Universitat Autònoma de Barcelona

Development of new immunostimulant and antimicrobial protein-based molecules from a One Health perspective

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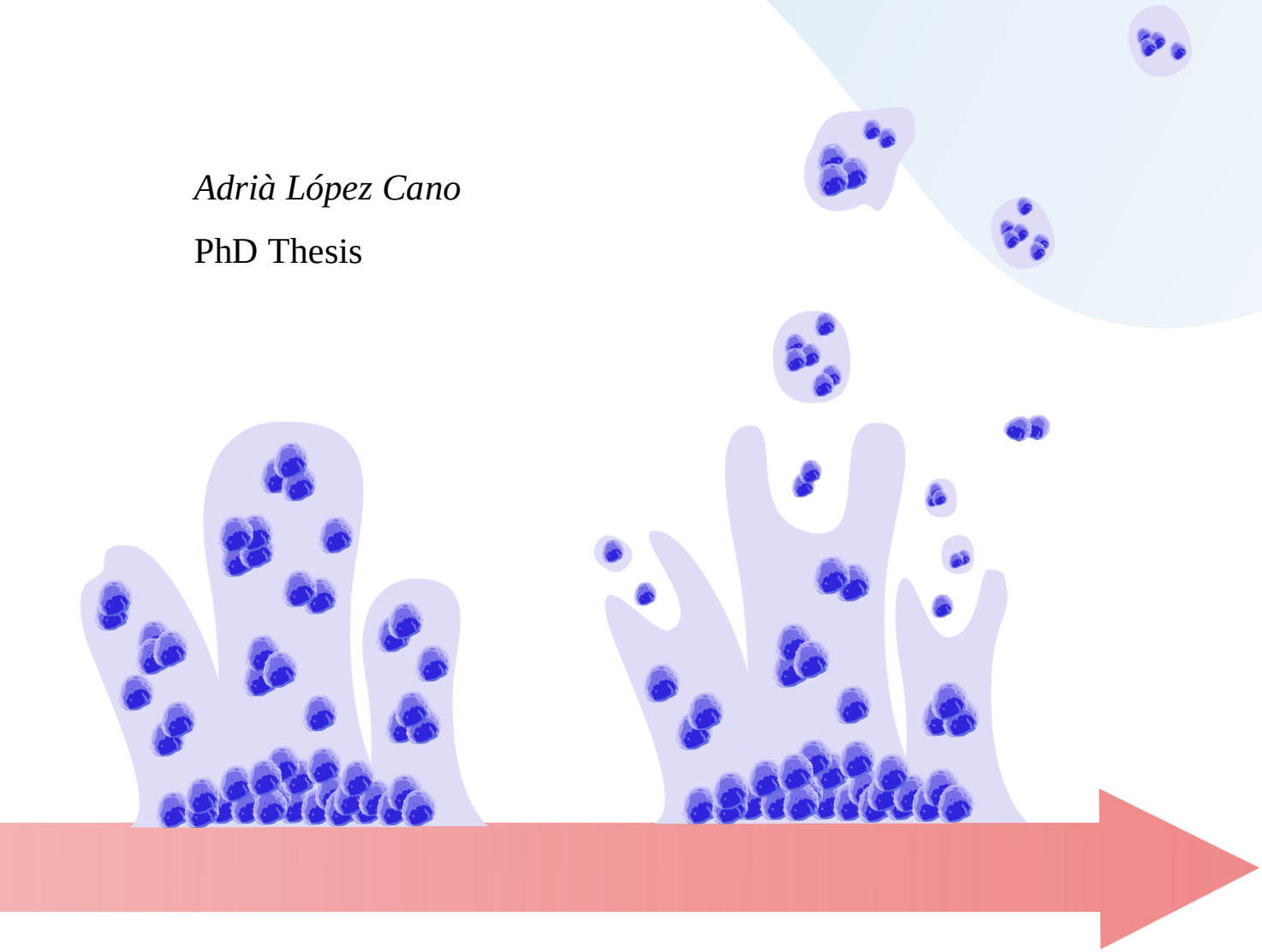
PhD Thesis



2022

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Ruminant Production Program, Institute of Agriculture and Food Research and Technology

PhD program in Biotechnology

Department of Genetics and Microbiology

Faculty of Biosciences

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Department of Genetics and Microbiology

Thesis submitted by Adrià López Cano as partial fulfilment of the requirements for the PhD Degree in Biotechnology by the Autonomous University of Barcelona

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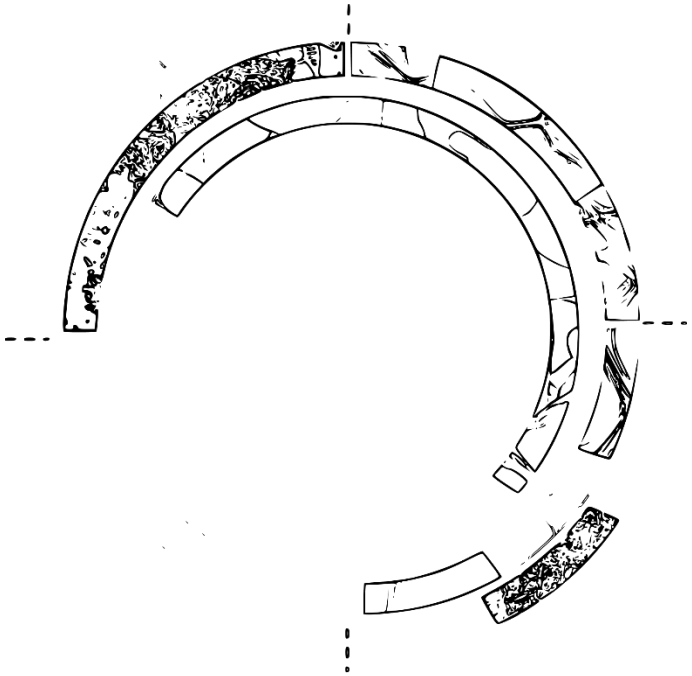
This work has been mainly conducted in the Institute of Agriculture and Food Research and Technology, under the supervision of the doctors Anna Arís Giralt and Elena Garcia Fruitós.

PhD advisor at UAB: Antonio Villaverde Corrales

A mis padres, a mi hermana y a ti, Mari.

“Everything is theoretically impossible, until it is done”

-Robert A. Heinlein



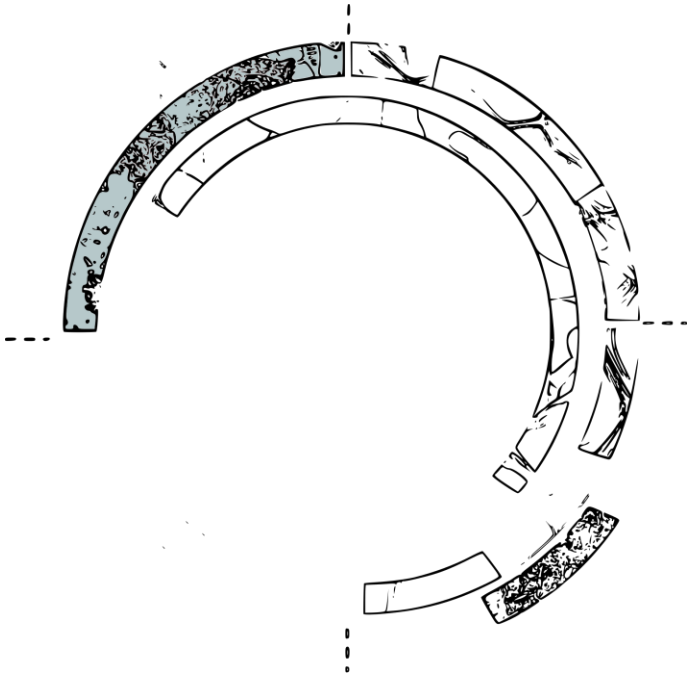
Contents

Summary

Antibiotics breakthrough is considered among the most remarkable hallmarks of modern medicine. However, antibiotic misuse and overuse have triggered the swift expansion of antimicrobial resistance (AMR), compromising both human and animal health. In this scenario, the World Health Organization called for addressing the AMR crisis using a multifaceted, transdisciplinary and integrative strategy, named One Health Approach. The development of alternative treatments to antibiotics is a critical aspect in the AMR fight. Within this framework, the Host Defense Peptides (HDPs) hold an outstanding duality as broad-spectrum antimicrobials -even against AMR bacteria-, and host immune regulation molecules, arousing the scientific community interest. Still, HDPs are generally chemically synthesized, but the high associated cost entails a major drawback for broader implementation. In this context, recombinant protein production emerges as an inexpensive source of antimicrobial protein-based compounds with noteworthy yields and straightforward scale-up. Although promising, recombinant HDP production is challenging due to the HDPs characteristics, being their small size, an early proteolytic degradation in the recombinant bacterial host, coupled with non-desirable recombinant host toxicity, the major obstacles for their recombinant production. With the aim of pursuing HDPs potential as the next generation antimicrobials, this thesis has been focused on the development of tunable HDPs-based antimicrobial drugs using and improving a recombinant production approach. As a first step, we have explored the most appropriate microbial cell factory for their production, taking into account the presence of conserved disulfide bridges in defensins, one of the most relevant HDPs family. In addition, to overcome proteolytic-related issues and potential toxicity for the bacterial producer, we have developed first-generation antimicrobial peptides in which HDPs have been fused to the Green Fluorescence Protein (GFP) carrier. Besides, we have also assessed alternative sources to purify the HDPs, considering their high aggregation ratio and inclusion bodies (IBs) formation. Our first findings pointed out that *Escherichia coli* BL21 is a suitable host for their production, achieving highly pure and active antimicrobial molecules. We have also demonstrated that IBs are a natural source of high-quality HDPs, developing also a free-detergent non-denaturing protocol to avoid unexpected activity losses. These results have encouraged us to construct a second-generation of HDP-based antimicrobial proteins, combining the most promising HDPs in a single polypeptide and removing the GFP carrier. Overall, we showed how these multidomain constructs hold an enhanced broad-spectrum antimicrobial activity and lower minimal inhibitory concentration (MIC) than their monodomain analogs, proving the synergic effect of combining different HDPs. Concurrently, we also tackled the AMR problems by using a complementary approach based on the development of novel cytokine-based IBs immunostimulants produced in *Lactococcus lactis*. The hypothesis was to increase the animal resilience to infections by activation of their immune system previous to critical productive moments, which will decrease the need of using antibiotic treatments. Interestingly, the first *in vitro* findings demonstrated the immunostimulant properties of the nanoparticulated porcine cytokines, although only a tendency was observed in preliminary *in vivo* experiments with piglets.

Resum [Catalan translation]

El descobriment dels antibiòtics és considerat una de les principals fites de la medicina moderna. No obstant això, l'abús i l'ús excessiu d'antibiòtics ha desencadenat una ràpida expansió de la resistència antimicrobiana (AMR), comprometent tant la salut humana com l'animal. En aquest escenari, l'Organització Mundial de la Salut va demanar que s'abordés la crisis de AMR mitjançant una estratègia multifacética, transdisciplinària i integradora, anomenada *One Health Approach*. El desenvolupament de tractaments alternatius als antibiòtics és un aspecte crític en la lluita contra la resistència antimicrobiana. Dins d'aquest marc, els pèptids de defensa de l'hoste (HDPs) presenten una dualitat destacada com antimicrobians d'ampli espectre -fins i tot contra els bacteris AMR-, i molècules de regulació immunitària de l'hoste, despertant l'interès de la comunitat científica. Tot i així, generalment els HDPs es sintetitzen químicament, provocant un alt cost associat que implica un gran inconvenient per la seva implementació a gran escala. En aquest context, la producció de proteïnes recombinats emergeix com una alternativa assequible per compostos antimicrobians basats en proteïnes amb uns rendiments notables i fàcil escalat. No obstant, la producció recombinant de HDPs és complexa degut a les seves característiques intrínseques, on la seva reduïda mida condiciona una primerenca degradació proteolítica en l'hoste bacterià recombinant, juntament amb una toxicitat no desitjada, esdevenen en conjunt els principals obstacles per la seva producció recombinant. Per tant, amb l'objectiu de trobar el potencial dels HDPs com a els agents antimicrobians de pròxima generació, aquesta tesi s'ha focalitzat en el desenvolupament de medicaments antimicrobians modificables basats en HDPs utilitzant i millorant l'aproximació de la seva producció recombinant. Com a primer pas, hem explorat quines fàbriques cel·lulars microbianes són més adients per la seva producció, sempre tenint en compte la presència de ponts de disulfur conservats en les defensines, una de les famílies més rellevants dels HDPs. A més, per fer front als problemes relacionats amb la proteòlisis i potencial toxicitat envers el productor bacterià, hem desenvolupat una primera generació de pèptids antimicrobians en els quals els HDPs s'han fusionat amb la *Green Fluorescence Protein* (GFP). Addicionalment, també hem considerat fons alternatives per purificar els HDPs tenint en compte la seva alta ratio d'agregació i formació de cossos d'inclusió (IBs). Els nostres primers resultats van indicar que *Escherichia coli* BL21 és un bon hoste per la seva producció, aconseguint molècules antimicrobianes amb gran activitat i puresa. També hem demostrat que IBs són una font natural de HDPs d'alta qualitat, desenvolupant específicament un protocol no desnaturalitzant lliure de detergents per evitar pèrdues d'activitat inesperades. Aquests resultats ens han encoratjat a construir una segona generació de proteïnes antimicrobianes basades en HDPs, combinant els HDPs més prometedors en un sol polipèptid i eliminant la GFP. En general, vam provar com aquestes construccions multidomini tenen un activitat antimicrobiana millorada d'ampli aspecte i una MIC més baixa que els seus anàlegs monodomini, demostrant l'efecte sinèrgic de combinar diferents HDPs. Al mateix temps, també hem abordat la problemàtica de AMR mitjançant un enfocament complementari, basat en el desenvolupament de nous IBs formats per citocines amb capacitat immunoestimulants produït en *Lactococcus lactis*. La nostra hipòtesis era augmentar la resiliència de l'animal a les infeccions mitjançant l'activació del seu sistema immunitari de forma prèvia a un moment de producció crític, reduint la necessitat de tractaments amb antibiòtic. Interessantment, les primeres troballes *in vitro* van demostrar les propietats immunoestimulants de les citocines nanoparticulades de porcí, encara que només és va observar una tendència en els experiments preliminars *in vivo* amb garrins.



Introduction

ANTIMICROBIAL RESISTANCE

Antimicrobial resistance (AMR) is a public health threat that has risen sharply in the last decades [1]. As a consequence of the overuse and misuse of antibiotics [2], combined with the lack of antimicrobial alternatives [3], resistant bacteria have emerged and worryingly expanded. AMR arises when pathogenic microorganisms are able to survive to one or even different antimicrobial drugs. Thus, these treatments become ineffective and infections caused by multiresistant microorganisms cannot be treated, causing severe illness or even the death of the infected organism [4, 5].

Although AMR emergency has become more recurrent in the last years, the existence of resistant bacteria was already described by Alexander Fleming, who discovered the first antibiotic compound -the penicillin- in 1928 [6]. In fact, AMR genes conferring drug resistance to bacteria were recovered in a wide variety of samples [7]: from 2,000 old glacial samples, cold-seep sediments of deep-sea to soil-dwelling actinomycetes. However, human action has driven the acceleration rate at which resistance events are developing and spreading, leading to the current global health crisis. In this context, the higher is the number of bacteria exposed to antibiotics, the more are the chances of developing resistance. Since then, newly discovered antibiotics and their resistant bacteria counterparts have competed in a furious race (Figure 1).

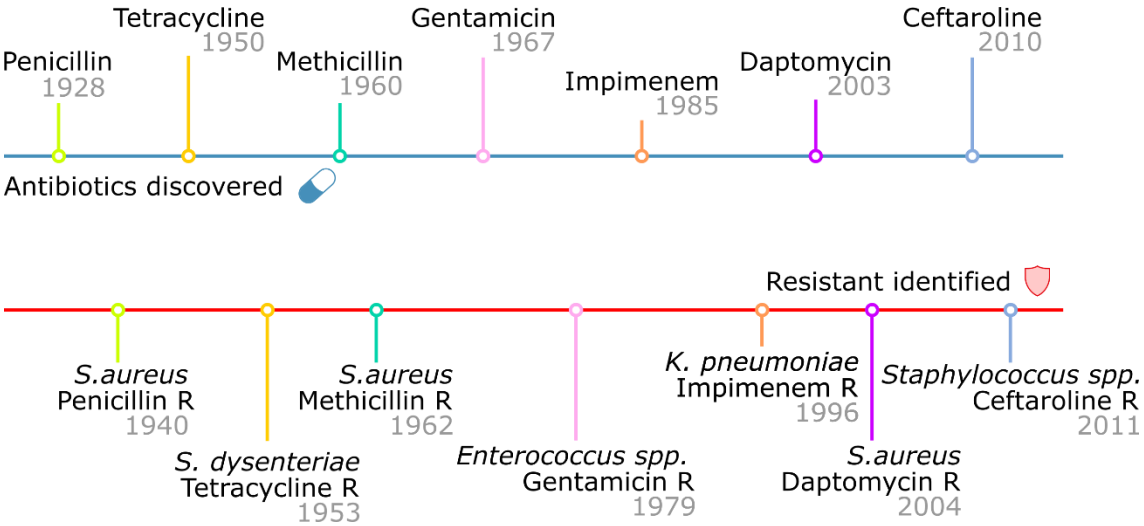


Figure 1. Timeline of antibiotic resistance. New antibiotic discoveries have been always narrowly associated with the emergence of resistant bacteria over time, where more extensive use is reflected in faster resistance development.

The selective pressure, which has been the cornerstone of the evolution, is the mechanism by which the AMR arises [8]. This bacteria diversity is a consequence of their exceptional genetic plasticity, allowing them to avoid or overcome threats that jeopardize their existence. From an evolutionary viewpoint, bacteria have two genetic strategies to evade antibiotic effects [9]: (i) acquisition of foreign AMR genes by horizontal gene transfer (HGT) of the environment and (ii) *de novo* mutations. HGT mechanism allows bacteria to adapt effortless to a constantly evolving environment. The HGT can be caused by different mechanisms (Figure 2) named (a) transduction, which involves the mobilization of bacterial genes, accidentally assembled in the bacteriophage capsid during replication, from one bacteria to another [10]; (b) conjugation, in which genetic material can be transferred from a donor to a recipient cell which is in close contact; (c) conjugative transposons that are able to move through bacteria by cell-to-cell contact, commonly carrying antibiotic resistance genes [11] and (d) transformation, based on the uptake of exogenous naked DNA from the environment [12]. In all cases, AMR genes can be easily spread among different bacteria communities.

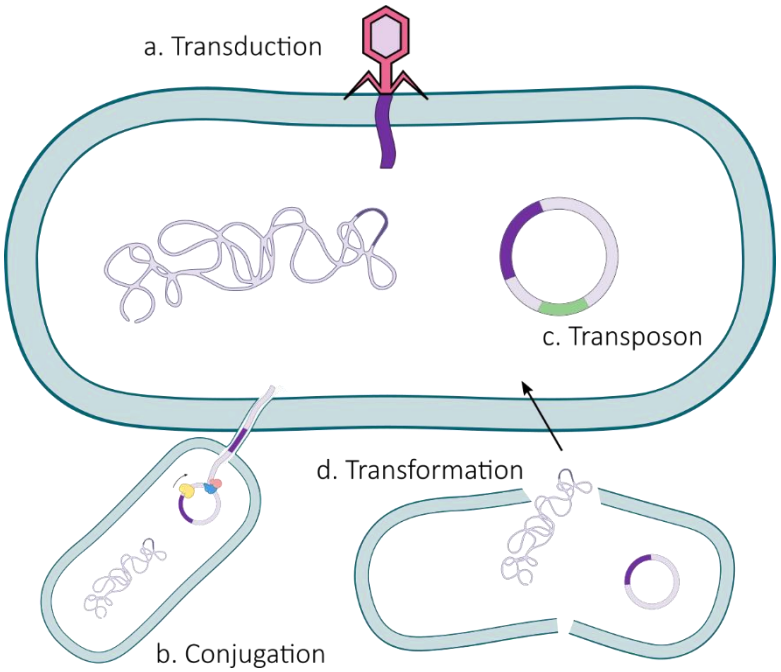


Figure 2. Schematic representation of the mechanism of horizontal gene transfer (HGT) in bacteria. HGT is performed through four principal mechanisms: transduction (a), conjugation (b), transposition (c) and transformation (d). After the DNA uptake, the transferred genetic material must be integrated into the bacteria genome through recombination, except for plasmids, which do not require integration into the host genome [13].

In reference to *de novo* mutations acquisition, although normal mutation rate is relatively low, when bacteria populations are subjected to selective pressure, they can largely increase their mutation rate to face off the challenge [14, 15]. In fact, sublethal levels of antibiotics can promote the expression of error-prone DNA polymerases, which repair antibiotic-related damage but with low fidelity and, hence, trigger mutations. Regardless AMR genes stem from intrinsic resistance, antibiotic-induced mutagenesis or HGT, this genetic material confers to the bacteria the capability to elude antibiotic presence, deploying a wide range of alternatives to overcome the threat (Figure 3). The mechanism by which bacteria are AMR can be classified into three main groups: (1) those that reduce intracellular concentration of the antibiotic through a low wall permeability of bacterium (Figure 3a) or with antibiotic efflux pumps (Figure 3b); (2) those that directly inactivate the antibiotic by hydrolysis or chemical modification (Figure 3c); and (3) those that generate antibiotic target modifications by genetic mutation or post-translational modification, avoiding target-antibiotic interaction (Figure 3d), synthesizing an alternative by-pass enzyme (Figure 3e) or upregulating target protein expression (Figure 3f).

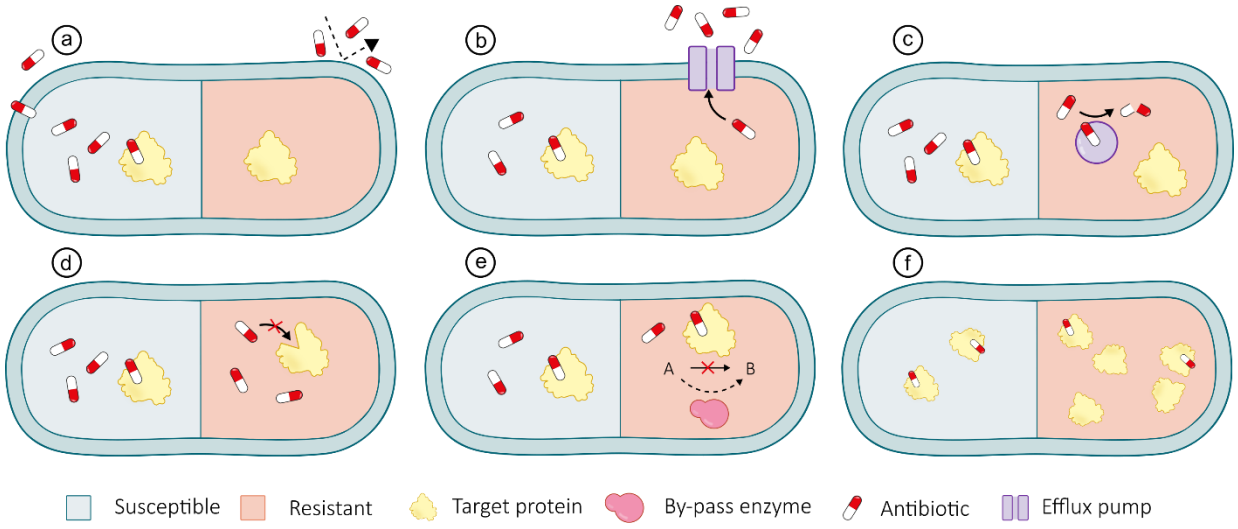


Figure 3. **Resistance mechanisms against antimicrobial compounds.** AMR mechanisms are represented, showing different mutations that converge in a drug resistant bacterium. AMR strategies can be summarized in: (a) reduce/block permeability, (b) activation of efflux mechanism, (c) antibiotic modification or degradation, (d) specific target site modification, (e) alternative metabolic pathways. and (f) alter gene expression of antibiotic target. Adapted from [16]

Once a resistant mutant sprouts, the antibiotic kills susceptible bacterial population, allowing resistant bacteria to spread and sharing the acquired mutational changes to their offspring and neighbors by HGT and inevitably increasing AMR gene pool.

Current State and Impact

The antibiotic-resistant crisis is reflected in at least 25,000 deaths per year in the EU [17] and 700,000 deaths per year globally that are attributable to AMR bacteria [18]. And all indicators point towards an even worse scenario in a close future. The World Heart Organization (WHO) estimates that without further strong actions, the AMR will cost 10 million lives per year by 2050 (Figure 4), being the leading cause of death surpassing cancer [1]. In addition, AMR has not only triggered a healthcare emergency but is also producing a heavy impact on the European economy. It is estimated that €1.5 billion are linked with higher health costs of treatment and productivity losses associated with persistent health problems every year [19]. The World Bank notices that associated drug-resistance infections may lead global economy to a strong financial crisis comparable with 2008 economic deceleration [20].

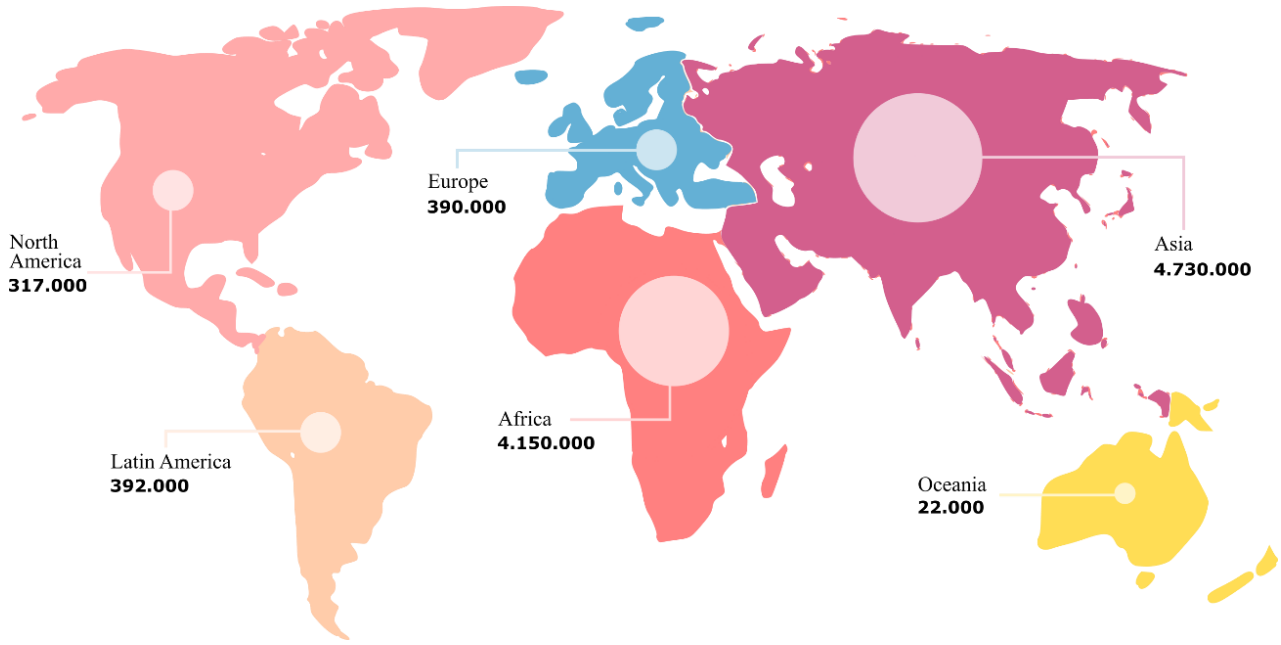


Figure 4. Estimated deaths caused by AMR each year by 2050. Adapted from: Review on Antimicrobial Resistance [1].

Thus, although the success of antibiotics is unquestionable, the arising of resistant and more worryingly multi-drug resistant (MDR) and extensively drug-resistant (XDR) bacteria have generated a challenge for global health [21]. The WHO considered a list of 12 bacteria affecting human health against new antimicrobial compounds are urgently needed [22]. Among them, the

six topping the list, and encompassed in the acronym ESKAPE [23, 24], are *Acinetobacter baumannii* carbapenem-resistant, *Pseudomonas aeruginosa* carbapenem-resistant, *Klebsiella pneumoniae* carbapenem-resistant, *Enterobacteriaceae* carbapenem-resistant, *Enterococcus faecium* vancomycin-resistant and *Staphylococcus aureus* methicillin-resistant (MRSA). These microorganisms are responsible for most of the nosocomial infections (defined as those infections developed in a patient during hospital care, which was not present or incubating at the time of admission [25]), being able to ‘‘escape’’ to the antimicrobial action of several therapeutic compounds through one or more of the mechanisms described before (Figure 3). In addition, over time, the overall number of effective antibiotics against ESKAPE is gravely diminishing. On the other hand, in veterinary medicine, the main pathogenic microorganism in livestock and domestic animal infections are *Campylobacter jejuni, Salmonella spp., Staphylococcus spp* (mostly MRSA), ESBL (extended-spectrum beta lactamases) Gram-negative bacteria and *Enterococcus spp* [26].

Contrary to expectations, the current pipeline of new antibiotics reflects a lack of novel molecules that are being developed [27]. Because of the poor commercially attractive market and the practical and regulatory barriers the antibiotic research and development is hampered, although now there are more needed than ever. Hence, there is a need to encourage drug developers to create long-term solutions. This can be done through incentives and development supports, which are pivotal to changing the landscape of antibiotics and their associated AMR organism.

TACKLING THE ANTIMICROBIAL RESISTANT BACTERIA FROM THE ONE HEALTH APPROACH

Wherever antimicrobials are used, bacteria become resistant and, consequently, they also act as reservoirs of AMR genes. This happens not only in hospitals and animal farms but also in the natural environment that is collaterally affected [28, 29]. As a result of bacteria genetic flexibility, their genes can easily shift between bacteria present in animals, humans and the environment. This means that actions taken in one area impact the others proportionately (Figure 5). Consequently, to tackle AMR, a harmonized, integrative and multisectoral approach is needed. The WHO has proposed the One Health approach [30] as a collaborative, multisectoral, and transdisciplinary approach -working at the local, regional, national, and global level- to achieve optimal health outcomes recognizing the interconnection between people, animals, plants, and their shared environment [31].

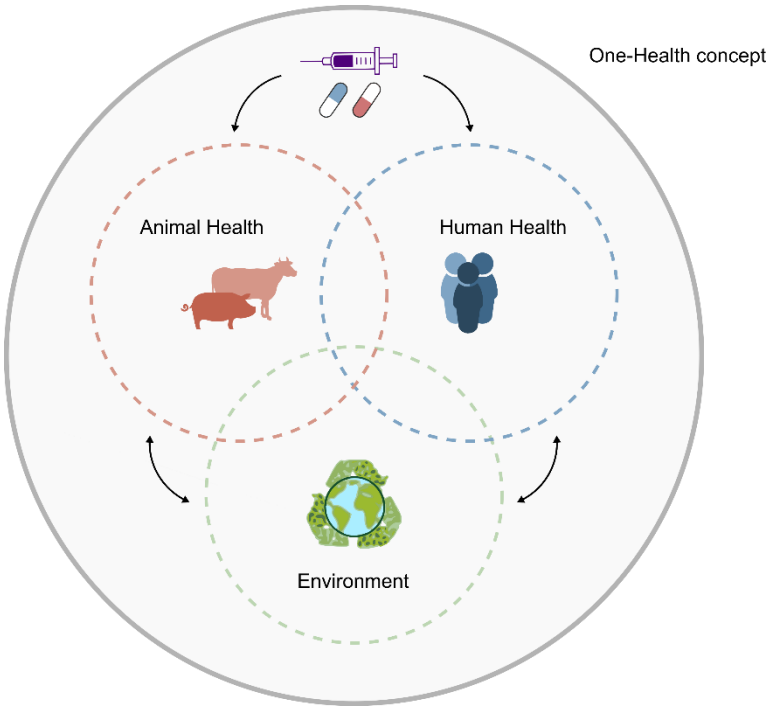


Figure 5. One Health diagram. The reciprocal dependency between humans, livestock and the environment plays a crucial role in the fight against AMR, requiring a coherent, coordinated, and effective response to address the challenge.

To work on that, the WHO has established a set of actions which can be summarized in (i) an enhanced microbial surveillance of established and emerging resistant microorganisms through the Antimicrobial Resistance Surveillance Networks; (ii) a strict control and monitoring of

antibiotic usage; (iii) the promotion of high standards of hygiene practices; and (iv) the development of new antimicrobial drugs with novel modes of action and reliable diagnostic tools [32].

Thus, the first step to address AMR problem is the establishment of a robust AMR surveillance network. For that, one of the goals of the European Antimicrobial Resistance Surveillance Network is to provide a complete image of the problem to define the adequate measures to cope with them [33]. To address that, different regional programs coordinated by WHO have been developed, such as Integrated Disease Surveillance and Response (IDSR) in Africa, Antimicrobial Resistance in the Eastern Mediterranean (ARMed) or Regional Program for Surveillance of AMR in the Western Pacific, connecting 111 countries with up to 1700 laboratories that have allowed the identification of critical parameters in the decay of antibiotic effectiveness [34].

The second approach in the fight against AMR relies on the control of antibiotic consumption in both human and veterinary medicine. One important action was already taken in Europe in the livestock context, banning the use of antimicrobials as growth promoters in 1999 [35]. Regulatory agencies in the United States or Asia have not totally banned the use of growth-promoting antibiotics. However, critical compounds for human therapy have been progressively withdrawn for this use [36]. In general terms, after the WHO warning about this topic, a reduction of up to 51% of countries that used antimicrobials as growth promoters were accomplished.

Although considerable steps have already been taken to reduce antibiotic consumption, a dissemination strategy for society is also important to reach this objective. In line with that goal, the antibiotic footprint initiative seeks to report average antibiotic consumption to maintain the population aware of misuse and overuse of antibiotics [37]. For example, a UK resident consumes twice antibiotics in relation to a Netherlands resident (8.3 versus 3.3g, respectively) [38] where the major variance contributors are not bacterial infections, rather differences in healthcare systems, patient behavior and awareness generate this antibiotic consumption gap. As a result, through consciousness campaigns and better access to evidence-based information, the total antibiotic consumption for human and veterinary use decreased by 6 and 35%, respectively [39]. Other examples of successful interventions with subsequent antibiotic reduction rely on: the restriction of fluoroquinolones use in Australia [40]; the implementation of rational antibiotic use campaign in China in 2011 (reduction 10% of prescribed antibiotics) [41]; yearly national antibiotic campaign in France since 2001 (reduction 27% of antibiotic prescription) [42] or the national program to contain antibiotic resistance in Sweden [43]. Remarkably, the application of antibiotic stewardship programs (ASPs) and specific action plans (following the WHO recommendations) have also resulted in a significant decrease of antibiotic consumption in the

hospital networks from Africa, Asia, Europe, Oceania and America [44-47]. As an example, Carling *et al*. demonstrated how a multidisciplinary antibiotic management program achieved a reduction of 22% in the use of parenteral broad-spectrum antibiotics, as well as the incidence of nosocomial infections by *Clostridium difficile* and resistant bacterial pathogens [48].

The third action involves the prevention, in which hygienic measures and sanitation are key factors to reduce infections and, in consequence, antibiotic use. As an example, the introduction of water and sanitation infrastructure in countries with limited resources could reduce up to 60% associated diarrhea cases and subsequent antibiotic treatment [49]. Besides, vaccination is also a preventive approach that has been shown to be effective to control infections [50]. For instance, a pneumococcal conjugate vaccine can prevent 11.4 million doses of antibiotics, reducing by 47% the antibiotics used to treat *S. pneumoniae* associated pneumonia [51]. However, in many cases, vaccines against pathogenic bacteria are quite inefficient or not commercially available due to their complexity [52]. In this context, it is necessary to develop novel antimicrobial drugs to treat infectious diseases, especially those caused by MDR bacteria for which antibiotics are not effective [53].

New Antimicrobial Agents

Early attempts to develop new therapies or improve the existing ones were frequently hampered by deficient investment, along with unfinished drug development and lack of clinical expertise in pharmacodynamics and pharmacokinetics, formulation, toxicology and manufacturing [53, 54]. Thus, the promotion of a new pipeline of therapies to face off drug resistant bacteria is crucial. Among the potential candidates, phage therapy, lysins, antibodies, probiotics, antimicrobial peptides or proteins are the most widely studied alternatives[54] .

Phage therapy

The therapeutic use of bacteriophages (also known as phages), which are virus that infect bacteria, was developed by Felix d'Hérelle in the old Soviet Union, roughly a century ago. It was proven to be an effective treatment against relevant bacterial infections, such as dysentery, skin infections, cholera, among others [55, 56]. Although the beginning of the antibiotic era resulted in a significant reduction of phage therapy, the arising of antibiotic resistant bacteria provided an optimal scenario for their reappearance.

Phage therapy relies on the application of bacteriophages to eradicate bacterial pathogens. Briefly, phages initiate the infection cycle through a specific receptor recognition by a lock-and-key

interaction. After adsorption, the virus injects genetic material into the host and afterward takes the control of cellular machinery to replicate itself. Finally, phage-delivered proteins are synthesized, lysing the cell and allowing new phages to restart the cycle [57] (Figure 6).

The extensive understanding of phage biology, genetics, and immunology makes them a viable alternative to antibiotics. Other potential advantages of phages can be summarized in (i) high target-specificity, protecting host-microbiota from undesirable side effects; (ii) self-limitation performance, since once the target is killed, their activity is stopped; (iii) low doses required due to phages are able to exponentially replicate in host bacteria; (iv) antibiofilm activity; (v) easy and cost-effective production; (vi) phages can be genetically engineered, widening their therapeutic uses; (vii) phage-antibiotic synergy with which the antibiotic doses can be limited; and (viii) phages undergo evolutionary events, this means that if resistance arises, phages mutate alongside bacteria, making them unique as a therapeutic compound. Nevertheless, some advantages might be a double-edged weapon. Phages specificity translates into a narrow spectrum of action, being essentially an accurate bacteria identification beforehand and occasionally using phage cocktails or engineered ones for better outcomes [58]. Moreover, because of their dynamic entity nature and activity, which is governed by the immune system of the patient, bacteriophages present a regulatory framework gap, hampering their widely market introduction [59, 60]. For these reasons, only a few products are in clinical trials [61] and only two candidates have reached phase 3 [62].

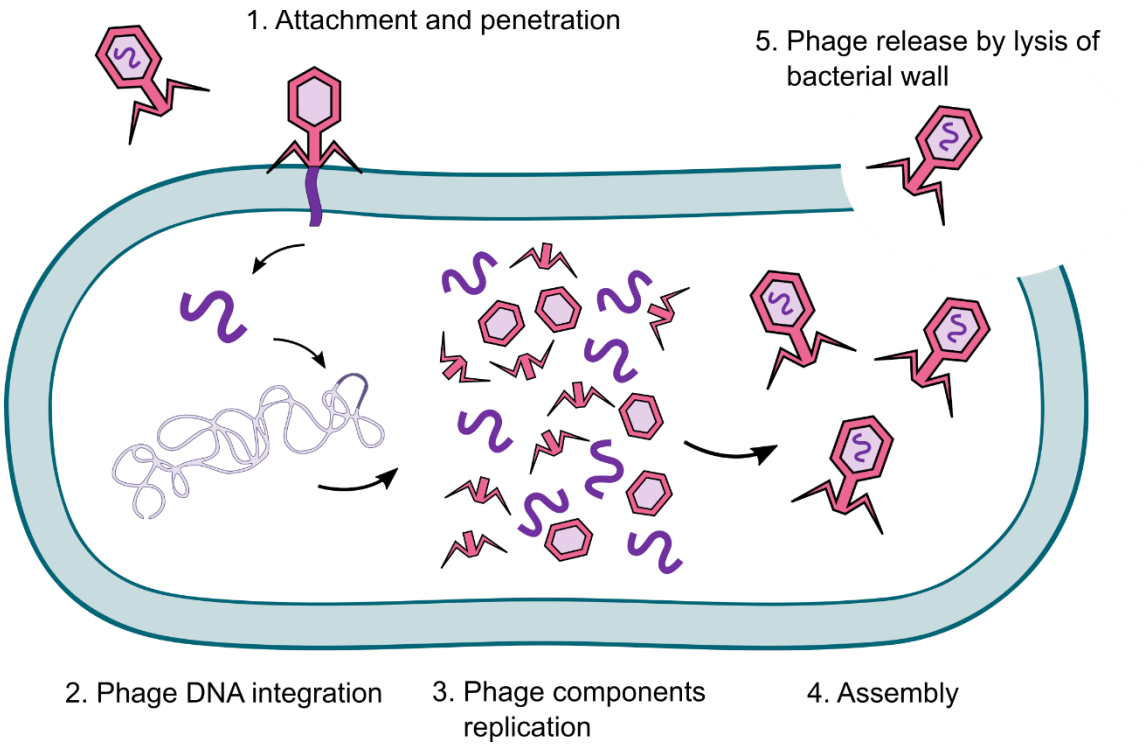


Figure 6. Lytic bacteriophage infection cycle. At the beginning of the cycle, the bacteriophage interacts with the surface of the specific host and then injects the viral genome through the cell wall and bacterial membrane (1). After that, the phage genome can be either integrated into the bacterial chromosome resulting in a prophage (2) until induction or directly taking the control of the bacterial machinery to synthesize new phage DNA and viral structural components for the building of new progeny (3). During the maturation phase, the different parts are assembled (4) resulting in fully infective phages and lastly, bacterial cell wall is disturbed and the bacteriophages are prepared to infect a new host (5).

Lysins

Lysins are bacterial cell-wall hydrolytic enzymes produced by bacteria and bacteriophages [63, 64], forming part of the phage-delivery protein system. In bacteria, these enzymes are involved in cell wall remodeling during cell division and bacterial killing of other potential competitors, whereas in phages they are synthesized in the last-stage of phage infection, enabling bacteria cell wall degradation and subsequently phages progeny release and propagation. These enzymes can selectively and rapidly kill target bacteria by peptidoglycan disruption with negligible impact in host microbiome, being another potential alternative candidate to treat bacterial infections [65]. Lysins are sorted according to the peptidoglycan structure that they target, being distributed in three major classes: glycosidases, amidases and endopeptidases [64]. In addition, lysins also differ in their host spectrum, with serovar-specific [66], multispecies [67] and multigenus [68] performance. One of the advantages of lysins are the minor chance of the bacteria to develop resistance due to selected targeting of highly conserved peptidoglycan components [69].

Lysins can be used alone [70], but also they have been tested together with antibiotics in a hybrid molecule, proving that both components act synergically against MDR bacteria [71]. However, their low or null performance of natural lysins against Gram-negative bacteria has limited the number of clinical trials that have been performed with these enzymes [59]. To cover this gap, this antimicrobial agent can be engineered, creating the second generation of chimeric lysins with altered catalytic activities or directed binding specificities, generating a tailored molecule with optimized antimicrobial activity, thermostability, specificity and efficiency against Gram-negative bacteria [72, 73]. In addition, a third generation of engineered lysins are being investigated with boosted pharmacokinetics and/or pharmacodynamics to altogether unlock the potential of lysins therapy [74].

Probiotics

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit on the organism [75]. This bacteria mixture supplement is able to pass through gastric and intestinal environments or be applied directly in targeted mucosa to finally interact with host intestinal microbiota, generating a wide range of beneficial effects [76].

Not all microbial species can be used as probiotics, being necessary a case-by-case evaluation [77]. The potential probiotic bacteria must be exempt from virulence factors and transferable antibiotic resistance genes [78], whereas should be able to coexist in balance with the preexisting microbiome. Generally, probiotics can improve digestive system functions, promoting beneficial microbiota, as well as reinforcing the immune system through prophylactic and therapeutic perspectives [77]. As prophylactic, probiotics can compete against pathogenic microorganism colonization and proliferation through available nutrient reduction and enhancing mucosal barrier activity. For that, probiotic bacteria can synthesize antimicrobial compounds as bacteriocins and peptides or inhibit pathogens by short-chain fatty acids (SCFAs) production, such as propionic, butyric or lactic acid [79]. In addition, probiotics can also stimulate the host immune system, activating innate and adaptive responses, such as increasing mucus secretion [80] and triggering cytokines and cationic antimicrobial peptides upregulation [79]. On the other hand, as therapeutic, probiotics are largely recommended to diminish infectious gastroenteritis symptoms [81], along with reducing antibiotic-associated diarrhea [82]. Therefore, although probiotics research area has significantly advanced with over 300 studies in clinical trials and several commercially available products [83, 84], some mechanism and interactions with host microbiota and the immune system remain unclear [79]. Strain-specific effects, doses and combination with other therapies will unlock probiotics potential, enabling their wider clinical use [85, 86]. In addition, probiotics are

more focused on preventive medicine or supplement of other treatments rather than therapy itself [80, 87].

Antibodies

Antibodies and antibody-derived molecules have been established as the cornerstone in protein-based therapeutic molecules [88]. Their well-defined structure and function interactions become them an excellent platform for protein engineering, generating a wide library of tailored molecules for each therapeutic purpose with a rational and safe use over time [89]. Antibodies have been widely applied against viral diseases, such as Hepatitis B or Ebola [90], for which antibiotics do not work. Remarkably, antibodies not only directly inactivate and subsequently opsonize a pathogen by attachment but also can neutralize their virulence factors and toxins [91, 92]. These features make them a flawless candidate to be also applied as prophylactic or therapeutic molecules against the disease-causing bacterial agents through a passive immunization strategy [93]. In addition, antibodies can also be used in combination with antibiotics (e.g., ciprofloxacin) to effectively tackle bacterial and MDR pathogens infections [94, 95]. However, although their narrow specificity avoids non-desirable host microbiome alterations and resistance events among non-targeted microorganisms, it is a strong obstacle to treating mixed infections. Besides, the use of unproductive platforms for the generation of these complex molecules, the high cost-effectiveness, and the need for greater clinical efficacy in some cases, affect significantly their wide commercialization [96]. Even so, the antibodies along with phages are the therapies better suited for short-term clinical implementation.

Despite their limitations, multiple antibody-based therapies against bacteria are in the early-stages of clinical trials, some in phases 2 and 3, such as Altasaph against *S. aureus* nosocomial infections or Pagibaximab directed against lipoteichoic acid (LTA) from Gram-positive bacteria [97]. And lastly, a few compounds have been approved for clinical use, such as monoclonal antibodies obiltoxaximab to opsonize *Bacillus anthracis* toxins and bezlotoxumab to prevent recurrent *Clostridium difficile* infections [98, 99].

Antimicrobial Proteins and peptides from Innate Immunity

Antimicrobial proteins belonging to innate immunity play a key role in epithelial surfaces homeostasis, preventing pathogen invasions as well as non-disturbing beneficial host microbiota [100]. They are composed by around 100-300 residues and mainly are synthesized in epithelial tissues – as skin, intestine, respiratory and reproductive tract – facing off continuously challenges

against bacteria, fungi, viruses, and parasites. Antimicrobial proteins can rapidly kill pathogenic microorganisms generally by membrane disruption or by their inactivation through essential trace elements chelation [101], exhibiting a broad-spectrum antimicrobial activity [102].

Among the different families that conform antimicrobial proteins, the followed enzymes are the most studied with a better understanding of their mode of action: lysozyme, secreted phospholipase A2 ($sPLA_2$), ribonucleases (RNases), and metal-chelating proteins (i.e., lactoferrin and calprotectin). Focusing on the lysozyme and $sPLA_2$ performance, both enzymes act by enzymatic disruption of the bacteria membrane. They target conserved cell wall/membrane structures and, as a result, microorganism resistance events are hampered [100]. Specifically, lysozyme hydrolyses the glycosidic linkages of cell wall peptidoglycans [103], whereas $sPLA_2$ generates bacteria-wall break by membrane phospholipids hydrolyzation [104]. Unlike lysozyme and $sPLA_2$, almost all RNases families display a non-enzymatic disruption of the bacterial envelope [105]. RNases cationic nature (i.e., eosinophil cationic protein -Rnase3-) allows them to interact with the negatively charged residues of bacteria membrane, generating membrane disturbance and subsequent lysis [106]. Lastly, lactoferrin and calprotectin do not perform a direct bactericidal activity, but through metal chelation, they can control the accessibility of essential trace elements (i.e., Zn, Mn, and Fe) and thus prevent bacterial growth and proliferation [100]. Furthermore, metal chelation can make bacterial pathogens more sensitive to host immune effectors, enhancing their clearance [107]. Hence, the understating of how antimicrobial proteins interact with host microbiome, the underlying mechanistic basis, and exploring the therapeutic delivery, potency and stability will be crucial for a market admission.

On the other side, antimicrobial peptides (AMPs) are small, cationic, and amphipathic molecules produced by multicellular organism as the first line of defense against pathogenic microbes [108, 109]. They are also defined as host defense peptides (HDPs) because of their pivotal role in the innate immunity system. In contrast to bacteriophages and antibodies, AMP exhibits a broad-spectrum activity against most pathogenic microorganisms, including Gram-positive and Gram-negative bacteria, enveloped viruses, parasites, and fungi [110]. In addition, their rapid antimicrobial action through conserved-target pathogens structures -hampering resistance events- and immunomodulation features make them valuable candidates as antibiotic alternatives. Generally, AMPs come from innate immunity, though, bacteriocins are a remarkable exception. They are peptides produced by bacteria to inhibit or kill closely related microorganisms, with the potential to cover a large field of medical applications such as skin and urinogenital infections, or herpes treatments [111, 112].

At length, the Host Defense Peptides are small peptides (12 to 50 amino acids) ribosomally synthesized with an overall positive charge at neutral pH due to their high proportion of positive

charged residues (mainly lysine and arginine) [113]. Moreover, their backbone is typically rich in hydrophobic residues. These two properties enable HDPs to fold into amphipathic secondary structures, which are able to interact with either bacterial membranes or cytoplasmatic targets (will be discussed in the following section) triggering rapidly and effective cell death.

Host Defense Peptides

Among antibiotic alternatives discussed before, HDP exhibit exceptional features to meet unmet medical requirements, such as new antibiotics for AMR pathogens, being their evaluation and development one of the scopes of this thesis. Interestingly, HDPs do not merely display a swift and broad-spectrum antimicrobial activity, but they can also modulate the immune response [114, 115], block viral infections [116], inhibit or eradicate pre-existing biofilm formation [117], and perform anticancer activity [118]. In addition, their short half-life, in contrast with conventional antibiotics, along with their targeting against bacterial conserved structures and the diversity of mechanism that they exhibit widely difficult the occurrence of resistances against HDPs [119].

The early findings of these peptides were identified in the 1980s, when cecropins A and B were described to be present in the hemolymph of silk moths as a defense to cope with pathogens [120]. In the following years, magainins detection in *Xenopus* frogs elucidated that HDPs play an important role in the innate immune system not only in vertebrates but in almost all forms of life [109]. With the subsequent discovery of a long list of HDPs -currently up to 3,700 [121] - throughout the six life kingdoms, they have been classified into different classes considering their length, secondary and tertiary structure, and amino acid backbone [122] (Table 1). The first group is α-helical peptides (i.e., LL-37, magainins, or cecropins), which predominantly have α-helix stabilizing residues like alanine, leucine, and lysine. A second group is β-sheet peptides (i.e., plectasin or human α- and β-defensins) that are stabilized by one to five disulfide bridges adopting predominantly β-sheet secondary structure, which is evolutionary conserved across plants, fungi, and vertebrate animals [123]. Another group is those with extended structures typically rich in glycine, proline, tryptophan, arginine, and histidine (i.e., indolicidin). And finally, there is a group of loop peptides with one disulfide bridge (i.e., bactenecin) and characterized by the non-existence of classical secondary structures.

Table 1. Classification of antimicrobial HDPs according to their structure

Structure	Features	Representatives	PDB ID	References
α-helical				
	Predominant α-helix structure			
	12-40 aminoacids length	Cecropins		[124]
	Rich in Ala, Leu, Lys	Magainins	2MAG	[125]
	Unstructured in aqueous solutions in	LL-37	2K6O	[126]
	absence of membrane interactions	Melittin	2MLT	[127]
β-sheet				
	Extended evolutionary conserved group			
	Predominant antiparallel β-sheet			
	secondary structure	Defensins	1DFN	[128]
	Stabilization by 1-5 disulfide bonds	Plectasin	3E7R	[129]
	Globular structure in aqueous	Protegrins	1PG1	[130]
	environments	Thanatin	8TFV	[131]
Extended				
	Rich in Pro, Gly, Trp and His			
	Lack of classical secondary structures			
	Highly flexible in solution	Indocilin	1G89	[132]
	Elevate antifungal activities	Histatins		[133]
Loop peptides	Presence of β-hairpin structures			
	Typically one disulfide bridge			
	formation that seems not to be crucial in			
	some HDPs antimicrobial properties	Bactenecin		[134]
	Broad mode of actions	Tigerinin		[135]

Among all the HDPs discovered so far, defensins and cathelicidins are the most remarkable families, where most of the research has been carried out.

Defensins

Defensins come from the Latin *defendo*, which means repel, pointing out their role in the prevention of infection and supporting their function as an essential element in the innate immune system. Defensins are a large family of small cationic peptides with exceptional antimicrobial and immunoregulation properties [136]. Yet, under physiological conditions, the antimicrobial activity of defensins has a supporting role, whereas immunoregulation function is predominant.

They are widely distributed in vertebrates, invertebrates (i.e., insects), fungus, and plants as well, with six cysteines forming disulfide bonds which are highly conserved [137, 138]. Their structure is generally stabilized through a β-sheet conformation, holding a significant structural homology across all the group due to an evolutionary relationship [139]. However, it is also worth noting that despite their conserved structure, defensins are exceptionally diverse. Their ability to dimerize, oligomerize, and multimerize on target molecules provides them a vast functional versatility [140]. Additionally, depending on the length, location, and connectivity of their six cysteine residues they can be classified in α-defensins, β-defensin, and θ-defensins (Figure 7).

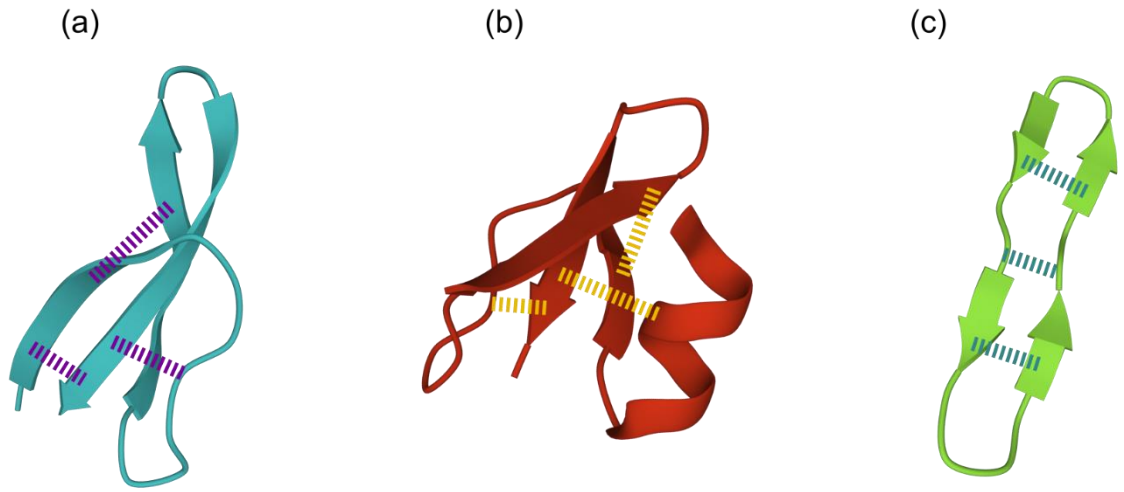


Figure 7. Three-dimensional structures of defensin peptides. (a) α-defensin HD5 (PDB: 2LXZ) [141] (b) β-defensin HβD1 (PDB: 1IJV) [142] (c) θ-defensin retrocyclin (PDB: 2ATG) [143]. Doted lines indicate disulfide bonds distribution within defensins groups. Images from the RCSB PDB (rcsb.org).

i. α-Defensins

Alpha defensins are short peptides -around 29-35 residues- normally synthesized as prepropeptides with a high arginine proportion. The six cysteine residues are linked at positions 1-6, 2-4 and 3-5, resulting in their characteristic structure. They are mainly produced by primates and rodents and, by contrast, they have not been described in cattle or swine.

In humans, six α-defensins have been described, named human neutrophil peptides (HNP) 1 to 4 (HNP1, HNP2, HNP3, and HNP4), human defensin 5 (HD5), and 6 (HD6) [144]. Both HD5 and HD6 are of enteric origin, constitutively expressed in Paneth cells and granulocytes within the small intestine epithelium [145]. After that, they are stored as propeptides in secretory vesicles, until an enzymatic cleavage activates them where required (Figure 8) [146]. HD5 exhibits a strong

broad-spectrum antimicrobial activity at very low molarity against *Escherichia coli, Listeria monocytogenes, Salmonella typhimurium,* and *Candida albicans* [147]. In addition, HD5 microbicidal activity is maintained under low pH and protease-rich environments, supporting their role as mucosal host defense [147]. HD6, in contrast to other defensins, lacks appreciable bactericidal activity and it is directly affected by pH and redox potential. However, this defensin affords protection against enteric pathogens by a unique strategy: HD6 can self-assemble into extracellular fibrils and nanonets (mesh-like) structures, trapping pathogenic microorganisms and thus regulating intestinal microbiota composition [148, 149].

HNPs are synthesized in neutrophil precursors cells, hence the name. Once neutrophils have activated, HNPs are discharged in inflammatory locations, taking place either pro- or anti-inflammatory responses because of the activation of specific intracellular signaling cascades in immune effector cells [150, 151]. The HNP1 is the most active human-α defensin and along with HNP2 and -3 display a high antimicrobial activity against Gram-negative and Gram-positive bacteria, including both intracellular and extracellular organisms, as well as fungi and some enveloped viruses [152]. Curiously, HNP4 preferentially kills Gram-negative bacteria, and unlike their homologs HNP1-3, they have a differential structure, sequence and it is present in lower amounts in neutrophils [153].

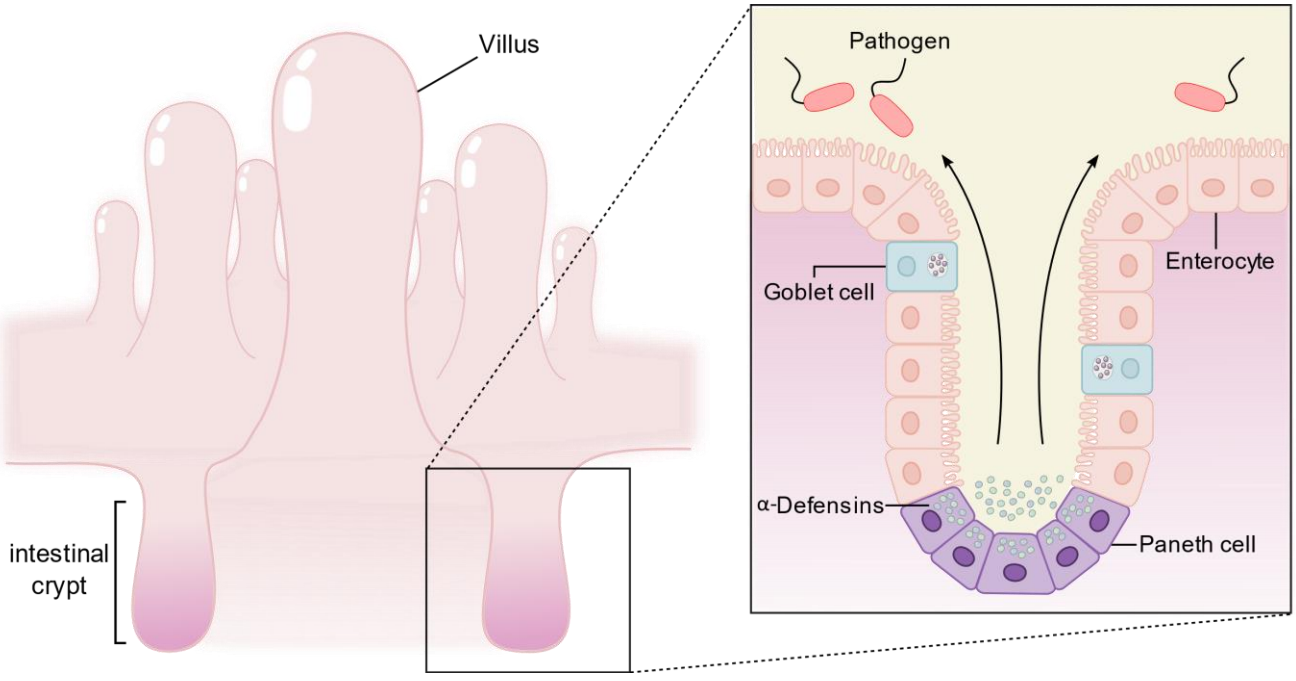


Figure 8. Paneth cells expression of α-defensins promotes barrier protection in the small intestine. In the small intestinal crypts, the constitutive expression of HD5 and HD6 α-defensins by Paneth cells provide intestinal homeostasis regulation and the preservation of host microbiome. In addition, when pathogenic bacteria are detected through pattern recognition receptors (NOD2) an upregulation of defensins expression takes place, enhancing the bacterial clearance and thus reestablishing the basal state. Adapted from [100].

ii. β-Defensins

Beta defensins also contain around 35 amino acids and six cysteine residues, but they differ from the α-defensins disulfide array pattern, being the cysteines connected at positions 1-5, 2-4 and 3-6 [154]. These defensins are expressed principally in leukocytes and epithelial cells, maintaining microbiome homeostasis [155]. In addition, β-defensins not only exhibit antimicrobial activity but also contribute together with α-defensins to the regulation of inflammatory responses [155], fertility, wound healing, plant and fish development, and cancer [156, 157].

In mammals, the first β-defensin described was the bovine tracheal antimicrobial peptide (TAP). This peptide shows antimicrobial properties against *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, and *E. coli* at low concentrations, ranging from 1.5 to 7 μM [158]. Along with that, a second bovine epithelial β-defensin, called lingual antimicrobial peptide (LAP), was later discovered [159]. LAP is a short, cationic peptide with microbicidal activity against Gram-positive and Gram-negative bacteria and fungi as well. Moreover, although LAP was first detected in the tongue, it is widely expressed in epithelial of mammary gland, intestinal and respiratory tract, acting in inflammation responses and resolving infections [159].

The first human β-defensin (HBD1) was discovered in 1995 [160], followed by HBD2, HBD3, and HBD4. HBD1 is thought to control microbiota on epithelial surfaces in absence of inflammation, being constitutively expressed. However, other defensins such as HBD2 are upregulated during inflammatory responses [161]. In addition, whereas HBD1 and HBD2 are highly active against Gram-negative bacteria (i.e., *E. coli* and *P. aeruginosa*), and yeast (i.e., *C. albicans*), the HBD3 also demonstrate bactericidal features against Gram-positive bacteria, such as *Streptococcus pyogenes*, MDR *S. aureus*, and even vancomycin-resistant *Enterococcus faecium* [146, 162].

iii. θ-Defensins

Theta defensins are expressed in the leukocytes and bone marrow of primates, but unlike α- and β-defensins they are not described in humans and evolutionary closed animal species (i.e., chimpanzee, gorilla, or bonobo). This defensins are structurally unique in animals, forming macrocyclic structures of 18 residues [140]. In addition to their antibacterial properties, theta-defensins have proved to be highly active against viral pathogens, as influenza A, dengue, HIV and SARS coronavirus. They are also strong pro-inflammatory cytokines inhibitors, having a great potential, among all defensins, to be applied as anti-infective and anti-inflammatory mediators [163-165]. Hence, their high stability, unique structure and several host defense activities make them a valuable therapeutic agent to be further considered.

Cathelicidins

Cathelicidins, together with defensins, represent a relevant part of the vertebrate's immune system [166]. They are stored in secretory neutrophils and macrophages granules as precursors, being necessary an enzymatic cleavage by neutrophil elastase to become fully mature peptides [167]. Besides their well-known antimicrobial properties, cathelicidins also stand out from their role in immune modulation, mediating inflammation response, cell proliferation and migration, wound healing, and angiogenesis [168].

This family of HDPs is characterized by a highly conserved pro-sequence domain termed *cathelin*, but is highly variable in the carboxy-terminal domain that performs the antimicrobial activity, both inter- and intraspecies [169]. Curiously, although 30-cathelicidin family peptides have been described in mammals, solely one named LL-37 has been detected in humans [170]. This cathelicidin is widely expressed in epithelial surfaces of the gastrointestinal tract, epididymis, lungs, as well as from B and T cells, natural killing cells, monocytes, macrophages, circulating neutrophils, and myeloid bone marrow cells [171, 172]. Moreover, several studies report their antimicrobial broad-spectrum [173], angiogenesis [174], wound healing [175], and large immunomodulation properties, including chemotaxis and highlighted anti-inflammatory attributes by LPS neutralization [176]. Hence, LL-37 ubiquitous presence and broad range of actions clearly reflect their pivotal role in the innate immune system.

Mechanism of Action of HDPs

The HDPs outstand for their duality, exerting either strong antimicrobial activities or an accurate modulation of the immune system when required.

Antimicrobial activity

Despite the observed molecular variety of the antimicrobial peptides discussed before -differing in size, sequence, overall positive charge, conformation and structure, hydrophobicity and amphipatichity- their antimicrobial activity can be generally attributed to two mechanisms of action:

i. Membrane-dependent interactions

For their direct antimicrobial activity, HDPs must interact with membranes, being the electrostatic interaction the major driven force [177]. It is generally assumed that the cationic residues of HDPs allow primary interaction with the anionic lipid headgroups of the bacterial and fungi walls

(specifically, the lipopolysaccharide (LPS) rich outer membrane from Gram-negative bacteria, the teichoic and teichuronic acids of Gram-positive bacteria, and mannoprotein phosphates on the outer surface of fungi [139]). Further, because of their amphipathic nature, HDPs can trigger modifications in the membrane structure, involving pore formation, modified curvature, and membrane potential disturbance. At length, hydrophobic amino acids -located on one edge of the molecule- can interact with lipids conforming membranes or membrane-like structures, whereas cationic and polar residues -sited in the opposite face- are exposed to an aqueous environment, stabilizing the resulting structure [178].

In these membrane-dependent interactions, different models of the permeabilization mechanism have been proposed (Figure 9). In the barrel-stave model, the hydrophobic peptide regions are perpendicularly aligned with the lipid core bilayer, generating a central transmembrane pore, which is stabilized by the exposed peptide hydrophilic region (Figure 9a). Similarly, in the toroidal-pore model peptide's helices are inserted into the membrane, triggering pore formation. However, in the toroidal model the peptides (i.e., magainins and protegrins), are always associated with the lipid head groups even when they are perpendicularly inserted, causing the bending of the lipid monolayers through the hole [179] (Figure 9b). This pore generation leads to membrane depolarization, HDP intracellular penetration, and cytoplasmatic content leakage, resulting in cell death [180]. Lastly, in the carpet model, a high peptide concentration is accumulated on the bilayer surface, but in contrast with the previous models, peptides are parallel-oriented in a carpet-like structure [181] (Figure 9c). HDPs accumulation make them behave as detergents, leading to micelles formation and consequent microbial death by membrane disruption [182].

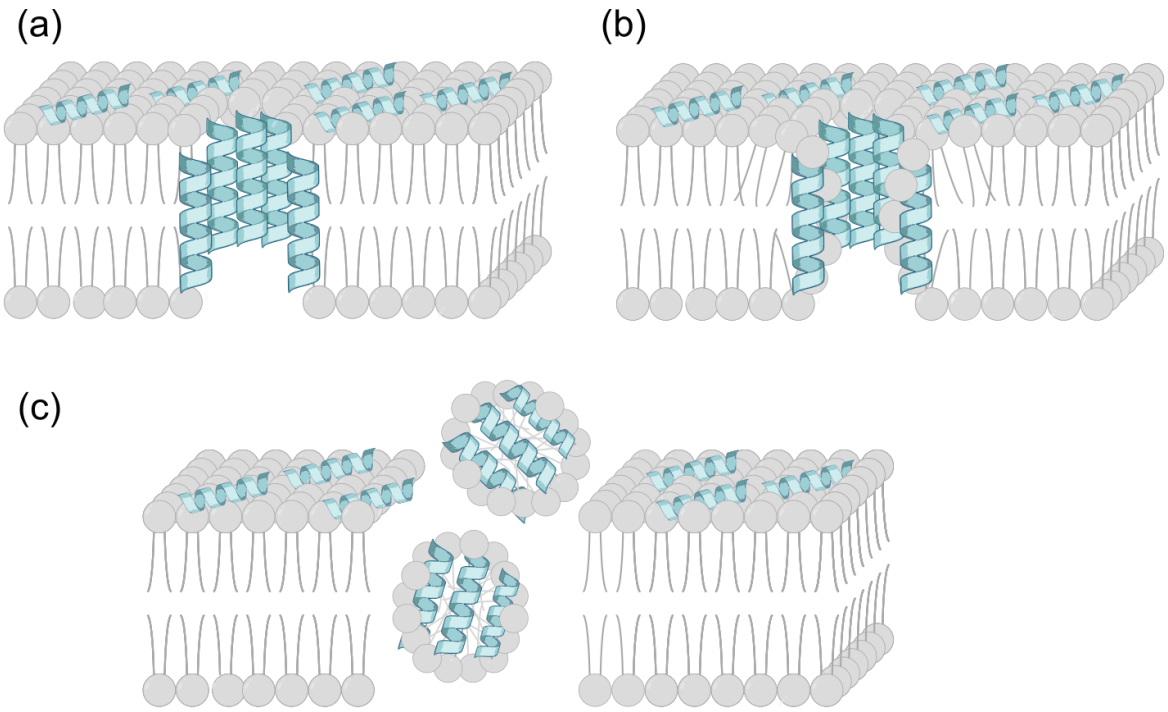


Figure 9. Models proposed for the membranolytic action of HDPs. (a) In the barrel-stave model, the HDP hydrophobic region interacts with the phospholipid bilayer, while the hydrophilic face forms a channel-like structure. **(b)** The toroidal model also destabilizes the membrane by pore formation, where constant interaction of the HDP with the membrane lipid core prompts its curvature. **(c)** In the carpet model, high peptide concentration triggers membrane collapse by micellization, acting as detergents.

ii. Non-membrane interactions

While HDPs are considered to perform their antimicrobial activity generally by membrane disruption, they can also kill or inhibit microbial cells in a non-membranolytic manner, and it has been theorized to be a mode of action equal to or even more significant than membrane permeabilization (Figure 10) [183]. Peptides can directly interact with bacterial structures such as ion exchange channels [137]. Moreover, in the case of peptides which also act by membrane interaction, they may either self-translocate through the bilayer or diffuse by pre-existing pores generated by themselves or other HDP. Once in the cytoplasm, HDPs can diffuse, addressing a wide range of intracellular targets. For example, proline-rich cathelicidins, such as PR-39, can interfere with bacterial proteins and the DNA synthesis pathway [184, 185]. Some HDPs are also known to inhibit bacterial enzymes, either by acting as a pseudo-substrate or by tight binding to their catalytic center [186]. *De facto*, histatins are capable to inhibit a trypsin-like proteinase from *Bacteriocides gingivalis* or inhibit other targets such as *C. albicans* mitochondria [187]. Several defensins have also been shown that regardless of their well-defined membrane lytic activities,

they also kill bacteria sequestering their cell wall precursors, such as lipid II for *S. aureus* and henceforth inhibiting cell wall synthesis [188, 189]. Thus, these mechanisms may in many cases be complementary rather than alternative, capturing the mechanistic complexity and diversity of HDPs in the innate immune system.

The main consideration that remains uncovered is how HDPs display selectivity against microbial cells, not affecting mammalian cells. For the group of non-membrane interacting peptides, their selectivity is mainly attributed to their specific targets, which are microbial-exclusive. Whereas for membranolytic peptides, it arises from specific microbial membrane characteristics. The absence of cholesterol and phosphatidylcholine as well as higher transmembrane potential and a larger presence of negatively charged phospholipids are essential for proper and targeted-directed HDP activity [139, 146].

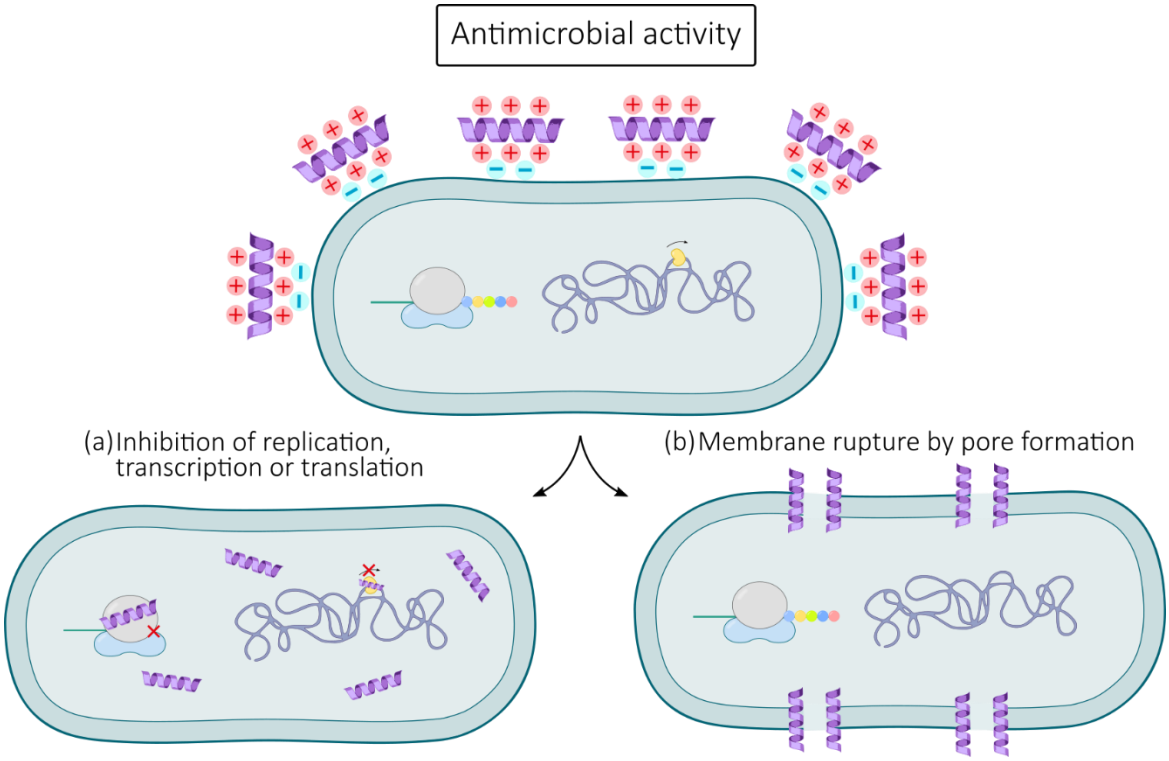


Figure 10. Antimicrobial dual mechanism of action of HDPs. The HDPs lead to microbial death by two different mechanisms that can be synergic. (a) non-membranolytic mechanisms, where peptides are translocated or diffused through lipid bilayer to address intracellular targets, inhibiting bacteria replication or protein both transcription and translation. (b) Membrane-lytic mechanisms that are based on pore formation and membrane disruption.

Immune system modulation

Although HDPs were initially explored for their antimicrobial activity, they are now widely recognized as key actors in host immune regulation. Their diverse immunomodulatory properties are involved in both the innate and adaptive immune responses, being these immunomodulatory features predominant in a physiological context [190]. *De facto*, they are able to modulate pro- and anti-inflammatory responses (Figure 11), chemoattraction, enhancement of intracellular and extracellular bacterial killing, activation and maturation of immune cells, and wound-healing [191-193]

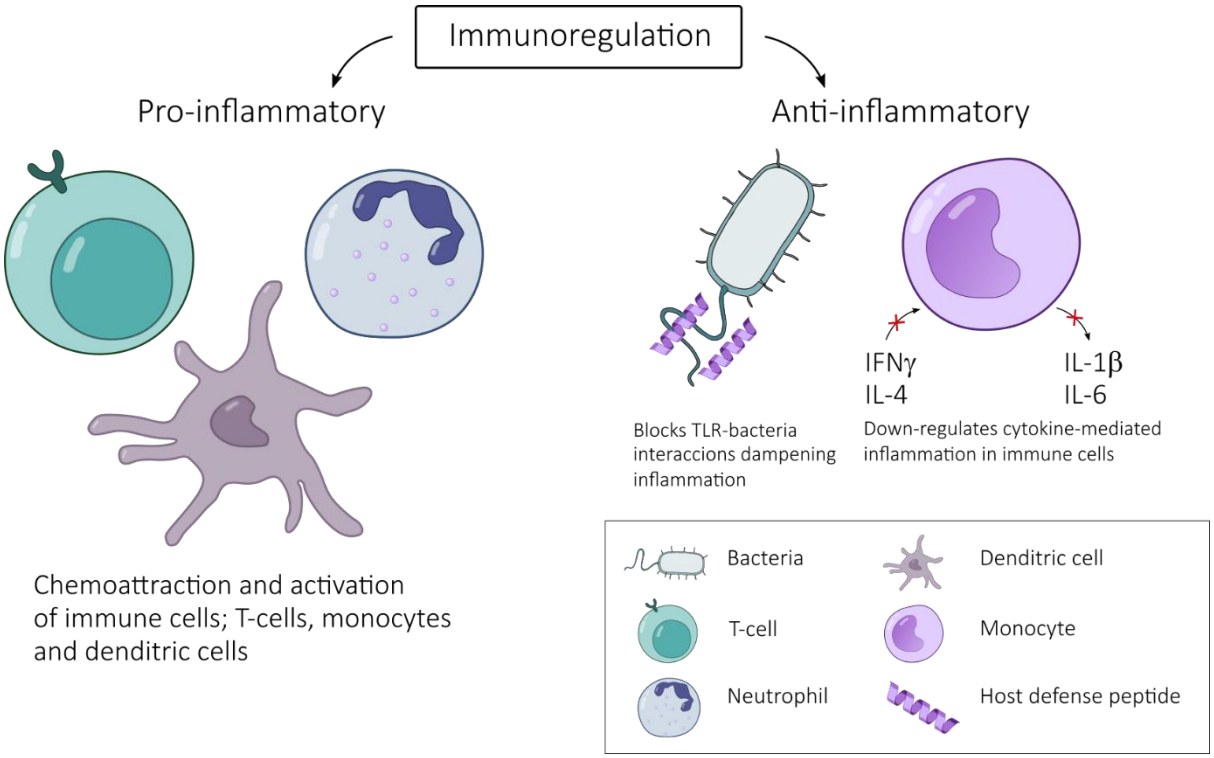


Figure 11. Immunomodulatory properties of HDPs. HDPs can modulate pro- and anti-inflammatory responses as the situation required. At the beginning of a pathogenic microbial invasion, HDPs chemoattract and promote dendritic cell maturation and macrophage differentiation to efficiently eliminate the threat of infectious agents. However, an excessive inflammation during infection resolution can trigger harmful side effects. For that, HDPs can block either antigenic elements of bacteria or the subsequent inflammatory signaling cascades, dampening inflammation. In addition, once the pathogen is eliminated, HDPs are involved in the downregulation of pro-inflammatory cytokines and thus promoting the host give back to a basal state.

During an infection, neutrophils and Paneth cells degranulate, releasing a high number of active forms of defensins and cathelicidins in the local environment. Defensins are involved in bacterial opsonization, mast cell degranulation, cytokine up- or down-regulation, chemotaxis of dendritic cells and monocytes, as well as mitogenesis and neovascularization [123]. As an example, HNP-1, HNP-2, and HNP-3 can stimulate IL-8 production, which is a strong neutrophil chemotactic cytokine, promoting the accumulation of neutrophils at infection sites. Thus, degranulation of the recruited neutrophils will increase defensins concentration and hence IL-8, resulting in a positive-feedback loop [194]. These defensins are also implicated in TNF-α and IL-1β upregulation while decreasing the production of anti-inflammatory IL-10 cytokine from monocytes [195].

On the other hand, the released cathelicidin LL-37 has exhibited a considerable immunomodulatory function *in vivo*, such as immune cells recruitment, anti-endotoxin activity, downregulation of pro-inflammatory mediators, such TNF-α and interleukin (IL)-12 in monocytes, macrophages, and dendritic cells [191]. Surprisingly, LL-37 inhibits inflammation activated by bacterial LPS, but holds the expression of chemotactic mediators [191]. Several studies report that this activity promotes a local immunity to infection while systemic hyperinflammation is prevented [191, 196]. In addition, the LL-37 also mediated the apoptosis of infected cells -enhancing pathogen clearance- and the degradation of dysfunctional cellular components after the end of the infections (autophagy) [197, 198]. Finally, to restore damaged tissues, this cathelicidin induces the chemotaxis of keratinocytes and metalloproteinase activation for extracellular matrix restructuration [199]. It is important to note that these HDPs immunomodulatory features are being in-depth analyzed to avoid the uncontrolled immune response that is generally related to critical patients infected with Coronavirus (SARS-CoV-2) [200]. In this regard, the combination of HDPs with antiviral drugs can provide efficient treatments against COVID-19 [201]. As an example, the LL-37 can suppress the expression of pro-inflammatory cytokines, regulating the inflammation and avoiding a systemic hyperinflammation, also known as cytokine storm [200].

Thus, their rapid and broad mode of action, targeting not only high conserved antimicrobial membrane components, but also intracellular essential elements for pathogenic microorganisms, as well as their multifunctional immune roles make HDPs exceptional antimicrobial molecules. Yet, although promising, only a few HDPs -as antimicrobials- reached the last phases of clinical trials (i.e., omiganan or pexiganan), where poor pharmacokinetics and/ or pharmacodynamics features, cytotoxic issues, or low antimicrobial activity in clinically relevant environments are the main challenges toward clinical application of HDPs [202]. On the other hand, the HDPs as immunomodulators is a relatively novel field, where several therapies are currently under clinical development, being the most promising ones in phases 2 and 3 of ongoing trials [115, 202, 203]. Yet the full potential of immunostimulation will be evaluated in the following section.

Immunostimulants

In the pursuit of new options to address antimicrobial resistance, strategies such as the use of phages, antibodies, antimicrobial proteins and, remarkably, HDPs have shown to be promising antimicrobial candidates. However, these molecules are solely a piece of the whole strategy at finding antibiotic alternatives, being the stimulation of the immune response an interesting complement to avoid antibiotic use. In this framework, the use of immunostimulants opens up the possibility to reinforce the immune system in essential timescales, decreasing infection vulnerability, as well as boosting resilience against potential pathogens.

The immune system is a versatile and complex network of biological processes that work simultaneously with a common purpose: to protect the host against harmful substances and pathogens. It has evolved alongside microbes do, overcoming potential threats that may jeopardize host health. This line of defense against external pathogenic microorganisms can be generally divided inro two types of responses: innate and adaptive immunity. Both differ in the activation time and its duration, the involved effector cells, and the degree of specificity and generated memory (Figure 12). However, an efficient immune response requires the coordinated activity of both immunities [115, 204].

Innate immunity is the evolutionary ancient strategy of the immune system that is crucial in plants, fungi, insects, and animal welfare [205]. They are integrated by anatomical and physiological barriers such as skin, mucosa, pH or temperature, molecules like enzymes as well or thoroughly discussed HDPs along with cells such as phagocytes, neutrophils, macrophages, and dendritic cells [206]. This immunity is characterized by triggering an immediate non-specific response, where antigen recognition is not mandatory. Consequently, innate immunity faces off effectively the potential threat, but it does not retain immunological memory [207].

The adaptive immunity is activated by innate immunity pathogen recognition. However, in contrast to the innate response, the adaptive response is defined by a slow activation (on the time scale of 3 days to a couple of weeks) with a high pathogen-specificity and more complex mechanisms. The major cell type involved, with distinctive properties of specificity, recognition, and memory are the lymphocytes. In addition, adaptive immunity comprises two different subresponses, usually interrelated: humoral (antibody-mediated) and cellular (cell-mediated) [208], for which will not go into detail.

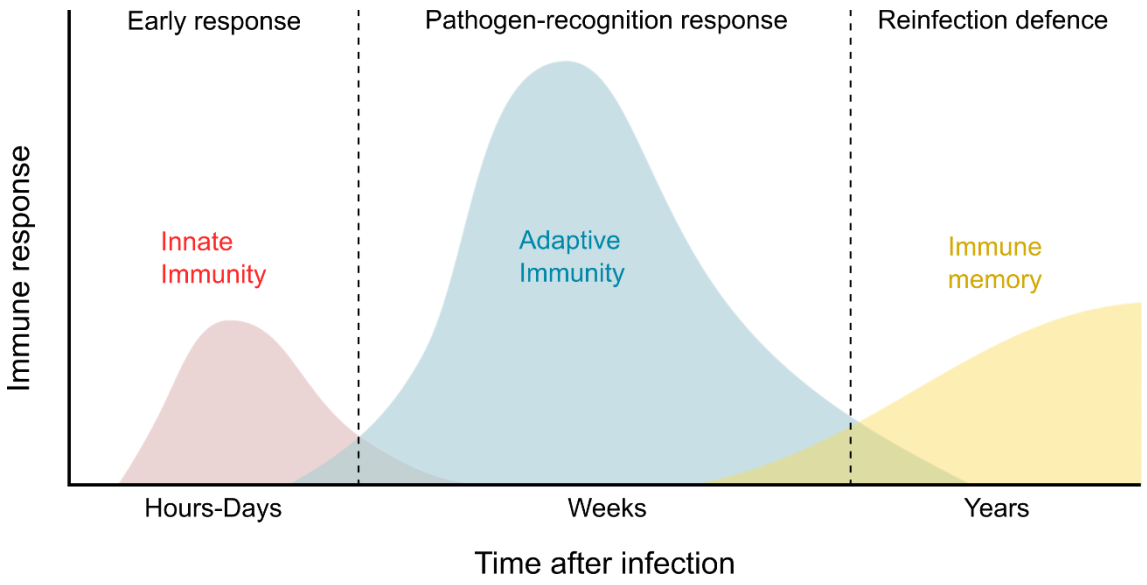


Figure 12. Sequential activation of the immune system during infection. After pathogen infection, the innate immunity displays an early (hours to days) non-specific response to neutralize the threat. If the pathogen persists, a potent tailored mechanism is triggered by the adaptive immunity. Once it is eradicated, specific immune cells involved in the response retain long-term pathogen memory. Thus, upon re-exposure to the same pathogen, memory cells prevent reinfection through a swift and targeted process. Adapted from [209].

Hence, when the host immune system is compromised, immunostimulants or immunopotentiators can be used to enhance its response capacity. This capability to stimulate the immune system is closely related to the compound secondary and tertiary structure, conformation, molecular weight, and solubility, underlying its complexity [210]. Moreover, it is important to stress that immune modulators hold an interesting duality in their application. (i) They can be utilized in a prophylactic manner, reinforcing the immune system before a potential challenge that can compromise host health [115]. For example, vaccines precisely stimulate the adaptive response to prevent a specific infection, whereas other immune modulators, such as cytokines, provide a broader range of protection to several challenging circumstances -that will be illustrated in the clinical application chapter. (ii) On the other hand, these compounds could be also useful to face off an established threat, enabling a prompt and effective resolution and diminishing associated side-effects. Some examples are the adjunctive therapies (not in vaccination), which enhance antibiotic or antiviral effectiveness or can be applied as treatment itself, such as the cytokines involved in cancer [210]. It is also worth mentioning that, unlike antibiotics and other treatments, immunomodulation targets the host rather than the pathogen, avoiding selective pressure for the evolution of antimicrobial resistance. Moreover, the non-specific nature of innate immune defenses denotes that their modulation will be reflected in broad-spectrum protection against a

wide range of pathogens, enabling prophylactic use and early treatment of problematic MDR infections [115].

The ability to modulate these immune responses, by suppression (i.e., COVID hyperinflammation) or boosting depending on the need, has been demonstrated to be a potent strategy [200, 211-214]. Diverse compounds including flavonoids, essential oils, polymers, cytokines, and synthetic sources are being studied as strategies to improve the immune response. Different approaches will be examined in this section, analyzing their strengths, weaknesses, and further clinical development.

Flavonoids

Plants have always been an important source of natural therapeutics. Among these components, flavonoids, which are low molecular weight phenolic compounds, are a remarkable group with a broad range of applications [215]. In nature, flavonoids are produced by plants as the first line of defense against bacteria and fungi [216]. Those selected for human health are associated with large health-promoting effects such as anti-oxidative, anti-inflammatory, immunostimulant, and microbicidal activities combined with anti-carcinogenic properties [217]. Diverse flavonoids are also linked with immunosuppressive effects through inhibition of mast cells and basophils degranulation, blocking allergic reactions [218], and inhibition of eosinophil-associated allergic inflammation and asthma [219]. Moreover, they can also modulate neutrophils, monocytes, or macrophages, either enhancing or dampening their activities as required [215].

Although flavonoids are mainly used to modulate the immune system, they also have antimicrobial activity by themselves. They exhibit a varied range of bactericidal mechanisms such as the inhibition of cell envelope and nucleic acid synthesis, inhibition of electron transport chain, inhibition of ATP synthesis, inhibition of bacterial motility, inhibition of quorum sensing, inhibition of biofilm formation, and inhibition of bacterial efflux pumps [220]. Moreover, flavonoids can inhibit MDR essential enzymes such as KAS III, responsible of fatty acid synthesis in methicillin-resistant *Staphylococcus aureus* [221].

Therefore, the diverse mechanism of action and the large collection of compounds make flavonoids a promising candidate for the development of new therapies. Nevertheless, most of the old research accessible is based on extracts, which are difficult to analyze [215]. In addition, further experiments of flavonoids interaction with receptor molecules during long-term treatments and exhaustive *in vivo* studies will be required to demonstrate their full potential [217].

Essential oils

Plant essential oils (EOs) are another group of plant-derived phytochemicals used for disease treatment or as health promoters. EOs are highly concentrated natural oils derived from plants that consist of aromatic, volatile, secondary plant metabolites [222]. There are over 3,000 EOs described, formed by a complex biochemical mixture. Their composition is largely influenced by plant variety, growth area, climatic changes, harvesting time, and storage conditions, affecting their biological activity [223].

The application of EOs is very diverse and highly dependent on the plant source. They are broadly used in cosmetic, food, and pharmaceutical industry due to their antiallergic, antioxidant, antidiabetic, antimicrobial, and underlined immunomodulatory properties [224-226]. Specifically, EOs are able to stimulate the immune system through multiple mechanisms: EOs from eucalyptus are reported *in vitro* as a promoter of phagocytic activity [227], whereas EO from *Schininus molle* increased tumor necrosis factor (TNF)-α and nitric oxide production, aiding microbial clearance [228]. Another essential oil from lavender has shown an increased phagocytic rate and up-regulation of ROS species *in vitro*. Curiously, EOs can act as pro- and anti-inflammatory molecules, stimulating the immune system and simultaneously mitigating an excessive inflammatory response, balancing the overall immune reaction [222].

Commonly, EOs are also used in aromatherapy, as purified extracts or single constituents, to treat and prevent diseases through topical or respiratory administration [229]. However, the selection of a suitable safe oil and dose determination is crucial to avoid undesirable side effects, specifically in children [223, 224]. Furthermore, a recent study has investigated the performance of EOs combined with antibiotics against MRSA, vancomycin-resistant enterococci (VRE), and extended-spectrum β-lactamase (ESBL)-producing *E. coli* with a promising outcome [230]. The remarkable synergistic effect of EOs with antimicrobial drugs allows a significant reduction in bacteria survival, modulating the sensitivity of MDR pathogens even when they are forming biofilms [230-232].

Although EOs demonstrate an excellent potential, the lack of clinical and toxicologic studies, together with the use of complex mixtures rather than isolated constituents, largely block global market entry [222, 224]. Furthermore, whereas herbal-derived immunostimulants are normally proposed for prophylaxis and resolution of moderate infections, such as bronchitis or recurrent urogenital infections, they are not a good alternative for severe bacterial and viral infections [210].

Polymers

In this class of compounds, the number of potential structures that may react with surface receptors of immune cells appears, at first glance, to be unlimited. Still, the relatively limited products that have finally reached the market indicate quite the opposite [210]. The first naturally occurring polymers investigated with considerable immunostimulant properties were polysaccharides. They are macromolecules formed by long chains of carbohydrates monomers -called monosaccharides- linked by glycosidic bonds [233]. Polysaccharides are synthesized by a large group of organisms, from plants, algae, bacteria, fungi to animals, differing in their monosaccharides building blocks. Their complex secondary and tertiary organizations allow them to fulfill many different functions, such as energy storage in plants or structural support of vegetal cells [234]. Besides, this versatile polymer is also capable to regulate smoothly innate and adaptive immune responses. Generally, polysaccharides can activate macrophages, lymphocytes and promote the secretion of immune-related molecules, such as cytokines or antibodies [235]. As an example, chitin-derivate chitosan can upregulate cytokine expression and enhance macrophage activation [236, 237]. Another well-known example is the β-glucans, which are also implicated in numerous pathways of host immune defense [238].

Polysaccharides have demonstrated desirable immunomodulation, biocompatibility, and biodegradability features, coupled with low toxicity and safety. But, it would be crucial to standardize purification protocols, synthesis, and characterization to achieve more robust conclusions. Further research in the clinical field will provide a better understanding, as well as increased safety and subsequent comprehensive application.

Cytokines

Cytokines are bioactive proteins of low molecular weight -around 10 to 25 kDa- with a pivotal role in immune regulation [239]. They are involved in cell growth and differentiation, modulation of inflammatory responses (Figure 13), chemotaxis, and tissue repair [240], to mention a few. The first insight that cytokines may be used as therapeutics was noted by Isaacs and Lindenmann, pointing out that cells previously treated with interferon (IFN) were resilient to viral infection [241]. So far, more than several hundred cytokines have been described and, currently, many of them are extensively used as adjuvants, immunostimulants, and therapeutics mostly in human medicine [242]. The effects of cytokines are mediated through high-affinity receptor binding (Figure 13). These cytokine receptors are displayed on the cell surface, but their amount can substantially differ depending on the immune cell is activated or not [239]. Thus, cytokines can trigger a broad range of responses based on the cytokine type, the receptor, and the target cell.

They are classified by their biological activity rather than the tertiary structure or amino acid sequence. Interleukins (ILs) are one of the most well-known group of cytokines, together with IFN and TNF. ILs are involved in pro-inflammatory responses (i.e., IL-1β, IL-6 or IL-8 are classical pro-inflammatory cytokines) but they can also lead to anti-inflammatory activities (i.e., IL-10 or IL-11) [243]. IL-1β and TNF-α typically upregulate pro-inflammatory genes, activating the cascade of inflammatory mediators and subsequent enhancement of endothelial adhesion and synthesis of chemokines [244]. The IL-6 are synthesized during inflammatory processes (as well as IL1-β and IL-8), and it is a potent stimulator of acute-phase proteins. In addition, IL-6 also upregulates IL-8 secretion and leukocyte recruitment [245]. However, IL-6 can also exhibit anti-inflammatory properties, blocking macrophage synthesis of IL-1β and TNF-α [243]. Finally, IL-8 is implicated in neutrophil chemotaxis and further degranulation, enhancing the inflammatory response [246]. On the other hand, anti-inflammatory cytokines are critical to balance the overall immune response, downregulating IL-1β, IL-8, or TNF-α production to avoid final toxic effects due to excessive inflammation. Lastly, IFNs are also an exceptional therapeutic family, clinically approved for the treatment of chronic hepatitis B, C, and cancer, such as malignant melanoma, or hairy cell leukemia [240]. Despite their versatile therapeutic properties, cytokine short half-life entails high doses administration, provoking toxicity side-effects in systemic applications and poor cost-effective performance [242]. To work on that, novel strategies to enhance cytokine stability are currently investigated to reduce treatment doses, therapy cost, and associated side-effects.

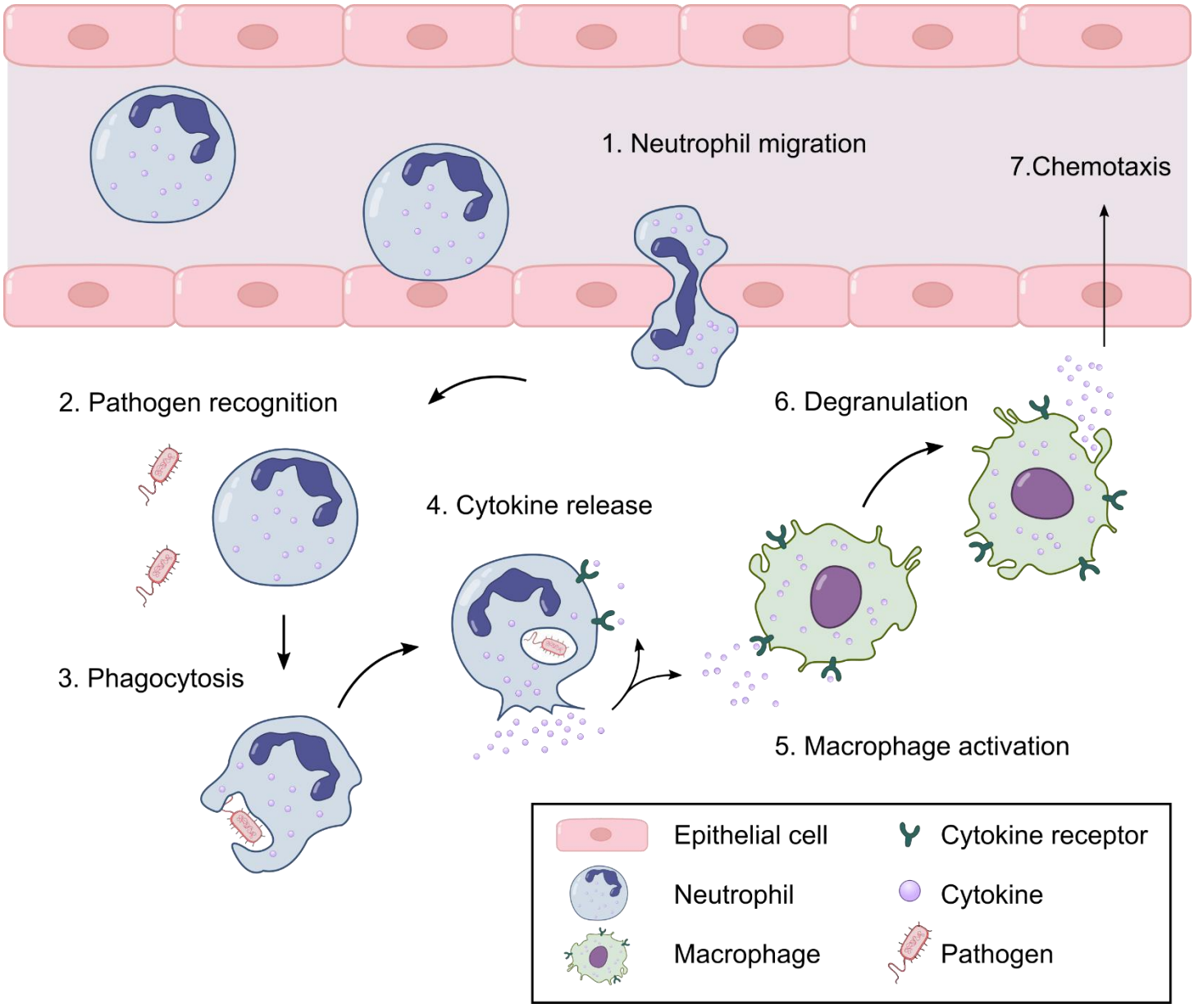


Figure 13. Schematic representation of cytokine-mediated response. During inflammation, bloodstream neutrophils interact with activated epithelial cells resulting in transendothelial migration (1). Once in the tissue, neutrophils recognize pathogen-associated molecular patterns (PAMPs) (2), triggering the activation of an array of responses, such as expression of receptors, cytoskeletal reorganization, or phagocytosis (3). In addition, neutrophils undergo degranulation of several compounds as reactive oxygen intermediates (ROIs) or pro-inflammatory cytokines, including IL1β, TNF-α, and IFN-γ (4). These cytokines can either display an autocrine effect (feedback loop), amplifying neutrophil function, or a paracrine effect through stimulating immune cells, such as macrophages (5). After that, activated macrophages also degranulate (6), releasing pro-inflammatory cytokines (IL1β, IL6, or TNF-α) and chemokines, recruiting further immune sentinel cells (7).

Synthetic sources

Synthetic immunostimulants have been developed recently to enhance immunostimulant pharmacokinetic and pharmacodynamic properties, avoiding early degradation, undesirable interactions, and subsequent side-effects. Among the numerous explored alternatives (synthetic oligonucleotides [247], self-assembled nano-stimulants [248], synthetic nanoparticles carrying antigens [249], or commonly synthetic derivates of natural compounds [250]) the innate defense regulator (IDR) peptides stand out from other competitors. They are synthetic immunomodulatory structures that are designed taking as reference sequences of natural HDPs. IDR are short cationic peptides with similar or enhanced HDPs immunomodulatory properties. They can also trigger macrophages differentiation, leukocyte recruitment, promote neutrophil degranulation, wound healing, and so forth [251-253]. But, unlike HDPs, IDR can be formed by non-natural amino acids, which substantially increase their stability (these amino acids are not recognized by host and pathogenic bacteria proteases). In addition, through iteration cycles, computational based approaches, and mathematical modeling it is possible to enhance, even further, their immunostimulatory and antimicrobial activities [115]. Therefore, IDR peptides are a robust candidate with improved pharmacokinetics and pharmacodynamics properties. However, associated cost of synthesis represents a considerable hurdle in their scale-up development that hinders a broadly therapeutic use.

ANTIMICROBIALS AND IMMUNOSTIMULATORS PRODUCTION

Before the evaluation of the activity of a protein or peptide of interest, and therefore its potential of applicability, it is necessary to make it. The selection of the production strategy should be based on the characteristics of the protein of interest, but also the associated production cost, yield needed, time required, and scale-up feasibility [254]. The two strategies that can be used for that are chemical synthesis and recombinant protein production, having each approach its strengths and drawbacks.

Chemical synthesis

Chemical synthesis allows the synthesis of peptides and proteins in a cell-independent manner. Unlike ribosome-mediated biosynthesis, chemical synthesis enables step-by-step control on protein composition, as well as the introduction of non-natural amino acids which are not recognized by host proteases, and allow to increase peptide stability [190, 255, 256]. Commonly, solid-phase techniques are the standard procedure for chemical synthesis, outlining their high velocity, easy automation, and straightforward product purification [257]. Briefly, the C-terminus end of the first amino acid is connected by a linker to a synthetic resin. To avoid undesirable reactions, protecting groups shield the N-terminus end along with aminoacidic reactive side-chain residues. Thereafter, subsequent N-protected amino acids are introduced to the attached amino acid through the removal of the N-terminal shield of the last residue, without disturbing side-chains protection groups. Repeated cycles of deprotection and coupling are stepwise performed until the desired peptide length is achieved and cleaved from the resin (Figure 14) [258].

Chemical synthesis has shown to be a useful tool in the research of antimicrobial peptides [259, 260]. However, the synthesis of peptides longer than 20 residues is difficult and alternative strategies like native chemical ligation must be applied for protein production [258, 261]. Moreover, the lack of stereoselectivity concerning enzyme biocatalysis -being necessary extra protection/deprotection reactions-, the environmental burden due to organic solvents during product synthesis, as well as the difficulties (sometimes impossibilities) to perform post-translationally modifications (PTMs), high production costs, and associated drawbacks for large peptides and protein production overall hinder a wider use [262].

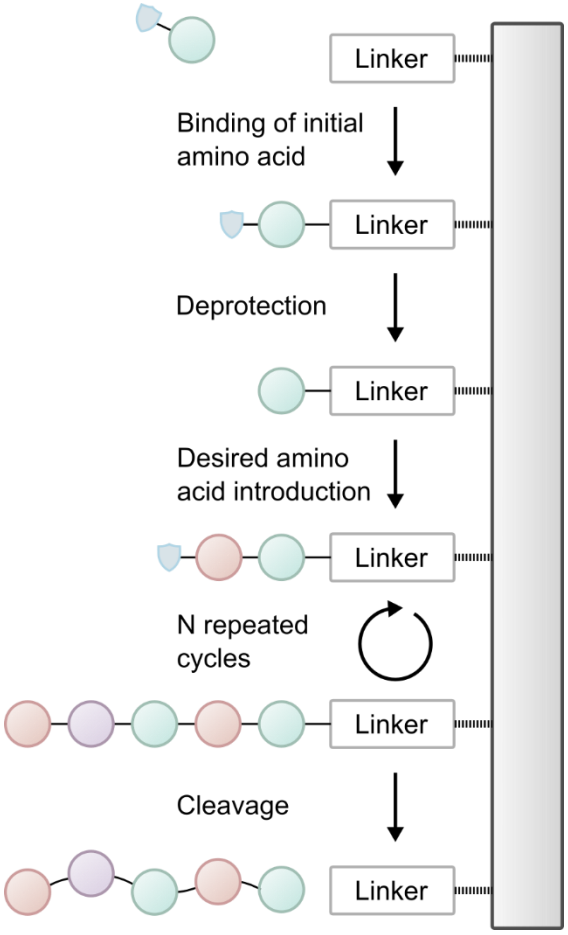


Figure 14. Overview of solid-phase peptides synthesis. Once the first amino acid is attached to the linker, in every cycle an N-terminus deprotection of the anchored nascent peptide is followed by the introduction of the desired N-protected amino acid until the complete sequence of the target peptide is achieved and then cleaved from the linker.

Recombinant protein production

The use of recombinant technologies for protein production has undoubtedly been a major breakthrough. Before their discovery, proteins of interest were purified from their natural sources, such as plant extracts or animals, in a time-consuming, variable, and costly way [263]. In addition, natural sources are associated with an inherent biological risk, derived from the potential pathogenic entities (i.e., virus, bacteria) present in the original host, which might contaminate the sample, being necessary strict quality controls. But in 1982 with the approval of the first recombinant protein (insulin), a new world of possibilities opened up [264]. Since then, the plethora of research in recombinant production was largely reflected in the development of a vast

number of molecular tools, production platforms, and strategies to produce even the more challenging compounds. Thus, recombinant protein production has not merely been established as an alternative to natural sources, but also to chemically synthesized peptides, showing an unparalleled versatility limited only by the imagination.

Protein cell biofactories

The discovery of recombinant DNA technology paved the way for using heterologous expression systems for recombinant protein production. Proteins produced using these systems are known as recombinant proteins because the gene sequences encoding them are recombined or engineered and artificially introduced inside a host cell [265].

The gene encoding the desired protein is first cloned into a suitable expression vector under the control of a promoter that regulates gene expression (Figure 15). Then, the plasmid vector is introduced into the selected host to overexpress the cloned gene. Secretion tags can be incorporated to the gene sequence to allow protein secretion into the media from where it can be purified. Otherwise, if protein remains inside the host cytoplasm, cell disruption is required before the protein purification step. For purification purposes, specific tags such as 6 histidine tag can be also added to the gene sequence.

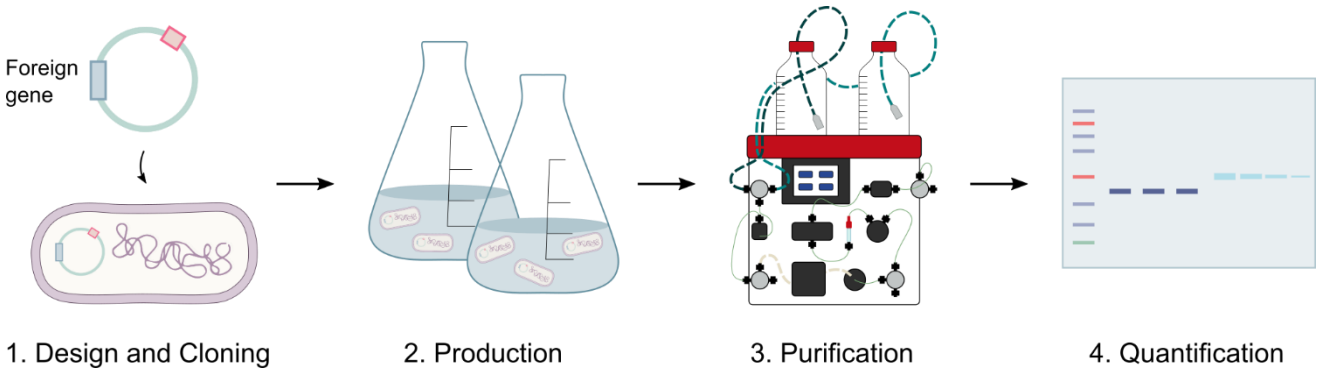


Figure 15 Recombinant protein production and purification scheme. First, an engineered plasmid that contains the encoded sequence for the target protein is introduced into the selected biofactory. Then, the culture grows until it reaches the desired biomass after protein production is induced. Finally, the protein is purified and quantified.

During recombinant protein production, non-native proteins are overproduced by a non-natural host. This drives the protein producer cell to a stressful condition with an overwhelmed metabolism. Under this situation recombinant proteins can aggregate or be degraded by proteases, and, in some cases, the protein can be even toxic for the host, provoking cell death or significantly shrinking production yields [266, 267]. For this reason, it is important to select the most appropriate recombinant platform among those currently available for protein production purposes. Bacteria, yeast, fungi, algae, insect cells, and mammalian cells can be used as protein cell biofactories [268]. In general terms, microorganisms are by far the most versatile and well-established recombinant protein production system. However, some specific protein features are crucial for host selection. For example, if PTMs (e.g., glycosylation) are essential for protein bioactivity, the use of eukaryotic cells is highly recommended. The Chinese hamster ovary (CHO) cells or HEK293 cell line derived from human embryonic kidney have been conventionally used for complex PTM protein expression. Nevertheless, high culture media cost, slow growth rates, difficult operation, and scale-up are their main weakness of this expression system [269].

Microbial cell factories

Genetic plasticity, high density cultures, fast growth kinetics, together with high production yields and inexpensive culture mediums are just some of the advantages that make microorganisms the first choice for recombinant protein production. In addition, among the different options, *E. coli* is indisputably the gold standard in this field, although other alternatives such as lactic acid bacteria (LAB) have also been consolidated as robust microbial cell factories [270].

- *Escherichia coli*

E. coli is a Gram-negative enterobacterium that is considered the workhorse in recombinant protein production [271]. Its well-characterized physiology, metabolism and genetics provide a large collection of molecular tools to work with [272]. *De facto*, almost a third of approved recombinant therapeutic proteins by Food and Drug Administration (FDA) and European Medicines Agency (EMA) are produced in *E coli* [273]. This organism offers a rapid and cost-effective method, giving yields of up to 25% of expressed recombinant protein of total biomass with a relatively simple scale-up process [267, 274]. In addition, its fast growth rate (around 20 min doubling time) and low-cost growth media, make this organism the cornerstone for heterologous protein production [275]. Currently, a wide range of expression vectors and *E.* coli commercial strains (each of them with a characteristic genetic background) are available on the market. *E.* coli BL21 (DE3) is one of the most used strains for protein production. Interestingly,

E. coli BL21 (DE3) strain is deficient in both Lon and OmpT proteases, which are involved in foreign protein degradation [276]. Moreover, plasmid loss is also blocked through *hsd*SB mutation, enhancing even more heterologous protein yield. But, although *E.* coli BL21 (DE3) and its derivates are the most employed strains for protein production, *E. coli* K-12 lineage is also useful for this purpose. For those proteins that have disulfide bonds, such as HDPs [277], Origami strains are a good alternative. These strains lack thioredoxin reductase (*trx*B) and glutathione reductase (*gor*) genes, where their more oxidized cytoplasm foster disulfide bond formation [278].

On the other hand, the expression plasmid, which is the outcome of the replicons combination (regulate plasmid copy number), promoters, selection marker, and multiple cloning sites [276], is also an important player. The pET collection is broadly used for recombinant protein production in *E. coli*, displaying complete solutions for high gene expression, heterologous production and subsequent purification with up to one hundred distinctive vectors [279]. Yet, other alternatives such as pQE vector -which was the original Hist-tag vectors-, pGEX vectors that allow glutathione S-transferase (GST) tagged proteins for purification or labeling purposes, as well as pLEX vectors which are regulated with a phage promoter and pBAD series that enable dual expression of recombinant proteins, are also largely employed [280, 281].

Therefore, unquestionably *E. coli* is quite an exceptional microorganism that agglutinate most of the needed features in a protein expression system. Still, their Gram-negative nature could be its main limitation, due to the presence of LPS in the outer membrane. This endotoxin is a conserved Gram-negative glycolipid that is recognized by the innate immune system, triggering a strong pro-inflammatory response [282]. Thus, when *E. coli* is used as a protein production platform, LPS is commonly found as a non-specific contaminant in the downstream protein purification. To address this, normally extra steps of purification are necessary to eliminate LPS [283]. In this context, an *E. coli* strain with a modified LPS (ClearColi ^{TM}) has been developed [284]. However, still be encouraging, further development in terms of safety is needed for a wider system application.

- Lactic Acid Bacteria (LAB)

Being the presence of LPS in the final protein product a shortcoming, the use of Gram-positive bacteria, which in contrast to Gram-negative bacteria are LPS-free, have been explored for recombinant protein production purposes [285]. Their engaging features for the expression of safe therapeutical compounds have generated a growing interest that will be examined throughout this section.

Lactic acid bacteria (LAB) constitute a heterogeneous group of Gram-positive bacteria that have been used in food fermentation of dairy products for a long time [286]. Besides, the current application of LAB in the food industry involves the production of vitamins, flavoring agents, or antimicrobial compounds such as bacteriocins. In addition, there is vast research on LAB as probiotics to improve both human and animal health. Due to their historically safe use and the absence of LPS in their cell walls, LAB has been Generally Recognized As Safe (GRAS) organism by FDA and fulfill criteria of the Qualified Presumption of Safety (QPS) according to the European Food and Safety Authority (EFSA) [287]. However, the use of these microorganisms as recombinant expression systems is a relatively novel area of study. During the last years, it has gained importance due to the need to find alternative microbial cell factories other than *E. coli* [285, 288].

Among all LAB species, *Lactococcus lactis* is an excellent alternative for recombinant protein production. *L. lactis* is a Gram-positive, spherical, non-sporulating, and facultative anaerobic gut bacteria [289]. Curiously, despite this microorganism has always been linked with dairy products, this bacterium was originally isolated from plants, waiting to be digested by ruminants and thus becoming fully active alongside the gastrointestinal tract [290]. This food-grade microorganism has undergone significant progress in the recombinant protein production field in the last two decades. Their genome was fully sequenced, and a comprehensive number of genetics tools were developed such as cloning protocols, expression vectors, and optimized mutagenesis systems [290-292].

Several expression systems have been developed for *L. lactis* heterologous protein expression, where inducible promoters are preferred rather than constitutive ones, providing a fine control to the user [289]. Among them, NICE ® (Nisin Controlled gene Expression) system developed by Kuipers and coworkers is by far the most chosen alternative [293]. In this approach, the *nisK* and *nisR* genes were isolated and introduced either into the chromosome of *L. lactis* subsp. *cremoris* MG1363, leading to the establishment of the most commonly used NZ9000 strain or into a plasmid such as pNZ9530 [294]. Briefly, nisin is able to interact with the membrane receptor NisK. Subsequently, NisK auto-phosphorylates itself and triggers an intracellular cascade, activating NisR by phosphorylation. Lastly, NisR induces the gene transcription downstream the PnisA promoter (Figure 16), which is allocated the foreign gene that codifies for the target protein [293]. Consequently, the plasmids for *L. lactis* usually incorporate the PnisA promoter, such as the extensively used pNZ8048 and its derivatives pNZ8148 and pNZ8150 [293].

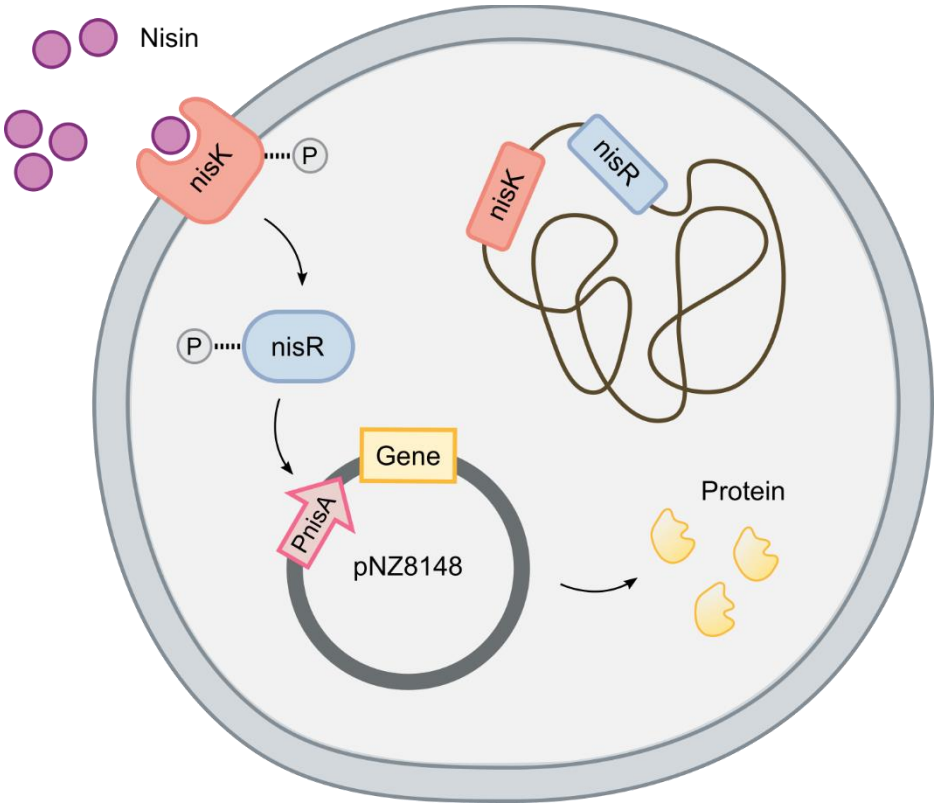


Figure 16. Nisin-controlled gene expression. After the nisin recognition by the nisK histidine-protein kinase, this membrane receptor undergoes an auto-phosphorylation, and afterward, the nisR mediator is phosphorylated as well. Once activated, NisR acts as a signal transductor, enabling the recognition of the PnisA promoter and hence the heterologous gene expression takes place to finally produce the desired protein.

Interestingly, the Gram-positive nature of *L. lactis* also enables a straightforward protein secretion through their unique cellular membrane, simplifying the subsequent purification steps [289]. For that, plasmids such as pNZ8110 integrate the signal sequence of the major secreted protein (USp45) of *L. lactis*, fostering an effective recombinant protein secretion. Along with that, NZ9000 *htrA* strain is deficient in the only reported cell surface protease HrtA, improving, even more, the achieved protein yields [295]. Furthermore, this expression platform facilitates disulfide bond formation, and just like *E. coli*, is an easily scalable process that uses inexpensive mediums, being a versatile and efficient microbial cell factory [285, 288].

Recombinant Protein formats

In nature, protein biosynthesis is a finely regulated process that provides the required components for proper protein folding. For that, the cell holds specific molecular machinery that guarantees

the folding quality in a soluble and bioactive form of the resultant polypeptide. Nevertheless, under recombinant protein production processes in bacterial hosts, the high quantity of the overproduced recombinant protein can lead nascent polypeptides to aggregation. Hence, the well-known soluble form is not the only format in which we can find the recombinant product, but also as bacterial protein aggregates, also known as Inclusion Bodies (IBs) [296].

Inclusion bodies

Inclusion bodies are protein-based aggregates naturally formed during heterologous protein production processes (Figure 17). These aggregates have been considered for years as undesirable by-products, chiefly structured by both mis- and unfolded proteins devoid of biological activity [297, 298]. However, when the first insights of bioactive and proper folded proteins forming IBs were reported, the IBs perception radically changed [296, 299, 300]. These fully active and properly folded polypeptides are embedded in an amyloidal structure, which acts as a scaffold [297]. Thus, nowadays, IBs are defined as functional and stable nanoparticles with a diameter ranging from 50 to 800 nm, mainly composed by the overproduced heterologous protein, which have formed not only in *E. coli* [301] but also in *L. lactis* [302] and other microbial expression systems such as *Pichia pastoris* [303].

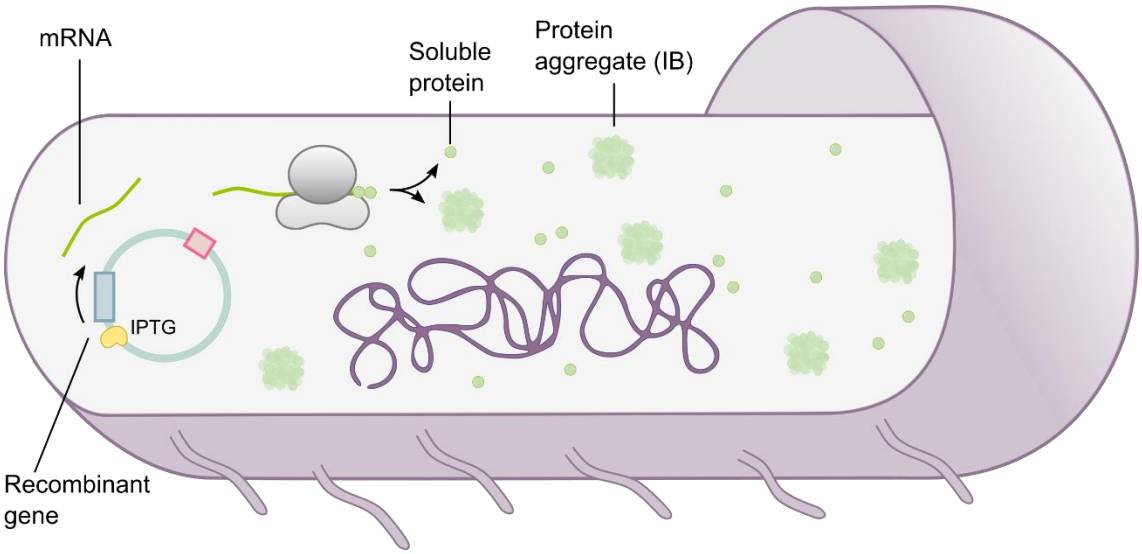


Figure 17. Inclusion bodies formation. Through the binding of the inducer (IPTG) to the plasmid promoter, the encoded downstream gene is transcribed to mRNA and then the ribosomes translate it into a polypeptide. Still, the metabolic burden produced by the overexpression of a foreign protein jointly with the surpassed quality control can lead protein to aggregate forming IBs.

IBs are particles that hold high mechanical stability together with slow-release properties, which has led to being used, once purified [304], in a wide range of applications. For instance, self-immobilized enzymes forming IBs have been exhibited as a robust alternative to classical carrier-dependent immobilized enzymes for biocatalysis processes [297]. Their intrinsic stability makes enzyme-based IBs simple to be recycled and effortlessly recovered by centrifugation [297, 305]. In addition, due to their intriguing properties, IBs have also been studied as a biomaterial. Their rough surface can mimic the natural mammal extracellular matrix, supporting cell attachment, proliferation, and hence tissue regeneration [306, 307]. Some researchers have also explored the immunostimulants properties of this adaptable biomaterial [308]. As previously mentioned, these aggregates are mainly formed by the overexpressed recombinant protein, yet, during its structuration small traces of the producer cell can be entrapped, such as nucleic acids, membrane debris, carbohydrates, and host proteins [309]. Taking advantage of this heterogenic nature, Torrealba *et al.* demonstrated that IBs have inherent immunostimulants properties, being applied effectively in aquaculture to prevent infections [308]. Although occasionally beneficial, these impurities represent a significant constriction for therapeutic approbation by regulatory authorities. In this framework, it is necessary to work on the production of next-generation "clean'' IBs. To address that, the use of aggregation tags -such as GFIL8 or ELK16- has been described as a useful approach to increase aggregation propensity of produced protein and, at the same time, diminish IBs impurities [310, 311]. Related to this, Roca-Pinilla and co-authors. proposed a novel type of aggregation-seeding domains based on protein-protein interaction through leucine zippers (LZ) [312]. In this study, the Jun and Fos LZ dimerization showed a more controlled GFP-based IBs formation that is reflected in improved protein quality, as well as the reduction of non-desirable contaminants. Going a step further, a recent study proved that *de novo* fabrication of artificial IBs, with chemically controlled components, enable free-impurity IBs that hold each of the relevant properties for their therapeutic application [313].

Remarkably, IBs are also capable to perform a natural sustained release of the embedded bioactive protein acting as a nanopill [309]. This feature confers to IBs an exceptional potential to be used in therapeutics as a drug delivery system (DDS) [314, 315]. For instance, this slow-release profile appears to be crucial in several fields, such as antibiotic-resistant bacteria fight. Recent findings pointed out that the generation of antimicrobial-based IBs enables a steady source of antimicrobial compounds, maintaining a therapeutic threshold against MDR bacteria [316]. Yet, the applications of IBs as DDS go far beyond that a clear-cut antimicrobial compound. Pesarrodona and co-workers also demonstrated that IBs can be positively applied for cancer therapy [317]. Concretely, they designed two novelty constructs conformed by the p31 protein that promotes apoptosis and Omomyc polypeptide, which hold antitumoral properties [318]. Each structure was fused to the tumor-homing peptide (FN) that exhibit a strong tropism against CD44 receptor (a

well-known described tumoral marker). Thus, both IBs were intratumorally injected in a mouse model of human breast cancer, where their promoted the destruction of $CD44^+$ cells, validating IBs as an antitumoral drug [317]. Along with that, IBs inherent amphiphilic nature promote their spontaneous uptake by mammalian cells, enabling intracellular protein release [297, 319].

Since IBs are self-assembling protein nanoclusters, largely composed of the heterologous expressed protein, they are also used as a source to obtain soluble proteins [320]. Traditionally, IBs solubilization protocols involved harsh detergents and chaotropic agents at high concentrations (6-8 M urea or guanidinium chloride) to entirely denaturalize the target polypeptide. After that, a refolding step to recover their native structure has been applied and the protein has been finally purified [321]. Still, the current understanding of its composition has led to the development of non-denaturing protocols to obtain soluble protein from these bioactive protein aggregates [322, 323]. This included the use of mild detergents (i.e., n-lauroylsarcosine) and organic solvents (i.e., *n*-propanol or isopropanol), often combined physicochemical adjustments -pH, temperature, or freeze-thaw cycles- [324-327]. These strategies enable a continuous release of the proper folded scaffold-embedded protein in a unique step, usually avoiding downstream refolding procedures [302, 323, 328].

In conclusion, IBs have widely proven to be considerably more than merely protein aggregates. Their appealing features open their applications to biomedical therapies, material sciences, and industrial fields or even as a rich source of soluble protein for its extraction.

Recombinant Antimicrobials and Immunostimulants

The development of new antimicrobials and immunostimulants arise as two possible solutions to fight against the crisis of AMR. However, production and testing of new molecules have intrinsic concerns such as high manufacturing costs, low biological stability and consequently, the need for high doses and potential toxicity must be resolved [190]. To address that, recombinant production opens a myriad of possibilities to overcome these drawbacks.

For example, the active forms of HDPs are commonly short -under 50 residues- and might be chemically synthesized. Nevertheless, their elevated production cost combined with the large volumes required during basic science studies and the clinical trial development makes chemical synthesis a poor choice [329]. In contrast, recombinant HDPs are produced at high yields with inexpensive culture media, especially when prokaryotic systems -such as *E. coli*– are used [275]. Moreover, recombinant production presents an easy scale-up procedure, adjusting working volumes according to the protein needed for each research phase.

In the case of the cytokines, their length -up to 80 residues- directly hampers chemical synthesis use as a strategy for its production. In contrast, the recombinant production of cytokines has been proven to be a feasible approach [240, 330]. Another potential drawback for the development of antimicrobials/immunostimulants and based therapies is their inherent low stability. To illustrate that, cytokines reduced half-life is a natural host protection mechanism, whereby the immune system finely regulates cytokine-mediated responses. On the other hand, HDPs stability is compromised due to their small size, facilitating their proteolytic degradation by host proteases during heterologous expression. Thus, for therapeutic applications, it is necessary to develop strategies to increase protein half-life, which will have an impact on the doses needed, the toxicity, and treatment cost. In this context, nanotechnology offers a wide range of possibilities [331-333].

Several studies have described the recombinant production of immunostimulants. Torrealba *et al*. proposed an exciting cytokine-based IBs [242]. They demonstrated that protein aggregation was an exceptional strategy to produce nanostructures in a cost-effective manner with outstanding immunostimulant properties, as well as mechanical and chemical extreme stability in this conformation. As a result, cytokine half-life was considerably enhanced, allowing the use of IBs as an efficient cytokine DDS, which provides exceptional *in vivo* immune protection [242]. In a similar context, Carratalá and co-authors designed a bovine interferon gamma (rBoIFN-γ) protein aggregates using aggregation-prone peptides (APPs) [330]. Concretely, the L6K2 addition to rBoIFN-γ leads to the structuration of rBoIFN-γ soluble nanoparticles, resulting in both production yields and biological performance improvement due to the stability of the cytokine in this nanoparticulate conformation. Moreover, other approaches demonstrated that it is also feasible to produce stable IFN-γ cytokine by protein engineering rely on disulfide bond incorporation, key amino acids substitutions, or sequence truncation, enhancing 4-folds its biological activity [334]. Going a step further, some researchers are developing a novel class of encouraging therapeutics based on antibody-cytokine fusion proteins -called immunocytokines. For that, recombinant cytokines are combined with specialized antibodies to boost their therapeutical index and reduce toxicity through antibody-dependent targeting [335, 336]. Therefore, increased stability and specificity is largely reflected in a gain of activity, administering a reduced amount of product and thus improving both tolerability and treatment cost.

In the case of antimicrobials based on HDPs, non-natural or D- amino acids introduction can substantially increase proteolytic stability, though this method is not compatible with biological expression systems [337]. Instead, the use of carriers in recombinant production is a widespread method largely reported in the literature [263, 338, 339]. Remarkably, these carriers protect labile peptides from proteolytic degradation, as well as mask HDPs toxicity towards the producer bacteria [340]. Some well-known examples of fusion carriers are the thioredoxin (commonly used

for defensins expressions), glutathione S-transferase (GST), small ubiquitin-related modifier (SUMO), and PurF fragment [341, 342]. Likewise, the HDPs production as IBs have also demonstrated to be a compelling strategy to overcome host toxicity issues, being HDP-based IBs an interesting format to be used either as antimicrobial itself or as an intermediate step to purify otherwise toxic soluble HDP [316]. On the other hand, in the case of cationic antimicrobial peptides, in similar fashion to cytokines, it has been reported that the structuration of soluble nanoparticles is an excellent approach to enhance its stability and hence biological activity [330, 343].

Even though for a first exploration proteins co-expressed with carriers are great alternatives, in clinical they are generally risky due to unspecific interactions or effects. In this context, several alternatives to remove carriers have been raised, such as enzymes or pH-dependent cleavage [344]. Still, a novel approach based on domains combination has demonstrated to be a powerful strategy to achieve high stable antimicrobial protein underpinned in HDPs [316].

This multidomain protein concept is not a novelty in nature, where this type of structuration predominates in the genomes from all three kingdoms of life, particularly 70% in eukaryotic polypeptides [345]. Concretely, domains have been defined as conserved, functionally independent protein sequences, which are self-stabilized and commonly fold independently [346]. Therefore, in natural molecular evolution, domains are used as building blocks, which can be combined to generate proteins with different functions across species.

Mimicking how nature works, throughout the DNA recombinant technology is possible to combine protein domains of multiple origins and engaging characteristics (i.e., antimicrobial properties) to create recombinant proteins with a plethora of possibilities. Briefly, the DNA sequence that encodes for each protein domain can be assembled synthetically in the needed order with a particular tandem domains [347]. A recent study illustrates how the combination of three peptidoglycan hydrolases derived from bacteriophages enhances antimicrobial activity against *S. aureus* [348]. Another research group investigated the recombinant expression of a cecropin B, a strong cationic antimicrobial, combined with the sericin, a natural protein biopolymer also with antimicrobial features [349]. The outcomes demonstrated that the chimeric sericin-cecropin exhibits a better antimicrobial performance against *E. coli* and *S. aureus* rather than its individual counterparts.

Bearing this in mind, the next generation of fully tunable, versatile, and enhanced antimicrobial multidomain polypeptides can be created by the combination of these ‘’Lego bricks’’ as desired [316]. Moreover, the size of the resultant recombinant protein makes it less susceptible to proteolytic degradation than producing small peptides recombinantly, and thus the presence of a protein carrier is not needed anymore [316]. A recent study of our group demonstrated that

multidomain antimicrobial proteins can be recombinantly produced in *E. coli*, showing bactericidal and anti-biofilm activity against AMR *K. pneumoniae, E. coli, Enterococcus spp.*, and *E. faecalis* [316]. Briefly, this study described the construction and production of the JAMF1 multidomain protein, which is formed through the combination of HD5, human $sPLA_2$, as well as the gelsolin-based bacterial binding domain and two leucine zippers domains (Jun and Fos) to promote aggregation [316]. Hence, the myriad of combinations that these multidomain compounds can hold provides a vast array of approaches on a unique molecule to address AMR.

CLINICAL APPLICATION

The last and more decisive step in the development of any therapeutic compound is its clinical validation. In the case of the recombinant AMPs, although numerous products have achieved advanced clinical trials, most of them have not been approved due to lack of efficiency in contrast with current treatments [83, 202]. Concerning immunostimulants based on cytokines, their scenario is slightly better, since several recombinant cytokine-based products have received marketing approval. Some examples are recombinant IL-2 (Proleukin) for metastatic renal cell carcinoma and melanoma treatment, recombinant IL-11 (Neumega) for chemotherapy-induced thrombocytopenia, recombinant IFN-α (Roferon-A and Intron-A) with antitumor and immunostimulant properties, TNF-α (Beromun) anti-tumoral or recombinant GM-CSF (Sargramostim) for leukemia and stem cell transplants [240, 350-352]. However, there are still a broad number of applications requiring the development of new antimicrobial and immunostimulant molecules.

At length, in animal production, diseases associated with stress periods, such as transport and weaning, are one of the main long-standing concerns that remain unresolved and where immunostimulants have emerged as an encouraging alternative. On the other hand, in human health, nosocomial infections are a serious challenge for the healthcare systems and could be tackled with the use of antimicrobial peptides. In addition, it is noteworthy to mention that those approaches suitable for animal production are potentially transferable to human health and vice versa.

Animal Production

Livestock sector has undergone fast-growing, which contributes 40% of the global value of agricultural output [353]. Still, frequently misuse and overuse of antibiotics in growth promotion, disease prophylaxis, or inadequate treatment have driven livestock to an unsafe path [354]. In some countries, around 80% of sold antibiotics are intended for animal agriculture [355]. Hence, the development of new treatments, fast diagnosis, alternative management, and nutritional strategies are needed to achieve the One Health standards. In this scenario, immunostimulants and new antimicrobials could considerably improve animal health and welfare.

Throughout farm animal production cycle, the transport between farms/ processing centers and the weaning process is associated with high stress levels, immunosuppression, metabolic dysregulation, and subsequent disease incidence [356, 357]. During the weaning period, significant nutritional adjustments are imposed on the animal, causing behavioral and

physiological alternation [357]. As a result, associated diarrhea can be triggered in part by enterotoxigenic bacteria and eventually may lead to mortality in cattle and piglets [358]. On the other hand, the Food and Agriculture Organization (FAO) describes animal transport as the perfect situation for spreading disease, where exhausted animals from different herds are confined together for long times in an inadequately ventilated and stressful atmosphere. Moreover, this prolonged transport also triggers a variety of respiratory diseases generated by endogenous microorganism that normally are not pathogenic for the host, but can be opportunistic under immunosuppression [359]. Consequently, the application of antibiotics and other therapeutics compounds was required, fostering AMR emergence. In addition, due to the lack of rapid diagnosis systems, and as a prophylactic measure, the entire herd have been frequently treated with antibiotics, worsening even more AMR [354, 360]. To address that, the use of immunostimulants to boost the immune system in challenging situations, such as transport and weaning, could prevent bacterial-associated diseases and hence antibiotic consumption.

Previous work in zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*) demonstrated the potential of bacterial IBs as protecting immunostimulants [242], which could be also transferred to livestock mammals. Within this general framework, recombinant cytokine-based IBs produced in *L. lactis* might be administered in specific scenarios to reinforce the animal immune system. Furthermore, due to the outstanding IB mechanical and chemical stability, they can be orally administrated [309, 361]. Indeed, other authors have explored as a proof-of-concept the immunostimulant potential of both INF-γ-based IBs and IBs formed by INF-γ fused with a cationic antimicrobial peptide (GWH1) in a mastitis mice model [362]. The results presented in this research indicate that both constructs decrease *E. coli* burden in the mammary gland, pointing out that direct immunostimulation or synergic antimicrobial/immune regulation are fitting approaches to cope with disease-causing pathogens.

Although recombinant immunomodulators approaches have been commonly explored in humans [240, 350, 351] still need some development for feasible animal applications, covering both the economic and field requirements.

Nosocomial infections

Nosocomial infections, also referred as healthcare-associated infections (HAI) are diseases acquired by patients during hospitalization [363]. Roughly one out of ten patients develop HAI, contributing to greater morbidity, mortality (doubled in the UCI patients with HAI [364]), extended hospital stays, and subsequent healthcare financial burden [365, 366]. In addition, due to the high prevalence of antibiotics treatment in hospitals, a significant number of HAI pathogens are dangerous MDR, among them the ESKAPE pathogens MRSA, *P. aeruginosa*, *Acinetobacter*

baumannii, or carbapenem-resistant *Klebsiella pneumoniae* [24, 367]. These pathogens may have several routes of transmission categorized as follows: surgical site infections (SSI), ventilator-associated pneumonia (VAP), central line-associated bloodstream infection (CLABSI), and catheter-associated urinary tract infection (CAUTI). In addition, although each pathway of transmission has distinct nature and thus associated pathogens, all of them generally display a common factor -the preexistence of a biofilm [24, 368].

Associated biofilm infections

Biofilms have been defined as a consortium of microorganisms associated within a surface and embedded in a self-produced matrix of polymeric substances [369]. Any surface, whether biotic or abiotic, are susceptible to biofilm foundation, indicating the ubiquitous nature of this complex macrostructure and the intricacy to eradicate them [370]. Furthermore, more than 60% of all microbial infections in humans are ascribed to preexisting biofilm formations [371]. In nosocomial infections, biofilms are one of the first clinical reasons of pathogenesis through device-related contaminations, infections on body surfaces or fomites [24, 372] (defined as a passive vector that, when is infected or exposed to virulent agents can transmit disease to another host, such as skin cells or bedding in the hospitals).

A rational comprehension of how biofilms are established is essential to design a better preventive approach. First, a conditioning film is generated due to the interaction of proteinaceous compounds with biotic or abiotic surfaces. Then, the conditioning film along with microbial features from planktonic pathogenic bacteria (i.e., pili, flagella, or fimbriae), results in microorganism attachment and biofilm foundation. After that, the self-production of insoluble extracellular polymeric substances (EPS) enables colonies growth, and the maturation of the biofilm takes place. Lastly, biofilm can detach part of their complex structure and thus colonize other surfaces, starting a new cycle [373, 374] (Figure 18).

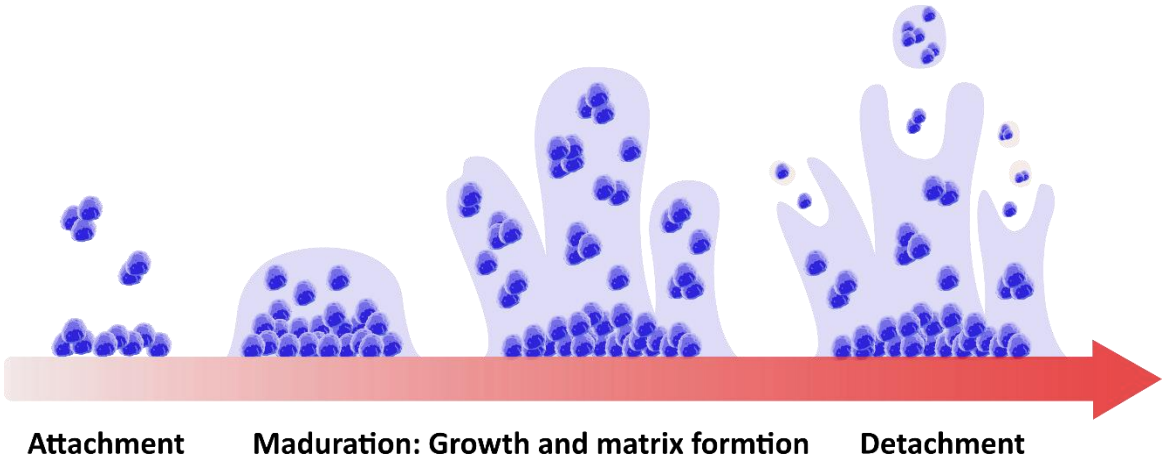


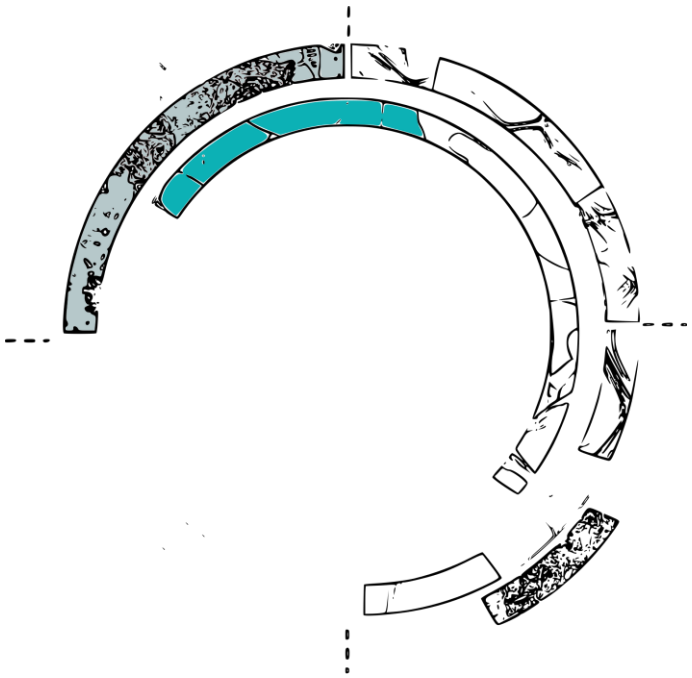
Figure 18. Development of a biofilm. Planktonic cells attach first to a conditioned surface, which leads to a primary colony formation. Then, cell-to-cell communication and quorum sensing signals resulted in the expression of biofilm-specific genes, driving the secretion of EPS and subsequent biofilm maturation. In the last stage, biofilm detachment provokes the shift of sessile cells to the motile form, being these spread to colonize a new surface.

Worryingly, these bacteria communities entrapped into the extracellular matrix have shown increased resistance to antimicrobial agents -up to 1000-fold [370]. Because of their slow growth, antimicrobial poorly diffusion in the EPS, common HGT events, and bacteria phenotypic modifications, antibiotics, or other therapeutic compounds turn out completely ineffective [375]. Besides, biofilm may also exist in a vast proportion of infections and not just in nosocomial ones, making their control and eradication even more crucial [370].

In this framework, the HDPs exhibit promising antibiofilm activities. They can act in the different stages of biofilm formation with several mechanisms of action, either inhibiting bacterial adhesion and quorum sensing or disrupting preexistent biofilms [376], even in MDR bacteria. Yet some researchers have examined the coating of susceptible biofilm formation surfaces [377, 378]. Yu *et al.* described a polymer brush layer with encouraging non-fouling and flexible qualities for peptide conjugation, which prevented bacterial adhesion up to 99.9% and inhibited planktonic growth by 70% [377]. Moreover, *in vivo* studies showed how coated catheters reduce bacterial adhesion by more than 4 logs and planktonic growth by 3 logs concerning conventional catheters [377]. Another study applied a new hybrid HDP called melamine, which is created by the combination of melittin and protamine. Interestingly, this peptide is stable to heat sterilization and it was tested in contact lenses, reducing considerably bacterial adhesion of *Staphylococcus* and *Pseudomonas* strains [378]. Among all the reviewed HDPs, the cathelicidin LL-37 displays an exceptional antibiofilm activity. The LL-37 can inhibit *P. aeruginosa* biofilms at 0.5 μg/mL, far

below the 64 μg/mL of planktonic cells minimal inhibitory concentration (MIC) and disperse preformed biofilms as well. To accomplish that, LL-37 down-regulates genes related to flagella and quorum sensing, hampering biofilm development [376]. Moreover, based on its natural antibiofilm properties, enhanced synthetic derived forms, such as peptide 1037, DJK-5, or LL7-31 have been engineered to address *Listeria monocytogens, E. coli, A, baumannii, K. pneumoniae*, and *Salmonella enterica* [379, 380]. Chereddy and co-authors also demonstrated that LL-37 can be incorporated into nanoparticles [381]. In this study, a poly lactic-co-glycolic acid (PLGA) has been used as a carrier for LL-37 sustained delivering, increasing, antimicrobial efficacy, wound healing, and neovascularization and thus blocking opportunistic biofilm instauration.

To conclude, other alternative anti-biofilm strategies based on AMPs seek to simulate a natural AMP-based system, where host immunity uses a combination of active molecules rather than a single approach. For example, Gordya *et al.* examined the activity of a natural assemblage of AMPs produced by maggots of *Calliphora vicina* [382]. The complex was discovered to contain AMP from defensins, cecropin, diptericin, and proline-rich peptides. Altogether, it exhibits a noteworthy cell killing and matrix destroying against *E. coli, A. baumanni*, and *S. aureus* biofilms, as well as lack of toxicity to human cells. Therefore, mimicking how natural AMP works, still using a single molecule, the domains for the recombinant HDP may rationally be chosen to tackle as efficiently as possible the potential threat in an adaptable approach also suitable for MDR bacteria.



Objectives

This work aims to develop new immunostimulant and antimicrobial molecules to overcome the threat of antibiotic resistance. On the one hand, it seeks to explore the potential of a new generation of recombinant antimicrobial proteins based on Host Defense Peptides (HDPs) as broad-spectrum therapeutics against antibiotic resistant bacteria. Complementarily, this thesis also aims to study the potential of cytokine-based nanoparticles (IBs) as immunostimulants to improve the resilience of animals during critical production stages.

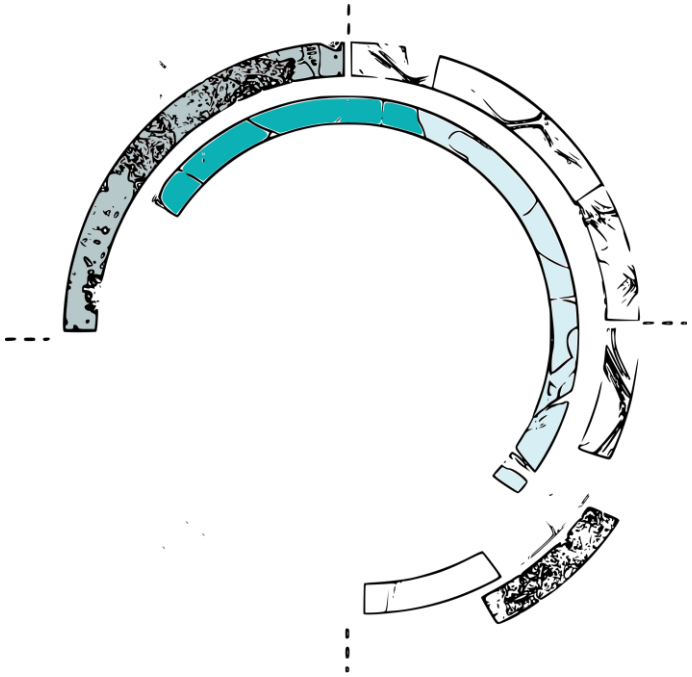
To accomplish our general objective, the following specific steps have been addressed:

1. To analyze the impact of the recombinant *Escherichia coli* strain on the production and bactericidal activity of both soluble and nanostructured α-defensin HD5 and β-defensin LAP, fused to GFP and using *E. coli* BL21 and Origami B strains as microbial cell factories. (Study 1)

2. To establish a general and optimal protocol for the purification of HDP-based proteins from IBs, comparing protein quality and bactericidal activity of HDP purified from the soluble fraction or by non-denaturing IBs solubilization protocols (Study 2)

3. To develop and analyze a new approach for the generation of multidomain antimicrobial proteins with improved bactericidal and anti-biofilm activity through the combination of different HDPs in a single polypeptide. (Study 3)

4. To explore the stability and *in vitro* and *in vivo* immunomodulation capacity of cytokine-based IBs produced in *L. lactis* GRAS recombinant platform. (Study 4)



Results

STUDY 1

EXPLORING THE IMPACT OF THE RECOMBINANT *ESCHERICHIA COLI* STRAIN ON α- AND β-DEFENSIN ANTIMICROBIAL ACTIVITY: BL21 VERSUS ORIGAMI STRAIN

Adrià López-Cano, Marc Martínez-Miguel, Imma Ratera, Anna Arís* and Elena Garcia-Fruitós*

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Preface

The treatment of bacterial-related infections is increasingly challenging by the swift expansion of antimicrobial resistant microorganisms, where firm actions must be taken right now in a coordinated, harmonized, and transdisciplinary manner as pursues the One Health approach. To address that, several research groups have been investigated encouraging alternatives to effectively face off resistant bacteria, being the host defense peptides (HDPs) one of the most promising options. HDPs are short, cationic, and amphipathic peptides with dual activity as antimicrobials and immunostimulants, widely labeled as natural antibiotics with proven efficiency against antimicrobial resistant bacteria. Moreover, several of these peptides comprise post-translational modifications, such as disulfide bond formation, which have been taken into account when produced as new antimicrobial molecules.

HDPs production has been predominantly carried out by chemical synthesis, where the high associated cost and technical limitations largely constrain their *in vivo* applications. Hence, this first study aims to explore the feasibility of the recombinant production of two relevant HDPs (human α-defensin 5 (HD5) and the bovine β-defensin lingual antimicrobial peptide (LAP)) and, on the other side, to evaluate their stability and performance when produced under both reducing and oxidizing cytoplasmic conditions using two *E. coli* strains. To our knowledge, disulfide bridges formation has been thoroughly explored in chemical synthetized HDPs, but much remains to do in recombinant production. Therefore, this research will provide us a better comprehension of the disulfide bridges role in recombinant HDPs and determine the impact of the producer strain on the HDPs conformation. Overall, we seek to establish the optimal production conditions in terms of redox environment to further produce all antimicrobial candidates used in this thesis.

EXPLORING THE IMPACT OF THE RECOMBINANT *ESCHERICHIA COLI* STRAIN ON α AND β-DEFENSIN ANTIMICROBIAL ACTIVITY: BL21 VERSUS ORIGAMI STRAIN

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Abstract

The growing emergence of microorganisms resistant to antibiotics has prompted the development of alternative antimicrobial therapies. Among them, the antimicrobial peptides produced by the innate immunity, which are also known as host defense peptides (HDPs), hold a great potential. They have shown to have activity against both Gram-positive and Gram-negative bacteria, including those resistant to antibiotics. These HDPs are classified into three categories: defensins, cathelicidins, and histatins.

Traditionally, HDPs have been chemically synthesized, but this strategy often limits their application due to the high associated production costs. Alternatively, some HDPs have been recombinantly produced, but little is known about the impact of the bacterial strain in the recombinant product. Since defensins have 3 disulfide bonds, this work aimed to assess if the *Escherichia coli* strain used as cell factory determine the activity and stability of recombinant HDPs. For that, an α-defensin (human α-defensin 5 (HD5) and β-defensin (bovine lingual antimicrobial peptide (LAP)) were produced in two recombinant backgrounds: *E. coli* BL21

strain, which has a reducing cytoplasm, and *E. coli* Origami B, being a strain with a more oxidizing cytoplasm. The first results showed that both HD5 and LAP fused to Green Fluorescent Protein (GFP) were successfully produced in both BL21 and Origami B strains. However, differences were observed in the HDP production yield and bactericidal activity, especially for HD5-based protein. The HD5 protein fused to GFP was not only produced at higher yields in the *E. coli* BL21 strain, but it also showed a higher quality and stability than that produced in the Origami B strain. Hence, this data showed that the strain had a clear impact on both HDPs quantity and quality.

Background

Infections caused by antibiotic resistant (AMR) bacteria are continuously growing and available drugs for their treatment are limited and, in some cases, nonexistent [1, 2]. The current situation has led the World Health Organization (WHO) to declare AMR as one of the top 10 global public health threats facing humanity [3]. To tackle this global challenge affecting both human and animal health, different groups are working on the generation of alternative antimicrobial therapies, including phage therapy [4], lysins [5], probiotics [6], antibodies [7], or antimicrobial proteins [8]. Among them, host defense peptides (HDPs) outstand for their broad-spectrum bactericidal activity [9, 10]. HDPs are short, cationic peptides which are naturally produced by the innate immunity of organisms of all life forms, being key molecules in the prevention of infections [11-13]. Besides, their fast and multiple mechanisms of action hamper the development of resistances [14-17].

The different HDPs have been classified into three groups: defensins, cathelicidins, and histatins [18, 19]. Defensins are one of the most remarkable group, widely distributed in animals and plants. Whereas invertebrate and plant defensins contain a common structure comprising an α-helix linked to a β-sheet by two disulfide bridges (CSαβ-motif) [20], mammalian defensins are characterized by an antiparallel β-sheet structure, stabilized by three disulfide bonds [13]. In addition, mammalian defensins are divided into α- and β-defensins, which mainly differ in length, location, and connectivity of their three pairs of intramolecular disulfide bonds, as well as in their unique consensus sequences [21]. The α-defensins, which are mainly produced by neutrophils and Paneth cells in the small intestine, are 29-35 residues long, containing six cysteines which are linked as follows: C1-C6, C2-C4, and C3-C5 [22-26], whereas β-defensins produced by epithelial cells are 38-42 residues long with C1-C5, C2-C4, C3-C6 pairs forming disulfide bonds [24-27]. The conserved cysteines of defensins have led to the conclusion that correct disulfide bond

formation could be critical for biological activity, structuration, and stability of these peptides [28].

Most studies done with defensins have used synthetic forms of these peptides. However, some of them have been also recombinantly produced [29-32]. Unlike chemically synthesis, recombinant production of peptides is an efficient and fully scalable process with no limits in peptide length [33-36]. Usually, when using the recombinant production strategy, defensins (and in general HDPs) are fused to carrier proteins to avoid proteolysis [37] and minimize the toxicity of these short peptides [38-40]. Although different production strategies have been explored to optimize defensins production, little is known about the disulfide bond formation of HDPs under recombinant conditions. This is particularly relevant in bacterial hosts and more specifically in *Escherichia coli*. *E. coli* has a reducing cytoplasmic environment maintained by the glutaredoxin and thioredoxin pathways, that hampers disulfide bond formation [41, 42]. Some groups have used commercial *E. coli* strains such as Origami (Novagen), in which the thioredoxin reductase (trxB) and glutathione reductase (gor) genes are deleted, to produce defensins in a more oxidizing environment. For example, Wang and coworkers have compared the production of human α-defensin 6 (HD6) in *E. coli* Bl21 and Origami strains, determining that higher production yields are reached when using Origami [43]. Other authors have proven that defensins produced in *E. coli* Origami are active against different pathogenic strains [44, 45]. However, any comparison of the quality (activity) of defensins produced in these two strains has been evaluated so far. Thus, in this study, we have determined the production yields and activity of one α- defensin and a β-defensin recombinantly produced in both oxidizing and reducing *E. coli* cytoplasm along. For that, we have used the soluble form of the human α-defensin 5 (HD5) and the β-defensin lingual antimicrobial peptide (LAP), but also the aggregated protein forming inclusion bodies (IBs). IBs are mechanically stable protein-based nanoparticles formed during recombinant protein production processes [46]. These aggregates have already been shown to be a low-cost drug delivery system for different applications including biomedicine, biocatalysis [47, 48], and also for antimicrobial therapy [49].

Results

Two different defensins, the human α-defensin 5 (HD5) and the β- defensin lingual antimicrobial peptide (LAP) were selected to perform this study (Table 1). Both HDPs, which are peptides with hydrophobic regions as well as positively charged amino acids, have been fused to Green Fluorescent Protein (GFP) as protein carrier.

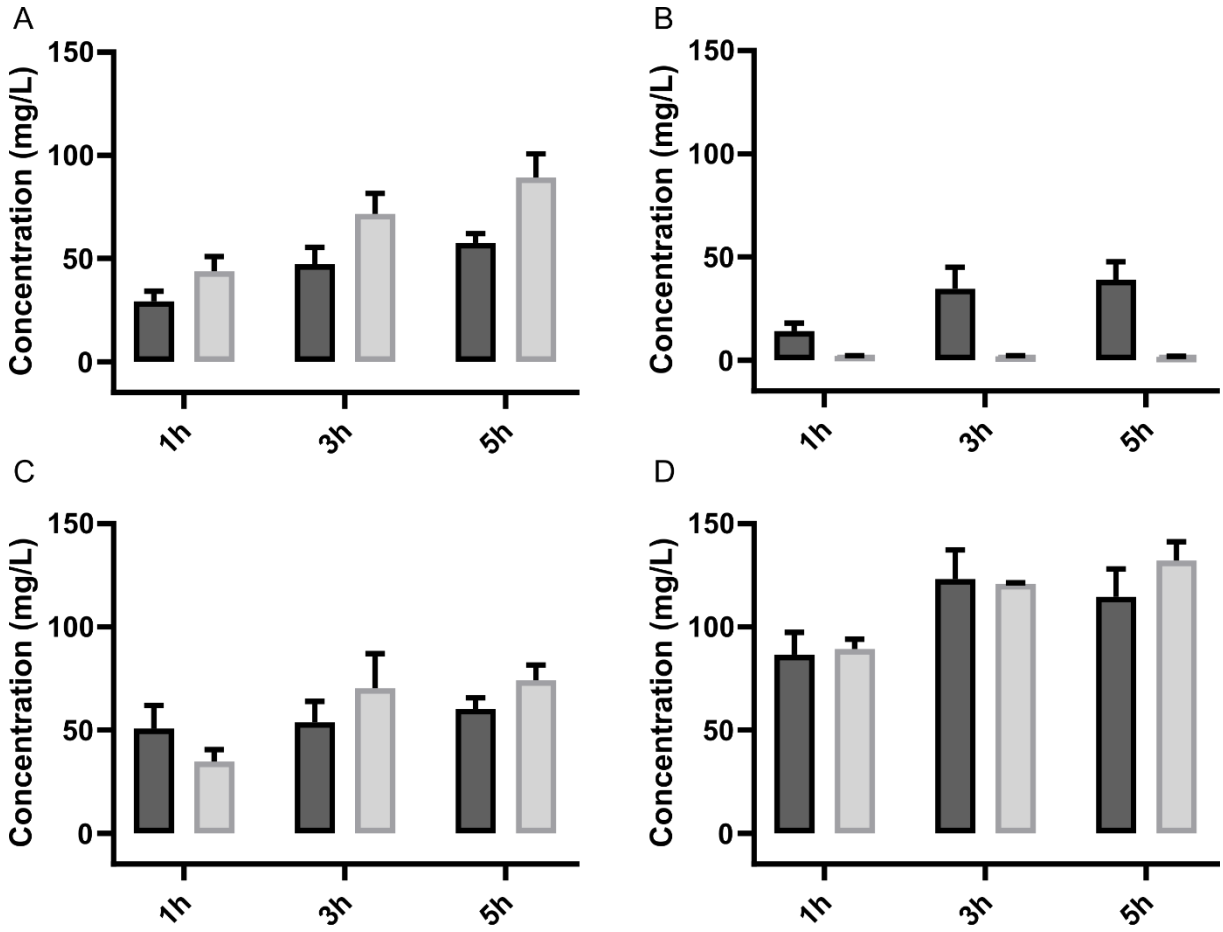
Table 1. LAP (V25-K64) and HD5 (A63-R94) sequences with the disulfide cysteine pairing. The proportion of hydrophobic residues, peptide M.W, net charge, and pI are also shown. HD5: human defensin 5; LAP: lingual antimicrobial peptide; M.W: molecular weight; pI: Isoelectric point.

Peptide	Sequence	Total residues (Hydrophobic)[a]	M.W (KDa)	Net charge	pI[b]
LAP β-defensin	VRNSQSCRRNKGICVPIRCPGSMRQIGTCLGAQVKCCRRK	40 (21)	4.46	+10	10.85
HD5 α-defensin	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR	32 (16)	3.59	+4	8.96

[a] The number of hydrophobic residues includes amino acids with aliphatic side chains.

[b] pI was theoretically calculated according to Expasy ProtParam tool.

Both HD5-GFP-H6 and LAP-GFP-H6 defensins were successfully produced in *E. coli* BL21 and Origami B strains, although the production profile was different depending on the HDP and the strain used (Figure 1A and B). In both cases, the proteins were produced soluble (Figure 1A and B) and insoluble (Figure 1C and D), but the aggregation ratio was higher for HD5-GFP-H6 and, especially when using the Origami B strain (Figure 1C). Soluble LAP-GFP-H6 had similar levels of production in both BL21 and Origami B strains, being in both cases time-dependent ($P<0.0001$) (Figure 1A top). By contrast, the production kinetics of HD5-GFP-H6 showed that the soluble form is produced at higher levels in BL21 than in the Origami B strain (Figure 1B top; $P= 0.040$). However, the aggregated form of both LAP-GFP-H6 and HD5-GFP-H6 showed no differences between strains (Figure 1 bottom).



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HDP	Aggregation ratio		*p*-value
	BL21	Origami	
LAP-GFP-H6	55.96 ± 3.79	46.39 ± 1.60	0.0586
HD5-GFP-H6	79.59 ± 3.39	98.37 ± 0.28	0.0062

Figure 1. Production kinetics and distribution of soluble (top) and IBs (bottom) of LAP-GFP-H6 (A, C) and HD5-GFP-H6 (B, D) antimicrobial proteins in mg/L culture at 1, 3, and 5 h in *E. coli* BL21 (dark grey) and Origami B (light grey) strains. The ratio of aggregation (at 3h) for each HDP and strain is indicated in Table (E).

Taking 3 h as the optimal production time, the two defensins were produced and purified in their soluble form and the antimicrobial activity was tested against two bacterial pathogens (Figure 2). Both defensins significantly reduced methicillin resistant *Staphylococcus aureus* -MRSA- (Figure 2A) and *Pseudomonas aeruginosa* (Figure 2B) survival, reaching values of survival decrease of up to 99% in both organisms. Comparing the activity of the proteins produced in a reducing environment (BL21 strain) and under more oxidizing conditions (Origami B strain), no differences were observed for LAP-GFP-H6 (Figure 2). However, HD5-GFP-H6 produced in

BL21 showed a higher bactericidal effect against both MRSA (Figure 2A) and *P. aeruginosa* (Figure 2B) than that produced in an oxidizing environment.

Aiming to analyze the protein quality also of the insoluble protein fraction of LAP-GFP-H6 and HD5-GFP-H6, bacterial IBs produced in both BL21 and Origami B strains, were purified, and their activity was also tested. The results shown in Figure 2C and 2D proved that defensin-based IBs had also antimicrobial activity to levels that are comparable with the soluble fraction (Figure 2A and 2B). As observed with the soluble form, LAP-GFP-H6 had the same activity against MRSA regardless of the producer strain, and HD5-GFP-H6 IBs showed higher bactericidal activity when they were produced in a reducing environment (BL21 strain) (Figure 2C, D).

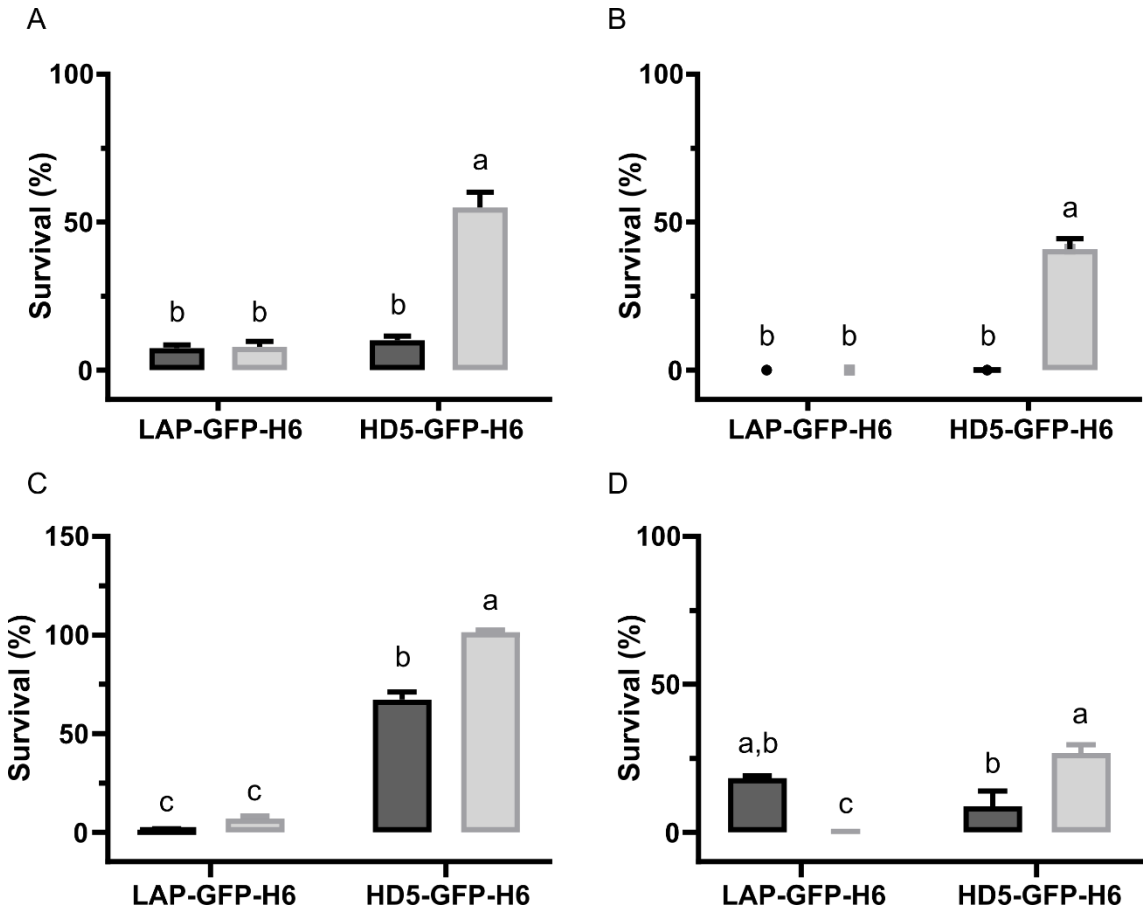

Figure 2. Bacterial survival of MRSA (A, C) and *P. aeruginosa* (B, D) in the presence of 5 μM of soluble LAP-GFP-H6 (A) and HD5-GFP-H6 (B) and insoluble (IBs) LAP-GFP-H6 (C) and HD5-GFP-H6 (D) produced in *E. coli* BL21 (dark grey) and Origami B (light grey). Different letters depict differences between proteins and producer strain (A) P=0.0024; (B) P<0.0001; (C) P=0.0108; (D) P=0.094.

The analysis of the presence of free cysteines in LAP-GFP-H6 and HD5-GFP-H6 produced in *E. coli* BL21 and Origami B strains revealed some differences (Figure 3). Surprisingly, both soluble HD5-GFP-H6 and HD5-GFP-H6 IBs had more free cysteines when using Origami as producer

strain than with BL21 strain (Figure 3). In the case of LAP-GFP-H6, no differences were observed between the protein produced in both strains, neither in the soluble form (Figure 3A) nor the IBs (Figure 3B).

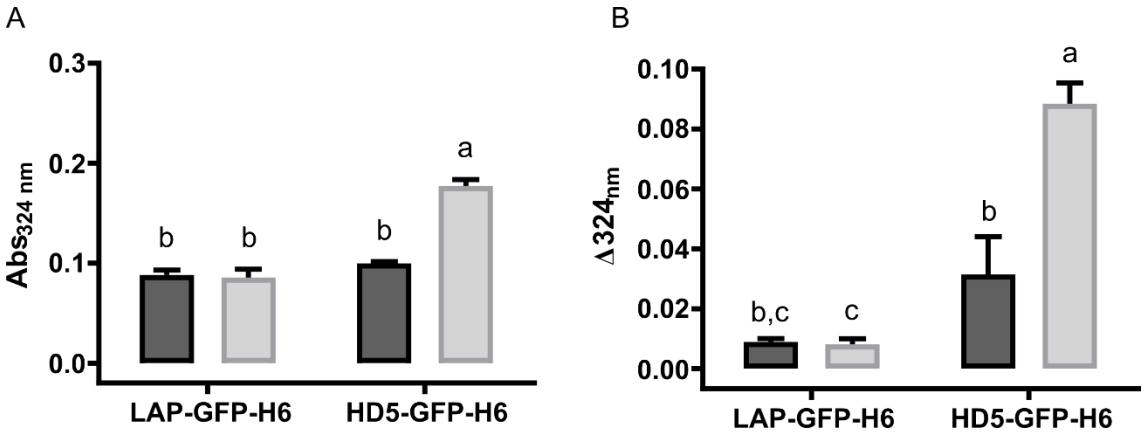

Figure 3. Analysis of free-cysteines in soluble **(A)** and insoluble (IBs) **(B)** LAP-GFP-H6 and HD5-GFP-H6 produced in *E. coli* BL21 (dark grey) and Origami B (light grey). Different letters depict differences between proteins and strains **(A)** *(P*=0.0008) **(B)** *(P*=0.0345).

In terms of protein stability, the analysis of soluble LAP-GFP-H6 and HD5-GFP-H6 at 37ºC showed that the producer strain had an impact on protein stability of the alfa-defensin (Figure 4B), while LAP-GFP-H6 performance was not affected by the background of the producer cell (Figure 4A).

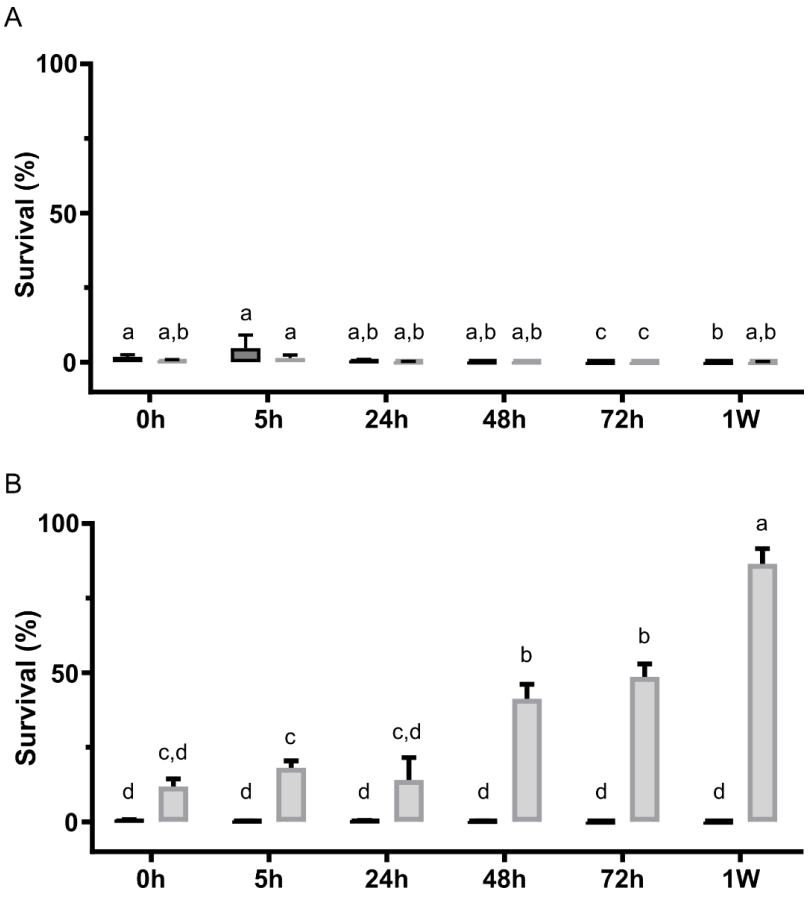

Figure 4. Antimicrobial activity of soluble (A) LAP-GFP-H6 and (B) HD5-GFP-H6 against *P. aeruginosa* at 5 μM after a 0, 5, 24, 48, 72 h and 1-week incubation at 37 ºC. Dark grey bars represent the HDPs produced in *E. coli* BL21 and light grey bars represent proteins from *E. coli* Origami B strain. Different letters indicate significant statistically differences between proteins and producer strains (A, B) $P<0.0001$. W: week.

Since all the defensins were fused to GFP as a reporter, we evaluated if specific fluorescence (Figure 2) could be correlated with the antimicrobial activity of both soluble (Figure 5A) and insoluble (Figure 5B) versions of the HDPs used. However, the results showed that there was no correlation between both parameters.

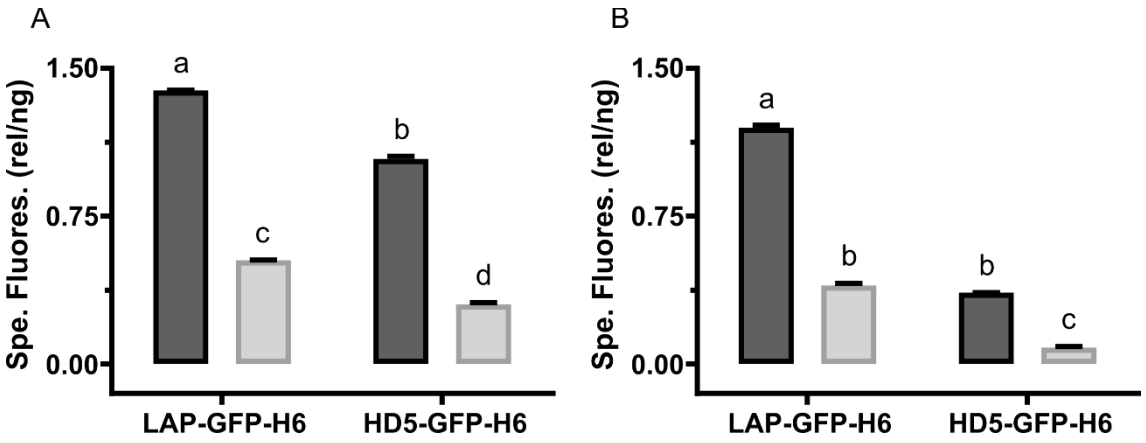

Figure 5. Specific GFP fluorescence (relative fluorescence units per ng of peptide) of soluble (**A**) and inclusion bodies (**B**) LAP-GFP-H6 and HD5-GFP-H6 produced in *E. coli* BL21 (dark grey) and Origami B (light grey). Different letters indicate statistical differences between proteins and strains **(A)** *(P<0.0001*) **(B)** *(P<0.0001*).

Discussion

The bactericidal capacity of defensins, and in general of HDPs, has aroused the interest of the scientific community for these short peptides [13, 50]. They have proven to have broad-spectrum activity against Gram-positive and Gram-negative bacteria, including against MDR microorganisms, making them a promising alternative to antibiotic therapy [51, 52]. Structurally, HDPs have 6 cysteines that form 3 conserved disulfide bonds. Different articles, in which chemically synthesized peptides have been used, reported contradictory information regarding the importance of disulfide bond formation in HDP bactericidal activity [53-58]. But in terms of recombinant protein production, little is known about the impact of the producer strain in the HDPs antimicrobial activity. Classical *E. coli* strains such as BL21 used as recombinant cell factories have a reducing cytoplasm, while the mutant strain *E. coli* Origami has an oxidizing intracellular environment which should favor disulfide bond formation [42]. Aiming to explore if the cytoplasmic environment of *E. coli* strains should be considered for the recombinant production of HDPs, in this work, we have studied the production and activity of two HDPs (an α- and a β-defensin) in two different cytoplasmic environments. The results proved that both production yields and protein activity are determined by the bacterial strain used, but also by the peptide (Figure 1 and 2). Whereas the β-defensin LAP fused to GFP was well produced (Figure 1A) and showed comparable activities when using both BL21 and Origami B strains (Figure 2), HD5-GFP-H6 showed significant differences when produced in the two different bacterial backgrounds (Figure 1B and Figure 2). The soluble form of the HD5-GFP-H6 showed a decrease in the production yields (Figure 1B top) and also a lower bactericidal activity when using an *E.*

coli strain with an oxidizing environment (Origami B) (Figure 2A, B). The greater activity of the soluble α-defensin produced in the BL21 strain against both Gram-positive and Gram-negative microorganisms indicated that, contrary to expectations, this strain produces a protein with better conformational quality than that produced by Origami B strain (Figure 2 A, B). This result matches with the free cysteine profile observed in Figure 3 A. The number of free cysteines when comparing the HD5-GFP-H6 produced in *E. coli* BL21 and Origami B strains is higher in the second case (Figure 3A), which is the protein that showed lower antimicrobial activity against the two pathogenic microorganisms tested (Figure 2A and B) and also lower stability (Figure 4). This data supports the findings published by other authors that described the importance of disulfide bond on α-defensins stability. Tanabe et al. and Maemoto et al. reported that the disruption of disulfide bonds of HD5 and mouse α-defensin cryptdin-4, respectively, increased peptide propensity to be proteolyzed and, in consequence, the activity of these peptide variants decreased [54-56]. Thus, this shows that disulfide bonds have an important role in protein stabilization. The protein stability analysis also showed that all the HDPs with low free-cysteines produced are highly stable, keeping the bactericidal activity for at least 1 week at 37ºC (Figure 4). This data is highly relevant in terms of applicability and storage of these bactericidal peptides.

In the same line, when the protein aggregates (IBs) were analyzed, we could observe that even though in all the cases IBs were formed (Figure 1 bottom and Table 1), the activity of HD5-GFP-H6 was again significantly higher when produced in BL21 strain (Figure 2C and D). In this context, it is interesting to underline that both soluble (Figure 2A and B) and insoluble proteins (Figure 2C and D) have the same behavior in terms of protein activity. This is in line with a previous publication in which it was described that protein conformational quality of both soluble and insoluble (IB) fractions takes place in parallel and those factors affecting the conformational protein quality of the soluble form also affect the IBs [57].

Besides, this study has also proven that the use of GFP is a good carrier protein for the production of HDPs, as other proteins such as thioredoxin, glutathione S-transferase (GST), small ubiquitin-related modifier (SUMO), or PurF fragment [58]. Indeed, GFP did not just protect the resultant HDP-based proteins from proteolytic degradation but also makes it easier to track the proteins during the whole production and purification process. However, the results shown in Figure 5 indicated that this fluorescent protein cannot be used as antimicrobial activity reporter, since the differences observed in bactericidal activity (Figure 2) did not correlate with differences in fluorescence emission (Figure 5).

Conclusions

This study proved that the strain used for the production of HDP-based proteins had an impact on both the production yields and the protein quality, being *E. coli* BL21 strain the strain with an optimal background for the recombinant production of HDPs.

Methods

Bacterial strains and medium

Escherichia coli BL21 (DE3) and Origami B (Tet^R, Kan^R) strains were used for heterologous protein expression. For the antibacterial assay, the strains used were *P. aeruginosa* (ATCC-10145) and methicillin resistant *S. aureus* (MRSA. ATCC-33592). *E. coli* strains were grown in Luria-Bertani (LB) medium, whereas *P. aeruginosa* and *S. aureus* were grown in Brain-Heart Infusion (BHI) broth (Scharlau, Barcelona, Spain).

Genetic construct design

Constructs consisting in the mature form of bovine lingual antimicrobial peptide (LAP; Uniprot entry Q28880, V25-K64) or human defensin 5 (HD5, Uniprot entry Q01523, A63-R94) were fused to green fluorescent protein (GFP) [53] using a linker sequence (SGGGSGGS) and named LAP-GFP and HD5-GFP, respectively. Each construct was C-terminally fused to a 6-histidine tag for purification and quantification purposes. LAP-GFP-H6 and HD5-GFP-H6 were codon-optimized (GeneArt®, Lifetechnologies, Regensburg, Germany) and cloned in pET22b (Amp^R) (Novagene, Darmstadt, Germany) vector. The plasmid with each construct (LAP-GFP-H6 HD5-GFP-H6) was transformed into competent *E. coli* BL21 and Origami B.

Kinetics of soluble protein and inclusion body production

E. coli BL21/pET22b cultures (0.5 L) with each antimicrobial fusion (LAP-GFP-H6, and HD5-GFP-H6) were grown O/N in shake flasks at 37º C and 250 rpm in LB broth with ampicillin at 100 μg/mL. *E. coli* Origami B/pET22b with each antimicrobial fusion (LAP-GFP-H6 and HD5-GFP-H6) were grown at same conditions with ampicillin, kanamycin, and tetracycline at 100, 25, and 12,5 μg/mL, respectively. The O/N were used as inoculum in fresh LB medium, starting at OD_{600}= 0.05. Recombinant protein expression was induced by 1 mM isopropyl- β-d-thiogalactoside (IPTG) when cultures reached an OD_{600}=0.4-0.6. Culture samples of 25 mL were taken at 0, 1, 3, and 5 h post-induction and they were collected by centrifugation at 6,000 x *g* for 15 min at 4º C. Pellets were resuspended in 500 μL PBS with EDTA-free protease inhibitor (Roche) and bacteria were disrupted by sonication (2 cycles of 3 min, 0.5s on, 0.5s off at 10% amplitude) (Branson SFX550 Sonifier). Soluble and insoluble fractions were separated by

centrifugation (15,000 x g, 15 min, 4º C). Quantifications of LAP-GFP-H6 and HD5-GFP-H6 in both BL21 and Origami strains were obtained by western blot using a monoclonal anti-His antibody (His-probe, Santa Cruz) and their purity was evaluated by coomassie blue staining assay. Both outcomes were evaluated by ImageJ software to determine protein quantity and purity.

Soluble antimicrobial protein purification

Cultures (1 L) of each fusion construct were grown and induced with IPTG as described in the previous section. After 3 h of production, the whole culture was harvested (6,000 x g, 15 min, 4º C). Pellets from 500 mL culture of LAP-GFP produced in both BL21 and Origami strains and HD5-GFP produced in BL21 strain were resuspended in 30 mL of binding buffer (500 mM NaCl, 20 mM Tris, 20 mM imidazole) with EDTA-free protease inhibitor (Roche). Bacteria were sonicated (4 cycles, 5 min, 0.5s on, 0.5s off at 10% amplitude, Branson SFX550 Sonifier) and centrifugated (15,000 x *g*, 45 min, 4º C), collecting the supernatant, which contains soluble protein. Culture samples (1 L) of HD5-GFP produced in Origami strain was harvested (6,000 x *g*, 15 min, 4º C) at 3h post-induction and the pellet was resuspended in 60 mL of PBS, sonicated as previously described, and centrifugated (15,000 x *g*, 45 min, 4º C). The supernatant was discarded and the pellet, containing the IBs, was washed with dH_2O and centrifugated (15,000 x g, 45 min, 4º C). Then, the supernatant was discarded, and the pellet was weighted, adding 40 mL of solubilization buffer (0.2 % N- lauroylsarcosine mild detergent, 40 mM Tris) per gram of pellet. Next, the pellet was solubilized for 40 h at RT continuously stirred. Solubilized protein was recovered after centrifugation (15,000 x *g*, 45 min, 4º C), and samples were equilibrated at 500 mM NaCl and 20 mM imidazole for purification.

All soluble proteins (obtained from supernatant or solubilized IBs) were filtered using a pore diameter of 0.2 μm and purified by Immobilized Metal Affinity Chromatography (IMAC) in an ÄKTA Start (GE Healthcare) using 1mL HiTrap chelating HP columns (GE Healthcare). Protein was loaded with binding buffer (20 mM Tris, 500 mM NaCl, 20 mM Imidazole) and eluted using a linear gradient with elution buffer (20 mM Tris, 500 mM NaCl, 500 mM Imidazole). Protein buffer exchange was done by dialysis in acetic 0.01% (v/v) O/N at 4 ºC with gentle agitation. The yield of purified soluble protein was determined by NanoDrop™, and the integrity and purity of the protein were analyzed by Western blot and Coomassie.

IB purification

As described before, after 3h post-induction, culture was harvested (6,000 x *g*, 15 min, 4º C). Supernatant was discarded and the pellet was stored at -80º C (minimum 16h). Then, cells were thawed at RT, sonicated (2 cycles, 1.5 min, 0.5s on, 0.5s off at 10% amplitude, Branson SFX550 Sonifier), and stored at -80º C O/N. Next, samples were thawed, and 0.2% (v/v) Triton X-100 (Sigma Aldrich) was added, incubating for 1 h at RT and 250 rpm, sample then was frozen at -

80º C. An extra frozen/thawed cycle was recommended. Next, a contamination control was performed, 100 µL of sample was platted on LB-agar plate and incubated at 37º C overnight (O/N). Freeze/thaw cycles were repeated until no viable bacteria were observed in control plates. Further, IBs were incubated with 250 µL NP-40 (ThermoScientific™) for 1h at 4º C and 250 rpm. Afterward, 0.6 µg/mL DNase I (Roche) and 0.6 µg/mL $MgSO_4$ were added, and sample were incubated 1h at 37º C and 250 rpm. Then, the IBs were collected by centrifugation (15,000 x *g*, 15 min, 4º C) and the supernatant was discarded. After, IBs were resuspended in lysis buffer (100mM NaCl, 50 mM Tris, 1mM EDTA, 0.5% Triton X-100), followed by a contamination control as previously described. Then, IBs were harvested (15,000 x *g*, 15 min, 4º C) and frozen -80º C after supernatant was removed. Finally, IBs were washed in 10mL PBS, aliquoted, and centrifuged (15,000 x g, 15 min, 4º C). Supernatant was removed and the pellets, which contain purified IBs, were kept at 80º C until use. Purity and quantity of purified IBs were assessed by Western Blot and Coomassie assay.

Antibacterial activity assay

Antimicrobial activity was determined with the Bactiter-Glo ™ Microbial Cell Viability kit (Promega). Briefly, an O/N culture of MRSA and *P. aeruginosa* was diluted 100-fold in 10 mM KPi (10mM), aliquoted in 150 µL eppendorf, and centrifugated (6,200 x g, 15 min, 4º C). Supernatant was removed and the bacteria pellet was resuspended in 150 µL of each treatment (acetic buffer -negative control-, soluble proteins (LAP-GFP-H6 and HD5-GFP-H6) at 5 µM and IBs (LAP-GFP-H6 and HD5-GFP-H6) at 5 µM. Samples were incubated in sterile polypropylene 96-well microtiter plate 5 h at 37º C. Next, 100 µL of each sample were mixed with the same volume of BacTiter-Glo™ reagent on sterile 96-well white opaque microtiter plate. Plates were incubated for 5 min and luminescence was measured in a microplate luminometer (LumiStar, Omega). The measured arbitrary luminescence values were normalized against the control (KPi treatment).

Fluorescence measurements

Fluorescence of the GFP fused with the antimicrobial peptides was recorded in a fluorescence spectrophotometer (LumiStar, Omega). LAP-GFP-H6 and HD5-GFP-H6 in both soluble and IBs format produced in both *E. coli* BL21 and Origami B strains were analyzed, being samples diluted when required. Samples were excited at 480 nm and the emission was recorded at 510 nm. Specific fluorescence was calculated using the amount of protein in each sample.

Sulfhydryl determination

Sulfhydryl's not-forming disulfide bonds were determined according a previously established protocol [59]. Briefly, the 4,4'-diothiodipyridine (DTDP) is small, amphiphilic, and lack of charge, allowing quickly react with poorly accessible sulfhydryls. Samples were diluted to final sulfhydryl concentration ≤ 40 μM in 1mL (calculated by protein moles x nº SH) and mixed with a 200 μL strong buffer (100 mM NaH_2PO_4, 0.2 mM EDTA, adjust 8.2 pH with NaOH). After the addition of 50 μL DTDP 4 mM DTDP, samples were vortexed and incubated for 5 min at RT. Next, the sample was read at A_{324} against a water blank. For the reagent blank (A_{324r}), 1mL potassium phosphate buffer was mixed with 200 μL strong buffer and 50 μL of DTDP reagent. For protein blank (A_{324p}), 50 μL of water was added instead of DTDP reagent in the sample with 200 μL strong buffer.

Statistical analysis

All experiments were performed in triplicate and represent as the mean of non-transformed data ± non-transformed standard error of the mean (SEM). Data were previously checked for normality (JMP, SAS Institute Inc.) and *p*-values and letters correspond to the ANOVA analyses and Tukey test analyses respectively, using transforming data when required.

Declarations

Competing interest

The authors declare that they have no competing interests.

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Authors' contribution

AL performed all the experiments, analysis and contributed in Writing-Original Draft. MM and IR contributed with the Sulfhydryl assay determination and subsequent analysis. AR and EG performed the conceptualization, supervision and Writing-Review & Editing. All authors read and approved the final manuscript.

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STUDY 2

SOLUBLE VS SOLUBILIZED RECOMBINANT PROTEINS, THE PURIFICATION PROTOCOL MATTERS

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Preface

The first outcomes of Study 1 pointed out that *E. coli* BL21 is a suitable host for HDPs recombinant production and it is also able to form disulfide bridge, which impact on both HDP stability and the antimicrobial activity against critical pathogenic bacteria. Study 1 also allowed us to determine that during recombinant production the HDPs were produced in both soluble and aggregated format. This is a common fact during recombinant production processes since the overwhelmed metabolism can favor protein aggregation and subsequent IB formation. IBs are protein nanoparticles that have been historically described as recombinant by-products devoid of biological activity. But, with the first insights of native and fully active protein embedded in these aggregated (mainly conformed by the overexpressed protein) the paradigm of IB underwent a radical shift, proving that they are structured by biologically active polypeptides. Thus, considering that the soluble HDPs are often toxic for the producer host, and might be produced at low yields, in this Study 2 we aim to evaluate the yields and quality of protein extracted from IBs through non-denaturing solubilization protocols. In addition, in this study we seek to compare the conformational quality of both soluble and IB solubilized protein, determining how the solubilization protocol impacts the HDP performance. In definitive, the purpose of Study 2 is to establish an alternative protocol for the purification of those challenging HDPs, whose production in the soluble form is limited.

SOLUBLE VS SOLUBILIZED RECOMBINANT PROTEINS, THE PURIFICATION PROTOCOL MATTERS

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Abstract

Recombinant protein production in bacterial cells is often accompanied by the formation of protein aggregates, known as inclusion bodies (IBs). Although several strategies have been developed to minimize protein aggregation, many heterologous proteins of pharmaceutical interest are produced in an aggregated form. In these cases, the purification of these proteins necessarily requires solubilization and refolding processes involving in many cases denaturing agents. However, the presence of biologically active and properly folded recombinant proteins forming IBs has driven the redefinition of the protocols used to obtain soluble protein avoiding any protein denaturation step. Among the different strategies described, the detergent n-lauroylsarcosine (NLS) has proven to be effective to solubilize proteins of interest. However, the impact of the NLS on the final protein quality has not been evaluated so far. For that, we compared the activity of the three antimicrobial proteins obtained from the soluble fraction and that of the solubilized forms isolated from IBs. Results proved that NLS efficiently solubilized proteins from IBs, but it had a negative impact on protein activity. Thus, a solubilization protocol without using detergents was evaluated demonstrating that this strategy efficiently solubilized proteins embedded in IBs while keeping their biological activity at levels comparable to the soluble counterpart. These results proved that the protocol used for IB solubilization has an impact on the final protein quality. Besides, IB properties make possible to solubilize aggregated proteins through a very simple step obtaining properly folded and active proteins.

Keywords: Inclusion bodies, protein quality, mild solubilization, n-lauroylsarcosine, soluble protein, solubilized protein

Introduction

Since the advent of recombinant DNA technologies, the recombinant protein production field has experienced a significant progress [1]. In this scenario, microorganisms are still one of the most widely used expression systems, being *Escherichia coli* by far the preferred choice [2]. Although some heterologous proteins of pharmaceutical or biomedical interest are mainly produced in a desirable soluble form, many others are produced as cytoplasmic aggregates also known as inclusion bodies (IBs) [3-6]. To avoid, or at least minimize, IB formation and increase the soluble protein fraction, different approaches have been proposed [7]. These strategies include the optimization of the expression conditions (i.e., temperature, inducer concentration or media composition), the use of solubility enhancing tags (i.e., maltose-binding protein (MBP), thioredoxin A (TrxA) or glutathione S-transferase (GST)), the secretion of the heterologous protein to the culture medium or *E. coli* periplasm, the co-expression of chaperones during the production process and the use of mutant strains [8].

However, in many cases, this is not enough to reach the desired soluble protein production yields. For these cases, different protocols have been developed for the extraction of soluble proteins from IBs. Traditionally, IBs have been solubilized applying harsh denaturing and high concentrations (6-8 M) of chaotropic agents such as urea or guanidine hydrochloride (GdnHCl), along with reducing agents like β-mercaptoethanol and dithiothreitol [9]. Consequently, the protein released from these aggregates undergoes a complete denaturation, being necessary a refolding step to recover the bioactive native conformation of the protein of interest [10]. But, the progress made over the last decade about IBs nature has evidenced that these protein aggregates are structured amyloid-like nanoparticles that contain biologically active and properly folded recombinant protein [11-14]. This has driven different groups to redefine the methodologies used to obtain soluble protein using IBs as protein source. *De facto*, the use of high concentration of chaotropic agents has been substituted by non-denaturing protocols that avoid fully denaturation and usually refolding steps [15-17]. The use of mild detergents like n-lauroylsarcosine (NLS) or lauroyl-L-glutamate takes advantage of IBs nature, enabling correctly folded protein release without the need of using costly and time-consuming refolding procedures [18, 19]. In addition, low concentrations of organic solvents, such as n-propanol, trifluoroethanol and isopropanol [20-23], as well as dimethyl sulfoxide (DMSO), have also been demonstrated to be suitable as IB solubilizing agents without affecting the native structure of the released protein [24]. These alcohols are well described for not only protecting but also promoting the secondary structure of the protein [25, 26]. Other approaches combine low amounts of denaturing reagents with the adjustment of either physical parameters, like heat [27], high hydrostatic pressure [28], and

freeze-thaw cycles [29] or chemical factors such as pH oscillations [30], to finally accomplish IBs protein solubilization in a non-denaturing manner.

Remarkably, despite the extensive description of novel solubilization methods and its inherent benefits, where NLS is one of the detergents most widely used, the comparison of the solubilized protein quality with its soluble counterpart remain unexplored, being this crucial to evaluate and validate the whole IB solubilization process. Hence, in this study, three proteins (lingual antimicrobial peptide (LAP), human α-defensin 5 (HD5), and human cathelicidin LL-37, fused to the Green Fluorescent Protein (GFP)) have been produced in *E. coli* and purified directly from the soluble fraction or using the IBs as a source of soluble protein using a mild solubilization protocol, seeking to compare if there is any impact of the protocol used in the final protein quality.

Materials and Methods

Bacterial strains and growth media

Escherichia coli BL21 (DE3) strain was used for recombinant protein production. The strain selected for antimicrobial activity evaluation was *E. coli* DH5α. Both strains were grown in Luria-Bertani (LB) medium.

Construction of expression plasmids

The active forms of bovine lingual antimicrobial peptide (LAP; Uniprot entry Q28880, V25-K64), human α-defensin 5 (HD5, Uniprot entry Q01523, A63-R94), and the cathelicidin LL-37 (Uniprot entry P49913, L134-S170) were fused to the green fluorescence protein (GFP) using the linker sequence SGGGSGGS. Each protein sequence was C-terminally fused to a 6 histidine (H6) tag for purification purposes. The resultant DNA sequences (LAP-GFP-H6 (32.53 kDa), HD5-GFP-H6 (31.79 kDa), and LL-37-GFP-H6 (32.66 kDa)) were chemically synthesized while optimizing codon usage for *E. coli* expression platform (GeneArt®, Life technologies, Regensburg, Germany). Each construct was cloned into a pET22b (Amp^R) vector and transformed by heat shock in competent *E. coli* BL21 (DE3) cells.

Protein production kinetics

E. coli BL21 (DE3)/ pET22b-LAP-GFP-H6, *E. coli* BL21 (DE3)/ pET22b-HD5-GFP-H6, and *E. coli* BL21 (DE3)/ pET22b-LL-37-GFP-H6 were grown overnight (O/N) in LB broth supplemented with ampicillin 100 μg/mL (for plasmid conservation) at 37 ºC and 250 rpm. O/N cultures were inoculated in 200 mL of LB media with 100 μg/mL ampicillin in 1 L shake flasks

(at an initial OD=0.05) and grown at 37 ºC and 250 rpm until reaching an OD_{600} of 0.4 – 0.6. Then protein expression was induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG). After that, cultures were grown at 37 ºC and 250 rpm and 25 mL samples were taken at 0, 1, 3, and 5 h post-induction. Then, cells were harvested and recovered by centrifugation at 6,000 x *g* for 15 min at 4ºC. These cultures were performed by triplicate.

To determine protein fractionation, pellets from 500 mL culture were resuspended in 30 mL phosphate buffered saline (PBS) with an EDTA-free protease inhibitor cocktail (cOmplete EDTA-free, Switzerland, Roche). Then, ice-jacketed samples were disrupted by sonication (2 cycles of 3 min at 10% amplitude under 0,5 s cycles) (Branson SFX550 Sonifier). The soluble and insoluble fraction was split by centrifugation (15,000 x *g*, 15 min, 4º C) and both fractions were stored at -80 ºC until quantification by Western blot and Coomassie (Supplementary Materials Figure 1).

Protein production and purification

For production purposes, two shake flasks of 2.5 L with 500 mL of LB media supplemented with 100 μg/mL of ampicillin were inoculated with O/N cultures at initial OD_{600} of 0.05 and each culture was incubated at 37 ºC and 250 rpm until reaching an OD_{600} of 0.4 – 0.6, when protein expression was induced with 1 mM of IPTG. After 3 h of induction, the whole culture was harvested by centrifugation at 6,000 x *g* for 15 min at 4ºC, the supernatant was discarded, and the pellet was stored at -80 ºC.

Pellets were resuspended in binding buffer (500 mM NaCl, 20 mM Tris, 20 mM Imidazole) with EDTA-free protease inhibitor (Complete EDTA-free, Switzerland, Roche) and disrupted by sonication (4 cycles, 5 min, at 10% amplitude under 0,5 s cycles) (Branson SFX550 Sonifier). After cell disruption, samples were centrifuged at 15,000 x *g*, 45 min, 4ºC and soluble and insoluble (pellet with inclusion bodies (IBs)) fractions were separated.

To solubilize protein from the insoluble fraction (IBs), the pellet obtained after centrifugation was washed with dH2O, centrifugated (15,000 x *g*, 45 min, 4 ºC) and the supernatant was discarded. Next, the pellet was weighted and 40 mL of solubilization buffer (0.2% n-lauroylsarcosine (NLS), 40 mM Tris) were added per gram of pellet. After that, the mixture was solubilized for 40 h at room temperature (RT) under gentle stirring. Further, the samples were equilibrated prior to the purification step adding 500 mM NaCl and 20 mM of imidazole. Finally, samples were centrifuged at 15,000 x *g*, 45 min, 4ºC, recovering the supernatant with the solubilized protein. Both soluble and solubilized protein were purified using the protocol described here below.

Samples were filtered (Ø 0.2 μm) and purified by Immobilized Metal Affinity Chromatography (IMAC) in an ÄKTA Start (GE Healthcare) using 1 mL HisTrap chelating HP columns (GE Healthcare). Protein was loaded into column with binding buffer (500 mM NaCl, 20 mM Tris, 20 mM Imidazole) and eluted with an increasing gradient of imidazole, mixing both binding and

elution buffer (500 mM NaCl, 20 mM Tris, 500 mM Imidazole). For those solubilized proteins, 0.2% NLS was also added to the binding and elution buffer. Finally, protein buffer exchange was performed with 5 mL HiTrap Desalting columns (GE Healthcare), using phosphate buffer (10mM KPi, 12.5 mM NaCl). The amount of purified protein was determined by NanoDrop™, and the integrity and purity by SDS-PAGE.

Antimicrobial activity assay

The effect of the different antimicrobial candidates was evaluated with the Bactiter-Glo™ Microbial Cell Viability kit (Promega). Briefly, the selected strain to assess the bactericidal activity (*E. coli* DH5α) was grown O/N at 250 rpm and 37 ºC and then diluted 1:100 in 10 mM KPi buffer. After that, 150 µL of the bacterial dilution were aliquoted and centrifuged at 6,200 x *g*, 15 min at 4 ºC. Following, the supernatant was removed, and the bacterial pellet was resuspended in 150 µL of either antimicrobial treatment (soluble or solubilized LAP-GFP, HD5-GFP, or LL-37-GFP) or KPi buffer as a negative control. Samples were incubated in sterile polypropylene 96-well (Costar) microtiter plate during 5 h at 37 ºC without agitation. After that, 100 µL of each well were transferred on sterile 96-well opaque microtiter plate (ThermoFisher) and mixed with 100 µL of the BacTiter-Glo™ reagent. The plate was incubated for 5 min and subsequently luminescence was measured using a microplate luminometer (LumiStar, Omega). The registered arbitrary luminescence was normalized against the control (KPi treatment).

Evaluation of N-lauroylsarcosine effect in soluble and solubilized protein

To evaluate the effects of NLS in the performance of the soluble and solubilized protein different conditions were assessed. With the soluble protein, two different binding buffers were examined for pellets resuspension before sonication, the standard buffer (500 mM NaCl, 20 mM Tris, 20 mM imidazole) described in protocol 1 (S) and another with the same composition plus 0.2% NLS, protocol 2 (S-NLS). For the solubilized proteins, three different protocols were evaluated, two with mild detergents (NLS) during solubilization, but differing in the purification buffer composition (protocol 3 (ST-NLS) and protocol 4 (ST-pNLS)). And the last protocol without using mild detergents (ST) with solely 40 mM Tris buffer was also used to solubilize proteins during 40 h at RT under gentle agitation. All the tested combinations are summarized in Table 1.

Table 1. Experimental conditions used to purify LAP-GFP-H6, HD5-GFP-H6, and LL-37-GFP-H6 from both soluble and insoluble fraction. S: soluble; ST: solubilized; NLS: n-lauroylsarcosine

Protocol nº	Sonication buffer	Purified fraction	Solubilization buffer	Purification buffers	
				Binding buffer	Elution Buffer
1- S	500 mM NaCl 20 mM Tris 20 mM Imidazole	Soluble	-	500 mM NaCl 20 mM Tris 20 mM Imidazole	500 mM NaCl 20 mM Tris 500 mM Imidazole
2- S-NLS	500 mM NaCl 20 mM Tris 20 mM Imidazole 0.2 % NLS				
3- ST-NLS	PBS	Insoluble	40 mM Tris, 0.2 % NLS	500 mM NaCl 20 mM Tris 20 mM Imidazole 0.2% NLS	500 mM NaCl 20 mM Tris 500 mM Imidazole 0.2% NLS
4- ST-pNLS				500 mM NaCl 20 mM Tris 20 mM Imidazole	500 mM NaCl 20 mM Tris 500 mM Imidazole
5- ST			40 mM Tris		

Statistical analysis

For all assays, each condition was performed in triplicate and the results are expressed as the means of non-transformed data ± standard error of the mean (SEM). Data were previously checked for normality (JMP, SAS Institute Inc.) and transformed when required. The p-values and letters correspond to the ANOVA and Tukey test analyses, respectively.

Results

The distribution of the three proteins used in this study (LAP-GFP-H6, HD5-GFP-H6, and LL-37-GFP-H6) in the soluble and insoluble fractions of recombinant *E. coli* cultures was determined (Figure 1A). LAP-GFP-H6 and LL-37-GFP-H6 proteins were equally distributed between both fractions, especially at longer production times (Figure 1A and 1C). By contrast, HD5-GFP-H6 was produced mainly insoluble, reaching aggregation values around 75-85 % (Figure 1B).

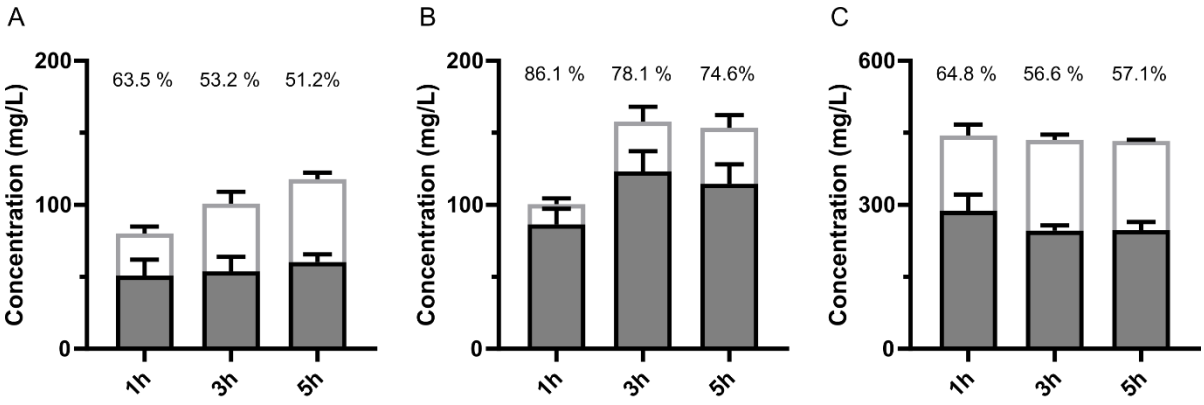

Figure 1. Production kinetics and soluble/insoluble protein distribution of LAP-GFP-H6 **(A)**, HD5-GFP-H6 **(B)**, LL-37-GFP-H6 **(C)** at 1, 3 and 5 h post-induction. The staked bars indicate the total amount of protein produced at each time distributed between aggregated fraction (grey) and soluble (white). Values of % aggregation are represented on the top of each condition. Error bars indicate the standard error of the mean (SEM).

Despite these fractioning differences, all the proteins were produced in sufficient quantity in both soluble and insoluble form, being possible to purify them from both fractions (cell cytoplasm and solubilized from IBs). The soluble form was purified using protocol 1 (Table 1) and the solubilized forms were purified after incubation of IBs with NLS to solubilize the protein forming protein aggregates (Table 1, protocol 3). In the purification process, LAP-GFP-H6 and HD5-GFP-H6 elution profiles were distributed in three peaks, while LL-37-GFP-H6 in two peaks, independently if the protein was obtained from the soluble (S) or insoluble fraction (ST-NLS) (Table 2). Analyzing the antimicrobial activity of the protein eluted in each peak, in general terms, the soluble protein was significantly more active than the protein purified from the solubilized IBs (Figure 2). This was particularly clear at 5 μM, where the highest activity was reached. Different elution peaks of the soluble version did not show differences in antimicrobial activity,

except for LL-37-GFP-H6 for which peak 1 was much more active at 5 μM (Figure 2). Although no important variances were observed for the activity of LAP-GFP-H6 and HD5-GFP-H6 elution peaks, protein yield revealed differences, being the protein amount of peak 2 the highest one for both proteins (Table 2).

Table 2. Peak distribution and yield of the protein obtained from the soluble fraction (soluble (S) and solubilized from IBs with n-lauroylsarcosine (ST-NLS). The % of elution buffer is indicated for each peak.

Protein	Format	Fraction	Yield (mg/L)	% B elution
LAP-GFP-H6	S	peak 1	0.62	15
		peak 2	7.02	27
		peak 3	1.86	49
	ST-NLS	peak 1	0.18	14
		peak 2	2.07	18
		peak 3	5.58	32
HD5-GFP-H6	S	peak 1	1.40	14
		peak 2	4.26	30
		peak 3	1.18	100
	ST-NLS	peak 1	1.93	14
		peak 2	4.20	24
		peak 3	1.89	40
LL-37-GFP-H6	S	peak 1	0.96	13
		peak 2	0.87	26
	ST-NLS	peak 1	5.57	10
		peak 2	1.37	25

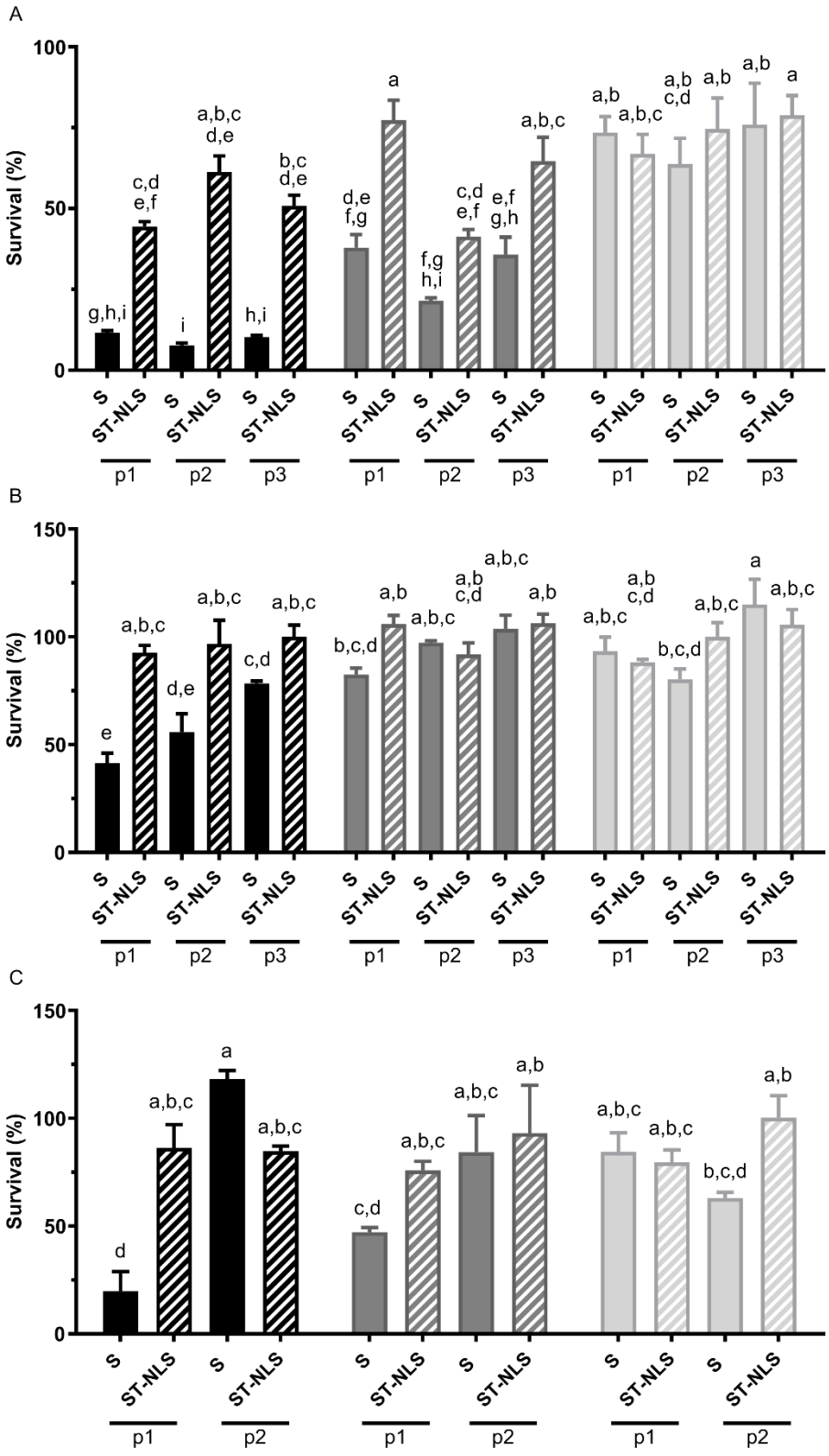

Figure 2. Antimicrobial activity of the different peaks (p1, p2 or p3) of LAP-GFP-H6 **(A)**, HD5-GFP-H6 **(B)**, and LL-37-GFP-H6 **(C)** against *E. coli* DH5α at 5 µM (black), 1 µM (grey) and 0.1 µM (light grey). The bars indicate the protein origin either soluble (S) represented with solid bars or solubilized with n-lauroylsarcosine (ST-NLS) with striped bars. Error bars indicate the standard error of the mean (SEM).

Different letters depict significant differences between format (S or ST-NLS), peak, and concentration A (P=0.05); B (P=0.0079); C (P<0.0001).

To determine if the mild detergent (NLS) used to solubilize the protein had a negative impact on the antimicrobial activity, IBs of two proteins (LAP-GFP-H6 and HD5-GFP-H6) were solubilized in Tris buffer without NLS (Table 1, protocol 5) and the solubilized (ST) protein activity was compared with that obtained from peak 2 of the soluble fraction (S) and the protein solubilized using detergent (ST-NLS) (Figure 3). Interestingly, the protein solubilized without any detergent (ST) had an activity comparable to that observed for the soluble version (S) either in LAP-GFP-H6 (Figure 3A) or even better for HD5-GFP-H6 (Figure 3B).

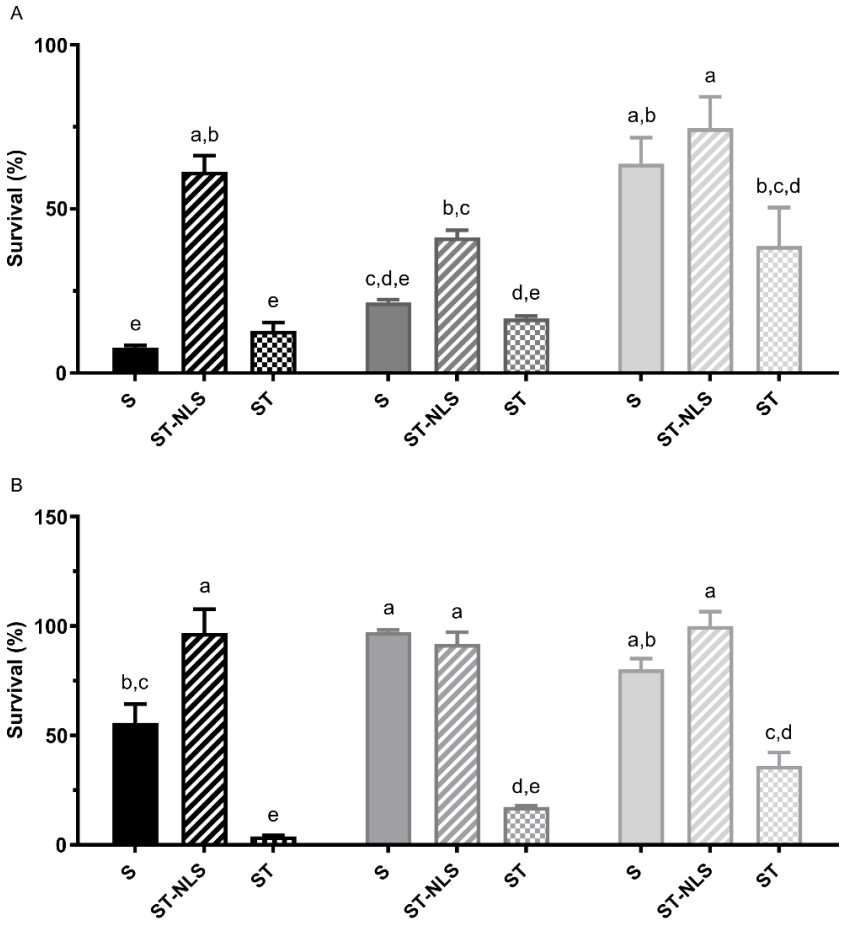

Figure 3. Bacterial survival (%) of *E. coli* DH5α in presence of LAP-GFP-H6 **(A)** or HD5-GFP-H6 **(B)** peak 2 at 5 μM (black), 1μM (grey) and 0.1 μM (light grey). The bars indicate the protein origin either soluble (S) represented with solid bars or solubilized with or without (ST) n-lauroylsarcosine (ST-NLS) represented with stripped or mosaic bars, respectively. Error bars indicate the standard error of the mean (SEM). Different letters depict significant differences between format (S, ST-NLS or ST) and concentration. A (P= 0.0001); B (P=0.002).

To validate these results, a final experiment comparing different combinations was performed. The activity of the soluble protein (S) purified using protocol 1 (Table 1), soluble protein purified with buffers containing NLS (S-NLS) (Table 1, protocol 2), solubilized protein using NLS in all the process (ST-NLS) (Table 1, protocol 3), solubilized protein with NLS but purified with buffers free of detergent (ST-pNLS) (Table 1, protocol 4) and solubilized and purified protein without NLS (ST) (Table 1, protocol 5) were compared (Figure 4). This experiment showed that only the soluble protein (S) and the solubilized without using detergent in the whole process (ST) showed good levels of activity (Figure 4). By contrast, when NLS was used in the solubilization and/or purification process the antimicrobial activity significantly decreased (Figure 4).

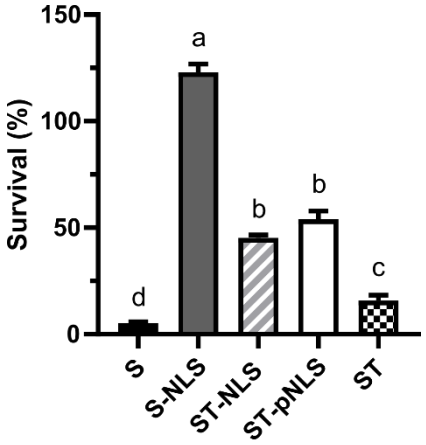

Figure 4. Bacterial survival (%) of *E. coli* DH5α in presence of LAP-GFP-H6 at 5 μM in different formats: soluble (S); soluble protein purified with NLS buffers (S-NLS); solubilized protein either with NLS in the whole process (ST-NLS) or solely during solubilization (ST-pNLS); and solubilized and purified free of NLS (ST). Error bars indicate the standard error of the mean (SEM). Different letters depict significant differences between treatments ($P < 0.0001$).

Discussion

Since many proteins of interest that are recombinantly produced aggregate forming IBs, their production in bacterial expression systems as soluble and native forms is often challenging. Different strategies are used to favor the production of these proteins in their soluble form [7, 8] but there are still many proteins only produced as IBs, being necessary to use protocols to extract soluble protein from IBs. Although denaturing and refolding procedures have been used for years, the presence of active protein forms in the IBs has evidenced the need to develop mild strategies for their recovery. Among the different strategies, NLS has been used for this purpose using both *E. coli* [19, 29, 31, 32] and *Lactococcus lactis* [16, 33] IBs. It has been proven to be a good strategy to obtain soluble and active protein from bacterial aggregates using a simple solubilization process. However, so far, no detailed comparison of the same protein obtained from the soluble and insoluble fraction has been published. Thus, in this work we have used three

different proteins that are produced in both soluble and aggregated forms (Figure 1) to compare the activity of each protein obtained from the soluble fraction or solubilized with NLS from IBs. In all the cases (LAP-GFP-H6, HD5-GFP-H6 and LL-37-GFP-H6) the purification profile of the protein obtained from the soluble fraction or from IBs, solubilized with NLS, was the same (Table 2). However, the antimicrobial activity revealed important differences (Figure 2). The soluble form (S) was highly active, especially at 5 μM whereas proteins purified from the solubilized fraction with NLS (ST-NLS) showed low levels of bactericidal activity (Figure 2). These results agree with those published by Peternel et al. where they showed that in two of the proteins used (GFP and His7ΔN6 TNF-α) a lower percentage of proteins extracted from IBs using NLS are active compared with the protein purified from the soluble cell fraction [17, 31]. Also, Tao and co-authors described a similar effect in GST with 0,3% NLS was used [34]. This indicated that NLS is interfering with the activity of the purified protein, which was proved by the negative impact of NLS traces on the protein activity (Figure 3 and Figure 4). After solubilization, proteins are usually purified and dialyzed using standard procedures, but it is known that, after dialysis, detergent traces can be still present in the solution, which could have an impact on the activity and safety of the purified protein. To minimize the detergent effect, other authors have previously proven that is possible to reduce NLS concentration reaching good levels of solubilization with NLS at 0.05 % [29]. However, the complete removal of the detergent during the solubilization process and its impact on protein quality were not tested before. Thus, we evaluated the IB solubilization using Tris buffer without any detergent (Table 1, protocol 5), demonstrating not only that the solubilized proteins (ST) showed an activity comparable (or higher) to the protein isolated from the soluble fraction (S), but also that the detergent is not necessary for the solubilization process (Figure 3). Although some proteins solubilized with NLS from IBs like G-CSF have been shown to keep their biological activity [19, 31], others, such as those described in this study and those previously reported by Peternel et al. [19, 31], are affected by the use of detergents.

These findings suggest that when using non-denaturing protocols for IB solubilization it is necessary to validate that the solubilization agent used does not interfere in the protein mode of action. For that, both protein yield, and protein activity need to be tested. Alternatively, for those proteins with an impaired activity when solubilized, a solubilization process could be applied without using detergent (Figure 4) [35]. In this study we have proven that through this simple process it is possible to obtain properly folded and active proteins from bacterial aggregates. Besides, in this study we also proved that the solubilization protocols without detergents used are effective in those termed classical IBs (produced at 37ºC) and not only for those produced at lower temperatures and described as non-classical IBs [36]. In agreement with that, Lu and Lin have

previously reported that the activity of epimerase recovered by mild solubilization from IB is identical when IBs are produced at 37ºC and 25ºC [35].

Conclusion

The comparison of the activity of different proteins either directly purified from the soluble fraction or the same proteins solubilized from IBs with a non-denaturing protocol done in this work proved that the solubilization agents can have a negative impact on protein activity. Thus, monitoring not only the purified protein yields but also protein activity it is necessary to determine the optimal protocol for IB solubilization.

Declaration of Interest

All authors contributed to manuscript revision, read, and approved the submitted version. The authors state no conflict of interest.

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Credit authorship contribution statement

Adrià López Cano: Investigation; Methodology, Analysis, Writing- Original Draft

Paula Sicilia: Investigation

Clara Gaja: Investigation

Anna Arís: Conceptualization, Supervision, Writing-Review & Editing

Elena Garcia-Fruitós: Conceptualization, Supervision, Writing-Review & Editing

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STUDY 3

A NOVEL GENERATION OF TAILORED ANTIMICROBIAL DRUGS BASED ON RECOMBINANT MULTIDOMAIN PROTEINS

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Preface

After demonstrating that HDPs can be produced recombinantly in *E. coli*, being fully active (Study 1), and establishing a production and purification protocol for those HDPs that are mainly produced as IBs (Study 2), we decided to expand the catalogue of antimicrobial HDPs candidates. For that, in Study 3 we aim to evaluate the production, activity, and characteristics of 5 HDPs (1st generation molecules), including a cathelicidin, α-defensins, and β–defensins. After an exhaustive screening, the most promising HDPs would be selected to be rationally combined, acting as building blocks, in multidomain proteins (2nd generation molecules). This strategy allows the development of novel tailored molecules where the selected fused domains may undergo synergistic effects outpacing the antimicrobial activity (reflected in a lower MIC and improved bacterial killing against relevant pathologic microorganisms) of their single domain counterparts. In addition, these multidomain polypeptides (second generation molecules) also enable the removal of the carrier protein (GFP) used to reduce the toxicity and avoid the proteolysis of the first generation HDP.

A NOVEL GENERATION OF TAILORED ANTIMICROBIAL DRUGS BASED ON RECOMBINANT MULTIDOMAIN PROTEINS

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Abstract

Antibiotic resistances have exponentially increased during the last years and the appearance of multi-drug resistant (MDR) bacteria, have rapidly raised. This has generated a global health crisis that requires urgent solutions. Among them, it is necessary to develop of new antimicrobial drugs to treat infectious diseases caused by MDR microorganism. Host Defense Peptides (HDPs) have a versatile role acting as antimicrobial peptides and as regulators of some innate immunity functions. The results shown by some previous studies using synthetic HDPs are only the tip of the iceberg since the synergistic potential of HDPs and their production as recombinant proteins are fields practically unexplored. The present study aims to move a step forward through the development of a new generation of tailored antimicrobials using a rational design of recombinant multidomain proteins based on HDPs. This strategy is based on a two-phase process, starting with the construction of 1st generation molecules using single HDPs and further selecting those HDPs with higher bactericidal efficiencies to be combined in the 2nd generation of broad-spectrum antimicrobials. As a proof of concept, we have isolated a new antimicrobial, named D5L37D5L37, equally effective against four relevant pathogens such as methicillin sensible

Staphylococcus aureus (MSSA), methicillin resistant *Staphylococcus aureus* (MRSA), methicillin resistant *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*, being MRSA and *P. aeruginosa* MDR strains. The low MIC values and versatile activity against planktonic and biofilm forms reinforce the use of this platform to isolate and produce unlimited HDPs combinations as new antimicrobial drugs by effective means.

Introduction

The discovery of antibiotics led to a golden age in human healthcare, providing a wide range of therapies to cope with bacterial infections [1, 2]. Since the breakthrough of penicillin in 1928, several classes of antibiotics were successfully introduced into clinical practices, but their effectiveness was steadily compromised by the expansion of antimicrobial resistant (AMR) bacteria [3]. As a result of prevalent and sometimes misuse of antibiotics the AMR and multi-drug resistant (MDR) bacteria have rapidly raised, generating a global health crisis affecting both human and animal health that requires urgent solutions [4, 5]. In this context, the search for new antimicrobial compounds has become imperative. Several approaches are under investigation, such as the use of enzymes, probiotics, antimicrobial peptides, or bacteriophages, to name a few. Among this vast array, the Host Defense Peptides (HDPs) or antimicrobial peptides from innate immunity have stood out over others due to their natural versatility [6-8]. HDPs are short (ranging from 12 to 50 amino acids) cationic and amphiphilic peptides, with a ubiquitous presence in nearly all biological kingdoms [9, 10]. These evolutionary conserved molecules have an essential role in the innate immune system, regulating a broad range of immunological responses [11]. Likewise, HDPs exhibit broad-spectrum activity against viruses, fungi, and bacteria, including MDR strains [1, 12] in both planktonic and biofilm forms [13, 14]. Unlike antibiotics, HDPs have a reduced half-life, which combined with their variety of mechanisms of action hamper the emergence of new resistances [15].

Historically, the HDPs have been categorized according to their secondary structures, length, and amino acid composition. Among the distinct families defensins and cathelicidins are by far the most distinguished [16]. Defensins are a large group of short cationic peptides widely distributed in multicellular organisms with six conserved cysteine residues that participate in disulfide bond formation [17]. In addition, according to the connectivity pattern of these cysteines, defensins are subdivided into: α-defensins, also known as cryptidins due to their ubiquitous presence in the intestinal crypts produced by Paneth cells and narrowly related with intestinal homeostasis through their strong microbicidal and immunomodulation action [16]; β-defensins, that are mainly expressed in leukocytes and epithelial cells [18], playing a pivotal role in preserving tissue

homeostasis by exerting a potent bactericidal and immunoregulatory activities [19], and finally θ-defensins, with a unique cyclic structure, which have only been described in non-human primates [20]. On the other side, the cathelicidins in conjunction with defensins, exert essential support in host immunity being the LL-37 the only cathelicidin described in humans [21]. These peptides are broadly expressed in neutrophils and macrophages, being released during inflammatory responses, where can act either directly resolving-infections by killing pathogenic bacteria or coordinating immune responses [13, 22].

HDPs production has been commonly carried out by chemical synthesis, although recombinant production has already been proven to be an alternative that allows producing these peptides through a scalable and cost-effective process, without limits in peptide length [23, 24]. However, when produced in recombinant hosts, HDPs need to be fused to a carrier protein [25] to protect the peptide from host proteases and mask their possible toxic effect on the producer cell [23]. The removal of the carrier protein involves extra steps in the downstream purification and hence yield reduction and additional cost [26]. In this scenario, a recent study carried out by Roca-Pinilla *et al.* demonstrated that the combination of different functional HDP-based domains in a single polypeptide enabled the synthesis of a potent antimicrobial protein without compromising host viability and without the need of using protein carriers [27]. The present study aims to move a step forward through the development of a new generation of tailored antimicrobials using a rational design of multidomain proteins. This strategy is based on a two-phase process, starting on the 1st generation of molecules produced from a library of HDPs fused to carrier fluorescent protein GFP. After their testing against planktonic and biofilm forms of target pathogens, the best performing HDPs are combined in the 2nd generation of chimeric molecules, where GFP is removed, and tactical linkers included, obtaining highly active and synergic HDP-based multidomain antimicrobial polypeptides.

Results

First generation of HDP-based antimicrobial proteins

In this study, the codifying region of five different HDPs, named human α-defensin 5 (HD5), human β-defensin 2 and 3 (HβD2, HβD3), bovine lingual antimicrobial peptide (LAP) β-defensin, and the cathelicidin LL37 were C-terminally fused to the GFP gene and to a His6 (H6)-tag (Fig. 1A) for the construction of the 1st generation Antimicrobials. These five constructs were successfully produced in the soluble fraction of recombinant *E. coli*. After IMAC purification, good yields and purity was achieved for all proteins (Table 1).

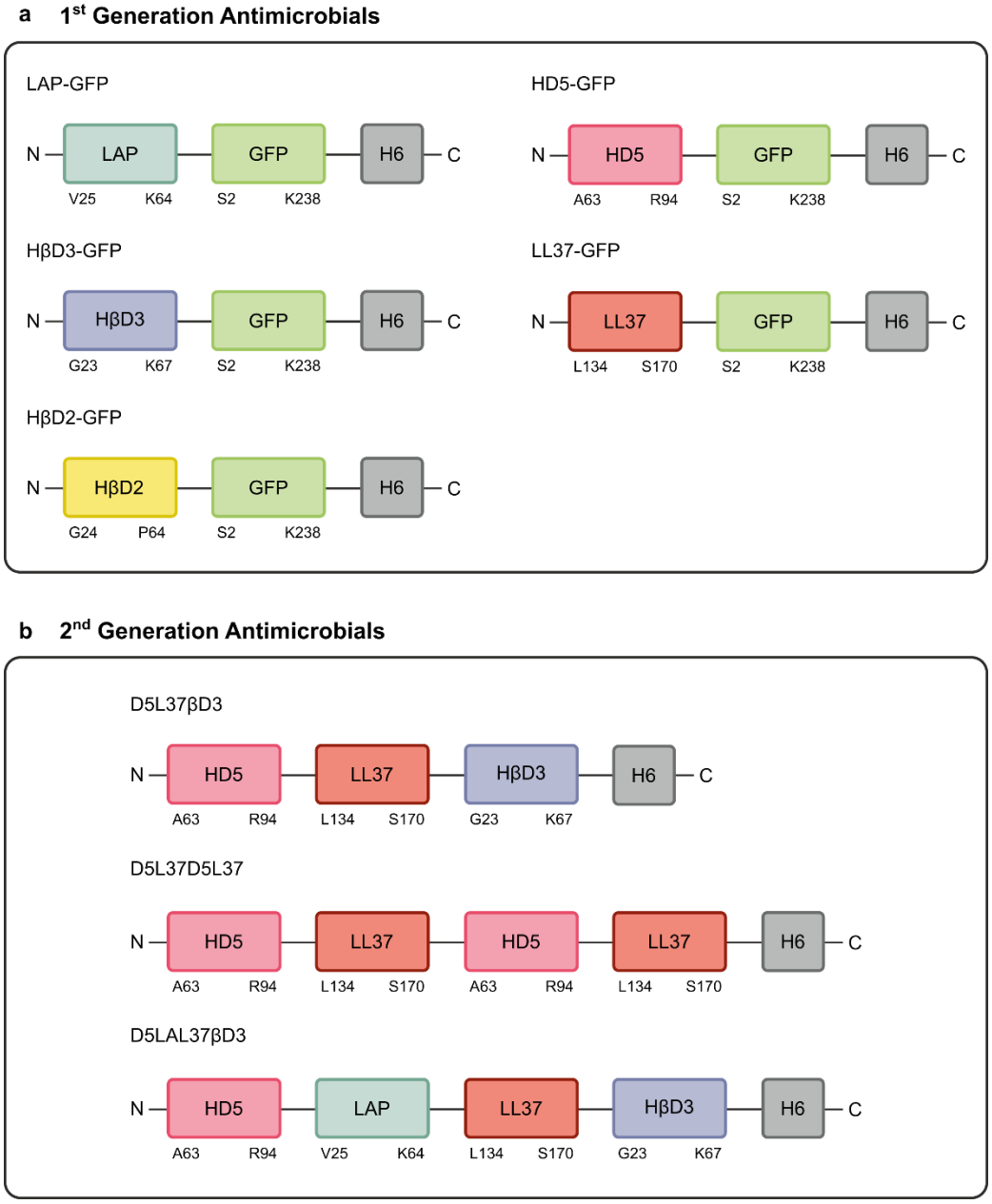


Figure. 1 | Schematic representation of both 1st and 2nd generation of antimicrobial proteins. a. The 1st generation constructs are constituted from N- to C-terminal by a single HDP-based domain (LAP, HβD2, HβD3, HD5, or LL37) fused to GFP gene. **b.** The 2nd Generation constructs are multidomain proteins combining HD5, LL37, and HβD3 domains (D5L37βD3), combining the last three with LAP (D5L37βD3) and using HD5 and LL37 tandem repetitions (D5L37D5L37). All constructs have a H6-tag at C-terminal for protein purification purposes.

Table. 1 | Antimicrobial protein yield (mg·L^{-1} culture) and purity (%) of soluble LAP, HβD2, HβD3, HD5, and LL37. [a] Yields calculated after protein purification.

Protein	Yield (mg L^{-1}) [a]	Purity (%)
LAP	23.4	>99
HβD2	3.48	97
HβD3	1.84	89
HD5	5.84	>99
LL37	1.74	58

The antimicrobial activity of 1st generation molecules was evaluated against both Gram-positive (methicillin sensible *Staphylococcus aureus* (MSSA), methicillin resistant *Staphylococcus aureus* (MRSA), and methicillin resistant *Staphylococcus epidermidis*) and Gram-negative bacteria (*Pseudomonas aeruginosa*), being MRSA and *P. aeruginosa* MDR strains. The testing of antimicrobial activity was done in three steps (1) wide screening assay (2) determination of the Minimal Inhibition Concentration (MIC) of HDPs selected in the first step and (3) biofilm eradication testing (Fig. 10). The wide screening assay focused on the selection of the most promising candidates against planktonic bacteria, was carried out at 5 µM, against two bacterial concentrations (10^5 and 10^3 cfu mL^{-1}). The concentration of 5 µM was chosen since it was determined as the most probable effective concentration of 1st generation Proteins (Fig S1, supplementary materials). The most active molecules were those based on HβD3 and HD5, reducing at least 3-log in all bacterial pathogens (Fig. 2) and 5-log in *S. epidermidis, and P. aeruginosa* (Fig. 2c, 2d). The LAP and HβD2-based proteins activity were strain-dependent, killing completely MSSA, *S. epidermidis* and *P. aeruginosa*, either at 10^5 and 10^3 cfu mL^{-1} (Fig. 2b, 2c and 2d, respectively), but showing lower performance against MRSA strain (Fig. 2c). On the other hand, the LL37-based construct only showed mild bactericidal effects against MRSA (Fig. 2a), *S. epidermidis* (Fig. 2c), and *P. aeruginosa* (Fig. 2d) at 10^5 cfu mL^{-1} and was not selected for the MIC determination.

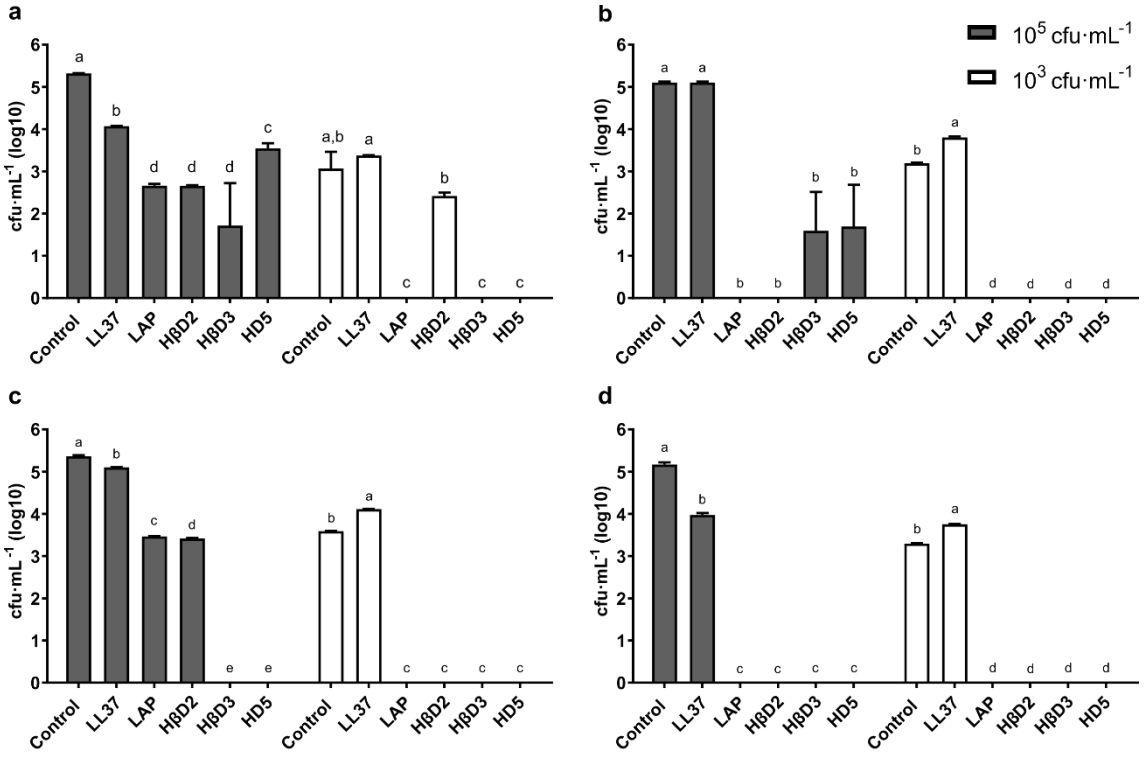
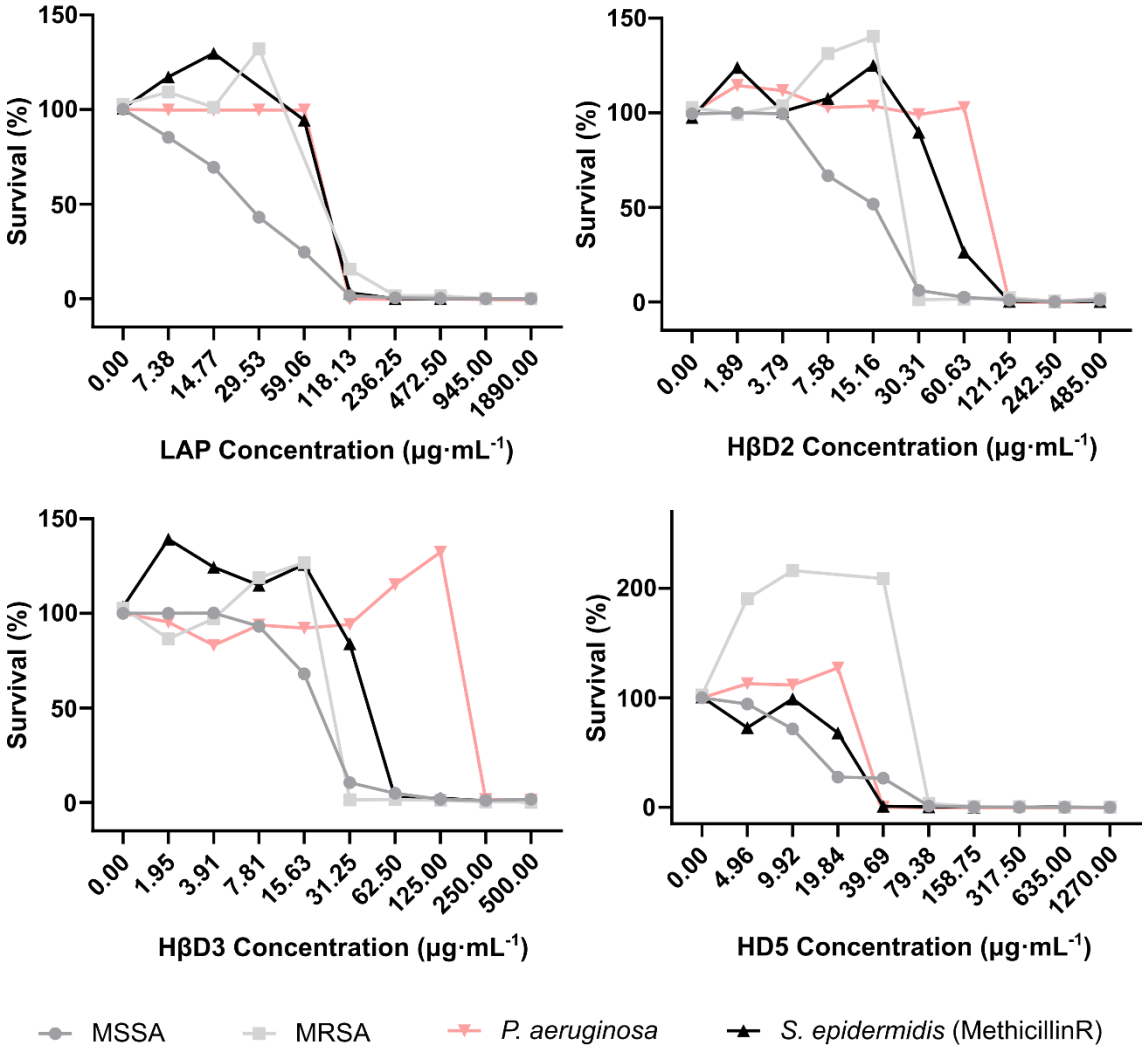


Figure. 2 | Antimicrobial activity of 1st generation constructs. Antimicrobial activity (reduction of log of cfu mL^{-1}) of the different 1st Generation constructs at 5 μM against **a** methicillin resistant *Staphylococcus aureus* (MRSA), **b** methicillin sensitive *Staphylococcus aureus* (MSSA), **c** methicillin resistant *Staphylococcus epidermidis* and, **d** *Pseudomonas aeruginosa*. The constructs were tested against an initial concentration of 105 cfu mL^{-1} (dark bars) or 10^3 cfu mL^{-1} (white bars). Data shown are the mean of a triplicate ± SEM. Different letters depict statistically significant differences (p<0.0001) examined by ANOVA and Tukey test analysis.

LAP-based construct had a MIC ranging from 236.25 μg·mL^{-1} against MRSA to 118.13 μg·mL^{-1} (Fig. 3) against MSSA, *S. epidermidis* and *P. aeruginosa* (Fig. 3b). HβD2 showed the same MIC value (121.25 μg·mL^{-1}) for Gram-negative *P. aeruginosa* and Gram-positive *S. epidermidis*. However, the MIC was much better against Gram-positive MRSA and MSSA, being 60.63 μg mL^{-1} and 30.31 μg·mL^{-1} respectively. HβD2-based protein had a high MIC of 250 μg·mL^{-1} for *P. aeruginosa* but it decreased considerably against Gram-positive MRSA, MSSA, and *S. epidermidis*, being 62.5 μg·mL^{-1}, 31.25 μg·mL^{-1}, and 62.50 μg·mL^{-1} respectively. Finally, the HD5 construct showed a similar performance against Gram-positive and Gram-negative strains, with MIC values between 79.38 μg·mL^{-1} against MRSA and MSSA and 39.96 μg·mL^{-1} against *S. epidermidis* and *P. aeruginosa* (Fig. 3b).

a



b

Strain	HDP							
	LAP µg·mL^{-1}	µM	HβD2 µg·mL^{-1}	µM	HβD3 µg·mL^{-1}	µM	HD5 µg·mL^{-1}	µM
Gram-positive								
Methicillin sensitive *S. aureus* (MSSA)	118.13	3.75	30.31	0.94	62.5	1.88	79.38	2.5
Methicillin resistant *S. aureus* (MRSA)	236.25	7.5	60.63	1.88	31.25	0.94	79.38	2.5
Methicillin resistant *S. epidermidis*	118.13	3.75	121.25	3.75	62.5	1.88	39.69	1.25
Gram-negative								
P. aeruginosa	118.13	3.75	121.25	3.75	250	7.5	39.69	1.25

Figure. 3 | Minimal inhibitory concentration of 1st generation antimicrobials. a. Minimal inhibitory concentration (MIC) assay of proteins based on LAP, HβD2, HβD3 and HD5 against methicillin resistant *Staphylococcus aureus* (■), methicillin sensitive *Staphylococcus aureus* (●), methicillin resistant *Staphylococcus. epidermidis* (▲) and *Pseudomonas aeruginosa* (▼). Each construct was tested at its maximum concentration and serial two-fold dilution to determinate MIC against the four tested microorganism. **b.** Summary of MIC values.

The last step along the evaluation of 1st generation antimicrobial potential was determining the capacity to eradicate bacteria biofilms. HβD3, HD5, and LL37-based proteins exhibited strong antibiofilm features in a dose-independent manner, reducing the biofilm survival almost by 100% in the three tested concentrations ($p<0.0001$) (Fig. 4). The biofilm eradication obtained by HβD2-based protein was also high but dose-variable since it worked at 1 and 10 μM but not at 5 μM. Finally, the morphological changes of *P. aerugionsa* and *MRSA* were assessed by electron microscopy after 5 min of incubation with 1st generation constructs. The bacteria controls without HDP-based proteins (Fig. 5a) exhibited smooth surfaces but those incubated with proteins appeared to clump and showed crenated surfaces for both HD5 and HβD3 (Fig 5b and 5c) along with the presence of sparse pores in the case of P. aeruginosa (Fig 5b). However, for LL-37 (Fig 5c) treatment cells appeared to be clumped and embedded in a whole layer of cell debris and a kind of mucus.

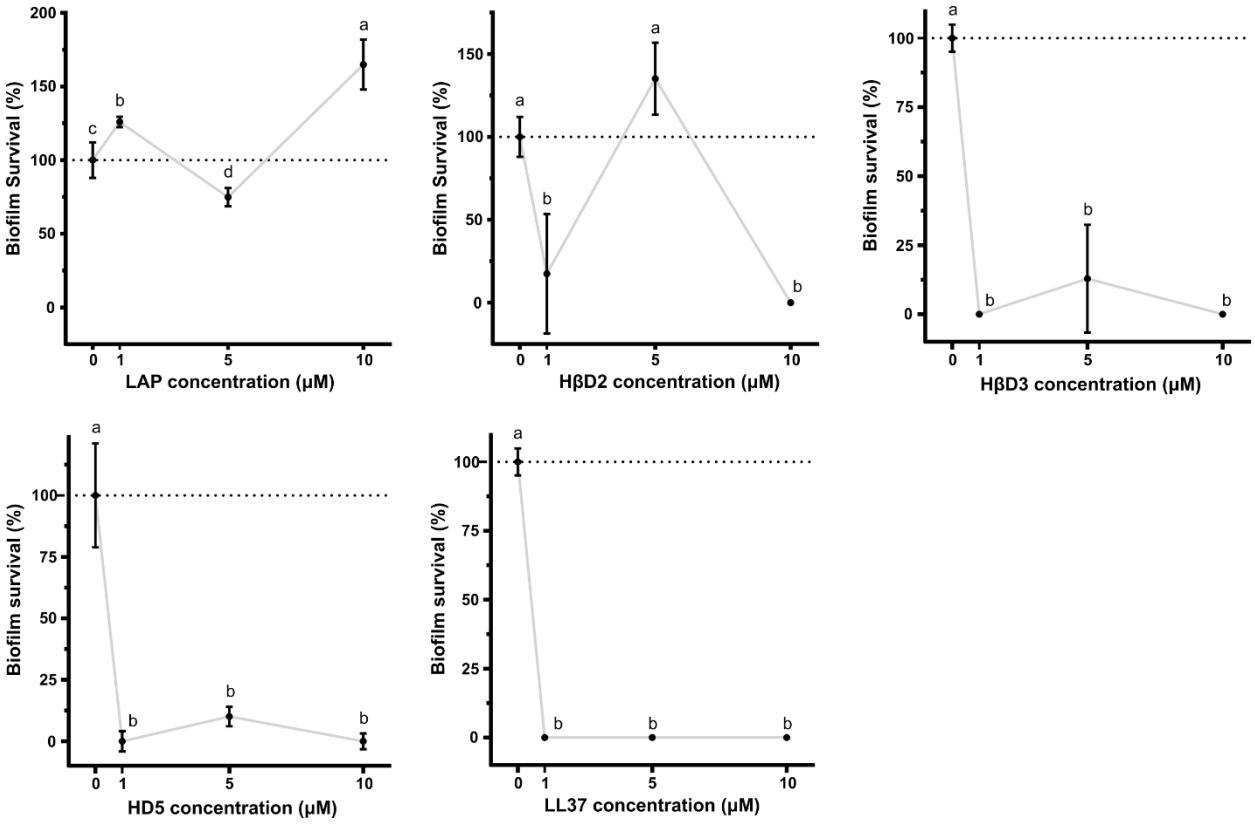
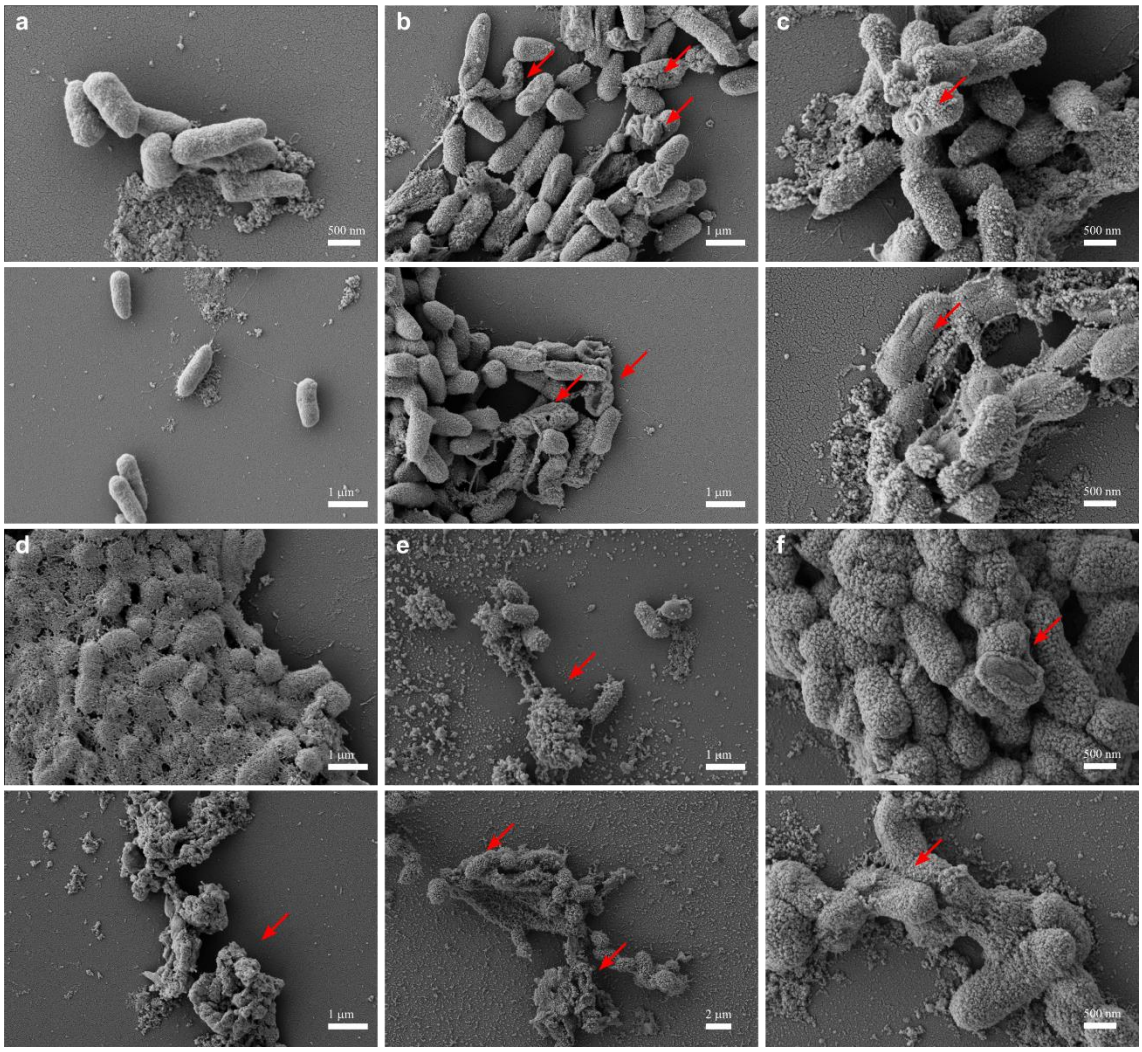


Figure. 4 | Antibiofilm performance of 1st generation molecules. Antibiofilm activity of the different 1st generation constructs at 10, 5 and 1 μM, against pre-formed biofilm of methicillin resistant *Staphylococcus aureus*. Data shown are the mean of triplicate ± SEM. Different letters represent statistically significant differences ($p<0.0001$) assessed by ANOVA and Tukey test analysis.

Pseudomonas aeruginosa



Methicillin resistant Staphylococcus aureus

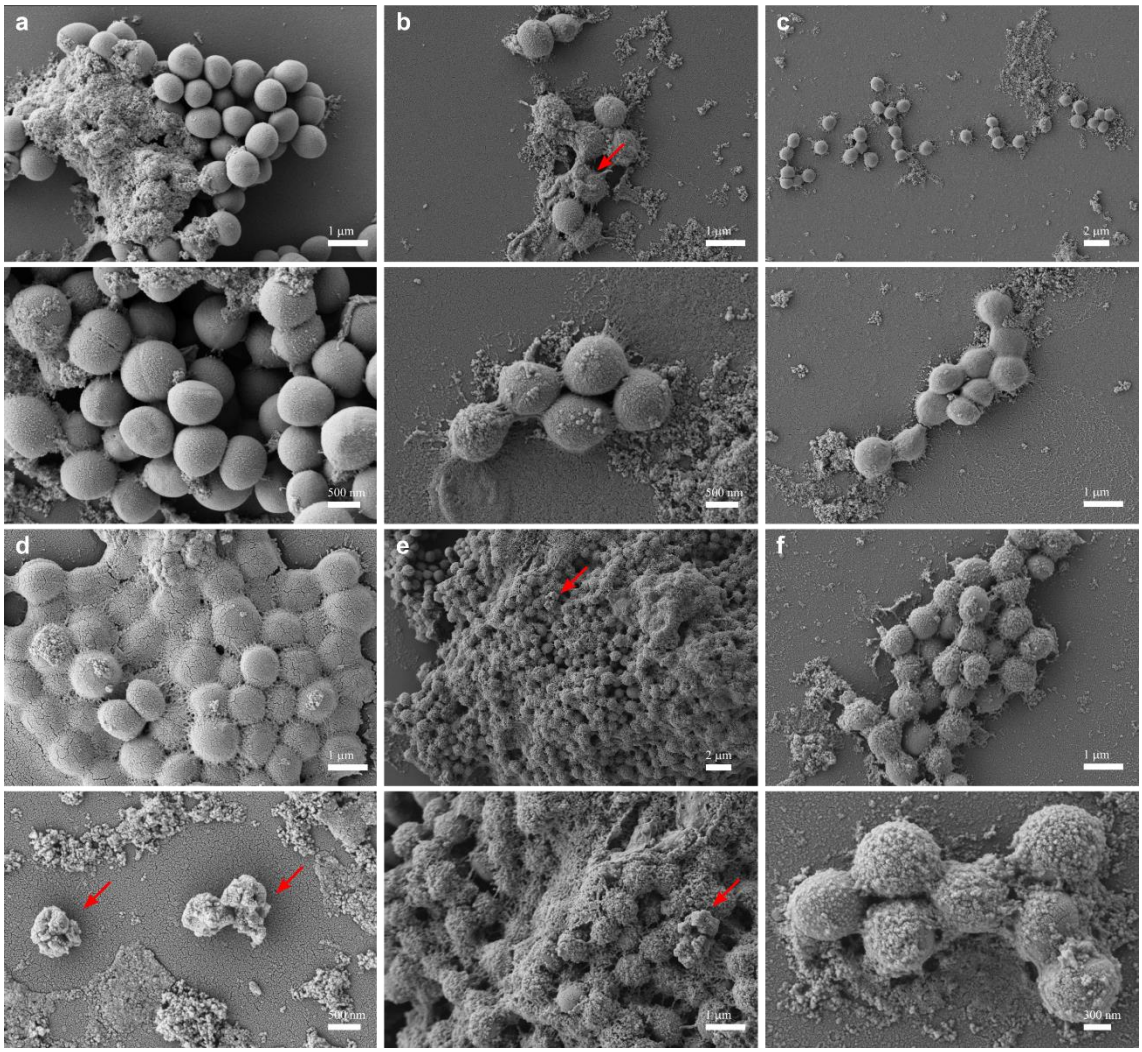


Figure. 5 | Analysis of the antimicrobial mechanisms by scanning electron microscopic (FE-SEM). FESEM cell integrity images of *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus* (MRSA) after **a.** control, **b.** HD5, **c.** HβD3, **d.** LL-37 1st generation constructs treatment and **e.** D5L37D5L37, **f.** D5L37βD3 multidomain proteins (2nd generation antimicrobial) treatment. All treatments were applied at 5 µM. Scale bar measure is indicated in each image. Red arrows pointed out relevant image areas.

Second generation of HDP-based antimicrobial proteins

After the three step-activity evaluation of first-generation proteins, HD5, LAP and HβD3 were selected for the modular protein design of the 2nd generation of antimicrobial proteins (Fig. 10). As a first proof of concept three proteins were constructed (Fig. 1B). The first construct, named D5L37βD3, was formed by the combination of the α-defensin HD5, the cathelicidin LL37 and the H□D3. The second construct, D5LAL37βD3, was structured like D5L37βD3 but with the

integration of the LAP flanked by HD5 and LL37. The last construct, D5L37D5L37. was designed by the duplication of the structural unit HD5 and LL37. All three constructs were produced successfully in *E. coli* at lower levels than 1st generation proteins but at good purity levels (Table 2). The D5L37D5L37 was purified from the soluble fraction and both D5L37βD3 and D5LAL37βD3 were solubilized from IBs and subsequently purified.

Table. 2 | Second generation antimicrobial protein yield (mg/L culture) and purity (%) of soluble D5L37βD3, D5L37D5L37 and D5LAL37βD3. [a] Yields calculated after protein purification.

Protein	Yield (mg L^{-1}) [a]	Purity (%)
D5L37βD3	0.44	97
D5L37D5L37	0.11	87
D5LAL37βD3	0.15	86

The antimicrobial potential of the 2nd generation molecules was evaluated also against the Gram-positive MRSA, MSSA, and *S. epidermidis* and Gram-negative *P. aeruginosa* at 10^5 cfu mL^{-1}. D5L37βD3 and D5L37D5L37 reduced 1.5-log the bacterial load of MRSA (Fig. 6a) and the total 5-log for MSSA (Fig. 6b), *S. epidermidis* (Fig. 6c), and *P. aeruginosa* (Fig. 6d) ($p<0.0001$). However, the construct D5LAL37βD3 did not show antimicrobial activity against the planktonic form any of the four tested pathogens (Fig. 6).

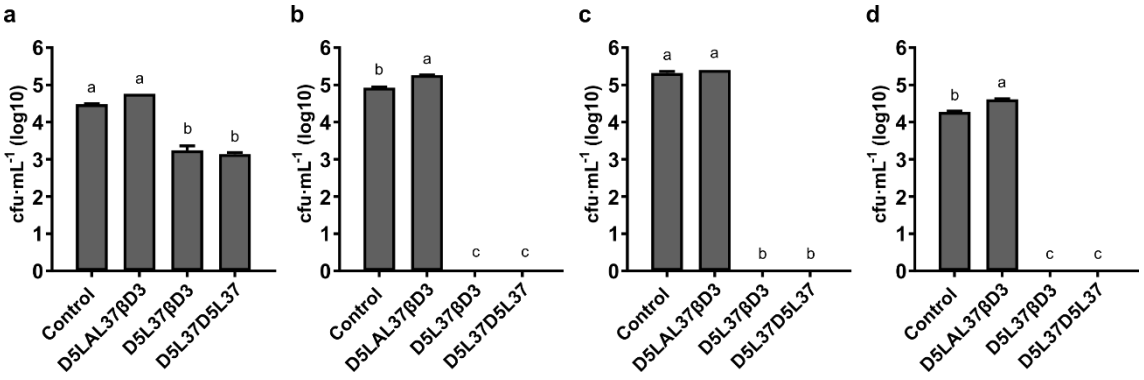
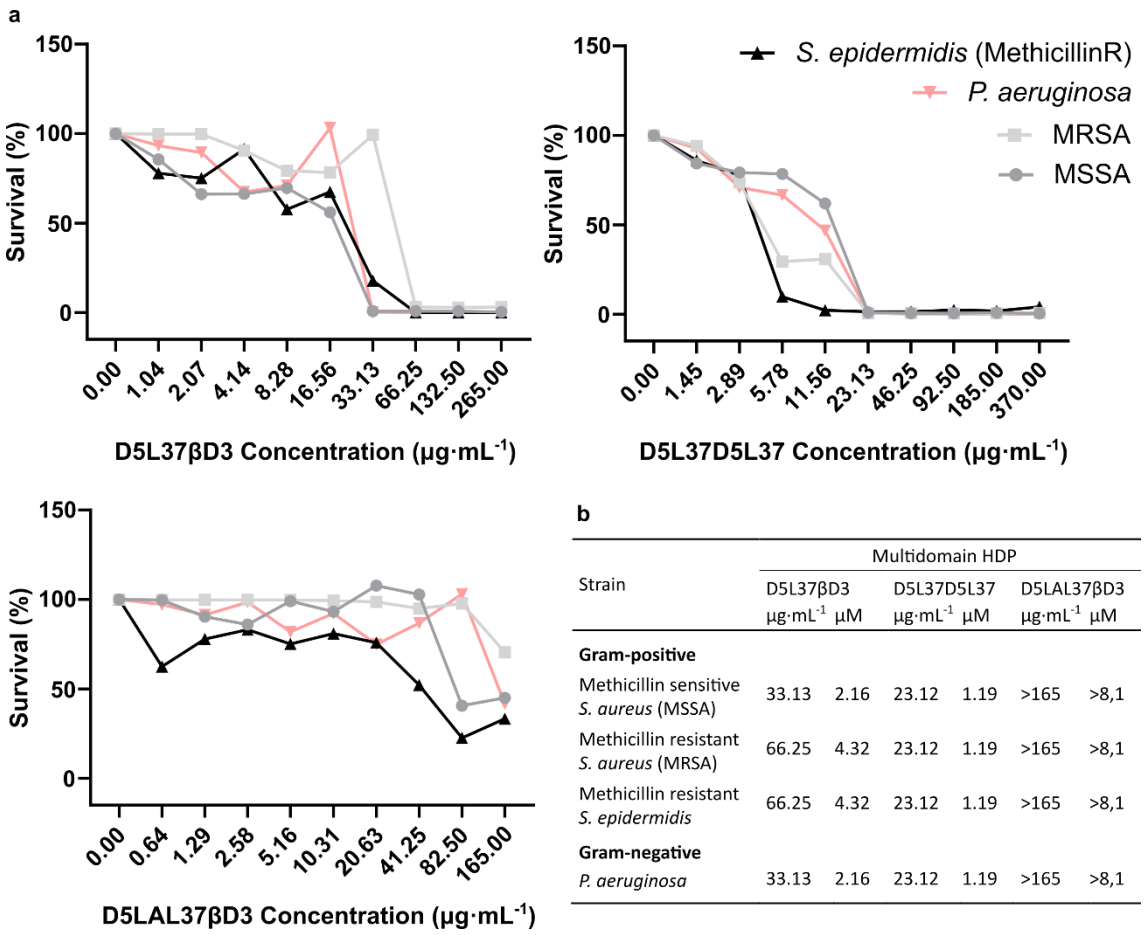


Figure. 6 | Bactericidal activity of second generation HDPs. Antimicrobial activity of D5L37D5L37, D5L37βD3, and D5LAL37βD3 multidomain constructs at 5 μM against **a**. methicillin resistant *Staphylococcus aureus* (MRSA), **b.** methicillin sensitive *Staphylococcus aureus* (MSSA) **c.** methicillin resistant *Staphylococcus. epidermidis* and **d.** *Pseudomonas aeruginosa*. All the constructs were tested against an initial 10^5 CFU/mL of each bacteria. Data shown are the mean of triplicate ± SEM. Different letters represent significant differences ($p<0.0001$) assessed by ANOVA and Tukey test analysis.

The MIC values of D5L37D5L37 (Fig. 7b) were the lowest achieved, being 26.88 μg·mL^{-1} for all tested organisms (MRSA, MSSA, *S. epidermidis*, and *P. aeruginosa*). D5L37βD3 construct had effectiveness against *S. epidermidis* and *P. aeruginosa* with a MIC of 31.25 μg·mL^{-1} in both cases and 62.50 μg·mL^{-1} for MRSA and MSSA (Fig. 7b). The MIC for the D5LAL37βD3 construct was greater than the maximum concentration that could be tested (Fig. 7a) and it was not possible to be determined. The final assay to evaluate the antimicrobial activity of 2nd generation proteins was the eradication of biofilms where the 3 proteins performed well either at 1 or 5 μM (Fig 8). The best biofilm inhibition rates (almost 100%) were achieved with D5LAL37βD3.



b

Strain	Multidomain HDP					
	D5L37βD3		D5L37D5L37		D5LAL37βD3	
	μg·mL^{-1}	μM	μg·mL^{-1}	μM	μg·mL^{-1}	μM
Gram-positive						
Methicillin sensitive *S. aureus* (MSSA)	33.13	2.16	23.12	1.19	>165	>8,1
Methicillin resistant *S. aureus* (MRSA)	66.25	4.32	23.12	1.19	>165	>8,1
Methicillin resistant *S. epidermidis*	66.25	4.32	23.12	1.19	>165	>8,1
Gram-negative						
P. aeruginosa	33.13	2.16	23.12	1.19	>165	>8,1

Figure. 7 | Optimized minimal inhibitory concentration of multidomain antimicrobial proteins. a MIC of the 2nd generation of antimicrobial constructs D5L37βD3, D5L37D5L37, and D5LAL37βD3 against methicillin resistant *Staphylococcus aureus* (■), methicillin sensitive *Staphylococcus aureus* (●), methicillin resistant *Staphylococcus. epidermidis* (▲) and *Pseudomonas aeruginosa* (▼). All constructs were evaluated at their maximum achieved concentration and a serial of two-fold dilution was performed to determinate MIC against the examined microorganism. **b**, MIC values summary.

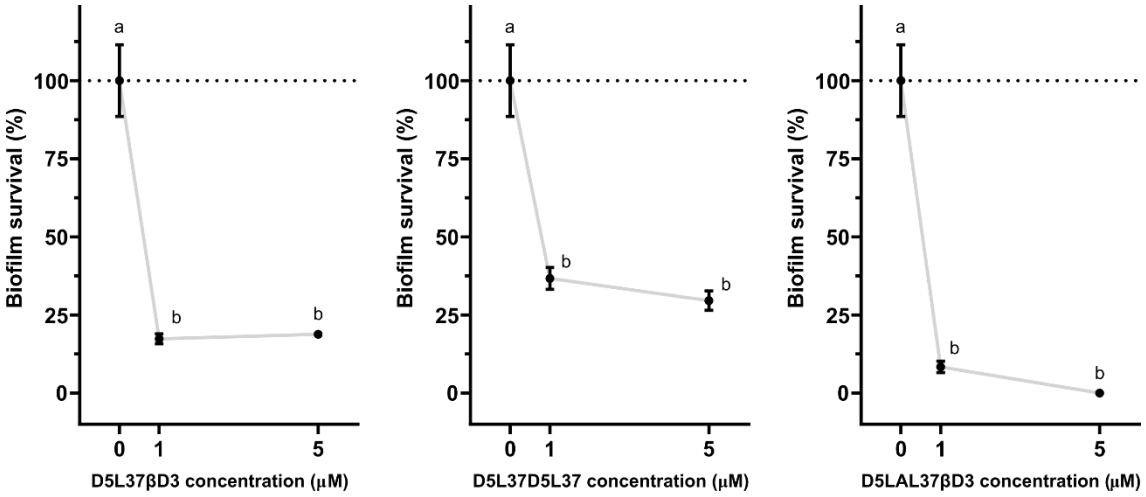


Figure. 8 | Antibiofilm performance of 2nd generation molecules. Biofilm eradication capacity of the different multidomain constructs D5L37βD3, D5L37D5L37, and D5LAL37βD3 at 10, 5, and 1 μM against MRSA pre-formed biofilms. Plots are the mean of triplicate ± SEM. Different letters indicate statistically significant differences (p<0.0001) assessed by ANOVA and Tukey test analysis.

To conclude, the morphological evaluation of *P. aerugionsa* and MRSA were analyzed by electron microscopy after 5 min of incubation with 2nd generation constructs. The non-treated bacteria (Fig. 5a) exhibited smooth surfaces in contrast with bacteria incubated with antimicrobials, which exhibited in the two tested strains rough and micelle-like surfaces for both D5L37D5L37 and D5L37βD3 multidomain proteins (Fig 5e and 5f).

Physicochemical characterization of 1st and 2nd generation of Antimicrobials

The physicochemical features of both generation HDP-based proteins were assessed by dynamic light scattering (DLS) analysis (Fig. 9a and b). The HβD3-based construct exhibited a predominant peak at 8.83 nm, whereas the HβD2, LAP, LL37, and HD5 showed a larger particle size, varying from 23.1 to 40.9 nm (Fig. 9a). The LL37, LAP, and HD5-based construct profiles also pointed out the existence of multiple populations in a dynamic equilibrium, generating the appearance of multiple peaks instead of one (Fig. 9a). The 2nd generation molecules presented heterogeneous profiles among them (Fig. 9b), where peaks ranged from 1.95nm for D5L37βD3 to 1,163 nm in the case of D5LAL37βD3. Protein D5L37D5L37 showed a predominant peak of 10.8nm more similar to those found in 1st generation.

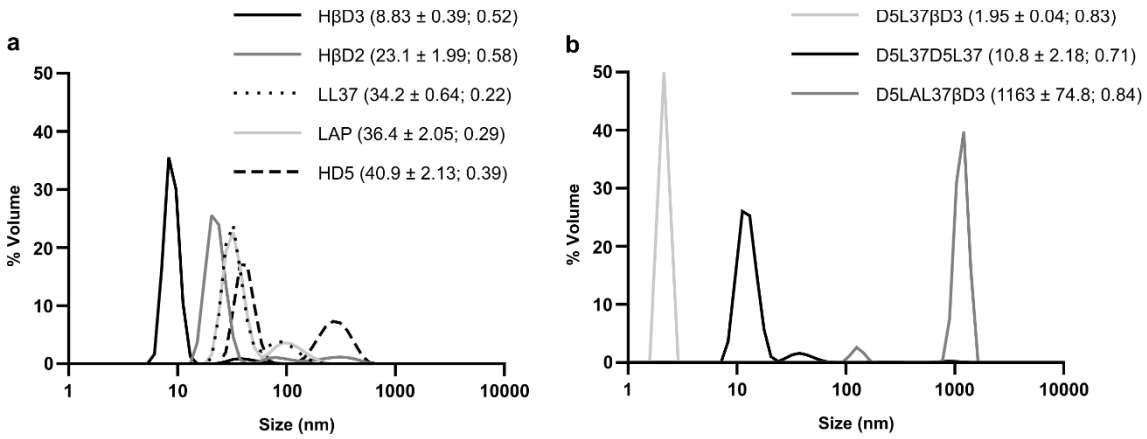


Figure 9. | Characterization of recombinant HDPs structuration. Size distribution plots of **a** 1st generation proteins based on HβD2, HβD3, LL37, LAP, HD5 and **b** 2nd generation proteins D5LAL37βD3, D5L37D5L37 and D5L37βD3. The mean size ± SEM and polydispersity index (PI) are indicated in brackets.

Discussion

In this study we have conceived a new strategy to customize the design of broad-spectrum HDP-based antimicrobials, even effective against multiresistant strains, exploiting the concept of recombinant multidomain proteins. The approach is based on a two-phase procedure (Fig. 10), starting with the construction of a 1st generation recombinant molecules based on a library of HDPs fused to the carrier protein GFP. The selection of the most active HDPs against the pathogens of interest is based on a triple activity assay against either planktonic or bacterial biofilm forms. The better performing HDPs proteins enter into phase 2, where they are combined in the same polypeptide without the carrier protein to give a 2nd generation antimicrobials, more potent and without any carrier protein (Fig. 10). A final triple activity screening allows the isolation of at least one broad-spectrum antimicrobial against the group of target pathogens (Fig.10).

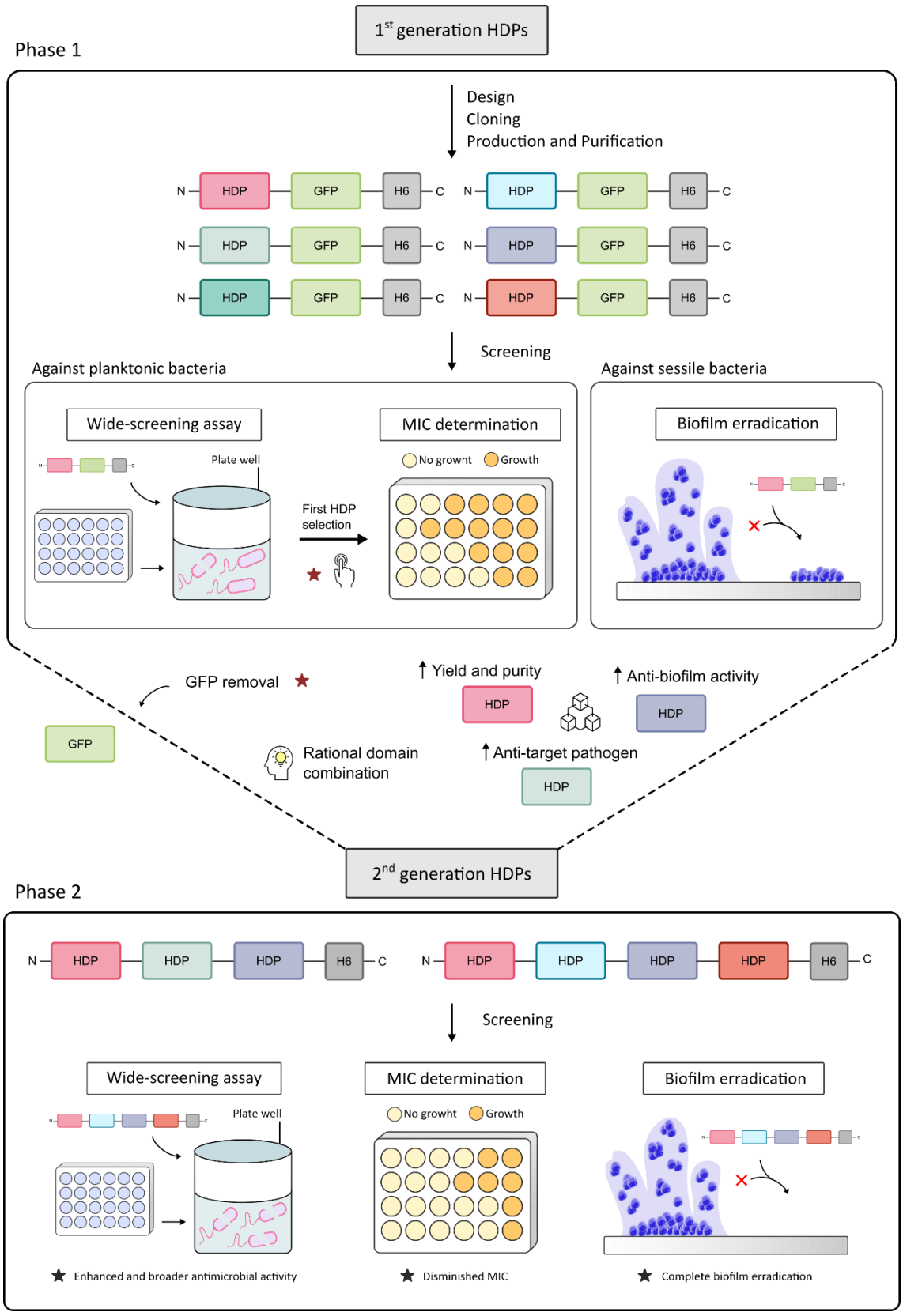


Figure 10. | Scheme depicting the followed approach for the generation of enhanced broad-spectrum antimicrobials. 1st generation of HDPs linked to a GFP carrier was evaluated in a triple assay, allowing the selection of the most promising to generate in phase 2 the 2nd generation of antimicrobials, devoid of a non-functional carrier, fully tunable and with enhanced antimicrobial features.

The results obtained herein proved that the 1st generation antimicrobials were successfully produced and mostly at good yields and purity (Table 1), whereas 2nd generation molecules, although maintaining good purity, were produced at lower yields (Table 2). This suggested that recombinant host toxicity was reduced in the 1st generation proteins, probably because of the carrier protein (GFP) presence, compensating the HDPs sequence. compensating the HDPs sequence. By contrast, the 2nd generation proteins did not present any carrier protein and hold several antimicrobial domains per polypeptide produced, which has a final negative impact on the protein yield. However, in spite of the lower yields of 2nd generation proteins, they are good enough to be produced and purified at reasonable and scalable levels. At the same time, these multidomain proteins avoid the need of using a carrier protein, which allows the selection of a final antimicrobial drug formed by only HDPs. Altogether this proved that a two-phase procedure is really worthy to take advantage of a carrier protein for a wide screening of HDPs against target pathogens to design multidomain proteins combining the most promising ones.

The antimicrobial activity obtained with the 1st generation molecules, except for HβD3, was not dependent on Gram-positive and Gram-negative microorganisms but has an effect which is pathogen-specific (Fig 2 and Fig. 3). Same profile was also confirmed in the 2nd generation of molecules. HβD3 showed a preferred antimicrobial activity against the Gram-positive MRSA, MSSA, and *S. epidermidis* in contrast to Gram-negative *P. aeruginosa* (Fig. 3b). These differences in performance might be supported by structural bacterial wall composition between Gram-positive and Gram-negative bacteria. However, the rest of HDPs were strain-specific probably because although the main mechanism of cell death is based on membrane disruption, the HDPs can also penetrate the bacterial cell wall and interfere with a vast array of intracellular targets [28], inhibiting DNA replication or bacterial protein synthesis, leading the cell to die.

In the first screening assay, two initial culture concentrations of bacteria (10^5 cfu·mL^{-1} and 10^3 cfu·mL^{-1}) were used and it could be observed that 10^5 cfu·mL^{-1} was the optimal one to finely evaluate the antimicrobial potential (Fig. 2). Although with most of the proteins a total killing could be observed at 10^3 cfu·mL^{-1}, in some cases the protein activity could be overestimated working at this concentration. This is the case of LAP-construct, which killed all the culture at lower culture density but showed less performance than other constructs at 10^5 cfu·ml^{-1} (Fig. 2). LL37 construct was discarded from this first screening assay, due to its low efficiency against planktonic cultures tested (Fig 2). Thus, the MIC of all the 1st generation molecules, except LL37, were determined (Fig. 3). The MIC assay determines the minimal concentration of an antimicrobial necessary to inhibit bacterial growth. It is a time-consuming assay and requires a greater amount of protein than testing proteins at only one concentration so we reasoned that for those constructs which does not inhibit 100% bacterial growth at 5 μM with an initial culture concentration of 10^3 cfu·mL^{-1} were not further evaluated with the MIC assay. However, for the

rest of the 1st generation molecules MIC determination allowed us to have a more accurate idea of their antimicrobial capacity (Fig. 3b). HDPs with similar activities in Fig. 2 showed clear differences in MIC values (Fig. 3b), proving that this assay is a complementary tool to evaluate antimicrobial capacity. For all MIC determinations, the selected protein concentration was 5 μM based on a curve representing the efficiency of a pull of first-generation proteins against several bacterial species (Fig 1S). The representation clearly showed that at 5 μM all proteins reached their maximum antimicrobial activity. HD5 and HβD3 1st generation constructs were the most potent antimicrobial domains from a broad-spectrum point of view, while LAP construct showed the lower activity (Fig. 3). HβD2-based construct performed very well against MSSA, but the MIC values for MRSA, *S. epidermidis*, and *P. aeruginosa* were worse than those obtained with HD5 and HβD3-based molecules (Fig 3).

The last activity assay performed with the 1st generation was the biofilm eradication, where all the proteins were tested independently of the results obtained with planktonic cells (Fig. 4). To perform this analysis, MRSA was chosen as an indicator strain for this assay because was the most consistent bacteria forming biofilms within all four pathogens (data not shown). Bacteria embedded in a biofilm undergo several phenotypic modifications, which in conjunction with their slow growth and poor diffusion of the antimicrobial compounds due to the extracellular matrix, altogether hamper bacterial killing. In accordance with this, despite the significant antimicrobial activity showed by LAP against planktonic bacteria (Fig. 2 and Fig. 3), it is not effective against biofilms (Fig. 4). On the contrary, HβD3 and HD5-based proteins, selected previously for their good activity in planktonic cultures (Fig. 2 and Fig. 3), had also good activity against biofilms (Fig. 4). Finally, the LL37 protein, which has a bad performance against planktonic cultures, was the best candidate against biofilms of MRSA. This difference in LL-37 performance can be attributed to its well-known activity affecting the quorum sensing of the biofilm and hence its development, together with this cathelicidin antibiofilm properties even at lower concentrations than MIC value [13]. In fact, the electron microscopy images (Fig 5) showed that LL-37 performed differently than other HDPs since the morphological aspect of treated bacterial cells was surprisingly different. The images suggested that LL37 is able to affect the whole culture at once but not from a single cell point of view.

Considering the results obtained from the triple activity assay (Fig. 10 Phase 1), the selected domains were both HβD3 and HD5 due to their potent antimicrobial (Fig. 2 and Fig. 3) and antibiofilm activities (Fig. 4), LAP, which had also good performance against planktonic bacteria (Fig. 2 and Fig. 3) and elevated productions yields (Table 1), and LL37, which exhibited the strongest antibiofilm properties (Fig. 4). Combining these HDPs, we evaluated three plausible multidomain candidates, still, the potentiality of this approach allows us to structure a myriad of

combinations, improving the performance in a rational strategy (Fig. 10 Phase 2). Thus, the 2nd generation constructs were D5L37D5L37, structured by the gathering of two HD5 and two LL37 motif, the D5L37βD3, linking up the HD5 and LL37 with de HβD3 and D5LAL37βD3, which was identical to D5L37βD3 with the addition of LAP domain flanked by HD5 and LL37 (Fig. 1). The domain combination triggered a synergistic effect, which is directly reflected in enhanced bactericidal activity (Fig. 6). In fact, the HD5 construct of the 1st generation was only able to reduce 3-log the bacterial survival of MSSA (Fig. 2), whereas the D5L37D5L37 construct showed a 5-log reduction in this strain (Fig. 6). In general, both D5L37D5L37 and D5L37βD3 exhibited a high antimicrobial performance against MSSA, *S. epidermidis*, and *P. aeruginosa*, whereas the MRSA strain was more resistant to the treatment (Fig. 6). This activity improvement can be clearly reflected in the MIC assay, where the values ranged from 62.50 to 26.88 $\mu g \cdot mL^{-1}$ (Fig. 7b). Remarkably, the construct D5L37D5L37 exhibited the lowest MIC values, perhaps indicating the role of domain repetitions in antimicrobial performance to be further evaluated extensively. *De facto*, when MIC values are expressed in molarity (Fig. 3 and Fig.7) it is possible to confirm the high efficiency of HDP-based recombinant proteins. The 2nd generation D5L37D5L37 protein was the best broad-spectrum antimicrobial selected, presenting MICs of 1.19 μM against all pathogens. This contrast with the MICs of best performing hybrid synthetic peptides already published which were 2 μM and 4 μM for *P. aeruginosa* and *S. aureus* respectively [29]. Surprisingly, the D5LAL37βD3 construct, although showed good anti-biofilm activity (Fig. 8), did not show any bactericidal activity against planktonic bacteria (Fig. 6 and Fig. 7) which indicates probably an incorrect domain structure or folding. Accordingly, the DLS peaks indicated that the 1st and 2nd generation proteins might be structured in dimers or oligomers, but D5LAL37βD3 presented a 1,163 nm peak, which indicated high aggregation that possibly impairs its activity.

Conclusion

In this study we have developed and proved a novel strategy to generate new broad-spectrum antimicrobials based on HDPs. A bi-phase strategy combines a first selection of single HDPs candidates (1st generation molecules) with good antimicrobial activity against a group of target pathogens with a second phase process in which selected HDPs are combine in a 2nd generation molecules to have a synergistic effect of the most active peptides in a single molecule. Their cost-effective recombinant production and the versatile function of HDPs of this new class of antimicrobials allows covering the gap of obtaining effective drugs against antibiotic resistant strains though an easily scalable platform.

Methods

Bacterial Strains

Escherichia coli BL21 (DE3) was used for recombinant protein expression. To evaluate antimicrobial activity the strains selected were methicillin-sensitive *Staphylococcus Aureus* (MSSA, ATCC-3556), methicillin-resistant *Staphylococcus Aureus* (MRSA, ATCC-33592), methicillin-resistant *Staphylococcus epidermidis* (ATCC-35984), and *Pseudomonas aeruginosa* (ATCC-10145). *E. coli* strains were grown in Luria-Bertani (LB) medium and MRSA, MSSA, *S. epidermidis*, and *P. aeruginosa* were grown in Brain-Hearth Infusion (BHI) broth (Scharlau).

Genetic construct design

The 1st generation of molecules was based on the mature sequences of lingual antimicrobial peptide (LAP; Uniprot entry Q28880, V25-K64), human β-defensin 2 (HβD2, Uniprot entry O15263, G24-P64), human β-defensin 3 (HβD3, Uniprot entry P81534, G23-K67), human α-defensin 5 (HD5, Uniprot entry Q01523, A63-R94), cathelicidin LL37 (Uniprot entry P49913, L134-S170) fused to the green fluorescence protein (GFP) gene through the linker sequence SGGGSGGS. The gene for 2nd generation construct D5L37D5L37 comprised the combination of the repeated HD5, LL-37 motif, forming HD5-LL37-HD5-LL37 construct. The gene encoding for D5LAL37βD3 consisted of the HD5, LAP, LL37, and HβD3 sequences and D5L37βD3 construct was identical to D5LAL37βD3 removing the LAP domain. The same linker sequence (SGGGSGGS) was used to connect domain-domain sequences in 2nd generation molecules but removing the GFP gene. All constructs were C-terminally fused to an H6-tag for protein purification and were codon-optimized for *E.* coli platform by GeneArt (GeneArt®, Life technologies, Regensburg, Germany), cloned into pET22b (AMP^{R}), and transformed by heat shock in competent *E. coli* BL21 (DE3) cells.

Antimicrobial protein production

Protein production cultures (1-2 L) were performed in erlenmeyer flasks containing 500 mL of LB medium supplemented with 100 μg/mL Ampicillin. Reinoculated flasks at OD600 0.05 were grown at 37 ºC and 250 rpm until reached an OD_{600}= 0.4-0.6. Then, protein expression was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cultures were grown 3h post-induction and cells were harvested by centrifugation (6,000 x g for 15 min at 4ºC). The resultant pellet was stored at -80 ºC until purification.

Soluble protein purification

For soluble protein, the whole cell pellet was resuspended in binding buffer (Tris 20 mM, pH 8.0, NaCl 500 mM and imidazole 20 mM) with EDTA-free protease inhibitor cocktail (Complete EDTA-free, Switzerland, Roche) and either sonicated for 1L 1st generation cultures (4 rounds, 5 min, at 10% amplitude under 0,5 s cycles) (Branson SFX550 Sonifier) or disrupted for 2L 2nd generation cultures (1 round, 20 KPsi) (Constant Systems CF1 disruptor). After that, cultures were centrifugated for 45 min at 15,000 x *g* and 4 ºC and the supernatant was recovered and filtered (Ø 0.2 μm). Resultant soluble protein was purified by Immobilized Metal Affinity Chromatography (IMAC) in ÄKTA Start (GE Healthcare) using 1mL HisTrap chelating HP columns (GE Healthcare). The selected fractions were dialyzed in 0.01% acetic O/N at 4ºC with gentle agitation. Protein quantity and integrity were determined by Western blot using an anti-His antibody (Santa Cruz Biotechnology) and Coomassie gels, further analyzed by ImageJ software [30].

Protein solubilization from IBs

D5LAL37βD3 and D5L37βD3 culture pellets were resuspended in PBS 1X with EDTA-free protease inhibitor cocktail (Complete EDTA-free, Switzerland, Roche) and disrupted, (1 round, 27 KPsi) (Constant Systems CF1 disruptor). Protein aggregates were centrifugated for 45 min at 15,000 x *g* at 4 ºC and the supernatant was discarded. Protein pellet was washed twice with distilled water and centrifugated 45 min at 15,000 x *g* at 4 ºC. Then, pellets were weighted and solubilized in 40 mM Tris pH 8 buffer (ratio 40 mL per gram of pellet) under non-denaturing conditions for 40 h at room temperature (RT) with gentle agitation and protease inhibitors. After the incubation, NaCl (500mM) and imidazole (20mM) were added to equilibrate the solubilized protein with the binding buffer used in ÄKTA and then samples were centrifugated at 45 min at 15,000 x g at 4 ºC. Further, supernatant was recovered and filtered (Ø 0.2 μm) to be subsequent purified by Immobilized Metal Affinity Chromatography (IMAC) using 1mL HisTrap chelating HP columns (GE Healthcare). Shorty, the sample was loaded with binding buffer (500 mM NaCl, 20 mM Tris, 20 mM Imidazole) and eluted with an increasing gradient of imidazole, mixing both binding and elution buffer (500 mM NaCl, 20 mM Tris, 500 mM Imidazole). The selected fractions were gathered and dialyzed against a 0.01% acetic buffer (w/v) O/N at 4 ºC with gentle agitation. Lastly, the next day protein was aliquoted, centrifugated 15 min at 15,000 x *g* at 4 ºC and stored at -80 ºC until use.

Broad Screening Antimicrobial Assay

Bacterial cell viability was evaluated with the BacTiter-Glo ™ Microbial Cell Viability assay (Promega). Shortly, an O/N culture of the selected strain (MRSA, MSSA, *S. epidermidis, or P.*

aeruginosa) was reinoculated in 10 mL of fresh BHI broth and grown at 250 rpm and 37 ºC until an exponential growth phase was reached (OD_{600}= 0.4-0.6). The cfu mL^{-1} was calculated for each strain (OD vs cfu mL^{-1} correlation equation) and diluted in 10 mM of KPi buffer at 10^6 and 10^4 cfu mL^{-1}, respectively. Then, 150 µL from the bacterial diluted stock (10^6 and 10^4 cfu mL^{-1}) was centrifugated at 6,200 x *g* at 4º for 15 min. The supernatant was removed, and the bacterial pellet was resuspended with 150 µL of either acetic acid 0.01% (negative control) or 5 µM of antimicrobial protein treatment and disposed in a sterile 96-well plate of polypropylene (Costar). After sample incubation for 5 h at 37 º C, 100 µL were withdrawn and mixed with 100 µL of the BacTiter-Glo™ reagent in an opaque microtiter plate (ThermoFisher). The plate was gently shaken and incubated for 5 min and then luminescence was measured using a microplate luminometer (LumiStar, BMG LABTECH). The registered arbitrary luminescence was normalized against the control. As a control, 100 µL of the sample incubation was serially diluted in ringer 0.9% NaCl (4 folds) and 100 µL was plated into BHI agar petri dishes to validate the bacterial viability of each well. All the conditions were plated in triplicate.

Determination of Minimal Inhibitory concentration (MIC)

The MIC of the different antimicrobial constructs was evaluated using a broth micro-dilution method with slight modifications. O/N cultures with test strains (MRSA, MSSA, *S. epidermidis*, or *P.* aeruginosa) in Mueller Hinton Broth Cation-adjusted medium (MHB-II, Sigma-Aldrich) were diluted in fresh MHB-II 10% (v/v) to contain 10^6 cfu mL^{-1} (colony forming units per mL). Then, 73 µL of bacterial suspension was dispensed in each well of a 96-well polypropylene microtiter plate (Costar) from column 2 to column 11. The first and the last column was used for medium sterility control and blank. Tested proteins and peptides were previously lyophilized and reconstituted in 0.01% acetic buffer to achieve a higher initial concentration. Then, a two-fold serial dilution of each tested protein in 0.01% acetic buffer was performed and 37 µL of treatment was added to each well from column 2 to 10, being column 11 a bacteria growth control (37 µL of 0.01% acetic buffer added). As a control, vancomycin antibiotic was used to validate this strategy for MIC values of MRSA, MSSA, *S. epidermidis* and meropenem for *P. aeruginosa* (Fig 2S). After antimicrobial treatment application, the plate was gently agitated and then incubated without agitation at 37 ºC for 24h. Bacterial viability was measured with the BacTiter-Glo ™ Microbial Cell Viability assay, mixing 100 µL of the incubated sample with 100 µL of the BacTiter-Glo™ reagent in an opaque microtiter plate, as previously described.

Biofilm Eradication Assay

Antibiofilm activity of each antimicrobial construct was assessed on pre-formed MRSA biofilms following the methodology described by Hancock *et al* [31] with certain adjustments. Briefly, an

MRSA O/N was grown in Tryptic Soy Broth (TSB, Thermo Fisher Scientific) at 37 ºC and 250 rpm. Then, fresh TSB supplement with 1% glucose (w/v) was inoculated with O/N culture at OD_{600} = 0.03. A total of 100 µL of bacterial dilution was added into 96-well microtiter plate and incubated at 37 ºC for 24 h without agitation to allow biofilm attachment and development (the wells in the edge of the plate were not used to growth biofilm and they are employed as media and evaporation control). After the incubation, the supernatant with planktonic bacteria was removed and the biofilm was rinsed per triplicate with 150 µL of fresh TSB. Then, 100 µL of sterile TSB was added to each well, followed by 100 µL of 20, 10, 2 µM of antimicrobial constructs to test the proteins at a final concentration of 10, 5, and 1 µM, or 100 µL of 0.01% acetic buffer as a control. The plate was again incubated 24 h at 37 ºC under static condition. The next day, supernatant was removed, and the wells were rinsed three times with 250 µL of Ringer solution (0.9% w/v NaCl), then fixated with 250 µL methanol for 10 min at RT. Methanol was discarded, and the plate was dried at 37 ºC for 15 min. Lastly, the plate was stained with 1% (v/v) crystal violet for 20 min at RT, washed three times with sterile distilled water, and the stained remaining biomass was resuspended in ethanol 70% v/v. The absorbance was recorded at 595 nm and the amount of biofilm eradication was calculated against the biofilm grown in the control. All measurements were done by triplicate in sterile conditions.

SEM imaging of antimicrobial effects

Ultrastructural effects of 1st and 2nd generation constructs were assessed in *P. aeruginosa* and MRSA cultures. Shortly, an O/N culture of both strains was 100-fold diluted in 10 mM KPi buffer. Then, 500 µL from the diluted bacteria was aliquoted and centrifugated at 6,200 x *g* at 4º for 15 min. The supernatant was removed, and the bacterial pellet was resuspended with 500 µL of antimicrobial construct at 5 µM or acetic acid 0.01 % (negative control). The treatments were disposed over cover glasses in a sterile 24-well plate and incubated 5 min at 37 ºC without agitation. After that, the supernatant was withdrawn and the samples were fixed with 500 µL of 2.5% (v/v) glutaraldehyde (Merck) in 100 mM of phosphate buffer for 2 h at 4 ºC. Following, the cover glasses were washed with 100 mM phosphate buffer and fixed with 1% (w/v) osmium tetroxide-potassium ferrocyanide for 2h. The samples were washed with miliQ water, dehydrated in a graded series of ethanol (50, 70, 90, 96, and 100% v/v) at RT and desiccated with hexamethyldisilazane (HMDS). Before the microscopy observation, samples were metal-coated and then observed in FESEM Merlin (Zeiss) operating at 3 kV.

DLS measurements

The volume size distribution of 1st and 2nd generation molecules was determined in a Zetasizer Pro (Malvern Instruments Ltd, Malvern, UK) by dynamic light scattering (DLS). A 100 µL

aliquot (stored at -80 ºC) was thawed and then centrifugated at 15,000 x *g* 15 min at 4 ºC to remove non-specific aggregates. Further, the supernatants were measured in triplicate, and the average size and polydispersity index (PI) were displayed as mean ± SEM.

Statistical Analysis

Results are expressed as the means of non-transformed data ± standard error of the mean (SEM). Data were obtained in triplicate and normality was checked using JMP software (SAS Institute Inc.), being transformed when required. The *p*-values (statistically significant when $P < 0.05$) and letters correspond to the ANOVA and Tukey test analyses, respectively.

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Author Contributions

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Writing-Original Draft: López-Cano, A., Garcia-Fruitós, E., Arís, A.
Writing-Review: Garcia-Fruitós, E., Arís, A

Competing Interest statement

All authors contributed to manuscript revision, read, and approved the submitted version. The authors state no conflict of interest.

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STUDY 4

POTENTIAL OF ORAL NANOPARTICLES CONTAINING CYTOKINES AS INTESTINAL MUCOSAL IMMUNOSTIMULANTS IN PIGS: A PILOT STUDY

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Preface

In the pursuit of antibiotic alternatives to cope with resistant and multidrug resistant bacteria, immunostimulants can play an essential role. As earlier mentioned, the HDPs hold immunostimulant features, being able to boost and modulate immune responses to efficiently take down disease-causing pathogens. Besides, immunostimulants can be applied synergically with other therapies, enhancing the bacterial clearance and comeback to the organism's basal state. Nevertheless, as a proof of concept, we decided to evaluate the potential of the immunostimulants using cytokines, which are pivotal molecular effectors of the immune system but do not have direct antimicrobial activity, as occurs with HDPs.

Previous studies carried out by Torrealba *et al*. [242] demonstrated the immunostimulatory potential of cytokines produced as protein nanoparticles (IBs), increasing substantially the survival of the treated fishes against an otherwise lethal bacterial infection. Hence, evaluating the multifaceted properties of the IBs, in the following study we have focused on their role as a drug delivery system (DDS), providing improved stability and biodistribution to the embedded compounds in this complex matrix. In addition, this nanostructuration is formed in one-step and cost/effective manner, making them even more engaging. Bearing in mind all these considerations, the aim of study 3 is to translate this approach to livestock animals, working on swine as a model. The target of our cytokine-based IBs was critical animal production stages, such as weaning, where the animal showed increased susceptibility to contract bacterial infections associated with productivity losses. Thus, immunostimulation prior to a well-known animal production cycle stressor could allow the animal to be able to face off opportunistic bacterial infections, avoiding antibiotic treatment and plausible resistance emergence.

POTENTIAL OF ORAL NANOPARTICLES CONTAING CYTOKINES AS INTESTINAL MUCOSAL IMMUNOSTIMUALNTS IN PIGS: A PILOT STUDY

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Simple Summary: Antibiotics are essential compounds to cope with bacterial infections. However, their inadequate and excessive use has triggered the rapid arising of antimicrobial resistant bacteria. In this scenario, the immunostimulants, which are molecules that boost the immune system, open up a new approach to face off this problem, enhancing treatment efficacy or preventing infections by immune system response. Cytokines are central effector molecules of the immune system, and their recombinant production and administration in animals could be an interesting immune modulation strategy. The aim of this study was the development of a highly stable nanoparticles format of porcine cytokines to achieve the immunostimulation of the intestinal mucosa in piglets. The outcomes of the present study proved how this approach was able to stimulate swine intestinal cells and macrophages *in vitro* and tended to modulate inflammatory responses *in vivo*, although further studies are required to definitively evaluate their potential in animals.

Abstract

Antimicrobial resistance is a global threat that is worryingly rising in the livestock sector. Among the proposed strategies, immunostimulant development appears as an interesting approach to increase animal resilience at critical production points. The use of nanoparticles based on cytokine aggregates, called inclusion bodies (IBs), has been demonstrated as a new source of immunostimulants in aquaculture. Aiming to go a step further, the objective of this study was to produce cytokine nanoparticles using a food-grade microorganism and to test their applicability to stimulate intestinal mucosa in swine. Four cytokines (IL-1β, IL-6, IL-8, and TNF-α) involved in inflammatory response were produced recombinantly in *Lactococcus lactis* in the form of protein nanoparticles (IBs). They were able to stimulate inflammatory responses in a porcine enterocyte cell line (IPEC-J2) and alveolar macrophages, maintaining high stability at low pH and high temperature. Besides, an *in vivo* assay was conducted, involving 20 piglets housed individually as a preliminary exploration of potential effects of IL-1β nanoparticles in piglet's intestinal mucosa after a 7-d oral administration. The treated animals tended to have greater levels of TNF-α in blood indicating that the tested dose of nanoparticles tended to generate an inflammatory response in the animals. Whether this response is sufficient to increase animal resilience needs further evaluation.

Keywords: Immunostimulant, Cytokines, Nanoparticles, Piglets, Antimicrobial Resistance

Introduction

Antibiotics are effective molecules to treat infectious diseases caused by bacteria. However, the overuse and misuse of these compounds have accelerated the emergence of antibiotic resistance, leading to the appearance of multiresistant bacteria that are easily transmitted between humans, animals, and the environment [1]. This has pushed the need to prioritize first-line antibiotics for human health, reducing their administration in livestock, and also to find new alternatives to the use of antibiotics to cope with resistant bacteria [2]. Antibiotic reduction in animal production mainly concerns preventive applications. In this context, new antimicrobial molecules are the focus of current research, but the use of immunostimulants to increase animal resilience at critical phases of animal production is a strategy that is also gaining interest [3]. For example, during transport, housing, or weaning processes livestock regularly suffer immunosuppression, metabolic dysregulation and, as a result, the development of concomitant diseases [4]. In this scenario, immunostimulants hold the potential to boost the immune response to act faster and more efficiently at mitigating opportunistic pathogen infections. Besides, immunostimulants administration to the mother could be a good strategy to increase the quality of the colostrum and

therefore the newborn immune status [5, 6]. Immunostimulants are substances (drugs or nutrients) that stimulate the complex and versatile biological network that compose the immune system. [7-9]. The development of immunostimulants in livestock is usually based on a non-specific activity for the activation of the innate immunity of the animal. Moreover, immunostimulants could be used as vaccine adjuvants, improving vaccine efficacy at stimulating specific immunity.

The application of immunostimulants at the gastrointestinal level is encouraging because they can be administrated as a feed additive that could target a wide variety of immune components involved in mucosal immunity and epithelial barrier function, comprising the microbiota, and extending its effect systemically [10]. In this context, compounds based on flavonoids, essential oils, probiotics, or prebiotics have been deeply explored for livestock applications [11]. However other molecules such as lipopeptidases, lipopolysaccharide (LPS), flagellin, CpG nucleotides, and cytokines are also attractive immunostimulants that have been less investigated for animal production [12].

Nanoparticles have been used as therapeutic agents in the human medical field for some time now, though their application in veterinary medicine and animal production is still relatively new. Torrealba *et al.* [13] proposed the use of nanoparticles based on cytokines aggregates named inclusion bodies (IBs) as a new source of immunostimulants. They proved that the use of IBs, which are highly stable nanoparticles, produced in a single-step and cost-effective way, showed an outstanding *in vivo* immune protection in fish against an otherwise lethal *Pseudomonas aeruginosa* challenge [13]. However, the use of these cytokine-based nanoparticles has not been investigated in other species. Thus, herein we have explored the concept of cytokine-based nanoparticles to boost the innate immunity driven by swine intestinal mucosa as a possible proof of concept to further develop applications focused on increasing animal resilience during stressful production periods.

Materials and Methods

Bacterial and culture strains

Lactococcus lactis subsp. cremoris NZ9000 [14] was used for heterologous protein expression. *L. lactis* was grown in M17 medium, supplemented with 0.5 % of glucose v/v (from now on GM17), as previously described [15]. Immunoassays were performed using intestinal porcine enterocytes cell line IPEC-J2 (DSMZ, German Collection of Microorganism and Cell Culture, Germany) cultured at 37 ºC and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with Fetal Bovine Serum (FBS) 10%, glutaMAX^{TM} 2mM (Thermo Scientific,

Applied Biosystems, Gibco), nystatin 0.5% v/v (Thermo Scientific, Applied Biosystems, Gibco), insulin-transferrin-selenium (Thermo Scientific, Applied Biosystems, Gibco), and penicillin-streptomycin (5.000 U/L, Thermo Scientific, Applied Biosystems, Gibco). Alveolar macrophages used in the immunoassays were isolated from pig bronchoalveolar fluid as previously described [16]. Briefly, after pig euthanasia, a bronchoalveolar lavage of the lungs was performed with 100 mL of sterile PBS supplemented with gentamicin at 70 μg/mL (Sigma-Aldrich, Madrid, Spain). Further, to collect the alveolar macrophages, the lavage fluids were centrifugated at 230 *x g* for 15 min, and cells were washed twice with DMEM containing gentamicin (50 μg/mL). Lastly, the alveolar macrophages concentration was adjusted to 1 x 10^7 cells/mL and aliquots were stored in DMEM with 10% of dimethyl sulfoxide (DMSO) and 20% of FBS.

Genetic construct design

Swine mature sequences of Interleukin-1β (IL-1β) (115-267, Uniprot entry P26889), Interleukin-6 (IL-6) (29-212, Uniprot entry P26893), Interleukin-8 (IL-8) (26-104, Uniprot Entry 26894), Tumor Necrosis Factor (TNF-α) (78-232, Uniprot Entry P23563) (using swine native sequences codon-optimized for its expression in *L. lactis*) and Green Fluorescence Protein (GFP) were chemically synthesized (GeneArt ®, Lifetechnologies, Regensburg, Germany). All of them were cloned in pMA-T (Amp^R) (GeneArt ®, Germany) vector. Each sequence was flanked by *NcoI* and *XbaI* restriction enzyme sequences, allowing subcloning of the genes in pNZ8148 (Cm^R; MoBiTech) vector, suitable for *L. lactis* expression system. All sequences also have a C-terminal 6 histidine tag for protein purification and quantification. Plasmids containing the sequences of interest were transformed into electrocompetent *L. lactis* NZ9000 strain, using a Gene Pulser (Bio-rad) at 2500V, 200 Ω, and 25 μF as described Cano-Garrido *et al* [17]**.**

Cytokine nanoparticles production and purification

Lactococcus lactis NZ9000/pNZ8148 containing each cytokine gene was grown overnight (O/N) at 30 °C in GM17, supplemented with 5 μg/mL of chloramphenicol (Cm). Next, fresh GM17 (5 μg/mL Cm) was inoculated with an O/N culture at an initial OD_{600} of 0.05. When cultures reached an OD_{600} = 0.4-0.6 were induced with 12.5 ng/mL of nisin, starting the heterologous gene expression. The recombinant proteins were produced along 3 h and bacteria were recovered by centrifugation at 6,000 *x g* for 30 min at 4 ºC. Then, supernatants were discarded, and bacterial pellets were resuspended in sterile PBS (ratio 30 mL PBS per 50 mL culture) and stored at -80 ºC until use. To purify cytokine-based nanoparticles, thawed bacteria were disrupted for 2 rounds at 40 KPsi (Constant Systems CF1 disruptor), ice-coated, and with protease inhibitors (cOmplete protease inhibitor cocktail EDTA-free, Roche). After a new freeze/thaw cycle, samples were incubated for 2 h with 0.01 mg/mL of lysozyme (Sigma-Aldrich) at 37 ºC and 250 rpm. A new

freeze-thaw cycle was followed by the addition of 4 µL/mL of Triton X-100 and subsequent incubation for 1 h at RT in an orbital rotator shaker. At this point, a sterility control was performed by plating a sample aliquot in agar-GM17 plates and incubating them O/N at 30 ºC. Further freeze-thaw cycles were carried out until no viable bacterial growth was detected. Following that, the mixture was incubated for 1 h with 0.25 µL of NP-40 per mL of sample at 4 ºC in a rotatory shaker, and then, 0.6 µg/mL DNase I and 0.6 mM $MgSO_4$ (Panreac, Barcelona, Spain) were added and incubated for 1 h at 37 ºC and 250 rpm. Samples were centrifuged at 6,000 *x g* for 30 min at 4 ºC. The pellet containing nanoparticles (IBs) was resuspended with 5 mL of lysis buffer (50mM Tris-HCl pH 8, 100 mM NaCl, 1 mM EDTA and Triton X-100 0.5% (v/v)) and frozen/thawed again. The resultant mixture was centrifuged at 6,000 *x g* for 30 min at 4 ºC and the pellet was resuspended in sterile PBS and aliquoted. Finally, a centrifugation at 15,000 *x g* for 30 min at 4 ºC was carried out, storing the IBs pellets at -80 ºC until use.

IBs aliquots were tested for sterility in agar-GM17 plates and incubating them overnight at 30 ºC. In addition, they were quantified by western blot using an anti-His antibody (Santa Cruz). Their purity was also evaluated by performing a Coomassie blue staining assay. Outcomes were analyzed by ImageJ software to determine both protein quantity and purity.

Immunoassays

IPEC-J2 cells were cultured at 37 ºC and 5% CO_2 until confluence and, after trypsinization, they were seeded in 24-well plates at a density of 100,000 cells/well. Alveolar macrophages were resuspended in DMEM medium (supplemented as explained before) and centrifuged at 560 x *g* for 10 min at 10 ºC. Then, the pellet was resuspended in fresh DMEM medium and seeded in 24-well plates at density of 100,000 cells/well. Then, prior to the immunoassay, medium was removed, followed by the addition of 300 µL of fresh medium and the resuspended cytokine-based IBs treatment in 200 µL of sterile PBS, reaching 500 µL/well. Each treatment was analyzed by sextuplicate. In both experiments, PBS, LPS, and GFP nanoparticles (IB^{GFP}) were used as negative control, positive control, and format control, respectively. The cultures were incubated for 16 h at 37 ºC and 5% CO_2. Supernatants from IPEC-J2 and alveolar macrophages were collected and kept at -80ºC and IPEC-J2 RNA was recovered using the TRIzol ® (Invitrogen) extraction method according to the manufacturer's instructions.

Gene expression analyses

RNA was quantified using NanoDrop™ device (ThermoFisher Scientific) and their integrity was analyzed by electrophoresis in 1.5% agarose gel. cDNA synthesis was performed using the PrimeScript RT reagent kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions. Besides, qPCR with SYBR green (SYBR Premix Ex Taq II, Perfect Real Time,

Takara Bio Inc,) was implemented on a BioRad real-time PCR thermocycler. Briefly, an initial denaturalization was performed at 95 ºC for 10 min. Next, 40 cycles of denaturalization at 95 ºC for 10 s and annealing/extension at 60 ºC for 30 s were performed. Finally, one cycle of 1 min at 95 ºC was carried out and the specificity of the amplified products was assessed by melting curve (61 cycles, thermal gradient of 65 to 95 ºC in 30 s). Several genes related to inflammatory profile (*β-defensin-1* (*BD1*), *β-defensin-2* (*BD2*), *IL-6, TNF-α* and intestinal integrity (*occludin and claudin-4* (*CLDN4*)) were analyzed in IPEC-J2 using the ribosomal protein L4 (*RPL4*) as housekeeping gene [18]. Primer sequences and parameters are reported in Table 1.

Table 1. Primers and PCR conditions (T º Annealing (ºC), optimal primer concentration (µM), and PCR product (bp)) for the selected target genes. Fw: Forward; Rv: Reverse; bp: base pairs

Target gene	Primer name	Sequence (5'-3')	Tº Annealing (ºC)	Conc (µM)	PCR product (bp)	Reference
Interleukin-6	IL6-Fw	CAAGGAGGTACTGGCAGAAA	60	0.25	185	
	IL6-Rv	CAGCCTCGACATTTCCCTTAT				
β-defensin 1	BD1-Fw	TGCCACAGGTGCCGATCT	60	0.25	81	
	BD1-Rv	CTGTTAGCTGCTTAAGGAATAAAGGC				
β-defensin 2	BD2-Fw	ACCTGCTTACGGGTCTTG	60	0.25	168	
	BD2-Rv	CTCTGCTGTGGCTTCTGG				
Tumor necrosis factor-α	TNFa-Fw	ATCGGCCCCCAGAAGGAAGAG	60	0.25	351	[19]
	TNFa-Rv	GATGGCAGAGAGGAGGTTGAC				
Claudin-4	CLDN4-Fw	CGCCCTCATCGTCATCTGTATC	60	0.25	121	
	CLDN4-Rv	GGCCACGATCATGGTCTTG				
Mucine-1	Muc1-Fw	GTGCCGACGAAAGAACTG	60	0.25	187	
	Muc1-Rv	TGCCAGGTTCGAGTAAGAG				
Occludin	Occludin-Fw	GCTTTGGTGGCTATGGAAGT	60	0.5	157	
	Occludin-Rv	CCAGGAAGAATCCCTTTGCT				
Ribosomal protein L4	RPL4-Fw	CAAGAGTAACTACAACCTTC	60	0.5	122	[18]
	RPL4-Rv	GAACTCTACGATGAATCTTC				
Glyceraldehyde 3-phosphate dehydrogenase	GDPH-Fw	GTCGGTTGTGGATCTGACCT	60	0.2	135	[20]
	GDPH-Rv	TCACAGGACACAACCTGGTC				
TATA-Box Binding Protein	TBP-Fw	AACAGTTCAGTAGTTATGAGCCAGA	63	0.2	153	[18]
	TBP-Rv	AGATGTTCTCAAACGCTTCG				

Enzyme-linked immunosorbent assay (ELISA)

The supernatants of both immunoassays were used for the determination of swine cytokines IL-6 and TNF-α secreted by the cultures under nanoparticles treatment using commercial ELISA kits (Kingfisher, London, UK) and following the manufacturer's instructions. Each sample was assayed in duplicate and diluted four times when required.

Temperature and pH IBs stability

Cytokine nanoparticles stability was tested mimicking swine gastrointestinal conditions and temperature experienced during their possible inclusion as a feed additive in piglets concentrate. To simulate gastrointestinal tract environment, nanoparticles were incubated for 2h at pH 4 and 37 ºC followed by 5h at pH of 6.5 and 37 ºC. On the other hand, to simulate the temperature potentially faced during the feed production process, the IBs were incubated for 1 min at 80ºC. Then, in both assays, a coomassie blue staining assay was performed to evaluate if the protein embedded was solubilized or degraded. For this, samples were centrifuged at 6,000 x *g* for 1 min and loaded on SDS-PAGE gel. Besides, an immunoassay using IPEC-J2 cells was conducted to evaluate if the nanoparticles immunogenicity was maintained after pH and temperature treatment.

In vivo assay

All animal experimentation procedures were approved by the Animal Ethics Committee (CEEAH) of the Universitat Autònoma de Barcelona (reference number: 9019/10548/2017) and were performed in accordance with the European Union guidelines for the care and use of animals in research (Directive 2010/63/EU).

A total of 20 piglets were selected for the study, ensuring the best litter homogeneity. Piglets were weaned around 21 days of age and were housed individually (1 animal/pen). All experimental basal diets were formulated to ensure piglet requirements. The feeding program included a creep feed (from weaning to 11 days after weaning (AW)), pre-starter feed (12-day AW to 27-day AW), and starter feed (from 28-day AW to 34-day AW) presented in mash form. Solid feed and water were offered *ad libitum* during the trials.

An initial phase of 11 days was conducted to acclimate the animals to the facilities and in the following week, the animals were submitted to an operant conditioning scheme to adapt them to the selected strategy of our target administration. Specifically, a round plate with 150 g of 0.5 M of sugar solution was offered every morning at 9:00 am until the animal finished its content

In the trial, 2 treatments were included (n=10 animals/each): control (animals received a sugar solution 0.5M) and treatment (animals received IL-1β nanoparticles in sugar solution 0.5 M.) The immunostimulant treatment based on IL-1β (20 µg/kg of BW) was applied for 7 days in a round plate following the trained routine previously described. Twenty-four hours after the last administration (d- 27), half of the animals of each treatment (n=5) were blood sampled and euthanized for tissue sampling. The rest of the animals were similarly sampled (for blood and tissues) 7 d after (d-34) the last administration. This 7-d sampling delay was decided in order to evaluate the effect of the nanoparticles slow-release over time in the piglet immune profile. The

concentration of inflammation-related proteins IL-8, IL-6, TNF-α, and IL-10 were quantified by ELISA in blood.

Statistical analysis

Immunoassays were performed per sextuplicate and cytokine stability experiments by triplicate, being all represented as the means of non-transformed data ± standard error of the mean (SEM). All data were tested for normality using JMP software (SAS Institute Inc.). Data was log-transformed when needed and analysed using the MIXED procedure of SAS (9.4, SAS Institute, Cary, NC). The model included treatment, day of tissue sampling and its interaction as main effect. Differences were declared significant at $P < 0.05$, and trends were discussed at $0.05 \leq P \leq 0.10$.

Results

Production and Characterization of Cytokine-based Nanoparticles

Four cytokines involved in inflammatory response, namely IL-1β, IL-6, IL-8, and TNF-α were produced recombinantly in *Lactococcus lactis* as protein nanoparticles (IBs). GFP was also produced as a non-immune related control nanoparticle. The protein yield of the nanoparticles and the estimated cytokine content are depicted in Table 2. The cytokine IL-8 was the best-produced nanoparticle whereas TNF-α was purified at such low levels that were not quantifiable by western blot. IL-1β, IL-6, and GFP control were produced at moderate yields ranging between 0.5 to 1.67 mg/L of culture. In all cases cytokines corresponded to 11 to 34 % of the nanoparticles composition, indicating that other proteins from the *L. lactis* host were also present.

Table 2. Cytokine IBs yields (mg/L culture) and recombinant protein content (%) of each cytokine nanoparticle produced in *L. lactis*. n.d: non-detected

Cytokine	Yield (mg/L) [a]	Recombinant Protein content (%)
Interleukin-1β	0.51 ± 0.20	16.19 ± 0.06
Interleukin-6	1.19 ± 0.12	34.40 ± 0.04
Interleukin-8	25.69 ± 3.49	23.39 ± 6.82
Tumor necrosis factor-α	n.d	n.d
GFP	1.67 ± 0.15	11.01 ± 1.39

[a] yield obtained after IBs purification process

Immunostimulation of swine intestinal cells and macrophages

The immunostimulation potential of the nanoparticles was tested on porcine intestinal cells and alveolar swine macrophages by monitoring the induction of TNF-α and IL-6 secretion (Table 3). The highest stimulation of alveolar macrophages was caused by IL-8 and IL-1β containing nanoparticles, boosting the secretion of TNF-α and IL-6, respectively. The positive control used was LPS and it performed equally to the IL-8 based nanoparticles (Table 3). The IL-6 and TNF-α cytokine-based nanoparticles did not increase the secretion of inflammation markers compared to basal levels of PBS treated cells or the negative control of GFP nanoparticles (Table 3). GFP-based nanoparticles slightly increased the basal levels of IL-6 secretion compared to PBS control (Table 3).

Table 3. Inflammatory response of alveolar macrophages and intestinal epithelial cell line of swine (IPEC-J2). The secretion of IL-6 and TNF-α (ng/mL) was evaluated by ELISA after treatment with 10 μg/mL cytokine-based IBs containing either IL-1β, IL-6, IL8, or TNF-α. GFP IB was used as a format control. LPS (10 μg/mL) and PBS were employed as positive inflammatory control and negative control, respectively. Means and Standard Error of the Mean (SEM) from non-transformed data are represented. Asterisk depict significant differences against PBS control; $p<0.0001$. n.d: non-detected.

Tissue	IB Treatment (10 μg/mL)	IL-6 secretion (ng/mL)	TNF-α secretion (ng/mL)
Alveolar macrophages	IL-1β	8.868 ± 0.182 *	n.d
	IL-6	n.d	n.d
	IL-8	0.031 ± 0	4.622 ± 0.109 *
	TNF-α	0.381 ± 0 *	1.206 ± 0 *
	GFP	2.235 ± 0.016 *	0.796 ± 0.091 *
	LPS	0.067 ± 0.010 *	5.277 ± 0.062 *
	PBS	0.036 ± 0.018	2.214 ± 0.061
Intestinal Epithelial cells (IPEC-J2)	IL-1β	21.125 ± 4.598 *	n.d
	IL-6	n.d	n.d
	IL-8	n.d	n.d
	TNF-α	0.300 ± 0.006	2.646 ± 0.055
	GFP	7.006 ± 0.403 *	n.d
	LPS	0.018 ± 0.004	n.d
	PBS	0.015 ± 0.003	n.d

On the other hand, IPEC-J2 intestinal cells showed a less reactive pattern than macrophages, and only IL-6 secretion was detected after stimulation with nanoparticles containing IL-1β (Table 3). Neither LPS at 10 μg/mL nor other cytokines induced any inflammation in the epithelial cells, although TNF-α nanoparticles slightly boosted epithelial TNF-α secretion (Table 3). In order to increase the sensitivity and have an idea of the effect of nanoparticles on intestinal epithelial cells, the gene expression of several genes involved in innate immunity was assessed (Figure 1). In this assay, the treatments were applied based on the total protein content of the nanoparticle rather than the cytokine concentration due to not only the cytokine embedded in the nanoparticle could trigger an inflammatory response. The results confirmed that IL-1β nanoparticles boosted an inflammatory response in epithelia, increasing gene expression of *TNF-α* (Figure 1). Moreover, the gene expression profile also confirmed that TNF-α based nanoparticles upregulated *TNF-α* and *CLDN4* genes whereas IL6-nanoparticles increased the expression of *BD2* and *CLDN4* genes (Figure 1). Herein PBS did not show any effect on gene expression while GFP induced *CLDN4* expression.

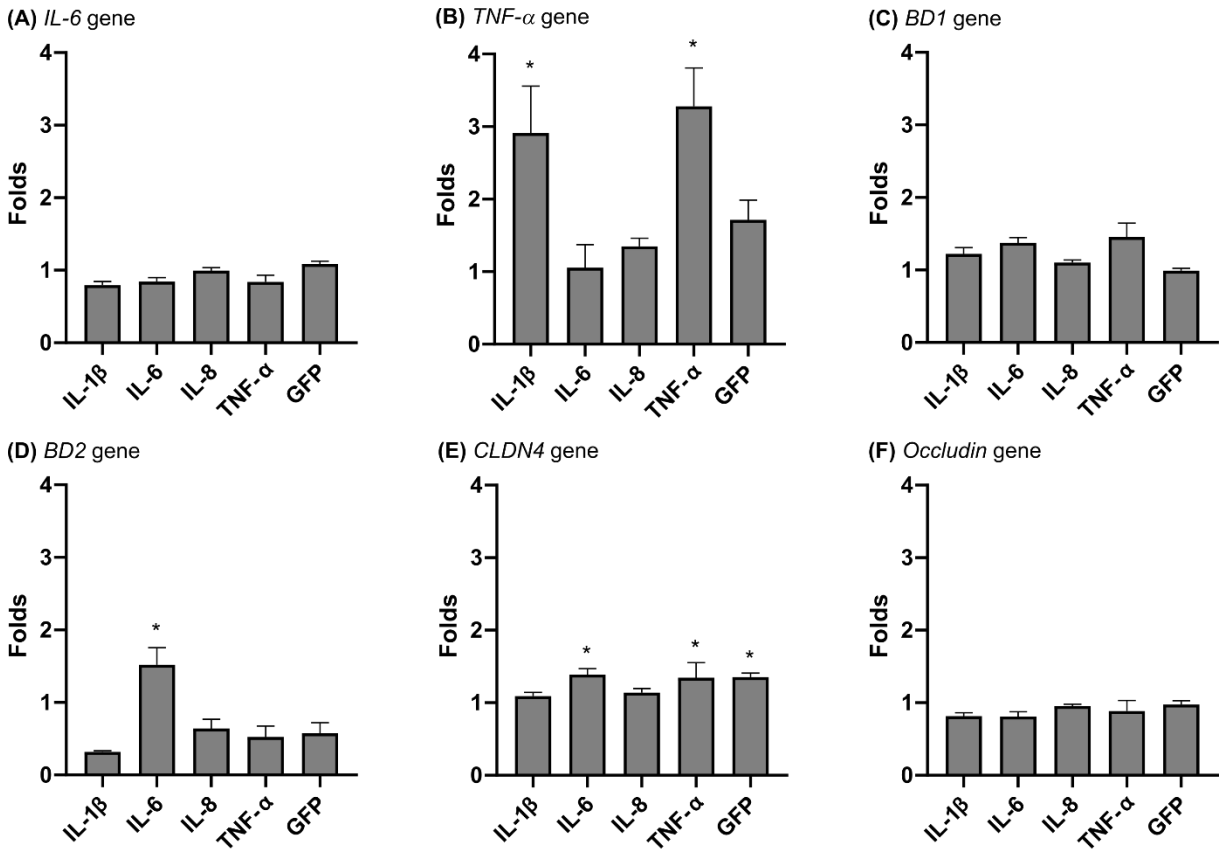

Figure 1. Analysis of gene expression of (A) *IL-6*, (B) *TNF-α*, (C) *BD1*, (D) *BD2*, (E) *CLDN4*, and (F) *Occludin*, in the IPEC-J2 cell line. Grey bars indicate the treatment with 6.25 μg total Protein /mL. GFP IB and PBS were used as a format control and negative inflammatory control, respectively. Error bars indicate the Standard Error of the Mean (SEM). Asterisk show statistically significant differences in expression

folds between PBS and treatments. (A) p=0.030; (B) p=0.0004; (C) p=0.0108; (D) p=0.0001; (E) p=0.0006; (F) p=0.5059

Temperature and pH stability of cytokine nanoparticles

The nanoparticles containing either IL-1β, IL-8, IL-6, TNF-α, or GFP were incubated at high temperatures and low pH to determine their stability. The fluctuation of protein content was determined in all cases except for TNF-α, which was not possible to quantify by Coomassie (Figure 2). In all scenarios, the protein content was maintained, and we did not register significant losses neither towards the soluble fraction nor degradation. The immunostimulation performance was assessed by *TNF-α* expression in epithelial. In all cases, the immunogenic activity was maintained except for TNF-α nanoparticles which lost activity after the temperature challenge (Figure 3).

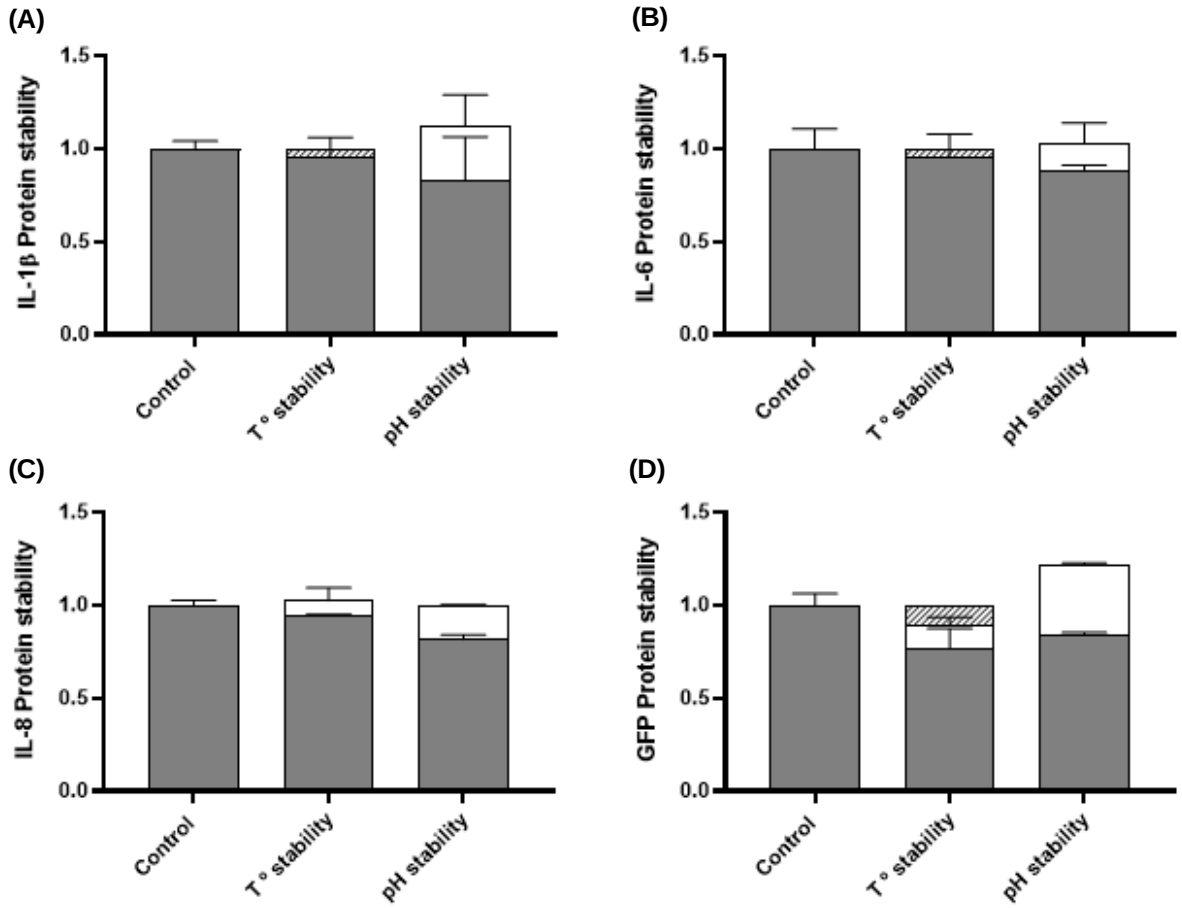

Figure 2. Protein distribution between soluble (white), insoluble (grey), or lost fractions (striped) after temperature and pH challenge in (A) IL1β, (B) IL6, (C) IL8, and (D) GFP-based IBs.

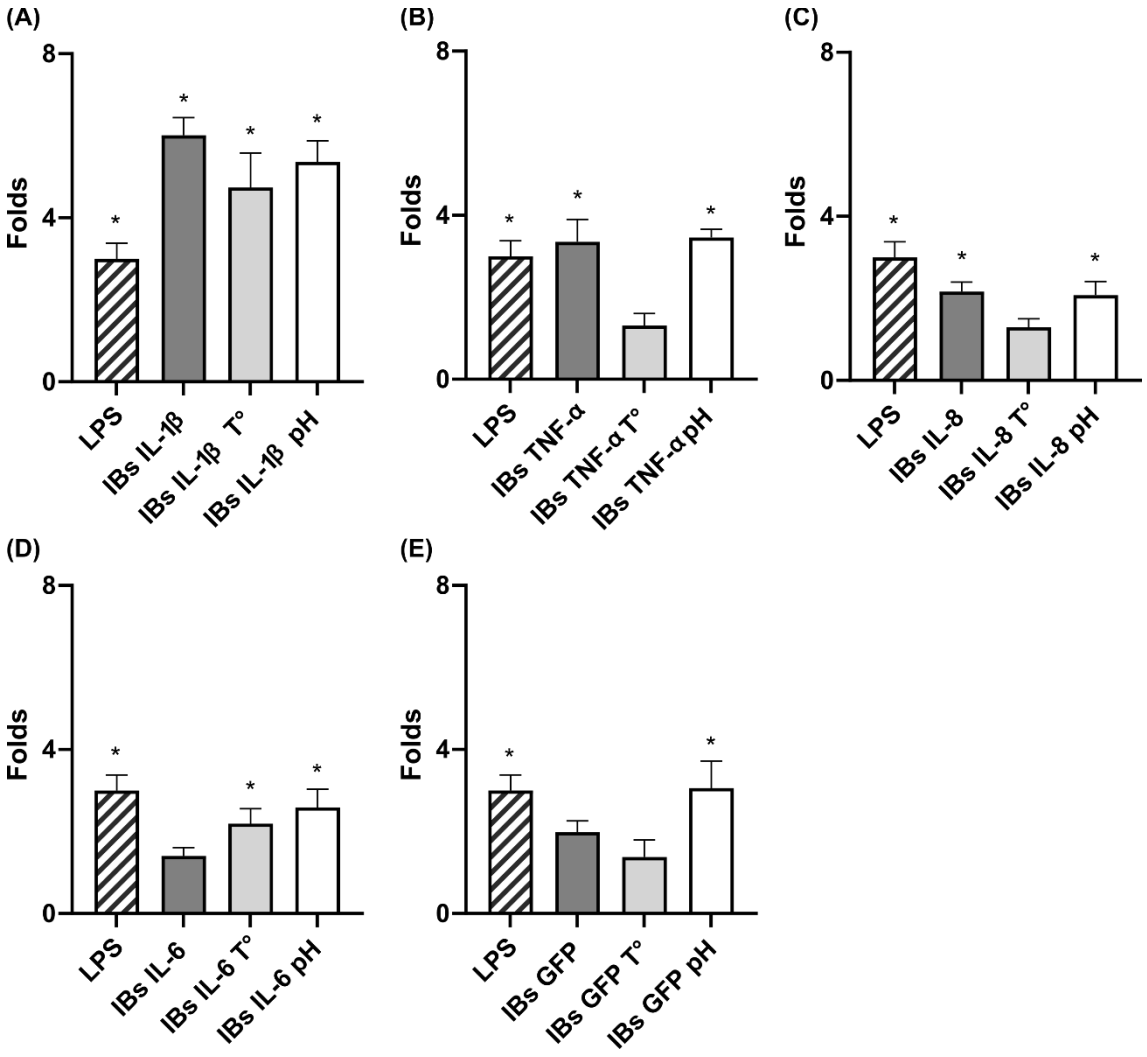

Figure 3. TNF-α gene expression after temperature and pH stability assay in IPEC-J2 cells treated by (A) IL-1β, (B) TNF-α, (C) IL-8, (D) IL-6, and (E) GFP-based nanoparticles. The bars of the mean and SEM of non-transformed data are represented. Asterisk display statistically significant differences in expression folds between PBS and treatments. (A) p=0.0001; (B) p=0.0001; (C) p=0.0003; (D) p=0.0002; (E) p=0.0019

Swine in vivo experiments

Since IL-1β nanoparticles showed adequate production yields (Table 2), fine modulation of inflammatory responses (Table 3, Figure 1), and intrinsic resistance to gastrointestinal (GIT) conditions, they were chosen to be tested *in vivo* in piglets. The results showed that 24-h after the last IL-1β nanoparticle administration (Table 4) none of the analyzed cytokines in blood showed significant differences with the control. Yet, TNF-α tended to increase at 7-d post-administration (d-34) (Table 4, P=0.0755) of IL-1β treatment compared to control piglets.

In samples of intestinal tissue, the immunostimulatory effect was assessed by gene expression of the extracted RNA from tissue explants after 24 hours (d-27) and 7-d (d-34) the administration of IL-1β nanoparticles (Table 5). No significant changes were observed in the ileum or jejunum for *TNF-α, IL-6, BD1, BD2, Muc1, CLDN4*, or *Occludin* genes in both evaluated sampling times.

Table 4. Cytokine determination in serum samples from *in vivo* swine treatments with IL-1β nanoparticles, and in the control treatment. The mean of each treatment and SEM are indicated. Highlighted results indicate a tendency. T: treatment; D: day.

	Day-27		Day-34		SEM	p value		
Cytokine	Control	Treatment	Control	Treatment		T	D	TxD
IL-8	0.066	0.097	0.089	0.251	0.080	0.116	0.476	0.112
IL-6	0.435	0.620	0.511	1.324	0.286	0.136	0.105	0.328
TNF-α	1.188	0.522	**0.570**	**54.106**	16.370	0.122	0.120	**0.076**
IL-10	0.350	0.680	0.300	3.202	1.569	0.854	0.697	0.900

Table 5. Cytokine gene expression analysis of ileum (A) and jejunum (B) after IL1β-based IBs treatment, and in the control treatment. The mean of each treatment and SEM are indicated. P-value <0.05 indicate statistical differences. W: week.

(A)

	Day -27		Day-34		SEM	p value		
Gene	Control	Treatment	Control	Treatment		T	D	TxD
TNF-α	2.8E-09	0.265	0.244	0.423	0.527	0.679	0.708	0.936
IL6	1.009	0.953	1.020	1.045	0.144	0.917	0.724	0.784
BD1	1.416	1.393	3.175	2.898	1.279	0.679	0.708	0.936
Muc1	1.092	0.873	1.594	1.165	0.251	0.215	0.133	0.680
CLDN4	1.139	0.677	1.064	0.970	0.173	0.129	0.539	0.304
Occludin	1.023	0.832	1.219	0.998	0.105	0.068	0.104	0.890

(B)

	Day -27		Day-34		SEM	p value		
Gene	Control	Treatment	Control	Treatment		T	D	TxD
TNF-α	1.030	1.153	0.948	0.807	0.113	0.938	0.077	0.261
IL6	1.039	0.964	0.877	0.783	0.144	0.566	0.251	0.950
BD1	1.707	1.761	1.990	5.290	2.130	0.917	0.362	0.321
BD2	1.118	1.555	1.550	3.406	0.922	0.225	0.155	0.497
Muc1	1.005	1.361	1.232	1.470	0.187	0.131	0.382	0.756
CLDN4	1.031	0.870	0.935	0.956	0.105	0.515	0.959	0.398
Occludin	1.017	1.001	1.116	1.085	0.106	0.827	0.401	0.944

Discussion

Torrealba *et al*. [13] showed that IBs, protein nanoparticles formed during recombinant protein production, presented excellent immunomodulatory properties able to protect fish against otherwise lethal bacterial challenges. Likely, the composition and structured organization of IB components (protein peptidoglycan, DNA, and RNA) make these protein biomaterials excellent immunogens [13]. Moreover, the authors showed that when the recombinant protein produced was a cytokine, such as TNF-α or CCL4, the nanoparticles were able to interact with relevant immune cells and tissues both when intraperitoneally injected or orally administrated and provide better protection levels compared to similar nanoparticles that included proteins without any specific immune function [21]. These conclusions pushed us to test this concept in swine production as an alternative approach to increase piglet's resilience during stressful periods and reduce the associated antibiotic use.

In the present study, *L. lactis* was the recombinant platform used to produce the cytokine-based nanoparticles since it is considered a Generally Recognized as Safe (GRAS) system and would facilitate its potential implementation as a feed additive for animal production [22]. Nanoparticles based on IL-1β were the only ones stimulating the immune response both in macrophages and intestinal epithelia by increasing IL-6 secretion above levels shown by control cells treated with PBS or GFP-nanoparticles. The IL-8 nanoparticles also stimulated alveolar macrophages by increasing TNF-α secretion but did not produce any effect on IPEC-J2 cells. LPS added at the same concentration as nanoparticles (10 μg/mL) increased TNF-α in macrophages but did not induce innate immunity in IPEC-J2 cells. This indicated that as expected the reactivity of macrophages was much greater than intestinal cells, although the latter was still able to respond to IL-1β nanoparticles stimulus by secreting pro-inflammatory cytokine IL-6. Gene expression in IPEC-J2 cells was evaluated, as this is considered to have a higher sensitivity than ELISA tests. We selected genes covering not only pro-inflammatory cytokines, such as *IL-6* and *TNF-α* but also Host Defense Peptides (HDPs) such as *β-defensin* 1 (*BD1*) and 2 (*BD2*) which play an important role in the innate immunity fighting against pathogens. Finally, two genes involved in the formation of tight junctions, *Occludin* and *CLDN4*, were selected since their increase prevents the entrance of pathogens inside the cells [23]. We indeed found an upregulation of *TNF*-α gene by IL-1β nanoparticles and an increase of *TNF-α* and *CLDN4* genes in cells treated with TNF-α nanoparticles. It was unexpected not detecting *IL-6* expression since it was well detected by ELISA but it is possible the timing of sampling for IL-6 expression analyses was not the optimum. Although *in vitro* we found a very interesting activity of TNF-α nanoparticles, it is important to state that the production yield of this nanoparticle was very low, which makes it difficult to consider as a candidate for further exploration. The nanoparticles based on IL-6 also stimulated

the expression of two genes in IPEC-J2, such as *CLDN4* and *BD2*, and since their yield was acceptable, they could be considered a possible candidate for future *in vivo* experiments. However, although IL-6 and IL-8 nanoparticles were able to stimulate macrophages, the IL-1β nanoparticles were able to induce both macrophages and intestinal cells, which makes them more attractive. Lastly, we also found that GFP nanoparticles induced a greater expression of *CLDN4* than PBS control. In a previous study, Torrealba *et al*. [21] also found unspecific immunogenicity responses *in vitro* using nanoparticles with control proteins such as iRFP, but, in the *in vivo* studies they demonstrated that the effect was better using nanoparticles containing immune relevant proteins such as cytokines [21]. It should be noted that all cytokine-based nanoparticles produced herein had a low purity of recombinant protein indicating that the immunogenicity was probably caused by a combination of several components embedded in the IBs. Stability experiments demonstrated that cytokine nanoparticles were highly stable, regarding both activity and protein content, at low pH plus physiologic temperature (37ºC), thus they could resist gastrointestinal conditions. Also, they supported high-temperature conditions usually used in animal feed preparation, which makes them suitable for possible applications as a feed additive.

Considering all the *in vitro* results, the IL-1β-based nanoparticles were chosen for a first proof-of-concept in a small number of piglets to assess if the immunostimulation was translated at the animal level. The dose applied was limited to the production that could be achieved at lab-scale and corresponded to a daily administration of 20 mg/kg of body weight for 1 week. This dose was considered reasonable since previous experiments with LPS at 2 µg/kg induced immunostimulation in piglets [24]. However, the effects detected in intestinal explants of piglets by gene expression were not significant for a broad-range of genes involved in mucosal immune response such as cytokines, mucins, tight junction proteins, or HDPs (Table 5). But, interestingly, the TNF-α concentration in the blood of animals treated with IL-1β nanoparticles tended to be greater at 7-d post-administration compared to control piglets. Since the number of animals was limited, it was difficult to obtain a significant effect, but the blood TNF-α concentrations tended to be 100 times greater than in controls (Table 4).

Other studies exploring alternative immunostimulants based on probiotics, such as *Bacillus subtilis* and lactic acid bacteria, have observed changes in gene expression at the intestinal level including a clear increase in *IL-6* gene expression [11]. In these cases, the administration of probiotics lasted for around 3 weeks so which could suppose a relevant difference, in conjunction with the selected strategy to trigger immunostimulation [11]. Another approach using non-viable microorganisms has been tested. Zhong *et al.* demonstrated that the intestinal mucosal and systemic immunity of early-weaned piglets were reinforced by heat-killed *Mycobacterium phlei*, but not by antibiotics [25]. However, there are also other studies exploring shorter treatments of

11 d using phytobiotics. For example, 10 mg/kg of *Capsicum Oleoresin*, *Garlic Botanical*, or *Turmeric Oleoresin* upregulated the expression of genes related to immune response in supplemented animals compared to the control [26].

Previous works have also explored the effect of immunostimulants on systemic immunity. However, in most cases, the focus has been the concentration of immunoglobulins which was not assessed in our case [27]. For example, the immune active protein lactoferrin has been studied on weaning piglets increasing PHA-stimulated lymphocyte proliferation, serum IgG by 16%, IgA by 17%, IL-2 by 14% ($P < 0.05$), serum iron values by 23% ($P < 0.01$) and decreasing the diarrhea ratio ($P < 0.05$) relative to the control on day 30 [28]. However, in the lactoferrin study, a much greater dose of 1 g/kg was administered for 15 and 30 days.

Conclusions

Immunostimulation is a compelling strategy to prevent non-desirable infections. This approach is underpinned on a proper application in the adequate animal production timescale. The preliminary outcomes demonstrated that our cytokine-based nanoparticles (specially IL-1β and IL-6) are able to immunostimulate *in vitro* swine intestinal cells and macrophages, even after a temperature and pH challenge. Going a step further, the selected cytokine for *in vivo* assays was IL-1β, but although it showed a good and stable *in vitro* performance IL-1β nanoparticles did not elicit significant effects *in vivo*. However, a tendency was observed to have immune stimulatory effect at systemic level which could increase the resilience of the animal to infections. It is possible that greater doses and longer treatments durations may be needed to detect a pronounced effect in the intestinal mucosa along with a comprehensive evaluation of the optimal treatment application timeframe.

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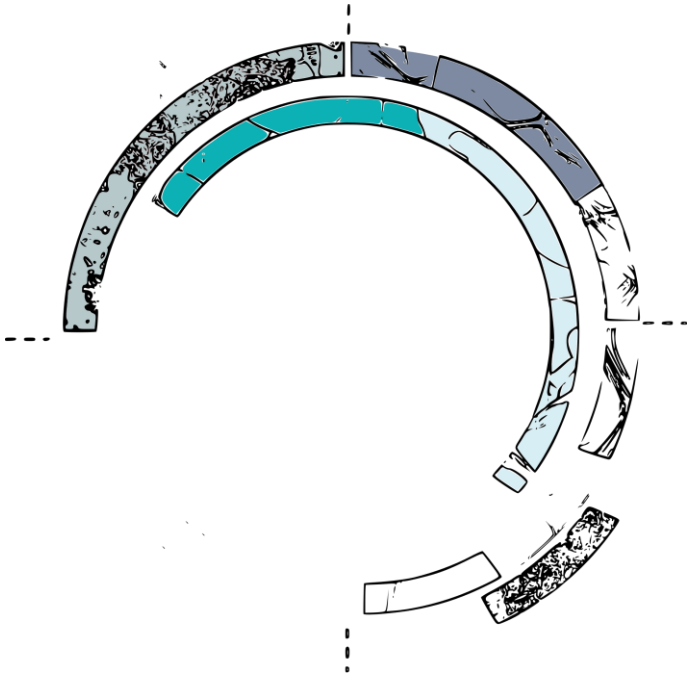


General discussion

RECOMBINANT HOST DEFENSE PEPTIDES AS A NATURAL ALTERNATIVE TO ANTIBIOTICS

Since resistance to conventional antibiotics is compromising the basis of our modern medicine, the scientific community is aiming their efforts in seeking promising alternatives against AMR bacteria [27, 54]. Among the options that the literature reported, which include probiotics [84, 383], antibodies [89], bacteriophages [56], lysins [70], and antimicrobial proteins and peptides [109, 384], this work has been focused on HDPs, which are a group of antimicrobial peptides produced by the innate immunity with a ubiquitous presence in all life kingdoms [108, 385]. Unlike other antimicrobial approaches, HDPs are molecules with a broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria [386], which makes them a highly versatile and global strategy to fight against MDR microorganisms [387]. Remarkably, these peptides not only efficiently kill planktonic bacteria but also are strongly effective against those embedded in a biofilm [110, 387]. Otherwise, lysins and bacteriophages are generally bacteria-specific [58, 74], while treatments based on antibodies display a reduced performance against complex mixed infections and those forming biofilms [388].

Impact of recombinant bacterial host on HDP production yields and activity

Currently, HDPs are mainly produced by chemical synthesis [389, 390]. Still, although this methodology has been demonstrated to be suitable for preliminary studies of antimicrobials , their associated cost, difficulties to perform PTM, and limitation on the peptide length are significant shortcomings that need to be addressed [262]. In this regard, the use of recombinant technologies for antimicrobial production provides a vast array of heterologous production platforms (i.e., bacteria, yeast, insect cells) and molecular tools to work with [391]. Recombinant production has emerged as a versatile approach to cover the chemical synthesis gaps, achieving high-quality products (in terms of purity and bioactivity) in a cost-effective way.

During recombinant production, several aspects have to be taken into consideration to optimize the whole process and the resultant product. The genetic background of the selected production strain, the expression plasmid, the gene promoter, the use of specific tags, and culture conditions (growth media, temperature, or culture agitation) are just a few variables to be considered [276]. Among them, it is known that the appropriate choice of the heterologous production host is pivotal, in terms of peptide production yield, production cost, and protein conformational quality [392, 393]. This is especially relevant for those proteins containing PTM such as disulfide bonds

[276]. In this sense, the defensins, which are one of the major families of HDPs, hold three conserved disulfide bridges in their structure, whose role on the activity is still not clear. Although studies done with chemically synthesized peptides show that disulfide bonds do not have a major role in the antimicrobial activity of HDPs [394-396], little is known about the recombinant versions. To cover this gap, Study 1 was focused on evaluating the impact of the selected microbial cell factory on the disulfide bond formation and protein yields and quality. For that, two *E.* coli strains, the well-known *E.* coli BL21 strain, and *E.* coli Origami B strain were considered for the production of two HDPs, an α-defensin (HD5) and a β-defensin (LAP), which both were fused to a GFP to avoid undesirable degradation. At length, *E. coli* Origami B is a genetically modified strain that lacks thioredoxin reductase (*trx*B) and glutathione reductase (*gor*) genes. Remarkably, these mutations trigger off a more oxidized cytoplasm, which should foster disulfide bridge formation [397].

Our first results proved that HDPs could be produced in both *E. coli* BL21 and Origami B strains. Concretely, the defensin HD5 and LAP were achieved at good yields and high purity in both tested strains (Study 1, Figure 1e). The reported yields are in line with the 4.8 mg/L of the hybrid AMP described by Xu and co-workers [398], the 4.1 mg/L of HβD2 reported by Diao *et al.* [399], or the 1.7 mg/L of LL-37 cathelicidin related by Li and co-authors [400]. These outcomes also stressed that the GFP is a proper carrier to recombinantly produce HDPs with low levels of toxicity, widely demonstrated in the subsequent production of the first generation of HDPs (Study 2, and Study 3). In fact, the GFP removal in the second generation of HDPs, which combine different HDPs in a single polypeptide, leads to a drastic drop in the protein yields (Study 3, Table 1 and Table 2), pointing out that GFP may mask non-desirable HDPs toxic effects. Remarkably, other authors explored alternative carriers or tags to improve HDPs soluble production and, at the same time, prevent non-desirable HDP host toxicity, being the thioredoxin and SUMO some of the most used for antimicrobial peptides production [340, 390]. However, it is important to stress that the GFP not merely stabilizes the resultant fusion protein, avoiding an early proteolytic degradation, but also enables a fluorometric track during the HDPs expression and downstream purification [401]. By contrast, other tags, such as the leucine zippers, prompted the peptide aggregation instead of their solubility, being an alternative strategy to overcome HDPs related toxicity [263, 312].

Surprisingly, contrary to our initial expectations, the more reducing *E. coli* BL21 cytoplasm is suitable to produce LAP defensin with the same antimicrobial activity as those formed in an oxidizing environment (Study 1, Figure 2a and C). The evaluation of the free cysteines did not show any significant difference between LAP-GFP-H6 produced in both *E. coli* BL21 and Origami B strains (Study 1, Figure 3). Thus, findings obtained with LAP-GFP-H6 allowed us to conclude that a standard reducing cytoplasmic environment, such as that of *E. coli* BL21, is

enough for the proper production and folding of HDPs. Indeed, evaluating the HD5, we unexpectedly noticed that those peptides purified from *E. coli* Origami B showed a higher proportion of free sulfhydryls than those produced in *E. coli* BL21 (Study 1, Figure 3), indicating the lack of disulfide bridge formation. As a consequence, the HD5-GFP-H6 from the Origami B strain displayed reduced stability (Study 1, Figure 4) along with diminished antimicrobial activity (Study 1, Figure 2) when compared to the protein produced in a reducing environment. These outcomes are in agreement with previous findings, where the absence of disulfide bridges attenuates the HDPs antimicrobial activity [396, 402]. Overall, with the findings of Study 1, we selected the *E. coli* BL21 strain, which has proven to be a suitable recombinant platform for the production of all HDPs analyzed throughout this thesis. Remarkably, this strain has been successfully used to produce HDPs fused to GFP (Study 1, Study 2, and Study 3) but also when forming multidomain proteins (Study 3).

Although *E. coli* seems to be a flawless candidate, it also has several bottlenecks to face up. As we noticed in the early evaluation of this microorganism, the high yields of heterologous protein produced often surpass the protein quality control machinery of *E. coli*, driving roughly 50 % and 80% of the LAP-GFP-H6 and HD5-GFP-H6, respectively, to aggregate in IBs (Study 1, Figure 1). This fact encourages us to explore the use of IBs as a natural source of soluble HDPs, especially for those HDPs with a high tendency to aggregate (Study 2 and Study 3).

Inclusion bodies as an alternative source of difficult-to-produce HDPs

The HDPs antimicrobial features generally trigger toxicity for the bacterial hosts, which is reflected in reduced growth profiles and protein yields, limiting the soluble production. In this context, even though the early mentioned carriers or tags might provide a plausible solution to the HDPs toxicity, our group has already proven in previous studies that the production of antimicrobial proteins as IBs minimize the host toxicity and early peptide proteolysis [312]. Moreover, in this research was also noted that IBs could be not merely a strategy to avoid toxicity but also a unique natural source of pure and active peptides that can be easily solubilized to obtain soluble antimicrobial proteins [312]. In this sense and aiming to take advantage of these protein nanoparticles, an exhaustive study to determine the optimal solubilization and purification protocol to isolate soluble antimicrobial proteins based on HDPs has been conducted in the framework of this thesis (Study 2)

Results of Study 1, Study 2, and Study 3 show that under recombinant protein production, most tested HDPs are partially produced as IBs. This aggregation can be understood as a general phenomenon since it occurs during antimicrobial protein production when HDPs are fused to the

GFP carrier (Study 1, Figure 1, Study 2, Figure 1) but also when HDPs are combined forming multidomain proteins (Annex 4, Table 1). Based on previous research [316, 322, 323], we selected a well-known mild detergent, named n-lauroylsarcosine (NLS) as our IB solubilization agent, and the solubilized protein of three defensins (LAP, HD5, and LL-37) fused to GFP was compared with their soluble form. As far as we know, for the first time, the quality of the same recombinant HDPs, isolated from the soluble fraction or solubilized from IBs, was evaluated.

To our surprise, even though the peptide yields and purification profile were similar for both soluble and solubilized forms (Study 2, Table 2), the antimicrobial activity was undoubtedly reduced in the solubilized form (Study 2, Figure 2). These results are in line with previous evidence, where those proteins solubilized with NLS exhibited a reduced activity regarding their soluble counterparts [323, 403]. However, these initial outcomes led us to question if this reduced antimicrobial activity was because of the quality of the HDPs embedded in the IBs or the solubilization protocol effect. To address this matter, we developed an alternative solubilization protocol without NLS and, interestingly, detergent-free solubilized HDPs showed a similar bactericidal activity than the purified peptides from the soluble fraction (Study 2, Figure 3 and Figure 4), demonstrating that previous lack of activity was associated to the use of this mild detergent. Broadly, the development of this protocol brings us a useful approach since it was decisive to isolate multidomain proteins with outstanding antimicrobial performance (Study 3). Moreover, this protocol may probably be applicable to other difficult-to-produce AMP, which low yields or high toxicity difficult their direct purification from the soluble fraction.

For decades solubilization protocols have been used to isolate soluble protein of pharmaceutical or biotechnological interest from IBs. Since IBs have been considered for years as waste products, lacking of biological activity, many of these strategies were based on hard denaturing processes (using chaotropic agents such as urea or GdCl at high concentration) followed by a refolding step. However, gradually, during the last years, different mild protocols have been developed for IB solubilization underpinned on the idea that these aggregates are protein nanoparticles containing important amounts of biologically active recombinant protein [322, 323, 326, 404]. Indeed, contrasted mild protocols have proven that the IBs solubilization does not require extra denaturation step, being proteins effortless released over time. In this context, the results of Study 2 go one step further, proving that mild solubilization agents are not necessary for protein solubilization, which presents a new scenario for all those proteins solubilized from IBs. Hence, this approach not only simplifies the purification process but also prevents a possible negative impact of the solubilizing agents on protein activity, as occurs with HDPs.

Concerning the detergent impact in the final solubilized products, we noticed in previous studies that the effect of the detergent in the biological activity is likely protein-specific [323, 405], thus

requiring to be evaluated case-by-case. A plausible preliminary explanation of the NLS effect could be an interference between the HDPs cationic charges and anionic residues of the target bacterial cell wall structures as occurs when using salt [147, 406] (Figure 19). At length, detergent impurities may modify irreversibility the HDPs native conformation [326], altering the exposed cationic residues and potential cell wall target interactions. This situation is particularly serious for those HDPs which exert their antimicrobial properties in a membranolytic way, while how does detergent affect HDPs that address intracellular targets will need more careful analysis.

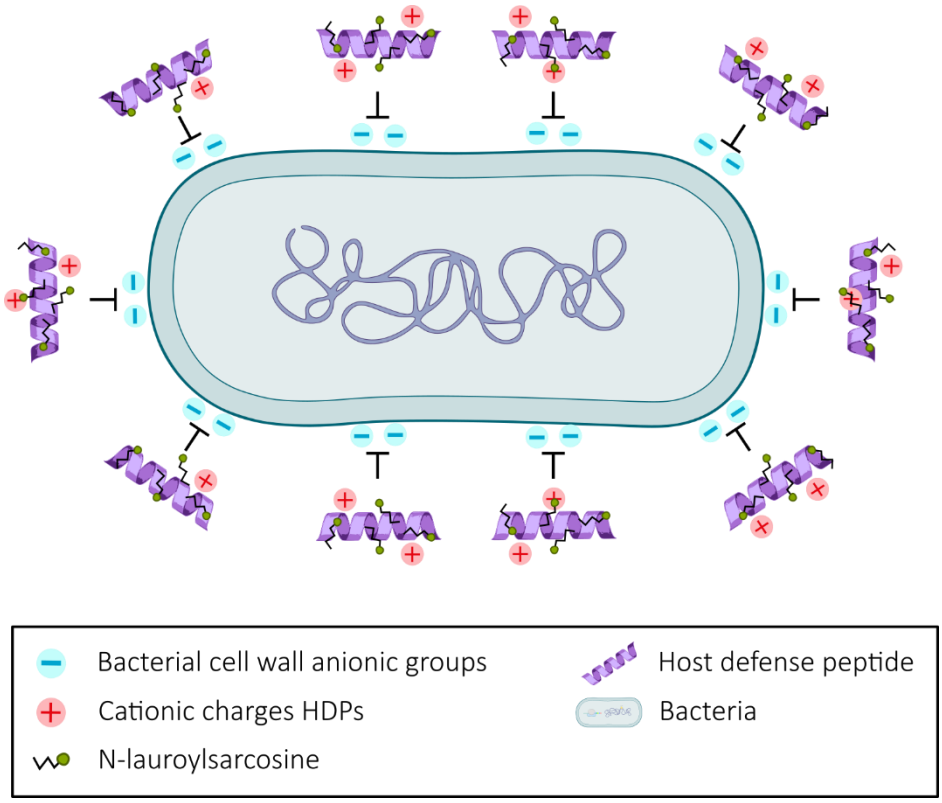


Figure 19. Interaction of NLS with HDPs. The HDPs exhibit an unparallel antimicrobial activity, yet some of them may undergo antimicrobial decreased performance after solubilization. Detergent impurities that remain after the whole downstream process might alter irreversibly the resultant protein conformation, overall charge, folding, or protein interaction with their target and thus diminishing antimicrobial HDP performance.

In summary, these study 2 findings are of greater relevance, demonstrating that IBs are a plausible source of highly active and correctly folded HDPs, which may be easily solubilized by novel detergent-free non-denaturing protocols.

Towards a novel generation of fully tunable antimicrobial proteins: the multidomain approach

We have proven that recombinant HDPs used (including LAP, HβD2, HβD3, HD5, and LL-37) hold an outstanding antimicrobial activity against both Gram-positive and Gram-negative bacteria in both planktonic (Study 1, Figure 2, Study 2, Figure 3, and Study 3, Figure 2) and biofilm-forms (Study 3, Figure 4). Still, it should be noted that depending on the tested HDP it showed better performance against Gram-negative or a specific Gram-positive bacteria (Study 3, Figure 2). For instance, HβD2 displayed a greater antimicrobial against methicillin-susceptible *S. aureus* and *S. epidermidis*, in contrast with anti-MRSA performance (Study 3, Figure 2). In this sense, the HβD3 also exhibited a better performance against *P. aeruginosa* and MRSA, pointing out that the HDPs could show strain-specific effects. Besides, these considerations can be clearly illustrated in the MIC values (Study 3, Figure 3b). Interestingly, our HDPs MIC data are consistent with those reported by Gaiser and co-workers [407]. In this study several frog defensins were tested against a large panel of pathogenic bacteria, ranging from up to 256 mg/L (bacteria not susceptible) to lesser than 8 mg/L, depending on both strain and tested peptide. Besides, Corrales-Garcia *et al.* [408] also described HβD2 MIC values of 51.6 mg/L against *P. aeruginosa* and *S. aureus*, which are only slightly different in the case of *P. aeruginosa* (121.25 mg/L, Study 3, Figure 3). Regardless, despite the MIC values were significantly higher than those reported for susceptible-bacteria antibiotics [409], this fact is not particularly relevant in MDR bacteria context, where the antibiotics do not work, and alternative therapies are on demand.

The first generation HDPs are only suitable for an initial screening since they have been produced fused to GFP, limiting their final applicability. Consequently, with the need to avoid the presence of the carrier protein for a possible HDP-based therapy and produce non-toxic and stables molecules in the recombinant cell factory, we defined a new strategy founded on the combination of the HDPs with higher activity and good production yields in a unique multidomain protein (Study 3, Table 2 and Figure 10). The objective of this second generation of multidomain HDPs was not solely to achieve a polypeptide large enough to avoid host proteolytic degradation without the need of using GFP, but also, at the same time, to improve the bactericidal and anti-biofilm activity of the resultant construct, which would combine the action of each individual HDPs in a synergistic way. Considering the bactericidal activity and the MIC, as well as the anti-biofilm activity of each HDP (Study 3, Figure 2, Figure 3, and Figure 4), those with better performance (LAP, HβD3, HD5, and LL-37) were selected to be combined in a single tailored multidomain molecule. Concretely, we designed the D5L37βD3 that is formed by the fusion of the α-defensin HD5, the cathelicidin LL-37, and the β-defensin HβD3; the D5LAL37βD3 structured like D5L37βD3 but with the addition of the LAP flanked by HD5 and LL37 and the last construct,

D5L37D5L37 arise from the combination of two copies of both HD5 and LL37 peptides. Our early findings revealed that the three designed multidomain molecules may trigger host toxicity since their production yields are drastically lower than the monodomain counterparts (Study 3, Table 1, and Table 2). But, remarkably, the antimicrobial activity and MIC evaluation showed that multidomain polypeptides (second generation HDPs) have a broader antimicrobial activity than monodomain proteins (first generation HDPs) (Study 3, Figure 3 and Figure 7).

These outcomes are of utmost relevance given that we have demonstrated the effectiveness of this strategy to fight against any bacterial infection. Outstandingly, the D5L37D5L37 have demonstrated to be the most effective multidomain protein, with equal inhibitory features in both Gram-positive and Gram-negative bacteria, whereas D5L37βD3 showed a decreased activity against MRSA and *S. epidermidis* (even being also great values) (Study 3, Figure 7b). Taken together, both multidomain polypeptides have considerably improved the overall MIC values of their monodomain counterparts. Still, the D5LAL37βD3 polypeptide did not exert any significant effects in none of the four microorganisms tested suggesting that some intrinsic structural and physical parameters are probably affecting the final activity of the construct.

Although major advancements have been accomplished in Study 3, improving the efficiency of the antimicrobial proteins through the multidomain approach, different aspects need to be further investigated. On the one hand, the outcomes of Study 3 noted the relevance of the specific position of each building block (HDP) in the resultant activity construct, being also important which HDPs are combined. In this connection, the HDPs selection, their position (N- or C- terminal), as well as the linker between domains and the neighboring HDPs can make a substantial difference in the multidomain performance. In fact, the D5L37βD3 polypeptide that exhibited superb bactericidal activity dropped their antimicrobial features drastically after the LAP domain combination (Study 3, Figure 6 and Figure 7), resulting in the ineffective D5LAL37βD3 multidomain protein. But, in a similar way that LL37 in the 1 st generation, although both exhibited a reduced activity against planktonic bacteria, this multidomain protein hold the best antibiofilm activity (Study 3, Figure 8). Consequently, in the future, *in silico* predictions and computational modeling would be helpful to optimize the molecule design [115]. On the other hand, the multidomain protein versatility and flexibility provide us a comprehensive-tailored platform to meet the needs of the healthcare sector. Interestingly, based on previous studies that demonstrate HDPs anti-viral features against human immunodeficiency virus (HIV), influenza, or coronavirus [410, 411], we decided to evaluate the HDPs activity against SARS-CoV-2 (Annex 1). The preliminary outcomes revealed a noteworthy anti-viral activity of roughly 50 % in the higher concentrations of LAP, HD5, HβD2, and HβD3 while non-toxicity effects were reported (Annex 1, Figure 1). Thus, multidomain proteins can be exceptionally useful to hold in a single molecule several modes of action, such as

exciting combinations of antiviral and antimicrobial HDPs, to address current and forthcoming health threats in a multifaceted way.

To conclude, the multidomain approach would allow designing tailored molecules for specific applications using HDPs as a building block. For example, combinations of antiviral and antibacterial activity, which would be a suited therapy for those infections comprising both infectious agents like the Bovine Respiratory Syndrome.

Based on the results obtained, it is possible to create molecules with antimicrobial activity (Study 3), targeting both planktonic and biofilm forms of a specific microorganism. This would be especially relevant to prevent or eradicate biofilms, such as those formed in medical devices (Figure 20), which are particularly important since a significant proportion of human disease and concretely nosocomial infections are triggered by the preexistence of a biofilm (Annex 2) [371].

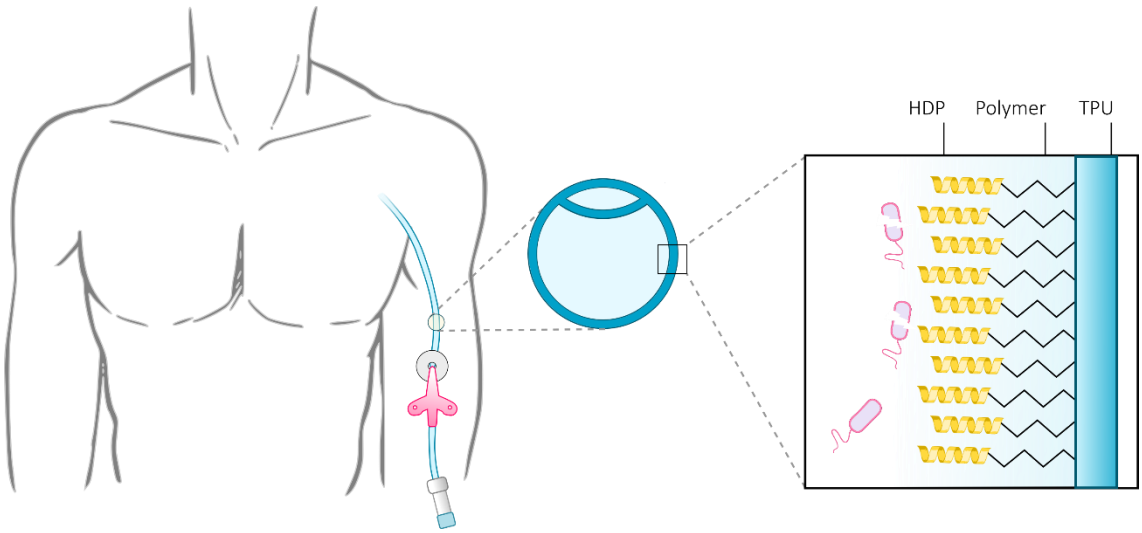


Figure 20. Catheter decorated surface by recombinant antimicrobial HDPs. Since HDPs are excellent compounds either directly killing planktonic cells or inhibiting/eradicating biofilms, they could be applied as a novel generation of improved catheter coating solutions, anchored through a polymer structure to provide enhanced activity to the medical TPU.

DEVELOPMENT OF NOVEL IMMUNOSTIMULANTS FOR LIVESTOCK

The strategies to reduce antibiotic consumption also include those approaches focused on the prevention of a potential infection. In light of these considerations, it is essential to combine a good preventive strategy with the development of new antimicrobial treatments [1, 32]. As a prophylactic measure, immunostimulants are applied to enhance the immune response when it is compromised or prior to a possible challenge. In our view, the development of new immunostimulants for livestock can boost the immune status of the animal before a significant challenge (i.e., transport, weaning). Thus, increased organism resilience may reduce the infectivity rates against a potential pathogen and, accordingly, the use of antibiotics [412] [413].

On this point, although we have demonstrated the HDPs antimicrobial and antiviral performance, which are in concordance with those already described, these peptides also hold encouraging immunostimulant features. Nevertheless, before looking closely at the HDPs immune modulation capabilities, we have developed a model to evaluate the potential of immunomodulatory molecules in the livestock framework. From this perspective, we have chosen inflammatory cytokines, which are molecules with a central effector role at stimulating the immune system. At length, this selection is based on the strong knowledge of the cytokine in the immunotherapy field, where it is largely reflected in the approved recombinant cytokine therapies in the market [240, 352], jointly with the previous studies carried out by Torrealba and co-workers [242, 308]. Briefly, they produced cytokine-based nanoparticles (IBs) such as TNF-α IBs and CCL4 IBs, proving that this strategy provides protection in fish against an otherwise lethal infection by *Pseudomonas aeruginosa* [242]. Taking this into consideration, we opted for the use of cytokine-based IBs instead of their soluble form. These multifaceted aggregates are naturally formed during recombinant production in a cost/effective and straightforward manner. In addition, their complex structure not only confers improved stability to the embedded peptides, being crucial to overcome short cytokine half-life concerns, but also provides an exceptional DDS, acting as a nanopill without the need for further encapsulation [314, 315].

These encouraging findings led us to explore the immunostimulant features of cytokines in the livestock context, particularly in piglets (Study 4). Our final objective was to test the immunostimulants, based on cytokine forming IBs, administered as a feed additive, stimulating the immune status of the swine intestinal mucosa in critical production stages, such as transport and allowing the animal to be able to respond rapidly to an infection threat. In this scenario, we recombinantly produced four porcine cytokines (IL-1β, IL-6, IL-8, and TNF-α) as protein

nanoparticles (IBs, Study 4, Table 2). For this study *L. lactis* was the selected microbial cell factory due to their extensive use for food industrial applications [289], being Generally Recognized as Safe (GRAS) organism by FDA, and fulfill criteria of the Qualified Presumption of Safety (QPS) [287]. Remarkably, these Gram-positive bacteria are an LPS-free expression system, providing endotoxin-free IBs and facilitating the potential implementation of immunostimulants as livestock feed additives [414].

As a preliminary exploration, we assessed *in vitro* the immunogenic features of the cytokine-based IBs using swine macrophages and swine intestinal epithelial cells IPEC-J2. Interestingly, the first outcomes stressed that not all the nanoparticles hold equal immunostimulant features, where IBs containing IL-1β were able to stimulate both cell types (Study 4, Table 3), whereas IL-8 and TNF-α nanoparticles solely stimulate alveolar macrophages (Study 4, Table 3). Probably, immunostimulation divergences may be caused not only by the cytokine tested but also because of the IBs composition, since the cytokine content of each nanoparticle is relatively low (Study 4, Table 2). Additionally, these findings were further confirmed in the gene expression analysis, where IPEC-J2 cells treated with the IL-1β nanoparticles showed an upregulated TNF-α expression (Study 4, Figure 1B). It is also important to mention that GFP (used as a format control) stimulates both cell types. These results are in line with previous reports where IBs formed by non-immunological proteins, such as GFP, can trigger unspecific immunological responses due to the IB format itself [308]. At length, even though IBs are structured predominantly by the overexpressed recombinant protein, during their formation other impurities can be trapped, such as DNA, RNA, or host proteins, where this heterogenic and complex nature confers to IB inherent immune modulation features [308].

Going a step further, to validate such immunostimulant nanoparticles as a suitable feed additive, we tested the cytokines-based IBs simulating the swine gastrointestinal conditions and the high temperatures reached during animal feed processing (Study 4, Figure 2). As expected, and in accordance with Torrealba and co-authors [242], the IBs containing cytokines demonstrated excellent stability at low pH and physiologic or feed processing temperature, holding unaltered both bioactivity and nanoparticle protein content (Study 4, Figure 2 and Figure 3). Likewise, Flores *et al.* [415] research also supported our findings, describing a highly active β-galactosidase IB in physiological conditions, in low pH (2.5) and also after a temperature challenge (65 ºC), acting as a reservoir of packed soluble enzyme. In addition, moving backwards to our study, these first *in vitro* results are of considerable relevance, demonstrating that *L. lactis* is a promising platform of cleaner IBs (LPS-free) with immunostimulant potential.

Given the performance of cytokines-based nanoparticles during *in vitro* assays, we selected the IL-1β IBs to move ahead in the *in vivo* analysis. Besides, as earlier mentioned, this treatment was

designed to be easily oral administrated in animal feeding, targeting swine intestinal mucosa to stimulate a protective immune response (Figure 21).

They were produced at reasonable yields (Study 4, Table 2), displaying a good modulation of inflammatory responses (Study 4, Table 2 and Figure 1), even after the stability challenge (Study 4, Figure 3).

Contrary to our *in vitro* findings, the results obtained from the *in vivo* trial were limited. They indicated that IBs containing IL-1β were unable to trigger an immune response in the examined intestinal tissues (ileum and jejunum), where none of the analyzed genes (including cytokines, HDPs, mucins or tight junction proteins) showed significant differences regarding the control animals (Study 4, Table 5). But, interestingly, the blood analysis of the treated piglets pointed out that the TNF-α concentration tended to be higher compared to control piglets (Study 4, Table 4). The divergence between the *in vitro* and *in vivo* results might be attributed to several factors. First, this trial was conducted with a relatively low number of animals (n=20), since it was designed as a preliminary experiment in piglets. Second, the selected immunostimulant dose and treatment duration may suppose a key point to consider. Besides, the choice of a proper sampling timeframe is essential to have a broader picture of the effects. In this regard, after the examination of previous IBs-related experiments [242, 416, 417], analyzing the sampling times according to the evaluated effect, we opted for sampling (blood and intestinal tissue) half of the animals 24 h after the last administration, and the rest were sampled similarly after 7 days the last IB administration. However, further experiments should include an improved sampling strategy to achieve more compelling results.

In sum, our preliminary findings of Study 4 denote that the use of cytokines as immunostimulants in a nanoparticulate format may be further studied and optimized, encouraging the exploration of HDPs as immune modulators [190, 418].

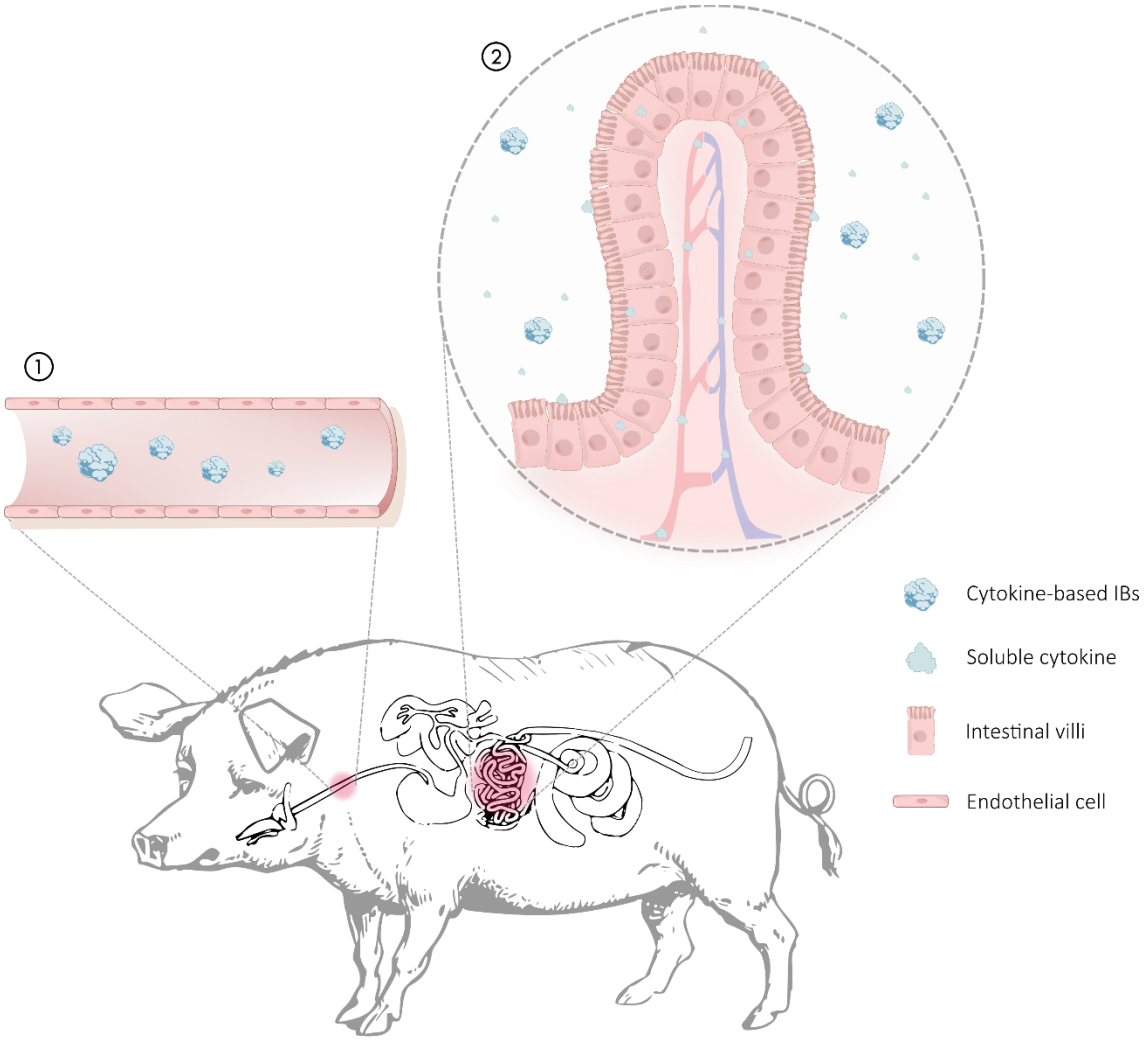


Figure 21. Cytokine-based nanoparticles conjectural mode of action. After the oral administration of the cytokine-based IBs, these aggregates are transported alongside the gastrointestinal tract (1), neither degrading nor losing biological activity due to its intrinsic temperature and pH stability previously assessed. When the nanoparticles reached the swine bowels (2), and as a consequence of a higher retention time, the IBs perform a drug delivery system, acting as a nanopill by the releasement of the embedded cytokine in the intestinal environment, triggering a broad range on inflammatory-related and host immune regulation responses.

To conclude, the following illustration (Figure 22) capture, in a graphical manner, the path travelled during this thesis, bringing to light the dual approach that our group proposed to address animal and human AMR bacteria from the One Health perspective.

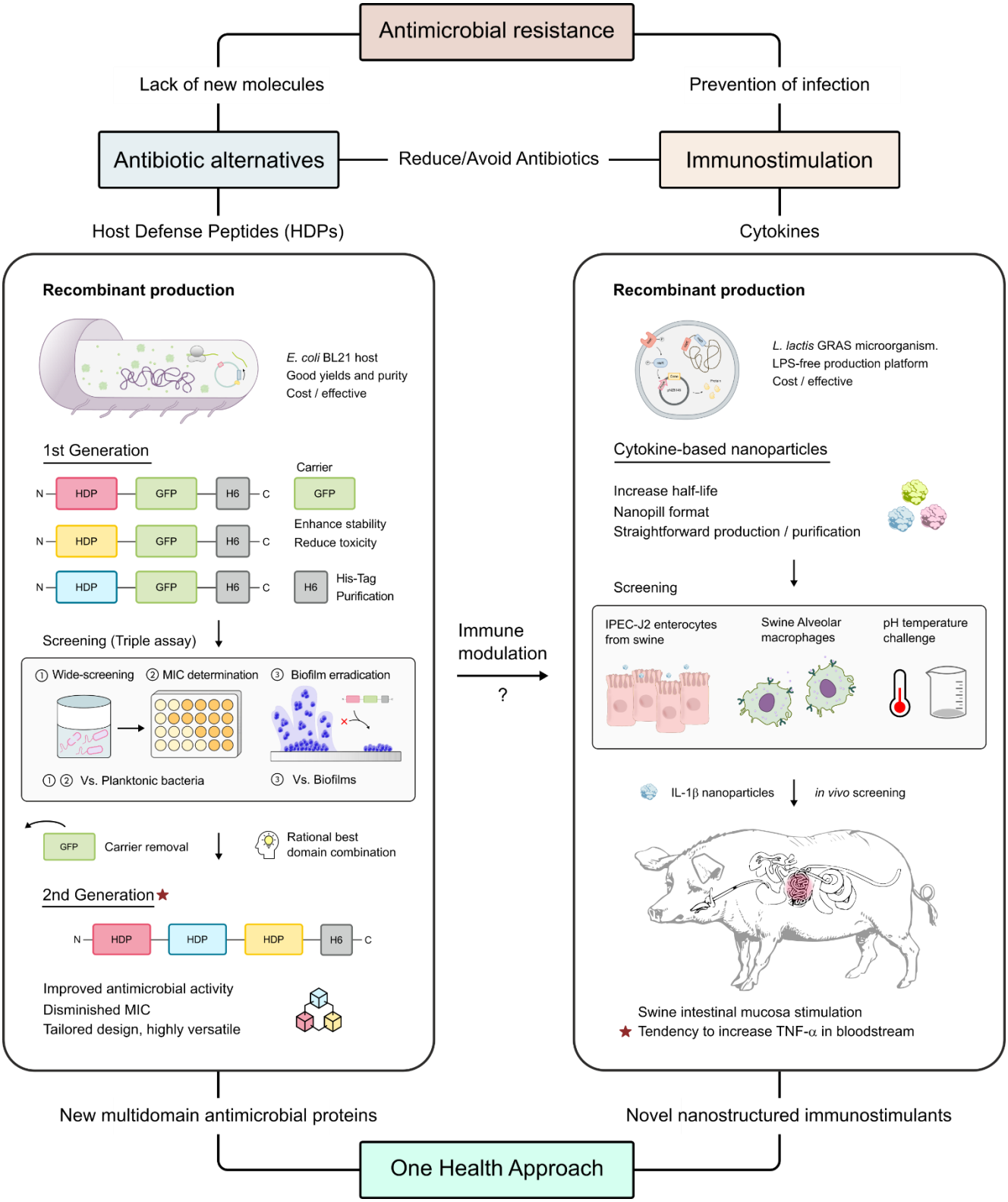
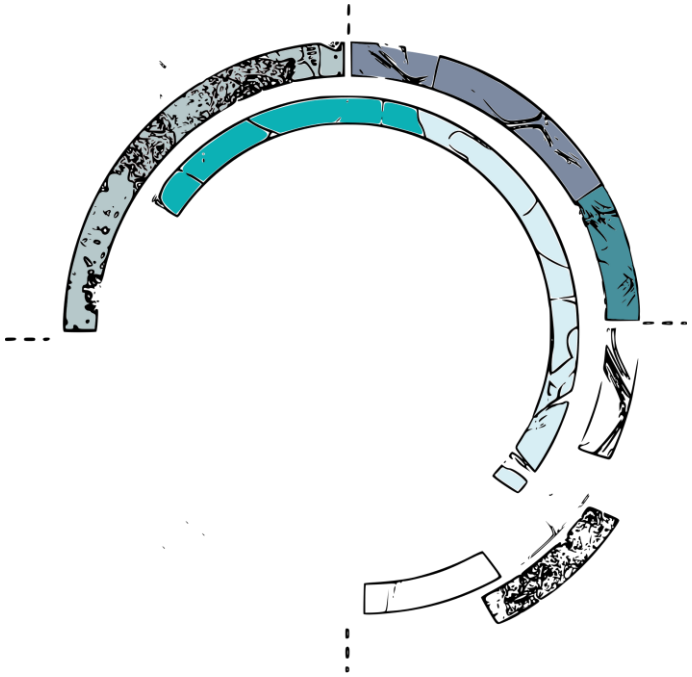


Figure 22. HDPs and immunostimulants against AMR. Illustration depicting the aim of this research and the approaches followed to cope with AMR. The development of an enhanced HDP generation and cytokine-based nanoparticles are two weapons against both AMR bacteria, affecting animal and human health.



Conclusions

In this thesis, we aimed to study the potential of a new generation of recombinant antimicrobial molecules based on Host Defense Peptides, and novel immunostimulants based on cytokine nanoparticles (IBs). Altogether, the results of this work can be summarized in the following conclusive statements:

1. *Escherichia coli* has been validated as a good alternative for the production of highly pure and active recombinant HDPs-based proteins. Concretely, the *E. coli* BL21 strain exhibited a suitable genetic background to achieve high quality (in terms of activity and stability) HDPs, better than those synthesized in *E. coli* Origami B strain.

2. The Green Fluorescent Protein (GFP) can be used as a protein carrier for the recombinant production of HDP-based proteins, avoiding early peptide degradation, and, at the same time, enabling fluorescence tracking during protein production and purification process.

3. The lack of disulfide bridges negatively impacts both HDP stability and antimicrobial activity against Gram-positive and Gram-negative bacteria.

4. Inclusion bodies (IBs) proved to be a rich source of high-quality HDPs-based antimicrobials (comparable with those purified from the soluble fraction), which could be efficiently solubilized through a newly developed, detergent-free non-denaturing protocol.

5. The use of n-lauroylsarcosine detergent during IB solubilization processes impaired the antimicrobial activity of solubilized HDP-based proteins.

6. A comprehensive and rational evaluation of individual HDP (1st generation molecules) production yields and bactericidal activity provides a solid basis for their combination in a new multidomain molecule (2nd generation molecules).

7. The multidomain approach can establish a flexible platform for the generation of broad-spectrum antimicrobial proteins, combining different HDPs as building blocks as favored to cope with a pathogenic microorganism.

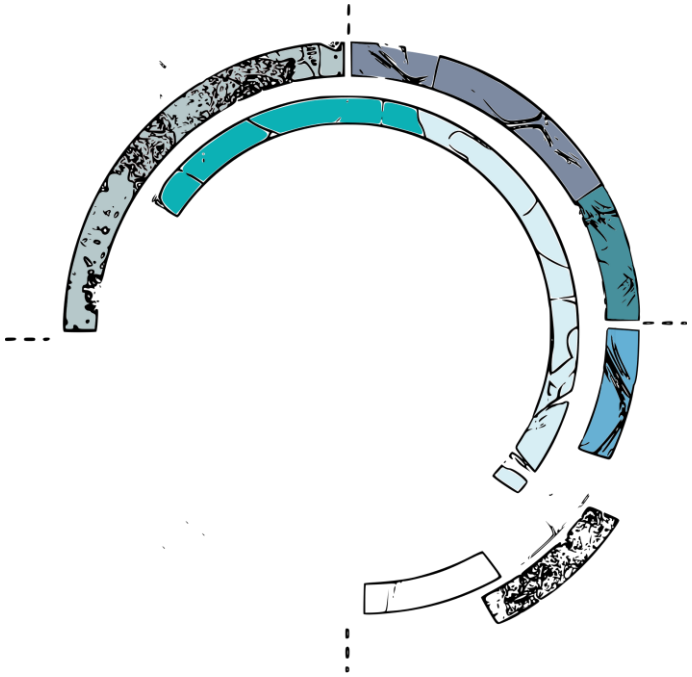
8. The multidomain constructs D5L37D5L37 and D5L37βD3 showed an increased broad-spectrum antimicrobial activity and lower MIC than their individual counterparts.

9. Differences in planktonic and anti-biofilm activities between multidomain antimicrobial proteins pointed out the relevance of the selected HDPs to be combined along with their order sequence in the final construct performance.

10. Protein nanoparticles containing cytokines (particularly porcine IL-1β) stimulated *in vitro* immunological responses in swine macrophages and swine intestinal epithelial cells IPEC-J2.

11. Cytokine-based IBs exhibited better stability than the soluble form, maintaining both activity and protein content after pH and temperature treatments that simulated the gastrointestinal tract and feed processing, respectively.

12. The oral administration of porcine IL-1β nanoparticles in swine tended to increase the TNF-α concentration in blood, but they were unable to trigger an immune response neither the ileum nor in the jejunum.



Annexes

ANNEX 1

Host defense peptides against SARS-CoV-2 virus

Background and objective

Currently, there is no effective and general treatment against infections caused by the SARS-CoV-2 virus. Thus, there is a strong need to find effective treatment strategies to stop the virus replication.

Host Defense Peptides produced by the innate immunity of all life forms possess a broad-spectrum therapeutic potential against different pathogenic agents including bacteria, fungi, and viruses (Barlow et al. 2014. Future Microbiol. 9(1):55-73). In terms of antiviral activity, previous data showed that HDPs have an important role during viral infections such as those caused by influenza virus (I-Ni Hsieh et al. 2016. Pharmaceuticals 9, 53) and HIV (Chang et al. 2005. J. Clin. Invest. 115: 765-773). Interestingly, Paneth cell-secreted HD5 efficiently bound and blocked ACE2 which locates on the surface of intestinal epithelial cells, lowering the recruitment of 2019-nCoV S1 (Wang et al. bioRxiv preprint Mar 2020). Besides, Zhao et al. found that a mouse β-defensin-4-derived peptide named P9 (Zhao et al. 2016 Sci Rep. 6:22008) lessened *in vitro* infectivity of MERS-CoV (strain hCoV-EMC/2012) or SARS-CoV (strainHKU398490) at non-toxic concentrations. Cathelicidin LL-37, the porcine cathelicidin Protegrin-1, and the ovine cathelicidin SMAP-29 also displayed potent antiviral activity towards human rhinovirus and their activity have been visible when either the virus is exposed to the peptides prior to cell infection or after cells have been infected (Sousa et al. 2017. Peptides. 95:76–83).

On the other hand, previous studies done by IRTA proved the capacity of recombinant microorganisms to produce HDP-based proteins (Roca-Pinilla et al. 2020. Microb. Cell Fact. 19(1):122; Garcia-Fruitós et al. PCT/EP2020/054235) using a 2-phases strategy. Phase 1 is based on the construction of the 1st generation of drugs composed of single HDPs fused to GFP carrier protein. After the selection of those HDPs more efficient against the target pathogen, Phase 2 combines selected HDPs in a single polypeptide (2nd generation drugs) without using any carrier protein. Thus, this project aimed to determine the potential of 1st generation of HDP-based proteins produced in recombinant bacteria to decrease SARS-CoV-2 infectivity.

Material & Methods

Production of 1st generation of HDP-based proteins against SARS-CoV-2 virus

Four HDPs (human alfa-defensin 5 (HD5), lingual antimicrobial peptide (LAP), human beta-defensin 2 (HβD2), and human beta-defensin 3 (HβD3)) were fused to a reporter protein (Green Fluorescent Protein -GFP-), to facilitate the expression and monitoring, and a histidine tag for protein purification. All these genes were chemically synthesized (Geneart) and cloned in pET22 expression vector. Each molecule was produced in *E. coli* BL21 using shake flasks at standard growth conditions and purified by IMAC as previously described (Roca-Pinilla et al. 2020).

In vitro activity of 1st generation of HDP-based proteins against SARS-CoV-2 virus

To test the antiviral activity of the HDPs against SARS-CoV-2, a constant concentration of a SARS-CoV-2 stock sequenced upon isolation was mixed with decreasing concentrations of the antiviral drugs and added to Vero E6 cells (Rodon et al. bioRxiv. Preprint. April 2020). To assess the potential drug-induced cytotoxicity, Vero E6 cells were also cultured with the same decreasing concentrations of the products (HDPs) in the absence of SARS-CoV-2. Cytopathic effects of the virus or products were measured at 3 days post-infection, using the CellTiter-Glo luminescent cell viability assay (Promega). Luminescence was measured in a Fluoroskan Ascent FL luminometer (ThermoFisher Scientific).

Results

HDP-based recombinant proteins reduced up to 50% the infection of human Vero-2 cells by SARS-CoV-2 virus in a dose-dependent manner (Figure 1a and 1b, left panel), but do not cause any toxic effects to cells (Figure 1a and 1b, right panel).

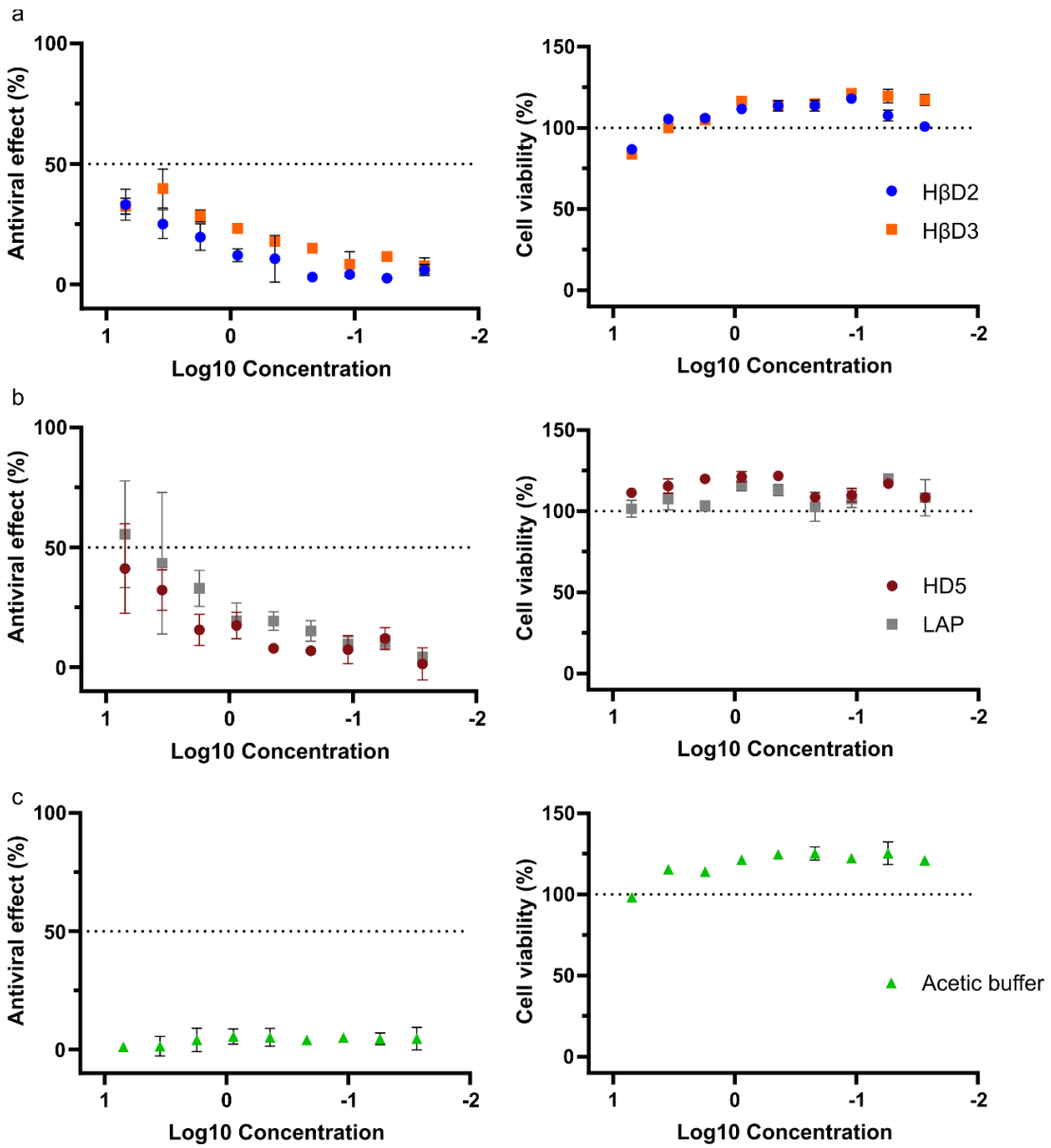


Figure 1. HDPs evaluation against SARS-CoV-2. Antiviral effect (left panels) and cell viability (right panels) after the two-fold diluted treatment series of (a) HβD2, HβD3, (b) HD5, LAP (c), and acetic buffer 0.01% (negative control). Assays were performed per duplicate and plotted as the mean value ± SD.

Conclusions and future perspectives

HDP-based proteins of 1st generation have shown to be promising candidates against SARS-CoV-2, decreasing up to 50% the infection of the virus. The efficiency could be even increased by testing more 1st generation drugs (based on HDPs with previous reported antiviral activity) and further construction of 2nd generation of molecules, where several HDPs are combined in the same polypeptide to work synergistically.

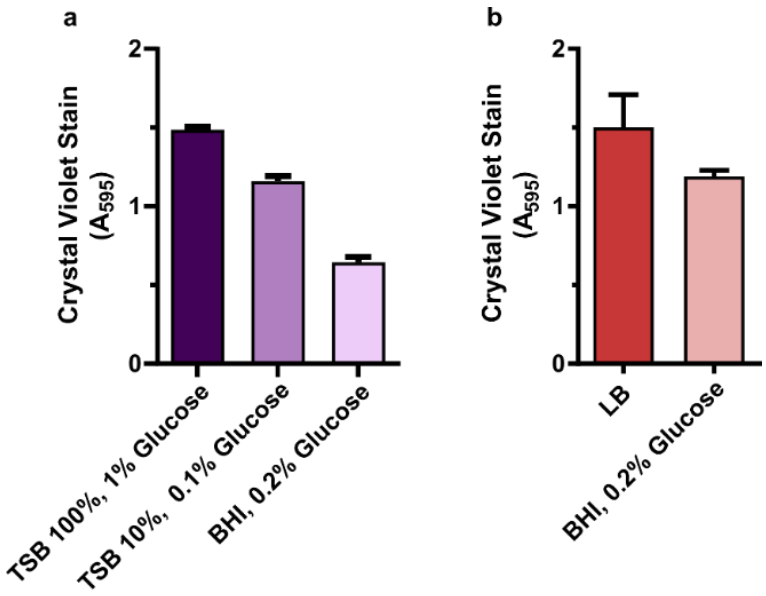
ANNEX 2

Functionalization of catheters with antimicrobial agents of broad-spectrum

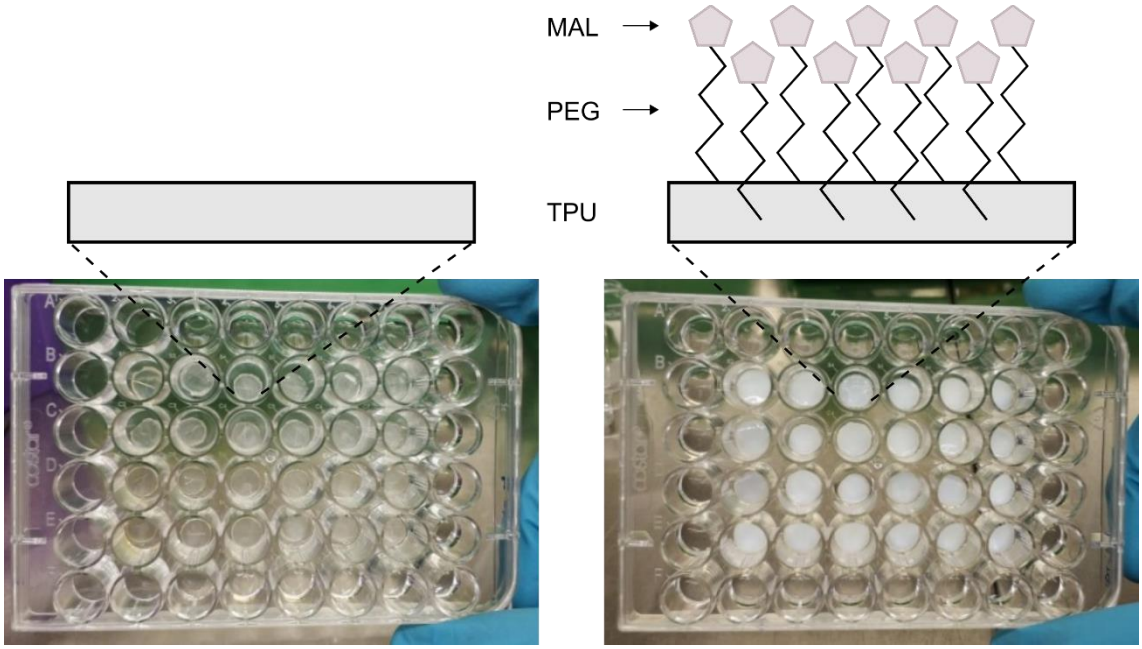
Background and objective

Nosocomial infections are generally associated with biofilm instauration of MDR bacteria in medical devices. Concretely, the most prevalent involved central line-associated bloodstream infection (CLABSI) and catheter-associated urinary tract infection (CAUTI). Hence, considering the superb antimicrobial activities of the extensively evaluated HDPs against both planktonic and biofilm bacteria, we are developing and testing a novel catheter functionalized with HD5-GFP-H6 defensin as a proof-of-concept. Briefly, a TPU surface, which is the material that catheters are made, is functionalized through the incorporation of polyethylene glycol (PEG) followed by a maleimide (MAL) molecule, allowing the covalent binding of an engineered HDPs forming self-assembled monolayers (SAM).

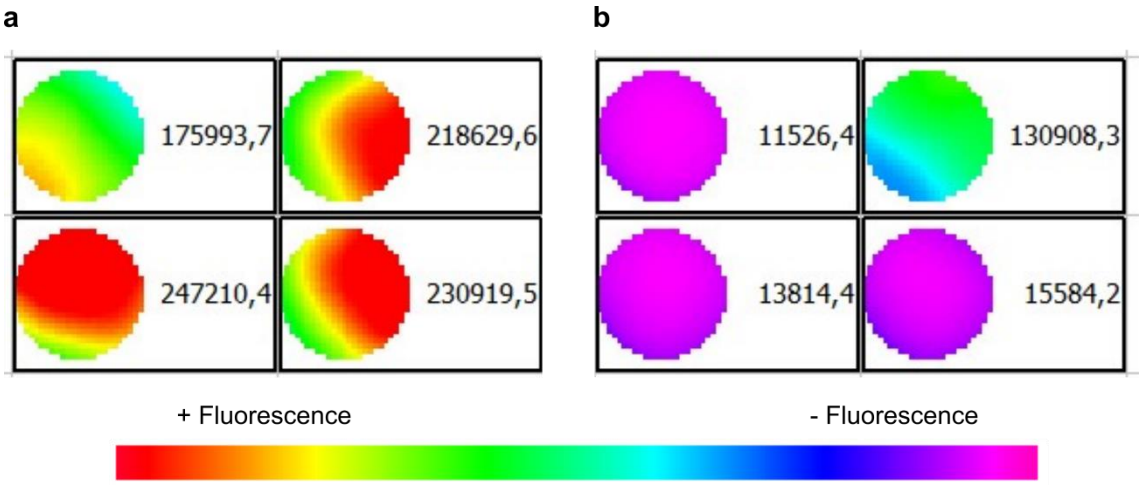
Results



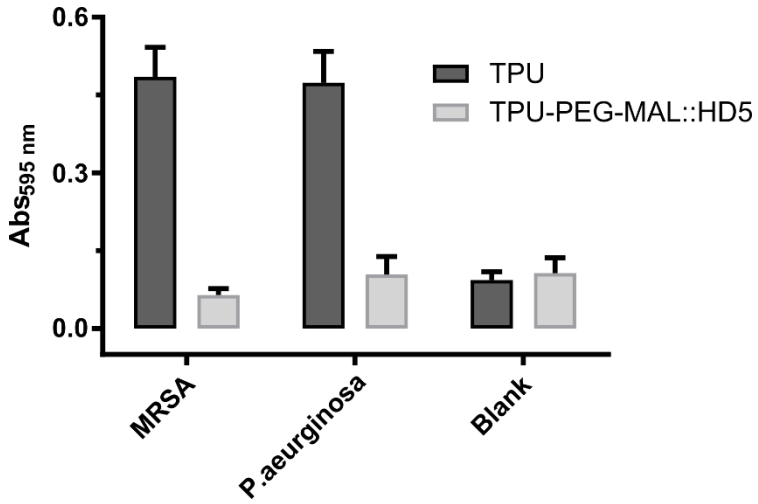
Supplementary Figure 1. Characterization of biofilm formation. Evaluation of biofilm formation of **a**. MRSA and **b**. *Pseudomonas aeruginosa* through crystal violet staining in various media formulation. TSB: tryptic soy broth; BHI: Brain-heart infusion broth; LB: Luria-Bertani broth.



Supplementary Figure 2. Circular TPU surface. Differences between non-functionalized TPU surface (left image) versus functionalized TPU with PEG and MAL (right image). PEG: polyethylene glycol; MAL: maleimide



Supplementary Figure 3. Analysis of HD5 attachment in TPU surface. a. Evaluation of the HD5-GFP-H6 distribution anchored in the circular surface of TPU-PEG-MAL through a fluorescence measurement of the GFP. **b.** control surface TPU-PEG-MAL. Data indicate the mean of RFU (relative fluorescence units) of a surface matrix analysis.



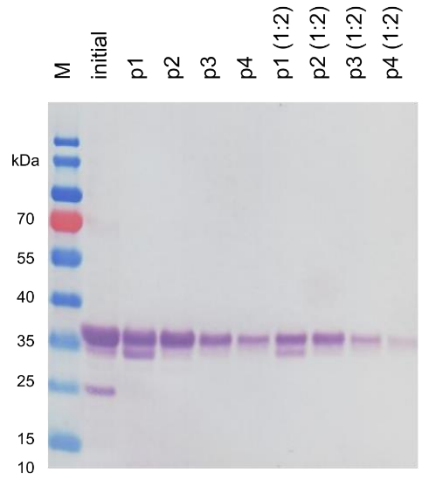
Supplementary Figure 4. HD5 biofilm inhibition. Biofilm inhibition against MRSA (Gram-negative) and *P. aeruginosa* (Gram-positive) bacteria of the anchored HD5-GFP-H6 antimicrobial peptide on the TPU-PEG-MAL surfaces (blak grey) in contrast with TPU control (ligh grey).

ANNEX 3: SUPPLEMENTARY MATERIAL IN STUDY 2

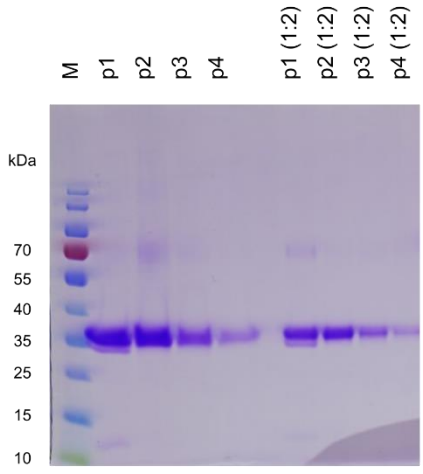
Soluble vs. solubilized recombinant proteins, the purification protocol matters

Adrià López-Cano[1], Paula Sicilia[1], Clara Gaja[1], Anna Arís[1*] and Elena Garcia-Fruitós[1*]

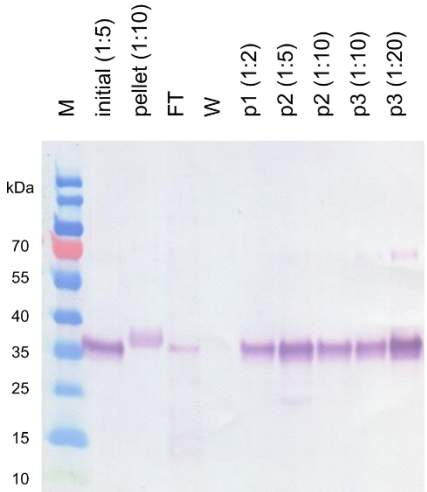
Supplementary figures. Western-blots (W.B) and Coomassie stained gels (Coom) of the soluble (S) and solubilized (ST-NLS) LAP-GFP-H6, HD5-GFP-H6, and LL-37-GFP-H6 from Study 2. Each peak (p) represents the different populations purified through the imidazole gradient during HDPs purification. Sample dilution (indicated in brackets) where applied when required. FT: flowthrough; W: wash; M: marker.



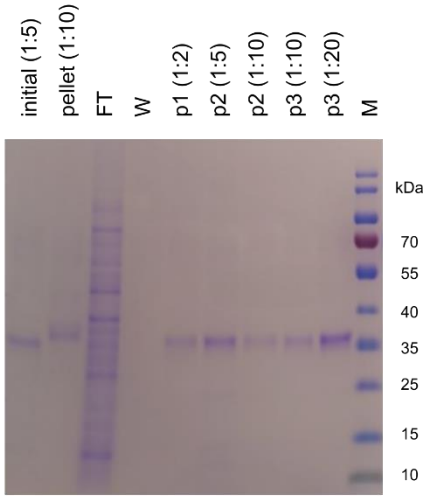
LAP-GFP-H6 W.B (S)



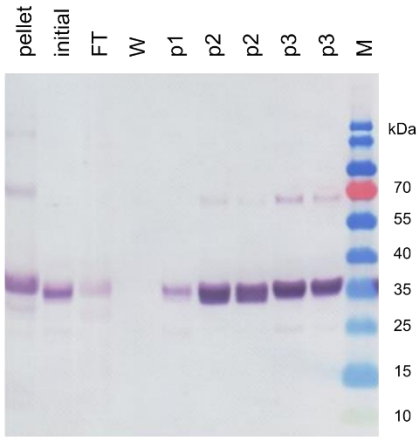
LAP-GFP-H6 Coom. (S)



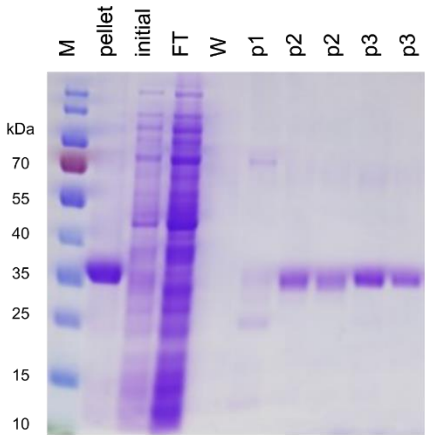
LAP-GFP-H6 W.B (ST-NLS)



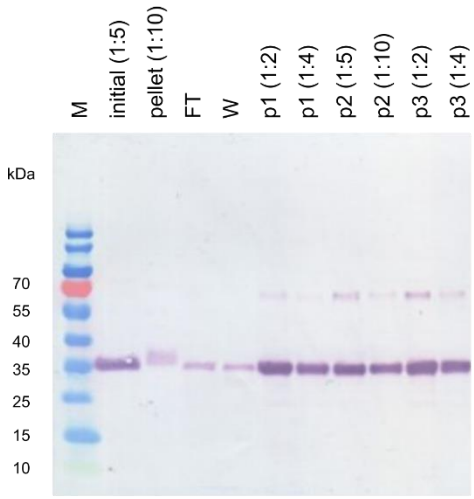
LAP-GFP-H6 Coom. (ST-NLS)



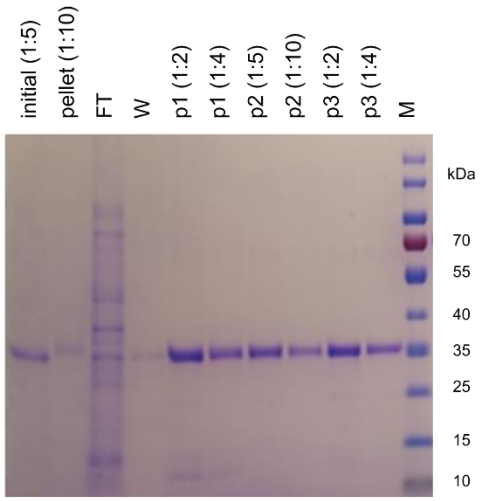
HD5-GFP-H6 W.B (S)



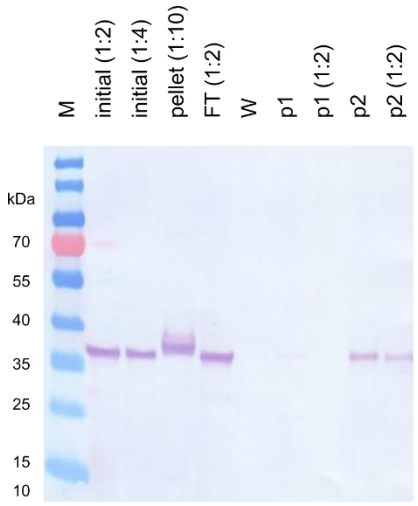
HD5-GFP-H6 Coom. (S)



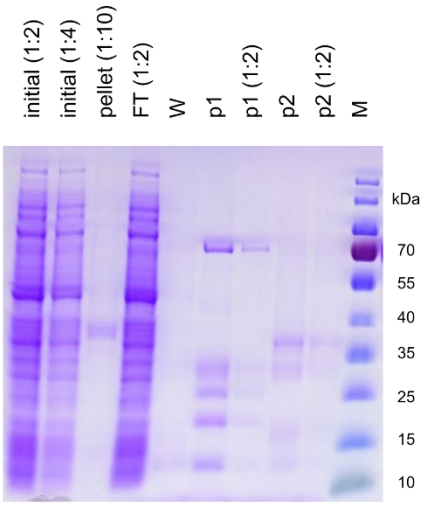
HD5-GFP-H6 W.B (ST-NLS)



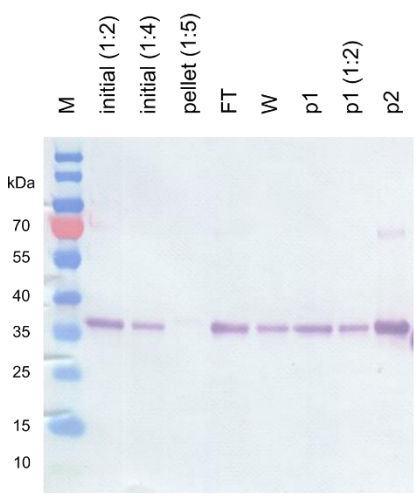
HD5-GFP-H6 Coom. (ST-NLS)



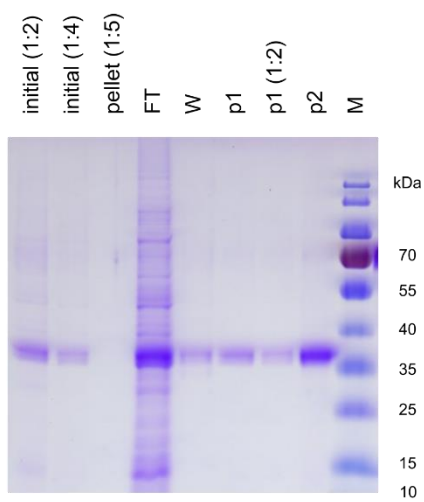
LL-37-GFP-H6 W.B (S)



LL-37-GFP-H6 Coom. (S)



LL-37-GFP-H6 W.B (ST-NLS)



LL-37-GFP-H6 Coom. (ST-NLS)

ANNEX 4: SUPPLEMENTARY MATERIAL IN STUDY 3

A novel generation of tailored antimicrobial drugs based on recombinant multidomain proteins

Adrià López-Cano, Neus Ferrer-Miralles[2,3], Julieta Sánchez[2], Anna Arís* and Elena Garcia-Fruitós*

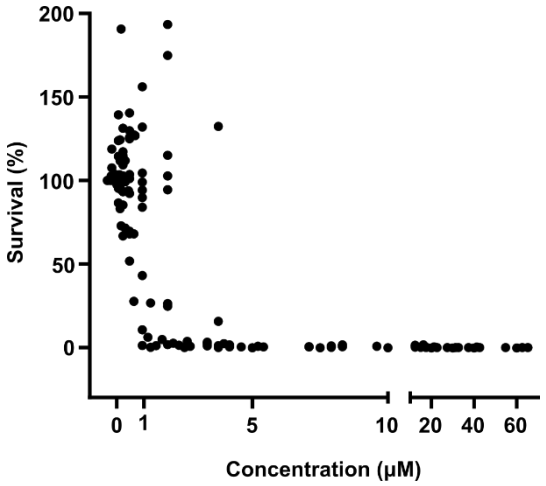


Figure. S1 | Dose-response determination of the 1st generation antimicrobials. MIC assay raw data of LAP, HβD2, HβD3 and HD5 against methicillin resistant *Staphylococcus aureus* (MRSA), methicillin sensitive *Staphylococcus aureus* (MSSA), methicillin resistant *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. Each point of the serial two-fold diluted antimicrobial concentration was illustrated to determine the optimal HDPs microbicidal concentration.

Table. S1 | HDPs aggregation ratio. Aggregation ratio of 1st and 2nd Generation of antimicrobial molecules. Data respresent the mean of triplicate ± SEM. n.d: non-determined

HDP	Aggregation ratio (%)
LAP	55.96 ± 3.79
HD5	79.59 ± 3.39
HβD2	13.08 ± 2.39
HβD3	71.95 ± 2.39
LL37	59.50 ± 2.64
D5L37D5L37	n.d
D5L37βD3	95.97 ± 4.03
D5LAL37βD3	100 ± 0.00

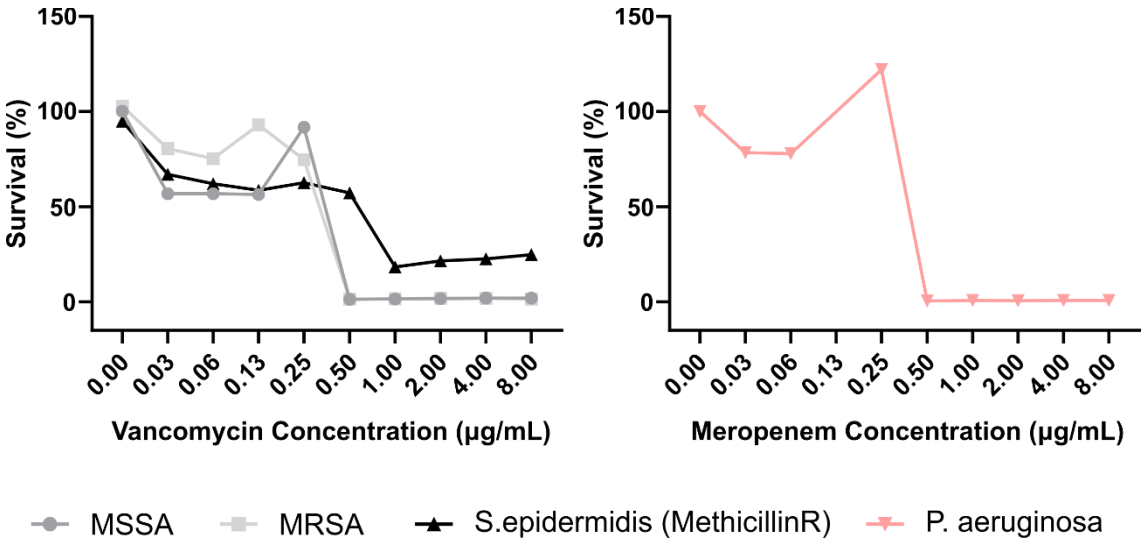
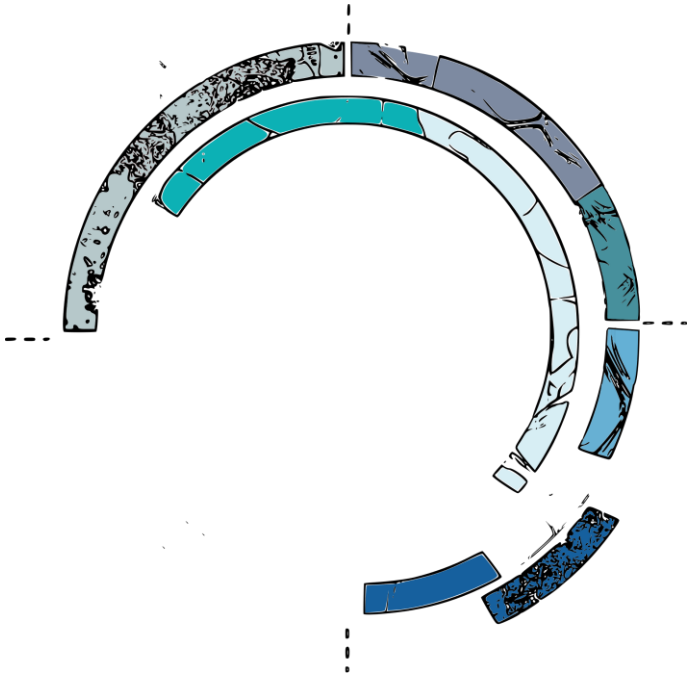


Figure. S2 | Minimal inhibitory concentration of relevant antibiotics. Minimal inhibitory concentration (MIC) assay of Vancomycin and Meropenem against methicillin resistant *Staphylococcus aureus* (■), methicillin sensitive *Staphylococcus aureus* (●), methicillin resistant *Staphylococcus. epidermidis* (▲) and *Pseudomonas aeruginosa* (▼), respectively. Each antibiotic was tested in a serial two-fold dilution to determine MIC against the four tested microorganisms, validating the strategy proposed.



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Sin duda alguna realizar un doctorado es una experiencia totalmente diferente a cualquiera que uno haya podido vivir. Se podía definir como una mezcla de incertidumbre y curiosidad (similar a aquella que puede tener una oveja por un león), pues en los comienzos uno siempre piensa que tampoco va a ser tan agotador y complejo (mentira). A medida que avanzas en la aventura (símil al círculo que se va rellenando de color en cada sección del trabajo) te vas dando cuenta que lo que antaño suponía un reto, ahora no es más que un mero obstáculo (los caminos de la investigación suelen ser como los de Jesucristo, inescrutables). Pero el doctorado siempre te acaba poniendo a prueba, ayudándote a conocer tus límites y lenta pero inexorablemente superándolos para poder continuar con el próximo desafío. Haciendo un paralelismo con la portada de esta tesis, se podría extrapolar a los organismos en suspensión como ese joven alumno perdido y zozobroso en busca de respuestas de como empezar su propio camino. En ese punto, si eres tan afortunado como yo, podrías encontrar a tus mejores guías, mis increíbles directoras y más que consagrado tándem Anna y Elena. Con ello, el joven alumno puede empezar a instaurarse en el que será su nuevo centro de investigación, donde dará sus primeros pasos y empezará a madurar, para finalmente (acabando con está metáfora) emprender un nuevo y emocionante comienzo.

Mi historia empieza en 2016 -cuaderno de bitácora, 4 años antes del COVID- cuando durante una jornada de seminarios (a cada cual más soporífero cabe destacar) mi futura directora, Anna Arís (a veces cariñosamente confundida como Anaís Arís), llamaría mi atención con una presentación de lo más motivadora. Rápidamente apunté su contacto al acabar el seminario, pero como ya suele suceder, no todo es tan sencillo. Por casualidades del destino, mi yo de 23 años decidió escribir un correo a anna.iris@, sin obtener ninguna respuesta (de lo contrario hubiera sido cuanto menos curioso), pero por suerte y después de un poco de búsqueda anna.aris@ recibió mi interés y a partir de aquí todo nos conduce a este punto final que son estos agradecimientos.

Anna, Elena, infinites gràcies per fer-ho tot tan fàcil, per sempre estar disponibles i fer aquests anys una vivència encara més extraordinària si cap. Durant aquests quatre anys m'heu fet reafirmar més encara la meva passió per la investigació, perquè amb vosaltres tot sembla més senzill. Realitzar aquest últim esprint que suposa la tesi no hagués sigut ni de tan bon tros una experiència tan enriquidora sense el vostre suport, surgències i aquest humor (afegir GIF minions here) que us caracteritza. Continuant per la vostra oficina, encara me'n recordo el primer dia que vaig anar a Torre Marimon, on la Marta ja em va dir que perquè no començava aquell mateix dia les pràctiques i que no feia falta deixar passar el Nadal. Sense cap mena de dubte, fas que el grup de Remugants sigui especial amb tu, donat que difícilment es poden trobar tan bones persones, sempre amb un Sí per davant si d'ajudar a un company es refereix. Ni parlem de la que possiblement és i serà la que més concursos oficials ha guanyat i guanyarà de Remugants.

D'altra banda, el millor duo en ~~swine production~~, dic ruminant beef production, Sònia i Maria. Tot i que quan no estàs familiaritzat et pugui sorprendre algun comentari de la Sònia, que a vegades fan cas molt omís a les famoses 3 portes, sense aquesta naturalitat i espontaneïtat res seria el mateix i et fa un component únic! Per no dir que sempre me'n recordaré que un cop dinant em vas donar la raó a un comentari que vaig fer, fet que probablement cap doctorant podrà repetir (o això espero). Pd: gràcies també per acceptar-me com a part de Remugants encara que hagi treballat com un tràsnfuga amb porcí. Maria, gràcies especialment a les teves signatures, que sembla que no, però, sense elles això mai s'hauria pogut escriure. Ara parlant seriosament, el teu enfocament que sempre li has donat a les teves reflexions o preguntes quan parlem sempre m'ha semblat del més enriquidor i m'ha permès poder obrir el meu camp de visió més enllà, per no dir que el average daily gain, entre d'altres, ara forma part d'aquest coneixement que mai oblidaré. Per última, Lourdes, encara que ets la nova incorporació, molta sort i felicitats per aquest nou any que estaràs amb nosaltres!

The serious business is about to start. Començant pels doctorats, Ramon tu vas ser el meu primer mentor i BrocTiter, d'alguna forma vaig agafar el relleu de la teva tesi i em vaig endinsar en el món antimicrobià. Com oblidar-se de Jonny a.k.a el planchao, les aventures per Boston + Nova York, el viatge Toscanes (les 581 multes de tràfic que ens van posar, suficient recaptació econòmica per asfaltar mitjà Italià i posar dreta la torre de Pisa), sembrar més plaques que dies té un any i el més important, per proclamar-me Stif I, espero que estiguis genial per Australià i que algun dia puguis guanyar-me algun combat a judo després de les 7 ó 8 derrotes al ring de Torre Marimon (tot totalment inventat). Laia, tot i que quan vaig començar no hi eres i que sempre em parlaven d'una noia molt riallera, ornitòloga de professió, amant de les orenetes i els estornells (consultar Gifre *et al* tesis, última pàgina), quan vas tornar tot el que deien es va quedar curt. Aquells berenars de miss Daisy, el siñor timmy i la siñora homer, els playmobils castellers i la teva energia cada matí era única, moltíssima sort per la teva aventura a Bèlgica! Ricky, també conegut com a Richi per Maria, quins bons records quan vaig perdre l'ànima per anar a veure les fagedes, caminant 342 km per muntanya ben lluny de qualsevol rastre de civilització. El segon BrocTiter de l'equació, com oblidar el sofà on vam dormir més doblegats que una cantonada de Boston Hills, les tardes d'AKTA amb la cançó de *under pressure* (per nosaltres *over pressure*), els seminaris que a tu per sort se't feien més curts que a ningú (sleepy Ricky), i en general els grans moments, especialment la "inflasión" i la preparació i edició de vídeos totalment random per tesis. Prepara't perquè l'altre mitja part que queda per asfaltar d'Italià la finançarem el pròxim març :).

Lucia, la recontraargentina (nota aclaratoria: en argentino poner recontra delante de cualquier palabra es sinónimo de éxito y de buen porvenir) que vino a cambiarlo todo, que instauró en la oficina el denominado *Eje del Mal*, la que le dio un nuevo significado al mítico buscaminas

versión argentina v.2.0. Eternamente agradecido por tus lecciones avanzadas de argentino, por enseñarnos que es una pileta, porque nos planteemos que un congelador ya no es un congelador, por tus historias y canciones de comer "asaditos" y por último por reducir nuestra productividad un 400% pero hacer que venir todos los días al trabajo sea sinónimo un gran día! Dra. Cristinovich & Català, molta sort (més encara que per qui hagi agafat el teu acudit anterior) en la teva primera i última matrícula (fora de termini segons els últims emails de Romualdo Giménez & Sons) i felicitats per la inscripció anual a l'Eje del mal. Esperem que quan Banyoles esdevingui ciutat important puguis començar els teus experiments i la millor de les sorts en el doctorat (si vols córrer en direcció contrària, *now or never*). Pd: Sergi el mateix missatge també t'aplica, sort amb el màster! Denise, muchísimas felicidades por Emma, por aguantarme los interrogatorios sobre el guaraní, guara qué? ¡guaraní! y por tus cariñosos macaco tan inconfundibles. Fent transició (mai millor dit), Marina, mai perdis aquest interès que tens en voler aprendre o conèixer una mica de tot el nostre món molecular i sort per aquests últims anys de doctorat! Menció especial a Clara, Jordi i Paula, els joves *esbirros* que vaig tenir la sort de poder compartir laboratori uns mesos.

Cesc, el mentor, l'apagafocs per excel·lència, el tècnic que feia funcionar els aparells només amb la seva presència, a.k.a Paco 3000. Diria que et trobaré a faltar, però tot sembla que encara tenim un any més per acumular noves vivències i que per fi tingui els meus temporitzadors en aquesta lluita interminable. Algú ha vist algun cop millor tàndem per evacuar un edifici? No ho crec... Per últim, però no menys important, els de camp: Anna, Xavi, Maria (no la que signa) Isis, (la boira de Lleida), sou un gran equip i un pilar fonamental pel grup, gràcies per deixar-me conèixer una mica més i aprendre dia rere dia (*specially on Mondays*) el que feu.

Gracias a mis amigos que me llevan soportando desde la infancia -que se dice pronto, especialmente a Fernando (buen abogado, mejor persona), Víctor, Sergi$_{profe}$, Dani, Gerard, Meritxell y también a aquellos que gracias a la Universidad pudieron formar parte, Raül (Jack) por aquel contrato con el At. Lubos que dio pie a todo, Miguel (Shoda), Joan, Meri, Queralt, Lidia y Laura, aún en la distancia, siempre Élite. Continuar con aquellos amigos inseparables de largas sesiones de juegos de mesa y fútbol (actualmente algo parecido al pádel), Xavi -no el de Catar-, Carlos, Adri y Madurell, gracias a todos por formar parte de este camino.

Por último, a mi familia, a mis padres, que me transmitieron los valores y la fuerza para conseguir aquello por lo que valía la pena esforzarse, por ser el mejor apoyo que un hijo pueda tener, el mejor faro y a la vez el mejor reflejo y sin ninguna duda mi fuente de inspiración. A mi hermana, de la que uno no se puede sentir más orgulloso y que siempre ha estado ahí en cada pequeño paso que he dado, seguro que lo harás igual de extraordinario con Abril.

Y a Mari, por ayudarme a ser quien soy, por guiarme, comprenderme y acompañarme durante ésta y otras mil aventuras sin dudarlo ni un momento, gracias de corazón.

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