within 30 days after patients having received health care (Haque, Sartelli, McKimm, & Bakar, 2018; WHO, 2002).

HCAIs rank among the top 10 leading causes of death and increased morbidity among hospitalized patients (Haque et al., 2018; WHO, 2002). The Center for Disease Control and Prevention (CDC) estimates that in the United States nearly 1.7 million hospitalized patients annually acquire HCAIs while being treated for other health issues and that more than 98,000 of these patients die due to HCAIs (Klevens et al., 2007).

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In Europe, more than 2.5 million new cases of HCAIs occur every year (Cassini et al., 2016).

HCAIs are usually associated to multidrug resistant bacteria due to the widespread use of antimicrobials for therapy or prophylaxis promoted by the healthcare environment (Khan, Baig, & Mehboob, 2017; WHO, 2002). Currently, antibiotic resistance is a massive public health challenge and besides increasing the incidence and negative impact of HCAIs, it greatly complicates treatment to a level that can go up to the inexistence of efficient antimicrobials against the bacterial etiological agents (Khan et al., 2017; WHO, 2002).

From the different types of HCAIs, bloodstream infections (BSIs) are among the most frequent and with highest mortality incidence rates (Haque et al., 2018; Khan et al., 2017). The pathogens responsible for these infections include mainly bacteria and less frequently viruses and fungal parasites (Khan et al., 2017). From these, *Enterococcus* (mainly *E. faecalis* and *E. faecium*) and *Staphylococcus* (mostly *S. aureus* and coagulase-negative staphylococci [CoNS]) account for the most common isolated pathogens (European Centre for Disease Prevention & Control, 2018; Haque et al., 2018; Horan, Andrus, & Dudeck, 2008; Khan et al., 2017). The emergence of antibiotic-resistant strains of these pathogens, such as the well-known methicillin-resistant *S. aureus* and vancomycin-resistant enterococci, increases their incidence and threatens the effective control of these bacteria with a consequently significant burden on the global healthcare system, particularly in low resource countries (Amin & Deruelle, 2015; Haque et al., 2018; Khan, Ahmad, & Mehboob, 2015; Khan et al., 2017). The CDC estimates that ~50% of the antibiotics that are prescribed are unnecessary (Colgan & Powers, 2001), which is one of the main causes of antibiotic resistance. In the absence of a fast and correct diagnosis, and giving the severity of infections, clinicians often resort to empirical broad-spectrum antimicrobials, with the corresponding associated consequences (Carlet et al., 2011). Moreover, the administration of an inadequate antimicrobial therapy within the first 24 hr causes a rapid decline in patient survival rates (Harbarth et al., 2003; Kumar et al., 2009).

Conventional culture methods are still the gold standard for diagnosis of BSIs, but their accuracy is affected or even hampered by the presence of antibiotics when samples are obtained during antimicrobial treatment. Moreover, the results obtained by these methods can take up to 72 hr to be conclusive due to the low levels of bacteria normally present in samples and their slow growth (Grace, Lieberman, Pierce, & Littenberg, 2001; Murray & Masur, 2012; Peters, Agtmael, Danner, Savelkoul, & Vandenbroucke-Grauls, 2004). Molecular methods, like those based on polymerase chain reaction (PCR), offer a fast alternative to conventional techniques and can overcome many of the pointed limitations, due to their specificity, high sensitivity, and enrichment culture avoidance (Peters et al., 2007; Wilson, 1997). However, there are many factors that can compromise these methods: the occurrence of PCR reaction inhibitors, especially in blood; the efficiency of DNA recovery is highly variable and dependent on the type of samples; and a high number of false positives occurs due to the presence of DNA from dead bacteria (Al-Soud & Rådström, 2001; Cangelosi & Meschke, 2014; Cogswell, Bantar, Hughes, Gu, & Philipp, 1996; Navarro, Segura, Jesus Castano, & Solera, 2006; Wilson, 1997). Immunoassays offer a simple and rapid detection of microorganisms but their efficiency is strongly dependent on the antibodies affinity and specificity towards the target pathogen with the consequent appearance of false-positive results due to the occurrence of antibody cross-reactions. Moreover, antibodies can be expensive and have a limited shelf-life with a stability that depends on the pH and temperature conditions (Velusamy, Arshak, Korostynska, Oliwa, & Adley, 2010).

Bacteriophages (phages) are bacterial viruses known for their high specificity against the target bacteria that can go up to the strain level. This specificity is conferred by the phage receptor binding proteins (RBPs), highly variable structures of the phage particle that are responsible for recognizing specific receptors on the cell surface (Casjens & Molineux, 2012). Consequently, RBPs are powerful tools for specific pathogen detection and have shown high potential in diagnostics (S. B. Santos, Costa, Carvalho, Nóbrega, & Azeredo, 2018; Simpson, Sacher, & Szymanski, 2016; Singh, Arutyunov, Szymanski, & Evoy, 2012; Sumrall et al., 2020). These proteins overcome some of the limitations of antibodies that hamper their use in in situ applications, for example, pH, temperature, and protease sensitivity, while exhibiting comparable or even superior specificity and affinity (S. B. Santos et al., 2018; Simpson et al., 2016; Singh et al., 2012). The intrinsic characteristics of RBPs make them attractive biorecognition elements on a variety of methodologies for the rapid and specific detection and identification of bacterial pathogens. Fluorescence spectroscopy has shown to be a useful analytical approach in many fields, particularly in the detection of bacteria from biological samples, improving diagnosis and clinical care (Shakibaie, Lamard, Rubinsztein-Dunlop, & Walsh, 2018).

In this study, we aimed at designing and developing a simple and fast spectrofluorometric multiplex assay based on phage RBPs to detect *Enterococcus* and *Staphylococcus* in blood samples. To accomplish this, we identified phage RBPs targeting specifically these two pathogens and fused them to different fluorescent proteins, detectable by spectrofluorometry, allowing to discriminate both bacteria in a single analysis.

2 | MATERIAL AND METHODS

2.1 | Bacterial strains and growth conditions

The bacterial strains used in this study (Table 1) comprised: 25 strains of *Staphylococcus (S. aureus*, *S. epidermidis*, *S. warneri*, *S. capitis*, *S. equorum*, *S. hominis*, *S. haemolyticus*); 23 strains of *Enterococcus (E. faecalis*, *E. faecium*, *E. gallinarum)*; 8 strains of other bacterial genera as *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. *E. coli* TOP10 and *E. coli* BL21 (DE3) were used for cloning and protein expression. Bacterial strains were routinely grown in Tryptic Soy Broth (TSB; VWR Chemicals) and in Lysogeny Broth (LB; Liofilchem) at 37°C in liquid medium (120 rpm) or in solid medium (through the addition of 12 g/L of agar; Liofilchem).

TABLE 1 Bacterial strains used in this study and binding ability of the RBPs towards those bacteria analyzed by epifluorescence microscopy and spectrofluorometry

	RBP GFP-*gp109*		RBP mCherry-*gp18*	
Bacterial strain	Micr	Spectr	Micr	Spectr
S. aureus Sa12	+	1.00	–	0.01
S. aureus Sa3	+	0.48	–	0.01
S. aureus Sa17	+	0.65	–	0.02
S. aureus Sa18	+/–	0.51	–	0.02
S. aureus Sa25	+	0.44	–	0.01
S. aureus 097	+	0.44	–	0.05
S. aureus C017	+	0.38	–	0.01
S. aureus C060	+/–	0.42	–	0.01
S. aureus C101	+	0.38	–	0.01
S. aureus C117	+	0.42	–	0.00
S. aureus C411A	+	0.47	–	0.01
S. aureus C577	+	0.46	–	0.04
S. aureus C610	+	0.40	–	0,01
S. aureus I366	+	0.59	–	0.01
S. aureus I642	+	0.55	–	0.01
S. epidermidis RP62A	+/–	0.60	–	0.01
S. epidermidis M129	+	0.98	–	0,01
S. epidermidis SECOM 020A.1	+	1.00	–	0.01
S. epidermidis IE186	+	0.99	–	0.01
S. epidermidis PT12003	+/–	0.19	–	0.01
S. warneri SECOM F16	+/–	0.16	–	0.01
S. capitis SECOM 052 A	–	0.13	–	0.01
S. equorum SECOM 060 A	+/–	0.38	–	0.01
S. hominis SECOM M11	+	0.42	–	0.01
S. haemolyticus SECOM 065 A.1	+/–	0.18	–	0.01
E. faecalis I809	–	0.01	+	1.00
E. faecalis LMV-034	–	0.01	+/–	0.13
E. faecalis LMV-036	–	0.01	+	0.99
E. faecalis LMV-038	–	0.01	–	0.13
E. faecalis LMV-039	–	0.01	+	0.99
E. faecalis LMV-040	–	0.02	–	0.19
E. faecalis LMV-056	–	0.03	+	0.91
E. faecalis I018	–	0.01	–	0.13
E. faecalis I640	–	0.03	+	0.71
E. faecalis I899	–	0.02	+	0.52
E. faecalis I900	–	0.01	+	0.52
E. faecalis I975	–	0.02	–	0.16
E. faecalis I980	–	0.04	+	0.70
E. faecalis 25	–	0.01	+	0.59
E. faecalis 27	–	0.02	–	1.00
E. faecalis U583	–	0.01	–	0.17
E. faecalis CECT 184	–	0.02	+	0.70
E. faecium LMV-037	–	0.02	–	0.05
E. faecium LMV-041	–	0.01	–	0.18
E. faecium I406	–	0.05	+	0.52
E. faecium I951	–	0.03	–	0.17
E. faecium LMV-042	–	0.01	+/–	0.51
E. gallinarum I936	–	0.01	–	0.05
E. coli 3	–	0.03	–	0.00
E. coli 6	–	0.04	–	0.05
K. pneumoniae 23	–	0.03	–	0.01
K. pneumoniae 24	–	0.04	–	0.01
A. baumannii 13	–	0.02	–	0.00
A. baumannii 14	–	0.01	–	0.01
P. aeruginosa PA01	–	0.03	–	0.01
P. aeruginosa H37783E2A-1	–	0.01	–	0.00

Note: For microscopy analysis, (+) indicates that cells are decorated with a high fluorescent intensity; (+/–) indicates that cells are decorated with a low fluorescent intensity; and (–) indicates that no fluorescent intensity was observed around the cells. For spectrofluorometric analysis, the fluorescent signal acquired (a.u.) was normalized against the signal obtained for the RBP incubated with the phage host strain. Normalized values <0.05 were considered negative.

Abbreviations: Micr, microscopy; RBPs, receptor binding proteins; Spectr, spectrofluorometry.

2.2 | Bioinformatic analysis of potential RBPs

The *E. faecalis* vB_EfaS_Max phage (Melo, Ferreira, Costa, Oliveira, & Azeredo, 2019) and the *S. aureus* vB_SauM-LM12 phage (Melo, Brandao, Akturk, Santos, & Azeredo, 2018) were selected from our collection due to their infection ability against *Enterococcus* and *Staphylococcus*. The phage genomes were sequenced and annotated (GenBank accession numbers MK360024.1 and MG721208.1, respectively) and searched for potential RBPs. These proteins were selected based on the existence of homologs resembling RBPs deposited on the National Center for Biotechnology Information (NCBI) nonredundant protein database identified through BLASTp (Altschul, Gish, Miller, Myers, & Lipman, 1990) and also on structure prediction through HHpred (Zimmermann et al., 2018). Moreover, predicted functional domains were identified through Motif Search (http://www.genome.jp/tools/motif), Pfam (Finn et al., 2013), and InterProScan (Jones et al., 2014). The molecular weight

and isoelectric point of the proteins were calculated using the Compute pI/Mw program ExPASy (Artimo et al., 2012).

2.3 | Cloning of the selected genes

The red fluorescent mCherry gene derived from the DsRed of *Discosoma sea anemones* and the *Aequorea coerulescens* green fluorescent protein (GFP) gene were inserted into the plasmid pET28a(+) (Novagen), between the *Nde*I and *BamH*I restriction sites conserving the plasmid N-terminal hexa-histidine (His)-tag sequence and originating the plasmids pET_mCherry (Akturk et al., 2019) and pET_GFP (S. B. Santos, Oliveira, Melo, & Azeredo, 2019), correspondingly. Primers with the desired enzyme restriction sites at the 5′-terminus were designed to amplify the selected genes (Table 2). Primer melting temperatures were calculated using OligoCalc (Kibbe, 2007) and the genes were amplified (annealing temperature of 55°C) with Phusion DNA Polymerase (Thermo Fisher Scientific) according to manufacturer's instructions. The corresponding phage DNA was used as template and the amplified DNA was digested with the corresponding restriction enzymes (*Sac*I and *Xho*I). The digested amplicons originated from the *E. faecalis* and *S. aureus* phages were respectively inserted into the pET_mCherry and pET_GFP (to fuse them with the fluorescent protein upstream, at the N-terminus) and ligated with the T4 ligase (Thermo Fisher Scientific) to obtain the different constructions, further used to transform *E. coli* TOP10 competent cells (Invitrogen). The resulting fused proteins were named as mCherry-*gp*17, mCherry-*gp*18, GFP-*gp*109, and GFP-*gp*111. Colonies were screened through colony PCR and positives were used for plasmid extraction and further confirmation through Sanger sequencing. Correct plasmids were used to transform competent *E. coli* BL21 (DE3) cells (Invitrogen).

2.4 | Expression and purification of the fused proteins

E. coli BL21 cells harboring the recombinant plasmids were grown at 37°C in LB medium supplemented with 50 mg/L of kanamycin until reaching an optical density (OD) at 620 nm ($OD_{620\,nm}$) of 0.6. Recombinant protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma-Aldrich), followed by incubation overnight at 16°C, 120 rpm. Cells were harvested by centrifugation (9,000 × *g*, 15 min) and further resuspended in phosphate lysis buffer (20 mM sodium dihydrogen phosphate, 500 mM sodium chloride, pH 7.4). Cell disruption was made by thaw-freezing (three cycles, from −80°C to room temperature) followed by a 5 min sonication (Cole-Parmer Ultrasonic Processor) for 10 cycles (30 s ON and 30 s OFF) at 40% amplitude. Soluble cell-free extracts were separated by centrifugation, filtered, and loaded on a 1 ml HisPur™Ni-NTA Resin (Thermo Fisher Scientific) stacked into a polypropylene column (Qiagen). After two washing steps with protein-dependent imidazole concentrations (lysis buffer supplemented with 20 mM imidazole in the first wash and 40 mM imidazole in the second wash), the protein was eluted with 300 mM imidazole. Protein fractions were observed through sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% wt/vol acrylamide), followed by Blue Safe staining (NZYTech). The purified proteins were concentrated and dialyzed against 0.1 M phosphate buffer pH 7.2 (PB) using the centrifugal filters Amicon Ultra 0.5 ml MWCO 10 KDa (Merck Millipore) and stored at 4°C. Protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific) with bovine serum albumin as standard.

2.5 | Functional analysis of the RBPs and specificity assays by epifluorescence microscopy

The binding ability of the different constructions (potential RBPs fused to the fluorescent proteins) was inferred by epifluorescence microscopy observations of the corresponding phage host cells (*E. faecalis* I809 or *S. aureus* Sa12) after incubation with the fused proteins (mCherry-*gp*17 and mCherry-*gp*18 or GFP-*gp*109 and GFP-*gp*111, respectively). The mCherry or GFP alone were used as negative controls to discard unspecific binding by the fluorescent proteins to *E. faecalis* or *S. aureus* cells.

TABLE 2 Primers used to amplify the selected genes and the respective restriction enzymes used

Primer name	Primer sequence (5′→3′)	Restriction enzyme
*gp*17.fw	GCCGCCGAGCTCATGAAAGAAAACTATAATTTTCTACGCTCATTTAC	*Sac*I
*gp*17.rev	CCGCCGCTCGAGTTATAGATATGTTTGTTCTAAAATACAATTCACTTC	*Xho*I
*gp*18.fw	GCCGCCGAGCTCATGGACTTTTACATTACAGATAGGACATTTAAG	*Sac*I
*gp*18.rev	CCGCCGCTCGAGCTATACTGAAAATATACTTGTCAATACCCATTTAG	*Xho*I
*gp*109.fw	GCCGCCGAGCTCATGGCATTAAATTTTACTACAATAACGGAAAAC	*Sac*I
*gp*109.fw	CCGCCGCTCGAGCTATGGCATATTAATACCTATAATTCTTGTAAC	*Xho*I
*gp*111.fw	GCCGCCGAGCTCATGGCATTTAATTACACGCCTCTTAC	*Sac*I
*gp*111.rev	CCGCCGCTCGAGCTAAAGTGTGTTAATTCCTGCTATTCTATATATAG	*Xho*I

Note: Restriction endonuclease sites are underlined.

Briefly, bacterial cells were grown in liquid TSB at 37°C until mid-log phase (OD_{620nm} = 0.3–0.4) and then the culture was centrifuged for 5 min at 9,000 × *g*, followed by resuspension in the same volume of PB. A volume of 500 µl of each bacterial suspension was centrifuged at 9,000 × *g* for 5 min. The pellet was resuspended in 20 µl of 5 µM purified protein and incubated for 30 min at room temperature. The cells were washed two times with PB by centrifugation to remove the unbound protein. The washed pellet was resuspended in 10 µl of PB and observed at the epifluorescence microscope equipped with U-RFL-T light source (Olympus BX51, magnitude ×1,000) in bright field (BF) and under the TRITC (530-550; LP-591) or fluorescein isothiocyanate (FITC; 470-490; LP-516) filters, depending on the fluorescent protein (mCherry or GFP, respectively). Control samples using PB instead of the fused recombinant proteins were prepared simultaneously.

The proteins mCherry-*gp*18 and GFP-*gp*109 were selected and tested for their specificity and sensitivity against the strains listed in Table 1, following the procedure described above.

2.6 | Spectrofluorometric assays

Overnight cultures of *E. faecalis* I809 and *S. aureus* Sa12 grown on TSB at 37°C, 120 rpm were centrifuged at 4,670 × *g* for 10 min and the cell pellets resuspended in 0.1 M PB (pH 7.2) setting the OD_{620} to 0.6. The bacterial suspensions (1 ml) were centrifuged at 9,000 × *g* for 5 min and concentrated 10 times in PB. Afterwards, a 120 µl reaction was set by adding 40 µl of one or both bacterial suspensions and 20 µl of one or both purified fused proteins (GFP-*gp*109 or mCherry-*gp*18) at a final concentration of 2.5 µM (when necessary PB was added to achieve the final reaction volume). The reaction was incubated at room temperature for 30 min. Cells were centrifuged (9,000 × *g* for 5 min) and then washed twice with PB at the same conditions to remove unbound protein and resuspended in 120 µl of PB. Each sample (100 µl) was analyzed in a 96-well black microplate through a BioTek™ Synergy™ H1 Hybrid Multi-Mode Microplate Reader with the BioTek Gen5 data analysis software. Excitation/emission (gain) was set to 470/510 nm (70) and to 570/610 nm (100) to analyze the protein fused to GFP and mCherry respectively, with the fluorescence intensity expressed in arbitrary units (a.u.). Two independent experiments with duplicates were performed for each sample and negative and positive controls were also included. Negative controls were: *S. aureus* Sa12 incubated with mCherry-*gp*18; *E. faecalis* I809 incubated with GFP-*gp*109; unstained bacteria; samples with only the fused proteins without bacterial cells. Positive controls comprised *E. faecalis* I809 with mCherry-*gp*18 and *S. aureus* Sa12 with GFP-*gp*109.

The proteins mCherry-*gp*18 and GFP-*gp*109 were tested as described above against the strains listed in Table 1 to assess their specificity and sensitivity on the developed spectrofluorometric assay.

To determine the detection limit, suspensions of *S. aureus* Sa12 and *E. faecalis* I809 were prepared as previously described and diluted into different concentrations, ranging from 1 to 10^8 colony-forming unit (CFU)/ml. Samples were treated and analyzed by spectrofluorometry, as mentioned above.

Assessing the detection of *S. aureus* and *E. faecalis* in blood was accomplished by artificially contaminate 5 ml of horse blood (Probiológica) with 1–5 CFU/mL. The spiked blood sample (as well as a negative control composed of noncontaminated blood) was incubated with 45 ml of TSB for ~15 hr at 37°C, 120 rpm. Afterwards, 1 ml of each sample was diluted ten times in ultrapure water to promote osmotic lysis of erythrocytes, centrifuged at 4,670 × *g* for 10 min and washed twice with 10 ml of PB at the same conditions. This suspension was then used to prepare the reaction mixture with the fused proteins (GFP-*gp*109 or mCherry-*gp*18), using the same procedure described above for the analysis of contaminated buffer samples.

2.7 | Statistical analysis

All results were analyzed by one-way analysis of variance test. The data are presented as means and standard deviations. Differences between samples were considered statistically significant for $p \leq .05$.

3 | RESULTS

3.1 | Bioinformatic analysis of potential RBPs

Tail proteins, mainly those from the minor tail, major tail, and tail fibers, are often associated with phage RBPs (Simpson et al., 2016). The genomic analysis of the *E. faecalis* vB_EfaS_Max and the *S. aureus* vB_SauM-LM12 phages enabled the identification of genes encoding those proteins, which may act as RBPs.

Concerning the *E. faecalis* phage, we have selected as potential RBPs two gene products (*gp*) that have homology with proteins from phage tails deposited at the NCBI nonredundant database. The *gp*17 has homology with tail/tail minor proteins from other *Enterococcus* phages but also with tail fibers from *Lactobacillus* prophages. Accordingly, HHpred found hits with a distal tail protein/RBP from *Lactococcus* phages (PDB 4V96_AT and 5LY8_A, at the N- and C-terminus, respectively). Domains from the Sipho_tail and phi3626_*gp*14_N families (which comprise tail component proteins from a number of phages) were also identified (Figure 1). The *gp*18, besides presenting many tail assembly protein homologs, also aligns with many minor tail proteins from other *Enterococcus* phages. In their great majority, and in contrast with *gp*17, the homologs align only at the N-terminus, showing that the C-terminus is highly variable, a common feature among RBPs (Dupont, Vogensen, Neve, Bresciani, & Josephsen, 2004; Santos et al., 2018; Singh et al., 2012). Moreover, the N-terminus presents an identifiable Prophage_tail superfamily and a Phage minor structural protein, N-terminal domain. The Prophage_tail superfamily is a family of prophage tail proteins that probably act as endopeptidases, which might suggest

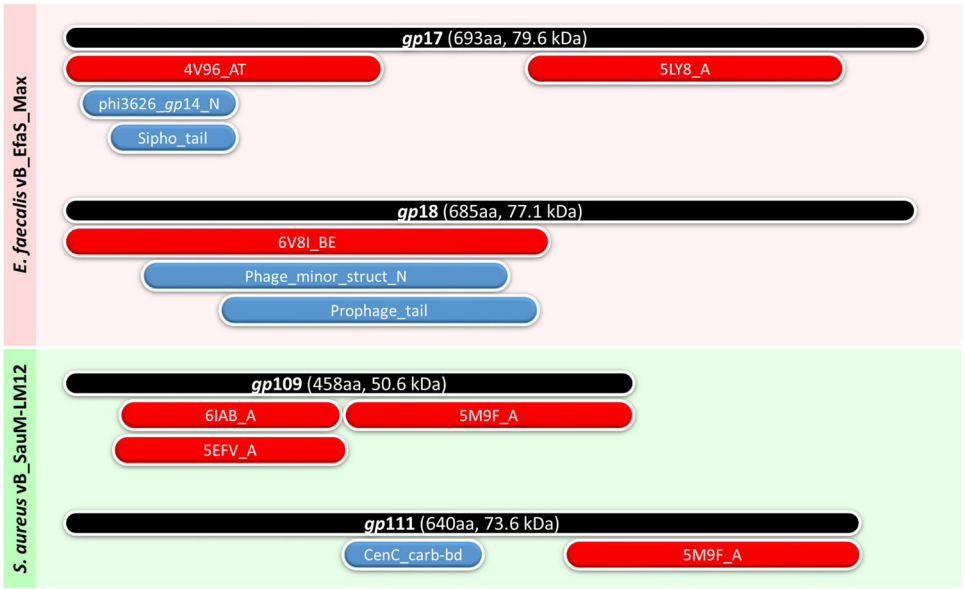

FIGURE 1 In silico analysis of the phage potential RBPs. Selected proteins are represented in black identified by their *gp* number, followed by their length (aa) and predicted molecular weight (kDa). Hits to protein families are denoted in blue with the family name. Homologous protein structures are denoted in red with the homologs Protein Data Bank (PDB) identification. The forms were drawn at a relative scale in their relative positions [Color figure can be viewed at wileyonlinelibrary.com]

that *gp*18 is likely a tailspike. Homology with distal tail protein/tail tip from a *Staphylococcus* phage (PDB 6V8I_BE) was also found with HHpred (Figure 1).

On the *Staphylococcus* phage genome, we have also selected two proteins based on the bioinformatics analysis. The *gp*109 revealed a number of tail fibers and some RBP homologs. PFAM and InterPro failed to identify any conserved domain, while HHpred found homology with the *Staphylococcus* phage K putative RBP (PDB 5M9F_A) at the C- terminus and with the *Staphylococcus* phage phi11 phage adsorption/wall teichoic acids interacting protein (PDB 5EFV_A) and the RBP of *Staphylococcus* phage P68 (PDB 6IAB_A) at the N-terminus (Figure 1). Carbohydrate-binding domain-containing proteins and some RBPs (all from other *Staphylococcus* phages) were found as homologs to the *gp*111. A carbohydrate-binding domain was found at the middle of the protein sequence and InterPro also identified domains related to carbohydrate-binding, cellulose-binding and galactose-binding (CenC_carb-bd). As with *gp*109, homology with the *Staphylococcus* phage K putative RBP (PDB 5M9F_A) at the C-terminus was identified with HHpred (Figure 1).

3.2 | Functional analysis of the selected potential RBPs

After the in silico identification of the potential RBPs, we have performed a functional analysis of these proteins to confirm their recognition binding ability.

Genes encoding hypothetical RBPs from the *Enterococcus* phage (*gp*17 and *gp*18) were cloned into pET_mCherry originating the fusion proteins mCherry-*gp*17 and mCherry-*gp*18, while those from the *Staphylococcus* phage (*gp*109 and *gp*111) were cloned into pET_GFP generating the fusion proteins GFP-*gp*109 and GFP-*gp*111. Such fusion recombinant proteins enabled to infer the binding ability of the potential RBPs through epifluorescence microscopy. If the tested protein is a RBP, it will bind to cells and due to the N-terminus fluorescent protein, the cells will be decorated with fluorescence, detectable via epifluorescence microscopy.

Functional analysis has shown that mCherry-*gp*18 (Figure 2ai) was able to bind to the *E. faecalis* I809 (the host for the *E. faecalis* phage vB_EfaS_Max) and that GFP-*gp*109 (Figure 2aii) recognized *S. aureus* Sa12 (the host for the *S. aureus* phage vB_SauM-LM12). Importantly, during this analysis, it was possible to notice that all target cells observed with the BF filter (Figure 2ai,ii BF) were fluorescently decorated when observed with the corresponding filter (Figure 2ai TRITC and ii FITC). Conversely, no fluorescence was observed when mCherry-*gp*17 and GFP-*gp*111 were incubated with their target cells, as well as the negative controls mCherry and GFP.

Considering these results, the proteins mCherry-*gp*18 and GFP-*gp*109 were selected to be used in the subsequent assays.

3.3 | Specificity and sensitivity assays by epifluorescence microscopy

Considering that we aimed at using the selected proteins (mCherry-*gp*18 and GFP-*gp*109) for diagnosis and in a multiplex approach, we have assessed the potential cross-reaction between these proteins as well as their specificity and sensitivity to the target bacteria. Through this evaluation, we observed that mCherry-*gp*18 and GFP-*gp*109 did not recognize *S. aureus* Sa12 or *E. faecalis* I809,

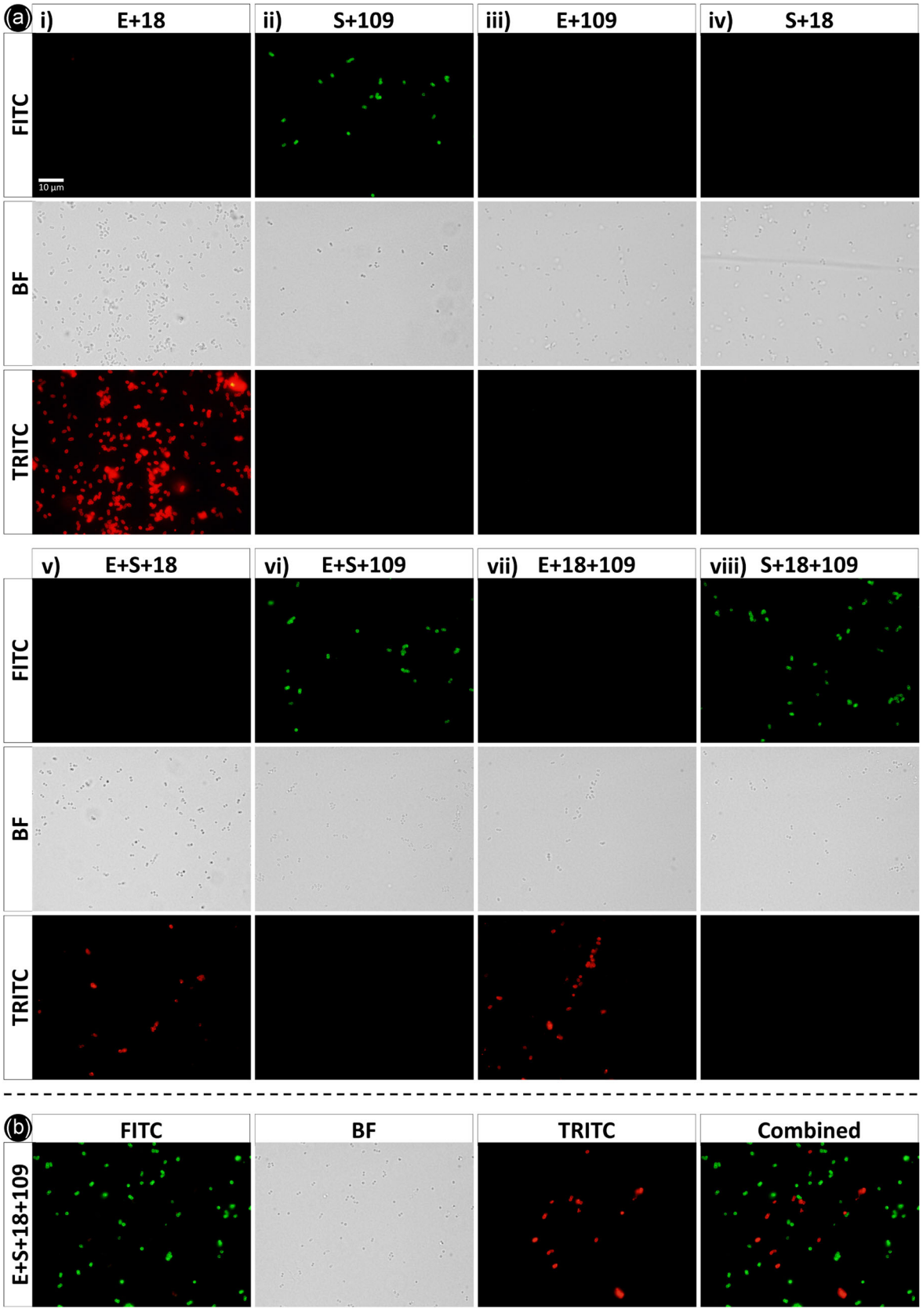

FIGURE 2 Functional analysis of the phage RBPs. (a) Bacterial suspensions of *Enterococcus faecalis* I809 and/or *Staphylococcus aureus* Sa12 were combined with mCherry-*gp*18 or GFP-*gp*109. (b) Bacterial suspensions of *E. faecalis* I809 and *S. aureus* Sa12 were combined with mCherry-*gp*18 and GFP-*gp*109 showing no cross-reactivity and the ability to detect and discriminate all bacterial cells. (E) *E. faecalis* I809; (S) *S. aureus* Sa12; (18) mCherry-*gp*18; (109) GFP-*gp*109; RBPs, receptor binding proteins [Color figure can be viewed at wileyonlinelibrary.com]

respectively (Figure 2aiii,iv). Also, when incubating both proteins with only *E. faecalis* I809 or *S. aureus* Sa12, no decorated cells were observed with the FITC (green) or TRITC (red) filter, respectively (Figure 2avii,viii). Following these results, a mixed culture of *E. faecalis* I809 and *S. aureus* Sa12 was incubated with mCherry-*gp*18 or GFP-*gp*109 and, as expected, only part of the population was decorated with red or green fluorescence, respectively (Figure 2av,vi). When both proteins were incubated with the mixed culture of *E. faecalis* I809 and *S. aureus* Sa12, all the cells observed with the BF filter were decorated with red or green fluorescence, without overlapping of both fluorescent proteins (Figure 2b).

The specificity and sensitivity of the RBPs were further assessed by testing a panel of 25 *Staphylococcus* and 23 *Enterococcus* strains as well as eight bacterial strains of other genera. The *Staphylococcus* phage RBP (GFP-*gp*109) was able to recognize all *Staphylococcus* strains with the exception of the *S. capitis* strain and did not bind to any non-*Staphylococcus* strain, accounting for a specificity of 100% and a sensitivity of 96%. The *Enterococcus* phage RBP (mCherry-*gp*18) was able to bind to most of the *E. faecalis* strains (11 out of 17) and to decorate two of five *E. faecium* strains (Table 1). No binding of mCherry-*gp*18 to *E. gallinarum* and to the other strains outside the *Enterococcus* genus was observed. This resulted in a specificity of 100% and a sensitivity of 57% (13 out of 23 strains) to the *Enterococcus* genus.

3.4 | Spectrofluorometric assays

The epifluorescence microscopy assays allowed to functionally analyze the identified *Enterococcus* and *Staphylococcus* phage RBPs and to assess their specificity and sensitivity against the target bacteria. Although this equipment enables to use the fusion proteins for the detection and identification of these two problematic pathogens, it is a time- and labor-intensive manual process, requiring expertise in microscopy observation. Conversely spectrofluorometric analysis is a simple methodology that does not require skilled technicians or expensive equipment. Consequently, we have designed a method based on this technique for the multiplex detection of *Enterococcus* and *Staphylococcus* using the phage fluorescent RBPs. First, it was important to determine the detection limit of the assay in terms of the number of decorated cells needed to obtain a significant signal. This was performed by analyzing 10-fold dilutions of bacterial suspensions from 1 to 10^8 CFU/ml decorated with the fusion RBPs. The results showed that 10^8 CFU/ml are needed for an unequivocal signal at the spectrofluorometer (Figure 3).

To assess the efficacy of the spectrofluorometric assay to detect *Staphylococcus* and *Enterococcus* cells, the same samples submitted to the epifluorescence microscopy (Table 1) were measured to determine the specificity and sensitivity of the assay and the quality and reproducibility of the fluorescent signal obtained. The results (Table 1) demonstrate that high green fluorescent signals were measured when the GFP-*gp*109 protein was tested against all *Staphylococcus* strains, including *S. aureus* and CoNS strains. Also, red fluorescent signals were observed when the mCherry-*gp*18 protein was tested with *Enterococcus* strains, including *E. faecalis* and *E. faecium*, with the exception of *E. gallinarum* I936 and *E. faecium* LMV-037, accounting for a sensitivity of 91%. When GFP-*gp*109 and mCherry-*gp*18 were incubated with bacterial strains not belonging to the *Staphylococcus* or *Enterococcus* genus respectively, no significant fluorescence signals were obtained. Regarding negative controls, when samples with unstained bacteria were analyzed, autofluorescence at a wavelength of 510 nm (green) and 610 nm (red) was not detected. Also, only residual fluorescent signals were observed when samples without bacterial cells and with only the recombinant fused proteins (after sample processing) were measured (Figure 4).

The ability of the spectrofluorometric assay to enable a multiplex detection of *Staphylococcus* and *Enterococcus* cells was performed first with bacterial suspensions in buffer to eliminate any interference from complicated matrices. From this analysis, it was possible to observe that an unequivocal positive signal was obtained only when the RBP and the target bacterium was present and only at the corresponding fluorescent RBP's wavelengths (Figure 4a). This indicates that a positive signal at a wavelength of 610 nm (red) was obtained

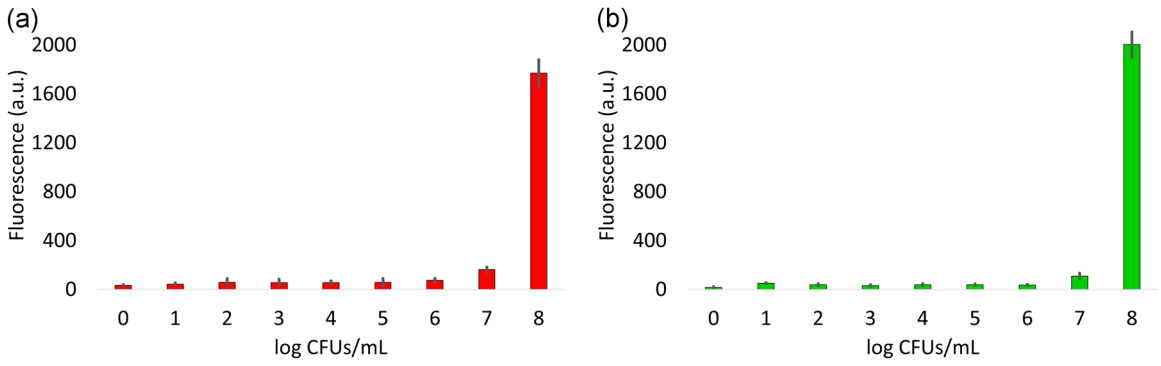

FIGURE 3 Detection limit of the spectrofluorometric assay. Serial 10-fold dilutions of bacterial suspensions were combined with the corresponding phage RBP and analyzed at the spectrofluorometer. (a) *Enterococcus faecalis* I809 with mCherry-*gp*18; (b) *Staphylococcus aureus* Sa12 with GFP-*gp*109. RBP, receptor binding protein [Color figure can be viewed at wileyonlinelibrary.com]

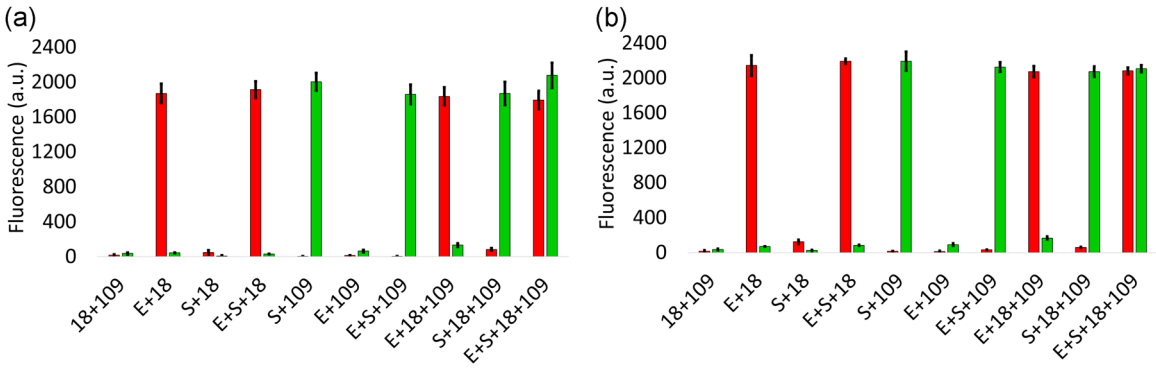

FIGURE 4 Specificity of the spectrofluorometric multiplex assays. Bacterial suspensions of *Enterococcus faecalis* I809 and/or *Staphylococcus aureus* Sa12 were combined with mCherry-*gp*18 and/or GFP-*gp*109. (a) Multiplex assays performed on buffer samples. (b) Multiplex assays performed on blood samples. (E) *E. faecalis* I809; (S) *S. aureus* Sa12; (18) mCherry-*gp*18; (109) GFP-*gp*109. GFP, green fluorescent protein [Color figure can be viewed at wileyonlinelibrary.com]

only when the *Enterococcus* mCherry-*gp*18 RBP and a sensitive *Enterococcus* strain was present in the sample, independently of the presence of other RBP. Accordingly, a positive signal at a wavelength of 510 nm (green) was obtained only when the *Staphylococcus* GFP-*gp*109 RBP and a sensitive *Staphylococcus* strain was present in the sample. For samples containing both bacteria with mCherry-*gp*18 or GFP-*gp*109, positive fluorescent red and green signals were detected, respectively. These signals were very similar to the values obtained for the individual bacterial detection, showing that the presence of both bacteria did not interfere with their individual detection. Similarly, when *Enterococcus* cells or *Staphylococcus* cells were incubated with both phage proteins, only the expected fluorescent signal was obtained (red or green, respectively). For the multiplex assay, in which a mixed culture of *Staphylococcus* and *Enterococcus* was incubated with both proteins, a high red and green fluorescence were attained, indicating the presence of both decorated cells (Figure 4a).

Considering the high incidence of these pathogenic bacteria in BSIs, we have assessed the applicability of this methodology in blood artificially contaminated with the target bacteria. The same rationale of the previous experiment in buffer was used herein (the same combinations between the RBPs and target bacteria). However, taking into account that the detection limit of the spectrofluorometric assay is 10^8 CFU/ml and that the concentration of pathogens present in a patient suffering from a BSI range from 1 to 10^3 CFU/ml (Bacconi et al., 2014), the assay in blood had to include an enrichment step, to detect roughly 1 CFU/ml of *E. faecalis* and *S. aureus* in 5 ml of blood. This procedure guarantees that the detection limit of the assay is assured (10^8 CFU/ml), enabling the bacterial detection within 1.5 hr.

The results obtained in the enriched blood samples (Figure 4b) were similar to the previous experiments with bacterial suspensions in buffer (Figure 4a). In fact, an unequivocal positive signal was measured only when the RBP and the target bacterium was present and only at the corresponding fluorescent RBPs wavelengths. Moreover, a multiplex detection of *Staphylococcus* and *Enterococcus* was successfully achieved on artificially seeded blood samples. A control sample of noncontaminated blood was submitted to the same procedure, resulting in a negative signal (<100 a.u.).

4 | DISCUSSION

The high incidence of HCAIs, namely, BSIs, worldwide coupled with their deleterious effects, make these infections a top leading cause of death with a significant burden both for the patient and public health. To counteract this, a fast and accurate identification of the etiological agent is required to design and apply an efficient treatment. However, the currently available methodologies are usually time-consuming, requiring at least 48 hr and can go up to a few days for the specific pathogen identification (Arabestani, Rastiany, Kazemi, & Mousavi, 2015; Opota, Croxatto, Prod'hom, & Greub, 2015). Moreover, they are not always accurate, detecting only 30–40% of all cases of sepsis (Arabestani et al., 2015), which leads to a high decrease in survival rates (Kumar et al., 2009). Therefore, there is an urgent demand for research and development of new diagnostic methods able to solve the problems of the existing ones, namely, accuracy and fastness. Such methods will prevent the common overprescription of antibiotics and their overuse-related problems (Garnacho-Montero et al., 2008).

Considering that BSIs are among the most frequent HCAIs and that *Enterococcus* and *Staphylococcus* are among the most common isolated bacteria (European Centre for Disease Prevention & Control, 2018), we have designed and developed a new methodology for the multiplex detection of these pathogens in blood samples based on phage RBPs as the biorecognition elements. Accordingly, we have searched for such proteins on the genome of phages infecting the target pathogens: *Enterococcus* and *Staphylococcus*. Bioinformatic analysis of genomes from *E. faecalis* phage vB_EfaS_Max (Melo et al., 2019) and *S. aureus* phage vB_SauM-LM12 (Melo et al., 2018) allowed the identification of four potential RBPs, based on the homology to other known proteins and conserved domains usually associated with RBPs. The selected proteins were further cloned and expressed fused to different fluorescent proteins. This approach enabled their use in a multiplex assay, allowing to differentiate the two target pathogens simultaneously in the same sample.

The functional analysis of the selected proteins, resulted in the identification of one RBP from each phage with the ability to bind to

each target pathogen (*gp*18 for *Enterococcus* and *gp*109 for *Staphylococcus*). Other studies have already reported the identification and structure of RBPs from *Staphylococcus* phages (Kizziah, Manning, Dearborn, & Dokland, 2020; Koç et al., 2016; Li et al., 2016). However, to the best of our knowledge, this is the first study in which the binding affinity (specificity and sensitivity) of RBPs from *Enterococcus* and *Staphylococcus* phages has been assessed. Such analysis has only been reported for RBPs from phages infecting other bacterial genera (Bielmann et al., 2015; Javed, Poshtiban, Arutyunov, Evoy, & Szymanski, 2013)

The fact that the other two proteins (*gp*17 and *gp*111) did not bind to the target hosts does not preclude that they do not have that role in the phage particle but may need other ancillary proteins or specific folding to be functional (Häuser et al., 2012; Santos et al., 2018). Also, phages may employ more than one RBP to attach their target bacterium and thus the identification of one RBP does not impair the existence of others (Häuser et al., 2012; Santos et al., 2018; Santos et al., 2011).

The potential use of RBPs for diagnosis of bacterial infections cannot be based only on their ability to bind to a single strain and consequently, we have performed specificity and sensitivity tests. In the microscopy assays, the *Enterococcus* phage RBP *gp*18 showed to be fully specific for the *Enterococcus* genus since it was not able to bind to any bacterial cell outside this genus. Moreover, *gp*18 recognized the majority of the strains of *E. faecalis* and some strains of *E. faecium*, which is undoubtedly important to detect enterococcal-associated infections that are mainly caused by these species (Kramer et al., 2018). The *Staphylococcus* phage RBP *gp*109 also showed 100% specificity for *Staphylococcus* and its sensitivity was even higher (96%) than the *gp*18, binding to all *Staphylococcus* strains, including *S. aureus* and CoNS (except the *S. capitis*), which are the most prevalent *Staphylococcus* species on BSIs (European Centre for Disease Prevention & Control, 2018). Overall, the RBPs *gp*18 and *gp*109 showed potential to be used for diagnosis of *Enterococcus* and *Staphylococcus* infections, respectively.

As RBPs, the cell binding domains (CBD) of phage endolysins (phage proteins responsible for cell lysis at the end of the lytic cycle) have proven successful as biorecognition elements but in the particular case of *Staphylococcus* phage endolysin CBDs they have been shown a specificity below 100% (Becker, Foster-Frey, Stodola, Anacker, & Donovan, 2009; Benešík et al., 2018). This is the case of the recently described CBD of *Staphylococcus* phage E-LM12, which besides its efficacious use in a new method for *Staphylococcus* detection in blood, demonstrated some binding affinity to two *Enterococcus* strains (Costa et al., 2020). Consequently, the increased specificity of the *Staphylococcus* phage RBP *gp*109 presents advantages as biorecognition molecules for diagnosis, potentially avoiding the occurrence of false positives since these proteins did not bind to any of the strains tested outside their target genus. The absence of cross-reaction between the two RBPs when used together against the panel of strains herein tested supports their application for the multiplex detection of *Staphylococcus* and *Enterococcus*.

Giving the potential of these proteins, we designed a simple multiplex diagnostic method that combines the RBPs as specific biorecognition molecules, the fluorescence of GFP and mCherry and a spectrofluorometer. Overall, the results from the spectrofluorometric assays confirmed that the *Enterococcus* phage RBP *gp*18 was specific for the *Enterococcus* genus since it was not able to bind to any bacterial strain outside this genus and also presented a high sensitivity to the *E. faecalis* and *E. faecium* species, binding to 100% and to 80% of the strains tested, respectively. The *Staphylococcus* phage RBP *gp*109 was specific for the *Staphylococcus* genus, being able to bind to all *Staphylococcus* strains tested.

When comparing the results obtained by this methodology with the microscopy analysis (Table 1), the sensitivity of the spectrofluorometric assay was higher. In fact, despite some *Enterococcus* species and the *S. capitis* strain have not shown fluorescence by microscopy, a fluorescence signal was detected by spectrofluorometric analysis. Although these bacteria presented a lower fluorescence intensity signal, it was noticeably higher than the negative controls, allowing the detection of the stained cells (91% of the *Enterococcus* and 100% of the *Staphylococcus* tested).

Analysis of the limit of detection of the spectrofluorometer revealed that a concentration of 10^8 CFU/ml is required for an unequivocal signal (Figure 3). Considering that 1–100 CFU/ml are usually present in circulation during a BSI (Skvarc, Stubljar, Rogina, & Kaasch, 2013), it implies the need for a sample enrichment step before application of the designed methodology. In fact, this is the reality of the great majority of the diagnostic methods developed to date and only a marginal number have been described to detect bacteria directly from blood without enrichment. Moreover, even these methods present some drawbacks, mainly associated with their high sensitivity (Peker, Couto, Sinha, & Rossen, 2018).

In the developed method, we were able to detect the presence of the target pathogens in blood samples with as just as 1–5 CFU/ml in <1.5 hr. For this, an enrichment step of 15 hr was required to achieve a bacterial concentration (approximately 10^8 CFU/ml) that could be undoubtedly detectable by spectrofluorometry. Although the enrichment step is undesirable for fastness, it prevents the occurrence of false negatives by guaranteeing the detection of viable cells. The time needed for bacterial enrichment is dependent on bacterial species, initial loads, physiological state, and on growth conditions used (Opota et al., 2015). The use of magnetic nanoparticles or microfluidic-based approaches to separate and concentrate bacterial cells before their detection can be applied to decrease the turnaround time of the spectrofluorometric method (Sande, Çaykara, Silva, & Rodrigues, 2020; Sumrall et al., 2020).

Another important fact of the designed method is the ability to detect and identify bacteria without compromising its viability. This allows the use of the identified bacterium for further antibiotic susceptibility tests, which is not possible with many other methods, such as those based on nucleic acids, that kill or degrade the bacterial cells. This is crucial to select the best antimicrobial and to provide an efficient therapy and reduce overprescription of antibiotics and associated adverse outcomes (Afshari, Schrenzel, Ieven, & Harbarth, 2012).

Overall, by combining the specific and sensitive binding ability of the identified phage RBPs, the fluorescence proteins and a

spectrofluorometer, we have designed and developed a new, accurate, reliable, simple, unskilled, and fast diagnostic method to be implemented in a diagnostic laboratory. The results validated these properties and the applicability of phage RBPs to detect *Enterococcus* and *Staphylococcus* easily and accurately by producing an unequivocal fluorescent signal. With this new methodology, we were able to detect low concentrations of the target pathogens in blood within 1.5 hr (excluding the enrichment step) using a simple and relatively inexpensive equipment. Moreover, we validated its use on blood samples, allowing for the individual and multiplex detection of *Enterococcus* and *Staphylococcus*, without the occurrence of false positives that could have occurred due to the autofluorescence of the blood components (Azevedo et al., 2011). Overall, the developed method presents several advantages over other reported diagnostic techniques and thus can be easily implemented in any laboratory and health care unit.

5 | CONCLUDING REMARKS

In this study, we have identified two phage RBPs for *Enterococcus* and *Staphylococcus* that demonstrated high affinity, specificity and sensitivity and thus present great potential to be used as biorecognition elements in the development of new methodologies for bacterial infections diagnosis. By fusing these promising proteins to fluorescent proteins and combining them with a spectrofluorometer able to detect the produced signal, we have developed a new method that enables the multiplex identification of *Enterococcus* and *Staphylococcus* from blood contaminated samples. This assay demonstrated to be accurate, reliable, simple, unskilled, relatively inexpensive, and fast, congregating thus many desirable properties for diagnostics that can potentially improve treatment and control of BSIs and thus decrease their negative impact worldwide.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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