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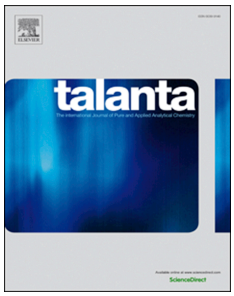

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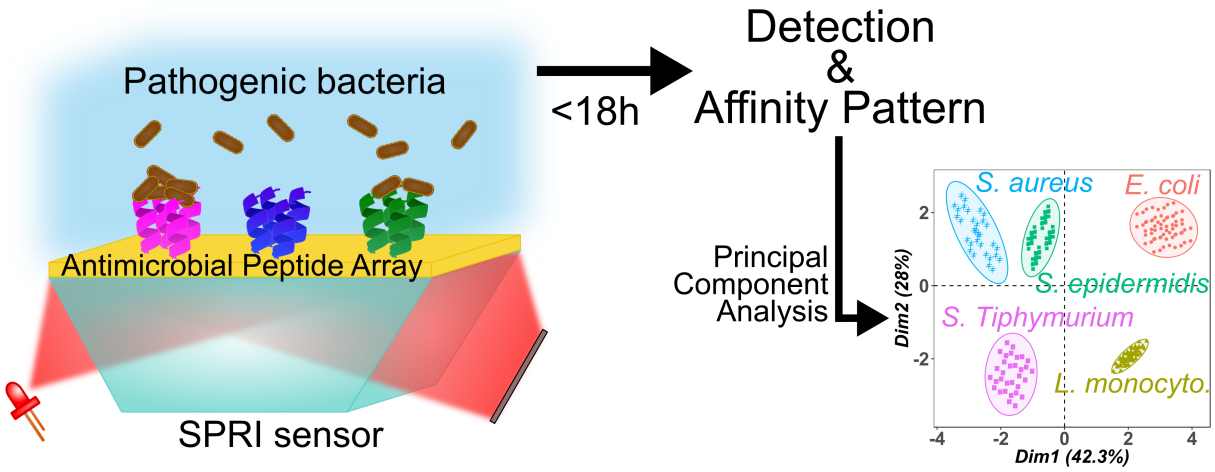

Antimicrobial peptide arrays for wide spectrum sensing of pathogenic bacteria

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Abstract

Fast detection of bacteria in samples presumed to be un-contaminated, such as blood, is of great importance. Indeed, rapid diagnosis allows the set-up of appropriate antibiotic treatment. Besides clinical issues, there are many other domains, such as food processing or drug manufacturing, where the strict absence of any bacteria has to be assessed. Because the bacterial load found in most contaminated samples is often below the limit of detection for currently validated assays, a preliminary enrichment step is required to allow bacterial multiplication before proceeding to the analysis step, whatever it might be – cultural, immunological or molecular methods. In this study, we describe the use of a biosensor for single-step bacteria detection. The whole analysis is performed in less than 20 hours, during the growth phase of the micro-organisms, using an array of antimicrobial peptides (AMPs) coupled with a surface plasmon resonance imager (SPRI). A wide range of bacterial strains are assayed, showing differentiated affinity patterns with the immobilized peptides, which are confirmed by multivariate analysis. This work establishes the evidence that antimicrobial peptides, mostly used so far in the antibiotic drug industry, are suited for the wide-spectrum detection of unknown bacteria in samples, even at very low initial loads. Moreover, the small set of AMPs that were assayed provided a specific affinity profile for each pathogen, as confirmed by multivariate analyses. Furthermore, this work opens up the possibility of applying this method in more complex and relevant samples such as foodstuff, urine or blood.

Keywords

Antimicrobial Peptides; Bacteria Detection; Pathogens; Surface Plasmon Resonance imaging

Introduction

The presence of bacteria in a normally sterile environment is a huge issue for human health, since it can cause severe infections. Whether they originate in foodstuffs or bodily fluids such as blood or urine, the rapid and sensitive detection of pathogenic microorganisms is thus of great importance. Although fast diagnosis is vital during an infection, the early detection of pathogens is made difficult because of their low concentrations. For this reason, all samples drawn from patients are first diluted and cultured in a liquid medium to increase the bacterial concentration, before proceeding to the analysis of the enriched samples. So far, culture-based techniques have remained the gold standard in the process of diagnosing bacterial infections, though several alternative methods have emerged in recent decades to devise faster and more sensitive ways to achieve bacterial detection [1]. Assays based on DNA amplification have paved their way to a large use in clinics [2]. In spite of their faster response, these assays are still time-consuming, quite expensive, and require a high level of skills. Moreover, such methods may give false-positive results due to the presence of residual DNA, released from killed pathogenic bacteria in some samples, for instance after antibiotic treatment. Mass spectrometry has also become widely used for the precise identification of strains [3]. However, it requires isolated colonies of the pathogen to be grown on a solid medium and thus can only be performed several days after enrichment of the sample. Owing to these considerations, new technologies enabling faster diagnostics are still emerging, promising simple and low-cost on-field implementation [4,5]. Amongst other alternatives under research, biosensors able to detect whole viable cells directly from a suspect sample are very promising. Nevertheless, each of these exciting alternatives still require a preliminary enrichment step to increase the initial bacterial concentration above their limit of detection (10^3 to 10^6 CFU.ml^{-1}, depending on the method). Surprisingly, only a few approaches couple the enrichment step with the detection step. From this perspective, surface plasmon resonance imaging (SPRI) is a promising method. Indeed, this technique is based on the optical detection of interactions at the surface of a metallic layer, thus enabling the monitoring of molecular interactions without labels. Although SPRI-based molecular detections are less sensitive than ELISA for biomolecular interaction studies, SPRI has been used in versatile applications from molecular studies to whole cell or living bacteria detection, taking advantage of its real-time and label-free multiplexing abilities [6,7]. This optical method has already proven its ability to successfully detect and identify pathogens starting from food matrices or blood spiked with low bacterial concentrations (few CFU.ml^{-1}), using arrays of proteins and antibodies [8–11]. Such one-step approach enables sensitive detection during the enrichment phase, with a wide range of probes used in parallel that hence allow a high selectivity. However, although antibodies or other protein probes have been proven to have high efficiency as ligands (enabling strain or even serotype identification) they are known to present several difficulties such poor stability, difficulty of production, high cost, and/or burdensome handling. It is the reason why new sets of probes have been benched by researchers, the most prominent being aptamers, molecularly imprinted polymers, and peptides [12]. Aptamers and molecularly imprinted polymers usually show high specificity and reliability [13,14]. Nevertheless, to achieve wide spectrum detection, the multiplexing of large series of probing ligands remains an issue, which limits the extent of wide spectrum detection.

Antimicrobial peptides (AMPs) are a subset of peptides presenting outstanding bactericidal activity [15,16]. Their binding to bacteria mainly relies on physico-chemical interactions, due to their polycationic or amphiphilic characteristics. Hence, they can easily attach to negatively charged lipopolysaccharides (LPS) anchored in the membrane of Gram-negative bacteria *via* electrostatic interactions [17,18]. Conformational changes of their structure can also mediate the attachment to bacterial outer walls [19]. Although AMPs have so far mainly been investigated to design new drugs or antimicrobial applications [20], they are also promising ligands as potential alternatives to antibodies as large spectrum recognition elements towards bacteria [21,22]. Indeed, they have naturally evolved to interact with a wide range of pathogenic bacteria and they are not only easy to synthesize but also easier to handle than antibodies for instance, since they can resist harsh chemical conditions and air-drying for storage.

Surprisingly, the use of AMPs as recognition elements has only rarely been commented on in the literature [23–26]. Those studies relied on the specific recognition of particular groups of bacteria, for instance targeting either a bacterial genus or a Gram class. Herein, we demonstrate the use of AMPs in a multiplexed fashion, with several different peptidic probes exposed to a contaminated sample. Thanks to the development of antimicrobial peptides arrays, we expand the spectrum of detectable bacterial strains in a single one-step assay. Wide-spectrum sensing of bacterial pathogens would hence be achieved. A set of AMPs inspired by previous works on biosensors (see *Table 1*), has been produced by solid-phase synthesis and arrayed on the gold surface of SPRI prisms. Such arrays were then assayed with a series of samples spiked with pathogenic bacteria at low levels. Those strains are representative of major species encountered in both foodborne infections and bacteremia. We were thus able to assess the performance of such peptide-based sensors for wide-spectrum detection of bacteria.

Material and methods

Reagents

Phosphate Buffered Saline (PBS), Dimethyl Sulfoxide (DMSO), Bovine Serum Albumin (BSA), glycerol and Tryptic Soy Broth (TSB) culture medium were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). The solid culture medium Tryptone Soy Agar (TSA) was bought from bioMérieux (Lyon, France). Ultra-pure water (18,2 $M\Omega$ of resistivity) was obtained from an ELGA PURELAB flex dispenser (Veolia Water, France).

Peptides

Name	Sequence	Targets	Technique	Ref.
Clavanin A	VFQFLGKIIHHVGNFVHGFSHVF–spacer–C–NH_2	*E. coli*, *S. aureus* & *S. Typhimurium*	Electrochemical impedance	[27]
Magainin 1	GIGKFLHSAGKFGKAFVGEIMKS-spacer–C–NH_2	*E. coli* & *S. Typhimurium*	Fluorescence	[28]
		E. coli & *Salmonella*	Electrochemical capacitance	[24]
		E. coli	Fluorescence	[29]
		E. coli	Field-effect transistor	[30]
Pediocin Ped3	GKATTCIINNGAMA–spacer–C–NH_2	*Listeria*	Microcantilevers	[31]

		monocytogenes		
PGQ	GVLSNVIGYLKKLGTGALNAVLKQ–spacer–C–NH_2	*E. coli*	Fluorescence	[32]
Leucocin A 24	C–spacer–SVNWGEAFSAGVHRLANGGNGFW–OH	*Listeria monocytogenes* & *E. coli*	Electrochemical impedance	[25, 33]
Control peptide	C–spacer–RGEWFWGNLVVSAASFGNHNAGG–OH	Scrambled version of Leucocin A 24 (newly introduced)		

Table 1. Set of arrayed antimicrobial peptides. Sequences are listed along with the bacteria they can detect, the detection techniques that they were used in and literature references for each. Spacer is corresponding to AEEA.

All peptides were synthesized by Smart Bioscience (Saint Égrève, France) through standard Fmoc solid-phase method. A (2-(2-(amino)ethoxy)ethoxy)acetic acid (AEEA) spacer was inserted between the peptide sequence and the additional terminal cysteine. When this cysteine amino acid was added at the C-terminus, the supplier amidated this latter to achieve higher synthesis yields, otherwise the terminal amino acid was left free. The sequences of the chosen peptides are listed in *Table 1*. The certificates of analysis furnished by Smart Bioscience are given in the Electronic Supplementary Information to confirm peptide purities and molecular weights.

Bacterial strains

The *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S. Typhimirium*), CIP104474, was obtained from the Pasteur Institute (Paris, France). *Listeria monocytogenes* strain belonging to the molecular serotype IVc was acquired from the Institut Scientifique d'Hygiène et d'Analyse (ISHA, Massy, France). It had been isolated from chicken meat. The methicillin-resistant *Staphylococcus aureus* subspecies *aureus* (MRSA), ATCC43300, the *Staphylococcus epidermidis* strain, ATCC12228, and the *Escherichia coli* serovar O1:K1:H7 (isolated from urine), ATCC11775, were all purchased from the American Type Culture Collection (Manassas, Virginia, USA).

Culture conditions

Prior to each experiment, an individual bacterial colony was isolated on a TSA plate and resuspended in 4 mL of sterile TSB. This bacterial culture was then incubated for 18 hours at 37℃ under constant agitation (180 rpm). Ten-fold s erial dilutions of the bacterial suspension were then performed in TSB. 100 μL of one of the ten-fold dilution series was used for sample spiking. The 10^{-5} and 10^{-6} dilutions were plated (100 μL on TSA plates, in triplicates) to determine the initial bacterial concentration through manual colony counting. For each assay, sterility controls were performed.

Peptide arraying

Lyophilized peptides were resuspended in DMSO at 1 $mg.mL^{-1}$ and stored at -80C. Each peptide sequence was diluted at 100 μM in PBS 1x, with 5% (v/v) of glycerol ahead of arraying biochips. SPRi-biochips bought from Horiba Scientific (Palaiseau, France) consisted of glass prisms coated with a thin gold layer for direct functionalization using thiol moieties (see fig. S1 in the Electronic Supplementary Information for more details). Peptide biochips were prepared

using a sciFLEXARRAYER (Scienion, Berlin, Germany), a piezo-dispenser allowing arraying of 4 nL droplets per spot. Each array resulted in 12 spots with a 450 μm diameter, and a 800 μm pitch between each spot. All peptides were systematically arrayed in duplicate. This step was performed at room temperature under a humid atmosphere (75% relative humidity). After arraying, prisms were incubated for 18 hours at 25°C, under 94% humidity, to allow the complete formation of self-assembled monolayers of peptides onto gold. Chips were then rinsed with ultra-pure water, dried under an argon flow for a few seconds, and stored up to several weeks at +4℃ until experiments. Effective surface functionalization was assessed by AFM comparison with bare gold surfaces (supplemental figure S2).

Biochip conditioning and sample processing

Prisms were systematically incubated with a sterile solution of PBS+1% BSA before each experiment to mimic interfering proteins of complex media such as blood, and to block non-specific interactions with gold. The surface of the prism was then rinsed with 3 mL of sterile PBS, before loading the biochip in the Surface Plasmon Resonance imager. 900 μL of sterile TSB were then injected in the culture chamber containing the AMP-array, followed by 100 μL of the bacterial dilution used for spiking. Interactions of bacteria on the surface of the prisms were observed in real-time with the SPRi-Lab+ system (Horiba Scientific, Palaiseau, France), at 37℃. The AMP-array was positioned so that its surface was vertical, on the sidewall of the culture chamber. This avoids any non-specific signal from sedimenting bacteria. Sensors were systematically discarded after use.

SPR data analysis

Real-time monitoring of the SPR signal began when the bacteria were spiked into the sample. Regions of interest (ROI) were defined on each duplicate of AMP spots, in order to monitor the temporal change of reflectivity. Kinetics data were directly collected and processed in R programming language. Duplicate spots of each peptide were averaged, corrected with respect to the reference signal, and plotted. Peptide-free gold ROIs, coated with BSA, were chosen as reference signals for non-specific interactions. Reflectivity shifts as a function of time for each ROI allowed the assessment of the presence of bacteria in samples without further processing of the data, by the observation of a temporal response shift. Kinetics data were used to generate a database of interaction patterns between peptides and bacteria. For each spot, the first derivative of the smoothed temporal data was calculated. The maximum value of this derivative, as well as the corresponding smoothed reflectivity shift, were recorded. Unsupervised multivariate analysis was performed on this database thanks to the FactomineR package, embedded in the R software [34]. Names of targeted bacteria were used as a descriptive variable, and therefore did not affect the results of the Principal Components Analysis (PCA). As data were heterogeneous, they were centered and reduced. On one hand, PCA score plots were used to distinguish results from one bacterial strain to another. On the other hand, they also permitted a high level of repeatability of the process from different duplicates of the same assay. Hierarchical Clustering on Principal Components (HCPC) was also performed on the outcome of the PCA. This statistical method ensured that targeted bacteria were indeed discriminated from each other, based on the Euclidean distance separating the replicates in the PCA results.

Results and discussion

Bacterial growth monitoring by SPRI

The set of five bacterial strains to be detected with the peptide array has been chosen to represent the diversity of pathogens frequently involved in bloodstream or urinary tract infections, as well as food poisoning. The set also reflects microbial morphological diversity, with both Gram-positive and negative species, including bacilli and cocci.

Growth kinetics monitored by SPRI of the bacteria are depicted in fig. *1*, datasets retrieved from those results allow defining characteristic detection times of bacteria (Supplemental Table S1) and affinity patterns with the peptides.

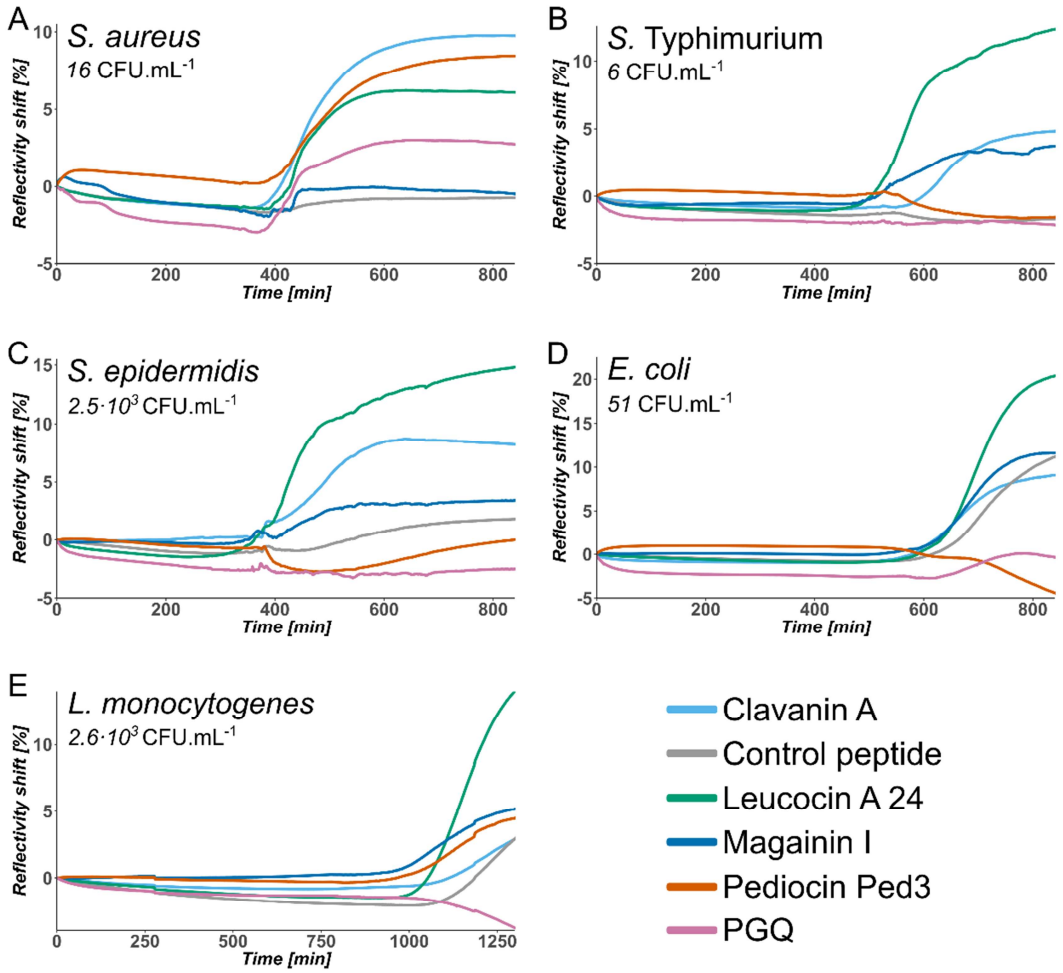

Figure 1. SPR kinetic data of the bacterial growth for five different bacterial strains cultured in tryptic soy broth at 37℃. Each curve is the averag e of duplicate spots for a given peptide arrayed on the sensor, with subtraction of the reference signal taken on peptide free gold.

AMP-based arrays have permitted the detection of pathogens present at low initial concentration in all samples, at most in 19 hours, and in even less than 12 hours in the majority of cases. All culture conditions being the same, variations of detection signal can come from dissimilarities in generation times of the different strains. Shortest detection times were for *S. aureus* and *S. epidermidis*, in about 6 to 7 hours. Similar conclusions with solid based culture methods are hitherto obtained in at least 24 hours. Compared to other biosensing methods using AMPs to analyze pre-enriched samples, we obtained a very low limit of detection of virtually one live bacterium per sample. The coupling of the enrichment step in our one-step process, not only improved the safety for the operator, but also significantly improved both the overall processing time and the limit of detection, by comparison to the two-steps methods described so far. Naturally, the lower limit of detection comes with a longer detection time to get bacterial concentrations sufficient for SPRI detection.

Individual peptide responses for bacterial pathogens detection

Peptides showed very distinct profiles of response depending on the targeted bacterial strains. Individual examination of their performances is thus interesting to understand underlying interactions with bacteria. Interestingly, Leucocin A 24 interacted strongly with all tested bacterial strains. Nonetheless, the scrambled version of Leucocin A 24, here used as a control peptide, displayed only weak to no interactions at all with any bacterial pathogen, suggesting that interactions of bacteria with tethered peptides are not only governed by physico-chemical mechanisms but more probably by structural interactions between peptides and bacterial membranes. Such observation has already been described for therapeutic peptides [35]. Clavanin A functionalized surfaces exhibited stronger SPRI responses with *Staphylococci spp.*, which are Gram-positive bacteria, although this peptide also enabled to successfully detect *Salmonella Typhimurium* and *E. coli*, although with weaker interactions. With our method, Magainin I only displayed mild interactions with all the tested bacteria, whatever their Gram coloration (except for *S. aureus* which gave a very low shift). On the contrary, Ped3, a Pediocin fragment, exhibited a moderate ability to detect *Listeria monocytogenes*, as previously described [31], but surprisingly Ped3 also displayed a potent interaction with MRSA in our assay. In the meantime it did not interact with *S. epidermidis*, although they belong to the same *genus*. This selective response might be caused by an association of the peptide with a peculiar receptor specific to the membrane of MRSA. PGQ displayed interactions with *E. coli*, which is consistent with the literature [32]. However it detected *S. aureus*, which is a Gram-positive strain, and had no apparent interactions with *Salmonella*, another Gram-negative bacterium, that also displays LPS, which is a hint that the interactions between PGQ and bacteria is not only mediated by interacting with the latter.

These results tend to show that AMPs keep a wide spectrum of affinity towards bacteria after tethering on a surface. Moreover, association of AMPs enabled to obtain various kinetic profiles depending on the bacterial strain. Interestingly, our SPRI detection method allowed to unravel interactions which remained unobserved with other methods [25]. This could be explained by the establishment of weaker interactions between bacteria and AMPs thanks to their longer exposure time to the cultured bacteria.

Multivariate analyses of kinetic results

Multivariate analyses were performed to objectively assess the discrimination values of our method. To do so, PCA was performed based on reflectivity shift and sensitivity values at the

maximum of the first derivative for each peptide (Figure *2A*). Each bacterial strain was processed once on an AMP micro-array, giving 64 sets of values, from all possible combinations of peptide duplicates responses at the surface. This combinatorial analyzing method ensured that statistical variability of the overall response was totally represented, and that cherry picking of data was avoided. Experiments on different bacterial strains with the same set of AMPs gave clustered results in the PCA 2D-score plot. Moreover, those clusters of data points were well segregated from each other according to the monitored pathogen: results were repeatable regardless of the set of replicates in the same monitoring. The first two principal components represented only 70.3% of the total variance. This meant that the data were of high dimensionality, thus explaining why such a good discrimination between bacterial strains could be obtained. However, those first two components were sufficient in our case to clearly distinguish between distinct bacterial strains. Such results underlined potential identification ability of the method, although more experiments would be required to create an even more exhaustive database. Results of the PCA were also used to perform a Hierarchical Clustering on Principal Components (fig. *2*B), leading again to a satisfying separation of the different strains in separate clusters. Such discriminated profiles came from the variety of affinities displayed by the peptides towards the tested bacteria. Due to different physicochemical properties and structures, each peptide interacted differently to Gram or genera, hence the overall combination discriminated the tested strains. Multivariate analyses with appropriate databases thus allow pathogen identification methods to be derived from the usual key-lock principle and enable cross-reactivity sensors as demonstrated by [36].

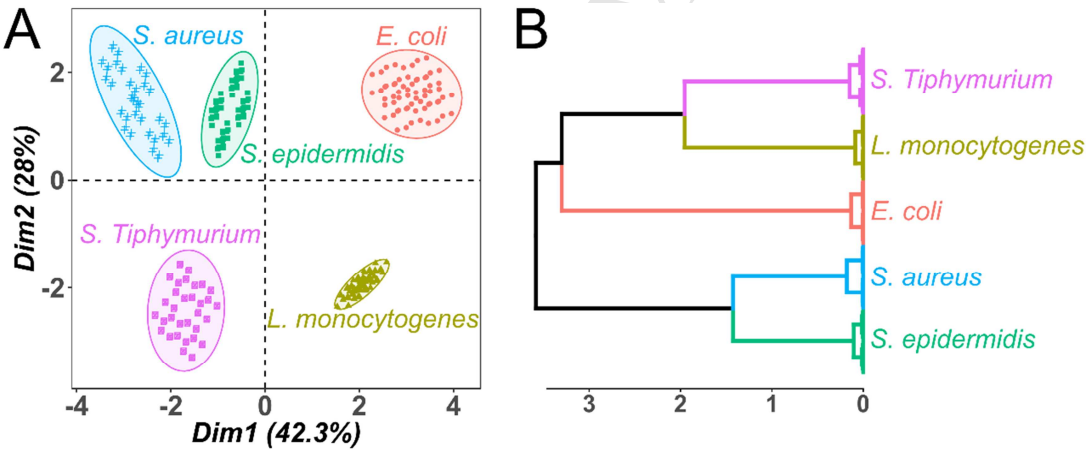

Figure 2. Multivariate data analyses for 5 bacterial strains processed on AMP-arrays. (A) 2D-score plot of the database generated from the kinetics of SPRI bacterial growth monitoring. Ellipses were annotated to represent the 95% confidence interval. (B) Hierarchical Clustering on Principal Components (HCPC) performed on the data from Fig. 2a. Distances between points are Euclidean.

In this work, we demonstrated the suitability of AMP-arrays as powerful ligands of bacterial pathogens. Traditionally studied in the field of pharmacology and antimicrobial applications, AMPs were revealed to be potent ligands for pathogens in biosensing applications. Not only they are well-adapted for the detection of bacteria in a standard culture medium such as tryptic soy

broth, but compared to antibodies, the biochip functionalization and operation were less tedious, thanks to peptide resistance to air-drying without loss of activity in the process.

The use of AMPs in a multiplexed fashion allowed a fast and simple assessment of the presence of pathogens even at low concentrations. Detection times ranged from 6 to 19 hours, in a one-step assay. This is shorter than standard methods in blood or foodstuffs, which require enrichment times of usually 24 hours before confirmation of the culture positivity. Each tested bacterial strain resulted in different kinetic responses on the part of the AMP-array. Ped3 even exhibited a near-specific behavior, interacting almost uniquely with MRSA. Further studies could assess this point, which would be of importance in the detection of this prominent pathogen. Our results, for instance for Leucocin A24 and its scrambled version, showed that the recognition of bacteria by AMPs is not uniquely driven by physico-chemical interactions, but also by their secondary structuration. Moreover, tethering of the AMPs on the surface of our sensor did not seem to alter this interaction with bacteria in solution. The study of the whole set of AMPs interacting with live bacteria gave clearly different affinity profiles. This combined approach was preferred to a biomolecular study to enable the identification of several bacterial pathogens, with a single AMP set. The response to polymicrobial infections, which represent only 10% of bloodstream infections [37,38], is still to be investigated to check how AMPs responses might be deciphered to identify two, or more, pathogenic strains present in the same sample.

Furthermore, the small set of AMPs we used provided very distinct affinity profiles towards different pathogens, as multivariate analysis confirmed. Arrays presenting a larger collection of peptides could hence enable the possibility to create interaction profile libraries and therefore the identification of bacterial genus, species or strain of the infecting pathogen in a sample.

To the extent of our knowledge, the system we described is the first one-step biosensor assay using different multiplexed AMPs and enabling detection of several viable bacterial strains in biomedically relevant concentrations along with a discrimination capacity in a label-free assay. In the near future, this approach could be applied for the detection of any pathogen in samples such as foodstuff, urine or blood without any pre-requisite knowledge of the threat.

If these auspicious results are confirmed on a larger scale, it could permit the development of a one-step assay – not only able to detect common pathogens overnight – but also to identify them in reference to a database of affinity patterns.

Color should be used for any figures in print

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Competing interests statement

The authors declare no financial or non-financial competing interests.

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Antimicrobial peptide arrays for wide spectrum sensing of pathogenic bacteria

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Highlights

- An antimicrobial peptide micro-array dedicated to bacteria detection is presented
- The same antimicrobial peptide set enables the detection of 5 pathogens
- The one-step detection of low bacterial loads is achieved in less than 20h
- Label-free assays are completed without requisite sample enrichment
- 6 different antimicrobial peptides enabled the pathogenic strain clustering