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Study of innate immunity-related proteins for the treatment of bovine infectious diseases

Ricardo Baltà Foix

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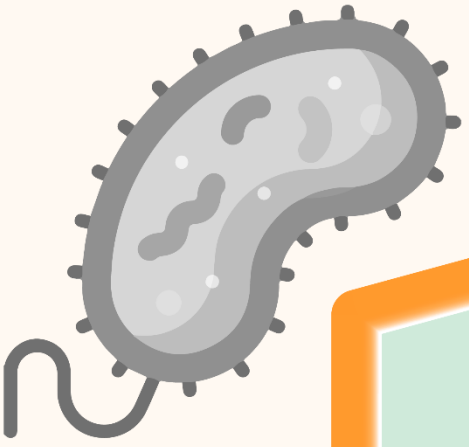

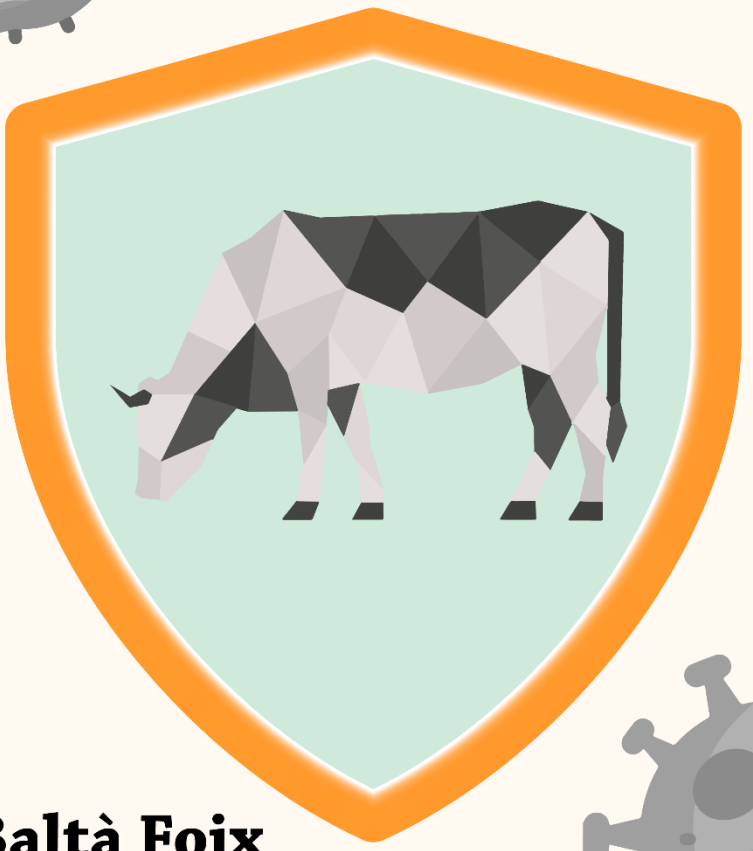

Ricardo Baltà Foix

PhD thesis

2023

PhD Thesis

Study of innate immunity-related proteins for the treatment of bovine infectious diseases

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Ruminant Production Program

Institut de Recerca i Tecnologia Agroalimentàries (IRTA)

PhD program in Biochemistry, Molecular Biology, and Biomedicine

Departament de Bioquímica i Biologia Molecular

Universitat Autònoma de Barcelona

2023

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PhD in Biochemistry, Molecular Biology and Biomedicine

Study of innate immunity-related proteins for the treatment of bovine infectious diseases

PhD Thesis, 2023

Thesis submitted by Ricardo Baltà Foix in candidacy to the PhD Degree in Biochemistry, Molecular Biology and Biomedicine by Universitat Autònoma de Barcelona

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This work has been mainly conducted in the Institut de Recerca i Tecnologia Agroalimentàries (IRTA), under the supervision of Anna Arís Giralt, PhD and Elena Garcia Fruitós, PhD

PhD advisor at UAB: Simó Schwartz Jr., MD, PhD

"The essential is invisible to the eyes"

Antoine de Saint-Exupéry – The Little Prince

A la Mamá, que siempre creyó

y sigue creyendo en mí

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SUMMARY

Infectious diseases (IDs) are a global challenge for human and animal health, particularly those caused by antibiotic-resistant microorganisms. In the specific case of the cattle industry, IDs not only impact on animal health and welfare, but also drastically reduce production and the economic margin of the sector. The use of antibiotics allows the control of part of IDs, but the constant emergence of resistant bacteria encourages finding new strategies to substitute antibiotics. On the other hand, the efficiency of existing vaccines is not sufficient to control the emergence of IDs, making it clear that alternative therapies are needed for the treatment of bovine IDs such as mastitis and bovine respiratory disease (BRD). Innate immunity provides us with an extensive assortment of molecules with high therapeutic potential. Among them, metalloproteinases, acute phase proteins, or cytokines have been studied in the last years with interesting results. Nevertheless, none of them have the functional versatility of Host Defense Peptides (HDPs), which have antibacterial, antiviral, antifungal and immunomodulatory activities. These peptides are mainly produced through chemical synthesis, which has important limitations in terms of cost and scalability to be applied *in vivo*. Recombinant protein production in bacteria has emerged as an ideal strategy for the obtainment of therapeutic innate immunity-related proteins at lower costs at industrial amounts. The bacterial specie most widely used for recombinant protein production purposes is *Escherichia coli*, but *Lactococcus lactis* has also shown to be a good alternative, being a lipopolysaccharide (LPS)-free system.

First, this thesis pursues to expand the available systems for the LPS-free recombinant protein production of immune-related protein, exploring the use of *Lactiplantibacillus plantarum*. The results obtained showed that *L. plantarum* can be used as a microbial cell factory as it occurs with *L. lactis*, reporting for the first time the formation of inclusion bodies (IBs) in this expression system. Secondly, suitable solubilization protocols to obtain soluble, pure, and active immune-related proteins from IBs have been developed. Finally, this thesis attains the production of recombinant bovine HDPs in LPS-free recombinant systems which show potent antibacterial and antiviral activities against bovine pathogens. The outcomes derived from this thesis will be the beginning of a new treatment for BRD and a promising alternative to the use of antibiotics in animal production.

RESUM EN CATALÀ

Les malalties infeccioses (MIs) són un repte global que afecta tant a la salut humana com a la salut animal, especialment aquelles causades per microorganismes resistents als antibiòtics. En el cas concret de la indústria bovina, les MIs no només impacten sobre la salut i el benestar animal, sinó també redueixen de forma dràstica la producció i el marge econòmic del sector. L'ús dels antibiòtics permet el control de part de les MIs però l'emergència constant de bacteris resistents a aquests impulsa trobar noves estratègies per substituir als antibiòtics. Per altra banda, l'eficiència de les vacunes existents no és suficient per controlar l'emergència de les MIs fent palès que es necessiten teràpies alternatives pel tractament de les MIs bovines com son la mastitis o la síndrome respiratòria bovina (SRB). La immunitat innata ens proporciona un ampli assortiment de molècules amb un alt potencial terapèutic. Entre elles, les metal·loproteinases, les proteïnes de fase aguda o les citocines s'han estudiat en els últims anys amb resultats interessants. No obstant això, cap d'elles té la versatilitat funcional que tenen els Pèptids de Defensa de l'Hoste (HDPs) ja que tenen activitat antibacteriana, antiviral, antifúngica i immunomoduladora. Aquests pèptids són principalment produïts mitjançant síntesi química, però és un procés que té importants limitacions en termes de costos i escalabilitat que dificulta l'aplicació d'aquestes molècules *in vivo*. La producció de proteïnes recombinants en bacteris ha emergit com una estratègia ideal per obtenir proteïnes terapèutiques relacionades amb la immunitat innata a baix cost essent possible la seva producció a escala industrial. L'espècie bacteriana més àmpliament usada per produir proteïnes recombinants és *Escherichia coli*, però *Lactococcus lactis* també ha mostrat ser una bona alternativa, essent a més un microorganisme lliure de lipopolisacàrid (LPS).

En primer lloc, aquesta tesi busca ampliar els sistemes lliures de LPS disponibles per a la producció de proteïnes recombinants relacionades amb la immunitat innata, explorant l'ús de *Lactiplantibacillus plantarum*. Els resultats mostren que *L. plantarum* pot ser usat com a fàbrica bacteriana igual que *L. lactis*, descrivint per primera vegada la formació de cossos d'inclusió (IBs) en aquest sistema d'expressió. En segon lloc, s'han descrit protocols adequats de solubilització per obtenir proteïnes relacionades amb la immunitat innata solubles, pures i actives a partir d'IBs. Finalment, aquesta tesi aconsegueix la producció de HDPs bovins recombinants en sistemes lliures de LPS, essent molècules

amb una potent activitat antibacteriana i antiviral contra patògens bovins. En global els resultats d'aquesta tesi suposen l'inici prometedor d'un nou tractament pel SRB i una alternativa viable al ús dels antibiòtics.

TABLE OF ABBREVIATIONS

Ab	Antibody
AMP	Antimicrobial Peptide
APP	Acute-Phase Protein
AP	Anionic Peptide
Bac	Bactenecin
BCV	Bovine Coronavirus
bG-CSF	Bovine Granulocyte Colony-Stimulating Factor
BHV-1	Bovine Herpesvirus Type 1
BMAP	Bovine Myeloid Antimicrobial Peptide
BNBD-1	Bovine β-Defensin 1
BNBD-2	Bovine β-Defensin 2
BNBD-3	Bovine β-Defensin 3
BNBD-4	Bovine β-Defensin 4
BP	Bacteriophage
BRD	Bovine Respiratory Disease
BRSV	Bovine Respiratory Syncytial Virus
BSA	Bovine Serum Albumin
BVDV	Bovine Viral Diarrhea Virus
CHO	Chinese Hamster Ovary Cells
CoV	Coronavirus
DMI	Dry Matter Intake
DRAMP	Data Repository of Antimicrobial Peptides
DTE	Difficult-To-Express
EFSA	European Food Safety Authority
ELP	Elastin-Like Polypeptide
FAO	Food And Agriculture Organization Of The United Nations
FBS	Fetal Bovine Serum
FDA	Food And Drug Administration (USA)
FESEM	Field Emission Scanning Electron Microscopy
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GdnHCl	Guanidine Hydrochloride
GFP	Green Fluorescent Protein
GRAS	Generally Regarded As Safe
GST	Glutathione S-Transferase
HBV	Hepatitis B Virus
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HD5	Human α-Defensin 5
HDP	Host Defense Peptide
HGT	Horizontal Gene Transfer
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
HSV	Herpes Simplex Virus
IB	Inclusion Body
ID	Infectious Disease
IMAC	Immobilized Metal Affinity Chromatography
IMI	Intramammary Infection
IV	Inactivated Virus
LAB	Lactic Acid Bacteria
LAP	Lingual Antimicrobial Peptide
LPS	Lipopolysaccharide

mAb	Monoclonal Antibody
MDR	Multidrug-Resistant
MI	Malaltia Infecciosa
MIC	Minimal Inhibitory Concentration
MLB	Modified-Live Bacteria
MMP-9	Matrix Metalloproteinase 9
MMP-9cat	MMP-9 catalytic domain
MMP	Matrix Metalloproteinase
MRSA	Methicillin-Resistant *Staphylococcus aureus*
M-SAA3	Mammary Serum Amyloid A3 Protein
NICE	Nisin Controlled Gene Expression
NID	Non-Infectious Disease
NK	Natural Killer cells
NLS	N-Lauroylsarcosine
O/N	Overnight
OD	Optical Density
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PEG	Polyethylene Glycol
PI-3	Parainfluenza-3 virus
PRR	Pattern Recognition Receptor
PT	Phage Therapy
PTA	Prone-To-Aggregate
PTM	Post-translational Modification
PVDF	Polyvinylidene Difluoride
QPS	Qualified Presumption of Safety
rAB	Recombinant Antibody
rboGMCSF	Recombinant Bovine Granulocyte-Macrophage Colony Stimulating Factor
RNase	Ribonuclease
RSV	Respiratory Syncytial Virus
RT	Room Temperature
RTI	Respiratory Tract Infection
SA	Specific Activity
SAAP	Surfactant-Associated Anionic Peptide
SARS	Severe Acute Respiratory Syndrome
SDS	Sodium Dodecyl Disulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error Of The Mean
SPA	Surfactant Proteins A
SPD	Surfactant Proteins D
sPLA2	Secreted Phospholipase A2
SapIP	Sakacin A Inducing Peptide
SppIP	Sakacin P Inducing Peptide
SPPS	Solid-Phase Peptide Synthesis
SRB	Síndrome Respiratòria Bovina
SUMO	Small Ubiquitin-related Modifier protein
TAP	Tracheal Antimicrobial Peptide
TBST	Tris Buffered Saline Tween
TEM	Transmission Electron Microscopy
WHO	World Health Organization

INTRODUCTION

INFECTIOUS DISEASES AND ONE HEALTH

Human and animal health have been always threatened by an uncountable number of diseases over the centuries. There exist many classifications, but all of them can be distinguished as non-infectious diseases (NIDs) or infectious diseases (IDs). The main difference between these two groups of diseases is their origin. NIDs are caused by conditions non-related to any type of pathogen, for instance, genetic abnormalities, cancer or cardiovascular disease. By contrast, IDs are caused by infections of one or a combination of pathogens in any part of the organism and are called "infectious" for their potential of being spread between individuals. Human beings have experienced many traumatic global health episodes, such as the three plague pandemics caused by *Yersinia pestis* in 541 AD, 1347, and 1894, the smallpox between 1870-1874, the measles in 1960 or the recent COVID-19 pandemic [1]. These events always have a common threat: the emergence of a new pathogen followed by the need to seek for a rapid and effective cure. Thus, this is a story that has been repeated over the centuries and it will be certainly repeated in the future.

Pathogens are microorganisms or agents that have the capability of causing diseases in their hosts. Pathogens from a huge variety of kingdoms and genera including bacteria, viruses, fungi and parasites have been described, and they affect humans, animals and plants. Microorganisms and viruses are present in all the environments of the planet and are part of most organisms. Fortunately, the bulk of microbes exposed to hosts has no negative effects. Only one in a billion microbial species is a human pathogen and nearly 1,400 have been described [2]. But, despite their low percentage, pathogens causing IDs are a major global threat. In 2019, one hundred out of every one-hundred and thousand people died due to infectious diseases globally [3]. Nowadays IDs are considered one of the main challenges for global economic, social and environmental growth.

Not only humans but livestock, domestic and wildlife animals are also affected by IDs. Especially in animal production the prevalence of IDs is higher than in other contexts [4]. The close contact between individuals and the mixture of animals from different origins makes spreading diseases easier. The bovine industry is not an exception, and the number of animals affected is increasing year by year. In this context, it is worth mentioning that it is being increasingly accepted that human, animal, and environmental health are closely connected. Every change in any of the parts could affect the others and

this idea has gained importance in the last years. Zoonotic diseases, antibiotic resistance or climate change are examples of this. Aiming to find integrative solutions for these global problems, the concept of One Health has emerged (**Figure 1**) [5]. It is based on the collaborative efforts of multiple disciplines to work jointly at the local, national and global levels to find strategies and solutions to improve human and animal health and also considering the environmental impact [6].

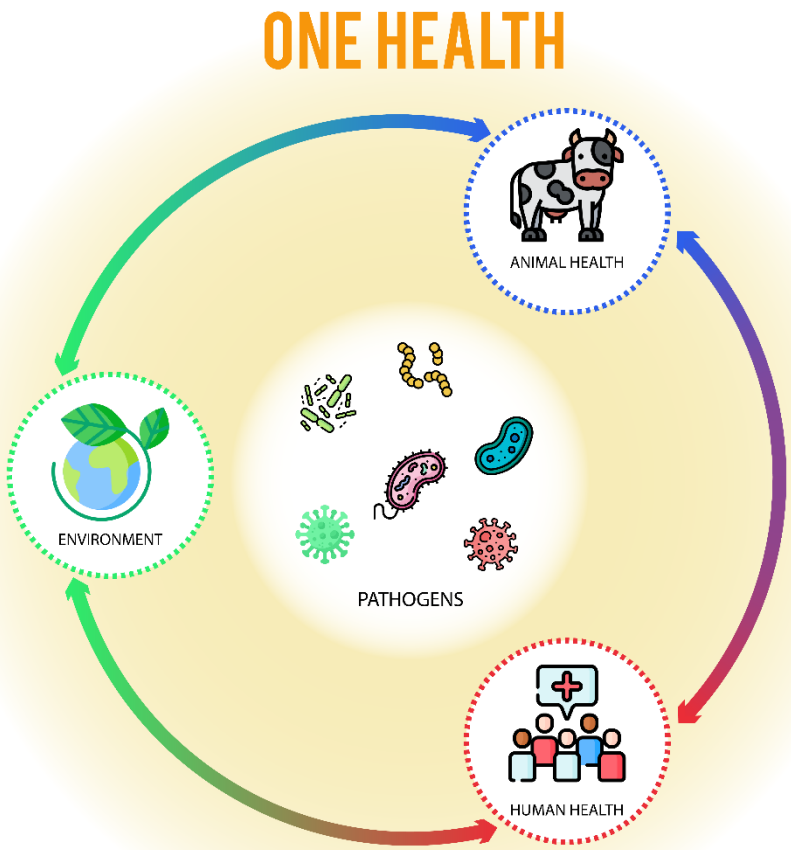

Figure 1. One Health scheme. The One Health concept encompasses the relationship between humans, animals and environment and their dependance. These connections manifest the importance of all partners in the fight against antimicrobial resistances, and stating the need of effective, consensuated and consistent actions to cope with this challenge.

Bovine infectious diseases

The livestock sector is a pillar of the global food system and a contributor to poverty reduction, food security, and agricultural development. According to the Food and Agriculture Organization of the United Nations (FAO), livestock contributes 40% of the global value of agricultural output [7]. Inside the livestock industry, cattle is one of the most important agricultural enterprises. Meat and milk from cattle and bison are estimated to account for approximately 45% of the global protein supply for humans [8]. Cattle, as humans, are affected by a huge number of diseases that go from affections of the digestive system to IDs of distinct origin [9]. Diseases represent an important challenge for bovine industry, significantly reducing the production and animal welfare as well as increasing the expense of animal maintenance, which overall have a negative impact for the producers.

Focusing on IDs, there exist more than 60 IDs affecting cattle worldwide [10]. The vast majority are caused by a unique or predominant pathogen, such as a bacterium, a virus, a fungus, or a parasite. However, some of these bovine diseases are caused by a combination of pathogens.

The bovine industry is divided into the dairy and beef industry, and each sector is affected by its specific IDs. Focusing on dairy cattle, mastitis is one of the most common and costly diseases in the dairy industry (**Figure 2**). It entails economic losses from 140€ in The Netherlands [11] to 450€ in Canada [12] per cow per year and raising up to 2 billion € annual costs only in the US [13]. It is caused by the persistent inflammation of the mammary gland consequence of intramammary infection (IMI). IMIs can occur during lactation in cows but they are more frequent during the dry period, a non-productive two-month period between lactations when the milking is stopped to prepare and regenerate the udder for the next lactation cycle [14–16]. This abrupt stoppage is associated with milk stasis and milk leakage in the udder, which significantly increases the possibility of IMIs and reduces animal welfare [17,18]. Moreover, if the IMIs persist or they are not well treated, the udder does not recover correctly, and that could affect the next lactations and reduce the milk yield and quality [19]. Mastitis is an example of a multi-pathogenic disease affecting cattle. Several types of bacteria can be involved in IMIs, being *Escherichia coli* [20], *Streptococcus uberis* [21], *Staphylococcus aureus* [22] or *Streptococcus agalactiae* the most frequent ones [23]. Although the treatment with

antibiotics is widely used for the prevention and control of infections in the mammary gland, their efficiency is decreasing due to the emergence of antibiotic resistances [24]. Thus, developing new treatments and strategies to tackle the mastitis issue is crucial for the improvement of the dairy sector productivity.

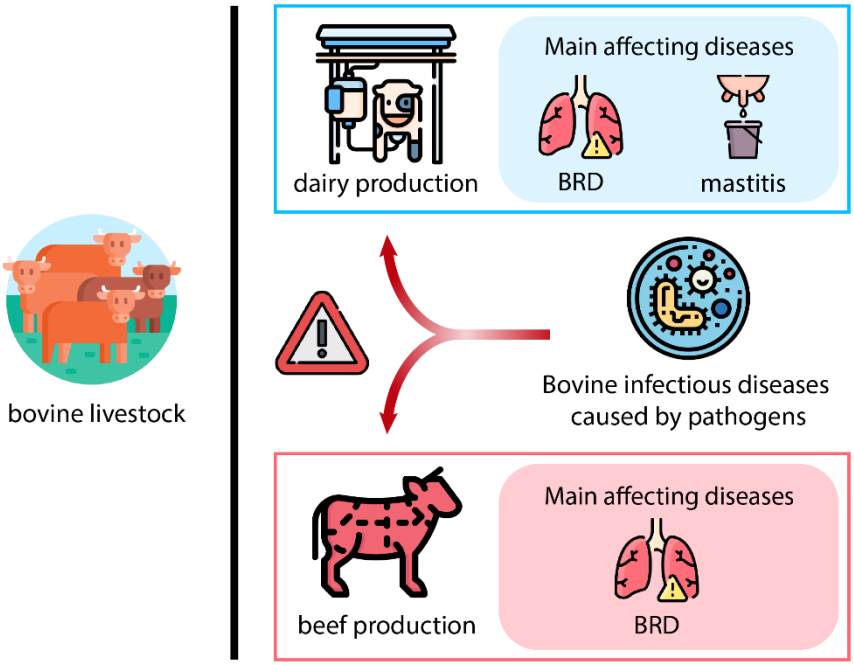

Figure 2. Bovine infections. Bovine livestock is affected by multiple pathogens, including bacteria and viruses at all levels in the industry. In the dairy industry, the most frequent and detrimental diseases are bovine respiratory disease (BRD) and mastitis. In the beef production industry, BRD is the most outstanding disease.

Bovine Respiratory Disease (BRD) is another example of ID affecting the bovine industry, beef and dairy,(25) caused by the combination of several pathogens, being in this case bacteria and also viruses (**Figure 2**). The effect of these infectious agents is usually combined with stress factors such as weaning, transportation, pooling of cattle from multiple sources, dusty conditions, parasitism, concurrent diseases, weather extremes and environmental factors [25]. This stressing situation directly affects the immunocompetence of the animals and increase their susceptibility to viral and bacterial infections which usually result in bronchopneumonia. The viral pathogens associated with BRD include bovine herpesvirus type 1 (BHV-1), parainfluenza-3 virus (PI-3), bovine viral diarrhea virus (BVDV) and bovine respiratory syncytial virus (BRSV). In addition, BRD is associated with several bacterial pathogens such as *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni* and *Mycoplasma bovis*. The

process starts with a primary viral infection, which facilitates the proliferation of opportunistic bacterial pathogens in the upper respiratory tract. Next, bacterial pathogens translocate into the lung and cause infections [26]. Thus, BRD is a multi-factorial and complex disease from a pathological, physical and physiological point of view [25,27]. The BRD treatment options are even less than those used for mastitis because of the higher variety of pathogens involved. Antibiotics do not have enough broader spectrum to act against all bacterial strains [27] and do not have activity against viruses. Moreover, the preventive strategies based on bacterial and viral vaccines do not achieve the expected results and need to be improved [28].

Bacteria and viruses as pathogens

The homeostasis of animals is constantly challenged by pathogens in the same way it occurs in humans. The infection of the host by pathogens always follows the same pathway: the encounter, the penetration, the dissemination and the multiplication [29]. Every step of this process disrupts different levels of the host defenses and every type of pathogen has its own strategies to achieve this disruption and impairs host homeostasis. Pathogens can directly damage tissues or cells during their replication or due to virulence factors such as the production of toxins. Another consequence of infections is a strong or excessive host immune response. Host defenses, during their efforts to reduce or eliminate the pathogen, can also harm its own cells and tissues. A disproportionate inflammatory response with immune cells (i.e., neutrophils) and inflammatory molecules (i.e., histamine) can impair the homeostasis in the infection site.

Although the microorganisms that originate IDs include bacteria, viruses, fungi and parasites, this thesis is focused on bacteria and viruses as the main causative pathogens of bovine diseases. Bacteria are prokaryotic organisms, which consist of a single cell with a cytoplasm containing DNA and organelles (**Figure 3**). The inner material is delimitated by a cell membrane and in most species, except a few such as *Mycoplasma*, by a complex cell wall (**Figure 3**). The most frequently observed genome structure is a double-stranded circular molecule of DNA, but there exist examples with more than one molecule of DNA and being lineal instead of circular. Some species also contain plasmids, which are small circular DNA molecules that can code for toxins or antibiotic-resistance genes. Outside the cell wall, some bacteria have flagella, pili or capsules. Flagella confers

motility, pili can adhere to host cell surface structures, and the capsule is a polysaccharide structure that surrounds the cell wall [30].

Most of bacteria are classified in two groups (Gram-positive or Gram-negative) based on the characteristics of their external layers outside the cell membrane (**Figure 3**). Gram-negative bacteria have a characteristic structure with a thin peptidoglycan cell wall and an outer membrane containing Lipopolysaccharide (LPS). LPS forms an external membrane that confers protection to Gram-negative bacteria and it is also an endotoxin that elicits a strong immune response. By contrast, Gram-positive bacteria have a thick peptidoglycan layer but they do not have the outer membrane or LPS. This greater cell wall is their main protection against external threats and counteracts the lack of LPS [29]. Not all bacteria are classified as Gram-positive or Gram-negative, some genus escape from that differentiation. Among them, the *Mycoplasma* genus is one of the most relevant. Their main characteristic is the lack of cell wall around their cell membranes (**Figure 3**). As cell membrane is the outer layer, they are pleomorphic and can adopt many different forms. *Mycoplasma* species are among the smallest organisms discovered with 0.2 – 0.3 µm in diameter. From a pathogenic point of view, *Mycoplasma* causes infections in different areas of human body, and some species can infect animals. Besides, they are resistant to many antibiotics, especially those that target the cell wall. In cattle, *Mycoplasma bovis* is a major pathogen causing respiratory disease, mastitis and arthritis [31].

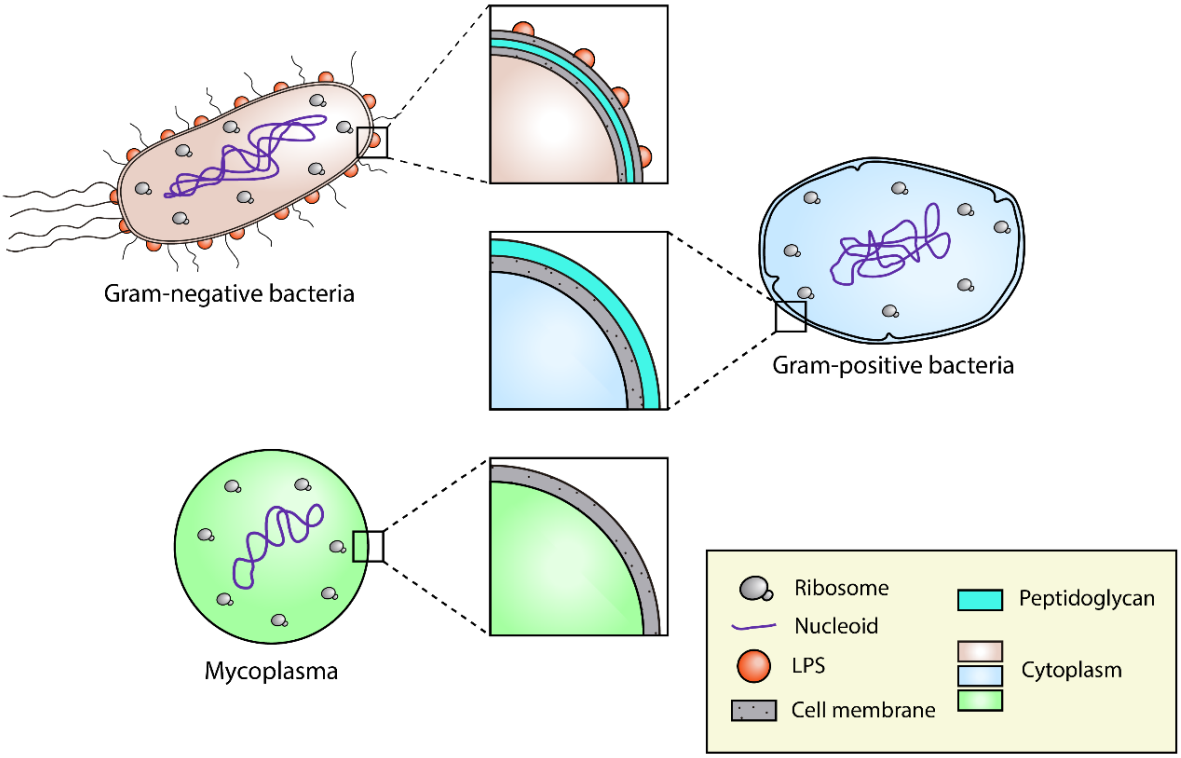

Figure 3. Structural comparison of the cell membrane of Gram-positive bacteria, Gram-negative bacteria and *Mycoplasma*. Upper panel corresponds to Gram-negative bacteria. Middle panel shows the structure of Gram-positive bacteria. Bottom panel corresponds to *Mycoplasma* membrane structure. All bacteria present an inner membrane that contains the cytoplasm. Gram-positive and Gram-negative bacteria present a peptidoglycan cell wall, whereas *Mycoplasma* has the simplest membrane structure. The cell wall in Gram-positive bacteria is thicker, making it harder to break. However, Gram-negative bacteria present an outer membrane, where lipopolysaccharides are embedded, along with pili and flagella which provide additional movement to these types of bacteria.

Viruses are completely different from bacteria conceptually, biologically, and structurally. First, they are not considered living microorganisms. They consist of a segment of nucleic acid (DNA or RNA), surrounded by a protein capsid and sometimes covered by an envelope (**Figure 4**). The viral genome can be single-stranded or double-stranded and may be linear, circular, or segmented. The capsid is composed of at least one protein and has three main functions: to protect the viral nucleic acid, to furnish recognition sites to receptors on the surface of the host cell, and to provide specialized proteins to penetrate through the cell surface membrane or to inject the infectious nucleic acid. Moreover, some viruses have a lipoprotein membrane, or envelope, derived mostly from the host membrane surrounding the capsid. The function of this envelope is closely related to the first recognition contact with host cell membrane receptors [32]. This simple viral structure does not permit them to survive for themselves and for that reason,

they are obligate parasites and are dependent on the cellular machinery for their replication. There exist viruses capable to infect living cells of animals, plants or bacteria with specific strategies adapted to each particular cell type [32].

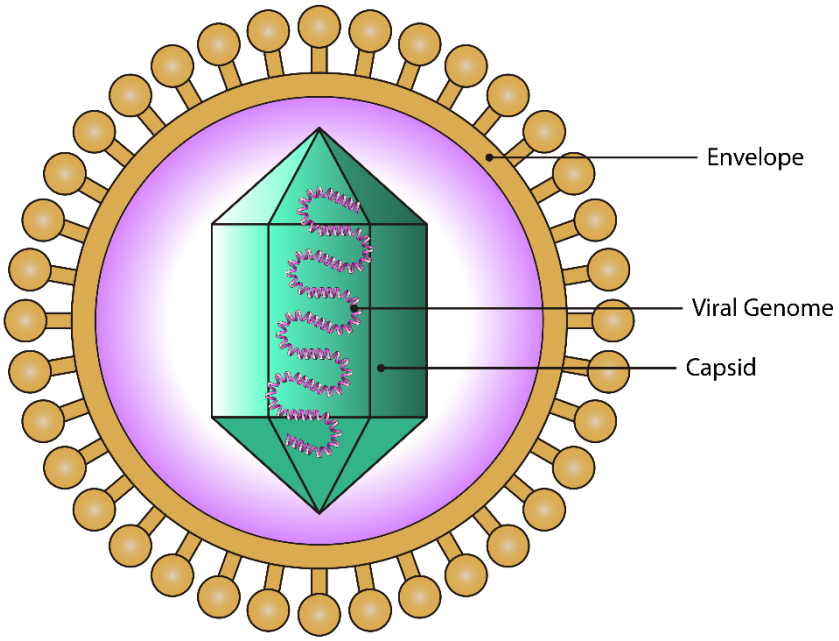

Figure 4. Structure of viral particles with envelope. Viruses present several types of structures. In the context of this thesis, the focus has been put on enveloped viruses. This type of virus consists in a capsid formed by proteins, that protect the viral genome in a first stage. They contain an additional cover, known as envelope, that corresponds to a lipidic membrane containing proteins that permits to the virus interacting with new hosts cells.

Treatments and preventive strategies

In the last century, the increasing knowledge about the infection capacity and infection mechanisms of bacteria and viruses has permitted the development of potent drugs, like antibiotics and antivirals, to directly act on the pathogens. Besides, this knowledge has been crucial to create new and effective preventive tools, like vaccines, to prepare the hosts and avoid future possible infections. All of them have reduced the impact of IDs for years. Nevertheless, pathogens have an extraordinary capacity for adaptation, continually challenging the effectiveness of the existing treatments and preventive strategies.

Antibiotics

Antibiotics are powerful drugs used to treat bacterial infections. Since the discovery of penicillin by Alexander Fleming in 1929 [33], more than 150 antibiotics have been found [34]. During the antibiotic era, thousands of lives have been saved, and human beings

have experienced exponential economic and social growth. Antibiotics have the capacity to either block bacterial growth and/or directly kill microorganisms. Their targets are specific sites present in the infecting organism but not in the host cells, i.e., the cell wall, cell membrane, nucleic acid synthesis pathway or ribosome, to name a few [30].

The use of antibiotics to treat bacterial infections has not only had an impact on human medicine but also on animal health and, consequently, affecting significantly in the bovine industry. The treatment and control of IDs in bovine livestock have allowed to increase milk and beef production, as well as to improve animal welfare. However, during the second half of the 20th century, the use of antibiotics was expanded to limits beyond the treatment of infections. It was discovered that in doses below the clinical ones, antibiotics had a growth-promoting effect in livestock [35]. This practice, and many other misuses, were spread globally contributing to the antibiotic resistance explosion [36]. Fortunately, at the end of the last century, different initiatives have been carried out for the control and reduction of the use of antibiotics. For instance, in Europe, the use of antibiotics as growth promoters was banned in 2006 [37] and in general the use of antibiotics has been controlled reserving the use of critical antibiotics for human therapy [38]. Although the use of antibiotics in veterinary medicine is being corrected, there is still much room for improvement. Nowadays, the most extended practice in bovine operations is the metaphylactic use of antibiotics [39]. It is based on the administration of antibiotics to a group of animals after diagnosing a clinical disease in part of the group, aiming to treat clinically sick animals and to control the spread of the disease to animals in close contact [40].

In the case of mastitis, direct intramammary administration of antibiotics has been applied for years to treat IMIs but also to prevent new IMI outcome during the dry period, most of the times without discriminating if there was or not an infection [41]. However, many countries have been trying to find effective alternatives to antibiotic use during cow dry period, avoiding economic losses to the farmer and accomplishing the One Health plan indications. Denmark and The Netherlands are two examples of countries that already banned the use of antibiotics during dry period unless the animals are sick [42]. Another strategy is the use of internal teat sealant without previous application of antibiotics. Teat sealant consists in the application of a soft viscose paste mimicking the

natural keratin plug that is formed when there is no lactation, impeding the entrance of pathogens into the mammary gland and reducing the risk of IMI [43].

The context of antibiotics and BRD is slightly different. Despite decades of research, the prevalence and challenges associated with BRD in bovine operations still remain. Antimicrobial metaphylaxis to cattle considered at high risk to develop clinical BRD signs has consistently been shown to reduce morbidity and mortality [44]. Besides, clinical diagnosis of BRD becomes very difficult by means of the current available tools. The BRD identification is totally subjective and does not supply robust signs of the illness [44]. Up today, metaphylaxis is the best option, but international and national policies reinforce finding alternatives to stop the widespread use of antibiotics in livestock farms [39,44,45]. Added to the difficulty of diagnosis, choosing the right antimicrobial becomes almost an impossible task due to the multipathogenic nature of BRD and the increasing appearance of antibiotic resistance. Moreover, antibiotics are not active against viruses requiring the development of additional therapies.

Despite all the advantages and improvements that the antibiotics use has provided as treatment for bovine infectious diseases such as mastitis and BRD, their overuse and misuse have contributed to the appearance of antibiotic resistance, being one of the most worrying threats nowadays and forcing the need to discover new and better therapies.

Antibiotic resistance

Antibiotic resistance is described as the ability of bacteria to avoid the effects of the drugs used to kill them or to stop their growth. Bacteria have always been rapidly adapted to any change in their surrounding environment. This fact is directly related to the acquisition of genes that confers them an evolutive advantage. Bacteria obtain this genetic improvement either through horizontal gene transfer (HGT) or through spontaneous mutation [46]. HGT is the transmission of genetic material between individuals without a parent-offspring connection. Spontaneous mutation refers to the appearance of *de novo* modifications in the bacterial genome due to errors in the replication process disrupting the natural genetic sequence. Considering this situation, the action of antibiotics has two main negative consequences: i) the reduction or elimination of sensible bacteria and ii) the growth of individuals with resistance genes (**Figure 5**). Antibiotics exert a boosted selective pressure on bacteria populations that would not occur under natural conditions [36]. This phenomenon is far from being an

anecdote. In humans 700,000 deaths per year are attributed to infections caused by antibiotic resistant bacteria [47], and estimations by 2050 are nearly 10 million deaths per year if the current use of antibiotics does not change [48].

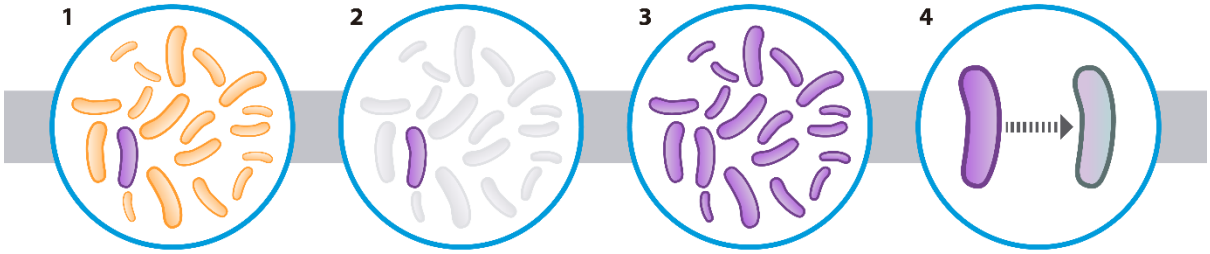

Figure 5. Selection of resistant bacteria. 1) In the bacterial population, there are punctual mutations that provide them of antibiotic resistance. 2) Addition of antibiotic cause the death of all sensitive bacteria and only resistant bacteria survive. 3) The surviving resistant bacteria continue dividing, generating a resistant bacteria population. 4) Resistant bacteria can spread the mutation through horizontal gene transfer (HGT) providing resistance to antibiotics to other bacteria.

According to estimates, 73% of all antimicrobials sold globally are used in food animals [49]. Counting chicken, cattle and pigs (93.75% of all food animals), the amount of active ingredients used was 93,309 tones, and an increase of 11.5% is projected by 2030 [50]. The real problem is hidden behind all the numbers and percentages, and it is that the current use of antibiotics is disproportionate. Treating all animals, sick and healthy ones, exerts a selective pressure that would be much lower if only infected animals would be treated. The emergence of resistant microorganisms is affecting livestock directly with an increasing prevalence in swine, poultry and cattle predominantly [51]. But, this also impacts on people working in farms and livestock holdings, who are in close contact with infected animals, as well as people living in the surroundings [50,52]. There exist 3 mechanisms described by which antibiotic use in animal production could impair human health: i) the direct infection with resistant bacteria by contact with livestock, or through ingestion of bacteria from contaminated meat or water, but without subsequent transmission between humans [53]; ii) a rupture in the species barrier by an animal pathogen followed by a sustained transmission in humans until one of these humans becomes ill [54,55]; iii) transfer of resistance genes arising in animal production and introduced into human pathogens by HGT [56–58].

In this scenario, World Health Organization (WHO) has provided guidelines considering the One Health approach to tackle the multifactorial problem of antibiotic resistance, with 5 main objectives: i) improving awareness and understanding the antibiotic resistance through better communication, education and training; ii) strengthening the knowledge through surveillance and research; iii) reducing the incidence of infection through effective sanitation, hygiene and infection prevention measures; iv) optimizing the use of antibiotics in human and animal health; v) increasing the investment in new medicines, diagnostic tools, vaccines and other interventions [59]. The consciousness about antimicrobial resistance issue and its connection with antibiotic usage is gaining importance, but a crucial part of the solution is the development of alternatives to antibiotics. In following sections, the most promising approaches are described. This point is decisive because there is an important innovation gap in the discovery of new antibiotics. The tough regulation, the lack of new molecules, the long process to raise the market and the lack of investment are hampering the research on new antibiotics [60].

The threat of antimicrobial resistance affects all strata of the natural world from humans to animals, and the environment surrounding them. Therefore, it is not a problem that can be solved with only one strategy or a unique perspective. A judicious usage and management of the antibiotics, the adoption of adequate preventive interventions and the improvement in the knowledge and discovering of novel classes of antimicrobials and alternatives will be vital to face this problem forcefully and effectively.

Antivirals

Currently, the most extended approach to control viral infections is the use of effective vaccines. However, vaccines are not a treatment, but a preventive tool used before infection. Thus, it will be commented more in detail in the following section.

This section is focused on antiviral drugs, which are those molecules used as a treatment to directly act on the life cycle of viral agents. Antiviral therapy seeks to eradicate the virus and prevent further viral invasion with a minimum or any impact on the host [61]. For that, the research of new antiviral drugs becomes very difficult due to the nature of the viruses. As viruses are mandatory intracellular parasites, it is an arduous mission to target the viral agent without harming or destroying the host cells. In addition, viruses are capable to develop new antiviral resistances, due to their high mutation rate [62]. Antivirals can be isolated from animals, plants, bacteria or fungi, and others must be

obtained by chemical synthesis [62]. They display different mechanisms of action, from enhancing the immune system blocking a specific enzyme or a particular step in the viral replication cycle [62,63]. Since the acceptance of the first antiviral drug, idoxuridine, by the US Food and Drug Administration (FDA) in 1963 [64,65], more than a hundred new antiviral molecules have been accepted. The increasing appearance of chronic viral infections such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), or hepatitis C virus (HCV) and the rise of new viruses like severe acute respiratory syndrome (SARS) coronaviruses, has accentuated the need to develop new antiviral agents.

Although antiviral-drug discovery has risen in the last decades, almost the totality of new molecules has been developed for human therapies [61]. The clinical use of antiviral drugs in animal medicine is not very common and it remains unexplored. There is a real concern about the use of antiviral agents in veterinary medicine for the possibility to develop resistances that could reduce the effect of the medications used against human viral pathogens [61]. In the veterinary medicine context, the economic viability of the treatments is essential. The amount of drugs required are much higher than those in human medicine. The efficiency must be maximized in trying to adjust the doses and reduce the cost. For that, the economic margin for the development of new antiviral drugs to treat viral IDs affecting animals is minimum. All together explains the poor current situation of antiviral drugs in animal health and why vaccines have been the preferred option in recent years.

Vaccines against bacteria and viruses

A vaccine is a biological product that gives active acquired immunity against a particular infectious agent to the organism that is receiving it. The functioning of a vaccine, from a conceptual point of view, is the administration of an agent to an organism with a subsequent immune reaction that will train the immune system to be aware of future infections. There exist several types of vaccines: i) inactivated vaccines; ii) live attenuated vaccines; iii) DNA or messenger RNA (mRNA) vaccines; iv) subunit, recombinant, polysaccharide and conjugate vaccines; v) toxoid vaccines vi) viral vector vaccines; and vii) virus-like particle vaccines (**Figure 6**) [66]. All of them are different strategies to accomplish the same objective, simulate the real disease without all the symptoms and the full pathogenicity. There exist vaccines against both bacteria and viruses.

Bacterial vaccines usually contain killed or attenuated bacteria that activate the immune system. Specific antibodies are produced against that bacteria and prevent the organism from future infections. Several vaccines against extracellular bacteria have been developed in the past and are still used successfully today, e.g., vaccines against tetanus, pertussis and diphtheria [67]. However, vaccination against bacterial infections is a challenge. In general, bacteria are more complex than other pathogens like viruses. They possess a higher variety of targets, and their immunogenicity, the capability to activate the immune system, is often unknown [67]. Because of that, choosing the right bacterial antigen to develop a new vaccine becomes a very arduous and tedious process. In addition, developing vaccines against intracellular bacteria is much more difficult than those just mentioned against extracellular bacteria. While induction of antibody production is usually sufficient against the second, T cell-mediated responses are needed to develop an effective defense against the first. Promoting cell-mediated immune responses often requires more potent antigens than those normally used as bacterial vaccines, for instance, non-living whole cells or bacterial subunits [67].

Viral vaccines are those preventive tools that have the goal of either inhibiting virus replication or killing infected cells [68]. They achieve this objective through the stimulation of an adaptive immune response. Immune responses target one or more viral antigens, and usually vaccines are composed of different versions of these antigens but without the capacity to develop the real disease. The first known vaccine used was a vaccine against smallpox, discovered and developed by Edward Jenner [69]. The smallpox vaccine was extraordinarily successful globally, and today is the only eradicated disease by immunization in the history of the humanity [70].

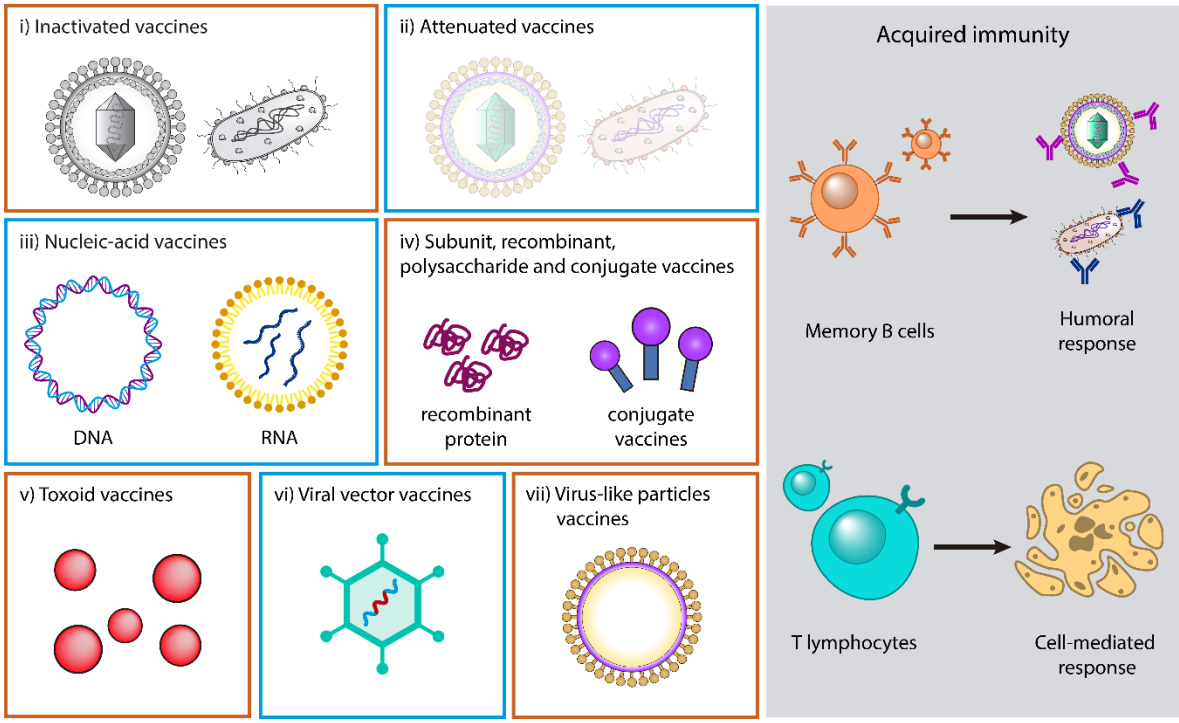

Figure 6. Types and classification of vaccines. There are different strategies to immunize organisms through vaccines: i) inactivated vaccines; ii) attenuated vaccines; iii) nucleic-acid vaccines, that can be made of DNA or RNA; iv) subunit, recombinant, polysaccharide and conjugated vaccines, v) toxoid vaccines; vi) viral vector vaccines; vii) virus-like particles vaccines. All of them activate the acquired immunity system through humoral and cell-mediated responses.

Vaccines have been very successful in human health context, but the number of vaccines used in animals is still low. However, examples like rabies and rinderpest vaccines have obtained promising outcomes and have demonstrated the real potential of vaccines applied to animal health [71]. Thanks to the Global Rinderpest Eradication Program, rinderpest may soon become the second disease (after smallpox) to be globally eradicated [71].

Regarding bovine sector, efforts have been made in the development of vaccines for preventing IDs. On the one hand, vaccines to prevent mastitis using whole killed bacteria cells are commonly used in dairy herds but with less than satisfactory outcomes in most cases [72]. There are commercial mastitis vaccines currently available for *E. coli*, *S. aureus*, and *M. bovis* [73] even so, there is still a lack of vaccines against many of the other pathogens that cause the disease. On the other hand, whole-herd vaccination against BRD pathogens is a common practice among producers and veterinarians [28]. Modified-live bacteria (MLB) [74] and inactivated virus (IV) [75] vaccines are commercially available. Nevertheless, the efficacy of the existing vaccines or the development of new ones needs

to be improved and reinforced by robust and contrasted vaccination protocols. An important number of studies has been reported in beef and dairy calves with modified-live virus (MLV) and attenuated virus and IV vaccines. They include at least one of the following agents, BHV-1, BVDV, BRSV, bovine coronavirus (BCV) and PI-3 virus, alone or in combination with *M. haemolytica* or *P. multocida* bacterins [28,76]. Although it has been observed a reduction in BRD morbidity and mortality in some cases, there are still many doubts about the moment and the type of vaccination. The responses are very different depending on the moment of vaccination, before or after weaning, and also depending on the via of administration (parenteral or intranasal). The immune system of the animals at the time of vaccination is far from optimal, and the immune response stimulated by the vaccination is not good enough in most cases [77]. Therefore, the standardization of vaccination protocols to obtain good immune responses is far from being accomplished.

NEW TREATMENTS AND PREVENTIVE STRATEGIES TO TREAT INFECTIOUS IDs

For years, research has been focused on the reinforcement of "classical antibiotics", producing very similar compounds with almost identical modes of action [60]. However, as it has been previously mentioned, the current antibiotic treatments are decreasing their efficacy due to the growing number of resistant bacteria affecting both human and animal health. Fortunately, some new sources of antimicrobial therapies and new preventive strategies are under investigation with potential applications to treat human and animal infections. Among them, antibodies, probiotics, bacteriophages, lysins and proteins, and antimicrobial peptides of innate immunity are the most promising ones. In parallel, research is also moving forward to the development of preventive strategies modulating the immune system as a strategy to minimize the risk of infection and in consequence the need of using antimicrobial therapies.

During the next sections, the different treatments and preventive strategies, as an alternative to antibiotics, are presented in more detail, highlighting their possible applications in human and animal health.

New treatments and preventive strategies

Antibodies

Antibodies are one of the therapeutic alternatives with the broadest application options. Their opsonization capacity permits them to bind and inactivate pathogens, their virulence factors or their toxins [78]. Until 2022, 11 antibody-based therapies for human infectious diseases have been approved in the European Union and the United States [79–83]. Besides, many other successful antibody-based therapies have been reported in the past decade against several types of pathogens (viruses, parasites and bacteria) [84]. The use of monoclonal antibodies (mAb) is being adressed for the prevention and treatment of bacterial infections caused by some of the most relevant bacterial pathogens. For example, interesting results against *Pseudomonas aeruginosa* have been reported, including infection reduction in mice [85], combined therapies of mAb with "classical antibiotics" in mice [86], and promising phase II clinical trials against the same pathogen [87]. The use of mAb has been proven as a treatment of *S. aureus*-induced pneumonia in mice as well [88], and good results have been obtained in the treatment of multidrug-resistant (MDR) *E. coli* infections *in vitro* and *in vivo* in mice [89]. Encouraging results have been accomplished using mAb for the prevention and treatment of viral infections, with clinical trials against Human Cytomegalovirus (HMCV), Influenza, HIV, Respiratory Syncytial Virus (RSV), Ebola and Rabies [90]. Moreover, new promising strategies are being developed to improve the existing mAb against viral pathogens [91,92]. Various combinations of mAbs have been 29ddressed to target Rabies virus, HIV, SARS-CoV, HBV, Ebola virus and Influenza virus [93]. Multivalent and multispecific mAbs are being explored to increase the binding capacity of the antibodies to viruses with a high number of epitopes like RSV or Herpes simplex virus (HSV) [93]. Single-domain antibodies, also known as nanobodies, are the latest approach trying to reduce the immunogenicity of the full-length mAb and presenting higher affinity and specificity for challenging viruses such as Influenza virus and HIV [94,95].

For these reasons, antibodies are within the approaches with more expectations to have a higher clinical impact. Their research is considered a low-risk area with a high degree of technical feasibility, a strong scientific basis, and a history of safe use. For example, a very distinctive advantage in front of other therapies is that administering a human antibody is much safer than administering attenuated or inactivated vaccine or

immunoglobulin preparations [96]. However, most of the technologies developed in the antibody context are only applied to humans and are not used in veterinary medicine [97]. The high production and development costs to obtain the necessary quantities to treat big animals limits their application in livestock. One of the most interesting approaches for the application of antibodies in animal IDs are the recombinant antibodies (rAbs). This technology is being developed for human diseases also, but other alternatives mentioned before are more advanced. But for veterinary medicine it represents an interesting alternative. There are several studies on pigs, cows, poultry and dogs using rAbs [97]. The main advantage compared to other technologies is the no usage of experimental animals for their production and its reduced cost. Moreover, the diversity of rAb is much wider compared to full and classic antibodies, with near to fifteen different types of rAbs. Although significant results have been obtained, most have not been developed enough to be approved. Further research and clinical trials are demanded to enable important findings to fulfill their potential to improve animal well-being [97].

Despite all the possible applications and strong advantages, antibody therapies also present several drawbacks that are hampering their use for the treatment of bovine IDs. Antibodies are much more expensive and difficult to produce than antibiotics so they are not yet a real alternative [98]. Besides, for viral infectious diseases, sometimes it becomes difficult to formulate a strategy to develop antibody drugs [90]. Viruses invade host cells to parasitize and replicate, having high ability to mutate and scape from immune system. The high specificity of Ab in some cases becomes a real drawback because with one of these minimum changes, the Ab loses its binding capacity to the pathogen. Furthermore, to use antibodies for therapy, it is essential to identify the epitope and to elucidate the mechanism by which the antibody affects the course of an infectious disease. Thus, research and development of antibody drugs require substantial cost and time in comparison with other approaches [99].

Bacteriophages

Bacteriophages (BPs), also known as phages, are viruses that infect and replicate in bacterial cells (**Figure 7**) [100]. They are very specie-specific [100] and, for that reason, they can infect and kill bacteria without any negative effect on human or animal cells [101]. For more than 100 years, research has attempted to use this property to treat pathogenic bacterial infections in people and animals. This application based on the use

of BPs to eliminate bacterial pathogens is known as phage therapy (PT). It presents advantages in comparison to antibiotics [101]: i) BPs are more specific than antibiotics, protecting host cells and commensal and symbiotic microbiota, conferring a higher safety and tolerability; ii) their administration is easier, as BPs do not need repeated administrations over several days (a low number of BPs is enough to attack a bacterial infection because once a BP infects a bacterial host, it replicates and grows exponentially); iii) BPs can be modified through genetic engineering to fight antibiotic resistance and to make these pathogenic strains susceptible again to classical antibiotics. In the last years, many potential clinical applications of BPs have been described in studies carried out in humans and animals [101]. Treatments based on phages against some of the most important bacterial pathogens, such as *P. aeruginosa*, *S. aureus*, *Vibrio cholerae*, *E. coli* and *Acinetobacter baumanni* are in development and are presenting promising results [101]. Moreover, there are 21 clinical trials based on the use of bacteriophages to treat bacterial infections [102]. Regarding the application of PT in animal health, many studies have been performed on livestock (e.g., pigs, cattle, chickens…) and companion animals [103]. More specifically, studies against *S. aureus* causing bovine mastitis have been done *in vitro* and *in vivo* [103–107]. Promising results in *S. aureus*-induced mastitis mice models have been obtained, proving that mixtures or cocktails of different BPs have higher antimicrobial activities than single-BP treatments [104]. Moreover, a reduction in *E. coli* gastrointestinal infections with oral, rectal and aerosol administration of BPs cocktails have been assessed [108].

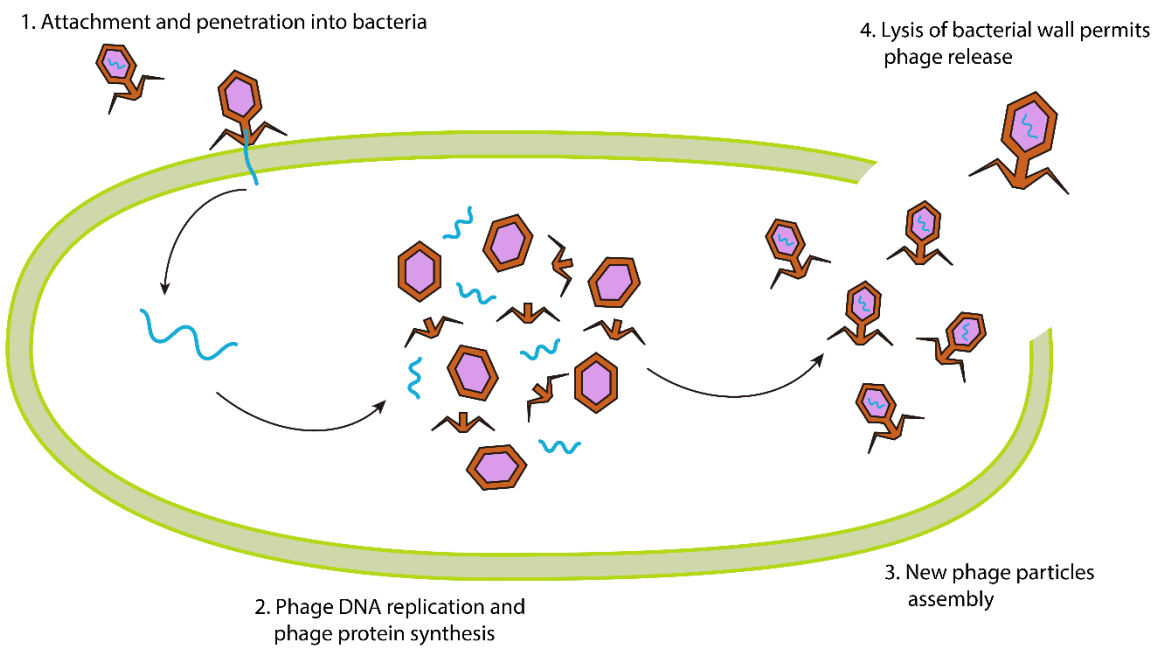

Figure 7. Lytic cycle of bacteriophages. 1) Bacteriophages attach to the specific host cell and injects the viral genome crossing the bacterial wall and the membrane. 2) Phage DNA replication is performed using the bacterial cell machinery as well as phage protein synthesis to build up a new generation of bacteriophages. 3) During the maturation of bacteriophages, different components are synthesized and assembled until generate a complete infective bacteriophage particle. 4) At last, bacteriophages promote the rupture of bacterial membrane and cell wall, releasing infective bacteriophages to infect new host cells.

Currently, there is no commercial product containing BPs applied for human or veterinary medicine. Nonetheless, there exist an important number of BPs-based preparations accepted for use in the elimination of pathogens from food products of animal origin like meat and meat-derived products, milk and dairy products, or plant origin products like fruits and vegetables [108,109]. Clear examples are the permissions of the Food Safety and Inspection Service of the USA to use phages in livestock prior to slaughter (e.g., phages specific for pathogenic strains of *E.coli* O157:H7 for use on beef hides) and in foods (e.g., phages specific for *Salmonella* on poultry or meat products derived from livestock) [108]. Besides, as the restrictions on the use of antibiotics in animal production are increasing, the marketing authorization of BPs-based preparations as substances generally recognized as safe (GRAS) is being demanded worldwide [108].

Despite all the advantages and progresses previously detailed, there exist also some obstacles to the clinical use of BPs as antimicrobial therapy [101]. The specific activity of BPs is an advantage but can become a drawback when broad-spectrum antimicrobial molecules are needed. Moreover, the BP genome usually contains integrase genes,

antibiotic-resistant genes, phage-encoded toxins or other bacterial virulence factors genes which could be a biosecurity matter. In addition, it is important to consider that bacteria could easily develop resistance mechanisms against BPs. Among them, there are found hiding, change or loss of the receptor, secretion of substances that prevent phage adhesion to the bacterial pathogen, activation of measures for blocking phage DNA injection into the cell and inhibition of phage replication and release [110]. Besides, phages are much more immunogenic than other antimicrobial drugs and are rapidly cleared from the blood due to the response of the immune system to them [101]. Finally, BPs could be a good alternative as a specific treatment against bacterial infections, but they do not present any application to treat viral infections.

Endolysins

Endolysins are hydrolytic enzymes produced by BPs [111] that specifically cleave the covalent bonds of peptidoglycan of the infected bacterial cell. After this cleavage, the progeny BP produced are released [112]. Thus, endolysins are promising antibacterial agents because they target highly conserved parts of the bacteria peptidoglycan without disturbing the normal flora and they have a low chance of bacterial resistance [113]. Another special feature of endolysins is their capacity to kill colonizing pathogens on mucosal surfaces [113], where few antimicrobials can develop their activity. This fact has important relevance because it is well known that pathogens form little reservoirs in the mucus can become the origin of future infections [114,115]. Other advantages of endolysins are their rapid bacterial activity within seconds of contact, the no reported resistance over number of treated generations (116–119), and a high antibiofilm activity [116]. Several studies have described the potent antibacterial activity of different endolysins *in vitro* and *in vivo* in mouse models against relevant Gram-positive pathogens such as *Enterococcus faecalis* [117], *Streptococcus pyogenes* [118] and methicillin-resistant *S. aureus* (MRSA) [118,119]. On the other hand, their most relevant drawbacks are their immunogenicity and side-effects [120,121], along with their production at large-scale [116]. Nevertheless, endolysins potential to treat Gram-positive bacterial infections is clearly demonstrated with 4 examples of phase I, II and III clinical trials and over 30 studies in preclinical phases [116]. Endolysins cannot target Gram-negative bacteria because they do not have the capacity to disrupt the outer cell membrane. However, different strategies are in development to solve this problem like chimeric endolysins

production adding binding domains or other molecules such as bacteriocins or antimicrobial peptides to pass through the outer membrane [122–124].

Almost the entirety of endolysins research is focused on human health applications for the treatment of bacterial infections. By contrast, very few investigations are developed for animal health. One example is the use of endolysins as a treatment of bovine mastitis [125]. Promising results have been obtained against *S. aureus in vitro* and *in vivo* [126,127]. In mastitis mouse models, significant reduction in bacterial cell counts have been achieved with intramammary infusions of endolysins [127]. Regarding trials in cow udders, results are too preliminary to get clear conclusions [128], but other seems to be more promising reinforcing the possibility to apply endolysins as a mastitis treatment [129]. In addition, *in vitro* studies incubating endolysins in cow milk at its specific conditions of ionic strengths, pH, and Ca^{2+} reduced bacterial cell counts of mastitis pathogens such as *S. agalactiae*, *S. dysgalactiae* and *S. uberis* [126].

Probiotics

Probiotics are microorganisms that, once administered at optimal doses, can confer health benefits on the organism [130]. They can improve digestibility, destroy bacterial pathogens that are causing disease, or produce vitamins, among many other bioactive compounds [130]. The action on bacterial pathogens follows two main strategies, competition for the same nutrients or production of antimicrobial peptides and toxins [131]. The use of probiotics to treat acute infectious diarrhea has been widely described in several studies, mainly in children [132–135], as well as their use to treat antibiotic-associated diarrhea [136,137]. Besides, probiotics can also be used as a prophylactic strategy. Their administration can enhance the epithelial barrier function, the innate immunity or even the adaptive immunity, which increase the protection of the organisms against pathogens. This prophylactic potential has been exploited for the prevention of antibiotic-associated diarrhea [138–140], including diarrhea caused by *Clostridium difficile*, one of the most worrying gastrointestinal pathogens nowadays [141]. Also, prevention of necrotizing enterocolitis [142] and sepsis [143] in premature infants have been assessed. In the case of viral infections, probiotic bacteria can inhibit the interaction between the virus and the host-cell receptor [144], can exert antiviral activity by direct probiotic-virus interaction, can produce metabolites with antiviral inhibitory activity, or can stimulate the immune system of the host [145,146]. Some studies have described the

beneficial effects of using a mixture of probiotics to reduce diseases caused by viruses [147]. Although they do not cure the affections, probiotic combinations are able to stimulate the immune system and reduce the duration of illness and the symptoms of the disease. We can find examples employed to prevent and reduce symptoms of infection caused by respiratory tract viruses, digestive viruses and viruses causing hepatitis, human papillomavirus, HIV and HSV in humans [147]. In respiratory viral infections, it has been demonstrated the beneficial impact of probiotic treatment on gut microbiota enhancement [146]. Probiotics are also useful for preventing gastrointestinal tract viral infections [148]. In this case, Lactic Acid Bacteria (LAB) strains exert protection against enteric viruses increasing the production of specific antibodies, shortening diarrhea episodes and stimulating the production of cytokines [149,150]. Regarding hepatitis infections, different studies support the idea of including probiotics to fight secondary effects of hepatitis C drug treatment. Modifying gut microbiota with probiotics can decrease endotoxins and derived compounds from opportunistic bacterial pathogens which are toxic and cause liver damage [151]. Finally, as HIV infections unbalances gut microbiota and the immune system of the host, it has been shown that probiotics could help to restore the gut microbiota and immunity, with beneficial impact on patients [152].

In animal health, the use of probiotics is a widely used strategy in poultry, ruminants and aquaculture for growth promotion, prevention of infections and maintenance of gut microbiota and animal welfare (**Figure 8**) [153]. More precisely, in ruminants, the yeast *Saccharomyces cerevisiae* [154] and LAB [155] are the most commonly used probiotics. *S. cerevisiae* exerts probiotic effects by interacting with the microbiota responsible for feed digestion and increasing the rumen microbial activity by stabilizing the rumen pH, preventing acidosis and stimulating the growth and activity of fiber-degrading bacteria (**Figure 8**) [154]. For its part, LAB enhances milk production by increasing dry matter intake (DMI), milk yield and milk fat, protein and lactose percentage [156]. A combined administration of different strains of *Lactobacillus*, *Enterococcus* and *Bifidobacterium* increases the ruminants' body weight (**Figure 8**) [157]. A mixture of *Lactobacillus* and *Propionibacterium* increases food digestibility improving the digestion of neutral detergent fiber and crude protein [158]. The supplementation of different *Lactobacillus* strains has been reported to enhance the immune system of young calves and brings down the occurrence of diarrhea [159]. *Lactobacillus* base teat spray can improve mammary gland condition and strengthen the functions of the teat sphincter [160].

It is clear that probiotics research has made a significant progress, with several probiotics commercially available [161]. However, some mechanisms and interactions with the immune system and host microbiota need to be further studied [162], as well as the combination with other therapies [78,163]. They show very interesting features in different fields of application, but it is important to highlight that they have more potential as preventive medicines or supplementing other treatments rather than as direct antimicrobial therapy [164,165].

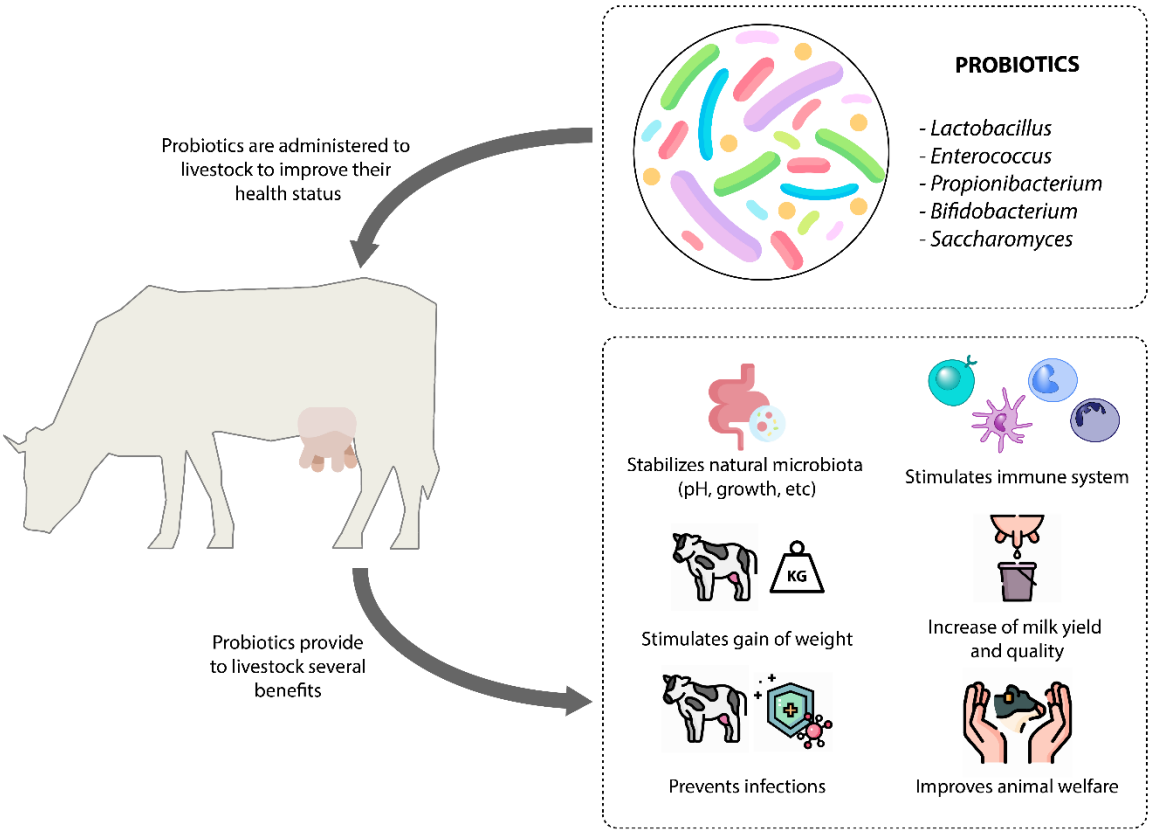

Figure 8. Probiotic as preventive treatment for animal health improvement. The use of *Lactobacillus, Enterococcus, Bifidobacterium* and *Propionibacterium* bacteria and *Saccharomyces* fungi as supplements for livestock provides with several benefits that improve animals welfare.

Immune system modulation

In previous sections direct antimicrobial therapies have been detailed as antibiotic substitutive molecules. Among them probiotics have been included, whose action goes beyond the direct antimicrobial activity against the pathogens. They have the capacity to modulate the host immune system in many ways, preparing the host to future or ongoing infections. The immune system of humans and animals has *per se* natural mechanisms that, with the right modulation, can have the potential to manage IDs. Moreover, in some situations like in stressed or immunocompromised animals, a boost in the immune response would help in the control of possible infections. Thus, the modulation of the immune system appears as a complementary strategy to antimicrobial therapies [166].

The immune system is a complex network comprised by different effectors from macroscopic level, such as organs, to microscopic scale, passing through cells and ending at molecular level. Its main objective is the defense of the body against harmful substances and pathogens. The barriers and mechanisms that defines the immune system are popularly divided in innate and adaptive immunity, but both are tightly connected. Innate immunity is the older arm of the immune system, composed of barriers (e.g., skin and mucosa), small molecules (e.g., proteins and peptides) and cells (e.g., macrophages and dendritic cells). It is so-called "innate" because it provides safety from pathogens without any external stimuli or warning of the environment. If the innate immune system detects a pathogen, it directly try to eliminate it from the host [167]. This immunity is characterized to have a rapid non-specific response. For its part, adaptive immunity is activated by innate immunity, and is defined by a slower activation that can start from 3 days to a couple of weeks after the pathogen recognition by the innate immunity. It has a higher pathogen specificity and entails more complex mechanisms. The major effectors are cells, specifically lymphocytes. More in deep, adaptive immunity is divided into humoral (antibody-mediated) and cellular (cell-mediated) responses which are related most of the times [168].

The immune response activated because of an infection involves all the mediators mentioned above depending on the moment or the casuistic of the infection. First, pathogens are detected by innate immune cells through pattern recognition receptors (PRRs). PRRs recognize molecules known as pathogen-associated molecular patterns (PAMPs) [169,170] as well as host molecules that indicate disease and cellular damage

[166]. Pathogen recognition activates the innate cellular defense mechanisms and the secretion of pro-inflammatory cytokines, driving further recruitment of immune cells and inducing systemic responses (e.g., fever). Moreover, PRRs pathogen recognition stimulates innate microbicidal mechanisms such as reactive oxygen species and antimicrobial peptides (AMPs) [166]. The adaptive immunity is activated later by a combination of innate signals and antigens. It is based mostly on B cells and T cells. B cells will produce antibodies to block or opsonize pathogens or toxins. T cells will kill infected host cells. The most important feature of adaptive immunity is the generation of memory B cells and T cells providing protection against future infections by pathogens with the same antigens.

In the last years, research in immune system modulation has grown exponentially and has shown the potential to control infections. One of the strategies with most promising results is the targeting of PRRs with agonists and antagonists. PRRs agonists drives the activation of innate immune cells, the production of pro-inflammatory cytokines and the induction of antimicrobial functions. PRRs antagonists performed the contrary action, promoting all the anti-inflammatory mechanisms. There exist a large number of studies in animal models showing that PRRs agonists can act as potent and safe vaccine adjuvants, inducing the clearance of existing bacterial infections [171–173] and promoting protection against future infections or colonization by pathogenic organisms [173–179]. In addition, several immunotherapies that target PRRs to treat microbial infections are in clinical phase, with clinical trials in phase II and III for the treatment of Human Papilloma Virus [180], leprosy [181], recurring respiratory tract infections [182,183], tuberculosis [181], sepsis [184,185], HIV and influenza [166]. The targeting of immune-related receptors is not the only strategy to modulate the immune system, there exist different strategies more focused on the direct use of molecules from the innate immunity, such as proteins or peptides, that will be detailed in the following section "Innate immunity-related molecules".

Regarding the current application of immune system modulation strategies to treat bovine IDs, specific approaches to prevent and treat mastitis [14] and BRD [186] have already been developed. In the case of mastitis, one of the most interesting strategies is the acceleration of the mammary gland involution at dry-off. Accelerating involution processes, closely related to the immune stimulation, the dry-off period could be

potentially reduced as well as the risks of IMIs appearance. The intramammary infusion of different types of molecules like LPS [187,188], concanavalin A [189] or *Panax ginseng* extract [190,191] has successfully activated the cow mammary gland immune response, triggering involution and conferring protection to new IMIs during dry-off. Other strategies involving the use of other molecules such as metalloproteinases [192,193] or acute-phase proteins (APPs) [194] are being developed with interesting results, but they will be explained more in detail in the following section "Innate immunity-related molecules". In BRD context, epithelial and immune cells prevent infection through mucociliary action and secretion of antimicrobial molecules and proinflammatory cytokines [186]. Neutrophils are essential for eliminating bacterial infections but also contribute to lung tissue destruction and inflammation. After infection, cattle are able to develop antibody and T-cell responses, but pathogens have the capacity to evade these immune responses. As of today, vaccine development continues to be the more active area of research related to immune system stimulation for BRD prevention. However, few promising alternative strategies are being developed to reduce the impact of BRD in bovine livestock [186]. Among them, the named innate immune system training is one of strategies more studied in the last years. It is based on the exposure of certain organisms or molecules to the innate immune system to enhance its state of responsiveness in the case of a second stimulus [195]. Mainly, monocytes, macrophages and natural killer (NK) cells are the inducers of this trained immunity based on a superior cytokine expression that enhances the capacity of innate immunity to prevent an infection. The process is independent of adaptive immunity but maintains a kind of "memory" through epigenetic changes in basal intracellular metabolic pathways, gene expression and cell physiology, conferring higher capacity to immune cells to respond in front of stimuli [195]. Several therapies have been developed with potential to train or enhance the innate immune system during times of stress when the immune system of the animals can be more suppressed or affected [195–199]. For instance, a DNA-based immunostimulant commercially known as Zelnate (Bayer Animal Health, Shawnee Mission, KS, USA) has shown reductions of experimental lung challenges with *M. haemolytica* in cattle [197] and reduction of mortality high-risk cattle after feedlot placement [196,198]. Another immunostimulant product based on a mycobacterium cell wall fraction named Amplimune (Novavive, Inc, located in Napanee, Ontario, Canada) prevents *E. coli*

infections in preweaned calves and also reduces the BRD incidence and mortality in beef calves [200].

Innate immunity-related molecules

As mentioned before, immune system is a complex network with an almost infinite list of effectors involved and interrelated at all levels and scales [167,168]. This section is focused mainly on proteins and peptides involved in the innate immunity of humans and animals. Their main functions in innate immunity and their therapeutic potential in IDs treatment will be detailed, with a special mention on animal health applications. Depending on their therapeutic target, we can differentiate two main groups: immunomodulators and antimicrobials (**Figure 9**).

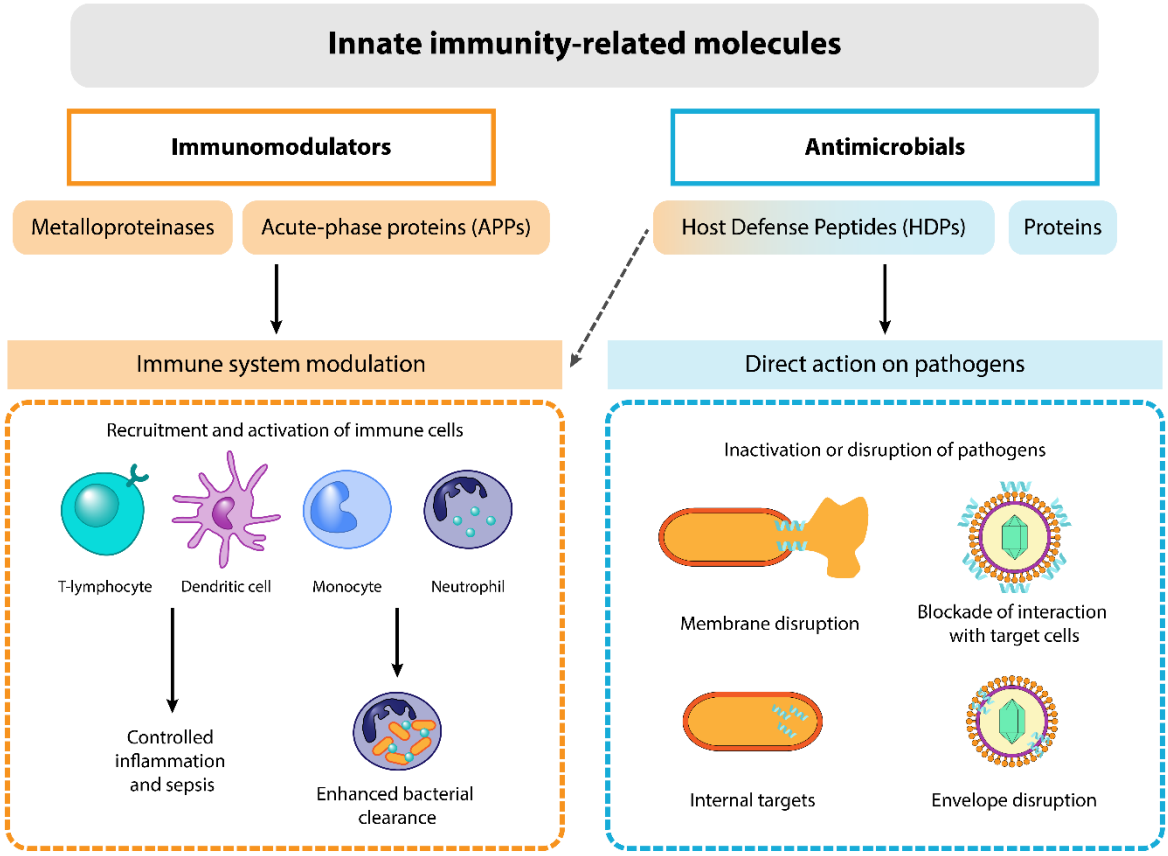

Figure 9. Innate immune-related molecules: classification and effects. There exist a huge variety of immune-related molecules. Some of them present immunomodulation activity, such as metalloproteinases or acute-phase proteins (APPs), that, under stress conditions produced by a pathogen, activate the recruitment and activation of immune cell system, including T-lymphocytes, dendritic cells, monocytes, and neutrophils, among others. This process leads to the control of inflammation and sepsis, as well as the clearance of bacterial pathogens. On the other side, antimicrobial polypeptides, including Host Defense Peptides (HDPs) and other proteins, causes a direct effect over pathogens. In the case of bacteria, HDPs can provoke membrane disruption or execute its

function by internal targets. In the case of viruses, they can block the interaction capacity with host cells, or, in the case of enveloped viruses, they might disrupt the envelope structure.

Immunomodulators

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a group of enzymes that break down proteins with the need of zinc or calcium atoms to work properly. They are normally found in the spaces between cells in tissues [201]. Matrix metalloproteinase 9 (MMP-9) or gelatinase B is one of the most widely studied, and as a metallopeptidase, it is capable to degrade structural components like collagen, gelatin or elastin and to cleave cell adhesions, surface receptors [202], cytokines and chemokines [203,204] and intracellular substrates [205]. Moreover, it is involved in chemotaxis, cell migration [206–208], and it also has a main role in the modulation of the integrity and permeabilization of many tissues. For that, it is crucial in several biological processes such as angiogenesis, growth, development, wound healing and cell migration [208–211].

In bovine mammary gland, MMPs play a major role during cow dry period [14]. Specifically, MMP-9 is secreted by different types of cells involved in mammary gland involution process, among them mammary epithelial cells, neutrophils, and infiltrating macrophages [212]. Once it is released to the extracellular environment it is responsible of breaking the adhesion molecules between cells and the basal membrane under the mammary epithelial cells [213]. Through this degradation, it promotes the remodeling of the mammary gland tissue and permeabilize the endothelia facilitating the recruitment of immune cells [214]. For that, different studies have been performed to expand the therapeutic potential of MMP-9, unmasking its real functions beyond the extracellular matrix degradation. These studies reinforce the concept that MMP-9 is highly involved in the remodeling acceleration and in the recruitment of neutrophils [215–217]. Its capacity as immune facilitator has been endorsed with *in vivo* results in mice models, mobilizing immune cells and stimulating cytokine expression [193,218]. Moreover, important efforts are being carried out on the production of MMP-9, mainly recombinantly, and on the exploration of different formats of this protein trying to maximize their potential as an immunomodulator therapy [192,193,218–222]. Those results and their implications will be explained more in depth in the section “Recombinant innate immunity-related proteins for the treatment of IDs”.

Acute-phase proteins

Acute-phase proteins (APPs) are a group of proteins that abruptly increase their plasma concentration in response to inflammatory signals, usually reacting to cytokines such as IL-1, IL-6 or TNF-α [223,224]. The APPs conformed the acute phase reaction, based on a wide range of responses such as fever, movement of peripheral leukocytes, hormone alterations and muscle protein depletion [225,226]. These processes take place in combination to minimize tissue damage while enhancing the reparation.

APPs are a broad group of proteins and their specific role in immune response is variable depending on the species. In cattle, and more specifically in mammary gland, APPs are fundamental to support inflammation. During an infection, they increase chemotaxis and pathogen recognition of immune cells (neutrophils and macrophages) and mammary epithelial cells [227]. In mastitis diagnostics, they are becoming important biomarkers of pathogen infections [227–229]. Differences in APPs levels can be associated with the presence of pathogens in the mammary gland [227–229]. Among APPs, mammary serum amyloid A3 (M-SAA3) is one of the best characterized APPs with a higher impact in mammary gland immune response. M-SAA3 is synthesized in the bovine mammary gland by mammary epithelial cells [230,231] and it is present during milk stasis, involution, infections and in colostrum [230,231]. Thus, M-SAA3 stands as a protein with a huge potential as a specific immunomodulator in the mammary gland. The intramammary infusion of M-SAA3 in cows entering dry-off, or during dry-off has been evaluated as a preventive strategy or therapy for mastitis. It has been described that through intramammary infusion of recombinant M-SAA3, involution biomarkers were activated [194]. Moreover, M-SAA3 has shown the capacity to opsonize Gram-negative bacteria [232] or to prevent intramammary infections in dairy cows at dry-off [233]. In addition, advances in the production and optimization of recombinant M-SAA3 has expanded the possibilities to obtain this protein for therapeutic purposes [221]. This has been further described in “Recombinant innate immunity-related proteins for the treatment of IDs”.

Antimicrobials

Proteins

Innate immunity-related antimicrobial proteins are essentially produced by epithelial cells. They can be found in intestine, skin, respiratory and reproductive tract, where they play a crucial role as defenses against pathogens [234]. Proteins from many different families with distinctive antimicrobial modes of action form part of this heterogeneous group. They exhibit broad-spectrum antimicrobial activity [235] and they generally disrupt the microbial membrane by electrostatic interactions or attack by enzymatic activity on their specific targets [236]. Although in some cases, they also have potent immune regulation capacity [234], in this section we will be focused only on their antimicrobial features.

Four representative examples of antimicrobial proteins are lysozyme, phospholipases, ribonucleases (RNases) and lactoferrin. All of them are enzymes but they exhibit their antimicrobial activity through four different modes of action. First, lysozyme hydrolyses the glycosidic linkages of bacterial peptidoglycans and it has been applied as growth promoter in livestock [237]. One example of phospholipases is the secreted phospholipase A2 ($sPLA_2$), which hydrolyses the membrane phospholipids generating a wall break in the bacteria [238,239]. Its mode of action seems to be more effective against Gram-positive bacteria because they lack the outer membrane that confers extra protection to the Gram-negative bacteria [240]. The antimicrobial activity of RNases is directly related to their cationic nature. They interact with the bacterial surfaces that are charged negatively producing bacterial cell disruption and death [241]. Lastly, lactoferrin presents a broader anti-infective activity, being antibacterial, antiviral, antiparasitic, anti-cancer and anti-allergic protein [242]. It has the capacity to hydrolyze RNA and inhibit the reverse transcription of retrovirus [242]. It can sequester free iron essential for bacterial growth and it can bind to bacterial cell walls forming peroxides that lead to bacterial lysis [242].

Although antimicrobial proteins have potent and broad antimicrobial activity, there exist many issues and challenges that are blocking their therapeutic use. There are still many questions and doubts about their interaction with the host microbiome, their complex regulatory networks and how dysregulation of these proteins expression and function could contribute to diseases [234,243].

Host Defense Peptides

Another main effector of innate immunity against infections, at least at the molecular level, are the antimicrobial peptides (AMPs), also known as Host Defense Peptides (HDPs) for their intrinsic role on the immune defenses of the host. The term Host Defense Peptides covers a tremendous variety of peptides present in all life kingdoms (animals, fungi, plants and bacteria) [244]. To date, the data repository of antimicrobial peptides (DRAMP) contains 6,105 AMPs [245], including natural and synthetic AMPs.

They are small peptides from 12 to 50 amino acids characterized for their positive net charge due to the high proportion of cationic amino acid residues in their sequence [246]. Their backbone also contains hydrophobic residues that combined with the positive ones confer them an amphipathic nature [247,248]. These two properties are the key for the broad antimicrobial spectrum of HDPs, which are active against Gram-negative and Gram-positive bacteria, including MDR strains, enveloped viruses, fungi and parasites [248,249]. Briefly, their mode of action consists of the interaction of the positive residues with the negatively charged membranes of the pathogens, triggering a membrane disruption and consequently pathogen death (bacteria, fungi and parasites) or inhibition of their pathogenic capacity (enveloped viruses) [246,250]. Their rapid antimicrobial action against highly conserved structures of the pathogens and their short half-life hamper the appearance of resistance [251]. Moreover, their functions go beyond the antibacterial activity, being able to modulate the immune system at different levels [252]. Therefore, HDPs, compared to the alternatives explained in previous sections, are one of the most complete and with a higher potential.

There exist different ways to classify the vast number of HDPs, but the most accurate from a descriptive point of view is based on their structure, considering their length, secondary and tertiary structure, and amino acid composition [253]. Four main groups are clearly identified: α-helical, β-sheet, looped peptides and extended structures. The α-helical peptides are characterized by their predominant secondary structure based on amphipathic α-helix with residues like alanine, leucine and lysine which confer stability. The β-sheet peptides are stabilized by one to five disulfide bridges permitting their β-sheet secondary structure. The looped peptides do not have a secondary structure, their folding consist in a single disulfide bridge. Last, extended peptides lack marked folding patterns but they are often rich in specific amino acids like glycine, proline, tryptophan,

arginine and histidine. Although their structural characteristics are clearly differentiated between the four groups, their biological functions are not directly related to their structures. There exist more ways to classify HDPs, but the two most relevant groups and most studied in different species are cathelicidins and defensins. The group of cathelicidins include peptides with α-helical structures, others with β-hairpins and some peptides rich in proline/arginine residues. For their part, defensins have two or more β-sheets stabilized by disulfide bonds. Thus, this two clusters of HDPs are more differentiated considering their biological activities instead of their structures.

Cathelicidins

Cathelicidins represent a relevant part of the immune system of vertebrates [254]. They are expressed mostly in neutrophils and macrophages, but also in epithelial cells as inactive precursors (prepropeptides) [255]. They are released extracellularly upon leukocyte activation and after being cleaved by neutrophil elastase [256]. Their structure is divided into two functional domains: the cathelin domain at the N-terminal, highly conservated inter-species, and the antimicrobial domain at the C-terminal, which shows high diversity, both interspecies and intraspecies [254,257–259]. The C-terminal domain can be α-helical, β-hairpin or proline/arginine rich [257]. Cathelicidins are well-known for their broad-spectrum antimicrobial activity but also from their role in immune modulation in processes such as inflammation response, cell proliferation and migration, wound healing, and angiogenesis [260].

Thirty cathelicidin families have been identified in mammals. Curiously, only one has been detected in humans, the LL-37 [261], but there exist many other examples in horses, pigs, cattle, among others [255]. Seven cathelicidins are described in cattle: cathelicidin BMAP-27, 28 and 34, bactenecins Bac1, Bac5 and Bac7, and indolicidin [262–265]. Bovine myeloid antimicrobial peptides (BMAP) contain an α-helical C-terminus which confers them antimicrobial activity against Gram-positive and Gram-negative bacteria, including MRSA and fungi [263,265]. Bactenecins (Bac) are characterized for having a polycationic aa sequence with a proline motif repeated [266], showing higher selectivity for Gram-negative than for Gram-positive bacteria [267]. Indolicidin for its part, is a tryptophan-rich peptide of 13 aa, with activity against bacteria such as *S. aureus* and *E. coli* [262], and fungi such as *Candida albicans* and *Cryptococcus neoformans* [268].

Antiviral activity of cathelicidins is not so well-described as defensins, but the interest in this potential activity is growing. The human cathelicidin LL-37 is the most studied with antiviral activities described against different enveloped viruses such as HIV [269], RSV [270], HSV [271] and dengue virus [272]. Besides, LL-37 has antiviral activity against non-enveloped viruses such as human rhinovirus [273]. Not at the same level, there exist few examples of bovine cathelicidins showing antiviral activity. Indolicidin and the bovine BMAP-18, a version of BMAP-27, presented antiviral activity against HIV [274,275]. Moreover, indolicidin and BMAP-28 are active against some types of HSV [271,276,277]. Their antiviral activity was showed when the virus was exposed to the peptides prior to cell infection and also after the infection of the cells. Despite the above-mentioned results, it is not very well defined how cathelicidins affect viruses. These are the most robust hypothesis: i) a direct effect on viral envelope; ii) effects on virus cell-binding and uptake; iii) modulation of systemic inflammatory response; iv) modulation of viral protein function; v) effects on viral uncoating or vi) modulation of PRR signaling [278].

Defensins

Defensins are a family of cationic and cysteine-rich peptides of 2-5 kDa found in vertebrates, invertebrates, fungi, and plants [279,280]. Six of those cysteines are highly conserved and form three disulfide bonds. Considering the arrangement of the disulfide bonds they are divided into three subtypes: α-, β- and θ-defensins [281]. The α-defensins are based on three β-sheet strands forming a β-hairpin through disulfide bonds [282]. β-defensins, for their part, contain both α-helix and β-sheet, and θ-defensins form macrocyclic structures. There exist differences on the defensin expression between species. For instance, humans only have α-defensins and β-defensins, cattle only have β-defensins [283], and θ-defensins are described in the leukocytes and bone marrow of some non-human primates [281]. In mammals, α-defensins are mainly produced by neutrophils, macrophages and intestinal Paneth cells, while β-defensins have a wider presence in leukocytes, epithelial cells, the respiratory, digestive and genitourinary tracts, the blood and urine [284].

Regarding their natural functions in the host, defensins have broader number of well-defined activities than cathelicidins with antibacterial, antiviral and immunomodulatory activities [285]. It has been described their antibacterial activity against Gram-negative

bacteria mainly through membrane disruption, and their activity against Gram-positive seems to be more structurally specific, because they have shown ability to binding to specific bacterial membrane structures [286]. Moreover, defensins are contrasted antiviral agents with activities against a broad range of viruses, among them HSV, HIV or RSV. Their antiviral modes of action are mainly the inhibition of viral infection through the binding to the envelope or the direct disruption of the viral envelope [287]. In addition to the two previously mentioned direct actions against pathogens, defensins are potent immunomodulators. They can induce inflammation and suppress inflammatory responses by acting on specific cells through distinct mechanisms [285].

Human α- and β- defensins are broadly studied [283], but the first β-defensin discovered was the bovine tracheal antimicrobial peptide (TAP). TAP is naturally produced by bovine airways an upregulated during lung tissue infections [288,289]. This peptide shows antimicrobial activity against relevant Gram-positive (i,e. *S. aureus*) and Gram-negative (i,e. *P. aeruginosa*, *Klebsiella pneumoniae*, and *E. coli*) bacterial pathogens [290]. Moreover, it has shown *in vitro* antibacterial activity against bovine respiratory pathogens such as *M. haemolytica*, *H. somni*, and *P. multocida* [291]. Another important bovine β-defensin is the lingual antimicrobial peptide (LAP). It was discovered later than TAP but its expression has been described in epithelial cells of the tongue and the mammary gland, and of the intestinal and the respiratory tract during infections [292]. Overall, the expression of β-defensins is up-regulated during infections in many bovine tissues such as viral respiratory infections causative of BRD [289] or bacterial IMIs involved in mastitis [293].

Other HDPs

Cathelicidins and defensins are the most remarkable families of HDPs with a higher percentage of the research focused on their characterization. Nevertheless, the innate immunity of mammals involves other families of peptides with crucial functions. Collectins represent an important group of innate immune molecules in the recognition of pathogen patterns [294]. They can be found in serum as well as in mucosal surfaces and for that they are highly expressed in lung tissues, especially during infections [295]. Among them, surfactant proteins A and D (SPA and SPD) are members of the collectin family expressed in the respiratory tract. Their main role is to enhance the opsonization

of bacteria, fungi and viruses facilitating the pathogen clearance from the lung tissue by the immune cells [296].

Another family of atypical AMPs are the anionic peptides (APs). In contrast to the vast majority of HDPs, their net charge goes from -1 to -7, but they are also short peptides no longer than 70 residues [297]. These APs have been identified in vertebrates, invertebrates and plants, showing important roles in the innate immune system. In the presence of Zn^{2+}, that acts as a cofactor, they perform antibacterial activity against Gram-positive and Gram-negative bacteria [249,298]. In cattle, one of the first APs reported were the surfactant-associated anionic peptides (SAAPs). It is thought that, in addition to their antibacterial activity, they may serve to synergize the action of other antimicrobial molecules such as cationic AMPs in respiratory tract [299].

Preclinical and clinical advances in HDP-based drug development and their limitations

The repertoire of functions shown by HDPs, at least *in vitro*, is unquestionable. In contrast, moving to the next level, the *in vivo* approaches and clinical trials, are currently a tough challenge. Their antibacterial and antiviral activities mainly rely on their net charges, but this advantage becomes a drawback in physiological conditions because they are salt-sensitive [300]. However, the research on animal models has shown promising results against bacterial, viral and fungal infections [301,302]. It is not clear if these effects are directly related to the antimicrobial activity of HDPs or if they are due to the effect of the peptides on host immunity, or a combination of both. Therefore, the mode of action of HDPs *in vivo* remains unclear and needs to be further described. Moreover, there exist several limitations that are hindering the advancement of HDPs' therapeutic way. The toxicity at the high concentrations needed to be active *in vivo*, the lack of suitable formulation and delivery to avoid degradation, as well as high production costs are the main examples of these limitations. Despite these obstacles, there exist several examples of HDPs arriving at the human clinical trial level that boost the hope on these therapeutic peptides [303–307].

RECOMBINANT PROTEIN PRODUCTION

The proteins and peptides mentioned in the previous sections can be found in nature in different organisms including animals, plants and fungi. For that, the first strategies used to obtain proteins and peptides for therapeutic purposes were based on the isolation from natural tissues or biological fluids where their expression had been described. This approach was totally inefficient and time-consuming because only minimal amounts of the molecule of interest were obtained from large amounts of samples [308,309]. Moreover, the biological risk of those samples was considerably high due to the potential presence of pathogens from the original host. Further on, chemical synthesis appeared, improving some aspects of the production of proteins and peptides. Especially in peptide production, solid-phase peptide synthesis (SPPS) is the fanciest option [307]. It permits the *de novo* design and production of almost any type of peptide, with extremely fine control of the aminoacidic sequence and the purity of its final products [310,311]. Moreover, the production of synthetic molecules mimicking the natural ones is feasible, being also possible to modify the sequence to improve their features [312–315]. Chemical synthesis has been optimized, but several drawbacks are hindering its use in a wider manner. Its production costs are high and even more for large amounts of molecules. Obtaining peptides longer than 20 residues is complex, requiring extra chemical processes not always feasible [316,317]. Indeed, chemical synthesis is unsuitable for producing bigger proteins or more complex peptides, which usually have secondary structures with multiple post-translational modifications (PTMs). These modifications sometimes are basic for the correct folding and final activity of the peptides [318].

The development of recombinant technologies solved many of these problems. Overall, the discovery of recombinant DNA technology has allowed the recombinant production of proteins and peptides of interest using cells as production factories. Besides, recombinant DNA opened the possibility to modify almost without limits any DNA sequence, subtracting or adding genetic information depending on the objective. Briefly, recombinant protein production consists in the introduction of a recombinant or engineered gene, which codifies for the protein of interest, in a cell to use its machinery for the protein production [319]. Through this simple process, it has been possible to overproduce many different proteins and peptides in large quantities, allowing their biological characterization and industrial and commercial exploitation [320,321]. Thus,

the cost, the time, the easy handling, and the possibility to produce large amounts of protein make recombinant production an optimal strategy compared with their alternatives. For that reason, recombinant protein production has been used for the development of new molecules and therapeutics for human and animal science. In fact, several recombinant products are already at the market, with examples like hormones, fibrolytic enzymes, antibodies, or cytokines used in animal production [322]. Since the approval of insulin in 1982 [323], more than 130 recombinant proteins have been approved by the US FDA for clinical use, and more than 170 recombinant proteins are produced and used in medicine worldwide [324,325].

Bacterial recombinant expression systems

There exist many different expression systems used for recombinant protein production purposes which are based on different cell types: bacteria, yeast, fungi, algae, insect and mammalian cells [319,326,327]. Although mammalian cells are the platform with the most complex and robust products [319], insect cells and yeast also present good performances. In terms of easy handling, fast growth rate, production and maintenance costs, bacteria are frequently the recombinant expression system of choice. By far, the most widely used bacteria is *E. coli*, but other bacteria such as Lactic Acid Bacteria (LAB) have gained importance in the last decades, with relevant examples such as *L. lactis* [328].

Escherichia coli as a protein expression system

E. coli is a Gram-negative enterobacterium, facultative anaerobic, rod-shaped and coliform with a fast growth rate (approximately 20 min for duplication) which only needs very low-cost media for its growth [320,329]. Its genetics, metabolism and physiology are very well-characterized and there exist many commercial strains and plasmids available for protein expression [330]. Overall, these different aspects have favored the extended use of this bacterium for recombinant protein production purposes [331–334]. Around 30% of the approved therapeutic proteins are currently being produced using it as a host [332]. Since 2018, 36 products have been approved for their therapeutic use in human health [335]. Historically, among the most relevant biopharmaceuticals, we find therapeutic molecules such as insulin (Humulin®, Eli Lilly) for the treatment of diabetes, interferon α2b (IntronA, Schering-Plough) for the treatment of cancer and hepatitis, or glucagon (Eli Lilly) for the treatment of hypoglycemia. However, this success in human health is far from being replicated in other fields. In animal production, promising

advances have been achieved on the recombinant production of hormones [336–339], xylanases [340,341], phytases [342] or antibodies [343] but none of them have reached the market yet as pure products.

Since *E. coli* is a Gram-negative bacteria, proteins produced in this expression system can contain LPS traces. LPS is an endotoxin and, in consequence, it can stimulate undesirable immune responses both *in vitro* and *in vivo*. Usually, LPS traces could be removed through extra purification steps, being an inefficient and time-consuming process [344]. Alternative strategies have been developed, for instance, the *E. coli* mutant strain ClearColi™ which has a modification in the LPS precursor [345]. ClearColi™ is able to produce endotoxin-free proteins at acceptable yields [346] but its slow growth rate and its high variability are relevant shortcomings. In addition, although the modified LPS of ClearColi™ does not stimulate immune responses in humans, it induces those reactions in other species such as mice [347], Chinese hamsters [348] or equines [349]. Hence, *E. coli* is an extraordinarily efficient platform for recombinant protein production but falls short at the later stages for its application in animal production.

Lactic Acid Bacteria (LAB) as a protein expression system

Lactic Acid Bacteria (LAB) are a group of bacteria formed by more than 60 genera, including *Lactococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Lactobacillus* and *Lactiplantibacillus* as the most popular. All of them are characterized by metabolizing carbohydrates and producing lactic acid [350,351]. In the search for alternative recombinant systems and trying to overcome the presence of LPS in the final protein products produced in *E. coli*, the group of Gram-positive LAB becomes an interesting option. It is worth mentioning that many of them are commensal bacteria present in the gut of humans and animals. For that reason, genera like *Lactococcus* or *Lactiplantibacillus* have the Generally Regarded As Safe (GRAS) status by the FDA and fulfill the criteria of the Qualified Presumption of Safety (QPS) according to the European Food Safety Authority (EFSA) [350,352]. The extended use of LAB in food fermentation of dairy products, their application in food preservation, as well as their vast features as probiotics are well known [353]. LAB were used as probiotics before being applied for recombinant protein production, and this application continues [354,355]. Linked to this application, LAB are known for being excellent live vectors for the delivery of proteins in the organism. For instance, *L. lactis* is among the most used strains [356–359], but there

already exist other examples such as *Lactiplantibacillus plantarum* [360] or some strains of the genus *Lactobacillus* applied for these delivery purposes [361]. LAB ability to express proteins anchored in their membrane has raised their potential to produce and express membrane proteins, one of the current challenges in biotechnology for the significance of these proteins as drug targets. LAB have been already used for the expression of antigens, antibodies, enzymes, and adhesion domains [362]. The display of these types of molecules in the LAB membranes has proven the possibility to enhance immune response and passive immunity [362]. Indeed, there are described examples of *L. plantarum* [360] and *L. lactis* [363] which induced specific immune responses, among many others [362]. Connected with that, the idea to use this type of protein expression as a vaccine is gaining importance [364,365].

Besides, in the last two decades, the research on the use of LAB for recombinant protein production to be further purified has evolved almost exponentially [366–372]. *L. lactis* is probably the LAB more studied as a recombinant cell factory [366,367,373,374]. It is a Gram-positive, spherical, non-sporulating, and facultative anaerobic commensal bacteria [366].There are several characteristics that make *L. lactis* an appealing expression system. Its small-sized genome (2.3 Mbp) was fully sequenced in 2001 [375], permitting a perfect knowledge of its genetic peculiarities. Related to that, a bulk of successful genetic engineering tools have been developed, such as strains, plasmids, and promoters with customizable options [369,376–378]. There exist constitutive promoters, which are not controlled by regulatory or growth conditions [379,380], and inducible promoters, those that express proteins after an induction [366]. The second ones has been more used than the first because they provide better control to the user on the protein expression. There exist inducible promoters regulated by zinc [381,382], pH [383], and xylose [384] used for protein expression in *L. lactis*. However, the most widely used is the nisin controlled gene expression (NICE) system (**Figure 10**) [377]. Nisin is an antimicrobial peptide, and its gene expression is regulated by nisR and nisK. NisK is a membrane histidine-protein kinase that acts as nisin receptor. Nisin reception activates nisR through a phosphorylation and the posterior transcription of the two promoters of the nisin gene cluster: PnisA and PnisF [385]. Protein expression based on NICE system needs plasmids with the PnisA promoter (i.e., pNZ8148 and pNZ8150 [378]) controlling the expression of the protein of interest and a nisin-negative *L. lactis* strain, such as *L. lactis* subsp. *cremoris* NZ9000, with the *nisK* and *nisR* genes inserted into its chromosome [377]. Thus, the addition

of nisin as inducer finally results in an activation of the expression of the heterologous protein.

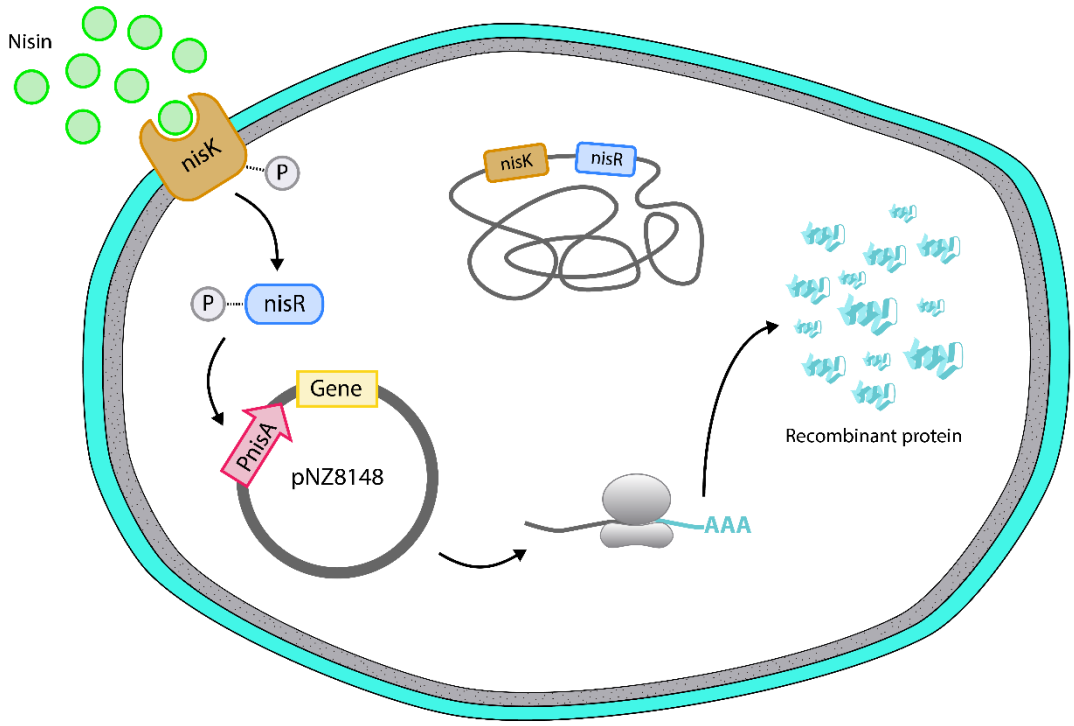

Figure 10. Nisin controlled gene expression (NICE) system in *L. lactis*. The addition of nisin into the culture medium promotes the activation of nisK histidine protein kinase (nisK), a membrane receptor. This interaction promotes the autophosphorylation, and consequently, the nisR mediator is as well phosphorylated. NisR activation promotes the signal transduction that facilitates the recognition of PnisA promoter and induce the recombinant expression of the heterologous protein.

Secreting proteins outside the cell sometimes is a better strategy than intracytoplasmic protein expression, because it simplifies purification processes, then the yields are higher [369]. In this context, *L. lactis* has a simple mechanism to secrete proteins outside the cell, being a mechanism much efficient than those found in *E. coli* where secreted proteins are mostly stuck in the periplasm [386]. Besides, this Gram-positive bacterium has only one major secreted protein, named Usp45 with its own signal peptide (SP) [367], which has been included in plasmids used with the NICE expression system such as pNZ8110, fostering an efficient recombinant protein secretion. Furthermore, *L. lactis* presents a single extracellular protease, HrtA, reducing the chances of heterologous protein degradation of the secreted products [387,388]. Connected with that, a strain which has

a deletion of the *hrtA* gene (NZ9000 *hrtA*) was developed improving the final yields of secreted recombinant proteins [388].

In this context, *L. lactis* has been used for the recombinant production of many different types of molecules [366]: enzymes with potential as thrombolytics [389], anti-inflammatory [359], immunomodulators [193,221] and sesquiterpene producers [390,391]. Furthermore, the production of cytokines such as IL-6 [392,393], IL-10 [394] and IFN-γ [395], as well as the expression of several antigens [363,396–399], APPs [221] or antimicrobials [400,401] has been widely demonstrated in this LAB. Interestingly, some of the previous examples have been produced for animal production application such as MMP-9, M-SAA3 or IL-6 that have showed promising immunomodulatory activities *in vitro* and *in vivo* [193,221,393]. *L. lactis*, besides all the tools and strategies named above, has an easily scalable and cost-effective production process which uses cheap culture mediums that do not need aeration during fermentation. That's why this LAB is suitable for further applications in the animal production industry where the costs must be extremely adjusted.

L. plantarum is another example of LAB with huge potential as a recombinant protein expression system. It is also a Gram-positive, plasmid-free, facultative heterofermentative organism found in many habitats, including dairy and meat products [402]. The level of knowledge about this bacterial strain is minor in comparison with *L. lactis*, but in the last years, different studies have demonstrated the unknown possibilities of *L. plantarum* in the context of recombinant protein production. There exist several strains such as *L. plantarum* NC8 [403], CD033 [404], WCFS1 [405], NCIMB8826 [406] or Pa21 [407]that have been explored for the development of vectors, promoters and expression systems [404–408]. As with *L. lactis*, we find constitutive promoters [404,405] and inducible promoters activated by heat shock [407] or by specific inducer molecules [408,409]. In addition, other inducible systems like NICE have been adapted to this LAB [406]. Among the existing expression systems, variants of pSIP vectors with inducible promoters of the bacteriocins sakacin A and sakacin P seems to be the most efficient and the more easily customizable [408–411]. The inducible promoters of pSIP vectors can be P_{sppIP} and P_{sppA}/P_{sppQ} and are activated by the externally addition of peptide pheromones named sakacin A or P inducing peptide (SapIP and SppIP). Using *L. plantarum*, it has already described the expression of enzymes such as chitinase [412], β-galactosidase

[413–415], aminopeptidase [409], reductase [416], β-glycosidase [417], decarboxylase [418] and β-glucuronidase [408,409], proving its feasibility and versatility.

Recombinant protein formats

The recombinant protein production process inside the bacterial producer cell can finish with the soluble recombinant protein either in the cytoplasm of the cell or secreted in the periplasm or in the media (**Figure 11**). However, during this production process many proteins aggregate in the bacterial cytoplasm forming protein aggregates known as Inclusion Bodies (IBs) (**Figure 11**). IBs' structure is formed by an amyloidal fibrillar scaffold of non-active protein conformations of the recombinant protein and, embedded in this scaffold, there are correctly folded and fully active recombinant proteins [419]. IBs' size ranges from 50 to 500 nm, and they are generally found in the bacterial cytoplasm distributed in the poles of the cells. These aggregates are mainly composed by the heterologous protein expressed, but they can also contain other recombinant cell components such as housekeeping proteins, nucleic acids, carbohydrates, or lipids. The formation of IBs has been described in recombinant bacterial expression systems such as *E. coli* [420] and *L. lactis* [222].

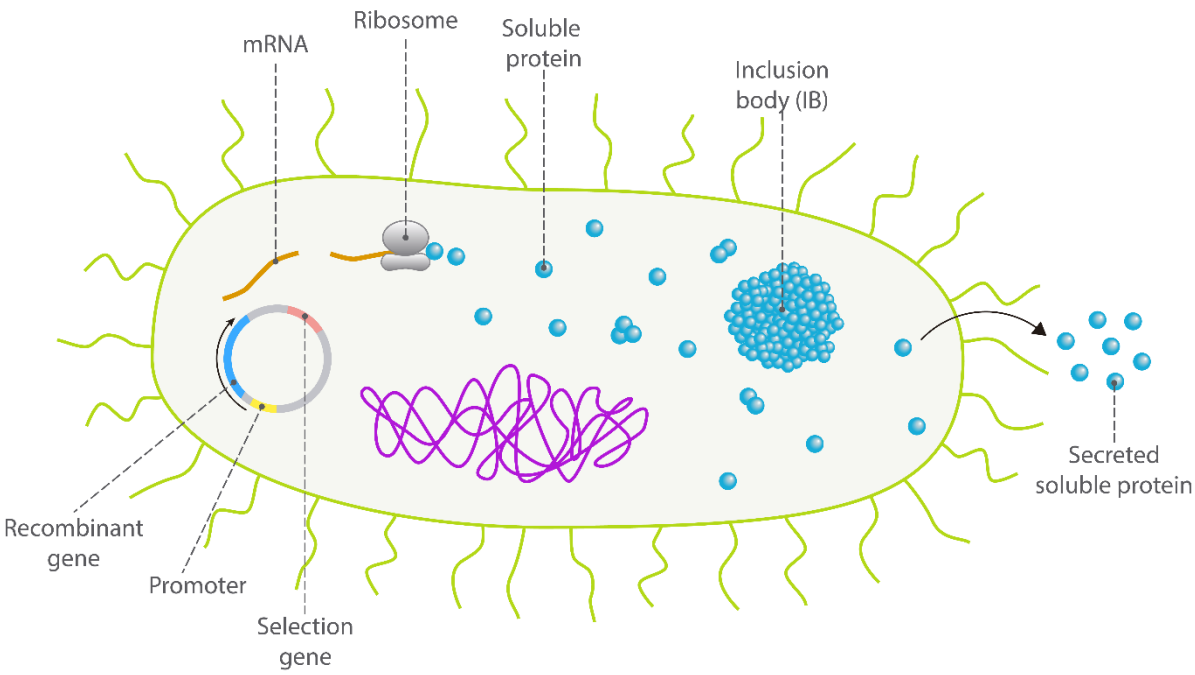

Figure 11. Recombinant protein production process and recombinant protein formats. Recombinant production of protein is induced by inductor that activates its specific promoter. The promoter activation induces the transcription of the recombinant protein gene into mRNA. The ribosome then captures mRNA and initiates the translation of mRNA into a polypeptidic chain that is folded with the

final soluble protein conformation. The soluble produced protein can be accumulated in the cytoplasm or secreted. Frequently, the overexpressed protein can be aggregated forming inclusion bodies (IBs).

It has been described that only 30 % of the cloned genes can be expressed in its soluble form, at least in *E. coli* [421]. Since the objective of the recombinant production processes is the obtainment of properly folded soluble protein, many efforts have been devoted to increase protein solubility during recombinant production processes [422,423]. Different strategies like codon-usage optimization, changes in culture conditions, the use of different plasmids or the addition of solubilization tags have been explored [424,425]. By means of these strategies, the solubility of many recombinant proteins has improved, but it is generally a protein-dependent process and sometimes none of them work, being not possible to increase the final soluble protein yields. When it is not possible to obtain the protein from the soluble fraction, IBs are used as source of recombinant protein. Traditionally, the protocols used for the solubilization of proteins from IBs have been based on the application of harsh detergents and chaotropic agents at high concentrations (e.g., urea or guanidine hydrochloride at 6-8 M) on the IBs. After this aggressive solubilization, the protein obtained is completely denatured, requiring refolding steps to recover its original structure and function. Theoretically, under favorable conditions, denatured protein should return to its original state, but at last the percentage of total correctly folded protein is low, being the refolding process a limiting step of the whole process. Despite the questionable efficiency of these protocols, they are widely used, even in the production of commercial products [426–428]. For years, it was believed that the protein inside the IBs was misfolded and dispossessed of any type of activity [429,430], but today it is known that this concept is totally wrong. Fortunately, the paradigm has changed, and now it is well described that IBs contain active and correctly folded proteins [431]. Considering that, alternative protocols have been developed in the last few years avoiding the denaturing and refolding processes to recover the protein embedded in the IBs [432,433]. The most successful strategy is the use of mild solubilization agents, such as the detergent n-lauroylsarcosine (NLS) or the organic compound dimethyl sulfoxide (DMSO), and organic solvents such as n-propanol and isopropanol. These agents can solubilize the recombinant protein from IBs but without disturbing its native structure [221,432,434,435]. After that simple solubilization step, the soluble protein can be easily purified from the supernatant by

standard chromatography procedures [221,435,436]. Hence, IBs are a solid source of soluble, pure and active protein.

IBs are far from being useless protein aggregates or only suppliers of soluble protein. It has been proven that they can be applied directly as an active protein format and they have demonstrated to be a valuable material in many biotechnological and biomedical applications [430] (**Figure 12**). Their porous and mechanically stable architecture confers them interesting properties such as high stability and slow protein release. In this context it is well described that IBs are able to slowly release the protein embedded in their amyloidal scaffold [437], which has proven to be a very interesting property for drug delivery in cancer [438] and in cell replacement therapies [437]. Besides, IBs have been used as immobilized biocatalysts [429,439,440], presenting more stable and robust features in comparison with their soluble counterparts. Another application is their use as scaffolds in tissue engineering since they support cell attachment, stimulate cell proliferation and differentiation, and improve tissue regeneration [441,442]. Focusing on IBs' application in the context of IDs, IBs formed by different types of immune-related proteins, such as cytokines or metalloproteinases, have proven to stimulate cytokines expression [393] as well as immune cell recruitment [193]. Moreover, the heterogenic composition of IBs and their nanostructuration also confer them immunostimulant properties proven in the development of vaccines in aquaculture [443–445] and as a mammary gland immunostimulant in dairy cows [192]. Also, they have been applied as antimicrobial nanoparticles against MDR bacteria [436].

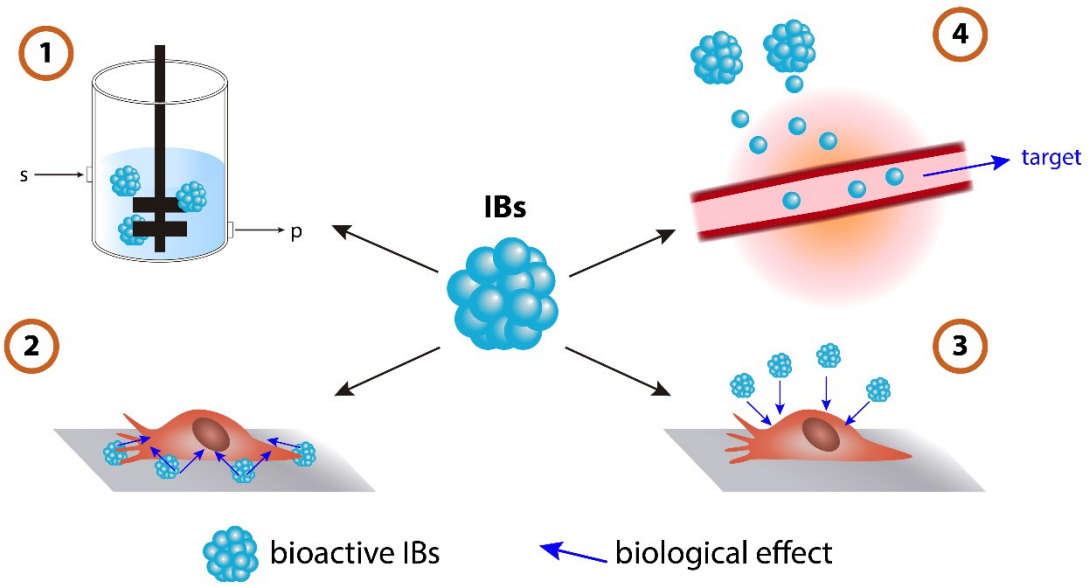

Figure 12. IBs applications. 1) IBs can be employed as natural immobilized biocatalysts to transform substrates into desired products. 2) IBs can be used as scaffolds to promote cell proliferation and differentiation. 3) IBs can be administered as free active nanoparticles to cultured cells. 4) IBs can be administered *in vivo*, where they will release soluble protein that can reach the target tissue travelling through the bloodstream.

RECOMBINANT INNATE IMMUNITY-RELATED PROTEINS FOR THE TREATMENT OF BOVINE IDs

In previous sections, it has been mentioned that different types of molecules that are part of the innate immunity have a huge potential to treat IDs. Among them, proteins and peptides with immunostimulant and antimicrobial functions are one of the most promising ones to develop a new antimicrobial and antiviral therapy. Interestingly, the recombinant production of some of these molecules has been already assessed, which prove that their production through recombinant technology is a feasible approach.

Recombinant immunomodulators for bovine IDs

Therapies based on different types of innate immunity-related molecules produced recombinantly have shown promising results. In the context of bovine IDs, studies on the application of recombinant APPs [194,233], enzymes [192,193,214,446], cytokines [447–450] have been developed. Mastitis arises as the most common target disease of these recombinant immunostimulants since it has a high economic impact in the dairy industry [11].

The use of immunomodulators is highly relevant during the cow dry period to accelerate the mammary involution and prevent days of milk leakage and the risk of IMIs that can

impair the next lactation of dairy cows. Different advances have been done in the study of recombinant M-SAA3 as a possible immunomodulating therapy for the prevention of mastitis during the dry period [194,221,233]. This APP has an important role in the innate immune response, and it is specifically produced in the mammary gland, being highly expressed in colostrum, during IMIs and at dry-off [230,231,451]. The recombinant expression of M-SAA3 has been described in *E. coli* [232,233] and in *L. lactis* [221]. In the case of *L. lactis*, a highly pure protein has been isolated from IBs [221]. In terms of activity, different immunomodulatory functions of that recombinant M-SAA3 have been described *in vitro*, including the increase of macrophage phagocytosis of bacteria [232], the stimulation of the cytokine expression in primary mammary cultures [233], and as a result of this immune-related activities, the reduction of bacterial populations in cultures infected by *S. aureus, Streptococcus uberis*, *Streptococcus dysgalactiae* and *E. coli* [233]. The capability of M-SAA3 to reduce those bacterial infections is especially relevant because these four pathogens are among the most frequently involved in IMIs [452]. Moreover, studies proving the intramammary infusion of recombinant M-SAA3 have shown an increase of the MMP-9 activity, the protein content in mammary secretions and cytokines expression [194]. All the results obtained are encouraging and show the potential of M-SAA3 produced recombinantly.

MMP-9, which has been previously mentioned to be stimulated with the administration of M-SAA3 in the mammary gland during dry-period, is a protein highly relevant for the involution process of the mammary gland [216,453]. Besides, MMP-9 has the capacity to recruit neutrophils [210,454] that can be very beneficial to fight against IMIs. For that, different studies exploring the recombinant production of MMP-9 and the characterization of its immune-related features have been performed. The production of recombinant MMP-9 in two LPS-free bacterial expression systems such as *Clearcoli* or *L. lactis* is widely described [214,219,221,222]. Avoiding the presence of potential immunostimulant molecules like LPS is crucial to obtain robust and reliable results in studies in which the immune-related activity is studied. Recombinant MMP-9 has a huge tendency to form IBs, showing values of 99% of aggregation [221,222]. Thus, the soluble protein has been isolated from IBs using non-denaturing protocols and the *in vitro* activity of MMP-9 has been proven in both soluble and IBs formats [219,221,222,446]. In *in vitro* assays, soluble MMP-9 is more active than IBs, but in *in vivo* experiments, using a mice model and also in dairy cows, IBs show a more sustained activity over time,

presenting higher recruitment of immune cells and higher stimulation of pro-inflammatory chemokines [192,193,214,446]. Although soluble protein seems to have a faster and more immediate action, MMP-9 IBs are a more stable format which is a relevant feature for *in vivo* applications.

Cytokines are small molecules mainly involved in cell-to-cell communication during the immune response. Moreover, they are crucial for the regulation of inflammatory and anti-inflammatory processes. These features fit perfectly with the immune needs of dairy cows affected by mastitis during lactation and dry periods. Thus, they have been studied as a mastitis treatment. For instance, the intramammary infusion of recombinant IL-2, IFN-γ, IL-1β and IL-8 has shown protection against IMIs caused by *S. aureus* or *E. coli* [447–450]. Other studies have been able to stimulate mammary gland involution by applying recombinant IL-8 and bovine granulocyte-macrophage colony stimulating factor (rboGMCSF) [455]. Another example is the use of a modified cytokine named pegbovigrastim and marketed under the name of Imrestor® since its approval in 2016 by FDA. Pegbovigrastim is based on the bovine granulocyte colony-stimulating factor (bG-CSF), an endogenous glycoprotein with bactericidal properties, covalently bound to polyethylene glycol (PEG), which increases its half-life. Its administration at calving reduces new IMIs during the first months of lactation [456]. However, the commercialization of this drug was suspended in 2018 due to several side-effects. Cows treated with it developed lameness in postpartum and abomasum dysplasia [457]. Despite this interruption, research is going on to overcome this drawbacks and to improve the efficiency of pegbovigrastim in dairy cows [458].

Recombinant antimicrobials for bovine IDs

The recombinant production of innate immunity-related molecules with antimicrobial activity, such as HDPs, might be a good approach to treat animal IDs such as BRD. The recombinant production processes of antimicrobial peptides based on HDPs is easily scalable and cost-effective [459]. Moreover, the possibility to produce long peptides and to achieve secondary structures and post-translational modifications (PTMs) crucial for the final activity of the peptides is an important advantage in front of other production strategies such as the chemical synthesis [460]. There exist many examples of recombinant HDP production using different expression systems [315] as detailed in **Table 1**. The most used are yeast, fungi, insect cells and bacteria, all of them with their

specific advantages and drawbacks (**Table 1**) [315,461]. Recombinant production in bacteria is the most extensively used strategy, as they are easy to manipulate, grow fast and use low-cost media. Yeasts for their part offer a good relation between the higher complexities of eukaryotic cells and the simplicity and cost-effective production process of prokaryotic systems. They grow faster than mammalian host (e.g., Chinese Hamster Ovary (CHO) cells) and secrete high amounts of heterologous recombinant proteins [462]. The use of fungi for the recombinant production of HDPs is not so well studied. However, their ability to perform PTMs and the easy possibility to scale-up the production processes with an adjusted budget has increased the interest in these recombinant hosts [463,464]. Insect cells would be the last example, offering PTMs such as glycosylation but their cultivation is more expensive than that of yeast and bacteria [465].

Table 1. Advantages and drawbacks of different expression systems to produce HDPs. Adapted from [315,466].

	Advantages	Drawbacks
Bacteria	• High expression levels • Low cost • Well established methodologies • IBs formation (less toxicity to host) • High purity	• Risk of degradation during production • No PTMs • HDPs might be toxic to host • LPS removal in Gram-negative
Yeast	• PTMs • Efficient secretion systems • High expression levels • Well known genetic regulation • Low cost	• Undesired hyperglycosylation • Fermentation • Hard to disrupt for protein releasing • HDPs might be toxic to host
Fungi	• PTMs • Efficient secretion systems • High expression levels • Low cost	• Not well established methodology • Spore formation • Longer culture times • HDPs might be toxic to host
Insect cells	• Genomic or plasmid expression • PTMs similar to mammalian cells	• High cost • Low yields • Difficult to scale-up

Focusing on the recombinant production of HDPs in bacteria, *E. coli* is the most widely used [422]. However, *Bacillus subtilis* and *L. lactis* are gaining importance in this field [315,400,466]. The use of LPS-free expression systems is not critical for the production and activity determination of antimicrobial proteins or peptides, but as we know, HDPs also have an immunomodulatory capacity and LPS traces could interfere with this activity. Thus, a further exploration of these LPS-free systems could have a high impact in this field of research. The biggest limitation of bacteria is their limited ability to make disulphide-bonds and their impossibility to do glycosylation and other PTMs [467–469]. Although some groups of HDPs have disulphide-bonds in their structure [470], it is not clear if they are crucial to reach the final conformation of HDPs. Another limitation of the recombinant production of HDPs is their small size, being highly degradable by proteases present in the cytoplasm of the producer cells. Moreover, they can be toxic for the producer cells due to their antimicrobial nature [471]. Aiming to address these two main drawbacks of HDPs recombinant production in bacteria, the expression of HDPs fused to a carrier protein is a widely used strategy. Remarkably, the production of HDPs fused to a bigger protein protects them from proteolytic degradation, as well as masks their toxicity towards the producer bacteria [472]. Some examples of carrier proteins are thioredoxin [473], glutathione S-transferase (GST) [474], small ubiquitin-related

modifier (SUMO) [475] or green fluorescent protein (GFP) [476]. Apart from avoiding degradation and reducing toxicity, the recombinant fusion protein production improves yields and becomes useful for preliminary characterization of different HDPs [476]. Nevertheless, the main issue of this strategy is the presence of a carrier which do not have antimicrobial activity. Thus, the removal of the carrier protein is almost mandatory to finely characterize the final antimicrobial product. This elimination requires expensive processes or reagents that sum costs and time to the production process [477,478]. To avoid some of these problems, there exists the possibility of including self-cleaving tags, such as inteins, as carrier proteins. [479]. Alternatively, an interesting approach for the recombinant production of HDPs is their production as multidomain proteins, which combine different HDPs in a single polypeptide, being not necessary to include a carrier protein [476]. The recombinant production of simple multidomain proteins formed by two [480] or several [476] HDPs have been proven. In both studies, the fusion of different types of HDPs in the same molecule expanded and increased the final antimicrobial activity compared with the same HDPs tested separately, including against MDR bacteria [476]. Another case of the combination of domains with different activities is the study performed by Roca-Pinilla and coworkers that joined two antimicrobial domains, the human α-defensin 5 (HD5) and the $sPLA_2$, with a bacteria-specific binding domain such as gelsolin, and two aggregation tags which allowed the expression of the fusion protein in *E. coli* [436]. This multidomain protein presented broad-spectrum antibacterial activity against *K. pneumoniae* quinolone and carbapenem-resistant. Similarly, in the context of vaccine development against *M. bovis*, different bovine HDPs were fused to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme highly involved in *M. bovis* virulence [481]. This protein chimera presented both antimicrobial and immunomodulatory activity, preserving the specific activity of each domain [481]. Besides, there is an example of a fusion of cecropin A and D and cationic elastin-like polypeptides (ELP) in a hybrid molecule expressed in *E. coli* [482]. ELP tags allow the HDP expression and the precipitation of the fused HDPs under certain temperature conditions simplifying the downstream processing and avoiding a further purification chromatography.

Recombinant production of HDPs as IBs has become another strategy to avoid toxicity on the producer bacteria [436,483,484]. HDP-based IBs are a versatile format because they can be applied directly as antimicrobials [436,470] but also, they are a trustworthy

source of soluble and active HDP by using the right solubilization protocol [484]. Besides, the antimicrobial potential of HDP-based IBs has been demonstrated against important Gram-positive pathogens such as MRSA and against Gram-negative bacteria such as *P. aeruginosa* [470]. Moreover, they have shown antibiofilm activity against biofilms formed by *K. pneumoniae* carbapenem-resistant [436].

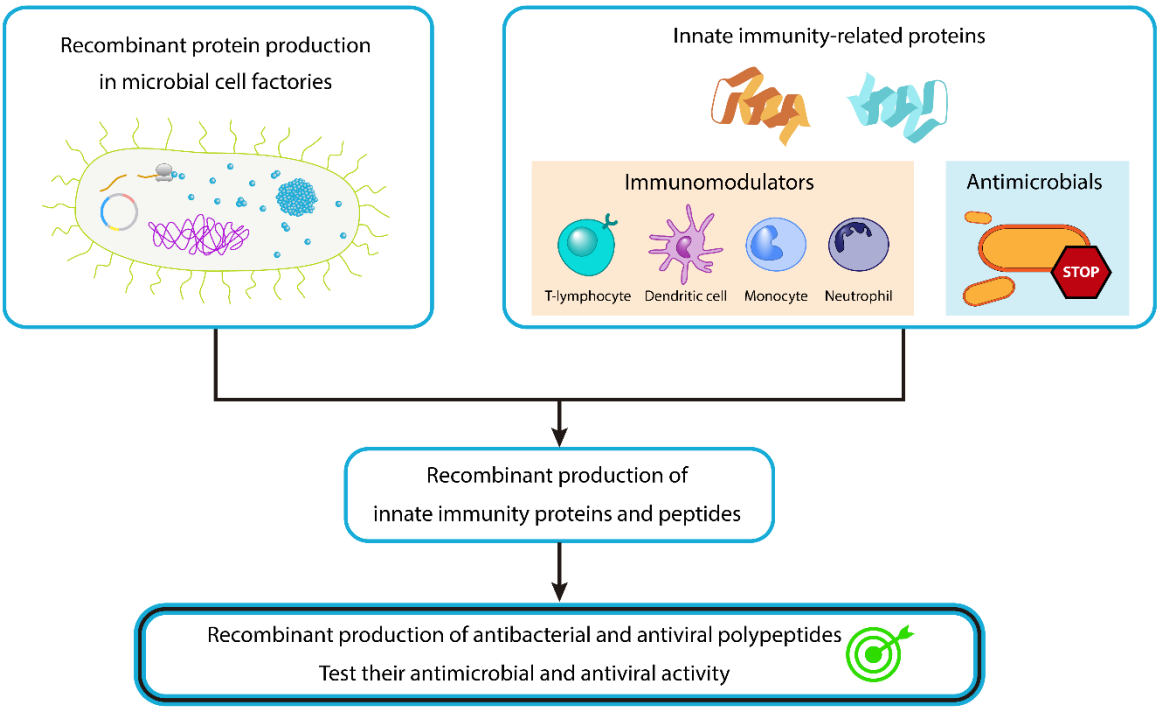

Figure 13. Schematic representation of the main thesis objectives. Taking advantage of the capacity of microbial cell factories to produce recombinant proteins, and in combination with a huge repertoire of innate immune-related protein, the main objective of this thesis is to produce recombinant immune-related proteins to test their potential as antimicrobial and antiviral molecules to treat IDs affecting ruminant production.

OBJECTIVES

This thesis aims to study the potential of innate immunity-related proteins recombinantly produced to develop a new antibacterial and antiviral treatment for bovine infectious diseases.

The main objectives to accomplish this challenge are herein detailed:

1. To study *Lactiplantibacillus plantarum* as an LPS-free recombinant expression system for the production of innate immunity-related proteins (Study 1).

2. To establish effective and suitable solubilization protocols for the isolation of soluble and active proteins from bacterial inclusion bodies (IBs) formed by different innate immunity-related proteins (Study 2).

3. To explore the recombinant production of bovine HDPs in the LPS-free expression system *Lactococcus lactis* and to determine their antibacterial and antiviral activity against bacteria and viruses causing BRD (Study 3/Annex 1).

RESULTS

STUDY 1

Lactiplantibacillus plantarum: a new example of inclusion body producing bacteria

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Accepted in Microbial Cell Factories, 2023 (Research article)

Preface

The recombinant protein production is a widely used, cost-effective, and easily scalable strategy for the obtainment of therapeutic molecules with an almost infinite number of applications. *Escherichia coli* is by far the bacterial workhorse of the field, being the first option to produce any type of protein of interest. Nevertheless, in some cases, the presence of lipopolysaccharide (LPS) in the final products limits their use. Therefore, during the last years, our research group has focused on exploring and characterizing Lactic Acid Bacteria (LAB) for the production of recombinant proteins. *Lactococcus lactis* has been the microbial cell factory studied so far. The current thesis is focused in the study of the production and use of innate immune-related proteins for the treatment and prevention of bovine infections and our first objective was to evaluate others LAB such as *Lactiplantibacillus plantarum* as recombinant systems to produce these proteins. We have used the innate immunity-related protein metalloproteinase-9 (MMP-9) as model protein. MMP-9 is a key enzyme during the mammary gland involution of the cow dry period which is also related to the risk to suffer intramammary infections. This is a prone-to-aggregate (PTA) protein and for that, its production in the soluble fraction becomes problematic in *E. coli* and *L. lactis*.

The results obtained in this first study have proven that MMP-9 is successfully produced in *L. plantarum*. However, as with *E. coli* and *L. lactis*, the protein is found in the aggregated fraction. Interestingly, this is the first time that inclusion bodies (IBs) formation is described in *L. plantarum*. The structural characteristics of the *L. plantarum* IBs obtained are resembling those found in *E. coli* and *L. lactis* aggregates, reinforcing the

idea that bacterial protein aggregation is independent of the bacterial strain used and is more linked to the overexpressed protein. Briefly, *L. plantarum* IBs contain biologically active recombinant protein, which is spontaneously and slowly released, demonstrating that these aggregates can be used as a starting material for the isolation of soluble, active, and high pure protein using a non-denaturing solubilization protocol. Thus, in this study, the characteristics and applicability of *L. plantarum* IBs have been unmasked and this has also amplified the variety of LPS-free bacterial expression systems available for the development of new recombinant protein-based therapies. However, this study also shows that MMP-9 protein yield obtained in *L. plantarum* is not higher than that achieved using *L. lactis* and since the last one presents a wider catalog of molecular tools, *L.plantarum* does not offer an extra advantage to take into consideration for further studies.

Lactiplantibacillus plantarum: a new example of inclusion body producing bacteria

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Abstract

Background

Lactic Acid Bacteria such as *Lactococcus lactis, Latilactobacillus sakei* (basonym: *Lactobacillus sakei*) and *Lactiplantibacillus plantarum* (basonym: *Lactobacillus plantarum*) have gained importance as recombinant cell factories. Although it was believed that proteins produced in these lipopolysaccharides (LPS)-free microorganisms do not aggregate, it has been shown that *L. lactis* produce inclusion bodies (IBs) during the recombinant production process. These protein aggregates contain biologically active protein, which is slowly released, being a biomaterial with a broad range of applications including the obtainment of soluble protein. However, the aggregation phenomenon has not been characterized so far in *L. plantarum*. Thus, the current study aims to determine the formation of protein aggregates in *L. plantarum* and evaluate their possible applications.

Results

To evaluate the formation of IBs in *L. plantarum*, the catalytic domain of bovine metalloproteinase 9 (MMP-9) protein has been used as model protein, being a prone-to-aggregate (PTA) protein. The electron microscopy micrographs showed the presence of

electron-dense structures in *L. plantarum* cytoplasm, which were further purified and analyzed. The ultrastructure of the isolated protein aggregates, which were smooth, round and with an average size of 250-300 nm, proved that *L. plantarum* also forms IBs under recombinant production processes of PTA proteins. Besides, the protein embedded in these aggregates was fully active and had the potential to be used as a source of soluble protein or as active nanoparticles. The activity determination of the soluble protein solubilized from these IBs using non-denaturing protocols proved that fully active protein could be obtained from these protein aggregates.

Conclusions

These results proved that *L. plantarum* form aggregates under recombinant production conditions. These aggregates showed the same properties as IBs formed in other expression systems such as *Escherichia coli* or *L. lactis*. Thus, this places this LPS-free microorganism as an interesting alternative to produce proteins of interest for the biopharmaceutical industry, which are obtained from the IBs in an important number of cases.

KEYWORDS: *Lactiplantibacillus plantarum*, inclusion bodies, recombinant proteins, active aggregates, protein aggregation

Background

Different recombinant protein production systems were developed with the appearance of recombinant DNA technology [1,2]. This technology opened a huge spectrum of possibilities, from the obtainment of proteins difficult to isolate from their natural origin to the *de novo* fabrication of polypeptides of interest. Different types of organisms are used for recombinant protein production purposes, including bacteria, yeast, fungi, algae, insect cells and mammalian cells [3]. For proteins that do not need specific post-translational modifications such as glycosylation, bacteria display the best performance-cost ratio as recombinant cell factories. More specifically, *Escherichia coli* has been by far the most used bacterium, due to low production costs, and a myriad of engineering tools that it offers as an expression system. Despite all the advantages, the lipopolysaccharides (LPS) present in the outer *E. coli* membrane is an important drawback since the presence of endotoxin or LPS traces in recombinantly produced proteins produces undesirable

inflammatory effects. Thus, extra purification steps, which are costly and low-yield procedures, are required to reduce or eliminate LPS from the final recombinant product [4–9]. In this scenario, alternative expression systems such as Lactic Acid Bacteria (LAB), which are LPS-free and are classified as Generally Regarded As Safe (GRAS) organisms by the Food and Drug Administration (FDA), have gained importance. The potential of this group of bacteria has been demonstrated with microorganisms such as *Lactococcus lactis, Latilactobacillus sakei* (basonym: *Lactobacillus sakei*) and *Lactiplantibacillus plantarum (*basonym: *Lactobacillus plantarum*), which have been used as recombinant cell factories [10–19], presenting similar performance to *E. coli* in many cases.

The possibility to produce and isolate recombinant soluble proteins from the cytoplasm fraction of *L. lactis* is well known, as well as the use of secretion strategies to recover the protein of interest from the culture media [10,12,19–22]. Although it is generally accepted that *L. lactis* does not form aggregates during recombinant production processes [12], the existence of protein aggregates or inclusion bodies (IBs) in the cytoplasm of this gram-positive microorganism has been documented [11,23].

IBs are protein deposits that are generated in recombinant bacteria under overexpression conditions [5]. Their size ranges from 50 nm to 500 nm, mainly presenting spherical shapes [23–25] and high stability [23,26–29]. However, some differences in size and surface functional group density have been described in IBs of the same proteins produced by different bacterial systems [26]. Besides, it has been broadly demonstrated that IBs are formed by active forms of the overexpressed protein, which are embedded in an amyloid-like structure [23]. The activity of these protein nanoparticles produced either in *E. coli* or *L. lactis* has led to study their applicability in biotechnology, material sciences and medicine, and as biocatalysts [30–32], scaffolds in tissue engineering [24], immunomodulators [28,29,33], antimicrobial agents [34], and drug delivery systems in cancer therapy [35–37]. Besides, IBs can be used as a source of soluble and active protein [5]. This strategy becomes very useful for the purification of proteins that are either difficult-to-express (DTE) or prone-to-aggregate (PTA), which present low yields when they are directly isolated from the bacterial cytoplasm in its soluble form.

Although soluble and aggregated forms have been very well-characterized in *E. coli* and in *L. lactis*, they have not been explored so far in other LPS-free systems like *L. plantarum*.

Thus, the current work aims to study and characterize the aggregation phenomena in *L. plantarum* to elucidate its capacity to produce IBs and their possible applications. For that, the 39 KDa catalytic domain of bovine metalloproteinase 9 (MMP-9) has been used as model protein. It is an enzyme involved in many biological processes such as extracellular matrix degradation, wound healing, angiogenesis, reproduction, growth, and tissue development [28,38]. Although the whole bovine MMP-9 has not been recombinantly produced so far, its catalytic fragment has been previously produced in *L. lactis* [23] and in *Clearcoli* and it has been described as a PTA protein [13].

Results

Solubility of MMP-9cat produced in *L. plantarum*

To analyze the MMP-9 catalytic domain (MMP-9cat) production and its solubility using *L. plantarum* as cell factory, we determined the percentage of protein in the soluble fraction and in the insoluble (or aggregated) fraction at 1, 3 and 5 h post-induction. The results obtained showed that MMP-9cat produced in *L. plantarum* has a high aggregation rate with 100% of protein aggregated at 1 h and 3 h post-induction, and 97.9% ± 1.1 at 5 h post-induction (**Figure 1.1**).

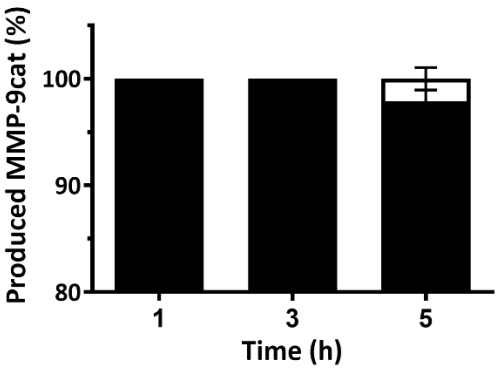

Figure 1.1. Percentage of aggregation of MMP-9cat produced in *Lactiplantibacillus plantarum* at 1,3 and 5 h post-induction. The aggregated MMP-9cat is represented in black and the soluble MMP-9cat is represented in white. Error bars indicate the standard error (SE).

Characterization of MMP-9cat IBs by electron microscopy

When *L. plantarum* cells were analyzed by Transmission Electron Microscopy (TEM), clear electron-dense structures with an average size of 250-300 nm were observed post-induction in the pole of the cells (**Figure 1.2**Error! No s'ha trobat l'origen de la referència.**B**). These aggregates observed after induction were not present in TEM micrographs of *L. plantarum* cells at 0 h (**Figure 1.2**Error! No s'ha trobat l'origen de la r eferència.**A**), proving that they were formed during recombinant production.

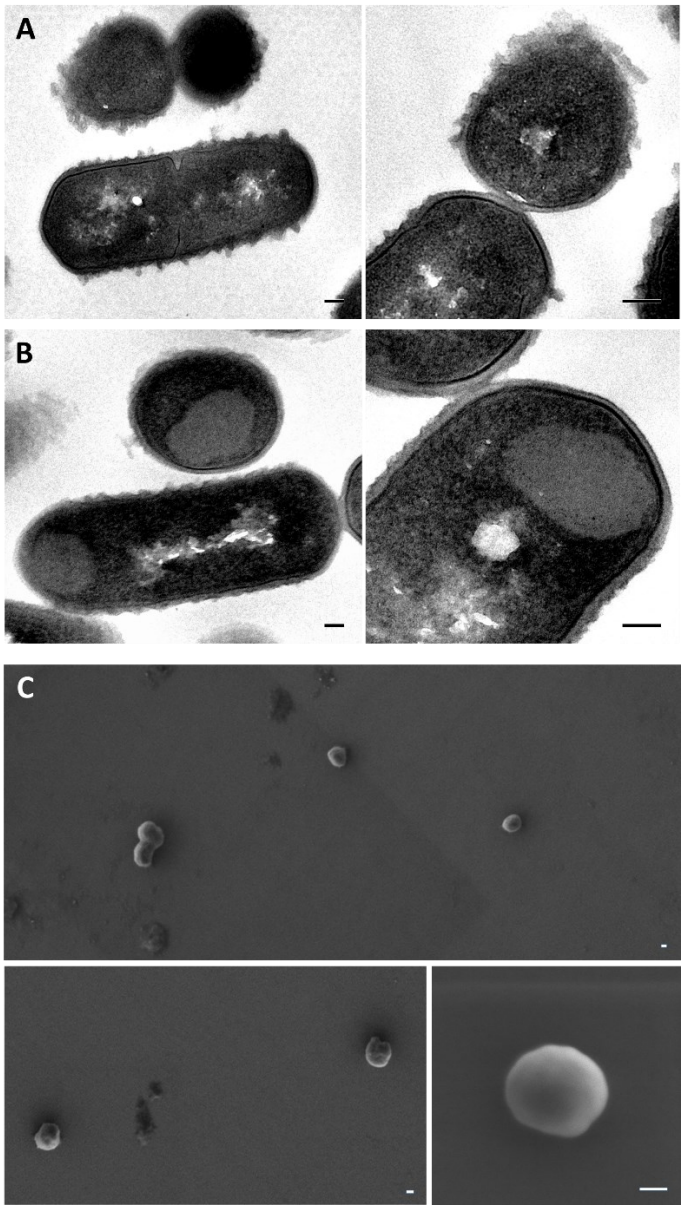

Figure 1.2. Electron microscopy characterization of MMP-9cat IBs in *Lactiplantibacillus plantarum* and purified MMP-9cat IBs. A) Representative TEM micrographs of ultrastructure of longitudinal and transversal bacilli sections at 0 h post-induction without the presence of MMP-9cat IBs and **B)** 3 h post-induction with the presence of MMP-9cat IBs. Bars size represents 100 nm. C) Representative

FESEM micrographs showing smooth surface, similar size, and round shape of purified MMP-9cat IBs. Bars size represents 100 nm.

After production, IBs were purified following standard protocols based on a cell disruption followed by several washing steps with the presence of mild detergents. The ultrastructural morphometry of purified IBs was characterized by Field Emission Scanning Electron Microscopy (FESEM) and micrographs showed smooth and round nanoparticles (**Figure 1.2**Error! No s'ha trobat l'origen de la referència.**C**) and confirmed t he average size of 250-300 nm shown in TEM micrographs (**Figure 1.2**Error! No s'ha trobat l'origen de la referència.**B**).

Solubilization and purification of MMP-9cat from IBs

After the characterization of IBs by electron microscopy, the next step was to confirm the presence of MMP-9cat inside the aggregates. Purified IBs were analyzed through Western Blot and Coomassie staining (**Figure 1.3Error! No s'ha trobat l'origen de la referència.**). The yield and purity were calculated by densitometry analysis (**Table 1.1**).

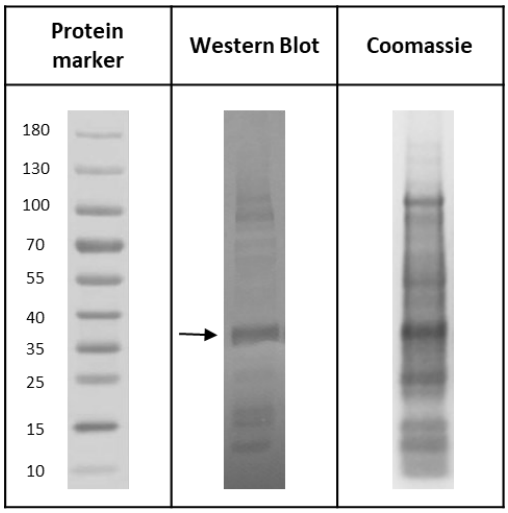

Figure 1.3. Confirmation of MMP-9cat presence in *Lactiplantibacillus plantarum* IBs by Western blot with Anti-His antibody and Coomassie staining. The arrow indicates the band with the theoretical size of the protein.

Table 1.1. Yield and purity of purified MMP-9cat IBs produce in L. plantarum.

	Yield	Purity
MMP-9cat IBs	4.56 mg/L	80.69 %

Aiming to determine if the aggregates produced in this recombinant cell factory were constituted of biologically active MMP-9cat, we analyzed their activity after a mild solubilization process. For that, recombinant expression of MMP-9cat in *L. plantarum* cells was induced with 50 ng/mL SppIP 3 h post-induction, bacterial cells were disrupted by a cell disruptor (**Figure 1.4Error! No s'ha trobat l'origen de la referència.**). Then, purified IBs were incubated at room temperature (RT) for 0, 24, 48 and 72 h and their activity was determined along the solubilization kinetics of MMP-9cat IBs (**Figure 1.4**Error! No s'ha trobat l'origen de la referència. **(5)**, **Figure 1.5**Error! No s'ha trobat l'origen de la referència.**A**). The activity assay proved that purified IBs were active, since although they showed activity at 0 h the specific activity significantly increased after the non-denaturing solubilization process (**Figure 1.5**Error! No s'ha trobat l'origen de la r eferència.**B**, $p<0.05$).

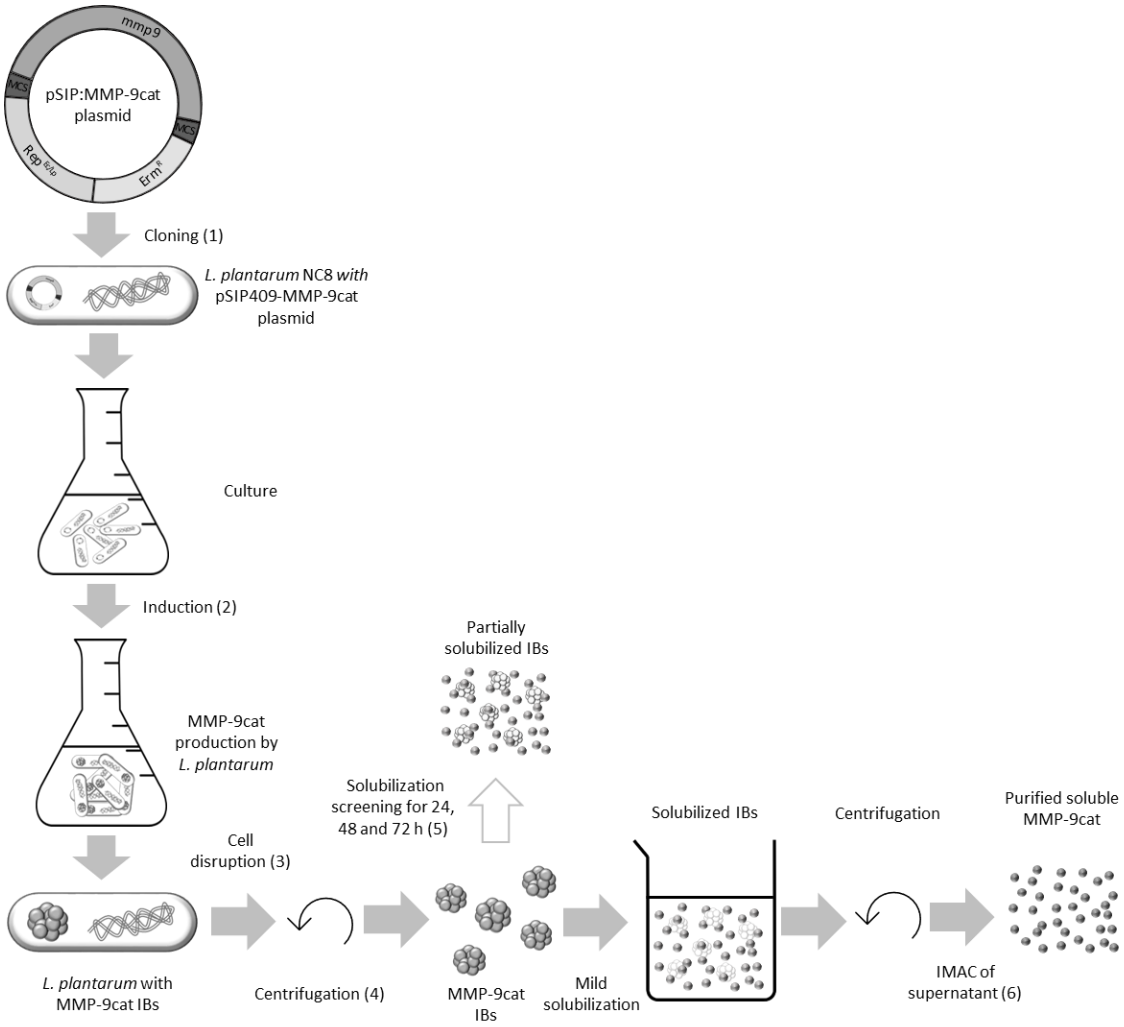

Figure 1.4. Schematic representation of production, isolation and solubilization of MMP-9cat IBs. *Lactiplantibacillus plantarum* NC8 cells were transformed with the pSIP:MMP-9cat plasmid carrying the catalytic fragment of the bovine MMP-9cat (1). Cells were grown in MRS medium, and the protein

production was induced with SppIP (2). After protein production, *Lactiplantibacillus plantarum* cells were disrupted (3) and centrifuged to recover the pellet containing MMP-9cat IBs (4). In an intermediate step, IBs were partially solubilized to evaluate their potential activity (5). Finally, the pellet was completely solubilized using a non-denaturing process and the soluble protein obtained after a centrifugation process was further purified by Immobilized metal affinity chromatography (IMAC) (6).

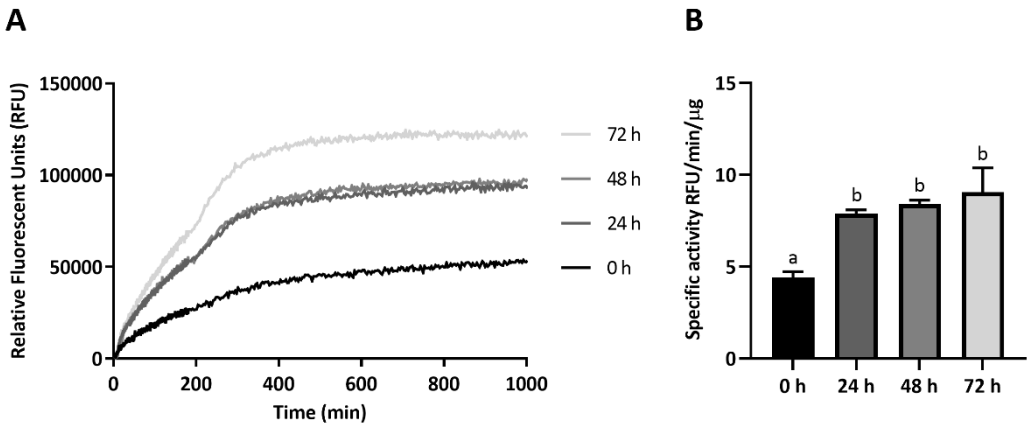

Figure 1.5. Enzymatic activity of MMP-9cat IBs solubilized. A) MMP-9cat activity kinetics of *Lactiplantibacillus plantarum* MMP-9cat IBs solubilized at RT for 0, 24, 48 and 72 h. **B)** Specific activity of the solubilized MMP-9cat IBs for 0, 24, 48 and 72 h. Different letters depict significant differences between the samples (p<0.05).

Taking 48 h as solubilization time, the protein solubilized from IBs was recovered and further purified by affinity chromatography (**Figure 1.4**Error! No s'ha trobat l'origen de l a referència. **(6)**) and the soluble protein obtained from the solubilized aggregates was further characterized. The purification chromatogram of the soluble MMP-9cat showed that the protein was eluted in 4 different peaks (**Figure 1.6**Error! No s'ha trobat l'origen de la referència.**A**). The final yield and the purity of each peak were quantified by Western Blot and Coomassie staining, respectively (**Figure 1.6**Error! No s'ha trobat l'origen de la r eferència.**B**). All the purified peaks contained pure protein at concentrations ranging from 0.21 to 1.90 mg/L, with peak 1 being the most concentrated (**Table 1.2**).

Soluble MMP-9cat and MMP-9cat IBs specific activity

The activity assay proved that soluble MMP-9cat was more active than IBs, which needed much greater concentrations to achieve similar activities (**Figure 1.7Error! No s'ha trobat l'origen de la referència.**). Peak 1 showed a specific activity higher than peaks 2, 3 and 4 (p<0.001), which did not statistically differ in activity (**Figure 1.7Error! No s'ha t robat l'origen de la referència.**). The specific activity of the 4 peaks of soluble MMP-9cat was also compared with that of the purified IBs, which presented a specific activity

similar to peaks 2, 3 and 4 (**Figure 1.7Error! No s'ha trobat l'origen de la referència.**). However, it is important to note that amount of IBs used in the assay is higher (100 μg) than that of the soluble version (1 μg).

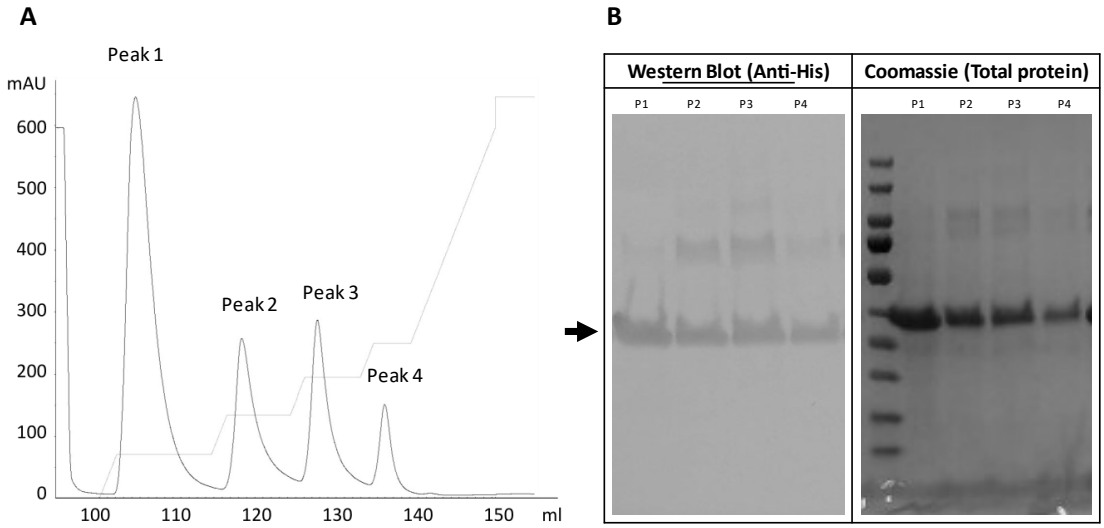

Figure 1.6. Characterization of soluble MMP-9cat after purification. A) Chromatogram of soluble MMP-9cat with four elution peaks. **B)** Western Blot with Anti-His antibody and Coomassie staining of the purified protein (P1: peak 1; P2: peak 2; P3: peak 3; P4: peak 4). The black arrow marks MMP-9cat bands.

Table 1.2. Yield and purity of soluble MMP-9cat 4 peaks.

	Yield	Purity
Peak 1	1.92 mg/L	>99%
Peak 2	0.70 mg/L	>99%
Peak 3	0.56 mg/L	>99%
Peak 4	0.21 mg/L	>99%

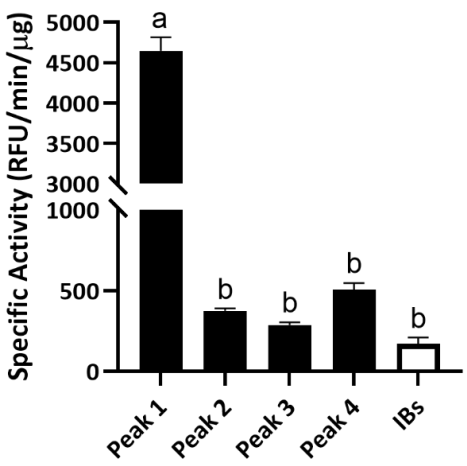

Figure 1.7. Specific activity of soluble MMP-9cat (1 µg) and MMP-9cat IBs (100 µg). The specific activity of the 4 peaks of soluble MMP-9cat (purified soluble MMP-9cat in Figure 1.4) is represented in black and the SA of IBs (MMP-9 cat IBs in Figure 1.4) is represented in white. Different letters depict significant differences between samples (p<0.001).

Discussion

Different authors have shown that *L. plantarum* can be used as recombinant expression system, obtaining products for different biological applications [17,18,39–43]. The expression of enzymes has been widely explored, obtaining high yields and good enzymatic activities of secreted α-amylases [39], β-galactosidases [18,41], chitinases [17], reductases [42] or decarboxylases [43]. Moreover, *L. plantarum* has been exploited as life vector for the surface displaying of foreign antigens, being able to induce good immune responses in chickens [44] or mice [45,46], to recruit T cells [47] or to stimulate monocytes *in vitro* [48]. Although these studies have proven that *L. plantarum* is an interesting bacterial expression system, the production and aggregation rates of PTA proteins have not been explored so far in this LPS-free bacterial cell factory. *L. plantarum* has been used on some occasions as an alternative to *E. coli*, to avoid or reduce the aggregation of proteins during the recombinant production process [49], but no studies focusing on the possible formation of IBs have been reported so far. Thus, in the present study, we used as model protein the catalytic domain of metalloproteinase 9 (MMP-9cat), which has previously been described to be a protein with a high tendency to aggregate [23]. As previously observed in *L. lactis* [23] and *Clearcoli* , when the catalytic domain of MMP-9 is produced in *L. plantarum* practically all the produced protein was found in the

aggregated fraction (**Figure 1.1** and **Figure 1.7Error! No s'ha trobat l'origen de la r eferència.Error! No s'ha trobat l'origen de la referència.**), which proved that the MMP-9cat's high tendency to aggregate is not expression system-dependent. When *L. plantarum* cells were analyzed under electron microscopy typical bacilliform cells were observed (**Figure 1.2**Error! No s'ha trobat l'origen de la referència.**A and B**) and after the i nduction of MMP-9cat expression, electron-dense structures were localized in the cell pole (**Figure 1.2**Error! No s'ha trobat l'origen de la referència.**B**). Thus, TEM micrographs c learly proved for the first time the formation of intracytoplasmic protein deposits in *L. plantarum* under overproduction conditions, as previously described in *E. coli* [50], *L. lactis* [23], and *P. pastoris* [51]. When these protein aggregates were purified, the morphology of this nanomaterial was analyzed by FESEM (**Figure 1.2**Error! No s'ha t robat l'origen de la referència.**C**). The ultrastructure of the protein aggregated formed in *L. plantarum* (**Figure 1.2**Error! No s'ha trobat l'origen de la referència.**C**) clearly r esembled that of the IBs formed in *E. coli* [25,52,53] and *L. lactis* [23,26], being smooth and round particles within the nanoscale range with a size around 250-300 nm. Although this size is slightly smaller than MMP-9cat IBs formed in *L. lactis*, it is a size in the range of the spectrum of IBs previously described in the literature [54]. Previous studies have shown that production conditions and strain can slightly impact on IB size and shape [23]. Thus, the size and shape of the IBs purified from *L. plantarum* are evidences of the consolidated aggregation process that occurs during the recombinant protein production in this GRAS expression system.

Besides, the protein yield and purity (**Figure 1.3**Error! No s'ha trobat l'origen de la r eferència.**B**) were comparable to other expression systems such as *E. coli* or *L. lactis*. Considering that all the MMP-9cat produced in *L. plantarum* aggregated, the insoluble fraction was further characterized in terms of protein activity. In previous studies, it has been widely demonstrated that IBs are active in their aggregated format [32,34,55] and capable to release the protein embedded inside them [56]. Thus, to evaluate the *L. plantarum* IBs characteristics, an initial solubilization kinetics test was done (**Figure 1.4**Error! No s'ha trobat l'origen de la referència. **(5)**), incubating IBs for 24, 48 and 72 h. The results showed that this simple solubilization process was enough to observe a significant time-dependent increase in the activity (**Figure 1.5Error! No s'ha trobat l 'origen de la referència.**), which indicated that protein bioavailability increases. That was the first evidence that the proteins embedded in *L. plantarum* IBs were active and

the first sign of protein release from these aggregates. This showed that *L. plantarum* IBs have the same properties as protein aggregates formed in other bacterial expression systems, having therefore the potential to be used as a source of soluble protein or as active nanoparticles in their aggregated form for a wide range of applications.

Taking it a step further, the solubilized protein from the IBs using a non-denaturing protocol was purified by affinity chromatography (**Figure 1.4**Error! No s'ha trobat l 'origen de la referència. **(6)**). Four different MMP-9cat peaks were eluted (**Figure 1.6**Error! No s'ha trobat l'origen de la referència.**A**) as described in previous studies where the same protein was produced in *Clearcoli* and *L. lactis* [13]. The yield and purity achieved (>99%) in the 4 eluted peaks in *L. plantarum* (**Figure 1.6A and B**) was comparable to that observed when MMP-9cat was produced in *L. lactis* [13]. However, in *Clearcoli* the purity and yields of the protein solubilized found in a previous study were lower [13]. In *L. plantarum*, the protein activity differed among the 4 peaks (**Figure 1.7Error! No s'ha trobat l'origen de la referència.**). Peak 1 of soluble protein showed an activity higher than that observed with peaks 2, 3 or 4, which showed similar levels among them (**Figure 1.7Error! No s'ha trobat l'origen de la referència.**). The same p rofile was observed in a previous study in *L. lactis*, where peak 1 showed the highest activity values [13]. In *Clearcoli*, however, peak 2 was the most active one and peaks 1, 3 and 4 showed just residual activity [13]. In terms of activity values, peak 1 of *L. lactis* [13] showed an activity approximately 2-fold higher than the presented by peak 1 in *L. plantarum* in this study (**Figure 1.7Error! No s'ha trobat l'origen de la referència.**). In t he case of *Clearcoli*, the activity values of peak 2, which was the most active one, [13] was significantly lower than those shown by *L. plantarum* and *L. lactis* [13]. Differences of activity observed in the different elution peaks could be attributed to different conformations of the protein eluted in each specific peak, indicating that proteins with different conformations are coexisting in the same protein aggregate.

We could also observe that the activity of MMP-9cat IBs produced in *L. plantarum* was much lower than that of the soluble version (**Figure 1.7Error! No s'ha trobat l'origen d e la referència.**). The amount of IBs analyzed in **Figure 1.7** were 100 µg while only 1 µg of the soluble protein was used, which meant that an amount 100-time higher of IBs is needed to reach the activity value of 1 µg of soluble MMP-9cat (**Figure 1.7Error! No s'ha t robat l'origen de la referència.**). This pattern is comparable to that seen in *E. coli* and *L.*

lactis with other proteins [13,57] showing that the protein embedded in IBs has different conformations and activities. Besides, it has already been described that although IBs have lower activity than their soluble counterpart, they have a huge potential when administered *in vivo* due to their stability and their capacity to slowly release the protein of interest [58].

Altogether we proved that *L. plantarum* IBs possess the same properties as protein aggregates formed in other bacterial expression systems, having the potential to be used as a source of soluble protein or as active nanoparticles in their aggregated form for a wide range of applications in biotechnology and biomedicine. The use of IBs as a source of soluble protein is an extremely useful approach when the protein of interest is not produced in a soluble form. These proteins can be easily purified in an active and highly pure form through a simple solubilization process, which has proven to be effective also for *E. coli* [34] and *L. lactis* [5] IBs. Therefore, *L. plantarum* arises as a new endotoxin-free environment system to isolate IBs to obtain soluble and functional proteins in any interested industry, which so far only uses *E. coli* IBs as the starting material to produce an important percentage of the commercialized recombinant proteins [59].

Conclusions

These results demonstrated the aggregation process in *L. plantarum* in general. The IBs produced by this expression system are another example that protein aggregation is not a specific phenomenon occurring only in *E. coli* and *L. lactis.* Moreover, the enzymatic activity of *L. plantarum* IBs has been demonstrated and, in consequence, this broadens the catalogue of possibilities to produce proteins of industrial interest using IBs as a source of soluble protein. Thus, *L. plantarum* gains relevance as a promising LPS-free recombinant protein expression alternative to *E. coli*.

Methods

Strains and media

The bacterial strains used in this study were *Lactococcus lactis subsp*. *cremoris* NZ9000 [60], *Escherichia coli* JM110 (Stratagene, Ref: 200239-11) and *Lactiplantibacillus plantarum* NC8 (CCUG 61730, Culture Collection University of Gothenburg, Sweden) [61]. *L. lactis* was grown in M17 media (Merck, Ref: 1151080500) supplemented with 0.5 % glucose at 30°C without shaking. *E. coli* was grown in Luria-Bertrani media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37°C with continuous shaking at 250 rpm. *L. plantarum* was grown at 30°C without shaking in MRS media without glucose (Conda, Ref: 1295) supplemented with 2 % glucose when necessary.

Plasmids and Cloning

The part of the gene codifying for the catalytic domain of bovine metalloproteinase MMP-9cat (*mmp9* gene fragment; Phe107-Pro449 NM_174744)) was obtained from *L. lactis*/pNZ8148:MMP-9cat (Cm^R, chloramphenicol resistant) previously developed by our group [5]. Plasmid pSIP409 (Erm^R, erythromycin resistant), kindly provided by Dr. Lars Axelsson [14], was used to clone *mmp9* gene fragment by replacing β-glucuronidase gene (*gus*), flanked by *NcoI* and *XbaI* restriction sites. Briefly, pSIP409 plasmid was transformed into *E. coli* JM110. For the isolation of both plasmids pNZ8148:MMP-9cat and pSIP409 the Spin Miniprep Kit (Qiagen, Ref: 27104) was used. For the purification of pNZ8148:MMP-9cat from *L. lactis* [5] an extra lysis step adding 10 mg/mL of lysozyme in the lysis buffer P2 for 1 h at 37°C was added being necessary for Gram-positive bacteria. Both plasmids (pNZ8148:MMP-9cat and pSIP409) were digested by FastDigest *NcoI* and

XbaI restriction enzymes (ThermoScientific, Ref: FD0574 and FD0684) for 1 h at 37°C. The *mmp9* gene fragment obtained after this digestion was ligated into digested pSIP409 (pSIP:MMP-9cat) by T4 DNA ligase (ThermoFisher, Ref: EL0011) for 1 h at 22°C. For this, 500 ng of vector and a 3:1 insert:vector ratio were maintained within a final volume of 20 µL. For its transformation in *L. plantarum*, ligation product was purified to eliminate ligation buffers and T4 enzyme using the Spin Miniprep columns (Qiagen), starting the procedure after the lysis step.

For *L. plantarum* electrocompetent cell preparation, cells were grown in MRS broth without glucose with 1 % glycine at 30°C overnight (O/N) in anaerobic conditions. The O/N culture was inoculated in fresh MRS media with 2 % glucose and 1 % glycine at an optical density (OD_{600nm}) of 0.25. The culture was incubated anaerobically at 30°C until the OD_{600nm} reached 0.6. The culture was harvested and chilled on ice for 20 min before starting washes. Then, the culture was centrifuged at 4,000 x *g* for 15 min at 4°C followed by two washes with an equal volume to that harvested, first with ice-cold 1mM $MgCl_2$ and second with ice-cold 30 % polyethylene glycol 1500 (PEG-1500). Finally, competent cells were resuspended in 1/100 initial volume of PEG-1500.

The transformation of *L. plantarum* was performed following the protocol described by Aukrust and Blom [61]. Briefly, 0.24 µg of the ligation product were added in 40 µL of electrocompetent cells and it was electroporated using a Bio-Rad GenePulser under the following electrical conditions: 1,500 V, 400 Ω, and 25 µF in pre-cooled 2 mm electroporation cuvettes. After the pulse, cells were restored in 1 mL of MRS with 2 % glucose for 2 h at 30°C [61] and plated in MRS 2 % glucose agar with 10 µg/mL Erm for selection of transformants.

MMP-9cat production in *Lactiplantibacillus plantarum*

L. plantarum hosting pSIP:MMP-9cat was cultured O/N under the conditions previously described. The O/N culture was inoculated in 200 mL shake flasks at OD_{600nm}=0.1. MMP-9cat production was induced by adding 50 ng/mL of sakacin P inducing peptide (SppIP) (GenScript, Sequence: MAGNSSNFIHKIKQIFTHR) [14] when the cultures reached OD_{600nm}=0.3. After the induction, cultures were grown for 5 h and 25 mL samples were taken at 0, 1, 3, and 5 h post-induction. Erm was used for plasmid maintenance at 10 µg/mL.

Samples were harvested by centrifugation at 6,000 *x g*, 4°C for 15 min. Pellets were suspended in 0.5 mL PBS 1X, jacketed in ice and disrupted by sonication with Branson Digital Sonifier SFX 550 for 3 rounds (0.5 s ON/0.5 s OFF, total time 1.5 min, 10 % A). After, soluble and insoluble fractions were separated by centrifugation at 15,000 *x g*, 4°C for 15 min. Insoluble pellets were suspended in 0.5 mL PBS 1X when needed for protein determination. The experiment was done by triplicate.

Protein solubility determination

The soluble and insoluble protein fractions were analyzed by Western blot. A 15 % denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was prepared. All samples were resuspended in PBS with Laemmli loading buffer (100 mM Tris base, 8 % glycerol, 55 mM SDS, 4 % β-mercaptoethanol, 1.6 M urea). Soluble and insoluble (inclusion bodies) fractions were boiled for 10 and 40 min before electrophoresis, respectively. Protein bands were electroblotted into polyvinylidene difluoride (PVDF) membranes at 2.5 A and 25 V for 10 min, followed by a blocking step with BSA O/N at 4°C (5 % BSA in TBST buffer: 10 mM Tris, 150 mM NaCl, 0.05 % Tween 20). Anti poly-histidine (Santa Cruz Biotechnology; mouse) was used as the primary antibody at a 1/1,000 dilution in BSA-TBST buffer, in which membranes were incubated for 2 h at RT, followed by 3 washes in TBST buffer. Then, membranes were incubated in a 1/20,000 dilution in TBST of an anti-mouse IgG-alkaline phosphatase (Sigma), used as secondary antibody, along 1 h at RT followed by 3 washes in TBST buffer. Protein bands were developed after adding the alkaline phosphatase substrate solution NBT/BCIP (Thermo Scientific). A protein marker, PageRuler™ Prestained Protein Ladder, has been loaded in all the gels (ref. 26616, ThermoFisher Scientific). Bands were quantified with a standard curve of purified soluble catalytic domain of MMP-9cat previously produced in *L. lactis* [5] Densitometry analyses were performed with ImageJ software.

MMP-9cat Inclusion Bodies production and purification in *Lactiplantibacillus plantarum*

Cultures of 1 L (1 L of media in 1 L bottles) of *L. plantarum*/pSIP:MMP-9cat were grown at 30°C without agitation and induced with 50 ng/mL SppIP at an OD_{600nm}=0.3. The whole volume was recovered after 3 h of production and centrifuged at 6,000 *x g* for 15 min at 4°C. Pellets of 500 mL of culture were suspended in 300 mL of PBS 1X with EDTA-free protease inhibitor cocktail (Roche). Bacteria were mechanically disrupted by a cell

disruptor (Constant Systems Ltd) with 2 cycles at 40 kpsi. Then, a freeze/thaw cycle was performed O/N at -80°C. After, lysozyme (50 mg/mL) at 0.01 mg/mL and mutanolysin (1,000 U/mL) at 1 U/mL were added and samples were incubated for 2 h at 37°C and 250 rpm. Just after that, samples were frozen at -80°C O/N and thawed the next day. Then, samples were washed with 4 µL/mL Triton X-100 for 1 h at RT and 100 µL of sample were plated onto MRS without glucose agar plates supplemented with 2 % glucose. Plates were incubated O/N at 30°C to evaluate the presence of viable cells. If bacterial colonies were observed, extra freeze/thaw cycles were performed until complete elimination of viable bacteria. Once no colonies were observed, the samples were washed in 0.25 µL/mL Nonidet P40 for 1 h at 4°C. After, they were then treated with 6 µL/mL of 0.1 M $MgSO_4$ and 6 µL/mL of 1mg/mL DNase and incubated at 37°C and 250 rpm for 1 h. Finally, samples were centrifuged at 6,000 *x g* for 30 min. Each pellet, containing IBs, of 50 mL of culture was resuspended in 5 mL of lysis buffer (Tris 50 mM, NaCl 100 mM, 1 mM EDTA) with added 0.05 % Triton X-100. Once suspended, samples were frozen O/N at -80°C and thawed the next day. After thawing, samples were centrifuged at 6,000 *x g* for 30 min and suspended in 5 mL of PBS. Finally, pure IBs were obtained after a centrifugation step at 15,000 *x g* for 15 min discarding the supernatant. IBs were quantified by Western blot using the protocol described in "Protein solubility determination".

Electron Microscopy

Ultrastructural characterization of intracellular and isolated IBs was performed with two high-resolution electron microscopy techniques. Imaging of intracellular IBs was performed with standard Transmission Electron Microscopy (TEM) procedures adapted to this type of sample [23,58,62,63]. Briefly, pellets of bacilli with and without IBs were fixed with 2.5 % glutaraldehyde in 0.1M phosphate buffer (PB) at pH 7,2, postfixed in 1 % osmium tetroxide containing 0.8 % potassium ferrocyanide in PB, dehydrated in acetone, embedded in Spurr resin and polymerized at 60°C during 48 h. Ultrathin sections (70 nm) obtained with an ultramicrotome UCT7 (Leica Microsystems) were placed in Cu grids (200 mesh) and contrasted following routine protocol of uranyl acetate and lead citrate solutions. Samples were observed in a TEM JEM 1400 (Jeol) equipped with an Erlangshen CCD camera (Gatan) and operating at 80kV.

Ultrastructural morphometry (size and shape) of nanoparticles was performed and characterized at nearly native state with field emission scanning electron microscopy

(FESEM). Drops of 20 µL of IBs sample were directly deposited on silicon wafers (Ted Pella Inc.) for 30 s and immediately observed without coating with a FESEM Merlin (Zeiss) operating at 1 kV and equipped with a high-resolution secondary electron detector.

Solubilization of MMP-9cat from IBs

MMP-9cat IBs were resuspended in Tris glycerol buffer (20 mM Tris HCl pH=8 and 5 % glycerol) at a concentration of 2 mg/mL. Aliquots of 50 µL containing 100 µg of MMP-9cat IBs were solubilized at RT for 0 h, 24 h, 48 h and 72 h. After solubilization, the mixture was recovered to determine the activity kinetics and the specific activity (SA) of the partially solubilized MMP-9cat IBs, containing a mixture of non-solubilized IBs and solubilized protein.

Production and purification of the solubilized MMP-9cat

A culture of *L. plantarum*/pSIP:MMP-9cat was induced for 3 h. The whole volume was centrifuged at 6,000 *x g* for 15 min and 4°C, and the pellets were frozen at -80°C. Pellets of 500 mL of culture were resuspended in 30 mL of PBS with EDTA-free protease inhibitor cocktail (Roche). Samples were subjected to 4 rounds of French Press disruption at 1,500 psi, intercalated by a minimum of 5 min repose in ice. After that, 0.05 mg/mL lysozyme (50 mg/mL) was added and samples were incubated for 2 h at 250 rpm and 37°C. The mixture was centrifuged at 15,000 *x g* for 45 min and 4°C, the supernatant was discarded and the pellet was suspended in the same volume of H_2O and centrifuged again at 15,000 *x g* for 15 min and 4°C. Pellets were weighted and solubilized in 0.2 % N-lauroyl sarcosine and 40 mM Tris solution at a ratio 1:40 (g:mL) for 48 h at RT under agitation. The supernatant was recovered through centrifugation at 15,000 x *g* for 45 min at 4°C.

NaCl and imidazole were added to the supernatant to equilibrate the samples with the binding buffer composition (20 mM Tris pH=8, 500 mM NaCl, 20 mM Imidazole). After that MMP-9cat was purified by Immobilized Metal Affinity Chromatography (IMAC) in an ÄKTA purifier FPLC (GE Healthcare) using 1 mL HiTrap Chelating HP columns (GE Healthcare). The selected fractions were dialyzed in Tris Glycerol buffer (20 mM Tris HCl pH=8, 5 % glycerol) O/N at 4°C and with gentle agitation. The amount of purified protein was determined by UV absorbance measured with Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific). The integrity of the proteins was analyzed by SDS-PAGE.

MMP-9cat purity determination

The purity of the soluble MMP-9cat purified by IMAC and the purified MMP-9cat IBs was determined by SDS-PAGE following a Coomassie staining (Bio-Rad Laboratories) of the gel. After the electrophoresis, the gel was stained for 1 h with gentle agitation. Then, the excess stain was removed with distaining solution (30 % v/v methanol and 7 % v/v acetic acid in distilled water). Densitometry analyses by ImageJ software were performed for purity calculation. Coomassie-stained bands were compared with bands obtained by WB using the protocol described in "Protein solubility determination". Coinciding bands in two techniques were considered as MMP-9cat and the rest of the bands stained were considered impurities. The purity percentage was calculated by dividing the densitometry outcomes of the bands of interest by the densitometry outcomes of all bands.

Activity of the solubilized MMP-9cat, MMP-9cat IBs and partially solubilized MMP-9cat IBs

The enzymatic activity of MMP-9 catalytic domain was determined by DQ gelatin™ assay (Invitrogen). Solubilized MMP-9cat samples and MMP-9 IBs samples were diluted in Tris Glycerol buffer (20 mM Tris HCl pH=8, 5 % glycerol) at specific concentrations depending on the samples. Soluble MMP-9cat samples were diluted at a concentration of 0.02 mg/mL and MMP-9cat IBs samples were diluted at a concentration of 2 mg/mL. Partially solubilized MMP-9cat IBs were totally recovered after solubilization times of 0, 24, 48 and 72 h. The concentration of MMP-9cat IBs before solubilization was 2 mg/mL. After samples preparation, 100 µL of Assay buffer (50 mM Tris HCl pH=7.4, 150 mM NaCl, 5 mM $CaCl_2$, 0.01 % Tween 20) were loaded in an opaque 96-well plate with transparent bottom. Then 50 µL containing either 1 µg of solubilized MMP-9cat or 100 µg of MMP-9cat IBs or partially solubilized MMP-9cat IBs were plated by triplicate. Finally, 50 µL of 0,005 mg/mL dye-quenched gelatin solution were added to each well. The degradation of the gelatin (emission of fluorescence) was measured in a Victor3™ Plate Reader (PerkinElmer's) at 495/515 nm (excitation/emission wavelengths) every 30 sec for 2 h for SA calculation, or every 3 min for 16 h for activity kinetics measurement. The SA of soluble MMP-9cat, MMP-9cat IBs and partially solubilized IBs was extracted from the kinetic data, by obtaining the initial velocity (relative fluorescence units per minute,

rfu/min) for each μg of MMP-9cat in the wells (rfu/min/μg). A model using R was done to adjust data.

Statistics

SA experiments were analyzed by ANOVA and Tukey's multiple comparisons test (GraphPad) with different letters depicting significant differences between samples. All experiments were performed in triplicate and represented as the mean of non-transformed data ± non-transformed standard error of the mean (SEM).

Acknowledgements

The authors are indebted to CERCA Programme (Generalitat de Catalunya) and the European Social Fund for supporting our research.

Author contributions

RB-F performed all the experiments, analyses and contributed in writing the original draft. CS-A, AL and LG-R contributed to the cloning, production, and purification processes, respectively. AS-C contributed with the electron microscopy analysis. AA and EG-F performed the conceptualization, supervision and writing—review and editing. All authors read and approved the final manuscript.

Funding

This work was funded by Ministerio de Ciencia, Innovación y Universidades Grants PID2019-107298RB-C21/AEI/10.13039/ 501100011033) to AA and EG-F. RB-F received a pre-doctoral fellowship from INIA (FPI-INIA, MINECO). The authors are also indebted to AGAUR for project 2021 SGR 01552.

Availability of data and materials

All data analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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STUDY 2

Time to rule out inclusion bodies denaturing protocols for spontaneous solubilization practices

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Preface

Either in standard expression systems, such as *E. coli*, or in non-conventional ones, such as *L. lactis* and *L. plantarum* (Study 1), the formation of IBs is a frequent phenomenon occurring under recombinant protein production conditions. Depending on the heterologous protein expressed, the percentage of aggregation may change, reaching values that can be close to 100 % in highly prone-to-aggregate (PTA) proteins like MMP-9. For this reason, different protocols have been developed for the recovery of soluble proteins from IBs. Among them, solubilization strategies including chaotropic agents like urea or guanidinium chloride at high concentrations are the most well-established and widely used to solubilize IBs followed by an inefficient refolding process to reach the protein native conformation. Alternatively, and considering that IBs have been proven to be biologically active and structurally organized protein aggregates, new and adapted protocols have been developed to solubilize the protein without the need of using complex denaturing-refolding procedures. One example is the use of low concentrations of mild detergents. However, previous studies of our group have proven that the presence of detergent traces after the solubilization process may impair the activity of the solubilized protein. Thus, this second study pursues the standardization of an appropriate screening assay to determine the optimal solubilization conditions for each protein without using solubilization products taking advantage of the IB ability to spontaneously release protein under certain conditions. The study demonstrates that it is possible to determine each protein's specific solubilization conditions (time and temperature) by performing an activity assay. Herein we use IBs of 3 innate immunity-related proteins as a model to demonstrate that after establishing the proper time and temperature conditions by monitoring activity it is possible to spontaneously release

soluble protein from any IBs in a standard buffer without adding any detergent or denaturing agent.

Time to rule out inclusion bodies denaturing protocols for spontaneous solubilization practices

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Abstract

The formation of inclusion bodies (IBs) in microbial cell factories is a very common process occurring during recombinant protein production. Different protocols have been developed for the extraction of soluble proteins from IBs using several strategies ranging from the use of harsh denaturing and high concentrations of chaotropic agents and reducing agents to the use of mild protocols based on the use of non-denaturing detergents. However, in the last years, the biological vision of IBs has changed and research studies have demonstrated that these protein aggregates contain biologically active and properly folded recombinant protein. This drives us to redefine the methodologies used so far to obtain soluble protein using IBs as a protein source. Hence, we propose the extraction of IBs protein by simple spontaneous solubilization of IBs as a widely applicable strategy to all kinds of recombinant proteins without the negative effects of detergents and chaotropic agents. The solubilization can be easily customized for each protein in terms of timing and incubation temperature by monitoring the protein activity of solubilized fraction with an activity assay that accounts for greater sensitivity than Western blot or standard protein detection protocols.

KEY WORDS: Inclusion Bodies, Solubilization, Activity Assay, Recombinant Production

Introduction

Recombinant proteins are becoming very important products with industrial, biomedical and agriculture applications (e.g., enzymes, antibodies, antimicrobials, growth factors, hormones, and vaccines). Microorganisms are the most used hosts for recombinant production due to the low cost associated with their fast growth and easily scalable procedures [1]. Nowadays it has been broadly accepted that during recombinant protein processes the formation of inclusion bodies (IBs) in microbial cell factories is a very common process, not only in the most used bacteria such as *Escherichia coli* but also in lipopolysaccharide (LPS)-free recombinant systems, such as *Lactococcus lactis* [2] and *Lactiplantibacillus plantarum* [3] or even in yeast such as *Pichia pastoris* [4]. IBs are protein-based aggregates naturally formed due to the cell stress associated with the overproduction of heterologous proteins. To avoid, or at least minimize, IB formation and increase the amount of soluble protein fraction, different approaches have been proposed. These strategies include the optimization of the expression conditions, the use of solubility-enhancing tags, 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 [5]. However, in many cases, this is not enough to reach the desired soluble protein production yield. Although IBs have long been considered to be residual by-products of such processes, during the last decade, it has been extensively demonstrated that they are protein-based nanoparticles with a huge potential in the biotechnological and biomedical context [6,7]. In fact, they can be directly used in a wide variety of applications such as anti-tumorals [8], biocatalysts of enzymatic reactions [9,10], immunostimulants [11] or antimicrobials [12]. However, IBs can contain impurities from producer cells such as nucleic acids, lipids, carbohydrates, and other proteins. Hence, this is the main reason why they are mainly used as a source of soluble protein, which is a purer protein format and a completely defined biological in terms of composition. It has been proven that proteins with difficult purification patterns can be obtained purer when extracted from IBs because these aggregates suppose a pre-concentrated sample of recombinant protein [13]. In this context, 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 [14]. After these treatments, the protein released from these aggregates undergoes a complete denaturation and requires a refolding protocol to recover its bioactive native conformation. However, in the last years, the biological vision of IBs has changed and research studies have demonstrated that these protein aggregates contain biologically active and properly folded recombinant protein [15]. This droved different groups to redefine the methodologies used to obtain soluble protein using IBs as a protein source. In this context, the use of a high concentration of chaotropic agents has been substituted by non-denaturing protocols that avoid complete denaturation and the need to apply refolding protocols. This has been achieved using mild detergents like n-lauroylsarcosine (NLS) or lauroyl-L-glutamate which enables the release of correctly folded protein from IBs [13,16–18]. As an alternative, low concentrations of organic solvents have also been demonstrated to be suitable as IB solubilizing agents [19]. Finally, combining low amounts of denaturing reagents with the adjustment of either physical parameters, like heat [20], high hydrostatic pressure [21], and freeze-thaw cycles [22] or chemical factors [23] have been used to accomplish solubilization of IBs in a non-denaturing manner.

Despite recently milder solubilization methods have been described, there are also some studies that demonstrate that non-denaturing detergents can impact the final protein quality and activity [24]. Although some proteins solubilized with NLS from IBs have been shown to retain their biological activity others are affected using detergents [17]. The main reason seems detergent traces difficult to be completely removed, which could impair the final biological activity of proteins recovered from IBs. One example are recombinant antimicrobial proteins whose activity is significantly reduced by residual traces of NLS. In these cases, our group has proven that IBs proteins can be spontaneously released in a detergent-free buffer while recovering the full activity of soluble proteins [17].

The present study makes a step forward in finding universal strategies for releasing IB proteins and ruling out the detergent containing IBs solubilization protocols. In summary, we describe a screening method to select the optimal conditions for the spontaneous solubilization of different types of recombinant proteins to be recovered from IBs by testing a broad range of solubilizing conditions and correlating them with the final specific activity.

Materials and Methods

Bacterial cultures and media

Lactococcus lactis sub. cremoris NZ9000 [25] was used for the expression of M-SAA3 and BMAP-27. *E. coli* BL21 (DE3) was used for the expression of JAMF-2. *L. lactis* strains were grown at 30°C in M17 media (Merck) supplemented with 0.5 % glucose and with 5 µg/ml Cm for plasmid maintenance. *E. coli* strain was grown in Luria-Bertrani (LB) medium with 100 µg/ml ampicillin for plasmid maintenance.

The strain used for eukaryotic cell culture infection was *Staphylococcus aureus*. The strains used for antimicrobial activity assays were carbapenem-resistant *Klebsiella pneumoniae* (KPC) and methicillin-resistant *Staphylococcus aureus* (MRSA). The three pathogenic strains were grown in Brain-Heart Infusion (BHI) broth (Scharlau).

Genetic construct design

The sequence of the goat mammary serum amyloid A3 (M-SAA3) was previously designed [26], cloned in the pNZ8148 plasmid (MoBiTech) and transformed in *L. lactis sub. cremoris* NZ9000 (NZ9000/pNZ8148:M-SAA3) [13]. The sequence of JAMF-2 was previously designed, cloned and transformed in *E. coli* BL21 (DE3) (*E. coli* BL21 (DE3)/pET22b:JAMF-2) [27]. The sequence of bovine myeloid antibacterial peptide 27 (BMAP-27) was based on the sequence UniProtKB - P54228 (CTHL6_BOVIN) fused to the Green Fluorescent Protein (GFP) gene through the linker sequence GGSSRSS. BMAP27-GFP sequence was cloned in the pNZ8148 plasmid (MoBiTech) (Cm^R) and transformed in *L. lactis sub. cremoris* NZ9000 (NZ9000/pNZ8148:BMAP27GFP) as previously described in [2]. All proteins had a 6 histidine (H6)-tag at C-terminal for protein purification purposes.

Eukaryotic cells cultures and media

Cell cultures used in this study were primary bovine mammary gland cultures and human mammary gland cell-line MCF-12A (ATCC® CRL-10782™). All cultures grew in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12) enriched with insulin (8 µg/ml), hydrocortisone (50 µg/ml), penicillin-streptomycin (100 U/ml), Fungizone™ (2.5 µg/ml), gentamycin (50 µg/ml) and 10 % fetal bovine serum (FBS). In the case of MCF-12A cell growth, epidermal growth factor (EGF) was added at 20 ng/ml. All cultures grew at 37°C and 5% CO_2.

M-SAA3, BMAP-27 and JAMF-2 IBs production and purification

For the production of both M-SAA3 and BMAP-27GFP IBs, cultures of 1 L of *L. lactis* NZ9000/pNZ8148:M-SAA3 or *L. lactis* NZ9000/pNZ8148:BMAP27GFP in 1 L bottles were grown at 30°C without agitation and induced with 12.5 ng/mL nisin at an OD_{600nm}=0.4-0.6. For the production of JAMF-2 IBs, cultures of 500 mL of *E. coli* BL21 (DE3)/pET22b:JAMF-2 in 2 L Erlenmeyer flasks were grown at 37°C and 250 rpm and induced with 1 mM isopropyl-β-d-thiogalactoside (IPTG) at an OD_{600nm}=0.4-0.6. The whole volume of the three cultures was recovered after 3 h of production and centrifuged at 6,000 *x g* for 15 min at 4°C. Pellets of 500 mL of culture were suspended in 300 mL of PBS with protease inhibitors (cOmplete™ EDTA-free, Roche). Bacteria were mechanically disrupted by a cellular disruptor (CF1 Model, Constant Systems Ltd) with 2 cycles at 40 kpsi for *L. lactis* and one cycle at 27 kpsi for *E. coli*. Then, a freeze/thaw cycle was performed O/N at -80°C to disrupt the partially digested bacteria and release the IBs protein content. After bacteria disruption, an extensive sample washing was performed in the presence of mild detergents. Firstly, lysozyme was added at 0.01 mg/mL and the mixture was incubated for 2 h 37°C at 250 rpm. Then samples were frozen at -80°C O/N and thawed the next day. IBs were then washed in 4 µL/mL of sample Triton X-100 for 1 h at RT. After that, 100 µL of the sample were plated in M17 agar plates supplemented with 0.5 % glucose and incubated O/N at 30°C for *L. lactis* or in LB plates and incubated O/N at 37°C for *E. coli* to evaluate the presence of viable cells. If bacterial colonies were observed, extra freeze/thaw cycles were performed to eliminate those bacteria. Once no colonies were observed, the IBs were washed in 0.25 µL/mL Nonidet P40 for 1 h at 4°C. The sample was then treated with $MgSO_4$ at 6 µL/mL from a stock of 0.1 M and with DNAse at 0.6 µL/mL from a stock of 1 mg/mL at 37°C and 250 rpm for 1 h. IBs were centrifuged at 6,000 *x g* for 30 min. Each pellet of 50 mL of culture was suspended in 5 mL of lysis buffer (Tris 50 mM, NaCl 100 mM, 1 mM EDTA) + 0.05 % Triton X-100. Once suspended, samples were frozen O/N at -80°C and thawed the next day. After thawing, the sample was centrifuged at 6,000 *x g* for 30 min and suspended in 5 mL of PBS. Finally, pure IBs were centrifuged at 15,000 *x g* for 15 min discarding the supernatant. The integrity and the amount of protein in the IBs were determined by SDS-PAGE, with a posterior densitometry analysis by ImageJ software.

Protein solubilization from IBs

JAMF-2 and BMAP-27GFP solubilization from IBs

Purified JAMF-2 and BMAP-27GFP IBs were suspended in 10 mM KPi buffer (K/PO_4 buffer pH 7.4) or acetic acid 0.01 % (v/v) respectively at a final concentration of 1 mg/mL. Then, different samples were incubated at 37°C, RT or 4°C for 16 h, 24 h or 48 h. After the incubation, the samples were centrifuged at 15,000 *x g* for 15 min and 4°C. The supernatants were recovered and frozen at -80°C until their use.

M-SAA3 solubilization from IBs

Purified M-SAA3 IBs were suspended in PBS at a final concentration of 150 µg/mL. Then, different samples of the suspension were incubated at 37°C 16 h, 24 h or 48 h. At the end of incubation, they were centrifuged at 15,000 *x g* for 15 min and 4°C. The supernatants were recovered and frozen at -80°C until their use.

Bactericidal assay against pathogenic bacterial strains

A bactericidal assay based on the BacTiter-Glo™ Microbial Cell Viability assay (Promega) was performed. Firstly, an O/N culture of the selected strain, KPC for JAMF-2 or MRSA for BMAP-27GFP, was diluted (10^6 CFU/ml) in 10 mM KPi buffer. Then, 150 µL from the bacterial diluted stock were centrifuged at 6,200 x *g* for 15 min and 4°C. The supernatant was removed and the bacterial pellet was suspended with 150 µL of either 10 mM KPi buffer (negative control) or the SN resulting from IBs solubilization for JAMF-2, or either acetic acid 0.01 % (v/v) (negative control) or the SN resulting from IBs solubilization for BMAP-27GFP. All samples were transferred to a sterile polypropylene 96-well plate (Costar) and were incubated for 5 h at 37°C. After, 100 µL were withdrawn and tested with 100 µL of the BacTiter-Glo™ reagent following manufacturer instructions.

Secretory Phospholipase A2 ($sPLA_2$) enzymatic activity assay

An enzymatic activity assay was performed based on $sPLA_2$ Assay Kit (Cayman) to quantify the enzymatic activity of the $sPLA_2$ domain of the solubilized JAMF-2. The enzymatic activity of the SN obtained after the solubilization of JAMF-2 IBs was analyzed for 20 min following manufacturer instructions.

Fluorescence determination

The fluorescence of the GFP of BMAP-27GFP solubilized from IBs was determined. 100 µL of every SN obtained after IBs solubilization were transferred to a black and opaque 96-well plate (Thermo Scientific). The fluorescence was measured by a LUMIstar Omega plate reader (BMG-LabTech).

Induction of cytokines expression in mammary gland primary cultures

Bovine mammary gland primary cultures were obtained as previously described [28] and expanded before the experiment. Once the culture was at 80 % of confluence, 44,000 cells/well were cultured in 24 wells plates with 500 µL of complete DMEM/F-12. The cells grew O/N at 37°C and 5 % CO_2. The day after, solubilized M-SAA3 was added to mammary gland primary cultures in a final volume of 1 mL (500 µL M-SAA3 + 500 µL medium), for an O/N incubation. At the end of the experiment, cells were washed twice with 500 µL of PBS and 500 µL of Trizol reagent (Thermo Fisher Scientific) were added to each well to collect and lysate the cells. The extraction of RNA was performed using the Trizol reagent (Thermo Fisher Scientific) and it was processed for qPCR analyses of IL-6, IL-8, IL-10 and IFN-γ expression. Relative gene expression was calculated using the $2^{\Delta Ct}$ method with RpS9 as reference gene.

***Staphylococcus aureus* infection inhibition assay in mammary gland cultures**

The mammary cells MCF-12A were grown at 80 % of confluence, 44,000 cells/well were cultured in 24 wells plates with 500 µL of complete DMEM/F-12. The cells grew O/N at 37°C and 5% CO_2. The day after, solubilized M-SAA3 was added to mammary gland cultures in a final volume of 1 mL (500 µL M-SAA3 + 500 µL medium) for an O/N incubation. After the incubation, the medium was discarded. The cells were washed twice with 500 µL of PBS. Then, the cells were infected with 500 µL of infection medium (*S. aureus* suspended in DMEM/F12 without additives) at 2 x 10^7 CFU/mL for 2 h at 37°C and 5 % CO_2. The cells were washed twice with 500 µL of PBS and incubated for 2.5 h at 37°C and 5 % CO_2 with 500 µL of gentamycin at 100 µg/mL. The cells were washed twice with 500 µL of PBS 1X, trypsinized with 150 µL of Trypsin-EDTA 10 min, neutralized with 300 µL of DMEM/F-12 with 10 % FBS and recovered. Then, the samples were centrifuged for 6.5 min at 200 *x g* and suspended in 500 µL of Triton 0.1 % for 10 min at 37°C. Finally,

each sample was diluted 1/100 in NaCl 0.9 %, plated in BHI-agar plates and incubated O/N at 37°C for viable cell counting.

Statistical analysis

All data were analyzed using an ANOVA that accounted for the effect of solubilization time (16 h, 24 h and 48 h) in comparison with the controls. Differences between the solubilization temperatures (37°C, RT and 4°C) were established using Tukey's multiple mean separation test. Results are expressed as the means of non-transformed data ± standard error of the mean (SEM).

Results and Discussion

The objective of this study was to prove that spontaneous solubilization protocols in standard buffer without the use of any detergent can be used as a universal protocol to easily recover pure and active soluble protein from IBs and to establish the optimal solubilization conditions for each protein of interest. For that, we screened different solubilization conditions considering temperature and timing as variables to be evaluated for each recombinant protein using an appropriate activity assay.

We used three model proteins named JAMF-2, BMAP-27GFP and M-SAA3. Protein M-SAA3 and BMAP-27GFP were produced using *L. lactis* as recombinant host whereas for JAMF-2 production *E. coli* was used. The multidomain protein JAMF-2 is an antimicrobial multidomain protein constructed by the combination of a human α-defensin-5 (HD5), a bacterial binding domain (gelsolin), and an enzymatic antimicrobial peptide ($sPLA_2$), flanked by two aggregation-seeding domains (a fragment of c-Jun and c-Fos leucine zippers at N- and C-terminal, respectively) in a single polypeptide [27]. JAMF-2 is a protein that is mainly produced as IBs (**Table 2.1**) that have previously been shown to be formed by biologically active protein [27]. The BMAP-27GFP is also an antimicrobial protein and it is based on the fusion of the bovine cathelicidin BMAP-27 and a carrier fluorescent protein (GFP). Finally, M-SAA3 is an acute phase protein from caprine mammary gland with the ability to modulate the innate immune response and prevent mammary infections [29]. Both BMAP-27GFP and M-SAA3 proteins showed moderate aggregation rates of 36% (**Table 2.1**).

Table 2.1. Description of the proteins produced, the expression system used, solubility and aggregation yield and protein yield reached using solubilization protocols using IBs as starting material.

	Expression system	Soluble protein	Insoluble protein	Yield
M-SAA3	*L. lactis*	64 %	36 %	0.34 mg/L
JAMF-2	*E. coli*	-	>99%	2.79 mg/L
BMAP-27GFP	*L. lactis*	64 %	36 %	2.76 mg/L

In agreement with what has been previously described, aggregation rates observed (**Table 2.1**) showed that this is a variable parameter that is protein dependent. Sometimes, as in the case of JAMF-2 (**Table 2.1**), the solubilization of IBs is the only viable approach to isolate the soluble protein of interest. However, in other situations in which aggregation rates are lower, and in consequence the presence of soluble protein in the bacterial cytoplasm is higher, the use of the soluble fraction can be used to purify the recombinant protein. However, high solubility rates do not guarantee the obtention of the soluble form of the protein of interest. M-SAA3 is an example of protein with a high solubility rate (Table 2.1) but with low production yields and which is copurified with other host proteins, having a final low-quality product [13]. In this case, the use of IBs as a source of recombinant protein allowed to obtain a highly pure soluble M-SAA3 through the IB solubilization using non-denaturing detergent [13].

Thus, to evaluate the optimal solubilization conditions of IBs of different proteins, a solubilization screening assay was performed using JAMF-2, BMAP-27GFP and M-SAA3 as model proteins. The experimental design of our study included a first step of IBs purification using standard protocols already defined [30]. These purified aggregates were then resuspended in the optimal buffer for each protein without adding any kind of denaturing agent or detergent (**Figure 2.1**). Herein, we used phosphate buffers (10 mM KPi buffer for JAMF-2 and PBS for M-SAA3) and acetic acid 0.01 % (v/v) for BMAP-27GFP because they were the most appropriate to perform the activity assays for the three model proteins. We first evaluated BMAP-27GFP and JAMF-2 IBs solubilization by monitoring the activity (antimicrobial assays) of protein present in recovered supernatant under different conditions (**Figure 2.2** and Figure 2.4**Figure 2.4**). The antimicrobial activity assays were also complemented with another kind of assays such

as those based on enzymatic (**Figure 2.5**) or fluorescent activity (**Figure 2.3**) for JAMF-2 and BMAP-27GFP, respectively. In the case of BMAP-27GFP the two activity assays tested, either antimicrobial assay against MRSA and fluorescence emission of GFP carrier protein, evidenced that maximum IBs solubilization was achieved at 37°C for 48 h (**Figure 2.2** and **Figure 2.3**). We did not test longer incubation times to avoid the risk of protein degradation and because the main goal of this study was to demonstrate that spontaneous solubilization is possible for all kinds of IBs and the protocol can be customized depending on each protein activity. The assay based on BMAP-27GFP fluorescence emission (**Figure 2.3**) was more sensitive, and also easier to carry out than the antimicrobial assay (**Figure 2.2**) because showed much greater differences between the activity of protein solubilized after 48 h (**Figure 2.3**) compared to 24 h or 16 h while these differences were not observed with the bactericidal activity (**Figure 2.2**). This suggested that for fusion proteins containing GFP as a carrier, the measurement of relative fluorescence should be enough to set up the best conditions for IBs solubilization. In the case of JAMF-2, the solubility efficiency was assessed by measuring the antimicrobial activity and the enzymatic activity of $sPLA_2$ domain (**Figure 2.4** and **Figure 2.5**) because it is a multidomain polypeptide that does not contain any carrier protein. The monitorization of the antimicrobial activity of the whole protein was tested against the target bacteria *K. pneumoniae* Carbapenem-resistant, while the activity of $sPLA_2$ domain was assessed by an enzymatic assay. The comparison of both assays showed that $sPLA_2$ activity was not sensitive enough (**Figure 2.5**), but bacterial survival indicated very well the release of active JAMF-2 protein from IBs (Figure 2.4**Figure 2.4**). In this case, the selected conditions were also 37°C but incubation time of 16 h was enough to obtain the maximum amount of solubilized protein (**Figure 2.4**). Herein, neither the RT nor 4°C was enough to solubilize JAMF-2 IBs, probably because the presence of jun and fos seeding domains that produced compact aggregates [27]. Depending on the recombinant protein one must evaluate the limitations of each activity assay and consider the possibility that no differences are observed because maximum threshold of the assay is reached. If this is the case, greater solubilization times could be used, also pondering if the protein is stable during more hours at 37°C. To elucidate if this was the case of JAMF-2 a Western Blot was performed but the protein could not be detected in solubilized supernatants and confirmed the need to perform activity assays to monitor the solubilizing rate (results not shown).

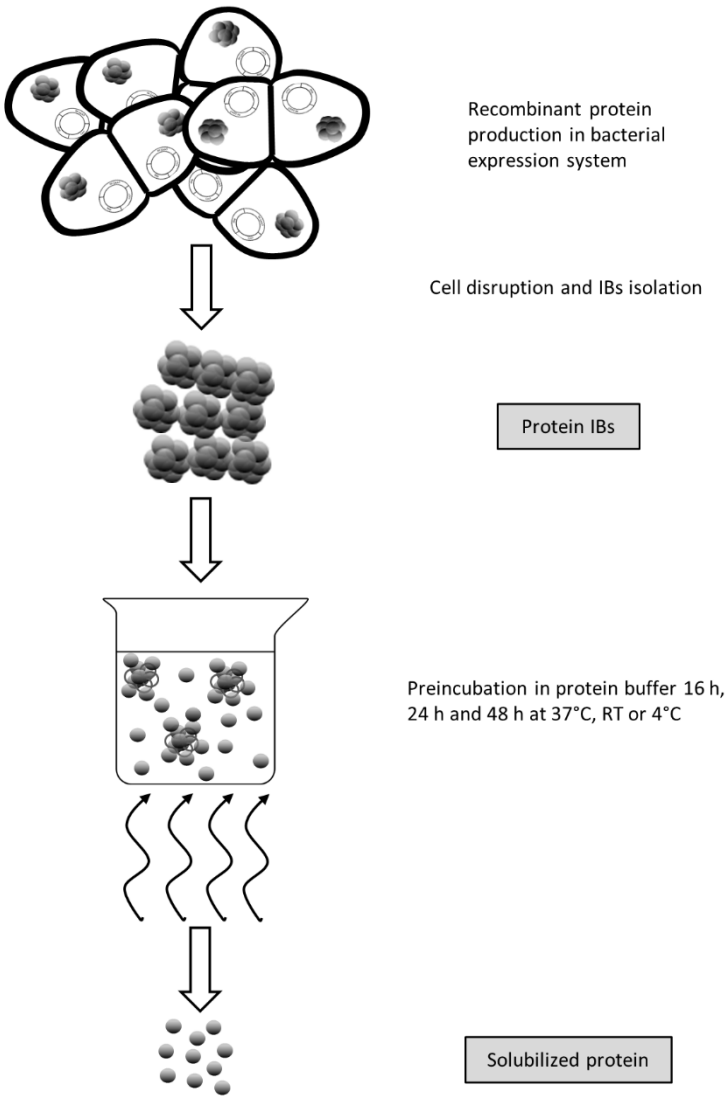

Figure 2.1. Schematic representation of the protein solubilization protocol.

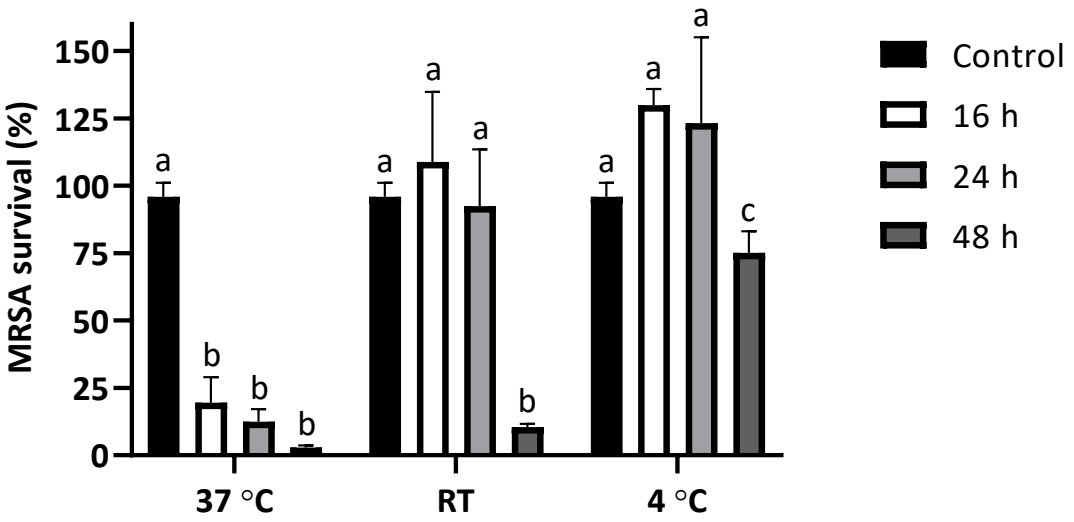

Figure 2.2. Antibacterial activity of BMAP-27GFP solubilized from IBs against methicillin-resistant Staphylococcus aureus (MRSA). Bars show MRSA survival percentage in presence of the different treatments. The results represented are significantly different from the control ($p<0.001$). Different letters depict significant differences among treatments ($p<0.001$). Error bars depict SEM of the different treatments.

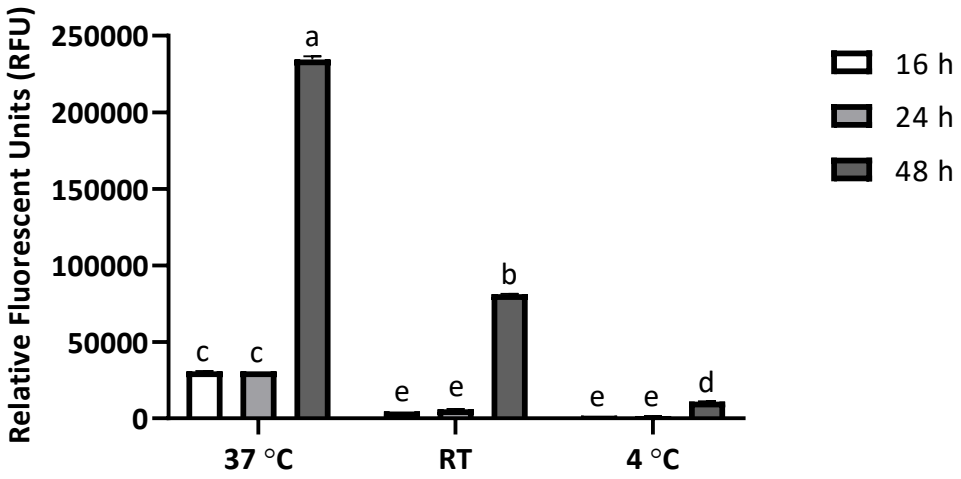

Figure 2.3. Fluorescence of BMAP-27GFP solubilized from IBs. Different letters depict significant differences among samples ($p<0.05$). Error bars depict SEM of the different samples.

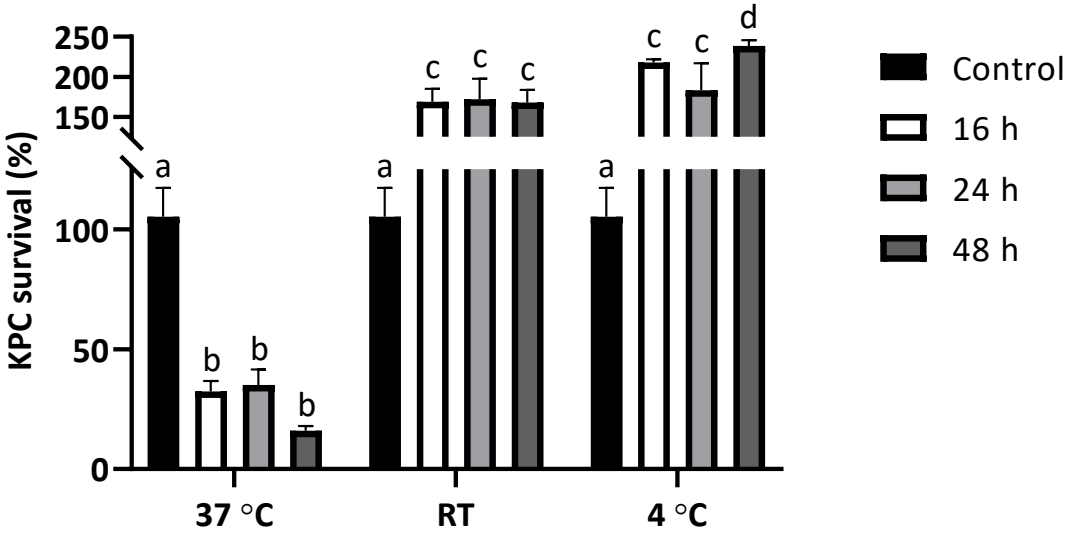

Figure 2.4. Antibacterial activity of JAMF-2 solubilized from IBs against *Klebsiella pneumoniae* Carbapenem-resistant (KPC). Bars show KPC survival percentage in presence of the different treatments. The results represented are significantly different from the control ($p<0.001$). Different letters depict significant differences among treatments ($p<0.001$). Error bars depict SEM of the different treatments.

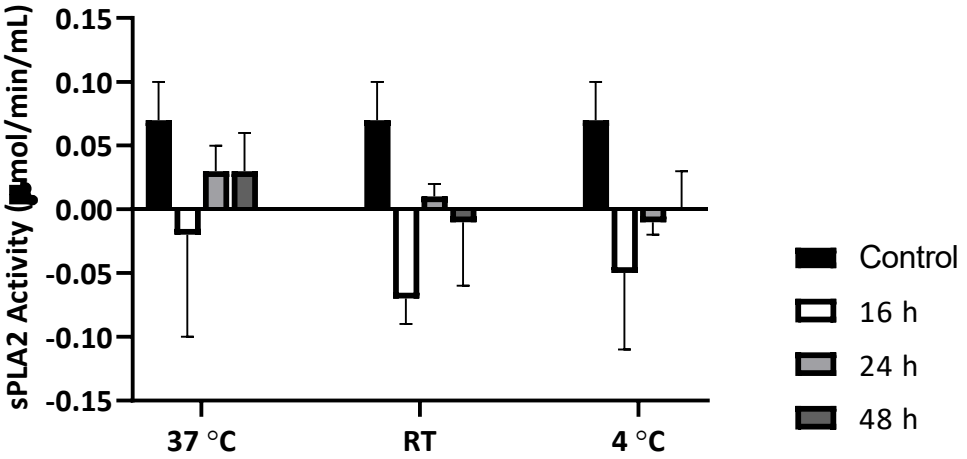

Figure 2.5. Enzymatic activity of JAMF-2 solubilized from IBs. Bars show the enzymatic activity of the Secretory Phospholipase A2 domain contained in JAMF-2 for 20 minutes. Error bars depict SEM of the different treatments.

Finally, the IBs of third model protein M-SAA3 were solubilized but using the temperature that best performed for other two proteins and increasing the number of activity assays using mammary cells. The release of IL-8, IL-6, IFN-γ and IL-10 (**Figure 2.6**) and inhibition of *S. aureus* invasion (**Figure 2.7**) were evaluated. Although the cytokine profile was similar in all the cases, the most sensitive assay was IL-8 because showed a

progressive increase of activity that could be correlated to protein solubilization at increasing the incubation time at 37°C (**Figure 2.6**).

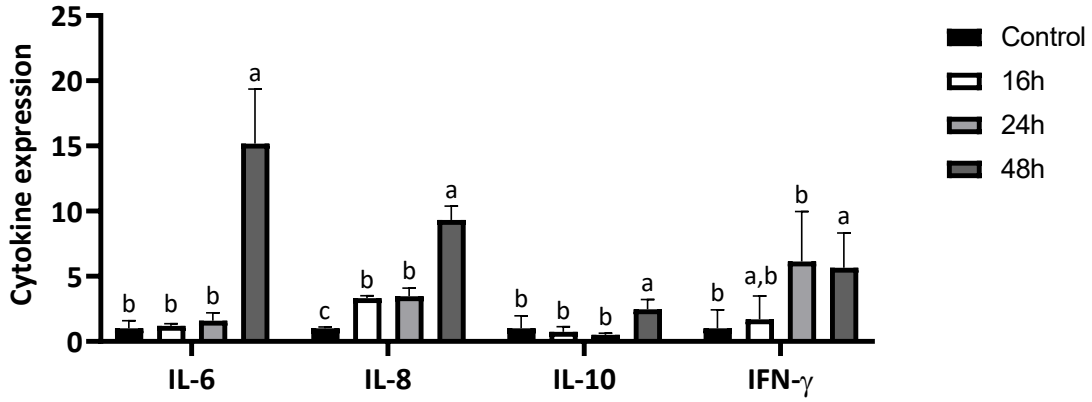

Figure 2.6. Activity determination of M-SAA3 solubilized from IBs. RT-PCR quantification of IL-6, IL-8, IL-10, IFN-γ expression in mammary epithelial cell culture. The results represented are significantly different ($p<0.01$) from the control. Different letters (a-c) denote differences ($p<0.05$) among treatments. Error bars depict SEM of the different treatments.

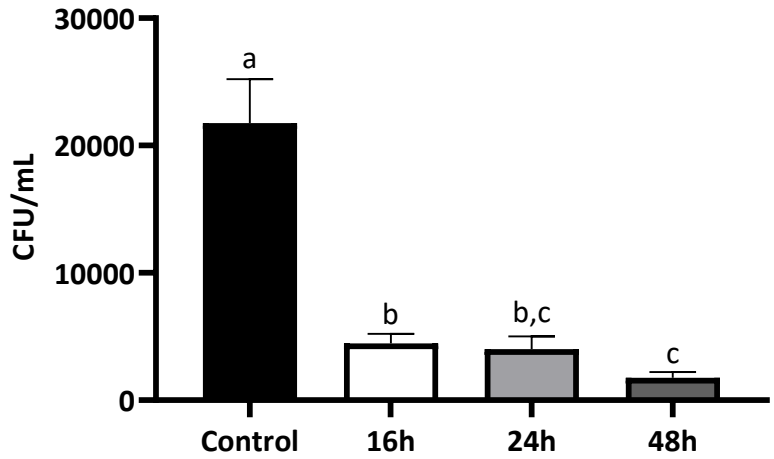

Figure 2.7. Antibacterial activity of M-SAA3 solubilized from IBs. Bacterial counts of infective bacteria (expressed as CFU/mL) associated with mammary gland cell cultures after infection with 2 x 10^7 CFU/mL of *S. aureus* and treated with solubilized M-SAA3 at 150 µg/mL. The results represented are significantly different ($p<0.001$) from the control. Different letters (a-c) denote differences ($p<0.05$) among treatments. Error bars depict SEM of the different treatments.

Conclusions

In conclusion, we clearly showed that IBs from three model proteins are very different among them and could be solubilized without the use of denaturing agents or non-denaturing detergents. The monitorization of protein activity and the screening of different solubilization conditions allowed to establish the optimum timing and temperature with greater sensitivity than Western Blot or standard protein detection protocols and ensure the customization of the protocol to achieve the maximum activity of the protein.

Acknowledgements

This work was funded by Ministerio de Ciencia, Innovación y Universidades Grants PID2019-107298RB-C21/AEI/10.13039/ 501100011033) to AA and EG-F. RB-F received a pre-doctoral fellowship from INIA (FPI-INIA, MINECO). The authors are also indebted to AGAUR for project 2021 SGR 01552. The authors are indebted to CERCA Programme (Generalitat de Catalunya) and the European Social Fund for supporting our research.

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STUDY 3

Recombinant host defense peptides with broad-spectrum antibacterial and antiviral activity: a potential solution against multi-etiological infectious diseases

Ricardo Baltà-Foix, Cristina Saubi, Adrià López-Cano, Jose Vicente Carratalá, Anna Arís* and Elena Garcia-Fruitós*

Preface

After exploring a new LAB expression system (STUDY 1 1) and optimizing protocols to recover the recombinant protein produced (Study 2), this work (Study 3) has focused to produce a new generation of antimicrobial and antiviral products to treat complex multi-etiological bovine diseases. For that, we designed, produced, and characterized the soluble form of a catalogue of immune-related proteins using *L. lactis*. Specifically, we have evaluated 9 different bovine Host Defense Peptides (HDPs) to test their antibacterial and antiviral activity against de main pathogens causing the bovine respiratory disease (BRD). BRD is one of the most relevant diseases in the bovine industry, involving bacterial and viral pathogens. The results obtained demonstrated, first of all, the versatility of *L. lactis* producing different types of immune-related proteins. Moreover, the antibacterial and antiviral activity of the recombinant proteins produced was confirmed, observing that some of the HDPs produced had a broad-spectrum activity. Thus, this demonstrates for the first time that is possible to produce a multifaceted treatment against a wide range of different pathogens: Gram-negative bacteria, bacteria lacking cell wall (*Mycoplasma* genera), and enveloped viruses using *L. lactis* as expression system. Therefore, the presented platform may be a promising option to obtain new treatments against complex IDs such as BRD.

Recombinant host defense peptides with broad-spectrum antibacterial and antiviral activity: a potential solution against multi-etiological infectious diseases

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Abstract

Infectious diseases caused by both bacteria and viruses, such as respiratory tract infections (RTI), are complex diseases, being the development of effective treatments an important challenge. The efficacy of vaccines, antivirals, and antibiotics is limited in these cases because they are specific for each pathogen. Moreover, the global health problem with antibiotic-resistant bacteria hinders the treatment when infecting bacteria are multi-drug resistant (MDR). Aiming to find an antibacterial and antiviral treatment for multi-etiological infections, a battery of Host Defense Peptides (HDPs), which are peptides from the innate immunity, have been recombinantly produced in *Lactococcus lactis*. Their activity has been further characterized against bacteria (*Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni* and *Mycoplasma bovis*) and viruses (bovine herpesvirus type 1 (BHV-1), parainfluenza-3 virus (PI-3) and bovine viral diarrhea virus (BVDV)) causing bovine respiratory disease (BRD). The bactericidal, bacteriostatic, antibiofilm and antiviral activities of HDPs were evaluated and Bac5, BNBD-1, BNBD-3 and LAP were identified as the most promising candidates since showed the broadest activity. Thus, this work proved that HDPs can be used as a broad-spectrum treatment against bacteria (with and without cell wall) and viruses, becoming a real alternative to treat multi-etiological infectious diseases caused by a combination of infectious agents. The results obtained postulate the strategy presented as a good platform to obtain new and potent treatments against complex diseases.

Keywords

Host Defense Peptides, Recombinant Proteins, *Lactococcus lactis*, broad-spectrum antimicrobial and antiviral activity

Introduction

On some occasions, pathogens from distinct groups coexist causing complex infections. An example are respiratory tract infections (RTI) in which both viruses and bacteria usually infect, becoming not only a leading cause of mortality worldwide in humans [1] but also an important challenge in animal health [2,3]. Infections caused by viruses and bacteria can be co-infections, which occur simultaneously, or superinfections, which are sequential [1]. Often, in the respiratory tract, there is a primary viral infection followed

by a bacterial secondary infection [4,5]. The most common scenario is the opportunistic appearance of bacterial strains which were not pathogenic before the viral infection. This secondary bacterial infection increases morbidity and mortality [6–8]. The singularity of these multiple infections reduces considerably the efficacy of the current treatments, such as antibiotics and vaccines, which are mainly directed against one pathogen. Moreover, since antibiotic resistance has exponentially increased during the last years, the treatment of these co-infections is especially critical when infecting bacteria are multi-drug resistant (MDR) bacteria. Thus, the discovery of new molecules not only with activity against MDR bacteria but also with broad-spectrum antimicrobial and antiviral activity represents an important challenge for the treatment of complex infections affecting both human and animal health.

Host defense peptides (HDPs) have emerged as a potential solution to tackle the challenge of infectious diseases. HDPs are short peptides (12-50 aa) produced by the innate immunity of animals and humans. Their cationic charge and amphiphilic structure allow them to interact with pathogens having a negative surface membrane charge such as bacteria, fungi or viruses [9–11]. Through this interaction, HDPs disrupt the membrane, increasing the permeability and finally resulting in bacterial cell death [12,13], being effective against both Gram-positive and Gram-negative bacteria, including MDR microorganisms [14,15]. A similar interaction with enveloped viruses has been described, by which HDPs reduced the virus infection capacity [11]. This dual antimicrobial and antiviral activity could be the most attractive feature of these peptides, postulating them as a promising treatment for multifactorial infectious diseases.

Chemical synthesis is the widest-used strategy to produce HDPs, but it presents important drawbacks especially for *in vivo* applications. Usually, the cost to obtain the necessary amounts of the final product is very high. Moreover, there exist limitations in the length of the peptide and to achieve posttranslational modifications (PTMs) that can impact on the final peptide activity [16–18]. Alternatively, recombinant production permits to easily scale up HDP production in a more cost-effective process [17,19,20]. Different research groups have proven the possibility to produce recombinantly soluble, pure, and active HDPs using *Escherichia coli* as expression system [17,20–24]. However, the presence of the endotoxin lipopolysaccharide (LPS) in Gram-negative bacteria such as *E. coli* requires additional steps in the purification process to obtain LPS-free proteins

[25]. This is a costly and time-consuming process, and, in consequence, other LPS-free recombinant systems are used. Among these alternative systems, the Gram-positive microbial host *Lactococcus lactis* is being widely explored for the production of different proteins of interest [26] such as enzymes [27–30], cytokines [31–34], antigens [35–38], acute phase proteins [39], among others [40].

The current work aimed to prove the HDPs dual antimicrobial and antiviral activity using as a model a multi-etiological disease. For that, a battery of HDPs was successfully produced in *L. lactis* and their activity was tested against bacteria and viruses involved in bovine respiratory disease (BRD), which is a complex respiratory infectious disease caused by several types of bacteria such as *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni* and *Mycoplasma bovis*, and viruses such as bovine herpesvirus type 1 (BHV-1), parainfluenza-3 virus (PI-3) and bovine viral diarrhea virus (BVDV) [41,42]. Thus, since nowadays, the existing vaccines against BRD pathogens present very low efficacy [43,44], and the current antibiotic treatments are decreasing rapidly their effect due to the appearance of AMR [45], this project aims to develop a new strategy to treat these complex infectious diseases.

Materials and Methods

Bacterial and viral strains

Lactococcus lactis subsp. cremoris NZ9000 [46] strain was used for recombinant protein expression. To evaluate the antimicrobial activity the strains selected were *Pasteurella multocida* (ATCC®15743™), *Mannheimia haemolytica* (ATCC®55518™), *Histophilus somni* (ATCC®700025™) and *Mycoplasma bovis* (ATCC®27368™). *L. lactis* strain was grown in M17 broth (Sigma-Aldrich) supplemented with 0.5 % glucose. *P. multocida and M. haemolytica* were grown in Brain-Heart Infusion (BHI) broth (Scharlau). *H. somni* was grown in BHI supplemented with 15 µg/ml Nicotinamide adenine dinucleotide (NAD) (Merck) and 15 µg/ml Hemin (Sigma-Aldrich). *M. bovis* was grown in Friis medium prepared in deionized water (*Mycoplasma* broth base 0.35 % (Oxoid), tryptone 1 % (Difco), peptone 0.53 % (BD-Difco), glucose 0.5 % (Sigma-Aldrich), CMRL medium 1066 10X 2 % (Gibco), Autolyzed Yeast Extract (Yeast Extract Solution) 3.5 % (Gibco), Yeastolate 0.2 % (Difco), Thallium acetate 0.01 % (Sigma-Aldrich), Phenol red 0.002 %

(Sigma-Aldrich), Horse Serum 10 % (Sigma-Aldrich) and Porcine Serum 10 % (Sigma-Aldrich), pH 7.2-7.5).

To evaluate the antiviral activity the strains used were bovine herpes virus type 1 (IZLER BHV1 VIR RE RSCIC 5), bovine parainfluenza 3 virus (IZLER PI-3VIR RE RSCIC 27), and bovine viral diarrhea virus (BVDV; NADL strain).

Eukaryotic cell culture for the viral infection

Cell culture used in this study was Madin-Darby Bovine Kidney cell-line (MDBK, ATCC CCL-22). MDBK cells grew in Minimum Essential Medium (Gibco) supplemented with 2 mM glutamine, 100 U/mL penicillin-streptomycin, 100 U/mL nystatin and 5% fetal bovine serum (FBS) pestivirus negative.

Genetic construct design of recombinant HDPs

The 9 recombinant HDPs used in this study were based on the sequences of Host Defense Peptides (HDPs) (

Table 3.1**Error! No s'ha trobat l'origen de la referència.**). All sequences were C-terminally fused to the Green Fluorescent Protein (GFP) gene through the linker sequence GGSSRSS and a 6 histidine (H6)-tag was added at C-term for protein purification. All constructs were codon-optimized for *L. lactis* by GeneArt (GeneArt®, Life technologies, Regensburg, Germany) and flanked by *NcoI* and *XbaI* restriction sites to be cloned in the pNZ8148 plasmid (MoBiTech) (Cm^R). Electrocompetent *L. lactis subsp. cremoris* NZ9000 cells were transformed by electroporation as previously described in [47].

Table 3.1. Host Defense Peptides used in the study.

Peptide name	Abbreviation	Category	UniProtKB code/sequence
Bactenecin 5	Bac5	Cathelicidin (HDP)	P19660 (CTHL2_BOVIN) 131-137
Bovine β-defensin 1	BNBD-1	β-defensin (HDP)	P46159 (DEFB1_BOVIN)
Bovine β-defensin 2	BNBD-2	β-defensin (HDP)	P46160
Bovine β-defensin 3	BNBD-3	β-defensin (HDP)	P46161 (DEFB3_BOVIN) 16-57
Bovine β-defensin 4	BNBD-4	β-defensin (HDP)	P46162 (DEFB4_BOVIN) 23-63
Lingual antimicrobial peptide	LAP	β-defensin (HDP)	Q28880 (LAP_BOVIN) 25-64
Surfactant-associated anionic peptide	SAAP	Surfactant-associated peptide	DDDDDDD
Surfactant protein D	SPD	Surfactant protein	P35246 (SFTPD_BOVIN) 21-369
Tracheal antimicrobial peptide	TAP	β-defensin (HDP)	D9J275 (D9J275_BOVIN)

Production and purification of HDPs

The 9 recombinant *L. lactis* NZ9000 carrying pNZ8148 plasmid codifying for the different HDPs were grown overnight (O/N) at 30°C in M17 broth supplemented with 0.5 % glucose and 5 µg/mL Cm for plasmid maintenance. The O/N cultures were inoculated in 1 L bottles with 1 L of fresh medium with Cm at an initial OD_{600nm} = 0.05. The recombinant protein expression was induced with 12.5 ng/mL nisin when these cultures reached an OD_{600nm} = 0.4-0.6. After 1 h of induction, for BNBD-1 and BNBD-4, and 3 h post-induction for the rest of proteins, the whole culture was harvested (6,000 x *g*, 15 min, 4°C). Pellets were frozen at -80°C until their use. Each pellet corresponding to 500 mL culture was thawed and resuspended in 30 mL binding buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, pH = 7.4) with EDTA-free protease inhibitor cocktail (Roche). Bacteria were subjected to 2 rounds of mechanical disruption at 40 kpsi in a cellular disruptor (CF1 Model, Constant Systems Ltd). The supernatant was recovered through centrifugation at 15,000 x *g* for 45 min at 4°C. After that, the supernatants were filtered using 0.2 µm filters (Merck) and purified by Immobilized Metal Affinity Chromatography (IMAC) in an ÄKTA start protein purification system (Cytiva) using 1 mL HisTrap™ HP columns (Cytiva). Proteins were loaded with binding buffer (20 mM Tris, 500 mM NaCl, 20 mM Imidazole,

pH = 7.4) and eluted using a linear gradient with elution buffer (20 mM Tris, 500 mM NaCl, 500 mM Imidazole, pH = 7.4). The selected fractions were dialyzed O/N in acetic acid 0.01 % (v/v) at 4°C with gentle agitation. The yield of purified soluble protein was determined by NanoDrop™ (Thermo Scientific), and the integrity and purity of the proteins were analyzed by Western Blot using a monoclonal anti-His antibody (His-probe, Santa Cruz) and by Coomassie blue staining assay. The two parameters were quantified by densitometry analysis with Image J software.

Antibacterial activity determination

Bactericidal assay against Gram-negative bacteria

The antimicrobial assay was based on the BacTiter-Glo™ Microbial Cell Viability assay (Promega). Firstly, an O/N culture of the selected strain (*P. multocida*, *M. haemolytica* or *H. somni*) was diluted (10^6 CFU/mL) in 10 mM potassium phosphate buffer. Then, 150 µL from the bacterial diluted stock were centrifuged (6,200 x *g*, 15 min, 4°C). The supernatant was removed and the bacterial pellet was resuspended with 150 µL of either 0.01 % acetic acid (negative control) or 5 µM of HDP treatment in 0.01 % acetic acid. All samples were disposed in a sterile 96-well plate of polypropylene (Costar) and were incubated for 5 h at 37°C. After, 100 µL were withdrawn and tested with 100 µL of the BacTiter-Glo™ reagent following manufacturer instructions.

Bactericidal assay against M. bovis

The bactericidal assay against *M. bovis* was similar to the protocol detailed in "Bactericidal assay against Gram-negative bacteria" with slight modifications. Briefly, the assay was initiated from a 72 h culture. Then, 150 µL from the initial culture were centrifuged (15,800 x *g*, 30 min, 4°C). The supernatant was removed, and the bacterial pellet was resuspended with 100 µL of 10 % Mueller Hinton Broth cation-adjusted medium (MHB-II, Sigma-Aldrich) and 50 µL of either 0.01 % acetic acid (negative control) or 15 µM of HDP treatment in 0.01 % acetic acid and disposed in a sterile polypropylene 96-well plate (Costar). The final working concentration of HDP was 5 µM. After sample incubation for 5 h at 37°C, 100 µL were withdrawn and tested with 100 µL of the BacTiter-Glo™ reagent following manufacturer instructions.

Minimal inhibitory concentration (MIC)

MIC was determined following performance standards for antimicrobial susceptibility testing with slight modifications [48]. Specifically, 10 % MHB-II (Sigma-Aldrich) was used to be more appropriate for testing cationic peptides and proteins. Another modification was the confirmation of the final MIC by an Optical Density read at OD_{600nm} with a microplate reader (LumiStar, Omega). Each construct was tested at a maximum concentration of 256 µg/mL (7.87 µM BNBD-1, 7.78 µM BNBD-2, 7.73 µM BNBD-3, 7.75 µM BNBD-4, 7.82 µM LAP, 7.90 µM TAP) and serial two-fold dilutions with a minimum concentration of 2 µg/ml (0.06 µM for all constructs) to determine MIC against *P. multocida*, *M. haemolytica* and *H. somni*.

Antibiofilm activity

Antibiofilm activity of each antimicrobial construct was assessed on preformed *M. haemolytica* biofilms following the methodology previously described [49]. Briefly, an O/N culture of *M. haemolytica* (10^8 CFU/mL) was diluted 1/200 in Tryptic Soy Broth (TSB) supplemented with 0.5 % glucose. Then, 200 µL/well of culture dilution were added to a non-treated and sterile 96 well plate and incubated for 24 h at 37°C. After the incubation, the media was gently removed using a vacuum aspirator followed by the addition of 100 µL of MHB 10 % and 100 µL of the specific construct at 10 µM. The final working solution of the constructs was 5 µM. The samples were incubated 24 h at 37°C. Next, the supernatant was gently removed using vacuum aspirator and the remaining *M. haemolytica* biofilms were fixed with 125 µL of methanol for 10 min. After fixation, the methanol was discarded and the plate was dried for 15 min at 37°C. Once the samples were dried, the remaining biofilms were stained with 125 µL of crystal violet (CV) 1 % for 20 min at RT. Next, the CV was discarded and the biofilms were washed 3 times with 125 µL H_2O mQ. Finally, the biofilms were dissolved with 100 µL of acetic acid 33 % and the absorbance was read in a microplate reader LUMIstar Omega (BMG Labtech) at 595 nm.

Antiviral activity determination

Antiviral assay against bovine herpesvirus type 1 (BHV-1)

MDBK cells (20,000 cells/well) were cultured in 96-well plate with opaque and white bottom (Costar) and incubated for 24 h at 37°C and 5 % CO_2. Then, a serial dilution of each HDP from 10 µM to 0.313 µM in culture medium without FBS (50 µL) was mixed with BHV-1 at 500 $TCID_{50}$/50 µL and incubated for 1 h at 37°C. After that, the medium was discarded and 50 µL/well of the mixture were applied to the cells and incubated for 1.5 h at 37°C and 5 % CO_2. The final working solutions of the constructs were from 5 µM to 0.156 µM. After, 50 µL of culture medium with 2 % FBS were added and the plates were incubated for 72 h at 37°C and 5 % CO_2. The antiviral activity of the recombinant proteins was measured by Viral ToxGlo™ reagent (Promega) with a luminometer Fluoroskan™ FL (ThermoFisher Scientific) following the manufacturer instructions.

Antiviral assay against parainfluenza 3 virus (PI-3)

The procedure to test the antiviral activity against PI-3 virus of the recombinant proteins was similar to the protocol detailed in "Antiviral assay against bovine herpesvirus type 1 (BHV-1)" but with one modification. After mixing the proteins and the PI-3 virus, the cells medium was discarded, and the mixture was added without any previous incubation. The rest of the protocol was developed as described above.

Antiviral assay against bovine viral diarrhea virus (BVDV)

MDBK cells (20,000 cells/well) were cultured in 96-well plate (Cultek) and incubated for 24 h at 37°C and 5 % CO_2. Then, a serial dilution of each HDP from 10 µM to 0.313 µM in culture medium without FBS (50 µL) was mixed with BVDV virus at 60 $TCID_{50}$/50 µL and incubated for 1 h at 37°C. The final working solutions of the constructs were from 5 µM to 0.156 µM. After that, 50 µL of cells medium were discarded, and 50 µL of mixture were applied to the cells and incubated for 72 h at 37°C and 5 % CO_2. After that incubation, medium was discarded, and cells were fixed by heat at 80°C for 24 h. The infection was revealed by an Immunoperoxidase Monolayer Assay (IPMA) against BVDV. Finally, 200 µL/well of acetic acid glacial were added and the absorbance was read at OD_{595nm}.

Cytotoxicity of HDPs

MDBK cells (20,000 cells/well) were cultured in 96-well plate with opaque and white bottom (Costar) and incubated for 24 h at 37°C and 5 % CO_2. Then, a serial dilution of each HDP from 10 µM to 0.625 µM in culture medium was applied to the cells and incubated for 72 h at 37°C and 5 % CO_2. The cytotoxicity was measured by Viral ToxGlo™ reagent (Promega) and using a luminometer Fluoroskan™ FL (ThermoFisher Scientific) following the manufacturer instructions.

Statistical Analysis

All experiments were performed in triplicate and data was analyzed by ANOVA analyses and Tukey's multiple comparisons test (GraphPad). The results are represented as the mean of non-transformed data ± non-transformed standard error of the mean (SEM). Asterisks correspond to significant differences between the samples and the control (*$p<0.05$ or **$p<0.01$ depending on the assay).

Results and Discussion

Production and purification of HDPs in *L. lactis*

Besides the specific anatomy of the respiratory tract to prevent any type of infection, pulmonary tissue secretes several HDPs which have a critical role against invading agents such as bacteria and viruses. The most important HDPs and active molecules produced by the innate immunity system described in bovine lung tissue are i) β-defensins 1 (BNBD-1), 2 (BNBD-2), 3 (BNBD-3) and 4 (BNBD-4); ii) lingual antimicrobial peptide (LAP) [50]; iii) tracheal antimicrobial peptide (TAP) [51]; iv) surfactant proteins (SP) [52,53]; v) anionic peptide (AP) [50,54–56]; and vi) cathelicidins such as Bac5 [57–59]. All of them are primarily expressed by ciliated respiratory epithelial cells (tracheal epithelium) of the respiratory tract or by neutrophils. Thus, all these HDPs with important functions at respiratory tract level were chosen to be recombinantly produced in *L. lactis* and further characterized (

Table 3.1**Error! No s'ha trobat l'origen de la referència.**).

After their production, the 9 selected HDPs were purified in their soluble form. The yields of all of them obtained at lab scale were comparable, except for SAAP protein which was produced at yields 10 times lower than the others (**Table 3.2Error! No s'ha trobat l'origen de la referència.**). In terms of purity, all of them reached high values, except BNBD-4 and SPD reporting purities of 80% and 75%, respectively. Considering the low purity of SPD protein together with the inconsistency in production and purification replicates (data not shown), this protein was discarded for further experiments. Although it has been broadly proven that HDPs can be recombinantly produced in different microbial expression systems including *E. coli*, *Bacillus subtilis*, *Pichia pastoris*, and *Saccharomyces cerevisiae*, in most of the cases using a fusion tag [17], there are just two examples of the use of *L. lactis* as expression system for antimicrobial peptides production [60,61]. Thus, the catalogue of HDPs produced in this study proved that *L. lactis* can be used as any other microbial expression system to produce these antimicrobial peptides. Compared to *E. coli*, the most common bacterial expression system, *L. lactis* has the advantage of being a GRAS microorganism which makes not necessary to include extra purification steps to remove residual LPS.

Table 3.2. Recombinant protein yield (mg/L culture) and purity (%) of soluble Bac5, BNBD-1, BNBD-2, BNBD-3, BNBD-4, LAP, SAAP, SPD, and TAP fused to GFP.

Peptide	Yield (mg/L)	Purity (%)
Bac5	1.00	100
BNBD-1	0.98	100
BNBD-2	0.86	95
BNBD-3	0.45	100
BNBD-4	0.38	80
LAP	1.19	100
SAAP	0.07	100
SPD	0.22	75
TAP	0.38	97

Antibacterial activity of recombinant HDPs

The produced and purified peptides were screened for their antibacterial properties against the most relevant bacterial pathogens involved in BRD [2], the multifactorial infectious disease chosen as model. Bacterial pathogens tested included *P. multocida*, *M.*

haemolytica and *H. somni*, all of them Gram-negative bacteria (**Figure 3.1**Error! No s'ha trobat l'origen de la referència.**A**), and *M. bovis* (**Figure 3.1**Error! No s'ha trobat l'origen de la referència.**B**), a cell wall-lacking bacterium. These bacterial strains are firstly commensal bacteria inside the respiratory tract, but after the primary viral infection they become opportunistic pathogens [62,63].

An antibacterial agent can be bacteriostatic, bactericidal or both regarding its action on the bacterial pathogen. The bacteriostatic activity of a drug is defined as its capacity to inhibit the growth of bacteria, while bactericidal activity is the ability to completely kill bacteria [64]. In this regard, the bactericidal activity of the produced HDPs was determined by BacTiter-Glo™ Microbial Cell Viability assay (Promega) and the bacteriostatic activity by MIC assay.

The results obtained in the bactericidal assay showed that all the HDPs tested killed *P. multocida* at a high extent, detecting only between 2 to 9 % of the cells at the end of the treatment (**Figure 3.1**Error! No s'ha trobat l'origen de la referència.**A**). The β-defensins B NBD-1, BNBD-3 and TAP were the best-performing HDPs, reducing *P. multocida* survival at percentages of 2.3 % ± 0.16, 3.2 % ± 0.24 and 2.8 % ± 0.16, respectively (**Figure 3.1**Error! No s'ha trobat l'origen de la referència.**A**). These 3 HDPs were also those best performing against *H. somni*, reducing the bacteria survival at 0.4 % ± 0.07, 0.4 % ± 0.06 and 2.5 % ± 1.55 with BNBD-1, BNBD-3 and TAP, respectively (**Figure 3.1**Error! No s'ha t robat l'origen de la referència.**A**). The results against *M. haemolytica* showed that BNBD-1 and BNBD-3 achieved the highest reductions with survival percentages of 0.7 % ± 0.15 and 0.6 % ± 0.24, respectively, although BNBD-4 and TAP showed also good bactericidal activity achieving survival reduction rates of 3.5 % ± 0.82 and 6.8 ± 0.98 respectively (**Figure 3.1**Error! No s'ha trobat l'origen de la referència.**A**). However, the results against c ell-wall-lacking bacteria showed a different profile (**Figure 3.1**Error! No s'ha trobat l'origen de la referència.**B**). Cathelicidin Bac5 and surfactant peptide SAAP worked better than others against *M. bovis* and survival percentages of 6.1 % ± 1.05 and 3.5 % ± 0.08 were detected respectively after the treatment (**Figure 3.1**Error! No s'ha trobat l'origen d e la referència.**B**). Interestingly these two HDPs did not exert important effect against two of the Gram-negative bacteria tested (**Figure 3.1**Error! No s'ha trobat l'origen de la referència.**A**). Although at a lower extent, BNBD-1 also showed acceptable efficiency against *M. bovis* with a survival percentage of 13.9 % ± 2.44 (**Figure 3.1**Error! No s'ha t

robat l'origen de la referència.**B**), being therefore the protein that showed good activity against all tested bacteria (**Figure 3.1**Error! No s'ha trobat l'origen de la referència.**A**).

On the other hand, we characterized the bacteriostatic activity by determining the MIC against the Gram-negative bacteria but excluding *M. bovis* because its low growth rate (**Table 3.3Error! No s'ha trobat l'origen de la referència.**). BNBD-3 was the HDP with the lowest MIC against *P. multocida* (64 µg/mL (1.93 µM)), whereas this MIC was also achieved by both BNBD-1 and BNBD-3 against *M. haemolytica.* Surprisingly none of the HDPs could reduce *H. somni* growth, at least at the maximum tested concentration of 256 µg/mL (7.73-7.90 µM). Also, the MIC values showed that TAP, despite having good bactericidal activity (**Figure 3.2Error! No s'ha trobat l'origen de la referència.**), had a limited bacteriostatic activity against *P. multocida* and *H. somni* (**Table 3.3Error! No s'ha t robat l'origen de la referència.**). These variations permitted to elucidate that each HDP had a specific mode of action which is strain dependent. Against *P. multocida*, BNBD-1 and BNBD-3 were clearly the HDPs with good bactericidal and bacteriostatic activities (**Figure 3.1**Error! No s'ha trobat l'origen de la referència. and **Table 3.3Error! No s'ha tr obat l'origen de la referència.**). These two β-defensins were also the HDPs with the highest activities against *M. haemolytica*. By contrast, against *H. somni*, BNBD-1, BNBD-3 and TAP performed the highest bactericidal activities, but none of them showed bacteriostatic activity. These results agree with previous publications in which different antimicrobial peptides with a limited bacteriostatic effect have shown a good bactericidal activity [65], while others that have similar bacteriostatic activities show differences in the bactericidal activity [66]. Therefore, unlike antibiotics which are either bacteriostatic or bactericidal, HDPs can have different activities (or even combined activities) that can vary depending on the pathogen.

The lack of bacteriostatic activity observed against *H. somni* could be due to the structural difference between the pathogenic bacterial strains tested. *P. multocida*, *M. haemolytica* and *H. somni* are members of the Pasteurellaceae family, but while the first two are encapsulated, the third is not [67]. Moreover, under certain growth conditions, *H. somni* produced an exopolysaccharide that forms an extracellular matrix conferring protection to bacterial cells [67,68]. The lack of a capsule and the presence of the exopolysaccharide could interfere with the interaction of the HDPs with the external layers of the bacteria.

Table 3.3. Minimum Inhibitory Concentration (MIC) of recombinant HDPs. MIC values for *P. multocida*, *M. haemolytica*, and *H. somni*.

	Pasteurella multocida		***Mannheimia haemolytica***		***Histophilus somni***	
	µg/ml	µM	µg/ml	µM	µg/ml	µM
BNBD-1	128	3.93	64	1.97	>256	>7.87
BNBD-2	>256	>7.78	>256	>7.78	>256	>7.78
BNBD-3	64	1.93	64	1.93	>256	>7.73
BNBD-4	128	3.87	>256	>7.75	>256	>7.75
LAP	>256	>7.82	>256	>7.82	>256	>7.82
TAP	>256	>7.90	128	3.95	>256	>7.90

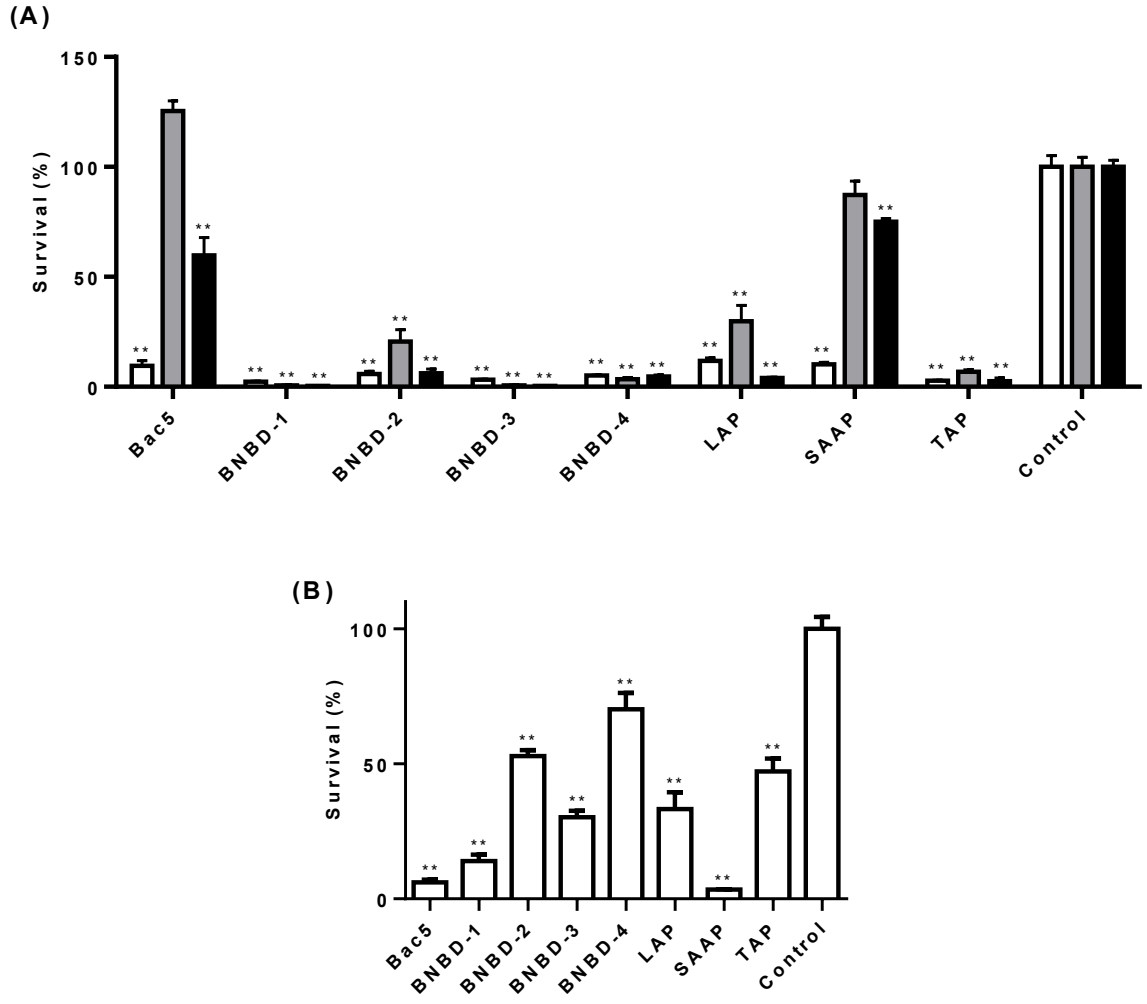


Figure 3.1. Antibacterial activity of recombinant HDPs. Antibacterial activity is represented as a reduction of bacteria survival percentage. HDPs were tested at a concentration of 5 µM. (A) *P.*

multocida survival is represented in white, *M. haemolytica* survival is represented in grey and *H. somni* survival is represented in black. (B) Antibacterial activity of recombinant HDPs against *M. bovis*. The data shown are the mean of a triplicate ± SEM. Asterisks depict significant differences in front of the control (**p-value≤0.01). Results without asterisks did not present significant differences.

Finally, the antibacterial profile was complemented with the antibiofilm activity of the produced HDPs. Biofilms represent one of the most important ways through which bacteria become more resistant to antibiotics [69], especially in RTI [70]. The antibiofilm activity of HDPs against biofilm formed by human pathogens is widely described [71]. Nevertheless, biofilms are an important problem in veterinary medicine, especially in livestock and in respiratory infections like BRD [72]. But there is not much research focused on biofilms affecting animals [73]. Thus, determining the capability of the recombinant HDPs to attack bacteria forming biofilms in BRD would add an extra value to their antimicrobial potential. The HDPs selected for these assays were Bac5, BNBD-1, BNBD-2, BNBD-3, LAP and TAP, considering their yields, their purity and their antibacterial activity presented in the previous assays. Since the peptide amount needed for the antibiofilm experiments was higher than for the antibacterial assays in planktonic form, BNBD-4 and SAAP were discarded because of their lowest yield and purity (**Table 3.2Error! No s'ha trobat l'origen de la referència.**). Taking *M. haemolytica* as the best-performing biofilm strain of the ones used, 5 out of 6 HDPs reduced the preformed biofilms up to 45 % (**Figure 3.2Error! No s'ha trobat l'origen de la referència.**). S pecifically, TAP presented the highest reduction, and the biofilm survival was 55.7 % ± 3.03. The biofilm percentage remaining with BNBD-1, BNBD-2, BNBD-3 and LAP was 76.3 % ± 5.33, 70.8 % ± 1.41, 59.9 % ± 1.82 and 65.6 % ± 1.40, respectively (**Figure 3.2Error! N o s'ha trobat l'origen de la referència.**). Bac5 did not reduce the biofilm at all, which agrees with the low bactericidal activity observed against *M. haemolytica* in **Figure 3.1**Error! No s'ha trobat l'origen de la referència.**A**. The antibiofilm activity presented by the recombinant HDPs could be an interesting starting point to potentiate the antibiofilm activity of HDPs in biofilms affecting livestock.

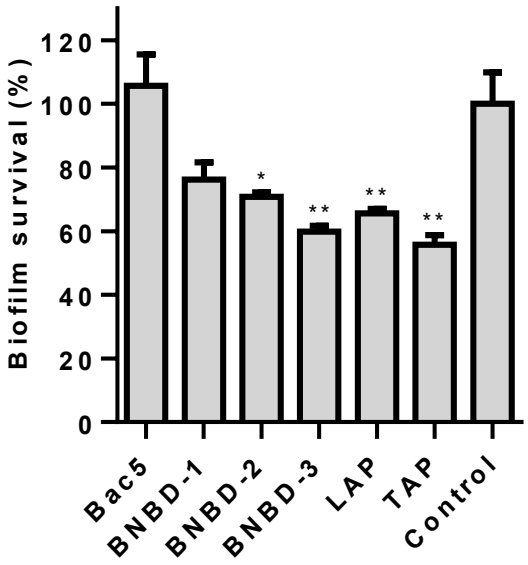


Figure 3.2. Antibiofilm performance of recombinant HDPs against *M. haemolytica*. Antibiofilm activity of HDPs at 5 µM against pre-formed biofilm of *M. haemolytica*. The results are represented in the percentage of biofilm survival. The data shown are the mean of triplicate ± SEM. Asterisks depict significant differences in front of the control (**p-value*≤0.05 and ***p-value* ≤0.01). Results without asterisks did not present significant differences.

These three different antibacterial activity assays performed allowed us to determine the overall antibacterial potential of the recombinant HDPs. The results indicated that although HDPs are cationic peptides presenting common antimicrobial mechanisms based on ionic interactions with negatively charged bacterial membranes and cell walls, their efficiency was clearly different (**Figure 3.1Error! No s'ha trobat l'origen de la r eferència.** and **Figure 3.2Error! No s'ha trobat l'origen de la referència.**). Probably the involvement of other mechanisms along with possible structural features behind the recombinant production are also influencing the final efficiency observed. Moreover, the mode of bacterial growth was also a critical parameter because overall BNBD-1 worked quite well against all bacteria growing in planktonic form, but it was not effective as others like TAP and LAP against biofilms (**Figure 3.2Error! No s'ha trobat l'origen de l a referència.**). Bacteria forming biofilms undergo several phenotypic modifications and this could impair bacterial killing capacity. Specifically, there is a slow bacterial growth, and the presence of an extracellular matrix also avoids the HDPs diffusion [74].

In general terms, we can conclude that β-defensins are more active than the cathelicidins or the surfactant proteins against the Gram-negative bacteria strains. Specifically, BNBD-1, BNBD-3 and TAP were the best candidates against the three strains reducing almost

totally the bacterial growth (**Figure 3.1**Error! No s'ha trobat l'origen de la referència.**A**). T he antibacterial activity of the β-defensins against Gram-negative bacteria has been widely proven in the last years [75–77], and the results obtained in this regard concur perfectly, proving the optimal design and the potent antibacterial activity of the β-defensins recombinantly produced also in *L. lactis*.

The behavior of HDPs with *M. bovis*, which is one of the toughest pathogens in the secondary bacterial infection causing BRD [2,78], was slightly different. *M. bovis* presents a strong and natural antibiotic resistance related to its lack of cell wall [79–81], and that is the key factor that hinders the discovery of effective treatments [81]. The cathelicidin Bac5 and surfactant peptide SAAP were the best candidates against this bacterium (**Figure 3.1**Error! No s'ha trobat l'origen de la referència.**B**). However, among the tested β -defensins, BNBD-1 also showed an important reduction of *M. bovis* survival, being postulated as the molecule with the broadest antibacterial activity. There exist very few examples of previous works describing HDP antibacterial activity against *Mycoplasma* strains [82,83], and that context confers high expectations for the antibacterial activity presented by the recombinant HDPs.

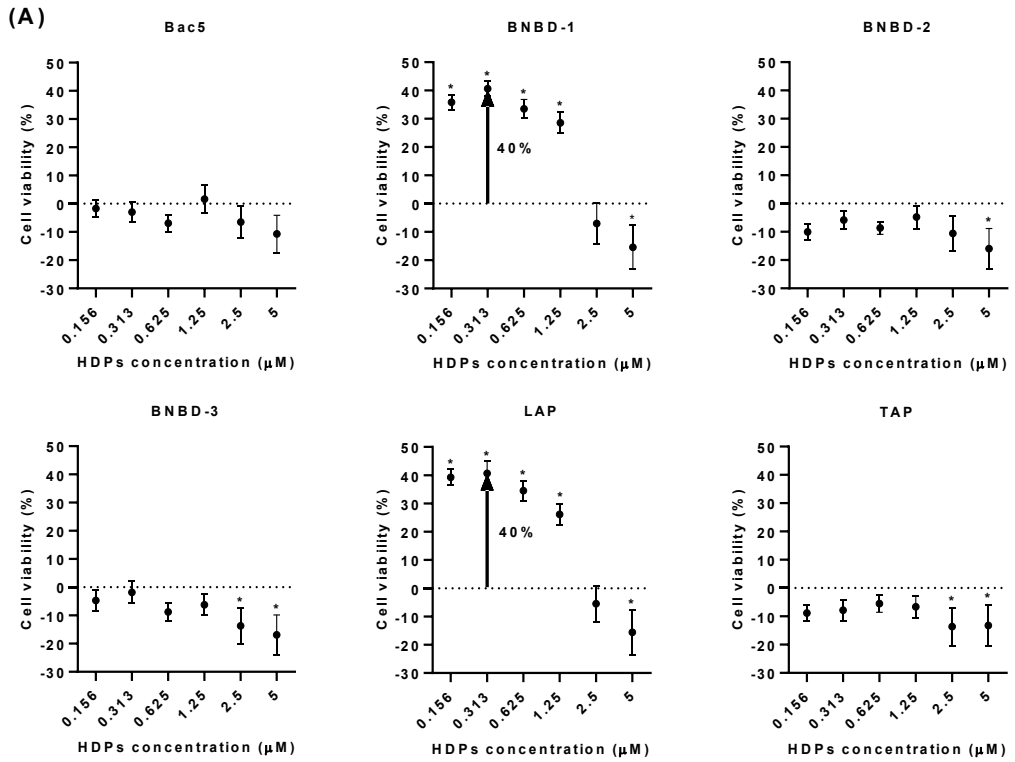
Antiviral activity of recombinant HDPs

It is well known that viruses play a key role in RTI, unbalancing the epithelial barrier and activating the antiviral immune response [1]. BRD is not an exception, because the primary action of BHV-1, PI-3 virus and BVDV, among others, in the respiratory tract of the animals, breaks into the cascade of following infections by other pathogens such as bacteria [2]. Because of that, several vaccines have been developed to prevent the disease [84–86] but most of them with partial success. This worrying situation becomes an excellent scenario for the search of new molecules or strategies to fight against viral pathogens. Thus, after the characterization of the HDPs antibacterial activity, six HDPs were selected to analyze their activity against three enveloped viruses involved in BRD. As indicated above, BNBD-4 and SAAP were discarded because of their lowest yield and purity (**Table 3.2Error! No s'ha trobat l'origen de la referència.**). The HDPs Bac5, B NBD-1, BNBD-2, BNBD-3, LAP and TAP were tested in MDBK cells infected with BHV-1, PI-3 virus or BVDV. The aim was to determine if the HDPs could reduce the virus culture infection, and consequently, increase the cell viability. Regarding the results against BHV-1, the cultures treated with β-defensins BNBD-1 and LAP recovered cell viability (**Figure**

3.3 Error! No s'ha trobat l'origen de la referència.**A**). Specifically, BNBD-1 increased 40.7 % ± 2.62 the cell viability at 0.313 μM, and LAP increased 40.6 % ± 4.35 the cell viability at the same concentration (**Figure 3.3**Error! No s'ha trobat l'origen de la referència.**A**). T he rest of the cultures treated with the other HDPs did not present significant changes (**Figure 3.3**Error! No s'ha trobat l'origen de la referència.**A**). Against the PI-3 virus, the c ultures treated with cathelicidin Bac5 and β-defensins BNBD-1 and LAP presented the highest cell viability (**Figure 3.3**Error! No s'ha trobat l'origen de la referència.**B**). The c athelicidin Bac5 increased 15.3 % ± 7.29 the cell viability at the concentration of 0.625 μM and the β-defensins BNBD-1 and LAP increased 10.7 % ± 2.35 (0.625 μM) and 17.6 % ± 1.82 (0.313 μM) the cell viability, respectively (**Figure 3.3**Error! No s'ha trobat l'origen d e la referència.**B**). The rest of the cultures treated with the other HDPs did not present changes (**Figure 3.3**Error! No s'ha trobat l'origen de la referència.**B**). Finally, against the B VDV, five out of the six HDPs tested incremented the cell viability of the treated cultures, and BNBD-1 achieved the best performance, with an increase of 36.7 % ± 2.41 in the cell viability at the concentration of 5 μM (**Figure 3.3**Error! No s'ha trobat l'origen de la r eferència.**C**). BNBD-2 increased 26.6 % ± 1.40 the cell viability at 5 μM, while BNBD-3 (5 μM), LAP (2.5 μM) and Bac5 (5 μM) increased the cell viability 11.8 % ± 1.77, 20.0 % ± 1.10 and 21.3 % ± 2.54, respectively. Cultures treated with TAP were the only samples that did not present changes (**Figure 3.3**Error! No s'ha trobat l'origen de la referència.**C**).

The antiviral activity of HDPs or other types of AMPs has been widely described [11,87], acting through different strategies depending on the virus and HDP class [87–89]. For instance, cathelicidins bind the viral envelope disrupting the membrane [90] and β-defensins bind viral glycoproteins inhibiting the infection [90]. The antiviral activity results shown by the recombinant HDPs against the enveloped viruses tested could be based on some of these previous effects described. The β-defensins BNBD-1 and LAP seem to be the most promising HDPs presenting the highest antiviral activity against the 3 viruses. Although the cathelicidin Bac5 showed good performances against PI-3 virus and BVDV as well. It is important to note that there are few studies describing the antiviral activity of *de novo* HDPs, and all of them are focused on synthetic peptides [91,92]. Here is presented a robust alternative platform for the recombinant production of new antiviral HDPs. Moreover, the antiviral activity shown by the recombinant HDPs reinforces the concepts described in the last years about the role of HDPs in viral infections [11,87–90]. The direct activity of the recombinant HDPs on the viruses opens

a variety of possibilities. For instance, a treatment based on a combination of the HDPs with the existing vaccines. While the lasts are a preventive tool to avoid future infections, the first could be a direct treatment once the viruses are already infecting the respiratory system. Moreover, they could be applied as a preventive tool also because it has been described their capacity to act on the host cell instead of on the viruses, inhibiting the viral infection [89].



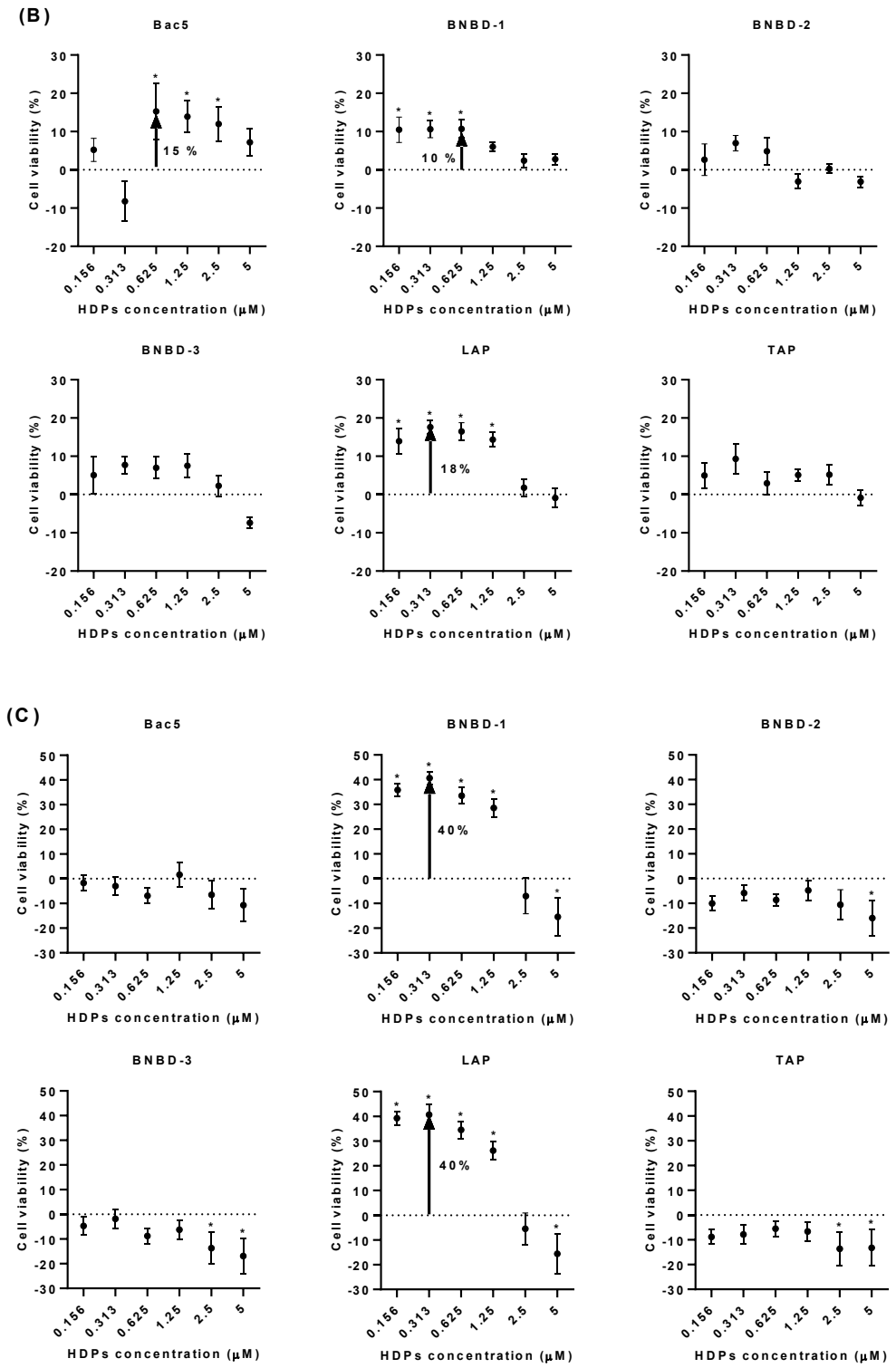


Figure 3.3. Antiviral activity of HDPs against viral infection of MDBK cells. The HDPs were tested at different concentrations ranging from 0.156 µM to 5 µM. **(A)** Antiviral activity in MDBK cells infected with BHV-1. **(B)** Antiviral activity in MDBK cells infected with PI-3 virus. **(C)** Antiviral activity in MDBK cells infected with BVDV. The results are represented as the increase or decrease of cell viability percentage, where the higher percentage of cell viability means the higher antiviral activity presented by the HDPs. The data shown are the mean of four replicates ± SEM. Asterisks depict significant differences in front of the control (**p-value*≤0.05). Results without asterisks did not present significant differences.

Overall, these results together proved for the first time that it is possible to have HDPs with broad-spectrum activity against different both bacteria and viruses (**Figure 3.4Error! No s'ha trobat l'origen de la referència.**), allowing the development of an HDP-based treatment for complex respiratory disease such as BRD. In general terms BNBD-1 showed the broadest antibacterial and antiviral effect. However, the broad screening of HDPs with potential activity done has also proven to be a very useful approach for the selection and combination, if necessary, of HDPs performing better against all the pathogens tested to develop a more efficient strategy against multi-etiological diseases. Specifically, in the case of BRD, a cocktail of BNBD-1, BNBD-3, TAP and Bac5 seems to be a promising combination (**Figure 3.4Error! No s'ha trobat l 'origen de la referència.**), having HDPs that also have antibiofilm activity (**Figure 3.2Error! No s'ha trobat l'origen de la referència.**). Thus, their combined effects need to be further characterized in future studies.

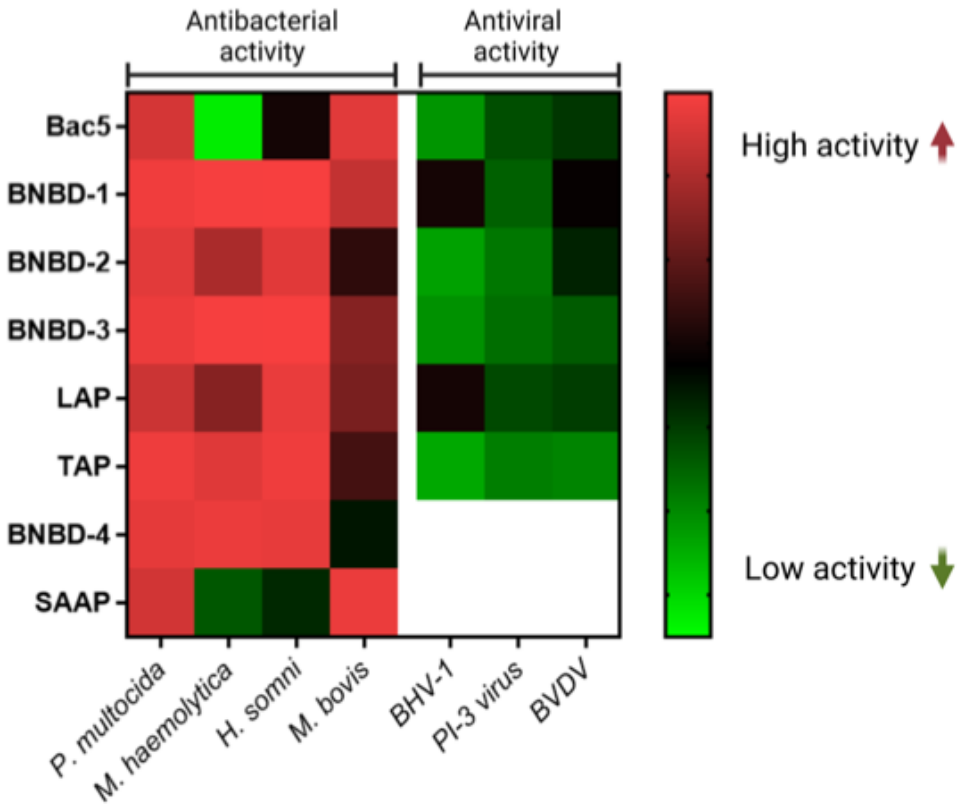


Figure 3.4. Schematic summary of the antibacterial activity against *P. multocida*, *M. haemolytica*, *H. somni*, *M. bovis* and the antiviral activity against BHV-1, PI-3 virus and BVDV of the HDPs analyzed in this study. The color scale indicates those peptides with higher activity (red) and those with lower activity (green).

In addition, in terms of applicability, the production process of the HDPs is easily scalable, an important advantage for future applications in animal health, where the drug amounts needed are much higher than those for human health. Besides the yields could be optimized for every specific HDP if needed, considering that the production and purification conditions presented in this regard are only a proof of concept to determine the antimicrobial activity of these recombinant HDPs.

Conclusions

For the first time, different recombinant HDPs produced in *L. lactis* have proven to have broad-spectrum antimicrobial activity against bacteria with cell wall, bacteria without cell wall and enveloped viruses. Bac5, BNBD-1, BNBD-3 and LAP are the most promising candidates with the broadest antibacterial and antiviral activities (**Figure 3.4Error! No s 'ha trobat l'origen de la referència.**) being BNBD-1 the molecule with the broadest activity spectrum. The results obtained postulate the strategy presented as a good platform to obtain new and potent antimicrobials. Furthermore, the recombinant HDPs obtained arises as a good alternative to treat multifactorial infectious diseases such as RTI.

Acknowledgements

This work was funded by Ministerio de Ciencia, Innovación y Universidades Grants PID2019-107298RB-C21/AEI/10.13039/ 501100011033) to AA and EG-F. RB-F received a pre-doctoral fellowship from INIA (FPI-INIA, MINECO) and AL-C and CS are supported with a pre-doctoral fellowship from Generalitat de Catalunya (FI-AGAUR). The authors are also indebted to AGAUR for project 2021 SGR 01552. The authors are indebted to CERCA Programme (Generalitat de Catalunya) and the European Social Fund for supporting our research.

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GENERAL DISCUSSION

Tackling the treatment of bovine IDs from a new perspective

The emergence of antimicrobial resistance is a naturally occurring phenomenon. Under any environmental pressure, natural or artificial, microorganisms develop adaptative strategies to overcome those difficulties and continue replicating. Thus, the overuse and misuse of antibiotics exert selective pressure on microorganisms, accelerating the appearance of resistant strains [485]. In consequence, this has a huge impact not only on humans but also on animals and the environment, being necessary to develop common strategies from a One Health perspective [47].

The impact of AMR in animal production is high, because of the huge amount of antibiotics used and the lack of alternatives to face-up IDs. This problem becomes more worrisome in the case of IDs caused by several etiologies. The bovine industry is a sector especially beaten by complex IDs. Diseases like mastitis or BRD, caused by several types of pathogens, are a real concern with high rates of morbidity and mortality [23,27]. The current treatments and preventive strategies, mainly against bacteria and viruses causing these IDs, are decreasing their efficacy or do not have the expected results. Hence, there is an urgent need to develop new treatments to substitute or improve the existing ones, and this thesis aimed to find a solution to treat multi-etiological diseases in cattle. When we searched among the possible alternatives, we realized that the immune system provides us with a vast number of molecules selected along evolution to fight all kinds of infections. This includes proteins and peptides with immunomodulatory and/or antimicrobial activity [243,244,250]. Some examples are MMPs, APPs, lysozyme, ribonucleases, phospholipases and HDPs. The functions of MMPs overpass the degradation of the extracellular matrix. They are involved in many aspects of inflammation and immunity, mainly regulating the activity of chemokines and resulting in the influx of immune effectors cells like neutrophils, monocytes and eosinophils [454]. For their part, APPs are contrasted modulators of the immune response [226], trapping microorganisms and having chemotactic effects on monocytes, leukocytes, and T cells [486]. In this context and considering the relevance of previous studies of our group demonstrating the immunomodulatory abilities of recombinant MMP-9 catalytic domain [192,214,220] and M-SAA3 [194,221,233], we chose them as model proteins for Study 1 and Study 2 of the current thesis. In addition, HDPs are the most versatile molecules of

the list enumerated above in terms of function and therapeutical potential [248,312,472,487]. They are quite unique for their broad antibacterial, antiviral, and immunomodulatory activities. Thus, we also selected some HDPs to evaluate their potential for the treatment of bovine IDs (Study 3, Annex 1).

Once the molecules to work with were chosen, the next challenge to address was the need for a cost-effective production strategy. This is especially relevant for those molecules to be used in the animal production industry, where economic margins are very tight, and the development of an efficient and cost-effective production process becomes a priority. Chemical synthesis has been a widely used strategy for peptide production such as HDPs for their further characterization. However, important limitations in terms of price or in the length of the synthesized peptide, as well as difficulties to perform PTMs, are major problems of this strategy [248,488,489]. By contrast, the production of these molecules by recombinant technologies seems to be a promising alternative. Recombinant protein production is well-established in the animal production field to obtain hormones for reproductive purposes and fibrolytic enzymes to improve animal performance, many of them already marketed (**Table 2**) [322]. There is a broad catalog of recombinant expression systems including mammalian cells, insect cells, algae, yeast, fungi, and bacteria [490,491], but so far mammalian cells and fungi are the first choices to produce the recombinant hormones and enzymes used in animal production [322]. However, the study of other cell factories such as bacteria and yeast has increased in the last decades [322,492,493] with the objective to minimize costs of production and broaden the use of recombinant proteins in animal production. In this context, the new molecules based on immune-related proteins that this thesis aimed to develop to treat multi-etiological bovine IDs has been produced in recombinant bacteria.

Table 2. Marketed recombinant hormones and recombinant enzymes used in animal production. Adapted from Gifre-Renom et al. [322].

Type of molecule	Cell factory	Companies
FSH	CHO cells	Merck, MSD, Merck, Venaxis, Nanocore Biotecnologia SA
LH	CHO cells	Venaxis, Merck
Xylanase	*Trichoderma reesei*	ABVista, Danisco Animal Nutrition
Xylanase	*Trichoderma citrinoviride*	Huvepharma
Xylanase	*Trichoderma longibrachiatum*	Danisco Animal Nutrition
Xylanase	*Aspergillus oryzae*	DSM-Novozymes
Xylanase	*Bacillus subtilis*	Beldem
β-Glucanase	*Trichoderma reesei*	ABVista
β-Glucanase	*Trichoderma citrinoviride*	Huvepharma
α-Amylase	*Bacillus licheniformis*	DSM-Novozymes
Xylanase, β-Glucanase	*Trichoderma reesei*	Aveve Biochem, DSM-Novozymes, Danisco Animal Nutrition
Xylanase, Amylase, Protease	*Trichoderma reesei*	Danisco Animal Nutrition
Xylanase, β-Glucanase, Pectinase	*Trichoderma reesei* *Aspergillus aculeatus*	Aveve Biochem
Xylanase, Amylase, Protease	*Trichoderma reesei* *Bacillus amyloliquefaciens* *Bacillus subtilis*	Danisco Animal Nutrition
Xylanase, β-Glucanase	*Aspergillus niger*	GNC Bioferm, BASF
Xylanase, β-Glucanase, Phytase	*Aspergillus niger*	BASF
α-Galactosidase, β-Glucanase	*Aspergillus niger* *Saccharomyces cerevisiae*	Biocon
Xylanase, β-Glucanase, Amylase	*Aspergillus niger* *Aspergillus oryzae*	GNC Bioferm
Xylanase, β-Glucanase	*Trichoderma longibrachiatum*	Danisco Animal Nutrition
Xylanase, β-Glucanase, Cellulase	*Trichoderma longibrachiatum*	Biovet
Pectinase, β-Glucanase	*Aspergillus aculeatus*	DSM-Novozymes

Abbreviations. Follicle stimulating hormone (FSH), Luteinizing hormone (LH), Chinese hamster ovary (CHO) cells.

Optimization of protein production strategies using recombinant bacteria to obtain new therapeutic molecules for animal health

E. coli has demonstrated to be an extraordinary bacterium for recombinant protein production purposes. There is an infinite number of tools available including expression vectors, production strains [320,422], and fermentation technologies [494,495], allowing the selection of the optimal strategy for the expression of each heterologous protein of interest. The recombinant production of immune-related proteins in bacterial expression systems is not an exception and *E. coli* is again the most widely used system. It is currently used to produce marketed therapeutical proteins like interferons, interleukins, tumor necrosis factor and antibodies [335]. Moreover, we can find examples in the literature of recombinant HDPs produced in *E. coli,* including defensins [474,496], cathelicidins

[471,497] and many other AMPs from different natures and origins [315]. In this context, previous studies of our group have also demonstrated the production of immune-related proteins such as APPs [232] or HDPs in *E. coli* [436,470,476,483,484]. The correct expression of M-SAA3 in *E. coli* BL21 has been proven [232], as well as its capacity to activate the involution of the mammary gland in dairy cows [194] and to control infections in mammary gland primary cultures [233]. Also, a recent study comparing the production efficiency of an α-defensin (human α-defensin 5, HD5) and a β-defensin (bovine lingual antimicrobial peptide, LAP) in two *E. coli* strains, BL21 and Origami, concluded that *E. coli* BL21 had the best performance in terms of protein yield and final antibacterial activity [470]. Moreover, different functional multidomain proteins formed by combinations of HDPs [476] or HDPs with other immune-based domains like phospholipases and bacterial binding domains were successfully produced in *E. coli* BL21 [436,483]. All of them showed antimicrobial effects against Gram-negative and Gram-positive bacteria, including multi-resistant strains and those forming biofilms [436,476,483].

However, the presence of LPS in *E. coli*-derived products continued hindering the production of immune-related proteins free from endotoxin traces. By contrast, LAB and *B. subtilis* have emerged as alternative LPS-free expression of proteins [366,371,498]. *B. subtilis* and *L. lactis* are two GRAS bacteria well known for their excellent ability to secrete proteins outside the cell [358,372,499–501], and they have also been widely characterized for intracytoplasmic recombinant protein production [221,366,502,503]. Although to a lesser extent than in *E. coli*, this type of bacteria has also been used to produce immune-related proteins. There exist some examples of antimicrobial protein production using *B. subtilis* [504–509] and some with *L. lactis* [221,393,400,401]. Regarding the production of immune-related proteins such as MMPs, APPs [221] and cytokines [393] our group also proved that *L. lactis* can be used as a successful recombinant production system. However, although yields obtained in *L. lactis* were good, they could be improved. For that reason, we decided to broaden the catalog of LPS-free systems for the production of immune-related proteins studying *L. plantarum* (STUDY 1). *L. plantarum* is also a GRAS bacterium belonging to the group of the LAB and its use as a recombinant expression system has experienced continuous growth in the last years. New inducible expression systems [407,510], expression vectors [408,410,411], promoters [405,409], origins of replication [404,511] and electroporation

protocols [512] have been developed. Besides, the expression of isomerases [513], β-glycosidases [417], β-galactosidases [413–415], β-mannanases [514], chitosanases [514], tannases [515], chitinases [412], oxalate decarboxylases [418], α-amylases [516], and reductases [416] have been described in this LAB. In Study 1, we demonstrated that *L. plantarum* was also a suitable recombinant expression system to produce immune-related proteins, achieving the correct expression of the MMP-9 catalytic domain (MMP-9cat). These results enlarged the robustness of *L. plantarum* to produce recombinant proteins, adding a new example to previous works on the heterologous expression of other enzymes. The obtained MMP-9cat yield of 3.39 mg/L (considering the values of the 4 peaks obtained as whole yield, **Table 1.2**Table 1.2. Yield and purity of soluble MMP-9cat 4 peaks.) was lower compared with the yield of 10.4 mg/L (doing the same calculations) obtained for the same protein produced in *L. lactis* [219] but higher compared to the 0.303 mg/L of another LPS-free strain like *Clearcoli* [219] (**Table 3**). Comparing the MMP-9cat yield with that obtained for other enzymes produced in *L. plantarum*, Nguyen and coworkers achieved yields of 5 mg/L of recombinant chitinase [412] whereas Curiel and coworkers got yields of 17 mg/L of a soluble tannase [515]. Thus, the yield of 3.39 mg/L of MMP-9cat is at comparable levels with them. In terms of protein purity, we reached high purity values (>99%) for the recombinant MMP-9cat produced in *L. plantarum* (Study 1), similar to other studies producing different types of enzymes [412,415,418]. However, in the Study 1 of this thesis the MMP-9cat (a DTE and PTA protein) produced in *L. plantarum* was isolated from IBs whereas the rest of the proteins previously produced in *L. plantarum* were isolated from the soluble fraction, either secreted [418,516] or purified from cell extracts [412,415,515]. This fact revalorizes the use of IBs as a source of soluble protein as a worthwhile strategy (**Figure 14**), providing similar yields than direct purifications of soluble protein from cell extracts or secretion media. Thus, this study has shown that it is possible to recover the soluble and active form of a PTA protein such as MMP-9 from *L. plantarum* IBs (**Figure 14**), as it has been described in *L. lactis* [221]. It is also appropriate to highlight that we have proven for the first time the formation of biologically active IBs in *L. plantarum* (**Figure 14**). This is of great relevance, considering that LAB has been usually presented as an alternative to *E. coli* to avoid the formation of these aggregates and they become another example of aggregation during recombinant production processes using bacterial expression systems [517]. The profile of 4 different peaks obtained after the purification

of MMP-9cat in *L. plantarum* was almost identical to that described in *L. lactis* [219]. The peak 1 in both expression systems presented the highest yield and activity (**Table 3**). In contrast, the profile obtained in *Clearcoli* was slightly different, with 4 eluted peaks as well, but the peak 2 had the highest yield and activity (**Table 3**) [219]. In terms of activity, the MMP-9cat produced in *L. lactis* was the most active, followed by the ones produced in *L. plantarum* and at last that of *Clearcoli* (**Table 3**). The 4 peaks of *L. lactis* MMP-9cat presented higher activities than those of *L. plantarum* and *Clearcoli*. Comparing the peaks with the highest activity of every expression system, the peak 1 from *L. lactis* was 4-fold more active than the *L. plantarum* peak 1 and 8-fold more active than the *Clearcoli* peak 2.

Table 3. Comparison of yield, purity and activity of purified soluble MMP-9cat produced in 3 different LPS-free bacterial systems. Data obtained from Study 1 and [219]**.**

L. lactis				
	Yield (mg/L)	**Total yield (mg/L)**	**Purity (%)**	**Activity (RFU/min/mg)**
Peak 1	6	10.4	97.4	16
Peak 2	3.1		97.9	10
Peak 3	1.1		98.2	7
Peak 4	0.2		97.3	3
Clearcoli				
	Yield (mg/L)	**Total yield (mg/L)**	**Purity (%)**	**Activity (RFU/min/mg)**
Peak 1	0.049	0.303	<50	<0.2
Peak 2	0.177		94.4	2.5
Peak 3	0.054		62.6	<0.2
Peak 4	0.023		79.6	-
L. plantarum				
	Yield (mg/L)	**Total yield (mg/L)**	**Purity (%)**	**Activity (RFU/min/mg)**
Peak 1	1.92	3.39	>99	4.6
Peak 2	0.70		>99	0.4
Peak 3	0.56		>99	0.3
Peak 4	0.21		>99	0.5

The outcomes presented by *L. plantarum* confirm its capacity to recombinantly produce proteins (Study 1) and expands the current options of available GRAS expression systems to produce immune-related proteins for animal production purposes (**Figure 14**). However, *L. plantarum* did not show significant advantages compared to *L. lactis.* In fact, the yields and activities performed by the protein produced in *L. lactis* were higher (**Table 3**). Moreover, there exist more available tools for recombinant protein production

in *L. lactis* including plasmids [374], strains [367] and a very efficient induction system, the NICE system [377]. For that reason, after performing Study 1 and comparing the results with previously published data we chose *L. lactis* as the expression system to carry out following studies (Study 2, Study 3 and Annex 1).

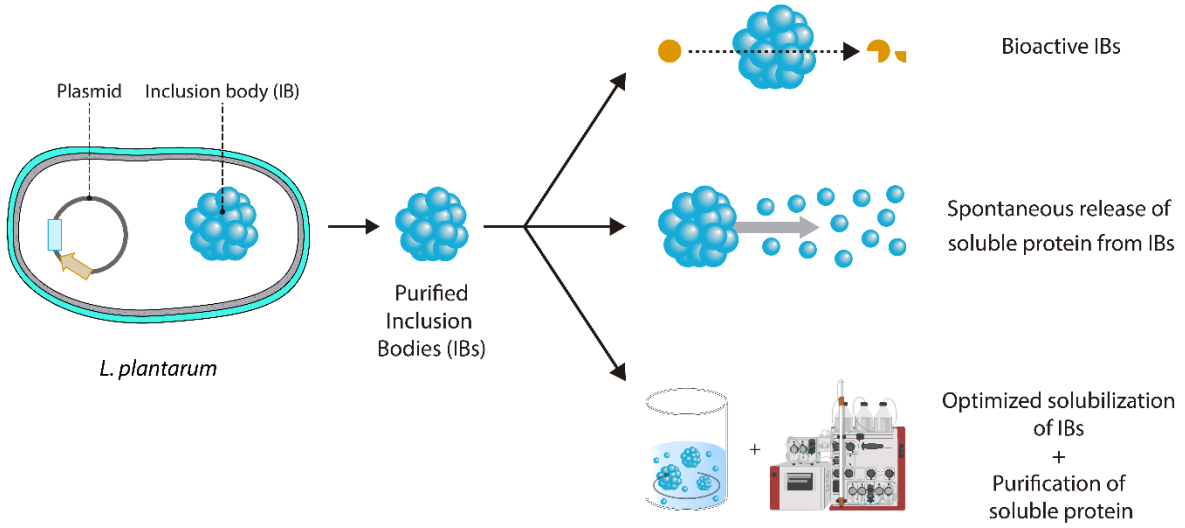

Figure 14. Production of IBs in *L. plantarum* and their final applications. The upper flowline represents that IBs are formed by active proteins, able to catalyze biological reactions. The middle flowline shows the spontaneous solubilization of IBs, permitting to obtain soluble and active protein. Finally, the bottom flowline represents the optimization of solubilization protocols explored, followed by additional purification steps, to gather soluble, active and pure recombinant proteins finally.

Although the selection of the most suitable recombinant expression system is the first step in a recombinant protein production process, this is not the only critical point. Even when a protein is successfully produced, it is often necessary to optimize specific steps of the production and purification processes to obtain the protein in the required conditions. During the protein production process, the recombinant product can be produced in its soluble form, either in the cytoplasm or secreted to the media if it has been designed for that, or aggregated forming IBs [431]. From our experience, and in contrast to the current belief, almost all recombinant proteins aggregate during the overproduction process, at least in a percentage. When proteins are mainly produced as IBs or the presence of other cytoplasmatic proteins makes the isolation of a pure soluble form almost impossible, it is necessary to use protocols for their recovery from IBs. Until a few years ago, the only protein form accepted as correctly folded and potentially active was the soluble form obtained directly from the cytoplasm or secreted in the media. The protein found in IBs was considered misfolded or non-usable. In this context, many

protocols were developed to solubilize protein from IBs with harsh detergents and chaotropic agents (e.g., urea or guanidine hydrochloride at high concentrations) followed by a protein refolding and a purification step [518]. But now, it is well known that IBs contain well-folded and fully active proteins [517,519,520]. Active IBs have been described in *E. coli* [437,521–524], in *L. lactis* [193,222,393,446], and also herein in *L. plantarum* (Study 1). Among these examples, two articles published by our group have shown that immune-related proteins form active IBs in *E. coli* [436] and in *L. lactis* [393]. Another example is MMP-9cat that when produced as IBs in *L. plantarum* (STUDY 1) has a similar activity to the MMP-9 IBs obtained in *L. lactis* [193,218,222]. Thus, simple protocols for the obtainment of functional and soluble protein from IBs but keeping the folded structure of the protein embedded in the aggregate are needed. The isolation of soluble and active protein using mild detergents was already described [221,483], and in fact, we applied these protocols in STUDY 1. However, the last findings of our group working on the solubilization of HDPs, noted an impairment of protein activity in some antimicrobial proteins (especially those based on HDPs) when mild detergents are used for their solubilization [484]. For that, in the current thesis, we wanted to make a step forward in the optimization of protein solubilization protocols using a detergent-free buffer, highlighting the potential of these protein aggregates for the obtainment of functional protein, well-folded and without impurities in cases where the isolation from the soluble fraction is not feasible (Study 1 and Study 2). In a previous work, López-Cano and coworkers demonstrated an optimal solubilization of HDP-based protein from *E. coli* IBs in a detergent-free buffer [484]. Following those results, Study 2 sought to perform wider testing of spontaneous IBs' solubilization protocols in detergent-free buffers, either using IBs formed in *E. coli* or in *L. lactis* but containing immune-related proteins which is the focus of this thesis. Specific conditions of temperature and solubilization time were established by using activity assays for 3 different proteins (**Figure 15**). The model proteins used in this Study 2 were the multidomain protein JAMF-2 expressed in *E. coli,* and the APP M-SAA3 and the cathelicidin BMAP-27 both expressed in *L. lactis*. JAMF-2 was chosen as an example of aggregation-prone protein forming IBs in *E. coli*. Previous works described the high aggregation percentage of the expressed protein [483]. Aiming to study also the behavior of aggregates formed in *L. lactis*, M-SAA3 was chosen as model protein for being a DTE protein [194,233,525]. Previously, M-SAA3 was produced in *L. lactis* and solubilized from IBs using the detergent NLS [221]. Finally, the cathelicidin

BMAP-27 was the third model protein used to study the solubilization of antimicrobial proteins based on HDPs embedded in IBs. Finding a suitable solubilization protocol for these antimicrobial proteins would be of great relevance as around 34% of the recombinant HDPs are extracted from IBs because of the impossibility to obtain them from the soluble fraction [315].

The three model proteins IBs used in Study 2 had different characteristics in terms of origin, structure, and activity. The solubilization screening performed was based on the specific activity of each protein since we demonstrated that it was more sensible and easier to perform than an SDS-PAGE or other protein quantification methodologies. Moreover, the activity gave us an idea of the functional recovered protein from the IBs. At least two activity assays were chosen for each protein trying to elucidate which was the most representative assay to detect solubilized and active protein (**Figure 2.2, Figure 2.3, Figure 2.4, Figure 2.5, Figure 2.6, Figure 2.7** and **Figure 15**). Focusing the screening on activity could also facilitate the implementation of the protocol in all kinds of labs producing a recombinant protein since having settled the protein activity assay is almost mandatory when one is interested in the obtention of the protein. We performed several activity assays including an antimicrobial activity assay (**Figure 2.2** and **Figure 2.4**), an enzymatic activity determination (**Figure 2.5**), the fluorescent emission determination (**Figure 2.3**), and the immunostimulatory activity detection (**Figure 2.6, Figure 2.7** and **Figure 15**). Regardless of their different nature and disparate running conditions, we have demonstrated that all of them are useful to monitor the activity of proteins solubilized from IBs. In the case of JAMF-2 the antimicrobial assay was more sensible than the enzymatic assay (**Figure 2.4** and **Figure 2.5**) but in the case of BMAP-27 the antimicrobial assay was less sensible than the fluorescence emission determination (**Figure 2.2** and **Figure 2.3**). In the same line, M-SAA3 was better monitored by its immunostimulatory functions than antimicrobial ones (**Figure 2.6, Figure 2.7** and **Figure 15**).

From Study 2 we also could extract general conclusions in terms of activity. The presence of a carrier protein such as GFP (used for BMAP-27 expression) offers the opportunity to follow up the solubilization profile of a fusion protein using the fluorescence emission with a good correlation with the active domain (**Figure 2.3**). Also, in the case of proteins sharing structural domains such as a carrier protein or those belonging to the same

generation of recombinant proteins with similar functions, the results can be extrapolated from previous studies and just perform the activity assay to corroborate the spontaneous solubilization process and adjust timing and temperature conditions if needed. The specific activity detected in Study 2 for the solubilized JAMF-2 was comparable to that previously determined by Roca-Pinilla and coworkers [483], but applying mild detergents to solubilize the protein. It is worth noting that in Study 2 of this thesis, the antibacterial activity was achieved with a simple and rapid solubilization. In contrast, the protocol applied in previous studies was longer and required extra agents and procedures such as mild detergents and chromatography purifications. The second model protein, BMAP-27, showed antibacterial activity against MRSA, a Gram-positive antibiotic-resistant pathogen, reaching almost a 99% of survival reduction (**Figure 2.2**). These results were highly comparable to those obtained in similar studies performed with the solubilized HDPs-based proteins without the use of any solubilization agent [484]. López-Cano and coworkers achieved similar growth reductions of *E. coli* strains by human α-defensin 5 (HD5) and bovine lingual antimicrobial peptide (LAP), both solubilized from IBs in detergent-free buffers [484]. Finally, the M-SAA3 activity observed in Study 2 was comparable with the previous results obtained with this protein. Parés et al. explored the immunostimulant activity of an M-SAA3 produced in *E. coli* and purified from the soluble fraction [233]. The highest M-SAA3 concentration (160 μg/mL) increased the IL-8 expression 3-folds approximately in primary mammary cell cultures. Then, Gifre-Renom and coworkers analyzed the activity of a solubilized (using NLS) and purified M-SAA3 produced in *L. lactis* at the highest working concentration of 90 μg/mL, achieving a 4-fold increase in IL-8 expression in bovine epithelial cells from the mammary gland [221]. The solubilized M-SAA3 obtained in Study 2 was able to stimulate the expression of IL-8 in the same cells 10 times more compared to the control (**Figure 2.6**). The antibacterial activity of M-SAA3 was already described by Parés and coworkers against several pathogens' causative of IMIs [233]. The highest M-SAA3 working concentration (160 μg/mL) reduced the presence of infective *S. aureus* in mammary primary cell cultures from 4.5 x 10^5 CFU/mL to 2.5 x 10^5 CFU/mL. In Study 2, the solubilized M-SAA3 reduced the infective *S. aureus* from 2.2 x 10^4 CFU/mL to 1.8 x 10^3 CFU/mL (**Figure 2.7**). Although the level of bacterial infection reduction was lower, the anti-infective activity of the solubilized M-SAA3 was demonstrated and at acceptable levels. In both assays performed, the M-SAA3 activity shown in Study 2 was highly

comparable (or even better) with the previous studies but applying a simpler solubilization protocol.

For the first time, a spontaneous solubilization process has been applied to IBs from different origins. The characterization of 3 proteins with different structures and activities has opened the application of agent-free solubilization protocols for a wide range of proteins difficult to obtain from the soluble fraction. It seems that for the three model proteins studied herein the 37°C is an optimum temperature, but not in all proteins it is necessary to incubate the suspension for up to 48 h (Study 2). In the case of JAMF-2, 16 h of solubilization has the same efficiency of activity recovery as 48 h (**Figure 2.4**). However, in the case of unstable proteins, where the temperature could impair protein stability, testing lower temperatures for the solubilization procedure could be a critical point to be considered.

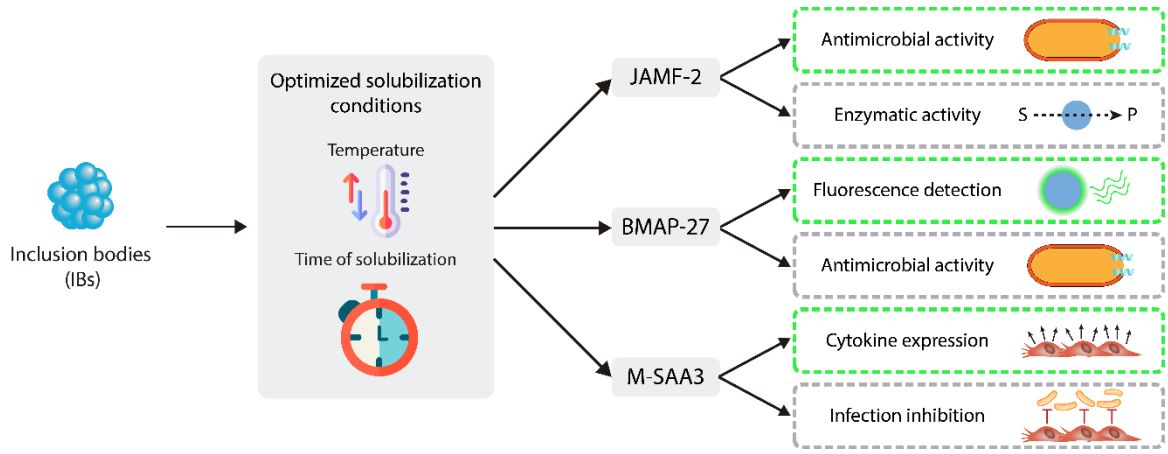

Figure 15. Schematic summary of the screening of solubilization protocols by monitoring the specific protein activity without requiring any solubilizing agent. Optimization of temperature and time of solubilization gather in a spontaneous release of soluble and active protein. These procedures were explored for JAMF-2, BMAP-27, and M-SAA3 proteins. Activity quantification was assessed by a specific activity test. The most suitable activity assays to monitor the protein solubilization are shown in green, whereas the specific activity tests that did not allow the correct monitorization of the protein solubilization are shown in grey.

The Study 1 and Study 2 allowed us to rationally choose a LAB-based expression system and to set up suitable protocols to recover protein either from soluble or insoluble fractions maximizing the yield and efficient cost for animal production applications. At that point, the Study 3 arises with the objective to design a new generation of proteins to treat complex IDs caused by bacterial and viral pathogens.

Recombinant antibacterial and antiviral HDPs

The treatment of multi-etiological diseases caused by different types of pathogens is an arduous challenge, especially when bacteria and viruses are involved because there is any drug able to kill both types of pathogens. Thus, in Study 3 of this thesis, we aimed to develop an all-in-one antibacterial and antiviral drug to treat BRD disease.

It has been previously highlighted in sections "*Host Defense Peptides*" and "*Recombinant antimicrobials for bovine IDs*" that HDPs have a huge potential for the treatment of bovine IDs. Their antibacterial and antiviral activity makes them quite unique [303,487]. Besides, HDPs have a clear advantage over conventional antibiotics since they have been shown to be associated with a lower emergence of bacterial resistance [526,527]. Hence, they were chosen in Study 3 as the most promising molecules in front of others like MMPs or APPs previously explored in our group [192,194,214,219–221,232,233].

The HDPs' antibacterial activity has been widely characterized since their discovery in 1980 [528]. The presence of negatively charged molecules in the outer layers of bacteria favors the interaction with the positively charged HDPs. The outer membrane of Gram-negative bacteria contains the LPS and the cell wall of Gram-positive is formed by the teichoic acids, both molecules with negative charges [529]. Thus, HDPs have high avidity to interact with these structures and disrupt the bacterial envelope [303]. Defensins and cathelicidins, being both HDPs, have shown activity against Gram-negative [255,530–532] and Gram-positive [255,533] bacteria. By contrast, the antiviral activity of HDPs was discovered later than the antibacterial one, but the knowledge in this field has increased exponentially and the antiviral activity of HDPs against several types of enveloped and non-enveloped viruses has been described [487]. Specifically, it seems that the mode of action against enveloped viruses is very similar to the charge-dependent interaction displayed against bacteria [534] and there exist HDPs active against HSV [276], HIV [269], RSV [270], influenza virus [535], adenoviruses [536], SARS-CoV-2 [537], MERS-CoV [538], rhinovirus [273] among others [278,287]. By contrast, the mode of action against non-enveloped viruses is not so well-understood. Different studies are trying to elucidate if HDPs act directly on the virions as for enveloped viruses or if their activity is a result from the immunostimulation that they promote converting the immune system in the direct antiviral actor [487,534,539]. Nevertheless, the two activities (antibacterial and antiviral) have not ever been studied in conjunction for pathogens involved in the same

pathology and Study 3 has demonstrated for the first time that a HDP or a combination of them (Annex 1) could be used against both bacterial and viral pathogens involved in the same multi-etiological ID. Among the bovine recombinant HDPs tested in the current thesis, we have observed activities against Gram-positive bacteria (Study 2), Gram-negative bacteria (Study 3), *Mycoplasma* species (Study 3) and enveloped viruses (Study 3), with some of the HDPs having 2 or more different activities. We find examples of HDPs in the literature with broad antimicrobial activities, being active against different strains of Gram-negative bacteria such as the human β-defensin-4 [540], against different strains of Gram-negative and Gram-positive bacteria such as the cecropin AD [541], or active against bacteria and yeast such as the cathelicidin-BF [504]. Also, there exist HDPs with broad antiviral activities as the short peptide P9 (derived from the mouse β-defensin-4) [538] or the human α-defensins [534] which are active against several enveloped viruses [538]. Nevertheless, some of the bovine recombinant HDPs tested in this thesis have not only presented broad antibacterial or antiviral activities but have joined both (**Figure 3.4**).

A detailed analysis of the HDPs activities show that they do not only have a broad-spectrum activity against both bacteria (**Figure 3.1, Figure 3.2** and **Table 3.3**) and viruses (**Figure 3.3**), but also that the activities reached are comparable to those previously described by other publications working with similar cathelicidins and β-defensins tested against single bacteria or viruses [476,504,534,538,542–544]. Moreover, in the case of bacteria, we observed both a bacteriostatic (inhibition of bacterial growth) and a bactericidal effect (bacteria killing) with some HDPs such as BNBD-1, BNBD-3, BNBD-4 and TAP (**Figure 3.1A** and **Table 3.3**). Although it has not been described before, the bacteriostatic activity of the recombinant HDPs tested in Study 3 (**Table 3.3**) was highly comparable with other recombinant and synthetic HDPs previously described and tested against other Gram-negative and Gram-positive bacterial strains [476,504,542–544]. Study 3 describes for the first time the MIC of BNBD-1, BNBD-3, BNDB-4 and TAP against Gram-negative bacteria involved in BRD such as *P. multocida* and *M. haemolytica,* ranging between 2-4 μM (**Table 3.3**). In previous studies, He and coworkers produced a cathelicidin in *B. subtilis* active against *E. coli* and *S. aureus* with MICs of 0.44 μM and 1.78 μM respectively [504]. We found another example in Li et al., who expressed also in *B. subtilis* two secreted AMPs with antibacterial activity against Gram-negative and Gram-positive pathogens with MIC values ranging from 7 to 45 μM

[542]. A similar screening was done in our group producing four HDPs fused to GFP in *E. coli* against different Gram-positive and Gram-negative bacteria. The MIC against both types of pathogens went from 1 to 8 μM [476]. Other examples of studies on HDPs' bacteriostatic activity have been performed using peptides chemically synthesized. Tan and coworkers characterized the antibacterial activity of five multidomain HDPs (combining magainins (amphibian AMPs), cecropins and cathelicidins) and the MIC against *E. coli* and *S. aureus* varied from 1 to 64 μM depending on the peptide and the pathogenic strain [543]. Xu and coworkers studied synthetic HDPs against several Gram-negative and Gram-positive pathogens and the MIC ranged from 0.5 μM to over 256 μM depending on the peptide and the pathogenic strain [544]. Thus, there exist many previous studies focused on the antibacterial characterization of recombinant and synthetic HDPs, but they presented less broad antimicrobial activities than the HDPs designed in Study 3 at similar working concentrations.

In terms of bactericidal activity, there exist many different methodologies for its specific characterization and there are not as well standardized as MIC protocols. Thus, it is worth mentioning that performing an explicit comparison becomes very arduous. Nevertheless, we focused the attention on the lowest working concentration and the percentage of survival reduction. Considering that, we obtained values at the same level or even better than those previously published [484,531,545–547]. The HDPs produced in Study 3 using *L. lactis* presented a high performance against the three Gram-negative pathogens tested, reaching values of 99 % viability reduction at a working concentration of 5 μM (**Figure 3.1A**). BNBD-1, BNBD-3 and TAP were the best killing the 3 bacterial strains with values ranging from 95 % to 99 % (**Figure 3.1A**). There exist previous studies with other recombinant HDPs produced in *E. coli.* Some of them described reductions of *E. coli* survival up to 90 % at a maximum working concentration of 6 μM [484,545]. Other studies described the bactericidal activity of the bovine β-defensin LAP and the human α-defensin 5 (HD5) against MRSA and *P. aeruginosa* killing the bacterial suspension over a 95 % at a working concentration of 5 μM [470]. The bactericidal activity of synthetic peptides have also been studied. For instance, the study of de Leeuw and coworkers described the bactericidal activity of a human α-defensin against *S. aureus* achieving bacterial reductions near 99 % at a working concentration of 5 μM [531]. Another example of a synthetic peptide, but this time with a broader bactericidal activity against Gram-negative and Gram-positive bacteria, is the α-helix-based AMP characterized by

Prada and coworkers, which killed MRSA and *E. coli* 35 % and 55 % respectively at 5 μM and achieved a 95 % reduction of both bacteria at 25 μM [546]. In addition, a battery of synthetic peptides was tested against several *E. coli* strains by Prada-Prada and coworkers [547]. They were able to kill *E. coli* until 99.9 % at concentrations ranging from 3 μM to 23 μM. Thus, in terms of bactericidal activity, the recombinant HDPs assessed in the current thesis had an efficient and broad performance against three different Gram-negative bacteria at an acceptable working concentration.

Besides, in Study 3 we also tested the antibiofilm effect of bovine recombinant HDPs (**Figure 3.2**). Determining their antibiofilm activity was highly relevant to do a complete characterization, especially in the context of BRD. Biofilms represent one of the main strategies of bacteria to resist the action of antimicrobials [548], and this bacterial structure is widely present in respiratory infections [549]. The antibiofilm activity of HDPs has been previously described [550]. Encouragingly, an acceptable reduction of *M. haemolytica* biofilms was achieved in Study 3, especially with TAP which reduced the biofilm formation near 50 % at 5 μM (**Figure 3.2**). In agreement with this, although at a lower level, these results concur with a previous work of our lab describing the antibiofilm activity of HDPs produced in recombinant *E. coli* [476] with which we obtained biofilm reduction values ranging from 75 % to 99 % at the same protein concentration. Antibiofilm effects of a fungal defensin-like peptide produced in *P. pastoris* was studied against MDR *S. aureus* biofilms, achieving reductions of 100 % at working concentrations ranging from 4 μM to 32 μM [551]. In addition, the antibiofilm activity of synthetic HDPs from different origins and species has been published [552–554]. Overall, *P. aeruginosa* is one of the most relevant biofilm former pathogen, and for that reason many studies are focused on it. The human cathelicidin LL-37 is by far the most studied among HDPs [555], but there exist more examples from mouse, bovine or amphibian. Chen and coworkers performed a wide screening characterizing the anti-P. *aeruginosa* biofilm activity of 13 HDPs [552]. The mouse cathelicidin CRAMP and the human cathelicidin LL-37 were able to reduce the 100 % of the preformed biofilms at 20 μM. A similar study by Overhage et al. compared the antibiofilm activity of the most relevant HDPs from human (LL-37), mouse (CRAMP) and bovine (indolicidin and Bac2A) against *P. aeruginosa* biofilms [553]. Whereas LL-37 and indolicidin reduced the biofilm biomass over an 80 %, CRAMP and Bac2A did not present any biofilm reduction. Furthermore, Luca and coworkers described the antibiofilm activity of an amphibian HDP which

reduced the 50 % of *P. aeruginosa* biofilms at 3 μM and the 100 % at 48 μM [554]. After all, the antibiofilm activity showed by the recombinant HDPs of the current thesis at 5 μM is not far from the existing studies on the antibiofilm activity of HDPs, although it needs to be better characterized and optimized.

Although the research on new antibacterial molecules is mainly focused on the development of alternatives to attack Gram-positive and Gram-negative bacteria, there are other bacteria not included in these groups, such as those belonging to *Mycoplasma* genus, which are also challenging and involved in syndromes such as BRD. Their pleomorphic structures without cell wall and only with the plasmatic membrane surrounding them, hinders the identification of suitable targets or strategies to disrupt them or inhibit their growth. Nowadays, classical antibiotics such as macrolides, tetracyclines, and fluoroquinolones are the first-line treatment for *Mycoplasma* infections [556,557]. However, strains with acquired resistance have emerged [558,559]. Although the most HDPs antibacterial activity described would not be useful against *Mycoplasma*, because of the negatively charged outer structures of Gram-negative and Gram-positive bacteria are lacking in *Mycoplasma*, there exist some studies describing the antimycoplasma activity of HDPs. Different examples of synthetic peptides with direct antimycoplasma activity have been published. For example, a potent activity of the human β-defensin 2 [560] and magainin-derived peptides [561] against *Mycoplasma pneumoniae* at 4 μM and 30 μM respectively has been described. Also, examples of antimycoplasma activity by recombinant HDPs have been reported. However, these examples only have described the HDPs' function as adjuvants in developing immune responses against *Mycoplasma* infections [481,562]. Thus, as far as we know, the direct antibacterial activity of recombinant HDPs against *Mycoplasma* genus has never been described. This fact reinforces the novelty of the antimycoplasma activity shown by the bovine recombinant HDPs of Study 3 at a concentration of 5 μM (**Figure 3.1B**), compared to the previous studies mentioned above with synthetic peptides [560,561]. There exist similar screenings to the one performed in Study 3 on the antimycoplasma activity of different synthetic HDPs [561,563]. Hayashi and coworkers described a significant reduction of *Mycoplasma* concentration by a group of magainin-derived peptides (a group of amphibian HDPs) at a working concentration of 30 μM [561]. For their part, Dassanayake et al. achieved reductions near the 90 % at the same concentration of 30 μM of NK-lysin peptides, whereas at the concentration of 5 μM the highest reduction was near

the 50 % [563]. Compared with those results, the antimycoplasma activities near the 99 % of the recombinant cathelicidin Bac5 and the anionic peptide SAAP are highly relevant (**Figure 3.1B**). It is important to note that, although Bac5 and SAAP presented moderate or low activities against Gram-negative bacteria (**Figure 3.1A**), they were the HDPs with the highest reductions of *M. bovis* growth (**Figure 3.1B**). This contraposition could be related to the mode of action of HDPs to attack *Mycoplasma*. The antimicrobial activity of HDPs against Gram-negative and Gram-positive bacteria is well-defined, but the one concerning *Mycoplasma* remains quite unexplored. Interestingly, it seems that they could interact with the phosphatidylcholine (PC) of the plasmatic membrane, because it has been described a direct connection with the interaction between AMPs and PC and the activity of this AMPs against *Mycoplasma* [564]. Moreover, it has been reported that exogenous PC is a very uncommon lipid in wall-covered bacteria. This fact could explain the low performances of Bac5 and SAAP against the Gram-negative bacteria (**Figure 3.1A**) but showing a good performance against *M. bovis*. By contrast, BNBD-1 showed acceptable activity for *Mycoplasma* and for the rest of bacterial pathogens (**Figure 3.1A** and **Figure 3.1B**, **Table 3.3**), indicating different mode of action than Bac5 and SAAP.

Finally in the context of complex IDs such as BRD, involving bacterial and viral pathogens, the activity of the bovine recombinant HDPs was also evaluated in Study 3 against three of the main viral pathogens causing BRD (**Figure 3.3**). Among the six HDPs tested, BNBD-1 was the best one, increasing cell culture viability up to 40 %, 10 %, and 37 % under infections by BHV-1, PI-3 virus and BVDV, respectively (**Figure 3.3**). LAP was the second most potent HDP increasing also a 40 %, 18 %, and 20 % the cell viability under viral infections by the three virus in the same order (**Figure 3.3**). Many different types of HDPs have antiviral features, but defensins take up a special place in this field [534,565]. The direct action of defensins on the virions, mainly disrupting the viral envelope and interrupting their replication cycle has been described [566]. Concretely, defensins have shown activities against relevant enveloped viruses such as HIV [567], HSV [568], influenza virus [535], adenoviruses [536], SARS-CoV-2 [537] or MERS-CoV [538]. Nevertheless, it is no so clear which is the exact mechanism by which defensins affect viruses or reduce the viral infection capacity [534]. It has been corroborated that there exists an interaction of defensins and viral particles, but not always the envelope seem to be structurally affected [569]. The composition of the envelope can be very heterogenic and can vary depending on the eukaryotic membrane that comes from, and for that

reason it is difficult to elucidate the peptide-envelope interaction. Moreover, defensins have shown other strategies beyond the direct peptide-virus interaction, inhibiting the viral infection in many other ways. They can block the binding of the virus with the receptors of the host cell [570] or to inhibit the viral fusion or penetration of host cell lipid bilayers [571]. The antiviral effects shown by the bovine recombinant β-defensins in Study 3 may be based on some of these mechanisms. Regarding the values of antiviral activity shown against BHV-1, the β-defensins BNBD-1 and LAP increased the cell viability a 40 % at the low concentration of 0.313 μM (**Figure 3.3A**). Against PI-3 virus, the BNBD-1 increased the cell viability a 10 % and the LAP a 18 % at a concentration of 0.313 μM (**Figure 3.3B**). Against BVDV, the β-defensins BNBD-1, BNBD-2, BNBD-3, and LAP increased the cell viability a percentage of 21 %, 37 %, 27 %, 12 % and 20 % respectively, at a working concentration of 5 μM except for LAP that had the highest activity at 2.5 μM (**Figure 3.3C**). A similar study by Quiñones et al. described the antiviral activity of recombinant human β-defensins 1, 2 and 3 against HIV-1 [567]. The recombinant HβD1, HβD2 and HβD3 reduced the viral infection a 20 %, 75 % and 80 % respectively, at the working concentrations of 10.4, 9.6 and 8 μM respectively. Another example published by Hazrati and coworkers described the antiviral activities of synthetic human β-defensins 1, 2, and 3, where HβD3 reduced the viral infection of HSV near a 60 % at the concentration of 1.3 μM, and the HβD1 and the HβD2 did not achieve viral reductions at any concentration [568]. Thus, the antiviral activity presented herein by the recombinant bovine β-defensins against the 3 enveloped viruses is at comparable levels with other studies with similar recombinant and synthetic β-defensins.

The antiviral activity of the recombinant cathelicidin Bac5 is not insignificant (**Figure 3.3**). It increased the viability of the cells infected by the PI-3 virus and BVDV in a 15 % and 21 %, respectively, at working concentrations of 0.625 μM and 5 μM (**Figure 3.3B** and **Figure 3.3C**). These results agree with other publications which described the antiviral activity of cathelicidins [270,273,278]. Focusing on synthetic bovine bactenecins, Shestakov et al. described the antiviral activity of four bovine bactenecin-derived peptides against HSV-2 [572]. Although they used high concentration (70 μM), the four peptides reduced the viral infection in values ranging from 70 % to 80 %. The human cathelicidin LL-37 and the mouse cathelicidin CRAMP are the most well-studied, presenting activity against several enveloped viruses such as Vaccinia virus (VV), RSV, Influenza, HIV, HSV and Dengue virus [278]. The mechanism of antiviral activity of

cathelicidins include direct damage to viral envelopes, inhibition of viral protein function and modulation of host cell responses to infection [278]. As a final example out of the classical classification in defensins and cathelicidins, we find the study of Wang et al. describing the antiviral activity of recombinant *de novo* designed peptides against paramyxoviruses and coronaviruses infections in values ranging from 20 % at 0.2 μM to 100 % at 25 μM [573]. Overall, the antiviral nature of the bovine recombinant HDPs is highly comparable to the previous studies on the antiviral functions of HDPs. Beyond this, there are not so many examples describing the antiviral activity of recombinant HDPs for bovine viruses [573–575] and this situation further increases the relevance of the results presented in this thesis.

Summing up the outcomes of Study 3, BNBD-1 has been postulated as the most versatile peptide showing activity against all the BRD bacteria and viruses tested (**Figure 3.1, Figure 3.2** and **Figure 3.3, Table 3.3**). Also, LAP has shown both antibacterial and antiviral activity (**Figure 3.1, Figure 3.2** and **Figure 3.3**). BNBD-3, for its part, has shown high anti-Gram-negative bacteria activity (**Figure 3.1** and **Figure 3.2**). And finally, Bac5 has joined anti-Gram-negative, antimycoplasma and acceptable antiviral activities (**Figure 3.1** and **Figure 3.3**). This four recombinant HDPs are the most promising among all the tested battery. But which is the next step? (**Figure 16**).

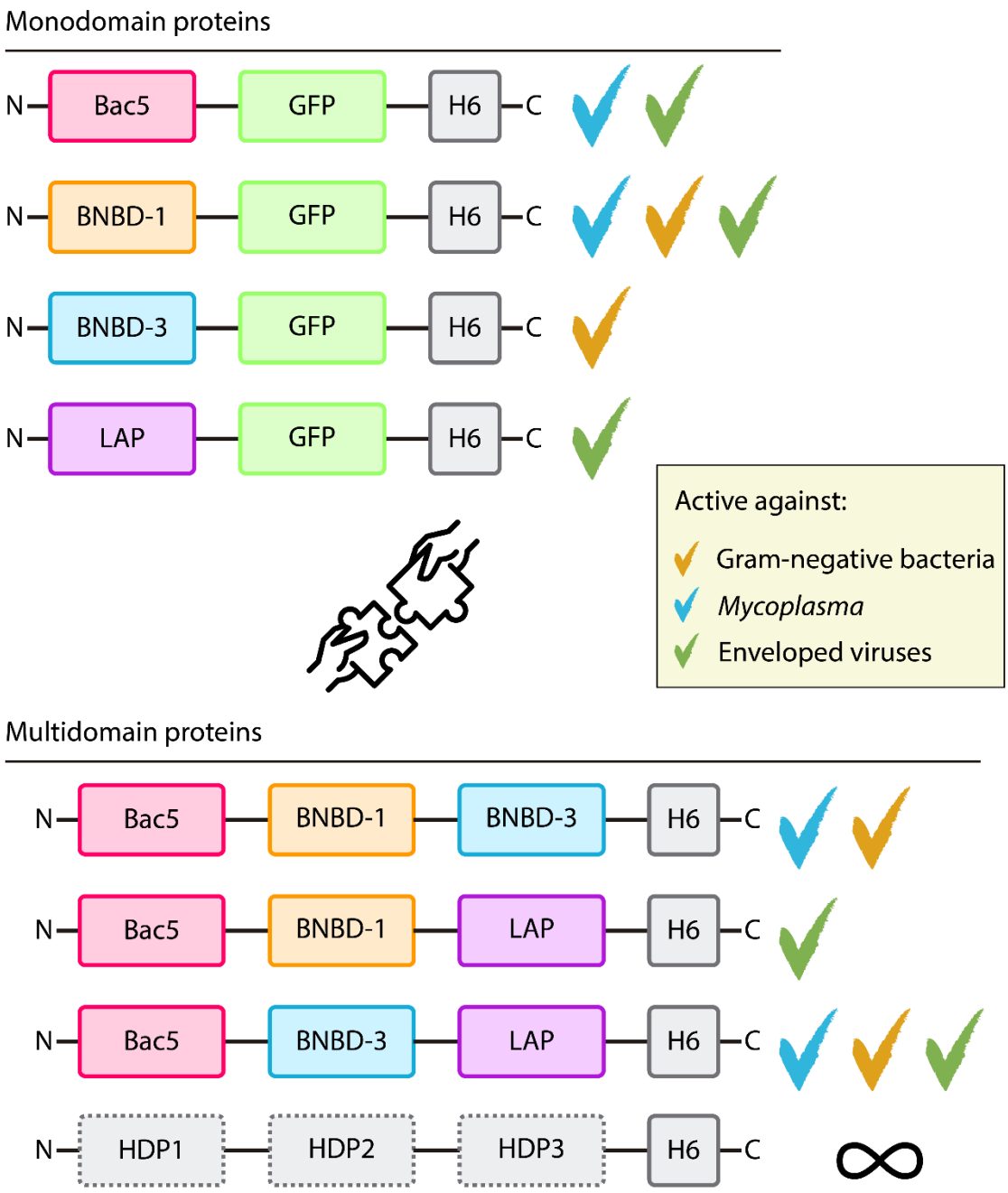

Figure 16. Recombinant HDPs schematic representation. Monodomain proteins formed by a single HDP are represented in the upper part. They comprise a HDP domain, followed by GFP (in green), which improves its solubility and allow their recombinant production. Moreover a 6-histidine tag (H6, in grey), is added at C-terminus end, allowing its purification. In the current scheme, Bac5, BNBD-1, BNBD-3 and LAP HDP domains are the most promising antimicrobial and antiviral candidates. A step forward in the development of new molecules consists in the design of multidomain proteins, combining diverse HDPs and a histidine tag (H6). In the current scheme, 3 examples of combinations are shown, but the possibilities are almost infinite. In the legend, there are shown colored ticks: orange tick corresponds to Gram-negative bacteria active proteins, blue tick corresponds to *Mycoplasma* active proteins and green tick corresponds to enveloped viruses active proteins.

The first possibility is to combine the selected HDPs using a kind of nanoparticle. In our case we have explored the possibility to combine several activities of recombinant HDPs by using polymer-based micelles (Annex 1).

Another approach could be a strategy previously established in our lab using HDPs produced in *E. coli* and selected to fight against nosocomial infections [476]. In that study the best-performing HDPs were combined in a single polypeptide removing the GFP carrier protein (used to express single HDPs) to evolve to a fully-multidomain protein with better activity (antibacterial activity against planktonic bacteria and biofilms) and best performances (lower MIC) [476]. We find similar approaches with synthetic peptides evaluated by separate (MIC from 1 to 256 μM) to select the best ones and form a multidomain peptide which improved the MIC from 1 to 4 μM [544].

Although the strategy to combine HDPs in the same polypeptide is promising, choosing the best HDPs is not the only point to consider, also the order matters. Recent studies of our group have described the importance of the correct order and combination of the different domains inside the final multidomain proteins [576]. Depending on that, the final activity could be altered. Hence, producing multidomain molecules formed by the same proteins but with different orders might be crucial to achieve the highest antimicrobial and antiviral activity.

The future applicability of these multifunctional proteins could be especially relevant on animal production, and more specifically in bovine industry. Preliminary results of *in vivo* experiments performed in calves (Annex 1) confirm the potential of bovine HDPs at controlling BRD-involved pathogens. Using drugs like these based on HDPs could reduce the application of metaphylaxis at farm and the risk to promote antimicrobial resistances. In addition, focusing the potential of recombinant HDPs from another perspective, the exploration of their immunomodulatory capabilities could be an interesting alternative on the reduction of the use of antibiotics in animal production. Preparing the immune system of the animals could avoid infections and the posterior use of antibacterial and antiviral drugs for the treatment of bovine IDs.

To conclude, though this thesis, *L. lactis* has proven to be a good alternative to produce immune-related proteins including MMPs (Study 1), APPs (Study 2) and HDPs (Study 2 and Study 3). Unlike chemically synthesized HDPs, the recombinant production has any limitation in terms of peptide length and scalability making the applicability of immune-

related proteins in animal production economically feasible. Besides, their production in a LAB allows to obtain LPS-free products, and this fact also directly reduces the costs, because no further purification processes are needed to eliminate LPS traces. In the context of the recombinant production of HDPs, the robustness showed by *L. lactis* is highly relevant. Overall, the results of Study 3 raised *L. lactis* as a highly versatile HDP producer, a feature not previously explored before in depth in this bacterial strain. Nine bovine HDPs including 1 cathelicidin, 6 β-defensins, 1 surfactant protein and 1 anionic peptide have been efficiently produced in *L. lactis* and purified at acceptable yields of 0.07 – 1.19 mg/L and with purities over 75% (Study 3). These encouraging results are not only a result of the bacterial expression system chosen, but also the result of the optimization of the whole recombinant protein production and purification strategy. To recombinantly produce HDPs, we bet for design of HDPs fused to the GFP which performed the function of *carrier* protein (Study 2 and Study 3), which has proven to be a good strategy to reduce protein toxicity and allow their recombinant production. Moreover, it seemed that the fusion of the GFP favored the solubility of the final products because all the HDPs produced were at least in 50 % soluble in the bacterial cytoplasm. However, in the case that the soluble protein was not enough we have developed a protocol to recover active protein from the IBs without the use of any denaturing agent or non-denaturing detergent (Study 2). Also, we have demonstrated the clear and broad-spectrum antibacterial and antiviral potential of recombinant HDPs, and that these peptides are among the most promising molecules from all reviewed and discussed through this dissertation for the treatment of bovine IDs (Study 3) (**Figure 17**).

It is worth mentioning that we have done a deep characterization at *in vitro* level and the potential of these molecules needs to be further explored. However, preliminary results of a first in vivo experiment, which have been included in Annex 1, are encouraging.

Hopefully the outcomes derived from this thesis will be the beginning of a new treatment for BRD and a promising alternative to the use of antibiotics in animal production.

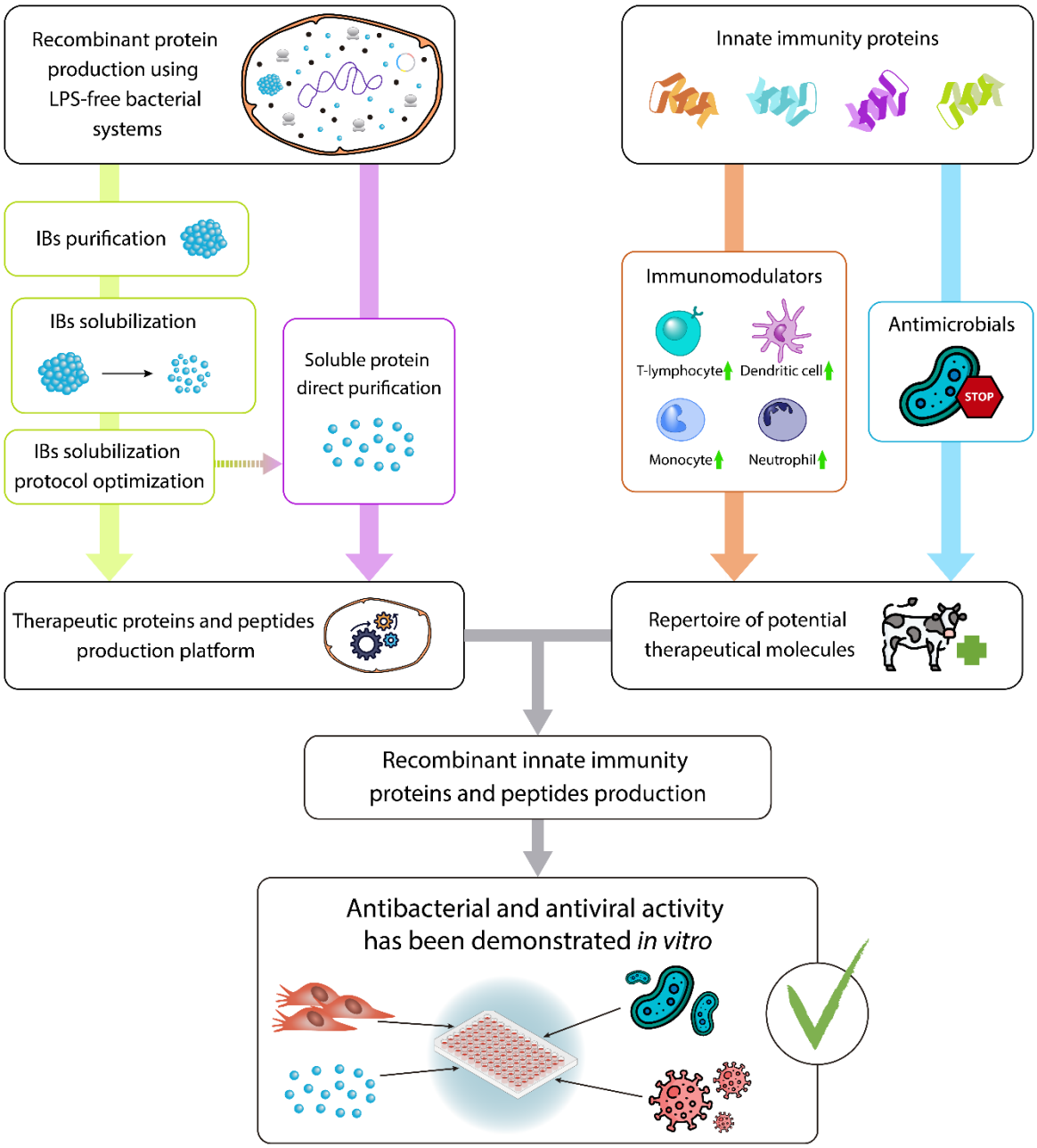

Figure 17. The production of recombinant proteins in LPS-free systems is an attractive approach in the field of biological drug development. *L. plantarum* has been proven as a good system to produce immune-related proteins. Moreover, the optimization of protocols for the obtention of soluble and solubilized protein (from IBs) without the use of any denaturing agents or detergents have been developed. The immune system is an inspiration for a wide catalog of new molecules to fight against infectious diseases. This thesis has demonstrated a clear potential of bovine host defense peptides (HDPs) against bacterial and viral pathogens in cattle, being a feasible alternative for the treatment of complex diseases such as BRD.

CONCLUSIONS

The overall results obtained and discussed in this thesis, focused on the study of recombinant innate immunity-related proteins for the development of new antibacterial and antiviral molecules as alternative treatments for bovine infectious diseases, can be summarized in the following conclusions:

1. *Lactiplantibacillus plantarum* has been validated as an LPS-free recombinant expression system for the production of innate immune-related proteins using MMP-9 as model protein.

2. Formation of inclusion bodies in *Lactiplantibacillus plantarum* has been described for the first time in this thesis. The aggregates formed under protein production conditions have structural and functional characteristics resembling those found in *Escherichia coli* and *Lactococcus lactis*. Briefly, *Lactiplantibacillus plantarum* inclusion bodies are electron-dense structures with an average size of 250-300 nm that contain active protein that can be spontaneously released.

3. The prone-to-aggregate protein MMP-9 has been successfully produced in *Lactiplantibacillus plantarum* but at lower yields than that previously described using *Lactococcus lactis*.

4. The optimization of solubilization protocols free of denaturing and non-denaturing agents has allowed the spontaneous release of soluble and active innate immunity-related proteins from inclusions bodies formed in *Escherichia coli* and *Lactococcus lactis*.

5. The use of specific activity assays has been stablished as a good screening strategy to monitor the protein solubilization from inclusion bodies and has allowed to determine the optimal solubilization conditions for each protein of interest.

6. *Lactococcus lactis* has been confirmed as a robust bacterial cell factory for the production of bovine innate immunity-related proteins, including metalloproteinases, acute phase proteins and Host Defense Peptides.

7. The fusion of candidate Host Defense Peptides with Green Fluorescent Protein has revealed as a good strategy to avoid peptide degradation and toxicity on the bacterial host, and also it has had a positive impact on peptide solubility.

8. Bovine recombinant Host Defense Peptides produced in *Lactococcus lactis* have shown a broad-spectrum activity against both bacterial and viral pathogens causing the Bovine Respiratory Disease. Particularly, tested proteins have displayed activity against relevant bovine pathogens including Gram-negative bacteria such as *Pasteurella multocida*, *Mannheimia haemolytica* and *Histophilus somni*, bacteria lacking cell wall like *Mycoplasma bovis* and three different enveloped viruses, bovine herpesvirus type 1, bovine parainfluenza 3 virus and bovine viral diarrhea virus.

9. Recombinant BNBD-3 has presented the broadest anti-Gram negative activity being active against the three Gram-negative bacteria tested, *Pasteurella multocida*, *Mannheimia haemolytica* and *Histophilus somni*.

10. Recombinant Bac5, and LAP have been postulated as Host Defense Peptides with both antibacterial and antiviral activities. Bac5 is active against *Pasteurella multocida*, *Mycoplasma bovis*, bovine parainfluenza 3 virus and bovine viral diarrhea virus. LAP is active against *Pasteurella multocida*, *Histophilus somni*, bovine herpesvirus type 1, bovine parainfluenza 3 virus and bovine viral diarrhea virus.

11. Among all candidates, β-defensin BNBD-1 has stand out as the most multi-functional recombinant Host Defense Peptide, being active against all evaluated bovine pathogens: *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, *Mycoplasma bovis*, bovine herpesvirus type 1, bovine parainfluenza 3 virus and bovine viral diarrhea virus.

12. The recombinant production of innate immunity-related proteins has arisen as a promising alternative to treat multifactorial bovine infectious diseases.

ANNEXES

ANNEX 1

The potential of Host Defense Peptides-based nanoparticles for the treatment of Bovine Respiratory Disease

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Introduction

Bovine respiratory disease (BRD) is a multi-factorial disease, usually resulting from the interaction of bacterial and viral agents, combined with stress factors such as weaning, transportation, pooling of cattle from multiple sources, dusty conditions, parasitism, concurrent diseases and weather extremes and environmental factors ultimately resulting in bronchopneumonia [1]. It has been and continues to be one of the largest causes of morbidity and mortality of dairy calves and feedlot cattle [2]. The viral pathogens associated with BRD include bovine herpesvirus type 1 (BHV-1), parainfluenza-3 virus (PI-3), bovine viral diarrhea virus (BVDV), and bovine respiratory syncytial virus (BRSV). In addition, BRD is associated with several bacterial pathogens, of which the major ones are: *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, and *Mycoplasma bovis*. Combined, the effects of viral infection facilitate the proliferation of opportunistic bacterial pathogens in the upper respiratory tract and translocation of these pathogens into the lung which cause infections to the compromised lung [3].

Specifically looking deeper in the lung, pulmonary innate immunity is the first and an important line of defense when an animal is fully immune-competent. Besides the specific anatomy of the respiratory tract to prevent any type of infection, pulmonary tissue secretes several active molecules which also have a critical role against invading agents such as bacteria and viruses. Alteration of regulation, expression, and function of these factors due to stressful situations during bovine production impairs immune response and may be important to disease development and resolution [4].

Vaccination protocols are part of a comprehensive herd health program and designed to stimulate immunity upon arrival to the feedlot and reduce or control disease outbreaks. However, there is neither a standardized vaccination protocol nor enough efficiency of vaccines, usually caused because of suboptimal age or health status of the animals. Besides vaccination, treatment and prevention of BRD are addressed with antibiotics. However, recent studies have shown the emergence of bacterial pathogens associated with BRD that are resistant to all classes of antibiotics used. Thus, although the antimicrobial use has progressively increased during last decades, the prevalence of BRD has also raised potentially due to the emergence of multidrug-resistant bacterial pathogens, which poses a serious threat to both animal and public health. At this point it

is important to stress that antibiotic resistance is associated to morbidity and mortality in animals with BRD, besides promoting an alarming increase of antimicrobial resistant bacteria, which would ultimately impact on animal productivity. In consequence, since there is an increase in the multidrug resistance towards these antimicrobials that are being used as metaphylaxis and considering the low efficiency of vaccination protocols used, the development of novel methods to mitigate bovine respiratory pathogens in cattle is urgently needed as alternatives to the current strategies.

Probiotic and essential oils, being two major natural antimicrobial sources, display the potential application of antimicrobial alternative agents against bovine respiratory bacterial pathogens but up to know a very limited effect has been demonstrated on the control of the disease [5–7]. By contrast, investigation in the field of Host Defense Peptides (HDPs), have raised a deep interest as a potential solution to the worldwide challenge imposed by BRD. HDPs are short peptides (12-50 aa) naturally produced by the innate immunity that possess a broad-spectrum therapeutic potential against bacteria, fungi, and viruses, including multidrug resistant (MDR) microorganisms and those present in biofilms. To date, over 6,000 antimicrobial peptides (AMPs) have been identified from species as widely different as amoeba, plants, birds, mammals and humans. These peptides interact with pathogens having a negative surface membrane charge, increasing the membrane permeability and finally resulting in cell death [8–10]. The mechanism for microbicidal activity is believed to be through membrane disruption; however, other mechanisms such as antiviral cell signaling have been suggested for defensins [11–13]. Although some of these peptides are expressed constitutively, most of them are induced by pathogen exposure. Importantly, the development of bacterial resistance against HDPs is expected to be rare, because they act rapidly against integral components of the bacterial structure [14].

For the first time, different bovine recombinant HDPs produced in *Lacotococcus lactis* have proven to have broad-spectrum activity against bacteria with cell wall, bacteria without cell wall and enveloped viruses (Study 3). Bac5, BNBD-1, BNBD-3 and LAP have been reported as the most promising candidates with the broadest antibacterial and antiviral activities, specifically against some pathogens involved in BRD such as *P. multocida*, *M. haemolytica*, *H. somni*, *M. bovis*, bovine herpesvirus type 1 (BHV-1), parainfluenza-3 virus (PI3) and bovine viral diarrhea virus (BVDV) (Study 3).

Despite HDPs show promising therapeutic activities, their short half-life, their *in vivo* toxicity (high doses needed), and also, their high production costs are important drawbacks that largely hinder their final *in vivo* application [15]. In this context, nanotechnology is a powerful tool to design and produce optimized drugs taking into consideration different parameters such as stability, toxicity, effectiveness, and delivery, which could be important requirements in the development of a new generation of drugs against BRD. Particularly, nanostructuration of such molecules into nanoparticles can provide the delivery system required to increase peptide stability while improving the therapeutic compound bioavailability. Among the milieu of possibilities concerning drug delivery, traditional technologies such as peptide conjugation to spectrum of polymers, can provide extra functionalities to the therapeutic agent. In this regard it is interesting the use of polymeric micelles (PM) made of the amphiphilic poloxamer Pluronic® F127. This is an already FDA approved excipient formed by poly (ethylene oxide)/ poly (propylene oxide)/ poly (ethylene oxide) (PEO-PPO-PEO) triblocks. In an aqueous environment, Pluronic® F127 form amphiphilic micelles consisting in a hydrophobic core, in which insoluble drugs can be loaded, and a hydrophilic shell able to carry a broad range of soluble molecules. Pluronic® F127 have been shown to supply, when displayed in a micelle form, stealth properties hiding the nanoparticle from the reticuloendothelial system and therefore reducing nanoparticle clearance [16]. Besides, its capacity to increase the solubility of other compounds when in the same formulation can prove useful if working with insoluble peptides. Finally, it is important to note that PEG, a building block of Pluronic® F127 that upon micellation composes the hydrophilic shell of the micelle, has been described as an enhancer of mucus penetration when placed onto nanoparticles surface [17].

In this study, we have tested the *in vitro* and *in vivo* activity of a combination of recombinant HDPs named Bac5, BNBD-1, BNBD-3 and LAP embedded in Pluronic® F127 PM to evaluate if they are able to control BRD pathogens and disease progression.

Materials and Methods

Bacterial and viral strains

Lactococcus lactis subsp. cremoris NZ9000 [18] strain was used for recombinant protein expression. To evaluate the antimicrobial activity *in vitro* the strain selected was *Pasteurella multocida* (ATCC®15743™). *L. lactis* strain was grown in M17 broth (Sigma-

Aldrich) supplemented with 0.5 % glucose. *P. aeruginosa*, *S. epidermidis* and *P. multocida* were grown in Brain-Heart Infusion (BHI) broth (Scharlau).

Production and purification of recombinant Host Defense Peptides

Four recombinant HDPs (Bac5, BNBD-1, BNBD-3 and LAP) C-terminally fused to the Green Fluorescent Protein (GFP) and a 6 histidine (H6)-tag (Bac5-GFP-H6, BNBD1-GFP-H6, BNBD3-GFP-H6 and LAP-GFP-H6), which designed as described in Study 3, were used in this study. The 4 *L. lactis* NZ9000 clones carrying pNZ8148 plasmid codifying for the 4 different HDPs were grown overnight (O/N) at 30°C in M17 broth supplemented with 0.5 % glucose and 5 µg/mL Cm for plasmid maintenance, as previously described (Study 3). The O/N cultures were inoculated in 5 L bottles with 5 L of fresh medium with Cm at an initial OD_{600nm} = 0.05. The recombinant protein expression was induced with 12.5 ng/mL nisin when these cultures reached an OD_{600nm} = 0.4-0.6. After 1 h of induction for BNBD-1 and 3 h post-induction for the rest of proteins, the whole culture was harvested (6,000 x g, 15 min, 4°C). Pellets were frozen at -80°C until their use. Each pellet corresponding to 500 mL culture was thawed and resuspended in 30 mL binding buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, pH = 7.4) with EDTA-free protease inhibitor cocktail (Roche). Bacteria suspensions were subjected to 2 rounds of mechanical disruption at 40 kpsi in a cellular disruptor (CF1 Model, Constant Systems Ltd). The supernatant was recovered through centrifugation at 15,000 x g for 45 min at 4°C. After that, the supernatants were filtered twice, using 0.45 µm and 0.2 µm filters (Merck) consecutively. After filtration, the sample was purified by Immobilized Metal Affinity Chromatography (IMAC) in an ÄKTA start protein purification system (Cytiva) using 5 mL HisTrap™ HP columns (Cytiva). Proteins were loaded with binding buffer (20 mM Tris, 500 mM NaCl, 20 mM Imidazole, pH = 7.4) and eluted using a linear gradient with elution buffer (20 mM Tris, 500 mM NaCl, 500 mM Imidazole, pH = 7.4). The selected fractions were dialyzed O/N in acetic acid 0.01 % (v/v) at 4°C with gentle agitation. The yield of purified soluble protein was determined by NanoDrop™ (Thermo Scientific). Finally, purified proteins were lyophilized O/N in a lyophilizer (Telstar) at -53°C and in vacuum conditions.

Protein loading in polymeric micelles (PM)

Proteins were loaded in PM formed by the amphiphilic polymer Pluronic® F127 (Sigma-Aldrich). PM were prepared by using the thin-film hydration procedure. Briefly, a mixture (8:2, w/w) of F127:F127-COOH were weighted and dissolved in a methanol:ethanol (1:1,

v/v) solution. Note that the carboxylated version of the polymer, F127-COOH, was synthesized in-house as previously described [19]. Solvent was removed by evaporation under vacuum conditions for 10 min, at 60 °C, and 200 rpm in a rotary evaporator IKA RV10; the film was further air-dried O/N at RT. For covalent binding of Bac5-GFP-H6, BNBD1-GFP-H6, BNBD3-GFP-H6 and LAP-GFP-H6, F127:F127-COOH films were hydrated in 0.8 mL MQ water by 5 min of vortex at RT and incubated with 1-etil-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma-Aldrich) at 10 µg/mL for 30 min at RT, under vigorous agitation. Then, Bac5-GFP-H6, BNBD1-GFP-H6, BNBD3-GFP-H6 and LAP-GFP-H6 were added to the PMs separately at a final total concentration of 20 µM and MQ water up to 1 mL and further incubated for 2 h at RT, under agitation. As a result, proteins were covalently bound between the –COOH terminals of the PM and the -NH2 groups present in the polypeptide sequence via amide formation. PM were finally freeze-dried and stored till further use.

Bactericidal assay against *P. multocida*

The antimicrobial assay was based on the BacTiter-Glo™ Microbial Cell Viability assay (Promega). Firstly, an O/N culture of *P. multocida* was diluted (10^6 CFU/mL) in 10 mM potassium phosphate buffer. Then, 150 µL from the bacterial diluted stock were centrifuged (6,200 x *g*, 15 min, 4°C). The supernatant was removed and the bacterial pellet was resuspended with 150 µL of either MQ water (negative control) or 20 µM or 5 µM of PM:HDP treatment in MQ water. All samples were disposed in a sterile 96-well plate of polypropylene (Costar) and were incubated for 5 h at 37°C. After, 100 µL were withdrawn and tested with 100 µL of the BacTiter-Glo™ reagent following manufacturer instructions.

Maxim tolerated dose (MTD) assay in mice model

Twenty-four BalB/cOlaHSd female mice were distributed in 8 groups (3 mice per group), G0a, G0b, G1, G2, G3, G4, G5, and G6 for the administration of the different treatments. Four lyophilized antimicrobial proteins, Bac5-GFP-H6, BNBD1-GFP-H6, BNBD3-GFP-H6 and LAP-GFP-H6 were resuspended in 10 mM HEPES to obtain a concentration of 20 µM (to administer mice in G1). Then, these HDPs were combined in equal parts to generate a single test compound named BNBD1- BNBD3-Bac5-LAP-GFP-H6 at its maximum dose. Once obtained the maximum concentration, 1/3 and 1/10 dilutions were prepared with the same buffer to administer mice in G2 and G3, respectively. In addition, sodium

chloride was administered to G0a mice as a negative control for free proteins. Four lyophilized antimicrobial proteins encapsulated in polymeric micelles, PM:Bac5-GFP-H6, PM:BNBD1-GFP-H6, PM: BNBD1-GFP-H6, and PM:LAP-GFP-H6 and one vial (lyophilized) of empty polymeric micelles were resuspended in 10 mM HEPES to obtain a concentration of 20 µM and 100 mg/mL of F127 for each of them. In this case, the PM were allowed to stabilize for 15 min and the four resuspended vials were mixed in equal parts. Once again, they were allowed to stabilize for 15-30 min and filtered with 0.22 µm to obtain test compound named PM:BNBD1-BNBD3-Bac5-LAP-GFP-H6 at its maximum concentration (to administer mice in G4). Then, 1/3 and 1/10 dilutions were prepared with the same buffer to administer mice in G5 and G6, respectively. In this case, empty polymeric micelles were used as a negative control. Briefly, PM-empty were resuspended in 10 mM HEPES to obtain a polymer concentration of 100 mg/mL of F127. The PM were allowed to stabilize for 15 min and filtered with 0.22 µm before administration of mice of G0b.

HDPs and PM:HDPs were administered to BalB/cOlaHSd female mice by intranasal (i.n.) route. Three different compound concentrations (20, 6.66 and 2µM) were tested, being 20 µM the maximum feasible dose common for all tested peptide formulations. To administer the mice by slow i.n. administration method, mice were slightly anesthetized with isoflurane before administration. 20 µL of saline, PM-empty, free formulation of HDPs (BNBD1-BNBD3-Bac5-LAP-GFP-H6) and PM:HDPs (PM: BNBD1-BNBD3-Bac5-LAP-GFP-H6) were given intranasally. To do so, a total of 8 administrations (4 per orifice) were given alternating nasal orifices (2.5 µL/each). Mice receiving the maximum concentration were euthanized 24 h post-administration, and lungs, trachea and nostril were collected, snapped-frozen and stored at -80°C. The welfare of the remaining animals was monitored for 16 days, to record any potential side-effects of the test compounds. No samples were collected from these later animals unless such side-effects were observed.

Experimental design in vivo assay with calves

The assay last 7 days, from day 0 to day 6. At day 0, a total of 42 crossbred unweaned male calves from different origins were selected in the rearing farm of Montgai (Lleida, Spain) by thoracic ultrasonography (TUS) which had a lung lesion score of 2 (scale explained in “thoracic ultrasonography (TUS)” section). After selection, the calve health scoring was determined (explained in “Calve health scoring” section). Then, calves were

transported to Torre Marimon facilities (Caldes de Montbui, Spain). At arrival all calves were weighted. At day 1, a blood sample from the jugular vein was collected using an 18 G needle and a 10-mL evacuated serum tubes (BD Vacutainer Becton Dickinson Co., San Agustin de Guadalix, Spain) for whole blood analysis detailed in "Hemogram" section. Nasal swab samples of the two nasal orifices were also collected for further metagenomics analysis of DNA bacteria and viruses (detailed in "Metagenomics" section). Following to swap samples, 21 calves were treated with 5 mL of 20 mM HEPES intranasally (2.5 mL/nasal orifice) and 21 calves were treated with 5 mL of PM:BNBD1-BNBD3-Bac5-LAP-GFP-H6 at 20 μM (2.5 mL/nasal orifice). After treatments, calve health scoring was determined and consumption of feed and straw was monitored for the following days (day 2, 3, 4, 5 and 6). At day 4, a TUS was performed to all calves to monitor lung lesions and rescore if necessary. The last study day, day 6, a blood sample from the jugular vein was collected again for whole blood analysis by hemogram, swab nasal samples were collected from the two nasal orifices for further metagenomics analysis of DNA bacteria and viruses (detailed in "Metagenomics" section), and a final TUS was performed (**Figure A1**).

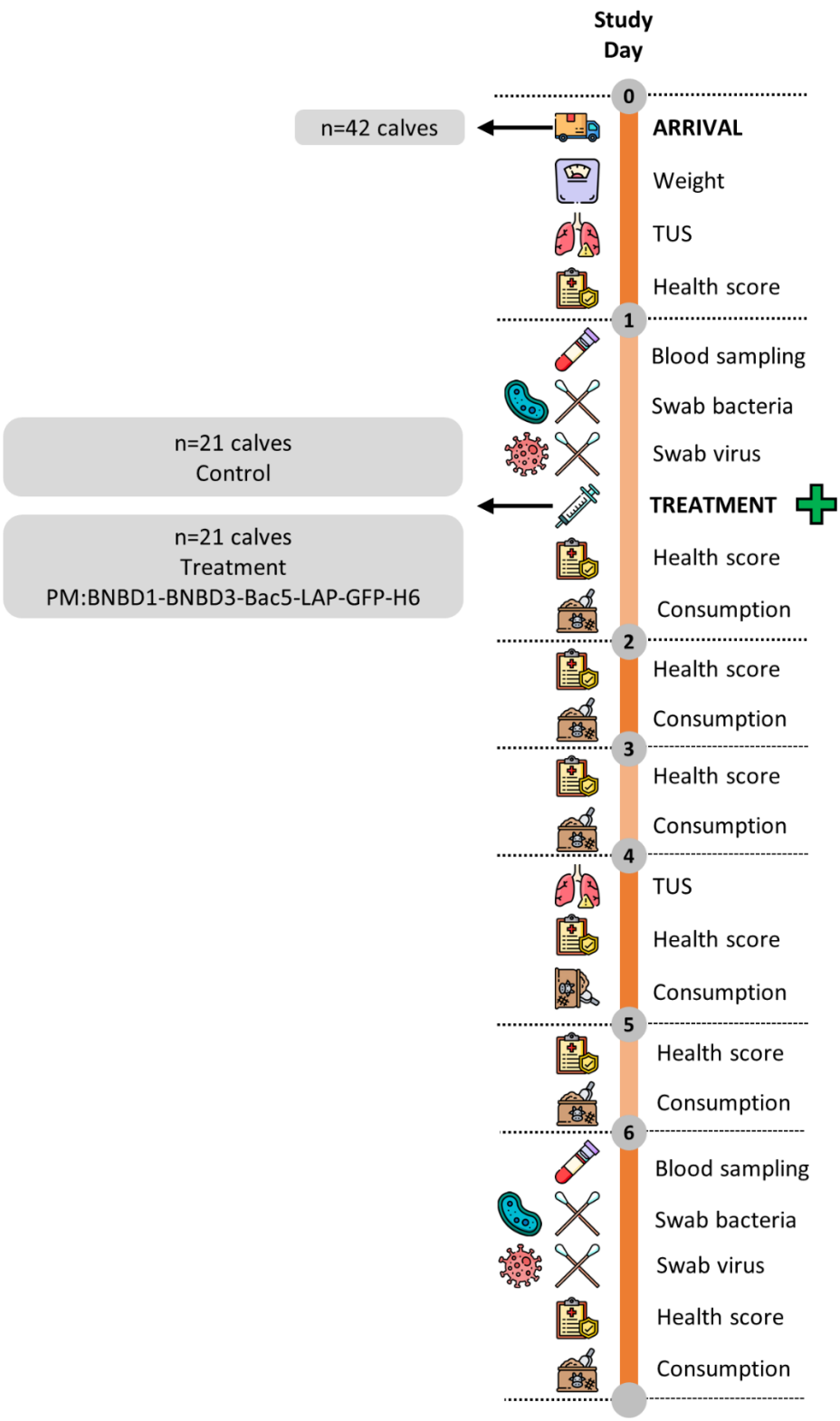

Figure A1. Timeline of the *in vivo* assay with calves. Schematic description of the different actions performed during the 7 days of study. Twenty-one calves were treated with 5 mL of 20 mM HEPES (control) and 21 calves were treated with 5 mL of a mixture of 4 Host Defense Peptides (HDPs) encapsulated in polymeric micelles (PM:BNBD1-BNBD3-Bac5-LAP-GFP-H6) and suspended in 20 mM HEPES (treatment). The final concentration of each protein was 5 µM and the total protein concentration was 20 µM. Abbreviations: Thoracic Ultrasonography (TUS).

Thoracic ultrasonography (TUS)

Lung lesions observed within the entire lung field were scored on a scale of 1 to 4 [20]. A score of 1 was attributed to lungs with no lesions or abnormalities, observing a healthy pleural surface or only an isolated comet tail within an image field. A score of 2 was given to lungs with multiple comet tails on the pleural surface and B-lines (coalescence of multiple comet tails) without significant lung consolidation. A score of 3 was assigned to lungs with one or more lung consolidation ≥ 1 cm. A score of 4 was assigned to lungs with extensive consolidations in one or more locations ≥ 6 cm, abscess within the lung parenchyma seen as an encapsulated fluid filled space, or significant pleural effusion.

Calf health scoring

Calf health scoring to evaluate clinical signs was based on seven items scored from 0 to 3: rectal temperature score, eye score, nasal discharge, ear score, cough, attitude and fecal score (**Table A1**) [21]. Then, the results of the different items were summed to obtain a total final health score. If this final health score was <5 it was renumbered as 0, and if the final health score was ≥5 it was renumbered as 1. Calves rescored as 0 were considered to be at good health status and calves rescored as 1 were considered to be at bad or worse health status.

Table A1. Calf health scoring criteria. Adapted from [20].

Calf health scoring criteria			
0	1	2	3
Rectal temperature score			
37.7–38.2°C	38.3–38.8°C	38.9–39.3°C	≥39.4°C
Eye score			
Normal	Small amount of ocular discharge	Moderate amount of bilateral discharge	Heavy ocular discharge
Nasal discharge			
Normal serous discharge	Small amount of unilateral cloudy discharge	Bilateral, cloudy, or excessive mucus discharge	Copious bilateral mucopurulent discharge
Ear score			
Normal	Ear flick or head shake	Slight unilateral droop	Head tilt or bilateral droop
Cough			
None	Induced single cough	Induced repeated coughs or occasional spontaneous coughs	Repeated spontaneous coughs
Attitude			
Calve reacts to external stimulus	Slow reactions to external stimulus	No reactions to external stimulus	No reactions to external stimulus and laying down
Fecal score			
Normal	Semi-formed, pasty	Loose, but stays on top of bedding	Watery, sifts through bedding

Hemogram

An hemogram of blood samples collected was performed using a hemogram HemaTrueHematology Analyzer (Heska, Loveland, CO) to quantify the whole blood cell numbers and percentages as well as other blood parameters. The quantified parameters were white blood cells (WBC) (cells/L and %), neutrophils (cells/L and %), lymphocytes (cells/L and %), monocytes (cells/L and %), eosinophils (cells/L and %), red blood cells (RBC) (cells/L), hemoglobin (HGB) (g/dL), hematocrit (HCT) (%), mean corpuscular volume (MCV) (fL), mean corpuscular hemoglobin (MCH) (pg), mean corpuscular hemoglobin concentration (MCHC) (g/dL), red blood cell distribution width (RDW-CV) (%), platelets (number/L) and mean platelet volume (MPV) (fL).

Metagenomics

DNA from nasal swap samples from 8 animals from each treatment (control and treatment) at day 1 and day 6 were extracted with the QIAamp PowerFecal Pro DNA Kit

(QIAGEN, Hilden, Germany) following manufacturer's instructions. Extracted DNA was sent to the Novogene for Shotgun-based Metagenomic Sequencing on an Illumina PE150 (Illumina, San Diego, CA, USA).

Statistics

Data from the bactericidal activity against *P. multocida* and mice body weight change during the MTD assay was analyzed by ANOVA assays. Data obtained from metagenomic analyses, calve health score and TUS score are represented by qualitative variable distribution and analyzed by LR-Chisquare.

Results and Discussion

The four recombinant HDPs (Bac5-GFP-H6, BNBD1-GFP-H6, BNBD3-GFP-H6 and LAP-GFP-H6) that have proven to have a broad-spectrum activity against bacteria and virus-causing BRD (Study 3) have been recombinantly produced, purified and covalently bounded to PM to increase its stability. A mixture of the four proteins bound to PM was prepared and their bactericidal activity against *P. multocida* was evaluated *in vitro* at 5 μM and 20 μM (**Figure A2A**). The results showed that the mixture of HDPs kept their activity after protein conjugation to micelles, with no differences observed among the two concentrations tested (**Figure A2A**). After, the maximum tolerated dose (MTD) of the 4 HDPs either in their free formulation or encapsulated in polymeric micelles (PM:HDP) was determined in mice after a single intranasal (i.n) administration (**Figure A2B**). HDPs and PM:HDP were tested at 20 μM, 6.66 μM (1/3 dilution) and 2 μM (1/10 dilution). Empty polymeric micelles were used as a negative control. The compounds were well tolerated at the moment of the administration. The body weight of the mice administered with 20 μM of HDPs and PM:HDPs was determined at the 24 h post-administration and the rest of the animals was determined along the study for 17 days to determine the adverse effects. None of the treated groups showed weight loss greater than 5 % (**Figure A2B**).

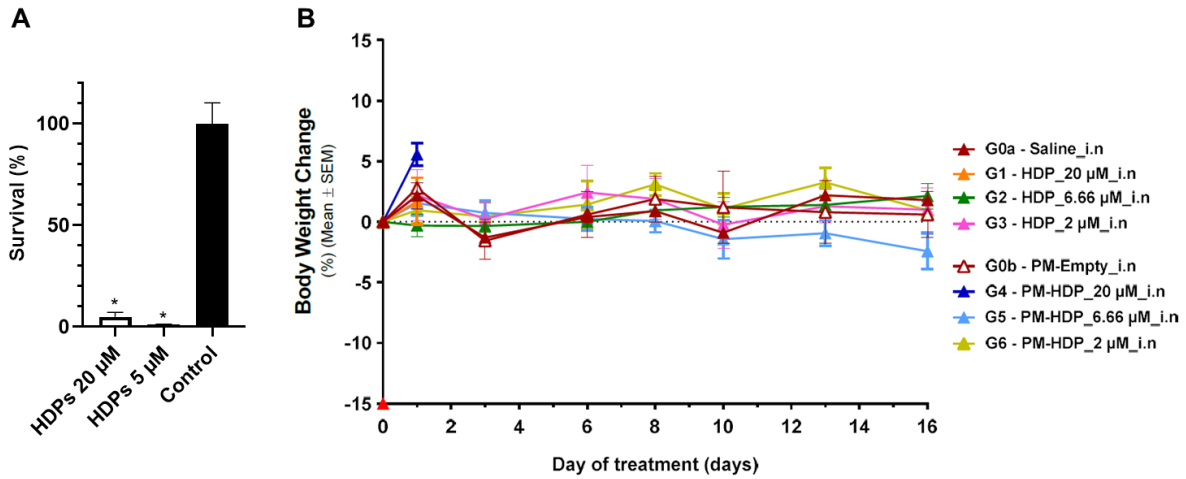

Figure A2. (A) Bactericidal activity against *P. multocida* of the 4 HDPs mixed at 20 µM and 5 µM. Asterisks depict significant differences compared with the control (*p*-value <0.001). (B) Body weight change during the MTD assay in mice model.

The lack of toxicity observed in MTD encouraged the evaluation of PM:HDPs at 20 µM in calves with a risk to have BRD. The animals were selected from commercial farms with a TUS score of 2, which corresponds to a lung with mild lesions or abnormalities but without clear consolidations indicative of BRD affectation. We selected animals with this TUS values to test the effect of the new treatment because the lung is already affected, but the disease is still not decontrolled, and, from an ethical point of view, at this point animals need to be treated with antibiotics. The results showed no differences between control and treated animals when clinical signs were evaluated by Calve health score neither at day 1 nor at day 6. In fact, no important changes were observed between day 1 and 6 in any calf included in the experiment (**Figure A3A**). However, although changes in the evolution of TUS score were observed over time, no significant differences were observed between the evolution of control or PM:HDPs treated calves (**Figure A3B**).

Taking more resolutive analyses based on metagenomics, the presence and absence of 3 bacterial pathogens involved in BRD were quantified from nasopharyngeal swabs. The results indicated significant differences between PM:HDPs animals compared to control ones. *Mycoplasma bovis* only appeared in Control animals at Day 6 but it was undetectable in any PM:HDPs treated animals (**Figure A4A**). In a similar manner, all Control animals presented *Pasteurella multocida* at day 6 while only half of PM:HDPs treated animals did (**Figure A4B**). Finally, no differences in the evolution of *Mannheimia haemolytica* between both treatments were observed (**Figure A4C**).

Overall, these results indicated the clear potential of HDP-based nanoparticles (PM:HDPs) at controlling the presence of BRD pathogens in the respiratory tract. So far, the evolution of clinical signs has not been correlated with this effect but encourages the execution of further experiments with a greater number of animals and longer experimental study monitorization.

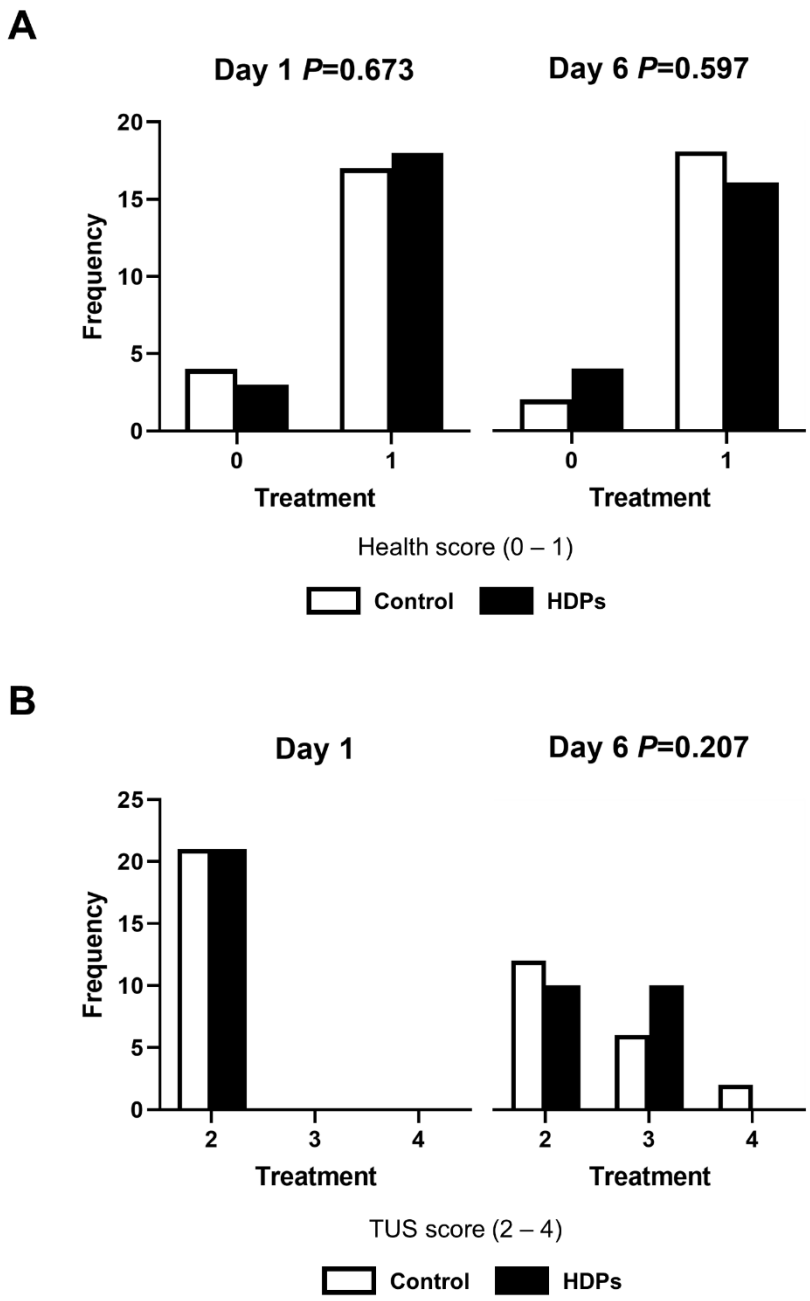

Figure A3. A) Frequency distribution analyses of the number of calves from control (white bars) or HDPs treatment (black bars) with a calve health score of 0 or 1 at day 1 or 6 of the experiment. B) Frequency distribution analyses of the number of calves from control (white bars) or HDPs treatment (black bars) with a TUS score of 2, 3 or 4 at day 1 or 6 of the experiment. The *P* values obtained in LR-Chisquare analyses are indicated at each sampling time, where $p<0.05$ is considered significant.

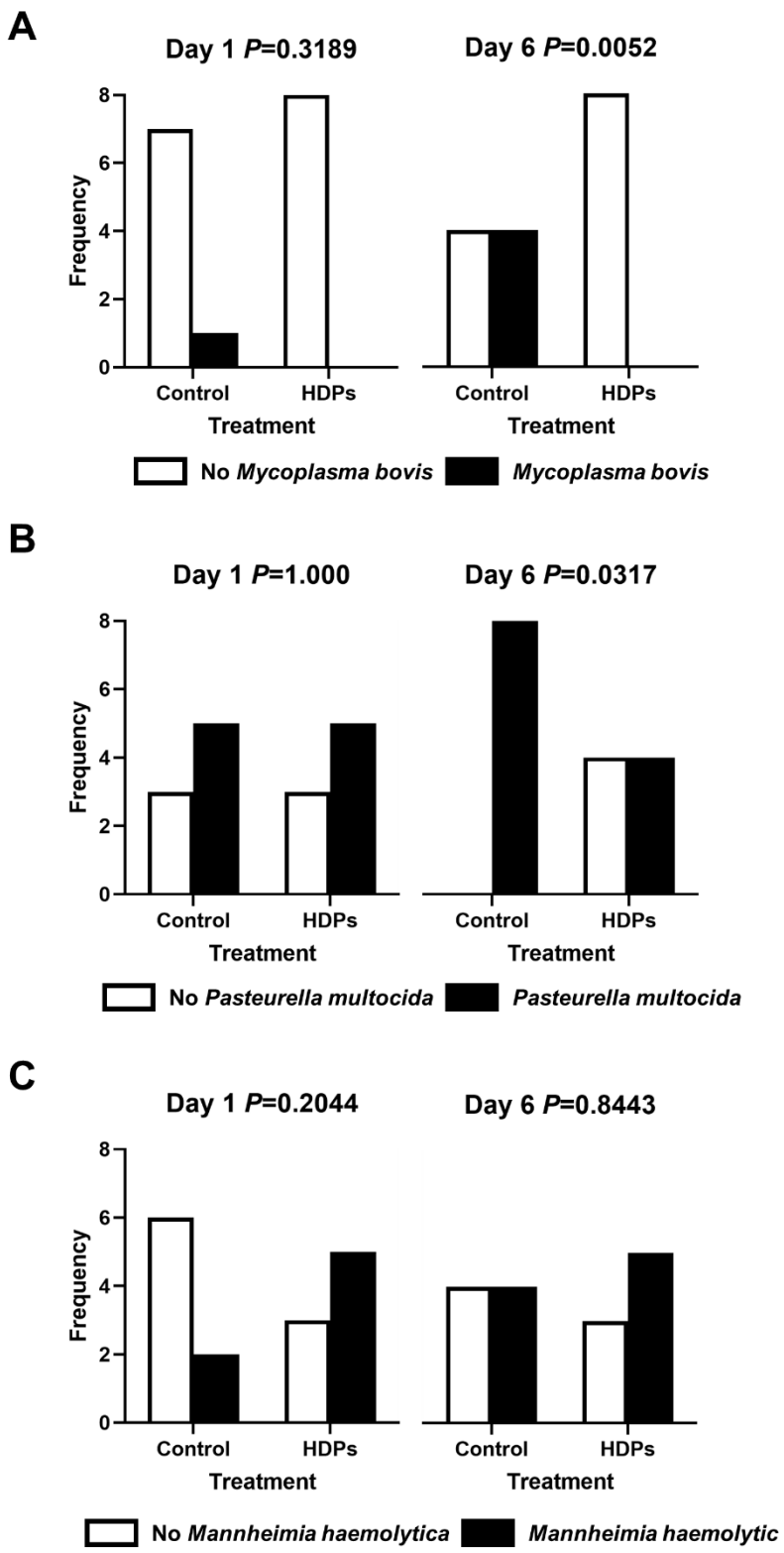

Figure A4. Frequency distribution analyses of absence (white bars) or presence (black bars) of *Mycoplasma bovis* (A), *Pasteurella multocida* (B) and *Mannheimia haemolytica* (C) in control and HDPs treated calves at day 1 or 6 of the experiment. The *P* values obtained in LR-Chisquare analyses are indicated at each sampling time, where p<0.05 is considered significant.

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ACKNOWLEDGEMENTS

Quan comences a escriure aquest apartat d'agraïments vol dir que ja s'acaba no? Ho dic perquè crec que no soc del tot conscient de que estigui acabant. De fet, encara no acabo d'assimilar que hagin passat 5 anys des de la meva arribada al grup de Producció de Remugants del IRTA. Però sí, sembla que fins aquí hem arribat. Hem arribat al final de la tesi doctoral del Ricardo Baltà Foix. I dic "hem" perquè tot i que surti el meu nom a la portada, la feina exposada en aquesta tesi no ha sigut resultat de la feina d'una sola persona. Jo sol no hagués pogut ni de bon tros, o almenys no hagués quedat igual de bé, això us ho asseguro. Per això m'agradaria agrair amb aquestes poques línies a tota la gent que d'alguna manera o altra ha estat involucrada en aquesta tesi i que sense ells hagués sigut no impossible però sí que molt més complicat. Espero no oblidar-me de ningú, i si és així i trobeu a faltar el vostre nom, demano perdó per endavant i rebeu el meu més sincer agraïment també.

Primer de tot, com no podia ser d'una altra manera, vull agrair-vos a vosaltres, Anna & Elena, TOT. Dic TOT perquè crec que no hi ha manera possible de resumir tot el que hem viscut durant aquests 5 anys. Agrair-vos la confiança cega que vau tenir al acceptar aquella sol·licitud "in extremis" a una veu que només havíeu escoltat per telèfon i que a sobre en l'últim moment us va dir que no havia arribat a temps a enviar la documentació per la beca. Encara al·lucino que l'acceptessin i que això permetés tot el que ha vingut després. Crec que encara avui cap dels 3 tenim una explicació per allò, però el que sí que sé és que me n'alegro moltíssim que passés així, i espero que per vosaltres també hagi valgut la pena. Moltíssimes gràcies per tots els consells que m'heu donat, sempre amb bones paraules i intentant trobar la millor solució en tot moment. Sempre posant per davant a les persones i amb una empatia que no és ni mig normal. De veritat, moltes gràcies per haver-me ensenyat i donat tant. I moltes més coses però hauria de fer uns agraïments a part. Sou un tàndem increïble! Ja anirem parlant eh Anna jejeje.

A tot l'equip de la part de camp de remugants. A la Maria per ser el pal de paller de la República Independent de Remugants. Pel teu pensament crític i analític sempre sabent com simplificar-ho tot i realment posar el focus on tocava i que ningú es despistés. He après molt de tu i t'agraeixo també que sempre hagis tingut present el benestar de tota la gent del grup per davant de tot. No deixis que es perdi mai aquest bon ambient. A la Sònia i a la Marta, i us poso juntes perquè l'ajuda que ens heu donat sobretot en aquest últim *in vivo* no es pot descriure de cap de les maneres. Moltíssimes gràcies per estar sempre

disposades a donar un cop de mà allà on calgui i quan calgui. Els vostres "zascas" a vegades tiren enrere, però qui us coneix bé sap que mai falleu i que sou dos dels pilars de Remugants. Al Xavi, l'Anna, el Guillem i la Lourdes. Especialment al Xavi i el Guillem per l'ajuda en l'últim assaig amb "els gorditos" com els hi diu la Marta (els vedells per la resta de gent), que sense vosaltres tampoc haguéssim arribat a bon port.

Als becaris precaris de l'altre despatx i al tècnic infiltrat entre ells. M'està agafant la son perquè ja porto una estona escrivint, però intentaré aguantar sense adormir-me fins al final com al seminaris...zzzz. Primer de tot a la Laiunski! El leprechaun furiós amiga dels ocells. La meva primera mestra a Torre Marimon i la que em va deixar el llegat de la MMP-9 i la M-SAA3. Moltes gràcies per acollir-me com un més des del primer dia i tenir la paciència per explicar-me totes les tècniques i experiments necessaris per treure suc a aquestes dues proteïnes, a vegades "maleïdes" perquè no sortia cap experiment. Sobretot les purificacions eternes al IBB amb la French Press i l'ÄKTA on vaig entendre des del principi que el doctorat no entenia d'horaris.

En segon lloc al nostre superheroi Ramon. Per les lliçons pràctiques de judo tant al despatx com a la gespa de Torre Marimon. T'agraeixo les contractures que em vas treure i les que em vas produir depenent de la clau que em toqués rebre. I com oblidar el moment "pulpo" Ricky a Boston, on la por a la teva força se'm va escapar pel lloc que no tocava... Moltes gràcies per tenir sempre disponible als teus Jun i Fos (bíceps dret i esquerre respectivament) per ajudar sempre que tenia dubtes.

Al bo d'en Cesquius. Has vist Cesc? La tesi més ràpida de la història, en 3 mesos l'hem enllestit i ja marxo jejeje. A tu no et queda massa per jubilar-te i marxar també no? Perdona, no podia faltar el "guiño" al nostre fòssil preferit ;). Moltes gràcies per tota l'ajuda i per estar sempre atent a qualsevol cosa que necessitéssim, amb un bon consell preparat o algun truquet que altre per sobreviure al laboratori. Vigila'm a tot aquest jovent que no es desmadrin i posa seny.

Al meu Broctiter, viralBro, labBro, gipsy queen, Master of disaster and of the cloning. Que haguéssim fet l'un sense l'altre eh Adri... Moltes gràcies primer de tot per convidar-nos al casament perquè va ser espectacular! Es va fer esperar però va valer la pena. La veritat és que no podria haver tingut un millor company d'alegries i desgràcies durant el doctorat. Sempre amb una broma preparada per fer l'anterior broma encara més grossa, o si alguna cosa sortia malament doncs riure i tornar-ho a intentar amb la mateixa alegria.

Sense els teus tocs especials a les tècniques de laboratori no m'haguessin sortit ni la meitat dels experiments.

El millor record que m'emporto amb vosaltres 4 és el viatge a Boston. Crec que allò va marcar un abans i un després en el nostre petit grupet. Sempre ho recordaré amb molt de carinyo i com un dels viatges més divertits que he fet mai. Moltes gràcies a tots 4.

A la Crisis! Ara sí que marxo de veritat eh Cris, però no pateixis que estàs en bones mans. No tinc cap dubte que quan acabis hauràs fet una tesi per emmarcar, perquè ho vals i tens un interès i una passió per la recerca que en poques persones he vist. Quin bon relleu has sigut quan semblava que Remugants s'anava buidant de doctorands. Digne successora dels 4 que he nombrat abans. Gràcies per preocupar-te sempre de com estava i per les nostres converses filosòfiques tan personals com laborals. Quin congrés que vam viure a la Toscana eh! Pensava que ningú em superaria posposant alarmes als matins, però tu ets una professional. Espero que a part de recordar-ho com el moment en que vas conèixer al totpoderós Hancock, també ho recordis igual que jo com un congrés on ens ho vam passar super bé. Molts ànims amb el que et queda, i per qualsevol dubte truca al Adrià vale?

Luchiii!!!! Que pensabas que ya no te escribía nada eh, seguro que te estabas metiendo conmigo por no ponerte la primera de la lista o por cualquier otra cosa. Lo siento, los argentinos siempre los últimos. Perdona, tenía que empezar con un poco de bullying que sino se me hacía raro. No, ahora de verdad. Muchísimas gracias por todo. Des de los monólogos eternos explicándome tus ligues hasta las conversaciones filosóficas interminables solucionando el mundo. Un poco como con Adrià, que hubiéramos hecho el uno sin el otro verdad? Seguramente nos hubiéramos vuelto locos o nos habríamos gastado mucho dinero yendo al psicólogo hahaha. Creo que ninguno de los dos estamos muy bien de la cabeza, pero me ha encantado compartir esta locura durante estos años. Detrás de todo el bullying sabes que tienes un amigo para lo que necesites.

Adri, Cris, Lucía y sleepy, hicky, peachy, etc. Ricky, el "Eje del mal" original. Quin quartet més xul·lo que hem format. Quan van marxar la Laia i el Ramon pensava que la millor època s'havia acabat, però la veritat és que la segona etapa ha sigut igual de bona o millor. Trobaré a faltar les nostres converses incoherents que només enteníem nosaltres, retroalimentant-nos amb les nostres pròpies bromes, alegries i desgràcies. Ha sigut una etapa MOLT guay!

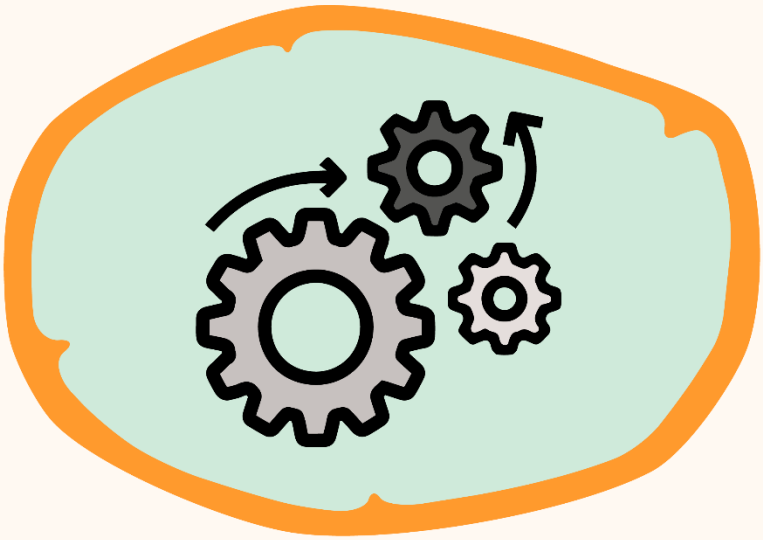
Denichi! Ni ahí luego, ya piró el terrrerrré. Seguro que no he utilizado correctamente ninguna de las dos expresiones, pero como viste en el vídeo de tu tesis hago lo que puedo imitando el acento paraguayo. Muchas gracias por preguntar siempre como iba la tesis y estar pendiente de tus amiguitos. Juanpa, Emma y tu sois una familia estupenda.

Marina. Al final sembla que presentarem gairebé a la vegada! Espero que acabi d'anar molt bé aquesta recta final. Molts ànims i molta força.

Aida i Sergi. Quins 2 bons fitxatges que han fet l'Anna i l'Elena. Si després del congrés de París no us vau espantar de lo bojos que estem tots, això vol dir que us agrada de veritat la Recerca. Us desitjo el millor a tots dos durant les respectives tesis.

A mi familia. Papá, Gonzalo, Sara, Begoña, Miguel Ángel, Guillermo, Irina, Juanjo, Mati y Víctor. A todos por interesaros siempre por cómo iban los experimentos con las vaquitas, como no os cansabais de decir. Especialmente en esta etapa final por entender de la importancia que tenía y apoyarme en todo momento. Y a mi madre allí donde esté, porque todo lo bueno que me ha pasado y me sigue pasando se que es porque ella cuida de mí.

I finalment, a tu Marta. Realment no cal que escrigui res perquè has viscut aquesta tesi amb la mateixa intensitat o més que jo. Només tu saps tot el que hem lluitat i patit per arribar fins aquí. El més segur és que sense tu no hagués pogut fer, o segurament encara aniria per la introducció. Saps de sobres el que t'agraeixo tota l'ajuda que m'has donat, la paciència i la comprensió. T'estimo infinit +1.



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