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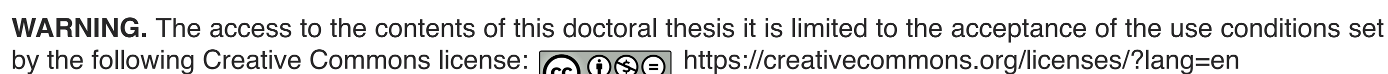
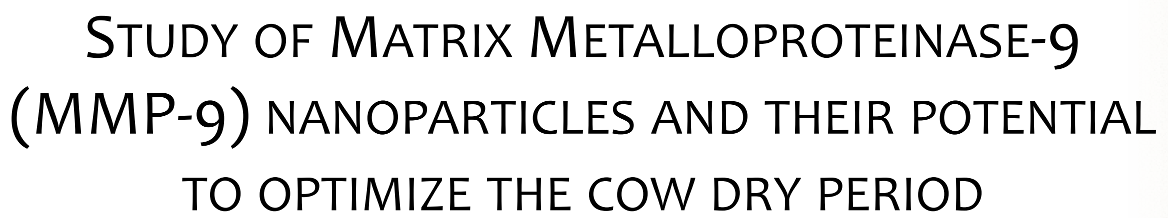
WARNING. The access to the contents of this doctoral thesis it is limited to the acceptance of the use conditions set by the following Creative Commons license:  https://creativecommons.org/licenses/?lang=en



Study of Matrix Metalloproteinase-9 (MMP-9) nanoparticles and their potential to optimize the cow dry period

Laia Gifre Renom

PhD Thesis

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PhD Thesis

STUDY OF MATRIX METALLOPROTEINASE-9 (MMP-9) NANOPARTICLES AND THEIR POTENTIAL TO OPTIMIZE THE COW DRY PERIOD

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Department of Biochemistry and Molecular Biology

Thesis submitted by Laia Gifre Renom as partial fulfilment of the requirements for the PhD degree in Biochemistry, Molecular Biology and Biomedicine by the Autonomous University of Barcelona.

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Als meus pares, als meus germans i a tu, Arnau,

i a tots aquells que m'heu fet riure de veritat.

"Little events, ordinary things, smashed
and reconstituted. Imbued with new meaning. Suddenly
they become the bleached bones of a story."

— Arundhati Roy, The God of Small Things

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Summary

There is a worldwide action plan under the motto One Health, calling for the urgent minimization in the use of antibiotics at all levels –human, animal and environmental– in the joint battle against the antimicrobial resistance (AMR) crisis that threatens global health. The development of new preventive strategies to treat infection diseases has become, therefore, an essential initiative since the use of preventive antibiotics is being banned. In dairy cattle, the dry period is a sensitive phase in which cows easily suffer of intramammary infections, or mastitis, due to the temporally inefficient immune condition in the mammary gland. In this context, alternatives are required in order to control and reduce mastitis incidence at dry-off and to diminish the need of antibiotics. Inclusion bodies (IBs), which are protein aggregates recombinantly produced in bacteria and with an easy and low-cost scale-up, have already demonstrated a great potential in research areas such as tissue engineering and cancer therapy. With the aim of exploring their potential into the animal production sector, this thesis has been focused to cover all steps in the way along the development, characterization and application of this novel and promising protein-based therapy in the dairy sector. Starting from the acquisition of soluble MMP-9 by solubilizing IBs using a newly described protocol for Generally Recognized as Safe (GRAS) bacteria protein-factories, we have been able to develop new MMP-9 delivery formats and to compare them with IB performances. The greater stability shown by IBs when compared with other formats brought us to consider their applicability *in vivo*. As a first step, their administration in a murine model revealed for the first time that their potential as an immunostimulant agent could be importantly dissected into two distinct effects. One effect, unspecific and ephemeral, was driven by the IB format whereas the other effect was MMP-9-specific and extended in time. These encouraged us to try to uncover what would be the effects triggered in their ultimate target, the bovine mammary gland at dry-off. Although MMP-9 had not a relevant specific role triggering the early stages of involution in this context, we observed that these protein aggregates accelerated from 3 to 6 days the onset of involution biomarkers, the release of natural bacteriostatic peptides such as lactoferrin, and the recruitment of immune cells in the mammary gland though an unspecific action. Therefore, these results consolidate MMP-9 IBs as a potent immunostimulatory –although unspecific– agent in the bovine mammary gland at dry-off, and reveal that new opportunities are yet to be explored in the use of protein nanoparticles to reach a more specific effect in the acceleration of the mammary gland involution and immune system.

Resum [Catalan translation]

Hi ha un pla d'acció mundial, sota el lema *One Health,* fent un clam urgent a minimitzar l'ús d'antibiòtics a tots els nivells –humà, animal i ambiental– en una batalla conjunta contra la crisi associada a la resistència als antimicrobians (AMR) que amenaça la salut global. El desenvolupament de noves estratègies preventives per tractar malalties infeccioses ha esdevingut, doncs, una iniciativa essencial des que l'ús preventiu d'antibiòtics està essent prohibit. En vaquí de llet, el període d'eixugat és una fase sensible durant la qual les vaques són especialment susceptibles a patir infeccions intramamàries, o mastitis, degut a l'estat immunitari temporalment ineficient en què es troba la glàndula mamària. En aquest context, calen alternatives per controlar i reduir la incidència de mastitis durant l'eixugat i, alhora, disminuir l'ús d'antibiòtics. Els cossos d'inclusió (CIs), agregats de proteïna produïts de manera recombinant en bactèries i fàcilment escalables a baix cost, ja han demostrat un gran potencial en àrees de recerca com l'enginyeria de teixits i la teràpia oncològica. Per tal d'explorar el seu potencial en l'àmbit de la producció animal, aquesta tesi s'ha enfocat a cobrir tots els passos cap al desenvolupament, la caracterització i l'aplicació d'aquesta teràpia recent i prometedora basada en proteïnes en el sector lleter. La posta a punt d'un protocol de solubilització dels CIs per l'obtenció de la Metal·loproteinasa-9 (MMP-9) en format soluble usant microorganismes classificats com a *Generally Recognized As Safe* (GRAS), ens ha permès desenvolupar nous sistemes d'alliberament de MMP-9 i comparar-los amb el comportament dels CIs. La gran estabilitat mostrada pels CIs comparada amb d'altres formats ens va fer considerar la seva aplicabilitat *in vivo.* Com a primer pas, l'administració d'aquests CIs en un model murí va suggerir, per primera vegada, que el seu potencial com a agent immunostimulant podia ser degut a la combinació de dos efectes diferents. Per una banda vam observar un efecte inespecífic i efímer, associat al propi format del CI, i per l'altra vam observar també un efecte específic de la MMP-9 que s'estenia en el temps. Això ens va encoratjar a estudiar quins serien els efectes en el teixit diana proposat en aquest treball, la glàndula mamària bovina en l'eixugat. La MMP-9 no va tenir un efecte específic rellevant accelerant els estadis més primerencs de la involució, però vam observar que aquests agregats proteics acceleraven de 3 a 6 dies l'augment en els nivells de marcadors d'involució, l'alliberament de pèptids bacteriostàtics naturals com ara lactoferrina, i el reclutament de cèl·lules immunitàries a la glàndula mamària, mitjançant un efecte inespecífic. Per tant, aquests resultats consoliden els CIs de MMP-9 com potents agents immunostimulants –tot i que inespecífics– a la glàndula mamària bovina durant l'eixugat, i revela que encara hi ha moltes oportunitats encara inexplorades en l'ús de nanopartícules proteiques, per assolir un efecte més específic en l'acceleració de la involució i el sistema immunitari a la glàndula mamària.

Introduction

DRY PERIOD IN DAIRY COWS

During the past 30 years, cow milk production yield has been almost doubled in many countries[1,2] through breeding selection of high-yielding dairy cows, being Holstein-Friesian cattle the highest milk producers outreaching 40 kg/cow/day. Concern is increasing on the metabolic challenges that the elevated production levels suppose for cows as these affect considerably the immune and reproductive performances, milk quality and animal welfare[3].

An ideal **lactation cycle** of a dairy cow lasts approximately 12 months, and is often divided in four stages: early, mid and late lactation, and the dry period[4]. Early lactation starts after calving and is an approximately 100-day period characterized by a negative energy balance due to high milk secretion and loss in body weight, although dry matter intake is high (Figure 1). The following 100 days define mid lactation, with stabilization in body weight due to a decrease in both milk production and dry matter intake. A marked reduction in milk secretion improves recovery of the cow body weight during late lactation, which is the next period and that lasts about-106 days[4].

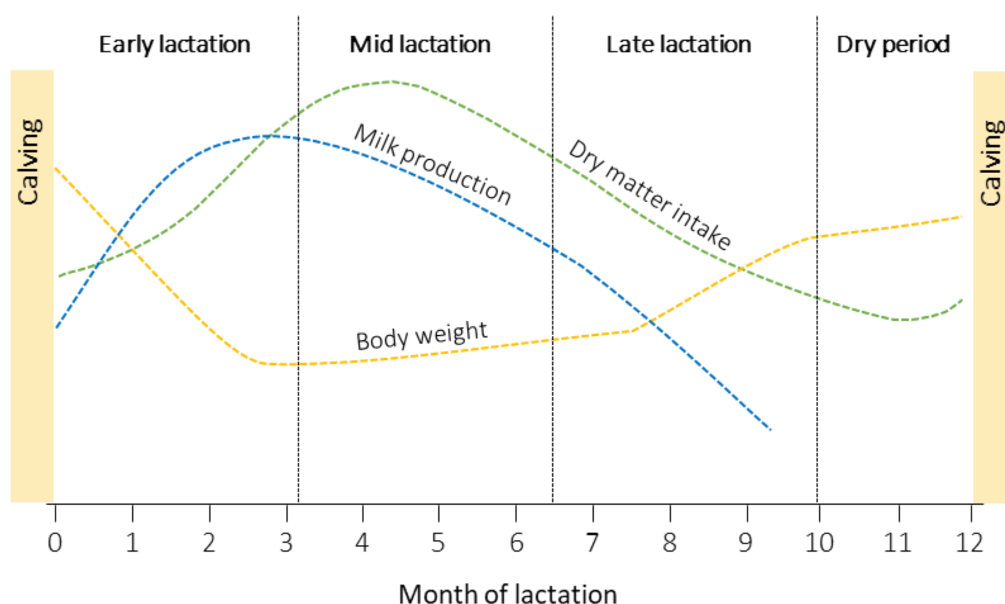

Figure 1. Scheme of the estimated changes in feed intake, milk yield and body weight along a 12-month lactation cycle in dairy cattle according to the Food and Agriculture Organization (FAO) of the United Nations. Adapted from FAO tutorial[4].

Dairy cows are inseminated during early lactation to promote consecutive pregnancies and lactation cycles. However, a resting milking period before calving, known as the **dry period** or dry-off, has been established to allow the involution and regeneration of the mammary gland tissue and to guarantee optimal milk yields in the next lactation. Because this is an unproductive season with an important economic impact in the dairy industry, its length and methods have been object

of study[5–11]. Therefore, it has been established that two months is an optimal length for drying-off, limiting the non-productive days[12,13] without impairing the cow health and welfare condition[14]. Milk cessation can be carried out either **abruptly** or **gradually.** An abrupt cessation consists in the stop of milking without previous changes neither in animal manage nor in nutrition regimes. Otherwise, gradual cessation consists in the reduction of milking before drying-off, combined or not with a reduction in caloric ingest to reduce milk production prior to dry-off. As will be detailed in "*Prophylactic antibiotic use at dry-off*" benefits or disadvantages between both have been long in debate and still conclusions about which is the best option cannot be generalized. An abrupt dry-off, however, has been in some cases recommended due to important health parameters described to be detrimental by a gradual cessation, such as increased risk of infections in multiparous cows[15] and an increased mobilization of fatty acids after gradual-linked procedures[16], as will be detailed in the mentioned section.

The dry period is a necessary and common practice in dairy farms, but at the same time it is a critical period associated to important **adverse effects**. **Milk stasis** is one of the main triggering factors of these adverse consequences: as milking stops –sometimes in still highly productive udders– milk accumulates leading to udder pressure, which may originate tissue damage and pain to the animal, and to **milk leakage**, delaying the formation of the keratin-based teat canal plug. Milk retained in the udder and leaking through the teat canal is, moreover, a potential nutritive media for microbes, and the absence of the teat-plug supposes an open access for the associated microorganisms, thus sensitising the mammary gland (MG) to **intramammary infections** (IMIs). In fact, owing to the tissue changes occurring in the MG at dry-off[5], IMI rates increase up to seven times during the early dry period compared to IMIs originated during lactation[17], and this increases the risk of clinical mastitis during the following lactation[18].

Bovine mastitis: causes and implications

Mastitis is the persistent inflammation of the mammary tissue and occurs mainly as a consequence of IMIs. It is considered the most costly disease in dairy worldwide[19] entailing economic losses from 140 € (The Netherlands)[20] to 450 € (Canada)[21] per cow per year due to milk production losses and animal management needs, and raising up to 2 billion € annual costs only in the US[22].

Bacteria are the foremost causatives of new IMIs, being **coliforms**, such as *Escherichia coli*, *Klebsiella* spp., and *Enterobacter* spp., and **streptococci** like *Streptococcus dysgalactiae* and *Streptococcus uberis,* the main pathogens causing bovine mastitis[23]. Although these have been traditionally classified as environmental pathogens, some of them have been recently described

to be able to be contagious as well, like *S. uberis*[24]. Other contagious bacteria, such as *Staphylococcus aureus* and *Streptococcus agalactiae,* are also important in the mastitis epidemiology, as these can spread from one MG to another and between animals[25], becoming persistent pathogens within herds[26].

Different pathogens trigger different immune responses in the MG[27,28]. Once bacteria enter the MG through the teat canal, an inflammatory response is activated through the recognition of pathogenic signals by the tissue (process detailed in the section *Immune condition in the udder: defeating an infection*). According to the pathogenic symptomatology manifested by the affected tissue, we divide the disease into **clinical mastitis** (CM) or **subclinical mastitis** (SCM):

- CM is mostly caused by coliforms and streptococci[23], and has clear symptoms, including severe inflammation in the udder, swelling, redness, heat and pain, as well as clotted milk with high **somatic cell counts** (SCC). SCC is a parameter that mainly scores immune cell infiltration in the MG secretion, being non relevant when SCC are low, usually from epithelia exfoliation, but critical when SCC rise above 200,000 cells per milk ml, as these may be leukocytes recruited in response to a threat and indicating that an inflammatory process is being triggered[29,30]. Moreover, the severity is also linked to the pathogen, being *E. coli* mastitis more severe but often resolved without needing a treatment, while *S. aureus* mastitis is associated to moderate symptoms but this may easily become **chronic**[28]. In addition, chronic mastitis is one of the core causes of culling in dairy farms (Figure 2), which is one of the costliest economic losses in this sector[20,21,31,32].

- SCM symptoms are not so evident and SCC is the only parameter that can be successfully used for its detection[30], being > 200,000 SCC / ml in milk indicative of SCM[29]. Mainly, SCM is caused by coagulase-negative staphylococci and different streptococci, as well as by *Corynebacterium* spp., *Pseudomonas* spp., *Pasteurella* spp. and *Listeria* spp. among others[28].

The probability to get infected depends on different aspects, starting from **environmental factors** like the farm hygienic condition[25,33,34] and the season of the year[35]. Also, relevant **cow factors** in this regard are: (i) **stage of lactation**, being the udder more susceptible to IMI during the dry period and early lactation[17]; (ii) **age and parity**, although it is not an absolute feature for all pathogens, it has been demonstrated that multiparous cows (two or more times calving) are more susceptible to *S. dysgalactiae* mastitis prevalence than primiparous (first time calving) cows[35,36]; (iii) **historic of somatic cell counts** (SCC) in milk has demonstrated to be critical for IMI predictability[29,30].

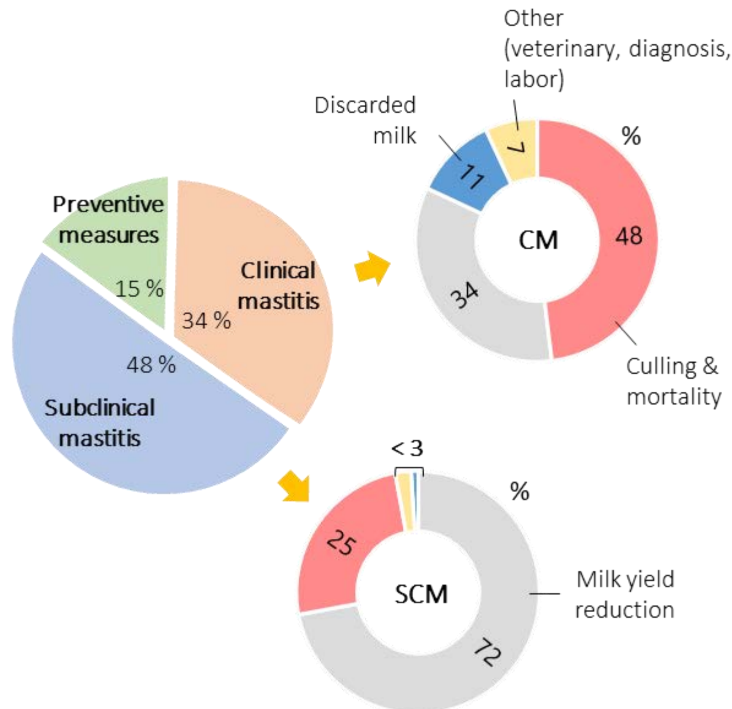

Figure 2. Costs associated to clinical and subclinical mastitis (CM and SCM) in a median herd in Canada (100 cows-year). The relative relevance of each mastitis type and of their principal causes are indicated. Diagram adapted from Aghamohammadi *et al.* 2018[21].

However, there are some pathogens able to escape the immune system, like *S. aureus*, which can form **small colony variants** with reduced growth rate and α-toxin production generating a mild IMI[37,38] and thus maintaining reduced SCC.

As it will be further detailed in the section "*Mastitis prevention within a global health alert context*", a control and reduction of mastitis in dairy cattle has been achieved through the prophylactic use of antibiotics[19,39]. However, the difficulty to anticipate some IMIs, together with the apparition of antimicrobial resistances, has impaired mastitis prevention and treatment. Taken together, these facts have accentuated the urgency to invest efforts to find new preventive approaches, starting from addressing the IMI risk origin in the dry period.

Molecular insight into the udder

From lactation to dry-off

Four MGs –referred as quarters– form the udder of a cow, being each of them independent compartments that will undergo along different development stages during the animal lifetime[40]. Each MG is composed by epithelial and myoepithelial tissues surrounded by fat forming a vascularised mammary parenchyma. This is structured in branches or ducts invaginated from the

teat canal and ending in lobules which terminate in alveoli (Figure 3). The lumen of this ducted circuitry is covered by **mammary epithelial cells** (MECs) that, in the alveoli level and due to hormonal changes occurring after parturition, differentiate into secretory cells which produce milk. In fact, alveologenesis occurs only after pregnancy, induced by the ovarian hormone **progesterone** and the pituitary hormone **prolactin** (PRL)[40].

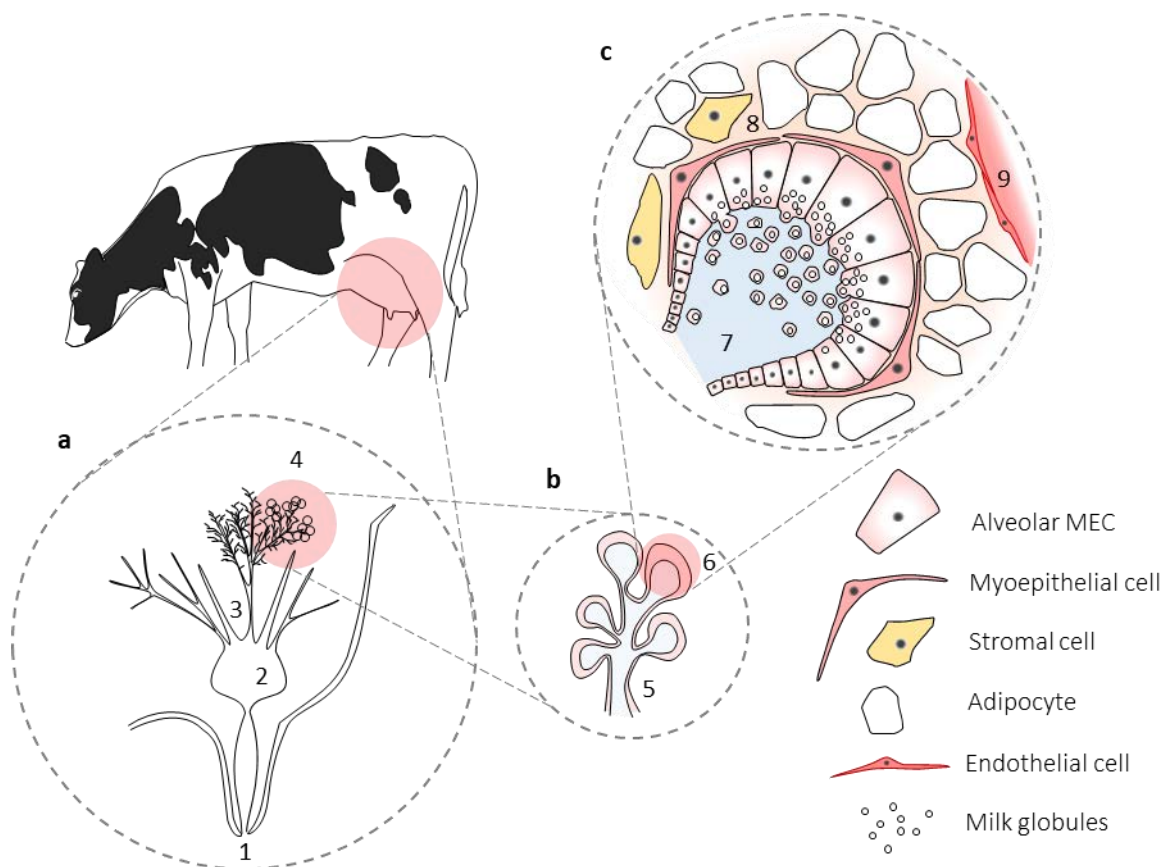

Figure 3. Illustration depicting the udder tissue structuration from the outside to the inside. **a.** Detail for one udder quarter showing the (1) teat canal, the (2) udder cistern, the (3) large milk ducts and the (4) alveoli formed in distal ends. **b.** Detail of one alveoli ramification through a (5) small milk duct, ending in several (6) alveolus. **c.** Insight in the alveolus cell organization composed by MECs, which form the (7) milk cavity or lumen where these secrete milk globules by apocrine secretion. The alveolus is at the same time surrounded by myoepithelial cells, adipocytes and other stromal cells within the (8) extracellular matrix and irrigated by the (9) bloodstream.

The binding of progesterone to its MEC intracellular receptors triggers the Receptor Activator of Nuclear factor kappa-B ligand (**RANKL**)/**RANK** signaling pathway, promoting epithelial proliferation and differentiation (mammary duct side-branching and alveologenesis)[41]. Later, at parturition, high levels of PRL bind to prolactin receptors (PRLR) in the membranes of MECs, activating downstream Janus kinases and Signal Transducer and Activator of Transcription 5 (**JAK2/STAT5**) signaling. At this stage, STAT5 promotes the expression of Serine/Threonine Kinase

AK1 gene (***Akt1***)[42], a molecule related with cell survival, and of milk protein genes like casein beta (*Csn2*) and whey acidic protein (*Wap*), beginning **lactogenesis**[43]. MGs are, thus, exocrine apocrine secretory glands, and milk is released into the alveoli lumen in evaginated vesicles containing part of the alveolar cell cytoplasm (Figure 3c).

In dairy cattle, MECs produce and secrete milk along approximately ten months from the date of calving, being the secretion rates extremely high during several months (see Figure 1). In natural conditions, a lack in milk demand after mammal weaning would promote a natural process of involution and regeneration of the MG. However, since in dairy cows there is a continuous milking, a **dry period** has been established before calving (and thus before starting the next lactation cycle) mimicking the natural scenario, with the final aim to recover the high performance of the tissue before a new lactation cycle begins.

Since animal health and welfare is a priority, optimizing the dry period conditions has been one of the most important challenges in the dairy industry with a dual objective, to reduce the risk of mastitis and to reduce the non-lactating period. However, understanding which are the main actors orchestrating the involution and regeneration processes is critical to do so.

Involution and regeneration of the mammary gland

Two months before calving, milking is ceased in dairy farms and the dry period starts. As a consequence of the pressure originated by **milk stasis** in the udder alveoli, programmed cell death or apoptosis[44] is promoted in the mammary epithelia and **involution** takes place, being a process that can be divided into two stages: a first **reversible phase** and a second **irreversible phase**[45]. While in mice the irreversible phase starts after 72 h of non-milking and it is characterized by a marked reduction in MECs number, in cow MGs it takes more than one week[42] to reach the irreversible phase (Figure 4), without an evident cell loss[46,47]. Indeed, after 14 and 28 days of non-milking it is still possible to partially restore milk production in cows by reinitiating milking[48].

During the first phase of involution, the increasing pressure in alveoli promotes changes in MECs intracellular signalling promoting-apoptotic pathways (Figure 5). High levels of tumour necrosis factor α (**TNF-α**) produced by secretory MECs and stromal cells activate the synthesis of Leukemia inhibitory factor-signal transducer (**LIF**)[49] and other cytokines, downregulating STAT5, which was supporting lactation, and activating **STAT3**[42]. Moreover, TNF-α amplifies apoptosis by permeabilizing lysosomal membranes[50]. STAT3, meanwhile, activates **acute phase proteins** and pro-inflammatory signals[51], like mammary serum amyloid A3 (**M-SAA3**)[52], downregulates the activated survival factor Akt1[53], and potentiates the synthesis of lysosomal **cathepsin proteases**[54].

Likewise, STAT3 has demonstrated to downregulate STAT5 through the promotion of Suppressor of cytokine signalling 3 (**SOCS3)** expression, which blocks more effectively STAT5 than it does with STAT3[42]. Altogether leads to a lysosomal-mediated cell death of MECs, and to an inflammatory response (detailed in the following section). We can say, thus, that involution starts with the switch from STAT5 (lactation) to STAT3 (apoptosis)[42].

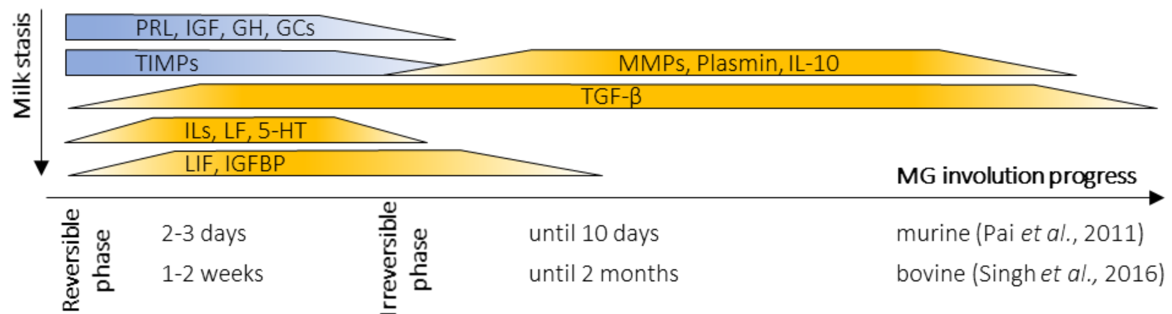

Figure 4. Scheme of the signaling changes along mammary gland (MG) involution. In blue, factors coming from lactation stages that are conditioning the onset of involution, on involution timescale; namely, systemic hormone and local tissue inhibitors of metalloproteinase (TIMP) levels. In orange, different patterns of expression of local factors involved in involution. In the murine model, the reversible phase takes about 72 h[55] while in dairy bovine this extends from 1 to 2 weeks[42]. Figure adapted from Pai *et al.* 2011[55].

Another important up-regulated player during early involution is the transforming growth factor β (**TGF-β**), which during lactation is inhibited by the high levels of PRL. During involution, TGF-β levels rise and become responsible for the **epithelial-mesenchymal transition** (EMT) process –understanding mesenchymal as cells conforming connective tissue and not parenchymal. During this process, MECs lose both cell polarity and adhesive molecules, such as integrins, facilitating tight junction reorganization and becoming **phagocytic**[56]. These new-skilled epithelial cells will contribute in the clearance of apoptotic cells, together with neutrophils and macrophages arriving chemoattracted by pro-inflammatory cytokines.

During the second involution phase, a prolonged withdrawal in PRL leads to the stimulation of mitochondria-mediated intrinsic apoptosis pathways, triggering irreversible changes in the MG leading to **tissue remodelling**. This reinforces the downregulating signals for the Akt survival pathway (Figure 5), also downregulating the IGF-mediated blockage of Matrix Metalloproteinase (**MMPs**) synthesis[57]. In addition, TGF-β, together with pro-inflammatory cytokines such as TNFα and interleukin 1β (IL-1β), have been described to promote the synthesis of MMPs such as **MMP-9**, through Smad and c-Jun N-terminal kinases (JNK) signalling pathways[58]. Thus, PRL withdrawal together with the increasing levels of MMP-9, derived from MG synthesis and degranulation of chemoattracted neutrophils, are likely to promote MMP-9 activity in bovine MECs. Nevertheless,

as far as we know few data is available about the signalling pathways promoting MMP-9 in the MG during bovine dry-off[59]. Be that as it may, high levels of MMPs and other proteases are found in the bovine MG at this point[60] surpassing tissue inhibitor of metalloproteinase (TIMP) levels and degrading extracellular matrix (ECM) components.

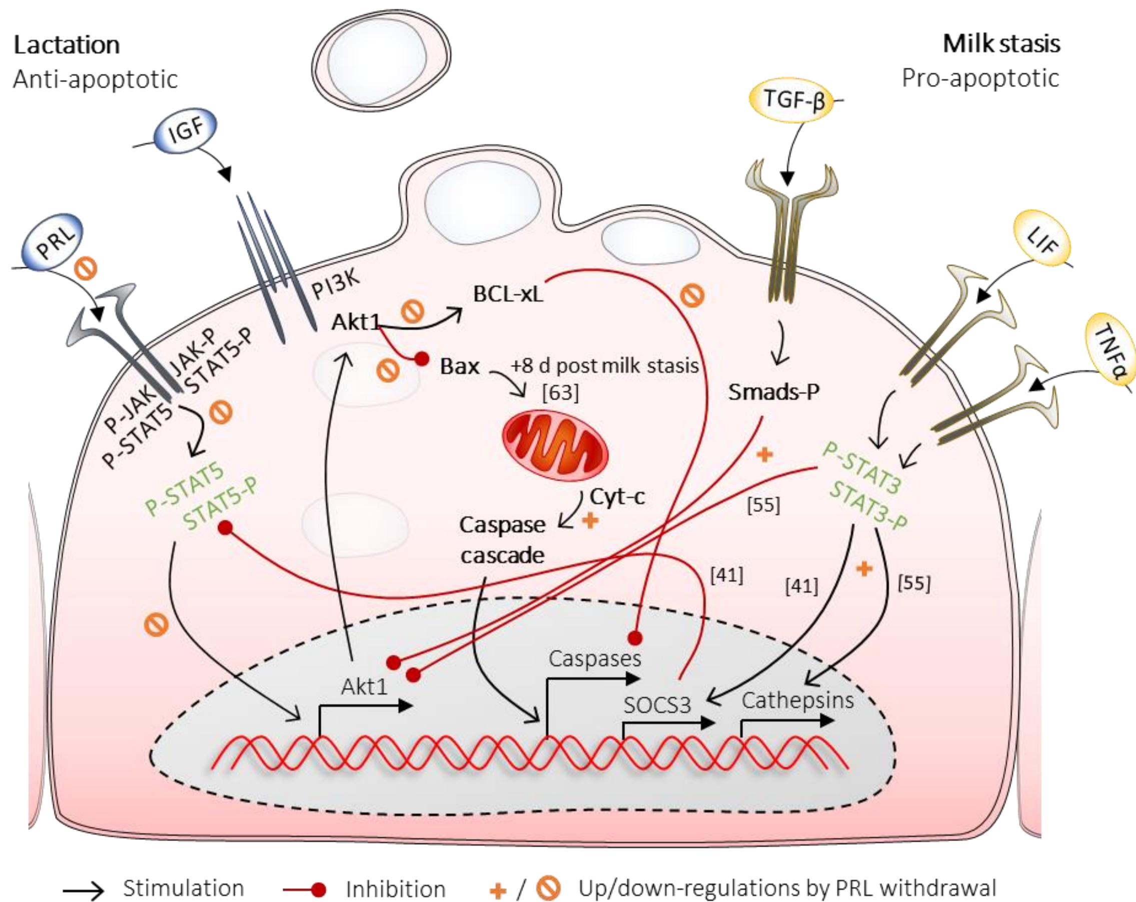

Figure 5. Simplified signal cascades at MEC turnover. During **lactation**, high levels of prolactin (PRL) and other factors (i.e. IGF, GH) promote cell survival. PRL upregulates STAT5 that signals in the nucleus (grey oval) to promote *akt1* expression. Akt1 binds PI3K, upregulated by IGF external signals, and inhibits Bax while stimulating BCL-xL that, in turn, blocks Caspase synthesis. At early dry-off, **milk stasis** increases levels of TGF-β, TNFα, LIF and pro-inflammatory cytokines, which activate pro-apoptotic factors through STAT3 and Smads. These can inhibit *akt1* expression and potentiate cathepsins (proteases). After one to two weeks from milking stop, **PRL withdraws** downregulating the survival pathways. Bax levels increase after 8 days (+8 d) in bovine[42], and permeabilize mitochondrial membranes favoring the release of cytochrome-c (Cyt-c), initiating the intrinsic mitochondrial apoptotic pathway through the **caspase cascade**. SOCS3, upregulated by STAT3, also blocks STAT5[42]. Altogether drives MECs into apoptosis. Signaling data not cited extracted from Stanford *et al.* 2013[61].

The **basement membrane** is the scaffold where MECs in the alveoli are prostrated and works as an impermeable barrier between the alveoli environment, rich in milk proteins such as caseins and lactose, and the interstitial fluid, rich in BSA, Na^+ and Cl^- [62]. When protease levels increase, thus, these degrade collagens and integrins finally provoking the alveoli basement membrane

breakdown and alveoli collapse[63]. Tissue permeabilization in the MG can be recognized by the presence of Na^+, BSA and SCC in MG secretions[64–66], among others.

In later stages, the clearance of apoptotic cells, milk residues and extracellular components by phagocytic cells promotes the replacement of inflammatory signals for anti-inflammatory and proliferative signals allowing the MG tissue to regenerate. This regeneration process includes ECM remodelling and parenchyma –epithelial and myoepithelial cells composing the functional component in the MG– regrowth[45] preceding the following **colostrogenesis**[67].

Immune condition in the udder: defeating an infection

Pathogen recognition

The first line of defence in the mammary gland is the teat canal. Besides from being a structural barrier, the teat canal is coated with antimicrobial substances such as keratin and bacteriostatic fatty acids impeding bacteria to pass through the teat canal into the gland cistern and set an infection easily. If pathogens still manage to infect the MG, different components from the **innate immune system** will take part to recognise them and to activate a pro-inflammatory response in the attempt to resolve the infection.

MECs, sentinel **macrophages** and **neutrophils** play an important role in the first detection of pathogen/microbial associated molecular patterns (PAMPs/MAMPs) by pathogen recognition receptors (PRRs). These molecular patterns can be lipopolysaccharide (LPS) motifs, bacterial elastases or exoenzyme S, recognized by a family of PRR called Toll-like receptors (**TLR**), or peptidoglycans from the gram-positive bacteria cell wall, recognized by other PRR named nucleotide-binding oligomerization domain- **(NOD-) like receptors**, among other antigens[68]. This interaction leads to a transcriptional signalling cascade, in which nuclear factor-κB (NF-κB) would activate the expression and secretion of **pro-inflammatory cytokines** and chemokines like TNF-α, IL-1, IL-8 (CXCL8) or CXCL2, among a long list[27], and promote the recruitment of more neutrophils and other immune cells to the inflammatory site (Figure 6). **Macrophages** can also recognise antigens through IgG receptors –a type of Fc receptors– and engulf them, further processing and presenting them in their surface to lymphocytes, acting as a linker between the innate and the adaptive immune responses.

Neutrophil migration and inflammatory response

After the release of chemoattractant cytokines, neutrophils are the fastest leukocytes migrating to the inflammatory site[69] due to their adhesion molecules –L-selectin and β2-integrin permitting **diapedesis**– becoming the predominant cell type in the infected MG. High amounts of these

polymorphonuclear (PMN) cells will start to recognise and **phagocyte** antigens through their IgG, IgM and complement receptors, leading to the activation of an intracellular **respiratory burst** to destroy engulfed microbes. Besides, monocytic, and later-on NK cells and cytotoxic lymphocytes, will secrete IFN-γ, increasing receptor-mediated phagocytosis in both neutrophils and macrophages[70,71].

By neutrophil degranulation, several bactericidal **Host Defence Peptides** (HDP) such as **β-defensins** will be released, being important innate defences in bovine mastitis[72]. Also, proteases and other enzymes will be released during degranulation. Through the liberation of MMPs, such as MMP-3 and MMP-9, endothelia increases its permeability allowing faster cellular migrations to the infection site[64] and potentiating a more robust local inflammatory process.

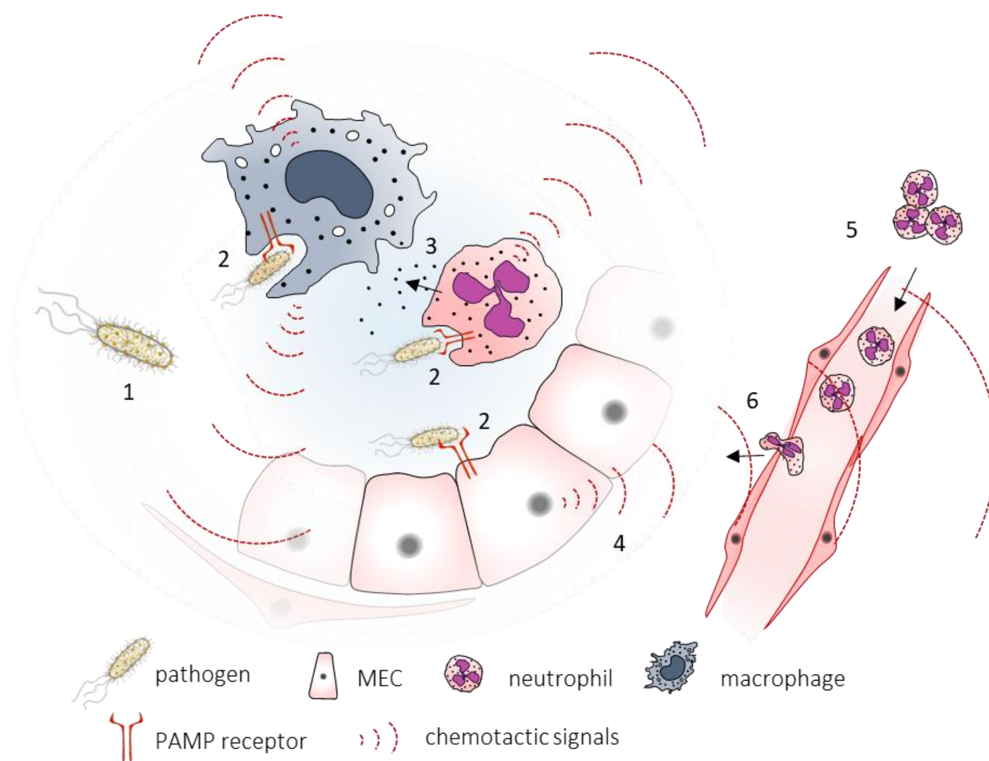

Figure 6. Illustration depicting the recruitment of immune cells in response to pathogen infiltration in the mammary gland (MG). When pathogens reach the MG lumen (1), these are detected by pathogen-associated molecular pattern (PAMP) receptors in membranes of resident cells and are engulfed by phagocytic cells (2). This recognition activates intracellular signalling cascades and trigger degranulation of neutrophils (3) and the release of pro-inflammatory cytokines and chemotactic signals (4), the call for new immune cells to move (5) to the threatening origin. Neutrophils are the first cells to arrive due to their capability to cross endothelia by diapedesis (6).

Innate soluble defences

Additional pro-inflammatory cytokines such as TNF-α will be secreted by the activated immune (neutrophils and macrophages) and epithelial cells, promoting an acute-phase reaction by

stimulating the release –in milk and blood– of acute-phase proteins such as SAA, haptoglobin, C-reactive protein and LPS-binding protein (LBP), as well as lactoferrin (LF).

- **Acute-phase proteins** (APPs) support inflammation through increasing chemotaxis and pathogen recognition capability in activated cells. These proteins are becoming important biomarkers in mastitis diagnostics as differences in APP levels can be associated to different causing pathogens[73–75]. As an example, low M-SAA3 levels are observed in mastitis caused by contagious bacteria (*S. aureus*) while higher levels are found in *E. coli*, *S. uberis* or *S. dysgalactiae* mastitis samples[75].
- **Lactoferrin** is one of the best-characterized mammal antimicrobials, expressed at high levels during MG involution and during infection. This antimicrobial has bacteriostatic and, for some pathogens, bactericidal properties through its capability to bind iron, which is required for bacterial proliferation[76].

Through all these innate mechanisms, infections are generally resolved, and the high levels of neutrophils enter programmed cell death and are phagocyted by macrophages, restoring their basal levels in the MG. However, at dry-off the immune system functionality is retarded due to pregnancy-related hormone levels, as will be detailed thereafter, as well as due to milk stasis, that collapse phagocytosis reducing macrophages contribution to resolve infection. If the infection persists, lymphocytes will be activated and an **adaptive immune response** will take place.

Adaptive response

Naïve lymphocytes are in constant recirculation. **Lymphocytes T** have a diverse repertoire of antigen recognising receptors or TCR in their surfaces, each specific for a unique antigen. Antigens are presented by macrophages, MECS or professional antigen-presenting cells (APC) such as **dendritic cells**, in association to major histocompatibility II (MHC-II) molecules. When one TCR recognises an antigen, the naïve T-cell will be activated, differentiated to helper $CD4^+$, and will start proliferating. A different subset of lymphocytes T are $CD8^+$ cells, which recognise antigens from altered self-cells –presented in association with MHC-I–, and undergo cytotoxic responses due to the expression of IFN-γ, or suppressor responses due to the expression of IL-4[77].

Lymphocytes B circulate as antigen presenting cells (APC), recognising and internalising new antigens to process and present them in the surface, until they are recognized by a T-helper cell becoming activated. In the MG, B-cells can differentiate into plasmatic cells *in situ* due to the direct recognition of certain antigens, such as LPS[78], and will produce and release specific

antibodies for the recognised antigen. Other lymphocytes can be differentiated in the canonical sites which are the lymph nodes and spleen. Antibodies will attach to the pathogen through their Fab fraction in a process called **opsonization**, which allows an MHC-I-independent cytotoxic activity, performed by a third non-immune lymphocyte subset that are the **NK cells**. These cells bind the free Fc fraction of the pathogen-attached antibodies lysing or promoting apoptosis to the opsonised cell though the liberation of several cytolytic and toxic molecules. Moreover, the above-mentioned macrophages and neutrophils will also be able to recognise the Fc fractions of the opsonising antibodies, permitting them to phagocyte the signalised pathogenic elements.

Resolution

Once the pathogen is eliminated, the excess in pro-inflammatory signals will lead to the synthesis of different pro-resolving **lipid mediators** such as lipoxins, resolvins, protectins and maresins as detailed by Buckley *et al.*[79]. Maresins, for example, will be responsible for the switch from macrophages pro-inflammatory phenotype (M1) to a **pro-resolving phenotype** (M2). Lipoxins and resolvins, by the way, will downregulate neutrophil transmigration and inhibit their chemotactic cytokines such as MCP-1, while upregulating inflammatory-antagonist cytokines such as IL-10 and TGF- β and anti-inflammatory cytokines, such as IL-6 and IL-4[79]**.** Through this, pro-inflammatory signals will be removed and PMN cells will enter into apoptosis and will be cleared by M2 macrophages, restoring the MG homeostasis[79]. If this process is impaired, tissue damage may occur due to chronic inflammatory responses, as it happens during prevalent subclinical mastitis[80].

Coexistence of dry-off and pregnancy

A particularity of dairy animals and one of the most critical causes increasing IMI risks during dry-off is that pregnancy and lactation occur simultaneously cycle after cycle. This determines important differences between the involution of the MG in dairy animals compared with the rest of mammals.

Aiming to mimic dairy cow MG conditions, a mouse model in which weaning is forced has been developed[81,82]. Using this dairy-like experimental model, it has been demonstrated that high levels of pregnancy-related hormones, such as progesterone (secreted by the ovaries and placenta during pregnancy), inhibited the MG involution[81,82]. Pregnancy impeded STAT5 inactivation thus inhibiting MECs apoptosis, observable in a lower decrease of these cells during involution[82], coinciding with previously reported data related to dairy cows[46]. Thus, progesterone,

together with PRL, which is also upregulating STAT5, are responsible for the systematic delay in the dry-off in dairy cows after milk cessation.

Another function of progesterone is the induction of **foetal tolerance**, due to a bias of the maternal immune response towards an anti-inflammatory phenotype[83]. This is important in order to avoid aborts, but also increases the animal susceptibility to pathogenicity during lactation months coexisting with pregnancy and, also, during the dry period. Thus, at dry off there is a delay in the arrival of phagocytes at the MG (6 to 7 days from dry off) and when they arrive, they are overwhelmed engulfing milk components and apoptotic cells, leaving the immune system further impaired[84]. This particular situation in dairy animals makes the bovine dry period especially critical and explains the high risk of IMI and an involution that occurs though a slower process than in other mammals.

MASTITIS PREVENTION WITHIN A GLOBAL HEALTH ALERT CONTEXT

Prophylactic antibiotic use at dry-off

Different approaches to reduce the high IMI risk and costs at dry-off have been largely studied. For example, as introduced in "*Dry period in dairy cows*", different studies have evaluated a gradual stop of milking as an alternative to the abrupt cessation[15,85]. Although with a gradual strategy milk is progressively reduced, it has been reported that, especially for multiparous cows, a gradual cessation of milking increases milk leakage and IMI incidence at calving[15]. It would be relevant to disclose whether an abrupt cessation of milking triggers a more important inflammatory response in the MG, favouring a faster involution in multiparous cows compared with gradual cessation. However, as far as we know, studies confirming this point are not yet available and differences after both dry-off strategies have not been demonstrated in somatic cell score (SCS) in the subsequent lactation[86].

Changing food routines to those with lower energy or with less palatable ingredients, in combination or not with a gradual stop of milking, has been also evaluated[16,87–89]. A reduction in milk productivity during or just before dry-off[88,90,91] is observed after these procedures. However, the relationship between limiting energy intake and IMI incidences is not clear and hunger supposes an additional stress to the dairy cow[89,92] that should be avoided. Moreover, stress, such as hunger, may increase the mobilization of fat and the production of non-esterified fatty acids (NEFAs) in blood[16], which further impairs the immune system[88,93].

Because of the high costs associated to IMI in dairy farms, and as a means of risk prevention, **dry cow therapy** (DCT) with intramammary **antimicrobials** (AMs) has been long considered essential to preserve udder health[94]. With the aim to prevent new IMI during the dry period, it is still being advised in some countries the administration of **preventive antimicrobials** at dry-off, irrespective to the presence or not of an infection (also called **blanket DCT**, BDCT). However, as will be highlighted in the next section, the apparition of antibiotic resistances has overturned the criteria whether this strategy should be used or not.

Facing the antimicrobial resistance crisis

The inappropriate use of AMs has leaded to an **antimicrobial resistance** (AMR) **crisis**, a dramatic global health threat that was declared "the most urgent global risk" in 2016 by the United Nations (UN) General Assembly[95]. **Resistances** to antimicrobials occur when microorganisms (i.e. bacteria, fungi, viruses and parasites), usually with a high capability to mutate and adapt to environmental changes, are exposed to a non-effective AM treatment (low doses or unfinished treatments). For example, if only one bacterium randomly mutates favorably to resist a specific AM and survives, an entire colony of this bacterium will be able to grow and spread and this AM will be nevermore useful to eliminate the new resistant bacteria strain. The situation can be repeated with a second AM, and successive, leading to **multi-resistant bacteria**, for which few alternatives are left to its elimination. According to a recent report of the UN Ad Hoc Interagency Coordinating Group on Antimicrobial Resistance, at least 700,000 people are dying every year due to drug-resistant diseases, being 230,000 of those deaths due to multidrug-resistant tuberculosis[96]. These numbers, however, are estimated to dramatically increase along the next years (Figure 7)[97,98].

Urgent global calls have been made by the World Health Organization (WHO) to stop over-using and misusing antimicrobials to preserve its efficiency, translated in 2015 in a Global action plan on AMR[99]. The European counterparts engaged in 2011 addressing the **EU One Health action plan against AMR**[95,100,101], recognising the interconnection between human, animal and environmental health, and describing a guideline to boost research initiatives to reduce AMRs and to enhance a better use of AMs in the EU. Because the animal sector is an important user of AMs, one of the measures taken is the **ban of preventive use of antibiotics** in groups of animals[95]. This has promoted real changes in the use of antibiotics in most countries, avoiding their preventive use, and respecting a restricted use of some critical antibiotics only for human application.

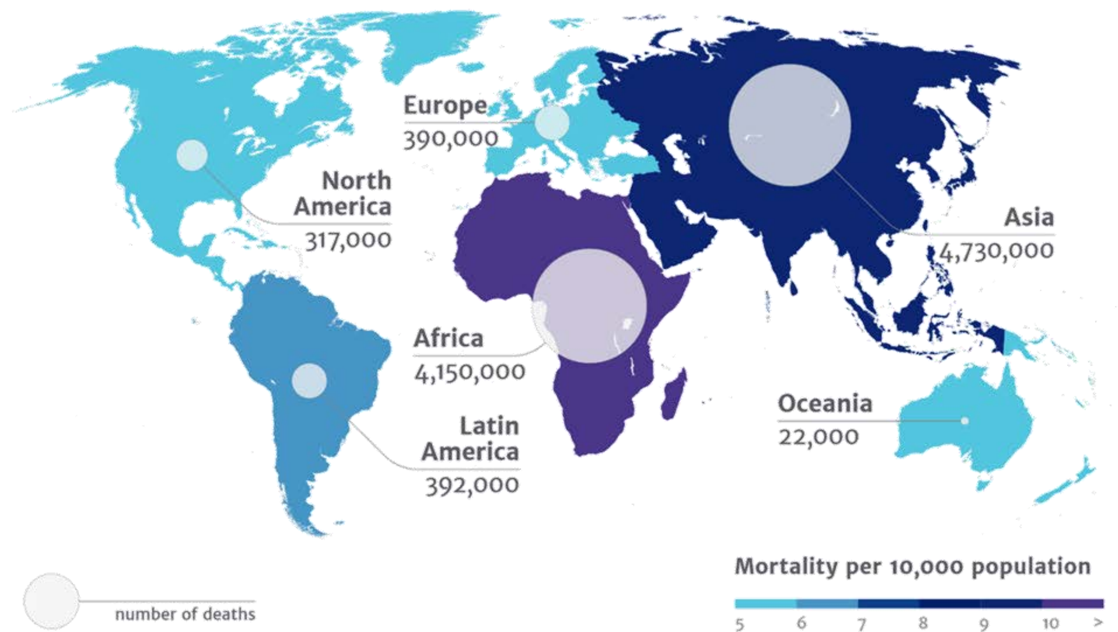

Figure 7. Worldwide estimated deaths by 2050 due to AMRs. Resistant bacteria are already causing 700,000 deaths each year globally and an abrupt scale-up is estimated in the next 30 years unless new practices and alternatives are accomplished. Image reprinted with permission from[97].

Thus, there is an urgent need to find **effective alternatives** to BDCT to ensure a proper management of the cows during the dry-off avoiding economical losses to the farmer and accomplishing the One Health plan indications. Some European countries, like Denmark and the Netherlands, already banned the use of BDCT in 1994 and 2012, respectively, and adopted a **selective dry cow therapy** (SDCT)[102]. Briefly, in the Netherlands, as it is also practiced in other countries such as UK and Germany[103,104], SCC in milk –along lactation or at the last milking before dry-off– is used as the main criterion to apply antimicrobial DCT only in positive cases. This has made possible to reduce the use of intramammary AMs during dry-off without detrimental effects in the udder health[102]. In Denmark they follow a two-step SDCT criteria, as SCC indicate the cows for which bacteria culture and PCR analyses should be done before drying-off, to further select which are the cows that will receive an AM treatment[105].

Spain has been one of the largest AM consumer for food-producing animals. Accordingly, and considering the eight European Surveillance of Veterinary Antimicrobial Consumption[106], SDCT in Spanish farms is being actively endorsed[103]. Indeed, the first results obtained in a Spanish multiple-farm study by Zoetis were presented in Sevilla on May this year in the XXIV International Congress of Bovine Medicine organized by ANEMBE (Asociación Nacional de Especialistas en Medicina Bovina de España). In this study they conclude that a 57 % reduction in the use of dry-

off antibiotics is possible without significant effects in udder health through the selection of risk cows by SCC[107].

Thus far, the use of strategies such as SDCT are of critical importance in the context of global resistance crisis. However, there are cases in which animals have a high predisposition to contract IMIs at every lactation cycle where SDCT is not enough, and alternatives must be found to avoid starting the dry period with unresolved subclinical infections. Thus, it is necessary to develop **complementary strategies** in animal production to guarantee an efficient production system. The use of an internal **teat sealant,** such as Orbiseal developed by Zoetis, is gaining popularity in dairy farms due to their success in reducing the IMI risk at dry-off[108,109]. This consists in the application of a soft viscose paste mimicking the natural keratin plug that is formed when there is no milk leakage, impeding the entrance of pathogens into the MG. Other interesting strategies aim to **optimize the MG involution process**, which is closely linked to the immune stimulation during the dry period. This would be of a great relevance, because through the acceleration of the involution processes, the dry-off length could be potentially reduced as well as the risks associated to mastitis and the use of AMs in dairy.

Accelerating mammary gland involution at dry-off

As reviewed by Zhao and coworkers in their recent publication[11], several strategies have been tested with the aim to accelerate the MG involution in bovine dry-off to reduce both the IMI risk and the non-productive period (Figure 8). These are briefly explained:

- Hormonal modulation: injections of **estradiol** to lactating cows have been reported to reduce milk production and to induce MG regression[110]. Also, PRL inhibition through **quinagolide,** a dopamine D2 receptor agonist, has been reported to reduce milk yields[111] as well as reducing proliferation and increasing apoptosis of MECs[112]. However, the dose regimen required (one or two injections per day) fades their applicability[88]. Moreover, another dopamine agonist, **cabergoline**, has demonstrated to reduce milk leakage and udder pressure through a single injection preceding drying-off[113,114], also involving matrix remodeling and accelerating involution[115,116].

- Intramammary infusions to increase permeability between MECs: **casein hydrolysates** infused through the teat canal in late-lactation cows altered the tight-junction integrity increasing LF concentrations in the lowered MG secretions[65]. **Calcium chelators** have been also infused and have demonstrated to destabilize tight junctions[117].

- Intramammary infusions to immunostimulate the MG: **LPS**[118,119], **concanavalin A**[120], ***Panax ginseng* extract**[121,122], and **M-SAA3**[123], are examples of infused molecules that successfully activate the cow MG immune response at dry-off. Also **chitosan,** which is a natural polysaccharide with immunostimulating properties that forms hydrogels at body temperature[124], has been proven to stimulate the immune system of dairy cows after infusion in the MG, triggering involution and conferring protection to new IMI during dry-off[125]. Some recombinant strategies, as will be detailed in "*Recombinant proteins as the alternative to deal with mastitis: immunostimulating strategies*", are gaining importance in this research field.

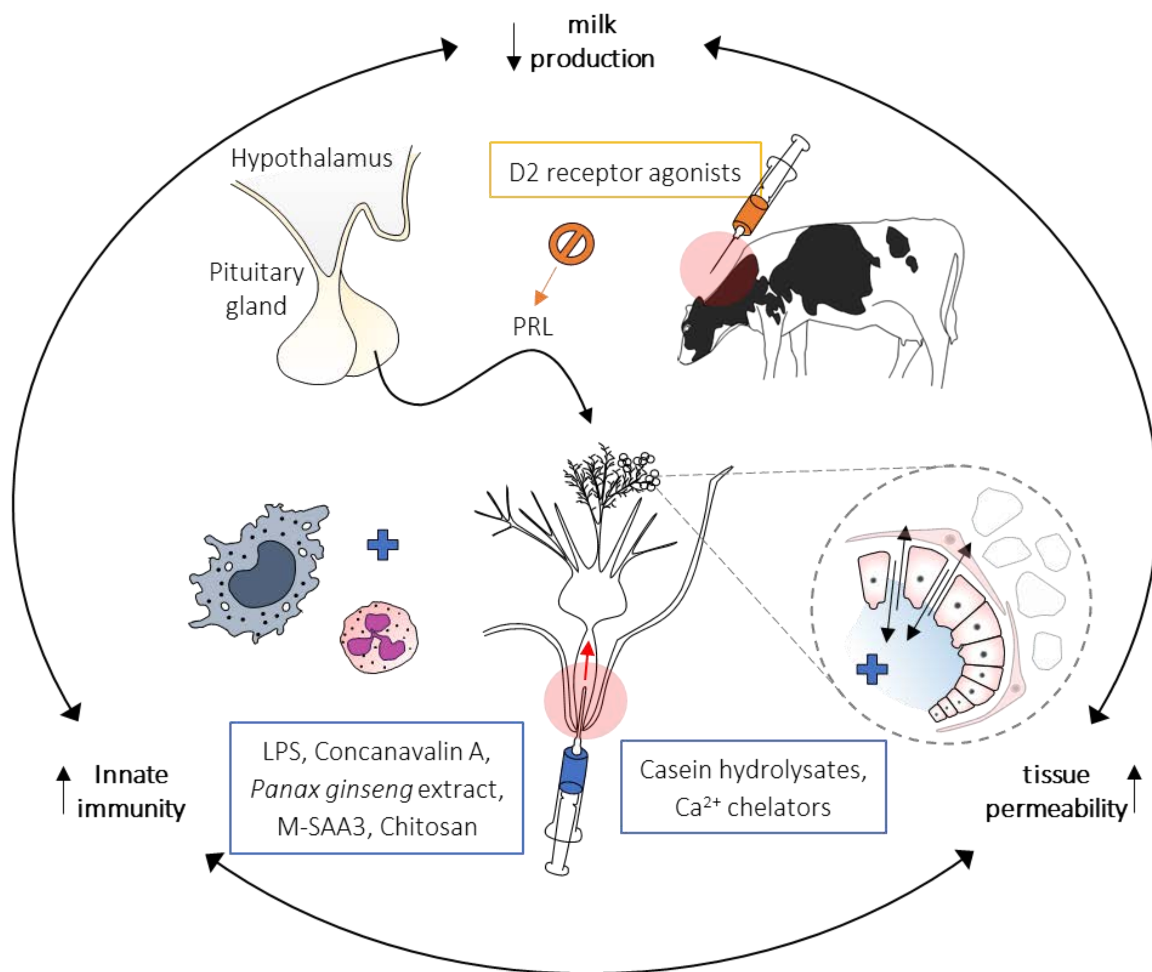

Figure 8. Different strategies are being explored to accelerate the MG involution in dairy cows with highly interconnected effects converging in a common response. In orange, injections with dopamine D2 receptor agonists such as quinagolide or cabergoline block lactogenic signals promoted by the pituitary hormone PRL. In blue, infusions in the MG of immunostimulant agents and tight-junction disruptors promote, respectively, the attraction of innate immune cells, like neutrophils (pink) and macrophages (blue), and the increase in tissue permeability.

Many efforts have been dedicated to find new strategies to optimize the dry period length and the cow condition in this problematic stage. Despite the good results obtained in some of the

cases, there is still a long way ahead in the research for alternatives and complementary strategies to antibiotics in the bovine mastitis battle to ensure an economical viable tool, easily implementable into the field. In this context, **biotechnological approaches** could have a relevant role to broaden the tackling toolbox.

NANOBIOTECHNOLOGY AND ANIMAL SCIENCE

The discovery of the DNA technology revolutionized science research and since then a wide catalogue of proteins has been recombinantly produced using cell factories[126]. Many of these **recombinant proteins** are being broadly used in human therapy and prophylaxis, like monoclonal antibodies Rituxan and Humira or the hematopoietic cytokine Filgrastim, which are some examples of **cancer-related** recombinant therapies[126]. Also, several proteins are being developed as improved **allergens**[127], **vaccination** strategies such as FluBlok against influenza virus[128], as well as **autoimmune-related** therapies like the case of the recombinant human insulin, which was the first commercialized recombinant protein (Humulin, Lilly and Genetech 1982) and for which diverse analogs have been developed to improve the treatment of type I diabetic patients[129,130].

In animal science, some recombinant proteins are being developed and some are already commercially available and used for animal production purposes, such as **reproductive hormones** rFSH by Nanocore Biotechnologia, and Luveris (rLH) by Merck[131,132], and **fibrolytic enzymes** like Rumistar (α-amylase) by DSM-Novozymes[133]. Moreover, several recombinant **vaccines**, such as the multimerized scFv against bovine Herpes virus-1, are being developed[134]. However, the costs associated to protein production and downstream purification processes to reach a final pure product sometimes limit their applicability. It would be relevant, in this sense, to uncover which are the main interests in the use of recombinant proteins in the animal sector, and how are those proteins being produced, to identify which are the bottlenecks that could be impairing their use. This would be essential for the development of strategies to reduce costs and make the affordability of the recombinant protein world a reality in animal science. In this context, **nanotechnology** could be a useful tool to consider, since it could allow to develop alternative protein formats to deliver the protein of interest with reduced costs. Developing more stable protein formats is one way to economize protein production costs, and in this context protein encapsulation, further detailed in "*Encapsulation of soluble proteins*", and the use of alternative formats such as **inclusion bodies** (IBs), detailed in "*Inclusion bodies as a new protein format*", may be interesting alternatives to the standard soluble formats.

Recombinant protein production

Recombinant proteins are proteins produced using host cells, or **heterologous expression systems**, as cell factories. These hosts may be prokaryotic cells like bacteria or eukaryotic cells like yeast, fungi, insect cells, or mammalian cells. Briefly, a gene of interest is cloned into a DNA vector or plasmid, and this is introduced in the host cytoplasm with the aim to overexpress the cloned gene using the host replicative machinery. Once produced, the recombinant protein can be obtained from the cell cytoplasm by cell disruption or alternatively can be recovered from the culture media if proteins are designed to be secreted by the host cell[135].

In animal production, the mostly used expression systems for commercially available recombinant proteins are mammalian cell hosts such as Chinese hamster ovary (CHO) cells[136], also popular to produce proteins for human therapy[126], and yeasts (*Komagataella pastoris*)[136]. Mammalian cells have the advantage to lack associated host toxins, as well as having similarity in post-translational modification processes compared to the natural producers for the protein of interest. Nonetheless, the costs associated to the use of eukaryotic cells are their main disadvantage, whereas the shortcoming for yeast is that although they do post-translational modifications, these are not equal to the ones performed by animals and human cells.

In academic research, ***Escherichia coli***, a Gram-negative bacterium, is still the first and most used expression system for recombinant production because of its simplicity, fast growth and high performance. Also, there is a broad knowledge about its genetic background and a plethora of commercially available mutant variations has been developed to work with[137]. A main limitation of this polyvalent bacterium, though, is the presence of **lipopolysaccharide** (LPS) in its cell wall. This endotoxin is difficult to remove and accompanies the recombinant protein even throughout purification processes. This is especially problematic when it is aimed to validate a recombinant protein for its immunomodulating functionality, as responses to the residual LPS interfere the experimental results. Aiming to cover this gap, a non-immunogenic *E. coli* mutant with a modified LPS precursor (lipid IV_A), named **ClearColi™** (Lucigen and Research Corporation Technologies) has been developed[138]. Although this strain was able to produce endotoxin-free soluble proteins and IBs with similar physicochemical properties as the previously reported and in sufficient yields[139], the slow growth rate of ClearColi™ is an important impairment. Moreover, it is worth to mention that even it does not trigger an endotoxic response in humans, lipid IV_A do trigger responses in other species cells such as mice, Chinese hamster and equine[138].

Gram-positive bacteria, as will be described in the following section, are microorganisms that do not contain LPS, and are being explored as promising alternatives to the costly processes of LPS removal needed when using *E. coli* (Figure 9).

Lactococcus lactis as a microbial cell factory

The **lactic acid bacteria** (LAB) group is a heterogenic group of bacteria that are mainly formed by the genus *Lactococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc* and *Lactobacillus*[140]. LAB have promising candidates to be used as cell factories for recombinant protein production[141]. In addition of being free of LPS by their Gram-positive condition, some LAB such as *Lactococcus lactis* or *Lactobacillus plantarum* have been generally recognized as safe (GRAS) microorganisms by the U.S. FDA, and fulfill the European criteria of the Qualified presumption of safety (QPS) according to the European Food and Safety Authority (EFSA)[142].

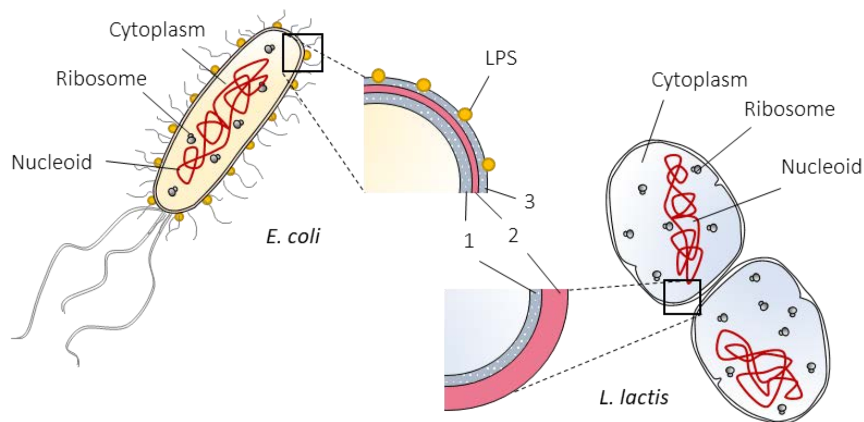

Figure 9. *Escherichia coli* and *Lactococcus lactis* structural comparison. Both bacteria have an inner membrane (1) surrounding the cytoplasm content, although the peptidoglycan wall (2) differs between them, being this thicker in Gram-positive bacteria, and more difficult to break. Gram-negative bacteria have an additional outer membrane (3) where endotoxin embeds, and flagella and pili increasing their displacement abilities.

The mostly used LAB for recombinant production is ***L. lactis***, the genome for whom was fully sequenced in 2001[143] making it a genetically well-known bacterium with different expression systems already developed[135,144]. In addition to the easy scaling-up by fermentation growth[145], another advantage that makes *L. lactis* a highly appreciated microbial factory is, indeed, that downstream purification processes can be highly simplified through driving the recombinant proteins into the medium, as *L. lactis* has only one endogenous major secreted protein (Usp45). To do so, the **Usp45 signal peptide** is tagged to the recombinant protein of interest[146]. This, added to its simple extracellular proteolytic system, consisting in a unique surface protease (HtrA), makes this microorganism an ideal secretory factory. Furthermore, to improve protein

production, a mutant strain lacking this protease has been developed[147,148].Also other protein secretion strategies have also been explored to increase production yields[149].

Both constitutive and inducible expression systems have been developed for *L. lactis*, being the inducible **NICE®** (Nisin Controlled gene Expression) system the most widely used[150]. This consists in a plasmid with a P_{nisA} promoter that is activated by the external administration of **nisin**, a food-grade inducer[150], permitting a controlled timing for protein production (Figure 10). Constitutive systems like SICE (Stress Controlled Expression) systems allow a sustained protein synthesis without requiring of an external inducer but a temperature increase (such as the physiological temperature found after an *in vivo* administration) or a pH decrease (such as those values found in the gastrointestinal tract) among others, as detailed by Cano-Garrido and coworkers[151]. Altogether has evoked *L. lactis* to be explored as a live vector for protein[152,153] and DNA[154] delivery purposes, becoming a potential new **live vaccine** approach[151,155,156].

Importantly, and as will be further detailed in the coming section, under overproduction conditions, *L. lactis* has also been described to form protein aggregates or **inclusion bodies (IBs)**[157], in the same way as it happens in *E. coli*. However, those IBs formed in *L. lactis* are LPS-free nanoclusters, that have also been demonstrated to be functional and have been well characterized[158]. Thus, *L. lactis* supposes an alternative bacterial expression system to *E. coli*, able to produce endotoxin-free recombinant proteins either in a soluble or aggregated format, being a cellular factory with high potential.

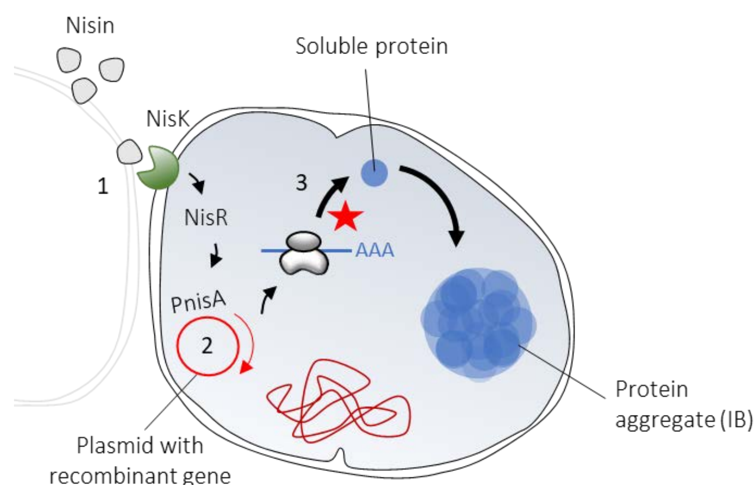

Figure 10. NICE system in *L. lactis* and split between the soluble and the insoluble (IB) protein fractions. Through the binding of nisin to the histidine-protein kinase NisK (1), the response regulator NisR is phosphorylated and leads the transcription of the promoter PnisA in the constructed plasmid. The recombinant gene, encoded downstream of the promoter, is transcribed to mRNA (2) and the host ribosomes will translate this to proteins (3). Due to overproduction stress (marked as a red star), the host machinery is overloaded and a fraction of the obtained proteins aggregate forming inclusion bodies (IBs).

Recombinant protein solubility using bacterial cell factories

As mentioned above, proteins produced using bacteria as heterologous expression systems are frequently divided in two different protein formats: (i) **soluble fraction,** which has traditionally been considered the properly folded and functional protein fraction, and (ii) **aggregated fraction** or IBs, which has long been treated as a disposable byproduct, considered to be formed by misfolded or partially folded proteins that aggregate[159].

Aiming to obtain high yields of soluble and functional protein, many efforts have been devoted to increase **protein solubility** during recombinant production processes[160,161]. These include strategies[160,162–164] such as codon-usage optimization, culture conditions testing, the co-expression of chaperones, evaluation of different plasmids, strains or expression systems, or even the addition of solubilization tags to the protein-coding sequence.

- Codon usage: codon bias occurs when the foreign mRNA contains rare codons for the host machinery that are misread by ribosomes, giving place to errors in the translated amino acid chain and leading, in some cases, to a low recombinant protein quality, yield and/or solubility[165]. **Codon optimization**, which is the substitution of rare codons by known codons translating to synonymous amino acids (silent mutagenesis), helped to overcome this bottleneck. However, some studies suggest that an excessive optimization may lead to an excessive speed in the translation machinery and to chain errors[166–168]. In multi-domain proteins, for example, slowing down the process in between domains, increasing the ribosome affinity to the mRNA, enhanced proper protein folding and solubility[169]. Thus, although optimizing the codon usage for each specific expression system can enhance protein solubility, this does not happen with all proteins and in the same grade and, in consequence, a case-by-case optimization study is recommended.

- Culture conditions: translation kinetics can be also modulated by using a low culture **temperature** while protein production is running. Through this, the overproduction-induced heat shock proteases are reduced[160,170], and the ribosome machinery is thought to reduce its speed action facilitating a more accurate recognition of mRNA triplets and a proper polypeptide construction[171] improving protein folding and solubility[172–176]. Moreover, when using bacteria that acidify culture media, like *L. lactis*, control of the **media pH** may enhance protein solubility and yield[150]. Besides, it has been described that IBs increase their compactness at low pH[177,178], being these more difficult to solubilize (this concept being further detailed in the next section) to extract soluble protein[179]. Moreover, increasing production **time lengths** and **carbon source availability**[160,161] may also promote protein

aggregation, thus tracing production kinetics and media composition is also recommended to find a compromise between yield and solubility.

- Protein secretion: the chosen plasmids can be also an ally to gain protein solubility. As already mentioned in the previous section *"Lactococcus lactis as a microbial cell factory"*, proteins can be tailored with **signal peptides** to be sent to the extracellular compartment. Through this, proteins do not accumulate inside the bacterial cytoplasm thus reducing their chances to aggregate[180].
- Co-expression of chaperones: **chaperone co-expression** along with the recombinant protein of interest has been reported to prevent IB formation in some cases[161,181]. One example is **DnaK chaperone**, which has been described to promote proteolysis of misfolded proteins and the reduction of IB formation[182,183]. Nonetheless, this chaperone is a polyvalent tool as it has also demonstrated to enrich IBs with native-like functional polypeptides[184]. However, adding chaperones is, once again, a strategy that needs to be explored case-by-case to determine its ability to enhance solubility[185,186].
- Mutant strains: especially for *E. coli*, many strains have been developed in order to improve protein production results[137]. Some of those are **C41(DE3)** and **C43(DE3)** *E. coli* mutants, designed to enhance protein solubility and useful to produce soluble membrane or globular proteins[161]. Also, **Origami™** strains, which have mutations in thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes, permit the formation of disulphide bonds in the cytoplasm[161] meliorating protein folding in some cases. Another example is **Rosetta™**, which contains the pRARE plasmid that encodes tRNAs for those rare codons that *E. coli* cannot correctly recognize, permitting a better expression of eukaryotic proteins with rare codons as an alternative to codon optimization[187].
- DNA modification: protein solubility can be also enhanced by the addition of **solubilizing tags** in the protein DNA sequence [163]. Three of the most widely known are maltose binding protein (MBP), *E. coli* N-utilizing substance A (NusA) and Thioredoxin tag (Trx), which are relatively large proteins that can act in a chaperone-like manner[163,188]. Also, the addition of transient pauses between protein domains has been reported to allow them to properly fold, slowing down translation and meliorating protein solubility[169].

Despite of the wide amount of options here exposed to increase solubility in overexpressed proteins using bacteria, sometimes none of them work, obtaining most of the protein in its aggregated version. In these cases, proteins can be solubilized from IBs.

Obtaining soluble protein from IBs solubilization

IBs can be **solubilized** becoming a source of soluble protein. This is a common practice when working with prone-to-aggregate proteins, as well as in large-scale protein production processes[189,190]. To disaggregate the proteins embedded in the IBs, a **solubilization** step followed by a **refolding** step have been classically combined to reach the final native-like and active protein conformation[191,192]. To do so, harsh detergents, such as Sodium dodecyl sulfate (SDS) or N-cetyl trimethyl ammonium chloride, and denaturing agents such as urea and guanidine hydrochloride have been successfully applied during the first step[193]. However, the refolding step of denatured proteins is a highly inefficient process, as some of the functional proteins are misfolded and/or degradated[190].

Nevertheless, the work done with IBs in the last two decades has driven to the discovery that these aggregates, far from being a disposable byproduct, contain properly folded and fully active proteins[159]. In this context, Peternel *et al.* developed a solubilization protocol for recombinant proteins produced in *E. coli* using a mild detergent (**N-lauroyl sarcosine**) and avoiding denaturing agents, observing that up to almost 97 % of correctly folded and fully active proteins could be detached from their aggregated state, avoiding downstream refolding processes[194]. Thus, this result proves that since the protein forming such aggregates is already folded, it is only necessary to use a mild solubilization process to release the protein of interest from these nanoclusters (Figure 11).

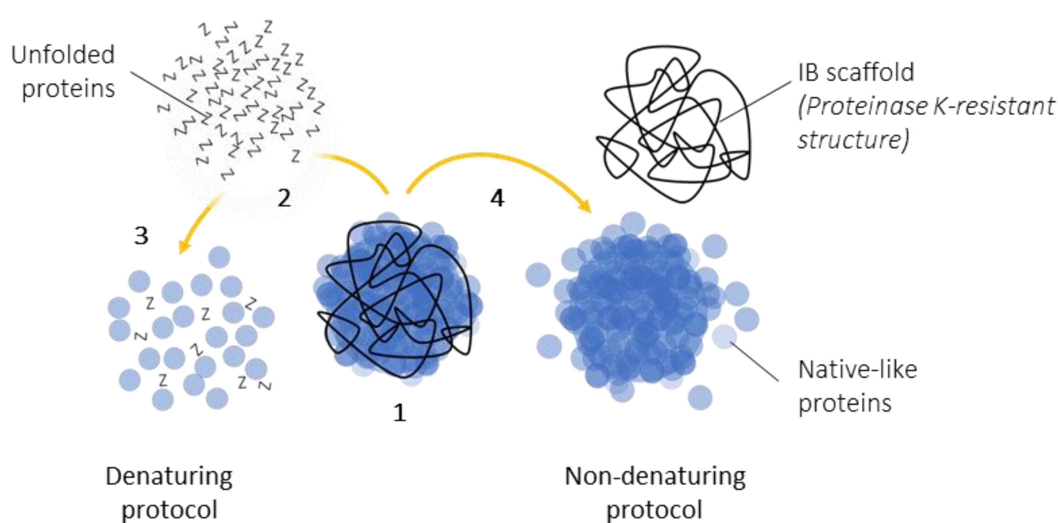

Figure 11. Supramolecular organization of IBs and different methods of solubilization to obtain soluble proteins. Functional proteins are embedded in IBs inside a proteinase-K resistant scaffold (1)[195]. Soluble proteins have been traditionally solubilized through harsh detergents and denaturing agents to unfold IBs (2) followed by a refolding step (3) implying protein loss along these procedures. Using non-denaturing procedures (4), first developed in 2008 by Peternel *et al.*, native-like proteins are released from the proteinase K- resistant scaffold without losing functionality[194]. Illustration adapted from Rinas *et al.*[196]

Inclusion bodies as a new protein format

Inclusion bodies have been defined as rough-surfaced amyloid-like protein nanoclusters, sizing from 50 to 800 nm in diameter, and spontaneously formed during recombinant protein production conditions in bacteria through an inexpensive, cost-effective, and fully scalable process[196,197]. As mentioned before, IBs are protein-based nanoparticles that contain correctly folded and **fully active proteins**[198]. In fact, IBs ultrastructure is composed by non-active proteinase K-resistant protein conformations forming an **amyloidal fibrillar scaffold**, acting as a sponge to native- and non-native-like proteinase K-sensitive protein conformations which fill in the gaps of the IBs matrix[195] (Figure 11). Although in between this protein mass there may be also unspecifically absorbed host debris like DNA and membrane fragments, lipids or even live cells escaping from disruption[199], these can be mostly removed through simple purification processes obtaining a relatively high purity in a stable protein material[200,201].

Because of their architecture, IBs provide **mechanical and chemical stability** to the forming proteins[159,195,196,202] resisting sonication and high-pressure homogenizing, lyophilization and freeze-thaw processes[203,204], added to the already mentioned heterogenic proteinase resistance[195]. Also due to this ultrastructure, it is possible to use IBs as a material that can **slowly release** the embedded proteins under physiological conditions becoming nanoparticles (or nanopills) with a high potential to be administered in a context in which a sustained release could be desirable[205].

Although the formation of IBs has been mainly associated to the recombinant protein production in *E. coli*, it has been observed that this aggregates are also formed in other bacteria when used as recombinant expression systems[206] such as *Corynebacterium glutamicum*[207] and *L. lactis*[157,158], as well as in yeasts[208] and mammalian cells (in this case, named aggresomes)[206].

Applicability of the IBs as a new protein format

Since IBs functionality has been widely reported, they have become a protein format with a high potential in a wide range of applications[196,206,209] (Figure 12) and their intrinsic properties have been exploited in many biological fields. For example, its rough surface favors mammalian cell adhesion and mechanically stimulates these cells to proliferate, making IBs a promising biomaterial to be used as **scaffold** for tissue engineering purposes[204,210]. Moreover, culturing mammalian cells on surfaces decorated with IBs formed by biologically relevant proteins, such as fibroblast growth factor (FGF-2), permits not only these cells to better proliferate due to IBs rough nature, but to have an extra effect on cell cultures due to the delivery of functional proteins

forming these nanoparticles[211–213]. Therefore, these combined effects are a cost-effective **bottom-up drug delivery** strategy especially relevant in regenerative medicine[211–213].

Interestingly, free IBs[205,214,215] or IBs decorating surfaces[211,213] can be **internalized** and can act intracellularly. Nevertheless, IBs can also act as a source for a **sustained release** of soluble and active proteins like **free nanopills**[205,214–216] bearing alternative delivery mechanisms for therapeutic uses, as observed for **cancer therapy**[216]. Briefly, subcutaneously injected CXCR4-targeting IBs demonstrated to slowly release soluble CXCR ligands to the bloodstream, successfully targeting and accumulating in tumor in a xenograft colorectal cancer mouse model[217]. Moreover, intratumorally injected IBs resulted in a stable *in vivo* **immobilized drug source** which promoted apoptosis of $CXCR^{+}$ cells and tumor ablation[218], also reported by Omomyc and p31 IBs against $CD44^{+}$ tumor cells in mouse models of human breast cancer[219].

Hrabárová and coworkers have also described the applicability of IBs in biocatalysis and biotransformation processes[220]. Enzyme-based IBs can work as **immobilized biocatalysts** due to the nature of this format, being a concept that has already been proven by IBs formed by D-amino acid oxidase, sialic acid aldolase, maltodextrin phosphorylase, and polyphosphate kinase[220,221], among others[206,220].

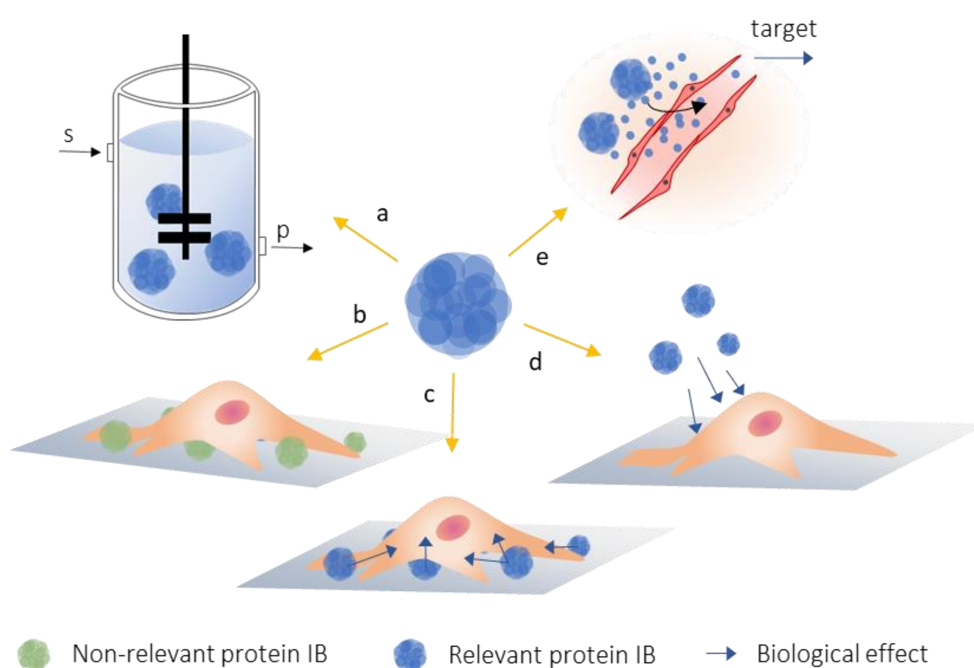

Figure 12. Explored applications of IBs. **a.** Enzyme-based IBs can be used as natural immobilized biocatalysts to transform substrates (s) to products (p). **b.** Immobilized in surfaces, IBs can be used as scaffolds improving mammalian cell adhesion in culture and promoting proliferation. **c.** Surfaces decorated with IBs composed by biologically relevant proteins have the additional asset of being bottom-up inducers for specific biological effects to the attached cells. **d.** Besides of their roles on surface, IBs can be also be administered as free nanoparticles. **e.** Injected *in vivo*, IBs can release soluble proteins that travel through the bloodstream to a target destination[217]. Adapted from Villaverde *et al.* 2015[206].

The heterogenic nature of bacterial IBs can be also an advantageous property of this protein format. As mentioned at the beginning of this section, although IBs are mainly composed by the overproduced recombinant protein, these can also contain traces of host components, like DNA fragments and proteins, including LPS in the case of *E. coli*[200,201]. This has evidenced, however, that IBs have intrinsic **immunostimulant** properties that can be used as **adjuvants** increasing vaccine efficiency in fishes, protecting them from infection[203]. Moreover, the specificity of this property can be enhanced by the production of cytokine-based IBs, also proven *in vivo* to protect zebrafish from infection[222].

Altogether composes a promising background to these nanoparticles, that can be further **tailored** if needed[223,224]. Also, for all the above mentioned, IBs have already demonstrated to be an interesting format in many relevant fields due to their intrinsic exploitable properties and this opens a promising opportunity for them to be explored as a new format also in animal production.

Encapsulation of soluble proteins

Other formats, different from IBs, have been explored in the last years to increase the bioapplicability of recombinant proteins. The use of nanotechnological platforms as protein carriers, or **nanocarriers** (NCs), has gained importance, becoming an alternative to overcome **drug delivery** bottlenecks like low permeability, *in vivo* short half-life or toxicity of the protein of interest[225]. Ideally, these nanodelivery vehicles are biocompatible, non-toxic and biodegradative materials which confer increased stability and improve pharmacokinetics to the encapsulated molecule[225]. NCs generally size less than 1 µm, and can be divided by their nature in, mainly, polymeric and lipidic nanoparticles.

- Polymeric NCs: **polymeric micelles** have spherical or cylindric structures composed of amphiphilic copolymers (i.e. Pluronic F-127) that aggregate generating a hydrophobic core where hydrophobic proteins can be carried, and a hydrophilic surrounding region. **Polymersomes** are more complex structures, formed by the same amphiphilic copolymers but these forming a bilayer. In this way, a hydrophilic core is formed where hydrophilic proteins can be carried as well. **Hydrogels** are also polymeric carriers, composed by a polymer component (i.e. chitosan[226] or hyaluronic acid) with several crosslinks that generate a water-swollen network. This particle can carry high loads of hydrophilic proteins.

- Lipidic NCs: **liposomes** are spherical lipid-based nanoparticles composed by a phospholipidic bilayer forming a hydrophobic core surrounded by the hydrophobic bilayer. This nanoparticle can carry both hydrophilic (core) and hydrophobic (bilayer) proteins.

RECOMBINANT PROTEINS AS THE ALTERNATIVE TO DEAL WITH MASTITIS

For the overall mentioned and considering that the dry period is a critical process for the dairy cow health involving high economical losses in this industry, the use of recombinant proteins appears to be an appealing alternative to optimize this period.

Antimicrobial strategies

One recombinant approach to tackle with mastitis is combating the causing pathogens by developing new antimicrobials with no previously reported resistances, avoiding the use of classic antibiotics.

An ideal recombinant AM would be efficient at killing the pathogen and would be quickly eliminated from the treated animal escaping from bacterial resistance mechanisms. Although this is obviously an ambitious goal, some **recombinant AM** peptides are being developed as potential new therapeutics against dairy cow mastitis pathogens. Some examples are the bacteriophage **endolysin PlyC**[227], the **scFc-Fc antibody** against *S. aureus*[228] or **chimeric phage lysins** which worked synergistically with lysostaphin in a murine mastitis model[229]. Also, **Pheromonicin-SA**, which is an engineered multidomain peptide with bactericidal effect against *S. aureus*, reported the stimulation of *S. aureus*-infected bovine MECs[230], and **bovine β-defensin 12**, expressed in *E. coli*, showed both antimicrobial potential against Gram positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria *in vitro*[231].

Also, although this study had been performed in goats, plasmids carrying genes for lactoferrin and bovine tracheal antibacterial peptides (TAPs), which are known antibacterial peptides, were transfected into MECs. When these MECs were injected into lactating goat MGs, the antibacterial peptides were secreted lasting from 3 to 6 days in milk secretions, which demonstrated these MECs to promote bacteriostatic activity against several pathogens[232].

Given the potential that the use of recombinant antimicrobial approaches has demonstrated, more research is needed to develop more strategies in this line and to evaluate their economic viability for animal production purposes.

Immunomodulating strategies

Considering the anti-inflammatory phenotype at gestation exposed in the section *"Coexistence of dry-off and pregnancy"*, an alternative recombinant strategy to fight against mastitis at dry-off would be the prevention of IMI using heterologous proteins to foster the MG immune system at

this stage. In the section "*Accelerating mammary gland involution after dry-off*" we have already introduced examples of immunomodulating agents that have been used to accelerate the MG involution in the attempt to reduce IMI risk; here we give insight into recombinant strategies being developed.

Most of the published work accounts for the use of **recombinant vaccines** against specific mastitis-associated pathogens (Table 1). However, the use of recombinant immunomodulators has also been addressed. Interestingly, a recombinant particle capable of preventing clinical mastitis by meliorating the immune condition in periparturient primiparous and multiparous cows was developed and marketed under the name of Imrestor® by Elanco after U.S. FDA approval in 2016. This particle, also named **pegbovigrastim**, consists in the recombinant bovine granulocyte colony stimulating factor (bG-CSF), an endogenous protein with pro-bactericidal properties, covalently bound to polyethylene glycol (PEG), a polymer which is well known to increase protein half-life in drug delivery applications. Through the administration of pegbovigrastim at calving, new IMIs during early lactation were reduced in a multicenter field study[233]. However, in 2018 Elanco suspended the commercialization of this drug because despite the benefits observed, lameness increases in postpartum without any apparent lesion and more cases of abomasum dysplasia were reported in treated cows[234]. Nevertheless the study to evaluate the potential for pegbovigrastim in dairy continues[235].

Another example of a potential recombinant immunostimulator during involution is the mammary serum amyloid A3 or **M-SAA3**. This acute-phase protein is synthesised in the bovine MG by MECs[52,236] early in milk stasis, during involution and during infection, and it is also present in bovine, equine and ovine colostrum[236]. Once in blood, low SAA levels (10 - 100 ng/ml) are associated to leucocyte chemotaxis while higher levels (1000-fold) may stimulate the proliferation of endothelial cells, cell adhesion and invasion, and angiogenesis[237]. Through the infusion of soluble M-SAA3 in the MG of dairy cows entering dry-off, involution biomarkers were activated[123]. Moreover, SAA proteins have been shown to be natural opsonins for Gram-negative bacteria, although the recombinant soluble M-SAA3 did not had the same effect in phagocytosis assays *in vitro*[238].

Moreover, **MMP-9**, which is related with the infiltration of immune cells and could have a relevant role at dry-off, has already been produced in the LPS-free bacterium *L. lactis,* and it has been described to be nearly 99 % produced in the IB format, which has been well characterized by Cano-Garrido and coworkers[158].

Table 1. Recombinant vaccines against bovine mastitis

Recombinant protein	Target	Model	Exp. system	Ref
IsdA (surface protein) and ClfA (clustering factor)-cholera toxin A_2/B chimeras	*S. aureus*	Holstein cows	ClearColi®	2018 [239]
CAMP (virulence factor) encapsulated in poly(lactic-co-glycolic acid) (PLGA) microspheres	*S. agalactiae*	Mice	*E. coli* Trans1-T1 (Transgen)	2017 [240]
SEA (Staphylococcal enterotoxin A) encapsulated in PLGA microspheres	*S. aureus*	Mice	*E. coli* BL21(DE3)	2012 [241]
TRAP (surface, stress-related protein)	Staphylococci	Holstein cows	*E. coli* BL21 (DE3)/pLysS	2011 [242]
SUAM (surface, adhesion molecule)	*S. uberis*	Holstein cows	*E. coli* OneShot TOP10®	2011 [243]
GapC/B chimera (surface proteins)	*S. aureus*	Mice	*E. coli* SG13009 (pREP4)	2006 [244]
D(1)D(3) FnBP (fibronectin binding protein domains) and ClfA combined with DNA vaccine	*S. aureus*	Holstein cows	*E. coli* DH5α	2004 [245]
GapC and Mig (surface proteins)	*S. dysgalactiae*	Holstein cows	*E. coli* BL21(DE3)	2004 [246]
CAMP-3 chimera (with epitopes from *S. uberis* and *S. agalactiae* CAMP-factors)	Heterologous bacterial challenge	Holstein cows	*E. coli* BL21(DE3)	2002 [247]

Matrix metalloproteinase 9

Also known as gelatinase B, MMP-9 is a multi-domain and zinc-dependent enzyme that, together with MMP-2 (gelatinase A), are the gelatinases from a family of 20 metallopeptidases that also includes collagenases, stromelysins, matrilysins, and membrane-type MMPs[248]. MMP-9 is composed of a signal peptide (removed during translation), a propeptide, a catalytic domain or active site, three fibronectin repeats and a Zn^{2+}-binding domain, as well as an O-glycosylated domain and four hemopexin domains.

MMP-9 degrades structural components like type IV collagen, gelatine or elastin, and cleaves cell adhesions and surface receptors[249], cytokines and chemokines[250,251], or even intracellular substrates[252], and therefore, it is involved in chemotaxis and cell migration[253–255]. Thus, this enzyme modulates the integrity and permeabilization in many tissues, being very relevant in

numerous biological processes like angiogenesis, growth and development, wound healing or cell migration[58,255–257]. Because this zymogen is involved in so many processes, a tight regulation of its activity is essential. Key mechanisms for the MMP-9 regulation are their expression as proenzymes, requiring of proteases for propeptide cleavage to be active, and the presence of inhibitors, like α2-macroglobulin and TIMPs[58].

Research in MMP-9 involvement during bovine dry-off: what do we know?

In the MG, **after PRL withdrawal** and due to apoptotic and pro-inflammatory signals (Figure 4), MMP-9 is secreted by MECs, neutrophils, and by infiltrating macrophages, endorsing tissue involution[59]. In the case of neutrophils, MMP-9 is reserved inside intracellular vesicles (the so-called tertiary granules) prepared for a quick **degranulation** within minutes. In result, high levels of extracellular MMP-9 during MG involution, together with MMP-2 and MMP-3, are thought to be responsible of **breaking tight junctions** between cells and other adhesion molecules, as well as the **basal membrane** supporting the alveoli MECs[258], promoting MG remodelling and permeabilizing the endothelia facilitating immune cells to recruit, as it happens during an inflammatory process[64].

Abundant research has focused on the role and regulation of MMPs in the MG since it was discovered their association with tumour growth and metastasis. However, all these studies have been conducted in mouse models while little data is available regarding to MMPs in bovine MG. In this sense, different studies have associated the increase in neutrophil-mediated endogenous MMP-9 to the onset of dairy cow MG involution mechanisms[60,259,260]. Tiantong and co-workers, for example, described an earlier expression of involution markers such as lactoferrin and caseins after intramammary infusions of an *Enterococcus faecium* SF6 preparation compared to non-treated quarters in Holstein cows[259]. They suggested the involvement of MMP-9 in such remodelling acceleration because they detected an increase both in this zymogen as well as in the recruitment of neutrophils[259]. Also, Yu *et al.* studied *in vitro* the molecular mechanisms of this association between neutrophil recruitment and degranulation in MG involution during milk stasis at dry-off, insisting in the remodelling properties of MMP-9 in the MG at this stage[260].

These results agree with the immunological context in the MG during involution, analysed in the section "*Molecular insight into the udder*", and suggest a potential point of action to be explored in the attempt to accelerate the MG involution and regeneration. However, little work has been done in this line to elucidate the real effects of MMP-9 in triggering these involution mechanisms in the cow MG, and more research is needed. In this sense, and given the recognized *in vivo*

advantages in the use of novel nanobiotechnological engineered protein formats such as IBs, the use of MMP-9 IBs could be a promising approach in the optimization of the dry period in dairy cows, looking forward to accelerating MG involution and regeneration and a reduction in the use of preventive antibiotic strategies.

Objectives

The aim of this study is to explore the potential of recombinant proteins embedded in protein-based nanoparticles, named inclusion bodies (IBs), in the animal production sector and, specifically, to elucidate the potential of MMP-9 IBs at accelerating the immune function and involution of mammary gland during the cow dry period.

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

1. To review the use of recombinant technologies in the animal production sector, as a way to focus on the specific needs and demands in the development of IBs as a novel livestock therapeutic strategy. (study 1)
2. To explore new protocols based on the use of IBs as a source to obtain proteins of interest, such as MMP-9, in a pure and soluble form using *L. lactis* bacterium as the recombinant host. (study 2)
3. To compare the functional performance and stability of different MMP-9 nanostructurations, including IBs, evaluating their suitability for *in vivo* applications. (study 3)
4. To test the immunogenicity of inactive MMP-9 IBs *in vivo,* discerning the real potential of MMP-9 to trigger an inflammatory response from the unspecific effect of the IB format. (study 4)
5. To validate the MMP-9 IBs potential as a nanomaterial to accelerate the mammary gland involution at dry-off in dairy cows. (study 5)

Results

STUDY 1

TRENDS IN THE RECOMBINANT PROTEIN USE IN ANIMAL PRODUCTION

Laia Gifre, Anna Arís, Àlex Bach and Elena Garcia-Fruitós

Microbial Cell Factories 16:40, 2017 (Review)

Preface

For close to half a century, biotechnology has enriched many research fields, from biomedical research to materials science. Among all the biotechnological advances, the discovery of the recombinant DNA technology boosted the emergence of a huge number of molecular tools providing plenty of applications. Specifically, this impulse in biotechnology has permitted to shoot up the yields for old and new druggable proteins through their recombinant production in cell factories. This aimed us to evaluate which was the impact of these recombinant technologies in the field of animal science and, more specifically, in animal production, since these proteins could become real strategies for still unresolved needs.

For our surprise, the use of recombinant technologies in the animal sector is noteworthy and greater than we expected, with a noticeable list of already commercialized recombinant hormones, fibrolytic enzymes and antibodies relevant for the improvement of livestock reproduction, feed and for prevention and therapy of diseases, respectively. Regarding the main expression systems used, we found out that due to their similarity in the post-translational machinery of natural sources, mammalian cells and yeasts are the main used cell hosts while bacteria are generally relegated to a more preliminary research. Thus, although there has been some transfer of biotechnological research to the livestock sector, there is a broad spectrum of possibilities still unexplored. In this context, the use of biotechnological approaches to make these recombinant products cost-effective and with improved characteristics such as higher half-life, lower toxicity and higher specific activity, among other parameters, would be very relevant. For that, the use of bacteria nanoparticles or inclusion bodies (IBs) is an interesting tool to be explored in animal production because there are increasing evidences of their potential. Several studies have validated IBs as an alternative protein format in many research fields, being a versatile and stable biomaterial, obtained through easy, scalable and, importantly, cost-effective procedures.

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Microbial Cell Factories

REVIEW Open Access

Trends in recombinant protein use in animal production

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Abstract
Recombinant technologies have made possible the production of a broad catalogue of proteins of interest, including those used for animal production. The most widely studied proteins for the animal sector are those with an important role in reproduction, feed efficiency, and health. Nowadays, mammalian cells and fungi are the preferred choice for recombinant production of hormones for reproductive purposes and fibrolytic enzymes to enhance animal performance, respectively. However, the development of low-cost products is a priority, particularly in livestock. The study of cell factories such as yeast and bacteria has notably increased in the last decades to make the new developed reproductive hormones and fibrolytic enzymes a real alternative to the marketed ones. Important efforts have also been invested to developing new recombinant strategies for prevention and therapy, including passive immunization and modulation of the immune system. This offers the possibility to reduce the use of antibiotics by controlling physiological processes and improve the efficacy of preventing infections. Thus, nowadays different recombinant fibrolytic enzymes, hormones, and therapeutic molecules with optimized properties have been successfully produced through cost-effective processes using microbial cell factories. However, despite the important achievements for reducing protein production expenses, alternative strategies to further reduce these costs are still required. In this context, it is necessary to make a giant leap towards the use of novel strategies, such as nanotechnology, that combined with recombinant technology would make recombinant molecules affordable for animal industry.

Keywords: Recombinant proteins, Animal production, Recombinant expression systems, Reproductive hormones, Fibrolytic enzymes, Therapeutic molecules

Background

Unquestionably, the production of recombinant proteins has become a reality thanks to the discovery of the recombinant DNA technology in the seventies. The implementation of this technology has made the production of most protein of interest recombinantly possible. Before this, proteins of interest were extracted from their natural sources through expensive processes and poor yields [1]. However, nowadays, scientists can routinely isolate or synthesize genes and clone them in a suitable expression system for production purposes at industrial scale. Although there is a wide range of cell factories that are currently used for recombinant protein production purposes, including bacteria, yeast, fungi, algae, insect cells, and mammalian cells [2], the bacterium *Escherichia coli* has become the workhorse in this field. This is not only due to the low production costs associated to this prokaryotic expression system, but also to the number of available tools that makes this process easy to implement. The first functional recombinant protein (somatostatin) was produced in 1977 using *E. coli* as cell host [3] and, just some years later, Genentech Inc. launched a recombinant human insulin also produced in *E. coli*.

However, despite the undeniable advances made in the recombinant protein production field, production processes, and more importantly downstream product processing, have important associated costs. This is particularly limiting for the production of recombinant proteins for animal science, where the development of low-cost products and strategies are a priority.

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Despite the existence of some limitations, the use of recombinant proteins in animal science has clearly increased in the last decades. Looking at the overall bibliography where recombinant proteins are being used, it comes out that one of the most studied field is the endocrine system [4–8]. Indeed, there are already some commercial recombinant hormones available and many groups are working on their implementation to improve reproduction of livestock. Many research studies also focus on less-demanded proteins that need specific customization of production procedures according to particular features. Recombinant engineered proteins are being widely explored for the design of both prophylactic treatments and therapeutic strategies. Also, several enzymes are being recombinantly produced with the aim of improving efficiency of feed conversion into edible products.

This article offers an overview of recombinant proteins produced in microbial cell factories, focusing in three fundamental pillars for animal production: (1) reproduction, (2) feed efficiency, and (3) health. This review seeks not only to draw a map of the current situation, but also to highlight the relevance that recombinant technologies could have in a near future for the animal sector. However, all the recombinant products involved in vaccination procedures have been excluded from this revision because they have been thoroughly covered in other articles and reviews [9–11].

Recombinant hormones in reproduction

Animal reproduction is one of the areas where production of recombinant protein is broadly used [4, 12, 13]. Reproductive hormones have a critical role in the regulation of the male reproductive function, female reproductive cycle, and the maintenance of pregnancy in the dams. In animal production, these hormones are used for two opposite purposes: enhancing female fertility by regulating ovulation and/or facilitating embryo implantation, and on the other hand improving meat quality by sterilized males.

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are gonadotropins secreted by the anterior pituitary gland when induced by the gonadotropin releasing hormone (GnRH), secreted by the hypothalamus [14–16]. These glycoproteins, together with the chorionic gonadotropin (CG) secreted by the placenta of primates and equids, are used in animal breeding management for superovulation purposes in females, and to stimulate testosterone production and spermatogenesis in males. On the other hand, inhibin, which is secreted by both male and female gonads, has a great importance because it exerts a negative feedback to the anterior pituitary lowering the secretion of gonadotropin and thus their effects.

At present, the most commonly used hormones for reproductive purposes are purified from animal-derived material such as pituitaries. Despite being a widespread practice, it has significant associated problems such as: (i) batch-to-batch inconsistencies leading to variations in the superovulatory responses between animals [17, 18], (ii) purity problems because of the presence of other hormones contaminating the sample [16], and (iii) the possible contamination with disease-transmitting agents (for a more detailed review see [13]). All these require the development and application of cumbersome purification protocols to concentrate and purify the protein to guarantee the quality of the final product. In this context, production of recombinant hormones appears as an attractive solution to overcome these drawbacks, having reproducible superovulation effects [12] by using considerably smaller doses than those utilized with animal-derived hormones [19].

Gonadotropins are structured in non-covalent heterodimers, composed of a common α subunit and a hormone-specific β subunit. To obtain a functional hormone, both subunits must be assembled together [14, 20] posing an important bottleneck for its recombinant production. However, and because the interaction between subunits is not an absolute requirement for receptor activation ([21] and references therein), functional single-chain gonadotropin analogs have been successfully developed recombinantly by merging their α and β subunit genes in a single sequence [21–24]. Nevertheless, and even if the steroidogenic response is achieved, the structural differences in the hormone analogs commonly imply differences in steroid secreted levels [21]. Furthermore, the 2 subunits of gonadotropins are glycosylated after translation generating pools of different glycoforms (gonadotropin varieties) with different half-lives and activity efficiencies [25, 26]. Thus, since reproductive hormones require a N-glycosylation [20], recombinant production has been carried out mainly in mammalian or insect cells [13, 27]. However, the production of proteins in large amounts in these eukaryotic systems is expensive, difficult, and time-consuming. Furthermore, protein hormones are usually produced at low yields. Alternatively, yeast (eukaryotic microorganisms), and in some cases *E. coli*, are being explored as a cost-effective and easy-to-work systems. *Pichia pastoris* (reclassified as *Komagataella pastoris*) is the most commonly used yeast in this context, because it efficiently secretes the protein produced and adds N-glycosylations. Even though *P. pastoris* can glycosylate proteins, it should be stressed that only one specific strain described

by Jacobs et al. is able to introduce a mammalian-type N-glycosylation [28].

Follicle stimulating hormone (FSH)

FSH acts in ovaries in conjugation with LH. They are responsible of stimulating the granulose cells and promoting follicle growth preceding the ovulation stage. Also, both gonadotropins stimulate the dominant follicle to ovulate. In males, FSH is responsible for stimulating the Sertoli cells in testes for spermatogenesis, together with testosterone secreted by the action of LH in Leydig cells.

Administering exogenous FSH has been a typical practice for promoting superovulation and spermatogenesis in different animal species, and due to the disadvantages associated with pituitary-extracted hormones, recombinant hormone formulations started to rise. There are commercial forms of FSH derived from pituitary glands such as Folltropin-V (Bioniche Animal Health-now Vetoquinol-) and Pluset (Calier), which also contain LH (Table 1). The commercially-available recombinant FSH used for animal follicular development and superovulation has been mostly produced by Chinese hamster ovary (CHO) cells. Some examples are Follistim (follitropin beta; Merck Serono (USA) -now Merck-), Puregon (follitropin beta; Organon B.V. (Europe) -now merged with MSD-) and Gonal-F (follitropin alpha; Merck). Also, AspenBio Pharma (named Venaxis, Inc. since 2012) tooks a relevant role as a supplier of bovine and equine single-chain and long-acting FSH analogs (BoviPureFSH™ and EquiPureFSH™) (Table 1). In some cases, human embryonic kidney (HEK) cells have been chosen as a cell factory to produce bovine FSH (Nanocore Biotecnologia SA) used to supplement culture medium for in vitro follicle development in mares [29], dogs [30], goats, and sheep [31] (Table 1). The equine reproduction research industry has clearly been a user and promoter of this drug to stimulate follicular growth [6, 19, 32, 33] and ovulation [34] in mares. Even so, although the human version of this long-acting and single-chain FSH, Elonva (Corifollitropin α; MSD) was already available in 1992 [35–38], the bovine and equine analogs did not reach the market until 2008 [6].

Although all commercial recombinant FSH are produced in mammalian cell lines and research is still being conducted in this area [39, 40], yeasts, which are relatively inexpensive and effective expression systems, are gaining importance in this field of study. The most used yeast to produce FSH is *K. pastoris.* Bovine, porcine, ovine, and primate recombinant FSH have been produced with *K. pastoris* in studies carried out to improve the yields with this affordable cell factory [41–44]. Although in these studies the activity was only tested in vitro, results indicated that proteins produced were functional and with a great potential to be applied in vivo. *K. pastoris* was also explored for the production of in vitro tested single-chain

Table 1 Marketed follicle stimulating hormone (FSH), Luteinizing hormone (LH) and chorionic gonadotropin (CG) for animal reproduction

Name	Cell factory/origin	Company
FSH		
Follistim®	CHO cells	Merck Serono (USA)-now Merck-
Puregon®	CHO cells	Organon B.V. (Europe) -now merged with MSD-
Gonal-F®	CHO cells	Merck
BoviPureFSH™	CHO cells	AspenBio Pharma (Venaxis, Inc. since 2012)
EquiPureFSH™	CHO cells	AspenBio Pharma (Venaxis, Inc. since 2012)
FSH	HEK cells	Nanocore Biotecnologia SA
Folltropin-V®	Pituitary gland	Bioniche Animal Health-now Vetoquinol-
Pluset®	Pituitary gland	Calier
LH		
BoviPureLH™	CHO cells	AspenBio Pharma (Venaxis, Inc. since 2012)
EquiPureLH™	CHO cells	AspenBio Pharma (Venaxis, Inc. since 2012)
Luveris®	CHO cells	Merck Serono (USA) -now Merck-
Pluset®	Pituitary gland	Calier
CG		
Pregnyl	Urine	Organon B.V. (Europe) -now merged with MSD-
Folligon®	Serum	MSD
Novormon® 5000	Chorion	Syntex
PG600®	Chorion and serum	MSD

ovine FSH analogs [45], as well as for the production of fish FSH that showed the capacity to stimulate steroidogenesis and ovarian development in vivo [46–48]. Moreover, eel FSH produced in *K. pastoris* has been proven in vitro, fostering steroidogenesis in immature eel testis tissue [49] and spermatogenesis [50] (for more information about recombinant fish gonadotropin development, see [27]). Also, the yeast *Hansenula polymorpha* (*Pichia angusta*) has been used to express bovine FSH, which has been successfully tested in vivo in mice for follicular growth purposes [51].

During the last decades, different strategies to increase FSH production in recombinant yeast have been evaluated including the co-expression of a disulfide isomerase [52], the co-expression of *Saccharomyces cerevisiae*-derived calnexin [51] or codon usage optimization [51, 52]. Medium optimization has also been deeply studied to optimize cell densities and production yields [53]. Thus far, although the yields achieved using both *K. pastoris* and *P. angusta* have notably been improved, they are still insufficient to be used for commercial purposes and further research is necessary in this context. Interestingly, a non-glycosylated form of recombinant bovine FSH produced in a bacterial expression system (*E. coli*) showed to be able to stimulate ovarian development in rats [54], emerging as a promising alternative to be further explored.

Luteinizing hormone (LH) and chorionic gonadotropin (CG)

In addition to stimulating ovulation in females, along with FSH, LH stimulates the following development of the corpus luteum. In males, LH is responsible for testosterone secretion in the testes by the Leydig cells, which in turn stimulate spermatogenesis in Sertoli cells. On the other hand, CG supports embryo implantation and pregnancy [20, 55]. In horses, both LH and CGβ subunit derive from the same gene, whereas in primates the two gonadotropins are derived from different genes although they share 80% of their amino acid sequence [55]. CGβ and LHβ subunits differ only in the length of their carboxyl terminal regions. The CG has a longer region because of a peptide called carboxyl terminal peptide (CTP) that provides the CG with more glycosylation places and prolong CG half-life by reducing its renal clearance [56]. These extra glycosylation sites have been fused to FSH [6, 19, 32–34] and LH [21, 24, 57–59], which do not naturally contain this sequence, to achieve long-acting hormones. This allowed the reduction in the number of injections for superovulation treatments [13, 36, 40]. Interestingly, given the similarities between the LH and CG, both hormones bind to the LH receptor.

Recombinant LH commercially-available used for animal reproduction purposes is also being produced in CHO cells (Table 1). As an example, the single-chain EquiPureLH (Venaxis) was used in mares in combination with EquiPureFSH for superovulation treatment [32], or in combination with a pituitary FSH (eFSH; Bioniche Animal Health) to study their effect in follicle and oocyte development [60]. Also, LH has been administered in mares as a model to treat the luteinizing unruptured follicle syndrome in humans [61]. Furthermore, the human-indicated Luveris (hLH; Merck) has been used for early embryonic development treatments in rabbits [62] combined with Gonal-F (see the FSH section), and in mice [63] combined with Pregnyl (hCG; Organon) (Table 1).

Both dimer- [64, 65] and single-chain forms [21, 24, 57–59, 66, 67] of LH and CG have been studied in CHO expression system. Also, human recombinant CG was obtained by CHO cell expression for the construction of a chimera hCG-boCTP used to study the potential of a CTP-like sequence present but not expressed in the β subunit of bovine (among other mammal species) LH [68].

Insect cells have been chosen as an alternative to the expensive CHO cells, for the recombinant equine LH and CG production [23, 69, 70] (for a review see [71]). Moreover, *K. pastoris* has been explored to produce recombinant human LH (hLH) and CG (hCG). Gupta et al. already described hCG production in 1999 [72] and some years later other authors successfully produced both hCG [73, 74] and hLH [74] in *K. pastoris*. Although the hLH expressed by *K. pastoris* showed to be less glycosylated and to have less affinity for the receptor than that naturally expressed in pituitary, it was fully active [74]. Gonadotropins from fish have also been produced in *K. pastoris*, showing the capacity to stimulate steroidogenesis in tilapia [27, 75]. *E. coli*-derived hCGβ was obtained for the first time in 1994 by Huth and coworkers [76]. Briefly, the β subunit of this hormone was recovered from purified and solubilized inclusion bodies (IBs), and refolded in vitro to conduct structural and biological studies. After dimerization with a urinary hCGα subunit, the resulting hormone activated ovulation in vivo in rats although its β subunit could not be glycosylated by *E. coli*. Thus, this study showed that it is possible to produce biologically-active hormones in a prokaryotic organism, which lacks the capacity to introduce post-translational modifications. In this context, Mukhopadhyay et al. also produced hCGβ in *E. coli* for vaccination purposes [77]. This, together with the fact that other hormones, such as growth hormones from different origins have been broadly studied in this recombinant system (buffalo [78], caprine [78], bovine [79], ovine [80] and porcine [81]), showing activities equivalent to those found in natural hormones, suggests that *E. coli* has a bold potential in this field. Although, thus far, mammalian cells have been

the gonadotropin producers *per excellence* [13, 27], articles published show that microbes can be used as a real alternative for the production of biologically active CG and LH through economic and facile processes [36–39, 49, 67, 70].

Inhibin

During the ovarian cycle, inhibin (which belongs to the TGFβ superfamily) is mainly secreted by the large developing follicles causing the atresia of the smaller ones [82, 83]. Its secretion in response to increasing levels of FSH in the gonads triggers a negative feedback to the anterior pituitary lowering FSH circulating levels. Therefore, inhibin regulates follicle development and ovulation rates in females, and spermatogenesis in males.

Yan et al. have extensively reviewed inhibin effects (and of its neutralization) on follicle and embryo development [84]. Superovulation treatments with exogenous gonadotropins result in increased numbers of developing follicles, which in turn lead to inhibin concentration rise in plasma [85] and a quantitatively and qualitatively reduced oocyte and embryo development [86]. Consequently, an immunization practice against inhibin, combined with a conventional superovulation protocol, has proven to enhance the quality of the resulting embryos both in vitro and in vivo [87–89]. This immunization has been achieved through the administration of exogenous inhibin leading to antibody production against this glycoprotein.

From a research perspective, recombinant inhibin or its α subunit used in a wide number of studies has been produced in bacteria, and more specifically in *E. coli* [90]. In this context, genetic engineering has been used to improve inhibin production in *E. coli* [91]. However, in many other cases inhibin has been produced in mammalian cells [16, 92]. Importantly, recombinant inhibin has been used as an antigen for the immunization against endogenous inhibin in hens [93, 94], cockerels [95], heifers [87, 89, 96], water buffaloes [88], guinea pigs [97], goats [98], and sheep [99–102]. Only in a minority of cases, the recombinant inhibin α subunit used is obtained synthetically [103–106] or purified from follicular fluid [107]. Thus, the production of inhibin, also known as the "superovulatory vaccine", has been extensively studied in recombinant bacteria. Although currently inhibin has not been marketed, the research done with this hormone is promising.

Recombinant fibrolytic enzymes

The efficiency of plant cell wall digestibility by endogenous enzymes in animals is low. Basically, most non-starch polysaccharides components present in the animal feed are indigestible by mammalian enzymes, which precludes a full recovery of the nutritional value of the diet. Furthermore, a fraction of the digestible nutrients (i.e., sugars, starch, fat, protein) becomes undigestible because are wrapped by non-starch polysaccharides [108, 109]. Thus supplementation of diets with exogenous enzymes to enhance animal performance has been a practice extensively used for decades to increase feed conversion rate (proportion of growth relative to the amount of feed consumed). Initially, fibrolytic enzymes were used essentially in non-ruminant animals (pigs and poultry), since it was believed that rumen proteases and ruminal microorganisms were able to efficiently degrade pectans, glucans, xylan, and cellulose. However, digestibility values in ruminants range between 35 and 65%, being widely accepted that the addition of fibrolytic enzymes in ruminant diets can notably increase feed conversion.

Enzymes used can be obtained from organisms able to naturally synthesize them such as fungi or bacteria [110, 111]. However, the obtained products contain an important fraction of impurities, being in many cases a mixture containing different interfering enzymatic activities. In this context, recombinant technology has been playing an important role since different fibrolytic enzymes can be produced separately using both homologous and heterologous protein expression hosts [110–115]. Some of these enzymes (β-glucanases, xylanases, mannanases, pectinases, and galactosidases) are used to specifically degrade feed components resistant to endogenous enzymes. Other enzymes, like phytases, are applied to inactivate antinutritional factors. Moreover, in some cases the supplementation with endogenous enzymes that are not produced at sufficient levels by the animal, such as proteases, lipases, and amylases are also used (Table 2). In general terms, unlike other applications previously mentioned, these enzymes are partially purified and commercialized as cellular extracts or culture supernatants that are directly used for feeding purposes, and thus commercial enzymes do not confer a single pure enzymatic activity (Table 1). However, some purification steps are required to eliminate any possible residues of genetically modified DNA and/or undesirable fermentation residues in the final product, but these purification processes are relatively simple.

Thus, although nowadays an important number of enzymes are commercially available for animal nutrition to improve animal productivity and the efficacy of utilization of natural resources, the development of optimized strategies for the production of fibrolytic enzymes is highly desirable. In this line, an extensive array of microorganisms, including bacteria (*E. coli*, *Bacillus subtilis*, and *Bacillus licheniformis*), yeast (*K. pastoris*), and fungus (*Trichoderma reesei* and *Aspergillus niger*), are

Table 2 Marketed carbohydrases

Name	Activity	Cell factory	Animal	Company
Xylanases				
Econase XT	Xylanase	*Trichoderma reesei* (GMO)	Poultry and pigs	ABVista
Danisco xylanase	Xylanase	*Trichoderma reesei* (GMO)	Poultry and pigs	Danisco Animal Nutrition
Hostazym X	Xylanase	*Trichoderma citrinoviride* (not GMO)	Poultry and pigs	Huvepharma
Porzyme®9300	Xylanase	*Trichoderma longibrachiatum* (not GMO)	Poultry and pigs	Danisco Animal Nutrition
Ronozyme WX	Xylanase	*Aspergillus oryzae* (GMO)	Poultry and pigs	DSM-Novozymes
Belfeed B 1100 MP	Xylanase	*Bacillus subtilis* (GMO)	Poultry and pigs	Beldem
Xylamax™	Xylanase	NA	Poultry	BRI
Beta-glucanases				
Econase®GT	β-Glucanase	*Trichoderma reesei* (GMO)	Poultry and pigs	ABVista
Hostazym C	β-Glucanase	*Trichoderma citrinoviride* (not GMO)	Poultry and pigs	Huvepharma
Amylases				
Roxazyme® Rumistar™	α-Amylase	*Bacillus licheniformis* (GMO)	Dairy cows	DSM-Novozymes
Multienzyme				
AvemIx®XG 10	Xylanase, β-glucanase	*Trichoderma reseei* (not GMO)	Poultry and pigs	Aveve Biochem
Roxazyme® G2	Xylanase, β-glucanase	*Trichoderma reseei* (not GMO)	Poultry and pigs	DSM-Novozymes
Axtra® XB	Xylanase, β-glucanase	*Trichoderma reesei* (GMO)	Poultry and pigs	Danisco Animal Nutrition
Axtra® XAP	Xylanase, amylase, protease	*Trichoderma reesei* (GMO)	Poultry and pigs	Danisco Animal Nutrition
AvemIx®02 CS	Xylanase, β-glucanase, pectinase	*Trichoderma reseei* (not GMO), *Aspergillus aculeatus* (not GMO)	Poultry and pigs	Aveve Biochem
Avizyme®	Xylanase, amylase, protease	*Trichoderma reesei* (GMO), *Bacillus amyloliquefaciens* (GMO), *Bacillus subtilis* (GMO)	Poultry	Danisco Animal Nutrition
Endofeed	Xylanase, β-glucanase	*Aspergillus niger* (not GMO)	Poultry	GNC Bioferm
Natugrain®	Xylanase, β-glucanase	*Aspergillus niger* (GMO)	Poultry	BASF
Natuphos® combi	Xylanase, β-glucanase, phytase	*Aspergillus niger* (GMO)	Poultry and pigs	BASF
Agal Pro BL	Alfa-galactosidase, β-glucanase	*Aspergillus niger* (not GMO), *Saccharomyces cerevisiae* (GMO)	Poultry	Biocon
Amylofeed	Xylanase, β-glucanase, amilase	*Aspergillus niger, Aspergillus oryzae* (not GMO)	Pigs	GNC Bioferm
Porzyme®9100	Xylanase, β-glucanase	*Trichoderma longibrachiatum* (not GMO)	Pigs	Danisco Animal Nutrition
Xybeten®	Xylanase, β-glucanase, cellulase	*Trichoderma longibrachiatum* (not GMO)	Poultry and pigs	Biovet
Ronozyme®VP	Pectinase, β-glucanase	*Aspergillus aculeatus* (not GMO)	Poultry and pigs	DSM-Novozymes
Rovabio®Excel	19 enzymes (xylanases, β-glucanase, and cellulases with other enzyme activities)	*Penicillium funiculosum* (not GMO)	Poultry and pigs	Adisseo
Ronozyme®Multigrain	Xylanase, β-glucanase	NA	Poultry and pigs	DSM-Novozymes
Ronozyme A	Amilase, β-glucanase	NA	Poultry and pigs	DSM-Novozymes
Cibenza® CSM	Xylanase, β-glucanase, α-galactosidase	NA	Poultry and pigs	Novus International

Xylanases, β-glucanases and α-amylases have one declared enzymatic activity, while in some cases some secundary activities are also present in the product

NA information not available

being explored for the production of enzymes with interest in the feed industry. All the strategies that are being explored aim at designing fibrolytic enzymes that meet the industry requirements, which include high production yields, low production costs, easiness to scale-up, high catalytic efficiency, and improved stability under different temperature and pH conditions. This includes the use of genetic and protein engineering approaches to produce highly-active enzymes, and variants with an increased resistance to temperature and proteolysis (in many cases derived from extremophile microorganisms), ultimately resulting in a greater stability in the

gastrointestinal tract. Research in this field is still underway and every year optimized processes and new products are developed and a large number of articles on this topic are published. This is particularly important considering that the global feed market is continuously growing. In particular, feed market is dominated by carbohydrases (being xylanases and beta-glucanases the most important) and phytases.

Carbohydrases: xylanases, beta-glucanases, and amylases

Xylanases can break down xylan, which is a major polysaccharide of hemicelluloses present in plant cells and in some algae. Thus, xylanases are widely used in animal feed to degrade complex hemicelluloses. Most of the xylanases used in feed industry for enzymatic treatment of animal feed are derived from those naturally produced in fungi [116–118]. Some examples are Danisco xylanases (Danisco Animal Nutrition) and Econase XT (ABEnzymes) that are produced in *T. reesei*, whereas Prozyme 9300 (Danisco Animal Nutrition) is produced in *Trichoderma longibrachiatum*, Ronozyme WX (DSM-Novozyme) in *Aspergillus oryzae*, and Hostazym X (Huvepharma) in *Trichoderma citrinoviride* (Table 2). There is also a commercial example of a recombinant xylanase produced in bacteria (Belfeed B 1100 MP, Beldem, *B. subtilis*) (Table 2).

However, as previously described, most of these commercial products are not pure enzymes, but a complex fermentation product that in some cases contains a mixture of different enzymatic activities that may have a synergistic effect (Table 2). In many cases, xylanase is combined with β-glucanase, whereas in others amylase, protease, pectinase, phytase, and/or α-galactosidase are also present in the mixture (Table 2).

β-Glucanases are enzymes capable of breaking down cellulose and have been used in poultry, pigs, ruminants, and fish since early 1980 to facilitate the bioconversion of cellulose to animal products (Table 2). On the other hand, α-amylases are used in dairy cow nutrition to increase feed efficiency and milk production [119–123]. The most widely used is Roxazyme® RumistarTM (DSM-Novozyme), which has been produced in a genetically-modified *B. licheniformis* (Table 2).

Given the importance of xylanases, β-glucanases, and α-amylases to improve the nutritional value of non-starch polysaccharides, and the increasing demand of more stable, highly-active, and non-expensive carbohydrases, different microbial hosts have been explored for their production. Although commercial carbohydrases are mainly derived from fungi, research in this field focuses in the development of bacterial and yeast-based production systems [124]. This is particularly evident for xylanases.

Considering that, in many cases, glycosylation is necessary to obtain functional and stable xylanases [125, 126], yeasts appear as the most promising heterologous expression host for their production as an alternative to fungi. Besides, some yeast have been accredited with the generally recognized as safe (GRAS) status by the American Food and Drug Administration (FDA), which brings additional value to this expression system. Altogether these advantages make yeast, and more specifically *K. pastoris*, the most widely used microorganism for xylanase production (extensively reviewed by [126]). Briefly, *K. pastoris* has been explored for the production of xylanases from *T. reesei* [127], *Aspergillus sulphureus* [128], *A. niger* [129, 130], and *Streptomyces* sp. S38 [131], among others [126]. Albeit at a lesser extent, *S. cerevisiae* has also been studied for the production of fungal xylanases [112, 132, 133]. Different enzymes in different yeast-based cell factories have been evaluated under diverse production conditions aiming to optimize enzyme production yields [133]. Li et al. and Fu et al., for instance, improved the production efficiency of the enzyme simply using an optimized sequence with the appropriate codon usage [128, 131]. On the other hand, Fang and collaborators described that *xylB* gene overproduced is not glycosylated, but it is still fully active and highly stable under different conditions [130]. Other fungal xylanases have also been shown to be non-glycosylated enzymes [118]. In line with this observation, different groups have described the production of catalytically-active eukaryotic xylanases in *E. coli* [134, 135]. Thus, *E. coli*, although to a lesser extent due to their lack of secretion system, has also been used to study different bacterial xylanases [136, 137]. Alternatively, other Gram-positive bacteria, such as *Lactobacillus* spp. and *B. subtilis*, also classified as GRAS organisms, have been used as cell factories for xylanase production purposes. Interestingly, these Gram-positive bacteria have a dual effect, since they are explored as probiotics to enhance gut health, but at the same time they are able to secrete recombinant enzymes of interest such as xylanases [138, 139]. In some cases, strategies to anchor xylanases in the bacterial cell wall have been explored [140]. Lastly, filamentous fungal expression systems (mainly *Aspergillus* spp. and *Trichoderma* spp.) have been also extensively studied for xylanase expression (reviewed in [116]) and, in fact, they are, as previously mentioned, the microorganism behind some products commercially available (Table 2). Other fungi such as *Thermoascus aurantiacus* have also been explored as potential cell factories for xylanase production [141]. Although fungi produce high levels of xylanase, they have two important limitations for industrial application, a reduced yield in fermenter conditions, and poor secretion efficiency.

Phytases

Non-ruminant intestinal microorganisms, in contrast to what occurs with ruminal bacteria, are unable to degrade phytate from plant-derived feedstuffs [142]. This is particularly important considering that phytate is the major source of phosphorous in animal diets. Thus, traditionally, the main phosphorous source in poultry, swine, and fish diets came from the inorganic phosphates via dietary supplementation. However, the excretion of excess phosphorous by animals fed supplemented diets was accumulated in the soil and water, creating a major environmental problem [143, 144]. This challenge was minimized with the commercialization in 1991 of the first recombinant phytase, which allowed avoiding the supplementation of diets with inorganic phosphorous and, consequently, decreasing phosphorus pollution in animal waste [145].

Since the development of the first commercial phytase product (Natuphos, BASF), others have been launched in the market and are nowadays available [113] (Fig. 1). Phytases that are available today are produced recombinantly in microbes including fungi (*T. resei, A. niger*, and *A. oryzae*) and yeast (*Saccharomyces pombe* and *K. pastoris*) (Fig. 1) and are widely used in diets for non-ruminant animals.

The market of feed enzymes and, more specifically of acidic phytases, has significantly grown in the last decades and its demand is estimated to continue growing in the next years. Phytases are enzymes with a large market (60% of the total feed enzyme market), which combine the capacity to improve feed efficiency with the advantage of reducing the phosphorus pollution. Due to the important phytase applicability in animal feeding, several groups are working on the design, production and characterization of phytases with optimized properties. Haefner et al., Lei et al., and Rao et al. have published extensive reviews describing all the advances in the production of phytases using recombinant cell factories [113, 142, 146].

Research on phytase synthesis has used fungi, bacteria, yeast, and plants [113, 142, 146–148] (Fig. 2). Among fungi, the genus *Aspergillus* has been the most widely explored for the isolation of phytases with interesting properties. Besides, different species from the genus *Bacillus*, as well as some *Lactobacillus* and *E. coli* have been deeply studied [147]. Although phytases were initially isolated from their natural origin, it is widely accepted that the levels of production in such wild type strains is too low. In this context, recombinant DNA technology has allowed to make a major step toward the production of phytases at high production levels using optimized cell factories. Summarizing, among all the recombinant cell factories reported in the literature used for the production of phytases, *K. pastoris* and *E. coli* appear as the most widely used microbial factories for research purposes, whereas *A. oryzae* is the preferred option among fungi (Fig. 2).

Currently, phytase research is still focused on the identification of new phytases, but more importantly there is a clear trend towards the optimization of key properties of the already described enzymes. For the development of a new generation of phytases as feed additives, genetic and protein engineering play a key role, since they are powerful tools to develop tuned phytase variants.

Considering that the action of phytases takes place in the stomach, one of the most important requirements for phytases is a high resilience at low pHs and resistance to proteolytic degradation. Aside from this high stability to the upper digestive tract conditions, phytases used to increase animal feed efficiency must resist high temperatures to cope with the conditions of the feed pelleting process. Obviously, it is also necessary to develop enzymes with a good catalytic efficiency and produced through cost-effective production processes [113]. Aiming to produce modified phytases with optimal

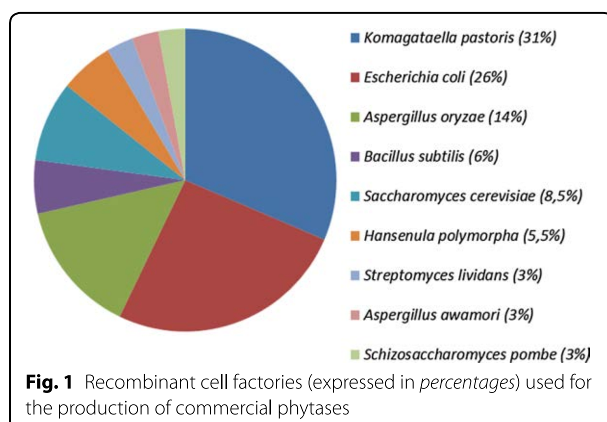

Fig. 1 Recombinant cell factories (expressed in *percentages*) used for the production of commercial phytases

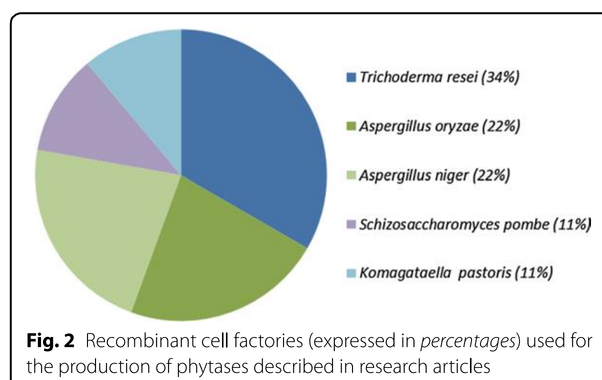

Fig. 2 Recombinant cell factories (expressed in *percentages*) used for the production of phytases described in research articles

properties, different expression systems are being evaluated, using the sequence of phytases from different sources as starting point for further improvements. It is important to note that different expression systems (combined with phytases from different origins) produce enzymes with different biophysical and biochemical properties. For instance, the molecular mass greatly depends on the phytase origin and on the glycosylation pattern. Because bacterial phytases do not have post-translational modifications, they are easier to produce and thus have an important advantage over those from other origins. Thermostability, catalytic performance, substrate specificity, and stability at acidic pHs are greatly influenced by both heterologous system and origin. Thus far, although important advances have been achieved in this field, no enzyme with all the optimal parameters has been developed [149]. Among all those that have been studied, enzymes derived from extremophylic organisms such as *Rhizomucor pusillus*, *Thermomyces lanuginosus*, *Aspergillus fumigatus*, *Peniophora lycii*, *Agrocybe pediades*, and *Ceriporia* sp., appear as the most promising candidates [150–154]. Resolution of the crystallographic structure of some phytases allows a better understanding of this enzyme, providing a good starting point to optimize the protein engineering process. Besides, sophisticated optimization of the condition for the growth processes are also contributing to maximize titers of the variant of interest [142, 155].

In short, fibrolytic enzymes extracted from their natural sources have a low productivity yield and poor thermal and pH stability. In this context, all the efforts have focused on the development of highly active enzymes able to support extreme environments and resistant to proteases. Importantly, this needs to be done through cost-effective and high-production processes to make the new enzymes a real alternative to the existing commercial ones. In this context, bacteria and yeast represent promising alternative microbial cell factories for the production of these enzymes [124].

Recombinant proteins for prevention and therapy

Recombinant antibodies

The use of passive immunization (administration of antibodies) for the control of infectious disease has been recognized as a successful approach in the modern production of a wide range of animals, including pigs, cattle, sheep, goats, poultry, and fish [156]. In contrast to vaccination or active immunization, administration of immunoglobulins establishes instant immunity and provides short-term protection with no induction of immunological memory. With multifactorial infectious diseases, especially those that have proven hard to control by vaccination, the potential of passive immunization is high. Moreover, this type of therapy may be considered as an alternative to antibiotics, whose use is starting to be limited due to concerns about potential development of antibiotic-resistant bacteria.

During the last decade, the development of recombinant antibody technologies has offered the possibility for developing highly specific pathogen-specific antibodies using a cost-effectiveness and reproducible technology [157]. Some studies have demonstrated the success of using recombinant antibodies in animal production. Transmissible gastroenteritis virus (TGEV) is a positive-strand RNA virus of the family *Coronaviridae*, infecting both enteric and respiratory tissues of pigs and causing a mortality rate close to 100% when newborn pigs are infected [158]. Single-chain fragments (scFv) obtained by joining the light- and heavy-chain variable regions (VL and VH) from a monoclonal antibody (mAb) reconstitute the original VL–VH association and retain the binding specificity of the original mAb in a single polypeptide [159]. To improve the affinity of monovalent scFv, dimeric single-chain mini-antibody molecules, named minibodies or SIPs (small immunoproteins), have been generated by connecting an scFv to the dimerizing domain of immunoglobulin heavy chains. These recombinant proteins are efficiently assembled and secreted in dimeric form by mammalian cells. In vivo protection experiments on newborn piglets have demonstrated a strong reduction of virus titers in infected tissues of animals orally treated with TGEV-specific SIPs [160].

On the other hand, available vaccines for bovine herpes virus 1 (boHV-1), which causes respiratory and genital diseases in cattle, do not confer adequate protection. Koti et al. developed a bovine scFv that has a proven specificity and in vitro neutralization activity against BoHV-1 [161]. *K. pastoris* was selected over bacterial expression systems available, since yeast has protein processing and post-translational modifications similar to those present in higher-order eukaryotes as well as providing high recombinant protein yield under the influence of *AOX1* promoter. In a posterior study, the authors demonstrated that scFvs against BoHV-1 with a short linker (2 amino acids) were capable of assembly into functional multimers that conferred high avidity, resulting in increased virus neutralization in vitro compared with that of monovalent scFv [162]. These studies need to be further expanded to experiments involving virus challenges to determine the efficacy of passive protective immunity provided by bovine scFv. However, since the virus neutralization ability of the scFv in vitro was comparable to the parental mAb against BoHV-1, which reduces mortality in rabbits infected with BoHV-1, there is a future potential to be used in infected animals, to treat semen preventing the spread of BoHV-1 infection, or even by

local application to treat infectious pustular vulvovaginitis caused by BoHV-1.

Another example is the foot-and-mouth disease virus (FMDV), which is a contagious viral disease that affects cloven-hoofed animals such as cattle, swine, and sheep with a potential for rapid spread. Emergency treatment by passive immunization can be used as an important control measure for FMDV outbreaks in FMDV-free regions such as the European Union. Harmsen et al. produced recombinant llama single-domain antibody fragments (VHHs) using recombinant strains of *S. cerevisiae* to confer rapid protection against FMDV by passive immunization in pigs [163].

VHHs have a number of advantages for therapeutic applications because they are well produced by microorganisms, have a high physicochemical stability and are well-suited for the construction of genetic fusions of several VHH domains [164]. Moreover, it is important to note that in that study no immunogenicity of VHHs was detected in treated pigs, which is an important aspect because passive immunotherapy can be complicated by the induction of an antibody response against the administered heterologous therapeutic recombinant antibody, especially when such antibodies are administered repeatedly.

From our knowledge, the only case of recombinant antibody produced thus far in *E. coli* as a potential therapy for animal production is related to the treatment of intrammamary infections. Bovine intrammamary infections are an important disease that causes large economical losses in the dairy industry and where passive immunization could be an interesting alternative, especially to treat infections such as those caused by *Staphylococcus aureus*, where vaccines do not confer adequate protection and the conventional antibiotic treatments have a limited success rate. Wang and collaborators constructed a recombinant scFv against fibronectin-binding protein A (FnBPA) and clumping factor A (ClfA), two important virulence factors in *S. aureus* infection [165]. However, future in vivo studies of the functionality of these scFvs are needed to confirm the potential of such scFvs.

Other therapies

Cytokines are small molecules, which act as intercellular communication signals and play a role in various aspects of the differentiation and maturation of immune system cells and the host response to infection. Although this network is complex, there is already available information on the role of specific cytokine in the modulation of the immune system in livestock as a preventive strategy of diseases or even controlling metabolic and physiological processes. There are many in vivo studies testing targeted recombinant cytokines that stimulate the immune system to fight intramammary infections during both the lactation and the dry (the last 2 months of pregnancy, when the cow does not lactate) periods in dairy cows. Intramammary infusion of recombinant IL-2, IFNγ, IL-1β, and IL-8 in the mammary gland of lactating cows have been shown to offer protection against *S. aureus* or *E. coli* infections [166–169]. Moreover some recombinant cytokines such as IL-8 and recombinant bovine granulocyte–macrophage colony stimulating factor (rboGM-CSF) [170] have been able to foster the involution of the mammary gland during the dry period (a period where tissue of mammary gland is involuted and regenerated in the preparation for the subsequent lactation).

Dairy cows often experience decreased immune function around the time of calving, typified by impaired polymorphonuclear neutrophil (PMN) function and increased incidence of disease. Subcutaneous injections of recombinant bovine granulocyte colony-stimulating factor covalently bound to polyethylene glycol (PEG rbG-CSF) dramatically increased circulating numbers of PMN [171]. Other applications concern to the improvement of reproductive performance of production animals using IFN-τ. Recombinant buffalo IFN-τ (buIFN-τ) increased in vitro buffalo blastocyst production rate [172] although intrauterine administration of liposomized bovine IFN-τ had no effect on the length of the estrous cycle and the lifespan of the corpus luteum in dairy cows [173]. However, Shirasuna et al. found that recombinant IFN-τ was associated to greater amounts of protein, IL-8, and neutrophils in the corpus luteum of pregnant cows [174]. Lastly, supplementing recombinant porcine leukemia inhibitory factor (poLIF) in the in vitro maturation medium can improve oocyte maturation [175].

The ability of IL-3 to stimulate the development of eosinophils makes it a particularly important candidate for therapeutic use to protect against parasites. Morris et al. demonstrated that in vivo administration of poIL-3 induced a significant increase in the number of eosinophils in the blood of pigs [176]. In a similar context, chicken IFN-γ (chIFN-γ) demonstrated reductions in intracellular sporozoite development in vitro without affecting sporozoite invasion of host cells. Furthermore, chickens treated with recombinant chIFN-γ showed decreased oocyst production and significant improvement in body weight gain following an *Eimeria acervulina* challenge infection [177, 178].

All these cytokines have been produced in several recombinant systems such as mammalian cells in the case of bovine IFN-τ (boIFN-τ) [173], bovine IL-2 (boIL-2) [167], porcine IL-3 (boIL-3) [176], poLIF [175], and chIFN-γ [177]. Insect cells have been chosen for the production of chIFN-γ [177], *Brevibacillus choshinensis* for boIL-8 [168], *K. pastoris* for boIL-2, IFN-γ, and GM-CSF

[170] and *E. coli* for buIFN-τ [172], boIFN-τ [174], and chIFN-γ [178].

Also in the context of the immune system modulation, the mammary serum amyloid A (M-SAA3) protein (an acute phase protein from the mammary gland) has been produced recombinantly in *E. coli* [179] and proposed as an immunostimulator of the mammary gland to fight against infections and enhance mammary involution during the dry cow period. The administration of M-SAA3 triggers an inflammatory response, the maturation of dendritic cells, and reduces the infection of mammary epithelia by pathogens such as *S. aureus*. Furthermore, relevant functions have been demonstrated in mammary function of dry cows such as the increase in neutrophil recruitment and of some key effectors of tissue involution such as metalloproteinase 9 (MMP-9) [180].

In summary, a new era of recombinant proteins, mostly key effectors in the immune system, opens the possibility to modulate physiological processes and prevent infections reducing the use of antibiotics in livestock and paving a safer and more productive future.

Future perspectives

The use of genetic and protein engineering techniques have led to a significant progress in animal production and it is starting to have a commercial impact in this field. Nowadays it is possible to design tailor-made sequences of enzymes, which in some cases combine specific properties of different enzymes in one molecule to obtain an optimal functional protein [181]. On the other hand, this technology allows the production of recombinant hormones through cost-effective processes using microbial cells as production hosts. In addition to this, novel strategies such as those based on passive immunization are gaining ground due to the broad range of possibilities that recombinant protein production offers. In this context, although important efforts have been done toward the minimization of recombinant protein production costs, currently, much remains still to be achieved. Cost effectiveness is particularly important in the context of animal production, where marginal returns are tight. Currently, the main restriction for the application of recombinant products in animals is still the cost associated to the production processes. Overcoming this bottleneck requires developing alternative strategies to further reduce the production costs of recombinant products and there is a wide range of unexplored strategies to improve recombinant production of proteins of interest for animal production.

The use of bacterial strains with an oxidizing cytoplasm, for example, represents a good approach to improve the production yields of proteins containing disulfide bonds. In this line of work, the development and optimization of production protocols for both bacteria and yeast and the use of genetic engineering to obtain proteins with improved stability is useful. On the other hand, though yeast and bacteria are being explored as alternatives for the production of many proteins of interest, the catalogue of other promising microorganisms for this purpose is limited. Lactic acid bacteria (LAB) are an attractive alternative for recombinant protein production, since they are GRAS organisms able to produce difficult-to-express proteins [182, 183]. Even though these microorganisms have been explored in some cases for animal production purposes, especially for the production of fibrolytic enzymes, broadening their field of application would be highly convenient. They do not only show the ability to produce recombinant proteins, but they also have interesting properties as probiotics. Besides, they are able to efficiently secrete the protein of interest, which reduces the purification costs of the product of interest, and also are used for surface display purposes [184]. Interestingly, surface display, which allows to naturally anchor the enzyme of interest to the cell envelop once it is produced by the recombinant cell, has already been proven to improve the stability of an endoglucanase produced in *K. pastoris* [185].

However, it is also necessary to think beyond these classical strategies and make use of novel approaches, such as nanobiotechnology, which has been explored in other fields of research. Considering that recombinant proteins are poorly used in animal production due to their normally high associated costs, new protein formats need to be explored. Among them, inclusion bodies (IBs), which are a low-cost, highly stable, and functional protein nanoparticles mainly containing the protein of interest overproduced in a recombinant system, represent a new and appealing protein format [186, 187]. Production of recombinant proteins as IBs allows the production of any protein of interest through a much more affordable process [186–189], which could open a wide range of possibilities in animal science. Thus far, in the context of animal production, IBs from *E. coli* have only been used as a source of protein. For that, as previously described, solubilization protocols using denaturants such as urea or guanidinium chloride followed by renaturation processes have been used to obtain properly folded and functional soluble proteins [76, 190, 191]. Nevertheless, IBs have never been explored as protein-based nanoparticles for animal reproduction, enhancers of feed efficiency, or treatment purposes, with only one exception. A recent article described for the first time that IBs formed by cytokines can successfully be used as a prophylactic measure, showing that zebra fish treated with IBs are protected against a lethal infection [192]. In the same way that cytokines have been successfully produced as IBs

for prevention purposes in fish, other proteins of interest (hormones, enzymes, and antibodies, among others) or other animal species could also be explored, unfolding enormous possibilities in this field. Contrarily to what has been widely believed, the formation of IBs does not only occur in *E. coli*, but in many other expression systems including yeast [193] and LAB [186, 194], meaning that their production can be conducted in a wide variety of microbial cell factories. Moreover, their size and shape are easily tunable.

Alternatively, protein encapsulation and/or coating could also be interesting nanobiotechnological approaches to increase protein stability, minimize doses and, consequently, reducing costs of proteins of interest for animal production. Nanoemulsions, liposomes, polymersomes, protein nanocapsules, polymeric nanoparticles, and hydrogel nanoparticles are some examples of different nanostructured systems used for protein encapsulation [195–198]. As an example, Diwan and collaborators described the encapsulation of gonadotropin-releasing hormone in polylactic-co-glycolic acid microspheres [199]. In another recent example, the use of gold nanoparticles has been studied to increase the stability and efficiency of a xylanase [200].

In summary, new protein formats such as IBs and encapsulation methods need to be further explored in animal science as attractive alternatives to make recombinant molecules affordable. Molecule stability and functionality can significantly be improved through these strategies.

Conclusions

Recombinant DNA technology allows modulating protein sequence, which therefore makes it possible to obtain recombinant products with improved properties compared with those isolated from their native hosts. This has helped to make a significant step forward in the development of recombinant products for a wide array of applications, including animal production, as reviewed in the text. A broad catalogue of microbial cell factories is being explored for the successful development of enzymes, hormones, and therapeutic molecules. Nevertheless, to continue advancing in this field of study, it is necessary to make a giant leap towards the use of novel strategies that combined with recombinant technology would allow the development of products with applicability in animal science. In this context, nanotechnology, and more specifically nanostructuration, could play a crucial role in the development of a new generation of recombinant biomolecules with affordable costs for animal industry.

Authors' contributions

EGF and AA conceived and designed the manuscript. LG, AA and EGF performed the bibliographic research, conceptualized and drafted the manuscript. AB outlined the structure and reviewed the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

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Study 2

A new approach to obtain pure and active proteins from *Lactococcus lactis* protein aggregates

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Preface

Given that (i) there is an increasing interest in the animal sector to face livestock-related challenges using recombinant technologies, that (ii) there is an urgent need to anticipate real alternatives to the use of preventive antibiotics for dry period IMIs in dairy cattle, and given that (iii) previous work in our lab indicated that two candidate proteins (M-SAA3 and MMP-9) related with the immune function of the cow MG are difficult to be recombinantly produced in a soluble and pure form, we aimed to explore a new protocol based on the use of IBs as a source of pure protein using a GRAS microorganism such as *L. lactis*. Moreover, obtaining the soluble and pure form of MMP-9 is an ineludible first step to have a control in the study of the real potential of MMP-9 IBs *in vivo*. For M-SAA3, very low yields and purity are achieved during heterologous production, while for MMP-9, practically the overall produced protein forms aggregates (IBs) being negligible the soluble fraction. These are common bottlenecks in recombinant protein production and, as an alternative, IBs could be used as the protein source to obtain the soluble counterparts.

It has already been reported that the use of the mild detergent N-lauroyl sarcosine to solubilize *E. coli* IBs gives greater functional protein yields than using classical denaturing conditions which include a highly inefficient refolding[194]. However, as this strategy has never been tested for *L. lactis* IBs and adding our need to obtain labile and prone-to-aggregate proteins, we decided to focus on the development of a new IBs solubilization protocol for this GRAS microorganism adapting it to obtain difficult proteins. After an optimization process, we succeed in obtaining pure, soluble and functional yields of prone-to-aggregate proteins from *L. lactis* IBs, as well as of labile proteins for which soluble and pure yields were never reached before. This advance, moreover, unlocks new opportunities in the study of novel formats of bovine MG-relevant proteins that could be interesting alternatives in bearing physiological conditions compared to the soluble formats.

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A new approach to obtain pure and active proteins from *Lactococcus lactis* protein aggregates

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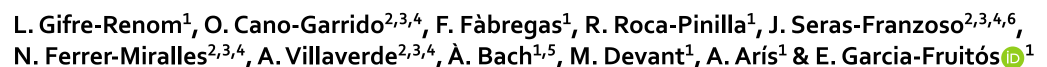

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The production of pure and soluble proteins is a complex, protein-dependent and time-consuming process, in particular for those prone-to-aggregate and/or difficult-to-purify. Although *Escherichia coli* is widely used for protein production, recombinant products must be co-purified through costly processes to remove lipopolysaccharide (LPS) and minimize adverse effects in the target organism. Interestingly, *Lactococcus lactis*, which does not contain LPS, could be a promising alternative for the production of relevant proteins. However, to date, there is no universal strategy to produce and purify any recombinant protein, being still a protein-specific process. In this context and considering that *L. lactis* is also able to form functional protein aggregates under overproduction conditions, we explored the use of these aggregates as an alternative source of soluble proteins. In this study, we developed a widely applicable and economically affordable protocol to extract functional proteins from these nanoclusters. For that, two model proteins were used: mammary serum amyloid A3 (M-SAA3) and metalloproteinase 9 (MMP-9), a difficult-to-purify and a prone-to-aggregate protein, respectively. The results show that it is possible to obtain highly pure, soluble, LPS-free and active recombinant proteins from *L. lactis* aggregates through a cost-effective and simple protocol with special relevance for difficult-to-purify or highly aggregated proteins.

Recombinant proteins represent a growing market and their applications are numerous for human medicine[1] and animal health and production[2]. To date, more than 400 recombinant proteins have been approved for human medicine and this number is expected to rise in the coming years[1,3]. Nowadays recombinant products can be based on naturally occurring biomolecules, but they can also be *de novo* designed proteins or even modular proteins with improved properties. However, proteins of interest are labile macromolecules and, in many cases, they are also prone-to-aggregate. In this context, the process to obtain recombinant soluble proteins is, in many cases, complex and time-consuming. Besides, nowadays there is no well-established universal protocol for the successful production and purification of recombinant proteins. All this has lead the research community to develop specific and in many cases cumbersome, protein-dependent production and purification strategies to reach the desired product. *Escherichia coli* has been, by far, the most widely used bacterium for recombinant protein production purposes. However, although the wide catalogue of available tools for protein production in this expression system, the presence of lipopolysaccharide (LPS) in its outer membrane limits the *in vivo* applicability of the recombinant product obtained from this Gram-negative bacterium. The presence of LPS in the recombinant product can trigger non-desired inflammatory responses once the protein has been administered, making the addition of extra purification steps essential to completely remove LPS from the recombinant product[4–6].

In the last decades, the use of alternative bacterial expression systems lacking endotoxins has significantly increased[7]. Among them, the Gram-positive *Lactococcus lactis* has been vastly studied[7–10]. This bacterium has been classified as a generally recognized as safe (GRAS) microorganism by the Food and Drug Administration

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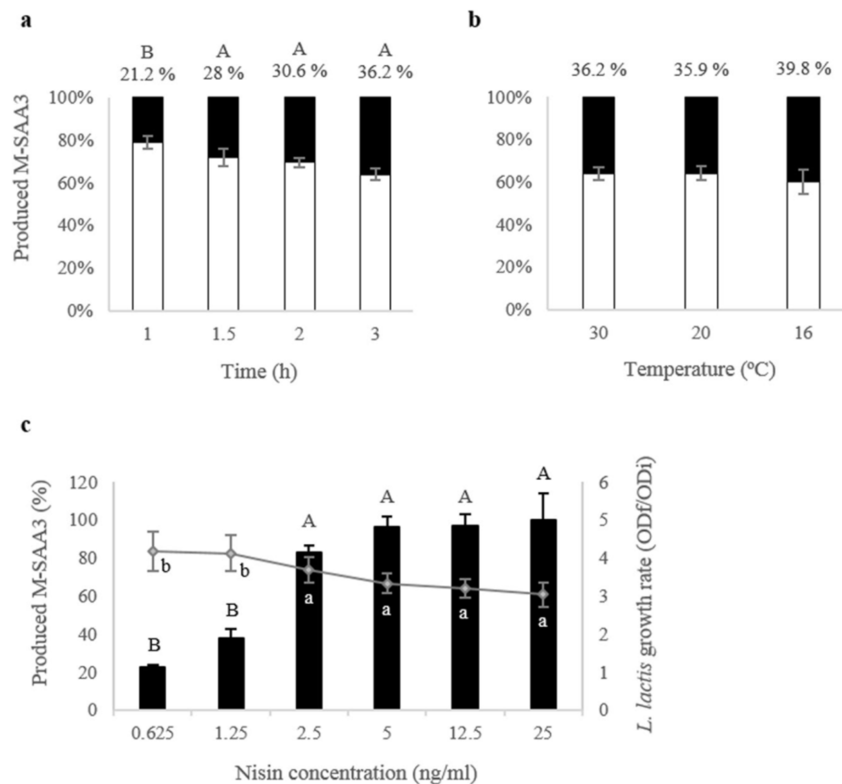

Figure 1. Productions of M-SAA3 in *L. lactis* after nisin induction under different conditions. (**a**) Kinetics for the produced M-SAA3 at 1, 1.5, 2 and 3 h post-induction at 30 °C. Different letters depict differences between production times ($P = 0.002$). The bars indicate the percentage of soluble (white) and of aggregated M-SAA3 (black). Error bars indicate the standard error (SE). Values for the % of aggregation are indicated on top of each bar. (**b**) Productions at different temperatures: 30, 20 and 16 °C. The bars indicate the percentage of soluble (white) and of aggregated M-SAA3 (black). Error bars indicate the standard error (SE). (**c**) Productions at different nisin concentrations, along 1.5 h and at 30 °C. The light grey line indicates *L. lactis* growth rates (final OD/OD at induction). The greatest production values of M-SAA3 were set as 100%. Different letters depict differences between nisin concentrations ($P = 0.002$) and growth rates ($P = 0.041$). Error bars indicate the standard error (SE).

(FDA) and thus, it represents a promising alternative to *E. coli* for recombinant protein production purposes[10]. Thus far, *L. lactis* has essentially been explored as a microbial cell factory for the production of soluble proteins, either intracellularly or secreted to the media[7,8,11–17]. However, recent studies show that this GRAS expression system does not differ from others in its capacity to form protein aggregates under overexpression conditions[9,18]. Different heterologous proteins produced in *L. lactis* have been found not only in their soluble form, but also as protein aggregates in the bacterial cytoplasm[19]. *L. lactis* aggregates (or inclusion bodies -IBs-), as it occurs with those found in other recombinant expression systems, are fully functional protein nanoclusters that are spontaneously formed under overproduction conditions[9,18,19]. The formation of such protein deposits is particularly relevant for those proteins difficult-to-purify and/or prone-to-aggregate[9,18]. In these cases, although the recombinant protein is free of LPS, the strategy used to produce and purify each specific protein is still largely protein-specific, as it occurs in other expression systems. Since these aggregates might be an alternative source of difficult-to-obtain proteins in *L. lactis* and considering the need to develop a universal protocol for the production and purification of LPS-free recombinant proteins, the objective of this study was to develop a broad-application strategy to extract functional protein from *L. lactis* aggregates. For that, two proteins have been used as model proteins in this work: mammary serum amyloid A3 (M-SAA3), a difficult-to-express protein[20,21] and metalloproteinase 9 (MMP-9), which is prone-to-aggregate[18]. M-SAA3 is an acute phase protein that participates in the innate immune response of the mammary gland. On the other hand, MMP-9 is an enzyme that degrades the extracellular matrix and is involved in the immune response and tissue remodeling.

Results

Characterization of the M-SAA3 production in *L. lactis*. In a first approach to evaluate the production profile of M-SAA3 in *L. lactis*, the production kinetics of this model protein was analyzed at 30 °C at different times post-induction. The separation of the soluble and the insoluble fractions of the cell lysate indicated that M-SAA3 was mainly produced in the soluble form (65–80%) in *L. lactis* cytoplasm, although protein aggregates (IBs) were also formed (Fig. 1a). Along time, there was a significant increase in the percentage of the aggregated M-SAA3 at 1.5, 2 and 3 h compared with 1 h post-induction ($P = 0.002$) (Fig. 1a). However, the percentage of

	1	2	3	4	5	6	7	8
Binding buffer (BB)	20 mM Tris pH = 8, 500 mM NaCl, 20 mM IMZ						20 mM Phosp. pH = 8, 1.5 M NaCl, 50 mM IMZ	20 mM Phosp. pH = 8, 500 mM NaCl, 20 mM IMZ
Elution buffer (EB)	20 mM Tris pH = 8, 500 mM NaCl, 500 mM IMZ	20 mM Tris pH = 8, 500 mM NaCl, 2 M IMZ	20 mM Tris pH = 8, 500 mM NaCl, 1 M IMZ				20 mM Phosp. pH = 8, 1.5 M NaCl, 1 M IMZ	20 mM Phosp. pH = 8, 500 mM NaCl, 1 M IMZ
Solubility Enhancer	.	.	0.5% Triton X-100 (toxic)	5% Glycerol		10% Glycerol		0.5% Tween-20
Protein loss	+++	+++	++	+++	+++	++	++	–
Cation Exchange	.	.	.	.	BB: 20 mM Tris pH = 7 EB: 20 mM Tris pH = 7, 1 M NaCl	.	.	BB: 20 mM Tris pH = 8 EB: 20 mM Tris pH = 8, 1 M NaCl
Purity (%)	13.8	12.4	54	15.2	30.4	20.8	ND	ND
Main Impurities	50–75 kDa	50–75 kDa	75 kDa	50 kDa	50 kDa	50–75 kDa	50–75 kDa	50–75 kDa

Table 1. Tested conditions in the process to optimize the purification of the soluble M-SAA3. IMZ: imidazole; Phosp.: phosphate buffer; pI: Isoelectric point; ND: non-detectable.

aggregation did not change when using different growing temperatures (~36% at 30 °C and 20 °C and 39.8% at 16 °C) (Fig. 1b).

The total amount of M-SAA3 produced improved by increasing the inducer concentration. Specifically, significant differences were observed using concentrations of 2.5, 5, 12.5 and 25 ng/ml nisin ($P = 0.002$) (Fig. 1c). Interestingly, *L. lactis* growth rate was lower in the last four nisin concentrations tested ($P = 0.041$), where the M-SAA3 production was enhanced, which indicates that growth rate can be negatively affected either by high nisin concentrations or by high production of M-SAA3 (Fig. 1c).

Thus, after this first screening, the production of soluble M-SAA3 at 30 °C at 1.5 h using 12.5 ng/ml nisin was established as the optimal condition.

M-SAA3 purification from the soluble fraction. As a starting point, the production and purification of soluble M-SAA3 following a standard protocol was assessed. For that, 1 l of *L. lactis* was grown at 30 °C and, after that, a purification trial using a standard immobilized metal affinity chromatography (IMAC) purification protocol for the isolation of cytoplasmic soluble proteins was performed (Table 1, condition 1). Under this condition, a large protein loss was observed during the purification process (Fig. 2, condition 1). Moreover, the eluted protein had low purity, being the presence of two proteins of around 50 and 75 kDa especially relevant (Fig. 2, condition 1). MALDI-TOF analyses revealed that these two main impurities were proteins from host bacteria and corresponded to *L. lactis* elongation factor TU (pI = 4.89; 43.2 kDa) and *L. lactis* elongation factor G (pI = 4.75; 77.9 kDa).

Aiming to optimize the purification process of the soluble protein M-SAA3, eight different strategies combining different imidazole concentrations and/or the addition of detergents or other solubility enhancers were tested (Table 1, conditions 2–8). In some cases, a cation-exchange step was added after the IMAC purification (Table 1, conditions 5 and 8) in order to increase the purity of the recombinant product. Some strategies such as conditions 3, 6, 7 and 8 reduced the protein loss, being this improvement particularly relevant for the condition 8. However, despite a substantial decrease in the amount of unbound protein, none of these strategies (conditions 3, 6, 7 and 8) significantly increased the purity of the final product. Besides, the incorporation of a purification step using a cation-exchange approach did not improve the result of the whole purification process. Thus, in general terms we can conclude that none of the tested strategies allowed to obtain highly pure M-SAA3 through an efficient purification process (Fig. 2).

Solubilization and purification of M-SAA3 aggregates. Once proven that soluble M-SAA3 produced in *L. lactis* cannot be successfully purified using conventional strategies under a trial-and-error process, we evaluated whether the extraction of soluble M-SAA3 from *L. lactis* aggregates could be an alternative. Considering that *L. lactis* aggregates are formed by biologically active proteins, a new, simple and non-denaturing protocol for the isolation of soluble and functional proteins using these bacterial aggregates as protein source was developed. Specifically, different washing steps with the use of a mild detergent for the efficient solubilization of M-SAA3 IBs, without using any denaturing agent, were combined (Fig. 3). Importantly, we proved that is possible not only to isolate soluble M-SAA3 protein from *L. lactis* aggregates, but also that the purified product has a high degree of purity (>98.5%) (Fig. 4a). In this context, it is important to emphasize that the recombinant product was purified in absence of the two major impurities (*L. lactis* elongation factor TU and elongation factor G) found in most of the conditions tested for the purification of soluble protein (Table 1). In the elution profile of the purification

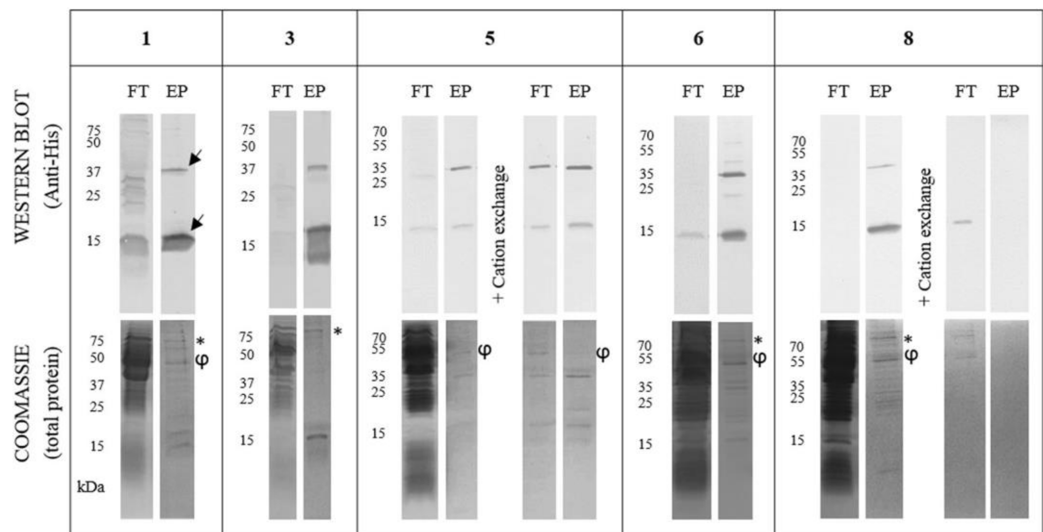

Figure 2. Protein gels for the soluble M-SAA3 IMAC purifications at different conditions (1, 3, 5, 6 and 8 in Table 1). Lane 1: flow through (FT) or non-bound protein, lane 2: eluted protein (EP). The eluted M-SAA3 is observed by Western blot in two bands, a monomer at 13–15 kDa and a dimer at 26–30 kDa (indicated by arrows). The obtained purities can be observed by Coomassie staining (* and φ indicate the main impurities). High-contrast was applied when necessary -only in Coomassie lanes- to allow a better display of the bands. Complete and original gels and blots for each condition can be found in the supplementary material (Supplementary Fig. 1).

of the solubilized M-SAA3, only two bands of 13–15 kDa and 26–30 kDa were detected, which corresponded to M-SAA3 monomer and dimer, respectively (Fig. 4a).

Activity of the solubilized and purified M-SAA3. An *in vitro* assay using bovine epithelial cells from the mammary gland was conducted to test the activity of the M-SAA3 obtained through the new purification method. The results of this assay showed that the M-SAA3 was fully active and a dose-dependent effect could be observed on the stimulation of interleukin 8 (*CXCL8*) expression (Fig. 5) after M-SAA3 treatment[21]. Specifically, a 1.6-fold increase of *CXCL8* expression compared with the PBS treatment was obtained by adding 9 µg/ml of M-SAA3 to the cells, whereas a 3-fold increase was noted for the treatment with 90 µg/ml of M-SAA3 ($P < 0.0001$).

IB solubilization of prone-to-aggregate proteins. To determine the efficacy of the new protocol with other difficult proteins, we assessed its effectiveness with a protein with a high tendency to aggregate, namely MMP-9. The aggregation rate in *L. lactis* of this protein has already been described, with reported values up to 100%[18]. Thus, we produced and purified MMP-9 aggregates, which were solubilized following the described protocol (Fig. 3). Again, soluble and highly pure proteins were obtained with this novel protocol. Specifically, a purity of 99% was observed by Coomassie staining for MMP-9 (Fig. 4b) and the activity of the soluble metalloproteinase obtained through the new method was tested. Specifically, the activity was tested by zymography, observing that soluble MMP-9 was active following a dose-dependent effect (Fig. 6).

Discussion

The production and purification of recombinant proteins is often a difficult and product-dependent process. The M-SAA3 is just one example amongst many other difficult-to-produce and difficult-to-purify proteins. Previous reports have shown that its production requires a difficult and a time-consuming process[20–22]. Moreover, results shown in this work reveal that none of the commonly used strategies to improve protein production and purification can be used for the isolation of M-SAA3 in its soluble form, using *L. lactis* as an intracytoplasmic expression system (Fig. 2). Another example of difficult proteins are MMPs, which have previously shown to have a strong tendency to aggregate in both *E. coli*[23–25] and *L. lactis*[18], reaching levels of up to 100% aggregation. Under these situations, which are common under recombinant production conditions, protein production and purification from the soluble fraction can become a long and unsuccessful trial-and-error process and alternative approaches are needed.

IBs are protein aggregates produced in recombinant bacteria under conditions of protein overproduction[26,27]. Their formation has been mainly described in *E. coli* cytoplasm, but increasing evidence shows that aggregation under these conditions occurs in different expression systems, including *L. lactis*[28]. These aggregates are composed mainly by the overexpressed protein in its active form and they are formed through a specific process[29,30], which means that they might drag just few proteins from the cell cytoplasm. Thus, due to their special features, IBs represent an appealing source of functional recombinant protein[31]. However, traditionally, the recovery of proteins from IBs produced by *E. coli* has been done using a two-step process, including a hard denaturation step (using agents like urea or guanidine hydrochloride) followed by, an often, non-efficient refolding process[32,33]. This

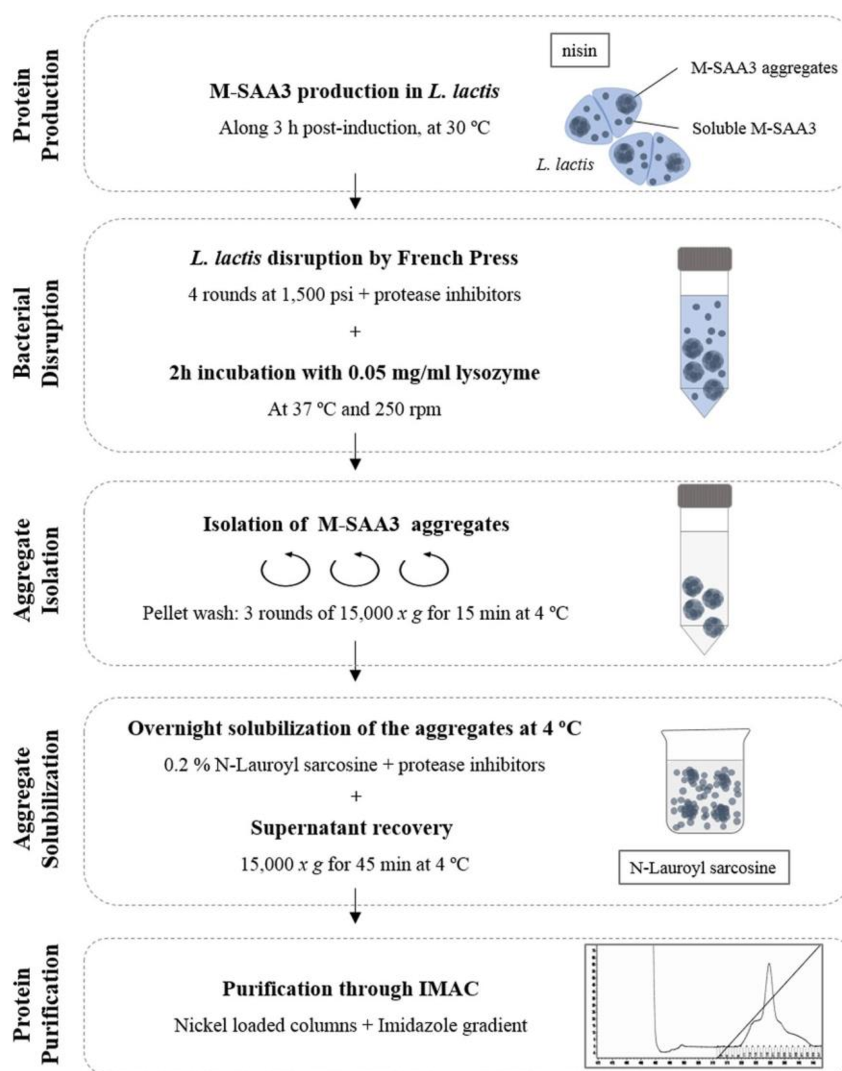

Figure 3. Schematic protocol for the solubilization of the M-SAA3 IBs produced in *L lactis*.

aggressive treatment disregards the IB nature and protein comprised in these aggregates is completely denatured. Thus, since these aggregates are formed by functional recombinant protein, the use of mild protocols to extract properly folded soluble proteins from IBs could be a promising strategy to avoid the use of denaturing agents. In this context, this approach has already been proven in *E. coli*[32,34], but no procedure has been developed thus far for the isolation of soluble and LPS-free proteins using *L. lactis* aggregates[14,18]. In the present work, we have developed a novel and straightforward procedure (Fig. 3) to obtain soluble, pure and biologically active proteins from *L. lactis* aggregates. Since the formation of MMP-9 IBs by *L. lactis* has been recently described[18] and the aggregation rates of the M-SAA3 can be increased by prolonging the production incubation time (Fig. 1a), we have used these proteins as models to evaluate *L. lactis* aggregates as a potential source of difficult proteins. Specifically, we have proven that it can be successfully applied with both difficult-to-express and difficult-to-purify proteins (Figs 4–6). On the one hand, we have shown that protein purity levels increase (Fig. 4) and the recovered protein is biologically active (Figs 5 and 6). Moreover, unlike protocols developed for the isolation of soluble proteins from *E. coli* IBs, this new procedure allows to isolate LPS-free proteins. Altogether gives consistency to the new method that can be applied as a unique approach to obtain pure yields of difficult-to-isolate proteins from bacterial aggregates in a soluble format and free of endotoxins. Thus, this approach is an appealing alternative to the extracytoplasmatic production[35] for the isolation of soluble proteins that are mainly produced as protein aggregates.

Conclusions

We have developed a new approach to obtain prone-to-aggregate and difficult-to-purify proteins in its soluble form using *L. lactis* as expression system. For the first time, we have proven that it is possible to use *L. lactis* aggregates as a source of fully functional proteins free of endotoxins. For that we have developed a protocol widely applicable for proteins that cannot be obtained through standard procedures without the need to add denaturing

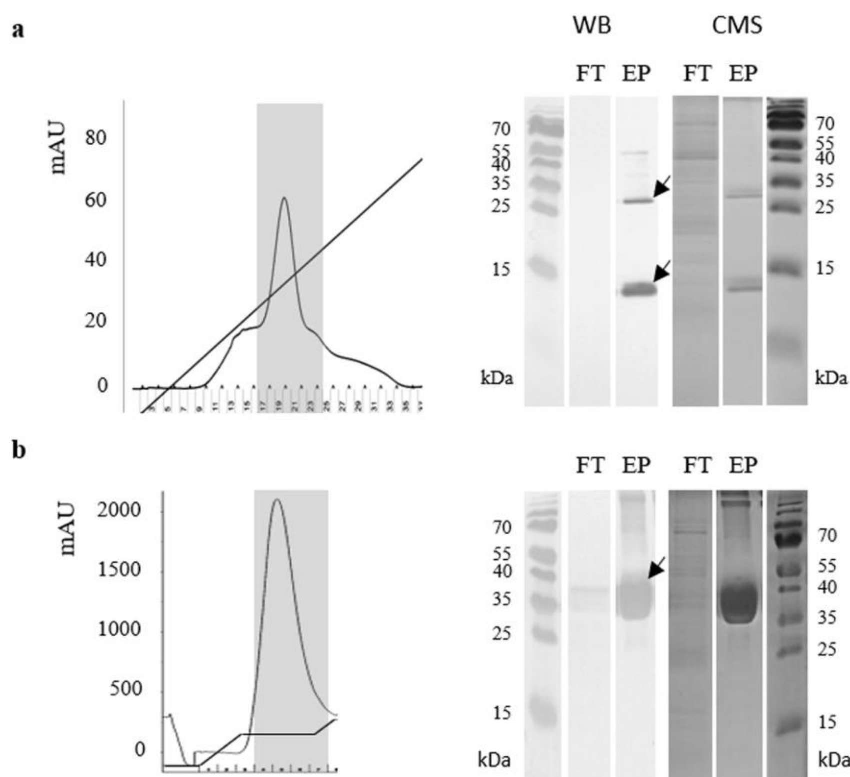

Figure 4. IMAC purification of the solubilized aggregates produced in *L. lactis*. In the left side, the chromatograms of the M-SAA3 (**a**) and MMP-9 (**b**) elutions. In grey, the selected fractions of the eluted proteins. In the right side, Western Blot and Coomassie staining for each eluted protein (EP) and flow through (FT). The arrows indicate the correspondent bands for each protein -the M-SAA3 (a) appears in a monomeric and a dimeric form, whereas MMP-9 (b) appears mainly as monomers-. Complete gels and blots can be found in the supplementary material (Supplementary Fig. 2).

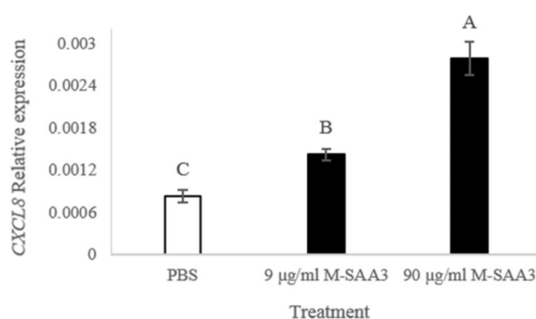

Figure 5. *CXCL8* gene expression by bovine epithelial cells from the mammary gland with solubilized and purified M-SAA3, obtained from *L. lactis* aggregates. *CXCL8* gene expression using 9 μg/ml and 90 μg/ml dose, using PBS as a control. Different letters depict differences between treatments ($P < 0.0001$). Error bars indicate the standard error (SE).

agents. The developed method represents an economical alternative that opens the door to the production of new recombinant proteins that, to date, could not be obtained.

Methods

Bacterial strains and plasmids. *Lactococcus lactis subsp. cremoris* NZ9000[36] and NZ9000 *clpP*$^-$ *htrA*$^-$ (*clpP-htrA*; erythromycin resistant (Em^R))[37,38] (kindly provided by INRA, Jouy-en-Josas, France; patent n° EP1141337B1) strains and the sequence of two proteins, the catalytic domain of bovine metalloproteinase 9 (MMP-9)[18] and the goat mammary serum amyloid A3 (M-SAA3)[21], were used in this study. Each protein sequence was flanked by *Nco*I and *Xba*I restriction sites and C-terminally fused to a His-tag for purification and quantification purposes. All genes were ligated into the chloramphenicol resistant (Cm^R) pNZ8148 plasmid (MoBiTech). The plasmid pNZ8148:SAA3 was transformed into electrocompetent *L. lactis* NZ9000, while

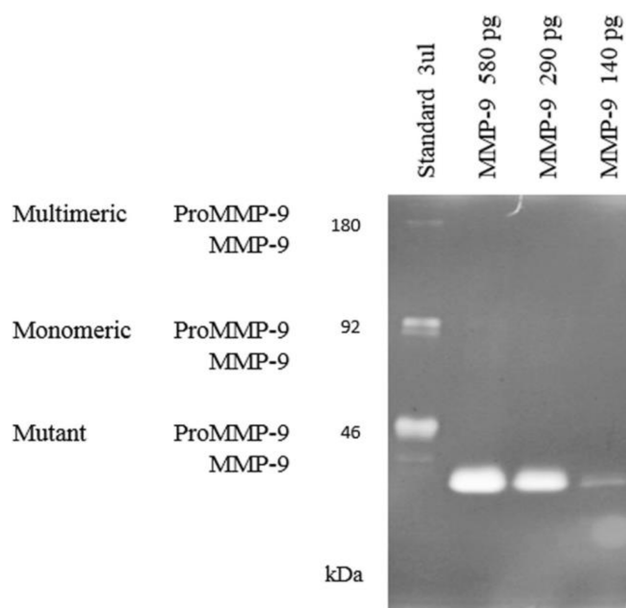

Figure 6. Zymography of MMP-9. Gelatin degradation can be observed using three different amounts of the solubilized MMP-9. Line 1: standard; line 2–4: different amounts of soluble MMP-9. The molecular weight of the solubilized MMP-9 (catalytic domain) is 39 kDa.

pNZ8148:MMP-9 was transformed in *L. lactis clpP-htrA*. For electroporation, a Gene Pulser (Bio-rad) at 2500 V, 200 Ω and 25 μF was used as detailed by Cano-Garrido *et al.*[18].

M-SAA3 protein production. *L. lactis* NZ9000/pNZ8148:SAA3 plasmid was grown overnight (O/N) at 30 °C in GM17 with 5 μg/ml Cm for plasmid maintenance. The O/N cultures were inoculated in fresh GM17 with Cm at an initial optical density at 600 nm (OD_{600}) of 0.1. When cultures reached an OD_{600} = 0.4–0.6, the recombinant protein expression was induced with 12.5 ng/ml nisin.

Culture samples of 25 ml were taken and centrifuged (6,000 × *g*, 15 min, 4 °C) at 0, 1, 1.5, 2 and 3 h post-induction for a protein kinetics analysis. Samples for a temperature-effect analysis were taken at 0 and 3 h post-induction from cultures grown at 30 °C and at 0 and 16 h post-induction from cultures grown at 20 and 16 °C. The inductor concentration-effect analysis was assessed by samples taken at 0 and 3 h post-induction with seven different nisin concentrations (0.625, 1.25, 2.5, 5, 12.5 and 25 ng/ml) in cultures grown at 30 °C. All the experiments were run in triplicate.

In all cases, pellets were resuspended in 500 μl PBS with an EDTA-free protease inhibitor cocktail (Roche) and bacteria were disrupted by sonication. Each sample was ice-coated and sonicated with 2 cycles of 1.5 min (0.5 sec cycles at 10% amplitude). The soluble and the insoluble protein fractions were separated by centrifugation at 15,000 × *g* for 15 min at 4 °C and the insoluble fraction was resuspended in the same buffer at the same initial volume.

Protein determination. The soluble and the insoluble protein fractions were analyzed by 15% denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). All samples were resuspended with Laemmli loading buffer (100 mM Tris base, 8% glycerol, 55 mM SDS, 4% β-mercaptoethanol, 1.6 M urea). Soluble fractions were boiled for 5 min and insoluble fractions for 40 min before electrophoresis. Protein bands were electroblotted into PVDF membranes at constant 250 mA and 100 V for 1 h, 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 (GE Healthcare; mouse) was used as the primary antibody at a 1/1,000 dilution in BSA-TBST buffer, in which membranes were incubated along 2 h at room temperature (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). Bands were quantified with a standard curve of T22-GFP-H6[39] and densitometry analyses with ImageJ software. The M-SAA3 aggregation rate (insoluble protein/total protein) in *L. lactis* was calculated for both the kinetic and the temperature experiments.

Production and purification of the M-SAA3 from the soluble fraction. Cultures of 1 l of the M-SAA3-producer *L. lactis* were grown at 30 °C and induced with nisin at an OD_{600} = 0.4–0.6, as previously described[14]. The whole volume was recovered after 1.5 h of production and centrifuged at 5,000 *x g* for 15 min at 4 °C. Pellets from 500 ml of culture were suspended in 30 ml of the binding buffer (20 mM Tris pH = 8, 500 mM NaCl, 20 mM imidazole) with an EDTA-free protease inhibitor cocktail (Roche). Bacteria were mechanically

disrupted by French Press (Thermo FA-078A) with 4 cycles at 1,500 psi in ice coating. Cell lysates were centrifuged at 15,000 × *g* for 45 min and the M-SAA3 in the soluble fraction was purified by Immobilized Metal Affinity Chromatography (IMAC) in an ÄKTA purifier FPLC (GE Healthcare) using 5 ml HiTrap Chelating HP columns (GE Healthcare).

Optimization of the purification process of the soluble M-SAA3. Eight different purification experiments were run to optimize the M-SAA3 final purity. Different imidazole concentrations (500 mM, 1 M and 2 M) were used in the elution buffer and none or different solubility enhancers were added to both the binding and the elution buffers (Triton X-100, glycerol, or Tween 20). Also, changes in the binding buffer were conducted: an increase of the imidazole concentration from 20 to 50 mM, a replacement of the Tris buffer for a phosphate buffer and an increase of the NaCl concentration from 500 mM to 1.5 M. All the tested combinations are detailed in Table 1.

The eluted peaks were dialyzed in PBS O/N, at 4 °C and with gentle agitation, unless a cation-exchange purification was scheduled. Cation-exchange chromatography was conducted in experiments 5 and 8 (see Table 1). The peaks obtained by the IMAC purification were dialyzed in 20 mM Tris pH = 7 in experiment 5, or in 20 mM phosphate buffer pH = 8 in experiment 8 (M-SAA3 isoelectric point is 8.67). In both cases, the dialyzed peaks were loaded in a negatively charged 1 ml SP FF column (GE Healthcare) and M-SAA3 was eluted by an increasing linear NaCl gradient to 1 M final concentration. Eluted samples were dialyzed in PBS as previously described.

The recovering efficiency and the purity of the eluted samples were determined by Western blot, as previously described[14] and by Coomassie staining.

Solubilization of M-SAA3 and MMP-9 from protein aggregates. Five liters of M-SAA3 were produced in *L. lactis* NZ9000 and 2 l of MMP-9 were produced in *L. lactis clpP-htrA*. In these cases, inductions were conducted for 3 h. The whole volumes were centrifuged at 6,000 x *g* and the pellets were resuspended in lysis buffer (20 mM Tris, 500 mM NaCl, 20 mM imidazole, 10% glycerol) in presence of protease inhibitors and in a ratio of 500:30 (ml:ml, culture:buffer). 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 was added and samples were incubated for 2 h at 250 rpm and 37 °C. Protein pellets were recovered and washed twice with distilled water. Pellets were weighted and solubilized in 0.2% N-lauroyl sarcosine in Tris solution at a ratio 1:40 (g:ml) as described by Peternel *et al.*[34] and adding protease inhibitors. The mixture was incubated O/N at 4 °C in agitation and the supernatant was recovered through centrifugation at 15,000 × *g* for 45 min at 4 °C for further purification.

Purification of the solubilized M-SAA3 and MMP-9. NaCl and imidazole were added to the solubilized proteins to equilibrate the samples with the binding buffer composition and IMAC purification was carried as previously described. Both the binding and the elution buffer contained 0.2% N-lauroyl sarcosine and the final imidazole concentration in the elution buffer was 1 M for the M-SAA3 and 500 mM for the MMP-9. The selected fractions were dialyzed in PBS O/N at 4 °C and with gentle agitation. The amount of purified protein was determined by Bradford's assay[40] and the integrity of the protein analyzed by SDS-PAGE.

Identification of the main contaminating proteins. The main contaminating bands were cut and sequenced by MALDI-TOF in the Servei de Proteomica i Biologia Estructural (sePBioEs, Autonomous University of Barcelona).

Activity of the solubilized M-SAA3. Mammary epithelial cells from primary cultures were obtained as described elsewhere[21] and seeded in 24-well plates at 44,000 cells/well. After 24 h incubation at 37 °C and 5% CO_2, wells were washed twice with warm PBS and 500 µl PBS or two doses (9 µg and 90 µg in 500 µl of PBS) of the solubilized and purified M-SAA3 were added to each well containing 500 µl of DMEM/F-12 medium with 8 µg/ml bovine insulin and 50 µg/ml hydrocortisone by sextuplicate. After 3 h of incubation at 37 °C, 5% CO_2, cells were gently washed with 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 *CXCL8* expression as described previously[21]. Relative gene expression was calculated using the $2^{\Delta Ct}$ method with ACTB as reference gene.

Activity of the solubilized MMP-9. MMP enzymatic activities were determined by zymography using a 10% SDS-PAGE gel with 1% gelatin under non-denaturing conditions. After that, the gel was incubated with developing buffer and dyed with Coomassie as detailed by Cano-Garrido *et al.*[18]. Densitometry analyses of the bands were performed with the Image J software. The standard (kindly provided by the Laboratory of Immunobiology of the Rega Institute for Medical Research, KU Leuven, Belgium) corresponds to a mixture of purified monomeric MMP-9, multimeric MMP-9 and a mutant MMP-9 with a domain deletion[41]. The enzyme precursors, proMMP-9, are present in this standard.

Statistical analysis. All data were analyzed using a mixed-effects model that accounted for the random effects of replicate (n = 3) and the fixed effects of treatment and/or time of sampling (JMP, SAS Institute Inc.). Sampling time entered the model as a repeated measure using an autoregressive covariance matrix. When more than 2 means were compared, differences were established using the Tukey's multiple mean separation test. Data were previously transformed to achieve a normal distribution when necessary. Results are expressed as the means of non-transformed data ± standard error of the mean (SEM).

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions

L.G., O.C.-G., F.F., R.R. and J.S.-F. performed the experiments and prepared the final data and figures. A.A. and E.G.-F. conceived the experiments and supervised the work. L.G., A.A. and E.G.-F. wrote the manuscript text with contributions from the other authors. N.F.-M, AV., M.D. and A.B. outlined the structure and reviewed the manuscript. All authors took part in the analysis of the data and approved the final version of the manuscript.

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STUDY 3

DON'T JUDGE A BOOK BY ITS COVER: THE BIOLOGICAL POTENTIAL HIDDEN IN INCLUSION BODIES

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Preface

MMP-9 is a relevant protein involved in tissue involution and in immune responses in the bovine MG at dry-off and could offer some potential as a new alternative drug to preventive antibiotics. Getting the soluble MMP-9 counterpart on our hands (study 2) awakened our interest to explore new options to exploit the MMP-9 potential. We aimed to obtain new protein formats based on biotechnological approaches and suitable for *in vivo* applications, widening the repertoire beyond bacterial IBs. Combining proteins with polymers or lipidic molecules can improve their half-life and permeability in a target tissue. Thus, thinking on strategies to hasten involution and the immune performance in the cow MG at dry-off, these vehicles could increase protein loads and/or temporally protect MMP-9 from proteases and inhibitors once in the target tissue, improving the soluble MMP-9 delivery efficiency. These nanocarriers, in addition, could bear different pharmacodynamics than IBs, being worth to compare them.

In the following study, thus, we obtained and characterized two differently ensembled MMP-9 nanoparticles combined either covalently or non-covalently to F127 amphiphilic nanopolymers forming two distinct polymeric-based micelles (PM-B and PM-C, respectively). Moreover, with the aim to uncover which are the potential pharmacokinetic differences between the soluble, aggregated (IBs) and nanopolymerized (PM-B and PM-C) MMP-9 formats, we enrolled these in an activity assay where their capability to degrade gelatin after being incubated in bovine serum was measured along time as a means of their functional stability. Interestingly, while soluble and nanopolymerized formats of MMP-9 showed an around 200-fold greater performance in terms of activity compared to MMP-9 IBs, the last offered better stability under *in vitro* conditions. Likewise, and contrasting with the *in vitro* low-active profile of IBs, when these were compared to the soluble counterpart under *in vivo* conditions, IBs demonstrated their potential providing a distinguished protein stability, importantly permitting the MMP-9 to extend its effects further than the soluble MMP-9 format.

DON'T JUDGE A BOOK BY ITS COVER: THE BIOLOGICAL POTENTIAL HIDDEN IN INCLUSION BODIES

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Abstract

Inclusion bodies (IBs) are protein nanoclusters obtained during recombinant protein production processes and several studies have demonstrated their potential as biomaterials for therapeutic protein delivery. Nevertheless, IBs have been, so far, exclusively sifted by their biological activity *in vitro* to consider in further protein-based treatments *in vivo*. Matrix metalloproteinase-9 (MMP-9) protein, which has an important role facilitating the migration of immune cells, has been used as model protein. The MMP-9 IBs were compared with their soluble counterpart and MMP-9 encapsulated in polymeric-based micelles (PM) through ionic and covalent binding. The soluble MMP-9 and the MMP-9-ionic PM showed the highest activity values *in vitro*. IBs showed the

lowest activity values *in vitro* but the specific activity evolution in 50% bovine serum at room temperature proved that they were the most stable format. The data obtained using an air-punch mice model showed that MMP-9 IBs presented the highest *in vivo* activity compared to the soluble MMP-9 which only was associated to a low and a transitory peak of activity. These results demonstrated that the *in vivo* performance is the addition of many parameters that not always correlate with the *in vitro* behavior of the protein of interest, becoming especially relevant at evaluating the potential of IBs as a protein-based biomaterial for therapeutic purposes.

Keywords: inclusion bodies, polymeric micelles, stability, matrix metalloproteinase-9, *in vitro*

Introduction

Inclusion bodies (IBs) are protein-based biomaterials naturally formed under recombinant protein production processes. Although they have for long been considered as 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[1,2]. Briefly, they have been described as a tunable and multieffector material used as cell culture substrate for tissue engineering purposes[3–6]. It has also been proven *in vitro* their application as nanopills for protein-based cell therapies[7,8]. *In vivo*, Unzueta and co-authors have observed that subcutaneously injected tumor targeted-IBs could release the forming protein, cross into the blood stream and accumulate in the tumor for sustained periods[9]. In addition, when intratumorally injected, IBs made of therapeutic proteins such as Omomyc and p31 induced tumor cells death[10], while cytokine-based IBs administered intraperitoneally conferred protection to zebrafish model from a lethal bacterial infection[11].

Although in most of the cases IBs have been produced using *Escherichia coli* as recombinant cell factory, their production has also been successfully done in lipopolysaccharide (LPS)-free recombinant systems such as *Lactococcus lactis*[12,13].

Despite the large number of studies related to IBs applications, their *in vitro* and *in vivo* performances have never been compared with other protein delivery formats to determine if protein activity is the only factor to consider in protein-based treatments. For that, we have chosen matrix metalloproteinase-9 (MMP-9) protein as model protein, which has an important role degrading the extracellular matrix (ECM) in contexts of tissue development, involution and remodeling, along with facilitating the migration of immune cells to the site of injury or inflammation[14,15]. Therefore, in the present work we have compared *in vitro* and *in vivo*

performance of our model protein when displayed as IB versus to their soluble counterpart and an alternative nanocarrier, consisting in PM loaded with MMP-9.

Results

Activity of MMP-9 nanoparticles

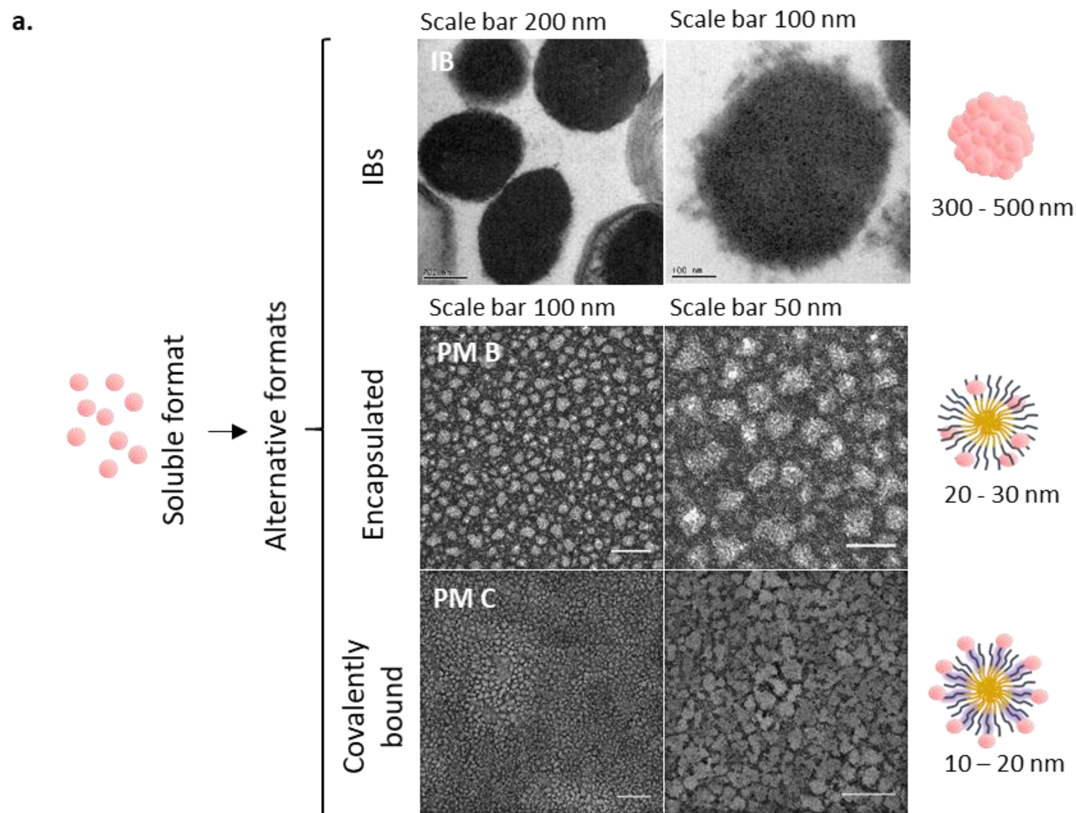
MMP-9 was successfully produced in *L. lactis* in its soluble form and as bacterial IBs, rendering nanoparticles in the range of 300 to 500 nm in diameter (**Figure 1a**). In a second step soluble MMP-9 was loaded in polymeric micelles using two different approaches: encapsulated by electrostatic interactions (named PM B) and covalently bound (named PM C) (Figure 1a). PM B and PM C, as it occurs with IBs, were pseudo-spherical nanoparticles with sizes of 20-30 and 10-20 nm, respectively (Figure 1a). Besides, as shown by the slight increase in N presence in PM B and PM C in comparison to empty PM (XPS table, Figure 1b), a certain amount of protein (major source of N) was effectively bound to the PM surface for both protein loading strategies. Analyzing MMP9 cargo in further detail we observed the encapsulation efficiency of MMP-9 within the polymer was around 80 %, being similar in covalent and ionic PM (Figure 1b). The percentage of MMP-9 forming IB nanoparticles achieved values of 96.5 % (Figure 1b).

The activity of MMP-9 in the different nanostructured formats was tested *in vitro* and compared with the soluble (non-nanoclustered) form (**Figure 2**). Although all forms were functional, we observed that the free MMP-9 and the MMP-9 encapsulated in PM B nanoparticles showed the highest activity values (Figure 2b). Polymeric micelles with covalently bound MMP-9 (PM C) showed a decrease of 10 % in the specific activity (Figure 2). But it is especially relevant to underline the low activity values of IBs, which had an activity 260 and 196 times lower than PM B and PM C, respectively (Figure 2b).

In vitro MMP-9 stability

Although IBs showed the lowest activity values (Figure 2b), the specific activity evolution in 50% bovine serum at room temperature, proved that they were the most stable format (**Figure 3**). The assessment of specific activity decrease revealed that soluble protein and polymeric nanoparticles lost almost all the activity after 2 h of incubation, while IBs kept a 28 % of the activity after 14 d of incubation (Figure 3).

a.



b.

XPS

(%)	C	O	N
Solubilized MMP-9	63.62	18.67	17.24
PM empty (A)	71.88	27.30	0.82
PM-MMP-9 (B)	70.93	27.63	1.44
PM-MMP-9 (C)	69.89	27.97	2.14

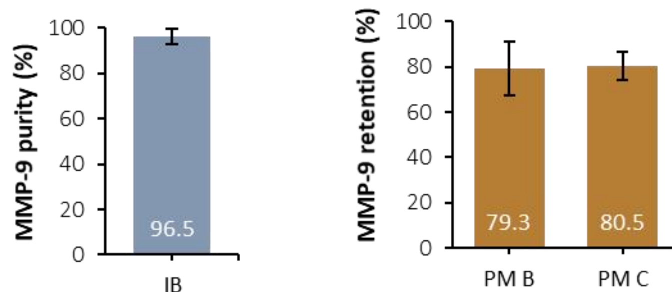


Figure 1. Description for the MMP-9 formats. **a.** Transmission electron microscopy (TEM) images for MMP-9 IBs (IB), non-covalent MMP-9-nanopolymers (PM B) and covalent MMP-9-nanopolymers (PM C). Pictures represent the format structures for the soluble proteins, IBs and Pluronic® F127 amphiphilic polymers. F127 hydrophobic chains are represented in yellow and hydrophilic chains in blue. In PM C, the carboxylated chains of polymer (F127:COOH) are represented as blue in purple shadow and correspond to 25 % of the total polymer (ratio 1:4). Soluble MMP-9 are represented as single pink spheres. **b.** XPS results summary showing elemental composition on the surface of MMP-9, non-loaded PM (A), and MMP-9 loaded PM by the two strategies previously mentioned (PM B and PM C). Panels below, on the left MMP-9 purity of IBs. On the right, protein encapsulation efficacy for both types of PM tested.

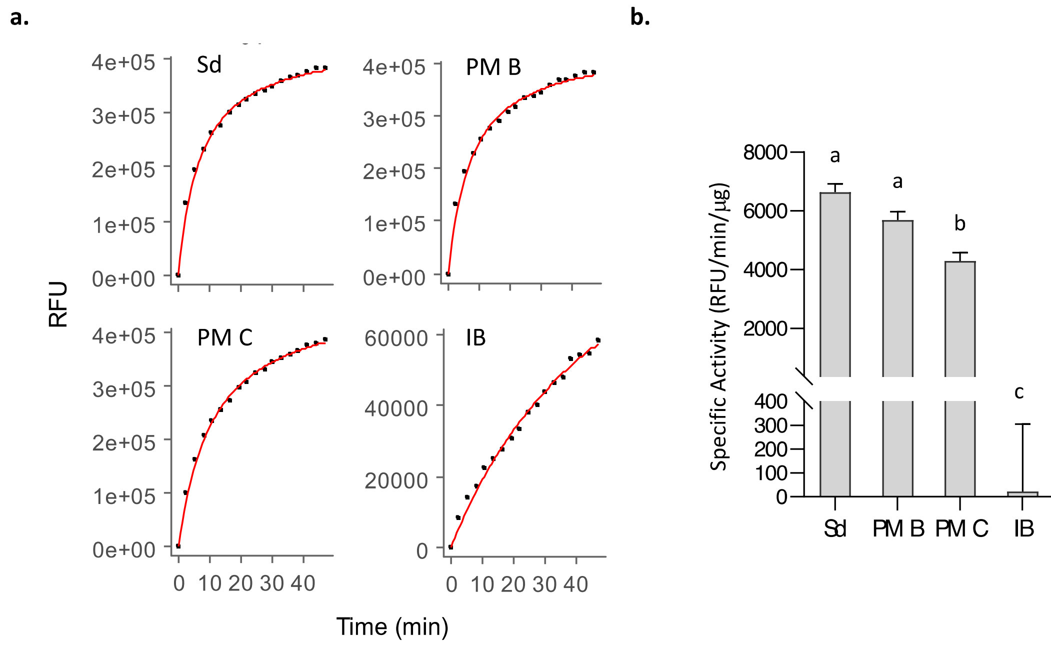


Figure 2. Initial MMP-9 activity (t = 0h) in PBS incubations for the analysed formats. **a.** DQ-gelatin degradation kinetics for one of the replicates of 10 µg of solubilized MMP-9 (Sd), 10 µg of non-covalent MMP-9-PM (PM B), 10 µg of covalent MMP-9-PM (PM C) and 100 µg MMP-9 IBs (IB). Fluorescence reads along time (dots) and R model curves (red line) are represented. RFU = Relative fluorescence units. **b.** Comparison of the specific activity (RFU corrected by time and MMP-9 µg) for the analysed formats. Means and SEM are represented. Data were analysed using a fixed effect model with format as fixed effect. Different letters depict significant differences (p = 0.0003).

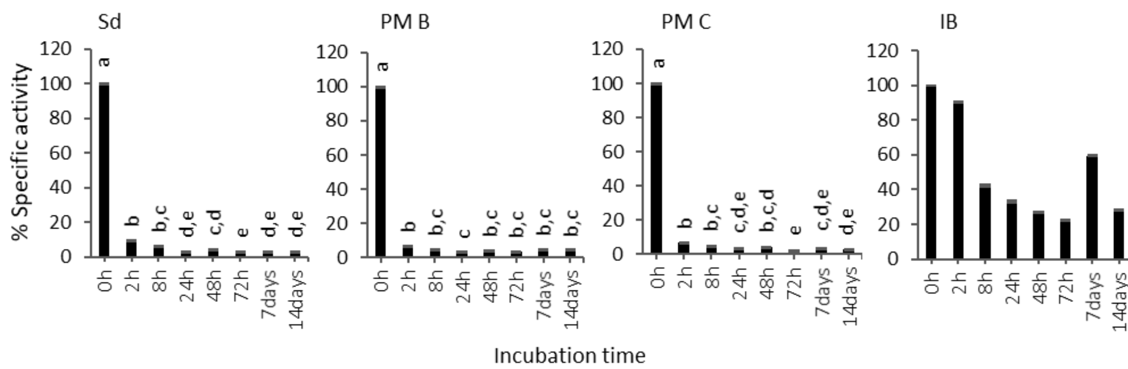


Figure 3. Evolution of the MMP-9 activity at different time points of incubation in serum 50 % (v/v). Non-transformed means and transformed SEM are represented. Data were analysed using a fixed effect model with format as a fixed effect. Different letters depict significant differences: solubilized MMP-9 (Sd), p < 0.0001; non-covalent MMP-9-nanopolymers (PM B), p = 0.0023; covalent MMP-9-nanopolymers (PM C), p < 0.0001; MMP-9 IBs (IB), p = 0.84.

In vivo MMP-9 activity

With the aim to determine if the results observed *in vitro* with MMP-9 IBs correlated with their performance *in vivo*, the soluble MMP-9, showing the highest biological activity (Figure 2), and MMP-9 IBs, showing the lowest activity but highest stability, were compared in a *mmp9* knock-out mice[16] following an air-punch experimental model[17]. Mutant MMP-9 IBs, which are non-functional MMP-9 IBs formed by an enzymatically inactive mutant form of MMP-9, were also administered to differentiate the specific MMP-9 effect from other components present in IBs but different from the recombinant MMP-9. After injections with DPBS (negative control), soluble MMP-9, MMP-9 IBs or mutMMP-9 IBs in mice air-pouches, exudates were recovered and centrifuged at 3 different time points (3, 24 and 48 h) to analyze the number of neutrophils that migrated to the site of injection due to the action of MMP-9 at degrading the extracellular matrix (ECM). At 3 h post-injections, there were no significant differences in neutrophils percentage for any of the MMP-9 treatments (soluble MMP-9, MMP-9 IBs, and mutMMP-9 IBs) (**Figure 4**). However, cell recruitment at 24 h was higher in those animals injected with both MMP-9 IBs and mutMMP-9 IBs in comparison with the DPBS control and the soluble MMP-9 (Figure 4). While neutrophil count values of those animals treated with mutMMP-9 IBs returned to basal levels at 48 h, the total cell recruitment levels were kept high in those exudates of animals injected with active MMP-9 IBs (Figure 4).

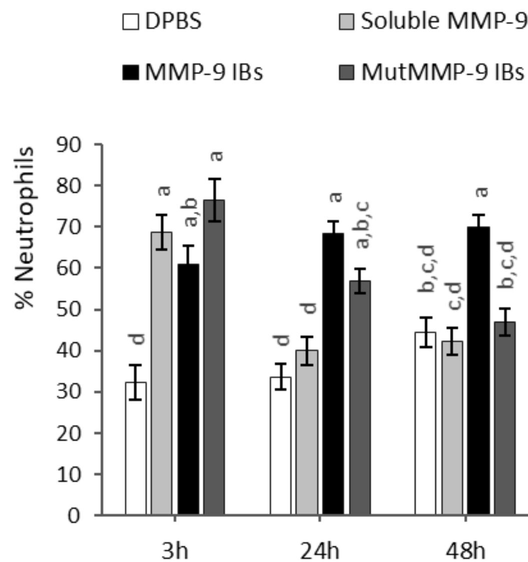


Figure 4. Relative quantification of neutrophils among total immune cell populations by cytospin centrifugation of exudates recovered after 3h, 24h and 48h after intradermal injection of Sb MMP-9, MMP-9 IBs and mutant MMP-9 IBs in air-pouches of *mmp9* K.O. mice. Percentages are relative to 100 cells counted by triplicate for each sample. Means and SEM from non-transformed data are represented. Different letters depict significant differences ($p < 0.0001$). Data were analyzed using a fixed effect model with treatment and time as fixed effects (n = 12 (3h), n = 24 (24h), n = 24 (48h)).

Discussion

Many examples have been published during the last decade proving that IBs are protein-based biomaterials with promising characteristics to be used for cell replacement therapies, for tissue engineering purposes, or even for cancer treatment[3-11], among other applications. However, their characteristics and their *in vitro* behavior have never been correlated with their *in vivo* performance compared with other standard formats. For that we have evaluated the functionality and stability of a protein with relevance in different fields such as MMP-9 produced in distinct formats: i) soluble (naked) form; ii) encapsulated in PM by ionic interactions or covalently bound to the PM surfaces (PM B and PM C nanoparticles, respectively); iii) IBs nanoparticles (Figure 1). Activity values bring out two important messages: MMP-9 IBs functionality is clearly lower than MMP-9 in its soluble form or as polymeric nanoparticles (Figure 2) and nano structuring of MMP-9 as PM B and PM C did not improve the functionality of our model protein (Figure 2). On the other hand, the stability analysis of MMP-9 in the different forms evaluated revealed that IBs are much more stable than soluble and MMP-9 PM B and PM C nanoparticles (Figure 3). Overall, in the line of the findings set out above, it becomes clear that in terms of activity the soluble version of MMP-9 is the best option (Figure 2), while the most stable format are IBs (Figure 3). Based on these results, we compared the *in vivo* response of mice treated with soluble MMP-9 and MMP-9 IBs to determine if the difference in stability showed by IBs could have relevance in a real context where proteases and other elements of a harsh environment are present. The data obtained using an air-punch mice model demonstrate that the *in vivo* performance is the result of many parameters that not always correlate with the *in vitro* behavior of the evaluated protein (Figure 4). Clearly, at the longest treatment time only those animals injected with IBs kept neutrophil levels higher than those treated with DPBS, being relevant that at 48 h the neutrophil proportion after MMP-9 IBs treatment was significantly higher than for mutMMP-9 IBs (Figure 4). The residual effect of mutMMP-9 indicates that there is a transitory and basal inflammatory effect due to an unspecific effect of protein nanocluster format, but the results distinguished clearly this background from the effect of heterologous protein embedded in the IBs. In addition, the effect of soluble MMP-9 was restricted to the first 3 h and disappeared at longer times indicating that despite the high activity of MMP-9 observed *in vitro* it is not translated to any relevant effect in an *in vivo* context.

Thus, as it has previously proven, IBs have a great potential, being in many cases totally or partially hidden when their action is evaluated *in vitro*. The results presented here prove that to observe a desired effect of the administered protein *in vivo* it is not only important their biological activity but also other parameters such as stability, format, and probably the slow-release properties that

IBs have, and that have been previously described[1,18]. In consequence, IBs are not only a promising format because it is produced through a cost-effective one-step process[18], but also because low amounts of functional protein in this format are enough to render desired significant response *in vivo*.

Conclusions

Although the *in vitro* results showed that IBs have much lower activity than soluble counterpart and other MMP-9-based nanoparticles, the *in vivo* mouse model indicates that this low *in vitro* activity is transformed in a relevant *in vivo* effect when administered to the animal. The results of this study showed that the evaluation of *in vitro* activity of IBs do not reflect their therapeutic potential, bringing to light the importance to integrate other parameters such as protein stability and the use of *in vivo* animal models when protein-based nanoclusters are evaluated as a therapeutic biomaterial.

Experimental section

Bacteria strains and plasmids

Lactococcus lactis subsp. *cremoris* NZ9000 *clpP*$^-$ *htrA*$^-$ (clpP-htrA; Em^R) strain[19,20] (kindly provided by INRA, Jouy-en-Josas, France; patent n. EP1141337B1), was used as the expression system for the production of the recombinant MMP-9 fragment Phe107-Pro449 (NCBI, NM_174744.2). Briefly, the gene was cloned into the pNZ8148 plasmid (Cm^R) and transformed into competent *L. lactis* ClpP$^-$ HtrA$^-$ bacteria as described elsewhere[21].

Protein production in *L. lactis*

Bacteria were grown overnight (O/N) at 30 ºC without shaking in M17 broth supplemented with 0.5 % glucose (M17G), 5 μg/ml chloramphenicol and 2.5 μg/ml erythromycin. Cultures were re-inoculated at a 0.05 initial OD_{660nm} and induced with 12.5 ng/ml nisin when OD_{660nm} achieved 0.4-0.6. Productions were sustained along 3 h after which cultures were centrifuged at 6,000 x *g* for 30 min at 4 ºC to recover bacteria. Pellets were frozen at -80 ºC until use.

Production and purification of soluble MMP-9

Soluble MMP-9 was obtained from the solubilisation of MMP-9 IBs produced in *L. lactis* as previously described[12]. Briefly, bacteria were suspended (30:500 PBS:culture, volume) and disrupted for 4 rounds by French press at 1,500 psi, ice-coated and with protease inhibitors

(Complete EDTA-free, Roche), followed by a 2 h incubation in 0.05 mg/ml lysozyme at 37 ºC in agitation. Lysates were pelleted (15,000 x *g* for 45 min at 4 ºC) and washed twice in mQ-H_2O, and pellets were incubated in solubilisation buffer (40 mM Tris pH=8, 0.2 % N-lauroyl sarcosine; 40:1 buffer:pellet, ml:g) for 40 h at room temperature (RT). The supernatant was recovered at 15,000 x *g* for 45 min at 4 ºC, filtered and purified through Immobilised Metal Affinity Chromatography (IMAC) using a 1-ml HisTrap column (GE Healthcare) in an ÄKTA purifier FPLC system (GE Healthcare). Binding and elution buffers consisted in 20 mM Tris pH=8, 500mM NaCl, 20 or 500 mM Imidazole respectively, and 0.2 % N-lauroyl sarcosine. Four MMP-9 peaks were registered by holding the Imidazole gradient when elution absorbance (mAU) increased (data not shown), and were dialysed separately, O/N, in PBS at 4 ºC in a stirrer. Possible precipitation was excluded (15,000 x *g* for 15 min at 4 ºC) and the purified soluble MMP-9 was aliquoted and stored at -80 ºC until use. The protein eluted in the first peak was selected for this study as the soluble MMP-9, and the purity, analysed by Coomassie blue staining, was of 97.4 %.

Purification of MMP-9 and mutMMP-9 IBs

MMP-9 IBs were recovered and purified as previously described[21]. Briefly, bacteria were suspended (30:50 PBS:culture, volume) and disrupted by French press –following above details– for 3 rounds, freeze/thawed (F/T) and agitated for 2 h in 0.01 mg/ml lysozyme at 37 ºC. After a new F/T, the solution was incubated at RT in 4 μg/ml Triton X-100 for 1 h in orbital agitation (OA) and aliquots were incubated O/N at 30 ºC in agar-M17G, intercalating F/T cycles until no bacteria colonies were grown. Afterwards, the solution was incubated in 0.25 μl of NP-40 per sample ml (μl/ml) for 1 h at 4 ºC and OA, and with 0.6 μl/ml $MgSO_4$ (stock 1 M) and 0.6 μl/ml DNAse I (stock 1 mg/ml) for 1 h at 37 ºC and OA. Pellets were obtained (6,000 x *g* for 30 min at 4 ºC) and suspended in 5 ml lysis buffer with 0.5 % Triton X-100 per each 50 ml culture, F/T, pelleted again and suspended in Dulbecco's Phosphate-Buffered Saline (DPBS, GIBCO). MMP-9 IB pellet aliquots were obtained (20,000 x g for 15 min) and stored at -80 ºC until use.

Polymeric Micelles (PM) synthesis

The purified soluble MMP-9 was loaded in PM formed by the amphiphilic polymer Pluronic® F127 (Kindly provided by BASF). Two different strategies were employed 1) the encapsulation of MMP-9 by electrostatic interaction with the polymer and 2) the covalent binding of the protein to a previously activated F127-COOH (synthesised as previously described[22]). In both cases, PM were prepared using the film hydration procedure. Briefly, 20 mg of F127 polymer for strategy 1) and a mixture (8:2, w/w) of F127: F127-COOH for strategy 2) were weighted and dissolved in a methanol:ethanol (1:1, v/v) solution. 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. The film was hydrated in 2 mL 20 mM Tris-HCl containing MMP-9 at 1 mg/mL for MMP-9 by 5 min of vortex at RT for protein encapsulation through electrostatic interactions. Self-assembled PMs were further dialyzed against 20 mM Tris-HCl in Float-A-Lyzer®G2 Dialysis devices with a 100 KD pore size (Spectrum Laboratories, Inc). For covalent binding of MMP-9 F127:F127-COOH films were hydrated in 1 mL 20 mM Tris-HCl as described above and incubated with 1-etil-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Sigma) at 10 µg/mL for 30 min at RT under vigorous agitation. Then 1 mL of MMP-9 solution at 2 mg/mL was added and further incubated for 2 h at RT under agitation. Finally, PM were dialyzed following the same procedure than the employed for PMs encapsulating MMP-9.

Transmission electron microscopy (TEM) imaging

The TEM images of MMP-9 IBs were obtained as previously described[21]. For PMs images, a 6 µL drop, 1:20 dilution, was gently placed over 400 mesh cupper grid, previously coated with carbon. Samples were incubated for 1 min at RT and liquid excess blotted with Whatman No.1 filter paper. Negative staining was performed by the addition of a 6 µl drop of uranyl acetate 2 % (w/v), 1 min at RT. Staining excess was removed as described above. PMs observation was carried out in a TEM Jeol JEM-1400 (Jeol Ltd) operated at 120 kV, Images were further processed using the software ImageJ NIH.

MMP-9 loading efficacy

MMP-9 loading efficacy in PM was determined by the comparison of the protein amount in the samples before and after the dialysis process described in the Polymeric Micelles Synthesis section. Protein amount was measured by bicinchoninic Acid (BCA) method following manufacturer's indications (Pierce). Plates were measured at 590 nm in an ELx800™ microplate reader (BioTek™).

XPS

XPS measurements were performed at room temperature with a SPECS PHOIBOS 150 hemispherical analyzer (SPECS GmbH, Berlin) in a base pressure of 5×10^{-10} mbar using monochromatic Al Kalpha radiation (1486.74 eV) as excitation source.

Purity determination

MMP-9 IBs quantification and purity were determined by Coomassie blue staining.

Protein stability assay

Soluble MMP-9, the ionic-bind and covalent-bind MMP-9 nanopolymers, and the MMP-9 IBs were incubated at 0.2 mg/ml in 50 % bovine serum or PBS at RT and samples were analysed for the MMP-9 capability to degrade a dye-quenched gelatin (DQ gelatin™, Invitrogen). Because the initial velocity of the gelatin catalysis by the MMP-9 IBs was lower than by the soluble or encapsulated MMP-9 (data not shown), after 0 h, 2 h, 8 h, 24 h, 48 h, 72 h, 7 days and 14 days of incubation, 100 or 10 µg of MMP-9 IBs or solubilised and encapsulated MMP-9, respectively, were plated in a black 96-well plate with transparent flat-bottom containing assay buffer (5 mM $CaCl_2$, 50 mM Tris pH 7.6, 150 mM NaCl, 0.01 % Tween20). Immediately before reading the plate, 0.25 µg of DQ-gelatine were added to the wells and the plate was bottom-read at a 495/515 excitation-emission wavelength in a Victor III multilabel counter (Perkin-Elmer) with repeated reads every 2.3 min along 1 h. For each kinetic, the specific activity was obtained through the initial velocity calculation (rfu/min) corrected by the MMP-9 µg in the respective well (rfu/min/µg), and the % of specific activity was obtained for each sampling time using the time 0 h in PBS as the reference.

In vivo comparison of the inflammatory response to solubilized MMP-9 versus MMP-9 IBs using a mouse model

Air pouches were provoked on the back of sixty-nine MMP-9 KO mice[16] by injecting 3 ml filtered air intra-dermally on days 0 and 3, as detailed in Vandooren *et al.*[17]. Forty µg of solubilized MMP-9 or MMP-9 IBs were injected into the pouches at day 6 in 200 µl DPBS, and air-pouch exudates were collected after 3, 24 and 48 h of the protein injections. Mice were euthanized with 40 mg/Kg Dolethal (pentobarbital) intraperitoneal, and the air-pouch exudates were obtained by injecting, massaging and recovering 2 ml PBS with heparin 20 U/ml, and repeating the process without taking out the needle with 3 more ml. Exudates were cooled on ice and viable cells were counted with a Neubauer chamber. About 50 to 80 x 10^3 cells per sample were centrifuged on microscope slides using a Shandon Cytospin 2 centrifuge (Thermo Shandon, Pittsburgh, PA, USA) at 75 x *g* for 10 min and preparations were fixed and stained with haematoxylin-eosin and analysed under a light microscope for immune cell populations.

Statistical analysis

For the stability assay, samples were incubated per duplicate. For the *in vivo* assay, 12 exudates were analysed after 3 h of protein injections, 24 after 24 h and 24 after 48 h. Variables were transformed to normalise data when necessary, and data were analysed using fixed effect models (JMP). For the stability assay, format or incubation time were used as fixed effects when appropriate. For the *in vivo* assay, treatment, exudate collecting time and their interaction were

used as fixed effects. Animals were not considered a random effect as no repeated measures were performed. Non-transformed means are represented in all graphs. Non-transformed SEM are represented in the stability assay graphs while transformed SEM are represented in the *in vivo* assay graphs. *P*-values and letters correspond to the Tukey test analyses using transformed data when required.

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Study 4

Protein-based nanomaterials: exploring their potential and inflammatory response *in vivo* as a new therapeutic format

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Preface

As advanced in the previous work (study 3), the stability shown by MMP-9 IBs suggested a remarkable potential for *in vivo* applications despite of the low initial activity of this format compared to the soluble counterpart. This encouraged us to deeply study the unspecific effect of the IB format when a protein related to inflammation (such as MMP-9) is embedded in the nanoparticle. Despite the enormous volume of research describing and tailoring IBs, and the intense explorations for their suitability in many relevant fields such as tissue engineering and tumor therapy, the immunological activity triggered *in vivo* due to the IBs intrinsic structure and composition has never been addressed before. Therefore, with the aim to elucidate which is the real potential of MMP-9 IBs in the activation of inflammatory responses *in vivo*, in this study we generated an inactive form of MMP-9 IBs. We injected soluble MMP-9, active MMP-9 IBs, and inactive MMP-9 IBs in dermal air-pouches of MMP-9 knock-out mice and measured inflammatory signs along different time points. Interestingly, the results in this work evidenced for the first time that IBs did trigger an initial format-related immune response *in vivo*, although this was transitory. Importantly, beyond 24 h, the unspecific effects triggered by inactive MMP-9 IBs disappeared, while those associated to the active MMP-9 IBs remained for longer time points. This outcome suggested that MMP-9 IBs had a relevant specific effect activating an inflammatory response in this model, strongly encouraging us to further transfer these results to the bovine dry period context.

PROTEIN-BASED NANOMATERIALS: EXPLORING THEIR POTENTIAL AND INFLAMMATORY RESPONSE *IN VIVO* AS A NEW THERAPEUTIC FORMAT

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Abstract

Bacterial inclusion bodies (IBs) are protein nanomaterials of few hundred nanometers formed during recombinant protein production processes in different bacterial hosts. IBs contain active protein in a mechanically stable nanostructured format that has been broadly characterized, showing a promising potential in different fields such as tissue engineering, protein replacement therapies, cancer, and biotechnology. However, the potential and the inflammatory response induced by these functional biomaterials formed by a therapeutic and immunomodulatory protein form is still an uncovered gap. For that, both active and inactive forms of the catalytic domain of a matrix metalloproteinase-9 (MMP-9) are produced as IBs and compared with the soluble form for dermal inflammatory effects in *mmp9* knock-out mice. MMP-9 IBs induce local neutrophil recruitment and increase pro-inflammatory chemokine levels lasting for at least two days and extending the effect of the soluble format. Interestingly, the effects trigger by the catalytically inactive mutant of MMP-9 IBs do not last more than 24 h. Therefore, it may be concluded that IBs are protein-based biomaterials that could be used for the delivery of therapeutic proteins such as immunomodulating proteins while preserving their stability in the

specific tissue without triggering important unspecific inflammatory response due to the protein format (IBs).

Keywords: inclusion bodies, matrix metalloproteinase-9, *mmp9* knock-out mice, functional nanomaterials, immunogenicity

Introduction

Recombinant proteins are used for a plethora of therapeutic applications, including cancer therapy, treatment of metabolic disorders, hormone substitution, infectious diseases, thrombolysis, and reproductive disorders, among others[1-3]. During recombinant protein production, bacterial host cells split the overproduced heterologous proteins in soluble and insoluble fractions. The insoluble fraction is also known as inclusion bodies (IBs), which are biophysically described as protein biomaterials with sizes ranging from 50 to 800 nm, and are easily formed during recombinant protein production[4,5]. During many years IBs have been considered as a waste product[6]. However, it has been gradually appreciated that IBs may be a promising alternative protein format *per se*[4,7]. Their structure and composition have been extensively characterized evidencing that proteins forming this rough nanomaterial[8] are biologically active[4-6]. It has also been documented that IBs are mechanically stable biomaterials with slow protein-release properties, easily produced and isolated through economically affordable processes. As a result of IB intrinsic properties, lacking in the soluble format, the applicability of IBs in biotechnology, material sciences, and medical purposes has been explored by different groups[4,7]. The nature of IBs, which combines biological activity and rough surfaces[8,9], has also allowed to successfully apply these as functional biomaterials for tissue engineering purposes[10,11]. Another explored application is the injection of targeted-IBs for cancer therapy, proving that these nanomaterials are a stable source for releasing functional proteins[12-14]. Besides, it has been shown that IBs can perform an active role as adjuvants for vaccination purposes[15,16], but the real influence of nanocluster format on inflammatory effects of IBs and their influence on immune-related therapies has never been assessed. Therefore, the exploration of this protein biomaterial as a new promising therapeutic format has still some uncovered gaps. With the aim to determine the potential of IBs to deliver an immune-related therapeutic protein *in vivo* and elucidate the possible side-effects of the format on the local inflammatory response, Matrix Metalloproteinase-9 (MMP-9) was used as a model protein. MMP-9 is an enzyme that has a relevant role in many biological processes such as wound healing, angiogenesis, reproduction, growth, and tissue development[17], and could be a very relevant drug in the context of several

diseases. It is mainly secreted by endothelial cells and neutrophils, and it is involved in the degradation and remodelling of the extracellular matrix and in chemotaxis[18]. Considering the relevance of MMP-9, we have used a catalytically active fragment of bovine MMP-9 and an inactive mutant of this enzyme produced in *Lactococcus lactis* as IBs[9,19] and tested them in a mouse model of skin inflammation. This study demonstrates that the IB format has only a limited inflammatory effect without interference with the specific activity of the model molecule embedded in the protein biomaterial.

Results and Discussion

On the basis of structural and functional data, we have produced both active (MMP-9) and inactive (or mutant -mutMMP-9-) forms of MMP-9 IBs which have almost identical structural feature[20,21] with the aim to evaluate the potential of IBs as a therapeutic and immunomodulatory protein biomaterial MutMMP-9 has a substitution of the essential glutamic acid in the Zn^{2+} binding domain for glutamine (E402Q) (**Figure 1a**). Soluble MMP-9 and IBs of MMP-9 and mutMMP-9 were produced in *Lactococcus lactis* and purified as detailed in Figure S1[9,19]. The biophysical properties of the IBs format are illustrated in Figure 1b: these consisted of electron dense nanoparticles that clustered in the host cells. The three MMP-9 recombinant forms (soluble MMP-9, MMP-9 IBs and mutMMP-9 IBs) were compared for catalysis. As expected, soluble MMP-9 presented higher gelatinolytic activity than MMP-9 in IBs and the mutMMP-9 IBs did not show activity (Figure 1c). These results were corroborated by gelatin zymography analysis (Figure 1d).

The air-pouch model in mice provides a tool to determine how a foreign substance or formulation thereof influences inflammatory reactions *in vivo*[22]. Thus, purified IBs of MMP-9 and mutMMP-9 were administered into air pouches of the mice model of skin inflammation[23] and soluble MMP-9 was used as positive control. We used *mmp9* knock-out mice for this application, in order to avoid artefacts caused by endogenous mouse MMP-9. Moreover, the fragment of MMP-9 produced lacks the hemopexin domain where Tissue inhibitors of metalloproteinases (TIMPs) bind. After injections with buffer DPBS, soluble MMP-9, MMP-9 IBs or mutMMP-9 IBs into the mice air-pouches, exudates were recovered at 3, 24 and 48 h to analyse the recruitment of myeloid cells. At 3 h post-injections, no significant differences in total cell counts were observed for any of the MMP-9 treatments (**Figure 2a**). However, cell recruitment at 24 h was significantly higher in the animals injected with IBs, while soluble MMP-9 treatment did not differ from the control (Figure 2a). Interestingly, after 48 h, the cells recruited in the air-pouches remained high

in mice injected with MMP-9 IBs while in mice treated with mutMMP-9 IBs total cell count values returned to basal levels (Figure 2a).

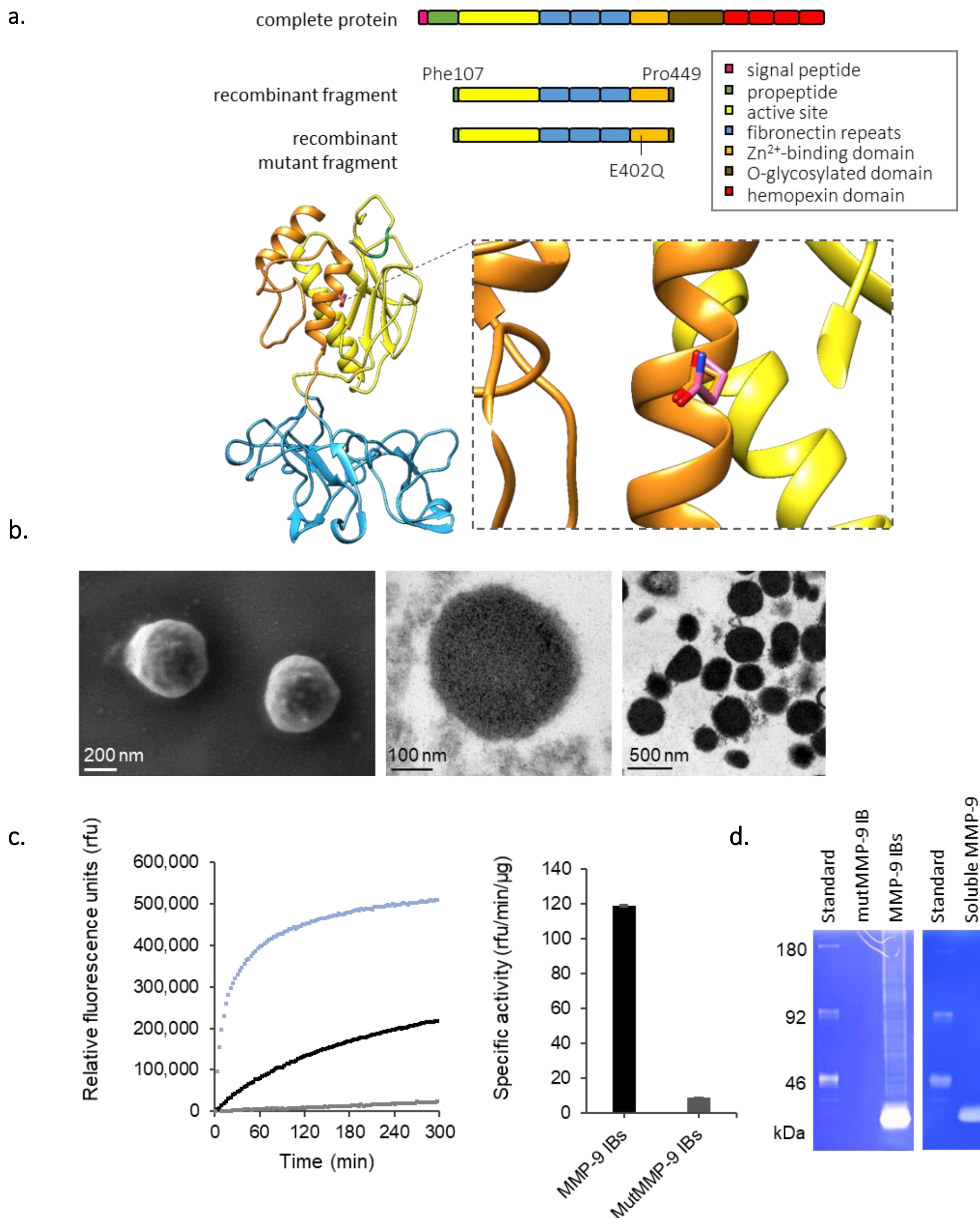

Figure 1. a. Recombinant bovine MMP-9. On top, domain structure of the complete bovine MMP-9 compared with the recombinantly expressed protein fragment (Phe107 – Pro449) and its proteolytic inactive mutant[20]. At the bottom, 3D model for P52176 (Phe107 to Gly444) with the mutated residue E402Q superposed (WT: Glutamic (E) colored in orange, mutant: Glutamine (Q) in purple). Left panel: full view of the generated models. Right panel: detail of the region with the mutated residue that impairs functionality in the mutMMP-9. **b.** SEM (left) and TEM (middle and right) images of the MMP-9 IBs obtained from *L. lactis*. **c.** Degradation kinetics for MMP-9 IBs (black), MutMMP-9 IBs (grey) and the soluble MMP-9 (blue) (left) and the corresponding specific activity (SA) for the IBs (right). The soluble MMP-9 SA was 2,282.8 ± 21.9 rfu/min/µg. Error bars indicate standard errors (n = 3). **d.** Zymography analysis showing that MMP-9 degrades gelatin in an electrophoresis gel.

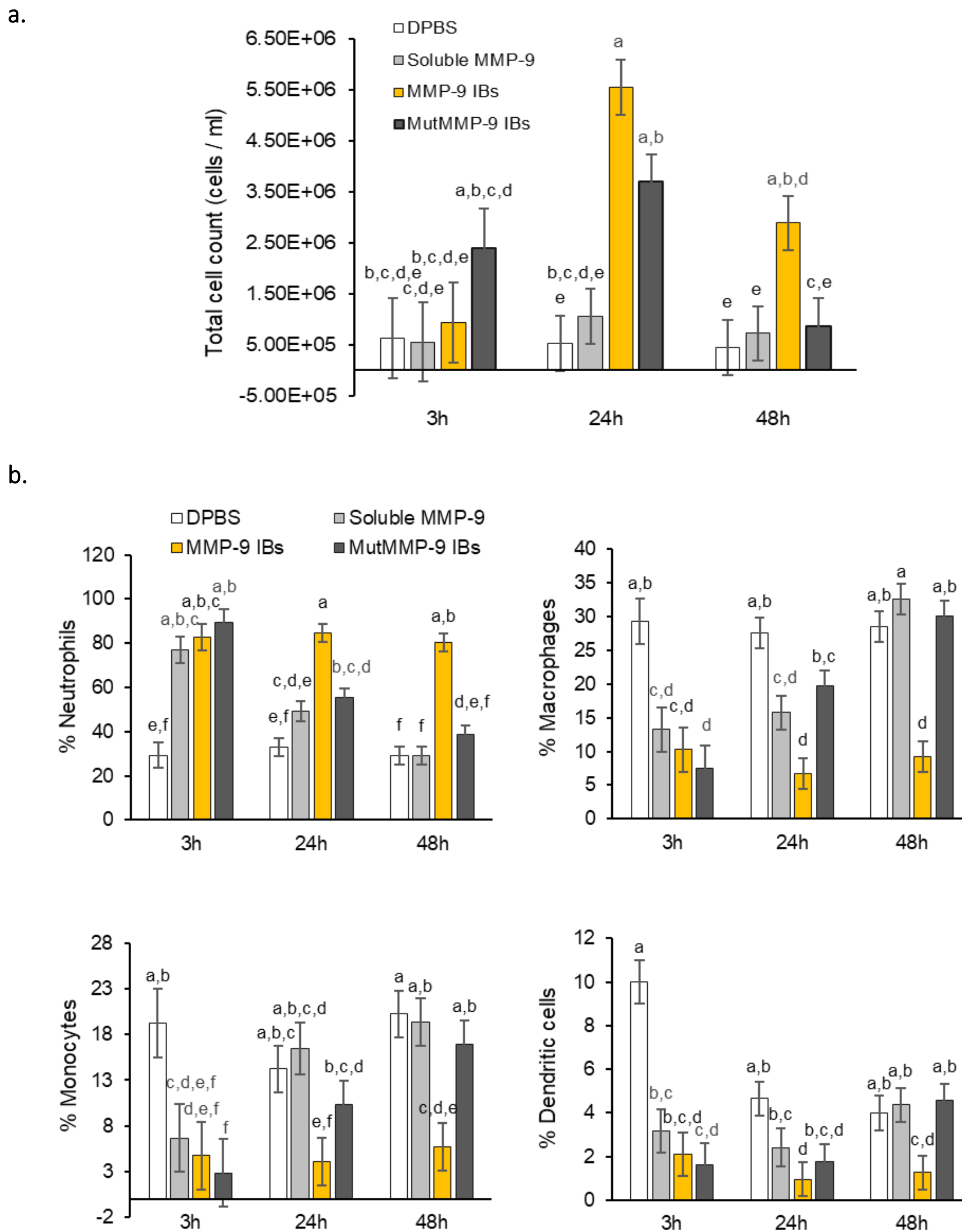

Figure 2. a. Total cell counts for the recovered exudates from the air pouches at 3, 24 and 48 h after injections with the four different treatments. Means and SEM (error bars) from non-transformed data are represented. Different letters depict significant differences ($p < 0.005$). **b.** Relative quantification of the different immune cell populations analyzed by flow cytometry. Means and SEM (error bars) from non-transformed data are represented. Different letters depict significant differences ($p < 0.0001$).

To determine the differences in recruited inflammatory cell, we quantified different cell populations (neutrophils, macrophages, monocytes, and dendritic cells) by flow cytometry (Figure 2b). At 3 h post-injections all MMP-9-based treatments showed a significant increase on

neutrophil percentages in agreement with they being the first cell type arriving after a challenge or tissue damage[24] (Figure 2b). This indicated a fast effect of MMP-9 in the soluble format and an inflammatory effect of IB nanocluster format. At that time point we cannot claim that MMP-9 activity in IBs is essential, since no significant differences were observed between MMP-9 IBs and mutMMP-9 IBs (Figure 2b). However, at longer times (24 and 48 h), only the animals injected with IBs kept neutrophil levels above those injected with DPBS buffer. Both MMP-9 IBs and mutMMP-9 IBs injections increased neutrophil recruitment after 24 h while after 48 h the granulocyte influx was only observed in the MMP-9 IBs treated pouches (Figure 2b). Macrophages, monocytes, and dendritic cells evolved differently from neutrophils, but all three cell populations came with similar profiles (Figure 2b). At 3 h after injections, and for all MMP-9 treatments, a decrease in macrophages, monocytes, and dendritic cells was observed compared to DPBS treatment. Interestingly, no differences were observed for mutMMP-9 IBs treated animals at 24 h and 48 h (Figure 2b). By contrast, exudates from animals treated with active MMP-9 IBs showed a decrease not only in macrophages, but also in monocytes and dendritic cells (Figure 2b) after all the time intervals.

In order to better evaluate whether the cell recruitment was a direct effect of the protein biomaterial or an indirect effect through induction of host-derived chemokines for the detected leukocytes, we studied major neutrophil and mononuclear cell chemokines. Supernatants from air-pouch exudates were analysed to determine levels of CXCL1 and CXCL2 (chemokines that recruit neutrophils), CCL2 (chemokines for monocytes and dendritic cells) and CCL3 (chemokines for macrophages) (**Figure 3**). At 3 h after injections, both MMP-9 IBs and mutMMP-9 IBs treatments significantly increased CXCL1 levels in comparison with the control and the soluble MMP-9 treated exudates (Figure 3a). In addition, mice treated with MMP-9 IBs showed a 2-fold increase in CXCL1 levels in the air pouch exudates in comparison with those of mice treated with mutMMP-9 IBs (Figure 2a), matching with the neutrophil-influx after 24 h (Figure 2b). After 24 and 48 h no differences were observed for CXCL1 in any of the conditions studied. MMP-9 IBs and the mutMMP-9 IBs had an impact on the levels of CXCL2 at 3 h (Figure 3b), whereas at later time points (24 and 48 h) only MMP-9 IBs treatment increased CXCL2 levels above the negative control and soluble MMP-9 (Figure 3b) corroborating the different behaviour of MMP-9 IBs compared to mutMMP-9 IBs (Figure 3b). Importantly, 24 h after the injection of soluble MMP-9 total cell counts of neutrophils, monocytes and dendritic cells returned to the levels of DPBS treated mice, whereas in the mice treated with MMP-9 IBs the modulation of the cell populations remained different, even after 48 h. This indicates that the soluble MMP-9 format is probably more labile than the MMP-9-based biomaterial. CCL2 profiles showed only increases at 3 h for

both MMP-9 IB- and mutMMP-9 IB-based treatments, which decreased after 24 h (Figure 3c). Similarly, CCL3 levels increased in pouches treated with MMP-9 IBs and mutMMP-9 IBs at 3 h in comparison with control and soluble MMP-9 (Figure 3d). After 24 h of injections, only the MMP-9 IBs maintained significantly high levels of CCL3 compared to DPBS, while the soluble MMP-9 treatment tended to be greater than DPBS (Figure 3d).

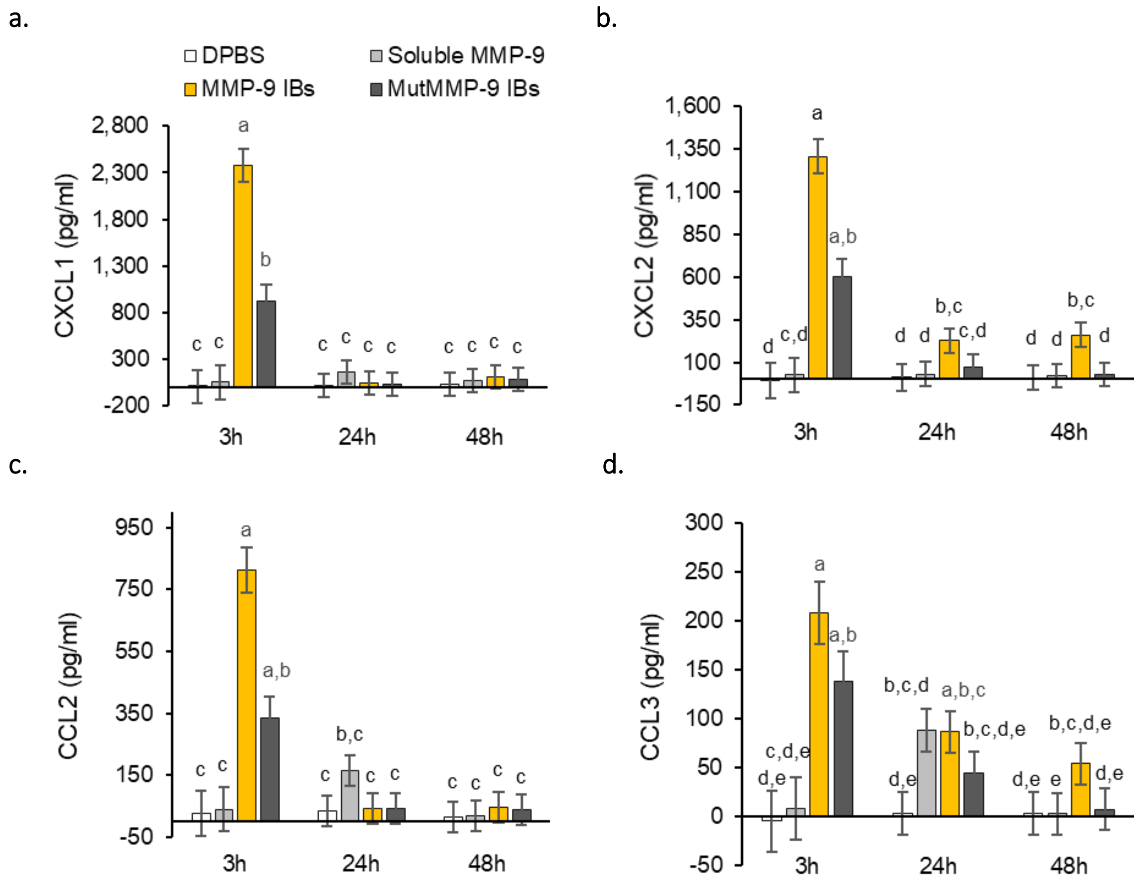

Figure 3. Pro-inflammatory chemokine concentrations in the recovered supernatants of the air pouches. **a.** CXCL1. **b.** CXCL2. **c.** CCL2. **d.** CCL3. Means and SEM (error bars) from non-transformed data are represented. Different letters depict significant differences ($p < 0.0001$ in a, b and c, and $p < 0.005$ in d).

There was a strong correlation between the chemokines at 3 h (Figure 3) and the neutrophil recruitment in the MMP-9 IBs and mutMMP-9 IBs treated air-pouches at 24 h (Figure 2b), that further reinforced an effect of active MMP-9 in the format of IB.

These results are relevant because we elucidate that the recombinant MMP-9 produced in *L. lactis* in the format of IB[9,19] triggers an inflammatory response due to its degradative capability and not because of the inflammatory response to the biomaterial itself. Thus, while the response triggered by the soluble MMP-9 disappears after 24 h, by injection of a similar amount of MMP-9 in the IB format, the MMP-9 remains active and its effects extend for more than 48 h. These

results indicated that IBs format provided stability to active MMP-9, protecting against degradation and therefore the practical use of this format should be considered. Moreover, this study demonstrates that, although the nature of the IB format, tested using inactive mutant MMP-9 IBs, transiently stimulates a local inflammatory response, it only remained for 24 h whereas active MMP-9 IB effect was still detected after 48 h.

Conclusion

Thus, we report for the first time that the IB format has only a limited inflammatory effect without interference with the specific activity of the model molecule in the protein biomaterial. In addition, we have shown that the recombinant protein embedded in IBs results in more sustained *in vivo* effects compared to the soluble (and standard) form. Although more examples will be needed, our work exemplifies the potential of this protein-based biomaterial as a new and promising delivery tool.

Experimental section

MMP-9 IBs production, purification and characterization

Bacteria strains and plasmids

Lactococcus lactis subsp. *cremoris* NZ9000 double mutant Em^R ClpP$^-$ HtrA$^-$ provided by INRA (France; patent n. EP1141337B1) was used in this study. A bovine MMP-9 fragment from Phe107 to Pro449 (NCBI, NM_174744.2), which comprises the catalytic, the fibronectin and the zinc-binding domains, was cloned into the plasmid Cm^R pNZ8148 (MoBiTech) and transformed into competent *L. lactis* ClpP$^-$HtrA$^-$ as described in Cano-Garrido *et al.*[9]. A similar MMP-9 cDNA fragment with a single amino acid substitution (E402Q[20,21]), which encodes a proteolytic inactive form of MMP-9 (named mutMMP-9 from now on), was cloned into the pNZ8148 plasmid and transformed into competent *L. lactis* ClpP$^-$HtrA$^-$. Both genes were C-terminally fused to a His-tag and were codon-optimized for *L. lactis* (Geneart)[9]. A scheme of recombinant MMP-9 structure and functionality is provided in Figure 1.

MMP-9 modelling

Protein structure homology models for *Bos taurus* MMP-9 selected region (from F107 to G444) were built with UCSF Modeller 9v13[25] for wild type (WT) (Uniprot code P52176) and mutant E402Q proteoforms, using the Crystal structure of human MMP-9 (PDB code 1L6J, chain A[26]

residues from G105 to G444) as template. Models were selected according to the best DOPE score[27]. Structure superposition was carried out with ProFit[28], using only the main chain atoms (N,CA,C,O).

MMP-9 and mutMMP-9 IBs production in L. lactis

Bacteria were grown without shaking at 30 ºC in M17 broth supplemented with 0.5 % glucose. Antibiotics were added for plasmid maintenance (5 µg/ml chloramphenicol and 2.5 µg/ml erythromycin). Re-inoculated cultures were induced to produce MMP-9 or mutMMP-9 with 12.5 ng/ml nisin at the OD_{600} of 0.4 - 0.6, and productions were performed along 3h. Cultures were recovered and centrifuged at 6,000 x *g* for 30 min at 4º C.

MMP-9 and mutMMP-9 IBs purification

After recombinant protein expression and recovery of the bacteria each 50 ml pellet was suspended in 30 ml sterile PBS and frozen O/N at -80 ºC. The following procedure aimed at the disruption of all the bacteria by several freeze-thaw cycles (F/T) and mechanical and chemical procedures. Briefly, thawed bacteria were subjected to 3 rounds of French Press (Thermo FA-078A) at 1,500 psi in the presence of protease inhibitors (Complete EDTA-free, Sigma). After a new F/T, samples were incubated for 2 h with 0.01 mg/ml lysozyme at 37 ºC and rotary shaking at 250 rpm. After another F/T, the lysate was incubated for 1 h with 4 µg/ml Triton X-100 at RT in an orbital rotator shaker. An aliquot of the lysate was plated on agar-M17 broth with 0.5 % glucose and incubated O/N at 30 ºC as a first sterility control. Additional F/T cycles were performed until no bacterial colonies were grown on the plates. Next, the lysates were incubated with 0.25 µl of NP-40 per ml of sample (0.25 µl/ml) for 1 h at 4 ºC in the orbital shaker, followed by 1 h with 0.6 µl/ml of $MgSO_4$ (stock 1 M) and 0.6 µl/ml of DNAse I (stock 1 mg/ml) at 37 ºC and 250 rpm. Lysates were centrifuged at 6,000 x *g* for 30 min at 4 ºC. Each pellet was suspended in 5 ml lysis buffer with 0.5 % Triton X-100 and F/T. Samples were centrifuged again and, under sterile conditions, the supernatant was discarded and pellets were suspended in 5 ml sterile GIBCO® Dulbecco's Phosphate-Buffered Saline (DPBS). The resulting lysate was aliquoted and centrifuged at 20,000 x *g* for 15 min, and the protein pellets were frozen at -80 ºC. Some aliquots were diluted in eukaryotic culture media, transferred into the wells of a 96-well plate, and incubated for 48 h at 37 ºC and 5 % CO_2 as a final sterility control.

Purification of soluble MMP-9

The soluble MMP-9 fraction and IBs were obtained as described[19] Briefly, cultures after 3 h of production were recovered and centrifuged at 6,000 x *g* for 15 min at 4 ºC. Each pellet from 500

ml cultures was suspended in 30 ml PBS with protease inhibitors and subjected to 4 rounds of French Press at 1,500 psi. Thereafter, lysates were incubated with 0.05 mg/ml lysozyme for 2 h at 37 ºC and 250 rpm. Pellets were obtained by the centrifugation of the lysates at 15,000 x *g* for 45 min at 4 ºC, and were washed in mQ-H_2O. After 15 min centrifugation, pellets were weighted and re-suspended in solubilization buffer (40 ml/g; 40 mM Tris pH=8 with 0.2 % N-Lauroyl sarcosine). The solution was covered and incubated on a magnetic stirrer O/N at 4 ºC. The obtained solution was centrifuged for 20 min at 20,000 x *g* and at 4 ºC and the supernatant was collected, filtered and further purified through Immobilized Metal Affinity Chromatography (IMAC). One ml HiTrap Chelating columns (GE Healthcare) were used in an ÄKTA purifier FPLC system (GE Healthcare), and both the binding and the elution buffer contained 0.2 % N-Lauroyl sarcosine. The MMP-9 was eluted by gradually increasing the imidazole concentration until 500 mM, and holding the gradient at each peak to allow a peak separation (data not shown). The eluted peaks were dialyzed O/N to PBS at 4 ºC with gentle agitation and centrifuged at 15,000 x *g* for 15 min at 4 ºC to remove possible precipitated protein. The supernatants were filtered under sterile conditions, aliquoted and stored at -80 ºC until use.

Protein determination by Western Blot and Coomassie blue staining analysis

The MMP-9 IBs and mutMMP-9 IBs were quantified by Western blot (ImageJ) analysis with the use of a soluble MMP-9 standard. The soluble MMP-9 peaks were quantified by Nanodrop (Thermo Scientific) indicating the MMP-9 parameters (MW: 39 kDa and ε: 70,080 $M^{-1}cm^{-1}$; ProtParam-ExPASy). The purity of MMP-9 IBs, mutMMP-9 IBs, and the soluble MMP-9 was analysed by Coomassie blue staining.

MMP-9 activity determination in vitro

DQgelatin™ assay: 10 μg MMP-9 IBs, mutMMP-9 IBs or soluble MMP-9 were plated in a transparent flat-bottom black 96-well plate in triplicate, at a final volume of 150 μl in assay buffer (5 mM $CaCl_2$, 50 mM Tris pH 7.6, 150 mM NaCl, 0.01 % Tween20). Immediately after adding 0.25 μg of dye-quenched gelatin (DQgelatin™, Invitrogen) per well, the plate was bottom-read every three minutes, overnight, in a fluorescence microplate reader (Victor III multilabel counter, Perkin-Elmer) at 495/515 nm (excitation/emission wavelengths). The specific activity of MMP-9 was extracted for each sample from the kinetic data, by obtaining the initial velocity (relative fluorescence units per minute, rfu/min) for each μg of MMP-9 in the wells (rfu/min/μg).

Gelatin zymography analysis: 7 μg MMP-9 IBs, 7 μg mutMMP-9 IBs and 0.6 ng soluble MMP-9 were loaded in non-denaturing conditions in 10 % SDS-PAGE gels with 1% gelatin. A standard mix

of MMP-9 was loaded as ladder, containing a multimeric (approximately 180 kDa), a monomeric (92 kDa) and a truncated (46 kDa) form of MMP-9[29,30]. After electrophoresis, gels were washed and incubated overnight with developing buffer at 37 ºC, and were further stained with Coomassie blue in 20 % acetic acid, followed by distaining incubations with strong and soft methanol-acetic acid solutions[30].

MMP-9 injection in mouse air-pouches

A total of sixty MMP-9 KO mice[23], males and females, were injected intra-dermally on the back with 3 ml of filtered air (0.2 µm filter) on days 0 and 3 to establish the air-pouch compartment, following the standardizations described in the work of Vandooren *et al.*[22]. Protein samples or 200 µl DPBS were injected at day 6. Protein injections consisted in 40 µg of MMP-9 (soluble or IBs) or mutMMP-9 (IBs) in 200 µl DPBS per injection. The soluble MMP-9 corresponded to the most active fraction (by DQgelatin™ assay) among the different peaks obtained in the chromatography profile (data not shown). At time intervals of 3, 24 and 48 h after injections, the mice were euthanized with an intraperitoneal injection of 40 mg/Kg Dolethal (pentobarbital) solution. The exudates in the air-pouches were collected by injecting 2 ml PBS with heparin 20 U/ml followed by a gentle massage on the air-pouch and the recovering of all the content. Without taking off the needle, the process was repeated with 3 ml additional PBS and the total volume was cooled on ice.

Exudate recovery and analysis

Exudate cell count analysis

The collected exudate volumes were centrifuged at 300 x *g* for 5 min in a swing-out cytocentrifuge and the supernatants were preserved at -80 ºC for cytokine analysis. The cell pellets were washed in 1 ml ACK buffer (Gibco, Life Technologies) to lyse erythrocytes. After centrifugation, the cells were resuspended in PBS and were counted in a Neubauer chamber after trypan blue staining to exclude dead cells.

Flow cytometry analysis

Immediately after viable cell counting, we incubated 5 to 10 x 10^5 cells in Fc-receptor-blocking antibodies and ZombieAqua-BV510 at RT for 20 min. A master-mix was prepared with the selected antibodies for the characterization of different cell populations: CD11b-APC (monocytes neutrophils, eosinophils, and dendritic cells), CD11c-BV711 (dendritic cells), Ly6C-FITC (monocytes, macrophages and neutrophils) and Ly6G-PE (neutrophils and eosinophils). All the antibodies were purchased from eBioscience. Cells were incubated with 10 µl of the master-mix

for 30 - 45 min at 4 ºC and protected from light and washed twice in PBS. Cells were fixed in 0.37 % formaldehyde and acquired in a fluorescence-activated cell sorting (FACS) Fortessa (BD Biosciences) flow cytometer. Data was analysed with the BD FACSDiva™ software.

After compensating the signals for each fluorochrome, live cells excluding doublets were selected. Briefly, cell debris were excluded gating FSC-$A^{mid\text{-}pos}$ SSC-$A^{mid\text{-}pos}$ quadrant in a dot plot. From this population, singlets were selected drawing a diagonal gate in a FSC-A vs. FSC-H plot, and excluding outlier events (doublet cells). Afterwards, from the last gate obtained, live cells were gated in a new plot selecting the ZombieAqua-BV510-A^{neg} cell population. From these cells, the CD11b-APC-A^{pos} population plotted vs. Ly6C-FITC-A was gated, and this gate was defined as monocytes, macrophages and neutrophils together (MMN, from now on). Also from live cells gate, neutrophils were selected gating the Ly6G-PE-A^{pos} CD11b-APC-A^{pos} population, and dendritic cells were selected gating the CD11c-BV711-A^{pos} CD11b-APC-A^{neg} population. Macrophages were selected as the Ly6C-FITC-A^{pos} Ly6G-PE-A^{neg} population. Finally, monocytes were defined as the difference between MMN population and the gates in isolated plots for neutrophils and macrophages. All gates were quantified as a percentage of events from total live cells.

Chemokine ELISA analysis

The presence of chemokines in the exudate supernatants from the air-pouch assay were analysed by ELISA (DuoSet ELISA, from R&D Systems). The chemokines studied correspond grossly to the recruitment of neutrophils (CXCL1/Gro-α/KC and CXCL2/Gro-β/MIP-2α), monocytes and dendritic cells (CCL2/MCP-1) and macrophages (CCL3/MIP-1α). The protocol was in accordance with the guidelines provided by the manufacturer, and all washing steps were done twice in PBS containing 0.05 % Tween20. Briefly, flat-bottom 96-well plates were coated with the pertinent capture antibody O/N at 4 ºC and washed. Plates were blocked with 0.5 % BSA containing 0.05 % Tween20 for 1 h at RT and washed again. Diluted samples were plated in duplicates and incubated O/N at 4 ºC and plates were washed. Plates were incubated for 2 h at RT with the provided biotinylated detection antibodies. After washing, streptavidin-HRP detection solution was added and incubated for 20 min at 37 ºC protected from light. Plates were washed and incubated for 20 min with substrate solution at RT and protected from light, and the reaction was stopped with stop solution provided in the kit. Reads were done in a plate reader at 450 nm and blank was subtracted to all wells. A sigmoidal standard curve equation and its R^2 were obtained using the commercial standards provided in each kit.

Statistical analyses

The animal experiments were executed three times with three mice per condition. Because the experimental variability was limited, the data of 3 to 6 animals per condition were pooled. Statistics were carried out with the total of sixty mice. Twelve exudates were obtained after 3 h of protein injection, 24 exudates were collected after 24 h, and 24 exudates after 48h. Variables were transformed to normalize data when necessary. Data were analysed using a fixed effect model (JMP). The model included the fixed effects of treatment and time, as well as their interaction, the experiments and the laboratories where the cytometry data was analysed. Animals were not considered a random effect as no repeated measures were performed. Means and standard deviations represented in graphs correspond to non-transformed data, while p-values and letters correspond to the Tukey test analyses using transformed data when required.

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Study 5

Elucidating the real potential of MMP-9-based nanoparticles at cow dry-off: pulling apart the MMP-9 and nanoparticle effects

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(In preparation)

Preface

Throughout the previous studies in this thesis, IBs have demonstrated to be a nanomaterial with a high stability in hostile conditions both *in vitro* (study 3) and *in vivo* (study 4), and with a high potentiality to be used as an immunostimulating agent (study 4). We also described, in mice, that unspecific effects due to the aggregated format were immediate and transitory, not lasting more than 24 h (study 4).

Early dry-off in dairy cows is a critical period in which these animals are under-protected by the immune system, being this affected by galactopoietic hormones such as PRL, and overloaded due to high phagocytic requirements to reabsorb milk and apoptotic cells in the MG. In a previous and recent work in our group, MMP-9 IBs showed a promising capability to immunostimulate MG involution at dry-off with a single infusion. Therefore, and conscious of the transient unspecific effect triggered by IBs in mice, we designed the study that follows, with the aim to elucidate which was the MMP-9 IBs real potential during the dry period: which was the MMP-9-specific contribution in this context? To answer this question, first we tested several doses of MMP-9 IBs to decide a minimal effective dose to perform our study. Secondly, active and inactive MMP-9 IBs were infused in the MG of cows entering at dry-off. Through this study, we confirmed the MMP-9 IBs potential as a local immunostimulatory treatment capable of accelerating the onset of involution biomarkers at dry-off. Also, we suggest a protective role in front to possible IMIs by the recruitment of immune cells and the liberation of defensive peptides in the MG such as lactoferrin. Nonetheless, our results demonstrated that these effects were not due to MMP-9 but due to the unspecific effects related to the intrinsic nature of IBs. Contrasting with our previous results in the mouse model, format-related effects of IBs extended along the 9 days of study in the MG context. Model intrinsic differences, gestational hormonal influence in dairy cows, and the MMP-9 relevance at dry-off are clues that are thoroughly discussed in this study. Thus, this work resolved our question about the nature of MMP-9 IBs effects in the MG at dry-off and opens the door to continue working in new tailoring possibilities for IBs with the look in this and other proteins to be explored.

Elucidating the real potential of MMP-9-based nanoparticles at cow dry-off: pulling apart the MMP-9 and nanoparticle effects

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Abstract

The dry period is crucial for dairy cows, which need a non-milking interval to guarantee an optimal milk production in the subsequent lactation. During this period, the mammary gland tissue involutes and after that the tissue regenerates. However, important bottlenecks are present at dry-off complicating the process resolution. High amounts of milk accumulated in the mammary gland, which may lead to discomfort and milk leakage, facilitate pathogen invasion to the mammary gland. This milk has to be reabsorbed involving a high phagocytosis requirement of leucocytes during mammary involution that, combined with a concomitant pregnancy, leads to a delay in the activation of the immune system, leading to unresolved intramammary infections. During decades, antibiotics have been routinely used as a preventive treatment at dry-off. Nevertheless, the alarming increase of antibiotic resistances opens a new scenario in which alternative strategies have to be developed. Matrix metalloproteinase 9 (MMP-9) is one of the enzymes able to degrade the extracellular matrix, being this degradation a signal involved in apoptosis and involution. Thus, we have based our strategy to optimize the dry period on the intramammary infusion of nanoparticles formed of recombinant MMP-9 which, in previous studies, have shown to induce the recruitment of immune cells. However, the dose effect and the dissection between specific and unspecific effects associated to the administration of this

protein format have not been explored thus far in cattle. The results obtained in this study show that among all the doses tested, which ranged between 12 and 0.012 mg, 1.2 mg was the lowest one able to induce a clear immune stimulation and to increase involution parameters. Moreover, the comparison of the effects triggered by the administration of an active and an inactive form of MMP-9 nanoparticles led to conclude that MMP-9-specific effects were limited. This indicates that the response observed in the bovine mammary gland by MMP-9 nanoparticles was mainly due to the protein format but not to the biological activity of the MMP-9 embedded in these nanoparticles. This is particularly relevant in the context of the mammary gland, which is highly immune-active and responsive, and where future treatments based on recombinant proteins could be suitable for the reduction of antibiotic use and prevention of antimicrobial resistances.

Keywords: Inclusion bodies, mammary gland, dry period, matrix metalloproteinase-9, bovine.

Introduction

The cow dry period is crucial to optimize the milk production in dairy cattle[1]. During this period, the mammary gland regresses and, after that, it proliferates and differentiates to allow optimal milk production in the subsequent lactation. However, the presence of galactopoietic hormones due to a concomitant pregnancy does not facilitate the beginning of the involution and delays the activation of the immune system, which orchestrates all this process[2]. Moreover, the high productivity in dairy cows leads to a dry-off in which high amounts of milk are accumulated in the mammary gland, exerting high intra-mammary pressure, causing discomfort[3], and milk leakage, which in turn maintains the teat canal opened and full of nutrients, increasing the risk of a pathogen invasion[4].

Involution of the mammary gland starts with a complex signaling pathway of cell factors, hormonal changes, and immune stimulation. The activation of the immune system at the beginning of the dry period recruits macrophages and neutrophils which could fight against a possible infection[5]. However, phagocytic activity against pathogens is diminished at dry-off as phagocytes are also focused in the tissue involution and engulfing milk fat, cell debris, and other compounds derived from milk and accumulated in the mammary gland[5]. In fact, it is assumed that the immune system does not reach effective protective levels until 8 d after dry-off in dairy cows[6], and during this first week there is a high risk to contract intramammary infections that will compromise the next lactation. To reduce the risk of mastitis, antibiotics are infused routinely into the mammary gland at dry-off. However, the preventive use of antibiotic has raised concern about the emergence of antibiotic resistances in many countries. In this context, there is need to

find new strategies to boost the immune system of the mammary gland and its involution at dry-off. In fact, several investigations indicate that the intramammary infusion of alternative molecules immunostimulate the mammary gland. Administrations of lipopolysaccharide (LPS)[7,8], concanavalin A[9], and *Panax ginseng* extract[10,11] are examples of infused molecules that successfully activate immune response of the mammary gland in cattle at dry-off. Also, chitosan, which is a natural polysaccharide with immunostimulating properties that forms hydrogels at body temperature[12], has been proven to stimulate the immune system of dairy cows after infusion in the mammary gland, triggering involution and conferring protection to new intramammary infections during dry-off[13].

Recently, new strategies based on the use of matrix metalloproteinase 9 (MMP-9) have been studied mainly to modulate infiltration of immune cells and involution at dry-off. Matrix metalloproteinase-9 is a tissue-remodeling enzyme that degrades the extracellular matrix (ECM) and, in the mammary gland, is physiologically released by mammary epithelial cells and neutrophils entering into the tissue during the involution process[14,15]. It has been previously demonstrated that the proteolytic degradation of the ECM is a key factor during the loss of mammary epithelial cells differentiated state and the induction of apoptosis and involution[16]. Hence, in a previous work we hypothesized that exogenous administration of a recombinant MMP-9 (rMMP-9), which was not sensible to tissue inhibitors of metalloproteinases 1 and 3 (TIMP1 and TIMP3), could be a strategy to accelerate tissue involution at dry-off. The administration of rMMP-9 into the mammary gland at dry-off was tested using two recombinant protein formats: a soluble form and a nanoparticulated format, also known as Inclusion bodies (IBs)[17]. IBs are protein-based nanoparticles of few hundred nanometers formed during recombinant protein production processes[18] in different bacterial hosts[19]. They have been described as nanoclusters of active protein in a mechanically stable format[18], showing a promising potential in different fields such as tissue engineering[20], protein replacement therapies[21], cancer treatments[22,23] and biotechnology[24]. By administering rMMP-9 IBs or nanoparticles at dry-off in dairy cows, an unexpected result was found since rMMP-9 nanoparticles accelerated the local immune response and mammary involution in a significant manner, whereas the soluble rMMP-9 only increased the endogenous MMPs without affecting general parameters of immune stimulation and mammary involution markers[17]. By contrast, another study conducted in rodents using rMMP-9 nanoparticles concluded that this format had only a limited unspecific inflammatory effect. Indeed, the rMMP-9 embedded in these nanoparticles produced a clear recruitment of murine immune cells, while an inactive form of these nanoparticles showed just a short-term effect[25]. These findings encouraged us to perform the present study, aimed to

elucidate whether the observed effects in the cow mammary gland by rMMP-9 nanoparticles were due to a different performance of rMMP-9 embedded in the nanoparticles compared with the soluble form, or due to the effect of the nanoparticle format itself. For this, we have determined *in vivo*, first, the lowest dose of rMMP-9 nanoparticles that boost innate immunity and mammary involution, and second, whether this was due to the nanoparticle format or to the inherent properties of the rMMP-9 comprised in this nanostructured format. Through the comparison of the effects triggered by the administration of an inactive form of rMMP-9 nanoparticles at dry-off with its active counterpart, the real potential of rMMP-9 nanoparticles during the cow dry period was distinguished.

Experimental section

Bacteria strains and plasmids

Lactococcus lactis subsp. *cremoris* NZ9000 double mutant ClpP- HtrA- (*clpP-htrA*; EmR) strain[26,27] (kindly provided by INRA (Jouy-en-Josas, France); patent n. EP1141337B1) was used to recombinantly produce the MMP-9 proteins used in this study. The genes encoding for an active bovine rMMP-9 fragment (Phe107 - Pro449, NCBI, NM_174744.2) and for the same rMMP-9 fragment with the E402Q single mutation[28,29], which makes it an inactive rMMP-9 proteoform, were cloned in pNZ8148 plasmid (CmR) and transformed into competent *L. lactis clpP-htrA* cells. Both genes were fused to a His-tag in the C-terminal and were codon-optimized (Geneart) for *L. lactis*[30].

Protein production in *L. lactis*

Both active and inactive rMMP-9 were produced in *L. lactis*, which was grown under static conditions at 30 ºC in M17 broth supplemented with 0.5 % glucose, 5 µg/ml chloramphenicol and 2.5 µg/ml erythromycin. Cultures were re-inoculated to an initial OD_{600nm} of 0.05 and protein expression was induced with 12.5 ng/ml nisin when the OD_{600nm} reached values between 0.4 and 0.6. The recombinant proteins were produced along 3 h and bacteria were recovered by centrifugation at 6,000 *x g* for 30 min at 4 ºC and stored at -80 ºC until use.

Purification of IBs

Pellets from 50 ml culture were suspended in 30 ml PBS, frozen/thawed at -80 ºC and disrupted with 3 French press (Thermo FA-078A) rounds at 1,500 psi, ice-coated, and with protease inhibitors (cOmplete protease inhibitor cocktail EDTA-free, Roche). After an additional freeze/thaw, suspensions were incubated with 0.01 mg/ml lysozyme for 2 h at 37 ºC and 250

rpm, and were frozen/thawed once again. After 1 h of incubation with 4 µg/ml Triton X-100 at room temperature (RT) at 250 rpm, samples were tested for sterility by plating an aliquot in agar-M17 plates with 0.5 % glucose and incubating them O/N at 30 ºC. Samples were frozen/thawed until no colonies were grown. Sterile suspensions were incubated with 0.25 µg/ml NP-40 for 1 h at 4 ºC and 250 rpm, and with 0.6 µg/ml $MgSO_4$ and 0.6 µg/ml DNAse I for 1 h at 37 ºC and 250 rpm. Then, samples were centrifuged at 6,000 *x g* for 30 min at 4 ºC. Pellets were suspended in lysis buffer with 0.5 % Triton X-100, at a ratio of 5 ml buffer for each 50 ml initial culture, freeze/thawed again, centrifuged, suspended in 5 ml sterile DPBS, and tested again for sterility. Samples were centrifuged at 15,000 *x g* 15 min 4 ºC and pellets were stored at -80 ºC until use. Aliquots of rMMP-9 IBs and mutant inactive rMMP-9 IBs were tested for purity through SDS-PAGE electrophoresis and Coomassie blue staining, and quantified using ImageJ software by using a solubilized rMMP-9 as the standard[31].

Protein infusions in cow mammary glands

Three experiments were performed (E1, E2 and E3); two experiments aimed to optimize the minimum effective protein dose to trigger an immune reaction in the mammary gland at dry off (E1 and E2), and the third aimed to dissect the effects triggered either by the protein activity or by the protein format (E3).

Udder quarter was used as an independent experimental unit, being 46, 44 and 30 quarters used for E1, E2 and E3 experiment, respectively (two animals were discarded due to mastitis / abortion, one for each E1 and E2). After the last milking before dry-off and just before protein infusions, 10 ml of mammary gland secretions (MGSs) were collected from all quarters as a day 0 sampling.

In the first experiment (E1), a range of different rMMP-9 IBs doses were infused into 10 quarters per treatment through the teat canal using sterile blunt tip cannulas immediately after day 0 MGSs collection. Namely, 1.2, 3, 6, and 12 mg of rMMP-9 IBs suspended in 10 ml saline solution were infused, and 10 ml saline solution infusions worked as negative controls. Following infusions, all quarters were treated with broad-spectrum antibiotics (Mamyzin secado®, Boehringer Ingelheim) following common production practices. At days 1, 3, 6, and 9 after protein infusions, 10 ml of MGSs were collected from each mammary gland. After the last sampling, all glands were sealed with teat sealant. All MGSs were analyzed in fresh for SCC (n = 9 observations), and aliquots were stored at -80 ºC until these were analyzed for Bovine Serum Albumin (BSA; n = 9), lactoferrin (LF; n = 8) and Sodium/Potassium (Na^+/K^+; n = 9) levels.

The second experiment (E2) aimed to test lower doses of rMMP-9 IBs. After collecting MGSs at day 0, 0.012, 0.12 and 12 mg of MMP-9 IBs and 10 ml of saline solution were infused into 11

quarters per treatment, and all of them were treated with antibiotics as described for E1. At days 1, 2, 3, 6, and 7 after infusions, MGSs were obtained from each quarter and after the last collection teat sealant was applied. These secretions were analyzed in fresh for SCC (n = 11) and BSA was analyzed from aliquots after preservation at -80 ºC (n = 11).

In the third experiment (E3), the minimum effective dose decided from E1 and E2 was used to compare the active and the inactive rMMP-9 IBs effects on the mammary gland at dry-off. Thus, rMMP-9 IBs and inactive rMMP-9 IBs (mutMMP-9 IBs) at selected dose, and 10 ml of saline solution were infused into 10 quarters per treatment and these were treated with antibiotics following previously detailed steps in E1. At days 1, 3, 6, and 9, after protein infusions, MGSs were obtained and analyzed in fresh for SCC (n = 10) and for immune cell populations (n = 10), and aliquots were stored at -80 ºC until analyzed for BSA (n = 10), LF (n = 8), Na^{+}/K^{+} (n = 10) and endogenous MMP-9 (n = 6).

Mammary secretion analyses

Somatic cell counts

Half ml of each MGS was mixed with half ml DPBS, inverted several times and centrifuged at 1,000 x *g* for 2 min at RT. Fat, located on top of the sample mixture, was removed by gently swirling a cotton swab around the top of the centrifuge tube. The supernatants were discarded without touching the cell pellets, and these were suspended in 1 ml DPBS and centrifuged again. Cells were washed twice, repeating the previous steps and were suspended in 0.5 ml DPBS. Cell suspensions were counted using a Scepter automatic cell counter (Merck Millipore). Cells were diluted when appropriate to obtain a best resolution in the Scepter histograms, and particle counts with diameters below 6 µm were discarded for all samples.

Immune cell populations

After SCC, cell suspensions were stored O/N at 4 ºC, and sent to the Veterinary Clinic Hematology Service at the Autonomous University of Barcelona (UAB, Barcelona, Spain) for the analyses of immune cell populations. Using a XN-1000 analyzer (Sysmex), white blood cells (WBC), polymorphonuclear cells (PMNC) and mononuclear cells (MNC) were differentiated and counted following morphological measurements by flow cytometry, selecting the body fluid mode.

Bovine Serum Albumin quantification

Bovine Serum Albumin (BSA) in MGSs was quantified following Ponchon *et al.*[32] protocol. One ml of each MGS was centrifuged at 1,000 *x g* for 10 min at RT and fat was removed with a swab as detailed in the section for SCC determination. A commercial BSA was used as the standard curve

and an eight-point serial dilution curve from 60 mg/ml was prepared. Two hundred µl of each supernatant were mixed in 450 µl dH_20 and 450 µl of Bromocresol Green working solution (consisting in three parts succinic acid at pH 4 and a part of bromocresol green sodium salt dissolved in 5 mM NaOH, and 0.8 % Brij L23). The solutions for all samples and standards were centrifuged at 1,900 *x g* for 10 min at RT and 150 µl of each supernatant were plated by duplicate in flat bottom transparent 96-well plates and were read at 640 nm. BSA concentrations in MGSs were interpolated to the 4-parametric standard curve.

Lactoferrin quantification

Whole MGSs were analyzed for lactoferrin concentrations following the Bovine Lactoferrin ELISA Quantitation Set from Bethyl Laboratories (USA). A commercial bovine lactoferrin calibrator was used as the standard curve, ranging from 500 to 7.8 ng/ml. After coating the plates with 0.01 mg/ml anti-Bovine lactoferrin, followed by a blocking step as indicated in the manufacturer protocol, samples diluted at 1/10,000 or 1/100,000 and standards were plated per duplicate and incubated for 1 h at RT. After several washes, HRP conjugated anti-Bovine lactoferrin detection antibody was added at a final concentration of 0.01 µg/ml and incubated for 1 h at RT. Following several washes, TMB substrate solution was added and reaction was stopped after 15 min with 0.18 M H_2SO_4. Plates were read at 450 nm and lactoferrin concentrations were interpolated from the 4-parametric standard curve.

Sodium and Potassium quantification

All samples were analyzed for sodium (Na^+) and potassium (K^+) concentrations at the Chemical Analysis Service at the UAB. An aliquot of 0.1 g of MGSs was diluted in Triton X-100 0.1 % (v/v). Clotted samples were previously digested in HNO_3 concentrate in a Ultrawave microwave digestion system (Milestone). Na^+ and K^+ levels were determined by inductively coupled plasma-Optical emission spectrometry (ICP-OES) using an ICP-OES (Optima 4300DV, Perkin-Elmer).

Endogenous MMP-9 zymography

Skimmed MGSs were quantified for endogenous MMP-9 activity through zymography analysis. Solubilized MMP-9[31] was used to prepare an eight-point standard curve ranging from 400 to 25 ng. Sample supernatants were diluted 1/10 and diluted samples and standards were loaded with non-denaturing loading buffer into 10 % SDS-PAGE gels containing 1 % porcine gelatin. Electrophoresis was run at constant 50 mA and gels were washed twice in 2.5 % Triton X-100, once in distilled water, and were incubated in static O/N at 37 ºC in developing buffer containing 50mM Tris pH 7.5, 200 mM NaCl and 10mM $CaCl_2$. Afterwards, Coomassie Blue staining was used to dye the gels for 2 h at RT and these were distained in a methanol-acetic solution. Degradation

bands were analyzed using ImageJ software and MMP-9 activity quantification was interpolated from the solubilized MMP-9 standard curve.

Statistical analyses

A total of 46, 44, and 30 quarters were used in E1, E2 and E3, respectively. For immune cell populations, outliers considered as 2-times standard deviation were discarded. Variables were log-transformed in E1, E2 and E3, or root-transformed for Na^+/K^+ in E1, to normalize data when necessary and data were analyzed using a fixed effects model using SAS 9.4 (SAS Inst. Inc., Cary, NC). Time, dose and the interaction between time and dose were included using an Anova nested, with quarters within cows and cows as random effects. Time was included as a repeated measure and for each analyzed variable, quarters within cows (the error term) was subjected to 2 variance-covariance structures: compound symmetry and autoregressive order 1. The covariance structure that yielded the smallest Schwarz's Bayesian information criterion was considered the most desirable analysis. Means and standard deviations represented in graphs correspond to non-transformed or back-transformed data, while p-values and letters correspond to the output from transformed data when required.

Results and discussion

Determining the minimal inflammatory dose of rMMP-9 nanoparticles

The highest tested dose (12 mg) at dry off was previously reported by Parés *et al.*[17] as a potent immunoestimulator of bovine mammary gland. Herein, in E1, three lower doses were evaluated together with the 12 mg dose. All the used doses enhanced the recruitment of immune cells into mammary gland (namely SCC) up to 6 days post dry-off (Figure 1a). At day 9, the myeloid cells recruited in the controls started to rise but were still below the doses of 12 and 6 mg (Figure 1a). Lactoferrin synthesis, whose rise is also associated to an augmented immune activity[33], increased significantly at day 1 post dry-off until day 3, in all the tested doses (Figure 1b). The involution markers BSA and Na^+/K^+, whose increase reflects the disruption of tight junction and the mixture of blood components in the milk secretion[34], followed similar patterns as lactoferrin with a marked increase yet at day 1 after infusions (Figure 1c and 1d). For all doses tested in E1 there was a shift in the levels of the analyzed parameters at day 1 and these values were kept above controls until day 3 or day 6 for BSA and Na^+/K^+, respectively (Figure 1c and 1d). Importantly, this experiment replicated the results observed by Parés *et al.*[17], consolidating the potential of rMMP-9 nanoparticles to locally stimulate the recruitment of immune cells in the mammary gland at

dry-off and to accelerate the onset of mammary gland involution biomarkers yet after 24 h of infusions.

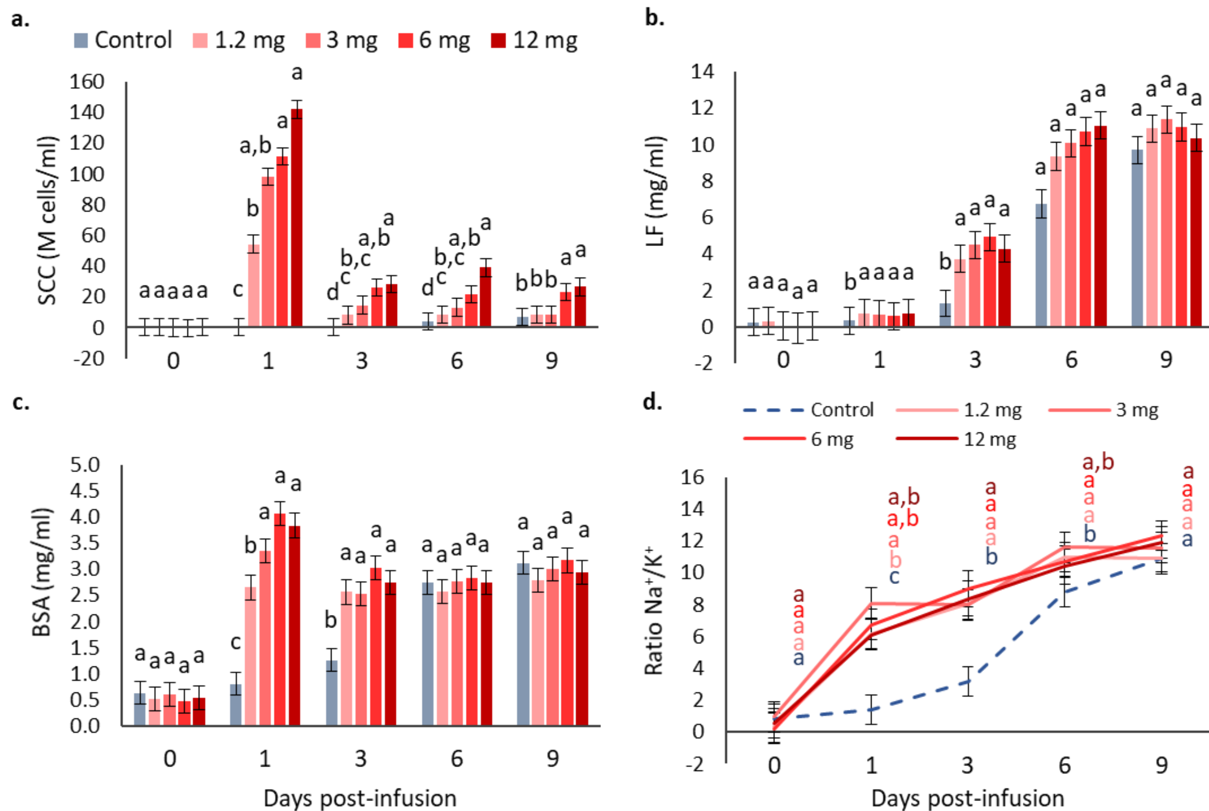

Figure 1. Mammary gland involution markers analyzed from MGSs for the tested doses of MMP-9 IBs (0 - control, 1.2, 3, 6, and 12 mg) along 0, 1, 3, 6, and 9 days post-infusion. Non-transformed means and SEM (error bars) are represented while p-values were obtained from transformed data, when necessary. Letters depict significant differences among treatments within time. **a.** Somatic cell counts (SCC) are expressed in million (10^6) cells per ml (M cells/ml); $p < 0.0001$. **b.** Lactoferrin (LF); $p < 0.0001$. **c.** Bovine serum albumin (BSA); $p < 0.0001$. **d.** Na^+/K^+ ratio. Lines and letters are colored using the same color pattern used in previous panels: control (blue, dashed line), active MMP-9 IB doses (red pantone); $p < 0.0001$.

Since all the tested doses in E1 (Figure 1) induced similar effects during the week post-dry off, either regarding the immune stimulation or involution parameters, E2 was carried out to test lower doses of rMMP-9 nanoparticles (Figure 2). In this case, there was a lack of effect of low doses only observing a slight increase over control quarters with the 0.12 mg (Figure 2). Given that the minimum amount of rMMP-9 nanoparticles eliciting a clear immunostimulating effect in the mammary gland was 1.2 mg (Figure 2), this dose was chosen to further analyze the observed effect in E3.

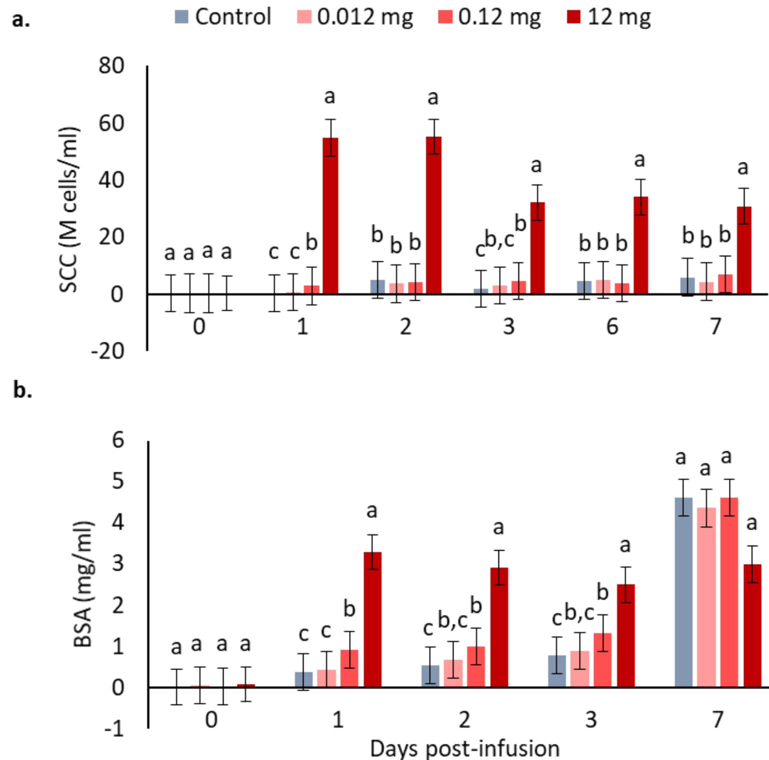

Figure 2. Mammary gland involution markers analyzed from MGSs for the tested doses of MMP-9 IBs (0 –control-, 0.012, 0.12, and 12 mg) along 0, 1, 2, 3, 6, and 7 days post-infusion. Non-transformed means and SEM (error bars) are represented while p-values were obtained from transformed data, when necessary. Letters depict significant differences among treatments within time. **a.** Somatic cell counts (SCC) are expressed in million (10^6) cells per ml (M cells/ml); $p < 0.0001$. **b.** Bovine serum albumin (BSA); $p < 0.0001$.

Differentiation of rMMP-9 and IB format effects

Aiming to determine whether the detected immune response triggered in the mammary gland by rMMP-9 IBs[17] (Figure 1) was only due to the nanoparticle format or whether the activity of the MMP-9 embedded in such nanoparticles was also relevant, as observed in mice[25], a third experiment (E3) was conducted. We compared the performance between rMMP-9 nanoparticles and the mutant and inactive rMMP-9 counterpart at the stablished dose of 1.2 mg in bovine mammary gland. Surprisingly, there was no difference between the performance of inactive or active rMMP-9 nanoparticles in the recruitment of immune cells, neither in the general (WBC) nor in mononuclear or polymorphonuclear cells, being these levels much greater than in control quarters for both treatments (Figure 3). The main recruited cells were neutrophils (determined as PMNCs, Figure 3d) as expected, since they are the first immune cell to arrive to the inflammatory site, and in agreement with the behavior previously observed in mice after the administration of rMMP-9 nanoparticles[25]. However, in the mentioned study using the mouse model, the inactive rMMP-9 nanoparticles only stimulated a slightly and transitory inflammatory

effect, while the active form had a clear and sustained effect over time[25]. Thus, the performance of mutant rMMP-9 nanoparticles was different in mouse intra-dermis model compared with the bovine mammary gland.

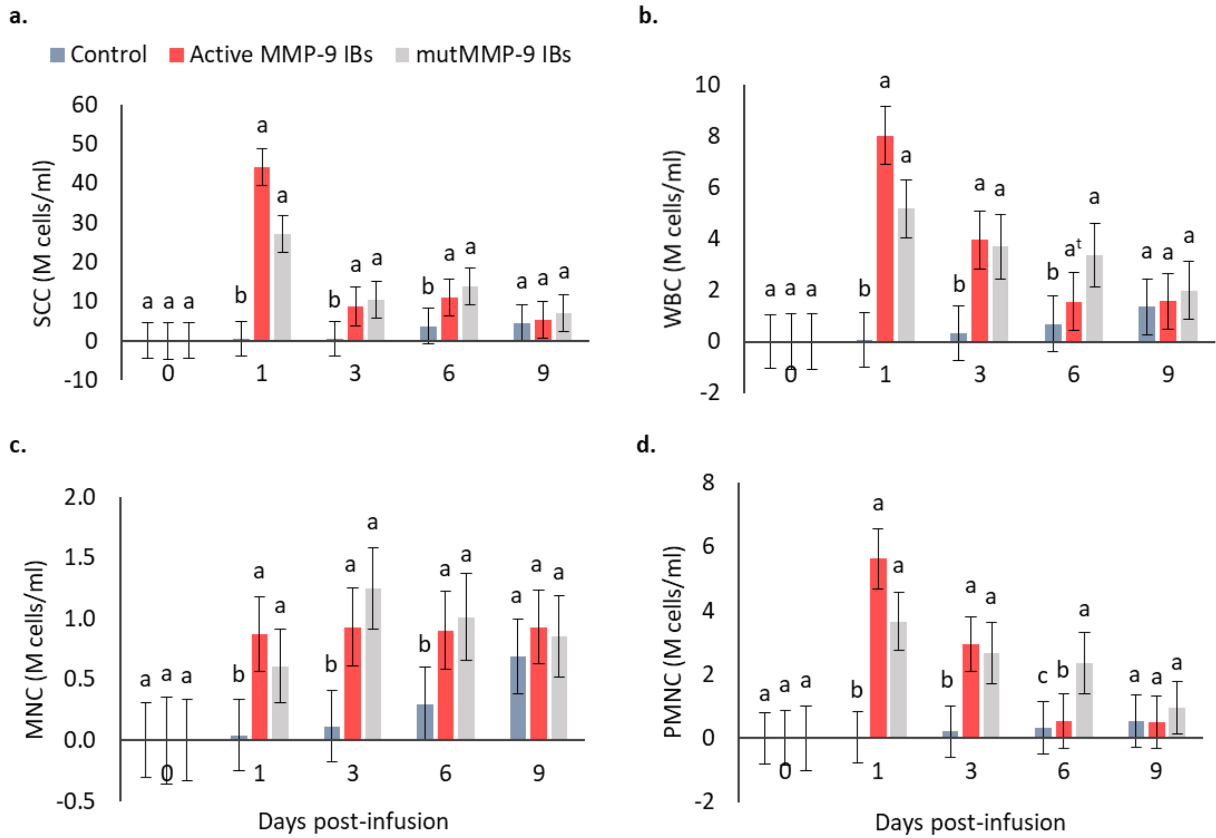

Figure 3. Mammary gland cell populations analyzed from MGSs for the 1.2 mg dose of both active MMP-9 IBs and inactive mutMMP-9 IBs and control, along 0, 1, 3, 6, and 9 days-post infusion. All cell measurements are expressed in million (10^6) cells per ml (M cells/ml). Non-transformed means and SEM (error bars) are represented while p-values were obtained from transformed data, when necessary. Letters depict significant differences among treatments within time. **a.** Somatic cell counts (SCC); $p < 0.0001$. **b.** White blood cells (WBC); $p < 0.0001$. a^t $p = 0.053$. **c.** Mononuclear cells (MNC); $p < 0.0001$. **d.** Polymorphonuclear cells (PMNC); $p < 0.0001$.

When other immune or involution parameters of bovine mammary gland were assessed, only in very few cases the active rMMP-9 nanoparticles had a slightly different performance compared to the inactive rMMP-9 form. Concretely, BSA levels were higher in days 1 and 6 in quarters treated with active rMMP-9 nanoparticles (Figure 4a), suggesting that MMP-9 had some activity behind the unspecific format effects. Also, Na^+/K^+ ratio significantly increased at day 6 and this was sustained at day 9 (Figure 4d). This indicates that the splitting time-point for both specific and unspecific effects of rMMP-9 IBs in bovine mammary gland at dry-off may occur later on, compared with the murine model[25]. However, during the analyzed time in E3, and as observed

for cellular recruitment, there were no rMMP-9-specific effects neither in lactoferrin nor in endogenous MMP-9 levels (Figure 4b and 4d).

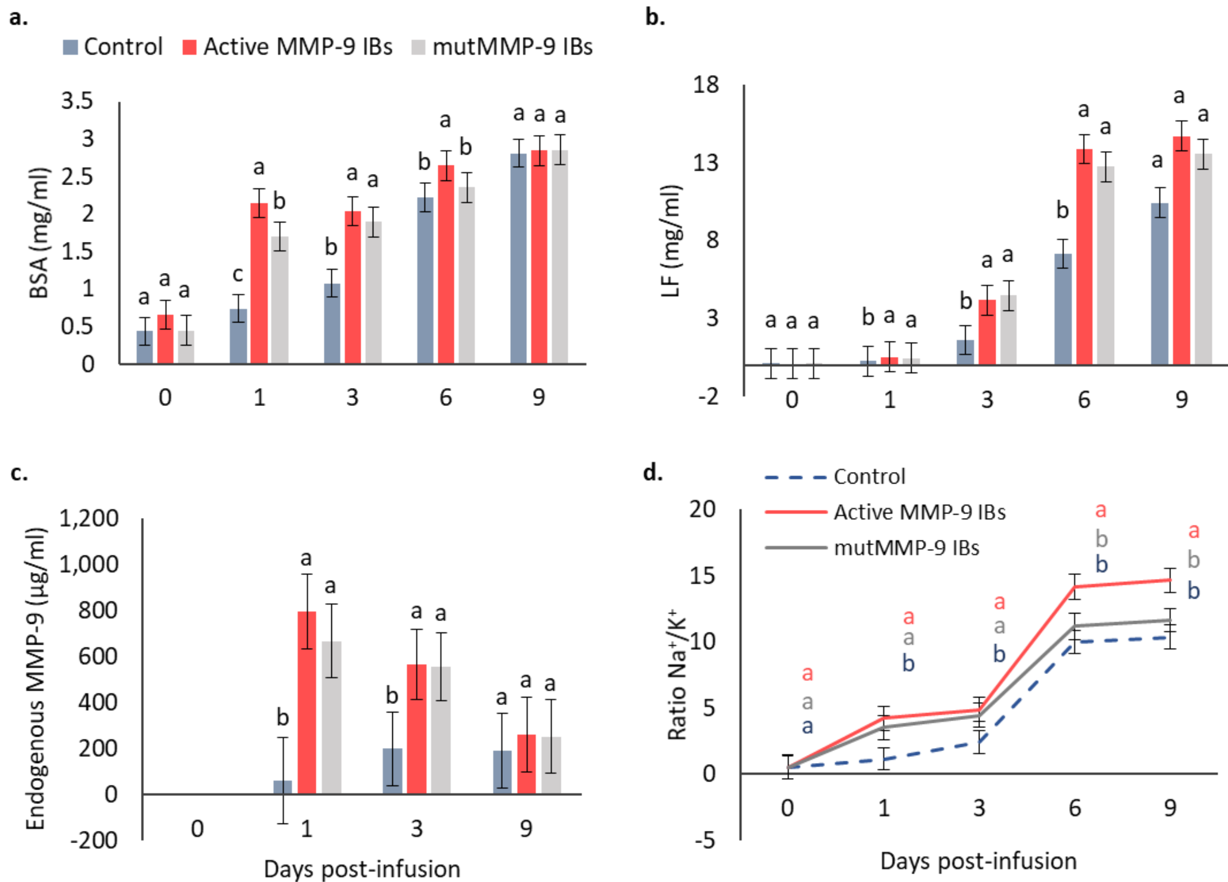

Figure 4. Mammary gland involution markers analyzed from MGSs for the 1.2 mg dose of both active MMP-9 IBs and inactive mutMMP-9 IBs and control, along 0, 1, 3, 6, and 9 days post-infusion. Non-transformed means and SEM (error bars) are represented while p-values were obtained from transformed data when necessary. Letters depict significant differences among treatments within time. **a.** Bovine serum albumin (BSA); $p < 0.0001$. **b.** Lactoferrin (LF); $p < 0.0001$. **c.** Endogenous MMP-9 was analyzed only for selected times: 0, 1, 3 and 9, days post-infusions; $p < 0.05$. **d.** Na^+/K^+ ratio; $p < 0.0001$.

Thus, these results confirm that the obtained effects in the bovine mammary gland were mainly due to the format but not due to the activity of the protein embedded in these nanoparticles. Again, this was an unexpected outcome because, in mice, the effect of MMP-9 could be clearly differentiated already at day 1 after injections from the inflammatory consequences of the nanoparticle format[25]. The question now is why do we observe such different effects in the two animal models? Divergence in the MMP-9 nanoparticle effects observed in mice model and in bovine mammary gland could be explained by important differences between both *in vivo* models. On the one hand, the mice model was knock-out for endogenous MMP-9, favoring a clear split between the MMP-9-specific effect from the format-linked unspecific effect. Nonetheless, in bovine, mammary gland endogenous MMP-9 seems not to have an important role at dry-off[17].

On the other hand, lactating and involuting mammary glands are very immune-active and responsive organs that, in fact, have been compared with strong mucosal immune programs[35]. This agrees with our results indicating that while in mice the nanoparticle unspecific effect was limited to 24 h, in the dairy cow mammary gland this was extended for a minimum of 9 days (except for Na^+/K^+ ratio, differenced at day 6, and the short specific effect in BSA). In this context, the same stimulus could trigger a higher inflammatory effect in cows than in rodents. Moreover, it has been demonstrated that soluble rMMP-9 does not exert any effect on immune and involution parameters at the beginning of bovine dry period[17]. This finding indicates that the protein embedded in the nanoparticles is not as relevant as in the mouse air pouches model, specifically designed to evaluate the MMP-9 activity and further infiltration of immune cells.

The effects of the nanoparticle format on the inflammation of a host were also previously studied by Torrealba *et al.*[36]. They have demonstrated that protein nanoparticles could induce inflammation in Zebrafish and act as an adjuvant[36], and this effect could be even increased through using nanoparticles composed by proteins with a relevant immune function, such as a cytokine[37]. Likewise herein, Torrealba *et al.* also described a dose-dependent effect when an unspecific protein like GFP was injected in Zebrafish, with a fast-induced immunoestimulating effect. This is in agreement with other studies performed previously with LPS[7,8], chitosan[13], *Panax ginseng* extracts[10,11], among others, in which a rapid immunostimulating effect is observed at dry-off.

Conclusions

Protein nanoparticles (IBs) trigger a clear immunostimulant effect in the bovine mammary gland at dry-off. Matrix metalloproteinase 9 protein forming such nanostructures has not a relevant effect in the context of the bovine dry-off. Thus, protein nanoparticles could be considered as an appealing strategy at bovine dry-off to accelerate this process and enhance the immune protection although MMP-9 protein itself does not provide any extra value during the first week of dry period.

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General discussion

POTENTIAL OF IBS AS A NEW FORMAT AND STRATEGY TO MEET THE NEEDS OF THE ANIMAL PRODUCTION SECTOR

Although we have focused our study in testing IBs as a strategy to solve the limitations during dairy cow dry-off, given their economic and global health impacts, IBs benefits and potential may go far beyond therein the animal production industry. Here, after an exhaustive examination of the matters and recombinant protein needs in livestock (study 1), one important curiosity appears to be underlined: there is still a broad spectrum of unexplored strategies, such as the use of IBs, that could be critical to overcome many of the sector demands.

Considering that achieving **affordable costs** is one of the main requirements for animal production developing therapies, a surprising observation is the wide use of the expensive-to-scale-up mammalian cell lines (CHO cells) as expression systems to produce many commercially available recombinant proteins in the animal sector (study 1, table 1). In this regard, N-glycosylations appear as the main limitation factor to reach functionality in many proteins even though the yeast *K. pastoris* –long cheaper– is capable to perform mammalian-like post-translational modifications using GlycoSwitch plasmids[263]. Moreover, since functional IBs are obtained with *K. pastoris*[208] and given their versatility to be used either as IBs or as a source of soluble protein –previously reported for *E. coli*[194] and inhere described using a new protocol for *L. lactis*[264] (study 2, figure 3)- it comes out that these are worth to be explored as a new biomaterial with interesting properties for animal production. Thus, IBs can be produced in *K. pastoris* for proteins requiring of N-glycosylations, or in bacteria if they do not. By using fermenters (bioreactors) these could become an alternative to obtain **higher yields** of proteins of great economical interest in animal production, such as recombinant reproductive hormones and antibodies for passive immunization, using simple and more affordable procedures.

IBs could be not only a strategy to obtain greater protein yields of highly pure soluble protein at low production costs but also can act as **protein stabilizers** in challenging conditions such as under extreme pH and temperatures[222] as well as under proteolytic activity, as observed for MMP-9 IBs (study 3, figure 2). This would be an especially relevant asset for those enzymes used in livestock nutrition as feed-additives that act in the gastrointestinal tract improving feed efficiency, such as the case of phytases. Although several approaches like the natural obtention of these enzymes from extremophile microorganisms (mainly fungi) have been already developed in this regard[265–269], higher yields could be achieved through their production as IBs in bacteria or other simple expression systems. Moreover, **purer yields** could be obtained compared to nowadays mixed

enzyme preparations, thus increasing the catalytic efficiency and reducing feed costs and, yet, adding the possibility to use these IBs as an alternative and more stable **delivery system**.

Moreover, given the **adjuvant-like** effect of IBs, suggested by inert protein-based and cytokine-based IBs used in zebrafish[203,222] and elucidated in mice and cattle by inactive MMP-9 IBs in this thesis work (study 4, figure 2 and figure 3 and study 5, figure 3, respectively), these particles offer a combination of easily tailorable and low-cost advantages for immune therapies.

UNLOCKING PROTEINS THROUGH *L. LACTIS* IBS SOLUBILIZATION TOWARDS NEW OPPORTUNITIES IN THE COW DRY-OFF OPTIMIZATION

Besides the needs in the animal production sector, in protein production research there are also some bottlenecks to face up, being one of the mostly requested the obtention of high-quality soluble proteins (pure and active) when we aim to isolate proteins of interest which are difficult-to-produce recombinantly. In our intention to characterize the potential of MMP-9 IBs as an immunostimulant to be used at cow dry-off, we encountered an important limitation: we needed a soluble version of MMP-9 to compare the protein performance in both formats but the MMP-9 is a **prone-to-aggregate** enzyme for which the soluble fraction during the overproduction process is practically undetectable[158].

With ***L. lactis*** as expression system, more and more strain and vector options are being developed[135,144], though there is still a long way to reach the inventory for *E. coli*. Fortunately, the study of *L. lactis* as cell factory is stepping up quickly because of its notable advantages due to the lack of LPS. Besides, functional endotoxin-free IBs, such as MMP-9 IBs, have been successfully produced in this bacterium[158]. Given that functional proteins were easily solubilized by non-denaturing protocols from *E. coli* IBs by Peternel and coworkers[194], this encouraged us to develop a similar protocol adapted to *L. lactis* IBs. However, we did not seek a specific protocol for MMP-9 IBs but a broad-spectrum protocol for all those difficult-to-obtain proteins (Figure 13). That is, even if we have a good cell host to produce our proteins, process plainness is not guaranteed at all and, indeed, the nature of these proteins unquestionably determines the difficulties found during production and downstream processes, forcing most protocols to be case-specific. M-SAA3 is a good example of this, because although this is relatively well expressed in *L. lactis*, probably due to its amyloid nature it binds at least to two host cytoplasmic proteins (study 2, figure 2), becoming so hard to reach pure yields from the soluble fraction not even trying several purification conditions (study 2, table 1). This took us to question either if this was an immediate interaction or if proteins do scape these unspecific bindings while aggregating in IBs, becoming

these a potential source of purer protein. Thus, aiming to obtain the soluble version of different type of difficult-to-obtain proteins (including those prone-to-aggregate and difficult-to-purify), we developed a universal solubilization protocol to extract the soluble form from *L. lactis* IBs of **challenging proteins** in terms of protein production and purification (study 2).

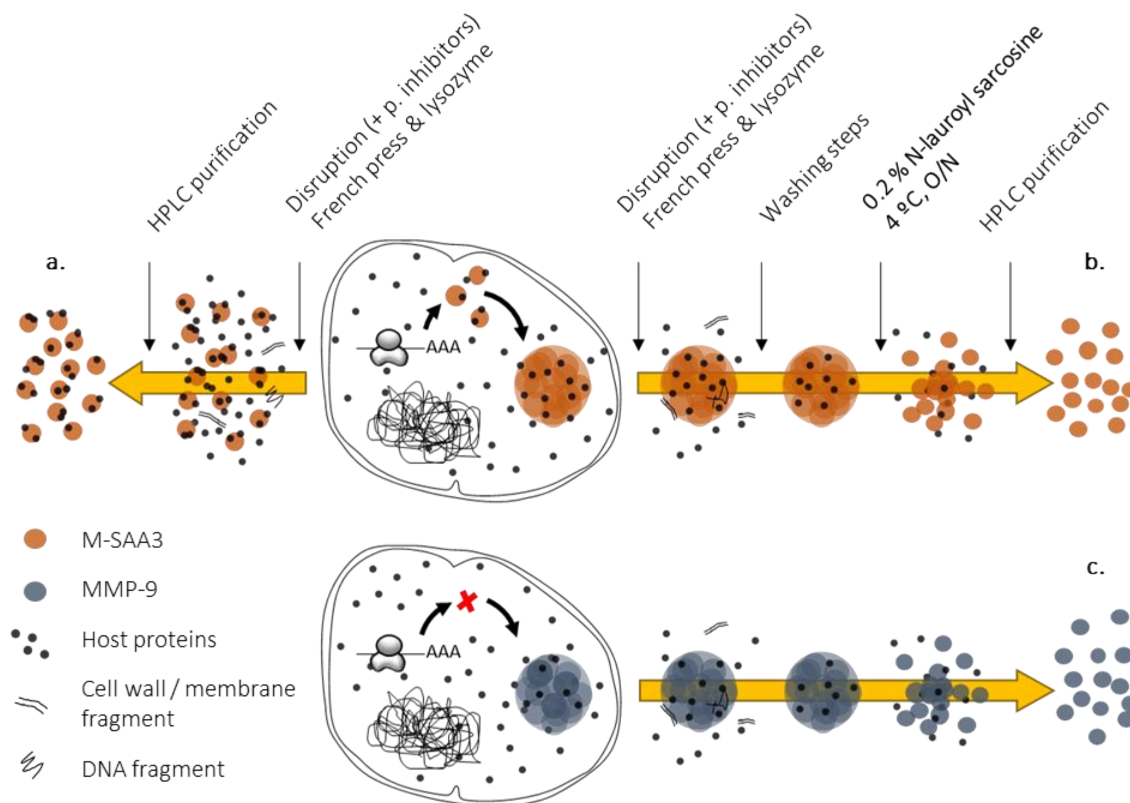

Figure 13. Purification strategies to obtain the soluble version of difficult proteins in *L. lactis*. **a.** Purification of M-SAA3 from the soluble fraction resulted in low purities, as two main host proteins were attached to it and eluted together during HPLC purification processes. **b.** Purification of M-SAA3 obtained through the solubilization of M-SAA3 IBs by using the new protocol resulted in pure yields of M-SAA3. **c**. Purification of MMP-9 was only possible through the solubilization of MMP-9 IBs, as no soluble fraction is obtained for this protein. Using the developed solubilization guidelines soluble and pure yields of MMP-9 were obtained.

Unlike *E. coli*, Gram-positive cell wall disruption by sonication is not efficient, but the use of high-pressure strategies like French press or cell disruptor, combined with an additional lysozyme incubation (study 2, figure 3), gives good results. Moreover, with the aim to preserve functionality of the solubilized proteins, especially those that are highly labile like M-SAA3, the use of protease inhibitors at disruption and an overnight solubilization at 4 ºC under non-denaturing conditions were stablished (study 2, figure 3) as an optimal strategy.

For our surprise, and after a preceding long and unsuccessful trial-and-error way along M-SAA3 purification from its soluble fraction, for the first time active and pure M-SAA3 was obtained through the new protocol developed in study 2 (figure 4 and figure 5). This reconfirms the

specificity of the aggregation process in the formation of bacterial IBs, composed mainly by the recombinant protein of interest even if these tend to bind, when soluble, to other proteins present in the cytoplasm.

Importantly, also the isolation of LPS-free soluble MMP-9 was possible by solubilizing *L. lactis* IBs through this easy and economical process, being this a critical step in the accomplishment of the present thesis work, and opening a wide range of interesting possibilities to be explored looking forward to elucidate the MMP-9 IBs real potential at cow dry-off. Briefly, through obtaining the solubilized MMP-9 we were able to compare not only the MMP-9 activity in the soluble format with that in MMP-9 forming IBs *in vitro* (study 3, figure 2; study 4, figure 1c) and *in vivo* (study 4, figure 2 and figure 3), but also to obtain the prime matter to construct new polymeric-based MMP-9 delivery strategies that helped us to understand the stability potential of MMP-9 IBs (study 3, figure 1).

In general terms, the development of this protocol gives to the scientific community an easy and affordable approach to obtain difficult proteins, whichever labile, "sticky" or prone-to-aggregate proteins, in a GRAS microorganism and preserving their activity. Moreover, this protocol may probably be transportable to other Gram-positive bacteria widening even more the recombinant protein research possibilities.

DISCOVERING MMP-9 IBS ADVANTAGES IN FRONT TO OTHER FORMATS: THE *IN VIVO* POTENTIAL HIDDEN UNDER *IN VITRO* CHARACTERIZATION

Obtaining the soluble counterpart of *L. lactis* MMP-9 IBs through the newly developed protocol (study 2) brought us to explore new ways of getting the most of it. Our intention in developing polymeric nano-encapsulated versions with the soluble MMP-9 (study 3, figure 1) was to obtain new formats with a meliorated stability compared to the nude solubilized format, with the final aim to compare them with the stability provided by IBs. This would open the spectra in the MMP-9 toolbox since different alternatives of MMP-9 delivery systems would be addressed.

The first result that stands out is the high initial specific activity observed for the free soluble MMP-9 together with the polymer-based MMP-9 nanoparticles, in comparison with the contrastingly low initial activity of MMP-9 IBs (study 3, figure 2). Although all formats have a common origin, the solubilized and the polymer-based MMP-9 activity is immediately observed in the assay performed, whereas that observed in the IBs is initially limited. Hence, these results probably indicate that protein is more accessible in the soluble-based formulations and, consequently, this also probably means being more exposed. In this regard, after incubation with

bovine serum, where proteases and probably TIMPs and other MMP-9 inhibitors may be present, the high activity of the soluble and polymeric formats of MMP-9 rapidly dropped whereas the activity in IBs showed no differences, remaining stable along the time course (study 3, figure 2). This goes in agreement with the **cotton-like structure** of IBs described by Peternel *et al.*[177,178] and validated by Cano-Garrido and coworkers[195], suggesting that, on the one hand, the heterogeneity of conformations coexisting in the IBs could explain the reduced initial specific activity compared to the soluble counterpart; and on the other hand, that the IB format supports protein integrity inside this scaffold permitting them to be active for an extended period.

In addition, and contrarily to our initial hypothesis, MMP-9 polymeric micelles did not offer any added stability compared to free MMP-9 under serum conditions (study 3, figure 2). Indeed, through this study we could observe that MMP-9 is a highly stable protein while it is not exposed to serum conditions, being this a possible explanation for the low efficiency of polymeric micelles to add any benefit to this protein observed in study 3. Thus, although F127 micelles were able to carry MMP-9, probably because this was localized in the hydrophilic area of the micelle, either encapsulated or covalently bound, the MMP-9 was fully accessible to external agents (Figure 14).

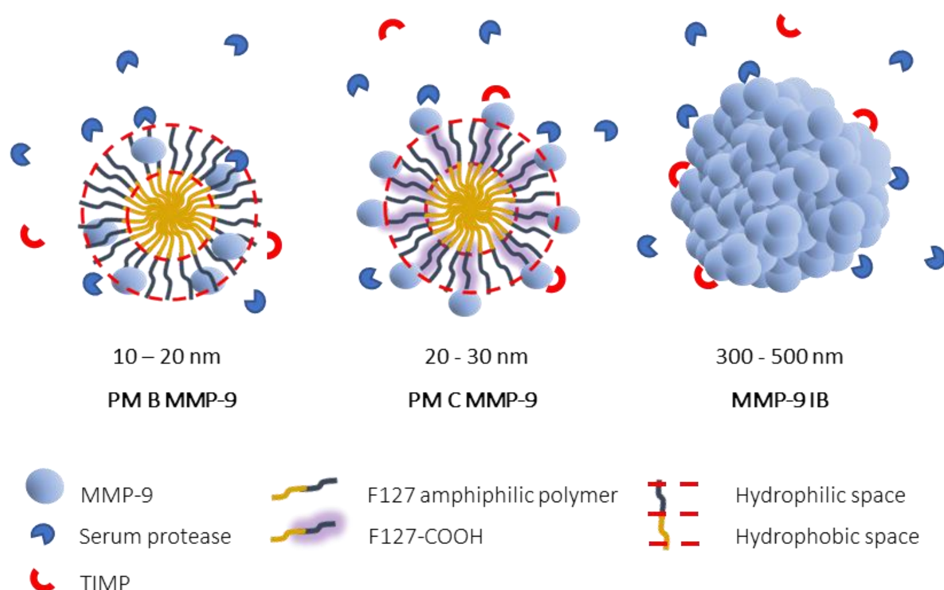

Figure 14. Illustration depicting the hypothesis about MMP-9 exposition to serum molecules for the different MMP-9 nanoparticulated formats. Sectioned nanoparticles are represented. While the MMP-9 in polymeric micelles (PM B, encapsulated; PM C, covalently bound) are situated in the hydrophilic surface and are accessible to serum proteases and TIMP molecules, in IBs the gross of MMP-9 is located in inner positions being only the surface MMP-9 exposed to external agents.

Since the soluble MMP-9 showed the highest specific activity but the IBs the highest stability *in vitro*, we aimed to explore what would be the performance of both formats *in vivo* (study 3 and

study 4). For that, MMP-9 knock-out mice were injected intradermally in air pouches with both formats of MMP-9 (soluble and IBs) and were analyzed under microscopy for neutrophil recruitment as an indicator of inflammatory stimulation (study 3, figure 4; additional analyses included in study 4). As expected, soluble MMP-9 triggered an immediate recruitment of neutrophils yet at 3 h, but this dropped after 24 h (study 3, figure 4; study 4, figure 2b). Meanwhile, despite the MMP-9 IBs low initial activity observed *in vitro*, these also triggered an equal neutrophil recruitment, first unspecific (also observed by inactive mutant MMP-9 IBs), but importantly, specifically sustained after 24 h and for a minimum of 48 h (study 3, figure 4; study 4, figure 2b).

These outcomes rise awareness in the importance of how cautiously we should interpret the results from *in vitro* assays with IBs, as these nanoparticles hide a high potential to be used *in vivo* by their intrinsic properties. On the one hand, in this study the **unspecific adjuvant-like effect** of IBs observed by Torrealba *et al.*[203] has been confirmed and successfully differentiated from the protein-specific activity –in this case MMP-9 (study 3, figure 4; study 4); this point being further discussed in the following section. On the other hand, our results are in agreement with other reports demonstrating the IBs **high stability** due to the above mentioned cotton-like structure[177,178,195]. Moreover, although we have not proven the MMP-9 IBs way of action, the low initial specific activity (study 3, figure 2), followed by the maintenance of MMP-9 functionality and effects for a prolonged period of time (study 3, figure 3 and figure 4; study 4), both point out the capability of IBs to **slow release** recombinant proteins under physiological conditions, in agreement with other studies[215,217,270]. It has been suggested that under physiological conditions, **chaperones**, which are common contaminants inside IBs[271] and also are present in cellular or tissue environments, would coordinate protein solubilization from IBs[271]. In this way, IBs have demonstrated to release functional proteins either in the bloodstream[217] after their subcutaneous injection in a mouse cancer model, as well as intracellularly[215,271] after their culture with mammalian cells, resulting in interesting bioapplications for this protein material.

THE IMPORTANT SPLIT OF MMP-9 IBS IMMUNOLOGICAL ACTION

So far, we have demonstrated that although MMP-9 IBs have little initial specific activity *in vitro* the activity of these aggregates becomes highly relevant under physiological conditions (study 3). In fact, many relevant research areas are exploring different *in vivo* contexts in which these aggregates are demonstrating a high potential, such as in oncology and tissue engineering, among others[206,209]. Concretely *in vivo*, IBs have been orally administered[214] and intravenously and intratumorally injected[219,272] in mice, and also intraperitoneally injected in zebrafish[203,222], and no

signs of secondary effects nor toxicity were detected in any of these models[214,219,222]. However, there are no studies determining which is the relevance of format-related **unspecific effects** of IBs. Indeed, there is just one previous study in which an unspecific effect was reported for IBs produced in *E. coli*. Specifically, in this study IBs formed by innocuous proteins (GFP) were injected to zebrafish demonstrating a **protective** effect in front of lethal infections[203].

The immunological stimulation by innocuous-protein IBs can be explained by their nature. Although the recombinant protein is the main component in IBs, when these are formed inside bacteria, different host molecules may unspecifically get trapped inside. These can be either DNA, RNA, bacterial proteins like chaperones, or lipids like LPS, and may activate unspecific immune reactions[196]. Specifically, IBs injected in zebrafish were produced in *E. coli* strains containing LPS or a modified non endotoxic LPS variant[203]. Interestingly, results reported that none of the impurities alone, not even LPS, provoked the protective unspecific immunostimulation, but the sum of them[203]. Moreover, when IBs were formed by **immune-relevant proteins** like CCL4 and TNFα, these offered even better protective effects[222]. However, the extent in which the specific and unspecific effects may contribute to these immunological effects has not been yet elucidated, being this an important point to be resolved.

Considering these previous results, we designed a study to dissect the *in vivo* specific and unspecific effects of IBs formed by an immune-related protein such as MMP-9. For that, we used MMP-9 IBs produced in *L. lactis* (study 4) where no LPS but probably other host elements such as nucleic acids, peptides or proteins are present, unspecifically aggregated and interfering in MMP-9 IBs purity (study 3, figure 1b). Moreover, two versions of MMP-9, active and inactive, were compared in this study.

Both active and inactive versions of MMP-9 IBs were produced in *L. lactis* (study 4, figure 1) and, interestingly, different immunostimulant effects were successfully elucidated after their injection in air-pouches in MMP-9 knock-out mice (study 4, figure 2 and figure 3) (Figure 15). On the one hand, a **non-specific** immune stimulating effect is observed yet after 3 h of injections for both IB treatments (study 4, figure 2b and figure 3), demonstrating that IBs produced in Gram-positive bacteria such as *L. lactis* have also an inflammatory/adjuvant potential as the observed by *E. coli*-produced IBs[203,222]. Also, although in this early time point (3 h) there is a slight specific inflammatory effect due to MMP-9, observed in an increased neutrophil proportion for the soluble MMP-9 treatment (study 4, figure 2b), this specificity cannot be determined for IB treatments, as the MMP-9 effect is probably masked by a greater unspecific effect due to the format. On the other hand, after 24 h the soluble MMP-9 effect disappears while the unspecific

effect of IBs is maintained at a cellular level (study 4, figure 2). The rapid clearance of the soluble MMP-9 effects goes in agreement with our previous observations *in vitro*, suggesting a lower half-life of soluble MMP-9 under physiological conditions due to its free/exposed nature (study 3, figure 2).

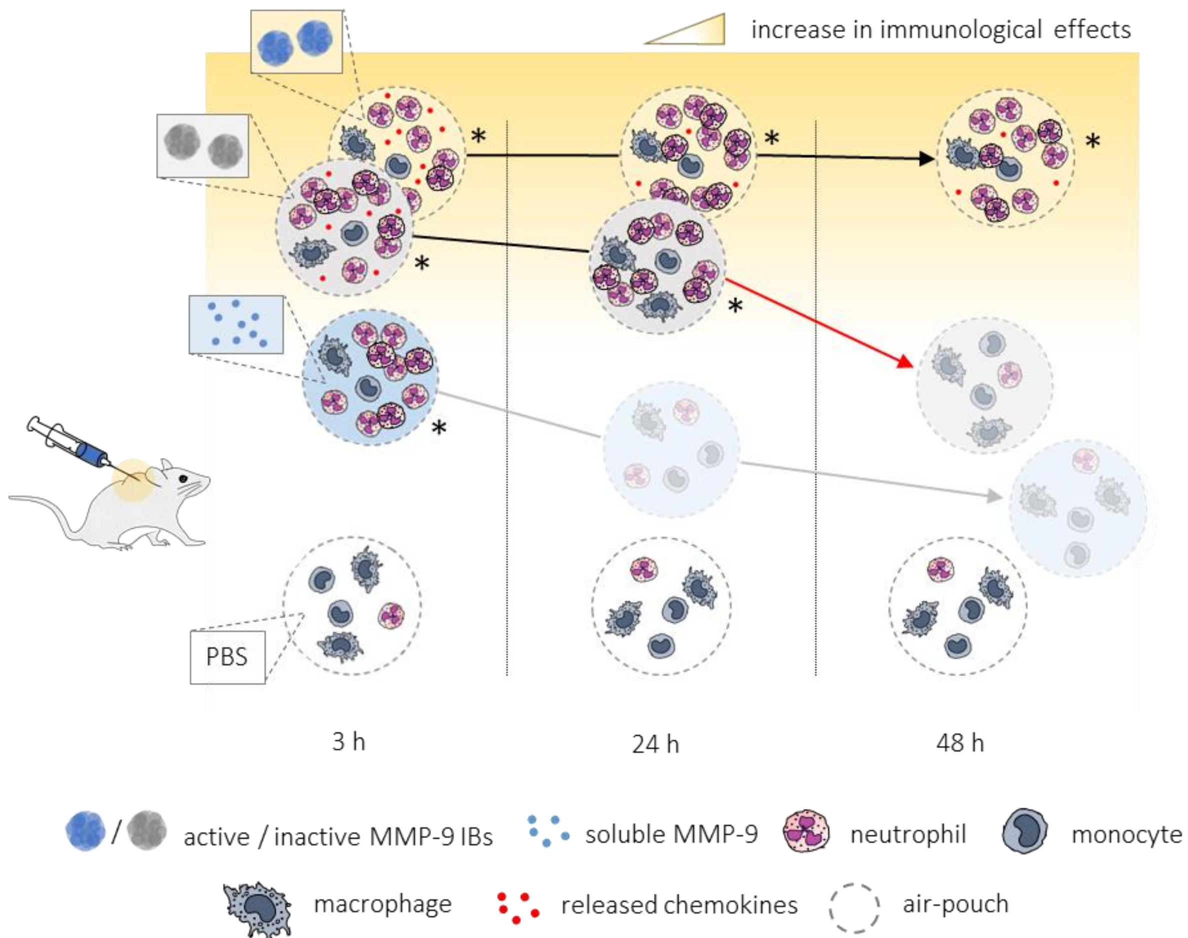

Figure 15. Illustration depicting the split between unspecific and specific immunological effects of MMP-9 IBs after their injection in dermal air-pouches in MMP-9 knock-out mice. Air-pouch contents are represented along time for each treatment (active MMP-9 IBs, inactive MMP-9 IBs, soluble MMP-9 and PBS as control; yellow-, grey-, blue- and white-colored air-pouches, respectively). At 3 h after injections, all treatments trigger the recruitment of neutrophils, reducing the abundance of other cells in proportion (mainly, monocytes and macrophages), while only IB-based treatments increase chemokine levels. Effects for the soluble MMP-9 are no longer detected while IB-based MMP-9 continue recruiting neutrophils after 24 h of injections. At this time-point, only active MMP-9 IBs continue increasing chemokine levels. After 48 h, the unspecific effects triggered by the inactive MMP-9 IBs are no longer detected (inflection phase indicated as a red arrow), whereas in air-pouches treated with active MMP-9 IBs, cellular and chemokine levels are still increased compared to the control group. Asterisks indicate differences in neutrophil recruitment compared to the control group.

Interestingly, after 48 h the active MMP-9 IBs still trigger both cellular and humoral immune responses, maintaining non-variable values in respect to the observed at 24 h for cell recruitment, neutrophil rates and CXCL2 neutrophil-chemoattractant levels (study 4, figure 2 and figure 3b).

In addition, at 48 h there is no evidence of any of the previously observed effects triggered by the inactive MMP-9 IBs, confirming that the IB unspecific effects are limited, lasting no more than 24 h in this mouse model (study 4, figures 2 and 3), and importantly confirming that 48 h-effects are due to an **MMP-9-specific** immune stimulation. The fact that both soluble and aggregated MMP-9 trigger specific *in vivo* effects with such a distinct pattern again matches with previous results on stability in serum (study 3, figure 2), supporting our statements pointing to a retarded increase in the availability of the MMP-9 forming IBs and/or its slow release.

These results take us back to the different *in vivo* studies in which functional IBs have been injected to study different specific effects, but their intrinsic unspecific effects corresponding to the IB format have not been determined. The observational windows for the IB-triggered effects in these assays differ depending on the targeted effect to be evaluated, ranging between few hours to 7 days after injections. For example, intratumorally injected T22-containing IBs in a colon cancer mouse model showed an increase in the cleavage of Caspase 3 initiating the apoptotic cascade after 5 h of injections, with apoptotic bodies being increased for a minimum of 7 days in consequence[272]. Similarly, when p31-based IBs were injected intratumorally in a breast cancer mouse model, tumor cell viability was reduced after 4 days of injections compared to the control group[219]. During this second experiment, unspecific effects existed, evidenced by the highest tested dose of GFP IBs which reduced tumor cell viability after 4 days of exposure (figure 2a in Pesarrodona *et al.*)[219]. Likewise, immunostimulant unspecific effects could be observed by non-relevant iRFP IBs in zebrafish after 24 h of injections. At this time, animal survival in front a challenge was comparable to the treatment with TNFα IBs and CCL4 IBs, and iRFP IBs protection sustained beyond 48 h with 50 % survival, while non-treated animals were ranging from 20 to 10 % beyond 48 h (figure 6 in Torrealba *et al.*)[222]. Thus, the effects observed by relevant-protein IBs in these studies are probably the sum of unspecific and specific effects at first instance and become solely specific when format-related effects end, as observed in our experiments (study 4).

These results are of great relevance because for the first time we have dissected both specific and unspecific immunostimulant effects triggered by IBs composed by an immune-related protein, being in this case MMP-9 IBs. Therefore, these two effects are (i) an earlier and limited immunostimulating unspecific format-related effect, and (ii) a later and prolonged MMP-9-specific effect. Moreover, it is worth to mention that the potential of MMP-9 as an immunostimulating agent has been evidenced in this model, upholding our hypothesis about using MMP-9 IBs to address the immune condition in the MG of dairy cows at dry-off.

MMP-9 IBs AS A NEW IMMUNOTHERAPY: FROM A MOUSE MODEL TO THE DRY-OFF CONTEXT IN DAIRY COWS

There are numerous publications supporting a remarkable role of MMP-9 in **tissue remodeling**[58,273,274] and in cell migration as a consequence of its tissue permeabilization and chemotactic effects[58,253–255]. It has been demonstrated that, in the dairy cow MG, MMPs and concretely MMP-9 may take part in inflammatory responses prompted due to damage, such as mastitis[275], or due to processes involved in MG involution during dry-off[60], as both neutrophil and epithelial MMP-9 levels substantially increase under these circumstances[259,276]. Rabot and co-workers also detected increased MMP-2 levels in endothelia of bovine MG blood vessels during late involution, although levels for MMP-9 were not analyzed[277]. An increase in MMP-2 and MMP-9 after milking cessation in dairy cows were also reported by Tremblay *et al.*[276]. It is undeniable, therefore, that these MMPs are upregulated during these processes. However, the specific mechanisms through which these proteinases are involved in bovine MG remodeling at dry-off are still unresolved[59,116,276]. Importantly, MMP-9 and MMP-2 are both **type IV collagenases** that degrade cell adhesions and collagen, which form a fibrous protein network that is critical to preserve the basal membrane integrity[258], the scaffold for MG alveoli.

Recombinant MMP-9 (herein referred as rMMP-9) was, thus, proposed by our group as a strategy to **accelerate tissue involution** at dry-off in dairy cows. Our final objective was to try to reduce the time window for increased IMI risks at the beginning of the dry period, promptly immunostimulating and helping the MG to **locally switch** from the gestational anti-inflammatory phenotype to the required **pro-inflammatory phenotype** at this stage. Parés *et al.* explored the administration of a single dose of rMMP-9 in IB format (12 mg) which accelerated the local immune response and involution of MG in a very significant and pronounced way (submitted work: annex 1). Meanwhile, soluble rMMP-9 only increased the endogenous MMP-9 activity, and more surprisingly, this happened without affecting general parameters of immune stimulation and mammary involution markers (submitted work: annex 1). This result placed squarely upon the table if the observed inflammatory effect was due to the MMP-9 or due to the IB format. At this point it is important to stress that in the present thesis we have demonstrated the specific effects of rMMP-9 embedded in the IBs in a knock-out mouse model (study 4). Thus, this encouraged us to move back to the **bovine model** to try to elucidate which specific and unspecific effects were triggered by rMMP-9 IBs regarding MG involution at **dry-off** (study 5).

Acceleration of mammary gland involution in dairy cows by infusing a single dose of 1.2 mg MMP-9 IBs at dry-off

Yet after 24 h post-infusion in MG with doses ranging from 1.2 to 12 mg of active rMMP-9 IBs in dairy cows, **tight junction disruption** and tissue permeabilization were demonstrated by increases in BSA, lactoferrin and Na^+ levels, as well as by **SCC increases** in MGSs (study 5, figure 1). This first result confirmed that rMMP-9 IBs can stimulate a fast inflammatory response in the MG as they did in the MMP-9 knock-out mouse model (study 4, figure 2a), and confirms the observations of Parés *et al.* study in the bovine MG context (submitted work: annex 1). Drying-off effects for non-treated animals began beyond day 3 and were clearly observed at day 6 from milking cessation (study 5, figure 1), in accordance with previous reports describing this as the normal condition after milk stasis[16,42,116,259,276,278]. Therefore, by infusing a single dose of 1.2 mg rMMP-9 IBs the onset of these effects could be successfully accelerated from 3 to 6 days respect to the control (study 5, figure 1). It is important to mention that Parés *et al.* accelerated MG involution infusing a high dose (12 mg) of rMMP-9 IBs (submitted work: annex 1), while here we have obtained similar results with a 10-times reduced dose. In fact, we analyzed even lower doses of MMP-9 IBs but although 0.12 mg rMMP-9 IBs increased BSA at 24 h and for 3 days (study 5, figure 2a), SCC were just slightly increased only for 24 h (study 5, figure 2b). Thus, 1.2 mg was considered the most suitable **minimal effective dose** of rMMP-9 IBs and was the chosen dose for the comparison of active and inactive IBs effect.

Context matters: MMP-9 IBs do not have MMP-9-specific activity during early mammary gland involution in dairy cows

Importantly, the results obtained from the first trial in which 1.2 mg of rMMP-9 IBs were infused in the MG (study 5, figure 1) were replicated (study 5, figure 3 and figure 4), consolidating the rMMP-9 IBs potential to locally stimulate the recruitment of immune cells in the MG at dry-off (study 5, figure 3) and to accelerate the onset of MG involution biomarkers (study 5, figure 4). As observed in the inflammatory responses in the mouse model (study 4, figure 2b), **neutrophils** were the main WBCs recruited in the MG due to rMMP-9 IBs infusions being their levels elevated along 6 days after infusions (study 5, figure 3d). This is especially relevant in terms of IMI prevention, as it has been described that the delay in neutrophil infiltration in the MG is one of the causes of high mastitis incidences at periparturient stages[260]. Levels in BSA, lactoferrin and the Na^+/K^+ rate did also increase for 3 to 6 days, and yet at 24 h of infusions (study 5, figure 4a, figure 4b and figure 4d), although Na^+/K^+ levels were not as pronounced in this trial (study 5,

figure 4d) as the observed in the first one (study 5, figure 1d) –suggesting that variability could be affecting this evaluation.

When we aimed to compare the effects by active rMMP-9 IBs and inactive mutant rMMP-9 IBs, however, all the analyzed parameters equally increased after both treatments compared to control groups (study 5, figure 3 and figure 4). Only a slight difference could be observed in BSA levels at day 6, and in the Na^{+}/K^{+} rate at days 6 and 9 after infusions, being the active rMMP-9 IBs still increasing these levels respect to the control group, but not the inactive rMMP-9 IBs. This evidenced the lack of a relevant MMP-9-specific effect of rMMP-9 IBs in bovine MG at early stages of dry-off. This could be explained either by a stronger unspecific effect covering possible specific effects, or either by the lack of –or by too slight– MMP-9 effects, at least at this stage of the dry period. Therefore, while in mice the rMMP-9 IBs unspecific effect was limited to 24 h (study 4, figure 2) and followed by a rMMP-9-specific effect, in the dairy cow MG at dry-off, format-related responses extended for a minimum of 9 days (study 5, figure 3 and figure 4) with no signs of a relevant rMMP-9-specific effect.

Different explanations emerge to resolve this divergence between the effects triggered by rMMP-9 IBs in both animal models.

- First, the mouse model was knock-out for **endogenous MMP-9** and, moreover, our rMMP-9 lacks the hemopexin domains where TIMP1 and TIMP3 bind to regulate MMP-9 effects. Thus, this model can be considered a "clean" and ideal model to study rMMP-9-specific effects in the IBs, which favored a proper split of both the specific and the unspecific effects in this format (study 4). Contrastingly, in bovine, high endogenous MMP-9 levels could be determined by zymography of MGSs for both IB treatments (study 5, figure 4c). These increases have been also reported after the infusion of molecules with immunostimulant effects, such as SF68 *E. faecium* preparations[259], or hormonal modifiers like the PRL inhibitor cabergoline[116], and have been mainly attributed to the degranulation of newly arriving neutrophils[115,259,260]. Nevertheless, recent results by Parés *et al.* (submitted work: annex 1) demonstrated that endogenous MMP-9 was not the trigger factor for the involution markers after rMMP-9 IBs infusions at dry-off. In this experiment, rMMP-9 in the soluble and IB formats were infused in the MG at dry-off and both triggered an increase in endogenous MMP-9, while only rMMP-9 IBs promoted involution markers, evidencing an unspecific format-linked effect and an apparent irrelevant role of MMP-9 at this stage. This irrelevance of MMP-9 as a trigger of involution can be also deduced by the static endogenous MMP-9 levels in the control group (study 5, figure 4c) while other involution markers start to rise

naturally, and also by the decrease of endogenous MMP-9 along time after the initial burst for the IB-treated groups (study 5, figure 4c), indicating an end of this transitory effect.

- Second, the bovine MG at dry-off is ruled by a **gestational hormonal condition**, meaning this that PRL levels, among other hormones such as progesterone, are maintaining the immune system in an anti-inflammatory phenotype during pregnancy. This may affect the MMP-9 activity during this stages, as PRL is known to downregulate MMP-9 expression in leucocytes[260] and MECs[279]. In fact, when PRL is therapeutically inhibited during bovine early dry-off, MG remodeling increases[116] suggesting that PRL has a role regulating MMP effects during this period. Although the concrete signaling mechanisms in this regard are still little known in bovine, IGF –upregulated by PRL– is known to inhibit MMP-9 synthesis though protein kinase C-α (PKCα) signaling[57]. Thus, it could be inferred that, until PRL withdrawal, MMP-9 could be blocked either by TIMPs or either by its repressed expression. Thus, even though we observed an immediate increase in endogenous MMP-9 levels after rMMP-9 IB infusions (study 5, figure 4c), this probably corresponded mainly to the burst from resident and newly arrived neutrophils (study 5, figure 3d) and did not extend beyond 3 days, fading out along with neutrophil infiltration. This suggests that although MMP-9 could be increased during early MG involution, its function is tightly regulated in this stage of the dry period in dairy cows. In this context, and although our rMMP-9 is resistant to the action of some TIMPs, it could not be excluded from this hormonal regulation since some inhibitors could still have an effect on rMMP-9 and MG is strongly linked to the cell survival signals still existing during the first week of dry period (see Figure 5).

- Third, the MG is a highly **immunoreactive tissue** that has been compared to strong mucosal immune reactions[280]. As it has been observed through the infusion of other immunostimulant agents[118,119,121,122,125], the MG is aware to recognize and respond to any intrusive substance. This can be also accounted during SCM, where SCC rapidly rise. This could explain why in other studies such as for GFP IBs administered in zebrafish, these exerted a clear dose-dependent protective effect[203] whereas in the MG, although dose-dependence existed, very different doses caused similar effects (increased levels) on some immune parameters (study 5, figure 1). Thus, when we say that the MG is under-protected of pathogenic invasions during gestation, this is not because of a lack in responsiveness but by its inefficacy due to the phagocytic overload caused by milk stasis. This physiological condition in the MG, therefore, would also explain the differences in immune responses by both tissues (namely, bovine MG and murine dermis) after rMMP-9 IB treatments.

Taken together, the results observed herein and in the paper of Pares *et al.* (submitted work: annex 1) point out that the nature of the chosen target, rMMP-9, was not relevant enough to specifically accelerate early involution in the cow MG. According to Ollier *et al.*, PRL withdrawal does not occur until around 10 to 12 days after milking cessation in dairy cows (figure 1 from paper)[281]. Thus, given that this withdrawal –as described in "*Involution and regeneration of the mammary gland*"– permits MMP-9 function and evolution of its effects only in later dry period stages, and since in our bovine *in vivo* study we have analyzed MG secretions along 9 days, we cannot conclude what would be the rMMP-9-specific effects of rMMP-9 IBs beyond this period. In this sense, it would be interesting to evaluate if rMMP-9 in the IBs would show up any relevant specific activity beyond the IB unspecific effects and PRL fall, in agreement with the rMMP-9 IBs effect-pattern observed in mice and considering the rMMP-9 IBs stability observed *in vitro* (Figure 16). If so, a promising dual potential of rMMP-9 IBs could be explored, not only by an early onset of MG involution due to unspecific effects (as proven), but also by a hypothetic greater tissue remodeling at later stages, maybe through synergistic effects with endogenous MMP-9 to accelerate and shorten the overall dry period length.

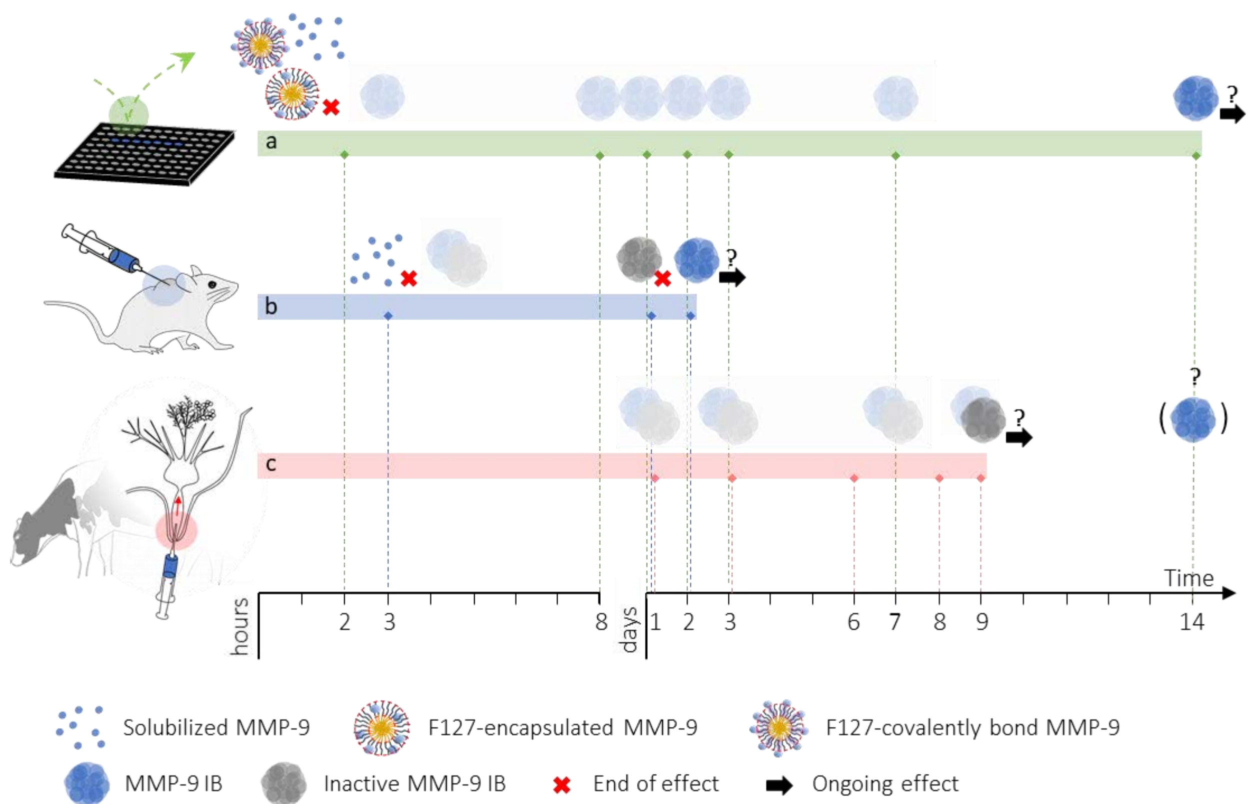

Figure 16. Sketch summarizing the functionality of MMP-9 along the different analyzed conditions –regarding MMP-9 format, testing model, and time course. **a.** *In vitro*, MMP-9 IBs demonstrated to be more stable under serum conditions than soluble-based formats (namely, solubilized MMP-9, F127-encapsulated MMP-9 and F127-covalently bond MMP-9). Concretely MMP-9 IBs could degrade dye-quenched gelatin along 14 days in the presence of serum while MMP-9 degradative capability was sharply reduced for

soluble-based formats yet after 2 h incubations. **b.** Injections into dermal air pouches in MMP-9 knock-out mice helped in elucidating the two-sided immunological effects of MMP-9 IBs. Unspecific effects (inactive MMP-9 IBs) were present along 24 h but were not detected beyond, whereas MMP-9 IBs specific effects were still detected after 2 days of injections. Solubilized MMP-9 had only an initial burst and these effects were not detected beyond 3 h. **c.** Infusions of MMP-9 IBs in their active and inactive forms in bovine mammary gland at dry off were decisive to determine that the acceleration of involution onset was due to unspecific effects in MMP-9 IBs and not because of MMP-9 activity itself. Active MMP-9 IBs did not had any relevant effect along the analyzed time points but their potential effects at later stages have not been determined.

Nevertheless, the results obtained in this study (study 5) clearly pull apart the relevance of rMMP-9 to accelerate the onset of MG involution in dairy cows. These become, however, the opening of new research paths looking forward to finding new proteins that could have a specific potential in this complex context. It would be intriguing to explore what would have happened if instead of infusing rMMP-9 IBs, we would have infused other IBs composed by proteins more relevant in early stages of MG involution, such as TGF-β, TNFα, LIF or even STAT3 IBs. Fortunately, the versatility of IBs permits plenty of tailoring options with low-costs, and, now that their immunogenicity has been elicited, soon strategies will be developed to obtain more accurate allies to prevent IMIs in dairy cattle, contributing to the One Health platform to fight the antimicrobial resistance battle.

THE IMMUNOSTIMULATING ABILITY OF IBS: WHAT ARE THE GOOD AND THE BAD NEWS?

An unignorable aspect stands out after the global sight of the results in this work: how beneficious can be the unspecific effects observed upon IB treatments? Although more research is needed to determine it, the answer must be linked to a more specific question: when is it required an inflammatory response and when could this become a complication? And, also, can we modulate IBs to reach more specific treatments?

It is important to have in mind the MG peculiarities regarding its high immune reactivity, and do not underestimate the potential of IBs as a new biomaterial suitable for other target organs or tissues. In the MG, IB-treatments trigger a clear immunostimulant effect. Overstimulating the immune system is not always desirable and can unquestionably trigger devastating side effects such as tissue degradation –when not required– and increased morbidity. In fact, this is what we encounter under unresolved infections such as mastitis cases, where high SCC evidence an immunostimulation, although its inefficiency permits some pathogens to escape causing tissue damage and/or chronic disease. However, during these threatened days at dry-off, triggering an

even more potent immune response may give an extra support to the "busy phagocytes" in the alveoli. Thus, under certain circumstances the IBs intrinsic inflammatory reaction could be the key to block aggravating situations. Nonetheless, in those cases in which prolongated inflammatory responses due to IBs could be detrimental, minimal effective doses should be carefully determined.

Controlling the immunogenic components in bacteria hosts that unspecifically aggregate in the IBs during their formation, would be also a critical step to take into account to reduce non-desired immune effects of these IBs. For that, some studies have been initiated in our group to look for a more specific aggregation process using tags mimicking natural protein dimers. Results, however, are still preliminary. On the other hand, the encapsulation of soluble or solubilized proteins in polymeric nanocarriers has just slightly been exploited here (study 3) regarding the stability of the recombinant protein. This approach could offer, however, other possibilities regarding to the reduction of the immunogenicity elicited during *in vivo* administration through building purer protein nanoparticles.

Conclusions

The overall results obtained and discussed in this thesis, aiming to study the potential of IBs in animal production and elucidate the role of MMP-9 IBs in the optimization of the dry period in dairy cows, can be summarized in the following conclusive statements:

1. Three major fields are currently exploring the use of recombinant proteins in the animal production sector. These are: (i) reproduction, where recombinant hormones are used to improve reproduction performances; (ii) nutrition, where recombinant enzymes are tested to improve nutrition efficiency, and (iii) health, where recombinant antibodies are explored as new agents for passive immunization therapies.

2. Many biotechnological approaches still remain to be explored in the animal production sector, where low-cost and easy to produce alternatives are required and for which inclusion bodies (IBs) emerge as a promising nanomaterial.

3. Highly pure, soluble and active proteins, difficult to obtain by traditional procedures –namely M-SAA3, a labile and "sticky" protein, and MMP-9, a prone-to-aggregate protein– can be successfully obtained from *L. lactis* IBs using a newly developed, non-denaturing and cautiously-conditioned IBs-solubilization protocol based on the use of the mild detergent N-lauroyl sarcosine at 0.2 %, in combination with protease inhibitors and optimal temperature and -time incubation conditions.

4. New MMP-9 nanocarriers have been obtained encapsulating or covalently binding soluble MMP-9 to micelles-forming F127 amphiphilic polymers.

5. F127-based nanocarriers neither modify the specific activity of MMP-9 nor confer greater stability in bovine serum compared to the already highly stable soluble MMP-9.

6. The MMP-9 IBs proved to be the most stable format, compared to other formats based on soluble MMP-9 –namely, solubilized MMP-9 and MMP-9 F127-based nanocarriers–, extending a minimum of 14 days their *in vitro* activity in 50 % bovine serum, and at least 2 days their effects in dermal air-pouches in mice.

7. The activity of IBs under *in vitro* conditions cannot always be used as a conclusive result and must be analyzed with caution: their full activity can only be determined by using *in vivo*-mimicking conditions where their particular properties, such as their capacity to slowly release the proteins forming these nanoparticles, may take place.

8. It is possible to split a reduced immunogenicity of the IB format from their protein-specific immune response comparing MMP-9 mutant with its active counterpart in an MMP-9 knock-out mice model.

9. The infusion of MMP-9 IBs in the mammary gland of dairy cows at dry-off accelerated from 3 to 6 days the onset of involution (BSA and Na^{+}) and protective (SCC, lactoferrin) biomarkers, being 1.2 mg the minimal effective dose.

10. Active and inactive MMP-9 IBs triggered similar effects in the mammary gland of dairy cattle, not observing a MMP-9-specific role at early involution and defining a 9-day extension of the unspecific format-related effects of IBs in the dairy cow model.

Annexes

ANNEX 1

IS MATRIX METALLOPROTEINASE-9 A TRIGGER FACTOR OF MAMMARY GLAND INVOLUTION AT DRY-OFF?

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Is matrix metalloproteinase-9 a trigger factor of mammary gland involution at dry-off?

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Abstract

The dry period is decisive for the milking performance of dairy cows. The promptness of mammary gland involution at dry-off affects not only the productivity in the next lactation, but also the risk of new intra-mammary infections. Matrix metalloproteinase-9 (MMP-9) is an enzyme present in mammary gland and it could have an active role during involution by disrupting the extracellular matrix, mediating cell survival and recruitment of immune cells. The objective of this study was to determine the potential of MMP-9 to accelerate mammary involution and boost the immune system at dry-off by infusing recombinant MMP-9 (rMMP-9) in a soluble or a nanostructured form in the mammary gland. Twelve Holstein cows were dried abruptly and received an intra-mammary infusion of either a soluble or a nanostructured form of rMMP-9. Saline solution was infused in the contralateral quarters as a negative control. Samples of mammary secretion were collected during the week following dry-off to determine somatic cell counts (SCC), metalloproteinase activity, and bovine serum albumin (BSA), lactoferrin, sodium (Na^+) and potassium (K^+) concentrations. Both, the soluble and the nanostructured forms of rMMP-9 increased endogenous metalloproteinase activity in the mammary gland compared with

controls. However, only the nanostructured form of rMMP-9 was able to support involution and immunity function. In comparison with saline controls, the nanostructured form of rMMP-9 increased SCC up to 400-fold and lactoferrin concentrations up to 1.8-fold. Furthermore, BSA and Na^+/K^+ (considered involution markers) in the udders treated with the nanostructured form of rMMP-9 increased 8-fold, compared with negative controls. The results demonstrated that MMP-9 does not have a key role orchestrating all the involution process in the cow mammary gland and that nanoparticulated format of rMMP-9 is related with the acceleration of tissular involution and effectors of innate immunity.

Keywords: Dry period, Mammary gland involution, MMP-9, Protein nanoparticle

Introduction

At the end of lactation and as calving approaches, pregnant dairy cows enter into a non-lactating (dry) period to optimize milk production in the subsequent lactation. During the dry period, the epithelial component of the mammary gland regresses, proliferates, and differentiates to allow optimal milk production in the subsequent lactation. Tissue regeneration of the mammary gland is necessary, and the omission of a dry period reduces milk production in the following lactation[1].

However, the strong presence of galactopoietic hormones due to a concomitant pregnancy could hamper the beginning of the involution and delays the activation of the immune system[2]. Furthermore, today's pregnant dairy cows are dried while producing ~25 kg/d of milk with some animals producing >35 kg/d[3]. These copious amounts of milk remaining in the mammary gland exert high intra-mammary pressure and may cause discomfort[4] and milk leakage[5]. In addition, high-producing dairy cows are more susceptible to intra-mammary infections during the early stages of the dry period[6].

Involution of the mammary gland starts with a complex signaling pathway of cell factors, hormonal changes, and immune stimulation. Activation of the immune system at the beginning of the dry period recruits blood leukocytes that progressively colonize the mammary gland after dry-off and phagocytize and destroy microorganisms[7]. However phagocytic activity against pathogens is diminished at dry-off as phagocytes engulf milk fat, cell debris, and other compounds derived from milk accumulation and their activity is not fully focused in the fight against pathogenic bacteria[7]. In fact, it is assumed that the immune system does not reach effective protective levels until 8 d after dry-off[8], and thus the health of the mammary gland is compromised during this period. To reduce risk of mastitis, antibiotics are used routinely into the

mammary gland at dry-off. However, this practice has been challenged due to concerns about potential emergence of antibiotic resistances.

Matrix metalloproteinase-9 (MMP-9) is a tissue-remodeling enzyme that is physiologically released by mammary epithelial cells and neutrophils entering into the mammary gland during the involution process[9,10]. Thus, MMP-9 is probably one of the enzymes involved in the breaking of the extracellular matrix of the mammary gland (ECM)[11], which could trigger a signal to the detached cells to enter into apoptosis supporting the tissue involution[12]. Moreover, the expression of MMPs, growth factors and cytokines is closely linked, but through a mechanism that is still widely unknown in the bovine mammary gland[13,14]. It has been previously demonstrated that the proteolytic degradation of ECM is a key factor through the loss of differentiated state and induction of apopotosis and involution[15]. Thus, we hypothesized that an intramammary administration of recombinant MMP-9 (rMMP-9) could represent an effective strategy to accelerate tissue involution at dry-off. Moreover, it could boost the infiltration of immune cells into the mammary gland and stimulate other active immune factors, reducing the need to use antibiotics at dry-off[16].

Traditionally, recombinant proteins have been produced and purified as soluble forms. Soluble forms can sometimes be unstable and have short lives because other enzymes from the tissue can degrade them. Also, the production and downstream processes to purify these soluble forms are usually costly. In order to overcome these obstacles, some research groups have produced recombinant proteins as self-organizing bacterial nanoparticles, also known as inclusion bodies, nanoclusters o nanopills[17, 18], which represent a low-cost source of slowly-releasable, highly-stable functional proteins[19, 20]. Thus, the objective of this study was to determine the potential of an intra-mammary infusion of MMP-9 as soluble and nanostructured recombinant forms to shorten the dry period length of the mammary gland of dairy cows after dry-off.

Experimental section

Bacterial strains, Plasmids, Recombinant Proteins and Growth Conditions

The production of recombinant MMP-9 (rMMP-9) was performed using *Lactococcus lactis* strain NZ9000 *clpP*$^{-}$ *htrA*$^{-}$ (*clpP-htrA*; Em^{R})[21, 22] (kindly provided by INRA, Jouy-en-Josas, France; patent nº EP1141337B1). The sequence of the catalytic domain of the bovine (*Bos taurus*) MMP-9 (from Phe107 to Pro449 NM_174744.2) was codon optimized for the expression in *L. lactis* and the cloning performed adding at the C-terminal a lysine plus a histidine tag to assist protein detection and purification (GeneArt, Invitrogen by Thermo Fisher Scientific, USA). Recombinant active

fragment of MMP-9 protein (39.6 kDa) was produced by expressing the encoding gene from the Cm^R pNZ8148 plasmid (NIZO) under the nisA promoter control[23]. This strain was cultured in shake flasks at 30 °C without shaking in M17 Broth with 0.5 % glucose supplemented. Antibiotics were used at the following concentrations: chloramphenicol (5 µg/ml) and erythromycin (2.5 µg/ml). Recombinant gene expression was induced by 12.5 ng/ml nisin (Sigma-Aldrich, Barcelona, Spain) during 3 h.

Purification and Quantification of Soluble and Nanostructured MMP-9 Proteins

Pellets of *L. lactis* cells were resuspended in phosphate-buffered saline (PBS) in the presence of protease inhibitors (Complete EDTA-Free, Roche) and frozen at -80 °C. After thawing, cells were disrupted at 1,500 psi (4 rounds for soluble protein and 3 for protein nanoparticles) in a French press (Thermo FA-078A). For the soluble MMP-9, 0.05 mg/ml lysozyme was added and the resulting mixture was incubated at 37 °C with shaking for 2 h. The lysate was centrifuged at 15,000 *x g* for 45 min. After that, soluble MMP-9 was recovered from the pellet as described in[24] and filtered through a 0.22 µm filter. The protein was purified by His-tag affinity chromatography using HiTrap Chelating HP 1 ml columns (GE Healthcare, Barcelona, Spain) with an ÄKTA purifier FPLC System (GE Healthcare). The purified soluble MMP-9 was analyzed by both SDS electrophoresis/Coomassie Brilliant Blue staining and Western blotting. Concentration was determined by Bradford's assay (BioRad). The isolation of MMP-9 protein nanoparticles was conducted following[17]. The yield of MMP-9 nanoparticles was determined by both SDS electrophoresis/Coomassie Brilliant Blue staining and Western blotting, using a standard curve of known amounts of a Green Fluorescent Protein (GFP) protein. Densitometry analyses were performed with the Quantity One software (BioRad). For Western blotting, a commercial monoclonal antibody against anti-His (#A00186-100 Genescript, Piscataway, USA) and an anti-mouse secondary antibody (#170-6516 Bio Rad) were used.

Electron microscopy (EM)

Nanoparticles were detected by field emission scanning microscopy (FESEM) and transmission electron microscopy (TEM). In the first technique, sample microdrops were deposited 2 min on silicon wafers (Ted Pella Inc., Redding, CA, USA) and then, air-dried. Nanoparticles micrographs were acquired at a nearly native state with a high-resolution in-*lens* secondary electron detector in a FESEM Zeiss Merlin (Zeiss, Oberkochen, Germany) operating at 2 kV. For TEM, nanoparticles samples were fixed with aldehydes and osmium, dehydrated, and embedded in Epon resin. Ultrathin sections were deposited on copper grids, and after contrast, observed with the electron microscope TEM Jeol JEM-1400 (Jeol Ltd., Tokyo, Japan).

Analysis of MMP-9 Activity

Metalloproteinase activity of rMMP-9 (soluble MMP-9 and MMP-9 protein nanoparticles -39,6 KDa-) and endogenous MMP-9 -92 KDa- present in mammary secretion samples were analyzed and compared by zymography as described elsewhere[24].

Intra-Mammary Infusions

This experiment was performed under the evaluation and permission of the Ethical Committee of IRTA, protocol number 8788. Twelve lactating Holstein cows (210-220 d pregnant, producing > 20 kg/d of milk during the last 3 d preceding dry-off, and with milk somatic cell counts < 200,000 cells/ml at dry-off) were enrolled in this study at dry-off. All cows were dried abruptly with no dietary intervention before dry-off and no change in milking routine or frequency. At dry-off time, cows received an intra-mammary infusion of either 0.75 mg of soluble rMMP-9 or 12 mg of MMP-9 nanoparticles in a total volume of 10 ml of 0.9 % NaCl sterile commercial saline solution. The actual enzymatic activity of the soluble and the nanostructured MMP-9 amounts used were identical as assessed by zymography. Treatments were randomly assigned to front or rear quarters to avoid potential milk dilution effects. Also, a negative control consisting of 10 ml of 0.9 % NaCl sterile commercial saline solution (Braun, Barcelona, Spain) was infused in the respective contralateral quarter. Then, broad-spectrum antibiotics (Mamyzin secado®, Boehringer Ingelheim, Barcelona, Spain) were locally administered following common production practices. No teat sealant was used. Milk or mammary secretion samples were obtained at days 0 (before last milking), 1, 2, 3, 6, and 7 post-drying at 08:00 h by manual milking. A fraction of mammary secretion samples was kept refrigerated until analyzed for somatic cell counts (SCC), and the remainder was kept frozen at -20 °C until subsequent analyses. Mammary biopsies from the rear quarters (n=12) treated with MMP-9 nanoparticles were obtained at day 9 after dry-off and conserved in RNAlater (Invitrogen, Madrid, Spain) for 24-72 h at 4 °C prior freezing at -80 °C until subsequent RNA extraction.

Biopsy Procedure

To perform the biopsies, cows were partially immobilized in a head bail. The rear part of the mammary gland was hair clipped and washed with a commercial povidone/iodine solution. All procedures beyond this point were carried in maximum sterility conditions possible. The biopsy area selected was at a midpoint on a rear quarter. The incision area was locally anesthetized with a subcutaneous injection of 1.5 ml (30 mg) lidocaine solution (Procamidor, Richter Pharma, Austria). An incision of 1.5-2.5 cm was made with a scalpel through the skin and gland capsule, avoiding the incision of large blood vessels. Then, a 6-mm trocar was introduced about 10 cm

deep into the mammary gland through the incision, the interior punch removed, and biopsy forceps introduced through the trocar canal into the mammary gland to collect a piece of tissue of about 3 mm^2. Pressure was applied to the biopsied area after the procedure for about 3-5 min. Last, skin was sutured with a non-absorbable multifilament suture (KRUUSE silk, Langeskov, Denmark). Samples were washed in PBS and conserved in 1 ml of RNAlater (Invitrogen) for transportation.

RNA Extraction and qPCR

The extraction of RNA was performed using the TRizol reagent (Thermo Fisher Scientific, Madrid, Spain). Then, RNA was retrotranscribed to cDNA using the PrimeScript RT reagent kit (Takara Bio Inc, Shiga, Japan) following manufacturer's instructions. The RNA purity was assessed by Nanodrop instrument at 260, 280, and 230 nm, obtaining 260/280 and 260/230 ratios between 1.9-2.0 and 2.0-2.2, respectively. Reaction of qPCR was performed in a MyiQ Single Color Real-Time PCR Detection System Thermocycler (Bio-Rad) with the SYBR Premix Ex Taq (Takara Bio Inc, Japan) and the conditions and primers specified in Table 1. The specificity of the amplification was evaluated by the single band identification at the expected molecular weight in 0.8 % DNA agarose gels and a single peak in the melting curve. The efficiency was calculated by amplifying serial 1/10 dilutions of each gene amplicon. A standard curve of Ct versus log concentration was plotted to obtain the efficiency, which was calculated using the formula $10^{1/slope}$, with an acceptable range of 1.8-2.2. A total reaction volume of 20 µl was used, containing 50 ng of cDNA, 10 µl of SYBER green fluorescent (Bio-Rad), and the optimized primer concentration for each gene (Table 1). The qPCR reactions were cycled as follows: an initial denaturing step of 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 15 s at optimized annealing temperature for each gene, 30 s at 72 °C and a final extension of 10 min at 72 °C. The resulting Ct values were used to calculate the relative expression of selected genes by relative quantification using *rps9* and *rps15* as housekeeping genes.

Mammary Secretion Analyses

Somatic cell counts were analyzed in fresh milk (last milking before dry-off) or mammary secretion samples within 4 h after extraction using a Scepter® cell counter (Merk-Millipore, Madrid, Spain) and 40 µm sensors. Briefly, 0.5 ml of mammary secretion was diluted 1:1 in PBS and cells were pelleted by centrifugation at 1,000 *x g* for 2 min. Cream and supernatant were discarded. Pellets were then washed 3 times in PBS (without resuspending the pellet), and then resuspended in 0.5 ml of PBS. In some cases, samples had to be diluted in PBS prior to analysis.

Lactoferrin concentration in skimmed milk (last milking before dry-off) and mammary secretions was measured by ELISA, using a commercial bovine lactoferrin ELISA kit (Bethyl Laboratories Inc., Montgomery, TX). The absorbance for each sample was measured at 450 nm using a Model 680 microplate reader (Bio-Rad).

The concentration of bovine serum albumin (BSA) in milk and mammary secretions was analyzed by a colorimetric assay as previously described[25], with some modifications. Briefly, 200 µl of skimmed milk or mammary secretion was mixed with 450 µl of distilled water and 450 µl of a solution containing 1 volume of 1.2 mM of bromocresol green dissolved in 5 mM NaOH, 3 volumes of 0.2 M succinic acid (pH 4.0), and 0.8 % Brij 35 detergent. After mixing by inversion and centrifugation at 1,900 *x g* for 10 min at room temperature, 150 µl of the supernatant were added to a 96-well microplate and the optical density was read at 655 nm using a Model 680 microplate reader (Bio-Rad).

Gelatinase activity was analyzed by zymography as described above. Samples were skimmed by centrifugation at 2,700 *x g* for 10 min. The fat layer was discarded with a swab and supernatant was diluted between 1:20 to 1:250 in PBS and mixed 1:1 with the loading buffer.

Sodium and potassium concentrations in milk secretion were analyzed by inductively coupled plasma-Optical emission spectrometry (ICP-OES) using an ICP-OES Perkin-Elmer Optima 4300DV after dilution with Triton X-100 0.1 % (v/v) instead of digested in a microwave oven as described in[26]. The method was validated before analyzing all samples by evaluating the repeatability of the results from 5 samples that were analyzed after digestion with microwaves or dilution in 0.1 % (v/v) Triton X-100.

Statistical Analysis

Each mammary quarter was the experimental unit. All data, except that pertaining to gene expression in mammary tissue at day 9 after dry-off, were analyzed using a mixed-effects model that accounted for the random effects of quarter within cow, cow, and block (enrollment week), and the fixed effects of treatment, day of sampling, and their 2-way interaction. Sampling time entered the model as a repeated measure using an autoregressive covariance matrix. Data were previously transformed to achieve a normal distribution when necessary. Data pertaining to gene expression in mammary tissue at day 9 after dry-off were analyzed with a similar model as described above but without the fixed effect of sampling time and the random effects of quarter within cow and cow.

Table 1. Primer sequences and amplification conditions for gene expression analysis.

Gene	Primer Sequences	Amplicon Size (bp)	Annealing temperature (Celsius)	Primer concentration (uM)
rps9 (HK)	Fw: 5’-CCTCGACCAAGAGCTGAAG-3’ Rv: 5’-CCTCCAGACCTCACGTTTGTTC-3’	63	57	0.125
rps15 (HK)	Fw: 5’-GCAGCTTATGAGCAAGGTCGT-3’ Rv: 5’-GCTCATCAGCAGATAGCGCTT-3’	150	57	0.125
casp	Fw: 5'-AAGCCATGGTGAAGAAGGAA-3' Rv: 5'-GGCAGGCCTGAATAATGAAA-3'	134	55	0.5
bax	Fw: 5’-AGTGGCGGCTGAAATGTT-3’ Rv: 5’-TTCTTCCAGATGGTGAGCG-3’	287	60	0.5
ki67	Fw: 5’-AACACCCAGTCGTGTTCGTT-3’ Rv: 5’-GAGCCTTCGGTTCTTCACGA-3’	130	62	0.5

Results

rMMP-9 production, activity determination and mammary gland infusion

Two different MMP-9 formats (soluble and nanostructured) were recombinantly produced in *L. lactis*. rMMP-9 nanoparticles produced herein were round, compact, negatively-charged and with a smooth surface and a 430 nm diameter (**Figure 1**). When comparing the rMMP-9 activity by zymography the soluble form showed about 16-fold greater activity than their nanostructured counterpart (data not shown). In consequence, equivalent metalloproteinase activity of either a soluble or a nanostructured form of rMMP-9 were infused in mammary gland quarters at dry off.

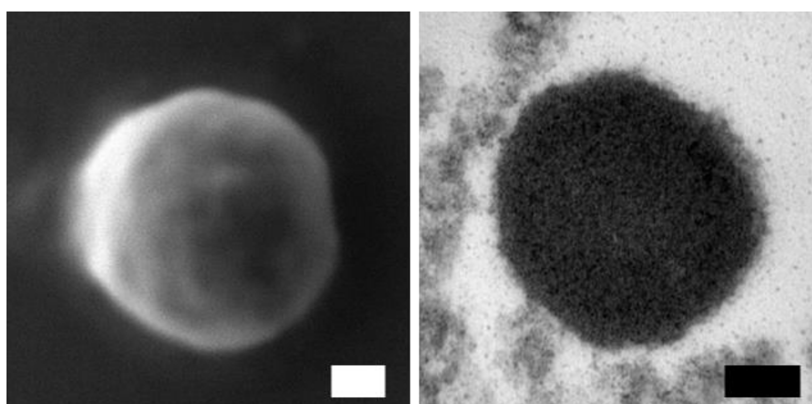


Figure 1. Field emission scanning electron microscopy (FESEM) micrographs of Matrix Metalloproteinase-9 (MMP-9) nanoparticles (left panel) and transmission electron microscopy (TEM) micrographs of MMP-9 nanoparticles (right panel). Scale bars: 100 nm.

Induced metalloproteinase activity in the mammary gland

Metalloproteinase activity in mammary secretion was evaluated by zymography after each treatment (**Figure 2**). The activity of rMMP-9 infused (soluble or nanostructured) was easily differentiated from the activity corresponding to the endogenous MMP-9 due to differences in the molecular weight between the 2 forms (92 kDa and 39.6 kDa, respectively). Overall, endogenous metalloproteinase activity increased as days evolved since dry-off (P < 0.001). At days 1 and 3, the activity was greater in soluble and nanostructured rMMP-9 treatments compared with their saline controls. The activity in quarters treated with soluble rMMP-9 was greater than in the quarters treated with nanostructured rMMP-9 at day 1, but at day 3, this activity was reversed (P < 0.0001).

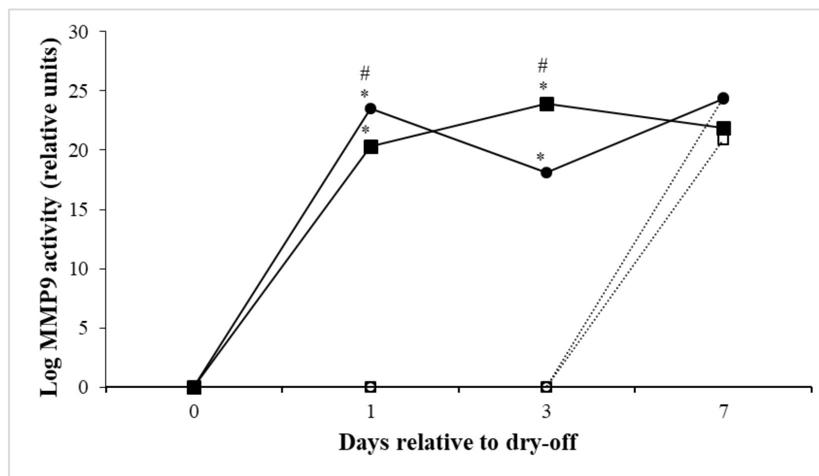


Figure 2. Endogenous matrix metalloproteinase-9 (MMP-9) activity in mammary secretions at 0, 1, 3, and 7 d after dry-off of pregnant dairy-cows analyzed by zymography. Continuous lines indicate MMP-9 treatments, discontinuous lines depict controls. Filled circles correspond to soluble MMP-9 treatment and empty circles to its control, filled squares represent MMP-9 nanoparticle treatment and empty circles to its control. Asterisks indicate significant differences (P < 0.05) between treatment and control whereas pound sign indicates differences between treatments.

Mammary immune response and involution markers monitorization after intramammary administration of rMMP-9

Somatic cell counts (SCC) in mammary secretions progressively augmented (P < 0.0001) as time elapsed since dry-off increased (**Figure 3A**). However, the increase in SCC was greater at all sampling times (P < 0.0001) in the quarters treated with rMMP-9 nanoparticles than in the other 2 treatments (Figure 3A), and SCC in milk secretion from quarters treated with soluble rMMP-9 or Control (saline) did not differ (Figure 3A, inset). From day 1 to 7, SCC increased (P < 0.001) by 157-fold in the mammary secretion from quarters infused with nanoparticles, whereas in the quarters treated with Control or soluble rMMP-9 the increase was 16- and 9-fold, respectively.

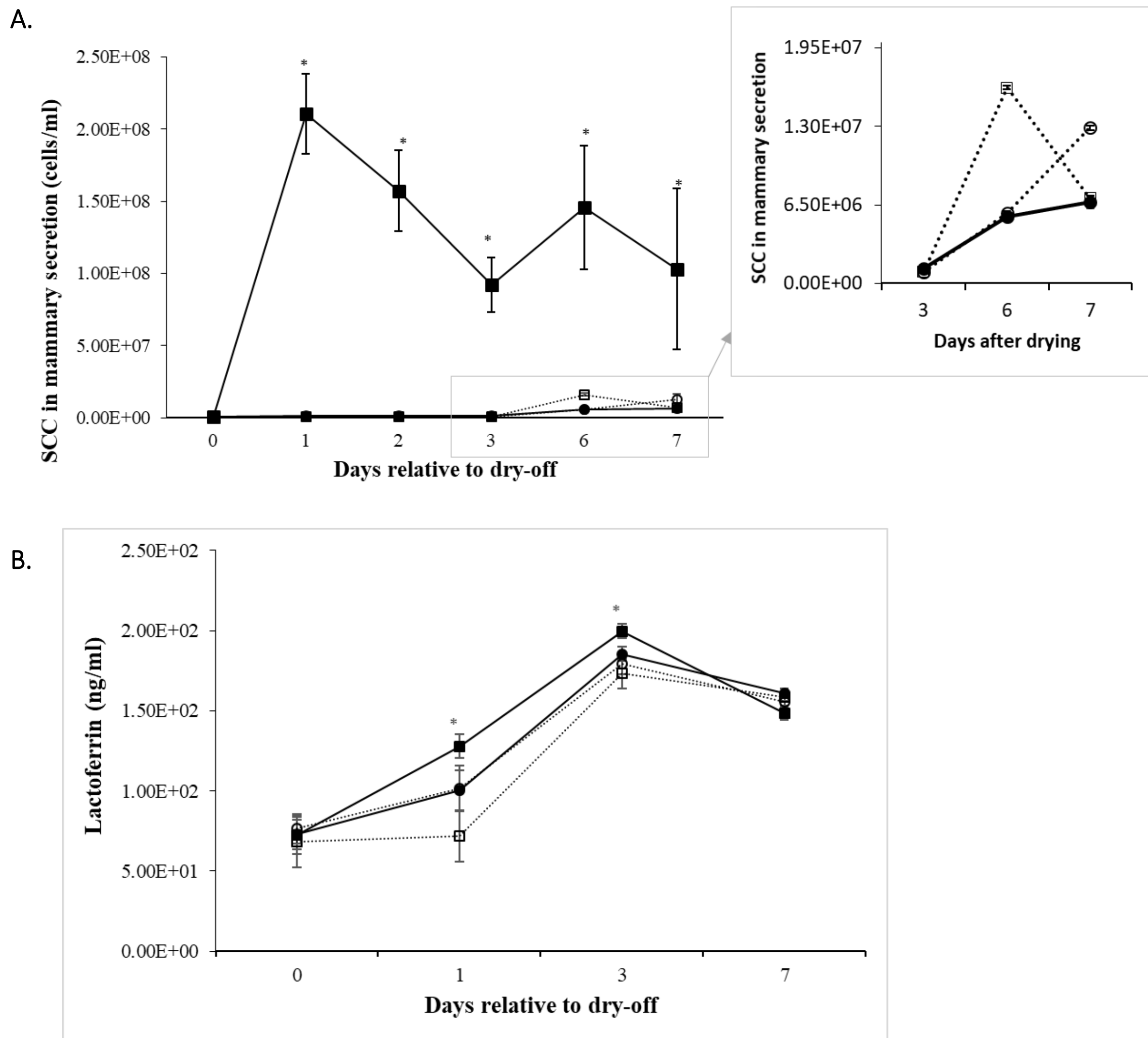


Figure 3. A. Progression of somatic cell counts (SCC) in mammary secretion at 0, 1, 2, 3, 6 and 7 d after dry-off of pregnant dairy-cows. Inset: SCC in milk secretion from quarters treated with soluble MMP-9 or Control (saline). **B.** Lactoferrin concentration in mammary secretion secretion at 0, 1, 3, and 7 d after dry-off of pregnant dairy-cows. In all panels continuous lines depict matrix metalloproteinase-9 (MMP-9) treatments and discontinuous lines indicate controls (saline). Circles correspond to soluble MMP-9 treatment and its control, squares represent MMP-9 nanoparticle treatment and its control. Asterisks indicate significant differences (P < 0.05) between treatment and control.

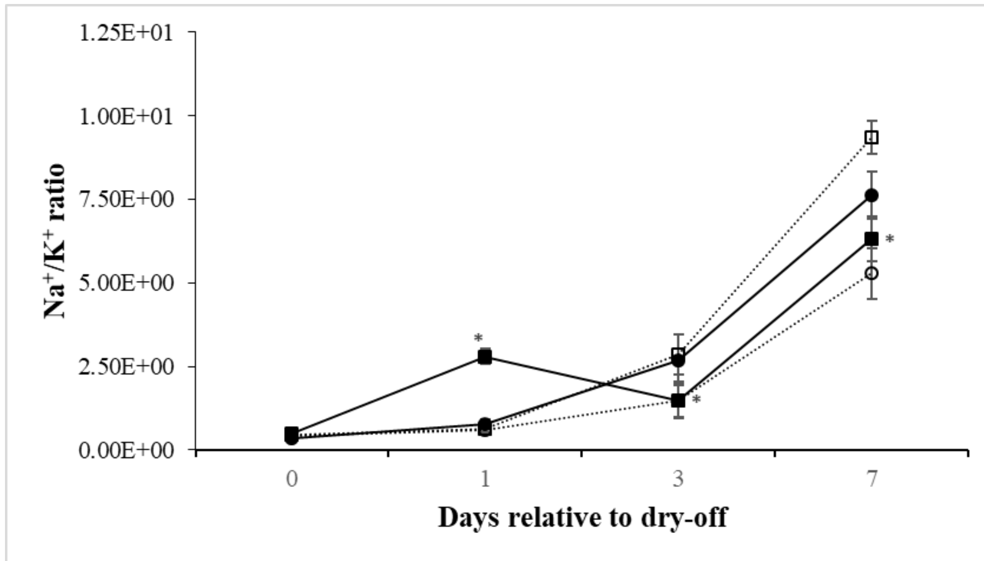
Lactoferrin concentration in mammary secretions increased (P < 0.0001) over time after dry-off (Figure 3B). Overall, mammary secretion from quarters treated with rMMP-9 nanoparticles had greater (P < 0.05) lactoferrin concentration than that from quarters on Control or treated with soluble MMP-9. There was also a positive interaction between treatment and time (P < 0.0001), with quarters treated with nanostructured rMMP-9 showing greater values of lactoferrin in mammary secretion at days 1 and 3 after dry-off compared with the other two treatments. Lactoferrin concentration in mammary secretions from quarters treated with saline (Control) or with the soluble form of MMP-9 did not differ at any sampling time during the week after dry-off.

Several parameters reflecting the tissular involution of the mammary gland were also evaluated (**Figure 4**). The sodium/potassium (Na^+/K^+) ratio in mammary secretion increased ($P < 0.0001$) as days since dry-off increased (Figure 4A). There was an interaction between treatment and time with mammary secretion from quarters treated with nanostructured rMMP-9 having a greater Na^+/K^+ ratio at day 1 compared with those treated with saline solution, but at days 3 and 7, the Na^+/K^+ ratio in mammary secretion from quarters treated with nanostructured MMP-9 was lower than in Control. No differences in the Na^+/K^+ ratio were detected in mammary secretion between quarters treated with soluble rMMP-9 or Control. The presence of BSA in mammary secretion followed a similar pattern than the Na^+/K^+ ratio (Figure 4B), and increased ($P < 0.0001$) within days after treatment. Concentration of BSA in mammary secretion from quarters treated with nanostructured MMP-9 was greater ($P < 0.0001$) than in the other two treatments on days 1, 2, and 3 after dry-off.

Cell survival and apoptosis

Expression of genes coding for the apoptosis marker *Caspase 3* and proliferation marker *Ki67* at day 9 increased by 1.4-fold ($P < 0.05$) and 1.9-fold ($P < 0.05$), respectively, when quarters were treated with rMMP-9 nanoparticles compared with Control (**Figure 5**). Also, a tendency ($P = 0.06$) towards an increased expression of the apoptosis marker *Bax* was observed in quarters treated with the nanostructured form of rMMP-9 in comparison with the Control.

A.



B.

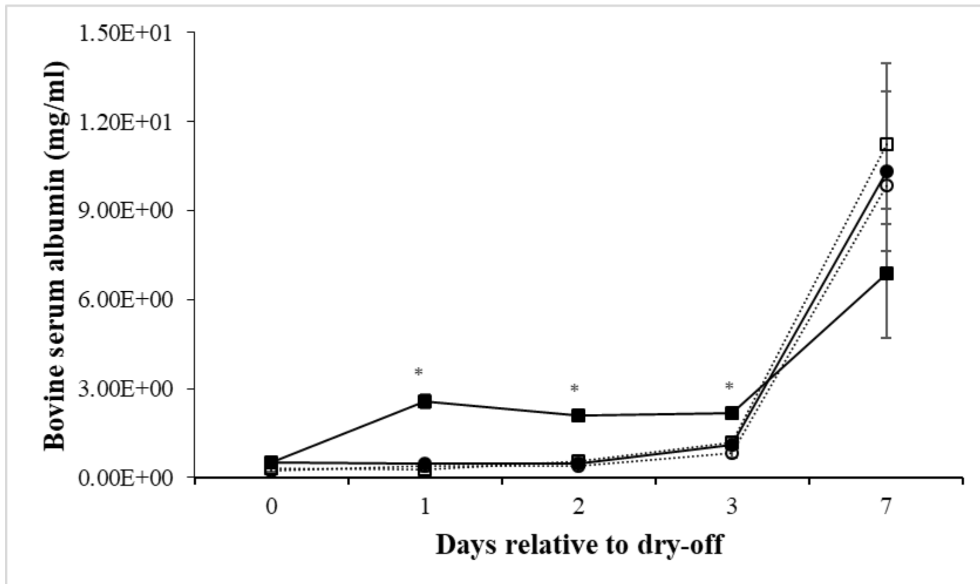


Figure 4. A. Sodium/potassium (Na^+/K^+) ratio in mammary secretion at 0, 1, 3, and 7 d after dry-off of pregnant dairy-cows. B. Bovine serum albumin (BSA) concentration in mammary secretion at 0, 1, 2, 3, and 7 d after dry-off of pregnant dairy-cows Continuous lines indicate matrix metalloproteinase-9 (MMP-9) treatments and discontinuous lines depict controls. Filled circles correspond to soluble MMP-9 treatment and empty circles to its control, filled squares represent MMP-9 nanoparticle treatment and empty squares to its control. Asterisks indicate differences ($P < 0.05$) between treatment and control.

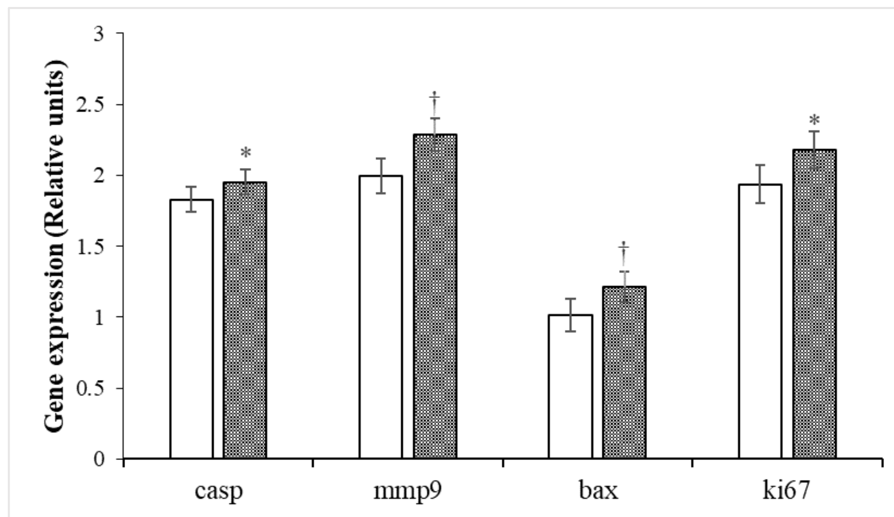


Figure 5. Gene expression profiling in mammary tissue at day 9 after dry-off. White bars correspond to controls and textured bars correspond to mammary quarters treated with MMP-9 nanoparticles. Asterisks indicate significant differences (P < 0.05) between treatment and control. t indicates a tendency to differ (P < 0.10) between treatment and control.

Discussion

A good strategy to accelerate the cow dry period would be to amplify the effects of some of the proteins that play important roles in the involution and remodeling processes through their exogenous administration. MMP-9 is increased during the involution of the cow mammary gland at dry-off and could potential have a key role boosting the mammary gland involution[9]. Epithelial cells and leukocytes, like neutrophils, can express and secrete MMP-9 as a proenzyme. When secreted to the ECM, and once in its active form, MMP-9 is able to degrade cell-matrix adhesions and cell-cell integrins, increasing permeability and mammary gland involution. Although physiologically in the mammary gland MMP-9 is secreted by the local immune cells or mammary epithelia, it can also be produced by recombinant DNA technologies and infused at dry-off aiming to accelerate the involution process.

In this study, we have recombinantly produced both soluble and nanoparticulated rMMP-9 in *L. lactis* to ensure that MMP-9 would be free of lipopolysaccharides and would not elicit any endotoxic response in the animal[27]. Although physiologically MMP-9 is secreted as a zymogen, and then activated by proteolysis[28], we have proven that it is possible to recombinantly produce an active MMP-9 domain in both soluble and nanostructured formats.

The analysis of milk secretion after the infusion of rMMP-9 in mammary gland quarters at dry-off revealed that metalloproteinase activity increased for both soluble and nanostructured rMMP-9

treatments compared to control quarters (Figure 2). Surprisingly we did not observe direct collagenase activity due to the rMMP-9, easily identified in zymography by a lower molecular weight band, but endogenous MMP-9 activity either coming from mammary tissue or neutrophil degranulation. The activity observed at day 1 was higher for soluble rMMP-9 treatment, while at day 3 the highest values were for rMMP-9 nanoparticles (Figure 2) quarters, which could probably be due to a slower and more stable release of rMMP-9 in the nanostructured than in the soluble form[20; 29]. Endogenous MMP-9 is secreted from epithelia o neutrophils as a zymogen and later activated by catalytic function or by other active metalloproteinases[30]. Thus, the increase in the MMP-9 endogenous activity (Figure 2) indicates that rMMP-9, regardless of whether is soluble or nanoparticulated, is functional once administered, activating endogenous pro-MMP-9.

A greater recruitment of immune cells (SCC) (Figure 3A) along with a concomitant increase in lactoferrin concentration (Figure 3B) at day 1 after dry-off was observed in the quarters treated with nanostructured rMMP-9. By contrast, in the soluble rMMP-9 or Controls quarters the increase started at day 3 achieving levels of SCC much lower (100 X) than those obtained with rMMP-9 nanoparticles (Figure 3A). These results indicate that only quarters treated with nanostructured rMMP-9 had an earlier activation of the immune system, compared with those treated with saline (Control) or the soluble form of rMMP-9 invalidating our hypothesis based on the increase of the MMP-9 activity at dry-off to boost the immune response. The rise in SCC in mammary secretion indicates a hastened recruitment of immune cells and the increase in lactoferrin concentration reflects a stimulation of other effectors of the innate immune system. The increased lactoferrin concentration in mammary secretions may have resulted from either an increased synthesis by mammary epithelial cells, or from a direct release by infiltrated somatic cells[31].

Tissue remodeling in the mammary gland is accompanied by a dissociation of the tight junctions, increasing the permeability between milk and blood stream. As a consequence, BSA and Na^+ from blood can be present in mammary secretion and are commonly used as involution markers[32]. Thus, the accelerated increase in both Na^+/K^+ ratio (Figure 4A) and BSA (Figure 4B) in mammary secretion from quarters treated with nanostructured rMMP-9 suggests that the involution was initiated earlier than with the soluble form of rMMP-9 and Control and was not directly linked with an increase of MMP-9 activity in the mammary gland that was also achieved in soluble rMMP-9 treatment. In line with the observations in mammary secretions, and based on the expression of different markers, it could be concluded that cellular apoptosis and proliferation were greater in quarters treated with nanostructured rMMP-9 than in Control (Figure 5) in which the immune response and involution markers were increased.

Thus, these results all suggest that the effect observed by the rMMP-9 nanoparticles is most likely due to an inflammatory response produced by the different components forming these protein-based nanoparticles (and not by the activity of rMMP-9), as previously described by the administration of different compounds such as *Panax ginseng*[33], chitosan[34], and LPS[35], among others.

Moreover, we can also conclude that although MMP-9 has a role in the mammary gland involution due its presence[12], it is not the main responsible to trigger all the mechanisms accompanying tissue involution process, because we do not observe any effect on SCC, lactoferrin, BSA and Na^+/K^+ ratio when soluble rMMP-9 is used.

Conclusions

Overall these results indicate that, contrary to the initial hypothesis made, the intramammary administration of rMMP-9 increases metalloproteinase activity in the tissue but does not accelerate neither tissue involution nor immune response at dry-off. An unexpected result was found in relation to the rMMP-9 nanoparticulated format per se which accelerates the local immune response and mammary involution in a very significant and pronounced fashion. This study leads us to further explore the nanoparticulated format to better dissect the effect due to the format and determine if it could be also an effect due to the recombinant proteins forming such nanoparticles, as it has been previously described in other applications[36].

Declarations

Ethics approval and consent to participate

All procedures were performed with the consent of the IRTA Ethics Committee.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

EGF and AA conceived and designed the experiment; SP, EGF, AB and AA performed animal experiment; SP and OCG performed laboratory analysis; SP, AB, EGF and AA analyzed data; SP, OCG, EGF and AA prepared the manuscript. All authors read and approved the final manuscript.

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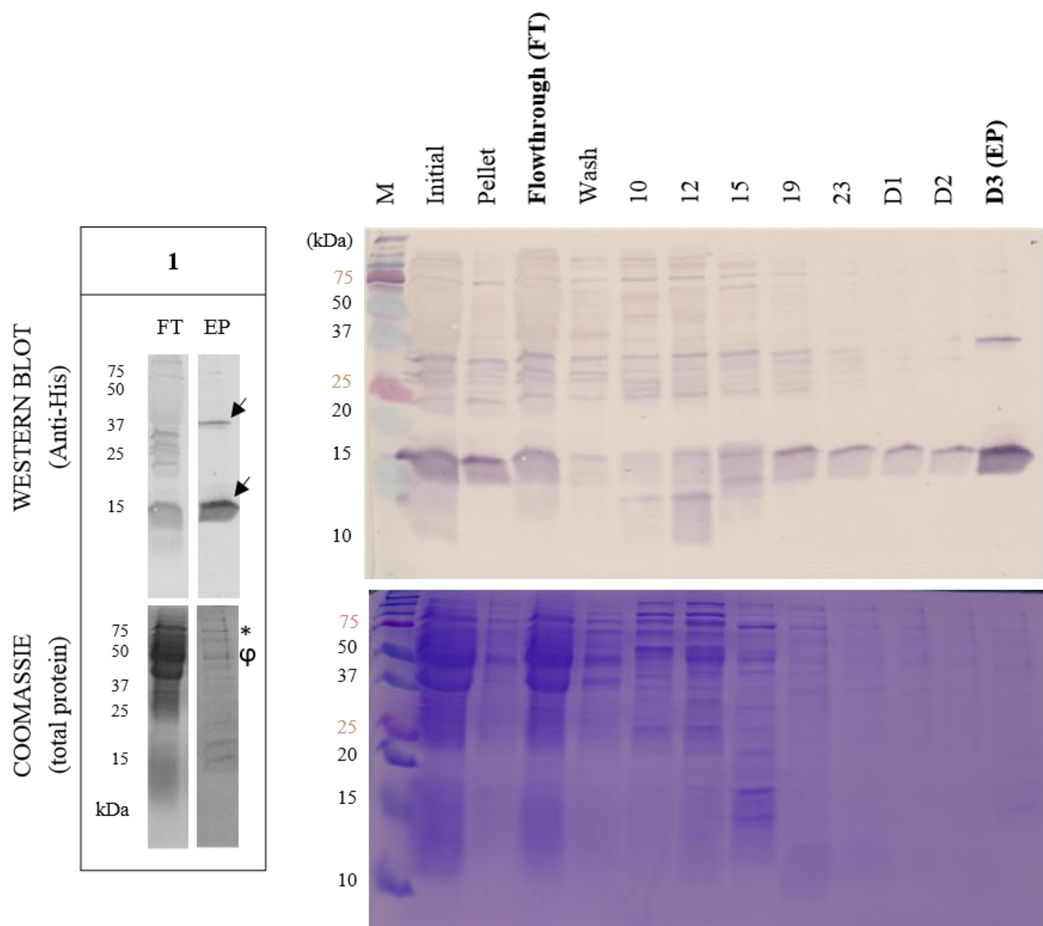
ANNEX 2: SUPPLEMENTARY MATERIAL IN STUDY 2

A new approach to obtain pure and active proteins from *Lactococcus lactis* protein aggregates

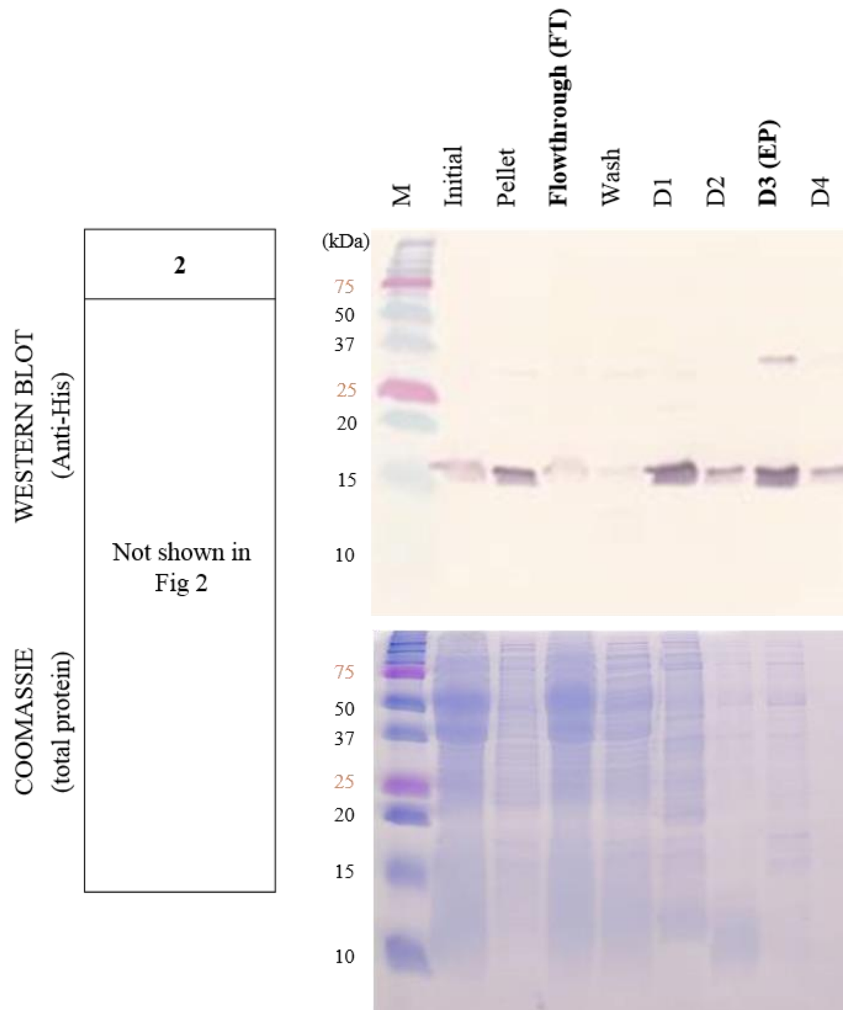
L Gifre-Renom, O Cano-Garrido, F Fàbregas, R Roca-Pinilla, J Seras-Franzoso, N Ferrer-Miralles, A Villaverde, À Bach, M Devant, A Arís and E Garcia-Fruitós

Supplementary Figure 1. Original western blots (top) and Coomassie stained gels (bottom) for the M-SAA3 purification conditions stated in Table 1. In the left, the respective cropped lanes shown in Figure 2 where high-contrast was applied when necessary -only in Coomassie lanes- to allow a better display of the bands. Numbered lanes correspond to eluted fractions along the imidazole gradient. *D* dialyzed samples; *EP* eluted protein; *FT* flow through; *M* protein marker.

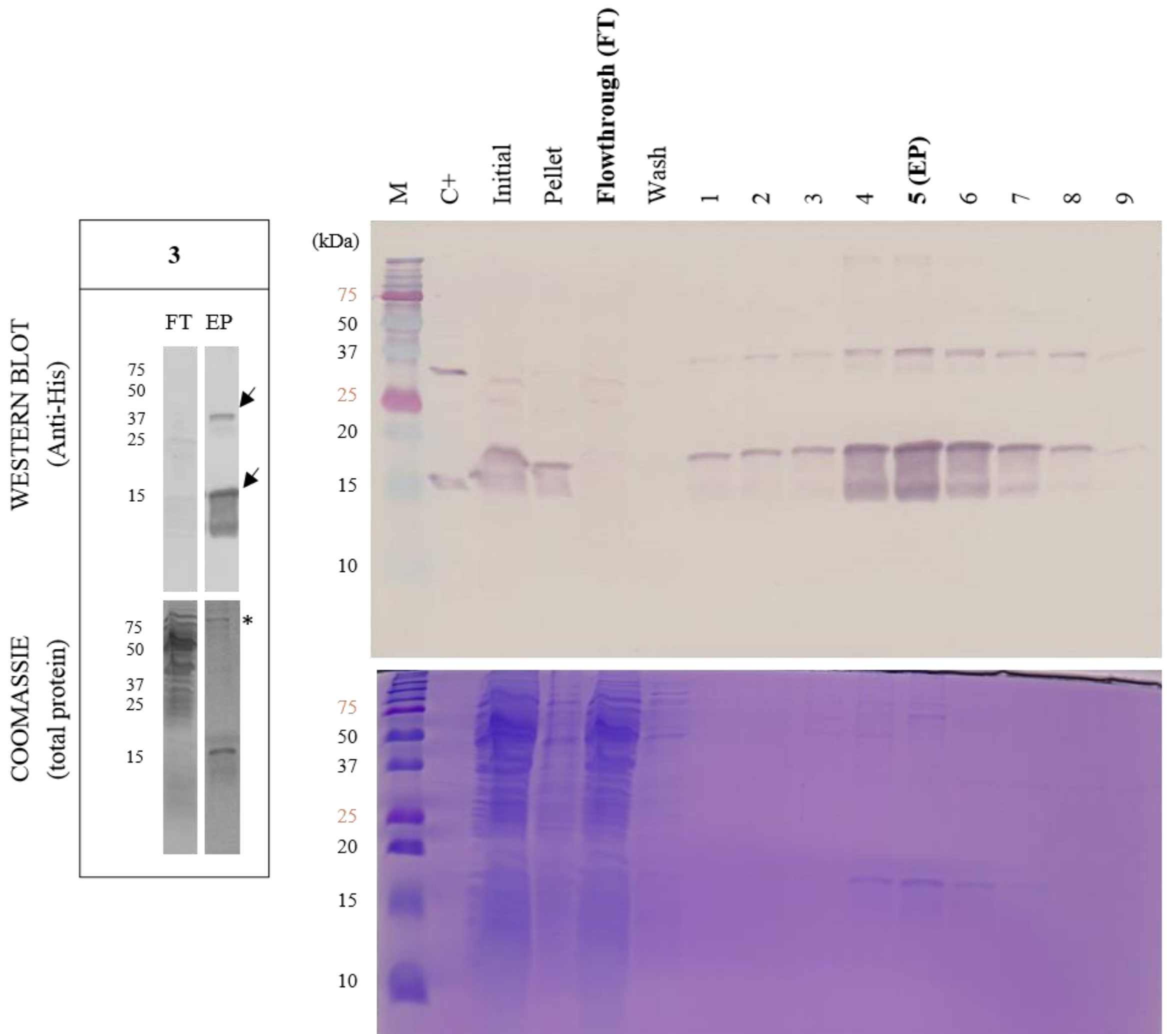
A. Condition 1



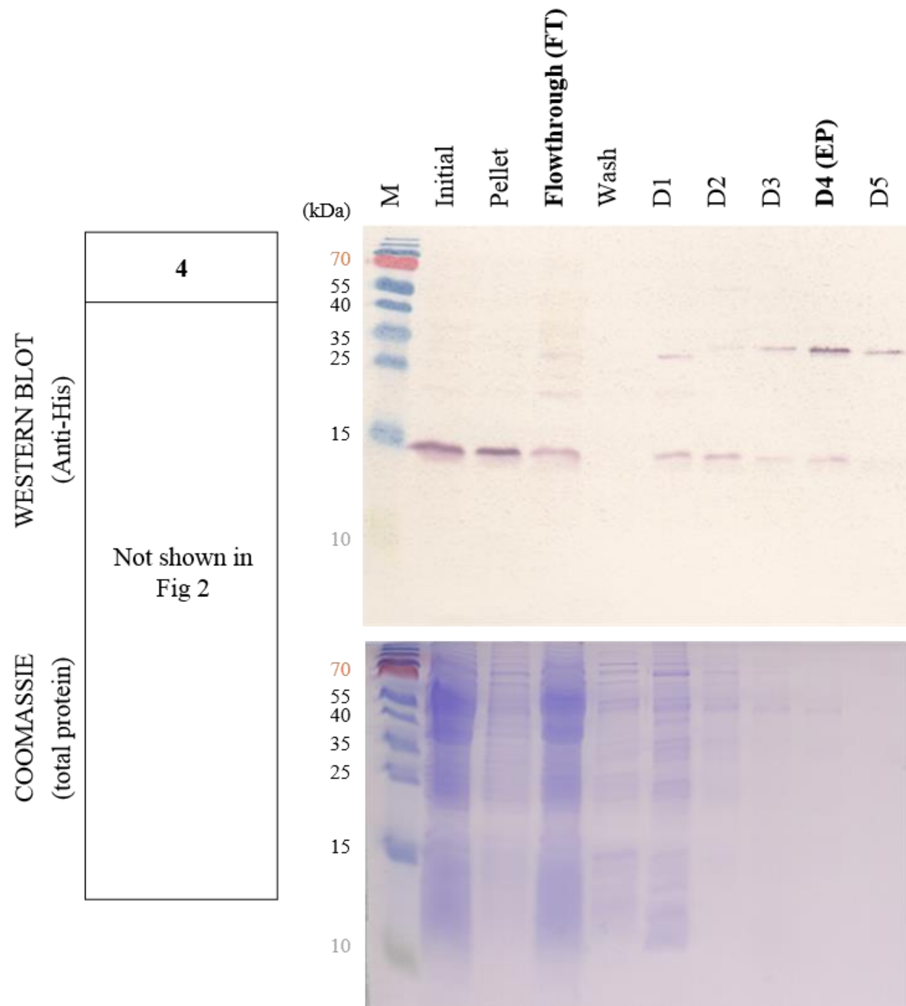
B. Condition 2



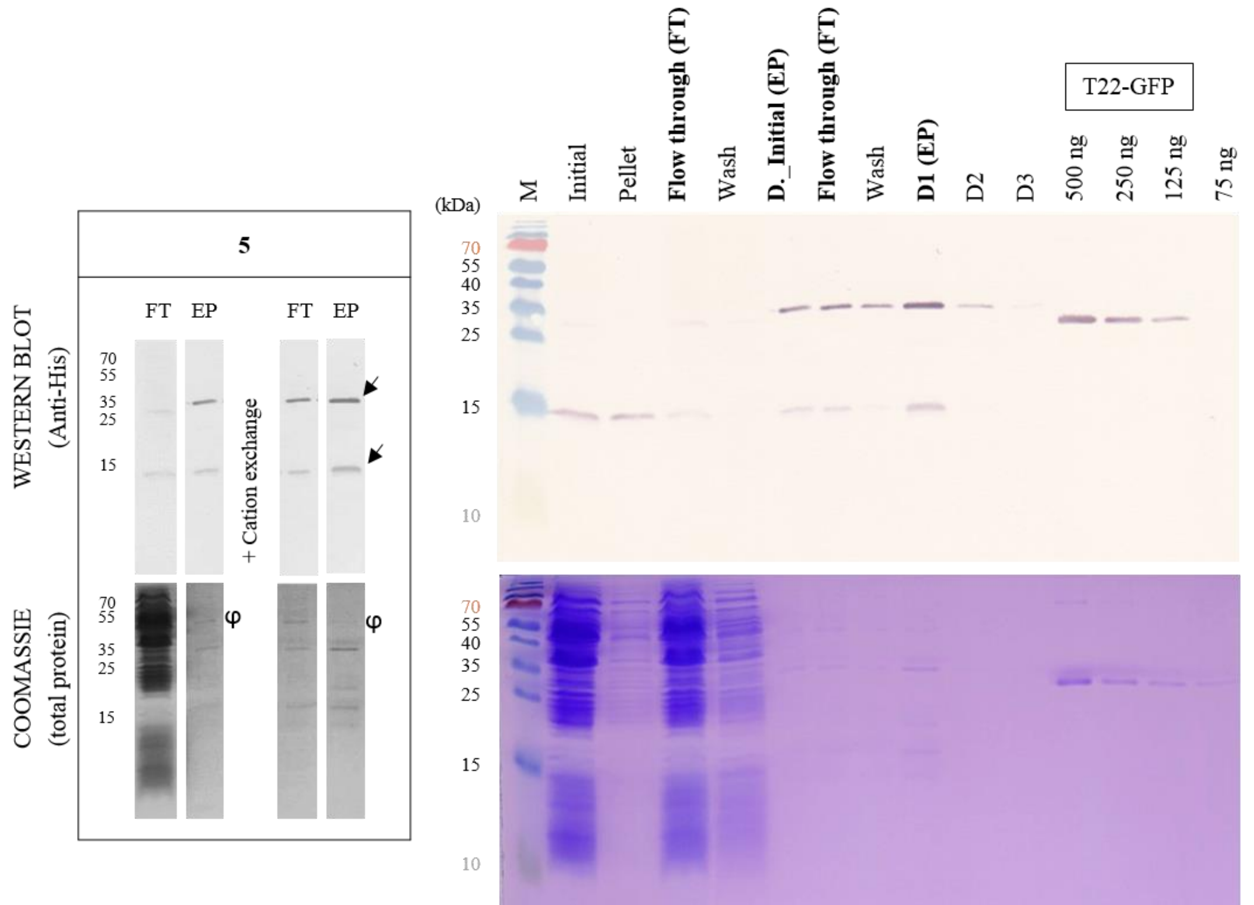
C. Condition 3



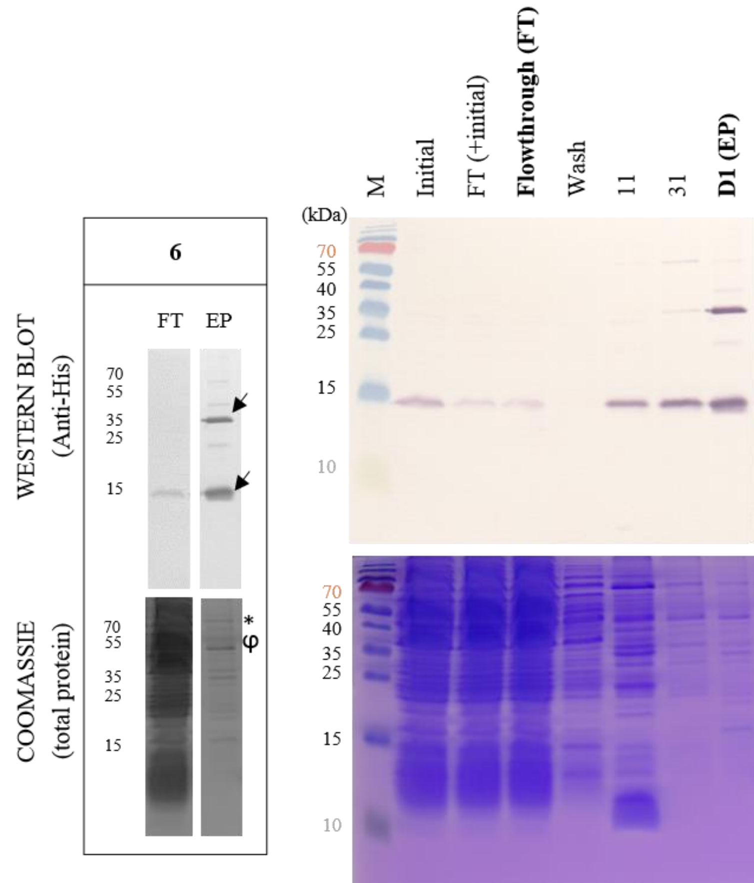
D. Condition 4



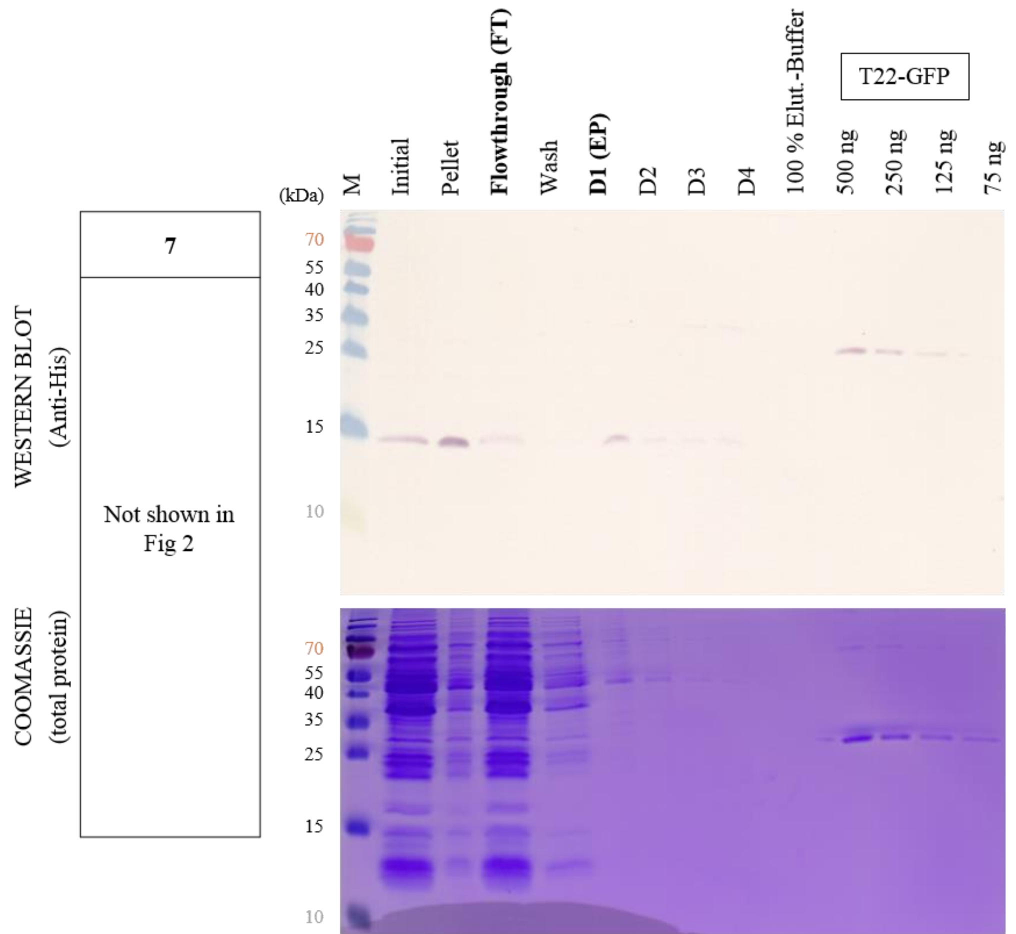
E. Condition 5



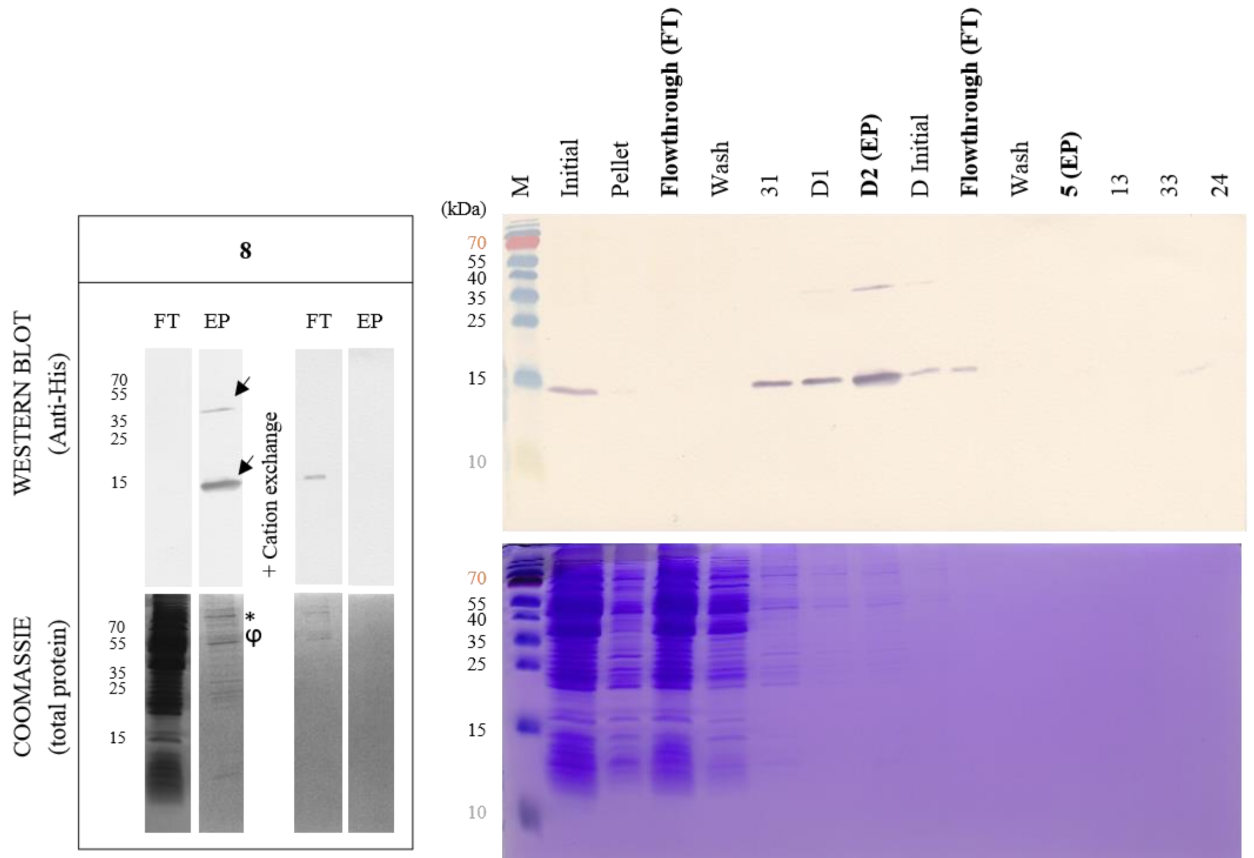
F. Condition 6



G. Condition 7

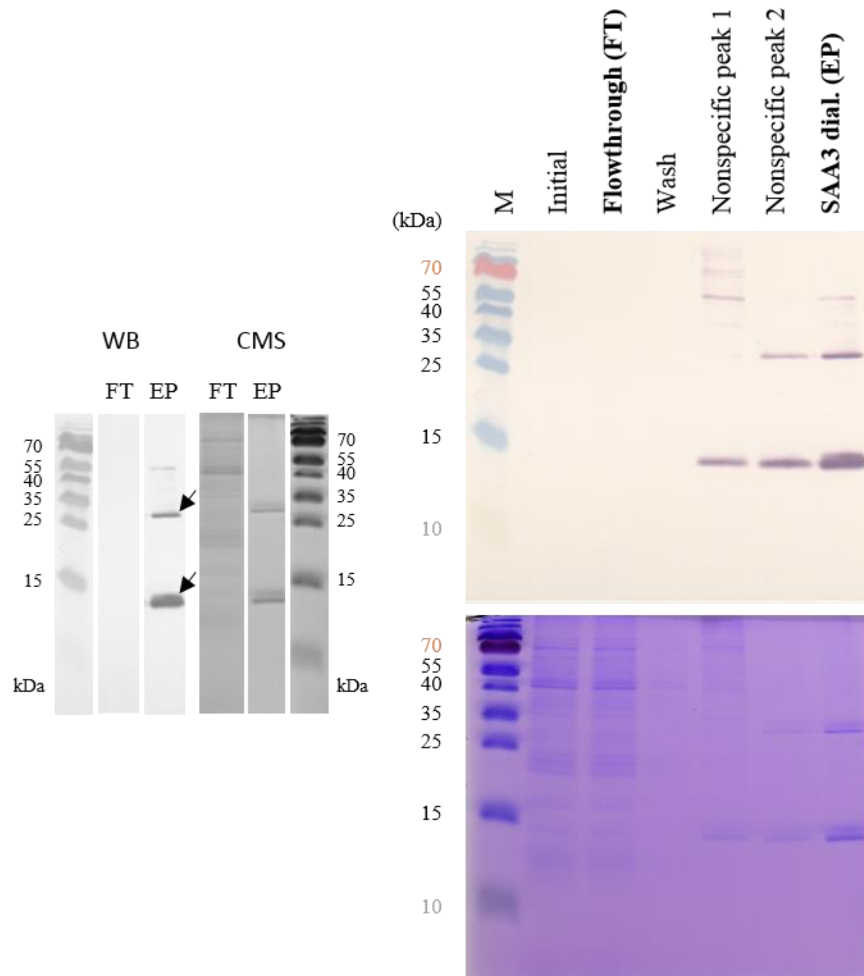


H. Condition 8

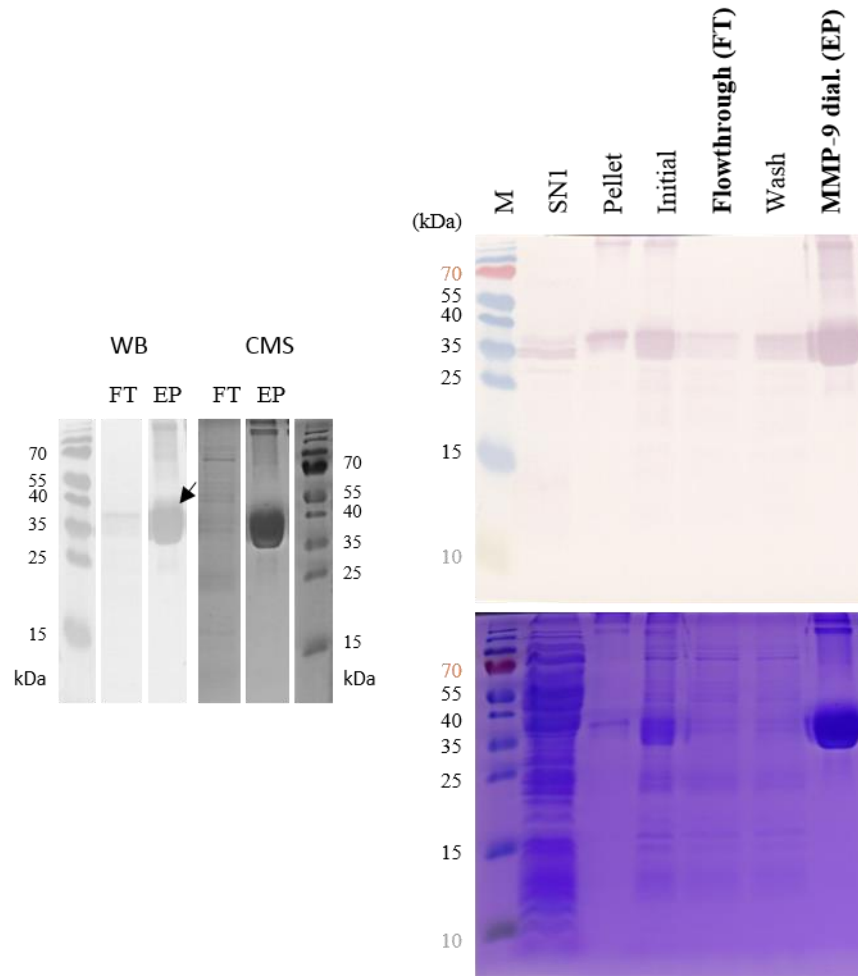


Supplementary Figure 2. Original western blots (top) and Coomassie stained gels (bottom) for the purification of the solubilized M-SAA3 and MMP-9. In the left, the respective cropped lanes shown in Figure 4. *CMS* Coomassie stained gel; *EP* eluted protein; *FT* flow through; *M* protein marker; *SN* supernatant; *WB* western blot.

A. Solubilized M-SAA3



B. Solubilized MMP-9



ANNEX 3: SUPPLEMENTARY MATERIAL IN STUDY 4

Protein-Based Nanomaterials: Exploring their Potential and Inflammatory Response *in vivo* in a Promising New Therapeutic Format

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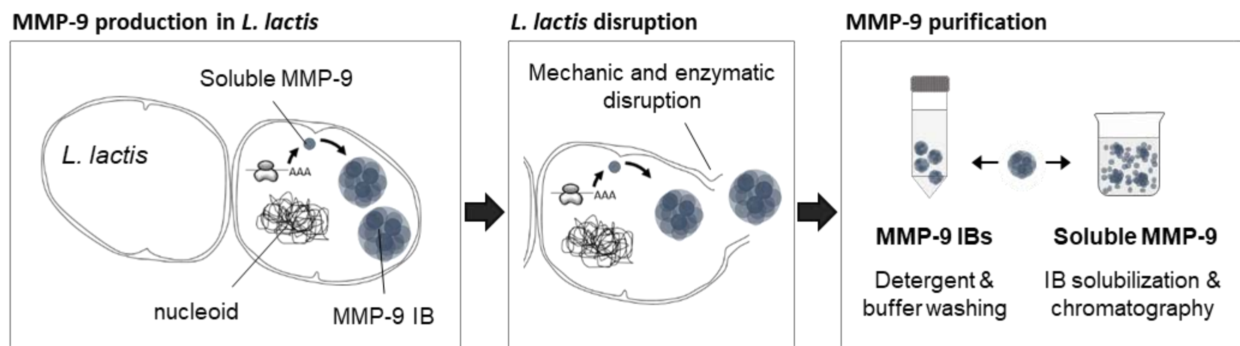
Figure S1. Schematic representation of the protocol used to obtain MMP-9 in *L. lactis* in both soluble and IB format, and protein functionality. Recombinant MMP-9 is intracellularly produced under the nisin-controlled gene expression (NICE) system in *L. lactis*. During the overproduction process MMP-9 is mainly accumulated as inclusion bodies (IBs). These protein aggregates are recovered after a mechanic and enzymatic disruption of the bacteria, and IBs are further purified through different detergent and buffer washing steps. The soluble MMP-9 is obtained through the IB solubilization followed by a chromatography purification step, as described elsewhere [Gifre-Renom *et al.*, Scientific Reports 2018].



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Laia Gifre Renom
PhD Thesis, 2019

Directors:
Anna Arís Giralt
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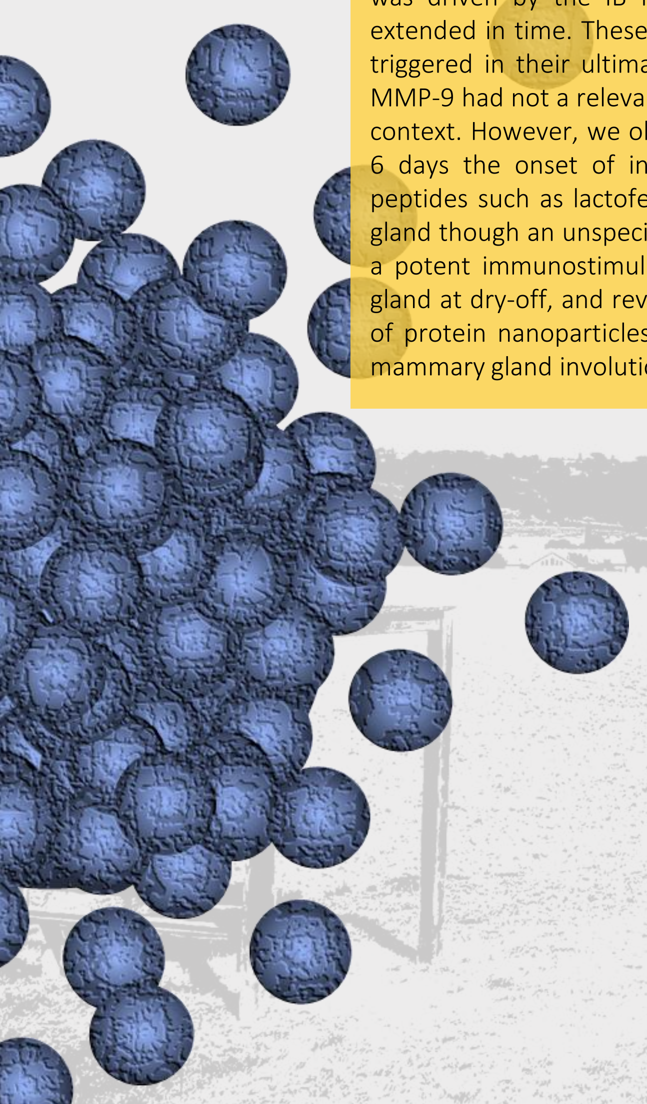
There is a worldwide action plan under the motto One Health, calling for the urgent minimization in the use of antibiotics at all levels –human, animal and environmental– in the joint battle against the antimicrobial resistance (AMR) crisis that threatens global health. The development of new preventive strategies to treat infection diseases has become, therefore, an essential initiative since the use of preventive antibiotics is being banned. In dairy cattle, the dry period is a sensitive phase in which cows easily suffer of intramammary infections, or mastitis, due to the temporally inefficient immune condition in the mammary gland. In this context, alternatives are required in order to control and reduce mastitis incidence at dry-off and to diminish the need of antibiotics. Inclusion bodies (IBs), which are protein aggregates recombinantly produced in bacteria and with an easy and low-cost scale-up, have already demonstrated a great potential in research areas such as tissue engineering and cancer therapy. With the aim of exploring their potential into the animal production sector, this thesis has been focused to cover all steps in the way along the development, characterization and application of this novel and promising protein-based therapy in the dairy sector. Starting from the acquisition of soluble MMP-9 by solubilizing IBs using a newly described protocol for Generally Recognized as Safe (GRAS) bacteria protein-factories, we have been able to develop new MMP-9 delivery formats and to compare them with IB performances. The greater stability shown by IBs when compared with other formats brought us to consider their applicability *in vivo*. As a first step, their administration in a murine model revealed for the first time that their potential as an immunostimulant agent could be importantly dissected into two distinct effects. One effect, unspecific and ephemeral, was driven by the IB format whereas the other effect was MMP-9-specific and extended in time. These encouraged us to try to uncover what would be the effects triggered in their ultimate target, the bovine mammary gland at dry-off. Although MMP-9 had not a relevant specific role triggering the early stages of involution in this context. However, we observed that these protein aggregates accelerated from 3 to 6 days the onset of involution biomarkers, the release of natural bacteriostatic peptides such as lactoferrin, and the recruitment of immune cells in the mammary gland though an unspecific action. Therefore, these results consolidate MMP-9 IBs as a potent immunostimulatory –although unspecific– agent in the bovine mammary gland at dry-off, and reveal that new opportunities are yet to be explored in the use of protein nanoparticles to reach a more specific effect in the acceleration of the mammary gland involution and immune system.



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